THE PRACTICALITY OF KARYOTYPE ANALYSIS AS A TAXONOMIC TOOL IN THE PLETHODONTIDAE (AMPHIBIA)

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An Abstract

Presented to

the Committee on Graduate Studies

Austin Peay State College

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

in Education

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by

Connie Sue Eatherly

August 17, 1967

ABSTRACT

A new technique for preparing chromosomes of a plethodontid salamander for karyotyping is described. The results of karyotypic comparisons between *Desmognathus fuscus conanti* and *Eurycea bislineata rivicola* showed taxonomically significant differences to exist, most notably in that *Desmognathus* lacked any acrocentric chromosomes while four such chromosomes were found in *Eurycea*. The techniques as used yielded acceptable preparations but at an unacceptable rate.

THE PRACTICALITY OF KARYOTYPE ANALYSIS AS A TAXONOMIC TOOL IN THE PLETHODONTIDAE (AMPHIBIA)

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To the Graduate Committee:

I am submitting herewith a thesis written by Connie Sue Eatherly entitled "The Practicality of Karyotype Analysis as a Taxonomic Tool in the Plethodontidae (Amphibia)." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts in Education, with a major in Biology.

David A. Snyder
Major Professor

We have read this thesis and recommend its acceptance:

Ellis B. Burns
Minor Professor

William H. Ellis

Accepted for the Committee:

William H. Ellis
Director of Graduate Studies

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CHAPTER I

INTRODUCTION

A karyotype is the systematic array of the chromosomes of a mitotic cell, depicting number, size, form and any other features that may typify the chromosome complement of a species.

Karyotype analyses have contributed to the elucidation of phylogenetic relationships within many groups and to the knowledge of isolating mechanisms between related groups of animals. Such analyses have been conducted on certain species of Amphibia, but this area has yet to be explored adequately in the Plethodontidae. Makino (1951) has reported chromosome numbers of certain species of Plethodon, Desmognathus and Eurycea. One reason for the few accounts of karyotypes of salamanders is the lack of a suitable technique for their preparation. Some common technical deficiences include the appearance of the chromosomes in more than one plane, inadequate spreading to allow counting and such poor texture as to render the preparation useless.

The purpose of this investigation was to determine the efficacy of karyotype analysis as a tool in plethodontid taxonomy by karyotyping several and varied representatives of the family and comparing the results for taxonomically significant differences.

CHAPTER II

REVIEW OF THE LITERATURE

Few chromosome studies have been conducted on salamanders. The oldest relating directly to my investigation were summarized by Darlington and LaCour (1947). They reported that chromosomes of the newt (Triturus cristatus), family Salamandridae, could be found in squashed sections of testes, with more satisfactory results obtained by using breeding animals.

Hungerford and DiBerardino (1958) demonstrated cytological effects of prefixation treatment on embryonic frog tail tips (Rana pipiens) placed in Niu-Twitty and modified Niu-Twitty solutions at thirty-seven degrees centigrade for twenty to sixty minutes to include swelling and disorganization of the spindle.

Weiler and Ohno (1962) found that intraperitoneal injection of colchicine (five-tenths milliliters of five percent aqueous solution) into the African water frog (Xenopus laevis) resulted in the appearance of more metaphases in spleenic tissue pretreated for fifteen minutes in distilled water.

Uzzell (1963) found regenerating tail tissue to be

more satisfactory than the original tail fin in chromosome studies of Ambystoma jeffersonianum. He found that such tissue yielded more mitotic figures and less melanin.

By maintaining leucocyte cultures at room temperature instead of thirty-seven degrees as is done for humans, Seto, Pomerat and Kezer (1964) demonstrated the karyotype of the mudpuppy (Necturus maculosus), and Becak, Becak and Nazareth (1966) that of the boa constrictor (Boa constrictor).

On the basis of the results of these studies an investigation was designed to determine the efficiency of karyotype analysis in plethodontid taxonomy.

