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ALLOZYME AND BASIDIOSPORE
CONTRIBUTIONS TO
PHYLOGENETIC STUDIES OF *AGARICUS* SPECIES.

A thesis
submitted in partial fulfillment
of the requirements for
the degree of
Doctor of Philosophy in Biochemistry
and Microbiology
at
Lincoln University
Canterbury, New Zealand
by
Anthony Mitchell

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1993

Abstract.

Abstract of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ALLOZYME AND BASIDIOSPORE CONTRIBUTIONS TO PHYLOGENETIC STUDIES OF *AGARICUS* SPECIES.

by Anthony Mitchell.

Department of Biochemistry and Microbiology.

A review of literature on the classification of the genus *Agaricus* showed a great deal of confusion exists regarding relationship between species. Traditional methods used in mycological systematics have been inadequate to resolve many problems found when classifying this genus. Thus recourse to new techniques are required.

As a result, methods were evaluated for the study of the allozymes of *Agaricus* species. Methods investigated included use of polyacrylamide gel electrophoresis and horizontal starch gel electrophoresis, composition of extraction buffers, quantity of mycelium needed for extraction, electrophoretic buffer systems, and staining systems. The method arrived at enabled successful resolution of 10 mycelial enzymes encoded by twelve polymorphic putative loci, Aat-1, Acp, β Glu, Gk-1, Gk-2, Gpt, Ha-1, Lap-1, Mdh-1, Mdh-2, Mpi, and Pgm-2.

An evaluation of allozymes from mycelium and basidioma parts revealed that additional allozyme information was available when more than one *Agaricus* tissue was tested. Mycelium was chosen because it could be produced under standardised and controlled culture conditions. From a comparison of shaken and stationary liquid culture, shaken culture was chosen because it allowed production of more mycelium at a faster rate. Of the conditions tested for enzyme preservation, storage at -80°C , of mycelium and extracted mycelial supernatant was successful for at least three months, and storage of

freeze dried mycelium was successful for two years at that temperature.

Depending upon species, the levels of mycelial enzyme activity may be influenced by culture method and period of incubation, and different electromorphs may be revealed by changing culture conditions. It was concluded that for allozyme analysis, a single standardised method should be used to grow, extract and store mycelium for the strains representative of the species investigated. Experiments also showed that for uniformity, mycelia should be of a similar stage of physiological development. It was considered that by decreasing differences due to cultural and electrophoretic methods, mycelial allozyme analyses and phylogenetic investigations based on such analyses will be improved.

The twelve putative loci, from eleven *Agaricus* species were successfully used as a source of data for exploring cladistic relationships of *Agaricus* species based upon Phylogenetic Analysis Using Parsimony (PAUP). *Leucoagaricus leucothites* and *Agrocybe parasitica* were used as outgroups for analysis. The results supported the contention that the traditional Section Bitorques, and the Section Agaricus, Group Campestris were each monophyletic. The monophyly of these traditional Sections was also supported.

Using computer image analysis basidiospore characters were measured with a greater speed and objectivity than has been possible using the light microscope. This method allowed the analysis of new variables, including area, and circularity, as well as length, breadth and elongation. PAUP was used to analyse the results obtained from the same eleven *Agaricus* species, again using *Leucoagaricus leucothites* and *Agrocybe parasitica* as outgroups. These analyses supported the phylogenetic usefulness of *Agaricus* basidiospores, and emphasised the need for the use of additional characters to improve the classification of this genus.

Key words; *Agaricus*, allozyme, basidiospore, classification, horizontal starch gel electrophoresis, image analysis, phylogenetic analysis, polyacrylamide gel electrophoresis, species.

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Definitions.

Allozyme: One of several forms of an enzyme coded for by different alleles at a locus.

Apomorphy: A character derived from its pre-existing generalised condition.

Electromorph: Allozyme information is not the same as studying DNA information directly, (see putative locus), thus the term electromorph is used in preference to allele.

Enzyme: A protein which is a catalyst, directly promoting only a limited range of reactions.

Homologous: Characters having a common origin.

Homoplasy: Agreement in the analogous rather than the homologous characters.

Isolate: Isolate was used to describe the culture derived directly from a field collected specimen.

Isozyme: One of several forms of an enzyme, being the product of more than one structural gene coding for a particular enzyme, and including all polymers of subunits produced by different gene loci or different alleles at the same locus,

Mycelium: The term mycelium was used to describe vegetative mass(es) of hyphae.

Nomenclature used to refer to enzymes and putative loci: The first letter of an enzyme's abbreviation was capitalised when this referred to the locus of the gene coding for that enzyme, e.g., acid phosphatase ACP, the ACP locus Acp. Hyphenated numerals referred to multiple loci, numbered from most cathodal to most anodal, e.g., loci for malate dehydrogenase MDH are given as Mdh-1, and Mdh-2. Electromorphs at a locus have been given a numeral based on their mobility from the

origin, from the most cathodal to the most anodal, e.g., Acp: 1, Acp: 2, Est-1: 1, Est-1: 2.

Putative locus: Because the determination of loci using allozymes is putative, the term putative loci may be used in preference to the term loci.

Strain: An isolate that had been asexually propagated and maintained in culture by serial transfer was termed a strain.

Synapomorphy: A character shared among a group of organisms which is found in their common ancestor and thought to have originated in that ancestor is termed synapomorphic (shared apomorphy).

Tissue(s): All macroscopic fungal structures consisting of aggregates of hyphae.

Abbreviations.

Chemicals.

ATP Na ₂	adenosine 5'-triphosphate
LDH	L-lactate dehydrogenase
MnCl ₂	Manganese(II) chloride 4-hydrate
MEA	Malt Extract Agar
MEB	Malt Extract Broth
MgCl ₂ · 6H ₂ O	Magnesium chloride 6-hydrate
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide; Thiazolyl blue)
MnCl ₂	Manganese(II) chloride 4-hydrate
Na	Sodium
NAD ⁺	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADP ⁺	β-nicotinamide adenine dinucleotide phosphate
NaEDTA	Ethylenediaminetetraacetic acid (sodium salt)
NaOH	Sodium hydroxide
Na phosphate	Sodium dihydrogen orthophosphate, anhydrous
PGI	Phosphoglucose isomerase
PMS	phenazine methosulfate
PVP	polyvinyl-pyrrolidone
TEMED	N, N, N, N-tetramethylethylenediamine
Tris	2-Amino-2(hydroxymethyl)-1,3-propanediol. crystallised.

Other.

CI	consistency index
g	gram
h.	hour
m	metre
μg	microgram
mA	milliamps
mg	milligram
min.	minute
μl	microlitre
ml	millilitre
mm	millimetre
Std	standard deviation
°C	degree Celsius
V.	volts

List of Chemicals.

All chemicals used were analytical grade where available.

Purchased from the Sigma chemical company, P.O. Box 14508, St. Louis, MO USA 63178-9916.

L-Alanine
 L-Amino acid oxidase (Type I; from venom of diamond back snake)
 N-(3-Aminopropyl)-morpholine
 ATP (Na₂) (adenosine 5'-triphosphate standard disodium salt)
 Bovine serum albumin
 N, N-Dimethylformamide-d₇
 Fast Blue BB Salt (purified grade, dye content minimum 80 %)
 Fast Blue RR Salt (crystalline)
 Fast Garnet GBC Salt (practical grade)
 Fructose-6-phosphate (disodium salt)
 Glucose-1-phosphate (disodium salt: hydrate, approximately 95 % (Grade VI)
 Glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*)
 Glyceraldehyde-3-phosphate dehydrogenase (porcine muscle)
 Glycyl-leucine
 α-Ketoglutaric acid (disodium salt 98%)
 LDH (L-lactate dehydrogenase)
 L-Leucine-β-naphthylamide (free base)
 β-D-Mannose-6-phosphate (barium salt)
 2-Mercaptoethanol (for electrophoresis. 98 % purity)
 4-Methylumbelliferyl β-D-xyloside
 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide
 4-Methylumbelliferyl β-D-glucoside
 MTT ((3-(4,5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide;
 Thiazolyl blue)
 NAD⁺ (β-nicotinamide adenine dinucleotide)
 NADH (β-nicotinamide adenine dinucleotide, reduced form)
 NADP⁺ (β-nicotinamide adenine dinucleotide phosphate)
 α-Naphthyl butyrate
 α-Naphthyl phosphate (disodium salt, crystalline)
 Peroxidase (crude form from horseradish)
 PGI (phosphoglucose isomerase: Type III from bakers yeast)
 D(-)-3-phosphoglyceric acid (disodium salt, crystalline)
 PMS (phenazine methosulfate)
 PVP (polyvinyl-pyrrolidone, soluble and insoluble forms)
 Pyridoxal-5-phosphate (approximately 98 %)
 Riboflavin
 (-) shikimic acid (crystalline)
 Starch, (potato hydrolysed for electrophoresis)
 TEMED (N, N, N, N-tetramethylethylenediamine)

Purchased from BDH Laboratory Supplies, Poole, Dorset, England. BH15 1TD.

Acetone
 Ammonium persulphate (peroxodisulphate)
 L-Aspartic acid
 Boric acid

— Bromophenol blue
 Citric acid
 Cysteine
 Dianisidine
 Dithiothreitol
 Ethanol (99.7-100 %)
 Formic acid (98-100 %)
 D-Glucose
 Glycine
 DL-Isocitric acid (trisodium salt)
 Lithium hydroxide
 Magnesium choride 6-hydrate
 Maleic acid
 DL-Malic acid
 Manganese(II) choride 4-hydrate
 N, N-Methylene bisacrylamide (for electrophoresis)
 Sodium acetate anhydrous
 Sodium dihydrogen orthophosphate, anhydrous
 Sodium hydroxide pellets
 Succinic acid

**Purchased from Oxoid. InterMed NZ Ltd., P.O. Box 33-268,
 Takapuna, Auckland.**

Malt extract agar
 Malt extract broth

**Purchased from SERVA. Scientific Supplies Ltd., P.O. Box 14-
 454, Auckland.**

Acrylamide

**Purchased from Boehringer Mannheim NZ Ltd. P.O. Box 26-051,
 Christchurch.**

NaEDTA (ethylenediaminetetraacetic acid, sodium salt)
 Tris (2-Amino-2(hydroxymethyl)-1,3-pripanediol. crystalline)

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Chapter 1.

General introduction.

The Eumycota or fungi, are an ancient group of organisms first reported along with the earliest land plants in Cambrian fossils, (Stevenson 1982a). The Eumycota have been divided into 5 Subdivisions by Hawksworth et al. (1983). The Subdivision Basidiomycotina, Class Hymenomycetes has in it approximately 750 genera, which have been divided into two Subclasses, namely the Phragmobasidiomycetidae, and the Holobasidiomycetidae, (Hawksworth et al. 1983). In the classification adopted by these authors there are nine orders in the Subclass Holobasidiomycetidae, including the order Agaricales Clements. The order Agaricales contains the type family Agaricaceae Cohn ss. Singer, which includes the type tribe Agariceae Patouillard, accommodating the type genus *Agaricus* L.: ex Fr.ss. Karst, (Cappelli 1984), which was officially recognised in the International code of Botanical Nomenclature (1972 p. 254.).

Traditionally, the classification of *Agaricus* species has been based upon morphological characters, (Heinemann 1978, Wasser 1980, Cappelli 1984), the genetic basis for which is often difficult to determine, (Cappelli 1984). The importance given to many of the more subtle morphological characters varies between mycologists, and is reflected in the large number of rearrangements of taxa that have occurred within the genus *Agaricus*, (Cappelli 1984). This genus has not been investigated using phylogenetic methods, and the latest classification of *Agaricus* species by Cappelli (1984) reflects an unknown quantity of phylogenetic information. For phylogenetic

analysis, the development of new characters and re-examination of existing ones is needed if there is to be worthwhile reappraisal of *Agaricus* classification.

In this thesis the technique of allozyme analysis, proposed by Richardson et al. (1986), Murphy et al. (1990), and May (1992) as a practical approach to generating phylogenetically useful information, has been investigated as one means of collecting data for phylogenetic study of *Agaricus* species. Many experimental procedures used for allozyme analysis of *Agaricus* species have not been fully evaluated, and standardisation of many of the techniques is necessary to at least decrease or at best eliminate sources of variability. Following the optimisation of methods the results of allozyme analyses of strains representative of eleven *Agaricus* species are reported and evaluated, and cladistics were used to explore the relationships of these species.

In addition to evaluating the usefulness of allozymes, another recently devised technique, image analysis, (Vezey et al. 1991, Cox and Thomas 1992), was used to obtain data on the morphology of basidiospores from *Agaricus* species. Computerised image analysis was investigated as a means of decreasing the time required for basidiospore measurement compared with the use of light microscopy, and as a means of increasing the numbers of variables measured. Cladistic methods were used to explore the phylogenetic usefulness of the basidiospore characters recorded to improve the definition of relationships between *Agaricus* species.

Finally some general conclusion have been drawn about the systematics of this scientifically and commercially important genus.

Chapter 2.

Literature review.

2.1. Introduction.

This investigation covered many disciplines, including systematics, traditional methods of classifying *Agaricus* species, and techniques of microbiology and biochemistry. It has therefore been necessary to review a wide range of literature in this Chapter, including;

- 1) principles of systematics, section 2.2,
- 2) development of *Agaricus* classification based on traditional methods, section 2.3,
- 3) basidiospore characters in *Agaricus* systematics, section 2.3,
- 4) the need for new methods and new characters, section 2.3,
- 5) principles of isozyme electrophoresis, and the application of this to *Agaricus* species, section 2.4, and 2.5,
- 6) the treatment of allozymes in phylogenetic studies, section 2.6,
- 7) the importance of morphology and allozymes as sources of comparative characters, section 2.7.

2.2. Main systematic philosophies.

According to Wiley (1981), systematics is the study of organismic diversity, involving the organisation of biological information or characters of organisms, with one or more character states. He contended that systematics is concerned with the use of characters to classify organisms into species, which are then combined into larger groups. The traditional classification convention is that of Linnaean hierarchy, in which the ultimate

category, the species, is successively grouped into genera, families, orders, classes, phyla, with intercalated categories such as subfamilies or subgenera. In addition to classification a major role of systematics is to determine relationships among groups of organisms or taxa, (Wiley 1981). The interpretation of the concept of relationship separates systematists into three philosophical schools, which are; 1) synthetic systematics, 2) phenetics, and 3) phylogenetic systematics. The way systematists view relationships may lead them to create different classifications of the same organisms, (Wiley 1981).

2.2.1. Synthetic systematics.

Synthetic systematics is based on the evolutionary history or phylogeny of organisms, but selectively incorporates other criteria, including overall similarity and habitat, into the classification system (Mayr 1942, Mayr et al. 1953, Simpson 1961, Mayr 1969). Synthetic systematists base their classifications on monophyletic groups, defined by them as groups of species evolved from a common ancestor, but not necessarily including all descendants from that ancestor, (Wiley 1981). For example, birds are excluded from the Reptilia, despite the fact that they are more closely related to crocodiles than crocodiles are to other reptiles (Wiley 1981).

For the evaluation of phylogeny, clear distinction has been made by synthetic systematists, (Mayr 1942, Mayr et al. 1953, Simpson 1961, Mayr 1969) between homologous characters, inherited with variable modification from a common ancestor, and analogous characters, bearing superficial similarity only.

— However, this distinction was considered subjective, by Sokal and Sneath (1963) and lead to the formulation of the phenetics.

2.2.2. Phenetics.

Pheneticists give equal value to every character and group organisms on their overall shared characters, (Sokal and Sneath 1963, and Sokal and Camin 1965, Sneath 1983). They do not distinguish between homologous and analogous characters, and may incorporate groups made up of descendants of unrelated ancestors in to their classifications, (Wiley 1981). Thus the groups of species included in a phenetic classification may not reflect phylogeny at all. The failure of phenetics to take account of evolutionary implications encouraged a return to methods that attempted to depict evolutionary history, (Heywood 1984), and resulted in the development of phylogenetics.

2.2.3. Phylogenetics.

This school, holds that phylogenetic inferences should be made based on the following axioms, (Wiley 1975): “(1) evolution occurs; (2) only one phylogeny of all living and extinct organisms exists, and this phylogeny is the result of genealogical descent; (3) characters may be passed from one generation to the next generation, modified or unmodified, through genealogical descent.”

Hennig (1966), in his development of phylogenetic principles, relied on the determination of homologous characters, and the separation of these characters into those that were primitive or plesiomorphic, and those that were derived or apomorphic. Phylogenetics relies on the technique whereby only

apomorphies shared by two or more species or synapomorphies are used to determine monophyletic groups of species (Hennig 1966). The same author described a group which did not include all descendants of an ancestor as paraphyletic. Thus what a phylogeneticists would refer to as a paraphyletic group defines what a synthetic systematist would consider monophyletic, (Wiley 1981).

Investigation of phylogenetic relationship at the species level was considered inappropriate by Hennig (1966), because at this level synapomorphy no longer defines monophyly. Hennig (1966), also contended that apomorphies which occur in a single taxon or autapomorphies, and shared plesiomorphic or symplesiomorphic characters, do not supply any phylogenetic information at all. According to phylogenetics, as presented by Hennig (1966), only monophyletic groups can form the basis of classifications which reflect the phylogeny of the taxa classified. A phylogeny may be depicted in a phylogenetic tree, defined by Wiley (1981) as "a branching diagram portraying the hypothesised genealogical ties and sequence of historical events linking individual organisms, populations or taxa".

Different phylogeneticists have produced different classifications of the same taxa because of the particular conventions they apply, (Wiley 1981). One such convention is termed subordinated classification, which names each branch point or level on a phylogenetic tree as a new rank in a classification, (Cracraft 1974). A subordinated classification from an asymmetrical phylogenetic tree may require a large number of

categories of organisms, but the number may be reduced if phyletic sequencing is applied, (Nelson 1972, 1974). In phyletic sequencing every branch point on a phylogenetic tree is not named, (Wiley 1981). Instead this method minimises the number of categories of organisms by grouping monophyletic groups together and listing them according to their branching sequence on the phylogenetic tree, (Wiley 1981).

Problems associated with the phylogenetic theory include homoplasies, defined by Hennig (1966), as apomorphies inconsistent with other apomorphies in their inference of monophyly. Many different methods have been developed to limit the problems associated with homoplasies, including maximum parsimony or cladistics, (Kluge and Farris 1969, Farris 1970, Fitch 1971), compatibility analysis, (Meacham and Estabrook 1985), and the statistical phylogenetic technique of maximum-likelihood estimation (Farris 1973, Felsenstein 1973). The most widely applied phylogenetic method has been cladistics, possibly because of the ready availability of computer programmes for carrying out cladistical techniques (Stewart 1993).

2.2.4. Choice and justification of systematic philosophy.

Justification for the use of one school of systematics over another reflects first the goals of the systematist, and second the systematists' views as to what constitutes an acceptable relationship. If a systematist's goal is to create a classification that reflects phylogeny, then the choice would be a system based on phylogenetic systematics, allowing estimates of relationship

acceptable to the systematist. Phylogeneticists, (Kluge and Farris 1969, Farris 1970, and Fitch 1971) have minimised the occurrence of homoplasies through cladistic methods which may be used to explore the data and by identifying weaknesses in the data, aid in redirecting study.

Justification for the use of cladistics over other phylogenetic methods has been based on the theory that it is an application of Popperian philosophy (e.g., Gaffney 1975, Wiley 1975). It is possible to test a cladistic classification by the analysis of synapomorphies in addition to those already used (Wiley 1975, Gaffney 1979). However, Bryant (1989) has argued that the role of synapomorphies was not to test a cladistic classification. To him synapomorphies represented the data base for the generation of a cladistic hypothesis.

It may be concluded that, while the use of cladistics may not be justified simply by claiming it is Popperian, this method still may be a logical choice over other systematic methods available. This is because cladistics is a useful technique for data exploration, when an analysis is required from which inferences may be made regarding common ancestry, evolutionary patterns, and monophyletic groups, (Wiley 1975).

2.2.5. Cladistics.

The theory of cladistics has evolved considerably from a 'minimum steps evolution method', developed by Camin and Sokal (1965), which decreased data inconsistencies by minimising the overall amount of evolutionary change needed to explain the

available data. Cladistics was further developed by Kluge and Farris (1969), Farris (1970), and Fitch (1971) into what became known as Fitch and Wagner parsimony or parsimony, a system which permitted character reversibility. Dollo parsimony, (Farris 1977) was also derived from cladistics, and only allowed each apomorphic character state to have a single origin, not permitting an apomorphy to originate via parallel evolution or convergence, and did not allow a character state to be lost and then regained. The applicability of one method of cladistics over another depends on the form of data being collected, and this topic will be further reviewed later in this chapter.

Cladistic analysis generates cladograms or trees, defined by Wiley (1981) as “a branching diagram(s) of entities based on the inferred historical connections between the entities as evidenced by synapomorphies”. The total number of evolutionary steps or transformations from one character state to another required to explain a given data set is said to reflect the overall amount of evolutionary change or total tree length, (Swofford and Olsen 1990). Based on the subordinate method of classification, it is usual for cladistic classifications to reflect tree structure, (Farris 1980).

2.2.5.1. Character polarity.

Through the process of distinguishing between plesiomorphies and apomorphies, information is gained on character polarity, (Wiley 1981). Attempts to gain such information have been carried out using many methods which have been reviewed by Stevens (1980). Two main methods

include; (1) outgroup method, (Watrous and Wheeler 1981, Wiley 1981, Maddison et al. 1984), and (2) the ontogenetic method (Nelson 1978, Nelson and Platnick 1981, Patterson 1982).

Outgroup method.

The outgroup method provides information on character polarity, and a rule defining the method was given by Watrous and Wheeler (1981), who stated that “when two or more character states occur within a group, the state occurring in related groups is assumed to be the plesiomorphic state”, and so the state restricted within the group is assumed to be apomorphic. The selection of an appropriate outgroup may be difficult if relationships of the taxon being investigated are not known, (Maddison et al. 1984). As a result, the selection of more than one outgroup for analysis has become common, (Maddison et al. 1984). To decrease the effects of several character states being present in many outgroups, Maddison et al. (1984), advocated the use of parsimony to determine relationships among the outgroups, prior to carrying out an analysis among the ingroup to determine a ‘globally parsimonious’ ingroup tree.

Methods not based on parsimony have been used in attempts to ease the effect of character states varying among outgroups, including the predominant state method, i.e., the state most commonly appearing in the outgroups is assumed plesiomorphic (Arnold 1981). As pointed out by Maddison et al. (1984), methods not using parsimony, such as the predominant state method, may lead to cladograms that are not “globally

parsimonious", i.e., not requiring the least number of homoplasy hypotheses within the ingroup and among the outgroups.

Ontogenetic method.

As does the outgroup method, the ontogenetic method also supplies information on character state polarity by assuming that "given an ontogenetic character transformation, from a character observed to be more general to a character observed to be less general, the more general character is primitive and the less general advanced" (Nelson 1978). Estimating character polarity from ontogenetic study may not be straightforward, as a change in the temporal occurrence of developmental stages or heterochrony, can cause error in character polarisation estimates (Gould 1977, Alberch 1985). Also, depending on the organisms under study, it may also not be possible to observe ontogenetic sequences easily.

Thus it may be concluded that the outgroup method is more appropriate for estimating character polarities in situations where heterochrony is suspected and especially when it is not possible to study organisms from different ontogenetic stages. However, depending on how much is known about the phylogenetic relationships within the taxon being studied, it may be difficult to select an appropriate outgroup.

2.2.5.2. Optimal cladograms and confidence in inferred cladograms.

Exact and heuristic algorithms have been developed to search for the most parsimonious trees. Algorithms for carrying out extensive searches of every possible tree, and branch and

bound searches are exact, while stepwise addition and branch swapping are heuristic algorithms (Swofford and Olsen 1990). Depending upon the search option chosen, analysis of large, (more than 20, depending on the data), numbers of organisms can become very time consuming when using cladistic computer programmes, (Stewart 1993). Several different techniques were reviewed by Stewart (1993) to aid in cladistic analysis of large numbers of organisms, including omission of problem organisms, breaking up data sets, and use of sub-tree definition. The appropriateness of each method will depend on the particular data set in question.

A test for the confidence of cladogram structure may be carried out using the bootstrap method, (Felsenstein 1985) in which cladistic analyses are carried out with random samples of characters, taken with replacement from the original data set. The confidence interval for a monophyletic group may be found from the number of times it occurs between bootstrap replications, (Felsenstein 1985). However, bootstrap analysis makes use of characters in their unweighted form, and if a decision has been made to weight characters based on their information content, as advocated by Bryant (1989), and Moran et al. (1990), use of bootstrap analysis may be inappropriate.

Jackknifing, proposed by Lanyon (1985), is a method of assessing tree stability by computing most parsimonious tree(s), each time leaving out one of the organisms, or one of the loci under investigation. It is possible to jackknife characters when equal character weights have been applied, because the ratio of

— weighted characters remains equal. However, for any given data set, jackknifing taxa may influence the number of informative characters, and this may affect the basis of the equal character weighting, of Moran et al. (1990), discussed later in this Chapter. Thus the application of a different set of character weights may be required each time a taxon is removed from the data set.

Errors in the search for the most parsimonious tree(s).

Systematic errors may occur because of unreliable data or sensitivity to specific taxa in the cladogram, and removal of a particular data set or taxon may cause the cladogram quality to change, as measured by the consistency index (Swofford and Olsen 1990). Rapidly evolving characters may also be a major source of systematic error, and by assigning lower weight to these characters the problem of chance convergences may be decreased (Swofford and Olsen 1990). However, assessment of rates of character evolution and of what weights to apply to which characters may seem as subjective.

2.3. Systematics of *Agaricus* species.

2.3.1. Introduction.

In this section a brief history is given of the classification of the species of *Agaricus*, and the systems of systematics that have been used during the definition of the genus. The value of characters used in the classification of *Agaricus* is also reviewed.

2.3.2. *Agaricus* species concepts and systematics.

Hypotheses concerning relationships of *Agaricus* species have been tested and modified since lamellate fungi were originally grouped in the genus *Agaricus* by Linnaeus (1753). Taxonomic species concepts such as the typological concept of Linnaeus (1753), relied on specimens of the same species matching morphologically with a type specimen. Almost a century passed before Fries (1821) revised the work of Linnaeus (1753), and created a tribe Psalliota, consisting of only a small portion of the original genus *Agaricus*. According to his definition, this tribe only included species which produced basidiomata with purple-brown basidiospores and free lamellae with annulate attachment. Generic recognition was given to Psalliota by Karsten (1879), and Donk (1962 and 1964) suggested a return to the name *Agaricus* for the genus and *Agaricus campestris* for the type species, (Anon. 1968). *Agaricus* species determination has been based mainly upon morphological characters, especially those of reproductive structures, (Cappelli 1984) in the tradition of taxonomic species concepts. However, taxonomic species concepts may be difficult to implement because of problems of environmentally, sexually, and developmentally induced

— variations in basidiocarp morphology, (Cappelli 1984, Hoiland 1983).

While the use of morphology has been most common for species definition within the genus *Agaricus*, (Cappelli 1984), attempts to define species limits have been made by Raper (1976), Elliott (1978), and Anderson et al. (1984), through the study of mating behaviour and mycelial intersterility. Their work was based on aspects of the biological species concept, (Mayr 1942), which relied on reproductive isolation to define species. Mycelial interfertility raised questions as to the accuracy of identification of *Agaricus* specimens, Raper (1976). Because of the unique sexual behaviour of certain fungi, Esser and Hoffmann (1977) modified Mayr's (1942) biological species concept for fungi as follows: "Populations belong to different biological species when failure to interbreed and to produce viable offspring in nature is not caused by genetic parameters operating in the completion of the sexual cycle." Practical application of the biological species concept may be difficult when criteria used to delineate species cannot be determined, such as occurs when 1) taxa are asexual, 2) populations have non overlapping ranges, and 3) it is difficult to determine the natural limits of populations and the extent of interbreeding.

Although investigations into reproductive isolation have aided in the definition of *Agaricus* species, application of a phylogenetic species concept for the definition of species has met with vigorous debate, (Nixon and Wheeler 1990), and a consensus has not yet been reached, (Baum 1992). A phylogenetic species

concept developed by Mishler and Donohue (1982), Mishler and Brandon (1987), and Cracraft (1990), was proposed by Vilgalys (1991) as a basis for use within *Collybia*. Vilgalys (1991) recognised that this species concept allowed for asexual forms and allopatric speciation, and therefore suggested that it was better for use with fungal species than the taxonomic and biological species concepts.

While the phylogenetic species concept proposed by Vilgalys (1991) takes account of some of the problems of other species concepts, it was based upon the results of authors such as Mishler and Brandon (1987) which have been criticised by Wheeler and Nixon (1990). Mishler and Brandon (1987) used monophyly to group organisms into species and applied ranking criteria, such as breeding barriers and synapomorphy patterns to distinguish which of the nested monophyletic groups represented species. However, phylogeneticists such as Hennig (1966), Platnick (1977), and Wheeler and Nixon (1990) have argued that monophyly only applies to groups of species and not to species. A most important objection by Wheeler and Nixon (1990) to the use of monophyly to group organisms into species is the occurrence of reticulate genealogies among populations. In such reticulate genealogies synapomorphy ceases to define monophyly, (Wheeler and Nixon 1990). The philosophical and practical limitations of using the monophyletic criterion for species recognition have been reviewed by Baum (1992).

The classification of species based on any of the above species concepts depends on the school of systematics applied, (Wiley 1981). The problem with the classifications of *Agaricus* species is that the criteria for classification has been unclear, and has varied between authors, (Cappelli 1984, Wasser 1989). The rearrangement of *Agaricus* species into different Groups and Sections by various mycologists depending upon their particular assessment of characters reflects how artificial the classifications have been, (Cappelli 1984, Wasser 1989). Thus the present classification by Cappelli (1984) does not necessarily reflect phylogeny at all. The problem of classification of the genus *Agaricus* is emphasised by the fact that nine major rearrangements within the genus were made from the early 1950's through to that by Cappelli (1984).

Phenetic systematics, a method reviewed in Section 2.2.2, has commonly been applied to Basidiomycetes, and examples were given in Wolfe (1984). While phenetic classifications do not aim to reflect phylogeny, they may be useful for assessment of overall similarity of taxa. *Agaricus* species relationships have been investigated by Raper and Kaye (1978), using phenetic techniques. Based on the method of single linkage clustering, (Sneath and Sokal 1973), Raper and Kaye (1978) used data on *Agaricus* species sexuality, interfertility patterns, macroscopic and microscopic morphology, growth and cultural characteristics, production of extracellular enzymes, and mycelial esterase isozyme patterns. They reported that specimens identified as *A. campestris* were present in three of six groups defined in their results, and similarity between specimens identified as different

- species was often supported up to 90 %. They concluded that species identifications based on basidiocarp characters were inaccurate, and their methods were useful for identifying problems with the available data.

While the use of phenetic systematics has most commonly been applied to Basidiomycetes, (Wolfe 1984), and phylogenetic relationships have rarely been taken into account when developing fungal classifications, (Wheeler and Blackwell 1984), an increasing number of phylogenetic studies of fungi have begun to appear among the phenetic studies. Examples of phylogenetic studies may be taken from the Ascomycete families, Capnodiaceae *sensu lato*, (Reynolds 1986), and Melampsoraceae (Hart 1988), and the genus *Trichoderma*, (Stasz et al. 1989). Further examples of phylogenetic studies include the Zygomycete family Endogonaceae, (Morton 1990), and both phenetic and phylogenetic studies of the *Dermocybe*, (Høiland 1983) and *Collybia*, (Vilgalys 1986 and 1991). These studies indicate the increasing interest in phylogenetic methodology in fungal systematics.

It may be concluded from the literature reviewed, that no single species concept is perfect, or is likely to meet the requirements of all mycologists. Definition of *Agaricus* species has relied on the taxonomic species concept, but a phylogenetic species concept is likely to be most applicable to fungi (Vilgalys 1991). However, there have been problems with the use of such a concept (Baum 1992), and its application to *Agaricus* species would be inappropriate at present because of the lack of consensus regarding this concept. Specific application of

phylogenetic systematics has not been made to *Agaricus* species, and their classification does not necessarily reflect phylogeny. It is important to point out that while a phylogenetic species concept may not presently be applicable for defining *Agaricus* species, this in no way negates the use of phylogenetic methodology for studying *Agaricus* species relationships.

2.3.3. Classification of *Agaricus* species investigated.

Morphological characters of *Agaricus* species have been presented and discussed in detail by many authors, e.g., Cappelli (1984), Heinemann (1974, 1977, 1978), Wasser (1989). Because of the large number of *Agaricus* species described (between 200 and 250, Bas 1991), this section of the literature review will be restricted to the eleven species studied during this research project. Descriptions prepared from field collected specimens of each of these species are given in Appendix 8. Unless otherwise stated, the most recent *Agaricus* classification proposed by Cappelli (1984) will be used to describe the relationships of the species investigated, Figure 2.1.

Many of the characters routinely used to describe *Agaricus* species are subtle and subjective, especially those concerned with odour and colour of closely related species, (Cappelli 1984), but also anatomical characters such as velar remnants, especially of the general veil, (Cappelli 1984). For example, floccules upon the lower stipe of *Agaricus campestris*, and to a lesser extent *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus*, may be difficult to determine unless fresh or well preserved specimens are

— available, (Cappelli 1984). Characters which do not vary within the genus, such as lamellae trama arrangement, basidiospore colour when mature, and lamellae insertion, (Wasser 1989) do not supply any useful phylogenetic information. Assessment of characters, such as stipe consistency, (Wasser 1989), basidiospore number per basidium, (Kerrigan and Ross 1986), and basidioma size, shape and colour, (Cappelli 1984), which may vary during basidioma development and/or are influenced by environment may be very difficult. Based on the survey of literature, it may be concluded that many characters are limited for use in a phylogenetic study of *Agaricus* species.

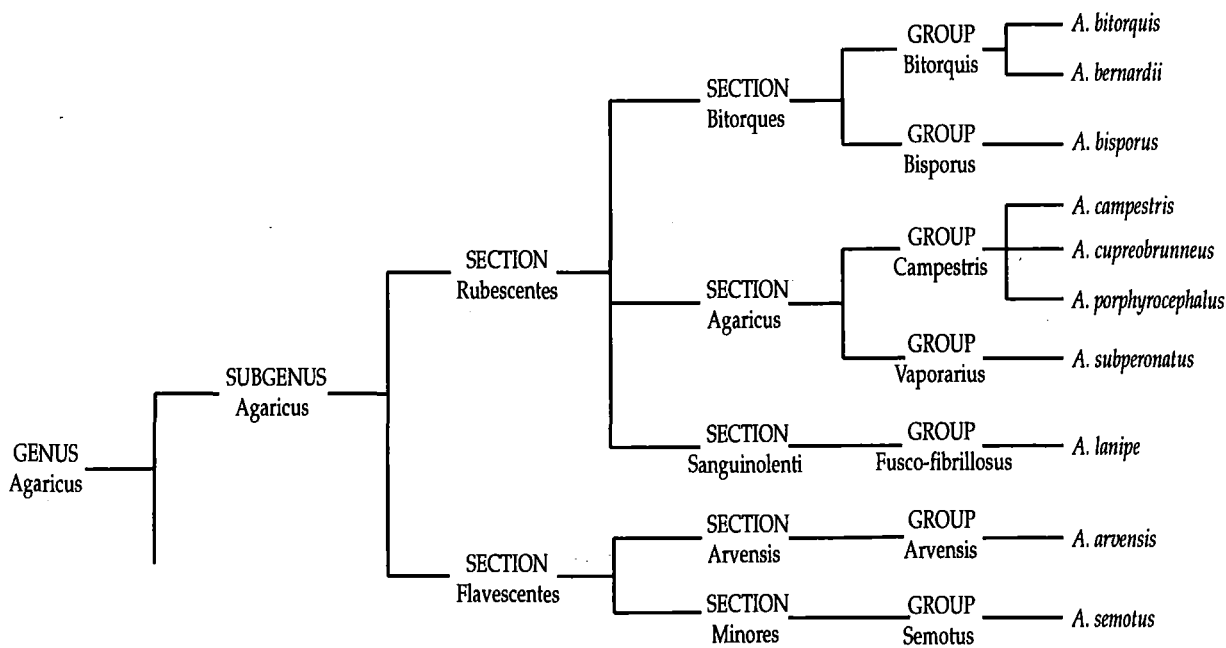


Figure 2.1. Classification of *Agaricus* species investigated, (Cappelli 1984).

However, some characters traditionally used for classification within the genus may have greater phylogenetic value than those discussed above. Three main characters used by Cappelli (1984) to distinguish the Section Rubescentes from the Section Flavescences, were; 1) the Schaeffer's cross reaction, 2) the alkali test, and 3), basidioma colour change when bruised or broken.

Schaeffer's cross reaction is a chemical test that involves streaking upon the pileus surface a solution of anilin water crossed by another line of concentrated HNO_3 . If an orange or red discolouration appears where the two reagents mix, then the result is positive. Species in the Section Rubescentes are usually negative, and those the Section Flavescences are usually positive, (Cappelli 1984). Application of this test may be limited to fresh specimens, as demonstrated by Freeman (1979), who found it did not work well with dried material. While the Schaeffer's cross reaction has been almost exclusively applied within the genus *Agaricus*, it may also be applied to species outside the Agaricaceae family, (Watling 1971). Thus if this character varies between *Agaricus* species and species from an outgroup, it may be a useful indicator of character polarity in an *Agaricus* cladistic study.

The alkali test involves the application of strong alkali, either NaOH or KOH to basidioma parts, (Singer 1975). This test readily differentiates between species in the large Section Flavescences, which react positively, turning yellow or orange, (Singer 1975) while species in the Section Rubescentes show no reaction. The fact that this character may be used to define

— species outside the genus *Agaricus*, (Singer 1975) implies that it may be a useful indicator of character polarity in an *Agaricus* cladistic study through the incorporation of an outgroup.

In the third test, basidioma colour when broken and bruised, species in the Section *Rubescentes*, generally remain unchanged or stain reddish when broken, while those of the Section *Flavescentes* generally exhibit a yellow colour, (Cappelli 1984). It was not determined from the literature if this character was shared with an outgroup, which implies it may not be a useful polarising character in cladistic analysis. Demonstration of homology for the character of colour change may be difficult, i.e., different biochemical pathways may be responsible for the same colour reaction, limiting the use of this character for cladistic analysis, although no evidence for this could be found in the literature.

Relationships within the Sections *Rubescentes* and *Flavescentes* are determined based on characters to do with the annulus or ring of veil remnants on the stipe of the mature basidioma. *Agaricus* species exhibit both a general veil covering the whole mushroom, and a partial veil covering the the lamellae from the stipe to the pileus margin, (Cappelli 1984). The partial veil of *Agaricus* species generally contributes more to the annulus than does the general veil, (Cappelli 1984). Whether the annulus is pendent (may be peeled upward from the stipe), or peronate (may be peeled downward from the stipe), is important as a character for use in *Agaricus* systematics, (Cappelli 1984). Species in the Section *Bitorques* possess a peronate annulus, while species

— in the Section *Agaricus* are defined as having a pendant annulus. Many possible outgroups share similar veil development with *Agaricus* species, including other Tribes within the Agaricaceae, making annulus pendant or peronate a suitable for character polarisation in cladistic analysis.

Two further characters have been used to discriminate species within the Sections *Rubrescentes* and *Flavescentes*. The first of these characters is presence or absence of cheilocystidia, (Wasser 1989). *Agaricus bisporus*, *Agaricus bernardii*, and *Agaricus bitorquis* have sterile lamellae edges with cheilocystidia, while *Agaricus campestris*, *Agaricus cupreobrunneus*, and *Agaricus porphyrocephalus* have fertile lamellae edges, with few or no cheilocystidia. Another character is the presence, (even if rudimentary) of a germ pore at the basidiospore apex, which has only been confirmed by Heinemann (1952, 1977) for *Agaricus campestris*, and *Agaricus aestivalis*, and described by him as rudimentary in *Agaricus cupreobrunneus* and *Agaricus ludoviei*.

Germ pores are present in basidiospores of the Tribes *Leucocoprineae*, and *Lepioteae*, also in the family Agaricaceae, and in many other families of the Agaricales, e.g., *Bolbitiaceae*, *Coprinaceae* and *Strophariaceae*, (Singer 1975). If the process of germ pore ontogeny is homologous within *Agaricus* and between *Agaricus* and an outgroup, the presence of a germ pore could be a useful polarising character for cladistic study. But, according to Pegler and Young (1971), the *Agaricus campestris* germ pore is present as a “result of a thinning of the exo-episporium with a corresponding thickening of the endosporium”, whereas the germ

— pore of *Leucoagaricus* represents a “discontinuous episporium”. Thus there may be problems in justifying the presence of a germ pore as a homologous character between species if the ontogenetic process leading to it is different.

Below the Sectional level of *Agaricus* classification are the Groups, (Cappelli 1984). The classification of the Groups containing species investigated will now be described, along with the main defining characters. *Agaricus bernardii* and *Agaricus bitorquis* have been placed together in the Group Bitorquis. Based upon the presence of two rings on the stipe, one from the partial veil and one from the general veil, *Agaricus bitorquis*, and to a lesser extent *Agaricus bernardii* may be clearly distinguished from other species by this character. They are thought to be more closely related to *Agaricus bisporus* as part of the Section Bitorques, than to *Agaricus campestris*, of the Section Agaricus. *Agaricus campestris*, *Agaricus cupreobrunneus*, and *Agaricus porphyrocephalus* belong to the Group Campestris. *Agaricus campestris* has been recognised by Wasser (1989), as one of the most diverse *Agaricus* species. According to him it is “ultrapolymorphic with more than 10 variants of different taxonomic significance”. This implies that extensive population surveys may be required to gain an accurate reflection of species variability.

Agaricus cupreobrunneus, first described as *Agaricus campestris* var. *cupreobrunneus*, (Shaeffer and Moeller 1938) was elevated to species rank by Pilat (1951). *Agaricus cupreobrunneus* was considered by Moser (1967, 1978) to be

— closely related to *Agaricus porphyrocephalus*, perhaps more so than to *Agaricus campestris*. Moser (1967, 1978) also suggested *Agaricus porphyrocephalus* and *Agaricus cupreobrunneus* may be a single species, but an analysis of specimens of each of these species by Wasser (1974, 1989), lead him to conclude that the specific rank was justified, based particularly on basidiospore size. This supported the conclusions of Bohus (1969), and Wasser (1989) who considered *Agaricus porphyrocephalus* to have smaller basidiospores than *Agaricus cupreobrunneus*, and placed emphasis on this character for species delimitation.

In the classification by Cappelli (1984), *Agaricus subperonatus* has been placed in the Group Vaporarius, of the Section Agaricus, and *Agaricus lanipes* in the Group Fusco-fibrillosus, Section Sanguinolenti. However, according to Wasser (1989), the position of *Agaricus lanipes* in the genus was disputable. This implies that other definitive characters are needed to place *Agaricus lanipes* accurately within the classification. The Sections Bitorques, Agaricus, and Sanguinolenti are all grouped in the larger Section Rubescentes, Subgenus Agaricus.

Agaricus arvensis and *Agaricus semotus* are each members of the large Section Flavescetes, and have traditionally been separated into different Sections and Groups based on their size, (Cappelli 1984). *Agaricus arvensis* is in the Section Arvenses, with other species having a pileus generally wider than 6 cm, and *Agaricus semotus* is in the Section Minores, with other species generally with pileus less than 6 cm across, (Cappelli 1984).

Agaricus arvensis is thought to be a polymorphic species, and Wasser (1989), listed three varieties, and likewise *Agaricus semotus* has been considered very variable (Cappelli 1984, Wasser 1989). This implies that population surveys may be required to establish within species variation for *Agaricus arvensis* and *Agaricus semotus*.

It may be concluded from the literature survey that the classification of *Agaricus* species has been based on few objectively determined characters, the number of which needs to be increased to allow phylogenetic study and a reassessment of the classification.

2.3.4. Basidiospores as a source of characters for *Agaricus* systematics.

The goal of this Section of the review is to assess the value of basidiospore characters for *Agaricus* systematics, and to evaluate problems affecting their use for systematics. Lange (1926) classified *Agaricus* species based on basidiospore size, but as Freeman (1979) pointed out, the system was not valid due to the overlap of this character between species. In spite of Lange's (1926) lack of success, more recent investigations have supported the use of basidiospore size for *Agaricus* species determinations, (Pegler and Young 1971, Raper and Kaye 1978, Wasser 1989). However, there was little discussion of the ranges of variation within and between species. Based on extensive basidiospore studies, Pegler and Young (1971) concluded that basidiospores were "the most fundamental of all characters in classification" of

— the species of the Agaricales, implying basidiospore variables were useful for systematics studies.

For descriptions of *Agaricus* species approximately 100 basidiospores from each of one to two specimens have commonly been measured, (Garibova and Safrai 1972, Wasser et al. 1976). However, for such descriptions “it is necessary to study a sufficiently large number of specimens taken from as many different locations as possible and from as many different environmental conditions”, (Parmasto and Parmasto 1987). According to Parmasto and Parmasto (1987), the study of basidiospores from too few specimens decreased the usefulness of species descriptions.

It is possible to measure the length and breadth of between 150-200 basidiospores per hour using light microscopy and manual methods, including time taken to prepare slides and enter data. If extensive basidiospore investigations are carried out to define species limits, it may be necessary to study thousands of basidiospores, requiring many hours of light microscopy for each specimen. As pointed out by Pegler and Young (1971), “the importance of the basidiospore has necessitated more sophisticated techniques in observation”. The use of a more efficient method of basidiospore measurement may allow the collection of more statistically meaningful basidiospore data sets. The measurement of basidiospores using automatic computer methods based on pixel counts, such as image analysis, (Cox and Thomas 1992, Vezey et al. 1991) would be faster and more objective than manual use of a micrometer.

While no literature could be found on the use of computer image analysis for the study of basidiospores, this method has been used by the mycologists Cox and Thomas (1992), as a method of classifying and measuring fungal pellets grown in liquid culture, and it may be possible to use this technique for the automated measurement of basidiospores. Thus an example of the way in which image analysis may be applied to systematics was given by Vezey et al. (1991), who studied images of pollen grains from three closely related Capparaceae species. Their use of phenetic methods enabled them to distinguish the species based on variables they had generated using image analysis. They recommended further investigation of larger numbers of taxa using image analysis and the application of cladistic methods.

Pegler and Young (1971) contended that homogeneity between basidiospore samples may be gained by collecting mature basidiospores for study from spore prints. However, Bresinsky et al. (1977), Watling (1977), and Clemencon (1979) showed that basidiospore size may be affected by environmental conditions. Furthermore, both Clemencon (1979), and Hoiland (1983) demonstrated that basidiospore size varied during basidioma development. Hoiland (1983) concluded that basidiospore variables "serve as reliable taxonomic characters in *Dermocybe*" when factors such as basidioma age had been taken into account. But, the selection of basidioma of similar developmental stage tends to be subjective because of environmental influence upon basidioma morphology. These factors limit the validity of basidiospore size comparisons between

specimens, especially those collected from geographically isolated populations and those subjected to differing environmental conditions.

Compared with basidiospore size, Garibova and Safrai (1972) and Wasser et al. (1976) placed higher priority on basidiospore shape as a useful character for *Agaricus* systematics. They used the ratio between average length and breadth, often referred to as the 'Q' or elongation value, (Watling 1977). However, Parmasto and Parmasto (1987) concluded from a review of the work by Garibova and Safrai (1972) and Wasser et al. (1976), that elongation value was not useful for *Agaricus* species delimitation because of overlapping results from closely related species.

This review shows that a consensus has not been reached regarding the value of basidiospore characters for *Agaricus* systematics. Investigation is required to assess faster, more objective methods for the measurement of basidiospore variables such as size and shape compared with light microscopy. Improved methods, such as image analysis, may allow extensive investigation of basidiospore variability within and between species during basidioma development and from different geographically isolated areas subject to different environmental influences. Information on *Agaricus* basidiospore variables generated through image analysis may be explored using cladistic techniques, as Vezey et al. (1991), suggested for Capparaeae species based on the data supplied through image analysis of pollen grains.

In conclusion, phylogenetic analysis of characters that have traditionally been used for *Agaricus* classification has not previously been carried out, possibly due to there being so few objectively determined characters. There has also been difficulty attributing a genetic basis to many characters, such as the form and colour of the pileus, which may be affected by variable environmental influence. Thus recourse to new methods of generating characters for *Agaricus* phylogenetic analysis is required to allow assessment of species relationships and re-evaluation of the present classification by Cappelli (1984).

2.4. Isozyme electrophoresis.

2.4.1. Introduction.

Developments in biochemical techniques, such as isozyme electrophoresis and direct study of DNA have provided more information on organisms than has previously been available, (Hillis and Moritz 1990). Isozymes are the product of more than one structural gene coding for a particular enzyme, and include all polymers of subunits produced by different gene loci or different alleles at the same locus, (Markert and Moller 1959). Isozyme electrophoresis provides a most important economical and efficient means of generating phylogenetically useful characters (Murphy et al. 1990, May 1992). The generally accepted, (Murphy et al. 1990, May 1992), independent genetic origin of characters generated from isozyme electrophoresis is an advantage over many *Agaricus* morphological characters, e.g., basidioma size, shape and colour, which may be influenced by variable environmental factors.

2.4.2. Isozyme technology.

Three important points regarding isozyme electrophoresis are highlighted in most introductory texts on the subject, e.g., Richardson et al. (1986), and are as follows; 1) the technique of electrophoresis brings about the movement of proteins through a support matrix under the influence of an electric field carried by ionic buffers, 2) those proteins that have a net positive charge migrate towards the cathode, and those with a net negative charge migrate towards the anode, 3) size and shape of the protein molecule affects the rate of migration, as well as charge.

Starch gel electrophoresis (SGE) was introduced by Smithies (1955) and subsequently polyacrylamide gel electrophoresis (PAGE) was developed by Chrambach and Rodbard (1971), leading to a convenient, cost-effective technique for the study of proteins. The matrix of starch and polyacrylamide maintains the position of the protein molecule after electrophoresis is terminated.

Isozyme electrophoresis relies on certain enzyme properties. Enzymes catalyse specific biochemical reactions when they are placed in contact with their substrate and appropriate co-factors. If a dye is included as a component of the enzymatic reaction, the position to which the protein has migrated in a gel may be seen, either as a 1) coloured band in normal light, or 2) fluorescent or de-fluorescent band under UV light. The use of stains to identify specific enzymes began with the work of Hunter and Markert (1957). As compared with general protein staining, isozymes may be revealed by staining for a specific enzyme, and many detection methods have been reported, (Harris and

Hopkinson 1976, Vallejos 1983, Cheliak and Pitel 1984, Murphy et al. 1990, Gabriel and Gersten 1992). As an example the staining system of phosphoglucomutase, (PGM), E.C. 5.4.2.2, is shown in Figure 2.2 (Harris and Hopkinson 1976).

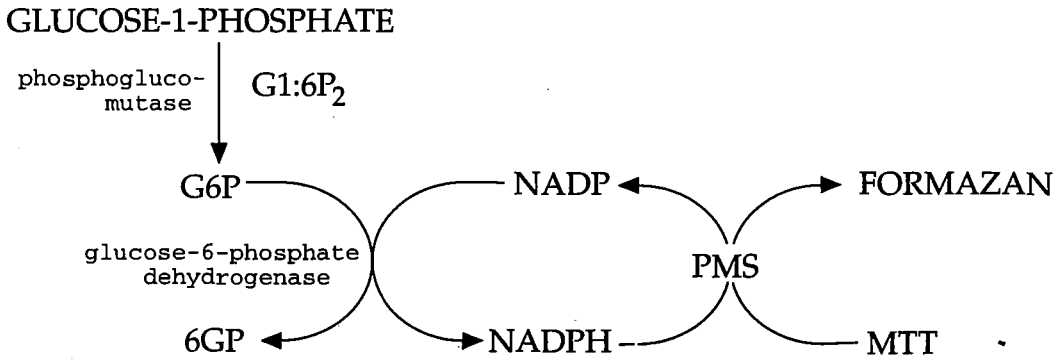


Figure 2.2. Staining reaction for the enzyme phosphoglucomutase (PGM) (Harris and Hopkinson 1976), which may be used to visualise PGM isozymes.

Isozymes are rarely used as sources of biological data for phylogenetic analysis, (Murphy et al. 1990) because without genetic interpretation, assumptions of character homology are likely to be inaccurate. However, after genetic interpretation of different alleles at a locus, a subset of isozymes, termed allozymes (Prakash et al. 1969) may be determined, (Harris and Hopkinson 1976). Allozymes can provide information on genetic variability which may be used as a direct source of biological data for phylogenetic systematics, (Baverstock et al. 1979, Crawford 1983, Buth 1984, Murphy et al. 1990).

Steps towards the phylogenetic use of allozymes from *Agaricus* species began with electrophoretic studies such as Paranjpe et al. (1979). They used PAGE to study strain variability

in *Agaricus bisporus* and evaluated isozyme banding patterns according to the presence or absence of particular bands with no regard for the genetic basis of these bands. Later, May and Royse (1981), using SGE, were the first to hypothesise a genetic basis for the specific enzyme banding patterns observed for the fungal species *Agaricus bisporus*, *Agaricus campestris*, and *Lentinus edodes*. The phylogenetic relationships of *Agaricus* species based on allozyme information remains to be evaluated, and depends on the genetic interpretation of isozyme banding patterns.

2.4.3. Genetic interpretation of isozyme banding patterns.

A comprehensive review of isozyme genetic interpretation was given by Harris and Hopkinson (1976), and other reviews also detailing methods of genetic interpretation have been published more recently, one specifically with reference to starch gel electrophoresis was by May (1992), and one with regard to the electrophoretic study of fungi by Micales et al. (1992).

The review that follows is based on the publication of Harris and Hopkinson (1976). Enzymes consist of 1 or more polypeptide chains or subunits. Isozyme bands observed after electrophoresis and staining are dependent upon the genotype of the individual, and the subunit number and structure for the enzyme being studied. An organism may be homozygous at a particular locus, and the two identical alleles will produce an identical polypeptide chain, seen as a single band after electrophoresis and staining. If an organism is heterozygous at a particular locus the two different alleles will code for two structurally distinct polypeptide chains,

— but the isozyme banding patterns produced depend upon the subunit nature of the enzyme. If the enzyme is monomeric, a simple two band pattern will be seen for a heterozygous individual after staining, each being a mixture of the two different subunits. That is, if a single polypeptide is needed to make the enzyme, and there are two different polypeptides available two forms of the enzyme may be made, each of which may be referred to as an allozyme. If the enzyme is multimeric, or made up of more than one subunit, the heterozygous individual will display additional ‘hybrid’ isozyme bands. For example, a dimeric enzyme will typically produce two homomeric isozyme bands, and one heteromeric ‘hybrid’ isozyme band for an heterozygous individual. That is, if two polypeptides are needed to make the enzyme, and there are two different polypeptides available, coded for by the different alleles, e.g., A and A’, they will combine in three different ways, represented by bands aa, aa’, and a’a’ in Figure 2.3.

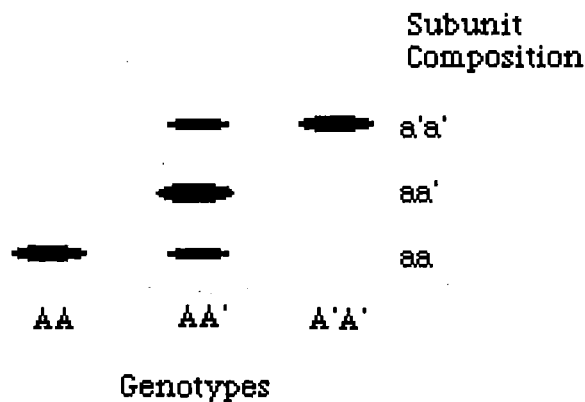


Figure 2.3. Expected banding patterns for a single locus with 2 alleles for a dimeric enzyme, (modified from May 1992).

The 3 bands for the heterozygous genotype AA', shown in Figure 2.3, which usually appear in the relative concentrations of

—1:2:1 may be compared with the 2 possible homozygous genotypes AA and A'A'. Only the isozyme bands, shown in Figure 2.3 to consist of aa and a'a' subunits may be considered allozymes and directly reflective of the different alleles, not the 'hybrid' isozyme band, shown in Figure 2.3 to consist of aa' subunits.

