TAXONOMIC STATUS OF GENERA IN THE "*NOWAKOWSKIELLA*" CLADE (KINGDOM FUNGI, PHYLUM CHYTRIDIOMYCOTA): PHYLOGENETIC ANALYSIS OF MOLECULAR CHARACTERS WITH A REVIEW OF DESCRIBED SPECIES

by

SHARON ELIZABETH MOZLEY

(Under the Direction of David Porter)

ABSTRACT

Chytrid fungi represent the earliest group of fungi to have emerged within the Kingdom Fungi. Unfortunately despite the importance of chytrids to understanding fungal evolution, the systematics of the group is in disarray and in desperate need of revision. Funding by the NSF PEET program has provided an opportunity to revise the systematics of chytrid fungi with an initial focus on four specific clades in the order Chytridiales. The "*Nowakowskiella*" clade was chosen as a test group for comparing molecular methods of phylogenetic reconstruction with the more traditional morphological and developmental character system used for classification in determining generic limits for chytrid genera. Portions of the 18S and 28S nrDNA genes were sequenced for isolates identified to genus level based on morphology to seven genera in the "*Nowakowskiella*" clade: *Allochytridium, Catenochytridium, Cladochytrium, Endochytrium, Nephrochytrium, Nowakowskiella,* and *Septochytrium*. Bayesian, parsimony, and maximum likelihood methods of phylogenetic inference were used to produce trees based on one (18S or 28S alone) and two-gene datasets in order to see if there would be a difference depending on which optimality criterion was used and the number of genes included. In addition to the

molecular analysis, taxonomic summaries of all seven genera covering all validly published species with a listing of synonyms and questionable species is provided to give a better idea of what has been described and the morphological and developmental characters used to circumscribe each genus. All of the isolates sequenced for the molecular portion of this study have been cryopreserved for future work and a review of cryopreservation of chytrid fungi is included. A modification of a previously published Q-tip method used to cryopreserve fungi for this study is also given.

INDEX WORDS: Chytrid, Fungi, *Allochytridium*, *Catenochytridium*, *Cladochytrium*, *Endochytrium*, *Nephrochytrium*, *Nowakowskiella*, *Septochytrium*, Taxonomic Summary, Morphology, Development, Genera, 18S nrDNA, 28S nrDNA, Bayesian, Parsimony, Maximum Likelihood, Cryopreservation, Q-tip, Glycerol, Liquid Nitrogen

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DEDICATION

I dedicate my dissertation to my wonderfully supportive husband, Zach Standridge, my family and my extended family that consists of my sister, Laurie Mozley, my mom, Chris Mozley, my dad, Ron Mozley, my grandmothers Sarah I. Mozley and Ethel Baker, my mother-in-law, Patty Standridge, my father-in-law, David Standridge, sister-in-law, brother-in-law, and nephew, Kay, Kirk, and Braxton Standridge and my grandmother-in-law, Betty Corn.

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CHAPTER 1

INTRODUCTION

Background on Chytrid Fungi and the "*Nowakowskiella*" Clade (Order Chytridiales, Phylum Chytridiomycota, Kingdom Fungi)

Chytrids compose a single phylum (Phylum Chytridiomycota) in the Kingdom Fungi with five orders, around a thousand species and about a hundred genera. Placement of chytrid fungi into the fungal kingdom is based primarily on ribosomal sequence data (Bowman et al. 1992, Bruns et al. 1991) but they also share certain characters exhibited by other fungi including cell wall composition, AAA lysine biosynthesis, and the use of glycogen as a storage product (Bartniki-Garcia 1970, Bartniki-Garcia 1987, Powell 1993). Chytrids themselves are unique in the fungal kingdom because they possess flagellated cells in one part of the life cycle. Zoospores of chytrid fungi represent the dispersal stage when the fungus is able to move through liquid to find a suitable host or substrate on which to continue the next generation. Most chytrid fungi produce uniflagellate zoospores with the flagellum posteriorly directed though insertion can occur anywhere on the zoospore body. In contrast to most of the uniflagellate species of chytrid fungi, certain members of the order Neocalimasticales produce zoospores with multiple flagella (Wubah et al. 1991). Chytrids are microscopic though growth can be seen when the numbers are increased on bait material or in pure culture. Found in both water and soil on a variety of organic substrates, chytrids are known to parasitize a wide variety of protists, insects, plants, animals and other fungi including other chytrids (Karling 1977, Sparrow 1960). Chytrids act as the primary decomposers of keratin (Ex. epidermis, hair, nails, scales, and feathers), chitin (Ex. insect

exoskeletons and other Fungi), and cellulose (Ex. vascular and non-vascular plants, algae, pollen) in nature and are considered to be ubiquitous in the environment and global in their distribution (Sparrow 1960). Though chytrids are not restricted to aquatic habitats as evidenced by species isolated from soil, the general impression is that chytrid fungi are found only in water. Discoveries of chytrid fungi in non-traditional environments such as the skin of amphibians, the rumen of herbivores, and the canopies of tropical forests suggest that researchers should examine other non-traditional habitats for chytrid fungi that may yield new undescribed species. In addition, there are many chytrids that cannot be brought into pure culture or cannot be maintained in pure culture. The unculturable isolates also represent a possible source of undescribed species. Each new species represents a missing piece in the evolutionary puzzle that once discovered can fill in the gaps and help answer questions concerning the history of the group, relationships among taxa, and development of characters.

Many different chytrid genera are present in the same or similar habitats and share a similar affinity for the same substrate or host. Similarity in choice of nutrient source has led to a great deal of similarity with respect to morphology and development. Both sets of characters (morphology and development) have traditionally been used for diagnosis of new taxa at the order, family, genus and species levels, for classification purposes and for the creation of keys. Unfortunately many species have incomplete descriptions lacking pertinent portions of the life cycle or life history, i.e. the morphological and developmental characters used for classification, leading to problems regarding placement for species considered to be validly described and exclusion from the formal classification for species labeled as questionable. The lack of a complete description is often coupled with the lack of cultures or type material with which to re-asses previous work. Isolation of a described species from an environmental sample is one way

of dealing with the lack of reference material but there is no guarantee that every described species can be found much less maintained in pure culture. In addition, recognition of the wide phenotypic plasticity exhibited by chytrids for certain morphological and developmental characters has led to the acknowledgement that the present system used to classify families, genera and species as put forth by Sparrow (1960) and Karling (1977) is artificial. Since there are possibly a large number of undescribed and unculturable chytrids and because it is not known which particular morphological and developmental characters can be used in a phylogenetic classification and which cannot, the big question in chytrid systematics then is how to create a phylogenetically valid classification scheme based on an incomplete set of taxa with characters that may or may not be phylogenetically valid. The lack of a phylogenetic classification system is a major impetus for carrying out a systematic revision of the Chytridiomycota. Revision of all the chytrid orders is needed but most especially for the order Chytridiales which represents the largest order in the phylum Chytridiomycota and has generally been used a dumping ground for anything that did not fit into one of the remaining four orders (Blastocladiales, Monoblepharidales, Neocallimasticales and Spizellomycetales).

A parsimony analysis by James et al. (2000) of sixty-four different chytrid 18S nrDNA sequences placed isolates in clades that matched an existing ordinal classification scheme based on zoospore ultrastructure (Barr 1990, Li and Heath 1993) for four of the five chytrid orders: Blastocladiales, Monoblepharidales, Neocallimasticales and Spizellomycetales. In contrast, the order Chytridiales was separated into four different clades (the "*Lacustromyces*" clade, the "*Rhizophydium*" clade, the "*Chytridium*" clade and the "*Nowakowskiella*" clade) by the 18S nrDNA (James et al. 2000). Each clade possessed a different subtype of the Chytridialean zoospore type with separation based mainly on differences in root morphology (Barr 2001). A

new NSF initiative to fund systematic work on understudied groups of organisms and to train a new generation of systematists in these groups was utilized to continue the work started by James et al. (2000) on the four clades of the Chytridiales. The NSF PEET program (Partnerships for Enhancing Expertise in Taxonomy) funded a Chytrid PEET grant at three different sites: the University of Alabama, the University of Maine and the University of Georgia. Each site was given a particular clade from the James et al. (2000) paper with the "*Nowakowskiella*" clade going to the University of Georgia.

The work presented in this study covers seven genera in the "*Nowakowskiella*" clade with the exception of the isolate labeled as *Diplochytridium lagenaria* as the genus *Diplochytridium* was determined to be taxonomically invalid (Blackwell and Powell 2002). All species of *Diplochytridium* were placed back into *Chytridium* and any discussion of *Chytridium lagenarium* (=*Diplochytridium lagenaria*) should be restricted until more species of the now defunct genus *Diplochytridium* can be sequenced. The possibility exists that species of *Diplochytridium* found on cellulosic substrates do belong in the "*Nowakowskiella*" clade but answering this question will require examination of more species than the one currently available in pure culture.

One of the goals of the PEET program and especially the Chytrid PEET grant is to support the production of monographs. Taxonomic summaries can be considered equivalent to monographs as they both contain the same kinds of information on a group of organisms such as species descriptions, geography and ecology but differ in that they cover a group still influx with respect to phylogeny. In the case of chytrid fungi, taxonomic summaries are desperately needed to collect all of the species described since the last monograph was published in 1960 and to get a better idea of the composition of genera and species. Species descriptions are scattered throughout various different journals and the current problematic status of chytrid systematics

requires a necessary review of previous descriptions before any real taxonomic decisions can be made. Chapter 2 contains taxonomic summaries for all seven recognized genera of the "*Nowakowskiella*" clade. Each taxonomic summary covers all of the validly published and currently accepted species of each genus with a listing of synonyms and questionable species. Included with each summary is a history of the genus, description of accepted species, a key to accepted species, a listing of references, and plates of drawings. The plates contain drawings of morphological characters noted in the key to species so that anyone who uses the key can have a search image for the character of interest when trying to make an identification.

Chapter 3 looks at the phylogenetic analysis of a two gene dataset for thirty-six isolates placed into different genera of the "*Nowakowskiella*" clade. The isolates were identified to genus based on certain morphological characters exhibited in pure culture. Only a few were identified to species level as many did not produce the various structures used in distinguishing species. Trees generated from the newest way to analyze phylogeneies, the Bayesian method of phylogenetic inference, were compared to trees produced from the more familiar parsimony and maximum likelihood methods. Analyses of single gene datasets (18S or 28S nrDNA alone) were also compared to the two-gene dataset using all three methods of phylogenetic analysis.

Chapter 4 provides a review of cryopreservation studies on chytrid fungi and a description of Q-tip cryopreservation method used to preserve all of the isolates sequenced in Chapter 3 plus other isolates identified as belonging to genera in the "*Nowakowskiella*" clade based on morphology. The reason for cryopreservation of all the isolates used in this study was to provide a culture resource for teaching and research including continued work funded by the Chytrid PEET grant. Inclusion of a cryopreservation study stemmed from the need to find the best way to store chytrid fungi long-term without loss of cultures. Long-term storage of chytrid

fungi is best achieved through cryopreservation as they do not do survive freeze drying and can easily be lost when maintained in broth or on agar media.

CHAPTER 2

TAXONOMIC SUMMARIES OF GENERA IN THE "*NOWAKOWSKIELLA*" CLADE

Introduction

The "*Nowakowskiella*" clade as characterized by ribosomal sequence data and zoospore ultrastructure (James et al. 2000) includes seven traditionally recognized genera and a species of *Chytridium* (Letcher and Powell 2002) not included in this treatment of the clade. The seven genera discussed here are as follows: *Allochytridium, Catenochytridium, Cladochytrium, Endochytrium, Nephrochytrium, Nowakowskeilla,* and *Septochytrium*. Current classification schemes (Sparrow 1960, Batko 1975, Karling 1977) either place *Cladochytrium* in a separate family based on type of discharge or in a family with *Nowakowskiella* and *Septochytrium* because of its polycentric nature. *Catenochytridium, Endochytrium,* and *Nephrochytrium* are in the same family but separate sub-families in all of the classifcation systems. *Allochytridium* was described in 1970 and the first species, *A. expandens*, is included in Karling (1977) and Batko (1975) but *A. luteum* Barr and Desaulniers (1987) is not as it was described much later.

Cladochytrium is the sole "inoperculate" genus while all the other genera are primarily operculate with some "endo-operculate" species in several genera. Inoperculate refers to the possession of a plug of gelatinous material at the tip of the discharge tube that dissolves before zoospore release. Operculate refers to the possession of a circular piece of wall material that acts like a lid at the tip of the discharge tube and can either be pushed off by the escaping zoospores or remain hinged at the discharge tube orifice as zoospores are released. Endo-operculation refers to the deposition of wall material down inside of the discharge tube that is either punctured or

pushed out by escaping zoospores. As stated above, *Cladochytrium, Nowakowskiella*, and *Septochytrium* represent the polycentric (multiple sporangia per thallus) members of the clade. All of the rest of the genera are monocentric (one sporangium per thallus): *Allochytridium*, *Catenochytridium*, *Endochytrium*, and *Nephrochytrium*. A common link for all of the species in this group is that they can be found or baited with cellulosic substrates (i.e. corn straw, onion skin, cellophane, green/yellow-green shoot material of vascular plants, green algae) from either water or soil. Position of the thallus relative to the substrate/host varies between species within each genus. Some species are considered to be epibiotic (sporangium and/or rhizoids sit on the surface of the substrate/host) while others are considered to be endobiotic (most of the thallus positioned on the inside of the substrate/host). Type of development is somewhat more complicated as evidence for the various types is lacking for many species. The polycentric species are most likely exogenous as the nucleus has to move out of the zoospore cyst in order to produced additional nuclei for multiple sporangia at different points on the rhizomycelium. For the monocentric species, the question of whether one is exogenous, endogenous or exo-endogenous (Barr 2001, Karling 1936) has not been determined since nuclear movement has not been shown for most species. Endogenous development retains the nucleus in the zoospore cyst and the cyst then enlarges to form a zoosporangium. Exogenous is the opposite of endogenous with the nucleus moving out of the zoospore cyst into a swelling in the germ tube that develops into a mature sporangium. Exo-endogenous development starts out like exogenous with the nucleus moving out of the zoospore cyst into a swelling in the germ tube but which then moves back into the cyst that will become the mature sporangium or in some species moves into a swelling budding off of the initial swelling in the germ tube. The initial swelling has been variously labeled prosporangium, apophysis, and sub-sporangial swelling but only recently has

there been an attempt to clarify the use of these terms (Letcher and Powell 2002). All three types of development are present throughout the phylum Chytridiomycota and are not useful phylogenetically above the level of genus (James et al. 2000). The same can be said of type of discharge, type of development, position with respect to interior or exterior of the substrate or host, and number of reproductive units per thallus. Though not informative at higher taxonomic levels, such characters may be useful at the genus level and below in combination with other characters but this has yet to be fully determined. Even though support for separation of the entire clade has not yet been satisfactorily achieved using sequence data (see Chapter 3 for an analysis of 18S and 28S nrDNA) it is still useful at this point to list the characters shared by all of the genera in the clade: 1) Zoospore ultrastructure - all the species that have been examined have a similar microtubular root structure different from the other clades that make up the order Chytridales (James et al. 2000), 2) Nuclear ribosomal sequence data, and 3) A predilection for plant shoot material or other cellulosic substrates (though some species have been isolated using chitin and keratin). The most likely taxonomic assignment of the clade would be as a family but further work is needed before a definite decision can be made.

Morphology Based Key to Genera in the "*Nowakowskiella*" Clade

1a. Monocentric.....2

1b. Polycentric.....3

2a. Non-apophysate zoosporangia.....5

2b. Apophysate zoosporangia.....6

3a. Zoosporangia inoperculate; most species have septate swellings.....*Cladochytrium*

3b. Zoosporangia exo-operculate and endo-operculate.....4

4a. Septations and constrictions along the rhizoids as well as delimiting the sporangia.....*Septochytrium*

4b. No septations or constrictions along the rhizoids; septa only present delimiting the sporangia.....*Nowakowskiella*

5a. Catenulations in the primary rhizoidial axes.....*Allochytridium*

5b. No catenulations in the primary rhizoidial axes.....*Endochytrium*

6a. Catenulations in the primary rhizoidial axes.....*Catenochytridium*

6b. No catenulations in the primary rhizoidial axes.....*Nephrochytrium*

Taxonomic Summary of the Genus *Allochytridium*

Taxonomic History of the Genus *Allochytridium*

Introduction

Ira Salkin erected *Allochytridium* in 1977 for an operculate, moncentric, eucarpic chytrid that formed sporangia from an expansion of a germ tube. At maturity, the sporangium fused with the zoospore cyst and Salkin viewed the combination of the exogenously formed sporangium and its subsequent fusion with the zoospore cyst as a unique method of development worthy of taxonomic recognition. Salkin followed the development of his chytrid in pure culture in a perfusion chamber, on agar media and on a natural substrate (onion skin). In using a pure culture and then following its development on multiple nutrition sources, he was able to identify two developmental pathways. One appeared 70-75% of the time and he considered it to be the primary pathway of development. In 1987, Barr described a second species, *A. luteum*, that resembled *A. expandens* with operculate, exongeously formed sporangia, catenulate rhizoids, an

arched wall separating the sporangium from the main rhizoid, and zoospore ultrastructure similar to Salkin's chytrid. Barr was able to separate *A. luteum* from A. expandens based on several distinguishing characters including difference in sporangium shape, color of the lipid globule, number of rhizoidial axes, number of discharge tubes/papilla, size of the operculum, and unique structures associated with the sporangium and/or the rhizoids. The rumposome in *A. luteum* is two –or three tiered versus open in *A. expandens*. Barr also noted differences in temperature maximum and nutritional requirements between the two species. At the species level, differences in color, shape, size, and number of different morphological characters can be enough to separate two species especially when they have been grown in pure culture on the same media under the same culture conditions to remove any environmental variables that might cause a difference in those characters.

Karling synonmized the genus with his *Karlingia* (Karling 1977b) because he did not agree with Salkin's separation of the *Allochytridium* based on the combination of sporangium development and fusion of the sporangium with the zoospore cyst at maturity. Blackwell and Powell (1999) reviewed Karlingia and found it to be an invalid genus, placing all endo-operculate and inoperculate species into *Rhizophlyctis*. Blackwell and Powell (2004) then placed all exo-operculate species of *Karlingia* (=*Rhizophlyctis*) that they considered to be valid into *Karlingiomyces*. Though they did not mention Karling's combination of *Karlingia expandens*≡*Allochytridium expandens* they did discuss the similarities between *Allochytridium* and *Karlingiomyces* and agreed that though similar with respect to morphology of the sporangium and the rhizoids, differences do exist that can be used to separate the two genera. They also considered Allochytridium as being distinct from other "rhizophlyctoid" genera when all taxonomic characters used to describe the genus are compared to the total characters of other

potentially similar "rhizophlyctoid" genera. In comparing *Allochytridium* to *Karlingiomyces*, *A. luteum* bears one discharge papillum versus one to several exhibited by *Karlingiomyces* though *A. expandens* can exhibit one to several depending on the nutrient source and number of surrounding thalli. Blackwell and Powell (2004) cite Salkin's description of *A. expandens* where only one discharge tube is produced based on the primary pathway of development. The two genera also differ in the method of sporangial development (with the exception of *K. exoperculatus* which also develops from the germ tube) and origin of the initial rhizoid group. Species of *Karlingiomyces* generally develop from the zoospore cyst whereas species of *Allochytridium* develop from an expansion of the germ tube. As for the origin of the initial rhizoid group, species of *Allochytridium* produce the initial grouping at the endobiotic tip of the germ tube differing from *Karlingiomyces*. Phylogenetic analysis of ribosomal genes places *Karlingiomyces* and *Allochytridium* into two separate and distinct clades suggesting that even with morphological similarity separation is warranted and supports the use of non-traditional morphological characters in classification.

The type of *Allochytridium*

Salkin did not formally designate *A. expandens* as the type for his new genus nor did he deposit any type material. Barr did designate type material for his species, *A. luteum* (Figs. 1-26 1987) including photographs of the development of *A. luteum* and TEM micrographs of the zoospore ultrastructure. Previous to his description of A. *luteum*, Barr reviewed the development and examined the zoospore ultrastructure of *A. expandens* with a then recently isolated culture that he believed to be *A. expandens* after observing its growth in pure culture and comparing it with Salkin's isolate which was still available at the time. Barr grew his *A. expandens* on an agar medium different from Salkin's so there is a difference in sporangium shape but other than that

the cultures seem identical. Since the description of *A. expandens* by Salkin, zoospore ultrastructure has gained great importance in chytrid systematics as a necessary taxonomic character. In accordance with Articles 8 and 9 of the International Code of Botanical Nomenclature (ICBN 2000), an illustration may be utilized as the type. Therefore, Salkin's photographs of the development of his isolate of *A. expandens* (Figs. 2-49) and Barr's (Figs. 1-38) including the TEM micrographs of the zoospore ultrastructure are accepted as the type for the genus *Allochytridium*. Salkin isolated his *A. expandens* from a roadside puddle next to the Green Valley exit, 1/4 mile north of Interstate 80, Solano Co. California. Barr's isolate of *A. expandens* was baited and brought into pure culture by Dr. Chris Lucarotti from soil collected in Virginia.

The Species of *Allochytridium*: Taxonomic Descriptions, Ecology and Distribution

1. *Allochytridium expandens* Salkin

American Journal of Botany 57: 656, fig. 2-49. 1977.

PLATE 1, figs. 1-4

Vegetative: Thallus epibiotic or interbiotic

Reproductive: Sporangium ampuliform (36-76 by 48-94), globose (52-85 in diameter), subglobose (48-100 by 61-120) (Barr describes as irregularly globose, ellipsoidal to sac-like structure) sessile; sporangial wall smooth; sporangium colorless.

Rhizoidial system: Primarily endobiotic, forming from the endobiotic tipe of the germ tube and from many points on the sporangium, extensive and profusely branched, with many regular and irregular nonseptate constrictions.

Zoospore, discharge: Operculum present, saucer shaped, 5-8um in diameter, persistant, one to several discharge tubes, discharge vesicle present, zoospores discharge as a mass, zoospores become motile about two minutes after discharge.

Zoospore, microscopic: Zoospores spherical, 6-8um in diameter, lipid globule one, colorless, flagellum 35-40um long.

Zoospore, ultrastructure: Barr (1986).

Resting spore: Salkin did not observe any resting spores in his California isolate but Barr observed resting spores on DT (dialysis tubing) produced by his Virginia isolate. The resting spores are globose, 6-32um in diameter, and have a slightly thickened wall with coarsely granular contents. Larger RS appear light brown under bright field.

Ecology and Distribution: Saprophytic on onion skin from roadside puddle, Salkin (loc. cit., California), boiled grass in soil-water suspension, Barr and Désaulniers, (1987: 439, Virginia), US.

2. *Allochytridium luteum* Barr and Désaulniers

Mycologia 79:195, figs. 1-26. 1987.

PLATE 1, figs. 5-6

Vegetative: Thallus epibiotic, interbiotic or endobiotic

Reproductive: Sporangium spherical(=globose), subspherical or pyriform,

Rhizoidial system: Rhizoids from a single axis or from two adjoining axes. The main rhizoid 4-15um in diameter with constricted points. Rhizoid system sparingly branched and very long. Rhizoids slender and terminating in fine ends about 0.5um in diameter.

Zoospore, discharge: Zoospores discharged in a highly evanescent vesicle and globose when actively swimming.

Zoospore, microscopic: 4-5.5um in diameter with a single, golden lipid globule (1.5-3.5um in diameter), flagellum 28-29um long.

Zoospore, ultrastructure: Barr and Désaulniers (1987).

Resting spore: small sporangia (less than 25um in diameter) became thick-walled on boiled grass and revived after being dried out for several weeks

Ecology and Distribution: Saprophytic on boiled grass from sandy soil, Barr and Désaulniers (loc. cit.), CANADA.

Taxonomic Key to Species of *Allochytridium*

All of the morphological characters used to create this taxonomic key are visible under a light microscope. Identification will require observation over several days in order to see the mature structures utilized in the key.

Key to the Species of *Allochytridium*

1a. Color of the lipid globule is luteous (golden)……...2a

1b. Lipid globule is colorless…….2b

2a. Nipple present at the point where the rhizoid and the sporangium meet (the rhizoid neck……3a

2b. Nipple not present on rhizoidial neck……..3b

3a. Very small operculum……..*Allochytridium luteum*

3b. Very large and noticeable operculum……*Allochytridium expandens*

Taxonomic Summary of the Genus *Catenochytridium*

Taxonomic History of the Genus *Catenochytridium*

Introduction

Catenochytridium was erected for a monocentric, eucarpic chytrid with a compound catenulate "apophysis" and which appeared to develop endo-exogenously on cellulosic substrates (Berdan 1939, 1941). Between 1939 and 1960 two more species and one variety of *Catenochytridium* were described. Hanson described the second species, *C. laterale* in 1944 and though she had her doubts as to the validity of the genus due to the similarities between *Catenochytridium* and *Chytridium* she felt that not enough was known about the two described species of the former and that there was too much variability in the later to warrant placement of her species in *Chytridium* or the invalidation of *Catenochytridium* at the time. *C. laterale* fit Berdan's diagnosis of the genus except for producing equal numbers of thalli with either a compound apophysis similar to *C. carolinianum* or a single apophysis reminiscent of the sub-sporangial swellings found in some *Chytridium* species. In 1953, Kobayasi and Ookubo described what they considered to be a form of *C. carolinianum* parasitizing a live specimen of the marine green alga *Cladophora japonica*. Kobayasi and Ookubo's chytrid (Fig. 4, 1953) looked very much like *C. carolinianum* but its mode of nutrition and choice of habitat differed from all the other previously described species prompting Sparrow to speculate that it might prove to be a separate species upon further study. In Sparrow's Aquatic Phycomycetes (1960), species were differentiated based on whether the apophysis was always compound or varied between being simple or compound, the position of the persistant zoospore cyst (from here on known as PZC) on the sporangium and the shape of the sporangium. Such a classification worked fine until 1965 when Sparrow described a new species from Hawaii that exhibited a "*Chytridium*-like" type of development (endogenous), monocentric and polycentric thalli, no PZC and a single rhizoidal axis. Sparrow considered the endogenous method of development and production of a single rhizoidal axis as reason enough to differentiate *C. oahuensis* from other

similar chytrid genera (*Truittella* and *Karlingiomyces*) and though the polycentric form of the thallus resembled *Septochytrium marylandicum* with the possibility that both genera were present in the baited gross culture, Sparrow felt that his species belonged in the genus *Catenochytridium*. *C. oahuensis* differed from the other species of *Catenochytridium* not only in developing endogenously but in that it did not have the characteristic persistent zoospore cyst, that it produced both monocentric and polycentric forms of the thallus, and that it formed cylindrical segments with septum-like divisions reminiscent of *Cladochytrium*. The need to expand the generic definition of the genus to include species with septate thalli did not bother Sparrow though he only suggested the need for expansion and did not make a formal emendment to the genus. Sparrow also did not include the occasional production of polycentric *Septochytrium*-like thalli in his description of *C. oahuensis* because of the possibility that there was indeed a *Septochytrium* mixed in with his monocentric *Catenochytridium* and while having genera with both monocentric and polycentric thalli was not unusual (i.e. *Septochytrium, Physoderma, Catenaria*), he may not have considered *Catenochytridium* as being a genus with both types of thalli and erring on the side of caution since he did not describe it from a pure culture. In addition, the lack of a persistent zoospore cyst though present in all of the previously described species did not affect Sparrow's decision to place his isolate in *Catenochytridium*. He stated that since Berdan did not use it to delimit the genus in her original description he did not need to consider it when placing a species into *Catenochytridium*. The next change to come involved Karling renaming *C. carolinianum* f. *marinum* (Kobayasi and Ookubo 1953) as *C. marinum* because of its aforementioned difference from all the other species in being marine and parasitic though without any further study of the original isolate or an other isolate (1977). The last described species, *C. hemicysti* Knox (Barr et al. 1987), developed in an exogenous-

endogenous manner (equivalent to Karling's endo-exogenous) and possessed catenulate segments in the rhizoids and a persistant zoospore cyst but differed from the other species in that the PZC split in two and did not thicken into a wart-like appendage on the zoosporangium. *C. hemicysti* also differed in having a flexuous operculum and in possibly possessing either a non-existent or highly evanesvent vesicle.

The type of *Catenochytridium*

Berdan designated her new species *C. carolinianum* as the type species of the genus when she erected *Catenochytridium* in 1939. Unfortunately even though *C. carolinianum* has been grown in both unifungal and pure culture (Berdan 1939, 1941, Barr et al. 1987) no type material has ever been deposited. Since the ICBN allows for the use of illustrations and photographs as type material, Berdan's illustrations (Fig. 1, 1939; Figs. 1-72, 1941) of *Catenochytridium carolinianum* is accepted as the holotype of the genus.

Terminology

Berdan described the type species, *C. carolinianum,* as having a catenulate, compound "apophysis" and developing in an "endo-exogenous" (sensu Karling 1936) manner. Hanson (1946) carried out a developmental study of *C. laterale* but also included a cytological study of both *C. laterale* and *C. carolinianum* to determine the position of the nucleus relative to the endobiotic rhizoids and apophyses, structures which mature before the epibiotic sporangium. Karling (1936) suggested in his study of *Chytridium lagenaria* that in "endo-exogenous" development the nucleus might migrate down into a swollen portion of the germ tube (defined as the apophysis or subsporangium) while the rhizoids and the apophysis/subsporangium mature. Once the rhizoids and the apophysis/subsporangium had reached maturity, the nucleus would then migrate back up into the zoospore cyst and begin multiplying as the cyst expanded to form

the epibiotic zoosporangium. Karling used both apophysis and subsporangium to refer to the same structure while Barr (1987, 1990, 2001) used the term "prosporangium" though later workers restricted the use of the term apophysis to a swelling beneath the sporangium associated with the movement of the nucleus out of the zoospore cyst (Letcher and Powell 2002). Hanson's cytological study of both *C. laterale* and *C. carolinianum* showed that this was not the case. In each species the nucleus remained in the zoospore cyst while the endobiotic parts of the thallus, the rhizoids and the "apophysis" developed to maturity. The sporangium then budded out of one side of the zoospore cyst while a portion of the cyst wall thickened and remained attached to the sporangium wall. Due to the lack of nuclear movement involved in the development of the swelling beneath the sporangium of each species, the type of development is not "endo-exogenous" but is simply "endogenous" with respect to nuclear movement. With respect to the position of the thallus parts relative to the surface of the host or substrate, Karling's definition of "endo-exogenous" development is flipped. When Karling refers to endogenous development he talks about the movement of nutrients and direction of growth into the host/substrateThe type of development in *C. laterale* and *C. carolinianum* where the endobiotic (exogenous *sensu* Karling) portions of the thallus develop before the epibiotic (endogenous *sensu* Karling) sporangium and where (minus the nucleus) into the germ tube may occur could be. Sparrow (1960) considered Karling's "endo-exogenous" method of development just a more "striking reversal" of the typical endogenous *Chytridium* method of development since in all forms of chytrid thallus development a partial transfer of the cytoplasmic contents of the zoospore cyst probably occurs to aid in formation of the endobiotic germ tube and subsequent rhizoids. With respect to *C. hemicysti*, the contents of the zoospore cyst at the beginning of development were reported to pass into a swelling of the germ tube which Barr et al. termed the prosporangium (Barr et al.

1987 noted in parentheses that a prosporangium was often called an apophysis) with the exception on occasion of a lipid globule though the movement of the nucleus was assumed but not confirmed with any cytological evidence. Since nuclear movement is unknown for *C. hemicysti, C. kevorkianii*, *C. marinum,* and *C. oahuensis*, a definitive judgement on whether the type of development for the genus should be characterized as either "exogenous-endogeous" or "endogenous" remains to be seen.

The Species of *Catenochytridium*: Taxonomic Descriptions, Ecology and Distribution

Currently the genus *Catenochytridium* contains six species: *C. carolinianum* Berdan (1939), *C. laterale* Hanson (1944), *C. kevorkianii* Sparrow (1952), *C. marinum* Karling (Kobayasi and Ookubo 1953) (1977), *C. oahuensis* Sparrow (1965), and *C. hemicysti Knox* (1987).

1. *Catenochytridium carolinianum* Berdan

American Journal of Botany 26:460, fig. 1. 1939.

American Journal of Botany 28, figs. 1-72. 1941.

PLATE 2, figs. 7-10

Vegetative: Thallus epibiotic, monocentric, eucarpic

Development: Zoospores come to rest on the surface of the substrate and produce a germ tube that penetrates into the substrate. The germ tube can then follow one of three paths: 1) swell to form an "apophysis" then produce rhizoids; 2) form rhizoids then produce a swelling right beneath the zoospore cyst inside of the host cell; 3) form a swelling and rhizoids simultaneously. Slowly all of the cytoplasm moves out of the zoospore cyst and into the "apophysis" and rhizoids. After formation of both the rhizoids and an "apophysis" secondary and tertiary swellings form in the rhizoids attached to the initial swelling and new rhizoidal branches are

formed. Before the extrametrical/epibiotic zoosporangium starts to form, the zoospore cyst is considered empty. Zoosporangium development begins when cytoplasm begins to move back up into the zoospore cyst. Cytoplasm moves from the tips of the rhizoids through the segments of the now catenulate apophysis into the germ tube and finally reaches the zoospore cyst. The cyst either breaks open so that the distal portion of the cyst is moved upward on the surface of the zoosporangium and appears as a brown or hyaline knob on the surface or is moved in its entirety upward and to the side of the developing zoosporangium as it bulges out between the surface of the host cell/substrate and the zoospore cyst. At maturity, the zoospores are discharged from the zoosporangium through an opening produced by an operculum that either remains attached like a hinged lid or is pushed off by the emerging mass of zoospores. According to Hanson (1944), the nucleus remains in the extrametrical zoospore cyst during the development of the intramatrical portions of the thallus (rhizoids and apophysis).

Reproductive: Sporangium spherical, subspherical, pyriform, obovoid, ovoid, elliptical, kidney-shaped or convoluted with pointed lobes, 8-40 x 8-75μm, hyaline in color; wall smooth and unornamented; Zoospore cyst usually persistent on both zoosporangia and resting sporangia, apical, slightly flattened, hyaline or amber and thick-walled, about 8μm in diameter.

Rhizoidal System: Primary sub-sporangial swelling commonly spherical to ovoid, 5.5-22μm; swellings in rhizoids 2-30 in number, arranged in 1-4 linear series attached to primary cell, spherical, ovoid, elliptical or irregularly elongate, joined by an imperceptible or an elongated isthmus; rhizoids including primary sub-sporangial swelling 55-800μm in extent; rhizoids 0.5 to 3u in diameter, becoming very finely branched, branching somewhat dichotomous.

Zoospore discharge: Operculum present, apical to sub-apical in position, circular in shape, 6-20μm in diameter, hinged to and persistent on empty sporangium; one discharge pore, no

papillae or tubes; zoospores emerge in a globular mass preceeded by a mass of clear cytoplasm and lay quiescent for a few moments before swimming away

Zoospore, microscopic: hyaline in color, spherical, 5-6μm in diameter; one lipid globule about 2.5μm in diameter; flagellum length 35-40μm

Zoospore, ultrastructure: not yet determined

Resting Spore: Epibiotic, spherical to ovoid, 8-40u, one large globule and a parietal layer of smaller globules, thick-walled, light to dark brown, smooth, no wall ornamentation, develop primarily intramatrically from one of the segments of the "apophysis" but can also form extramatrically from a developing zoosporangium. During germination the resting spore acts as a prosporangium from which material moves out of the spore either through the original germ tube (if formed from the primary apophysis) or a pore created at some point in the wall of the resting spore (if formed from another portion of the vegetative thallus). The zoosporangium develops the same as stated above and zoospores are released in the same manner.

Ecology and Distribution: From dead grass found in a collection of algae from a pasture, saprophytic in leaves of wheat, corn, rye, oats and various grasses, Berdan (*loc. cit.*: North Carolina, New York, and Canada); Sparrow (1960: 557) Karling (1941a:387; 1941b:108; 1942c:620; 1948c:509) United States.

2. *Catenochytridium hemicysti* Knox

Mycologia 79:588, figs.1-25. 1987.

Chytridium hemicysta Knox, Biosystematic Studies of Aquatic Phycomycetes: Chytridiales and Blastocladiales. Ph.D. Thesis, p. 12, Pl. 1-3, pp. 74-78. 1971.

PLATE 2, figs. 11-12

Vegetative: Thallus epibiotic, monocentric, eucarpic

Development: Zoosporangium develops from a splitting of the zoospore cyst after formation of a swelling in the germ tube. The zoospore encysts on the surface of the substrate and produces a germ tube that burrows into the substrate or agar. Cytoplasm from the zoospore cyst moves into the germ tube and a swelling forms in the germ tube some distance from the zoospore cyst. Once the swelling appears, rhizoids arise from multiple points on the swelling (Barr calls this a prosporangium). Cytoplasm then flows from the swelling in the germ tube back into the zoospore cyst. The cyst splits releasing cytoplasm that is covered by a hyaline substance. Barr hypothesized that the hyaline material is either incorporated into the maturing zoosporangium wall or is dissolved.

Reproductive: Sporangium spherical to sub-spherical, (8-)18-55(-80)µm in diameter; Persistent zoospore cyst ruptures in half when protoplasm from the prosporangium (=apophysis) is discharged to form the sporangium and remains loosely attached at the base of the developing sporangium.

Rhizoidal System: Prosporangium variable in shape, 10-20µm in diameter; Rhiziods strangulate, having a catenulate appearance, and sparingly branched.

Zoospore discharge: Discharge apparatus single, comprising a persistent, flexuous operculum, (1-)8-20um, that folds following zoospore release; zoospores escape in a globular mass which fragments almost at once and the zoospores drift apart over a period of several minutes before the become motile.

Zoospore, microscopic: hyaline in color, spherical, 4-5(-6.5)µm in diameter in diameter; 2, 3, or 4 (rarely one) refractive globules; flagellum length 26-30µm

Zoospore, ultrastructure: not yet determined

Resting Spore: Epibiotic in place of the zoosporangium or endobiotic within the apophysis; thought to be formed asexually.

Ecology and Distribution: From a moribund desmid in a pond water sample, saprophytic on dead algae, pine pollen, cellophane and on nutrient agar, Knox (*loc. cit.*: Riopel's Pond, University of Virginia Mountain Lake Biological Station, Virginia) United States.

3. *Catenochytridium kevorkianii* Sparrow

Revista De La Sociedad Cubana De Botánica 9:70, figs. A-E. 1952.

PLATE 2, figs. 13-14

Vegetative: Thallus monocentric, eucarpic

Development: Not available.

Reproductive: Zoosporangium predominanatly citriform at maturity, 40-43μm high by 35-36μm in diameter, colorless or faintly amber colored; wall smooth and unornamented; Persistent zoospore cyst is a basal, thick-walled, amber colored protrusion 5-8μm in diameter on the zoosporangium.

Rhizoidal System: Primary "apophysis" irregularly pyramidal, 12-21μm in greatest width, its broadest face usually adjacent to the base of the sporangium; catenulate rhizoidal segments thin-walled, variable in size and number, arising from one or opposite faces of the primary "apophysis" and terminating distally in a main branching rhizoidal axis; rhizoidial system not extensive, branching dichotomous.

Zoospore discharge: Operculum present, apical, 12-13 μm in diameter, zoospores escape through a broad pore formed upon the dehiscence of the operculum and remaining in a compact quiescent group before assuming motility

Zoospore, microscopic: Zoospore ellipsoidal, 9 x 5μm; a single conspicuous hyaline lipid globule present.

Zoospore, ultrastructure: Not available.

Resting Spore: Not observed.

Ecology and Distribution: Saprophytic on cellophane bait from roadside soil, Sparrow (*loc. cit.*), CUBA.

4. *Catenochytridium laterale* Hanson

Torreya 44:32. 1944.

American Journal of Botany 33:390,392; figs. 1-25. 1946.

PLATE 2, figs. 15A-G

Vegetative: Thallus epibiotic, monocentric, eucarpic

Development: Zoosporangium buds out of the side of the zoospore cyst after formation of the primary sub-sporangial swelling and rhizoids. Hanson determined that the nucleus remained in the zoospore cyst during development of the primary sub-sporangial swelling and rhizoidial system contradictory to Karling's hypothesis (1936) that it moved in and out of the zoospore cyst in exo-endogeous development.

Reproductive: Sporangium oval 16-46μm x 1-62μm; spherical 12 x 44μm; pyriform12-48μm x 18-71μm; cylindrical 15-25μm x 61-93μm; lobed 28-63μm x 88-160μm when developed intramatrically (endobiotic), hyaline in color; wall smooth and unornamented; Zoospore case always persistent on sporangium, thickened, bulbous, never flattened, amber to dark brown in color, rarely apical or lateral but remaining like a basal protuberance on the sporangium.

Rhizoidal System: Predominant primary sub-sporangial swelling spherical, ovoid or lobed, up to 27-30μm in diameter; swellings in rhizoids 1-7 in number, arranged in 1-4 linear series

attached to the primary sub-sporangial swelling laterally or apically so that they emerge between the primary sub-sporangial swelling and the sporangium, rarely emerging from the base of the primary sub-sporangial swelling, often completely lacking; rhizoidial system (including primary sub-sporangial swelling) up to 224μm in extant, becoming finely branched, branching dichotomous.

Zoospore discharge: Operculum present, apical, sub-apical or lateral, 7.5-15 μm in diameter, generally persistant on empty sporangium; one discharge pore, no papillae or tubes; zoospores ooze out into a globular mass and lay quiescent for a short time before they gradually begin to move and separate and shortly thereafter they become very active and swim away.

Zoospore, microscopic: hyaline in color, spherical, 2.9-4.5μm in diameter; 2, 3, or 4 (rarely one) refractive globules; flagellum length 26-30μm

Zoospore, ultrastructure: not yet determined

Resting Spore: Endobiotic, develops from the primary swelling of the germ tube, ovoid or lobed, up to 27-30μm in diameter, one or two large globules surrounded by multiple smaller globules, thick-walled, golden in color, no wall ornamentation, germination unknown.

Ecology and Distribution: From decaying vegetable debris in soil, saprophytic on grasses, bleached corn leaves, onion, and cellophane, Hanson (*loc. cit.*: Connecticut) United States.

5. *Catenochytridium marinum* (Kobayasi and Ookubo) Karling nom. nov.

Chytridiomycetarum Iconographia p. 166, Pl. 73 fig. 29. 1977.

Synonmy: *Catenochytridium carolinianum* forma marinum Kobayasi and Ookubo, Bulletin of the National Science Musuem (Tokyo) 33:57. fig. 4. 1953.

PLATE 3, figs. 16

Vegetative: Thallus epibiotic, monocentric.

Development: No development mentioned by Kobayasi and Ookubo but as shown in fig. 4 (pg. 57, 1953) the mature thallus looks much like *C. carolinianum* with possible similarities in development.

Reproductive: Sporangium spherical, ovoid, or kidney-shaped, 40-60μm high, and 15-36μm in diameter, hyaline in color; wall smooth and unornamented, somewhat thick-walled (1.5-2.0 μm thick).

Rhizoidal System: Basal segment ovoid or oblong; apophyses composed of 2 or 3 linear series with 2-6 catenluate segments each, ovoid, fusoid or irregularly elongate, terminally attenuated; rhizoids originate from apical and intermediate segments of apophyses, simple or dichotomously branched, not so long, length of rhizoidal system (including apophyses) 40-60μm.

Zoospore discharge: Zoospores and discharge not observed; two or more papillae; orifice circular, 5-10μm in diameter, operculum not mentioned.

Zoospore, microscopic: hyaline in color, spherical, 2.9-4.5μm in diameter; 2, 3, or 4 (rarely one) refractive globules; flagellum length 26-30μm

Zoospore, ultrastructure: Not available.

Resting Spore: Not observed.

Ecology and Distribution: On *Cladophora japonica* in seawater, Kobayasi and Ookubo (*loc. cit.*), JAPAN.

