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Sex Chromosome Associated Satellite DNA: Evolution and Conservation

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Abstract. Satellites visible in female but not in male DNA were isolated from the snakes *Elaphe radiata* (satellite IV, $p = 1.708\ g \cdot cm^{-3}$) and *Bungarus fasciatus* (BK[1] minor, $p = 1.709\ g \cdot cm^{-3}$). The satellites cross hybridize. Hybridization of ^3H labelled nick translated BK minor satellite DNA with the total male and female DNA and/or chromosomes in situ of different species of snakes revealed that its sequences are conserved throughout the snake group and are mainly concentrated on the W chromosome. Snakes lacking sex chromosomes do possess related sequences but there is no sex difference and visible related satellites are absent. The following conclusions have been reached on the basis of these results. 1. The W chromosome associated satellite DNA is related to similar sequences scattered in the genome. 2. The origin and increment in the number of the W satellite DNA sequence on the W chromosome is associated with the heterochromatinization of the W. 3. Satellite sequences have become distributed along the length of the W and resulted in morphological differentiation of sex chromosomes. 4. Evolutionary conservation of W satellite DNA strongly suggests that functional constraints may have limited sequence divergence.

Introduction

In our earlier study (Singh et al., 1976) we showed that when total DNA of *E. radiata* was centrifuged to equilibrium in Ag^+/Cs_2SO_4 gradients, four major bands were seen in the female and three in the male. Two satellites (I and II) were common to both male and female without any apparent quantitative difference whereas, satellite III appeared as a prominent peak in the female and was poorly represented in the male. We demonstrated by in situ hybridization that satellite III is mainly concentrated on the W sex chromosome and its sequences are conserved throughout the snake group excepting the primitive family Boidae in which these sequences are absent. Satellite IV which was

[1] BK = Banded Krait = *Bungarus fasciatus*

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confined to the ♀ could not be isolated because of its minute quantity in the genome and the limited quantity of female DNA available to us at that time. However, a strong correlation between the appearance of a satellite DNA (satellite III) on the W chromosome and morphological differentiation of sex chromosomes in snakes prompted us to isolate satellite IV because of its apparent female specificity, with the hope that its origin and evolution might contain clues to the evolutionary significance of satellite DNA in general and its role, if any, in sex determination and differentiation in particular. In the present communication we present the analysis of apparently female specific satellite DNA isolated from a non-poisonous species of snake, *E. radiata* (sat. IV) and from a poisonous species of snake, *B. fasciatus* (BK minor). Sequences of these satellites are very similar. We will therefore be concentrating mainly on BK minor.

Materials and Methods

Extraction of DNA. The following are the species which have been used in the present investigation. *Eryx johni johni, Xenopeltis unicolor,* belonging to the family Boidae; *Elaphe radiata, Ptyas mucosus* and *Natrix piscator* (Colubridae); *Vipera russelli russelli* (Viperidae); *Enhydrina schistosa* (Hydrophiidae) and *Bungarus caeruleus, Bungarus fasciatus, Bungarus walliwall, Naja naja naja* (Elapidae) were procured from India; *Notechis scutatus,* tiger snake (Elapidae) from Australia and *Naja naja oxiana* (Elapidae) from Iran. DNA was extracted from blood, liver, kidney, heart tissues and gonad from each individual separately as described earlier (Marmur, 1961) with the inclusion of repeated phenol-chloroform, RNAase and Pronase treatment.

Isolation and Purification of Satellite DNA. The procedure described earlier (Jensen and Davidson, 1966) was used for satellite DNA isolation. *E. radiata* ♀ satellite IV was isolated at a Ag^+ to DNA ratio of 0.20 (Singh et al., 1976) by Cs_2SO_4 gradient centrifugation. Satellites III and IV band so closely that it is very difficult to purify them. However, when the crude preparation of satellite III and IV, after thorough dialysis in 0.1 M Na_2SO_4 is recycled on Cs_2SO_4 gradients at a Ag^+ to DNA ratio of 0.20 the two satellites band widely apart in the gradient which enables their purification by successive CsCl centrifugations in the M.S.E. 8×40 Ti rotor for 80 h at 32 K r.p.m. at 25° C. *B. fasciatus* minor satellite DNA was however, isolated at a Ag^+ to DNA ratio of 0.25 by successive Cs_2SO_4 and CsCl gradient centrifugations. The buoyant densities were determined in neutral CsCl in the Spinco model E analytical centrifuge at +25° C, 44,000 r.p.m. for 20 h using *Micrococcus lysodeikticus* DNA ($\rho = 1.731$ g·cm^{-3}) as density marker. The buoyant densities were, IV = 1.708 g·cm^{-3} and minor = 1.709 g·cm^{-3} respectively.

Labelling of Satellite DNA. The satellite DNA was [^{3}H] labelled by means of the nick translation procedure (Rigby et al., 1977). Equimolar (15–20 μM) of [^{3}H]-dATP (spec. act. 30 Ci/m mol), [^{3}H]-dTTP (spec. act. 30 Ci/m mol), [^{3}H]-dGTP (spec. act. 16.4 Ci/m mol) and [^{3}H]-dCTP (spec. act. 20 Ci/m mol) and 1–2 μg of satellite DNA was used for each reaction.

Filter Hybridization. Male and female DNAs of various species of snakes were denatured and loaded onto filters (HAWP 0.45 μ 13 mm) (Gillespie and Spiegelman, 1965). Each filter contained 0.05 μg of total DNA with 2 μg of *Micrococcus lysodeikticus* DNA as a carrier. Filters containing *M. lysodeikticus* DNA (2 μg/filter) served as controls. The Denhardt procedure (1966) was adapted for DNA-DNA hybridization on filters. The hybridization was carried out for 2 h at 60° C (T_{opt}) (Singh et al., 1979a), nick translated satellite DNA concentration of 2.4×10^{-2} μg/ml in $3 \times$ SSC. After hybridization, filters were washed twice in $2 \times$ SSC 15 min each, dried and counted. Counts hybridized are the average of 3 filters of male, female and control expressed in the Table 1.

Dissociation of DNA-DNA Hybrids. DNA-DNA hybrids were formed at optimal temperature in $3 \times$ SSC and the hybrids were dissociated as described in the legend of Fig. 14.

Hybridization to CsCl Gradient Fractions. Hybridization of *B. fasciatus* minor (BK minor) nick translated satellite DNA to CsCl gradient fractions of *E. radiata* ♀ and *X. unicolor* ♀ was carried out as follows. 40 µg of total DNA with 20 µg *M. lysodeikticus* DNA in 4 ml 0.1 × SSC were made to an initial density of 1.700 $g \cdot cm^{-3}$ with CsCl and centrifuged for 48 h at 42 K r.p.m. in the M.S.E. 10 × 10 rotor. Five drop fractions were collected into 0.5 ml of 0.1 × SSC. 30 µl were withdrawn from each fraction and loaded onto filter discs. The discs were hybridized at T_{opt} (60° C) for 3 h at nick translated DNA concentration of 0.03 µg/ml in 3 × SSC (Denhardt, 1966).

In situ Hybridization. The procedure described by Jones (1973) was used with minor modifications. Slides were heat denatured (Singh et al., 1977). Nick translated DNA was denatured in 0.1 × SSC by heating to 100° C for 5 min, chilled, frozen down rapidly, lyophilized and diluted just before use to the desired volume and salt concentration. 5 µl of nick translated satellite DNA (1.7 µg/ml, spec. act. $2\text{–}4 \times 10^7$ counts/min/µg) was used on each slide. Hybridization was carried out at 60° C (T_{opt}) (Singh et al., 1979) for 3 h. Hybridized slides were treated with nuclease S_1 type III, 6 units/slide in 5 µl of nuclease S_1 buffer (1 mM Z_nSO_4 + 0.1 mM NaCl + 30 mM Na acetate pH 4.5) for 1 h at 39° C, thermally washed in 2 × SSC at 55° C for 1–2 h, washed overnight in 2 × SSC at 4° C, passed through alcohol series and air-dried.

