THE USE OF PHYSICAL MAPS OF MITOCHONDRIAL AND RIBOSOMAL DNA TO DETERMINE EVOLUTIONARY RELATIONSHIPS AMONG ZOOSPORIC FUNGI

by

© Sally Ann McNabb

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To
the memory of my father
Otto Gruenke

You always believed in me.
Thanks.

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ABSTRACT

Mitochondrial DNA (mtDNA) from 14 species and ribosomal DNA (rDNA) from 26 species of zoosporic fungi were examined to explore the possibility of using restriction maps to determine evolutionary relationships. mtDNA restriction maps of *Aplanopsis*, *Leptolegnia*, *Pythium*, and *Sapromyces* were found to contain inverted repeats (IRs). *Phytophthora* and *Apodachlya* did not contain IRs. Approximate mitochondrial complexity (genome size minus one arm of the IR) in all Oomycetes investigated is very uniform, ranging from 36.2 to 45.3 kb with an average value of 39.9 kb. This uniformity is in contrast to the nonuniformity observed in higher fungi. Within the Hyphochytriomycetes, an IR was present in *Hyphochytrium* and genome complexity (40 kb), was identical with the average value for Oomycetes. In *Rhizidiomyces*, complexity was found to be 50 kb with no IR. The occurrence of a typical IR in the Hyphochytriomycetes indicates that the character is ancestral for the Oomycetes and that its loss can be treated as a derived character for the purpose of phylogenetic analysis. Regularities in the detection of the IR in Oomycetes are consistent with accepted taxonomic groupings.

Physical maps of the rDNA repeat unit were constructed for representative zoosporic fungi and the cladistic status of each restriction site was determined (as ancestral, derived, or shared-derived) at each evolutionary level. Combinations of shared-derived sites were used to postulate phylogenetic groupings. Results suggest that within the Oomycetes two major subclasses exist, the Peronosporomycetidae and the Saprolegniomycetidae. *Lagenidium*, *Phytophthora*, and *Pythium* form a single group within the Peronosporomycetidae. The Saprolegniales and Leptomitales are closely related to each other within the Saprolegniomycetidae as are two Omycetes of doubtful

status, *Verrucalvus* and *Pachymetra* (Sclerosporales). *Sapromyces elongatus* was found to be within the Saprolegniomycetidae subclass but distinct from other orders. The Hyphochytriomycetes form their own group, but are more akin to the Oomycetes than to the Chytridiomycetes. The Chytridiomycetes form a separate, distant group. *Zoophagus insidians* was found not to be an Oomycete. Interestingly, several cases of length heterogeneity of the rDNA repeat were noted.

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LIST OF ABBREVIATIONS

bp - base pairs

cm - centimetre(s)

cpDNA - chloroplast DNA

DNA - deoxyribonucleic acid

EDTA - ethylenediamine-tetra-acetic acid

EtBr - ethidium bromide

Fig(s) - figure(s)

g - gram(s)

h - hour(s)

IGS - intergenic spacer

IR - inverted repeat

kb - kilobase pairs

L - litre(s)

LrRNA - large subunit ribosomal RNA

M - molar

mA - milliamp(s)

mg - milligram(s)

min - minute(s)

mL - millilitre(s)

mm - millimetre(s)

mM - millimolar

mtDNA - mitochondrial DNA

NTS - nontranscribed spacer

rDNA - ribosomal DNA

RNA - ribonucleic acid

rpm - revolutions per minute

rRNA - ribosomal RNA

SDS - sodium dodecyl sulphate

SrRNA - small subunit ribosomal RNA

Tris - Tris (hydroxymethyl) aminomethane

tRNA - transfer RNA

ug - microgram(s)

uL - microlitre(s)

V - volts

INTRODUCTION

INTRODUCTION

The current classification of fungi has been based primarily on morphological criteria. Physiological and biochemical characteristics have also been used but have left numerous unsolved evolutionary problems. Due to the relatively simple morphology of fungi there are problems in defining morphological discontinuities. Also the lack of a good fossil record has hindered evolutionary studies. The examination of nucleic acids has therefore become important in determining evolutionary relationships among fungi. DNA-DNA homology studies, sequence comparisons of highly conservative genes, and comparisons of mitochondrial genomes are some of the different approaches which have been utilized.

In this thesis two molecular approaches have been applied to phylogenetic analysis of zoosporic fungi. These are i) mitochondrial DNA restriction maps and ii) ribosomal DNA repeat unit maps. Analysis of mitochondrial DNA is appealing because the genome is usually small enough to be studied in its entirety. rDNA is appealing because of its highly repetitive nature and its small size, characteristics which make it very accessible. The combination of these two approaches takes advantage of the different evolutionary rates of the two molecules. mtDNA sequences evolve much more rapidly than do the highly conserved ribosomal genes of the rDNA repeat unit.

Traditionally all the zoosporic fungi have been grouped together in the subdivision Mastigomycotina within the Eumycota (Sparrow, 1973). The Mastigomycotina are generally considered to be polyphyletic. The higher fungi are thought to have evolved from an ancestral Chytridiomycete (Cavalier-Smith 1986). The Oomycetes and Hyphochytriomycetes differ from other fungi in their

systems of enzyme regulation and cell wall composition (Lejohn 1971) and are thought to have evolved from a heterokont alga (Cavalier-Smith 1986). In this thesis this problem is briefly addressed.

The study presented here concentrates primarily on the Oomycetes, although species of the Hyphochytriomycetes and Chytridiomycetes were also examined. Evolutionary relationships at two levels, within the classes of the Mastigomycotina and between the classes of the Mastigomycotina, were explored. rDNA repeat unit physical maps have proved particularly useful for grouping organisms within a class. The taxonomy of a number of Oomycetes of uncertain classification was confirmed, while for one species, *Zoophagus insidians*, the current classification was shown to be inappropriate. mtDNA maps were found to be more appropriate for differentiating closely related species within a genus. Overall, mitochondrial genome organization was useful at the level of class because it led to the establishment of a pattern of genome organization for Oomycetes/Hyphochytriomycetes that is different from that seen in higher fungi including the Chytridiomycetes.

HISTORICAL

HISTORICAL

1. Mitochondrial Genome

1.1 Introduction

There is a great deal of diversity in the physical form, organization, size, and base composition of mitochondrial genomes (Wallace 1982, Gray 1982). The mode of evolution of mtDNA is quite different in plants, animals, and fungi. Metazoa have small uniform genome sizes of 16 to 19 kb (Avise and Lansman 1983) and populations of mtDNA molecules within individuals and species are homogeneous. Plant mitochondrial genomes are very large (220 - 2400 kb) (Ward et al. 1981), non-uniform, and individuals have heterogeneous populations of mtDNA molecules. Among fungi, molecular homogeneity is almost as strict as it is among the metazoa, but nonuniformity of sizes is like that of plant mtDNA. Interestingly, the range of fungal mitochondrial genome sizes is intermediate between the metazoan size and the lower limit of plant mitochondrial sizes (Grossman and Hudspeth 1985).

Mitochondrial genomes vary not only in size but also in physical form. Physical maps indicate that most mitochondrial genomes exist in a circular form. However, several linear genomes have been discovered. The protists *Paramecium aurelia* (Goddard and Cummings 1977) and *Tetrahymena pyriformis* (Goldbach et al. 1979), the yeasts *Candida rhagii* (Kovac et al. 1984) and *Hansenula mrakii* (Wesolowski and Fukuhara 1981), and the slime mould *Physarum polycephalum* (Kawano et al. 1982) have linear mitochondrial genomes. In plants, mitochondrial genomes consist of a large circular master chromosome which can be subdivided into various subgenomic circles (Lonsdale 1984).

Mitochondrial genomes, despite their physical diversity, are functionally conservative, coding for a similar complement of genes. The genes commonly coded by all mitochondrial genomes include small and large ribosomal genes, transfer RNA genes, the three largest subunits of cytochrome oxidase (COI, COII, and COIII), the apoprotein of cytochrome b (cytB), and subunit 6 of the ATPase synthase complex (Wallace 1982, Gray 1982). In addition to these genes, most mitochondrial genomes possess other coding functions. Some of those which have been identified include the *var1* gene in yeast and the 5S RNA gene which is unique to plant mitochondrial genomes. Those which have not been identified are known as unidentified reading frames (URFs). Gene order in metazoa is highly invariable with all vertebrates displaying the same order while invertebrates have a different gene order (Clary and Wolstenholme, 1985). Gene order in plants and fungi is highly variable (Wallace 1982, Palmer 1985).

1.2 Fungal Mitochondrial Genomes

Fungal mitochondrial genomes are very diverse (Wallace 1982). Size and gene order are extremely variable characteristics while gene complement and shape are less variable. Until recently, most studies concentrated on the Ascomycetes, especially the yeast *Saccharomyces cerevisiae*, and the filamentous fungi, *Neurospora crassa* and *Aspergillus nidulans*. Several mitochondrial genomes from Ascomycetes have been almost totally sequenced. Therefore, much is known about gene complement and organization in these organisms in addition to size and circularity. Recently, however, fungal studies have expanded to include a number of Basidiomycetes, Chytridiomycetes, and Oomycetes. Although studies in these groups lag far behind those within the Ascomycetes, some interesting observations have been made.

Perhaps the most conservative characteristic of fungal mitochondrial genomes is circularity. Almost all fungi possess circular mitochondrial genomes.

Only two exceptions have been reported in the Eumycotina, these being the yeasts *Hansenula mrakii* (Wesolowski and Fukuhara 1981) and *Candida rhagii* (Kovac et al. 1984) which have linear mitochondrial genomes. The slime mould, *Physarum polycephalum* also has a linear genome (Kawano et al. 1982).

The gene complement of fungi is also quite conservative, although beyond the genes universally encoded by mitochondrial genomes (see Historical section 1.1) variability does exist. ATPase subunit 8 has been found in many fungal mitochondrial genomes. This finding is similar to the situation in mammalian mitochondrial genomes which all possess genes for ATPase 8 (Grossman and Hudspeth 1985). Yeasts encode the functional gene for ATPase subunit 9 while *Aspergillus* and *Neurospora* encode an intact but nonfunctional ATPase 9, the functional gene being nuclear (Van den Bogaart et al. 1982, Brown et al. 1984). *S. cerevisiae* encodes the *var1* gene whose product is a protein of the small ribosomal subunit (Hudspeth et al. 1982). Genes homologous to *var1* have been found in a number of other fungi: *Torulopsis glabrata* (Ainley et al. 1985), *Coprinus stercorarius* but not *C. coprinus* (Weber et al. 1986), and in *Achlya ambisexualis* and *Achlya heterosexualis* (Shumard et al. 1986). *Neurospora* and *Aspergillus* encode the S-5 gene whose product is functionally similar to the *var1* gene but whose sequence is nonhomologous to *var1* (Burke and RajBhandary 1982). Besides these identified polypeptide coding genes, open but unidentified reading frames (URFs) have been found in all fungal mtDNAs which have been sequenced. Some of these may represent maturases. The number and position of URFs are highly variable and differences may exist even within different strains of the same species, indicating possible differences in gene complement in very closely related organisms (Grossman and Hudspeth 1985).

Studies on gene order have largely been confined to the Ascomycetes, where a great deal of diversity has been found. Variability exists even between

species of the same genus as has been observed for *S. cerevisiae* and *S. exiguss* (Grossman and Hudspeth 1985) and for *Brettanomyces* species (Hoeben and Clark-Walker 1986). Little is known of gene order within the Basidiomycetes although two species of *Coprinus* have significantly different gene orders (Weber et al. 1986). However, in the Chytridiomycetes gene order is conserved between two different genera, *Allomyces arbuscula* and *Blastocladiella emersonii* (Borkhardt et al. 1987). Amongst the Oomycetes gene maps have been constructed for *Achlya ambisexualis* and *Achlya heterosexualis* (Shumard et al. 1986) and gene order has been found to be conserved in these species. Partial gene maps (SrRNA, LrRNA, cytB, and COII) have been mapped in *Phytophthora megasperma* f. sp. glycinea, *Phytophthora megasperma* f. sp. medicaginis, and Phytophthora parasitica (Förster et al. 1987). Again gene order has been found to be conserved among these species. Comparison of *Phytophthora* and *Achlya* gene maps is inconclusive since only a few genes have been mapped for *Phytophthora.* However, the order of cytB and COII would appear to be different. Interestingly, however, the SrRNA and LrRNA genes are found adjacent to one another in both genomes, indicating that some similarity in gene order may exist.

Fungal mitochondrial genomes are extremely variable in size. They range in size from the compact genome of 17 kb of *Schizosaccharomyces pombe* (Zimmer et al. 1984) which is comparable to mammalian genome sizes, to the large 176.3 kb genome of *Agaricus bitorquis* (Hintz et al. 1985) which approaches the typical plant mitochondrial genome size. An extensive list of mitochondrial genome sizes is shown in Table I illustrating the variability that exists amongst various fungal groups.

The most widely studied group of fungi is the Ascomycotina where the pattern is one of striking nonuniformity of genome size. Sizes range from 17 -

Table I Sizes of Fungal Mitochondrial Genomes

Taxon	Mitochondrial Genome Size (kb)	References
Ascomycotina - filamentous		
Aspergillus nidulans	31.5	Stepien et al. 1978
Cephalosporium acremonium	27	Minuth et al. 1982
Claviceps purpurea	45	Tudzynski et al. 1983
Cochliobolus heterostrophus	115	Garber and Yoder 1984
Neurospora crassa	63.5	Taylor and Smolich 1985
Podospora anserina	94	Cummings et al. 1979
Penicillum chrysogenum	48.3 - 49.2	Smith et al. 1984
Histoplasma capsulatum class 1	47	Vincent et al. 1986
Histoplasma capsulatum class 2	38	Vincent et al. 1986
Histoplasma capsulatum class 3	33	Vincent et al. 1986
Ascomycotina - yeasts		
Brettanomyces custersii	101.1	Hoeben and Clark-Walker 1986
Brettanomyces custersianus	28.5	Hoeben and Clark-Walker 1986
Brettanomyces naardenensis	41.7	Hoeben and Clark-Walker 1986
Eeniella nana	34.5	Hoeben and Clark-Walker 1986
Dekkera bruxellensis	85.0	Hoeben and Clark-Walker 1986
Dekkera intermedia	73.2	Hoeben and Clark-Walker 1986
Hansenula mrakii	55	Wesolowski and Fukuhara 1981
Hansenula petersonii	42	Falcone 1984
Hansenula wingei	25.5	O'Connor et al. 1975
Kluyveromyces lactis	37	Groot and Van Harten-Loosbrock 1980
Saccharomyces cerevisiae	68 - 78	Sanders et al. 1977
Saccharomyces exiguus	23.7	Clark-Walker et al. 1983
Saccharomycopsis (Yarrowia) lipolytica	44 - 48	Kuck et al. 1980 Wesolowski et al. 1981
Schizosaccharomyces pombe	17 - 19	Del Giudice et al. 1981 Zimmer et al. 1984
Deuteromycotina		
Torulopsis glabrata	18.9	Clark-Walker and Sriprakash 1981
Kloeckera africana	27.1	Clark-Walker et al. 1981
Candida albicans	41	Wills et al. 1985
Candida rhagii	30	Kovac et al. 1984
Candida maltosa	52	Kunze et al. 1986
Basidiomycotina		
Agaricus brunnescens	98.3	Hintz et al. 1985
Agaricus bitorquis	148.5 - 173.6	Hintz et al. 1985
Coprinus cinereus	43.3	Weber et al. 1986
Coprinus stercorarius	91.1	Weber et al. 1986
Schizophyllum commune	50.3 - 52.2	Specht et al. 1983
Ustilago cynodontis	76	Mery-Drugeon et al. 1981
Zygomycotina		
Phycomyces blakesleaanus	22	Grossman and Hudspeth 1985

Table I Sizes of Fungal Mitochondrial Genomes (Continued)

Taxon	Mitochondrial Genome Size (kb)	References
Mastigomycotina		
Chrytridiomycetes		
Allomyces anomalus	49.9 - 51.2	Borkhardt et al. 1987
Allomyces arbuscula	47.1 - 48.3	Borkhardt et al. 1987
Allomyces catenoides	55.1	Borkhardt et al. 1987
Allomyces macrogynus	55.9	Borkhardt and Delius 1983
Allomyces moniliformis	49.9	Borkhardt et al. 1987
Blastocladiella emersonii	35.5	Borkhardt and Olson 1986
Oomycetes		
Achlya ambisexualis	49.8 - 51.1	Hudspeth et al. 1983 Shumard et al. 1986
Achlya heterosexualis	51.3 - 50.7	Shumard et al. 1986
Achlya klebsiana	50.4	Boyd et al. 1984
Phytophthora infestans	36.2	Klimczak and Prell 1984
Phytophthora megasperma	41 - 45.3	Förster et al. 1987
Phytophthora parasitica	39.5	Förster et al. 1987
Pythium ultimum	57	Grossman and Hudspeth 1985
Saprolegnia ferax	46.5	Grossman and Hudspeth 1985
Myxomycetes (Slime Moulds)		
Physarum polycephalum	69	Kawano et al. 1982

101 kb, slightly greater than a six-fold range. Even within a genus, mitochondrial genome sizes are extremely variable. In *Saccharomyces*, sizes vary by at least three-fold (23.7 - 78 kb) and within the genus *Brettanomyces* the variation is almost four-fold (28.5 - 101.1 kb) (See Table I for references). Among the Basidiomycetes where six species have been studied, the pattern is again one of nonuniformity (43.3 - 176.3 kb), a four-fold variation in sizes. As with the Ascomycetes, variability even within the same genus is very pronounced (98.3 - 176.3 kb for *Agaricus* species and 43.3 - 91.1 kb for *Coprinus* species) (See Table I for references). Information on the Zygomycetes is not available. In the Mastigomycotina, studies have been limited to two genera of the Chytridiomycetes (*Allomyces* and *Blastocladiella*) and in the Oomycetes to the orders Saprolegniales and Peronosporales. Overall for the Mastigomycotina, sizes range from 35.5 to 57 kb, only a two-fold size variation (See Table I for references). In the Chytridiomycetes, two genera have been explored and sizes range from 35.5 kb (*Blastocladiella emersonii*) to 55.9 kb (*Allomyces macrogynus*) which is similar to the range within *Allomyces* itself (39.3 - 55.9 kb). In the Oomycetes the range is 36.2 to 57 kb. Within the genus *Achlya*, mitochondrial genome size is quite invariable (49.8 - 51.3 kb), while in *Phytophthora*, sizes are slightly more variable (36.2 - 45.3 kb).

Various proposals have been put forth to account for the high variability in fungal genome size (Taylor 1986). One possible source of variation is differences in gene complement. For instance, the *var1* gene is mitochondrial in *Coprinus stercorarius* but appears to be nuclear in *Coprinus cinereus* (Weber et al. 1986). This difference could be accounted for by gene transfer from the nucleus or by gene transfer out of the mitochondrion. A model has been proposed for gene transfer between mitochondrial and nuclear genomes (Obar and Green 1985). Although transfer could occur in either direction, transfer from the

mitochondrial to the nuclear genome was seen as the most probable event. In plants, transfer of chloroplast sequences to mtDNA has been observed (Stern and Palmer 1986). Also in the plant *Oenothera*, a sequence from the nuclear SrRNA gene has been found in the mtDNA (Schuster and Brennicke 1987). These findings indicate that transfers between the different genomes of an organism are possible. Another source of size variation would involve duplication of a portion of mtDNA such as that seen in *Achlya*, *Kloeckera*, and *Candida* which have inverted repeats (Hudspeth et al. 1983, Clark-Walker et al. 1981, Wills et al. 1985) or in the form of short direct repeats as seen in *Aspergillus niger* (Brown et al. 1983) and *Neurospora crassa* (Yin et al. 1981). One other possible explanation for size variations is that since in some fungal mitochondrial genomes 50 - 80% of the genome lacks a coding function, deletions of large blocks of noncoding regions may account for some of the variability (Grossman and Hudspeth 1985).

1.3 Summary of use of mtDNA for evolutionary studies

Mitochondrial genomes can be used in evolutionary studies in a number of ways (reviewed in Taylor 1986). Comparison of restriction length polymorphisms (RFLPs), comparison of restriction map sites, and comparison of gene maps are all methods that have been used to obtain evolutionary information among fungi. Studies have shown that RFLPs are primarily useful for population studies, or in comparisons between closely related species due to the high degree of size variability seen between the mtDNA of most fungal species. Comparison of restriction map sites is generally useful for closely related species while comparisons of gene order and gene complement are more useful among more distantly related taxa.

2. Inverted repeats

Large inverted duplications of a portion of the genome occur relatively infrequently in mitochondrial genomes, although they have been reported in a number of unrelated organisms. Significant inverted repeats (greater than 1 kb) have been found in the protozoan, *Tetrahymena pyriformis*, the plant, *Zea mays*, and the fungi, *Kloeckera africana*, *Candida albicans*, and *Achlya* (see Table II for references and size details).

Large inverted repeats (IRs) are, however, the norm in chloroplast genomes. For example, angiosperm chloroplast DNAs (cpDNA) typically contain IRs of 20 to 28 kb (Whitfeld and Bottomley 1983). They are present in the cpDNA of all Angiosperms except one section of a subfamily of the legume family Fabaceae. IRs have also been found in a Gymnosperm, a fern, a bryophyte, *Chlamydomonas* and *Clorella* (green algae), *Cyanophora paradoxa* (an alga of uncertain classification), and the alga *Olisthodiscus luteus* (a Chromophyte). These inverted repeats range in size from 10 to 76 kb (Palmer 1985).

In land plants there are three properties common to all cpDNA inverted repeats. These include an asymmetrical location in the genome (IRs divide the genome into small and large single-copy regions), the presence of a complete set of rRNA genes, and uniformity in orientation of the rRNA genes (the 23S gene towards the small single-copy region and the 16S gene further from the single-copy region). In the green alga *Chlamydomonas*, the IR differs slightly. In *Chlamydomonas* the IR has an almost central position and the order of the 16S and 23s genes appears to be inverted with respect to the order found in plant cpDNA. Given the similarities of IRs and their widespread presence in cpDNAs, it is likely that the IR in cpDNA is an ancestral trait of land plants and the green algae (Palmer 1985).

In Angiosperms, the group in which chloroplast genomes have been studied most widely, a distinct pattern has emerged. Chloroplast genome sizes range from 120 to 217 kb and the range of inverted repeat sizes is 10 to 76 kb. However, if one repeat is factored out, the range in sequence complexity is only 40 kb (110 - 150 kb). This means that most of the size variability in angiosperm cpDNA results from expansion or contraction of the IR, not changes in sequence complexity (Palmer 1985, Palmer et al. 1987). That the inverted repeat expands to engulf more of the genome is supported most strikingly by the example of geranium, which contains a 76 kb repeat packed with many genes that are in single copy regions of other cpDNA (Palmer et al. 1987).

IRs seem to possess some general characteristics not dependent on the type of genome with which they are associated. A common feature of IRs, whether chloroplast or mitochondrial, appears to be the presence of rRNA genes (see Table II). In plant and algal chloroplasts the IR contains the complete rRNA operon with the exception of *Olisthodiscus luteus* whose IR contains no ribosomal genes. The IR of the protozoan *Tetrahymena pyriformis* contains the LrRNA gene. In fungi, the IR of *Kloeckera africana* contains a portion of the LrRNA gene while IRs of *Achlya* contain both the LrRNA and SrRNA genes. Another common feature of IRs is their ability to undergo intramolecular recombination. In both cpDNAs (Palmer 1985), fungal mtDNAs (Hudspeth et al. 1983, Boyd et al. 1984), and maize mtDNA (Lonsdale et al. 1984) intramolecular recombination has been found to occur between the IRs. In cpDNA and *Achlya* mtDNA the genomes consist of equimolar populations of two molecules which differ only in the orientation of one single-copy region with respect to the other (flip-flop isomerism). In maize the situation is more complex due to a number of direct repeats which also undergo recombination. Although flip-flop isomerism has not been observed for all genomes with IRs, intramolecular recombination

Table II Inverted repeats in chloroplast and mitochondrial genomes

Taxon	Repeat size (kb)	Flip-flop Isomerism	Ribosomal Genes on Repeat	Location of Repeat	References
Chloroplast DNA					
Chlorophytes and *Cyanophora*	10 - 76	Yes	4.5S, 5S, LrRNA, SrRNA	Asymmetrical	Palmer 1985
Olisthodiscus luteus	22	Unknown	None	Unknown	Palmer 1985
Mitochondrial DNA					
Plants					
Zea mays	14	Yes	Unknown	Asymmetrical	Lonsdale et al. 1984
Fungi					
Kloeckera africana	4.3	Unknown	Incomplete LrRNA	Tail-tail	Clark-Walker et al. 1981
Candida albicans	5	Unknown	Unknown	Asymmetrical	Wills et al. 1985
Achlya species	10.6	Yes	LrRNA, SrRNA	Asymmetrical	Hudspeth et al. 1983 Boyd et al. 1984 Shumard et al. 1986
Protozoa					
Tetrahymena pyriformis	2	Unknown	LrRNA	Terminal	Goldbach et al. 1979

probably does occur (Palmer 1985). However, such recombination is often difficult to detect due to technical difficulties. To reveal isomerism, restriction enzymes which do not cleave in the IR but cleave both single-copy regions must be found. One other general characteristic of IRs is that they are always identical within an individual organism presumably due to a copy-correction mechanism which maintains sequence identity between the repeats on a molecule (Palmer 1985).

3. Ribosomal DNA Repeating Units

3.1 Introduction

The ribosomal RNA (rRNA) genes exhibit dosage repetition, meaning that they occur in multiple copies within a genome (Long and Dawid 1980). Dosage repetition occurs because a single copy gene could not provide enough of the product required by the cell in an appropriate time. Such repetition is displayed primarily by RNA genes involved in protein synthesis. These include the 5S RNA gene, tRNA genes and the rRNA genes. The rRNA genes commonly exist in the form of tandem repeats, each repeat unit comprised of a highly conserved gene region containing the large (25-28S), the small (17-18S), and the 5.8S ribosomal RNA genes and a nonconservative spacer region. The rRNA genes of eukaryotes vary considerably in their repeat number, ranging from 40 to 19,300 copies per haploid genome, the redundancy correlating approximately with genome size (Long and Dawid, 1980). The highly repetitive nature of the rRNA genes has aided in their study and isolation, and along with the highly conservative nature of the rRNA genes, has made them popular for evolutionary studies.

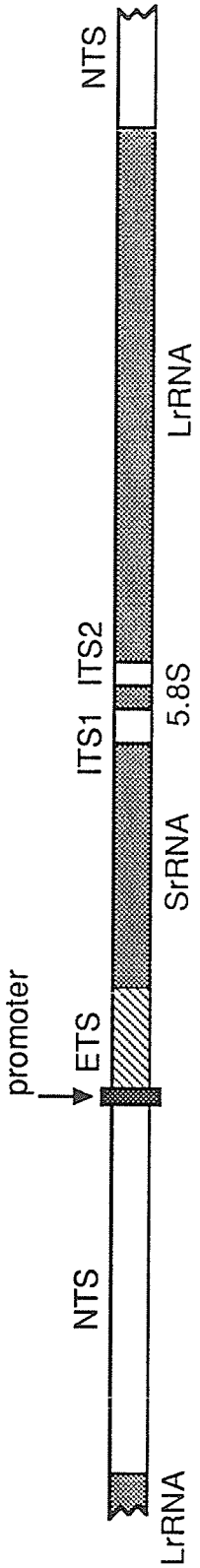
3.2 Location and Structure of Ribosomal DNA repeating units.

The rDNA repeat units of most eukaryotes occur as blocks of tandem arrays on one or more chromosomes and are associated with nucleolar organizing

regions (Mandel 1984). In a number of cases rDNA repeats are extrachromosomal and this situation may be relatively common among unicellular eukaryotes. In *Tetrahymena*, *Physarum* and *Dictyostelium* the extrachromosomal rRNA repeat units are in the form of palindromic molecules each containing two transcription units, with the transcription units directed outwards. The palindromes of *Physarum* and *Dictyostelium* are especially long, namely 60kb and 88kb respectively (Long and Dawid, 1980). In *Naegleria gruberi*, a soil amoeba, the rRNA genes are on a 14 kb plasmid (Clark and Cross 1987). In *Paramecium tetraurelia* the repeats are on either linear or circular extrachromosomal molecules in tandemly repeated units (Long and Dawid 1980).

The typical integrated rDNA repeat unit consists of a transcription unit coding for a 35-45S precursor rRNA which alternates with a spacer region known as the nontranscribed spacer (NTS). The transcription unit contains the 17-18S, 5.8S and 25-28S rRNA genes separated from one another by internal transcribed spacers (ITS) and preceded by an external transcribed spacer (ETS) as shown in Fig 1. The transcription unit is transcribed by RNA polymerase I and the spacers are removed post transcriptionally to produce the mature rRNAs. Transcription proceeds from the 5' end of the ETS and proceeds in the sequence: 18S - ITS1 - 5.8S - ITS2 - 28S (Long and Dawid 1980, Mandel 1984). The ribosomal genes are highly conserved. The large rRNA gene is the most variable, ranging in size from 3.4 - 4.7 kb, while the small rRNA gene is less variable, ranging in size from 1.75 - 1.87 kb (Noller 1984, Huysmans and DeWachter 1985). The 5.8S gene is about 160 bp long (Erdmann and Wolters 1986). Both the large and small rRNA genes have highly conserved regions of primary sequence and secondary structure alternating with variable regions that differ in size, base composition and secondary structure (Gerbi 1986). The ITS, ETS, and NTS, are highly divergent in sequence and size (Mandel 1984).

