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Chromosome Evolution in Amphibia

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About half of all currently existing species of Amphibia have been cytogenetically analysed. These studies were primarily conducted with the conventional methods of cytogenetics. The modern techniques of chromosome banding have only been used on a very limited number of species. Nonetheless, the comparative cytogenetics of Amphibia already permit a closer insight into the operant laws of karyological evolution than was achieved in any other class of vertebrates. At the same time, however, the variable parameters playing a part in the chromosome evolution of individual amphibian groups also become apparent. The present review is to provide a summary of the lawful and variable parameters so far established with either conventional as well as modern cytogenetic methods.

Phylogeny and systematics of the Amphibia

Before going into the results of the chromosome studies on Amphibia, a short summary of the phylogeny and systematics of these vertebrates would be advantageous. According to the current view of vertebrate evolution, Ichthyostega from the late upper Devon is regarded as one of the first amphibian-like tetrapods. Ichthyostega represents a transitional stage between the crossopterygian fishes of the suborder Rhipidistia and the early amphibians. The adaptations necessary for a terrestrian way of life (atmospheric breathing, prevention of dessication, tetrapod locomotion) was accomplished by the amphibians during the Carboniferous and Permian periods. In these periods, several groups differentiated (Ichthyostegalia, Rhachitomi, Stereospondyli, Embolomeri, Lepospondyli), of which the Lepospondyli are considered the ancestors of the modern Amphibia (for reviews see Romer, 1966; Schmalhausen, 1968).

Unfortunately, very few fossils exist of the time between the well-documented paleozoic and the recent Amphibia. Therefore, it has not been possible to reconstruct the immediate ancestry of the Amphibia. The development of the abundance of forms among the recent amphibians is thought to have begun in the Tertiary. The Amphibia in existence today can be classified into 3 orders (Anura, Urodela and Apoda), about 28 families, approximately 320 genera and about 3000 species. Whereas the Apoda with their 160 species occur exclusively in the tropical regions of America, Africa and Asia, and the Urodela with their roughly 350 species are restricted to the temperate and sub-tropical regions of the northern hemisphere, the Anura with their 2500 species are almost ubiquitous (Ziswiler, 1976).

The classical cytogenetic studies on Amphibia

The early investigations of the 3 amphibian orders (Anura, Urodela and Apoda) were performed with the conventional methods of cytogenetics on uniformly stained chromosomes (Fig. 1). The comparative criteria used for this primarily were the number and morphology of chromosomes (relative length, centromeric position, arm ratio) as well as the number and positions of the secondary constrictions. The results obtained in the course of these studies yielded the first indications of the processes which had occurred during the chromosomal evolution of the Amphibia. Thus Morescalchi (1971, 1973) was able to demonstrate impressively that in all 3 amphibian orders during their evolution from primitive to highly specialized genera the number of microchromosomes and telocentric chromosomes was reduced in favor of large metacentric or

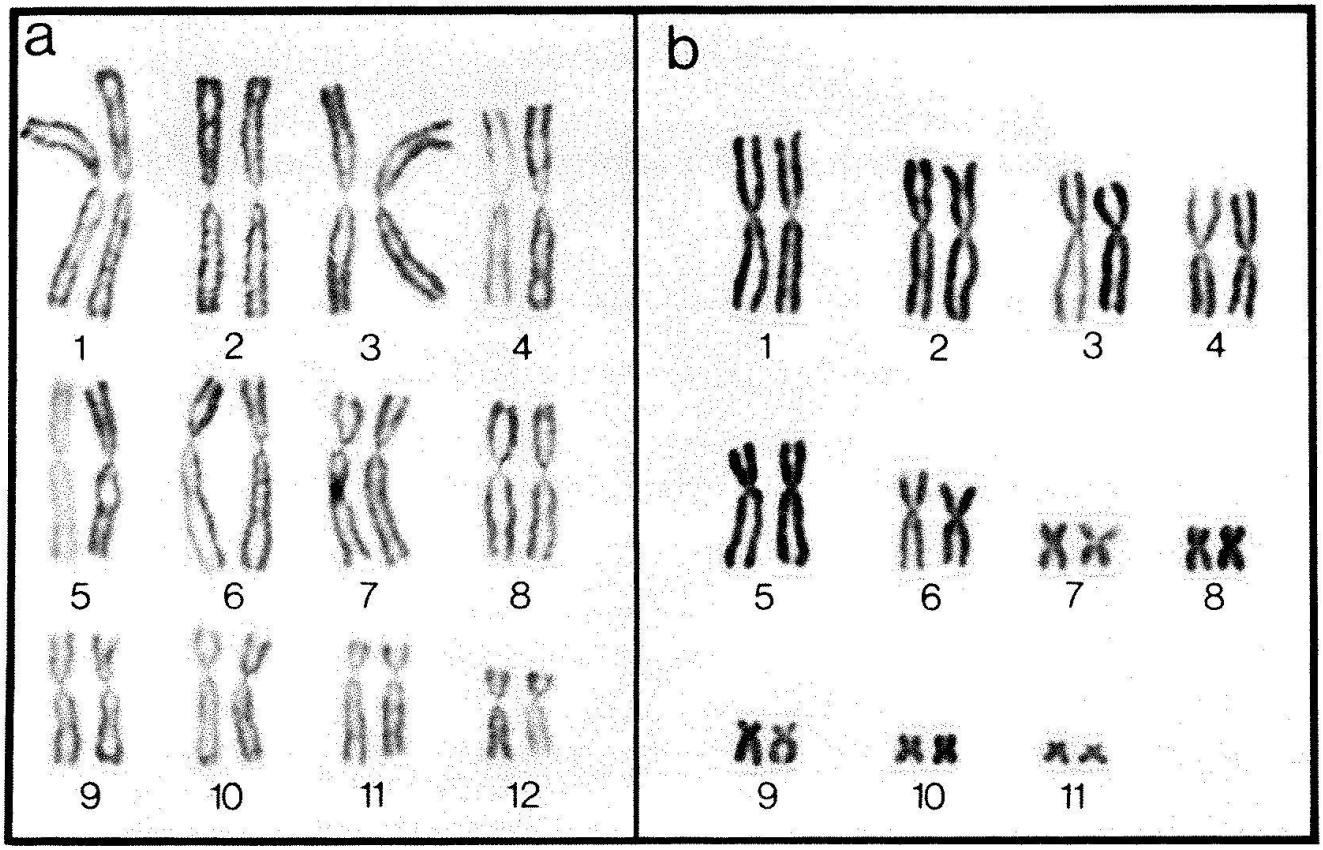


Fig. 1. Conventionally stained chromosomes (aceto-orcein) of highly developed species of Urodela and Anura. (a) Triturus alpestris and (b) Bufo bufo. Note that all chromosomes are meta- or submetacentric. Telocentric chromosomes, whose centromeres are localized at one end of the chromosomes, no longer occur in these species.

submetacentric chromosomes. The evolution of the karyotypes from the primitive genera, which still possess microchromosomes and telocentric chromosomes alongside a few meta- or submetacentric chromosomes, to the more highly evolved genera with exclusively metacentric chromosomes can be explained by centric fusions and tandem translocations taking place between the chromosomes (Morescalchi, 1973). This successive reduction of the chromosome number in the variously highly evolved families of Urodela, Anura and Apoda is described in the following three paragraphs (see also the reviews by Morescalchi, 1971, 1973, 1975, 1979).

Urodela

The order of the recent Urodela is divided into 8 families. The two primitive families Hynobiidae and Cryptobranchidae comprise very generalized species with a relatively high chromosome number ($2n = 40$–64). Microchromosomes (less than 2 μm in length) and telocentric chromosomes are exceptionally numerous. The number of chromosome arms ("nombre fondamental" of Matthey, 1945) was determined to be between 62 and 90. The species of these two families have retained the most primitive karyotypes known in Urodela. Cytogenetically, the family Proteidae constitutes the next higher stage of evolution. The chromosome number of the Proteidae ($2n = 38$, $NF = 64$–68) is clearly intermediate between that of the primitive Hynobiidae and Cryptobranchidae and that of the more highly evolved families of Urodela. The Proteidae are distinguished from the primitive families by the lack of microchromosomes and from the more highly evolved families by the presence of telocentric chromosomes. The species of the highly evolved families Ambystomatidae, Plethodontidae and Amphiumidae possess $2n = 28$ or 26 meta- or submetacentric chromosomes and a $NF = 56$–52. These karyotypes have developed due to the complete loss of microchromosomes (tandem translocations) and the telocentric chromo-

somes (centric fusions). Finally, the chromosome number is yet further reduced in the family Salamandridae (2n = 24, 22; NF = 48, 44). All chromosomes are large, meta- or submetacentric elements (Fig. 1a). Cytogenetically, the Salamandridae must be considered the most highly evolved family of the recent Urodela. The only exceptions to the relationships between number, morphology and chromosome size as described above are the species of the family Sirenidae (Siren intermedia, S. lacertina and Pseudobranchus striatus). Although these, like the primitive families of Urodela, possess a relatively high number of chromosomes (2n = 46, 52, 64), almost all chromosomes are meta- or submetacentric; furthermore, there are no microchromosomes. It is considered probable (see below) that these species constitute tetraploid Urodela, which have originated from one of the highly evolved families (Morescalchi and Olmo, 1974).

Anura

The oldest family of the Anura are the Ascaphidae with only one living species in North America (Ascaphus truei) and three species in New Zealand (Leiopelma). Ascaphus truei is distinguished by a high chromosome number with telocentric chromosomes as well as microchromosomes. Since in this species 2n = 44 and 2n = 46 chromosomes have been determined although the number of chromosome arms is always NF = 56 (Morescalchi, 1967; Bogart, 1970), centric fusions must be regarded responsible for the discrepancy in the chromosome number. The genus Leiopelma is karyologically at a much higher stage of development than Ascaphus. Thus L. hochstetteri possesses 2n = 22 macrochromosomes (10 meta- and 12 telocentric) and between 0 and 12 supernumerary microchromosomes occurring in variable numbers. In L. hamiltoni and L. archeyi only 18 macrochromosomes (16 meta- and 2 telocentric) are found (Morescalchi, 1968; Stephenson et al., 1972; 1974). These examples illustrate, that the reduction of the chromosome number which occurred during the phylogeny from the primitive to the highly evolved families can by all means take place within one family. The ancient family Discoglossidae in considered transitional between the primitive family Ascaphidae and the higher evolved anuran families. The karyological evolution in this family has led to the loss of the microchromosomes. There are only few telocentric chromosomes remaining in some species (Alytes obstetricans, Discoglossus pictus). The chromosome number of the species investigated to date fluctuates between 2n = 24 and 2n = 48, the number of chromosome arms between NF = 48 and 72. The also very ancient family Pipidae comprises very specialized genera (Pipa, Xenopus, Hymenochirus, Pseudhymenochirus). Their karyotypes differ at the intergeneric level (2n = 20–36), with some species showing telocentric chromosomes and microchromosomes. The number of chromosome arms varies between NF = 30 and 72. In one species, Pipa parva, all 30 chromosomes are telocentric; this situation, however, very probably does not reflect the original stage, having instead developed from centric fissions of meta- and submetacentric chromosomes (Morescalchi, 1968). Several tetraploid and hexaploid species were discovered in the genus Xenopus (see below). The karyological heterogeneity in the family Pipidae is thought to be related to the antiquity of this Anura. The species of this family possibly followed a different path of karyological evolution than did the higher Anura. In the relatively ancient family Pelobatidae and the highly evolved families Leptodactylidae, Dendrobatidae, Hylidae, Bufonidae, Microhylidae, Ranidae and Rhacophoridae the chromosome number is with some exceptions reduced to 2n = 26 to 22. The chromosomes are largely meta- and submetacentric, very rarely telocentric (Figs. 1b, 5–8). Microchromosomes are present only in the rarest of cases (see B-chromosome of Rana temporaria, Fig. 8a). In several families, the successive reduction of the chromosome number can still be observed in a number of species. Thus in the family Ranidae there are species with 2n = 26, 24, 22 and even with only 14 chromosomes (Arthroleptis stenodactylus, A. poecilonotus). Cytogenetic analyses on other Ranidae could possibly reveal karyotypes constituting a transition between species with 2n = 22 chromosomes and Arthroleptis with 2n = 14.

