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Meiosis, Blade Development and Sex-Determination in *Porphyra umbilicalis* (L.) J. Agardh from Avonport, Nova Scotia Canada.

by

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Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

August, 1991

ISBN 0-315-71481-6

To my parents Marilyn and Robert Mitman, and my wife Kimberley.

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ABSTRACT

Recently, conclusive evidence of sexual reproduction, and the discovery that meiosis occurs in the germinating conchospores for several species of *Porphyra* C. Ag. has renewed interest in studying this genus. The intriguing sexually divided morphology of *Porphyra umbilicalis* (L.) J. Agardh from the mudflats of Avonport, Nova Scotia, prompted me to hypothesize that sex expression in *P. umbilicalis* and other striped *Porphyras* may result from genetic segregation of a pair of sex-determining alleles during early sporeling development, presumably at meiosis.

The present thesis constitutes an investigation of this hypothesis using *P. umbilicalis*. Meiosis in the germinating conchospore resulting in a meiotic tetrad was confirmed by karyological observations. Second, stable color mutants of *P. umbilicalis* were used as genetic markers demonstrating that the bulk of the mature blade is derived from the two anticlinally divided upper cells of the germinating sporeling. Last, by comparing cell lineage boundaries laid down by the segregation of color mutations at meiosis with those defined by sexual differentiation on the same fronds, strong evidence is given that sex-determination is also controlled by segregating genetic factors with no genetic linkage between any of the color mutations and the putative sex-determining alleles.

ACKNOWLEDGEMENTS

I would like to thank Dr. John van der Meer for his constant encouragement and advice throughout this study. Thanks are also due to Jane Osborne for her help in the lab.

Laboratory facilities were provided by the Institute of Marine Biosciences, National Research Council of Canada.

Special Thanks go to my wife Kimberley for her patience, enduring constant neglect, and many missed vacations as I completed my Thesis.

Finally, much thanks go to Dr. Harry K. Phinney for getting me interested in phycology in 1978 on the Oregon coast, and changing my life forever.

I. INTRODUCTION

Porphyra, a genus in the Bangiales includes 60-70 reported species which range from tropical to polar seas throughout the world (Cole 1991). Its life cycle is usually a biphasic alternation of generations between macroscopic gametophytic blades and microscopic sporophytic conchocelis filaments. Gametophytes are seasonal, occur as mono- or distromatic blades, and are anchored by a rhizoidal holdfast. This macrophyte grows primarily in the intertidal zone (Bold and Wynne 1985), although some species do occur in deeper water and may grow to 75 cm in length. The conchocelis occurs as pink anastomosing filaments either shell-boring or epilithic, growing throughout the year. Four species of *Porphyra* are reported to occur in the Maritimes: *P. leucosticta* Thur in Le Jol., *P. linearis* Grev., *P. miniata* (C. Ag.) C. Ag., and *P. umbilicalis* (L.) J. Ag. (South 1976). In addition to these, however, there are several poorly classified forms of *Porphyra spp.* that may fit into one of the above species or be entirely different.

The genus *Porphyra* has always interested phycologists, since Kathleen Drew-Baker's discovery, in which she established that the

previously classified *Conchocelis rosea* Batt. was actually the sporophyte stage in the life history of *Porphyra* (Drew 1949). In addition, it was only recently that Hawkes (1978) provided elegant documentation of sexual reproduction through use of light and electron microscopy. Until this report, the union of gametes was assumed, without proof, and remained controversial. As a result of these observations, other phycologists began a re-examination of other species.

Wang and Xu (1984) observed cell fusion of the spermatium to the prototrichogyne, and also observed degenerating chloroplasts in the fertilization canal of *P. haitanensis* T. J. Chang et B. F. Zheng. In addition, Cole and Conway (1980) observed nuclear fusion in eight species of *Porphyra*: (*P. abbottae* Krish., *P. kanakaensis* Mumford, *P. miniata*, *P. nereocystis* Anders, *P. perforata* J. Ag., *P. pseudolanceolata* Krish., *P. schizophylla* Hollb., and *P. variegata* (Kjellm.) Hus), and also acknowledged observations of sexual reproduction made by other workers: *P. tenera* Kjellman (Tseng and Chang 1955a, 1955b; Yabu 1969; Kito 1978), *P. yezoensis* Ueda (Yabu and Tokida 1963; Migita 1967; Kito 1978), *P. purpurea* (Roth) C. Ag. (Giraud and Magne 1968), *P. gardneri* (Smith et Hollenberg) Hawkes (Hawkes 1978), *P. nereocystis*

Anders (Hawkes 1978), and *P. pseudolinearis* Ueda (Kito 1978).

The site or temporal position of meiosis in the life history remains controversial and has long eluded phycologists. This is principally due to four factors: small chromosome size, inadequate staining techniques, poor photographic documentation, and inaccurate interpretation because of the first three factors. In spite of this, many researchers were certain they had determined the correct site of meiosis; unfortunately, not everyone agreed on the same site.

There were basically four schools of thought:

1. Meiosis occurred in the first division of the fertilized carpogonium (Ishikawa 1921; Dangeard 1927; Tseng and Chang 1955a, 1955b);
2. Meiosis occurred in a conchosporangial branch during conchospore formation (Migita 1967; Giraud and Magne 1968; Kito 1974, 1978);
3. Meiosis did not occur at all in some species and the life cycle was apomeiotic and apogamic with an alternation of generations (Krisnamurthy 1959; Conway and Cole 1973, 1977; Coll and Oliveira 1977, 1989; Freshwater and Kapraun 1986); and
4. Meiosis occurred in the germinating conchospore (Miura and coworkers, reported by Miura and Merrill (1982); Ma and Miura 1984;

Ohme *et al.* 1986; Burzycki and Waaland 1987; Ohme and Miura 1988; Tseng and Sun 1989).

Recently, various researchers have helped to resolve this dilemma. During their studies with natural color mutants of *P. yezoensis*, Miura and Kunifuji (1980) observed that heterozygous conchocelis from crosses between different color mutants produced many blades that were color chimeras. They initially hypothesized that this might be due to meiosis occurring during conchospore formation, resulting in abnormal multinucleate spores. However, this explanation was inadequate, because it could not account for the high frequency of sectored plants. Miura and coworkers, reported by Miura and Merrill (1982), soon after established that the explanation for these results was that meiosis occurred in the germinating conchospores, and the chimeras were a result of the four-celled sporeling being a meiotic nuclear tetrad. Since then, meiosis in the germinating conchospores has been established or confirmed for *P. yezoensis* (Ma and Miura 1984; Ohme and Miura 1988; Tseng and Sun 1989), *P. torta* (Burzycki and Waaland 1987), and *P. tenera* (Tseng and Sun 1989). All of these reports support the view that meiosis occurs in the germinating conchospores, and this may prove to be the case for

many other species of *Porphyra*.

Spore terminology for the Bangiophycidae is in transition. Unlike the Florideophycidae which have specialized reproductive structures, the Bangiophycidae have very minor differences between vegetative and reproductive structures (Dixon 1973). Also, the terminology used to describe the life cycles is borrowed from other algal groups as well as mixed with terms unique to this subclass. For instance, when encountering the term "monospore", it is often difficult to ascertain whether the spore description refers to asexual or sexual spores, even after careful analysis of the text. In this thesis I will be using the terminology for spore types as proposed by Guiry (1991) which is both clear and precise.

Drew (1956) based spore classification in the Bangiophycidae on three different spore types :

Type I-formation of monospores from differentiated sporangia;

Type II-formation of monospores from undifferentiated vegetative cells; and

Type III-formation of a number of spores by successive divisions of a mother cell.

This classification was based on sporangial developmental patterns and spore form. Guiry (1991), however, reclassifies these previous three types of spores into four different types by also considering spore origin. According to Guiry (1991), type III spores from Drew's (1956) classification should first be subdivided into two categories: asexual and sexual spores.

Asexual spores are formed as the products of vegetative cells, and endospores are the only representative derived from type III. These spores regenerate the same phase and result from consecutive internal mitotic cell divisions within the sporangial walls. The second category is sexual spores. These spores develop as the products of a sexual fusion. There are two types: (1) zygotospores (formerly carpospores) - spores which are a result of fertilization and subsequent mitotic cell divisions to form spores within the carpogonial wall; and (2) carpospores - spores which are also a result of sexual fusion, but spore formation is external to the carpogonium. The only example of this second type in the Bangiophycidae is *Rhodochaete parvula* Thuret.

Finally, there are two additional asexual spore types. First, and most confusing is the term "monospore". This word has been used by

phycologists to include just about anything "spore-like" from spermatia to contaminating cyanobacteria. Guiry (1991) clarifies the term by defining monospores as mitospores produced in either the gametophyte or sporophyte which will regenerate the like phase, and having only one spore formed per sporangium as a common feature. By definition this also includes the term "aplanospores" as used by other researchers (Guiry 1991). The last of the four spore types in the Bangiophycidae is the conchospore. These are rectangular, deeply pigmented cells produced in branches of the conchocelis phase in some members of the Bangiophycidae. Conchospores, germinate into the gametophyte (blade) phase.

Very little solid information is available for species of *Porphyra* in the Maritimes except for *P. linearis* which was briefly studied by Bird *et al.* (1972), and Bird (1973). Also, Yabu (1978) determined the haploid and diploid chromosome numbers of *P. leucosticta, P. umbilicalis P. linearis,* and an unclassified species, *Porphyra sp.*, for the Maritimes, but there were uncertainties in specimen identification even for the named entities (Yabu 1978).

The species I chose for my study was tentatively identified as *P. umbilicalis* (*in herb.* NRCC No. 10599) which grows on the mudflats near Avonport, Nova Scotia, at the head of the Bay of Fundy. Although this plant has been classified in Nova Scotia as *P. umbilicalis* it may actually prove to be a new or different species and should be further studied taxonomically. The gametophytic blade is found growing epilithically on small stones imbedded in the mud and on sandstone outcroppings, and often grows to a length of 0.5 m. It is abundant from August through October, and the conchocelis (thin form) is found growing epipelically between the loose sandstone and mud particles during the entire year.

The gametophytes occur as monoecious distromatic blades with usually only one stellate chloroplast per cell. Monospores have not been observed from either the conchocelis or gametophytes. The thallus has an interesting longitudinally divided morphology. As blades mature, they display a reddish-brown male half and a greenish-brown female half, with a clear line of demarcation. This intriguing divided morphology prompted me to hypothesize that if meiosis takes place in the germinating conchospore, sex expression in *P. umbilicalis* and other striped species

may result from genetic segregation of a pair of sex-determining alleles during early sporeling development, presumably at meiosis. The present thesis constitutes an investigation of this hypothesis.

The objectives of the research were fourfold. First, to make karyological observations and determine if meiosis occurs during conchospore germination. Second, to generate and characterize stable color mutants of *P. umbilicalis* to be used as genetic markers. Third, to trace the fate of individual cell lineages in the developing blade using these mutants as tissue markers. Last, by comparing cell lineage boundaries laid down by the segregation of color mutations at meiosis with those defined by sexual differentiation on the same fronds, to ascertain if sex determination was also controlled by segregating genetic factors. In connection with the last point, a sub-objective was to determine if there was genetic linkage between any of the color mutations and the putative sex-determining alleles.

II. MATERIALS AND METHODS

A. Culture Conditions

Plants of *Porphyra umbilicalis* were collected from mudflats at Avonport, Nova Scotia (Figure 1) and transported back to the Institute for Marine Biosciences (IMB) laboratory in Halifax for processing. This involved the separation and scoring of spermatangial, carpogonial and monoecious thalli. Zygotosporangial sectors were subsequently dissected from monoecious and carpogonial thalli and used for the initiation of conchocelis cultures using the following procedure. The plant sectors were cleaned of any visible epiphytes by brushing, followed by a rinse (30 seconds) in tap water. Sectors were wrapped in paper towels, placed in a polyethylene bag and held at 5 °C overnight as a pretreatment to promote release of zygotospores. The following day, individual sectors were transferred to polystyrene petri dishes (Labtek #4026, 100 x 25 cm) half-filled with GeO_2 medium to prevent diatom growth (McLachlan 1973). Thalli were maintained at 15 °C with a photoperiod of 16:8 h

L:D under cool-white 40W fluorescent lights at an approximate photon fluence rate of 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$. Over the next few days, these cultures were checked periodically for released zygotospores. Conchocelis cultures were initiated from zygotospores collected by means of a pasteur pipet with a drawn capillary tip to reduce contamination from epibionts such as microalgae and protozoans. A few zygotospores were transferred to individual petri dishes with GeO_2 medium and returned to the culture chamber.

After two weeks the GeO_2 medium was replaced with modified D-11 medium (Table 1) (Chen 1988) and the dishes were returned to the same culture chamber. Modifications to the original D-11 medium formula were made as shown in Table 1. For 20 L of modified D-11 medium the specified amount of stocks were added, one at a time and in order, to 20 L of seawater with continuous mixing by aeration. Then, medium was sterilized by filtration through separate 0.30 μm and 0.22 μm Millipore filters.

After about one month, when conchocelis had grown to a size of 2 mm or more in diameter, fragments were transferred to individual petri dishes containing sterile filtered SWM-3 [without added soil or liver

extract, and without tris buffer (McLachlan, 1973)] or D-11 medium to continue growth. Conchocelis cultures were maintained under growth conditions as previously described, with changes of medium every two weeks. When clumps of conchocelis had grown to at least 5 mm in diameter, some of them were removed for conchospore induction experiments. The remainder were maintained in culture as stocks.

Nine different induction combinations were tested; three temperatures (10, 13, and 15 °C) with 8:$\overline{16}$, 12:$\overline{12}$ and 16:$\overline{8}$ h L:D photoperiods at an approximate photon fluence rate of 40 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} as measured by a quantum meter (Tables 2 and 3). Microscopical observations were made several times per week to determine the suitable conditions for conchosporangial production, and release of conchospores (Tables 2 and 3). After determining these conditions, conchocelis filaments ($\approx$5 mm diameter balls) were transferred to small plastic polystyrene dishes (Corning #2500, 35 x 10 mm) containing modified D-11 or SWM-3 medium. Each of these small dishes contained a glass ring, which positioned the conchocelis over a coverslip on the bottom of the dish (Figures 5 and 6). Germinating conchospores attached to the coverslips and could easily be collected. Before use, both rings and

coverslips were washed in 95% ethanol, 5.0% HCl in distilled water, and washed several times in distilled water to remove any residues which might be harmful to the developing spores. They were also autoclaved before use, as was all labware, to prevent contamination.

Coverslips with attached conchospores were collected daily and transferred individually to larger petri dishes with modified D-11 or SWM-3 medium. The sporulating conchocelis was transferred to new, sterile, petri dishes with glass rings as previously described. The subcultures of germinating zygotospores were observed and photographed daily using an inverted microscope (Olympus IMT-2) to determine early developmental growth patterns. Also, cultures were examined and photographed using differential interference contrast (DIC) illumination on a Reichert Polyvar research microscope.

The developing blades were cultured at 15 °C with an 16:8 h L:D photoperiod, at an approximate photon fluence rate of 40 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}, and the medium was changed every two weeks. When blades attained a length of 1 cm or more, some were transferred to larger containers to grow to reproductive maturity. The plants were cultured in 8 L aspiration bottles (Canlab B7581-8L) at 15 °C with a 16:8 h L:D

photoperiod at an approximate photon fluence rate of 60 μmol $\cdot$ $m^{-2} \cdot s^{-1}$, changing the medium, and autoclaving the bottles, every two weeks. Some *P. umbilicalis* blades were grown to reproductive maturity during the summer months in 23 L buckets of flowing seawater at 15 °C in a greenhouse at the IMB Aquaculture Research Station, located near Sambro, Nova Scotia. These plants were supplemented with additional nitrogen (1.0 mM) and phosphate (0.1 mM), and agitated by aeration from the bottom of the bucket as previously described by van der Meer and Chen (1979).

Crosses were performed by excising the appropriate male and unfertilized female sectors of wild-type (wt) or mutant plants. These sectors were then co-cultured under the same conditions as for plants grown in the 8 L aspiration bottles until zygotosporangia appeared on the female plant. Female plants were then removed to separate petri dishes and cultured under the same photoperiod and temperature conditions, but at an approximate photon fluence rate of 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$. Plants were checked weekly, changing media until zygotospores were released. Spores were then pipetted into separate petri dishes and cultured (modified D-11) into reproductively mature conchocelis as previously

described. If female sectors did not release zygotospores, they were maintained in culture, until the zygotospores eventually germinated *in situ* forming conchocelis that could be subcultured. Conchocelis from crosses was induced to release conchospores from conchosporangia, as previously described. Spores were allowed to develop into small blades which were then classified for genetic analysis.

B. Chromosome Staining

Spermatangia, vegetative cells and conchocelis of *P. umbilicalis* were all fixed prior to staining. Samples were placed in 2 parts 95% ethanol:1 part glacial acetic acid for 12-24 hours. Afterwards, they were transferred to 75% ethanol and stored in a refrigerator (at least 24 hours) at 8 °C until required for staining.

Three different procedures were tested for staining chromosomes of *P. umbilicalis*. In the first two methods, chromosomes were stained by modified procedures after Austin (1959) using acetocarmine, and after Wittman (1965) using iron-hematoxylin-chloral hydrate. Chromosome

counts were conducted using both staining procedures without prior dehydration of material from storage. In addition, material was gently squashed with a wedge-shaped plastic rod following staining.

The third method of staining used the fluorescent dye DAPI (4',6-diamidino-2-phenylindole, Sigma # D 1388). The material was first rehydrated (75, 50, 30, 15% ethanol up to distilled water), and stained in 50 μg/mL DAPI in McIlvaine's Buffer, pH 4.0 for 10 minutes. It was left in the buffer to destain for one hour. Samples were then mounted in the buffer and examined (modified from Hull 1982).

Stained cells were examined and photographed under oil immersion with a Zeiss Universal research microscope using the Neofluar 63X oil, 1.25 N.A., and Neofluar 100X oil objectives, with a tungsten-halogen illuminator. DAPI stained material was examined with a mercury arc epi-illuminator with the 48 77 15 Zeiss filter set. Photographs were taken using Ilford XP1 400 film by a Mamiya 645 1000S medium format camera adapted to this microscope.

C. Mutagenesis

Three different chemical mutagens were tested for their effectiveness in inducing colour mutants in *P. umbilicalis*: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and N-methyl-N'-nitro-N-nitrosoguanidine (NG) (Sigma M7629). The following concentrations of each were used: EMS (0.2 M and 2.5 M); MMS (0.025 M and 0.25 M); and NG (25 $\mu g \cdot mL^{-1}$ and 50 $\mu g \cdot mL^{-1}$). All three mutagens were used at the various concentrations dissolved in SWM-3 medium at room temperature. NG, however, was first mixed as a concentrated stock at 2.5 $mg \cdot mL^{-1}$ in 160 mL of 0.02 M citrate buffer at pH 5.0 (160 mL 0.1 M $H_3C_6H_5O_7 \cdot H_2O$, 260 mL 0.1M $Na_3C_6H_5O_7 \cdot 2H_2O$, 1680 mL H_2O) under dim light in a fume hood, and stored frozen at -15 °C in 2.0 mL aliquots.

Mutagens were applied to *P. umbilicalis* in three ways. In the first method, pieces of thallus approximately 1 cm^2 were very finely minced on a piece of Plexiglass with 20 single edged razor blades held tightly together. The resulting fragments were then transferred to a petri dish and treated with mutagen solution. Each concentration of the three

different mutagens was applied for a variety of treatment times from 0 to 90 minutes. After treatment, *Porphyra* fragments were washed by decanting and replacement three times with SWM-3 medium. They were then cultured at 15 °C with an 8:$\overline{16}$ h L:D photoperiod, at an approximate photon fluence rate of 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$, with renewal of medium (SWM-3) every week. The growing fragments were examined periodically, and mutant sectors with altered color were excised and maintained in culture under the same conditions.

For the second method, recently germinated conchospores or young haploid sporelings, consisting of a few to several cells attached to coverslips, were treated as in the first method. They were cultured and later examined for color mutant blades or sectors. In the last method, conchosporangial conchocelis was treated with mutagens. Small tufts of conchocelis approximately 5 mm in diameter were treated as in the first two methods. The tufts were then washed by decanting 3 times in SWM-3 medium and cultured under conditions promoting conchospore formation and release (13 °C with an 8:$\overline{16}$ h L:D photoperiod, at an approximate photon fluence rate of 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$) in small dishes with glass rings and coverslips. The medium was changed weekly and

coverslips with released conchospores were removed to larger dishes. As these spores developed into blades, any mutant plants or plants with mutant sectors were transferred to separate dishes and grown to maturity (using the same culture conditions as for the blades).

III. RESULTS

A. Culture Conditions

1. Conchocelis

Conchocelis was maintained in culture between 13-15 °C with 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ of cool white fluorescent light at a 16:8 h L:D photoperiod. Temperatures of 20 °C and 10 °C were tested, but 20 °C caused cultures to die, and 10 °C slowed growth. Light intensities higher than 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ caused conchocelis to form compact clumps that grew poorly, and lower light intensities slowed growth.

