A DEVELOPMENTAL AND PHYSIOLOGICAL COMPARISON OF TWO MATING STRAINS OF *ZYGNEMA CIRCUMCARINATUM* CZURDA

by

Richard Dobbin Miller

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF BIOLOGICAL SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN BOTANY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 7 3

THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Richard Dobbin Miller

entitled A DEVELOPMENTAL AND PHYSIOLOGICAL COMPARISON OF TWO MATING STRAINS OF *ZYGNEMA CIRCUMCARINATUM* CZURDA

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Robert W. Hoshaw — June 11, 1973

Dissertation Director — Date

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

Robert W. Hoshaw	June 11, 1973
Kaoru Nagatsuka	June 11, 1973
James W. O'Leary	June 11, 1973
Robert L. Mellor	July 18, 1973

*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Richard D. Miller

ACKNOWLEDGMENTS

First, I wish to express my appreciation to Dr. Robert W. Hoshaw for his guidance and advice during my graduate training.

I also wish to thank Dr. Kaoru Matsuda, Dr. James O'Leary, Dr. Robert Harris, and Dr. O. G. Ward for their help during this investigation and for serving upon my graduate committee.

To my loving wife Brenda, whose patient understanding aided me during my years of graduate study, I dedicate this manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

A genetic, physiological, and morphological comparison was made of the mating types 698 1A and 698 1B of *Zygnema circumcarinatum* Czurda obtained from the Culture Collection of Algae and Protozoa at Cambridge University. Data for the comparisons were obtained by means of genetic backcross, thin-layer chromatography, colorimetry, disc electrophoresis, and light microscopy.

The basis of heterothallism in the mating types was determined by backcrossing F_1 progeny to the parental types. Results showed that three detectable classes of individuals existed. These classes contained individuals identical to the two parental types in regard to mating compatibility and another class that conjugated with both parental types. This suggests the probable existence of a fourth undetermined class that would not conjugate with either parental type. The presence of more than two classes of offspring and the ratio of the classes show that heterothallism in *Z. circumcarinatum* is determined by two or more loci on the same chromosome. A hypothetical model based upon a two-gene theory of heterothallism has been constructed and relates to the data from the present investigation and to prior morphological studies in which supernatants from conjugating cultures induced conjugation in a single mating type.

Inheritance of cell width in *Z. circumcarinatum* was determined by analysis of F_1 progeny. Distribution of cell widths among the F_1 progeny suggests the involvement of polygenes in cell-width inheritance

whose polygenic character appears additive. A polygenic mode of inheritance of cell width in *Z. circumcarinatum* is in agreement with the mode of cell-width inheritance found in *Zygnema* sp. (strains 921 and 922) from the Culture Collection of Algae at Indiana University.

Comparison of the mating types by thin-layer chromatography and colorimetry in regard to pigmentation showed the two mating types differed in amounts of chlorophylls present per cell but did not differ in the types of pigments present. Electrophoretic banding patterns of filament extracts showed the two mating types represent two dynamic systems in regard to catalase, nonspecific esterase, and acid phosphatase isozymes. The patterns of the mating types differed in cultures of different ages, in cultures grown in different environments, and in sexual versus asexual cultures. These differences suggest that electrophoretic banding patterns of the mating types do not constitute a reliable taxonomic tool for distinguishing the mating types of the species.

Mucilaginous sheath morphology was followed through the vegetative and sexual stages of the life cycle. Morphologically, mating types differed in production of mucilaginous sheath. Sheath-width data support earlier physiological studies on the production of sheath in sexual and asexual cultures and are in agreement with observations dealing with epiphyte attachment to old or sexual filaments of Zygnemataceae in nature. Reduction in sheath width occurs when sheath degradation exceeds sheath synthesis. It was observed that sexual induction and attainment of old age are both accompanied by a similar reduction in sheath width in *Z. circumcarinatum*. Continued sheath formation is related to preventing mating type induction until such time as sheath degradation exceeds

sheath synthesis. An observation relating sheath-width differences between the mating types to the ability of one of the types to exhibit "creeping" up a glass surface in a moist atmosphere provides further evidence that sheath width represents a valid characteristic separating the mating types.

The results of the present investigation show that the mating types are distinct genetically in regard to heterothallism, physiologically in regard to isozyme banding patterns and pigment content, and morphologically in regard to sheath formation. Therefore, they represent two genetically and physiologically distinct strains within the species.

INTRODUCTION

The experimental organism *Zygnema circumcarinatum* Czurda is a freshwater, filamentous alga in the division Chlorophyta. This species is vegetatively typical of the genus and is characterized by two stellate chloroplasts per cell with each chloroplast containing one pyrenoid. Between the chloroplasts, the nucleus occupies a median position in the cell. The cells are arranged in unbranched filaments which are covered by a conspicuous pectose sheath that varies in thickness during the life cycle of the organism.

Czurda (1930) originally isolated and described *Z. circumcarinatum*. His description states that typically the cells are 20 to 22 μ wide, conjugation is isogamous, and sexually the species is atypical with regard to the other members of the genus. In this latter regard both gametes are expelled from the ends of the conjugation tubes, where they form zygotes in the growth medium. Mature zygospores are brownish orange and are circumscribed by a conspicuous ridge.

Z. circumcarinatum is one of only three species in the family Zygnemataceae thus far demonstrated to be heterothallic (Starr, 1964). According to Randhawa (1959), 580 species have been described for the family Zygnemataceae, and to date only species of *Zygnema* have been shown to be heterothallic.

Since its initial description, *Z. circumcarinatum* has been the experimental organism for only three investigations (Jost, 1953; Gauch, 1966; Hoshaw, 1968). Jost (1953), in the first investigation following

the description of the species, gave an extensive account of the interactions of environmental parameters on its growth and reproduction in axenic culture. He was able to follow the morphological events associated with sexual induction and defined the optimum conditions for laboratory culture and sexual induction. Yet, he was unable to germinate zygospores in the laboratory.

Gauch (1966) provided the first basis for genetic study of the mating types of *Zygnema*. His work concerned the mating compatibility of intercrosses among the various available mating types. Although his experiments were primarily concerned with the genetics of strains 921 and 922 from the Culture Collection of Algae at Indiana University (Starr, 1964), they also dealt briefly with spore germination. He made a provisional statement to the effect that both mating types are perpetuated in the algal progeny of *Z. circumcarinatum*.

Hoshaw (1968) described a hypothetical model based upon morphological observations for sexual induction at the molecular level in *Z. circumcarinatum*. In his study sexual induction was obtained in one mating type by the use of supernatants from a mixed conjugating culture. Because of this phenomenon, it was inferred that heterothallism is of a "weak nature" in *Z. circumcarinatum*.

These three works and the original description constitute the available information on *Z. circumcarinatum* and generally testify to the paucity of information on the species of *Zygnema*. Studies of *Zygnema* have historically centered on taxonomy (Transeau, 1934; Randhawa, 1943; Transeau, 1951). Morphological and ecological studies relevant to *Zygnema* have usually been confined to works supportive of taxonomy or to

mere mention within species lists of particular habitats (Tiffany, 1926; Transeau, 1934; Taft, 1944). Studies dealing with the cytology, physiology, and genetics of *Zygnema* are particularly scarce.

Despite the scarcity of physiological and genetic studies within the filamentous algae, methods exist in the field of mycology that can be easily adapted to the study of *Zygnema*. Gauch's work (1966) was a forerunner in the adaptation of fungal techniques to the study of *Zygnema*. He successfully applied the fungal technique of the Riddell slide, which allows long-term microscopic observations of *Zygnema*. Raper (1959) in his review on the sexual processes of fungi cites references to genetic analysis that can be applied to heterothallism in algae. The morphological similarities existing between the conjugating algae and members of the phycomycetes make the adaptation of fungal techniques of these organisms particularly useful in genetic analysis of *Zygnema*.

Electrophoretic banding patterns of proteins have been used successfully in fungal taxonomy and ecology to differentiate species and ecotypes (Clare, 1963; Clare and Zentmyer, 1966; Macko, Novacky, and Stahmann, 1967; Hall, Zentmyer, and Erwin, 1969). Similarly, electrophoretic analyses of protein banding patterns have been used to separate mating types of fungi (Hall, 1967).

The rationale for use of electrophoretic studies in problems concerning taxonomy and morphogenesis was put forth by Hall (1967) in his work on the fungal genus *Fusarium*. He states: "The sequence of amino acids is, as far as we know, the only genetically determined property of a protein and this sequence determines a protein's size, shape, and amphoteric properties. As these are the characters that determine

electrophoretic mobility, there is a theoretical basis for attempting to apply gel electrophoresis of proteins to taxonomic studies."

The rationale just described can be applied to problems concerning the separation of mating types in *Zygnema*. Following this rationale, electrophoretic techniques have been applied to developmental studies in slime molds and bacteria to trace alterations of protein constituents during morphogenesis (Baillie and Norris, 1963; Solomon, Johnson, and Gregg, 1964). The application of gel electrophoretic techniques to plant materials is reviewed by Brewbaker et al. (1968) and by Ornstein (1964). These works provide information on methods and equipment for gel electrophoresis of flowering plant material and are applicable to gel electrophoresis of algae.

The electrophoretic technique as applied to algal proteins is just beginning to be used as a taxonomic and morphological tool (Derbyshire and Whitton, 1968; Thomas and Brown, 1970; Lindsey, 1972). Modifications of existing techniques of protein electrophoresis to specific algal systems has yet to be refined.

The scarcity of genetic information for the Zygnemataceae has resulted in part from the inability of zygospores produced in the laboratory to germinate. This inability to obtain germination of zygotes has limited genetic study designed to trace the inheritance of genetic factors. Smith (1950) acknowledged this immature state of *Zygnema* genetics when he noted that "all studies on sex have been based upon conjugation in filaments of unknown parentage."

Gauch (1966) successfully applied a means of zygospore germination in *Zygnema* that had been used with success in the desmid *Netrium*

(Biebel, 1964). By drying and rewetting mature zygospores, Gauch was able to germinate *Zygnema* zygospores. The obstacle that had previously prevented genetic analysis in *Zygnema* was thus removed.

Prasad and Godward (1966), while examining material from the Cambridge Culture Collection of Algae and Protozoa, determined that *Z. circumcarinatum* has a haploid chromosome number of 19. This species possesses the smallest number of chromosomes of any member of the genus thus far studied. A low chromosome number enhances the possibility of finding linked loci as compared to an alga like *Netrium digitus*, which has a haploid chromosome number of 150 (Biebel, 1964).

The manipulation of culturing techniques to initiate morphological changes in algae has been reviewed by Coleman (1961). Initiation of conjugation in *Zygnema* has been attempted by manipulation of nutrient content, depletion of nitrogen source, increased CO_2 tension, and manipulation of light intensity (Jost, 1953; Prasad, 1963). Increased CO_2 concentration and elimination of nitrogen from the medium are ineffective in inducing conjugation in *Z. circumcarinatum*. In *Z. circumcarinatum* transfer to 0.1 dilute medium induces sexual stages (Jost, 1953).

The determination of the genetic bases of heterothallism has been investigated in the Zygnematales for the Desmidiaceae in *Cosmarium* (Starr, 1954) and *Closterium* (Lippert, 1967) and for the Mesotaeniaceae in *Spirotaenia* (Hilton, 1970) and *Netrium* (Biebel and Reid, 1965). Gauch (1966) provided the preliminary basis for the study of heterothallism in the Zygnemataceae through his work with *Zygnema* species 921 and 922 and through his mating compatibility studies among all the available heterothallic strains. In his preliminary study on the genetics of *Zygnema* he

did not attempt a backcross with the progeny of *Z. circumcarinatum* to determine the genetic basis of heterothallism.

