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Evolution of Sex-chromosomes and Formation of W-chromatin in Snakes*

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Abstract. The analysis of sex-chromosome complexes and formation of W-chromatin in 16 species of snakes of the families *Boidae*, *Colubridae*, *Elapidae*, and *Hydrophiidae*, reveal three very pertinent facts. First, the snakes exhibit various states of the differentiation of the Z and W chromosomes, apparently according to the evolutionary status of the families, being homomorphic in primitive families and well differentiated in highly evolved ones. Second, the demonstration of a heteropycnotic body in the interphase nuclei of the families of a large number of species of snakes has definitely shown that the nuclear sexing is possible not only in those species of snakes where the W chromosome is morphologically distinguishable from the Z, but also in those species where it is not so, but shows an asynchrony in the replicating pattern of W. It is suggested that development of allocycly rather than establishment of structural changes is the first step in the differentiation of the W from the Z in snakes. Third, the absence of coexistence of nucleolus and W-chromatin in a condensed state in the interphase nuclei of different tissues in a few species of snakes reported in this paper suggests that the W-chromatin is responsible for the synthesis of the nucleolus in these snakes.

Introduction

Ray-Chaudhuri, Singh, and Sharma (1970) have reported that the W chromosome in the females of the common Indian Krait, *Bungarus caeruleus* of the family *Elapidae* forms a characteristic heteropycnotic body in the nucleus at the interphase stage, not unlike the sex-chromatin in female mammals. This species has a multiple sex-chromosome complex having $Z_1Z_1Z_2Z_2$ males and Z_1Z_2W females (Singh, Sharma, and Ray-Chaudhuri, 1970). The W chromosome is the largest element in the chromosome complement and therefore can be identified easily. This enabled us to follow its DNA replication timing through tritiated thymidine incorporation and we found that it synthesises its DNA asynchronously at the late S phase. The heteropycnosis of the W extends up to the prometaphase stage and therefore the "hot" W can be traced right from the metaphase stage back to the interphase nuclei through

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the prometaphase stage. We have designated this body in the interphase nuclei as W-chromatin in order to distinguish the same from sex chromatin in mammals which is formed by the X-chromosome.

Snakes exhibit various states of the differentiation of the Z and W chromosomes apparently according to the evolutionary status of the families, being homomorphic in primitive families and well differentiated in highly evolved ones (Beçak and Beçak, 1969). Encouraged by our discovery of sexual dimorphism in the somatic cells of *Bungarus* and a few other species, we extended our analysis of chromosome constitution to sixteen more species of Indian snakes of the families *Boidae, Colubridae, Elapidae,* and *Hydrophiidae* in order to study the evolution of the sex chromosome complex in primitive and more advanced families of snakes and to correlate the phenomenon of differentiation with the female specific heteropycnotic body if and when detectable in the interphase nuclei of the somatic cells.

Materials and Methods

The following are the species whose sex chromosome constitution and the behaviour of the W-chromatin are reported in this paper. Family—*Boidae*: *Eryx johni johni, E. conicus;* Family—*Colubridae*: *Ptyas mucosus, Coluber fasciolatus, Boiga forsteni, B. trigonata, Natrix piscator, N. stolata, Lycodon aulicus, Cerberus rhynchops, Gerardia prevostiana*; Family—*Elapidae*: *Bungarus caeruleus, Naja naja naja, Naja naja kaouthia*; Family—*Hydrophiidae*: *Hydrophis spiralis, Enhydrina schistosa.*

The chromosome preparations have been made from bone marrow, spleen and short term leucocyte cultures following the air-drying technique. For leucocyte culture, blood was drawn directly from the heart of living snakes. After its withdrawal the snakes were injected intraperitonially with 0.25 ml colcemid per kg of body weight for chromosome preparation done 4 hours after the injection, from bone marrow and spleen cells.

For the study of the W-chromatin, cells from brain, kidney, leucocyte culture, liver, spleen, intestine and ovary were directly fixed in acetoalcohol without any pretreatment and slides were prepared by the air drying procedure. Feulgen, pyronin Y-methyl green and Carbol fuchsin stains were used for studying the relationship between the W-chromatin and the nucleolus if any. Photography was done with a Carl-Zeiss photomicroscope.