Conchocelis germinated from zygotospores as very thin filaments (< 5 μm), but within 2-3 weeks, thicker filaments (10-15 μm) characteristic of early conchosporangial production developed (Figure 3). This thicker "conchosporangial" morphology persisted under the standard growing conditions (13-15 °C at 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ of cool white fluorescent light and a 16:8 h L:D photoperiod). It did not revert back to

the thinner vegetative filaments, unless light and temperature were greatly reduced (0-5 °C at 5 μmol · m^{-2} · s^{-1} of cool white fluorescent light at 8:16 h L:D photoperiod). The thin form of conchocelis grew very slowly under these conditions, small clumps taking several months to attain a size of 1 mm in diameter. The thick form of conchocelis, under standard culture conditions grew somewhat faster, with clumps attaining 1 mm or more in diameter after one month.

Two important aspects of the culture work were to determine suitable conditions for inducing formation of conchospores (Table 2), and to determine conditions for regulating release of conchospores from the conchocelis. This information was important because substantial conchospore formation and release could occur as early as two weeks following the germination of zygotospores, whereupon the small conchocelis clump became so decimated that it died (Figure 4). For mature larger conchocelis at 15 °C, only about a one week induction period was required for sporulation. A reliable, continuous supply of conchospores was necessary for mutagenesis experiments, studies of early developmental patterns, chromosome studies to determine the position of meiosis in the life cycle, and genetic analyses of F_1 gametophytic blades.

A good regime for conchospore release was determined to be 13 °C with 40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ of cool white fluorescent light at a 16:$\overline{8}$ h L:D photoperiod (Table 3). A small amount of agitation and moderate temperature increase seemed to stimulate release. This was supplied while carrying cultures from the incubator to the microscope. Although some conchospore release occurred at other conditions, the releases were sporadic and the numbers of spores released were low (Table 3).

2. Blades

Suitable culture conditions for blade growth, carpogonia formation, spermatangia formation, fertilization, and zygotosporangia formation were determined to be 15 °C with a 16:$\overline{8}$ h L:D photoperiod at an approximate photon fluence rate of 60 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ of cool white fluorescent light. Blades grew between 10-20 °C, but growth was slower at the upper and lower extremes of this temperature range. Vegetative growth of blades also occurred in photoregimes of 8:$\overline{16}$ and 12:$\overline{12}$ L:D; however, growth

was slower, and blades never became sexually mature. Blades grew vigorously at 15 °C under natural light and long days (> 16:8 h L:D) in bucket cultures at the IMB Aquaculture Research Station and quickly reached sexual maturity. This was invaluable for the purpose of scoring sectors for genetic analysis. Unfortunately, such plants harboured too many epibionts to be reestablished in the laboratory for controlled crosses.

3. Life Cycle

Completion of the life cycle in culture required a minimum of 9-12 months. Conchospores, settled on coverslips, required 2-3 months in culture to grow into small blades. At a length of approximately 1 cm they were transferred to 8 L aspiration bottles to continue growth to sexual maturity. The small blades required another 2-3 months of growth in culture before a cross could be attempted. After fertilization of carpogonia and subsequent development of zygotosporangia, the zygotospores sometimes were not released from the blade for six months or more. It appeared that the release process did not always work

properly in culture, with the blades sometimes having to deteriorate before the spores were released. Finally, conchocelis usually required about three months of vegetative growth before conchospores could be collected without totally losing the filaments to sporulation. Thus, the time required to complete the life cycle (conchocelis to conchocelis) in culture was usually about nine months to one year. Completion of the life cycle generally took even longer for color mutants, especially if they were slow-growing. For instance, crosses involving the red mutant took nearly two years to produce next generation of conchocelis.

B. Nuclear Division and Chromosomes

1. Chromosome Stains

Staining *P. umbilicalis* with acetocarmine proved to be ineffective for visualizing nuclei in any cell type of the life history. On the other hand, Wittman's iron-hematoxylin-chloral hydrate and DAPI were both quite useful. Wittman's was reliable for staining blade material, in particular developing spermatia. The mitotic figures appeared dark and

distinct on a clear background of cytoplasm. Unfortunately, when cells of the conchocelis and germinating conchospores were stained, the cytoplasm became so dark that chromosomes could not be distinguished. However, nuclei of these two cell types fluoresced bright bluish-white following staining with DAPI. There was some interference from chloroplast fluorescence, but in most instances chromosomes were quite distinguishable from the cytoplasm and organelles (Figures. 11-34). Cells from vegetative blades, spermatia, vegetative conchocelis and germinating zygotosporangia are represented in this study.

2. Chromosome Analysis

In *P. umbilicalis* gametophytes, five chromosomes were observed in various stages of nuclear division (Figures. 1-15). Mitotic figures were regularly observed in the rapidly dividing cells of the developing spermatangia, independent of the time of fixation (Figure 11). Occasionally, mitotic figures were also observed in the vegetative cells of blades. In contrast, meiotic figures in germinating conchospores were

abundant only when material was fixed in late afternoon. Ten chromosomes were counted in nuclear divisions of conchocelis filaments, but observations of dividing nuclei were rare.

Many cytological observations were made for the germinating conchospores of *P. umbilicalis* using the fluorescent dye DAPI. It was determined that the germinating conchospores are the site of meiosis. This was concluded primarily from the observations described below.

The nuclei of the conchosporangia and recently released spores were in interphase and contained a large central nucleolus (Figure 13). Chloroplast DNA in these cells was often observed as blue-white circular rings (Figure 12). Nuclear division did not begin until the spore attached to the substrate and produced a germination tube (Figure 3). Subsequently, meiosis I was initiated and was coincident with the first periclinal division of the cell. Initial karyokinesis commenced with early meiotic prophase; the nucleus migrated to a central location in the cell and the nuclear membrane dissipated (Figure 14). This permitted the nucleoplasm to expand into the cell as the chromatin condensed and became granular (Figures 15-16). Next, during the zygotene stage, the chromatin condensed even further and five vaguely defined chromosome

bodies began to become visible (Figure 17). As the cells entered the diplotene stage, five discrete chromosome bodies were clearly apparent (Figure 18). In diakinesis, the chromosomes became highly condensed, virtually spherical in shape, and the double nature of the bivalent chromosomes was evident (Figures 19-21).

Observations were made of metaphase I (Figures 22 and 23), and at anaphase I the chromosomes separated into two sets of five which migrated to opposite ends of the cell (Figures 24-26). During late meiotic telophase I, one set migrated to the base of the germination tube, whereas the other migrated to the apex of the cell (Figure 27). Subsequently, a periclinal cytokinesis occurred, the chromosomes dispersed during interkinesis, and the germling had two interphase nuclei (Figure 28). At this point, meiosis II proceeded. Prophase II (Figure 29) could begin in either of the two cells; however, the upper cell usually divided before the lower (basal) cell (Figure 30). Subsequent stages of meiosis II followed: metaphase II in both cells (Figure 31), and anaphase II in both cells (Figure 32). In the upper cell, the nuclei usually migrated to opposite sides of the cell, followed by an anticlinal cytokinesis (Figure 33). Rarely, the nuclei migrated to the top and bottom of the cell and

was followed by a periclinal division (Figures 35 and 36: B"). In some sporelings, meiosis II did not occur in the nucleus of the basal cell or it was delayed (hours to weeks). When division of the basal cell occurred, the nuclei migrated to opposite ends of the cell; one migrated down into the germ tube, whereas the other migrated towards the upper cell, followed by a periclinal cytokinesis (Figure 34). When both the upper and lower nuclei of a two celled sporeling immediately completed meiosis, the resulting four nuclei and cells constituted a meiotic tetrad.

C. Mutagenesis

1. Mutagens and Mutagen Treatment

Mutagenesis treatments with EMS and MMS on germinating conchospores, minced blades and conchocelis proved to be ineffective. *P. umbilicalis* yielded no mutants even though time/concentration combinations were applied to the point of lethality. Callus-like growths grew from the minced blades, but this was due exclusively to the mechanical disruption of the blade's normal development, not

mutagenesis, because the same results were obtained for minced, non-mutagenized blades grown in culture as controls.

NG proved to be a more potent mutagen for *P. umbilicalis*, yielding a small number of mutants. Mutagen treatments at 50 $\mu g \cdot mL^{-1}$ for 45 minutes were determined to be effective for minced blades. Milder treatments with NG (25 $\mu g \cdot mL^{-1}$ for 0-90 minutes) produced no mutants in these trials. At 50 $\mu g \cdot mL^{-1}$, the maximum number of mutants was detected for 45 minute treatments, with fewer mutants found for 30 and 60 minutes. Below 30 minutes no color mutations were observed and treatments exceeding 60 minutes were lethal. The time/concentration combination found to be optimum in these trials was used for all subsequent treatments to obtain mutants.

2. Outcome of Mutagenesis Treatments

About one month after the mutagen treatment, sectors or small groups of cells having mutant color began to appear within, or growing out from, the small tissue fragments. The mutant sectors were grown to

a size of several millimeters, and then excised from the surrounding tissue with a scalpel. Any remaining wild-type tissue was carefully trimmed from the mutant sectors as growth continued. The majority of mutant sectors grew slowly and without the trimming were quickly overgrown by the surrounding normal tissue. Unfortunately, these color mutant sectors never grew directly into blades and after reaching a size of about 1 cm^2, they reverted completely to calli (Figure 9). Color mutant calli were maintained in culture, where they grew vegetatively, continuously increasing their numbers by fragmentation. Occasionally, a blade-like growth would develop from such a callus (Figure 10) and then it could sometimes be grown to sexual maturity in aspiration bottles. However, this was not an efficient procedure, because these small blades ($\approx$ 1cm) often reverted completely back to clumps of calli. In fact, blades obtained from callus in this manner occasionally reverted to callus, regardless of size. Even though 35 separate color mutant calli were collected, and many attempts were made to obtain blades from each isolate, only three color mutant blades were ever induced to grow to sexual maturity: GREEN, $PINK_1$ AND $PINK_2$.

Treatment of germinating conchospores and small embryonic blades with NG produced only two mutants in several trials (50 $\mu g \cdot mL^{-1}$ for 45 minutes). Surprisingly, these two mutants appeared in the same petri dish as single-color, mutant blades: yellow and red. Both of these mutants matured as males and were crossed to normal females to study their inheritance and to maintain the mutant genotypes in the heterozygous conchocelis generation, from which they could be recovered for subsequent crosses.

Treating conchosporangial conchocelis produced no mutants, even though conchocelis was exposed to the point of lethality (50 $\mu g \cdot mL^{-1}$ for 90 minutes). At lower time/concentration combinations, sporulation was arrested for about three weeks, but eventually resumed, yielding only normal blades for the life of the culture.

D. Blade Development Patterns

As conchospores grew into blades, the general pattern of development was observed by Nomarski light microscopy. The following

descriptions will refer to Figures 35 and 36. Dark arrows represent the dominant developmental patterns, and hollow arrows represent infrequent alternatives. Before any germination could occur at all, the spores had to attach to the coverslips. The cells that did not attach firmly either died, or occasionally developed into callus-like growths. After attachment, the cells began to change shape until they became pyriform (Figures 35A and 36A). The first cytokinesis of the young sporeling was always periclinal, dividing the cell in half with an upper cell (Figure 36B: A_0) and a basal cell (Figure 36B: B_0). The next division was usually either another periclinal division of the basal cell (Figure 36B": B_1, B'_1 and Figure 36C: B_1, B'_1) or an anticlinal division of the upper cell (Figure 36C: A_1, A'_1). It was observed that the upper cell (Figure 36B: A_0) usually divided first (Figure 36: C, D_1, D_2, E) whereas the basal cell might not divide until hours to weeks later (Figure 36B': B_0). The basal cells, after additional anticlinal and periclinal divisions, became pyriform with very long extensions. This area developed into the small ($\approx$ 1 mm) holdfast region of the mature blade. In contrast, the two upper cells, underwent rapid anticlinal and periclinal cell divisions until they made up the bulk of the blade (Figure 36 C, D_1,D_2, E). In addition, from the genetic nature of

the meiotic tetrad of cells formed in the first divisions (Figure 36C), it became clear that each of the first two upper cells (Figure 36C: A_1 and A'_1) produced separate and distinctive cell lines, causing the young blades to have an anticlinally divided appearance (Figure 36E). The two original basal cells also produced two separate cell lines resulting in a periclinal demarcation of tissue in the basal region (Figure 36E). The results of these division patterns can be discerned in small F_1 blades from a mutant x wild type cross (Figure 37 and 38).

Plants in culture did not all follow the "normal" pattern of development. Rarely, the cells would divide periclinally for the first two divisions (Figure 36: B", and then the top one or two cells would divide anticlinally and go on to develop into a normal blade. Occasionally, during normal development, one of the upper cells died, possibly due to bacterial infection, or did not develop for some other reason. In such cases, the remaining upper cell produced a blade by itself. In culture, many blades also developed abnormally forming undifferentiated callus-like growths (Figure 9). Such "callus" never formed normal blades; however, malformed, blade-like growths, which rarely became sexually mature developed sporadically. Undisturbed cultures grew and developed

very well, but when young germlings were examined by microscopy or manipulated, it seemed to interfere with their normal development. Therefore, individual sporelings could not be observed for very many successive divisions. The developmental patterns described above are partly based on a composite of observations of many individual separate plants (Figures 35 and 36).

The inferences drawn about meiosis from microscopic examination of nuclear division in germinating conchospores and of early cell division patterns in the development of sporeling blades were further supported by observations on the genetic transmission of color mutations in crosses (wt x yellow and wt x red). The segregation of the color alleles strongly correlated with the observations on chromosome behaviour and cell division patterns. Very young plants from heterozygous conchocelis were distinctly mosaic in appearance, often showing 4 clear sectors (2 mutant in color and 2 wild type), which is in keeping with the conclusion that the first two nuclear divisions of the developing gametophyte are meiotic in nature (Fig 37 and 38). Each of these four sectors presumably developed from one of the four cells in the initial tetrad generated by meiosis. As the plants grew larger, the sectors on the small holdfast derived from the

two lower cells of the tetrad became increasingly difficult to distinguish. In contrast, sectors derived from the upper two cells of the tetrad constituted the bulk of the mature blade. Interestingly, most young plants from crosses had a periclinal (transverse) separation of color sectors on the holdfast portion of the plant and an anticlinal (longitudinal) division of color sectors in the blade. Fronds in which the blade was one uniform color with the alternate color in the holdfast region were much less frequent. These observations, indicate that segregation of the color-determining alleles for these mutants occurred predominantly at the second meiotic division.

E. Genetic Analysis of Crosses.

1. Sex-determining Alleles

Mature fronds of *Porphyra umbilicalis* are characteristically longitudinally divided into male and female halves. This feature, taken in conjunction with the developmental pattern established during the initial cell divisions of the conchospore, strongly suggests that primary sex

determination is controlled by a gene locus through a pair of alleles that segregate at meiosis. To obtain more information on the inheritance of these alleles, wild plants collected from nature and progeny from crosses were scored for their sexual phenotype (Figures 39 & 40). In characterizing the transmission pattern of the sex-determining locus, analysis was restricted to results from crosses between plants having identical color genotypes. This precaution avoids any potential interference that segregation of different color types might have on the transmission and expression of the sexual phenotypes.

Initial observations were made on a collection of plants taken from the field. Nearly all of these (96.4%; Fig. 39A and Table 5A) had sectored ♂/♀ fronds, presumably the same as plants in the previous gametophytic generation. Unsectored, single-sex plants occurred 3.6% of the time in a 1♀:1♂ ratio. Taken together, there was a perfect 1:1 segregation for male to female sectors (327♀:327♂), in keeping with the hypothesis of a nuclear sex-determining locus.

Considering that observations on early developmental patterns indicated that the blade portion of the gametophytic thallus generally is derived from the upper two cells of the initial meiotic tetrad, sexually

sectored plants would have to arise by second division segregation of the sex-determining alleles. Thus, the frequency of sectored plants relates directly to the frequency of second division segregation of the sex-determining alleles. This provides a measure of the amount of genetic crossing over between the sex locus and the centromere of the chromosome on which it resides, and thus an estimate of the position of this locus relative to the centromere. The genetic recombination frequency (in percent) equals half the percentage of second division segregation, thus the proposed gene for sexual determination is calculated to be 48.2 (96.4/2) centimorgans from the centromere (Table 5B). This result indicates the locus is not genetically linked to the centromere, but rather is located a considerable but undetermined distance away.

Using frequency of sectored fronds to measure second division segregation and, from that, the genetic distance, makes the assumption that the unsectored, single-sex blades represent first division segregation. This is almost certainly an oversimplification; a substantial proportion of the apparent cases of first division segregation may arise by other mechanisms. Death of one of the upper cells in the tetrad could lead to a single-sex thallus even though it started as a potentially sectored plant.

An abnormal cell division pattern in early development might do the same. In addition to these, it must be recognized that certain types of double crossover would undo the effect of the initial crossover and thus lead to undetected crossover events and a corresponding underestimation of crossing over frequency. All these factors would lead to an underestimation of the genetic distance and thus the 48.2 centimorgans calculated above has to be considered a minimum estimate.

The second set of data analyzed for sexual segregation was from the cross GREEN♀ X GREEN♂ which, along with all the other crosses, was performed in culture (Figure 39B). Green F_1 generation blades were usually sectored ♀/♂ (89.9%), identical to the parental type, and the remaining 10.1% unsectored plants encompassed 4♀ and 6♂ plants (Figure 39B & Table 5A). Since total male and female sectors occurred in nearly a perfect 1:1 ratio (93♀:95♂), the hypothesis of Mendelian segregation of sex-determining alleles is again supported. Second division segregation for the sex-determining locus in this cross was 89.9%, again indicating that crossing-over between the sex locus and the centromere occurs with a very high frequency. The smaller percentage of unsectored plants may represent first division segregation, or, as

mentioned above, an abnormal division pattern or death of one or more cells of the tetrad due to culture conditions. This cross indicates the gene for determining the sexual phenotype is a minimum of 45.0 centimorgans from the centromere and therefore almost certainly genetically unlinked (Table 5B).

A small number of plants from the YELLOW♀ X YELLOW♂ cross were also scored for sexual phenotype (Figure 39C and Table 5A). A high proportion of the yellow F_1 gametophytic blades were again sectored ♀/♂ (83.3%). The few unsectored blades encompassed 1 female and 4 male plants. Overall, the male and female sectors occurred in nearly a 1:1 ratio (26♀:30♂) and the hypothesis of Mendelian segregation of sex-determining alleles is supported.

Sectored blades represented 83.3% of the total sampled and provide yet another estimate of the percentage of second division segregation for the sex-determining locus. As described above, the 16.7% unsectored blades represent first division segregation or abnormal pattern formation. In this cross, the gene determining sexual phenotype is estimated to be at least 41.6 centimorgans from the centromere (Table 5B). Because of the smaller number of plants, this estimate is probably the least accurate.

Quantitative data were not recorded for the last two crosses and the results are based on only a small number of blades occurring in conchocelis culture. These observations were important however, because they support the results of the previous three crosses. Plants from the $PINK_1$♀ X $PINK_1$♂ cross usually produced stable pink blades that were sectored ♀/♂ (Figure 39D) and blades from the RED♀ X RED♂ cross usually produced blades sectored into a darker red (presumably female) half and a lighter red (presumably male) half (Figure 40). These observations are consistent with the case for second division segregation of alleles for sex.

2. Color-determining Alleles

The five color mutants (YELLOW, RED, GREEN, $PINK_1$, and $PINK_2$), generated from mutagenesis experiments, were grown to sexually mature blades. These plants were crossed to wild type and to each other in order to characterize the mutations genetically by determining their transmission pattern. A summary of conchocelis and F_1 blade phenotypes

from these crosses is presented in Table 4, and a detailed analysis is presented in the following text.

a. Yellow Mutant

The YELLOW♀ X YELLOW♂ cross produced yellow conchocelis from germinated zygotospores and the resulting conchospores developed into yellow blades in the F_1 generation (Figure 39C and Table 5A). The yellow mutant was very stable and never mutated to another color or reverted to wild type (Figure 49).

The next cross examined for information about the yellow mutation was WT♀ X YELLOW♂ (Figure 41). Conchocelis from germinated zygotospores was usually (>99%) yellowish in color (yellower than wild type conchocelis, but less yellow than conchocelis from a YELLOW♀ X YELLOW♂ cross). This indicates that the yellow phenotype has some degree of dominance over wild type in the 2N sporophyte. Conchospores from the heterozygous conchocelis mostly germinated into color-sectored blades (Figures 37 and 48). Only two colors appeared in blades of the F_1

gametophyte generation: yellow and wild type. Individual sporelings frequently exhibited 4 well-defined sectors (two yellow and two wild type), although those in the holdfast region became difficult to distinguish as the blades enlarged. When small plants were scored simply as sectored or unsectored for a particular color, the total number of sectors or plants for each color type occurred in a nearly perfect 1:1 ratio (yellow 333 + 61 = 394; and wt 333 + 56 = 389). From these data it is apparent that the yellow color results from a mutation in a single nuclear gene.

Two classes of blades resulted with regards to color segregation pattern. Sectored yellow/wild type and unsectored yellow or wild type (Figure 41 and Table 5A). Sectored blades represented 74% of the total sampled and reflect the percentage of second division segregation occurring following crossing-over between the mutant locus and the centromere at meiosis. The remaining 26% of blades that were single color plants (yellow or wild type) probably arose from a number of mechanisms as discussed previously for the sexual phenotypic segregation. Since death of part of the meiotic tetrad, or abnormal development was quite common in culture, it is likely even more plants

would have been sectored under more natural conditions. Therefore, the gene determining the yellow phenotype is a minimum of 37.0 centimorgans from the centromere and, at most, only loosely linked to it (Table 5B).