Figure 1. General organization of a typical rDNA repeat unit. NTS, nontranscribed spacer (this term has has been replaced by the term intergenic spacer - IGS); ETS, external transcribed spacer; ITS, internal transcribed spacer; SrRNA, small subunit ribosomal RNA; LrRNA, large subunit ribosomal RNA.


NTS
LrRNA
ITS2
ITS1
5.8S
SrRNA
promoter
ETS
NTS
LrRNA

The size of the repeating units vary considerably between species, ranging between 6.8 and 44 kb (Gerbi 1986). Some of the variability in size is due to variability within the gene region either in the ITS or in the size of the LrRNA gene. Intervening sequences (introns) are known to exist in the LrRNA genes of some species. In the dipteran insects *Drosophila*, *Calliphora*, and *Sciara* some LrRNA genes are interrupted while others are not and it is not known whether the interrupted genes are transcribed (Long and Dawid 1980). In some protozoans the LrRNA gene is very fragmented. In *Crithidia fasciculata* and *Trypanosoma brucei* the LrRNA gene contains 5 introns (Spencer et al. 1987, White et al. 1986). Some species of *Tetrahymena* contain one intron while *Physarum polycephalum* contains two introns in the LrRNA gene (Nielson et al. 1985, Nomiyana et al. 1981). Although the presence of introns and consequent differences in gene lengths can account for some of the variation observed in rDNA repeats, most of the size variations in rDNA repeating units can be attributed to spacer size differences, especially in the NTS.

3.3 Properties of the NTS

NTS regions of different organisms are not conservative at a sequence level except possibly within closely related species. For example, the NTS of human rDNA shows some homology with the NTS of certain primates (Sylvester et al. 1986). Even within one individual the NTS can exhibit considerable variability. Heterogeneity in length, nucleotide sequence, and base modification have been observed amongst the NTSs of different rDNA repeat units isolated from an individual organism.

The most striking class of NTS heterogeneities are the length heterogeneities which are very common among higher eukaryotes. A feature of many NTSs is the presence of many short subrepetitive elements. Length heterogeneities are thought to arise by unequal crossing over between

subrepeats leading to losses or gains in repeat unit sizes by multiples of the subrepeat size. In plants these subrepeats are quite common and are generally between 100 and 200 bp long, have a species specific sequence, and flank the 3' end of the LrRNA gene (Rogers and Bendich 1987). The most pronounced case of this stepwise type of length heterogeneity has been observed in *Vicia faba* where more than 20 size classes have been observed (Rogers et al. 1986).

Although subrepeats may be involved in generating length heterogeneities, in some instances subrepetitive elements have also been found which do not seem to produce significant heterogeneity. Subrepeats have been found in plants with homogeneous repeat units (Rogers and Bendich 1987). In the house cricket, which has a very long NTS, (43 kb out of which 29 kb has been cloned and found to be very repetitive) areas with subrepeats have been found not to display significant length heterogeneities (Sharp et al. 1986). Also, where length heterogeneities appear to be linked to subrepetitive elements, not all possible size classes are represented equally. For example in *Calliphora erythrocepha* the subrepeats are 350 bp long but there are only two major classes differing by 1 kb in length (Schafer et al. 1981).

It should also be noted that subrepeats may also be heterogeneous. In maize the subrepeats are only 85 - 90% homologous (Toloczyki and Feix 1986). In pea some repeats have extra restriction sites (Jorgensen et al. 1987) and in the insect *Calliphora erythocepha* subrepeats show sequence variation (Schafer et al. 1981). In some organisms the NTS may contain several classes of subrepeats which may or may not be related to one another in sequence. Wheat, house cricket, *Drosophila*, *Xenopus*, and sea urchin are some examples of organisms with more than one class of subrepeat in the NTS (Lassner et al. 1987, Sharp et al. 1986, Grimaldi and Di Nocera 1988, Morgan and McMahon 1980, Simmen et al. 1985).

Length heterogeneities also exist that do not seem to involve subrepetitive elements and may be due to insertions or deletions of unique sequences of DNA. For example, this type of length heterogeneity bas been found in the NTS of pumpkin and wheat (Siegel and Kolacz 1983, May and Appels 1987).

Other types of heterogeneity found in the NTS include sequence and/or base modification heterogeneities. These types of heterogeneities have also been found in the gene region but to a much lesser extent (Kolosha et al. 1986, Popodi et al. 1985). Such heterogeneities are often detected by restriction analysis and thus may be difficult to differentiate. Sequence and/or base modification heterogeneity has been found in onions, peas, sea urchins, pumpkins, and humans, to name a few examples (Maggini and Carmona 1981, Jorgensen et al. 1987, Simmen et al. 1985, Passanti et al. 1983, Siegel and Kolacz 1983, Sylvester et al. 1986).

At first the NTS was thought to serve only as a spacer region. Recently however, evidence has accumulated that suggests that regions of the NTS may act as enhancers of transcription of pre-rRNA. Enhancers have been found in the NTSs of *Saccharomyces cerevisiae*, rat, *Xenopus*, and *Drosophila* (Cassidy et al. 1986, Dixit et al. 1987, Labhart and Reeder 1984, Grimaldi and Di Nocera 1988). In *Xenopus* and *Drosophila* enhancement involves subrepetitive elements and transcription of the NTS (De Winter and Moss 1986, Grimaldi and Di Nocera 1988). Therefore, the term NTS is a misnomer, and consequently, the term IGS (intergenetic spacer) has been proposed and is replacing NTS in current usage.

3.4 Fungal ribosomal DNA repeat units

Fungal ribosomal DNA repeat units share the structure common to most eukaryotes. The repeat units exist in tandem arrays and contain the large, small, and 5.8S rRNA genes. Some yeasts and Basidiomycetes possess unusual repeats in that the 5S gene is also encoded on the repeat unit (Table III) but is not part of

the rRNA transcription unit. In *S. cerevisiae* the 5S gene is transcribed from the strand opposite to that from which the pre-rRNA is transcribed. The yeast 5S gene is transcribed by RNA polymerase III, unlike the pre-rRNA which is transcribed by RNA polymerase I (Planta and Raue 1988). Basidiomycetes in the order Agaricales also seem to possess the 5S gene on the rDNA repeat unit (Pukkila and Cassidy 1986, Buckner et al. 1987). The organization of the 5S gene differs from that in yeasts in that in Agaricales the 5S gene is transcribed from the same strand as the pre-rRNA. An exception to this is a species of *Coprinus*, *C. comatus*, where an inversion seems to have occurred so that the 5S gene is transcribed from the opposite strand (Pukkila and Cassidy 1986).

The sizes of rDNA repeat units vary between species of a genus but are generally consistent within closely related strains of a species (Lachance et al. 1986, Russell et al. 1984, Vincent et al. 1986, Vaughan Martini et al. 1987, Magee et al. 1987). Within the Ascomycetes sizes of the repeats vary between 6.9 - 11.1 kb while in the Basidiomycetes the sizes range from 8.8 - 10 kb (See Table III for references). The fungal rDNA repeat units are generally thought to be homogeneous in length within an individual isolate. The one definite exception to this is *Yarrowia lipolytica* in which length heterogeneity has been found and characterized. Several different strains of *Y. lipolytica* were found to have between two to five classes of repeat units by examination of digests of genomic DNA hybridized to a rDNA probe (Fournier et al. 1986). The NTSs from clones representing the two major size classes of rDNA repeat units from one strain have been sequenced. The NTS was shown to contain many sequence repetitions in both repeat unit types. The subrepeats have a periodicity of 140-150 bp and are not identical but have a high degree of sequence homology. Although both classes of NTS have the same organizational structure (two unique sequence regions flanking a repetitious region), the differences between the NTS of the

Table III Characteristics of Fungal Ribosomal DNA Repeat Units

Taxon	Size (kb)	5S gene	Length heterogeneity	References
Ascomycotina				
Aspergillus nidulans	7.8	No	No	Borsuk et al. 1982
	7.7	No	No	Lockington et al. 1982
Neurospora crassa	9.2	No	-	Russell et al. 1984
	8.7	No	-	Chambers et al. 1986
Neurospora intermedia	8.4	No	-	Chambers et al. 1986
Neurospora sitophila	8.4	No	-	Chambers et al. 1986
Cephalosporium acremonium	9.2	-	No	Carr et al. 1987
	8.0	-	-	Jarai et al. 1987
Histoplasma capsulatum				
strain class 1	6.9	-	-	Vincent et al. 1986
strain class 2	7.3	-	-	Vincent et al. 1986
strain class 3	8.4	-	-	Vincent et al. 1986
Schlerotinia species	8.2 - 11.3	-	-	Kohn et al. 1988
Saccharomyces cerevisiae	9.3	Yes	No	Bell et al. 1977
Saccharomyces carlsbergensis	9.1	Yes	-	Veldman et al. 1981
Saccharomyces rosei	8.5	Yes	-	Verbeet et al. 1983
Kluyveromyces lactis	8.6	Yes	-	Verbeet et al. 1984
Hansenula wingei	11.1	Yes	possibly minor	Verbeet et al. 1984
Yarrowia lipolytica	8.9	No	possibly very minor	Clare et al. 1986
Yarrowia lipolytica	7.7,8.7	No	Yes	van Heerikhuizen et al. 1985
Deuteromycotina				
Clavispora opuntiae	7.6	-	possibly one strain	Lachance et al. 1986
Clavispora lusitaniae	9.0	-	-	Lachance et al. 1986
Candida albicans	12.7	Yes	Yes in two strains	Magee et al. 1987
Candida stellatoides	-	Yes	No	Magee et al. 1987
Candida guilliermondii	-	Yes	No	Magee et al. 1987
Candida tropicalis	-	Yes	No	Magee et al. 1987
Candida glabrata	-	Yes	No	Magee et al. 1987
Torulopsis utilis	11.9	Yes	No	Tabata 1980

Table III Characteristics of Fungal Ribosomal DNA Repeat Units (Continued)

Taxon	Size (kb)	5S gene	Length heterogeneity	References
Basidiomycotina				
Coprinus cinereus	9.3	Yes	-	Cassidy et al. 1984
Coprinus comatus	-	Yes	-	Pukkila and Cassidy 1986
Coprinus atramentarius	-	Yes	-	Pukkila and Cassidy 1986
Coprinus micaceus	-	Yes	-	Pukkila and Cassidy 1986
Agaricus bisporus	-	Yes	-	Pukkila and Cassidy 1986
Flammulina velutipes	-	Yes	-	Pukkila and Cassidy 1986
Tryomyces unicolor	9.2	-	-	Kwok et al. 1986
Phanerochaete chrysosporium	10	-	No	Raeder and Broda 1984
Sporotrichum pulverulentum	10	-	No	Raeder and Broda 1984
Schizophyllum commune	9.2 - 9.6	Yes	-	Specht et al. 1984 Buckner et al. 1979
Puccinia graminis	9.0	-	No	Klassen unpublished
Ustilago hordei	9.3	-	No	Klassen unpublished
Agrocybe pediades	9.4	-	-	Rehner personal communication
Tilletia controversa	8.8	-	No	Klassen unpublished
Zygomycotina				
Mucor racemosus	10.2	Yes	-	Cihlar and Sypherd 1980
Oomycetes				
Achlya ambisexualis	*>24 (10.7)	Yes	No	Rozek and Timberlake 1979

*See text for explanation (Discussion 2.1)

two repeat classes are primarily not due to variation in the number of subrepeats but involve other types of sequence divergence (van Heerikhuizen et al. 1985). The pattern of heterogeneity is therefore unlike the pattern observed in plants. The finding of repetitive regions in the NTS of *Y. lipolytica* is in contrast to findings for *S. cerevisiae* where only a limited number of short direct repeats (up to 16 bp) have been found and where there are no significant length heterogeneities. *S. cerevisiae* does not contain any repetitive sequences comparable to the subrepeats found in *Y. lipolytica* or the subrepeats found in some other organisms displaying length heterogeneities (Skryabin et al. 1984).

While *Y. lipolytica* is the only fungal species reported to have major length heterogeneities, there have been several other reports of possible length heterogeneities. Two strains of *Candida albicans* seem to each have two major size classes (Magee et al. 1987). In *Hansenula wingei*, *Coprinus cinereus*, and one strain of *Clavispora opuntiae*, minor length heterogeneities may exist as indicated by minor bands hybridizing to rRNA probes (Verbeet et al. 1984, Cassidy et al. 1984, Lachance et al. 1986). Further investigation would be necessary to confirm that these minor bands are due to length heterogeneities in the NTS.

3.5 The use of ribosomal repeat units for phylogenetic studies.

The sequencing of the ribosomal genes is an approach which has been commonly used in evolutionary studies. The 5S and 5.8S genes have been used extensively since their size is conducive to sequence analysis (Hori and Osawa 1986, Olsen and Sogin 1982), but it has become apparent that there are a number of limitations to the use of such small genes (Walker 1985). The 18S gene has also been popular for evolutionary studies since it contains more information than the 5S or 5.8S genes but is easier to sequence than the larger 28S gene (Sogin et al. 1986[a]). The 28S gene may be particularily promising for such studies due to a number of alternating highly conserved and highly variable

domains. Sequencing highly conserved domains may be useful among highly divergent groups while sequencing variable domains may be useful among closely related organisms (Baroin et al. 1988). Although sequencing methods are becoming much more rapid, obtaining entire sequences of genes (especially the 28S gene) from a large number of organisms is very time consuming.

Generally restriction maps of rDNA repeat units have been considered to be primarily useful for the identification of fragments suitable for cloning and sequencing. Although many maps of repeat units are available few have been compared and assessed for their usefulness in phylogenetic analysis. There are, however, a few examples of such analysis that are worthy of brief mention.

One such example has been the construction of restriction maps of the 28S gene portion of the repeat in anurans (frogs). These maps were shown to have potential phylogenetic implications at the levels of suborder, family and genus (Hillis and Davis 1987). In that study alignment of conserved sites led to the identification of regions of variability that corresponded to previously described divergent domains. Phylogenetic inferences could then be made on the basis of position and size of certain deletions or insertions associated with the variable domains or by the presence or absence of a restriction site at a particular location. Another benefit gained from the restriction mapping was that the identification of the variable domains could prove useful in the selection of portions of the 28S gene to be subjected to sequence analysis.

rDNA repeat unit maps have been used for phylogenetic analysis of two species of yeast within the genus *Clavispora*. *C. opuntiae* (ten strains) and *C. lusitaniae* (nine strains) were shown to constitute well-defined separate species through the methods of cluster analysis, and principal-component analysis of rDNA restriction maps. It was also shown that the results obtained through such analysis were consistent with physiological criteria for species delineation

(Lachance et al. 1986). The method not only showed species discontinuity within the genus *Clavispora* but also allowed assessment of intraspecific relationships.

Among seventeen *Tetrahymena* species, restriction maps of rDNA were found useful in generating a scheme of species associations (Nielson et al. 1985). In this study associations were based solely on variations of a few restriction sites (sites for three enzymes) within the transcribed region. Although firm conclusions about evolutionary relationships could not be drawn from such simple maps, the fact that associations were obtained that agreed with previously described groupings illustrated the usefulness of this method.

In a study on *Mus musculus* (mouse), the rapid evolutionary rate of the NTS was taken advantage of to classify subspecies (Suzuki et al. 1986). Individual mice were shown to possess heterogeneous rDNA repeat units. Restriction maps of the 5' end of each major repeat unit type (referred to as haplotype) were constructed. Each subspecies of mice was shown to possess a characteristic combination of haplotypes which could be detected by simple restriction analysis. Restriction patterns could then be used to classify wild subspecies and to trace the origins of laboratory mouse strains.

There are a few cases in which rDNA repeat maps have been used in comparative studies. Comparison of the repeat unit maps of wheat, pumpkin and pea involving at least 25 restriction sites suggested that pea and pumpkin were more closely related to one another than either was to wheat. (Jorgensen et al. 1987). Several members of the *Saccharomycetoideae* have been compared to *S. carlsbergensis/S. cerevisiae* (the maps for these two were identical). Relatedness to *S. cerevisiae* was deduced to be in the descending order of *S. rosei*, *K. lactis* and *H. wingei* (Verbeet et al. 1984). The rDNA maps of three heterothallic species of *Neurospora* which were difficult to distinguish morphologically were compared (Chambers et al. 1986). Maps of *N. crassa*, *N. intermedia*, and *N.*

sitophila were found to differ by no more than one site in the gene region but several differences were found in the NTS region. rDNA maps of a number of vertebrates have been constructed and compared showing that a number of sites are highly conserved in all vertebrates (Tanhauser et al. 1986). These maps have not been used, however, to group organisms. The examples sited above indicate that comparison of rDNA maps have potential as tools for phylogenetic analysis. This potential has largely been unexplored to date.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Fungal cultures

Pythium diclinum (4110a), *Pythium torulosum* (4212b), *Aplanopsis terrestris* (3102b), *Phytophthora cryptogea* (JM10), *Sapromyces elongatus*, *Apodachlya pyrifera* (502a), *Apodachlya brachynema* (501a), *Pythium paddicum* (480a), *Pythium anandrum* (4401d), *Aphanomyces stellatus* (301a), and *Leptolegnia caudata* (351d), were provided by M.W. Dick from the Aquatic Phycomycete Culture Collection, University of Reading, Reading, England. *Pythium irregulare* (174), *Saprolegnia diclina* (314), *Hyphochytrium catenoides* (217), *Rhizidiomyces apophysatus* (296), *Spizellomyces punctatus* (117), *Spizellomyces plurigibbosus* (33), *Gaertneriomyces semiglobiferus* (43), and *Chytridium confervae* (97) were provided by Dr. D.J.S. Barr, Biosystematics Research Institute, Ottawa, Canada. *Achlya klebsiana* was described earlier (Boyd et al. 1984).

2. Prepared DNA

Purified ribosomal or total DNA from *Saprolegnia ferax*, *Leptomitus lacteus*, *Lagenidium giganteum*, and *Zoophagus insidians*, was a generous gift from Dr. M. E. S. Hudspeth, University of Northern Illinois, USA. Total DNA or DNA enriched for ribosomal DNA for *Verrucalvus flavofaciens*, *Pachymetra chaunorhiza* and *Zoophagus insidians* were prepared by Abbes Belkhiri in M.W. Dick's laboratory, University of Reading, Reading, England.

3. Bacterial cultures and plasmids

The plasmid *pMAK*, a recombinant plasmid containing the large rRNA gene from *Achlya klebsiana*, was constructed by D. Boyd as previously described (Boyd 1986). *pMAK* was derived from the vector *pBR322* (Bolivar et al. 1977) and a fragment of mtDNA from *A. klebsiana* containing the LrRNA gene. *pMAK* was carried by the bacterial host *E. coli* HB101 (Boyer and Roulland-Dussoix

1969). *E. coli* C600SF8 carrying the plasmid *pMF2* was a gift from Robert L. Metzenberg (Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin). *pMF2* was derived from *pBR322* and contains a 6.5 kb insert from the *Neurospora crassa* rDNA repeat unit in the ampicillin resistance gene. The 17S, 5.8S, and 26S ribosomal RNA coding regions are fully represented in *pMF2* (Free et al. 1979).

4. Culture media

PYG Broth	3.0 g dextrose
	1.0 g yeast extract
	1.0 g peptone
	1000 mL distilled water
PYGS Broth	3.0 g glucose
	1.25 g peptone
	1.25 g yeast extract
	1.36 g K_2HPO_4
	0.71 g Na_2HPO_4
	0.12 g $MgSO_4$ $7H_2O$
	0.07 g $CaCl_2$ $2H_2O$
	200 uL of 10 mg/mL bromocresol purple
	1000 mL distilled water
YpSs Broth	4.0 g yeast extract
	15.0 g soluble starch
	1.0 g K_2HPO_4
	0.5 g $MgSO_4$
	1000 mL distilled water
YpSs Agar	Same as YpSs Broth plus 20.0 g agar

5. Growth of cultures

Inoculation cultures were prepared from stock axenic cultures (Clark and Dick 1974, Dick 1965) as follows. For *A. terrestris*, *P. cryptogea*, *P. paddicum*, *P. irregulare*, *P. diclinum*, *P. torulosum*, *P. anandrum*, *S. diclina*, *A. klebsiana*, and *L. caudata* an agar plug was transferred to a Petri plate containing V8 broth (2.5% V8 juice), and allowed to grow until a thick mycelial mat formed. The mat was transferred to 500 mL of V8 broth in a 1 L flask, shaken vigorously and the contents dispensed into Petri plates. These were allowed to grow until thick mats formed. Six mats were used to inoculate 2 L baffle flasks containing 500 mL of PYG medium (Griffin et al. 1974) which were allowed to grow for three to five days with vigorous agitation. Room temperature (22 - 26^{o}C) was used as the growth temperature for all the above listed cultures except *A. terrestris* which was grown at 18 - 20^{o}C. *A. pyrifera*, *A. brachynema*, and *S. elongatus* were grown in petri plates containing PYGS pH 6.6 (Gleason 1968) and then used to inoculate shaker flasks containing PYGS. It was sometimes necessary to maintain the pH of cultures of *S. elongatus* by addition of NaOH. Cultures were shaken on a rotary shaker at 150 rpm at 28^{o}C during the incubation period. Mycelium was harvested by vacuum filtration through Whatman no. 1 filter paper. The mycelial mat was rinsed thoroughly with distilled water and then with isolation medium. The mycelium was stored at -40^{o}C when not used immediately. *H. catenoides* was grown in Petri plates on 1/4 YpSs agar and *R. apophysatus* on 1/2 YpSs agar (Emerson, 1941). Plates were inoculated from a sporulating culture, flooded with 5 mL of distilled water and incubated at room temperature until confluent growth occurred. The surface of the plate was scraped aseptically with a spatula and the dislodged material added to 500 mL of YpSs broth in a 2 L baffle flask. The flask was incubated at 28^{o}C on a rotary shaker at 150 rpm for 16 h. Cells were harvested by

filtration as described above. *S. punctatus*, *S. plurigibbosus*, and *C. confervae* were grown on plates of 1/2 YpSs agar while *G. semiglobiferus* was grown on plates of 1/2 YpSs plus 1% NaCl and handled as described above for *H. catenoides* and *R. apophysatus*, except that inocolum was grown in 500 ml PYG broth instead of YpSs.

6. Isolation of DNA

MtDNA was isolated from purified mitochondria of *R. apophysatus* by the method described previously (Boyd et al. 1984). All other DNA was prepared by phenol extraction and CsCl-bisbenzimide density gradient centrifugation as described by Garber and Yoder (1983) and as outlined below.

6.1 Buffers

Isolation Medium

0.35 M sucrose

50 mM EDTA

10 mM Tris

pH 7.4

20 ug/mL proteinase K added immediately before use

Lysis Buffer

150 mM NaCl

50 mM EDTA

10 mM Tris

pH 7.4

20 ug/mL proteinase K added immediately before use

TE Buffer

10 mM Tris-HCl

1 mM EDTA

pH 7.6

6.2 Method

Mycelial mats were frozen in liquid nitrogen and broken into small pieces with a mortar and pestle. Pieces of frozen mycelium (about 10 g) were added to liquid nitrogen in a precooled steel blender, and blended for about 30 s before the next addition of frozen mycelium. After all mycelium had been added, blending was continued for about 10 min until cells were broken. Liquid nitrogen was added as needed. Generally 20-40 g of frozen mycelium were used for a typical isolation.

After blending the liquid nitrogen was allowed to evaporate and the mycelium powder was gradually added to 100-150 mL isolation buffer and stirred vigorously until totally resuspended (about 10 minutes). The suspension was centrifuged at 3500 rpm for 10 min at 4^{o}C in a Sorvall SS-34 rotor. The supernatant was poured through layers of cheesecloth and centrifuged at 34,500 rpm in a Beckman 60Ti rotor for 30 min at 4^{o}C. The resulting pellets were resuspended in a total volume of 2.5 mL of ice-cold lysis buffer and homogenized gently with a tissue grinder to remove any lumps. The resulting suspension was densely turbid and was put into a 15 mL glass corex tube so lysis could be observed. The volume was estimated and SDS was added to a final concentration of 2%. The tube was mixed gently by rocking to promote lysis. If lysis was poor the tube was incubated for up to 40 min at 65^{o}C. The tube was then placed on ice for 5-10 min and centrifuged at 7,000 rpm for 10 min at 4^{o}C in a Sorvall SS-34 rotor.

The supernatant was phenol extracted by adding an equal volume of phenol and mixing by inverting the tube several times. The tube was allowed to sit several minutes and then centrifuged at 10,000 rpm for 10 min at room temperature in a Sorvall SS-34 rotor. The upper aqueous phase was placed in a clean tube and phenol extractions repeated until the interface was clear following centrifugation (3-4 times). The aqueous phase was then extracted once or twice with phenol/chloroform/isoamyl alcohol (25/24/1) and once with chloroform/isoamyl alcohol (24/1).

The aqueous phase was then transferred to a fresh tube, the volume determined, and CsCl added to give a refractive index of approximately 1.398 (approximately 1.25 g CsCl to 1 mL DNA suspension). Bisbenzimide was added to a final concentration of 25 ug/mL. The solution was loaded into a 16 x 79 mm polyallomer centrifuge tube, overlayered with paraffin oil to fill the tube, and centrifugation carried out at 50,000 rpm in an 80 Ti rotor (Beckman) for at least 16 h at 20^{o}C.

DNA bands were viewed under ultraviolet light and collected by puncturing the centrifuge tube with an 18 guage needle at the top and about 1/8 inch beneath the band and allowing the DNA to drip into a microfuge tube. The collected DNA bands were then extracted several times with an equal volume of isopropanol saturated with CsCl. Three volumes of TE buffer were added to the DNA solution to dilute the CsCl. The DNA was precipitated by adding .08 volumes of 5 M LiCl and two volumes ethanol. After 2 h at 20^{o}C the DNA was centrifuged at 10,000 rpm for 15 minutes in a Sorvall SS-34 rotor. The pellet was dried and resuspended in 50-200 uL of TE buffer.

7. Hybridizations

pMF2 plasmid DNA was prepared from *E. coli* C600SF8 (Free et al. 1979) and *pMAK* plasmid DNA from *E. coli* HB101 (Boyd 1986) using the preparative

method of Birnboim and Doly (1979). Plasmid DNA was nick translated according to the method of Rigby et al. (1977). Southern blots were prepared using Gene Screen Plus (New England Nuclear Research Products) according to the manufacturer's instructions. Prehybridization was done at 60°C by agitating the membrane for six hours in prehybridization fluid (1% SDS and 1 M NaCl). The probe was denatured and then added to the prehybridization fluid. Incubation was carried out at 60°C with constant agitation for 16-24 hours. After hybridization the filters were washed twice in 2x SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate) at room temperature for 10 minutes each. The filters were washed twice in 2x SSC and 1% SDS at 60°C with constant agitation for 30 minutes. Autoradiography was carried out for 24-48 hours at -70°C.

8. DNA digestion and electrophoresis

Restriction enzymes were obtained from Boehringer Mannheim or Bethesda Research Laboratories (BRL) and digestions were performed according to the manufacturer's instructions or using the Maniatis three buffer system (Maniatis et al. 1982). Electrophoresis was carried out in Tris/borate buffer (0.089 M Tris, 0.089 M boric acid, 2.5 mM EDTA) using 0.5 - 1.2% agarose submarine gels. The BRL 1 kb and high molecular weight (HMW) ladders were used as standards for determining fragment sizes. Gels were stained for 20 minutes in 0.5 ug/mL ethidium bromide so that the DNA bands could be seen under ultraviolet light.

9. Field inversion gel electrophoresis (FIGE)

FIGE (Carle et al. 1986) was performed by using a conventional Biorad DNA "subcell" apparatus with a 15 x 15 x 0.4 cm horizontal 1% agarose slab gel. The buffer (0.5 x TBE, 0.05 ug/mL ethidium bromide) (1 x TBE: 0.089 M Tris, 0.089 M boric acid, 2.5 mM EDTA) was circulated over the gel at 150 mL/min and the temperature was regulated by circulation through a glass coil submerged

in a water bath at 20°C. The gel was run at 8.33 V/cm (250 V) with a buffer volume such that the current was 100 mA. Current from a power supply was routed through a KA14DG 6V relay (Electronics designed by Richard Hamel, University of Manitoba) which was controlled from the printer port of an IBM PC computer using a simple program in BASIC. Samples were run in the forward direction for 10 minutes before the pulsing program was activated. For resolution of bands in a narrow size window, a fixed forward and reverse pulse time was used, but for increased resolution over a wide range of sizes, a ramp of continuously increasing pulse times was used. Forward pulses were always twice as long as reverse pulses.

10. Localization of mitochondrial large ribosomal gene

The large ribosomal gene was localized by probing blots of restriction digestions from each species with a recombinant plasmid probe (*pMAK*) from *Achlya klebsiana* mitochondrial DNA containing the large ribosomal gene. The probe and hybridization methods were as described above.

