

THE CHROMOSOMES OF THE TWO OREGON  
SUBSPECIES OF CROTALUS VIRIDIS (RAFINESQUE)

by

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INTRODUCTION

Modern views on the relation of chromosomes to taxonomic problems may be said to begin with the studies of McClung. As early as 1905, McClung (20, p. 326) reported differences in chromosomes among the species of several Orthopteran genera. He advanced the idea that fundamental knowledge of species would come "by a comparison of the germ cells and body characters in nearly related species, by observing the differences in germ cells of individuals that vary from the type of the species, or finally by experimentally disturbing the normal conditions in the germ cells and observing the effect upon the body." McClung attempted to demonstrate that specific chromosome architecture accompanied the various somatic characters commonly utilized in classification. Again in 1914, McClung (21) published a more extensive treatment of Orthopteran chromosomes which paralleled his earlier work. In the same year, Metz (22) reported a comparison of the chromosomes in five species of Drosophila. Metz proposed a simple dichotomous relationship among the species and thus postulated an evolutionary series of chromosome changes.

Robertson (27) in 1916, following the research techniques of McClung, investigated several species of insects. He theorized that many variations in chromosome number among nearly related species could be explained by a fusion of non-homologous chromosomes. A fundamental number could then be obtained for a related group of organisms by counting the number of chromosome arms appearing in the caryotype. Matthey (15), writing in 1939, added support to Robertson's fundamental number concept by reporting a case of possible centric fusion in certain lizards. The value of chromosome caryotypes to taxonomy has been discussed by Wickbom (31). He contends that cytological information should be established as a definite criterion in phylogenetic classification.

It is now generally agreed that any significant change in chromosome structure may affect the genetic constitution of the individual and may also induce phenotypic alteration. The recent work of Ford et al. (5) and Tjio et al. (29) with human chromosomes has shown that chromosome number and morphology are directly related to several pathological syndromes. It is evident that many evolutionary changes that occur in the chromosomes are sub-microscopic and will require new and finer methods of analysis. White (30) has presented

extensive data in favor of the cytogenetic concept of evolution.

Sajiro Makino (11), in his 1951 chromosome atlas, lists the chromosome numbers for 99 species of Reptiles, but for only twenty-five of possibly three thousand species in the suborder Ophidia. Of the genera reported, there are no representatives of the true rattlesnakes.

Loyez (9) was among the first to investigate Reptilian chromosomes. His observations on the chromosomes of the lizard, Anguis fragilis, were included in a developmental study of the ovary. Loyez's proposed haploid chromosome number of 12 was disputed by Trinci (11, p. 242) in 1908 who reported the number to be 18 in the primary oöcyte. The number was finally resolved as 22 by Margot (13) in 1946.

The most controversial problem appearing in the literature on Reptile chromosomes has been that of sex determination. The Japanese school, lead chiefly by Makino, Momma, Nakamura, and Oguma, has reported numerous instances of female heterogamety among the Chelonia and Squamata (see Makino's atlas, 11). Matthey, Risley, and Margot have opposed this concept. Matthey and Van Brink (18; 19) using a new chromosome squash technique, have demonstrated female homogamety in several species of Lacertilia. Risley (26) and Matthey (16, p. 54) have



found even chromosome numbers in both male and female *Chelonia*. Risley has expressed the belief that environmental factors may be capable of directing sex differentiation in turtles. Matthey (16, pp. 48-54) in re-checking the early work of Painter (25), Thatcher (28), Dalcq (4), and Jordan (6) has succeeded in showing complete male homogamety in contradiction to the original papers. Matthey (17) asserts that Reptilia like the Anamniota do not possess heterochromosomes at the morphological level. The controversy is still not completely settled, however, since recent evidence tends to support the views of Matthey.

The first account of ophidian chromosomes was published by Thatcher (28) in 1922. Although certain aspects of his work on the gartersnake were erroneous, it created interest in further investigation. The following list is a tabulation to date of the published reports on ophidian chromosomes, including the species, investigators, and dates:



## LIST OF PUBLISHED OPHIDIAN CHROMOSOME NUMBERS

## Suborder Ophidia

## Family Colubridae

<i>Bungarus multicinctus</i>	2n-36	Nakamura	1935
<i>Coelopeltis lacertina</i>	2n-42	Matthey	1931
<i>Coronella austriaca</i>	2n-36	Matthey	1931
<i>Dinodon rufozonatum</i>	2n-46	Nakamura	1935
<i>Elaphe climacophora</i>	2n-36	Nakamura	1935
<i>Elaphae quadrivirgata</i>	2n-36	Nakamura	1935
<i>Heterodon platyrhinos</i>	n-20-22	Edgren	1953*
<i>Holarchus formosanus</i>	2n-36	Nakamura	1935
<i>Laticauda semifasciata</i>	2n-38	Nakamura	1935
<i>Macropistodon rudis carinatus</i>	2n-46	Nakamura	1935
<i>Naja naja atra</i>	2n-38	Nakamura	1935
<i>Natrix tigrina</i>	2n-40	Nakamura	1928
<i>Ptyas mucosus</i>	2n-34	Bhatnagar	1957
<i>Tarbophis fallax</i>	2n-36	Matthey	1931
<i>Thamnophis butleri</i>	2n-37	Thatcher	1922
<i>Tropidonotus natrix</i>	2n-36	Matthey	1931
<i>Tropidonotus viperinus</i>	2n-36	Matthey	1931
<i>Zamenis gemonensis</i>	2n-36	Matthey	1931
<i>Zoacys nigrorginatus oshimai</i>	2n-36	Nakamura	1935

## Family Viperidae

<i>Vipera aspis</i>	2n-42	Matthey	1933
<i>Vipera berus sachaliensis</i>	2n-36	Makino and Momma	1949

## Family Crotalidae

<i>Agkistrodon acutus</i>	2n-36	Nakamura	1935
<i>Agkistrodon halys blomhoffii</i>	2n-36	Nakamura	1935
<i>Pelias berus</i>	2n-36	Matthey	1933
<i>Trimeresurus flavoviridis</i>	2n-36	Momma	1948
<i>T. gramineus stejnegeri</i>	2n-36	Nakamura	1935
<i>Trimeresurus mucrosquamatus</i>	2n-36	Nakamura	1935
<i>Trimeresurus okinavensis</i>	2n-36	Makino and Momma	1949

\*This observation was incidental and is not reliable.