A major unanswered question about *Z. circumcarinatum* is the basis underlying the distinction of the presumed isomorphic mating types as reproductive units. Research on this question has not been conducted because of problems associated with obtaining a complete life cycle for the species. The techniques for sexual induction and germination of zygospores are now available (Jost, 1953; Gauch, 1966).

The hypothesis constructed and tested by the present investigation can be stated as follows:

> The morphologically identical mating types of *Zygnema circumcarinatum* are distinguishable on physiological and genetic bases.

To test the validity of this hypothesis, investigations were designed to (1) determine the genetic bases for heterothallism in *Z. circumcarinatum* by genetic backcross; (2) determine the degree of physiological separation of the mating types through the life cycle of the organism by comparison of electrophoretic isozyme patterns; (3) compare the quantity and type of pigment content of the mating types by means of thin-layer chromatography and colorimetry; and (4) compare morphologically the mating types to determine whether they are isomorphic.

MATERIALS AND METHODS

Culture Methods

Axenic cultures of the mating types of *Zygnema circumcarinatum* Czurda (strains 698 1A and 698 1B) for this investigation were obtained from the Collection of Algae and Protozoa at Cambridge, England. Filaments were maintained in axenic culture on agar slants and in liquid medium, both employing Godward's medium (Godward, 1942) fortified with 1 ml per liter with Holm-Hansen and Lewin trace element solution (Clay, 1972). This medium is designated hereafter as full strength medium.

Filaments were cultured in 100 ml of sterile medium in 250-ml Erlenmeyer flasks stoppered with cotton plugs. The flasks were placed in a Percival controlled-environment chamber under cool-white fluorescent tubes at a light intensity of 3000 lux on a 12:12-hr light-dark cycle at $20^{\circ} \pm 2^{\circ}C$. Prior to inoculation or transfer the filaments were chopped with razor blades to facilitate handling and to secure equal aliquots of material for each flask (Rosowski and Hoshaw, 1970). Vegetative filaments were maintained under these culture conditions throughout the investigation. These culture conditions will be referred to hereafter as standard conditions and will differ only in concentration of medium.

Genetic Analysis

Sexual reproduction (conjugation) was obtained by mixing the two mating types in a 0.1 dilute medium prepared by mixing 9 parts sterile distilled water to one part Godward's medium and trace element solution.

This medium will be designated hereafter as dilute medium. Matings were made in 1 ml of this dilute medium placed in inverted glass caps commonly used to cover culture test tubes. Each glass cap was covered by a 22-sq-mm Corning cover glass held in place by a seal of petroleum jelly between the cover glass and the glass cap.

Conjugation occurred within 48 hr after the mating types were mixed. Newly formed zygotes were allowed to mature in dilute medium for a period of 2 months, at which time mated cultures were transferred to Petri plates of full strength medium solidified with 1.5% agar. The agar plates were dried for a week, during which time all vegetative cells were killed, leaving only viable zygospores, parthenospores, and akinetes (Fig. 11, p. 38).

Zygospore germination occurred 1 week after the dried plates were rewetted with full strength medium. Germling filaments were removed from the plates with a micropipette. Each germling was examined first to determine whether it originated from a zygospore and not an asexual spore, and secondly care was taken to observe that only one germling was transferred to each tube of fresh medium.

The criteria for a germinated zygospore were size and shape of the adhering spore wall and presence of empty gametangia on two sides of the germling. These criteria distinguished zygospore germlings from any asexual germlings and ensured the isolation of only progeny from conjugation. Only one of the four products of zygotic meiosis apparently survives in *Zygnema* with the production of a single germling from this meiotic product.

To ascertain the optimum cell stage for isolating germlings, 10 germlings each were isolated at the 1, 2, 3, 4, 6, and 15-celled stages. The germlings were inoculated into test tubes containing 10 ml of full strength medium. After 30 days under standard conditions, the clones were assayed for growth by observing a tuft of filaments when tubes were held in front of a lighted surface. The results of this determination are shown in Figure 1 (p. 17).

The germlings used in the genetic analysis were isolated and transferred to test tubes of full strength medium and later to 250-ml flasks of full strength medium. One month after isolation, the clones had grown sufficiently to provide material for backcrossing to the parent mating types. Sixty-three clones out of 125 were chosen at random for genetic analysis. The clones were designated GZ (germinated zygospore) and numbered 1 through 63; they are designated hereafter by GZ and their appropriate number.

The GZ clones were backcrossed to both parental mating types by mixing in dilute medium. Analysis of clones was conducted for the ability to mate with mating type 698 1A (hereafter referred to as 1A) or to the mating type 698 1B (hereafter referred to as 1B). Mating-type designations were then assigned to the clones. A clone mating with 1A was thus assigned mating type B. The results of the backcrosses of the 63 progeny appear in Table 1 (p. 18).

Gauch (1966) had used cell width as a genetic marker in *Zygnema* strains 921 and 922. To determine if cell width could function as a genetic marker in *Z. circumcarinatum*, measurements of cell width were made. The cell width of each of the 63 clones was determined by measurement

of a total of 50 cells from 10 different filaments from each clonal culture. Cell width data are presented in Figure 2 and Table 2 (pp. 19-20).

Electrophoretic Analysis

A problem encountered early in the electrophoretic study of the mating types was the selection of a reliable method for protein extraction. To resolve this problem, vegetative cultures were subjected to different methods of protein extraction and the amount of protein per milligram of fresh weight of material was determined. The entire contents of flasks were harvested by means of vacuum suction through a 15-μ pore piece of Nytex cloth that was stretched across a funnel inserted in the top of a vacuum flask. These samples of filaments were weighed and subjected to the eight methods of protein extraction described below. Protein content of the extracts was determined by the method of Lowry et al. (1951), and the results are shown in Table 3 (p. 21).

(1) Grinding . . . . . .The filaments were ground using a mortar and pestle for 5 min in 15 ml of 126 B buffer.

(2) Grinding with glass beads . . .The filaments were ground in a mortar and pestle with glass beads for 5 min in 15 ml of 126 B buffer.

(3) Blending . . . . . .The filaments were decanted to a Waring blender and chopped at full speed for 2 min in 15 ml of 126 B buffer.

(4) Blending with acetone. . . .The filaments were decanted to a Waring blender and chopped at full speed for 2 min in 15 ml of 100% acetone.

(5) Liquid N . . . . . .The filaments were frozen in liquid nitrogen in a mortar and were shattered by hitting with the pestle

followed by grinding in 15 ml of 126 B buffer.

(6) Sonication . . . . .The filaments were subjected to 5 min of sonication in 15 ml of 126 B buffer in an "Ultrasonic Disontegrator Model System 80."

(7) Pressure bomb . . .The filaments were placed under 1400 psi pressure of N in 15 ml of 126 B buffer in a Paar Disruption Bomb for 30 min and were returned suddenly to atmospheric pressure.

(8) Freeze and thaw. . .The filaments were frozen in 15 ml of 126 B buffer in liquid N and were allowed to thaw undisturbed.

Of the eight methods just described, the pressure bomb extraction method was selected for all subsequent protein extractions.

To determine the degree of physiological separation that exists between mating types 1A and 1B, a series of disc electrophoretic studies was conducted. Vegetative cultures were transferred to fresh full strength medium and grown under standard conditions. At 7, 14, and 21 days following transfer, the contents of the flasks were harvested by vacuum onto 15-μ-pore sized Nytex cloth (Kressilk Products, Monterey Park, California), were mixed with 15 ml of 126 B buffer (Brewbaker et al., 1968), and were ruptured in a Paar Disruption Bomb. The soluble proteins were separated from the cellular debris by centrifugation, and the extracts were frozen. These proteins were separated by means of acrylamide disc electrophoresis on a 7% gel following the method of Brewbaker et al. (1968). The resulting gels were stained for nonspecific esterases, catalases, and acid phosphatases, and the results are shown by zymograms in Figures 3, 4, and 5 (pp. 23, 24, and 25).

Fourteen-day-old cultures of both mating types were transferred separately to dilute medium. These cultures were sampled, ruptured, and extracted at 21 days following the method described in the preceding paragraph to determine the effect a shift to dilute medium conditions has on the mating types. Figures 3, 4, and 5 show the electrophoretic comparison of the 21-day-old cultures grown in dilute medium with the same age cultures grown in full strength medium.

Fourteen days following transfer, cultures of both mating types were mixed and induced to conjugate. These cultures were sampled, ruptured, and extracted on the 21st day to determine the isozyme patterns that result with mixing of the mating types. The zymograms in Figure 6 (see page 28) show the electrophoretic comparison of banding patterns of the mated cultures designated 1A × 1B with a mixture of equal amounts of 21-day-old cultures of 1A and 1B that had been grown separately (1A + 1B).

To determine the daily variability of isozyme patterns, 17-day-old cultures of both mating types were sampled and extracted at 0, 8, and 22 hr during the 17th day. These extracts were subjected to electrophoresis and stained for nonspecific esterases and for acid phosphatases. The results of this separation are given in Figure 7 (see page 30).

All electrophoretic separations were conducted at 2° to 4°C in a walk-in refrigerated room. Gels, buffers, and stains were prepared and used according to the method of Brewbaker et al. (1968) without modification. Each gel received 200 µg of protein extract before electrophoresis. The extracts themselves were made 0.5 molar with sucrose to enable the

layering of the protein extract on the gels. Electrophoresis was accomplished by use of 4 mA of direct current per tube with a running time of approximately 3 hr. The exact running time was determined by observing the migration of a bromophenol blue indicator through the gels. A Heathkit regulated H.V. Power Supply Model IP-17 was used as the power source for electrophoresis. Destaining of gels was accomplished by diffusion in plastic trays containing 7% acetic acid.

Gels were examined by placing each gel on a Logan Magna-View Slide Sorter and Viewer Model 1050. The positions of the bands were measured with a metric ruler. These measurements were used to prepare a scale drawing of each gel and expressed the value of the midpoints of the bands. No measurements were taken of the widths of the individual bands. Rf values for the bands were calculated using the bromophenol blue indicator as the migratory front, and these values were used to produce the zymograms of Figures 3, 4, 5, 6, and 7. The patterns are the mean midpoint values for the bands obtained from the various runs.

Pigment Analysis

Mating types 1A and 1B maintained under standard conditions often appeared to vary in their greenish color when cultures were viewed macroscopically. This color difference led to an investigation of the pigment content of the mating types.

Qualitatively the pigment content was assayed by means of thin layer chromatography on silica plates. Pigments were extracted by grinding filaments in 80% acetone in a mortar and pestle. The resulting pigment extracts were spotted onto the silica plates by means of a pulled

micropipette. Chromatograms were developed with a light petroleum ether: isopropanol: water solvent system (100:10:25) as described by Klein and Klein (1970). Figure 8 (see page 33) shows a comparison of the chromatograms for the pigment content of the mating types.

A quantitative determination of the pigment content was obtained by extraction of the pigments in 80% acetone followed by the measurement of optical density at wavelengths of 645 nm, 663 nm, and 452 nm, and concentration was calculated by the method of Arnon (1949). All pigment extracts were obtained by sampling cultures of equal age. These measurements were standardized on a per cell basis by chopping the filaments prior to extraction in a Petri dish with razor blades, followed by counting the cells in a hemacytometer counting chamber. Ten counts of each sample were made prior to pigment extraction to minimize error. The results of the quantitative pigment analysis are shown in Table 9 (see page 32).

Morphological Analysis

Concurrent with sampling of cultures during the life-cycle stages for use in the electrophoretic analysis, filaments were preserved in FAA for a morphological comparison of the mating types. The cells were examined under oil immersion with a Zeiss Phase Contrast Microscope Model GFL. Measurements were made of cell width, cell length, width of mucilaginous sheath, cell-wall width, and distance between pyrenoids.

