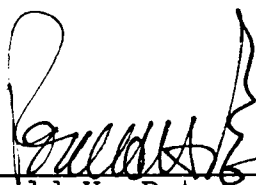


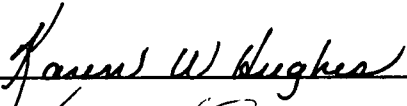
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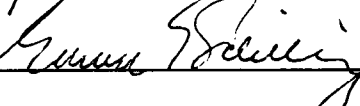
I am submitting herewith a dissertation written by Qiuxin Wu entitled " Systematics in the genus *Clavicornia* (Basidiomycotina, fungi): morphology, incompatibility and phenoloxidase isozymes." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.



Ronald H. Petersen, Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the Council:



Vice Provost
and Dean of The Graduate School

SYSTEMATICS IN THE GENUS *CLAVICORONA*
(BASIDIOMYCOTINA, FUNGI):
MORPHOLOGY, INCOMPATIBILITY AND PHENOLOXIDASE ISOZYMES

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Qiuxin Wu
August 1991

To my mother

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ABSTRACT

The genus *Clavicornia* includes about 12 species of wood-rotting basidiomycete fungi distributed worldwide. The purposes of this study were to explore the potential of cultural morphology, incompatibility tests and isozyme electrophoresis in combination with comparative fruitbody morphology to solve systematic problems in *Clavicornia*.

One hundred and five monokaryotic and/or dikaryotic isolates representing nine taxa of *Clavicornia* were obtained from China, Europe, Japan, New Zealand, North America and Puerto Rico. These nine morphologically based taxa include a newly proposed species from Japan and four species from New Zealand. Morphological descriptions of these nine taxa are presented based on macro- and micromorphological characters.

The potential value of cultural morphology and spot tests for phenoloxidase activity was analyzed. Culture mat morphology developed on PDA and MYG was useful for delimiting species. Descriptions of culture mat morphology are furnished for each analyzed species. Spot test results showed that all *Clavicornia* species have laccase activity on at least one medium during development in culture. All tested species showed tyrosinase activity on PDA and MYG, but not on MA. These results indicated that laccase and tyrosinase spot tests may be characteristic for the genus

and only of very limited value in separating species. Self-crosses of single-spore isolates revealed that seven of these nine taxa were governed by a homogenic bifactorial incompatibility system. Intercollection matings indicated that collections from the same morphospecies were either fully compatible or shared common mating type alleles, regardless of morphological variation, whereas collections from different morphospecies were completely incompatible. Morphological delimitation of species in this study is basically congruent with biological species in this genus. Partial compatibility, however, was found among allopatric populations in one species.

Electrophoretic analysis showed that extracellular laccase banding patterns were mostly simple and reproducible in the genus. Banding patterns were different between morphospecies but consistent within species, indicating that laccase electrophoresis may be of significance in the systematics of *Clavicornia*. In contrast, electromorphic patterns for tyrosinase were variable in certain species, but specific for others.

A final section summarizes the results from various methods for each species, and discusses the roles and validity of each method in the systematics of *Clavicornia*.

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CHAPTER I

INTRODUCTION

A. TAXONOMIC HISTORY OF THE GENUS *CLAVICORONA*

Although the first *Clavicornona* species was described in 1794 by Persoon as *Clavaria pyxidata*, the name *Clavicornona* was not proposed until 1947. Before then most *Clavicornona* species had been classified as part of the genus *Clavaria*, like other clavarioid fungi, based on their branched fruitbodies and other macroscopic morphological characters. Three species of *Clavicornona*, however, have been treated as members of other genera such as *Craterellus* and *Physalacria*. Doty (1947) recognized that these fungi constituted a distinct group based on peculiar branching, small, white spores and gloeocystidia, and he proposed a new generic name, *Clavicornona* (type species *Craterellus taxophilus* Thom) to include *C. pyxidata* (Fries) Doty, *C. taxophila* (Thom) Doty, *C. cristata* (Kauffm.) Doty and *C. coronata* (Schw.) Doty. Corner (1950) accepted the genus as a natural group and added five species, *C. candelabrum* (Mass.) Corner, *C. colensoi* (Berk.) Corner, *C. javanica* (Sacc. & Syd.) Corner, *C. mairei* (Batt.) Corner and *C. tuba* (Heim) Corner, and later, another, *C. dichotoma* (Corner, 1970). Leathers and Smith (1967) accepted the genus and described two species, *C. divaricata* Leathers & Smith and *C. avellanea* Leathers &

Smith, as well as proposing a new combination, *C. piperata* (Kauffm.) Leathers & Smith.

Leathers and Smith (1967) also proposed dividing the genus into three sections based on reactions of basidiospores in Melzer's reagent.

Dodd (1970) performed extensive nomenclatural and taxonomic studies on *Clavicornia* for his Ph. D. dissertation and later published the results (Dodd, 1972). He recognized eleven species and proposed two subgenera:

Subg. *Clavicornia*; unbranched fruitbodies, on litter and duff.

≡ Sect. *Clavicornia* Leathers & Smith (1967)

Subg. *Ramosa* Dodd, branched fruitbodies, on wood.

≡ Sect. *Pyxidospoggus* Leathers & Smith (1967).

≡ Sect. *Coronatae* Leathers & Smith (1967).

Dodd's classification reflected morphological and ecological differences between the two groups in the genus. Furthermore, Jülich (1981) accepted these two groups as separate genera: a) *Clavicornia* s. Jülich, with clavate, truncate, unbranched basidiocarps, inamyloid spores, growing on leaf litter etc. but not on wood; and b) *Artomyces* Jülich, with in almost all cases pyxidately branched basidiocarps, strongly amyloid spores and habit on wood, including most *Clavicornia* species accepted originally by Doty. In this study, however, broad interpretation of *Clavicornia* is used.

Delimitation of species in *Clavicornia* has been difficult because there is continuity of morphological variation among species, lack of distinguishing characters and developmental variation of the fruitbody. Furthermore, in various areas of the world, mycologists have used the same names to represent different organisms (i. e., *C. pyxidata* has been reported from several continents). As a result, there has been confusion in taxonomy, based solely on morphological characters. Application of experimental criteria to the systematics of the genus, therefore, is significant.

B. INCOMPATIBILITY AND FUNGAL SYSTEMATICS

Historical Review

The history of research on fungal compatibility may be divided into three stages: 1) pioneering work; 2) development and synthesis and 3) application.

Pioneering work started at the beginning of the twentieth century and represented the early knowledge of fungal life cycle and plasmogamy (Buller, 1909), conjugate division and clamp connections (Bensaude, 1918; Kniep, 1912), fungal sexual patterns (Bensaude, 1918), heterothallic phenomena (Blakeslee, 1904) and genetic basis of incompatibility (Kniep, 1922).

The first case of obligatory cross-mating in fungi was

reported by Blakeslee (1904) for *Rhizopus nigricans*. He introduced the term "heterothallism" to describe the phenomenon that cross-mating between two self-sterile strains was required in the process of sexual reproduction. In higher fungi, Bensaude (1918) revealed for the first time the origin of the dikaryotic association and presented a description of conjugate division and formation of clamp connections in *Coprinus fimetarius*. Kniep (1920) made several important discoveries, namely that basidiospores initially contained only a single nucleus, but when monosporous strains were paired, clamped, dikaryotic mycelia were established in certain pairings. Kniep (1919) suggested that pairs of alleles, labelled Aa and Bb, were the causal factors for the mating pattern in *Schizophyllum commune*. Burgeff (1920) used the term "bipolar sexuality" to define mating patterns in the Mucorales while Bauch (1930) applied the term "tetrapolar sexuality" to the four mating-type system in the smut, *Ustilago longissima*.

In the second stage, stimulated by the pioneering work, many reports were published showing that fungal incompatibility systems occurred in all major groups of fungi: in water molds (Couch, 1926); in yeasts (Winge & Laustsen, 1939); in Euascomycetes (Shear & Dodge, 1927); in the rust fungi (Craigie, 1927) and in the hymenomycetes (Arnold, 1935; Barnett, 1937). These works on fungal incompatibility led to theoretical summarization and reviews

which cover this field very comprehensively and contain references to original publications include those by Whitehouse (1949), Raper (1954, 1960, 1963, 1966), Lewis (1954, 1956), Burnett (1965), Papazian (1958), Esser (1962, 1966), Raper and Esser (1964), and Esser and Kuenen (1976).

The third developmental stage of fungal incompatibility research was characterized by the application of mating tests to the study of speciation and evolution and by the provision of experimentally based criteria for fungal systematics. Since the middle of the 1960s, fungal incompatibility tests have been used widely in various groups of basidiomycetes for delimitation of species. Examples can be found in literature by Anderson & Ullrich (1979), Bandoni (1963, 1965), Berthier (1974), Boidin (1977), Boidin & Lanquetin (1965, 1973), Duncan (1972), Esser & Hoffman (1976), Ginns (1985), Hallenburg (1984), Kemp (1974, 1975), Wong (1985), Wong & Wells (1985). Extensive reviews on the role of incompatibility tests in the study of fungal speciation and evolution have been given by Burnett (1983), Bresinsky et al. (1977, 1987) and Esser & Hoffman (1976).

Principles of Fungal Incompatibility

The following features in the typical fungal life cycle are important in incompatibility systems: 1) limitless growth potential of the monokaryon makes it possible to test

incompatibility through monokaryotic mycelium conjugation; 2) formation of a dikaryotic mycelium is essential for the formation of basidiomata; 3) an prolonged dikaryotic stage makes it easy to observe the nuclear condition; and 4) in most basidiomycetes, the existence of a dikaryon can be inferred by the presence of clamp connections.

The majority of basidiomycetes are characterized by heterothallism, in which compatible mating occurs only between two mycelia that arise from different haploid monospores. Specific genes (= incompatibility factors) determine the pattern of mating in the absence of any morphological differentiation. Incompatibility, as defined by Esser (1966), "is a genotypically determined inhibition of karyogamy within a sexually reproducing system of monoecious individuals." Two basic types of incompatibility in the fungi have been recognized: 1) homogenic incompatibility, in which sexual interaction is prevented by like alleles in the two presumptive mates; and 2) heterogenic incompatibility, in which sexual interaction is prevented by unlike alleles. Most known incompatibility phenomena belong to homogenic incompatibility, which may be controlled by either one factor, termed a "bipolar" or "unifactorial" mechanism, or two factors, termed a "tetrapolar" or "bifactorial" mechanism.

In a bipolar incompatibility system, there are only two mating type alleles determining sexual behavior. They

generally designated as + and - or A and a. All mycelia with the same mating type are incompatible. Fertilization occurs only between different mating types.

In a tetrapolar incompatibility system, mating behavior is determined by two unlinked genes, called A and B. Each of these two loci is multi-allelic, resulting in four mating types, A_1B_1 , A_2B_2 , A_1B_2 , and A_2B_1 . Four types of mating reactions that result from all possible combinations of the four mating types are as follows (Esser, 1966):

- 1) mycelia with different A and B factors undergo plasmogamy and nuclear exchange and migration; dikaryon usually exhibits clamp connections;

- 2) mycelia with common A and different B factors undergo plasmogamy, nuclear exchange and migration, but no clamp connections are formed. Contact zone macromorphology of this type of mating reaction is usually termed "flat;"

- 3) mycelia with common B and different A factors undergo exchange of nuclei but no nuclear migration occurs because of the prevention of fusion of clamp connections to the incipient penultimate cell. Contact zone macromorphology of this type of mating reaction is usually termed "barrage;"

- 4) mycelia with common A and common B factors, hardly undergo plasmogamy and exchange of nuclei; nuclear migration and clamp connections do not occur.

Intercompatibility and Interfertility

In mating reactions, A and B factors control formation of the dikaryotic hyphae, i. e. control intercompatibility (or conversely, interincompatibility). Clamp connections can be used as evidence of successful plasmogamy and dikaryon formation and, therefore, as an indication of intercompatibility. However, only after fruiting, karyogamy, meiosis, and basidiospore germination take place and progeny are viable and normal can it be proven that mated strains were interfertile (Boidin, 1986). Because there are other factors involved in interfertility, Esser and Hoffman (1976) proposed a "fruiting initiation locus" (fi) which interferes with dikaryotic fruiting. Chase and Ullrich (1990) determined that there were five genes determining "intersterility" in *Heterobasidion annosum*. Since most fungi possess a prezygotic isolation mechanism, interincompatibility tests can be used as an indication of intersterility.

Application of Incompatibility to Fungal Systematics

Fungal incompatibility has been used as a means of delimiting species in fungal systematics. The methods of incompatibility tests are relatively simple and can provide experimental evidence to systematics. A species defined by interincompatibility is termed a "biological species," in contrast to species defined mainly by morphological

characters, termed a "morphospecies". Collections which are completely interincompatible are considered to be in different biological species, while collections which are entirely intercompatible are believed to belong in one biological species. Boidin (1986) summarized uses of incompatibility tests in fungal systematics: 1) to determine if morphospecies are reproductively isolated; 2) to determine the range of morphological variation within a population or species; 3) to demonstrate reproductive isolation between different species; 4) to determine the geographic distribution of a species; and 5) to demonstrate gradual speciation.