Autoradiography. Hybridized slides were coated with Ilford K2 nuclear emulsion, exposed for 20 days at 4° C, developed in Kodak D19b developer at 5–6° C for 12 min and fixed in Ilford Hypam for 6 min diluted with distilled water (1:4). Slides were stained in Giemsa (2 ml Giemsa + 50 ml of buffer) for 20–40 min, rinsed in buffer pH 6.8 and air-dried. Photographs were taken on Agfa Gevaert Copex Pan 35 mm film using a Zeiss Research microscope with camera attachment.

Chromosome Preparations. Chromosomes were prepared either from monolayer cultures established from lung cells using MEM culture medium supplemented with 10% fetal calf serum or from short-term leukocyte cultures by the usual air-drying procedure. Cultures were treated with colchicine (0.015 µg/ml) for 4 h, 0.075 M KCl for 8–10 min and fixed in 1:3 acetic acid alcohol. Slides were stored in cold room.

C-Banding Procedure. For the staining of constitutive heterochromatin chromosomes were treated for 1 h in 0.2 N HCl at room temperature, 4–10 min in 5% aqueous solution of Barium hydroxide octahydrate at 50° C, 1–5 h in 2 × SSC at 60° C (Sumner, 1972) and stained for 1–5 h in buffered Giemsa (1 ml Giemsa + 50 ml buffer pH 6.8).

Results

Satellite DNA and Sex

When total male and female DNA of BK was centrifuged to equilibrium in Ag^+/Cs_2SO_4 gradients at an Ag^+/DNA phosphate ratio (RF) of 0.25 in the analytical ultracentrifuge both contained an identical amount of a prominent satellite band (major) but the female contained an additional minute satellite band very close to the main band (minor) on the heavy side (Fig. 1) and therefore presumably concentrated on the W chromosome. Similarly *Elaphe* satellite IV was observed only in the female (Singh et al., 1976). Here we are mainly concerned with the BK minor and *Elaphe* satellite IV.

Similarity Between E. radiata Satellite IV and B. fasciatus Minor Satellite DNA

The following evidence strongly suggested that *Elaphe* satellite IV and BK minor satellite DNA sequences were very similar. 1. Both the satellites were

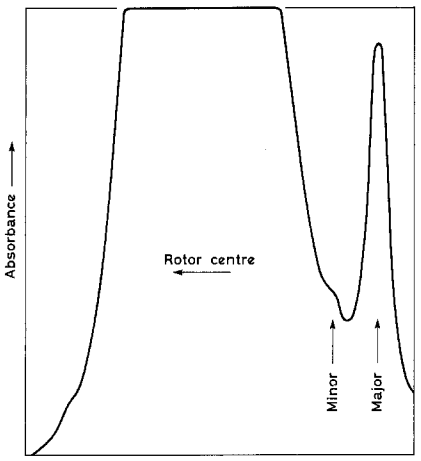

Fig. 1. Analytical equilibrium density gradient centrifugation of total DNA of BK ♀ in Ag^+ Cs_2SO_4 gradient. 70 µg of DNA was centrifuged to equilibrium in Cs_2SO_4/Ag^+ gradients at a Ag^+ to DNA-phosphate ratio (R_F) of 0.25 in the Spinco model E analytical centrifuge at 25 C, 44 K rpm for 20 h. Two satellite bands (major and minor) were seen at the heavy side of the gradient which were isolated by successive Cs_2SO_4 and CsCl centrifugations. The buoyant densities were, major = 1.700, minor = 1.709 $g \cdot cm^{-3}$ respectively

present in the female and apparently absent in the male. 2. Their buoyant densities (p = 1.708 $g \cdot cm^{-3}$ and 1.709 $g \cdot cm^{-3}$ respectively) were nearly identical. 3. Hybridization of nick translated BK minor satellite DNA with the *Elaphe* satellite IV loaded onto millipore filter and vice-versa showed strong hybridization. 4. In situ hybridization of radio labelled probes of both of the satellites with the chromosomes of *E. radiata* ♀ revealed an identical pattern.

Differences Between E. radiata Satellite III and B. fasciatus Minor Satellite DNA

The buoyant density of BK minor satellite DNA (p = 1.709 $g \cdot cm^{-3}$) was significantly different from the buoyant density of *E. radiata* satellite III (p = 1.700 $g \cdot cm^{-3}$) which suggested that the sequences of the two satellites were different. This was substantiated by hybridizing nick translated BK minor satellite DNA with the *Elaphe* satellite III DNA loaded onto millipore filter and vice-versa which failed to reveal any substantial cross hybridization.

Conservation and Sex Specificity of Apparently Female Specific BK Minor Satellite DNA

Presence of BK minor satellite DNA in the ♀ and its apparent absence in the male seemed likely to be due to its concentration of the W chromosome. By analogy with the conservation of the *E. radiata* satellite III DNA sequences throughout the sub-order Ophidia, the possibility of similar conservation of these sequences during the evolution of the W chromosome was checked by cross hybridization of BK minor nick translated satellite DNA with total amounts of male and female DNA (0.05 µg/filter) of *E. johni johni* (Boidae), *P. mucosus, N. piscator* and *E. radiata* (Colubridae), *B. caeruleus, B. walliwall, B. fasciatus, N. scutatus* (Elapidae), *V. russelli russelli* (Viperidae) and total

Table 1. Hybridization of *B. fasciatus* ♀ minor nick translated satellite DNA with the total male and female DNA of various species of snakes, representing different evolutionary states of sex chromosome differentiation, bound to millipore filters. The details are given in materials and methods. The counts hybridized are the average of 3 filters. The relative length of the W chromosome is expressed as percent of the total chromosome length of the male haploid set, including the microchromosomes. The values are the means of 5 metaphase plates corrected to their nearest integers

Species	Family	Counts per min. hybridized			Ratio of ♀/♂ counts of respective species	State of sex chromosomes	% relative length of the W chromosome
		Sex		Control			
		♂	♀				
1. *Eryx johni johni* (Sand boa)	Boidae	717	744	119	1.03	Undifferentiated (primitive)	–
2. *Ptyas mucosus* (Rat snake)	Colubridae	1,852	4,165	116	2.25	Morphologically undifferentiated (intermediate)	8
3. *Elaphe radiata* (Tree snake)	Colubridae	2,429	3,536	137	1.46	Morphologically undifferentiated (intermediate)	8
4. *Natrix piscator* (Water snake)	Colubridae	2,496	5,042	116	2.02	Differentiated	6
5. *Bungarus caeruleus* (Common Indian Krait)	Elapidae	1,861	6,681	166	3.59	Differentiated	11
6. *Bungarus walliwall* (Krait)	Elapidae	–	5,566	137	–	Differentiated	12
7. *Bungarus fasciatus* (Banded Krait)	Elapidae	1,108	1,742	59	1.57	Differentiated	4
8. *Notechis scutatus* (Tiger snake)	Elapidae	2,963	10,253	158	3.46	Differentiated	Not known
9. *Naja naja oxiana* (Cobra)	Elapidae	–	2,348	55	–	Differentiated	7
10. *Vipera russelli russelli* (Russell's viper)	Viperidae	1,621	1,932	158	1.19	Differentiated	5