6. *Catenochytridium oahuensis* Sparrow

Mycopathologia et Mycologia Applicata 25:136, figs. 67-70. 1965.

PLATE 3, figs. 17-20

Vegetative: Thallus epibiotic, monocentric, occasionally polycentric, eucarpic

Development: Sparrow did not give a formal description of development, only stating that his isolate developed in a "*Chytridium*-like manner". Since *Chytridium* development is considered endogenous then by extrapolation *C. oahuensis* probably develops in the same way as *C. laterale*.

Reproductive: Sporangium predominantly ovoid, nearly spherical to somewhat pyriform, 22-35μm high by 15-30μm in diameter, hyaline in color; wall smooth and unornamented.

Rhizoidal System: Predominant primary sub-sporangial swelling spherical, ovoid or lobed, up to 27-30μm in diameter; swellings in rhizoids 1-7 in number, arranged in 1-4 linear series attached to the primary sub-sporangial swelling laterally or apically so that they emerge between the primary sub-sporangial swelling and the sporangium, rarely emerging from the base of the primary sub-sporangial swelling, often completely lacking.; rhizoidial system (including primary sub-sporangial swelling) up to 224μm in extant, becoming finely branched, branching dichotomous.

Zoospore discharge: Operculum present, apical, 4-5 μm in diameter; one apical, hyaline papilla about 4-5μm in diameter present on the sporangium before discharge; one small, somewhat elevated discharge pore formed upon the dehiscense of the operculum; zoospores escape through the discharge pore and remain in a compact quiescent group before assuming motility.

Zoospore, microscopic: Spherical, 4-5μm in diameter; one minute hyaline globule; posterior flagellum.

Zoospore, ultrastructure: Not available.

Resting Spore: Not observed.

Ecology and Distribution: Saprophytic on cellophane bait from soil, Sparrow (*loc. cit.*) US.

III. Taxonomic Key to Species of *Catenochytridium*

All of the morphological characters used to create this taxonomic key are visible under a light microscope. Identification will require observation over several days in order to see the mature structures utilized in the key.

Key to the Species of *Catenochytridium*

1a. Organism parasitic on *Cladophora japonica*…….....*Catenochytridium marinum*

1b. Organism saprophytic on cellulosic substrates…….……2

2a. Persistent zoospore cyst absent. Septations in the rhizoids…….....*Catenochytridium oahuensis*

2b. Persistent zoospore cyst present. No septations in the rhizoids……..3

3a. Persistent zoospore cyst at the apex of the sporangium…….....*Catenochytridium carolinianum*

3b. Persistent zoospore cyst at the base of the sporangium…….4

4a. Persistent zoospore cyst remains thin-walled as the sporangium matures……*Catenochytridium hemicysti*

4b. Persistent zoospore cyst becomes thickened as the sporangium matures…….5

5a. One small lipid globule in the released zoospore…….....*Catenochytridium kevorkianii*

5b. Multiple lipid globules in the released zoospore…….....*Catenochytridium laterale*

Taxonomic Summary of the Genus *Cladochytrium*

Taxonomic History of the Genus *Cladochytrium*

Introduction

Cladochytrium was erected by Nowakowksi (1876) for a polycentric, endobiotic chytrid with an extensive and highly branched rhizomycelium. Nowakowksi described two species: *Cladochytrium elegans* and *Cladochytrium tenue*. Both species produced swellings along the rhizomycelium but differed on whether the swellings were septate (C. tenue) or non-septate (*C. elegans)*. *C. elegans* also differed from *C. tenue* in that it produced exo-operculate sporangia while *C. elegans* was inoperculate. Schroeter (1892) later separated *C. elegans* from *Cladochytrium* and placed it in a new genus, *Nowakowskiella*, because of its exo-operculate condition. Zopf and de Wildeman added three new species to *Cladochytrium* between 1884 and 1896. All three are considered questionable either because they lack details of the life cycle that would clearly place them in *Cladochytrium* or they are most likely identical with species already described. *C. polystomum* strongly resembles *C. replicatum* but Zopf only made drawings of what he saw without any written description that was not added until later by Fischer (1892). Fischer's description only places the species into the genus but does not give any differing characteristics or method of discharge. Sparrow questioned the validity of *C. polystomum* and equated the sizes of structures with *C. replicatum*, suggesting that *C. polystomum* was probably *C. replicatum* differing only in the possession of multiple discharge tubes and non-proliferating zoosporangia (1960). *C. cornutum* de Wildeman and *C. irregulare* de Wildeman are both considered questionable. Discharge was not observed for *C. cornutum* which possessed non-septate swellings and Sparrow postulated that it was possibly a parasitized *Phlytochytrium planicorne*. *C. irregulare* was described as producing a branched rhizomycelium with very large

(200-235µm) sporangia but no zoospores were observed. Sparrow noted that the sporangia resembled those of *Mitochytridium* and rightfully questioned its validity due to the lack of information on zoospore type. Sparrow did not include *C. aneurae* Thirumalachar because it was not aquatic (1960). The next species to be described was *C. replicatum* by Karling in 1931. *C. replicatum* is the best characterized species of the genus (Couch 1939, Karling 1935, Sparrow 1933) and the most common (see species description below for a listing of locations) with a very recognizable reddish-orange lipid globule in the zoospore and septate intercalary swellings. *C. replicatum* produces the typical inoperculate, polycentric thallus with septate intercalary swellings typified by *C. tenue*. The next two species, *C. crassum* and *C. hyalinum*, are more like species of *Nowakowskiella* with non-septate swellings but possess inoperculate zoosporangia characteristic of *Cladochytrium*. Hillegas (1941) felt that because *C. crassum* had a more coarse and extensive rhizomycelium than previously described species (at that point *C. tenue* and *C. replicatum*), trabeculae in the filaments (also seen in *Septochytrium*) of the rhizomycelium, and possessed non-septate intercalary swellings it should be regarded as a distinct species. Hillegas also noted that the tip deliquesces before the zoospores are released and the viscous material which surrounds the zoospores upon release is formed beneath the inner membrane that separates the zoospores from the outside environment. This differed from *C. hyalinum* where the tip softens and a plug of material fills the pore into which the zoospores are later released. No material is formed beneath the inner membrane in *C. hyalinum*. Berdan described *C. hyalinum* in 1941 and separated it from other species of *Cladochytrium* as it produced non-septate intercalary swellings and zoospores larger (*C. hyalinum* = 11µm) than *C. tenue* (4.5-5.5µm) to which she closely allied *C. hyalinum* due to the shared lack of pigmentation. *C. replicatum* is the only species with a pigmented lipid globule in the zoospore as all other described species (save for *C.*

polystomum and *C. nowakowski=C. replicatum*) have a hyaline (colorless) lipid globule. In addition, *C. tenue*, *C. taianum*, and *C. setigerum* all produce septate intercalary swellings similar to the swellings of *C. replicatum* effectively separating these species from *C. crassum* and *C. hyalinum*. Shen and Siang described *C. taianum* from grass collected in a swamp in China. *C. taianum* is very similar to *C. tenue* in every respect save for a difference in zoospore size with *C. taianum* producing larger zoospores (11µm) than *C. tenue* (4.5-5.5µm). Shen and Siang cited the larger zoospores as the reason for separating *C. taianum* from *C. tenue* and the non-apophysate zoosporangia and septate intercalary swellings as reasons for separating it from *C. hyalinum*. The last two characters could also be used to separate *C. taianum* from *C. crassum*. *C. setigerum* which is extremely similar to the exo-operculate *Nowakowskiella atkinsii* produces setae on zoosporangia derived from swellings in the rhizomycelium which both Karling and Sparrow considered as incipient sporangia and not as intercalary swellings as seen in other species of *Cladochytrium*. Karling and Sparrow's distinction of the swellings that do not produce zoosporangia as aborted incipient sporangia instead of as intercalary swellings (in the case of *C. setigerum* they can be septate) is confusing as in all other species of the genus swellings that do not form sporangia are not considered aborted sporangia but simply as swellings. A detailed ultrastructural and cytological study would need to be carried out in order to determine if there is an actual difference in the aborted incipient sporangia of *C. setigerum* and the septate intercalary swellings of other species of *Cladochytrium*. *C. setigerum* as it is currently described does not produce septate intercalary swellings characteristic of *Cladochytrium*. Following after *C. setigerum*, *C. aurantiacum* Richards (1956) made pigmented zoospores like *C. replicatum* but differed in that the swellings and zoospores were larger, accumulated hyaline lipid globules instead of pigmented ones and were fewer in number in the rhizomycelium than in *C.*

replicatum. Richards stated that his species produced larger zoospores but comparison of the actual size ranges reveals that *C. replicatum* (4-7.3µm) overlaps with *C. aurantiacum* (6-7µm) which is only at the higher end of the range for *C. replicatum* but not larger. The possibility exists that *C. aurantiacum* is only a morphological variant of *C. replicatum* with swellings that are more spherical in shape and a difference in pigmentation of lipid globules stored in the rhizomycelium. At this point, *Cladochytrium* contained six inoperculate species, three species with septate intercalary swellings, three with non-septate intercalary swellings leaving the inoperculate nature of all six species as the main character separating *Cladochytrium* from *Nowakowskiella*. The last three species assigned to *Cladochytrium* include *C. novoguineense* Kobayasi and Konno (1971), *C. indicum* Singh and Pavgi (1971), and *C. salsuginosum* Batko and Hassan (1986). *C. indicum* Singh and Pavgi resembles *C. aurantiacum* in that it produces zoospores with a bright yellow lipid globule but differs from both *C. aurantiacum* and the only other pigemented species, *C. replicatum*, in having larger zoosporangia and resting spores (Zoosporangia: 8-18µm for *C. replicatum*, 18-24µm for *C. aurantiacum* and 32.5-100µm for *C. indicum*). No resting spores were observed for *C. aurantiacum* but the resting spores of *C. replicatum* (9-21µm) are smaller than the resting spores of *C. indicum* (32.5-100µm). *C. indicum* also differs from all the other species in the genus because it was found using snake skin as bait. Snake skin is a keratin based substrate and represents a departure from the more traditional cellulosic substrates on which species of *Cladochytrium* are generally found. Singh and Pavgi noted that the morphology of the rhizomycelium was different from *C. replicatum* and *C. aurantiacum* (Compare PLATE 4 Figs. 22-23 and Fig. 39 to PLATE 5 Fig. 51). The filamentous portions of the rhizomycelium of *C. indicum* are broader than either *C. replicatum* or *C. aurantiacum*. The swellings of *C. indicum* are also more ovoid and less elongate when compared

to the spherical or ellipsoidal swellings of *C. aurantiacum* and *C. replicatum*, respectively. *C. novoguineense* produces smooth walled resting spores derived from intercalary or terminal swellings similar to *C. crassum*, *C. hyalinum*, *C. salsuginosum*, *C. tenue*, *C. taianum*, and the smooth walled resting spores of *C. replicatum*. *C. novoguineense* zoospores (4-6.5µm) fall into the same size range as *C. crassum, C. tenue,* and *C. replicatum* but differs from all the other species in producing zoosporangia as multiple chambers at the tips of branches (see PLATE 5, Figs. 43-44) though *C. salsuginosum* appears in drawings to also produce multi-chambered zoosporangia. Multi-chambered zoosporangia are also seen in *Nowakowskiella elongata* but this species is endo- and exo-operculate as opposed to the inoperculate *C. salsuginosum*. *C. novoguineense* also differs in that no swellings are seen in the rhizomycelium which is uncharacteristic for species of *Cladochytrium*. *C. salsuginosum* Batko and Hassan is a hyaline polycentric, inoperculate chytrid with non-septate swellings isolated from a brackish water sample baited with onion skin (1986). Batko and Hassan noted the larger zoospore of their chytrid as compared to the other two hyaline, non-septate species, *C. crassum* and *C. hyalinum* and distinguished their species further from *C. crassum* based on differences in the rhizomycelium. *C. crassum*'s rhizomycelium was more coarse and exhibited trabeculae in the filaments versus *C. salsuginosum* which was more delicate like *C. tenue* and did not have trabeculae. *C. hyalinum* differed because it had apophysate zoosporangia with a more elongate morphology than the non-apophysate spherical or lobate zoosporangia of *C. salsuginosum*.

Being polycentric and inoperculate are the two main characters that tie all species of *Cladochytrium* together. In the family Cladochytriaceae as defined by Karling (1977) there are five inoperculate, polycentric genera: *Amoebochytrium Cladochytrium*, *Coenomyces*, *Physocladia*, and *Polychytrium*. *Amoebochytrium, Physocladia* and *Polychytrium* are all

monotypic genera with one species. 18S nrDNA place *Physocladia* and *Polychytrium* into two separate clades with other genera in the Chytridiales but not in the same clade as *Cladochytrium* (James et al. 2000) supporting a separation of the genera using sequence data. Differences in zoospore ultrastructure *Polychytrium* (Longcore unpublished) and the "*Nowakowskiella*" clade (based on Barr 1986; Barr and Desaulniers 1987; Barr, Desaulniers, and Knox 1987; Lucarotti 1981) reinforce separation as suggested by the 18S nrDNA data. *Polychytrium* also differs morphologically with its distinct tuberculate zoosporangia. *Physocladia* is predominantly extramatrical, produces septa in the rhizoidial filaments and has no swellings in the rhizomycelium as oppposed to the more endobiotic *Cladochytrium* with swellings but no septa (save for the septate swellings). *Amoebochytrium* differs in the fact that its zoospores do not have flagella. Lack of flagella is an usual condition for chytrid fungi and would be worth examining ultrastructually. In contrast, all known species of *Cladochytrium* produce zoospores with a single flagellum. The last genus, *Coenomyces*, differs from *Cladochytrium* with respect to its zoospore and thallus morphology. *Coenomyces* produces definite cross walls that divide the mycelium into long segments reminiscent of septa found in other fungal phyla. The *Coenomyces* zoospore body is oval and tapers toward the flagellum in a manner very similar to the zoospore of *Thalassochytrium gracilariopsidis* (Nyvall, Pedersén, and Longcore 1999). Based on zoospore ultrastructure, sequence data and certain morphological characters (differences in zoosporangial morphology and septation of the rhizomycelium) *Cladochytrium* can be distinguished from *Polychytrium* and *Physocladia*. Lack of flagella distinguishes *Amoebochytrium* from *Cladochytrium* while *Coenomyces* differs because it produces a tubular, septate mycelium more similar in organization to hyphae of the more derived fungal phyla (Ascomycota, Basidiomycota, Zygomycota, and Glomeromycota) and possible differences in zoospore ultrastructure.

The type of *Cladochytrium*

Cladochytrium tenue Nowakowski is the designated type species for the genus (Clements and Shear 1931) but like many chytrid genera no type material from the original culture is currently available. According to ICBN (2000) articles 8.1, 9.1, and 9.2 illustrations can be used as type material for taxa with no extant holotype material available and as such we designate *Cladochytrium tenue* Nowakowski (1876), Cohn Beirt. Biol. Pflanzen 2:92, pl. 6, figs. 6-13 as the lectotype.

Terminology

The term trabeculae refers to invaginations of the cell wall along the filaments of the rhizomycelium. The exact nature of trabeculae in chytrid fungi has not been determined so it is not certain if they represent the beginnings of septa or something else.

The Species of *Cladochytrium*: Taxonomic Descriptions, Ecology and Distribution

1. *Cladochytrium aurantiacum* Richards

Trans. Brit. Mycol. Soc. 39: 264, figs. 1-3. 1956.

PLATE 4, figs. 21-23

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia formed either from swelling in the rhizoid or enlargement of a segment of septate intercalary swelling while the other segment either remains at the base or also develops into a zoosporangium; Zoosporangia spherical to sub-spherical (18-24µm in diameter), irregularly shaped when occuring in cells of substrate; Zoosporangia proliferating.

Rhizoidial System: Rhizomycelium profusely developed, about 2µm broad; few irregular non-septate intercalary swellings and septate intercalary swellings with 1 to 2 septa, 10-20µm in diameter with hyaline lipid globules.

Zoospore discharge: Zoospores released when tip of discharge tube deliquesces and forms a temporary globular mass before swimming away; Narrow discharge tube 8-12µm long.

Zoospore microscopic: Inoperculate; zoospores spherical, 6-7µm in diameter, single large lipid globule (3.7-4.2µm in diameter); long posteriorly directed flagellum.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores not observed.

Ecology and Distribution: Saprophytic in leaves, stems, roots of *Avena* and *Triticum* (loc. cit.), ENGLAND.

2. Cladochytrium crassum *Hillegas*

1941. Mycologia. 33: 618, figs.

PLATE 4, figs. 24-31

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia formed either from swelling in the rhizoid or enlargement of a segment of septate intercalary swelling while the other segment either remains at the base or also develops into a zoosporangium; Zoosporangia terminal or intercalary, variously shaped, commonly spherical to slightly pyriform (11x20µm – 30-43µm); seldom proliferating and non-apophysate.

Rhizoidial System: Rhizomycelium well developed, extensive, coarse, filaments as small as 1.5µm in diameter with trabeculae; Numerous non-septate intercalary swellings, fusiform to globose (3.85x15µm – 18x25µm).

Zoospore discharge: Zoospores released when tip of discharge tube/papilla deliquesces, form a temporary globular mass imbedded in “slime” before becoming amoeboid or swimming away; Discharge tube occasionally reaches 27x74µm or papilla, both usually single.

Zoospore microscopic: Inoperculate; zoospores hyaline, spherical to slightly pyriform, 4.9-6µm in diameter, single large lipid globule (2-2.75µm in diameter); posteriorly directed flagellum 25-35µm long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores spherical (9.35-23µm), fusiform (10x26µm – 14.8x23.1µm); wall light brown, thick (1.5µm); germination unknown.

Ecology and Distribution: Saprophytic on decaying vegetation, Hillegas (loc. cit.), in rotting oat leaves, onion skin, and cellophane from moist soil, Karling (1941b:108; 1942:620; 1948c: 509), US; vegetable debris, Karling (1945:34), BRAZIL.

3. *Cladochytrium hyalinum* Berdan

Amer. J. Bot. 28: 425, figs. 1-84. 1941.

PLATE 4, figs. 32-37

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia formed either from swelling in the rhizoid or enlargement of a segment of septate intercalary swelling while the other segment either remains at the base or also develops into a zoosporangium; Zoosporangia terminal or intercalary, spherical (15-40µm), sub-spherical, ovoid, pyriform, irregular, branched and lobed or greatly elongated (4-40µm x 12-100µm); proliferating and apophysate.

Rhizoidial System: Rhizomycelium extensive, branching, main axes 1.5-3.5µm in diameter; Spindle-shaped, elongate (5-8µm x 10-17µm), oval (6-8µm x 9-12µm), spherical or oblong intercalary swellings often transversely septate with two and sometimes three divisions.

Zoospore discharge: Inoperculate; Zoospores are released after tip of discharge tube deliquesces and form a temporary globular mass imbedded in "slime" before swimming away; Usually one discharge tube, 2-6µm x 2-10µm.

Zoospore microscopic: Zoospores hyaline, spherical, 8-10µm in diameter, single large lipid globule; single posteriorly directed flagellum, 40-50µm long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores spherical (12-18µm), sometimes oval, pyriform, elongated (10-12µm x 25-28µm), subtended by several small, thin-walled cells (look like an intercalary swelling with multiple divisions); wall smooth, hyaline, multi-layered; functioning as a prosporangium upon germination.

Ecology and Distribution: Saprophytic in grass leaves, Berdan (loc. cit.), in rotting oak leaves, Karling (1941:108), bleached grass leaves, Karling (1941a:108; 1942:620; 1948c: 509), US; cellophane, corn leaves, Shanor (1944:332), MEXICO; onion skin and corn leaves, Karling (1945a: 34), BRAZIL; grass, Haskins (1946:135), ENGLAND.

4. *Cladochytrium indicum* Singh and Pavgi

Hydrobiologia. 37: 37, figs. 1-7. 1971.

PLATE 4, figs. 38-41.

Vegetative: Thallus polycentric, endobiotic.

Reproductive: Zoosporangia formed from swelling in the rhizoid Zoosporangia intercalary, globular to variable in shape (32.5-100µm in diameter), hyaline, smooth and thin-walled.

Rhizoidial System: Extensively branched rhizomycelium with irregular intercalary swellings; Intercalary swellings do not possess internal divisions or septations but appear to be separated from the rest of the thallus by cross-walls at each end.

Zoospore discharge: Inoperculate; Zoospores are released after the tip of the discharge tube deliquesces and form a small quiescent mass at the discharge orifice before swimming away; Discharge tubes are small, smooth and thin-walled (11.3-40 x 8.8-15µm).

Zoospore microscopic: Zoospores 3.3-6µm; single posteriorly directed flagellum, 5.5-11 x 0.3µm.

Zoospore ultrastructure: Not available.

Resting Spore: Resting sporangia same size and shape as zoosporangia, 32.5-100µm and globular to variable in shape; developing from intercalary swellings after zoosporangia have formed; pale amber yellow and medium thick-walled; germination unknown.

Ecology and Distribution: On snake skin bait from soil, Singh and Pavgi (loc. cit.), INDIA.

5. *Cladochytrium novoguineense* Kobayasi and Konno

Bull. Nat. Sci. Mus. Tokyo. 14:377, figs. 3C-I. 1971.

PLATE 5, figs. 42-47

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia terminal or intercalary, pyriform, urceolate or ellipsoidal if terminal, clavate, elongate, spherical or irregular, intercalary zoosporangia frequently arranged in chains, 13-25µm in diameter in broadest part, 25-75µm long; Wall smooth, hyaline.

Rhizoidial System: Rhizomycelium profuse, branched, 1.8µm in diameter; No intercalary swellings.

Zoospore discharge: Low papilla or long to short regular or irregular stout discharge tube which either penetrates surface of substrate or goes into another cell (in decaying plant shoot material).

Zoospore microscopic: Zoospores spherical, 4-6.5µm in diameter, single small lipid globule less than1µm in diameter; flagellum 28-35µm long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores terminal or intercalary, spherical, ovate or fusiform (12-20µm in diameter and 17-25µm long); wall smooth, hyaline, 1-2µm thick; germination unknown.

Ecology and Distribution: Saprophytic on cellophane and welsh onion skin from soil, Kobayasi and Konno (loc. cit.), JAPAN.

6. *Cladochytrium replicatum* Karling

Amer. J. Bot. 18: 538, Plates 42-44. 1931.

Cladochytrium nowakowskii Sparrow, Amer. J. Bot. 18: 619, Plate 45, figs. H-N. 1931.

Entophlyctis aurantiaca Scherffel, in Domján, Folia Cryptogam. 2:26, Plate 1, figs. 50-51, 57-59, 72-72, 75. 1936.

Synonomy: *Cladochytrium aureum* Karling, Bull. Torrey Bot. Club. 76:298. 1949. Sydowia. 20: 130. 1967.

PLATE 5, figs. 48-56

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia generally terminal on short lateral branches, predominantly spherical, ovoid, pyriform, irregular or symmetrical (8-18µm in diameter); Wall thin, smooth, colorless; Zoosporangia proliferating.

Rhizoidial System: Rhizomycelium delicate, much branched, extensive, main axes 1.5-3.5µm in diameter; Septate intercalary swellings at frequent intervals.

Zoospore discharge: Zoospores released when tip of discharge tube deliquesces, form a temporary globular mass before becoming amoeboid or swimming away; Several narrowly cylindrical discharge tubes of variable length.

Zoospore microscopic: Inoperculate; zoospores spherical, 4-7.3µm in diameter, single large lipid globule, cadmium-orange or golden-brown in color; long flagellum.

Zoospore ultrastructure: Lucarotti, C. 1981*.

*Preliminary rDNA sequence data suggest that Lucarotti's C. replicatum is a *Nowakowskiella*. A re-examination of the zoospore ultrastructure is warranted.

Resting Spore: Resting spores borne like zoosporangia, predominantly spherical (9-21µm in diameter); wall smooth or spiny, colorless with a large cadmium-orange or golden-brown globule; functioning as a prosporangium or zoosporangium upon germination.

Ecology and Distribution: Saprophytic in a wide variety of plant shoot material and artificial media, Karling (loc. cit.; 1935, 1937b), parasitic in Spirogyra crassa, Oedogonium sp., Coleochaete sp., cultivated on maize-meal agar, Sparrow (loc. cit.), saprophytic in Elodea canadensis, decaying grass culms, Sparrow (1933c: 524), artificial media, Couch (1939a) Karling (1941a:387), in rotting oat leaves, Karling (1941b:108), Karling (1942: 620), on onion skin and bleached grass leaves, Karling (1948:509), from water and soil containing animal and vegetable debris, Karling (1949c:209), saprophytic on vegetable debris, Sparrow (1952d:768), US; saprophytic in Elodea canadensis, grass, Sparrow (1936a: 453), plant debris, Richards (1956:263), ENGLAND; Typha leaves, Scherffel (in Domjan, 1936), HUNGARY; decaying plant shoot material, Sparrow (1952a: 39), CUBA; Shanor (1944: 331), MEXICO; Karling (1945a:35), BRAZIL.

7. *Cladochytrium salsuginosum* Batko and Hassan

Acta Mycologica. 22: 189, figs. 1-9. 1986 (1988).

PLATE 5, figs. 57-63

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia intercalary or terminal, mostly nearly spherical to broadly ovate (21-34.5µm in diameter and 30-41µm long), sometimes very irregular and lobate; Wall smooth, colorless; Zoosporangia proliferating, secondary sporangia smaller.

Rhizoidial System: Rhizomycelium cobwebby, delicate, prostrate, extensive, branching, filaments thin, mostly 1.5-3.1µm in diameter, rarely thicker, up to 4.5µm, thin-walled or wall slightly thickened; Non-septate intercalary swellings, fusiform, 9-13.5µm long and 4.5-6.8µm wide,

Zoospore discharge: Zoospores released when tip of papilla deliquesces, form a temporary globular mass before swimming away; One short dome-shaped or cylindrical papilla, up to 4.5µm high and 4.1µm in diameter.

Zoospore microscopic: Inoperculate; zoospores hyaline, spherical, 9.6-10.5µm in diameter, single large lipid globule, 4.2-5.1µm in diameter; flagellum 54µm long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores more or less elliptical, intercalary, 12-18.8µm long and 7.5-9.8µm wide; wall smooth, light brown, moderately thickened; germination unknown.

Ecology and Distribution: Saprophytic on onion skin bait from brackish water sample, Batko and Hassan (loc. cit.), POLAND.

8. *Cladochytrium setigerum* Karling

Bull. Torrey Bot. Club. 78: 38, figs. 1-8. 1951.

PLATE 5, figs. 64-68

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia formed either from swelling in the rhizoid or enlargement of a segment of septate intercalary swelling while the other segment either remains at the base or also

develops into a zoosporangium; zoosporangia commonly intercalary, spherical (13.2µm – 28.5µm), oval (11-27µm x 19-44µm), occasionally elongate and constricted; wall bearing 10-50 simple or branched setae (5.5-30µm long x 1.7-2.4µm in diameter).

Rhizoidial System: Rhizomycelium extensive, delicate, branched, 1.7-2.8µm in diameter; Few non-septate intercalary swellings, fusiform, elongate or irregular (6-10µm wide x 8-18µm long).

Zoospore discharge: Zoospores released when tip of discharge tube/papilla deliquesces, form a temporary globular mass before swimming away; Single conical or hemispherical papilla or a short discharge tube (7-9µm x 14-20µm).

Zoospore microscopic: Inoperculate; zoospores spherical, 3-3.4µm in diameter, single small lipid globule, colorless, 0.8-1.2µm in diameter; flagellum 16-19µm long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores not observed.

Ecology and Distribution: Saprophytic on cellophane from soil, Karling (loc. cit.), US.

9. *Cladochytrium taianum* Shen and Siang

Sc. Repts. Nat. Tsing Hua Univ., Ser. B: Biol. And Psych. Sci. 3: 183, fig. 5.

1948.

PLATE 6, figs. 69-70

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia formed either from swelling in the rhizoid or enlargement of a segment of septate intercalary swelling while the other segment either remains at the base or also develops into a zoosporangium; zoosporangia terminal or intercalary, variable in shape and size, spherical, sub-spherical, ovoid (22-42µm in diameter); zoosporangia non-apophysate.

Rhizoidial System: Rhizomycelium extensively branched and anastomosed; numerous fusiform septate, rarely more than two septa per swelling.

Zoospore discharge: Zoospores released when tip of papilla deliquesces; single papillum present.

Zoospore microscopic: Inoperculate; zoospores hyaline, spherical, 11µm in diameter, single large lipid globule, 7.2µm in diamter; posteriorly directed flagellum.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores terminal or intercalary, mostly spherical (13-14µm in diameter) with a single central lipid globule (8.2µm in diameter); wall smooth, about 1µm thick; germination not mentioned.

Ecology and Distribution: Saprophytic on decaying grass leaves from swamp, Shen and Siang (loc. cit.), CHINA.

10. *Cladochytrium tenue* Nowakowski

Beirt. Biol. Pflanzen 2:92, pl. 6, figs. 6-13. 1877.

PLATE 6, figs. 71-78

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia formed either from swelling in the rhizoid or enlargement of a segment of septate intercalary swelling while the other segment either remains at the base or also develops into a zoosporangium; Zoosporangia 66µm in diameter, spherical (8-30µm), somewhat pyriform, broadly oval or slightly citriform (8-18µm x 10-22µm); Wall smooth, colorless; Zoosporangia proliferating, secondary sporangia smaller.

Rhizoidial System: Rhizomycelium extensive, branching, main axes 1.5-3.5µm in diameter; Spindle-shaped, elongate (5-8µm x 10-17µm), oval (6-8µm x 9-12µm), spherical or oblong intercalary swellings often transversely septate with two sometimes three divisions.

Zoospore discharge: Zoospores released when tip of discharge tube/papilla deliquesces, form a temporary globular mass imbedded in "slime" before becoming amoeboid or swimming away; Low papilla or long to short regular or irregular stout discharge tube which either penetrates surface of substrate or goes into another cell (in decaying plant shoot material).

Zoospore microscopic: Inoperculate; zoospores spherical or oval, 4.5-5.5µm in diameter, single large lipid globule; posteriorly directed flagellum 25-28µm long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores spherical (8-16µm), oval (10-12µm x 14-16µm), or broadly fusiform; wall smooth, colorless; functioning as a prosporangium upon germination.

Ecology and Distribution: Saprophytic in decaying *Alcorus calamus, Iris pseudoacorus, Glyceria spectabilis*, Nowakowski (loc. cit.), soil, Remy (1948: 214), GERMANY; Hippuris vulgaris, de Wildman (1895b: 91), FRANCE; leaves of "Massette," Constantineanu (1901: 385), RUMANIA; Acorus calamus, Sparrow (1943: 309), grass leave bait from soil, Karling (1948:510), US; vegetable debris, Karling (1945:33), BRAZIL; from soil, Gaertner (1954: 22), EGYPT.

Questionable species not included either because they were not completely described or because they most likely resemble previously described species (Sparrow 1960):

Cladochytrium aneurae Thirumalachar

Cladochytrium cornutum de Wildeman

Cladochytrium irregulare de Wildeman

Cladochytrium polystomum Zopf

Taxonomic Key to Species of *Cladochytrium*

All of the morphological characters used to create this taxonomic key are visible under a light microscope. Identification will require observation over several days in order to see the mature structures utilized in the key.

Key to the Species of *Cladochytrium*

1a. Found on cellulosic substrates or hosts……..2

1b. Found on snake skin…….....*Cladochytrium indicum*

2a. Swellings present in rhizomycelium.....3

2b. Swellings absent in rhizomycelium; multi-chambered zoosporangia.....*Cladochytrium novoguineense*

3a. Intercalary swellings septate.....4

3b. Intercalary swellings non-septate.....7

4a. Zoospore pigmented cadmium-orange or golden brown.....5

4b. Zoospore hyaline.....6

5a. Lipid globules in swellings hyaline.....*Cladochytrium aurantiacum*

5b. Lipid globules in swellings cadmium-orange or golden brown.....*Cladochytrium replicatum*

6a. Zoospores 4.5-5.5μm in diameter.....*Cladochytrium taianum*

6b. Zoospores 11μm in diameter.....*Cladochytrium tenue*

7a. Zoosporangia bearing simple or branched setae.....*Cladochytrium setigerum*

7b. Zoosporangia unornamented, smooth.....8

8a. Resting spores smooth and hyaline; produced by multi-celled swellings.....*Cladochytrium hyalinum*

8b. Resting spores smooth and light brown.....9

9a. Trabeculae in the filamentous portions of the rhizomycelium.....*Cladochytrium crassum*

9b. No trabeculae in the filamentous portions of the rhizomycelium; spherical to irregularly lobed zoosporangium.....*Cladochytrium salsuginosum*

Taxonomic Summary of the Genus *Endochytrium*

Taxonomic History of the Genus *Endochytrium*

Introduction

The genus *Endochytrium* (Sparrow 1933) was erected for an intramatrical, operculate, uniflagellate chytrid parasitic on *Cladophora* with ovate, sub-spherical or pyriform sporangia and a single oil globule in the zoospore. Sparrow's initial description of *Endochytrium ramosum* noted morphological similarities to species of *Entophlyctis* and without any further explanation stated that it possessed a "radically" different type of development. Sparrow did not observe resting spores and he made no mention of the number of sporangia per thalli but an examination of his figures (Sparrow 1933 Plate 2) show mutliple sporangia with elongated and possibly intertwining rhizoids inside cells of the *Cladophora* host. In 1928 and again in 1931, Karling isolated a similar fungus from the same host (*Cladophora* sp. - though not the same material) and after following the development of his chytrid both on host material and on agar plates, Karling felt that he had the same species as Sparrow's *E. ramosum* with one major difference. Karling's described his isolate as producing only monocentric thalli while Karling interpreted Sparrow's *E. ramosum* as producing polycentric thalli based on Sparrow's drawings of *E. ramosum*. Karling

then emended Sparrow's generic description of *Endochytrium* to reflect the monocentric nature of his isolate, named it *Endochytrium operculatum* and synonomized *E. ramosum* with *E. operculatum*. Karling also synonymized *Rhizidium operculatum* (de Wildeman) Minden (1911), an unidentified species of *Entophlyctis* described by Karling (1931), and *Entophylctis maxima* Dangeaerd (1932). Sparrow (1943, 1960) disagreed with Karling and removed *E. ramosum* from synonomy with *E. operculatum* noting the differences in resting spore size and wall ornamentation (Hillegas 1940, Karling 1937) as his reasons for separation. Sparrow also retained *E. operculatum* as a separate species and amended his original description of *E. ramosum* to include only monocentric thalli and a description of larger, smooth walled resting spores produced from his isolate of *E. ramosum*. Sparrow also questioned Karling's synonomy of Dangeaerd's chytrid since the type of discharge was not observed and the species could easily be placed in either *Entophlyctis* or *Endochytrium*. Karling's next contribution to the genus came with the description of *E. digitatum* in 1938. *Endochytrium digitatum* differed from all previously described species in producing one to several blunt digitations on the base of the zoosporangium or the main axis of the rhizoidal system and light to medium brown resting spores. In 1941, Karling placed Scherffel's *Entophylctis pseudodistoma* into *Endochytrium* because it was operculate instead of being inoperculate, a characteristic of species in the genus *Entophylctis*. At the time of its description, Scherffel's chytrid differed from other species of *Endochytrium* by having irregularly spaced reflexed scales or columns on the surface of a resting spore that either partially or completely filled the structure from which it developed (Sparrow uses the phrase "sporangium-like structure in his translation of Scherffel's latin diagnosis). Dogma (1969) described the last two species of Endochytrium from baited gross cultures of submerged plant material, forest soil and leaf litter. *E. multiguttulatum* was found by baiting

cultures of decayed Sphagnum moss and other plant debris with lens paper, cellophane and onion skin. Dogma described *E. multiguttulatum* as a new species of *Endochytrium* because it was operculate, monocentric, endobiotic, possessed non-apophysate zoosporangia and resting spores, developed in an *Entophlyctis*-type manner and exhibited a unique origin of the rhizoidal axes near the persistant, thickened zoospore cyst. With respect to differences at the species level, Dogma again cited the unique insertion of the epibiotic rhizoids near the zoospore cyst along with multiple lipid globules in the zoospores and laterally inserted flagella as his reasons for separating E. multiguttulatum from all other species in the genus. The second species Dogma described, *E. cystarum*, resembled *E. multiguttulatum* and other members of the genus with respect to development but differed in possessing very simple and reduced rhizoids arising from multiple points on the zoosporangium, smaller zoospores with a single lipid globule and resting spores that were either spiny or smooth walled. Dogma also noted a persistant zoospore cyst present on both the zoosporangia and resting spores of *E. cystarum* and pointed out that *E. pseudodistomum* also had the same structural feature. Interestingly enough in his drawings of *E. multiguttulatum*, Dogma drew a structure that could be interpreted as a persistant zoospore cyst on the resting spores. Karling also noted that sometimes the zoospore cyst remained intact on zoosporangial thalli of *E. operculatum* as well as on the resting spores.

The type of *Endochytrium*

The first species of *Endochytrium* to be described was *E. ramosum* by Sparrow (1933) but Sparrow did not officially designate his species as the type specimen (see Aquatic Phycomycetes 1960) nor did he deposit any type material. According to ICBN (2000) articles 8.1, 9.1, and 9.2 illustrations can be used as type material for taxa with no extant holotype

material available and as such we designate *Endochytrium ramosum* Sparrow (1933), Amer. J. Bot. 20:63-77, pl. 2, figs. A-G as the lectotype.

II. The Species of *Endochytrium*: Taxonomic Descriptions, Ecology and Distribution

Currently there are six accepted species of *Endochytrium* and one questionable species. *E. ramosum* Sparrow, *E. operculatum* (de Wildeman) Karling, *E. digitatum* Karling, *E. pseudodistomum* (Scherffel) Karling, *E. multiguttulatum* Dogma, and *E. cystarum* Dogma are the six accepted species. *E. oophilum* Sparrow has remained questionable since Sparrow first described it in 1933.

1. *Endochytrium cystarum* Dogma

Arch. Mikrobiol. 66:207, pl. 2, figs. 26-39. 1969.

PLATE 7, figs. 79-89

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Zoosporangium formed by enlargement of the germ tube; attached to a persistant, epibiotic, thin-walled, colorless (2-2.5μ in diameter) zoospore cyst by a 3-15μ long germ tube.

Reproductive: Zoosporangium hyaline, smooth, thin-walled, spherical (13.5-90μ in diameter), oval or irregular (24-75μ broad by 18.5-54μ high).

Rhizoidial System: Rhizoids depauperate, poorly developed, arising from one to four places on the surface of the zoosporangium, sometimes lacking, very delicate, thin-walled, colorless, simple or branched twice, up to 2.5μ broad at the point of origin.

Zoospore discharge: Zoospores released en masse by the dehiscence of 4.0-6.0μ diameter opercula capping one to five papillae or discharge tubes 8.0-27μ x 6.5-17μ; held quiescent for some time at the orifice by the extruded gelatinous substance formed beneath the operculum. Endooperculum and vesicle not formed.

Zoospore microscopic: Zoospores hyaline, spherical, 2.2-2.6μ in diameter, with a small centric lipid globule 0.6μ in diameter, a faint arc-like body (nuclear cap?), and a posteriorly attached flagellum 17μ long.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores borne like the zoosporangium, spherical or oval (15-23.5 x 10-13.5μ with globular content and light brown 1.5-2.0μ thick wall with or without blunt spines or tubercles 2.0-7.0μ x 1.5-2.0μ at the base; germination not observed.

Ecology and Distribution: On lens paper and cellophane bait from forest soil and leaf litter samples, Dogma (loc. cit.), US.

2. *Endochytrium digitatum* Karling

Mycologia. 30: 307, figs. 20-37. 1938.

PLATE 7, figs. 90-99

Vegetative: Thalli numerous, endobiotic, monocentric and eucarpic.

Development: Zoosporangia develop exogenously from a swelling in the germ tube and delimited from the rhizoidal system by a septum at maturity. Karling considered it to be the same as *E. operculatum* but this species lacks a persistant zoospore cyst as seen in *E. operculatum*.

Reproductive: Zoosporangia hyaline, smooth, except for one to several blunt digitations at or near the base; elongate and obclavate (11 x 44μ - 18 x 35μ), pyriform (15 x 22μ - 71 x 120μ), obpyriform, subspherical, irregular, somewhat triangular and lobed, with 1-4 usually one, single or branched, straight, curved, undulating, or coiled, tapering exit tubes, 5-18μ in diameter and 10-275μ in length, which may occasionally extend 88μ beyond the surface of the host wall.

Rhizoidial System: Rhizoidal system well developed and richly branched, extending sometimes for a distance of 550μ, smooth or irregular in contour, 2.7-5μ in diameter and occasionally digitate at the base.

Zoospore discharge: Operculum spherical or slightly oval, 3.3-5.5μ; Zoospores emerge fully formed and singly, and lying quiescent in a globular mass a short while before becoming motile, intermittently amoeboid.

Zoospore microscopic: Zoospores hyaline with a clear refractive globule 1.6-2.2μ in diameter, spherical, 4.4-5.5μ, posteriorly uniflagellate

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores smooth, light to medium brown, oval, subspherical (16 x 18μ - 10 x 15μ), spherical (20μ), obpyriform, with a 1.75-2.5μ thick wall and a large refractive globule usually surrounded by several small ones; germination unknown.

Ecology and Distribution: Saprophytic in dead internodes of Chara coronata, Nitella flexilis, and other algae, Karling (loc. cit., New Jersey, New York), US.

3. *Endochytrium multiguttulatum* Dogma

Arch. Mikrobiol. 66:204, pl. 1, figs. 1-19, pl. 2, figs. 20-25. 1969.

PLATE 7, figs. 100-108

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Zoosporangium formed by enlargement of the distal portion of the germ tube of an encysted zoospore, the proximal portion of the germ tube usually persisting as as a thick-walled (5-18μ by 2-10μ) appendage capped by a portion of the persistant zoospore cyst (2-4μ in diameter).

Reproductive: Zoosporangium usually large and coarse, spherical (83-150µpyriform (15 x 135µ high by 9-112µ broad) or irregular; wall smooth, colorless at first, soon thickening to 1.3-1.7µ becoming golden brown or amber and crusty with age.

Rhizoidial System: Rhizoids arise near zoospore cyst, mostly epibiotic, extensive, wavy, coiling or twisting, up to 23µ broad at point of origin, with thick, golden-brown wall, trabeculate at the proximal and vacuolate at the distal end.

Zoospore discharge: Zoospores are released en masse by the dehiscense of a convex, 3-22.5µ diameter operculum and remain quiescent for a period of time at the orifice of the zoosporangium embedded in a gelatinous, extruded substance formed beneath the operculum ; One to four discharge tubes (10-142µ x 7-27µ) or short papilla present per zoosporangium;

Zoospore microscopic: Zoospores hyaline, spherical, 6-7µ in diameter, with five to ten minute refractive globules, and a laterally inserted, posteriorly directed, 38-40µ long flagellum that is retracted by vesiculation.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop from zoosporangia; Spherical (13.5-38µ in diameter broadly ellipsoid (30-38µ x 20-22µ) with 4.5 thick golden-brown walls exhibiting numerous low, sharp or blunt bullations; germination not observed.

Ecology and Distribution: Saprophytic on lens paper, cellophane, and onion skin bait in dish cultures of decaying Sphagnum and other plant debris, Dogma (loc. cit.), US.

4. *Endochytrium operculatum* (de Wildeman) Karling

Amer. J. Bot. 24: 353, figs. 1-53. 1937.

Rhizophylctis operculata de Wildeman, Ann. Soc. Belge Micro, 19:103. 1895.

Rhizidium operculatum (de Wildeman) Minden, Krpt. Fl. Mark Brand., 5:374. 1911.

Entophylctis sp. Karling (pro parte), Amer. J. Bot. 18:448. 1931.

