

THE UNIVERSITY OF MANITOBA

REGULATORY ISOZYMES OF THE OOMYCETES

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ABSTRACT

About 50 species of lower fungi Oomycetes were examined by the technique of polyacrylamide gel electrophoresis to determine what the electrophoretic properties of the isozymes of GDH, LDH, IDH, and MDH are. Six allosteric activators, NADP^+ , P-enolpyruvate, GTP, ATP, UTP and short chain acyl-CoA derivatives of enzyme activity were used as a second parameter to distinguish the GDH isozymes of these fungal species. LDH isozymes were compared by electrophoresis and their response to the allosteric inhibitor, GTP. Citrate served a similar role for GDH isozymes. From the results obtained, it was concluded that the GDH isozymes of Saprolegniales were fairly similar. The GDH isozymes of species of Peronosporales were more sensitive to the effects of the allosteric modulators than the Saprolegniales, and the isozymes of two species of Leptomitales tested were insensitive to any allosteric modulation. The electrophoretic mobilities of D(-)lactic dehydrogenases were similar in the species of Achlya, Saprolegnia, Protoachlya and Isoachlya. There were moderate differences among the LDH isozymes of Aphanomyces, Aphanopsis, Thraustotheca and Leptolegnia species. The LDH isoenzyme of Sapromyces elongatus was

unique among all others. It was the slowest moving of all isozymic species in the electric field and, in addition, the enzyme was present at a concentration that is 50 to 100 times higher than that found in all other species. LDH of 11 species of genus the Pythium displayed great variation in electrophoretic mobilities and were the most sensitive to inhibition by GTP. The isozyme patterns of NADP-linked isocitric dehydrogenases of Oomycetes, with only few exceptions, showed two isozyme bands. They were electrophoretically similar in the major genera. In contrast, multiple isozymes of NAD-linked malic dehydrogenases in the Oomycetes, varied with from one to five isozymes in the different species. The nature of the isozyme patterns for GDH, LDH, IDH and MDH indicated that members of the 50 Oomycetes tested bear close biochemical affinities with each other. The results did point out some subtle similarities between species placed in different genera and, vice versa, marked differences between species assigned to the same genus. For example Aphanopsis terrestris and Aphanomyces cladogamus have closer biochemical affinities than they do to members within their respective genera.

The controlled conditions of growth and standardized biochemical studies have provided useful data in the study of sympatric and allopatric relations between fungal groups.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CoA	Coenzyme A
DEAE	Diethylaminoethyl cellulose
EDTA	Ethylenediaminetetraacetic acid
GDH	Glutamic dehydrogenase
GTP	Guanosine triphosphate
IDH	Isocitric dehydrogenase
MDH	Malic dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
PEP	Phosphoenolpyruvate
PMS	Phenazine methosulphate
TRIS	Trishydroxymethylaminomethane
UTP	Uridine triphosphate

PREFACE

This thesis was written as an expansion of a series of papers that were presented to the Canadian Journal of Microbiology and accepted in November 1973 for publication in that journal. This step was taken to reduce the delay that frequently occurs between the preparation of a thesis and abstraction of material therefrom for publication.

In view of the nature of the investigation, the electropherogram patterns of isozymes of the 50-odd Oomycetes species studied are presented as illustrations and tabular summaries of their relative mobilities. The latter approach, affords an easy and rapid comparison of the electrophoretic properties of all the homologous isozymes of a particular order or genus. Similarly, data on allosteric activation and inhibition of some isozymes are represented as histograms and as tabular summaries of the relative activities obtained.

SECTION I

HISTORICAL

Literature Review and Analysis

1. Evolutionary Relations

On current genetic concepts, the evolution of species is due to mutations in the nucleotide base sequence of their DNA. Perpetuation of such mutations depends on the survival of the protein product during natural selection. Thus, diverging species may differ with respect to protein molecules that were initially homologous. While amino acid sequences spell out the details of similarity, a more convenient criterion of homology among proteins is the net charge of the molecules which is readily measured by its mobility in an electric field.

Ingram (23) among others has affirmed by experiment that proteins that differ by a single amino acid residue derived through a single gene mutation may show differing electrophoretic mobilities provided there is an overall charge change. Thus, a crude extract comprising a mixture of proteins when electrophoretically fractionated on a suitable medium, is expected to produce a pattern of bands diagnostic for the species. Homology between the bands of different species theoretically

provides an estimate of their genetic similarity. This is clearly demonstrated in the studies on the fractionation of blood plasma, egg white, and crude tissue extracts of animals (13).

It is generally agreed that determination of the sequences of amino acids in homologous proteins from different organisms is likely to give a better insight into evolutionary relations between the organisms than would other data from studies of low molecular weight substances, electrophoretic and serological properties (14, 5, 41, 15, 24).

This opinion is not totally flawless. Certainly sequence studies are very important and other cellular entities are generally further removed from the genome than proteins. But such substances arose through evolutionary pressures and their value lies in an understanding of the advantages they impart. Evolutionary pressures do not act in isolation on an individual compound whether it be protein or other molecules or compounds (37, 26).

They act on biochemical pathways in which related compounds in the cell affect the fitness of the individual in its environment. A change in the amino acid sequence in a given protein may therefore confer little or no advantage or disadvantage to the organism.

But some sequence changes in proteins do have an evolutionary advantage. For example, Kaplan demonstrated adaptively useful variations in the activity of lactic dehydrogenases in the wing muscle of birds of different flight habit (24). Therefore, by combining sequence data with other properties of proteins, it helps to throw a better light on evolutionary processes and kinship among species.

2. Biochemical Controls

Turning attention to cellular control mechanisms, phylogenetic studies can run into serious difficulties as evinced by the classical work of Stanier on the utilization of benzoic acid and catechol by Pseudomonas and related species. Stanier showed that although the pathways might be biochemically homologous in all respects including induction and regulation of enzymes, the individual strains of the same organism could contain serologically, hence genetically, different enzymes (7). The same phenomenon is known to occur in fungi and may ultimately be found to apply to every biochemical sequence in different taxa. A comprehensive analysis is found in the review by LéJohn (28).

3. Biochemistry and Microbial Taxonomy

If two organisms are grown in exactly the same conditions and they differ by at least one enzyme, then they are evolutionary divergent. One way of detecting gross differences in protein composition is by electrophoresis on polyacrylamide gels. A few biochemical taxa have been examined. Their protein profiles are usually quite distinctly different. For example, strains belonging to the genera Streptococcus, Staphylococcus and Proteus display specific profiles (27). In some cases, strains belonging to different species also display a typical profile. More specific and limited knowledge of phylogenetic relations and evolutionary changes based on protein analyses is recounted in the review article by Garber and Rippon (17).

4. Biochemistry and Control

The biochemical and physiological properties of microbial populations have provided several criteria for systematic analyses at the levels of species and genera. Take for example the fermentation of sugars and acid production; the assimilation of organic carbon or nitrogen;

the use of biosynthetic pathways and their associated enzymes; and the regulation of pathways and their associated enzymes which are among the more obvious characteristics. But according to Corvan (12) "the microbial species does not exist; it is impossible to define except in terms of a nomenclatural type and it is one of the greatest myths of microbiology". To offset this gloomy situation, he suggested instead that "better characterization and separation of microbial units will depend on the progress made by biochemists in analyzing the different parts of microbes and substituting a biochemical characterization for the old biological ones based on simple morphology, staining and sugar reactions, serology, and so on".

Biochemical reactions involving specific substrates, the assimilation of organic compounds as sources of energy, bacteriophage specificity, and serological relations were used as taxonomic criteria very early even before they could be linked to their genetic determinants. But with the advent of the one-cistron-one polypeptide chain concept, characters with taxonomic value came to be regarded as expressions of fundamental differences or similarities in the organization of genetic information in taxa. This was then related to differences or similarities among particular proteins. Practical techniques were later developed to detect the relation between the characters

expressed and their genotype. Electrophoresis evolved as an easy method to compare proteins and enzymes for taxonomic value.

4. (A) Zone Electrophoresis

The purpose of using zone electrophoresis is to provide a method of characterizing proteins and enzymes from different sources such as cell-free extracts or culture filtrates by comparing their number, location (anodic or cathodic) and relative mobility in terms of distance of migration from the origin. An identical electrophoretic mobility does not confirm an identity of proteins or enzymes. Also, biochemical characters including electrophoretic patterns of enzymes are subject to the same limitations as more conventional taxonomic characters used in taxonomic studies. Biochemical characters have been useful in clarifying taxonomic problems in some groups but not in others. Similarly, electrophoretic mobilities of enzymes and proteins may be expected to be of value for some systems not in others. The more difficult the systematic problem, the more likely that its solution by molecular or morphological means will be equally difficult. However, since the protein product being analyzed is a

direct result of the activity of a few cistrons, the protein systematist is close to the genotype while still retaining the fundamental basis of the phenotype concept.

Homologous enzymes from related species may have different electrophoretic patterns. This may be due to any of a number of reasons. 1. The species may be a valid one but due to occasional hybridization gene flow has been permitted to proceed in both directions. This is introgressive hybridization. 2. The species may be polymorphic and this includes intraspecific categories. 3. Polymorphism may have existed in the ancestor and maintained during the evolution of the related species. 4. Convergent evolution could have occurred by mutations in related species so that enzymes with identical electrophoretic mobilities arose. Therefore, a reasonable survey of strains of a species as well as species of a genus is essential to evaluate the value of electrophoretic patterns as adjuncts in a system survey of a group of organisms. In addition it is valuable to study several homologous enzymes in the same population to reduce the change of obstruction in data interpretation by that rate mutation leading to identity in electrophoretic pattern between species.

4. (B) Methodology in Electrophoretic Analysis

In separating a mixture of charged compounds such as proteins or enzymes, a direct current is applied to the system for a set period. Separation is achieved into discrete bands because the net charge depends on the amino acids of the primary structure of the polypeptide, pH of the buffer system and other environmental factors such as support matrix etc. The shape and dimensions of the proteins and enzymes play a role in their electrophoretic mobility, so that the pore size of the solid supporting medium will have a sieving effect of importance. Supporting media that have been used vary from paper, cellulose acetate membranes to gels prepared from agar, starch or polyacrylamide (42). Most of the supporting systems have limitations because only a small amount of the material can be applied to them. But they have the advantage of being convenient and rapid. This is particularly true of cellulose acetate membrane. For larger sample analyses, a variety of gels may be employed. Gels are useful because they can be dissected to yield a number of slices that can be analyzed further. To distinguish small differences in electrophoretic mobilities, the same gel must hold all the samples and then be analyzed under the same conditions of electrophoresis and detection.

The choice of buffers for preparing gels is empirical for pH and components. Low ionic strength buffers lead to rapid migration of proteins and enzymes, fuzzy zones and some heating of the gel.

Procedures for obtaining cellular extracts depend on the system to be studied and the objectives. When extracts from a large number of cultures are to be surveyed, crude extracts may suffice and the procedures should be relatively rapid and efficient. They should also allow against serious denaturation, degradation and inactivation in the case of enzymes. Since crude extracts of cells contain numerous proteins and small molecules, it may be necessary to pre-test them by crude fractionation and dialysis or even heating. The sites of the separated enzymes may be detected by immersing the gel in a solution containing the appropriate stain.

A problem is that the relative concentrations of proteins or enzymes in the extracts may be too high so that two enzymes in the same extract differing only slightly in their relative electrophoretic mobilities may yield a single detectable activity site. Such problems may be resolved by appropriately diluting the extract or adding different inhibitors to the gel or reaction mixture to resolve the bands. Some more resolution may be achieved

if the pH of the buffer used to prepare the gel, voltage or amperage of the current, the duration of the time for separation, the length of the gel slab and its concentration are all varied.

When Hunter and Markert in 1957 (22) used a histochemical technique to detect the sites of enzyme activity in gels, the pattern of such gels gave birth to a zymogram. Thus, enzymes with similar or identical activity for a specific substrate but differing in their electrophoretic mobility came to be termed isoenzymes (isozymes) by Markert and Moller in 1959 (31). By coupling the enzymatic reaction to another reaction system that produces a coloured compound, the sites of migration of the enzyme can be seen directly and compared. Since it is the product of the reaction, not the protein that is detected by such a technique, very little protein is required to obtain good zymograms.

The principle involved in preparing zymograms may be illustrated in a single case, that of lactic dehydrogenase. When the gel carrying the electrophoresed enzymes is immersed in the reaction mixture containing NAD^+ as coenzyme, the sites of LDH activity catalyze the dehydrogenation of lactate to produce pyruvate. The H^+ liberated reduced NAD to NADH which in turn is oxidized to NAD^+ by phenazine methosulphate in the reaction mixture.

The H^+ liberated from NADH reduces the colourless tetrazolium salt to an insoluble coloured formazan. Such a system was used for all the enzymes analyzed in this thesis.

4. (C) Protein Profiles and Zymograms in Taxonomy of Fungi

Chang, Srb and Steward (9) distinguished four species of Neurospora by their protein profiles. But, interestingly, a mutant strain of N. crassa gave a different profile from its wild type derivative. Therefore, a survey of strains is essential to study intraspecific variation. Clare (10) showed that six species of Pythium gave protein profiles exhibiting little intraspecific variation and the profiles were useful in characterizing each species. Compared to other fungi such as Pullaria, Fusarium and Saccharomyces, each species displayed its own characteristic electrophoretic fingerprint. Clare and Zentmeyer (11) and Gill and Powell (18) have observed similar relations for three species of Phytophthora. Among Ceratocystis, a difficult genus, Stipes (39) showed that there were significant intraspecific variations using four species.

Novacky and Macko (34) found some 23 common sites in profiles for extracts for 2 physiological races of Puccinia graminis var. tritici and they claimed to have distinguished the three races on the basis of a single gene difference. Since no mention was made of the number of replicates used per race, the claim cannot be assessed at all.

Zymogram analyses have been done for malate dehydrogenase, isocitric dehydrogenase, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGD) from seven strains of N. crassa (40). No significant zymogram differences were detected whether the cells were grown in the same medium or different media. Clare and Zentmeyer (11) in their studies of Phytophthora strains, found that in two strains of P. palmivora, one of P. citrophthora and three of P. cinnamoni, the zymograms for G6PDH and 6PGD were dissimilar only with respect to P. palmivora. Those of P. cinnamoni were identical. If the strains were grown in different extracts and analyzed after different periods of time, differences were not found for P. palmivora in contrast to the zymograms for P. citrophthora.

Nealson and Garber (32) compared the zymograms for esterase, phosphatase and leucine aminopeptidase from 32 strains (15 spp.) of Aspergillus. Although the strains

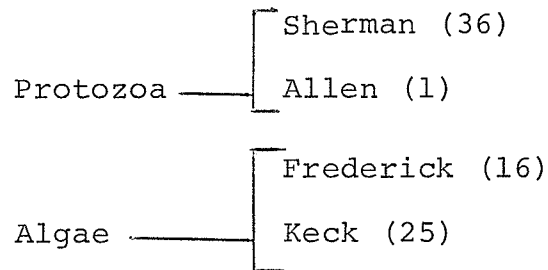
showed different patterns of esterase and phosphatase activity, it was possible to detect invariant sites for strains assigned to the same species. A wider study conducted by McClements and Garber 1967 in an unpublished report indicated that 77 to 79 strains of A. nidulans displayed identical esterase and phosphatase zymograms.

Other microorganisms

Related studies on bacteria, protozoa and algae will not be discussed in this review because the studies reported in this thesis were confined to fungi. Suffice to say that the results obtained for each phylum compare very well with those discussed for the fungi. Useful information for the interested reader can be obtained from the following reports.

Bacteria	Huisinigh and Durbin (21)
	Roltem and Ragin (35)
	Goltlieb and Hepdon (19)
	Lund (30)
	Norris and Burges (33)
	Cann and Wikox (6)
	Green and Goldbert (20)
	Bowman et al (4)

Sherman (36)



5. General Comments

The increasing interest in using enzymes and proteins to yield profiles or zymograms by electrophoresis for taxonomic objectives makes this a useful tool in assessing phylogeny. But this should not be taken as liberty for wandering away from the established taxonomic criteria in identifying strains or species.

Intraspecific variation for protein profiles and zymograms must be established by surveying a large number of strains and species. When invariant sites are detected for all strains tested, then such sites must be considered as species-specific. Such a case has been brought to the foreground by LéJohn (29) in his studies of glutamic dehydrogenases of fungi. All so-called "lower fungi" which are coenocytic have only an NAD-specific glutamic dehydrogenase whereas "higher" fungal forms (non coenocytes) carry both the NAD and NADP-specific enzymes.

When each species of a genus yields a characteristic array of invariant sites, it is possible to const-

ruct an atlas of invariant sites for the genus that would extend the phylogenetic links of species. When strains of sites provides a useful "fingerprint" for identification without recourse to a battery of cumbersome tests and analyses. But the paucity of data precludes an evaluation of the relative worth of zymograms and protein profiles as taxonomic tools at this time.

With these limitations and historical procedure, the zymogram analysis of enzymes of the TCA cycle of the Oomycetous fungi was undertaken and the findings form the bases of 3 papers to be published in the Canada Journal of Microbiology in 1974, and expanded upon here in this thesis. For this reason, the thesis is compiled in sections, each section covering the detailed elaboration of the papers.

6. General Biology of the Oomycetes

The Oomycetes consist of fungi that reproduce both asexually (production of biflagellate zoospores) and sexually (production of antheridia and oogonia). The most advanced Oomycetes are terrestrial obligate parasites starting and completing their entire life history in the host. Under these conditions, they depend on the wind for dissemination of the spores but can also

utilize the water dispersal route if the need arises. This is a strong indication that they have ancestral affinities with aquatic forms.

The Oomycetes are constituted by 4 orders of fungi - Saprolegniales, Leptomitales, Lagenidiales and Peronosporales. Only the Peronosporales have well-established economic importance to man. A few species of Saprolegniales can attack economically useful plants and a few parasitize fish causing serious necrotic and other lesions. The Peronosporales constitute some of the more destructive parasites known to man. These range from Plasmopara viticola that causes mildew of grapes of Phytophthora infestans that scourge which causes late blight of potatoes.

Classification of the Oomycetes (38) is dependent upon the following morphological features. The Saprolegniales are regarded as fungi that are either holocarpic or eucarpic. The zoospores may be di-or mono-planetic but rarely aplanetic. If the hyphae are constricted and the thallus eucarpic, Leptomitales is indicated. The Lagenidiales are entirely holocarpic and the Peronosporales eucarpic. Both orders, however, have species that may reproduce asexually by zoospores that form within a sporangium or inside an evanescent vesicle which is derived from the sporangium.

These fungi are ubiquitous and, being members of the obsolete "class" Phycomycetes, are therefore coenocytic. Thus, the vegetative cells of the Oomycetes show considerable diversity of form ranging from the presumably primitive holocarpic cell of the Lagenidiales to a eucarpic highly branched Peronosporales. It is thought that nutritionally, the Leptomitales may be more closely related to the Peronosporales than to the Saprolegniales where structural phylogenetic considerations would place them (8.3).

A totally different conflict exists about the ancestral roots of these fungi. The bulk of the argument favours the idea that they are early diversions from the Chrysophyta (algae) (2).

The main features are that these organisms have the plant-type lysine biosynthetic route, have cellulosic cell walls, and the zoospores with whiplash and primitive flagella are similar to the heterokont unicellular algae. Also, in view of their unique modes of enzyme regulation (29) and their peculiar enzyme distribution patterns evinced by LéJohn's studies, it was most essential to conduct detailed studies of the polyacrylamide protein 'fingerprint' of these fungi and relate the zymograms to the enzyme controls elucidated. Also vital is a comparison of this data with information on homologous enzymes from fungal species that are excluded from this class.

The content of this thesis indicates that only part of this goal was met because the studies were confined to a cluster of Oomycetes represented by members of the Peronosporales, Leptomitales and Saprolegniales. Extension to higher forms of coenocytic fungi (Zygomycetes) remains incomplete.

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SECTION II

COMPARATIVE ALLOSTERY OF NAD-SPECIFIC GLUTAMIC DEHYDROGENASES OF OOMYCETES

INTRODUCTION

Van Niel (13) observed that for any biochemical character to be useful in the elucidation of evolutionary relations it must be poised between the extremes of biochemical unity and diversity. Vogel (14) applied this concept with tremendous success in his analysis of the distribution of the α , ϵ -diaminopimelic acid (DAP) and α -aminoadipic acid (AAA) lysine biosynthetic pathways in fungal and other living organisms. He found that, without exception, the Oomycetes harbour the DAP biosynthetic path, and all other fungi, the AAA path.