A problem with the genetic interpretation of isozyme banding patterns is that the symmetrical isozyme pattern for AA' heterozygous genotype shown in Figure 2.3, will only occur if equal contributions of enzyme activity are made by each of the polypeptides coded for by the different alleles. Thus during genetic interpretation, the presence of asymmetrical isozyme patterns may make it difficult to assess which of the isozyme bands actually represent allozymes. Asymmetrical isozyme patterns can result from the unequal contribution of polypeptides to the enzyme activity, which can occur at differing rates of synthesis, differing stabilities, or differing modification effects post isozyme formation. Allozymes wrongly determined due to misinterpretation of asymmetric isozyme banding patterns, may introduce error into a phylogenetic analysis based on such information. Thus great care is needed when attempting to genetically interpret asymmetric patterns for phylogenetic analysis.

2.4.4. Limitations of isozyme electrophoresis.

Systematists using allozyme data, e.g., Selander et al. (1971), and Buth (1979), make the assumption that differences in allozyme mobility reflect the encoding DNA sequence, (May 1992). Different allozymes at the same locus for any two individuals are

—assumed to be genetically based and heritable in a simple Mendelian fashion, (May 1992). Breeding studies are useful to check the genetic basis for allozymes, especially if patterns depart from expectations, (Richardson et al. 1986). But depending on the organism and/or time restrictions imposed on a research programme breeding studies are not always practical, (Richardson et al. 1986).

The problem of not detecting all genetic variability using electrophoresis is a limitation to the accuracy of the methods, (Ferguson 1988). Because there can be many codons for the same amino acid, and amino acid exchange on the exterior of a protein only represents a charge change approximately one third of the time, the genetic difference may not be detected in allozyme mobility, (Ferguson 1988). Limits to the number of loci and polymorphic allozymes per locus may also limit the use of the isozyme technique, (Murphy et al. 1990) in that there may simply not be enough information available upon which to base a study.

Isozyme electrophoresis can show bands of dye deposition, known as artifacts, which do not reflect the genetic constitution of the study organism, (Murphy et al. 1990). The main cause for these artifacts is postranslational modifications, these represent variation from co-dominant allozyme expression which may occur during polymerisation, termination and/or processing of the final protein product, (Murphy et al. 1990). Artifacts representing postranslational or other enzyme activity which do not fit simple Mendelian and biochemical models, may sometimes be detected by observing the relative speed of dye deposition, thus artifacts

—often appear after the inherited allozymes have become visible, (Richardson et al. 1986). Null-alleles or reduced/absent enzyme expression occur when an allele does not produce a protein product or when it produces a non-functional protein product, (Richardson et al. 1986). It may be concluded that null-alleles and artifacts may complicate genetic interpretation of isozyme patterns, and consequently decrease the confidence in a phylogenetic study incorporating organisms displaying such limiting factors.

Despite the limitations of isozyme electrophoresis, the technique has been established as one of the most useful for study of systematics and population genetics of extant organisms (Ferguson 1988, May 1992). Because allozyme information is not the same as studying DNA information directly, for the purpose of this thesis the term electromorph, first introduced by King and Ohtu (1975) will be used in preference to allele. Allozyme loci are acknowledged to be only putative, and the term putative loci will be used in preference to the term loci, (Murphy et al. 1990).

2.5. Applications of the isozyme electrophoretic technique to *Agaricus* species.

2.5.1. Techniques associated with polyacrylamide gel electrophoresis (PAGE) and horizontal starch gel electrophoresis (HSE) of *Agaricus* mycelial allozymes.

2.5.1.1. Introduction.

A considerable body of literature has been amassed concerning the techniques involved, but it may be conveniently divided into the following topics;

- 1) extraction buffers,
- 2) weight of mycelium required for analysis,
- 3) electrophoretic methods, and their applicability,
- 4) modifications of electrophoretic methods affecting allozyme resolution,
- 5) mycelial allozyme putative loci,
- 6) intra and inter species polymorphism for putative loci.

2.5.1.2. PAGE of *Agaricus* species.

The methods used for PAGE are discussed in Chapter 3. PAGE investigations of *Agaricus* species have enabled the adequate resolution of at least nine enzymes, there being little difference between the methods used, see Table 2.1. Excluding the study by Raper and Kaye (1978), PAGE studies noted in Table 2.1 have only been used to investigate enzymes for *Agaricus bisporus*. Provided these enzymes can be found in other *Agaricus* species, it may be concluded that the use of PAGE to generate

allozyme data for phylogenetic analysis may be possible. However, no further information could be located in the literature on the use of PAGE for study of *Agaricus* species.

Extraction buffer	Percent acrylamide in running gel	Running gel buffer	Running conditions	Enzymes adequately resolved	Reference
0.1 M Tris-Borate, EDTA, pH 8.9, 5 % sucrose.	7.5	0.1 M Tris-borate, EDTA. pH 8.9	4 h. 15 V cm ⁻¹	esterase	Raper and Kaye (1978)
0.1 N Tris-HCl, pH 8.0, and 0.1 Na phosphate pH 6.0	7.5	0.1 M Tris-HCl, pH 8.6	?	cytochrome oxidase, peroxidase, tyrosinase	Paranjpe et al. (1979)
0.005 M Tris-HCl, pH 8.0, 0.01 M 2-mercapto-ethanol	6	0.1 M Tris-citrate, pH 8.5	110 V	esterase, alcohol dehydrogenase, malate dehydrogenase, cytochrome oxidase	van-Loon et al. (1986)
?	?	?	220 V 30 mA	esterase	Liming et al. (1987)
0.05 M Tris-HCl, pH 7.1 with and without 20 mM NAD ⁺	5 ----- 7.5	0.05 M Tris-HCl, pH 8.8	3.5 h. 200 V	β-glucosidase, glutamate pyruvate transaminase , peptidase. ----- alcohol dehydrogenase, mannosephosphate isomerase .	Kerrigan and Ross (1989)

Table 2.1. Methods of *Agaricus* polyacrylamide gel electrophoresis. Question mark (?) means information unavailable.

2.5.1.3. HSE of *Agaricus* species.

Since the early 1980's May and Royse have used HSE to study allozymes of *Agaricus bisporus* and *Agaricus campestris*, and the allozymes of species from other Basidiomycete genera (May and Royse 1981, 1982a, 1982b, 1988, May et al. 1988, Royse and May 1982a, 1982b, 1989, Royse et al. 1983a, 1983b). As set out in Table 2.2, their studies provide a list of possible enzymes and electrophoretic conditions that might be used for further investigation. Table 2.2 gives the enzyme abbreviations used throughout this thesis.

Table 2.2. List of the enzymes, abbreviations, enzyme commission numbers (E.C. No.), fungal genus studied, appropriate buffer systems used, and the reference. Enzymes not found in vegetative mycelium have not been included. All studies were intra specific except for all those to do with *Pleurotus* that were concerned with inter species relationships.

<u>Recommended name</u>	<u>Abbr.</u>	<u>E.C. No.</u>	<u>Genus</u>	<u>Buffer*</u>	<u>Reference</u>
Acid phosphatase	ACP	3.1.3.2	<i>Agaricus</i>	S4	Royse and May (1989)
			<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
			<i>Volvariella</i>	C	Royse <i>et al.</i> (1987)
Adenosine deaminase	ADA	3.5.4.4	<i>Agaricus</i>	C	May and Royse (1981)
			<i>Volvariella</i>	M	Royse and May (1982a) Royse <i>et al.</i> (1987)
Alcohol dehydrogenase	ADH	1.1.1.1	<i>Agaricus</i>	M	Royse and May (1989)
			<i>Pleurotus</i>	S4	Royse and May (1982a) May and Royse (1988)
Alkaline phosphatase	AKP	3.1.3.1	<i>Lentinus</i>	C	Royse <i>et al.</i> (1983a)
Aspartate aminotransferase	AAT	2.6.1.1	<i>Agaricus</i>	C	Royse and May (1989)
				C, R	May and Royse (1981)
					Royse and May (1982a)
			<i>Pleurotus</i>	C	May and Royse (1988)
					May <i>et al.</i> (1988)
			<i>Lentinus</i>	C	Royse <i>et al.</i> (1983a)
					Royse <i>et al.</i> (1983b)
			<i>Volvariella</i>	C	Royse <i>et al.</i> (1987)
Catalase	CAT	1.11.1.6	<i>Agaricus</i>	?	Royse and May (1989)
Diaphorase	DIA	1.6.4.3	<i>Pleurotus</i>	R	May <i>et al.</i> (1988)
			<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
			<i>Volvariella</i>	R	Royse <i>et al.</i> (1987)

Esterase	EST	3.1.1.1	<i>Agaricus</i>	S4	Royse and May (1989)
			<i>Pleurotus</i>	R	May <i>et al.</i> (1988)
			<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
			<i>Volvariella</i>	C	Royse <i>et al.</i> (1987)
Formate dehydrogenase	FDH	1.2.1.2	<i>Agaricus</i>	C	Royse and May (1982a)
Fumarase	FUM	4.2.1.2	<i>Pleurotus</i>	S4	May <i>et al.</i> (1988)
			<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
Glucose-6-phosphate dehydrogenase	G-6-P	1.1.1.49	<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
Glucokinase (Hexokinase)	GK	3.7.1.1	<i>Agaricus</i>	C	May and Royse (1981)
			<i>Lentinus</i>	C	Royse and May (1982a) Royse <i>et al.</i> (1983a)
Glucosephosphate isomerase	GPI	5.3.1.9	<i>Agaricus</i>	M	May and Royse (1981)
			<i>Lentinus</i>	C	Royse and May (1982a)
			<i>Volvariella</i>	C	Royse <i>et al.</i> (1983a) Royse <i>et al.</i> (1987)
α -Glucosidase	α GLU	3.2.1.20	<i>Agaricus</i>	?	Royse and May (1989)
β -Glucosidase	β GLU	3.2.1.20	<i>Agaricus</i>	R	May and Royse (1981)
			<i>Lentinus</i>	C	Royse and May (1982a) Royse <i>et al.</i> (1983a)
Glucuronidase	GLUR	3.2.1.31	<i>Agaricus</i>	?	Royse and May (1989)
Glutamate dehydrogenase	GDH	1.4.1.2	<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a) Royse <i>et al.</i> (1983b)
Glutamate pyruvate transaminase	GPT	2.6.1.2	<i>Agaricus</i>	M	Royse and May (1989)
				M	May and Royse (1981)
			<i>Pleurotus</i>	C	Royse and May (1982a) May and Royse (1988) May <i>et al.</i> (1988)

			<i>Volvariella</i>	M	Royse <i>et al.</i> (1987)
Glutathione reductase	GR	1.6.4.2	<i>Pleurotus</i>	R	May <i>et al.</i> (1988)
			<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
Guanine deaminase	GDA	3.5.4.3	<i>Agaricus</i>	?	Royse and May (1989)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.2.1.12	<i>Agaricus</i>	?	Royse and May (1989)
Hexose aminidase	HA	3.2.1.30	<i>Agaricus</i>	?	Royse and May (1989)
			<i>Pleurotus</i>	R	May <i>et al.</i> (1988)
Inorganic pyrophosphatase	IP	3.6.1.1	<i>Pleurotus</i>	S9	May and Royse (1988)
Isocitrate dehydrogenase	IDH	1.1.1.42	<i>Agaricus</i>	?	Royse and May (1989)
Lactate dehydrogenase	LDH	1.1.1.27	<i>Agaricus</i>	?	Royse and May (1989)
			<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)
Leucine aminopeptidase	LAP	3.4.11.1	<i>Pleurotus</i>	R	May <i>et al.</i> (1988)
			<i>Volvariella</i>	R	Royse <i>et al.</i> (1987)
Malate dehydrogenase	MDH	1.1.1.37	<i>Agaricus</i>	C	May and Royse (1981)
			<i>Pleurotus</i>	C	Royse and May (1982a)
			<i>Lentinus</i>	C	May and Royse (1988)
					May <i>et al.</i> (1988)
					Royse <i>et al.</i> (1983a)
Malic enzyme	ME	1.1.1.40	<i>Agaricus</i>	C	May and Royse (1981)
			<i>Lentinus</i>	C	Royse and May (1982a)
					Royse <i>et al.</i> (1983a)
Mannitol dehydrogenase	MAND	(?)	<i>Agaricus</i>	C	May and Royse (1981)
					Royse and May (1982a)

Mannosephosphate isomerase	MPI	5.3.1.8	<i>Agaricus</i>	M	May and Royse (1981)
			<i>Lentinus</i>	M	Royse and May (1982a)
			<i>Volvariella</i>	M	Royse <i>et al.</i> (1983a) Royse <i>et al.</i> (1987)
4-methylumbelliferyl phosphatase	MUP	3.1.3.-	<i>Pleurotus</i>	R	May <i>et al.</i> (1988)
Nothing dehydrogenase	NDH	1.1.1.-	<i>Pleurotus</i>	S4	May <i>et al.</i> (1988)
Nucleoside phosphorylase (or Purine-nucleoside phosphorylase)	NP	2.4.2.1	<i>Pleurotus</i>	S4	May <i>et al.</i> (1988)
			<i>Volvariella</i>	C	Royse <i>et al.</i> (1987)
Peptidase with glycyl-leucine	PEP-GL	3.4.11-13	<i>Agaricus</i>	R	May and Royse (1981)
			<i>Pleurotus</i>	S4, R	Royse and May (1982a)
			<i>Lentinus</i>	R	May <i>et al.</i> (1988)
			<i>Volvariella</i>	R	Royse <i>et al.</i> (1983a) Royse <i>et al.</i> (1987)
Peptidase with leucyl-glycyl-glycine	PEP-LGG	3.4.11-13	<i>Pleurotus</i>	S4, R	May <i>et al.</i> (1988)
Peptidase with leucyl-leucyl-leucine	PEP-LLL	3.4.13.13	<i>Agaricus</i>	M	May and Royse (1981)
			<i>Lentinus</i>	M	Royse and May (1982a)
			<i>Volvariella</i>	R	Royse <i>et al.</i> (1983b) Royse <i>et al.</i> (1987)
Peptidase with phenyl-alanyl-proline	PEP-PAP	3.4.11-13	<i>Pleurotus</i>	S4, R	May <i>et al.</i> (1988)
		3.4.13.1	<i>Volvariella</i>	M	Royse <i>et al.</i> (1987)
Phosphoglucomutase	PGM	2.7.5.1	<i>Agaricus</i>	S9	Royse and May (1989)
			<i>Pleurotus</i>	S4,R	May and Royse (1988)
				C	May <i>et al.</i> (1988)

		5.4.2.2	<i>Volvariella</i>	R	Royse <i>et al.</i> (1987)
Phosphogluconate dehydrogenase	PGD	1.1.1.46	<i>Agaricus</i>	C	May and Royse (1981)
			<i>Pleurotus</i>	C	Royse and May (1982a)
		1.1.1.44	<i>Lentinus</i>	R	May and Royse (1988)
			<i>Volvariella</i>	R	May <i>et al.</i> (1988)
Phosphoglycerate kinase	PGK	2.7.2.3			Royse <i>et al.</i> (1983a)
			<i>Agaricus</i>	C	Royse <i>et al.</i> (1987)
			<i>Pleurotus</i>	C	May and Royse (1981)
			<i>Lentinus</i>	C	Royse and May (1982a)
Pyruvic kinase	PK	2.7.1.40			May <i>et al.</i> (1988)
			<i>Agaricus</i>	?	Royse <i>et al.</i> (1983a)
					Royse <i>et al.</i> (1983b)
Shikimic kinase	SK	?	<i>Agaricus</i>	?	Royse and May (1989)
Shikimic dehydrogenase	SKDH	1.1.1.25	<i>Pleurotus</i>	C	May and Royse (1988)
Superoxide dimutase	SOD	1.15.1.1			May <i>et al.</i> (1988)
			<i>Agaricus</i>	M, R, C	May and Royse (1981)
			<i>Lentinus</i>	M	Royse and May (1982a)
			<i>Volvariella</i>	C	Royse <i>et al.</i> (1983a)
Triosephosphate isomerase	TPI	5.3.1.1			Royse <i>et al.</i> (1983b)
			<i>Pleurotus</i>	S4	Royse <i>et al.</i> (1987)
					May <i>et al.</i> (1988)
Xanthine dehydrogenase	XDH	(?)	<i>Lentinus</i>	R	Royse <i>et al.</i> (1983a)

A question mark (?) means that the information could not be found in the literature.

* S4; Selander et al. (1971): electrode- tris base (0.223 M), citric acid (0.094 M), adjusted to pH 6.3 with NaOH; gel- tris base (8 mM), citric acid (3mM), adjusted to pH 6.7 with NaOH.

S9; Selander et al. (1971): electrode- tris base (0.1 M), maleic acid (0.1 M), NaEDTA (0.01 M), MgCl (0.01 M), NaOH (0.124 M); gel- a 1 : 19 dilution of the electrode buffer was used for gel preparation. (pH 8.0)

C; Clayton and Tretiak (1972) modified by May and Royse (1981): electrode- citric acid (0.04 M), adjusted to pH 6.1 with *N* -(3 aminopropyl)-morpholine; gel- a 1 : 9 dilution of this electrode buffer was used for gel preparation.

R; Ridgway et al. (1970): electrode- lithium hydroxide (0.06 M), boric acid (0.3 M); gel- tris base (0.03 M), citric acid (5 mM), lithium hydroxide (0.6 mM), boric acid (3 mM). (pH 8.2)

M; Markert and Faulhaber (1965) : stock solution ; tris (0.9 M), boric acid (0.5 M), EDTA (0.02 M), pH 8.7; electrode- a 1:5 dilution of stock solution was used for the electrode buffer (pH 8.6); gel- a 1:20 dilution of stock solution was used for the gel buffer (pH 8.4).

May and Royse (1981) and Royse and May (1982a) screened *Agaricus bisporus* tissues for the presence of over 90 enzymes, and found 20 to be active. Of the 20 enzymes they found to be active, only GPI and MDH were found to be monomorphic within *Agaricus bisporus*, while AAT, ADH, FDH, GDH, GK, GPT, MPI, PEP-LLL were polymorphic. The other 10 enzymes for which they found activity were not described further. For the phylogenetic analysis of *Agaricus* species, the 2 enzymes they found to be monomorphic within *Agaricus bisporus* may be more useful than those they found to be polymorphic, because low polymorphism within species relative to between species is desirable for species level phylogenetic studies (Baverstock and Moritz 1990). Of the enzymes May and Royse (1981) and Royse and May (1982a) found to be polymorphic, only MPI, PEP-LLL, GPT, and ADH electromorphs were genetically interpretable and of sufficient resolution to enable their use as markers. If they also found the monomorphic enzymes, GPI and MDH, to be genetically interpretable, and equal to MPI, PEP-LLL, GPT, and ADH in resolution, this implies that of the 20 enzymes they found to be active in *Agaricus bisporus*, only 6 were genetically interpretable and of sufficient resolution for use.

An additional 37 enzymes were screened for activity in *Agaricus bisporus* mycelial extracts by Royse and May (1989), who found no activity for twenty undescribed enzymes, and poor resolution for GAPDH, HA, and IDH. Royse and May (1989) were first to report *Agaricus bisporus* mycelial ACP, EST, and PGM activity, which they reported to be polymorphic. They found another 11 enzymes to be monomorphic; α GLU, CAT, GLUR, GDA,

LDH, MDH, ME, PEP-PAP, PGD, PK, and SK, but did not report on the resolution. These monomorphic enzymes may be especially useful for *Agaricus* phylogenetic analysis in addition to GPI and MDH, (May and Royse 1981).

Thirteen of the enzymes May and Royse (1981) and Royse and May (1982a) found to be active in *Agaricus bisporus* were also found to be active in *Agaricus campestris* (May and Royse 1982b). Of these thirteen enzymes, adequate resolution was only noted by May and Royse (1982b) for nine; AAT, BGLU, DIA, GDH, GPI, MDH, MPI, PEP-GL, and PEP-LLL. No monomorphic enzymes were reported for *Agaricus campestris* and intra-species allozyme variability may generally be greater than for *Agaricus bisporus* (May and Royse 1982b). Levels of allozyme polymorphism for *Agaricus* species other than *Agaricus bisporus* and *Agaricus campestris* have not been published.

Comparison of the results reported in May and Royse (1981), (1982b), and Royse and May (1982a) shows that of the enzymes screened, only four; GPI, MDH, MPI, PEP-LLL may be sufficiently resolved for accurate scoring in both *Agaricus bisporus* and *Agaricus campestris*. Ferguson (1988), suggested that a minimum of ten loci are necessary for interspecific studies. Thus it may therefore be concluded that further allozyme study is needed to adequately resolve a sufficient number of putative loci from *Agaricus* species for phylogenetic analysis.

2.5.2. Choice of material for analysis.

2.5.2.1. Introduction.

Both the quantity and quality of the allozyme information available for a phylogenetic analysis may be affected by the choice of material selected for study. The goal of this review was to determine if there were any reports in the literature to indicate; 1) if the morphological part and developmental stage of *Agaricus* species have been shown to differ in allozyme information, 2) the methods of standardisation necessary when selecting material for analysis, and 3) the most appropriate material for analysis.

2.5.2.2. Effect of tissue.

The amount of allozyme information available on an *Agaricus* species may be increased by increasing the number of morphological parts studied, because genetic factors vary depending on the part investigated, (Richardson et al. 1986). Some *Agaricus bisporus* enzymes found in both basidioma parts and mycelium have been reported polymorphic, e.g., cytochrome oxidase (CTO), (Paranjpe et al. 1979), and ADH (May and Royse 1982a). Therefore electromorph information available on *Agaricus bisporus* derived by screening both basidioma and mycelial extracts for CTO and ADH is greater compared with studying only one of these tissues. This may be compared with *Agaricus bisporus* enzymes found to be identically expressed in pileus and mycelium, e.g., GPT, MPI, PEP-LLL, and AAT, (May and Royse 1982a), for which the screening of either part alone will provide the maximum quantity of electromorph information.

Certain enzymes have been reported present in the basidioma parts but not in the mycelium, e.g., ADA, DIA, GDH, LAP, ME, PGD, PGK for *Agaricus bisporus*, and DIA, and GDH for *Agaricus campestris*, (Royse and May 1982a, May and Royse 1982b). Enzymes have also been reported present in mycelium but not in basidioma parts, e.g., FDH for *Agaricus bisporus*, and ADH, GK, GPT, and PGK for *Agaricus campestris*, (Royse and May 1982a, May and Royse 1982b). Thus additional electromorph information may be gained by screening particular morphological parts for enzymes shown to be active only in those parts.

Isozymes specific to particular tissues have been reported among several Basidiomycete genera, e.g., Okunishi et al. (1979), found a variation in the distribution of EST isozymes between mycelia, primordia, stipe, pileus and hymenia of *Lentinus edodes*, *Coprinus kimurae* and *Polyporellus brumalis* basidiomata.

2.5.2.3. Developmental stages.

The study of developmental stages may supply more phylogenetically useful information compared with that obtainable from a single stage, (Kluge 1985). This is because genetic control factors of an organism change during development, (Richardson et al. 1986). However, unless the developmental stages of the organisms under study can be recognised, there are likely to be problems in standardising results. Hennig (1966) advocated the study of taxa of a similar developmental stage, as a source of characters for phylogenetic study, thereby achieving standardisation of study material.

Isozyme electrophoresis has been used by Paranjpe et al. (1979) in a study of different basidioma developmental stages. They suggested that during basidioma development from mycelium, additional forms of CTO were induced. A 'mushroom', isozyme study by Ingebrigtsen et al. (1989), presumably of *Agaricus bisporus*, although this was not explicitly stated, showed that catechol oxidase activity varied with the developmental stage while dihydroxy-o-phenylalanine oxidase, and tyrosinase activities decreased during development. They also showed that the number of SOD isozymes increased from 1-2 for immature basidiomata to 2-4 during the elongation and maturity stages, and variable numbers of SOD isozymes were present during basidioma senescence.

The stage of development of basidiomata of *Agaricus* species collected from the field for analysis has not been noted, (Royse and May 1982a, and May and Royse 1982b), which indicates a lack of standardisation and potential problems in interpretation of results. It may be possible that certain enzymes, e.g., GK, GPT, and PGK, not found in the basidioma parts of *Agaricus campestris*, (May and Royse 1982b), but found in basidioma parts of *Agaricus bisporus*, (Royse and May 1982a), were influenced not by species difference, but by the stage of basidiomata development when harvested.

Because determination of stage of development of basidiomata of *Agaricus* species or specimens of the same species from the field is subjective, the use of mycelium from specimens

grown under standardised controlled conditions would allow a more objective estimate of developmental stage.

2.5.2.4. Conclusions.

From the information in the literature it would appear that prior to beginning an allozyme study of *Agaricus* species it may be necessary to investigate the effect of different tissues and different stages of development on allozymes. Because mycelium from *Agaricus* species may be grown in controlled and standardised conditions, the use of cultured mycelium may prove to be more appropriate for study than basidiomata parts collected from the field.

2.5.3. *Agaricus* culture methods.

Culture of *Agaricus* mycelia from field collected specimens for allozyme analysis has been carried out using a variety of methods e.g., agar culture (van Loon et al. 1986), stationary liquid culture (May and Royse 1981), and shaken liquid culture (Raper and Kaye 1978, Paranjpe et al. 1979). In this review, the effect of culture methods used to grow mycelium for allozyme analysis will be examined.

The most common mycelial culture method for Basidiomycete allozyme analysis has been stationary liquid culture, e.g., *Agaricus* (May and Royse 1981), *Pleurotus* (Kulkarni et al. 1986, May and Royse 1988, and Magae et al. 1990), and *Lentinus* (Toyomasu and Zennyozzi 1981, Royse et al. 1983a, 1983b, and Ohmasa and Furukawa 1986). For mycelial production, agar culture has been recognised as being inefficient

—in terms of mass of mycelium produced over time compared with stationary liquid culture (Royse and May 1982a).

The successful use of liquid culture may vary between species. While Raper and Kaye (1978) found many *Agaricus* species grew poorly in shaken liquid culture, Paranjpe et al. (1979), who used similar growing conditions, did not report any problems with this technique for *Agaricus bisporus*. Mycelium of ectomycorrhizal fungi have been reported to grow faster in shaken liquid culture when compared with stationary liquid culture (Sasek 1989). Similarly, an increase in *Lentinus edodes* mycelial dry weight per ml was reported by Song et al. (1987), when shaking frequency was increased from 0 to 150 rpm.

It may be concluded that adequate comparison of the effect of culture techniques for *Agaricus* mycelial production could not be found in the literature.

2.5.4. Allozyme variation between replicate cultures.

Evers and Ross (1983) did not find any isozyme variation between mycelia of the same strain grown in replicate culture vessels. Theirs was the only reference found in the literature that made mention of this topic.

2.5.5. Effect of method and length of storage on *Agaricus* mycelial allozymes.

2.5.5.1. Introduction.

A study of the literature on storage of *Agaricus* mycelia used for allozyme analysis revealed that a variety of methods and time periods have been used, e.g., Liming et al. (1987) stored vacuum filtered mycelium at 4°C for 67 days, while van Loon et al. (1986) stored extracted mycelial supernatant for an unreported period at -72°C, and Vilgalys (1991) stored freeze dried mycelium at -20°C for 4-8 weeks. In this review, the effect of mycelial storage methods used for allozyme analysis will be discussed.

2.5.5.2. Vacuum filtered mycelium

Liming et al. (1987) reported that EST activity of fresh or vacuum filtered *Agaricus bisporus* mycelium decreased with storage from 1 to 67 days at 4°C. They considered only fresh mycelium to be suitable for studying this enzyme. From their results it may be concluded that 4°C was not a suitable temperature for adequate *Agaricus bisporus* enzyme preservation. However, without evaluation of storage temperatures other than 4°C, it would be inappropriate to conclude that only fresh mycelium is suitable for study.

Several studies have used fresh mycelium stored at -70 °C or below, e.g., Kulkarni et al. (1986), Royse et al. (1983a, 1983b), and Royse and May (1987, 1989), but none of these studies reported on the success or otherwise of this method for particular

enzymes. Therefore the effectiveness of storing vacuum filtered mycelium before analysis at -70°C or less can not be assessed from the literature.

2.5.5.3. Extracted mycelial supernatant.

Agaricus bisporus mycelium was extracted prior to storage of supernatant for an unreported period at -72°C by van Loon et al. (1986). Compared with the freshly extracted mycelium, they reported lower ADH and MDH activity in stored mycelial supernatant, but they did not report any loss of EST and cytochrome oxidase (CTO) resolution using stored extracted mycelial supernatant. Because they found only some enzymes to be preserved, it may be concluded that the effectiveness of storing extracted mycelial supernatant may vary depending on the enzyme investigated.

2.5.5.4. Freeze dried mycelium.

The freeze drying of mycelium before analysis was reported to cause reduced activity of CAT and GDH (Hanafusa 1973 and Chaillot 1976). Similar problems were also encountered by Vilgalys (1991) who found that freeze drying of *Collybia dryophila* mycelium and storage at -20°C was successful for 4-8 weeks, but did not preserve enzyme activity over "prolonged" periods. However, Evers and Ross (1983) reported that MDH activity from freeze dried mycelia stored at -20°C for undefined periods, was similar to those of fresh mycelia of *Coprinus congregatus* and *Schizophyllum commune*. Differences between the results of Vilgalys (1991) and Evers and Ross (1983), who both used PAGE, may have been due to species and/or length of the

storage periods. It may be concluded that a consensus has not been reached as to the effectiveness of storing freeze dried mycelium.

2.5.6. Effect of culture method and harvest time on mycelial enzymes.

Effect of culture method.

Standardisation of results and retention of potentially useful phylogenetic information within and between studies may depend on the form of mycelium studied, (Okunishi et al. 1979 and Liming et al. 1987). Differences in EST banding patterns were found between mycelium grown in shaken liquid culture and aerial mycelium cultured on agar for *Lentinus edodes*, *Coprinus kimurae* and *Polyporellus brumalis* by Okunishi et al. (1979). Differences were also obtained by Liming et al. (1987) for *Agaricus bisporus* EST banding patterns for mycelium grown in shaken compared with stationary liquid cultures.

Use of different forms of mycelium has expanded the number of detectable enzymes in an allozyme study of *Agaricus bisporus* by Kerrigan and Ross (1989). Surface and submerged mycelium was used and they reported that specific forms of mycelium were more useful for the resolution of certain allozymes than were others. Increasing the number of detectable enzymes through the use of different forms of mycelium may be an advantage for phylogenetic studies based on information generated from a small number of putative loci.

The quantity of mycelium alone has often been the main factor for determining the choice of culture method. For example, Royse and May (1982a) used stationary liquid culture because it allowed them to "produce a larger amount of vegetative mycelium than could be practically produced on agar plates". Because of the electromorph differences reported by Okunishi et al. (1979), and Liming et al. (1987), it would appear that the quantity of mycelium produced should not be the only priority when considering different mycelial forms produced by different methods.

Effect of harvest time.

Liming et al. (1987) reported the development of additional EST bands in *Agaricus bisporus* over time in shaken and stationary liquid cultures. The development of additional EST bands was slower in stationary liquid culture than in shaken culture, and by 30 days after inoculation stationary liquid cultures showed similar patterns to those that had been grown in shaken culture for 10 days. It may be concluded from the work of Liming et al. (1987), that mycelial allozyme changes may occur during incubation, and if unaccounted for, such differences may be confused with those due to species differences. This may introduce errors into phylogenetic analysis based on such information.

In allozyme studies of *Agaricus* species the general practice has been to harvest mycelium from culture at a time when a quantity of mycelium had developed that satisfied the needs of the investigators, e.g., May and Royse (1981) harvested standing

liquid cultures "after the mycelium had grown for approximately 2-4 weeks", and Kerrigan and Ross (1989) harvested stationary cultures, (except for biweekly manual agitation) after 3-6 weeks of incubation. Until further research on mycelial allozyme variability during incubation is carried out, there is cause for concern over the use of randomly selected mycelial harvest dates, as used by May and Royse (1981), and Kerrigan and Ross (1989).

Two main conclusions may be drawn from the literature; 1) a standardized form of mycelium and time of mycelial harvest may be important to ensure the phylogenetic usefulness of data, and 2) the effects of using different forms of mycelium need investigation.

2.6. Allozymes in phylogenetic studies.

Allozymes were commonly analysed phylogenetically after transformation into genetic distances, fitting a phylogenetic tree to a matrix of pair-wise dissimilarities among taxa, (Swofford and Olsen 1990). However, information was lost during this transformation, weakening the argument for the use of distances over other data for phylogenetic analysis, (Farris 1981). The use of allozyme character data has largely replaced distance data for phylogenetic analyses, (Swofford and Olsen 1990) but use of character data remains problematic, as shown below.

2.6.1. Cladistic analysis of allozyme character data.

Cladistic analyses of allozyme characters has generally been based on qualitative binary coding by treating the electromorph as the character, and its presence or absence as the character state

—(Mickevich and Johnson 1976). They suggested that electromorph absence may be defined as the frequency of the electromorph less than an arbitrary cut off point, the placement of which is subjective. As an alternative to treating the electromorph as the character, electromorph frequency may be considered an independent character, (Buth 1979 and Simon 1979). Characters are assumed to be independent homologous variables, thus avoiding the need for statistical covariance analysis among characters, (Swofford and Olsen 1990). However treatment of electromorphs using discrete character coding methods violates of the assumption of independence of characters (Mickevich and Mitter 1981, 1983). If electromorph frequencies at a putative locus are constrained to sum to one for each hypothetical ancestral taxon, (the additivity requirement), and the frequency of an electromorph increases, the frequency of at least one other electromorph must decrease, (Swofford and Olsen 1990). Some allozyme analyses based on the independent allele model of Mickevich and Mitter (1981) may produce hypothetical ancestral taxa with no electromorphs at all for some putative loci, negating evolutionary interpretation of tree branch lengths, (Swofford and Berlocher 1987).

The problems associated with the independent allele model lead to the recognition of the putative locus as the character (Mickevich and Mitter 1981, Buth 1984). Arrays of electromorph frequencies were recognised by them as multi-states, in which frequencies at each putative locus constrained to the additivity requirement. However, when many different electromorphs occur in varying arrangements between taxa, the number of unique

—electromorph arrays may approach or equal the number of taxa (Swofford and Olsen 1990), negating the use of this method.

Four main interrelated problems concerning the use of the independent allele model for coding allozyme data have been discussed by Murphy (1993). The first of these problems is the parallel loss of relatively primitive alleles, where the loss of an electromorph may be considered as a synapomorphy. Therefore he concluded that, as suggested by Hecht and Edwards (1976), the loss of an allele should not be given the same weight as the gain of an allele. The second problem discussed by Murphy (1993) (1993) was, that of unequal weighting of polymorphic loci. He suggested that tree structure may be affected by the number of electromorphs per putative locus, concluding that the “greater the number of alleles at a locus, the greater the weight of that locus”. This problem was also recognised by Moran et al. (1990), who developed a method whereby each electromorph was weighted as a character by $1/n$, where n was the number of electromorphs at the putative locus. Other problems discussed by Murphy (1993) that may affect character polarisation and tree structure include character conflicts arising when no electromorphs are shared with the outgroup, and polymorphisms in the outgroup resulting in false hypotheses. He concluded that the independent allele model was not valid for analysis of allozyme data, even when the procedure had been modified to remove one or more of the inherent problems, and recommended the use of his mutation model for coding ordered electromorph arrays. However, his model is likely to work best on putative loci with only two electromorphs, as the increased complexity with any greater

—number of electromorphs makes it impractical, (Rodrigo 1993, pers. comm.).

Methods in character-based analyses have been developed that do not need electromorphs to be recorded as discrete states, instead these methods rely on the electromorph frequencies (Rodgers 1984, 1986, Swofford and Berlocher 1987). However, Crother (1990) suggested that electromorph frequencies could not be synapomorphic, as they fluctuate in populations over time. Thus frequency data themselves may be inappropriate for phylogenetic analysis, even though appropriate analytical methods have been developed, (Crother 1990).

From the literature it can be concluded that the use of allozymes for phylogenetic analysis has been an extremely dynamic field. While there are problems with the independent allele model for phylogenetic analysis of allozymes, (Swofford and Olsen 1990, Murphy 1993), it is an established method, (Murphy 1993) that will continue to be used until a viable alternative has been provided. However, as reported by Murphy (1993), it is important to recognise the limitations of the method and the consequences for phylogenetic inference based upon its use.

2.6.2. Allozyme analysis using Dollo parsimony.

Application of Dollo parsimony, (Farris 1977) to allozyme analysis minimises the total number of electromorph losses, and does not allow for independently originating electromorphs, (Olmstead 1989). This may be compared with Fitch and Wagner parsimony, (Kluge and Farris 1969, Farris 1970, and Fitch 1971)

—which minimises the total number of gains or losses, (Olmstead 1989). Dollo parsimony has been preferred over Fitch and Wagner parsimony for allozyme analysis by Olmstead (1989) because he contended that it reflects more accurately the transfer of electromorphs during speciation. His argument was based on two hypotheses, the first was also an axiom for phylogenetic systematics generally which says that species inherit alleles from their ancestors, and the second says that the parallel loss of alleles is relatively more common than is parallel origin through mutation, (Olmstead 1989). The application of Dollo parsimony to allozyme data appears logical considering it is likely to be easier to lose an allele than it is to gain one. While only single nucleotide deletions may result in the parallel loss of an allele, it is necessary for the entire nucleotide arrangement of an allele to be duplicated for parallel origin to occur.

2.6.3. Allozyme analysis and the effects of sample size.

Adequate sample size for an allozyme based systematic study depends upon; 1) specimen availability (Hillis 1987), 2) the level of allozyme variability within the taxa being studied, (Baverstock and Moritz 1990), and 3) the type of allozyme data being collected and any transformations performed, (Swofford and Berlocher 1987), e.g., discrete character and electromorph frequencies transformed into a genetic distance measures.

The most frequent argument against small numbers of specimens is that electromorphs of low frequency are less likely to be detected when using small numbers than when larger sampling strategies are employed, (Swofford and Berlocher 1987).

The use of a single specimen from a one population of a species was shown by Nei (1978) and Gorman and Renzi (1979), to have minimal effect on genetic distances calculated from electromorph frequencies, given that a relatively large number of putative loci were examined. However, Archie et al. (1989), in a re-examination of the work of Gorman and Renzi (1979) concluded that small sample size did decrease the confidence in genetic distances. They recommended a pilot study using "large" numbers of organisms to estimate genetic variability, and if this was not possible they recommended screening "reasonable samples (at least 20 individuals) for as many taxa as possible...". Swofford and Berlocher (1987) emphasised the importance of sample size specifically when using presence/absence binary coding of electromorphs for cladistic analysis, contending that these analyses may be more affected by small sample size than those using genetic distances.

Collections of small numbers of specimens from many populations in combination with the examination of a "large" number of putative loci, (minimum of 10) has been advocated by Buth (1984), Richardson et al. (1986), and Ferguson (1988). These authors based their recommendations upon the existence of fixed electromorph differences, described by Swofford and Berlocher (1987) as follows; "...at any given locus, most pairs of taxa either display similar allelic frequency configurations or else approached complete differentiation." However, debate by Swofford and Berlocher (1987) has caused a re-evaluation of fixed differences. They argued that fixed differences shown in studies using single locus genetic similarities, (Nei 1972, Rogers 1972)

were an artifact of the methods employed. Thus it may not now be justified to base the use of small sample size upon the fixed differences argument.

From the literature reviewed here, it may be concluded that, depending on specimen availability, a pilot study should be used to determine the variability present in the taxa prior to a phylogenetic study based on allozyme information. This is especially relevant when it is planned to carry out presence/absence electromorph coding. Sample size may be based on an understanding of the variability present in the taxa under investigation. The lower the level of variability, the fewer the samples needed.

2.6.4. Allozyme analysis and fungal systematics

Phenetic application of the unweighted pair group method of arithmetic averages (UPGMA), (Sokal and Sneath 1963), to the clustering of genetic distances, usually those derived by Nei (1972) or Rodgers (1972), have been commonly applied to fungal systematic studies using isozyme techniques, e.g., for *Agaricus bisporus*, (Royse and May 1982b, 1989), *Lentinus edodes*, (Royse and May 1987), the genus *Morchella*, (Gessner et al. 1987, Royse and May 1990), the genus *Pleurotus* (May and Royse 1988), the genus *Leptographium*, (Zambino and Harrington 1992).

However, Stasz et al. (1989), used isozyme technology in combination with cladistic analysis in a study of the genus *Trichoderma*. Based on the broad grouping of strains according to species identified based on morphology, they concluded that their

methods enabled further testing and modification of traditional species relationship hypotheses.

2.7. Comparison of allozymes and morphology in systematics.

It is a requirement that characters used for phylogenetic analysis have explicit and independent genetic basis, (Wiley 1981). This may be possible to demonstrate for allozymes, (May 1992), but is often not possible for morphological characters, (Patterson 1982), under the control of many alleles and influenced to varying degrees by the environment, (Hoiland 1983). For example it is important to assess the effects of environmentally induced morphological variation, a factor frequently noted among *Agaricus* basidiomata found in the field, (Cappelli 1984).

Mickevich and Johnson (1976), reported that following phylogenetic analyses of carefully selected morphological and allozyme characters, a direct comparison of hypotheses of relationships can be made from the different character types. They suggested that such a comparison allows evaluation of the relative merits of the different data sets and methods of analysis. It was pointed out by Mickevich and Johnson (1976) that comparison of cladograms based on different forms of data with traditional classifications does not directly estimate congruence, as most classifications are based on a combination of systematic techniques.

Olmstead (1989) listed three main advantages of using both allozymes and morphology for phylogenetic inference, these were

—“1) two data sets may help resolve relationships in which a single data set is not conclusive...; 2) an alternative estimate of phylogeny, derived from an independent data set, can expose cases of parallel or convergent evolution that would otherwise may not be apparent; 3) a phylogeny based on molecular characters allows one to interpret phenotypic evolution without the circularity of interpreting morphological change from a phylogeny based on morphology.” He concluded that neither allozymes, nor morphology were able to supply sufficient phylogenetically useful information to allow adequate resolution of relationships of *Scutellaria angustifolia* (Lamiaceae) and proposed a phylogeny based on the results of both allozymes and morphology.

Agreement is expected between different data sets analysed using phylogenetic methodology, especially in the absence of homoplasy, (Hennig 1966,), but congruence has been shown even in the presence of significant homoplasy, (Mickevich and Johnson 1976). However, other cladistic analyses by Luykx et al. (1990), incorporating information on both allozymes and morphology have found most parsimonious cladograms not to be in agreement, each supporting different monophyletic groups. They combined the allozyme and morphology data, as compared with forming a consensus between the differing phylogenies, a method advocated by Hillis (1987).

It may be useful to analyse both allozyme and morphological data sets from *Agaricus* species, for which some

agreement is expected. But care is especially needed when assessing appropriate morphological characters.

2.8. General conclusions from the literature.

The use of cladistics is logical when an analysis is required from which inferences may be made regarding common ancestry, evolutionary patterns, and monophyletic groups. The outgroup method is appropriate for estimating character polarities where heterochrony is suspected and/or when it is not possible to study organisms from different ontogenetic stages. The selection of an appropriate outgroup depends on the state of knowledge regarding the relationships of the taxa of interest.

No single species concept is perfect or is likely to meet the requirements of all mycologists. The continued reliance of *Agaricus* classifications on the taxonomic species concept has resulted in difficulties in determining species. However, the application of a phylogenetic species concept to *Agaricus* species would be inappropriate at present because of the lack of consensus regarding this concept.

Because traditional methods are slow, investigation is required to assess faster, more objective methods of measuring basidiospore variables such as size and shape compared with light microscopy. Improved methods, such as computer image analysis, may allow extensive investigation of basidiospores from *Agaricus* species. The additional information obtained from such a technique would allow better assessment of the phylogenetic value of basidiospore characters.

To improve upon the present classification by Cappelli (1984), it is important to be able to assess the phylogenetic relationships of *Agaricus* species. As shown above, for phylogenetic analysis, there is difficulty using characters traditionally considered important for defining *Agaricus* species, because there are few characters which may be objectively determined with a clear genetic basis unaffected by variable environmental influence. Thus new methods need to be evaluated for the generation of characters from *Agaricus* species.

Further studies into the use of allozymes for interspecific investigations are needed to adequately resolve a sufficient number of putative loci from *Agaricus* species for phylogenetic analysis. Before embarking on such a study, investigation is needed into; 1) the effect of culture techniques for the production of *Agaricus* mycelium, 2) methods of storing mycelium before allozyme analysis, 3) mycelial allozyme differences over time in culture.

The use of allozymes for phylogenetic analysis has been an extremely dynamic field. In the absence of a valid alternative, the independent allele model may be used to code electromorphs for phylogenetic analysis, provided the inherent problems with the method are recognised and where possible reduced. Of the cladistic methods available, Dollo parsimony is the most logical choice for allozyme studies. Depending on specimen availability, a pilot study should be used to determine the variability present in the taxa prior to a phylogenetic study based on allozyme

information. The study of fungal systematics using cladistic analysis of allozyme variation, may allow a better estimate of species relationships than has been previously available.

In conclusion it appears likely that analysis of both allozyme and morphological data sets from *Agaricus* species may allow a better estimate of phylogeny than would be available from one of these methods.

Chapter 3.

Methods.

3.1. General Introduction.

The review of literature indicated that many characters traditionally used for the study of *Agaricus* species are of limited use in a phylogenetic study. Thus to improve the validity of the classification the number of phylogenetically useful characters needs to be increased. The study of allozymes, (Murphy et al. 1990, May 1992) may be one method of doing this.

Many of the experimental procedures used for allozyme analysis of *Agaricus* species have not been fully investigated. Therefore, it was necessary to standardise the experimental techniques, and to eliminate as many of the potential sources of error as possible. While allozymes have been used to study *Agaricus* species, particularly *Agaricus bisporus*, using both PAGE, (Kerrigan and Ross 1989), and HSE, (Royse and May 1989), further investigation is required to adequately resolve a sufficient number of putative loci for phylogenetic study, and therefore methods used in PAGE and HSE were evaluated.

Because allozymes have been reported to vary depending on the tissue, (Royse and May 1982a, May and Royse 1982b) allozymes from mycelia and basidioma tissues of *Agaricus bisporus* and *Agaricus bitorquis* were investigated as a source of greater phylogenetic information compared with mycelium alone. As was shown in the literature review, the determination of the developmental stage for basidiomata collected from the field is

subjective, the use of mycelium grown under standard controlled conditions was considered more appropriate.

An adequate comparison of culture techniques for *Agaricus* mycelial production could not be found in the literature, and a decision as to the most appropriate culture method was based on an evaluation of growth rates of *Agaricus bitorquis* in shaken and stationary liquid culture. Mycelial allozyme variation between replicate cultures of the same strain was in need of investigation as only Evers and Ross (1983) were found to have reported on this. Thus, EST variation between *Agaricus bisporus* mycelia grown in different culture vessels in shaken submerged liquid culture was evaluated.

From the review of literature it was found that a consensus had not been reached as to the most appropriate method of storing mycelium before analysis, thus the effects of storage treatments were investigated. Mycelial allozymes are affected by time of incubation, (Liming et al. 1987), and variation may occur between forms of mycelium grown using different culture methods, (Kerrigan and Ross 1989). Therefore an evaluation was carried out to assess the effect these variables may have on the phylogenetic usefulness of data obtained. This was followed by an attempt to estimate similar physiological stages for strains investigated, in an effort to decrease the effects of mycelial age on allozyme expression.

3.2. Culture methods.

Standard conditions and terms relating to culture methods were used throughout this Chapter;

- 1) culture methods, including sterile techniques, used were outlined in Booth (1971),
- 2) sterilisation was carried out by autoclaving at 96-110 kPa and 121 °C, for 20 min.,
- 3) incubation was carried out in the dark at 23-27 °C,
- 4) % dry weight of mycelium was considered to be approximately 10 % that of wet weight,
- 5) fresh mycelium was used to describe freshly harvested mycelium (wet weight),
- 6) liquid nitrogen used to freeze tissues prior to storage or analysis was -196 °C.

3.2.1. Agar culture.

Malt Extract Agar (Oxoid) (MEA), 12 ml, was added to each petri dish used for mycelial culture, and 15 ml used for slopes.

3.2.2. Isolation of cultures.

Basidiomata were collected from the field before bacterial decomposition or major infestation by insect larvae had occurred. Each pileus was broken in half, and a piece approximately 2-3 mm³ was removed with a scalpel from just above the point where the stipe joined the pileus. The piece was placed onto the surface of a MEA plate, and incubated 10-15 days before sub-culturing.

3.2.3. Maintenance of cultures.

Cultures were maintained on MEA slopes. They were incubated for 10-15 days at 23-27°C before being held at 8-12°C. They were subcultured at 6 monthly intervals. Subcultures were

deposited in the International Collection of Microorganisms from Plants, (ICMP) held at Landcare Research New Zealand Ltd., Private Bag 92170, Auckland. ICMP Landcare research accession numbers, original culture name and source are described for each isolate in Appendix 1.

3.2.4. Liquid culture.

Unless otherwise stated, 250 ml Erlenmeyer flasks were used for liquid culture, each contained 100 ml Malt Extract Broth (Oxoid) (MEB). They were plugged with cotton wool, capped with aluminium foil, and autoclaved. Each flask was inoculated with two mycelial plugs, taken from the edge of a growing agar culture with an 8 mm diameter cork borer. When shaken cultures were required the inoculated flasks were placed on a Gallenkamp orbital shaker, SGM 300, set to rotate at 80 rpm. For greater mycelial production 5 litre flasks containing 500 ml of MEB were used. Ten mycelial plugs were used to inoculate each flask, which was then treated as for culture in 250 ml flasks. The mycelium in shaken culture was kept submerged by manually swirling flasks every 3 days to remove any mycelium that was adhering to the sides of the flasks.

3.2.5. Harvesting mycelia.

Except where reported otherwise, incubation of liquid cultures was for 10 days. Mycelium was collected by vacuum filtration of the medium through Whatman No. 1 filter paper using a Buchner funnel, 90 mm diam., into a one litre flask. The mycelium was removed from the filter paper with a spatula and

transferred to a tared small plastic cup, in which it was weighed before extraction in preparation for electrophoresis or storage.

3.3. Mycelial allozyme study using PAGE.

3.3.1 Introduction.

PAGE was investigated as a method to supply allozyme information on *Agaricus* species. At least one strain was tested from each of the species listed in Appendix 1 for enzymes presented in Table 2.1. The methods described below, were used as a basis for investigation. Methods evaluated, included type of extraction buffer, gel concentration and gel components.

3.3.2. Methods.

3.3.2.1. Extraction of fresh mycelium for analysis.

Mycelial extraction and electrophoresis was carried out at 8-12°C. Harvested, weighed mycelium was frozen with liquid nitrogen in an agate mortar and ground with an agate pestle. An equal volume of pH 7.1, 0.05 M Tris-HCl extraction buffer, (May and Royse 1981), was added to the ground mycelium, which was macerated with 20-25 mg 40-100 mesh acid rinsed sand. The resulting homogenate was centrifuged at 10000 X G for 12 min., in a Heraeus Christ Biofuge A, and the supernatant either stored (Section 3.8.2) or used within 1 h. for electrophoresis.

3.3.2.2. Gel preparation, electrophoresis and staining.

A LKB Bromma 2001 vertical electrophoresis unit, and a LKB Bromma 2197 constant power supply unit were used for PAGE. The acrylamide stock solution for PAGE contained 30 g acrylamide, 0.8 g bisacrylamide, and 100ml water. Running gel buffer was 1.5 M Tris-HCl, pH 8.8. Running gel contained 3.0% acrylamide; 14.75 ml water, 3.75 ml running gel buffer, 10 ml acrylamide stock, 1.5 ml of 1.5 % ammonium persulphate and 15 μ l N, N, N, N-tetramethylethylenediamine, (TEMED). Stacking gel buffer was 0.5 M Tris-HCl, pH 6.8. Stacking gel contained 9 ml water, 5 ml stacking gel buffer, 2.5 ml acrylamide stock, 1.0 ml of 1.5 % ammonium persulphate and 15 μ l TEMED. Upper glycine buffer was 1.5 g Tris and 7.2 g glycine in 500 ml water, pH 8.3. Gels were prepared on the day of use. Enzymes were run towards the anode at 30 mA and 15°C, and the running time was generally 5 h. When electrophoresis had been stopped, gels were placed into individual trays, internal volume 150 x 150 x 80 mm. An enzyme stain was then poured into each tray containing a gel. Gels were allowed to incubate, at 30°C in the dark for up to 30 min., or until electromorphs became visible.

3.3.3. Evaluation of PAGE.

Because preliminary experiments did not give adequate enzyme resolution, modifications to the system were tested. These included testing the following buffer systems in addition to those described by May and Royse (1981);

- (1) 0.1 M Tris-HCl, pH 8.0 (Paranjpe et al. 1979),
- (2) 0.005 M Tris-HCl, pH 8.0 (van Loon et al. 1986),
- (3) 0.05 M Tris-maleate, pH 8.0 (Barnes 1993),

The following additions were made to the extraction buffer of May and Royse (1981), described in Section 3.3.2.1;

- (1) 20 mM NAD⁺ (Kerrigan and Ross 1989),
- (2) 12 mM cysteine-HCl (Cheliak and Pitel 1984),
- (3) 0.01 M 2-mercaptoethanol (van Loon et al. 1986),
- (4) a mass of hydrated insoluble PVP equal to that of the sample (modified from Cheliak and Pitel 1984),
- (5) 1 mM dithiothreitol (Cheliak and Pitel 1984),
- (6) 0.01% soluble PVP (Cheliak and Pitel 1984),
- (7) 0.05 % bovine serum albumin (Cheliak and Pitel 1984).

Modifications of the gel described in Section 3.3.2.2, included;

- (1) gel concentrations of 5 % and 7.5 % acrylamide, (Kerrigan and Ross 1989), 10 % acrylamide, (Barnes 1993).
- (2) linear gradient gels from 5-15 % acrylamide, and from 5-20 % acrylamide (Barnes 1993). This was specifically used to try and separate MDH isozyme smears or areas of enzyme activity diffused such that genetic interpretation was impossible.
- (3) the replacement of ammonium persulphate in the stacking gel with 0.004 % riboflavin, (Hames and Rickwood 1981).

Except for EST, none of the changes listed above to the PAGE techniques described in Section 3.3.2.2 resulted in adequate resolution of the enzymes tested. While enzyme activity was found, the main problem was failure of the electromorphs to

separate. However, use of PAGE as described in Section 3.3.2.2, for the study of EST consistently produced sharp single bands of excellent resolution using the extraction buffer and staining solution set out below;

- (1) the extraction buffer used by May and Royse (1981), or this buffer plus 12 mM cysteine-HCl, (Cheliak and Pitel 1984), and a mass of hydrated insoluble PVP equal to that of the sample, (modified from Cheliak and Pitel 1984), and
- (2) the following staining solution: 0.1 M Na phosphate, pH 6.2; 3ml, 1% α -naphyl butyrate in acetone; 100 mg Fast Blue RR Salt, (Vallejos 1983). As little as 25 μ l of the supernatant collected from extracted mycelium was necessary for excellent EST resolution.

EST banding patterns were frequently complex, preventing genetic interpretation. However, single EST electromorphs were observed for *Agaricus bisporus* and *Agaricus bitorquis*.

3.3.4. Conclusions.

From all 50-60 electrophoretic runs made using different extraction buffers and 6 gel modifications with 13 fungal cultures, only EST isozymes were adequately resolved for the strains investigated. It was concluded that PAGE was inappropriate for the generation of information for phylogenetic study. However, PAGE may be useful for the study EST variation in *Agaricus bisporus* and *Agaricus bitorquis*.

3.4. Mycelial allozyme study using HSE.

3.4.1 Introduction.

Investigations by May and Royse (1981, 1982b), and Royse and May (1982a) on *Agaricus bisporus* and *Agaricus campestris* using HSE found only four enzymes to be adequately resolved for both species. It was hoped that through an evaluation of the electrophoretic methods, conditions for the resolution of a greater number of enzymes from a wider range of *Agaricus* species could be determined.

At least one strain was tested from each of the species presented in Appendix 1. The enzymes listed in Table 2.2, and the methods described below, were used as a basis for investigation. Methods evaluated included;

- (1) extraction buffers,
- (2) quantity of mycelium necessary for extraction,
- (3) electrophoresis buffer systems,
- (4) period of electrophoresis prior and subsequent to wick removal, and
- (5) staining systems.

Extraction of fresh mycelium was as carried out as described for PAGE, Section 3.3.2.1, until test results indicated modification of this method was desirable, Section 3.4.3.1.

