A KARYOTYPIC STUDY OF TWO SYMPATRIC SPECIES OF TURBELLARIANS (PHAGOCATA GRACILIS AND P. MORGANI) OF BRIGHAM BRANCH OF THE CUMBERLAND RIVER DRAINAGE

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An Abstract

Presented to the

Graduate and Research Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

David R. Cheatham, D.D.S.

July 1986

ABSTRACT

Two species of planarians, Phagocata gracilis and P. morgani were positively identified through anatomic features of the reproductive apparatus.

Karyologic studies were done. Phagocata gracilis possessed 38 chromosomes, (2N=38). Two pairs were particularly large chromosomes. There were nine pairs of metacentrics, six pairs of submetacentrics, and four pairs of acrocentrics.

Phagocata morgani provided no suitable chromosome spreads for karyotypic analysis but counts of 42 and approximately 56 chromosomes could be made, representing a polyploid condition.

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To the Graduate Council:

I am submitting herewith a Thesis written by David R. Cheatham entitled "A Karyotypic Study of Two Sympatric Species of Turbellarians (Phagocata gracilis and P. morgani) of Brigham Branch of the Cumberland River Drainage." I have examined the final copy of this paper for form and content, and I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Floyd M. Ford
Major Professor

We have read this thesis and recommend its acceptance:

David W. Bath
Second Committee Member

Floyd L. Brown
Third Committee Member

Accepted for the Council:

William T. Ellis
Dean of the Graduate School

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The writer would like to express gratitude to Dr. Clay Chandler of Middle Tennessee State University for his assistance in the identification of planarian species studied in this manuscript and to Dr. Nicole Gourbault of the National Museum of Natural History of France who provided important information that made this study possible. The reading and correction of this manuscript by Dr. Daniel Bath and Mr. Floyd Brown were greatly appreciated. Special thanks are in order to Mr. Billy D. Cole whose expertise in tissue micro-technique was invaluable and of course, to Dr. Floyd Ford, whose inspiration shall never be forgotten.

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CHAPTER I

INTRODUCTION

Flatworms are worm-like invertebrates with bilaterally symmetrical bodies that are elongated and dorsoventrally compressed. Planaria, as the free-living group is called, belong to the Phylum Platyhelminthes and the Class Turbellaria. Although the variety of species are common to many North American streams and lakes and despite many studies on chemical effects and regeneration of severed parts, little work has been done with the karyology of the many species.

Dr. Nicole Gourbault of the National Museum of Natural History of France has worked extensively with chromosome studies of European planarians. The karyological methods of this study were based largely on her research (Chandler, 1980). This study involved analyzing the karyotypes of two species of the Genus Phagocata (P. gracilis and P. morgani) that occur sympatrically in Brigham Branch, a tributary of Long Creek (Cumberland River drainage) in south-central Stewart County, Tennessee.

Karyology is defined as the study of an organism's chromosomes. A karyotype is the systematic arrangement of chromosome photomicrographs according to an accepted

standard classification. Any chromosome features of a particular mitotic cell such as size, number, and form may at once be seen.

Karyotyping has played an important role in studying the relationship of organisms from a phylogentic standpoint. This importance in species identification of Platyhelminthes has not been fully realized. Little emphasis has been placed on their karyology until relatively recently. The lack of appropriate technique for chromosome study no doubt has played a role in admonishing researchers to look for other suitable methods of classification. The handling of tissue is quite critical and chromosome squashes are often inappropriate for study due to inadequate spreading which disallows counting and karyotypic evaluation.

A conventional approach to flatworm identification and classification has been used for a number of years. It is widely agreed that anatomic variations in reproductive systems of the different species is marked and provides a reliable basis for discrimination. As many species appear similar by their external characteristics, it is not possible to positively identify flatworms in the field and confirmation by studying microscopic anatomy is advisable.

Species identification in this study was determined via sagittal sections of sexually mature specimens that had been fixed whole. Sections were serial, five to seven

microns apart. This permitted an in-depth study of the anatomy of the reproductive apparatus which is the key to positive identification.

CHAPTER II

REVIEW OF THE LITERATURE

The Genus Phagocata

Haldeman (1840) first described Phagocata gracilis naming it Planaria gracilis. Leidy (1847) used the generic name Phagocata as a subgenus of Haldeman's animal. The name saw little usage until it was resurrected much later by Hyman (1937). Dahm (1949, 1958) introduced the name into the European literature, but some Europeans felt the older designation of Fonticola was more appropriate for similar worms native to that continent. There have been a number of Asian worms described and many having a strong similarity to the members of Phagocata of North America have been placed under this genus (Kawakatsu, 1969).

The original classification of Phagocata gracilis was based largely on its polypharyngy. Another worm also called Planaria gracilis (Girard, 1850) exhibited polypharyngy but has since been shown by anatomical features to be a distinctly different species, P. woodworthi (Hyman, 1937).

Leidy (1851) described Planaria truncata, an unpigmented worm of small size. This may have been the first attempt to name Phagocata morgani (Stevens and Boring, 1906) but the connection seems to be unclear. The names, however, are now used synonymously in the literature.

The genus Phagocata is represented by a large number of species. Kenk (1976) lists detailed descriptions of 13 North American species. Since that time he has described at least four more; P. carolinensis, Kenk (1979), P. holleri, Kenk (1979), P. hamptonae, Kenk (1982), P. procera, Kenk (1984).

