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On the Arrangement of Chromosomes in the Elongated Sperm Nuclei of Anura (Amphibia)

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Abstract. The position of specific constitutive heterochromatic chromosome regions within the elongated sperm nuclei of eight species of Anura was examined with Q- and C-banding. These species differ widely with regard to the number, size and position of the brightly fluorescing heterochromatic regions. The empirical frequency distributions determined for the heterochromatic regions relative to the longitudinal axis of the sperm nuclei were compared with random frequency distributions calculated on the basis of two spatial models. None of the specifically stained heterochromatic regions occupy any definite preferential position within the sperm nuclei. In two instances, a specific sequence of the heterochromatic regions within the sperm nuclei could be excluded. The type of chromosomal arrangement within the elongated sperm nuclei of Anura is discussed on the basis of the distribution patterns obtained.

Introduction

Various cytogenetic methods permit the localization of particular chromosomes or chromosome regions in haploid nuclei during the various stages of spermiohistogenesis. Whenever a non-random arrangement of all or of individual chromosomes is determined in these investigations, there are mainly two causes for this: 1) The centromeres do not distribute themselves equally within the haploid nucleus of the spermatid after anaphase II of meiosis, but instead maintain their anaphasic position throughout the entire spermiohistogenesis. This leads to a conspicuous polarization of the centromeres and the chromosome arms. 2) One or more of the centromeres and chromosome arms arrange themselves in a specific nuclear position during spermiohistogenesis. Such an arrangement requires a directed movement of chromosomes within the spermatid nucleus. These two types of non-random chromosome arrangement in spermiohistogenesis could be determined in several species of vertebrates and invertebrates (for review see Macgregor and Walker, 1973; Walker and Macgregor, 1976; Schmid and Krone, 1976).

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Species with very elongate and thin sperm nuclei and low chromosome numbers are particularly suited for such investigations. The chromosomes are forced into a linear arrangement by the extreme elongation of the spermatids. Deviations from the random distribution of the chromosomes along the longitudinal axis of the sperm nuclei can thus be determined much more easily than in the small, asymmetrical or ellipsoid sperm nuclei of other species. The most important prerequisite is a suitable marker-technique, by which entire chromosomes or specific chromosome regions within the spermatid- and sperm-nuclei can be localized (e.g., in situ hybridization with cRNA complementary to satellite DNA or ribosomal DNA; specific staining of constitutive heterochromatin with fluorochromes or C-banding). All these conditions are found in the spermiohistogenesis of the Amphibia. Most species of Amphibia have a low chromosome number, large spermatids easily identifiable in every stage of differentiation and elongate mature sperm nuclei.

Using the in situ hybridization technique, Macgregor and Kezer (1971) were able to demonstrate, that a high density satellite DNA is localized on all centromeres of the chromosomes of *Plethodon cinereus cinereus* (Amphibia, Urodela). The centromeres labeled by this method are always found in the basal quarter of the nuclei during the entire spermiohistogenesis and in mature sperm. No centromeres were localized in the anterior (acrosomal) part of the nuclei (Macgregor and Walker, 1973). This led to the conclusion, that in *P.c. cinereus* each chromosome is arranged in a U-shaped configuration, with its centromere at the base of the sperm nucleus and its arm extending forward along the longitudinal axis of the nucleus. Investigations concerning the position of the constitutive heterochromatic chromosome regions in the sperm nuclei of 8 further species of Urodela (genera *Triturus, Salamandra, Cynops* and *Ambystoma*) yielded very similar results (Schmid and Krone, 1976). In these species, all centromeres remain in the basal part of the spermatid nuclei as well, and the chromosome arms extend toward the acrosome. During spermiohistogenesis, however, a pericentrically located heterochromatic chromosome region migrates from the basal to the anterior part of the nucleus and there induces the evagination and elongation of the spermatid (acrosomal chromocenter). This polarisation of a single heterochromatic chromosome region could also be observed in the elongate spermatids of the rooster *(Gallus domesticus)* (Dressler and Schmid, 1976).

In the present investigation, the position of particular heterochromatic chromosome regions in the elongated sperm nuclei of appropriate species of frogs and toads (Anura) was examined. The main purpose was to analyze whether heterochromatic regions localized centrically, interstitially and terminally on the chromosomes are distributed in a random or non-random manner within the sperm nuclei. The fluorochrome quinacrine mustard and the C-band method were used for the specific labeling of the heterochromatic regions.

Material and Methods

Animals. Eight species of Anura were selected for this investigation (Table 1). The American and African species were obtained from specialized animal dealers, kept in aquaterraria at 18–20° C and fed regularly with flies.

Chromosome Preparations. The frogs and toads received an intraperitoneal injection of 0.25–1.0 ml of a 0.3% colchicine solution 14–16 h before they were sacrificed. Metaphase chromosomes were prepared from bone marrow and blood cultures according to the usual methods (Schmid, 1978a).

Sperm Preparations. The freshly excised testes were minced as finely as possible in a petri dish with a pair of fine scissors. Using Pasteur pipettes, the cells were then vigorously resuspended

Table 1. Summary of material studied

Family	Species	Origin[a]	♂ animals analysed	Sperm nuclei analysed	2n	Position of Q-bright heterochromatin on chromosomes[b]		
						terminal	interstitial	centric
Bufonidae	*Bufo fowleri*	N.Am.	1	100	22	10p, 10q	8p, 11p	
	Bufo terrestris	N.Am.	1	75	22	7p, 9p, 11p	8p, 9q	
	Bufo mauritanicus	N.Afr.	1	40	22	10p	4p, 6pq, 7pqq, 9pq, 11pq	
	Bufo poweri	S.Afr.	4	50	20		4q, 5p, 6p, 7pq, 8p, 8pq	
Pelobatidae	*Pelobates syriacus*	S.E.Eur.	3	200	26	9q	10q	
Ranidae	*Rana catesbeiana*	N.Am.	1	200	26			
Rhacophoridae	*Kassina wealii*	S.Afr.	3	60	24			1, 6, 8, 10, 11, 12
	Leptopelis bocagei	S.Afr.	1	200	22		5p	

[a] N.Am. = North America; N.Afr. = North Africa; S.Afr. = South Africa; S.E.Eur. = South East Europe (Balkans)

[b] p = short arm; q = long arm

several times in 10 ml of a 0.075 M KCl solution. This suspension was centrifuged either directly afterwards, or after 45 min at room temperature (1000 rpm, 10 min). The sediment was carefully fixed with ice cold acetic acid – methanol (1:3) solution. The remaining suspension, containing almost exclusively mature sperm, was centrifuged a second time for 10 min at 1500 rpm and the sperm sediment was finally fixed. The material was resuspended in 1–3 ml fixative, and 3–6 drops of this suspension were dropped on slides. The chromosome- and sperm preparations were either allowed to dry at room temperature (for later C-banding) or briefly heated over a flame (for Q-banding and ammoniacal $AgNO_3$-staining).

Staining. Chromosome- and sperm-preparations were stained for 10 min in aqueous solutions of quinacrine mustard (0.05 mg/ml), washed in phosphate buffer (pH 5.5) and mounted in saturated saccharose solution. The microscopic analysis of the fluorescence patterns was performed with a Zeiss fluorescence microscope, equipped with an HBO 50W/AC lamp, BG 12 exciting filter and a 53/44 barrier filter. For demonstrating constitutive heterochromatin, the preparations were denatured in a saturated $Ba(OH)_2$ solution according to the method of Sumner (1972). A denaturation of 5 min duration at 30° C was the prerequisite for an optimal staining of all heterochromatic bands on the chromosomes and of the heterochromatic blocks in the sperm nuclei. The nucleolus organizer regions on the chromosomes were stained according to the ammoniacal $AgNO_3$ method of Goodpasture and Bloom (1975).