3.4.2. Methods.

3.4.2.1. Gel preparation.

Eleven percent gels were prepared on the day of use, prior to extraction of samples, by mixing 25 g starch, (potato hydrolysed for electrophoresis), with 75 ml of buffer in a 1 litre conical flask. A further 150 ml of buffer was heated to boiling, and added to the unheated starch-buffer suspension while constantly swirling the flask. The mixture was then immediately heated, with swirling approximately every 5 sec., until boiling. The boiling starch solution was degassed under a vacuum created by a water aspirator for 1 min. Two open horizontal Perspex forms, internal volume 220 x 110 x 3 mm were used for starch gel preparation, Figure 3.1. They were clipped one above the other to

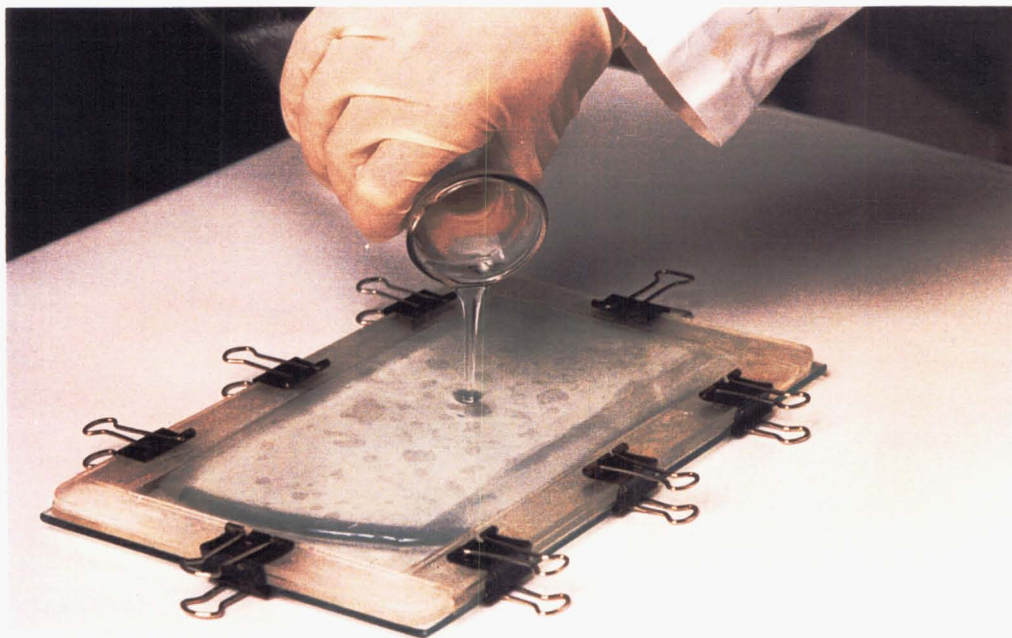


Figure 3.1. Perspex forms, held in place with clips to a glass plate for starch gel preparation. The starch gel forms were placed on a flat, horizontal surface, and the de-gassed starch-buffer solution rapidly poured in.

a glass plate 240 x 130 x 5 mm. The-hot starch solution was poured into the forms which had been placed on a flat, horizontal surface, Figure 3.1. The gel was allowed to cool to room temperature for at least 1 h. before use. The gel forms were removed, and the gel laid onto the surface of the cooling pad of a LKB Bromma 2117 Multiphor II horizontal electrophoresis unit.

3.4.2.2. Gel loading and electrophoresis.

A cut was made across the gel, 25 mm from the cathodal side. Muslin, soaked in buffer, was used to connect the buffer compartments and the gel. A LKB Bromma 2197 constant power supply was used. Prior to the application of samples, pre-electrophoresis, (Gordon 1972) was carried out for 1 h. at 400 V. to facilitate subsequent even sample migration.

MFS filter paper No. 526 was cut to create sample wicks 5 x 15 mm. The wicks were then loaded with 50 μ l of sample, and an additional two wicks were loaded with 0.1 % bromophenol blue as a dye marker to trace electrophoretic migration. The gel was gently pulled apart at the cut, and loaded wicks inserted, 5 mm wick edges evenly pushed down to the cooling plate surface, Figure 3.2. After the proteins had moved from the sample wicks into the starch, as indicated by the marker, electrophoresis was temporarily terminated, the wicks removed, and the gel pushed firmly back together.



Figure 3.2. Individual filter paper wicks, loaded with 50 μ l sample extract supernatant, were inserted into the starch gel.

3.4.2.3. Gel slicing and staining.

After electrophoresis, the gel was sliced horizontally into 2 pieces, Figure 3.3 a, each approximately 3 mm thick. To mark the sides of the gels, the left hand bottom corner was cut away. The slices were separated, Figure 3.3 b, and placed in individual trays, internal volume 260 x 115 x 20 mm, with the sliced surface of the gel facing upward. The appropriate enzyme stain was poured into each tray, and the gels allowed to incubate, at 30°C in the dark, for up to 30 min. or until electromorphs became visible.

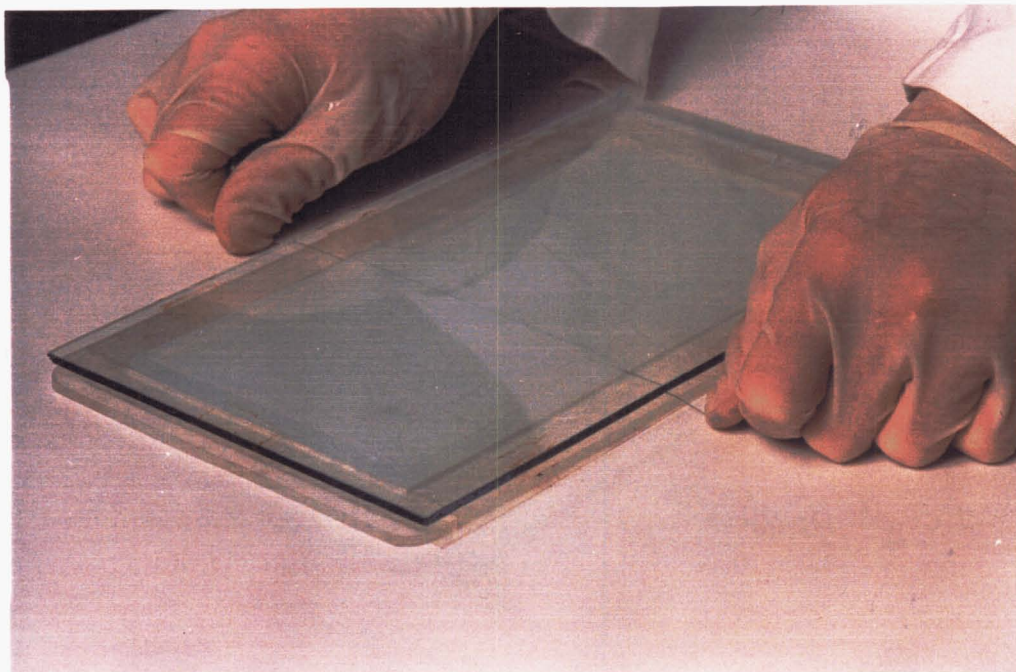


Figure 3.3 a. Gel cutting procedure; 1) the starch gel was placed on a flat surface, 2) one of the perspex forms used during gel preparation was replaced over the gel, 3) a glass plate was placed on top of the gel to hold it down, 4) a piece of wire was pulled horizontally through the gel, along the surface of the perspex form.

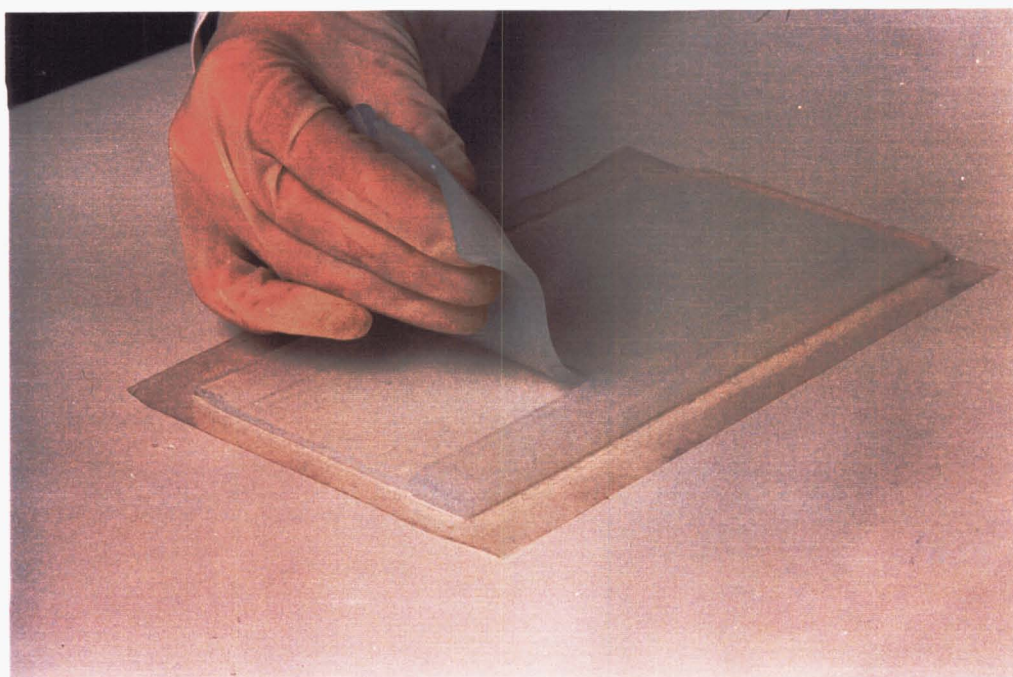


Figure 3.3 b. The two horizontally cut halves of the starch gel were easily separated for subsequent enzyme staining, each half approximately 3 mm thick.

3.4.3. Evaluation of methods and results.

3.4.3.1. Extraction buffers.

Extraction buffers tested for PAGE, Section 3.3.3, were also evaluated for HSE. It was found that the addition of NAD^+ to all extraction buffers hindered the separation and overall migration of electromorphs at all putative loci investigated.

Modified extraction buffer A.

The resolution of all enzymes in Table 3.1, and those in Appendix 2, including succinate dehydrogenase (SUDH), and EST, was improved by the addition of 12 mM cysteine-HCl, (Cheliak and Pitel 1984), and a mass of hydrated insoluble PVP equal to that of the sample, (modified from Cheliak and Pitel 1984) to the extraction buffer used by May and Royse (1981).

Modified extraction buffer B.

The resolution of AAT, and LAP was improved through the addition of both 1 mM dithiothreitol, (Cheliak and Pitel 1984); 0.01% soluble PVP, (Cheliak and Pitel 1984), and 0.05 % Bovine Serum Albumin, (Cheliak and Pitel 1984), to the modified extraction buffer A.

3.4.3.2. Quantity of mycelium extracted.

A range of fresh mycelial wet weights from 0.2-2.0 g, were extracted separately using the method described in Section 3.3.2.1, with the modified extraction buffers described for the individual enzymes in Section 3.4.3.1.

Table 3.1. Enzymes, abbreviations, enzyme commission number (E.C. No.), appropriate buffer systems, running conditions, and reference for staining solutions used in this study. All staining solutions were made up in 100 ml distilled water. Where indicated, staining solutions include 30mg NADH, 30mg NADP⁺, 30mg NAD⁺, 4mg PMS, 20mg NBT, or 20mg MTT.

<u>Enzymes</u>	<u>Abbr.</u>	<u>Loci Studied</u>	<u>E.C. No</u>	<u>Buffer System^a</u>	<u>Conditions</u>	<u>Staining solution^b</u>
<u>Oxidoreductases</u>						
Malate dehydrogenase	MDH	Mdh-1 Mdh-2	1.1.1.37	C	110V, 70mA 7h.	0.1 M Tris, pH7.5; 3ml, 1 M, DL-malic acid, pH 7.5; NAD ⁺ , MTT, PMS.
<u>Hydrolases</u>						
Acid phosphatase	ACP	Acp	3.1.3.2	S4	100V, 60mA 5h.	50mM Na acetate, pH 5.5; 1ml, 1M, MgCl ₂ .6H ₂ O; 3ml, 0.5 % Na a-naphthyl phosphate in 50% acetone; 50mg Fast Garnet GBC Salt.
β-Glucosidase	βGLU	βGlu-2	3.2.1.21?	S4	100V, 60mA 5h.	50mM Na acetate pH 4.3. 15 mg 4-methylumbelliferyl β-D-glucoside in 50% acetone. Observed under long wave UV light.
Hexoseaminidase	HA	Ha-1	3.2.1.52?	C	110V, 70mA 5h.	50mM Na acetate pH 4.3. 15 mg 4-methylumbelliferyl N-acetyl-β-D-glucosaminide.
Leucine aminopeptidase	LAP	Lap-1	3.4.11.1	S4	100V, 60mA 7h.	0.13 M Tris-0.055 M citric acid, pH 5.3; 40mg L-leucine-β-naphthylamide; 2ml 1M MgCl ₂ .6H ₂ O; 35mg Fast Garnet GBC Salt.

<u>Enzymes</u>	<u>Abbr.</u>	<u>Loci Studied</u>	<u>E.C. No</u>	<u>Buffer System^a</u>	<u>Conditions</u>	<u>Staining solution^b</u>
<u>Transferases</u>						
Aspartate aminotransferase	AAT	Aat-1	2.6.1.1	C	110V, 70mA 7h.	<u>Solution A</u> ; 0.2 M Tris, pH 8.0; 200mg α -ketoglutarate ; 600mg aspartic acid. <u>Solution B</u> ; 20mg pyridoxal-5-phosphate; 150mg Fast Blue BB Salt. Mix solution A at least 15min. before adding to solution B.
Glucokinase (Hexokinase)	GK	Gk-1 Gk-2	2.7.1.2?	C	110V, 70mA 6h.	50 mM Tris, pH 8.4; 2 ml, 1M, $MgCl_2 \cdot 6H_2O$; 180mg D-glucose; 130mg ATP Na_2 ; 15mg $NADP^+$; MTT; PMS; 25 units Glucose-6-phosphate dehydrogenase (add just before incub.).
Glutamate pyruvate transaminase	GPT	Gpt-1	2.6.1.2	M	200V, 60mA 5h.	0.2 M Tris, pH 8.0. 40mg L-alanine; 20mg α -ketoglutaric acid; 15mg NADH; 150 units LDH. Deflourescent.
Phosphoglucomutase	PGM	Pgm-2	2.4.2.2?	S9	80V, 130mA 7h.	0.1 M Tris, pH 7.5; 1ml, 1 M, $MgCl_2 \cdot 6H_2O$; 150mg, glucose-1-phosphate; 15mg $NADP^+$; MTT; PMS; 40mg glucose-6-phosphate dehydrogenase (add just before incub.).
<u>Isomerases</u>						
Mannosephosphate isomerase	MPI	Mpi-1	5.3.1.8	S2	200V, 60mA 5h.	5ml, 0.2 M Tris, pH 8.0, 1:4 solution with water; 50mg β -D-mannose-6-phosphate; 100 units PGI; 80 units glucose-6-phosphate dehydrogenase; 5mg $NADP^+$; MTT; PMS.

^a Buffer system.

S2; Described by Selander et al. (1971) as follows: Stock solution A; lithium hydroxide (0.03 M), boric acid (0.19 M); pH 8.1: Stock solution B; Tris base (0.05 M), citric acid (8 mM), lithium hydroxide; pH 8.4: Electrode; Undiluted stock solution A: Gel; 1:9 mixture of stock solution A:B, final pH 8.3, was used for gel preparation.

S4; Described by Selander *et al.* (1971) as follows: Electrode; Tris base (0.223 M), citric acid (0.094 M), adjusted to pH 6.3 with NaOH: Gel; tris base (8 mM), citric acid (3mM), adjusted to pH 6.7 with NaOH.

S9; Described by Selander et al. (1971) as follows: Electrode; Tris base (0.1 M), maleic acid (0.1 M), NaEDTA (0.01 M), MgCl (0.01 M), adjusted to pH 7.4 with NaOH: Gel; a 1 : 9 dilution of the electrode buffer was used for gel preparation.

C; Described by Clayton and Tretiak (1972) modified as follows: Electrode; citric acid (0.04 M), adjusted to pH 8.2 with N -(3-aminopropyl)-morpholine: Gel; a 1 : 9 dilution of this electrode buffer was used for gel preparation.

^b Staining solution.

Source of stain solutions; MDH, ACP, AAT, GK, PGM (Vallejos 1983), GPT, LAP (Allendorf et al. 1977), β GLU, HA, LAP, (Cheliak and Pitel 1984). Stain solutions may have been modified from those given. All gels were incubated at 30°C in the dark.

Samples were electrophoresed, and the gels stained for all the enzymes listed in Table 3.1. Adequate enzyme resolution was obtained by using equal parts of mycelium and extraction buffer for extraction. As a result of these experiments it was found that 0.2 g fresh mycelium was an easily manageable mass that could be used without any loss in electromorph intensity compared with larger amounts.

Experiments were also conducted to find out if the volume of supernatant applied to the gel affected the result. Supernatant volumes, 15-80 μ l, collected from freshly extracted mycelium were electrophoresed and the gels stained for the enzymes in Table 3.1. As little as 25 μ l of supernatant was necessary for the resolution of ACP, while the all other enzymes listed in Table 3.1 required 50 μ l, for adequate resolution. As a result of these experiments it was decided to use 50 μ l extracted mycelial supernatant, and the problem of higher activity for ACP was solved by decreasing the quantity of the stain ingredients Fast Garnet GBC Salt, i.e., 50 mg compared with the 100 mg commonly recommended by other workers, (Vallejos 1983).

3.4.3.3. Electrophoresis buffers.

As a result of experiments testing buffer systems it was found that they were critical for allozyme resolution, and buffer systems that were different or modified from those reported in Table 2.2, were often more appropriate for the resolution of certain enzymes, Table 3.1. Specifically, β GLU, and LAP were better resolved using the S4 buffer system, Table 3.1, compared with the R buffer system, Table 2.2. However, the separation of

LAP and β GLU electromorphs was greater using the R buffer system compared with the S4 buffer system. Poor separation of both LAP and β GLU electromorphs using the S4 buffer system led to difficulties in assessing relative electromorph positions, Section 4.2. The C buffer system, for AAT, GK, HA, and MDH produced greater electromorph separation at pH 8.2, Table 3.1, than at pH 6.1, commonly used in the studies presented in Table 2.2.

3.4.3.4. Period of electrophoresis prior to sample wick removal.

When intending to study any of the enzymes listed in Table 3.1, except AAT and LAP, sample wicks were removed after 3-5 min. of electrophoresis or as soon as the dye marker had fully entered the gel from the origin. This was critical to avoid band smearing for β GLU, ACP, and HA. Bands of AAT, and LAP, were denser, showing much more activity, and little smearing when sample wicks were left in for 10-15 min. or until the dye marker had migrated approximately 1 cm into the gel from the origin.

3.4.3.5. Staining systems

Results of experimentation testing buffer pH, quantity of substrate, and the dye compounds indicated that staining systems were very important for allozyme visualisation, Table 3.1. Use of a stain solution buffered to pH 5.3 (Cheliak and Pitel 1984), with 40 mg L-leucyl- β -naphthylamide per 100 ml solution, and 35 mg Fast Garnet GBC Salt improved LAP visualisation compared with the stain used by May and Royse (1981). They used a stain solution buffered to pH 7.0, with 10 mg L-leucyl- β -naphthylamide per 100 ml solution and 20 mg Fast Garnet GBC Salt. β -GLU

-staining solution buffered to pH 4.3 improved β -GLU visualisation compared with the solution used by May and Royse (1981) which had a final pH of 8.0.

3.4.3.6. Enzyme systems which may be useful for future investigations.

As a result of these experiments it was found that there were present in addition to those listed in Table 3.1, a number of enzyme systems requiring modification to facilitate their use in future study of *Agaricus* systematics, which could also be useful, unmodified, for the study of a limited number of species; these are described in Appendix 2.

3.4.4. Discussion and conclusions.

3.4.4.1. Extraction buffers.

Cysteine-HCl, plus insoluble PVP were also useful additions to the extraction buffer used by May and Royse (1981) because of the enzyme protection they provided, (Barnes 1993). The inclusion of dithiothreitol, soluble PVP, and BSA, in the extraction buffer may have improved AAT and LAP resolution because, Barnes (1993) indicated that 1) dithiothreitol acts to decrease oxidation of thiols which form cross links with enzymes rendering them inactive, 2) Soluble PVP absorbs phenols and decreases enzyme denaturation, 3) BSA acts as a decoy for proteases and absorbs quinones.

3.4.4.2. Quantity of mycelium -extracted.

Extraction of 0.2 g of fresh mycelium with 200 μ l extraction buffer was sufficient to obtain enough enzyme for resolution. From each extracted mycelial sample, a minimum of 50 μ l supernatant was needed for electrophoresis.

3.4.4.3. Electrophoretic buffers.

Considering the differences between the R and S4 buffer systems, Table 2.2, the most likely reason for the greater separation of *Agaricus* mycelial LAP and β GLU electromorphs, achieved using the R buffer system, (electrode buffer pH of 8.3, and a gel buffer pH of 8.4) compared with the S4 buffer system, (electrode buffer pH of 6.3, and gel buffer pH of 6.7), was the difference in pH between these systems.

Use of the C buffer system at pH 8.2 increased electromorph migration and improved the resolution for AAT, GK, HA, and MDH when compared with pH 6.1 used by May and Royse (1981). Because a higher pH may cause greater electromorph mobility, the pH of 8.2 for the C buffer system may have been responsible for the demonstration of a second AAT putative locus for *Agaricus bisporus* during this study, where as only one had been previously reported for this species, (May and Royse 1982a). Using the C buffer system at pH 6.1 *Agaricus campestris* Mdh-1 electromorphs, were reported to migrate cathodally, while other electromorphs for this putative locus remained at the origin (May and Royse 1982b). Further migration of MDH electromorphs for *Agaricus* species with a C buffer pH of 8.2 should allow increased scoring accuracy for MDH electromorphs compared with a pH of

6.1. That Mdh-1 electromorphs no longer remained close to the origin at the higher pH, is an example of this phenomenon.

3.4.4.4. Period of electrophoresis prior to sample wick removal.

It was found to be important to control the length of time prior to sample wick removal as this factor was shown to influence directly the clarity of electromorphs after staining. *Agaricus* mycelial enzymes with higher activity levels, prone to smearing, e.g., ACP, β GLU, and HA, required less time of electrophoresis prior to sample wick removal compared with enzymes with less activity, e.g., AAT, and LAP.

3.4.4.5. Staining systems.

The staining system for LAP adopted in this research project was improved compared with that used by May and Royse (1981), who were able to detect LAP activity for *Agaricus bisporus* pileus and stipe extracts only. Compared with differences in buffer systems, changes to the stain solution contributed significantly to the successful resolution of LAP. The most effective staining system included; 1) a stain solution buffered to pH 5.3, compared with pH 7.0 used by May and Royse (1981). 2) use of twice the amount of substrate compared with their study, and 3) use of the dye Fast Garnet GBC Salt compared with their use of Black K Salt. Staining buffer pH was also a significant factor in obtaining adequate β -GLU resolution. The pH of the β -GLU staining buffer used in this study was pH 4.3, compared with 8.3 used by May and Royse (1981) and Royse and May (1982a).

3.4.4.6. Mycelial enzymes suitable for full character analysis of strains.

Criteria for the selection of enzymes suitable for full character analysis of all strains included;

- 1) adequate resolution of bands,
- 2) repeatability,
- 3) genetically interpretable banding patterns, and
- 4) electromorph similarities and differences between species.

Based on the results of this investigation, ten mycelial enzymes encoded by twelve polymorphic putative loci, Aat-1, Acp, β Glu, Gk-1, Gk-2, Gpt, Ha-1, Lap-1, Mdh-1, Mdh-2, Mpi, and Pgm-2, Table 3.1, were selected for full character analysis of all strains. This was despite a maximum of four enzymes being reported to be resolved in both *Agaricus bisporus* and *Agaricus campestris*, using HSE (May and Royse 1981, 1982b, and Royse and May 1982a).

Based on the adequate resolution of ten mycelial enzymes using HSE, compared with one using PAGE, it was concluded that HSE was the more effective method of resolving mycelial allozymes from *Agaricus* species. Compared with PAGE, HSE allowed for greater electrophoretic buffer manipulations which were critical for successful enzyme resolution. All further studies were conducted using HSE. However, as indicated in Section 3.3.3, PAGE was useful for resolving EST isozymes, and PAGE was used for this purpose, in addition to HSE, later in this Chapter.

3.4.4.7. Enzyme systems which may be useful for future investigations.

SUDH may hold the most promise for future use in mycelial allozyme study of *Agaricus* species, Appendix 2. SUDH on occasion showed very clear electromorphs, and in particular, further evaluations of gel and electrode buffers should be carried out in an attempt to find a system(s) that allow(s) repeatable high resolution and increased electromorph separation compared with those found in this study.

EST, reported by Royse and May (1989) to be useful for the study of *Agaricus bisporus* isolates may be as useful for within species study of a number of other *Agaricus* species, in particular *Agaricus bitorquis*, Appendix 2.

Although, ADH, GPI, PEP-GL, and PGK have been reported in *Agaricus bisporus* (e.g., Royse and May 1982a), and *Agaricus campestris* (May and Royse 1982b), only ADH was found by Royse and May (1982a) to be of sufficient resolution for the study. They reported some *Agaricus bisporus* isolates repeatedly displayed no ADH activity, as was found for the *Agaricus bisporus* isolates used in this study, Appendix 2. While PEP-LLL was found by Royse and May (1982a) and May and Royse (1982b), to be useful for the study of *Agaricus bisporus* and *Agaricus campestris*, PEP-GL was only reported by to be useful for the study of *Agaricus campestris*, (May and Royse 1982b).

3.5. A comparison of allozymes obtained from different tissues.

3.5.1. Introduction.

Allozyme analysis of the different tissues of an organism can often supply more phylogenetically useful information than can any single part, (Richardson et al. 1986). Thus allozyme polymorphism may be seen to occur when basidioma tissues in addition to mycelium are studied, as shown by Paranjpe et al. (1979) for CYTO and May and Royse (1982a) for ADH.

However, Paranjpe et al.(1979) and Ingebrigtsen et al. (1989) showed that isozymes may also vary depending on basidioma stage of development. The selection of basidiomata from the field at similar developmental stage is subjective, and this limits the use of basidiomata parts from field collections for analysis. With future research it may become possible to recognise similar developmental stages between basidiomata, especially those produced under controlled/standarised conditions. Thus further research into allozyme variation between tissues is needed.

The goal of this study was to evaluate the allozyme variability between mycelium, pileus, lamellae, and stipe tissues using HSE. Due to the periodic availability of basidiomata in the field, storage of freeze dried basidiomata for analysis was also investigated.

3.5.2. Methods.

Five replicate shaken cultures each of *Agaricus bisporus* X20, and *Agaricus bitorquis* K46 were grown as described in Section 3.2.4. Mycelium from each flask was harvested separately, as described in Section 3.2.5. Each replicate was freeze dried separately, and stored at -80° , using methods described for storage experiments later in this Chapter. Mycelium samples were removed from storage after 30 days for analysis.

In another experiment, three fresh basidiomata of commercially cultivated *Agaricus bisporus* X20, and *Agaricus bitorquis* K46, Appendix 1, were used for analysis. They were separated into pileus, lamellae and stipe, and each tissue was extracted separately using the method described in Section 3.3.2.1, with the modified extraction buffers described for the individual enzymes in Section 3.4.3.1. Basidiomata were not available for *Agaricus bisporus* A5.2, a commercial strain which was no longer being cultivated locally.

For comparison with fresh basidioma tissues, 3 whole basidiomata of the two species were frozen in liquid nitrogen and placed into a Cuddon freeze drier at -22°C to -18°C and less than 1 Torr vacuum pressure for 48 h. When removed from the freeze drier, basidiomata were stored at -80°C . They were removed after 30 days storage, separated into pileus, lamellae and stipe, and extracted for analysis as for freeze dried mycelium, using methods described later in this Chapter.

All extracts from freeze dried mycelium, fresh and freeze dried basidiomata parts were electrophoresed using HSE, and stained for EST, using the S4 buffer system and the staining solution described in Appendix 2, and for ACP, LAP, MDH, and AAT, using the conditions set out for the individual enzymes in Table 3.1.

Thus for each of *Agaricus bisporus* and *Agaricus bitorquis*, replicates of each of the following treatments were used for electrophoresis. Each replicate was kept separate.

- 1) freeze dried mycelium stored for 30 days at -80°C.
- 2) freeze dried pileus, stored for 30 days at -80°C.
- 3) freeze dried lamellae, stored for 30 days at -80°C.
- 4) freeze dried stipe, stored for 30 days at -80°C.
- 3) fresh pileus.
- 4) fresh lamellae.
- 5) fresh stipe.

3.5.3. Results.

No difference in the allozymes present was noted for any treatment of either species for LAP and EST. However, allozyme differences were noted for the other enzymes investigated. The following descriptions are given to illustrate these results.

Acid phosphatase (ACP)

Two electromorphs, shown in Figure 3.4, for *Agaricus bisporus* lamellae extracts were typical of heterozygotes and a monomeric enzyme, the most cathodal of these electromorphs was Acp: 1, and the most anodal was Acp: 3. All other *Agaricus bisporus* samples showed, Figure 3.4, single Acp: 1 electromorphs.

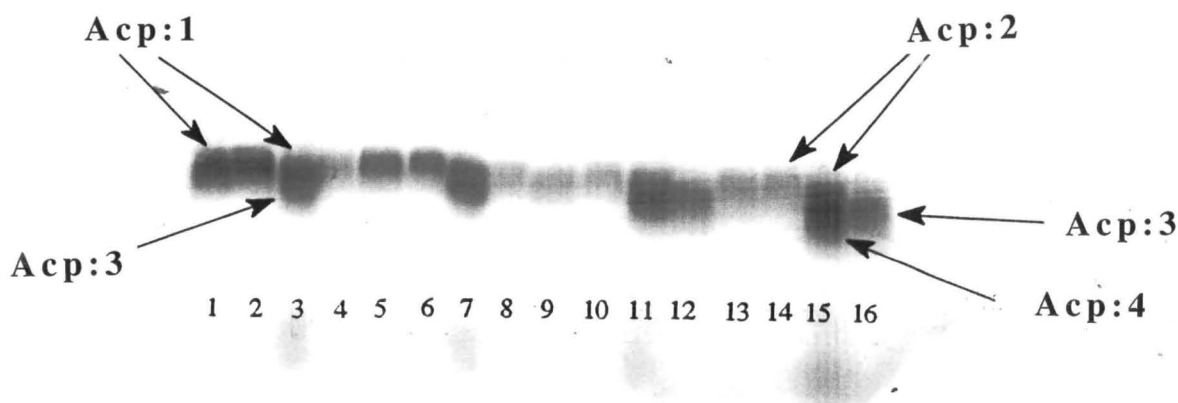


Figure 3.4. Acid phosphatase (ACP) activity for different basidioma tissues and vegetative mycelium for *Agaricus bisporus* X20, and *Agaricus bitorquis* K46. The following abbreviations were used; X20 = *Agaricus bisporus* X20, K46 = *Agaricus bitorquis* K46, M = vegetative mycelium, S = stipe, L = lamellae, P = pileus, Fresh = freshly harvested material, Stored = freeze dried material stored at -80°C . Lanes 1- 16 were as follows; 1) X20 S Fresh, 2) X20 P Fresh, 3) X20 L Fresh, 4) X20 M Fresh, 5) X20 S Stored, 6) X20 P Stored, 7) X20 L Stored, 8) X20 M Stored, 9) K46 S Fresh, 10) K46 P Fresh, 11) K46 L Fresh, 12) K46 M Fresh, 13) K46 S Stored, 14) K46 P Stored, 15) K46 L Stored, 16) K46 M Stored.

Two electromorphs were shown in Figure 3.4, for the *Agaricus bitorquis* lamellae extracts, typical of heterozygotes and a monomeric enzyme, the most cathodal of these electromorphs was Acp: 2, and the most anodal was Acp: 4. While both *Agaricus bitorquis* stipe and pileus extracts showed, Figure 3.4, single Acp: 2 electromorphs, the mycelial extracts of this strain showed a single Acp: 3 electromorph.

Malate dehydrogenase (MDH).

Mdh-1 showed a single identical electromorph for all *Agaricus bisporus* extracts, Figure 3.5.

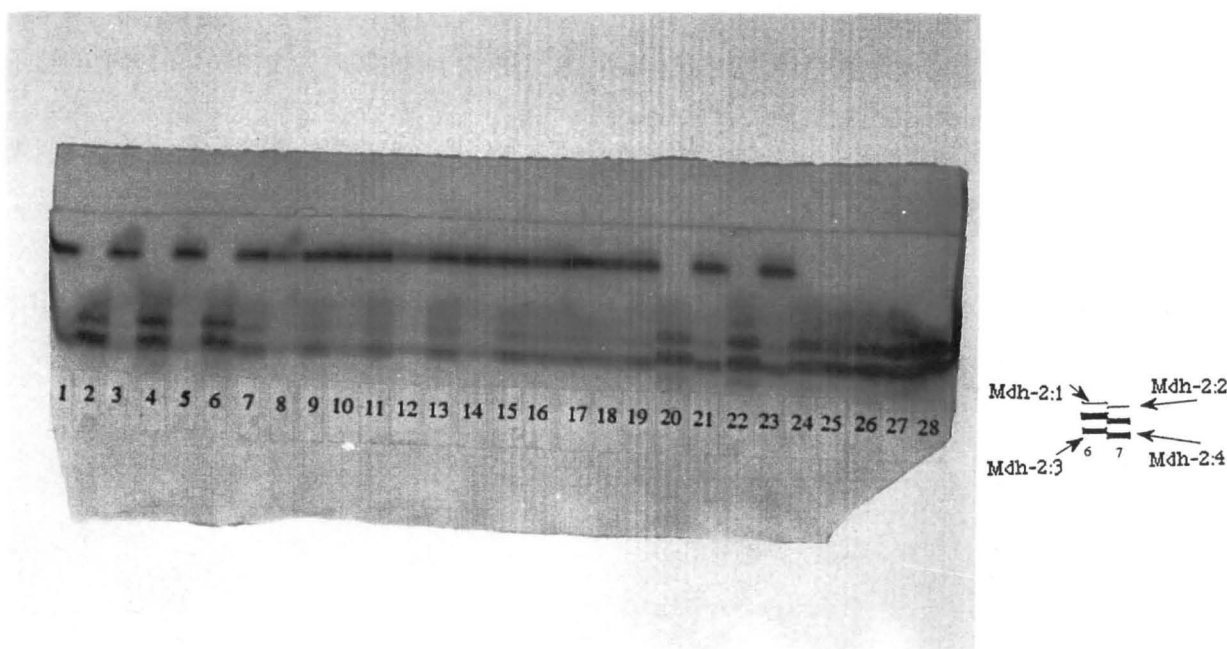


Figure 3.5. Malate dehydrogenase (MDH) activity for different basidioma tissues and vegetative mycelium for *Agaricus bisporus* X20, and *Agaricus bitorquis* K46. The following abbreviations were used; X20 = *Agaricus bisporus* X20, K46 = *Agaricus bitorquis* K46, M = vegetative mycelium, S = stipe, L = lamellae, P = pileus, Fresh = freshly harvested material, Stored = freeze dried material stored at -80°C . Lanes 1-28 were as follows; 1) X20 M Stored, 2) K46 P Fresh, 3) X20 P Stored, 4) K46 L Fresh, 5) X20 P Stored, 6) K46 L Fresh, 7) X20 M Stored, 8) X20 S Stored, 9) X20 L Stored, 10) X20 P Stored, 11) X20 L Stored, 12) X20 S Stored, 13) X20 M Stored, 14) X20 P Stored, 15) X20 M Stored, 16) X20 L Stored, 17) X20 M Stored, 18) X20 L Stored, 19) X20 M Stored, 20) K46 L Stored, 21) X20 M Stored, 22) K46 M Fresh, 23) X20 M Stored, 24) K46 S Stored, 25) K46 P Stored, 26) K46 L Stored, 27) K46 M Fresh, 28) K46 M Stored.

Agaricus bisporus mycelial and lamellae extracts showed, Figure 3.5, asymmetric Mdh-2 electromorph patterns. The most anodal and weakest *Agaricus bisporus* mycelial and lamellae electromorph, Mdh-2: 2, e.g., lanes 7 and 9, Figure 3.5, was increased in density as a result of deliberate over staining. This was carried out to increase the dye deposition for weak *Agaricus bisporus* pileus and stipe samples, e.g., lanes 8 and 10, Figure 3.5. Only the most cathodal electromorph, Mdh-2: 4, in the heterozygous pattern of the other parts could be clearly determined for *Agaricus bisporus* pileus and stipe extracts, e.g., lanes 8 and 10, Figure 3.5.

Agaricus bitorquis extracts showed no activity at Mdh-1, which was interpreted as a null 'allele'. *Agaricus bitorquis* basidioma extracts, and mycelial freeze dried extracts showed an asymmetric heterozygous pattern at Mdh-2. A band more anodal than Mdh-2: 4 was found in extracts of *Agaricus bitorquis* fresh mycelia, i.e., lanes 22 and 27, Figure 3.5. Further tests must be carried out before this most anodal band may be accounted for.

Aspartate aminotransferase (AAT).

Similar results were obtained for this enzyme, but are not illustrated. *Agaricus bisporus* and *Agaricus bitorquis* mycelial extracts shared a single electromorph, Aat-1: 1. A second *Agaricus bitorquis* mycelial electromorph, Aat-2: 1, and was less mobile compared with the second *Agaricus bisporus* mycelial electromorph, Aat-2: 2. Extracts of *Agaricus bisporus* stipe, pileus, and lamellae also exhibited Aat-2: 2. This electromorph was the only one observed for *Agaricus bisporus* stipe extracts. However,

the *Agaricus bisporus* pileus and lamellae extracts each showed different individual electromorphs of greater migration compared with Aat-2: 2, at what may have been a third putative locus. This electromorph, Aat-3: 1 from the pileus extracts was of less mobility compared with that from the lamellae extracts, Aat-3: 2.

3.5.4. Discussion and conclusions.

Based on the effective preservation of enzymes found in basidiomata, freeze dried, and stored at -80°C , it was concluded that basidiomata may be collected from the field and stored for analysis for a period of at least one month. This may allow flexibility in future experiments using basidiomata parts for analysis, making it unnecessary to analyse specimens on the day they are collected.

Allozyme variation between tissues was observed for Aat-1, Aat-3, and Mdh-2 for *Agaricus bisporus*, and at Aat-1, Aat-3, and Acp for *Agaricus bitorquis*. EST and LAP electromorphs were monomorphic for the different tissues of both species. The general conclusion from this experiment was that *Agaricus* tissues may express different amounts of activity, and/or different loci, and/or different alleles. These results do not agree with those of May and Royse (1982a), who found that except for Adh, all other loci including; Gpt, Mpi, Pep-LLL-1, Pep-LLL-2, and Aat were expressed identically in *Agaricus bisporus* pileus or mycelial extracts. It is not known why they did not find allozyme variation at Aat, unless their methods inhibited electromorph separation or resolution. Concerning AAT resolution, it is possible that their use of an electrode buffer pH of 6.1, compared with pH 8.2, used in

this study may have inhibited their electromorph separation. However, it is unlikely this would have prevented the observation of allozyme polymorphism between tissues.

The enzymes studied were found to be present in all tissues investigated, unlike other enzymes studied by Royse and May (1982a), and May and Royse (1982b), reported by them to be present in the basidioma tissues but not in the mycelium or *vice versa*, of *Agaricus bisporus* and *Agaricus campestris*. However, the additional electromorph information that may be gained by screening particular tissues for enzymes shown only to be active in those tissues is a possible source of phylogenetic information which is in need of further investigation.

In *Agaricus* species, compared with study of mycelium alone, more allozyme information may be gained from the study of tissues. Thus it would be of interest to carry out a study of allozyme variability during the tissue development. Such research could make use of established cultivation techniques for *Agaricus bisporus* and *Agaricus bitorquis*, where basidiomata of these species can be grown under controlled conditions, allowing harvest and analysis at similar stages of growth. Subsequent to research of this kind, it may be possible to recognise specific ontogenetic stages in both cultivated and field collected specimens, thereby allowing incorporation of allozyme data from specific tissue types of specific ontogenetic stage. Research is needed on the relevance of the ontogenetic principle, (Section 2.2.5.1), as applied to allozyme changes during Basidiomycete life

cycle, to evaluate it's merits for phylogenetic analysis within this group.

Until research has been carried out on allozyme changes during basidioma development, compared with basidioma tissues, it is logical to use mycelium grown under standardised and controlled conditions for analysis.

3.6. Growth rates of *Agaricus bitorquis* K46 in shaken and stationary liquid cultures.

3.6.1. Introduction.

Many methods have been used for the culture of *Agaricus* mycelia for allozyme analysis, e.g., agar culture (van Loon et al. 1986), stationary liquid culture (May and Royse 1981), and shaken culture (Raper and Kaye 1978, Paranjpe et al. 1979). It is generally recognised that agar culture is an inefficient method of producing mycelial mass compared with liquid culture (Royse and May 1982a). However, adequate comparison of *Agaricus* stationary liquid culture with shaken culture could not be found in the literature. As a result it was decided to evaluate the relative efficiencies of these culture methods, with respect to yield over time. *Agaricus bitorquis* K46 was used as the test organism.

3.6.2. Methods.

Twelve replicate liquid cultures of *Agaricus bitorquis* K46 were prepared, as described in Section 3.2.4. Six replicates were incubated as stationary cultures, and six as shaken cultures. The first mycelial harvest of a flask from each treatment was 14 days

after inoculation, and subsequent harvests were every 3 days until 29 days after inoculation. Mycelial wet weight was noted.

3.6.3. Results

Six times as much mycelium was produced in shaken cultures compared with that which grew in stationary conditions. Growth of mycelia in shaken culture was exponential over the period of the experiment, but not so in stationary liquid culture, Figure 3.6. Mycelium in stationary conditions reached a maximum

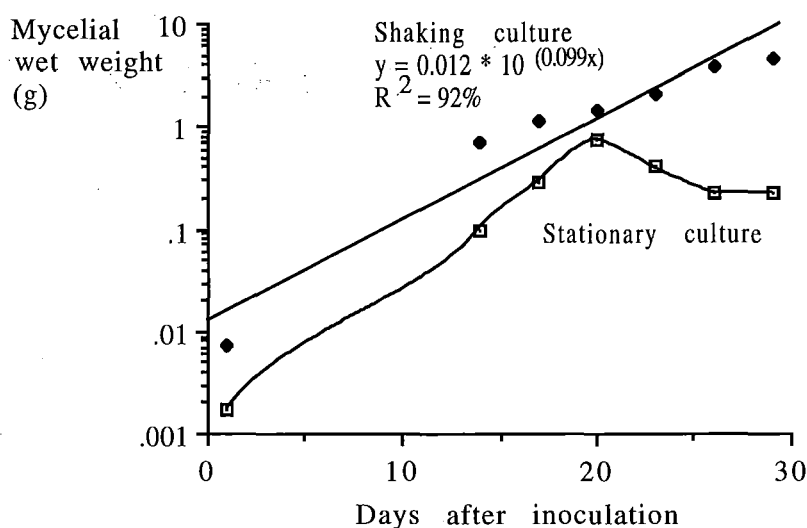


Figure 3.6. Growth of *Agaricus bitorquis* (K46) in 100 ml Oxoid Malt Extract Broth at 25°C, using stationary and shaken, 80 rpm, conditions.

wet weight of 0.75 g 20 days after inoculation, which represented the stationary phase, (Moore-Landecker 1972) of the growth curve, and an average production rate of 37 mg/day. Maximum wet weight was not achieved for mycelium grown in shaken culture, i.e., the growth curve stationary or autolysis phase was not reached prior to the termination of the experiment. The last

recorded wet weight on 29 days after inoculation was 4.65 g, with an average production rate of 160 mg/day.

3.6.4. Discussion and conclusions.

Shaken culture of *Agaricus bitorquis* produces more mycelium at a faster rate than stationary liquid culture. These results are supported by the work of Song et al. (1987) in their study of *Lentinus edodes* and Sasek (1989) for ectomycorrhizal fungi.

The report by Raper and Kaye (1978) of “poor” growth of *Agaricus* species in liquid shaken medium is inconsistent with the findings of this study, and could have been due to their use of different species, different growth medium, and possible differences in rate of shaking, compared with this study. However, they did not define their term “poor” growth, and there is no way of determining if the results found for shaken culture in this investigation are what they would have called “poor”.

3.7. Investigation of EST electromorph variation between the mycelium from replicate cultures of *Agaricus bisporus* A5.2 in shaken submerged liquid culture.

3.7.1. Introduction.

Evers and Ross (1983) were the only authors found in the literature to have reported on isozyme variation between the mycelium of the same strain grown in replicate culture vessels. Thus it was necessary to determine if replicate cultures of mycelium isolated from a single basidioma grown under identical conditions exhibit identical electromorphs. Both PAGE and HSE were used for this investigation, and EST was chosen as the enzyme for study because it was well resolved using both of these methods.

3.7.2. Methods.

Ten liquid cultures of *Agaricus bisporus* A5.2 were grown, as described in Section 3.2.4, from one isolate taken from a single basidioma pileus. Mycelium from each flask was harvested and extracted separately using the method described in Section 3.3.2.1, with the modified extraction buffer A described in Section 3.4.3.1. The supernatants from the extractions were electrophoresed and gels stained for EST using the PAGE methods described in Section 3.3, and the HSE methods described in Appendix 2 with the S4 buffer system.

3.7.3. Results.

A single putative locus, Est-1, was resolved, with a single electromorph, and it was found that flask to flask *Agaricus bisporus* mycelial electromorph variation did not occur. Some smeared activity was detected for *Agaricus bisporus* at a second EST putative locus using HSE which was not detected using PAGE, but this was not considered significant.

3.7.4. Conclusions.

Replicate cultures did not vary, and in subsequent experiments it was considered to be justified if multiple cultures of each *Agaricus* strain were pooled for use.

3.8. Effects of storage treatments on mycelial enzymes.

3.8.1. Introduction

A method for control and/or elimination of enzyme degradation in storage would avoid the need for harvest and extraction of a large quantity of fresh mycelial samples on the day of analysis. To be effective, the selected method of mycelial storage should allow the maximum number of clearly resolved putative loci to be determined and accurately scored for later phylogenetic analysis. Great variation in methods of mycelial storage for allozyme analysis, with few direct comparisons, were found in the literature, (Hanafusa 1973 and Chaillot 1976, Evers and Ross 1983, van Loon et al. 1986, Liming et al. 1987, Vilgalys 1991).

The goal of these experiments was to evaluate the effects of storage on allozyme pattern and resolution. Because of the on going nature of these storage experiments, photographs were not taken to illustrate results.

3.8.2. Effects of freezing mycelia and mycelial extract.

3.8.2.1. Methods

All electrophoretic runs included an extract of fresh *Agaricus bisporus* A5.2 mycelium grown, as described in Section 3.2.4, as a control. Thirty replicate flasks of *Agaricus bisporus* A5.2 and *Agaricus bitorquis* K46 were grown for analysis in shaken culture, as described in Section 3.2.4.

For each species, mycelia from 15 flasks were harvested separately and placed in individual Eppendorf tubes and frozen with liquid nitrogen before being stored at -80°C.

The fresh mycelia from the remaining 15 flasks of both species were harvested and extracted separately using the method described in Section 3.3.2.1, with the modified extraction buffer A described in Section 3.4.3.1. Aliquots of supernatant, 50 μ l, were prepared in triplicate from the mycelium recovered from each flask. Each aliquot, contained in individual Eppendorf tubes, was frozen with liquid nitrogen, before being stored at -80°C.

Replicates of mycelia and supernatant of both species were removed from the freezer for electrophoresis at 7 day

intervals for a total of 28 days. After 28 days in storage, the intervals were increased to 14 days for a further 70 days storage.

Mycelium from the fresh *Agaricus bisporus* A5.2 control, and the mycelium that had been stored from each species was extracted in the same manner. The supernatants retrieved from these extractions were analysed beside samples of supernatant which had been stored frozen. HSE was carried out using the S4 buffer system and the EST staining solution described in Appendix 2, and the conditions set out for ACP and MDH in Table 3.1.

Thus both *Agaricus bisporus* A5.2 and *Agaricus bitorquis* K46, a replicate of each of the following treatments was removed after 7, 14, 21, 28, 42, 56, 70, 84, and 98 days after storage for analysis.

- 1) mycelium frozen in liquid nitrogen and stored at -80°C.
- 2) mycelium extracted, and the supernatant frozen in liquid nitrogen and stored at -80°C.

Freshly harvested mycelium was also extracted for analysis at the same time.

3.8.2.2. Results

Allozyme expression remained identical between stored mycelium, stored supernatant, and freshly extracted mycelium for each strain using the same enzyme indicator over the entire length of the storage experiment.

3.8.2.3. Discussion and conclusions

Agaricus bisporus A5.2 and *Agaricus bitorquis* K46 mycelia may be stored fresh or extracted prior to the storage of small

quantities of supernatant, ideally enough for single applications, for up to 3 months at -80°C . This may be accomplished without loss of resolution for at least ACP, EST, and MDH. However, further experimentation is required to assess the the effect of freezing mycelium or mycelial extract from other species and on a greater range of enzymes.

The results show that mycelia may be successfully stored for analysis for longer periods than those routinely used by others, such as Royse and May (1989), who stored mycelium for up to 1 month in vapor-phase liquid nitrogen. The results reported here are supported by the work of van Loon et al. (1986), who used stored mycelial supernatant at -72°C without any adverse effects on EST. But the successful MDH preservation observed in this study was not supported by van Loon et al. (1986), who reported decreased MDH activity in mycelial supernatant stored at -72°C . However, these authors did not supply information on the length of storage time, and direct comparison cannot be made.

3.8.3. Effects of mycelial freezing and freeze drying.

3.8.3.1. Methods

All electrophoretic runs included an extract of fresh *Agaricus bisporus* A5.2 mycelium grown, as described in Section 3.2.4, as a control. Seven grams wet weight of *Agaricus bisporus* A5.2 mycelium which had been grown in shaken culture, as described in Section 3.2.4, was frozen with liquid nitrogen and placed into a Cuddon freeze drier at -22°C to -18°C and less than 1

Torr vacuum pressure overnight. When removed from the freeze drier, mycelium was weighed into 30 aliquots, each of 20-23 mg. Each aliquot of mycelium was placed into a 5 ml polystyrene test tube, 75 x 12 mm, with a polystyrene bung (Lab Serv, LBS 503), and stored at -80°C.

After 30 and 60 days in storage, triplicate samples of *Agaricus bisporus* A5.2 were removed from -80°C, extracted as described below, and electrophoresed. Gels were stained for EST using methods described for PAGE in Section 3.3, and using the S4 buffer system and staining solution described in Appendix 2 for HSE. Gels used for HSE were also stained for ACP and MDH, using the conditions set out for these enzymes in Table 3.1. Every third day in storage, from day 60 to day 90, and once every 30 days, from day 90 until day 180 the process was repeated.

HSE of the same *Agaricus bisporus* A5.2 mycelium was carried out after 2 years storage at -80°C and stained for EST, ACP and MDH using the same buffer systems and staining solutions.

Thus two replicates of freeze dried *Agaricus bisporus* A5.2 mycelium were removed 30, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, 120, 150, 180 days after storage for analysis using PAGE and HSE. And samples were removed from storage after 2 years for HSE only. The replicates were kept separate.

3.8.3.1.1. Extraction of freeze-dried mycelium

To each tube of freeze dried mycelium, was added 200 μ l, pH 7.1 extraction buffer A containing 0.05 M Tris-HCl, plus 12 mM cysteine-HCl and 200 mg of hydrated insoluble PVP, Section 3.4.3.1. Tubes were immediately placed in crushed ice. The mixture was homogenised for 10 sec. using a Janke and Kunkel, Ultra Turax homogeniser, and after blending the tube was immediately placed back into crushed ice until centrifugation for 20 min. at 6000 x G in a Heraeus Christ Minifuge. The supernatant containing the soluble enzymes was used for electrophoresis.

3.8.3.3. Results

Two putative loci were determined for *Agaricus bisporus* mycelial EST, but the more anodal of these, Est-2, was not scorable due to poor resolution. Both species shared a single Est-1 electromorph, designated Est-1: 2.

Using PAGE, until 81 days of storage no difference was detected in the appearance of the single Est-1: 2 electromorph. The main single Est-1: 2 electromorph remained after 87 days in storage using PAGE, but several subsidiary bands developed compared with the freshly harvested mycelia; 2 of higher and 1 of lower mobility. All 3 subsidiary bands were slower to develop than the main single electromorph shared with the freshly harvested mycelium, and were visually lighter in density. The 3 subsidiary bands remained after 90 days in storage, and were of slightly greater density than on day 87, but still lighter than the main single electromorph, which remained constant in density.

Subsidiary EST bands were not resolved using HSE. The resolution of the single Est-1: 2 electromorph, common throughout this experiment using HSE and PAGE remained comparable and constant between the methods.

The single Est-1: 2 electromorph was still clearly resolved from freeze dried mycelium and HSE 2 years after storage and no difference in the resolution and identity of ACP and MDH electromorphs was observed between freshly harvested mycelium and mycelium stored after 2 years.

3.8.3.4. Discussion and conclusions

Storage of freeze dried *Agaricus bisporus* mycelium at -80°C preserved sufficient EST ACP, and MDH activity to enable accurate scoring for up to two years using HSE. The successful mycelial enzyme preservation achieved using the storage methods in this study is supported by Evers and Ross (1983), who reported that MDH activity from freeze dried mycelia stored at -20°C for undefined periods. However it was not supported by the results reported by Hanafusa (1973) and Chaillot (1976) and Vilgalys (1991), who found enzyme activity to decrease with the freeze drying of mycelium. The decrease of enzyme activity reported by them may have been due to species differences compared with this study and that of Evers and Ross (1983). The 3 subsidiary EST bands found using PAGE were interpreted as storage artifacts, as they were slower to develop than the main single electromorph (Richardson et al. 1986), and they only appeared after 87 days in storage.

— Thus it is concluded that this method of mycelial storage may be suitable for preserving the activity of a range of enzymes for considerable periods, especially when using HSE. However, the possibility of artifacts developing during storage may present a problem when PAGE is used, and further investigation of this phenomenon is required to evaluate the extent of artifact development and resolution. Further analyses are required to assess the effect of storing freeze dried mycelium on other enzymes.

3.9. Effect of culture method and harvest time on mycelial enzymes.

3.9.1. Introduction.

Isozyme banding patterns have been shown by Liming et al. (1987) to change during mycelial development in culture, and at different rates depending on culture method. Differences in mycelial enzymes have also been found by Okunishi et al. (1979), Liming et al. (1987) and Kerrigan and Ross (1989), to occur in different forms of mycelium with different culture methods .

The influence of culture method and harvest time on mycelial allozyme expression were investigated. The importance of standardising and clearly reporting culture methods for allozyme analyses was evaluated. HSE was used in this experiment as it allowed the resolution of a greater number of *Agaricus* enzyme systems than did PAGE, and use was made of stored freeze dried mycelium.

3.9.2. Methods.

Agar culture.

Twenty agar plates of *Agaricus bisporus* A5.2, and *Agaricus bitorquis* K46, were prepared as described in Section 3.2.1. To facilitate removal of the mycelium, sheets of Cellophane, ICI England, were cut into discs that would fit inside the petri dishes. The discs were sterilised by autoclaving interleaved with Whatman No 1 filter paper to prevent them from sticking together. The sterile discs were placed on the surface of the solidified agar, and the inoculum was placed on the cellophane. Mycelium was allowed to grow for 20 days after inoculation before being harvested by removing the cellophane disc from the agar plate and scraping off the mycelium with a spatula. The mycelium, was pooled, freeze dried, and stored at -80°C.

Liquid culture.

Thirty six liquid cultures were prepared for each species, as described in Section 3.2.4, half were kept stationary, while the others were shaken. For this experiment flasks that were shaken were not periodically manually swirled to remove mycelium from the sides, in a deliberate attempt to grow mycelium both submerged and partially exposed to the air on the sides of the flasks, Figure 3.7.

Three replicate flasks of each treatment were harvested 10, 20, and 30 days after inoculation. Mycelia from individual flasks were harvested and kept separate. Surface and submerged mycelia were separately harvested from stationary flasks.