11. Construction of physical maps of mitochondrial DNA

Mapping of the mtDNA was done directly by analysis of single and double restriction digests of the DNA in all cases except for *R. apophysatus* and *H. catenoides*. Preliminary digestion of the mtDNA of both these organisms revealed that the band patterns were too complex to be mapped by analysis of single and double digestions. The method used involved radioactive labelling of a set of restriction fragments and use of each fragment as a hybridization probe to detect overlapping fragments from other sets of restriction fragments. *Eco*RV fragments from *R. apophysatus* and *Bam*HI fragments from *H. catenoides* mtDNA molecules were found to be most suitable as mapping probes. Two methods of preparing the probes were used. In the first method the set of *Eco*RV fragments was nick translated (Rigby et al. 1977) and run on an agarose

gel. Bands were cut out of the gel and the agarose plugs were melted in SSC (0.15 M sodium chloride, 0.015 M sodium citrate), boiled for 10 min to denature the DNA, and then added to the hybridization fluid. The second method involved digestion (*Bam*HI) and electrophoresis of unlabeled DNA followed by purification of individual bands from the agarose using GENECLEAN (BIO 101, Inc.), following the manufacturer's instructions. DNA from each band was then nick translated and used as a probe. The latter method was more tedious but results were generally better since the probes were free of agarose and more radioactive. Hybridizations were done at 65^{o}C as previously described, except that an additional two washes (.1 X SSC for 30 min at room temperature) were done before autoradiography.

12. Restriction mapping of ribosomal DNA repeat unit

The DNA used for mapping consisted of purified rDNA or DNA enriched for rDNA from CsCl gradients, or total bulk DNA purified by phenol extraction. Mapping was done by analysis of single and double restriction enzyme digestions of the DNA preparations containing the rDNA repeating unit. Where rDNA preparations were relatively pure, rDNA was analysed directly by examination of restriction digestions from ethidium bromide-stained gels. Restriction digestions of bulk DNA were visualized by hybridization to *pMF2*.

RESULTS

RESULTS

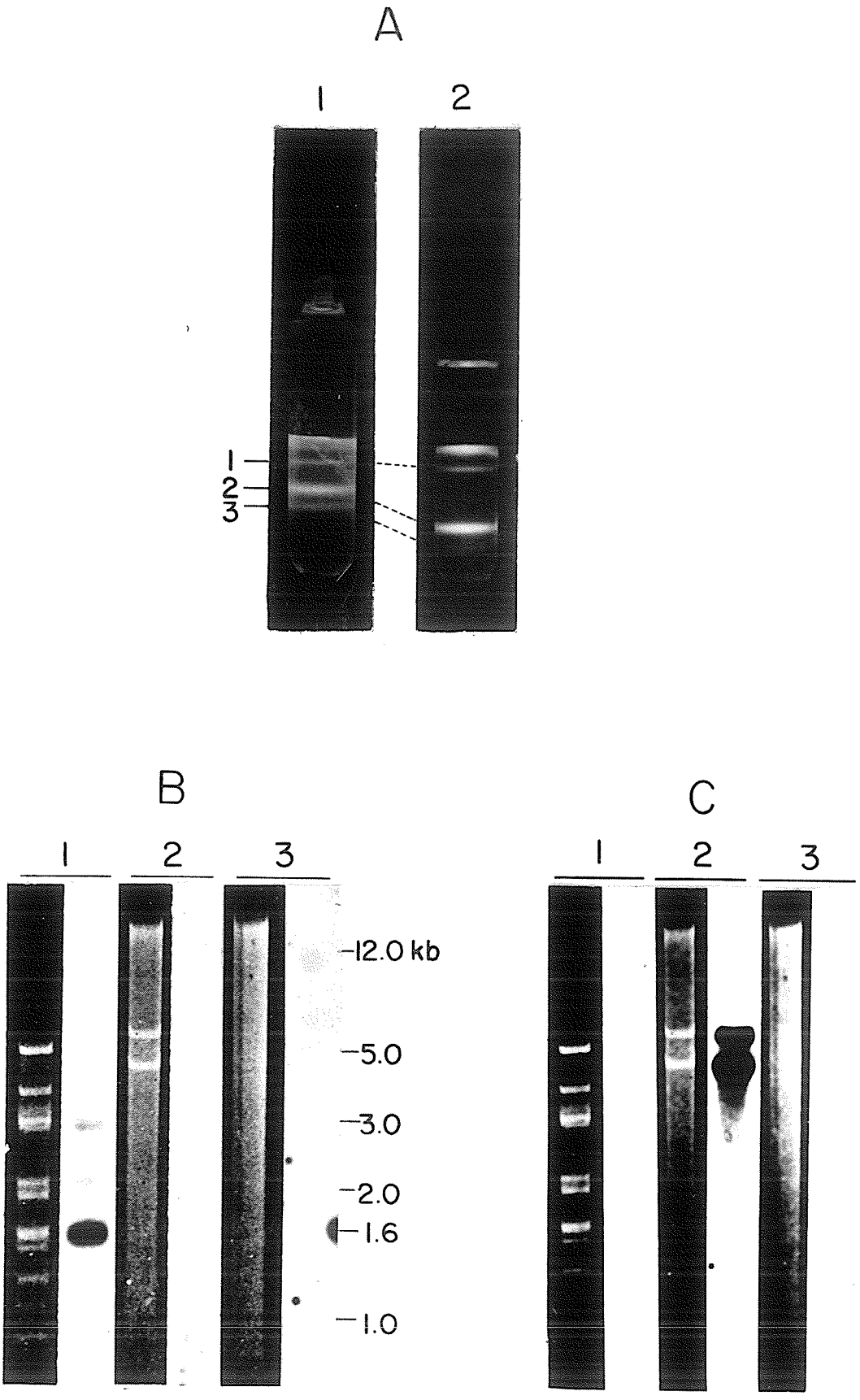
1. Isolation and identification of DNA bands from CsCl-gradients.

The fractionation of *Pythium* DNA by means of CsCl-bisbenzimide gradients resulted in the resolution of three bands as shown for *P. diclinum* and *P. torulosum* (Fig 2A). The top and bottom bands fluoresced blue while the middle band fluoresced green. This separation and colouration pattern has been observed for all species of *Pythium* studied to date. The width and intensity of each band on the gradient varied between different species and even between different preparations of the same species. In general the middle band was the broadest and most intense, but the relative amount of DNA recovered from each band could not be predicted from these appearances. The colour differences and lack of linearity between fluorescence and DNA concentration in bands may be due to spectral shifts occurring when bisbenzimide binds to DNA molecules of varying AT richness (Muller and Gautier, 1975).

DNA recovered from the three bands was digested with *Hin*dIII and the fragments separated by agarose gel electrophoresis (Fig 2B and 2C). DNA from the top and middle CsCl bands produced discrete restriction bands, indicating low complexity, while DNA from the bottom band produced no discernible restriction bands, indicating that the DNA was of high complexity, characteristic of chromosomal DNA.

The uppermost band in the CsCl-bisbenzimide gradient tube was shown to be mitochondrial by hybridization to a portion of the mtDNA of *A. klebsiana.* The probe (*pMAK*) was constructed by the ligation of a 4.9 kb *Bcl*I fragment from *A. klebsiana* mtDNA (Boyd et al. 1984) into the tetracycline resistance gene of *pBR322.* The cloned *Bcl*I fragment is totally within the inverted repeat region

Figure 2. Identification of ribosomal and mitochondrial DNA in *Pythium* from CsCl-bisbenzimide gradients. A: Typical banding patterns of *Pythium* DNA on CsCl-bisbenzimide gradients. 1) *P. diclinum*, 2) *P. torulosum.* B: *Hin*dIII digestion of DNA bands of *P. diclinum* as shown in A and hybridization to the mitochondrial probe *pMAK.* Lane 1, top band; lane 2, middle band; lane 3, bottom band. Size estimations were based on the Bethesda Research Laboratories 1 kb ladder. C: *Hin*dIII digestions of DNA bands of *P. diclinum* as in B, but hybridized to the ribosomal probe *pMF2.* Lanes are as in B. Sizes are not shown, but are as shown in B.


A
1
2
1
2
3
B
1
2
3
12.0 kb
5.0
3.0
2.0
1.6
1.0
C
1
2
3

in *Achlya* and overlaps a region of the mtDNA which contains the large ribosomal gene. The results of attempted hybridization of the probe to *Hin*dIII digests of DNA from all three CsCl-bisbenzimide bands is shown in Fig 2B. Strong hybridization of the probe to several bands of DNA from the top CsCl-bisbenzimide band confirms the mitochondrial origin of that band. The low buoyant density of *Pythium* mtDNA in CsCl-bisbenzimide is in agreement with findings in other fungi (Garber and Yoder 1984, Raeder and Broda 1984, Specht et al. 1983).

Satellite bands similar to the middle band found for *Pythium* species have been described for other fungi and have in several cases been found to consist of rDNA repeating units (Garber and Yoder 1983, Raeder and Broda 1984, and Specht et al. 1984). To determine whether this was the case for *P. diclinum*, the gel (Fig 2C) was blotted to hybridization membrane and the DNA probed with *pMF2*, a derivative of *pBR322* containing coding regions of the rDNA repeating unit from *Neurospora crassa* (Free et al. 1979). The results (Fig 2C) indicate that nearly all of the DNA of *P. diclinum* homologous to *N. crassa* rDNA resides in the 5.7 and 4.6 kb *Hin*dIII fragments derived from the middle CsCl band. Faint hybridization to the bottom band DNA at 5.7 and 4.6 kb indicates either that the bottom band was contaminated with middle band DNA or that a small fraction of the rDNA remains associated with chromosomal DNA. The middle band in *Pythium* species is far more prominent than corresponding bands from other fungi (Garber and Yoder 1983).

Other fungal species investigated in this study produced only two major DNA bands on CsCl density gradients, both bands fluorescing blue in color. The top band in each case was shown to be mitochondrial in origin by hybridization to the mitochondrial probe *pMAK*. The bottom band was in some cases very broad, and if collected as one fraction, produced no discernible bands indicating

that it was high complexity chromosomal DNA. However, if this band was collected in several fractions, the upper fraction of the band often contained DNA enriched for rDNA so that discrete bands were produced upon restriction. This might indicate that a satellite band could have formed if the gradient conditions had been altered to achieve better resolution of bands varying only slightly in AT content.

2. Field inversion gel electrophoresis

In order to derive physical maps by the double digestion method, the sizes of restriction fragments must be accurately determined. Fragments up to 10 kb in size were determined by conventional electrophoresis, but larger fragments, which are often generated during digestion of 50 kb mitochondrial genomes, were determined by pulsed field electrophoresis. In order to resolve fragments between 10 and 50 kb by using the conditions described under Materials and Methods, it was determined that pulse times of 0.2 to 0.6 sec in the forward direction and half these times in the reverse direction were needed. Constancy of temperature and voltage gradient were critical because the window of resolution depended on these parameters. Agarose concentration was not important. A ramp of pulse times from 0.2 to 0.6 seconds was used to increase resolution of bands in the entire range from 10 to 50 kb in a single gel. Typical results using this technique are shown Fig 3.

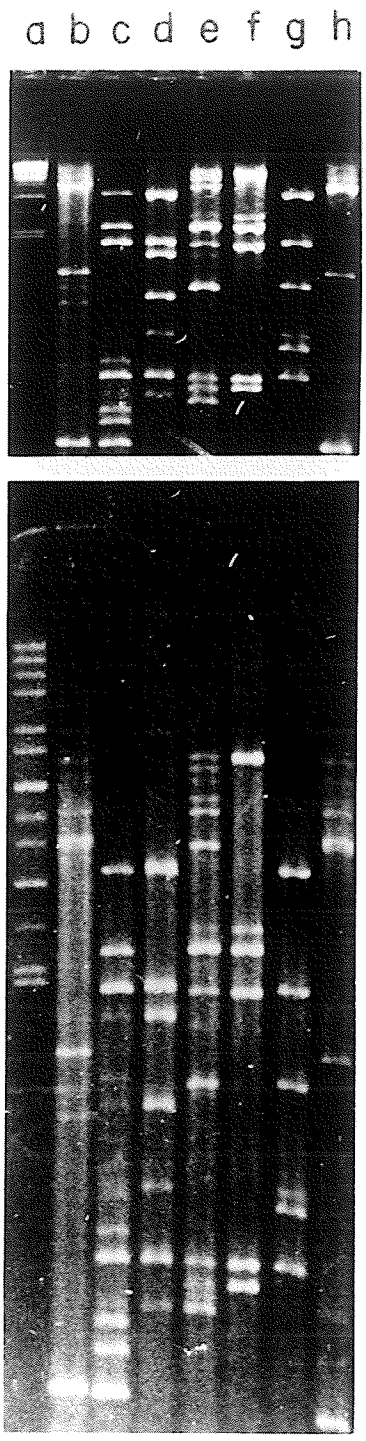
3. Physical maps of mtDNA

3.1 Oomycetes mtDNA maps

3.1.1 Saprolegniales - *Aplanopsis terrestris* and *Leptolegnia caudata*

Physical maps for three species of *Achlya* have been reported (Hudspeth et al. 1983, Boyd et al. 1984, Shumard et al. 1986). To extend the survey of mitochondrial genomes within the Saprolegniales, mtDNA was isolated from *A. terrestris* and a physical map constructed (Fig 4). The genome was found to be a

Figure 3. Conventional and field inversion gel electrophoresis (FIGE) of restriction fragments of *A. terrestris* mtDNA. Top: Conventional electrophoresis in 0.8% agarose at 75 V for 6 h. a) Bethesda Research Laboratories high molecular weight ladder b) *Sst*I c) *Sst*I + *Eco*RV d) *Eco*RV e) *Sst*I + *Bgl*II f) *Bgl*II g) *Eco*RV + *Sal*I h) *Sst*I + *Sal*I. Bottom: Field inversion gel electrophoresis of the same gel performed after conventional electrophoresis. The gel was run at 250 V in circulating 0.5 X TBE buffer. Pulsing protocol consisted of a continuous ramp of pulse times beginning with 0.2 sec in the forward direction and 0.1 sec in reverse and ending with 0.6 sec forward and 0.3 sec reverse. The run time was 16 h. Sizes of BRL standards in lane a are 48.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4 (bright), 17.1, 15.0, 12.2 (bright), 10.1, 8.6, and 8.3 kb.

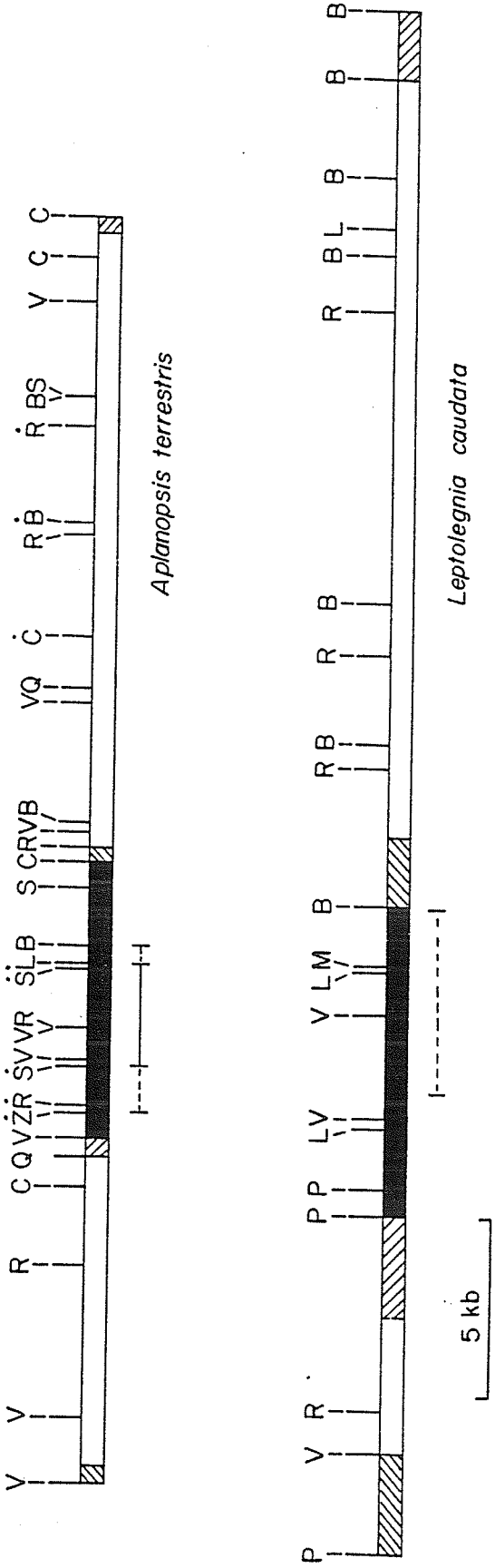

a b c d e f g h

44.5 kb circle with an inverted repeat (IR) of 8.0 to 8.9 kb placed so that the molecule is divided into smaller and larger single-copy regions. Although this genome size (45 kb) is significantly smaller than that of *A. klebsiana* (50 kb), the position and size of the IR are similar, and eight of the restriction sites in the IR region could be aligned with corresponding sites in *Achlya* mtDNA (Fig 4). This high level of homology implies that the LrRNA gene is near the large single-copy region, as it is in *Achlya* (a comparison of Oomycete maps show that there is a highly conserved *Sst*I site adjacent to a *Sal*I site towards one end of the LrRNA gene). Hybridization with *pMAK*, however, indicated a central position within the IR (Fig 4). This discrepancy may be due to the fact that the LrRNA is not well defined within *pMAK* and that location of the gene by hybridization is approximate.

To extend the survey of Saprolegniales, the mtDNA map for *L. caudata* was determined (Fig 4). The genome size was 52.4 kb with an IR of 8.7 to 13.5 kb. As a minimum of sites were used to obtain results rapidly, the size of the IR was determined with reduced accuracy, but the general proportions of single-copy and IR regions was consistent with that of the other members of the Saprolegniales. The genome size was slightly greater than that for *A. klebsiana.* The position of the LrRNA gene was not determined by hybridization. However the position of this gene can be determined by alignment with *Achlya* maps and identification of highly conserved sites within the gene (*Sal*I adjacent to *Bam*HI, Shumard et al. 1986).

Flip-flop isomerism as has been found in *Achlya* species (Hudspeth et al. 1983, Boyd et al. 1984, Shumard et al. 1986) was not investigated in *A. terrestris* or *L. caudata.* In order to identify flip-flop isomerism by restriction mapping it is necessary to use an enzyme or combination of enzymes which cut in both the small and large unique regions but not in the repeats. None of the enzymes

Figure 4. Linearized maps of mtDNA in Saprolegniales: *A. terrestris* and *L. caudata* shown without one arm of the minimum inverted repeat (IR). Solid shading indicates minimum extent of the IR and cross-hatching indicates uncertainity in the maximum extent of the IR. Open bars indicate regions of unique sequence. The solid line underneath the *A. terrestris* map indicates restriction fragments which hybridize strongly to *pMAK*, a plasmid containing the large RNA gene from *Achlya klebsiana* mtDNA. The dots above the restriction sites on the *A. terrestris* map indicate sites which can be aligned with sites in *Achlya* species (Shumard et al. 1986, Boyd et al. 1984). Dashed lines indicate fragments hybridizing weakly to *pMAK*. The dashed line under the *L. caudata* map indicated the position of the LrRNA gene based on alignment with *Achlya* maps (Shumard et al., 1986). Restriction enzyme symbols: A - *Ava*I, B - *Bgl*II, C - *Cla*I, E - *Bst*EII, H - *Hin*dIII, L - *Sal*I, M - *Bam*HI, P - *Pvu*II, Q - *Pst*I, R - *Eco*RI, S - *Sst*I, V - *Eco*RV, Z - *Sst*II.


Aplanopsis terrestris
Leptolegnia caudata
5 kb

used to map either *A. terrestris* or *L. caudata* were suitable to detect flip-flop isomerism. However, the two repeat arms in both species are identical indicating that recombination has been occurring, presumably by the flip-flop mechanism.

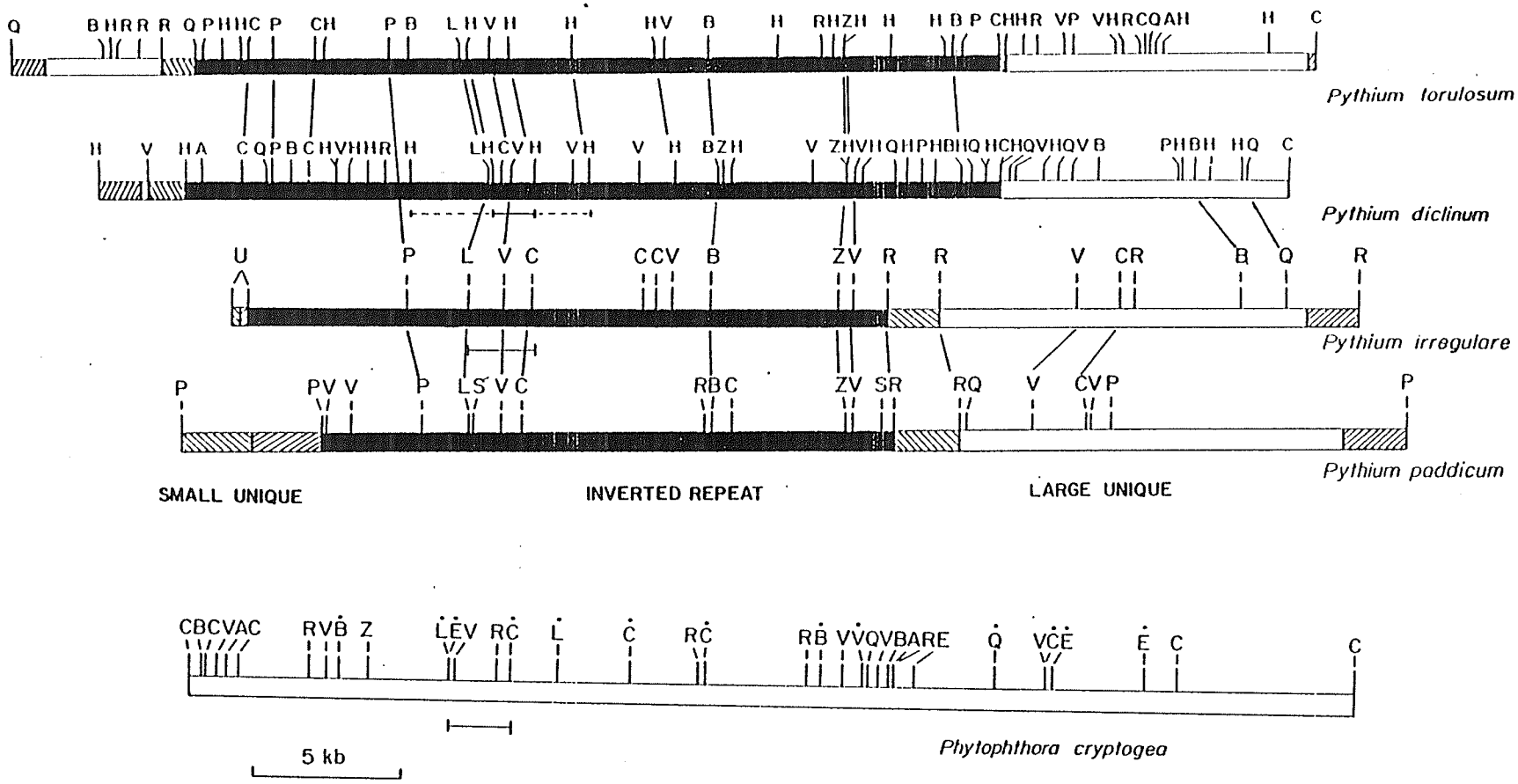
3.1.2 Peronosporales - *Pythium* species and *Phytophthora cryptogea*

Pythium diclinum, *Pythium torulosum*, *Pythium paddicum* and *Pythium irregulare*

Physical maps of *P. diclinum* and *P. torulosum* mtDNA were constructed with ten different restriction enzymes. *Hin*dIII produces more than 30 bands from both *P. diclinum* and *P. torulosum* mtDNA, many of which are less than 1 kb in size. Consequently, some of the smaller *Hin*dIII fragments which were not cut in any double digestion were not included on the maps. However, since *Hin*dIII sites helped to define the minimum and maximum lengths of the repeats, those sites which could be mapped with reasonable certainty were included.

Aligned physical maps for *P. diclinum* and *P. torulosum* are presented in Fig 5, each one showing only one of the repeat arms. The maps do not have discrete termini and are obviously circular. The size of *P. diclinum* mtDNA was found to be 70.0 + 1.9 kb while *P. torulosum* mtDNA was found to be slightly larger -- 73.0 + 1.5 kb. The size of the repeat region in *P. diclinum* was 28.2 to 29.5 kb while that of *P. torulosum* was found to be 27.6 to 29.1 kb. The sequence complexity of the remaining unique regions of each of the two mitochondrial genomes is then only about 12.4 and 16.5 kb respectively. This is less than half the complexity of the nonreiterated regions of *Achlya* mtDNA (Hudspeth et al 1983, Boyd et al 1984). As these two species, *P. diclinum* and *P. torulosum*, are closely related (M.W. Dick, personal communication), two other species were investigated (Fig 5). *P. paddicum* was found to have a 61.3 kb circular mitochondrial genome with a large IR (19.4 - 24.1 kb) and a reduced or absent

Figure 5. Linearized maps of the mtDNA in Peronosporales: *P. torulosum*, *P. diclinum*, *P. irregulare*, *P. paddicum*, and *P. cryptogea*. See the legend in figure 4 for interpretation. The symbol U above the small unique region in the *P. irregulare* map indicates uncertainty about whether this region contains the terminus of a linear molecule or whether it contains sites for all the enzymes used in constructing the map. Alignment of conserved sites between *Pythium* species are indicated by solid lines. Dots above the enzyme symbols in the *P. cryptogea* map indicates those sites which appear to be homologous to corresponding sites in the published mtDNA maps of *P. megasperma* and *P. parasitica* (Förster et al., 1987).


Pythium torulosum
Pythium diclinum
Pythium irregulare
Pythium paddicum
SMALL UNIQUE
INVERTED REPEAT
LARGE UNIQUE
Phytophthora cryptogea
5 kb

small single copy region. *P. irregulare* mtDNA was very similar (60 kb size, 21.7 - 23.8 kb IR). The region containing the LrRNA gene was identified by hybridization of *pMAK* to the central region of the IR in *P. irregulare* and *P. diclinum*. Location of the LrRNA gene in *P. paddicum* and *P. torulosum* was on the basis of alignment with the other two *Pythium* maps. The *Sal*I and *Eco*RV sites in this region are conserved in all four *Pythium* species. Preliminary alignment of maps shows that similarity is most pronounced along the IR, where the single *Bgl*II and *Sst*II sites are also conserved in all *Pythium* species.

Flip-flop isomerism such as that seen in *Achlya* (Boyd et al. 1984) was not investigated in *Pythium* due to the small size of the unique regions and the absence of suitable restriction sites. The two repeat arms in both species are identical, however, indicating that recombination has been occurring, presumably by the flip-flop mechanism.

The restriction data obtained for *P. irregulare* (table IV) may indicate that this genome is unusual. Many digests were found to total to a size larger than the 60 kb size shown in Fig 5. In each restriction digest a band approximately twice the size of a smaller band was found. When the "half size" bands were omitted from the summation of fragment sizes a consistent value of approximately 60 kb was obtained using various restriction enzymes. The larger bands always mapped in the same position as the bands half their size, the smaller band ending at the small unique region and the larger band spanning the small unique region.

One possible explanation for this data would be partial digestion of the DNA possibly due to some unusual configuration of the DNA in the small unique region. However this would mean that the small (0.5 kb or less) unique region would have to contain sites for all enzymes mapped. This possibility seems rather unlikely. Another more plausible explanation which would fit the

Table IV *P. irregulare* mtDNA digests suggesting molecule may exist in two forms (sizes in kb)

	*Cla*I	*Sal*I	*Pvu*II	*Bgl*II	*Eco*RV
	19.6#	44	49	34.0#	11.5
	16.2	16.2#	11.7#	19.5	8.5
	16.2	7.85*	5.7*	17.5*	8.5
	9.6*			10.3	7.7
	3.7				6.05
	3.7				6.05
					5.90
					5.90
					<1.0#
Totals	69.0	68.05	66.4	81.3	61.1

bands generated assuming a circular version of the map
* bands generated assuming a linear version of the map

data presented in Table IV and the map in Fig 5 would be that the molecule exists in both a linear and circular form. The IRs would be at the termini of the linear form and adjacent to each other in the circular from. A very tiny small unique region may or may not exist.

The one digest that does not seem to fit this theory is the *Eco*RV digest which sums to only 61 kb without excluding any bands. The smallest band given in Table IV has not been mapped and is not shown in Fig 5. There is an *Eco*RV site near the end of the IR in *P. paddicum* and therefore it is quite likely that there is an *Eco*RV site near the end of the IR in *P. irregulare* as well. The small band in Table IV may actually be generated from the circular version of the map. The less than 0.5 kb band predicted for the linear version of the map may have been too small to have been observed on the gels run. Thus the *Eco*RV digest could be considered consistent with the other data presented.

It is not known which form or if both forms coexist in vivo. Although linear mitochondrial genomes are rare at least two other examples of fungi with linear mitochondrial genomes have been reported (Wesolowski and Fukuhara 1981, Kovac et al. 1984).

Phytophthora cryptogea

Phytophthora mitochondrial genomes differ from those in *Pythium* in that they do not have the IR (Klimczak and Prell 1984, Förster et al. 1987). This was confirmed in *P. cryptogea*, which was found to have a circular mtDNA of 41 kb with no IR (Fig 5). The LrRNA region was identified by alignment to the *Phytophthora* species mapped by Förster et al. (1987). Using that region as a reference point, it was possible to align a 4 kb region containing the sequence *Sal*I - *Bst*EII - *Cla*I - *Sal*I with the *P. infestans* map (Klimczak and Prell 1984), and with the *P. megasperma* and *P. parasitica* maps (Förster et al. 1987). There are 13 sites (indicated by dots above enzyme symbols in Fig 5) in a 28 kb

central region of our *P. cryptogea* map that align with other *Phythophthora* mtDNA maps (Dr. D. Maxwell, personal communication). This is roughly the same region which in *Pythium* represents one arm of the IR.