The idea has been taken to a different level of resolution, working on the premise that an analysis of the adaptive nature of the biochemical pattern may produce a finer resolution of the relations that exist between species. A general survey of the experimental findings in support of this notion has been presented elsewhere by LéJohn and associates (9, 10).

The present investigations were initiated to examine the problem of intra- and inter-generic relations of the Oomycetes at the level of enzyme regulation and electrophoretic property of a series of strategically-located enzymes in metabolism. Because regulatory glutamic dehydrogenases may have a common ancestral gene thread (3)

and, in fact, do have the same substrate (either NAD^+ or NADP^+), these types of enzymes were selected for use in the characterization study.

It is shown here that examination of taxonomic correlations between species based on enzyme regulation may be a very useful guide to the study of allopatric and sympatric speciation within fungal groups.

MATERIALS AND METHODS

Source of Cultures. All fungal cultures were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands. The fungal species are listed in Table 1.

Culturing of Organisms. The organisms were grown in a standard medium composed of Bacto-peptone, 1 g, yeast extract, 1 g, D-glucose, 3 g, and 0.2 ml of Bacto beef extract, all in 1 litre of distilled water. This medium was used even though several of the species could grow on a defined medium of glucose and salts plus organic nitrogen and sulphur as supplements.

Cells were obtained from large-scale cultures prepared either as 3 litre cultures in glass carboys grown under forced aeration (3 litres/min), or as stationary 1-litre cultures in 2-litre flasks both types of cultures grown at 28°C . The culture growth phase varied from 2 to 8

Table 1. Oomycetes Species Information

Organism

Saprolegniales

<u>Achlya</u> <u>ambisexualis</u> (male)	CBS	100.50
<u>A.</u> <u>ambisexualis</u> (female)	CBS	101.50
<u>A.</u> <u>americana</u>	CBS	100.52
<u>A.</u> <u>dubia</u>	CBS	101.38
<u>A.</u> <u>colorata</u>	CBS	102.37
<u>A.</u> <u>bisexualis</u> (male)	CBS	102.50
<u>A.</u> <u>bisexualis</u> (female)	CBS	103.50
<u>A.</u> <u>crenulata</u>	CBS	104.50
<u>A.</u> <u>intricata</u>	CBS	106.50
<u>A.</u> <u>flagellata</u>	CBS	107.35
<u>A.</u> <u>racemosa</u>	CBS	108.35
<u>A.</u> <u>radiosa</u>	CBS	109.35
<u>A.</u> <u>heterosexualis</u> (male)	CBS	420.65
<u>A.</u> <u>heterosexualis</u> (female)	CBS	419.65
<u>Isoachlya</u> <u>eccentrica</u>	CBS	211.35
<u>I.</u> <u>unispora</u>	CBS	213.35
<u>I.</u> <u>intermedia</u>	CBS	278.52
<u>I.</u> <u>toruloides</u>	CBS	279.52
<u>Protoachlya</u> <u>paradoxa</u>	CBS	150.45
<u>Aphanomyces</u> <u>laevis</u>	CBS	107.52
<u>A.</u> <u>cladogamus</u>	CBS	108.29

Table 1. Continued

Organism

<u>Aphanomyces euteiches</u>	CBS 110.28
<u>A. stellatus</u>	CBS 578.67
<u>Thraustotheca clavata</u>	CBS 343.33
<u>Aphanopsis terrestris</u>	CBS 548.67
<u>Leptolegnia caudata</u>	CBS 559.67
<u>Saprolegnia asterophora</u>	CBS 180.44

Saprolegniales

<u>Saprolegnia lapponica</u>	CBS 284.38
<u>S. ferax</u>	CBS 305.37
<u>S. litoralis</u>	CBS 306.37
<u>S. mixta</u>	CBS 307.37
<u>S. terrestris</u>	CBS 308.37
<u>S. monoica</u>	CBS 327.35
<u>S. delica</u>	CBS 342.65
<u>S. megasperma</u>	CBS 386.52
<u>S. furcata</u>	CBS 542.67

Peronosporales

<u>Pythium ultimum</u>	CBS 111.65
<u>P. debaryanum</u>	CBS 114.19
<u>P. heterothallicum</u> (male)	CBS 143.69
<u>P. heterothallicum</u> (female)	CBS 202.68

Table 1. Continued

Organism

<u>Pythium butleri</u>	CBS	164.68
<u>P. splendens</u>	CBS	191.25
<u>P. intermedium</u> (+)	CBS	221.68
<u>P. intermedium</u> (-)	CBS	222.68
<u>P. undulatum</u>	CBS	323.47
<u>P. sylvaticum</u> (male)	CBS	452.67
<u>P. sylvaticum</u> (female)	CBS	453.67

Leptomitales

<u>Sapromyces elongatus</u>	CBS	453.67
<u>Apodachlya brachynema</u>	CBS	111.39

days except in the case of Leptolegnia and Sapromyces whose growth period lasted for as long as 15 to 18 days.

Preparation of Cell-free Extracts. Mycelia was collected by filtration of the cell suspension through Whatman No. 1 filter paper, washed with 1 litre of distilled H₂O, and sucked dry by applying vacuum pressure of 20 mm Hg to the hyphal mat. The wet weight of the cell mat was determined and a suitable fraction cut for preparation of cell extract. The wet weight (taken as 1 volume) of the cell mat was determined and 2 volumes of a buffer composed of 10 mM Tris-acetate, pH 7.2, 1 mM KCl, 1 mM MgCl₂, and 0.1 mM Na₄EDTA added to the mycelia placed in a Waring Blender. The mycelia was disrupted by blending at 4°C for 2 min followed by sonication for 15 min in the cup of a Raytheon 10 kc sonicator at 10°C. Cell debris was removed by centrifugation at 48,000 x g for 10 min at 4°C. The supernatant was saved and the cell debris discarded. Small molecules were removed from the supernatant by treatment with Sephadex-G25, and the extract concentrated 2 to 3-fold with aquacide II at 4°C. This preparation was used as the source of enzymes for electrophoresis and enzyme analysis. All extracts were used freshly prepared. The amount of extract applied to the polyacrylamide gel slab was related to the enzyme activity. These activities were

kept constant as far as possible.

Vertical Gel Electrophoresis. Vertical gel slab electrophoresis was carried out using the model E-C 454 equipment of E-C Apparatus Corporation designed after Jordan and Raymond (5). A total of 16 samples were analysed simultaneously by this method. For each run, a control sample from a previous study was used to achieve uniformity in comparing electrophoretic mobilities of proteins on gels made at different times since such gels cannot have the same porosity.

Gel slabs 6 mm thick, were made according to the directions of E-C Apparatus Technical Bulletin 128. The gel solutions were prepared as follows. Cyanogum-41 consisting of 95% acrylamide and 5% bisacrylamide, was chemically polymerised with TMED (N,N,N',N', tetramethylethylenediamine) and ammonium persulphate. In all cases, 5% cyanogum-41 gel was used, and the composition of the ingredients were 10 g cyanogum-41, 0.2 ml TMED, 0.2 g ammonium persulphate in 200 ml of buffer. The composition of the buffer used varied depending upon the enzyme whose activity was to be determined. Consequently, the buffer used in electrophoresis is specified in the appropriate figure legend.

Electrophoretic runs varied between 3 hr and 6 hr, at 10°C.

Detection of Enzyme Activity on Gels. NAD-specific Glutamic Dehydrogenase. It had been established previously (9) that all of the glutamic dehydrogenases in the Oomycetes are NAD-linked, and that they are stimulated to react with their substrates if NADP^+ is present. The stimulation affects both oxidative demination and reductive amination reactions, although the effect on the latter is more prominent. Activity staining was performed on gels in both directions of the reaction. In the negative stain technique, the reactants consisted of: NADH, 1 mM; NADP^+ , 1 mM; α -keto-glutarate, 10 mM; NH_4^+ , 100 mM, phenazine methosulphate, 0.16 mM; nitroblue tetrazolium, 0.43 mM; and Tris-HCl, pH 9, 100 mM. In the positive staining technique, the reactants were: NAD^+ , 2 mM; NADP^+ 1 mM; L-glutamate, 100 mM; phenazine methosulphate, 0.16 mM; nitroblue tetrazolium, 1.43 mM; Tris-acetate, pH 9, 100 mM.

The gels were immersed in the reaction solutions and incubated at 30°C in the dark for 2 hr.

Spectrophotometric Assays. The method described by LéJohn et al (8) was followed. The term "activation ratio" signifies the ratio of enzyme activity in the presence of

an activator (V_a) and in its absence (V_o).

Percent inhibition was computed from the equation:

$$\% \text{ Inhibition} = \frac{V_o - v_i}{V_o} \times 100$$

where v_i is the activity in the presence of inhibitor, and v_o activity in its absence.

Because the K_m values for the substances of the enzyme from all the different species were not predetermined, these activation and inhibition values are somewhat empirical. They are all related to one fully-characterized system, that of glutamic dehydrogenase from P. debaryanum where the enzyme has been purified and its kinetic properties studied (8).

Protein Determination. The colorimetric method of Lowry et al (11) was used with bovine serum albumin as standard. The colour intensity was determined with a Klett colorimeter using Klett No. 62 filter.

Source of Chemicals. Electrophoresis supplies were obtained from Eastman Kodak Co., Rochester, New York. Biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo., from PL-Biochemicals, Milwaukee; and from Calbiochem., California. Nutrients for growth studies were purchased from Difco Laboratories, Detroit, Michigan.

RESULTS

Analysis of Related Species. Stebbins (12) has proposed a useful concept of the term "species" in which he assumes that in the absence or rarity of gene interchange, organisms that are separated primarily by morphological and physiological characteristics may be considered to be species. Based on this concept, I examined about 50 species of the class Oomycetes of the so-called lower fungi for their isozyme patterns of glutamic dehydrogenase. The results obtained were organized into four categories based largely on genera as shown in Figs. 1 to 5 and Tables 2 and 3.

The isozyme patterns for the Saprolegniales which constitute the largest order examined are shown in Figs. 1, 2, 3 and 4. Fourteen species of the genus Achlya were studied, and the isozyme patterns are shown in Fig. 1. There were 3 pairs of sexually distinct species among the 14. The enzyme activity staining procedure involved the negative staining technique since the allosteric modulators effects are stronger on this reaction. The electrophoretic mobilities of all the isozymes of glutamic dehydrogenase in this genus are similar. The sexually distinct pairs

Fig. 1. Electrophoretic patterns of NAD-dependent glutamic dehydrogenase isozymes of 14 species of Achlya. Electrophoresis was for 4 hr at 10°C in a buffer composed of 5.52 g barbital diethylbarbituric acid, 1 g Tris-base in 1 litre of H₂O, adjusted to pH 8 if necessary. Arrows indicate position of tracking dye.

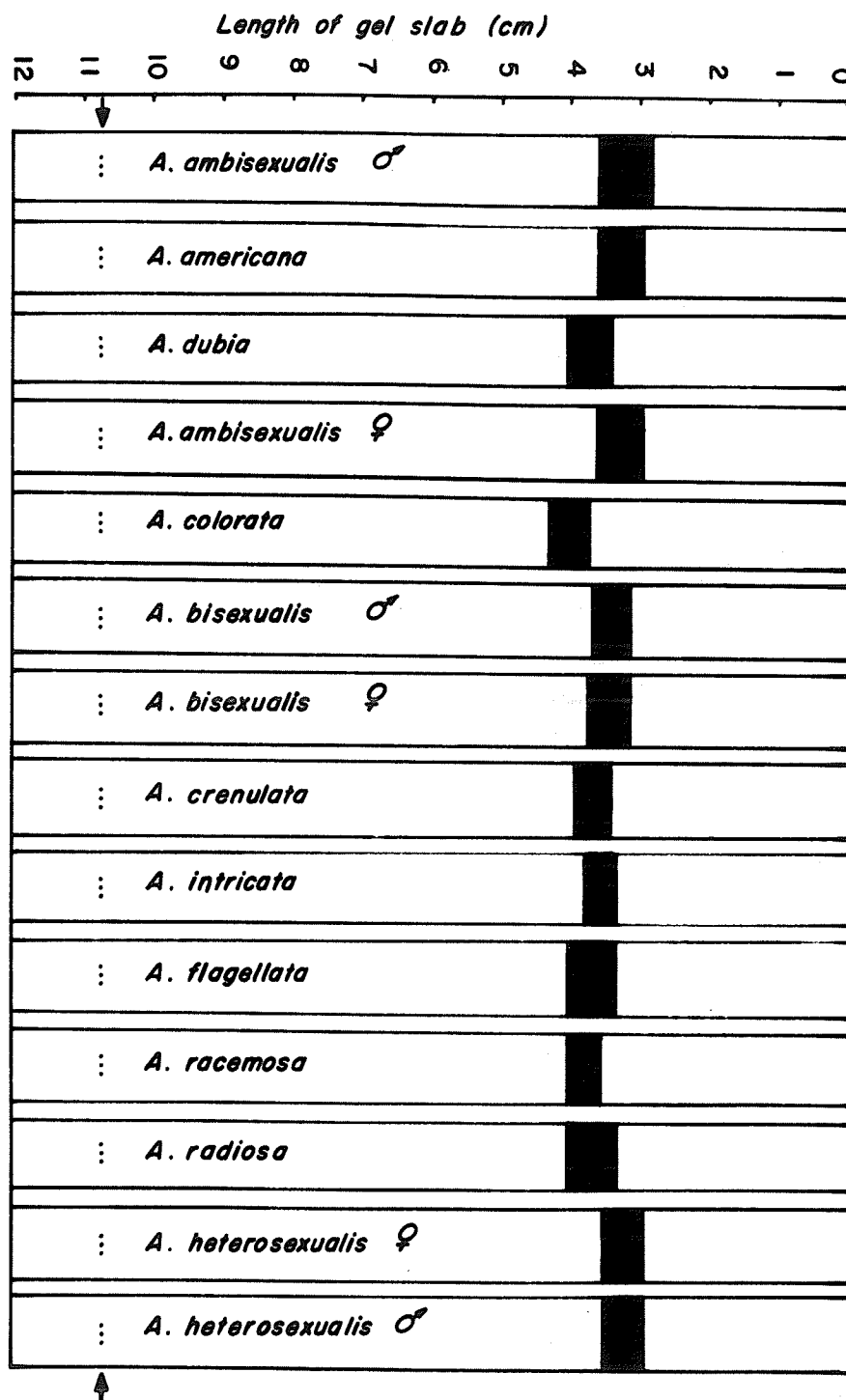


Fig. 2. Electrophoretic patterns of NAD-dependent glutamic dehydrogenase isozymes of 10 species of Saprolegnia. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

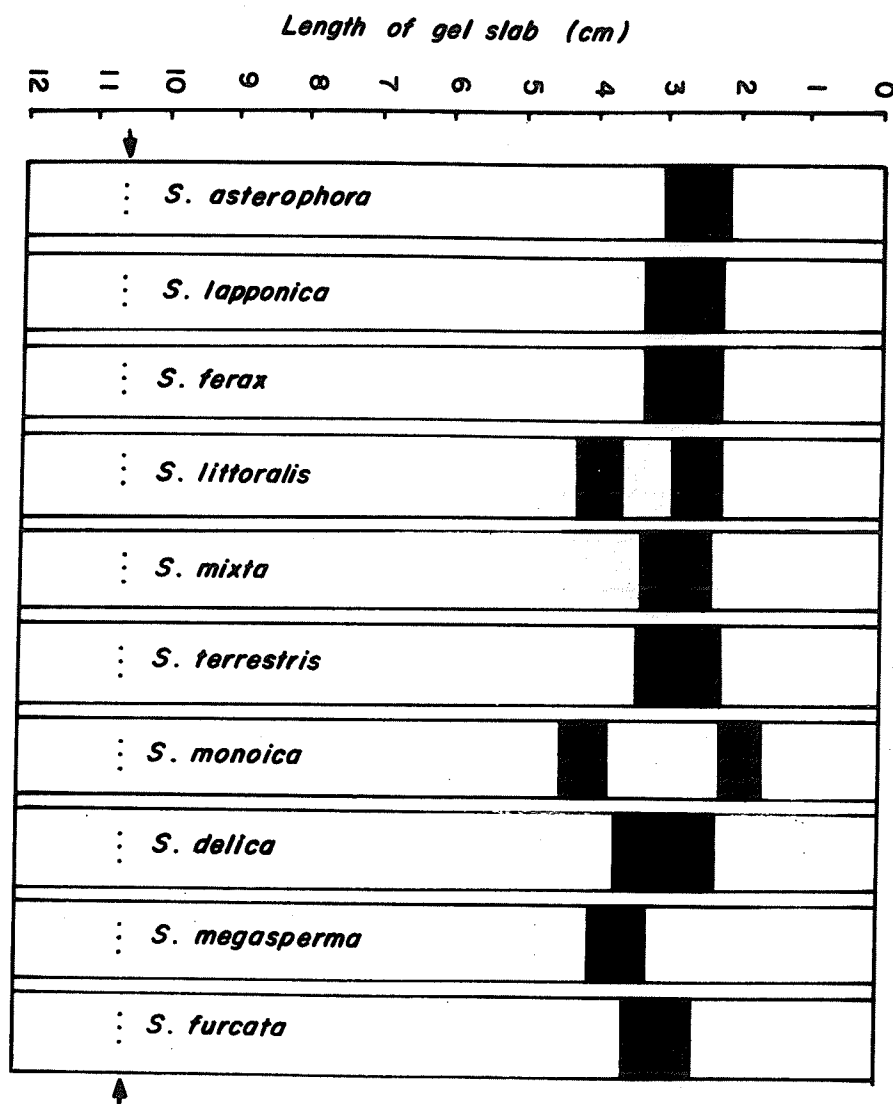


Fig. 3. Electrophoretic patterns of NAD-depenedent glutamic dehydrogenase isozymes of Aphanomyces spp., Aphanopsis, Thraustotheca and Leptolegnia. Electrophoreses was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

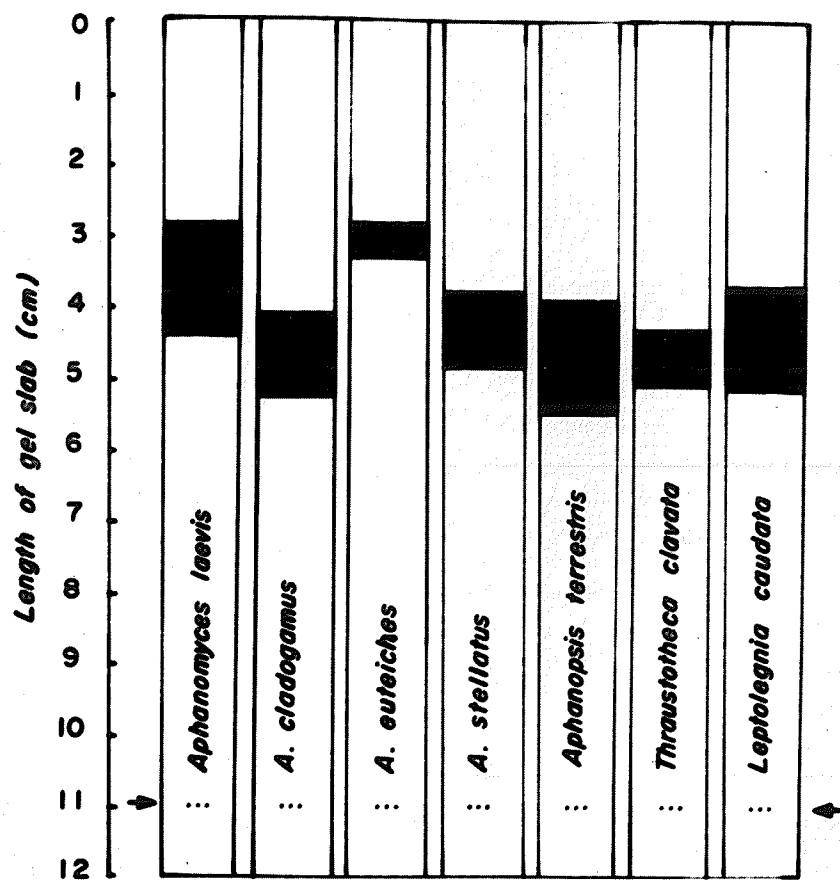


Fig. 4. Electrophoretic patterns of NAD-dependent glutamic dehydrogenase isozymes of some Saprolegniales (left panels) and Leptomitales (right panels). Electrophoresis was for 4 hr. at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

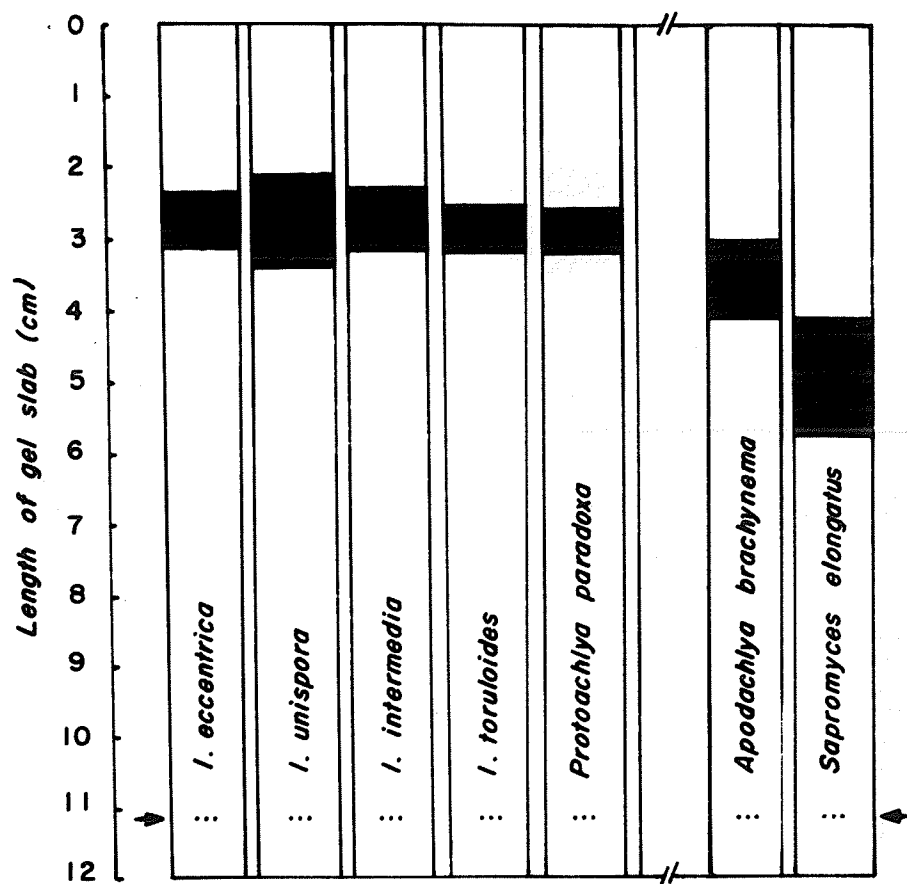


Fig. 5. Electrophoretic patterns of NAD-glutamic dehydrogenase isozymes of 11 species of *Pythium*. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate positions of tracking dye.