Karyology

There is a paucity of information in the literature concerning karyological analysis of planarians. In Chromosome Numbers in Animals Makino (1951) lists karyological data for ten species of planaria. Five of these belonged to the genus Planaria representing diploid chromosome numbers of 6 to 24. The remaining five species had diploid numbers of 12 and 16. No species of Phagocata were entered in Makino's atlas and possibly none had been examined until Whitehead (1965) analyzed P. morgani and P. velata from populations in Cattaraugus County, New York. From one P. morgani population she reported a diploid number of 14, the basic genome consisting of one large, four medium sized and two small chromosomes. Sexually mature worms were noted often from this location. Specimens from two other sampling locations (different springs) revealed a much higher number of chromosomes which was estimated to be 35.

The nucleus size of these cells was noted to be larger than those from the first location. She speculated that this might represent a polyploid population. No signs of sexual maturity were noted in worms at either of these locations.

Mitotic stages in Phagocata velata were found to have approximately 60 chromosomes suggesting polyploidy and two other species, Dugesia tigrina and Cura foremanii, were found to have diploid numbers of 16 and 12 respectively (Whitehead, 1965). C. foremanii was one of the earliest reported karyotypes and is one of ten listed by Makino (1951).

Dahm (1949, 1958) has studied four European species of Phagocata in depth. P. vitta has 34 chromosomes and was found to exhibit polyploidy.

Ball and Gourbault (1975) have noted that polyploidy has been observed in most European species of Phagocata studied, including P. albissina and P. ochridana, having 36 and 32 chromosomes, respectively.

Ball and Gourbault (1975) described and karyologically analyzed P. fawcetti, a new species. They found a diploid complement of 38. The karyotype revealed one pair of chromosomes which was more than 1.5 times the size of the next largest pair. They reasoned that this peculiarity of having one much larger pair would preclude polyploid conditions in this species. The remaining 17 pairs

gradually decreased in size, most of them being metacentrics. A few chromosomes were found to be submetacentric with a centromeric index of less than 37.

Ball, Gourbault, and Kenk (1981) karyologically analyzed Phagocata velata and Hymanella retenuova, populations from temporary waters of eastern North America. P. velata was found to have a high degree of polyploidy with a diploid number of 12. H. retenuova was found to have a diploid number of 38. Both of these species were noted to have one pair of chromosomes at least twice the length of the second largest pair. These especially large chromosomes were designated "marker" chromosomes as they were instrumental in identifying polyploid populations. The number of large pairs corresponds exactly to the different levels of polyploidy.

The literature does not support previous karyological research on Phagocata gracilis. No karyologic studies have been reported to date.

CHAPTER III

METHODS AND MATERIALS

Description of the Sampling Population

Brigham Branch is a northerly flowing spring in south-central Stewart County, Tennessee, which terminates in Long Creek approximately one and one-half miles upstream from where that tributary enters the Cumberland River. The total length of Brigham Branch itself is less than one half mile originating in a wooded field's edge and coursing through pastureland. There is a varied flora and fauna owing largely to the spring's ready access to sunlight and agricultural run-off over most of its course.

Three species of planarians have been collected from Brigham Branch. Phagocata morgani, an unpigmented worm, was noted commonly near the spring's mouth in small groups or singularly beneath rocks and submerged tree leaves. This species exhibited sexual maturity infrequently. P. gracilis, a polypharyngeal form with brown pigmentation, was the most numerous planarian and seemed to congregate in large numbers on the undersides of rocks at all locations along the spring's course. The large majority (approximately 75%) of the members of this species were sexually mature. Dugesia tigrina was an infrequent inhabitant and appeared only in warmer water downstream.

Six sexually mature individuals of each species were collected and prepared for microtome sectioning according to Humason (1972) as follows: The animals were placed in formo-acetic acid for tissue fixation for 24 hours followed by 70% ethyl alcohol for one hour. They were then placed in 85%, 95%, and 100% ethyl alcohol concentrations for periods of 30 minutes each. This was followed by placement in melted paraffin for 30 minutes. The animals were then embedded and microtome sections were made at five and seven microns for Phagocata morgani and P. gracilis, respectively. Sections were affixed to glass slides with adhesive Mayer's albumin and run through xylene and ethyl alcohol solutions of 100%, 95%, and 70% for two to three minutes each. Slides were then placed in tap water for three minutes, hematoxylin for two to five minutes, tap water again for three minutes, and Scott's solution for three minutes. Counterstaining was done with Eosin Y for one minute. Slides were placed in each of the following solutions for approximately three minutes; tap water, 70% ethyl alcohol, 95% ethyl alcohol, 100% ethyl alcohol, and xylene. Coverslips were applied with adhesive.

Slides were examined by phase contrast microscopy using a 10x objective and 8x ocular. Photomicrographs were taken through a 10x objective and 8x ocular using Kodachrome ASA-64 film, size KR 135-20.

Technique for Positive Identification of Species

To the experienced planarian collector, species may often be identified in the field. The general body morphology, and nature of movement and particular habitat may lend clues as to the proper identification. However, the collector with little experience may not rely upon such criteria. Serial sagittal sections of five-seven microns of the fixed whole planaria allows anatomical observation of the sexual apparatus which serves as the key to positive taxonomic identification. For this reason only sexually mature specimens were used. Larger body size and sexual maturity ordinarily correspond positively, therefore, only the larger animals from these populations were collected.

All freshwater planarians are hermaphroditic; that is, both male and female genitalia can be found in the same individual. The ovaries may be found bilaterally in the anterior portion of the animal adjacent to the nerve cord. From each ovary extends an oviduct posteriorly. Numerous yolk glands branch out from the oviduct.

Many testicular follicles may be found bilaterally between the ovaries and the level of the pharynx. The testis are connected to a vas deferens which runs posteriorly and parallel to the nerve cord.