Examples of the application of mating tests in fungal systematics can be found in various groups. Traditionally, the name *Armillaria mellea* represented a group of fungi which differed in morphological and ecological characters but were all included in one morphospecies. Korhonen (1978) discovered that there were at least five intersterility groups in European *A. mellea* and that these intersterility groups exhibited differences in macro- and micromorphological characters and living habitat. Anderson and Ullrich (1979) found that "*A. mellea*" consisted of ten intersterility groups, i. e. ten biological species (later corrected into nine). Using restriction fragment length polymorphism (RFLP) techniques, Anderson et al. (1987) analyzed nuclear and mitochondrial DNA of 16 isolates

representing eight biological species and found that there were differences **between** the biological species but not **within** biological species. *Collybia dryophila* is a widely distributed and variable complex, presenting difficulty in distinguishing similar species. Vilgalys and Miller (1983), using mating tests combined with morphological studies, found that there were four biological species corresponding to previously described morphospecies. Bresinsky et al (1987) demonstrated by using mating test and other methods that in *Pleurotus*, reproductive isolation occurred at a primary stage of speciation. Hallenberg (1984, 1985, 1986, 1988) published results of incompatibility tests on Corticiaceae from Europe and North America. In many cases, collections from North America and Europe were intercompatible. Some cases of intersterility were accompanied by morphological differences of basidiomata. More examples can be found in the publications of Lemke (1969), Ullrich (1973), Ainsworth et al. (1989), Galland et al (1979), Fries (1985) and Esser & Hoffman (1977).

Mating systems in *Clavicornia*

There are few reports on the mating system of *Clavicornia*. Dodd (1972), using a few single-spore isolates from one fruitbody, reported that *Clavicornia pyxidata* exhibited tetrapolar with homogenic incompatibility. James (1983) confirmed the tetrapolarity of this species and

reported multi-allelic phenomena in the species by conducting intercollection matings. However, mating systems in other species have remained unknown and mating tests have not been used for systematic purposes in *Clavicornia*.

C. APPLICATION OF ISOZYME PATTERNS TO SYSTEMATICS

Occurrence, Properties and Function of Laccase

Laccase-catalyzed reactions were first reported by Yoshida (1883) in the darkening and hardening of latex from lacquer trees, and the enzyme laccase was named by Bertrand (1895). The presence of laccase in fungi have been probably first reported by Laborde (1897). Since then, the presence and properties of laccase in many plants and fungi have been described. It has now been shown that laccase is produced by numerous fungi in the Ascomycetes, Basidiomycetes and Fungi Imperfecti, either in extracellular or intracellular form. Most fungal laccase is excreted by hyphae into the medium, i. e. extracellular. Extracellular laccases are commonly present in white-rot fungi and in many mycorrhizal fungi, at least at some stage of fungal development.

Laccase (EC 1. 10. 3. 1) is an oxygen oxidoreductase acting on various *p*-diphenols, *p*-phenylenediamines and related substances as donors, with molecular oxygen as the acceptor, according to the general equation: $p\text{-diphenol} + \frac{1}{2} \text{O}_2 \xrightarrow{\text{laccase}} p\text{-quinone} + \text{H}_2\text{O}$. Specifically, laccase catalyzes

the aerobic oxidation of various monophenols, triphenols, and ascorbic acids as well as *o*- and *p*- diphenols. Related phenolic compounds include guaiacol, hydroquinone, catechol, pyrogallol and *p*-phenylene diamine. Laccase differs from tyrosinase in that laccase does not oxidize L-tyrosine and *p*-cresol, whereas tyrosinase shows no action on hydroquinone. Laccase and tyrosinase oxidation were evaluated for six spot test reagents in a 72-fungal specimen sample by Marr (1979). Results indicated that pyrogallol, guaiac and guaiacol spot test reagents were non-specific for laccase and tyrosinase whereas L-naphthol was laccase-specific and phenol and *p*-cresol reagents were tyrosinase-specific.

The pH optimum for enzyme activity varies for different laccases. The pH optimum for *Ganoderma* laccase is around 5.4, for example, whereas that for *Botrytis* is 4.7 and for *Polyporus* around 4.0. Most laccases have a pH optimum range of 4.0-6.0 and are unstable at alkaline pH. All laccases so far investigated are glycoproteins (Mayer, 1979). The molecular weight of various laccases differs; for example, 67,000 for *Lactarius* (Iwasaki et al., 1967), 63-65,000 for *Russula* (Matsubara & Iwasaki, 1972) and 100,000 for *Agaricus bisporus* laccase (Wood, 1980). All laccases contain copper, so purified fungal laccases are normally blue in color. Research on laccase in *Agaricus bisporus* showed that the enzyme contained 15% carbohydrate and two atoms of copper

per molecule (Wood, 1980). In an assay for laccase, syringaldazine has been successfully used for the quantitative determination of the fungal enzyme (Grassin & Dubourdieu, 1989).

The well-known function of laccase is related to lignin degradation by white rot fungi. Laccase is specifically present in white-rot fungi but absent in brown-rot fungi. White-rot fungi decompose both cellulose and lignin and brown-rot fungi degrade cellulose only. As a substrate of white-rot fungi, lignin is found within the cell walls, where it is intimately interspersed with hemicellulose, forming a matrix that surrounds the orderly cellulose microfibrils. In wood, lignin in high concentration is the glue that binds contiguous cells, forming the middle lamella. As described by Kirk (1987a), the mycelium of white-rot fungi secrete enzymes that degrade lignin and wood components when introduced to the plant cell lumen. The possible role of phenol-oxidizing enzymes in lignin degradation by white-rot fungi have been discussed (Kirk, 1971; Mayer, 1987). According to Kirk, there seem to be several reasons why phenol-oxidases have been considered in connection with lignin breakdown: "(1) the fact that white-rot fungi, which decompose lignin, produce extracellular phenol-oxidizing enzymes that have been easy to detect in most cases, whereas the taxonomically closely related brown-rot fungi, which alter but do not decompose

lignin, apparently do not produce the enzymes or at least not extracellularly; (2) the fact that lignin is a phenolic material and should therefore be substrate for phenol-oxidizing enzymes; and (3) the awareness for many years that lignin decomposition is primarily an oxidative process and consequently that phenol oxidases act in the right direction." There are certain observations supporting the participation of laccase in lignin decomposition. For instance, a laccase-less mutant of *Sporotrichum pulverentum* could not degrade lignin. However after laccase was added to the lignin-containing culture medium, this mutant was able to degrade lignin. It was concluded that products of lignin oxidation catalyzed by added laccase might regulate formation of the lignin and polysaccharide-degrading enzymes (Ander & Eriksson, 1976). It is now believed that fungal laccase catalyzes demethylation of lignin and related compounds and this reaction is thought to be an initial step in lignin biodegradation. Laccase also catalyzes the side-chain elimination which is also an important reaction in decomposition of lignin (Reinhamar, 1984).

Laccases have also been implicated in the pigmentation of fungal spores (Clutterbuck, 1972) and in the development of fruitbodies of fungi (Wood, 1985) such as *Schizophyllum commune* (Leonard & Phillips, 1973).

Occurrence, Properties and Function of Tyrosinase

The enzyme tyrosinase was discovered by Bertrand (1896). Tyrosinases from fungi have been extensively studied (Angleton & Flurkey, 1984; Kanagy *et al.*, 1988; Khan & Ali, 1986; Moore *et al.*, 1989; Goodenough, 1978; Pomerantz, 1964; Robb & Gutteridge, 1981; Sharma & Ali, 1981; Strothkamp *et al.*, 1976), with respect to substrate specificity, state of copper, effect of inhibitors, mechanism of action and developmental variations.

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing bifunctional oxidase catalyzing the *o*-hydroxylation of monophenols to diphenols and the oxidation of *o*-diphenols to corresponding quinones. Mushroom tyrosinase is a protein possessing both monophenolase and *o*-dihydric phenolase activity. Tyrosinase oxidation of spot-test reagent shows that phenol and *p*-cresol reagents are tyrosinase-specific (Marr, 1979).

Tyrosinase has multiple forms and may be monomeric, dimeric, tetrameric and pentameric. The size of a single polypeptide chain varies from a M_r of 30,000 to about 70,000, mostly a unit of M_r 40,000 to 48,000. Mushroom tyrosinase is unique in containing nonidentical subunits termed H and L (Robb, 1984). Tyrosinase contains at least 2 mol of copper per mol enzyme.

Tyrosinase in fungi occurs chiefly at fruitbody

formation stage of the life cycle and is responsible for pigmentation of the fruitbodies.

Application of Isozyme Electrophoresis in Fungal Systematics

The term isozyme refers to all functionally similar forms of enzymes, including all polymers or subunits produced by different gene loci or by different alleles at the same locus (Markert & Moller, 1959). Isozyme electrophoresis techniques have been widely used in biosystematics to determine the amount of genetic variation among organisms at the molecular level. It is assumed that changes in the mobility of enzymes in an electric field reflect changes in the encoding DNA sequence and that differences in banding patterns are genetically based and heritable. It is also assumed that enzyme expression is codominant (Hillis & Moritz, 1990).

Isozyme electrophoresis techniques have been applied to systematics in higher basidiomycetes, mostly in the genus *Agaricus* (May & Royse, 1982a,b; Royse & May, 1982a,b), and reviewed by Franke (1973) and Micales et al. (1986). Blaich & Esser (1975) found that intra-specific laccase patterns were highly congruent whereas significant variation in enzyme patterns occurred even between closely related species of the same genus. Isozyme analysis of extracellular laccases of *Agaricus* spp. on polyacrylamide gels also revealed patterns of activity which were

dissimilar between species but largely invariable within species (Kerrigan & Ross, 1988). In the above two reports, it was concluded that laccase electrophoretic patterns may be of systematic value at the species level but not above species. No report has been published, however, on laccase electrophoresis patterns of the genus *Clavicornia*, and, therefore, its value for systematics in the genus has remained unknown. Although tyrosinase has been studied extensively in respect to its properties and mechanism of action, no clear connection between isozyme electrophoretic patterns and systematics has been established. This may be because of the changing patterns of tyrosinase during fungal development (Ingebrigtsen et al., 1989; Moore & Flurkey, 1989). Nevertheless, comparison of tyrosinase isozyme patterns from different organisms under controlled electrophoretic conditions may reveal whether it is of systematic value.

Polyacrylamide Gel Electrophoresis (PAGE)

In this research study, polyacrylamide gel electrophoresis was used both because of the availability of the equipment and supplies and also because of the advantages it offered. Polyacrylamide gels are formed by the catalytic polymerization of monomeric forms of acrylamide and bisacrylamide. It contributes a molecular sieving effect and can accurately separate proteins on the basis of both

size and electromagnetic charge. Comparing to starch gel electrophoresis, polyacrylamide gel can be prepared from highly purified reagents in a reproducible manner so polymerization conditions can be standardized. In addition, polyacrylamide gel is chemically inert, stable over a wide range of pH, temperature and ionic strength, and is transparent (Hames, 1984). Because of the advantages of polyacrylamide gel, it has been widely used for electrophoresis of most proteins.

D. GENERAL PURPOSE OF THE STUDIES

The general purpose of biosystematics is to provide an index of understanding a biotic system based on similarity and diversity. This index should reflect evolutionary, geographic and phylogenetic effects of nature on organisms. These effects may be represented in morphological, cytological, physiological and genetic characters. Any characteristics of an organism may prove valuable in the practice of systematics. Because the taxonomy of *Clavicornia* has been largely based on morphology, which in some situations has subsequently resulted in ambiguous or controversial classification, the introduction and application of experimental criteria was thought to have the potential to contribute to a better understanding of systematics within the genus. Specifically, therefore, the

purposes of this study were as follows:

1. To examine incompatibility systems in the genus and to determine mating relationships of geographic populations as well as to determine the distribution of biological species;
2. To explore the potential of using laccase and tyrosinase isozyme electropherogram as criteria in the systematics of the genus;
3. To evaluate morphological characters in comparison with mating tests and results of enzyme analysis; and
4. To explore the potential of culture morphology in the systematics of the genus.

CHAPTER II

MATERIALS AND METHODS

A. RESEARCH MATERIALS

Basidiome collections and accompanying notes and cultures were obtained from China, Japan, New Zealand, North America and Puerto Rico. Collections selected for mating tests, culture studies and/or isozyme analysis are listed in Appendix A. Field numbers were used in all studies except where otherwise indicated. Specimens were preserved in the Herbarium of the University of Tennessee under TENN numbers. A concordance of field numbers and TENN numbers is listed in Appendix B.

B. METHODS FOR MORPHOLOGICAL STUDIES

Fresh basidiome collections were gathered from several locations from various parts of the world. In North America, several field trips were conducted in the southern Appalachian Mountains with a concentration in east Tennessee, western North Carolina and northern Georgia, specifically the Great Smoky Mountains National Park (GSMNP) and areas near Highlands Biological Station (Macon Co., North Carolina); northern and central New York state; and Madison, Wisconsin. In Asia, two field trips were conducted

in the Chang Bai Mountains of northeast China, and in Yunnan, in southwest China. The collections from Japan and New Zealand were contributed mainly by Dr. Petersen, and collections from California were contributed by Dr. D. L. Largent.

In the field, fresh specimens were photographed in color (35 mm ASA 200 Ektachrom film) with a grey card for light comparison. After spore-print gathering and tissue culture work as described below, field notes, including those on fruitbody size, color, stature, odor and taste were compiled in field books. Notes on color followed Kornerup & Wanscher's (1978) terminology, with reference to Ridgway's (1912) which are cited in quotation marks. Fresh specimens were then dried thoroughly over warm air and preserved in the Herbarium of University of Tennessee (TENN) for laboratory examination.