A single wild type conchocelis was also observed in this cross (Figure 41). This wild type conchocelis subsequently released conchospores that developed as WT ♀/♂ sectored blades or as WT (♀ or ♂) sexually unsectored blades. This rare, apparent reversion to wild-type conchocelis may have been due to any of a number of genetic mechanisms including somatic mutation, gene conversion, or mitotic recombination in diploid tissue. Only one occurrence of such wild type conchocelis was ever observed, and it is not possible to determine which of these mechanisms was the actual process.

b. Red Mutant

WT♀ X RED♂ was one of the first successful crosses accomplished in culture, and even though the red mutant grew

extraordinarily slowly, a substantial number of F_1 gametophytes were scored (Figure 42A and Table 5A). Released zygotospores germinated into red conchocelis, which indicates a high degree of dominance for the red phenotype in the 2N sporophyte. Red/wild type sectored blades or unsectored blades of either color were obtained from conchospores in the F_1 generation (Figure 47). The individual color sectors of small plants (<1 cm) were scored as follows: red 269 + 35 = 304, and wild type 269 + 29 = 298. Since only two colors were observed in the cross, and sectors of the two types occurred in nearly a 1:1 ratio, the red color, like the yellow phenotype results from a single mutation of a nuclear gene. Here again, observations of 2 red and two wild type sectors on individual small plants add strong support to this conclusion.

Two classes of blades were scored with respect to color segregation pattern: Sectored red/wild type and unsectored red and wild type (Figure 42A and Table 5A). Small sectored blades represent 80.8% of the sample and indicate a high percentage of second division segregation for the mutant locus. The remaining 19.2% single color plants may represent first division segregation, or more likely developmental

anomalies due to culture conditions, especially in view of the slow growth rate of the red mutant sectors.

Evidence for abnormal development was observed when plants from this cross were grown to sexual maturity; 3.8% of the sexually mature blades were sectored WT♀/WT♂ and 96.2% were either WT♀ or WT♂ (89WT♀:89WT♂) (Tables 5A and 5). None of the red sectors that occurred 1:1 alongside normal sectors in the upper portion of the small blades (< 1cm) (Figure 42A and Table 5A), ever appeared in large blades (> 15cm). Because of the slow growth rate of the red sectors, they were invariably overgrown by normal sectors. However, if it is assumed that an equal number of RED♀/RED♂ blades would have resulted from first division segregation as WT♀/WT♂ blades, this would lead to a higher second division segregation estimate than the 80.8% measured above: (100% - (3.8% RED♀/RED♂ (estimate) + 3.8% WT♀/WT♂) = 92.4%). Consequently, the gene for red phenotype was calculated to be between 40.4 and 46.2 centimorgans from the centromere and essentially unlinked (Table 5B).

c. Green Mutant

The reciprocal crosses WT♀ X GREEN♂ and GREEN♀ X WT♂ were the next to be examined (Figures 42B and 42C). In the first cross, WT♀ X GREEN♂, discharged zygotospores consistently germinated into wild type conchocelis. Subsequently, the conchocelis produced conchospores which upon release germinated exclusively into wild type blades which were usually sectored ♀/♂, or more rarely, were unsectored (♀, ♂) (Figure 49). It was difficult to determine anything from this cross by itself, other than that the green mutation was not inherited for some reason. Most of the F_1 blades were sectored ♀/♂ in this cross indicating that the putative sex-determining alleles were behaving normally.

GREEN♀ X WT♂ proved to be a very valuable cross for several reasons (Figure 42C). First, the conchocelis produced from germinated zygotospores for this cross was nearly always green (>99%), and conchospores from this green conchocelis consistently produced only green blades. This is the same result as found for the GREEN♀ X GREEN♂ cross (Figure 39B) except that the conchocelis in that cross was always green. The differences in results for the reciprocal crosses,

and the fact that there was no color segregation on the fronds in either cross indicate that the green mutation is non-Mendelian, with nearly exclusively maternal transmission, suggesting a plastid location for the defective gene.

One very significant result from the GREEN♀ X WT♂ cross was the repeated appearance of a small percentage (<1%) of wild-type conchocelis which produced wild type gametophytes. This could represent male transmission of the wild type chloroplast genome by chloroplast transfer through the spermatia. Another explanation would be the possibility that the green mutant is unstable and reverts to wild type at a low frequency. This explanation, is very unlikely, since a somatic reversion from green was never observed during the six years in culture. In addition, neither wild-type conchocelis nor wild type blades were ever observed from the GREEN♀ X GREEN♂ cross. The green mutation appears very stable and thus the appearance of wild type conchocelis by paternal transmission of chloroplast genes appears much more likely than reversion.

These reciprocal crosses provide another, independent estimate of second division segregation of sex-determining alleles. The high

percentage (85.5%) of sectored GREEN ♀/♂ blades Figure 42C and Table 5A) place the sex-determining locus at least 42.8 centimorgans from the centromere which is in agreement with the results already discussed in the section on sex-determining alleles (Table 5B).

The next two crosses analyzed were also reciprocals, GREEN♀ X RED♂ and RED♀ X GREEN♂ (Figure 43A and 43B). Conchocelis from the GREEN♀ X RED♂ cross was green, consistent with the hypothesis of a non-Mendelian green mutation. Had enough 2N conchocelis been generated, the appearance of paternally derived non-green plastids might have been expected in some of the conchocelis. This cross is puzzling in one aspect; even though the conchocelis produced conchosporangia, spores were never released and blades were never observed during more than six years in culture.

The reciprocal cross, RED♀ X GREEN♂, produced zygotospores which germinated into red conchocelis, as was expected based on previous cross results. The phenotypes of the F_1 blades, however, were not the wild type/red sectored blades expected, but rather red sectored (lighter/darker (♀/♂?)) and red unsectored blades. These results cannot be explained readily and the cross must be re-examined (only six slow-

growing red blades were ever produced).

The GREEN♀ X YELLOW♂ cross produced predominately (>99%) green conchocelis. Since, the green and yellow mutations had already been characterized, segregation in this cross should follow those same patterns. Indeed, the results were consistent with results from the WT♀ X YELLOW♂ cross, with the exception that the non-Mendelian green was present simultaneously in the background. Thus, in the F_1 green/light-yellow gametophytes (Figure 51), the green sectors are the same as the green parent ([G]Y^+), and light-yellow is a double mutant ([G]Y), where [G] represents the chloroplast gene producing green phenotype, Y is the nuclear mutation producing a yellow phenotype and Y^+ is the corresponding normal allele. The ratio of green sectors to light-yellow sectors was nearly 1:1 (99:101) and is clearly a confirmation of results from the WT♀ X YELLOW♂ cross. The non-Mendelian green was transmitted predominately maternally as previously observed.

Sectored green/light-yellow blades represented 90.5% of the offspring sampled, which gives another estimate of the minimum gene-centromere distance for the yellow mutation as 45.2 centimorgans which agrees reasonably well with the estimate derived from the WT♀ X

YELLOW♂ cross (Table 5A).

One additional observation from this cross was the appearance of yellow conchocelis. The blades produced were a yellow-orange color instead of the expected yellow/wild type. The yellow conchocelis may be due to a spontaneous mutation, particularly since only one clone of this yellow conchocelis was ever observed. These observations were not followed further due to time limitations.

d. $PINK_1$ Mutant

GREEN♀ X $PINK_1$♂ and $PINK_1$♀ X GREEN♂ were the next two crosses examined (Figure 45A and 45B). Conchocelis from zygotospores of the GREEN♀ X $PINK_1$♂ cross was green, but blades have not yet been observed. Zygotospores from the reciprocal cross produced pink conchocelis, with resulting conchospores germinating into pink ♀/♂ sectored blades or occasionally unsectored pink (♀ or ♂) blades. These two crosses further verify the non-Mendelian inheritance pattern of the green mutant and a high frequency of second division segregation for the sex-determining alleles. In addition, these two crosses present evidence

that the $PINK_1$ mutant is also non-Mendelian in nature.

$PINK_1$♀ X WT♂ was almost a duplicate of the previous cross, except that the non-Mendelian green chloroplast mutation was not present. All F_1 gametophytic plants were pink. Female/male sectored pink blades were produced more frequently than single sex blades (Figure 45D). There was no evidence for genetic segregation within the fronds. These results in conjunction with the $PINK_1$♀ X $PINK_1$♂ and $PINK_1$♀ X GREEN♂ crosses (Figure 39D and 45B), which also produced the same results, support the hypothesis that $PINK_1$ is non-Mendelian and again draw attention to a high frequency of second division segregation for the sex-determining locus.

e. $PINK_2$ Mutant

The final cross analyzed to determine the transmission patterns for color-determining alleles was $PINK_2$♀ X WT♂ (Figure 46). Zygotospores from this cross consistently germinated into pink conchocelis which indicates a high degree of dominance of the $PINK_2$ phenotype in the 2N sporophyte. Only two colors appeared in the F_1

gametophytic blades, pink and wild type. The individual color sectors of large (>15cm) plants were scored as follows: pink (325 + 6 = 331) and wild type (325 + 4 = 329) (Figure 46). Sectored pink/wild type plants were present at a frequency of 97% (Figure 46 & Table 5A). Since, the sectored blades represent the percentage of second division segregation for the $PINK_2$ gene, this places the $PINK_2$ gene a minimum of 48.5 centimorgans from the centromere (Table 5B). Since the two colors segregated nearly 1:1, and the blades were usually sectored, the $PINK_2$ mutation must result from a single mutation in a nuclear gene.

In contrast to the GREEN♀ X $PINK_1$♂ cross, GREEN♀ X $PINK_2$♂ zygotospores germinated into a greenish-pink conchocelis that was likely a double mutant consisting of the non-Mendelian green and the dominant effect of the Mendelian $PINK_2$ (Figure 45C). Unfortunately, blades have not been obtained to date.

3. Linkage Analysis of Sex and Color-determining Alleles.

Individual sex-determining alleles and color-determining alleles were examined separately in the previous sections in order to determine

their respective patterns of expression and transmission. Here, color and sex-determining alleles will be examined together to test for genetic linkage, expressed by having a specific sex and a specific color appearing together more frequently than expected in a sector of F_1 gametophytic blades.

Three different crosses were examined to establish whether there was any genetic linkage between sex and color-determining alleles. For analyses of this type it was very difficult to obtain data because sectors of different colors in the same blade grew at different rates, one often outgrowing the other and forming most of the blade. The minor component sometimes disappeared completely early in development, or was present only as a small sector that failed to mature.

In the first cross, WT♀ X YELLOW♂ (Table 6), the individual sectors were scored as follows: parental (2(17 WT♀/YELLOW♂) + 16 WT♀ + 24 YELLOW♂ = 74) and recombinant (2(18 YELLOW♀/WT♂) + 23 YELLOW♀ + 27 WT♂ = 86). These values appear fairly even, with recombinants actually more frequent than parentals. Mendelian 1:1 segregation of parentals:recombinants was tested by chi-square for one degree of freedom ($X_{[1]}^2 = 0.90$). This

indicates a probability of greater than 30% and is not considered significant. Thus from the cross, WT♀ X YELLOW♂, it appears clear that the sex locus is not linked to the yellow locus.

The second cross analyzed for linkage between color and sex-determining alleles was $PINK_2$♀ X WT♂ (Table 6). The individual sectors were scored as follows: parental (2(120 $PINK_2$♀/WT♂) + 2 $PINK_2$♀ + 1 WT♂ = 243) and recombinant (2(205 WT♀/$PINK_2$♂) + 3 WT♀ + 4 $PINK_2$♂ = 417). These values appear to be skewed, with recombinants nearly double the parentals. It is likely this result was due to scoring errors. It was observed that when pink plants were female they became less pink, and when wild type plants were male they became more pink, often making it difficult to distinguish $PINK_2$♀/WT♂ from WT♀/$PINK_2$♂ (Figure 50). This would certainly explain the skew towards WT♀/$PINK_2$♂. The correct ratio between the two types would have to be determined from new data.

The final cross analyzed for linkage between color and sex-determining alleles was WT♀ X RED♂ (Figure 42A). Only wild type sectors were scored from this cross because the red mutant grew very slowly, and reached sexual maturity only under low light levels. In

addition, red sectors were always overgrown by adjacent wild type sectors as the plants developed. Even so, it was still possible to score for linkage, because if one of the two apical cells was wild type, the alga survived and grew to maturity as either male or female. It was assumed that the missing sector was red and of the opposite sex, since second division segregation of red and wild type in small plants was at least 80.8% (Table 5A). The sectors were scored as follows: parental (89 WT♀ + 7 WT♀ = 96) and nonparental (89 WT♂ + 7 WT♂ = 96). This is quite obviously a 1:1 ratio; thus, from the cross WT♀ X RED♂, it is clear the sex locus is also not linked to the red locus.

F. Coincidence of Color and Sex Boundaries

This analysis was performed in order to test whether color and sex boundaries coincide when both appear to undergo second division segregation in F_1 gametophytic blades. This represents a critical test of the hypothesis that sex-determination is controlled by genetic alleles segregating at meiosis. If color and sex boundaries do not always coincide, the hypothesis would be refuted.

Most of the data was collected from the WT♀ X YELLOW♂ and $PINK_2$♀ X WT♀ crosses (Table 6, Fig. 41 and Fig. 46). These plants were preferable to others attempted, because for the most part, they developed to sexual maturity without one sector overgrowing the other. The WT♀ X RED♂ (Table 6 and Fig. 42A) was not used for this analysis, although it was presumed that the 7 WT♀/WT♂ sectored blades were produced by first division segregation of color alleles, with the red sectors becoming assimilated into the holdfast. The WT♀ X YELLOW♂ cross produced a total of 35 sexually mature female/male sectored blades, and the $PINK_2$♀ X WT♂ cross produced 325 female/male sectored blades in the F_1 generation. Although the results from the $PINK_2$♀ X WT♂ cross were skewed (because of color scoring errors) for the linkage analysis, they were completely acceptable for observations pertaining to the coincidence of color and sex boundaries. For the 260 color sectored thalli examined from these two crosses, and also scattered observations from other crosses (Figs. 48-51), the color and sex boundaries always coincided perfectly.

G. Illustrations and Figures

Table 1. Composition of Modified D-11 Culture Medium

Nutrient Stocks	Final Concentration	ml Stock/L Seawater
1.0 M $NaNO_3$	1.0 mM	1.0 mL
0.25 mM $NaH_2PO_4 \cdot H_2O$	0.5 mM	2.0 mL
0.1 M $Na_2SiO_3 \cdot 9H_2O$	0.1 mM	1.0 mL
P-1 Metals[a]	-	2.0 mL
1 mM FeEDTA	2.0 μM	2.0 mL
Vitamins[a]	-	1.5 mL
0.25 M NH_4NO_3	0.5 mM	2.0 mL
0.1 M $MgSO_4 \cdot 7H_2O$	20.0 μM	0.2 mL
5 mM $Na_2MoO_4 \cdot 2H_2O$	10.0 μM	2.0 mL
2.5 mM KCL	0.5 μM	0.2 mL
10^{-4} M Pyridoxine HCL	2.0 nM	0.025 mL
10^{-4} M Phenylacetic acid	2.0 nM	0.025 mL
10^{-3} M ρ-Hydroxy-phenylacetic acid	25.0 nM	0.025 mL
Seawater	-	1000mL

[a]For stock refer to SWM-3 medium (McLachlan, 1973).

Table 2. Conchospore induction for *Porphyra umbilicalis* conchocelis at 40 μmol · m^{-2} · s^{-1} of cool white fluorescent light

Temperature (°C)	Photoperiod		
	16:$\overline{8}$	12:$\overline{12}$	8:$\overline{16}$
10	++	+	-
13	+++	++	+
15	+++	++	+

Table 3. Conchospore release for *Porphyra umbilicalis* conchocelis at 40 μmol · m^{-2} · s^{-1} of cool white fluorescent light

Temperature (°C)	Photoperiod		
	16:$\overline{8}$	12:$\overline{12}$	8:$\overline{16}$
10	+	-	-
13	+++	+	-
15	+	+	-

\- = ABSENT
\+ = PRESENT
++ = COMMON
+++ = ABUNDANT

Figure 1. *P. umbilicalis* blades on mudflats at Avonport, Nova Scotia.

Figure 2. *P. umbilicalis* (field) showing divided nature of monoecious blade (upper female half, lower male half). Ruler = 15 cm.

1

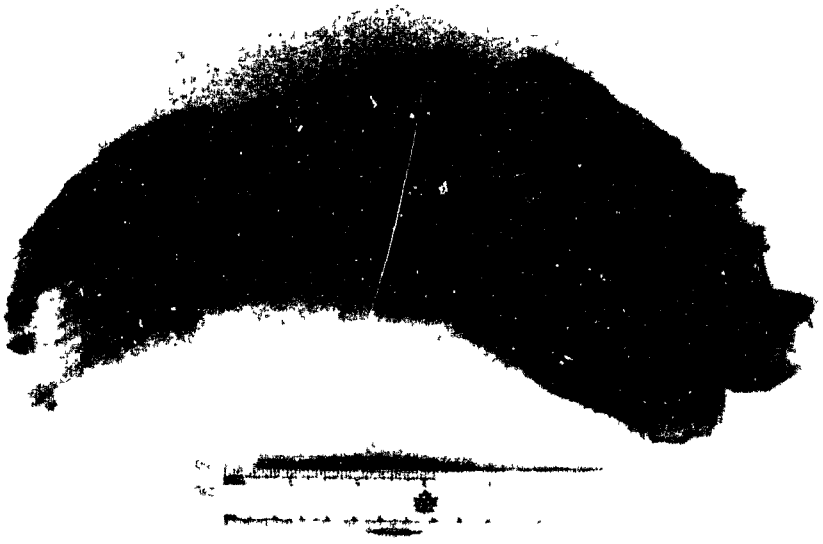

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Figure 3. Germinating conchocelis (two weeks old), thin filaments showing the development of thicker conchosporangial filaments.

Scale = 100 μm.

Figure 4. Sporulating conchocelis (1 month old). Note that the conchosporangial filaments have all disintegrated due to sporulation.

Scale = 100 μm.

Figure 5. Conchocelis in small petri dishes, with glass ring and coverslip.

Scale = 3.5 cm.

3

4

5

Figure 6. Conchocelis in small petri dish, with glass ring and coverslip (closeup). Scale = 3.5 cm.

Figure 7. Blades growing in 8 L aspirator bottles (left and right), and aerated 500 mL flask. Scale = 15 cm.

6

7

Figure 8. *P. umbilicalis* (culture) showing divided nature of monoecious blades (left side: female, right side: male). Ruler = 15 cm.

Figure 9. Green calli grown in culture. Scale = 0.5 cm.

Figure 10. Green callus (arrow) producing a blade. Scale = 0.5 cm.

8

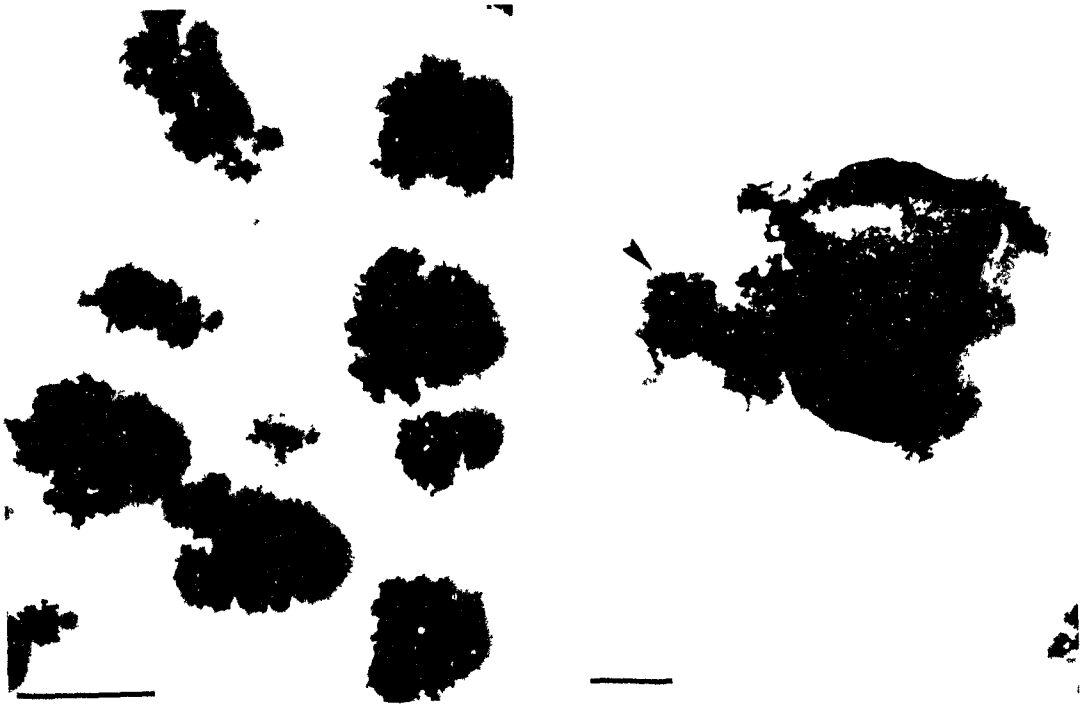

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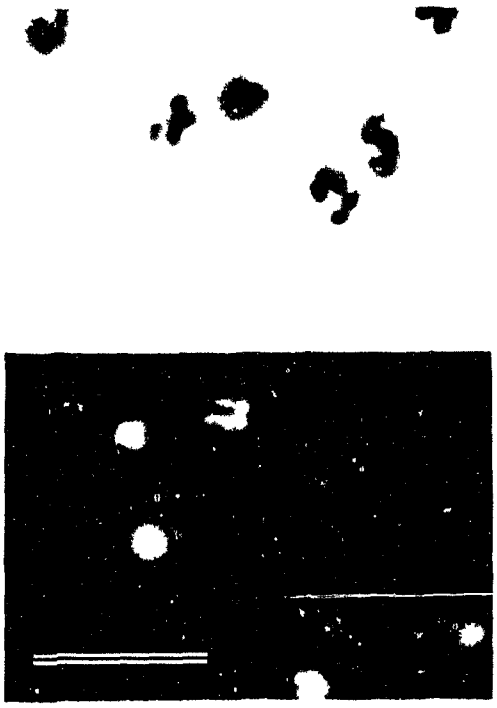
Figure 11. Mitotic spermatagonial metaphase (upper photo: Wittman's stain, lower photo: DAPI stain. Scale = 5 μm.