The problem of phylogenetic relationship among the Ophidia has been treated from the cytological viewpoint by Matthey (16, pp. 226-232) and Nakamura (24). Both investigators have applied Robertson's fundamental number concept and agree to an ancestral prototype of 24 V-shaped chromosomes. White (30, p. 180) has reported that snakes, unlike the lizards, show little chromosomal variation and suggests that more data are necessary to warrant any definite conclusions.

## MATERIALS AND METHODS

Within the boundaries of the state of Oregon are found two subspecies of the rattlesnake Crotalus viridis (Rafinesque). The two subspecies listed by Klauber (8, pp. 25-55) are Crotalus v. lutosus Klauber, the Great Basin Rattlesnake, and Crotalus v. oreganus Holbrook, the Northern Pacific Rattlesnake. The range of the subspecies lutosus is south and east of an imaginary line connecting Upper Klamath Lake, Fort Rock, Burns, and Council (Idaho). This line represents the approximate area of intergradation in southeastern Oregon. The lutosus population also extends into the states of Utah, Arizona, Nevada, and California. The subspecies oreganus extends west of the line to the Coast Range; however, it is not found in the Cascade Mountains. Oreganus ranges north to Idaho, Washington, and British Columbia and south to Kern and San Luis Obispo counties of California.

This cytological study constitutes the first investigation of the genus Crotalus, and the first study of the chromosomes of any of the New World Crotalidae. It was the purpose of this study to establish the chromosome number for the species and also to compare the chromosomes of the two subspecies to see if any significant morphological differences might occur.



In total, 39 specimens were collected during the months of April through June of 1959. Three female oreganus were collected from the Monroe area early in April, and four male specimens were obtained from Riddle, Oregon, early in June. In May, 32 lutosus and intergrades of the two subspecies representing both sexes were collected from the Malheur National Wildlife Refuge in southeastern Oregon. Of the total specimens collected, 10 males were found to be suitable for investigation of spermatogenesis and caryotype analysis.

The meiotic male testes were used exclusively throughout the study as the source of chromosome material. The animals were killed by decapitation and the testes removed immediately. Testicular material was prepared for observation by the paraffin section technique and also by the squash method. One testis of each pair was placed in Newcomer's solution to be later embedded in paraffin, sectioned, and stained regressively with Heidenhain's iron hematoxylin. The tissue was sectioned at 6, 8, and 10 microns. The remaining testis was sliced longitudinally and pretreated in distilled water for a period of 5-10 minutes after the method of Makino and Nishimura (12). Permanent squash preparations were then made using the following modification of the acetic-orcein method outlined by Darlington and LaCour (3, pp. 153-155):



The sliced tissue was transferred into 1% orcein in 45% propionic acid and minced to form a fine suspension. After a period of 5-10 minutes, a drop of stain containing particles of tissue was placed on a clean slide, further minced, and squashed hard between slide and albumenized coverslip. The coverslip with adhering tissue was soaked off by inverting the slide in a 10% propionic acid bath. The coverslip was then passed through two 5 minute changes of 1% propio-orcein, dehydrated through a standard series of alcohols, cleared in xylol and mounted on a clean slide with "Permunt."

A second method of squash preparation similar to the above technique was found to produce excellent results.

The procedure may be summarized as follows:

Following the distilled water pretreatment, the tissue was macerated in a solution containing 9 parts 2% propio-orcein to 1 part 1N HCl. The suspension was then flamed over a spirit lamp until vapors appeared. The solution was not allowed to boil. A drop of the suspension was placed on a clean slide and a permanent squash prepared as outlined above.

The most important result of the water-pretreatment in the squash technique is the dissolution of the spindle which allows the chromosomes to be spread evenly under pressure. Of the two methods of squash preparation, the HCl technique produced the best defined figures. It was found, however, that the two methods differed with respect to chromosome swelling. In all cases requiring measurement, non-HCl preparations were used in order to standardize the results.

The skin of each animal was spread on absorbent paper to dry. Kodachrome transparencies were taken of each fresh skin, and after complete press drying, the skins were photographed using high contrast microfile film. The methods of cytological analysis employed in this study may be outlined briefly as follows:

1. Chromosome number. Accurate counts of the diploid chromosome number for each subspecies were made at the spermatogonial mitotic metaphase. Counts of the haploid condition were made at late-diplotene and diakinesis of the meiotic prophase I, at anaphase I, and at anaphase II. Over 75 counts were made, drawn with the aid of a Leitz-Wetzlar camera lucida, and photographed with kodachrome or microfile film using a 35mm Exacta camera. All microscopical observations were made at an original magnification of 1552 X using an A0 Spencer Microstar research microscope with Koehler illumination provided by a Spencer Ortho-Illuminator.

2. Chromosome morphology. General observations on chromosomal size, shape, and centromere position were made at all pertinent stages and recorded by camera lucida drawings and photomicrographs. Metacentric and acrocentric chromosomes could best be distinguished at anaphase I.

3. Chromosome idiograms. Idiograms were constructed to compare the lengths and relative centromere positions

between the chromosome pairs of the two subspecies. The idiogram for each subspecies was based on the average measurements of five well spread spermatogonial mitotic metaphase figures. It was decided to use only the macrochromosomes for idiogram and chiasma frequency analysis.

4. Chiasma frequency. Chiasmata were studied in the diplotene stages of meiotic prophase and a chiasma frequency chart was compiled from the macro-chromosome data. Of the ten rattlesnakes studied, it was found that only five were suitable for chiasma frequency study. For each individual, the average chiasma frequency was computed from observations on ten cells. Three lutosus and two oreganus are represented.