CHAPTER III

MATERIALS AND METHODS

All techniques mentioned in this chapter were employed on several species of Plethodontidae. Desmognathus fuscus conanti and Eurycea bislineata rivicola were the only forms for which suitable karyotypes were obtained. Unsuitable preparations were made from the following animals: Eurycea lucifuga, Eurycea longicauda longicauda, Eurycea bislineata wilderae, Pseudotriton ruber ruber, Plethodon cinereus cinereus and Plethodon glutinosus glutinosus. All specimens were collected in Tennessee.

Preliminary Evaluation of Techniques

Several methods were evaluated in preparing the chromosomes of the salamanders, as outlined in the following paragraphs.

Darlington and LaCour's (1947) acetic-lacmoid method (substituting orcein for lacmoid) yielded chromosomes visible at polar metaphase but tightly spaced in the center of the spindle.

Culture of peripheral blood was attempted, as follows: Upon anesthetization with tricaine methanesulfonate (MS 222,

Sandoz) in 250 parts tap water, approximately twenty-five-hundreths milliliters (ml.) of blood was drawn from the animal's heart and placed in a twelve ml. centrifuge tube containing two-tenths ml. of heparin. To the heparinized blood was added a nutrient fluid of eighty-five percent Earle's solution, five-tenths percent lactoalbumin hydrolysate, ten percent calf serum, five percent whole egg ultrafiltrate and two percent antibiotics (streptomycin and penicillin, Difco). Cultures were incubated at twenty-six degrees centigrade without agitation for ten days. Forty hours before the culture was harvested, colcemid, in the final concentration of thirty-five-hundreths micrograms per ml. of culture was added to the centrifuge tube. The cells were then harvested according to the method of Moorhead (1960).

Peripheral blood cultures were also established, maintained and harvested according to Moorhead (1960) with modifications of temperature and duration of incubation. Some cultures were incubated at thirty-seven degrees centigrade for three days and others at twenty-six degrees centigrade for three to six days.

An attempt to culture solid tissue according to the method described for human tissue in a series of papers by Evans and Earle (1947), Earle, Schilling and Shelton (1950), Evans and Earle (1951), Earle and Sanford (1951), Sanford and

Earle (1951) was made using tissue from the tail of Desmognathus.

An attempt to aspirate bone marrow from the long bones of the legs, as suggested by Tjio and Whang (1965), yielded insufficient quantities of material for study.

Direct preparations of liver, spleen, tail, ovaries and testes of the salamanders were made using Weiler and Ohno's (1962) method.

A New Modification of Moorhead's Technique

A modification of Moorhead's (1960) harvest technique developed by Bunting (personal reference) and myself was used with spleenic and testicular tissues. The technique, with modifications incorporated, was as follows: The animal was injected intraperitoneally with colcemid (N-desacetyl-N-methyl-colchicine, demecolcin CIBA) two hours prior to sacrifice by decapitation. The spleen and testes were immediately removed and placed in a twelve ml. centrifuge tube containing five ml. of five percent solution of trypsin in Earle's solution with the pH adjusted to seven and four-tenths with sodium bicarbonate. The cells were macerated with a micro-spatula and two drops of heparin added to the tube. The cells were suspended in the trypsinized Earle's solution by repeated aspiration with a siliconized, flamed, Pasteur's pipette and the centrifuge tube placed in a water bath at thirty-seven degrees centigrade for

thirty minutes. The cells were then resuspended in the trypsinized solution, centrifuged at 2000 revolutions per minute (RPM's) for five minutes and the supernatant decanted. The cells were then washed and suspended in five ml. of standard Earle's solution, centrifuged at 2000 RPM's and the supernatant decanted, leaving one ml. of solution in the tube. The cells were then suspended in one ml. of solution. Three ml. of sterile distilled water were added, dropwise, to insure the suspension of the cells in the water. The cells were resuspended in the water by a repeated pipetting, and the centrifuge tube placed in a water bath at thirty-seven degrees centigrade for five minutes. Then the cells were again suspended in the water by a single gentle pipetting, centrifuged at 2000 RPM's for five minutes and the supernatant decanted. The volume in the tube was then brought to two ml. by the slow addition (pipetting it dropwise down the side of the tube) of one part glacial acetic acid and three parts methyl alcohol, at which point three additional ml. were added more rapidly, bringing the final volume to five ml. The cells were left in the fixative, unagitated, for thirty minutes.