Directly behind the pharynx or pharynges may be found the copulatory apparatus. Owing to the general dorsoventral

compression of the body and the relative substantial size of the apparatus, sexually mature specimens may often be identified in the field by viewing the planaria in the location of the copulatory organs. In species lacking substantial pigmentation (as with Phagocata morgani) the sex organs may be seen dorsally through the body wall. Species with darker pigmentation such as P. gracilis, etc., may be noted as sexually mature by locating the genital aperture or gonopore just posterior to the pharyngeal opening on the ventral side of the animal.

Though much anatomical variation may be found from one species to the next a brief description of the typical reproductive system is in order. The genital aperture leads into the genital atrium, a cavity which may be further subdivided into a male atrium which encloses the penis papilla, the female atrium into which the openings of the female complex empty, and a common atrium. The male atrium houses the penis which is divided into an anterior bulb and posterior papilla. The paired sperm ducts combine to form a vas deferens which opens into the seminal vesicle. An ejaculatory duct communicates between the seminal vesicle and the cavity of the male atrium.

The oviducts unite to form a common oviduct which usually opens into the dorsal aspect of the atrium.

Directly anterior to the penis bulb a bursa may be found in most species. A bursal duct runs dorsally on the midline or eccentrically to empty into the atrium.

Squash Technique

Gourbault's technique for preparation of planarian chromosomes was utilized (Chandler, 1980). Three days prior to chromosome viewing nine worms of each species were collected from Brigham Branch. Each was cut longitudinally with a razor blade into two pieces and placed into petri dishes containing water transported from the habitat. Seventy-two hours were allowed for regeneration of severed parts.

Worm sections were placed in petri dishes containing 0.3% colcemid solution for four hours. The regenerating areas of each section were removed with a razor blade and each was placed on a slide with one drop of water. This drop of water was pipeted away and to each section of worm was added one drop of 2% acetic acid. After a period of five minutes the acetic acid was removed and two drops of orcein-lactic were added. The slides were placed in a humidity chamber, consisting of a shallow glass dish with cover, for one half hour.

Slides were removed and placed on several thicknesses of paper towels. Coverslips were placed on worm sections and squashes were made with pressure applied from the heel

portion of the hands with care being taken not to let coverslips move. The edges of the coverslips were then sealed with synthetic mounting medium.

Slides were examined by phase contrast microscopy using a 100x objective and 8x ocular. Numerous slides were scanned for suitable metaphase chromosome spreads. Photomicrographs were taken through a 100x objective and 8x ocular.

Photomicrographs of the most clearly dilineated metaphase spreads were chosen and negatives were used to make slides for a projector. These were projected onto a wall and carefully outlined with pencil onto white butcher paper. Chromosomes were blackened with an India ink pen and cut separately so as to facilitate the arrangement of karyotypes.

CHAPTER IV

RESULTS

Species Identification

Identification of anatomical features required close inspection of several sections of each individual animal of each species.

Approximately 75% of the Phagocata gracilis, Figure 1, population was sexually mature. Six worms were chosen for fixation and subsequent microtome slicing of seven microns each. Figure 2 shows the muscular penis bulb (PB) and elongated penis papilla (PP) and large bursa (B) together with bursal duct (BD), chief anatomical landmarks for identification of this species. The ejaculatory duct (ED) is convoluted and appears as separate chambers in sagittal sections. Anterior and posterior seminal vesicles (ASV, PSV, respectively) are clearly evident as is the male atrium (MA). The approximate location of the gonopore (GP) is designated. Anteriorly can be seen the multiple pharnges (Ph) and posterior to the reproductive apparatus are located the intestines (I). Figure 3 is a sketch adaptation of P. gracilis, sagittal section from Fresh-water Planarians (Turbellaria) of North America, Kenk (1976).

Figure 1. Enlarged Photograph of Phagocata gracilis.

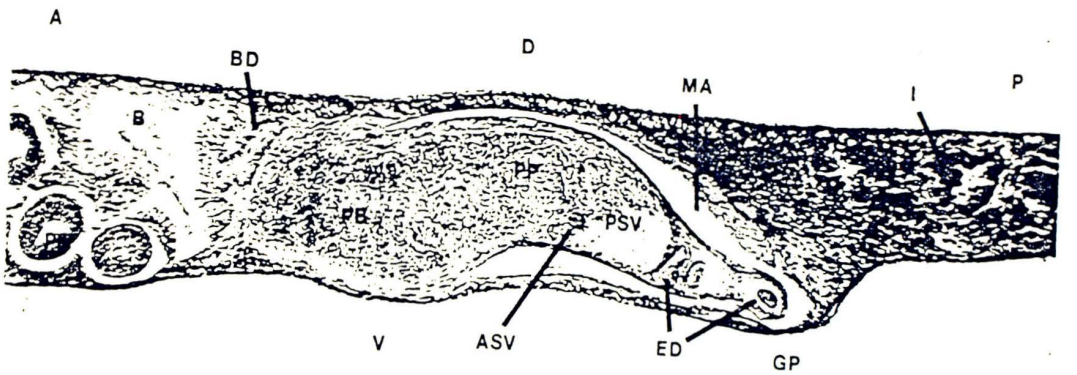

Figure 2. Sagittal Section of Reproductive Apparatus of Phagocata gracilis.