Results

A detailed report of the karyotypes of the various species of snakes whose sex chromosome constitution are herein discussed will form a separate communication to be published elsewhere. The somatic chromosome numbers of some of the species have been reported by Singh, Sharma, and Ray-Chaudhuri (1970). In this paper, we shall restrict ourselves to the description of the morphology of the sex chromosomes with particular reference to the W chromosome.

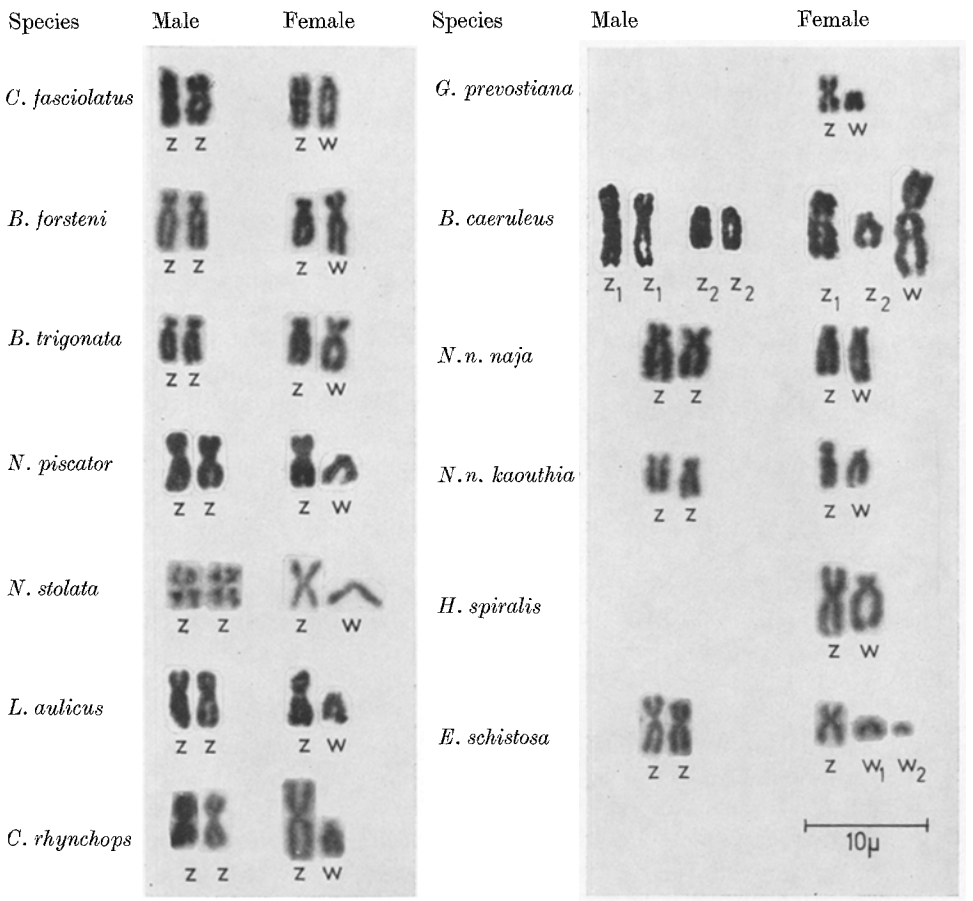

Fig. 1. Sex chromosomes of the males and females of 13 species of Indian snakes

Sex-Chromosomes

In Fig. 1 we have illustrated the sex-chromosomes of the males and females of 13 species of Indian snakes. In all of them, the males are homogametic and the females, heterogametic. In three other species included in this study viz., *Eryx johni johni* and *E. conicus* of the primitive family *Boidae* and in *Ptyas mucosus* of *Colubridae* both sexes are homomorphic in their sex chromosome constitution. *Bungarus caeruleus* has $Z_1Z_1Z_2Z_2$ males and Z_1Z_2W females (Singh, Sharma, and Ray-Chaudhuri, 1970) while in *Enhydrina schistosa* the sex chromosome complex in males is ZZ while in females it is ZW_1W_2 (Singh, in press). The sex chromosomes of 11 other species are ZZ and ZW in the males and females respectively (Fig. 1).