Figure 12. Meiotic interphase in a germinating conchospore. Note circular chloroplast nucleoids around the central nucleus. Figures 12-34 were stained with DAPI. Scale = 10 μm.

Figure 13. Meiotic interphase in two germinating conchospores. Scale = 10 μm.

Figure 14. Early meiotic prophase I in a germinating conchospore. Note condensing chromatin threads. Scale = 10 μm.



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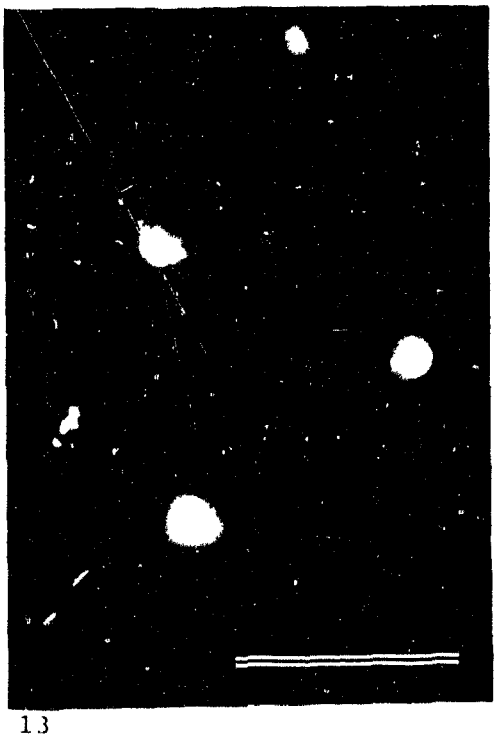

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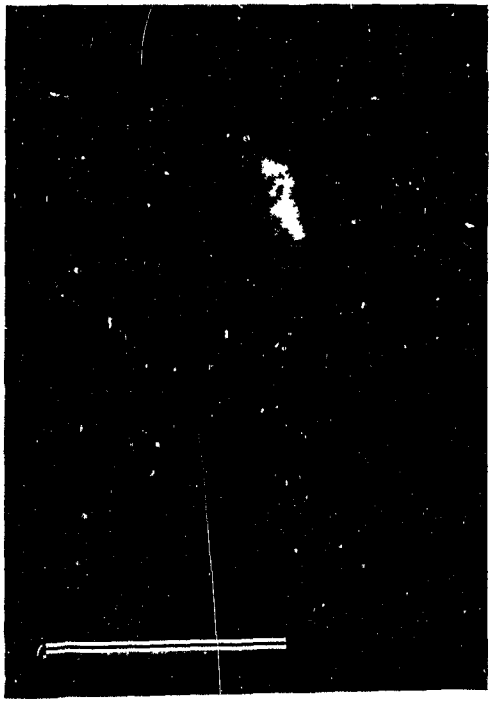

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Figure 15. Early meiotic prophase I in a germinating conchospore. Note chromatin threads condensing into chromosomes.

Scale = 10 μm.

Figure 16. Early meiotic prophase I in a germinating conchospore. Note chromatin threads condensing into chromosomes.

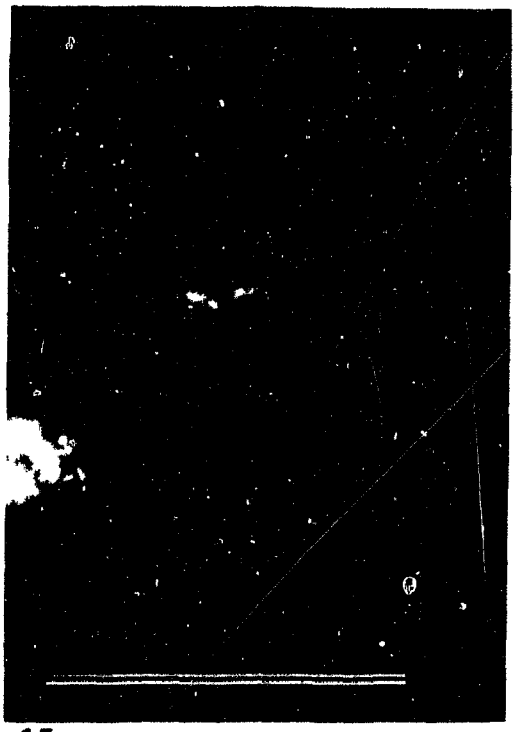
Scale = 10 μm.

Figure 17. Zygotene stage of meiotic prophase I in a germinating conchospore. Note formation of five individual chromosomes.

Scale = 10 μm.

Figure 18. Diplotene stage of meiotic prophase I in a germinating conchospore. Note formation of five distinct chromosomes.

Scale = 10 μm.



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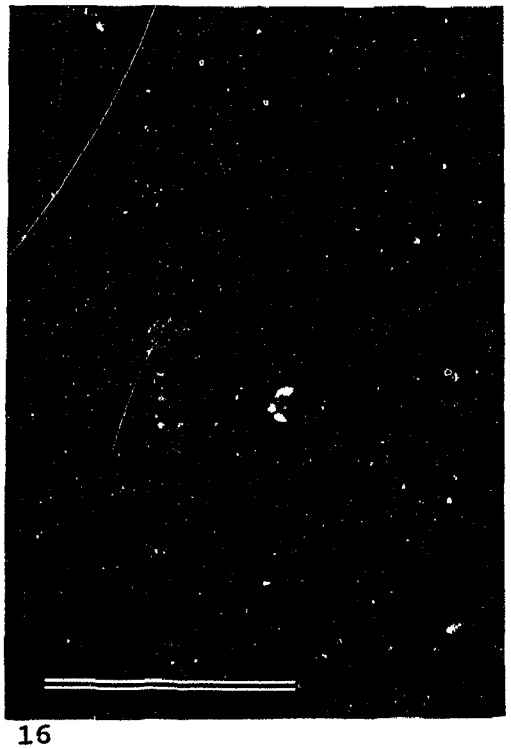

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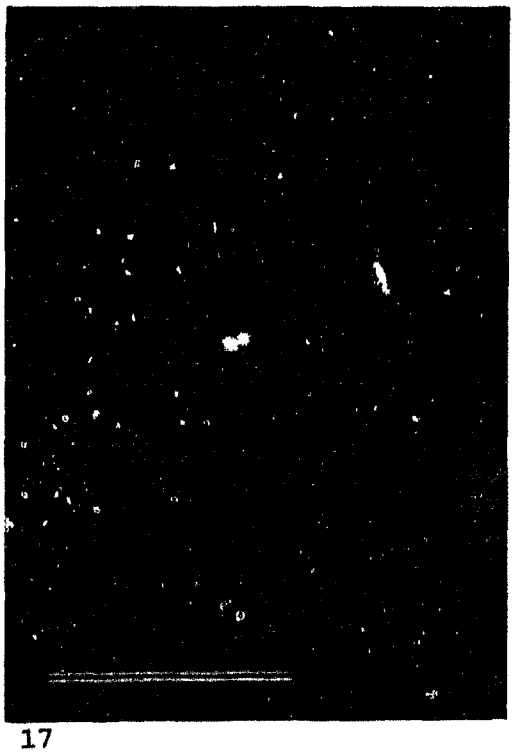

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Figure 19. Diakinesis stage of meiotic prophase I in a germinating conchospore. Note double nature of the five chromosomes.

Scale = 10 μm.

Figure 20. Diakinesis stage of meiotic prophase I in a germinating conchospore (squash). Note double nature of the five chromosomes.

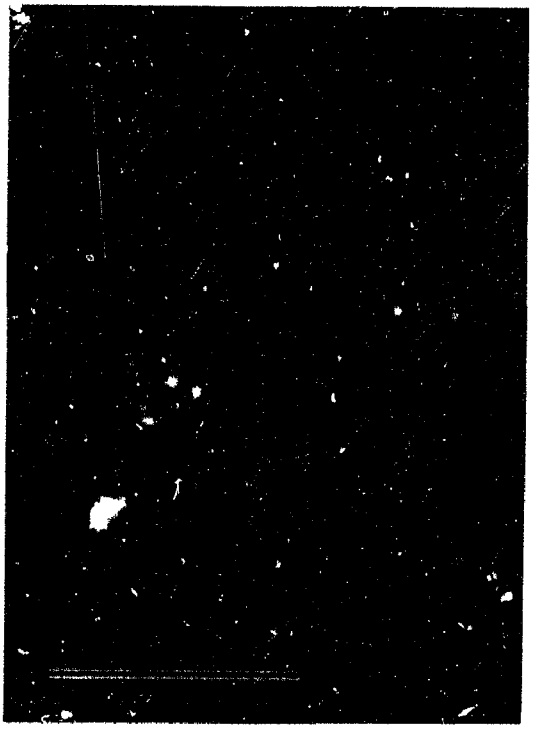
Scale = 10 μm.

Figure 21. Diakinesis stage of meiotic prophase I in a germinating conchospore. Note double nature of the five chromosomes.

Scale = 10 μm.

Figure 22. Early meiotic metaphase I in a germinating conchospore.

Scale = 10 μm.



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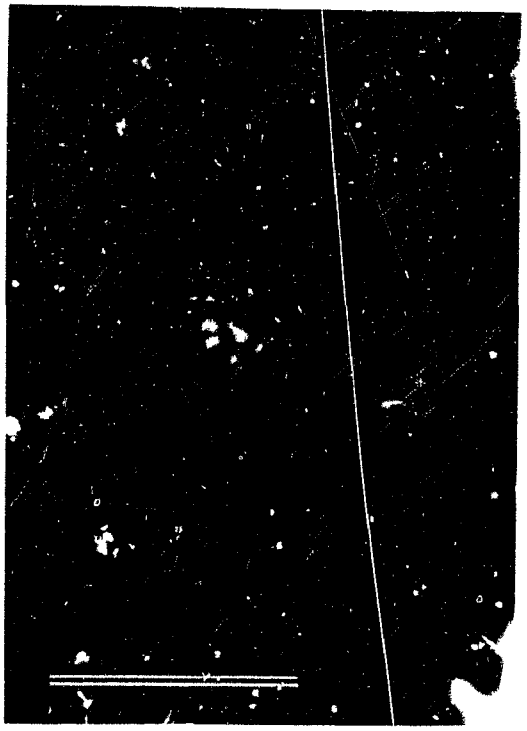

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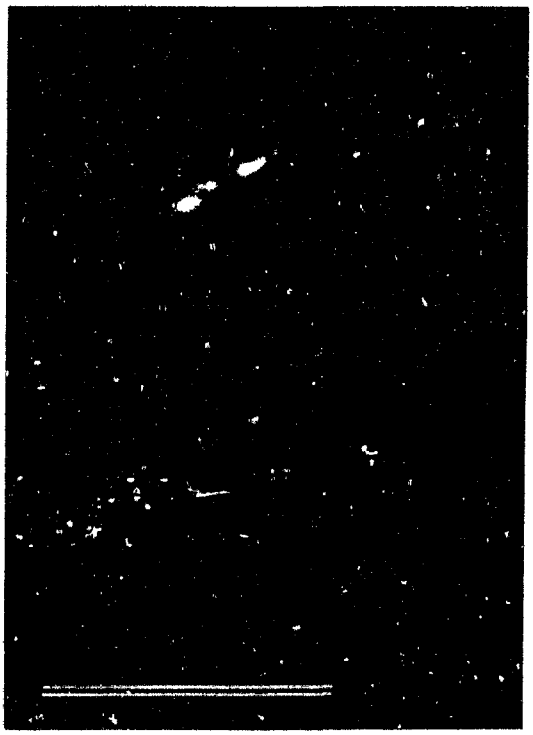

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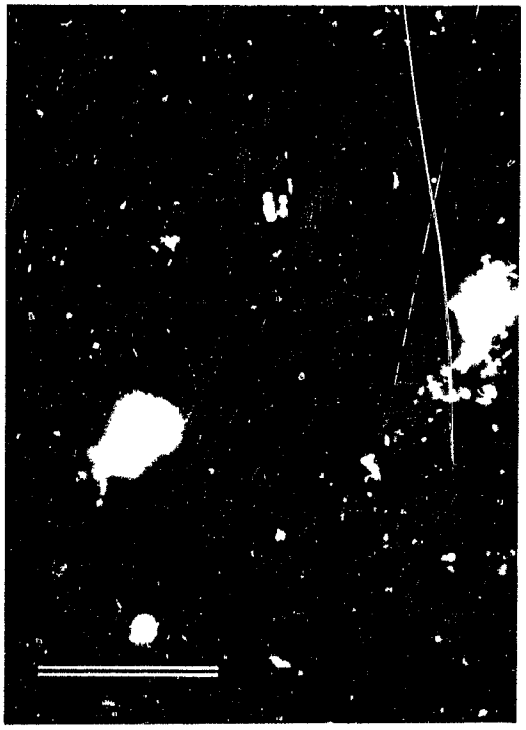


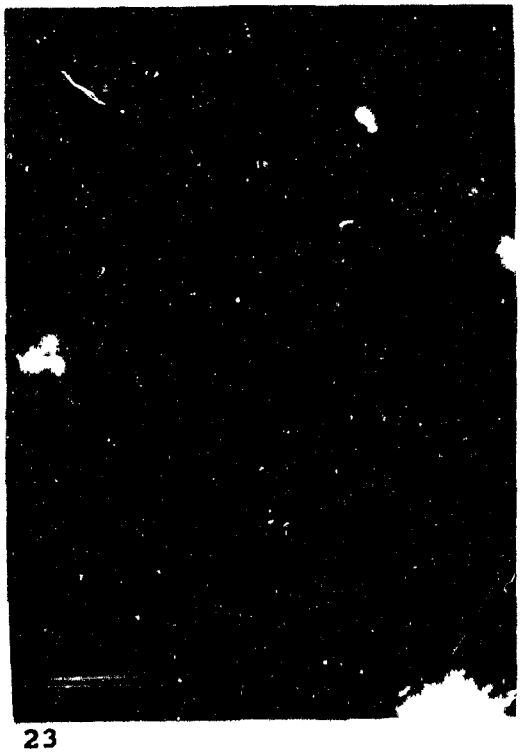
22

Figure 23. Meiotic metaphase I in a germinating conchospore. DAPI staining. Scale = 10 μm.

Figure 24. Early meiotic anaphase I in a germinating conchospore. Note separation of sister chromatids. Scale = 10 μm.

Figure 25. Meiotic anaphase I in a germinating conchospore. Scale = 10 μm.

Figure 26. Late meiotic anaphase I in a germinating conchospore. Scale = 10 μm.



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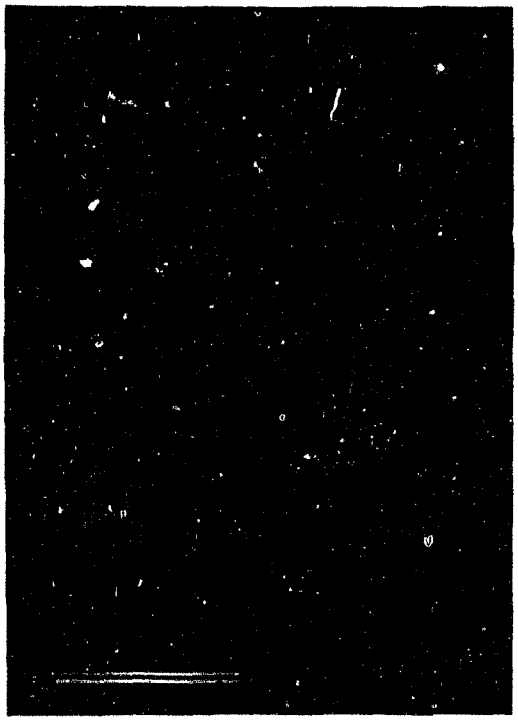

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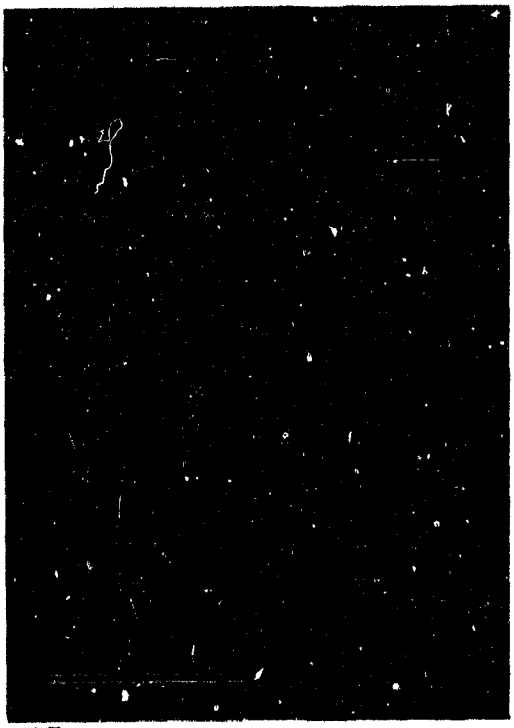

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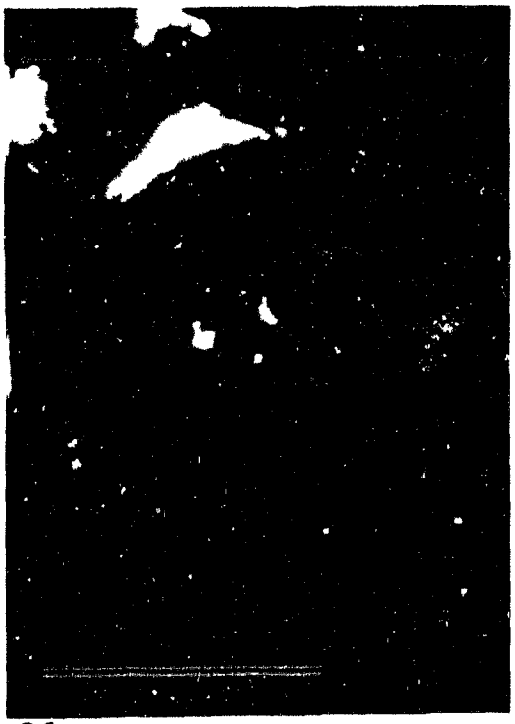


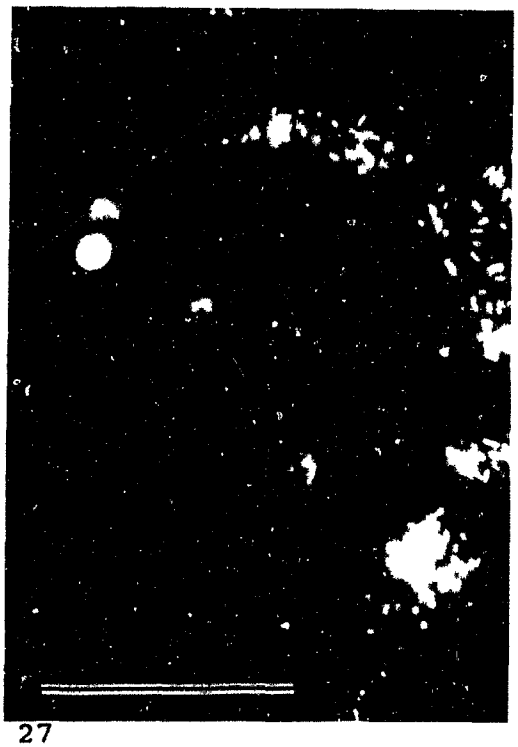
26

Figure 27. Late telophase I in a germinating conchospore. Note reorganization of nuclei. Scale = 10 μm.

Figure 28. Interkinesis in a two-celled sporeling. Cytokinesis has occurred. Scale = 10 μm.

Figure 29. Late prophase of the second meiotic division in a two-celled sporeling. Scale = 10 μm.

Figure 30. Late prophase of the second meiotic division in a three-celled sporeling (upper two cells) and interkinesis (lower cell). Same plant shown in different focal planes (left and right). Scale = 10 μm.