Entophylctis maxima Dangeaerd, Le Bot. 24:242. 1932.

Endochytrium ramosum Sparrow, Amer. J. Bot. 20:72. 1933.

PLATE 7, figs. 109-118

Vegetative: Thallus monocentric, eucarpic, usually endobiotic

Development: Zoosporanium develops exogenously from a swelling in the germ tube.

Reproductive: Sporangia hyaline, smooth, almost spherical (4-140µm), broadly and narrowly pyriform (5x7µm – 60x150µm), ovate, egg- or spindle-shaped, elongated, tubular and cylindrfical, occasionally obclavate or irregular and plurilocular with one to several thick exit papillae or tapering tubes of varying length, 15 to 75µm, and diameter of 5-20µm

Rhizoidial System: usually extensively developed, branched, coarse and irregular, often invading several adjacent host cells and attaining a diameter of 2-10µm at point of insertion on sporangium.

Zoospore discharge: Operculum is spherical, 4-8µm or oval 4x5µm – 6x7µm.

Zoospore microscopic: Zoospores are hyaline, spherical or slightly oval, 3-5µm, with a clear lipid globule in the center

Zoospore ultrastructure: Not available

Resting Spore: Hyaline or occasionally with a faint yellow tinge, predominantly spherical (4.5-18µm), oval or slightly ellipsoidal (5x7µm – 12x16µm), smooth, rough or warty; Germinates via formation of a pore in the resting spore wall though which contents of the spore are extruded to form a sporangium flush with the outer wall of the resting spore or at a distance from the surface of the resting spore at the tip of a tube and delimited by a cross wall (Hillegas 1940).

Ecology and Distribution: Saprophytic or weakly parasitic in various algae, tissues of Charophytes and other vascular plants and cysts of monadineae, Karling (loc. cit.), US; de Wildeman (loc cit.), FRANCE.

5. *Endochytrium pseudodistomum* (Scherffel) Karling

Mycologia 33:357. 1941.

Entophylctis pseudodistoma Scherffel, in Domján, Folia Cryptogamica 2:46, pl. 1, figs. 64,66,68,70,76-81, 85-87, 99, 100, 111. 1935.

PLATE 8, figs. 119-125

Vegetative: Thallus monocentric, eucarpic, endobiotic.

Development: Zoosporanium develops exogenously from a swelling in the germ tube.

Reproductive: Zoosporangia hyaline, 17.5-25.4μ high by 15-25.4μ wide. Persistant zoospore cyst remains connected to zoosporangium by the germ tube.

Rhizoidial System: Rhizoids originate from the base of the sporangium, large and highly branched, thick, extensive.

Zoospore discharge: Operculum present; Discharge tube usually forms next to the germ tube, rarely out of the side of the sporangium, length 5-15μ, width 5-7.5μ; Zoospores are released en masse and remain still at the orifice of the zoosporangium for a short period of time before slowly separating and swimming away. The operculum is either pushed off or folded back as a hinged lid.

Zoospore microscopic: Zoospores hyaline, 5-7.5μ in diameter with a single lipid droplet 1.2-2.5μ in diameter,

Zoospore ultrastructure: Not available.

Resting Spore: Endobiotic, develop from zoosporangia also with a persistant zoospore cyst; scales on surface of outer resting spore wall either curved or s-shaped; brown in color at maturity; subspherical (17.5-22.5μ); thick-walled; possess a large, eccentric lipid droplet 11μ in diameter.

Ecology and Distribution: Saprophytic in senescent Zygmena and Spirogyra filaments, Scherffel (loc. cit.), HUNGARY.

6. *Endochytrium ramosum* Sparrow

Amer. J. Bot. 20:72. Plate 2, figs. A-G. 1933.

PLATE 8, figs. 126-130

Vegetative: Thallus endobiotic, monocentric, eucarpic

Development: Not available

Reproductive: Sporangium ovoid, subspherical, or sometimes pyriform, up to 35μm in diameter; wall thin, smooth, colorless

Rhizoidial System: Rhizoids extensive, irregularly and profusely branched, ramifying through many cells, often broad (up to 10μm in diameter) at the basal or somewhat lateral place of attachment on the sporangium wall

Zoospore discharge: Sporangia produce a single, short broad discharge tube that just penetrates the wall of the substrate or host; Zoospores are released upon the dehiscence of an operculum at the tip of the discharge tube and remain motionless in an ellipsoidal mass at the orifice for a short period of time. Operculum is 7μm in diameter.

Zoospore microscopic: Zoospores spherical or somewhat elongate, 3-5μm in diameter, posteriorly uniflagellate, with a single colorless centric lipid globule,

Zoospore ultrastructure: Not available

Resting Spore: Spherical, 20-35µm in diameter, with a smooth faintly brownish wall 2.5-3µm thick; Contains a large central and several small peripheral lipid globules. Germination not observed.

Ecology and Distribution: Weakly parasitic or saprophytic in *Cladophora* sp., Sparrow (loc. cit.), US; Vegetable material, Shanor (1944:331), MEXICO; Saprophytic on grassleaf bait, from soil, Sparrow (1952: 69), CUBA.

Taxonomic Key to Species of *Endochytrium*

All of the morphological characters used to create this taxonomic key are visible under a light microscope. Identification will require observation over several days in order to see the mature structures utilized in the key.

Key to the Species of *Endochytrium*

1a. Zoospore with multiple small lipid globules.....*Endochytrium multiguttulatum*

1b. Zoospore with a single large lipid globule.....2

2a. Rhizoids poorly developed with few branches.....*Endochytrium cystarum*

2b. Rhizoids highly developed with many branches.....3

3a. Zoosporangia one to several blunt digitations at the base of the sporangium or on the main axis of the rhizoidal system.....*Endochytrium digtatum*

3b. Zoosporangia smooth, ovate, spherical, pyriform or elongate.....4

4a. Zoospore cyst persistant on zoosporangium.....*Endochytrium pseudodistomum*

4b. Zoospore cyst evancescent.....5

5a. Resting spore wall smooth.....*Endochytrium ramosum*

5b. Resting spore wall smooth, warty or rough.....*Endochytrium operculatum*

Taxonomic Summary of the Genus *Nephrochytrium*

Taxonomic History of the Genus *Nephrochytrium*

Introduction

Currently the number of species in *Nephrochytrium* varies depending on which publication one uses: Sparrow's Aquatic Phycomycetes listed 3 (1960), Batko's Zarys Hydromikologii listed 6 (1975), and Karling's Chytridiomycetarum Iconographia listed 5 (1977). One new species was described in 1983 bringing the total number to seven though there is no current publication that lists all seven species. The species listed in Sparrow's monograph follow Karling's original 1938 definition of the genus but species described after 1960 expanded the generic concept so that it overlaped with the genus *Diplophlyctis*. The primary goal of Batko's book was to provide a list of aquatic fungi found in Poland not as a monograph. Karling meant his publication only to be a pictoral reference to chytrid genera and the most common species in each genus leaving Sparrow's publication as the only monograph for chytrid fungi.

Even though Karling (1977) asserted that his publication was not a monograph the entry for *Nephrochytrium* listed reversals of previous taxonomic decisions by other authors and included an expanded definition of the genus with characters belonging to species in the genus *Diplophlyctis*. Batko's list moved species in and out of the genus making taxonomic changes with no textual explanation. Karling's taxonomic changes mirrored Batko's suggesting that Karling agreed with Batko even though he did not cite Batko's 1975 publication. Most of the confusion in deciding which genus to place newly described species revolved around similarities in morphology and development between *Nephrochytrium* and *Diplophlyctis* coupled with disagreements over the operculation status of "endo-operculate" species. Three species *(N. appendiculatum* Karling*, N. stellatum* Couch*, and N. aurantium* Whiffen) have only once been

questioned as to their generic status but were left within the genus due to lack of information. In 1938, Karling erected the genus *Nephrochytrium* for a monocentric chytrid with occasional intercalary swellings in the rhizoids and which formed both sporangia and resting spores as outgrowths of what he termed an "apophysis". Similarities in appearance of the sporangia and zoospores to *Diplophlyctis intestina* led Karling to initially consider the chytrid to be a member of the genus *Diplophlyctis* (1938 Karling). A later examination of his drawings and notes revealed a difference in development of the sporangia and resting spores between *D. intestina* and his new isolate. In *D. intestina*, the sporangia and resting spores develop as enlargements of the germ tube whereas Karling's isolate as stated above produced sporangia and resting spores as outgrowths of an "apophysis". Karling also cited the presence of occasional swellings in the rhizoids and the thickened persistent zoospore cyst as further differences between the two chytrids and decided to erect a new genus named for the typical kidney shape of the sporangia and resting spores. The only thing missing from Karling's description was the type of discharge mechanism utilized by his fungus, as he was unable to observe the initial stages of release. Later the same year, Couch described a monocentric operculate fungus that shared a similar type of development and morphology with Karling's fungus. Couch cited these similarities as a basis for placing his isolate within *Nephrochytrium* and named it *N. stellatum* due to the stellate morphology of the resting spores. Though Couch's fungus did not produce the typical kidney shaped sporangia and resting spores as found in *N. appendiculatum*, both types of sporangia formed by an outgrowth of what both authors termed an apophysis while the zoospore cyst thickened and remained attached to the apophysis by the initial germ tube. These characteristics are readily apparent in Couch's drawings of *N. stellatum* (Couch 1938). In 1941, Whiffen described a new monocentric operculate chytrid with the same type of development as both *N.*

appendiculatum and *N. stellatum*. *N. aurantium* Whiffen also produced a thickened persistant zoospore cyst but differed from the previous two species in producing a spherical apophysis, lacking a distinct isthmus between the apophysis and the sporangium, and in producing orange colored zoospores.

Sparrow circumscribed the genus in 1943 to include intramatrical, monocentric, eucarpic forms with variously shaped apophysate zoosporangia and resting spores that developed as outgrowths of an apophysis. The operculation status of the genus was left open because Karling's description of the type species, *N. appendiculatum*, did not have any information on whether the species was operculate or inoperculate. Sparrow simply stated that the zoosporangia had "one or more exit papillae or tubes of varying length," a statement taken directly from Karling's description paper of *N. appendiculatum*. In his revision of the Aquatic Phycomycetes in 1960, Sparrow did not change his statement concerning the lack of information on the operculation status of the type species and again wondered if *N. aurantium* and *N. stellatum* maybe should be placed in their own genus. Karling eventually stated in one of his later publications that *N. appendiculatum* was indeed operculate based on his re-isolation of *N. appendiculatum* while working in India (Karling 1964, 1966). In order to reinforce his view of the operculate nature of *Nephrochytrium*, Karling (1967) stated in one of his paper's on the zoosporic fungi of New Zealand that: "… In establishing the genus in 1938, the author [Karling] failed to mention the presence of an operculum but subsequent studies by Couch (1938), Whiffen (1941), and the author [Karling] (1964,1966) have shown that the sporangia are operculate." Karling cites Couch's and Whiffen's descriptions of their *Nephrochytrium* species as support for the type species being operculate as well as re-citing his 1964 paper and a 1966 survey paper that simply cited his 1964 paper as evidence for operculation (no drawings in the paper). Since Karling's

original culture of the type (or any culture isolated by Karling of the type) is not available for examination it is impossible to say with any degree of certainty whether or not his fungus was indeed operculate.

In contrast to the three previously mentioned species, *N. amazonenesis* Karling, *N. buttermerense* Willoughby, *N. complicatum* Willoughby, and *Diplophlyctis sexualis* Haskins have all been moved back and forth by different authors between *Nephrochytrium* and *Diplophlyctis*. After the first edition of Sparrow's Aquatic Phycomycetes was released in 1943 Karling described another species of *Nephrochytrium* in 1944. *N. amazonenesis* was observed in moist soil samples collected in Brazil. Karling described his species as being "endo-operculate" with the operculum forming down inside of the discharge tube instead of on the surface as seen in "exo-operculate" species (Karling 1944, Dogma 1973). Until he observed the "sunken operculum" he considered the isolate to be a species of the inoperculate genus *Diplophlyctis* because it strongly resembled members of the genus in both its development and morphology. *Diplophlyctis* species generally (see Sparrow 1960 for a discussion on the variability in *Diplophlyctis* development) develop with the sporangium forming first followed by the apophysis whereas *Nephrochytrium* is the exact opposite with the apophysis developing first followed by the sporangium. Karling's description of *N. amazonenesis* development clearly resembled the development of a *Diplophlyctis* but he suggested that further study was needed to confirm exactly how the sporangia developed even though he had not observed any sporangia budding out from an apophysis in his study of the species. He felt that development might not be as diagnostic for the genus as operculation when *N. amazonenesis* and other species were studied in greater detail. Sparrow (1960) did not share Karling's view of "endo-operculation" as being a type of operculation and citing the similarity in development moved Karling's fungus to

Diplophlyctis renaming it as *D. amazonense* (Karling) Sparrow. Sparrow agreed with Haskins (1950) observation that "endo-opercula" only occurred in aging cultures despite Karling's description of the *N. amazonenesis* "endo-operculum" as being a regular occurrence in cultures both young and old. Karling's fungus also differed from other species of *Nephrochytrium* in that it did not have a persistent zoospore cyst.

In 1961, Willoughby used the presence of a persistent zoospore cyst to place two monocentric, eucarpic, and apophysate chytrids into *Nephrochytrium*: *N. complicatum* and *N. buttermerense.* Both chytrids were isolated from lake mud collected from the Lake District of England but *N. complicatum* was inoperculate and grew on chitin while *N. buttermerense* exhibited "endo-opercula" similar to that found in Karling's *N. amazonensis* and grew on cellulose. Willoughby did acknowledge some embarrassment in the fact that one of the new species was inoperculate; a condition which violated the genus description of *Nephrochytrium.* In 1968, Dogma isolated a chytrid from Michigan (USA) that he felt clearly resembled Willoughby's *N. buttermerense* in having a persistent zoospore cyst, an endo-operculum, and the same type of development. After a detailed morphological study of his Michigan isolate, Dogma (1969) amended *Diplophlyctis* to include species with a persistent zoospore cyst and renamed *N. buttermerense* Willoughby as *Diplophlyctis buttermerense* (Willoughby) Dogma. He also agreed at the time with Sparrow (1960) and Haskins (1950) that an "endo-operculum" did not constitute a true operculum, i.e. "a discrete and integral part of the sporangial wall" and considered both Karling's and Willoughby's species to be inoperculate (Dogma 1973, Sparrow The Fungi Chapter 6 pg.87-92).

N. complicatum Willoughby was described in the same paper as *N. buttermerense* but differed from *N. buttermerense* in that it produced inoperculate sporangia, a character consistent

with *Diplophlyctis* not *Nephrochytrium*. Though both Karling (1967) and Dogma (1969) had suggested moving *N. complicatum* to *Diplophlyctis* because it was most likely inoperculate they lacked the necessary material to make a definite decision. Willoughby never observed actual zoosporangia releasing but reported in the text of his paper that he did see "dehisced sporangia [that] suggested the dehiscence mechanism was inoperculate" and later stated in the species description that "zoospores are released when the tip of the exit tube dissolves." Willoughby's assumption and the lack of material available for further study prevented elucidation as to the true nature of *N. complicatum's* discharge method. Shortly after Dogma tranferred *N. buttermerense* to *Diplophlyctis*, he isolated a species resembling *N. complicatum* from water and mud samples collected in Michigan and England. As with *D. buttermerense*, Dogma carried out a morphological study on the isolate and showed it to be inoperculate. This prompted Dogma to officially transfer the species to *Diplophlyctis* as *D. complicata* (Willoughby) Dogma (Dogma 1974, Index of Fungi 1975).

In his 1975 publication on the aquatic fungi of Poland, Zarys Hydromikologii, Batko retained *D. amazonense* (Karling) Sparrow and *D. buttermerense* (Willoughby) Dogma in *Nephrochytrium*. Batko also moved *Diplophlyctis sexualis* Haskins into *Nephrochytrium* and renamed the species *Nephrochytrium sexuale* (Haskins) Batko. Batko gave no reason for his taxonomic decisions in the text so one can only hypothesize as to why he thought these changes were necessary. He may have simply disagreed with Sparrow and Dogma's view on "endo-operculation" as being inoperculate and disregarded the movement of Karling and Willoughby's species into *Diplophlyctis* (Longcore pers.comm.). As for moving *D. sexualis*, Batko may have been influenced by Haskin's description of the fungus as having "endo-opercula" in older cultures. Haskin stated that in older cultures after the gelatinous tip had dissolved the membrane

covering the contents of the sporangium thickened and was either pushed out like a cap or ruptured upon release. Haskin's description mirrored Karling and Dogma's description of "endo-opercula" in their species (1944, 1974). Karling's 1977 Chytridium Iconographium also retained *D. amazonenesis* (Karling) Sparrow and *D. buttermerense* (Willoughby) Dogma in *Nephrochytrium* and similar to Batko suggested the inclusion of *Diplophlyctis sexualis* Haskins because it exhibited "endo-operculation". Karling also kept Dogma's species with a persistant zoospore cyst in *Diplophlyctis*. Karling (1977) emphatically stated that *Nephrochytrium* "included operculate species whose zoosporangia bud out as discrete entities from an apophysis or prosporangium, but [it] is emended to include other operculate species such as *N. amazonenese* Karling and *N. buttermerense* Willoughby in which formation of the zoosporangium out of an apophysis is not sharply defined." Retaining *N. amazonenese*, *N. buttermerense*, and *D. sexualis* widened the *Nephrochytrium* generic concept with respect to type of development and operculation to include species with a *Diplophlyctis* type of development and endo-operculation.

S.K.M. Hassan described *Nephrochytrium bipes* in 1983 as a monocentric, eucarpic, apophysate chytrid isolated from pond water on onion skin. As with Willoughby and Karling before him, Hassan did not observe actual zoospore release but he did note that in developing sporangia the "discharge papilla or tube" became filled with a hyaline portion of the sporangial contents that supported a "thin-shallow endo-operculum". He also noted that empty zoosporangia appeared to have "a slightly thickened rigid wall" with "fragments of the endo-operculum within the evacuation tube" and concluded that the endo-operculum was probably partially dissolved or perforated during release and then pushed out of the discharge tube. The possibility exists that *N. bipes* is indeed endo-operculate but Batko's drawings and pictures are inconclusive and there is

no culture of the fungus available for comparison. The author of this paper has seen pictures of the type material but they are also inconclusive with respect to determining the release mechanism. The method of development is similar to that seen in the exo-operculate *Nephrochytrium* species: a "sack-like" apophysis develops first followed by the sporangium that buds laterally off of the apophysis. Hassan's paper also cryptically states that type of development was no longer significant in distinguishing *Diplohlyctis* from *Nephrochytrium* because of the discovery of *D. complicata* that developed like a species of *Nephrochytrium*. Wiloughby's paper does not give a complete enough description of the development in the text to determine if his fungus developed like a *Nephrochytrium* or not but the drawings do suggest a *Diplohlyctis* type of development. In Willoughby's Plate 112 b-j, the sporangium forms from a swelling of the germ tube followed by a second swelling at the junction between rhizoids right beneath the sporangium. In this case the sporangium develops before the apophysis. Dogma's isolate of *D. complicata* developed in the same manner though Willougby did not report formation of a bi-lateral apophysis as seen by Dogma for some thalli of his isolate. If one followed Dogma, Haskin and Sparrow's view of "endo-operculation" as being inoperculate then *N. bipes* would belong in *Diplohlyctis* but with a *Nephrochytrium* type of development. On the other hand, if we followed Karling, Willoughby and Hassan's view of "endo-operculation" then *N. bipes* would remain in *Nephrochytrium*. Sparrow's view that "endo-operculation" is something that occurs in many chytrids and primarily in older cultures (Sparrow 1960) is based on studies of several chytrids but the possibility that some chytrids are only "endo-operculate" and do not exhibit any other form of release has not been fully examined. Genera could be composed of species with different release mechanisms allowing for the inclusion of endo-operculate species with exo-operculate species as seen with *Nowakowskiella*. Based on this line

of reasoning, *N. bipes* could be retained within the exo-operculate genus *Nephrochytrium*. Though Sparrow (1960) stated that the order of development for different parts of the thallus vary in *Diplophlyctis*, an examination of species descriptions show that the sporangium either forms before or at the same time as a secondary swelling (or apophysis) beneath the sporangium never after as in *Nephrochytrium*. So while endo-operculation and the possession of a persistant zoospore cyst may cross generic boundaries type of development does not. Species in which the sporangium forms after the apophysis or subsporangial swelling could also be relegated to *Nephrochytrium*. Unfortunately, rDNA sequence data (Chapter 2) suggests that the genus is polyphyletic making any circumscription based on the current suite of morphological and developmental characters phylogenetically invalid.

The goal of this summary is to provide a description of all species in one publication. Unfortunately, an accurate determination of taxonomic status is beyond the scope of this paper and should not be made until all described species are brought into pure culture and material can be obtained for sequencing and for examination of zoospore ultrastructure.

The type of *Nephrochytrium*

Karling (1938) clearly stated that *Nephrochytrium appendiculatum* was the type species for the genus but did not deposit any type material. *N. appendiculatum* has been reported several times since its original description by Karling (Sparrow and Barr 1955; Karling 1941, 1964, 1967) and its habit of growing saprophytically on *Chara* and *Nitella* should make it easy to find but so far none of the chytrids isolated from Charophytes or other sources by the author of this paper or other chytrid workers resemble Karling's species exactly as it is described. Lacking a culture that matches Karling's description, drawings serve as a reasonable replacement. According to ICBN (2000) articles 8.1, 9.1, and 9.2, illustrations can be used as type material for

taxa with no extant holotype material available and as such we designate *Nephrochytrium appendiculatum* Karling (1938), Amer. J. Bot. 25:211-215, figs. 1, 2A-V as the lectotype.

Terminology

Unfortunately as with many things in chytrid taxonomy type of development is not free from controversy. Karling (1936) and Sparrow (1936) originally defined an apophysis as a "swelling of the germ tube upon exogenous migration of the nucleus from the zoospore cyst/case into the germ tube". Powell and Letcher (2002) agreed with Karling (1936) and Sparrow (1936) and only considered a swelling beneath a sporangium an apophysis if it is connected to a nuclear event, i.e. movement of the nucleus. Anything else should be considered simply a sub-sporangial swelling and not homologous to an apophysis. In *Nephrochytrium*, the nucleus is known to move out of the zoospore cyst and develop in a swelling of the germ tube off of which the sporangium buds and into which the cytoplasm moves before zoosporogenesis occurs. In this case the initial swelling could be considered an apophysis. In *Diplophlyctis* species where the swelling of the germ tube serves as the sporangium the secondary swelling beneath it would not be considered an apophysis but simply a sub-sporangial swelling. *Nephrochytrium* could then be defined as a monocentric, eucarpic, apophysate operculate/endo-operculate genus with an apophysis first/sporangium second type of development while *Diplophlyctis* constitutes a monocentric, eucarpic, inoperculate/endo-operculate genus with a sporangium first/sub-sporangial swelling second type of development. For *Diplophlyctis* species in which the second swelling appears at the same time as the sporangium the second swelling could be considered an apophysis since its development is not separated from the movement of the nucleus. On the other hand, if the nucleus remained in the upper part that eventually became the sporangium the bottom part could

be considered a sub-sporangial swelling since the nucleus never technically enters that part of the thallus.

The Species of *Nephrochytrium*: Taxonomic Descriptions, Ecology and Distribution

1. *Nephrochytrium amazonensis* (Karling) Batko

Zarys Hydromikologii p. 220, fig. 359. 1975.

Diplophlyctis amazonense (Karling) Sparrow, Aquatic Phycomycetes 2nd ed., p. 388. 1960.

Nephrochytrium amazonensis Karling Amer. J. Bot. 36:352, figs. 1-28. 1944.

PLATE 9, figs. 131-144

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Zoosporangium formed by enlargement of the germ tube followed by formation of the apophysis as a second swelling above the zoosporangium.

Reproductive: Zoosporangium hyaline, smooth, pyriform (12-30 x 50-140μm), almost spherical (10-60μm in diameter), obclavate, flattened and often somewhat kidney-shaped; No persistant zoospore cyst; Apophysis oval (5-12 x 8-22), flattened, obpyriform, almost spherical.

Rhizoidial System: Rhizoidal system arising from base of apophysis, extending over a radius of 80-400μm, main axis up to 8μm in diameter, richly branched.

Zoospore discharge: Zoospores when released form a globular mass at discharge tube orifice before swimming away; Tip of discharge tube swells and softens to form a plug of hyaline material followed by formation of endo-operculum; Endo-operculum formed down inside of discharge tube, shallow, saucer-shaped, deeper bowl- or cup- and occasionally somewhat cone-shaped, 4-7μm in diameter; Discharge tube short (5x10μm) or elongate (5-7μm x 20-130μm).

Zoospore microscopic: Zoospores 5-6.5µ in diameter, with a large lipid globule; flagellum 35-38µm in length.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop from base of apophysis; Usually oval and somewhat bean-shaped (20-28µm x 30-40µṃalmost spherical (15-35µm in diameter), sometimes irregular; Wall dark brown, 2-3µm thick, usually spiny, sometimes verrucose or covered with numerous short setae, rarely smooth; function as prosporangium in germination.

Ecology and Distribution: Saprophytic in decaying vegetable debris from river water, Karling (loc. cit.), BRAZIL.

2. *Nephrochytrium appendiculatum* Karling

Amer. J. Bot. 25:211, figs. 1, 2A-V. 1938.

PLATE 9, figs. 145-160

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Apophysis develops as a swelling in the germ tube followed by the zoosporangium which develops by budding out of the apophysis.

Reproductive: Zoosporangium hyaline, smooth, sub-spherical, flattened, depressed, usually somewhat kidney-shaped (8x14µm – 18x30µm); Persistant zoospore cyst present on zoosporangium and resting sporangium, becomes thick-walled, amber colored as sporangium matures; Apophysis elongate, transverse, usually spindle-shaped and medianly constricted.

Rhizoidial System: Rhizoidal system arising from apophysis, extending over a radius of 600µm, main axis up to 5-6µm in diameter, richly branched, occasional intercalary swellings 4-8µm in diameter.

Zoospore discharge: Zoospores when released form a globular mass at discharge tube orifice, remain quiescent for a short period of time before swimming away; Exo-operculum present (Karling 1964, 1966); 1-3 papillae or discharge tubes of varying length.

Zoospore microscopic: Zoospores hyaline, spherical, 3.5-4.5μm in diameter, with a large lipid globule; flagellum 40μm in length.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop from base of apophysis; Usually somewhat kidney-shaped, flattened, depressed, occasionally obpyriform (10x18μm – 17x26μm); Wall thick, light to dark amber, smooth with one or more lipid globules; germination not observed.

Ecology and Distribution: Saprophytic in Chara and Nitella, Karling (loc. cit.), Karling (1941b:108), Sparrow and Barr (1955: 555), US.

3. *Nephrochytrium aurantium* Whiffen

Amer. J. Bot. 28:41, figs. 1-30. 1941.

PLATE 9, figs. 161-169

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Apophysis develops as a swelling in the unbranched germ tube followed by the zoosporangium which develops by budding out of the apophysis.

Reproductive: Zoosporangium spherical, cylindrical, or much lobed (12-54μm x 16-62μm); Persistant zoospore cyst present, attached to apophysis by germ tube, orange-brown color; Apophysis (6-23μm x 7-30μm) typically spherical.

Rhizoidial System: Rhizoidal system branches before apophysis appears in germ tube, colorless, extensive, much branched, continuous with apophysis.

Zoospore discharge: Zoospores when released form a globular mass at discharge tube orifice before swimming away; Exo-operculum pushed out by emerging zoospores; One or more papillae or short discharge tubes.

Zoospore microscopic: Zoospores 4-4.8μm in diameter with a large orange-colored lipid globule.

Zoospore ultrastructure: Not available.

Resting Spore: Not observed.

Ecology and Distribution: Saprophytic on grass leaves from water, Whiffen (loc. cit.), Karling (1941a: 387), Karling (1942: 620), US.

4. *Nephrochytrium bipes* Hassan

Nova Hedwigia 38:732, figs. 18-28. 1983.

PLATE 9, figs. 170-172

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Apophysis develops as a swelling in the germ tube followed by the zoosporangium which develops by budding out of the apophysis.

Reproductive: Zoosporangium smooth, spherical (22-116μm), ovate; persistent zoospore cyst present, 2-4.5μm across, connected to apophysis by narrow isthmus or directly appressed to apophysis, thickened germ tube also present; apophysis sack-like or more or less cylindrical (6.2-19.5μm)

Rhizoidial System: Rhizoidal system arising from base of apophysis, two initial rhizoidal axes, 2-19.5μm thick at point of origin.

Zoospore discharge: Zoospores when released form a globular mass at discharge tube orifice before swimming away; Endo-operculum formed down inside of discharge tube, either partly

dissolved or perforated during release, then pushed off outside; Usually one broad, short discharge tube (10-17μm in diameter).

Zoospore microscopic: Zoospores 3.2-3.9 in diameter, slightly oval, ovaoid to subspherical, hyaline, with a large lipid globule (0.9-1.0μm in diameter); posterior flagellum.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop from base of apophysis; spherical, cylindrical, or much lobed; Wall thick, smooth; Germination unknown.

Ecology and Distribution: Saprophytic on onion skin bait from pond water, Hassan (loc. cit.), POLAND.

5. *Nephrochytrium buttermerense* (Willoughby) Batko

Diplophlyctis buttermerense (Willoughby) Dogma, Arch. Mikrobiol. 66:210, Plate III, figs. 40-49. 1969.

Nephrochytrium buttermerense Willoughby, Nova Hedwigia 3:441, Plates 114-116, 1961.

PLATE 10, figs. 173-186

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Zoosporangium and apophysis formed by enlargement of the germ tube simultaneously.

Reproductive: Zoosporangium hyaline, smooth, spherical (40-70μm in diameter), oval (20-90μm x 15-70μm), almost obclavate, flattened and often somewhat kidney-shaped; Persistant zoospore cyst thin walled, incorporated into one of the rhizoidial axes, spherical (4-5μm in diameter), oval (5-6μm long x 4-4.5μm wide), positioned 6-25μm from apophysis, sometimes as

far as 60µm; Apophysis campanulate (13-25µm high x 9-30µm wide), wall unevenly developed, hyaline, thick, up to 7.5µm across.

Rhizoidial System: Rhizoidal system formed by one to several coarse, branched rhizoidial axes arising from base of apophysis.

Zoospore discharge: Zoospores when released form a globular mass at discharge tube orifice before swimming away; Tip of discharge tube swells and softens to form a plug of hyaline material followed by formation of endo-operculum; Endo-operculum formed down inside of discharge tube, up to 15µm beneath pore, pushed out and carried out by emerging mass of zoospores; One to two discharge tubes, 22-70µm long, sometimes 180µm long, 9-16µm wide.

Zoospore microscopic: Zoospores 5-6µm in diameter, with a large hyaline lipid globule (2.5-3.0µm in diameter); posterior flagellum 23-30µm in length.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop like zoosporangia with a persistant zoospore cyst attached; always spherical; Wall golden brown, spiny; germination unknown.

Ecology and Distribution: Saprophytic on cellophane bait from submerged mud samples, Willoughby (loc. cit.), ENGLAND; On onion skin from water samples with decaying vegetation, Dogma (1969:210), US.

6. *Nephrochytrium sexuale* Haskins

1950. Mycologia. 42: 772, figs.

PLATE 10, figs. 187-191

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Zoosporangium and apophysis formed simultaneously by enlargement of the zoospore cyst and end of a branch in the rhizoidal system, respectively.

Reproductive: Zoosporangium hyaline, smooth, spherical (50-170μm in diameter), variously shaped; No persistant zoospore cyst.

Rhizoidial System: Rhizoidal system arising from base of apophysis, stout, extensive and much branched.

Zoospore discharge: Zoospores when released form a temporary globular mass at discharge tube orifice before swimming away; During discharge, tip of discharge tube either swells and softens to form a plug of hyaline material followed by formation of an endo-operculum that is either pushed out or ruptured or the tip simply deliquisces; Endo-operculum when formed is positioned down inside of discharge tube, shallow, saucer-shaped to cone-shaped with long spine; One to several discharge tubes, short and broad, or occasionally very long up to 150μm.

Zoospore microscopic: Zoospores spherical to sub-spherical, 5-6μm in diameter, with a single large lipid globule; flagellum 30-40μm in length.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop from base of apophysis; Spherical (16-23μm in diameter); Wall dark brown, thick and layered, densely spiny, spines short and stout to long and hair-like; formed either asexually or through sexual fusion of gametangial male and female thalli, female thalli remain abortive unless fertilized, male thalli consist of a gametangium, apophysis and rudimentary sparsely branched rhizoidial system, gametangium hyaline, thin-walled, containing 4 to many gametes, gametes encysting in-situ, produceing fine sparsely branched rhizoid-like germination tubes which penetrate gametangium wall to anastomose with rhizoidial system of female thallus, through which the contents of the male thallis moves to enter the female thallus and immediately develops into a normal, brown, spiny-walled resting spore; function as prosporangium in germination.

Ecology and Distribution: Saprophytic in decaying vegetable debris, boiled maize leaves, cellophane, and lens paper submerged in water, Haskins (loc. cit.), CANADA, US.

7. *Nephrochytrium stellatum* Couch

Amer. J. Bot. 25: 507, figs. 1-34. 1938.

PLATE 10, figs. 193-207

Vegetative: Thallus endobiotic, monocentric, eucarpic.

Development: Apophysis develops as a swelling in the germ tube followed by the zoosporangium which develops by budding out of the apophysis.

Reproductive: Zoosporangium hyaline, smooth, disc-shaped, circular or irregular with a basal columella which bulges into sporangium; Persistant zoospore cyst present; Apophysis (8.4-12.8μm x 14.7-20μm) basal, globose, ovoid, or sometimes lobed, connected to sporangium by a narrow isthmus; Zoosporangium and apophysis have an hour-glass-like appearence with the zoosporangium being the larger part of the hour-glass; Persistant zoospore cyst pyriform, thick-walled, yellowish, attached to apophysis by isthmus.

Rhizoidial System: Rhizoidal elaborately developed, much branched, terminal branches with blunt tips, attached to apophysis near isthmus or more often to isthmus by one main rhizoidial axis.

Zoospore discharge: Zoospores when released form a temporary globular mass at discharge tube orifice before swimming away; Exo-operculum always formed at tip of discharge tube; Discharge tube formed near columella, usually not tubular but penetrating through surface of substrate.

Zoospore microscopic: Zoospores spherical, 5μm in diameter, with a single large lipid globule; flagellum 35-40μm in length.

Zoospore ultrastructure: Not available.

Resting Spore: Resting spores develop from base of apophysis; nearly spherical but slightly wider than tall (10-30μm wide x 11-29μm long usually 18-21μm wide x 16-19μm long); Wall near amber brown, 2.8-4μm thick, usually with 8-12 large, rounded protuberances, rarely smooth, at maturity possessing one large lipid globule surrounded by a layer of smaller spherical lipid globules; apophysis empty, basal, usually barrel-shaped (12-19μm wide x 8-15μm long but much smaller in depauperate specimens), or sometimes irregular, walls hyaline or nearly so, about 2.5μm thick beneath resting sporangium portion but thinning to a mere membrance at the basal rhizoidial end; persistant zoospore cyst thick-walled and yellowish, remaining attached to base of apophysis; germination not observed.

Ecology and Distribution: Saprophytic in Nitella hyalina, Couch (loc. cit.), US.

Note: Not included but worth mentioning is *Diplophlyctis complicata* (Willoughby) Dogma. *D. complicata* was first described as *Nephrochytrium complicatum* by Willoughby in 1961. Dogma (1974) later isolated twelve cultures (all derived from a single-spore) from water and mud samples collected in Michigan and from Willoughby's original collection site in England using the same type of bait (processed shrimp exo-skeleton). Dogma identified all twelve cultures as *Nephrochytrium complicatum*. Based on a complete examination of the life cycle of four of the twelve single-spored isolates, Dogma re-described *Nephrochytrium complicatum* and transferred the species to *Diplophlyctis*. All four isolates that Dogma studied were clearly inoperculate and he used this character as the primary reason for moving Willoughby's chytrid into *Diplophlyctis*. Other characters that Dogma used as reasons for placement in *Diplophlyctis* included an *Entophlyctis*-type of development and growth on chitin. Other species of *Nephrochytrium* prefer cellulosic substrates so growth on chitin represents a unique preference for a different kind of

substrate. Whether or not this reflects an actual taxonomic difference is not known. Neither Dogma's nor Willoughby's cultures are available for comparison or for examination of more phylogenetically useful sequence and zoospore ultrastructure characters.

Taxonomic Key to Species of *Nephrochytrium*

All of the morphological characters used to create this taxonomic key are visible under a light microscope. Identification will require observation over several days in order to see the mature structures utilized in the key.

Key to the Species of *Nephrochytrium*

1a. Zoosporangia exo-operculate.....2

1b. Zoosporangia endo-operculate.....4

2a. Lipid globule of zoospore bright orange in color.....*Nephrochytrium aurantium*

2b. Lipid globule of zoospore hyaline, colorless.....3

3a. Resting sporangia stellate with large rounded protuberances; zoosporangia spherical or irregular …….....*Nephrochytrium stellatum*

3b. Resting sporangia and zoosporangia kidney-shaped.....…….....*Nephrochytrium appendiculatum*

4a. Resting sporangia smooth.....*Nephrochytrium bipes*

4b. Resting spore spiny.....5

5a. Connection (isthmus) between sporangia and apophysis narrow.....*Nephrochytrium amazonensis*

5b. Connection (isthmus) between sporangia and apophysis broad.....6

6a. Resting sporangia dark brown; formed either asexually or after fusion of gametangial thalli.....*Nephrochytrium sexuale*

6b. Resting sporangia golden brown; only formed asexually.....*Nephrochytrium buttermerense*

Taxonomic Summary of the Genus *Nowakowskiella*

Taxonomic History of the Genus *Nowakowskiella*

Introduction

In 1876, Nowakowksi described two species of a new chytrid genus (*Cladochytrium elegans* and *Cladochytrium tenue*). Both were polycentric and produced swellings along the rhizomycelium. *C. elegans* differed from *C. tenue* in that it produced operculate (same as exo-operculate) sporangia while *C. elegans* was inoperculate. Schroeter (1892) later separated *C. elegans* from *Cladochytrium* and placed it in a new genus, *Nowakowskiella*, because it was exo-operculate and not inoperculate like *C. tenue* and renamed it *Nowakowskiella elegans*. Over the last century sixteen new species and two varieties have been described worldwide for the genus *Nowakowskiella*. The most common features for species of *Nowakowskiella* include exo- or endo-operculation, non-septate swellings and polycentric thalli. Species in *Nowakowskiella* also produce apophysate and non-apophysate sporangia that may or may not be internally proliferating. Differing views on the status of endo-operculation as either being equivalent to exo-operculation or as a rare condition of aging cultures resulted in a shuffling of species from *Nowakowskiella* to *Cladochytrium* (Haskins and Weston1950, Sparrow 1960). Karling documented endo-operculation in all of his described species (*N. granulata, N. macrospora, N. multispora, N. sculptura*) with some being completely endo-operculate and others exhibiting a mix of endo-operculate and exo-operculate zoosporangia. Other species of *Nowakowskiella* have been described as being endo-operculate (*N. keratinophila, N. methistemichroma, N. moubasheriana*) but were published long after Sparrow's monograph, Aquatic Phycomycetes,

and have only been isolated once. Sparrow's difference of opinion on endo-operculation led to his removal of *N. granulata* from *Nowakowskiella* into *Cladochytrium* because it was not clearly exo-operculate. Karling (1966) later placed *N. granulata* back into *Nowakowskiella* based on a study of over 200 dehisced sporangia all of which were clearly endo-operculate and not inoperculate. Another morphological difference that intergrades somewhat between the two genera is presence or absence of septa in the swellings (also known as intercalary swellings, spindle organs or turbinate cells – see Terminology for a short discussion) along filaments of the rhizomycelium. For most species of *Cladochytrium* the intercalary swellings are septate with a wall dividing the swellings into two or more separate compartments. The opposite is true for species of *Nowakowskiella* with most species exhibiting non-septate intercalary swellings. The generic definition of *Cladochytrium* (Sparrow 1960) lists both irregular swellings (= non-septate intercalary swellings) and septate turbinate cells (=septate intercalary swellings). *Nowakowskiella* is defined as having "irregular swellings, occasionally septate turbinate cells" (Sparrow 1960). Five of the thirteen species of Nowakowskiella are either described as being occasionally septate or have one or more septate swellings in drawings and despite the slight overlap between genus descriptions Whiffen (1943) may have been on the right track with regards to suggesting that swelling morphology might be a better character for distinction than method of operculation. The molecular analysis presented in this study (Chapter 2) groups species with non-septate swellings including the type species, N. elegans, into *Nowakowskiella* and not into *Cladochytrium* (Chapter 2) with a high level of clade support.

The type of *Nowakowskiella*

Nowakowskiella elegans is the designated type species for the genus (Clements and Shear 1931) but like many chytrid genera no type material from the original culture is currently

available. *N. elegans* is a commonly isolated and easily identified species and there are many isolates in pure culture available for examination. In addition, sequence data is available that supports previous taxonomic work based on zoospore ultrastructure (Lucarotti 1981, James et al. 2000, and this publication) and can be used for comparison of any isolates brought into pure culture. Because zoospore ultrastructure (Lucarotti 1981), rDNA sequence data (Genbank Accession numbers AF164281-2), and photographs (Lucarotti 1978) are available for the N. elegans culture BK50-1, this culture is designated as the lectotype. BK50-1 can be obtained growing in live culture from Barbara Waaland, University of California at Berkeley or from the University of Georgia Cryopreserved Chytrid Collection (Dr. David Porter, Department of Plant Biology, University of Georgia, Athens, GA).

Terminology

Many different terms have been used to describe the swellings that occur along the filaments in the rhizomycelium of polycentric chytrids. The swellings have been variously termed intercalary swellings, irregular swellings, spindle organs, and turbinate cells. Terms referring to the swellings as cells or organs do not accurately describe these structures since they are not always physically separated from the rest of the thallus (but see Karling 1931 for a different position). Irregular best defines the shape and frequency of occurence but not position in the thallus so it does not completely define the structure. The best term to use would be intercalary swelling as it most accurately describes an occasional inflation of the filaments in the rhizomycelium. The term intercalary swelling should be used irrespective of whether all the swellings of a single thallus are septate or not. In the past, authors have used irregular swelling to define the non-septate swellings and intercalary for the septate swellings as they are most likely the same structures and should not be labelled differently. Swellings that are septate should be referred to

as septate intercalary swellings and non-septate swellings should simply be labeled as non-septate intercalary swellings.

The Species of *Nowakowskiella*: Taxonomic Descriptions, Ecology, and Distribution

Currently there are thirteen recognized species and one variety. Few have been brought into pure culture and most were described from gross culture. Species of *Nowakowskiella* are commonly found in soil and water worldwide primarily on celluosic substrates (especially cellophane bait) with two exceptions: *N. keratinophila* was found on insect wings and *N. pitcairnensis* according to Karling (1968) preferred hemp seeds as bait and would not grow at all on cellophane.

1. *Nowakowskiella atkinsii* Sparrow

Journal of the Washington Academy of Sciences. 40: 52, figs. 25, 26. 1950

PLATE 11, figs. 208-209

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia at the tips of more or less elongated branches, rarely intercalary, predominantly spherical and 13-20µm in diameter or somewhat pyriform, often apophysate; the wall bearing a variable number of somewhat thickened flexous setae, 9-16µm long.

Rhizoidial System: Thallus extensive, much branched, bearing zoosporangia, occasional irregular non-septate intercalary swellings and rarely very large (35-40µm x 18-25µm) setigerous 1- or 2-celled intercalary swellings.

Zoospore discharge: Zoospores emerge through a lateral or sub-basal slightly elevated pore upon the dehiscence of an operculum 8µm in diameter and remaining in a compact quiescent group before assuming motility

Zoospore microscopic: Zoospores slightly ovoid, 5 by 3µm with a single hyaline globule and posterior flagellum

Zoospore ultrastructure: Not available.