3.1.3 Leptomitales - *Apodachlya pyrifera* and *Apodachlya brachynema*

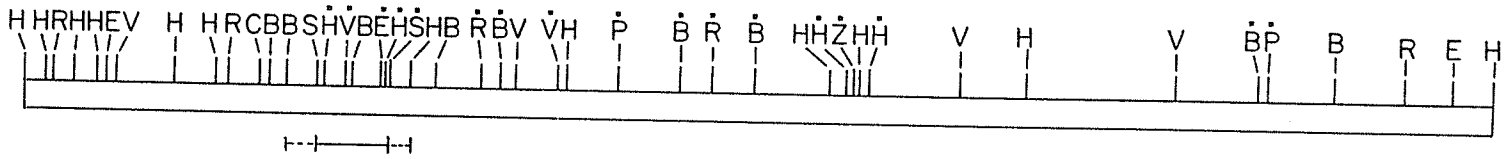
A. pyrifera and *A. brachynema* were investigated as representatives of the Leptomitales. Both genomes were found to be circular with sizes of 39 and 36 kb respectively, and containing no IRs (Fig 6). The LrRNA region was located in *A. pyrifera* by hybridization to *pMAK*. Alignment of the two maps was possible on the basis of 17 apparently homologous sites, five of them within the LrRNA region. The conserved region (indicated with dots above enzyme symbols in Fig 6) is like the conserved region in *Phythophthora* in that it is confined to a contiguous region of the map which has the LrRNA gene at its leftward end.

3.1.4 Rhipidiales - *Sapromyces elongatus*

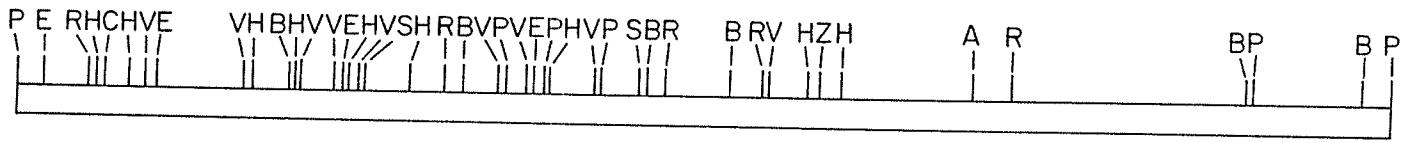
S. elongatus has been reclassified and now is considered to be a member of the Rhipidiales (Dick et al. 1984). Its mitochondrial genome was found to be a 51 kb circle with a 14.6 - 16.2 kb IR (Fig 6). The region hybridizing to the LrRNA probe is adjacent to the small single copy region, an arrangement not yet found in any other member of the Oomycetes.

Two orientational isomers of the mtDNA have been observed. A double digest with enzymes *Pst*I and *Pvu*II cut in both unique regions but not the repeats. Four bands were produced which appeared to be in half molar concentration with respect to other bands (Fig 7). These results can be explained by assuming the existence of two orientational isomers as has been observed in *Achlya* species (Hudspeth et al. 1983, Boyd et al. 1984). These four bands were present in equal intensity indicating that the two isomers are present in equal proportions.

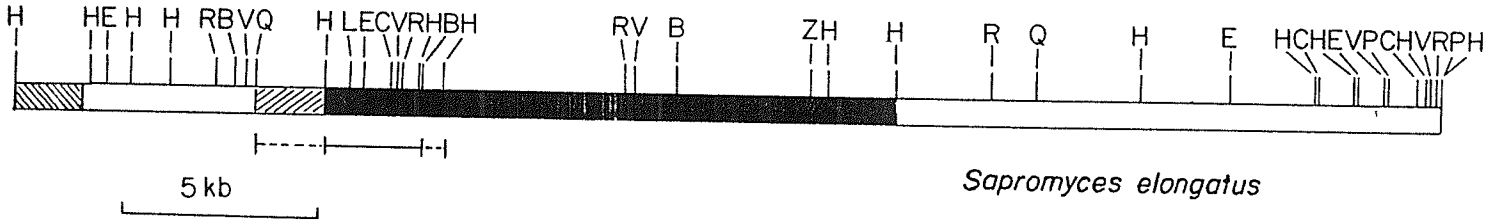
Figure 6. Linearized maps of the mtDNA in *A. pyrifera*, *A. brachynema*, and *S. elongatus*. See legend of figure 4 for interpretation. Dots above the enzyme symbols indicate sites which correspond to putative homologous sites in the *A. pyrifera* and *A. brachynema* maps.



Apodachlya pyrifera

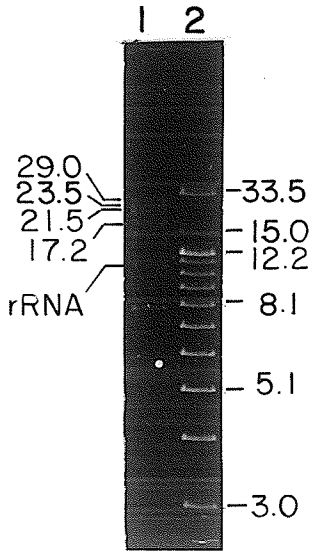


Apodachlya brachynema



Sapromyces elongatus

Figure 7. Digest of *Sapromyces elongatus* mtDNA to visualize flip-flop isomerism. 1. *Pst*I X *Pvu*II digest of *S. elongatus* mtDNA. 2. Bethesda Research Laboratories 1 kb ladder plus a lambda *Xho*I digest. Electrophoresis was through a 0.6% agarose gel. The four bands in lane 1 for which sizes are given are presumed to result form the presence of two orientational isomers. The band at approximately 10 kb is ribosomal DNA containing the mitochondrial DNA preparation. In addition to the bands seen on this gel, there is a 1.45 kb band which ran off the bottom.


1
2
29.0
23.5
21.5
17.2
rRNA
33.5
15.0
12.2
8.1
5.1
3.0

3.2 Hyphochytriomycetes - *H. catenoides* and *R. apophysatus*

The physical maps of mtDNA from the two species of Hyphochytriomycetes are presented (Fig 8). *H. catenoides* was found to have a circular 54 kb mtDNA with a 13.5 - 14.5 kb IR. Approximate genome complexity (genome size minus one arm of the IR) was 39.5 - 40.5 kb. The LrRNA gene region was found adjacent to the small unique region. The difference in size between the small and large unique regions was not as pronounced as it is in Oomycetes which have the IR. Two orientational isomers of the *H. catenoides* mitochondrial genome were observed by examination of an *Eco*RV digest which shows four half molar bands (Fig 9). *Eco*RV has multiple sites in the unique regions but no sites in the IR. This finding is consistent with the flip-flop isomerism found in Oomycete mitochondrial genomes possessing IRs. *R. apophysatus* had a circular genome of 50 kb with no IR. The LrRNA region was located on the map, but no obvious restriction site alignment with the *H. catenoides* map was possible.

3.3 Chytridiomycetes - *Spizellomyces punctatus* and *Spizellomyces plurigibbosus*

Physical maps of the mtDNA of *S. punctatus* and *S. plurigibbosus* were not constructed. However restriction profiles were done to get a size estimation and to look for double bands which would indicate the presence of an IR (see Table V for data). The size of the mitochondrial genome is roughly estimated at 62.2 kb in *S. punctatus* and 63 kb in *S. plurigibbosus*. Restriction profiles were not suggestive of an IR although some double bands were observed in a HindIII digest of *S. plurigibbosus* mtDNA. The possibility of an IR cannot be ruled out without construction of a detailed restriction map.

Figure 8. Linearized maps of mtDNA of *H. catenoides* and *R. apophysatus* shown without one arm of the minimum inverted repeat (IR). See legend in figure 4 for interpretation.

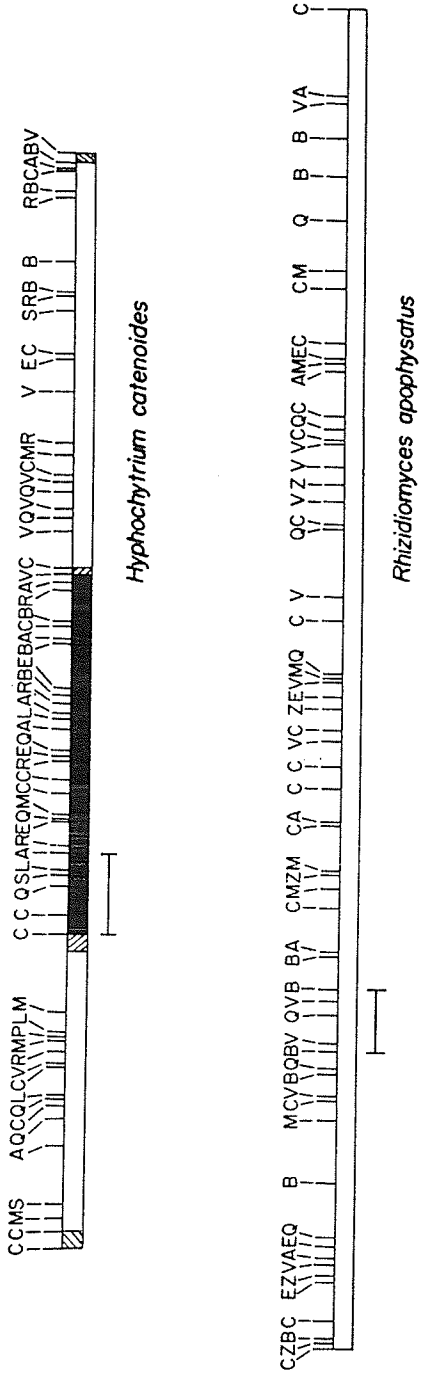

CCMS AQCQLCVRMPLM C C QSLAREQMCCREQALARBEBACBRAVC VQVQVCMR V EC SRB B RBCABV
Hyphochytrium catenoides
CZBC EZVAEQ B MCVBQBV QVB BA CMZM CA C C VC ZEVMQ C V QC VZY VCQC AMEC CM Q B B VA C
Rhizidiomyces apophysatus

Figure 9. Digest of *Hyphochytrium catenoides* mtDNA to visualize flip-flop isomerism. 1. Bethesda Research Laboratories 1 kb ladder. 2. Bethesda Research Laboratories high molecular weight ladder. 3. *Eco*RV digest of *H. catenoides* mtDNA. The four bands in lane 3 for which sizes are given are presumed to result from the presence of two orientation isomers. Electophoresis was through a 0.8% agarose gel at 300 V using FIGE.

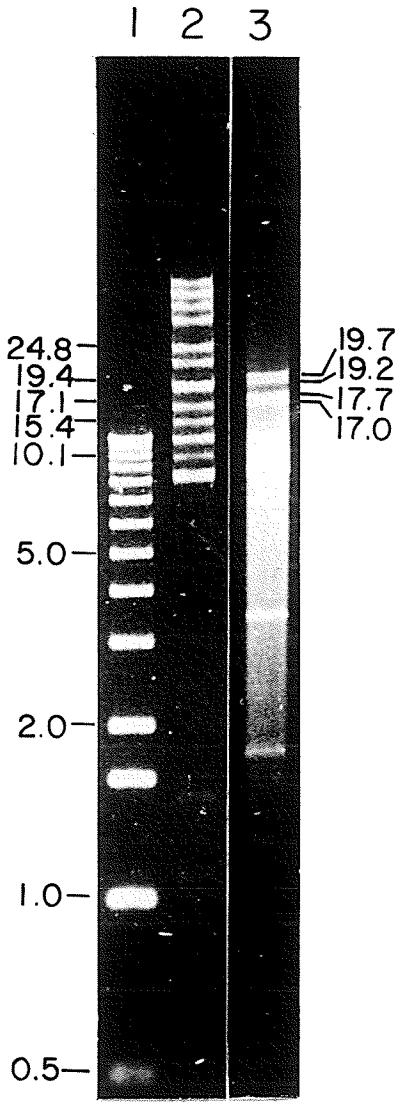

1
2
3
24.8
19.4
17.1
15.4
10.1
5.0
2.0
1.0
0.5
19.7
19.2
17.7
17.0

Table V Mitochondrial DNA Restriction Digestions of *Spizellomyces*

A. *Spizellomyces punctatus* mitochondrial digests

	*Eco*RV	*Pvu*II	*Bam*HI	*Bgl*II
	19.7	28.9	21.5	13.0
	11.0	11.9	21.0	9.8
	8.9	7.2	6.45	8.1
	7.0	6.0	3.60	4.1
	5.3	3.85	3.20	3.2
	4.2	2.90	3.15	3.2
	2.08	1.37	1.94	2.42
	1.94	1.37	1.85	2.32
	1.34		1.83	2.00
	1.30			1.92
				1.27
				1.15
				0.94
				0.87
				0.87
Totals	62.76	63.49	64.52	58.24

Average: 62.2 kb

B. *Spizellomyces plurigibbosus* mitochondrial digests

	*Hin*dIII	*Eco*RI
	10.2	8.3
	10.2	7.6
	7.0	6.8
	5.9	6.6
	4.8	5.3
	4.05	4.7
	3.95	4.4
	3.25	4.25
	2.8	4.10
	1.75	2.40
	1.75	1.97
	1.60	1.85
	1.30	1.33
	1.17	1.17
	1.06	1.08
	0.80	0.95
	0.72	0.87
Totals	62.3	63.67

Average: 63 kb

4. Physical maps of rDNA

4.1 General Characteristics of rDNA repeat unit maps

Maps of rDNA were constructed for 26 species of fungi using the enzymes *Ava*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hin*cII, *Pst*I, *Sst*I, *Sst*II, *Xba*I, and *Bam*HI. When rDNA was not sufficiently purified to construct maps directly from digests visualized on EtBr stained gels rDNA bands were visualised by hybridization to the probe *pMF2*. Typical results of this hybridization are shown in Fig 10. Maps constructed by hybridization to the probe *pMF2* may not be complete in the IGS region since any bands totally within the IGS do not hybridize to the probe and therefore cannot be mapped by these methods. Physical maps of the fungi included in this study indicate that discrete termini do not exist, and thus that the molecule is either circular or consists of a number of tandem repeating segments. To show that tandem repeating units were present the rDNA of *P. diclinum* was partially digested with *Bgl*II, which cuts the sequence only once. A ladder of approximately 10, 20, and 30 kb bands was discernible on a 0.4% agarose gel (Fig 11). This indicates that at least three tandem repeats exist. This finding is consistent with other studies on rDNA which are commonly found in the form of tandem repeats (Long and Dawid 1980). Other characteristics (such as chromosomal or extrachromosomal location, redundancy within the cell) of the rDNA repeat were not investigated. The sizes of the repeat units ranged between 7.6 kb in *Zoophagus insidians* and approximately 15 kb in *Leptolegnia caudata* (see Table VI for complete listing).

The physical maps were aligned on the basis of a highly conservative 6 kb region which hybridizes to *pMF2*, the probe containing the gene region of the *N. crassa* rDNA repeat (Free et al. 1979). The maps are not drawn to reflect very slight size variations between conserved fragments in the gene region among

Figure 10. Digestion of *A. pyrifera* DNA from the bottom band of a CsCl-bisbenzimide gradient and hybridization to the ribosomal probe *pMF2*. A: Ethidium Bromide stained gel of *A. pyrifera* bottom band DNA Lanes: 1) and 15) Bethesda Research Laboratories 1 kb ladder; 2) *Bgl*II X *Eco*RI; 3) *Bgl*II; 4) *Eco*RI; 5) *Eco*RI X *Hin*dIII; 6) *Bgl*II X *Hin*dIII; 7) *Hin*dIII; 8) *Hin*dIII X *Pst*I; 9) *Pst*I; 10) *Pst*I X *Bgl*II; 11) *Pst*I X *Eco*RI; 12) *Pst*I X *Eco*RV; 13) *Eco*RV; 14) *Eco*RV X *Hin*dIII. B) Autoradiogram of Southern blot of gel in A hybridized to the ribosomal probe *pMF2*.

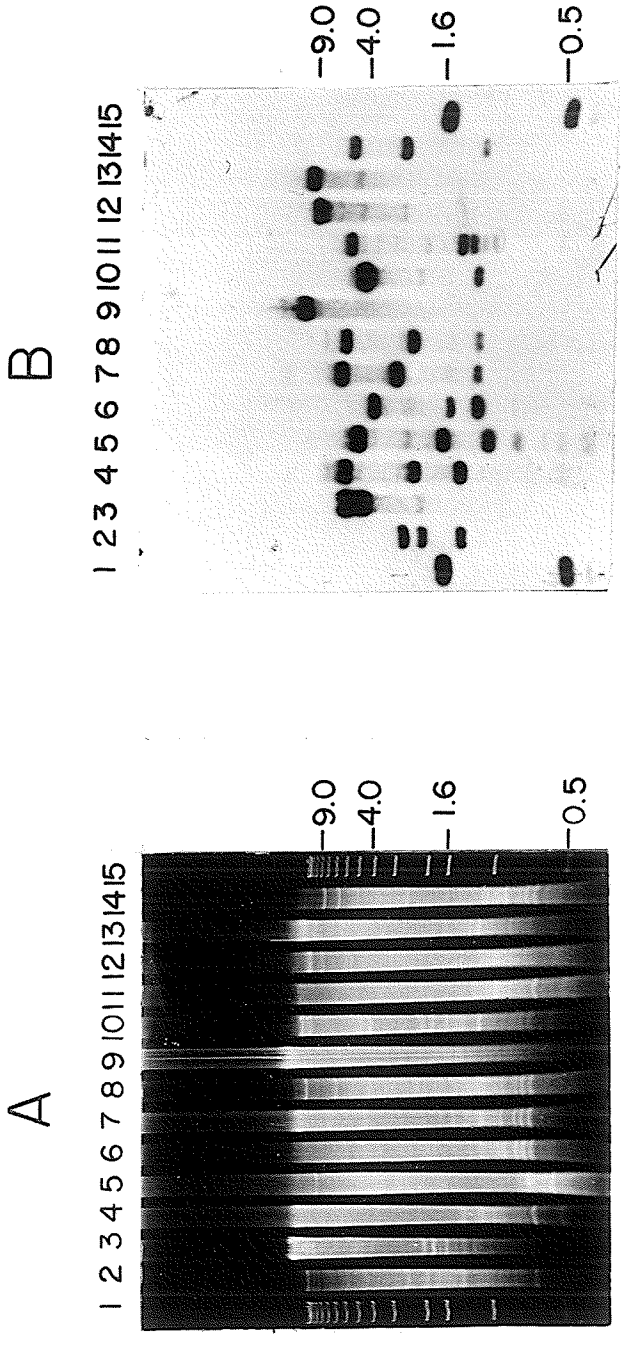

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
9.0
4.0
1.6
0.5
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
9.0
4.0
1.6
0.5

Figure 11. Partial *Bgl*II digestion of purified rDNA of *P. diclinum.* Lanes: 1) Bethesda Research Laboratories 1 kb ladder; 2) Lambda DNA digested with *Bgl*II; 3) Lambda DNA digested with *Sal*I; 4) Lambda DNA unrestricted; 5) *Bgl*II digestion of *P. diclinum* rDNA for 10 min; 6) *Bgl*II digestion of *P. diclinum* rDNA for 30 min; 7) Digestion of *P. diclinum* rDNA for 60 min. White dots indicate position of bands in partial digests - sizes are approximately 30, 20 and 10 kb.

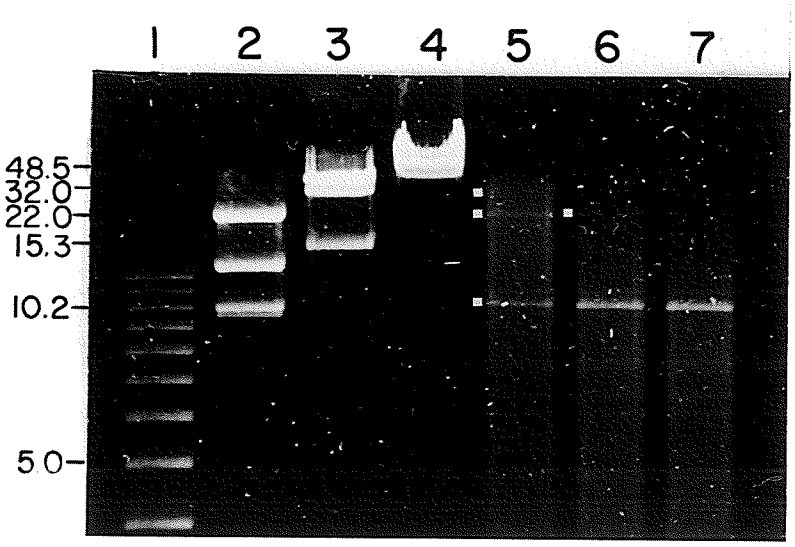

1
2
3
4
5
6
7
48.5
32.0
22.0
15.3
10.2
5.0

Table VI Repeat Unit Sizes of Zoosporic Fungi

Taxon	Species	Size (kb)	Proposed Classification	
			Order	Subclass
Oomycetes				
Peronosporales	*Pythium diclinum*	10.3	Pythiales	Peronosporomycetidae
"	*Pythium torulosum*	10.4	"	"
"	*Pythium paddicum*	10.6	"	"
"	*Pythium anandrum*	10.6	"	"
"	*Pythium irregulare*	11.7	"	"
"	*Phytophthora cryptogea*	11.2	"	"
"	*Zoophagus insidians*	7.6	Higher Fungi	
Lagenidiales	*Lagenidium giganteum*	10.6	Pythiales	Peronosporomycetidae
Saprolegniales	*Saprolegnia ferax*	10.1 - 10.7	Saprolegniales	Saprolegniomycetidae
"	*Saprolegnia diclina*	11.5	"	"
"	*Achlya klebsiana*	9.9	"	"
"	*Aplanopsis terrestris*	9.8	"	"
"	*Aphanomyces stellatus*	9.6	"	"
"	*Leptolegnia caudata*	> 14	"	"
Leptomitales	*Leptomitus lacteus*	10.5	Leptomitales	"
"	*Apodachlya pyrifera*	9.6	"	"
"	*Apodachlya brachynema*	10.0	"	"
"	*Sapromyces elongatus*	11.1	Rhipidiales	"
-	*Verrucalvus flavofaciens*	9.7 - 11.3	Sclerosporales	"
-	*Pachymetra chaunorhiza*	10.5	"	"
Hyphochytriomycetes				
	Rhizidiomyces apophysatus	10.0 - 14.0		
	Hyphochytrium catenoides	11.6		
Chytridiomycetes				
-	*Spizellomyces punctatus*	10.8	Spizellomycetales	
-	*Spizellomyces plurigibbosus*	9.3	"	
-	*Gaertneriomyces semiglobiferus*	8.2	"	
Chytridiales	*Chytridium confervae*	8.8	Chytridiales	

various species. Slight variations do exist but since these variations have not been precisely mapped the maps are all drawn with conserved sites exactly aligned (except where insertions or deletions are substantial). The gene locations were identified on the basis of highly conservative ancestral sites which exist for all three genes (SrRNA, 5.8S, and LrRNA) which are encoded by the repeat (see Discussion for explanation of ancestral sites). The IGS regions of the maps generally could not be aligned except in the cases of a few closely related species which will be discussed in more detail elsewhere. The presence of the 5S gene within the rDNA repeat unit was not investigated in this study.

4.2 Oomycete rDNA repeat unit maps

Peronosporales. Physical maps of rDNA from five *Pythium* species and *Phytophthora cryptogea* were constructed and a consensus map was constructed for the gene region (Fig 12). There were no *Bam*HI sites in *P. diclinum*, *P. torulosum*, and *P. paddicum*. The size of the rDNA repeating unit ranges from 10.3 kb (*P. diclinum*) to 11.7 kb (*P. irregulare*), with an average of 10.8 kb. No length heterogeneities were observed for the Peronosporales species surveyed. However, a partial digest of *P. irregulare* with the *Ava*I suggests that there may be a subrepeat within the IGS with a periodicity of between 400 and 500 bp and possessing and *Ava*I site (data not shown). Further experimentation is necessary to confirm the presence of this subrepeat and to determine the exact size.

Saprolegniales/Leptomitales. The maps for six members of the Saprolegniales and three members of the Leptomitales were constructed and were found to be identical in the gene region of the repeat (Fig 13 and 14). There were no *Pst*I sites in *A. klebsiana*, *A. stellatus*, or *L. caudata*. *Bam*HI was not investigated in any of these species. Two of the maps in this group are unusual in that length heterogeneity is present. The rDNA repeat unit of *S. ferax* exists in three versions having sizes of 10.1, 10.5, and 10.7 kb. The

Figure 12. rDNA repeat unit maps of selected members of the Peronosporales. Maps are shown for *P. torulosum* (10.4 kb), *P. diclinum* (10.3 kb); *P. paddicum* (10.6 kb); *P. irregulare* (11.75 kb); *P. anandrum* (10.6 kb); and *Phytophthora cryptogea* (11.2 kb). The solid bars indicate the appoximate boundaries of the SrRNA, 5.8S, and LrRNA genes. The map at the bottom of the figure respresents a consensus for the approximately 6 kb highly conserved gene region derived from the other Peronosporales maps. The vertical lines beneath the individual maps correspond to the sites shown on the consensus map while species-specific sites are shown by the vertical lines with symbols on top of the map. Consensus sites absent in a particular species are marked whith a (-) above the enzyme symbol. Sites for which the positions have only tentatively been mapped are shown with 50% shaded symbols.

Peronosporales

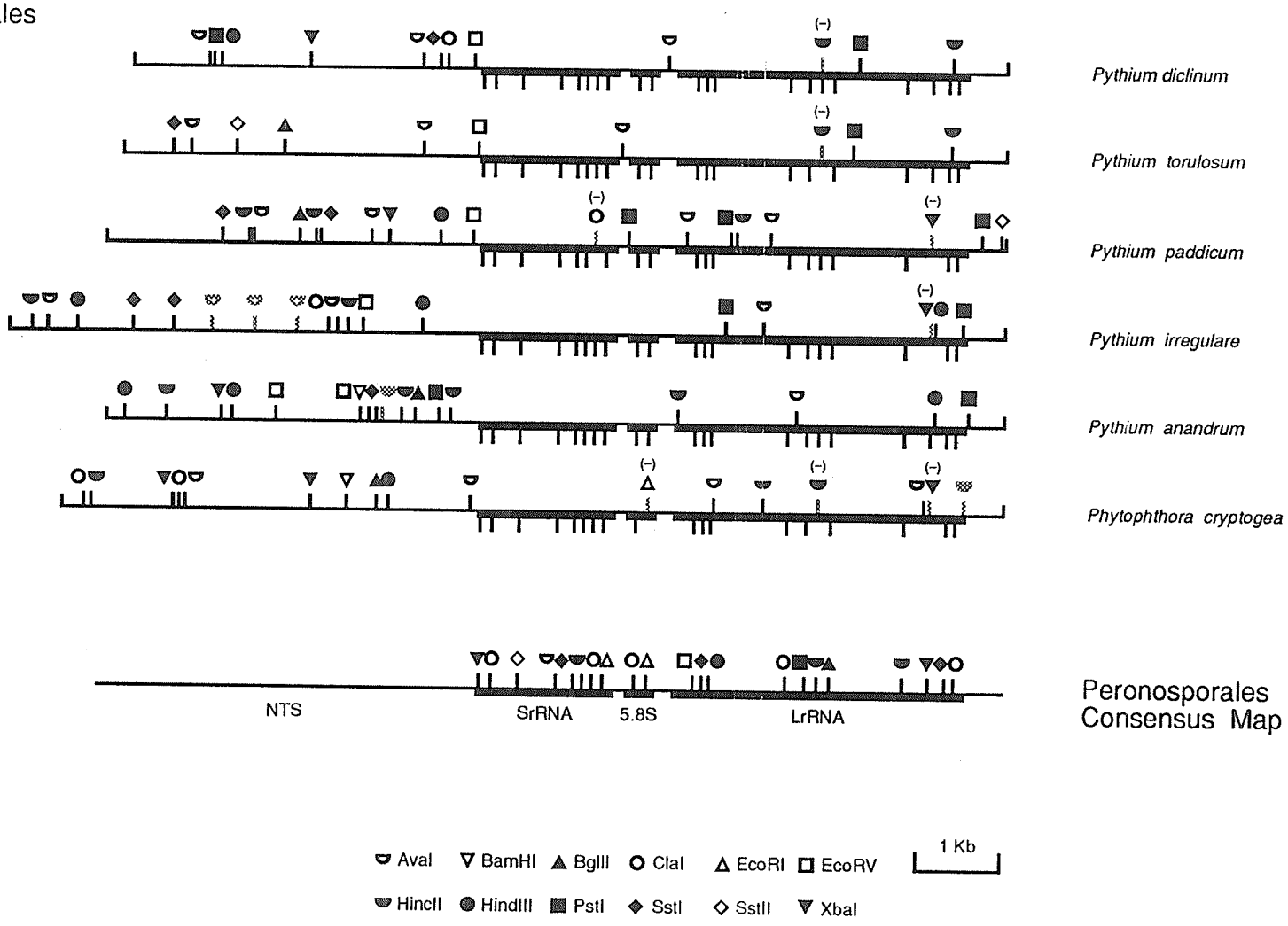


Figure 13. rDNA repeat unit maps of selected members of the Saprolegniales. Maps are shown for *S. diclina* (11.5 kb), *S. ferax* (10.2 kb), *Achlya klebsiana* (9.9 kb), *A. terrestris* (9.8 bk), *A. stellatus* (9.6 kb), and *L. caudata* (14.7 kb). See Fig 12 for interpretations. The regions marked with a wavy line and marked HET represent regions of heterogeneity.