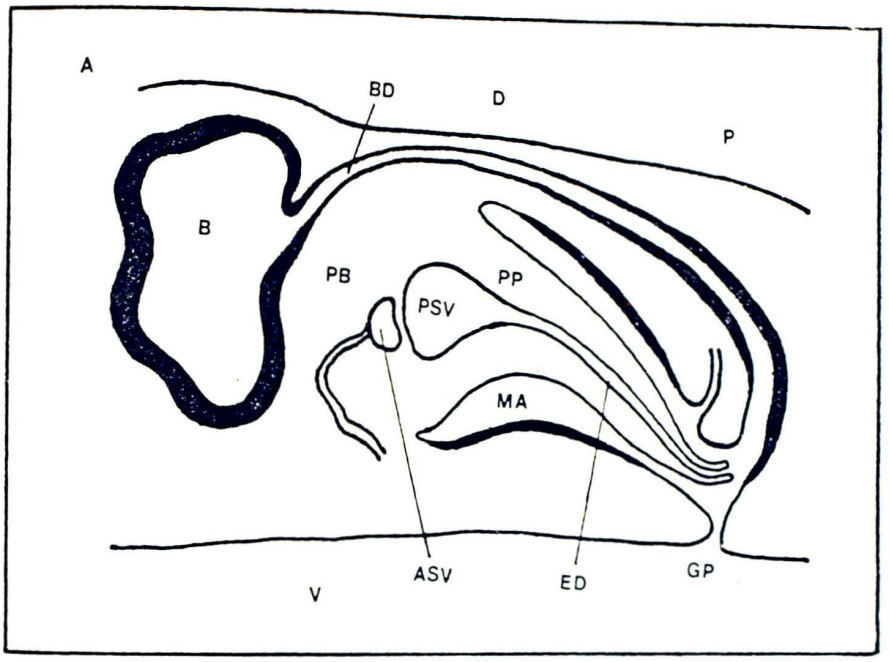

Figure 3. Sketch of Sagittal Section of Reproductive Apparatus of Phagocata gracilis.

No distinct seminal vesicle is found in Phagocata morgani (Figure 4). In Figure 5 may be noted a remnant of the bursa (B) which was not prominent in either of the six animals. The penis papilla (PP) is characterized as rather elongate with bulb indistinct and rounded papilla. The tip of the papilla is especially noteworthy for this species bearing a muscular wart-like epithelium (MP). The male atrium (MA) is distinct dorsally and ventrally forming a rather large common atrium posteriorly, which exits the body via the gonopore (GP). Figure 6 is a sketch adaptation of P. morgani, sagittal section, from Fresh-water Planarians (Turbellaria) of North America, Kenk (1976).

Karyology

A number of suitable metaphase spreads of Phagocata gracilis were photographed, example Figure 7. This species exhibited a diploid number of 38, (2N=38). Relative total lengths of each chromosome were calculated as well as relative lengths of long versus short arms. Arm ratios were determined by dividing the length of the long arm by that of the short arm. The value obtained served to categorize chromosomes into three groups based on centromere position: Those with arm ratios between one and thirty-three thousandths were classified as metacentric, those between one and thirty-four-thousandths and one and ninety-

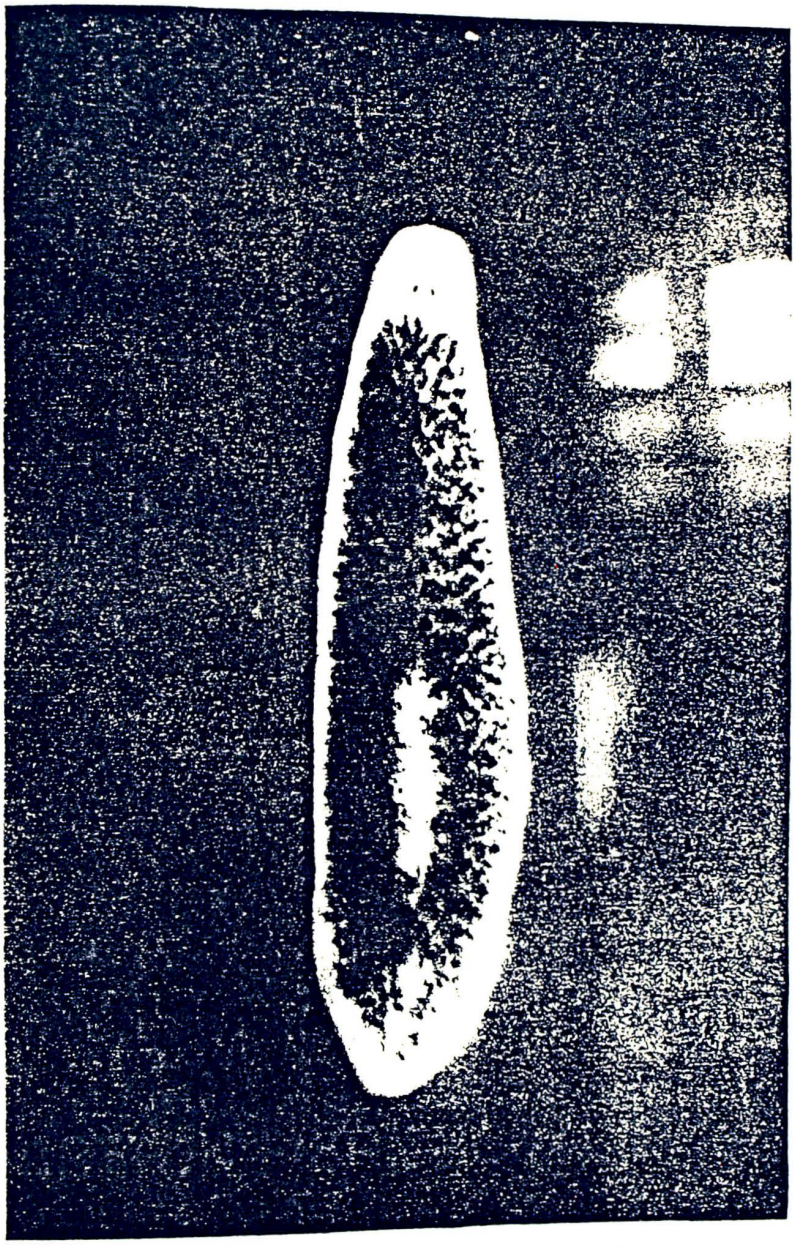

Figure 4. Enlarged Photograph of Phagocata morgani.