As the cells were being fixed, slides were readied for tissue by washing according to a three step method devised by Bunting (personal reference): (1) the slides were soaked for fifteen minutes in fifty ml. of warm tap water containing

three drops of detergent; (2) each slide was rinsed twenty times in warm tap water and placed in a Copley jar of tap water; (3) each slide was rinsed twenty times in distilled water and placed in a Copley jar containing distilled water.

After fixation the cells were suspended in the five ml. of fixative, centrifuged at 2000 RPM's for five minutes and the supernatant decanted. One ml. of fresh fixative was added to the centrifuge tube and the cells suspended in the fresh solution. A slide from the Copley jar was dipped successively into two beakers of distilled water and the suspension pipetted onto it. The slide was tapped firmly, endwise, on a pad of blotting paper to aid the spreading of the suspension, then passed slowly through a Bunsen flame. Each slide was then air-dried for a minimum of forty-eight hours, hydrolyzed in 1N HCl for twelve minutes at fifty-six degrees centigrade, stained by immersion for eight seconds in one ml. of Bleu de Unna (phenol-sulphone-phtaleine) in fifty ml. of distilled water and mounted in Permount.

In some instances tissues treated as above were taken from animals which had been injected with two-tenths ml. of chorionic gonadotropin (Antuitrin "S", 200 units per ml.) twenty-two hours prior to the injection of twenty-five-hundreths ml. of five-tenths percent colchicine.

In other cases the toes of a hind foot of the animals

were amputated prior to tissue treatment with the modified Moorhead (1960) harvest technique to accelerate mitotic activity in the spleen. This process had been shown to produce satisfactory results in spleenic tissue of Xenopus laevis as described by Mikamo and Witschi (1966).

Observations of Preparations

The prepared slides were examined by phase contrast microscopy using a Zeiss 100x objective and 8x ocular.

Photomicrographs were made with a Zeiss Photomicroscope (Number 62829) through a 100x objective and 8x ocular. Kodak Kodabromide high contrast copy film with a Tungsten ASA-64 was used. Contrary to normal procedure, Kodak stopbath was used (diluted as recommended) in the development of the film to prevent an undesirable staining caused by the fixer. Photomicrographs were printed on Kodabromide (Grade A-3) paper.

Pictures of the chromosomes were cut from the photomicrographs and arranged in order of decreasing lengths. Measurements were made from the photos in millimeters. Using a flexible ruler the actual lengths were calculated in microns based upon the degree of magnification acheived in the photographing and printing and the arm ratios determined. Each chromosome was given a specific number correspondent to its

position in a ranked table of measurements. The number of metacentric, submetacentric and acrocentric chromosomes was determined based on centromere position. The karyotypes were then compared for significant differences.

CHAPTER IV

RESULTS

Preliminary Evaluations

The squash method of Darlington and LaCour (1947) as used in this investigation yielded visible chromosomes in about five percent of the slides, but they were too tightly fixed in the center of the spindle for counting. The chromosomes swelled after prefixation as suggested by Hungerford and DiBerardino (1958) but still did not spread. Mitotic activity and therefore the frequency of metaphases was increased by using regenerating tissue from the tail of the salamanders as suggested by Uzzell (1963).

Cultures of peripheral blood according to Moorhead (1960), Seto (1964) and Becak (1966) showed no apparent growth of cells and certainly no mitotic activity.