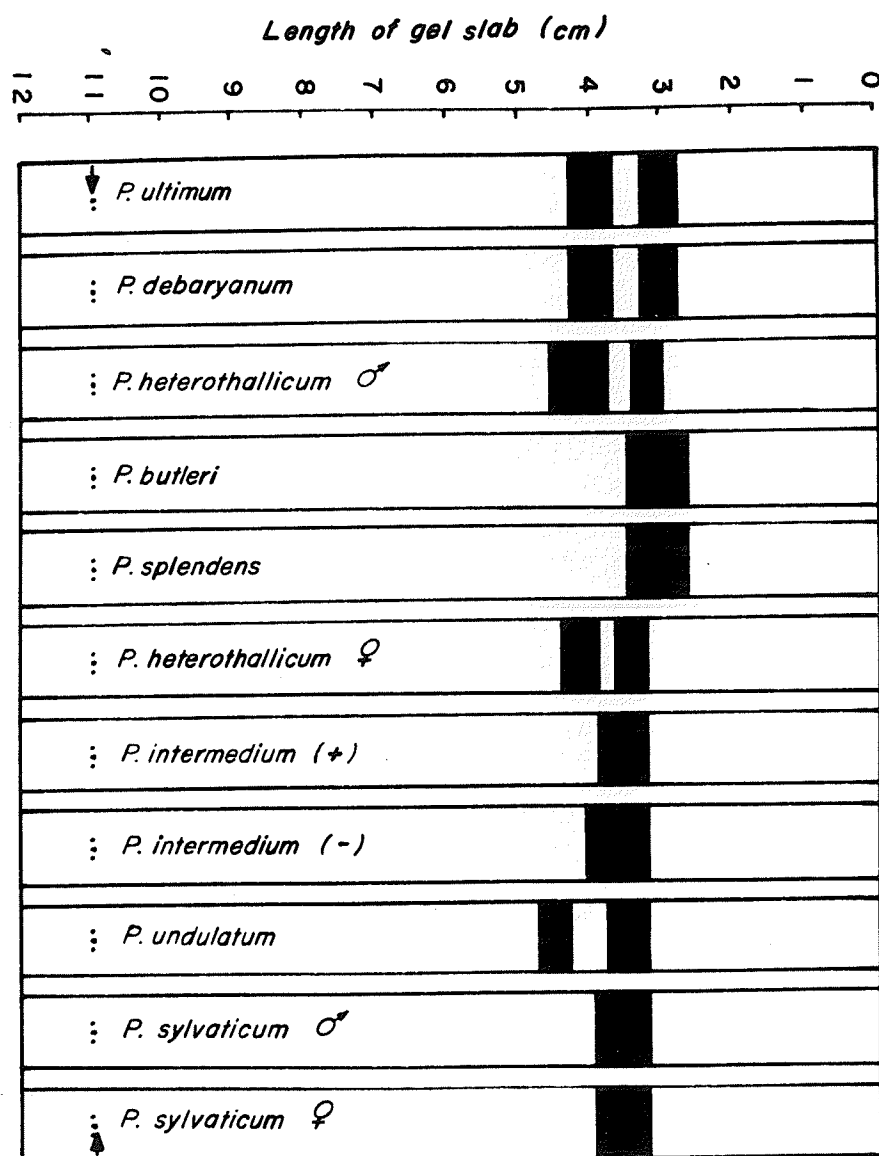


Table 2. Relative mobilities (Re) of isozymes glutamic dehydrogenases of Saprolegniales in an electric field. Experimental procedure described in "Materials and Methods".

Organism	Re (cm)
<u>Achlya</u> <u>ambisexualis</u> ♂	3.3
<u>A.</u> <u>ambisexualis</u> ♀	3.3
<u>A.</u> <u>americana</u>	3.4
<u>A.</u> <u>dubia</u>	3.8
<u>A.</u> <u>colorata</u>	4.1
<u>A.</u> <u>bisexualis</u> ♂	3.5
<u>A.</u> <u>bisexualis</u> ♀	3.5
<u>A.</u> <u>crenulata</u>	3.8
<u>A.</u> <u>intricata</u>	3.7
<u>A.</u> <u>flagellata</u>	3.8
<u>A.</u> <u>racemosa</u>	3.9
<u>A.</u> <u>radiosa</u>	3.8
<u>A.</u> <u>heterosexulis</u> ♂	3.3
<u>A.</u> <u>heterosexulis</u> ♀	3.3
<u>Saprolegnia</u> <u>asterophora</u>	2.1
<u>S.</u> <u>lapponica</u>	2.8
<u>S.</u> <u>ferax</u>	2.8

Table 2 continued

Organism	Re (cm)
<u>Saprolegnia littoralis</u>	2.6 4.0
<u>S. mixta</u>	2.9
<u>S. terrestris</u>	2.9
<u>S. monoica</u>	1.9 4.2
<u>S. delica</u>	3.1
<u>S. megasperma</u>	3.6
<u>S. furcata</u>	3.1
<u>Aphanomyces laevis</u>	3.7
<u>A. cladogamus</u>	4.7
<u>A. euteiches</u>	3.1
<u>A. stellatus</u>	4.4
<u>Aphanopsis terrestris</u>	4.8
<u>Thraustotheca clavata</u>	4.8
<u>Leptolegnia caudata</u>	4.5
<u>Isoachlya eccentrica</u>	2.8

Table 2 continued

Organism	Re (cm)
<u>Isoachlya unispora</u>	2.8
<u>I. intermedia</u>	2.8
<u>I. toruloides</u>	2.9
<u>Protoachlya paradoxa</u>	2.9

Table 3. Relative mobilities (Re) isozymes of glutamic dehydrogenases of Peronosporales and Leptomitales in an electric field. Experimental procedure described in "Materials and Methods".

Organism	Re (cm)
Peronosporales	
<u>Pythium</u> <u>ultimum</u>	3.1 4.0
<u>P.</u> <u>debaryanum</u>	3.1 4.0
<u>P.</u> <u>heterothallicum</u> ♂	3.2 4.2
<u>P.</u> <u>heterothallicum</u> ♀	3.5 4.2
<u>P.</u> <u>butleri</u>	3.1
<u>P.</u> <u>splendens</u>	3.1
<u>P.</u> <u>intermedium</u> (+)	3.6
<u>P.</u> <u>intermedium</u> (-)	3.7
<u>P.</u> <u>undulatum</u>	3.5 4.6
<u>P.</u> <u>sylvaticum</u> ♂	3.6
<u>P.</u> <u>sylvaticum</u> ♀	3.6
Leptomitales	
<u>Apodachlya</u> <u>brachynema</u>	3.6
<u>Sapromyces</u> <u>elongatus</u>	4.9

of A. ambisexualis and A. heterosexualis had enzymes that co-electrophoresed. The isozymes from the male and female strains of A. bisexualis were identical but differed slightly from the isozymes of ambisexualis.

Electrophoretic mobilities of the isozymes from species of Saprolegnia (see Fig. 2) were comparable to those of Achlya. One interesting point is that S. littoralis and S. monica have 2 isozymes of glutamic dehydrogenase per species. Both isozymes from S. monica are electrophoretically different from the enzyme of all the other species tested. The faster moving component from S. delica and S. littoralis appear to be identical. The slower moving isozyme from S. littoralis is more closely related to the enzymes of the other species.

The isozymes from Aphanomyces spp., show significant intrageneric and enter generic differences (see Fig.3). The isozymes of the Isoachlya and Protoachlya species tested are similiar to those of the Saprolegnia and Achlya species tested in terms of electrophoretic mobilities (see Fig. 4, left panel).

Only two species of the Leptomitales were studied and their isozymes differed markedly (see Fig. 4, right panel).

As will be shown later, allosteric modulators have very little influence upon these enzymes.

Multiple species of glutamic dehydrogenase isozymes occurred with greater frequency among the representatives Peronosporales tested (Fig. 5). Of the 11 species of Pythium examined, 5 had a pair of isozymes per species. The male and female forms of P. heterothallicum were among the five, and none of the other sexually distinguishable forms displayed this characteristic.

Enzyme Regulation. It has been established by LéJohn that the glutamic dehydrogenases of the Oomycetes are subject to complex multivalent controls (7, 8, 9). An attempt was made at this juncture to correlate the allosteric control patterns with the taxonomic distribution of the various species.

Six compounds, NADP^+ , P-enolpyruvate, GTP, ATP, UTP, and short chain acyl-CoA derivatives were used as activators, and citrate as an inhibitor to determine whether the enzyme has metallo-enzyme properties. These activity tests were performed on the reductive amination reaction only.

Members of the genus Pythium have isozymes that are activated by all 6 activators, and inhibited by

citrate. As shown in Fig. 6 and Table 4, NADP^+ was the strongest activator eliciting an activation ratio of 10 or more in the female strain of P. heterothallicum. P-enolpyruvate and GTP were also very strong activators of this strain. In contrast, enzyme from the male strain of P. heterothallicum was only half as responsive to these modulators. These two species were shown in Fig. 5 to harbour a pair of isozymes per species. It is tempting to speculate that one of the two isozymes of the male strain may be insensitive to the modulators.

One significant point of interest is the closeness in individual activation patterns of the isozymes from the different sexual strains. P. intermedium, (+), and (-), and P. sylvaticum, male and female, have isozymes that respond more to NADP^+ , GTP and UTP, than to P-enolpyruvate, ATP and CoA.

Members of the Genus. Achlya species rank second among the Oomycetes in their sensitivity to allosteric modulators. Fourteen species were tested and only 2, A. racemosa and A. colorata were found to have isozymes that responded to all the modulators (Fig. 7 and Table 6). Although the female strain isozyme of A. ambisexualis responded very well to all the modulators except

Fig. 6. Activation of NAD-dependent glutamic dehydrogenase isozymes of various species of Pythium by NADP^+ , P-enol-pyruvate, GTP, ATP, UTP and acetyl-CoA (CoA). The ratio v_e/v_o represents the increase in enzyme activity over control where no activator is present. The assay was carried out spectrophotometrically in a 3-ml reaction solution consisting of 30 mM NH_4^+ , 10 mM α -ketoglutarate, 0.167 mM NADH, 33.3 mM Tris-acetate buffer, pH 8 with or without one of the following activators 1 mM NADP^+ ; 0.5 mM P-enol-pyruvate; 0.33 mM GTP; 1 mM ATP; 1 mM UTP; and 0.33 mM acetyl-CoA. Reaction rate was determined from the change in absorbance at 340 nm. v_e is the rate in the presence of one activator and v_o is the rate without an activator.

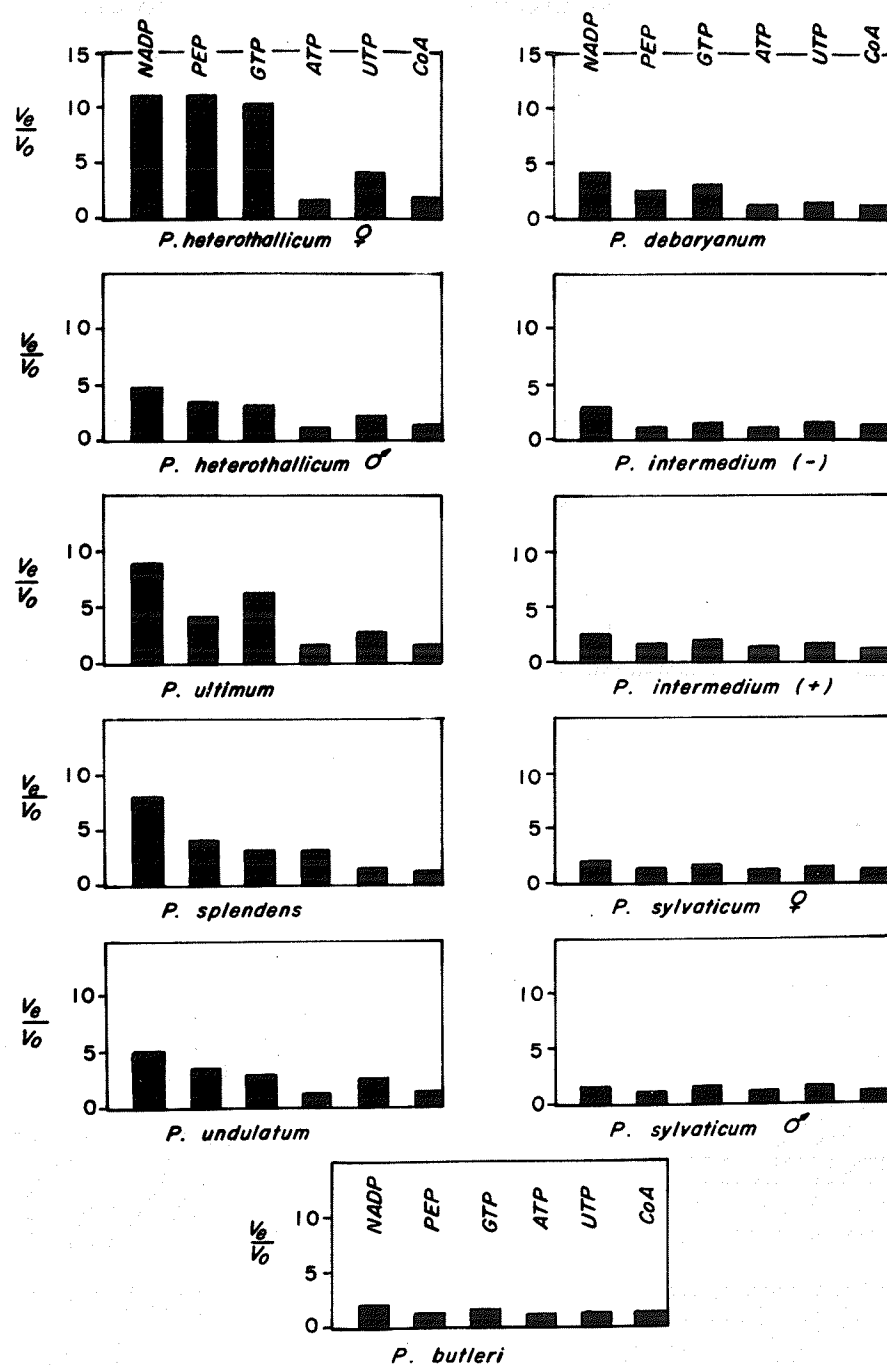


Table 4. Activation of NAD-dependent glutamic dehydrogenase isozymes of Peronosporales by NADP^+ , P-enolpyruvate, GTP, ATP, UTP and acetyl-CoA (CoA). The ratio V_a/V_o represents the increase in enzyme activity over control where no activator is present. The assay was carried out spectrophotometrically in a 3-ml reaction solution consisting of 30 mM NH_4^+ , 10 mM α -ketoglutarate, 0.167 mM NADH, 33.3 mM Tris-acetate buffer, pH 8 with or without one of the following activators 1 mM NADP^+ ; 0.5 mM P-enolpyruvate; 0.33 mM GTP; 1 mM ATP; 1 mM UTP; and 0.33 mM acetyl-CoA. Reaction rate was determined from the change in absorbance at 340 nm. V_a is the rate in the presence of one activator and V_o is the rate in the absence of an activator.

		Modulator effect, V_a/V_o					
		NADP^+	PEP	GTP	ATP	UTP	CoA
<u>Pythium</u>	<u>heterothallicum</u> ♀	11.0	11.0	10.0	1.7	3.9	1.8
<u>P.</u>	<u>heterothallicum</u> ♂	4.8	3.5	3.0	1.3	2.0	1.5
<u>P.</u>	<u>ultimum</u>	8.8	4.2	6.2	2.0	2.7	1.5
<u>P.</u>	<u>splendens</u>	8.0	4.0	3.3	3.1	1.4	1.4
<u>P.</u>	<u>undulatum</u>	5.1	3.4	2.9	1.5	2.5	1.3
<u>P.</u>	<u>debaryanum</u>	3.7	2.3	2.8	1.5	1.4	1.6
<u>P.</u>	<u>intermedium</u> (-)	2.8	1.5	1.5	1.3	1.4	1.4
<u>P.</u>	<u>intermedium</u> (+)	2.5	1.6	1.9	1.4	1.6	1.4
<u>P.</u>	<u>sylvaticum</u> ♀	1.8	1.4	1.6	1.3	1.5	1.3
<u>P.</u>	<u>sylvaticum</u> ♂	1.7	1.4	1.7	1.3	1.5	1.3
<u>P.</u>	<u>butleri</u>	2.1	1.5	1.8	1.5	1.5	1.5

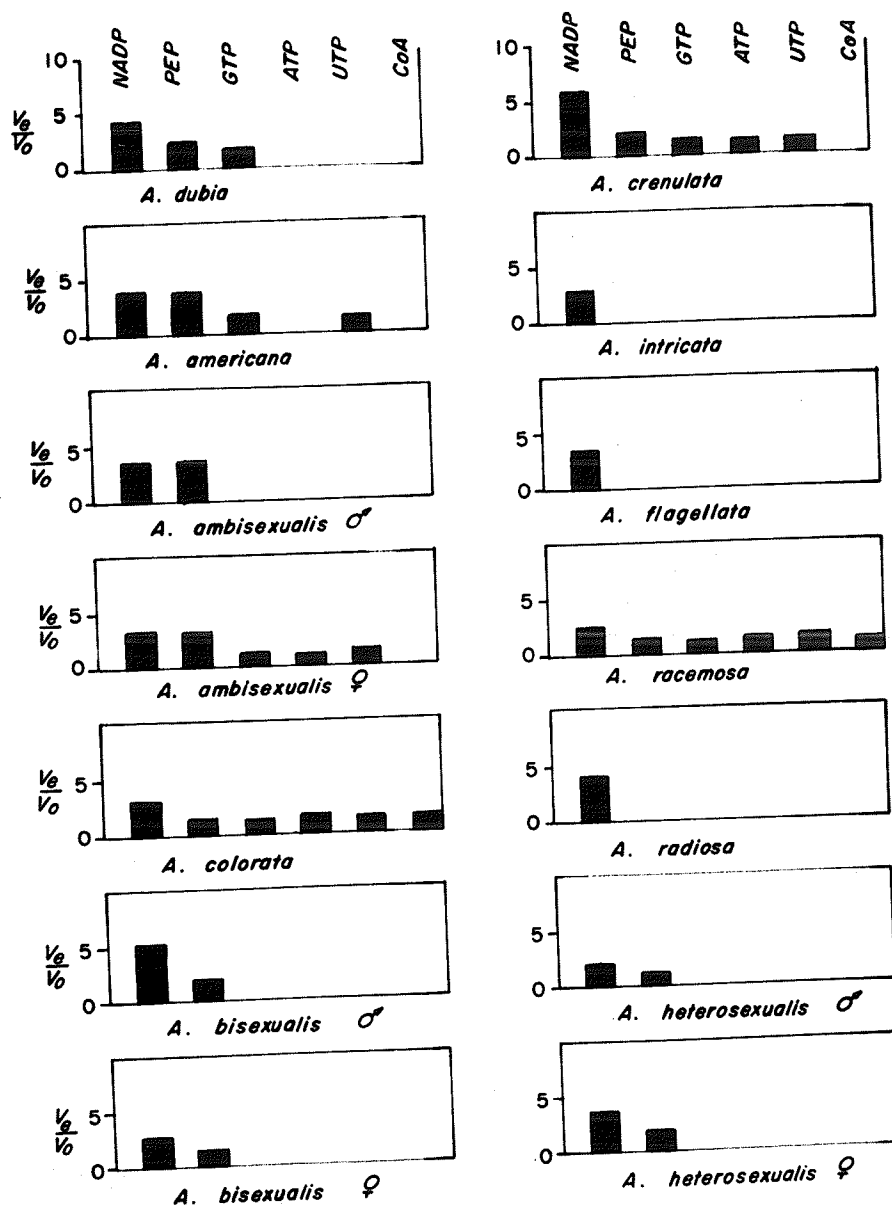
CoA, the male strain enzyme was insensitive to all but NADP^+ and P-enolpyruvate. In contrast, the male and female strains of A. bisexualis have similar activation patterns, being comparable to the male strain of A. ambisexualis.