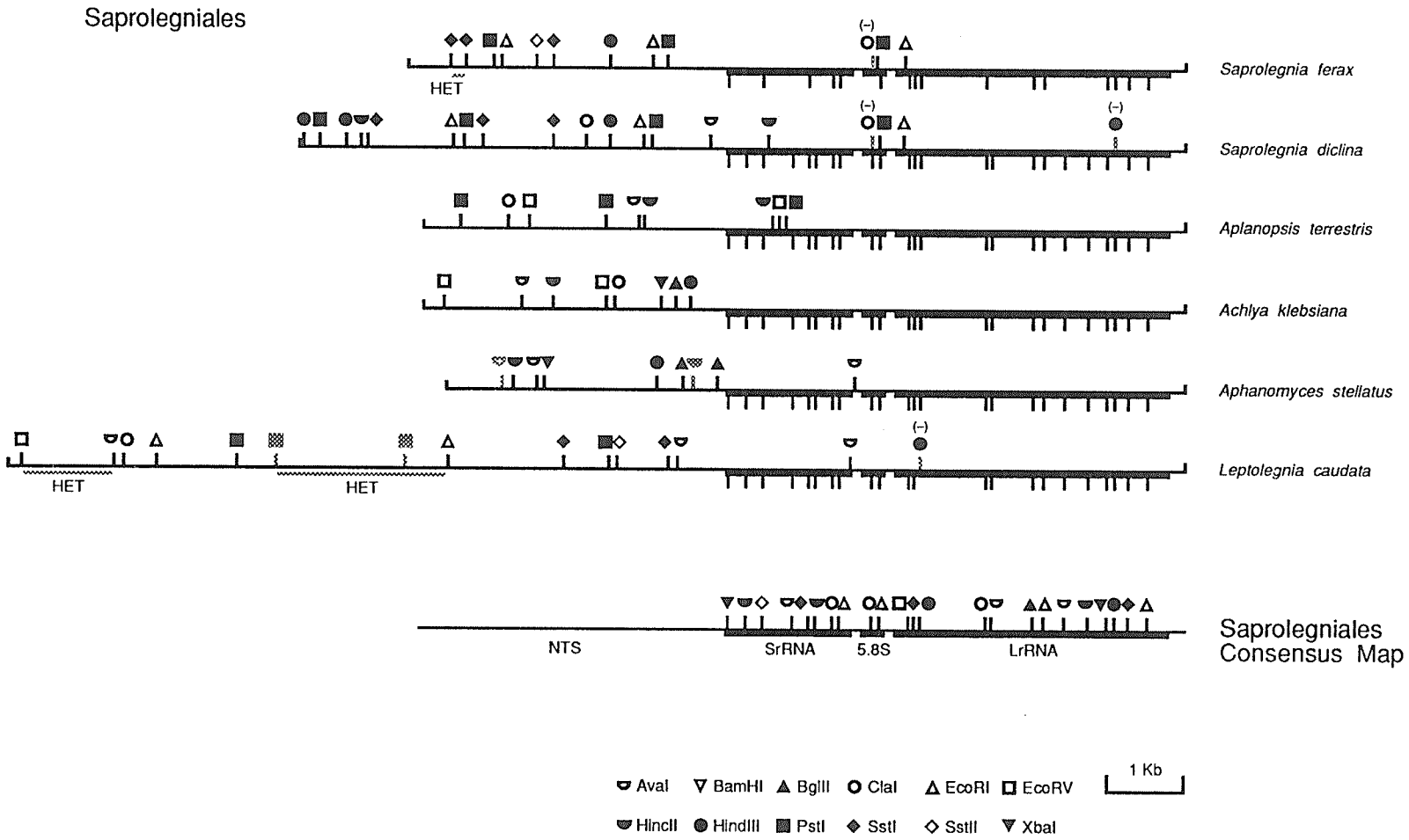
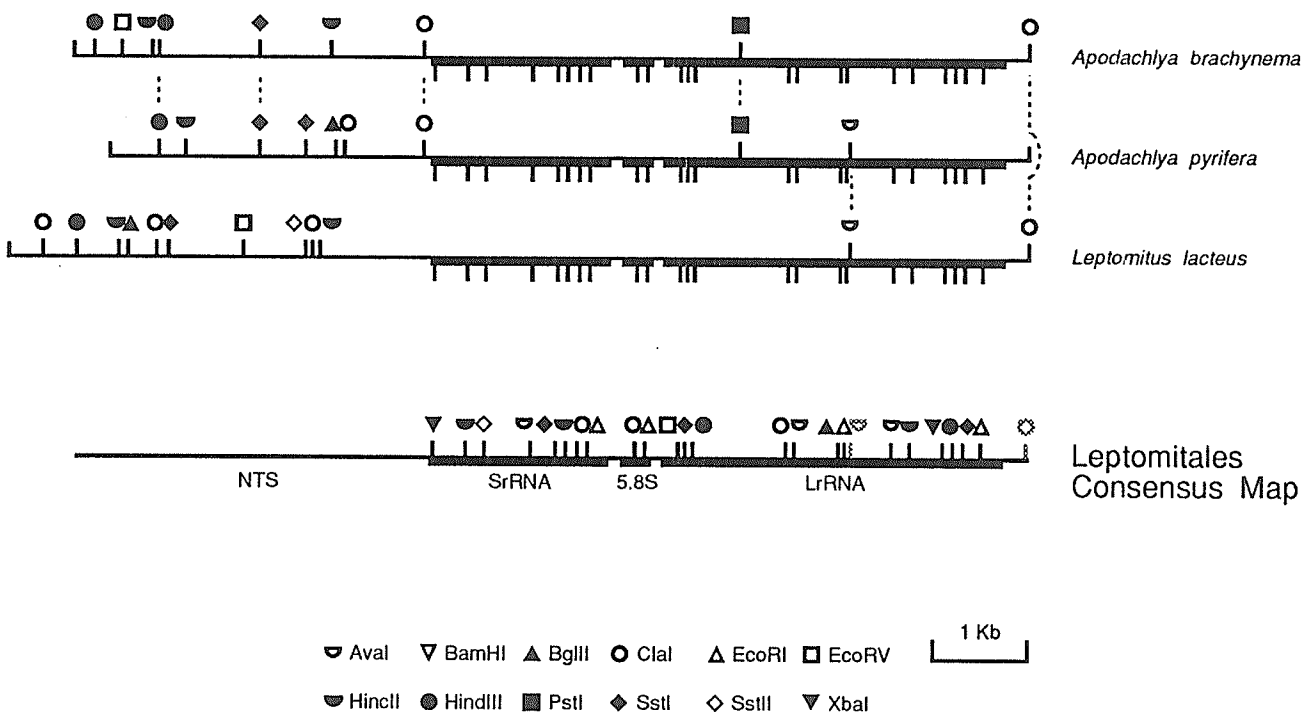

Saprolegniales
(-)
HET
Saprolegnia ferax
(-)
(-)
Saprolegnia diclina
Aplanopsis terrestris
Achlya klebsiana
Aphanomyces stellatus
(-)
HET
HET
Leptolegnia caudata
NTS
SrRNA
5.8S
LrRNA
Saprolegniales
Consensus Map
AvaI
BamHI
BglII
ClaI
EcoRI
EcoRV
HincII
HindIII
PstI
SstI
SstII
XbaI
1 Kb

Figure 14. rDNA repeat unit maps of selected members of the Leptomitales. Maps are shown for *Apodachlya brachynema* (10.0 kb), *Apodachlya pyrifera* (9.6 kb), and *L. lacteus* (10.5 kb). shared-derived sites between species are shown with wavy lines. See Fig 12 for interpretations. Sites in the consensus which potentially may be Leptomitales specific sites are shown in 50% shading.

Leptomitales

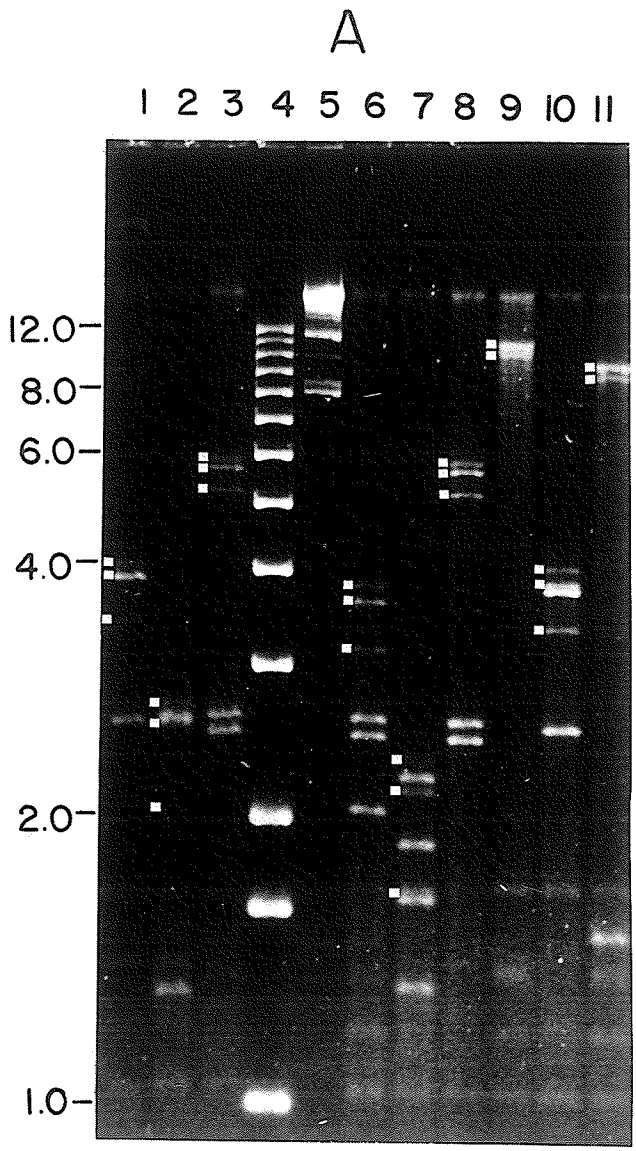


differences can be accounted for entirely by noting differences in the sizes of the *Sst*I fragment at the 5' end of the IGS (Fig 15). There may also be a region of heterogeneity in the *S. diclina* map but the heterogeneity is probably very slight (two versions of an *Sst*I band differing by 0.2 kb and therefore hard to detect - data not shown). The *L. caudata* map is unusual in that it has two regions of heterogeneity which result in several versions of the map (Fig 16). There is a region of heterogeneity near the 5' end of the IGS which is probably due to the presence of 500 - 600 bp subrepeats. There are at least three major versions of this region of the map and probably several minor versions which are not shown. There is probably another region of heterogeneity in the central portion of the IGS but this region of heterogeneity has not been characterized. The *L. caudata* map is also unusual in that its repeat size is very large. The exact size of the *L. caudata* repeat is unknown due to the heterogeneity but its longest version is probably greater than 15 kb. The two *Saprolegnia* maps are extremely similar. Four sites in the IGS can be aligned while another three sites can be aligned, assuming an inversion has taken place (Fig 17). All other species surveyed within the Saprolegniales/Leptomitales have homogeneous repeats with sizes ranging between 9.4 - 11.5 kb. The average size of the repeat for the Saprolegniales/Leptomitales group when *L. caudata* is excluded is 10.2 kb.

4.3 Hyphochytriomycete rDNA repeat unit maps

Maps of the rDNA repeats of two members of the Hyphochytriomycetes were constructed, that of *Hyphochytrium catenoides* and that of *Rhizidiomyces apophysatus*. These maps are shown in Fig 18. The repeat unit of *H. catenoides* was found to be 11.6 kb. *R. apophysatus* displays heterogeneity in the IGS region which made the size of the repeat difficult to determine. The minimum size of the *R. apophysatus* is probably at least 10 kb. *H. catenoides* has an insertion in the 5' end of the LrRNA gene as compared to *R. apophysatus*. Due to a large

Figure 15. Heterogeneity of the rDNA repeat unit of *S. ferax*. A: Ethidium Bromide stained gel of purified *S. ferax* rDNA. Band representing the three size classes of the repeat unit are indicated with white dots and sizes for each of these bands are indicated below under the appropriate lane number. In lanes where only two bands are indicated, the bands for the largest versions have not been separated by the gel. 1) *Hin*dIII: 4.1, 3.95, 3.5 kb; 2)*Hin*dIII X *Pst*I: 2.72, 2.57, 2.07 kb; 3) *Pst*I: 6.0, 5.75, 5.3 kb; 4) Bethesda Research Laboratories 1 kb ladder; 5) Bethesda Research Laboratories high molecular weight ladder; 6) *Pst*I X *Bgl*II: 3.80, 3.65, 3.25 kb; 7) *Pst*I X *Eco*RI: 2.38, 2.12, 1.70kb; 8) *Pst*I: 5.9, 5.75, 5.25 kb; 9) *Eco*RV: 10.5, 10.0 kb; 10) *Eco*RV X *Hin*dIII: 4.05, 3.85, 3.45 kb; 11) *Eco*RV X *Bgl*II: 9.4, 8.8 kb. B: restriction map of *S. ferax* showing the three length variants and localizing the heterogeneity within the IGS.


A
1 2 3 4 5 6 7 8 9 10 11
12.0
8.0
6.0
4.0
2.0
1.0

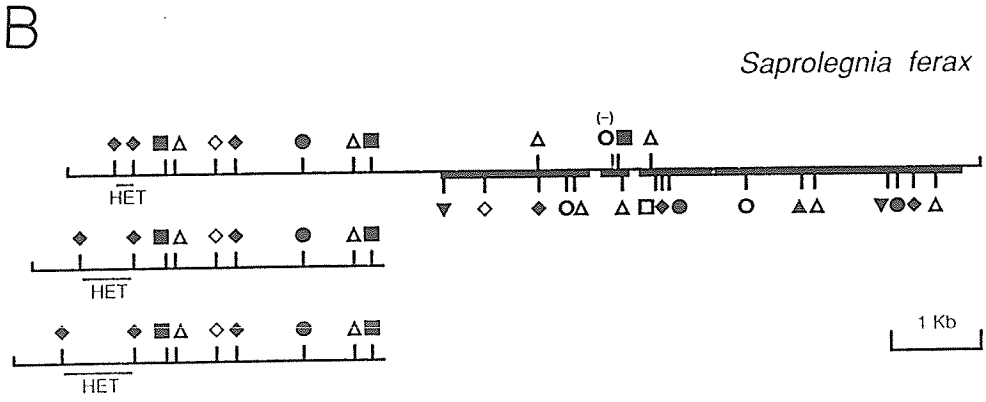
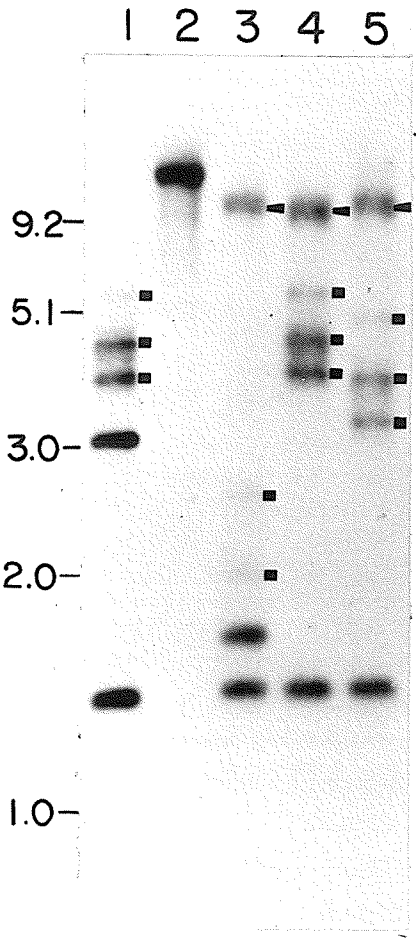

B
Saprolegnia ferax
(-)
HET
HET
HET
1 Kb

Figure 16. Heterogeneity of the rDNA repeat unit of *L. caudata*. A: Autoradiogram of a southern blot of a gel of partially purified *L. caudata* rDNA. Bands representing the three major size classes of heterogeneity in the 5' end of the IGS are indicated by the small black rectangles and their sizes given with the appropriate lane number. The fuzzy bands marked with the black arrows are thought to be a composite of several size classes and represent the uncharacterized region of heterogeneity in the central portion of the IGS. These bands are in the range of 9 kb in size and therefore the various size classes have not been adequately separated on this gel. 1) *Cla*I X *Pst*I: 5.6, 4.4, 3.8 kb; 2) *Pst*I: size not determined; 3) *Cla*I X *Hin*dIII: largest version not visible, 2.6, 2.02 kb; 4) *Cla*I: 5.6, 4.5, 3.9 kb; 5) *Cla*I X *Bgl*II: 4.9, 3.9, 3.35 kb. B: restriction map of *L. caudata* showing the three major length variants and localization of the heterogeneity in the 5' region of the IGS. The approximate location of the other region is shown. However, the length variations in this region are unknown.

A



B

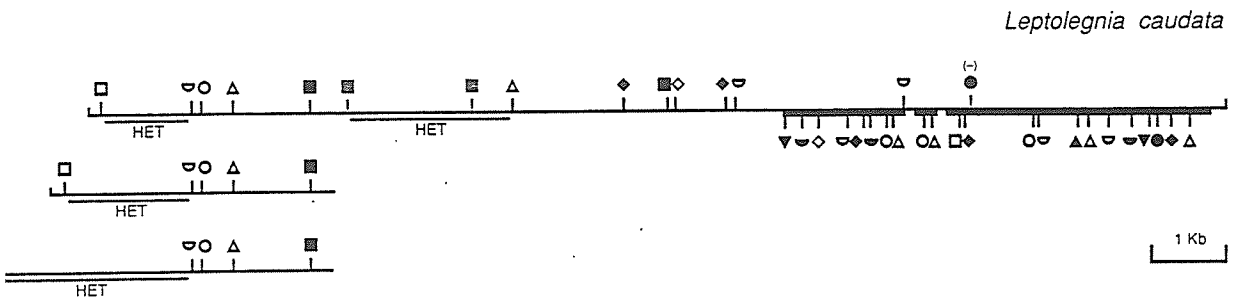


Figure 17. Alignment of the rDNA repeat unit maps of *S. ferax* and *S. diclina.* Alignment of sites in the IGS region of the two species are indicated by vertical dashed lines. An inversion event is assumed in the alignment of an *Sst*I-*Pst*I- *Eco*RI constellation.

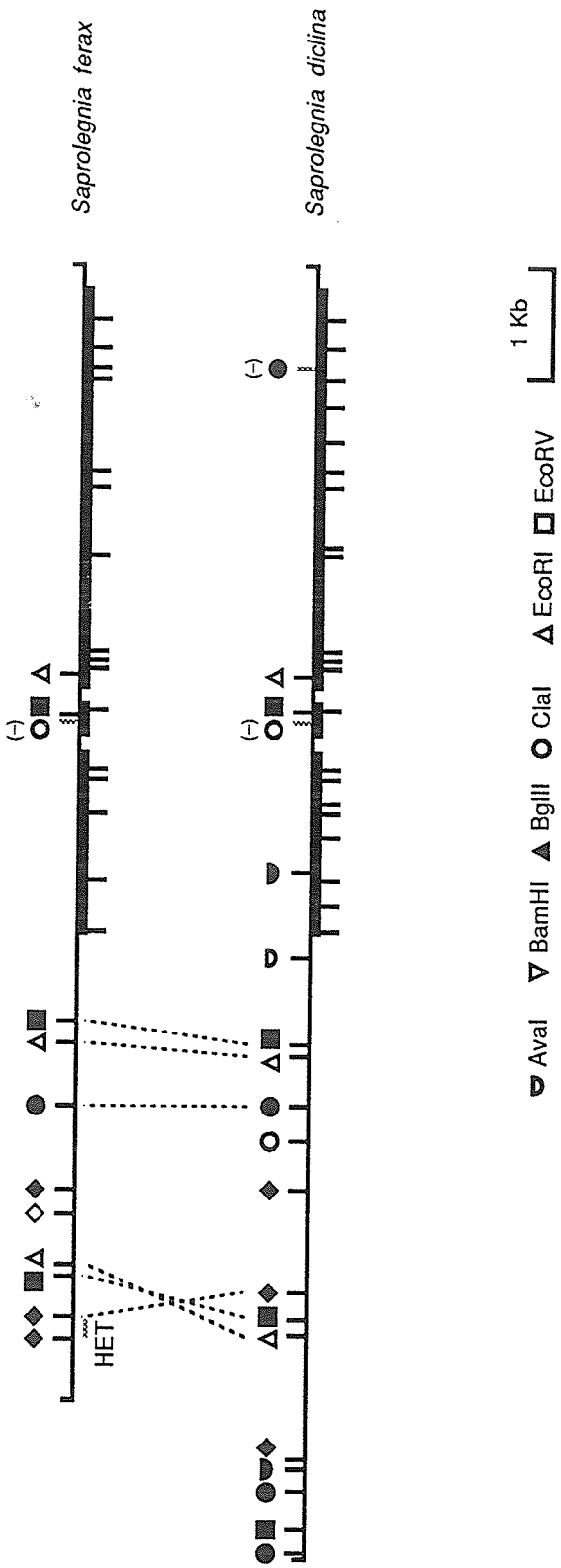
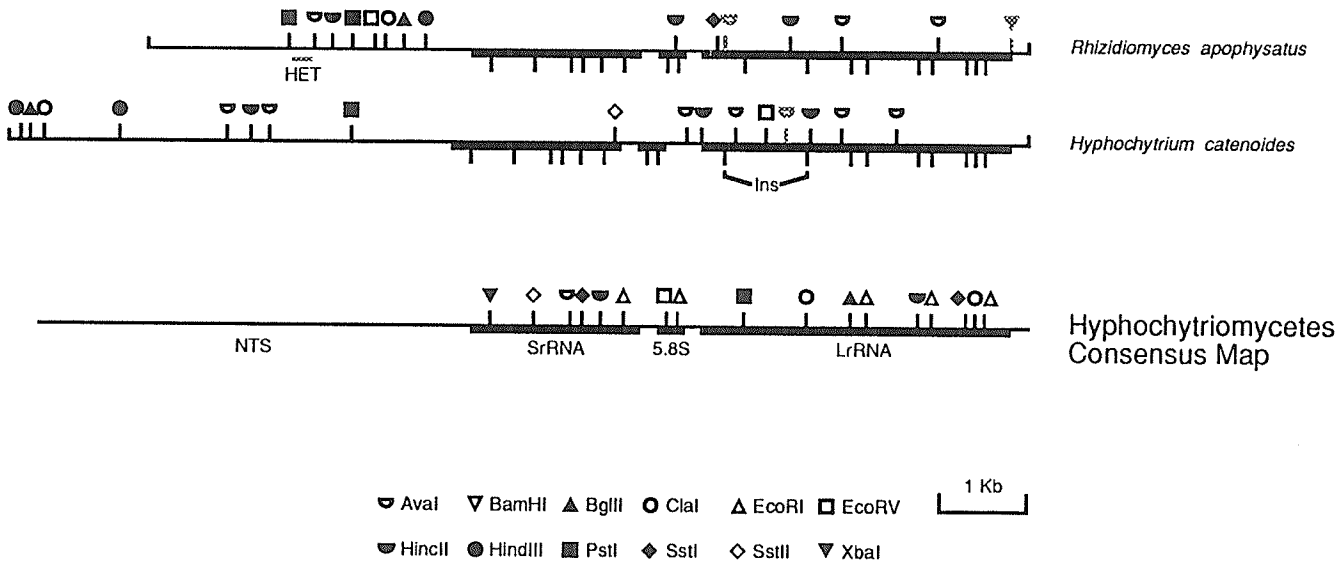

Saprolegnia ferax
Saprolegnia diclina
HET
(-)
(-)
1 Kb
AvaI
BamHI
BglII
ClaI
EcoRI
EcoRV
HincII
HindIII
PstI
SstI
SstII
XbaI

Figure 18. rDNA repeat unit maps of Hyphochytriomycetes. Maps are shown for *H. catenoides* (11.6 kb) and *R. apophysatus* (10.0 kb - minimum size version). Interpretation is as in Fig 12. A region of insertion in the LrRNA gene of *H. catenoides* as compared to *R. apophysatus* is indicated by Ins. The precise location of the insertion is unknown. The region of heterogeneity in *R. apophysatus* is indicated with a wavy line and HET.

Hyphochytriomycetes



number of *Ava*I and *Hin*cII sites in these maps not all *Ava*I and *Hin*cII sites have been mapped. Only those sites which have been mapped with a reasonable degree of certainty are shown in Fig 18. These include the sites that are important for comparison with the Oomycete consensus (Fig 25). The enzyme *Bam*HI has not been investigated in the Hyphochytriomycetes.

4.4 Chytridiomycete rDNA repeat unit maps

Maps for four species of Chytridiomycetes have been constructed (Fig 19), three species were from the Spizellomycetales and one from the Chytridiales. *Ava*I and *Hin*cII sites have only been mapped in *S. punctatus*. There are no EcoRV sites in any of the Chytridiomycetes surveyed thus far, and the enzyme *Bam*HI has not been investigated. The map of *C. confervae* which is in the order Chytridiales is a preliminary map but is included since it can be considered an outgroup for the Spizellomycetales. The sizes of rDNA repeat units in the Chytridiales range from 8.2 to 10.8 kb.

4.5 Species of uncertain classification rDNA repeat unit maps

Sapromyces elongatus. The *S. elongatus* map is shown in Fig 20. The enzyme *Bam*HI has not been investigated. The size of the repeat is 11.1 kb.

Lagenidium giganteum. The map for *L. giganteum* is shown in Fig 21. The enzymes *Ava*I and *Hin*cII have not been mapped completely and therefore some sites shown are tentative. Partial digests with *Hin*cII indicate that although the rDNA repeat units are homogeneous, the IGS may contain subrepeats with a periodicity of 500 bp similar to the possible *Ava*I subrepeats in *P. irregulare* . The size of the rDNA repeat unit is 10.6 kb.

Verrucalvus flavofaciens/Pachymetra chaunorhiza. Maps of the repeat unit of *V. flavofaciens* and *P. chaunorhiza* are shown in Fig 22. The maps for these two organisms are extremely similar differing only in the IGS region and even the IGS regions are extremely similar sharing three sites. *V. flavofaciens* has no

Figure 19. rDNA repeat unit maps of selected Chytridiomycetes species. Maps shown are *S. punctatus* (10.8 kb), *S. plurigibbosus* (9.3 kb), *G. semiglobiferus* (8.2 kb), and *C. confervae* (8.8 kb). Interpretation is as in Fig 12. Regions of insertions in *S. punctatus* and *S. plurigibbosus* with respect to other maps are identified with Ins.

Chytridiomycetes

Spizellomycetales

Chytridiales

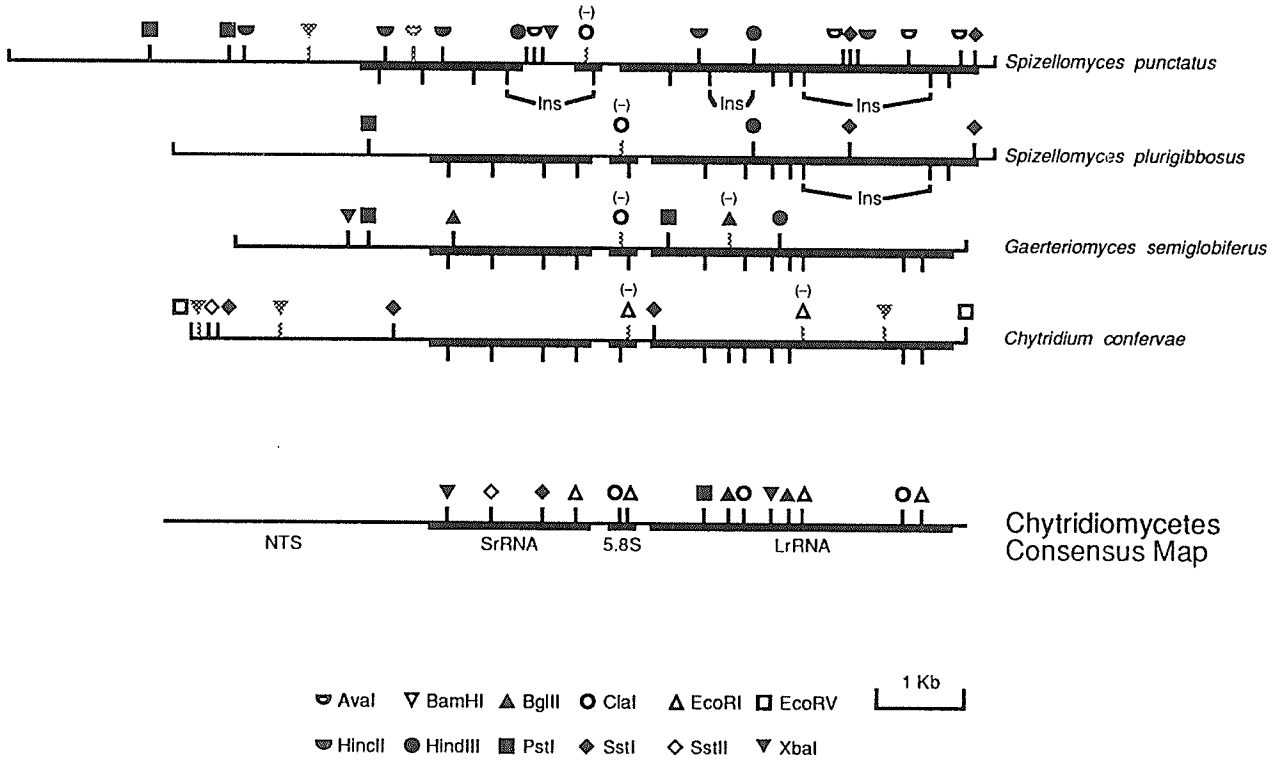
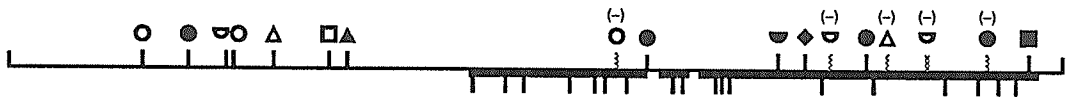


Figure 20. rDNA repeat unit map of *S. elongatus* (11.1 kb). Interpretation is as in Fig 12. The consensus map for Leptomitales is also shown since *S. elongatus* was formerly classified in this order.