Metaphase chromosomes obtained from testicular material, prepared by the method of Bunting (personal reference) and myself, yielded diploid and haploid sets from Desmognathus.

Using the method of Weiler and Ohno (1962) diploid sets of Eurycea were obtained.

Karyotypes of Desmognathus and Eurycea

Metaphase chromosomes obtained from testicular material of Desmognathus and Eurycea are shown in Figures 2 and 3, respectively; their karyotypes are shown in Figures 4 and 5. The relative lengths of individual chromosomes are expressed as a percentage of total length of the haploid genome. Chromosomes with an arm ratio between one and one and thirty-three-hundreths (long arm/short arm) were classified as metacentric, those between one and thirty-four-hundreths and one and ninety-nine-hundreths as submetacentrics and those over two as acrocentrics. These classifications are indicated in Table 1.

Desmognathus had a diploid number of twenty-eight (2N=28) confirming that listed by Goin and Goin (1962). The haploid number was fourteen (N=14), Figure 1, one-half the diploid complement. The twenty-eight chromosomes of Desmognathus regularly formed fourteen bivalents in spermatogenic metaphases as shown in Figure 1. To facilitate comprehension and comparison of karyotypes, each of the chromosomes of Desmognathus, after having been ranked and numbered in order of decreasing size, was placed into one of five groups. Group I consisted of the five longest pairs, all of which were metacentric. Group II contained one pair of metacentrics and four pairs of submetacentrics. Group III contained a pair of

TABLE 1

Average Values of Relative Chromosome Lengths and Arm Ratios of *Desmognathus fuscus conanti* and *Eurycea bislineata rivicola*

Chromosome Number	Taxon	Relative Total Length	Arm Ratio	Centromere Position
1	D	17.64	1.17	M
	E	11.89	1.45	SM
2	D	15.61	1.29	M
	E	11.41	1.58	SM
3	D	11.15	1.14	M
	E	11.89	1.56	SM
4	D	10.51	1.33	M
	E	9.29	1.88	SM
5	D	10.41	1.31	M
	E	9.78	1.11	M
6	D	10.14	1.02	M
	E	9.61	1.29	M
7	D	10.14	1.33	M
	E	8.47	1.74	SM
8	D	9.79	1.33	M
	E	8.47	1.62	SM
9	D	9.77	1.16	M
	E	9.12	1.09	M
10	D	8.86	1.23	M
	E	7.66	1.00	M
11	D	6.94	1.33	M
	E	7.00	1.00	M
12	D	8.13	1.03	M
	E	7.49	1.20	M
13	D	7.40	1.42	SM
	E	7.00	1.00	M
14	D	6.12	1.70	SM
	E	6.52	1.08	M
15	D	5.56	1.83	SM
	E	6.84	1.03	M
16	D	5.39	1.76	SM
	E	6.52	1.00	M

TABLE 1 (continued)

Chromosome Number	Taxon	Relative Total Length	Arm Ratio	Centromere Position
17	D	5.11	1.35	SM
	E	6.52	1.00	M
18	D	5.11	1.38	SM
	E	5.54	1.04	M
19	D	4.57	1.90	SM
	E	5.86	1.75	SM
20	D	4.38	1.89	SM
	E	5.86	1.39	SM
21	D	5.75	1.80	SM
	E	4.23	1.68	SM
22	D	4.47	1.68	SM
	E	4.23	1.45	SM
23	D	4.75	1.09	M
	E	4.89	1.11	M
24	D	4.11	1.25	M
	E	4.23	1.29	M
25	D	3.83	1.69	SM
	E	3.91	4.36	A
26	D	2.92	1.87	SM
	E	2.93	4.08	A
27	D	1.82	1.18	M
	E	2.93	2.68	A
28	D	1.55	1.16	M
	E	2.90	2.54	A

Figure 1. Haploid Chromosomes of Desmognathus fuscus conanti in Diakinesis Stage Showing Fourteen Bivalents.