Resting Spore: Not observed.

Ecology and Distribution: Saprophytic on cellophane in soil from serpentine savanna, Sparrow (loc. cit.), CUBA.

2. *Nowakowskeilla elegans* (Nowakowski) Schroeter

Mycologia. 69: 43, figs. 16-24. 1973.

Schroeter, in Engler und Prantl, Natürlichen Pflansenfam. 1: 82. 1892 (1893).

Synonomy:

Cladochytrium elegans Nowakowski (pro parte), in Cohn, Beitr. Biol. Pflanzen. 2: 95. 1876.

Nowakowskeilla endogena Constantineanu, Rev. Gén. Bot. 13: 387. 1901.

Nowakowskeilla profusa Karling, Bull. Torrey Bot. Club. 68: 386. 1941.

Nowakowskeilla profusa f. constricta Kobayasi and Konno, Bull. Nat. Sci. Mus. Tokyo. 14: 380. 1971.

Nowakowskeilla delica Whiffen, J. Elisha Mitchell Sci. Soc. 59: 37. 1943.

Nowakowskeilla crassa Karling, Bull. Torrey Bot. Club. 76: 294. 1949.

PLATE 11, figs. 210-211

Vegetative: Thallus polycentric, epibiotic and endobiotic.

Reproductive: Zoosporangia hyaline, thin-walled, apophysate or non-apophysate; spherical, subspherical, ovoid, ampulliform, obclavate, pyriform, elongate, cylindrical, or reniform or irregular; spherical ones 10-45µm in diameter, others extremely variable, 15-63 long x 9-47µm in diameter; often clustered, sometimes in linear fashion, but in some substrates, scattered, and produced sparingly along the rhizomycelium.

Rhizoidial System: Rhizomycelium extensive, sparse or dense, the degree of branching and length of filaments dependent on substrate. Rhizoids coarse or delicate, long, sparingly branched, or short and dendriform; hyaline, extremely variable in diameter, but up to 21µm in some substrates; usually with ellipsoidal, fusiform, or subglobose non-septate intercalary swellings, these sometimes poorly defined; septate intercalary swellings present or absent; main axes tapering to long, delicate rhizoids on some substrates, but to short, tapering and sinuous ones on others; often radiating from central clusters or linear rows of sporangia on the substrates.

Zoospore discharge: Zoospores released through a short, broad exit tube (usually one, rarely two) or a papilla, the apex often closed by a hyaline, gelatinous plug; Zoospores either already cleaved when released or cleave up after release; After discharge, zoospores remain motionless at orifice of sporangium for a short period, then swim away; imbedded in a gelatinous matrix prior to becoming motile.

Zoospore microscopic: Operculum 5-7.5µm in diameter, usually exo-operculate, occasionally endo-operculate; zoospores spherical or oval, 3.5-7µm in diameter, single posteriorly directed flagellum; containing a small refractive body (lipid globule).

Zoospore ultrastructure: Lucarotti, C. 1981.

Resting Spore: Resting spores present or absent; when present, terminal or intercalary, spherical, subspherical, ovoid, ellipsoidal, or fusiform, rarely irregular; formed by enlargement and maturation of intercalary swellings in the rhizomycelium; wall smooth, thick, bright golden yellow, or pale yellow, occasionally very pale brown; content at first vacuolate, then refringent; germinating by forming a simple (rarely branched) short or long exit tube, and producing zoospores endogenously; zoospores identical to those from zoosporangia, escaping through a pore on the dehiscence of an operculum, clustering at the exit orifice presumably in a matrix,

then assuming motility; in some instances functioning as a prosporangium; spherical (11-)14-18(-29) µm in diameter, others (11-)13-21 (-26) µm long by (9-)12-16(-23) µm in diameter.

Ecology and Distribution: Nowakowski (loc. cit.); Decaying vegetable debris from swamp and stream water samples, Karling (1941: 386, Virginia), US; Decaying vegetable debris from small brook water sample, Karling (1949: 294, Maryland), US; Rotting oat leaves in water trough, Karling (1941: 108, Texas), US; On *Paspalum* grass, Sparrow (1965: 121, Michigan), US; On various baits from moist soil sample, Karling (1944: 388), BRAZIL; On bleached corn leaves, cellophane, and fibrin film from dry soil, Karling (1966: 65), INDIA; On boiled cellophane, lens paper, and bleached grass leaves from soil, Johnson (1973: 1339), ICELAND; On cellophane in soil, Kobayasi and Konno (1973:500), JAPAN.

Note: *Nowakowskeilla elegans (=profusa)* f. constricta Kobayasi and Konno is not considered a true variety as the description and drawings appear identical to *N. elegans*.

3. *Nowakowskeilla elongata* Karling

Bulletin of the Torrey Botanical Club. 71: 375. figs. 30-44. 1944

PLATE 11, figs. 212-214

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia terminal and intercalary, rarely apophysate, sometimes with a basal 1-3 septate sterile portion, straight, elongate-clavate (8-40µm x 20-820µm), cylindrical with swollen apex, curved or coiled (5-20µm x 30-900µm), pyriform (15-44µm x 20-70µm), globose (10-70µm in diameter), or irregularly oval, occasionally proliferating.

Rhizoidial System: Rhizomycelium hyaline, profuse, copiously branched; filaments 1-6 in diameter, broad non-septate intercalary swellings present at various increments along the

rhizoids, oval (5-13µm x 7-15µm), or broadly fusiform (4-8µm x 8-17µm), or globose (5-15µm), often with multiple lipid globules.

Zoospore discharge: Operculum apical, convex, 4-8µm in diameter, rarely persistent

Zoospore microscopic: Zoospores globose, 2-2.5µm in diameter, when released form a temporary globular mass at the orifice of the discharge tube.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spore formed from the intercalary enlargements, hyaline, smooth, globose (16-24µm in diameter), oval (14-16µm x 18-22µm), citriform, with a large lipid globule up to 15 in diameter or sometimes completely filling the resting spore body, wall thickened, upon germination functioning as a prosporangium and forming a thin-walled evanescent sporangium.

Ecology and Distribution: Saprophytic in decaying vegetable debris and on corn leaves and onion skin from soil, Karling (loc. cit.), BRAZIL; On cellophane, R. M. Johns (Sparrow 1960: 580), UNITED STATES.

4. *Nowakowskeilla granulata* Karling

Bull. Torrey. Bot. Club. 71: 374, figs. 1-29. 1944.

Synonomy: *Cladochytrium granulatum* (Karling) Sparrow, 1960. Aquatic Phycomycetes, pg. 469.

Nowakowskiella granulata Karling. 1966. Beihefte zur Sydowia, Annales Mycologici, Ser. II, Beiheft VI. The Chytrids of India with a Supplement of other Zoosporic Fungi. pg. 66.

PLATE 11, figs. 215-217

Vegetative: Thallus polycentric, endobiotic.

Reproductive: Zoosporangia terminal or intercalary, mostly non-apophysate, globose (12-35µm in diameter), pyriform (12-22µm x 15-30µm), oval (10-18µm x 12-25µm), or occasionally irregular; Also can be non-septate or with one to three septa; For septate sporangia the apical end is usually inflated or swollen while the basal portions can simply be inflated or develop into additional zoosporangia.

Rhizoidial System: Profusely developed, much branched, hyaline when young, brownish and thick-walled with age, slender parts 1.5-7µm in diameter, expanded parts mostly nonseptate, oval (6-8µm x 9-11µm), boradly fusiform (5-9µm x 8-13µm), almost globose (6-10µm in diameter), or irregular

Zoospore discharge: One to three discharge papillae, 3 x 5µm, or one terminating a more or less elongate discharge tube which at its tip bears a plug of opaque gelatinous material and within it on the surface of the contents an oval, discoid, crateriform, saucer-, bowl-, cup-, or cone shaped endo-operculum, 3-7µm in diameter.

Zoospore microscopic: Zoospores globose, 5-6.6µm in diameter, with numerous golden-brown lipid globules of uniform size, flagellum about 35 in length.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spores formed from the intercalary swellings, with a smooth,hyaline 1.5-2µm thick wall, globose (15-24µm in diameter), oval (15 x 20µm), with a large (12µm in diameter) lipid globule and numerous smaller ones, germination unknown

Ecology and Distribution: Saprophytic in decaying vegetable, Karling (loc. cit.), BRAZIL.

5. *Nowakowskeilla hemisphaerospora* Shanor

American Journal of Botany. 29: 174, figs. 1A-F, 2-36. 1942.

PLATE 12, figs. 218-222

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia usually terminal on short lateral branches or occasionally intercalary, smooth, hyaline, operculate, quite variable in shape and size but commonly ovoid, ellipsoid, or pyriform, usually 7.5-14.2µm x 9.5-28.4µm (commonly 11.5 x 17.2µm) with one to several exit papillae or tubes, apical, subapical, or lateral, varying in length up to 18µm and usually about 4.0-4.7µm in diameter

Rhizoidial System: Rhizomycelium much branched, extensive, hyaline; individual filaments quite variable in diameter; non-septate intercalary swellings numerous or very much scattered.

Zoospore discharge: Operculum circular, 3.1-3.5µm in diameter, either carried away by emerging zoospores or less often remaining attached to the sporangium after discharge; At maturity, zoospores are discharged into a hyaline matrix (Shanor (1942) did not equate the hyaline matrix with Sparrow's vesicle).

Zoospore microscopic: Zoopores hyaline, spherical or ovoid, 4.4-6.3µm in diameter, with a single lipid globule; single posteriorly inserted flagellum, 32.5-40µm in length.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting bodies (Shanor's term) terminal or less often intercalary, usually somewhat ellipsoidal, containing one to four thick-walled hyaline resting spores and a corresponding number of empty cells. Resting spores uniform in size, 8.5-12.6µm x 11.6-15.6µm (commonly 11.0 x 14.2µm) usually somewhat hemispherical in shape; Lipid globule in mature resting spores large with several smaller ones commonly surrounding it; Germination unknown.

Ecology and Distribution: Saprophytic on cellophane, grass leaves, corn seedling leaves, filter paper, and lens paper in water samples, Shanor (loc. cit., Illinois), US; On grass bait and cellophane in soil, Karling, Patterson, and Johns (1965:121, Michigan), US; (Hawaii), US; On

cellophane in soil, Kobayasi and Konno, (1973:499), JAPAN; MEXICO; ENGLAND; CUBA; NEW ZEALAND; COOK ISLAND; SINGAPORE.

6. *Nowakowskeilla keratinophila* Hassan and Batko

Acta Mycologica. 22: 194, figs. 1-7. 1986 (1988).

PLATE 12, figs. 223-226

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia 24.2-73.4µm in diameter and 25.5-43.6µm long with small apohyses, sometimes not apophysate, moderately abundant, regular, mostly ovoid, broadly ovoid or obpyriform; apophysis more or less hemispherical, 9-11.5 in diameter, wall thinner than upon sporangium.

Rhizoidial System: Rhizomycelium profuse, extensive, branched, 2.4-7.1µm in diameter, thread-like and mostly isodiametric, aseptate, with numerous non-septate intercalary swellings 9.1-12.8µm long and 5.9-9.7µm wide and scarcely branched thin rhizoids; wall slightly thickened, content hyaline, homogenous.

Zoospore discharge: Zoosporangia produce moderately prominent conical papilla ended at maturity by a shallowly dome-shaped sunken operculum 4.6-4.8µm in diameter.

Zoospore microscopic: Zoospores spherical, 7.5-9.1µm in diameter with flagellum up to 49 long and possessing a single eccentric hyaline lipid globule 2.2-3.8µm in diameter, and clear round zone in the coarsely granular content, fully formed inside the zoosporangium and liberated collectively or singly after the dehiscence of the endo-operculum.

Zoospore ultrastructure: Unknown

Resting Spore: Unknown

Ecology and Distribution: On snake skin from brackish water sample, Hassan and Batko (loc. cit.), POLAND;

7. *Nowakowskeilla macrospora* Karling

American Journal of Botany. 32:29, figs. 1-30. 1945.

PLATE 12, figs. 227-229

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia terminal or intercalary, hyaline, smooth, usually apophysate, often slightly flattened and elongated transversely to apophysis, spherical (14-40µm), oval (18-25 x 23-30µm), pyriform (8-30µm x 18-55µm), or elongate (10-20µm x 30-60µm), often with an elongate neck, 5-8µm x 20-60µm; apophysis oval or nearly spherical, 8-20µm in diameter, oblong, clavate or elongate.

Rhizoidial System: Rhizomycelium profuse, richly-branched, fairly coarse, filaments 2-6µm in diameter; nonseptate intercalary swellings oval (8-16µm x 12-15µm), broadly spindle-shaped (5-7µm x 12-15µm), elongate and fusiform (6-10µm x 15-30µm), or slightly irregular.

Zoospore discharge: Operculum usually though not always slightly sunken, often apiculate and somewhat hat-shaped, 5-8µm in diameter; Zoospores slowly ooze out when released and form a globular mass at exit orifice, then separate slowly before swimming away.

Zoospore microscopic: Zoospores spherical, 10-12µm with a large (3-5µm), somewhat disc-shaped lipid globule and numerous minute lipid globules at posterior end; flagellum 38-42µm long.

Zoospore ultrastructure: Unknown

Resting Spore: Two types of resting spores are formed: 1) Resting spores derived from sporangia which become brown, thick-walled in old cultures and upon germination function as

either sporangia or prosporangia, 2) Resting spores derived from intercalary swellings that become faintly yellowish-brown in color and function as prosporangia in germination (means a sporangium buds out from a hole in the surface of the resting sporangium and zoospores are formed in the budding sporangium), spherical (12-22µm), oval (15-18µm x 20-25µm), with a large lipid globule surrounded by numerous smaller ones, wall smooth, 1.5-2µm thick.

Ecology and Distribution: Saprophytic in decayed vegetable debris from water and moist soil samples, Karling (loc. cit.), BRAZIL;

8. *Nowakowskeilla methistemichroma* Batko and Hassan

Sydowia. 35:27, figs. 1-12. 1982.

PLATE 12, figs. 230-232

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia apophysate, abundant, variable in form and size, most often bottle-like, with nearly spherical base and elongated neck and then 52-70µm long including the neck and 36-44µm wide, or elongated, sac-like to more or less cylindrical, up to 168µm long, or with transversely swollen, spheroidal or subspherical basal part up to 34µm wide and 38µm high when measured with out the neck etc. The discharge tube usually rather long, up to 84µm long, and narrow, 8µm in diameter; more rarely sporangia with only the shorter, more or less conical papilla 7µm high and 8µm wide at the base. Apophysis thin-walled, hyaline, most often hemispherical or slightly drop-like, basal or markedly shifted laterally, 6-12-22µm high and 9-12-22µm in diameter at the widest part (usually on the plane of contact with zoosporangium)

Rhizoidial System: Rhizomycelium profuse, extensive, branched, 2-5µm in diameter, thread-like and mostly isodiametric, rarely with irregular, elongated, sac-like extensions, non-septate, with numerous non-septate intercalary swellings 8-22µm long and 6-12µm wide, and very thin,

scarce rhizoids; wall of the intercalary swellings and filaments slightly thickened and reddish or light reddish-brown; intercalary swellings mostly regular distributed along the rhizomycelial strands but sometimes radially arranged and forming loose clusters.

Zoospore discharge: Operculum dome-like, conical, hat-like or in the form of mushroom's pileus, thickened, colorless, 4-8µm diameter and 1-2µm high, slightly sunken, and usually not protruding above the orifice of the discharge tube; Operculum pushed out from tube or papilla before liberation of the first zoospore (Endo-operculation A, Dogma 1973), then zoospores leave sporangium rather quickly, one after another, passing rapidly through the discharge tube and then form a temporary motionless cluster at the discharge tube orifice but the remaining zoospores come through the narrow tube extremely slowly, sometimes taking ten minutes.

Zoospore microscopic: Zoospores spherical, 8.5-10µm in diameter with long flagellum up to 50µm; Lipid globule big, mostly more or less central but often slightly shifted to the flagellum base, 3-3.5µm in diameter, fully formed inside the sporangium before release; Lipid globule of zoospores inside zoosporangium colorless or very slightly greyish, strongly refractive, plastic, changing its shape to rod-like during movement through the discharge tube; After release, zoospore body and lipid globule round up and rest for a short time (about 30 seconds), motionless, and during this time the color of the lipid globule quickly changes to light but bright lemon-yellow; After activation of the zoospore this color is more intensified, so that in the older free-swimming zoospore the globule becomes perfectly yellow.

Zoospore ultrastructure: Unknown

Resting Spore: Resting spores formed in abundance in older cultures, more or less globular, rounded, hyaline, smooth, 28µm in diameter. Germination unknown.

Ecology and Distribution: On onion skin bait from stagnant water, Batko and Hassan, (loc. cit.), POLAND.

9. *Nowakowskeilla moubasherana* Hassan

Acta Mycologica. 19: 80, figs. 1-18 and Plate 1. 1983.

PLATE 13, figs. 233-237

Vegetative: Thallus polycentric.

Reproductive: Zoosporangia mostly terminal and apophysate, usually more or less rounded, from spherical to broadly pyriform, 45-54µm in diameter and up to 60µm long, but very often bigger and elongated transversely to apophysis, then 50-122µm in diameter and 48-109µm long, or 96µm in diameter and 37µm high; Apophysis thin-walled and very elongated, narrow and indistinct, up to 8µm in diameter, or nearly spherical or pyriform, 16-18µm in diameter, 21µm long, or sometimes transverse, over 30µm in width by 10µm high.

Rhizoidial System: Rhizomycelium profuse, moderately branched, rather fine, filaments 1-3µm in diameter, thin-walled, with mostly non-septate, thin-walled intercalary swellings 11-30µm long and 8-16µm in diameter; some larger septate intercalary swellings, 28-52µm long and 18-23µm in diameter, possess one or two very thin traverse septa; Rhizoids very thin, abundantly branched, arising from the filaments as well as from the swellings.

Zoospore discharge: Operculum external, dish-like, shallow, thin-walled, up to 12µm in diameter; The discharge tube mostly lateral but often apical, rather long, up to 76µm and 9-14(-24) µm in diameter, usually isodiametric and straight, but sometimes locally swollen or curved or on occasion short and papillum-like; Zoospores slowly ooze out and form a globular mass at exit orifice, or on occasion individual zoospores escape and swim away.

Zoospore microscopic: Zoospores 9-10µm in diameter, with large, 5-6 µm in diameter, slightly yellowish to dirty greenish, anterior plastic lipid globule, flagellum up to 40µm long.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spores from apophysate zoosporangia become light brown, thick-walled, verrucose and dormant, usually over 30µm in diameter; Resting spores with unknown origin are smooth, angular, hyaline, up to 30µm in diameter, formed collectively inside very thin-walled evanescent containers. Germination unknown.

Ecology and Distribution: Saprophytic on onion skin in sphagnum-bog water, Hassan (loc. cit.), POLAND.

10. *Nowakowskeilla multispora* Karling

1964. Sydowia. 17: 314, figs.

PLATE 13, figs. 238A-C

Author's Diagnosis: Zoospores 3-3.9µm, highly abundant resting spores (as compared to other described species up to 1964)

Vegetative: Thallus polycentric, endobiotic.

Reproductive: Zoosporangia usually terminal, sometimes intercalary, non-apophysate, hyaline, smooth, predominantly fusiform, 12-16 x 20-32µm in diameter, frequently elongate and almost cylindrical, ovoid or spherical, 8-26µm in diameter.

Rhizoidial System: Rhizomycelium profuse, highly branched, extensive, filaments 2-5µm in diameter; non-sepate intercalary swellings numerous and frequently in tandem, narrowly ovoid, fusiform, 10-15 x 17-30µm in diameter, or elongate, 8-10 x 17-22 in diameter

Zoospore discharge: Operculum present; zoosporangia either exo-operculate or endo-operculate; Long discharge tubes present on zoosporangia.

Zoospore microscopic: Zoospores small, 3-3.9µm in diameter, spherical with a single minute lipid globule, flagellum 12-14µm long.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spores unusually abundant, usually intercalary, formed by transformation of intercalary swellings into fairly thick-walled, hyaline, smooth, almost spherical 15-30µm, broadly to narrowly ovoid 12-15 x 15-30µm, oblong or elongate 8-10 x 17-22µm in diameter with truncate ends, containing numerous large lipid globules, function as prosporangia in germination.

Ecology and Distribution: Saprophytic in bleached corn leaves and cellophane from non-brackish soil, Karling (loc. cit.), INDIA.

11. *Nowakowskeilla multispora* var. *longa* Kiran

Acta Botanica Indica. 20: 303, figs. 1-8. 1992.

PLATE 13, figs. 239-241

Vegetative: Thallus polycentric

Reproductive: Zoosporangia terminal, intercalary, non-apophysate, smooth, hyaline, when free spherical, ovoid, pyriform, oblong or elongate, 14-21 x 12-35µm, some zoosporangia internally proliferating.

Rhizoidial System: Rhizomycelium profuse, richly branched, filaments 2-6µm in diameter, bearing numerous non-septate intercalary swellings narrowly ovoid, fusiform, 8-12 x 16-25µm, and fine rhizoids.

Zoospore discharge: Zoosporangia predominantly exo-operculate, discharge tubes 1-6 per zoosporangium, sometimes branched, 3-7µm in diameter, 5-90µm long, zoospores released fully formed in a quiescent globular mass at the mouth of the discharge tube before swimming away.

Zoospore microscopic: Zoospores oval, 6-7μm in diameter with a single lipid globule, flagellum 15-20μm long.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spores not observed.

Ecology and Distribution: Saprophytic in decomposing leaf litter, insect wings, and cellophane paper from pond water, Kiran (loc. cit.), INDIA.

Note: Kiran's variety differs from the species noted by Karling (1966) in that it produces exo-operculate zoosporangia as opposed to Karling's chytrid which produced endo-operculate zoosporangia. Kiran cited the extremely long, branched discharge tubes as his reason for noting it as a variety of N. multispora and did not note the difference in operculation. The possibility exists that N. multispora is both exo- and endo-operculate though Kiran's variety has larger zoospores (6-7 vs. 3-3.9 for N. multispora) and difference in zoospore size has been used as a basis for creating new species (ex. N. macrospora). Traditionally varieties or forms (formae specialis) are isolates of the same species of a fungal pathogen on different hosts and are not usually defined by differences in morphology or substrate though this seems to be the case for some varieties in chytrid fungi (ex. Catenochytridium carolinianum f. marinum). Kiran's variety was isolated from both cellulosic substrates (decomposing leaf litter and cellophane paper) and insect wings. Insect wings would represent a different substrate than the cellulosic substrate Karling used to isolate N. multispora and could also be used to erect a new species comparable to past descriptions. Unfortunately creating a new species at this time would be of little value because no culture exists for comparison and it is the opinion of the author that new species should only be erected for species growing in pure culture.

12. *Nowakowskeilla pitcairnensis* Karling

Nova Hedwigia. 15: 191, Plate 21(1), figs. 1-19. 1968.

PLATE 13, figs. 242-246

Vegetative: Thallus polycentric, endo- and epibiotic.

Reproductive: Zoosporangia terminal or intercalary, non-apophysate to uni- or bi-apophysate, broadly or narrowly pyriform (18-20 x 25-30µm), ovoid (20-24 x 26-31µm), subspherical (16-29µm), or sometimes irregular in shape

Rhizoidial System: Rhizomycelium endo- and epibiotic, extensive, richly branched, filaments 2.5-4µm in diameter, bearing numerous rhizoids at irregular intervals along its length; Intercalary swellings infrequent, sometimes septate, usually occuring in tandem, 6-12µm in diameter.

Zoospore discharge: Exo-operculum apical, shallow, saucer-shaped, 2.8-3.5µm in diameter by 3.5-4µm high; Lip or rim of exit orifice flaring out at discharge; Zoospores emerge in a globular mass, elongating immediately after emerging but becoming spherical when actively motile.

Zoospore microscopic: Zoospores 3-3.2µm in diameter, with a hyaline, 0.8-1.2µm in diameter lipid globule, flagellum 22-25µm in length.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spores terminal or intercalary, spherical (10-13µm in diameter), ovoid (10-15 x 12-18µm), fusiform (5-11µm in diameter x 14-18µm long). Content dense, coarsely granular; Wall light brown, smooth and moderately thick; Functioning as a prosporangium in germination.

Ecology and Distribution: Saprophytic on hemp seeds and rarely in bleached corn leaves from soil, Karling (loc. cit.), PITCAIRN ISLAND, OCEANIA.

13. *Nowakowskeilla ramosa* Butler

Memoirs of the Department of Agriculture in India. 1: 141, figs. 3-10. 1907.

Karling, J.S. Brazilian chytrids. I. Species of Nowakowskiella. Bull. Torrey Bot. Club, 71: 384. 1944.

PLATE 14, figs. 247-250

Vegetative: Thallus polycentric

Reproductive: Zoosporangia terminal or intercalary, apophysate or non-apophysate, apophysis when present usually sub-spherical and up to 11µm in diameter; Zoosporangia spherical 20-50µm, or pyriform 15-30 x 25-40µm, or oval 15-20 x 22-30, or elongate or slightly irregular.

Rhizoidial System: Rhizomycelium hyaline, profuse, richly-branched, occasionally septate; filaments 1.5-8µm in diameter, occasionally anastomosing; intercalary swellings oval 4-6 x 6-10µm, or broadly fusiform 5-7 x 12-16µm, or almost spherical 6-9µm, or elongate.

Zoospore discharge: Operculum oval or circular, 4-6µm in diameter; 1-3 short papillae or discharge tubes up to 100µm long; Zoospores form a globular mass at exit orifice immediately after emerging but soon separating and swimming away.

Zoospore microscopic: Zoospores spherical, 6.6-8.8µm in diameter, with a large, 3µm, plastic lipid globule, flagellum 36-40µm long.

Zoospore ultrastructure: Unknown.

Resting Spore: Resting spores formed from intercalary swellings and short lateral branches, spherical or slightly angular, 15-26µm, hyaline to yellowish in color, with numerous

Ecology and Distribution: Saprophytic on rotting stems of *Triticum vulgare*, Butler, (loc. cit.), INDIA; tissues of Typha, Domján (as *Nowakowskiella endogena*; 1936: 51, pl. 1, figs. 89, 98, 108-109, 112-124, 126-127, 133-136, 143-150, 156-159, 166, 168-169, 175-179), HUNGARY;

rotting oat leaves, Karling (1941c:107), bleached grass leaves and cellophane from moist soil, Karling (1942: 620), Roberts (1948:154, fig. 1), nucules or moribund *Chara* sp., Johns (pers. comm. with Sparrow 1960), Sparrow and Barr (1955: 555), UNITED STATES; decaying grass leaves, Karling (1944a: 384, fig. 69), BRAZIL; from soil, Gaertner (1954: 22), EGYPT, SOUTH AFRICA.

14. *Nowakowskeilla sculptura* Karling

1961. Trans. Brit. Mycol. Soc. 44: 453, figs.

PLATE 14, figs. 251-257

Vegetative: Thallus polycentric, endobiotic

Reproductive: Zoosporangia terminal or intercalary, usually non-apophysate, subspherical (16-36µm), ovoid (18-24µm x 26-38µm), broadly or narrowly pyriform (10-22 x 30-40), oblong or slightly irregular.

Rhizoidial System: Rhizomycelium profuse, richly branched, filaments 2-8µm in diameter, intercalary swellings unusually profuse in cellophane, usually non-septate, ovoid (8-10 x 14-16), broadly fusiform (7-9µm x 12-15µm), elongate, and sometimes slightly irregular.

Zoospore discharge: Primarily endo-operculate but occasionally exo-operculate, operculum oval or circular and disk-shaped, 5-8µm in diameter, one to two short or long exit tubes per sporangium, emerging zoospores push out operculum which can remain on top or side of the expanding mass of zoospores.

Zoospore microscopic: Zoospores spherical, 3-3.8µm with one hyaline lipid globule (0.8-1.2µm in diameter), flagellum 16-18µm long.

Zoospore ultrastructure: Unknown

Resting Spore: Resting spore frequently occuring in clusters and borne usually on a pseudoparenchyma, hyaline, spherical to subspherical (13-25µm), ovoid (12-20µm x 18-26µm), usually with highly sculptured walls, sometimes warty, verrucose, or smooth; functioning as prosporangia in germination

Ecology and Distribution: Saprophytic in decaying debris in soil, Karling (loc. cit.), UNITED STATES

Taxonomic Key to Species of *Nowakowskiella*

Currently there are thirteen recognized species and one variety. Few have been brought into pure culture and most were described from gross culture. Interestingly enough, species of *Nowakowskiella* are commonly found in soil and water worldwide primarily on celluosic substrates especially cellophane bait with two exceptions: N. keratinophila was found on insect wings and N. pitcairnensis according to Karling (1968) preferred hemp seeds as bait and would not grow at all on cellophane. The morphological characters used to create this key are visible through a light microscope but will require following the life cycle from zoospore to resting sporangium which can take one to several weeks depending on the species and medium used. The main problem with many of the morphological characters used to make the key is the use of size ranges to differentiate between species. Reliance on sizes of structures to determine species level differences is problematic with chytrid fungi as it has been shown that size can differ greatly depending on the type of nutrient source used, temperature, pH and salinity (Booth 1971, Miller 1976). Cultures would need to be grown on the same type of media for comparison between species and on different media to determine the range possible for a single species. As only a handful of cultures are available for determination of the more taxonomically reliable ultrastructural and molecular characters, a true understanding of the importance of the size range

for various structures like zoospores and sporangia will remain unknown. Lacking a suitable replacement size ranges will continue to be used for convenience. Batko and Hassan (1982) published a key using a similar set of morphological characters but did not include *N. keratinophila* or *N. multispora* var. longa as they were described after publication of the key. Batko and Hassan also included *N. crassa*, *N. delica*, and *N. profusa* in their key. No mention is made of Johnson's synonmy of all three species with N. elegans (Johnson 1977) so either Batko and Hassan (no mention is made of Johnson's work) did not agree with Johnson or were not aware of the publication.

Key to the Species of *Nowakowskiella*

1a. Zoospores with numerous small golden-brown lipid globules.....*Nowakowskiella granulata*

1b. Zoospores with at least one large lipid globule.....2

2a. Multiple swellings in a row (looks like beads on string); grows on hemp seeds.....*Nowakowskiella pitcairnensis*

2b. Single swellings throughout rhizomycelium.....3

3a. Resting spores produced.....4

3b. Resting spores not produced.....10

4a. Resting spores develop from pseudoparenchymatous divisions of intercalary swellings…….5

4b. Resting spores develop from single intercalary swellings or terminal branches.....6

5a. Resting spores smooth walled or verrucose.....*Nowakowskiella ramosa*

5b. Resting spores highly sculpted, with pegs or smooth.....*Nowakowskiella sculptura*

6a. Zoosporangia endo-operculate and exo-operculate.....7

6b. Zoosporangia endo-operculate or exo-operculate.....8

7a. Zoospores with one large lipid globule and multiple small lipid globules in the posterior end of the zoospore body; zoospores 10-12µm.....*Nowakowskiella macrospora*

7b. Zoospores with a single large lipid globule; zoospores 3-3.9µm.....*Nowakowskiella multispora*

8a. Lipid globule in zoospore pigmented.....9

8b. Lipid globule in zoospore hyaline (colorless).....12

9a. Lipid globule bright lemon yellow upon release; endo-operculate.....*Nowakowskiella methistemichroma*

9b. Lipid globule yellowish or dirty-green; exo-operculate.....*Nowakowskiella moubasheriana*

10a. Present on cellulosic substrates.....11

10b. Present on keratin substrates.....*Nowakowskiella keratinophila*

11a. Setae on zoosporangia and intercalary swellings.....*Nowakowskiella atkinsii*

11b. No ornamentation on zoosporangia or swellings; long, branched exit tubes.....*Nowakowskiella multispora* var. *longa*

12a. Resting sporangium divided into empty portions and resting spore material.....*Nowakowskiella hemisphaerospora*

12b. Resting sporangium completely filled by resting spore material.....13

13a. Sporangia elongate and divided with septa into multiple chambers.....*Nowakowskiella elongata*

13b. Sporangia spherical, pyriform, ovoid or oblong.....*Nowakowskiella elegans*

Taxonomic Summary of the Genus *Septochytrium*

Taxonomic History of the Genus *Septochytrium*

Introduction

Septochytrium was erected in 1939 for an operculate, monocentric/polycentric fungus with constrictions, septations and intercalary swellings in the rhizomycelium (Berdan 1939). *Septochytrium variabile* Berdan received its species epithet from the extremely variable shape and size of the zoosporangium. Though similar in appearance to species of *Nowakowskiella*, Berdan felt that her fungus did not belong in that genus because it did not produce the internally proliferate, apophysate sporangia characteristic of *Nowakowskiella* and the rhizomycelium of her species was endobiotic whereas at the time one of the two described species of *Nowakowskeilla* produced both endobiotic and epibiotic thalli (sporangia and rhizomycelium). Later species of *Nowakowskeilla* were noted to grow inside of the substrate or more commonly on the surface in an epibiotic fashion (Sparrow 1960). Berdan cited the size, abundance and "character" of the resting spores as well as the formation of constrictions, septations and trabeculae in the rhizoids and not the swellings as the primary reasons for placing her fungus into a new genus. Karling described the next species, *S. macrosporum,* in 1942. *S. macrosporum* possessed a "coarse rhizomycelium" as reported by Berdan for her species but according to Karling even though it lacked any septations or trabeculae in the rhizoids the "structure, development and organization [were] fundamentally the same" as *S. variabile*. Another species, *S. plurilobulum* Johanson, was described in 1943 as having the characteristic coarse rhizomycelium with septations and trabeculae but which differed in having multiple lipid globules in the zoospores and irregularly lobed greenish-grey resting spores. In 1951, Karling described *S. marylandicum* placing it in the genus because it possessed the familiar coarse rhizomycelium with occasional septations or

trabeculae but which differed because it was endo-operculate (the operculum was positioned down inside the discharge tube instead of on the surface), had long exit tubes, and zoospores with multiple lipid globules. Karling was probably not aware of Johanson's *S. plurilobulum* (1943) that also produced zoospores with multiple small lipid globules (Sparrow 1960) when he described his fungus. The last species to be described was *S. willoughbyi* by Dogma (1973). Dogma renamed an isolate identified earlier by Dr. L. G. Willoughby as *S. marylandicum* (1964). Dogma cited the production of larger zoospores and monocentric resting spore thalli as his reasons for renaming Willoughby's isolate as a new and separate species. The main hurdle in resolving the phylogenetic position of the genus *Septochytrium* and the validity of the described species is the lack of culture material. Not having a culture to work with means no comparison can be made with the written description and determining new characters like DNA or zoospore ultrastructure is impossible unless the species can be re-isolated from the same location. Three species possess zoospores with multiple small lipid globules (*S. marylandicum, S. plurilobulum, S. willoughbyi)* while *S. macrosporum* has one large globule surrounded by several smaller globules and *S. variabile* usually has one globule though Berdan did report zoospores with multiple globules (1942). The difference in zoospore ultrastructure may be indicative of difference in relationship as has been seen with other chytrid genera (Barr 1980, Letcher et al. 2004) but cannot be determined without a living culture. In the description of *S. macrosporum*, Karling stated that the rhizomycelium was "rarely septate" even though later in the paper he remarked that he never observed any constrictions or septations in the rhizoids. Karling also noted that the rhizoids of *S. marylandicum* were "occasionally septate or trabeculate." Karling's drawings of *S. marylandicum* show a few septations in the rhizoids while none appear in the figures of *S. macrosporum*. The problem with the complete absence of constrictions or septations

in the rhizoids is that Berdan used the presence of these structures to separate *Septochytrium* from other similar polycentric genera (i.e. *Cladochytrium, Megachytrium, Nowakowskiella*). Sparrow (1960) noted that both *S. macrosporum* and *S. marylandicum* resembled species of *Nowakowskiella* (operculate/endooperculate, polycentric, non-septate swellings) but refrained from actually placing them into *Nowakowskiella* and removing them from *Septochytrium*. Whether or not there is an acceptable range of unconstricted to usually constricted rhizoids in species of *Septochytrium* remains to be seen. Other characters may be required to delimit the genus from other polycentric genera without definite constrictions in the rhizoids but which also exhibit structures like trabeculae in their rhizomycelium (Karling 1977). Further work on defining the constrictions/septations/trabeculae including development and composition will be needed to determine their utility at the generic level.

The type of *Septochytrium*

Septochytrium variabile was designated as the type species but no type material was ever deposited. When Berdan erected the genus in 1939 the ICBN did not require typification in order for a new taxon to be considered validly published. Typification including the deposition of type material was not required until 1953. Berdan did publish a more detailed observation of the development of the original culture derived from a single spore (Berdan 1942). According to ICBN articles 8.1, 9.1, and 9.2 illustrations can be used as type material for taxa with no extant holotype material available and as such we designate *Septochytrium variabile* Berdan Amer. J. Bot. 29:260-270, figs. 1-52 as the lectotype.

The Species of *Septochytrium*: Taxonomic Descriptions, Ecology and Distribution

Septochytrium at present consists of five species: *S. macrosporum, S. marilandicum, S. plurilobulum, S. variabile and S. willoughbyi*.

1. *Septochytrium macrosporum* Karling

Amer. J. Bot. 29: 616, figs. 1A-H, 2-15.

PLATE 15, figs. 258A-D.

Vegetative: Thallus predominantly polycentric, occasionally monocentric

Development of the Zoosporangium: Zoospores germinate on the surface of the substrate and produce one to several germ tubes which penetrate the surface and begin to branch. A fusiform swelling appears in the germ tube either before or after branching begins. The rhizomycelium continues to develop with swellings formed at various intervals. The swellings can either remain as non-septate swellings or develop into zoosporangia or resting spores. Zoosporangia can also form by a swelling of the tip of a branch.

Reproductive: Zoosporania formed at the tips of branches (terminal) or from intercalary swellings, delimited from rhizomycelium by true septa at maturity, non-apophysate, spherical (15-280μ), pyriform (30-50μx40-75μ), obpyriform, broadly fusiform, obclavate, uteriform, and sometimes irregular, wall hyaline and smooth.

Rhizoidal System: Unusually coarse rhizomycelium with no pseudosepta or trabeculae in the tenuous portions of the rhizomycelium (Karling put in his description that the rhizomycelium was “rarely septate” but stated in the body of the paper that he never observed any pseudosepta or trabeculae in the rhizomycelium). Rhizoids thick-walled, 5-15μ in diameter, numerous and richly branched. Intercalary swellings broadly or narrowly spindle-shaped, elongate, fusiform and irregular.

Zoospore discharge: Operculum 9-16μ in diameter. Zoospores are released fully formed into a globular mass that remains quiescent at the exit orifice for a few mintues after discharge before swimming away. Zoospores can be intermittently amoeboid.

Zoospore, microscopic: Zoospores spherical, 11-13μ (average 12.2μ) with one large globule (2.5-3.5μ) and three to six minute refractive globules.

Zoospore, ultrastructure: Nucleus appears obpyriform in median, longitudinal views and tapers toward the point of attachment of the flagellum. The upper one-third of the nucleus is enveloped by a densely-stainable lunate body or nuclear cap as described for *Cladochytrium replicatum* (Karling 1937)

Resting Spore: Resting spores develop from intercalary swellings, either smooth-walled or covered with coarse, simple or branched pegs or filamentous extensions, 4-18μ long. Shapes range from oval (30-50μx50-75μ), spherical (25-115μ) and color ranges from yellow to light brown. Wall is 4-6μ thick. Contents are coarsely granular with one to several large refractive granules. Germination unknown.

Ecology and Distribution: Saprophytic in Kleenex cleansing tissue, vegetable debris collected from ponds, brooks, lakes, swamps etc., Karling, (loc. cit.) US.

2. *Septochytrium marilandicum* Karling

Bulletin of the Torrey Botanical Club. 78:39, figs. 9-30. 1951.

PLATE 15, figs. 259-260

Vegetative: Polycentric. No monocentric thalli were observed by Karling (1951)

Development of the Zoosporangium: According to Karling the development of the rhizomycelium, intercalary swellings, and sporangia are “fundamentally similar to that of other species of Septochytrium as well as of Cladochytrium and Nowakowskiella.” He made no other mention of the details of his species development.

Reproductive: Sporangia often oval (30-60μx40-80μ), broadly pyriform, occasionally spherical (25-60μ), usually with their long axis at right angles to the concomitant rhizoidal axis, endo-

opercula common, rarely apophysate, usually with one or two exterior exit tubes, simple or branching, straight, winding, or tortuous, 6-18μ wide, 18-1400μ long.

Rhizoidal System: Rhizomycelium profuse, much branched, coarse, 8-17μ in diameter, bearing slender rhizoids, with occasional anastomoses; rarely septate or trabeculate, with large, broadly or narrowly fusiform or variously shaped intercalary enlargements.

Zoospore discharge: The tip of the discharge tube dissolves at the same time as the endo-operculum develops further down inside of the tube. The endo-operculum shape ranges from slightly conical to shallow saucer or deeply bowl-shaped or almost hemispherical and is 6-18μ in diameter. During discharge, the endo-operculum is pushed out by the zoospores and may disappear from view. The zoospores emerge in a quiescent mass that starts to disperse when the zoosporangium becomes half empty. The dimensions of the discharge tube are 6-18μ wide and 18-1400μ long.

Zoospore, microscopic: Zoospores are spherical, 3.8-4.7μ in diameter and have multiple minute refractive granules. The flagellum is 24-27μ in length and is posteriorly inserted and directed during swimming.

Zoospore, ultrastructure: Not available.

Resting Spore: No resting spores were reported by Karling (1951).

Ecology and Distribution: Saprophytic in onion skin from moist soil, Karling (loc. cit.) US.

3. *Septochytrium plurilobulum* Johanson

Amer. J. Bot. 30:619, figs. 1A-Z. 1943.

PLATE 15, figs. 261-263

Vegetative: Thallus endobiotic, polycentric.

Development of the Zoosporangium: Zoospores germinate on the surface of the substrate and produce one to several germ tubes which penetrate the surface and begin to branch. A fusiform swelling appears in the germ tube either before or after branching begins. The rhizomycelium continues to develop with swellings formed at various intervals. The swellings can either remain as non-septate swellings or develop into zoosporangia.

Reproductive: Zoosporangia formed terminally or from intercalary swellings, hyaline, smoothe, spherical (11-113μ in diameter average 45-55μ), oval, ellipsoidal, broadly pyriform, with one to several short broad (7μ), or long (33-38μ) exit tubes.

Rhizoidal System: Rhizomycelium coarse, irregular and extensive with septations and trabeculae extending partially or completely across the tenuous portions of the rhizoidal branches.

Zoospore discharge: Operculum oval, spherical (3.75-7μ in diameter). Zoospores are released fully developed in a globular mass that remains quiescent for a short period at the exit orifice before swimming away.

Zoospore, microscopic: Zoospores have several small refractive globules that give the spores a grayish granular appearance. Zoospores are oval to spherical, 7-8μ with an average size of 7.7μ.

Zoospore, ultrastructure: Not available.

Resting Spore: Resting spores are usually terminal, irregular, deeply lobed, 15-43.5μ in greatest diameter; greenish gray in color with a dark, smooth, thick wall (2.62-7μ) and containing one to several small or large refractive globules. During germination, the contents of the resting spore slowly emerge to form an evanescent, thin-walled zoosporangium on the surface of the spore.

Ecology and Distribution: Saprophytic in leaves of striped maple, corn and other grass, Johanson, (loc. cit), US.

4. Septochytrium variabile *Berdan*

Amer. J. Bot. 26: 461, fig. 2. 1939.