## OBSERVATIONS

Late in May of 1959, the collected rattlesnakes were sexed and the males selected for dissection at approximately weekly intervals. It was thought that such a procedure would permit the observation of developing stages of spermatogenesis. A total of 110 slides were prepared and the following general observations made:

1. May 29. Mitotic spermatogonial divisions and early meiotic prophase stages were prominent in the subspecies lutosus.
2. June 11. Excellent mitotic and meiotic stages of division were found in oreganus, including diplotene, diakinesis, and metaphase I.
3. June 18. Two lutosus were observed to have all stages of mitosis and meiosis up to anaphase I.
4. June 30. All major meiotic stages were observed in oreganus including some early spermiogenesis.
5. July 16. Very few mitotic figures were observed after this date in lutosus. The testes were engorged with sperm and of little value for chromosome investigation.

Comparisons of the chromosome karyotypes of the two subspecies were made on the basis of the following



## cytological data:

1. Chromosome number
2. Chromosome morphology
3. Chromosome idiograms of the diploid set
4. Chiasma frequency.

CARYOTYPE OBSERVATIONS

1. Crotalus viridis lutosus. The chromosome number for this subspecies was found to be 36 in all individuals, with a haploid number of 18. Of the 36 elements, ten are V-shaped, six rod-shaped, and 20 dot-like. The V and rod-shaped chromosomes may be denominated macro-chromosomes and the smaller dot-shaped bodies micro-chromosomes. At the spermatogonial metaphase plate, the 16 macro-chromosomes are located at the periphery while the 20 micro-chromosomes are distributed in the center of the complex. Plate I, a camera lucida drawing of a typical mitotic metaphase, demonstrates the number and positions of the diploid chromosomes as prepared by the squash technique. With reference to the idiograms presented in Plate II, it may be noted that among the V-shaped chromosome pairs, 1 and 2 are the largest, 3 is medium sized, and pairs 4 and 5 are the smallest. Of the two pairs

## PLATE I

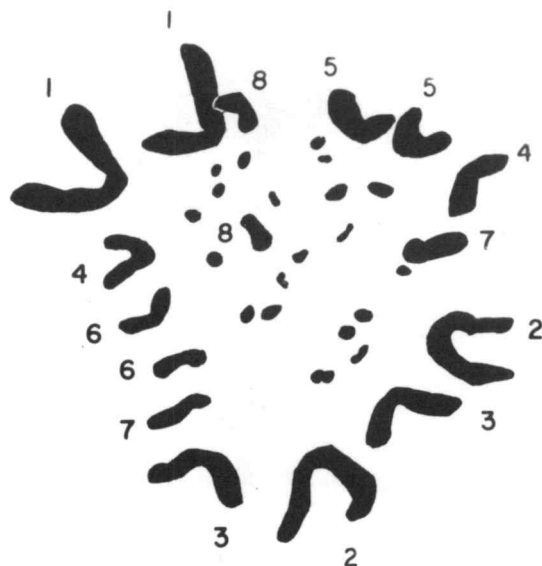
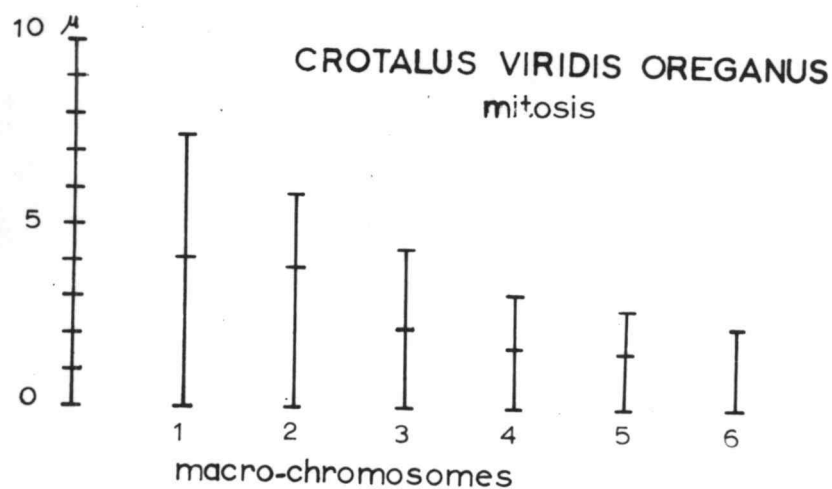
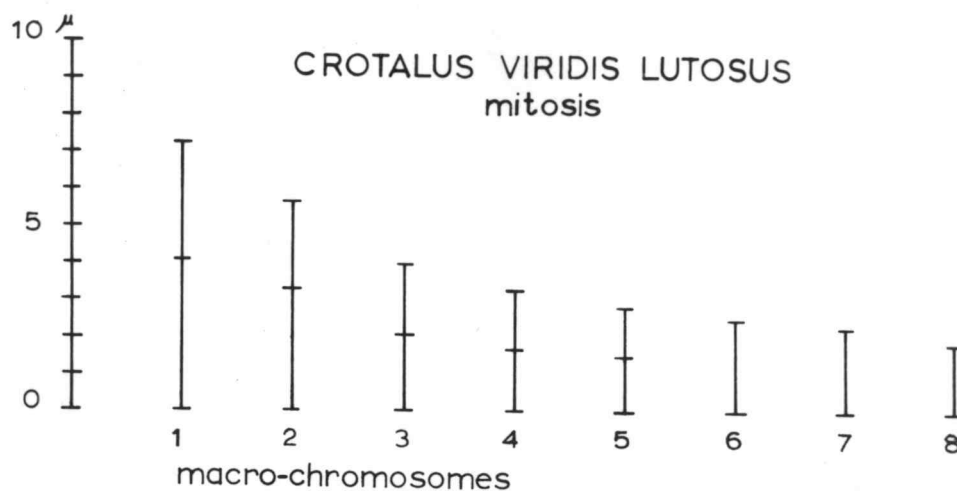
*Crotalus viridis lutosus*

PLATE II  
CHROMOSOME IDIOGRAMS



of largest chromosomes, pair 2 is definitely J-shaped or sub-metacentric. The smallest pair of metacentric chromosomes, pair 5, is generally larger than the longest pair of rods. These smallest metacentric chromosomes occasionally appear as curved rods; however, their metacentric nature is apparent in the anaphase I division when as bivalents they display four arms of almost equal length. The three pairs of short rods are considered to be acrocentric, having only two arms in the anaphase I. Due to their small size, no centromere measurements were attempted. The entire chromosome set forms a gentle idiogram curve from the largest to the smallest having no breaks or severe steps. It must be remembered, however, that the idiograms depict only the macro-chromosomes, and the inclusion of the micro-chromosomes would definitely show a sharp decrease in size as may be noted in Plate I. No evidence was found for heterochromosomes. The average chiasma frequency was found to be 21.2 with a range of 20-23.

