THE CHROMOSOMES OF THE NORTHWEST AMBYSTOMID SALAMANDERS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

THE CHROMOSOMES OF THE NORTHWEST AMBYSTOMID SALAMANDERS

CHAPTER I

INTRODUCTION

Ever since early chromosome research produced such epoch-making studies as those of McClung (24, pp. 304-340), Boveri (2, pp. 181-268), Janssens (20, pp. 387-411), and Agar (1, pp. 1-44), the chromosomes have occupied one of the central places in evolutionary thought. That the determination of chromosome numbers and morphology might be of value in taxonomic studies of plants and animals has not been well recognized however until comparatively recent times. Though the early work listed above was done on zoological material most recent advances in cytology have been in the main part based on botanical studies. The morphology and numbers are now known for a large number of plant families, but modern cytological studies on animals are less extensive. Using some of the special techniques developed by the botanists, animal students are now beginning to increase our knowledge of more and more animal groups. At the present time the literature reveals a rather large number of studies on the cytology of insects, especially the various orthopteran groups. Vertebrates have been much less studied. Makino (26, p. xiii) lists only 108 species of amphibians that have had their chromosome numbers studied and recorded. Careful morphologic studies

in this and other vertebrate groups are, however, even more rare.

That an increase in our knowledge of the chromosome cytology of all organisms is important cannot be doubted. As early as 1905 McClung (24, pp. 304-340) professed the belief that careful comparison of the germ cells of nearly related species would be of extreme value in establishing evolutionary relationships. McClung's three lines of investigation: (1) comparison of caryotypes in related species, (2) comparison between the caryotypes of aberrant individuals and the normal chromosome set of the species, and (3) experimental alteration of the caryotype, have all proved fruitful methods of inquiry and have set the broad outlines for many subsequent studies.

In his compilation, An Atlas of Chromosome Numbers in Animals, Makino (26, pp. 1-290) lists the chromosome numbers and some other cytological details for 3317 species of animals. Of these 2754 are invertebrates (mostly insects) and 563 are studies of vertebrate species. At the time Makino's Atlas was published in 1951, 54 species of Caudata had been investigated. Since 1951 a few workers have been adding to this list. Here in America the studies of Kezer (22, pp. 1-55) have added significantly to the knowledge of the plethodontid salamanders. Other families of Caudata have not been systematically studied however, and data on

most forms are either completely lacking or only sketchy.

White, in his excellent book Animal Cytology and Evolution (37, pp.1-435), documents the evidence to support the hypothesis that the chromosomes constitute the physical basis of heredity and also furnish the material source of evolutionary changes. Since this is the case any information about differences in numbers and shapes of chromosomes between closely related species throws new light on problems of taxonomy. In the same sense characteristics of the chromosomes of whole groups have a bearing on the differentiation of the higher categories of our classification as well as the problem of evolutionary patterns, plasticity, and adaptiveness. It was with the belief that a comparative study of the chromosomes of a family unit occuring in Oregon might help elucidate some of the problems of comparative evolution, as well as the recognition of the need for studies on the cytology of these heretofore uninvestigated species, that the present research was undertaken.

A careful search of the literature reveals that only three of the twenty-two recognized species of the family Ambystomidae have been investigated as regards general chromosome morphology or chromosome number. Studies on the axolotl, Ambystoma mexicanum, and on the siredon, Ambystoma tigrinum, have been conducted by a number of workers. The early studies such as those by Jenkinson (21, pp. 408-482),

Muckerman (31, pp. 233-252), and Mack (25, pp. 119-127) present conflicting data as to the number of chromosomes characteristic of these species. Indeed, in some cases it seems that some of these investigators may have been confused as to whether they were actually studying material from the axolotl or the siredon. The methods used by these early workers explain to a great extent the variations in their results. Not until 1919 did really significant work appear. At that time Parmenter (33, pp. 169-226) presented an excellent study of both chromosome numbers and morphology in the somatic mitoses of Ambystoma tigrinum. Parmenter established that the diploid number of this species was 28 and made measurements and comparisons of all the chromosome pairs. The more recent work of Galgano (17, pp. 171-200), Carrick (5, pp. 63-74), and Prokofieva (34, pp. 148-164) confirm Parmenter's results for this species. Prokofieva reported finding variable numbers of chromosomes for this species, either 24 or 28, in various mitotic figures. On the basis of Parmenter's careful study and the subsequent discussion by Wickbom (38, pp. 241-346) of this discrepancy it seems evident that the 24 diploid number which, by the way, was also reported by Muckerman and Mack in earlier studies must be due to some misinterpretation of material. A careful scrutiny of the cytological technique employed in these investigations in question reveals possible reasons

for error.

Wickbom (38, pp. 247-248) and Fankhauser and Humphrey (14, pp. 367-374) have shown that Ambystoma mexicanum has, like Ambystoma tigrinum, a diploid somatic number of 28. Fankhauser, in a series of important investigations, has shown that polyploid individuals occur naturally in this species, although it is doubtful that many survive to maturity. The work of Wickbom has established some of the details of the morphology of the somatic chromosome set. Humphrey (19, pp. 33-66) using the axolotl proved in a classic genetic study that sex determination in this species is of the ZW type even though morphologically distinguishable sex chromosomes are absent.

In 1938 Creighton (6, pp. 497-504) investigated chromosome structure in Ambystoma maculatum (punctatum), but made no chromosome counts for the species nor did she contribute significantly to the knowledge of the gross chromosome morphology of this species. A curious situation exists in the literature. Wickbom (38, p. 257) credits Creighton with describing a diploid number of 28 chromosomes for this species, and cites the paper referred to above. After several careful readings of the citation this writer could find no mention of any counts by Creighton. It would thus seem that Wickbom is in error and unless Creighton made this count available in some unpublished source the

credit for determining the chromosome number for Ambystoma punctatum should go to Henley and Costello (18, pp. 94-95). This discrepancy in the literature should be clarified; previous similar mistakes have led to confusion both in priority and in accuracy. Henley and Costello investigated this species during a study of natural polyploidy in amphibian populations. From a number of mitotic figures in tail clips a diploid number of 28 was recorded. Unless it can be shown otherwise, these investigators should rightly be credited with this discovery. Kezer (22, p. 39) reported a haploid number of 14 for Ambystoma jeffersonianum.

Thus, previous to this study only Ambystoma tigrinum, Ambystoma mexicanum, Ambystoma jeffersonianum, and Ambystoma maculatum had been investigated cytologically and of these the last two named only as to chromosome number. The other two North American genera, Dicamptodon and Rhyacotriton had not been studied.

The paper of Carrick (5, pp. 63-74) has been the only one to date that dealt with meiotic chromosomes. Carrick did not use the methods employed in this investigation however, and this may in part explain some of the faulty conclusions contained in his paper. These will be discussed in more detail later. This study, then, presents the first use of modern squash preparation techniques on this family of salamanders, and represents the first in a series of in-

vestigations to be undertaken of the cytology of the entire ambystomid group.

CHAPTER II

METHODS AND MATERIALS

There are five species of ambystomid salamanders found within the confines of the state of Oregon. One of these species, Ambystoma tigrinum, is found only in limited numbers in the extreme northeast corner of the state. Since the studies of Parmenter (33, pp. 169-226), and Carrick (5, pp. 63-74) deal with the cytology of this species, and since it does not occur in the western part of the state it was omitted from the study.