PLATE 15, figs. 264-266

Vegetative: Thallus endobiotic, sporangia sometimes rupturing the surface of the substrate but rhizomycelium always endobiotic, polycentric with occasional monocentric thalli

Development of the Zoosporangium: Zoospore germinates on the surface of the substrate and produces usually one germ tube that penetrates the surface. Soon after the germ tube enters the substrate, production of the rhizomycelium begins followed by a swelling of the germ tube. The initial swelling in the germ tube will eventually become the primary sporangium though it may also remain only as a swelling while other swellings develop into zoosporangia. As the primary sporangium enlarges, branches of the rhizomycelium continue to elongate and develop. The zoospore cyst on the surface of the substrate either persists after the primary sporangium matures or quickly disappears. Once the primary sporangium has started to develop, other swellings in the rhizomycelium appear. Some will become zoosporangia while others will form resting spores but most remain as simple swellings that become empty of cytoplasm as the thallus matures. When the thallus has fully matured, true septa separate the zoosporangia and resting spores from the rhizomycelium but the constrictions, partial septations, and trabeculae characteristic of this species (and the genus) can be found throughout the rhizomycelium during the entire course of development. The swellings remain non-septate and can be found as a single swelling in a branch of the rhizomycelium or as a continuous series.

Reproductive: Sporangia hyaline to pale brown, wall smooth when young but becoming striated or layered at maturity and wrinkled when empty; Shapes range from spherical (4-150μ in diameter; often 75-150μ with an average of 45-60μ) with a very short, broad papilla or neck, to

ovate, egg-shaped, broadly pyriform (10x15µ - 180x220µ; commonly 100x150µ) with a neck 4µ-60µ wide, obclavate to flask-shaped (2x6µ-35x360µ), or bell-shaped, irregular, flattened and depressed with one (rarely several) broad exit papilla or neck (Berdan preferred this term to "exit tube") of varying diameter and length.

Rhizoidal System: Endobiotic rhizomycelium has constrictions and septations or trabeculae extending partially or entirely across the rhizoids. Coarse, extensive (20µ to 1cm in diameter), and highly branched. Insertion points number from 1 to 12 on the sporangia with the diameter at the point of insertion 0.4-10µ.

Zoospore discharge: A thin ring appears around the operculum just before release to indicate that discharge is about to occur. When discharge does occur, the operculum can either be pushed up and carried off with the emerging zoospores or can remain hinged to the zoosporangium. The zoospores emerge in a globular mass through the circular orifice created by the operculum and are preceeded by the hyaline material visible just beneath the operculum before release. The mass of zoospores remains quiescent for a short period before it begins to loosen up and zoospores start to swim off. The circular orifice measures 1-16µ in diameter or can be oval shaped (4x6µ-6x10µ).

Zoospore, microscopic: Zoospores hyaline, spherical to oval, 4-6µ, with a single refractive globule, 0.7-3µ (usually 2µ) in diameter; flagellum length 30-45µ.

Zoospore, ultrastructure: Stained zoospores resemble Blastocladialean zoospores with a obyriform nucleus that tapers toward the flagellum and a cap of ribosomes

Resting Spore: Resting spores light to dark amber, spherical (4-60µ), ovoid (4x6µ-50x65µ), or elongated (10x35µ), thick-walled, smooth, layered or with outer coat rather irregular, usually with one large refractive globule and numerous small ones. Resting spores function as

prosporangia at germination. Zoosporangia may either develop in contact with the resting spore or at the end of a tube. Shape of the zoosporangia varies depending on where it forms. If it forms in contact with the resting spore the shape can be spherical, pyriform or ovate whereas if it forms at the end of a tube its shape can range from being oval, rounded pyriform, clavate or obclavate. The tube can be wide and saccate or narrow and long with twists and coils (7-26μx10-450μ).

Ecology and Distribution: Saprophytic on various grasses, wheat, rye, oats, corn leaves and narcissus root tips, Berdan (loc. cit.) US, CANADA; Karling (1941: 387, 1942: 620, 1948: 510) US; grass leaf bait from soil, Sparrow (1952: 69) CUBA.

5. *Septochytrium willoughbyi* Dogma

Nova Hedwigia. 24: 367. figs. 1-22. 1973.

PLATE 16, figs. 267-270

Vegetative: Zoosporangial thallus polycentric and infrequently monocentric. Resting spore thalli monocentric.

Development of the Zoosporangium: On polycentric thalli, zoosporangia are formed by the direct enlargement of the ellipsoid, intercalary swellings. On monocentric thalli, the zoosporangia develop by the unequal enlargement of the germinated zoospore cyst.

Reproductive: Polycentric zoosporangia spherical (23-56μ in diameter), oval or pyriform (23-65μ high by 20-40μ wide) or ellipsoid (50-60μ by 10-25μ), non-appendiculate (According to Dogma's description and drawings, the initial swelling which usually does not develop into a sporangium retains a thickened, colorless remnant of the zoospore cyst that Dogma termed the appendiculum and is also found on the single sporangia of monocentric thalli). Zoosporangia are colorless to grey, become crustose or rugulose with age, and persist after zoospore discharge.

Rhizoidal System: Rhizomycelium extensive, coarse, up to 6µ in diameter, trabeculate to rarely septate, with ellipsoidal swellings, 18-25 by 10-15µ.

Zoospore discharge: The endo-operculate zoosporangia release zoospores by the dehiscence mechanism Type II (Endo-Operculation-A). All of the zoospores are released as a globular mass in a passive manner that remains temporarily at the mouth of the discharge tube and bound by a gelatinous substance formed beneath the endo-operculum. The zoospores become motile and disperse a short time later but do not swarm in a collective manner as seen with other chytrid zoospores. The endo-operculum is convex, pyramidal or umbonate in shape, 5-12µ in diameter, and positioned just below the rim of the exit orifice of either a short (i.e. discharge papilla) or long branched or unbranched exit tube which can reach a length of 180µ.

Zoospore, microscopic: Zoospores 5.5-6.5µ in diameter (average 6.2µ), spherical, with 6-15 hyaline, refractive globules, a large vacuole and a posterior flagellum 30-32µ long.

Zoospore, ultrastructure: Not available.

Resting Spore: Monocentric resting spore thalli formed by the direct and unequal expansion of the zoospore cyst or the expansion of a germ tube produced by the zoospore cyst. Angular or spherical in shape (10-20µ in diameter), oval or ellipsoid (13-30 by 8-22µ). Wall yellow in color with two layers and contents compact, yellowish, consisting of globules around a central vacuole. Resting spores function as prosporangia during germination. Germ sporangia superficial, formed outside and through a pore on the wall of the germinated resting spore, thin-walled, with a single discharge tube or papilla, endo-operculate and discharging zoospores like vegetative zoosporangia.

Ecology and Distribution: Isolated using lens paper, onion skin and cellophane from soil, Dogma (loc. cit.), BRAZIL; On cellophane from forest soil, Willoughby (1964), ENGLAND.

Taxonomic Key to Species of *Septochytrium*

All of the morphological characters used to create this taxonomic key are visible under a light microscope. Identification will require observation over several days in order to see the mature structures utilized in the key.

Key to the Species of *Septochytrium*

1a. Zoospores possess one single large globule…….....*Septochytrium variabile*

1b. Zoospores posses multiple globules…….....2

2a. Constrictions, septations or trabeculae absent from the rhizomycelium…. …….*Septochytrium macrosporum*

2b. Constrictions, septations or trabeculae present in the rhizomycelium…….....3

3a. Zoospores 3.8-4.7µ in diameter…….*Septochytrium marilandicum*

3b. Zoospores larger than 5µ in diameter…….....4

4a. Zoospores 7-8.7µ in diameter, multilobed resting spores on polycentric thalli……*Septochytrium plurilobulum*

4b. Zoospores 5.5-6.5µ in diameter, resting spores on monocentric thalli……..*Septochytrium willoughbyi*

PLATE 1

Figs.1-4 *Allochytridium expandens* Salkin (Karling 1977)

Fig.1 Spherical zoospore with a large hyaline lipid globule.

Fig. 2 Content of zoospore cyst has passed into the expanding spherical zoosporangium. Rhizoids have begun to branch at this stage of development.

Fig. 3 Mature zoosporangium on agar with several constricted segments of catenulate, rhizoidal axes arising from the periphery.

Fig. 4 Empty irregular, elongate zoosporangium on agar and onion skin with an exo-operculum at the discharge orifice.

Figs. 5-6 *Allochytridium luteum* Barr and Desaulniers (Barr and Desaulniers 1987)

Fig. 5 A mature sporangium with a discharge papillum and an exo-operculum off to the side.

Fig. 6 Young germling with a swelling in the germ tube that will eventually develop into a zoosporangium. The zoospore cyst is still present attached to the germ tube and rhizoids have already started to branch.

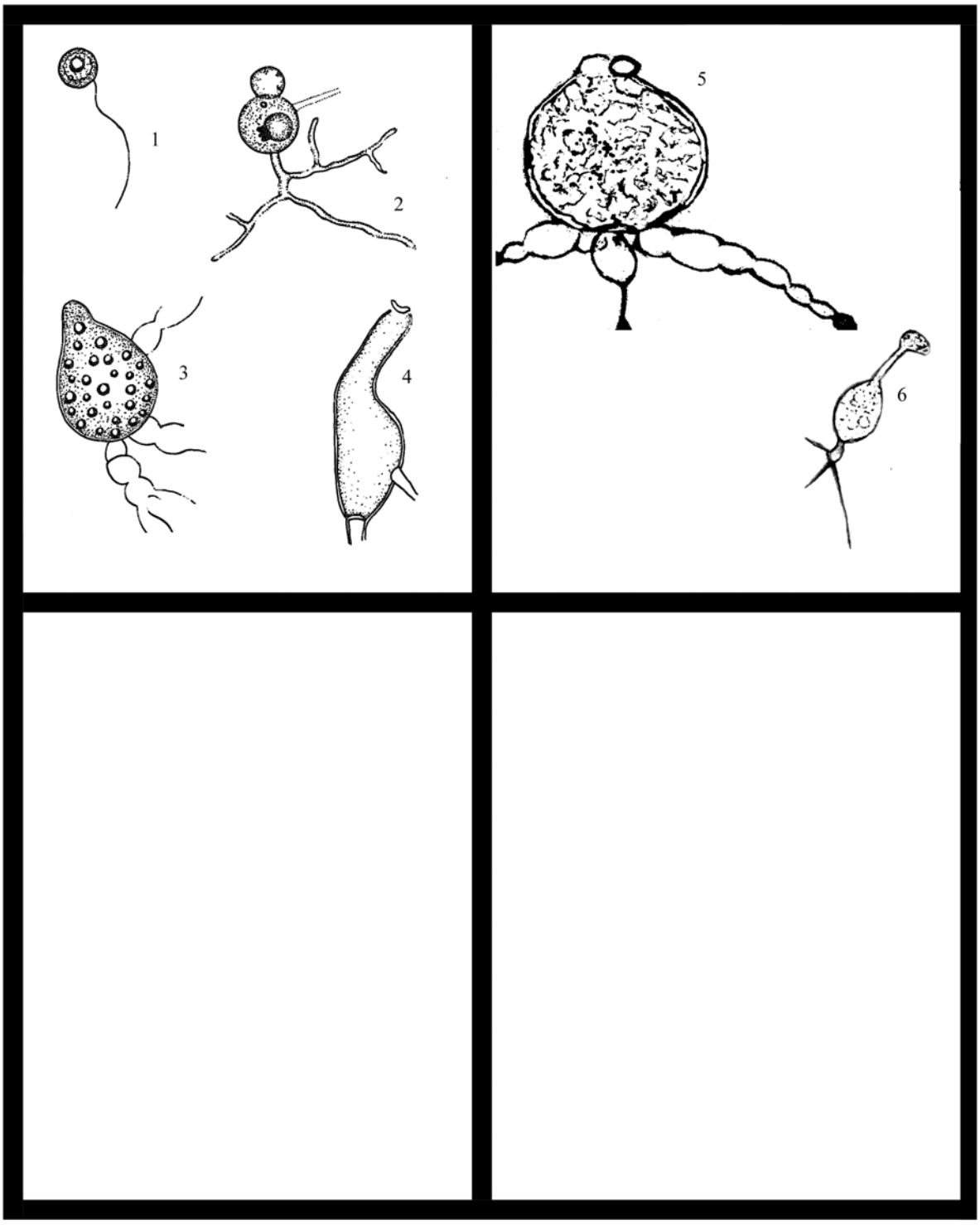


PLATE 1: Figures 1-6, *Allochytridium*

PLATE 2

Figs. 7-10 *Catenochytridium carolinianum* Karling (Karling 1977).

Fig. 7-8 Spherical zoospores, 5-6μm in diameter with hyaline lipid globules

Fig. 9 Mature endobiotic resting spore with a pale to dark-brown smooth wall and filled with lipid globules. Formed from one segment of a compound apophysis.

Fig. 10 Zoosporangial thallus with an endobiotic compound catenulate apophysis and rhizoids, an epibiotic operculate zoosporangium and a globular mass of discharged zoospores.

Figs. 11-12 *Catenochytridium hemicysti* Knox (Barr and Desaulniers 1987).

Fig. 11 One part in the development of the thallus showing rough surfaces of the developing zoosporangium.

Fig. 12 A mature sporangium with a smooth wall.

Figs. 13-14 *Catenochytridium kevorkianii* Sparrow (Karling 1977).

Fig. 13 Discharge of zoospores from an exo-operculate zoosporangium.

Fig. 14 Thallus with a citiriform operculate zoosporangium, thinned-walled catenulate segments, and basal zoospore cyst.

Fig. 15 *Catenochytridium laterale* Hanson (Karling 1977).

Fig. 15A Initial stage of zoospore discharge from an exo-operculate zoosporangium.

Fig. 15B-D Mature endobiotic spherical and irregular resting spores with smooth golden-colored walls and filled with lipid globules. Developed asexually by encystment of the primary segment of the apophysis. The thick walled portion of the zoospore cyst persistent as an appendage.

Fig. 15E Mature epibiotic zoosporangium with a conspicuous discharge papillum, a thickened portion of the zoospores cyst at the base, and primary and secondary segments of the apophysis.

Fig. 15F Spherical zoospore from living material with one large and two smaller hyaline lipid globules.

Fig. 15G Fixed and stained zoospore with a large nuclear cap enveloping the clear nucleus.

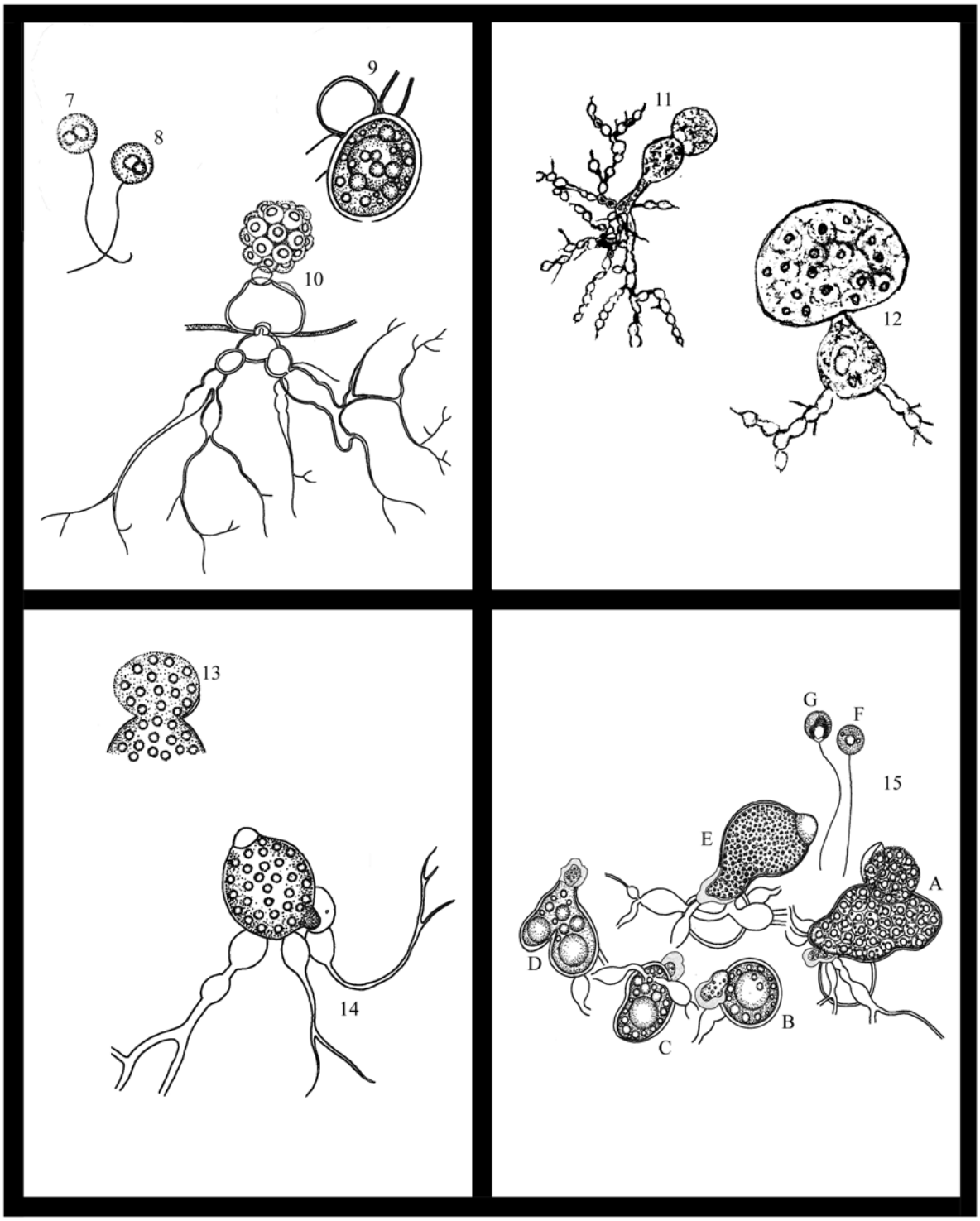


PLATE 2: Figures 7-15, *Catenochytridium*

PLATE 3

Fig. 16 *Catenochytridium marinum* Kobayasi and Ookubo (Karling 1977).

Fig. 16 Thallus of *Catenochytridium marinum* on *Cladophora japonica*.

Figs. 17-20 *Catenochytridium oahuensis* Sparrow (Karling 1977).

Fig. 17 Discharge of zoospores from an exo-operculate zoosporangium. The exo-operculum is off to the side of the discharge orifice. Zoospores remain in a compact group before gaining motility and swimming away.

Fig. 18 Discharge of zoospores. The single small, hyaline lipid globule is visible in the zoospore at the edge of the vesicle.

Fig. 19 Unusual polycentric *Septochytrium*-like thallus with three zoosporangia and occasionally septate, cylindrical, constricted rhizoidial segments.

Fig. 20 Usual monocentric thallus with a globular zoosporangium and cylindrical constricted rhizoidial segments.

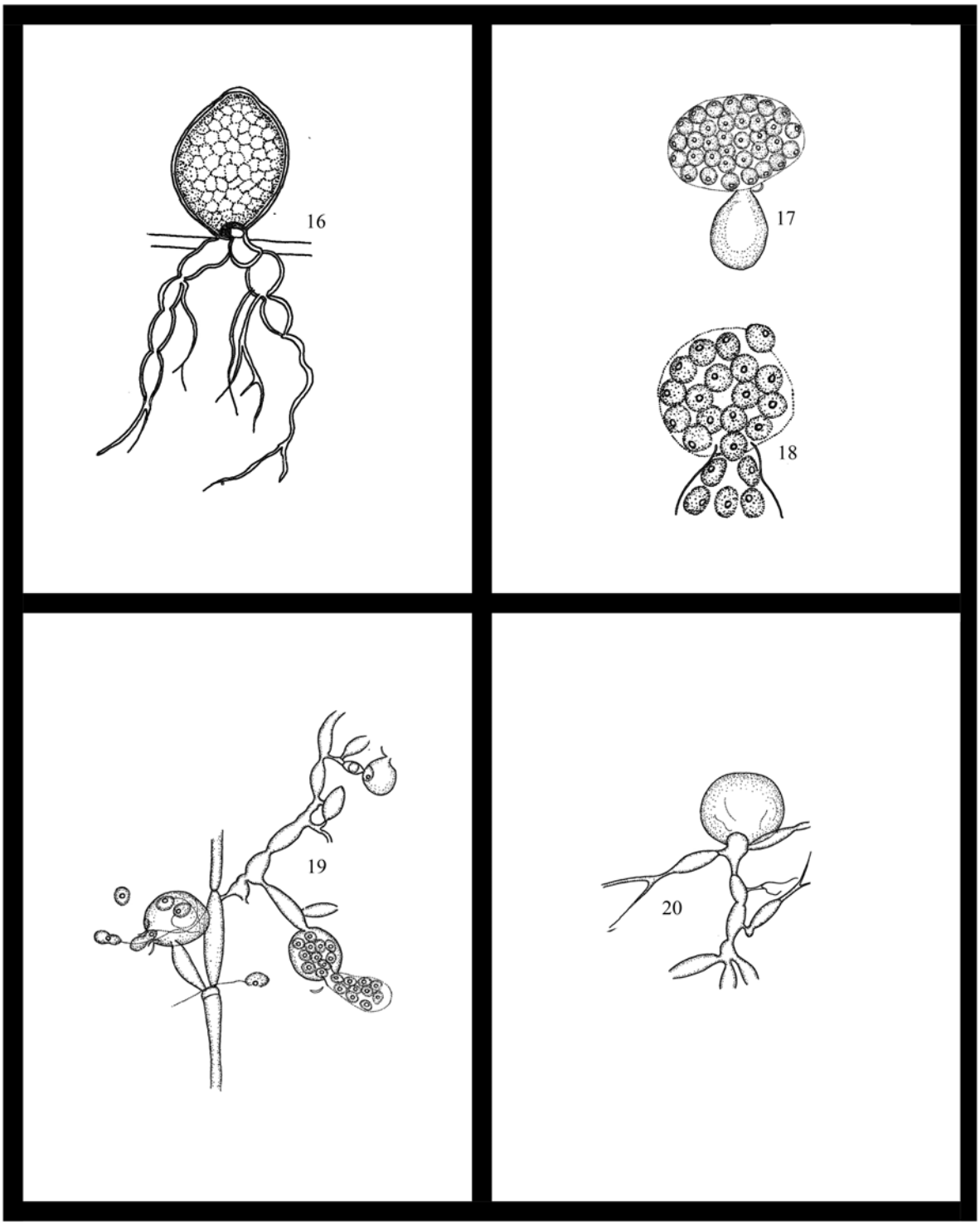


PLATE 3: Figures 16-20, *Catenochytridium*

PLATE 4

Figs. 21-23 *Cladochytrium aurantiacum* Richards (Karling 1977)

Fig. 21 Spherical zoospore with a single golden-orange lipid globule.

Fig. 22A, B Variations in size and shape of sepate intercalary swellings.

Fig. 23 Portion of the rhizomycelium showing a sepate intercalary swelling, a non-septate intercalary swelling and a developing zoosporangium.

Figs. 24-31 *Cladochytrium crassum* Hillegas (Karling 1977)

Fig. 24 Spherical Zoospores with a hyaline refractive globule Portion of the course rhizomycelium with trabeculae

Fig. 25 Portion of the course rhizomycelium with non-septate swellings and trabeculae. Later germination stage with primary spindle organ

Fig. 26 Enlarged portion of the course rhizomycelium with trabeculae.

Fig. 27 Mature zoosporangium with a plug of opaque material in the discharge orifice.

Fig. 28 Vesicle at the mouth of the discharge orifice.

Fig. 29 Discharge of zoospores from inoperculate zoosporangium.

Fig. 30-31 Intercalary resting spores with a smooth light-brown wall and filled with lipid globules.

Figs. 32-37 *Cladochytrium hyalinum* Berdan (Karling 1977)

Fig. 32 Spherical zoospore with a single hyaline lipid globule.

Fig. 33 Developing zoosporangium. Cleavage furrows have appeared that will eventually form individual zoospores. Changes in shape of the refractive globule

Fig. 34 Discharge of zoospores from an inoperculate zoosporangium. A septate intercalary swelling is visible below the zoosporangium. Rhizoids can be seen branching off of the swelling.

Fig. 35 Common proliferation of zoosporangia.

Fig. 36 Germination of a resting spore through a pore in the resting spore wall. The developing zoosporangium is flush with the surface of the resting spore.

Fig. 37 Intermediate stage in the formation of resting spores. The multiple lipid globules will soon coalesce into one large lipid globule.

Figs. 38-41 *Cladochytrium indicum* Singh and Pavgi (Singh and Pavgi 1971)

Fig. 38 Motile zoospore with a single lipid globule.

Fig. 39 Portion of the rhizomycelium with non-septate intercalary swellings and a developing zoosporangium in the middle.

Fig. 40 Mature inoperculate zoosporangium discharging zoospores.

Fig. 41 Mature resting spore formed from an intercalary swelling.

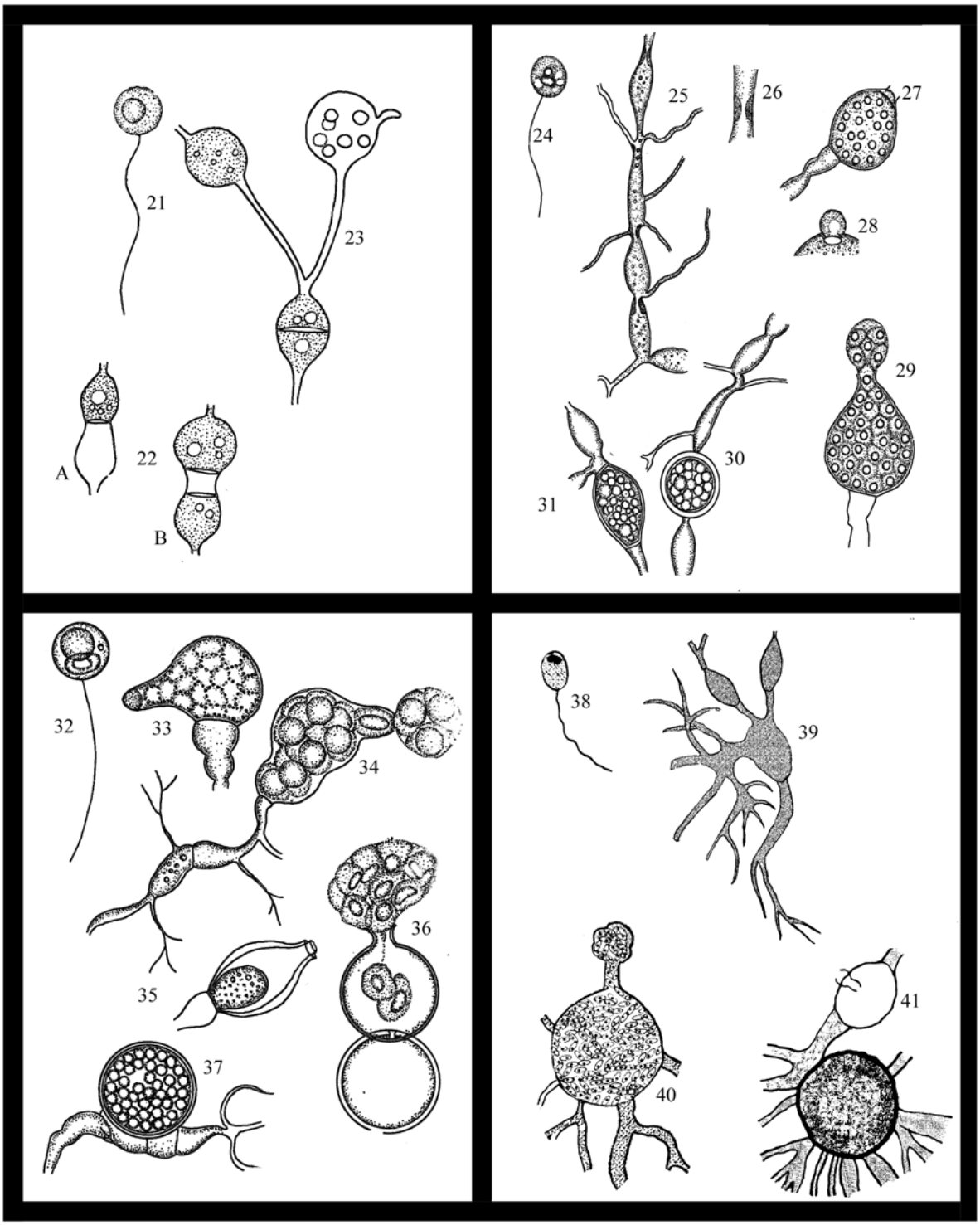


PLATE 4: Figures 21-41, *Cladochytrium*

PLATE 5

Figs. 42-47 *Cladochytrium novoguineense* Kobayasi and Konno (Kobayasi and Konno 1971)

Fig. 42 Irregular zoosporangium with a deliquescing tip.

Fig. 43 A multi-chambered urceolate zoosporangium. Nearby zoospores show a single lipid globule.

Fig. 44 Multi-chambered, irregular zoosporangium.

Fig. 45-47 Resting spores.

Figs. 48-56 *Cladochytrium replicatum* Karling (Karling 1977)

Fig. 48 Spherical zoospore with a single golden-orange lipid globule.

Fig. 49 Mature terminal zoosporangium shortly before cleavage, with a tapering discharge tube.

Fig. 50 Discharge of zoospores from an inoperculate zoosporangium.

Fig. 51 Portion of a rhizomycelium showing a nucleus in the upper division of a septate intercalary swelling. The nucleus of the lower division has migrated down into another swelling. Drawing gives the overall appearance minus zoosporangia of the rhizomycelium.

Fig. 52 Mature resting spore.

Fig. 53 Fixed and stained uninucleate resting spores formed in a septate intercalary swelling.

Fig. 54 Germination of a spiny resting spore.

Fig. 55-56 Variations in sizes and shapes of septate intercalary swellings.

Figs. 57-63 *Cladochytrium salsuginosum* Batko and Hassan (Batko and Hassan 1986)

Fig. 57 Motile zoospore with a single lipid globule.

Fig. 58 Spherical free-formed immature zoosporangium with thin rhizoids.

Fig. 59 Zoosporangium with a group of recently discharged zoospores.

Fig. 60 Clusters of empty zoosporangia connected via thin filaments of the rhizomycelium.

Fig. 61 Two zoosporangia flush with each other. The top zoosporangium is empty while the bottom one is ready to discharge.

Fig. 62 Three irregular zoosporangia developing on the surface of a substrate.

Fig. 63 Thin filaments of the rhizomycelium bearing a string of mature zoosporangia.

Figs. 64-68 *Cladochytrium setigerum* Karling (Karling 1977).

Fig. 64 Spherical zoospore with a minute hyaline lipid globule.

Fig. 65 Zoosporangium discharging zoospores with the setae omitted.

Fig. 66 Enlarged portion of the rhizomycelium showing setae on the tenous filaments.

Fig. 67 Portion of the delicate rhizomycelium and two setigerous zoosporangia.

Fig. 68 Zoosporangium with branched and unbranched setae.

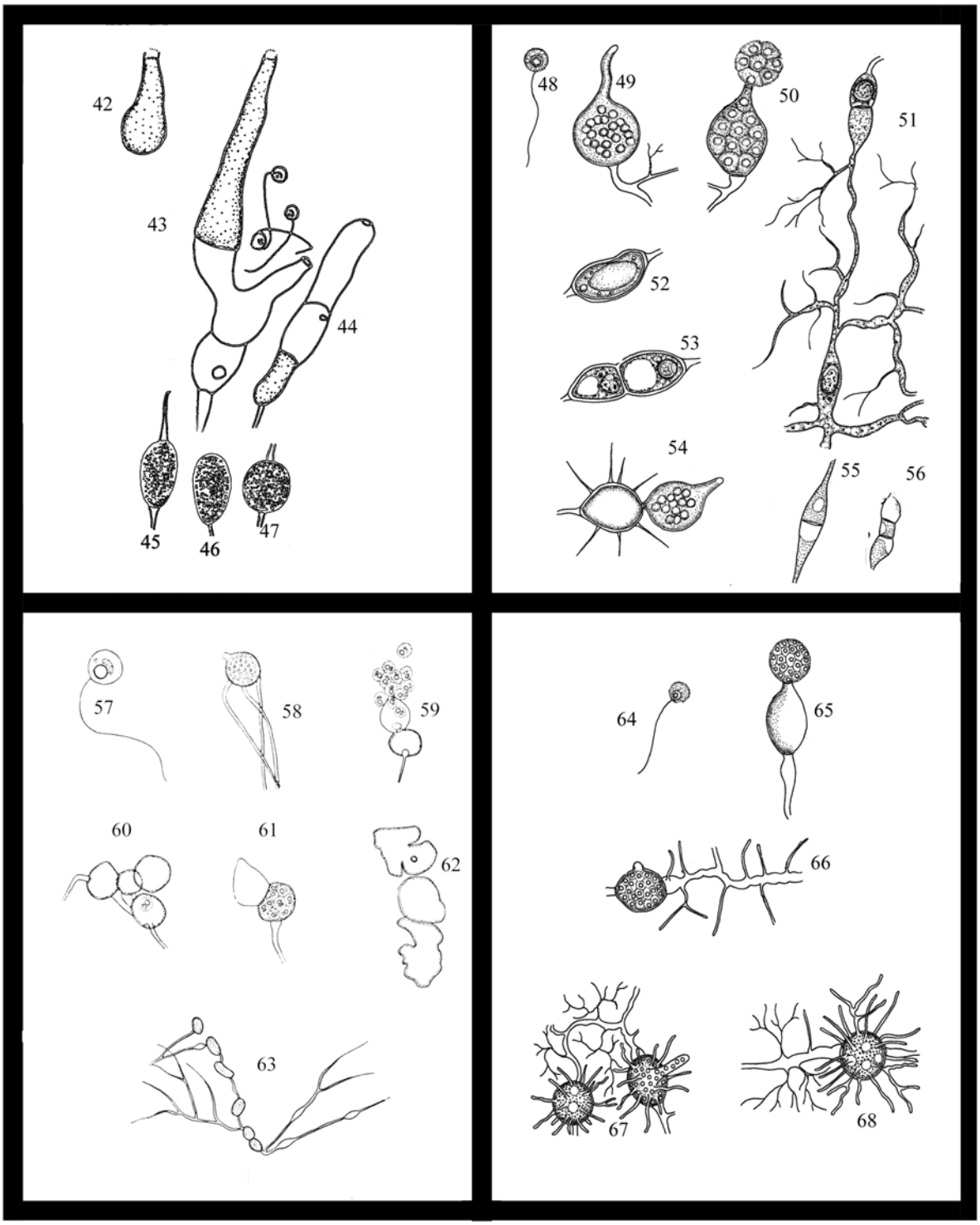


PLATE 5: Figures 42-68, *Cladochytrium*

PLATE 6

Figs. 69-70 *Cladochytrium tainum* Shen and Siang (Karling 1977).

Fig. 69 Spherical soospore with a single hyaline lipid globule.

Fig. 70 Portions of the rhizomycelium showing septate intercalary swellings and two resting spores with a single large lipid globule.

Figs. 71-78 *Cladochytrium tenue* Nowakowski (Karling 1977).

Fig. 71 Spherical zoospore with a hyaline lipid globule. Portion of a rhizomycelium with a tenuous branched filaments.

Fig. 72A-C Proliferating intercalary swellings. A zoosporangium is budding out of the central division in Fig. 72A.

Fig. 73 Septate and non-septate intercalary swellings.

Fig. 74 Discharge of zoospores from an inoperculate zoosporangium.

Fig. 75 Portion of the rhizomycelium with a mature terminal zoosporangium, a zoosporangium developing from an intercalary swelling, septate and non-septate intercalary swellings and fine rhizoids branching off of the main filaments of the rhizomycelium.

Fig. 76 Portion of the rhizomycelium with tenuous branched filaments, rhizoids, non-septate intercalary swellings, and immature zoosporangia.

Fig. 77 Germination of resting spores. One resting spore has a stalked zoosporangium.

Fig. 78A-D Variations in the size and shape of resting spores formed in the intercalary swellings. Figs. 78A is not quite mature and shows multiple lipid globules whereas the mature resting spores seen in Figs. 78B-D exhibit the typical single large lipid globule.

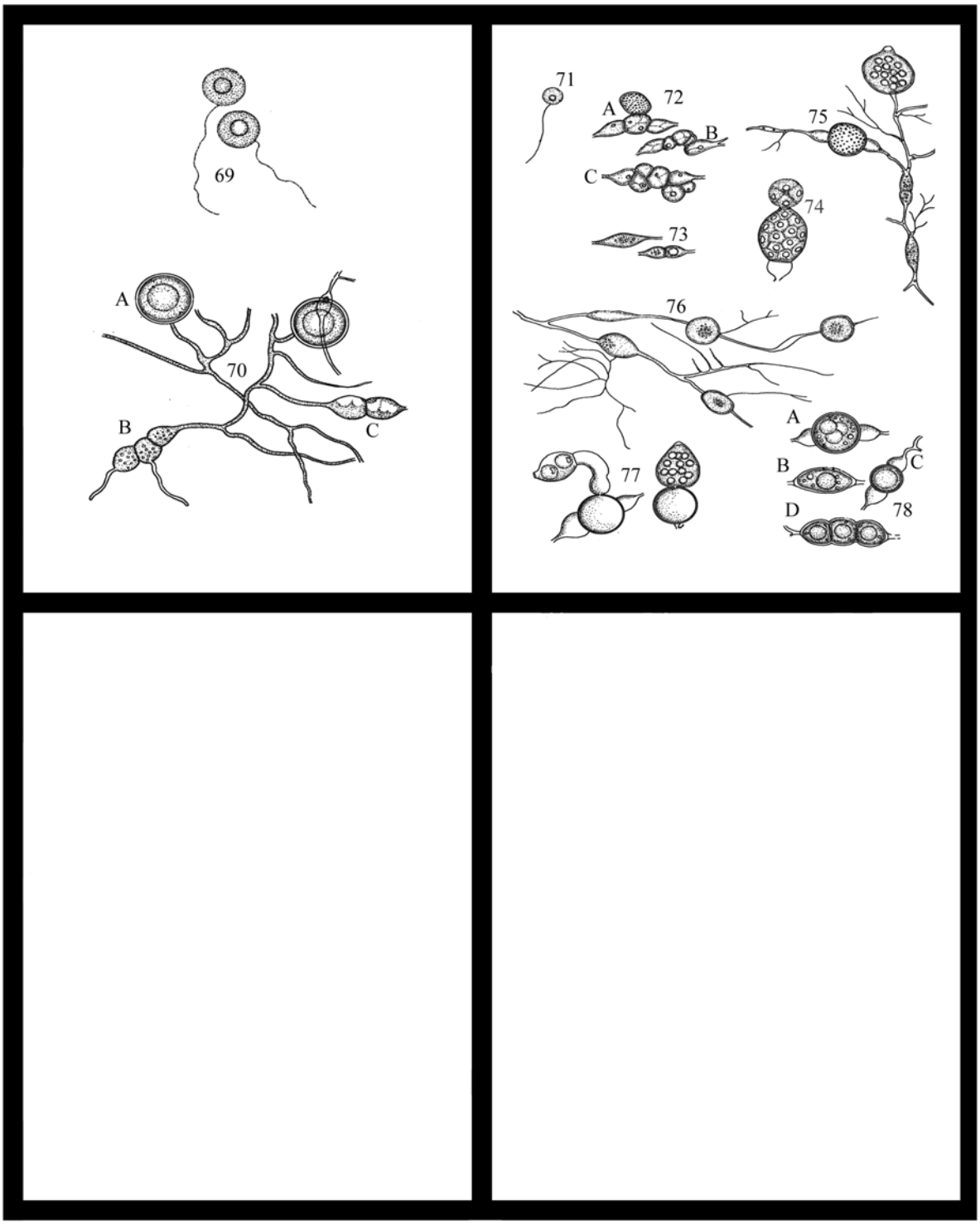


PLATE 6: Figures 69-78, *Cladochytrium*

PLATE 7

Fig. 79-89 *Endochytrium cystarum* Dogma (Karling 1977).

Fig. 79 Spherical zoospore with a minute hyaline lipid globule.

Fig. 80 Developmental stage of zoosporangium by local enlargement of the germ tube.

Fig. 81 Immature zoosporangium with zoospore cyst still attached via a portion of the germ tube. Depauperate rhizoids arising from two points on the developing zoosporangium.

Figs. 82-84 Mature zoosporangia with one to two discharge papillae, small, depauperate rhizoids, and a zoospore cyst attached via a portion of the germ tube. Empty zoosporangium with 2 exit canals and 1 papilla

Fig. 85 Development stages of resting spores with persistent zoospore cyst and germ tube.

Fig. 86 Empty zoosporangium with two discharge tubes and one discharge papillum. Exo-opercula can be seen floating near the discharge orifices.

Figs. 87-89 Mature thick-walled resting spores.

Figs. 90-99 *Endochytrium digitatum* Dogma (Karling 1977).

Fig. 90 Spherical zoospore with a hyaline lipid globule.

Figs. 91-93 Young irregular and digitate zoosporangia with irregular apophysis-like swellings in Figs. 91 and 93.

Fig. 94 Elongate irregular zoosporangium with a long, irregular and coiled discharge tube and an irregular apophysis.

Fig. 95 Irregular end of a discharge tube.

Fig. 96 Discharge of zoospores from an irregular zoosporangium with a long coiled discharge tube and a visible portion of the rhizoidial system.

Fig. 97 Ovoid zoosporangium with a straight tapering discharge tube.

Fig. 98 Deeply lobed mature zoosporangium with a short neck.

Fig. 99 Mature resting spore with a smooth wall and a large lipid globule surrounded by a few smaller ones.

Figs. 100-108 *Endochytrium multiguttulatum* Dogma (Karling 1977).

Fig. 100 Motile zoospore with multiple lipid globules.

Fig. 101 Zoosporangia with a discharge papillum.

Fig. 102 Zoosporangia with a tapering discharge tube.

Fig. 103-104 Zoosporangia with conspicuous golden brown thick walled persistent germ tubes.

Fig. 105 Discharge of zoospores. Exo-operculum is pushed off by the emerging mass of zoospores.

Fig. 106 Empty non-rhizoidial sporangium with hairs arising from the thickened portion of the zoospore cyst. Exo-operculum floating just above the discharge orifice.

Fig. 107 Optical section of resting spore.

Fig. 108 Surface view of resting spore.

Figs. 109-118 *Endochytrium operculatum* Karling (Karling 1977)

Fig. 109 Living zoospore greatly enlarged with a single large lipid globule followed by a fixed and stained zoospore with a conspicuous nuclear cap over the clear nucleus.

Fig. 110 Mature zoosporangium with rhizoids arising at two places on the periphery.

Fig. 111A-C Some variations in the shapes of zoosporangia.

Fig. 112 Discharged mass of zoospores. Early germination stage of a fixed and stained zoospore

Fig. 113 Almost empty zoosporangium with rhizoidal axes arising from four places on its lower periphery.

Fig. 114 Mature warty resting spore with a central lipid globule surrounded by smaller lipid globules.

Fig. 115 Bi-nucleate vacuolated resting spore with an undulating outer wall.

Fig. 116 Early stage of resting spore germination. Developing zoosporangium bi-nucleate.

Fig. 117 Later stage of resting spore germination.

Fig. 118 Mature smooth walled resting spores with a central lipid globule surrounded by smaller lipid globules.