Rhipidiales



Sapromyces elongatus

Leptomitales
Consensus Map

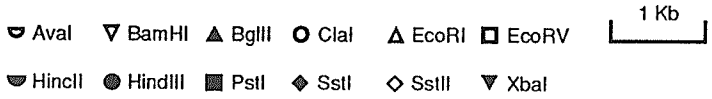
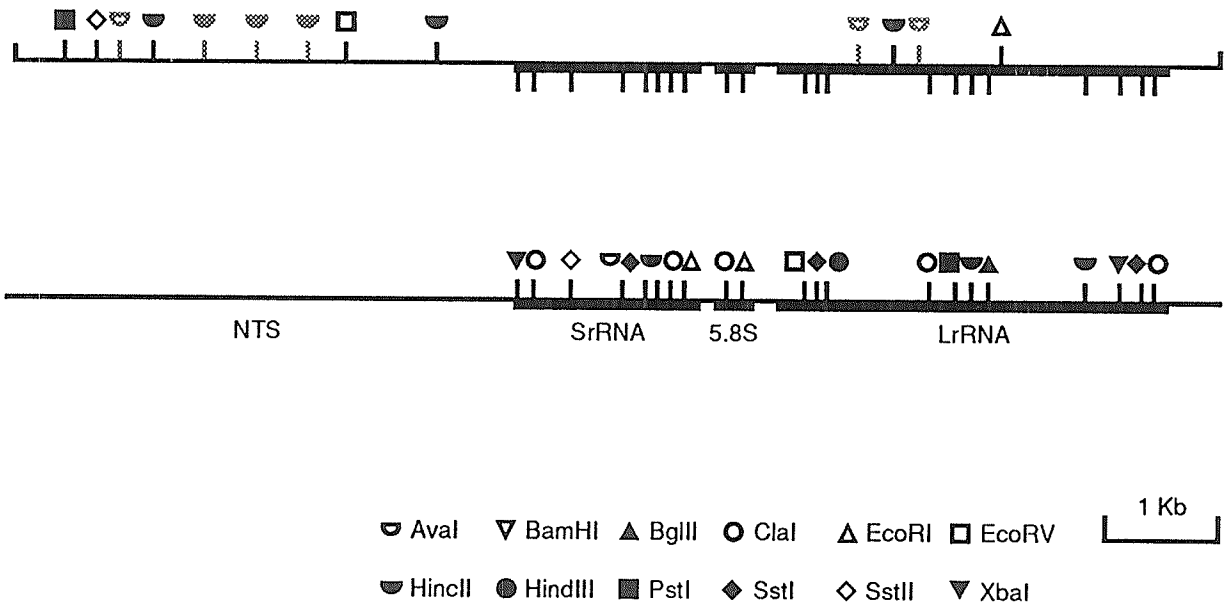


Figure 21. rDNA repeat unit map of *L. giganteum* (10.6 kb). Interpretation is as in Fig 12. The Peronosporales consensus map is shown below the *L. giganteum* map to illustrate that *L. giganteum* conforms to this consensus.

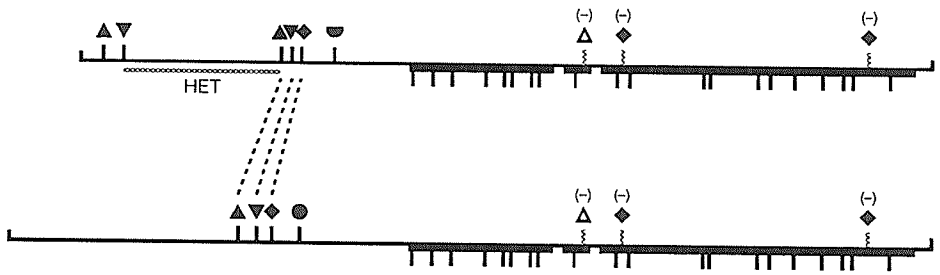


Lagenidium giganteum

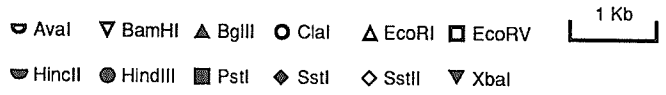
Peronosporales
Consensus Map

Figure 22. rDNA repeat unit map of *V. flavofaciens* (9.7 kb - short version) and *P. chaunorhiza* (10.5 kb). Interpretation is as in Fig 12. Length heterogeneity in *V. flavofaciens* is indicated by the wavy line and HET. The consensus map for Saprolegniales/Leptomitales is also included to show the similarity of *V. flavofaciens/P. chaunorhiza* to these groups. Alignment of sites between the two species in the IGS region is shown by dashed lines.

Sclerosporales



Saprolegniales / Leptomitales
Consensus Map

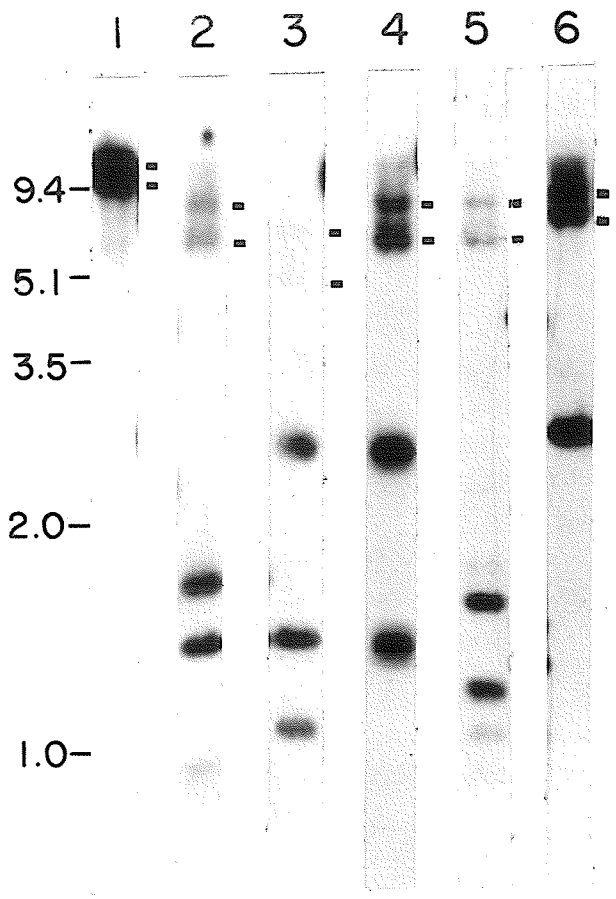


*Bam*HI sites, and neither *V. flavofaciens* nor *P. chaunorhiza* have *Pst*I sites. The enzymes *Ava*I, *Hin*cII, and *Bam*HI were not mapped in *Pachymetra.* However, due to the extreme similarity to *Verrucalvus flavofaciens* these sites are assumed to be the same in *P. chaunorhiza* as in *V. flavofaciens*, at least in the gene region. *Verrucalvus flavofaciens* displays heterogeneity in the IGS (Fig 23) while *Pachymetra chaunorhiza* does not. The major size classes of the repeats for *V. flavofaciens* are 9.7 and 11.3 kb. Numerous minor size classes probably also exist. The repeat unit in *P. chaunorhiza* is 10.5 kb.

Zoophagus insidians. The map for *Zoophagus insidians* is shown in Fig 24. Two sources of DNA were used and the maps for both sources were found to be identical. The enzymes *Ava*I and *Hin*cII have not yet been mapped. There are no *Eco*RV sites in this map. The size of the *Zoophagus repeat* unit is 7.6 kb.

Figure 23. Heterogeneity of the rDNA repeat unit of *V. flavofaciens*. A: autoradiograph of a southern blot of a gel containing restrictions from the bottom band of *V. flavofaciens* DNA. Bands generated from the two major size classes are indicated with the small black rectangles. Sizes of these bands are indicated below. 1) *Eco*RV: size not determined, 9.8 kb; 2) *Eco*RV X *Eco*RI: 7.8, 6.1 kb; 3) *Eco*RI X *Sst*I : 6.5, 5.0 kb; 4) *Eco*RI: 7.7, 5.9 kb; 5) *Eco*RI X *Hin*dIII: 7.4, 5.8 kb; 6) *Hin*dIII: 8.7, 7.1 kb. B: rDNA repeat unit map showing the two major size classes and the position of the heterogeneity within the IGS.

A



B

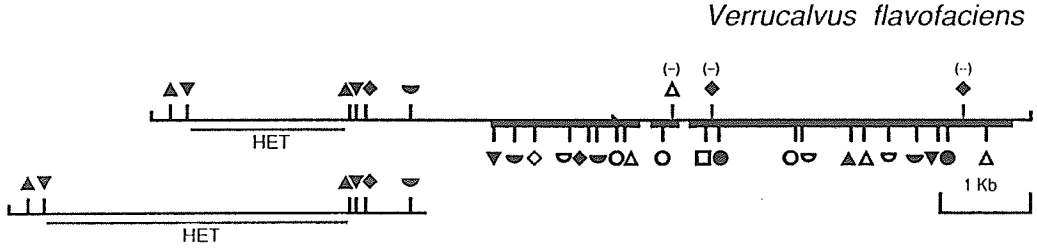
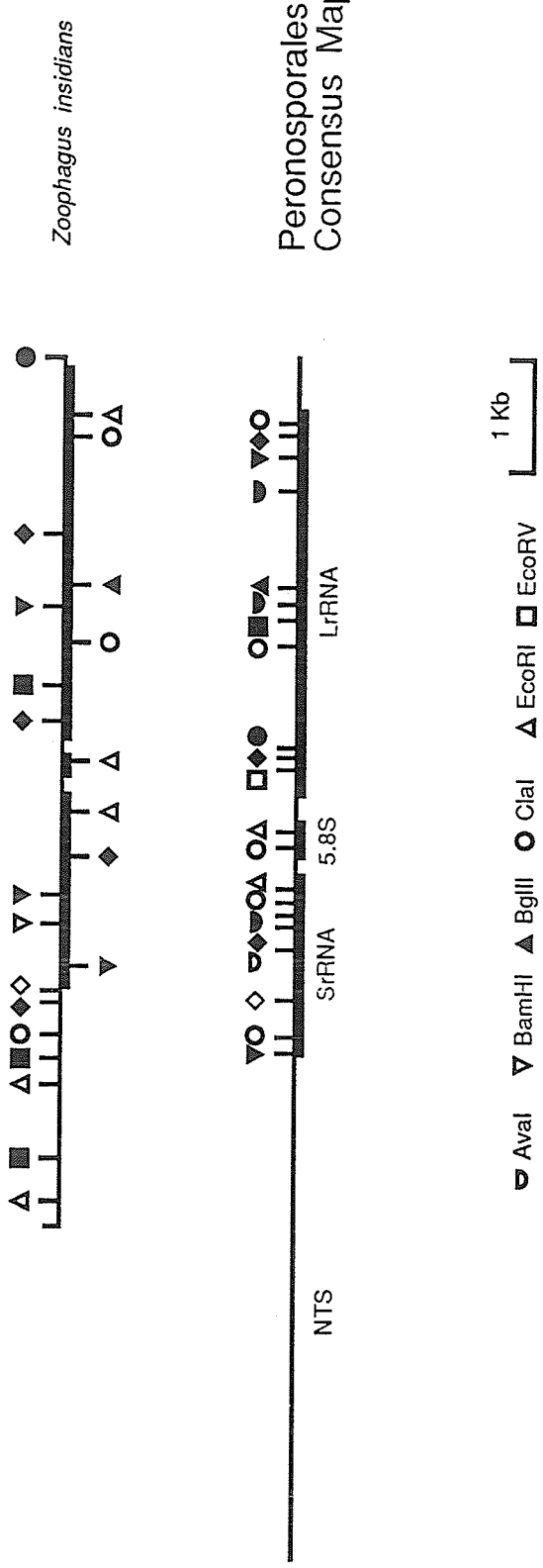


Figure 24. rDNA repeat unit map of *Z. insidians* (7.6 kb). Interpretation is as in Fig 12. The consensus map for the Peronosporales has been included to show the dissimilarity between *Z. insidians* and the Peronosporales to which this species had previously been classified.


Zoophagus insidians
Peronosporales
Consensus Map
NTS
SrRNA
5.8S
LrRNA
1 Kb
AvaI
BamHI
BglII
ClaI
EcoRI
EcoRV
HincII
HindIII
PstI
SstI
SstII
XbaI

DISCUSSION

DISCUSSION

1. Discussion of mitochondrial physical maps

All currently available physical mapping data from investigations of Oomycete, Hyphochytriomycete, and Chytridiomycete mitochondrial genomes is presented in Table VII. Partial data for *Saprolegnia ferax* and *Pythium ultimum*, which presumably have IRs, and for *Leptomitus lacteus*, which does not, are not included (Grossman and Hudspeth 1985, Shumard and Hudspeth 1986). From the fungi surveyed in this study, several features of mitochondrial physical maps of the Mastigomycotina have become apparent.

All the maps, with the possible exception of *P. irregulare*, show that the genomes are circular as are almost all other mitochondrial genomes. The *P. irregulare* mitochondrial DNA may exist as a linear molecule or as a combination of linear and circular molecules. All enzymes seem to have sites in the 0.5 kb small unique region (Fig. 5), indicating that the small unique region may not actually exist and that this region may be the termini of the map. This would be highly unusual since linear mitochondrial genomes have only been reported twice previously in fungi (Kovac et al. 1984, Wesolowski and Fukuhara 1981) and are rare in other groups (Wallace 1982, Grey 1982). If *P. irregulare* does have a linear mitochondrial genome, one could postulate that if the IR grows to encompass the entire unique region the molecule then linearizes. It is perhaps significant that *Tetrahymena pyriformis* has a linear mitochondrial genome with terminal inverted repeats (Goldbach et al. 1979). Further studies are necessary to determine whether the *P. irregulare* mitochondrial genome is really linear and what the significance of the linearity may be.

Table VII Mitochondrial genome organization in Zoosporic Fungi (Sizes given in kb)

	Genome size	Small single-copy	Large single-copy	Inverted repeat	Complexity	Position of LrRNA[1]	References
Saprolegniales							
Achlya ambisexualis E-87[2]	49.8	6.3 - 8.3	20.5 - 21.0	9.9 - 11.2 (10.6)	37.7 - 40.2 (40.0)	large	Hudspeth *et al.*, 1983;
Achlya heterosexualis 1-4[3]	51.3	6.3 - 8.3	20.5 - 21.0	9.9 - 11.2 (10.6)	39.2 - 41.7 (41.5)	large	Shumard *et al.*, 1986
Achlya klebsiana	50.7	8.2 - 9.8	20.7 - 21.5	9.7 - 10.9 (10.3)	39.8 - 41.0 (40.4)	large	Boyd *et al.*, 1984
Aplanopsis terrestris	45.0	9.0 - 9.9	18.0 - 18.9	8.1 - 9.0 (8.6)	36.0 - 36.9 (36.5)	large[4]	this thesis
Leptolegnia caudata	52.4	3.8 -9.5	21.6 - 25.5	8.7 - 13.5 (11.1)	38.9 - 43.7 (41.3)	large	this thesis
Peronosporales							
Phytophthora infestans	36.2	---	---	---	36.2	---	Klimczak & Prell, 1984
Phytophthora cryptogea	40.7	---	---	---	40.7	---	this thesis
Phytophthora megasperma f. sp. *glycinea*	45.3	---	---	---	45.3	---	Forster *et al.*, 1987
Phytophthora megasperma f. sp. *medicaginis*	41.0	---	---	---	41.0	---	Forster *et al.*, 1987
Phytophthora parasitica	39.5	---	---	---	39.5	---	Forster *et al.*, 1987
Pythium diclinum	70.0	0.4 - 2.9	10.6 - 10.7	28.2 - 29.5 (28.9)	40.5 - 41.8 (41.2)	central	this thesis
Pythium torulosum	73.0	3.9 - 6.2	10.9 - 11.6	27.6 - 29.1 (28.4)	43.9 - 45.4 (44.7)	central	this thesis
Pythium paddicum	61.3	0 - 4.8	13.1 - 17.7	19.4 - 24.1 (21.8)	37.2 - 41.9 (39.6)	central	this thesis
Pythium irregulare	60.0	0 - 0.5	12.4 - 16.1	21.7 - 23.8 (22.7)	36.2 - 38.3 (37.3)	central	this thesis
Leptomitales							
Apodachlya pyrifera	40.0	---	---	---	40.0	---	this thesis
Apodachlya brachynema	36.4	---	---	---	36.4	---	this thesis
Rhipidiales							
Sapromyces elongatus	53.0	4.7 - 8.5	14.3	15.1 - 17.0 (16.1)	36.0 - 37.9 (37.0)	small	this thesis

Table VII Mitochondrial genome organization in Zoosporic Fungi (Continued) (Sizes given in kb)

	Genome size	Small single-copy	Large single-copy	Inverted repeat	Complexity	Position of LrRNA[1]	References
Hyphochytriomycetes							
Hyphochytrium catenoides	54.0	10.4 - 11.6	15.1 - 15.6	13.5 - 14.5 (14.0)	39.5 - 40.5 (40.0)	small	this thesis
Rhizidiomyces apophysatus	50.0	---	---	---	50.0		this thesis
Chytridiomycetes							
Spizellomyces punctatus	62.2	---	---	---	62.2	---	this thesis
Spizellomyces plurigibbosus	63.0	---	---	---	63.0	---	this thesis
Allomyces anomalus	49.9 - 51.2	---	---	---	49.9 - 51.2	---	Borkhardt *et al.*, 1987
Allomyces arbuscula	47.1 - 48.3	---	---	---	47.1 - 48.3	---	Borkhardt *et al.*, 1987
Allomyces catenoides	55.1	---	---	---	55.1	---	Borkhardt *et al.*, 1987
Allomyces macrogynus	55.9	---	---	---	55.9	---	Borkhardt & Delius, 1983
Allomyces moniliformis	49.9	---	---	---	49.9	---	Borkhardt *et al.*, 1987
Blastocladiella emersonii	35.5	---	---	---	35.5	---	Borkhardt & Olsen, 1986

[1] large - adjacent to the large single-copy region
small - adjacent to the small single-copy region
central - in center of inverted repeat

[2] *A. ambisexualis* 734 was also investigated and found to be very similar to E-87.

[3] *A. heterosexualis* 8-6 was also investigated and found to be very similar to 1-4.

[4] See text for interpretation.

In all the mitochondrial genomes where inverted repeats have been found both copies of the repeat are identical with respect to restriction sites. This suggests that homologous recombination is occurring between the repeats and that the repeats are kept identical by the copy choice correction mechanism (Palmer 1985). The presence of equimolar proportions of isomers differing from one another by the orientation of one unique region with respect to the other (flip-flop isomerism) has been previously reported in *Achlya* (Hudspeth et al. 1983, Boyd et al. 1984), and in chloroplasts which have IRs (Palmer 1985). Such isomerism can be explained by assuming homologous recombination between the two repeats. The finding of flip-flop isomerism in *Sapromyces elongatus* and *Hyphochytrium catenoides* is further evidence that this is a general feature of IRs. Flip-flop isomerism was not investigated in the *Pythiums*, *Aplanopsis terrestris*, or *Leptolegnia caudata* due to a lack of appropriate restriction enzyme sites.

From a comparison of all the maps it can be seen that in general, maps from closely related species within a genus can be aligned (Fig 5). Attempts at alignment at the genus level are hampered by the use of different restriction enzymes for mapping. However, a comparison of *A. terrestris* with *A. klebsiana* shows alignment in the IR and the large unique region of the map (Fig 4). Other than this example alignment between genera has not been demonstrated except within the highly conserved LrRNA gene which has the highly conserved SalI-SstI constellation. Among the *Pythiums*, *P. torulosum* and *P. diclinum* have similar sized IRs and share many sites in common. *P. irregulare* and *P. paddicum* also seem to share a similar IR size and show conservation of sites, especially in the IR region. Only a few restriction sites are shared between all four species (Fig 5). In orthodox taxonomy, *P. diclinum* and *P. torulosum* are considered to be closely related. Similarly, *P. irregulare* and *P. paddicum* are probably more

closely related to one another than to either *P. diclinum* or *P. torulosum* (M.W. Dick, personal communication). Therefore, the mitochondrial restriction map data presented here supports the current taxonomic classification of these *Pythiums*. Maps of the rDNA repeats (to be discussed later) also support this grouping of *Pythium* species. In *Achlya* species, a similar level of restriction site alignment and IR size has been observed (Boyd et al. 1984, Shumard et al. 1986). These *Achlya* species are also close within the genus *Achlya* (Dick 1973). At the species level, the presence and size of the inverted repeat may be useful in confirming recent divergence along with restriction site conservation.

The most striking feature of the *Pythium* maps is the size of the inverted repeats, 28 kb in *P. diclinum* being by far the largest found in any mitochondrial genome. The largest previously discovered mitochondrial repeat is the 14 kb inverted repeat in maize (Lonsdale et al. 1984). In fungi the largest previously discovered repeat is the 10 - 12 kb inverted repeat in *Achlya* (Hudspeth et al. 1983, Boyd et al. 1984). Such large inverted repeats are, however, commonly found in the DNA of chloroplasts. In chloroplast genomes, the inverted repeats commonly range in size from 20 - 28 kb (Whitfeld and Bottomley 1983). The largest inverted repeat in chloroplast DNA reported to date is the 76 kb repeat of geranium (Palmer et al. 1987). Although this repeat is considerably larger than the repeats reported here, the repeats in *Pythium* represent a similar percentage of the total genome. In geranium the repeats represent 70% of the total genome. In *Pythium* the percentage of the genome represented by the repeats ranges between at least 80% in *P. paddicum* to 60% in *P. diclinum*. This is close to twice the fraction of the genome which is contained in the repeats of the Saprolegniales (minimum values of 33 - 40%).

In *Achlya* the IR contains the small and large ribosomal RNA genes (Hudspeth et al. 1983). Fungal genomes do not generally show the extreme

mitochondrial genome compression found in metazoa. Therefore since the fraction of the mitochondrial genome included by the inverted repeats in *Pythiums* is much higher than in *Achlya*, it is highly likely that genes other than the ribosomal RNA genes have been absorbed into the inverted repeats.

A comparison of all the Oomycete maps (Table VII) reveals one striking feature of the Oomycete mitochondrial genome. This is conservation of genome complexity. When the size of one arm of the IR is subtracted from genome size, an upper limit to genome complexity is obtained. Small scale reiteration of less than 1 kb is not detected by physical mapping so that the actual genome complexities may be slightly lower. There is no evidence that significant reiteration occurs in fungal mtDNA. When no large IR is found, genome complexity is taken to be equivalent to genome size. Comparison of approximate mitochondrial genome complexities shows that this parameter is remarkably uniform among the Oomycetes. Average complexity for Oomycetes is 39.9 kb with values ranging between 36.2 (*P. infestans*) and 45.3 kb (*P. megasperma* f. sp. glycinea). Uncertainty in determining genome size by means of physical mapping of 50 kb circles of DNA is generally about 3% (Hudspeth et al. 1983, Boyd et al. 1984), and uncertainty in the size of IRs is generally less than 1.5 kb. The cumulative effect of these two sources of error should not influence complexity calculations by more than 3 kb.

The uniformity of mitochondrial genome complexities among Oomycetes is in contrast to the nonuniformity seen among Ascomycotina, Basidiomycotina, and even Chytridiomycetes. Two species of *Saccharomyces* may differ by 48 kb (Grossman and Hudspeth 1985), two of *Agaricus* by 78 kb (Hintz et al. 1985), two of *Coprinus* by 47.8 kb, and two of *Allomyces* by 17 kb (Borkhardt et al. 1987). Within the entire class Oomycetes the complexity difference between extreme cases is only 9.1 kb (*P. infestans/P. megasperma*). The fact that the entire

Oomycete range is equivalent to the range within one genus may mean that genome complexity changes rapidly, but that it is constrained within strict upper and lower limits.

This pattern of mitochondrial genome organization and evolution (presence of IRs, uniform complexity, homogeneous populations of molecules) is unlike that of any other known mitochondrial system. It is most like the pattern observed in angiosperm chloroplast genomes. These genomes are about three times the size of Oomycete mitochondrial genomes but otherwise they share their homogeneity, their IR structure, and their uniform complexity (Palmer 1985). In both genomes the IRs are similarly placed within the map, are associated with ribosomal genes, and may differ greatly in size. The huge IR structures in *Pythium*, which may comprise about 80% of the genome (Fig. 5) have their parallel in geranium chloroplast DNA, which is also dominated by its IR structure (Palmer et al. 1987). The fact that genome complexity conservation and the IR occur together in both angiosperm chloroplast DNA and Oomycete mtDNA may indicate that the IR influences genome size changes or else that organelles with constrained genome sizes tend to develop the IR structure.

If the IR constrains genome complexity, then its absence should correlate with deviations from the norm. *Phytophthora* species, which lack the IR, have mtDNA complexities ranging from 36.2 to 45.3 kb, with an average value of 40.5 kb. *Pythium* species, which have the IR, range from 37.3 to 44.7 kb with an average of 40.7 kb. No significant deviation occurs that can be correlated with the presence or absence of the IR. Unless the IR was recently lost in *Phytophthora* or gained in *Pythium* it appears that the IR does not influence complexity conservation. Leptomitales, which lack the IR, also do not deviate significantly from the Oomycete norm (Table VII).

The other alternative is that genetic systems in which complexity is constrained, perhaps by the nature of the recombination system, also have the fortuitous tendency to develop or maintain the IR structure. In these systems the genome is changed by the incorporation of variable amounts of a fixed sequence into the IR (always including the rRNA genes), but not by the acquisition of new sequences. Reiteration of genes may be adaptive (Hudspeth et al. 1983), but it has been shown in *Chlamydomonas* chloroplasts that deletion of one set of rRNA genes does not alter the ribosome supply (Myers et al. 1982). The fact that the IR is found in some Oomycete groups and not in others also suggests that it is not a primary feature of the Oomycete mitochondrial genetic system.

Available data on mitochondrial genomes has reached the point where it is possible to assess the value of the presence or absence of the IR as a trait useful for phylogenetic studies. The large, chloroplast-like IR has been found almost exclusively in Oomycetes. IRs in *Tetrahymena* (Goldbach et al. 1978), *Kloeckera* (Clark-Walker et al. 1981), and *Candida* (Wills et al. 1985) have not been shown to be comparable to IRs in Oomycetes. The limitation of the trait to a taxonomic group indicates that its presence is systematic and not just a scattered phenomenon that appears at random. Moreover, regularities within the Oomycetes are becoming evident. All members of the Saprolegniales so far investigated have an IR of about 10 kb in size with the large rRNA gene adjacent to the large single-copy region (Fig 4, Table VII). The size and position of small and large single-copy regions is also very similar in all species. All *Pythium* species have an IR which is larger than that in the Saprolegniales with a small single-copy region which is very much reduced. The large rRNA gene is located centrally (Fig 5, Table VII). The size of the IR in *Pythium* species may be somewhat variable as indicated by the 7 kb difference between *P. torulosum* and *P. paddicum* and by the report that *P. ultimum* has a genome

size of 57 kb (Grossman and Hudspeth 1985). However, conservation of the size of the IR in some *Pythium* species may indicate a close taxonomic relationship as discussed previously. No *Phytophthora* species has the IR and genome size is as variable as is possible within the Oomycete constraints discussed above. Three species of Leptomitales also do not have the IR, namely the two species of *Apodachlya* in this thesis and *Leptomitus lacteus* (Shumard and Hudspeth 1986). *Sapromyces elongatus*, which has recently been removed from the Leptomitales and placed in the Rhipidiales (Dick et al. 1984), has an IR of intermediate size (between the Saprolegniales and *Pythium* species) and is unusual in that the LrRNA gene is adjacent to the small single-copy region. Thus, the regularities seen within the Oomycetes are consistent with accepted taxonomic distinctions.