In contrast to this process of successive

reduction of the chromosome number, there are also isolated groups among the higher evolved Anura in which telocentric chromosomes develop from meta- or submetacentric chromosomes by way of centric fissions. This process increases the chromosome number. The subfamily Eleutherodactylinae from the very species-rich family Leptodactylidae can serve as an example for this. As in many other highly evolved Anura, the original karyotype in this group is supposed to be $2n = 26$ chromosomes (Morescalchi, 1973; 1979). Whereas the chromosome number was reduced to $2n = 22$, 20 and 18 by way of centric fusions and tandem translocations in some species, it was increased to $2n = 30$, 32, 34 and 36 by way of centric fissions in others. It is interesting to note that the higher chromosome numbers are found in the species from Central- und northern South America, where the Eleutherodactylidae are currently undergoing an exceptionally strong radiation and speciation.

Apoda

Only 12 of the 160 species comprising the order Apoda have been cytogenetically analysed at present (see reviews by Seto and Nussbaum, 1976; Morescalchi, 1979). The chromosome numbers determined to date range between $2n = 20$ and 42 ($NF = 40$–52). As in Urodela and Anura, there also exists a trend in the karyological evolution of Apoda to reduce the number of telocentric chromosomes and microchromosomes. Two species of the primitive family Ichthyophiidae have been studied (Ichthyophis glutinosus and I. orthoplicatus). These have $2n = 42$ chromosomes ($NF = 52$). 10 chromosome pairs are meta- and submetacentric, the others are telocentric or microchromosomes. In the more advanced families Typhlonectidae, Caeciliidae and Dermophiidae, the diploid chromosome number is lower. The number of telocentric chromosomes and microchromosomes is reduced in favor of the large meta- and submetacentric chromosomes. Thus in Caecilia occidentalis and Siphonops paulensis (Caeciliidae) there are only $2n = 24$ ($NF = 48$) and in Chthonerpeton indistinctum (Typhlonectidae) there are only $2n = 20$ ($NF = 40$) chromosomes left.

Polyploidies in Amphibia

The early cytogenetic investigations have already shown polyploidies to be very frequent in Amphibia. The number of observations about polyploid species, populations or single individuals increases rapidly. In no other class of vertebrates are naturally occurring polyploids so frequent as in Amphibia. Polyploidies are only described in a few fish species of the Chondrostei and Teleostei and a few reptiles of the Squamata. In the Urodela, triploidies and tetraploidies can very simply be experimentally induced by subjecting the eggs to temperature or pression shocks. These physical factors prevent the formation of the second polar body. The use of female animals from the newt species Pleurodeles waltlii (Salamandridae) with a marker chromosome showing a pericentric inversion permitted an interpretation of the origin of triploidy and the other types of ploidies (Ferrier and Jaylet, 1978). It was also possible to create triploid animals in the Anura (Xenopus laevis) by means of pression to the fertilized eggs (Tompkins, 1978). It is probable, that a certain number of embryos in the progeny of many Anura and Urodela are polyploid, since such animals have been found whereever large number of offspring were analysed (Fig. 7j).

Only in the Apoda have no polyploidies been observed to date; then again, there have hardly been enough cytogenetic studies performed on them.

The naturally occurring polyploid species or populations of Anura and Urodela discovered hence are listed in the Table 1. The first report of a tetraploid population of Anura was made on the South American species Odontophrynus americanus (Beçak et al., 1966). Investigations on a great number of animals from Brasil, Argentina and Uruguay have established that O. americanus only occurs in the diploid $2n = 22$ or tetraploid $4n = 44$ state (Bogart, 1967; Barrio and Rinaldi de Chieri, 1970; Barrio and Pistol de Rubel, 1972). Observations of the meiotic stages in the tetraploid animals in some cases revealed quadrivalent pairings involving the four supposedly homologous chromosomes (Bogart and Wassermann, 1972; Beçak et al., 1967). In the octoploid species Ceratophrys ornata, it was even pos-

Tab. 1. Polyploid species or populations found in Anura and Urodela.

Order	Family	Species	Diploid	Polyploid	Reference
Anura	Pipidae	Xenopus vestitus	–	4n = 72	1
		Xenopus wittei	–	4n = 72	2
		Xenopus ruwenzoriensis	–	6n = 108	3
	Ceratophrydidae	Ceratophrys ornata	2n = 26	8n = 104	4
		Odontophrynus americanus	2n = 22	4n = 44	5
	Leptodactylidae	Pleurodema bibroni	–	4n = 44	6
		Pleurodema kriegi	–	4n = 44	6
	Hylidae	Hyla versicolor	–	4n = 48	7
		Phyllomedusa burmeisteri	2n = 26	4n = 52	8
	Bufonidae	Bufo danatensis	–	4n = 44	9
		Bufo viridis	2n = 22	4n = 44	10
		Bufo sp.	2n = 20	4n = 40	11
	Ranidae	Rana esculenta	2n = 26	3n = 39	12
		Pyxicephalus delalandii	2n = 26	4n = 52	11
		Dicroglossus occipitalis	2n = 26	4n = 52	11
Urodela	Ambystomatidae	Ambystoma platineum	–	3n = 42	13
		Ambystoma tremblayi	–	3n = 42	13
		Ambystoma texanum x A. laterale	–	3n = 42	14
	Sirenidae	Siren intermedia	–	4n = 46	15
		Siren lacertina	–	4n = 52	15
		Pseudobranchus striatus	–	4n = 64	15

(1) Tymowska et al., 1977. (2) Tymowska and Fischberg, 1980a. (3) Tymowska and Fischberg, 1980b. (4) Barrio and Rinaldi de Chieri, 1970a. (5) Beçak et al., 1966; Bogart and Wassermann, 1972. (6) Barrio and Rinaldi de Chieri, 1970b. (7) Wassermann, 1970. (8) Beçak et al., 1970; Batistic et al., 1975. (9) Pisanetz, 1978. (10) Mazik et al., 1976. (11) Bogart and Tandy, 1976. (12) Günther, 1970, 1975. (13) Uzell, 1963. (14) Downs, 1978. (15) Morescalchi and Olmo, 1974.

sible to demonstrate octovalents, hexavalents, quadrivalents and bivalents. On the other hand, the meiosis of the tetraploid Pleurodema, Xenopus, Bufo and Dicroglossus exclusively revealed bivalents (see references in Table 1). Then again, the hexaploid Xenopus and the tetraploid Pyxicephalus exhibit some few multivalents. Multivalent pairings have generally been considered evidence for chromosomal homology. The question of whether the polyploid species are autopolyploids (evolved by polyploidization from a single species) or allopolyploids (evolved from interspecies hybrids and secondary polyploidization) is comprehensively reviewed by Bogart (1980). It is probable that in the polyploid species of Amphibia a "diploidization-process" similar to the one in polyploid fish species becomes effective, causing a divergence in the development of chromosomes and genes initially identical. This diploidization is propelled by centric fusions, inversions and reciprocal translocations. After the "diploidization-process" is completed, the initial polyploidy can no longer be readily recognized on the basis of the chromosome morphology or the pairing configuration of the meiotic chromosomes. Among the Urodela, there exists the all-female triploid populations of Ambystoma, which reproduces gynogenetically. They require sperm nuclei from a related diploid bisexual species for the activation of the eggs. The 3 species of the urodelan family Sirenidae (Siren intermedia, S. lacertina and Pseudobranchus striatus) also seem to show traces of a tetraploid condition; most of their chromosomes can be arranged in quartets of similar length and morphology (Morescalchi and Olmo, 1974).

Genome sizes in the Amphibia

The polyploidies described in the last section can also be perceived by determining the DNA-content with cytophotometric methods. The nuclear DNA amounts of

twelve species and subspecies of Xenopus, which were determined by Thiébaud and Fischberg (1977), can serve as an example of this. The lowest DNA amount (3.5 picograms DNA/diploid nucleus) was found in X. tropicalis, which possesses only 20 chromosomes. The species with 36 chromosomes has DNA amounts of 6.35–8.45 pg. The DNA value is roughly doubled (12.8 pg and 12.5 pg) in the two tetraploid species X. vestitus and X. wittei with their 72 chromosomes. Finally, the highest DNA content (16.2 pg) was found in the tetraploid species X. ruwenzoriensis. This example shows how the genome can increase through the multiplication of whole sets of chromosomes.

With regard to the genome size, the Amphipia, along with the lung fish (Dipnoi) are distinctly different from the rest of the vertebrates. Whereas in other vertebrates the DNA content per diploid nucleus is around 10 picograms or below, the Amphibia display values considerably above this figure (Fig. 2). In addition to this, there exists in the Amphibia and Dipnoi a very high inter-specific variability of the genome sizes. In Urodela, the highest DNA values were found in species from the families Amphiumidae, Proteidae and Sirenidae (90–189 pg) and the lowest in the families Plethodontidae and Salamandridae (30–86 pg). A common characteristic of the Urodela with the maximum genome sizes seems to be a larval or semi-larval mode of existence. This has led several authors to see a correlation between the genome size and the length of the larval period in Amphibia (Commoner, 1964; Goin et al., 1968). The genome sizes would accordingly have both an "adaptive" function as well as an evolutionary significance (Morescalchi, 1973; 1975). The highest DNA values of the Anura occur in the primitive family Discoglossidae (10–21 pg), the lowest in the Pelobatidae (1.6–8.9 pg). Those species with the extremely low genome sizes (genus Scaphiobus of North America) go through the extraordinarily short embryonic period of only 2 (!) days: this can also serve as a confirmation of the correlation between the genome size and the length of the larval period.