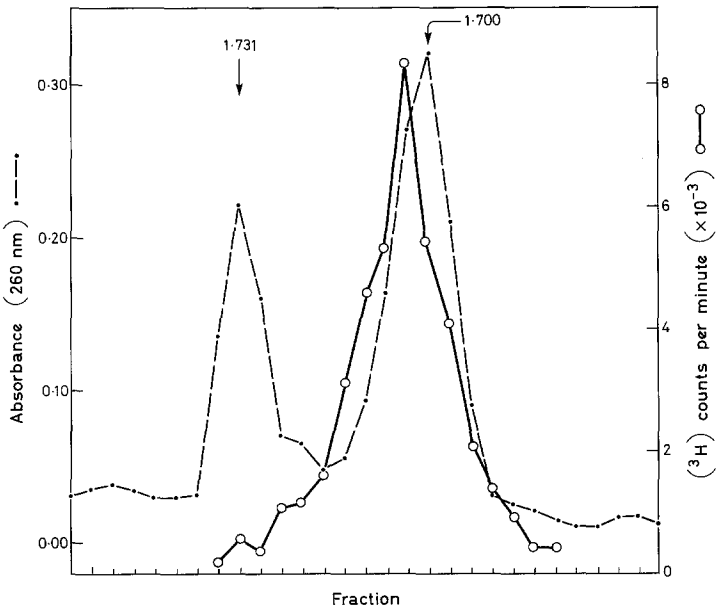

Fig. 2. Hybridization of *B. fasciatus* minor (B.K. minor) nick translated satellite DNA to CsCl gradient fractions of *E. radiata* ♀ DNA showing hybridization with a distinct component (probably satellite IV) on the heavy side of the main band. The sex chromosomes in this species are homomorphic but differentiated at molecular level

female DNA of *N. naja oxiana* (Elapidae) bound to millipore filters. There were significant counts above the background level on the female as well as male filters of *E. johni johni* with no significant sex difference (Table 1). However, twice as many counts were observed on the female filters as on the male of *P. mucosus* and *N. piscator;* 1.5–1.6 times as many counts on the female filters as on the male of *E. radiata* and *B. fasciatus;* 3–4 times as many counts on the female filters as on the male of *B. caeruleus* and *N. scutatus* and 1.2 times as many counts on the female filters as on the male of *V. russelli russelli* (Table 1). There were significantly high counts on the female filters of *B. walliwall* and *N. naja oxiana* (Table 1). However, no males of these species were examined. The high number of heterologous hybrid counts obtained (Table 1) shows the presence of related sequences in a large number of species belonging to various families (Boidae, Colubridae, Elapidae and Viperidae). Hybridization of BK minor satellite DNA to male DNA in these species is in contrast to the result of analytical centrifugation of male and female DNA of *B. fasciatus* which shows the apparent absence of BK minor in the male (Fig. 1). The same is true for *E. radiata* satellite IV (Singh et al., 1976).

Hybridization of BK minor nick translated satellite DNA across an isopycnic neutral CsCl gradient of *E. radiata* female DNA showed hybridization with a distinct DNA component (presumably satellite IV) at the heavy side of the main band (Fig. 2). Hybridization across an isopycnic neutral CsCl gradient of *X. unicolor* female DNA however, revealed hybrid formation at the buoyant density corresponding to the main band DNA ($p = 1.700\ g \cdot cm^{-3}$) (Fig. 3). This

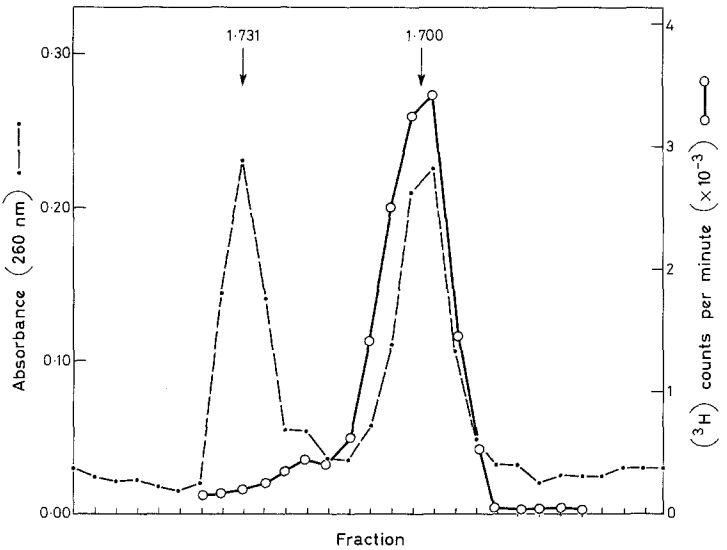

Fig. 3. Hybridization of B.K. minor nick translated satellite DNA to isopycnic neutral CsCl gradient fractions of a primitive snake *X. unicolor* belonging to the primitive family Boidae in which sex chromosomes are in primitive state of differentiation. Note hybrid formation at the buoyant density corresponding to the main band DNA ($p = 1.700\ g \cdot cm^{-3}$)

is a species of snake in which sex chromosomes are in a primitive state of differentiation and there is no sex difference in the number of hybrid counts obtained on filters (see *E. johni johni*, Table 1). This suggests that in primitive snakes satellite sequences similar to BK minor satellite DNA do not band as a distinct satellite component and probably are dispersed in main band DNA.

Chromosomal Location of BK Minor Satellite in situ

The above studies revealed that sequences related to BK minor satellite DNA are conserved throughout the sub-order Ophidia and the difference in their content between male and female seemed likely to be due to their concentration on the W chromosome. Accordingly we hybridized BK minor nick translated satellite DNA with the chromosomes of *E. johni johni* female of the primitive family Boidae, of *N. piscator* female, *P. mucosus* female and *E. radiata* female (family Colubridae), of *B. caeruleus* female *B. walliwall* female, *B. fasciatus* female, *N. naja naja* female and *N. naja oxiana* female (family Elapidae), of *E. schistosa* female (family Hydrophiidae) and of *V. russelli russelli* female (family Viperidae). With the exception of *E. radiata, B. walliwall* and *N. naja oxiana* which are included in the present study (Figs. 4–6), karyotype analyses of these species have been described earlier (Singh et al., 1968a, 1968b, 1970; Singh, 1972a, 1972b, 1974). More than 100 hybridized metaphase spreads were analysed in each case. In the case of *E. johni johni* female, grains were scattered on most of the chromosomes with no sign of any differential concentration on a particular chromosome (Fig. 7). This is in agreement with the absence of sex difference in the hybrid counts obtained on filters (Table 1) and hybridiza-

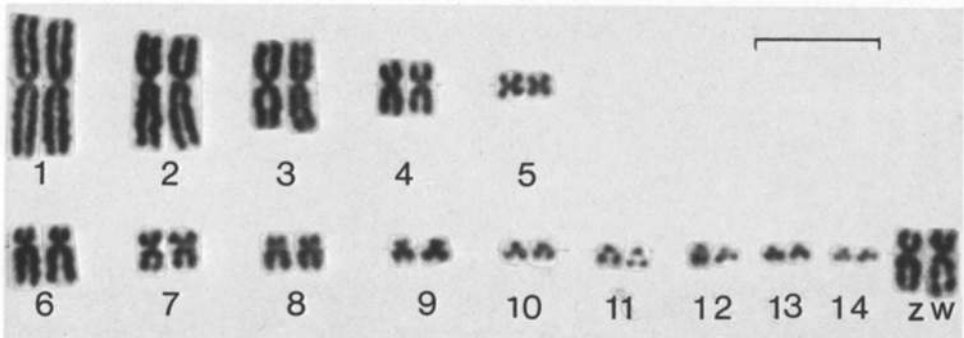

Fig. 4. Female karyotype of *Elaphe radiata* (2n=30) from short term leukocyte culture showing homomorphic Z and W chromosomes. Bar=10 μ