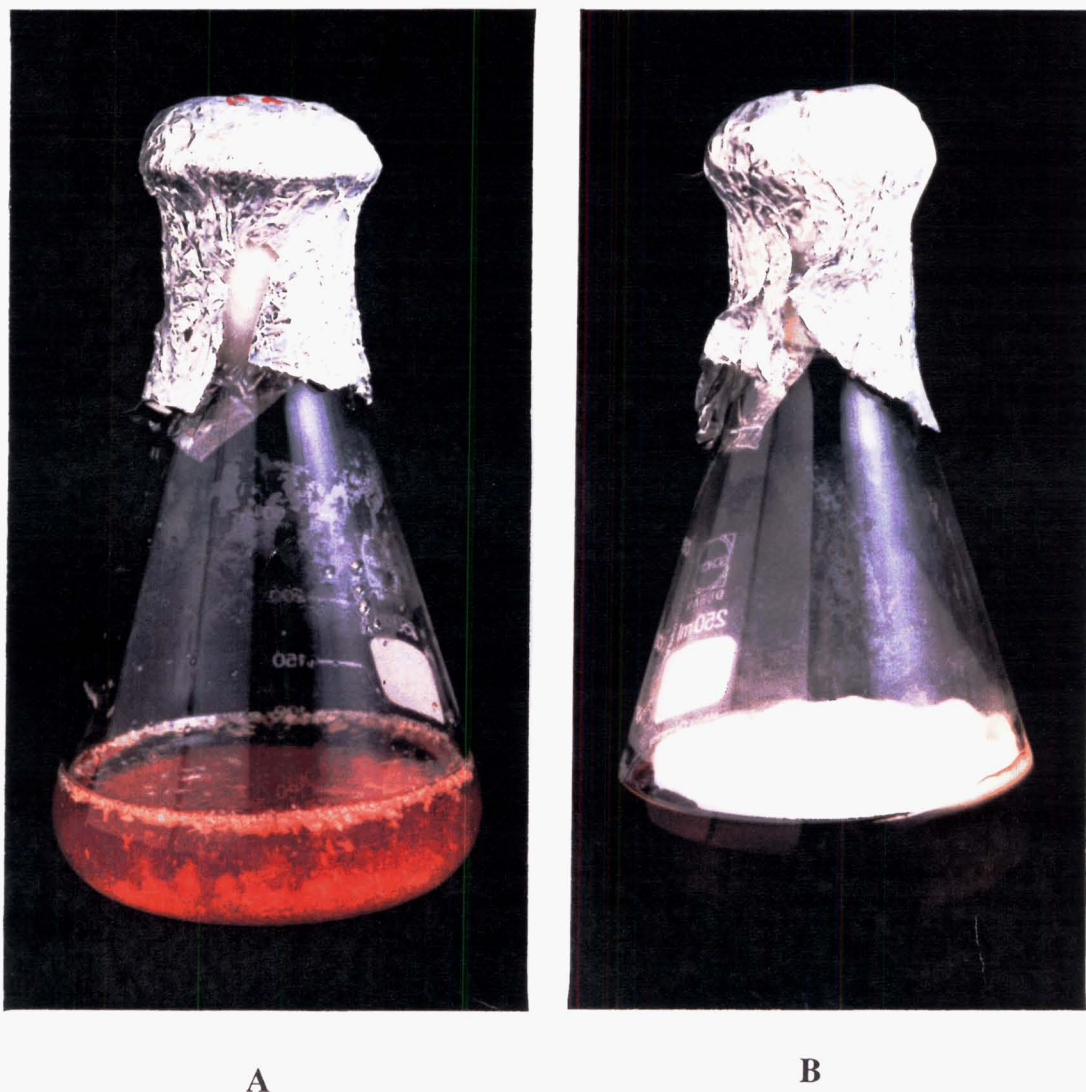


Figure 3.7. *Agaricus bisporus* A 5.2 growing in 250 ml Erlenmeyer flasks, in 100 ml Oxoid Malt Extract Broth medium at 25°C in the dark. (A) after shaking at 80 rpm for 20 days after inoculation showing build up of mycelium on the side of the flask, at the medium-air interface. (B) stationary conditions for 20 days after inoculation showing surface mycelium.

For flasks that were shaken, submerged mycelium, and that which had grown attached to the sides were also separated. Mycelia were freeze dried, (Section 3.8.3) before being stored at -80°C .

Replicates, 20-23 mg each, of each freeze dried form of mycelium were extracted using methods described in Section 3.8.3.1.1, electrophoresed using HSE, and stained for EST, ACP, LAP, and MDH, using methods described for the individual enzymes in Table 3.1.

Thus three replicates of each of the following treatments were harvested 10, 20, and 30 days after inoculation. Each replicate was kept separate.

- 1) Surface mycelium from agar culture,
- 2) Surface mycelium from stationary liquid culture,
- 3) Surface mycelium grown attached to the sides of the flasks in shaken culture,
- 4) Submerged mycelium from stationary liquid culture,
- 5) Submerged mycelium from shaken culture.

3.9.3. Results

No variation was noted between replicates from any one harvest date. Submerged mycelium harvested from stationary liquid culture of both species did not show any enzyme activity. The results set out below exclude this treatment.

Single Acp and Lap-1 putative loci and electromorphs, were resolved for all *Agaricus bisporus* A5.2 and *Agaricus bitorquis* K46 mycelial treatments.

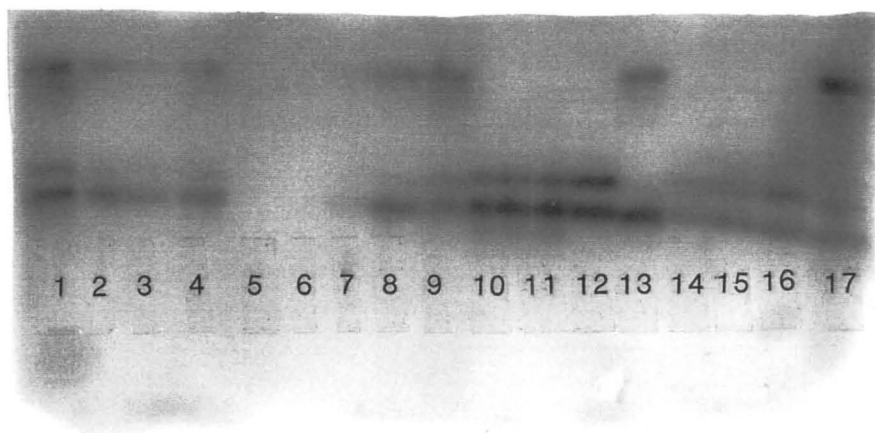


Figure 3.8. Malate dehydrogenase (MDH) activity for different forms of *Agaricus bisporus* A5.2, and *Agaricus bitorquis* K46, vegetative mycelia; harvested at 10 (harvest 1), 20 (harvest 2), and 30 (harvest 3) days after inoculation in shaken, 80 rpm, and stationary Oxoid Malt Extract Broth culture, and agar culture. A5.2 was used as a mycelial marker, cultured for 10 days after inoculation in shaken submerged conditions, and stored freeze dried at -80°C . Lanes 1 to 17 are as follows; 1) A5.2 marker, 2) A5.2 agar cultured mycelium, 3) A5.2 harvest 3, stationary surface mycelium, 4) A5.2 harvest 2, stationary surface mycelium, 5) A5.2 harvest 3, stationary submerged mycelium 6) A5.2 harvest 3, shaken submerged mycelium, 7) A5.2 harvest 2, shaken submerged mycelium, 8) A5.2 harvest 1, shaken submerged mycelium, 9) A5.2 freeze dried mycelial marker, 10) K46 agar cultured mycelium, 11) harvest 3, surface of stationary mycelium, 12) K46 harvest 2, surface of stationary mycelium, 13) A5.2 mycelium taken from the sides of a shaken flask used for culture, 14) K46 harvest 3, shaken submerged mycelium, 15) K46 harvest 2, shaken submerged mycelium, 16) K46 harvest 1, shaken submerged mycelium, 17) A5.2 freeze dried mycelial marker.

For Acp and Lap-1, *Agaricus bitorquis* K46 electromorphs were of the same density within and between mycelial treatments. While electromorphs for this species were the same density for any one mycelial treatment at other putative loci investigated, e.g., Mdh-2, Figure 3.8, they varied in density between mycelial treatments. Differences in electromorph density between *Agaricus bisporus* A5.2 mycelial treatments at all putative loci investigated were the same.

Agaricus bisporus A5.2 mycelium harvested from shaken submerged culture showed decreasing levels of enzyme activity with time. At 10 days after inoculation electromorphs were clearly visible, at 20 days electromorphs were weaker, and at 30 days electromorphs were often not visible. *Agaricus bisporus* A5.2 mycelium from the following treatments all showed activity equal to that of mycelium from shaken submerged culture at 10 days after inoculation.

- 1) the sides of shaken culture flasks harvested 20 and 30 days after inoculation,
- 2) the surface of stationary liquid cultures harvested 20 and 30 days after inoculation, and
- 3) agar cultures harvested 20 days after inoculation.

Agaricus bitorquis K46 did not show decreasing enzyme activity over time at any of the putative loci investigated for mycelium grown using either of the liquid culture methods. Two MDH putative loci were resolved for *Agaricus bisporus* A5.2 and *Agaricus bitorquis* K46, as reported in Section 3.8.2.2. The MDH putative loci for the different treatments of these species is shown in Figure 3.8.

Agaricus bisporus A5.2 Est-1 showed a single electromorph Est-1: 2, and smeared, unscorable activity at Est-2, Figure 3.9. The *Agaricus bisporus* A5.2 Est-1: 2 electromorph was also exhibited by *Agaricus bitorquis* K46. For the *Agaricus bitorquis* K46 mycelium grown in shaken submerged liquid culture, a second more cathodal electromorph was evident, Est-1: 1. *Agaricus bisporus* A5.2 surface mycelium, including that from agar culture, stationary liquid culture, and the edge of shaken culture, incubated for 20-30 days after inoculation showed Est-1: 2 activity as strongly as for the submerged mycelia of this species harvested at 10 days after inoculation. However the non-submerged mycelium for *Agaricus bitorquis* K46 showed Est-1: 2 only weakly compared with mycelium of this species grown in submerged shaken culture.

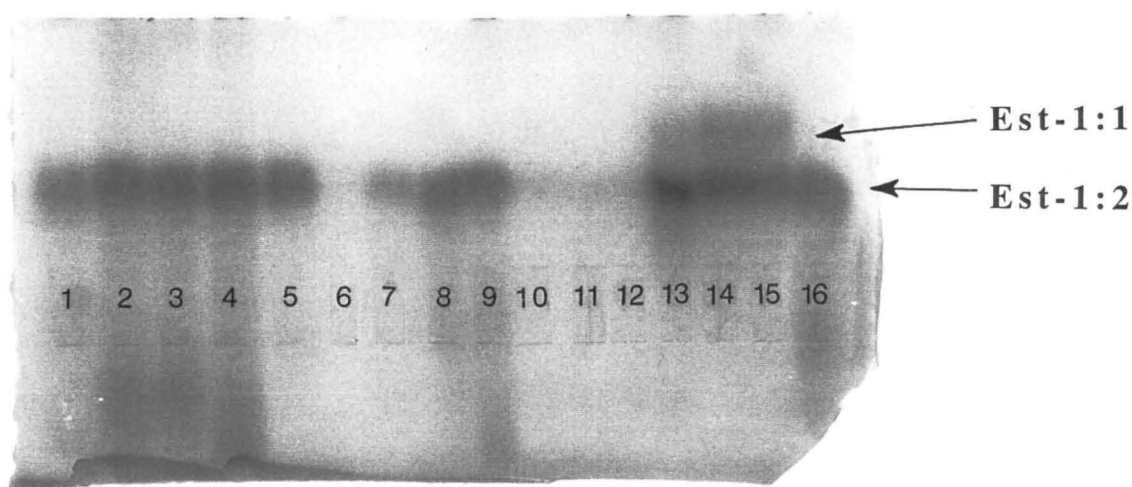


Figure 3.9. Esterase activity for different forms of *Agaricus bisporus* A5.2, and *Agaricus bitorquis* K46, vegetative mycelia, harvested at different times after inoculation in shaken, 80 rpm, and stationary Oxoid Malt Extract Broth culture. An additional electromorph is shown for K46 shaken submerged cultures at the Est-1 putative locus compared with the non-submerged K46 form of mycelium, due to the different expressions of the enzyme in the different forms of mycelium. Lanes 1 to 16 are as follows; 1) A5.2 marker, 2) A5.2 agar cultured mycelium, 3) A5.2 harvest 3, stationary surface mycelium, 4) A5.2 harvest 2, stationary surface mycelium, 5) A5.2 mycelium taken from the sides of a shaken flask used for culture, 6) A5.2 harvest 3, shaken submerged mycelium, 7) A5.2 harvest 2, shaken submerged mycelium, 8) A5.2 harvest 1, shaken submerged mycelium, 9) A5.2 freeze dried mycelial marker, 10) K46 agar cultured mycelium, 11) harvest 3, surface of stationary mycelium, 12) K46 harvest 2, surface of stationary mycelium, 13) K46 harvest 3, shaken submerged mycelium, 14) K46 harvest 2, shaken submerged mycelium, 15) K46 harvest 1, shaken submerged mycelium, 16) A5.2 freeze dried mycelial marker.

3.9.4. Discussion and conclusions

It is not known why the submerged mycelium harvested from stationary liquid culture showed no enzyme activity, although it might be assumed that this mycelium received relatively little oxygen compared with the surface mycelium, which may have caused it to die.

Approximately equivalent activity was noted for all *Agaricus bisporus* A5.2 enzymes investigated from submerged shaken mycelium grown for 10 days after inoculation compared with surface mycelium from agar culture, stationary liquid culture, and the edge of shaken culture, incubated for 20-30 days after inoculation. This may have been due to shaken cultures producing more mycelium at a faster rate than stationary liquid culture, as shown for *Agaricus bitorquis* K46 in Section 3.6. It could be postulated that the faster physiological development observed in Section 3.6 for mycelium grown in shaken submerged liquid culture compared with stationary liquid culture, corresponded with faster enzyme production.

The decrease in *Agaricus bisporus* A5.2 enzyme activity in submerged shaken culture may have been a result of the stage of physiological development. The absence of *Agaricus bisporus* A5.2 mycelial enzyme activities at 30 days after inoculation corresponds with the stationary phase, (Moore-Landecker 1972) when hyphal autolysis and enzyme breakdown could be occurring. It is interesting to note that *Agaricus bitorquis* K46 showed no decrease in enzyme activity in submerged shaken culture. This may be explained by the fact that this strain is shown not to have

reached the stationary phase 30 days after inoculation in experiments reported in the next Section of this Chapter. It may be concluded that relative physiological rates of development between species may affect enzyme production.

No change was found in the number of *Agaricus bisporus* A5.2 mycelial EST electromorphs over time in this study, a result which differs from that of Liming et al. (1987) who reported increasing numbers of these electromorphs over time for other strains. Differences between their results and those found in this study may have been due to 1) their use of PAGE, 2) strain differences, 3) differences in liquid culture shaking speed.

While changes in enzyme activity did not occur within mycelial treatments over time for *Agaricus bitorquis* K46, mycelium grown in shaken submerged culture compared with non-submerged culture did show more EST activity and the presence of the additional Est-1 electromorph Est-1: 1. This may be explained by the faster physiological development in shaken submerged culture compared with non submerged culture, reported in Section 3.6. The absence of Est-1: 1 in mycelium grown in stationary surface culture may have been due to weak expression caused by inhibited mycelial development compared with mycelium grown in shaken submerged culture.

Although differences in the number of EST electromorphs between mycelium cultured on agar compared with submerged liquid culture have been reported by Okunishi et al. (1979) for species of other genera, it has not previously been reported for

Agaricus bitorquis. Depending upon species the levels of mycelial enzyme activity may be influenced by culture method and period, and the presence of previously undetected mycelial electromorphs may be revealed through the use of different culture methods. If standardised culture methods and harvest times are not used, the phylogenetic usefulness of data may be reduced by; 1) misinterpreting the lack of enzyme activity for a particular mycelial sample as a 'null allele', when the enzymes activity is simply low due to the stage of mycelial development, 2) the presence or absence of electromorphs being dependant upon the culture method used.

However, standardisation of culture method must take into account the growth habit of the strain under investigation. If one strain in liquid culture has a surface growing habit and another a submerged growing habit, the lack of oxygen for the submerged culture may prevent the expression of some forms of an enzyme leading to incorrect hypotheses of genetic difference. Fortunately all strains investigated in this study expressed the same growth habits with all of the culture methods investigated.

Thus it can be concluded that it is important to standardise and clearly report the fungal growth habits and culture methods used to enable correlation of results between similar allozyme studies. Conclusions drawn from comparing studies using different forms of mycelium, may only be considered tentative. Thus van Loon et al. (1986), who used agar culture and made direct comparisons with the study of Royse and May (1981), who

used stationary liquid culture, may not be justified in comparing the results. However, further research is needed to test allozyme variation between different forms of mycelium. With consideration given to culture period, it may be possible to increase the quantity of phylogenetically useful data through the use of different forms of mycelium in a manner similar to Kerrigan and Ross (1989), who in an allozyme study of *Agaricus bisporus* used different forms of mycelium from surface and submerged cultures for investigation of different allozymes.

For comparative work, it is not recommended that mycelium be harvested for analysis at random periods after inoculation, as has been the practice in many studies, e.g., 2-3 weeks, (Royse and May 1981), and 3-6 weeks, (Kerrigan and Ross 1989). However, the generation of more phylogenetic information may be possible by the use of different but defined culture periods compared with a single harvest time, and further work on the evaluation of allozyme variation during incubation is needed.

However, when comparing different species, it may not be possible to ensure that samples have either the same form of mycelium or equal physiological stage due to the variable growth habits of the fungi. But in the absence of information on the differences in mycelial allozyme production using different culture methods over time, mycelium from different species should be grown for analysis using a single standardised method. Cultures should be observed daily during incubation and if necessary action should be taken to aid the production of a standard form of mycelium. For example the manual swirling of

shaken submerged liquid cultures may be carried out to prevent the development of different forms of mycelium, a stratagem resorted to in other sections of this project. Results of an experiment designed to find a method to define physiological stage for mycelium grown in submerged shaken culture are reported in the following Section 3.10.

3.10. Estimation of physiological stage.

3.10.1. Introduction.

The growth rate of mycelium in liquid culture follows a pattern, (Moore-Landecker 1972) from inoculation through a lag period to growth and finally to senescence. This pattern of growth may be depicted by curve, (Moore-Landecker 1972). At any point on this growth curve, the physiological stage of mycelial development in culture is reflected by mycelial mass. Harvesting mycelia of different species at a similar physiological stage may reduce allozyme variability. This may be achieved by harvesting mycelia at approximately the same point on their growth curves prior to maximum dry weight, when hyphal autolysis, (Moore-Landecker 1972) and enzyme breakdown could be occurring simultaneously.

3.10.2. Methods.

A strain was chosen at random to be representative of each species, as listed in Appendix 3 which contains the raw data for this experiment. One hundred and seventeen flasks were prepared, as described in Section 3.2.4. Nine flasks of each of *Agrocybe parasitica*, *Leucoagaricus leucothites*, and eleven species

of *Agaricus* were grown in shaken submerged culture. A single flask of each species was harvested every 3 days until 30 days after inoculation, the first harvest was usually at 6 days after inoculation, as described in Appendix 3. Harvested mycelium was freeze-dried and weighed.

3.10.3. Results.

Weight of freeze-dried mycelium was plotted for each species against days after inoculation, as shown in Figure 3.10. Linear regressions were fitted to points arbitrarily designated as being representative of the most rapid period of growth. This phase did not include the lag phase, (Moore-Landecker 1972) at the beginning of the growth curve, or the stationary phase, (Moore-Landecker 1972) towards the end of the growth curve.

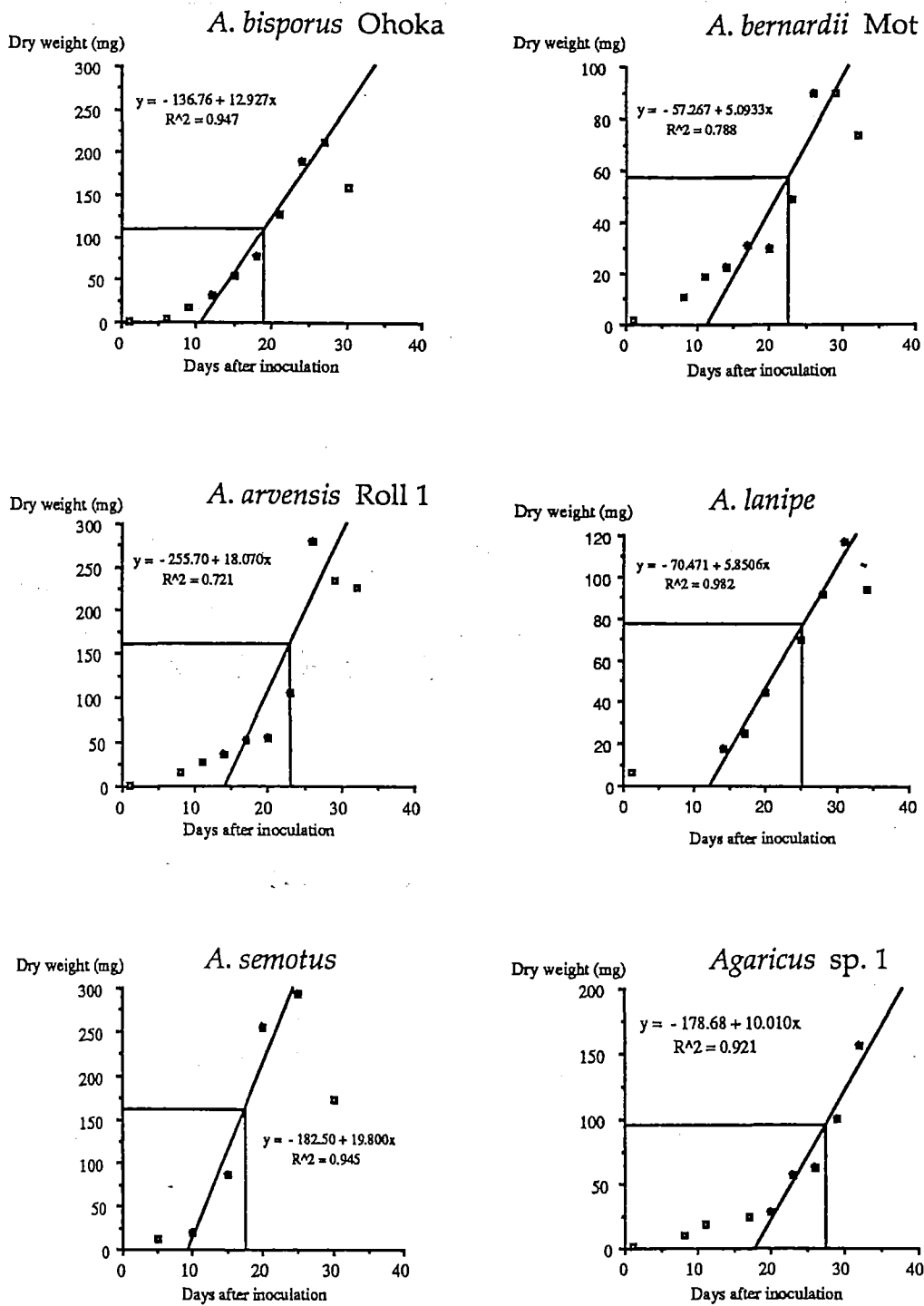
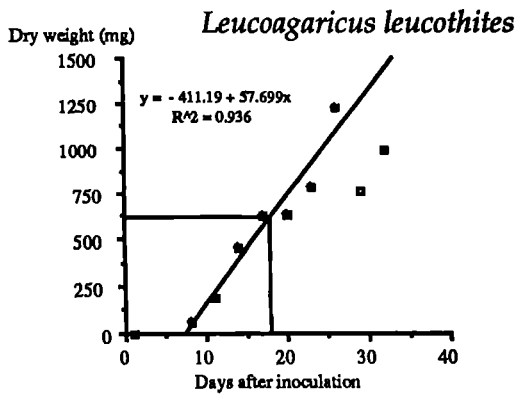
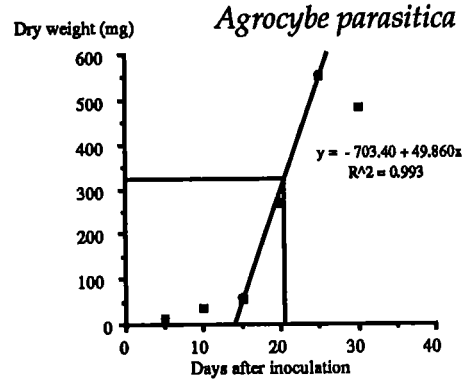
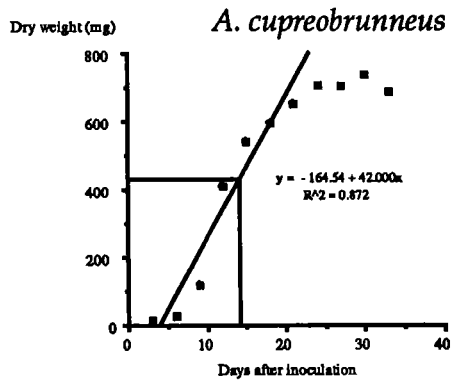
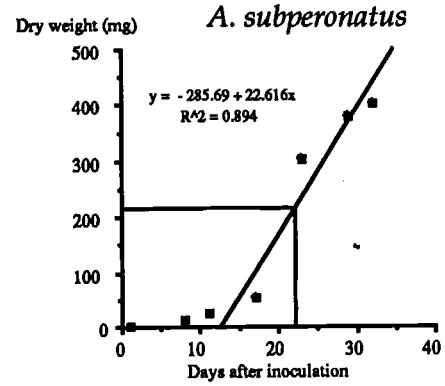
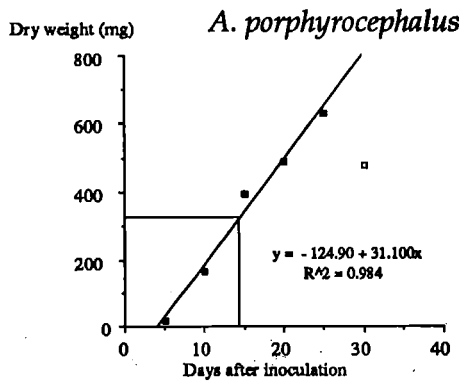
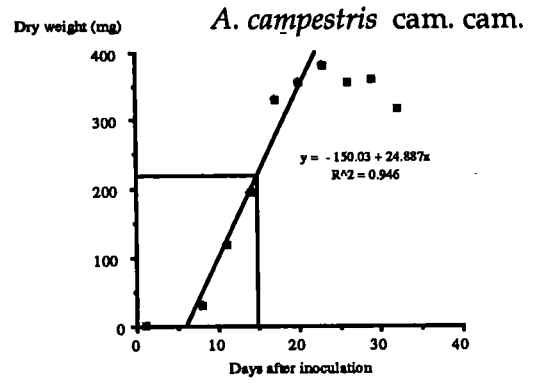
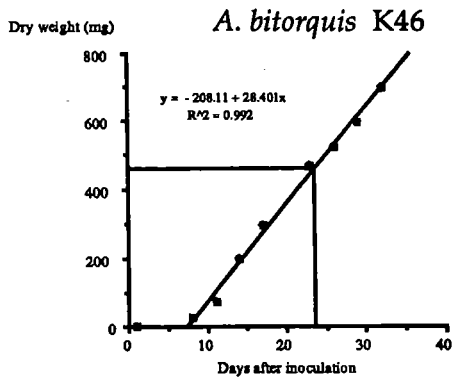


Figure 3.10. Growth curves for strains representative of eleven *Agaricus* species, *Leucoagaricus leucothites* and *Agrocybe parasitica*. Linear regressions were fitted to points arbitrarily designated as being representative of the most rapid period of growth, and the point mid way up each linear regression was used to decide harvest day and mycelial dry weight at harvest for each strain.



As shown in Figure 3.10, the point mid way up each linear regression line was used to indicate harvest day and mycelial dry weight at harvest for each strain. Table 3.2 summarises the information contained in the graphs depicting growth curves shown in Figure 3.10. *Agaricus bitorquis* K46, *Agaricus* sp. 1, and *Agaricus subperonatus* did not reach a stationary phase, (Moore-Landecker 1972) in their growth, but linear regressions were fitted to the points above the lag phase, (Moore-Landecker 1972) for each of these species, and a point mid way along each of these curves was used to determine harvest days and mycelial dry weight at harvest in the same way as for the other species. It is acknowledged that the estimates of similar physiological growth stage for these species may not have been as accurate compared with the other species examined. Differences in the number of days after inoculation, prior to the time arrived at to estimate a similar physiological stage for all cultures ranged from 14 for *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus* to 27 for *Agaricus* sp.1, (Table 3.2). Differences in mycelial production per flask at the time when similar physiological stage had been reached ranged from 57 mg dry weight of mycelium for *Agaricus bernardii* to 610 mg dry weight of mycelium for *Leucoagaricus leucothites*, (Table 3.2).

Species ^a	Harvest day ^b	Mycelial freeze dried weight (mg) at harvest ^b
<i>Leucoagaricus leucothites</i>	18	610
<i>Agaricus bitorquis</i> ^c	24	430
<i>Agaricus cupreobrunneus</i>	14	420
<i>Agaricus porphyrocephalus</i>	14	330
<i>Agrocybe parasitica</i>	21	320
<i>Agaricus subperonatus</i> ^c	23	225
<i>Agaricus campestris</i>	15	220
<i>Agaricus arvensis</i>	23	160
<i>Agaricus semotus</i>	18	160
<i>Agaricus bisporus</i>	19	110
<i>Agaricus</i> sp.1 ^c	27	90
<i>Agaricus lanipes</i>	25	78
<i>Agaricus bernardii</i>	23	57

^a strains representative of each species were incubated at 25°C and shaken at 80 rpm in 100 ml Oxoid Malt Extract Broth contained in 250 ml Erlenmeyer flask. Strains are listed in decreasing order according to weight of freeze-dried mycelium per flask at the harvest dates.

^b Harvest day after inoculation and freeze dried mycelial weight were estimated median values of the most rapid period of growth. This is the time arrived at to estimate similar physiological stage for all cultures

^c Strains that did not reach the stationary phase on their growth curves.

Table 3.2. Harvest dates representing points of similar physiological stage for 11 strains representing the *Agaricus* species studied, *Leucoagaricus leucothites*, and *Agrocybe parasitica*.

3.10.3. Discussion and conclusions.

The harvest dates determined by this study provide an estimate of when the strains are at a similar physiological stage, which should mean that the allozymes produced will be similar in number and resolution, thereby decreasing error due to age.

Growth rate information for species, such as the time taken for nutrients to become limiting for growth, could be used as a character for phylogenetic analysis. It would be possible to group the species in Table 3.2 according either to the days after inoculation they take to reach similar physiological stage or

according to the production of dry weight mycelium up to that time. However, while general conclusions as to relative physiological rates between species may be gained through analysis of a representative strain, a far more rigorous examination of variability within species is needed before such information could be used directly in a phylogenetic study. However, it is clear that it is important to conduct surveys to establish the extent of variation in physiological growth rate within and between species.

3.11. General conclusions.

3.11.1. Electrophoretic methods.

1) Except for EST, HSE was more effective than PAGE for the resolution of *Agaricus* allozymes, possibly due to greater range of electrophoretic buffer variations possible with HSE compared with PAGE. HSE was therefore the method of choice when analysing the allozyme variability of *Agaricus* species.

2) The resolution of all enzymes was improved by the addition of 12 mM cysteine-HCl, plus a mass of hydrated insoluble PVP equal to that of the sample, to the extraction solution used by May and Royse (1981). The inclusion of 1 mM dithiothreitol, (Cheliak and Pitel 1984), 0.01% soluble PVP, (Cheliak and Pitel 1984), and 0.05 % bovine serum albumin, (Cheliak and Pitel 1984) in the extraction buffer improved AAT and LAP resolution. Thus it has been shown that it is possible to increase the quantity of allozyme information regarding *Agaricus* species by modifying the extraction buffer systems.

3) The weight of mycelium to be extracted was established by the finding that it was necessary to have approximately 50 μ l supernatant for electrophoresis. As a result, routinely 0.2 g wet weight mycelium or 20-23 mg freeze dried mycelium was used for extraction with 200 μ l of extraction buffer. Experiments showed that the quantity of supernatant applied to the gel for electrophoresis was critical for the resolution of enzymes.

4) Use of the S4 buffer system, Table 3.1, provided greater resolution of *Agaricus* mycelial LAP and β GLU compared with the R system. But migration and separation of electromorphs was less satisfactory because of the lower pH of the system. Use of the C buffer system at pH 8.2, Table 3.1, increased electromorph migration and improved the resolution for AAT, GK, HA, and MDH compared with pH 6.1, and may have been responsible for the demonstration of a second AAT putative locus for *Agaricus bisporus*, where only one had been previously reported for this species. Modification of the electrophoretic buffer systems was critical for the resolution of LAP, β GLU, AAT, GK, HA, and MDH.

5) *Agaricus* mycelial enzymes with higher activity levels, which were prone to smearing, e.g., ACP, β GLU, and HA, required shorter electrophoresis time prior to sample wick removal compared with enzymes with less activity, e.g., AAT, and LAP. Thus knowledge of the relative activity levels of the enzymes under investigation was found to be useful for the resolution of these enzymes.

6) Modifications to the staining techniques which were significant to the success of the LAP system included 1) use of a stain solution buffered to pH of 5.3, 2) increasing the quantity of L-leucyl- β -naphthylamide, 3) use of the dye Fast Garnet GBC Salt. A staining buffer pH 4.3 was a significant factor in obtaining adequate β -GLU resolution. Thus modifications of existing staining systems can be useful for improving the visualisation of enzymes.

7) Using HSE, and manipulation of the electrophoretic methods enabled the successful resolution of ten mycelial enzymes encoded by twelve polymorphic putative loci, Aat-1, Acp, β Glu, Gk-1, Gk-2, Gpt, Ha-1, Lap-1, Mdh-1, Mdh-2, Mpi, and Pgm-2 for strains representative of eleven *Agaricus* species, *Leucoagaricus leucothites*, and *Agrocybe parasitica*. Standard conditions for the study of these enzymes using HSE are contained in Table 3.1. Electromorph similarities and differences between strains at the twelve putative loci indicated they may be useful for phylogenetic analysis.

3.11.2. A comparison of allozymes obtained from different tissues.

Compared with mycelium alone, additional allozyme information was revealed when other tissues of *Agaricus* were tested. As a result of these experiments, it appears that further study of allozyme variability during the development of the basidioma tissues is needed. Until this research has been carried out, mycelium for analysis grown under standardised and controlled conditions is most appropriate.

3.11.3. Growth rates of *Agaricus bitorquis* K46 in shaken and stationary liquid culture.

The most effective culture method, in terms of mass of mycelium produced over time was shaken culture, which produced more mycelium at a faster rate than stationary liquid culture.

3.11.4. Investigation of EST electromorph variation between replicate cultures of *Agaricus bisporus* A5.2 mycelia in shaken submerged liquid culture.

Replicate cultures did not vary in EST pattern or resolution, and multiple cultures of each *Agaricus* isolate could be pooled for use in subsequent experiments.

3.11.5. Effects of storage treatments on mycelial enzymes.

Storage at -80°C of *Agaricus bisporus* A5.2 and *Agaricus bitorquis* K46 mycelia and extracted mycelial supernatant did not cause any significant changes in resolution of ACP, EST, and MDH for at least 3 months using HSE. Storage of freeze dried *Agaricus bisporus* A5.2 mycelia for two years did not cause loss of resolution these enzymes using HSE. Further experimentation is required to assess the effect of storage on specimens representative of other species and a greater range of enzymes. To ensure standardisation only one storage method should be used in any study. Further investigation is required into artifact development during the storage of freeze dried mycelium for PAGE analysis of EST.

3.11.6. Effect of culture method and harvest time on mycelial enzymes.

Depending upon species, the level of mycelial enzyme activity may be influenced by culture method and length of incubation. Compared with a single culture method, additional electromorphs may be revealed through the use of different culture methods. The failure to use standardised culture method and incubation time could influence the phylogenetic usefulness of data generated from mycelia, and could account for some of the differences reported in the literature. For allozyme analysis, a single standardised method should be used to grow mycelium for each strain, and mycelia should be of similar physiological development. However, these conditions are difficult to achieve due to the variable growth habits of fungi.

3.11.7. Estimation of physiological stage.

It was possible to provide an estimate of similar physiological stage between strains representing each species by studying their growth in liquid culture. This was achieved by harvesting each strain at a stage half way through its most rapid period of growth. It is suggested that this technique should decrease error due difference in physiological age for mycelial allozyme analyses and phylogenetic investigations based on such analyses.

3.11.8. Overall conclusion.

An evaluation of experimental procedures for allozyme analysis of *Agaricus* species, showed many potential sources of error could be decreased and/or eliminated. Using the methods

arrived at in this investigation it should be possible to screen strains, representing different *Agaricus* species for their allozyme variability at up to twelve putative loci, and obtain valid results that can be used in interspecific comparisons. Based on information generated from such analyses it may be possible to evaluate better the phylogenetic relationships of *Agaricus* species.

Chapter 4.

Allozymes of *Agaricus* species.

4.1. Introduction.

A standard method arrived at as a result of the experiments described in Chapter 2 is given in Appendix 4. This method was used for screening 40 fungal strains representative of 11 *Agaricus* species, *Agrocybe parasitica* and *Leucoagaricus leucothites*, Appendix 1. The allozyme information obtained was used for cladistic analysis of *Agaricus* species, using *Leucoagaricus leucothites* and *Agrocybe parasitica* as outgroups.

Each enzyme and encoding putative locus or putative loci is briefly described and discussed, with particular attention being paid to difficulties of genetic interpretation. A character matrix was formed representing electromorph presence and absence for all strains at all putative loci and is presented in Appendix 5. Abbreviations used to indicate lanes of sample migration on gel photographs presented in Section 4.2.2, have been used in the character matrix, Appendix 5.

4.2. Results and discussion.

1) Malate dehydrogenase (MDH).

Two MDH putative loci, Mdh-1 and Mdh-2, were exhibited for all strains, except *Agrocybe parasitica*, which showed weak, unresolvable Mdh-1 activity, and activity in 2 further zones interpreted as Mdh-2 and Mdh-3, as shown in Figure 4.1.

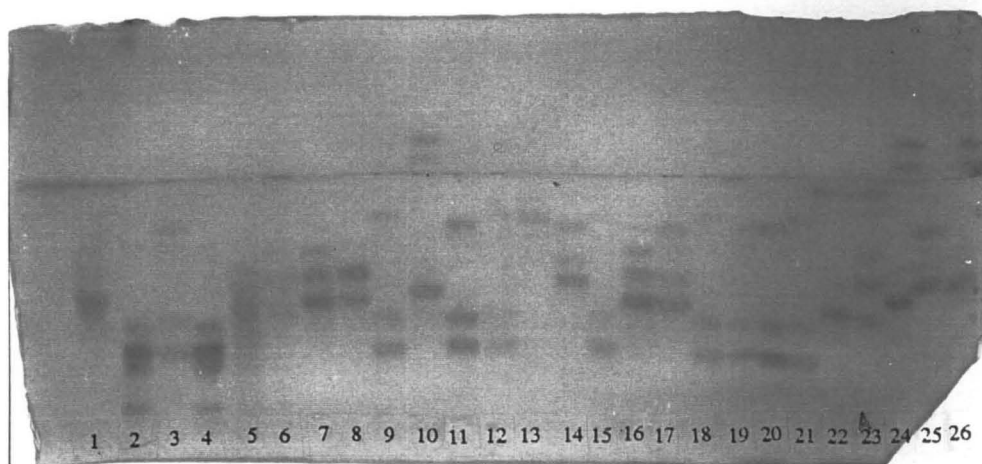


Figure 4.1. Typical malate dehydrogenase (MDH) activity, for a range of strains. Lanes 1-26 were as follows; 1) *Agaricus* sp. 1, 2) *Agrocybe parasitica*, 3) *Agaricus cupreobrunneus*, 4) *Agrocybe parasitica*, 5) *Leucoagaricus leucothites*, 6) *Agaricus bernardii* Mot, 7) *Agaricus campestris* Halls2, 8) *Agaricus campestris* var. *campestris*, 9) *Agaricus bisporus* A5.2, 10) *Agaricus lanipe*, 11) *Agaricus arvensis* Roll1, 12) *Agaricus bisporus* A5.2, 13) *Leucoagaricus leucothites* 14) *Agaricus semotus*, 15) *Agaricus bisporus* A5.2, 16) *Agaricus campestris* Halls2, 17) *Agaricus campestris* Halls1.2, 18) *Agaricus bisporus* A5.2, 19) *Agaricus cupreobrunneus*, 20) *Agaricus porphyrocephalus*, 21) *Agaricus bisporus* A5.2, 22) *Agaricus bitorquis* Ropley, 23) *Agaricus bernardii* Mot, 24) *Agaricus lanipe* 25) *Agaricus semotus*, 26) *Agaricus lanipe*.

Designation of bands as electromorphs was made difficult due to commonly occurring asymmetric heterozygous patterns for Mdh-2, e.g., *Agaricus campestris* cam.cam, Figure 4.1, lane 8. While asymmetric ADH heterozygous banding patterns have been reported for *Agaricus bisporus* (Royse and May 1982a, Kerrigan and Ross 1989), they have not previously been reported for *Agaricus* MDH banding patterns. *Agaricus arvensis*, *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus* displayed the same Mdh-2 bands, in the same asymmetric heterozygous pattern, as reported for *Agaricus bisporus* earlier, (Section 3.8.2.2). *Leucoagaricus leucothites* and *Agaricus* sp.1 each typically showed a 5 band pattern, although the most anodal of these for both species is not shown clearly in Figure 4.1. The MDH banding patterns for *Leucoagaricus leucothites* and *Agaricus* sp.1 were considered representative of overlapping putative loci. Of the 5 MDH bands for *Leucoagaricus leucothites* and *Agaricus* sp.1 the most anodal, the most cathodal, and the middle band were designated as electromorphs.

2) Acid phosphatase (ACP).

ACP may function as a monomeric or a dimeric enzyme depending upon the 'tissue' sampled, (Murphy et al. 1990). Except for *Agaricus bitorquis* Ropley, all mycelial samples analysed in this study showed single ACP electromorphs, Figure 4.2. *Agaricus bitorquis* Ropley appeared as a two electromorph heterozygote pattern, Figure 4.2, lanes 27 and 28. This implies that ACP activity from mycelium of this strain behaved as a monomer. This conflicted with the report by Royse and May (1989), that *Agaricus bisporus* mycelial ACP activity was dimeric. The observation of

double bands for *Agaricus bisporus* X20 and *Agaricus bitorquis* K46 lamellae in Section 3.5, also supported the contention that ACP may behave as a monomeric enzyme.

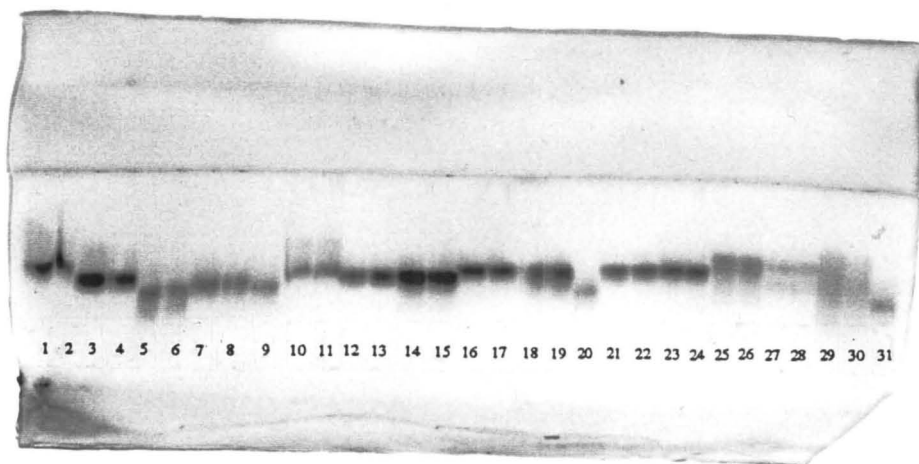


Figure 4.2. Typical acid phosphatase (ACP) activity for a range of strains. Lanes 1-31 were as follows; 1) *Agaricus campestris* Halls2, 2) *Agaricus campestris* Halls2, 3) *Agaricus cupreobrunneus*, 4) *Agaricus cupreobrunneus*, 5) *Agaricus bisporus* Ohoka, 6) *Agaricus bisporus* Ohoka, 7) *Agaricus subperonatus*, 8) *Agaricus subperonatus*, 9) *Agaricus bisporus* A5.2, 10) *Leucoagaricus leucothites*, 11) *Leucoagaricus leucothites*, 12) *Agaricus cupreobrunneus*, 13) *Agaricus cupreobrunneus*, 14) *Agaricus campestris* Halls1.4, 15) *Agaricus campestris* Halls1.4, 16) *Agaricus campestris* cam.cam, 17) *Agaricus campestris* cam.cam, 18) *Agaricus campestris* Halls1.2, 19) *Agaricus campestris* Halls1.2, 20) *Agaricus bisporus* A5.2, 21) *Agaricus campestris* Halls2, 22) *Agaricus campestris* Halls2, 23) *Agaricus campestris* Halls1.5, 24) *Agaricus campestris* Halls1.5, 25) *Agaricus arvensis* Roll1, 26) *Agaricus arvensis* Roll1, 27) *Agaricus bitorquis* Ropley, 28) *Agaricus bitorquis* Ropley, 29) *Agaricus bernardii* Mot, 30) *Agaricus bernardii* Mot, 31) *Agaricus bisporus* A5.2.

3) β -Glucosidase, (β GLU).

Kerrigan and Ross (1989) suggested this enzyme behaved as both a monomer and as a tetramer. During this study the observation of 2 electromorph heterozygote patterns, Figure 4.3, were common for this enzyme, supporting the hypothesis that it behaves as a monomer.

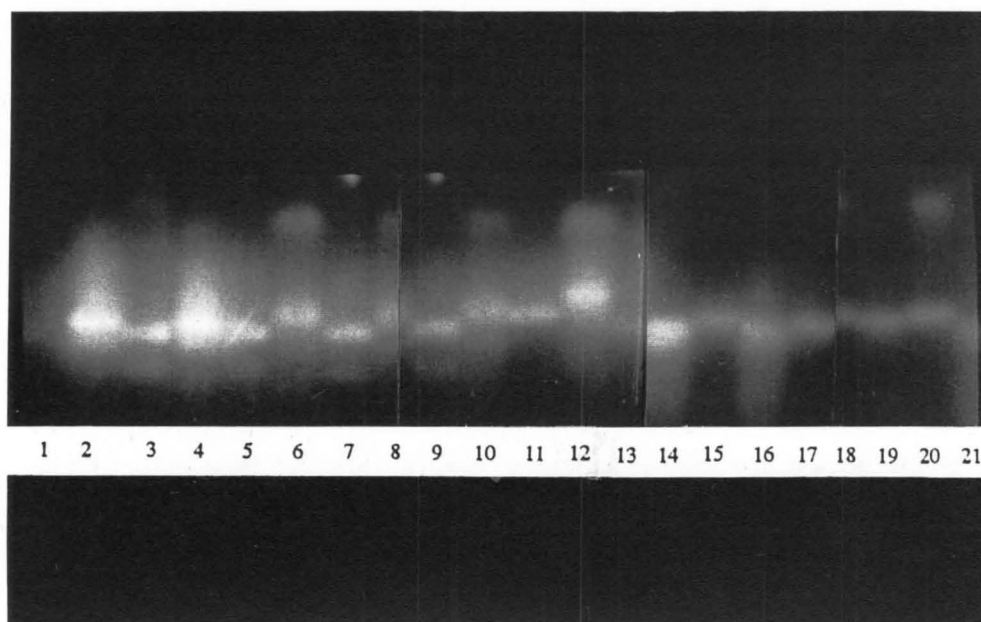


Figure 4.3. Typical β -glucosidase (β GLU) activity for a range of strains. Lanes 1-20 were as follows; 1) *Agaricus bisporus* B90SS#8, 2) *Leucoagaricus leucothites*, 3) *Agaricus bisporus* B90SS#8, 4) *Leucoagaricus leucothites*, 5) *Agaricus bisporus* A5.2, 6) *Leucoagaricus leucothites*, 7) *Agaricus bisporus* A5.2, 8) *Leucoagaricus leucothites*, 9) *Agaricus bisporus* X20, 10) *Agaricus bitorquis* 34782, 11) *Agaricus bisporus* Brown, 12) *Agaricus subperonatus*, 13) *Agaricus bisporus* B90SS#8, 14) *Agaricus campestris* Halls2, 15) *Agaricus bisporus* B90SS#8, 16) *Agaricus campestris* Halls2, 17) *Agaricus bitorquis* 34782, 18) *Agaricus campestris* Halls2, 19) *Agaricus bitorquis* 34782, 20) *Agaricus bisporus* B90SS#8,

While most species demonstrated 2 zones of β GLU activity, interpreted as 2 putative loci, *Agaricus bernardii* 34741, and 34751 showed 3 zones of activity, each with 2 electromorphs, which have been interpreted as heterozygous patterns at 3 putative loci. *Agaricus bisporus* β GLU was found to be reproducible and reliable using PAGE, (Kerrigan and Ross 1989), but not using HSE (Royse and May 1982a). However β GLU has been used to study *Agaricus campestris* successfully (May and Royse 1982). It was found during this study that β GLU was difficult to score accurately for all strains due to the following problems. Firstly, electromorphs could be extremely close together, e.g., Figure 4.3, lanes 13 and 15. Secondly, electromorph variation was observed between electrophoretic runs for some samples: e.g., using the same batch of freeze dried and stored mycelium, over a 3 day period with an electrophoretic run carried out each day with the same conditions the following events occurred: Day 1) a heterozygous banding pattern was observed for *Agaricus bitorquis* 34782, day 2) only the most cathodal electromorph of the heterozygous pattern observed on day 1 was detected, day 3) only the most anodal electromorph of the heterozygous pattern observed on day 1 was detected. To be certain about electromorph position in situations where the heterozygous banding pattern varied between electrophoretic runs, the heterozygous condition needed to be observed at least once, and reliable standards be included either side of variable strains before accurate interpretation could be carried out. Thus standards were used to assess each variable strain.

4) Hexoseaminidase, (HA).

Mycelial HA was successfully used for the first time to study genetic variability of *Agaricus* mycelial extracts, Figure 4.4.

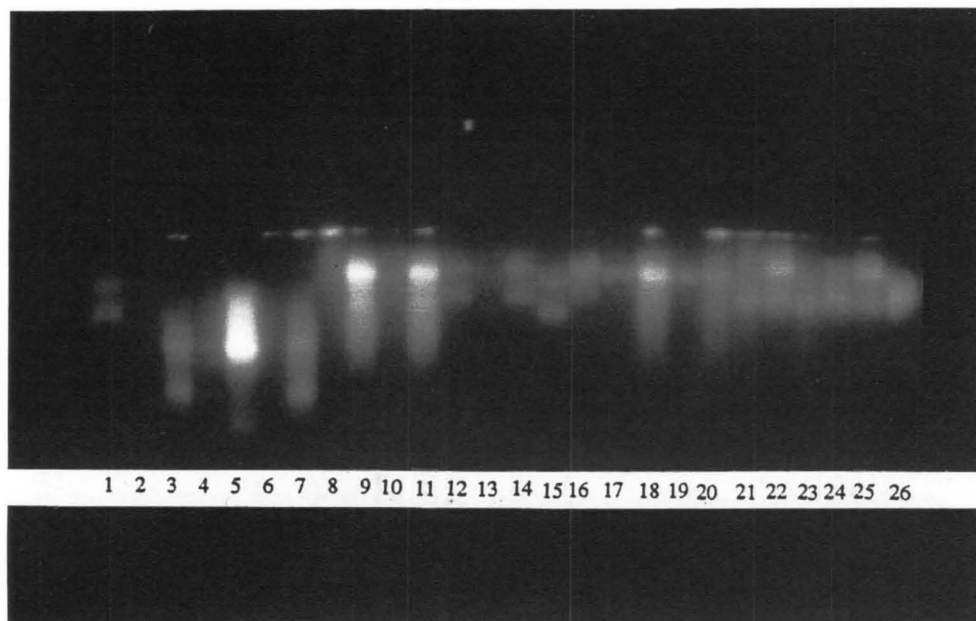


Figure 4.4. Typical hexoseaminidase (HA) activity for a range of strains. Lanes 1-26 were as follows; 1) *Agaricus campestris* Halls2, 2) *Leucoagaricus leucothites*, 3) *Agaricus subperonatus*, 4) *Agaricus bitorquis* K46, 5) *Agaricus bitorquis* Ropley, 6) *Agaricus bisporus* A5.2, 7) *Agaricus* sp. 1, 8) *Agaricus campestris* Halls1.2, 9) *Agaricus porphyrocephalus*, 10) *Agaricus campestris* Halls1.2, 11) *Agaricus porphyrocephalus*, 12) *Agaricus campestris* cam.cam, 13) *Agaricus campestris* Halls1.2, 14) *Agaricus campestris* cam.cam, 15) *Agaricus campestris* Halls2, 16) *Agaricus subperonatus*, 17) *Agrocybe parasitica*, 18) *Agaricus cupreobrunneus*, 19) *Agrocybe parasitica*, 20) *Agaricus cupreobrunneus*, 21) *Agaricus arvensis* Roll1, 22) *Agaricus semotus*, 23) *Agaricus arvensis* Roll1, 24) *Agaricus bernardii* Mot, 25) *Agaricus semotus*, 26) *Agaricus bernardii* Mot.

Two putative loci were obvious for HA, and the observed banding patterns were typical of a monomeric enzyme. Genetically interpretable Ha-1 activity was demonstrated by all species

except *Agaricus bisporus*, *Agaricus bitorquis*, and *Agaricus* sp.1, which showed uninterpretable Ha-2 activity due to smearing, Figure 4.4. However, *Agaricus bitorquis* 34782 showed clear genetically interpretable Ha-1 activity, and no Ha-2 activity whatsoever, suggesting a different genetic constitution for this strain in comparison with the others of the species. Royse and May(1989), also found *Agaricus bisporus* HA activity to be limited.

5) Leucine aminopeptidase, (LAP).

LAP was successfully used for the first time to study genetic variability for *Agaricus* mycelial extracts, Figure 4.5. A single putative locus was found to code for this enzyme for all strains studied except *Agrocybe parasitica*, which showed only very weak and unresolvable Lap-1 activity. *Agrocybe parasitica* was the only strain to demonstrate Lap-2 activity, which was much stronger than the Lap-1 activity of any other strain investigated. Observation of both single and double Lap-1 electromorph patterns, Figure 4.5, implied this enzyme behaved as a monomer, as has been previously reported for *Agaricus bisporus* pileus and stipe extracts (May and Royse 1981). As was reported in the investigation of different tissues, Section 3.5, all *Agaricus bisporus* strains were found to be monomorphic for Lap-1, and no other species was demonstrated to share this electromorph. Further tests are needed to establish if this putative locus can be a genetic marker for this species.