C. fasciolatus ($2n = 36$) females have a W with a subterminal centromere while the Z is submetacentric. Both of them are almost equal in size and as, postulated by Ohno (1967), their differentiation might be supposed to have originated through the occurrence of a single pericentric inversion. The Z chromosome stands out distinct in metaphase preparations of both males and females by its size and centromeric position. The somatic metaphase chromosomes of *B. forsteni* ($2n = 36$) are illustrated in Fig. 2a–d. The W in this species is a submetacentric chromosome and is definitely bigger than the Z (Fig. 2a), although it is not possible to identify the Z chromosome definitely in all plates because of the presence of two other chromosomes of the same size and morphology. In some metaphase plates, however, one pair of chromosomes of the same size as the Z has a secondary constriction very near the primary one. They may therefore be regarded as a pair of autosomes, making the third one the Z (Fig. 2a). In those plates where the secondary constrictions are not visible (Fig. 2b) or in plates where only one chromosome is seen with a secondary constriction (Fig. 2c) the Z cannot be identified at all. In rare instance the W chromosome shows a secondary constriction (Fig. 2d). The sex chromosomes of *B. trigonata* ($2n = 36$) are exactly similar to what has been described for the other congeneric species both for the morphology of Z and W as well as for the presence of secondary constrictions. The Z and W chromosome of *N. piscator* ($2n = 42$) were described by Singh, Sharma, and Ray-Chaudhuri (1968). The Z is a metacentric chromosome although the two arms are distinctly unequal and the W is much smaller in size and is acrocentric. The Z and the W chromosome, as the case may be, can be distinguished in every metaphase plate of either sex by their size and centromeric position. In *N. stolata* ($2n = 36$) the Z is almost a perfectly metacentric element while the W is acrocentric, similar in structure to the W in the above congeneric species. The Z is submetacentric in *L. aulicus* ($2n = 36$) while the W is acrocentric with a very short second arm and is much smaller compared to Z. *C. rhynchops* ($2n = 36$) has a metacentric Z while the W is acrocentric with a distinct but small second arm. Males of *G. prevostiana* ($2n = 36$) were not available to us for study. Study of female plates leads us to conclude provisionally that the Z in this species is metacentric and the W is about half of its size and is acrocentric. *B. caeruleus* has a multiple sex determining mechanism. The W is the largest member of the complement and is a submetacentric chromosome. In the common Indian Cobra, *N.n. naja*, there is no heteromorphic chromosome pair either in the males or in the females. The sex chromosomes, therefore, could not be identified morphologically. Such a situation was not expected in the highly evolved family *Elapidae* to which the species belongs. We therefore, undertook the study of the DNA