Measurement of the mucilaginous sheath was accomplished after the preserved material was placed in a drop of India ink. The width of the sheath was measured as the distance from the outside of the cell wall

to the India ink boundary. Figures 9 and 10 (see page 36) show the alteration of sheath width during the culturing cycle. The distance between the two pyrenoids was measured from the center of one pyrenoid to the center of the other pyrenoid. Measurement of cell-wall width was aided by immersion of cells in a solution of ruthenium red for several minutes prior to mounting in India ink. The ruthenium red stained the outer pectose layer of the cell wall pink and distinguished it from the mucilaginous sheath.

The results of the morphological comparisons are given in Tables 10 and 11 (see page 35). All measurements are mean values plus or minus standard error of the mean for a 50-cell sample from 10 filaments of each culture age.

OBSERVATIONS AND RESULTS

Genetic Analysis

No attempts previously have been made to determine the genetic bases of heterothallism in any species of the Zygnemataceae. By combining the methods of sexual induction developed by Jost (1953) with the methods of zygospore germination developed by Gauch (1966), it was possible to recover genetic progeny of known parentage for genetic analysis in *Z. circumcarinatum*. Conjugation was induced by mixing the mating types in dilute medium, and zygospores were obtained and allowed to mature for two months in this same medium. Germination of zygospores was obtained by drying and rewetting these zygospores. Clones of zygospore germlings were established and were used one month later as the experimental material for genetic analysis.

The effect of isolation and transfer on the viability of the young germlings was determined to find the optimum time for isolation of germlings. Ten germlings of various cell lengths were isolated, transferred to full strength medium, and grown for four weeks. Cultures were then examined to determine whether the germlings survived the isolation and transfer. Results of this zygospore germling viability test are presented in Figure 1.

A genetic analysis was used to determine the bases underlying heterothallism in *Z. circumcarinatum* by backcrossing the F_1 generation of the known mating (1A × 1B) to the parental types. The results of this analysis are presented in Table 1. Results show that three classes of

offspring are identifiable. These classes, designated A, B, and AB, represent individuals that mated with parent 1B, parent 1A, or both parents, respectively. The presence of the two AB clones and the nonmating clone is highly significant in the interpretation of the basis of heterothallism.

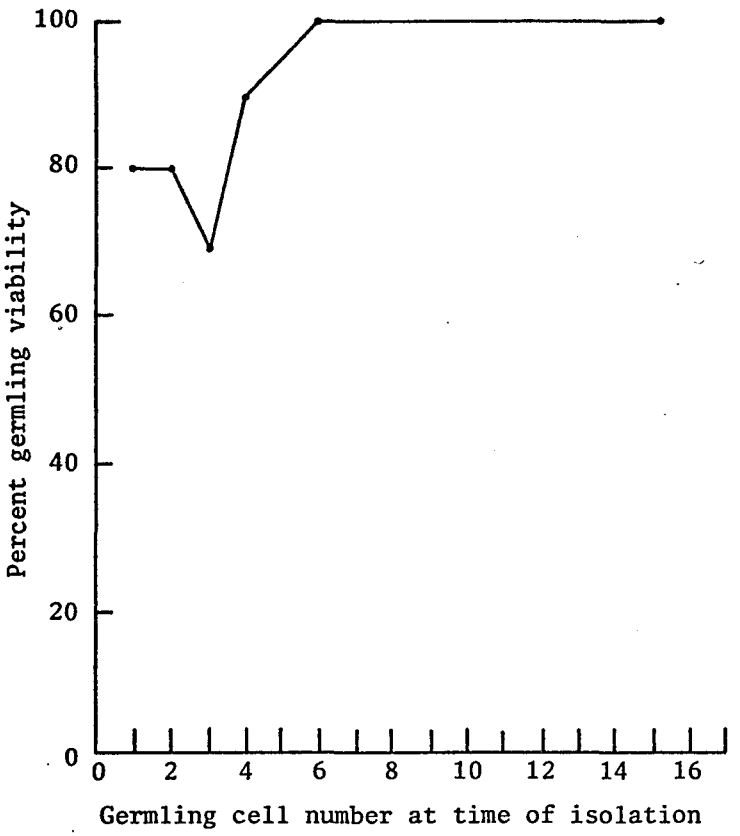

Figure 1. Percent Viability of Germlings Isolated at Various Cell Stages.

Table 1. Mating Compatibility and Mating Type Inheritance of 63 Clonal F_1 Isolates of *Zygnema circumcarinatum*.

\+ Sexual compatibility as indicated by conjugation.
\- Sexual incompatibility as indicated by no conjugation.

Clone	Mates with 1A	Mates with 1B	Mating type designation	Clone	Mates with 1A	Mates with 1B	Mating type designation
GZ 1	-	+	A	GZ 33	+	-	B
GZ 2	+	-	B	GZ 34	+	-	B
GZ 3	-	+	A	GZ 35	-	-	-
GZ 4	+	-	B	GZ 36	-	+	A
GZ 5	-	+	A	GZ 37	+	-	B
GZ 6	+	-	B	GZ 38	-	+	A
GZ 7	+	-	B	GZ 39	+	-	B
GZ 8	+	-	B	GZ 40	+	-	B
GZ 9	+	-	B	GZ 41	-	+	A
GZ 10	-	-	-	GZ 42	-	+	A
GZ 11	-	+	A	GZ 43	+	-	B
GZ 12	+	-	B	GZ 44	-	+	A
GZ 13	+	-	B	GZ 45	+	-	B
GZ 14	+	-	B	GZ 46	+	-	B
GZ 15	-	+	A	GZ 47	+	-	B
GZ 16	+	+	AB	GZ 48	-	+	A
GZ 17	-	+	A	GZ 49	-	+	A
GZ 18	+	-	B	GZ 50	-	+	A
GZ 19	+	-	B	GZ 51	+	-	B
GZ 20	-	+	A	GZ 52	+	-	B
GZ 21	+	-	B	GZ 53	+	-	B
GZ 22	+	-	B	GZ 54	-	+	A
GZ 23	-	-	-	GZ 55	+	-	B
GZ 24	+	-	B	GZ 56	-	+	A
GZ 25	-	+	A	GZ 57	-	+	A
GZ 26	-	+	A	GZ 58	+	-	B
GZ 27	+	-	B	GZ 59	+	-	B
GZ 28	-	+	A	GZ 60	-	+	A
GZ 29	-	+	A	GZ 61	-	+	A
GZ 30	+	+	AB	GZ 62	+	-	B
GZ 31	+	-	B	GZ 63	+	-	B
GZ 32	-	+	A				

Gauch (1966) in *Zygnema* sp. 921 and 922 obtained evidence that cell width is determined by two gene loci. As a part of the genetic analysis in *Z. circumcarinatum*, cell widths were measured and analyzed for the F_1 clonal isolates by measuring 50 cells from 10 separate filaments of each clone. The results of these measurements are presented in Figure 2 and Table 2 and show that the majority of clonal widths are grouped around the parental cell width value of 23 μ (Figure 2). However, three clones were significantly different from the clones grouped around the parental value, and these will be discussed later.

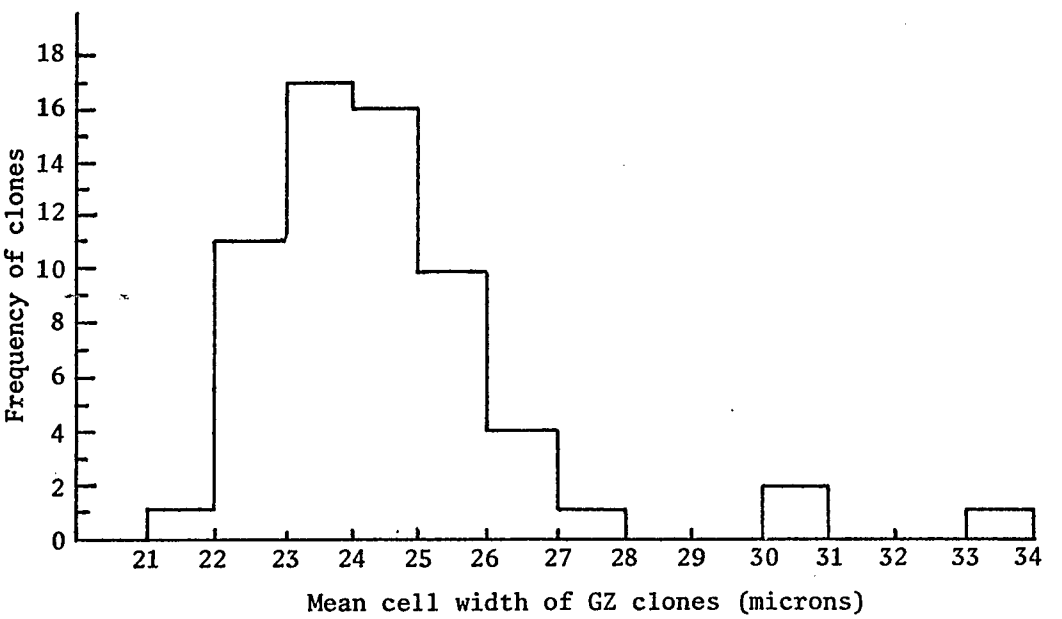

Figure 2. Frequency Histogram of Germinated Zygospore Clone Widths.

Table 2. Mating-Type and Cell-Width Inheritance in F_1 Isolates of *Zygnema circumcarinatum*.

Clone	Mating type designation	Mean cell width ± S.E. (μ)	Clone	Mating type designation	Mean cell width ± S.E. (μ)
GZ 1	A	24.4 ± .1	GZ 33	B	24.6 ± .2
GZ 2	B	22.1 ± .1	GZ 34	B	23.9 ± .1
GZ 3	A	24.8 ± .1	GZ 35	-	30.8 ± 1.0
GZ 4	B	24.9 ± .1	GZ 36	A	24.3 ± .1
GZ 5	A	23.6 ± .3	GZ 37	B	24.9 ± .5
GZ 6	B	25.6 ± .2	GZ 38	A	25.7 ± .5
GZ 7	B	24.8 ± .1	GZ 39	B	22.3 ± .0
GZ 8	B	23.7 ± .1	GZ 40	B	23.8 ± .1
GZ 9	B	24.3 ± .1	GZ 41	A	25.3 ± .1
GZ 10	-	26.7 ± .7	GZ 42	A	23.7 ± .1
GZ 11	A	22.7 ± .1	GZ 43	B	21.9 ± .1
GZ 12	B	24.3 ± .3	GZ 44	A	25.3 ± .1
GZ 13	B	22.9 ± .1	GZ 45	B	22.6 ± .1
GZ 14	B	24.5 ± .1	GZ 46	B	22.4 ± .1
GZ 15	A	25.7 ± .8	GZ 47	B	33.0 ± .3
GZ 16	AB	23.3 ± .2	GZ 48	A	23.3 ± .7
GZ 17	A	24.5 ± .1	GZ 49	A	23.0 ± .1
GZ 18	B	25.9 ± .1	GZ 50	A	23.4 ± .1
GZ 19	B	23.1 ± .1	GZ 51	B	25.4 ± .2
GZ 20	A	24.2 ± .1	GZ 52	B	23.6 ± .6
GZ 21	B	22.7 ± .6	GZ 53	B	22.1 ± .1
GZ 22	B	25.1 ± .2	GZ 54	A	22.7 ± .1
GZ 23	-	24.0 ± .1	GZ 55	B	30.5 ± .4
GZ 24	A	23.5 ± .1	GZ 56	A	24.2 ± .1
GZ 25	A	24.8 ± .8	GZ 57	A	24.8 ± .1
GZ 26	A	23.5 ± .1	GZ 58	B	22.5 ± .1
GZ 27	B	26.4 ± .7	GZ 59	B	23.3 ± .1
GZ 28	A	25.4 ± 1.2	GZ 60	A	23.3 ± .1
GZ 29	A	26.0 ± .1	GZ 61	A	23.9 ± .1
GZ 30	AB	27.1 ± .1	GZ 62	B	22.9 ± .1
GZ 31	B	25.6 ± .5	GZ 63	B	23.5 ± .7
GZ 32	A	26.4 ± .7			

Electrophoretic Analysis

Prior to the electrophoretic study of the mating types of *Z. circumcarinatum*, a reliable means of protein extraction was required. Eight methods of extraction were tested to determine which method allowed for the optimum extraction of *Zygnema* proteins. A comparison of these extraction methods is summarized in Table 3. The comparative results are the averages of two extractions for each method. The bomb extraction method yielded the highest quantity of extractable protein. All methods were assigned a percent efficiency based on the bomb extraction method representing 100% efficiency.