All the isozymes of the 14 Achlya spp. were activated by NADP^+ , and only those of three species, A. intricata, A. flagellata, and A. radiosa were not activated by P-enolpyruvate.

LéJohn has argued (7) that the role of UTP in glutamic dehydrogenase activation may be to facilitate biosynthesis of amino sugars in species that lack chitin. On that premise, most of the species of Achlya examined in this study should have some chitinous material as a component of their walls.

Genus Saprolegnia. Among the 10 species tested, only the S. asterophora isozyme responded to all 6 activators (Fig. 8 and Table 5), although the S. ferax isozyme while insensitive to ATP, but responded to all other modulators. The other Saprolegnia glutamic dehydrogenase isozymes of the other Saprolegnia species were activated by NADP^+ but only S. monoica and S. megasperma enzymes could interact with P-enolpyruvate as well.

Fig. 7. Activation of NAD-dependent glutamic dehydrogenase isozymes of various species of Achlya with NADP^+ , P-enolpyruvate, GTP, ATP, UTP and acetyl-CoA (CoA). See Fig. 6 legend for other details.



Although S. littoralis and S. monoica displayed a pair of electrophoretically distinct isozymes per species, S. littoralis was one of the two species (the other being S. delica) that failed to respond in any way to all the activators.

Other Saprolegniales. None of the species found in the genera, Aphanomyces, Isoachlya, Protoachlya, and Thraustotheca contained enzymes that showed complete sensitivity to all the modulators (Fig. 9 and Table 5). NADP^+ was the only activator that interacted with all the isozymes. None of the species in this group interacted with UTP implying that they may contain chitin in their walls. Furthermore, I. intermedia only responded to CoA.

The Leptomitales. Only two species, Sapromyces elongatus and Apodachlya brachynema had isozymes that failed to respond to all activators but NADP^+ . The interaction with this modulator was very weak.

Influence of Citrate. Glutamic dehydrogenase of bovine liver is considered to be a metalloenzyme (4) although this point has been disputed because there is less than one mole of Zn^{++} per mole of protein. In the cases we have been studying (6, 8) the fungal enzymes

Table 5. Activation of NAD-dependent glutamic dehydrogenase isozymes of Saprolegniales by NADP^+ , P-enolpyruvate, GTP, ATP, UTP and cetyl-CoA (CoA). The ratio V_a/V_o represents the increase in enzyme activity over control where no activator is present. The assay was carried out spectrophotometrically in a 3-ml reaction solution consisting of 30 mM NH_4^+ , 10 mM α -ketoglutarate, 0.167 mM NADH, 33.3 mM Tris-acetate buffer, pH 8 with or without one of the following activators 1 mM NADP^+ ; 0.5 mM P-enolpyruvate; 0.33 mM GTP; 1 mM ATP; 1 mM UTP; and 0.33 mM acetyl-CoA. Reaction rate was determined from the change in absorbance at 340 nm. V_a is the rate in the presence of one activator and V_o is the rate in the absence of an activator.

		Modulator effect, V_a/V_o					
		NADP^+	PEP	GTP	ATP	UTP	CoA
<u>Achlya</u>	<u>dubia</u>	4.0	2.0	1.5	-	-	-
<u>A.</u>	<u>americana</u>	3.8	3.7	1.5	-	1.4	-
<u>A.</u>	<u>ambisexualis</u> ♂	3.6	3.6	-	-	-	-
<u>A.</u>	<u>ambisexualis</u> ♀	3.4	3.4	1.3	1.4	1.4	-
<u>A.</u>	<u>colorata</u>	3.0	1.3	1.3	1.3	1.3	1.3
<u>A.</u>	<u>bisexualis</u> ♂	5.0	1.3	-	-	-	-
<u>A.</u>	<u>bisexualis</u> ♀	2.3	1.2	-	-	-	-
<u>A.</u>	<u>crenulata</u>	6.0	2.0	1.4	1.4	1.4	-
<u>A.</u>	<u>intricata</u>	3.0	-	-	-	-	-
<u>A.</u>	<u>flagellata</u>	3.5	-	-	-	-	-
<u>A.</u>	<u>racemosa</u>	2.6	1.4	1.3	1.4	1.4	1.4
<u>A.</u>	<u>radiosa</u>	4.0	-	-	-	-	-
<u>A.</u>	<u>heterosexualis</u> ♂	1.8	1.3	-	-	-	-

Table 5 Continued

	Modulator effect, V_a/V_o					
	NADP ⁺	PEP	GTP	ATP	UTP	CoA
<u>Achlya heterosexalis</u> ♀	3.7	1.7	-	-	-	-
<u>Saprolegnia asterophora</u>	2.0	1.7	1.7	1.5	1.5	1.3
<u>S. lapponica</u>	3.3	-	-	-	-	-
<u>S. ferax</u>	3.1	1.4	1.2	-	1.3	1.2
<u>S. mixta</u>	2.0	-	-	-	-	-
<u>S. terrestris</u>	3.0	-	-	-	-	-
<u>S. monoica</u>	3.5	1.3	-	-	-	-
<u>S. megasperma</u>	2.6	1.3	-	-	-	-
<u>S. furcata</u>	3.0	-	-	-	-	-
<u>S. litoralis</u>	-	-	-	-	-	-
<u>S. delica</u>	-	-	-	-	-	-
<u>Aphanomyces laevis</u>	3.0	1.2	-	-	-	-
<u>A. cladogamus</u>	1.4	1.2	-	-	-	-
<u>Isoachlya eccentrica</u>	3.5	-	-	-	-	-
<u>I. unispora</u>	2.6	1.2	-	-	-	-
<u>I. intermedia</u>	3.2	1.4	-	-	-	1.2
<u>I. toruloides</u>	3.4	1.3	1.2	-	-	-
<u>Thraustotheca clavata</u>	2.4	-	-	-	-	-
<u>Aphanopsis terrestris</u>	2.4	-	-	-	-	-
<u>Protoachlya paradoxa</u>	2.1	1.2	1.2	-	-	-

Fig. 8. Activation of NAD-dependent glutamic dehydrogenase isozymes of various species of Saprolegnia by NADP^+ , P-enolpyruvate, GTP, ATP, UTP and acetyl-CoA (CoA). See Fig. 6 legend for other details.

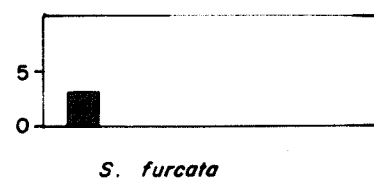
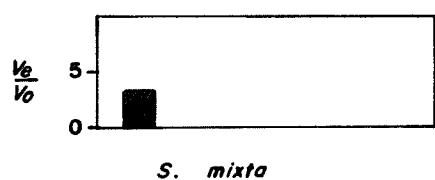
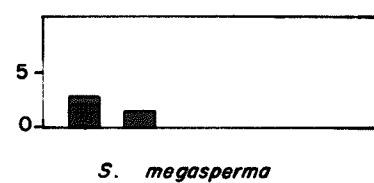
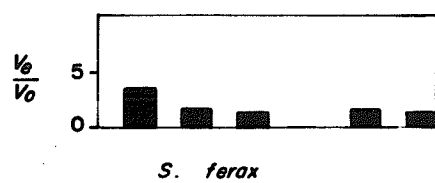
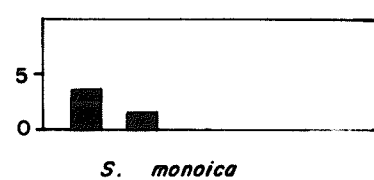
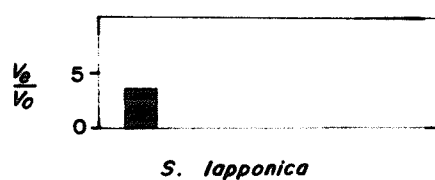
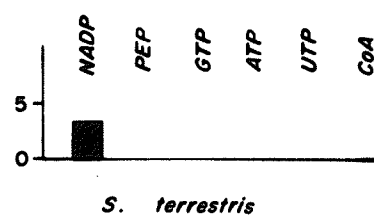
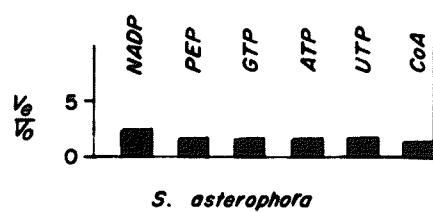
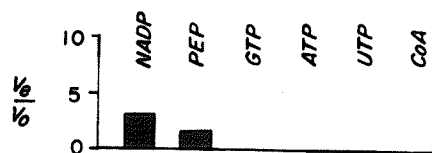
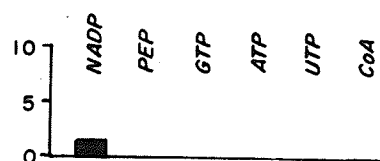
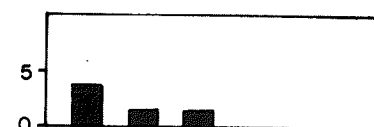
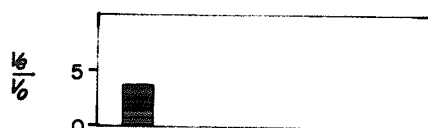
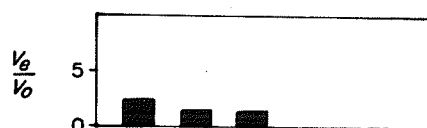
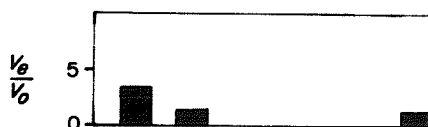


Fig. 9. Activation of NAD-dependent glutamic dehydrogenase isozymes of various species of Aphanomyces, Isoachlya, Thraustotheca and Protoachlya by NADP^+ , P-enolpyruvate, GTP, ATP, UTP and acetyl-CoA (CoA). See Fig. 6 legend for other details.

*Aphanomyces laevis**Thraustotheca clavata**A. cladogamus**A. terrestris**Isoachlya eccentrica**I. unispora**Protoachlya paradoxa**I. intermedia**I. toruloides*

do seem to be inhibited by metal ion chelators such as EDTA and citrate. The influence of citrate on the isozymes of glutamic dehydrogenase in the Oomycetes was restricted to the Peronosporales in the present study. Inhibition by citrate was very strong in most cases. The isozymes of sexually distinct but related species were inhibited to about the same extent in every case. For example, P. sylvaticum isozymes were inhibited 85% by citrate. Both male and female strains showed the same sensitivity (Table 6).

There was no correlation between the activation and inhibition patterns of the enzymes from the various species, other than the tenuous fact that those isozymes that were more responsive to the allosteric activators were less sensitive to the inhibitor citrate. This feature, however, may be due to effects of residual activation caused by small endogenous molecules that may have escaped elimination during treatment of the cell extracts.

The basic conclusion is that the enzyme of all species of the genus Pythium studied here may be metallo-enzymes or may at least have some metal ion functional in their catalytic activity.

Table 6. Inhibition of NAD-dependent glutamic dehydrogenase isozymes of Pythium spp. by citrate.

Organism	% Inhibition
<u>Pythium ultimum</u>	40
<u>P. debaryanum</u>	40
<u>P. heterothallicum</u> ♂	64
<u>P. heterothallicum</u> ♀	67
<u>P. butleri</u>	82
<u>P. splendens</u>	31
<u>P. intermedium</u> (+)	65
<u>P. intermedium</u> (-)	80
<u>P. undulatum</u>	63
<u>P. sylvaticum</u> ♂	87
<u>P. sylvaticum</u> ♀	85

DISCUSSION

The results obtained from this study indicate that there is no direct correlation between electrophoretic mobility of glutamic dehydrogenase isozymes of the Oomycetes and their allosteric properties. The differences in the degree to which the various isozymes respond to the activators must therefore be due to subtle 'local' changes in the active sites where these modulators bind.

Some useful information, however, was gained from these analyses. First, the integrated approach of looking at gross form, function and regulation of isozymes intra- and inter-generically has provided support for the concept of form and function. In the majority of cases, species placed within the same genus possess isozymes that are uniformly similar in electrophoretic mobility and regulatory patterns. For example, within the genus Pythium, there is no deviation from the pattern in which NADP^+ , P-enolpyruvate, and GTP are the strongest activators. Also, all modulators affect all the isozymes. Isozymes of the genus Achlya have a more diffuse regulatory pattern even though the electrophoretic mobilities are not very different. There are but 2 instances within the 14 species where all 6 activators work on the isozymes. Nevertheless, most of the activators, with

the exception of NADP^+ , are not strong modulators of the enzymes of A. colorata and A. racemosa.

Second, one sees an increase in complexity of the regulatory properties of the isozymes as the taxonomic limb is traversed from the Saprolegniales to the Peronosporales. Mycologists generally agree that the Peronosporales constitute the most advanced form of the Oomycetes, being largely terrestrial obligate parasites (1). On this premise, the increased complexity in the regulation of the glutamic dehydrogenases in species of this order may signify that they may be more highly adapted forms, but not necessarily more evolutionarily advanced.

It is interesting to note that the isozymes of species of the genus Pythium are the most prone to control by UTP. These organisms constitute the majority of the Oomycetes tested which are plant parasites. It could be that their association with plants has led to a selection for species that could utilize the metabolic capacity of the green plants to synthesize amino sugars with a concomitant loss of their own ability. Consequently, the free-living forms may not be able to synthesize chitin.

The third, and perhaps most important point, is that the allosteric controls on the glutamic dehydrogenase isozymes of the Oomycetes is far from uniform. In a few cases, none of the activators affect the enzyme. The most common pattern is that activation is biased in favour of NADP^+ , P-enolpyruvate, and GTP. While isozymes of Pythium spp react with all activators, those of Achlya spp. fall in the next category; mostly reactive but with some enzymes insensitivity to some modulators. Isozymes of Saprolegnia spp., Protoachlya, Isoachlya spp., Aphanomyces spp., Thraustotheca and Leptolegnia are the least sensitive and display the greatest variation - indicative of an uncertainty that is reflected in their taxonomic characterization. A system whereby taxonomic identification of fungal species suspected of being in this class of Oomycetes may be approached through an examination of the regulatory properties of one or more enzymes using crude cell-free extracts of the organism may therefore be profitable.

Speciation

The ability to grow a large number of taxonomically-related species of the Oomycetes in the same laboratory

environment and nutrient, provided me with an experimental system by which I could look at the problem of speciation in fungi with a little more care. This topic is sorely neglected in fungal studies (2), probably due to the continuing debate on what is a fungal species. By definition, speciation is the originating of species through the action of isolation mechanisms on populations.

There is, of course, no evidence in this work to conclude that the physiological speciation observed through enzyme regulation studies is a consequence of sympatric or allopatric speciation. One can suggest that due to the terrestrial habit of Pythium spp., their enzyme control patterns are markedly different from those of the aquatic genera Saprolegnia, Achlya, and other genera of the order Saprolegniales because of allopatric speciation. However, since the same controls are manifested in some species of all the fungal groups tested, it may be that these differences arose between strains or groups of strains not through isolation by distance but through micro-habitat localization which would support the concept of sympatric speciation. It is worth noting that certain Pythium spp. are not entirely terrestrial for it is known that they can produce zoospores given the right conditions. Zoospores are commonly produced by aquatic species of the

Oomycetes, probably as a mode of dispersal of the progeny, and this forms a vital part of their life cycle.

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SECTION III

COMPARATIVE ALLOSTERY OF D (-) LACTATE DEHYDROGENASES OF OOMYCETES

INTRODUCTION

As part of a continuing programme of studies on the use of enzyme regulation and other physical characterization of proteins to elucidate what may be the underlying biochemical bases for speciation in fungal populations, enzymes that are located at strategic positions in metabolism were selected for such studies. LéJohn had shown in a general survey that the Oomycetes constitute a class of fungi with interesting biochemical novelties (2). Besides the novel feature that they and all other members of the obsolete class "phycomycetes" (1) contain only the NAD-linked glutamic dehydrogenase (2), they also harbour only the D(-)lactic dehydrogenase enzyme form which is subject to a unique form of control (3). GTP functions as an allosteric inhibitor, binding cooperatively with NADH and D(-)lactate, but not NAD^+ and pyruvate. A plausible physiological reason for this control was proposed (3).

The present study was undertaken to determine how extensive this control system is among the Oomycetes, and to evaluate the possibility that it could be used as an important parameter to support the morphologically-based separation of the species. Rather than depend on just

enzyme function alone, the electrophoretic properties of the enzymes were utilized as a physical parameter in the characterization.

It is shown here that, by and large, the present taxonomic division of the Oomycetes is entirely justified. The biochemical approach does permit one to recognize some interrelations between species that may not otherwise have been possible.

MATERIALS AND METHODS

The materials used and the procedures followed for the growth, harvesting, and preparation of fungal cultures for enzyme analysis have been enumerated in Section II of this thesis.

Detection of Enzyme Activity on Gels. D(-)lactic dehydrogenase isozymes were detected by staining the gels with a reaction solution composed of: 1 mM NAD^+ ; 100 mM D(-)lactate (lithium salt), 0.30 mM nitro-blue tetrazolium; 0.163 mM phenazine methosulphate; and 50 mM Tris-HCl, pH 8. The reaction was allowed to proceed for 2 hr at 30°C in the dark.

Spectrophotometric Assays. The method described by LéJohn (3) was followed. Percent inhibition of enzyme activity by GTP was determined from the equation:

$$\% \text{ Inhibition} = \frac{v_o - v_i}{v_o} \times 100$$

where v_i is the activity of the enzyme in the presence of GTP, and v_o , the activity in its absence. Since the individual K_m values for the substrates of the enzyme were not determined, the inhibition values obtained are empirical. They are all related to the single fully-characterized system, that of lactic dehydrogenase from P. debaryanum where the enzyme has been purified and its kinetic properties studied (3).

Protein Determination. The colorimetric method of Lowry et al (4) was used with bovine serum albumin as standard. The colour intensity was evaluated with a Klett colorimeter using Klett No. 62 filter.

Source of Chemicals. Photographic and electrophoresis supplies were obtained from Eastman Kodak Co., Rochester, New York. Biochemicals were obtained from: Sigma Chemical Co., St. Louis, Mo.; Calbiochem., Calif.; and PL-Biochemicals, Milwaukee. Nutrients for growth studies were purchased from Difco Laboratories, Detroit, Michigan.