Figure 2. Diploid Chromosomes of Desmognathus fuscus conanti.

Figure 3. Diploid Chromosomes of Eurycea bislineata rivicola.

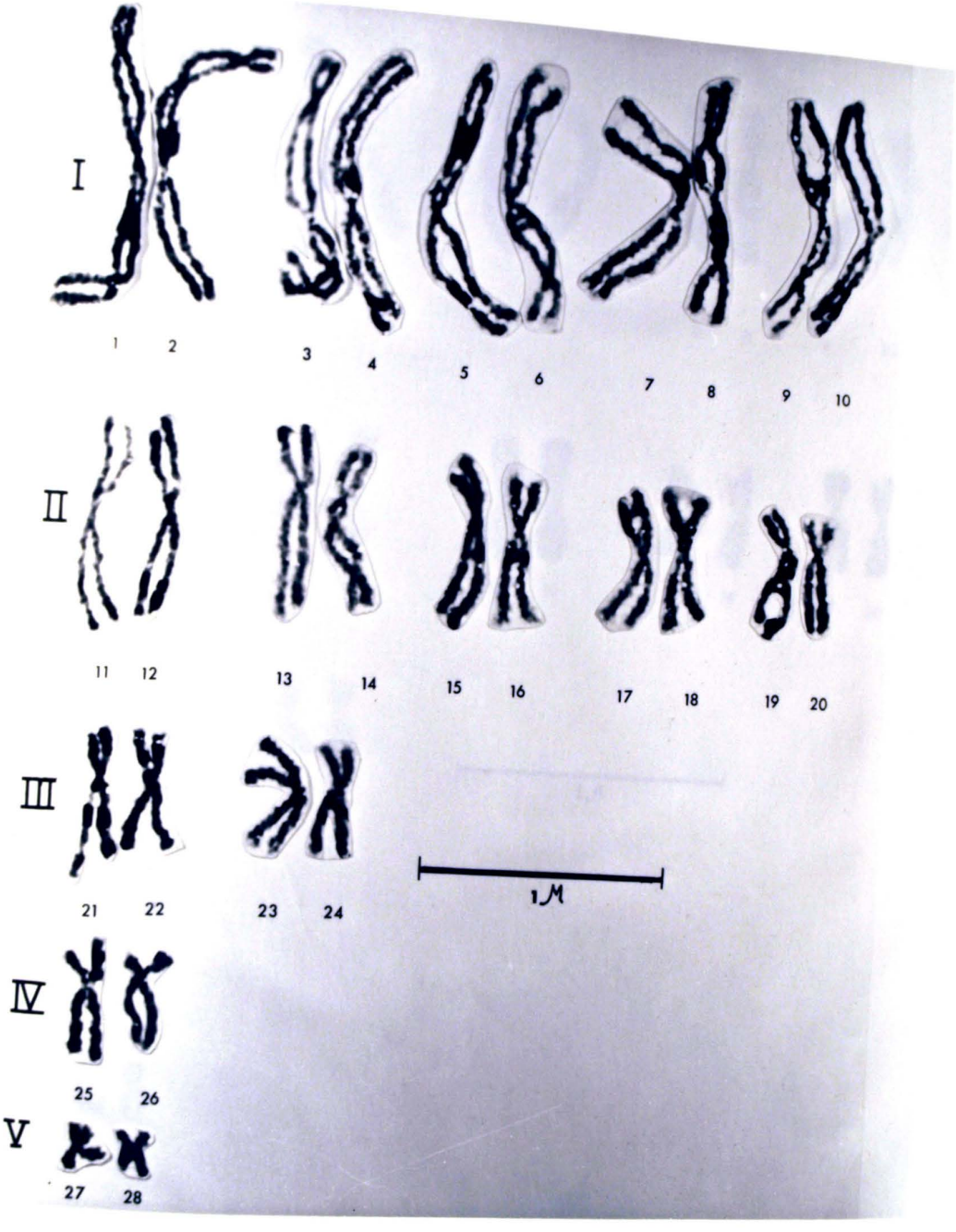

Figure 4. Karyotype of Desmognathus fuscus conanti.