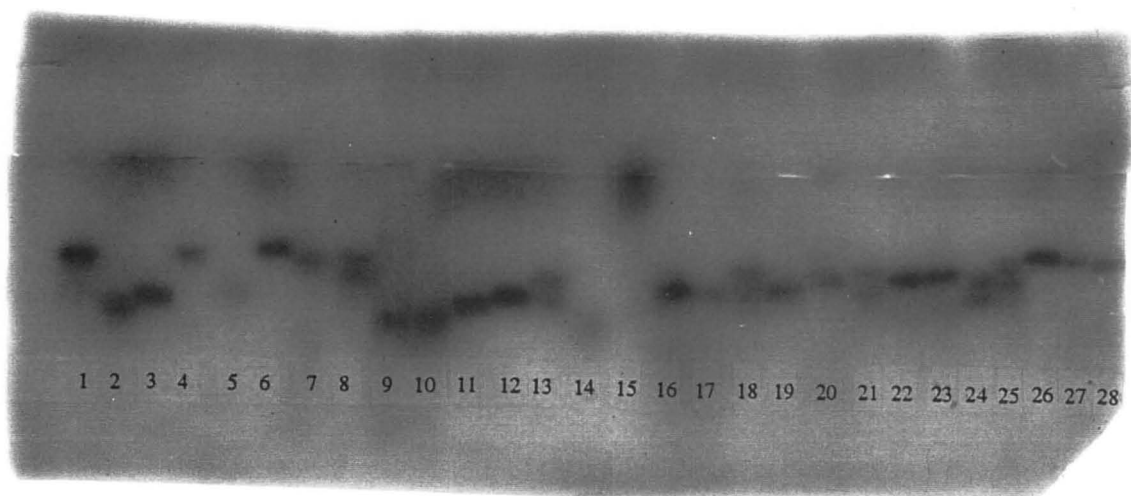


Figure 4.5. Typical leucine-amino peptidase (LAP) activity for a range of strains. Lanes 1- 28 were as follows; 1) *Agaricus porphyrocephalus*, 2) *Leucoagaricus leucothites*, 3) *Agaricus subperonatus*, 4) *Agaricus campestris* Halls1.5, 5) *Agaricus* sp.1, 6) *Agaricus campestris* Halls2, 7) *Agaricus lanipe*, 8) *Agaricus cupreobrunneus*, 9) *Agaricus semotus*, 10) *Leucoagaricus leucothites*, 11) *Agaricus subperonatus*, 12) *Agaricus bitorquis* 34725, 13) *Agaricus bitorquis* Ropley, 14) *Agaricus bitorquis* K46, 15) *Agaricus bernardii* Mot, 16) *Agaricus bisporus* Ohoka, 17) *Agaricus bisporus* A5.2, 18) *Agaricus cupreobrunneus*, 19) *Agaricus arvensis* Roll1, 20) *Agaricus lanipe*, 21) *Agaricus cupreobrunneus*, 22) *Agaricus lanipe*, 23) *Agaricus porphyrocephalus*, 24) *Agaricus bitorquis* Ropley, 25) *Agaricus cupreobrunneus*, 26) *Agaricus campestris* Halls2, 27) *Agaricus campestris* cam.cam, 28) *Agaricus campestris* Halls1.5.

6) Aspartate aminotransferase, (AAT).

This study revealed 2 AAT putative loci for mycelial extracts of *Agaricus bisporus* and *Agaricus bitorquis*, one close to the origin, Aat-1, and another having greater migration, Aat-2, Figure 4.6.

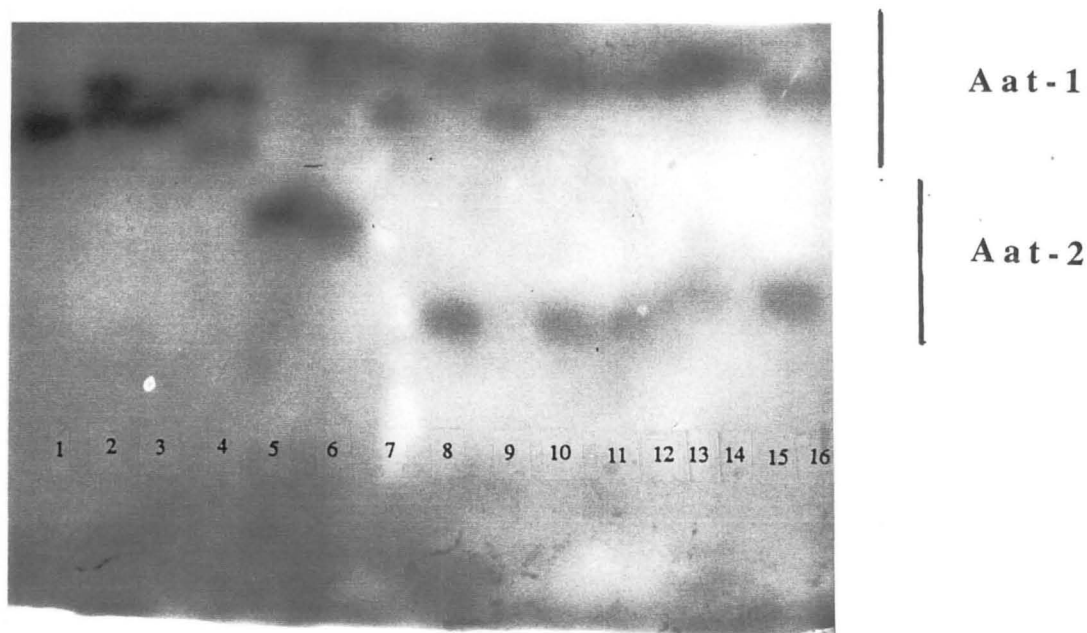


Figure 4.6. Typical aspartate aminotransferase (AAT) activity showing the presence of 2 putative loci for *Agaricus bisporus* and 2 electromorph patterns common at Aat-1. Lanes 1-14 were as follows; 1) *Agaricus arvensis* Nect3, 2) *Agaricus* sp.1, 3) *Agaricus arvensis* Rb1, 4) *Agaricus bitorquis* 34782 5) *Agaricus bitorquis* 34725, 6) *Agaricus bitorquis* 34780 7) *Agaricus subperonatus*, 8) *Agaricus bisporus* X20, 9) *Agaricus subperonatus*, 10) *Agaricus bisporus* X20, 11) *Agaricus bisporus* 28#SS502, 12) *Agaricus bisporus* Brown, 13) *Agaricus bisporus* 24663, 14) *Agaricus bisporus* B90#SS8, 15) *Agaricus bernardii* 34741, 16) *Agaricus bisporus* B90#SS8.

Strains of all other species showed activity for Aat-1 only. This was the putative locus used for cladistic analysis. Two AAT putative loci have been reported for *Agaricus campestris* by May and Royse (1982b), but they only used Aat-2. May and Royse (1982a) only reported a single AAT putative locus with 2 electromorphs for *Agaricus bisporus*. Faster AAT band migration was noted in this investigation using an electrode buffer pH of 8.2, in comparison with pH 6.1 used by May and Royse (1982a and 1982b) to study *Agaricus bisporus* and *Agaricus campestris* respectively. When conditions used by May and Royse (1982a) were repeated in this study, Aat-2 was found to be closer to the origin than shown in Figure 4.6, making Aat-1 difficult or impossible to observe. This implies that May and Royse (1982a) may have studied the putative locus designated here as Aat-2 for *Agaricus bisporus*. Support for this conclusion comes from the position of the Aat-2 electromorph for 28#SS502 which migrated further than that for B90#SS8, as reported by May and Royse (1982a) for their single putative locus. Whereas, the electromorphs for these strains were found to be identical at Aat-1 in this study. The AAT putative locus studied by May and Royse (1982a, 1982b), and designated here as Aat-2, was reported by them to be dimeric. The dimeric nature of this enzyme at Aat-2 was supported during this study by the infrequent observation of 3 band heterozygote patterns. However, electromorphs observed in this investigation at Aat-1 consistently produced either 1 or 2 band patterns typical of a monomeric enzyme, which is shown in Figure 4.6.

7) Glucokinase (Hexokinase), (GK).

Two putative loci were found to code for this enzyme in all but *Agaricus arvensis*, which showed three putative loci for the 'Nect' strains. Gk-1 was consistently easier to score accurately than Gk-2 because of electromorph smearing. For some species, i.e., *Agrocybe parasitica* and *Leucoagaricus leucothites*, uncertainty about the relative positions of electromorphs at Gk-2 lead to the conclusion that this putative locus should only be used for phylogenetic analysis with caution. Further modification of techniques is needed to clarify the electromorph content of Gk-2.

8) Glutamate pyruvate transaminase, (GPT).

Only homozygous banding patterns were detected for this dimeric enzyme, which was supported by May and Royse (1981). As reported by May and Royse (1982a), both *Agaricus bisporus* 22#SS502, and B90#SS8 shared the same electromorph, found to be most common among the strains tested for this species. Judging by the relative GPT electromorph positions between strains, more than 1 putative locus may be represented, although accurate determination of number of GPT putative loci could not be made. As a result, GPT activity was treated as a single putative locus for cladistic study.

9) Phosphoglucomutase, (PGM).

Three PGM putative loci were demonstrated only for *Agaricus semotus* and *Agaricus* sp.1. Pgm-1 was found to be cathodic. *Agaricus bitorquis* Ropely showed activity for Pgm-1 and Pgm-2, but all other species showed activity at Pgm-2 only, Figure 4.7. Pgm-2 was used for cladistic analysis.

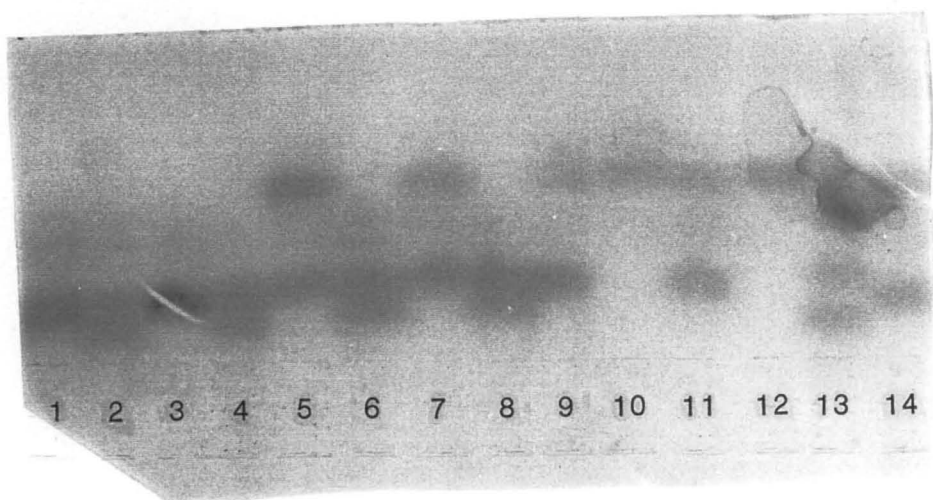


Figure 4.7. Typical phosphoglucmutase (PGM) activity for a range of strains. Lanes 1-14 were as follows; 1) *Agaricus campestris* cam.cam, 2) *Agrocybe parasitica*, 3) *Agaricus campestris* Halls1.5, 4) *Agrocybe parasitica*, 5) *Agaricus bisporus* A5.2, 6) *Agrocybe parasitica*, 7) *Agaricus bisporus* X20, 8) *Agrocybe parasitica*, 9) *Agaricus bisporus* Ohoka, 10) *Agaricus subperonatus*, 11) *Agaricus bisporus* Ohoka, 12) *Agaricus subperonatus*, 13) *Leucoagaricus leucothites*, 14) *Agaricus bisporus* A5.2

10) Mannosephosphate isomerase, (MPI).

All species, except *Agrocybe parasitica* demonstrated Mpi-1 activity. The electromorph for *Agrocybe parasitica* was positioned well below those for the other species, in what may have been another putative locus, Mpi-2. According to May and Royse (1982a), *Agaricus bisporus* strains commonly displayed null alleles for MPI. The only *Agaricus bisporus* strain to show MPI activity in this study was bisOhoka, the other strains for this species presumably displayed null alleles. May and Royse(1982a)

reported MPI activity for *Agaricus bisporus* strains 22#SS502, and B90#SS8, but despite repeated attempts this result could not be repeated in this study. This result can not be explained, except for the slight chance that mutation at the locus coding for MPI had occurred since their analysis or if the cultures screened in this study were not in fact the same ones used by May and Royse(1982a).

4.3. Cladistic analyses of data generated from mycelial allozyme study of all strains.

4.3.1. Data analyses.

Discrete character parsimony analyses using Phylogenetic Analysis Using Parsimony, (PAUP 3.0 Macintosh version; Swofford 1990) were carried out on the electromorph characters determined in the previous section, (Section 4.2) . Based on the independent allele model of Mickevich and Mitter (1981), electromorphs were coded as characters, and their presence and absence was coded as discrete character states, see Appendix 5 for character matrix. Dollo-up character types were used as this character type is most appropriate for the study of allozymes, (Olmstead 1989).

Unless otherwise stated, each character (excluding uninformative characters constant between taxa) was weighted by $1/n$ where n was the number of characters at a putative locus. Thus the problem of unequal weighting of polymorphic putative loci was decreased, (Moran *et al.* 1990). No limit was set to the number of trees retained in the memory during any search.

When heuristic searches were made the branch swapping option with tree bisection-reconnection (TBR) was used, and all minimal trees were saved (MULPARS). *Agrocybe parasitica* and *Leucoagaricus leucothites* were used as outgroups for analyses. When multiple equally parsimonious trees were saved from a search, 50 % majority rule consensus trees were constructed, (Margush and McMorris 1981) to assess robust groups of taxa. Consistency indices (C.I.) and tree lengths are reported for all PAUP analyses, (Swofford 1990). Jackknifing (Lanyon 1985) of taxa and putative loci was used to investigate the stability of the most parsimonious tree(s).

Preliminary analyses were carried out with individual strains treated as separate taxa, and heuristic searches were used to find the most parsimonious tree(s). The use of equally weighted characters was compared with unweighted characters during preliminary analyses. Strains were pooled for analysis according to species determined using traditional methods primarily based on the taxonomic species concept. Exact branch and bound searches were used to find the most parsimonious tree(s) when species were analysed with data from individual pooled strains.

4.3.2. Results.

4.3.2.1. Preliminary analyses.

Jackknifing taxa and putative loci showed that the data set was sensitive to both these variables. The tree structure of Figure 4.8, shown Section 4.3.2.1.1, was often affected by taxon or

putative locus removal, e.g., removal of some putative loci, e.g., *Mpi*, caused the most parsimonious tree to become asymmetrical, with taxa from the *Campestris* group becoming most highly derived. However, it was usual for taxa to group according to species during the Jackknifing of putative loci. The Jackknifing of some taxa did not have as pronounced an effect on tree structure as it did with others, and through elimination it was possible to show which were the troublesome taxa. These taxa vastly increased tree length or caused major changes to tree structure.

The inclusion of *Agaricus bernardii* Mot consistently caused dramatic changes in tree structure, lower consistency indices and longer tree lengths in comparison with those analyses that omitted this strain. Thus this strain was eventually omitted from further analysis, a practice which has been generally accepted in such circumstances, (Stewart 1993). *Agaricus bitorquis* 34782 consistently grouped with *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus*, and was significantly removed from the other *Agaricus bitorquis* strains. Based on the cladistic position of *Agaricus bitorquis* 34782, it was not considered justified to include this strain with the other *Agaricus bitorquis* strains when they were pooled, and hence forth it was considered an individual. Other strains were also troublesome, lacking sufficient synapomorphies to clearly define relationship, including those for *Agaricus arvensis*, *Agaricus semotus*, *Agaricus subperonatus*, *Agaricus lanipes* and *Agaricus* sp.1.

4.3.2.1.1. Analyses of strains forming robust groups.

Strains of *Agaricus bisporus*, *Agaricus bitorquis*, *Agaricus bernardii*, *Agaricus campestris*, *Agaricus cupreobrunneus*, and *Agaricus porphyrocephalus* were analysed. A single weighted most parsimonious tree was found, 275 (unweighted) steps long, CI = 0.360, Figure 4.8. The low CI value was considered a result of the high number of taxa. When no character weighting was applied 4 equally parsimonious trees were found, each 272 steps long, CI = 0.364. Only minor isolate differences were observed between the trees found using unweighted putative loci and that found when putative loci were equally weighted.

A full apomorphy list for the tree shown in Figure 4.8 is given in Appendix 6. A parsimony length of 12 characters determined the monophyly of all ingroup taxa, 11 determined the monophyly of taxa from the traditional Section Bitorques, and 11 the monophyly of taxa from traditional Section Agaricus, Appendix 6.

In the description of the of the tree in Figure 4.8 that follow, use of the term 'monophyletic' was restricted to groups of species, as has been generally accepted in phylogenetics, (Hennig 1966, Platnick 1977, and Wheeler and Nixon 1990). Except for *Agaricus bitorquis* 34782, strains formed groups with others of the same species. Strains of *Agaricus bisporus*, *Agaricus bitorquis*, and *Agaricus bernardii* formed a large monophyletic group. *Agaricus bisporus* and *Agaricus bitorquis* also formed a monophyletic group. *Agaricus bisporus* bisOhoka was the first of the strains of this species to diverge. Next to diverge were the sister taxa

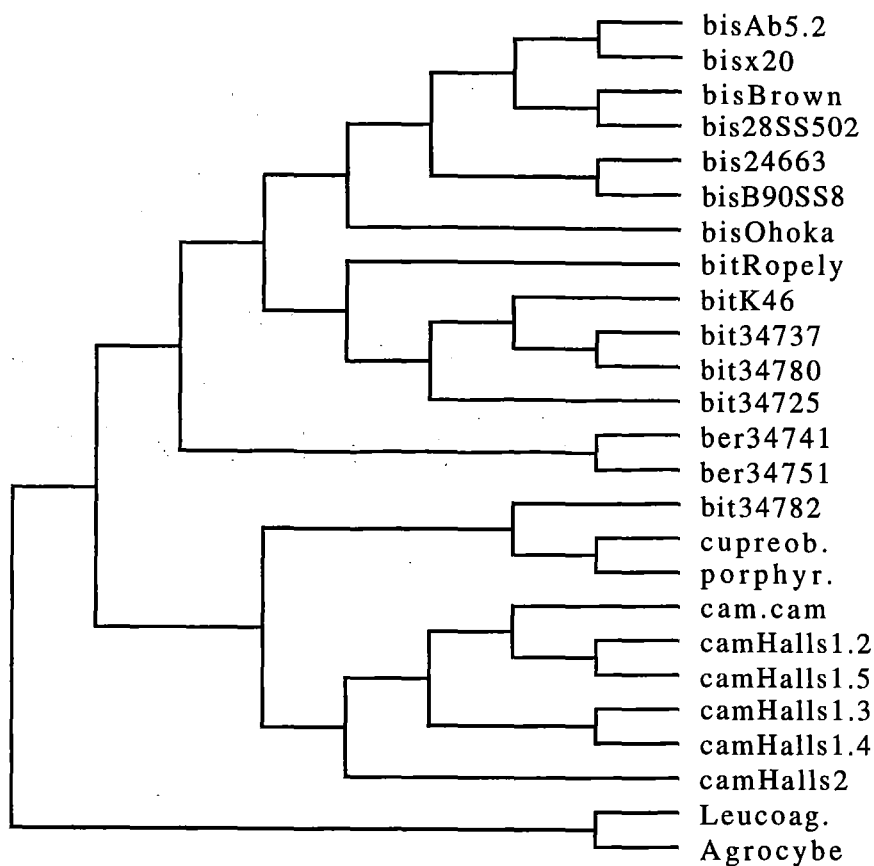


Figure 4.8. Single most parsimonious tree, 275 (unweighted) steps, CI = 0.360. Prefixes represented taxa as follows, bis. *Agaricus bisporus*, bit. *Agaricus bitorquis*, ber. *Agaricus bernardii*, cupreob. *Agaricus cupreobrunneus*, porphyr. *Agaricus porphyrocephalus*, cam. *Agaricus campestris*, Leucoag. *Leucoagaricus leucothites*, and *Agrocybe parasitica*.

Agaricus bisporus bis24663, and bisB90SS8. Most derived of the *Agaricus bisporus* strains were the sister taxa *Agaricus bisporus* bisA5.2 and bisx20, grouping with *Agaricus bisporus* bis28SS502 and bisBrown. The earliest of the *Agaricus bitorquis* strains to diverge was bitRopley, followed by bit34725, and then bitK46. Forming sister taxa *Agaricus bitorquis* bit34737, and 34780 diverged last of the strains of this species. The two *Agaricus bernardii* strains ber34741, and 34751 were sister taxa, ancestral to the monophyletic *Agaricus bisporus* and *Agaricus bitorquis*.

Agaricus bitorquis 34782, *Agaricus cupreobrunneus*, *Agaricus porphyrocephalus*, and *Agaricus campestris* strains formed a monophyletic group distinct from the others. *Agaricus bitorquis* 34782 grouped with the sister taxa *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus*. Of the *Agaricus campestris* strains, camHalls2 was first to diverge. The next strains of this species to diverge were the sister taxa camHalls1.3, and camHalls1.4. *Agaricus campestris* cam.cam then diverged, prior to the sister group camHalls1.2 and camHalls1.5.

4.3.2.3. Analyses of pooled strains.

Strains were pooled according to species, to allow exact branch and bound searches and use of jackknifing to assess the stability of the most parsimonious tree(s).

4.3.2.3.1. Pooled strains forming robust groups.

A single most parsimonious tree, $CI = 0.645$, was found when strains from 4.3.2.1.1 were pooled according to species, and *Agrocybe parasitica* designated as an outgroup, Figure 4.9. The CI value for this tree was higher than that shown in Figure 4.8. The most likely reason for this was the reduced number of taxa in comparison with the tree shown in Figure 4.9. Jackknifing putative loci showed that the removal of *Mdh-2*, *Lap*, *Gk-2*, *Pgm*, or *Mpi* caused *Agaricus bisporus* to form a monophyletic group with *Agaricus bitorquis*, in the same way that occurred when strains were analysed as individual taxa, Figure 4.8. However, the removal of any of the other 7 putative loci did not affect the structure of the tree shown in Figure 4.9. A full apomorphy list for the tree shown in Figure 4.9 is given in Appendix 7.

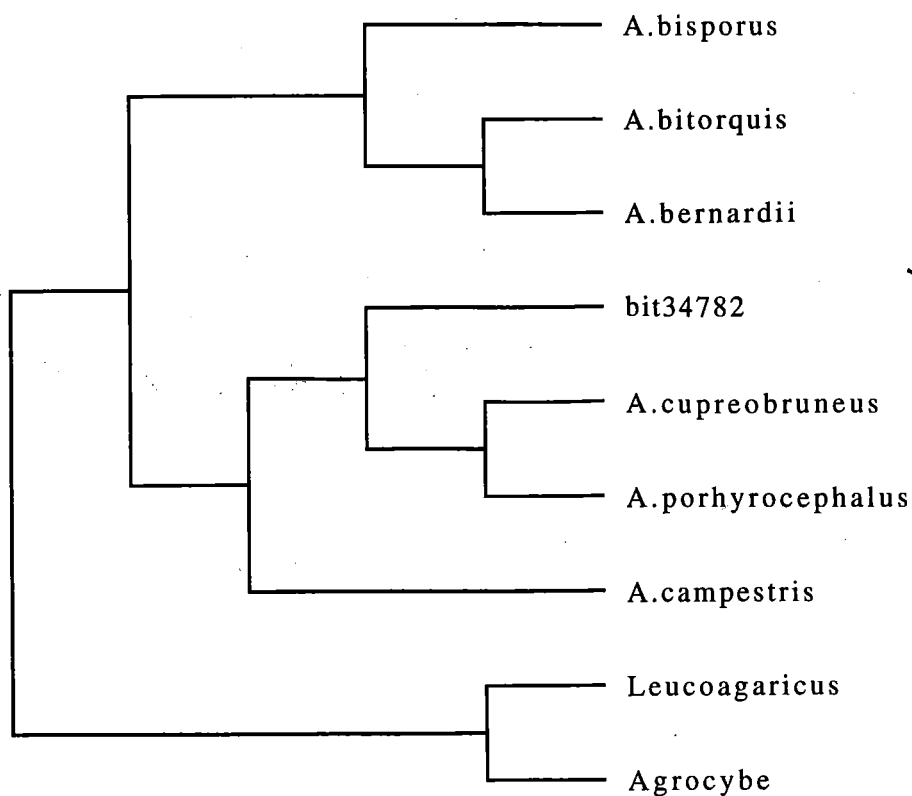


Figure 4.9. Single most parsimonious tree reflecting relationships of *Agaricus* species, 171 (unweighted) steps long, CI = 0.645. bit34782 = *Agaricus bitorquis* 34782, *Leucoagaricus* = *Leucoagaricus leucothites*, and *Agrocybe* = *Agrocybe parasitica*.

4.3.2.3.2. Pooled strains not forming robust groups.

Repeated analyses were carried out using weighted putative loci and branch and bound searching to evaluate cladistic relationships for *Agaricus arvensis*, *Agaricus semotus*, *Agaricus subperonatus*, *Agaricus lanipe* and *Agaricus* sp.1 within the tree structure shown in Figure 4.9. Analyses incorporated these species, one at a time, into the data set used for Section 4.3.2.3. It was found that by jackknifing putative loci most of these species could be moved from one monophyletic group to another leaving little doubt that the results of the allozyme analysis were not useful for resolving the relationships of these taxa. Sample analyses are given in Appendix 8.

4.3.3. Discussion.

Support for the use of allozymes as a source of phylogenetic information on *Agaricus* species is shown by the results presented in Figure 4.8, where strains may be seen to group according to species identified based on the taxonomic species concept, Appendix 9. Use of Dollo parsimony and the application of equal character weights, depending upon the number of useful characters per putative locus, as shown in Figures 4.8 and 4.9, revealed two main monophyletic species groups directly corresponding to traditional classification, i.e., Section Bitorques, containing *Agaricus bisporus*, *Agaricus bitorquis*, and *Agaricus bernardii*, and Section Agaricus, group Campestris, containing *Agaricus campestris*, *Agaricus cupreobrunneus*, and *Agaricus porphyrocephalus*.

— This study offered support for the proposition that *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus* should be placed together in a monophyletic group distinct from *Agaricus campestris*, as suggested by Moser (1967, 1978). Based upon a study of morphological characters he contended that these two species may in fact be regarded as a single entity. However, the fact that electromorphs may not have been detected for these species due to small sample size, i.e., single specimens for both *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus* should be noted. Further tests are needed to evaluate the specific nature of these taxa by screening specimens from many populations of both *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus*. The validity of the phylogenetic relationships implied in Figures 4.8 and 21 will remain justified only by continued study employing reappraisal of techniques, and the generation of additional characters to those already used.

Raper (1976) questioned the identification of *Agaricus* specimens as a result of her interfertility analyses. The results of this study cast further doubt over the identification of her *Agaricus bitorquis* stock 2, designated here as *Agaricus bitorquis* bit34782. Originally strain bit34782 was collected by H. Visscher and M. Vaandrager in 1968, and identified based on basidioma morphology by Bas, (Raper 1976), which was later confirmed by Singer, (Raper 1976), based on basidiospores and photographs. However, in this study strain bit34782 consistently showed affinities to the monophyletic group containing species from the traditional Section *Agaricus*, Group *Campestris*, rather than the monophyletic group containing species from the Section *Bitorques*.

Of particular importance was the possession of Ha-1 electromorphs by *Agaricus bitorquis* bit34782, when none of the other *Agaricus bitorquis* strains showed activity for this putative locus. Based upon the evidence presented in this study it was concluded that *Agaricus bitorquis* bit34782 has stronger affinities with *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus* than with any other species examined. Future examination of this strain should be directed towards confirming the identification of *Agaricus bitorquis* bit34782.

While jackknifing putative loci for the pooled strain data set did not affect the monophyly of the Bitorques section, it uncovered instability within the section. The use of only two *Agaricus bernardii* strains in this study may have been insufficient to accurately resolve the relationship of the species. Screening a larger number of strains for this species may resolve the relationship between *Agaricus bernardii* and the other members of the Section Bitorques using the methods described in this thesis.

The present allozyme data set was not useful in accurately assessing the cladistic positions for *Agaricus subperonatus*, *Agaricus lanipe*, *Agaricus arvensis*, *Agaricus semotus*, *Agaricus* sp.1, Appendix 8. The most likely reason for the lack of resolution for these species is that they are distantly related to the seven taxa studied in Section 4.3.2.3.1. However, tentative associations may be postulated from the exploratory analyses set out in Appendix 8. *Agaricus subperonatus*, while most likely ancestral to the other species, as shown in Figure 1, Appendix 8, did have some affinity

to the group containing *Agaricus campestris*, Figure 2, Appendix 8. The possible position implied for *Agaricus subperonatus* in Figure 2, Appendix 8 reflects traditional classification of *Agaricus subperonatus* within the Section *Agaricus*, Group *Vaporarius*. *Agaricus campestris*, *Agaricus cupreobrunneus*, and *Agaricus porphyrocephalus*, also in the Section *Agaricus* are traditionally classified in the Group *Campestris*, distinct from the Group *Vaporarius*.

Based on the information available, it was not possible to postulate even a tentative relationship for *Agaricus lanipeŕ*, traditionally classified in the Section *Sangulinolenti*. Similarly *Agaricus arvensis* and *Agaricus semotus*, and *Agaricus* sp.1 appeared too distantly related to determine their phylogenetic position based upon the present data set. It is interesting to note the position of *Agaricus semotus* in Figure 5, Appendix 8, where it is clearly shown to be quite separate from the other species, consistent with traditional classification, (Cappelli 1984). While affinities between *Agaricus semotus* and *Agaricus campestris*, Appendix 8, may well have implied convergences, they may equally be evidence of early relationship between these taxa.

The development of phylogenetic techniques is an extremely dynamic field, especially in relation to the use of allozyme characters (Swofford and Olsen 1990). Future analyses of the present data set using different methods may allow resolution of additional species relationships. Coding allozyme data according to their presence or absence may be difficult to justify, as discussed by Murphy (1993), even when the application of equal

character weighting has been implemented, e.g., Moran et al. (1990).

4.3.4. Conclusions.

The results described above show that information gained from the study of *Agaricus* allozymes may be used to explore the cladistic relationships of closely related species. It was found that the use of pooled strains for analysis provided increased stability of taxon relationships, as indicated by jackknifing taxa and putative loci. The results supported the contention that the traditional Section Bitorques, and the Section Agaricus, group Campestris were each monophyletic. The monophyly of these traditional Sections was also supported. Tentative support was found for the monophyly of *Agaricus bitorquis* with *Agaricus bernardii*, within the Bitorques section, and for *Agaricus cupreobrunneus* with *Agaricus porphyrocephalus* within the Agaricus section.

Relationships of more distantly related species could not be accurately assessed, including *Agaricus arvensis*, *Agaricus semotus*, *Agaricus subperonatus*, *Agaricus lanipes*, and *Agaricus* sp.1. However tentative support was given for the inclusion of *Agaricus subperonatus* within the Section Agaricus. It would also appear that increased sample sizes, and evaluation of alternative coding methods for the data may aid in resolving the relationships of *Agaricus bisporus*, *Agaricus bitorquis*, and *Agaricus bernardii* with one another, and may allow the correct placement of other species, especially *Agaricus subperonatus*.

— Thus in summary it may be concluded that *Agaricus* mycelial allozymes may be successfully used to explore the cladistic relationships of closely related species. Monophyly was supported for species from the traditional Sections Bitorques, and Agaricus Group Campestris. Determination of the cladistic relationships of other species from Section Agaricus, was not possible. Neither was it possible to resolve the relationships of species from the traditional large Section Flavescentes.

Chapter 5.

Basidiospore study.

5.1. Introduction.

From the literature reviewed in Chapter 2, it can be concluded that only a limited amount of morphological divergence has occurred between closely related *Agaricus* species, and there has been a great deal of difficulty in character assessment. For these reasons additional phylogenetically useful characters and the development of more objective methods of assessing existing characters would be highly desirable for the preparation of a more effective classification of *Agaricus* species. As an example, problems associated with the study of basidiospores, discussed in the review of literature, Chapter 2, led to the conclusion that new methods for their measurement could be of considerable advantage to fungal systematists.

In this study image analysis of basidiospores was evaluated for increased speed of measurement compared with the use of light microscopy, and the use of characters which are not easy to measure manually. The results of image analysis were applied in an evaluation of the phylogenetic value of basidiospore variables from *Agaricus* species. A preliminary cladistic analysis was carried out using basidiospore variables and a limited number of other morphological and developmental characters. Basidiospores of the same 11 *Agaricus* species examined in Chapter 4, *Leucoagaricus leucothites* and *Agrocybe parasitica* were analysed. *Leucoagaricus leucothites* and *Agrocybe parasitica* were used as outgroups for analysis.

5.2. Preparation of basidiospores for analysis.

Details of species investigated are presented in the descriptions in Appendix 9. Basidiospore deposits or prints were made from each of 3 specimens of each taxon by placing the hymenial side of the fresh basidioma on a sheet of white paper, placing glass over the top and leaving them overnight (Stevenson 1982a). Basidiospores from an area of 5 mm x 10 mm, were collected from each basidiospore print, and rehydrated for 1 h. in an Eppendorf tube containing 0.5 ml of distilled water. The contents of each tube were then mixed for 1 min. with a Chiltern vortex shaker to suspend basidiospores. From each basidiospore suspension, a 10 μ l aliquot was pipetted onto a glass slide, and to increase the contrast between basidiospores and background, 5 μ l lacto phenol cotton blue, (Cruickshank et al. 1965) was added. The mixture was covered with a cover slip which was pressed down gently. Excess suspension was removed with tissue paper. Basidiospore images were magnified x100, x200, and x300 with an Olympus BH-2 microscope equipped with an JVC ccd video camera, with a Nikon, AF MICRO NICCOR 60 mm, 1:2.8 lens. Each field of view containing 10-30 well separated basidiospores was recorded for 5-10 sec. using a Panasonic NV-J1 VHS video cassette recorder (VCR) and viewed using an AWA Colourtrack T.V. monitor (DMQ-1422). A double coated Fuji Super VHS PRO (SE 120) videotape was used. Measurements of more than 100 basidiospores were recorded from each basidiospore suspension.

5.3. Image processing and analysis.

For image processing, the basidiospore images were replayed using a Hitachi DA4 VCR and viewed with an JVC Colour/RGB T.V. monitor (TM-150 PSN). Images were passed into the image analysis system via an I.DEN digital Time Base Corrector (TBC), which electronically corrected improper tuning of the sync signals created by mechanical and electronic errors in the VCR. Image quality was optimised and maintained throughout the analysis by adjusting the TBC once prior to all basidiospore measurements. The TBC images were composited via a RGB transcoder (Electrocraft) to the computer. Operation of image analysis menus was achieved using a IBM compatible '386' computer and a mouse (Logitech). The image screen had a 512 x 512 pixel format which was manipulated using the light pen (Joyce Loeb).

A Magiscan computer (Joyce Loeb) digitised the basidiospore images using the software program GENIAS Ver 3.5 (Joyce Loeb). Calibration of a known distance in μm was entered using the light pen and the scale factor calculated was then used in the analysis transforming pixel units into μm . In this study the known distance was the image of a graticule ($100 \times 0.01 = 1 \text{ mm}$, Graticules Ltd. Tonbridge, Kent, England), recorded at the same magnification as the basidiospores. The steps carried out for calibration are detailed in Appendix 10. Variation in calibration accuracy was assessed and found to be negligible.

After calibration, a task list was defined for basidiospore measurements. Main features of the task list included; (1)

contrast setting between basidiospores and the background; (2) thresholding, which was used to separate the basidiospore images from the background; (3) separation of touching basidiospores; (4) deletion of objects (such as background noises, overlapping basidiospores, and basidiospores viewed from the apex) was defined by size; and (5) basidiospore measurements. Basidiospore measurements included; 1) length (μm); 2) breadth (μm); 3) elongation: length/breadth; 4) circularity: $4\pi \times (\text{area})/(\text{perimeter})^2$; and 5) area: (μm^2), determined from the number of pixels inside the boundary. Perimeter was the sum of the distances between the mid points of the vectors forming the boundary of the basidiospores. Length, breadth, and area were considered size variables, while elongation and circularity measured shape. Thresholding and deletion of objects was carefully controlled for each fungal specimen and corrected if necessary.

5.3.1. Results.

Including time taken to record basidiospores, image processing and analysis, it was estimated that 1500-2000 basidiospores could be measured for all 5 variables per hour. Of the three magnifications tested, x100 provided insufficient magnification, with too few pixels per image, and x400 gave weak contrast between background and basidiospore wall. Thus x200 was considered the most appropriate magnification, and this was used for all images. Mean values for each of the basidiospore variables were recorded and are shown in Table 5.1. The range of values is given in the descriptions of the field collected specimens contained in Appendix 9.

Taxa ^a	Length (Std ^b)	Breadth (Std)	Elongation (Std)	Circularity (Std)	Area (Std)
arvensis	7.1 (0.59)	5.7 (0.42)	1.26 (0.129)	0.92 (0.067)	29.5 (2.93)
semotus	6.4 (0.6)	4.5 (0.47)	1.48 (0.206)	0.91 (0.087)	20.5 (2.64)
bisA5.2	7.4 (0.57)	5.8 (0.42)	1.28 (0.133)	0.94 (0.030)	32.6 (3.02)
bisOhoka	7.7 (0.85)	6.1 (0.52)	1.27 (0.231)	0.90 (0.080)	34.0 (4.23)
bitRopley	7.4 (0.52)	6.0 (0.43)	1.25 (0.115)	0.91 (0.054)	32.0 (2.95)
berMot	7.8 (0.84)	6.1 (0.57)	1.29 (0.133)	0.92 (0.064)	34.9 (4.68)
cam.cam	8.0 (0.92)	5.5 (0.66)	1.48 (0.206)	0.88 (0.081)	32.0 (5.16)
camHalls1	8.2 (1.13)	5.8 (0.74)	1.43 (0.204)	0.87 (0.103)	33.7 (5.79)
camHalls2	7.7 (0.92)	5.8 (0.58)	1.33 (0.169)	0.92 (0.093)	32.2 (4.24)
cupreo.	8.3 (1.11)	5.6 (0.68)	1.49 (0.212)	0.86 (0.117)	33.5 (5.37)
porphyr.	8.5 (0.86)	5.7 (0.7)	1.26 (0.129)	0.87 (0.094)	35.1 (4.76)
lanipeξ	8.2 (0.91)	5.8 (0.58)	1.57 (0.155)	0.90 (0.064)	34.9 (5.59)
Agaricus1	8.8 (0.91)	6.1 (0.79)	1.46 (0.169)	0.88 (0.085)	39.8 (6.1)
subpero.	8.4 (1.48)	6.1 (0.62)	1.43 (0.204)	0.88 (0.109)	37.1 (6.52)
Leucoag.	8.5 (0.99)	5.9 (0.46)	1.46 (0.202)	0.89 (0.047)	36.8 (5.4)
Agrocybe	10.7 (1.04)	6.8 (0.59)	1.57 (0.155)	0.86 (0.050)	54.9 (8.07)
Sp ^c	0.87	0.58	0.177	0.079	4.74

^a Taxa abbreviations; *Agrocybe* = *Agrocybe parasitica*, *arvensis* = *Agaricus arvensis*, *cupreo.* = *Agaricus cupreobrunneus*, *Agaricus1* = *Agaricus* sp.1, *lanipeξ* = *Agaricus lanipeξ*, *porphyr.* = *Agaricus porphyrocephalus*, *subpero.* = *Agaricus subperonatus*, *semotus* = *Agaricus semotus*. The following prefixes were used to define species; *bis* = *Agaricus bisporus*, *cam* = *Agaricus campestris*, *bit* = *Agaricus bitorquis*, *ber* = *Agaricus bernardii*, *Leucoag.* = *Leucoagaricus leucothites*.

^b Std = standard deviations are given in ().

^c Sp = the standard deviation of the pooled mean.

Table 5.1. Mean values for 5 basidiospore variables generated using image analysis.

5.4. Use of basidiospore characters for cladistic analysis.

Cladistic analysis is based on the assumption of character independence, (Wiley 1981), and measurements of some basidiospore variables, such as length and breadth were positively correlated, (Parmasto and Parmasto 1987). Thus Principal Component Analysis (PCA), was used for variable reduction and for generating new uncorrelated variables for cladistic analysis, (Wiley 1981).

5.4.1. PCA correlation interpretation.

The 5 basidiospore variables measured for each taxon were reduced to 3 important principal components. Table 5.2 shows the eigenanalysis of the correlation matrix for the principal component analysis.

Proportion ^a		53.8%	31%	14%	1%	0.2%
X _i :	Variable	pc1	pc2	pc3	pc4	pc5
X ₁ :	Area	-0.53	0.248	-0.424	0.667	0.183
X ₂ :	Breadth	-0.32	0.67	0.14	-0.523	0.394
X ₃ :	Circularity	0.403	0.182	-0.852	-0.276	-0.044
X ₄ :	Elongation	-0.315	-0.668	-0.223	-0.299	0.562
X ₅ :	Length	-0.595	-0.105	-0.158	-0.342	-0.702

^a Proportion of variation explained by each principal component.

Table 5.2. Eigenanalysis of the correlation matrix.

Taxa separation based on principal component (pc) 1, pc 2, and pc 3 is shown in Figure 5.1.

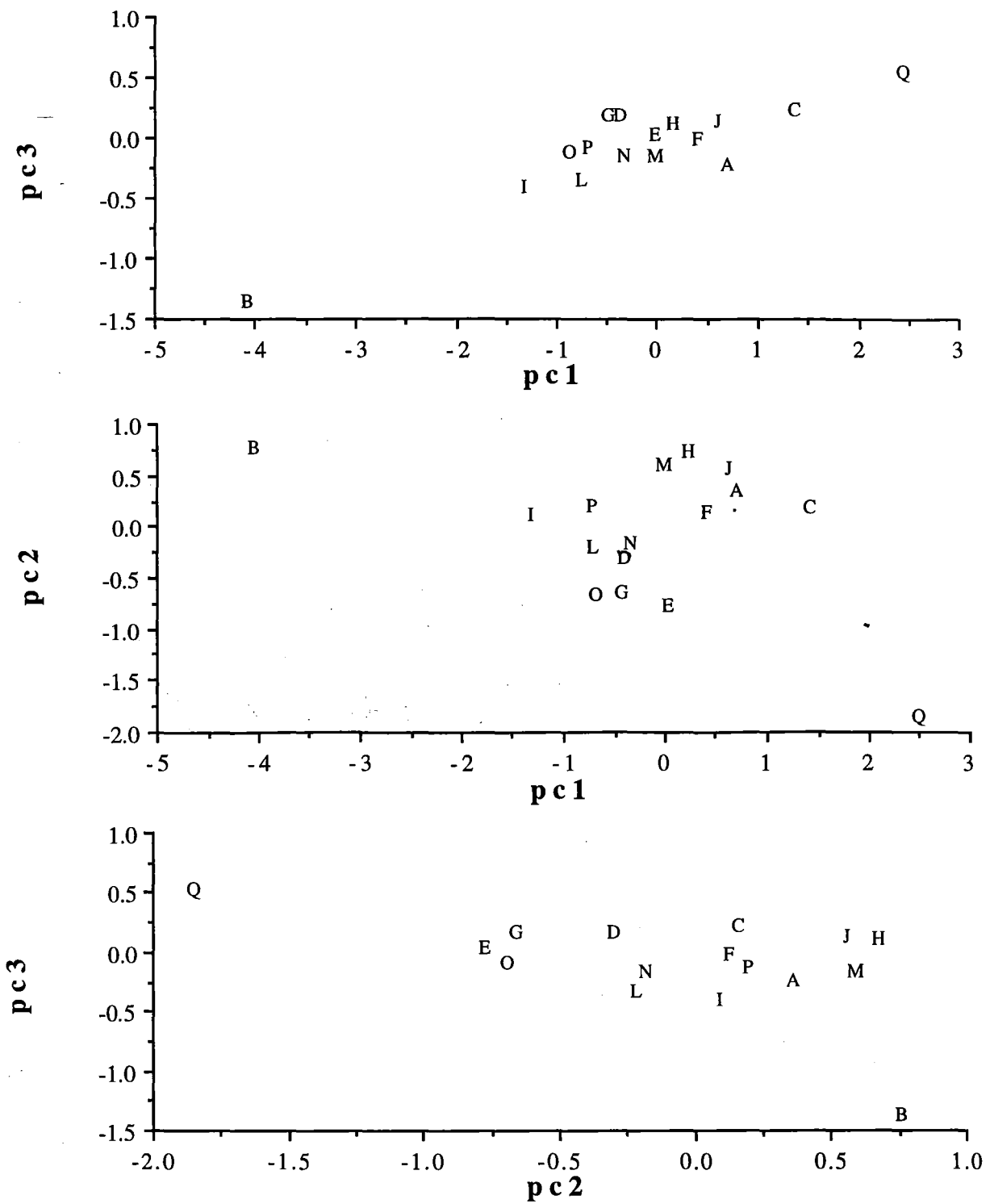


Figure 5.1. Plot of pc 2 vs pc 1, pc3 vs pc1, and pc3 vs pc2, of all taxa. Taxa are as follows: A= *Agaricus bisporus* A5.2, B= *Agrocybe parasitica*, C= *Agaricus arvensis*, D= *Agaricus campestris* Halls1, E= *Agaricus campestris* cam.cam, F= *Agaricus campestris* Halls2, G= *Agaricus cupreobrunneus*, H= *Agaricus bisporus* Ohoka, I= *Agaricus* sp.1, J= *Agaricus bitorquis* Ropley, L= *Leucoagaricus leucothites*, M= *Agaricus bernardii* Mot, N= *Agaricus lanipe*, O= *Agaricus porphyrocephalus*, P= *Agaricus subperonatus*, Q= *Agaricus semotus*.

Despite the fact that the components primarily distinguish *Agrocybe parasitica* (B), and *Agaricus semotus* (Q), from the remaining taxa, they were included in the principal component analysis because their removal resulted in minimal changes to the eigenvectors and the proportion of variation explained by each vector.

The first principal component (pc1) explained 54 % of the total variation in the sample and hence was the main source of variation among the basidiospores. Coefficients of the variables (X_i) in this component decreased in magnitude in the following order; length (X_5); area (X_1); circularity (X_3); breadth (X_2); and elongation (X_4). Except for the value of circularity (X_3), the coefficients were negative, hence the size measuring variables, area (X_1), breadth (X_2), and length (X_5), and the shape measuring variable elongation (X_4) were all contributing in the same direction to pc 1. This combination of variables separated the basidiospores according mainly to their size which was determined by length (X_5) and area (X_1). The highest values for pc 1 were for small basidiospores, e.g., *Agaricus semotus* (Q), and *Agaricus arvensis* (C), and the lowest values for large basidiospores, e.g., *Agrocybe parasitica* (B), and *Agaricus* sp.1. The larger basidiospores tended to be those with low values for circularity and the smallest basidiospores tended to be those with high values for circularity, explaining the positive coefficient for circularity in pc 1.

The second principal component (pc2) accounted for 31% of the total variation, and was equally determined by elongation (X_4)

and breadth (X_2), with negative and positive signs respectively. The coefficients of area (X_1), circularity (X_3), and length (X_5) were of little importance to this component because of their relatively low magnitudes. Thus pc 2 separated the basidiospores according to their form (size and shape). A high value was obtained for the broad basidiospores with low elongation (globose), e.g., *Agaricus bernardii* (M), and *Agaricus bisporus* (H), and *Agaricus bitorquis* (J). A low value was obtained for narrow basidiospores with a high elongation (ellipsoid), e.g., *Agaricus semotus* (Q), *Agaricus campestris* (E), *Agaricus cupreobrunneus* (G), and *Agaricus porphyrocephalus* (O). Although *Agrocybe parasitica* (B) had the highest elongation, it also had the highest breadth, causing it to group with *Agaricus bernardii* (M), and *Agaricus bisporus* (H), and *Agaricus bitorquis* (J).

The third principal component, pc 3, explained 14 % of the sample variation and was determined mainly by circularity (X_3), and to a lesser extent by area (X_1), separating the basidiospores by shape. The lowest values for pc 3 were taken from basidiospores with high circularity (X_3), and/or area (X_1) measurements, e.g., *Agaricus bisporus* (A), *Agaricus* sp.1, *Leucoagaricus leucothites* (L), and *Agrocybe parasitica* (B). The largest values for pc 3 were taken from basidiospores with both low circularity (X_3) and area (X_1) measurements, e.g., *Agaricus semotus* (Q).

The fourth and fifth principal components, pc 4 and pc 5, accounted for 1 % and 0.2 % of the total variation among the basidiospores respectively and were therefore negligible.

5.4.2. Segment coding of principal basidiospore components.

Basidiospore measurements and principal components were continuously variable quantitative characters. Cladistic analysis of this type of character requires coding into discrete character states, (Chappill 1989). In her review of coding procedures Chappill (1989) suggested that segment coding provided the least amount of distortion. Segment coding involves dividing the range of values into a number of equal range segments using the pooled mean to define the segments (Chappill 1989). Thus preliminary cladistic analysis was carried out using segment coded principal basidiospore components, Table 5.3, section 5.4.4. According to Chappill (1989), it is acknowledged that "all available methods of coding quantitative characters for phylogenetic analysis result in some distortion of relationships between taxa".

5.4.3. Phylogenetic value of principal basidiospore components.

A measure of the phylogenetic value of the principal basidiospore components may be gained by assessing their fit to the best representation of phylogeny available, as indicated by the consistency index, (Rodrigo 1993, pers. comm.). This may be achieved by mapping the characters of interest onto an independently derived tree using Phylogenetic Analysis Using Parsimony, (PAUP 3.0 Macintosh version; Swofford 1991). The segment coded principal basidiospore components, Table 5.3, section 5.4.4, were mapped onto the independently derived tree shown in Figure 4.9, section 4.3.2.3.1, depicting the relationships

of *Agaricus* species generated from allozyme information on strains pooled according to species.

The ingroup shown in Figure 4.9, section 4.3.2.3.1, consisted of strains representative of each of the following; *Agaricus bisporus*, *Agaricus bitorquis*, *Agaricus bernardii*, *Agaricus bitorquis* bit34782 (basidiospore data not available for this taxon), *Agaricus cupreobrunneus*, *Agaricus porphyrocephalus*, and *Agaricus campestris*, and the outgroups were *Leucoagaricus leucothites* and *Agrocybe parasitica*. Some species were represented by more than one taxon in Table 5.3, section 5.4.4, and the coded basidiospore principal components from the following were used for the mapping process; *Agaricus campestris* cam.cam for *Agaricus campestris*, *Agaricus bisporus* bisOhoka for *Agaricus bisporus*, and *Agaricus bitorquis* bitRopley for *Agaricus bitorquis*.

The results that show how the principal basidiospore components were mapped onto the tree derived in section 4.3.2.3.1, are presented in Appendix 11. Results indicated that pc1, CI = 0.778, and pc3, CI = 1.000, acted as synapomorphies for the ingroup. Pc1, CI = 0.778, also contributed to the definition of two other monophyletic groups; the first including *Agaricus bisporus*, *Agaricus bitorquis* and *Agaricus bernardii*, and the second containing *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus*. The monophyly of the group including *Agaricus bitorquis* bit34782, *Agaricus cupreobrunneus*, *Agaricus porphyrocephalus*, and *Agaricus campestris* was supported by pc2, CI = 0.750. Principal basidiospore components behaved as

autapomorphies as follows; *Agaricus bernardii*, pc1, CI = 0.778, *Leucoagaricus leucothites*, pc2, CI = 0.750, and *Agrocybe parasitica*., pc1, CI = 0.778, pc2, CI = 0.750, and pc3, CI = 1.000.

The good fit of the principal basidiospore components, as indicated by their high CI values, (not less than CI = 0.750), onto the independently derived tree, indicated basidiospores contain valuable phylogenetic information. This conclusion lead to the decision to continue investigation of the phylogenetic relationships of *Agaricus* species based on basidiospore components.

5.4.4. Preliminary cladistic analysis.

Selected morphological, chemical and developmental characters described in the literature review, Chapter 2 were analysed with the coded basidiospore principal components, Table 5.3. Characters were treated as unordered, and analysed as multistates using Phylogenetic Analysis Using Parsimony, (PAUP 3.0 Macintosh version; Swofford 1991). A base weight of 1000 was used to scale for equal character weighting regardless of the number of states, e.g., binary characters were assigned a weight of 1000, 3 state characters a weight of 500 etc, (Swofford 1991).

Leucoagaricus leucothites and *Agrocybe parasitica* were used as outgroups for analysis. The branch and bound search option was used, with no limit set to the number of trees retained in the memory during any search.

Taxa ^a	Characters ^b									
	1	2	3	4	5	6	7	8	9	10
arvensis	A	A	A	A	B	A	B	I	D	B
semotus	A	A	A	A	B	A	B	K	A	A
bisA5.2	B	B	B	B	B	A	B	H	E	C
bisOhoka	B	B	B	B	B	A	B	H	E	C
bitRopley	B	B	B	B	B	A	A	H	E	C
berMot	B	B	B	B	B	A	B	G	E	C
cam.cam	B	B	B	A	A	B	B	G	C	C
camHalls1	B	B	B	A	A	B	B	G	D	B
camHalls2	B	B	B	A	A	B	B	H	D	C
cupreo.	B	B	B	A	A	B	B	F	C	B
porhyro.	B	B	B	A	B	B	B	F	C	C
lanipe§	B	B	B	A	B	A	B	G	D	C
subpero.	B	B	B	A	B	A	A	F	D	C
Agaricus1	B	B	B	A	B	A	B	E	D	D
Leucoag.	B	B	?	B	A	A	B	F	D	D
Agrocybe	B	B	?	B	A	A	C	A	F	F

^a Taxa abbreviations; *Agrocybe* = *Agrocybe parasitica*, *arvensis* = *Agaricus arvensis*, *cupreo.* = *Agaricus cupreobrunneus*, *Agaricus1* = *Agaricus* sp.1, *lanipe* = *Agaricus lanipe§*, *porhyr.* = *Agaricus porphyrocephalus*, *subpero.* = *Agaricus subperonatus*, *semotus* = *Agaricus semotus*. The following prefixes were used to define species; *bis* = *Agaricus bisporus*, *cam* = *Agaricus campestris*, *bit* = *Agaricus bitorquis*, *ber* = *Agaricus bernardii*, *Leucoag.* = *Leucoagaricus leucothites*.

^b Characters;

- 1) Schaeffer's cross reaction positive (A), negative (B),
- 2) KOH positive (A), KOH negative (B),
- 3) basidioma colour change reddish (A), or yellowish (B) upon cutting and bruising,
- 4) annulus pendant (A) or peronate (B),
- 5) presence, even if rudimentary (A), or absence (B), of basidiospore germ pore,
- 6) cheilocystidial presence (A) or absence (B),
- 7) basidioma development hypogean (A), epigean (B), other (C),
- 8) basidiospore principal component 1,
- 9) basidiospore principal component 2,
- 10) basidiospore principal component 3.

a question mark (?) indicates missing data.

Table 5.3. Chemically, morphologically and developmentally based character matrix for taxa representative of eleven *Agaricus* species, *Leucoagaricus leucothites* and *Agrocybe parasitica*.

When multiple equally parsimonious trees were saved from a search 50 % majority rule consensus trees were studied, (Margush and McMorris 1981). Analysis was carried out utilising all characters presented in Table 5.3. Analysis revealed 6 equally parsimonious trees, each with an unweighted length of 29, and CI = 0.795, Figure 5.2.

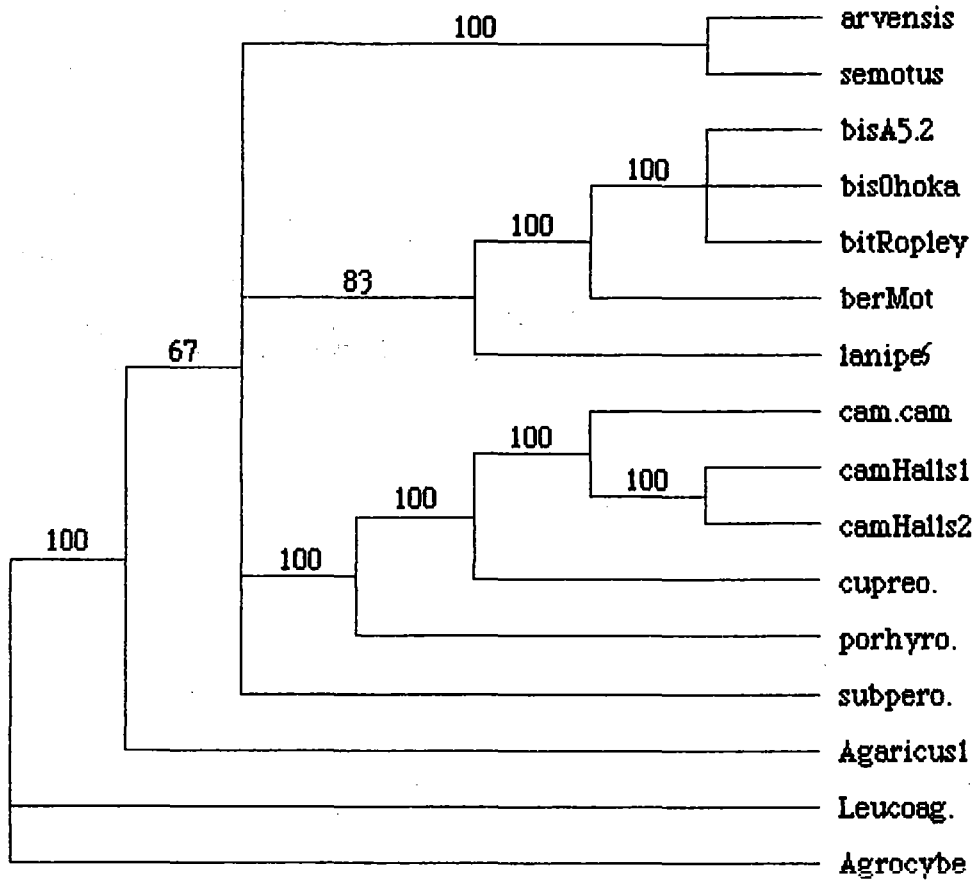


Figure 5.2. Majority rule consensus of 6 equally parsimonious trees, unweighted length 29, CI = 0.795. Numbers indicate the percentage of the 6 most parsimonious trees giving support for the group. Taxa abbreviations; Agrocye = *Agrocye parasitica*, arvensis = *Agaricus arvensis*, cupreo. = *Agaricus cupreobrunneus*, Agaricus1 = *Agaricus* sp.1, lanipe = *Agaricus lanipe6*, porhyr. = *Agaricus porphyrocephalus*, subpero. = *Agaricus subperonatus*, semotus = *Agaricus semotus*. The following prefixes were used to define species; bis = *Agaricus bisporus*, cam = *Agaricus campestris*, bit = *Agaricus bitorquis*, ber = *Agaricus bernardii*, Leucoag. = *Leucoagaricus leucothites*.