RESULTS

Electrophoretic Studies

The genus Pythium. Eleven species of Pythium were analysed for their lactic dehydrogenase isozyme patterns. The results, shown in Fig. 1 and Table 1 illustrate some interesting features. The following species, P. ultimum, P. intermedium (+), and P. intermedium (-) have identical pairs of isozymes. This feature is absent in all other species of the Oomycetes tested, except of Leptolegnia of the Saprolegniales. The mating pairs of P. sylvaticum have isozymes that have coincident electrophoretic mobilities. Those of the male and female components of P. heterothallicum migrate differently from each other and this is in direct contrast to the patterns observed for all other mating pairs tested. The faster moving of the two isozymes of P. ultimum is similar to the enzyme from P. debaryanum. Isozymes from P. butleri, male and female components of P. heterothallicum, and P. undulatum bear no electrophoretic relation with each other or with isozymes of any of the other Pythium spp.

The genus Aphanomyces. The results of Fig. 2 and Table 2 display not only the isozyme patterns for species of Aphanomyces, but also single species of Aphanopsis, Thraustotheca, and Leptolegnia. Only Leptolegnia caudata contained two LDH isozymes. The isozymes of Aphanomyces stellatus and Aphanopsis terrestris are electrophoretically similar although they belong to different genera. None of the species of the genus Aphanomyces contained LDH isozymes with identical electrophoretic properties, and, in turn, they showed little correlation with isozymes of the other species mentioned in Fig. 2.

The genus Achlya. The LDH isozymes of 14 species of the genus Achlya examined had practically identical electrophoretic mobilities (Fig. 3 and Table 2). They moved considerably faster than the isozymes of the other species illustrated in Fig. 1 and Fig. 2. There was no indication of multiple isozyme forms in any of the 14 species tested. In contrast to the situation observed for the sexually different forms of P. heterothallicum, none of the physiological mating of different Achlya species (A. bisexualis, A. ambisexualis, and A. colorata) display isozymes that are different from each other. In fact, they are all of the same electrophoretic mobility.

9.

Fig. 1. Electrophoretic patterns of NAD-dependent D(-)lactic dehydrogenase isozymes of all species of Pythium. Electrophoresis was for 4 hr at 10°C in a buffer composed of 5.52 g barbital diethylbarbituric acid, 1 g Tris base in 1 litre of H₂O, pH 8. Twenty ml barbital buffer was added to the polyacrylamide solution. Arrows indicate position of tracking dye.

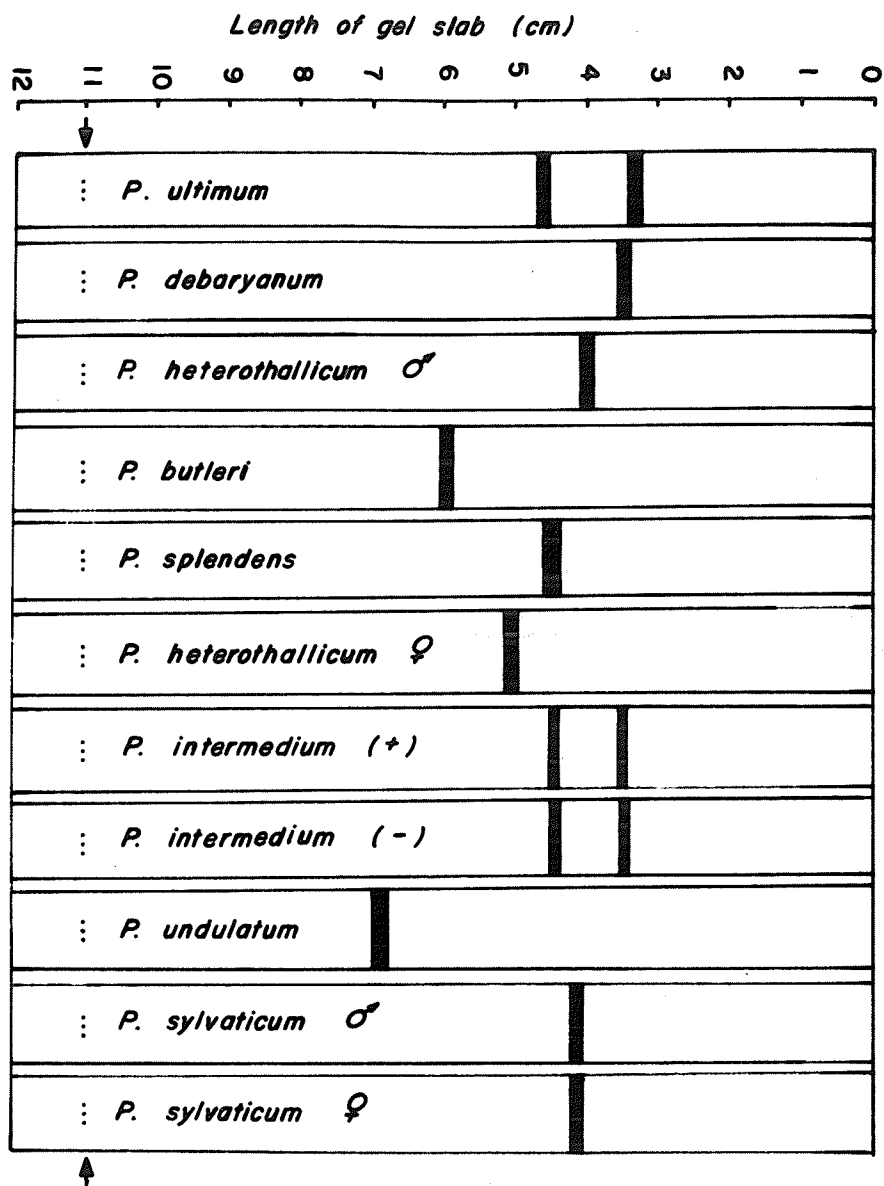


Fig. 2. Electrophoretic patterns of NAD-dependent D(-)lactic dehydrogenase isozymes of Aphanomyces, Aphanopsis, Thraustotheca and Leptolegnia spp. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend.

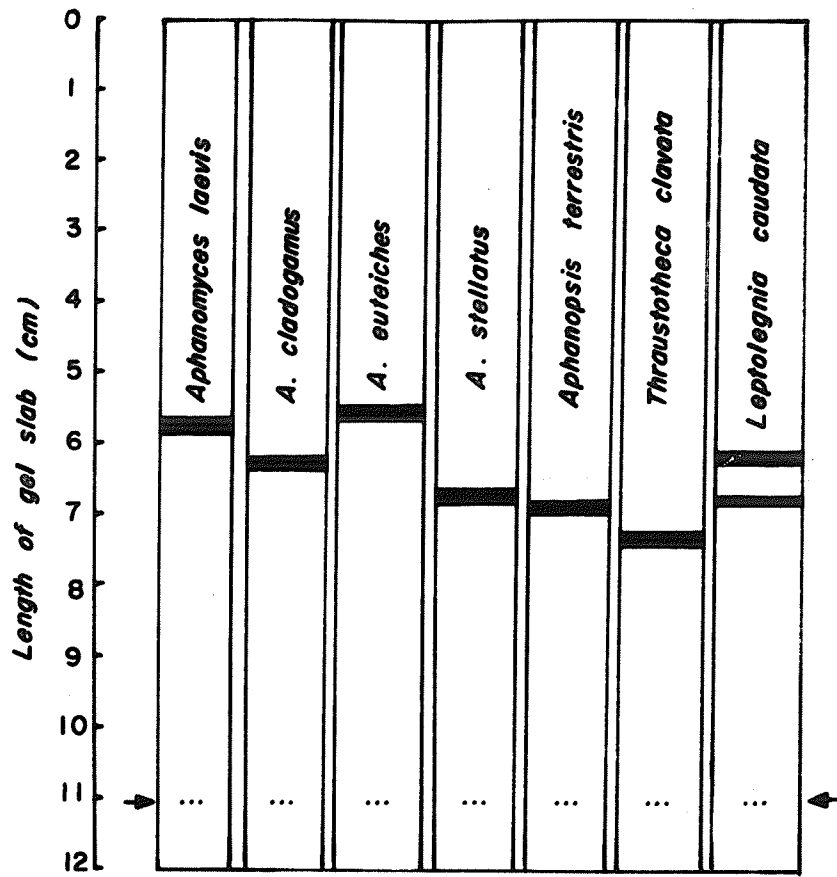


Table 1. Electrophoretic mobilities (Re) of D(-) lactic dehydrogenase isozymes of members of the Peronosporales and Leptomitales. Experimental details given under "Materials and Methods".

Organism	Re	(cm)
Peronosporales		
<u>Pythium ultimum</u>	3.3	4.7
<u>P. debaryanum</u>	3.5	
<u>P. heterothallicum</u> ♂	4.0	
<u>P. heterothallicum</u> ♀	5.1	
<u>P. butleri</u>	6.0	
<u>P. splendens</u>	4.5	
<u>P. intermedium</u> (+)	3.5	4.5
<u>P. intermedium</u> (-)	3.5	4.5
<u>P. undulatum</u>	6.9	
<u>P. sylvaticum</u> ♂	4.2	
<u>P. sylvaticum</u> ♀	4.2	
Leptomitales		
<u>Apodachlya brachynema</u>	7.0	
<u>Sapromyces elongatus</u>	0.8	

The genus Saprolegnia. The differences in the electrophoretic mobilities shown by LDH isozymes of the Saprolegnia spp. are small but noticeable and reproducible (Fig. 4 and Table 2). In general, their mobilities matched those of the Achlya IDH isozymes.

The genus Isoachlya. The species of Isoachlya and Protoachlya have LDH isozymes with electrophoretic mobilities similar to those of the Achlya spp. (Fig. 5 left panel and Table 2). The enzymes from I. eccentrica and I. unispora are slightly different from the isozymes of the other two Isoachlya spp. Their R_e values are identical at 6.5 whereas the others migrate farther to 6.8 and 6.9 cm.

The genera Apodachlya and Sapromyces. Only a single species from each genus was analysed in these studies. The isozymes were totally distinctive, that of Sapromyces elongatus being cathodic, barely migrating from the negative pole. The R_e value was determined as 0.8 cm in contrast to the R_e of Apodachlya brachynema LDH of 7 cm (Fig. 5, right panel and Table 1).

The R_e values for the isozymes of each genus are given in Table 1; and Table 2. Since two isozymes have been found in 3 species of Pythium, the averages estimated have been adjusted to compensate for this feature.



Fig. 3. Electrophoretic patterns of NAD-dependent D(-)lactic dehydrogenase isozymes of 14 species of Achlya. Electrophoresis was for 4 hr at 10 C in a buffer described in Fig. 1 legend.

Fig. 4. Electrophoretic patterns of NAD-dependent D(-)lactic dehydrogenase isozymes of 10 species of Saprolegnia. Electrophoresis was for 4 hr at 10 C in a buffer described in Fig. 1 legend.

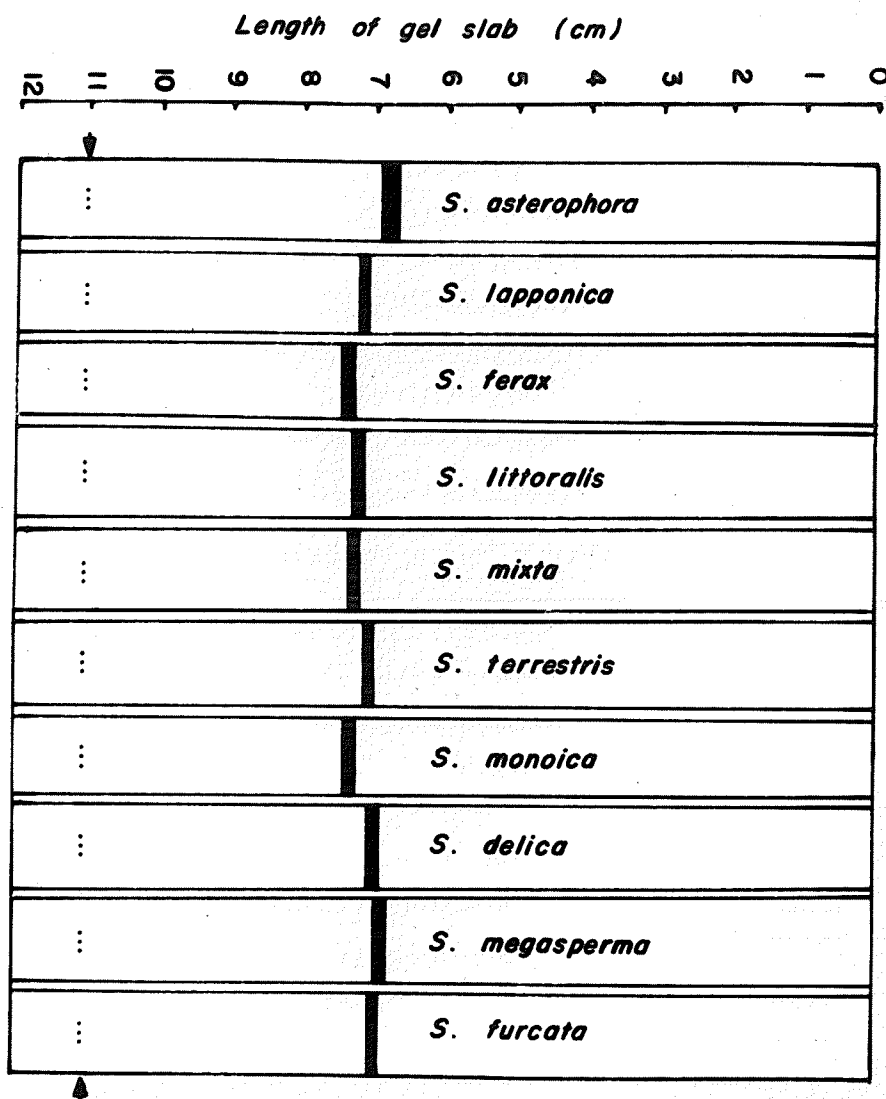


Fig. 5. Electrophoretic patterns of NAD-dependent D(-)lactic dehydrogenase isozymes of some Saprolegniales (left panels) and Leptomitales (right panels). Electrophoresis was for 4 hr at 10 C in a buffer described in Fig. 1 legend.

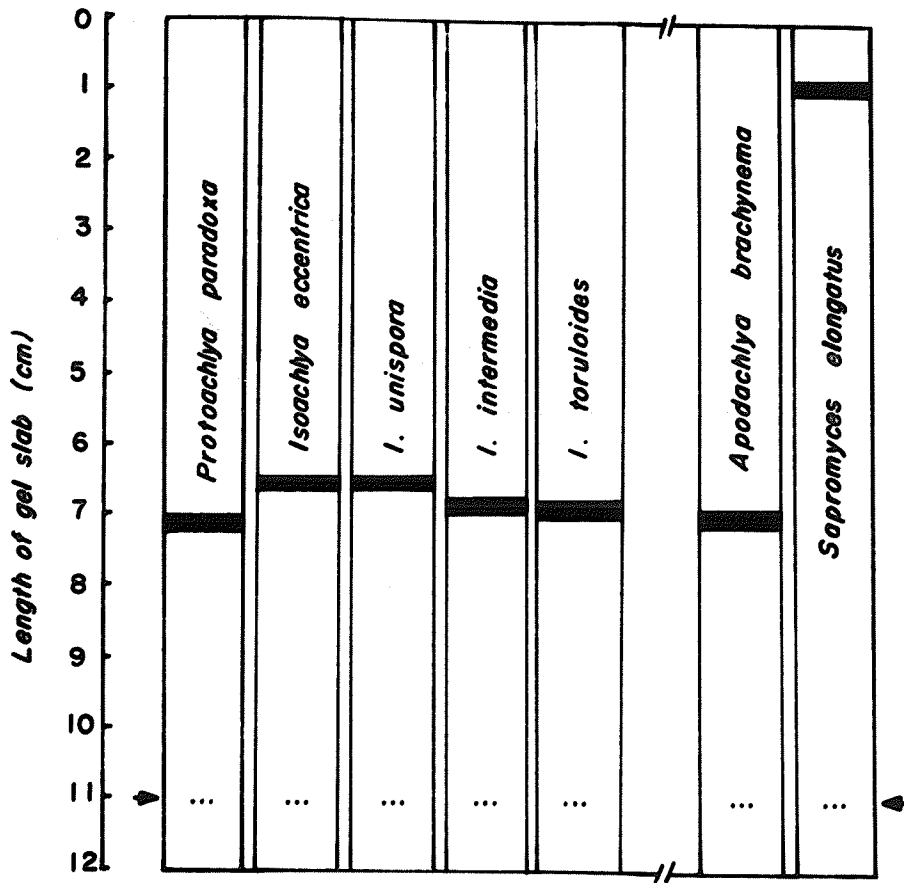


Table 2. Electrophoretic mobilities (R_e) of D(-) lactic dehydrogenase isozymes of members of the Saprolegniales. Experimental details given under "Materials and Methods".

Organism	R_e (cm)
<u>Achlya</u> <u>ambisexualis</u> ♂	6.8
<u>A.</u> <u>ambisexualis</u> ♀	6.8
<u>A.</u> <u>americana</u>	6.8
<u>A.</u> <u>dubia</u>	6.7
<u>A.</u> <u>colorata</u>	6.8
<u>A.</u> <u>bisexualis</u> ♂	6.8
<u>A.</u> <u>bisexualis</u> ♀	6.8
<u>A.</u> <u>crenulata</u>	6.7
<u>A.</u> <u>intricata</u>	6.5
<u>A.</u> <u>flagellata</u>	6.6
<u>A.</u> <u>racemosa</u>	6.6
<u>A.</u> <u>radiosa</u>	6.6
<u>A.</u> <u>heterosexualis</u> ♂	6.8
<u>A.</u> <u>heterosexualis</u> ♀	6.8
<u>Saprolegnia</u> <u>asterophora</u>	6.8
<u>S.</u> <u>lapponica</u>	7.1
<u>S.</u> <u>ferax</u>	7.3

Table 2. Continued

Organism	R_e (cm)
<u>Saprolegnia</u> <u>littorolis</u>	7.2
<u>S.</u> <u>mixta</u>	7.3
<u>S.</u> <u>terrestris</u>	7.0
<u>S.</u> <u>monoica</u>	7.3
<u>S.</u> <u>delica</u>	6.9
<u>S.</u> <u>megasperma</u>	6.8
<u>S.</u> <u>furcata</u>	6.9
<u>Protoachlya</u> <u>paradoxa</u>	7.1
<u>Isoachlya</u> <u>eccentrica</u>	6.5
<u>I.</u> <u>unispora</u>	6.5
<u>I.</u> <u>intermedia</u>	6.8
<u>I.</u> <u>toruloides</u>	6.9
<u>Aphanomyces</u> <u>laevis</u>	5.8
<u>A.</u> <u>cladogamus</u>	6.3
<u>A.</u> <u>euteiches</u>	5.6
<u>A.</u> <u>stellatus</u>	6.8
<u>Aphanopsis</u> <u>terrestris</u>	6.9
<u>Thraustotheca</u> <u>clavata</u>	7.4
<u>Leptolegnia</u> <u>caudata</u>	6.2 6.8

It is assumed that the 11 species with 14 isozymes represent 14 species and 14 isozymes. In actual fact, computation of the R_e values as slow-moving components in combination with the others, or as fast-moving component only, surprisingly, produced closely-related R_e values. Whereas the average value for Pythium spp. is 4.5, the values for the Saprolegniales (excluding Saprolegnia spp.) fell between 6.4 and 6.9. The average for Saprolegnia spp. was 7.13 making them the least cathodic proteins.

GTP Regulation

The conditions for studying the allosteric inhibitory effects of GTP on these enzymes have been described in detail (3). Because crude enzyme preparations are being used, the inhibition values obtained in this series of analysis should be considered as empirical.

The inhibitory effects of GTP were studied over a range of NADH concentrations (0.016 mM to 0.05 mM) where the inhibition is most pronounced and interaction is cooperative in nature (3). As illustrations of these inhibition curves would be futile and superfluous, the results of GTP at the highest and lowest NADH concentrations are recorded in Table 3 as percent inhibition.

This value was obtained using the formulation given in "Materials and Methods".

For ease of comparison, the species are listed in the same order in which their isozyme patterns are presented in the electropherograms of Fig. 1 to Fig. 5.

Isozymes of Pythium spp. were the most sensitive to GTP inhibition. They also do provide some interesting features for speculation. First of all, the isozymes of the mating types, P. heterothallicum, P. intermedium, and P. sylvaticum were about the most sensitive to GTP effects. Increasing the concentration of NADH from 0.016 mM to 0.05 mM could only reverse GTP inhibition by 47% in one case, that of P. heterothallicum (male). Very little reversal of the inhibition occurred in the other cases.

In contrast, the homothallic species had enzymes that were also very sensitive to GTP inhibition, but this effect was adequately antagonized by high concentrations of NADH. In the case of P. ultimum, inhibition was reduced by as much as 66%.