Measurements Performed on the Sperm Nuclei. The sperm nuclei of *Bufo fowleri, B. terrestris, B. mauritanicus, B. poweri, Rana catesbeiana, Kassina wealii* and *Leptopelis bocagei* usually lie on the slides in extended shape (Figs. 1, 4, and 5). The exact position of the brightly fluorescing heterochromatic blocks within the sperm nuclei could therefore be determined very easily with an ocular micrometer. The very long sperm nuclei of *Pelobates syriacus*, however, frequently lie on the slides in complex configurations (Fig. 2e). The position of the brightly fluorescing heterochromatin in these sperm nuclei was thus merely classified as belonging to the anterior, central or posterior third of the respective nuclei.

Results

1. The Constitutive Heterochromatin of Mitotic Metaphase Chromosomes

The eight species of frogs and toads (Table 1) were selected from 70 different species of Amphibia for the special staining faculties or the chromosomal position of their constitutive heterochromatin. With the exception of *Rana catesbeiana,* all the species examined display heterochromatic chromosome regions which fluoresce distinctly brighter than the euchromatic segments (Figs. 1–5). The euchromatic segments all fluoresce with uniform intensity; no multiple banding patterns, as they are usually observed on the chromosomes of other vertebrates, can be demonstrated on amphibian chromosomes (Schmid, 1978a, b). The comparison of quinacrine-stained and C-banded karyotypes shows, that the brightly fluorescing heterochromatic regions are always C-band positive as well (Fig. 1a–d). The remaining C-band positive heterochromatic regions exhibit, for the most part, a less intense fluorescence than the euchromatin. This variable staining characteristic of heterochromatin with quinacrine mustard has been described in detail on the basis of the karyotypes of 35 amphibian species (Schmid, 1978a, b, Schmid et al., 1979).

The species differ widely with regard to the number, size and position of the brightly fluorescing heterochromatic regions (Table 1, Figs. 1–5). The karyotypes of *Bufo fowleri* and *B. terrestris* (Fig. 1a, b) exhibit both terminal and interstitial, those of *B. mauritanicus* and *B. poweri* (Fig. 1c, d) almost exclusively interstitial brightly fluorescing heterochromatin. In *Kassina wealii,* on the other hand, the brightly fluorescing heterochromatin is confined to the centric regions of the chromosomes (Fig. 5a). The only fluorescing heterochromatic region in the karyotype of *Leptopelis bocagei* is immediately adjacent to the nucleolus organizer region (NOR) in the short arm of chromosome 5, as shown by comparison with the $AgNO_3$-staining (Fig. 4a, b). The position of the brightly fluorescing blocks within the sperm nuclei of this species (Fig. 4c) thus simultaneously indicates the position of the NOR. The direct staining of the inactive NOR in the mature sperm nuclei using the $AgNO_3$-method is not possible (Schmid et al., 1977; Hofgärtner et al., 1979). *Pelobates syriacus* only has two brightly fluorescing heterochromatic regions (Fig. 2a). The terminal heterochromatin in the long arm of chromosome 9 is distinctly larger than the interstitial heterochromatin in the long arm of chromosome 10. By this means, these two heterochromatic regions can also be distinguished in the spermatids and sperm nuclei (Fig. 2b–e).

Certain heterochromatic regions in *Rana catesbeiana* can be selectively stained by prolonged denaturation of the chromosomes in the saturated $Ba(OH)_2$ solution. A denaturation of 5 min at 30° C is the prerequisite for an optimal staining of all heterochromatic regions (Fig. 3a). After a denaturation of 10 min, all heterochromatic regions, with the exception of a pericentric block on chromosome 7 and an interstitial block on chromosome 8, lose a great deal of their distinctness (Fig. 3b). After a denaturation of 15–20 min, only the heterochromatic regions on the chromosomes 7 and 8 are visible in the mitotic as well as meiotic metaphases (Fig. 3c–f). For this present investigation, a male animal

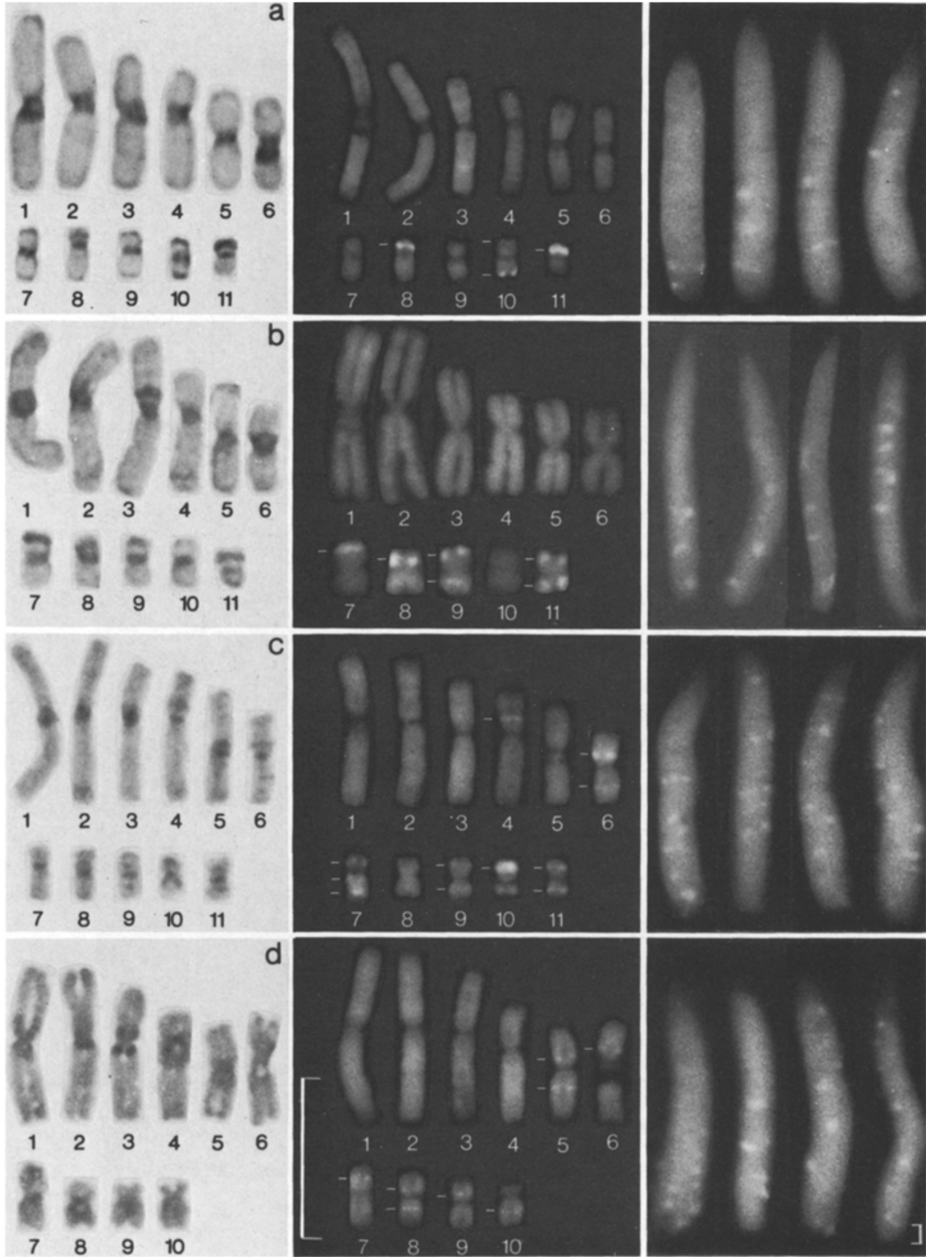

Fig. 1a–d[1]. Haploid male karyotypes and mature sperm nuclei of **a** *Bufo fowleri*, **b** *B. terrestris*, **c** *B. mauritanicus*, and **d** *B. poweri* showing C-bands (left) and quinacrine staining (middle and right). Only a few of the C-band positive heterochromatic chromosome regions show a bright fluorescence (arrows). The acrosomal regions of the sperm nuclei are at the top of each picture. Due to the hypotonic treatment, the sperm nuclei are somewhat distended, permitting a better differentiation of the individual brightly fluorescing heterochromatic regions