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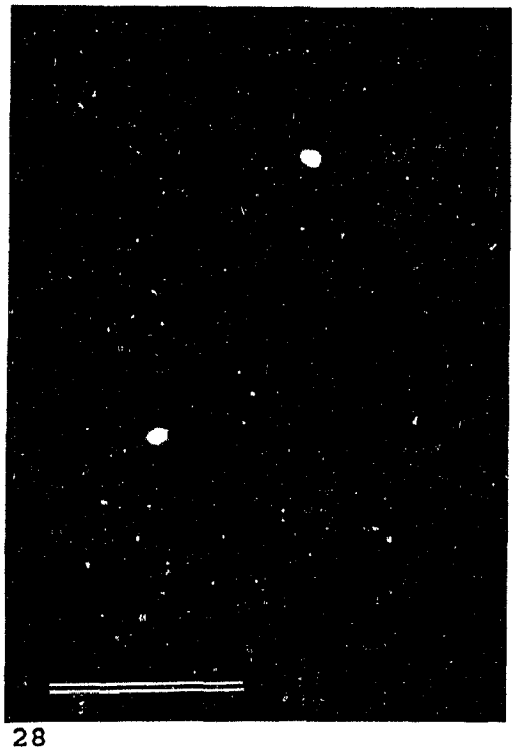

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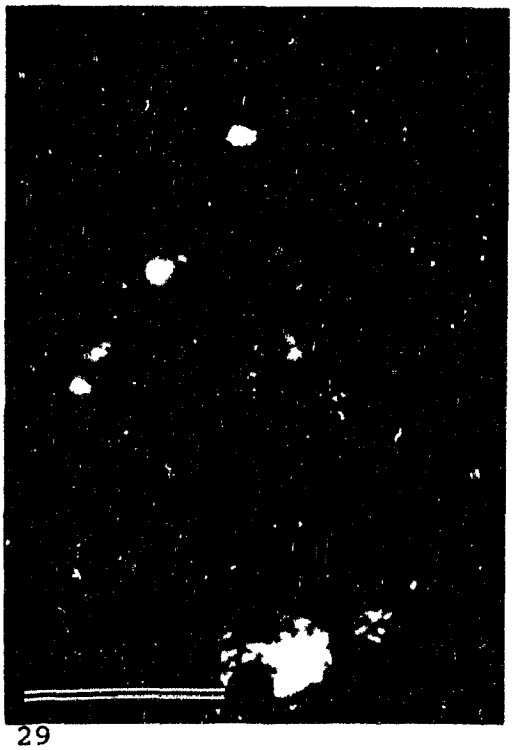

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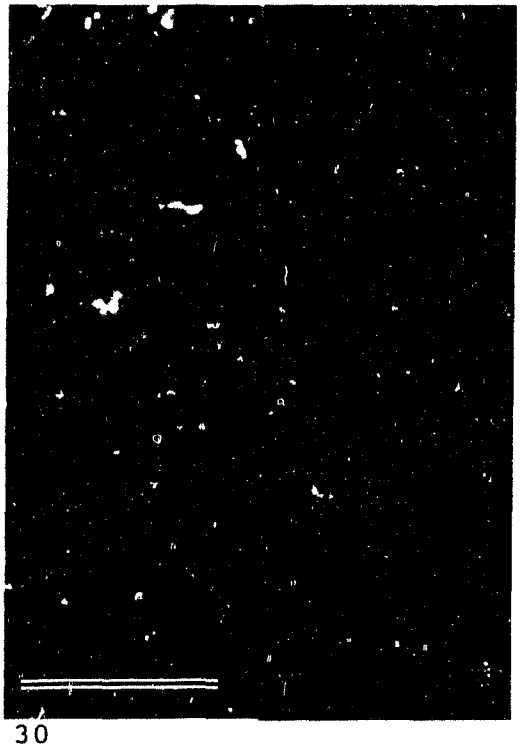


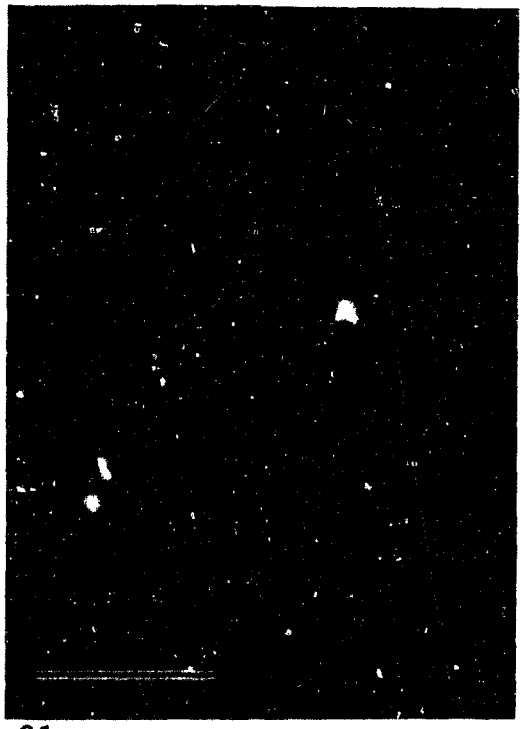
30

Figure 31. Metaphase of the second meiotic division in a two-celled sporeling. Scale = 10 μm.

Figure 32. Anaphase II in a two-celled sporeling.
Scale = 10 μm.

Figure 33. Completion of meiosis in the upper two cells, interkinesis in the bottom cell, in a three-celled sporeling.
Scale = 10 μm.

Figure 34. Meiotic nuclear tetrad in a three-celled sporeling. The upper cell has not yet finished cytokinesis. Scale = 10 μm.



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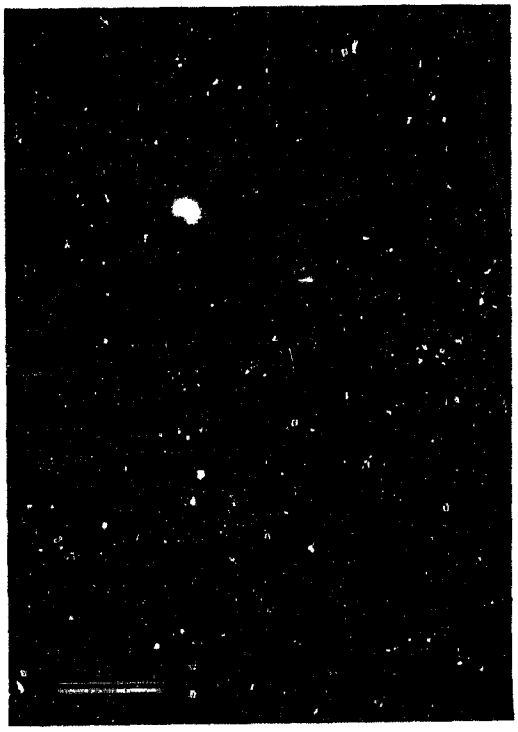

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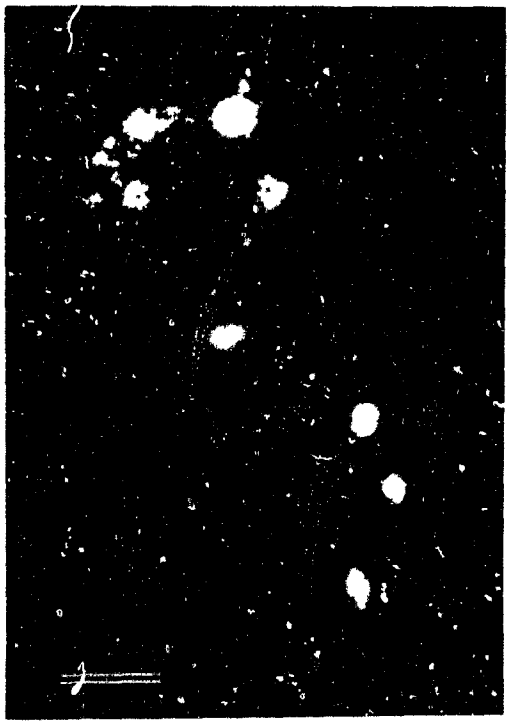

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Figure 35. Early developmental patterns of germinating sporelings (DIC illumination). Dark arrows represent the dominant developmental patterns, and hollow arrows represent infrequent alternatives.

Scale = 10 μm.

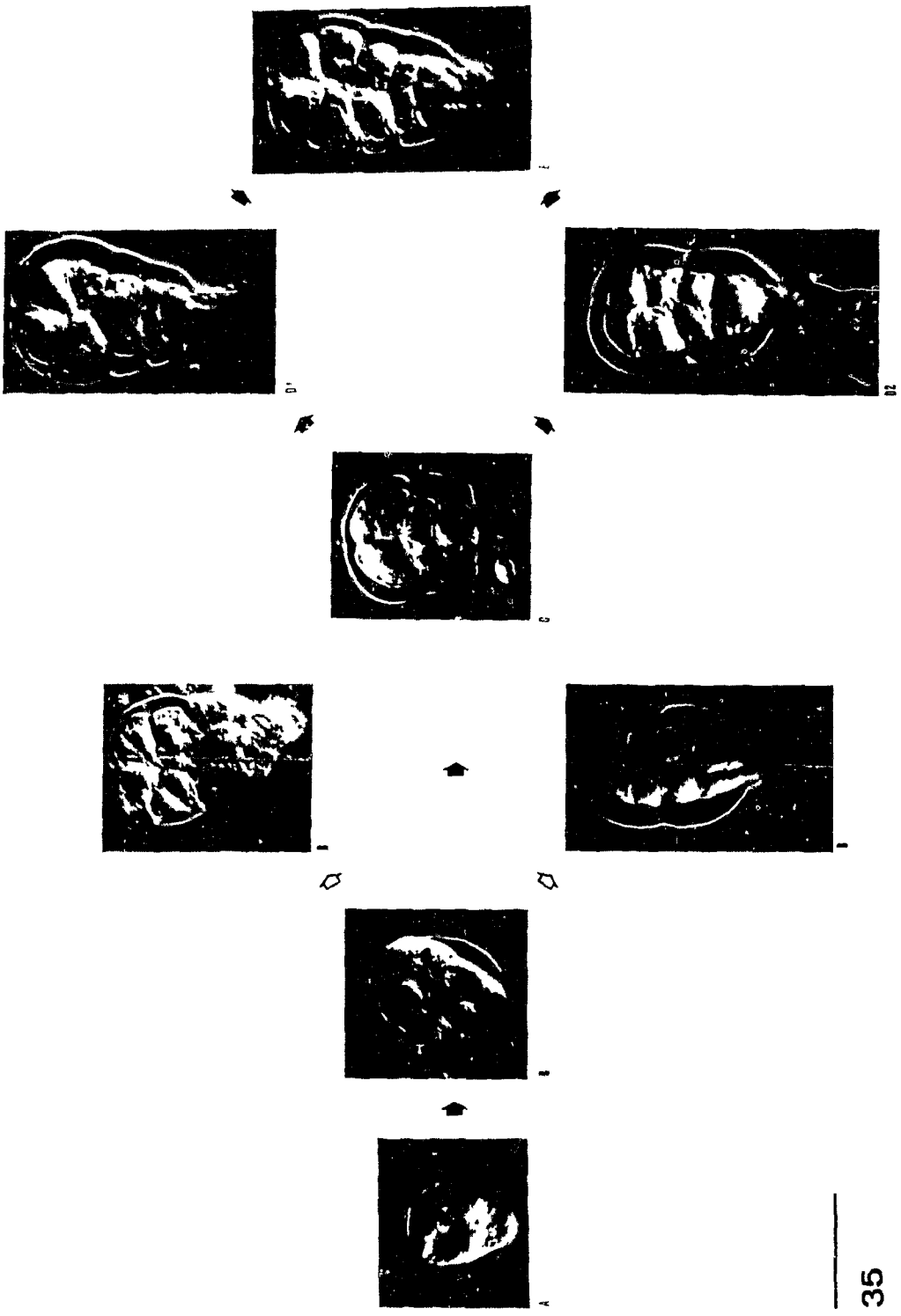

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Figure 36. Early developmental patterns of germinating sporelings (DIC illumination). Dark arrows represent the dominant developmental patterns, and hollow arrows represent infrequent alternatives. A and A' represent the upper two cells of the meiotic tetrad respectively and their subsequent divisions. B and B' represent the two lower cells of the meiotic tetrad respectively and their subsequent divisions. Subscript numbers represent cell divisions: 0 = original upper and lower cells,

1 = first division, 2 = second division, 3 = third division.

Scale = 10 μm.

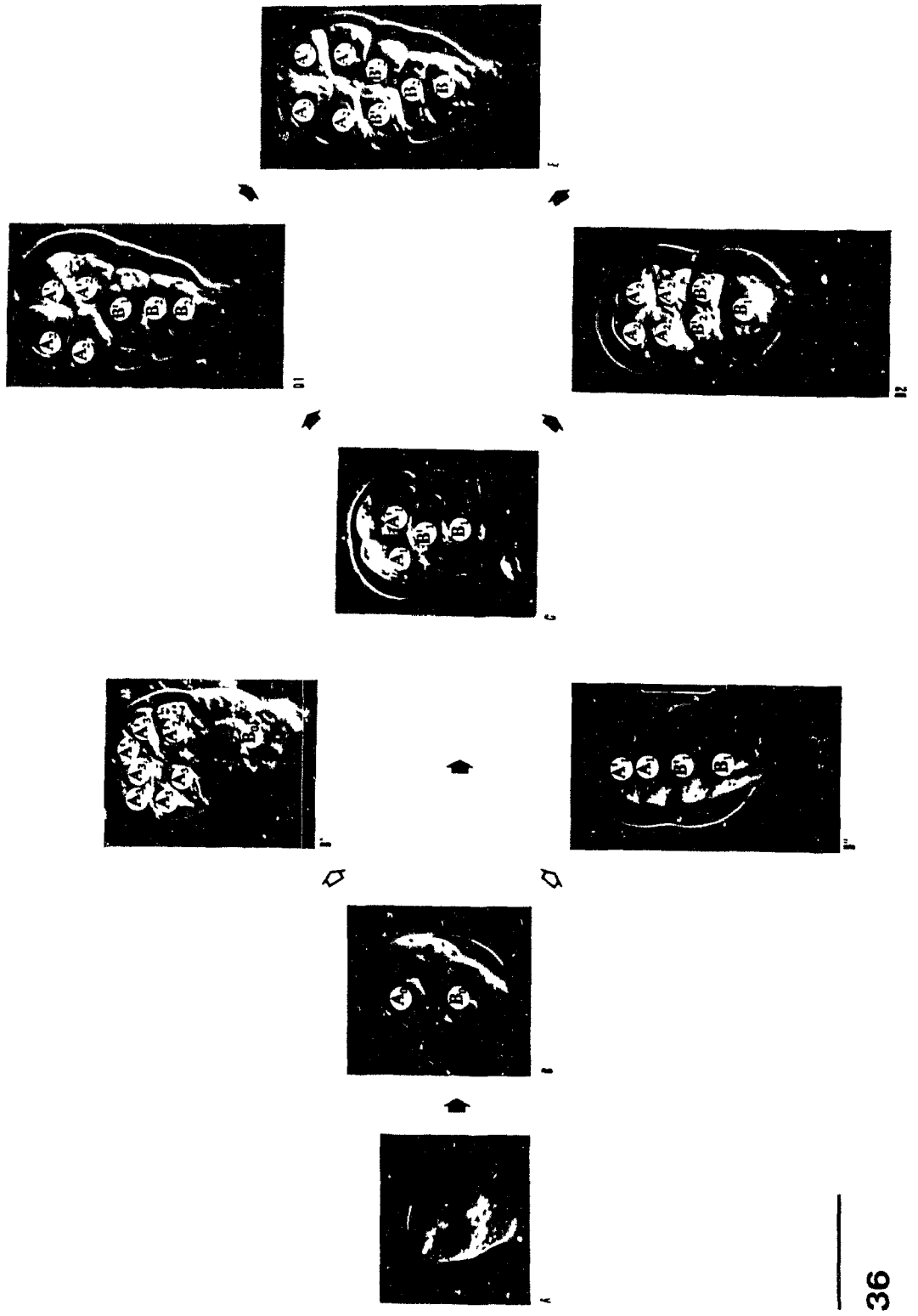

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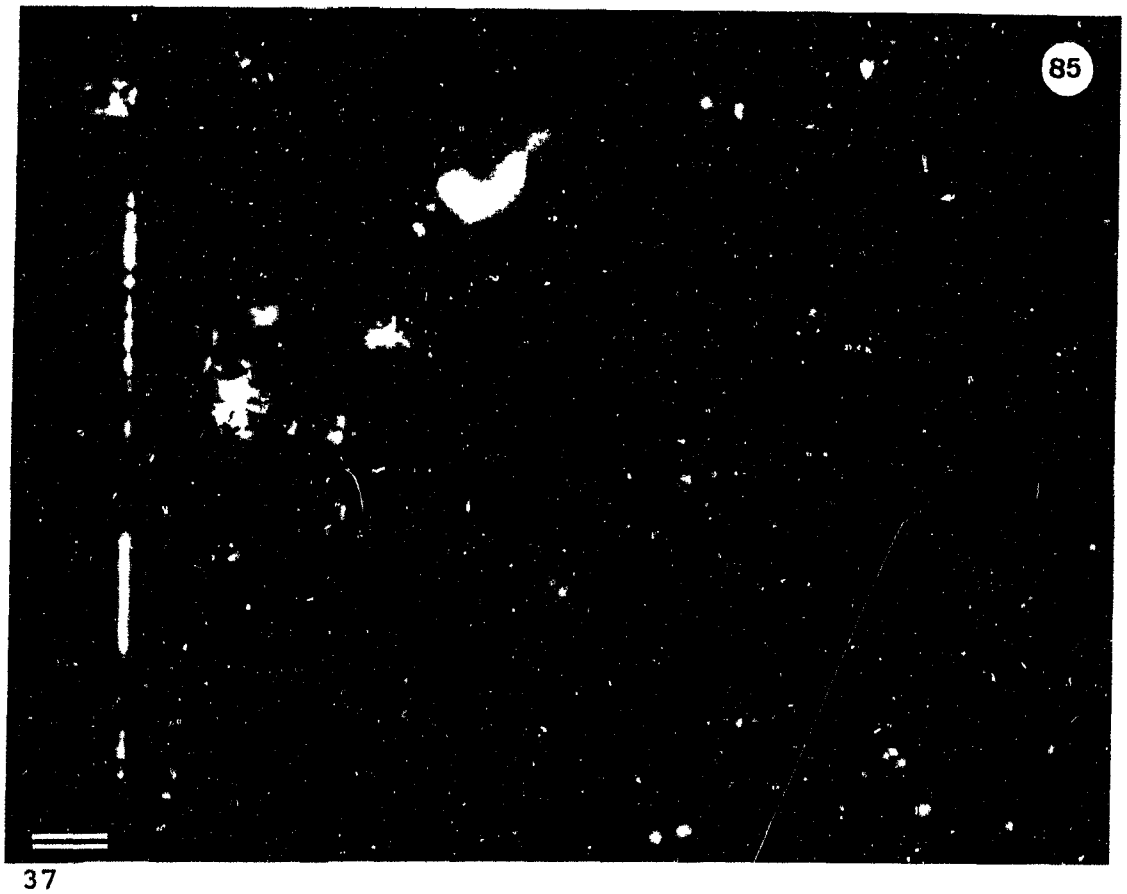
Figure 37. Early blade development of WT♀ X YELLOW♂ showing segregation pattern of mutant and normal sectors.

Scale = 1 mm.

Figure 38. Interpretation of early blade development of WT♀ X YELLOW♂ showing segregation pattern of mutant and normal sectors.

Y = yellow sector, R = wt sector, dashed lines represent division between two putative sectors of the same color. Scale = 1 mm.