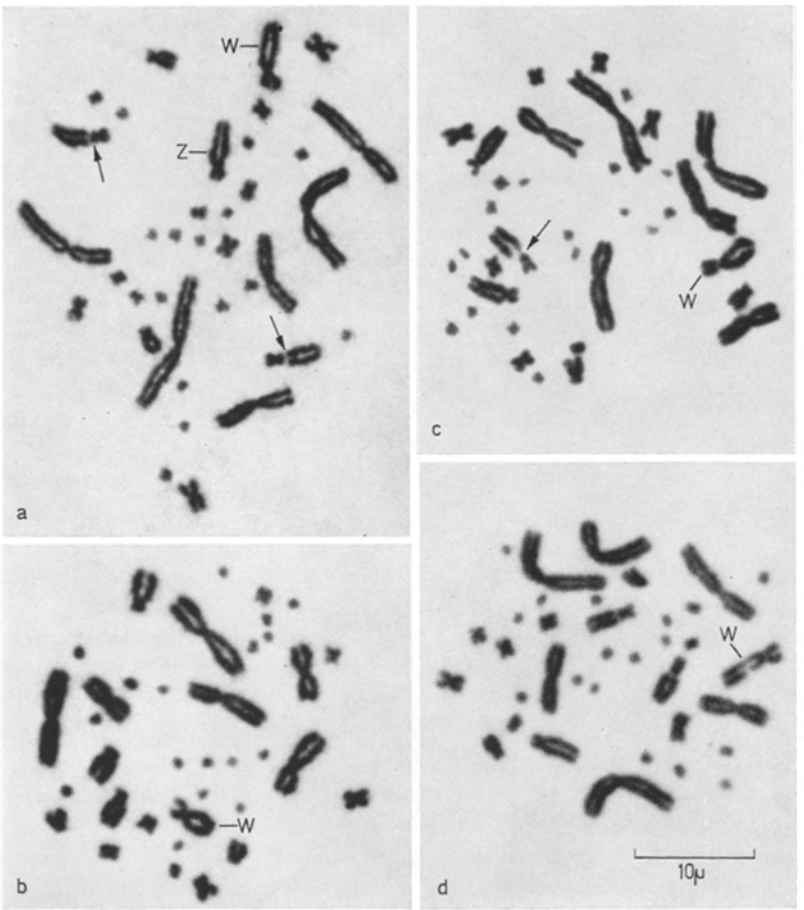

Fig. 2. a Metaphase plate from leucocyte culture of *B. forsteni* female, showing secondary constrictions in a pair of autosomes. b Metaphase plate of *B. forsteni* female showing no secondary constriction at all. c Metaphase plate of *B. forsteni* female showing secondary constriction in one autosome only. d Metaphase plate from leucocyte culture of *B. forsteni* female showing secondary constriction in W chromosome

replicating pattern with tritiated thymidine and were able to identify the W by its early replicating property (Ray-Chaudhuri, Singh, and Sharma, 1970). The W, thus identified, is a submetacentric chromosome with a distinct short arm. The second subspecies of Indian Cobra, viz, *N. n. kaouthia* ($2n = 38$), studied by us curiously shows a morphologically distinct W in the females which is definitely shorter than the Z and is an acrocentric chromosome with a distinct but

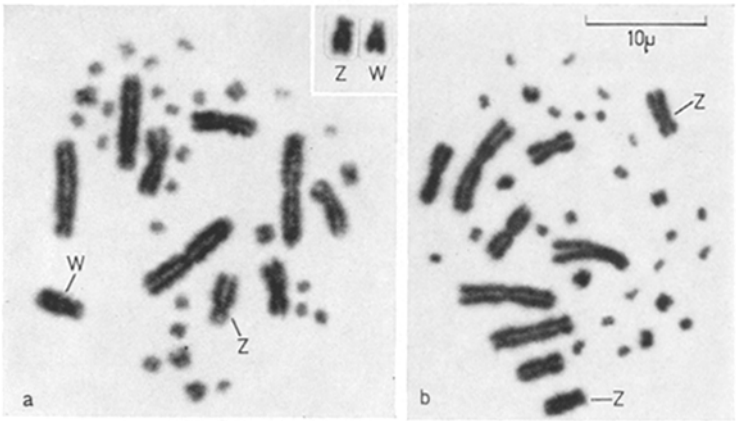

Fig. 3. a Female metaphase plate of *N. n. kaouthia* from leucocyte culture. ZW equal in size and slightly un-equal are shown in the inset. b Metaphase plate from leucocyte culture of *N. n. kaouthia* male

short second arm (Fig. 3a). The Z is submetacentric with a distinct but short second arm and a pair of these chromosomes is quite clear and characteristic in male plates (Fig. 3b). In a single female specimen however, out of five studied, the Z and W in about 60% cells are of equal size (Fig. 3a) and in the rest the W appears slightly shorter (Fig. 3a, inset). This appears to be a case of polymorphism of the W in the population of *N. n. kaouthia* from which we obtained the specimens. More specimens of this type are needed to confirm the hypothesis.

In *H. spiralis* ($2n = 32$) the Z and W are well differentiated, the former is metacentric with unequal arms and the latter subtelocentric and distinctly smaller than the Z. *E. schistosa* has a multiple sex chromosome complex being ZW_1W_2 in the females ($2n = 33$) and ZZ in the males ($2n = 32$). The Z in this species is submetacentric and W is telocentric without any visible second arm and much shorter than the Z. The W_2 is a microchromosome and cannot be identified easily.