There arises the question of the other factors, besides polyploidization, on which the exceptionally great inter-specific and inter-familial differences of the genome sizes in the Amphibia could be based. It has been shown, mainly by the kinetics of renaturation of DNA, that the genomes of all eucaryotes are comprised of single-copy ("unique") sequences, of intermediately repeated and of highly repeated sequences (Britten and Kohne, 1968). The various species differ from each other mainly in the quantity of the intermediately and highly repeated sequences of DNA. The renaturation kinetics performed with the DNA of some species of Anura and Urodela permit the conclusion that the quantity of intermediately and highly repeated sequences is greater in those species with a larger genome (Britten and Davidson, 1971; Straus, 1971; Morescalchi and Serra, 1974; Mizuno and Macgregor, 1974). Baldari and Amaldi (1976) compared the genomes of two species each of Anura and Urodela with highly varied DNA content: Xenopus laevis (6 pg DNA) with Bufo bufo (14 pg DNA) and Triturus cristatus (46 pg DNA) with Necturus maculosus (104 pg DNA). It turned out that within each subclass the two species analysed possess about the same absolute amount of single-copy sequences. The differences in the total nuclear DNA could be accounted for by the quantitative differences of the repetitive sequences. Furthermore, it could be shown that the great difference in the DNA content between the Anura and Urodela involves all sequence classes in parallel. There is at present no convincing interpretation concerning the function of the sizeable amounts of repetitive DNA in the genomes of the eucaryotes. The mechanism, besides polyploidization, by which the amount of nuclear DNA can increase is a repeated tandem gene duplication (Ohno, 1970). Since these duplications can proceed uniformly within the entire genome, this would alter only the chromosome length, but not the chromosome number.

In favorable instances, the highly repetitive DNA can be separated from the rest of the DNA by equilibrium density gradient centrifugations of the DNA in Ag^{+}-Cs_2SO_4 or CsCl in analytical or preparative ultracentrifuges. Due to the high proportion of AT- or GC-base pairs, the highly repetitive DNA then appears as a light or heavy "satellite", respectively. The satellite DNAs constitute a

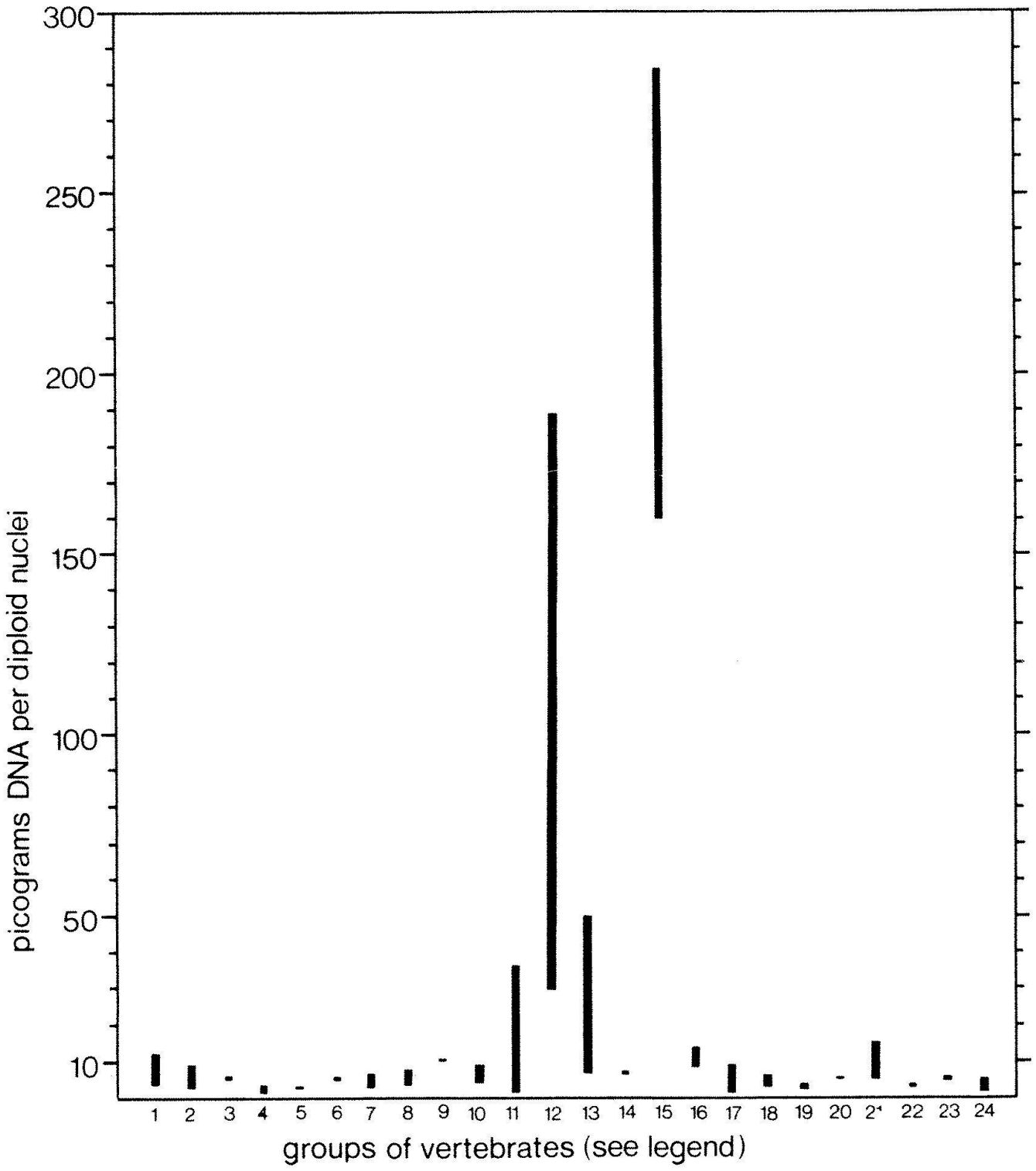


Fig. 2. Minimum and maximum DNA contents in vertebrates. The data are compiled by Morescalchi (1977). Note the extremely high DNA values and interspecific variability in the Dipnoi (15) and the Amphibia (11, 12, 13). Mammalia: (1) Eutheria. (2) Marsupialia. (3) Monotremata. Aves: (4) Carinatae. (5) Ratitae. Reptilia: (6) Crocodilia. (7) Ophidia. (8) Sauria. (9) Rhyncocephalia. (10) Chelonia. Amphibia: (11) Anura. (12) Urodela. (13) Apoda. Osteichthyes: (14) Latimeria. (15) Dipnoi. (16) Polypterini. (17) Teleostei. (18) Chondrostei. (19) Holostei. Chondrichthyes: (20) Batoidea. (21) Selachii. (22) Holocephali. Agnatha: (23) Myxinoidea. (24) Petromyzonta.

readily available and pure fraction of DNA, whose chromosomal location can be made readily visible by the so-called in situ hybridization of radioactive complementary RNA or DNA (for reviews see Gall and Pardue, 1969; Hennig, 1973, 1976). The experiments using in situ hybridization have demonstrated that satellite DNA is located predominantly in the constitutive heterochromatin of the chromosomes (see next section). Thus satellite DNAs were localized in the constitutive heterochromatin in several species of the genus Plethodon by Macgregor (1973) and in Xenopus (Anura, Pipidae) by Pardue (1974).

Banding methods in amphibian cytogenetics

The methods of chromosome analysis newly developed in the past decade on the one hand permit exact studies on the karyological relationships among the species, and on the other hand they produce precise information on the fine structure of the chromosomes. In a few cases, it was even possible to obtain information about the function of specific chromosome regions. An almost complete review of these new methods was written by Bostock and Sumner (1978) and Dutrillaux (1975). These methods were primarily developed on the metaphase chromosomes of mammals and later used with great success on the lower vertebrates as well. A preliminary account of the vertebrate species studied shows that in comparison with the enormous number of publications on mammalian species, there are only few completed studies on amphibians or other lower vertebrates (reptiles and fish) available to date. However, with these modern methods of cytogenetics a great number of data about these organisms will become known in the next years.

DNA-base specific fluorochromes, which produce characteristic transverse bands along the metaphase chromosomes of higher vertebrates are increasingly used to characterize the karyotypes. Each chromosome pair exhibits its own pattern of transverse bands. This permits a more precise identification of chromosomal rearrangements (inversions, translocations, etc.) which took place in the course of karyological evolution than was possible with conventional methods. Such DNA-base specific fluorochromes are, for instance, the AT-specific quinacrine mustard and Hoechst 33258 or the GC-specific mithramycin and chromomycin A_3. Those banding patterns produced by the staining with the AT-specific fluorochromes (Q-bands) are usually exactly the reverse of the patterns produced with the GC-specific fluorochromes (R-bands). The use of these fluorochromes on the chromosomes of the lower vertebrates (amphibians, fish) showed a remarkable difference to the results obtained on the chromosomes of the higher vertebrates. None of the euchromatic chromosome regions of any of the amphibian species examined to date revealed any such multiple banding patterns. The euchromatin mainly fluoresces with a uniform intensity (Fig. 3a–d). All of those chromosome regions found to fluoresce significantly stronger or weaker than the euchromatin (Fig. 3a–d) could, after closer analysis, be identified as constitutive heterochromatin. It was also not possible to induce multiple bands in the euchromatic chromosome regions (G-bands) by enzymatically treating amphibian chromosomes with trypsin (Seabright, 1971) or pronase (Dutrillaux et al., 1971). The lack of such multiple Q-, R- and G-bands in the euchromatic regions of amphibian chromosomes can be partially attributed to the extremely strong spiralisation of the chromosomes in the metaphases. The distances between the individual bands in the euchromatin of the amphibian metaphase chromosomes is probably too slight to be resolved with a light microscope. Thus, the comparison between the chromosomes of man (as a representative of the mammals) and several Anura showed the average DNA-content per µm chromosome length to be a factor of 1.5 to 3 higher in the metaphases of Anura (Schmid, 1978a). It is also possible, however, that those additional factors thought responsible for the occurrence of the banding patterns in higher vertebrates (Comings et al., 1975) are missing on the amphibian chromosomes. Staining the amphibian chromosomes with the various fluorochromes also revealed yet other significant differences to the chromosomes of the higher vertebrates. Thus, in all families of Anura and Urodela examined to date, a remarkably large number of species was found possessing chromosomes with quina-