The four ambystomid salamanders occuring regularly in western Oregon are the northwestern salamander (Ambystoma gracile), the long-toed salamander (Ambystoma macrodactylum), the Pacific giant salamander (Dicamptodon ensatus), and the Olympic salamander (Rhyacotriton olympicus). These four species comprised the animals used for the present study. The group consisted of three genera and thus presented the opportunity to compare animals of differing genera as well as two of the same genus. These species presented the further advantage that they had not yet been investigated and that they showed a variety of adaptations to different ecologic niches. Detailed species descriptions of this group can be found in the recent work of Stebbins (36, pp. 32-41).

Collections were made by the writer and other members of the Zoology Department of Oregon State College over a period of eight months, beginning in February of 1955 and continuing through September of that same year. Since the chief source of chromosome material was the meiotic male testis it was necessary to collect over this span of time in order to determine the times of spermatogenesis. After each collection some of the animals were examined cytologically. Fresh collections were made at periodic intervals. All specimens were preserved, cataloged, and coded. Individuals that provided favorable material could be referred to by means of a code system written on the slide preparations with a diamond pencil. Females, and males not in spermatogenesis, can be found in the Oregon State College Natural History Museum herpetological collection; specimens from which slides were made are in the investigator's possession.

The material used for this study consisted of embryos, testes, and regenerating liver tissue from the above listed species as follows:

Species	Number of Specimens	Tissue
Ambystoma gracile	6	Embryo tail clips, whole mounts
" "	6	Embryo tail clips, squashes
" "	3	Regenerating liver tissue, sections

Species	Number of Specimens	Tissue
Ambystoma gracile	8	Testes, sections and squashes
Ambystoma macrodactylum	6	Embryo tail clips, whole mounts
" "	6	Embryo tail clips, squashes
" "	8	Testes, sections and squashes
Rhyacotriton olympicus	11	Testes, sections and squashes
Dicamptodon ensatus	3	Testes, sections and squashes
" "	1	Regenerating liver tissue, squashes

In the case of Ambystoma macrodactylum the establishment of a laboratory colony proved to be helpful as the colony could be sampled at regular intervals and a large stock collected when these animals were relatively easy to obtain. Squash preparations of testis material were also made available by J. Kezer of the University of Oregon.

The larval tail clips used in this study were handled in two different ways. One, after the method of Fankhauser (13, pp. 22-23), consisted of removing the tails of larvae at the hind limb bud stage and fixing them in Bouin's fluid, then staining with either iron hematoxylin or aceto-orcein. The other method consisted of removing the tail and placing it directly on a slide in aceto-orcein and removing the spinal cord so that only the thin tail epidermis remained.

Squashes were then made by a modification of the aceto-orcein squash method for chromosomes as outlined by Darlington and La Cour (8, p. 120). This squash method proved to be best for well spread mitotic figures.

Testis material was prepared in two ways. First, whole testes were fixed in Newcomer's fluid, then imbedded in paraffin, and later sectioned at 15 microns and stained with iron hematoxylin. Secondly, the whole testes or parts of testes were macerated to form a suspension of cells which were in turn prepared by the aceto-orcein squash method of Darlington and La Cour referred to above. Some excellent squash preparations were also obtained with an iron hematoxylin stain. The method used for these iron hematoxylin squashes was as follows:

> The testis material was macerated to form a suspension of cells and this suspension was then fixed in a 10% acetic acid solution and squashed between a slide and an albumenized cover slip. The cover slip was then soaked off in a 15% solution of acetic acid. The squashed cells remained fixed to the cover slip. The material was then washed in two or three changes of water and then stained by the usual regressive iron hematoxylin staining method. The schedule included five minutes in iron mordant followed by several rinses and then ten minutes in Heidenheim's hematoxylin. After rinsing and allowing to blue up the material was destained for five to ten minutes in picric acid. This was followed by rinsing, dehydration, and mounting in balsam. The results proved to be very satisfactory.

In an effort to obtain mitotic material from species in which the larval tail clip method seemed impractical experi-

ments were carried out to determine the feasibility of using regenerating liver tissue as a source of mitotic figures. The method proved to be of some success and can be summarized as follows:

> Neotenic Ambystoma gracile larvae and adult Dicamptodon ensatus were used for the experiments. First an incision about 1 centimeter in length was made ventrally in the region of the liver. The liver was then partially exposed from the body cavity and approximately 70% by weight was snipped off and the remaining liver was returned to the body cavity. The wound was then stitched and the animal returned to the aquarium or the refrigerator. At three-day intervals the animals were injected with colchicine and after a ten-hour lapse the liver was removed and either fixed in Bouin's fluid or prepared by the aceto-orcein squash method.

This regenerating liver yielded good metaphase plates and was suitable for chromosome counts. This material was also useful in making comparisons of mitotic and meiotic figures as well as comparisons of spermatogonial mitoses and regular somatic mitoses. Colchicine seemed to cause some shrinking of the chromosomes leaving these specimens unsuitable for morphologic studies.

Slides prepared by the above cytological methods were all coded for easy reference to the source animal and then analyzed. For all of the examinations of the slide material a Tiyoda Zeiss-type research microscope was used. Koehler illumination was provided by a Spencer Advanced Research Illuminator. A Spencer camera lucida was used for drawing

and photomicrographs were made on microfile film with a 35mm camera. Photomicrographs and camera lucida drawings were made at an original magnification of either 400x or 1000x.

The cytological data collected for this study were as follows:

1. Counts of chromosome numbers. At least 100 counts were made for each species and the results recorded. In each case counts were made from both mitotic and meiotic material and included diplotene, metaphase I, anaphase I, metaphase II, and anaphase II figures. Mitotic metaphases or anaphases were also examined. The majority of the material used in establishing chromosome numbers for the various species was from the testis; however other tissues gave identical results.

2. General chromosome morphology. Individual sets of chromosomes were either photographed (when well spread) or drawn out by camera lucida. Thus, the general pattern of longer and shorter chromosomes, including centromere position, could be established. From a number of such sets a representative group of idiograms portraying the length and relative position of the centromere for each member of the caryotype was prepared for analysis. Measurements were made by the use of an ocular micrometer to establish the actual size of the chromosomes.

3. Ratio of longest chromosome to shortest. From the idiograms described above it was possible to establish a comparative ratio of the longest chromosome member and the shortest for each species. This was found to show a significantly constant relationship and thus seemed to be a suitable diagnostic tool for purposes of species comparison. The value of this ratio agrees with the work of Makino (27, pp. 153-160).

4. Chiasmata frequency. From material showing the diplotene stage of the meiotic prophase I, chiasmata frequency could be determined and used for comparative purposes. Twenty-five counts were made of comparable diplotene stages and the average of these counts as well as the variational range was graphed. Furthermore, it was thought that the number of chromosomes showing a minimal number of chiasmata (in this case, two)might be of comparative value so this was established for each member of the group. Some general observations on the degree of terminalization at metaphase I were also recorded.

The above data were established for each species studied and used to determine what patterns of caryotype change might have taken place within this group.

The idiogram prepared by Wickbom (38, p. 251) for the species Ambystoma mexicanum and those prepared by Parmenter (33, p. 249) for Ambystoma tigrinum were studied and in-

cluded in a limited way in the final analysis of the material.

CHAPTER III

PRESENTATION OF DATA

For purposes of comparison the following data were obtained for each of the species investigated:

1. Chromosome numbers
2. Chromosome morphology of the haploid set
3. Chromosome idiograms for the haploid set
4. Ratios of the longest chromosome to the shortest
5. Chiasmata frequency
6. Times of spermatogenesis

Observations on Chromosomes

1. Ambystoma macrodactylum - The haploid chromosome number for this species was 14, the diploid number 28. These numbers were determined from over one hundred counts from both mitoses and meioses. No evidence for heterochromosomes was found. This species has nine metacentric chromosomes and five submetacentric ones. If the haploid set is arbitrarily divided into longer and shorter groups a formula of 5M, 2S, 4m, 3s can be obtained, where M stands for metacentric, and S stands for submetacentric chromosomes. The ratio of the longest chromosome to the

shortest chromosome of the set is 3.9. From twenty-five counts the average chiasmata frequency was found to be 59.9 with a range of variation at middle diplotene from 58 to 62.