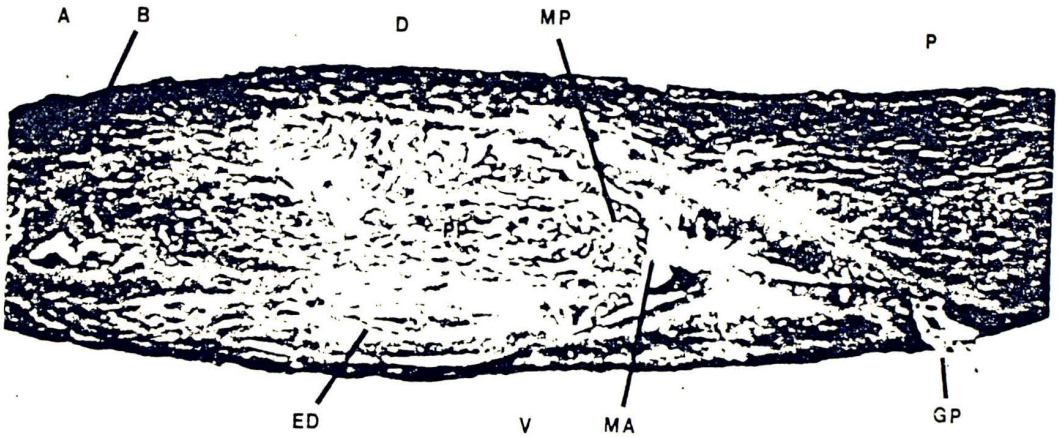

Figure 5. Sagittal Section of Reproductive Apparatus of Phagocata morgani.

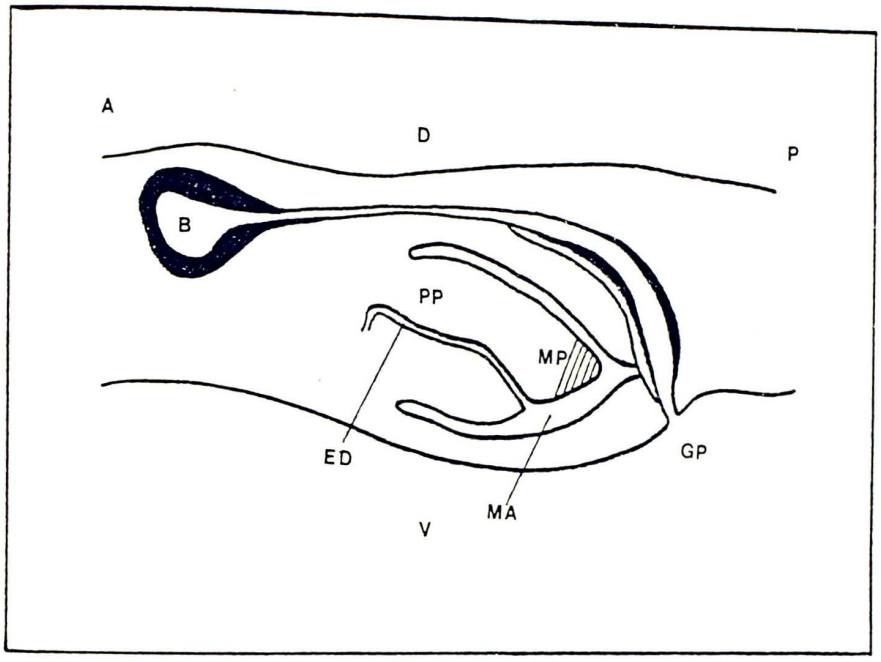

Figure 6. Sketch of Sagittal Section of Reproductive Apparatus of Phagocata morgani.