Another interesting point is the fact that P. heterothallicum (male) had an enzyme with twice the specific activity of the female strain. While the female strain isozyme is completely inhibited by GTP,

at all NADH concentrations that of the male strain is only approximately 50% inhibited at high NADH concentrations. It would have been logical to speculate on the possibility that the male strain may have duplicate enzymes, one of which is sensitive to GTP while the other is not but, since GTP inhibits the enzyme activity by 90% at low NADH concentration and only one enzyme activity band is detected in polyacrylamide gels, this is an unlikely hypothesis. But note that the isozymes of these strains are unique in that they are the only mating pair isozymes with very different electrophoretic mobilities (Fig. 1). Obviously, some chemical differences does exist between the proteins of these two strains, and this is reflected at the level of enzyme regulation.

The isozyme of P. splendens was also relatively insensitive to GTP inhibition. At low NADH levels, the enzyme was only 54% inhibited, and at high NADH concentrations, it was only inhibited by 32%.

Isozymes from species of Saprolegnia were the next group that showed some reasonably high sensitivity to GTP inhibition. Only in the case of S. mixta can one observe 100% inhibition by GTP at the lowest concentration. In other cases, inhibition was as low as 16% and as high as 67%. Inhibition values are not given for 3 species

Table 3. GTP inhibition of NAD-dependent D(-)
lactic dehydrogenase isozymes of
Oomycetes.

Organism	% Inhibition of Control Activity	
	0.0167 mM NADH	0.05 mM NADH
Peronosporales		
<u>Pythium</u> <u>ultimum</u>	87	35
<u>P.</u> <u>debaryanum</u>	92	68
<u>P.</u> <u>heterothallicum</u> (male)	89	53
<u>P.</u> <u>heterothallicum</u> (female)	100	100
<u>P.</u> <u>butleri</u>	93	95
<u>P.</u> <u>spendens</u>	55	32
<u>P.</u> <u>intermedium</u> (+)	100	99
<u>P.</u> <u>intermedium</u> (-)	97	95
<u>P.</u> <u>undulatum</u>	88	77
<u>P.</u> <u>sylvaticum</u> (male)	100	90
<u>P.</u> <u>sylvaticum</u> (female)	100	96
Saprolegniales		
<u>Achlya</u> <u>ambisexualis</u> (male)	60	57
<u>A.</u> <u>ambisexualis</u> (female)	33	25
<u>A.</u> <u>americana</u>	50	60
<u>A.</u> <u>dubia</u>	50	25
<u>A.</u> <u>colorata</u>	25	0
<u>A.</u> <u>bisexualis</u> (male)	25	17
<u>A.</u> <u>bisexualis</u> (female)	67	50

Table 3. Continued

Organism	% Inhibition of Control Activity	
	0.0167 mM NADH	0.05 mM NADH
Saprolegniales		
<u>Achlya crenulata</u>	17	17
<u>A. intricata</u>	-	50
<u>A. flagellata</u>	100	33
<u>A. racemosa</u>	50	33
<u>A. radiosa</u>	33	33
<u>A. heterosexuais</u> (female)	67	55
<u>A. heterosexuais</u> (male)	50	50
Saprolegniales		
<u>Saprolegnia asterophora</u>	17	50
<u>S. lapponica</u>	-	100
<u>S. ferax</u>	57	40
<u>S. litoralis</u>	-	-
<u>S. mixta</u>	100	20
<u>S. terrestris</u>	90	60
<u>S. monoica</u>	33	12
<u>S. delica</u>	-	-
<u>S. megasperma</u>	67	43
<u>S. furcata</u>	50	20

Table 3. Continued

Organism	% Inhibition of Control Activity	
	0.0167 mM NADH	0.05 mM NADH
Saprolegniales		
<u>Aphanomyces laevis</u>	0	0
<u>A. cladogamus</u>	60	4
<u>A. euteiches</u>	33	0
<u>A. stellatus</u>	0	0
<u>Aphanopsis terrestris</u>	33	17
<u>Thraustotheca clavata</u>	-	-
<u>Leptolegnia caudata</u>	-	50
<u>Isoachlya eccentrica</u>	-	-
<u>I. unispora</u>	71	57
<u>I. intermedia</u>	60	50
<u>I. toruloides</u>	50	46
<u>Protoachlya brachynema</u>	60	17
Leptomitales		
<u>Apodachlya brachynema</u>	41	22
<u>Sapromyces elongatus</u>	33	20

because an inordinately large amount of crude extract was required to obtain a reasonable reaction rate necessary in such studies. These rates could not be reliably compared with rates obtained when approximately the same amount of protein in cell extracts are used.

The isozymes of the *Achlya* species were all moderately sensitive to GTP inhibition, ranging from 16% inhibition to 100% in a single case that of, A. flagellata. In the majority of cases, inhibition was about 33% to 50%. There was no coincidence in the inhibitory effects of GTP on the isozymes of the mating pairs - A. ambisexualis, A. bisexualis, and A. heterosexulis. Since their electrophoretic mobilities are similar, the significance of this is difficult to assess. Because the specific activities of the LDH's from all species of the Saprolegniales were considerably lower than those of the Peronosporales, the concentration range of NADH used was 0.033 mM to 0.1 mM.

Inhibition of the isozymes of Isoachlya spp., Protoachlya, Aphanomyces spp., Leptolegnia sp., and Aphanopsis sp. by GTP was about the same as determined for *Achlya*. Inhibition greater than 60% was rare. Thraustotheca clavata, like some of the species of Saprolegnia, and

Isoachlya eccentrica, had low specific activity enzymes which made this type of study difficult.

Sapromyces elongatus was unique among all the Oomycetes. The specific activity of the enzyme was about 50 to 100 times greater than that of any other species tested. Its slow mobility in the electric field has already been mentioned. Under the assay conditions, GTP inhibition was 33% at low NADH, and 20% at high NADH concentration. This compares favourably with the isozyme from Apodachlya brachynema which, although it migrated in the same way as the Saprolegniales in electrophoresis, was inhibited 40% and 22% at the low and high concentration of NADH by GTP.

DISCUSSION

These studies provide ample support for the suggestion put forward recently by LéJohn that the Oomycetes contain only the D(-) form of NAD-linked lactate dehydrogenase, and that this enzyme is subject to allosteric control by GTP (2, 3). In one of those reports, LéJohn (30) pointed out that this enzyme may serve a catabolic function so that its physiological function is carefully regulated by GTP moderating the rate at which NADH is oxidized. This is borne in some 40 of the 49 species tested.

In turn, as in the previous study on glutamic dehydrogenases of the Oomycetes (Section II of the thesis), the crucial guide to the question of speciation in these population of morphologically-related fungi, comes from studies on the regulation of the enzyme activity. One finds that the more advanced Oomycetes, Pythium spp., provide the more varied isozyme patterns and also they are the most sensitive to allosteric control by GTP. Again, the allosteric sensitivity becomes less marked in the Saprolegniales - an order that constitutes the largest number of genera in these fungi. Whether this means that the Saprolegniales have been exposed to a wider

range of micro-habitats so that they are gradually adapting and losing some fundamental regulatory features of their enzymes is difficult to evaluate. The fact that members of the Peronosporales are mostly terrestrial forms cannot be invoked as an explanation, for it is known that species of both orders inhabit terrestrial and aquatic environments. Members of the both orders are parasitic on plants although the majority of species of Saprolegnia infect fish and fish eggs whereas species of Pythium generally infect higher plants.

Electrophoretic variation of the LDH isozymes is more pronounced in species of Pythium and Aphanomyces. It is interesting to note that these two genera generally infect plants, the latter infecting roots of vascular plants such as sugar beets and peas (1).

The existence of multiple isozyme species in a few cases in the genus Pythium cannot be explained on the basis of hybrid forms of the enzyme due to interaction of different subunits. If there were only 2 types of subunits in a dimer protein, we would expect to see 3 isozymic bands unless one of the 3 is inactive. However, both isozymic species of the enzyme in P. ultimum and P. intermedium (both (+) and (-) mating types), are inhibited by

GTP (Table 2). One could not invoke a theory that one of the isozymes arise so as to circumvent the physiological control imposed on lactose metabolism by GTP. If these are indeed different proteins, not hybrids, then there must be two genes for this enzyme in these cells, and one must have been altered sufficiently so that its electrophoretic mobility is different. Since the identical form is detected in different species - P. ultimum, P. intermedium, and P. debaryanum (based on electrophoretic mobility), and all other species do not seem to have multiple isozymes of LDH, it is unlikely that their formation is a consequence of hybrid formation from different subunits.

Interestingly, the isozymes from P. intermedium are considerably more sensitive to GTP inhibition than those from P. ultimum and P. debaryanum. In fact, the latter are not fully inhibited by GTP while the former are totally inhibited even at high concentrations of NADH. These isozymes are therefore not as similar as the electrophoretic patterns might suggest. The parameter of enzyme regulation is therefore a more sensitive parameter than gross physical properties to detect speciation.

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SECTION IV

ISOZYME PATTERNS OF MALIC AND ISOCITRIC DEHYDROGENASES

INTRODUCTION

Plant protein and plant enzymes have been used in studies on variation, both within and between species (2). All of these studies relied on the use of electrophoresis to attain physical separation of the protein species under the same conditions. When fungal species are sexually compatible, one expects then that their genetic products (proteins) would also show very close similarities which can be detected by this method of analysis. If non-sexually compatible species show such similarities, then one could make a strong case in support of the idea that such species are more closely-related taxonomically than imagined.

In the previous reports (Sections II, III of this thesis) enzyme regulation was used as an added parameter to bolster conclusions regarding species variation and similarity. No reliable enzyme control systems have been found for the two enzyme types, isocitric and malic dehydrogenases, used in this study. I have, therefore, to rely solely on electrophoretic mobility correlations for this analysis and consider as significant only those relations that are reiterated in more than one way.

MATERIALS AND METHODS

The materials used and the procedures followed for the growth, harvesting, and preparation of fungal cultures for enzyme analysis on polyacrylamide gels have been outlined in Section II of this thesis.

Detection of Enzyme Activity on Gels. NAD-linked malic dehydrogenase, and NADP-linked isocitric dehydrogenase were detected by staining the gels after electrophoresis with the following reaction solutions. For malic dehydrogenase, the reaction mixture was 1 mM NAD^+ ; 50 mM L-malic acid; 0.31 mM nitro-blue tetrazolium; 0.16 mM phenazine methosulphate; and 50 mM Tris-HCl, pH 8. For isocitric dehydrogenase, the reaction solution was: 0.26 mM NADP^+ ; 0.5 mM DL-(threo)-isocitrate (Na salt); 1 mM MnCl_2 ; 0.46 mM phenazine methosulphate; 0.21 nitro-blue tetrazolium; and 100 mM Tris-HCl, pH 8. The reactions were allowed to proceed for 1 hr at 30°C in the dark before the gels were removed from the staining solution.

Spectrophotometric Assays. The specific activities of crude enzyme preparations at 25°C were determined spectrophotometrically using a Gilford model 2400 recording spectrophotometer. The reaction mixture for malic dehydrogenase consisted of 0.167 mM oxaloacetate, 0.05 mM NADH,

66.67 mM Tris-acetate, pH 8 and enzyme protein in a total volume of 3 ml. The reaction mixture for isocitric dehydrogenase contained 0.167 mM NADP^+ , 3.33 mM isocitrate, 1 mM MnCl_2 , 66.67 mM Tris-acetate, pH 8 and enzyme protein in a total volume of 3 ml.

Protein Determination. The colorimetric method of Lowry et al (1) was used with bovine serum albumin as standard. The colour intensity was evaluated with a Klett colorimeter using Klett No. 62 filter.

Source of Chemicals. Photographic and electrophoresis supplies were purchased from Eastman Kodak Co., Rochester, New York. Biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. and Calbiochem., Calif. Nutrients for growth studies were obtained from Difco Laboratories, Detroit, Michigan.

RESULTS

Isocitric Dehydrogenase Isozymes

The Genus Achlya. The results displayed in Fig. 1 and Table 1 indicate that the electrophoretic mobilities of the isocitric dehydrogenase isozymes of the genus *Achlya* are fairly similar. The relationship that exists between the isocitric dehydrogenase isozymes based on this parameter is quite similar to that noted for the glutamic dehydrogenase isozymes of these same species (see Section II of thesis). Not only are the mating pair isozymes coincident in electrophoretic mobility (but showing interspecies differences), their relative migration rates bear the same correspondence with each other. As an example, the isocitric dehydrogenase isozymes of *A. heterosexuais* mating pair are the slowest in migrating from the anode. The same situation was noted with the glutamic dehydrogenases (see Section II of thesis). The only exception is that the isocitric dehydrogenase isozyme of *A. radiosa* migrated the fastest whereas the glutamic dehydrogenase isozyme of *A. colorata* is the least anodic.

The Genus Saprolegnia. Whereas the average Re value for the isozymes of *Achlya* is 3.97, that for the *Saprolegnia* spp. is 4.65 (Fig. 2 and Table 1) indicating that the isocitric dehydrogenases of these two genera are different. The summary of Re values for the isozymes are recorded in Table 1. There was more uniformity in the mobility of the isozymes with the *Saprolegnia* spp. than within those of the *Achlya* spp.

The genus *Aphanomyces* and other genera. Four species of *Aphanomyces*, were tested and one of these, *A. cladogamus*, had 2 isocitric dehydrogenase isozymes (Fig. 3 and Table 1). The second isoenzyme was a faster moving component with an Re value of 6.1 compared to the average of 4.5 ± 0.106 (S.D.). In this regard, the slower migrating isozymes are comparable to the *Saprolegnia* isozymes.

In the same Fig., we can compare the isozymes pattern of a species of *Aphanopsis*, the only other species that has two isocitric dehydrogenase isozymes. Whereas the slow-moving component is similar to the isozyme species of the species of *Aphanomyces*, *Thraustotheca*, and *Leptolegnia* tested, the faster-moving component is different from the second isozyme of *A. cladogamus* is 5.6. No great significance can be placed on the fact that these are the only 2 species of all members of the diverse genera examined

that display dual isocitric dehydrogenase isozyme content for it will become clear that in many other respects, the two species are different.

The genera Isoachlya and Protoachlya. The five species of these two genera examined (Fig. 4 and Table 1) possess isozymes that fall into 3 categories. Four species of Isoachlya were examined and the isozymes of I. eccentrica and I. unispora are similar but very different from the isozymes of I. intermedia and I. toruloides, which are themselves of identical mobilities. The electrophoretic mobility of Protoachlya paradoxa is intermediate between those of the two Isoachlya pairs.

Two species of the Leptomitales were analysed, and the electrophoretic patterns of the isocitric dehydrogenase isozymes are shown on the right panels of Fig. 4 and in Table 2. These two species, Sapromyces elongatus and Apodachlya brachynema, continued to show, as in the other enzyme analyses, that they are quite different. The R_e for S. elongatus was 4.6, and that for A. brachynema, 2.8.

The genus Pythium. In the other studies, species of Pythium had displayed the greatest variation in electrophoretic mobilities of their isozymes (Sections II and III of thesis). In contrast, the isocitric dehydrogenase isozymes of Pythium spp. were very similar (Fig. 5 and Table 2). There was coincidence in the electrophoretic mobilities of the homologous mating pairs - P. hetero-

Fig. 1. Electrophoretic patterns of NADP-dependent isocitric dehydrogenase isozymes of 14 species of Achlya. Electrophoresis was for 4 hr at 10°C in a buffer composed of 5.52 g barbital diethylbarbituric acid, 1 g Tris base and H₂O to 1 litre. Twenty ml of buffer added to polyacrylamide gel solution. Arrows indicate position of tracking dye.

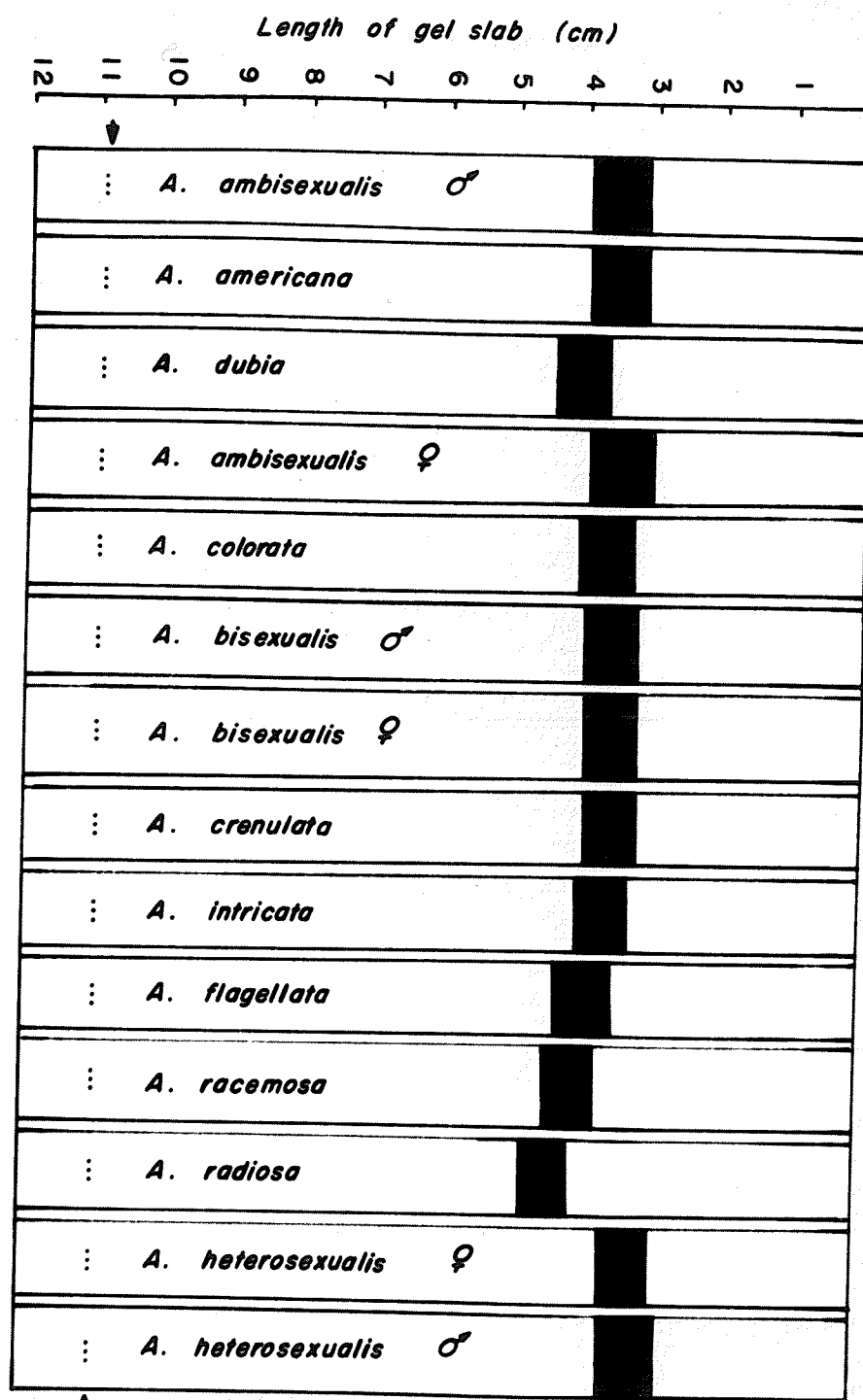
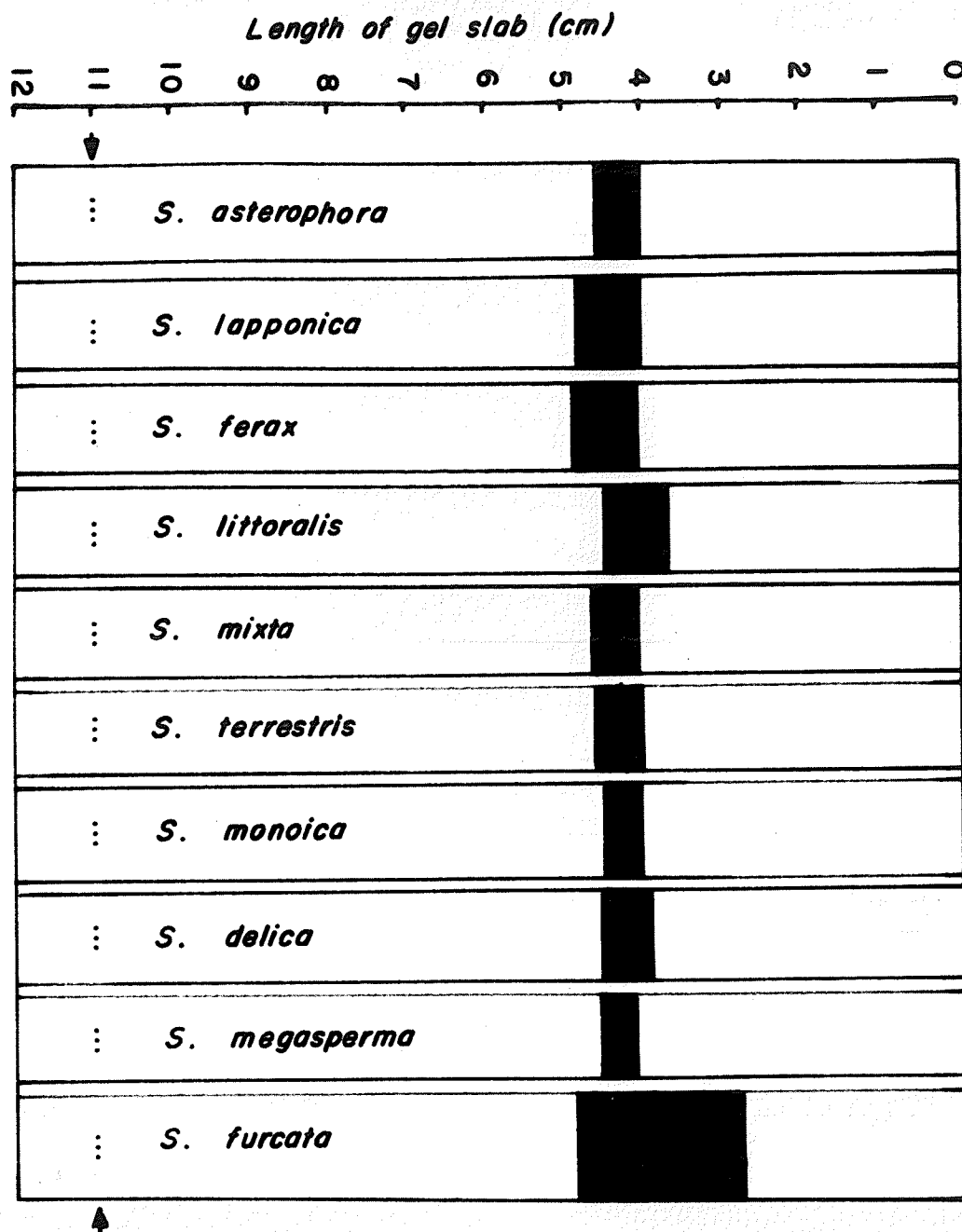


Fig. 2. Electrophoretic patterns of NADP-dependent isocitric dehydrogenase isozymes of 10 species of Saprolegnia. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend.