It was of interest to determine whether detectable physiological differences existed between the two mating types in regard to different aged cultures grown under identical conditions, in regard to cultures of the same age grown under different medium conditions, and in regard to mated (sexual) versus unmated (asexual) cultures.

Table 3. Efficiency of Eight Methods of Protein Extraction in *Zygnema circumcarinatum*.

Extraction method	Protein extracted, mg protein/mg fresh wt	Percent efficiency
Bomb	.0168	100.0
Liquid N: Grinding	.0100	59.5
Blender	.008[illegible]	47.5
Blender: Acetone	.0059	35.0
Mortar & pestle	.0038	22.6
Mortar & pestle: Glass beads	.0033	19.6
Freeze rupture	.0033	19.6
Sonication	.0009	5.3

Disc gel electrophoresis was used to distinguish qualitatively the differences in enzyme patterns (i.e., physiological separation) existing between the mating types at various times during the culturing cycle (Figures 3, 4, and 5). The differences between the mating types grown in dilute medium were determined to see whether detectable physiological changes occur in catalases, acid phosphatases, and nonspecific esterases during the shift of medium conditions that in mixed cultures would initiate conjugation. Differences are shown in Figures 3, 4, and 5, for catalases, esterases, and acid phosphatases, respectively. Tables 4, 5, and 6 present the mean Rf values for the isozyme bands. The results indicate that at all times and under all conditions tested, except for the acid phosphatase sample on the 14th day, detectable differences did exist in the isozyme patterns of the mating types.

An electrophoretic comparison of a mixed culture versus an equal amount of unmated material of each mating type was used to show the qualitative change in physiology occurring when the mating types were mixed. The results are shown in Figure 6; Table 7 presents the mean Rf values for the isozyme bands. All results indicate that isozyme transitions do occur in all the systems studied when the mating types are mixed and conjugation follows.

Both mating types were assayed at various times during the 17th day to ascertain the amount of internal variability within the mating types regarding isozyme patterns. The results of this electrophoretic analysis are given in Figure 7 and Table 8. Results indicate that internal daily variability is insufficient to account for the changes of banding patterns observed in the above-mentioned electrophoretic studies.

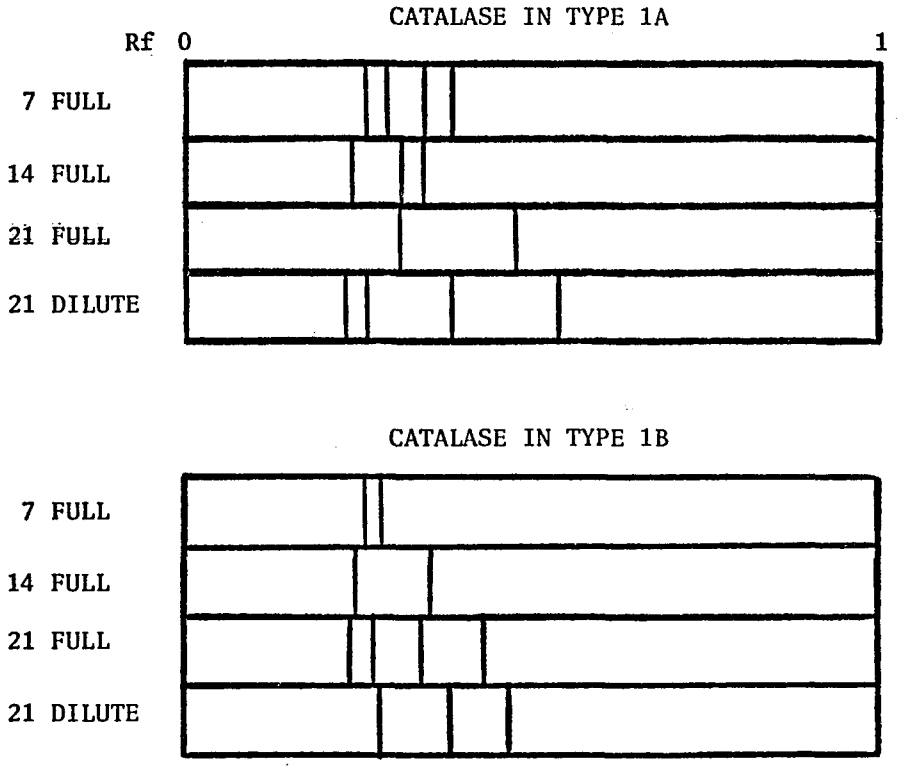

Figure 3. Catalase Isozyme Banding Patterns at Various Times During the Culturing Cycle.

Zymograms are from 7, 14, and 21-day cultures in full strength media and 21-day cultures in dilute media. Values are the means from three separate electrophoretic determinations.

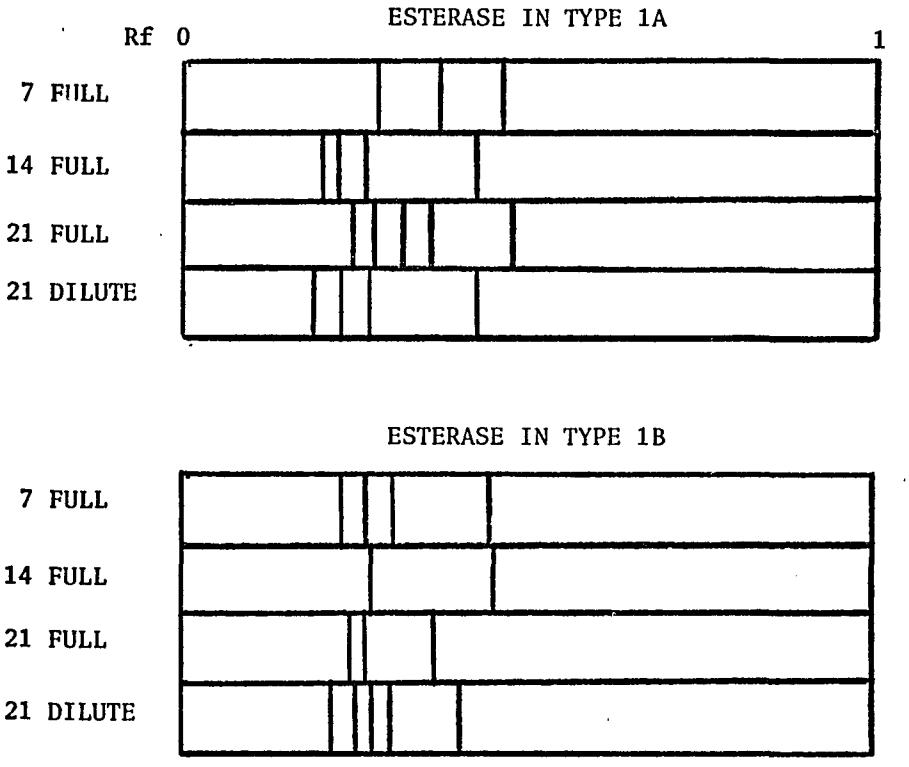

Figure 4. Esterase Isozyme Banding Patterns at Various Times During the Culturing Cycle.

Zymograms are from 7, 14, and 21-day cultures in full strength media and 21-day cultures in dilute media. Values are the means from three separate electrophoretic determinations.

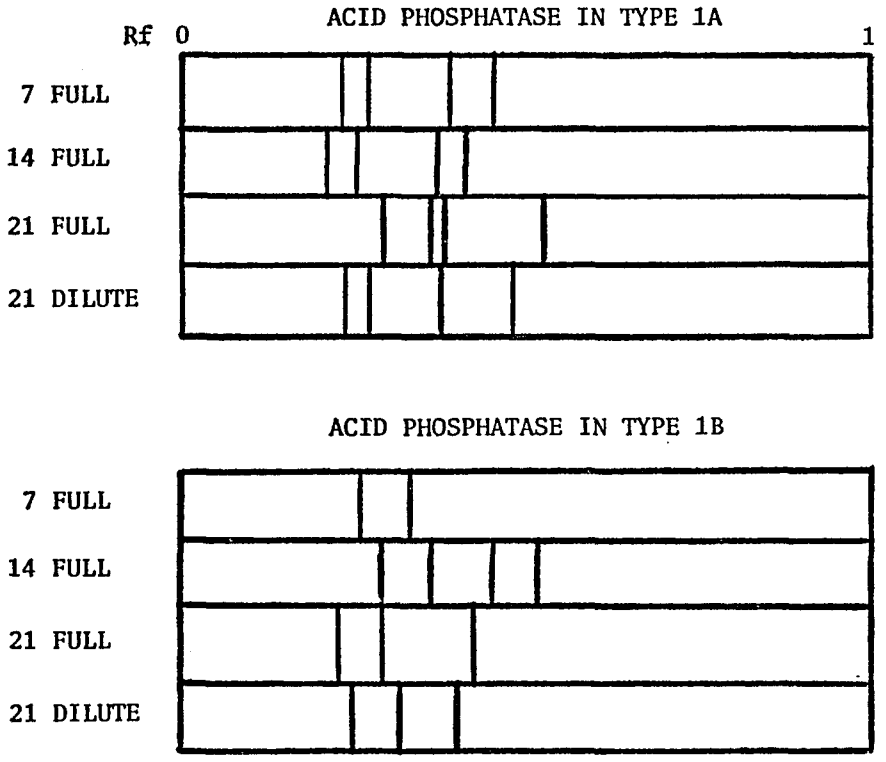

Figure 5. Acid Phosphatase Banding Patterns at Various Times During the Culturing Cycle.

Zymograms are from 7, 14, and 21-day cultures in full strength media and 21-day cultures in dilute media. Values are the means from three separate electrophoretic determinations.

Table 4. Mean Rf Values of Catalase Isozyme Bands of the Mating Types at Three Times During the Culturing Cycle.

Mean Rf values of bands are averages from three determinations. Bands were designated A, B, C, to show order of bands in regard to origin. Band A migrated the least from the origin of any of the bands.

Mating type	Age, days	Strength of medium	Mean Rf value of bands			
			A	B	C	D
1A	7	Full	.262	.293	.347	.386
1A	14	Full	.239	.314	.344	--
1A	21	Full	.310	.478	--	--
1A	21	Dilute	.231	.262	.386	.540
1B	7	Full	.259	.283	--	--
1B	14	Full	.246	.355	--	--
1B	21	Full	.239	.269	.344	.432
1B	21	Dilute	.286	.381	.468	--

Table 5. Mean Rf Values of Esterase Isozyme Bands of the Mating Types at Three Times During the Culturing Cycle.

Mean Rf values of bands are averages from three determinations. Bands were designated A, B, C, to show order of bands in regard to origin. Band A migrated the least from the origin of any of the bands.

Mating type	Age, days	Strength of medium	Mean Rf value of bands				
			A	B	C	D	E
1A	7	Full	.280	.374	.464	--	--
1A	14	Full	.197	.217	.259	.423	--
1A	21	Full	.243	.271	.313	.347	.473
1A	21	Dilute	.186	.229	.272	.425	--
1B	7	Full	.230	.262	.305	.445	--
1B	14	Full	.271	.448	--	--	--
1B	21	Full	.245	.262	.367	--	--
1B	21	Dilute	.211	.244	.267	.299	.394

Table 6. Mean Rf Values of Acid Phosphatase Isozyme Bands of the Mating Types at Three Times During the Culturing Cycle.