The regularities indicate that the presence of the IR is an ancestral character for Oomycetes and that the absence of the IR is derived. It is more likely that the IR was lost in *Phytophthora* species and in the Leptomitales than that it appeared independently in the Saprolegniales, in *Pythium* species, and in *S. elongatus*. This tentative inference leads to the prediction that groups closely allied to the Oomycetes will be found to have the IR in their mitochondrial genomes. Studies of 5S and 16S rRNA sequences indicate possible relationships between Hyphochytriomycetes, crysophyte algae, and Oomycetes (Wolters and Erdmann 1986, Gunderson et al. 1987). The possibility of IRs being present was therefore investigated in the Hyphochytriomycetes, *Rhizidiomyces apophysatus* and *Hyphochytrium catenoides*.

An IR was found in the mtDNA of *H. catenoides*. The size and position of this IR is remarkably like that in Oomycetes. Also, approximate genome complexity, calculated by subtracting the size of one arm of the IR from genome size, is identical to that of the Oomycetes (Table VII). The LrRNA gene region is adjacent to the small unique region, similar to the arrangement in

Sapromyces elongatus. The small unique region is somewhat larger than is typical for Oomycetes, especially for *Pythium* species, where the small unique region is very small or absent. The other species of Hyphochytriomycetes investigated, *R. apophysatus*, does not have the IR and has a genome size of 50 kb (equivalent to an approximate genome complexity of 50 kb), which is significantly above the average for Oomycetes and which falls outside the range of known Oomycete mtDNA complexities (36.2 - 45.3 kb).

Comparison of the two Hyphochytriomycete maps leads to a number of observations. First, the physical maps are not similar enough to allow alignment. It is, however, well established on the basis of ultrastructural studies of the flagellar apparatus and on chemotaxonometric data that the two species are more closely related to each other than to any other fungus (Barr 1981, Cooney and Barr 1985, Barr and Allan 1985). We have also seen this relationship in a comparison of ribosomal DNA physical maps (to be discussed later). The lack of an obvious alignment between the two maps is consistent with the state of affairs within the Oomycete group. In general, maps of mtDNA from species within the same genus can be aligned (shown for *Pythium*, *Phytophthora*, *Apodachlya*, and *Achlya*), but as discussed previously, alignments above the species level are generally not observed (Boyd et al. 1984, Shumard et al. 1986, Förster et al. 1987, Fig. 4, Fig 5, and Fig 6). The second difference between the two Hyphochytriomycete maps is that one has an IR of the Oomycete type while the other does not. Among Oomycetes, presence or absence of the IR has so far been found to be consistent at the level of genus. All *Pythium* species have a large IR and all *Phytophthora* species have none (Table VII). Thus, the two Hyphochytriomycetes do not exhibit any mtDNA differences inconsistent with their accepted classification.

The third difference between the two mitochondrial genomes may be more significant. Although the two genome sizes are similar (*H. catenoides*: 54 kb, *R. apophysatus*: 50 kb), their approximate genome complexities, calculated by subtracting one arm of the IR from genome size, differ by about 10 kb (*H. catenoides*: 39.5 - 40.5 kb, *R. apophysatus*: 50 kb). A comparison of seventeen species of Oomycetes from seven genera is given in Table VII. The average genome complexity was found to be 39.9 kb with a range from 36.2 - 45.3 kb. Of the seventeen species surveyed ten species had IRs and seven did not. It appears that the complexity difference between the two Hyphochytriomycetes is at least as large or larger than that between the members of the most extreme pair in a much larger sample of Oomycetes. The likely conclusion is that mitochondrial genome complexity is less conserved among Hyphochytriomycetes than it is among Oomycetes. The genome complexity of *H. catenoides* is identical to the average value for Oomycetes while the value for *R. apophysatus* falls significantly above the Oomycete range. This may mean that the evolutionary forces keeping Oomycete mtDNA complexities near to 40 kb are operating more effectively in *H. catenoides* than in *R. apophysatus*.

The presence of the IR in the mtDNA of *H. catenoides* and its striking resemblance to the IRs of Oomycetes supports the idea that the evolutionary origin of the IR predates the isolation of the Oomycetes as a group. This finding allows the treatment of the IR as an ancestral character in the phylogenetic study of Oomycete groups. Any modification or loss of the IR can then be seen as a derived character significant for phylogeny. Enough Oomycete mtDNA maps are known so that shared derived characters related to mtDNA organization can be discerned. All *Pythium* species share an extremely large and variable IR and the reduced small unique region. All *Phytophthora* species share the absence of the IR, as do the Leptomitales. All Saprolegniales share the

relatively small IR with the LrRNA gene adjacent to the large unique region. Further investigation of gene complement within the IR should yield more shared derived characters useful for phylogenetic analysis.

The survey of Chytridiomycete mtDNA is too small to analyze for evolutionary trends since maps of only two genera are known and sizes for three (Table VII). However, genome size variability may be more constrained in Chytridiomycetes as opposed to Basidiomycetes or Ascomycetes, since so far only less than a two-fold variation in size has been observed. Also gene order is conserved between the genera *Allomyces* and *Blastocladiella* (Borkhardt and Olsen 1986), a finding in contrast to the pattern in Ascomycetes (Grossman and Hudspeth 1985). As more species of Chytridiomycetes are surveyed, it should be interesting to see if any trends exist in Chytridiomycete mitochondrial genomes which could be useful in phylogenetic analysis.

2. Discussion of rDNA repeat physical maps

2.1 General characteristics of the rDNA repeat

The physical maps of Mastigomycotina rDNA repeats reported in this thesis conform closely to those published for other true fungi in size and gene arrangement. An investigation of the rDNA of *Achlya ambisexualis* E87 (Rozek and Timberlake 1979) produced results which indicated that this Oomycete might have a very long (estimated at 40 kb) extrachromosomal rDNA molecule, similar to that found in *Dictyostelium* (Maizels 1976). This conclusion was based on the observation that *Pst*I and *Bam*HI produced fragments greater than 24 kb which hybridized to the ribosomal RNA probe that was used. I have found a normal tandem 9.9 kb repeat in *A. klebsiana* which lacks *Pst*I sites (*Bam*HI has not been used.) If there were no *Pst*I or *Bam*HI sites in *A. ambisexualis* rDNA, the rDNA would be included with other high molecular-weight DNA in *Pst*I and *Bam*HI gel profiles, accounting for the results observed. Failure to detect the

rDNA repeat in the physical map of *A. ambisexualis* may have been due to an unfortunate choice of endonucleases and limitations in the accuracy of mapping by the method used. The size of the repeat unit is probably 10.7 kb as defined by a *Sal*I band which is likely to contain the whole repeat unit.

Most Mastigomycotina have an rDNA repeat with a size falling within the range 9 to 12 kb (Table VI). This is similar to the range of sizes for Ascomycotina and Basidiomycotina, although the repeats for Ascomycotina seem to be slightly smaller on the average than repeats in other groups (see details and references in Table III). *L. caudata*, however, is atypical in this respect, having a repeat size of 15 kb or more (Fig 16). A similar atypically large rDNA repeat has been found in *Pythium aphanidermatum* (Belkhiri and Dick 1988). Increased size of the repeat in *L. caudata* is entirely due to an enlarged IGS region.

Heterogeneity in the size of the IGS is rarely observed in fungi. In the Ascomycete *Yarrowia lipolytica* two IGS size classes (7.7 and 8.7 kb) have been reported in the same organism and it has been shown that differences reflect complex sequence differences and not just variability in families of short repeats (Van Heerikhuizen et al. 1985). Heterogeneity has now been found in a number of Oomycetes (*S. diclina* , *L. caudata*, *V. flavofaciens* (Fig.15, 16 and 22), *Pythium ultimum*, and certain strains of *Phytophthora cryptogea* (G. Klassen, unpublished results). Heterogeneity has also been found in the Hyphochytriomycete, *R. apophysatus* (Fig. 17). In *Pythium ultimum* the heterogeneity resembles that found in plants. Heterogeneity in *Pythium ultimum* is very pronounced, with a large number of size classes all differing from one another by increments of approximately 350 bp. This suggests that the IGS contains 350 bp subrepeats. In the other species displaying heterogeneity only a few size classes are usually present and the heterogeneity has not been

characterized. In certain *Phytophthora cryptogea* strains and *R. apophysatus*, the size classes seem to differ in a stepwise fashion as seen in *Pythium ultimum*, indicating that subrepeats may also be present. In *R. apophysatus* the subrepeats appear to be very large (900 bp) and also form a uniform ladder as in *P. ultimum* (G. Klassen, unpublished results). Also in *P. irregulare* and *L. giganteum* which have homogeneous rDNA repeats a number of subrepeats may exist of approximately 500 bp. Although the presence of subrepeats in *P. irregulare* and *L. giganteum* awaits confirmation through further experimentation, the presence of subrepeats in plants with homogeneous rDNA have been observed (Rogers and Bendich 1987). These results therefore indicate that plant-like subrepeats may not be uncommon in the rDNA of lower fungi. In *S. diclina*, *L. caudata*, and *V. flavofaciens*, even ladders were not observed. However, the data is consistent with subrepeats of approximately 200 bp in *S. diclina* and 500 bp in *L. caudata*, although some steps of the ladder do not seem to be represented. Data is still insufficient to conclude decisively whether the observed heterogeneity is like that found in *Yarrowia lipolytica* or like that found in plants and *Pythium ultimum*. Interestingly, *Pythium ultimum* has two regions of heterogeneity in the IGS as does *L. caudata* (Fig 16 and G. Klassen, unpublished results). The presence of plant like heterogeneity in Oomycetes/Hyphochytriomycetes and the apparent absence of such heterogeneity in higher fungi may support the argument for a separate ancestry for these two groups. Examination of rDNA repeats for the presence of heterogeneity in chrysophyte algae, which are thought to have a common ancestry with the Oomycetes/ Hyphochytriomycetes, may help resolve this issue (Cavalier-Smith 1986).

2.2 Identification of sites ancestral within the Eumycota

Comparison of the eumycota maps available to date shows that some sites are highly conserved (Fig. 26). A further search of published 18S, 28S, 5.8S gene

sequences and other eukaryotic ribosomal repeat maps has revealed the presence of some sites that are highly conserved throughout evolution. Use of different sets of restriction enzymes by various researchers has hampered compilation of data on repeat unit maps but some generalizations can be made. Sites that appear at the same positions in each of the genes in several different taxons are considered to be ancestral sites. Positions of ancestral sites in the SrRNA gene are given in relation to sites in the sequence for *S. cerevisiae* (Rubstov et al. 1980). The positions in the 5.8S gene refer to the alignment published by Erdmann and Wolters (1986). The positions of ancestral sites in the LrRNA gene are given in reference to those from *S. carlsbergensis* (Veldman et al. 1981). A discussion of ancestral sites is given below.

In the SrRNA gene, four sites are highly conserved within the Eumycota. The *Xba*I site at the 5' end of the gene (position 159) appears to be a universal eukaryotic site since it has been found in almost all eukaryotes whose sequences have been determined (Huysmans and DeWachter 1986). This site has been found in all fungi examined. The *Sst*II site (position 572) is an extremely stable site present in eukaryotes, prokaryotes, chloroplasts, and mitochondria (Huysmans and DeWachter 1986). This site is present in all fungi except *Zoophagus insidians* (Fig 24) and *Puccinia graminis* (G. Klassen, unpublished results). Further into the gene is an *Sst*I (position 1230) which is present in most fungi except some Basidiomycetes (G. Klassen, unpublished results). This site also appears to be present in plants (Huysmans and DeWachter 1986) and the algae *Chlamydomonas* and *Ochromonas* (Gunderson et al. 1987). The *Eco*RI site at the 3' end of the gene (position 1564) is another universal eukaryotic site. (Huysmans and DeWachter 1986). It has been found in all fungi except *N. crassa* (Sogin et al. 1986) and some Basidiomycetes (G. Klassen, unpublished results).

In the 5.8S gene two sites have been identified that can be considered ancestral. The first is a *Cla*I site (position 34). This site is found in plants and *Chlamydomonas reinhardii* (Erdmann and Wolters 1986). In fungi, this site appears to be somewhat labile being present in some Ascomycetes (Erdmann and Wolters 1986), most Oomycetes (Figs 12, 13, and 14), some Chytridiomycetes (*C. confervae* - Fig 19), and some Basidiomycetes (G. Klassen, unpublished results). However it is absent in other Chytridiomycetes (Spizellomycetales - Fig 19), some Basidiomycetes (G. Klassen, unpublished results) and Hyphochytriomycetes. The other ancestral site in the 5.8S gene is an *Eco*RI site (position 80). This site appears primarily in fungi (Erdmann and Wolters 1986) and can be considered a stable fungal site, being present in the majority of members of all fungal groups studied (Fig 26).

In the LrRNA gene at least four ancestral sites have been determined. The first is a *Cla*I site in the 5' portion of the gene (position 882). This site has been found in the Ascomycete, *S. carlsbergensis* (Veldman et al. 1981), in all Oomycetes, Hyphochytriomycetes, and Chytridiomycetes (Fig 18, 19, and 25), as well as some, but not all, Basidiomycetes (G. Klassen, unpublished results). The most conservative site in the LrRNA gene is a *Bgl*II site near the middle of the gene (position 1431). This site appears to be a highly conservative eukaryotic site found in animals, (Tanhauser et al. 1986), plants (Jorgensen et al. 1987, Tucci and Maggini 1986) and *Physarum polycephalum* (Otsuka et al. 1983). It has been found in all fungi studied (Fig 26, and references from Table VI). Another ancestral but more labile site is the *Cla*I site towards the 3' end of the gene (position 2760). This site has been found in the Ascomycete, *S. carlsbergensis* (Veldman et al. 1981), some Oomycetes, Hyphochytriomycetes, Chytridiomycetes, (Fig 18, 19, and 25), and in the Basidiomycete, *Tilletia controversa* but not other Basidiomycetes (G. Klassen, unpublished results). The fourth site is an *Eco*RI

site (position 2900) near the 3' end of the LrRNA gene which is a universal eukaryotic site found in plants, animals and most fungi (Jorgensen et al. 1987, Tucci and Maggini 1986, Tanhauser et al. 1986, references in Table III and Fig 26).

There are three other sites in the LrRNA gene which are possibly ancestral for Eumycota. The first of these is a *Xba*I site (position 1216) which is present in Ascomycetous yeasts (Verbeet et al. 1984), Chytridiomycetes (Fig 19) and Basidiomycetes (G. Klassen, unpublished results). This site has not been found in any Oomycetes or Hyphochytriomycetes (Fig 18 and 25). This may mean that this site was ancestral to all Eumycota but was lost in Oomycetes/Hyphochytriomycetes, or that it was gained in an ancestor common to higher fungi. Alternatively the distribution of this site may reflect a polyphyletic ancestry among fungi.

Another potentially ancestral site is an *Eco*RI site just downstream of the highly conserved *Bgl*II site. This site is commonly found in Oomycetes, Hyphochytriomycetes, and some Chytridiomycetes. This site has not been found in Ascomycetes (Russell et al. 1984, Verbeet et al. 1984) or Basidiomycetes (G. Klassen, unpublished results) (Fig 26). This may reflect a loss of this site in these groups. Alternatively the distribution of this site could suggest a relationship between the Oomycetes, Hyphochytriomycetes, and Chytridiomycetes. The study of more fungal groups as well as algal groups which have been proposed to be related to Oomycetes/Hyphochytriomycetes may help resolve these issues.

Finally there is an *Hinc*II site (position 2172) which may be ancestral. This site has been found in Oomycetes, Hyphochytriomycetes, and possibly some Chytridiomycetes (*S. punctatus* - Fig. 19). It is also present in the Ascomycete *N. crassa* (Russell et al. 1984) and *S. carlsbergensis* but not in other yeasts (Verbeet

et al. 1984). Analysis of *Hinc*II sites in more fungal groups should aid in determining whether or not this is an ancestral Eumycota site.

2.3 Analysis of ancestral and derived sites within zoosporic fungi

2.3.1 Mastigomycotina

Among the Mastigomycotina only one potentially derived site has been found. This site is the *Eco*RI site just downstream of the universal *Bgl*II site in the LrRNA gene which, as discussed above, may be an ancestral Eumycota site which has been lost in the higher fungi. Even within the Mastigomycotina this site is labile, being absent in Peronosporales and Rhipidiales (*S. elongatus*) within the Oomycetes, and absent in the Chytridiales (*C. confervae*) within the Chytridiomycetes. It therefore seems plausible that this site was present in an ancient eukaryotic progenitor and then lost in the Ascomycotina and Basidiomycotina rather than derived in the Mastigomycotina. This one site therefore should not be considered evidence for a common ancestry between classes of the Mastigomycotina.

2.3.2 Chytridiomycetes

Only four members of the Chytridiomycetes have been surveyed. These include three fungi from the order Spizellomycetales and one fungus from the order Chytridiales. Chytridiales and Spizellomycetales represent divergent orders of the Chytridiomycetes (Barr 1983). Therefore sites found in both these orders can be considered ancestral to the entire class. Two sites in the LrRNA gene have been identified which appear to be Chytridiomycete specific sites. One is a *Pst*I site towards the 5' end of the gene which is present in all four species surveyed. There is also a *Pst*I site near the 5' end of the gene in Hyphochytriomycetes (Fig. 18). However, it is doubtful whether this is the same site since it appears to be closer to the 5' end of the gene and has a larger *Cla*I to *Pst*I spacing in Hyphochytriomycetes than in Chytridiomycetes. The other

Chytridiomycete derived site is a *Bgl*II site about 200 bp downstream of the *Pst*I site. This Chytridiomycete *Bgl*II site is absent in *G. semiglobiferus* but its presence in two species of *Spizellomyces* and in *C. confervae* suggest that this is an ancestral Chytridiomycete site which has been lost in *Gaertneriomyces.*

Within the order Spizellomycetales three sites have been identified which are common to *Spizellomyces* (Table VIII, Fig 19). A *Hin*dIII site 400 - 500 bp upstream of the universal *Bgl*II site is found in *S. punctatus* and *S. plurigibbosus* but not in *G. semiglobiferus.* There are also two *Sst*I sites found in *S. punctatus* and *S. plurigibbosus*, one about 600 bp downstream of the universal *Bgl*II site and one at the extreme 3' end of the gene. Since *Sst*I sites have not been mapped in *G. semiglobiferus*, it is not known whether these *Sst*I sites are specific for the genus *Spizellomyces* or whether they are common to the order Spizellomycetales. There also seems to be an approximate 300 bp insertion in the 3' end of the LrRNA gene specific to the genus *Spizellomyces.* The ancestral *Cla*I, *Eco*RI sites at the 3' end of the gene are displaced by this amount in *S. punctatus* and *S. plurigibbosus* when aligned with other fungal rDNA maps. This insertion may be useful as a *Spizellomyces* diagnostic character. These results suggest that rDNA mapping may be useful in differentiating between genera within the Spizellomycetales.

In *C. confervae* the ancestral *Eco*RI site is absent in the 5.8S gene, but unlike the situation in the other Chytridiomycetes the *Cla*I site is present. Also in *C. confervae* the *Eco*RI site adjacent to the universal *Bgl*II site is absent, unlike the situation in the Spizellomycetales. Further studies will be necessary to establish whether these are general features of the order Chytridiales. These sites may reflect differences between the Chytridiales and Spizellomycetales which may prove useful in following the evolution of the different orders of Chytridiomycetes.

Table VIII Number of Differences and Similarities Between Species of the Chytridiomycetes Within the Conservative Gene Region

	[1]*S. punctatus*	*S. plurigibbosus*	[2]*G. semiglobiferus*	*C. confervae*
[1]*S. punctatus*		4	1	0
S. plurigibbosus	2		1	0
[2]*G. semiglobiferus*	7	5		0
C. confervae	11	9	9	
Consensus	6	4	5	5

The numbers in the top half of the table refer to the number of shared-derived characters (gains or losses of restriction sites) in the pairwise comparison of species within the Chytridiomycetes. The bottom half of the table shows the total number of differences in the species being compared form the consensus map in Fig 19.

[1]Does not include *Ava*I and *Hin*cII sites since these were not mapped in other species.

[2]Does not include data for *Sst*I sites which were not mapped in this species.

2.3.3 Oomycetes and Hyphochytriomycetes

Three sites are shared between the Oomycetes and the Hyphochytriomycetes which may indicate common ancestry. These are an *Ava*I site about 450 bp downstream of the universal *Sst*II site and a *Hin*cII site just past the ancestral *Sst*I site in the SrRNA gene. The third site is a *Sst*I site towards the 3' end of the LrRNA gene. Since these three sites are on two different genes and since none of them have been found in other fungal groups these sites would seem to indicate a common ancestry for the Oomycetes and the Hyphochytriomycetes. Interestingly, the *Hin*cII site mentioned above is also found in the chrysophyte alga *Ochromonas danica* and the SrRNA sequence shows a high degree of homology with the *Achlya* SrRNA sequence. This supports the idea of a common ancestry for Oomycetes and chrysophyte algae (Gunderson et al. 1987). Study of rDNA repeat maps or sequence analysis of more chrysophyte algae may help confirm a relationship between these organisms and Oomycetes/ Hyphochytriomycetes.

2.3.4 Hyphochytriomycetes

Three Hyphochytriomycete-specific sites have been determined. These are an *Eco*RV site in the 5.8S gene upstream of the conserved *Eco*RI site. The same *Eco*RV site is found in ciliates and some angiosperms. Ascomycetes have a sequence which with a single bp change would give an *Eco*RV site at this position (Erdmann and Wolters 1986). The somewhat labile *Cla*I site of the 5.8S gene is missing from the Hyphochytriomycetes. In the LrRNA gene there are two Hyphochytriomycete-specific sites. These are a *Pst*I site towards the 5' end of the gene and an additional *Eco*RI site in the 3' portion of the gene. There are possibly some *Ava*I and *Hin*cII sites in the LrRNA gene shared by Hyphochytriomycetes (an *Ava*I site just upstream of the universal *Bgl*II site in

the LrRNA gene is probably a Hyphochytriomycete-specific site) but the mapping of these enzymes is too incomplete to make accurate comparisons.

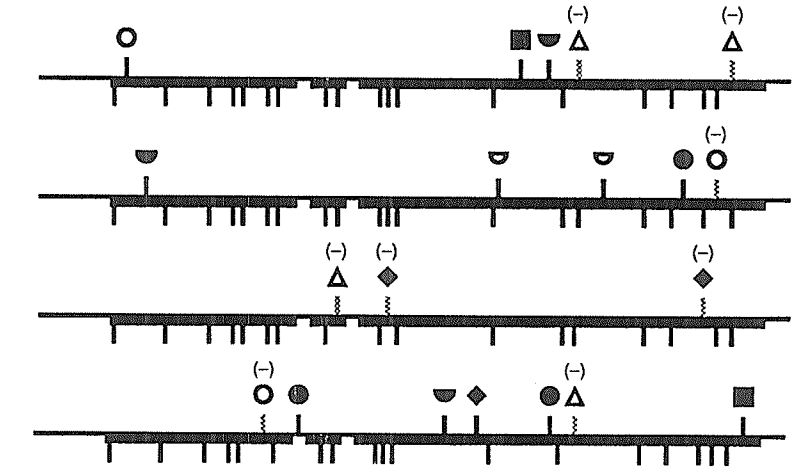
2.3.5 Oomycetes

A number of sites specific to the class Oomycetes have been determined (Fig 25). In the SrRNA gene there is *Cla*I site just upstream of the ancestral *Eco*RI at the 3' end of the gene. This *Cla*I site has been found in all Oomycetes except *P. paddicum* and *S. elongatus*. In the LrRNA gene four sites have been identified as ancestral to the class. These include a cluster of *Eco*RV, *Sst*I, and *Hin*dIII sites near the 3' end of the gene. These sites appear in all Oomycetes except that the *Hin*dIII site is absent in *L. caudata* and the *Sst*I site is absent in *P. chaunorhiza* and *V. flavofaciens*. The other Oomycete-specific site is a *Xba*I site towards the 3' end of the gene. This site is absent in certain Peronosporales.

Peronosporales. By comparison of the Peronosporales consensus with other Oomycete maps (Fig 25) two Peronosporales-specific sites can be identified. These are the *Cla*I at the 5' end of the SrRNA gene and the *Pst*I site near the middle of the LrRNA gene. There are also two sites which are characteristically absent in Peronosporales, the *Eco*RI site near the middle of the LrRNA gene which is present in Saprolegniales/Leptomitales, Hyphochytriomycetes, and Chytridiomycetes (Fig 25 and 26), and the near universal *Eco*RI site at the 3' end of the LrRNA gene. Also in the Peronosporales the Oomycete-specific *Xba*I site at the 5' end of the large rRNA gene is absent in some species. This site is present in all Saprolegniales/Leptomitales, *S. elongatus*, *V. flavofaciens*, *P. chaunorhiza*, and in *L. giganteum* and therefore is probably ancestral for Oomycetes. Its absence in only a few Peronosporales may reflect a recent loss and may be useful in grouping species within the Peronosporales.

Figure 25. Comparison of the major orders of the Oomycetes and construction of an Oomycete consensus map. The consensus maps for Peronosporales, Saprolegniales/Leptomitales, Sclerosporales, and Rhipidiales are as given in Fig 12, 13, 14, 20, and 21, except that the sites ancestral to all maps are shown beneath the map while sites diagnostic for the order are shown above the map. A consensus for the class Oomycetes is shown at the bottom.

Oomycetes


(–)
(–)
Peronosporales
(–)
Saprolegniales / Leptomitales
(–)
(–)
(–)
Sclerosporales
(–)
(–)
Rhipidiales
NTS
SrRNA
5.8S
LrRNA
Oomycete
Consensus Map
AvaI
BamHI
BglII
ClaI
EcoRI
EcoRV
HincII
HindIII
PstI
SstI
SstII
XbaI
1 Kb

If each of the Peronosporales maps is compared to the consensus, differences between the consensus map and each individual range from 4 in *P. anandrum* to 10 in *P. paddicum* (Table IX, Fig 12) thereby indicating there is considerable variability within the order. Morphological criteria (M.W. Dick, unpublished results) suggest that there are perhaps five major centers of speciation within *Pythium*. *P. anandrum*, *P. torulosum/P. diclinum* and *P. irregulare* correspond to three of these groups. Molecular divergence data can be used to begin to test this suggestion. The maps shown in Fig 12 illustrate the potential of rDNA restriction mapping for grouping species within the genus *Pythium*. Two groups are emerging based on shared derived-sites. *P. diclinum* and *P. torulosum* share 4 sites and contain only two differences in the compared region. This would indicate that the two species are distinct but quite closely related. The other group includes *P. irregulare*, *P. anandrum*, and *P. paddicum*. Shared derived sites between these species range from 1 to 4 sites while differences range from 5 to 12 sites (Table IX). The large number of differences between members of this group probably indicate that this is a somewhat divergent group and study of more *Pythium* species will probably lead to the identification of subgroups within this group. *P. cryptogea* does not show greater divergence from the various *Pythium* species than members of the different *Pythium* groups show from one another (Table IX). Taxonomically this could be taken as providing a prima-facie case for examining the generic boundaries within the Pythiaceae and for considering a sub-division of *Pythium*.

Saprolegniales/Leptomitales. The Saprolegniales/Leptomitales consensus shows that there are several Saprolegniales/Leptomitales-specific sites Fig 25. One of these sites is an *Ava*I site slightly downstream from the middle of the LrRNA gene. An *Ava*I site appears in a similar position in the Chytridiomycete, *S. punctatus* (Fig 19), but because of the dissimilarities of these two maps in

Table IX Comparison of sites of Peronosporales

	P. diclinum	*P. torulosum*	*P. irregulare*	*P. anandrum*	*P. paddicum*	*P. cryptogea*	[1] *L. giganteum*
P. diclinum		4	0	0	1	0	0
P. torulosum	2		0	0	1	0	0
P. irregulare	10	10		2	4	0	0
P. anandrum	9	9	5		1	0	0
P. paddicum	13	13	7	12		0	0
P. cryptogea	11	11	11	12	16		0
[1] *L. giganteum*	5	5	7	6	12	8	
Consensus	5	5	5	4	10	8	1

The numbers in the top half of the table refer to the number of shared-derived characters (gains or losses of restriction sites) in the pairwise comparison of species within the Peronsporales. The bottom half of the table shows the total number of differences in the species being compared from the consensus map in Fig 12.

[1] These numbers do not include *Ava*I and *Hinc*II sites as they have not been fully mapped in *L. giganteum.*

other respects this similarity is likely to be of little taxonomic significance. This site is therefore useful as a diagnostic site for the Saprolegniales/Leptomitales. Another site is the *Ava*I site slightly upstream of the middle of the LrRNA gene. This site has not been found in other organisms and should be considered diagnostic for this group. The *Hin*dIII site near the 3' end of the LrRNA gene is potentially a third diagnostic site. This site is absent from the *S. diclina* map but its presence in the closely related species *S. ferax* (see discussion below) suggests that this site is a general feature of the Saprolegniales/Leptomitales and its absence in *S. diclina* represents an isolated loss. This *Hin*dIII site may also be present in *P. irregulare* and *P. anandrum* (Fig 12) and thus may be of limited value as a diagnostic character. It is unclear whether the presence of this *Hin*dIII site in certain *Pythium* species indicates that this is actually an ancestral Oomycete site which is easily lost or whether this site has been gained independently in some *Pythiums*. There is also a *Hin*cII site near the 5' end of the SrRNA gene which has been found in all the members of the Saprolegniales/Leptomitales for which this enzyme has been mapped. This site has been found in the Rhipidiales, so although not useful as a Saprolegniales/Leptomitales diagnostic site, its absence in Peronosporales can be considered a diagnostic character for that order (Fig 25).