2. Ambystoma gracile - The haploid chromosome number for this species was 14, the diploid number 28. As in the preceding species no evidence for heterochromosomes was found. Ambystoma gracile has nine more or less metacentric chromosomes and five definitely submetacentric ones. The set has a formula of 5M, 2S, 4m, 3s. The ratio of the longest chromosome to the shortest is 3.7. The average chiasmata frequency was 59, with a range of variation from 55 to 63.

3. Dicamptodon ensatus - For this species the haploid number was also 14, the diploid number 28. Once again no evidence for morphologically distinct sex chromosomes was found. This species possesses nine metacentric chromosomes and five submetacentric ones. The formula is 5M, 2S, 4m, 3s. For Dicamptodon the ratio of the longest chromosome to the shortest is 4.0. The average chiasmata frequency was 58.2 with a range of variation from 54 to 63.

4. Rhyacotriton olympicus - Unlike the three species listed above Rhyacotriton has a haploid chromosome number of 13, and a diploid number of 26. Like the species above no evidence of heterochromosomes was found. The morphology of the haploid set reveals eight metacentric chromosomes and five submetacentric ones. The formula for this species is 4M, 2S, 4m, 3s. The ratio of the longest chromosome to the shortest is 2.9, significantly lower than the other species. The chiasmata frequency was also lower, being 39, with a range of variation from 36 to 42. The number of bivalents showing only two of chiasmata was five.

The main part of these cytological data is presented in graphic fashion in Plates II, III, IV, and V in Chapter IV. For each characteristic it can be noted that Rhyacotriton olympicus is divergent from the other species of the group.

Observations on Times of Spermatogenesis

In addition to the cytological data just presented, information on the time of spermatogenesis and the meiotic cycle in the forms investigated should prove of interest to

anyone contemplating further studies on these species. One of the most vexing problems of this entire study was the determination of the proper time to collect animals in order to obtain testes in florid meiosis. Data for times of spermatogenesis in these western forms were entirely lacking in the literature. In fact, the only discussion of spermatogenesis in the ambystomids was that of Carrick (5, pp. 63-74) on Ambystoma tigrinum and his study employed laboratory animals from a colony in England.

Dr. James Kezer of the University of Oregon reported that he had some material on Dicamptodon ensatus, Ambystoma gracile, and Ambystoma macrodactylum which had been prepared in August, 1952, and which showed more or less meiotic activity. This material was taken from higher altitudes and it was suggested that late June or early July might be a good time to procure specimens. However, some of the plethodontid salamanders undergo spermatogenesis in the early or late spring. Because of the possible variability of the species and because it was of critical importance to make all the collections by Fall, 1955, collecting was begun in late February, 1955, and continued through September of that year. This collecting experience revealed the following:

1. Ambystoma gracile - During the early spring the testes are reduced in size and the sperm

ducts are full of mature sperm. By May the testes begin to enlarge and smears reveal some spermatogonial mitoses. Material collected from Owl Creek near Corvallis showed little activity during May and June. However, by the middle of July the meiotic wave had entered the testes and good squashes could be made from the neotenic larvae used. At this time the testes are enlarged and prominent. Until the males show the full size testes, which are approximately 12 mm. long and 3.5 mm. wide, there is little likelihood of finding meiosis. Specimens collected from the middle of July until the middle of August showed meiotic activity. By the end of August the meiotic wave had passed through the testes and only spermatids, sperm, and Sertoli cells were in evidence. An adult male collected in January, 1956, had an enlarged testis gorged with mature sperm. Evidently after the breeding season in early spring the testes degenerate until June when they begin to enlarge again. The best time, then, to collect and utilize Ambystoma gracile for cytological studies is during July and

the first part of August.

2. Ambystoma macrodactylum - As in Ambystoma gracile squash preparations were made from animals over a long period of time before good material was found. The testis is greatly reduced in the early part of the spring and is not at full size until the latter part of June. At this time the testes enlarge, reaching a size of approximately 10 mm. in length and 3 mm. in width. July is the best month for obtaining material for cytological purposes. However, this is not a favorable time of the year for finding these animals in the Willamette Valley. During April while the animals were leaving the breeding ponds the writer collected a number of adults and placed them in a colony that was kept out-of-doors in a special box filled with moist soil. Earthworms and insects were introduced for food and the colony was sampled at weekly or biweekly intervals. Stomach analyses showed that the animals were eating well and conditions seemed fairly normal. By the first of July these animals were in spermatogenesis

and excellent material was obtained from this source. Once again, July and August are the best times for utilizing Ambystoma macrodactylum for studies of spermatogenesis.

3. Dicamptodon ensatus - No Dicamptodon ensatus was collected before the latter part of July. At that time nine animals were collected from Parker Creek on Mary's Peak. Such are the vagaries of research that eight of the nine turned out to be females and the ninth an immature male. On the fifth of August twelve animals were collected from the same site and three were in spermatogenesis (only three were adult males). Of these three, one was just completing the meiotic wave, one just beginning, and one showed a high degree of meiotic activity throughout the testis. The testes averaged about 23 mm. in length and 45 mm. in width. It would seem that July and August would be the best times for obtaining this species also. Neotenics of this form might also be utilized since they may be easier to obtain in many places.