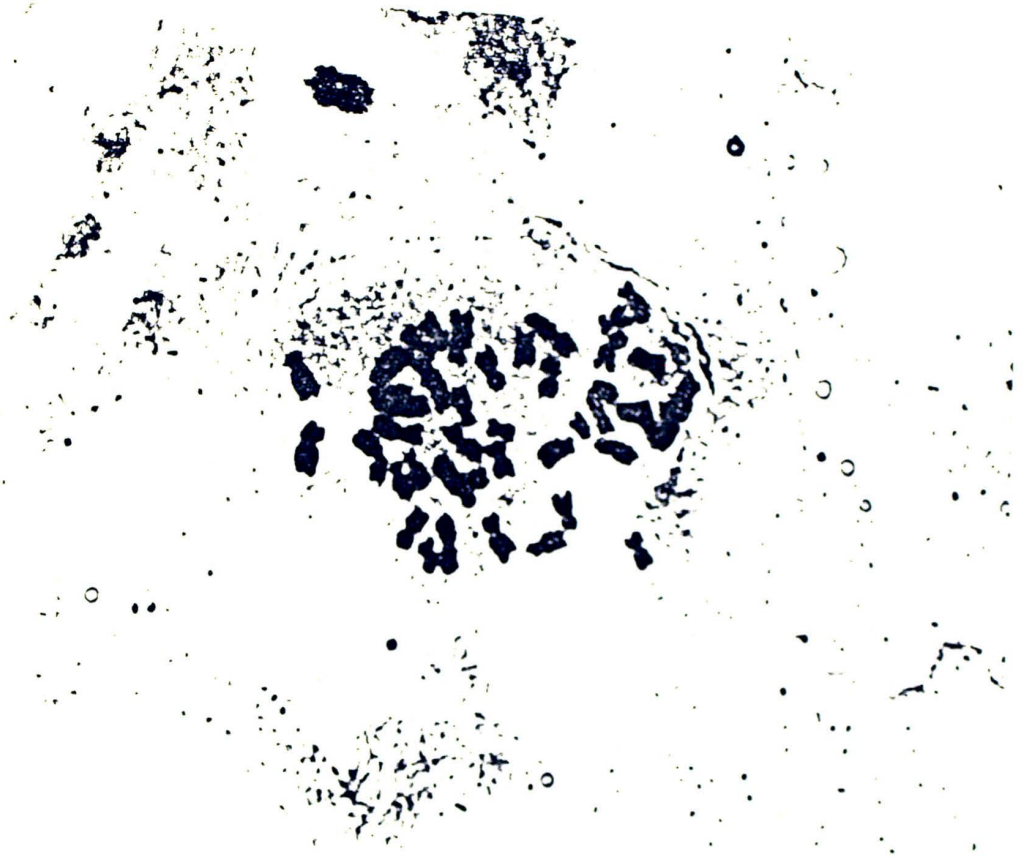

Figure 7. Squash of Phagocata gracilis.

nine thousandths as submetacentrics, and those over two as acrocentrics. Data can be found in Table 1.

Phagocata gracilis had nine pairs of metacentrics, six pairs of submetacentrics, and four pairs of acrocentrics. Chromosomes one and two were considerably larger than the remaining seventeen with number one larger than number two. Figure 8 is a karyotype based on data found in Table 1. An idiogram is also provided for this species (Figure 9).

No metaphase spreads of Phagocata morgani suitable for karyotyping were obtained (Figures 10 and 11). There appeared to be marked distortion of chromosomes and a lack of appropriate spreading.

TABLE 1

Average Values of Relative Chromosome Lengths and Arm Ratios of *Phagocata gracilis*

Chromosome Number	Relative Total Length	Arm Ratio	Centromere Position
1	3.075	1.085	M
2	2.528	1.793	SM
3	1.723	1.210	M
4	1.620	2.560	A
5	1.578	1.740	SM
6	1.545	1.176	M
7	1.416	1.043	M
8	1.348	1.058	M
9	1.338	3.316	A
10	1.265	1.321	M
11	1.203	1.560	SM
12	1.126	1.601	SM
13	1.125	1.064	M
14	1.108	2.011	A
15	1.053	1.449	SM
16	1.043	1.045	M
17	1.006	2.021	A
18	0.960	1.866	SM
19	0.945	1.329	M

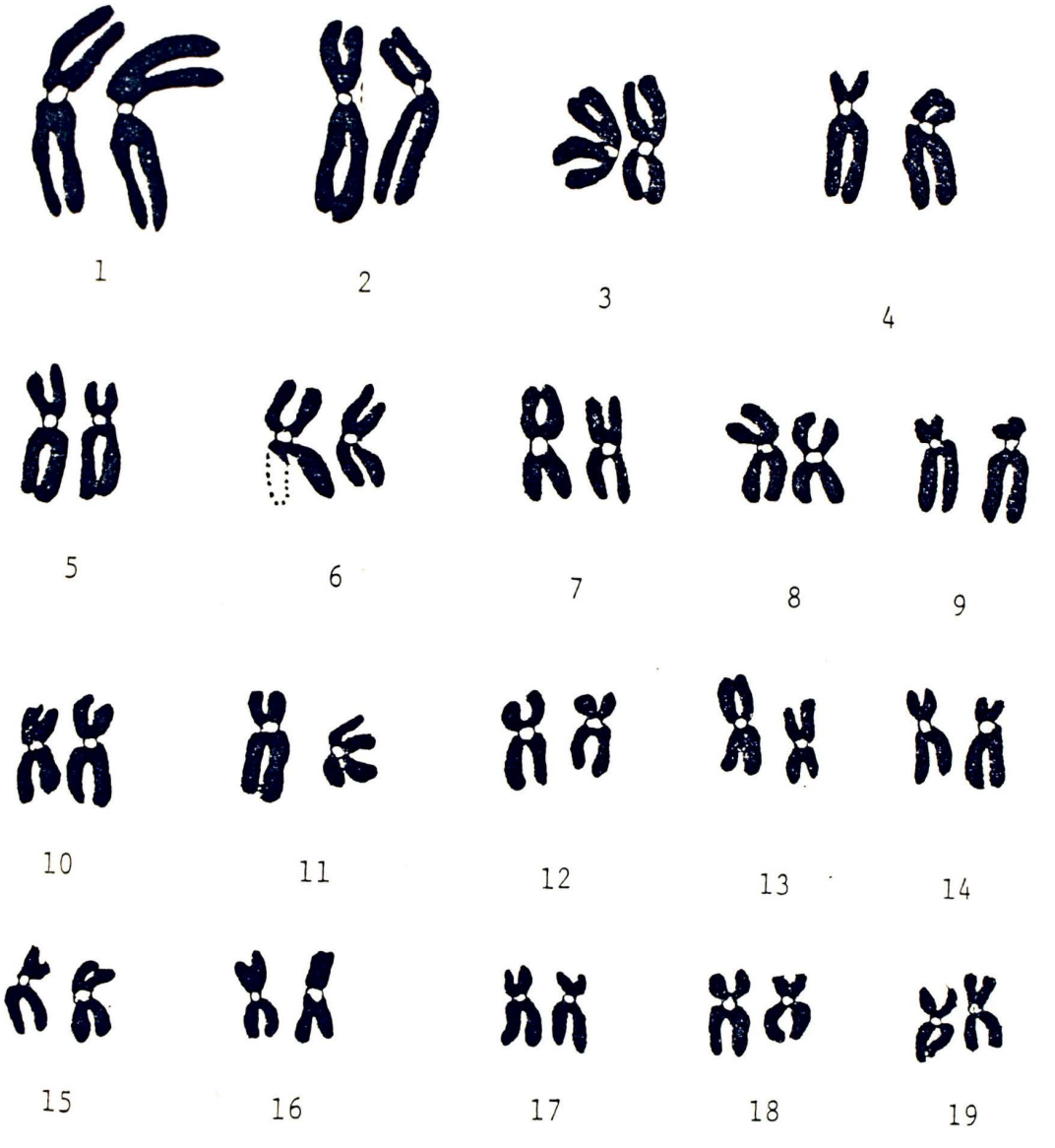

Figure 8. Karyotype of Phagocata gracilis.