5.

Fig. 3. Electrophoretic patterns of NADP-dependent isocitric dehydrogenase isozymes of Aphanomyces, Aphanopsis, Thraustotheca and Leptolegnia spp. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

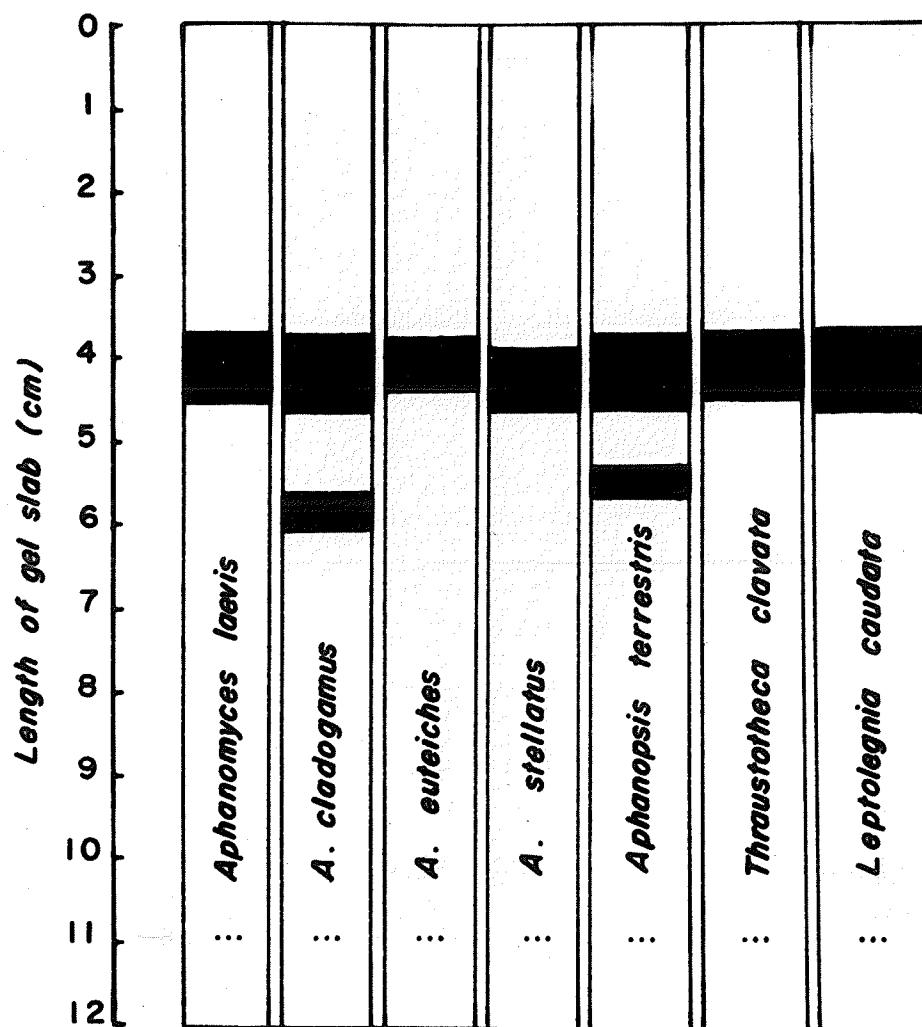


Fig. 4. Electrophoretic patterns of NADP-dependent isocitric dehydrogenase isozymes of some Saprolegniales (left panels) and Leptomitales (right panels). Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

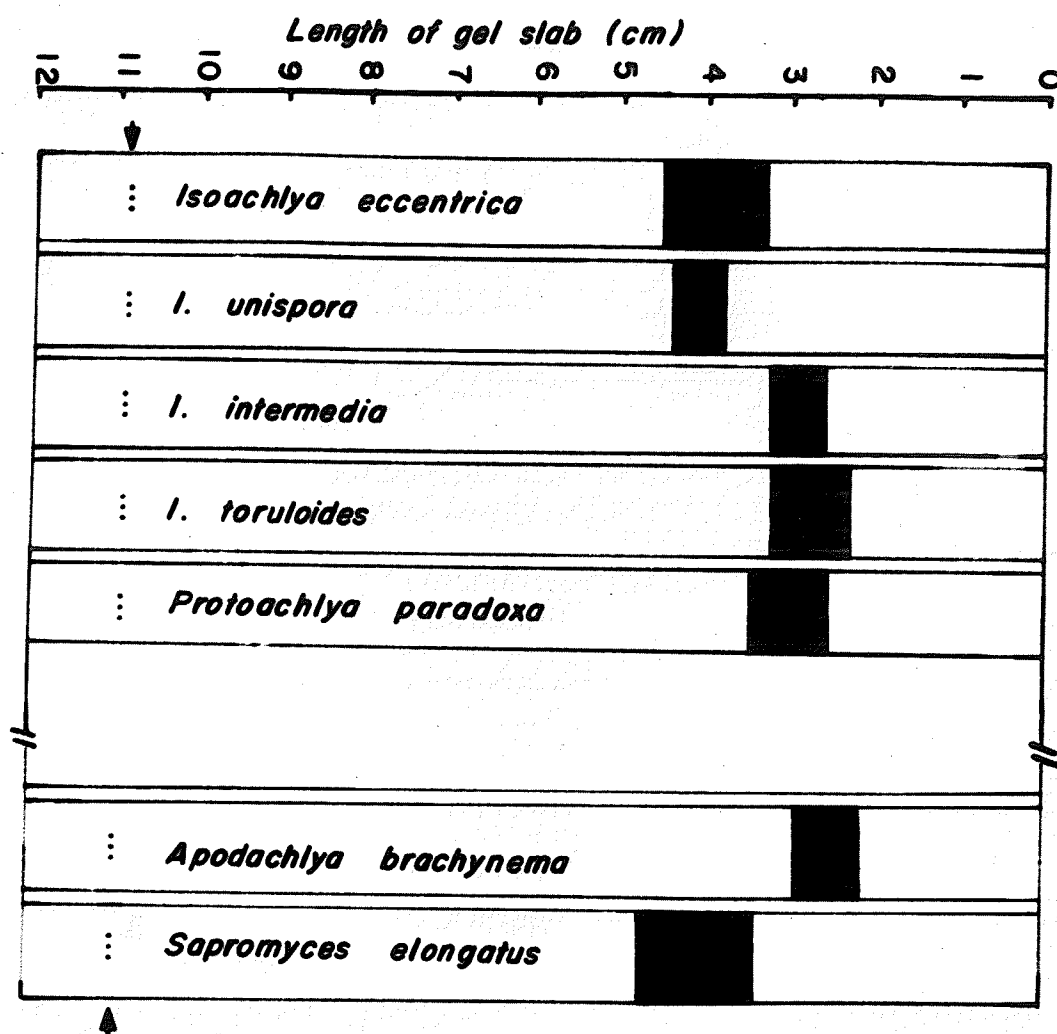




Fig. 5. Electrophoretic patterns of NADP-dependent isocitric dehydrogenase isozymes of 11 species of Pythium. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

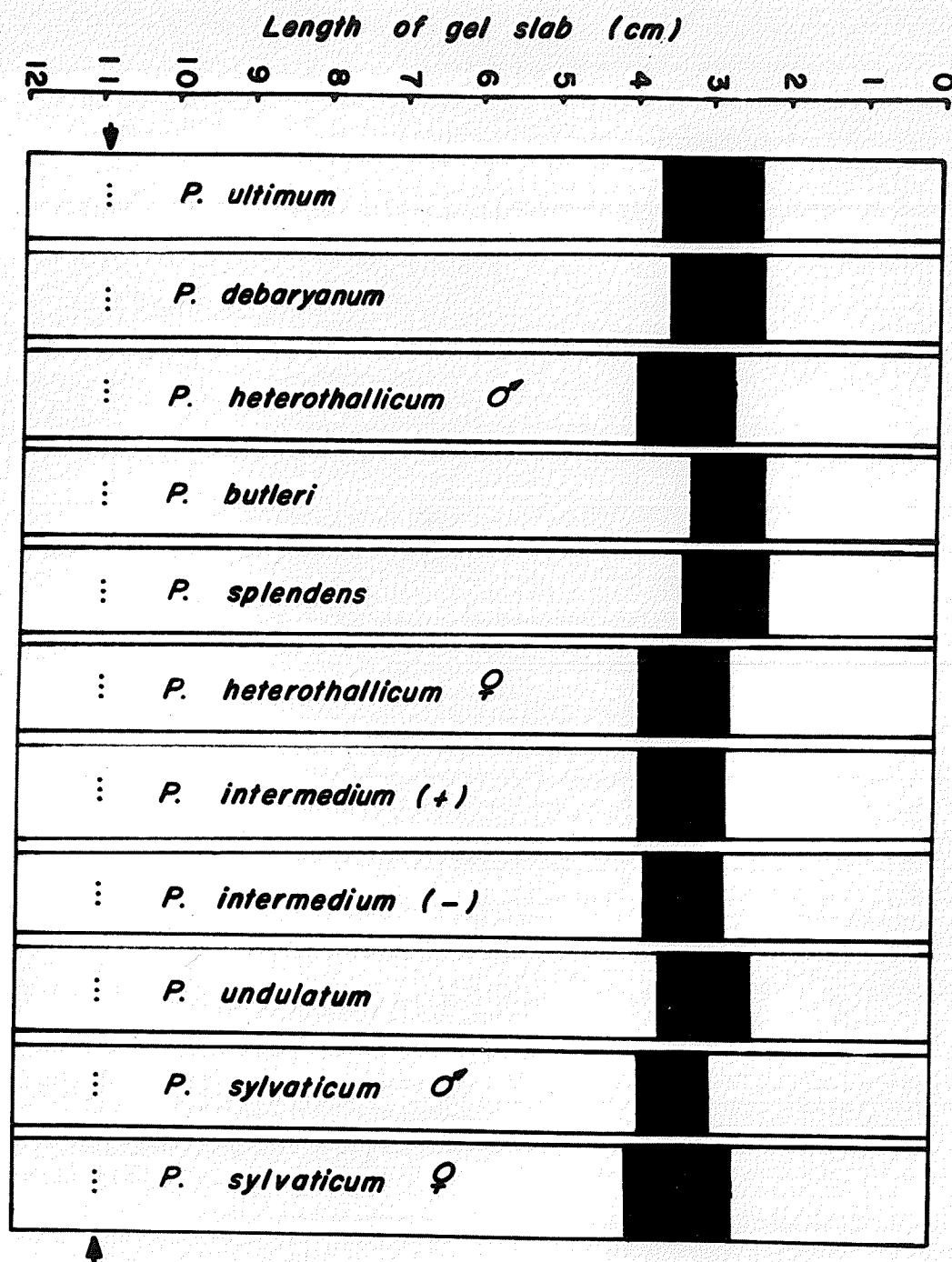


Table 1. Relative electrophoretic mobilities (R_e) of NADP-specific isocitric dehydrogenase isozymes of the Saprolegniales. Experimental procedure described in "Materials and Methods".

Organism	R_e (cm)
<u>Achlya ambisexualis</u> ♂	3.6
<u>A. ambisexualis</u> ♀	3.5
<u>A. americana</u>	3.6
<u>A. dubia</u>	4.1
<u>A. colorata</u>	3.7
<u>A. bisexualis</u> ♂	3.6
<u>A. bisexualis</u> ♀	3.6
<u>A. crenulata</u>	3.6
<u>A. intricata</u>	3.7
<u>A. flagellata</u>	3.9
<u>A. racemosa</u>	4.1
<u>A. radiosa</u>	4.5
<u>A. heterosexuais</u> ♂	3.3
<u>A. heterosexuais</u> ♀	3.2
<u>Saprolegnia asterophora</u>	4.3
<u>S. lapponica</u>	4.5
<u>S. ferax</u>	4.5
<u>S. littoralis</u>	4.1

Table 1. Continued

Organism	Re (cm)
<u>Saprolegnia mixta</u>	4.4
<u>S.</u> <u>terrestris</u>	4.3
<u>S.</u> <u>monoica</u>	4.3
<u>S.</u> <u>delica</u>	4.3
<u>S.</u> <u>megasperma</u>	4.4
<u>S.</u> <u>furcata</u>	3.9
<u>Isoachlya eccentrica</u>	4.0
<u>I.</u> <u>intermedia</u>	2.9
<u>I.</u> <u>unispora</u>	4.1
<u>I.</u> <u>toruloides</u>	2.8
<u>Protoachlya paradoxa</u>	3.0
<u>Aphanomyces laevis</u>	4.2
<u>A.</u> <u>cladogamus</u>	4.3 5.9
<u>A.</u> <u>euteiches</u>	4.1
<u>A.</u> <u>stellatus</u>	4.3
<u>Aphanopsis terrestris</u>	4.2 5.1
<u>Thraustotheca clavata</u>	4.1
<u>Leptolegnia candata</u>	4.2

Table 2. Relative electrophoretic mobilities (R_e) of NADP-specific isocitric dehydrogenase isozymes of Peronosporales and Leptomitales. Experimental procedure described in "Materials and Methods".

Organism	R_e (cm)
Peronosporales	
<u>Pythium ultimum</u>	3.0
<u>P. debaryanum</u>	2.9
<u>P. heterothallicum</u> ♂	3.3
<u>P. heterothallicum</u> ♀	3.3
<u>P. butleri</u>	2.7
<u>P. splendens</u>	2.8
<u>P. intermedium</u> (+)	3.3
<u>P. intermedium</u> (-)	3.3
<u>P. undulatum</u>	2.9
<u>P. sylvaticum</u> ♂	3.3
<u>P. sylvaticum</u> ♀	3.3
Leptomitales	
<u>Apodachlya brachynema</u>	2.5
<u>Sapromyces elongatus</u>	4.1

thallicum (male and female), P. intermedium (+) and (-), and P. sylvaticum (male and female). In fact, the R_e values for all 6 species were almost identical.

P. ultimum and P. debaryanum isozymes showed a similarity that has characterized these two species in all of the isozyme studies. P. ultimum glutamic dehydrogenase isozymes were identical to the isozymes of P. debaryanum (see Section II of thesis). They were distinctive among species of Pythium because they were two of five species that showed the existence of dual isozymes of glutamic dehydrogenase in each species, and only these two had coincident isozyme pairs. Similarly, P. butleri and P. splendens isocitric dehydrogenase isozymes are coincident as we saw for their glutamic dehydrogenase isozymes (Section II).

In many ways then, the similarity that is demonstrated between species at one level extends to other areas as well. Whether the close metabolic link between the isocitric and glutamic dehydrogenases is the reason for this physical correlation is difficult to determine at this point. This is a plausible motion because the electrophoretic properties of the D(-)lactic dehydrogenases of these same species differ considerably (Section III).

Malic Dehydrogenase Isozymes

The isozyme patterns for malic dehydrogenases of the Oomycetes produce the most complex patterns of all. Not only do we find a wide variation in the electrophoretic mobilities, but each genus has species that contain multiple forms of isozymes.

The genus Achlya. The only features of consistency that are demonstrated in the isozyme patterns shown in Fig. 6 and Table 3 are as follows. (a) The mating types A. ambisexualis (male and female) have corresponding isozyme pairs which differ from the matched forms for A. heterosexalis (male and female). (b) The isozyme pairs for A. bisexualis (male and female) do not correspond and show some significant differences. (c) None of the other species contain isozymes whose mobility patterns correspond. (d) Only A. racemosa did not show the typical dual isozymic forms characteristic of the other species of Achlya. Whether this is because they have close electrophoretic mobilities and are not separable as such is difficult to determine. It is also entirely possible that some of the broad isozyme bands of some species contain two or more species that cannot be easily resolved by this technique. The reason for

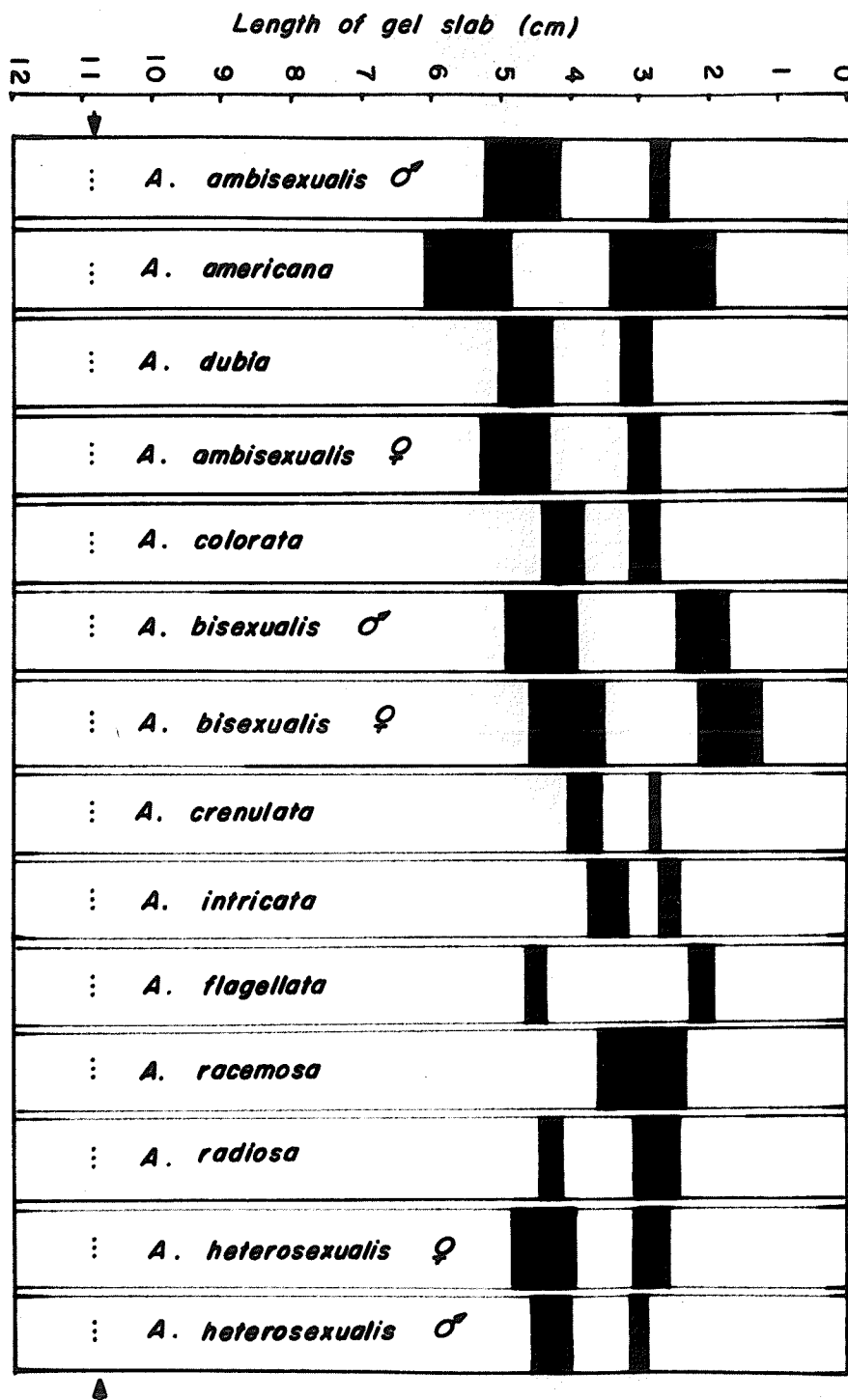
this conjecture is seen in the next series of electrophoretic analyses.