4. Rhyacotriton olympicus - This species shows

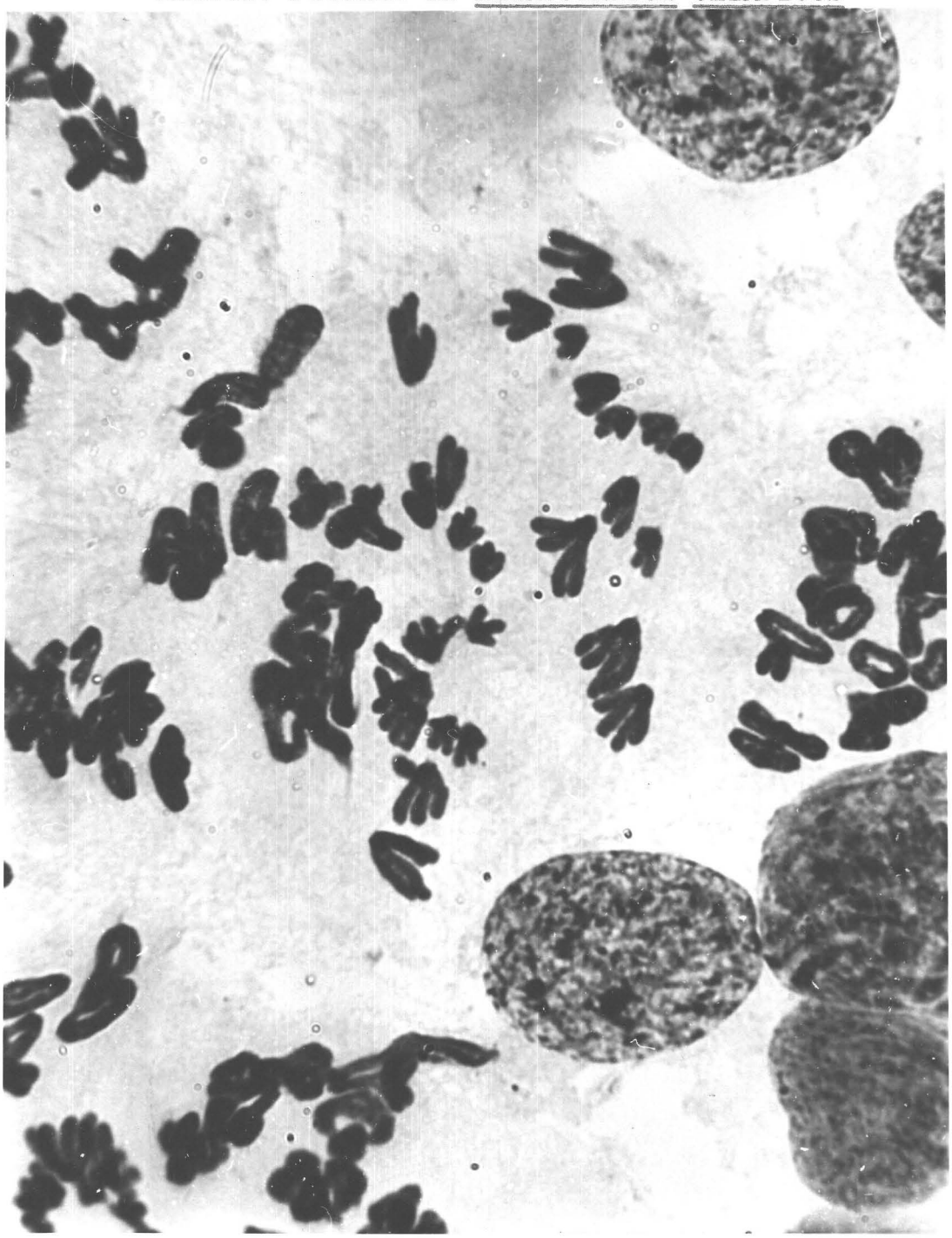
a much earlier time of meiotic activity than any of the other species and also a longer and more varied period during which individuals may be in spermatogenesis. In general the older individuals are through the meiotic cycle about the time the younger males are entering it. Individuals studied in early April showed sperm present but no meiotic activity. The testis does not seem to regress in size to the extent it does in the other species. In this species the testis is darkly pigmented and more ovoid. The average size is 5 mm. in length and 2 mm. in width. By the first of May the animals are in spermatogenesis, most individuals examined being in the height of activity by June 1. Some animals sampled on July 13 showed various stages of development, from those in spermatogenesis to those in which the process was completed. One animal collected by Dr. Storm in November, 1954, whose testes had been fixed in Newcomer's fluid by Dr. Dornfeld, was found to show no meiotic activity but did show a great deal of sperm. It can be concluded from

the above that this species can best be studied during May and June, somewhat earlier than any of the other species studied.

Plate I shows the florid nature of the meiotic figure made available for study by the aceto-orcein squash method. Since meiosis appeared to follow the usual pattern in all of the species studied no further observations need to be presented here. Spermatogenesis has been described in detail for many amphibians; the work of Burger (3, pp. 450-488; 4, pp. 489-513), Janssens (20, pp. 387-411), Carrick (5, pp. 63-74), and Wickbom (38, pp. 341-346) is especially applicable to these forms. No differences were found to exist in any of the present species.

PLATE I

MEIOTIC FIGURES IN RHYACOTRITON OLYMPICUS



CHAPTER IV

DISCUSSION

Of the twenty-two recognized species of the family Ambystomidae the caryotypes of four species had been determined previous to this investigation. These are summarized in Table 1.

TABLE 1

CHROMOSOME NUMBERS IN AMBYSTOMID SALAMANDERS

Species	Haploid Number	Diploid Number	Investigator
Ambystoma mexicanum	--	28	Wickbom
Ambystoma tigrinum	14	28	Parmenter; Carrick
Ambystoma maculatum	--	28	Henley and Costello
Ambystoma jeffersonianum	14	--	Kezer

The use of meiotic material in this investigation has established the haploid number of 14 for Ambystoma gracile, Ambystoma macrodactylum, and Dicamptodon ensatus. Rhyacotriton olympicus, however, has a haploid number of 13. No evidence for heterochromosomes was found in either meiotic or mitotic material. Carrick (5, p. 65) reported, "I have found the haploid number (of Ambystoma tigrinum) to be 14,

with the addition of an X-chromosome which normally divides in the first maturation division and remains whole in the second." Elsewhere in this paper Carrick talks of a questionable autosome and of a diploid number of 28 for this species. The fact that no sex chromosomes were found in the present study sheds considerable doubt on Carrick's observation of them in such a closely related species. As a matter of fact, it supports the opposite view of Wickbom, Matthey, and others that the Caudata do not show morphologically distinct sex chromosomes. The excellent study by Humphrey (19, pp. 33-66) in Ambystoma mexicanum gives genetic proof of a ZW type of sex inheritance in these forms and supports the findings of the present study on the question of heterochromosomes in this group.

As was pointed out in an earlier chapter the previous work done on ambystomid chromosome numbers has shown a high degree of variability. Review of the methods used by previous investigators does much to explain the discrepancies that exist in the literature. The methods used here give clearly spread figures which leave no doubt in their analysis. For this reason it seems there can be no question that the numbers shown in Table 2 are the correct numbers for these species. The term "fundamental number" as used in this table refers to the number of chromosome arms. Metacentric chromosomes have two arms; acrocentric

are considered to have only one arm. In these species all chromosomes are metacentric.

TABLE 2

CHROMOSOME NUMBERS IN NORTHWEST AMBYSTOMIDS

Species	Haploid Number	Diploid Number	Fundamental Number
Ambystoma gracile	14	28	56
Ambystoma macrodactylum	14	28	56
Dicamptodon ensatus	14	28	56
Rhyacotriton olympicus	13	26	52

In view of the fact that Matthey (28, pp. 167-169) has stated that differences in numbers of chromosomes in salamanders correspond very well with familial differences, the diploid number of 26 with the corresponding haploid 13 established here for Rhyacotriton olympicus is of importance. Two hypotheses seem to present themselves when this chromosome number for Rhyacotriton olympicus is considered. Perhaps this species belongs in a new and separate family group since this is the first time the diploid number of 26 has been recorded for any Caudata. On the other hand, it may be that this merely represents an evolutionary offshoot not divergent enough to enjoy a separate family status but still indicating a genus rather remote from the main group of ambystomids. A question that immediately arises at this

point is how important is chromosome number to phylogeny. In many groups of organisms variation of chromosome number even within a single genus is not uncommon. This does not seem to be true of the Caudata however. The question of the taxonomic status of Rhyacotriton olympicus will be considered in more detail in the next chapter, after other data have been examined.

Chromosome Morphology

Matthey (29, pp. 184-185) reports that the general pattern of caudate chromosome morphology is one of large two-armed or V-shaped figures at the metaphase plate. In the more primitive families there is also an inner group of micro-chromosomes. In more specialized families the micro-chromosomes are lost and chromosome number is reduced. The material studied here is not at variance with this general description of the entire group. Plate II shows nicely spread anaphase II figures for each species and gives a good picture of the haploid set of each species at a comparable meiotic stage. Since this stage shows the morphology of the condensed chromosomes more clearly than any other it was used for the morphological analysis.

One problem inherent in the method used was that of variability due to different squash pressures and the possibility of comparing slightly different stages. Fixation did not seem to be always constant either; sometimes the

PLATE II

THE HAPLOID CHROMOSOMES

Ambystoma gracile n = 14

Rhyacotriton olympicus n = 13

Ambystoma macrodactylum n = 14

Dicamptodon ensatus n = 14

chromosomes seemed more swelled than other times. This variability thus diminishes the chances for and the validity of any microquantitative comparisons.