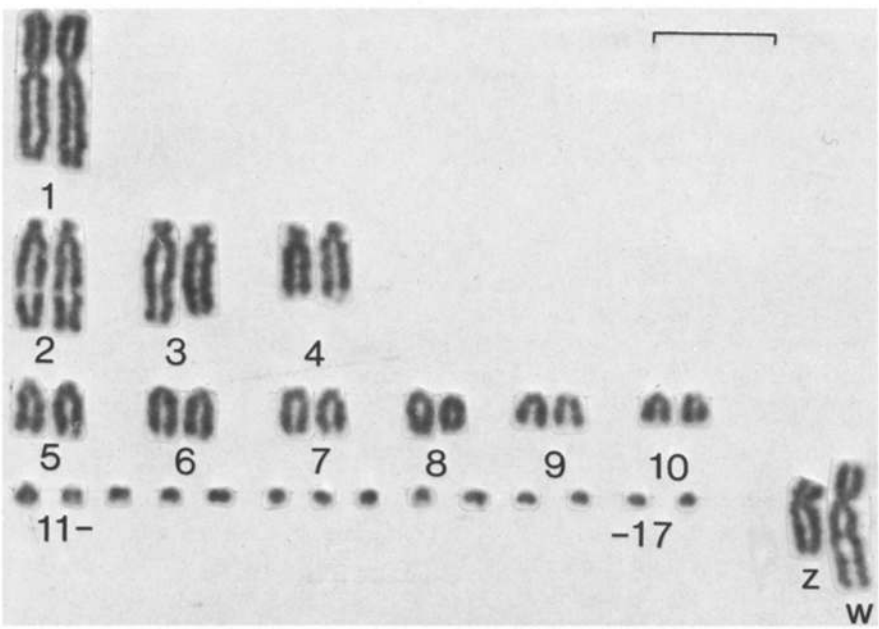

Fig. 5. Female karyotype of *Bungarus walliwall* (2n=36, 22 macro and 14 microchromosomes) from short term leukocyte culture showing differentiated sex chromosomes. The W chromosome is unusually large. Bar=10 μ

tion at the buoyant density corresponding to the main band DNA across an isopycnic neutral CsCl gradient (Fig. 3). In the case of *N. piscator* and *P. mucosus,* grains were highly concentrated along the entire length of the W chromosome (Fig. 8 b, d), which could unequivocally be identified by C-banding (Fig. 8 a, c). On longer exposure scattered grains were also observed on most of the chromosomes (Fig. 8 b, d). It should be pointed out that unlike the highly differentiated W chromosome of other species, the W chromosome in

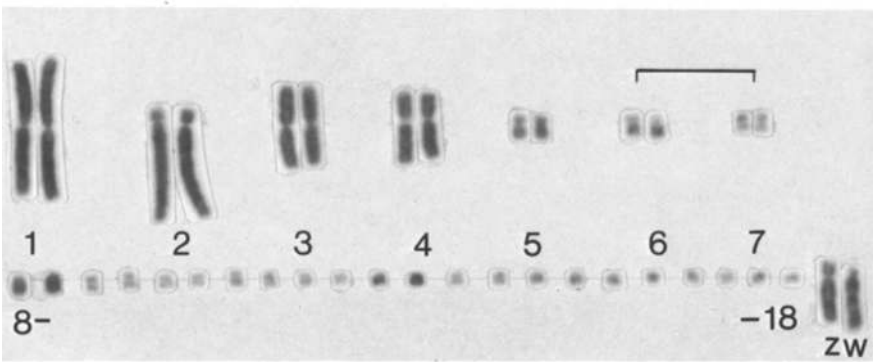

Fig. 6. Karyotype of *Naja naja oxiana* female from short term leukocyte culture (2n = 38, 16 macro and 22 micro-chromosomes). The Z and the W are similar in size but different in morphology. Bar = 10 μ

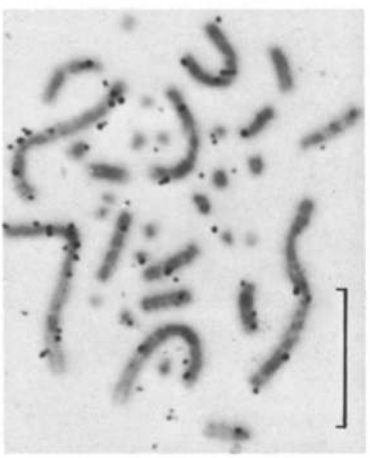

Fig. 7. Cross hybridization of BK minor nick translated satellite DNA with the metaphase chromosomes of *Eryx johni johni* ♀ (Boidae) after 20 days exposure. Note the absence of any differential concentration of grains on a particular chromosome. Bar = 10 μ

P. mucosus represents an intermediate state between non-differentiated and highly differentiated sex chromosomes (Singh et al., 1976). In this species the Z and the W chromosomes are homomorphic (Singh, 1972a) and the W chromosome does not show very intense C-banding (Fig. 8c). In spite of their otherwise 'primitive' features, for example absence of asynchrony in DNA replication or formation of a W chromatin body in interphase nuclei (Ray-Chaudhuri and Singh, 1972; Ray-Chaudhuri et al., 1971), the sex chromosomes in this species as revealed by in situ hybridization studies are differentiated at the molecular level. In *Elaphe radiata,* unlike the other snake species, there is no sharp distinction between the macro and microchromosomes. Like *P. mucosus,* in this species sex chromosomes are morphologically indistinguishable (Fig. 4) but could unequivocally be identified by C-banding, which intensely stains the W chromosome along with the centromeric regions of all other chromosomes (Fig. 9a). In this species grains were highly concentrated in an intercalary region

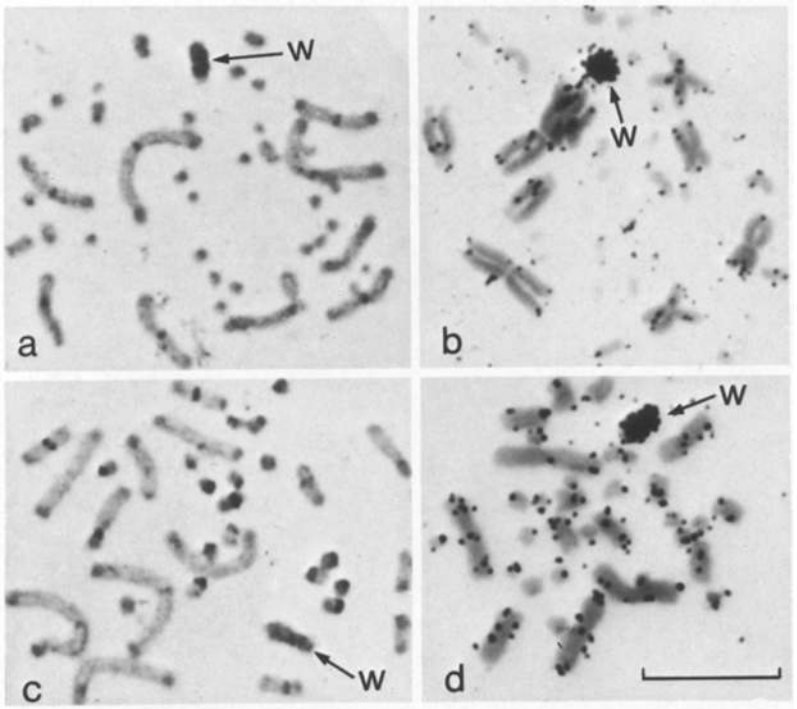

Fig. 8a–d. Cross hybridization of BK minor nick translated satellite DNA with metaphase chromosomes of **b** *Natrix piscator* ♀ (Colubridae), **d** *Ptyas mucosus* ♀ (Colubridae) after 20 and 25 days exposure, respectively. Grains are highly concentrated all along the W chromosome and are also present but scattered on most of the chromosomes. **a and c** Metaphase chromosomes showing C-banding. **a** *Natrix piscator* ♀, **c** *Ptyas mucosus* ♀. Apart from centromeric C-banding in other chromosomes the entire W chromosome is differentially stained. Bar = 10 μ