[1] Bars in Figures 1–5 represent 10 μm

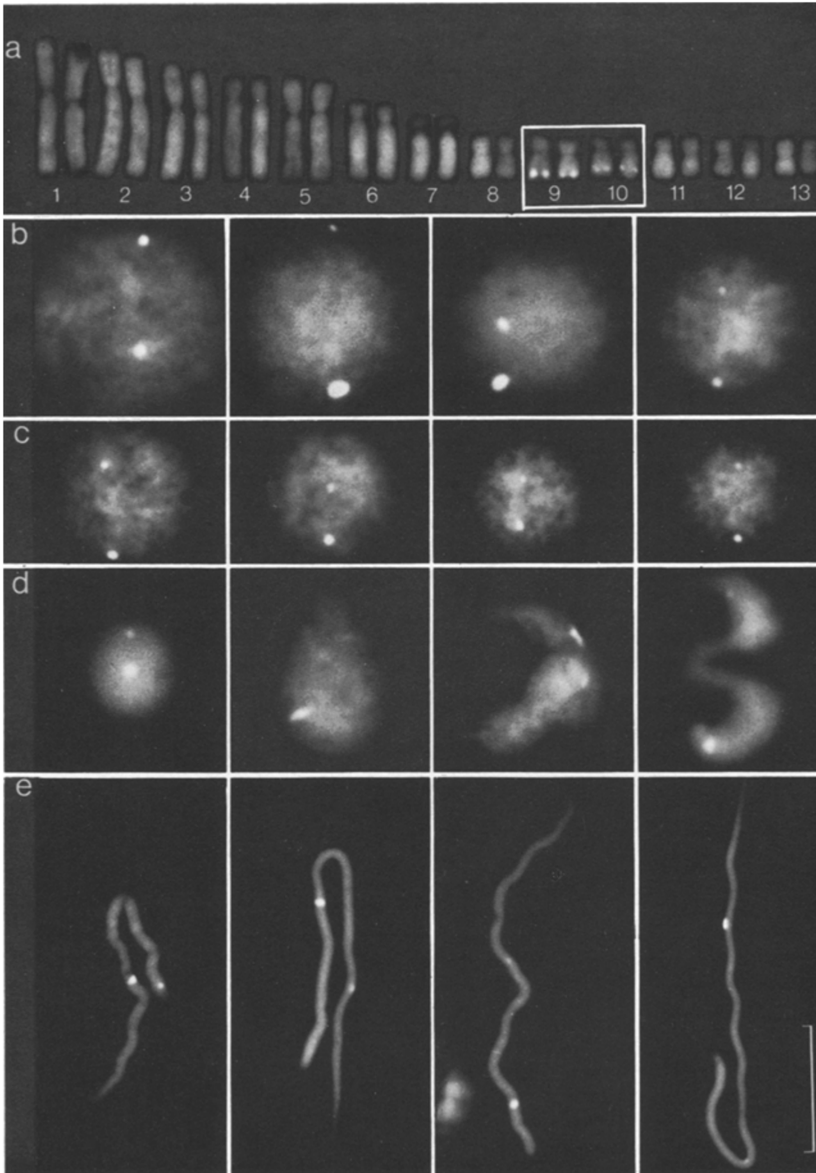

Fig. 2a–e. Quinacrine-stained karyotype (**a**) and the successive stages in spermiohistogenesis (**b–e**) of *Pelobates syriacus* demonstrating the position of the brightly fluorescing heterochromatin. **b, c** Progressive reduction in spermatid volume in the early phases of spermiohistogenesis. **d** Evagination of the nuclear membrane at the prospective acrosomal pole and elongation of the spermatids. **e** Mature sperm nuclei. The large, brightly fluorescing heterochromatic region of chromosome 9 can be differentiated from the smaller one on chromosome 10 in all spermatid- and sperm nuclei. Note the peripheral position preferred by the telomeric heterochromatin of chromosome 9 within the spermatid nuclei and the random sequence of the two heterochromatic regions in the mature sperm nuclei

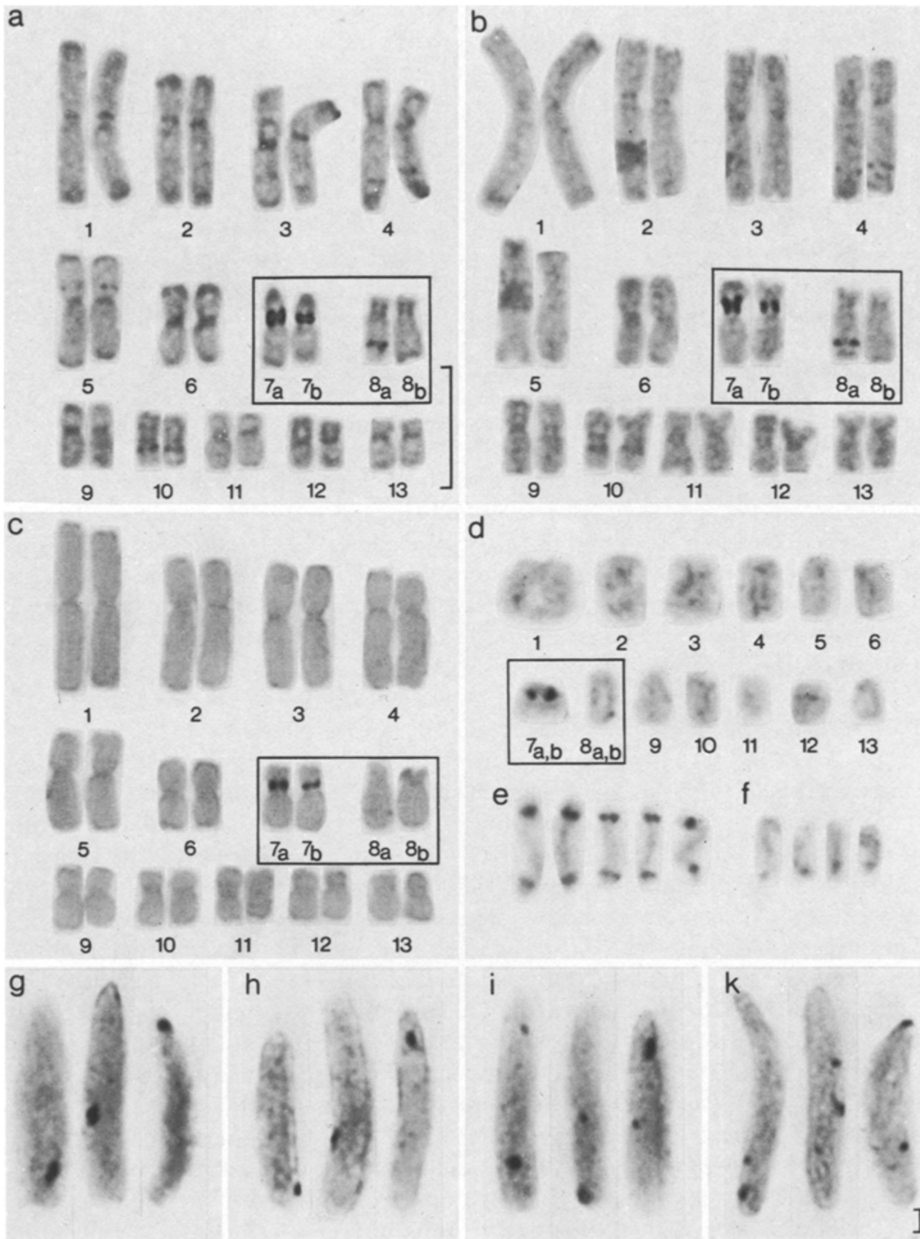

Fig. 3a–k. Karyotypes and mature sperm nuclei of the male *Rana catesbeiana* examined showing the varying sensitivity of individual C-bands following pre-treatment with $Ba(OH)_2$ of various duration. **a** 5 min denaturation; the karyotype shows the maximum number of C-bands. **b** 10 min denaturation; the pericentric C-band on chromosome 7 and the interstitial C-band on chromosome 8 (both heteromorphic) remain clearly visible, whereas all other C-bands appear pale. **c** 20 min denaturation; except for the C-bands on chromosomes 7 and 8, no constitutive heterochromatin is stained. **d** Chromosomes of diakinesis exhibiting the heteromorphic C-band regions on the bivalents 7 and 8. **e, f** Various examples of end-to-end paired bivalents 7 and 8. **g–k** The four types of sperm nuclei found correspond to the random segregation of chromosomes 7a/7b and 8a/8b in meiosis. Note the random sequence of the two heterochromatic regions in **i** and **k**