Mean Rf values of bands are averages from three determinations. Bands were designated A, B, C, to show order of bands in regard to origin. Band A migrated the least from the origin of any of the bands.

Mating type	Age, days	Strength of medium	Mean Rf value of bands			
			A	B	C	D
1A	7	Full	.233	.270	.390	.452
1A	14	Full	.216	.251	.371	.414
1A	21	Full	.293	.361	.386	.525
1A	21	Dilute	.238	.268	.378	.482
1B	7	Full	.259	.331	--	--
1B	14	Full	.286	.358	.450	.516
1B	21	Full	.229	.289	.426	--
1B	21	Dilute	.253	.321	.400	--

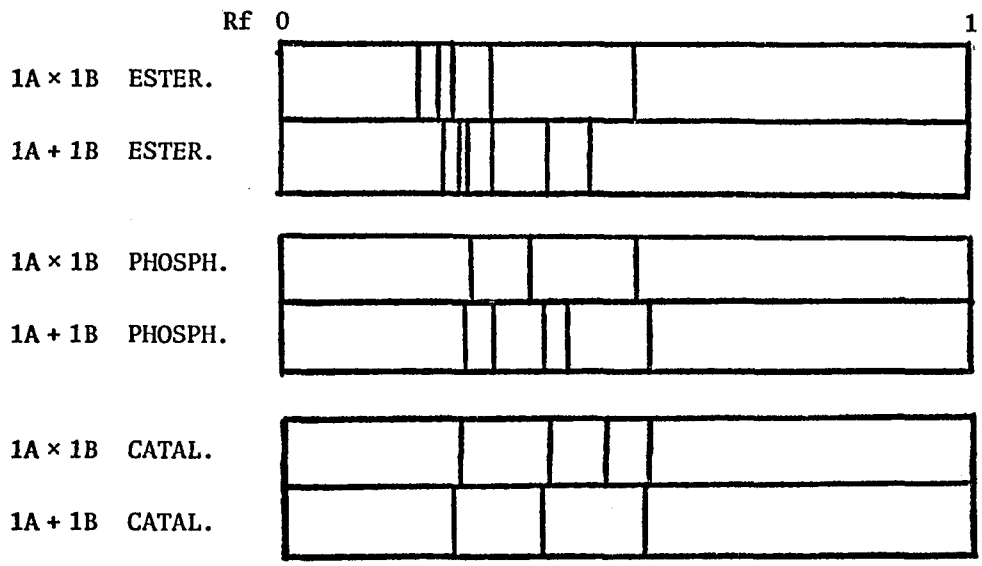

Figure 6. Isozyme Banding Patterns of Mixed and Simulated Mixed Cultures.

Zymograms are from cultures mixed together and induced to conjugate (1A × 1B) and control cultures that were mixed prior to extraction (1A + 1B). Values are means from three separate electrophoretic determinations.

Table 7. Mean Rf Values of Isozyme Bands in Mixed and Simulated Mixed Cultures.

Mean Rf values of bands are averages from three determinations. Bands were designated A, B, C, to show order of bands in regard to origin. Band A migrated the least from the origin of any of the bands.

Mating type condition	Isozyme class	Mean Rf values of bands						
		A	B	C	D	E	F	G
1A × 1B	Catalase	.260	.393	.468	.535	--	--	--
1A + 1B	Catalase	.250	.379	.530	--	--	--	--
1A × 1B	Esterase	.200	.232	.253	.307	.517	--	--
1A + 1B	Esterase	.211	.242	.260	.270	.310	.390	.450
1A × 1B	Phosphatase	.276	.359	.516	--	--	--	--
1A + 1B	Phosphatase	.265	.310	.380	.415	.530	--	--

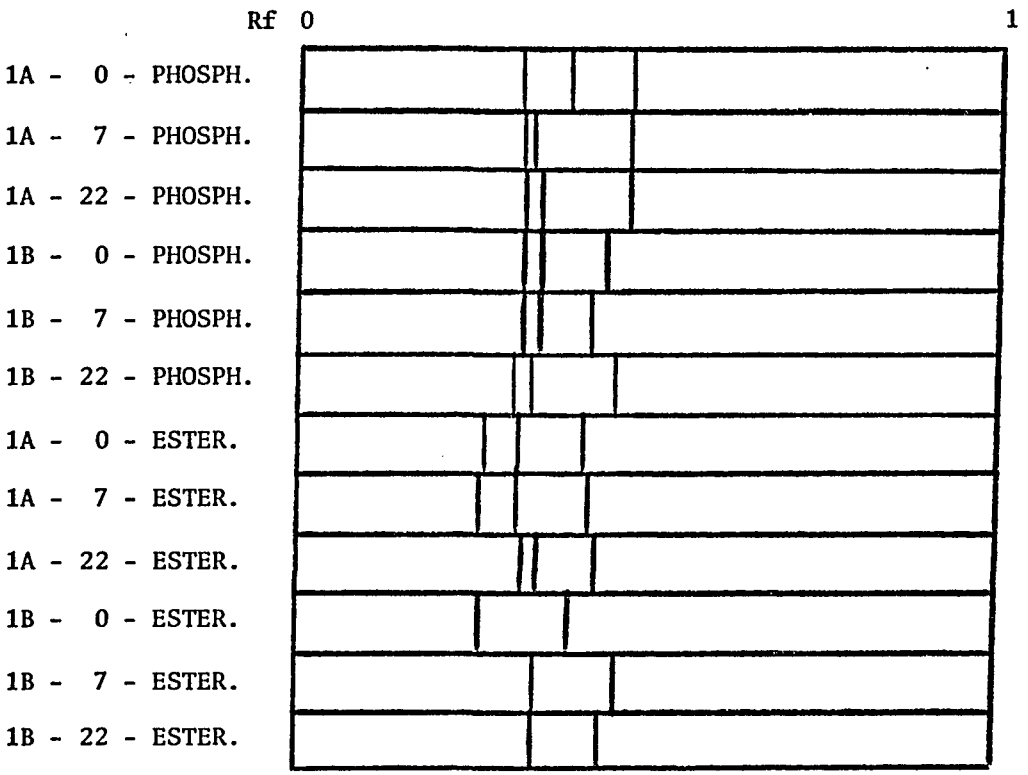

Figure 7. Isozyme Banding Patterns at Various Times During the 17th Day.

Zymograms are from 0, 7, and 22-hr extracts taken during the 17th day. Values are means from three separate electrophoretic determinations.

Table 8. Mean Rf Values of Isozyme Bands at Various Times During the 17th Culturing Day.

Mean Rf values of bands are averages from three determinations. Bands were designated A, B, C, to show order of bands in regard to origin. Band A migrated the least from the origin of any of the bands.

Mating type	Time, hrs	Isozyme class	Mean Rf values of bands		
			A	B	C
1A	0	Phosphatase	.317	.387	.469
1A	+7	Phosphatase	.311	.328	.459
1A	+22	Phosphatase	.318	.334	.456
1B	0	Phosphatase	.312	.335	.427
1B	+7	Phosphatase	.317	.336	.408
1B	+22	Phosphatase	.302	.321	.447
1A	0	Esterase	.259	.306	.399
1A	+7	Esterase	.254	.309	.404
1A	+22	Esterase	.318	.334	.425
1B	0	Esterase	.260	.380	--
1B	+7	Esterase	.332	.446	--
1B	+22	Esterase	.331	.423	--

Pigment Analysis

Cultures of mating type 1A consistently appeared greener than cultures of mating type 1B. To determine whether this difference in coloration was a characteristic of the mating types, a quantitative and qualitative analysis of the pigment content of the mating types was made. The results of the qualitative analysis by thin layer chromatography are shown in Figure 8. The quantitative amounts of chlorophyll pigments were determined according to the method of Arnon (1949). A comparison of total chlorophyll, chlorophyll a, and chlorophyll b content of the mating types is shown in Table 9. These values were obtained according to the following relationships:

$$\text{Total chlorophyll mg/l} = 20.2\ (O.D._{645}) + 8.02\ (O.D._{663})$$
$$\text{Chlorophyll a mg/l} = 12.7\ (O.D._{663}) - 2.69\ (O.D._{645})$$
$$\text{Chlorophyll b mg/l} = 22.9\ (O.D._{645}) - 4.68\ (O.D._{663})$$

Results indicate that mating type 1A contains more chlorophyll on a per cell basis than mating type 1B and that this physiological parameter is a valid characteristic separating the mating types.

Table 9. A Comparison of Pigment Content in Mating Types 698 1A and 698 1B of *Zygnema circumcarinatum*.

Mating type	Total chlorophyll (mg/cell)	Chlorophyll a (mg/cell)	Chlorophyll b (mg/cell)
1A	4.77×10^{-8}	2.61×10^{-8}	2.18×10^{-8}
1B	2.99×10^{-8}	1.37×10^{-8}	1.66×10^{-8}

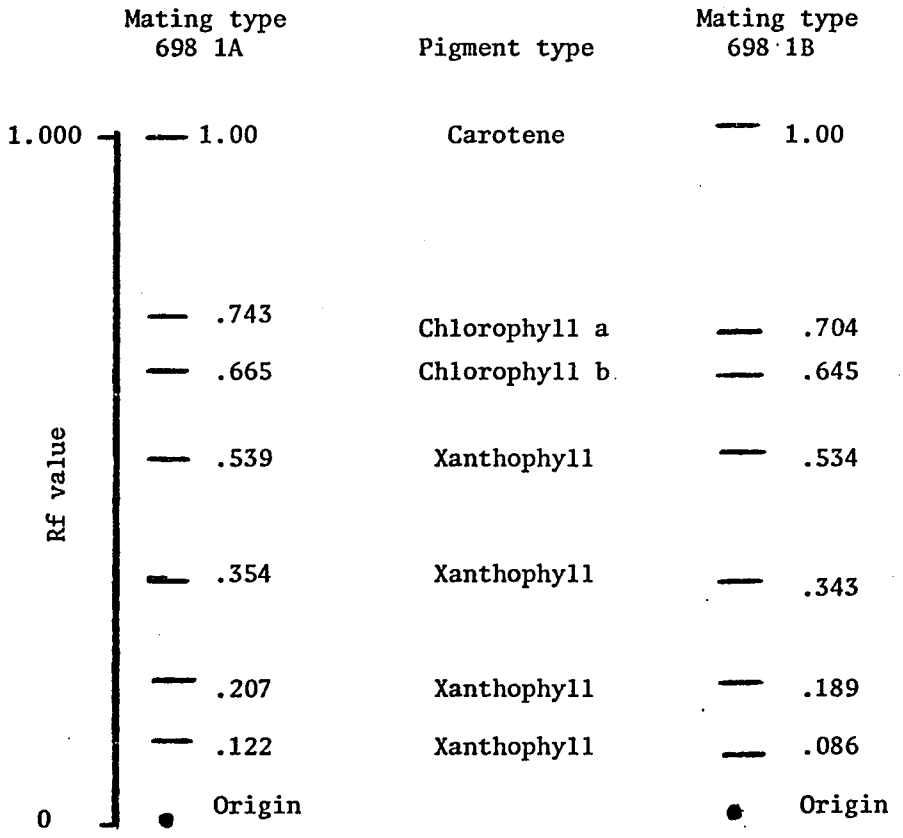

Figure 8. Comparison of Thin-Layer Chromatograms of Mating Types 698 1A and 698 1B of *Zygnema circumcarinatum*.

Morphological Analysis

Cell morphology is the sum total of all the genetic and physiological processes occurring within the cell. To determine whether the mating types differed morphologically, the following cell parameters were measured: cell width, cell length, cell-wall width, sheath width, and distance between the pyrenoids. Table 10 presents the averages of 50 measurements made of these parameters for the two mating types in dilute and full strength media. Table 11 and Figures 9 and 10 show the variation of sheath width during the culturing cycle.