W-Chromatin

The interphase nuclei from various tissues, viz, brain, kidney, blood, liver, spleen, and intestinal epithelium, of both sexes were studied in order to detect, if possible, the W-chromatin in the cells from the female snakes. In all those species where the W is differentiated from the Z either by its morphology or allocycly in DNA replication, a definite positive heteropycnotic body is detectable in all tissues examined for the purpose. This body, however, was not seen in all cells of the same tissue. No W-chromatin could be seen in any of the above tissue of *E. j. johni*,

Table. *Frequencies in percent of the W-chromatin in the interphase nuclei of various tissues of female snakes*

Species	Brain	Kidney	Leucocyte	Liver	Spleen	Intestinal epithelium
Boidae						
E. j. johni	—	—	—	—	—	—
E. conicus	—	—	—	—	—	—
Colubridae						
P. mucosus	—	—	—	—	—	—
C. fasciolatus	60	5	20	10	?	?
B. forsteni	65	30	55	18	40	?
B. trigonata	70	35	40	20	36	?
N. piscator	40	35	40	30	20	25
N. stolata	30	30	?	10	10	?
L. aulicus	35	30	40	15	18	20
C. rhynchops	40	35	35	20	?	?
G. prevostiana	75	70	50	20	?	?
Elapidae						
B. caeruleus	80	80	80	60	60	80
N. n. naja	60	60	40	20	35	30
N. n. kaouthia	60	5	30	10	?	?
Hydrophiidae						
H. spiralis	40	10	70	30	?	?
E. schistosa	20	30	35	?	?	?

E. conicus and *P. mucosus*. These are the very species where the sex-chromosomes are not detected by using any of the above criteria.

The frequency of nuclei having detectable W-chromatin varies in different tissues of the same species and is most common in the brain cells in almost all the species studied (Table). In Fig. 4a–m are illustrated the interphase nuclei from females showing W-chromatin in various tissues. It will be evident from the photographs that the demonstration of this body is unequivocal and that it cannot be confused with other heterochromatic blocks which may be present in the nuclei. Moreover, like the sex-chromatin in the female mammals, this body is generally associated with the nuclear membrane.

In the sea snakes, *E. schistosa* we should expect two W-chromatin bodies corresponding to the two W chromosomes. They are however, very small telocentric chromosomes. Moreover, the W_2 as pointed out earlier, belongs to the micro group of chromosomes. Even if the minute W_2 forms a heteropycnotic body it would be almost impossible to