2. Crotalus viridis oreganus. The chromosome number for this subspecies was also found to be 36 in all individuals, with a haploid number of 18.



The general morphology is similar to that of the subspecies lutosus. The idiograms shown in Plate II reveal very little discrepancy between the corresponding chromosome lengths and centromere positions of the two subspecies. The average chiasma frequency was found to be 21.1, with a range of 20-23.

The summarized data on chromosome measurements for both subspecies is given in Table I. Table II gives the frequency of chiasmata for ten late-diplotene stages observed in each of five specimens.

TABLE I  
CHROMOSOME MEASUREMENTS

Subspecies	Chromosome pair	Average length	Centromere * position
<u>C. v. lutosus</u>	1	7.25 $\mu$	3.31 $\mu$
	2	5.73	2.43
	3	4.00	1.92
	4	3.26	1.52
	5	2.83	1.28
	6	2.53	
	7	2.27	
	8	1.87	
<u>C. v. oreganus</u>	1	7.40	3.36
	2	5.71	2.11
	3	4.29	1.98
	4	3.10	1.50
	5	2.70	1.20
	6	2.21	
	7	1.94	
	8	1.82	

\* Measured from end of short arm

TABLE II  
CHIASMA FREQUENCY

Subspecies	Specimen	Number of Chiasmata				Mean
		20	21	22	23	
<u>C. v. lutosus</u>	3	2	5	2	1	21.2
	4	1	6	2	1	21.3
	6	2	5	2	1	21.1
<u>C. v. oreganus</u>	2	3	6	1	-	20.8
	7	2	3	3	2	21.5

INCIDENTAL OBSERVATIONS ON SPERMATOGENESIS

A photomicrograph of a spermatogenetically active testis tubule of Crotalus viridis oreganus is shown in Plate XII. The usual distribution of maturation stages is evident, with spermatogenesis originating in the peripheral epithelium and meiotic divisions proceeding towards the lumen. Specimens dissected late in July show numerous spermatozoa in the lumen.

Early stages of meiotic prophase are shown in Plates V to IX. All represent material prepared by the propio-orcein squash technique. In order of sequence these include leptotene, amphotene, pachytene, and diplotene. Only late-diplotene and diakinesis were specifically utilized in the present study of the Crotalus caryotypes.

Optimum spermatogenesis in the Oregon rattlesnakes was observed to occur late in June. At that time, all maturation stages were evident and an abundance of diplotene figures enabled satisfactory chiasma frequency determinations.



## DISCUSSION

A search of the literature has revealed caryotypic chromosome studies for only seven species of the entire family Crotalidae. The two genera reported represent only the Asiatic species of Aqkistrodon and Trimeresurus. The chromosomal formula in each case is 10 V's, 6 R's, and 20 D's. In no instance has a significant caryotypic variation been described for the family Crotalidae. Differences have been described, however, within the families Colubridae and Viperidae. A study of the reported fundamental numbers shows a remarkably close relationship among even the most divergent ophidian caryotypes.

The most common chromosome number encountered in the Ophidia is  $2n=36$ . Macropistodon rudis carinatus, a member of the Colubridae, has the highest reported ophidian chromosome number of 46. The formula for this species is 2 V's, 14 R's, and 30 D's, which, resolved to the fundamental number of 48, compares favorably with the ophidian range of 40-50. The lowest chromosome number has been recently reported by Bhatnagar (1) in the Indian Rat Snake. This species has a chromosome number of 34 in which two of the dot-like chromosomes have been lost. Nakamura (23), has reported the highest fundamental

number for Ophidia in the Colubrid, Natrix tigrina. The formula of 10 V's, 6 R's, and 24 D's gives the fundamental number of 50.

Based on the knowledge of fundamental number and basic chromosome morphology, both Matthey (16, p. 231) and Nakamura (24, p. 393) have shown that the evolution of chromosome complexes in snakes is consistent with the classification proposed by Boulenger (2). It is agreed that Proteroglypha, Opisthoglypha, and Amblycephalidae have been derived from Aglypha, while Viperidae and Crotalidae originated from Opisthoglypha. Keenan (7) and Nakamura (24, p. 392) have suggested a prototype fundamental number of 48 for the Ophidia; the caryotype consisting of 24 V-shaped chromosomes. Thus it has been theorized that the present chromosomal complexes have arisen from the prototype through such processes as centric fusion, fragmentation, translocation, and duplication.

An investigation of both mitotic and meiotic cell divisions in Crotalus viridis lutosus and C. v. oreganus revealed a diploid chromosome number of 36 with a haploid number of 18 for both subspecies. The formula of 10 V's, 6 R's, and 20 D's yielded a fundamental number of 46. In no case was any evidence found of heterochromosomes in the male. Historically, Matthey (14), in

his first investigation of Vipera aspis, reported an XO sex complex for the male, but in 1931 affirmed an XX male homogamety. These data for Crotalus viridis agree in every respect with the results obtained in Old World Crotalidae by Nakamura (24) and Makino and Momma (10). It is clear that the Old World pit vipers and moccasins are cytologically closely related to the New World Crotalidae of the genus Crotalus. Plate III, figure 1 shows a diplotene configuration of the caryotype that is consistent throughout the species studied. The two largest metacentric chromosomes form multiple ring tetrads while the medium sized pair forms a key-like figure of three chiasmata. The remaining five macro-chromosomes, including the two smallest metacentrics, form conspicuous equatorial rings or doughnuts. Due to the small size of the dots, no chiasmata could be established in these, however, figures 1 and 2 of Plate III show the paired nature of the tiny elements. Apparently, excessive pressure on this slide caused the separation of the dots and also ruptured some of the ring tetrads of the macro-chromosomes.