An interesting difference between the mating types was observed during an attempt to increase the growth rate of filaments by aeration. During an experiment, chopped algal filaments were introduced into 2800-ml Fernbach culture flasks containing 100 ml full strength medium. The flasks were equipped with a glass tube through which cotton-filtered air bubbled to aerate and stir the cultures. After one month under these conditions, both mating types exhibited dense growth with filaments of mating type 1A growing to the top of the culturing flask in an undulating pattern. This "creeping," undulating pattern of filaments extended higher than any splash zone created by bursting air bubbles that could have carried filaments, and this phenomenon was not seen in mating type 1B. It is inferred that mating type 1A differs in some unknown way from 1B to allow for the "creeping" of filaments in the moist atmosphere of the flask.

Table 12 presents a morphological comparison of the mating types in regard to sheath width of the filaments grown under the previously mentioned conditions in which 1A exhibited "creeping" while 1B did not.

Table 10. Morphological Comparison of Mating Types 1A and 1B of *Zygnema circumcarinatum*.*

Mating type and strength of medium	Cell length, μ	Cell width, μ	Distance between pyrenoids, μ	Cell-wall width, μ
1A Full	34.0 ± .2	23.0 ± .8	13.9 ± .5	1.5 ± .0
1A Dilute	33.8 ± .3	27.5 ± .5	14.8 ± .3	1.5 ± .0
1B Full	31.1 ± .7	23.6 ± .8	14.0 ± .2	1.5 ± .0
1B Dilute	38.7 ± 1.1	27.5 ± .4	18.3 ± .3	1.5 ± .0

*Mean ± S.E.

Table 11. Comparison of Sheath Width in the Mating Types of *Zygnema circumcarinatum*.*

Mating type	Strength of medium	Culture age, days	Sheath width, μ
1A Full	Full	7	6.3 ± .1
1A Full	Full	14	13.3 ± .6
1A Full	Full	21	11.4 ± .5
1A Dilute	Dilute	21	6.0 ± .1
1A Full	Full	39	6.8 ± .4
1A Full	Full	114	7.0 ± .4
1B Full	Full	7	7.7 ± .5
1B Full	Full	14	8.8 ± .4
1B Full	Full	21	6.5 ± .1
1B Dilute	Dilute	21	6.0 ± .1
1B Full	Full	113	6.8 ± .5

*Mean ± S.E.

Figure 9. Comparison of Sheath Widths of the Mating Types During the Culturing Cycle.

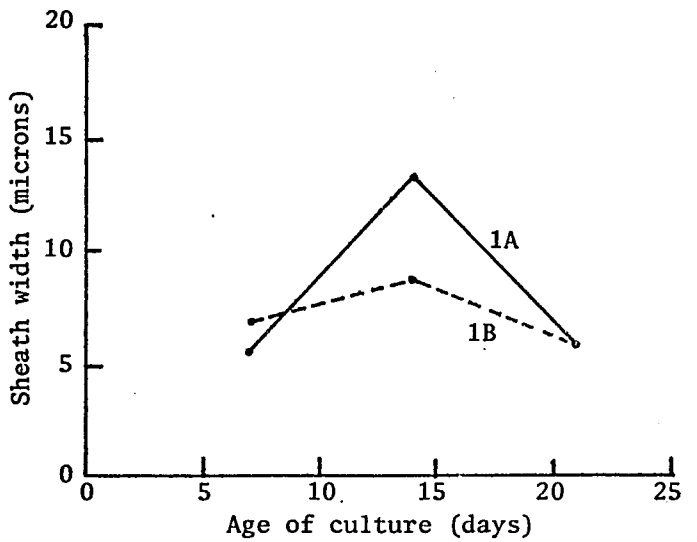

Figure 10. Comparison of Sheath Widths of Mating Types Following Transfer to Dilute Medium on the 14th Day.

Table 12. Comparison of Sheath Width Between Mating Types 1A and 1B of *Zygnema circumcarinatum* Grown under Aeration with "Creeping" Filaments of 1A from the Sides of the Culture Vessel.*

Mating type	Source of material	Mean sheath width, μ (50-cell sample)
1A	Sides of flask (creeping)	9.5 ± .5
1A	Medium	8.8 ± .5
1B	Medium	8.3 ± .5

*Mean ± S.E.

The filament samples were taken from 1A and 1B filaments growing in the liquid medium for comparison with 1A filaments "creeping" on the sides of the flask.

Photomicrographs of significant morphological stages encountered during this investigation are shown in Figure 11. These photomicrographs show a typical vegetative filament mounted in India ink, conjugation with newly formed zygotes, mature zygospores, a zygospore germling, a typical akinete, and a parthenospore.

Figure 11. Morphological Stages in the Life Cycle of *Zygnema circumcarinatum*.

A. Mating strains 698 1A and 698 1B conjugating, showing zygospore formation between the filaments and the resulting empty gametangia. 478 X.

B. Mature zygospores following a two-month maturation period in the conjugation medium. 173 X.

C. Zygospore germling isolated at the five-celled stage. 123 X.

D. Typical vegetative filament mounted in India ink to delineate the mucilaginous sheath. 173 X.

E. Comparison of a mature zygospore with a cylindrical shaped asexual spore (akinete). 217 X.

F. Asexual parthenospore resulting from a gamete failing to escape from the gametangium during conjugation. 217 X.

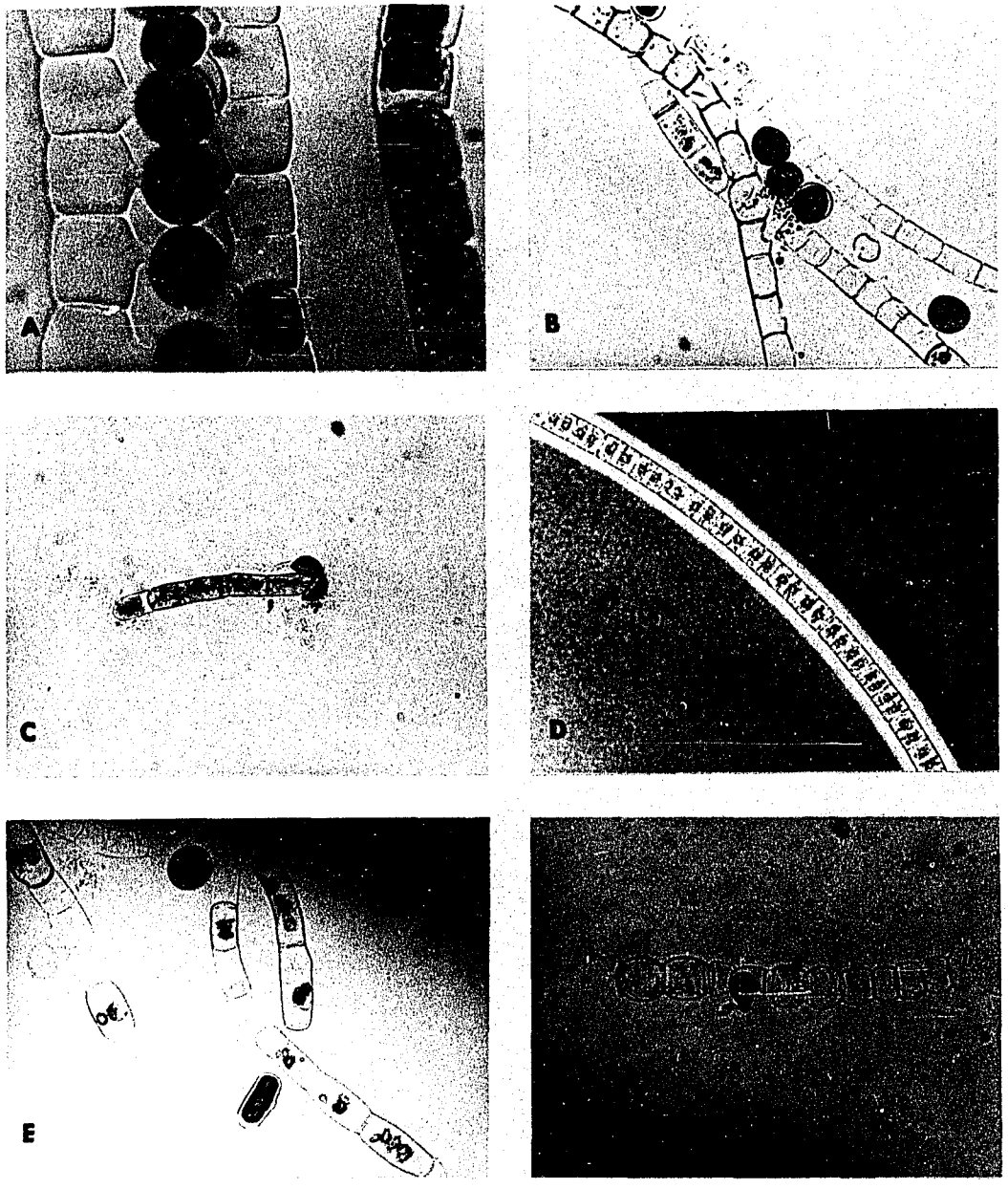

Figure 11. Morphological Stages in the Life Cycle of *Zygnema circumcarinatum*.

DISCUSSION

Genetic Analysis

Cultures of the filamentous, green heterothallic alga *Zygnema circumcarinatum* provide excellent experimental material for determining the genetic and physiological bases of morphology. Heterothallism, as far as is known in the Zygnemataceae, is of extremely rare occurrence. An understanding of the genetic basis of heterothallism can be used as a reference point for genetic mapping in this alga. In addition, this understanding could provide insight into physiological and genetic questions dealing with mode of mating-type recognition, mating-type inheritance, and physiological separation of mating types. Jost (1953) has shown that *Z. circumcarinatum* can be sexually induced under laboratory conditions, and Gauch (1966) further has shown that zygospores produced in the laboratory can be germinated and that offspring can be recovered. This knowledge was applied in this study to perform genetic backcrosses as a means to gain an understanding of the basis of heterothallism.

Heterothallism can be viewed as involving a single gene difference, two or more genes involved on the same chromosome, or two or more genes involved on different chromosomes. The genetic analysis of mating type by means of backcrossing F_1 progeny provides a means of viewing the relationship between the heterothallic mating types. The results of this study show that three classes of individuals, designated A, B, or AB, are detectable by genetic backcross (Table 1). The majority of individuals were assigned to one or the other of the parental mating types

(A or B) on the basis of mating compatibility. The presence of a smaller third class (AB), which mated with both parental types, points to the probable existence of a fourth undetermined class that could contain individuals genetically unable to mate with either mating type. Since only the ability to conjugate was detectable, it was impossible to determine whether these nonmating individuals were genetically incapable of conjugation. Attempts were made to induce conjugation in the nonmating clones and all of these failed. This suggests, but does not prove, the existence of the fourth class since lack of mating ability may be related to various factors, such as the internal physiology of the filaments or age of the cultures, and may not be due to strictly a genetic factor. For this reason the existence of the fourth nonmating class can only be inferred, and the number of individuals in this class, if produced, should approximate the number existing in the AB class.

In a haploid organism like *Zygnema* the occurrence of only two classes of offspring would provide evidence for heterothallism determined by one genetic factor. Similarly, if four classes were detected, this would suggest two or more loci being involved. Therefore, the observed and postulated existence of four classes of offspring indicates that mating type in *Z. circumcarinatum* is determined by two or more genetic factors. Since the ratio of the progeny departed significantly from a 1:1:1:1 ratio, it is suggested that the genetic factors involved are not segregating independently but are linked.

Thus the results suggest a hypothesis that heterothallism in this species is determined by two genetic factors, A and B. Each mating type possesses one but not both of these factors. Mating type 1A possesses a

functional A locus and a nonfunctional or impaired B locus. Conversely, mating type 1B possesses a functional B locus and a nonfunctional or impaired A locus. Both products of gene action (gene products) from a functional A and functional B locus must be present in the medium to induce conjugation.