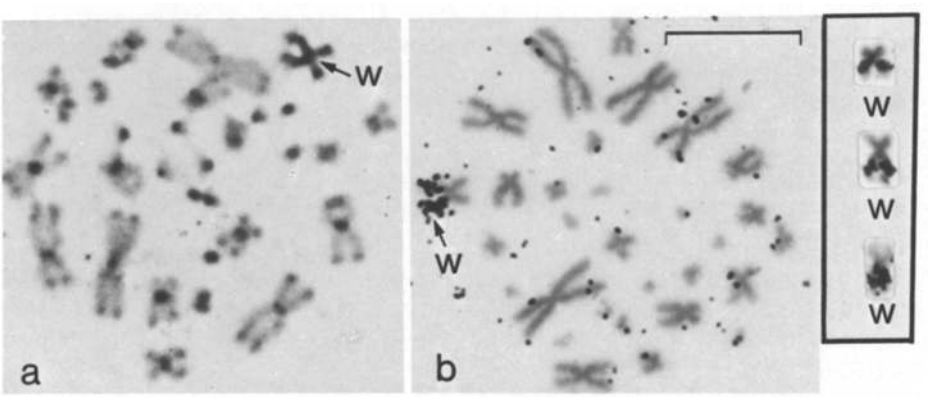

Fig. 9. a Female metaphase chromosomes of *Elaphe radiata* showing C-banding. The centromeric region of all the macro and the microchromosomes are darkly stained excepting one of the macrochromosomes which is entirely C-band positive. This is the W chromosome which otherwise cannot be distinguished from the Z. **b** Cross hybridization of B.K. minor nick translated satellite DNA with the metaphase chromosomes of *Elaphe radiata* ♀ after 25 days exposure. Grains are mainly concentrated in a small intercalary region of the long arm of a macrochromosome (better shown in the inset from 3 different metaphase plates). On the basis of its size and morphology this appears to be the same chromosome which is entirely C-band positive (see **a**). There are some grains scattered on other chromosomes also. Bar = 10 μ

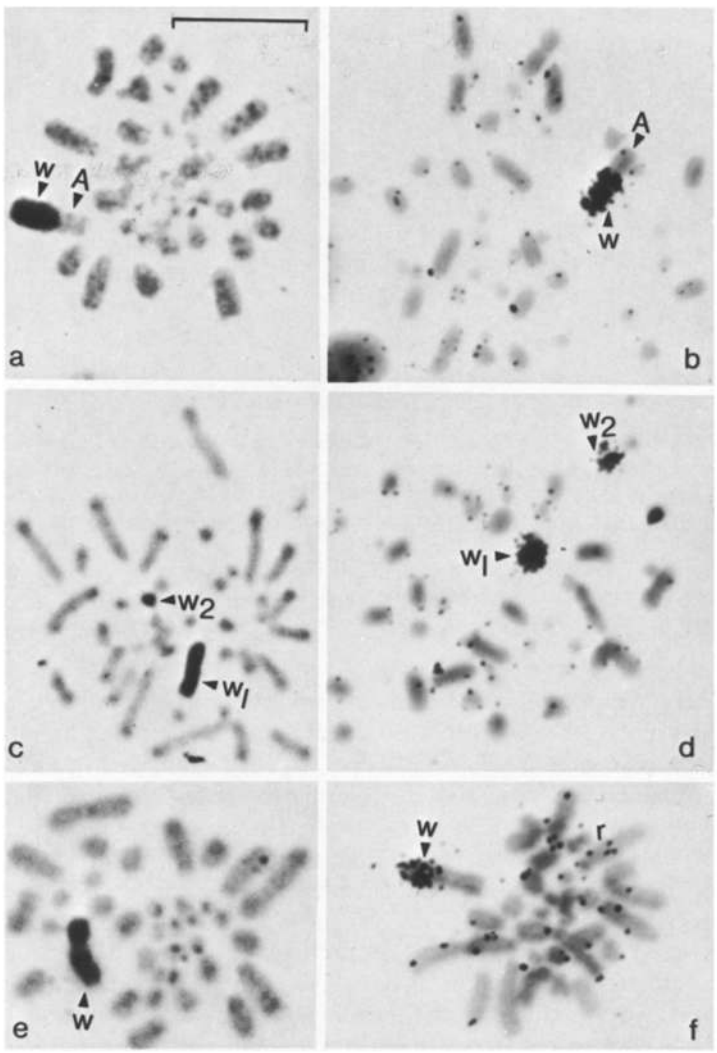

Fig. 10a–f. Cross hybridization of BK minor nick translated satellite DNA with metaphase chromosomes of **b** *Bungarus caeruleus* ♀ (Elapidae), **d** *Bungarus caeruleus* ♀ (polymorphic), **f** *Bungarus walliwall* ♀ (Elapidae) after 20 days exposure. Grains are mainly concentrated in the long arm of the W chromosome in **b**, all along the W_1 and W_2 chromosomes in **d** and in the long arm of the W chromosome in **f**. There are scattered grains on other chromosomes also. **a, c and e** Metaphase chromosomes showing C-banding. **a** *Bungarus caeruleus* ♀. C-banding is exclusively localised in the long arm of the W chromosome. This species has a multiple sex chromosome constitution of $Z_1Z_1Z_2Z_2♂/Z_1Z_2W♀$ type (Singh et al., 1970). C-band negative short arm of the *W* chromosome (*A*) represents a translocated autosome which does not seem to be affected by the constitutive heterochromatin of the long arm of the W chromosome. **c** *Bungarus caeruleus* ♀ polymorphic individual. In this case the original W chromosome has undergone fission resulting into two W chromosomes (W_1 and W_2) both of which are entirely C-band positive (Singh et al., 1979) and hybridize with B.K. minor nick translated satellite DNA probe suggesting that both the W chromosomes are similarly constituted. **e** *Bungarus walliwall* ♀. Apart from minute centromeric region of 4-pairs of microchromosomes the unusually large W chromosome is entirely C-band positive. BK minor satellite DNA sequences however, show hybridization only to a portion of the long arm of the W chromosome (see **f**). Bar = 10 μ

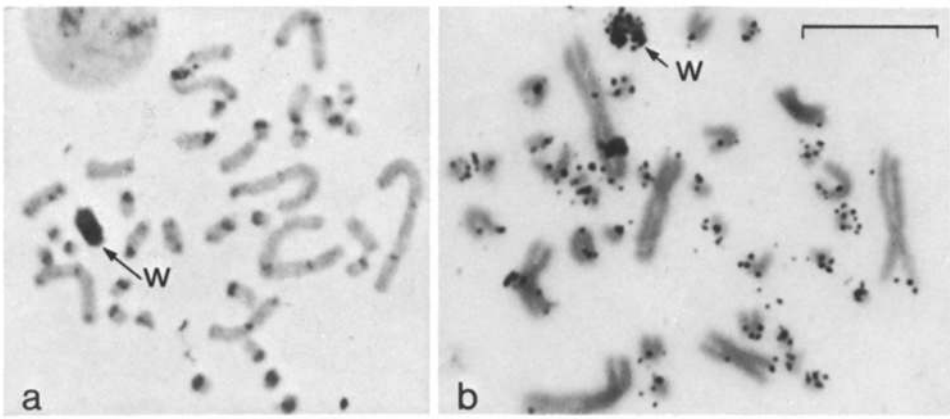

Fig. 11 a and b. In situ hybridization of BK minor nick translated satellite DNA with metaphase chromosomes of **b** *Bungarus fasciatus* ♀ (Elapidae) (the same species) after 20 days exposure. Grains are highly concentrated along the lengths of the W chromosome. There is concentration of grains in centromeric region of most of the microchromosomes. This is because of slight contamination of a major autosomal satellite DNA (BK major) which is exclusively localised in the microchromosomes (Singh et al., 1979). **a** *Bungarus fasciatus* ♀ metaphase chromosomes showing C-banding. Note the darkly stained entire W chromosome apart from centromeric C-banding in other chromosomes. Bar = 10 μ

of the long arm of the W chromosome. The short arm and adjacent and distal regions of the long arm of the W chromosome were devoid of any grains (Fig. 9b and the W chromosome in the inset). The extent of grain distribution on the W chromosome in this species is compatible with the low ratio of female/male hybrid filter counts (Table 1). *B. caeruleus* is a poisonous species of snake in which females are polymorphic (Singh et al., 1979b). In one type of female a medium sized macrochromosome is translocated with the W chromosome resulting in a multiple sex chromosome constitution of Z_1Z_2W ($2n=43$). In this case grains were exclusively localized on the C-band positive long arm of the W chromosome, representing the original W, leaving the short arm of the W chromosome, corresponding to the translocated autosome, completely unlabelled (Fig. 10a, b). In another type of female grains were distributed along the entire length of one macro and one microchromosome which were entirely C-band positive representing the W_1 and W_2 sex chromosomes respectively (Fig. 10c, d) (Singh et al., 1979b). Hybridization of W specific satellite DNA probe to both of the Ws (W_1 & W_2) suggests their similar constitutions. In *B. walliwall* the entire W and centromeric regions of 4 pairs of microchromosomes were C-band positive (Fig. 10e). Hybridization was mainly confined to the distal region of the long arm of the W chromosome (Fig. 10f). In the case of *B. fasciatus* grains were highly concentrated on the W chromosome (Fig. 11b) which could unequivocally be identified by C-banding (Fig. 11a). Grains were also observed in the C-band positive centromeric regions of most of the microchromosomes (Fig. 11b). These are the sites where BK major autosomal satellite DNA sequences are localised (Singh et al., 1979a). This suggested contamination of BK minor with BK major sequences. This was confirmed