An idiogram comparison of the macro-chromosomes was made in order to establish the relative chromosome dimensions and centromere positions. The micro-chromosomes



Plate III

- Figure 1. Late-diplotene stage of Crotalus v. lutosus showing five equatorial ring tetrads.
- Figure 2. Late-diplotene stage of Crotalus v. oreganus showing paired nature of the dot-like chromosomes.
- Figure 3. Spermatogonial mitotic metaphase of Crotalus v. lutosus.
- Figure 4. Spermatogonial mitotic metaphase of Crotalus v. oreganus.

## PLATE III

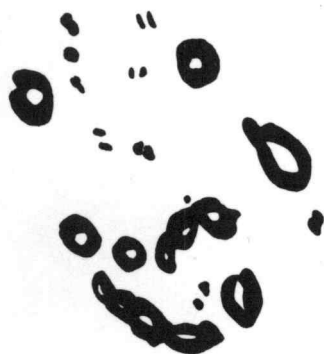


Figure 1



Figure 2



Figure 3



Figure 4

demonstrated little morphological differences, and the centromere positions could not be positively identified. In the case of the eight pairs of macro-chromosomes, centromeric positions were plotted for only the five metacentric members. It was found that the mitotic chromosome dimensions of the two subspecies were identical. The maximum variation between any corresponding pair was 0.3 of one micron noted in pair number 7. This minute variation may be of no significance considering the fact that all metaphase chromosomes were undoubtedly not in the same stage of foreshortening when measured. The idiogram presented in Plate I clearly shows the relative size relationships among the V-shaped chromosomes to be similar to those described by Nakamura (24) for Old World Crotalidae.

Chiasma frequency is generally recognized as a valid criterion for the comparison of karyotypes (cf. Wickham, 31). Many subtle subspecific differences may first become apparent as changes in chiasma frequency and localization, as a result of internal chromosomal rearrangement. No significant differences were, however, found between the chiasma frequencies of the two subspecies of Crotalus viridis. This is not surprising in view of the congruity in chromosome morphology, length,



and centromere position. Subspecific distinction in the present case is evidently below the level of visible chromosome changes. It was observed that some terminalization of chiasmata occurred during the course of diplotene. To minimize error due to terminalization, all chiasmata counts were made at the relatively stable late-diplotene stage preceeding diakinesis. Due to the virtual impossibility of observing early diplotene phases, no attempt was made to analyze the degree of terminalization.

## SUMMARY AND CONCLUSIONS

1. The caryotypes of the two Oregon subspecies of the rattlesnake were compared cytologically using the propio-orcein squash technique. The source of chromosome material was exclusively the meiotic testes.
2. Crotalus viridis lutosus and Crotalus v. oreganus have identical chromosome numbers, these being 36 for the diploid set and 18 for the haploid.
3. The chromosomal formula for both subspecies is 10 V's, 6 R's, 20 D's, giving a fundamental number of 46. This chromosome formula has been found in all Crotalidae investigated, both Old World and New.
4. No evidence for heterochromosomes was found in either subspecies.
5. Idiogram comparison reveals no significant differences between chromosome lengths or centromere positions. The chromosomes range in length from 8.5 to 0.4 microns. Among the V-shaped chromosomes, one pair was found to be sub-metacentric or J-shaped.
6. Chiasma frequency counts at late diplotene for the macro-chromosomes averaged 21.2 for C. v. lutosus and 21.15 for C. v. oreganus. Evidence for terminalization was noted during the course of diplotene. The two largest metacentric chromosomes show multiple ring tetrads at

diplotene; the medium sized pair resembles a key and has three chiasmata; the remaining five macro-chromosomes form single ring tetrads.

7. The caryotype evidence supports a close relationship of the genus Crotalus to the Old World Crotalidae.

8. Spermatogenesis in the rattlesnakes investigated reached the peak of development during the month of June.



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## APPENDIX

## EXPLANATION OF PLATES

## PLATE IV

- Figure 1. Pressed skin of Crotalus v. lutosus.  
Figure 2. Crotalus v. oreganus.  
Figure 3. Crotalus v. lutosus.  
Figure 4. Crotalus v. lutosus.

## PLATE V

- Figure 1. Mitotic metaphase plate of C. v. lutosus.  
Figure 2. Anaphase I division of C. v. oreganus.

## PLATE VI

- Figure 1. Early meiotic prophase showing nucleolus and chromocenters.  
Figure 2. Early leptotene stage.

## PLATE VII

- Figure 1. Mid-leptotene stage of meiotic prophase.  
Figure 2. Amphitene stage showing unpaired areas between homologous chromosomes.

## PLATE VIII

- Figure 1. Pachytene stage of meiotic prophase.  
Figure 2. Early diplotene stage.

## PLATE IX

Figure 1. Early diplotene stage showing early development of chiasmata.

Figure 2. Mid-diplotene stage.

## PLATE X

Spermatogonial metaphase figures showing the metacentric and sub-metacentric nature of the large V-shaped chromosomes.

## PLATE XI

Diakinesis figures confirming the haploid number of 18.

## PLATE XII

Longitudinal section of testis showing developing stages of spermatogenesis. Note the progressive stages occurring from tubule periphery to mature spermatozoa in the lumen.