The suggestion that these products are present in the medium rather than confined to the cell comes from the work of Hoshaw (1968). He observed that a supernatant from a conjugating mixed culture of *Z. circumcarinatum* could stimulate zygote production in a culture of a single mating type. This phenomenon can be viewed from the standpoint that in a conjugating mixed culture both mating types are present. Therefore, the present work suggests that genetic products A and B would be produced and released into the medium. A single mating type, when placed in the supernatant from such a conjugating culture, would be exposed to both gene products and thus would conjugate.

It is hypothesized that, during meiosis, genes usually segregate to perpetuate the two mating types. Occasionally a crossover occurs between the A and B loci, as shown diagrammatically in Figure 12. The crossover results in the production of individuals with either a functional A locus and a functional B locus or both a nonfunctional A locus and a nonfunctional B locus. Since only one of the products of zygotic meiosis survives in *Zygnema*, the probability of either of these recombinate individuals surviving is equal. Surviving functional AB individuals are able to induce conjugation with both of the parental lines. Nonfunctional ab individuals would be unable to mate with either parent since both gene products would not be present in the medium to induce conjugation.

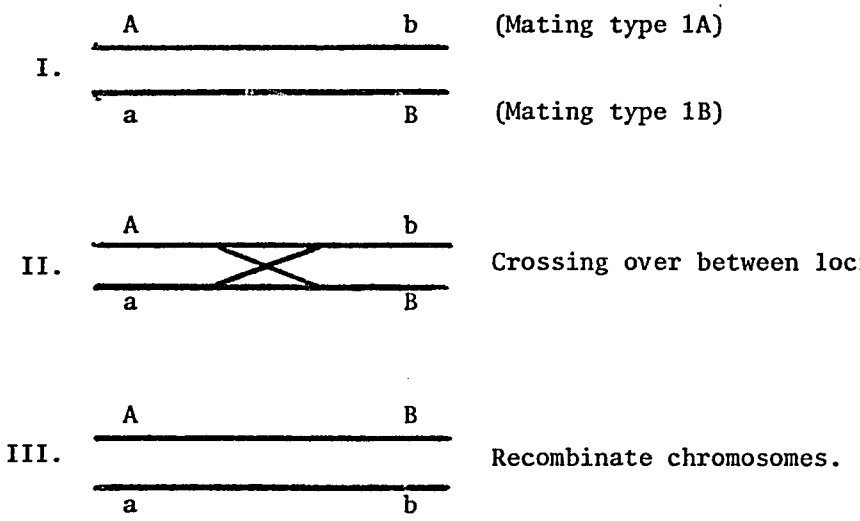

Figure 12. Hypothesized Mode of Origin of AB and Nonmating Clones.

I. Hypothesized parental genotypes in regard to heterothallism.
II. Crossing over between A and B loci during zygotic meiosis.
III. Recombinate chromosomes that can give rise to a nonmating individual (ab) or an individual capable of mating with both parents (AB).

Further support for such a hypothesis of heterothallism in *Z. circumcarinatum* has come from examination of the isolated clones GZ 16 and GZ 30, which are designated type AB. Clone GZ 30 was observed to be homothallic and conjugates with itself. This observation agrees with the hypothesis for heterothallism stated in the previous paragraphs because clone GZ 30 would be genetically able to elaborate both gene products A and B, making it capable of a homothallic response.

The two-gene theory of heterothallism proposed here for *Z. circumcarinatum* is not in agreement with the work on heterothallism in the Desmidiaceae and the Mesotaeniaceae. In *Cosmarium* (Starr, 1954), *Netrium* (Biebel and Reid, 1965), and *Closterium* (Lippert, 1967), heterothallism has been reported as a single gene difference. The data from the present study would be in agreement with data reported for other members of the Zygnematales except for the appearance of the two AB clones. Experiments were performed to prove that the AB clones were not contaminated with filaments of both mating types. To do this, unialgal isolates were made of both AB clones, and these isolates conjugated with both parents and thus confirmed the proposed AB genotype. This suggests the interpretation of heterothallism in *Z. circumcarinatum* as a phenomenon of two or more genes.

The GZ clones also were examined to determine the mode of inheritance of cell width (Table 2). It can be seen that the majority of the clones are grouped around the 23-24 μ interval and are distributed between 21 and 34 μ (Fig. 2). Both parental cell widths are within the 23-24 μ interval (Table 10), and thus the GZ clones are grouped around the parental values.

Two possible explanations are proposed for the distribution of the clonal widths around the parental values. The first of these proposes that the distribution of the clones is due predominately to genetic influence. Under this interpretation, cell width in *Z. circumcarinatum* would be determined by polygenes whose influence appears to be additive. A polygenic mode of inheritance would produce a distribution similar to the one shown in Figure 2 (Strickberger, 1968).

A second interpretation involves the influence of environment upon the clones. If the environment greatly influences cell width, a distribution similar to that shown in Figure 2 also would result even though width is not controlled by a polygenic mode of inheritance (Strickberger, 1968). Uspenskii (1934) noted that cell width in *Spirogyra* can be made to fluctuate appreciably above and below normal values by increasing and decreasing the growth rate. A difference in growth rate between the clones caused by some environmental influence could account for the observed distribution of cell widths in *Z. circumcarinatum*. However, all the clones were maintained under standard conditions in full strength Godward's medium and trace element solution and were simultaneously transferred to fresh medium. Since the clones were cultured under the same environmental regime, it is inferred that the environmental influences on the cultures were similar for all cultures. Because of this similarity of environment for all cultures, it is concluded that the distribution of cell width should be attributed predominately to the influence of polygenic factors. This interpretation agrees with Gauch (1966), who suggested that inheritance of width in *Zygnema* strains 921 and 922 was determined by two or more loci.

Cell width is an important taxonomic characteristic in the Zygnemataceae (Randhawa, 1959). The occurrence of a polygenic mode of inheritance in cell width for *Z. circumcarinatum* and in *Zygnema* strains 921 and 922 casts doubt on the validity of using cell width as a taxonomic character in these species and may well cast doubt on the use of cell width as a taxonomic characteristic for all members of the Zygnemataceae.

Three GZ clones analyzed during the cell-width study were significantly different from the clones grouped around the parental values. Of these, GZ 35 and GZ 55 were significantly different from GZ 47, and all three taken together constituted two additional groups. The two distinct groups represented by clones GZ 35, GZ 55, and GZ 47 are not thought to be members of the polygenic distribution of cell widths. These three clones are taken as exceptions and are inferred to be due to some as-yet undetermined influence. Polyploid series are known to exist in the Zygnemataceae (Allen, 1958). The existence of polyploidy in these three isolates of Z. *circumcarinatum* could provide a plausible explanation for these exceptional clones.

Manipulation of zygospore germlings to establish the GZ clones is a laborious task that is prerequisite to any genetic study. To minimize this task it is important to isolate germlings at the cell stage that will increase the probability of attaining viable clones. Results of the preliminary germling viability test indicate that the isolation of filaments composed of six cells or more is best for the establishment of clones (Fig. 1). Isolation of filaments at the six-celled stage also is easier than isolation of shorter filaments from the standpoint of actual manipulation and viewing of the isolated filaments to ensure unialgal cultures. Germling filaments of 20 cells or more are usually found free from the spore wall and gametangia, and are thuse useless for genetic purposes since the parentage of the filament is unknown.

It is noteworthy that germlings of three-celled length were of rare occurrence whereas germlings of one- two-, four-, and six-celled stages occurred frequently. This suggests that cell division is initially

synchronous, but synchrony begins to diminish after the third division. Jost (1953) has presented evidence for the existence of "hot spots" of division along mature filaments with resultant undulating movements of *Zygnema* filaments on agar. He related this phenomenon to lack of division synchrony along the length of the filament. It is interesting that this lack of division synchrony seems to appear as early as the third division of the germling.

Electrophoretic Analysis

Prior to electrophoretic analysis of protein content it was necessary to find a reliable means of protein extraction for *Zygnema*. Eight methods of extraction were tested (Table 3). The most efficient extraction was attained by cell disruption employing nitrogen in a Paar Disruption Bomb. This method was more efficient than disruption employing liquid nitrogen and grinding. However, either of these methods provided adequate cellular extracts for electrophoretic analysis. All other methods tested yielded extractable protein in quantities too small for use in electrophoretic analysis.

The electrophoretic separation of the protein extracts taken at 7, 14, and 21 days of the culturing cycle showed that at any one time the mating types differed regarding the number of isozymes present for a particular enzyme system (Figs. 3, 4, and 5). The only exception to this was seen in the 14th-day sample grown in full strength medium and run for acid phosphatase. At this time both mating types showed similar banding patterns in number and position of acid phosphatase isozymes.

It is difficult at this time to assign significance to the disappearance or occurrence of particular bands. What is significant is that the two mating types can be thought of as two physiologically separate and dynamic systems. Under the same growth conditions, these two systems elaborate electrophoretically different types and numbers of isozymes to catalyze what are inferred to be similar physiological responses to identical environments.

Transfer of filaments from full strength medium to dilute medium at 14 days with mixing of mating types induces sexual stages. Transfer of filaments to dilute medium without mixing of mating types was found to cause changes in both position and number of bands for the enzyme systems studied (Figs. 3, 4, and 5). Whether any of these transformations are directly related to the attainment of sexual potential observed in a mixed culture can only be inferred. However, it is important to note that in this transition both mating types continue to act as physiologically separate organisms.

The effect that the mixing of mating types has on culture physiology is shown in Figure 6. In two of the three systems studied, mixing of the mating types brought about a reduction in the number of isozymes present. Catalases were the only isozymes that increased in number when compared to comparably conditioned but unmixed (asexual) cultures.

To ascertain the possible significance of isozyme changes it was necessary to test the internal variability of these enzyme systems. This was done to determine whether the changes in isozyme patterns observed during the 21-day study were due to variability that exists daily within the isozyme population of the organism (Fig. 7). It was observed that

internal daily variability does seem to affect the position of a particular band but has nothing to do with the number of isozyme bands present. Therefore, any transition that is marked by the disappearance or new occurrence of a particular band should be looked upon with more significance than the mere shifting of position of a band.

Banding patterns of the mating types of Z. *circumcarinatum* varied from one time to another in unmixed cultures, varied in regard to different environmental factors (dilute versus full strength medium), and varied in regard to sexual versus asexual cultures. Since physiologically the mating types differed in isozyme patterns in regard to age, growth condition, and sexual versus asexual cultures, it is inferred that the mating types exemplify two dynamic systems. Further study should be directed to determine the exact alterations of protein populations in these two dynamic systems and relate these changes to specific morphological modifications of the species.

Since the mating types possess little isozyme pattern similarity during the culturing cycle, it seems unlikely that electrophoretic banding patterns can be used successfully as a taxonomic tool in Z. *circumcarinatum* to differentiate it from other species of the genus. However, it is possible to set forth strict culturing conditions that at a specific sampling time will provide banding patterns to distinguish the two mating types.

To obtain a valid basis for taxonomic distinctions based upon electrophoretic banding patterns it is important to sample algae at comparable stages of development. The evidence presented here indicates that differences exist throughout the vegetative and sexual portions of

the life cycle. The only life cycle stage that could be inferred to be physiologically stable enough for use as a point of reference is the resting zygospore. If reliable electrophoretic taxonomic data are to be obtained, it is likely they will come from analysis of zygospores. However, it is difficult, if not impossible, to obtain zygospores in large enough quantity for electrophoretic analysis.

Pigment Analysis

The pigment content of the mating types was investigated to see whether differences in these cellular components existed between the mating types. Thin-layer chromatography of the pigments showed no discernible difference in types of pigments present (Fig. 8). Quantitatively, however, there existed a difference in the amount of chlorophyll present per cell. Actively growing cultures of mating type 1A consistently contained more chlorophyll per cell than cultures of mating type 1B (Table 9). This difference substantiates the original observation of differences in culture coloration.