5.5. Discussion and conclusions.

Image analysis was used successfully to make measurements of basidiospores from *Agaricus* species. This method was approximately ten times faster than the traditional light microscopy method. The speed of the operation and the quantity of data it was possible to collect using image analysis would be a major advantage for extensive investigations of basidiospore variability within and between *Agaricus*, species.

Generation of new characters for *Agaricus* phylogenetic analysis is of particular importance for this morphologically simple genus as by increasing the quantity of information available for phylogenetic study, the reliability of the classification may be improved. Image analysis has enabled the measurement of new basidiospore variables, for example area and circularity measurements, which played important roles in the first and third basidiospore principal components respectively. Based on the principal component analysis it was concluded that the measurement of basidiospore area and circularity contributed significantly to the separation of *Agaricus* species, and their measurement is likely to be equally useful in other studies of the genus.

Image analysis may be an appropriate technique for the study of spores from many other fungal species. The techniques described in this investigation could be applied directly to any type of spore lacking surface ornamentation. For spores with surface ornamentation, such as those within the Russulaceae (Pegler and Young 1971), image analysis may provide an

important method of not only objectively measuring the size and shape of basidiospores, but of assessing the importance of the ornaments in systematic studies.

The phylogenetic usefulness of the principal components of basidiospores was demonstrated by mapping these characters onto the independently derived tree found during cladistic analysis of allozyme information. The significant phylogenetic value of basidiospores from *Agaricus* species was noted by Pegler and Young (1971), Raper and Kaye (1978), and Wasser (1989), but their studies did not use phylogenetic techniques, and a direct comparison with their work is not possible. Further studies are needed to determine basidiospore variability within species. Image analysis may be directed towards population surveys to establish basidiospore variability within species by sampling specimens from many different locations and environmental conditions, (Watling 1977, Bresinsky et al. 1977, Clemencon 1979, Parmasto and Parmasto 1987). Image analysis may also be of use to test further the extent of basidiospore variation which Clemencon (1979), and Hoiland (1983) suggested occurred during basidioma development. A survey of basidiospores from a wider selection of outgroup species is required to establish greater character polarisation than was achieved using *Leucoagaricus leucothites* and *Agrocybe parasitica* in this study. Possible outgroups for future analyses may include species from tribes within the family Agaricaceae other than Agariceae, e.g., the Leucocoprineae, Lepioteae, and Cystodermateae.

— The characters analysed were insufficient to resolve cladistic relationships between the 3 main monophyletic groups and *Agaricus subperonatus*, (Figure 5.2) and it would be inappropriate to make inferences on character evolution based on the analysis carried out in this investigation. A far more detailed study of characters traditionally used for *Agaricus* systematics will be required before the relationships between these monophyletic groups can be inferred from cladistic analysis. However, it is interesting to note the similarities between the consensus tree, (Figure 5.2), and the classification arrived at by Cappelli (1984). The two groups defined by cladistic analysis, containing *Agaricus bisporus*, *Agaricus bitorquis*, and *Agaricus bernardii* were similar to the Section Bitorques, and the group containing representatives of *Agaricus campestris* var. *campestris*, *Agaricus cupreobrunneus*, and *Agaricus porphyrocephalus* was similar to the Group Campestris of the Section Agaricus.

This study represents an exploration of a technique that could be applied in *Agaricus* phylogenetic analyses using morphological characters. The basidiospore analysis methods described here could be used for extensive studies of many species and represents an important addition to traditional methods of analysis. No other study on *Agaricus* species using image analysis to investigate basidiospores is recorded in the literature, and this appears to be the first to have applied phylogenetic methodology to basidiospore characters.

Chapter 6.

General discussion.

A review of literature shows that the classification of *Agaricus* species is not stable. Authors over the last 200 years have endeavored to produce a workable system, however even the proposal by Cappelli (1984) is not completely satisfactory. This is because *Agaricus* species are determined based on many subjective characters, such as distribution of velar remnants and colour change of cut basidioma tissues, and their classification has not been based on phylogenetic relationship. The basis of this thesis is that new techniques are available that may allow an improved system of classification of *Agaricus* species.

The study of allozymes is an established method for creating characters for use in systematics, (Murphy et al. 1990, May 1992). In fungal systematics this technology has been used to study a number of genera, including *Trichoderma*., Stasz et al. (1989), and *Leptographium*, (Zambino and Harrington 1992), and several authors, such as Royse and May (1982a), van-Loon et al. (1986), Liming et al. (1987), and Kerrigan and Ross (1989), have applied the technique to *Agaricus* species. However, from the literature it is clear that there are difficulties when using this technique to study *Agaricus* species. These included problems due to; 1) electrophoretic methods limiting interspecific comparison, 2) the effect of culture techniques used for production of *Agaricus* mycelium for analysis, 3) methods of storing mycelium for allozyme analysis, and 4) mycelial allozyme changes during incubation.

Clearly before the technique could be applied satisfactorily it was necessary to clarify the situation regarding the difficulties listed above, and as a result, a series of experiments were entered into, (Chapter 3). The results of these experiments showed that certain conditions were required for achievement of reliable results. The use of HSE, modified extraction buffers, a mass of mycelium equal to the amount of extraction buffer, modified electrophoretic buffer systems and staining solutions were required for the resolution of the 10 mycelial enzymes chosen for study. It was found that replicates of shaken submerged cultures could be pooled, and stored before analysis at -80°C for at least 3 months either as mycelium, fresh or freeze dried, or as extracted mycelial supernatant. It was also found that to enable increased reliance on the results of allozyme analysis, a single standardised culture method should be used to grow the mycelium for each strain, and mycelia should be of similar physiological development. While these conditions are difficult to achieve due to the variable growth habits of the fungi, it was possible to arrive at an estimate of similar physiological stage between strains representing each species by harvesting each strain at a stage half way through its most rapid period of growth.

Allozyme analyses were undertaken for eleven *Agaricus* species and the results which are presented in Chapter 4 reveal the similarities and differences between species. These results were used as the basis for cladistic analyses of the genus *Agaricus*, the results of which supported many of the traditional concepts concerning the relationships in this genus, particularly the close relationship of species studied from the traditional Sections Bitorques, and *Agaricus* Group *Campestris*.

Another new technique, one not previously used for collecting data relating to *Agaricus*, is image analysis of basidiospores. The size and shape of basidiospores have been recognised as an important feature for many years, (Pegler and Young 1971, Wasser et al. 1976, Raper and Kaye 1978, Garibova and Safrai 1972, Wasser 1989). Generally however, because techniques using light microscopy are tedious in the extreme, there have been insufficient numbers of basidiospores measured to test character significance.

By digitising basidiospore images using the software program GENIAS Ver 3.5 (Joyce Loebl), it was possible to measure the length, breadth, elongation, circularity, and area of 1500-2000 basidiospores per hour. Approximately 100 basidiospores were examined from each of three specimens of eleven *Agaricus* species. These results were used to test the phylogenetic value of *Agaricus* basidiospore variables, and to provide additional data that could be incorporated into cladistic analysis. Basidiospore variables were found to be phylogenetically useful, and cladistic analysis supported conclusions made regarding the monophyly of species studied from the traditional sections Bitorques, and *Agaricus* group *Campestris* based on allozyme analysis.

The use of allozymes compared with morphology allowed the generation of more polymorphic characters for the study of *Agaricus* species. However, the phylogenetic positions of many of the species still remain in question. It may be possible to resolve the relationships of these species by; 1) examination of other enzymes, 3)

increasing the sample size for each of the species; and 4) using a phylogenetic technique other than cladistics, such as compatibility analysis, (Meacham and Estabrook 1985), and statistical phylogenetics, e.g., maximum-likelihood estimation (Felsenstein 1973, Farris 1973). While the comparison of these different analytical measures was beyond the scope of this thesis, they represent possibilities for future investigations. The use of other techniques may also provide information suitable for phylogenetic analysis of *Agaricus* species, e.g., use of RFLP's (Loftus et al. 1988, Summerbell et al. 1989), double stranded RNA (Deahl et al. 1987), 5S r RNA nucleotide sequences (Huysmans et al. 1983), and genome characterisation, (Arthur et al. 1982, Hintz et al.^(d)1988).

Research on allozyme and morphological data sets from *Agaricus* species may help resolve relationships in which neither single data set was conclusive. Further evaluation of many of the other characters routinely used to describe *Agaricus* species is needed to increase the objectivity with which they are defined, and to assess characters such as basidiocarp colour reactions and the presence of a germ pore at the basidiospore apex. The use of image analysis for objective study of many fungal characters, including both macroscopic and microscopic characters represents a great opportunity for future investigations.

Although the experiments undertaken show that much remains to be done to provide an adequate system of classification of the genus *Agaricus*, it is clear that new methods and techniques can greatly assist in bringing this about.

References.

- Alberch, P. 1985. Problems with the interpretation of developmental sequences. *Systematic Zoology*. 34: 46-58.
- Allendorf, F. W., Mitchell, N., Ryman, N., and Stahl, G. 1977. Isozyme loci in brown trout (*Salmo trutta* L.): detection and interpretation from population data. *Hereditas*. 86: 179-190.
- Anderson, J. B., Petsche, D. M., Herr, F. B., and Horgen, P. A. 1984. Breeding relationships among several species of *Agaricus*. *Canadian Journal of Botany*. 62: 1884-1889.
- Anonymous. 1968. Report of the committee for fungi and lichens. *Taxon*. 17: 580-581.
- Archie, J. W., Simon, C., and Martin, A. 1989. Small sample size does decrease the stability of dendrograms calculated from allozyme -frequency data. *Evolution*. 43: 678-683.
- Arnold, E. N. 1981. Estimating phylogenies at low taxonomic levels. *Zeitschrift fuer Zoologische Systematik und Evolutionsforschung*. 19: 1-35.
- Arthur, R., Herr, F., Straus, N., Anderson, J., and Horgen, P. 1982. Characterisation of the Genome of the Cultivated Mushroom *Agaricus brunnescens*. *Experimental Mycology*. 7: 127-132.
- Barnes, M. F. 1993. Leaf peroxidase and catechol oxidase polymorphism and the identification of commercial apple varieties. *New Zealand Journal of Crop and Horticultural Science*. 5: 207-210.
- Bas, C. 1991. A short introduction to the ecology, taxonomy and nomenclature of the genus *Agaricus*. In: *Genetics and breeding of Agaricus*. Proceedings of the first international seminar on mushroom science. Mushroom experimental station, Horst, the Netherlands, 14-17 May, 1991, (ed., L, J, D, van Griensven). Pudoc. Wageningen, Netherlands. pp. 21-24.
- Baum, D. 1992. Phylogenetic species concepts. *Trends in Ecology and Evolution*. 7: 1-2.

Baverstock, P. R., and Moritz, C. 1990. Sampling design. In: Hillis, D. M., and Moritz, C. (Ed.). *Molecular systematics*. Sinauer Associates, Inc. Sunderland, Massachusetts, U.S.A. pp. 13-24.

Baverstock, P. R., Cole, S. R., Richardson, B. J., and Watts, C. H. S. 1979. Electrophoresis and cladistics. *Systematic Zoology*. 28: 214-219.

Bohus, G. 1969. *Agaricus* studies II. *Annales Historico-Naturales-Musei Nationalis Hungarici*. 61: 151-156.

Booth, C. 1971. *Methods in Microbiology*. 4. Academic Press. London.

Bresinsky, A., Hilber, O., and Molitoris, H. P. 1977. The genus *Pleurotus* as an aid for understanding the concept of species in basidiomycetes. In: *The species concept in Hymenomycetes*, (ed., Clemencon, H.). J Cramer, Vaduz. pp. 229-250.

Bruns, T. D., White, T. J., and Taylor, J. W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics*. 22: 525-564.

Bryant, H. A. 1989. An evaluation of cladistic and character analyses as hypothetico-deductive procedures, and the consequences for character weighting. *Systematic Zoology*. 38: 214-227.

Buth, D. G. 1979. Biochemical systematics of the cyprinid genus *Notropis*. I. The subgenus *Luxilus*. *Biochemical Systematics and Ecology*. 7: 69-79.

Buth, D. G. 1984. The application of electrophoretic data in systematic studies. *Annual Review of Ecology and Systematics*. 15: 501-522.

Camin, J. H., and Sokal, R. R. 1965. A method for deducing branching sequences in phylogeny. *Evolution*. 19: 311-326.

Cappelli, A. 1984. *Agaricus* L.: Fr. ss. Karsten, (Psalliota Fr.). M. Candusso, Stampato in Italia.

Chaillot, B. 1976. Study by electron paramagnetic resonance of charges arising in catalase after freezing and lyophilization. *Kholodil'naya Tekhnika*. 1: 44-45.

Chappill, J. A. 1989. Quantitative characters in phylogenetic analysis. *Cladistics*. 5: 217-234.

Cheliak, W. M., and Pitel, J. A. 1984. *Techniques for starch gel electrophoresis of enzymes from forest tree species*. Information report P1-X-42. Petawawa National Forestry Institute. Canadian Forestry Service.

Chrambach, A., and Rodbard, D. 1971. Polyacrylamide gel electrophoresis. *Science*. 172: 440-451

Clayton, J. W., and Tretiak, D. N., 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. *Journal of the Fisheries Research Board Canada*. 29: 1169-1172.

Clemencon, H. 1979. Biometrische Untersuchungen zur Variabilität der Basidiosporen. *Beihefte Sydowia Annales. Mycologici Serie II*. 8: 110-138.

Cox, P. W., and Thomas, C. R. 1992. Classification and measurement of fungal pellets by automated image analysis. *Biotechnology and Bioengineering*. 39: 945-952.

Cracraft, J. 1990. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: *Speciation and its consequences*, (eds., D. Otte., and Endler, J. A). Sinauer Associates, Inc., Sunderland, Massachusetts. pp. 28-59.

Cracraft, J. 1974. Phylogenetic models and classification. *Systematic Zoology*. 23: 71-90.

Crawford, D. J. 1983. Phylogenetic and systematic inferences from electrophoretic studies. In: *Isozymes in plant genetics and breeding*, part A. (eds., S. D. Tanksley, and T. J. Orton). Elsevier Science Publishers B. V., Amsterdam. pp. 257-287.

Crother, B. I. 1990. Is "some better than none" or do allele frequencies contain phylogenetically useful information? *Cladistics*. 6: 277-281.

Cruickshank, R., Duguid, J. P., and Swain, R. H. A. 1965. *Medical microbiology. A guide to the laboratory diagnosis and control of infection*. E and S Livingstone Ltd. Edinburgh and London.

- Deahl, K. L., San Antonio, J. P., and Civerolo, E. L. 1987. Electrophoretic analysis of double-stranded RNA in stocks of cultivated mushroom (*Agaricus brunnescens*). *Plant Disease*. 71: 430-433.
- Donk, M. A. 1962. The generic names proposed for Agaricaceae. *Nova hedwigia beigefte*. 5.
- Donk, M. A. 1964. Nomina conservanda proposita. *Regnum Vegetabile*. 34: 32.
- Elliott, T. J. 1978. Comparative sexuality in *Agaricus* species. *Journal of General Microbiology*. 107: 113-122.
- Esser, K., and Hoffmann, P. 1977. Genetic basis for speciation in higher basidiomycetes with special reference to the genus *Polyporus*. In: *The species concept in Hymenomycetes*, (ed., Clemencon, H). J Cramer, Vaduz. pp. 189-201.
- Evers, D. C., and Ross, I. K. 1983. Isozyme patterns and morphogenesis in higher Basidiomycetes. *Experimental Mycology*. 7: 9-16.
- Farris, J. S. 1970. Methods for computing Wagner trees. *Systematic Zoology*. 19: 83-92.
- Farris, J. S. 1973. A probability model for inferring evolutionary trees. *Systematic Zoology*. 22: 250-256.
- Farris, J. S. 1977. Phylogenetic analysis under Dollo's law. *Systematic Zoology*. 26: 77-88.
- Farris, J. S. 1980. The efficient diagnoses of the phylogenetic system. *Systematic Zoology*. 29: 386-401.
- Farris, J. S. 1981. Distance data in phylogenetic analysis. In: *Advances in cladistics: Proceedings of the first meeting of the Willi Hennig society*, (eds., V, A, Funk, and D, R, Brooks). The New York Botanical Garden, Bronx, New York. pp. 3-23.
- Felsenstein, J. 1973. Maximum likelihood and minimum steps methods for estimating evolutionary trees from data on discrete characters. *Systematic Zoology*. 22: 240-249.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*. 39: 783-791.

- Ferguson, A. 1988. Isozyme studies and their interpretation. In: *Prospects in systematics*, (ed. D. L. Hawksworth). Clarendon Press, Oxford. The systematics association special volume 36:184-201.
- Fitch, W. M. 1971. Toward defining the course of evolution: minimal change for a specific tree topology. *Systematic Zoology*. 20: 406-416.
- Freeman, A. E. H. 1979. *Agaricus* in the southeastern United States. *Mycotaxon*. 8: 50-118.
- Fries, E. 1821. *Systema Mycologicum*. 1. Cited in; Freeman, A. E. H. 1979. *Agaricus* in the southeastern United States. *Mycotaxon*. 8: 50-118.
- Gabriel, O., and Gersten, D. M. 1992. Staining for enzymatic activity after gel electrophoresis, I. *Analytical Biochemistry*. 203. 1-21.
- Gaffney, E. S. 1975. A phylogeny and classification of higher categories of turtles. *Bulletin. American Museum of Natural History*. 155: 387-436.
- Gaffney, E. S. 1979. An introduction to the logic of phylogeny reconstruction. In: *Phylogenetic analysis and paleontology*, (eds. J. Cracraft and N. Eldredge). Columbia University Press, New York. pp. 79-111.
- Garibova, L. V., and Safrai, A. I. 1972. On some taxonomic characteristics of *Agaricus* Fr. species. *Mikologija i Fitopatologija*. 6: 440-443. Cited in; Parmasto, E., and Parmasto, I. 1987. Variation of basidiospores in the Hymenomycetes and its significance to their taxonomy. *Bibliotheca Mycologia*. Band 115. J. Cramer. Berlin, Stuttgart.
- Gessner, R. V., Romano, M. A., and Schultz, R. W. 1987. Allelic variation and segregation in *Morcella deliciosa* and *M. esculenta*. *Mycologia*. 79: 683-687.
- Gordon, A. H. 1972. Electrophoresis of proteins in polyacrylamide and starch. North Holland Publishing Company, Amsterdam.
- Gorman, G. C., and Renzi, J. Jr. 1979. Genetic distance and heterozygosity estimates in electrophoretic studies: Effects of sample size. *Copeia*. 2: 242-249.

Gould, S. J. 1977. *Ontogeny and phylogeny*. Belknap Press of Harvard University Press. Cambridge, Massachusetts.

Hames, B. D., and Rickwood, D. 1981. Gel electrophoresis of proteins: A practical approach. IRL Press Ltd. London.

Hanafusa, N. 1973. Freezing and drying of enzyme protein. *Bulletin. Institut International du Froid. Annexe. 5*: 9-18.

Harris, H., and Hopkinson, D. A. 1976. *Handbook of enzyme electrophoresis in human genetics*. North Holland Publishing Company, Amsterdam. pp. 1-12.

Hart, J. A. 1988. Rust fungi and host plant coevolution: do primitive hosts harbor primitive parasites? *Cladistics. 4*: 339-366.

Hawksworth, D. L., Sutton, B. C., and Ainsworth, G. C. 1983. *Ainsworth and Bisby's Dictionary of the fungi (including the lichens)*, seventh edition, Commonwealth mycological institute, Kew, Surrey.

Heinemann, P. 1952. Etudes sur le genre *Agaricus* Fr. S. Stricto. *Bulletin Societe Mycologique de France. 62*. 382-385. Cited in; Pegler, D. N. and Young, T. W. K 1971. Basidiospore morphology in the Agaricales. *Nova hedwigia beigefte. 35*.

Heinemann, P. 1974. Quelques *Agaricus* de Nouvelle-Zelande. *Bulletin. Jardin Botanique National de Belgique. 44*: 355-366.

Heinemann, P. 1977. *Les Psalliotes*. Les Naturalistes Belges. Bruxelles.

Heinemann, P. 1978. Essai d'une cle de determination des genres *Agaricus* et *Micropsalliota*. *Sydowia. 6-37*.

Hennig, W. 1966. *Phylogenetic Systematics*. University of Illinois Press, Urbana, Illinois.

Heywood, V. H. 1984. The current scene in plant taxonomy. In: *Current concepts in plant taxonomy*, (eds., V, H, Heywood, and D, M, Moore). Academic Press. U.K.

Hillis, D. M. 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics. 18*: 23-42.

Hecht, M. K., and Edwards, J. L. 1976. The determination of parallel or monophyletic relationships: the proteid salamanders-a test case. *American Naturalist. 110*: 653-677.

Hillis, D. M., and Moritz, C. 1990. *Molecular systematics*. Sinauer Associates, Inc. Sunderland, Massachusetts, U.S.A.

Hintz, W. E. A., Anderson, J. B., and Horgen, P. A. 1988.^(a) Physical mapping of the mitochondrial genome of the cultivated mushroom *Agaricus brunnescens* (= *Agaricus bisporus*). *Current Genetics*. 14: 43-49.

Hintz, W. E. A., Anderson, J. B., and Horgen, P. A. 1988.^(b) Relatedness of three species of *Agaricus* inferred from restriction fragment length polymorphism analysis of ribosomal DNA repeat and mitochondrial DNA. *Genome*. 32: 173-178.

Hoiland, K. 1983. *Cortinarius* subgenus *Dermocybe*. *Opera Botanica*. 71 : 1-112. Copenhagen.

Hunter, R. L., and Markert, C. L. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science, New York*. 125: 1294-1295.

Huysmans, E., Dams, E., Vandenberghe, A., and De Wachter, R. 1983. The nucleotide sequences of the 5S rRNAs of four mushrooms and their use in studying the phylogenetic position of basidiomycetes among the eukaryotes. *Neucleic Acids Research*. 11: 2871-2880.

Ingebrigtsen, J. Kang, B., and Flurkey, W. H. 1989. Tyrosinase activity and isozymes in developing mushrooms. *Journal of Food and Science* 54: 128-131.

International code of Botanical Nomenclature. 1972. VIII Fungi. p 254.

Karsten, P. 1879. Rysslands, Finlands och den Skandinaviska halfons Hattsvampar. Florre delen. Skifsvampar. *Bidrag till Kannedon av Finlands Natur och Folk*. 32.

Kerrigan, R. W., and Ross, I. K. 1984. Basidiospore number variation in *Agaricus*. In: *Cultivating edible fungi*, (eds., P. J. Weust., D. J. Royse., and R. B. Beelman). Elsevier. Amsterdam. pp 155-162.

Kerrigan, R. W., and Ross, I. K. 1989. Allozymes of a wild *Agaricus bisporus* population: new alleles, new genotypes. *Mycologia*. 8: 433-443

Jodon, M. H., Royse, D. J., Antoun, G. G., May, B. 1986. Use of isozyme analysis to determine genetic similarity in the paddy straw mushroom *Volvariella volvacea*. *Phytopathology*. 76: 654.

- King, J. L., and Ohtu, T. 1975. Polyallelic mutational equilibria. *Genetics*. 79: 681-691.
- Kluge, A. G. 1985. Ontogeny and phylogenetic systematics. *Cladistics*. 1:13-27.
- Kluge, A. G., and Farris, J. S. 1969. Quantitative phyletics and the evolution of anurans. *Systematic Zoology*. 18: 1-32.
- Kulkarni, R. E., Kamerath, C. D., and Allred, K. L. 1986. Genetic diversity between isolates of *Pleurotus ostreatus* as revealed by isozyme analysis. In: *Cultivating edible fungi*. 1987. (eds., P. J. Wuest, D. J. Royse, and R. B. Beelman). Elsevier publishers. Amsterdam, Oxford, New York, Tokyo. pp. 171-181.
- Lange, J. E. 1926. Studies in the agarics of Denmark. Part 6: Psalliota. *Dansk Botanisk Arkiv*. Bd. 4, Nr. 12. 1-11.
- Lanyon, S. 1985. Detecting internal inconsistencies in distance data. *Systematic Zoology*. 34: 397-403.
- Liming, S., Xiulian, X., and Xiafen, L. 1987. The technique of studying esterase isozymes from mycelia of cultivated strains of *Agaricus bisporus*. *Acta Agriculturae Shanghai*. 3: 39-46.
- Linnaeus, C. 1753. *Species Plantarum*. 2. Cited in; Freeman, A. E. H. 1979. *Agaricus* in the southeastern United States. *Mycotaxon*. 8: 50-118.
- Loftus, M. G., Moore, D., and Elliott, T. J. 1988. DNA polymorphisms in commercial and wild strains of the cultivated mushroom, *Agaricus bisporus*. *Theoretical and applied genetics*. 76: 712-718.
- Luykx, P., Nickle, D. A., and Crother, B. I. 1990. A morphological, allozymic, and karyotypic assessment of the phylogeny of some lower termites (Isoptera: Kalotermitidae). *Proceedings. Entomological Society of Washington*. 92: 385-399.
- Maddison, W. P., Donoghue, M. J., and Maddison, D. R. 1984. Outgroup analysis and parsimony. *Systematic Zoology*. 33: 83-103.
- Magae, Y., Haga, K., Taniguchi, H., and Sasaki, T. 1990. Enzymes of strains of *Pleurotus* species (Basidiomycetes) compared by electrophoresis. *Journal of General and Applied Microbiology*. 36: 69-80.

Margush, T., and McMorris, F. R. 1981. Consensus n-trees. *Bulletin of Mathematical Biology*. 43: 239-244.

Markert, C. L., and Faulhaber, I. 1965. Lactate dehydrogenase isozyme patterns of fish. *Journal of Experimental Zoology*. 159: 319-332.

Markert, C. L., and Moller, F. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species-specific patterns. *Proceedings. National Academy of Sciences (United States of America)*. 45: 753-763.

May, B., and Royse, D. J. 1981. Application of the electrophoretic methodology to the elucidation of genetic life histories of edible mushrooms. *Mushroom Science*. 11: 799-817.

May, B., and Royse, D. J. 1982a. Confirmation of crosses between lines of *Agaricus brunnescens* by isozyme analysis. *Experimental Mycology*. 6: 283-292.

May, B., and Royse, D. J. 1982b. Genetic variation and joint segregation of biochemical loci in the common meadow mushroom *Agaricus campestris*. *Biochemistry and Genetics*. 20: 1165-1173.

May, B., and Royse, D. J. 1988. Interspecific allozyme variation within the fungal genus *Pleurotus*. *Transactions of the British Mycological Society*. 90: 29-36.

May, B., Henley, K. J., Fisher, C. G., and Royse, D. J. 1988. Linkage relationships of 19 allozyme encoding loci within the commercial mushroom genus *Pleurotus*. *Genome*. 30: 888-895.

May, B. 1992. Starch gel electrophoresis of allozymes. In: *Molecular genetic analysis of populations*, (ed. A. R. Hoelzel). IRL Press at Oxford University Press, Oxford, New York, Tokyo.

Mayr, E. 1942. *Systematics and the origin of the species*. Columbia University Press, New York.

Mayr, E. 1969. *Principles of systematic zoology*. McGraw and Hill, New York.

Mayr, E., Linsley, E. G., and Usinger, R. L. 1953. *Methods and principles of systematic zoology*. McGraw and Hill, New York.

Meacham, C. A., and Estabrook, G. F. 1985. Compatibility methods in systematics. *Annual Review of Ecology and Systematics*. 16: 431-446.

Micales, J. A., Bonde, M. R., and Peterson, G. L. 1992. Isozyme analysis in fungal taxonomy and molecular genetics. In: *Handbook of applied mycology volume 4: Fungal Biotechnology*, (eds., D. K. Arora., R. P. Elander., and K. G. Mukerji). Marcel Dekker, Inc. New York, Basel, Hong Kong. pp. 57-79.

Mickevich, M. F., and Johnson, M. S. 1976. Congruence between morphological and allozyme data in evolutionary inference and character evolution. *Systematic Zoology*. 25: 260-270.

Mickevich, M. F., and Mitter, C. 1981. Treating polymorphic characters in systematics: a phylogenetic treatment of electrophoretic data. In: *Advances in cladistics: Proceedings of the first meeting of the Willi Hennig society*, (eds., V, A, Funk, and D, R, Brooks). The New York Botanical Garden, Bronx, New York. pp. 45-58.

Mickevich, M. F., and Mitter, C. 1983. Evolutionary patterns in allozyme data: A systematic approach. In: *Advances in cladistics: volume 2, Proceedings of the second meeting of the Willi Hennig society*, (eds., N, I, Platnick and V, A, Funk). Columbia University Press, New York. pp. 169-176.

Mishler, B. D., and Brandon, R. N. 1987. Individuality, pluralism, and the phylogenetic species concept. *Biology and Philosophy*. 2: 397-414.

Mishler, B. D., and Donohue, M. J. 1982. Species concepts: a case for pluralism. *Systematic Zoology*. 31: 491-503.

Moore-Landecker, E. 1972. Fundamentals of the fungi. Prentice-Hall, Inc., Englewood Cliffs, N.J.

Moran, G. F., Bell, J. C., and Prober, S. 1990. The utility of isozymes in the systematics of some Australian tree groups. *Australian Systematic Botany*. 3: 47-57.

Morton, J. B. 1990. Evolutionary relationships among arbuscular mycorrhizal fungi in the Endogonaceae. *Mycologia*. 82: 192-207.

- Moser, M. 1967. *Die Roehrlinge und Blaetterpilze (Agaricales)*. 3. Auflage, Jena: Gustav Fisher. Bd. 2b. T2, Kleine Kryptogamenflora. Begrundet von H. Gams. Stuttgart.
- Moser, M. 1978. *Die Roehrlinge und Blaetterpilze (Polyporales, Boletales, Agaricales, Russulales)*. 4. Auflage. Gustav Fisher. Bd. II b/2, Kleine Kryptogamenflora. Begrundet von H. Gams. Stuttgart.
- Murphy, R. W. 1993. The phylogenetic analysis of allozyme data - invalidity of coding alleles by presence absence and recommended procedures. *Biochemical Systematics and Ecology*. 21: 25-38.
- Murphy, R. W., Sites, J. W. Jr., Buth, D. G., Haufler, C. H. 1990. Proteins I: Isozyme electrophoresis. In: *Molecular systematics*, (eds., D. M. Hillis, and C. Moritz). Sinauer Associates, Inc. Sunderland, Massachusetts, U.S.A. pp. 45-126.
- Nei, M. 1978. Genetic distance between a small number of individuals. *Genetics*. 89: 583-590.
- Nelson, G. A. 1972. Phylogenetic relationship and classification. *Systematic Zoology*. 21: 227-231.
- Nelson, G. A. 1974. Classification as an expression of phylogenetic relationship. *Systematic Zoology*. 22: 344-359.
- Nelson, G. A. 1978. Ontogeny, phylogeny, paleontology and the biogenetic law. *Systematic Zoology*. 27: 324-345.
- Nelson, G. A., and Platnick, N. 1981. *Systematics and biogeography: Cladistics and vicariance*. Columbia University Press, New York.
- Nixon, K. C., and Wheeler, Q. D. 1990. An amplification of the phylogenetic species concept. *Cladistics*. 6: 211-233.
- Ohmasa, M., and Furukawa, H. 1986. Analysis of esterase and malate dehydrogenase isozymes of *Lentinus edodes* by isoelectric focusing for the identification and discrimination of stocks. *Transactions. Mycological Society of Japan*. 27: 79-90.
- Okunishi, M., Yamada, K., and Komagata, K. 1979. Electrophoretic comparison of enzymes from basidiomycetes in different stages of development. *Journal of General Applied Microbiology*. 25: 329-334.
- Nei, M. 1972. Genetic distance between populations. *American Naturalist*. 106: 283-292.

- Olmstead, R. 1989. Phylogeny, phenotypic evolution, and biogeography of the *Scutellaria angustifolia* complex (Lamiaceae): inference from morphological and molecular data. *Systematic Botany*. 14: 320-338.
- Paranjpe, M. S. Chen, P. K., and Jong, S. C. 1979. Morphogenesis of *Agaricus bisporus*; changes in proteins and enzyme activity. *Mycologia*. 71: 469-478.
- Parmasto, E., and Parmasto, I. 1987. Variation of basidiospores in the Hymenomycetes and its significance to their taxonomy. *Bibliotheca Mycologia*. Band 115. J. Cramer. Berlin, Stuttgart.
- Patterson, C. 1982. Morphological characters and homology. In : *Problems in phylogenetic reconstruction*, (eds. K. A. Joysey and A. E. Friday). Systematics association special volume 21. Academic Press, London. pp. 21-74.
- Pegler, D. N. and Young, T. W. K. 1971. Basidiospore morphology in the Agaricales. *Nova hedwigia beigefte*. 35.
- Pilat, A. 1951. The Bohemian species of the genus *Agaricus*. *Acta mus. nat. Prag*. VIIB, 1: 3-142.
- Platnick, N. 1977. Monotypy and the origin of higher taxa: a reply to E. O. Wiley. *Systematic Zoology*. 28: 537-546.
- Prakash, S., Lewontin, R. C., and Hubby, J. L. 1969. A molecular approach to the study of genetic heterozygosity in natural populations, IV. Patterns of genetic variation in central, marginal and isolated populations of *Drosophila pseudoobscura*. *Genetics*. 61: 841-858.
- Raper, C. A., and Kaye, G. 1978. Sexual and other relationships in the genus *Agaricus*. *Journal of General Microbiology*. 105: 135-151.
- Raper, C. 1976. Sexuality and life-cycle of the edible, wild *Agaricus bitorquis*. *Journal of General Microbiology*. 95: 54-66.
- Reynolds, D. R. 1986. Foliicolous Ascomycetes 7. Phylogenetic systematics of the Capnodiaceae. *Mycotaxon*. 27: 377-403.
- Richardson, B. J., Baverstock, P. R., and Adams, M. 1986. *Allozyme electrophoresis. A handbook for animal systematics and population studies*. Academic Press. Sydney.

- Ridgway, G. J., Sherburne, S. W., and Lewis, R. D. 1970. Polymorphism in the esterases of Atlantic Herring. *Transactions. American Fisheries Society*. 1: 142-151.
- Rodgers, J. S. 1972. Measures of genetic similarity and genetic distance. *Studies in Genetics VII*. University of Texas Publication 7213: 145-153.
- Rodgers, J. S. 1984. Deriving phylogenetic trees from allele frequencies. *Systematic Zoology*. 33: 52-63.
- Rodgers, J. S. 1986. Deriving phylogenetic trees from allele frequencies: A comparison of nine genetic distances. *Systematic Zoology*. 35: 297-310.
- Rodrigo, A. 1993. Personal communication. Zoology Department, Auckland University, Auckland, New Zealand.
- Royse, D. J. and May, B. 1982a. Use of isozyme variation to identify genotypic classes of *Agaricus brunnescens*. *Mycologia*. 74: 93-102.
- Royse, D. J. and May, B. 1982b. Genetic relatedness and its application in selective breeding of *Agaricus brunnescens*. *Mycologia*. 74: 569-575.
- Royse, D. J. and May, B. 1990. Interspecific allozyme variation among *Morchella* spp. and its inferences for systematics within the genus. *Biochemical Systematics and Ecology*. 18: 475-479.
- Royse, D. J., and May, B. 1987. Identification of shiitake genotypes by multilocus enzyme electrophoresis. *Biochemical Genetics*. 25: 705-716.
- Royse, D. J., Spear, M. C., and May, B. 1983a. Cell line authentication and genetic relatedness of lines of the shiitake mushroom, *Lentinus edodes*. *Journal of General Applied Microbiology*. 29: 205-216.
- Royse, D. J., Spear, M. C., and May, B. 1983b. Single and joint segregation of marker loci in the shiitake mushroom, *Lentinus edodes*. *Journal of General Applied Microbiology*. 29: 217-222.
- Royse, D. J., and May, B. 1989. Identification and use of three new biochemical markers in *Agaricus bisporus*. *Agricultural Biology and Chemistry*. 53: 2861-2866.
- Royse et al. 1987. This reference was incorrect. Please refer to Jodon et al. 1986.

- Sasek, V. 1989. Submerged cultivation of ectomycorrhizal fungi. *Agriculture, Ecosystems and Environment*. 28: 441-447.
- Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E., and Gentry, J. B. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old field mouse (*Peromyscus polionotus*). *Studies in Genetics*. VI. University of Texas Publication. 7103. pp. 49-90.
- Selander, R. K., Smith, M. H., Yang, S., Johnson, W. E., and Gentry, J. B. 1971. IV. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. variation in the old field mouse (*Peromyscus polionotus*). *Studies in Genetics* VI. University of Texas Publication. pp. 49-90.
- Shaeffer, J., and Moeller, F. H. 1938. Beitrag zur Psalliota Forschung. *Annales Mycologici*. 36: 64-82.
- Simon, C. M. 1979. Evolution of periodical cicadas: phylogenetic inferences based upon allozyme data. *Systematic Zoology*. 28: 22-39.
- Simpson, G. G. 1961. *The principles of animal taxonomy*. Columbia University Press. New York.
- Singer, R. 1975. *The Agaricales in Modern Taxonomy* (3rd edn.). Strauß and Cramer, GmbH, D-6901, Leutershausen. Germany.
- Smithies, O. 1955. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochemical Journal*. 61: 629-641.
- Sneath, P. H. A. 1983. Philosophy and method in biological classification. In: *Numerical taxonomy*, (ed. J. Felsenstein). Springer Verlag. Berlin.
- Sneath, P. H. A., and Sokal, R. R. 1973. *Numerical taxonomy*. Freeman. San Francisco.
- Sokal, R. R., and Camin, J. H. 1965. The two taxonomies: areas of agreement and conflict. *Systematic Zoology*. 14: 176-195.
- Sokal, R. R., and Sneath, P. H. A. 1963. *The principles of numerical taxonomy*. Freeman, San Francisco.

- Soltis, D. E., Haufler, C. H., Darrow, D. C., and Gastony, G. J. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *American Fern Journal*. 73: 9-27.
- Soltis, D. E., Haufler, C. H., Darrow, D. C., and Gastony, G. J. 1983. Starch gel electrophoresis of ferns: grinding buffers, gel and electrode buffers, staining schedules. *American Fern Journal*. 73: 9-27.
- Song, C. H., Cho, K. Y., and Nair, N. G. 1987. A synthetic medium for the production of submerged cultures of *Lentinus edodes*. *Mycologia*. 79. 866-876.
- Stasz, T. E., Nixon, K., Harman, G. E., Weeden, N. F., and Kutter, G. A. 1989. Evaluation of phenetic species and phylogenetic relationships in the genus *Trichoderma* by cladistic analysis of isozyme polymorphism. *Mycologia*. 81: 391-403.
- Stevens, P. F. 1980. Evolutionary polarity of character states. *Annual Review of Ecology and Systematics*. 11: 335-358.
- Stevenson, G. 1982a. *Field guide to Fungi*. University of Canterbury Publication No. 30.
- Stevenson, G. 1982b. A parasitic member of the Bolbitiaceae. *New Zealand Journal of Forestry*. 27: 130-133.
- Stewart, C. B. 1993. The powers and pitfalls of parsimony. *Nature*. 361: 603-607.
- Summerbell, R. C., Castle, A. J., Horgen, P. A., and Anderson, J. B. 1989. Inheritance of restriction fragment length polymorphisms in *Agaricus brunnescens*. *Genetics*. 123: 293-300.
- Swofford, D. L. 1991. PAUP: Phylogenetic Analysis Using Parsimony, Version 3.0. Computer programme distributed by the Illinois Natural History Survey, Champaign, Illinois.
- Swofford, D. L., and Olse, S. H. 1987. Inferring evolutionary trees from gene frequency data under the principle of maximum parsimony. *Systematic Zoology*. 36: 293-325.

- Swofford, D. L., and Olsen, G. J. 1990. *Phylogeny reconstruction*. In: *Molecular systematics*, (eds., D. M. Hillis, and C. Moritz). Sinauer Associates, Inc. Sunderland, Massachusetts, U.S.A. pp. 411-501.
- Toyomasu, T., and Zennoyzi, A. 1981. On the application of isozyme electrophoresis to identification of strains in *Lentinus edodes* (Shiitake). *Mushroom Science*. 11: 675-684.
- Vallejos, C. E. 1983. Enzyme activity staining. In: *Isozymes in plant genetics and breeding*, part A. (eds., S. D. Tanksley, and T. J. Orton). Elsevier Science, Amsterdam. pp. 469-516.
- Vallejos, E. 1983. Enzyme activity staining. In: *Isozymes in plant genetics and breeding*, part A. (eds., S. D. Tanksley, and T. J. Orton). Elsevier Science Publishers B. V., Amsterdam. pp. 469-515.
- van Loon, P. C. C., Radermakers, G. M., Griensven, L. J. L. D. 1986. De herkenning van champignonrassen op basis van isoenzym patronen. *Champignoncultuur*. 30: 79-87.
- Vezey, E. L., Skvarla, J. J., and Vanderpool, S. S. 1991. Characterizing pollen sculpture of three closely related Capparaceae species using quantitative image analysis of scanning electron micrographs. In: *Pollen and Spores*. Systematics Association Special Volume No. 44, (eds., S. Blackmore and S. H. Barnes). Clarendon Press, Oxford. pp. 291-300.
- Vilgalys, R. 1986. Phenetic and cladistic relationships in *Collybia* sect. *Levipedes* (Fungi: Basidiomycetes). *Taxon* 35: 225-233.
- Vilgalys, R. 1991. Speciation and species concepts in the *Collybia dryophila* complex. *Mycologia*. 83: 758-773.
- Wasser, S. P. 1974. Note on Agaricales of the steppe zone of the Ukraine, new or little-known for flora of the Soviet Union. In: *Novitates Systematicae Plantarum Vascularium et non Vascularium*. Kiev. pp. 166-171. Cited in; Wasser, S. P. 1989. *Tribe Agariceae Pat. of the Soviet Union*. Koeltz Scientific Books. Koenigstein. Germany.
- Wasser, S. P. 1980. Flora Fungorum RSS Ucrainicae. Kiev. Cited in; Cappelli, A. 1984. *Agaricus* L.: Fr. ss. Karsten, (Psalliota Fr.). M. Candusso, Stampato in Italia.

Wasser, S. P. 1989. *Tribe Agariceae Pat. of the Soviet Union*. Koeltz Scientific Books. Koenigstein. Germany.

Wasser, S. P., Garibova, L. P., and Mokeeva, V. L. 1976. Morphometry of spores and substantiation of a new system in the genus *Agaricus* Fr. emend. Karst. *Acta Botanica Academiae Scientiarum Hungaricae*. 22: 249-258. Cited in; Parmasto, E., and Parmasto, I. 1987. Variation of basidiospores in the Hymenomycetes and its significance to their taxonomy. *Bibliotheca Mycologia*. Band 115. J. Cramer. Berlin, Stuttgart.

Watling, R. 1971. Chemical Tests in Agaricology. In: *Methods in Microbiology*. 4: 367-597.

Watling, R. 1977. An analysis of the taxonomic characters used in defining the species in Bolbitiaceae. In: *The species concept in Hymenomycetes*, (ed., H. Clemençon). J. Cramer, Vaduz. pp. 11-44.

Watrous, L. E., and Wheeler, Q. D. 1981. The out-group comparison method of character analysis. *Systematic Zoology*. 30: 1-11.

Wheeler, Q. D., and Nixon, K. C. 1990. Another way of looking at the species problem: a reply to De Queiroz and Donoghue. *Cladistics*. 6: 77-81.

Wheeler, Q., and Blackwell, M. 1984. Cladistics and the historical component of fungus-insect relationships. In: *Fungus-insect relationships; perspectives in ecology and evolution*, (eds., Q. Wheeler, and M. Blackwell). pp. 5-41.

Wiley, E. O. 1975. Karl R. Popper, systematics, and classification: A reply to Walter Bock and other evolutionary taxonomists. *Systematic Zoology*. 24: 233-244.

Wiley, E. O. 1981. *Phylogenetics: the theory and practice of phylogenetic systematics*. John Wiley and Sons, New York.

Wolfe, C. B, Jr. 1984. A numerical taxonomic analysis of the tribe Ixechineae (Boletaceae). *Mycologia*. 76: 140-147.

Zambino, P. J., and Harrington, T. C. 1992. Correspondence of isozyme characterization with morphology in the asexual genus *Leptographium* and taxonomic implications. *Mycologia*. 84: 12-25.

Appendix 1.

Isolates used in this study have been deposited in the culture collection held at Landcare Research, Private Bag 92170, Auckland, New Zealand. Numbers of original culture names, sourced from the American Type Culture Collection and from Dr D. J. Royse, are accession numbers for the respective culture collections from which they originated. Cultures given by D. J. Royse are also held in the Pennsylvania State University Culture Collection. Map references are from Topographical Maps, New Zealand Map Series (NZMS) or Info map series (not abbreviated), produced by the Department of Lands and Survey; V. D. Ward, Government Printer, Wellington, New Zealand.

All isolates not obtained from culture collections were collected by the author during the autumn of 1990, except for *Agaricus semotus* and *Agaricus porphyrocephalus*, which were collected by the author 22.5.92. Prof. P. Heinemann, c/o the Director of Jardin Botanique National de Belgique, Chateau de Bouchout, B.1860 Meise, identified to species the specimens from which the following isolates were taken; ICMP 11645 *Agaricus bisporus* Ohoka Red, ICMP 11654 *Agaricus bitorquis* Ropley, ICMP 11655-11657 *Agaricus campestris* Halls1, ICMP 11661 *Agaricus cupreobrunneus*, and ICMP 11664. *Agaricus lanipe?* Isolates not obtained from culture collections or identified by Prof. P. Heinemann, were identified by the author.

International collection of microorganisms from plants, (ICMP) Landcare research accession numbers. Original culture name and Source are described for each isolate as follows:

ICMP 11623. *Agaricus arvensis*, (Roll1). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36; 35-36N, 63-64E Rolleston (Roll) Lincoln.

ICMP 11623. *Agaricus arvensis*, (Roll2). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36; 35-36N, 63-64E Rolleston (Roll) Lincoln.

ICMP 11625. *Agaricus arvensis*, (Roll3). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36; 35-36N, 63-64E Rolleston (Roll) Lincoln.

ICMP 11626. *Agaricus arvensis*, (Roll4). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36; 35-36N, 63-64E Rolleston (Roll) Lincoln.

Note: All *Agaricus arvensis*, (Roll) cultures were isolated from specimens that were growing approximately 5-10m apart.

ICMP 11628. *Agaricus arvensis*, (Nect1). Basidioma culture isolated from single specimen in bare soil under nectarine(Nect) trees. NZMS260 M36; 28-29N, 66-67E, Horticultural Research Area, block C, Lincoln University.

ICMP 11629. *Agaricus arvensis*, (Nect2). Basidioma culture isolated from single specimen in bare soil under nectarine(Nect) trees. NZMS260 M36; 28-29N, 66-67E, Horticultural Research Area, block C, Lincoln University.

ICMP 11630. *Agaricus arvensis*, (Nect3). Basidioma culture isolated from single specimen in bare soil under nectarine(Nect) trees. NZMS260 M36; 28-29N, 66-67E, Horticultural Research Area, block C, Lincoln University.

Note: All *Agaricus arvensis*, (Nect) cultures were isolated from specimens that were growing approximately 20m apart.

ICMP 11631. *Agaricus arvensis*, (Rb1). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36; 39-40N, 85-86E, immediately east of traffic island, Provincial State Highway 74. Christchurch.

ICMP 11632. *Agaricus arvensis*, (Rb2). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36; 39-40N, 85-86E, immediately east of traffic island, Provincial State Highway 74. Christchurch.

ICMP 11633. *Agaricus arvensis*, (Rb3). Basidioma culture isolated from single specimen in a lawn. NZMS260 M36;

39-40N, 85-86E, immediately east of traffic island,
Provincial State Highway 74. Christchurch.

ICMP 11634. *Agaricus arvensis*, (Rb4). Basidioma culture
isolated from single specimen in a lawn. NZMS260 M36;
39-40N, 85-86E, immediately east of traffic island,
Provincial State Highway 74. Christchurch.

Note: All *Agaricus arvensis*, (Rb) cultures were isolated from
specimens growing in a single 10m² patch.

ICMP 11635. *Agaricus bisporus*, (28SS#502 Penn. State. Uni.
culture collection). Gift from D, J, Royse; Pennsylvania State
University, University Park, Pennsylvania 16802.

ICMP 11636. *Agaricus bisporus*, (B90SS#8 Penn. State. Uni.
culture collection). Gift from D, J, Royse; Pennsylvania State
University, University Park, Pennsylvania 16802.

ICMP 11641. *Agaricus bisporus*, (24663 American Type Culture
Collection).

ICMP 11642. *Agaricus bisporus*, (Sinden Hauser A5.2
commercial strain). Courtesy of Meadow Mushrooms Ltd.
Prebbleton, Christchurch.

ICMP 11643. *Agaricus bisporus*, (Sinder Hauser X20 commercial
strain). Courtesy of Meadow Mushrooms Ltd. Prebbleton,
Christchurch.

ICMP 11644. *Agaricus bisporus*, (Brown). Basidioma culture
isolated from single specimen in a fairy ring in tussock
grassland, with saline soils near Lake Ellesmere, NZMS260
M36; 18-19N, 75-76E. SW of Motukarara Gun Club.

ICMP 11645. *Agaricus bisporus*, (Ohoka). Basidioma culture
isolated from single specimen, one of many in a patch, in a
goat shelter area. NZMS 260 M35; 62-63N, 72-73E. Ohoka,
Canterbury

ICMP 11646. *Agaricus bernardii*, (34741 American Type Culture
Collection)

ICMP 11647. *Agaricus bernardii*, (34751 American Type Culture
Collection).

- ICMP 11648. *Agaricus bernardii*, (Mot). Basidioma culture isolated from single specimen in fairy ring N°1, tussock grassland with saline soils near Lake Ellesmere. NZMS260 M36; 18-19N, 75-76E. SW of Motukarara Gun Club.
- ICMP 11649. *Agaricus bitorquis*, (34725 American Type Culture Collection)
- ICMP 11650. *Agaricus bitorquis*, (34737 American Type Culture Collection)
- ICMP 11651. *Agaricus bitorquis*, (34780 American Type Culture Collection)
- ICMP 11652. *Agaricus bitorquis*, (34782 American Type Culture Collection)
- ICMP 11653. *Agaricus bitorquis*, (K46 commercial strain).
Courtesy of Morton Mushrooms Ltd, Springston,
Christchurch.
- ICMP 11654. *Agaricus bitorquis*, (Ropley). Basidioma culture isolated from a single specimen, one of many in a patch in front lawn of 11 Ropley St, Amberly.
- ICMP 11655. *Agaricus campestris*, (Halls1.1). Basidioma culture isolated from single specimen in fairy ring N°1 in cow paddock. NZMS260 M36; 34-35N, 74-75E, SE of Sabys Rd, Halswell.
- ICMP 11656. *Agaricus campestris*, (Halls1.2). Basidioma culture isolated from single specimen in fairy ring N°2 in cow paddock. NZMS260 M36; 34-35N, 74-75E, SE of Sabys Rd, Halswell.
- Culture lost. *Agaricus campestris*, (Halls1.3). Basidioma culture isolated from single specimen in fairy ring N°3 in cow paddock. NZMS260 M36; 34-35N, 74-75E, SE of Sabys Rd, Halswell.
- ICMP 11657. *Agaricus campestris*, (Halls1.4). Basidioma culture isolated from single specimen in fairy ring N°4 in cow paddock. NZMS260 M36; 34-35N, 74-75E, SE of Sabys Rd, Halswell.
- Note: All *Agaricus campestris*, (Halls1) fairy rings were between 10-20m apart in a single paddock.

- ICMP 11659. *Agaricus campestris*, (Halls2). Basidioma culture isolated from single specimen in fairy ring in sheep paddock. NZMS260 M36; 34-35N, 74-75E, E of Provincial State Highway 75, near bend.
- ICMP 11660. *Agaricus campestris* var. *campestris*, (cam.cam). Basidioma culture isolated from single specimen, one of many in a patch, in a lawn. NZMS260 M36; 28-29N, 66-67E, biological husbandry area, Lincoln University.
- ICMP 11661. *Agaricus cupreobrunneus*, (cupreo.). Basidioma culture isolated from single specimen in a fairy ring on lawn. 113 New Brighton Rd. Christchurch.
- ICMP 11662. *Agaricus porphyrocephalus*, (porphyro.). Basidioma culture isolated from single specimen in a fairy ring, in a lawn. 260-Q07, PT R07; 89-90N, 12-13E. Tanihua Lodge, Whangarei.
- ICMP 11663. *Agaricus semotus*, (semotus). Basidioma culture isolated from single specimen, one of many in a patch in humus under *Leptospermum ericoides*, Kanuka. Info map 260-Q07, PT R07; 09-11N, 23-25E. Pukenui Walkway, Kamo, Whangarei.
- ICMP 11664. *Agaricus lanipe*§ (lanipe§). Basidioma culture isolated from single specimen, one of many in a patch under conifer with bark mulch, Mona Vale, Christchurch. Spring and Autumn.
- ICMP 11665. *Agaricus* sp.1, (Agaricus1). Basidioma culture isolated from single specimen, one of many in a patch under a *Arucaria araucan*, Monkey Puzzel tree. Front lawn 166 Queensbury St, Christchurch.
- ICMP 11666. *Agaricus subperonatus*, (subpero.) Basidioma culture from single specimen, one of many in bare soil under *Populus nigra*, Poplar, and *Cupressus macrocarpa* tree. NZMS260 M36; 29-30N, 66-68E. Shelter belt Lincoln University Arable Farm block A3. Basidiocarps found in Autumn the entire 100m length of the Poplar shelter belt.
- ICMP 11667. *Leucoagaricus leucothites*, (leucoag.). Basidioma culture from single specimen, one of many in cow paddock, Taitapu. NZMS260 M36/5.2 (002); 43 40, 172 33.

Collected by unknown member of the public, precise location uncertain. Christchurch.

ICMP 11668. *Agrocybe parasitica*, (*Agrocybe*). Basidioma culture from single specimen, one of many, Deans Bush, on *Plagianthus* sp. Christchurch.

Appendix 2.

From the results of experiments carried out in Chapter 3 it was concluded that several enzymes showed promise for future study, but were not presently considered suitable for full character analysis of all isolates. Table 1 lists these enzymes, their abbreviations, E.C numbers, and the buffer systems and staining solutions evaluated. Brief descriptions of these enzyme systems will now be given.

Enzymes studied for which activity had not previously been reported for *Agaricus*.

Succinate dehydrogenase (SUDH): Activity was found in range of *Agaricus* species, *Agrocybe parasitica* and *Leucoagaricus leucothites* Figure 24. TBE buffer system provided the most distinct SUDH electromorphs, at 100V, 60 mA for 5 h. Figure 24 illustrates the resolution possible for SUDH. Accurate scoring of SCDH electromorphs was made difficult as a result of: 1) a high level of resolution was not readily repeatable. 2) electromorphs for the different species were very closely positioned with one another, Figure 24. 3) electromorphs faded in light within 5-15 min. Two loci were present for SUDH, and the observed banding patterns were typical of a monomeric enzyme. Between species polymorphisms were evident for SUDH. SOD activity was often revealed while scoring SUDH electromorphs over a light box.

Table 1. Enzymes, abbreviations, enzyme commission number (E.C. No), buffer systems tested, staining solution composition and reference for source of staining solution used. All staining solutions were made up in 100 ml distilled water. Where indicated, staining solutions include 30mg NADH, 30mg NADP⁺, 30mg NAD⁺, 4mg PMS, and 20mg MTT.