Examination of fruitbodies for microscopic characters was made utilizing a Wild research microscope, magnifying up to 3125X. Phase contrast lenses were used for morphological observation. Small macroscopic structures (axils, apices, basal mat etc.) were observed utilizing a dissecting microscope. Color slides of fruitbodies were also observed under the dissecting microscope. Tramal tissues were observed by making squash mounts in 3% KOH solution with fragments from different parts of basidiocarps and examined under phase contrast microscopy, usually unstained. Dubious

gloeoplerous hyphae were observed by staining with sulfo-benzaldehyde (SBZ) solution.

Spores were observed and measured in 3% KOH solution and spore amyloidity was visualized in Melzer's reagent. Usually, upper branches and/or apices gave larger numbers of spores. Spore length and width were measured for each spore in profile and 20 spores were measured per specimen. The E value (length divided by width), median E value (Em) and median length (Lm) were calculated for each specimen. The unusual highest and /or lowest measures shown by less than 3 spores out of 20 spores measured are indicated in parentheses.

All macroscopic and microscopic characters were noted on a record table sheet. Routine identifications were based on Dodd's (1972) and Petersen's (1988) keys and descriptions.

C. METHODS FOR CULTURE STUDIES

Cultures for mat analysis were either from basidiome tissue or of polypore origin. To establish tissue cultures, a piece of inside branch (about 2 mm long) of freshly collected fruitbody was excised with a sterile forceps and placed vertically on malt extract agar (MA, Appendix C) so that the tramal tissue faced the agar. Streptomycin sulfate was sometimes added to the medium after

sterilization. After the mycelium started growing out, agar blocks with mycelium were transferred into slanted test tubes containing MA agar. To make polyspore cultures, spore prints were collected following the methods described in mating study methods below. After spore germination, inoculum blocks bearing many spores were transferred into slanted test tubes containing MA agar. The germinated mass of spores was assumed to contain all genotypes. All tissue/polyspore cultures were stored in the dark at 4°C. Each isolate was marked with the same number as the original basidiome collection. Isolates selected for culture studies are listed in Appendix A.

Culture studies were carried out following standard methods described by Nobles (1965) and Stalpers (1978). Stock cultures were inoculated separately on MA agar and incubated in the dark at 22-24°C for each isolate of each species. After 2 weeks, most isolates grew enough to obtain a number of inoculum plugs. In some slow-growing species, 4 weeks growth was required for sufficient development of the advancing zone. From each actively growing culture, nine 5 mm square inoculum plugs were removed from the advancing zone and transferred to the edge of 100 X 15 mm Petri dishes containing MA, Potato Dextrose Agar (Difco, PDA) and Malt Yeast Glucose Agar (MYG) (Appendix C), mycelium side down. Three replicates of each isolate on each medium were incubated at 22-24°C in the dark with

brief exposure to light once a week for culture growth-rate measurements. Growth rates were measured every seven days and recorded on a pre-made form for all replicates of all cultures. Culture mat macromorphology and advancing zone micromorphology were examined and recorded at week II using the terminology of Nobles (1965) and Stalpers (1978) for one replicate of each isolates. Aerial and submerged hyphal micromorphologies were recorded at week VI also following standard terminology. Culture study results are presented in Chapter III B.

D. METHODS FOR INCOMPATIBILITY STUDIES

Field Work

In the field, fruitbodies of *Clavicornia* were collected extensively and characterized following the procedure described above. Fresh fruitbodies were transported to the laboratory as soon as possible.

Spore Print Collection

Spore prints were obtained by excising a few hymenium-bearing branches from freshly collected fruitbodies and suspending them (using petroleum jelly) from the inside cover of either a sterile empty Petri dish (35 x 10 mm) or a medium-sized Petri dish (60 x 15 mm) with MA, placed at an angle to allow for wider spore dispersal. After spore

prints were visible, the fruitbody pieces were removed from the inside of the Petri plate and spore prints were retained for isolating purpose.

Single-spore Isolation

From spore prints in empty Petri dishes, spores were gathered in a sterile water film in a bacteriological loop and suspended in a test tube containing sterile water. Two subsequent dilutions were made from this suspension and a few drops of each dilution were decanted onto a series of Petri dishes with MA. The dishes were wrapped with Parafilm and placed at room temperature under ordinary laboratory conditions to allow spore germination. For spore prints on agar, the plates were wrapped and kept under laboratory conditions to allow spores to germinate. All spore-containing plates were inspected twice a day for spore germination, utilizing a Wild stereo dissecting scope.

After spores germinated and grew out, about 60 μm , single germlings were harvested and placed approximately 1 cm apart in a large Petri dish (100 X 15 mm) containing MA. After colonies attained about 4 mm diam, they were transferred to slants of MA and allowed to grow for 2-several weeks. Well-established single-spore isolates were then stored at 4°C for subsequent mating tests.

All cultures and mating pairs were grown at room temperature (21-24°C) under typical laboratory light

conditions.

Long Term Storage

For highly stable and low mutable storage, duplicates of stock single-spore isolates were also stored using the methods of Maekawa *et al.* (1988). Mycelia with attendant agar blocks were suspended in A-MM vials containing 10% glycerol and stored at -70°C. Cultures can be stored using this method for years without transfer.

Intracollection Matings

Intracollection matings were carried out to ascertain the compatibility system of each collection. Before mating tests, each single-spore isolate was transferred into a Petri dishes of MA and allowed to grow up to provide inocula. These single-spore isolates were examined for absence of clamp connections to make certain of a monokaryotic nuclear condition.

Pairings between single-spore isolates were made by placing two inoculum blocks (about 3 mm square) about 1 cm apart in the center of a Petri dish (60 x 15 mm) of MA. Matings were allowed to grow until hyphae from each donor contacted (3 weeks or more), matings were examined microscopically for clamp connections, barrage reaction or flat phenomena between pairs. Establishment of dikaryotic hyphae was indicated by the formation of clamp connections

and was interpreted as evidence of a compatible mating. Incompatible matings were indicated by absence of clamp connections. A "barrage" reaction was considered to be an indicator of common-B matings and "flat" morphology was considered to an indicator of common-A matings. For some extremely slow-growing species, matings were carried out by placing two inoculum blocks together side by side, so that less growth time was required.

All matings were examined microscopically for presence of clamp connections. In species where clamp connection were of sufficient size, microscopic observations were made directly at the zone of contact using a Wild compound light microscope equipped with a 25X ocular lens and a 10X objective. In species where clamp connections were not easily detected, a small portion of contact zone mycelium was removed, stained with phloxine, and viewed at 1000X. For matings in which clamp connections were hardly found, mycelium was mounted with DAPI (4'-6-diamidino-2-phenylindole) solution and observed under epifluorescence microscopy for paired nuclei. Peripheral hyphae of each isolate in compatible matings were also examined for presence or absence of clamp connections, as indirect evidence of nuclear migration.

For this dissertation, mating results are presented in grids. Compatible matings which formed abundant clamp connections all over the mating plates, are symbolized by a

(+). Otherwise, presence of clamp connections were indicated by a (+) in the center of a square and presence or absence of clamp connections on the peripheral hyphae of each isolate were indicated by normal (+; clamp connections present) or (-; clamp connections absent) on the upper left or lower right corner of a grid corresponding to the isolate number on the left side or bottom of the mating grid. Barrage phenomena, with or without pseudoclamp connections, were symbolized by a (B) and flat phenomena were indicated by an (F). Columns and rows from the resulting grid were adjusted by clustering like mating types to elucidate the mating system pattern. Furthermore, mating time, mating morphology, nuclear migration and some other characters were recorded.

Ten single-spore isolates were paired in all combinations. When necessary, more isolates were tested until the four mating types were obtained. Subsequently, four single-spore isolates were selected as testers based on mating types, fast and healthy growth, and ability to form abundant clamp connections.

Inter-collection Matings

To test inter-collection compatibility, testers from one collection were paired with testers from another collection in all possible combinations, following methods described in intracollection matings. When necessary, more

single-spore isolates from each collection were paired to investigate population variations. Alleles at each mating type were then assigned to respective strains on the basis of mating reactions of paired colonies.

Inter-collection mating pairs were selected among: 1) collections from the same log to test natural distribution of alleles; 2) collections of the same morphospecies from different geographical areas to determine distribution of biological species; 3) collections of the same morphospecies but with distinct morphological differences to test the range of morphological variation; 4) collections from certain similar morphospecies to determine the presence of genetic isolation; and 5) collections from certain sympatrically distributed species to investigate speciation and species delimitation.

E. SPOT TEST METHODS

Combined with culture studies, spot tests for presence or absence of laccase and tyrosinase were carried out on tissue/polyspore cultures (on MA, PDA and MYG) of selected collections representing most species studied. The following reagents were employed in spot tests: 1) Syringaldazine (Harkin & Obst, 1973) as a specific substrate of laccase activity; 2) *p*-cresol (Marr, 1979) as a specific substrate for tyrosinase; and 3) distilled water and 95%

ethanol as controls.

At week II and week VI, one replicate of each isolate on each medium was recorded, macroscopically and microscopically, for culture morphology. Simultaneously, the same replicate was used in spot tests for laccase and tyrosinase activity. In spot tests, four pie-shaped culture wedges with advancing zone as the marginal arc were cut out from each culture replicate and placed in a separate 35 X 10 mm Petri dish. Each culture wedge was then flooded with a different test reagent and reactions were recorded at 10, 30 and 60 minutes, and at 3 and 24 hours. In syringaldazine tests, positive (+) reactions were indicated by a purple-pink coloration. In *p*-cresol tests, positive reactions were indicated by brown coloration. Negative reactions (-) were indicated by no color change or the same color as in water and ethanol. Three plus marks (+++) were donated to strongest reactions in which the entire wedge changed to very deep color rapidly. Two plus marks (++) were assigned to less strong reactions in which most of wedge changed color and one plus mark (+) to weak reactions in which only a small part of the culture wedge changed color. Data obtained from spot tests are presented in Chapter III D.

F. METHODS FOR ISOZYME ANALYSIS

Sample Cultures

Samples were prepared by inoculation of a 5 mm plug of a actively growing tissue culture (or polyspore culture) on MA into 25 ml of potato-dextrose-yeast-mineral broth medium (PDYB, Appendix C) in a 125 ml flask. All flasks were sealed with cotton ball and aluminum foil and placed under ordinary laboratory conditions of temperature and light. After sufficient stationary incubation (usually 6 weeks, but with some species requiring several months) samples were collected: media for laccase analysis and mycelium for tyrosinase analysis. The samples were either used immediately or stored at -70°C for up to several months before use. Laccase activity was detected in aseptically withdrawn samples of medium, using o-tolidine (modified from Leslie & Leonard, 1979) as a chromogenic substrate. Tyrosinase activity was detected in supernatant obtained by grinding mycelium in sodium phosphate buffer (see below for details) with L-Dopa (Angleton et al, 1984) as substrate.

Gel preparation

Vertical polyacrylamide slab gels measuring 16 x 14 x 0.2 cm were used. Non-frosted gel glass was washed with soap and water and then rinsed thoroughly. Glass was not touched with finger after this point. Plates were then dried.

Two spacers (2 mm thick) were placed between glass plates and secured with clips. The bottom of the plates were taped to hold the agarose and then the whole apparatus was heated at 60°C oven for three minutes. The edges were sealed by adding melted 2% agarose solution along the inside edges with a pipette, followed by cooling.

For stock solutions and concentrations see Appendix D.

The separating gel (10%, Vol 45 ml) was made by combining 15 ml 30% acrylamide stock solution and 11.25 ml 4X separating gel buffer (0.2 M Tris-HCl, pH=8.8) in 18.75 ml distilled water, degassing the solution in a vacuum flask for at least 15 minutes, and then adding 240 μ l 10% ammonium persulfate which was prepared immediately before using and 10 μ l TEMED. The solution was swirled gently without introducing oxygen and poured between the glass plates, allowing enough room for a comb (3 cm). A few drops of butanol were added to the central top of the gel without disturbing the gel so that the entire surface was covered evenly with butanol. The separating gel was retained at room temperature for one hour to allow polymerization and then either used immediately or stored at 4°C overnight.

The stacking gel (5%, Vol 15 ml) was made by combining 2.5 ml 30% acrylamide stock with 3.75 ml 4X stacking gel buffer (0.2 M Tris-HCl, pH=6.8) in 8.75 ml distilled water, degassing as above for 15 minutes, then adding 90 μ l 10% ammonium persulfate and 3.75 μ l TEMED. The butanol on top of

the separating gel was removed with a Pasteur pipette. A small amount of acrylamide solution was added and sloshed back and forth to remove residual butanol and then removed with a pipette. A comb was inserted and the rest of the acrylamide solution was added until full. Butanol was then added as above. The whole gel was placed at room temperature and allowed to polymerize for 1 hour.

Laccase Sample Preparation

Samples for laccase electrophoretic analysis were prepared by combining 25 μ l of sample culture medium with 25 μ l of sucrose sample buffer (10% sucrose in 1X upper electrode buffer), and 1 μ l 0.4% bromphenol blue was added as a tracking dye. The sample was either used immediately or frozen at -70°C .