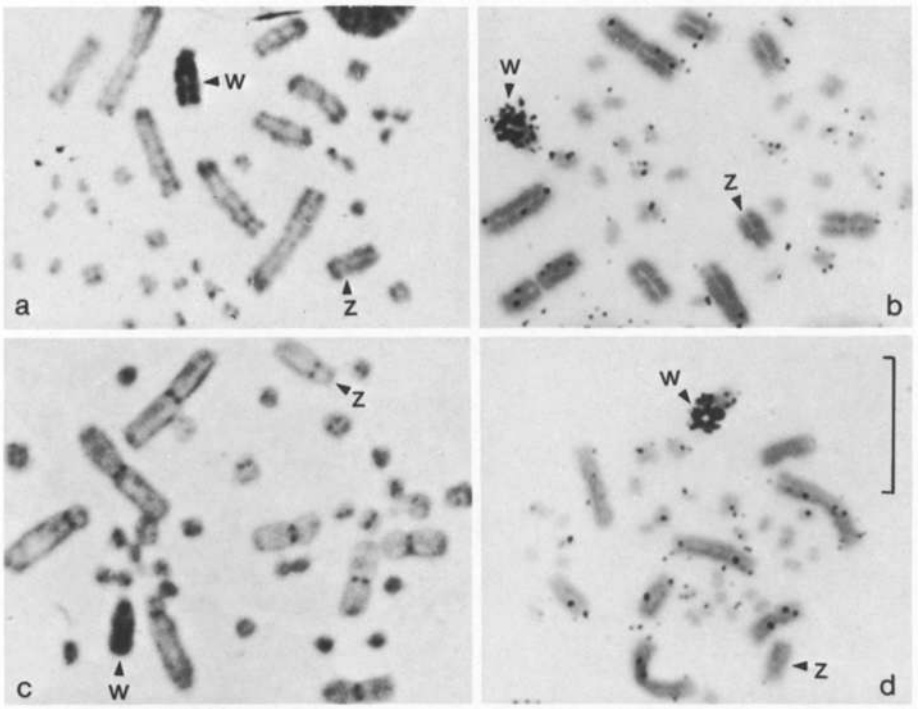

Fig. 12a–d. Cross hybridization of BK minor nick translated satellite DNA with metaphase chromosomes of **b** *Naja naja naja* ♀. **d** *Naja naja oxiana* ♀ after 20 days exposure. Grains are predominantly concentrated in the long arm of the sub-telocentric W chromosome leaving the short arm of the W unlabelled. There are some grains scattered on most of the chromosomes also. **a and c** Metaphase chromosomes showing C-banding. **a** *Naja naja naja* ♀ (Elapidae) **c** *Naja naja oxiana* ♀ (Elapidae). Apart from centromeric C-banding in other chromosomes the entire W chromosome is C-band positive in both the species. Bar = 10 μ

by hybridization of BK major satellite DNA loaded onto filters with nick translated BK minor satellite DNA in solution and vice versa. The contamination was less than 2% (not shown). BK major satellite DNA sequences are not conserved even in closely related species (unpublished data). Heterologous hybridization data therefore, are not affected by this slight contamination. In the case of *N. naja naja* and *N. naja oxiana* grains were predominantly concentrated in the long arm of the sub-telocentric W chromosome, leaving the minute short arm of the W unlabelled (Fig. 12b, d) though in both species the entire W chromosome was positively stained by C-banding (Fig. 12a, c). It should be mentioned that in *N. naja naja* sex chromosomes are homomorphic (Singh, 1972a) but the W chromosome could be identified by its characteristic pattern of early DNA synthesis (Ray-Chaudhuri et al., 1970), C-banding and hybridization with the sex chromosome specific DNA probe suggesting that it is differentiated at the molecular level. In *V. russelli russelli* the W chromosome is unequivocally identified by its minute size, morphology and early DNA replication pattern (Ray-Chaudhuri and Singh, 1972). Grains were mainly concentrated

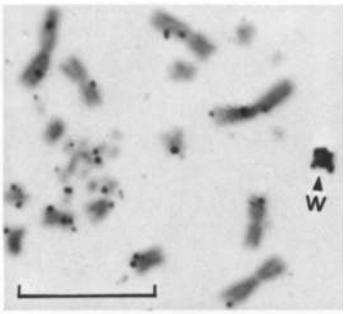

Fig. 13. Cross hybridization of BK minor nick translated satellite DNA with metaphase chromosomes of *Vipera russelli russelli* ♀ (Viperidae) showing high concentration of grains over the entire W chromosome which is one of the smallest W chromosome in the snakes known so far. There are few scattered grains on other chromosomes also. Bar = 10 μ

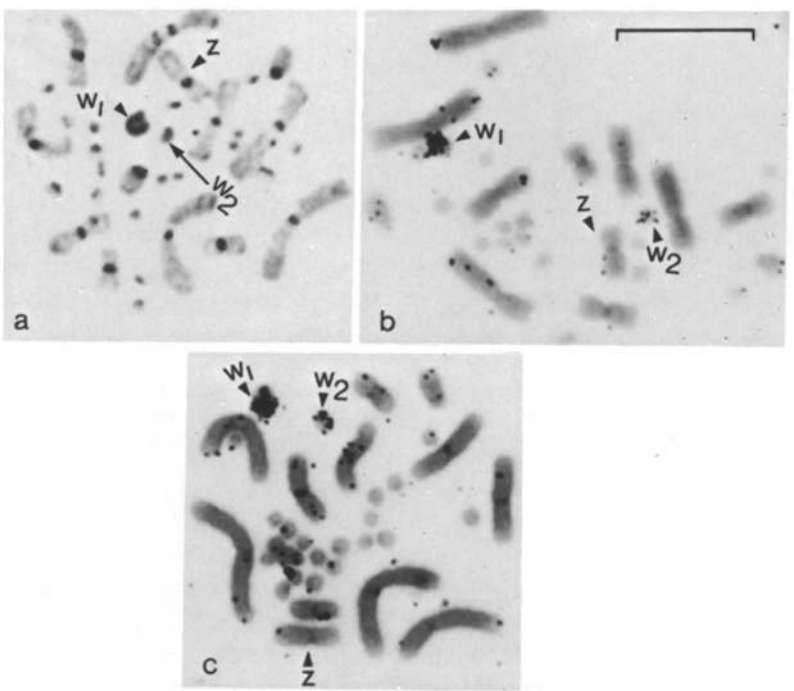

Fig. 14 a–c. Cross hybridization of BK minor nick translated satellite DNA with metaphase chromosomes of *Enhydrina schistosa* ♀ (Hydrophiidae). **b and c** Grains are predominantly concentrated on a small macro and a minute microchromosome which are recognised as W_1 and W_2 chromosomes respectively. There are few grains scattered on other chromosomes also. **a** Female metaphase chromosomes of *E. schistosa* showing C-banding. Centromeric region of all the macro and the microchromosomes are prominently C-band positive excepting the W_1 and the W_2 which are entirely darkly stained. The W_1 and the W_2 show hybridization along their length with B.K. minor satellite DNA probe suggesting that both are similarly constituted. Bar = 10 μ