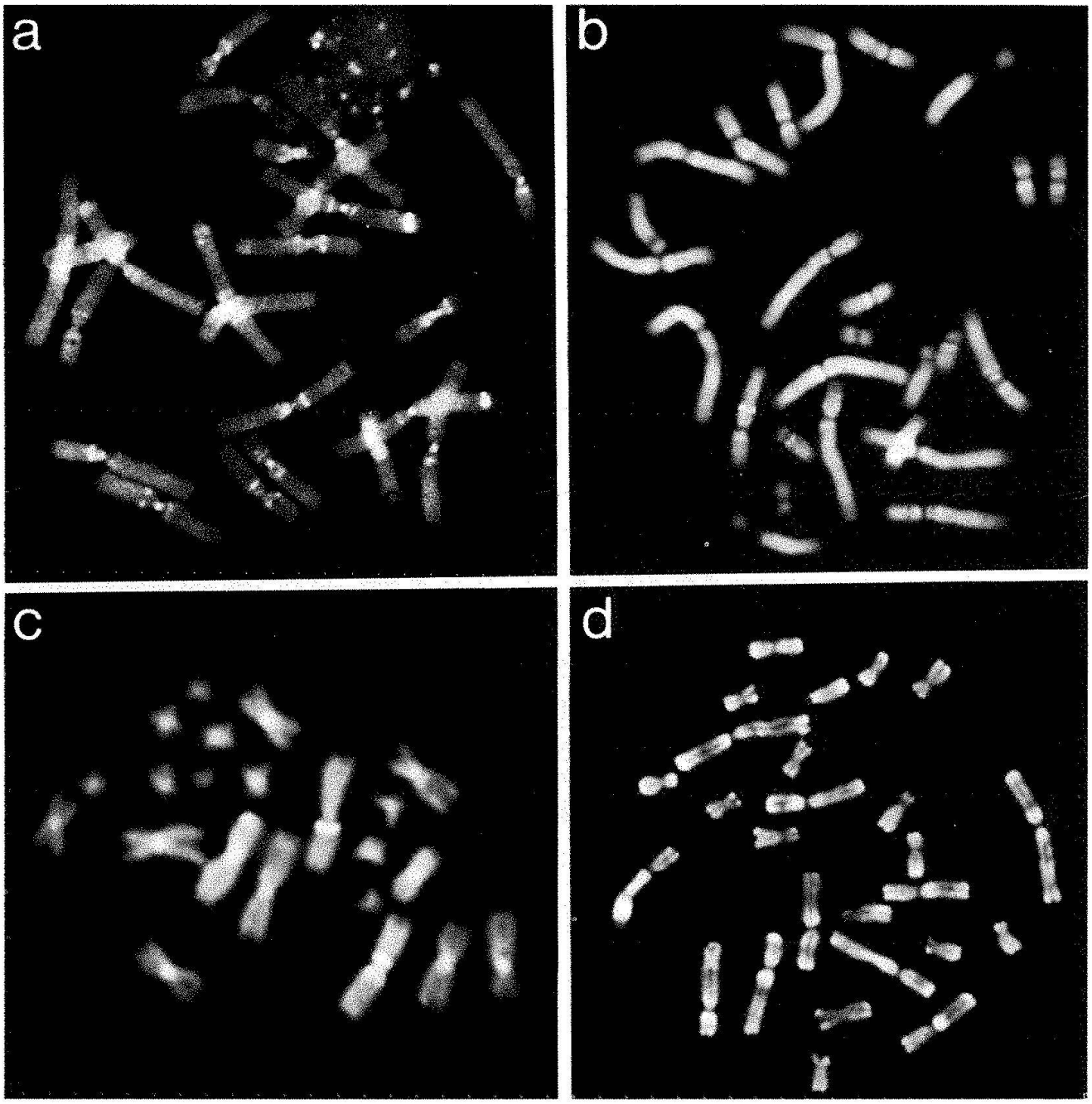


Fig. 3. Metaphases of four amphibian species stained with (a, b) quinacrine mustard and (c, d) mithramycin. (a) Triturus alpestris (Urodela, Salamandridae). (b) Pachymedusa dachnicolor (Anura, Hylidae). (c) Bufo americanus (Anura, Bufonidae). (d) Rana catesbeiana (Anura, Ranidae). Note the very brightly fluorescing AT-rich heterochromatic regions in (a, b) and GC-rich heterochromatic regions in (c, d). The euchromatic chromosome regions fluoresce with uniform intensity by comparison.

crine-bright and consequently AT-rich constitutive heterochromatin (Schmid, 1978a, b, 1980; Schmid et al., 1979). The quinacrine mustard-stained chromosomes of Triturus alpestris (Fig. 3a), Pachymedusa dachnicolor (Fig. 3b) and Bufo americanus (Fig. 4b) are shown as examples for this. Species with AT-rich heterochromatic chromosome regions are very rare in mammals (Jalal et al., 1974). Most of the constitutive heterochromatic chromosome regions of the Amphibia are GC-rich, as the stainings with the GC-specific fluorochromes mithramycin and chromomycin A_3 revealed (Figs. 3c, d; 4c, f). In the karyotypes of all amphibian species investigated, the regions fluorescing the brightest with GC-specific fluorochromes (Fig. 4k) are the nucleolus organizer regions (see below). The nucleolus organizers of mammals do not display any remarkably bright fluorescence

following treatment with these two GC-specific fluorochromes (Schweizer, 1976; Schnedl et al., 1977). It is conceivable that several factors in the Amphibia join to cause the mithramycin- or chromomycin A_3-fluorescence of the nucleolus organizers: the very high number of ribosomal genes (e.g. about 450 repeating units in the nucleolus organizers of Xenopus laevis; Brown and Dawid, 1968), the higher spiralization of the chromatin and a very high fraction of GC-base pairs in the sequences separating the ribosomal genes from each other ("spacer sequences": about 73% GC-content in X. laevis; Brown et al., 1972).

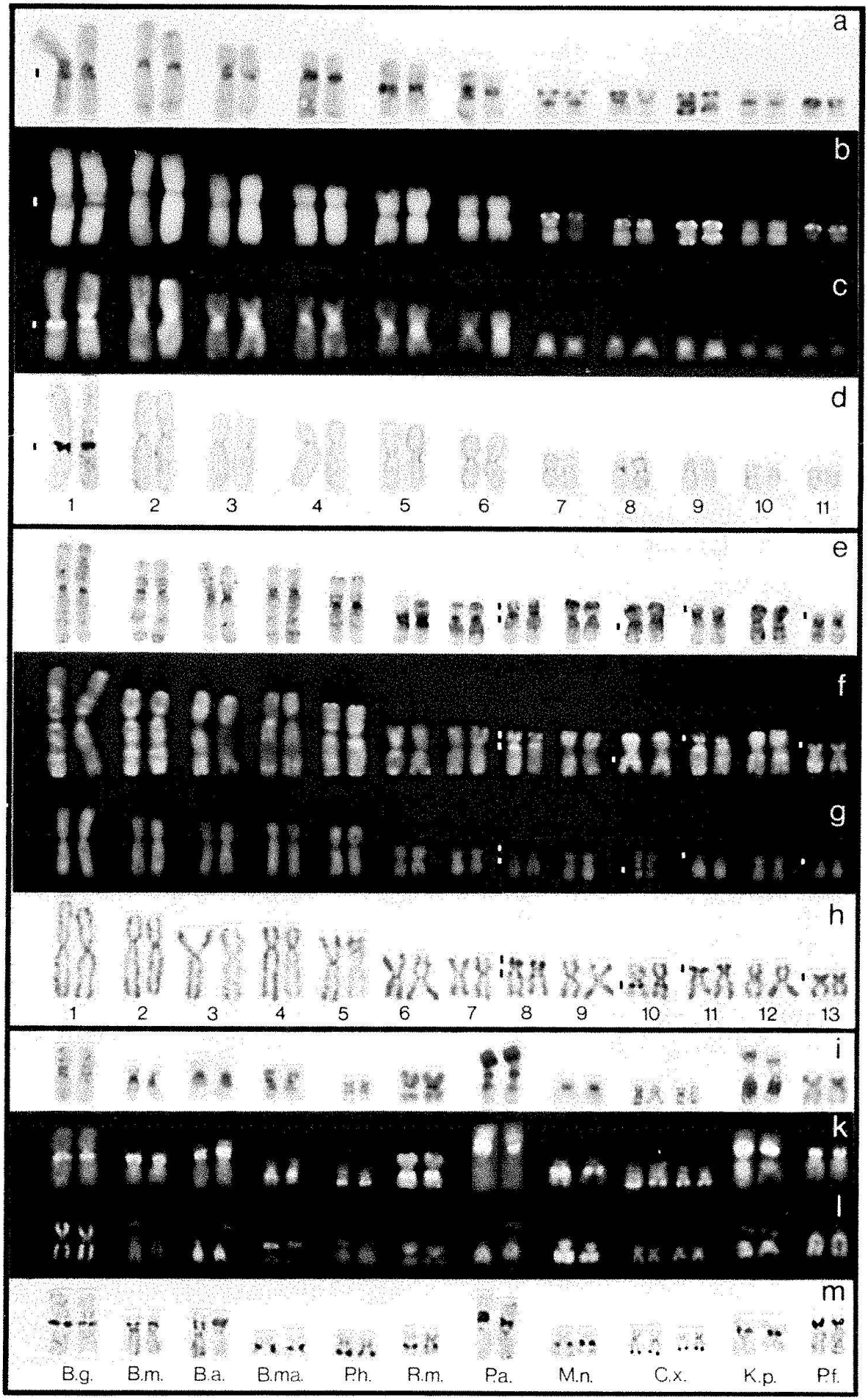
The most customary method for staining all heterochromatic chromosome regions, independent of their AT- or GC-content, is probably the denaturation-reassociation-technique (C-band-technique; see Bostock and Sumner, 1978). Since this staining is done with common Giemsa, the chromosomes can be analysed under the bright-field microscope (Fig. 4a, e, i). The number heterochromatic regions on the amphibian chromosomes is, compared with the chromosomes of other vertebrates, relatively high (see below). Since in addition the diploid chromosome number of Amphibia rarely exceeds the value of $2n = 30$ in most species, the chromosomes can be fairly well identified with these C-bands. The comparison between the C-bands and the banding patterns induced with AT- and GC-specific fluorochromes permits definitive statements about the distribution of the constitutive heterochromatin as well as its chemical composition (Schmid, 1980a). A summary of the staining reactions of the constitutive heterochromatin with the different banding methods and the base compositions deduced therefrom is given in table 2.

The $AgNO_3$-staining method developed five years ago (Goodpasture and Bloom, 1976) serves to specifically demonstrate the nucleolus organizer regions in the interphase cell nucleus and on the metaphase chromosomes (Fig. 4d, h, m). The term nucleolus organizer region denotes that position of the genes responsible for the formation of nucleoli in the interphase nucleus (synthesis of ribosomal 18s and 28s RNA). These nucleoli are occasionally apparent as secondary constrictions in the chromosomes. $AgNO_3$-staining does not label the ribosomal genes (rDNA) itself, but rather ribonucleoproteins, which accumulate at the nucleoli of the interphase cell nuclei and in the nucleolus organizer regions of the metaphase chromosomes which had been active in the preceding interphase (Schwarzacher et al., 1978). Homologous chromosomes rather frequently have considerable differences in the size of their silver-stained nucleolus organizers (Fig. 4d, h, m). The position of the nucleolus organizers and their number in the karyotypes of Amphibia will be discussed further on in the paper.

Tab. 2. Summary of the differential stainability of the various chromatin categories in 26 species of Anura investigated and the DNA base compositions deduced therefrom.

Chromatin class	Differential stainability*)						Presumed DNA base composition
	C-bands (Giemsa)	Fluorochromes AT-specific DAPI	Hoechst 33258	Quinacrine mustard (Q-bands)	GC-specific Mithramycin	Chromomycin A_3	
Euchromatin	−	−	−	−	−	−	
Heterochromatin	+	+	+	+	−	−	AT-rich
	+	−	−	−	+	+	GC-rich
	+−	−	−	−	+	+	GC-rich
	+	−	−	−	−	−	AT + GC-rich (?)
Nucleolus organizer regions	+ or −	−	−	−	++	++	GC-rich

*) Differential stainability implies a positive reaction of the chromatin of the metaphase chromosomes. The euchromatin shows a uniform and weak reaction to all stains (−). Some C-bands show a very weak reaction to the denaturation-reassociation technique; this class is designated with +−. These data were taken from Schmid (1980a).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B.g. B.m. B.a. B.ma. Ph. R.m. Pa. M.n. C.x. K.p. Pf.