Tyrosinase Sample Preparation

Samples for tyrosinase electrophoretic analysis were prepared according to the following procedures: 1) from the sample cultures, pieces of the mycelial mat (enough to fill 1/2 of a microfuge tube) were removed and blotted dry; 2) 1X 25 mM sodium phosphate buffer (pH=6.0) was added to the mycelium and macerated manually with a grinding tube, kept in ice; 3) the suspension was poured into a microfuge tube and centrifuged at 10,000 g at 4°C ; 4) 25 μ l supernatant and 25 μ l 50% glycerol were combined, and 1 μ l 0.4%

bromphenol blue was added as tracking dye. The remaining supernatant was stored at -70°C for up to several months.

Standard Sample

Electrophoretic mobility is subject to variation caused by any minor change in parameters of electrophoresis, such as Ph of solutions, temperature and voltage. A standard sample was applied to each gel for the purpose of comparison and calculation of relative mobility. *Clavicornia pyxidata* strain ATCC No. 24439 isolated from the representative specimen by Dodd (1972) was chosen as the standard sample and applied in each gel for both laccase and tyrosinase analysis.

Electrophoresis

The butanol on top of the stacking gel buffer was removed by upper electrode buffer and the comb was removed after marking the wells with a glass-marking pen. Three hundred milliliters 1X upper electrode buffer (50 mM Tris + 383 mM glycine, pH=8.3) was poured into the top container of the electrophoresis instrument. Wells (except the first and last wells) were loaded with 50 μ l samples + sample buffer including a standard sample added to each gel. Three hundred milliliters 1X lower electrode buffer (63 mM Tris-HCl, pH=7.5) was poured into the bottom container of the electrophoresis apparatus. The loaded gel was placed in a

cold room and electrodes attached to the power source, - on top and + on bottom. Voltage was first adjusted to 150 volts until the tracking dye reached the bottom of the wells and then decreased to 50 volts until the tracking dye reached the bottom of the separating gel (approx. 10 hours).

Staining for Laccase and Tyrosinase Activity

After the tracking dye reached the bottom of the separating gel, gels and clips were removed from the electrophoresis chamber and spacers were pulled out gently. Plates were pried apart with a spatula. The stacking gels were cut off and a corner of the separating gel near lane one was cut off to identify orientation of the gel. The gel was then immersed in approximately 200 ml of staining solution. Bands of protein with laccase activity were visualized in a 10 % ethanolic solution of o-tolidine buffered to pH 4.7 with sodium acetate, modified from Leslie & Leonard (1979) (appendix C). Bands of protein with tyrosinase activity were located by immersing the gel in 0.1 M sodium phosphate buffer (pH 6.0) containing 1 mM L-Dopa and 1 g catalase per 100 ml staining solution (Ingebrigtsen *et al.*, 1984) (see appendix D). Bands appeared after 20 minutes.

Gel Photographing

After the bands were fully developed and before they faded, the gel was placed on a light box and photographed at $t=1/15$ seconds, $f=5.6$. Polaroid 55 P/N, black-and-white film was used.

Gel Recording

After staining, band and tracking dye positions were measured and recorded directly. The origin of migration was noted as the interface between stacking and separating gels. Relative mobility (R_f) was measured at the midpoint of the band divided by the dye front mobility and calculated as band mobility. Relative mobility of a standard sample (*Clavicornia pyxidata*, 24439) was calculated and indicated as R_s . Internally calibrated mobility (R_i) was then calculated as $R_i = R_f/R_s$. The R_i value of the standard sample was 1.0.

Determination of Developmental Variation

Laccase and tyrosinase activities were suspected to change during cultural development. Broth cultures were sampled weekly starting at the third week till the 12th week. Collected samples were stored at -80°C . All samples gathered from different developmental stages were co-run together with the calibration standard sample.

CHAPTER III

RESULTS

A. MORPHOLOGICAL DESCRIPTION OF SPECIES

Clavicornia candelabrum (Masse.) Corner. 1950. Ann. Bot.,
Mem. 1, 286, figs. 2F, 19, 20, 22, 23, 106; pl. 15,
fig. 5.

≡ *Clavaria candelabrum* Masse. Kew Bull. Misc. Inform.
1899: 172. 1901.

Fruitbodies up to 3 X 2.5 cm, slender, pyxidately
branched, densely gregarious, arising from brown mycelial
mat; stipe short, up to 3 mm diam; stipe and lower branches
brownish orange (7C4); ("wood brown," "fawn color,"
"fuscous," "army brown," "benzo brown"); upper branches
sahara (6C5) to greyish orange (6B4) upward ("avellanus,"
"cinnamon buff," "light cinnamon drab"); branching up to 3
ranks; apices bluntly coronate, concolorous with upper
branches or paler; consistency tough; drying brownish black
or black; taste quickly acrid, sometimes also bitter; odor
typical.

On rotting wood, including *Leptospermum*.

Trametal hyphae of three types: 1) inflated, 4-15 μ m
diam, clamped, hyaline to yellowish, brown to reddish brown
when aggregated, densely packed, parallel, hard to separate,
thin- to slightly thick-walled, (wall up to 1.0 μ m thick);

2) thick-walled (wall up to 2 μm thick) generative hyphae, 3-8(-15) μm diam, somehow semitransparent, clamped, yellow, sometimes roughened; and 3) gloeoplerous hyphae abundant, 4-15 μm diam; contents yellowish, dense, refractive, stained black in SBZ.

Subhymenium approx. 20 μm thick, not obviously gelatinous. Hymenium more than 20 μm thick; basidia 15-20 X 4-5 μm , clavate, hyaline, clamped at base, 4-sterigmate; sterigmata up to 4 μm long; gloecystidia ventricose, projecting up to 30 μm beyond hymenium.

Spores 4.0-5.0 X 2.0-3.0 μm ($E=1.33-2.0$; $E_m=1.61$; $L_m=4.3$ μm), broadly elliptical, weakly amyloid, minutely roughened, uniguttulate; apiculus typical of the genus.

Discussion: This description is based on collections 2681, 2679, 2802 and 2807 from New Zealand. These collections share the following common characters: 1) very densely gregarious habitat; 2) relatively small, milk-chocolate brown to dark brown fruitbodies drying brownish black or black; 3) tough consistency, drying hard; 4) aggregated hyphae reddish brown; 5) some hyphae roughened, thick-walled; 6) basidia short and broad; 7) closely similar spore measurement. Except for small differences, such as notation of "drab" coloration in 2681 and bitter taste of 2679, these collections are virtually identical and constitute a good group. This group has been identified as

C. candelabrum based on Dodd (1972) and Petersen's (1985) description of the type specimen.

Corner (1950) reported "skeletal hyphae" in this species. Dodd (1972) described that these were actually secondarily thick-walled generative hyphae, based on examination of the type specimen. Petersen (1985) also studied the type specimen and stated that the so-called thick-walled hyphae might be collapsed tramals. In my study, thick-walled hyphae were observed, but not typical thick-walled generative or skeletal hyphae. The hyphae were yellowish, thick-walled and also roughened, semitransparent, and seem to be gloeoplerous hyphae without contents, but not stained in SBZ. These hyphae have been assumed to correlate with the thick-walled hyphae noted by Dodd.

Dodd (1972) also reported that the hymenium and subhymenium of *C. candelabrum* were gelatinized. However, Petersen (1985) concluded from the type specimen that such gelatinized hymenium was a phenomenon of poor drying. Gelatinized hymenium is not observed in the new collections although it has proved to be present in other species. In this study, the above collections from New Zealand listed above has been considered to be *C. candelabrum*. Further studies of all taxa in New Zealand, employing modern biological methods, are needed.

Clavicornia colensoi (Berk.) Corner, Ann. Bot. Mem. 1: 287,
1950.

≡ *Clavaria colensoi* Berk. in Hooker, Flora Novae-
Zealandiae, p. 186, 1855.

Fruitbodies up to 2.5 cm high, 1.5 cm wide, slender, delicate, divaricately and pyxidately branched, gregarious to cespitose, arising from an off-white mycelial mat; stipes short, up to 2.5 mm long, 1 mm wide, or absent, red-haired (6C4), ("avellaneous"), to light brown (6D5); branches in 2-4 ranks, greyish orange (6B4) to pale orange (5A3) ("pale ochraceous buff" to "tilleul buff"); internodes diminishing gradually; apices paler, orange white (6A2) to white, coronate and cuspidate; texture pliable; fruitbody drying dark brown; taste quickly acrid; odor typical.

On rotten wood, including *Nothofagus* and *Leptospermum*.

Tramal hyphae 4-15 μm wide, thin- to slightly thick-walled (wall up to 0.5 μm thick), inflated, clamped, densely interwoven, not gelatinous, hyaline, yellowish brown when aggregated. Subhymenium approx. 20 μm thick; hyphae 2-6 μm wide, clamped, tightly interwoven; gloeoplerous hyphae abundant, 4-15 μm wide, with refractive yellowish contents, elongated, stained black in SBZ, clamped at base, usually ending in gloeocystidia in hymenium.

Hymenium up to 25 μm thick; basidia 15-20 x 3.5-5.0 μm , clavate, thin-walled, hyaline, 4-sterigmate;

sterigmata up to 4 μm long, slightly curved; gloeocystidia 4-13 wide, inflated, with yellowish refractive contents, projecting up to 30 μm beyond basidia, ventricose; leptocystidia not observed.

Spores 4.5-6.0 x 3.0-4.0 μm ($E=1.25-1.83$; $E_m=1.48$; $L_m=5.2 \mu\text{m}$), elliptical to broadly ovoid, amyloid, white in mass, thin-walled, minutely roughened, uniguttulate in contents; apiculus typical of the genus.

Discussion: This description is primarily based on collection No. 2644, with supplementary reference to nos. 2555, 2637, 2685, 2667, 44117 (TENN) and 55203 (Buchanan). In the field, these collections were characterized by small, pale ochraceous buff fruitbodies which dried brown to dark brown. Microscopically, aggregated hyphae are yellowish brown; hyphae are not obviously thick-walled; and subhymenium and hymenium are not gelatinized. Collection 2685 was classified into this species because of its overall similarities. The spores of 2685, however were smaller than the remaining collections ($E_m = 1.65$; $L_m = 4.8 \mu\text{m}$).

Clavicornia colensoi has been described only from New Zealand (Dodd, 1972; Petersen, 1988). However, the morphology of *Clavicornia* collections from New Zealand are so similar that species delimitation has been very difficult and therefore has resulted in taxonomic confusion (Corner, 1950; Dodd, 1972; Petersen, 1988). In this study, all

collections from New Zealand are classified into four groups according to minute morphological differences. Based on my understanding of the species, the following species names represent the four groups: *C. colensoi*, *C. candelabrum*, *C. piperata* and *C. turgida*. For comparison, see Table 1 under *C. turgida*.

Clavicornia cristata (Kauffm.) Doty. Lloydia 10: 40, fig. 1. 1947.

= *Craterellus cristatus* Kauffm. Pap. Michigan Acad. Sci. (1929) 11: 172, figs. 3. 1930.

Fruitbodies up to 8 mm high, 1 mm wide, narrowly coniform, with broad coronate apices, rarely branched, gregarious, arising from a small, brown mycelial patch, light yellowish brown above, light greyish yellowish brown downwards; texture tough, pliable; taste unknown.

On rotting *Quercus* wood.

Trametal hyphae 4-10 μm wide, thin-walled, hyaline, clamped, inflated, not gelatinous. Subhymenial hyphae 2-5 μm wide, clamped, tightly interwoven; gloeoplerous hyphae abundant, 3-6 μm diam, elongated, with yellowish, refractive contents, stained black in SBZ, extending into hymenium.

Hymenium more than 35 μm thick; basidia 25-35 X 4-5 μm , clavate, thin-walled, clamped at base, 4-sterigmate; sterigmata up to 4 μm long, slightly curved;

gloeocystidia up to 5 μm wide, mucronate or clavate, projecting up to 5 μm beyond basidia; leptocystidia rare, unbranched, filamentous, 2 μm wide, projecting up to 10 μm beyond basidia.

Spores 6.0-7.0 X 2.0-2.5 μm ($E=2.4-3.5$; $E_m=2.9$; $L_m=6.3$ μm), elongate-elliptical to allantoid, thin-walled, hyaline, uniguttulate, white in mass, amyloid, minutely roughened; apiculus typical.

Discussion: This species is distinct from all others because of: 1) small, unbranched basidiocarp on rotten wood; 2) elongate basidia; and 3) elongate elliptical basidiospores. Spores of this species are quite peculiar, with $E_m=2.9$. In this study, collection no. 8974 from California was identified as *C. cristata*. Two culture collections from Sweden were contributed without specimens.