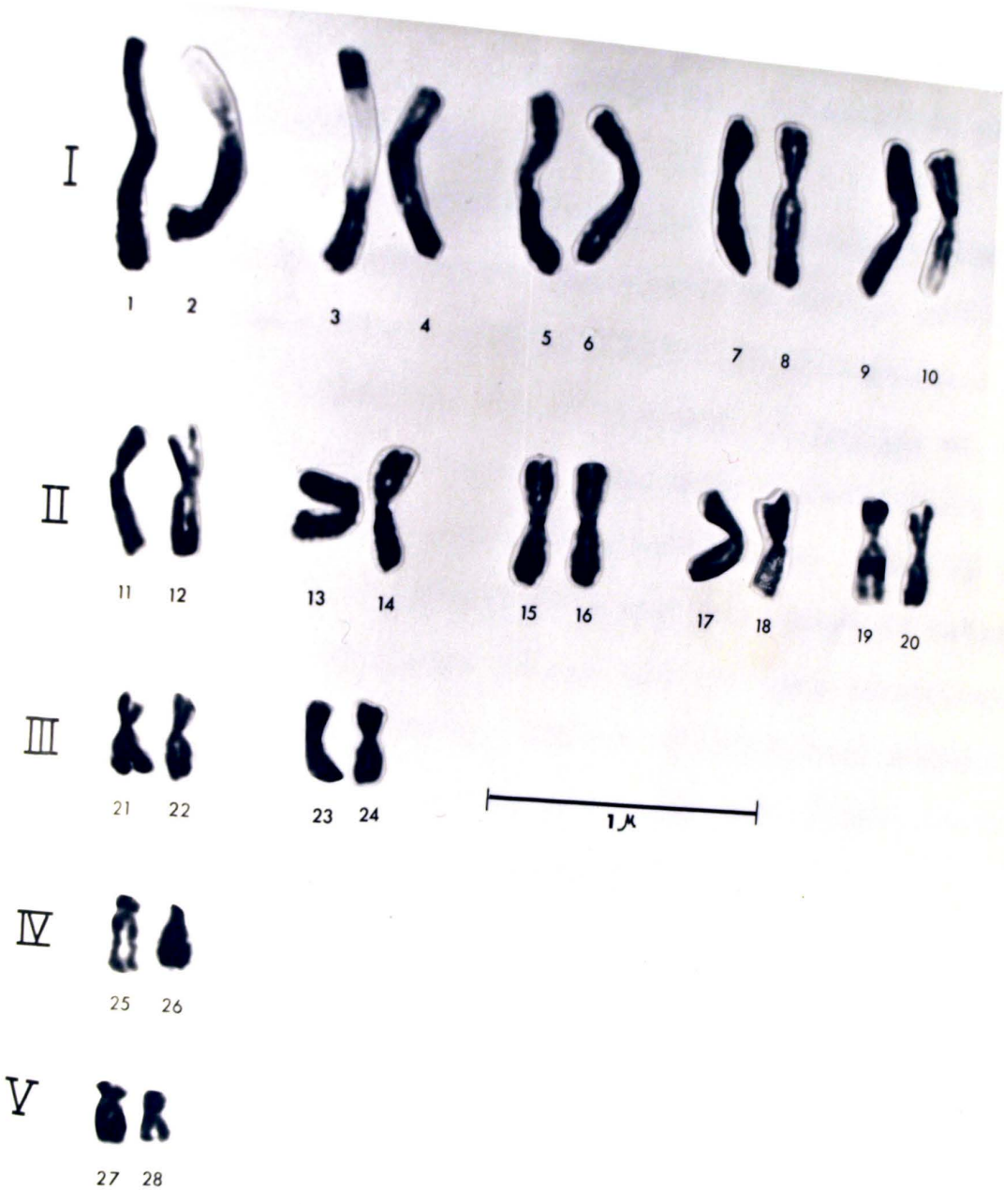

Figure 5. Karyotype of Eurycea bislineata rivicola.

metacentrics and a pair of submetacentrics. The pair in Group IV was submetacentric and in Group V was a small pair of metacentrics. No significant secondary constrictions were observed in Desmognathus.