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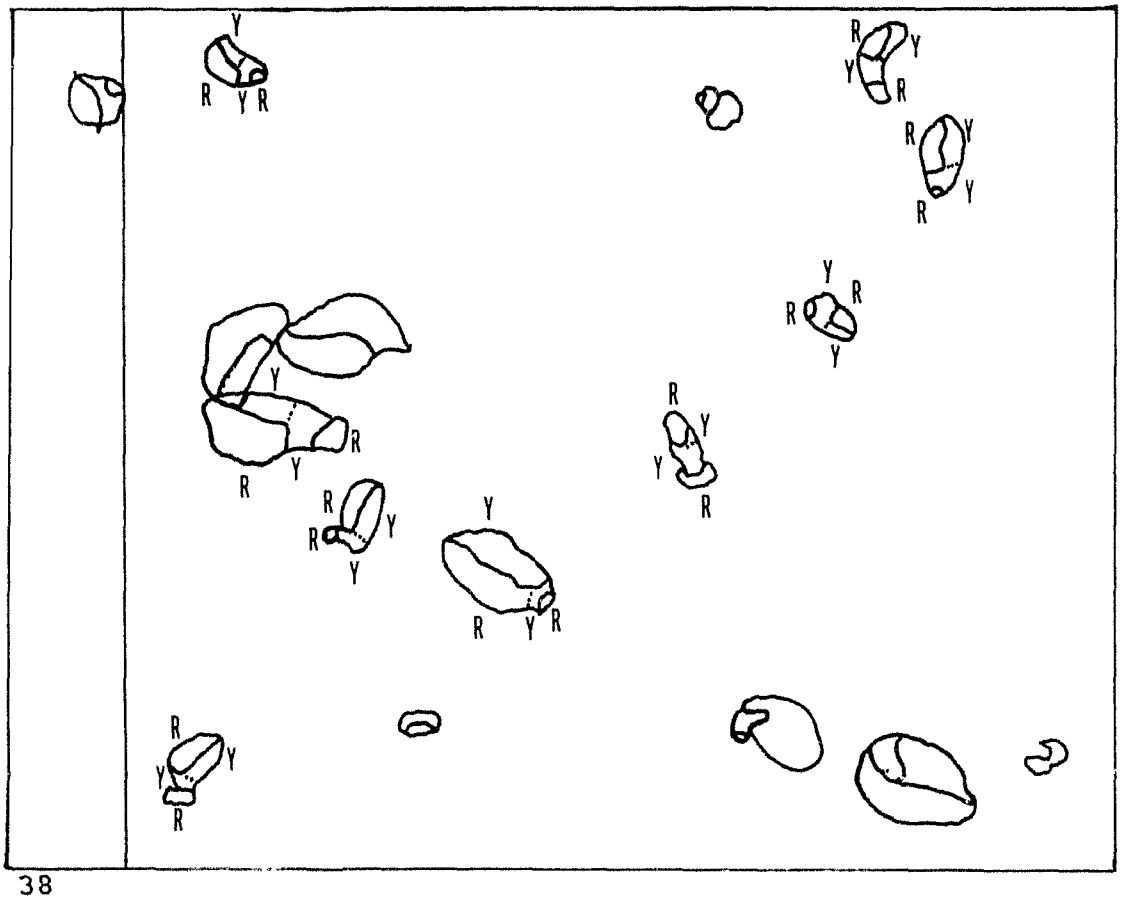

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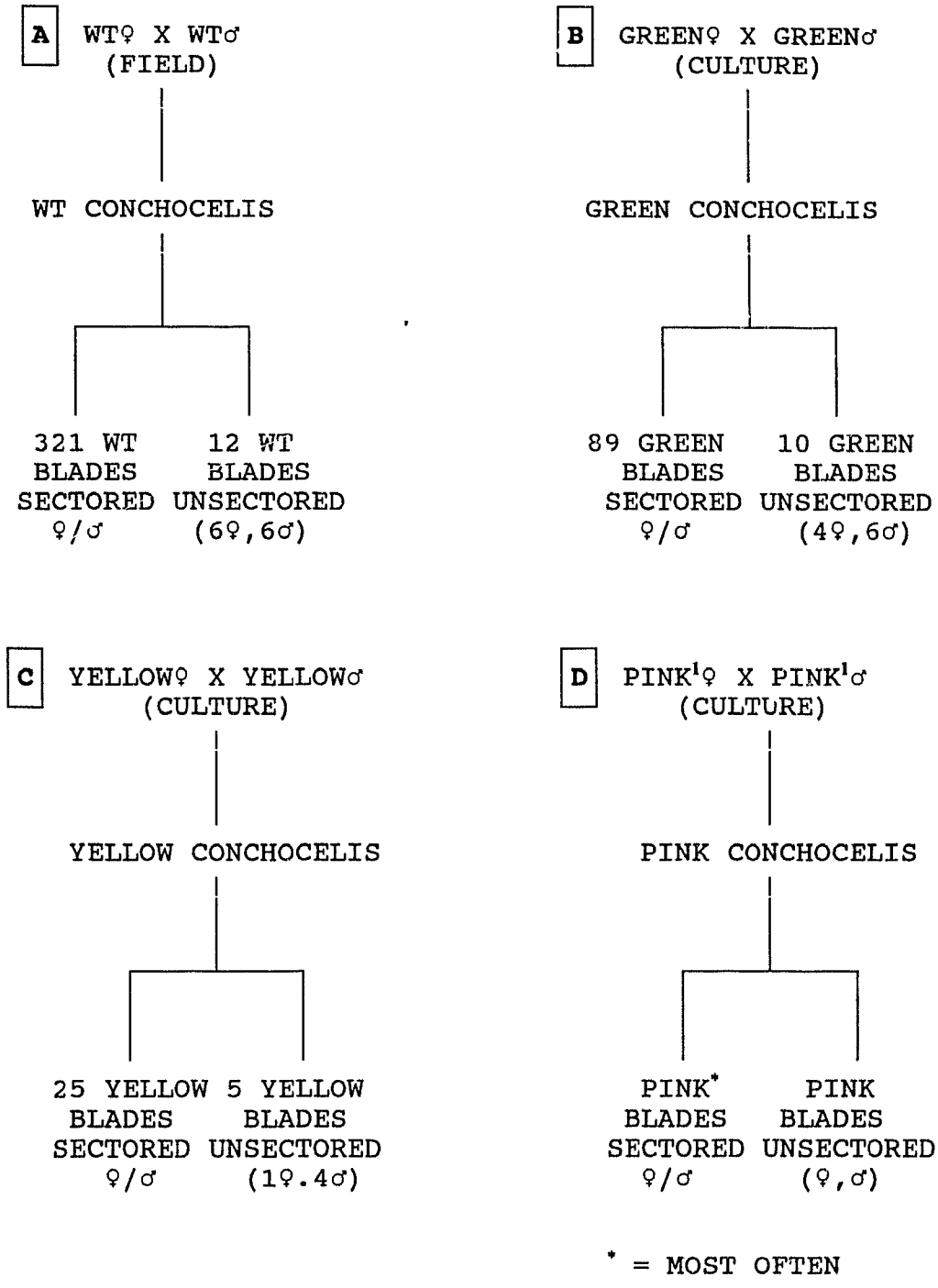

Figure 39. A summary of the transmission patterns observed for proposed sex determining alleles.

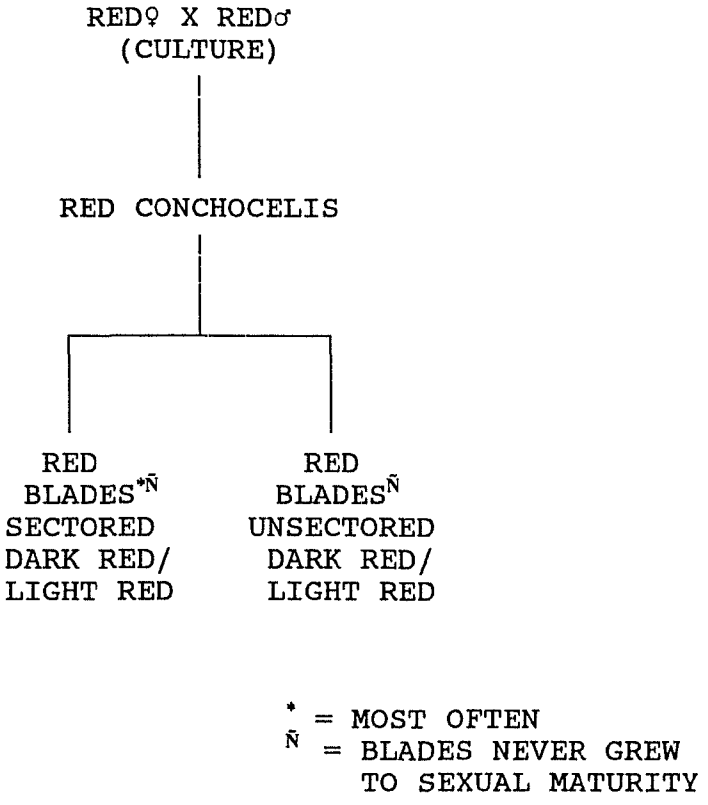

Figure 40. A summary of the transmission patterns observed for proposed sex determining alleles (continued).

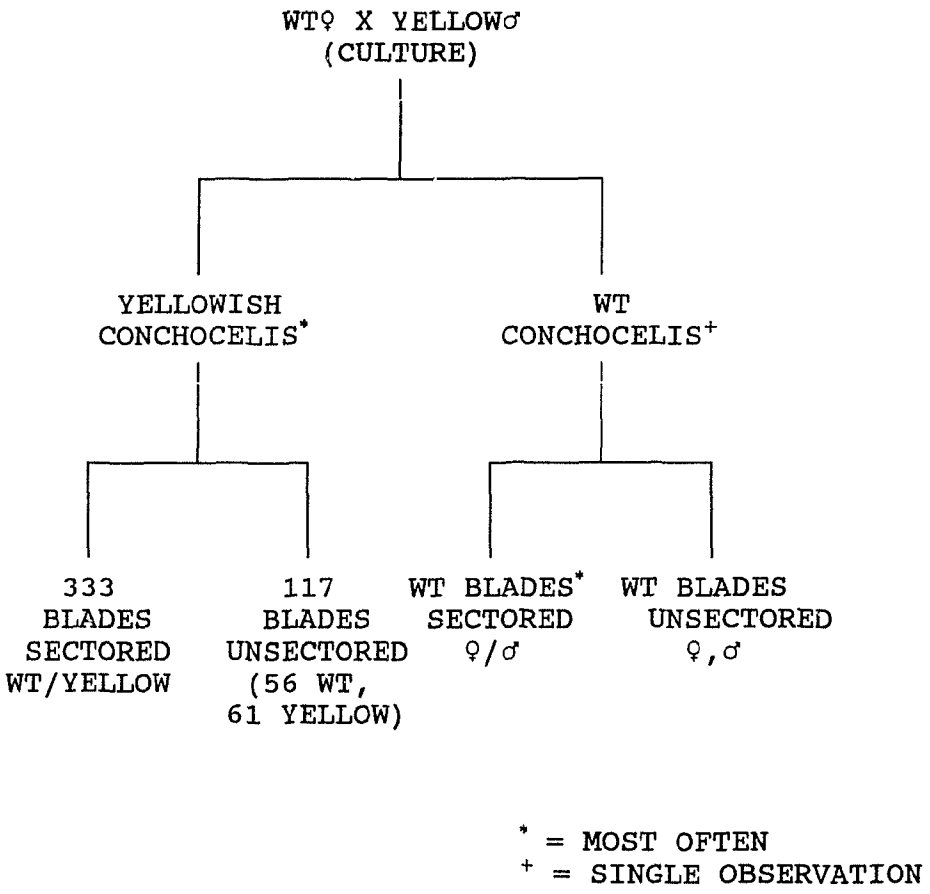

Figure 41. A summary of the transmission patterns observed for proposed color determining alleles.

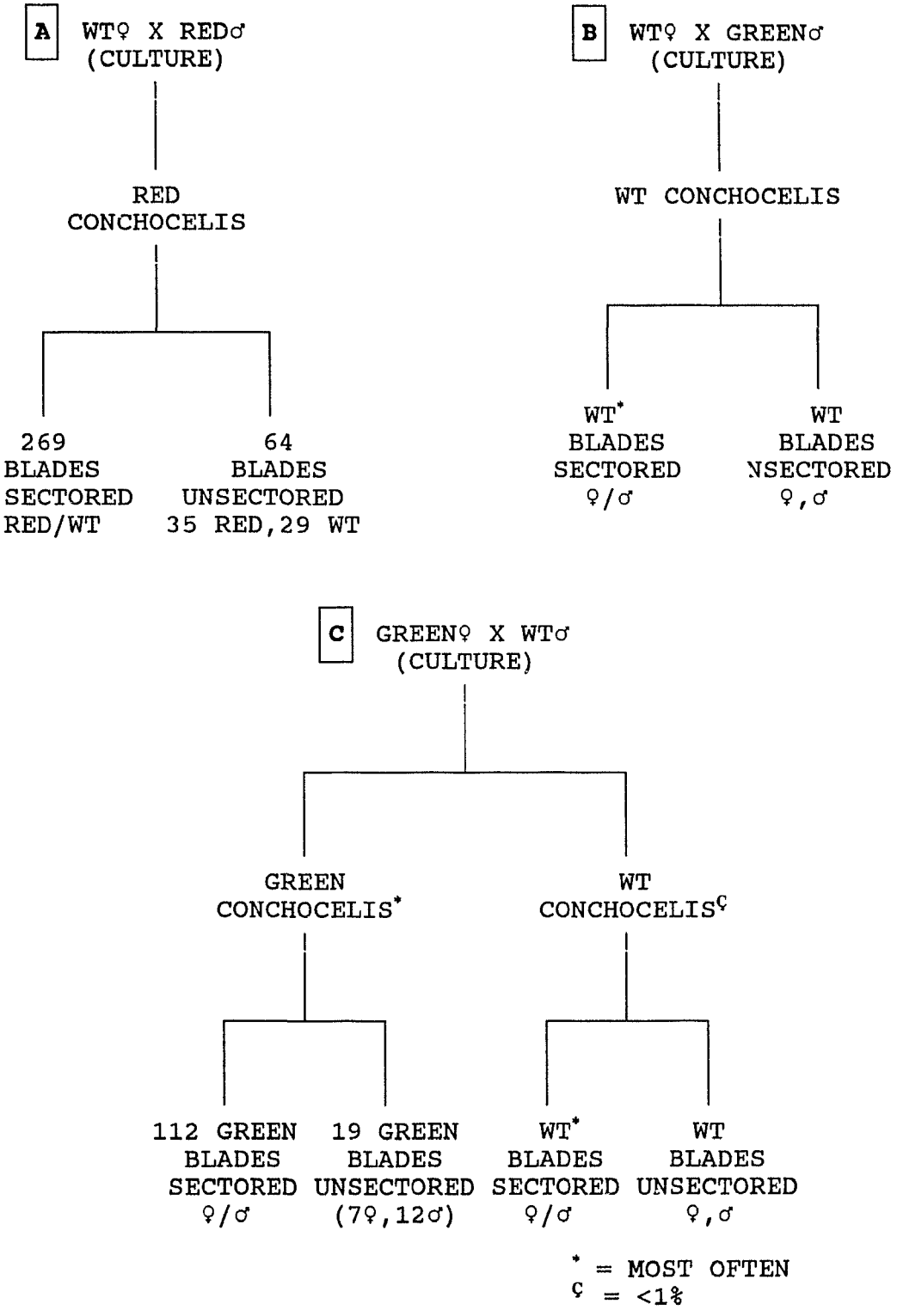

Figure 42. A summary of the transmission patterns observed for proposed color determining alleles (continued).

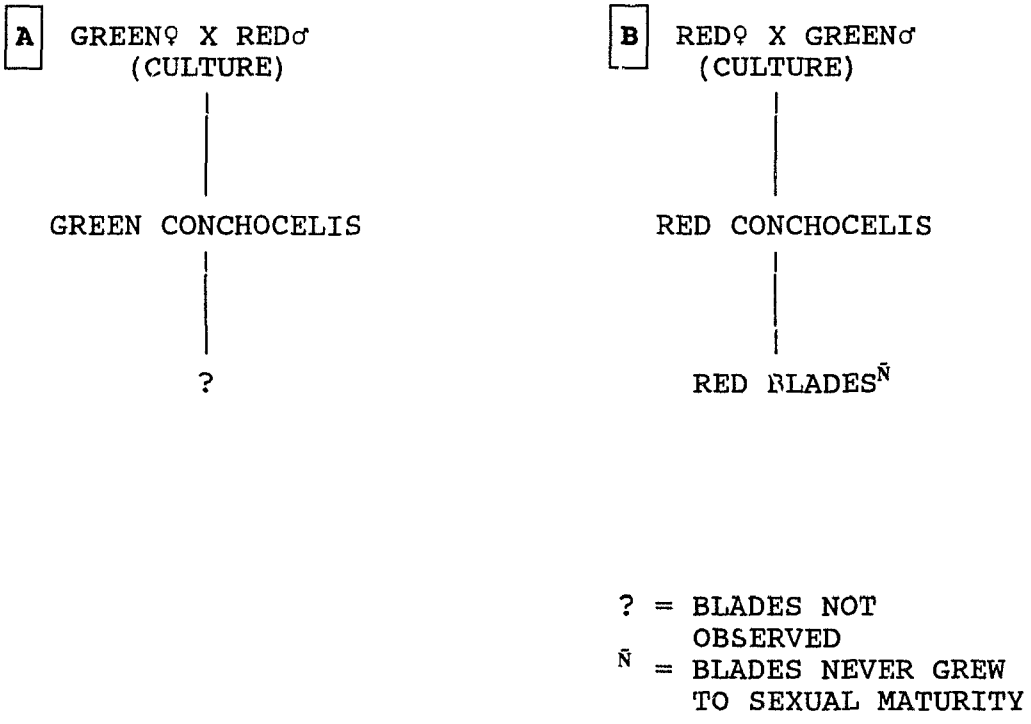

Figure 43. A summary of the transmission patterns observed for proposed color determining alleles (continued).

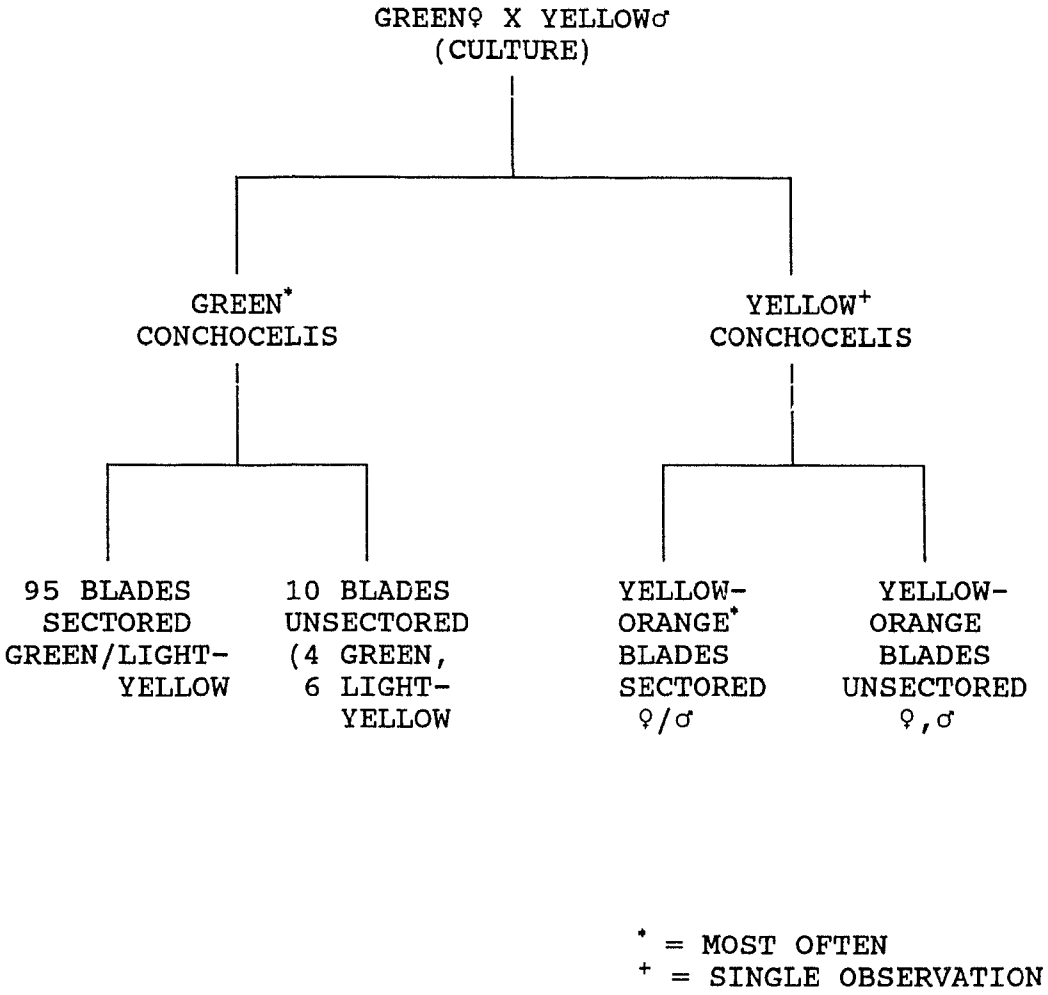

Figure 44. A summary of the transmission patterns observed for proposed color determining alleles (continued).