Clavicornia microspora sp. nov. (Fig. 1)

Basidiomata circa 9 cm alta, 6 cm lata, caespitosa, pyxidata; ramulis, gracilis, infra luteo-albis, supra dilute ochraceo-bubalinis vel dilute subroseo-cinnamominis; apicibus coronatis, acutis, pallide subroseo-cinnamominis; sapore infirme acerbis. Hyphae 1.5-10 μm latae, subinflatae, parietibus tenuibus vel parum incrassatis instructae, fibulatae. Hymenium ad 20 μm crassum. Basidia 10-18 X 3-4.5 μm , clavata, fibulata. Gloeocystidia 3-4.5 μm

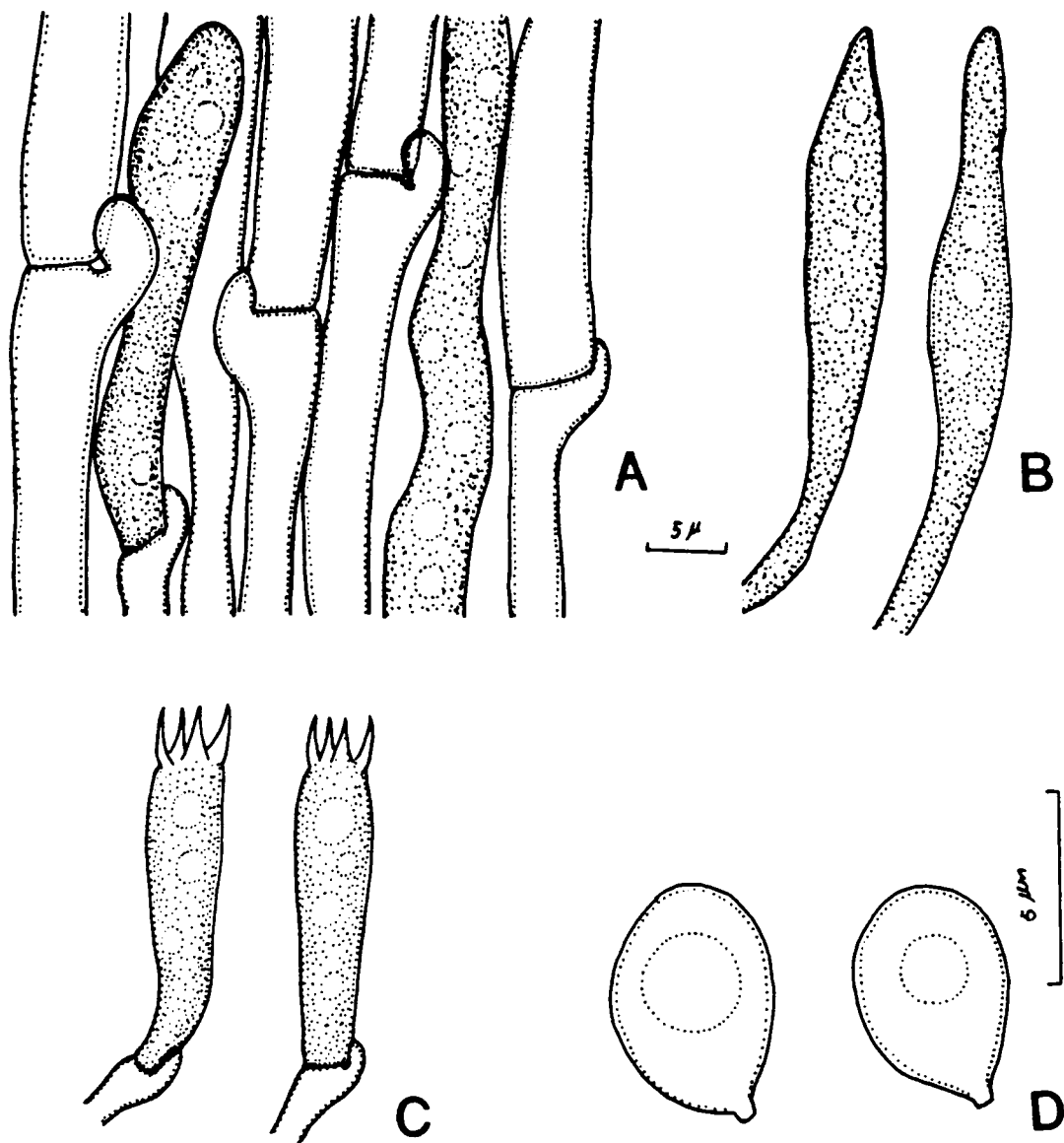


Fig. 1. *Clavicornia microspora* - A) tramal hyphae with gloeoplerous hyphae; B) gloeocystidia; C) basidia; D) basidiospores.

lata, cylindrica vel ventricosa. Leptocystidia 3-6 μm lata, filamentosa. Sporae 3.0-4.0 X 2.0-2.5 (-3.0) μm , anguste ovatae vel ellipsoideae, amyloideae, prope laevigatae, apiculo obliquo instructae.

HOLOTYPUS: Japan, Mashiko town, 89 IX 29, Coll. R. H. Petersen, 2349 (TENN 48856)

Fruitbody 7-9 cm high, 4-6 cm wide, typically pyxidately and very finely branched, cespitose; stipes rare, branched at very base; basal mycelial patch brown ("pecan brown"); branches in up to 7 ranks; lower branches yellowish white (2A2), pale yellow (2A3) to pastel yellow (2A4), upward "pale ochraceous buff" to "pale pinkish cinnamon," with slight tint of flesh or pink; branches slender, crowded; apices coronate, cuspid, last 2-4 mm light orange (5A5) ("pinkish buff" to between "vinaceous buff" and "light pinkish cinnamon"); texture drying very fragile; flesh white, inamyloid; taste slowly, weakly acrid.

On rotten wood

Tramal hyphae 1.5-10 μm wide, inflated, hyaline, clamped, thin- to slightly thick-walled (wall up to 0.7 μm thick), very tightly packed, parallel, not gelatinous. Subhymenial hyphae 1.5-3.5 μm wide, thin-walled, clamped, tightly interwoven, hyaline; gloeoplerous hyphae abundant, 3-8 μm wide, clamped at base, with yellowish, refractive contents, elongated,

ending in hymenium as gloeocystidia, staining black in SBZ.

Hymenium up to 20 μm thick, not gelatinized; basidia 10-18 X 3-4.5 μm , thin-walled, hyaline, clamped at base, clavate, (2-)4-sterigmate; sterigmata 2-4 μm long, slightly curved; gloeocystidia 3-4.5 μm wide, projecting up to 16 μm beyond basidia, cylindrical or ventricose; leptocystidia 3-6 μm wide, filamentous, hyaline, projecting up to 13 μm beyond basidia.

Spores 3.0-4.0 X 2.0-2.5 (-3.0) μm ($E=1.2-2.0$, $E_m=1.5$, $L_m=3.4$ μm), narrowly ovate to ellipsoid, amyloid, white in mass, nearly smooth, uniguttulate; apiculus <0.5 μm long, typical of the genus.

Discussion: This description is based on collection no. 2349 from Japan, originally identified as *C. pyxidata*. Fruitbodies of this collection are characterized by crowded, pyxidate, fine branches with light orange colored apices; drying fragile; extremely abundant gloeoplerous hyphae; short basidia and very small spores. This species is similar to Chinese *C. pyxidata* (no. 1541) in its crowded, pyxidately branched fruitbody. However, the light orange color of apices of no. 2349 are quite peculiar and its spores are shorter and broader than those of no. 1541 ($E_m=1.8$, $L_m=4.1$ μm). Subsequent mating tests and isozyme electrophoresis provide additional evidence that the Japanese collection represents a different species from *C.*

pyxidata.

Clavicornia microspora is also similar to *C. candelabrum* in that both have light colored apices and short basidia. Dodd (1972) suggested that *C. candelabrum* was distinct from others in the genus because of the presence of thick-walled hyphae in the basidiomata. Petersen (1985) examined the type specimen of *C. candelabrum* and found no thick-walled hyphae. If thick-walled hyphae are absent in *C. candelabrum*, these two species are even more similar. Based on the description of the type specimen given by Petersen (1985), *C. candelabrum* had small fruitbodies (20 X 12 mm dry) which are brown-black and cartilaginous after drying. In contrast *C. microspora* has relatively large, crowded-branched fruitbodies and remained yellowish color when drying. Spores of no. 2349 seem smaller than those of *C. candelabrum*, but more careful examination of *C. candelabrum* is still needed.

Clavicornia microspora is different from *C. turgida* in the lighter color of basidiomata, slender branches, hyphal texture that is not gelatinous and smaller spores. *Clavicornia dryophila* is characterized by trumpet-like branches, presence of chlamydospores and larger basidiospores, which differ from *C. microspora* (Maas Geesteranus, 1973).

Clavicornia piperata (Kauffm.) Leathers & Smith, Mycologia
59: 461, 1967.

= *Clavaria piperata* Kauffm., Pap. Mich. Acad. Sci. 8:
146, 1928.

Fruitbodies up to 7 x 5 cm, gregarious, pyxidately branched, arising from off-white mycelial patches; stipe up to 1 cm long, 5 mm wide, light brown (7D5; "avellanceous," "wood brown"); major branches few; middle branches up to 3 mm wide, flesh colored (6B3; "pinkish buff," "avellanceous," "wood brown") upwards; old branches light brown (7D5); branching in 3-4 ranks; internodes diminishing gradually; apices small, orange-white (6A2; "pale pinkish cinnamon," "avellaneous"), coronate; texture pliable; drying dark brown to brownish black; taste quickly acrid; odor typical.

On rotten wood, including *Nothofagus*.

Tramal hyphae 4-20 μm diam, inflated, hyaline, yellowish when aggregated, densely packed, parallel, not gelatinous, clamped, thin- to slightly thick-walled (wall up to 0.5 μm thick). Subhymenium up to 15 μm thick, not gelatinous; subhymenial hyphae 1.5-5.0 μm diam, tightly interwoven, clamped, thin-walled, with dense contents; gloeoplerous hyphae 4-7 μm diam, abundant, elongated, with yellowish refractive contents, staining in SBZ, ending as gloeocystidia in hymenium.

Hymenium 25-30 μm thick; basidia 20-25 x 4-6 μm , clavate, thin-walled, clamped at base, 4-sterigmate;

sterigmata 4-5 μm long, straight to slightly curved; gloeocystidia clavate, lanceolate, projecting up to 30 μm beyond basidia; leptocystidia up to 2.5 μm wide, filamentous, straight to rarely branched, hyaline, thin-walled, projecting up to 20 μm beyond basidia.

Spores 5.0-6.0 X 4.0-5.0 μm ($E=1.0-1.5$; $E_m=1.19$; $L_m=5.8$ μm), subglobose to broadly ovate, thin-walled, white in mass, uniguttulated, amyloid, obviously roughened; apiculus typical.

Discussion: This description is based on collection nos. 2822, 2672 and 55204 (Buchanan). As in *C. pyxidata* and *C. colensoi*, fruitbodies of *C. piperata* are variable in color, pale when young or in very poor light, darker when mature or exposed. Therefore, fruitbody color as a character must be accompanied with other microscopic characters, among which spore dimensions were found to be useful. Spores of these collections are obviously larger and broader than any other New Zealand collections. Dodd (1972) and Petersen (1988) believed that *C. piperata* had larger fruitbodies and larger spores than those of *C. colensoi*. Neither of these species have distinct thick-walled tramal hyphae and gelatinized hymenium, which are the distinguishing characters of other two species from New Zealand, *C. turgida* and *C. candelabrum*. In this study, the delimitation of *C. piperata* is congruent to Petersen's (1988), in which spore

dimensions were reported as approximately 1 μ m larger than those given by Dodd (1972). Dodd measured spores in Melzer's reagent, while in this study, spores were measured in 3% KOH which might expand spores to their actual size. More detailed comparison is given in Table 1 under the description of *C. turgida*.

Clavicornia pyxidata (Pers. : Fr.) Doty, Lloydia 10: 43,
fig. 3, 1947.

≡ *Clavaria pyxidata* Pers., Neues Mag. Bot. 1:117, 1794.

≡ *Clavaria pyxidata* Pers. : Fries. Syst. mycol. 1: 470,
1821.

Fruitbodies 3-10 cm high, pyxidately branched, arising from a small, white to brownish mycelial patch, gregarious to caespitose, variable in color according to age and/or individuals, from light yellow (2A5) to brown (6E8). Stipe usually short, light brown (6D8) to greyish orange (6B4); branches in up to 6 ranks, light yellow (2A5) ("maize yellow," "greyish yellow," "pale orange-yellow," "naples yellow") when young, fading when old to pale yellow (2A3) in upper branches, downward brownish orange (6C8; "cinnamon-buff"), grayish-orange (6B5; "vinaceous-cinnamon," "wood brown," "clay color," "sayal brown," "isabella color," "chamois") to brownish orange (6C8), cinnamon (6D6; "tawny-olive"), when very old becoming completely brown; primary branches 1-3 mm wide; secondary branches usually

<1 mm wide; apices pale yellow (2A3) to yellowish white (2A2; "maize yellow"), coronate to cuspidate; texture tough, pliable; flesh white, inamyloid; taste slowly acrid to very acrid.

On rotten deciduous and conifer wood.

Tramal tissue aerenchymatous; tramal hyphae 4-15 μm wide, hyaline, inflated, clamped, thin- to slightly thick-walled (wall up to 0.5 μm thick); gloeoplerous hyphae 3-10 μm wide, with yellowish refractive contents, elongated, ending as gloecystidia in hymenium. Subhymenial hyphae slender, 1.5-4.5 μm wide, tightly interwoven, clamped, thin-walled, with rich contents.

Hymenium 20-30 μm thick; basidia 17-25 x 3.5-4.5 μm , thin-walled, clavate, clamped at base, (2-)4-sterigmate; sterigmata 2.5-4.0 μm long, slightly curved; gloecystidia 3-6 μm wide, cylindrical to ventricose, projecting 7-20 μm beyond basidia; leptocystidia 3-6 μm wide, filamentous or ventricose, hyaline, projecting up to 10 μm beyond basidia.