<u>Recommended name</u>	<u>Abbr.</u>	<u>E.C. No.</u>	<u>Buffers@ Tested</u>	<u>Staining solution# composition</u>	<u>Source of stain solution</u>
<u>Oxidoreductases</u>					
Alcohol dehydrogenase	ADH	1.1.1.1	TBE, C, S2	0.1M Tris, pH 7.5; NAD ⁺ ; MTT; PMS; 6ml ethanol (just before incub.).	Vallejos (1983)
Formate dehydrogenase	FDH	(?)	TBE, C, S2	0.1 M Tris, pH 7.5; 100mg formic acid NAD ⁺ ; MTT; PMS.	Royse and May (1982a)
Isocitrate dehydrogenase	IDH	1.1.1.42	TBE, C S2	0.1M Tris, pH 7.5; 1 ml 1M MnCl ₂ ; 100mg DL-isocitric acid (Na ₃); NADP ⁺ ; MTT; PMS.	Vallejos (1983)
Shikimic dehydrogenase	SKDH	1.1.1.25	TBE, C S2	0.1 M Tris, pH7.5; 100mg shikimic Acid; 15mg NADP ⁺ ; MTT; PMS.	Vallejos (1983)
Succinate dehydrogenase	SUDH	1.3.99.1	TBE, C, S2	50mM Na phosphate, pH 7.0; 80 mg ATP; 450mg succinic acid; 800mg EDTA (Na ₂); NAD ⁺ ; MTT; PMS.	Vallejos (1983)
Superoxide dimutase	SOD	1.15.1.1	TBE, C, S2, BS7	<u>Solution A</u> ; 50 mM Na phosphate, pH 7.5; 200mg MTT. <u>Solution B</u> : 50 mM Na phosphate, pH 7.5; 0.4ml TEMED; 1mg riboflavin. First, soak gels in solution A for 20 min. Then drain off solution A and pour on solution B and incubate under illumination.	Vallejos (1983)

<u>Recommended name</u>	<u>Abbr.</u>	<u>E.C. No.</u>	<u>Buffers Tested</u>	<u>Staining solution# composition</u>	<u>Source of stain solution</u>
<u>Hydrolases</u>					
Esterase (butylesterase)	EST	3.1.1.1	S4, TBE, C	0.1M Na phosphate, pH 6.2; 3ml, 1% α -naphthyl butyrate in acetone; 100mg Fast Blue RR Salt.	Vallejos (1983)
Peptidase with glycyl-leucinez	PEP-GL	3.4.11-13	S4, TBE, C, BS7	Solution A: 50 mM Na phosphate, pH 6.5; 80mg glycyl-leucine. Solution B; 3ml N, N, dimethylformamide; 10mg dianisidine Solution C; 30 units L-amino acid oxidase; 80 units peroxidase. Dissolve the tripeptide in warm buffer for 10-20min. Pour solution A into B and mix into flask containing solution C.	Vallejos (1983)
β -D-Xylosidase	β XYL	3.2.1.37	S4, C	50mM Na acetate pH 4.3; 15 mg 4-methylumbelliferyl β -D-xyloside.	Vallejos (1983)
<u>Transferases</u>					
Phosphoglycerate kinase	PGK	2.7.2.3	C	5ml, 0.2 M Tris, pH8.0, 1:4 solution with water 10 mg D(-)-3-phosphoglyceric acid; 15mg NADH; 10mg ATP; 5mg $MgCl_2$; 1mg EDTA; 100 units glyceraldehyde-3 phosphate dehydrogenase.	Allendorf et al. (1977)
<u>Isomerases</u>					
Glucose-6-phosphate isomerase	GPI	5.3.1.9	TBE, C, S2	0.1 M Tris, pH 7.5; 1ml, 1M, $MgCl_2 \cdot 6H_2O$; 80mg fructose-6-phospahte (Na_2); 20mg $NADP^+$; MTT; PMS; 20 units glucose-6-phosphate dehydrogenase.	Vallejos (1983)

@ S4; Described by Selander et al. (1971) as follows: Electrode; 0.223 M tris, 0.094 M citric acid, adjusted to pH 6.3 with NaOH. Gel; 8 mM tris, 3 mM citric acid, adjusted to pH 6.7 with NaOH.

TBE; Described by Murphy et al. (1990) as Tris-borate-EDTA 1, which is a modification of the M buffer system described by Markert and Faulhaber (1965). Stock solution; 0.90 M Tris, 0.50 M boric acid, 0.02 M EDTA (Na₂), adjusted to pH 8.6 with NaOH. Electrode: Anode; 1:6 dilution of stock solution, Cathode; 1:4 dilution of stock solution. Gel; 1:19 dilution of stock solution.

C; Described by Clayton and Tretiak (1972) modified as follows: Electrode: 0.04 M citric acid, adjusted to pH 8.2 with N -(3-aminopropyl)-morpholine: Gel- a 1 : 9 dilution of this electrode buffer was used for gel preparation.

S2; Described by Selander et al. (1971) as follows: Stock solution A; 0.03 M lithium hydroxide, 0.19 M boric acid, pH 8.1: Stock solution B; 0.05 M tris, 8 mM citric acid, lithium hydroxide, pH 8.4. Electrode; Undiluted stock solution A. Gel; 1:9 mixture of stock solution A:B, final pH 8.3, was used for gel preparation.

BS7; Buffer system 7 described by Soltis et al. (1983). Electrode; 0.038 M lithium hydroxide, 0.188 M boric acid, adjusted to pH 8.3, adjusted with dry components. Gel; 0.045 M tris, 0.007 M citric acid, 0.004 M lithium hydroxide, 0.019 M boric acid, adjusted to pH 8.3 with NaOH.

#All gels were incubated at 30° in the dark. Staining solutions may have been modified from that of the source given.

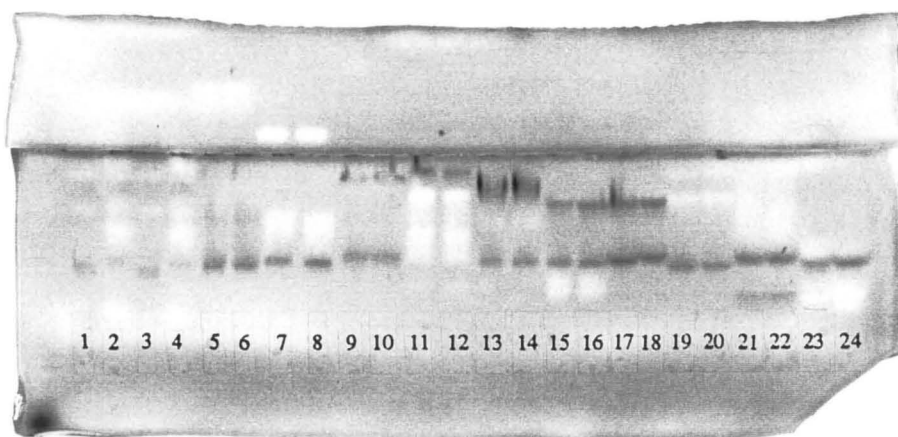


Figure 1. SUDH activity for 12 different strains. Lanes 1-24 were as follows; 1) *Agaricus campestris* Halls2, 2) *Agrocybe parasitica*, 3) *Agaricus campestris* Halls2, 4) *Agrocybe parasitica*, 5) *Leucoagaricus leucothites*, 6) *Leucoagaricus leucothites*, 7) *Agaricus subperonatus*, 8) *Agaricus subperonatus*, 9) *Agaricus bitorquis* Ropley, 10) *Agaricus bitorquis* Ropley, 11) *Agaricus arvensis* Roll1, 12) *Agaricus arvensis* Roll1, 13) *Agaricus cupreobrunneus*, 14) *Agaricus cupreobrunneus*, 15) *Agaricus campestris* var. *campestris*, 16) *Agaricus campestris* var. *campestris*, 17) *Agaricus campestris* Halls1.5, 18) *Agaricus campestris* Halls1.5, 19) *Agaricus lanipe*, 20) *Agaricus lanipe*, 21) *Agaricus bernardii* Mot, 22) *Agaricus bernardii* Mot, 23) *Agaricus bisporus* Ohoka, 24) *Agaricus bisporus* Ohoka. Achromatic bands shown on the gel were caused by SOD activity.

β XYL: Activity was found in a range of *Agaricus* species and *Leucoagaricus leucothites* using the C buffer system, at 110 V, 70

mA for 5 h, Figure 25. Electromorph patterns were very similar to those of β GLU, which may indicate a lack of specificity for the same enzyme, i.e., having a broad number of closely related substrates. It was not possible to determine if β XYL and β GLU produced exactly the same banding patterns because of the inherent problems associated with these enzymes, including electromorph variation between electrophoretic events. Even though banding patterns were not always repeatable, the observation of either 1 or 2 band patterns were consistent with a monomeric enzyme.

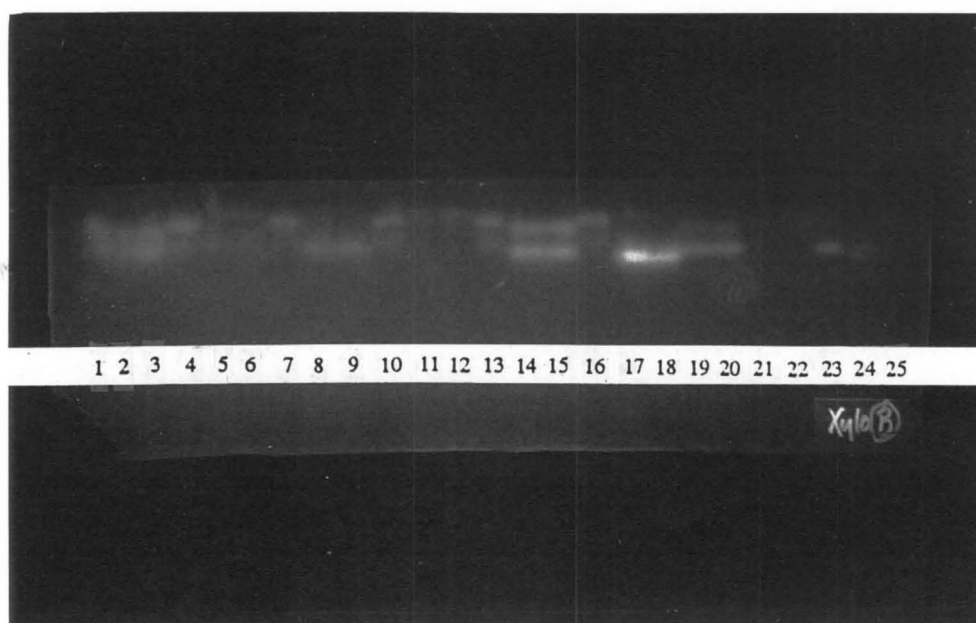


Figure 2. β XYL activity for 12 different strains. Lanes 1-25 were as follows; 1) *Agaricus bisporus* A5.2, 2) *Agaricus bisporus* Ohoka, 3) *Agaricus bisporus* Ohoka, 4) *Agaricus bisporus* A5.2, 5) *Agaricus bitorquis* Ropley, 6) *Agaricus bitorquis* Ropley, 7) *Agaricus bisporus* A5.2, 8) *Agaricus arvensis* Roll1, 9) *Agaricus arvensis* Roll1, 10) *Agaricus bisporus* A5.2, 11) *Agaricus bernardii* Mot, 12) *Agaricus bernardii* Mot, 13) *Agaricus bisporus* A5.2, 14)

Leucoagaricus leucothites, 15) *Leucoagaricus leucothites*, 16) *Agaricus bisporus* A5.2, 17) *Agaricus campestris* Halls2, 18) *Agaricus campestris* Halls2, 19) *Agaricus campestris* var. *campestris*, 20) *Agaricus campestris* var. *campestris*, 21) *Agaricus subperonatus*, 22) *Agaricus campestris* Halls1.5, 23) *Agaricus cupreobrunneus*, 24) *Agaricus* sp.1, 25) *Agaricus bisporus* A5.2.

IDH: TBE buffer system provided the most distinct IDH electromorphs, and polymorphic activity between species was demonstrated. Activity was detected for *Agaricus arvensis* Roll1, *Agaricus bernardii* Mot, *Agaricus bisporus* A5.2, *Agaricus bitorquis* Ropely, *Agaricus campestris* cam.cam and Halls2, *Agaricus cupreobrunneus*, *Agaricus* sp.1, *Agaricus subperonatus*, *Agrocybe parasitica* and *Leucoagaricus leucothites*. Poor separation and clarity of electromorphs prevented genetic interpretation.

Enzymes studied for which activity had previously been reported for *Agaricus bisporus* and *Agaricus campestris*.

ADH: TBE buffer system provided the most distinct ADH electromorphs, with between species polymorphisms evident. Activity was found for *Agaricus arvensis* Nect 1, Nect2, and Rb2, *Agaricus bernardii* 34741, *Agaricus bitorquis* 34782, *Agaricus campestris* cam.cam and Halls2, *Agaricus cupreobrunneus*, *Agaricus porphyrocephalus*, *Agaricus semotus*. No activity was found for *Agaricus arvensis* Roll 1, Roll4, and Nect 3, *Agaricus bernardii* Mot, *Agaricus bisporus* A5.2, Ohoka, 22#SS502, and B90#SS8, *Agaricus bitorquis* K46, 34737, and Ropely, *Agrocybe*

parasitica and *Leucoagaricus leucothites*. Generally the ADH electromorphs were smeared, and genetic interpretation difficult.

GPI: The TBE and S2 buffer systems, rarely provided reasonable electromorph separation for for most species, but usually resulted in smeary activity zones. The C buffer system, after 5 h. run time at 110 V and 50 mA resulted in reasonably clear electromorphs that were retained too close to the origin for reliable genetic interpretation. Attempts to shift these electromorphs down from the origin included combinations of the following; 1) increasing the run time, 2) decreasing the gel buffer concentration, and 3) increasing the current. All attempts were unsuccessful, resulting in smeared and weak electromorphs that could not be reliably scored. Between species polymorphisms were evident for GPI.

PEP-GL: The most distinct PEP-GL electromorphs were gained through the use of the BS7 buffer system. Between species polymorphisms were evident for PEP-GL. This enzyme was investigated because strains infrequently showed clear banding patterns. However, consistent problems of smeared banding patterns were not solved.

PGK: Electromorph resolution for most species was unacceptable at what may have been 2 PGK loci. However, *Agaricus cupreobrunneus* and *Agaricus porphyrocephalus* shared an electromorph, which was more cathodal compared to another electromorph shared by *Agaricus campestris*, cam.cam and *Agaricus subperonatus*. *Agaricus bitorquis* K46 showed a clearly

defined electromorph of greater migration than the isolates already described. An electromorph for *Agaricus bitorquis* Ropley was anodal compared with any other electromorph, and may have been coded for by a separate locus.

Enzymes studied for which activity had previously only been reported for *Agaricus bisporus*.

EST: Activity was found for all *Agaricus* species, *Agrocybe parasitica* and *Leucoagaricus leucothites* using the buffer and stain systems in Table 2. Between species polymorphisms were evident for EST. Problems with this enzyme included smeared and complex banding patterns for many species, preventing genetic interpretation.

FDH: Conditions allowing accurate genetic interpretation of FDH electromorphs were not determined. Activity for this enzyme was shown for *Agaricus bernardii* Mot, *Agaricus bisporus* A5.2, *Agaricus bitorquis* Ropley, *Agaricus campestris* cam.cam and Halls2, *Agaricus cupreobrunneus*, *Agaricus lanipe*, *Agaricus subperonatus*, *Agrocybe parasitica* and *Leucoagaricus leucothites*. Between species polymorphisms were demonstrated for this enzyme. Detection of 1 and 3 banding patterns were consistent with a dimeric enzyme.

SOD: Conditions allowing accurate genetic interpretation of SOD electromorphs were not determined. *Agaricus arvensis* consistently showed good SOD activity with all buffer systems

tested, Table 2. The most distinct SOD activity was obtained, not when it was specifically stained for, but when scoring other tetrazolium based enzymes over the light box, i.e., the active ingredients for SOD staining are found in all tetrazolium based stains, such as SUDH, shown in Figure 1.

Appendix 3.

Mycelial growth with time in 100 ml Oxoid Malt Extract broth in 250 ml Erlenmeyer flasks, incubated at 25°C with shaking, 80 rpm in the dark. Data in this Appendix was used for the construction of growth curves in Chapter 3, shown in Figure 3.10.

* DAI	Weight of freeze dried mycelium (mg) for species A to M												
	A	B	C	D	E	F	G	H	I	J	K	L	M
5					28.1					13.5	18.2	23.6	
6													
7													
8	16.3	10.7	30.0	30.0		1.3	10.7	13.6	66.4				
9					120.3								
10										36.2	24.3	174.4	
11	27.9	18.8	119.2	73.4		4.0	19.2	26.0	191.1				
12					410.2								
13													
14	36.2	22.6	194.0	200.7		104.0			465.0				18.7
15					706.7					56.4	82.9	398.2	
16													
17	51.5	31.3	382.2	295.6		329.0	24.9	55.3	628.7				25.2
18					597.0								
19													
20	54.8	29.8	357.4			416.0	29.0		635.2	270.2	267.4	497.8	44.8
21					656.9								
22													
23	106.0	49.3	331.5	525.1		577.5	57.3	303.9	782.3				
24					708.5								
25										555.1	294.5	644.5	78.2
26	280.0	90.0	357.0	470.0		470.7	632.0		1228.1				
27					642.9								
28													90.3
29	234.9	90.0	361.0	596.0		584.7	101.2	379.4	764.5				
30					738.0						175.7	482.3	
31													116.5
32	226.0	73.9	318.8	699.4		519.7	157.2	402.9	991.7				
33					689.4								
34													92.8
35							204.6			485.0			

* DAI = Days after inoculation

Species names

A *Agaricus arvensis* Roll

C *Agaricus campestris* cam.cam

E *Agaricus cupreobrunneus*

G *Agaricus* sp.1

I *Leucoagaricus leucothites*

K *Agaricus semotus*

M *Agaricus lanipes*

B *Agaricus bernardii* Mot

D *Agaricus bitorquis* K46

F *Agaricus bisporus* Ohoka

H *Agaricus subperonatus*

J *Agrocybe parasitica*

L *Agaricus porphyrocephalus*

Appendix 4.

For experiments in Chapter 4, involving the comparison of allozymes from strains representing eleven species of *Agaricus*, *Leucoagaricus leucothites*, and *Agrocybe parasitica*, the following methods were used to ensure as far as possible that all procedures were standardised.

Culture methods.

Isolation of cultures and maintenance of cultures were carried out as described in Sections 3.2.2, and 3.2.3. The strains listed in Appendix 1, were grown in shaken submerged culture until the required physiological stage had been reached, (Sections 3.2.4 and 3.10.). Five litre flasks, each containing 500 ml of medium were used for *Agaricus arvensis*, *Agaricus semotus*, *Agaricus bisporus* *Agaricus* sp.1, *Agaricus lanipe*, and *Agaricus bernardii*, and replicate cultures were pooled for use. Mycelium was prevented from adhering to the sides of the flasks by manually swirling the flasks every 3 days, (Section 3.9) to decrease possible effects caused by different forms of mycelium.

Approximately 50 g fresh mycelium from each strain was harvested, (Section 3.2.5), frozen with liquid nitrogen and placed into a Cuddon freeze drier at -22°C to -18°C and less than 1 Torr vacuum pressure overnight. When removed from the freeze drier, mycelium was weighed into approximately 100 mg aliquots. Each aliquot of mycelium was placed into a 5 ml polystyrene test tube, 75 x 12 mm, (Lab Serv, LBS 503), each with a polystyrene

stopper, and stored at -80°C for analysis over a period of 3 months, (Section 3.8.3).

Mycelial extraction.

Modified extraction buffer A; a 100 ml solution of pH 7.1, 0.05 M Tris-HCl extraction buffer, (May and Royse 1981), plus 12 mM cysteine-HCl, (Cheliak and Pitel 1984) was prepared 10 min. prior to extraction. A minimum of 0.2 g wet weight mycelium was extracted with an equal mass of hydrated insoluble PVP, (modified from Cheliak and Pitel 1984), and an equal volume of extraction buffer when it was intended to study the enzymes listed in Table 3.1, (excluding LAP and AAT), and SUDH and EST given in Appendix 2.

Modified extraction buffer B, which was modified extraction buffer A plus 1 mM dithiothreitol, (Cheliak and Pitel 1984), 0.01% soluble PVP, (Cheliak and Pitel 1984), and 0.05 % bovine serum albumin, (Cheliak and Pitel 1984), was used for the resolution of LAP and AAT.

Mycelium was extracted using the methods described in Section 3.8.3.1.1 (mycelium and extraction solution components proportionally increased), and the electrophoretic conditions and staining solutions listed in Table 3.1. Equal quantities of mycelium and extraction buffer were used for extraction with a minimum of 50 μ l supernatant needed for electrophoresis.

Electrophoresis.

Gel preparation and gel loading were as described in Sections 3.4.2.1 and 3.4.2.2, and electrophoretic conditions were as described in Table 3.1. All strains listed in Appendix 1 were screened for MDH, ACP, β GLU, HA, LAP, AAT, GK, GPT, PGM, and MPI.

As electromorph migration rates varied between electrophoretic runs, depending upon conditions such as differences in ostensibly similar gels, different strains with apparently identical electromorphs were repeatedly run next to each other on successive gels for comparison until electromorph similarity or difference had been established.

Gel slicing and staining was as described in Section 3.4.2.3, and staining systems for the individual enzymes were as described in Table 3.1. When it was intended to stain for ACP, β GLU, and HA, sample wicks were removed as soon as the sample had migrated into the gel from the origin. But when it was intended to stain for AAT, and LAP, sample wicks were removed 10 minutes after the start of electrophoresis.

Strains.\ Loci.	Mdh-1	Mdh-2	Acp	β Glu-2	Ha-1	Lap-1	Aat-1	Gk-1	Gk-2	Gpt	Pgm-2	Mpi
<i>Agaricus arvensis</i>												
arvRoll1	00000101	0000001000010	00010000	00100000000000??	10000?0000	0000010000	00001010000	00000100000	00000000000	000000100	0100000000000	00010000000
arvRoll2	00000101	0000001000010	00010000	00100000000000??	1000010000	0001000000	00001010000	00000100000	00000000000	000000100	0100000000000	?????000000
arvRoll3	00000101	0000001000010	00010000	00100000000000??	1000010000	0001000000	00001010000	00000100000	00000000000	000000100	0100000000000	00010000000
arvRoll4	00000101	0000001000010	00010000	00100000000000??	1000000000	0001000000	00001010000	00000100000	00000000000	000000100	0100000000000	00010000000
arvNect1	00000101	0000001000010	00010000	0010000000000100	??????????	0000010000	00001010000	00100001000	?010000100	000000100	0100000000000	01010000000
arvNect2	00000101	0000001000010	00010000	0010000000000100	1000000000	0001010000	00001010000	00100001000	?010000100	000000100	0100000000000	01010000000
arvNect3	00000101	0000001000010	00010000	0010000000000100	??????????	0000010000	00001010000	00100001000	?010000100	000000100	0100000000000	01010000000
arvRb1	00000100	0000001000010	00010000	0010000000000??0	1000000000	0001000000	00001010000	00100000000	00000000000	000000100	0100000000000	00010000000
arvRb2	00000101	0000001000010	00010000	0010000000000??0	??????????	0001000000	00001010000	00100000000	00010000000	000000100	0100000000000	00010000000
arvRb3	00000100	0000001000010	00010000	0010000000000??0	??????????	0001000000	00001010000	00100000000	00000000000	000000100	0100000000000	00010000000
arvRb4	00000101	0000001000010	00010000	0010000000000??0	1000000000	0001000000	00001010000	00100000000	00010000000	000000100	0100000000000	00010000000
arv34807	00000101	0000001000010	00010000	00100000000001000	??????????	0001000000	000010?0000	00100000000	00000000000	000000100	0100000000000	00010000000
<i>Agaricus bisporus</i>												
bisAb5.2	00010000	0000001000010	00000010	0000100000010000	0000000000	0000100000	00100000000	00000000100	00001100000	010000000	00100000000100	00000000000
bisx20	00010000	0000001000010	00000010	0000100000010000	0000000000	0000100000	00100000000	00000000100	00001100000	010000000	00100000000100	00000000000
bisOhoka	00010000	0000001000010	00000010	0000000010000100	0000000000	0000100000	00100000000	00000000100	00001100000	100000000	00100000000100	00000100000
bisBrown	00010000	0000001000010	00000001	0001000001000000	0000000000	0000100000	00100000000	00000000000	00001100000	010000000	0010000000000	00000000000
bis24663	00010000	0000001000010	00000010	0000000010000100	0000000000	0000100000	00100000000	00000000000	00001100000	010000000	0010000000000	00000000000
bisB90#SS8	00010000	0000001000010	00000010	0000000010000100	0000000000	0000100000	00100000000	00000000000	00001100000	010000000	0010000000000	00000000000
bis28#SS502	00010000	0000001000010	00000001	0000100000010000	0000000000	0000100000	00100000000	00000000100	00001100000	010000000	0010000000000	00000000000
<i>Agaricus bitorquis</i>												
bitRopely	00100000	0100000100000	000100?0	0100000100001000	0000000000	0010010000	000?0000000	000000000101	00100000000	000000000	10000010001000	00100000000
bitK46	00000000	00000100000100	00000001	00000000100?0000	0000000000	0000000010	00100000000	000000000010	000000001000	001000000	0000000010000	00100000000
bit34725	00000000	00000100000100	00000010	00000000100?0000	0000000000	0000010000	10000000000	001000000001	000000001100	001000000	00000000001000	000000010000
bit34737	00000000	00000100000100	00000010	0000000001000100	0000000000	0000000000	00010000000	000000000010	000000001000	??????????	00000000100000	000000010000
bit34780	00000000	00000100000100	00000010	0000000001000100	0000000000	0000000001	01000000000	000000000010	000000001000	??????????	00000000100000	000000010000
bit34782	00001000	00000010000010	00000010	1000010000000000	0001000001	1000000000	00010000000	00001000000	?010000000	000001000	00000000000100	000000010000
<i>Agaricus bernardii</i>												
berMot	00100000	0100000100000	00000000	010010000000?00	1000010000	0000010000	00?100000?0	00000010000	001000000100	000000001	0010000?0000	00000000010
ber34741	00001000	0100000100000	00000010	1001000000001001	0010000100	0000010000	00110000000	00100000000	00100100000	000000100	0100000000000	000000?000
ber34751	00001000	00000010000010	00000010	1001000000001001	0010000100	0000010000	00010000000	00100000000	00?000100000	000000100	0100000000000	000000010000
<i>Agaricus campestris</i>												
cam.cam	00000100	0100000100000	00001000	0000000000100000	01000001000	1000000000	000100000010	01010000000	01010000000	000001000	00000000000100	000000001000
camHalls2	00000100	0100000100000	00001000	0100000100000000	0001000001	1000000000	000100000010	10000001000	01000000000	000000100	00000000000100	000000010000
camHalls1.2	00000100	0100000100000	00001000	0000000100000000	1000100000	??????????	000000000010	00010000000	?0000000000	0000001000	00000000000100	????????????
camHalls1.3	00000100	0100000100000	00000100	????????????????	00100000010	1000000000	00000000000	000000010000	00000000000	000001000	00000000000100	????????????
camHalls1.4	00000100	0100000100000	00000010	0000000000100000	????????????	????????????	????????????	00100000000	01010000000	0000001000	00000000000100	????????????
camHalls1.5	00000100	0100000100000	00001000	0000000100000000	10000001000	1000000000	000000000010	00010000000	00000000000	000001000	00000000000100	000000010000
<i>Agaricus cupreobrunneus</i>	00001000	0000001000010	00000100	0000000000000100	0010010000	1001000000	00000?01000	010000010000	01000000000	000001000	0000000010000	00001001000
<i>Agaricus porphyrocephalus</i>	00001000	0000001000010	00001000	0000000000010000	0010010000	0100000000	00000?01000	00001000000	00010000000	000001000	0000000001000	000000110000
<i>Agaricus lanipe\$</i>	11000000	0000001000000	00000100	0100000000000000	1000000000	0100000000	000000010000	000000010001	00100000000	000000100	0010001000000	????????????
<i>Agaricus</i> sp.1	00000010	000001000100000	10000100	0000010000000000	0000000000	0000010000	000100010000	000000000011	000000000011	000000100	01000000000100	10000000000
<i>Agaricus subperonatus</i>	00000100	0?00000000000	00000010	0000000001000000	0100100000	00000001000	010000100000	0000?00000	?0000000000	000100000	0010000000000	00001000000
<i>Agaricus semorus</i>	00000100	1000000100000	00000010	0100000000000000	1000010000	0000000100	000100000010	00010000000	00010000000	000000100	00010000100000	000000000100
<i>Leucoagaricus leucothites</i>	00000000	00010000101000	?0000000	00000000001000010	0001000000	00000000100	000000000???	?0000000000	00000?0000	000000010	00000000000100	000001000000
<i>Agrocybe parasitica</i>	00000?00	00000000100001	?0000000	00000000000000100	0010000000	0000000000	000000000???	000000001000	00000?0000	000010000	00000000000101	000000000001

Character matrix for electromorph presence and absence at 12 putative loci for strains representing eleven *Agaricus* species, *Leucoagaricus leucothites* and *Agrocybe parasitica* determined during experimentation in Chapter 4. Note; One, (1) represents the presence of an electromorph, zero, (0) represents the absence of an electromorph, and a question mark, (?) represents missing data.

Appendix 6.

Apomorphy lists for the tree presented in Chapter 4, Figure 4.8, generated from preliminary cladistic analysis of strains.

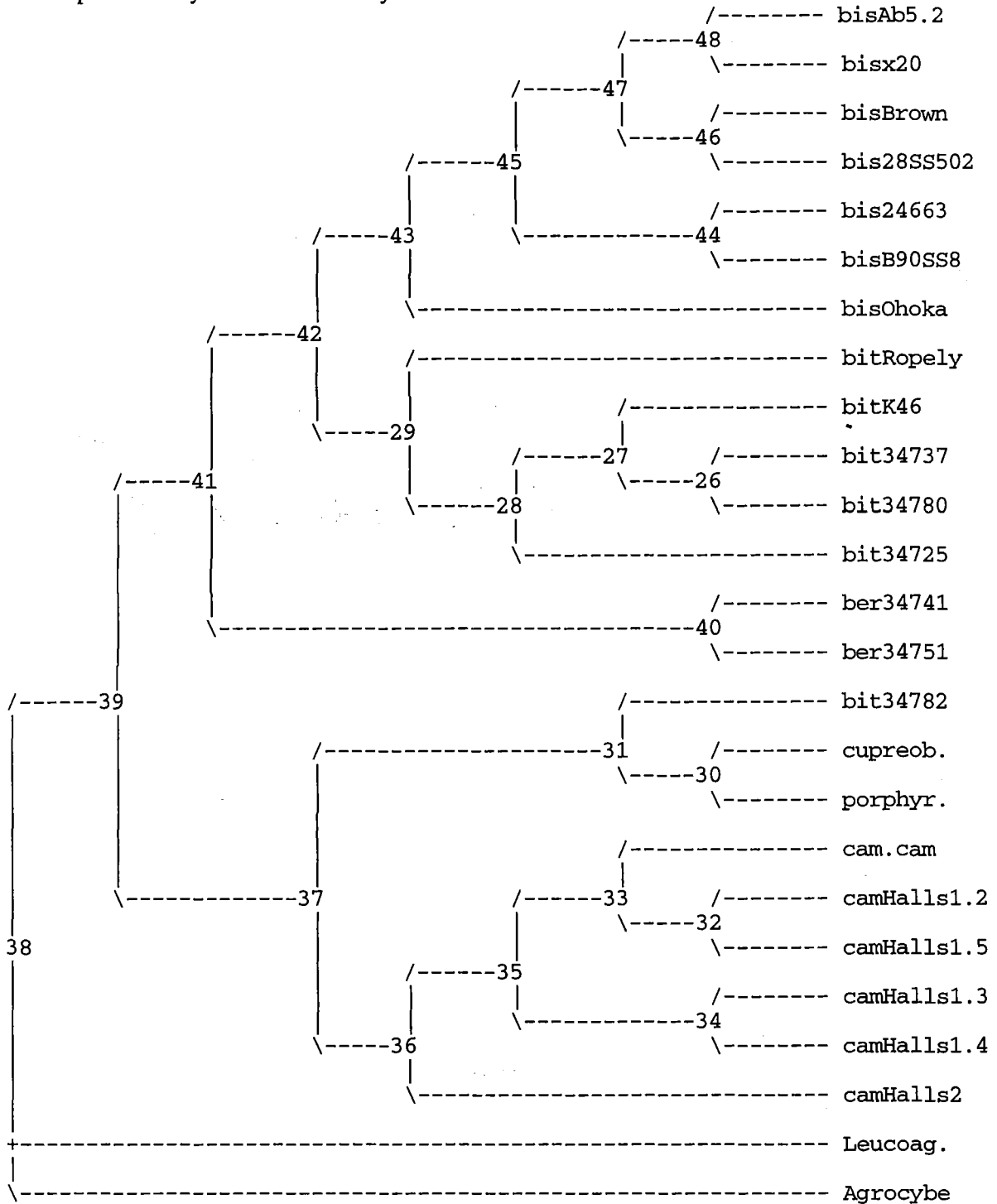


Figure 1. Single most parsimonious tree, CI = 0.379, numbers indicate nodes as a reference for apomorphies. Prefixes represented taxa as follows, bis. *Agaricus bisporus*, bit. *Agaricus bitorquis*, ber. *Agaricus bernardii*, cupreob. *Agaricus cupreobrunneus*, porphyr. *Agaricus porphyrocephalus*, cam. *Agaricus campestris*, Leucoag. *Leucoagaricus leucothites*, and *Agrocybe* represented *Agrocybe parasitica*.

Apomorphy lists:

Branch	Character	(Unweighted)		Change
		Steps	CI	
node_38 --> node_39	5.Mdh-1:5	1	0.333 0 ==> 1	
	10.Mdh-2:2	1	0.200 0 ==> 1	
	15.Mdh-2:7	1	0.250 0 ==> 1	
	20.Mdh-2:12	1	0.250 0 ==> 1	
	27.Acp:6	1	0.111 0 ==> 1	
	30.bGlu:1	1	0.250 0 ==> 1	
	31.bGlu:2	1	0.167 0 ==> 1	
	68.Aat:4	1	0.125 0 ==> 1	
	78.Gk-1:3	1	0.111 0 ==> 1	
	104.Gpt:7	1	0.250 0 ==> 1	
	128.Mpi:6	1	0.143 0 ==> 1	
	129.Mpi:7	1	0.167 0 ==> 1	
node_39 --> node_41	28.Acp:7	1	0.250 0 ==> 1	
	33.bGlu:4	1	0.167 0 ==> 1	
	39.bGlu:10	1	0.167 1 ==> 0	
	41.bGlu:12	1	0.250 0 ==> 1	
	42.bGlu:13	1	0.200 1 ==> 0	
	48.Ha:4	1	0.250 1 ==> 0	
	60.Lap:6	1	0.333 0 ==> 1	
	67.Aat:3	1	0.167 0 ==> 1	
	89.Gk-2:3	1	0.333 0 ==> 1	
	92.Gk-2:6	1	0.500 0 --> 1	
	127.Mpi:5	1	0.200 1 ==> 0	
node_41 --> node_42	5.Mdh-1:5	1	0.333 1 ==> 0	
	29.Acp:8	1	0.143 0 ==> 1	
	30.bGlu:1	1	0.250 1 ==> 0	
	37.bGlu:8	1	0.333 0 ==> 1	
	38.bGlu:9	1	0.125 0 ==> 1	
	47.Ha:3	1	0.200 1 ==> 0	
	66.Aat:2	1	0.125 0 ==> 1	
	84.Gk-1:9	1	0.500 0 ==> 1	
	104.Gpt:7	1	0.250 1 ==> 0	
node_42 --> node_43	4.Mdh-1:4	1	1.000 0 ==> 1	
	10.Mdh-2:2	1	0.200 1 ==> 0	
	16.Mdh-2:8	1	0.200 1 ==> 0	
	31.bGlu:2	1	0.167 1 ==> 0	
	59.Lap:5	1	1.000 0 ==> 1	
	60.Lap:6	1	0.333 1 ==> 0	
	68.Aat:4	1	0.125 1 ==> 0	
	78.Gk-1:3	1	0.111 1 ==> 0	
	86.Gk-1:11	1	0.200 1 ==> 0	
	89.Gk-2:3	1	0.333 1 ==> 0	
	91.Gk-2:5	1	1.000 0 ==> 1	
	112.Pgm:6	1	1.000 0 ==> 1	
	119.Pgm:13	1	0.200 1 ==> 0	
	129.Mpi:7	1	0.167 1 ==> 0	
node_43 --> node_45	99.Gpt:2	1	1.000 0 ==> 1	
	128.Mpi:6	1	0.143 1 ==> 0	
node_45 --> node_47	27.Acp:6	1	0.111 1 ==> 0	
	34.bGlu:5	1	0.500 0 ==> 1	
	37.bGlu:8	1	0.333 1 ==> 0	
	40.bGlu:11	1	0.500 0 ==> 1	
	41.bGlu:12	1	0.250 1 ==> 0	
	66.Aat:2	1	0.125 1 ==> 0	
node_47 --> node_48	29.Acp:8	1	0.143 1 ==> 0	
	33.bGlu:4	1	0.167 1 ==> 0	
	38.bGlu:9	1	0.125 1 ==> 0	

node_47 --> node_46	28.Acp:7	1	0.250	1	==>	0
	120.Pgm:14	1	0.167	1	==>	0
node_46 --> bisBrown	34.bGlu:5	1	0.500	1	==>	0
	40.bGlu:11	1	0.500	1	==>	0
node_46 --> bis28SS502	33.bGlu:4	1	0.167	1	==>	0
	38.bGlu:9	1	0.125	1	==>	0
node_45 --> node_44	29.Acp:8	1	0.143	1	==>	0
	33.bGlu:4	1	0.167	1	==>	0
	38.bGlu:9	1	0.125	1	==>	0
	120.Pgm:14	1	0.167	1	==>	0
node_44 --> bis24663	28.Acp:7	1	0.250	1	==>	0
	67.Aat:3	1	0.167	1	==>	0
node_44 --> bisB90SS8	27.Acp:6	1	0.111	1	==>	0
	66.Aat:2	1	0.125	1	==>	0
node_43 --> bisOhoka	27.Acp:6	1	0.111	1	==>	0
	29.Acp:8	1	0.143	1	==>	0
	33.bGlu:4	1	0.167	1	==>	0
	38.bGlu:9	1	0.125	1	==>	0
	66.Aat:2	1	0.125	1	==>	0
	98.Gpt:1	1	1.000	0	==>	1
node_42 --> node_29	15.Mdh-2:7	1	0.250	1	==>	0
	20.Mdh-2:12	1	0.250	1	==>	0
	27.Acp:6	1	0.111	1	==>	0
	33.bGlu:4	1	0.167	1	==>	0
	92.Gk-2:6	1	0.500	1	==>	0
	120.Pgm:14	1	0.167	1	==>	0
	125.Mpi:3	1	0.333	0	==>	1
	128.Mpi:6	1	0.143	1	==>	0
node_29 --> bitRopely	3.Mdh-1:3	1	1.000	0	==>	1
	25.Acp:4	1	1.000	0	==>	1
	29.Acp:8	1	0.143	1	==>	0
	38.bGlu:9	1	0.125	1	==>	0
	57.Lap:3	1	1.000	0	==>	1
	66.Aat:2	1	0.125	1	==>	0
	67.Aat:3	1	0.167	1	==>	0
	78.Gk-1:3	1	0.111	1	==>	0
	108.Pgm:2	1	1.000	0	==>	1
	110.Pgm:4	1	1.000	0	==>	1
	116.Pgm:10	1	1.000	0	==>	1
	129.Mpi:7	1	0.167	1	==>	0
node_29 --> node_28	10.Mdh-2:2	1	0.200	1	==>	0
	14.Mdh-2:6	1	1.000	0	==>	1
	16.Mdh-2:8	1	0.200	1	==>	0
	19.Mdh-2:11	1	1.000	0	==>	1
	31.bGlu:2	1	0.167	1	==>	0
	84.Gk-1:9	1	0.500	1	==>	0
	89.Gk-2:3	1	0.333	1	==>	0
	94.Gk-2:8	1	1.000	0	==>	1
	100.Gpt:3	1	1.000	0	==>	1
node_28 --> node_27	60.Lap:6	1	0.333	1	==>	0
	63.Lap:9	1	0.500	0	==>	1
	78.Gk-1:3	1	0.111	1	==>	0
	85.Gk-1:10	1	1.000	0	==>	1
	86.Gk-1:11	1	0.200	1	==>	0
	117.Pgm:11	1	1.000	0	==>	1
node_27 --> bitK46	28.Acp:7	1	0.250	1	==>	0
	38.bGlu:9	1	0.125	1	==>	0
	41.bGlu:12	1	0.250	1	==>	0
	66.Aat:2	1	0.125	1	==>	0
	68.Aat:4	1	0.125	1	==>	0
	129.Mpi:7	1	0.167	1	==>	0
node_27 --> node_26	29.Acp:8	1	0.143	1	==>	0
	37.bGlu:8	1	0.333	1	==>	0

	67.Aat:3	1	0.167	1	==>	0
	119.Pgm:13	1	0.200	1	==>	0
	125.Mpi:3	1	0.333	1	==>	0
node_26 --> bit34737	66.Aat:2	1	0.125	1	==>	0
node_26 --> bit34780	63.Lap:9	1	0.500	1	==>	0
	64.Lap:10	1	1.000	0	==>	1
	68.Aat:4	1	0.125	1	==>	0
node_28 --> bit34725	29.Acp:8	1	0.143	1	==>	0
	38.bGlu:9	1	0.125	1	==>	0
	41.bGlu:12	1	0.250	1	==>	0
	65.Aat:1	1	1.000	0	==>	1
	66.Aat:2	1	0.125	1	==>	0
	67.Aat:3	1	0.167	1	==>	0
	68.Aat:4	1	0.125	1	==>	0
	95.Gk-2:9	1	1.000	0	==>	1
	125.Mpi:3	1	0.333	1	==>	0
node_41 --> node_40	27.Acp:6	1	0.111	1	==>	0
	31.bGlu:2	1	0.167	1	==>	0
	44.bGlu:15	1	1.000	0	==>	1
	52.Ha:8	1	1.000	0	==>	1
	86.Gk-1:11	1	0.200	1	==>	0
	111.Pgm:5	1	1.000	0	==>	1
	119.Pgm:13	1	0.200	1	==>	0
	120.Pgm:14	1	0.167	1	==>	0
	128.Mpi:6	1	0.143	1	==>	0
node_40 --> ber34741	15.Mdh-2:7	1	0.250	1	==>	0
	20.Mdh-2:12	1	0.250	1	==>	0
node_40 --> ber34751	10.Mdh-2:2	1	0.200	1	==>	0
	16.Mdh-2:8	1	0.200	1	==>	0
	67.Aat:3	1	0.167	1	==>	0
node_39 --> node_37	26.Acp:5	1	0.250	0	==>	1
	54.Ha:10	1	0.333	0	==>	1
	55.Lap:1	1	0.500	0	==>	1
	77.Gk-1:2	1	0.167	0	==>	1
	82.Gk-1:7	1	0.200	0	==>	1
	86.Gk-1:11	1	0.200	1	==>	0
	88.Gk-2:2	1	0.250	0	==>	1
	90.Gk-2:4	1	0.200	0	==>	1
	103.Gpt:6	1	0.500	0	==>	1
	119.Pgm:13	1	0.200	1	==>	0
	130.Mpi:8	1	0.200	0	==>	1
node_37 --> node_31	10.Mdh-2:2	1	0.200	1	==>	0
	16.Mdh-2:8	1	0.200	1	==>	0
	31.bGlu:2	1	0.167	1	==>	0
	78.Gk-1:3	1	0.111	1	==>	0
	80.Gk-1:5	1	0.500	0	==>	1
	104.Gpt:7	1	0.250	1	==>	0
node_31 --> bit34782	26.Acp:5	1	0.250	1	==>	0
	35.bGlu:6	1	1.000	0	==>	1
	39.bGlu:10	1	0.167	1	==>	0
	42.bGlu:13	1	0.200	1	==>	0
	47.Ha:3	1	0.200	1	==>	0
	77.Gk-1:2	1	0.167	1	==>	0
	82.Gk-1:7	1	0.200	1	==>	0
	127.Mpi:5	1	0.200	1	==>	0
	128.Mpi:6	1	0.143	1	==>	0
	130.Mpi:8	1	0.200	1	==>	0
node_31 --> node_30	30.bGlu:1	1	0.250	1	==>	0
	48.Ha:4	1	0.250	1	==>	0
	50.Ha:6	1	1.000	0	==>	1
	54.Ha:10	1	0.333	1	==>	0
	68.Aat:4	1	0.125	1	==>	0
	72.Aat:8	1	1.000	0	==>	1

	118.Pgm:12	1	1.000	0	==>	1
	120.Pgm:14	1	0.167	1	==>	0
node_30 --> cupreob.	26.Acp:5	1	0.250	1	==>	0
	39.bGlu:10	1	0.167	1	==>	0
	58.Lap:4	1	1.000	0	==>	1
	80.Gk-1:5	1	0.500	1	==>	0
	90.Gk-2:4	1	0.200	1	==>	0
	128.Mpi:6	1	0.143	1	==>	0
	129.Mpi:7	1	0.167	1	==>	0
node_30 --> porphy.	27.Acp:6	1	0.111	1	==>	0
	42.bGlu:13	1	0.200	1	==>	0
	55.Lap:1	1	0.500	1	==>	0
	56.Lap:2	1	1.000	0	==>	1
	77.Gk-1:2	1	0.167	1	==>	0
	82.Gk-1:7	1	0.200	1	==>	0
	88.Gk-2:2	1	0.250	1	==>	0
	127.Mpi:5	1	0.200	1	==>	0
	130.Mpi:8	1	0.200	1	==>	0
node_37 --> node_36	5.Mdh-1:5	1	0.333	1	==>	0
	6.Mdh-1:6	1	1.000	0	==>	1
	15.Mdh-2:7	1	0.250	1	==>	0
	20.Mdh-2:12	1	0.250	1	==>	0
	30.bGlu:1	1	0.250	1	==>	0
	36.bGlu:7	1	0.333	0	==>	1
	42.bGlu:13	1	0.200	1	==>	0
	74.Aat:10	1	0.500	0	==>	1
	127.Mpi:5	1	0.200	1	==>	0
	128.Mpi:6	1	0.143	1	==>	0
node_36 --> node_35	31.bGlu:2	1	0.167	1	==>	0
	48.Ha:4	1	0.250	1	==>	0
	54.Ha:10	1	0.333	1	==>	0
	104.Gpt:7	1	0.250	1	==>	0
node_35 --> node_33	27.Acp:6	1	0.111	1	==>	0
	47.Ha:3	1	0.200	1	==>	0
	51.Ha:7	1	0.500	0	==>	1
	79.Gk-1:4	1	0.500	0	==>	1
	82.Gk-1:7	1	0.200	1	==>	0
node_33 --> cam.cam	36.bGlu:7	1	0.333	1	==>	0
	46.Ha:2	1	1.000	0	==>	1
	78.Gk-1:3	1	0.111	1	==>	0
	129.Mpi:7	1	0.167	1	==>	0
node_33 --> node_32	39.bGlu:10	1	0.167	1	==>	0
	45.Ha:1	1	1.000	0	==>	1
	68.Aat:4	1	0.125	1	==>	0
	77.Gk-1:2	1	0.167	1	==>	0
	88.Gk-2:2	1	0.250	1	==>	0
	90.Gk-2:4	1	0.200	1	==>	0
node_32 --> camHalls1.2	49.Ha:5	1	1.000	0	==>	1
	51.Ha:7	1	0.500	1	==>	0
	78.Gk-1:3	1	0.111	1	==>	0
node_32 --> camHalls1.5	79.Gk-1:4	1	0.500	1	==>	0
	130.Mpi:8	1	0.200	1	-->	0
node_35 --> node_34	26.Acp:5	1	0.250	1	==>	0
	77.Gk-1:2	1	0.167	1	==>	0
node_34 --> camHalls1.3	53.Ha:9	1	1.000	0	-->	1
	68.Aat:4	1	0.125	1	-->	0
	74.Aat:10	1	0.500	1	-->	0
	78.Gk-1:3	1	0.111	1	==>	0
	88.Gk-2:2	1	0.250	1	==>	0
	90.Gk-2:4	1	0.200	1	==>	0
node_34 --> camHalls1.4	36.bGlu:7	1	0.333	1	-->	0
	82.Gk-1:7	1	0.200	1	==>	0
node_36 --> camHalls2	27.Acp:6	1	0.111	1	==>	0

	39.bGlu:10	1	0.167	1	==>	0
	47.Ha:3	1	0.200	1	==>	0
	76.Gk-1:1	1	1.000	0	==>	1
	77.Gk-1:2	1	0.167	1	==>	0
	78.Gk-1:3	1	0.111	1	==>	0
	90.Gk-2:4	1	0.200	1	==>	0
	103.Gpt:6	1	0.500	1	==>	0
	130.Mpi:8	1	0.200	1	==>	0
node_38 --> Leucoag.	12.Mdh-2:4	1	1.000	0	==>	1
	18.Mdh-2:10	1	1.000	0	==>	1
	42.bGlu:13	1	0.200	1	==>	0
	43.bGlu:14	1	1.000	0	==>	1
	47.Ha:3	1	0.200	1	==>	0
	62.Lap:8	1	1.000	0	==>	1
	86.Gk-1:11	1	0.200	1	==>	0
	105.Gpt:8	1	1.000	0	==>	1
	120.Pgm:14	1	0.167	1	==>	0
	121.Pgm:15	1	1.000	0	==>	1
node_38 --> Agrocybe	16.Mdh-2:8	1	0.200	1	==>	0
	17.Mdh-2:9	1	1.000	0	==>	1
	21.Mdh-2:13	1	1.000	0	==>	1
	39.bGlu:10	1	0.167	1	==>	0
	48.Ha:4	1	0.250	1	==>	0
	83.Gk-1:8	1	1.000	0	==>	1
	102.Gpt:5	1	1.000	0	==>	1
	119.Pgm:13	1	0.200	1	==>	0
	122.Pgm:16	1	1.000	0	==>	1
	127.Mpi:5	1	0.200	1	==>	0
	133.Mpi:11	1	1.000	0	==>	1

Appendix 7.

Apomorphy lists for the tree presented in Chapter 4, Figure 4.9, generated from cladistic analysis of pooled strains forming robust groups.

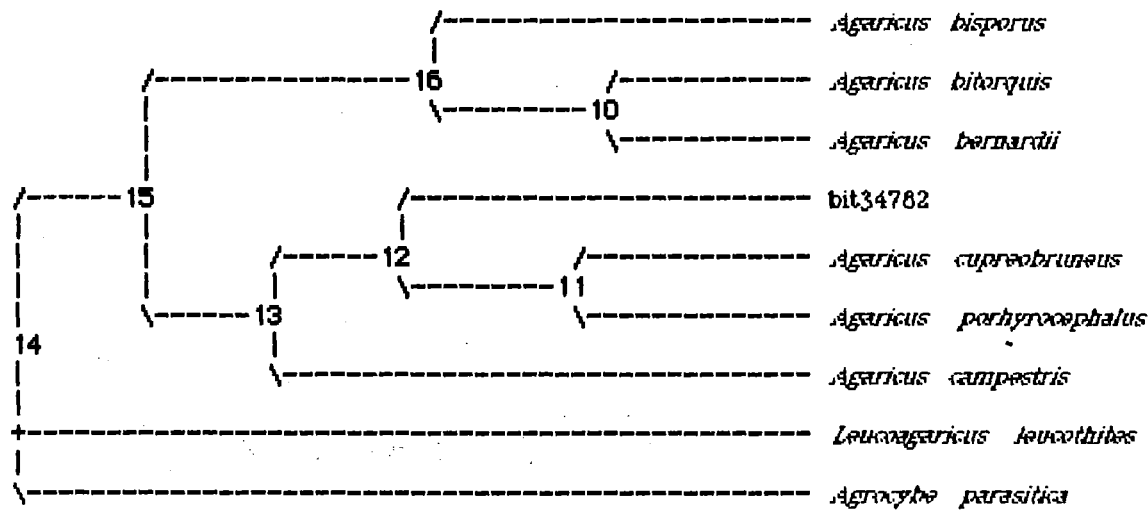


Figure 27. Single most parsimonious tree, CI = 0.645. *Agaricus bitorquis* 34782 = bit34782, numers indicate nodes as a reference for apomorphies.