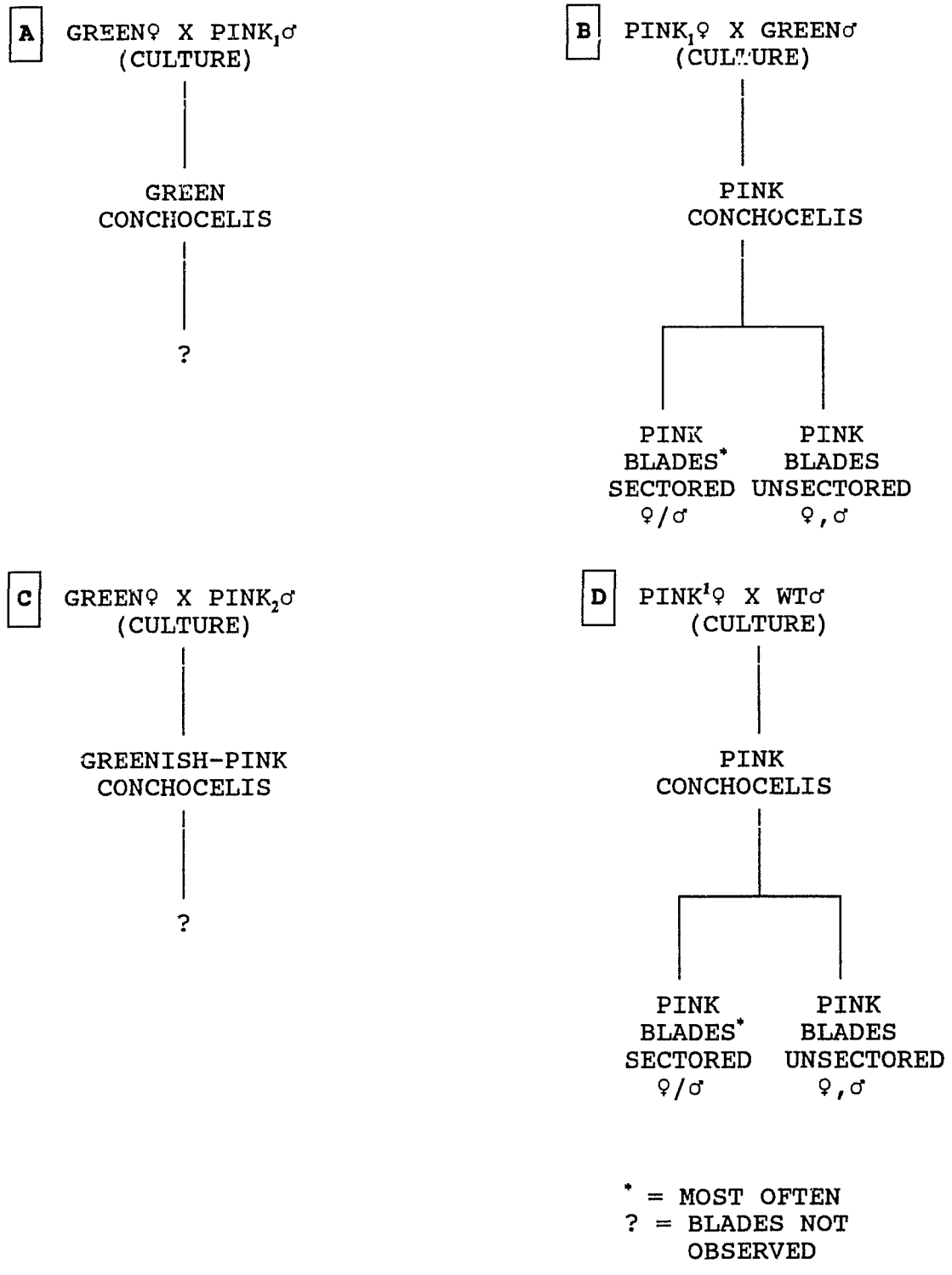

Figure 45. A summary of the transmission patterns observed for proposed color determining alleles (continued).

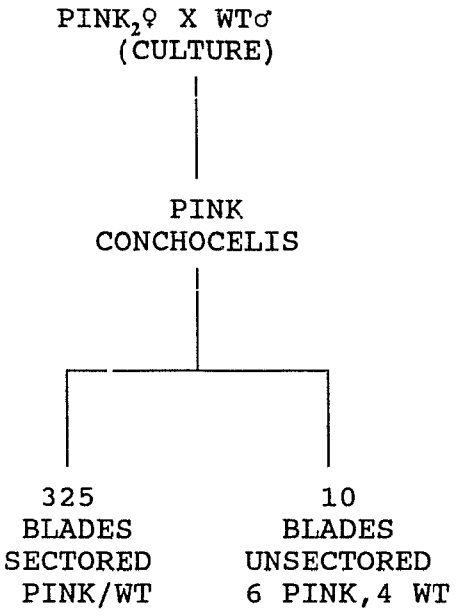

Figure 46. A summary of the transmission patterns observed for proposed color determining alleles (continued).

Table 4. Summary of conchocelis and F_1 blade phenotypes from crosses in culture

Cross	Conchocelis Phenotype	F_1 blade Phenotype
YELLOW♀ X YELLOW♂	YELLOW	YELLOW♀/YELLOW♂^{L}
WT♀ X YELLOW♂	YELLOWISH (WT)$^{+}$	WT/YELLOW^{L} (WT♀,WT♂)$^{+L}$
WT♀ X GREEN♂	WT	WT♀/WT♂^{L}
GREEN♀ X WT♂	GREEN (WT)^{Ç}	GREEN♀/GREEN♂^{L}, (WT♀/WT♂)^{ÇL}
GREEN♀ X RED♂	GREEN	?
GREEN♀ X GREEN♂	GREEN	GREEN♀/GREEN♂^{L}
GREEN♀ X $PINK_1$♂	GREEN	?
GREEN♀ X $PINK_2$♂	GREENISH-PINK	?
GREEN♀ X YELLOW♂	GREEN (YELLOW)$^{+}$	GREEN/LIGHT-YELLOW,^{S} (YELLOW♀/YELLOW♂)$^{+L}$
$PINK_1$♀ X $PINK_1$♂	PINK	PINK♀/PINK♂^{L}
$PINK_1$♀ X GREEN♂	PINK	PINK♀/PINK♂^{L}
$PINK_1$♀ X WT♂	PINK	PINK♀/PINK♂^{L}
$PINK_2$♀ X WT♂	PINK	PINK/WT^{L}
RED♀ X RED♂	RED	DARK RED/ LIGHT RED^{ÑS}
WT♀ X RED♂	RED	RED/WT^{S}
RED♀ X GREEN♂	RED	RED^{ÑS}

^{S} = SMALL PLANTS < 1CM
^{L} = LARGE SEXUALLY MATURE PLANTS > 15CM
? = BLADES NEVER OBSERVED
$^{+}$ = SINGLE OBSERVATION
^{Ñ} = BLADES NEVER GREW TO SEXUAL MATURITY, BUT DARK RED IS PRESUMED ♀ AND LIGHT RED PRESUMED ♂
^{Ç} = <1%

Table 5a. Percentage of sectored blades from crosses

Cross	Sectored	Unsectored	Total	% Sectored
WT♀ X WT♂ (FIELD)[L]	WT♀/WT♂ 321	WT♀,WT♂ 12	333	96.4
$PINK_2$♀ X WT♂ (CULTURE)[L]	PINK/WT 325	PINK,WT 10	335	97.0
WT♀ X RED♂ (CULTURE)[S]	RED/WT 269	RED,WT 64	333	80.8
WT♀ X RED♂ (CULTURE)[L;]	WT♀/WT♂ 7	WT♀,WT♂ 178	185	3.8
WT♀ X YELLOW♂ (CULTURE)[S]	WT/YELLOW 333	WT,YELLOW 117	450	74.0
WT♀ X YELLOW♂ (CULTURE)[L;;]	WT/YELLOW 35	WT,YELLOW 90	125	28.0
YELLOW♀ X YELLOW♂ (CULTURE)[L]	YELLOW♀/ YELLOW♂ 25	YELLOW♀, YELLOW♂ 5	30	83.3
WT♀ X GREEN♂ (CULTURE)[L]	GREEN♀/ GREEN♂ 112	GREEN♀, GREEN♂ 19	131	85.5
GREEN♀ X GREEN♂ (CULTURE)[L]	GREEN♀/ GREEN♂ 89	GREEN♀, GREEN♂ 10	99	89.9
GREEN♀ X YELLOW♂ (CULTURE)[S]	GREEN/ LIGHT-YELLOW 95	GREEN, LIGHT-YELLOW 10	105	90.5

[S] = small plants < 1cm
[L] = large sexually mature plants > 15cm
[;] = based on surviving wt plants and wt portions of sectored plants
[;;] = based on surviving portions of plants

Table 5b. Recombination analyses from crosses

Cross	% Recombination
WT♀ X WT♂ (FIELD)^{L}	48.2 SEX LOCUS→CENTROMERE
$PINK_2$♀ X WT♂ (CULTURE)^{L}	48.5 $PINK_2$ LOCUS→CENTROMERE
WT♀ X RED♂ (CULTURE)^{S}	40.4 RED LOCUS→CENTROMERE
WT♀ X YELLOW♂ (CULTURE)^{S}	37.0 YELLOW LOCUS→CENTROMERE
YELLOW♀ X YELLOW♂ (CULTURE)^{L}	41.6 SEX LOCUS→CENTROMERE
GREEN♀ X WT♂ (CULTURE)^{L}	42.8 SEX LOCUS→CENTROMERE
GREEN♀ X GREEN♂ (CULTURE)^{L}	45.0 SEX LOCUS→CENTROMERE
GREEN♀ X YELLOW♂ (CULTURE)^{S}	45.2 YELLOW LOCUS→CENTROMERE

^{S} = small plants < 1cm
^{L} = large sexually mature plants > 15cm

TABLE 6. A summary of the co-transmission patterns observed for proposed sex and color determining alleles in sexually mature blades (linkage analysis).

WT♀ X YELLOW♂

	WT♀/YELLOW♂		YELLOW♀/WT♂		TOTAL
BLADES SECTORED WT/YELLOW	17		18		35
	YELLOW♀	YELLOW♂	WT♀	WT♂	TOTAL
BLADES UNSECTORED WT,YELLOW	23	24	16	27	90

$PINK_2$♀ X WT♂

	PINK♀/WT♂		WT♀/PINK♂		TOTAL
BLADES SECTORED PINK/WT	120		205		325
	PINK♀	PINK♂	WT♀	WT♂	TOTAL
BLADES UNSECTORED PINK,WT	2	4	3	1	10

WT♀ X RED♂

	WT♀/WT♂	WT♀	WT♂	TOTAL
SELECTED WILD TYPE SECTORS; SECTORED AND UNSECTORED FOR SEX	7	89	89	185

Figure 47. F_1 gametophyte blade from the WT♀ X RED♂ cross. Color of the left half is wt, and color of the right half is red. Scale = 1 mm.

Figure 48. F_1 gametophyte blade from the WT♀ X YELLOW♂ cross. The phenotype of the left half is yellow male and the phenotype of the right half is wt female. Note coincidence of sex and color boundaries. Scale = 15 cm.

Figure 49. Top left: F_1 gametophyte blade from the YELLOW♀ X YELLOW♂ cross. Phenotype is yellow male (bulk of blade) and yellow female (upper left wedge laying over the rest of the blade). Top right: F_1 gametophyte blade from WT♀ X WT♂ in culture. Phenotype is upper half male and lower half female. Bottom: F_1 gametophyte blade from the GREEN♀ X GREEN♂ cross. Phenotype is upper half female and lower half male. Scale = 15 cm.

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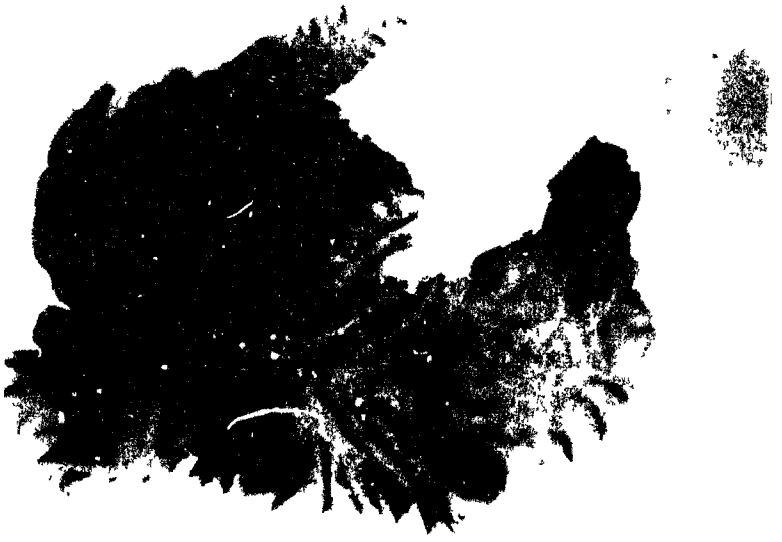
Figure 50. F_1 gametophytes of the PINK$_2$♀ X WT♂ cross. Left: Phenotype is left side wt male and right side pink female. Right: Phenotype is right side wt female and left side pink male. Note coincidence of sex and color boundaries. Scale = 10 cm.

Figure 51. F_1 gametophyte of the GREEN♀ X YELLOW♂ cross. Phenotype is upper half light-yellow male and lower half green female. Note coincidence of sex and color boundaries.

Scale = 15 cm.

50



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IV. DISCUSSION

A. Culture Conditions

Determination of good growth conditions for *P. umbilicalis* in culture was critical for the present study. It has become evident that as the spores of certain algae mature in nature, environmental signals may trigger their maturation and release. These signals may include pH, nutrients, desiccation, light intensity, salinity, temperature, photoperiod, or possibly some of these factors in combination or in a specific sequence. Although it was not the objective of this study to perform growth experiments, it was nevertheless essential to determine appropriate culture conditions for growth and manipulation of the life cycle in *P. umbilicalis* in order that its genetics and development could subsequently be studied.

Culture medium selection is one of the first considerations when growing algae of any kind. Many different enriched media exist, and every individual laboratory seems to make its own changes to these

established media. In addition, individual algal species often respond differently to specific media with respect to growth and sporulation. Such was the case in this study. Initially, various media that were available at IMB were tested, and eventually it was determined that sterile filtered SWM-4 [without added soil or liver extract, and without tris buffer (McLachlan, 1973)] produced the best growth. However, subsequent to these trials, D-11 medium was suggested (L. C.-M. Chen, personal communication) as a good medium for *Porphyra* culture. After some modifications (Table 1), this medium replaced SWM-4 in these studies and proved to be an exemplary culture medium for growth and manipulation of the life cycle of *P. umbilicalis*.

One modification that was made to the SWM-4 medium was that the optional tris (0.0-5 nM) was not added to the medium. While tris offered a good degree of buffering, it promoted excessive bacterial growth which decreased the vitality of the cultures. In order to alleviate pH increases in rapidly growing cultures, or in cultures with high plant density, the enriched seawater medium was simply replaced frequently. SWM-4 and D-11 in contrast to SWM-1, SWM-2 AND SWM-3 are sterile-filtered (McLachlan 1973). This avoids phosphate, silicate or

trace metal precipitation which may sometimes occur during autoclaving (Hamiliton 1973).

Another important aspect pertaining to culture conditions was the determination of appropriate conditions for the manipulation of the life cycle. The control of conchospore induction (Table 2) and release (Table 3) in the sporophyte phase, and the growth of gametophytes to reproductive maturity, was critical to the subsequent study of the genetics and development in *P. umbilicalis*.

Many researchers have investigated conchospore induction and release in *Porphyra* species. Although, many environmental signals may be involved, light intensity, photoperiod and temperature are considered most important (Kurogi and Akiyama 1966, Dring 1967, 1984, Rentschler 1967, Kapraun and Luster 1980, Freshwater and Kapraun 1986, Kapraun and Lemus 1987, Waaland *et al.* 1990). In this study, light intensity was only investigated sufficiently to determine appropriate levels for conchocelis (40 μmol $\cdot$ $m^{-2} \cdot s^{-1}$) and blade growth (60 μmol $\cdot$ $m^{-2} \cdot s^{-1}$). The interaction between temperature and photoperiod was also briefly investigated. Maximum conchospore induction and release were found to be dependent on certain combinations of these two factors. It

appeared that conchospore induction in *P. umbilicalis* was a result of a long day (LD) photoperiod response (Table 2). This is consistent with reports of previous investigators who demonstrated that conchospore induction in other species of *Porphyra* is due to either a LD or a short day (SD) photoperiodic response, depending upon the species examined (Dring 1967, 1984, Rentschler 1967). The conditions for conchospore formation in *P. umbilicalis* are not surprising since they correspond to summertime conditions when thalli appear in nature.

In contrast to spore formation, substantial conchospore release occurred as result of a LD response in combination with a specific temperature (13 °C). A decrease in temperature was not necessary for spore release, as it is for some other species of *Porphyra* (Kurogi and Hirano 1956, Chen *et al.* 1970, Miura 1975, Avila *et al.* 1986, Melvin *et al.* 1986). Only the temperature of 13°C was critical for conchospore release, and after about a one week induction period, sporulation occurred. This requirement is similar to that observed by Bird *et al.* (1972) for *P. linearis*, where a specific temperature was also critical for conchospore release. Since there is considerable fluctuation in temperature during the summer, both above and below 13°C, and during

each tidal cycle, spore release in nature is readily triggered at periods that coincide with the appearance of blades during the summer months.

Determination of conditions needed for blade growth to reproductive maturity from conchospores was also essential to this study. It was important in order to study blade development patterns, perform controlled crosses using mutant blades, and to obtain large fronds needed for genetic analysis. One of the considerations in culturing *Porphyra* blades was the selection of an appropriate culture vessel. Aspirator bottles proved to be ideal. When an air line was connected to the bottle outlet, it supplied the vigorous aeration necessary to circulate the nutrient medium and fronds. These bottles kept the plants in constant circulation without having them float to the surface or getting them tangled on internal tubing, both common problems in other vessels.

In culture it appeared that LD photoperiod and nutrient supply were the most important conditions necessary for plants to reach sexual maturity. Blades grown in flow-through bucket culture at the IMB Aquaculture Research Station developed to sexual maturity much more rapidly than those grown in the laboratory. This more rapid growth may be due to the constant influx of nutrients and CO_2, maintenance of pH,

flushing of bacteria and wastes and natural sunlight. Some of this could be achieved in culture, but only with weekly culture changes. It is not surprising that the culture conditions necessary to induce sexual development coincide with the summertime appearance of spermatia, carpogonia and zygotospores in *P. umbilicalis* blades in nature.

B. Nuclear Division and Chromosomes

In general, species of *Porphyra* have small numbers of chromosomes. Most observations (45) indicate N=3 with some reports having N=2 (10), N=4 (14), N=5 (14), N=6 (1), N=7 (1), and N=12-13 (1) (Cole 1991, Patwary and van der Meer in manuscript). In *P. umbilicalis* a chromosome number of n=5 was observed in the gametophyte. This agrees with Yabu's observations (1978) from Nova Scotia. In contrast, Kito *et al.* (1971) observed n=4 in *P. umbilicalis* from Nova Scotia, and this is generally considered to be the more common haploid number (Kapraun and Freshwater 1987). Furthermore, different haploid chromosome numbers have repeatedly been reported for

supposedly the same species. Clearly, published chromosome numbers are not by any means a reliable criterion for taxonomy in species of *Porphyra*.

Observations of germinating conchospores in *P. umbilicalis* demonstrate that meiosis I occurs in the first division of the developing blade, and meiosis II occurs in the second division. All of the observations in this study indicate that released conchospores are in interphase and meiosis does not begin until spore germination. These results are consistent with recent observations made by other researchers: *P. yezoensis* (Ma and Miura 1984; Ohme and Miura 1988; Tseng and Sun 1989); *P. torta* Krish. (Burzycki and Waaland 1987); *P. tenera* (Tseng and Sun 1989).

Although many previous workers concluded that the temporal position of meiosis occurred at various sites, all observations in the current study indicate that meiosis occurs exclusively in the germinating conchospore. This is generally consistent with Ma and Miura (1984), and Ohme *et al.* (1986); however present observations do not support the idea that meiosis is initiated in the conchosporangial branch and concludes during conchospore germination.

In any event, there is insufficient, recent, reliable evidence confirming these previous observations or hypotheses that meiosis originates in the conchosporangia. Possibly, certain observations of meiotic pairing may be misinterpretations of somatic pairing which has been reported for *Bangia* Lyngb. (Cole *et al.* 1983), conchocelis filaments of *P. tenera* (Sun and Tseng 1987), and the conchosporangial branches of *P. torta* (Burzycki and Waaland 1987). Moreover, Burzycki and Waaland (1987) and Guiry (1991) suggest that meiosis may not be a fixed event and could vary according to species or growing conditions, and could either occur or begin in the conchosporangium. This hypothesis seems likely, especially if conchosporogenesis is completed and sporulation is delayed or arrested due to unsuitable culture conditions. Meiosis might be initiated in the conchosporangium and possibly become arrested at some point until normal sporulation can be initiated. For instance, Bird *et al.* (1972) noted that deterioration of the conchosporangial wall was often necessary for sporulation in *P. linearis* in culture and may sometimes be the case for other algae. Giraud and Magne (1968) describe their observations of meiosis in a conchosporangial filament of *P. umbilicalis* (as *P. umbilicalis* var.

laciniata), but also conceded that their interpretation was difficult because of the absence of morphological details and this putative site of meiosis should be studied further.

Even though meiosis has now been observed to take place entirely in the germinating conchospores for four species of *Porphyra*, there is not enough evidence to conclude that it is "universal for all monoecious species in the genus" as Tseng and Sun (1989) suggest. However, it may well be a very common feature shared by many monoecious or dioecious (none have been examined yet) species of *Porphyra*.