Spores 4.0-5.0(-6.0) X 2.0-2.5(-3) μm (E=1.6-2.6; Em=1.8; Lm=4.7 μm), elliptical, white in mass, amyloid, thin-walled, minutely asperulose; apiculus 0.5 μm .

Discussion: Collections of *C. pyxidata* were obtained from the southern Appalachian Mountains, including the Great Smoky Mountains National Park (GSMNP), Macon Co., especially

Highlands in North Carolina and nearby areas in Georgia, northeast New York state and Madison, Wisconsin. Two collections were gathered from the Changbai Mountains, northeast China and Simao, southwest China.

One of the important characters in identifying *C. pyxidata* is the narrow basidiospores, with average length/width ratio as 1.8-2.0. Wide variation in fruitbody size, color and branching, however, has led to taxonomic confusion (Dodd, 1972). Collection no. 1706 and 1190 from New York State showed "pale ochraceous buff" color over tips and upper branches, greyish orange color (6C4), "clay color" to "sage brown" downward, and single, slender, elongated stipes with fine, 4-6 ranked branches. Collection no. 56655 from North Carolina had "cream buff" apices, "tawny-olive" branches and "light brownish olive" stipes, while another collection (no. 56653) from North Carolina showed "maize yellow" in apices, "naples yellow" in middle branches and "isabella color" to "chamois" at base. Collection no. 1607 from GSMNP was characterized by small basidiocarps (3 X 2 cm) and greyish yellow (4B4) colored branches. Twenty collections from a restricted area near Madison, Wisconsin, were representatives of the large population abundantly occurred in these areas. Basidiocarps of those collections varied from 5 cm to 20 cm high, light yellow to light brown-colored in apices, cream-white to greyish brown over the branches. The two collections from China (no. 1449 and

1541) also showed differences. Fruitbodies of no. 1449 were relatively small and wholly "pale pinkish cinnamon" colored, with no sign of yellow, and rather open branching. On the other hand, no. 1541 had very abundant, compact and delicate branches in up to 8 ranks, major branches sahara or red-haired, and upper branches yellowish white to pale yellow. An even more distinct character of 1541 was the gelatinized hymenium, which is an important character of *C. turgida*, although all other characters suggested that 1541 belonged to *C. pyxidata*.

Because of morphological variation, it became important to conduct mating tests to assess the range of morphological variation and geographical distribution.

Clavicornia turgida (Lev.) Corner, 1957. Darwiniana 11: 195.

≡ *Clavaria turgida* Lev. Ann. Sci. Nat. Bot., Ser. 35: 155, 1846.

Fruitbodies up to 5.5 X 3 cm, somewhat stout, pyxidately branched, gregarious, drying dark brown to black; stipe short, brownish orange (6C4) to cinnamon (6D5) ("light drab", "clay color"); branches in 3-4 ranks, light orange (5A4) to brownish orange (6C5; "light ochraceous buff," "vinaceous buff"); internodes diminishing gradually; apices cream colored (4A3) or brownish orange (7C4; "cream buff," or "wood brown"), sometimes minutely bruised to light brown (7D4; "natal brown"), coronate;

consistency fleshy, not tough; taste acrid; odor typical.

On rotting wood, including *Leptospermum* and *Nothofagus*.

Tramal hyphae 4-12 μm diam, inflated, clamped, thin- to slightly thick-walled (wall < 1 μm thick), hyaline, yellowish when aggregated, more or less gelatinous; gloeoplerous hyphae abundant, 3-12 μm diam, with dense refractive contents, yellowish, staining black in SBZ. Subhymenium 20 μm thick, gelatinized.

Hymenium about 20 μm thick, gelatinized; lower 3/4 part of individual basidium not distinct; basidia up to 20 X 5-6 μm , 4-sterigmate; sterigmata 4-5 μm long; gloecystidia not observed; leptocystidia up to 5 μm diam, filamentous, projecting up to 8 μm from hymenium.

Spores 4.5-6.0 X 2.5-4 μm ($E=1.25-1.8$; $E_m=1.53$; $L_m=4.9$ μm), broadly elliptical, thin-walled, obviously asperulous, uniguttulate, amyloid; apiculus typical.

Discussion: This description is based primarily on collection 2682, with reference to 2651 and 44103 (TENN, as *C. piperata*) from New Zealand. This species is characterized by the gelatinized subhymenium and hymenium. Under magnification, the gelatinized hymenium seems to be glued together so that individual basidia are not distinguishable and only the top 1/4 of basidia can be distinguished. A gelatinized hymenium is one of the diagnostic characters for *C. turgida*, according to Dodd

(1972), who examined the type specimen. Moreover, *C. turgida* is characterized by stout fruitbodies; yellowish white color when and young, becoming tan or brown when mature; broad basidia; and spore dimensions.

The character of a gelatinized hymenium has been used by some authors and overlooked by others. It is still doubtful whether this character can be used as a species-level criterion. The collections cited above all exhibited gelatinized hyphae and shared other characters as well, although color of fruitbodies may have varied a bit from collection to collection. It is common in this genus, however, that fruitbody color varies according to age and exposure to light. All in all, the gelatinized hymenium is considered to be a key character of these collections, identified as *C. turgida*. On the other hand, all collections of *C. pyxidata* possess non-gelatinized hymenium except for collection from China (no. 1541) whose hymenium was obviously gelatinized.

The following four species are easily confused both in literature description and in fresh specimens: *C. colensoi*, *C. candelabrum*, *C. piperata* and *C. turgida*. They all occur in New Zealand. Based on collections from New Zealand and previous work, a comparison of these four species is given in Table 1.

Table 1. Morphological comparison of New Zealand collections

eiphet	colensoi	candelabrum	piperata	turgida
Fruitbody	small, slender	small, slender	large, stout	large, stout
Color of upper branches	cream colored	fawn or avellannous	pinkish- buff avellannous	vinaceous buff cream buff
Habitat	gregarious	very densely gregarious	gregarious	gregarious
Drying color	dark brown	brownish black or black	dark brown	dark brown or black
Hyphae	no distinct thick- walled hyphae	some hyphae distinctly thick- walled	no distinct thick- walled hyphae	same as <i>piperata</i>
Hymenium	not gelatinized	not gelatinized	not gelatinized	gelatinized
Spores	Lm = 5.2 μ m Em = 1.48	Lm = 4.3 μ m Em = 1.61	Lm = 5.8 μ m Em = 1.19	Lm = 4.9 μ m Em = 1.53
Coll. numbers	2555, 2644 2667, 2637 44117 55203 (B)	2679, 2681 2802, 2807	2822, 2672 55204 (B)	2651, 2682 44103

Clavicornia taxon 1 (collection 1615)

Fruitbodies up to 4.5 cm high, 3.5 cm wide, more or less stout, pyxidately branched, arising from a small, off-white basal mycelium; stipe very short or absent, brown at very base; branches relatively stout, camel brown (6D4), elongate; in 3 ranks, internodes diminishing gradually; apices coronate and cuspidate, camel brown (6D4); texture strongly gelatinous, dry cartilaginous, dark brown; flesh paler than surface; taste very slowly acrid; odor typical of the genus.

On rotten conifer wood.

Tramal hyphae 4-8 μm wide, inflated, hyaline, thin- to slightly thick-walled (wall $< 1 \mu\text{m}$ thick), clamped, gelatinized; subhymenium hyphae 3-4 μm wide, clamped, hyaline, tightly interwoven, thin-walled; gloeoplerous hyphae abundant, 4-7 μm wide, elongated, with yellowish, refractive, multiguttulate contents, staining black in SBZ.

Hymenium 20-25 μm thick; basidia 15-20 X 4-5 μm , clavate, thin-walled, clamped at base, 4-sterigmate; sterigmata 3-4 μm long, slightly curved; gloecystidia up to 4 μm wide, cylindrical, rarely enlarged distally, projecting up to 18 μm beyond basidia; leptocystidia up to 5 μm wide, filamentous, projecting up to 23 μm beyond basidia.

Spores (3.8-)4-4.5 X 3-4 μm ($E = 1-1.35$, $E_m = 1.23$,

Lm = 4.0 μm), subglobose to broadly ovoid, thin-walled, amyloid, uniguttulate, smooth to minutely roughened, white in mass; apiculus up 1 μm long, typical of the genus.

Discussion: This description was based on a single collection from North Carolina (no. 1615). While fruitbodies of *C. pyxidata* were extensively collected from North Carolina, this collection was very distinct because of its evenly camel-brown colored fruitbodies, and more or less stout branches. Microscopically, this collection is characterized by gelatinous hyphal texture, short and broad basidia and broad spores ($E_m=1.23$, $L_m=4.0 \mu\text{m}$). The spores of no. 1615 are broader than those of *C. pyxidata* ($E_m=1.8$) and smaller than those of *C. piperata* ($L_m=5.83 \mu\text{m}$), *C. colensoi* ($L_m=4.82 \mu\text{m}$), *C. candelabrum* ($L_m=4.3 \mu\text{m}$) and *C. turgida* ($L_m=5.0 \mu\text{m}$). The most similar species is *C. turgida* which also shows stout, brownish fruitbodies, but *C. turgida* has gelatinized subhymenium and hymenium which is not exhibited by collection 1615.

Previously, Dodd (1972) reported a single collection from North Carolina (CUP-Atkinson 10700) and concluded that it was *C. turgida*. According to him, in that collection the subhymenium was gelatinous (he did not mention the hymenium) and the apices were not cup-shaped, but pointed. In collection no. 1615, the subhymenium and hymenium were not gelatinous, but tramal hyphae were so and most apices

were coronate with a few pointed apices, and most branches pyxidate with a few exceptions that were dichotomous. Atkinson 10700 and 1615 are probably identical and represent a new species. If cultures were available from South America and Asia, it would be very interesting to perform mating tests among collections identified as *C. turgida* with cultures from New Zealand to determine if they represent distinct biological species.

A collection from India (TENN 44243), identified as *C. turgida*, gave similar spore dimensions, 4-5(-6) X 3-4 μm ($E=1-1.67$, $E_m=1.25$, $L_m=4.35 \mu\text{m}$), but the fruitbody color notes accompanying the specimen read "white throughout," which is different from the camel brown color of no. 1615. The Indian collection is similar to collection no. 3456 from Puerto Rico in being white throughout, and in spore dimensions, but no. 3456 showed no sign of gelatinous tissues.

***Clavicornia* taxon 2** (collection 3456)

Fruitbodies obovate in outline, up to 3.0 cm high, 2.5 cm wide, gregarious, arising from "vinaceous buff" or "fawn color", small mycelial patches; stipe short, up to 5 mm long, 6 mm wide, white to "vinaceous buff" at very base; branches in 4 ranks, polychotomous, pure white, turning "tilleul buff" where bruised, off-white when dried; flesh white; apices pure white, coronate, cuspidate; texture soft

when dried; odor weakly typical; taste quickly intensely acrid, persisting on tongue and lips.

On rotten hardwood stick.

Contextual tissue aerenchymatous; tramal hyphae of upper branches and stipe inflated, thin-walled, hyaline, 4-15 μm diam, clamped, closely adherent but not gelatinous; gloeoplerous hyphae abundant in tramal tissue and subhymenium, but not penetrating hymenium, 4-14 μm diam, elongate; contents yellowish brown, dense, staining black in sulfobenzaldehyde. Subhymenium and hymenium together up 40 μm thick; basidia 20-30 X 4-5.5 μm , thin-walled, hyaline, clamped at base, 4-sterigmate; sterigmata 4-5 μm long, acute.

Spores 4-5.5 X 3-4 μm ($E=1.14-1.67$; $E_m=1.54$; $L_m=4.82$ μm), elliptical, amyloid, thin-walled, asperulous; apiculus minute, <0.5 μm .

Discussion: This description was based on a single specimen from Puerto Rico, collection no. 3456. The pyxidate branches, gloeoplerous hyphae, and small, amyloid spores are typical of the genus, but collection 3456 is characterized by its pure white fruitbodies and obviously asperulous and strongly amyloid spores. Spore dimensions of collection 3456 are close to those of *C. colensoi* and *C. piperata*, but basidiocarp color is different. *C. pyxidata* varies in color during development, but it never pure white as this

collection. Moreover, *C. pyxidata* produce narrower spores.

The most similar species may be *C. turgida*. According to Dodd's (1972) description, basidiocarps of *C. turgida* are "white or yellowish white, becoming dingy to brownish white". Collection no. 1615 has close color to "brownish white". One possibility is that collection 3456 may be an immature stage of *C. turgida*, but it showed no sign of "toughly gelatinous" texture as in *C. turgida*.

Collection 3456 may represent a distinct species, but more specimens and taxonomic studies are needed to determine its status. No species name is given to the collection at this time.

B. RESULTS OF CULTURE STUDIES

Cultural morphology in the taxonomy of higher basidiomycetes has been reported in a few groups (Campbell & Petersen, 1975; Desjardin, 1990; Mueller, 1982, 1984; Mueller & Fries, 1985). Dodd (1972) briefly described the culture characters of *C. pyxidata*, and James (1983) studied the physiology of fruitbody formation in the same species. The following studies on cultural characters of several *Clavicornia* species were carried out to determine the potential value of culture morphology in the systematics of *Clavicornia*.