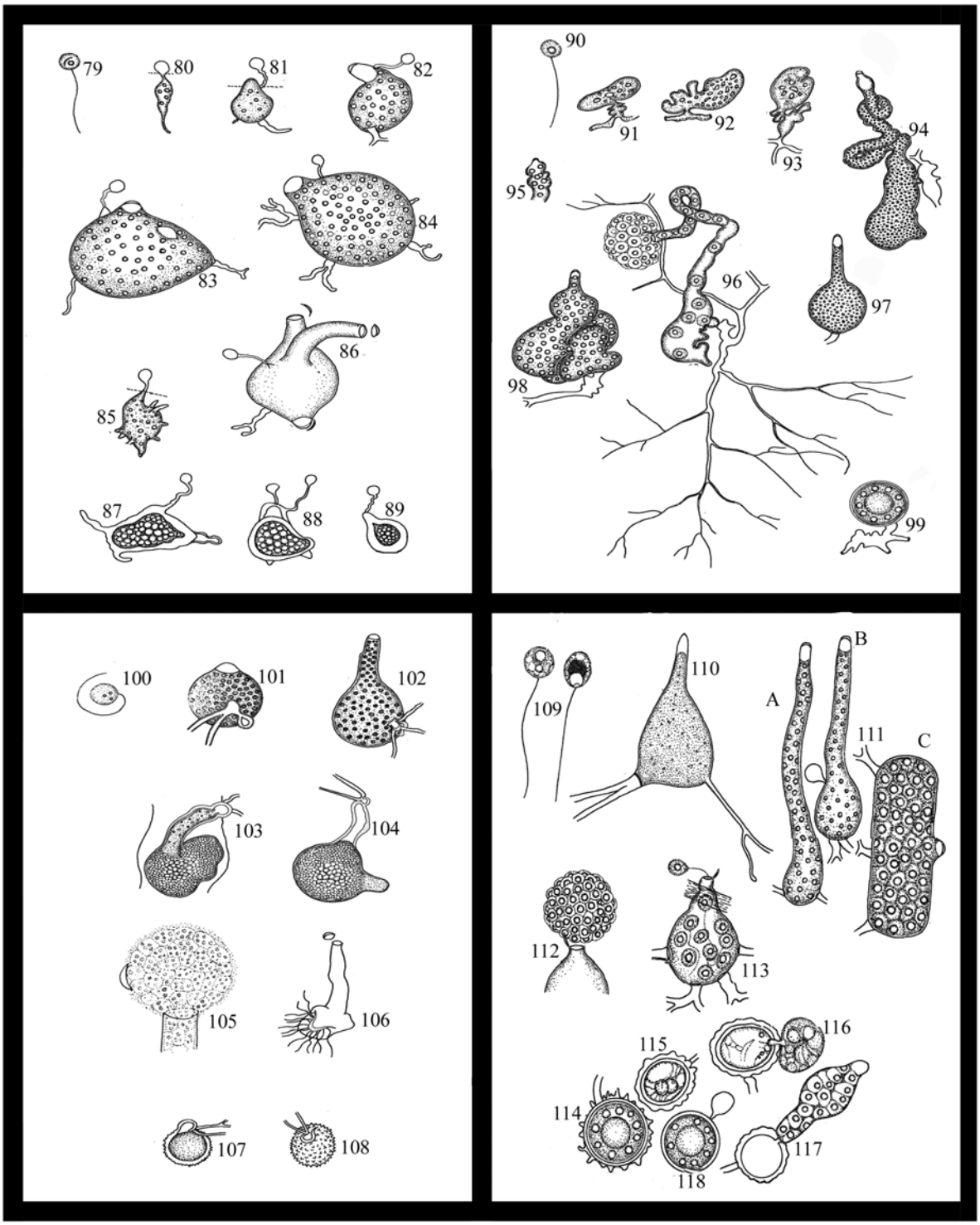


PLATE 7: Figures 79-118, *Endochytrium*

PLATE 8

Figs. 119-125 *Endochytrium pseudodistomum* (Scherffel) Karling (Karling 1977).

Fig. 119 Zoospores with a hyaline lipid globule.

Fig. 120 Zoosporangium and a part of the rhizoids in a *Spirogyra* cell.

Fig. 121-122 Mature zoosporangia with short and long discharge tubes, respectively.

Fig. 123 Tip of discharge tube with attached exo-operculum.

Fig. 124 Young resting spore formed apparently by the contraction of the contents of a sporangium-like vesicle.

Fig. 125 Mature resting spore formed endogenously in a sporangium-like vesicle. Outer wall covered with reflexed scales.

Figs. 126-130 *Endochytrium ramosum* Sparrow (Karling 1977).

Fig. 126 Portion of a Cladophora filament with three zoosporangia and coarse rhizoids.

Fig. 127-129 Stages of zoospore discharge from exo-operculate zoosporangia. Exo-operculum gets pushed off to the side but the emerging zoospore mass but not far from the discharge tube orifice.

Fig. 130 Zoospore dispersal. Zoospores enlarged out of proportion to the size of the zoosporangium.

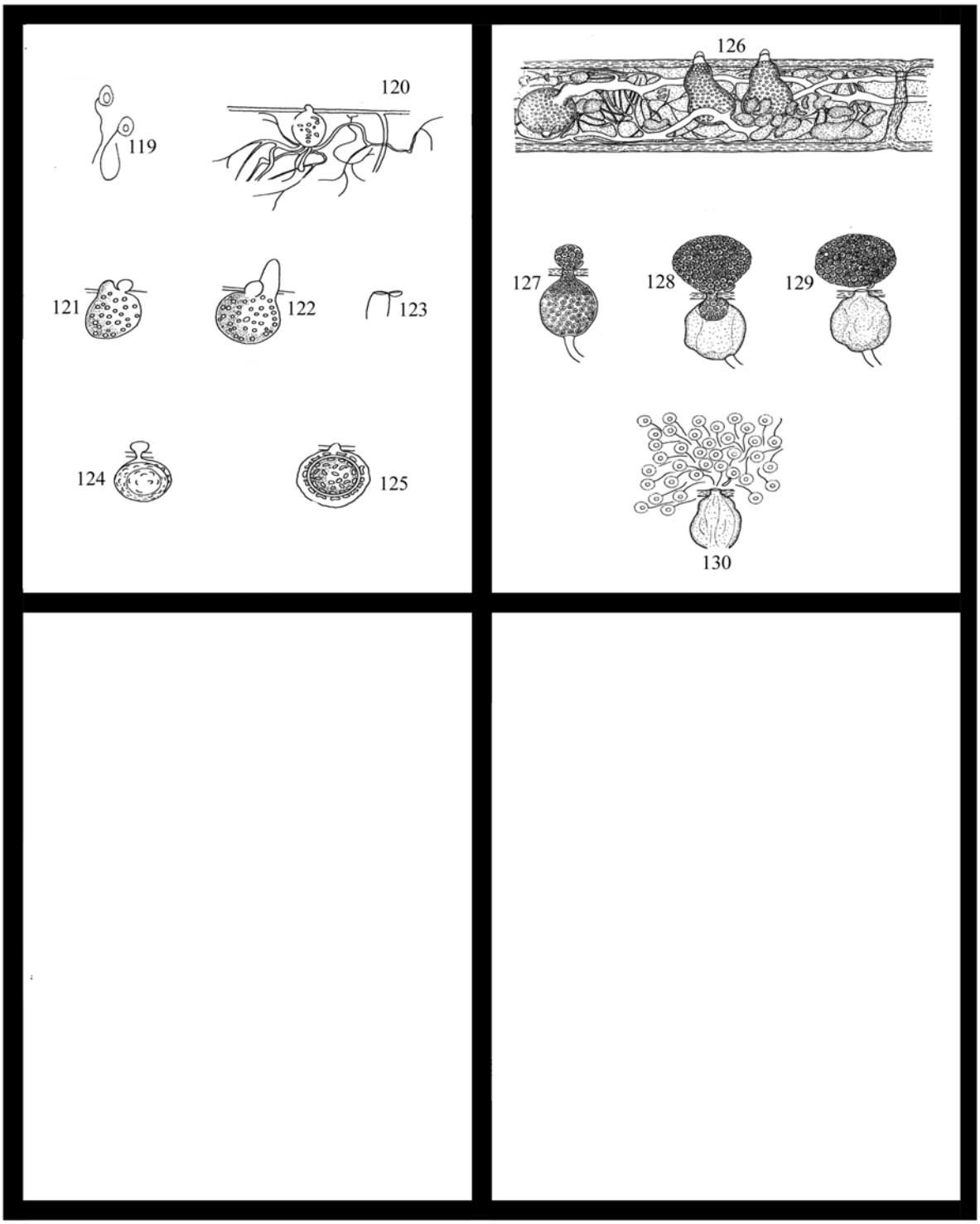


PLATE 8: Figures 119-130, *Endochytrium*

PLATE 9

Figs. 131-144 *Nephrochytrium amazonense* Karling (Karling 1977).

Fig. 131 Spherical zoospore with a hyaline lipid globule.

Fig. 132 Elongate zoospore with a hyaline lipid globule.

Fig. 133 Portion of a young sporangial thallus.

Fig. 134 Young sporangial thallus with zoospore cyst still attached via the germ tube and showing branched rhizoids.

Fig. 135 Variations in the shapes of discharged endo-opercula.

Fig. 136 Young zoosporangium with the discharge tube forming at the apex.

Fig. 137 Tip of a discharge tube with an endo-operculum beneath a plug of gelatinous material.

Fig. 138 Small zoosporangium shortly before dehiscing. Plug of gelatinous material has disappeared.

Fig. 139 Discharge of zoospores with the endo-operculum on the surface of the expanding mass of zoospores.

Figs. 140-143 Variations in the shapes, sizes, and character of the outer wall of mature resting spores

Fig. 140 Smooth walled resting spore

Fig. 141 Verrucose or spiny resting spore

Fig. 142 Hairy (lots of spines) resting spore

Fig. 143 Spiny resting spore but fewer spines than Fig. 142

Fig. 144 Germination of a resting spore. The resting spore acts as a prosporangium during germination as the developing zoosporangium buds out through a pore in the resting spore wall and is sessile on the surface of the resting spore.

Figs. 145-160 *Nephrochytrium appendiculatum* Karling (Karling 1977).

Fig. 145 Spherical zoospore with a large hyaline lipid globule.

Fig. 146 Germinating zoospore.

Fig. 147 Germ tube of germling branching dichotomously.

Fig. 148-149 Formation of the constricted apophysis at the junction of the rhizoidial branches.

Fig. 150 Zoospore cyst still attached to the developing apophysis via the germ tube.

Fig. 151 Rudiment of zoosporangium budding out of the apophysis.

Fig. 152 Developing zoosporangium budding out of the apophysis.

Fig. 153 Occasional fusiform swelling in rhizoid.

Fig. 154 Later stage of zoospore release characterized by the expanding zoospore mass.

Fig. 155 Initial discharge of zoospore. Operculum at side of emerging zoospore mass.

Fig. 156 Mature zoosporangium with two short discharge tubes. Zoospore cyst thick-walled and dark.

Fig. 157 Tip of exo-operculate discharge tube.

Fig. 158 Young resting spore budding out of an apophysis.

Fig. 159-160 Variations in shapes and sizes of mature dark walled resting spores.

Figs. 161-169 *Nephrochytrium aurantium* Whiffen (Karling 1977)

Fig. 161 Spherical zoospore with a single lipid globule.

Fig. 162 Formation of apophysis as a swelling in the germ tube. The zoospore cyst is at the top while rhizoids appear branched at the bottom. Germinated zoospore with a long fairly thick germ tube

Fig. 163-164 Stages in the formation of a spherical apophysis with a dark-walled empty zoospore cyst on the surface. Dichotomous branching of germ tube; small rhizoid at apex

Fig. 165 Fully formed spherical apophysis filled with lipid globules.

Fig. 166 Same apophysis as seen in Fig. 165 but 5 1/2 hours later with the zoosporangium budding out from the surface.

Fig. 167 Same thallus as seen in Fig. 165 but 17 hours later. Vacuolate zoosporangium fully formed with a discharge papillum in surface view.

Fig. 168 Discharge of zoospores from the conical discharge papillum. Exo-operculum at the side of the expanding zoospore mass.

Fig. 169 Same thallus as seen in Fig. 165 but 19 hours later. Apophysis is now empty and the zoosporangium is fully formed and ready to discharge zoospores. The exo-operculum is in the center of the discharge papillum.

Figs. 170-172 *Nephrochytrium bipes* Hassan (1983).

Fig. 170 Motile zoospore with a single lipid globule.

Fig. 171 Mature zoosporangium in the course of zoosporogenesis.

Fig. 172 Mature resting spore.

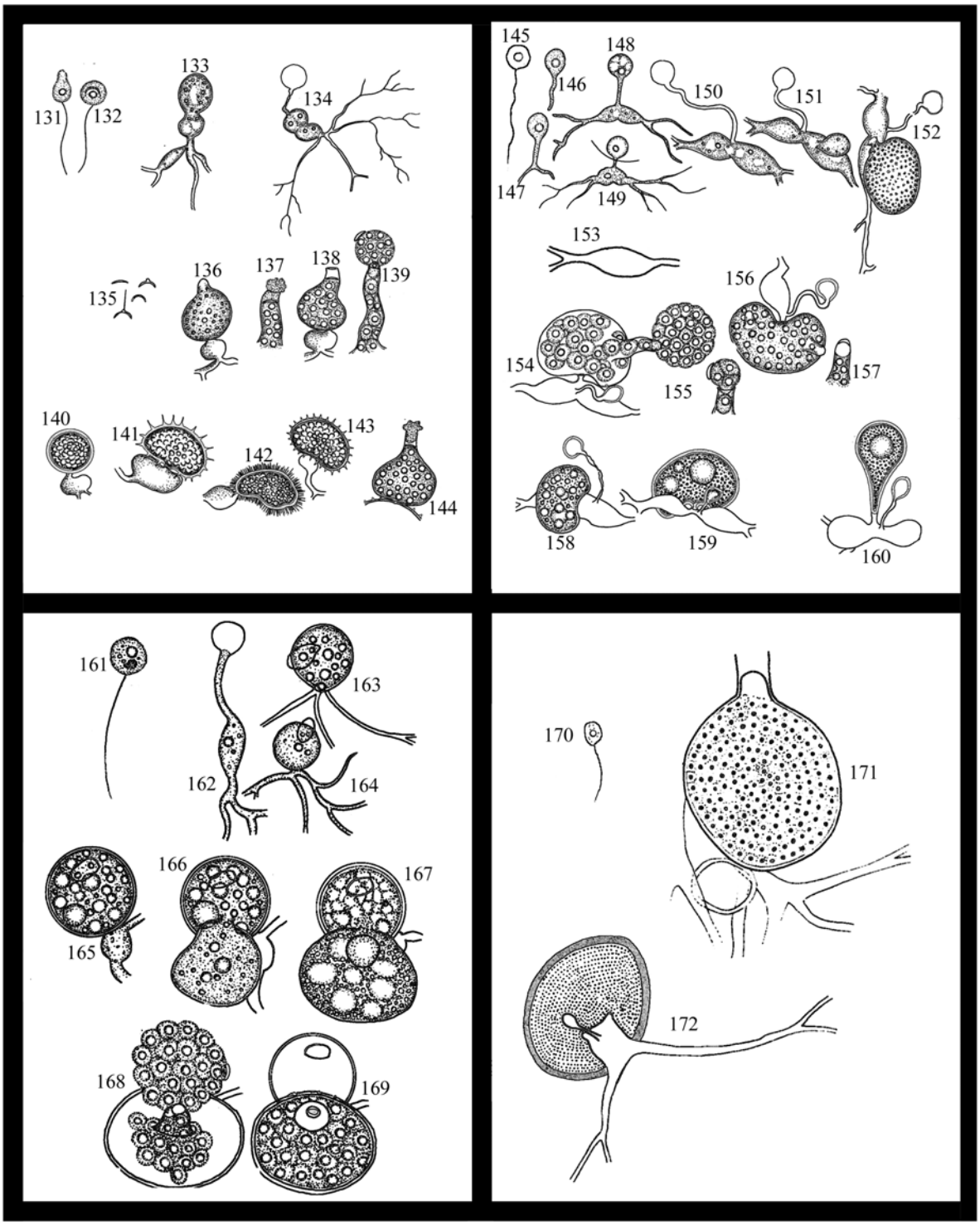


PLATE 9: Figures 131-172, *Nephrochytrium*

PLATE 10

Figs. 173-186 *Nephrochytrium buttermerense* (Willoughby) Batko (Karling 1977).

Fig. 173 Spherical zoospore with a single lipid globule.

Figs. 174-180 Represent stages in the development of a zoosporangium from a germling (Fig. 173) to a mature zoosporangium (Fig. 180).

Fig. 174 Zoospore cyst at the tip of a long germ tube, rhizoids, and a dumb-bell shaped swelling that will eventually form a zoosporangium subtended by an apophysis.

Fig. 175 Young zoosporangium continuous with a broad apophysis. Persistent zoospore cyst attached to the main rhizoidial axis with another rhizoid coming out on the opposite side.

Fig. 176 Later stage of Fig. 175. The apophysis wall is beginning to thicken.

Fig. 177 Later stage of Fig. 176. Incipient zoosporangium still continuous with the broad apophysis.

Fig. 178 Zoosporangium delimited from the thick-walled apophysis. The zoospore cyst is connected to the rhizoidial axis and bears a long curved, branched rhizoid.

Fig. 179 Small zoosporangium with short discharge tube before deliquescence of its tip. Zoospores have yet to cleave-up.

Fig. 180 Two mature zoosporangia borne on a branched apophysis. Endo-opercula down inside discharge tubes.

Fig. 181 Mature endooperculate zoosporangium with a large thick-walled apophysis. Persistent zoospore cyst attached to the rhizoidial axis by a long branched rhizoid.

Fig. 182 Small empty zoosporangium with an unusually long discharge tube.

Fig. 183 Empty zoosporangium with two discharge tubes.

Fig. 184 Tip of discharge tube with portion of the endo-operculum down inside the tube.

Fig. 185 Empty tip of discharge tube with reflexed wall material at its discharge orifice and portion of the endo-operculum down inside the tube.

Fig. 186 Tip of discharge tube with beaked endo-operculum.

Figs. 187-191 *Nephrochytrium sexuale* Haskins (Karling 1977)

Fig. 187 Germinated zoospore on boiled maize leaf showing a long germ tube, branching rhizoid, and a swelling that will eventually develop into a zoosporangium.

Fig. 188 Almost mature zoosporangium, apophysis and rhizoids.

Fig. 189 Plug of gelatinous material in discharge tube.

Figs. 190A-B Endo-operculate discharge tubes with beaked endo-opercula.

Fig. 191 Five spiny to hairy resting spore with apophyses connected by filaments to male gametes (?) in a male (?) thallus.

Figs. 193-207 *Nephrochytrium stellatum* Couch (Karling 1977).

Fig. 193 Motile zoospore with single lipid globule.

Fig. 194 Early stage in zoospore germination; initial swelling that eventually becomes the apophysis is formed first.

Fig. 195 Empty zoospore cyst and young apophysis.

Fig. 196 Zoosporangium budding out of the apophysis near the point of union of the apophysis and rhizoid.

Fig. 197 Fully grown zoosporangium with an discharge papillum in surface view near the zoospore cyst.

Fig. 198 Discharge of zoospores from a small zoosporangium. Exo-operculum carried off by expanding mass of zoospores.

Fig. 199 Large mature zoosporangium with a slit-like basal papillum.

Fig. 200 Portion of resting-spore thallus with apophysis, rhizoids and empty zoospore

Fig. 201 Apophysis elongating. The basal portion appears vacuolated.

Fig. 202, 203 Formation of a smooth walled resting spore in the upper end of an elongated apophysis.

Fig. 204, 205 Similar to stages seen in Figs. 202-203 in the development of the resting spore but with an undulate outer wall.

Fig. 206, 207 Mature stellate resting spores.

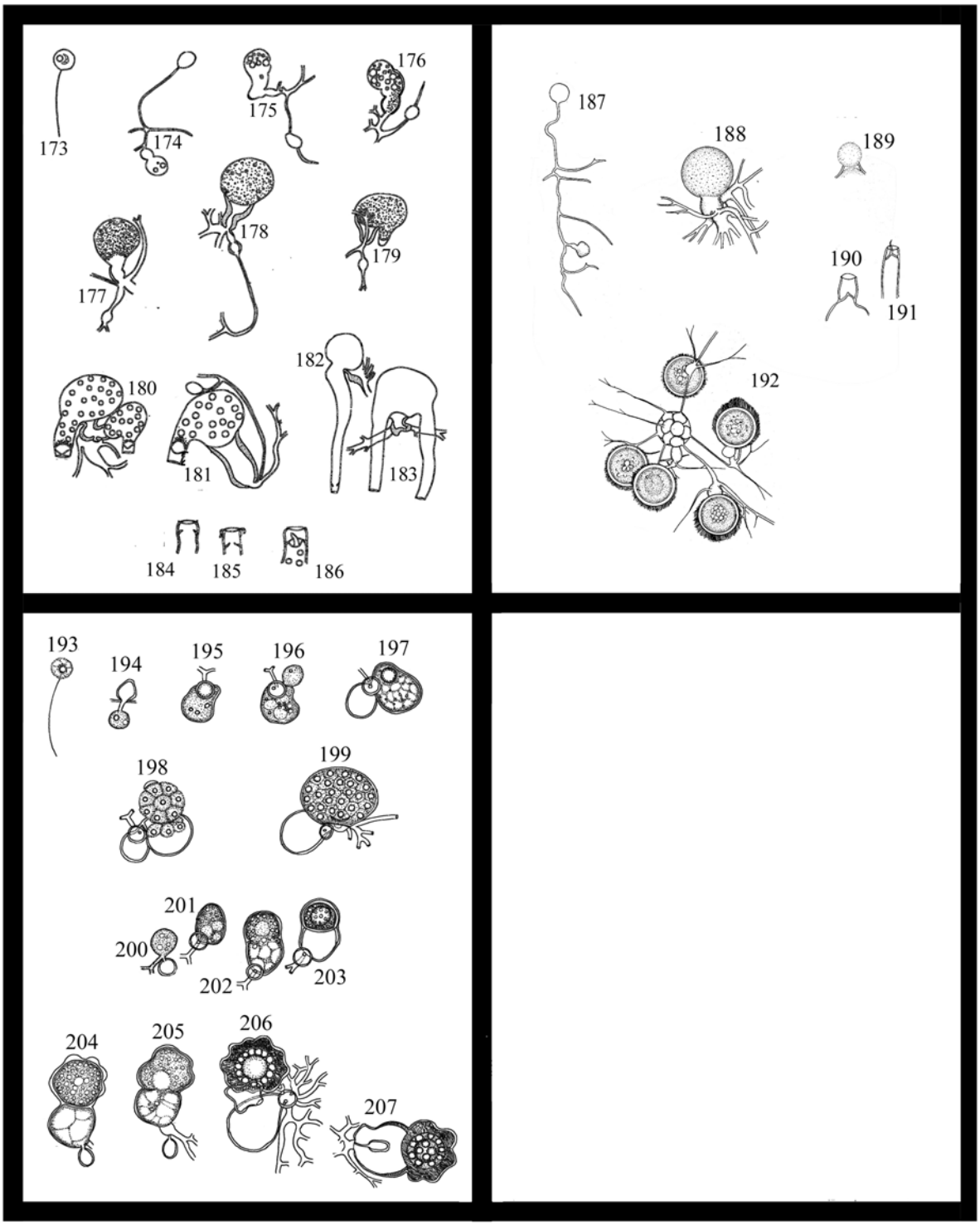


PLATE 10: Figures 173-207, *Nephrochytrium*

PLATE 11

Figs 208-209 *Nowakowskiella atkinsii* Sparrow (1950).

Fig. 208 Thallus bearing one discharged zoosporangium and one mature zoosporangium with a clear area underneath the exo-operculum.

Fig. 209 Portion of the rhizomycelium bearing three discharged zoosporangia and two non-septate swellings.

Figs. 210-211 *Nowakowskiella elegans* (Nowakowski) Schroeter (Karling 1977).

Fig. 210 Portion of the rhizomycelium with one empty proliferated zoosporangium, one empty zoosporangium with the exo-operculum still attached, and four undischarged mature zoosporangia, rhizoids, and non-septate intercalary swellings.

Fig. 211 Intercalary smooth-walled resting spore with a large central lipid globule.

Figs. 212-214 *Nowakowskiella elongata* Karling (Karling 1977).

Fig. 212 Motile zoospore with a single lipid globule.

Fig. 213 Portion of the rhizomycelium showing variations in the shape and structure of the elongated and multi-chambered zoosporangia along with the non-septate intercalary swellings and rhizoids branching off the main filaments of the rhizomycelium.

Fig. 214 Mature spherical resting spore.

Figs 215-217 *Nowakowskiella granulata* Karling (Karling 1944)

Fig. 215 Spherical zoospore with finely granular contents.

Fig. 216 Portion of the rhizomycelium showing a mostly empty zoosporangium with the endo-operculum floating near the discharge orifice, a mature zoosporangium with a plug of hyaline material, rhizoids branching off the main filaments of the rhizomycelium and non-septate intercalary swellings.

Fig. 217 Mature resting spore.

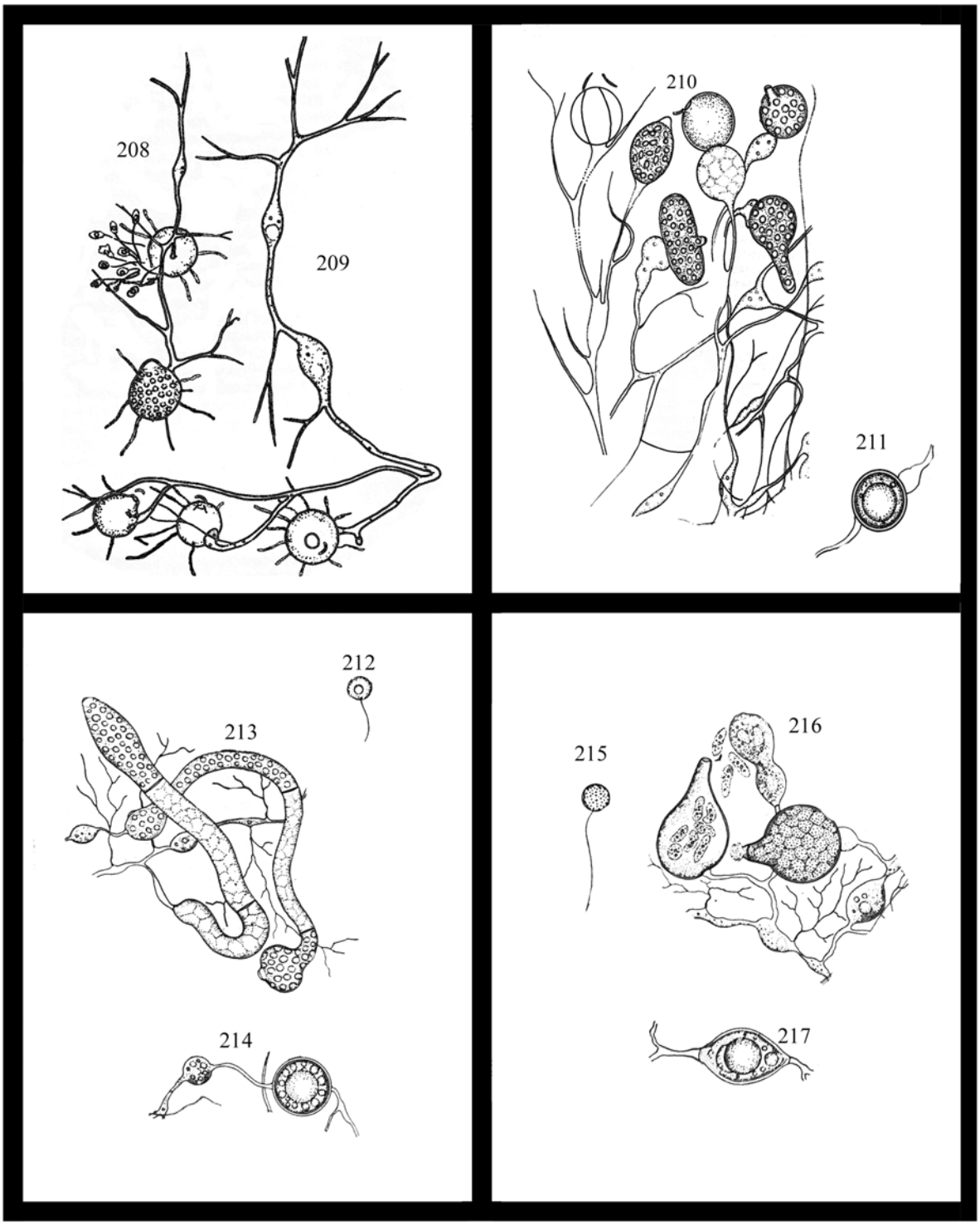


PLATE 11: Figures 208-217, *Nowakowskiella*

PLATE 12

Figs. 218-222 *Nowakowskiella hemisphaerospora* Shanor (Shanor 1942, Karling 1977)

Fig. 218 Motile zoospore with a single lipid globule.

Fig. 219 Close-up of discharge orifice showing hinged exo-operculum.

Fig. 220 Zoospore release from an exo-operculate zoosporangium.

Fig. 221 Germinating resting bodies. The resting spore container acts as a partial prosporangium.

Fig. 222 Hemispherical resting body with a single resting spore.

Figs. 223-226 *Nowakowskiella keratinophila* Hassan and Batko (Hassan and Batko 1986).

Fig. 223 Motile zoospore with a single lipid globule.

Fig. 224 An immature zoosporangium with an exposed endo-operculum.

Fig. 225 Mature zoosporangium slowly releasing zoospores.

Fig. 226 Rhizomycelium with numerous non-septate intercalary swellings.

Figs. 227-229 *Nowakowskiella macrospora* Karling (Karling 1977).

Fig. 227 Motile zoospore with a single lipid globule.

Fig. 228 Portion of rhizomycelium with a mature endo-operculate, apophysate zoosporangium.

Fig. 229 Ovoid resting spore with large and small lipid globules.

Figs. 230-232 *Nowakowskiella methistemichroma* Batko and Hassan (1982).

Fig. 230 Motile zoospore with a single lipid globule.

Fig. 231 Appearance of a mature zoosporangium with a short discharge tube before dehiscence.

Fig. 232 Thallus with developing resting sporangia.

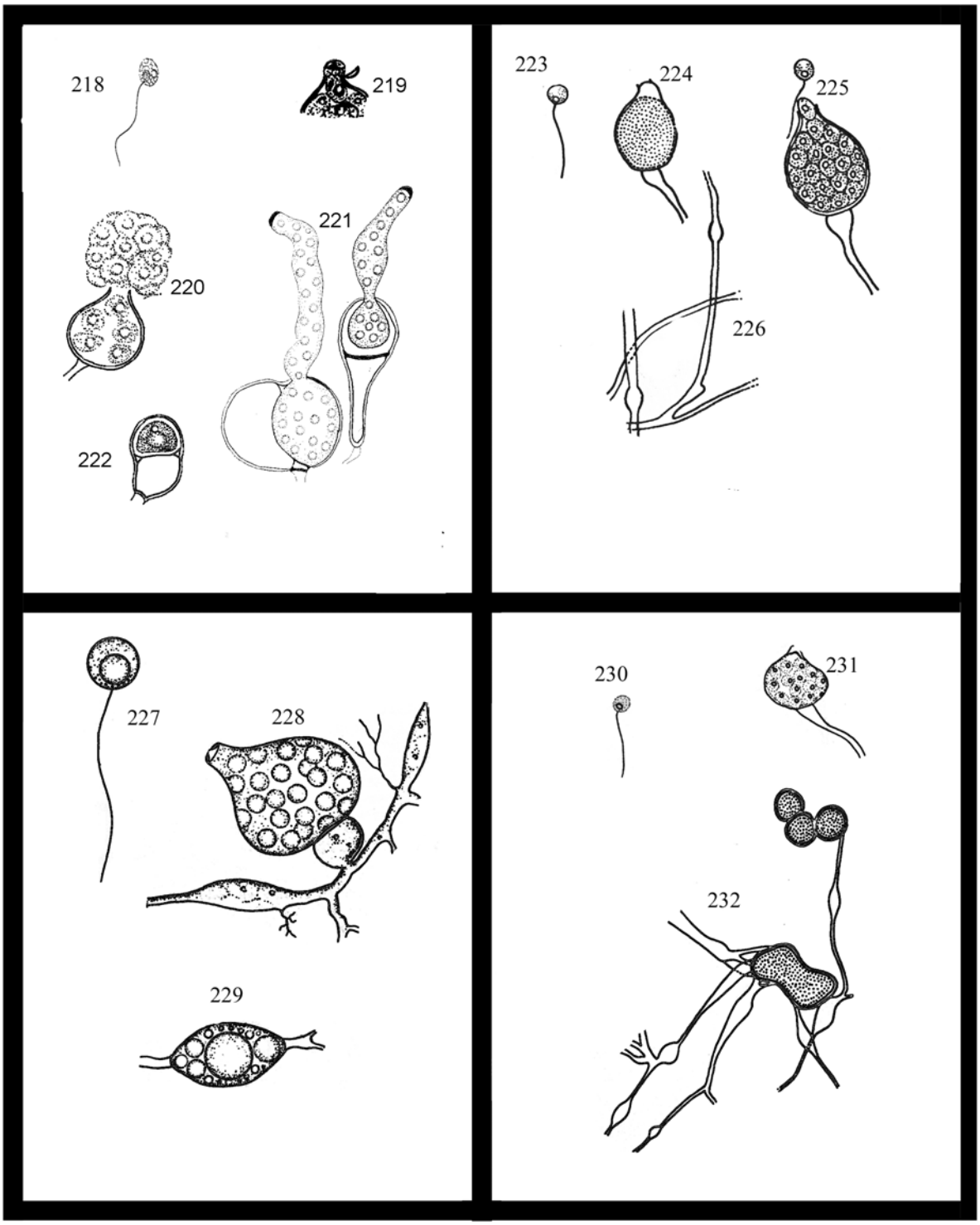


PLATE 12: Figures 218-232, *Nowakowskiella*

PLATE 13

Figs. 233-237 *Nowakowskiella moubasheriana* Hassan (Hassan 1983).

Fig. 233 Exo-operculate zoosporangium during zoospore release.

Fig. 234 Branched rhizomycelium with non-septate and septate intercalary swellings.

Fig. 235 Mature zoosporangium.

Fig. 236 Brown thick-walled resting spore.

Fig. 237 Smooth, hyaline resting spores.

Fig. 238A-C *Nowakowskiella multispora* Karling (Karling 1977)

Fig. 238A Exo-operculate zoosporangia

Fig. 238B Resting spores

Fig. 238C Non-septate intercalary swellings

Figs. 239-241 *Nowakowskiella multispora* var. *longa* (Karling) Kiran (Kiran 1992)

Fig. 239 Rhizomycelium with an immature zoosporangium

Fig. 240 Mature zoosporangium with two long exo-operculate discharge tubes

Fig. 241 Mature exo-operculate zoosporangium

Figs. 242-246 *Nowakowskiella pitcairnensis* Karling (Karling 1977).

Fig. 242 Exo-operculate zoosporangium.

Fig. 243 Exo-operculate zoosporangium during zoospore release. Single lipid globule is visible in the emerging zoospores and in the zoospores in the zoosporangium next to the bi-septate swelling.

Fig. 244 Bi-septate swelling.

Fig. 245 Catenulate swelling - looks like beads on a string.

Fig. 246 Resting spores.

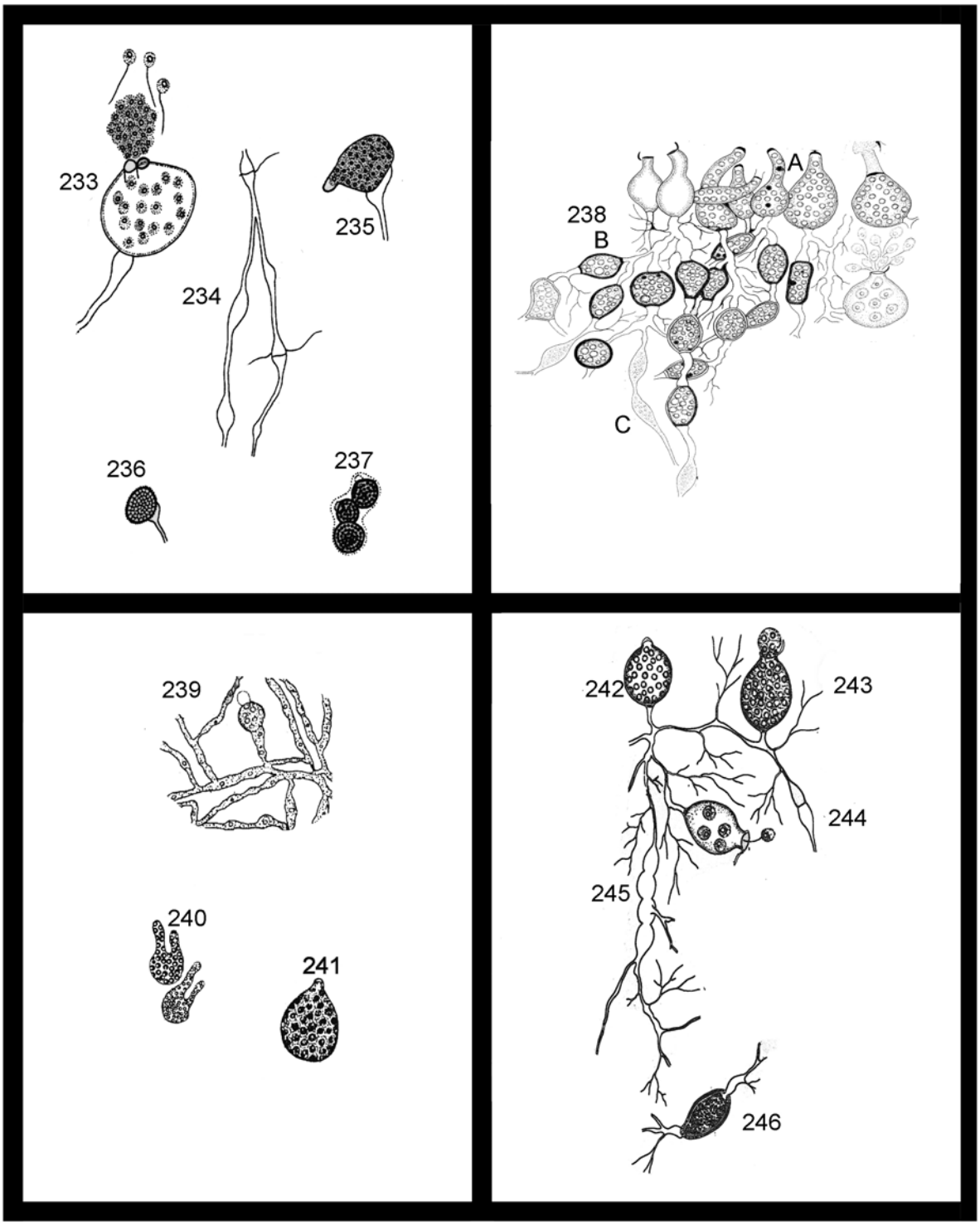


PLATE 13: Figures 233-246, *Nowakowskiella*

PLATE 14

Figs. 247-250 *Nowakowskiella ramosa* Butler (Karling 1944).

Fig. 247 Zoospore with a single lipid globule.

Fig. 248 Exo-operculate zoosporangia.

Fig. 249 Resting spores derived from pseudoparenchymtous divisions of swellings.

Fig. 250 Intercalary zoosporangium.

Figs. 251-257 *Nowakowskiella sculptura* Karling (Karling 1977).

Fig. 251 Zoospore with a single lipid globule.

Fig. 252 Rhizomycelium growing in cellophane showing mostly non-septate intercalary swellings with one exception.

Fig. 253 Mature zoosporangium with an endo-operculum.

Fig. 254 Endo-operculum being pushed out by emerging zoospores.

Fig. 255 Division or proliferation of intercalary swellings. Some swellings have pegs that will eventually develop into resting spores.

Fig. 256 Pseudoparenchyma bearing a smooth, a sculptured and a verrucose resting spore.

Fig. 257 Smooth-wall resting spore that has germinated and formed an endo-operculate zoosporangium.

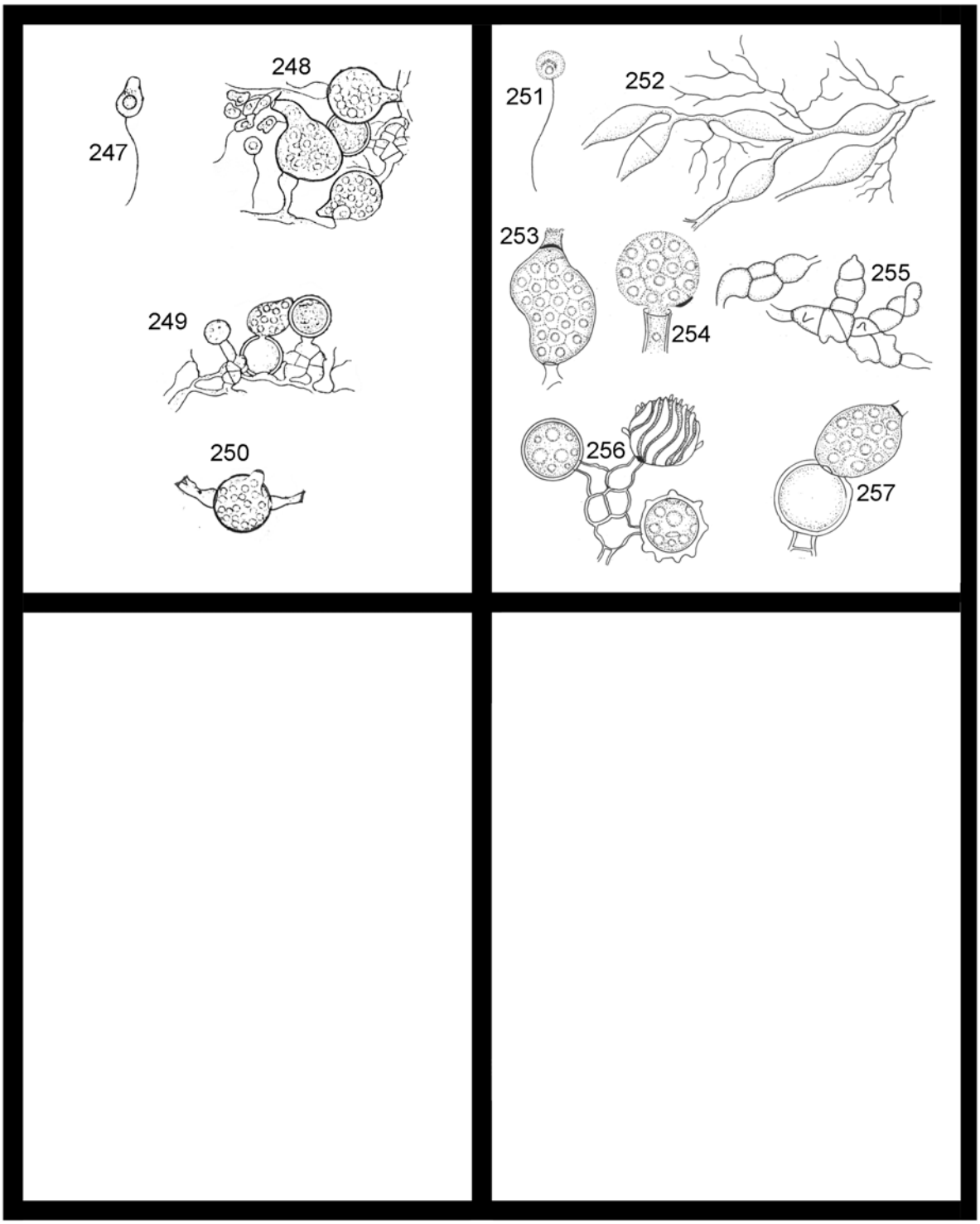


PLATE 14: Figures 247-257, *Nowakowskiella*

PLATE 15

Figs. 258A-D *Septochytrium macrosporum* Karling (Karling 1977)

Fig. 258A Exo-operculate zoosporangium.

Fig. 258B Motile zoospore.

Fig. 258C Non-septate intercalary swelling.

Fig. 258D Intercalary resting spore.

Figs. 259-260 *Septochytrium marilandicum* Karling (Karling 1977).