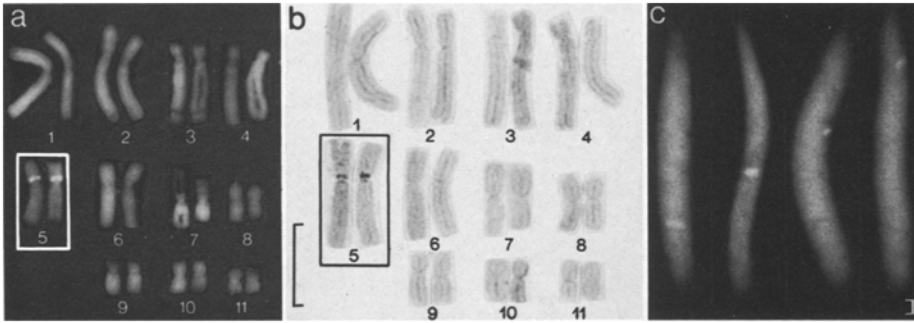

Fig. 4a–c. Karyotypes and mature sperm nuclei of *Leptopelis bocagei* showing quinacrine staining (left and right) and Ag-stained NORs (middle). The brightly fluorescing heterochromatin in the short arms of chromosome pair 5 is localized immediately adjacent to the NORs. Note the variable position of the heterochromatic block within the sperm nuclei

displaying a heteromorphism between the homologous heterochromatic regions on the chromosomes 7 and 8 was selected (Figs. 3a–f): the pericentric heterochromatic regions on the homologous chromosomes 7 differed distinctly in size, and only one of the homologous chromosomes 8 demonstrated the interstitial band in the long arm. These three variously large heterochromatic regions could also be selectively stained in sperm nuclei after denaturing for 15–20 min, and could thus be localized exactly (Fig. 3g–k).

2. The Number of Heterochromatic Regions in Sperm Nuclei

The sperm nuclei of the species examined should exhibit just as many heterochromatic regions as there were determined for the haploid karyotypes of these species. In the overwhelming majority of the sperm nuclei, however, the analysis with quinacrine mustard revealed fewer brightly fluorescing blocks than are theoretically expected (Fig. 6a–e). The most plausible explanation for this is, that several heterochromatic chromosome regions fuse to form larger chromocenters during spermiohistogenesis. In *Bufo mauritanicus* and *B. poweri* on the other hand, sperm nuclei were found displaying more heterochromatic blocks than the haploid karyotypes (Fig. 6b, c). It is possible, that there are some very small brightly fluorescing heterochromatic regions at other sites than those described on the chromosomes of these two species (Table 1), which are not detectable on the highly contracted metaphase chromosomes. But it is also feasible, that a few of the brightly fluorescing segments of the metaphase chromosomes in fact consist of several, closely adjacent heterochromatic regions; once the chromosomes lose their contracted configuration after meiosis, the distance between these would become greater, and more heterochromatic regions would become apparent in the sperm nuclei than in the metaphases.

3. The Arrangement and Sequence of Specific Heterochromatic Regions in Sperm Nuclei

a) Pelobates syriacus. The successive stages of the spermiohistogenesis of *P. syriacus* are compiled in Figure 2. As in all other Amphibia, spermatid volume

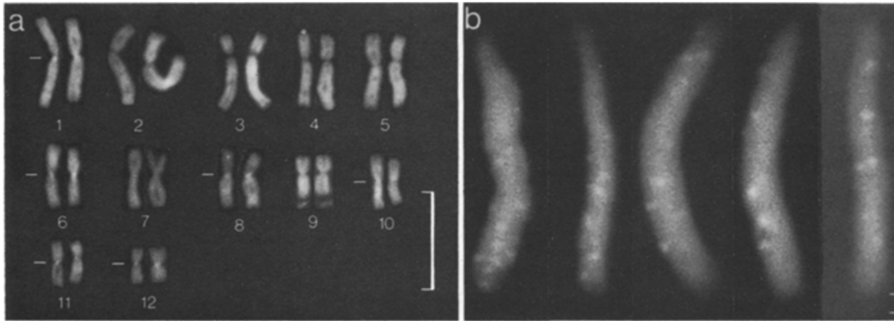

Fig. 5a and b. Quinacrine stained karyotype and mature sperm nuclei of *Kassina wealii*. The brightly fluorescing heterochromatin is confined to the centric regions of the chromosomes (arrows)

Table 2. The position of the brightly fluorescing heterochromatic regions of the chromosomes 9 and 10 in 200 mature sperm nuclei of *Pelobates syriacus*

Hetero-chromatic region on chromosome	Frequency of heterochromatic blocks in three regions of the sperm nuclei			Expected frequency per third[a]	χ^2 (df=2)[b]
	basal third	central third	acro-somal third		
9	87	89	24	66.6	41.0 ($P<0.001$)
10	76	62	62	66.6	1.97 ($P<0.50$)

[a] Total of heterochromatic blocks found: 3
[b] df = degrees of freedom

distinctly decreases during early spermiohistogenesis, whereby nuclear chromatin becomes highly condensed (Fig. 2b, c). In the ensuing phase of elongation, the nuclear membrane evaginates at one point, and the spermatids take on a pear-shaped form (Fig. 2d). The mature amphibian sperm nuclei acquire their characteristic shape by the elongation of this nuclear evagination and the progressing condensation of the chromatin.

The two brightly fluorescing heterochromatic regions of chromosomes 9 and 10 could be identified in all stages of spermiohistogenesis of *P. syriacus* (Fig. 2b–e). The telomeric heterochromatin of chromosome 9 in the early, round spermatid nuclei is preferentially situated near the nuclear periphery. Conversely, the interstitial heterochromatin of chromosome 10 occupies random positions within the nuclei (Fig. 2b, c). In mature sperm nuclei, the heterochromatin of chromosome 9 is localized significantly more often in the basal and central third of the nuclei, whereas the heterochromatin of chromosome 10 occurs with equal frequency in all the three nuclear regions (Table 2). In the very long and thin sperm nuclei, the two heterochromatic blocks are always arranged in a linear fashion and are separated by distances of varying lengths (Fig. 2e). No specific sequence could be determined; each of the two brightly fluorescing blocks can maintain the anterior (acrosomal) as well as posterior (basal) position in the sperm nucleus. In the 200 sperm nuclei analysed, the heterochromatin of