A formula-comparison of the haploid set of each species is shown in Table 3. The formula employs the letters M and S to indicate longer metacentric and submetacentric chromosomes and the same letters in lower case to indicate shorter ones. The set was arbitrarily divided as to longer chromosomes and shorter ones.

TABLE 3

FORMULAE OF THE HAPLOID SET OF CHROMOSOMES

Species	Formula
Ambystoma gracile	5M, 2S, 4m, 3s
Ambystoma macrodactylum	5M, 2S, 4m, 3s
Dicamptodon ensatus	5M, 2S, 4m, 3s
Rhyacotriton olympicus	4M, 2S, 4m, 3s

The results reported here are in fairly good agreement with the work of Parmenter (33, pp. 169-249), Wickbom (38, pp. 250-254), and Prokofieva (34, pp. 148-169). It should be remembered however that this previous work done on the genus Ambystoma utilized mitotic metaphase figures. Perhaps the squash method used here gives more accurate results when used to study morphology. For example,

attempts by Wickbom and Prokofieva to formulate the haploid set for Ambystoma mexicanum reveal divergent morphologic patterns. How real these divergences are is difficult to determine because of the differing techniques employed. In the future comparisons should be made from material that has been treated in a uniform manner.

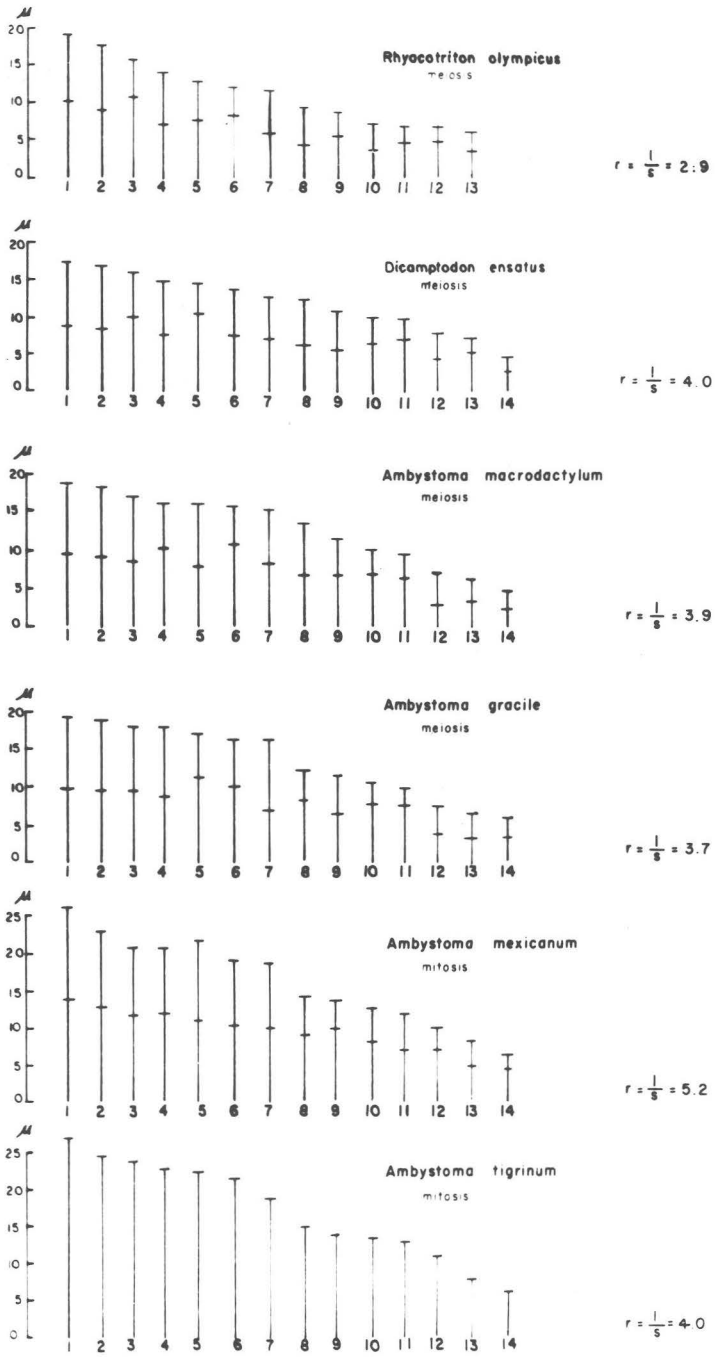
Idiograms

From photomicrographs and camera lucida drawings an idiogram for each species was prepared to show the dimensions of the chromosomes and the positions of the centromere in an average haploid set. These idiograms, as well as one adapted from Parmenter (33, p. 248) for Ambystoma tigrinum and one from Wickbom (38, p. 251) for Ambystoma mexicanum, are presented in Plate III. In each case the chromosomes are arranged and numbered in order of descending chromosome length. This plate illustrates a fundamental characteristic of caudate chromosome morphology. When arranged in this fashion the chromosomes form a steadily falling curve. Salientia, on the other hand, show a sharp step in the middle of the curve, possibly indicating an incipient division of the set into a group of large chromosomes, and a group of small ones. This has been discussed in detail by Wickbom (38, p. 326).

As will be seen from the idiograms the chromosomes of the species studied are large; indeed, the Caudata as a

PLATE III

CHROMOSOME IDIOGRAMS



group possess some of the largest of all known chromosomes. The length ranges to almost 30 microns at the stage measured. The idiograms also clearly show the relative lengths of the arms of each chromosome.

Ratio of Longest Chromosome to Shortest

From the idiograms it was possible to make a number of comparisons both within the set of each species as well as between species. The most significant of these was the ratio of the longest chromosome of the set to the shortest. This is shown in Plate III and also in Table 4. It may be noted that while the other species studied are very similar in this ratio, Rhyacotriton olympicus is significantly lower.

TABLE 4

RATIO OF LONGEST CHROMOSOME TO SHORTEST CHROMOSOME

Species	Longest chromosome in microns	Shortest chromosome in microns	Ratio $\frac{l}{s}$
R. olympicus	19	6.5	2.9
D. ensatus	18	4.5	4.0
A. gracile	20	5.4	3.7
A. macrodactylum	19.5	5.0	3.9
A. tigrinum	39	9.8	4.0
A. mexicanum	26	5.0	5.2

It is interesting to observe that while the material of Wickbom and Parmenter is from mitotic material this ratio is rather close to that obtained for the species studied here. A check was made of this comparison for mitotic metaphase for Dicamptodon ensatus, and for meiotic anaphase I, metaphase II, and anaphase II for Rhyacotriton olympicus and Ambystoma macrodactylum. In each case, although the actual length of the chromosomes varied with the degree of forshortening in the various stages, this ratio remained within plus or minus 0.3 of the values recorded in the table above. The results as presented appear significant without subjecting them to statistical analysis.

Chiasmata Frequency

One of the tools that has proven to be of value in comparative cytology studies is the comparison of the chiasmata frequency of the caryotype of each species studied. Chiasmata frequency at metaphase I was used by Kezer (22, pp. 1-55) in his studies of the plethodontid salamanders. Wickbom (38, pp. 241-346) used chiasmata frequency at diplotene in his investigation. In some species it has been shown that as diplotene progresses the chiasmata terminalize and at metaphase of the first division are greatly reduced in number. In other species terminalization before metaphase is negligible, although chiasmata sometimes shift more distally in the bivalent.