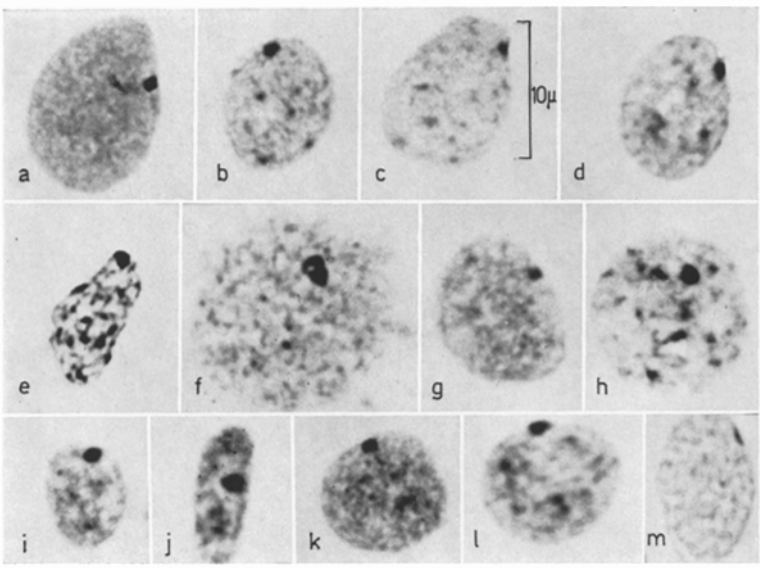

Fig. 4a–m. W-chromatin in interphase nuclei of various species of snakes. a Interphase nucleus from leucocyte culture of *C. fasciolatus* female. b Interphase nucleus from kidney of *B. forsteni* female. c Interphase nucleus from liver of *B. trigonata* female. d Interphase nucleus from kidney of *N. piscator* female. e Interphase nucleus from spleen of *N. stolata* female. f Interphase nucleus from brain of *L. aulicus* female. g Interphase nucleus from brain of *C. rhynchops* female. h Interphase nucleus from leucocyte culture of *G. prevostiana* female. i Interphase nucleus from kidney of *B. caeruleus* female. j Interphase nucleus from intestinal epithelium of *N. n. naja* female. k Interphase nucleus from kidney of *N. n. kaouthia* female. l Interphase nucleus from leucocyte culture of *H. spiralis* female. m Interphase nucleus of *E. schistosa* female from leucocyte culture showing only one small W-chromatin

distinguish the same among the coarse heterochromatin blocks which are always present in the interphase nuclei. The best cells for the detection of this body in this species are leucocytes but here also the frequency of W-chromatin positive cells is only about 35%. In these cells there is invariably only one heteropycnotic body, presumably formed by the relatively bigger W_1 characteristically located near the nuclear membrane.

Nucleolus

We had earlier observed an association between the nucleolus and the W-chromatin in *Bungarus caeruleus* (Ray-Chaudhuri, Singh, and Sharma, 1970). This led us to study the nucleolus and its relationship with the W-chromatin, if any, in the present series of snakes and we could not detect any nucleolus associated with the W-chromatin