Several observations can be made with respect to comparisons within the Saprolegniales/Leptomitales group. In the 6 kb gene region, differences between the consensus map and each individual map range from 0 to 5 (Table X, Fig 13 and 14). Variability between different genera range from 1 to 8 differences, based on pairwise comparisons. This is considerably less variability than was observed within the Peronosporales (Table IX). This suggests that the Peronosporales are a more divergent group than the Saprolegniales/Leptomitales and may indicate the need to revise the taxonomy of the Peronosporales. In

Table X Comparison of sites of Saprolegniales/Leptomitales

	S. diclina	[1]*S. ferax*	*A. klebsiana*	*A. terrestris*	*A. stellatus*	*L. caudata*	*A. pyrifera*	*A. brachynema*	*L. lacteus*	*V. flavofaciens*	*P. chaunorhiza*	*S. elongatus*
S. diclina		3	0	1	0	0	0	0	0	0	0	1
[1]*S. ferax*	1		0	0	0	0	0	0	0	0	0	0
A. klebsiana	5	3		0	0	0	0	0	0	0	0	0
A. terrestris	6	5	3		0	0	0	0	0	0	0	0
A. stellatus	6	3	1	4		1	0	0	0	0	0	0
L. caudata	7	4	2	5	1		0	0	0	0	0	0
A. pyrifera	8	5	3	6	4	5		2	1	0	0	0
A. brachynema	8	6	3	6	4	5	2		1	0	0	0
L. lacteus	7	4	2	5	3	4	3	3		0	0	0
V. flavofaciens	8	6	3	6	4	5	6	6	5		3	0
P. chaunorhiza	8	6	3	6	4	5	6	6	5	0		0
S. elongatus	13	10	10	13	11	12	13	13	12	13	13	
Consensus	5	3	0	3	1	2	3	3	2	3	3	10

The numbers in the top half of the table refer to the number of shared-derived characters (gains or losses of restriction sites) in the pairwise comparison of species within the Saprolegniales/Leptomitales. The bottom half of the table shows the total number of differences in the species being compared from the consensus map in Fig 13.

[1]These numbers do not include *Ava*I and *Hinc*II sites since these enzymes have not been mapped in *S. ferax*.

general, different genera do not share sites other than those in the consensus, although some exceptions to this do exist. *L. caudata* and *A. stellatus* share one derived site as do *S. diclina* and *A. terrestris.* The other exception to this is *L. lacteus* which shares one site each with *A. pyrifera* and *A. brachynema.* The two *Apodachlya* species share a *Cla*I site at the extreme 5' end of the SrRNA gene and a *Pst*I site within the first third of the LrRNA gene. As well, certain sites in the IGS can possibly be aligned. This indicates that these two Apodachlya species are closely related. *A. brachynema* and *L. lacteus* have a *Cla*I site at the extreme 3' end of the LrRNA gene or slightly beyond. *A. pyrifera* and *L. lacteus* share an *Ava*I site in the middle of the LrRNA gene. As more Leptomitales species are studied it may actually be that the sites shared by *L. lacteus* with *Apodachlya* species are actually Leptomitales-specific sites which have been lost in certain *Apodachlya* species. These sites may therefore help to form a consensus for Leptomitales which is slightly different from the Saprolegniales.

The two *Saprolegnia* species are strikingly similar, sharing three differences with the consensus map near the 5' end of the LrRNA gene. In addition, four sites in the 3' region of the NTS of the two species can be aligned (*Sst*I, *Hin*dIII, *Eco*RI, *Pst*I) and it is possible that the central regions of the two NTSs could be aligned if the segment were inverted (*Eco*RI, *Pst*I, *Sst*I). This is shown in Fig 17. These results indicate that these two species are probably very closely related.

2.3.6 Species of Uncertain classification

Sapromyces elongatus.

The *S. elongatus* rDNA repeat has none of the sites considered to be diagnostic for Peronosporales nor does it have the sites that are diagnostic for Saprolegniales/Leptomitales. It also does not have the Oomycetes specific *Cla*I site near the 3' end of the SrRNA gene although it does have all the other

Oomycetes specific sites. It does have the near-universal *Eco*RI site at the 3' end of the LrRNA gene which is missing in the Peronosporales. It also has the *Hinc*II site at the 5' end of the SrRNA gene which is found in all Saprolegniales/Leptomitales. The sites unique to *S. elongatus* (*Hin*dIII at 3' end of SrRNA, *Hin*dIII in middle of LrRNA, *Pst*I at 5' end of LrRNA) are not found in other Oomycetes with the exception that the *Pst*I site is found in three species of *Pythium* (*P. irregulare*, *P. anandrum*, *P. paddicum*). The size of the repeat (11.1 kb) is typical of Oomycetes.

Lagenidium giganteum

The map for *L. giganteum* was compared with existing Peronosporales maps (Fig 21). The *L. giganteum* map differs from this consensus map by the addition of a single *Eco*RI site near the middle of the LrRNA gene and possibly some *Ava*I and *Hinc*II sites which have not yet been mapped. The two Peronosporales specific sites (*Cla*I at 5' end of SrRNA, *Pst*I near middle of the LrRNA) are also present in *L. giganteum.* The additional *Eco*RI site coincides with an *Eco*RI site that is found in the Saprolegniales/Leptomitales group. This site is not found in any of the Peronosporales maps as has been discussed above. The other *Eco*RI site that is absent in the Peronosporales (the *Eco*RI site at the 5' end of the LrRNA gene) is also absent in *L. giganteum.* A *Hinc*II site present in the central portion in the gene corresponds with a *Hinc*II site found in *P. irregulare*, *P. anandrum*, *P. paddicum*, *P. ultimum*, and *P. paroecandrum* (Fig 12 and G. Klassen, unpublished results). The presence of this site in *L. giganteum* probably indicates that it is ancestral to Peronosporales but has been lost in some *Pythiums* and *Phytophthora.* The size of the *L. giganteum* repeat (10.6 kb) falls very near to the average value reported for Peronosporales (10.7 kb). In a recent revision of Oomycete taxonomy (Dick et al. 1984) it was proposed that the order Peronosporales be reserved for obligate parasites of dicotyledons and

that a new order, the Pythiales be created which would include the families Pythiaceae, and Lagenidiaceae. The results in this study are therefore consistent with such a classification of *L. giganteum* within the Pythiales.

Verrucalvus flavofaciens/Pachymetra chaunorhiza

V. flavofaciens has been described by Dick et al. (1984) and is thought to be related to the Saprolegniales. *P. chaunorhiza* is a newly described species similar to *V. flavofaciens* (M.W. Dick, personal communication).The maps for these two organisms are extremely similar differing only in the IGS region and even the IGS regions are extremely similar, sharing three sites. This suggests that these two species are very closely related. When compared to the Saprolegniales/Leptomitales consensus these two maps were found to share all the Saprolegniales/Leptomitales specific traits. However they both differ from the Saprolegniales/Leptomitales consensus by the absence of the *Sst*I site at the 5' end of the LrRNA gene (an Oomycete specific site), the *Sst*I site at the 3' end of the LrRNA gene (an Oomycete /Hyphochytriomycete site) and the *Eco*RI site in the 5.8S gene (a fungal ancestral site). These differences may substantiate the separation of the species into a separate order, the Sclerosporales (Dick et al. 1988). The extreme similarity of the two species however in both the gene region and the IGS is comparable only to similarities found between species within the same genera (*S. diclina* and *S. ferax* (Fig 17), *A. pyrifera* and *A. brachynema* (Fig 14), and *Neurospora* species (Russell et al. 1984, Chambers et al. 1986). One would wonder if *P. chaunorhiza* and *V. flavofaciens* should also be considered species of the same genus. Morphologically these species are extremely similar differing only in that *V. flavofaciens* forms sporangium while *P. chaunorhiza* does not (M.W. Dick, personal communication) The size of the repeats for *V. flavofaciens* and *P. chaunorhiza* are within the range of the Saprolegniales/Leptomitales.

Zoophagus

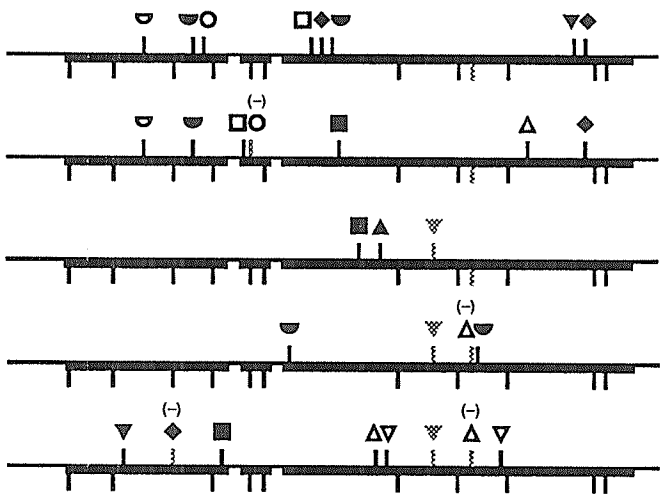
The rDNA repeat unit map for *Zoophagus insidians* was compared to the consensus map for the Peronosporales (Fig 24), the taxon to which it had previously been classified (Sparrow 1973). No Peronosporales-specific sites were found. In fact when compared to consensus maps for the various subdivisions of the Eumycota (Fig 26) the only sites shared between *Z. insidians* and the Oomycetes/Hyphochytriomycetes are those that have been shown to be ancestral for Eumycota. When compared to consensus maps for higher fungi some similarities do exist. The presence of the *Xba*I site in the central portion of the LrRNA gene, which is found in Chytridiomycetes, some Ascomycetes, and Basidiomycetes, suggests that *Z. insidians* should be grouped with the higher fungi. In addition to this *Xba*I site, *Zoophagus* has an extra *Xba*I site in the SrRNA gene in a position similar to the extra *Xba*I site in the SrRNA gene of Basidiomycetes (G. Klassen, unpublished). *Z. insidians* has a *Pst*I site in a position corresponding to the *Pst*I site found in the 3' half of the LrRNA gene of Chytridiomycetes. As well, an *Sst*I site found in the central portion of the LrRNA gene of *Z. insidians* corresponds in position to a *Sst*I site in *Spizellomyces*. These results all suggest that *Z. insidians* is not an Oomycete but rather should be grouped with the higher fungi. These rDNA findings are consistent with morphological observations that report that *Zoophagus* forms conidia-like spores, produces a brown water soluble diffusible pigment like *Fusarium* and has no real mycelial network (M.W. Dick, personal communication). Although molecular data strongly supports removal of *Zoophagus* from the Oomycetes, its taxonomic position within the higher fungi cannot be determined until more complete information on rDNA consensus sites have been determined for more groups within the higher fungi.

Figure 26. Comparison of Eumycota consensus maps to one another and to *Z. insidians*. The Oomycete, Hyphochytriomycete and Chytridiomycete consensus maps were taken from Fig 25, 18, and 19. The Ascomycete and Basidiomycete consensus maps are based on published rDNA maps from the literature (Table III for references) and from unpublished results of G. Klassen. Sites shown above the line are diagnostic at the subdivision level, while sites below the maps are presumed to be ancestral to all Eumycota. The *Xba*I and *Eco*RI sites are shown in 50% shading, since it is not known whether these sites are ancestral or derived (see text for explanation).

Mastigomycotina

Ascomycotina

Basidiomycotina

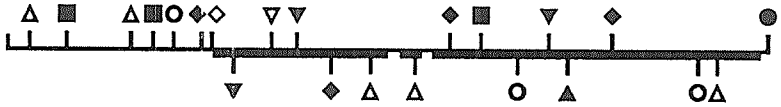


Oomycete

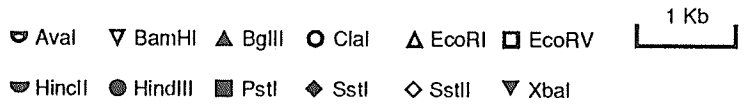
Hyphochytriomycete

Chytridiomycete

Eumycota
Consensus



Zoophagus insidians



3. Construction of a phylogenetic tree and its implications for taxonomy

A phylogenetic tree for fungi has been constructed in Fig 27 based on the molecular data presented in this thesis. This tree represents only one of the possible interpretations of the data presented. The tree shown supports the theory that Oomycetes and Hyphochytriomycetes are phylogenetically distant from the higher fungi. This interpretation is based on the presence of a *Xba*I site which is found centrally located only in the LrRNA gene of higher fungi. The presence of an IR and constraints on genome complexity in mtDNA which are characteristic of the Oomycetes/Hyphochytriomycetes are also important criteria for this interpretation. The *Xba*I site is shown as a derived character for the higher fungi in this tree but it could equally well be considered a loss for the Oomycetes/Hyphochytriomycetes. This version of the tree assumes that the *Eco*RI site in the centre of the LrRNA gene was ancestral to all Eumycota and was later lost in the branch leading to the Ascomycetes and Basidiomycetes as has been discussed previously. A different tree would result if this site was considered a derived site for the Mastigomycotina rather than an ancestral Eumycota site (Fig 28). In this case the *Xba*I site is considered to be ancestral but lost in Oomycetes/Hyphochytriomycetes after they diverged from the Chytridiomycetes. This possibility is in need of further exploration. Data on complexity and organization of mtDNA might prove useful in this respect since the limited data available indicates that Chytridiomycete mtDNA may be more variable than Oomycete mtDNA but more constrained than that in Ascomycetes or Basidiomycetes.

Within the Oomycetes two major branches of the tree are observed. These could be considered to correspond to the subclasses Peronosporomycetidae and Saprolegniomycetidae proposed by M. W. Dick (Dick et al. 1988). The Peronosporomycetidae branch consists of *L. giganteum*, *Phytophthora cryptogea*,

Figure 27. Phylogenetic tree of fungi based on rDNA repeat unit and mtDNA restriction mapping with emphasis on relationships within the Oomycetes. The base of the tree assumes the presence of sites as shown for the Eumycota rDNA consensus map in Fig 26 and the absence of an inverted repeat (IR) in the mitochondrial genome. Shared gains of characters are indicated by (+), and shared losses by (-). Species shown in parentheses were not included in the study presented in this thesis but are included in the phylogenetic tree based on other unpublished result of G. Klassen. The supercripts next to the site names indicate the position of the site within the gene region of the repeat unit: a) 5' end of the SrRNA gene, b) central portion of the SrRNA gene, c) 3' end of the SrRNA gene, d) 5.8S gene e) 5' end of the LrRNA gene, f) central portion of the LrRNA gene, g) 3' end of the LrRNA gene.

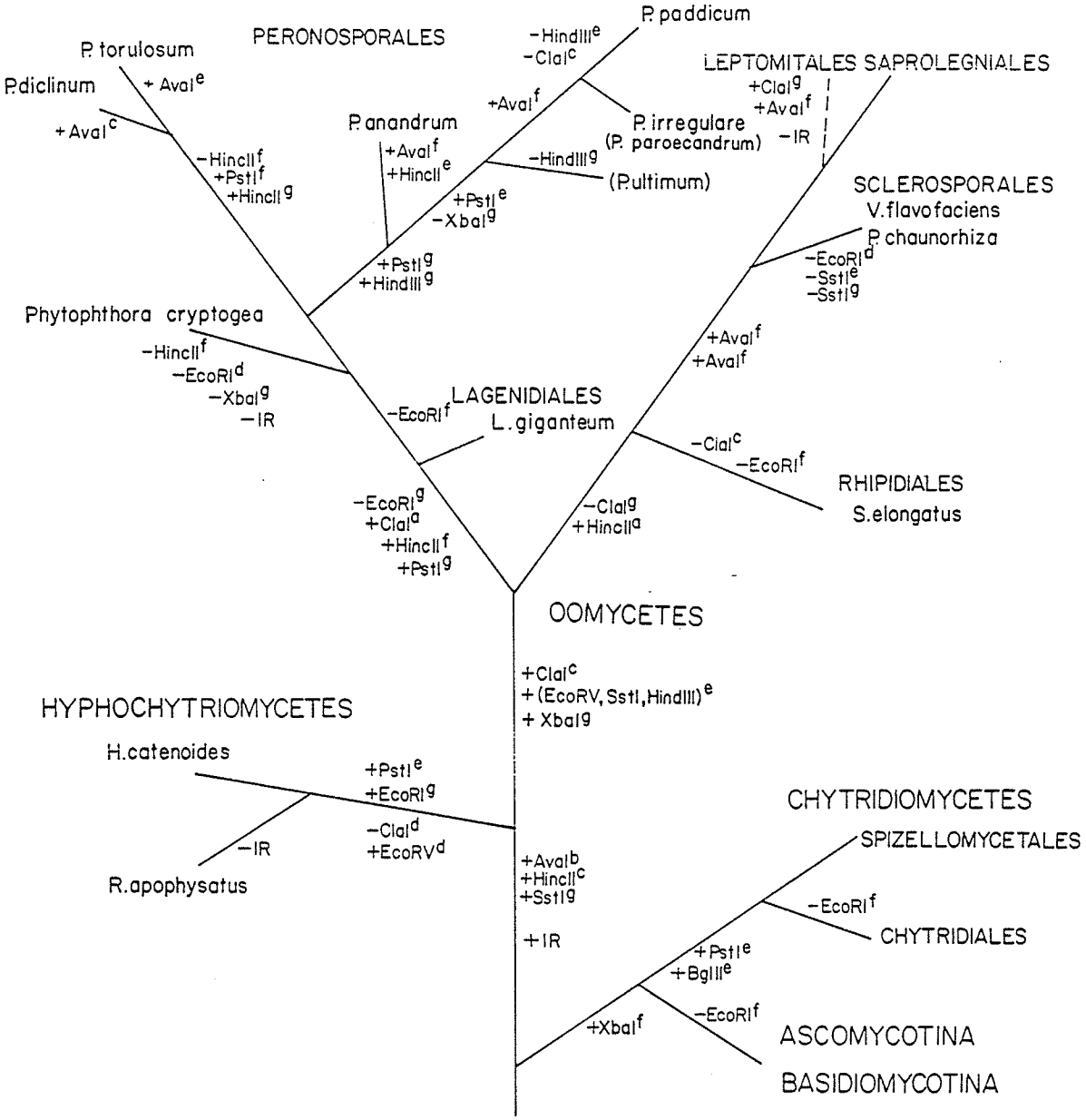
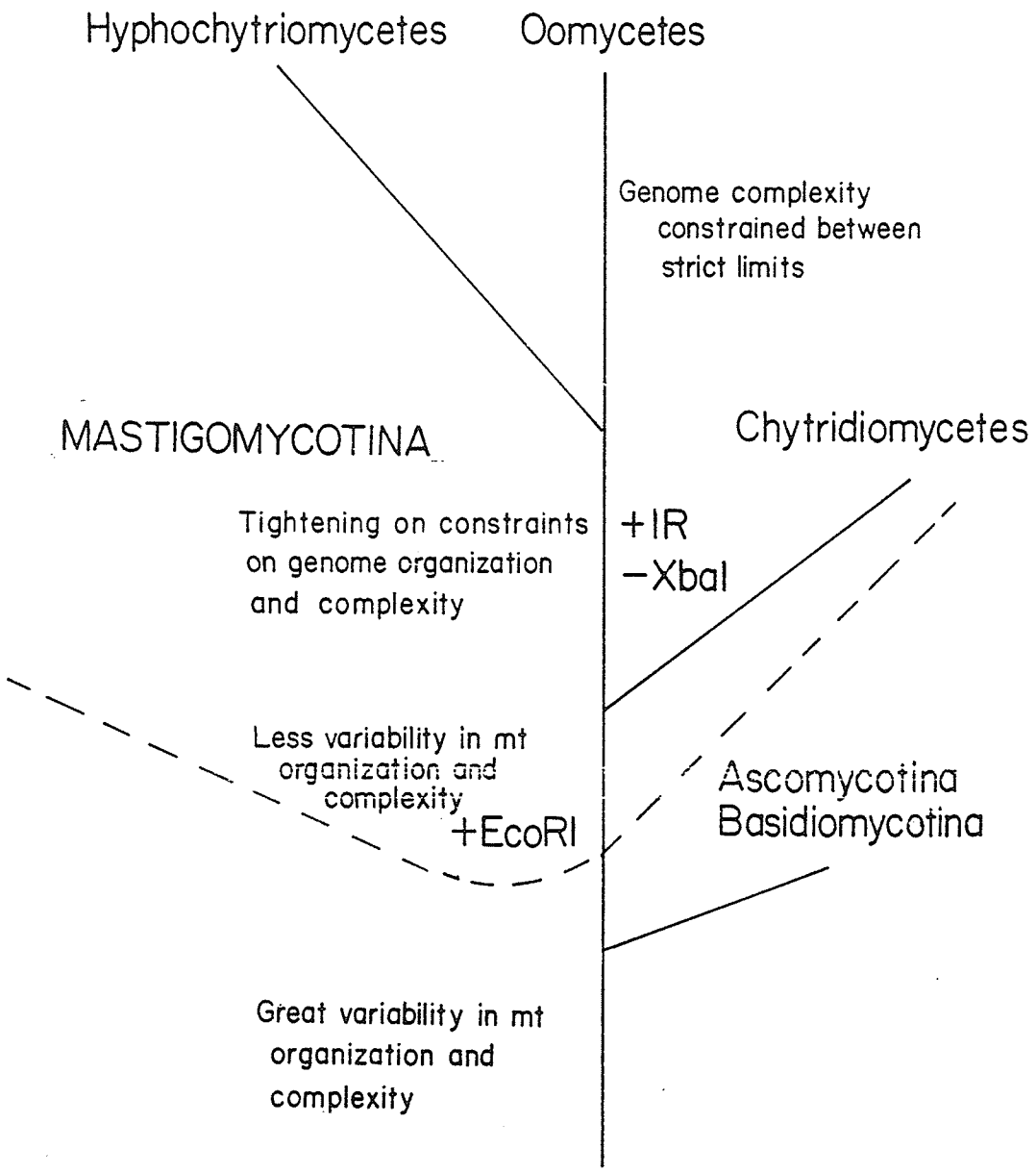

PERONOSPORALES
P. torulosum
+ AvaI e
P.diclinum
+ AvaI c
-HincII f
+PstI f
+HincII g
P. anandrum
+AvaI f
+HincII e
+PstI e
-XbaI g
+PstI g
+HindIII g
-HindIII e
-ClaI c
P. paddicum
+AvaI f
P. irregulare
(P. paroecandrum)
-HindIII g
(P.ultimum)
LEPTOMITALES SAPROLEGNIALES
+ClaI g
+AvaI f
-IR
SCLEROSPORALES
V.flavofaciens
P. chaunorhiza
-EcoRI d
-SstI e
-SstI g
+AvaI f
+AvaI f
Phytophthora cryptogea
-HincII f
-EcoRI d
-XbaI g
-IR
-EcoRI f
LAGENIDIALES
L. giganteum
-ClaI c
-EcoRI f
RHIPIDIALES
S.elongatus
-EcoRI g
+ClaI a
+HincII f
+PstI g
-ClaI g
+HincII a
OOMYCETES
+ClaI c
+(EcoRV, SstI, HindIII) e
+ XbaI g
HYPHOCHYTRIOMYCETES
H.catenoides
+PstI e
+EcoRI g
-ClaI d
+EcoRV d
-IR
R.apophysatus
+AvaI b
+HincII c
+SstI g
+IR
CHYTRIDIOMYCETES
SPIZELLOMYCETALES
-EcoRI f
CHYTRIDIALES
+PstI e
+BglII e
-EcoRI f
+XbaI f
ASCOMYCOTINA
BASIDIOMYCOTINA

Figure 28. Alternate phylogenetic tree for subdivisions of the Eumycota. In this version of the phylogenetic tree The *Xba*I site in the central portion of the LrRNA gene is considered to be ancestral for Eumycota and the *Eco*RI site slightly further downstream is considered to be derived in Mastigomycotina (See text and Fig 26 and 27 for further interpretation).


Hyphochytriomycetes
Oomycetes
Genome complexity
constrained between
strict limits
MASTIGOMYCOTINA
Chytridiomycetes
Tightening on constraints
on genome organization
and complexity
+IR
−XbaI
Less variability in mt
organization and
complexity
+EcoRI
Ascomycotina
Basidiomycotina
Great variability in mt
organization and
complexity

and *Pythium* species, representing the order Pythiales. The Saprolegniomycetidae branch consists of the orders Rhipidiales, Sclerosporales, Saprolegniales, and Leptomitales. Within the Saprolegniomycetidae two branches are observed, one for the order Rhipidiales (*S. elongatus*) and the other containing the orders Leptomitales, Saprolegniales, and the Sclerosporales. The Saprolegniales and Leptomitales are closely related, based on rRNA data and it could be suggested that these should be combined into one order. The major difference between these orders, loss of the IR in the Leptomitales, is similar to the situation seen in Pythiales where *Phytophthora* has lost the IR or in the Hyphochytriomycetes where *R. apophysatus* has lost the IR. Loss of IR may therefore not be criterion enough for separation of orders. Within the Peronosporomycetidae, *L. giganteum* is seen to be the most primitive member having retained the ancestral *Eco*RI site in the middle of the LrRNA gene. The *Pythiums* form a divergent group. Two major branches are shown here. *Phytophthora* is no more divergent from the *Pythiums* than they are from one another based on rDNA data. This data, however, is limited to only one species of *Phytophthora* and therefore should be regarded with caution. Hypothetically, *Phytophthora* could be shown on the same branch as *P. diclinum /P. torulosum* through the common loss of the *Hinc*II site in the central portion of the LrRNA gene. It is shown on its own branch, however, on the basis of mitochondrial data which shows that *Phytophthora* species have lost the IR while *Pythium* species have retained the IR. The loss of the *Hinc*II site in *Phytophthora* is then considered to have occurred independently of that in *P. diclinum/P. torulosum.*

The phylogenetic tree in Fig 27 indicates a monophyletic origin for Oomycetes and Hyphochytriomycetes. It supports the separation of Rhipidiales from the Leptomitales. It also suggests that the Saprolegniales/Leptomitales should be merged into one order or that criteria for separating orders should be

re-examined. It confirms that the Sclerosporales and Saprolegniales are closely related (Dick 1989). It supports the inclusion of *Lagenidium giganteum* in the Pythiales. The multibranching of the genus *Pythium* suggests that the boundaries of this genus should be re-examined.

4. Concluding Remarks

A number of conclusions can be drawn from the work presented in this thesis. First of all, the mitochondrial and ribosomal data generally complement one another. mtDNA analysis, by direct comparison of restriction sites is useful for comparing closely related species within a genus but not for comparisons between genera. Structural arrangements such as gene order (position of LrDNA gene within the IR) and size of repeat may also be useful for comparisons between species or closely related genera. The presence of an IR and genome complexity may be useful at higher taxonomic levels and has been shown to be helpful in relating Oomycetes and Hyphochytriomycetes and separating them from higher fungi. rDNA mapping is generally not useful for establishing relationships between closely related species within a genus due to the highly conservative nature of the gene region. However maps of the IGS, which evolves very quickly, may be useful at this level. Since purified rDNA cannot always be isolated and since interspecific rDNA probes do not hybridize to the IGS region, mitochondrial studies are probably more appropriate at this level. Alignment of rDNA maps between different genera and higher taxons is useful for taxonomic analysis since consensus maps for various taxons can be established and used to confirm existing taxonomic arrangements or to place doubtful organisms.

Neither mtDNA analysis or rDNA mapping is really adequate to establish very distant evolutionary relationships. Direct comparison between rDNA maps at higher levels is complicated by the necessity to survey large numbers of members from various branches of a taxon to get an idea of the variation

within the taxon. Also at higher levels it is difficult to determine whether a site in a similar position in two individuals is a derived shared site, co-evolution, or a different site in the same general vicinity.

When studying evolutionarily distant organisms, sequence analysis is still the method of choice since the presence of highly conservative domains usually allows sequences to be aligned with little ambiguity. For taxonomic surveys, however, rDNA mapping has several advantages over sequencing. rDNA mapping can generally be done rapidly on preparations of whole cell DNA which means it is possible to survey a large number of organisms in a relatively short period of time as compared to sequencing. rDNA includes information about three genes whereas sequence comparisons are usually based on only one gene. rDNA maps also have the advantage that analysis is relatively simple. Finally where information from rDNA maps is inadequate, alignment of sites with other maps can pinpoint regions of variability or stability for use in sequencing. A major disadvantage of rDNA mapping is that results are dependent on the appropriate choice of restriction enzymes. Availability of a few gene sequence within the taxon of interest can aid considerably in making wise choices of enzymes. Consequently, the two techniques each have their place in evolutionary studies.

The mitochondrial and rDNA data presented in this thesis suggest that Oomycetes and Hyphochytriomycetes share a common ancestor. If these fungi have indeed evolved from a heterokont alga (Cavalier-Smith 1986) then one would expect that rDNA maps of heterokont algae will share sites with the Oomycetes/Hyphochytriomycetes which are not found in the higher fungi. One would also expect constraints on genome complexity and the presence of IRs in organisms proposed to have a common ancestry with these fungi. The establishment of more complete rDNA consensus maps for higher fungi should

also help to resolve evolutionary relationships among fungi. In the meantime the use of rDNA mapping has helped to confirm the taxonomic placement of some zoosporic fungi, suggested areas where revisions need to be considered, and has also helped place some fungi of taxonomic uncertainty into the scheme.

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