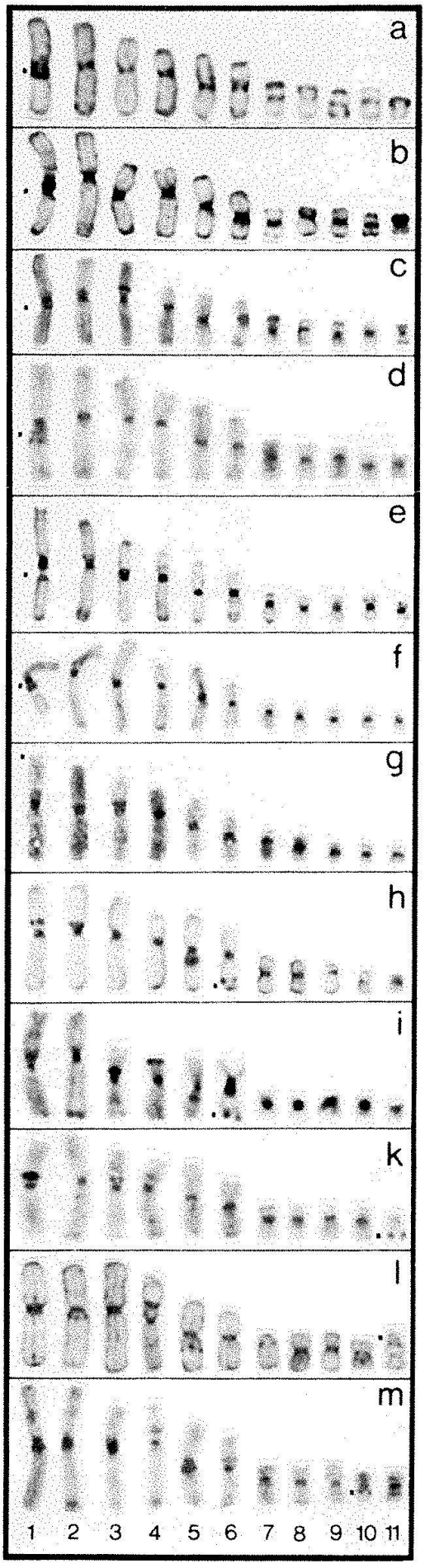
Constitutive heterochromatin

There are several characteristic features of the constitutive heterochromatin of the eucaryotic chromosomes: late DNA replication in the S-phase, condensed state in interphase nuclei, very large amounts of repetitive DNA sequences, differential staining with banding techniques, enhanced frequency of spontaneous or induced breakages, low crossover frequency in meiosis and genetic inertness. However, it is established that no class of constitutive heterochromatin possesses all of these features. It can therefore not be excluded that some of the heterochromatic chromosomal regions in the amphibian species studied to date were not revealed by the banding techniques used. A summary of the differential staining of the various chromatin classes with the different staining techniques employed is shown in table 2.

The amphibian chromosomes differ from most karyotypes in the other classes of vertebrates in the high frequency of constitutively heterochromatic regions. On most of the chromosomes analyzed the number of heterochromatic bands (C-bands) is largely independent of the length or the DNA-content of the chromosomes (Schmid, 1978a, b). The small chromosomes can contain just as many or even more heterochromatic regions as the largest chromosomes of the karyotypes (Fig. 5). This holds true for the highly evolved as well as for the primitive groups of Amphibia. Three heterochromatic regions can almost always be discerned on the chromosomes of the Anura: the centric region and the two telomeres. There are in addition occasionally 1–3 interstitial heterochromatic bands. The nucleolus organizer regions are always associated with the constitutive heterochromatin.

The interspecies-comparisons of the chromosomal position of the constitutive heterochromatin are of great importance. It has been shown, that although many species within each genus have a uniform basic banding pattern, there are always strong deviations from it in individual species (Schmid, 1978a, b; King, 1980). Thus, for instance although the chromosome morphology between the species from the genus Bufo with its $2n = 22$ chromosomes is very similar in general, the occurrence of large, brightly fluorescing heterochromatic regions (Fig. 4b) is restricted entirely to the chromosomes of the North American species. Xenopus laevis and X. muelleri also exhibit few differences with regard to chromosome morphology, yet only X. muelleri possesses extraordinarily large AT-rich heterochromatic regions (Pardue, 1974). Rana erythrea (Fig. 5u) can be mentioned as a further example, possessing significantly more interstitial heterochromatin than any of the other Rana species (Fig. 5 o–u). One of the more unusual features of Australian Hylidae of the genus Litoria is the shared similarity in gross chromosome morphology between the species, contrasted to the extensive C-banding variation (King, 1980). These examples were chosen to demonstrate that the heterochromatic patterns on the chromosomes of an amphibian species can change very rapidly. A connection between the heterochromatic pattern on the chromosomes and any evolutionary parameters could, except for the heteromorphic sex chromosomes (see below), not be established. It is absolutely possible that the distribution and amount of constitutive heterochromatin are very variable properties, which have significantly contributed to the chromosomal isolation mechanisms in evolution.

Fig. 4. Comparison of various banding methods performed on the chromosomes of several species of Anura. (a–d) Diploid karyotypes of Bufo americanus. (e–h) Diploid karyotypes of Rana erythrea. (i–m) Chromosome pairs of 11 anuran species on whom the nucleolus organizer regions are stained. Bufonidae: (B.g.) Bufo garmani. (B.m.) B. marinus. (B.a.) Bufo arenarum. (B.ma.) B. mauritanicus. (P.h.) Pedostibes hosii. Ranidae: (R.m.) Rana macrodon. (P.a.) Pyxicephalus adspersus. Rhacophoridae: (C.x.) Chiromantis xerampelina. Microhylidae: (K.p.) Kaloula pulchra. Pelobatidae: (P.f.) Pelobates fuscus.
(a, e, i) C-bands after the denaturation-reassociation-method. (b, g, l) Q-bands after direct staining with quinacrine mustard; note the brightly fluorescing (AT-rich) heterochromatic regions. (c, f, k) Direct staining with mithramycin; note the brightly fluorescing (GC-rich) heterochromatic regions. Compare the opposite banding patterns of the quinacrine- and mithramycin-stained heterochromatic bands. (d, h, m) $AgNO_3$-staining for the specific labeling of the nucleolus organizer regions. Note that the nucleolus organizer regions which stained strongly with Ag show an extremely bright fluorescence with mithramycin (c, f, k). In (a–h) the nucleolus organizers are marked by dots to the left of the corresponding chromosomes.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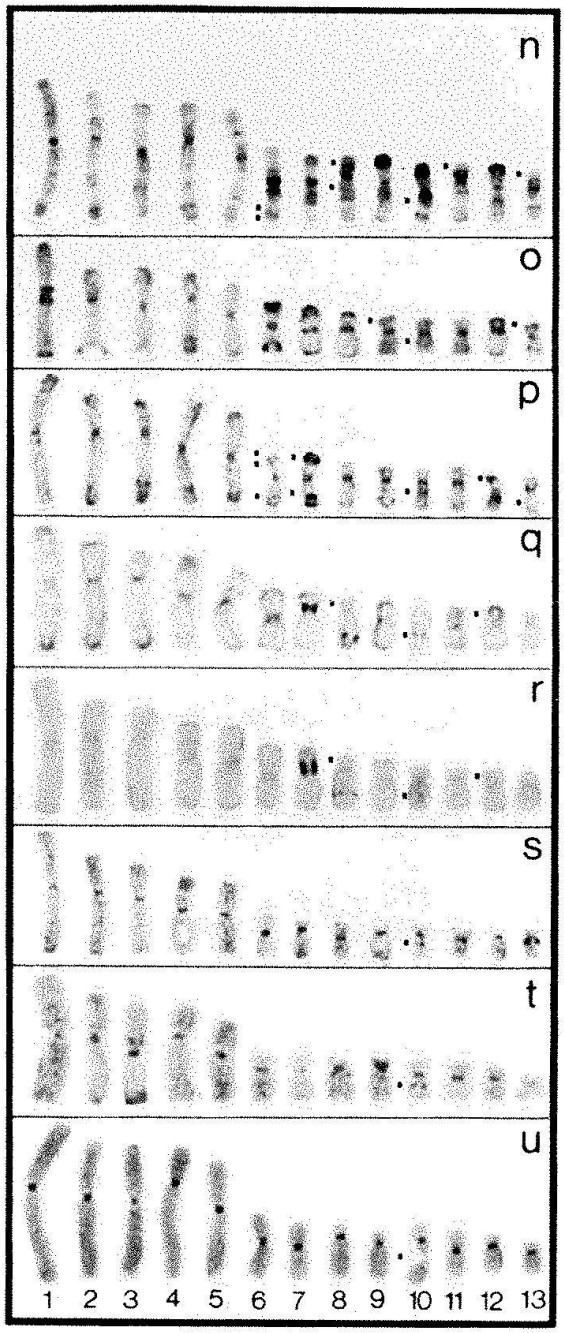
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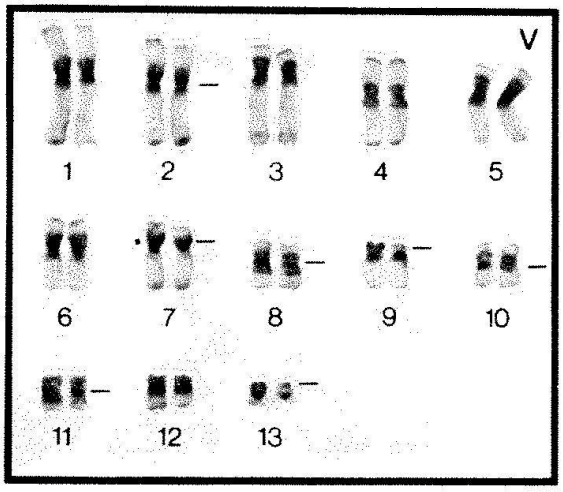
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Considerable differences can be found between the heterochromatic regions of homologous chromosomes in individual animals (Schmid, 1978a, b). This intra-individual variability is a general property of constitutive heterochromatin and is also found in all other vertebrate classes. It is attributed to unequal cross-over between the heterochromatic regions in meiosis. Fig. 5v shows several such variable heterochromatic regions in the karyotype of Pachymedusa dachnicolor. If, however, these heteromorphisms are restricted to one and the same chromosome pair in only one sex of a certain species, they may also be heteromorphic sex chromosomes (see below).