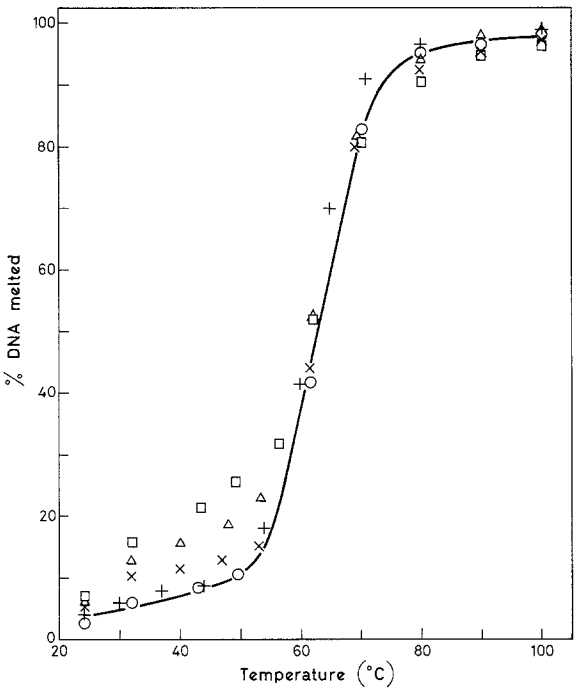

Fig. 15. Melting temperature profiles of DNA-DNA hybrids of B.K. minor nick translated satellite DNA and total ♀ DNA of ○—○ *Bungarus fasciatus* (Elapidae); □ *Xenopeltis unicolor* (Boidae); × *Vipera russelli russelli* (Viperidae); △ *Elaphe radiata* (Colubridae); + *Natrix piscator* (Colubridae). 0.05 µg of total female DNA of different species of snakes and 2 µg of *micrococcus lysodeikticus* DNA as a carrier were loaded onto millipore filter and hybridized with nick translated BK minor satellite DNA at optimal temperature (60° C) for 2 h at nick translated satellite DNA concentration of 2.4×10^{-2} µg/ml in 3 × SSC. The Denhardt procedure (1966) was used. After hybridization filters were washed twice in 2 × SSC, 15 min each and vacuum dried. To determine the temperature at which 10% of the hybrid were dissociated from the filter (T_m), the filters were placed in 1 ml of 1 × SSC and heated in temperature increments of approximately 8° C. After 5 min at each temperature, the released DNA was recovered by trichloroacetic acid precipitation (by using bovine serum albumin 0.1 mg/ml as a carrier), loaded on millipore filter and counted (Birnstiel et al., 1972)

along the length of the W chromosome (Fig. 13). Some grains were observed scattered on other chromosomes also. In *E. schistosa,* in addition to being scattered on most of the chromosomes, grains were concentrated on W_1 and W_2 chromosomes (Fig. 14b, c) which were also found to be entirely C-band positive (Fig. 14a). This strongly substantiates the view that multiple W chromosomes have originated by centromeric dissociation of the original W chromosome and that the W_1 and W_2 are similarly constituted (Singh, 1972a, 1972b).

Filters containing total ♀ DNA of *X. unicolor, E. radiata, N. Piscator, B. fasciatus* and *V. russelli russelli* hybridized with BK minor nick translated satellite DNA were used to construct melting profiles of the hybrid in 1 × SSC. The homologous hybrid yielded a sharp monophasic melting curve consistent with a single component. 50% of the homologous, as well as heterologous hybrids melted at 64° C suggesting that many of the heterologous hybrid molecules

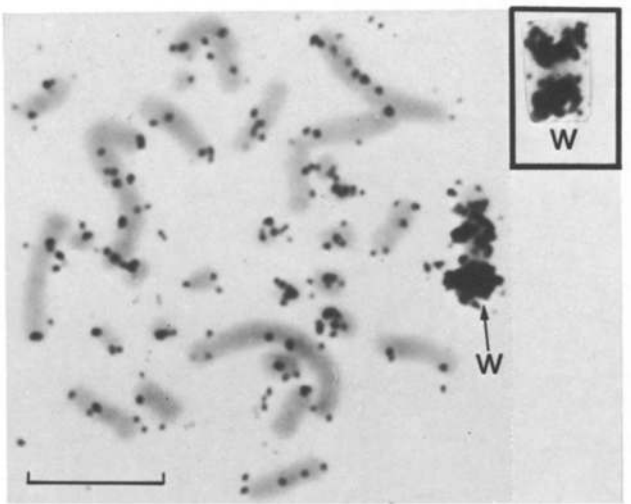

Fig. 16. In situ hybridization of nick translated *Elaphe radiata* ♀ satellite III DNA with metaphase chromosomes of the same species after 20 days exposure. Note conspicuous hybridization in telomeric region of both the arms of the W chromosome. The W chromosome in the inset from another metaphase plate demonstrates this pattern more clearly. There are scattered grains and other chromosomes also. BK minor satellite DNA sequences are localised only in a minute inter-calary region of the long arm of the W chromosome in this species (see Fig. 9b). Bar = 10 μ

are well matched (Fig. 15). However, the melting profile at lower temperatures reflect stability differences between homologous and heterologous hybrids consistent with nucleotide divergence (Fig. 15). Melting profiles of male and female homologous hybrids (male profile not shown) were identical. This suggests that BK minor sequences are conserved irrespective of their chromosomal locations.

Discussion

The nucleotide sequences represented in *Elaphe radiata* female satellite IV/BK minor satellite DNA are recoverable only from females but hybridize to male DNA on filters. This is perhaps because their minute quantity in the male is beyond optical resolution or because interspersion in main band DNA does not permit separation as a satellite band. Another explanation is that these sequences are present only in the W chromosome and the hybridization which we see to males DNA and to DNA of both sexes of primitive snakes reflects a relatively low homology with some other non satellite repeated DNA. However, this latter explanation seems to be less likely because all of the homologous and heterologous hybrids examined yielded a sharp monophasic melting profile suggesting that most of the heterologous hybrid molecules are quite well matched.

Evolution of W Satellite

The evolution of W satellite DNA can be inferred from its cross hybridization with the female DNA of primitive snakes, *E. johni johni* and *X. unicolor,* having undifferentiated sex chromosomes and from the scattered pattern of distribution of hybrid molecules in situ in most of the macrochromosomes of these species. Leaving aside the question of origin of these sequences in the primitive snake (see for example models by Smith, 1976; Keyl, 1965; Maio et al., 1977), it is clear that geometric increments in the amount of this sequence have subsequently occurred on a macrochromosome which we now identify as the W sex chromosome. Our in situ hybridization studies have shown hybridization of BK minor nick translated satellite DNA along the entire length of the W chromosome in most of the species with a few exceptions particularly *E. radiata.* In this species the Z and the W chromosomes are homomorphic but the W chromosome is distinguished by its characteristic C-banding. BK minor satellite DNA sequences hybridize only to a minute intercalary region of the long arm of the W chromosome (Fig. 9). Similarly *E. radiata* satellite III, another W specific satellite DNA, which hybridizes along the entire length of the W chromosome in most of the species (Singh et al., 1976), shows hybridization in discrete telomeric regions of both the arms of the W chromosome in *E. radiata* (Fig. 15). These observations suggest that the origin and tandem multiplication of satellite DNA in a specific region of the W chromosome may be the initial step of the evolutionary process of sex chromosome differentiation. During the course of evolution these sequences on the W chromosome were also distributed throughout its length by internal rearrangements (e.g., inversion, unequal sisterchromatid exchange). This is suggested by the fact that in highly evolved species of snakes, BK minor and *E. radiata* satellite III DNA sequences also are distributed along the length of the W chromosome (Singh et al., 1976 and present investigation) indicating that these two W satellites are now intimately interspersed.