The genus Saprolegnia. Again, the existence of multiple forms of malic dehydrogenase isozymes in certain species becomes manifest in the genus Saprolegnia (Fig. 7 and Table 3). In one case, that of S. delica, 3 isozymes are detected. A very different situation is recorded for four other species, S. ferax, S. mixta, S. terrestris, and S. furcata. Wherein only a single isozyme species with close electrophoretic mobilities is detected in each case. Typically, the isozyme bands are very broad, in contrast to the width of the bands for the other species - again raising the possibility that multiple isozymes may exist but they are not resolvable under these conditions.

The genus Aphanomyces and Others. In the genus Aphanomyces, we have the first and only case in this study where five malic dehydrogenase isozymes exist in a single species (Fig. 8 and Table 3). A variety of one to five isozymes are found in four Aphanomyces spp. The single Aphanopsis sp. examined has only one isozyme.

Very little correlation could be envisaged between the isozyme patterns of these species for Thraustotheca and Leptolegnia malic dehydrogenase isozymes display no correspondence with any of the others as well.

Fig. 6. Electrophoretic patterns of malic dehydrogenases isozymes of 14 species of Achlya. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.



9.

Fig. 7. Electrophoretic patterns of malic dehydrogenase isozymes of 10 species of Saprolegnia. Electrophoresis was 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

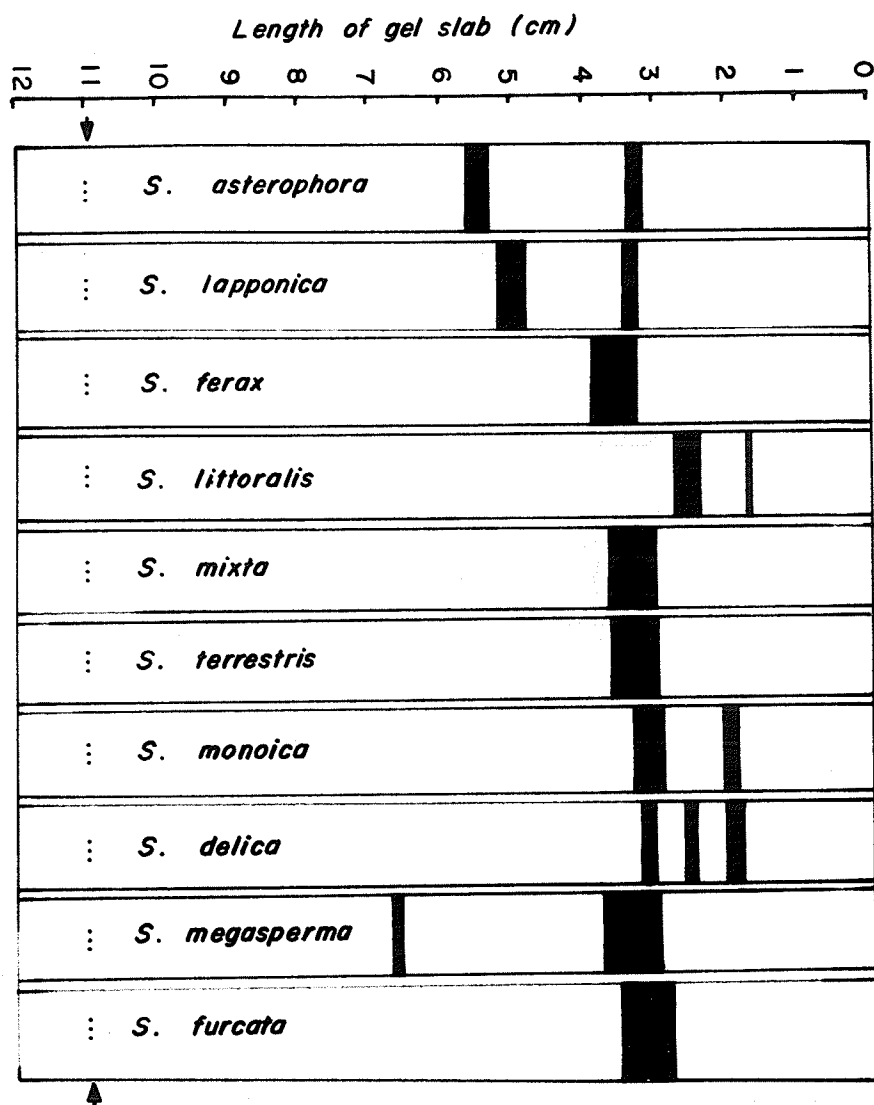


Table 3. Relative electrophoretic mobilities (Re) of Malic dehydrogenase isozymes of the Saprolegniales. Experimental procedure described in "Materials and Methods".

Organism	Re	(cm)
<u>Achlya ambisexualis</u> ♂	2.8	4.8
<u>A. ambisexualis</u> ♀	3.0	4.9
<u>A. americana</u>	2.8	5.6
<u>A. dubia</u>	3.1	4.2
<u>A. colorata</u>	3.0	4.2
<u>A. bisexualis</u> ♂	2.1	4.5
<u>A. bisexualis</u> ♀	1.7	4.2
<u>A. crenulata</u>	2.8	3.9
<u>A. intricata</u>	2.6	3.5
<u>A. flagellata</u>	2.1	4.5
<u>A. racemosa</u>	3.0	-
<u>A. radiosa</u>	2.7	4.3
<u>A. heterosexuais</u> ♀	2.8	4.4
<u>A. heterosexuais</u> ♂	3.0	4.3
<u>Saprolegnia asterophora</u>	3.3	5.5
<u>S. lapponica</u>	3.4	5.1
<u>S. ferax</u>	3.7	-
<u>S. littoralis</u>	1.7	2.6

Table 3. Continued

Organism	Re	(cm)
<u>Saprolegnia mixta</u>	3.4	
<u>S. terrestris</u>	3.4	
<u>S. monoica</u>	2.0	3.2
<u>S. delica</u>	2.0	2.6 3.2
<u>S. megasperma</u>	3.5	6.7
<u>S. furcata</u>	3.2	
<u>Aphanomyces laevis</u>	1.4	
<u>A. cladogamus</u>	0.7	1.0 3.2 3.6 3.9
<u>A. euteiches</u>	1.8	4.1
<u>A. stellatus</u>	1.8	3.2 4.1
<u>Aphanopsis terrestris</u>	4.1	
<u>Thraustotheca clavata</u>	2.4	4.5
<u>Leptolegnia candata</u>	1.2	2.7
<u>Isoachlya eccentrica</u>	4.2	
<u>I. unispora</u>	4.2	
<u>I. intermedia</u>	3.6	4.2
<u>I. toruloides</u>	3.6	4.2
<u>Protoachlya paradoxa</u>	2.7	5.1

The genera Isoachlya and Protoachlya. As in the case of isocitric dehydrogenase isozymes, the malic dehydrogenase patterns for the four species of Isoachlya correspond in pairs. I. eccentrica and I. unispora have single isozyme species with coincident electrophoretic mobilities. I. intermedia and I. toruloides each have a pair of isozymes that were also coincident in their mobilities. Isozymes of Protoachlya were very different from those of Isoachlya as shown in Fig. 9 and Table 3 (left panel).

The two species of the Leptomitales, Apodachlya brachynema and Sapromyces elongatus have malic dehydrogenase isozyme patterns that, like all other features studied, were different. Whereas A. brachynema displayed 2 isozyme species, S. elongatus appeared to have only one form (Fig. 9, right panel and Table 4). A firm conclusion could not be reached on the nature of the enzyme from Sapromyces because of the fact that it did not migrate significantly from the anode and the activity band was so broad.

The genus Pythium. Malic dehydrogenase isozymes of Pythium spp. are shown in Fig. 10 and Table 4. A close relationship can be noted between the enzymes of P. ultimum and P. debaryanum. In all respects, then, except for LDH

Fig. 8. Electrophoretic patterns of malic dehydrogenase isozymes of Aphanomyces, Aphanopsis, Thraustotheca and Leptolegnia spp. Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.

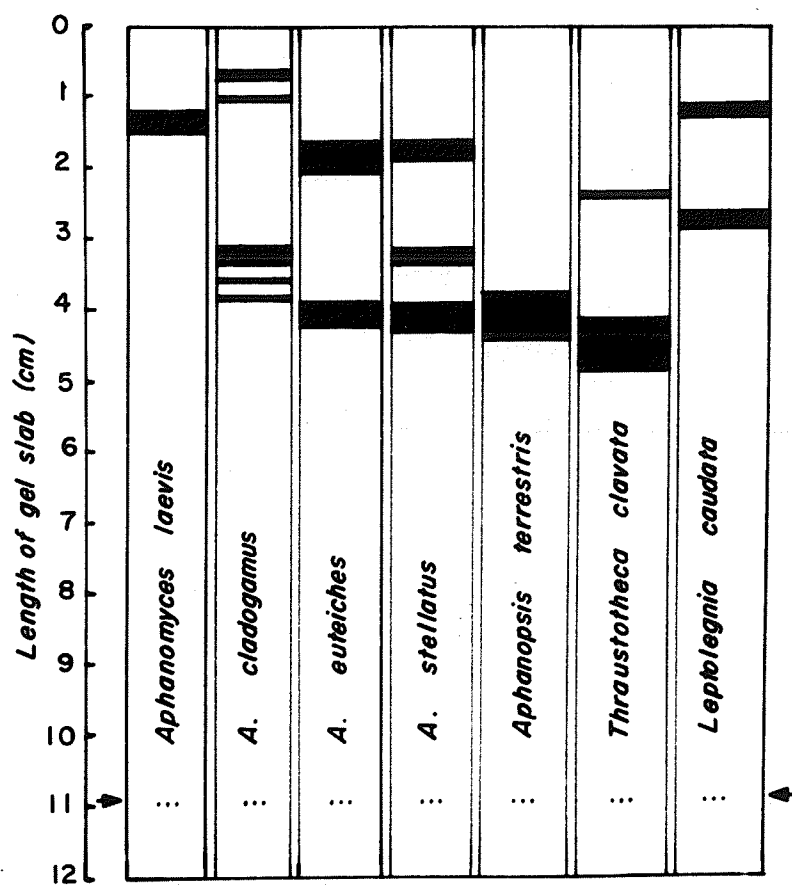
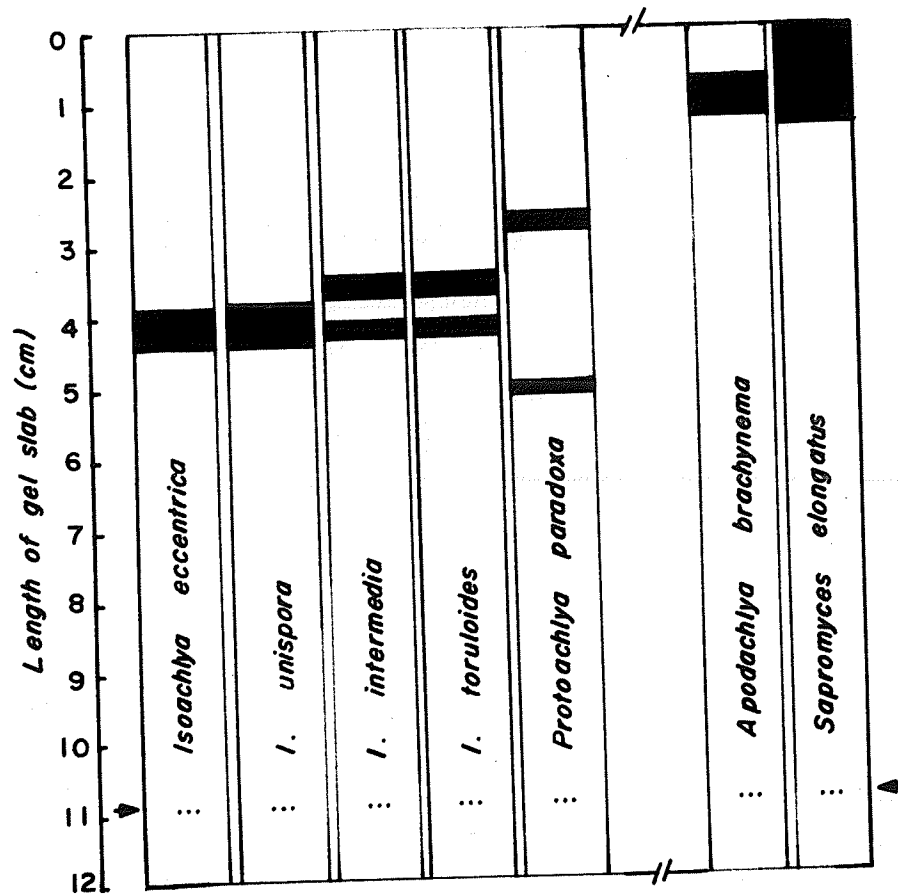


Fig. 9. Electrophoretic patterns of malic dehydrogenase isozymes of some Saprolegniales (left panels) and Leptomitales (right panels). Electrophoresis was for 4 hr at 10°C in a buffer described in Fig. 1 legend. Arrows indicate position of tracking dye.



isozymes, indications are that these two species are very closely-related.

The mating types of *Pythium* have malic dehydrogenase isozymes that are closely related with each other. All species contained two malic dehydrogenase isozymes. Unlike the species of the Saprolegniales, the isozymes of the Peronosporales did not show very great disparity in their relative mobilities which indicates they are possibly more closely-related as a group.



Fig. 10. Electrophoretic patterns of malic dehydrogenase isozymes of 11 species of Pythium. Electrophoresis was for 7 hr at 10°C in a buffer composed of 34.55 g Tris, 5.25 g glycine, pH 9.3 and H₂O to 1 litre. Twenty ml of buffer added to gel solution. Arrows indicate position of tracking dye.

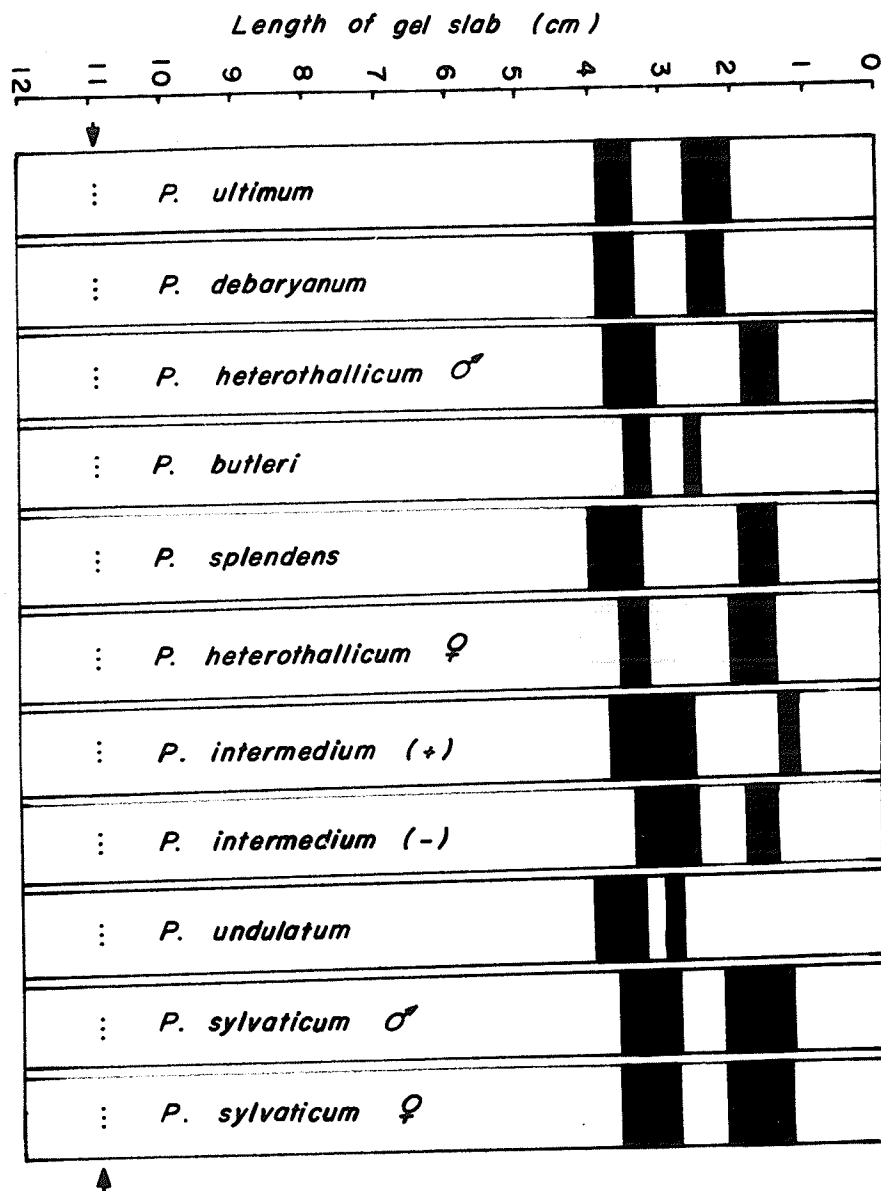


Table 4. Relative electrophoretic mobilities (Re) of malic dehydrogenase isozymes of the Peronosporales and Leptomitales. Experimental procedure described in "Materials and Methods".

Organism	Re	(cm)
Peronosporales		
<u>Pythium ultimum</u>	2.4	3.7
<u>P. debaryanum</u>	2.4	3.7
<u>P. heterothallicum</u> ♂	1.6	3.5
<u>P. heterothallicum</u> ♀	1.8	3.4
<u>P. butleri</u>	2.6	3.4
<u>P. splendens</u>	1.7	3.7
<u>P. intermedium</u> (+)	1.3	3.2
<u>P. intermedium</u> (-)	1.7	3.0
<u>P. undulatum</u>	2.9	3.7
<u>P. sylvaticum</u> ♂	1.7	3.3
<u>P. sylvaticum</u> ♀	1.7	3.3
Leptomitales		
<u>Apodachlya brachynema</u>	1.0	
<u>Sapromyces elongatus</u>	0.8	

DISCUSSION

Because of the existence of multiple bands of malic dehydrogenases in the Oomycetes, and because of the extreme variation in the isozyme patterns, no clear-cut conclusions regarding speciation among this fungal population based on this parameter could be obtained. This difficulty becomes more pronounced since we do not know of any useful system of enzyme regulation that could be used as a second distinguishing feature.

No doubt the malic dehydrogenases represent both mitochondrial and cytosol types. Where only one isozymic species is detected, we must assume that either one of the two types has not been solubilized or else that it is unstable under the conditions of analysis. The third explanation which has already been elaborated upon is that the isozymes have not been separated by electrophoresis.

The remarkable correlations detected for the 4 species of Isoachlya should prompt more investigation into further characterization of these species.

Even in the studies on the isozymes of glutamic dehydrogenase (Section III of thesis) we had seen that Aphanomyces cladogamus and Aphanopsis terrestris have a

close affinity. We find more support for this belief in the isozyme patterns obtained for malic and isocitric dehydrogenases of these same two species. It is unlikely that it is pure coincidence to find that these two species are the only ones with two isozymes of isocitric dehydrogenase of all 49 Oomycetes species examined. The only exception is that there is a significant difference in the LDH isozymes of these two species which could be accounted for if a mutation has affected the structure of one of these enzymes. This then, is the only reasonable instance we have in these studies in which we can use electrophoretic property to detect a moderate form of speciation in the same genus.

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