The species studied here show no great reduction in chiasmata from diplotene to metaphase I. In no case was any significant reduction in chiasmata from early to late diplotene demonstrated. For this study only chromosome sets in middle diplotene were used for comparing chiasmata frequency. In making counts of chiasmata frequency great care must be used to guarantee that the material is interpreted correctly. It is the author's opinion that at least in some studies overlaps in long diplotene bivalents have been mistaken for chiasmata. This would lead to a report of high chiasmata frequency. For example, if counts in Rhyacotriton olympicus are made in late diplotene or diakinesis five synaptic figures distinctly show a minimum number of chiasmata of two per bivalent. This is characteristic for this species. Studies of early diplotene do not always show these five characteristic figures; instead some bivalents appear as figure-eights. Careful analysis of these bivalents reveals that they are not bivalents with three chiasmata but only apparently so. This sort of overlapping is common throughout the set and if misinterpreted could lead to much higher counts of chiasmata frequency than actually exist.

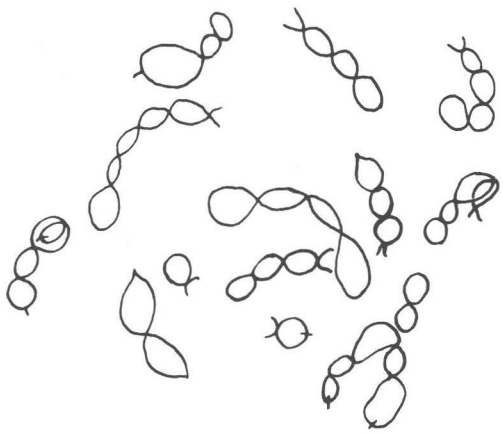
The determinations made here were done under oil at a magnification of 1500 diameters and camera lucida drawings were made of a characteristic caryotype for each species

(see Plate IV). Since some variation in chiasmata does exist from cell to cell at least twenty-five determinations were made from as many different individuals as possible and these determinations averaged. The average for each species as well as the limits of variation was graphed for analysis. The results can be seen in Plate V. They seem to substantiate the other findings of this investigation in showing that Rhyacotriton olympicus has a significantly lower chiasmata frequency than any of the other three species studied. Since none of the earlier studies on Ambystoma has included an analysis of chiasmata frequency no comparisons with earlier work could be made. The writer hopes to continue the analysis of this family and in time should have all the members of the group investigated.

Future work should also include subspecific analysis as regards chiasmata frequency. For the present it is interesting to note that the material from Rhyacotriton olympicus which was obtained in the zone of intergradation between the two subspecies of this species from such widely separated places as Mary's Peak, Falls City, and the Columbia River Gorge failed to show any differences. This was also true of material from Dicamptodon ensatus which came from Mary's Peak and the Oregon Caves, and for Ambystoma macrodactylum from the Willamette Valley and Crater Lake. Further exhaustive microquantitative studies may

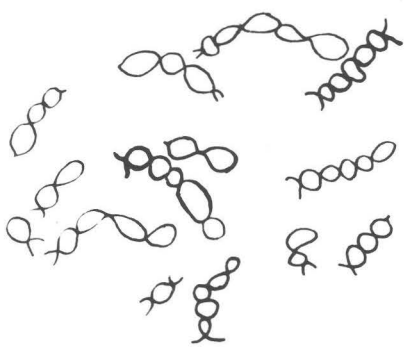
PLATE IV

CHIASMATA AT DIPLOTENE

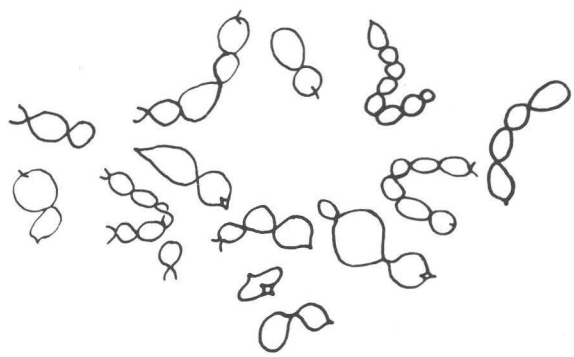


Dicamptodon ensatus X:ta = 58

Rhyacotriton olympicus X:ta = 40



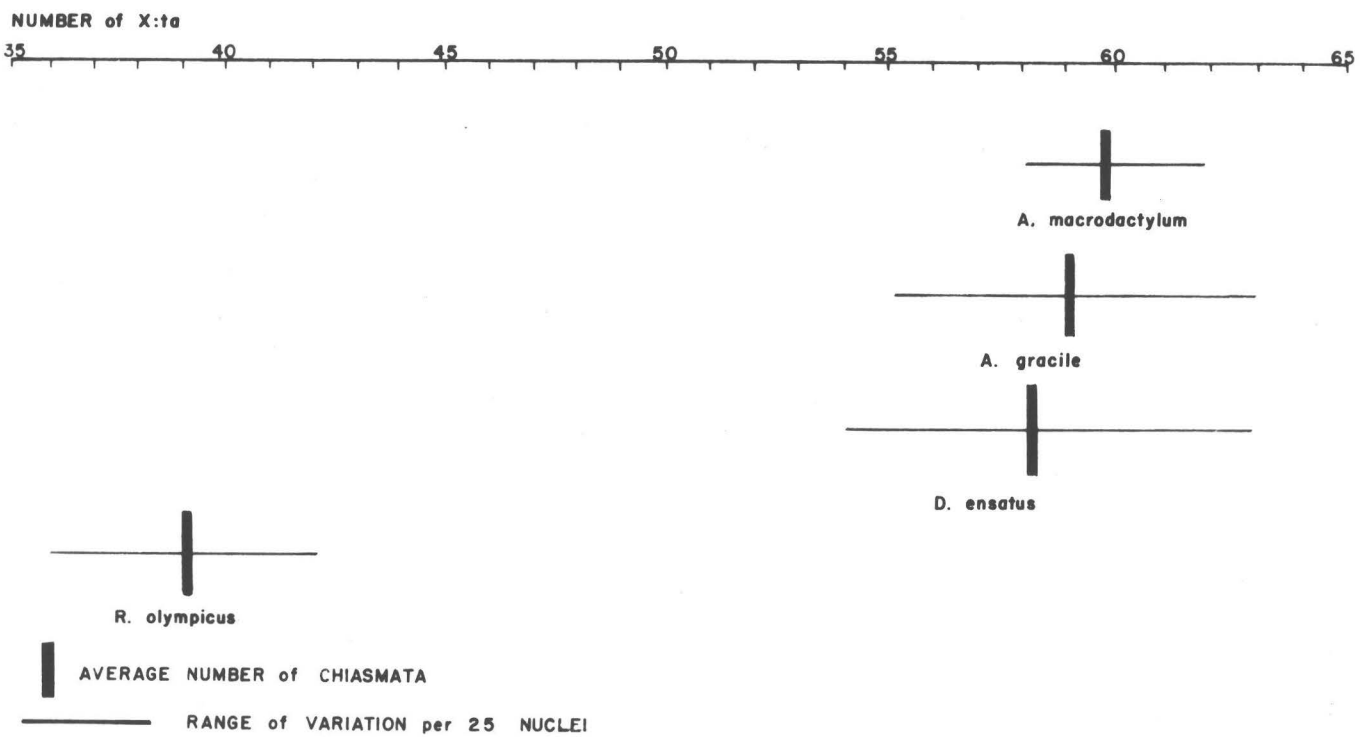
Ambystoma gracile X:ta = 60



Ambystoma macrodactylum X:ta = 59

PLATE V

CHIASMATA FREQUENCY



shed more light on the value of this determination in the cytological analysis of subspecies. It is of utmost importance in species comparisons.