Evolution of Sex Chromosomes

The chromosomal sex determining mechanism evolved very early in vertebrate evolution, probably more than 300 million years ago. Yet both the morphological appearance of chromosomes and the behaviour of sex-linked genes indicate that in certain lines of vertebrate evolution, a pair of chromosomes which evolved as determiners of the opposite sexes in other species (the X and the Y in male heterogamety, as well as the Z and the W in female heterogamety) remained largely structurally homologous to each other. In these less evolved vertebrates, sex is apparently determined by a few pairs of alleles which are largely under environmental control (Ohno, 1967; Bull and Vogt, 1979). Differentiation of the chromosomes bearing sex determining genes, resulting in the evolution of distinct Z and W and X and Y chromosomes, must have resulted from restrictions on recombination between the originally homologous chromo-

somes, thus reinforcing the isolation of the sex gene alleles and stabilising the sex ratio. Structural rearrangements or genes specifically suppressing crossing-over in the sex bivalents have been suggested as the cause of such restriction on crossing-over (Ohno, 1967; Bowen, 1965). The investigation of Ray-Chaudhuri et al. (1971) however, led to the conclusion that the primary step of the W chromosome differentiation was the heterochromatinization of one of the two sex chromosomes. This view has been substantiated by recent observations of Schmidt et al. (1979) that heterochromatinization of telomeres in the long arm of one homologue of the sex chromosome pair in the male is accompanied by a reduced crossing-over frequency between the long arms in male meiosis of *Triturus alpestris alpestris* and by complete abolition of crossing-over between the long arms of *Triturus vulgaris vulgaris*. In our earlier studies we showed that distribution of specific satellite DNA in the W chromosome precedes morphological differentiation of sex chromosomes in snakes suggesting that this is an early step in the process (Singh et al., 1976). The origin of satellite DNA in a discreet region of the W chromosome may be the initial step of the differentiation process.

Why Have Not All Species Developed Chromosomal Sex?

Chromosomal sex determination has led to the genetic deterioration of major genes of the Y and the W chromosomes leaving their alleles on the X and the Z chromosomes respectively in the hemizygous condition which necessitated the evolution of dosage compensation in Drosophila and mammals. If a discrete gene locus was involved in sex determination it is not obvious why structural isolation should have occurred between the X and Y, Z and W and why only some vertebrates have evolved in this manner. A theoretical possibility would be that the sex determining alleles in vertebrates in certain cases covered a larger segment of the chromosomes involved than in others. This could have arisen from gene duplication or from a change in the intronic structure of single alleles. Such alterations would tend to disturb the sex ratio by increasing the probability of crossing-over within the gene or within the gene cluster. This might either destroy the functions of the sex genes or produce a population with a range of sex gene dosage and would be moderated and perhaps prevented by the supervention of mechanisms which limited crossing-over in such chromosomes. The mechanisms leading to these initial alterations in the sex genes themselves may have been those which give rise to repeated sequences generally (e.g. Smith, 1976; Maio et al., 1977), so that the reduplication of these genes could have paralleled the generation of satellite DNA, which in turn, may have modified the recombinational structure of these chromosomes leading to isolation and the accelerated accumulation of satellite DNA by such chromosomes. Ohno (1967) has suggested that the sex genes of mammals are multiple and it is a common finding that sex chromosomes are heterochromatic. Multiple sex genes and satellite DNA may, according to this model, have evolved in synchrony. Similar tendencies, which we must suppose exist in all chromosomes, would be checked because of the non-adaptive drift in other important genes and chromosomes which would result from cessation of crossing-over. A chromo-

some or chromosomal region prevented from crossing-over with its homologue and kept permanently heterozygous, tends to accumulate mutations which are neutral because they can never become homozygous (Muller, 1914, 1918; Charlesworth, 1978), such mutation causing the loss of the function of a locus from wild type to recessive alleles is unopposed by natural selection. Over a long evolutionary period of time, the W or the Y chromosome or their differential segment, becomes fixed for recessive loss of functional alleles at most of their loci depending upon functional constraints. Because of the above consequences of sex chromosome differentiation it appears that only those species which succeeded in accumulating opposing sex determining factors on a particular pair of homologous chromosomes, the genes of which could functionally adapt to function in a permanent heterozygous condition, were able to develop chromosomal sex determination. There is no direct evidence for this view. However, the remarkable genetic conservation of the X chromosome in mammals (Ohno, 1967) and the Z chromosome in snakes and birds (Singh, 1972a; Ohno, 1967) which have successfully evolved chromosomal sex determination, and extremely variable and inconsistent occurrence of the X chromosome in some species of lizards and its absence in others, and absence of differentiated sex chromosomes even in the same species in different population (Singh, 1974; Gorman, 1973) suggesting their failure to evolve stable chromosomal sex determination could be consistent with the above view.

W Satellite Content in Relation to the Size of the W Chromosome

Contrary to the remarkable conservation of DNA content of the Z chromosome the relative length of the W chromosome varies between 4% and 27% in different species of snakes (Singh, 1972a). Is there any relationship between BK minor satellite DNA content and relative length of the W chromosome? It is apparent from Table 1 that the ratio of ♀/♂ counts hybridized per unit of DNA bound onto millipore filter generally varies according to the size of the W chromosome but this variation does not always seem to be directly proportional to the size of the W chromosome. For instance the relative length of the W chromosome (including the microchromosomes) of *E. radiata* and *N. piscator* is 8% and 6% respectively whereas the ratio of ♀/♂ hybrid counts is 1.46 and 2.02 respectively. Similarly the relative length of the W chromosome of *V. russelli russelli* and *B. fasciatus* is 5% and 4% respectively whereas, the ratio of ♀/♂ hybrid counts on filters is 1.19 and 1.57 respectively. These observations suggest that some other DNA component(s) is perhaps largely responsible for variation in size of the W chromosome. In *B. walliwall* this indeed appears to be the case. In this species the relative length of the metacentric W chromosome is 12% which is entirely C-band positive. Sequences similar to *E. radiata* satellite III (unpublished observation) and BK minor satellite DNA (present study) are exclusively concentrated in the long arm of the W chromosome. The short arm of the W may contain repetitive DNA sequences specific for the species which may account for the large size of the W chromosome. It should be pointed out that the W chromosome in this species is larger than the Z which represents 9% of the haploid length. A similar situation exists in *B. caeruleus*

where the W chromosome occupies 11% of the haploid length and is considerably larger than the Z which represents 9% of the total haploid length. Occurrence of a W chromosome larger than the Z has been considered to be relatively primitive state of sex chromosome differentiation and to be the result of accidental crossing-over between the Z and the W chromosome (Ohno, 1967). The present studies, however, have shown the presence of unusually large W chromosome in two poisonous species of snakes belonging to highly evolved family Elapidae. The W chromosome in both species is highly differentiated both morphologically as well as at the molecular level. These observations make earlier interpretations unlikely and alternatively suggest that the origin and tandem multiplication of novel repetitive DNA sequences on the W chromosome are responsible for its large size.

In summary the present observations reveal the following pertinent facts with regard to the evolution of BK minor satellite DNA and to the differentiation of sex chromosomes.

1. Sequences of BK minor satellite DNA are present scattered in the genome of both sexes in snakes belonging to the family Boidae in which sex chromosomes are in a primitive state of differentiation, suggesting that W satellite originated from pre-existing similar sequences dispersed in the genome (Salser et al., 1976).

2. Once satellite DNA originated in a specific region of the W chromosome its tandem multiplication followed by internal chromosomal rearrangements seems to have distributed these sequences along the length of the W chromosome. This is associated with the heterochromatinization of the W and morphological differentiation of sex chromosomes.

3. Unusually long evolutionary persistence of BK minor satellite DNA suggests that functional constraints may have restricted sequence divergence.

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