Morphological Analysis

Morphology has as its basis the genetic and physiological events of the cell. Within the cells of *Z. circumcarinatum* the genetic bases for sexual differentiation and cell width already have been discussed. The morphological parameters of cell-wall width, cell length, sheath width, and distance between pyrenoids were also investigated (Tables 10 and 11).

Although most of the parameters studied point to the mating types as morphologically identical, there were some obvious exceptions.

In dilute medium the two mating types were found to differ in cell length. Similarly, distance between pyrenoids differed as a result of the difference in cell length in dilute medium. An interesting difference also existed in cell width of both mating types grown in full versus dilute conditions. The increase in width in dilute medium could possibly be related to a slowed growth rate and could thus exemplify in *Zygnema* the phenomenon observed by Uspenskii (1934) in *Spirogyra*, where modification of cell width resulted from an alteration of growth rate.

Of most interest was the morphological comparison of the mucilaginous sheath that surrounds the filaments and gives them their slippery, "slimy" feel. Three authors (Tiffany, 1923; Smith, 1950; Yamashita, Takahashi, and Sasaki, 1968) have noted that the chemical and morphological constitution of the sheath varied with filament age. It was significant therefore to follow the formation of sheath through the organism's life cycle (Fig. 9). It was observed that both mating types undergo an increase in sheath width immediately upon transfer to fresh medium. The sheath width of mating type 1A increased more initially than 1B and remained wider until the 39th day. Of special interest is the reduction of sheath width that occurs after 14 days. This time coincides with the time at which the species reaches its greatest sexual potential. Older accounts have related continued sheath formation to prevention of attachment of epiphytes onto *Zygnema* in natural habitats (Tiffany, 1923). One can speculate that an increasing sheath also could prevent the attachment of the mating types prior to conjugation in axenic culture or in nature. If this is true, then it is only when the loss of sheath exceeds its synthesis that filaments become capable of conjugation.

Figure 10 shows the change occurring in sheath formation during sexual induction when the filaments are transferred from full strength to dilute medium on the 14th day. Sexual induction brings about an abrupt decrease in the sheath width of 1A. This decrease in sheath size can be related to sheath degradation exceeding production or the discontinuation of sheath production followed by continued degradation. It should be noted that sexual induction causes a decrease in sheath width to a point where the sheath width of both mating types is equal. This width approximates the decrease in sheath width attained in old filaments.

Yamashita et al. (1968) found that a decrease from 30% to 18% of total insoluble sheath polysaccharide occurs during sexual induction in *Spirogyra* sp. The results presented here for *Z. circumcarinatum* grown in axenic culture are in agreement with the observations of Yamashita and other investigators who have observed that members of the Zygnemataceae are usually free of epiphytes until either sexual induction or old age ensues. Older cultures (>40 days) of both 1A and 1B differed little in sheath width, and in these sheath width decreases to a minimum. However, it appears that mating type 1A undergoes the greater increase in sheath width of the two mating types.

The observations described relative to "creeping" are thought to be related to sheath formation. Filaments (1A) with the thicker sheath crept up the sides of the culture flask. Whether this is a cause or an effect is speculative. However, only mating type 1A was observed to creep. The width of the sheath associated with "creeping" in 1A is greater than the maximum sheath width ever observed for 1B (Table 11). Therefore, if creeping is dependent upon a minimum sheath width greater

than 8.8 μ and less than or equal to 9.5 μ (Table 12), the width for non-creeping and creeping 1A, respectively, then 1B would be unable to creep since its maximum observed sheath size of 8.8 μ was below the minimum hypothesized for creeping. Only one other account of creeping has come to this author's attention. This account (Langer, 1930) deals with creeping in *Spirogyra nitida*, in which erect tufts of filaments were formed in a moist atmosphere on agar. *Spirogyra* and *Zygnema* appear similar in regard to sheath morphology. Perhaps these two accounts are observations of related phenomena.

The mucilaginous sheath is a conspicuous morphological character in *Zygnema*, worthy of additional study. Isolation or production of a "sheathless" mutant of *Zygnema* might assist in the design of experiments on the significance of production and participation of the sheath in the life cycle.

Genetically in regard to mating type, physiologically in regard to pigment content and isozyme content, and morphologically in regard to sheath, the mating types of *Z. circumcarinatum* can be regarded as two distinguishable units. Hoshaw (1968) observed that 1A was the first of the mating types to show any morphological changes during conjugation and that 1A precedes 1B in the occurrence of every stage associated with conjugation. It appears therefore that certain genetic and physiological differences are associated with the separation of the mating types into reproductive units.

Future investigations of this genus will no doubt elucidate additional physiological and genetic differences between the mating types. Genetic methods are now available to investigate the bases of morphology

in the filamentous conjugating algae. The use of a genetic approach to developmental problems in *Zygnema* offers the best available method to date for elucidating the role of genetics and physiology on the morphology of this alga or any algal species in which a complete life cycle can be induced and in which detectable differences exist in morphology or physiology of the species to serve as genetic markers.

REFERENCES CITED

Allen, M. A. 1958. The biology of a species complex in *Spirogyra*. Ph.D. Dissertation. Indiana University, Bloomington, 240 pp.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts: Polyphenolexidase in *Beta vulgaris*. Plant Physiol. 24:1-15.

Baillie, A., and J. R. Norris, 1963. Studies of enzyme changes during sporulation in *Bacillus cereus*, using starch gel electrophoresis. J. Appl. Bact. 26:102-106.

Biebel, P. J. 1964. The sexual cycle of *Netrium digitus*. Am. J. Bot. 51:697-704.

Biebel, P. J., and R. Reid. 1965. Inheritance of mating type and zygospore morphology in *Netrium digitus* var. *lamellosum*. Proc. Pa. Acad. Sci. 39:134-137.

Brewbaker, J. L., M. D. Upadhya, Y. Makinen, and T. MacDonald. 1968. Isoenzyme polymorphism in flowering plants. III. Gel electrophoretic methods and applications. Physiol. Plant. 21:930-940.

Clare, B. G. 1963. Starch gel electrophoresis of proteins as an aid in identifying fungi. Nature 200:803-804.

Clare, B. G., and G. A. Zentmyer. 1966. Starch gel electrophoresis of proteins from species of *Phytophthora*. Phytopathol. 56:1334-1335.

Clay, W. F. 1972. Nucleic acid metabolism in *Chlamydomonas moewusii* G. Ph.D. Dissertation. University of Arizona, Tucson. 90 pp.

Coleman, A. W. 1961. The role of modern culture methods in the study of algal life cycles. Quart. Rev. Biol. 36:247-253.

Czurda, V. 1930. Experimentelle Untersuchungen über die Sexualitätsverhaltnis der Zygnemalen. Beih. bot. Zentralbl. 47:15-68.

Derbyshire, E., and B. A. Whitton. 1968. A disc electrophoretic study of proteins of blue green algae. Phytochemistry 7:1355-1360.

Gauch, H. G. 1966. Studies on the life cycle and genetics of *Zygnema*. M.S. Thesis. Cornell University, Ithaca, New York. 91 pp.

Godward, M. B. E. 1942. The life-cycle of *Stigeoclonium amoenum* Kutz. New Phytol. 41:293-301.

Hall, R. 1967. Proteins and catalase isoenzymes from *Fusarium solani* and their taxonomic significance. Austral. J. Bio. Sci. 20: 419-428.

Hall, R., G. A. Zentmyer, and D. C. Erwin. 1969. Approach to taxonomy of *Phytophthora* through acrylamide gel-electrophoresis of proteins. Phytopathol. 59:770-773.

Hilton, R. L. 1970. A cytological and morphological study of the alga *Spirotaenia condensata* Brébisson. Ph.D. Dissertation. University of Arizona, Tucson. 87 pp.

Hoshaw, R. W. 1968. Biology of the filamentous conjugating algae, pp. 135-184 *in* D. F. Jackson, ed. Algae, Man, and the Environment. Syracuse University Press, Syracuse, New York.

Jost, D. N. 1953. Studies on the vegetative growth and sexual reproduction of *Zygnema circumcarinatum* Czurda. Ph.D. Dissertation. Harvard University, Cambridge, Mass. 238 pp.

Klein, R. M., and D. T. Klein. 1970. Research Methods in Plant Science. The Natural History Press, Garden City, New York. 756 pp.

Langer, S. 1930. The creeping of filaments of *Spirogyra nitida*. Biol. Abstr. 7:2230. (Abstr.)

Lindsey, J. P. 1972. Protein differences in the vegetative and sexual stages of *Vaucheria sessilis*. M.S. Thesis. University of Arizona, Tucson. 50 pp.

Lippert, B. E. 1967. Sexual reproduction in *Closterium ehrenbergii*. J. Phycol. 3:182-198.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Macko, V., A. Novacky, and M. A. Stahmann. 1967. Protein and enzyme patterns from uredospores of *Puccinia graminis* var. *tritici*. Phytopathol. Z. 58:122-127.

Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. N. Y. Acad. Sci. 121:321-349.

Prasad, B. N. 1963. On conjugation in *Zygnema cruciatum* (Vaucher) Agardh. Phykos 2:19-21.

Prasad, B. N., and M. B. E. Godward. 1966. Cytological studies in the genus *Zygnema*. Cytologia 31:375-391.

Randhawa, M. S. 1943. A critical review of some recently created new species of Indian Zygnemales. Proc. Indian Acad. Sci. Sect. B. 18:73-81.

Randhawa, M. S. 1959. Zygnemaceae. Indian Council of Agricultural Research, New Delhi. 478 pp.

Raper, J. R. 1959. Sexual versatility and evolutionary processes in fungi. Mycologia 51:107-124.

Rosowski, J. R., and R. W. Hoshaw. 1970. Cultivation of a filamentous alga in quantity on agar plates. J. Phycol. 6:219-221.

Smith, G. M. 1950. The Fresh-Water Algae of the United States. 2nd ed. McGraw-Hill Book Co., Inc., New York. 719 pp.

Solomon, E. P., E. M. Johnson, and J. H. Gregg. 1964. Multiple forms of enzymes in a cellular slime mold during morphogenesis. Devel. Biol. 9:314-326.

Starr, R. C. 1954. Inheritance of mating type and a lethal factor in *Cosmarium botrytis* var. *subtumidum* Wittr. Proc. Nat. Acad. Sci., U.S. 40:1060-1063.

Starr, R. C. 1964. The culture collection of algae at Indiana University. Am. J. Bot. 51:1013-1044.

Strickberger, M. W. 1968. Genetics. The Macmillan Co., New York. 868 pp.

Taft, C. E. 1944. New species of Zygnemataceae. Ohio J. Sci. 44:238.

Thomas, D. L., and R. M. Brown. 1970. New taxonomic criteria in the classification of *Chlorococcum* species. III. Isoenzyme analysis. J. Phycol. 6:293-299.

Tiffany, L. H. 1923. A physiological study of growth and reproduction among certain green algae. Ph.D. Dissertation. Ohio State University, Columbus. 99 pp.

Tiffany, L. H. 1926. The filamentous algae of northwestern Iowa. Trans. Am. Microsc. Soc. 45:69-132.

Transeau, E. N. 1934. The genera of the Zygnemataceae. Trans. Am. Microsc. Soc. 53:201-207.

Transeau, E. N. 1951. The Zygnemataceae. Ohio State University Press. Columbus. 327 pp.

Uspenskii, E. E. 1934. Transformation of certain hereditary forms into other ones and the arising of new forms in the genus *Spirogyra*. Biol. Abstr. 11:724. (Abstr.)

Yamashita, T., S. Takahashi, and K. Sasaki. 1968. Biochemical, histochemical, and structural changes during conjugation process of *Spirogyra*. Bot. Mag., Tokyo 81:210-218.