The number of bivalents showing a minimum of chiasmata might also prove to be a valuable comparative quantity. In the present investigation it proved to be of value. As previously recorded Rhyacotriton olympicus had five bivalents showing this minimum number. This variation alone did much to explain the lower chiasmata frequency in Rhyacotriton, though the fact that this species had one less bivalent also helped to account for the lower number. The use of bivalents with a minimum of chiasmata for comparative purposes had the further advantage that this datum was easy to determine and thus fulfilled the requirement of a good and simple diagnostic character.

It can be noted from the discussion in this chapter that Rhyacotriton olympicus is divergent from the other species of the unit for each characteristic studied.

CHAPTER V

CHROMOSOMES AND THE PHYLOGENY OF THE CAUDATA

Speculations on the phylogeny of the Caudata have been made by Dunn (10, pp. 1-441) and by Noble (32, pp. 1-577). Noble has proposed a scheme based on life history, comparative anatomy, and similarity of habits. From these data he felt that the Hynobiidae are the most primitive of all the living salamander groups. The Cryptobranchidae are closely related to the hynobiids, perhaps representing "permanent larvae" of some unknown hynobiid. The Ambystomidae are also rather primitive and could have evolved directly from the hynobiids. The Salamandridae do not show close relationships to any existing genus of hynobiid or ambystomid and probably arose from some pre-hynobiid stock. Nevertheless, they are related to these two families. Amphiuma, although perhaps more similar to the salamandrids than to any other family, is not directly derived from any recent genus. The Plethodontidae have arisen from early mountain brook salamandrids, and the Proteidae seem to occupy an isolated position among the salamanders. The phylogenetic relationships of the Sirenidae to any of the above groups are unknown since they have no close affinities to any other modern group. For a diagrammatic representation of this proposed scheme see Plate VI.

PLATE VI

THE PHYLOGENY OF THE CAUDATA

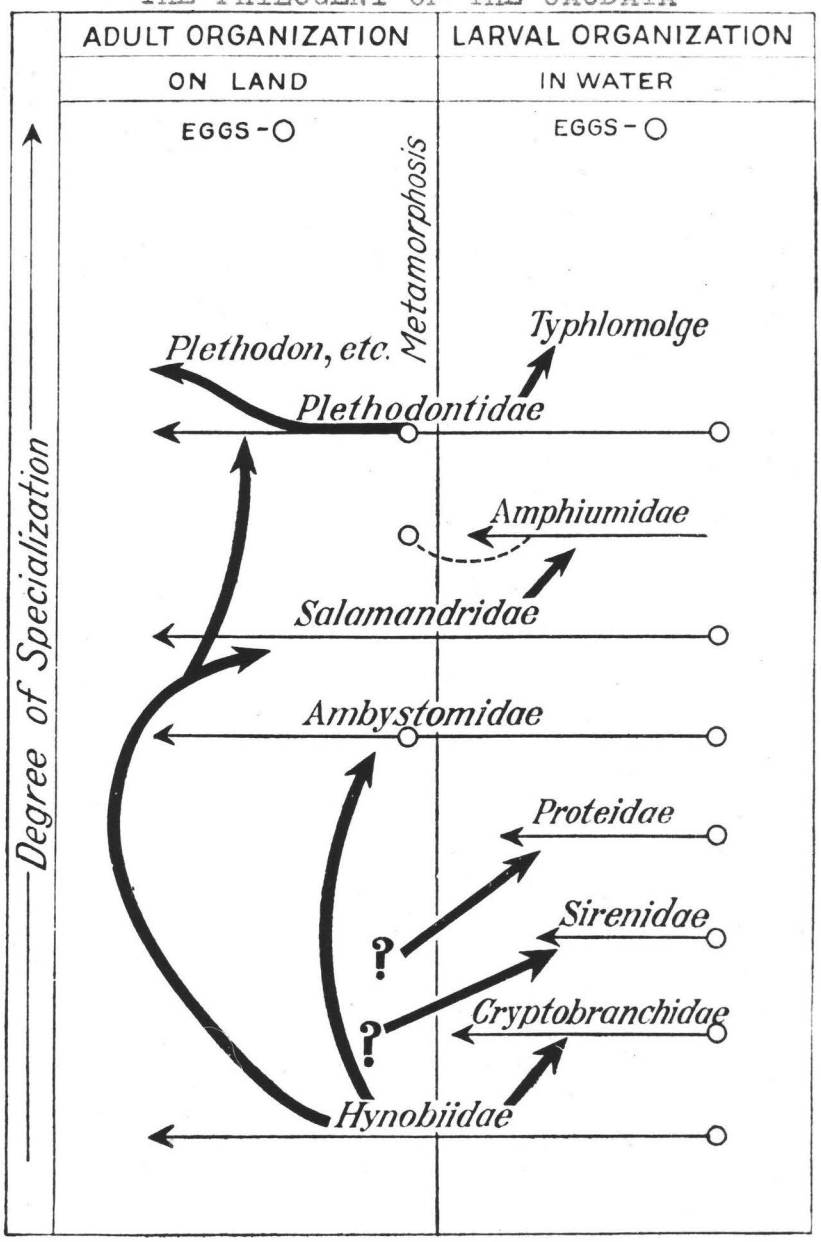


FIG. 145. Diagram illustrating the phylogeny of the urodeles. The heavy black arrows indicate the phylogenetic relations. The narrow, horizontal arrows represent the ontogeny of the various families. The degree of metamorphosis of the hyobranchial apparatus is employed as the chief criterion of metamorphosis in this diagram. The "permanent larvae" are not closely related but have been derived from different groups. (Adapted from Noble)

Noble has further speculated that among the Ambystomidae, the genera Dicamptodon and Rhyacotriton are more closely related to each other than to any members of the genus Ambystoma. He states that Rhyacotriton olympicus, the only species of this genus, is a dwarf derivative of Dicamptodon.

The cytology of the various families of Caudata is less than complete. However some data on the majority of the families are available. In many cases this consists of only chromosome counts without any detailed study of the chromosome morphology. The Sirenidae, Proteidae, and Amphiumidae have been studied scarcely at all. Matthey (28, p. 168) summarized the knowledge to date in a short listing as follows:

Family	2n	Fundamental Number
Hynobiidae	40-62	76
Cryptobranchidae	62-64	74
Proteidae	?	?
Ambystomidae	28	56
Salamandridae	22-24	48
Amphiumidae	?	?
Plethodontidae	28	56

As was mentioned in Chapter IV the term "fundamental number" refers to the number of chromosome arms. This term has been proposed by Matthey (29, pp. 176-185) to emphasize that in groups where chromosome number sometimes varies the number of chromosome arms is nevertheless constant. Thus oftentimes this "nombre fondamental" is of value in ascertaining relationships that otherwise might be unclear.

It is interesting to note that the Dipnoi have a fundamental number of 76, the same as the supposedly primitive hynobiids. It is assumed from this and other evidence that there has been a reduction of the number of chromosomes and of the fundamental number in the evolution from the primitive to the more specialized forms. Both losses of chromosomes and centric fusions have been used to explain possible mechanisms by which the changes in chromosome numbers have come about. It will be noted from the above listing that up to the time of Matthey's paper, which was published in 1951, intrafamilial variations had been found only among the Hynobiidae, Cryptobranchidae, and Salamandridae, and for the first two families at least centric fusions seemed to be a good explanation of these variations since the fundamental number was constant. All other families were characterized by a single modal number. On the basis of this evidence Matthey (28, p. 169) states, "A chromosomal discontinuity corresponds to the familial discontinuity of the systematicians; within the families, the fundamental homologs of the chromosomes are respected; there have been no great chromosome mutations but essentially gene mutations. The chromosomal formula gives an objective value to the notion of the family."