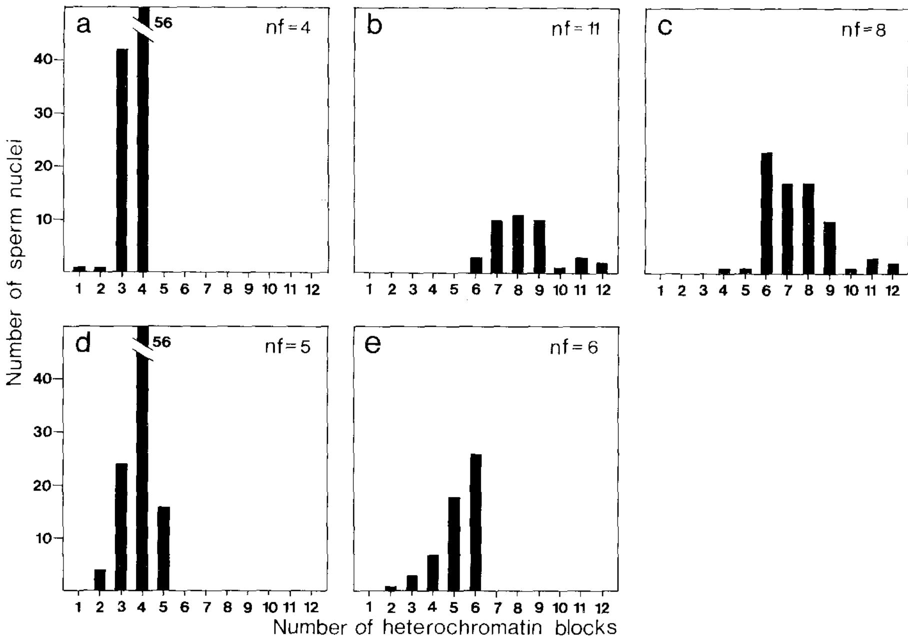

Fig. 6a–e. The number of brightly fluorescing heterochromatic regions in the mature sperm nuclei of **a** *Bufo fowleri*, **b** *B. mauritanicus*, **c** *B. poweri*, **d** *B. terrestris* and **e** *Kassina wealii*. *nf* Number of brightly fluorescing chromosome regions determined in the haploid karyotypes

chromosome 9 was situated in the anterior portion of 82 nuclei (41%), and the heterochromatin of chromosome 10 was found in the front part of 118 nuclei (59%).

b) Rana catesbeiana. The animal examined demonstrated heteromorphisms of the homologous heterochromatic regions of chromosomes 7 and 8 (Fig. 3a–f). Due to the random segregation of chromosomes in the first meiotic division, four types of sperm with variously large heterochromatic blocks should develop. These were in fact found by selective staining of the heterochromatin (Fig. 3g–k). In 51% of the 200 sperm nuclei examined, a single heterochromatic block was demonstrated (Fig. 3g, h); chromosome 7a (large heterochromatic region) or else 7b (medium-sized heterochromatic region) and chromosome 8b (no heterochromatin) must lie in these sperm nuclei. The other 49% of the sperm nuclei regularly displayed one large or medium-sized and one very small heterochromatic region (Fig. 3i, k); this is the result of the joint segregation of chromosomes 7a or 7b with chromosome 8a. A more detailed analysis of the two latter sperm types revealed, that the heterochromatic regions on the chromosomes 7 and 8 are not arranged in a particular sequence within the sperm nuclei. In 53% of the nuclei, the heterochromatic region of the chromosomes 7a or 7b were localized in the anterior (acrosomal) position (Fig. 3i, k). All three heterochromatic regions are much more frequently situated in the basal and central third than in the acrosomal third of the sperm nuclei (Table 3).

Table 3. The position of the C-band positive, heterochromatic regions of the chromosomes 7a, 7b and 8a (see Fig. 3) in 150 mature sperm nuclei of *Rana catesbeiana*

Heterochromatic region on chromosome	Frequency of heterochromatic blocks in three regions of the sperm nuclei			Expected frequency per third[a]	χ^2 (df = 2)[b]
	basal third	central third	acrosomal third		
7a	40	22	14	25.3	14.01 ($P<0.001$)
7b	28	20	14	20.6	4.78 ($P<0.10$)
8a	32	24	14	23.3	6.97 ($P<0.05$)

[a] Total of heterochromatic blocks found : 3
[b] df = degrees of freedom

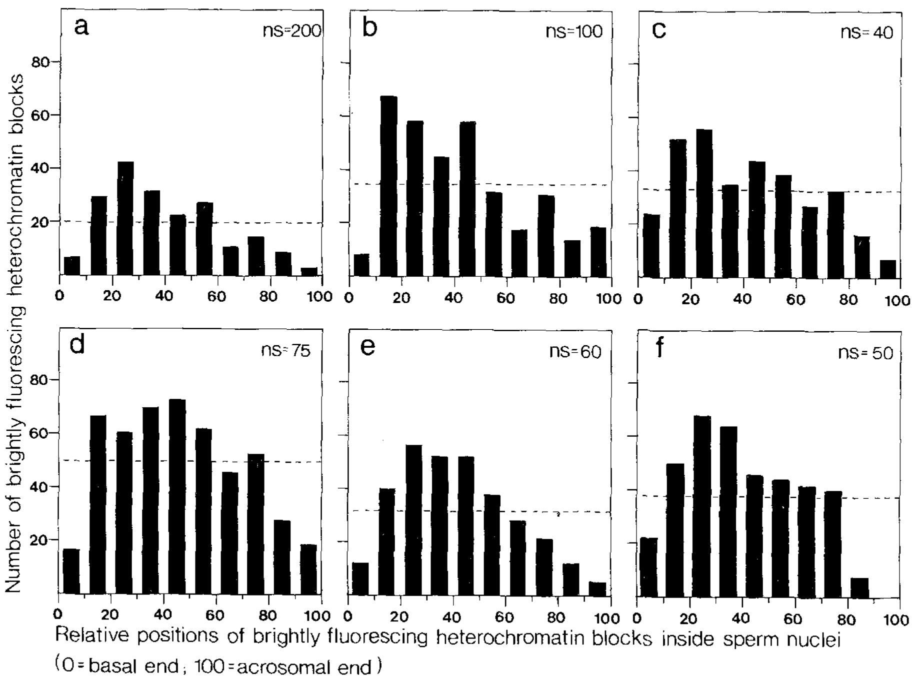

Fig. 7a–f. The frequency of the brightly fluorescing heterochromatic regions on the longitudinal axis in the mature sperm nuclei of **a** *Leptopelis bocagei*, **b** *Bufo fowleri*, **c** *B. mauritanicus*, **d** *B. terrestris*, **e** *Kassina wealii* and **f** *B. poweri*. n = number of sperm nuclei analysed. The intermittent horizontal line designates the value determined for a random distribution of the heterochromatic regions within the sperm nuclei (total number of the heterochromatic blocks divided by 10 segments of equal length)

c) Leptopelis bocagei. One single, very brightly fluorescing block corresponding to the heterochromatic region in the short arm of chromosome 5 was determined in all sperm nuclei analysed (Fig. 4). Since this heterochromatin is situated immediately adjacent to the nucleolus organizer region (NOR), the fluorescing block simultaneously indicates the position of the NOR within the mature

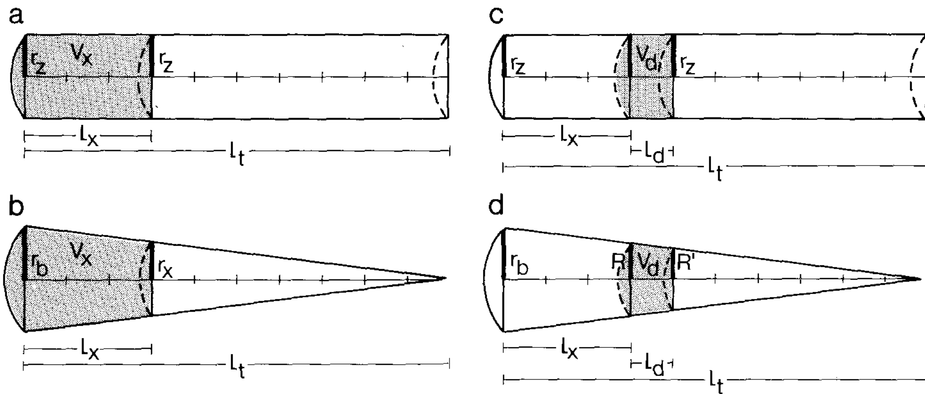

Fig. 8a–d. Diagrammatic representation of a cylinder (**a, c**) and a cone (**b, d**), used as spatial models in the calculation of the random distributions of heterochromatic regions within the sperm nuclei. For better display, the models are shown as longitudinal sections. **a, b** Model for the calculation of the frequencies of heterochromatic regions within a defined distance $l_{(x)}$ from the base of the sperm nuclei. **c, d** Model for the calculation of the frequencies of heterochromatic regions in various sections of the sperm nuclei. For the explanation of symbols refer to the calculations in the text

sperm nucleus. This region of constitutive heterochromatin could be demonstrated in every position within the sperm nuclei (Fig. 4c), although on the average it was localized more frequently in the basal half of the nucleus (Fig. 7a).