Eurycea had a diploid number of twenty-eight (2N=28) also. This number confirmed that listed by Makino (1951). The haploid number was not determined. For comparison with Desmognathus I classified the chromosomes of Eurycea as was done for Desmognathus. Group I consisted of three pairs of submetacentrics and two pairs of metacentrics. Group II contained one pair of submetacentrics and four pairs of metacentrics. Group III consisted of one pair of metacentrics and one pair of submetacentrics. Groups IV and V each contained one pair of acrocentric chromosomes. No significant secondary constrictions were observed in Eurycea.

CHAPTER V

DISCUSSION AND CONCLUSIONS

I found the squash technique of Darlington and LaCour (1947) unsuitable for determining the karyotypes of Desmognathus and Eurycea due to inadequate spreading and poor rendition of chromosome texture. Of the many modifications designed to allay the problem of inadequate spreading, none were successful, and the technique was therefore abandoned.

Blood culture techniques incorporating elements of methods used on both humans and amphibians were useless because of a lack of either cell growth or division.

The method which produced the best results with Eurycea was that of Weiler and Ohno (1962) with no modifications, although it produced no results with Desmognathus. The method which ultimately produced the best results with Desmognathus was the modification of Moorhead's (1960) leucocyte harvest technique, as devised by Bunting (personal reference) and myself.

Distortion of the chromosomes was inevitably produced by the fixation process. The degree of coiling of the several chromosomes may have been different at the time each was fixed, thereby affecting the measurements and possibly the determinations of the arm ratios. Colchicine and/or colcemid

may have affected chromosome lengths.

The chromosomes of Desmognathus differed from those of Eurycea in several respects, as follows: The chromosomes of Desmognathus averaged larger though due to the vagaries encountered in determining chromosome lengths, such measurements are of little but comparative value. One of the most taxonomically useful differences noted between the karyotypes was the absence of what were believed to be real acrocentrics in Desmognathus, confirmed by Goin and Goin (1962), and the presence of two such pairs in Eurycea. An interesting difference between the five pairs in Group II of the karyotypes was the presence of four pairs of submetacentrics and one pair of metacentrics (the largest of the group) in Desmognathus, whereas in Eurycea there were four pairs of metacentrics and one pair of submetacentrics, the deviant pair in this case being the smallest of the group.

This investigation has demonstrated karyotype analysis to be efficacious in plethodontid taxonomy. The methods used in determining the karyotypes need further refinement and modification to insure a greater frequency of usuable preparations. Until such an increased efficiency in chromosome preparation of plethodontid salamanders is realized, the practicality of this tool in the study of their taxonomy will be but slight.

CHAPTER VI

SUMMARY

The karyotypes of Desmognathus fuscus conanti and Eurycea bislineata rivicola, family Plethodontidae, order Urodela, are described. Both animals possessed a diploid chromosome complement of twenty-eight.

Techniques used in preparation of the material involved a new modification of Moorhead's (1960) harvest technique for Desmognathus and the unmodified technique of Weiler and Ohno (1962) for Eurycea.

The karyotypes were compared for taxonomically significant differences the most notable of which was the presence of acrocentric chromosomes in Eurycea and the absence of such in Desmognathus.

Karyotype analysis was shown to be efficacious as a tool in the taxonomy of the plethodontid salamanders, but for practical application a technique yielding a higher percentage of acceptable preparations is needed.

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