These statements must be kept in mind when the significance of the chromosome number found to be constant in Rhyacotriton olympicus is considered. The two members of

the genus Ambystoma, and Dicamptodon ensatus studied here have caryotypes very similar to each other and as far as can be ascertained from the literature, very similar to other ambystomids investigated. When these species are compared with Rhyacotriton olympicus however, it can be noted that there are fundamental differences in chromosome number, ratios of longest chromosomes to shortest, chiasmata frequency, and timing of spermatogenesis. Here then, the cytological evidence would point to a chromosomal discontinuity, which in turn might point to an actual familial discontinuity. If this were the case then it would seem the Rhyacotriton olympicus should be separated from the family Ambystomidae, and placed in a new one. In the light of the incompleteness of our present knowledge it would be wise however to investigate this whole line of reasoning much more thoroughly before any taxonomic changes are attempted.

At this point it might be of some significance if a few observations on the comparative anatomy and ecology of these species were presented. There are several characteristics in which the first three named animals studied are more similar and in which Rhyacotriton olympicus proves to be divergent. For example, the testes of Ambystoma macrodactylum, Ambystoma gracile, and Dicamptodon ensatus are all non-pigmented and though of varying size all are of the same general shape and appearance. Rhyacotriton

olympicus, on the other hand, has more oval testes that are darkly pigmented. Whereas the time of spermatogenesis is July to August for the first three species, Rhyacotriton olympicus enters spermatogenesis in May. Rhyacotriton olympicus has only rudimentary lungs, no nasals, the larvae lack a body fin, and have very short bushy gills; the adults have a more tubular tail instead of the laterally compressed one characteristic of the other species, the eye is much larger in proportion to the head, and the male possesses prominent lobes on either side of the vent. These rectilinear lobes are not found in the other species.

From the above morphologic as well as cytological evidence it can be deduced that Noble's supposition that Dicamptodon and Rhyacotriton are more closely related to each other than to any of the members of the genus Ambystoma does not seem plausible. In the light of the new evidence presented here there is also no good reason to support his hypothesis that Rhyacotriton olympicus is a dwarf form of Dicamptodon ensatus. In fact there is no evidence that these two species are closely related at all.

The affinities of Dicamptodon and Rhyacotriton were explored from a comparative anatomy point of view by Eaton (12, p. 182). Eaton pointed out that Rhyacotriton has little superficial resemblance to Dicamptodon and that Rhyacotriton is "structurally one of the most peculiar of all salamanders." Eaton presents further evidence which

he considers to be proof that these two species are not at all closely related. In fact he feels that Rhyacotriton olympicus has evolved as a genus from one of the two northwestern Ambystoma. The present study supports the first conclusion of Eaton but not the second. On the basis of the cytological evidence this writer would suggest that Rhyacotriton olympicus must have evolved at an early time and is not closely related to any other living ambystomid.

At a fairly early stage in this investigation an attempt was made to explain the difference in the chromosome number of Rhyacotriton olympicus on the basis of a centric fusion of two chromosomes of the usual ambystomid 14, thus giving 13 to this species by this process. In view of the lack of any acrocentric chromosomes and because of other difficulties there seemed to be no basis for such an explanation. When chiasmata frequency was compared it seemed to point to the fact that Rhyacotriton olympicus was fundamentally different from any of the other species and any splitting off from the present-day ambystomids would have to be discounted. It was not possible to explain this chromosome difference by proposing a loss of one chromosome either. For in each case it would be hard to explain the great difference in chiasmata frequency. This illustrates that chiasmata frequency is a most valuable cytological tool, for without the knowledge of the difference in the frequency of chiasmata, one might be tempted to construct a

rather pat explanation of chromosome evolution. From the evidence at hand it is probably most prudent to state only that Rhyacotriton olympicus is the most divergent cytologically of the unit here studied, and that possibly its taxonomic status should be reviewed.

This investigation should conclusively clear up another problem that is found in the literature. In 1917 Gaige (16, pp. 1-3) described a new salamander from the state of Washington which was put into the genus Ranodon. In 1920 Dunn (11, pp. 55-59) put this salamander in the family Ambystomidae and revised its name to Rhyacotriton olympicus. The present study supports the evidence that this species is definitely not one belonging to the genus Ranodon since Ranodon is a genus in the family Hynobiidae, a family which shows a much higher chromosome number than Rhyacotriton olympicus. Thus the cytological evidence supports the work of Dunn.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. Aceto-orcein and iron hematoxylin squash preparations and tissue sections were used to study the chromosomes of four Northwest ambystomid salamanders. The primary source of chromosomes was the meiotic testis, but larval tail clips and regenerating liver tissue were also used.

2. Ambystoma gracile, Ambystoma macrodactylum, and Dicamptodon ensatus have a haploid number of 14, and a diploid number of 28. Rhyacotriton olympicus has a haploid number of 13, and a diploid number of 26.

3. No evidence was found for heterochromosomes for any of these species.

4. Formulae for the haploid set of each species have been presented.

5. The ratio of the longest chromosome of the haploid set to the shortest is almost the same for each species with the exception of Rhyacotriton olympicus which has a significantly lower ratio.

6. Chiasmata frequency at middle diplotene averages about 60 per caryotype in Ambystoma gracile, Ambystoma macrodactylum, and Dicamptodon ensatus. For Rhyacotriton olympicus the average caryotype has a chiasmata of 40.

7. The number of bivalents showing a minimum of chiasmata is two for each species except Rhyacotriton

olympicus in which the number of bivalents showing a minimum of chiasmata is five.

8. The times of spermatogenesis for each species except Rhyacotriton olympicus are July and August. In Rhyacotriton spermatogenesis occurs earlier, in May and June.

9. Ambystoma macrodactylum, Ambystoma gracile, and Dicamptodon ensatus all possess the characteristic modal number of chromosomes reported for the family Ambystomidae. The morphology of the chromosome set and the chiasmata frequency during diplotene are all similar. In addition, the ratio of the longest chromosome to the shortest chromosome of the set is approximately the same for each of these species. All of these species have only two bivalents showing a minimum (2) of chiasmata. Thus, the evidence points out that these species are fairly closely related and most differences between them have been due to gene mutations, rather than chromosome changes.

10. Rhyacotriton olympicus possesses a new and unique number of chromosomes as compared to other Caudata. The chiasmata frequency at diplotene is significantly lower than that of any other member of the group studied. The ratio of the longest chromosome to the shortest chromosome of the set is significantly less than that of the other species. It has five bivalents showing a minimum (2) of chiasmata. All of this evidence indicates that Rhyacotriton

olympicus is highly divergent when compared to other ambystomids. In fact this information indicates the need for a possible revision of the taxonomic status of this genus.

11. Contrary to Noble, there seems to be little or no evidence to support the hypothesis that Rhyacotriton olympicus and Dicamptodon ensatus are more closely related to each other than to the members of the genus Ambystoma. Furthermore, his speculation that Rhyacotriton is a dwarf derivative of Dicamptodon seems untenable. Both cytological and morphological evidence confirm this.

12. On the point above, Eaton's idea that the two above-mentioned species are not closely related is supported but his further speculation that Rhyacotriton olympicus evolved from either Ambystoma macrodactylum or Ambystoma gracile is not.

13. The evidence from the chromosome number of Rhyacotriton confirms the earlier decision of Dunn that this species did not belong to the genus Ranodon which was the genus to which it was first assigned.

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