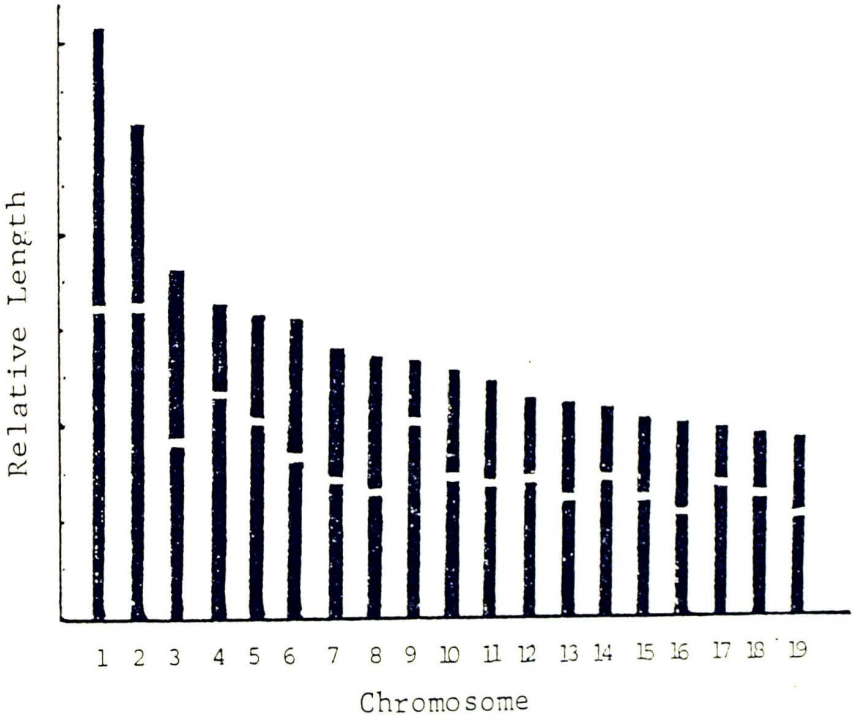

Figure 9. Idiogram Analysis of Phagocata gracilis Based on Data in Table 1.

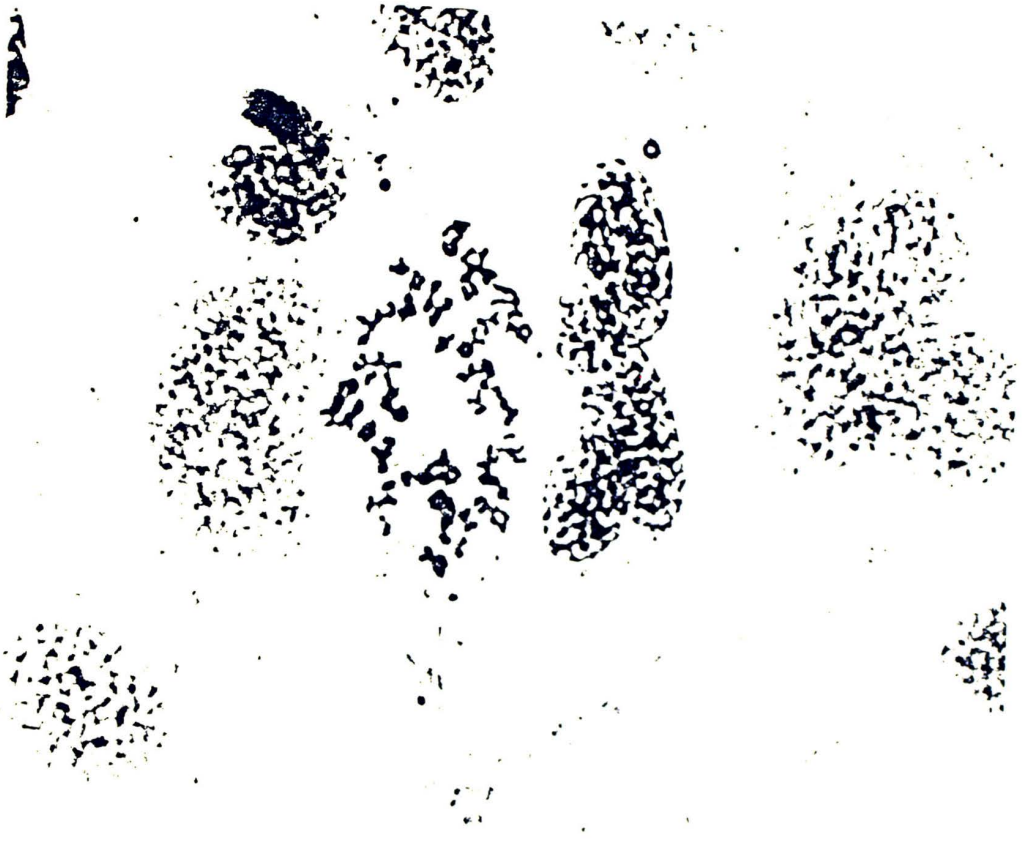

Figure 10. Squash of Phagocata morgani with Approximately 42 Chromosomes.