Fig. 259 Motile zoospore with numerous lipid globules.

Fig. 260 Mature endo-operculate zoosporangia and intercalary swellings.

Figs. 261-263 *Septochytrium plurilobulum* Johanson (Karling 1977).

Fig. 261 Motile zoospore with numerous lipid globules.

Fig. 262 Portion of rhizomycelium bearing zoosporangia, one of which is releasing zoospores.

Fig. 263 Deeply lobed resting spores.

Figs. 264-266 *Septochytrium variable* Berdan (Karling 1977).

Fig. 264 Monocentric thallus with an immature sporangium. Zoospore case is still attached to the base of the zoosporangium.

Fig. 265 Mature resting spore.

Fig. 266 Mature thallus of *Septochytrium variabile* showing its polycentric nature. The large central body is a young primary zoosporangium. Rhizomycelium with swellings and secondary zoosporangia radiate out from the central zoosporangium.

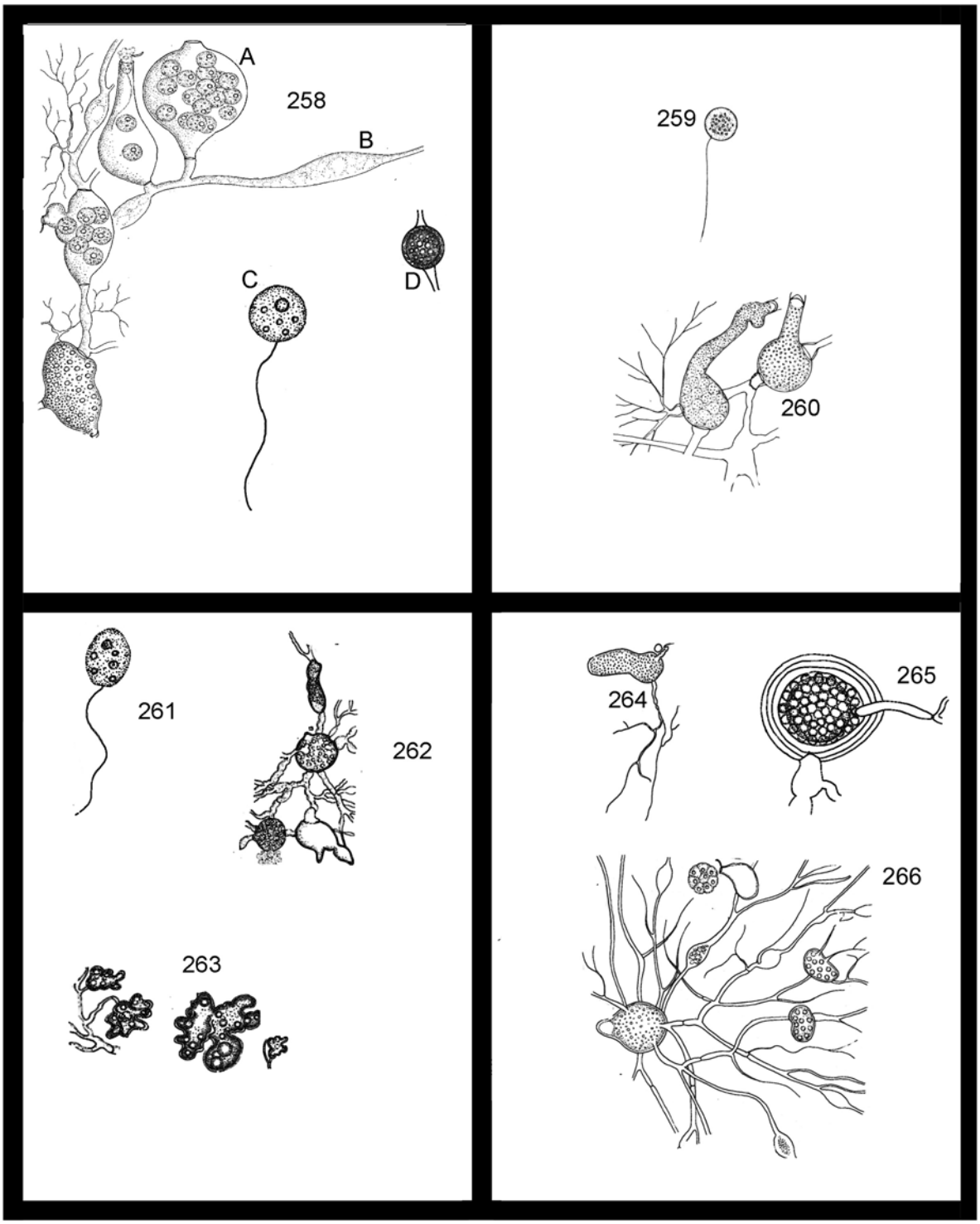


PLATE 15: Figures 258-266, *Septochytrium*

PLATE 16

Figs 267-270 *Septochytrium willoughbyi* Dogma (redrawn from Dogma 1973).

Fig. 267 Motile zoospore with multiple lipid globules.

Fig. 268 Portion of rhizomycelium with intercalary and terminal zoosporangia.

Fig. 269 Mature resting spore formed by encystment of primary swelling.

Fig. 270 Immature thallus with non-septate intercalary swellings. Some of the swellings will eventually develop into zoosporangia. The primary swelling retains a thickened remnant of the zoospore cyst.

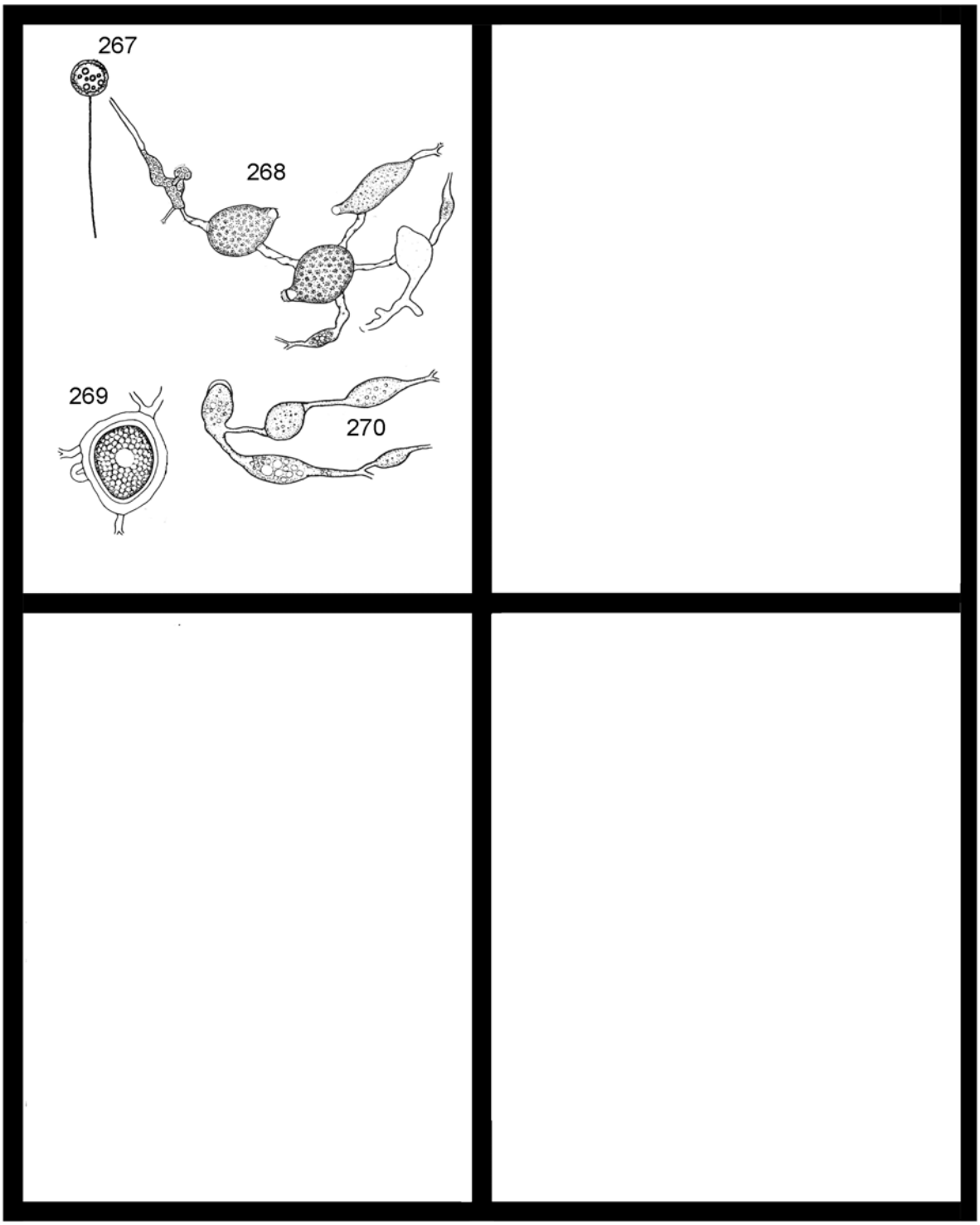


PLATE 16: Figures 267-270, *Septochytrium*

CHAPTER 3

MOLECULAR PHYLOGENY OF GENERA IN THE "*NOWAKOWSKIELLA*" CLADE BASED ON 18S AND 28S NUCLEAR RIBOSOMAL DNA

Introduction

Despite the regular inclusion of chytrid fungi in numerous molecular phylogenetic analyses, only a few representative sequences have been sampled for each analysis. The sequences have primarily been used for the determination of relationships between the major fungal phyla, multi-Kingdom analyses or as members of an outgroup for a non-chytrid ingroup (Bowman et al.1992, Berbee et al. 1993, Keeling et al. 2000, Lutzoni et al. 2004). In contrast, chytrid specific analyses looking at relationships between and among the different taxonomic ranks are few in number (James et al. 2000, Chambers 2003, Letcher et al. 2004). Chytrid families, genera and species are widely known to be artificial constructs based on morphological and developmental characters that are not phylogenetically useful as currently implemented (Booth 1971, Miller 1976, Karling 1977).

Genera in the "*Nowakowskiella*" clade are good examples of the problems with chytrid classification and taxonomy. Grouped together based on 18S nuclear ribosomal DNA (nrDNA) and zoospore ultrastructure (James et al. 2000), genera in the clade do exhibit different thallus morphologies and different developmental pathways but with a certain amount of overlap making it difficult to state clear distinctions between genera and species. Genera currently placed in the "*Nowakowskiella*" clade include *Allochytridium, Catenochytridium, Cladochytrium, Endochytrium, Nephrochytrium, Nowakowskiella*, and *Septochytrium*. Prior to the molecular

phylogenetic analyses of James et al. (2000), different classification schemes put forth by Sparrow (1960), Karling (1977) and Barr (1980) separated the genera into different families and subfamilies. Sparrow's monograph (1960) included all of the genera except for *Allochytridium* (Salkin 1970) and separated them into two series based on difference in method of discharge. *Cladochytrium* as the only inoperculate genus was placed into the Series Inoperculatae while the rest of the genera were put in the Series Operculatae and separated into different families and subfamilies based on difference in number of sporangia per thallus (monocentric vs. polycentric), position of the sporangium relative to the surface of the substrate/host (endobiotic vs. epibiotic) and certain morphological features (i.e. presence/absence of an apophysis). Karling (1977) organized genera into families and subfamilies based on the same morphological and developmental characters as used by Sparrow but without the emphasis on operculation. He placed *Nowakowskiella*, *Cladochytrium* and *Septochytrium* into the same family (Cladochytriaceae) but classified *Allochytridium* and *Catenochytridium* into a separate family (Rhizideaceae) and further subdivided the two genera into different subfamilies. *Endochytrium* and *Nephrochytrium* were also placed into different subfamilies within yet another family (Entophlyctaceae). *Allochytridium* though not included in Sparrow (1960) because it was described after 1960 was allied by Barr (1986, 1987) with the genera *Nowakowskiella* and *Cladochytrium* based on zoospore ultrastructure (Lucarotti 1981). Barr also described a species of *Catenochytridum, C. hemicysti*, with the same type of zoospore as *Nowakowskiella* and *Cladochytrium* though he only noted the similarities and never formally grouped *Catenochytridium* or *Allochytridium* with *Nowakowskiella* and *Cladochytrium*. Barr (1980) did formally group *Cladochytrium* and *Nowakowskiella* together because of similarities in zoospore ultrastructure but separated *Endochytrium* into a different family based on two species

descriptions as no ultrastructural information was available. Reinforcement of taxonomic decisions based on the zoospore ultrastructure data provided by 18S and 28S nrDNA analyses (James et al. 2000, Letcher et al. 2004, Chambers 2003) gives a more stable basis for classification and allows for the creation of a framework on which to map morphological and developmental characters. Such a framework will also prove useful in defining monophyletic clades that can be recognized as formal taxonomic units and aid in revising the current artificial classification system now in use.

The goal of this study was to determine relationships between genera in the "Nowakowskiella" clade by analyzing a combined dataset of 18S and 28S nrDNA sequences using the Bayesian Metropolis-coupled Markov Chain Monte Carlo (B-MCMCMC) method of phylogenetic inference (Huelsenbeck et al. 2001) as implemented in the program MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001). As with maximum likelihood, Bayesian inference of phylogeny uses a model of sequence evolution but is a more computationally efficient method especially when using a complex model for estimating phylogenies with the added bonus of posterior probabilities. Posterior probabilities derived from B-MCMCMC analyses (BPP) provide a measure of clade support as they represent the actual number of times in the trees sampled that a clade appeared and are generated along with the trees unlike bootstrap proportions (BP) which require a separate run that takes considerably more time than required to generate trees (Hall 2001). Though BPP have been shown to overestimate support under certain circumstances (Douady et al. 2003), Bayesian analyses of sequences have been shown to recover a greater number of correct internodes with a high level of support compared to maximum likelihood and parsimony (Alfaro et al. 2003).

Materials and Methods

Cultures

Thirty-nine taxa were used in this study (See Table 3-1). Most of the cultures were kindly provided and identified (designated JEL in Table 3-1) by Dr. Joyce Longcore, University of Maine with a few isolated by the author (designated SEM in Table 3-1) and others (Jonathan Hulvey (JH) and the Berkeley Microgarden (BK)). Genomic DNA was isolated from chytrid thalli grown in liquid media and mycelium growing in agar. Three methods were used for isolating DNA: Method 1: Standard CTAB extraction (Zolan and Pukkila 1986); Method 2: 4µl of a cell lysate was pipetted directly into a 0.5ml tube containing a PCR reaction mix (See below). The lysate was produced by placing a $1cm^3$ square from cultures growing in agar or a small amount of thalli growing on the surface into 100µl of sterile ddH_20 and grinding with a Teflon dremel tool at speed one for 30sec or grinding using a sterile Kontes pestle for 30sec; Method 3: DNA extracted using Method 2 then blotting 4µl of a cell lysate onto a 1/8 in diameter circle of Isocode paper (previously sold by Schleicher-Schuell Inc. but discontinued and is comparable to FTA Cards sold by Whatman Inc.), punched out with a sterilized 1/8in metal hole punch, then dried at 50^0C for 20min. Once dried the Isocode paper was put into a 1.5ml microcentrifuge tube containing 500µl of sterile ddH_20 and vortexed three times for a total of 5sec for each sample. The paper was then blotted on the side of the tube to remove excess water and placed directly into a 0.5ml tube with a PCR reaction mix (See below).

PCR and Sequencing Techniques

PCR reaction conditions were as follows: (1) warm start at 94^0C for 3 min; (2) 35 amplification cycles of 94^0C for 1 min s, 50^0C for 30 s, 72^0C for 1 min; (3) an indefinite hold at 4^0C. PCR reaction mix for chemically extracted DNA was based on a recipe provided by the Vilgalys lab

(Duke University): 5.875µl sterile ddH_20, 2.5µl 10X Roche PCR Buffer, 4µl 1.25mM dNTPs, 1.25µl of 10µM Primers, and Roche Taq (5U/µl) 0.125µl (RedHot Taq from ABGene was used in the Vilgalys lab). PCR reaction mix for the Isocode samples (Method 2) and direct extract samples (Method 3) were as follows: 29.5 sterile ddH_20 (for Method 2) and 25.5 sterile ddH_20 (for Method 3), 5µl 10 mg/ml BSA, 5µl 10X Roche PCR Buffer, 5µl 1.25mM dNTPs, 2.5µl of 10µM Primers, and Roche Taq (5U/µl) 0.5µl. PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics Corporation, Indianapolis, IN). NS1/NS4 were used to amplify the first 1200 bp of the 18S nrDNA gene. NS1, NS2, SR1.5, and NS4 were used for sequencing (NS primers came from White et al. and SR1.5 came from James et al. 2000). For the 28S gene, 5.8S and LR7 were tried initially but did not work with all templates so LROR and LR5 were then used for PCR. The first 600-700 bp of the 28S nrDNA gene were obtained using LROR and LR5. LROR and LR5 were also used for cycle sequencing (http://www.biology.duke.edu/fungi/mycolab/primers.htm). Cycle sequencing was carried out using both Big Dye v2.0 and v3.0 on a GeneAmp Thermocycler using the programs specified in the BigDye manual for each version. Cycle sequencing products were cleaned using the Ethanol/Sodium Acetate protocol in the ABI Big Dye v3.0 manual. Sequencing was performed on ABI 310 capillary sequencer at the Plant Biology Genetic Analysis Facility at the University of Georgia, Athens, GA and on an ABI 96-capillary machine at Duke University, Durham, NC. Contigs were assembled and edited with Sequencher™, version 4.1.2 (Gene Codes Corporation, Inc. 2000). Text files of sequences from Sequencher were created and initially aligned using Clustal X then imported into SeqAl v. 2.0a11 Carbon (Rambaut 1996) and re-aligned and edited by eye.

Data analysis

Bayesian MCMCMC analysis was performed with MrBayes v3.0b4 (Hulsenbeck and Ronquist 2001) on a Macintosh G4 400MgHz Powerbook. Outgroup sequences (JEL136, JEL93, JEL190) were provided by Dr. James Chambers, University of Alabama. After determining the number of generations required to reach stationarity, a run with 500,000 generations starting with a random tree was executed. PAUP* 4.0b10 PPC (Swofford 2002) was used to generate the majority rule tree from 5000 trees in the MrBayes tree file minus the first 500 trees which were discarded as burn-in before stationarity was reached. The analysis was performed assuming the general time reversible model (Rodriguez et al. 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate catagories (GTR + I + G). Modeltest 3.06 (Posada and Crandall 1998) was used to select the GTR model. No molecular clock was assumed. Trees were prepared using TreeView (Page 1996). Parsimony and maximum likelihood analyses were also run for comparison on the two gene dataset as well as the single gene datasets (18S or 28S alone) and to see if a difference in optimality criterion would affect the grouping of sequences. Parsimony analyses was performed with TBR branch swapping over one-hundred heuristic searches with 1000 random taxon sequence addition replicates. Maximmum likelihood also utilized the GTR model of sequence evolution. Both parsimony and likelihood analyses were carried out using PAUP* 4.0b10 PPC (Swofford 2002) on a Macintosh G4 400MgHz Powerbook.

Results

Both the single gene and two gene datasets from all three optimality criteria (Bayesian, Parsimony, and Maximmum Likelihood) showed that *Cladochytrium*, *Nowakowskiella*, and *Endochytrium* are monophyletic (Figs. 3.1 thru 3.9). On the Bayesian trees *Cladochytrium*,

Nowakowskiella, and *Endochytrium* received a 100% BPP (shown only on the combined two gene dataset Fig. 3.1). The branching order of each clade differed some depending on which optimality criterion was used. The *Cladochytrium* clade branched off before any other clade in all of the bayesian and parsimony runs but not with maximum likelihood where it came out between the two *Catenochytridium* clades, after *Chytridium lagenarium* or after the *Catenochytridium-Nephrochytrium-Endochytrium* clades. Isolates identified as being species of *Catenochytridium* based on morphology in pure culture were divided up into two separate but closely related clades, *Catenochytridium* Clades I and II (Figs. 3.1, 3.2, 3.6, 3.7, 3.9). *Catenochytridium* Clade I contained sequences for *Septochytrium* and *Allochytridium*. The two sequences fell out together in all of the analyses (Figs. 3.1 thru 3.9) with high BPP support in the bayesian combined dataset tree (Fig. 3.1). Isolate JEL 24 flipped back and forth between Clade I and branching off before the *Nephrochytrium-Endochytrium* clade in the 28S parsimony tree or the Cladochytrium clade in the 18S maximum likelihood tree. *Catenochytridium* Clade II (Figs. 3.1, 3.2, 3.6, 3.7, 3.9) grouped with the *Nephrochytrium-Endochytrium* clade with high BPP support in the bayesian combined dataset tree (Fig. 3.1) but in the 28S bayesian, parsimony and maximum likelihood trees isolate JEL 44 branched off before the *Nephrochytrium-Endochytrium* clade. The *Nephrochytrium-Endochytrium* clade is present in all of the trees but its position differs depending on optimality criterion. In the 28S maximum likelihood and parsimony trees (Figs. 3-5 and 3-8), the *Nephrochytrium-Endochytrium* clade splits *Catenochytridium* Clade II but in all other trees it falls out after *Catenochytridium* Clade II. The two *Nephrochytrium* species appear at two widely separate positions on all of the trees with high support at each position (98% BPP and 100% BPP respectively) in the bayesian combined dataset tree. Isolates were placed into *Nowakowskiella* if they produced non-septate swellings as zoosporangial

production could not be induced in pure culture. Production of septate swellings was used to put isolates into *Cladochytrium* along with inoperculate zoosporangia as many of these isolates did make zoosporangia in pure culture. Placement of isolates in *Nephrochytrium* was based on the presence of an apophysis beneath the zoosporangium as opposed to the lack of an apophysis which was used to place isolates into *Endochytrium*. If an isolate produced multiple highly enlarged swellings (considered to be a compound apophysis) in the initial rhizoidial axes arising from a monocentric (single) zoosporangium then they were labeled as isolates of *Catenochytridium*. The one isolate of *Allochytridium expandens* came from ATCC. The culture was deposited by Dr. Don Barr and used for examination of the zoospore ultrastructure. Dr. Barr had compared it to Salkin's original isolate and found it to be the same. The author observed its development in pure culture and found that it matched Salkin's description of *A. expandens*. *Septochytrium variabile* was isolated and identified by Dr. Joyce Longcore based on growth in pure culture. The mature thallus with multiple zoosporangia matched the original description (Berdan 1939).

Discussion

Combining portions (see Figs. 3.1, 3.6, and 3.9 for combined dataset trees) of the 18S and 28S ribosomal sequences gives greater resolution than analysis of either gene alone (see Figs. 3.2, 3.3, 3.4, 3.5, 3.7 and 3.8 for individual 18S and 28S nrDNA trees). *Catenochytridium* represents two closely related but separate clades with one clade containing species from *Septochytrium* and *Allochytridium*. The grouping of *Septochytrium* with *Catenochytridium* is somewhat surprising given its polycentric nature which would have suggested an alliance with either *Nowakowskiella* or *Cladochytrium* and more likely with *Nowakowskiella* due the presence of non-septate swellings in the rhizomycelium of several *Septochytrium* species. JEL192 *Allochytridium*

expandens Salkin in pure culture looks like a smaller more angular version of *Catenochytridium carolinianum* Berdan (type species of *Catenochytridium*) and produces similar catenulate swellings (Fig. 3.12) in the rhizoids extending from the zoosporangium so its grouping with *Catenochytridium* is not surprising and does not support generic separation of the two species currently placed in the genus. A highly supported *Nowakowskiella* consists of several closely related groups of isolates most of which were only identified to genus and not to species because they do not produce any distinguishing characters in pure culture such as resting sporangia or zoosporangia. All of the isolates placed in *Nowakowskiella* produce non-septate swellings (Fig. 3.11) suggesting that this character could be used as a basis for inclusion in the genus and for separation from *Cladochytrium* over the type of operculation. *Cladochytrium* is the one genus where all the isolates fall into one highly supported clade and all produce the characteristic septate swellings found in *Cladochytrium replicatum* (Fig. 3.10) supporting the use of septate swellings to delimit the genus from *Nowakowskiella*. More species of *Endochytrium* (Fig. 3.14) need to be brought into pure culture for sequencing since most of the isolates appear to be identical and are probably the same species. Though at present *Endochytrium* represents a well-supported clade of non-apophysate, monocentric species with the inclusion of one species of *Nephrochytrium*. The two widely separated species of *Nephrochytrium* suggest that this particular genus is polyphyletic and that possession of an apophysis (Fig. 3.13) does not warrant generic distinction. *Septochytrium* groups in *Catenochytridium* Clade I with *Allochytridium* and several isolates of *Catenochytridium*. Producing polycentric and monocentric thalli is not a unique character among members of the Chytridiomycota as it is seen in *Physoderma* (Sparrow 1960) but the thalli of *Septochytrium* differed enough to warrant creation of a new genus at the time it was described (Berdan 1939). Since there is only one isolate in the tree a formal

taxonomic decision cannot be made at this time but the bayesian analysis does suggest that *Septochytrium* is not a valid genus and that possession of two different thallus types is probably not a valid character at the generic level.

Table 3.1. Cultures Used For Sequencing. The Strain IDs are as follows: JEL refers to cultures isolated and provided by Dr. Joyce E. Longcore. SEM refers to cultures isolated by the author. JH refer to cultures isolated by Jonathan Hulvey. All sequences will be deposited into Genbank and given an accession number.

Species	Strain ID	Accession Number
Allochytridium expandens	JEL 192	XXXXXX
Catenochytridium sp.	JEL 24	XXXXXX
Catenochytridium sp.	JEL 145	XXXXXX
Catenochytridium sp.	JEL 44	XXXXXX
Catenochytridium sp.	JEL 331	XXXXXX
Cladochytrium sp.	JEL 304	XXXXXX
Cladochytrium sp.	JEL MBNA	XXXXXX
Cladochytrium sp.	JEL 153	XXXXXX
Cladochytrium sp.	SEM 13	XXXXXX
Cladochytrium replicatum	JEL 303	XXXXXX
Cladochytrium replicatum	JEL 325	XXXXXX
Endochytrium sp.	JEL 27	XXXXXX
Endochytrium sp.	JEL 70	XXXXXX
Endochytrium sp.	JEL 72Orange	XXXXXX
Endochytrium sp.	JEL 75	XXXXXX
Endochytrium sp.	JEL 324	XXXXXX
Endochytrium sp.	JEL 295	XXXXXX
Endochytrium sp.	JEL 296	XXXXXX
Karlingiomyces sp.	JEL 93	XXXXXX
Nephrochytrium aurantium	JEL 36	XXXXXX
Nephrochytrium sp.	JEL 327	XXXXXX
Nowakowskiella elegans	BK 50-1	XXXXXX
Nowakowskiella hemisphaerospora	BK 85-6	XXXXXX
Nowakowskiella sp.	JEL 46	XXXXXX
Nowakowskiella sp.	JEL 78	XXXXXX
Nowakowskiella sp.	JEL 127	XXXXXX
Nowakowskiella sp.	JEL 133	XXXXXX
Nowakowskiella sp.	JEL 156	XXXXXX
Nowakowskiella sp.	JEL 157	XXXXXX
Nowakowskiella sp.	JH CC	XXXXXX
Nowakowskiella sp.	JH CC2	XXXXXX
Nowakowskiella estuarensis Hulvey	JH Saltgrass	XXXXXX
Nowakowskiella sp.	JH Fresh	XXXXXX
Polychytrium aggregatum	JEL 190	XXXXXX
Rhizophydium brooksianum	JEL 136	XXXXXX
Septochytrium variabile	JEL 177	XXXXXX
Chytridium lagenarium	JEL 72White	XXXXXX
Unknown N-Clade Member	JEL 296	XXXXXX

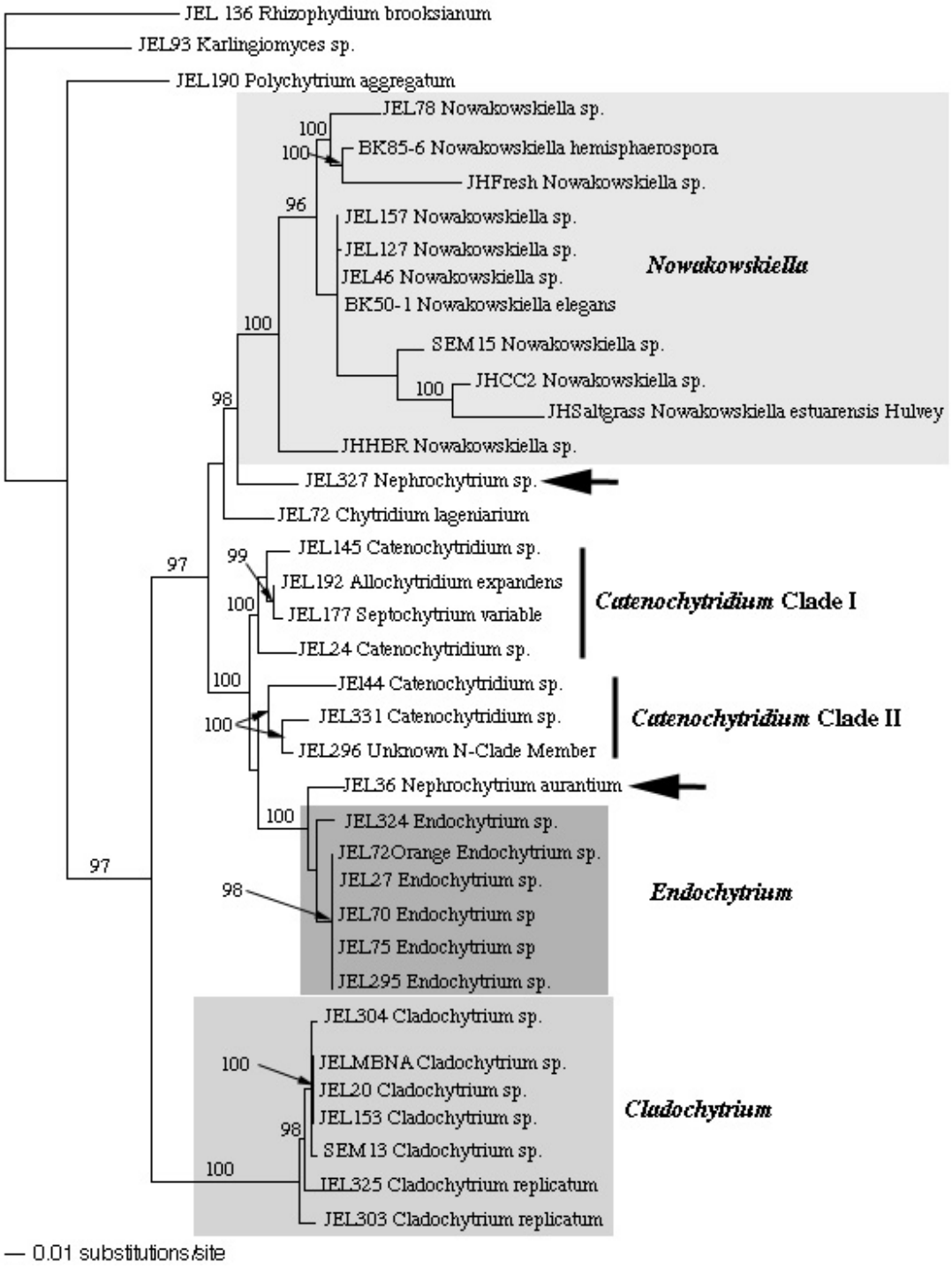


Fig. 3.1. Bayesian Tree based on 18S and 28S nrDNA.

Fig. 3.1. Bayesian Tree based on 18S and 28S nrDNA. For this tree and each tree following, large arrows point out different positions of the two isolates of *Nephrochytrium* and the monophyletic genera of *Cladochytrium*, *Endochytrium*, and *Nowakowskiella* are shaded in grey. The two Catenochytridium clades are highlighted by vertical lines in each tree to show the difference in position. The combined 18S and 28S Bayesian tree is 50% majority rule tree created in PAUP*4.0 based on 4510 trees from a 500,000 generation run.

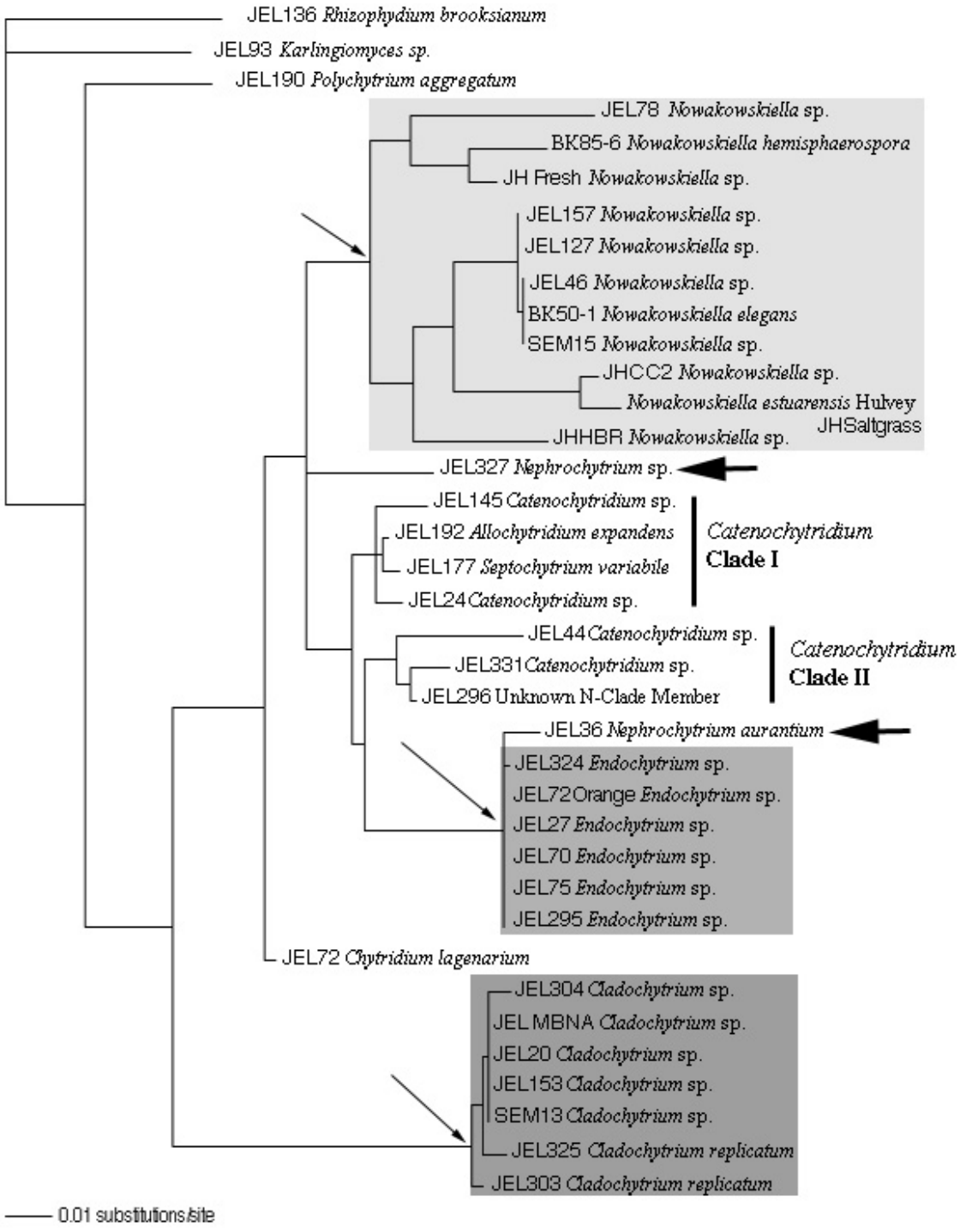


Fig. 3.2. Bayesian tree based on 18S nrDNA.

Fig. 3.2. Bayesian tree based on 18S nrDNA. The 18S Bayesian tree is 50% majority rule tree created in PAUP*4.0 based on 4510 trees from a 500,000 generation run.

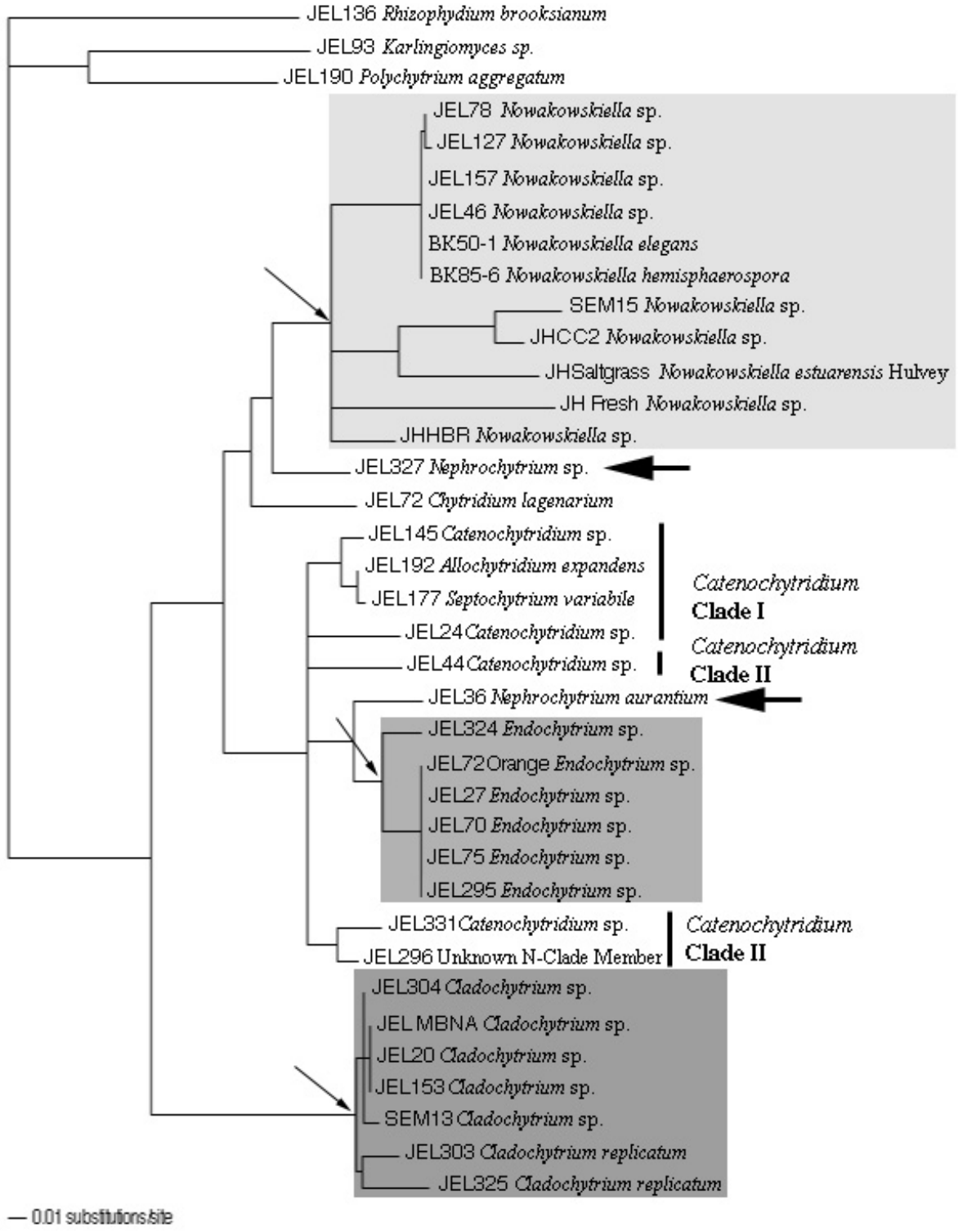


Fig. 3.3. Bayesian tree based on 28S nrDNA.

Fig. 3.3. Bayesian tree based on 28S nrDNA. The 28S Bayesian tree is 50% majority rule tree created in PAUP*4.0 based on 4510 trees from a 500,000 generation run.

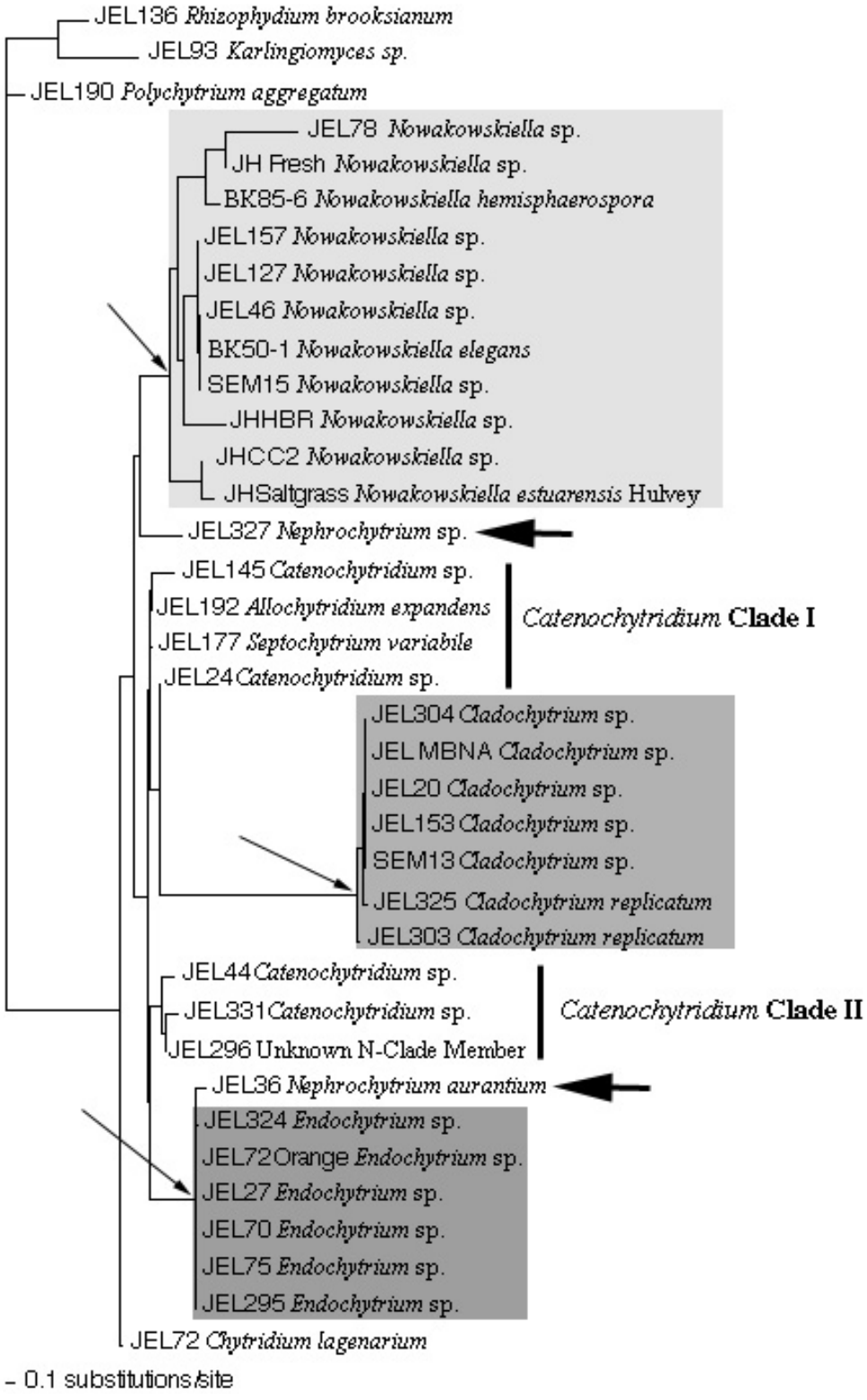


Fig. 3.4. Maximum likelihood tree based on 18S nrDNA.