There are also reports that some species of *Porphyra* are apomeiotic and apogamic: e.g., *P. brumalis* Mumford (Mumford and Cole 1977), *P. carolinensis* Coll et Cox (Freshwater and Kapraun 1986; Kapraun and Freshwater 1987), *P. drewii* (Coll and Oliveira 1989), *P. fucicola* Krish. (Krishnamurthy 1984), *P. leucosticta* (Coll and Oliveira 1977), *P. maculosa* Conway (Conway *et al.* 1975) *P. papenfussi* Krish. (Conway and Cole 1973), *P. pseudolanceolata* (Conway *et al.* 1975; Krishnamurthy 1984), *P. rosengurtii* Coll et Cox (Kapraun and Freshwater 1987; Kapraun and Luster 1980), *P. spiralis* var. *amplifolia* Oliveira Filho et Coll (Kapraun and Freshwater 1987), *P. variegata*

(Mumford and Cole 1977). These reports raise questions, in light of the recent, fully documented reports of sexual reproduction in other species. Even though these species have spermatia, and carpogonia, and release "spores" (=zygotospores? unfertilized carpogonia?) that develop into conchocelis; according to the researchers, all phases are haploid. A hypothesis for this phenomenon is that certain species with southern and northern distributions lack sexual reproduction (Kapraun and Freshwater 1987). However, this apparent disfunction of sex may be attributable to other factors and should be further examined; especially since many of these reports were made before it was determined that the position of meiosis is in the germinating conchospore.

There are some additional alternative possibilities to consider concerning apomeiosis. First, fertilization is often a difficult event to detect both in the field and in culture. This is substantiated by the many misinterpretations made previously regarding fertilization in the Bangiophycidae (Dixon 1973). Second, the temporal position of meiosis is often difficult to determine and even distinguish from mitosis (as is evidenced by inaccurate interpretations of somatic pairing). Third, several researchers have observed that occasionally unfertilized

carpogonia germinate into haploid conchocelis (Krishnamurthy 1959; Polne-Fuller and Gibor 1984; L. C.-M. Chen (pers. com.)). Finally, chromosome observations are incomplete for cells of different phases in these reports (Tseng and Sun 1989), with much of the existing photographic documentation ambiguous.

Other species of *Porphyra* certainly should be critically examined or re-examined to ascertain the presence or absence of meiosis in the germinating conchospores before any "universal" generalizations are made. Although chromosome observations support meiosis in the germinating conchospores for some species, the introduction of color mutant markers certainly would serve to substantiate the temporal position of the reduction division. Subsequent sections of this thesis will discuss using color mutants as a genetic tool, which in conjunction with microscopy can be used to ascertain and confirm the position of meiosis, as well as help follow the subsequent blade development of any species with markers.

C. Mutagenesis

Mutations can be separated into two categories, spontaneous and induced. Spontaneous mutations are randomly produced in nature as the result of nucleotide sequence changes in genes. In the Rhodophyta, there are many examples of spontaneous color mutations collected from nature or arising in culture (van der Meer 1991). However, this is not the case for *Porphyra* species. Only seven naturally occurring color mutations have ever been reported thus far, all in *P. yezoensis* (Miura 1975, 1977; Migita and Fujita 1983;). Detailed analyses have determined that there are four genes in three linkage groups. Alone or in combinations, the alleles of these genes determine the phenotypes for wild type, red, yellow, light red, light green and light yellow (Miura 1985). In *P. umbilicalis* thousands of plants were examined, both from the field and in culture and no spontaneous color mutations were ever observed.

Induced mutations are produced as the result of a specific chemical or physical agent (mutagen) altering the DNA structure. Many color and morphological mutations have been isolated for the Rhodophyta,

predominately by van der Meer (1991) as a result of treatment with chemical mutagens (EMS, MMS, and NG). Katayama (1983, 1984) tested the effect of a number of compounds (EMS, NG, 4-Nitroquinoline, Nitrous Oxide, N-Nitroso-N-methylurethanol(NMU), Mercury Chloride, Sodium Azide, Diethyl Sulfonate, Phenylmercuric acetate, Colchicine, β-Indoleacetic Acid, Cumarine, Urethane and Gibberellin A_3) on the conchocelis stage of *P. yezoensis* f. *narawaensis*, *P. yezoensis*, and *P. tenera*. Although he produced some putative mutant blades, from treatment with NG (100 μgmL^{-1}), they grew slowly with irregular cell divisions and enlarged to only a few millimeters in length before they died. He also produced green conchocelis from treatments with NMU (100 μgmL^{-1}), but it was never isolated from wild type and was eventually overgrown by wild type. In addition, even though some green mutant blades were produced from this mixed conchocelis, they never grew any larger than 2 cm (Katayama 1984). Finally, many of his putative mutants consisted of gametophytes with unusually large cells. However, this is unlikely a mutagenic effect since such cells were also common in many untreated control calli of *P umbilicalis*.

Of the three mutagens, EMS, MMS, and NG, used to treat *P. umbilicalis*, only NG was effective in this study. In contrast to Katyama's experiments (1983, 1984), haploid thalli were treated in addition to diploid conchocelis. The only color mutants detected were from treatments of minced blades and germinating conchospores. This result is not surprising since recessive mutants and mutant sectors would preferentially be detected in the haploid gametophytes where they are not masked by wild type alleles. In addition, treatment of minced thalli and nascent sporelings from germinating conchospores may allow better penetration of the mutagen into the cells because of disrupted or thinner cell walls respectively. Furthermore, since the nascent sporelings are the site of meiosis, mutations are more likely to persist as cell lines and constitute a sizable sector on the developing blade.

Much of the success in producing and growing mutant blades of *P. umbilicalis* to maturity was due to the application of the proper time/concentration combinations during mutagenesis, followed by immediate transfer to appropriate culture conditions. These conditions included:

1. Trimming off wild type tissue from mutant sectors so they were not overgrown;

2. Culturing small cell clusters of mutant tissue into calli, and then maintaining them in culture until blades were produced (sometimes several years); and

3. Frequent medium changes for blades grown from calli, to prevent reversion back to calli.

It is not surprising that Katayama had difficulty growing his putative mutations to maturity, for some of his treatments used long treatment times (to 20 days), high concentration of mutagen (100-200 μgmL^{-1}), multiple combinations of mutagens, and finally, he did not excise putative mutant sectors from wild type.

P. umbilicalis proved to be quite resistant to mutagenesis from treatments with EMS and MMS and displayed no visible effects except lethality upon prolonged exposure. Only NG proved effective enough to produce a small number of color mutants. All three of these mutagens are classified as alkylating agents, which can induce mutations in at a high frequency even in nonreplicating DNA. EMS and MMS alkylate guanine or thymine so that the altered base, pairs incorrectly, producing a

transition mutation (AT-GC and GC-AT) (Russell 1991). In addition, these mutagens may also ethylate guanine, causing the base to be removed by depurination. Mutation results from faulty repai with any of the four available bases filling the gap (Tamarin 1991). Similarly, NG may cause specific mispairing by methylation (alkylation) of guanine and subsequent mispairing with thymine which will produce a GC-AT transition after two replication cycles (Prescott *et al.* 1990). However, by a different reaction, NG may also cross link DNA strands causing the faulty region to be excised by DNase. This action causes either point mutations or deletions (Brock 1991).

My results indicate that NG was the most effective mutagen tested for *P. umbilicalis*. This is consistent with van der Meer's (1991) observation that NG appears to be one of tne more potent mutagens for the Rhodophyta; however, he found EMS and MMS also to be useful. Moreover, NG has proved to be a more effective mutagen than EMS and MMS in many other species that are resistant to mutagenesis eg. cyanobacteria (Lewin, 1976). The mechanisms are obscure, but apparently different genes are preferentially mutated by one chemical over another for unknown reasons (Rothwell 1988).

D. Blade Development Patterns

The present study established the early pattern of development in *P. umbilicalis* by cell lineage analysis using color mutants introduced as genetic markers in conjunction with microscopic observations. The germinating conchospore, after two consecutive divisions, usually results in a meiotic tetrad consisting of two upper anticlinally divided cells and two lower periclinally divided cells. Each individual cell of the tetrad subsequently divides to produce a distinct sector in the developing blade. Consequently, through the introduction of the color marker, the inferences made by microscopic observations could be confirmed by observations of genetic segregation.

The pattern of thallus development in *P. umbilicalis* is almost entirely derived from the arrangement of the two upper anticlinally divided cells. These two cells divide to make up the bulk of the blade (composed of two sectors), whereas the two lower periclinally arranged cells only divide sufficiently to develop into a small rhizoidal area (composed of two sectors). Four distinct sectors were often observed in small blades, but as they matured the rhizoidal region was obscured.

Similarly, blade development in *P. yezoensis*, which in contrast germinates as a linear array, is also dependent upon the two upper cells in most cases. However, as a result chimeral thalli (from heterozygous conchocelis) are horizontally striped instead of vertically divided as in *P. umbilicalis*. Both of these observations are consistent with meiosis occurring in the germinating conchospore.

E. Genetic analysis of crosses

1. Sex-determining alleles

Sexual reproduction in *Porphyra* species is now considered to be widespread (Cole and Conway 1980, Ma and Miura 1984, Burzycki 1987, Tseng and Sun 1989). Although, there are still some reports of alternation of generation without change in ploidy (Freshwater and Kapraun 1986; Kapraun and Luster 1980), or older reports of no alternation of generations at all - *P. subtumens* J. Ag. (Laing 1928; Conway and Wylie 1972), *P. sanjuanensis* Krish. (Krishnamurthy 1969; Mumford 1973; Conway *et al.* 1975), such reports should be

independently confirmed. In view of the recent elegant demonstration of sexual reproduction in a number of species, and taking into consideration the complexities of determining culture conditions suitable for completing life histories, further study of apparently deviant species is essential for a more secure understanding of reproductive options in the genus.

Most species of *Porphyra* are monoecious, with both spermatia and carpogonia produced on the same thallus. There are three general patterns which may occur as blades mature sexually. For some species (eg. *P. yezoensis*), a fine-grained mosaic of small, scattered patches of spermatia and carpogonia occur throughout the fertile portions of the thallus. In other species, (eg. *P. leucosticta*), there is a coarser mosaic with spermatia and carpogonia in larger, often parallel, blocks of cells (5-20 mm). Finally, there are a number of species in which the thallus becomes longitudinally divided, with male and female tissue confined to separate halves of the thallus; this is the situation for *P. umbilicalis*.

Mature plants of *P. umbilicalis* often have a divided appearance because the colors of the male and female tissue differ substantially. The male side becomes increasingly yellow with the onset of maturity due to degeneration of chloroplasts in the spermatia, whereas the female side

becomes dark red due to maturing carpogonia and zygotosporangia. The distinct, often central position of the demarcation between male and female tissue and the regularity with which such blades develop suggested that the mechanism for their development was probably different from that producing the finer grained mosaic patterns in other species.

Taking into consideration recent observations of meiosis in the germinating conchospores of three species of *Porphyra*, it became apparent that the bisected thalli of certain species might result directly from the genetic segregation of a pair of sex- determining alleles during the initial divisions of the sporelings. All that would be necessary to generate sexually bisected plants would be to have meiosis in the germinating conchospores, as found by the Japanese researchers, followed by a congruous cell division pattern in the germinating sporeling.

At this point it became important to establish whether sex was determined by genetic segregation of sex-determining alleles as hypothesized, or alternatively, whether striped blades occurred as a result of a sex determination mechanism activated soon after meiosis. That is, cells might become developmentally fixed for one sex or the other at an

early stage, still resulting in striped plants whose line of demarcation did not originate from meiotic segregation.

Evidence for segregation of sex-determining alleles is shown clearly by the results obtained from selfs of color mutants (green and yellow) and plants collected from the field. Consistently, these plants developed primarily as ♀/♂ bisected blades identical to the parental type. Moreover, male and female sectors taken collectively always occurred in a nearly perfect 1:1 ratio indicating sex determination is by means of a single pair of Mendelian factors. Furthermore, the high frequency of second division segregation indicates that the locus for sex determination is a considerable distance from the centromere. Consequently, crossing over between the centromere and the sex locus nearly always occurs. These results in conjunction with observations of early blade development, strongly support the hypothesis of Mendelian segregation of sex-determining alleles. In fact, it was the segregation of male and female sectors observed initially in plants from the field that inspired this study.

The frequencies of female/male sectored plants, and of color sectored plants, observed in this study were very high, and generally

exceeded the expected maximum frequency of second division segregation in tetrads. Based on theoretical calculations, a maximum of 66.7% rather than 100% second division segregation is expected for unlinked loci on the same chromosome (Mather 1935). This reduction is due to the effect of undetected double and multiple crossover events between the two loci, in this case a gene locus and the chromosome centromere. A single crossover event between the centromere and the locus generates a second division tetrad pattern, but some of these are never realized because a second or third crossover event restores the chromatids to a first division segregation pattern.

From the results observed in this study, it appears that the expected "suppression" of second division tetrads by double or multiple crossover events does not occur, or is greatly weakened, in *Porphyra umbilicalis*. In genetic terms this may be due to a regional suppression of double crossovers, known as "chiasma interference." In other words, the presence of the first crossover event reduces the probability of a second crossover in that region. The strength of this suppression can vary with species and chromosome region. Chisma interference of this type has recently been reported to occur between alleles and the centomere in

studies with the Medaka *(Oryzias latipes)* (Naruse *et al.* 1988). The very high observed frequency of second division segregation in *Porphyra* may be an adaption to promote a high frequency of sectored plants in nature, which would facilitate fertilization of carpogonia.

The results discussed above do not by themselves disprove sex determination by another mechanism. However, evidence was obtained from color mutant crosses that further verified genetic segregation of sex-determining alleles. Genetically characterized color mutants were used in this study as marker genes which enabled the identification of blocks of cells derived from individual haploid nuclei produced in meiosis. The blades were subsequently grown to sexual maturity so that a comparison could be made between striping caused by the segregation of the color markers and the striping caused by sexual segregation. In every observation of blades grown to maturity, the boundary between male and female tissue blocks coincided exactly with the boundary between color markers. These observations were collected predominately from sexually mature F_1 gametophytes produced by the WT♀ X YELLOW♂ and $PINK_2$♀ X WT♂ crosses. Comparable observations on coincidal boundaries were also made for some in a few F_1 gametophytes from the

GREEN♀ X YELLOW♂ cross.

F_1 gametophytes from some of the color mutant crosses were difficult to maintain in culture because one sector (from one of the two upper cells of the tetrad) would grow more quickly than the other and overgrow it. Consequently, although small blades could be scored quite easily for color-determining alleles, sexually mature blades retained only a single-color sector. In nearly every observation, single color-blades developed as a single sex, either entirely male or entirely female, in nearly a 1:1, ratio which was the expected outcome for sex-determination by meiotic segregation. There was no convincing evidence that sex-determination was initiated subsequent to meiosis by some other differentiation mechanism. One case occurred in which 7 WT♀/WT♂ sectored blades developed from the WT♀ X RED♂ cross, which might at first sight be taken as evidence of sex-determination <u>within</u> a single color-sector and hence not due to meiotic segregation of sex-determining alleles. However, these plants are readily explained as products of first division segregation for the color alleles with the two wild type alleles going to the upper cell in the first meiotic division. Second division

segregation of the sex-determining alleles in the second meiotic division would generate the observed striped plants.

In summary, the results strongly support the hypothesis that the development of *P. umbilicalis* as a monoecious longitudinally divided (♀/♂) blade is due to genetic segregation of sex-determining alleles during meiosis in the germinating conchospore.

In contrast, sex-determination in *P. yezoensis*, the only other species of *Porphyra* studied using color mutations as markers (Miura and Kunifuji 1980; Ohme *et al.* 1986), clearly occurs subsequent to meiosis during blade development, although the mechanism responsible for forming the fine-grained mosaic of small male and female sectors in this type of plant remains to be elucidated.

Eventually, it would be interesting to study some of the dioecious species of *Porphyra* to determine the means by which sex is determined in these species. Single-sex plants may result from meiosis in the conchosporangia with usually only one of the nuclei surviving in the spore, or from a linear tetrad in which only the top cell forms the blade, with the lower three cells forming the holdfast. These are only two possibilities amongst several that would yield separate male and female

plants.

My observations from herbaria and the field indicate that occasionally longitudinally striped female/male plants can be detected from populations usually described as dioecious. Some of these striped species may have a tight linkage between the centromere and the sex-determining locus, resulting in predominantly first division segregation at meiosis and hence predominantly single-sex fronds. The occasional striped blade could be produced by second division segregation or developmental anomalies (eg. having one of the basal cells, presumably of the opposite sex, becoming part of the developing blade).

Another possibility is that monoecious striped blades may be protandrous, with the spermatia forming a deliquescing longitudinal band. Consequently, when the carpogonial tissue becomes fertilized, the plant would appear entirely female, and if it has not been fertilized the plant might appear entirely male, thus, dioecious. This may be the situation for some of the presumed dioecious species. In any event, the dioecious species of *Porphyra* certainly warrant additional developmental study.

2. Color-determining Alleles

Stable color mutants of *P. umbilicalis* were fundamental to this thesis research because they were used as genetic markers to confirm the position of meiosis in the germinating conchospore, interpret blade development, determine timing of sex-determination and seek genetic linkage between a marker gene and putative sex locus. In addition, the characterization of these mutants by analysis of their transmission patterns (Mendelian or non-Mendelian) proved interesting in itself.

Although at least 35 separate color mutant calli were produced through mutagenesis, most never developed into blades capable of sexual reproduction. Only five color phenotypes were grown to sexual maturity. All of the selected mutations are stable. Unstable mutations would have been selected against as a consequence of their becoming overgrown by adjacent reverted tissue subsequent to mutagenesis. YELLOW, RED AND $PINK_2$ exhibit Mendelian transmission patterns as a result of mutations in individual nuclear genes, and also exhibit a high proportion of second division segregation in crosses. This behaviour mirrors that for

sexual phenotypic segregation and thus makes these mutants ideal markers for use in boundary analysis during sexual differentiation. The remaining two mutations, GREEN and $PINK_1$, displayed a non-Mendelian inheritance pattern. These two pigmentation mutations show cytoplasmic maternal transmission, indicating that the defective gene is most likely in the plastid genome.

An interesting observation was made involving one of the non-Mendelian mutants. The expected result from germinated zygotospores produced by the GREEN♀ X WT♂ cross is green conchocelis, which subsequently produces conchospores that germinate into green blades. However, wild-type conchocelis (that later produced wild type gametophytes) appeared a number of times in culture. Since the green mutation was otherwise extremely stable in culture, I consider the generation of wild type conchocelis to represent the first evidence in the Rhodophyta of male transmission of a chloroplast genome by spermatia.

Although never before observed in the red algae, biparental plastid inheritance in the vascular plants is quite common and has been observed in about one-third of the species studied so far, in both the Anthophyta

and Coniferophyta. This has been confirmed through genetic analysis and also by observations of paternal proplastids in mature pollen grains and fertilized zygotes (Kirk and Tilney-Bassett 1978).

Further support for biparental transmission in *P. umbilicalis* results from cytological observations in the Rhodophyta. Transmission of the chloroplast genome has always been assumed to be maternal because the occurrence of viable plastids in spermatia from the Florideophycidae is rare (Kugrens and West 1972; Scott and Dixon 1973). However, recent investigations of the Bangiophycidae have yielded evidence for small persistent plastids and proplastids within the spermatia of *Bangia* (Cole and Sheath 1980) and also within the fertilization canal of *Porphyra gardneri* (Hawkes 1978) and *P. haitanensis* (Wang and Xu 1978).

Although these thesis results provide qualitative evidence of biparental plastid gene transmission for *Porphyra,* further quantitative confirmations from the GREEN♀ X WT♂ cross are required. Paternal plastid transmission involving the GREEN and $PINK_1$ crosses should also be detectable a small percentage of the time, but was not observed during this study.

3. Linkage Analysis of Sex and Color-determining Alleles

The final component of this genetic analysis was to determine whether the alleles for sex determination and color determination were transmitted independently or show some genetic linkage to each other. The patterns of transmission from previous results indicate that both sex and color-determining alleles independently show high frequencies of second division segregation with respect to the centromere which somewhat increases the likelihood they might be linked. Demonstration of genetic linkage between color and sex loci would provide convincing evidence for the existence of segregating sex-determining alleles.

The analysis of the linkage data was rather straight forward. The yellow locus is completely unlinked from the sex-determining locus since the parental and recombinant sectors occurred in equal frequency. The same was true for the red locus, even though only the wild type sectors could be scored on mature plants. These results indicate that the color and sex loci are located on different chromosomes, or far apart on the same chromosome. Thus, unfortunately, the hoped-for genetic linkage

was not found, which was not at all surprising considering the limited number of loci tested.

F. Coincidence of Color and Sex Boundaries

F_1 gametophytic blades from WT♀ X YELLOW♂, $PINK_2$♀ X WT♀ and other scattered crosses always showed a perfect coincidal alignment of the color and sex boundaries. These results strongly support the hypothesis that sex-determination, just as color-determination, is tied to meiotic segregation of genetic alleles. Although all of the observations are most readily explained on the basis of meiotic segregation of sex and color-determining alleles, there remains one developmental alternative which cannot be completely excluded using the available data. It could be proposed that sex-determination becomes set at the four cell stage of sporeling development by the same kind of mechanism that, in other species, acts later in development to produce fine-grained mosaics of female and male tissue. In other words, the striped plants are simply a very coarse mosaic resulting from very early

"random" differentiation into female and male cells, which just happens to coincide in timing with meiotic segregation of chromosomes. Such a developmental model would have to explain how the top two cells of the tetrad regularly differentiate into female and male sectors giving the appearance of second division segregation. Meiotic segregation of sex-determining alleles is by far the most straightforward mechanism to explain all the observations. Taken together, the genetics, cytological and developmental data presented in this thesis provide very strong evidence that primary sex-determination in *P. umbilicalis* is controlled by segregation of alleles during meiosis in the germinating conchospore.

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