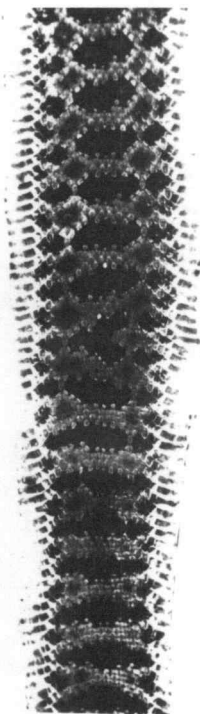


Figure 1

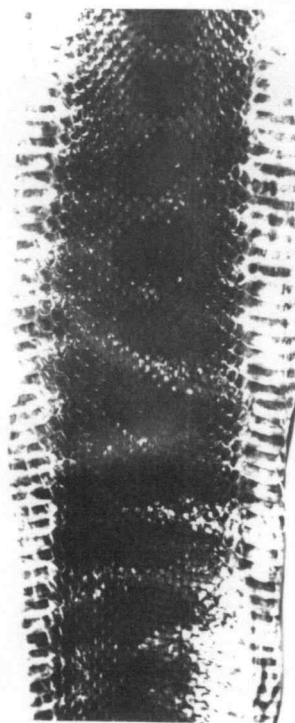


Figure 2

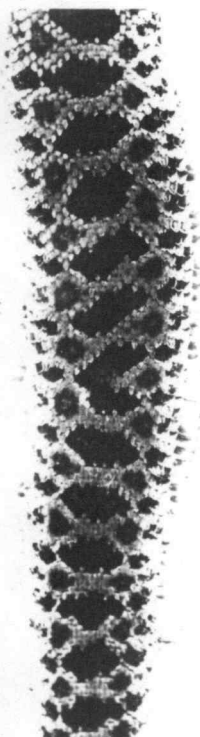


Figure 3

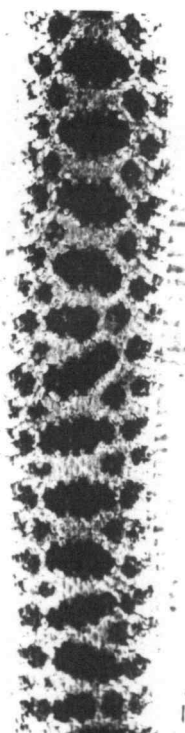


Figure 4