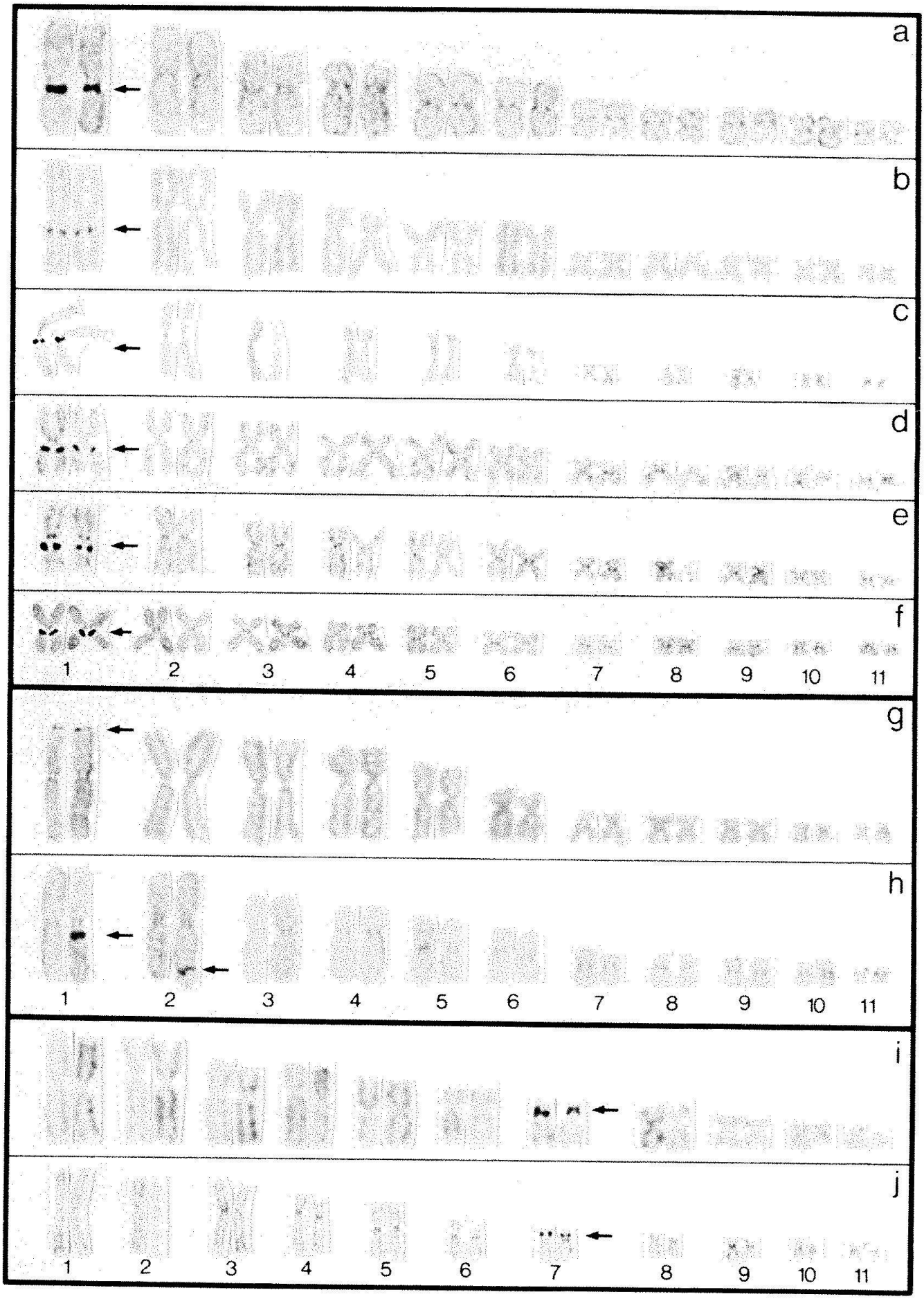
As already described for the chromosomes of other vertebrates, the constitutive heterochromatin in Amphibia is extremely heterogeneous. Thus, a denaturation in alkali for different lengths of time can be used for demonstrating specific heterochromatic regions, as is shown in the example of Rana catesbeiana (Fig. 5q, r).

The analysis of urodelan chromosomes with the C-band technique and fluorochromes has hitherto been limited almost entirely to species of the family Salamandridae (genus Triturus) (Mancino et al., 1977; Ragghianti et al., 1978; Schmid and Krone, 1976; Schmid et al., 1979). In these species, the heterochromatic regions are primarily concentrated at the centric, para-centric and interstitial portions of the chromosomes (Fig. 9c–f). On the other hand, important experiments are being at this time performed on mitotic chromosomes and lampbrush chromosomes of the female meiosis of Triturus with the in situ hybridization technique of intermediately and highly repetitive DNA. This permits the precise determination of the chromosomal position (highly repetitive DNA is preferentially localised in the constitutive heterochromatin) of these DNA sequences (Macgregor and Andrews, 1977; Macgregor, 1979).

Nucleolus organizer regions (NORs)

The $AgNO_3$, staining method permits a quick and easy localization of the chromosomal NORs and thus opens a new way for interspecies comparison. It is feasible that karyotypes with one single homologous pair of NORs are more primitive than those, in which the NORs are scattered over several chromosomes (Hsu et al., 1975). Accordingly, the number of NORs has remained constant in the anuran family of Bufonidae despite multiple speciation (Fig. 6, 7). Although the NORs have reached the most chromosome pairs as whole packages by means of translocations (Fig. 6h) and inversions (Fig. 6g), they were not distributed in the genome as smaller units. In the karyotypes of species belonging to the same or closely related species-groups, the silver-stained NORs are always localized in the same chromosome regions. In North American toads (Fig. 6a–f) the pericentric region on the largest chromosome appears to be preferred as NOR. One single pericentric inversion on the same chromosome seems to have shifted the NORs in a terminal position (Fig. 6g). In contrast, the NORs in the South American toads are localized in the short arms of the chromosomes 7 (Fig. 6i, j), and are situated in different chromosomes in the Eurasian species (Fig. 7a–e). The NOR-carrying chromosomes No. 6 in those African toads with $2n = 20$ chromosomes (Fig. 7g–i) could have originat-

Fig. 5. The distribution of the constitutive heterochromatin (C-bands) on the chromosomes of various species of (a–m) Bufonidae, (n–u) Ranidae and (v) Hylidae. (a–u) Haploid karyotypes and (v) diploid karyotype. (a) Bufo americanus. (b) B. fowleri. (c) B. terrestris. (d) B. valliceps. (e) B. compactilis. (f) B. punctatus. (g) B. boreas. (h) B. bufo. (i) B. viridis. (k) B. calamita. (l) B. parvus. (m) B. mauritanicus. (n) Rana erythrea. (o) R. sphenocephala. (p) R. palustris. (q, r) R. catesbeiana. (s) R. ridibunda. (t) R. temporaria. (u) R. esculenta and (v) Pachymedusa dachnicolor. The points on the left side of the chromosomes mark the position of the nucleolus organizer regions (compare with Figs. 6–8). Note the frequent localization of the constitutive heterochromatin in the centric regions and telomeres of the chromosomes. The nucleolus organizers are always associated with heterochromatin. (q, r) Demonstrates the different sensitivity of individual C-bands by prolonged pretreatment with alkali in Rana catesbeiana: (q) 5 min denaturation of the chromosomes in $Ba(OH)_2$ results in a maximum number of C-bands; (r) 20 min denaturation leaves only the C-bands in chromosomes No. 7 and 8 remaining. (v) In the diploid karyotype of Pachymedusa dachnicolor those heterochromatic regions with variations between the homologous chromosomes are indicated by arrows.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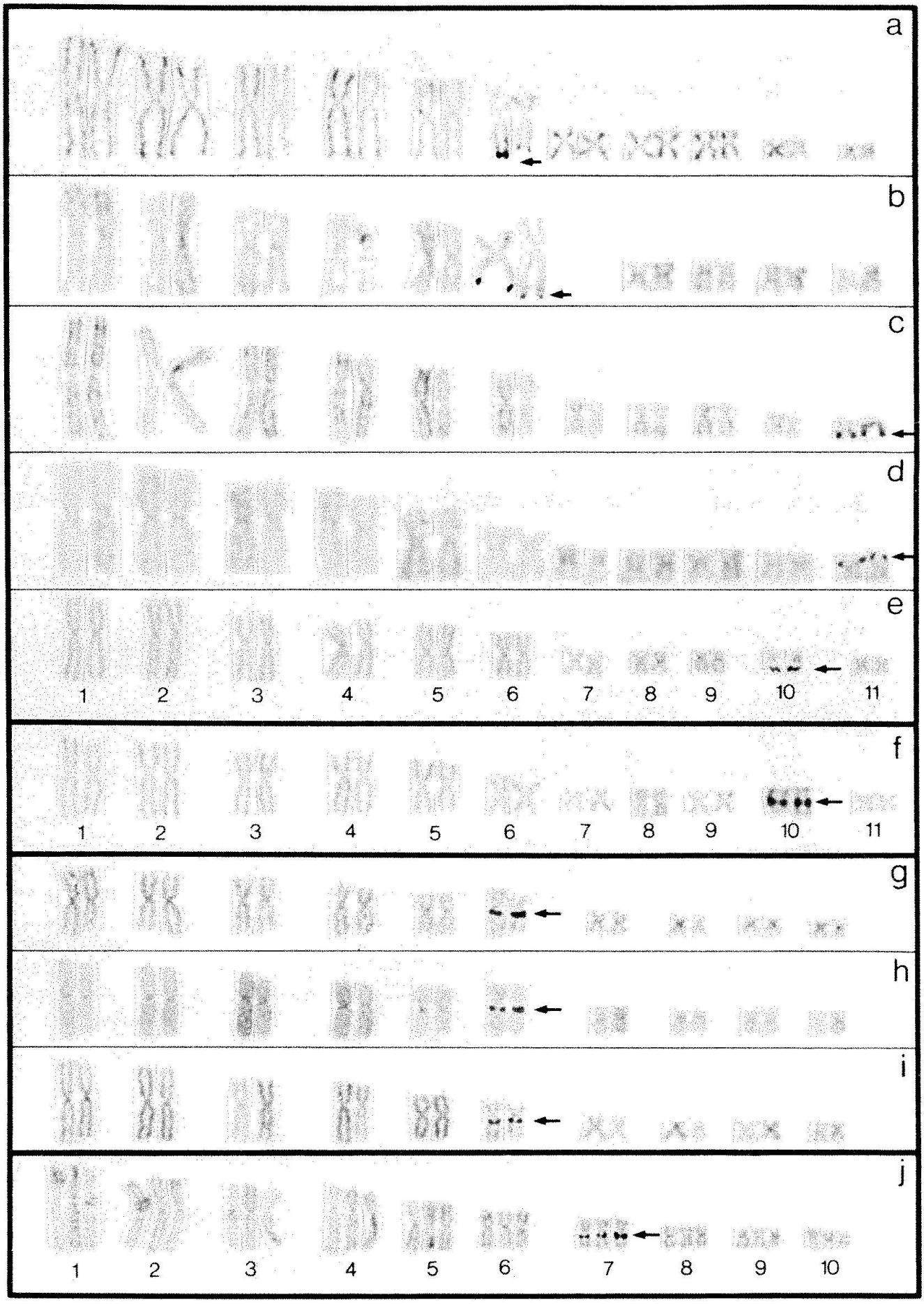
ed from the African species with $2n = 22$ chromosomes (Fig. 7f) by way of a fusion of the two small chromosomes, one of which contained the NOR. The genus Rana shows that other situations can also exist in the distribution of the NORs: most of the frogs of this genus have 26 metacentric and submetacentric chromosomes with 5 larger and 8 smaller pairs. They all have a pair of large NORs in the long arm of chromosome No. 10 (Fig. 8a–g). This position of the NORs would accordingly have remained preserved during evolution. Surprisingly, however some species have between 2 and 5 pairs of additional small NORs on the various smaller chromosome pairs (Fig. 8d–g). Since the morphology of the chromosomes remained very constant during the evolution of the Rana-karyotypes and since the large "standard NORs" in the chromosome 10 were preserved, one can duly ask in what way the additional NORs could have come to be in so many different positions in the karyotypes. Translocations and inversions could hardly have been responsible, as these would have altered the karyotypes of most species in the most various manners. One possible explanation is the mechanism of amplification of ribosomal cistrons in the Amphibia. It is known that during oogenesis the 18s and 28s rDNA is selectively amplified and used for the extrachromosomal nucleoli in the oocyte. There also exists the possibility, although as yet without experimental evidence, that these extrachromosomal rDNA might become reintegrated at various additional sites on the chromosomes (Buongiorno-Nardelli et al., 1972; Hourcade et al., 1974). This reintegration does not alter the morphology of the chromosomes. These examples show that a careful analysis of the localization of NORs can pose new questions about the evolution of amphibian karyotypes.