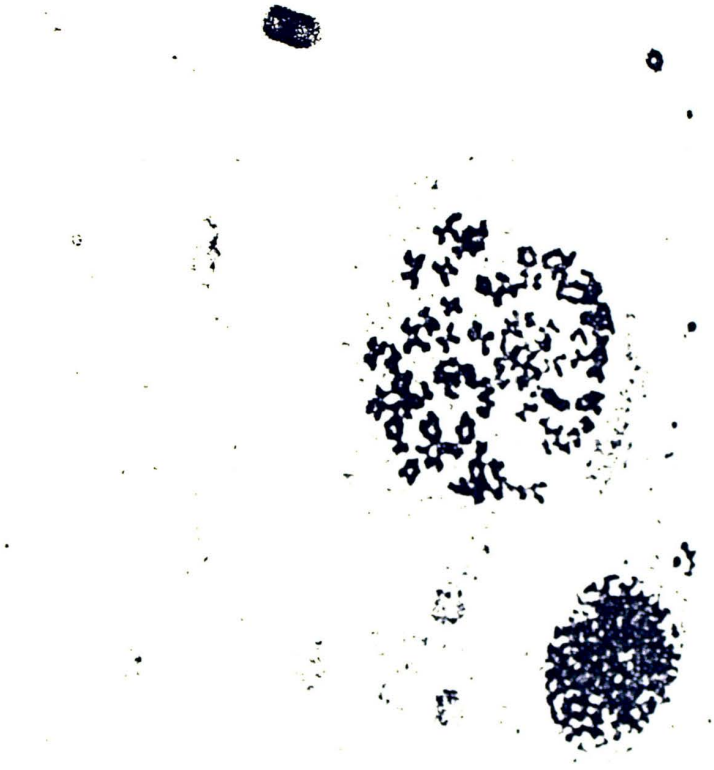

Figure 11. Squash of Phagocata morgani with Approximately 56 Chromosomes.

CHAPTER V

DISCUSSION OF RESULTS

The squash technique of Gourbault (Chandler, 1980) worked well for Phagocata gracilis and numerous metaphase spreads of good quality were photographed. This species exhibited a diploid chromosome number of 38, (2N=38). In this respect P. gracilis appears to be similar to P. fawcetti (Ball and Gourbault, 1975) in which 2N=38. P. gracilis also had two large pairs of chromosomes with pair number one being larger than number two. However, this difference was not nearly 1.5 times the size of the next largest pair as in P. fawcetti, and the term "marker chromosomes" though possibly applicable in reference to P. gracilis, does not represent the great and obvious difference noted in P. fawcetti.

The remaining 17 pairs of chromosomes in both species gradually decreased in size in similar fashion. P. fawcetti had mostly metacentric chromosomes with a few submetacentrics. P. gracilis had nine pairs of metacentrics, six pairs of submetacentrics, and four pairs of acrocentrics.

There is no evidence to suggest that this population of Phagocata gracilis represented polyploidy as the chromosome number, (2N=38), was constant with all observed

metaphase spreads and chromosome pair number one was clearly evident with no multiplicity.

Suitable metaphase spreads for karyotypic purposes of Phagocata morgani were not obtained. There appeared to be marked distortion of chromosomes and a lack of appropriate spreading. Variations in squash technique were implemented but there was no improvement in the quality of squashes. These variations included running the procedure at different times of day in order to coincide with possible circadian rhythms and varying the concentrations of colcemid, acetic acid, and orcein-lactic solutions and time lengths of treatment with each. The degree of coiling may have been different at different times of chemical fixation thereby affecting the lengths of chromosomes. This would explain the fact that a wide range of chromosome lengths in different cells were observed with this species.

Appropriate spreading of chromosomes in Phagocata morgani was complicated by the increased number of chromosomes and their apparent small size. An estimation of chromosome count could be obtained and there appeared to be significant differences in different squashes lending evidence to the fact that this represented a polyploid condition. Some cells appeared to have 42 chromosomes whereas others appeared to have approximately 56. Whitehead (1965) reported polyploid populations with chromosome

numbers of 35, the haploid genome being seven. From polyploid populations she reported no sexually mature specimens. Signs of sexual maturity of this species in Brigham Branch were rare.

CHAPTER VI

SUMMARY

Two species of planarians, Phagocata gracilis and P. morgani, that occur sympatrically in Brigham Branch, a tributary of Long Creek (Cumberland River drainage), in south-central Stewart County, Tennessee were positively identified and karyologic studies were done.

Species were identified by anatomical analysis of the reproductive apparatus via serial sagittal microtome sections of whole fixed sexually mature specimens. Kenk (1976) provided the anatomic descriptions necessary for identification.

Phagocata gracilis possessed a diploid number of 38, (2N=38) with two pairs of particularly large chromosomes, pair number one being significantly larger than pair number two. There were nine pairs of metacentrics, six pairs of submetacentrics, and four pairs of acrocentrics.

Phagocata morgani provided no suitable chromosome spreads for karotyping due to marked distortion and a lack of appropriate spreading. However, approximate counts could be made. Some spreads had 42 chromosomes whereas others appeared to have approximately 56. This may well represent a polyploid condition.

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APPENDICES

APPENDIX A

Orcein-lactic solution used in karyological technique was made as follows:

lactic acid, pure	15 parts
acetic acid, glacial	35 parts
distilled water	50 parts
orcein, synthetic	1 gm

APPENDIX B

Abbreviations used in denoting features in diagrams of sagittal sections and histologic photomicrographs of planarian reproductive organs are as follows:

A - Anterior
ASV - Anterior Seminal Vesicle
B - Bursa
BD - Bursal Duct
D - Dorsal
ED - Ejaculatory Duct
GP - Gonopore
I - Intestines
MP - Muscles of Penis
P - Posterior
PB - Penis Bulb
Ph - Pharynges
PP - Penis Papilla
PSV - Posterior Seminal Vesicle
V - Ventral