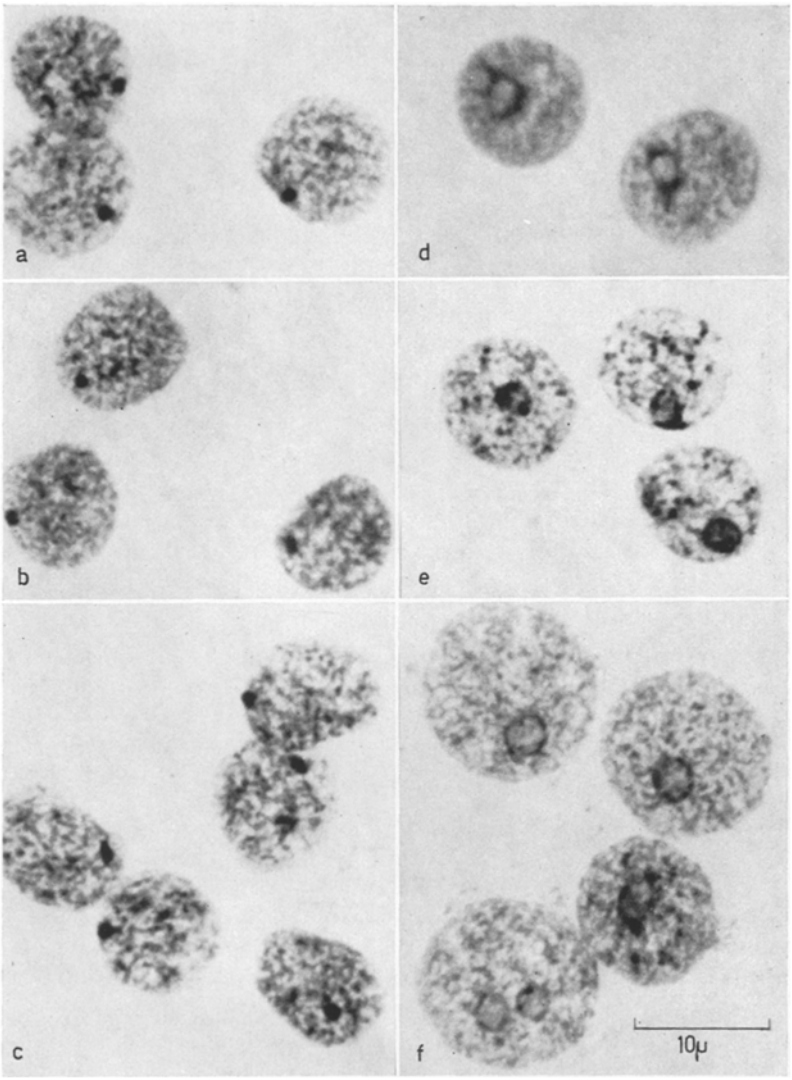

Fig. 5. a Interphase nuclei from kidney cells of *B. forsteni* female showing the presence of prominent W-chromatin and absence of conspicuous nucleoli. b Interphase nuclei from kidney cells of *B. trigonata* female showing the presence of conspicuous W-chromatin and absence of a prominent nucleolus. c Interphase nuclei of *N. n. kaouthia* female from kidney cells showing prominent W-chromatin. d Interphase nuclei from kidney cells of *B. forsteni* female showing the absence of W-chromatin and the presence of a prominent nucleolus. e Interphase nuclei of *B. trigonata* female from kidney cells with a very conspicuous nucleolus. The W chromatin is completely absent. f Interphase nuclei from kidney cells of *N. n. kaouthia* female showing the presence of a prominent nucleolus and absence of W-chromatin

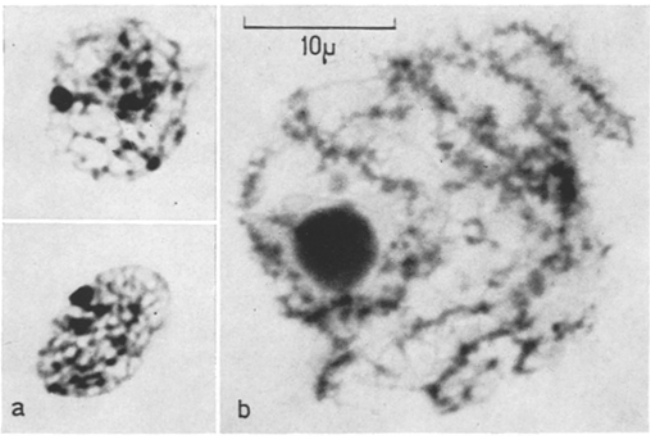

Fig. 6. a Early oocytes of *H. spiralis* with W-chromatin. b Late oocyte of *H. spiralis* with nucleolus

in any other snake species so far. A secondary constriction was found in the W chromosome in *B. forsteni* and if this region is nucleolus organizing then we should get a female specific nucleolus in this species. But its specific demonstration in the female is complicated by the presence of secondary constrictions in a pair of autosomes as well which may be regarded as potential nucleolus organisers in both sexes. As a matter of fact the kidney cells in both males and females of this species contain prominent nucleoli which are easily seen even in carbol fuchsin stained slides. This was confirmed by pyronin Y-methyl green techniques.

We observed however an indirect relationship between the W-chromatin and the nucleolus in the kidney cells of *N. n. kaouthia*, *B. forsteni*, *B. trigonata*, *C. rhynchops*, *H. spiralis*, and *C. fasciolatus*. In each of these species two types of kidney cells were seen in the females, one type with prominent nucleoli and the other without them. Those cells which do not have a nucleolus have a prominent W-chromatin body (Fig. 5a–c) whereas cells with nucleoli do not show W-chromatin (Fig. 5d–f).

In *Coluber fasciolatus* the heteropycnotic body is almost undetectable in the kidney cells and methyl green pyronin-Y staining revealed the presence of a prominent nucleolus instead of the heteropycnotic body. In *Cerberus rhynchops* the situation is slightly different in the sense that in two female specimens out of four studied the heteropycnotic body was very prominent in the kidney, liver and brain cells and no prominent nucleolus was present in them. In two other individuals,

in the same tissue, the heteropycnotic body was almost totally absent and the nucleoli were very prominent and even detectable in carbol fuchsin stained slides. This was also confirmed by pyronin staining. The oocytes were specifically studied in *H. spiralis* for the heteropycnotic body as well as for the nucleolus. In early oocytes there is a definite heteropycnotic body. In these cells the nucleolus can not be detected even with pyronin staining (Fig. 6a). But as the oocytes grow larger and enter meiosis, the heteropycnotic body becomes inconspicuous and a prominent nucleolar body is formed (Fig. 6b). The nucleolus gradually increases in size as prophase advances.