To determine the morphological variation of cultures on

various culture media, preliminary tests were undertaken by employing the following media: Malt Extract Agar (MA), Potato Dextrose Agar (PDA), Media 597 (M597; from ATCC), Malt Yeast Glucose (MYG), and YpSs agar. (Appendix C). All available (at that time) dikaryotic isolates representing six *Clavicornia* species were examined (Table 2).

Some degree of growth were observed on all media except YpSs, on which all *Clavicornia* species showed no or little growth (0-0.2 cm/4 wks). On the other four media, the same isolates showed differing growth rates. For example, for *C. pyxidata* isolate 24439, growth rates from high to low were MA $\approx$ PDA > MYG > M597 $\approx$ MYG, while for *C. colensoi* isolate 44120, growth rates from high to low were MA $\geq$ PDA > M597 $\approx$ MYG. For most isolates, culture morphology and growth rate on M597 and MYG were similar. Growth rate of isolates on MA and PDA were close, but culture morphology was different.

Table 2. Isolates of Certain *Clavicornia* Species in Preliminary Culture Studies

Species Name	Collection Numbers
<i>C. colensoi</i>	44120, 44117
<i>C. cristata</i>	DLL8974
<i>C. microspora</i>	2349, 5352
<i>C. pyxidata</i>	22501, 56655, 56662, 56665, 56667, 56668, 1606, 1607, 1617, 1650, 1661, 1190, 1449, JPL479, 1704
<i>C. turgida</i>	44103
Taxon 1	1615

Generally, isolates of the same species showed similar culture morphology and/or growth rate on each medium, while isolates of different species showed great variability on each medium, especially on PDA and MYG. MA was a traditional medium used in standard keys (Nobles, 1948, 1965; Stalpers, 1978). PDA-grown culture morphology was confirmed to have taxonomic value (Desjardin, 1990). MYG was a medium for *Clavicornia* utilized by James (1983). For the above reasons, three media, MA, PDA and MYG, were employed for culture studies of 27 isolates representing six *Clavicornia* species (Appendix A). Macro- and micromorphologies of dikaryotic cultures on each medium were examined.

1. Culture Characters of *Clavicornia*

a. Macromorphological Characters

Mean growth rate

Growth of each replicate of each culture on each medium was measured at one week intervals for a period of six weeks or until the plates were covered. On all three media, growth rates of isolates within a given species were very similar. For *C. pyxidata*, for example, growth rates of 14 isolates ranged from 6.0 to 7.0 cm at Week IV, and plates were more or less covered at Week V, except isolate (1449),

which covered plates at Week VI. In contrast, growth rates of isolates from different species were different on each medium, as shown in Fig. 2.

On MA (Fig. 2, A), mean growth rate ranged from 1.7 cm at Week VI to covering plate (total 9.0 cm, but actual growth diameter 7.5-8.0 cm) at Week V. Isolates of *C. pyxidata*, *C. microspora* and *C. turgida* exhibited relatively high growth rate while isolates of *C. cristata* grew much slower. *Clavicornia microspora* was the only species which produced fruitbodies on MA within five weeks.

On PDA (Fig. 2, B), mean growth rate ranged from 1.0-8.0 cm at Week VI. Isolates of *C. microspora* exhibited lower growth rate than those of *C. pyxidata*. *Clavicornia turgida* grew much slower on PDA than on MA.

On MYG (Fig. 2, C), cultures grew similar to those of PDA, except that *C. colensoi* exhibited a lower growth rate than 1615, which representing taxon 1. *Clavicornia cristata* was still slowest.

Color of culture mat

Culture mat coloration ranged from white, cream to yellow, greyish orange and fawn. In some species, coloration changed during culture development, usually white or cream when young but developing brown pigment in older regions. Sometimes pigments formed patches. Culture mat color was recorded at Week VI.

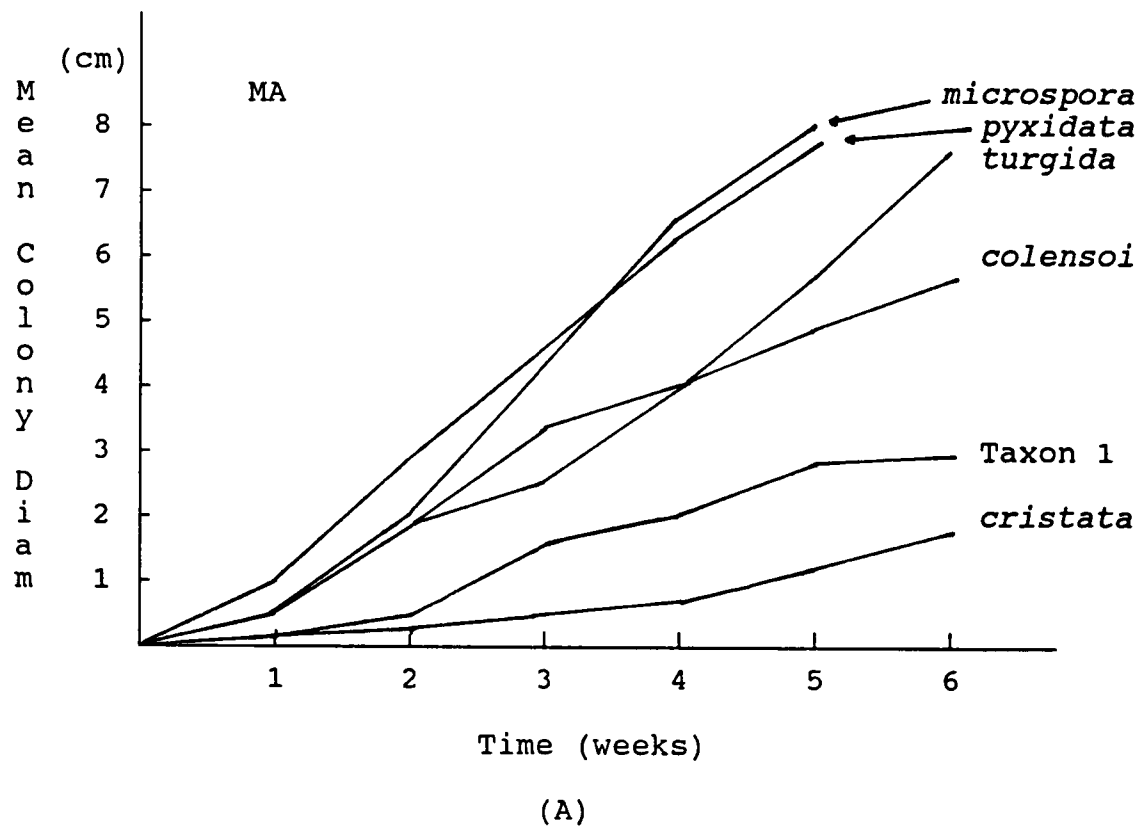


Fig. 2. Mean growth rate of *Clavicornia* species on: A) MA; B) PDA; and C) MYG.

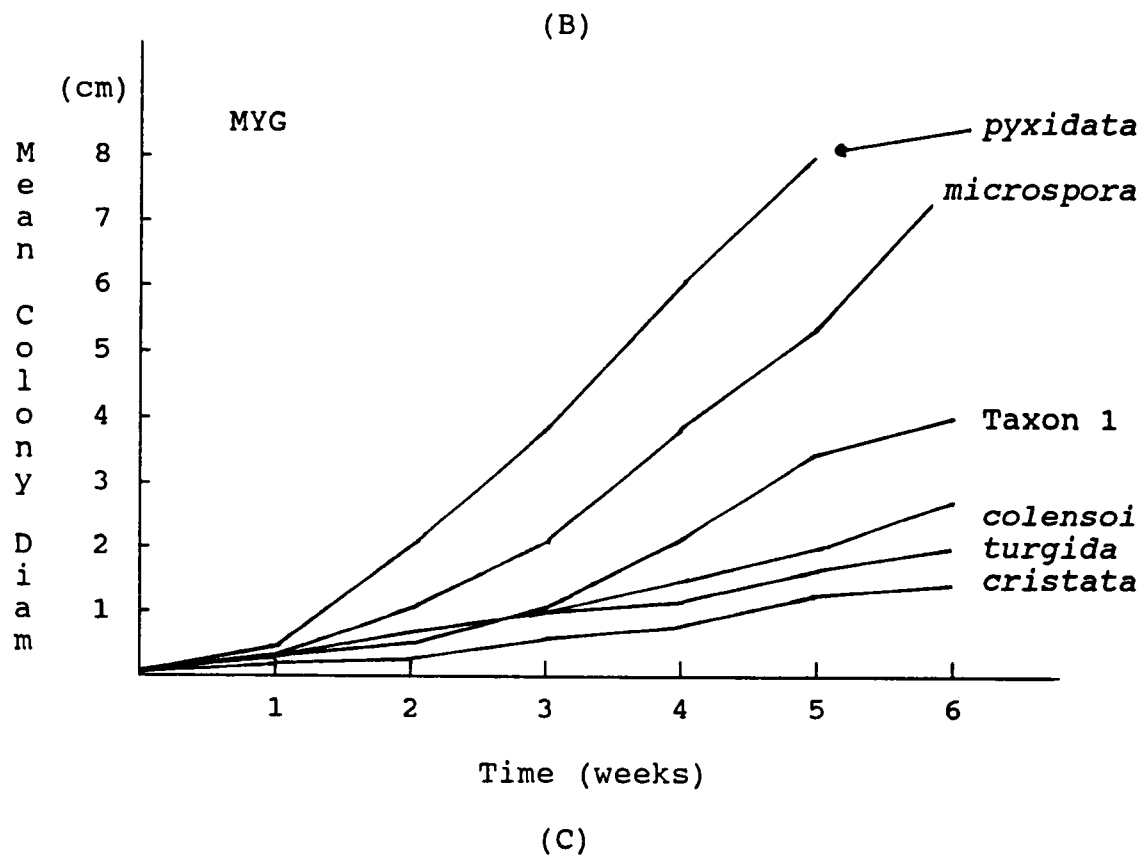
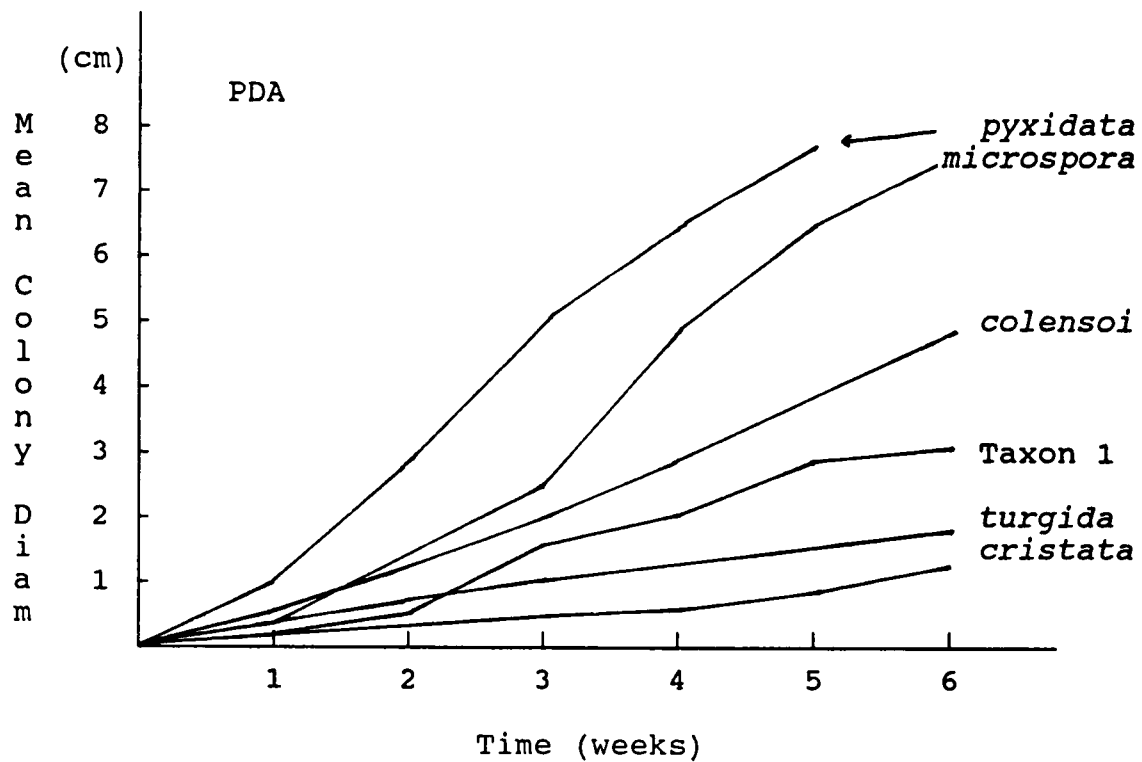


Fig. 2 (Continued)

On MA, there was little difference in culture mat coloration which could separate species. Culture mat color for most species was white or cream, except that *C. colensoi* isolates produced light yellow pigments.

On PDA and MYG, culture mat color varied for different species, basically bright yellow in *C. colensoi*, cream colored in *C. cristata* and *C. turgida*, pale orange in *C. microspora*, brownish orange in *C. pyxidata* and fawn colored in Taxon 1. Color of culture mat on PDA and MYG was of potential use in taxonomy of *Clavicornia*.