4. The Distribution of the Heterochromatic Regions Relative to the Longitudinal Axis of the Sperm Nuclei

The exact positions of the brightly fluorescing heterochromatic regions were determined with an ocular micrometer in a great number of mature sperm. In all cases, this procedure showed more heterochromatic regions to be localized in the basal half of the sperm nuclei than in their anterior half (Fig. 7). The frequency distributions empirically determined for heterochromatic regions on the longitudinal axis of the sperm nuclei were compared to random frequency distributions theoretically obtained. The random distributions were calculated for two spatial models, a cylinder and a cone (Fig. 8). Graphing the random distributions calculated for each of the two models in a co-ordinate system, two curves are found constituting the limits of an area (Figs. 9 and 10). The actual form of anuran sperm nuclei, however, conforms neither to an ideal cylinder nor to a cone, but is somewhere between these two models. If the distribution of heterochromatic regions within the sperm nuclei is random, then the values found must lie within the area delimited by the values calculated for the cylinder and cone model (Figs. 9 and 10). The equations for random distribution in a cylinder and a cone are derived in the following:

a) The Frequency of Heterochromatic Regions Within a Defined Distance $l_{(x)}$ from the Base of the Sperm Nuclei (see Fig. 8a, b). The probability $P_{(x)}$, that a heterochromatic block is within the partial volume $V_{(x)}$ of a cylinder with a total volume $V_{(t)}$ is described by:

$$P_{(x)}=\frac{V_{(x)}}{V_{(t)}}=\frac{\pi r_z^2 l_{(x)}}{\pi r_z^2 l_{(t)}}=\frac{l_{(x)}}{l_{(t)}}. \tag{1}$$

The probability, that all n brightly fluorescing heterochromatic blocks are localized within this partial volume is:

$$P_{(x)}=\left(\frac{l_{(x)}}{l_{(t)}}\right)^x. \tag{2}$$

The probability, that a heterochromatic block is localized within the truncated volume $V_{(x)}$ of a cone with a total volume $V_{(t)}$ is accordingly:

$$P_{(x)}=\frac{V_{(x)}}{V_{(t)}}=\frac{\frac{1}{3}\pi r_b^2 l_{(t)}-\frac{1}{3}\pi r_x^2(l_{(t)}-l_{(x)})}{\frac{1}{3}\pi r_b^2 l_{(t)}}=1-\frac{r_x^2(l_{(t)}-l_{(x)})}{r_b^2 l_{(t)}}. \tag{3}$$

The relationships between the radii $r_{(b)}$ and $r_{(x)}$ as well as the lengths $l_{(x)}$ and $l_{(t)}$ are described by the equations:

$$\frac{r_b}{l_{(t)}}=\frac{r_x}{l_{(t)}-l_{(x)}}, \tag{4}$$

$$r_x=\frac{r_b}{l_{(t)}}(l_{(t)}-l_{(x)}). \tag{5}$$

By substituting equation (5) into equation (3), the calculation of the probability $P_{(x)}$ is simplified to read:

$$P_{(x)}=1-\frac{\left(\frac{r_b}{l_{(t)}}(l_{(t)}-l_{(x)})\right)^2(l_{(t)}-l_{(x)})}{r_b^2 l_{(t)}}=1-\left(\frac{l_{(t)}-l_{(x)}}{l_{(t)}}\right)^3. \tag{6}$$

Finally, the probability, that all n brightly fluorescing heterochromatic blocks are localized within this truncated cone is:

$$P_{(x)}=\left(1-\left(\frac{l_{(t)}-l_{(x)}}{l_{(t)}}\right)^3\right)^n. \tag{7}$$

To calculate the distribution frequencies in the cylinder- and cone model, values for the total length $l_{(t)}=1$ and for the variable length $l_{(x)}=0$, 0.1, 0.2, ..., 1.0 were inserted in the equations (2) and (7). The maximum number of brightly fluorescing blocks found in the sperm nuclei of each species was inserted for n.

b) The Frequency of Heterochromatic Regions in Various Sections of the Sperm Nuclei (see Fig. 8c, d). If the brightly fluorescing heterochromatic blocks are randomly distributed, then the average number of blocks $N_{(d)}$ within the segment $V_{(d)}$ of a cylinder with a total volume $V_{(t)}$ is:

$$N_{(d)}=\frac{V_{(d)}}{V_{(t)}}n=\frac{\pi r_z^2 l_{(d)}}{\pi r_z^2 l_{(t)}}n=\frac{l_{(d)}}{l_{(t)}}n. \tag{8}$$

The average number of heterochromatic blocks $N_{(d)}$ within the segment $V_{(d)}$ of a cone with a total volume $V_{(t)}$ is accordingly:

$$N_{(d)}=\frac{V_{(d)}}{V_{(t)}}n=\frac{\frac{1}{3}\pi l_{(d)}(R^2+RR'+R'^2)}{\frac{1}{3}\pi r_b^2 l_{(t)}}n=\frac{l_{(d)}(R^2+RR'+R'^2)}{r_b^2 l_{(t)}}n. \tag{9}$$

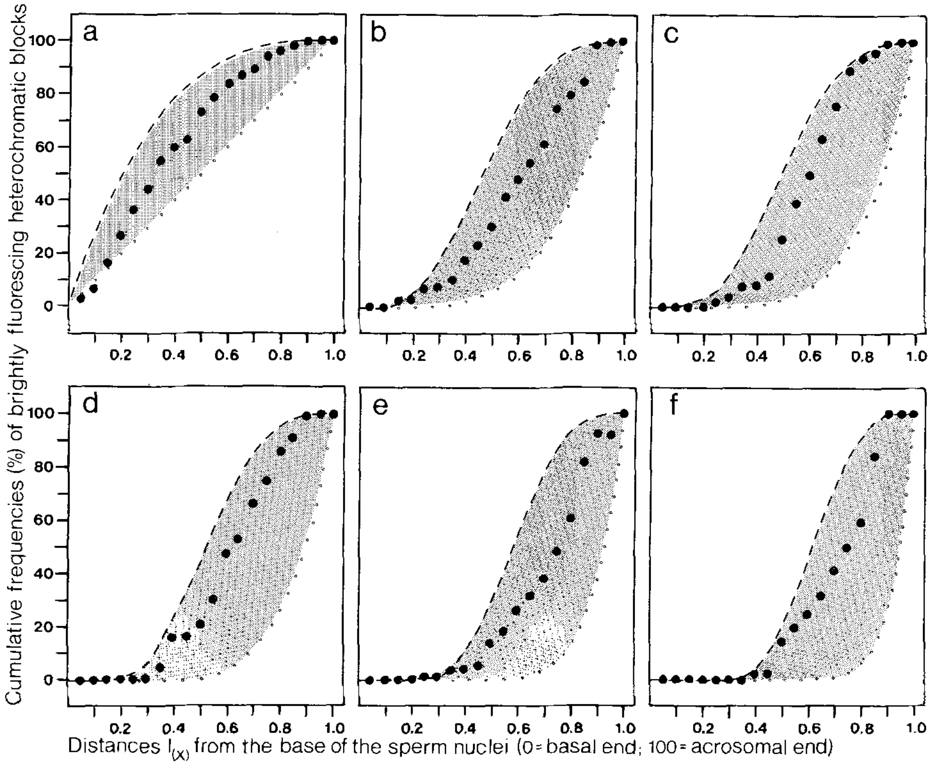

Fig. 9a–f. Cumulative frequencies (%) of brightly fluorescing heterochromatic regions within a defined distance $l_{(x)}$ from the base of the sperm nuclei. ● Observed frequencies. --- Random frequency distributions calculated according to the cone model. ····· Random frequency distributions calculated according to the cylinder model. **a** *Leptopelis bocagei*. **b** *Bufo fowleri*. **c** *B. terrestris*. **d** *Kassina wealii*. **e** *B. poweri*. **f** *B. mauritanicus*. Note that in all species the frequencies observed lie within the area (grey) delimited by the random frequency distributions