Fig. 3.4. Maximum likelihood tree based on 18S nrDNA. The 18S Maximum likelihood tree was generated in PAUP*4.0 based on an initial set of trees created using parsimony and neighbor joining. This tree had a likelihood of -1480.90208.

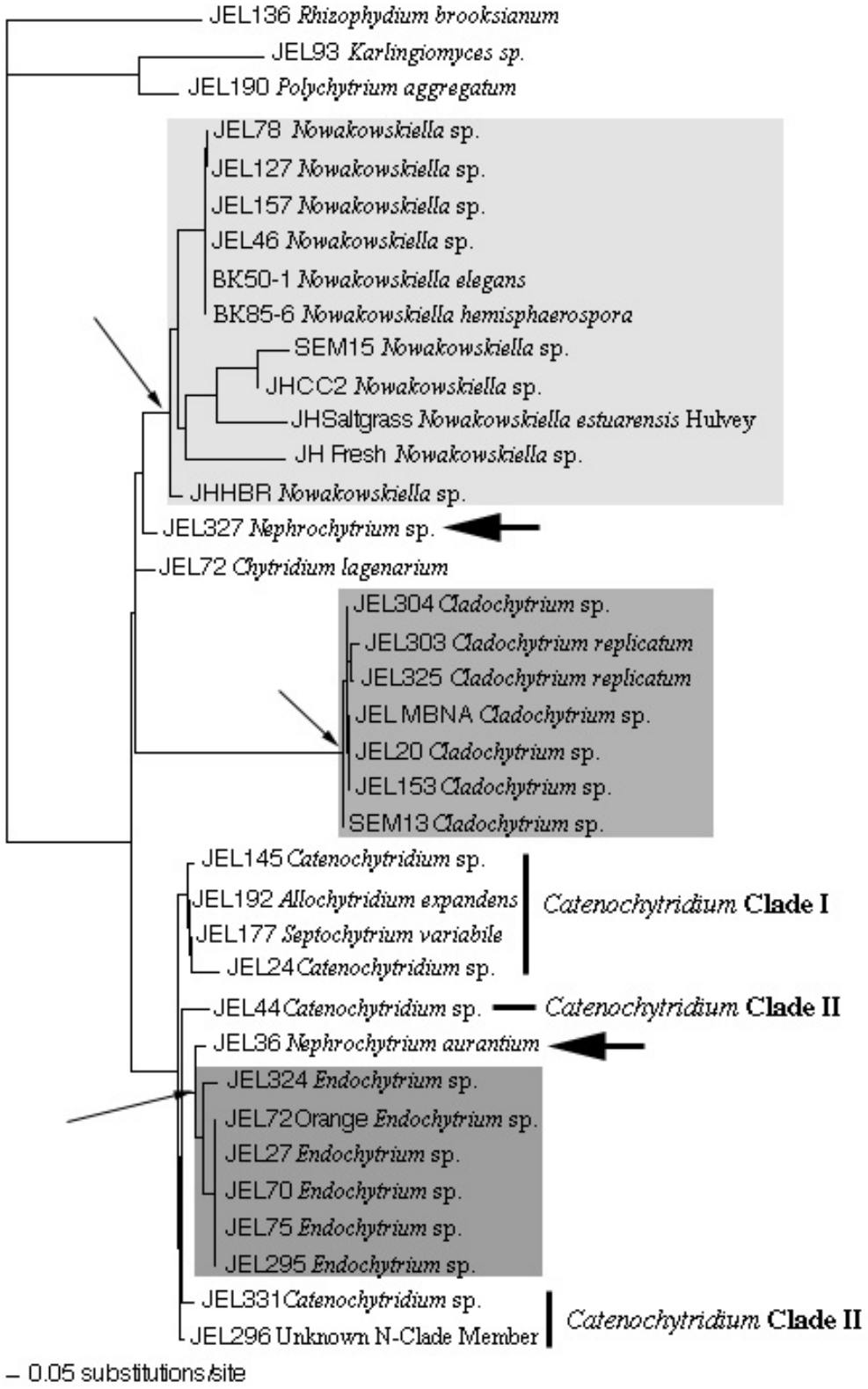


Fig. 3.5. Maximum likelihood tree based on 28S nrDNA.

Fig. 3.5. Maximum likelihood tree based on 28S nrDNA. The 28S Maximum likelihood tree was generated in PAUP*4.0 based on an initial set of trees created using parsimony and neighbor joining. This tree had a likelihood of -2003.79569.

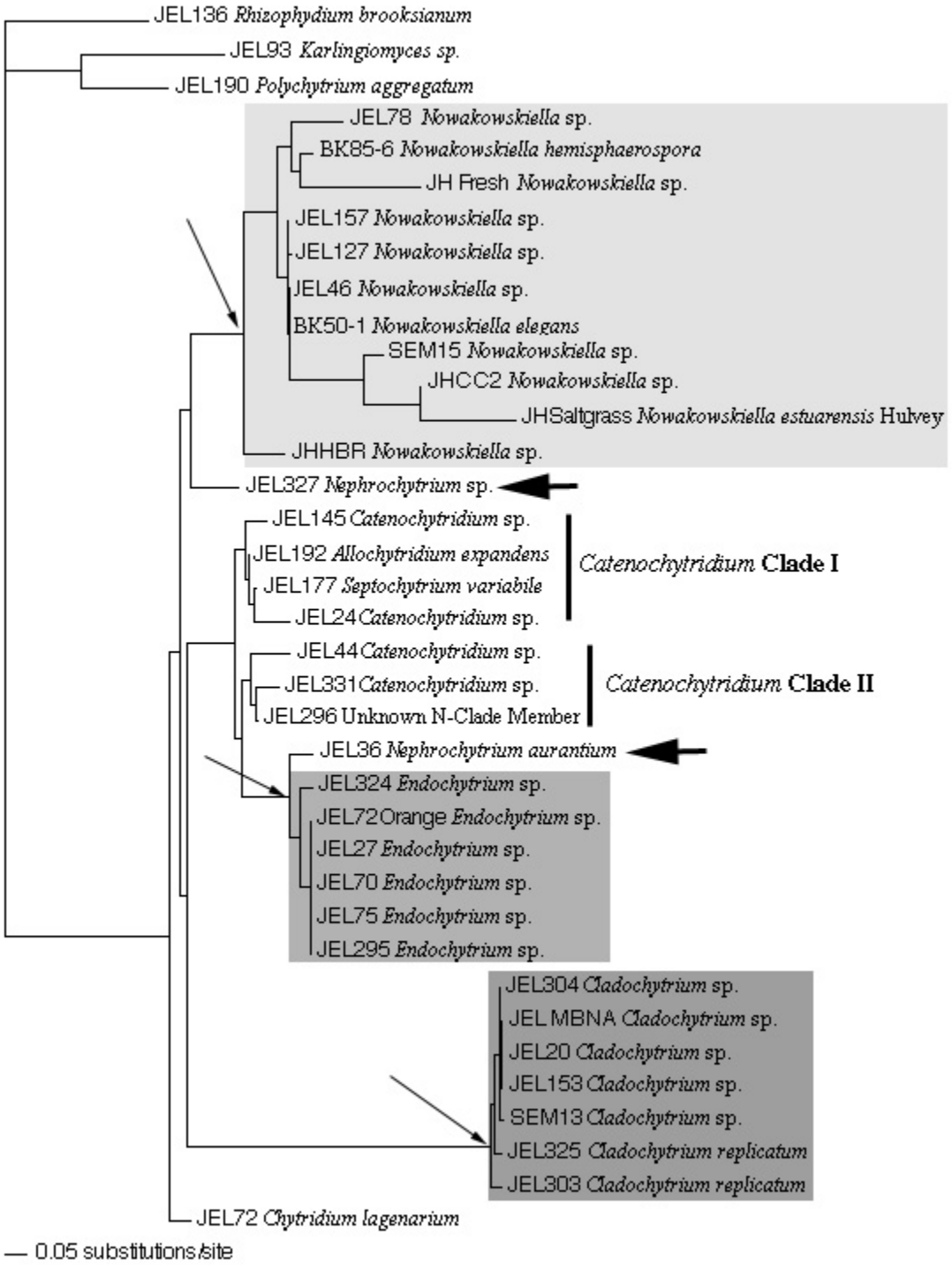


Fig. 3.6. Maximum likelihood tree based on 18S and 28S nrDNA.

Fig. 3.6. Maximum likelihood tree based on a combined dataset of portions of the 18S and 28S nrDNA. The combined maximum likelihood tree was generated in PAUP*4.0 based on an initial set of trees created using parsimony and neighbor joining. This tree had a likelihood of -3485.78730.

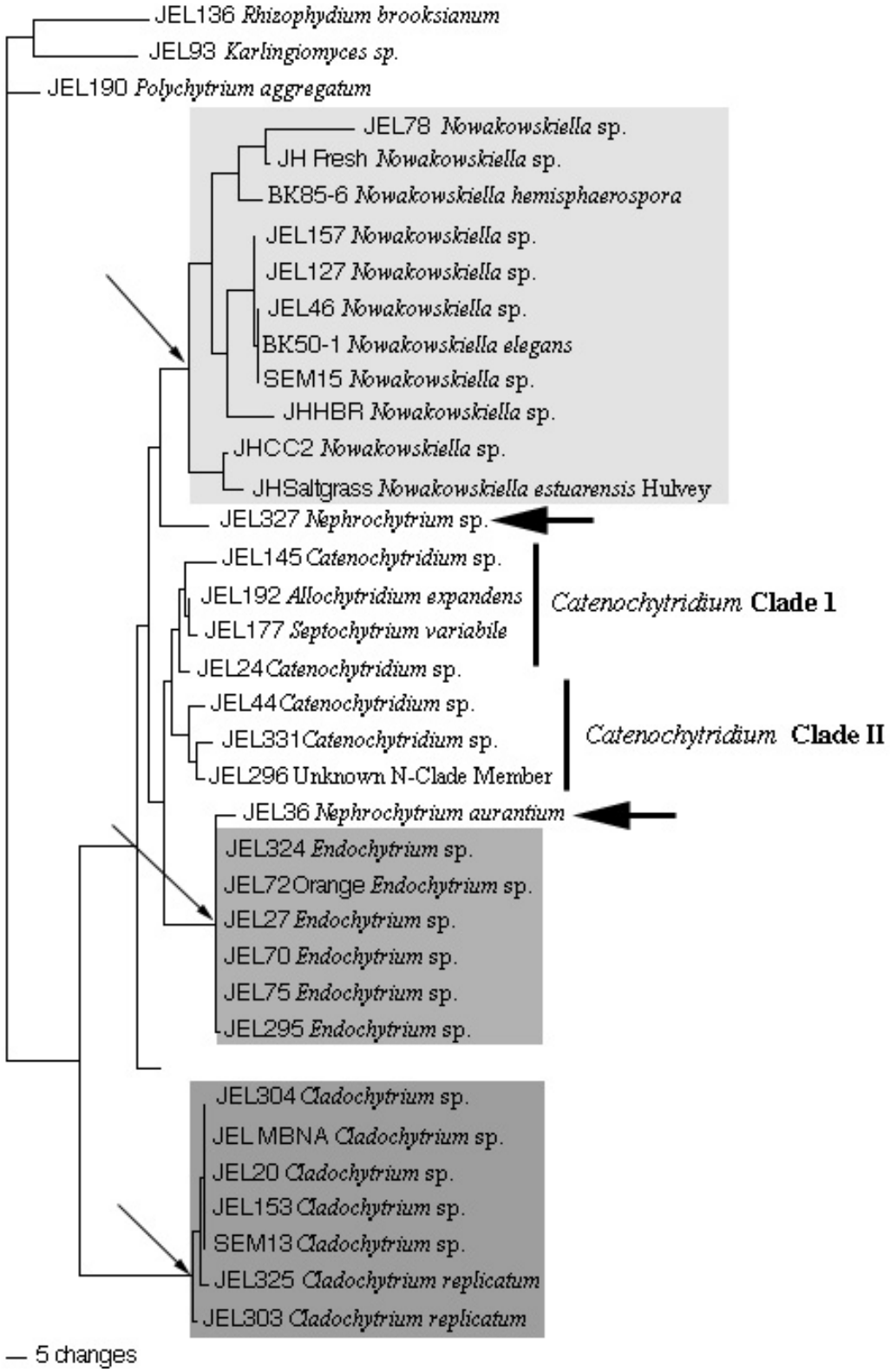


Fig. 3.7. Parsimony tree based on 18S nrDNA.

Fig. 3.7. Parsimony tree based on 18S nrDNA. The 18S parsimony tree was created in PAUP*4.0. This tree is one of thirteen most parsimonious trees generated after running 1000 random sequence additions. Length=307, CI=0.502, RI=0.808, HI=0.498.

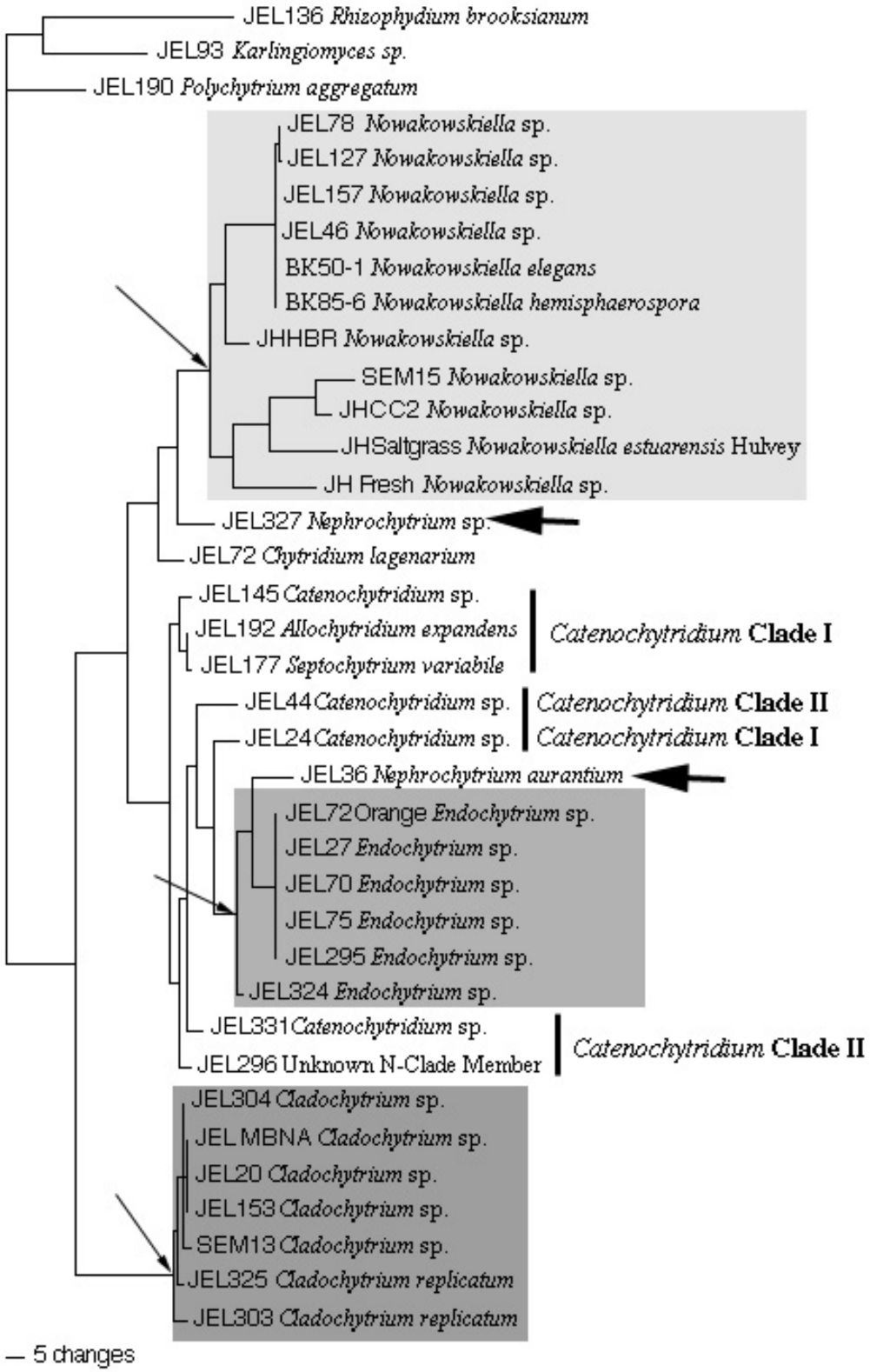


Fig. 3.8. Parsimony tree based on 28S nrDNA.

Fig. 3.8. Parsimony tree based on 28S nrDNA. The 28S parsimony tree was created in PAUP*4.0. This tree is one of four most parsimonious trees generated after running 1000 random sequence additions. Length=446, CI=0.511, RI=0.797, HI=0.489.

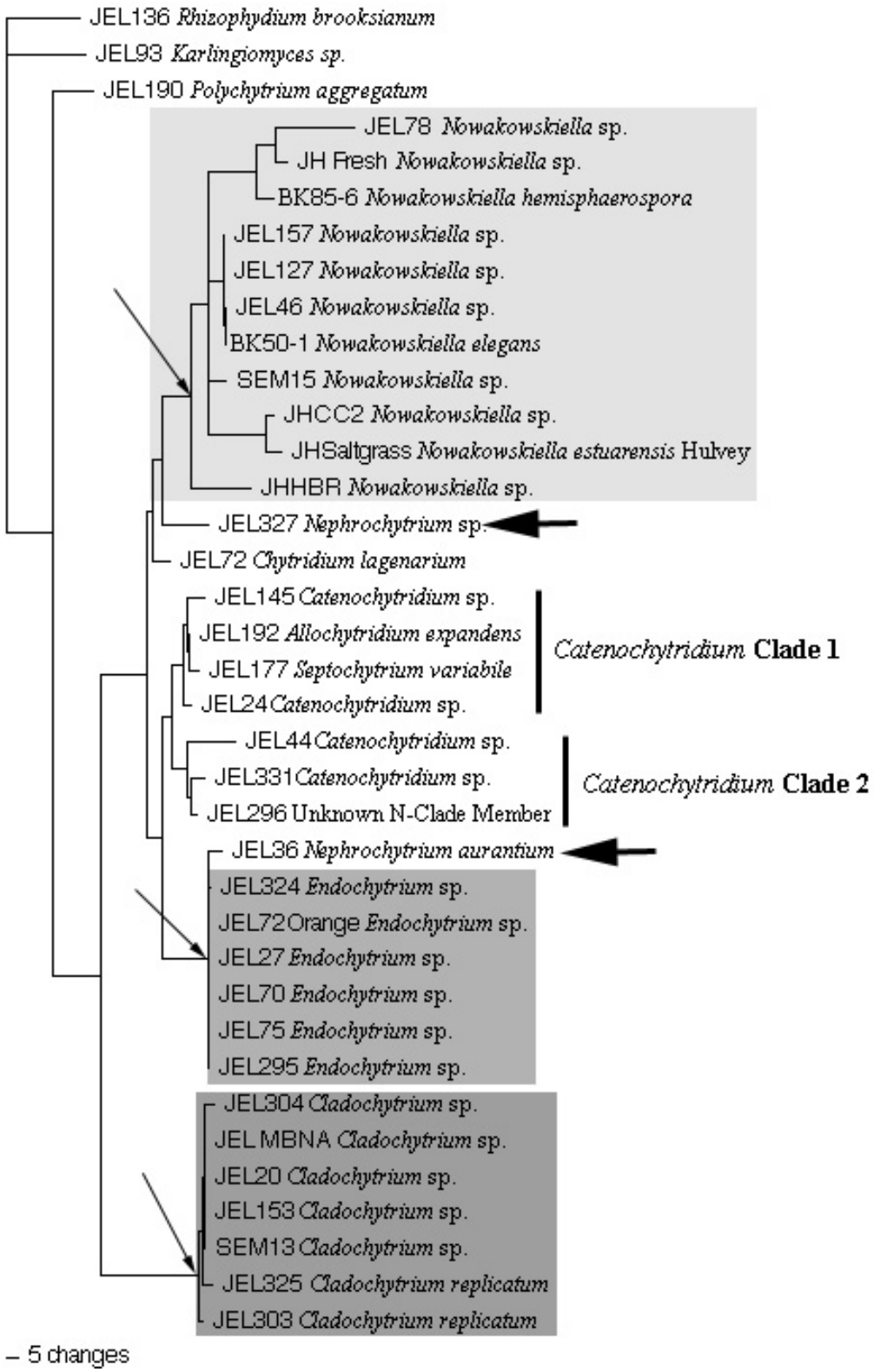
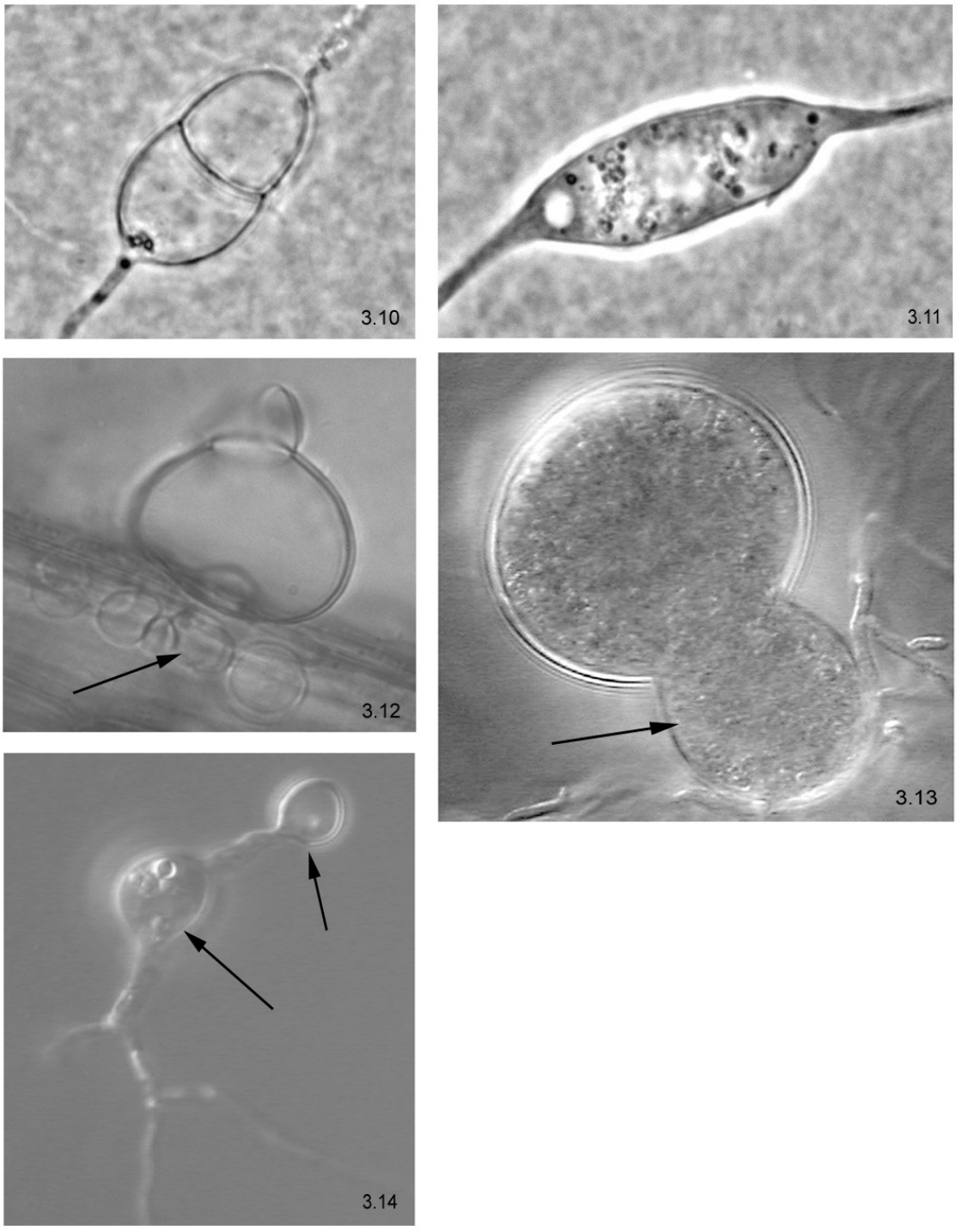


Fig. 3.9. Parsimony tree based on 18S and 28S nrDNA.

Fig. 3.9. Parsimony tree based on a combined dataset of 18S and 28S nrDNA. The combined parsimony tree was created in PAUP*4.0. This tree is one of two most parsimonious trees generated after running 1000 random sequence additions. Length=791, CI=0.483, RI=0.781, HI=0.517.



Figures 3.10 – 3.14. Images of morphological characters used to taxonomically separate genera in the "*Nowakowskiella*" Clade.

Figures 3.10 – 3.14. Images of morphological characters used to taxonomically separate genera in the "*Nowakowskiella*" Clade. Fig. 3.10 Septate swelling of a species of *Cladochytrium*. Fig. 3.11 Non-septate swelling of a species of *Nowakowskiella*. Fig. 3.12 Catenulate rhizoid produced by a species of *Catenochytridium*. In this case the rhizoids are inside a decaying plant acting as a substrate. Catenulations or multiple swellings in the initial rhizoids seen in *Catenochytridium* (Fig. 3.12) are also found in *Allochytridium*. Fig. 3.13 Immature zoosporangium of a species of *Nephrochytrium*. Possession of an apophysis is the morphological character used to separate *Nephrochytrium* from *Endochytrium*. Fig. 3.14 Three-day old germling of *Endochytrium*. The germling has an incipient non-apophysate sporangium (longer arrow) and a persistent zoospore cyst (shorter arrow) characteristic of the genus. Figs. 3.10-3.11, 3-13-3.14 by the author and Fig. 3.12 courtesy of Dr. Joyce E. Longcore, University of Maine.

CHAPTER 4

CRYOPRESERVATION OF CHYTRID FUNGI

Introduction

At present, a limited number of cryopreserved cultures of chytrid fungi can be found in a small number of culture collections including the Canadian Collection of Fungal Cultures (CCFC) and the American Type Culture Collection (ATCC). Dr. Joyce E. Longcore (University of Maine) curates the largest collection of non-cryopreserved chytrid fungi maintained on agar slants and in liquid broth totaling about 400 cultures with almost 1/3 representing strains of *Batrachochytrium dendrobatidis* Longcore, Pessier et Nichols. One goal of the Chytrid PEET (NSF DEB-9978094) was to establish an easily maintained cryopreserved culture collection that would provide cultures for systematic, ecological, physiological, and biotechnological studies especially for species not preserved anywhere else. Most chytrid fungi (Kingdom Fungi, Phylum Chytridiomycota) are not thought to survive dessication or extremely dry conditions very well (except see Gleason et al. 2004 for a few species shown to survive drying) and therefore all known attempts at preservation of chytrid fungi have led to cryopreservation in cryoprotectant liquids (Boyle et al. 2003, Barr and Babcock 1994, Hohl and Iselin 1987, Sakurada et al. 1995, Smith and Thomas 1998, Smith 1982, Smith and Onions 1983). Cryopreservation allows for the maintenance of a much larger number of cultures with fewer personnel. Problems often encountered with maintaining live cultures on agar media or in broth that are not encountered with cryopreservation include a lack of long-term culture viability and genetic instability. Another advantage of cryopreservation is that the culture stock can be replenished by re-freezing

a thawed culture without any appreciable loss of pathogenicity or sporulation ability. As such, cryopreserved cultures represent the best method for long-term preservation of biological material for this particular group of fungi. Different methods and protocols have been used to cryopreserve chytrid fungi with varying results (Barr and Babcock 1994, Boyle et al. 2003, Kuznetsov 1981, Sakurada et al. 1995, Smith and Thomas 1998, Smith 1982, Smith and Onions 1983, Yarlett et al. 1986). Unfortunately despite the importance and usefulness of the publications cited above papers covering cryopreservation of fungi often do not list them among their citations or even cover the Chytridiomycota as a fungal taxonomic group (Nakasone et al. 2004, Tan 1997, Tan and van Ingen 2004). A search of several literature databases (Web of Science, Current Contents, BIOSIS) found that most cryopreservation studies focus on the Ascomycota and Basidiomycota followed by studies of the Zygomycota and Oomycota (Kingdom Chromista). The goal of this paper then is two-fold: 1) Present a method for cryopreservation of chytrid fungi utilized for Chytrid PEET systematic studies and 2) Provide a much needed review of cryopreservation studies on chytrid fungi with pertinent citations.

Materials and Methods

A test of two cryopreservation methods was conducted to see which would work better for members of the "*Nowakowskiella*" clade before committing to one method for all of the cultures in the UGA collection. A common skim milk plus glycerol method was used first (Table 4-1). The cryoprotectant was composed of 10% glycerol and 8.5% skim milk. 1.5ml of cryoprotectant was pipetted into a 1.8ml Sarstedt external threaded cryotube and a small cube of agar with inoculum (culture growing in or on the agar) was added. The cryotubes were placed in a Nalgene Cryo -1^0C/minute Freezing Container (Cat. No. 5100-0001, Nalgene Nunc Int.). The cryocontainer was then put into a -80^0C freezer for three days. After three days, the cryotubes

were moved into a liquid nitrogen (LN_2) dewar for long-term storage. Thawing cultures required the use of a water bath at 43^0C. The cryotubes were quickly removed from the LN_2 dewar and carefully lowered into the water bath (to prevent injury from exploding tubes due to LN_2 seepage into the tubes) and removed once the contents were visibly thawed. The contents of the cryotubes were dumped out onto an agar plate and the agar block was then moved to a separate plate. Both plates were flooded with 1ml of ddH_2O or Dilute Salts (Machlis 1953).

For the Barr and Babcock method (Tables 4-2 and 4-3), 10 Q-tip heads were placed into a 250ml Erlenmeyer Flask with 100ml of YPD broth (Barr and Babcock 1994) then autoclaved and allowed to cool down before inoculation. Barr and Babcock suggested using 10 to 20 Q-tips but a preliminary growth test determined that growth was inhibited if more than 10 tips were used. The cotton tips were cut from the paper or plastic stems before use. Cultures were incubated at room temperature for 3-5 days. For cryopreservation, 1ml of 10% glycerol was used as the cryoprotectant. One Q-tip head was placed into each 1.8ml Sarstedt cryotube and the cryotubes were placed into the Nalgene cryocontainer that was then put into a -80^0C mechanical freezer for three days. After three days the tubes were moved to an aluminum cane and placed into a LN_2 dewer. Cultures were thawed in a 35^0C water bath and the Q-tip was placed into 100ml of YPD. Barr and Babcock stored their cultures in the vapor phase but in this study the cultures were stored in canisters immersed in LN_2.

The chosen method for cryopreservation of "*Nowakowskiella*" clade cultures (see Table 4-4 for a complete list) involved using a slightly modified version of Barr and Babcock (1994). Ten Q-tips were placed in a 125ml Erlenmeyer flask filled with 75ml of liquid media. Once growth was visible to the naked eye on the Q-tips, six tips were chosen and each tip was placed in a separate 1.8ml cryotube to which 1ml of 10% glycerol was added. The tubes were placed in

the Nalgene cryocontainer that was then put into an -80^{0}C freezer for three days as stated above. After three days, the tubes were transferred to an aluminum cane and put into a LN_2 dewer at -196^{0}C for long-term storage. For each culture a single cane was labeled and loaded with six cryotubes. Thawing involved the use of a 35^{0}C water bath. Tubes were immersed to the edge of the cap in the water bath until the contents were thawed enough to pour out into a 125ml Erlenmeyer flask containing liquid media (about 30sec to 1min). Growth often took up to four weeks after being thawed but varied between species with some only taking two weeks before growth was visible. Once growth appeared the cultures were transferred to agar plates with antibiotics. Antibiotics (Penicillin and Streptomycin at a concentration of 0.25g/L) were added to the agar media to prevent growth of potential bacterial contaminants. Cultures were then transferred to non-antibiotic agar media after a certain amount of growth was visible on the antibiotic media. For cultures that did not grow on the Q-tips a 1cm^3 square of agar was frozen.

Results

The skim milk/glycerol cryopreservation method (Table 4-1) worked well for the monocentric cultures *Chytriomyces hyalinus* (G75-1), *Nephrochytrium aurantium* Whiffen (JEL36), and *Endochytrium* sp. (JEL75) as they were all successfully revived but SEM14 either did not survive or could not be revived by plating. *Cladochytrium replicatum* (SEM14) did survive freezing and thawing with the Barr and Babcock method but *Endochytrium* sp. (JEL75) did not grow in either of the two trials though later attempts were successful with a larger amount of inoculum and a different medium (not presented here). For the modified Barr and Babcock method used to cryopreserve members of the "*Nowakowskiella*" clade, some of the isolates grew normally on Barr's YPD but others preferred the liquid version of the agar medium on which they were maintained in live culture. The various media used for live cultures included mPMTG

(Longcore 1992), PMTG (Barr 1986), and DSS (Recipe from Dr. J. E. Longcore). Most of the monocentric species were easy to freeze as they grew well on the Q-tips and were easily revived after being frozen. The polycentric species proved more difficult as some did not grow in liquid media and did not grow on the Q-tip. In such cases, small squares of agar containing mycelia and/or sporangia were frozen. Test vials were thawed and while the more recalcitrant polycentric species did survive the number of vials recovered were fewer than for those species that were frozen on a Q-tip. Light microscopic examination of thawed cultures did not reveal any changes in morphology. At the time of freezing, the Q-tip cultures contained a mix of mature and immature thalli (not shown) which most likely contributed to growth after thawing by producing viable zoospores. Rhizomycelium in agar blocks were able to restart tip growth after thawing as rhizomycelial strands were seen extending out into the uncolonized portions of the agar block and in most cases while growing in liquid media after being thawed the strands completely covered the block. The blocks were then placed onto solid agar plates and the rhizomycelium extended out in all directions into the agar media.

Discussion

Chytrids in the University of Georgia Chytrid Culture Collection represent primarily a single clade, the "*Nowakowskiella"* clade (James et al. 2000). Members of the clade exhibit a range of morphological types (polycentric, monocentric) and growth habits (soil, water, decaying vegetation) and in culture some readily colonize agar and liquid media while others are much more restricted in their growth. The Q-tip provides a large amount of surface area for colonization and the more thalli or rhizomycelium present the greater the likelihood that some will survive the freezing/thawing process. As stated in Barr and Babcock (1994) the Q-tip is easier to work with than small agar blocks and can be placed in liquid media that disperses the

cryoprotectant. Though the cryoprotectant (in this case 10% glycerol) allows the culture to survive freezing, once thawed it can become toxic. Dispersal of the cryoprotectant in liquid aids in revival of the culture and is the reason for the use of liquid media instead of agar plates in the first step of the thawing process. The only difference seen in survival occurred between monocentric and polycentric species. Monocentric species were easy to freeze and thaw while the same was not true for some polycentric species. Ensuring survival for polycentric species that did not grow on a Q-tip will require freezing more tubes to increase the odds of a viable one. In comparison to previously published cryopreservation methods of chytrid fungi, the most common factors that resulted in a successful revival included using a relatively young culture (less than a week old) and a 10% solution of the chosen cryoprotectant into which small plugs of agar with rhizomycelium, pellets of sporangia, zoospore suspensions, or inoculated cotton Q-tips were placed. Glycerol, glycerol and skim milk, dimethyl sulfoxide (Me_2SO = DMSO), Me_2SO and fetal calf serum, and ethylene glycol have all been used as cryoprotectants for chytrid fungi with the Me_2SO/fetal calf serum and glycerol being the best for aerobic chytrids and Me_2SO or ethylene glycol as the best for anaerobic rumen chytrids (Barr and Babcock 1994, Boyle et al. 2003, Sakurada et al. 1995, Yarlett et al. 1986). The rate of freezing did not vary much ranging from a 1^0C/min freezing rate with the Nalgene cryocontainer in a -80^0C freezer to a 1.5^0C/min freezing rate in a programmable freezer or for the anaerobic chytrids placement directly into CO_2 for 24hrs then stored at -84^0C. For the cultures used in this study 10% glycerol worked best with cultures growing on cotton Q-tips in liquid media and blocks of rhizomycelium in agar and a freezing rate of 1^0C/min using the Nalgene cryocontainer in a -80^0C freezer. The difference in culture material, cryoprotectant, and method of freezing exhibits the need for tailoring the cryopreservation method for the culture of interest. Methods that work for one species may not

necessarily work for another and need to be experimented with to find the best parameters for successful freezing and revivial.

Table 4.1. Cultures Frozen with Skim Milk and Glycerol. After being thawed: + culture grew, - culture did not grow, na not available which means could not grow up enough to freeze, nt not thawed so tube is still frozen, nr not recorded.

	3 Day		4 Day		5 Day	
Culture	Tube 1	Tube 2	Tube 1	Tube 2	Tube1	Tube2
G75-1 - *Chytriomyces hyalinus*	+	nt	+	nt	+	nt
SEM14 - *Cladochytrium replicatum*	-	nt	-	nt	-	nt
JEL36 - *Nephrochytrium aurantium*	+	nt	+	nt	+	nt
JEL75 - *Endochytrium* sp.	na	na	na	na	+	na

Table 4.2. Cultures Frozen with Barr and Babcock Method Trial 1.

Culture	Age of culture when frozen	Tube 1	Tube 2
G75-1 - *Chytriomyces hyalinus*	10 days	+	nt
SEM14 - *Cladochytrium replicatum*	10 days	-	nt
JEL36 - *Nephrochytrium aurantium*	18 days	+	nt
JEL75 - *Endochytrium* sp.	na	na	na

Table 4.3. Cultures Frozen with Barr and Babcock Method Trial 2.

Culture	Age of culture when frozen	Tube 1	Tube 2
G75-1 - *Chytriomyces hyalinus*	6 days	+	nt
SEM14 - *Cladochytrium replicatum*	6 days	+	nt
JEL36 - *Nephrochytrium aurantium*	nr	+	nt
JEL75 - *Endochytrium* sp.	na	na	na

Table 4.4. University of Georgia Chytrid Cultures Frozen with modified Barr and Babcock Method. Species are listed in the first column followed by a culture number and whether or not it is monocentric (M) or polycentric (P).

Species	Culture Number	M/P
Chytriomyces hyalinus G75-1	G75-1	M
Nowakowskiella sp.	TX#1	P
Caternaria sp.	JEL339	P
Cladochytrium sp.	SEM11	P
Cladochytrium sp.	SEM13	P
Cladochytrium replicatum	SEM14	P
RT Cladochytrium sp.	SEM15	P
Cladochytrium sp.	PL18	P
Nowakowskiella sp.	JH_SaltgrassCA	P

Nowakowskiella sp.	JH_Fresh	P
Nowakowskiella sp.	JH_JTC	P
Nowakowskiella sp.	JH_CC	P
Nowakowskiella sp.	JH_CC2	P
Nowakowskiella sp.	JH_HBR	P
Nowakowskiella sp.	GTC	P
Cladochytrium sp.	JH_17	P
Cladochytrium sp.	JH_5	P
Cladochytrium sp.	MBNA Clado	P
Nowakowskiella sp.	10	P
Cladochytrium sp.	20	P
Nowakowskiella sp.	21	P
Nowakowskiella sp.	22	P
Catenochytridium sp.	24	M
Endochytrium sp.	27	M
Catenochytridium sp.	28	M
Nephrochytrium aurantium	36	M
Catenochytridium sp.	44	M
Nowakowskiella sp.	46	M
Endochytrium sp.	49	P
Endochytrium sp.	50	M
Endochytrium sp.	51	M
Cladochytrium sp.	58	P
Endochytrium sp.	70	M
Chytridium lagenarium	72	M
Cladochytrium sp.	74	P
Endochytrium sp.	75	M
Nowakowskiella sp.	78	P
Cladochytrium sp.	110	P
Nephrochytrium sp.	125	M
Nowakowskiella sp.	127	P
Septochytrium sp.	140	M/P
Cladochytrium sp.	144	P
Catenochytridium sp.	145	M
Cladochytrium sp.	152	P
Cladochytrium sp.	153	P
Nowakowskiella sp.	154	P
Nowakowskiella sp.	157	P
Septochytrium variabile	177	M/P
Cladochytrium replicatum	180	P
Unknown	187	M
Septochytrium variabile	191	M/P
Allochytridium expandens	192	M
Unknown	279	M

Endochytrium sp.	295	M
Unknown	296	M
Cladochytrium sp.	303	P
Cladochytrium sp.	304	P
Endochytrium sp.	324	M
Cladochytrium sp.	325	P
Nephrochytrium sp.	327	M
Diplophylctis sp.	331	M
Nowakowskiella sp.	335	P
Nowakowskiella sp.	85-5	P
Nowakowskiella hemisphaerospora	85-6	P
Nowakowskiella elegans	50-1	P

CHAPTER 5

CONCLUSIONS

Based on the molecular analysis in Chapter 3, the "*Nowakowskiella*" clade can be circumscribed using portions of the 18S and 28S nrDNA in addition to zoospore ultrastructure (Barr 1986, Barr and Désaulniers 1987a, Barr and Désaulniers 1987b, Lucarotti 1981) and the preference for cellulosic substrates. In the latest classification schemes put forth by Barr (1980), only *Cladochytrium* and *Nowakowskiella* are grouped together based in part on morphological and developmental characters. All the other genera in the "*Nowakowskiella*" clade are separated into different families and subfamilies (Karling 1977) using the same morphological and developmental characters. A further examination of cultures is needed to determine what morphological and developmental characters could be used to circumscribe the "*Nowakowskiella*" clade, distinguishing it from all other clades of chytrid fungi. Pure cultures of isolates belonging to the clade along with described species not already in pure culture will be necessary to get an idea of the full range of phenotypic expression of such characters. Characterization of the cultures used in this study will aide in the diagnosis of the "*Nowakowskiella*" clade as a possible family in the Chytridiales or in a new order if the Chytridiales is restricted only to the "*Chytridium*" clade (James et al. 2000). The clade will most likely be turned into a family but this needs greater sequence support before it can be validated.

The taxonomic summaries in Chapter 2 provide the necessary information on species described (including a listing of synonyms, questionable species, and species described since 1960 after publication of the last Chytrid monograph) for all seven genera with up-dated keys

including species described since 1960 and a listing of pertinent citations in the reference section. Valid publication of the summaries in a referred journal will allow for an up-to-date listing of all described species and a discussion of the current taxonomic status of each genus including problems relating to terminology and character overlap. In addition, the summaries represent the first step on the road to revising Sparrow (1960) which is the only comprehensive monograph of chytrid fungi available.

Bayesian analysis of a combined 18S and 28S rDNA dataset supports previous work by James et al. (2000) in that all the genera listed are related and belong in the clade. In addition, *Cladochytrium*, *Nowakowskiella*, and *Endochytrium* are monophyletic. The morphological characters such as swelling septation and non-apophysate zoosporangia can be used to identify isolates in culture and are phylogenetically useful at the genus level. *Allochytridium*, *Catenochytridium*, *Nephrochytrium*, and *Septochytrium* are not monophyletic. Isolates identified from pure culture as *Catenochytridium* fell into two clades. Clade I contained both *Septochytrium* and *Allochytridium* while Clade II only contained *Catenochytridium* isolates. The two clades are very close to each other though different analyses (Parsimony, ML, Bayesian) either make them sister to each other or groups Clade II with the larger *Nephrochytrium*-*Endochytrium* clade. The exact reasons for the splitting of the *Catenochytridium* clade are not known and will require further sequencing to work out. More isolates similar to the ones described are needed to provide support for subsuming *Septochytrium* and *Allochytridium* into *Catenochytridium* and for the polyphyletic *Nephrochytrium*.

A short review of cryopreservation protocols used on chytrid fungi showed that chytrids can be cryopreserved a number of different ways with varying levels of success. This is the first time a comprehensive review of chytrid cryopreservation methods has been presented.

Cryopreservation of chytrid fungi in the University of Georgia Chytrid Fungal Collection used a modified Q-tip method (Barr and Babcock 1994) that worked better in ensuring survival than a more traditional skim milk method. A listing of cultures available for teaching and research is provided.

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