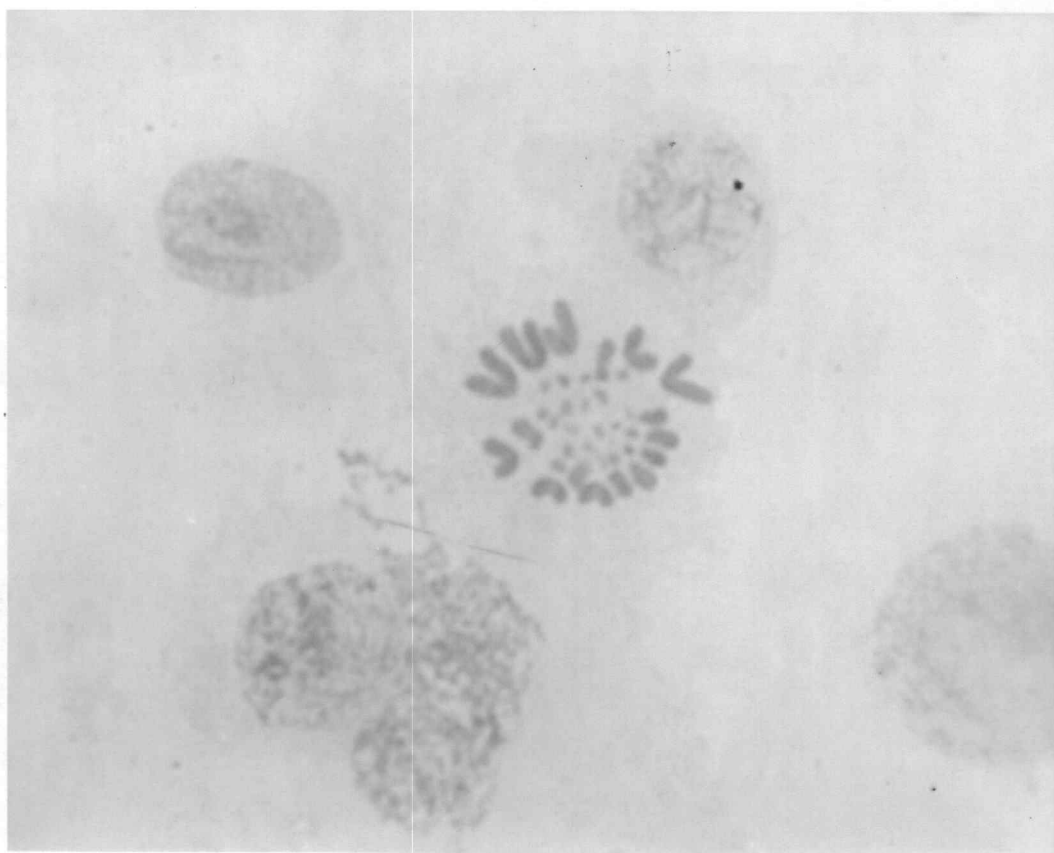


Figure 1

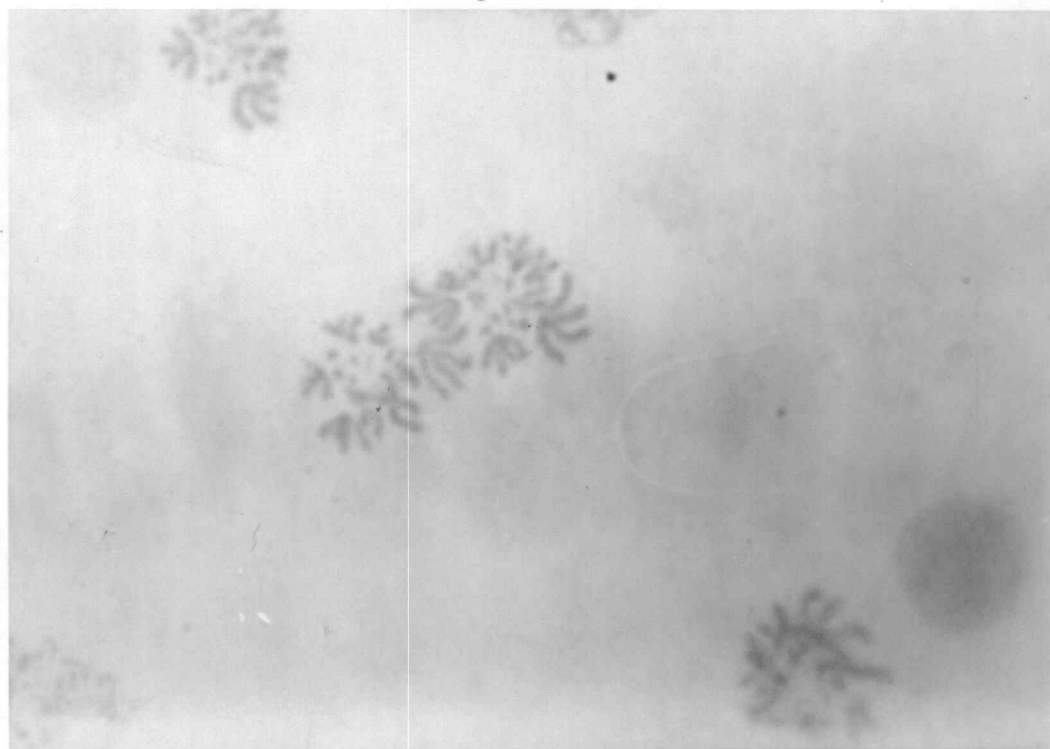


Figure 2

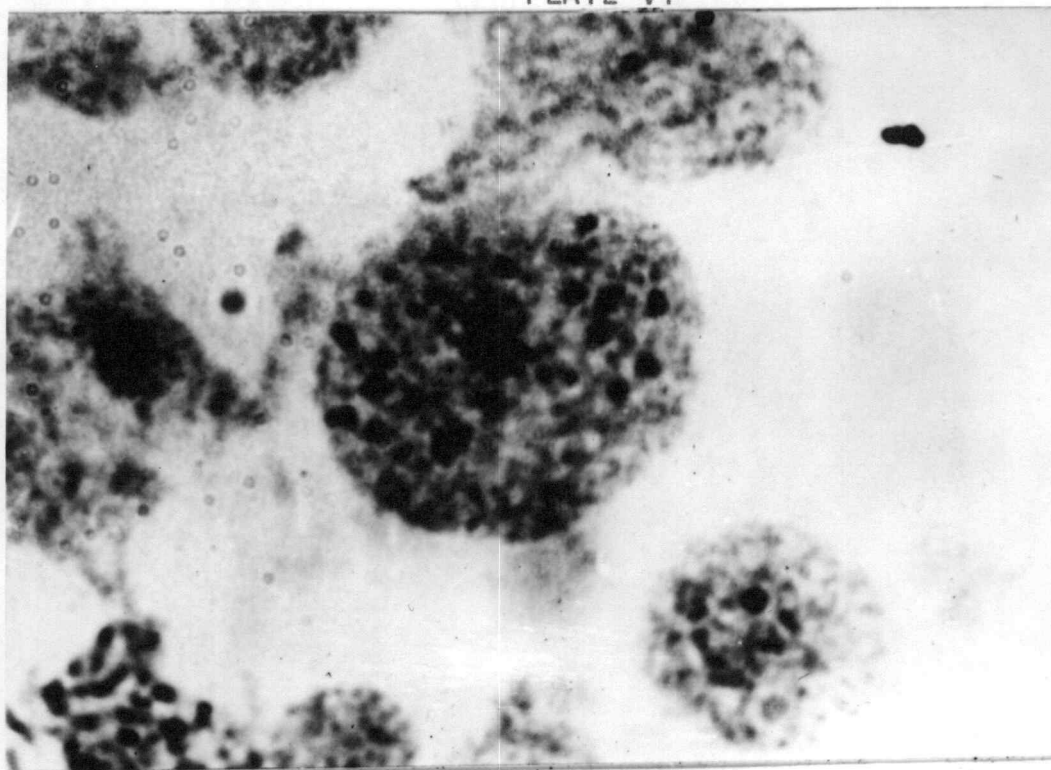


Figure 1

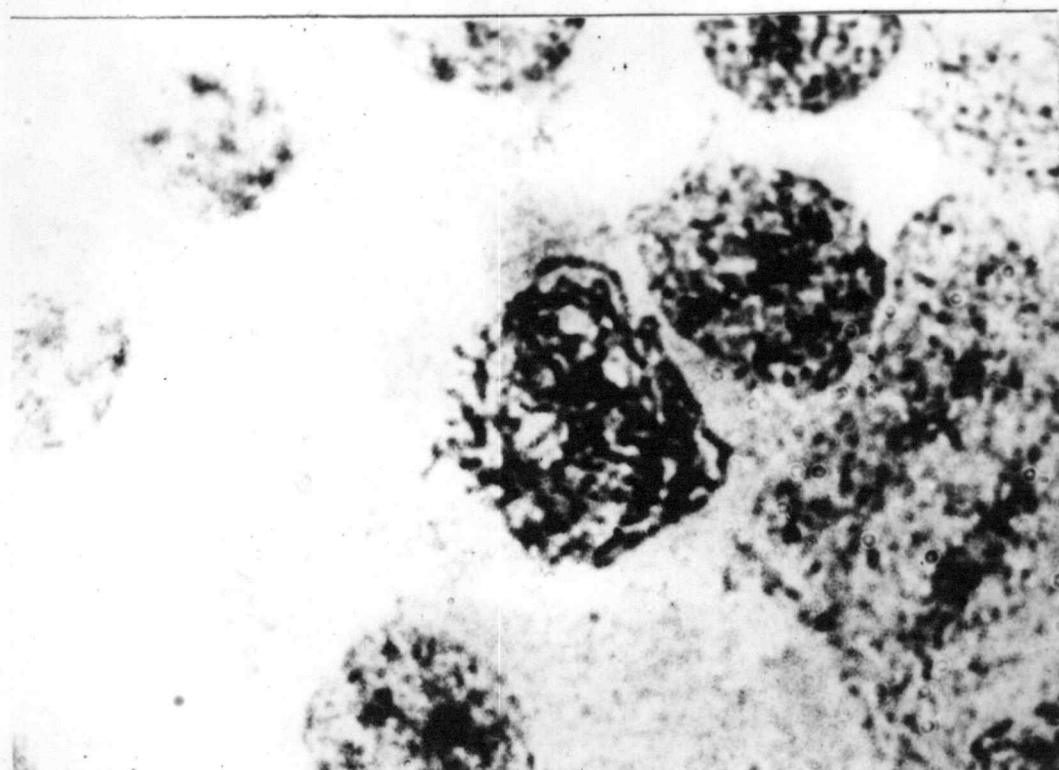


Figure 2



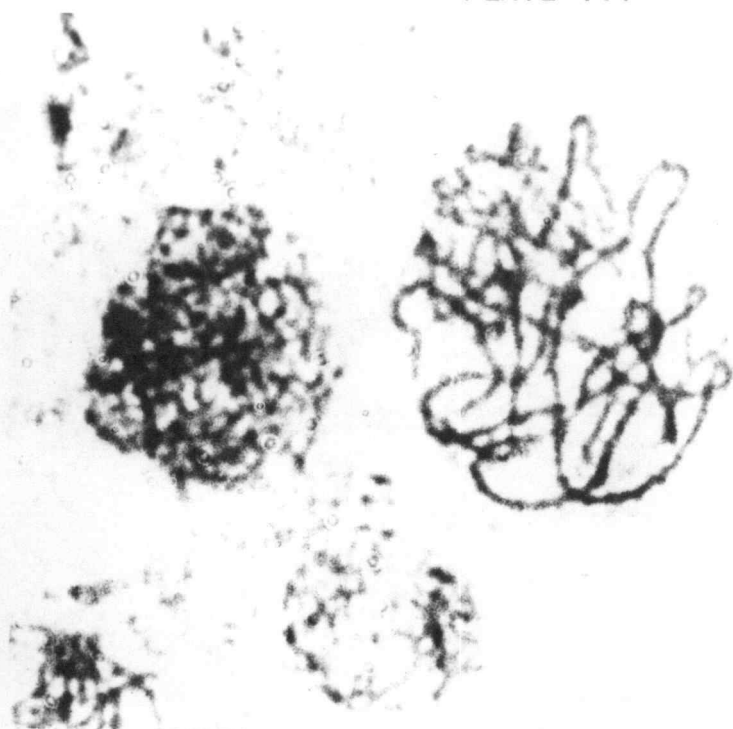