The relationships between the radii $r_{(b)}$, R and R′, as well as the lengths $l_{(t)}$, $l_{(d)}$ and $l_{(x)}$ are described by the equations:

$$\frac{r_b}{l_{(t)}} = \frac{R}{l_{(t)} - l_{(x)}} = \frac{R'}{l_{(t)} - (l_{(x)} - l_{(d)})}, \tag{10}$$

$$R = \frac{r_b(l_{(t)} - l_{(x)})}{l_{(t)}} \qquad R' = \frac{r_b(l_{(t)} - l_{(x)} - l_{(d)})}{l_{(t)}}. \tag{11}$$

By substituting the equations (11) into equation (9), the calculation of the average number of heterochromatic blocks $N_{(d)}$ is simplified to read:

$$N_{(d)} = \frac{(l_{(t)} - l_{(d)})^2 + (l_{(t)} - l_{(d)})(l_{(t)} - l_{(x)} - l_{(d)}) + (l_{(t)} - l_{(x)} - l_{(d)})^2}{l_{(t)}^3} l_{(d)} n. \tag{12}$$

To calculate the average numbers of heterochromatic blocks within the various sections of the sperm nuclei, values for the total length $l_{(t)} = 1$, for the variable length $l_{(x)} = 0$, 0.1, 0.2, ..., 0.9, and for the length of the sections $l_{(d)} = 0.1$ were substituted in equations (8) and (12). The

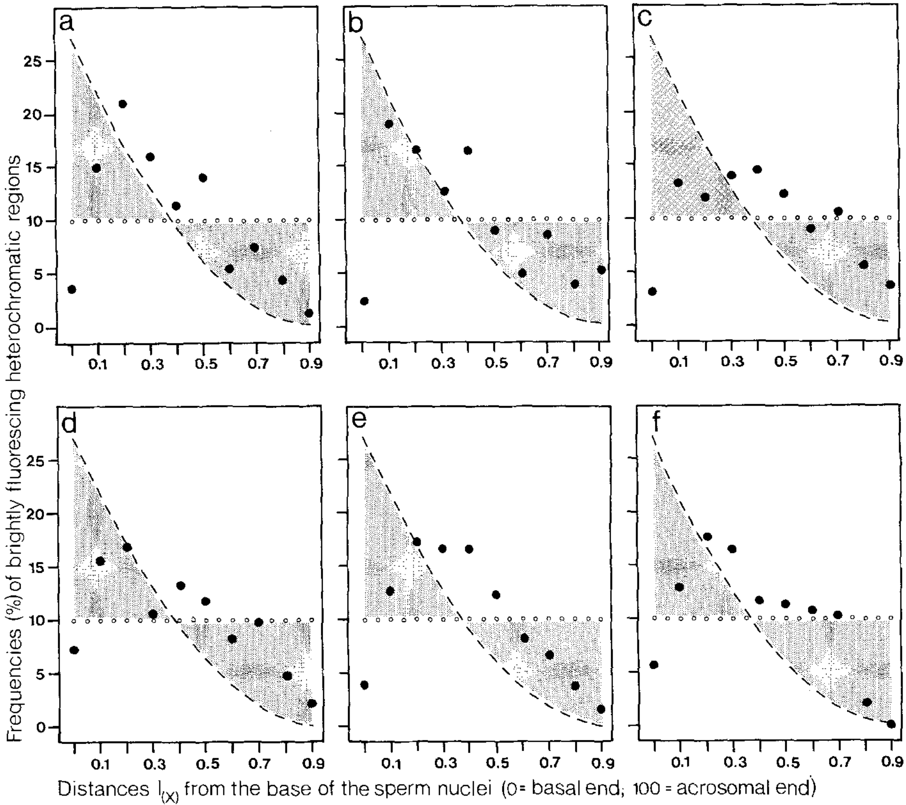

Fig. 10a–f. Frequencies (%) of brightly fluorescing heterochromatic regions in 10 successive segments of equal length ($l_{(d)} = 0.1$) within the sperm nuclei. The segments are localized at a distance $l_{(x)} = 0$, 0.1, 0.2, ···, 0.9 from the base of the sperm nuclei. ● Observed frequencies. --- Random frequency distributions calculated according to the cone model. ····· Random frequency distributions calculated according to the cylinder model. **a** *Leptopelis bocagei*. **b** *B. fowleri*. **c** *B. terrestris*. **d** *B. mauritanicus*. **e** *Kassina wealii*. **f** *B. poweri*. The observed frequencies of heterochromatic regions in the acrosomal segments are within the area (grey) delimited by the random frequency distributions, but deviate from the calculated frequencies in the central and in the most basal segments

maximum number of brightly fluorescing blocks found in the sperm of each species was inserted for n.

The frequencies obtained for heterochromatic regions within a distance of $l_{(x)}$ to the base of the sperm nuclei show a good correspondence with the random distributions calculated from equations (2) and (7) (Fig. 9). Beyond this, the graphs show the theoretical values for the cone model to yield a better approximation of the data obtained than do the values for the cylinder model. Regular small deviations exist, on the other hand, between the frequencies of heterochromatic regions in the various sections of the sperm nuclei calculated from equations (8) and (12), and their respective empirical values (Fig. 10). These deviations are found in the sperm nuclei of all species investigated (Fig. 10a–f), independent of whether the fluorescing heterochromatic blocks are localized in terminal, interstitial or centric positions on the chromosomes (Table 1).

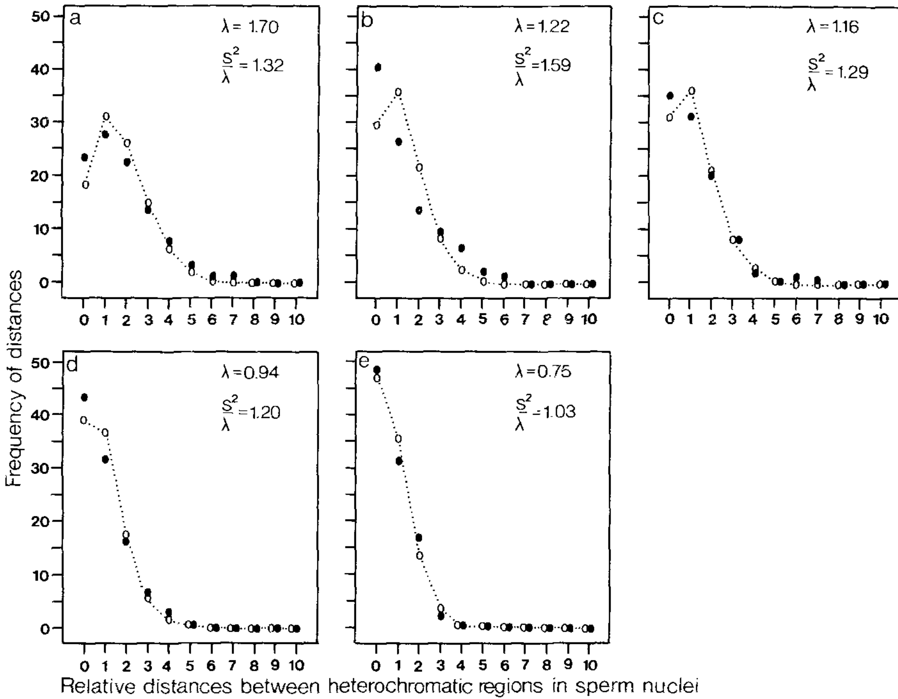

Fig. 11 a–e. Distribution of distances between heterochromatic regions in mature sperm nuclei. ● Observed frequencies. ○ Frequencies corresponding to the Poisson distributions for the same mean λ. s^2 = variance. **a** *Bufo fowleri.* **b** *B. poweri.* **c** *Kassina wealii.* **d** *B. terrestris.* **e** *B. mauritanicus*

5. *The Distance Between Heterochromatic Regions in Sperm Nuclei*

The distances between the brightly fluorescing heterochromatic blocks within the sperm nuclei were tested to see whether their values are random, or whether certain distances are regularly favored.