Apomorphy lists:

Branch	Character	(Unweighted)		
		Steps	CI	Change
node_14 --> node_15	10.Mdh-2:2	1	0.333	0 ==> 1
	15.Mdh-2:7	1	0.333	0 ==> 1
	20.Mdh-2:12	1	0.333	0 ==> 1
	27.Acp:6	1	0.333	0 ==> 1
	30.bGlu:1	1	0.200	0 ==> 1
	31.bGlu:2	1	0.250	0 ==> 1
	68.Aat:4	1	0.333	0 ==> 1
	104.Gpt:7	1	0.250	0 ==> 1
	128.Mpi:6	1	0.200	0 ==> 1
	129.Mpi:7	1	0.333	0 ==> 1
node_15 --> node_16	28.Acp:7	1	1.000	0 ==> 1
	29.Acp:8	1	0.500	0 ==> 1
	33.bGlu:4	1	0.500	0 ==> 1
	37.bGlu:8	1	0.500	0 ==> 1
	38.bGlu:9	1	0.500	0 ==> 1
	39.bGlu:10	1	0.250	1 ==> 0
	41.bGlu:12	1	1.000	0 ==> 1
	42.bGlu:13	1	0.200	1 ==> 0
	48.Ha:4	1	0.333	1 ==> 0
	66.Aat:2	1	0.500	0 ==> 1
	67.Aat:3	1	1.000	0 ==> 1
	84.Gk-1:9	1	0.500	0 ==> 1

	92.Gk-2:6	1	0.500	0	-->	1
	27.Mpi:5	1	0.200	1	==>	0
node_16 --> A.bisporus	4.Mdh-1:4	1	1.000	0	==>	1
	10.Mdh-2:2	1	0.333	1	==>	0
	16.Mdh-2:8	1	0.333	1	==>	0
	30.bGlu:1	1	0.200	1	==>	0
	31.bGlu:2	1	0.250	1	==>	0
	34.bGlu:5	1	1.000	0	==>	1
	40.bGlu:11	1	1.000	0	==>	1
	47.Ha:3	1	0.250	1	==>	0
	59.Lap:5	1	1.000	0	==>	1
	68.Aat:4	1	0.333	1	==>	0
	86.Gk-1:11	1	0.250	1	==>	0
	91.Gk-2:5	1	1.000	0	==>	1
	98.Gpt:1	1	1.000	0	==>	1
	99.Gpt:2	1	1.000	0	==>	1
	104.Gpt:7	1	0.250	1	==>	0
	112.Pgm:6	1	1.000	0	==>	1
	119.Pgm:13	1	0.250	1	==>	0
	29.Mpi:7	1	0.333	1	==>	0
node_16 --> node_10	20.Mdh-2:12	1	0.333	1	==>	0
	27.Acp:6	1	0.333	1	==>	0
	60.Lap:6	1	1.000	0	==>	1
	78.Gk-1:3	1	1.000	0	==>	1
	89.Gk-2:3	1	1.000	0	==>	1
	120.Pgm:14	1	0.333	1	==>	0
	128.Mpi:6	1	0.200	1	==>	0
node_10 --> A.bitorquis	3.Mdh-1:3	1	1.000	0	==>	1
	14.Mdh-2:6	1	1.000	0	==>	1
	15.Mdh-2:7	1	0.333	1	==>	0
	19.Mdh-2:11	1	1.000	0	==>	1
	25.Acp:4	1	1.000	0	==>	1
	30.bGlu:1	1	0.200	1	==>	0
	33.bGlu:4	1	0.500	1	==>	0
	47.Ha:3	1	0.250	1	==>	0
	57.Lap:3	1	1.000	0	==>	1
	63.Lap:9	1	1.000	0	==>	1
	64.Lap:10	1	1.000	0	==>	1
	65.Aat:1	1	1.000	0	==>	1
	85.Gk-1:10	1	1.000	0	==>	1
	92.Gk-2:6	1	0.500	1	==>	0
	94.Gk-2:8	1	1.000	0	==>	1
	95.Gk-2:9	1	1.000	0	==>	1
	100.Gpt:3	1	1.000	0	==>	1
	104.Gpt:7	1	0.250	1	==>	0
	108.Pgm:2	1	1.000	0	==>	1
	110.Pgm:4	1	1.000	0	==>	1
	116.Pgm:10	1	1.000	0	==>	1
	117.Pgm:11	1	1.000	0	==>	1
	125.Mpi:3	1	1.000	0	==>	1
node_10 --> A.bernardii	29.Acp:8	1	0.500	1	==>	0
	31.bGlu:2	1	0.250	1	==>	0
	37.bGlu:8	1	0.500	1	==>	0
	38.bGlu:9	1	0.500	1	==>	0
	44.bGlu:15	1	1.000	0	==>	1
	52.Ha:8	1	1.000	0	==>	1
	66.Aat:2	1	0.500	1	==>	0
	84.Gk-1:9	1	0.500	1	==>	0
	86.Gk-1:11	1	0.250	1	==>	0
	111.Pgm:5	1	1.000	0	==>	1
	119.Pgm:13	1	0.250	1	==>	0
node_15 --> node_13	26.Acp:5	1	0.333	0	==>	1
	54.Ha:10	1	0.500	0	==>	1

	55.Lap:1	1	0.500	0	=> 1
	77.Gk-1:2	1	0.333	0	=> 1
	82.Gk-1:7	1	0.333	0	=> 1
	86.Gk-1:11	1	0.250	1	=> 0
	88.Gk-2:2	1	0.500	0	=> 1
	90.Gk-2:4	1	0.500	0	=> 1
	103.Gpt:6	1	1.000	0	=> 1
	119.Pgm:13	1	0.250	1	=> 0
	130.Mpi:8	1	0.333	0	=> 1
node_13 --> node_12	5.Mdh-1:5	1	1.000	0	=> 1
	10.Mdh-2:2	1	0.333	1	=> 0
	16.Mdh-2:8	1	0.333	1	=> 0
	31.bGlu:2	1	0.250	1	=> 0
	80.Gk-1:5	1	0.500	0	=> 1
	104.Gpt:7	1	0.250	1	=> 0
node_12 --> 34782BIT	26.Acp:5	1	0.333	1	=> 0
	35.bGlu:6	1	1.000	0	=> 1
	39.bGlu:10	1	0.250	1	=> 0
	42.bGlu:13	1	0.200	1	=> 0
	47.Ha:3	1	0.250	1	=> 0
	77.Gk-1:2	1	0.333	1	=> 0
	82.Gk-1:7	1	0.333	1	=> 0
	127.Mpi:5	1	0.200	1	=> 0
	128.Mpi:6	1	0.200	1	=> 0
	130.Mpi:8	1	0.333	1	=> 0
node_12 --> node_11	30.bGlu:1	1	0.200	1	=> 0
	48.Ha:4	1	0.333	1	=> 0
	50.Ha:6	1	1.000	0	=> 1
	54.Ha:10	1	0.500	1	=> 0
	68.Aat:4	1	0.333	1	=> 0
	72.Aat:8	1	1.000	0	=> 1
	118.Pgm:12	1	1.000	0	=> 1
	120.Pgm:14	1	0.333	1	=> 0
node_11 --> A.cupreobruneus	26.Acp:5	1	0.333	1	=> 0
	39.bGlu:10	1	0.250	1	=> 0
	58.Lap:4	1	1.000	0	=> 1
	80.Gk-1:5	1	0.500	1	=> 0
	90.Gk-2:4	1	0.500	1	=> 0
	128.Mpi:6	1	0.200	1	=> 0
	129.Mpi:7	1	0.333	1	=> 0
node_11 --> A.porhyrocephalus	27.Acp:6	1	0.333	1	=> 0
	42.bGlu:13	1	0.200	1	=> 0
	55.Lap:1	1	0.500	1	=> 0
	56.Lap:2	1	1.000	0	=> 1
	77.Gk-1:2	1	0.333	1	=> 0
	82.Gk-1:7	1	0.333	1	=> 0
	88.Gk-2:2	1	0.500	1	=> 0
	127.Mpi:5	1	0.200	1	=> 0
	130.Mpi:8	1	0.333	1	=> 0
node_13 --> A.campestris	6.Mdh-1:6	1	1.000	0	=> 1
	15.Mdh-2:7	1	0.333	1	=> 0
	20.Mdh-2:12	1	0.333	1	=> 0
	30.bGlu:1	1	0.200	1	=> 0
	36.bGlu:7	1	1.000	0	=> 1
	42.bGlu:13	1	0.200	1	=> 0
	45.Ha:1	1	1.000	0	=> 1
	46.Ha:2	1	1.000	0	=> 1
	49.Ha:5	1	1.000	0	=> 1
	51.Ha:7	1	1.000	0	=> 1
	53.Ha:9	1	1.000	0	=> 1
	74.Aat:10	1	1.000	0	=> 1
	76.Gk-1:1	1	1.000	0	=> 1
	79.Gk-1:4	1	1.000	0	=> 1

	127.Mpi:5	1	0.200	1	==>	0
	128.Mpi:6	1	0.200	1	==>	0
node_14 --> Leucoagaricus	2.Mdh-2:4	1	1.000	0	==>	1
	18.Mdh-2:10	1	1.000	0	==>	1
	42.bGlu:13	1	0.200	1	==>	0
	43.bGlu:14	1	1.000	0	==>	1
	47.Ha:3	1	0.250	1	==>	0
	62.Lap:8	1	1.000	0	==>	1
	86.Gk-1:11	1	0.250	1	==>	0
	105.Gpt:8	1	1.000	0	==>	1
	120.Pgm:14	1	0.333	1	==>	0
	121.Pgm:15	1	1.000	0	==>	1
node_14 --> Agrocybe	16.Mdh-2:8	1	0.333	1	==>	0
	17.Mdh-2:9	1	1.000	0	==>	1
	21.Mdh-2:13	1	1.000	0	==>	1
	39.bGlu:10	1	0.250	1	==>	0
	48.Ha:4	1	0.333	1	==>	0
	83.Gk-1:8	1	1.000	0	==>	1
	102.Gpt:5	1	1.000	0	==>	1
	119.Pgm:13	1	0.250	1	==>	0
	122.Pgm:16	1	1.000	0	==>	1
	127.Mpi:5	1	0.200	1	==>	0
	133.Mpi:11	1	1.000	0	==>	1

Appendix 8.

Each of the following species were introduced, one at a time, into the pooled strain data set from Chapter 4, Section 4.3.2.3.1. Cladistics were used to explore potential relationships of these species based the allozyme data set. Jackknifing loci affected the structure of the most parsimonious trees found.

A position for *Agaricus subperonatus*.

A single most parsimonious tree, Figure 1, generated when all loci were analysed simultaneously was also found when Mdh-1, Acp, Ha, Lap, Gk-1, Gk-2, or Pgm was removed during Jackknifing.

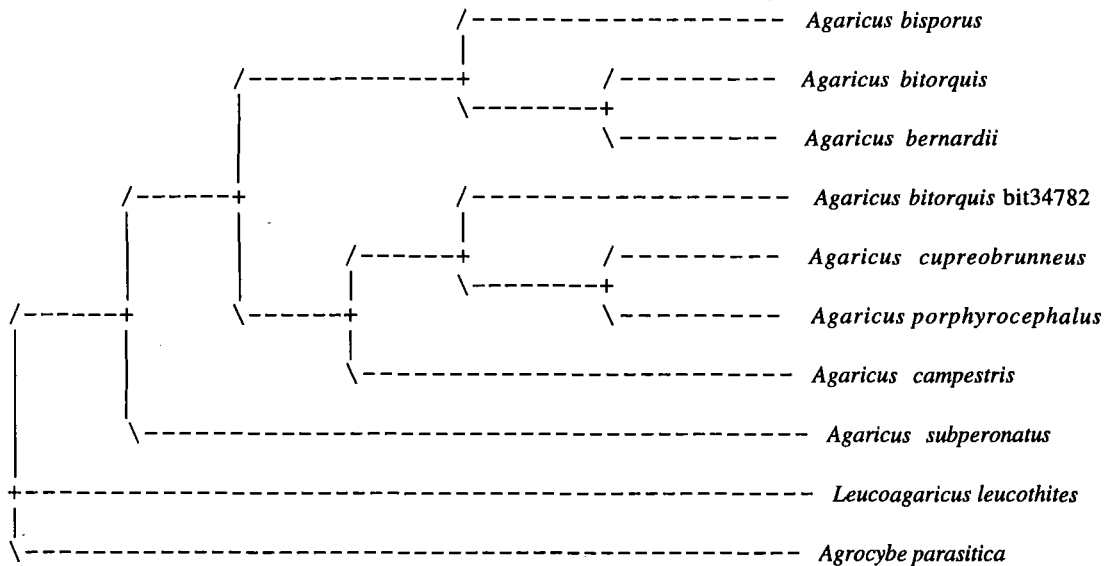


Figure 1. Placment for *Agaricus subperonatus*. Single most parsimonious tree found when all 12 loci were included in the analysis, or when Mdh-1, Acp, Ha, Lap, Gk-1, Gk-2, or Pgm was removed during Jackknifing.

If Mdh-2, bGlu, Aat, Gpt, or Mpi was excluded from the data set a single most parsimonious tree was found with the structure shown in Figure 2.

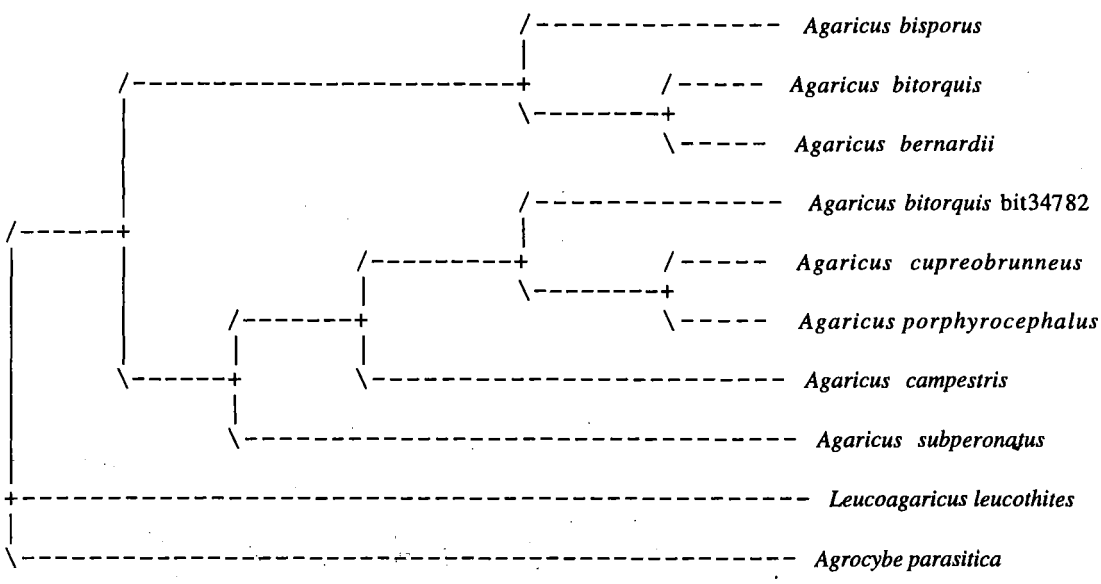


Figure 2. Placment for *Agaricus subperonatus* Single most parsimonious tree generated when Mdh-2, bGlu, Aat, Gpt, or Mpi was removed during Jackknifing.

A position for *Agaricus lanipe*.

When all loci were analysed simultaneously an unresolved tricotomy occurred between 1) *Agaricus lanipe*, 2) species of the group including *Agaricus campestris*, and 3) species of the group including *Agaricus bisporus*. This same tricotomy was found when Mdh-1, Mdh-2, Acp, Ha, Aat, Gpt, Pgm, or Mpi was removed during Jackknifing. When β Glu or Gk-2 was removed during Jackknifing *Agaricus lanipe* was included as an ancestral taxon monophyletic with the group including *Agaricus campestris*. If Lap or Gk-1 were removed during Jackknifing *Agaricus lanipe* was included as an ancestral taxon monophyletic with the group including *Agaricus bisporus*.

A position for *Agaricus arvensis*.

When all loci were included in the analysis simultaneously or when Acp, bGlu, Ha, Aat, or Mpi was removed during Jackknifing an asymetrical single most parsimonious tree was found, Figure 3.



Figure 3. Placment for *Agaricus arvensis*. Asymetrical single most parsimonious tree generated when all 12 loci were included in the analysis or when Acp, bGlu, Ha, Aat, or Mpi was removed during Jackknifing.

When Mdh-1, Mdh-2, Lap, or Gpt was removed during Jackknifing a single most parsimonious tree which implied monophyly between *Agaricus arvensis* and the Bitorques section was found, Figure 4.

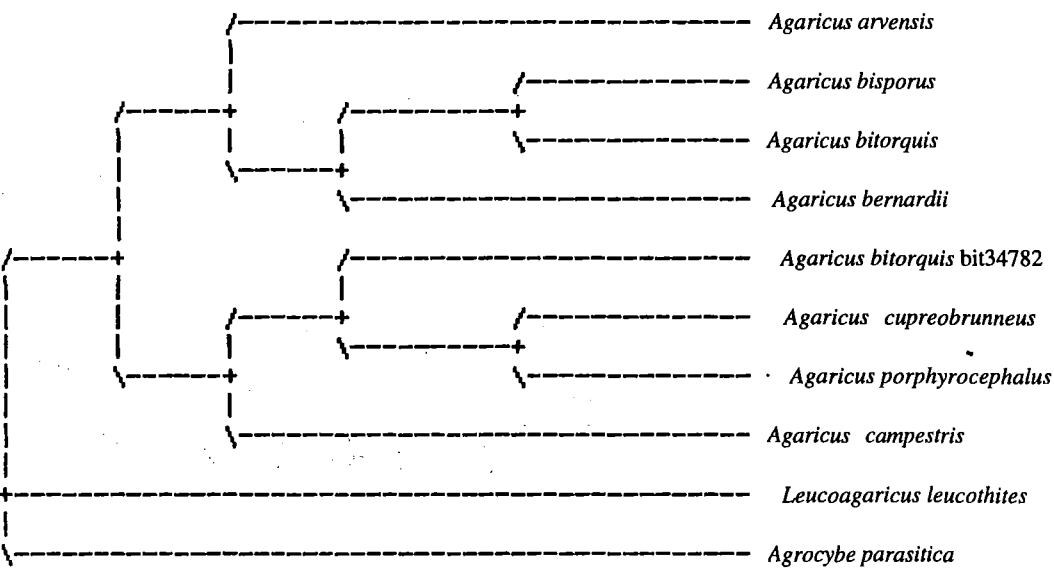


Figure 4. Placment for *Agaricus arvensis*. Single most parsimonious tree found when Mdh-1, Mdh-2, Lap, or Gpt was removed during Jackknifing.

A position for *Agaricus semotus*.

A single most parsimonious tree, Figure 5, generated when all loci were analysed simultaneously was also found when Mdh-1, Ha, Gk-1, Gk-2, Gpt, or Pgm was removed during Jackknifing.

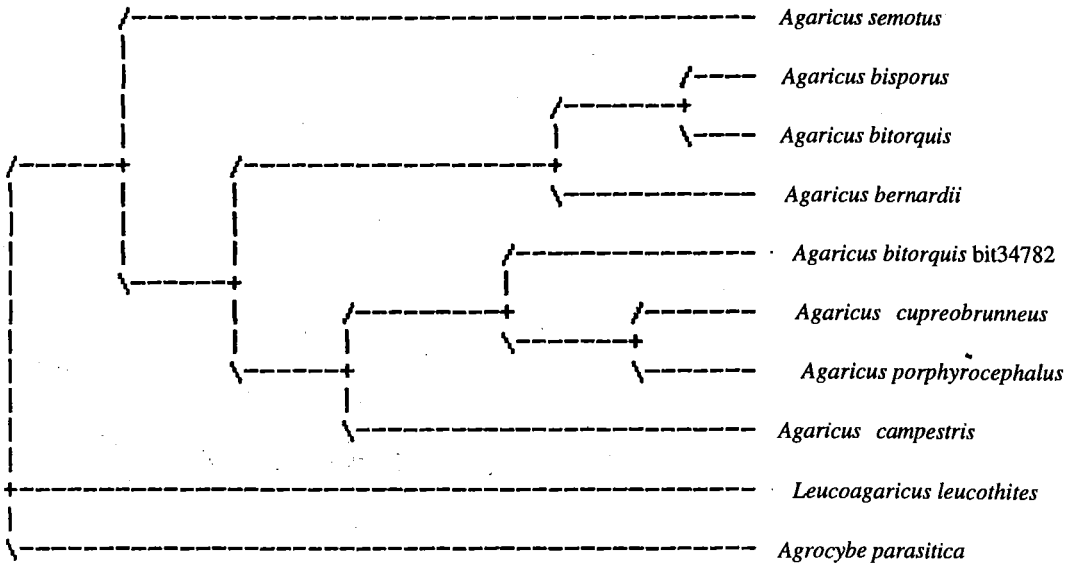


Figure 5. Placement for *Agaricus semotus*. Single most parsimonious tree found when Mdh-1, Ha, Gk-1, Gk-2, Gpt, or Pgm was removed during Jackknifing.

Removal of Mdh-2, Lap, or Mpi during Jackknifing resulted in closer association of *Agaricus semotus* with *Agaricus campestris*, and removal of Acp, bGlu or Aat drastically affected tree structure, causing *Leucoagaricus leucothites* to join the ingroup.

Appendix 9.

Descriptions of field collected specimens used in this study. Abbreviations used in this thesis for strains are given in brackets at the end of each description. The classification used is by Cappelli (1984). Basidiospore sizes were generated by image analysis, (Chapter 5). The range of basidiospore length and breadth is given for each species, with the mean values underlined. All specimens were collected by the author during the autumn of 1990, except for *Agaricus semotus* and *Agaricus porphyrocephalus*, which were collected by the author 22.5.92. Prof. P. Heinemann, c/o the Director of Jardin Botanique National de Belgique, Chateau de Bouchout, B.1860 Meise. identified to species the following; *Agaricus bisporus* (Ohoka), *Agaricus bitorquis* (Ropley), *Agaricus campestris* (Halls1), *Agaricus cupreobrunneus*., and *Agaricus lanipe*. Specimens not identified by Prof. P. Heinemann, were identified by the author.

Family Agaricaceae, Tribe Agariceae. Genus *Agaricus*.
Flavescentes Moller et J. Schaeffer, Section *Arvensis* Konrad et Maublanc Sin.: *Flavescentes* Moller et J. Schaeffer p.p sensu Moser, Group *Arvensis*.

Agaricus arvensis Schff. : Fries. Pileus 4-12 cm diam., thick, campanulate when young, later convex-applanate, surface silky smooth, colour when young white, brusing yellowish. Margin thin, inrolled when young, later straight, with remnants of veil. Lamellae insertion free, thin, crowded, of several lengths, colour when young white to yellowish, edge pale and sterile, trama arrangement parallel to irregular. Basidia 4 spored, 15-30 x 8-10 μm , clavate, sterigmata 3-5 μm . Cheilocystidia 15-40 x 5-10 μm , broadly clavate, hyaline, abundant. Basidiospore print brown, size 6.6-7.1-7.7 x 5.3-6.7-6.1 μm , elongation 1.1-1.3-1.4, elipsoid, colour brown, refractive centre (1-2 droplets), apiculus lateral, germ pore not visible. Stipe 5-15 x 1.5-3 cm, central, solid to fistulose, equal, cylindrical, base round, surface smooth, colour above annulus white and below annulus amber yellow, brusing intensifies yellowing. Annulus decending, may be cog wheel, colour above white with

yellowing along the edge, surface silky smooth, colour below amber yellow to white, surface powdery. Flesh white, little effect upon cutting, yellowing upon brusing, smell of anise, and taste sweet. Schaeffer's cross reaction positive. Habitat; generally in lawn, but also in bare soil under fruit trees, e.g., Nectarine trees, usually gregarious. (Roll, Nect., Rb)

Section Minores Fries, Group Semotus.

Agaricus semotus Fr. Pileus 1.5-4.0 cm diam., thin, convex when young, later applanate, center may have slight umbo, surface dark brown scales on a white surface, scales dense at the center and decreasing in density toward the edge, brusing brown. Margin inrolled when young, later straight, smooth edge, with remnants of veil. Lamellae insertion free, thin, crowded, of several lengths to alternate, colour fawny pink when young, later brown/pink, pale sterile edge, trama arrangement parallel to irregular. Basidia 4 spored, 12-20 x 5-7 μm , clavate, sterigmata 3-4 μm . Cheilocystidia 15-29 x 6-8 μm , clavate, abundant, hyaline. Basidiospore print dark brown, size 5.7-6.4-6.9 x 4.0-4.5-4.9 μm , elongation 1.3-1.5-1.7, elliptical, pale brown, refractive centre (2-3 droplets), apiculus lateral, germ pore not visible. Stipe 2-4 x 0.4-1.0 cm, central, fistulose, cylindrical, club shaped with swollen sometimes twisted base, surface smooth to silky, white above annulus and yellow below annulus, especially yellowing towards base, brusing yellow, base with mycelial cords. Annulus pendant, thin, may be ephemeral, white above, yellow below, silky surface. Flesh white, becoming yellow at the base upon cutting or brusing, smell of anise, and taste sweet. Schaeffer's cross reaction positive. Habitat; in soil under *Leptospermum ericoides*, Kanuka. (semotus)

Rubescentes Moller, Section Bitorques (Kuhn. et Rom. ex Hein.)
Bon et Cappelli 1983 Sin.: Edules (Moller) sensu Moser-
Duploannulatae Wasser. Group Bitorquis.

Agaricus bitorquis (Quel.) Sacc. Pileus 3-6 cm diam., thick, fleshy, convex when young, later broadly convex-applanate, surface silky to fibrillose, colour when young white, brusing

brownish, soil particles often adhering to surface. Margin thin, inrolled edge, with remnants of veil when young. Lamellae insertion free, thin, crowded, colour when young pink, later dark brown, edge pale and sterile, trama arrangement parallel to irregular. Basidia 2-4 spored, 22-38 x 5-11 μm , , sterigmata 4-8 (10) μm . Cheilocystidia 12-37 x 6-11 μm , abundant, broad clavate, colour hyaline to fawn. Basidiospore print dark brown, size 6.9-7.4-7.9 x 5.5-6.0-6.4 μm , elongation 1.1-1.3-1.4, elliptical, colour pale brown to brown, refractive centre (1-2 droplets), apiculus lateral, germ pore not visible. Stipe 2.5-4.5 x 1.0-3.0 cm, central, solid, equal to widening at center, cylindrical, base narrowing, surface smooth to fibrillose, colour white above annulus and browning below annulus, bruising brown. Annulus double, lower brownish peronate annulus more visible than upper white pendant annulus, surface fibrillose. Flesh slowly reddening with cutting or bruising, smell and taste pleasant. Schaeffer's cross reaction negative. Habitat; hypogaeal in lawn around *Arucaria araucana*, Monkey puzzle stump. (bitRopley)

Agaricus bernardii (Quel.) Sacc. Pileus 6-20 cm diam., thick, fleshy, hemespherical when young, later broadly convex-applanate, may be centrally depressed, surface scaly, scales may become very pronounced during dehydration, colour when young whitish, later brownish, bruising reddish. Margin thin, inrolled and broken edge, with remnants of veil. Lamellae insertion free, thin, crowded, colour when young pale greyish, latter brown to dark brown, edge pale and sterile, trama arrangement parallel to irregular. Basidia 4 spored, 14-25 x 4-7 μm , clavate, sterigmata 4-5 (8) μm . Cheilocystidia 17-30 x 4-8 μm , abundant, broadly clavate, colour hyaline to cylindrical. Basidiospore print dark brown, size 7.0-7.9-8.7 x 5.5-6.1-6.7 μm , elongation 1.1-1.3-1.4, spherical to globose, colour pale brown to brown, refractive centre (single droplets), apiculus lateral, germ pore not visible. Stipe 4-8 x 4-5 cm, central, solid, cylindrical, base round to narrowing, surface smooth, colour above annulus white and below annulus white to brownish, bruising reddish. Annulus peronate, may be double, thin, colour above white, below brown close to stem, surface smooth. Flesh quickly becoming red with cutting or bruising, smell unpleasant, like fish,

taste unpleasant. Schaeffer's cross reaction negative. Habitat; saline, sandy soils of tussock grassland close to coast. (berMot)

Group Bisporus.

Agaricus bisporus var. *bisporus* (J. Lge) Imb. Pileus 5-8 (12) cm diam., thick, convex, fleshy, brown scales on a white surface, bruising redish. Margin thick, inrolled when young, later straight, with remnants of veil. Lamellae insertion free, thin, crowded of several lengths, convex, colour when young pink, later brown, edge pale and sterile, trama arrangement parallel to irregular. Basidia 1-2-3-4 spored, 15-25 x 9-11 μm , broadly clavate, sterigmata 3-5 μm . Cheilocystidia 30-80 x 7-9 (12) μm , clavate, colour hyaline, abundant. Basidiospore print brown, size 6.8-7.7-8.5 x 5.6-6.1-6.6 μm , elongation 1.0-1.3-1.5 broadly elliptical to elliptical, colour fawn to pale brown, refractive centre (2 droplets), apiculus lateral, germ pore not visible. Stipe 33-5 x 1-3 cm, central, solid to fistulose, cylindrical, base round to narrowing, surface smooth, colour above annulus white and below annulus white to brownish, bruising causing slight reddening. Annulus peronate, thick and broad, bifurcated edges, colour above white, below brown close to stem, white towards the edges, surface smooth. Flesh reddening with cutting or bruising, smell and taste pleasant. Schaeffer's cross reaction negative. Habitat among wheat straw and goat droppings in paddock. (bisOhoka)

Section *Agaricus* Linneo: Fries ss. Karsten Sin.: *Campestris* Konrad et Maublanc - *Rubescens* Moller sensu Moser. Group *Campestris*.

Agaricus campestris var. *campestris* L.: Fr. Pileus 6-10 cm diam., medium thickness, fleshy, spherical when young, then convex-applanate, surface silky smooth, white when young, latter pinkish, bruising red. Margin inrolled when young, latter straight, with remnants of veil. Lamellae insertion free, thin, crowded, pale pink when young, latter stronger pink then brown, edge concolourous and fertile, trama arrangement parallel to irregular. Basidia 4 spored, 25-40 x 10-15 μm , broadly clavate, sterigmata 2-

5 μm . Basidiospore print dark brown, size 7.1-8.0-8.9 x 4.8-5.5-6.1 μm , elongation 1.3-1.5-1.7, ovoid to ellipsoid, colour brown, refractive centre (1-2 droplets), apiculus lateral, germ pore present. Stipe 5-8 x 1-1.5 cm, central, solid, cylindrical, narrowing at base, surface smooth, floccose at apex, colour white above and below annulus, bruising reddish. Annulus thin, wide, pendant, brownish above, white below, surface silky. Flesh white, quickly reddening upon cutting or bruising, smell and taste pleasant. Schaeffer's cross reaction negative. Habitat; lawn. (cam.cam)

Agaricus campestris. Pileus 2-5 cm diam., medium thickness, fleshy, spherical when young, then convex, often centrally depressed, surface scaly in centre, streaked towards the margin, white when young, latter yellowish. Basidia 2-4 spored, 17-35 x 6-9 μm , clavate, sterigmata 3-5 (10) μm . Basidiospore size camHalls1; 7.1-8.2-9.3 x 5.1-5.8-6.5 μm , camHalls2; 6.7-7.7-8.6 x 5.2-5.8-6.4 μm , elongation camHalls1; 1.2-1.4-1.6, camHalls2; 1.2-1.3-1.5, elliptical. Stipe 1.5-3.0 x 1.0-1.5 cm, very pointed sometimes twisted base, yellow below annulus, bruising yellow. Annulus thin, pendant, often flocculose. Flesh becoming pale brown upon cutting or bruising. Habitat; cow and sheep paddocks. (camHalls1 and camHalls2).

Agaricus cupreobrunneus (Moll.) Boh. Pileus 3-7 cm diam., medium thickness, convex when young, then broadly convex to applanate, surface with light brown scales, scales dense at the center and decreasing in density toward the edge, bruising brown to reddish. Margin inrolled when young, later inrolled to straight, often with remnants of veil. Lamellae insertion free, thin, crowded, pink when young, later dark brown, edge concolourous and fertile, trama arrangement parallel to irregular. Basidia 4 spored, 15-34 x 10-11 μm , clavate to broadly clavate, sterigmata 3-5 μm . Basidiospore print brown, size 7.2-8.3-9.4 x 4.9-5.6-6.3 μm , elongation 1.3-1.5-1.7, pale brown to brown, refractive centre (1-2 droplets), apiculus lateral, germ pore not visible. Stipe 2-4 x 0.8-1.5 cm, central, solid to fistulose, cylindrical, narrowing toward the based, surface smooth and white above the annulus, floccose and

brownish below the annulus, browning when bruised. Annulus pendant, broad, brown above, white below, surface streaked. Flesh white, slowly reddish upon cutting or bruising, smell and taste pleasant. Schaeffer's cross reaction negative. Habitat; lawn. (cupreo.)

Agaricus porphyrocephalus Moll. Pileus 4.5-6.5 cm diam., thick, fleshy, convex when young, then broadly convex, often with central depression, surface dark purple/brown scales on lilac surface, scales less thinly distributed towards the edge, bruising dark red. Margin inrolled when young, later inrolled to straight, uneven edge, with remnants of veil. Lamellae insertion free, thin, crowded, pink when young, later reddish brown, edge concolourous and fertile, trama arrangement parallel to irregular. Basidia 4 spored, 15-35 x 8-10 μm , broad clavate to clavate, sterigmata 2-5 μm . Basidiospore print brown, size 7.7-8.5-9.3 x 4.9-5.7-6.4 μm , elongation 1.1-1.3-1.4, elliptical to ovoid, colour pale brown to dark brown, refractive centre (2 droplets), apiculus lateral, germ pore not visible. Stipe 3.5-4.5 x 1.2-1.8 cm, central, solid to fistulose, cylindrical, may be wider at centre, narrowing towards the base, surface may be streaked below the annulus, dark pinkish above annulus, purplish below annulus, bruising dark purple/brown. Annulus pendant, thin, purple/brown, ephemeral. Flesh white, slowly reddish upon cutting or bruising, smell and taste pleasant. Schaeffer's cross reaction Habitat in fairy ring amongst *Paspalum* grass. (porphyr.).

Group Vaporarius.

Agaricus subperonatus (J. Lge) Sing. Pileus 4-9-15 cm diam., very thick, fleshy, hemispherical when young, then broadly convex to applanate, surface smooth to streaky, white when young, later buff to fawny coloured scales develop, soil particles often adhering to surface. Margin very thick and inrolled, with remnants of veil. Lamellae insertion free, thick, crowded of several lengths, may be grown together, white to pink when young, then umber, pale sterile edge, trama arrangement parallel to irregular. Basidia 4 spored, 19-40 x 6-11 μm , clavate to broadly clavate, sterigmata 5-6 μm (very

hyaline and difficult to see). Cheilocystidia 17-45 x 7-15 (20) μm , clavate to broadly clavate, hyaline to fawn, abundant. Basidiospore print dark brown, size 6.9-8.4-9.8 x 5.5-6.1-6.7 μm , elongation 1.2-1.4-1.6 μm , ellipsoid to subglobose and globose, fawn, refractive centre (1-3 droplets), apiculus later, germ pore not visible. Stipe 3-6 x 2-3 cm, central, solid, cylindrical, swollen sometimes pointed base with mycelial cords, surface fibrillose, white to buff above and below annulus. Annulus pendant, white to buff above and below, surface smooth. Flesh white becoming senna to vinacious buff upon cutting or brusing, smell and taste pleasant. Schaeffer's cross reaction negative. Habitat hypogean, in bare soil beneath *Populus* shelter belt, and *Cupressus macrocarpa*. (subpero.).

Section Sanguinolenti (Moller et J. Schaeffer 1938) Singer 1951 Sin.: Rubescentes Moller 1950 sensu Moser 1967. Group Fusco-Fibrillosus.

Agaricus lanipeſ (Moll. et J. Schff.) Sing. Pileus 6-12 (25) cm diam., thick, fleshy, convex when young, then convex-applanate, may have depressed center, dark brown scales on paler brown surface, lighter brown scales when young, brusing dark brown. Margin thin, inrolled, uneven edge, with remnants of veil. Lamellae insertion free, thin, crowded, of several lengths, pink when young, later chocolate brown, with pale sterile edge, trama arrangement parallel to irregular. Basidia 4 spored, 12-27 x 8-10 μm , broadly clavate, sterigmata 2-4 μm . Cheilocystidia 12-27 x 7-10 μm , broadly clavate, hyaline to fawn. Basidiospore print dark brown, size 7.3-8.2-9.1 x 5.2-5.8-6.4 μm , elongation 1.4-1.6-1.7 μm , colour brown, apiculus lateral, germ pore not visible. Stipe 5-8 x 2-5 cm, central, solid, cylindrical, swollen base, with mycelial cords, surface white and fibrillose above the annulus, white with brown streaks below annulus, brusing brown. Annulus pendant, wide, brown above, white below. Flesh white, becoming pinkish to brown upon cutting or brusing, smell and taste pleasant. Schaeffer's cross reaction negative. Habitat under large conifer growing through fine bark mulch. (lanipeſ.).

Not identified to species.

Agaricus sp.1. Pileus 4-6-8 cm diam., thick, fleshy, convex when young, then convex to applanate, surface scaly, evenly distributed, colour when young brown, then dark brown, brusing no reaction to light reddening. Margin thick, smooth edge, no remnants of veil. Lamellae insertion free, thin, equally arranged, convex, colour when young pink, then brown, edge pale and sterile, trama arrangement parallel to irregular. Basidia 1, 2, 4 spored, 10-18 x 7-8 μm , clavate to broadly clavate, sterigmata 4-8 (10) μm . Cheilocystidia 15-35 x 9-10 (15) μm , clavate, colour hyaline, pale brown, abundant. Basidiospore print dark brown, size 7.9-8.8-9.8 x 5.3-6.1-6.9 μm , elongation 1.3-1.5-1.3, elliptical, pale brown to dark brown, refractive centre (1-2 droplets), apiculus lateral, germ pore not visible. Stipe 3-4-5 cm, central, equal to swollen at the base, cylindrical, base roundish, surface powdery to floccose, colour above and below annulus white, brusing no reaction to light reddening. Annulus large and decending, thick, colour white, surface powdery, smooth. Flesh colour white, no reaction upon cutting, no reaction to light reddening upon brusing, smell and taste pleasent. Schaeffer's cross reaction Habitat in lawn, close to mature *Arucaria arancana*, Monkey puzzel tree. (*Agaricus*1).

Tribe *Leucocoprineae*. Genus *Leucoagaricus*.

Leucoagaricus leucothites (Vitt.) Wasser syn. *Lepiota leucothites*, *Lepiota pudica*, *Lepiota naucina*. Pileus 5-10 cm diam., thick, fleshy, convex when young, then convex to applanate, surface smooth, silky, and white. Margin straight, uneven, with remnants of the veil. Lamellae insertion free, thin, colour white when young, fawn when mature, edge pale and sterile, trama arrangement irregular, almost parrallel. Basidia 4 spored, 20-30 x 8-10 μm , broadly clavate, sterigmata 4-5 μm . Cheilocystidia 20-100 x 10-18 μm , clavate, colour, hyaline, abundant. Basidiospore print white, size 7.5-8.5-9.5 x 5.4-5.9-6.3 μm , elongation 1.3-1.5-1.7, elliptical to ovoid, colour hyaline to fawn, apiculus lateral, germ pore present. Stipe 5-10 x 0.8-2 cm, central, fistulose, cylindrcial, base round and swollen with mycelial cords, surface silky, white above and below the annulus when young, later becoming brown below the annulus,

especially towards the base. Annulus large, thin, almost completely free of the stem, concolorous with the pileus surface, becoming brown at the edges, may be ragged at the edges, surface silky. Flesh colour white, becoming brown upon cutting or brusing, smell and taste pleasant. Habitat in cow paddock. (Leucoag.).

Family Bolbitiaceae. Genus *Agrocybe*.

Agrocybe parasitica Stevenson. Pileus 4-25 cm diam., thick, convex when young, then broadly convex to applanate, surface smooth and silky, pale to almost cream when young, later clay buff, brusing brown. Margin inrolled when young, connected long lasting partial veil, breaks way leaving ragged edge with remnants of veil. Lamellae insertion adnexed to adnate, thick, crowded, of several lengths, colour when young pale cream, later brown, edge pale and sterile, trama arrangement parallel. Basidia 1-2-4 spored, 15-25 x 7-10 μm , clavate, sterigmata 7-8 μm . Cheilocystidia 22-50 x 8-10 μm , broadly clavate to pyriform, hyaline, abundant. Basidiospore print umber, size 9.7-10.7-11.8 x 6.3-6.8-7.4 μm , elongation 1.4-1.6-1.7, elliptical to oval, fawn to pale brown almost yellowish, refractive centre (1-2 droplets), apiculus lateral, germ pore present. Stipe 8-20 x 1.0-2.5 cm, central, solid, equal, cylindrical, generally curved at base depending upon route of exit from substrate, surface concolourous with pileus surface, smooth, may be fibrous and tough in massive specimens, brusing brown. Annulus wide, apical, brown above, buff below. Flesh white, becoming pale brown upon cutting or brusing, smell and taste almost none when young, later sweet. Habitat; a heart rot of native hardwood trees, e.g., *Plagianthus betulinus*, Manatu, *Bellschiedia tawa*, Tawa, and exotic trees such as *Populus* spp. A more extensive list of host species was given by Stevenson (1982b). Inoculum source; basidiospores entering via a wound large enough to expose dead heart wood. (Agrocybe).

Appendix 10.

The calibration and thresholding procedures used for image analysis and processing carried out in Chapter 5.

Calibration of pixel units into μm .

The steps involved in the calibration are listed over the page. Capital letters in () indicate the keyfunction of the keyboard, whereas CAPITAL print indicate the menu/option selected. Computer commands are written in {}, and 'simple quotes' show what was typed in this study. Finally, [] are used where an explanation was added.

After starting Magiscan, (I)mage Analysis was chosen from the first MAIN MENU which allowed selection of the (G)eneral Image Analysis Software. From the GENIAS TASK MODE the (I)nteractive was chosen to open the second MAIN MENU for the actual calibration:

MAIN MENU

(E)nvironment

ENVIRONMENT

(I)mage source

IMAGE SOURCE

(M)onochrome

(Q)uit

ENVIRONMENT

(N)o. of camera '1'

(Q)uit

MAIN MENU

(C)apture [scale of the graticule]

CAPTURE

(Q)uit & photo

MAIN MENU

(E)nvironment

ENVIRONMENT

(C)alibrate

CALIBRATION

(L)ight pen calibration {calibration units: ' μm '}{point distance in μm : '100'}

{1st point with pen}

{2nd point with pen [100 μm distant]}

{space "accepts", or repeat}

units = μm

scale = 0.2612 [automatically calculated]

(P)oint distances [to check the accuracy of the calibration]

POINT DISTANCES

{1st point with pen}

{2nd point with pen}

(A)ccept

{1st point with pen}

{2nd point with pen}

(A)ccept

[may be repeated several times]

(Q)uit

CALIBRATION

(Q)uit

ENVIRONMENT

(S)ave

SAVE ENVIRONMENT

(C)hange name {name of file: 'calibration x200.env'}

ENVIRONMENT

(Q)uit

MAIN MENU

(Q)uit {re-initialize: 'yes'}

Setting up the tasklist.

After starting Magiscan, (I)mage Analysis was chosen from the first MAIN MENU which then allowed selection of the (G)eneral Image Analysis Software. From the GENIAS TASK MODE, (D)efine tasklist was chosen, the output task file was named 'tasklist A.tsk' under (C)hange name, which opened the second MAIN MENU. This was used to set up the automatised detection and measurement of the spores, as listed below:

MAIN MENU

(E)nvirionment

ENVIRONMENT

(L)oad [calibration file]

(C)hange name {type the name of the calibration file:
'calibration x200.env'}

(Q)uit

MAIN MENU

(C)apture [spore image]

CAPTURE

(D)efine table [to manipulate the contrast between background
and the objects measured]

INPUT TABLE SETTINGS

(A)djust limits {input table limits: low '100'}
{input table limits: high '255'}

(P)arabola

(I)nvert

(Q)uit

CAPTURE

(Q)uit & photo

MAIN MENU

(P)ointset

DEFINE POINTSET

(E)ntire image

(Q)uit

MAIN MENU

(T)hreshold

THRESHOLD

(M)anual {slice: low '160'}
{slice: high '255'}

(Q)uit

MAIN MENU

(B)inary ops

BINARY OPS

(O)bject based ops

OBJECT BASED OPS

(F)ill objects

(S)eparate objects {how many erosions per step: '2'}
{fill objects: 'no'}
{minimum circularity for object to be indivisible: '0.95'}

```

(D)elete objects
  DELETE OBJECTS
  (A)rea      {minimum: '25'}
               {maximum: '40'}
  (E)xclusive
  (Q)uit
OBJECT BASED OPS
(D)elete objects
  DELETE OBJECTS
  (B)readth    {minimum: '0'}
               {maximum: '3'}
  (I)nclusive
  (Q)uit
OBJECT BASED OPS
(D)elete objects
  DELETE OBJECTS
  (L)ength     {minimum: '14'}
               {maximum: '50'}
  (I)nclusive
  (Q)uit
OBJECT BASED OPS
(Q)uit
BINARY OPS
(Q)uit
MAIN MENU
(M)easure
  FIELD MEASUREMENTS
  (O)bject measurements
  (S)ingle image [inversion]    {nesting levels [0 = no nesting] '0'}
  MEAUREMENTS 1
  (A)rea
  (B)readth
  (C)ircularity
  (L)ength
  (*)-others
  MEASUREMENTS 2
  (E)longation
  (Q)uit
  MEASUREMENTS 1
  (Q)uit
  FIELD MEASUREMENTS
  (Q)uit, to measure {any key}
MAIN MENU
(Q)uit {re-initialize: 'yes' [saves automatically the task list]}

```


The taskfile 'tasklist A.tsk' shown above was used in this original form for all fungi, except for *Agrocybe parasitica*, *Agaricus semotus* and *Agaricus subperonatus*, for which modifications were made, Table 1.

	Others ^a original tasklist	<i>Agrocybe</i> <i>parasitica</i>	<i>Agaricus</i> <i>semotus</i>	<i>Agaricus</i> <i>subperonatus</i>
Threshold ^b				
Low	160	145	160	170
High	255	255	255	255
Delete objects				
Area ^c				
minimum	25 ex ^d	30 ex	15 ex	30ex
maximum	50 ex	70 ex	30 ex	65 ex
Breadth ^e				
minimum	0 in ^f	0 in	---	---
maximum	3 in	4 in	---	---
Length ^e				
minimum	14 in	---	---	12 in
maximum	50 in	---	---	50 in

^a other fungi included *Agaricus arvensis*, *Agaricus bisporus* X20, *Agaricus bisporus* Ohoka, *Agaricus bernardii*, *Agaricus bitorquis* K46, *Agaricus bitorquis* Ropley, *Agaricus campestris* Halls1, *Agaricus campestris* Halls2, *Agaricus campestris* var. *campestris*, *Agaricus cupreobrunneus*, *Agaricus lanipe*, *Agaricus porphyrocephalus*, *Agaricus subperonatus*, *Leucoagaricus leucothites*.
^b threshold ranged from 0-255
^c area in μm^2
^d ex = exclusive (objects with measurements outside the given range were deleted)
^e breadth and length in μm
^f in = inclusive (objects with measurements inside the given range were deleted)

Table 1. Modifications to original taskfile for *Agrocybe parasitica* *Agaricus semotus* *Agaricus subperonatus*.

Appendix 11.

Demonstration of the phylogenetic value of the segment coded basidiospore principal components from Chapter 5.

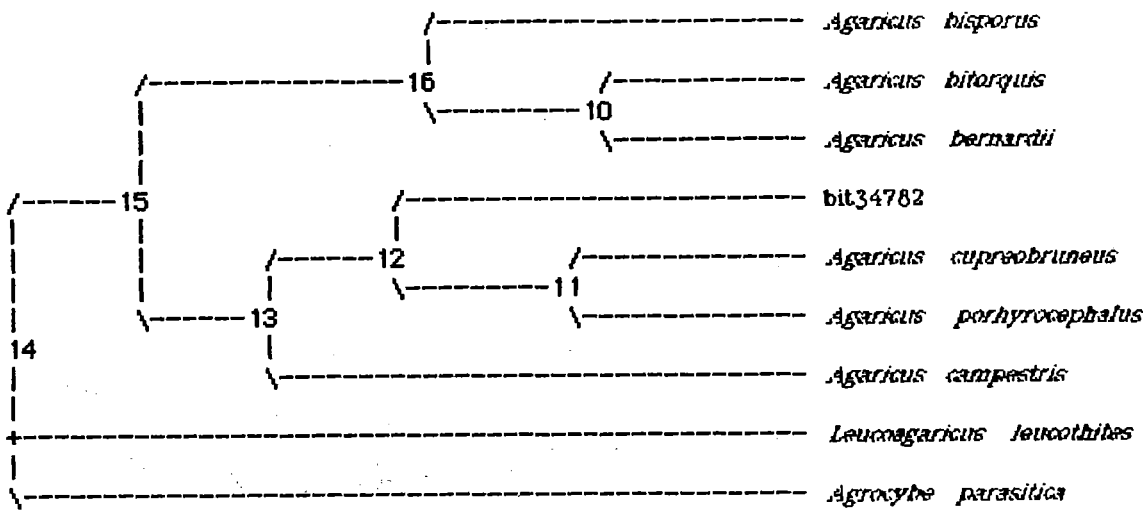


Figure 1. Single most parsimonious tree, CI = 0.645 derived from allozyme characters, Chapter 4. bit34782 = *Agaricus bitorquis*, numbers indicate nodes as a reference for apomorphies.

Apomorphy lists, including those for the basidiospore principal components mapped on to the tree in Figure 1.

To integrate the alphabetical coding of the basidiospore principal components (PC 1, PC 2, PC 3) into the Phylogenetic Analysis Using Parsimony, (PAUP; Swofford 1991) computer programme, the presence/absence electromorph coding was as follows; A = electromorph absence, and B = electromorph presence.

* Excluded character(s) not included in length calculations.

Branch	Character	(Unweighted)		
		Steps	CI	Change
node_14 --> node_15	10.Mdh-2:2	1	0.333	A ==> B
	15.Mdh-2:7	1	0.333	A ==> B
	20.Mdh-2:12	1	0.333	A ==> B
	27.Acp:6	1	0.333	A ==> B
	30.bGlu:1	1	0.200	A ==> B
	31.bGlu:2	1	0.250	A ==> B
	68.Aat:4	1	0.333	A ==> B
	104.Gpt:7	1	0.250	A ==> B
	128.Mpi:6	1	0.200	A ==> B
	129.Mpi:7	1	0.333	A ==> B
	*134.PC1	1	0.778	F ==> G
	*136.PC3	1	1.000	D ==> C
node_15 --> node_16	28.Acp:7	1	1.000	A ==> B

	29.Acp:8	1	0.500	A	=> B
	33.bGlu:4	1	0.500	A	=> B
	37.bGlu:8	1	0.500	A	=> B
	38.bGlu:9	1	0.500	A	=> B
	39.bGlu:10	1	0.250	B	=> A
	41.bGlu:12	1	1.000	A	=> B
	42.bGlu:13	1	0.200	B	=> A
	48.Ha:4	1	0.333	B	=> A
	66.Aat:2	1	0.500	A	=> B
	67.Aat:3	1	1.000	A	=> B
	84.Gk-1:9	1	0.500	A	=> B
	92.Gk-2:6	1	0.500	A	=> B
	27.Mpi:5	1	0.200	B	=> A
	*134.PC1	1	0.778	G	=> H
node_16 --> A.bisporus	4.Mdh-1:4	1	1.000	A	=> B
	10.Mdh-2:2	1	0.333	B	=> A
	16.Mdh-2:8	1	0.333	B	=> A
	30.bGlu:1	1	0.200	B	=> A
	31.bGlu:2	1	0.250	B	=> A
	34.bGlu:5	1	1.000	A	=> B
	40.bGlu:11	1	1.000	A	=> B
	47.Ha:3	1	0.250	B	=> A
	59.Lap:5	1	1.000	A	=> B
	68.Aat:4	1	0.333	B	=> A
	86.Gk-1:11	1	0.250	B	=> A
	91.Gk-2:5	1	1.000	A	=> B
	98.Gpt:1	1	1.000	A	=> B
	99.Gpt:2	1	1.000	A	=> B
	104.Gpt:7	1	0.250	B	=> A
	112.Pgm:6	1	1.000	A	=> B
	119.Pgm:13	1	0.250	B	=> A
	29.Mpi:7	1	0.333	B	=> A
node_16 --> node_10	20.Mdh-2:12	1	0.333	B	=> A
	27.Acp:6	1	0.333	B	=> A
	60.Lap:6	1	1.000	A	=> B
	78.Gk-1:3	1	1.000	A	=> B
	89.Gk-2:3	1	1.000	A	=> B
	120.Pgm:14	1	0.333	B	=> A
	128.Mpi:6	1	0.200	B	=> A
node_10 --> A.bitorquis	3.Mdh-1:3	1	1.000	A	=> B
	14.Mdh-2:6	1	1.000	A	=> B
	15.Mdh-2:7	1	0.333	B	=> A
	19.Mdh-2:11	1	1.000	A	=> B
	25.Acp:4	1	1.000	A	=> B
	30.bGlu:1	1	0.200	B	=> A
	33.bGlu:4	1	0.500	B	=> A
	47.Ha:3	1	0.250	B	=> A
	57.Lap:3	1	1.000	A	=> B
	63.Lap:9	1	1.000	A	=> B
	64.Lap:10	1	1.000	A	=> B
	65.Aat:1	1	1.000	A	=> B
	85.Gk-1:10	1	1.000	A	=> B
	92.Gk-2:6	1	0.500	B	=> A
	94.Gk-2:8	1	1.000	A	=> B
	95.Gk-2:9	1	1.000	A	=> B
	100.Gpt:3	1	1.000	A	=> B
	104.Gpt:7	1	0.250	B	=> A
	108.Pgm:2	1	1.000	A	=> B
	110.Pgm:4	1	1.000	A	=> B
	116.Pgm:10	1	1.000	A	=> B
	117.Pgm:11	1	1.000	A	=> B
	125.Mpi:3	1	1.000	A	=> B
node_10 --> A.bernardii	29.Acp:8	1	0.500	B	=> A

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31.bGlu:2      1  0.250 B ==> A
37.bGlu:8      1  0.500 B ==> A
38.bGlu:9      1  0.500 B ==> A
44.bGlu:15     1  1.000 A ==> B
52.Ha:8        1  1.000 A ==> B
66.Aat:2       1  0.500 B ==> A
84.Gk-1:9      1  0.500 B ==> A
86.Gk-1:11     1  0.250 B ==> A
111.Pgm:5      1  1.000 A ==> B
119.Pgm:13     1  0.250 B ==> A
*134.PC1       1  0.778 H ==> G
node_15 --> node_13
26.Acp:5       1  0.333 A ==> B
54.Ha:10       1  0.500 A ==> B
55.Lap:1       1  0.500 A ==> B
77.Gk-1:2      1  0.333 A ==> B
82.Gk-1:7      1  0.333 A ==> B
86.Gk-1:11     1  0.250 B ==> A
88.Gk-2:2      1  0.500 A ==> B
90.Gk-2:4      1  0.500 A ==> B
103.Gpt:6      1  1.000 A ==> B
119.Pgm:13     1  0.250 B ==> A
130.Mpi:8      1  0.333 A ==> B
*135.PC2       2  0.750 E ==> C
node_13 --> node_12
5.Mdh-1:5      1  1.000 A ==> B
10.Mdh-2:2     1  0.333 B ==> A
16.Mdh-2:8     1  0.333 B ==> A
31.bGlu:2      1  0.250 B ==> A
80.Gk-1:5      1  0.500 A ==> B
104.Gpt:7      1  0.250 B ==> A
node_12 --> 34782BIT
26.Acp:5       1  0.333 B ==> A
35.bGlu:6      1  1.000 A ==> B
39.bGlu:10     1  0.250 B ==> A
42.bGlu:13     1  0.200 B ==> A
47.Ha:3        1  0.250 B ==> A
77.Gk-1:2      1  0.333 B ==> A
82.Gk-1:7      1  0.333 B ==> A
127.Mpi:5      1  0.200 B ==> A
128.Mpi:6      1  0.200 B ==> A
130.Mpi:8      1  0.333 B ==> A
node_12 --> node_11
30.bGlu:1      1  0.200 B ==> A
48.Ha:4        1  0.333 B ==> A
50.Ha:6        1  1.000 A ==> B
54.Ha:10       1  0.500 B ==> A
68.Aat:4       1  0.333 B ==> A
72.Aat:8       1  1.000 A ==> B
118.Pgm:12     1  1.000 A ==> B
120.Pgm:14     1  0.333 B ==> A
*134.PC1       1  0.778 G --> F
node_11 --> A.cupreobruneus
26.Acp:5       1  0.333 B ==> A
39.bGlu:10     1  0.250 B ==> A
58.Lap:4       1  1.000 A ==> B
80.Gk-1:5      1  0.500 B ==> A
90.Gk-2:4      1  0.500 B ==> A
128.Mpi:6      1  0.200 B ==> A
129.Mpi:7      1  0.333 B ==> A
*136.PC3       1  1.000 C ==> B
node_11 --> A.porhyrocephalus
27.Acp:6       1  0.333 B ==> A
42.bGlu:13     1  0.200 B ==> A
55.Lap:1       1  0.500 B ==> A
56.Lap:2       1  1.000 A ==> B
77.Gk-1:2      1  0.333 B ==> A
82.Gk-1:7      1  0.333 B ==> A
88.Gk-2:2      1  0.500 B ==> A

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	127.Mpi:5	1	0.200	B ==>	A
	130.Mpi:8	1	0.333	B ==>	A
node_13 --> A.campestris	6.Mdh-1:6	1	1.000	A ==>	B
	15.Mdh-2:7	1	0.333	B ==>	A
	20.Mdh-2:12	1	0.333	B ==>	A
	30.bGlu:1	1	0.200	B ==>	A
	36.bGlu:7	1	1.000	A ==>	B
	42.bGlu:13	1	0.200	B ==>	A
	45.Ha:1	1	1.000	A ==>	B
	46.Ha:2	1	1.000	A ==>	B
	49.Ha:5	1	1.000	A ==>	B
	51.Ha:7	1	1.000	A ==>	B
	53.Ha:9	1	1.000	A ==>	B
	74.Aat:10	1	1.000	A ==>	B
	76.Gk-1:1	1	1.000	A ==>	B
	79.Gk-1:4	1	1.000	A ==>	B
	127.Mpi:5	1	0.200	B ==>	A
	128.Mpi:6	1	0.200	B ==>	A
node_14 --> Leucoagaricus	2.Mdh-2:4	1	1.000	A ==>	B
	18.Mdh-2:10	1	1.000	A ==>	B
	42.bGlu:13	1	0.200	B ==>	A
	43.bGlu:14	1	1.000	A ==>	B
	47.Ha:3	1	0.250	B ==>	A
	62.Lap:8	1	1.000	A ==>	B
	86.Gk-1:11	1	0.250	B ==>	A
	105.Gpt:8	1	1.000	A ==>	B
	120.Pgm:14	1	0.333	B ==>	A
	121.Pgm:15	1	1.000	A ==>	B
	*135.PC2	1	0.750	E ==>	D
node_14 --> Agrocybe	16.Mdh-2:8	1	0.333	B ==>	A
	17.Mdh-2:9	1	1.000	A ==>	B
	21.Mdh-2:13	1	1.000	A ==>	B
	39.bGlu:10	1	0.250	B ==>	A
	48.Ha:4	1	0.333	B ==>	A
	83.Gk-1:8	1	1.000	A ==>	B
	102.Gpt:5	1	1.000	A ==>	B
	119.Pgm:13	1	0.250	B ==>	A
	122.Pgm:16	1	1.000	A ==>	B
	127.Mpi:5	1	0.200	B ==>	A
	133.Mpi:11	1	1.000	A ==>	B
	*134.PC1	5	0.778	F ==>	A
	*135.PC2	1	0.750	E ==>	F
	*136.PC3	2	1.000	D ==>	F