Sex chromosomes in Amphibia

Although numerous careful cytogenetic studies were performed with conventional cytogenetic methods (for reviews see Morescalchi, 1973; Singh, 1974) and with modern techniques, heteromorphic sex chromosomes could only be determined in very few species. These were all discovered in the last two years. It can be said in advance, that no uniform type of sex determination exists for either Anura or Urodela. Both orders exhibit the XX/XY-type as well as the ZZ/ZW-type of chromosomal sex determination.

In the Urodela, sex-specific chromosomes were determined in the family Salamandridae. Thus, in Triturus alpestris and T. vulgaris (Fig. 9c–f) the male animals have one heteromorphic chromosome pair, of which only one homologue displays heterochromatic telomeres in the long arms; the telomeres of the other homologue are euchromatic. In the female animals, this chromosome pair is found to be homomorphic and contains no telomeric heterochromatin (Fig. 9c–f). The cross-over frequency between the heteromorphic chromosome arms in the male meiosis of these species is highly reduced. In the male meiosis of Triturus vulgaris, the heteromorphic chromosomes No. 5 form bivalents with chiasmata exclusively in the short arms (Fig. 10a, b). After the chiasmata terminalize in the late diakinesis stage, the short arms of chromosomes No. 5 are always paired end-to-end, whereas the heteromorphic long arms point into opposite directions. During the meiosis of Triturus alpestris, the heteromorphic chromosomes 4 form bivalents with chiasmata in the long and short arms (Fig. 10e, f), as well as bivalents with the chiasmata exclusively in the short arms (Fig. 10c, d). Whenever chiasmata are present on both chromosome arms, the paired

Fig. 6. $AgNO_3$-stained nucleolus organizers (see arrows) in the diploid karyotypes of (a) Bufo americanus, (b) B. fowleri (c) B. terrestris, (d) B. valliceps, (e) B. compactilis and (f) B. punctatus. All Bufo-species in (a–f) have their nucleolus organizers in the proximal portion of the long arms of the chromosomes No. 1. By way of a pericentric inversion, these nucleolus organizers can reach the telomeres of the short arms, as is the case in (g) Bufo boreas. Row (h) shows an exceptional karyotype of a Bufo americanus in which a reciprocal translocation had ocurred between one chromosome No. 1 and 2: this places one of the nucleolus organizers onto a different chromosome. The karyotype of this animal was also examined by C-banding, which confirmed the reciprocal translocation. All Bufo-species in (a–h) are from North America. The two species in (i) Bufo arenarum and (j) B. marinus on the other hand are from South America; their nucleolus organizers are localized in the short arms of the chromosomes No. 7.



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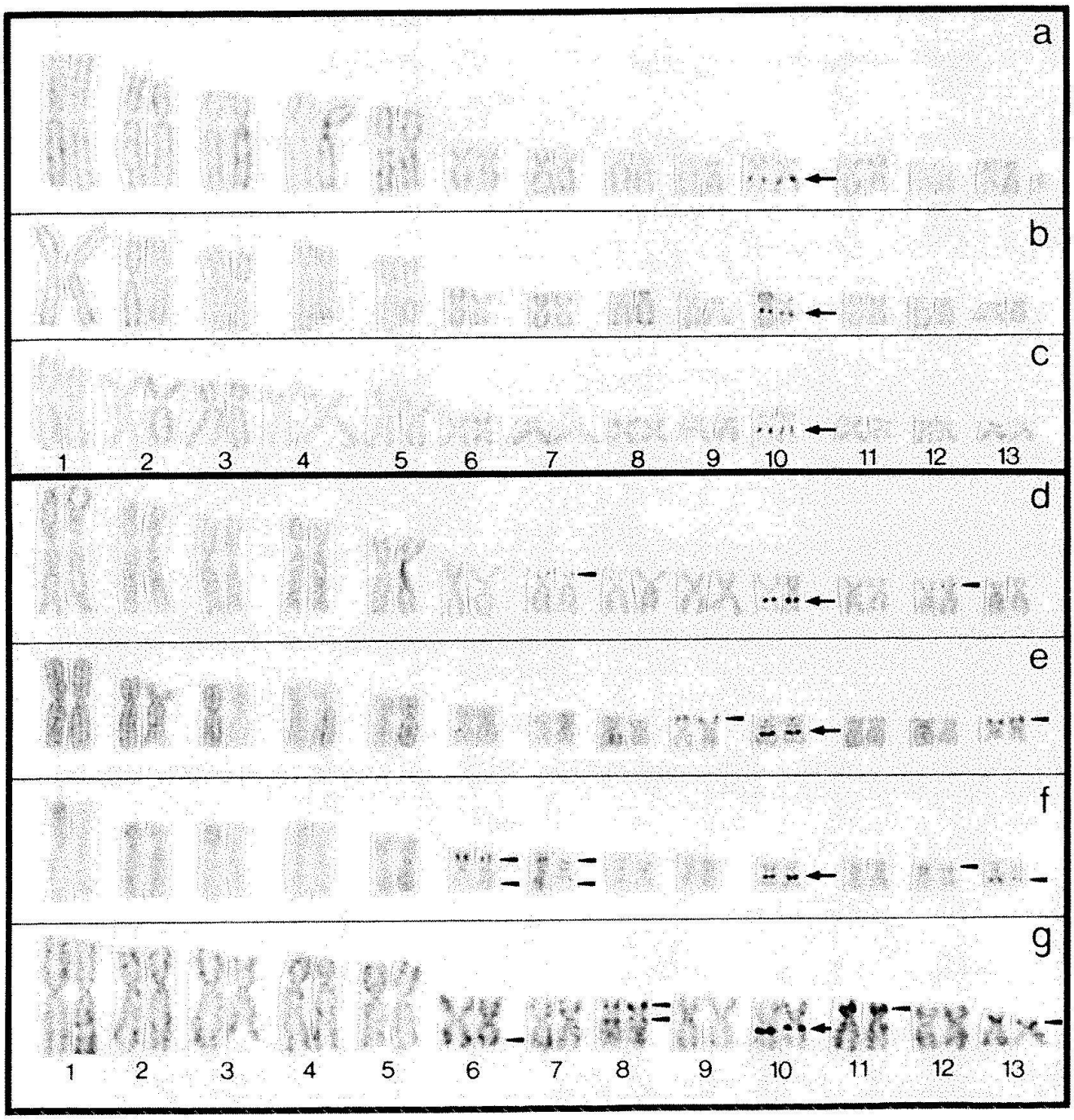
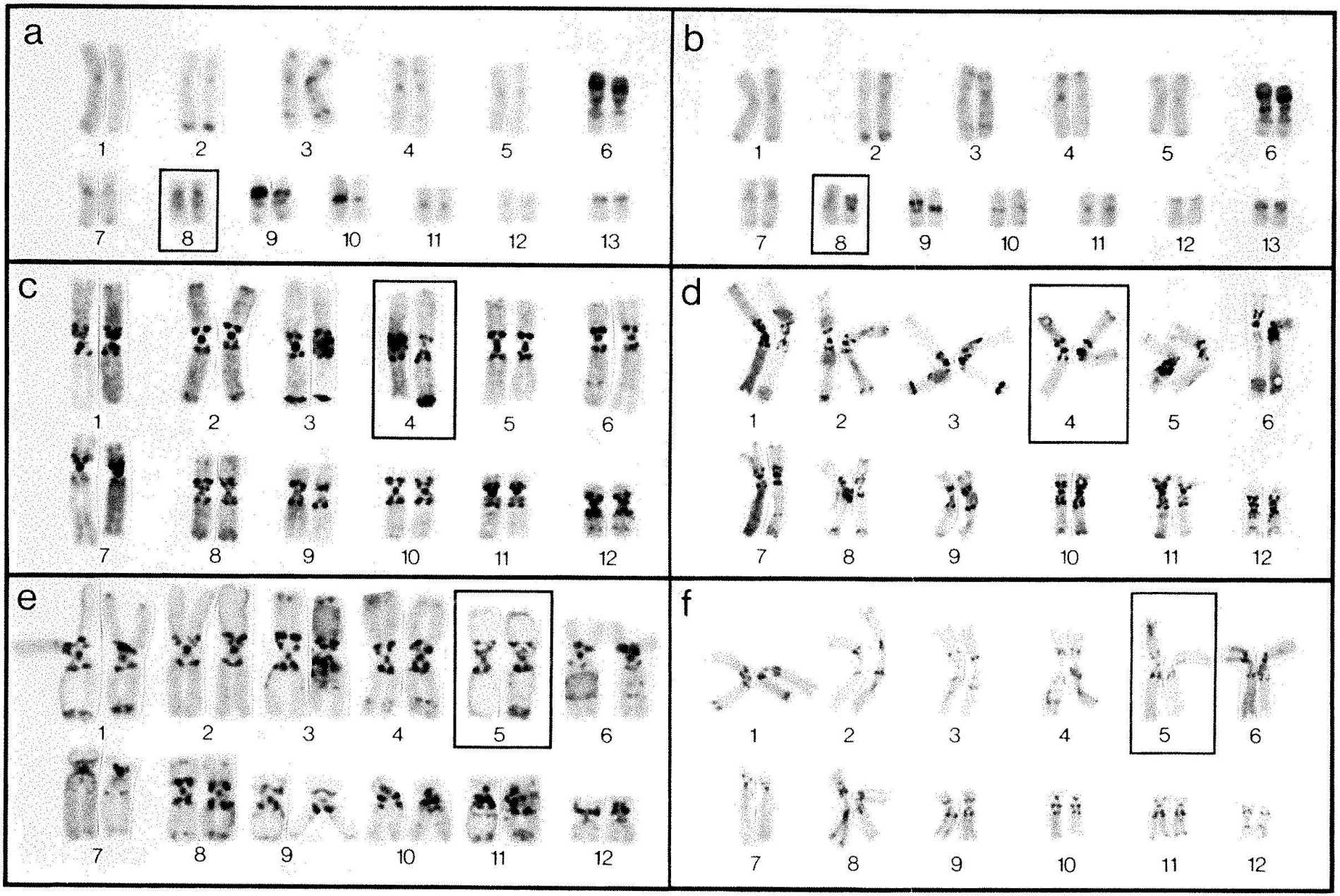


Fig. 8. $AgNO_3$-stained nucleolus organizers (see arrows) in the diploid karyotypes of (a) Rana temporaria, (b) R.esculenta, (c) R.ridibunda, (d) R.catesbeiana, (e) R.sphenocephala, (f) R.palustris and (g) R.erythrea. The species (a–c) are indigenous to Central Europe, the species (d–f) to North America and (g) to Southeast Asia. All species have a large nucleolus organizer in the long arm of chromosome No. 10. In addition to this, this species in (d–display small nucleolus organizers in several chromosome pairs. Also note the supernumerary B-chromosome in the karyotype of (a) Rana temporaria.