To do this, the individual distances between successive blocks were measured in all sperm nuclei analysed. The values obtained were divided into 10 categories ($X_1 = 0.01$–0.14, $X_2 = 0.15$–0.24, ..., $X_{10} = 0.95$–1.0). Whenever two or more blocks were fused, they were apportioned to the category $X_0 = 0$. The frequencies obtained for these distances are shown in Fig. 12. The expected values for the individual categories $P_{(x/\lambda)}$ can be calculated from the mean values of the distances λ according to the Poisson-distribution:

$$P_{(x/\lambda)} = \frac{\lambda^x e^{-\lambda}}{x!} \quad x = 0, 1, 2, \ldots, 10; \ \lambda > 0. \tag{13}$$

The quotient of the variance s^2 and the mean value λ suffices to compare the distribution of distances obtained with the respective Poisson-distributions:

$$\frac{s^2}{\lambda} = \frac{\Sigma x_i^2 f_i - (\Sigma x_i f_i)^2/n}{(n-1)\lambda} \tag{14}$$

$X_i = X_0, X_1, \ldots, X_{10}$
f_i = frequencies of X_i
n = number of sperm nuclei analysed.

If the quotient is $\lesssim 10/9$ it can be assumed that the distribution obtained can be approximated by a Poisson-distribution (Sachs, 1969).

The values in Figure 11 show, that the Poisson-distributions are suited for the description of the empirical distributions of the distances between the heterochromatic blocks. The agreement of theoretically calculated frequencies with those actually obtained is especially good for medium and greater distances (categories X_3–X_{10}). In almost all cases, however, the frequency of small distances (categories X_1 and X_2) was found to be somewhat lower, and the frequency of the X_0-category was found to be greater than expected. These deviations can be sufficiently explained by the fusion of heterochromatic blocks. Closely adjacent blocks have a greater tendency to fuse with each other; thus, values originally from the categories X_1 and X_2 are transferred to the X_0-category. The distances between brightly fluorescing heterochromatic blocks in the sperm nuclei of the species investigated are therefore randomly distributed and are only influenced by the fusional tendencies of these blocks.

Discussion

The investigation has shown that the specifically stained heterochromatic chromosome regions do not occupy any definite preferential position in the sperm nuclei of Anura. The constitutive heterochromatin of the centromeres *(Kassina wealii)* and of the nucleolus organizer regions *(Leptopelis bocagei)*, as well as the interstitial and terminal heterochromatic chromosome regions *(Bufo)* are largely randomly distributed. Moreover, a specific sequence of heterochromatic regions in mature sperm nuclei could be excluded with certainty in two cases *(Rana catesbeiana, Pelobates syriacus)*. Although a statistically higher frequency of heterochromatic regions could be determined for the basal sperm sections in Anura, this difference is not as conspicuous by far as it is in the sperm nuclei of Urodela (Macgregor and Kezer, 1971; Macgregor and Walker, 1973; Schmid and Krone, 1976). These findings lead to the conclusion, that most, if not all, chromosome arms become randomly distributed during the spermiohistogenesis of Anura. Earlier investigations of the chromosomes in the elongated sperm nuclei of Anura were exclusively concerned with the sequences for (18S+28S) rRNA and 5S rRNA of *Xenopus laevis.* In this species, the sequences for (18S+28S) rRNA are localized in the nucleolar constriction on the short arm of the submetacentric chromosome 12 and the sequences for 5S rRNA are at or near the telomere of the long arm of many, if not all, chromosomes. The in situ hybridization with (18S+28S) r-cRNA or (18S+28S) rRNA show the position of the nucleolus organizer to be marked by a small cluster of silver grains which has no fixed position along the length of the sperm nuclei (Pardue and Gall, 1972; Macgregor and Walker, 1973). The hybridization with 5S cRNA or 5S RNA produced a series of silver grain clusters in a pattern which seems to be entirely random (Pardue et al., 1973). These results likewise indicate, that the arrangement of the chromosomes within the sperm nuclei of Anura is not a constant one.

The distribution patterns of the heterochromatic regions found in mature

sperm nuclei exclude a U-shaped arrangement of the chromosome arms. If in all sperm nuclei the centromeres would always be localized on the basal region, and the telomeres on the acrosomal region of the nuclei, then the statistical analysis of the position of the brightly fluorescing blocks would yield a constant pattern with accordingly great deviations from the calculated random values. A strict tandem arrangement of the chromosomes is also improbable, because the heterochromatic regions are localized in the basal sperm segments with a slightly higher degree of frequency than in the acrosomal segments, and because the distances between the heterochromatic regions are randomly distributed.

An alternative model of chromosome arrangement in elongated sperm nuclei, which is also best suited to explain the present results, was suggested by Walker and Macgregor (1976). According to this, there is a correlation between the arrangement of the chromosomes in the sperm nuclei and the type of condensation undergone by the nucleoprotein during spermiohistogenesis. In the sperm nuclei of those species, whose nucleoprotein condensates in a granular pattern, the chromosome arms lie alongside one another. The position of the chromosomes along the longitudinal axis of the sperm nuclei, however, can vary greatly. Very large chromosomes maintain their U-shaped arrangement from the second meiotic anaphase in the sperm nuclei. On the other hand, the position of medium-sized and small chromosomes can shift in a random fashion during spermiohistogenesis, although the chromosome arms remain parallel to the longitudinal axis of the sperm nuclei. The nucleoprotein does in fact condense in a granular pattern in the spermiohistogenesis of Anura, as the electronmicroscopic investigations on sperm nuclei of *Bufo, Rana* and *Xenopus* have confirmed (Burgos and Fawcett, 1956; Zirkin, 1971; Walker and Macgregor, 1976). Conversely, the chromosome arms in those nuclei exhibiting fibrous or lamellar condensation of the nucleoprotein are arranged in tandem. This is known to be the case in the elongated sperm nuclei of several species of insects (for review see Walker and Macgregor, 1976). No amphibians, however, whose nucleoprotein is condensed in form of fibers or lamellae during spermiohistogenesis, have yet been found. The frequent fusion of heterochromatic chromosome regions observed in anuran sperm nuclei appears to be a general feature of constitutive heterochromatin, since it has already been proven to occur in the tissues of several species of vertebrates (Hsu et al., 1971; Rae and Franke, 1972; Schmid et al., 1975; Dressler and Schmid, 1976). The occurrence in constitutive heterochromatin of satellite DNA composed of highly repetitive sequences suggests that DNA of similar base composition and sequence might mediate heterochromatin fusion, as discussed by Hsu et al. (1971), Barr and Ellison (1972) and Natarajan and Ahnström (1973).

A specific chromosome region causing the evagination and elongation of the spermatids (acrosomal chromocenter), as it occurs in the spermiohistogenesis of several Urodela (Schmid and Krone, 1975, 1976), could not be established for the Anura investigated. This does not necessarily mean, however, that no such chromosome region with the same function exists in the anuran genome, because the method of determination depends entirely on the linkage of constitutive heterochromatin with this chromosome region.

Although the present results indicate a random distribution of chromosomes within the sperm nuclei of Anura, they naturally do not preclude the possibility that individual *non*-heterochromatic chromosome regions must maintain a specific position during spermiohistogenesis and/or in the mature sperm nuclei. Using specific labelling methods, a further analysis of as many different chromosome regions as possible can yield considerable information about the arrangement of chromosomes within the sperm nuclei.

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