Discussion and Conclusions

Our demonstration of a heteropycnotic body in the interphase nuclei of the females of a large number of species of snakes has definitely shown that nuclear sexing is possible not only in those species of snakes where the W chromosome is morphologically distinguishable from the Z, but in those species where it is not so, but shows an asynchrony in the DNA replicating pattern of W. The claim is clearly substantiated from our demonstration of W-chromatin in the Indian Cobra, *N. n. naja*. We could demonstrate unequivocally that the heteropycnotic body in the interphase nuclei of the somatic cells of the females of *B. caeruleus* is formed by the condensation of the W chromosome because of its distinctive morphology in the chromosome complement and have designated this body as W-chromatin (Ray-Chaudhuri, Singh, and Sharma, 1970). Although the evidences are not as direct as in the above species, there is hardly any doubt that the conspicuous heteropycnotic bodies demonstrated in the somatic interphase cells of the females of various species reported herein are also similarly formed by the W chromosomes of the respective species. They may, therefore, also be designated as W-chromatin.

From the absence of coexistence of nucleolus and W-chromatin in a condensed state in the interphase nuclei of the liver and kidney cells in a few species of snakes reported in this paper we like to suggest that the W-chromatin is perhaps responsible for the synthesis of nucleolus in these snakes. The difficulty of observing the W-chromatin in cells with well developed nucleolus may be due to its active state of synthesis and therefore, may be present in an extended form. It seems likely that the W at least in these species contains constitutive heterochromatin which has been found associated with nucleoli in mouse liver and brain cells which also contain constitutive heterochromatin (Yasmineh and Yunis, 1970). The elegant technique of hybridization of nucleic acid in cytological preparations demonstrated by Gall (1969) has already demonstrated ribosomal RNA

within the body of the nucleolus in *D. hydei* and at one end of the X chromosome and one end of the C chromosome in *Rhynchosciara hollaenderi* (Pardue, Gerbi, Eckhardt, and Gall, 1970). We feel that the above hybridization technique may reveal *r*-DNA in the nucleolus organizing W-chromosomes of snakes.

Our studies on the sex chromosome complex of various species of Indian snakes confirm in general the evolutionary trend of the morphological differentiation of the Z and W chromosomes suggested by Beçak and Beçak (1969). Ohno (1967) has discussed in detail the mechanism of evolution of sex chromosomes in snakes. He pointed out that the Z and W in *Ophidia* are homomorphic in the primitive family of *Boidae*. We have also studied several species of this family and support the hypothesis. We further add that not only the Z and W are homomorphic, they are isocyclic in their DNA replication pattern and the W does not form a heteropycnotic body in the interphase cells in the somatic tissue. In the family *Colubridae*, Ohno (1967), points out that there are many species in which the Z and W are equal in size but they differ by a pericentric inversion. This we also confirm from our studies on *C. fasciolatus* where the most simple hypothesis for the differentiation of W will be a pericentric inversion. It may be pointed out here, that the change is just not that simple. The W chromosome has already undergone heterochromatization in this species since we find that there is W-chromatin in the interphase nuclei in all tissues examined. That inversion is not the first step in the differentiation of W can be clearly shown from the study of the Z and W in the Indian Cobra, *N. n. naja*. In spite of the fact that this species belongs to a highly evolved family, *Elapidae*, it has homomorphic sex chromosomes in both sexes. Here, the W in the female not only forms a characteristic W-chromatin in the interphase nuclei but is also allocyclic in its DNA replication pattern when compared with the Z chromosome. We therefore, believe that heterochromatization, whatever it may mean in molecular terms, rather than any kind of structural change is the first step in the differentiation of W in snakes. Prevention of crossing over between the homomorphic Z and W is essential for their gradual differentiation and from that point of view Ohno's (loc. cit.) inversion hypothesis is attractive. But, development of allocycly in one of the two chromosomes can also conceivably reduce the frequency of crossing over between them. We therefore presume that differentiation at the molecular level rather than at the morphological level is important in the evolution of W in snakes.

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