Figure 1

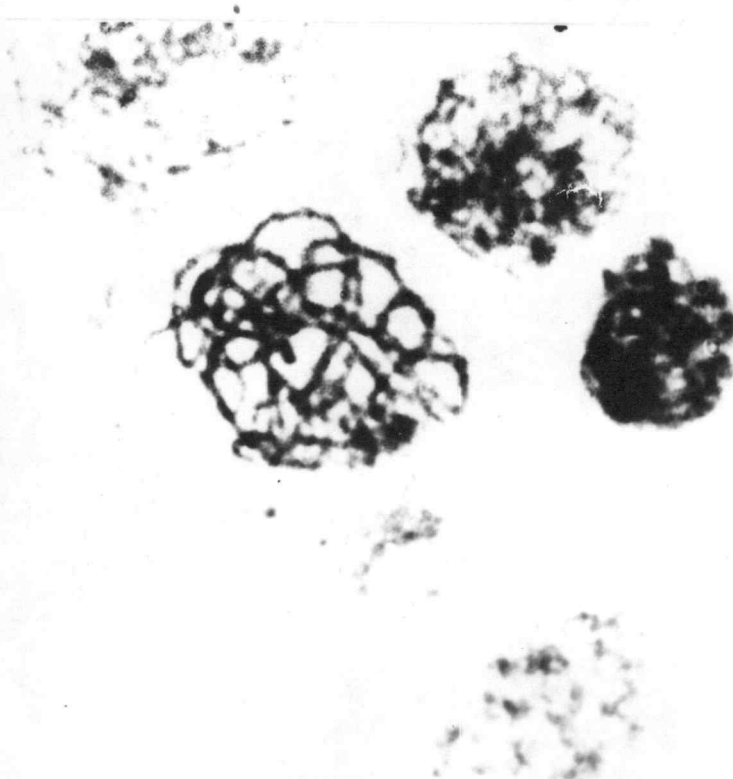


Figure 2



Figure 1

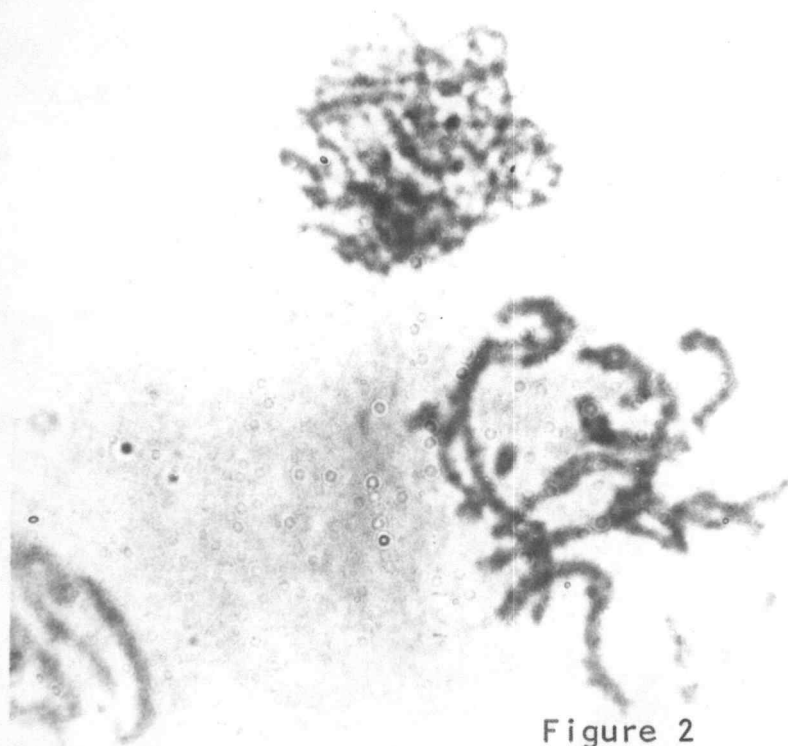


Figure 2

## PLATE IX

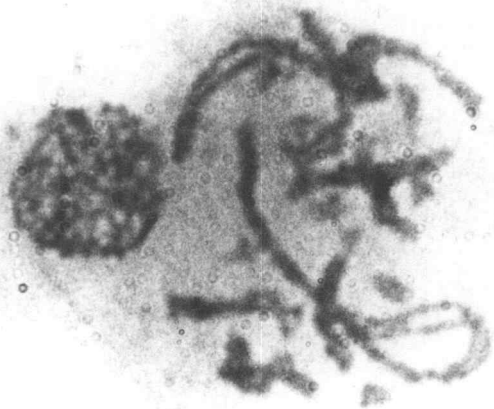


Figure 1



Figure 2