Fig. 7. $AgNO_3$-stained nucleolus organizers (see arrows) in the diploid karyotypes of (a) Bufo viridis, (b) B.bufo, (c) B.calamita, (d) B.parvus and (e) Pedostibes hosii. These species are indigenous to the Eurasian region. They each possess one pair of nucleolus organizers. (f) Bufo mauritanicus is an African species possessing, like the Bufo-species in America, Europe and Asia $2n = 22$ chromosomes. The African species (g) Bufo garmani, (h) B.poweri and (i) B.regularis on the other hand only have $2n = 20$ chromosomes. In all three species, the nucleolus organizers are localized in the long arms of the chromosomes No. 6. Row (j) shows an exceptional triploid karyotype found in an animal classified as Bufo poweri (?). Note that the nucleolus organizers in this animal are localized in chromosome No. 7.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chromosomes 4 form ring-like bivalents after the chiasmata have terminalized (Fig. 10f). Whenever the chiasmata are located exclusively on the short arms, however, end-to-end paired bivalents, whose heteromorphic regions point into opposite directions, must develop (Fig. 10d). The homology between the long arms of the sex-specific chromosomes No. 4 of Triturus alpestris would therefore have been largely preserved. The homology of the long arms of the sex-specific chromosomes No. 5 of Triturus vulgaris, on the other hand, would be completely lost. The differentiation of the long chromosome arms has not influenced the homology between the short arms of the sex-specific chromosomes of these species. The C-band patterns and the pairing configurations of the sex-specific chromosomes in the male meiosis indicate that these urodelan species possess an XX/XY-type of sex determination. A review of the literature on experimental species hybrids, on the gonadic structure of haploid and polyploid animals and on sex-linked genes provided further evidence in favour of male heterogamety in Triturus (Schmid et al., 1979). Another case of XX/XY-type of sex determination was found in the family Proteidae: in the neotenic, perennibranch species Necturus maculosus, Sessions (1980) was able to demonstrate highly differentiated sex chromosomes in the males. The X and Y chromosomes differ greatly in size and morphology, the X being one of the largest biarmed chromosomes in the karyotype, the Y on the contrary being diminutive and composed almost entirely of constitutive heterochromatin. In the family Plethodontidae, certain neotropical bolitoglossines (Thorius, Oedipina and Chiropterotriton) have heteromorphic XX/XY-sex chromosomes (Léon and Kezer, 1978). Contrasting with this situation, there is a ZZ/ZW-type of sex determination without heteromorphic somatic sex chromosomes in Pleurodeles waltlii (Salamandridae) and Ambystoma mexicanum (Ambystomatidae), as shown by the analysis of the sex of the progeny of sex-reversed females (Gallien, 1954; Humphrey, 1945).

In the Anura, the cytogenetic studies on the occurrence of heteromorphic sex chromosomes are marked by conflicting results. Although sex chromosomes were described in a total of 15 anuran species in the studies performed with the conventional techniques, none of these findings could stand up to a detailed re-examination with the improved methods (Singh, 1974; Schmid, 1980b). Non-cytogenetic methods showed that there is no uniform type of genetic sex determination in Anura. Thus, in Xenopus laevis, female heterogamety was clearly determined by analysing the sex of the progeny of sex-reversed females (Chang and Witschi, 1955). According to the study of Ponse (1942) on hermaphrodite animals, the females of Bufo bufo also seem to be heterogametic. On the other hand, investigations of the sex of parthogenetically bred frogs and sex-reversal experiments in Rana and Bombina have always shown the male sex to be heterogametic (Kawamura and Nishioka, 1977). The only certified heteromorphic sex chromosomes in Anura to date were found in the South African frog Pyxicephalus adspersus (Ranidae) (Fig. 10a, b). All of the male animals of this species hitherto examined had ZZ sex chromosomes and all female animals had ZW sex chromosomes (Schmid, 1980). The W chromosome is considerably smaller than the Z chromosome and consists to a great portion of constitutive heterochromatin. The fact that of the heteromorphic sex chromosomes found in Amphibia to date it is always the Y- or W sex chromosome and never the X- or Z sex chromosome which constitutes the derived chromosome (with an increased amount of constitutive heterochromatin) indicates that they went through the

Fig. 9. Examples of heteromorphic sex chromosomes in Amphibia. The karyotypes show the pattern of the constitutive heterochromatin (C-bands). Male karyotypes are displayed in the left half, female karyotypes on the right. The sex chromosomes are framed. (a, b) Pyxicephalus adspersus from South Africa. (c, d) Triturus alpestris and (e, f) Triturus vulgaris from Central Europe. Note in (b) the highly heteromorphic sex chromosomes. No. 8 in the female sex of Pyxicephalus adspersus (ZW sex chromosomes). In (c) Triturus alpestris and (e) T. vulgaris the heteromorphism of the sex chromosomes is restricted to a small region of the telomeres in the long arms of the chromosomes No. 4 and No. 5, respectively (XY sex chromosomes). This small heteromorphism does not exist in the female karyotypes (d, f).

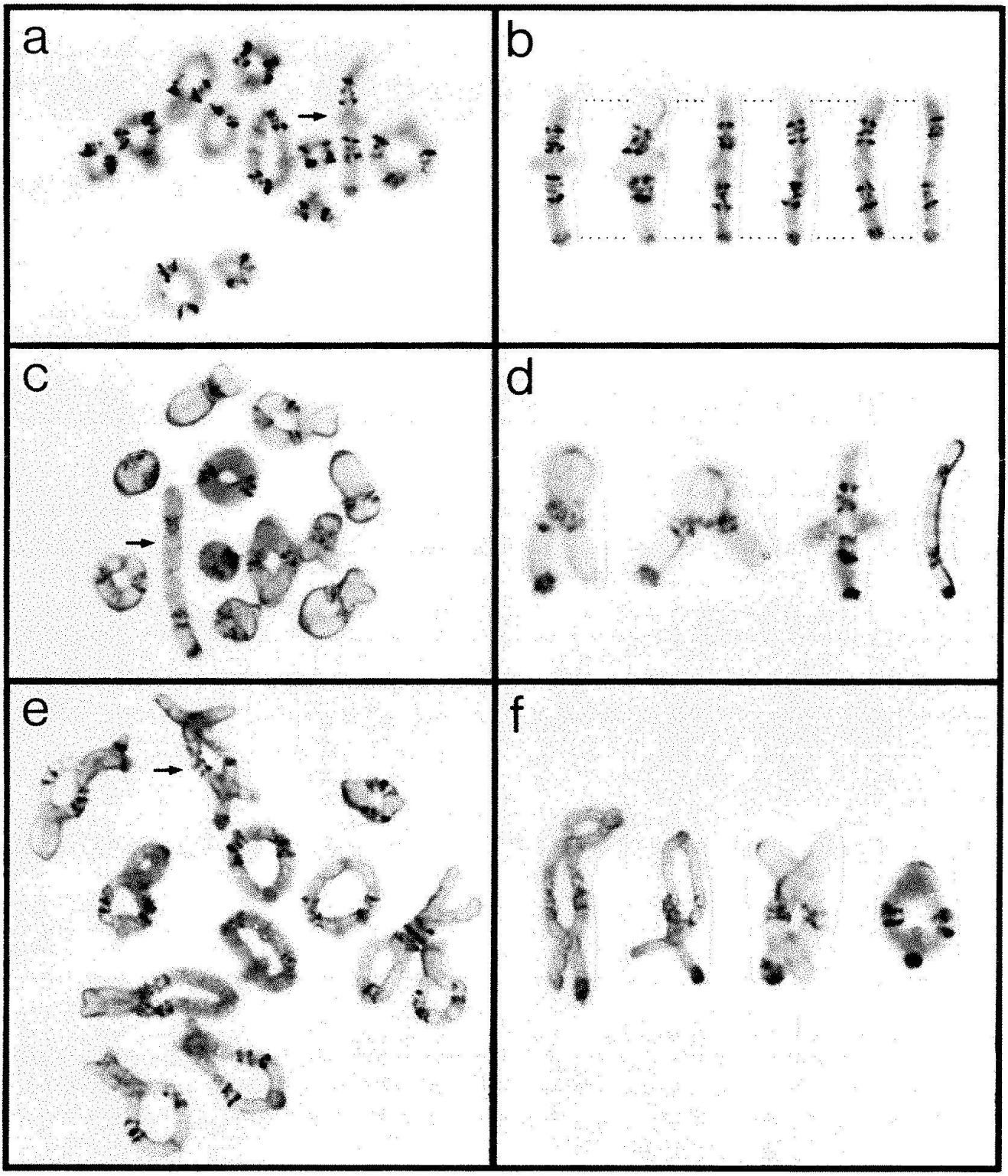

Fig. 10. Meiotic chromosomes from the male diakinesis of (a, b) Triturus vulgaris and (c–f) Triturus alpestris. The chromosomes exhibit the pattern of constitutive heterochromatin (C-bands). (a, c, e) represent complete diakineses, (b, d, f) each show several examples of paired XY sex chromosomes from different diakineses. In (a, b) Triturus vulgaris the XY sex chromosomes always pair end-to-end with their short arms, while the heteromorphic long arms point into the opposite direction. This is also the case in a part of the XY sex chromosomes from (c, d) Triturus alpestris; in some diakineses of this species, however, an additional chiasma is localized in the long arms of the XY bivalent (e, f). This permits the conclusion that the genetic homology still existing between the long arms of the sex chromosomes of Triturus alpestris is greater than that between the sex chromosomes of Triturus vulgaris.

same stages in their morphological and molecular differentiation as did the sex chromosomes of snakes, birds and mammals (Singh et al., 1976, 1980).

Rates of chromosomal- and protein evolution in Amphibia

Wilson et al. (1974) compared the relative rates of protein (albumin) evolution and chromosomal evolution in Bufonidae, Ranidae, Hylidae (i.e. highly evolved Anura) and mammals. It was found that the average rate of change in the chromosome number was about 20 times faster in mammals than in frogs. Mammals differing in only 6 albumin units had a 50% chance of having a different chromosome number, whereas in frogs the same amount of molecular difference occurred between species of identical chromosome number. Assuming that the rate of mutant substitutions in albumin was the same in mammals as in frogs (1.7 units per 10^6 years), the estimated rate of change in the chromosome number was calculated to be 1 every 3.5×10^6 years in mammals, whereas in frogs it was only 1 every 70×10^6 years (!). Since, however, these investigations were exclusively conducted on those families of Anura with extremely conservative karyotypes, they are not necessarily representative of the Amphibia. Those groups distinguished by a very variable diploid chromosome number should also be studied under this aspect. Our understanding of the chromosomal evolution in Amphibia will in the coming years be considerably expanded by the combination of the cytogenetic data and serological and biochemical methods of investigation.

Acknowledgments

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