Texture of culture mat

In cultures of *Clavicornia*, texture ranged from silky, cottony, woolly to subfelty, felty, zonate, lacunose or crustose. Texture of most isolates was silky, occasionally woolly or zonate on MA, making it very difficult to separate species. On PDA and MYG, texture of culture mat was diagnostic at species-level, and at different development stages of single isolates. All textures described below were noted at Week VI.

Reverse color

Reverse color was defined for color changes in the agar underneath the mycelium, induced by the fungus (Stalpers, 1978). In the majority of isolates in *Clavicornia*, the agar was not pigmented except under or adjacent to the inoculum

plug. Isolates of Taxon 1 (1615), however, produced pigment which darkened the agar on PDA, but not on MA or MYG.

Odor of cultures

Some isolates of *Clavicornia* on agar had a distinct odor, ranging from lemonscented to earthy, while some isolates had no distinctive odor. In *C. pyxidata* and *C. microspora*, the odor of culture mat on MA was like lemon. The latter produced a special odor like medicine on PDA.

Fruitbody formation in culture

Only MA-grown isolates of *C. microspora* produced fruitbodies in culture within five weeks. The fruitbodies were branched, slender and tan colored. Spores were shed to the top of plate.

b. Micromorphological Characters

Tissue types

Culture mat tissue types were described for aerial mycelium and submerged mycelium using terms amended by Desjardin (1990). Advancing zone mycelia in all species and all media tested were similar, characterized by hyaline, thin-walled, and clamped hyphae. Aerial mycelium was more or less differentiated and formed definable tissues described as *textura angularis*, *textura globulosa*, *textura*

intricata, or *textura epidermoidea*. Tissue types of most isolates grown on MA were similar, but varied on PDA and MYG.

Special differentiated hyphae

Basic generative hyphae in culture mats of *Clavicornia* were 1.5-6 μm diam, thin-walled, slightly inflated, constantly clamped and hyaline. Beside these, the following differentiated hyphal types were observed: 1) thick-walled generative hyphae, with wall up to 1 μm thick, usually intercalary, as in *C. microspora*; 2) spherical swollen hyphae, up to 15 μm diam, terminal or intercalary, usually thin-walled, as in *C. pyxidata*; 3) hyphal knots, observed in *C. microspora* and *C. turgida*; 4) verticillate clamps, i. e. two or three clamp connections at a single septum, and sprouting clamps, i. e. a lateral hypha grew out from a clamp, both were produced only in culture of *C. cristata*; and 5) gloeoplerous hyphae, more or less inflated, with oily, yellowish contents, stained black in SBZ, present in all isolates of *Clavicornia*.

MA-grown isolates usually had less differentiation in hyphal structure than PDA- or MYG-grown isolates, and consequently were difficult to separate based solely on hyphal structures.

Crystals

Crystals were abundant in certain species (*i. e.*, *C. microspora*), rare in other species, (*i. e.*, *C. pyxidata*), and absent in *Clavicornia* Taxon 1. They were usually produced around submerged hyphae, but occasionally on aerial hyphae, as in *C. microspora*. Crystals were of different shapes, parallelogram-shaped (common) and needle-shaped (in *C. turgida*). It is not known whether crystal production is stable enough to be of taxonomic use.

2. Description of Culture Morphology

Culture morphology of MA-, PDA- and MYG-grown isolates representing six *Clavicornia* species are described separately below. Terminology utilized is from Nobles (1965) and Stalpers (1978). Color terms are from Kornerup and Wanscher (1978). Results of spot tests for laccase and tyrosinase, performed at Week II and Week VI, were recorded for each species on each medium. Results of mating tests discussed in Chapter III C, were also recorded for each species. Subsequently, the Nobles Species Code (Nobles, 1965), Stalpers Species Code (Stalpers, 1978) and supplemental codes by Boidin and Lanquetin (1983) and Desjardin (1990) were determined on each species for each medium. Code numbers for PDA- and MYG-grown isolates which are different from those for MA-grown isolates were

indicated by underlining.

Clavicornia colensoi

Isolates examined: 44117 (TENN), 44120 (TENN), 2555, 2644.

Medium: MA. Mean growth rate: (n = 4 X 3) 1 wk, 0.5 cm; 2 wks, 1.8 cm; 3 wks, 3.3 cm; 4 wks, 3.8 cm; 5 wks, 4.8 cm; 6 wks, 5.5 cm. **Macromorphology:** Advancing zone appressed, margin even; aerial mycelium poorly developed, silky, yellow, or with a few white radiating strands; reverse unchanged; odor like lemon or not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-3 μ m diam, hyaline, infrequently branched, non-amyloid, thin-walled, clamped; aerial hyphae rare, similar to advancing zone hyphae; submerged hyphae 1.5-3 μ m diam, hyaline, dextrinoid, *textura intricata*, frequently branched, clamped; gloeoplerous hyphae rare, with granular, dense, yellowish content; crystals parallelogram-shaped, few, 5-10 μ m long. **Phenoxidase Reactions:** Week II: Laccase (+), Tyrosinase (-). Week VI: Laccase (+), Tyrosinase (-). **Nobles Code:** 2a. 3. 11. 14. 15. 26. 37. 38. 47. 50. 55. 60. **Stalpers Code:** 1. 9. 13. 14. 15. 20. 35. 37a. 39. 45. 52. 73. 83. 89. 94. 95. 97. 99.

Medium: PDA. Mean growth rate: (n = 4 X 3) 1 wk, 0.3 cm; 2 wks, 1.2 cm; 3 wks, 2.0 cm; 4 wks, 2.8 cm; 5 wks, 3.8 cm; 6 wks, 4.7 cm. **Macromorphology:** Advancing zone

appressed; margin even or slightly waved, hyphae hyaline, silky; aerial hyphae cottony or wooly; white at first, then packed to felty, with yellow colored zones and white to creamy patches; reverse unchanged; odor like cleaned, raw fish. **Micromorphology:** Advancing zone hyphae 1.5-4 μm diam, hyaline, thin-walled, clamped, infrequently branched, non-amyloid; spherical swollen hyphae present; aerial hyphae 2-9 μm diam, thin- to slightly thick-walled (wall up to 1 μm thick), hyaline, clamped; submerged hyphae of three types: 1) thin-walled, 2-4 μm diam, infrequently branched, hyaline, clamped, elongate, *textura intricata*; 2) thick-walled (wall up to 1 μm thick), 2-5 μm diam, intercalary, refractive; and 3) gloeoplerous hyphae, 3-8 μm diam, yellowish, with dense, granular contents, clamped at both ends.

Phenoloxidase Reactions: Week II: Laccase (+), Tyrosinase (+); Week VI: Laccase (+), Tyrosinase (+). **Nobles Code:** 2. 3. 9. 11. 14. 15. 26. 37. 38. 47. 53. 55. 60. **Stalpers Code:** 1. 2. 9. 13. 14. 15. 21. 25. 35. 37a. 39. 48. 45. 53. 73. 83. 89. 94. 95. 97. 99.

Medium: MYG. **Mean growth rate:** 1 wk, 0.3 cm; 2 wks, 0.5 cm; 3 wks, 1.0 cm; 4 wks, 1.5 cm; 5 wks, 2.0 cm; 6 wks, 2.7 cm. **Macromorphology:** Advancing zone appressed; margin even or slightly waved, silky; aerial mycelium cottony or woolly, slightly raised at first, then packed to felty, crustose, light yellow; reverse unchanged; odor earthy. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam,

hyaline, clamped, infrequently-branched, with occasional spherical cells; aerial mycelium of young woolly areas of densely interwoven hyphae, 2-3 μm diam, typically infrequently-branched, non-diverticulate, hyaline, inamyloid, clamped, thin-walled (wall up to 0.5 μm thick); aerial mycelium of older regions forming a poorly- to well-developed layer, composed of brown, dextrinoid hyphae, with wall up to 1 μm thick, loosely interwoven or tightly adherent and lacking interhyphal spaces; crystals parallelogram-shaped crystals present, up to 15 μm long. Submerged hyphae 2-4.5 μm diam, inamyloid, hyaline, thin- to slightly thick-walled (wall < 0.5 μm). Gloeoplerous hyphae 3-5 μm diam, typical. Thick-walled hyphal fragments the same as on PDA. **Phenoloxidase Reactions:** Week II: Laccase (-), Tyrosinase (+); Week VI: Laccase (-), Tyrosinase (+). **Nobles Code:** 2a. 3. 9. 11. 14. 15. 26. 37. 38. 47. 53. 55. 60. **Stalpers Code:** 2. 9. 13. 14. 15. 21. 25. 35. 37a. 39. 48. 45. 53. 73. 83. 89. 94. 95. 97. 99.

Discussion: Cultures of *C. colensoi* were characterized by: 1) light yellow coloration; 2) woolly to felty aerial mycelium on PDA and MYG; and 3) thick-walled hyphae. *Clavicornia colensoi* cultures on MA were different from those of *C. turgida* in yellow coloration and no growth zone formation.

Clavicornia cristata

Isolates examined: DLL8974, GB1700.

Medium: MA. Mean growth rate: (n = 2 X 3) 1 wk, 0.1 cm; 2 wks, 0.2 cm; 3 wks, 0.5 cm; 4 wks, 0.7 cm; 5 wks, 1.2 cm; 6 wks, 1.7 cm. **Macromorphology:** Advancing zone submerged; margin slightly fringed; hyphae silky; aerial mycelium cottony, cream colored; reverse unchanged; odor not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-3 μ m diam, hyaline, infrequently-branched, loosely interwoven, non-amyloid, thin-walled, clamped; aerial hyphae 2-5 μ m diam, thin-walled, infrequently branched; sprouting-clamps present. Submerged hyphae 1.5-3 μ m diam, hyaline, non-dextrinoid, loosely interwoven, frequently branched, more or less diverticulate, clamped. Gloeoplerous hyphae present, with granular, dense content. Bipyrarnidal crystals abundant, 5-10 μ m long. **Phenoloxidase Reactions:** Week II: not tested. Week VI: Laccase (+), Tyrosinase (-). **Nobles Code:** 2a. 3. 5. 11a. 15. 26. 36. 38. 47. 54. 60. **Stalpers Code:** 1. 10. 13. 21. 31. 37a. 39. 45. 52. 73. 80. 83. 90. 94. 95. 97. 99.

Medium: PDA. Mean growth rate: (n = 2 X 3) 1 wk, 0.1 cm; 2 wks, 0.2 cm; 3 wks, 0.4 cm; 4 wks, 0.5 cm; 5 wks, 0.8 cm; 6 wks, 1.1 cm. **Macromorphology:** Advancing zone submerged; margin fringed; hyphae hyaline, silky; aerial hyphae cottony or woolly at top, packed to felty and crustose at bottom, cream colored; reverse dark ruby colored

(12F4) in part; odor not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-4 μm diam, hyaline, thin-walled, clamped, infrequently branched, non-amyloid; outer aerial hyphae 2-4 μm diam, thin-walled, hyaline, clamped, frequently branched, with sprouting clamps; spherical swollen hyphae present, up to 10 μm diam, slightly thick-walled (wall < 1 μm), intercalary or terminal; aerial hyphae at agar surface 2-4 μm diam, densely adherent, *textura epidermoidea*, brownish. Submerged hyphae 1.5-3 μm , thin-walled, non-amyloid, loosely interwoven, with a few verticillate and sprouting clamps. **Phenoloxidase Reactions:** Week II: not tested; Week VI: Laccase (-), Tyrosinase (+). **Nobles Code:** 2b. 3. 5. 11. 15. 26. 37. 39. 47. 54. 60. **Stalpers Code:** 2. 10. 13. 21. 25. 28. 31. 38e. 39. 41. 42. 45. 53. 73. 80. 83. 90. 94. 95. 97. 99.

Medium: MYG. **Mean growth rate:** (n = 2 X 3) 1 wk, 0.1 cm; 2 wks, 0.2 cm; 3 wks, 0.5 cm; 4 wks, 0.6 cm; 5 wks, 1.0 cm; 6 wks, 1.1 cm. **Macromorphology:** identical with PDA. **Micromorphology:** as on PDA. **Phenoloxidase Reactions:** Week II: not tested; Week VI: Laccase (+), Tyrosinase (+). **Nobles Code:** 2. 3. 5. 11. 15. 26. 37. 39. 47. 54. 60. **Stalpers Code:** 1. 2. 10. 13. 21. 25. 28. 31. 38e. 39. 41. 42. 45. 53. 73. 80. 83. 90. 94. 95. 97. 99.

Discussion: Cultures of *C. cristata* were characterized by extremely slow growth rate on MA, PDA and MYG, presence of verticillate and sprouting clamp connections. Isolate

D118974 produced dark ruby coloration on PDA and MYG, but isolate GB1700 did not produce such pigments. For both isolates, Laccase activity was detected on MYG, but not on PDA.

Clavicornia microspora

Isolates examined: 2349.

Medium: MA. **Mean growth rate:** (n = 3) 1 wk, 0.5 cm; 2 wks, 2.0 cm; 3 wks, 4.3 cm; 4 wks, 6.5 cm; 5 wks, plate covered, fruitbodies produced. **Macromorphology:** Advancing zone appressed; margin even to more or less fringed (comparing to *C. pyxidata*); aerial mycelium poorly developed, silky, hyaline; reverse unchanged; odor typical, like lemon. **Micromorphology:** Advancing zone hyphae 1.5-5 μm diam, hyaline, infrequently branched, non-amyloid, thin-walled, clamped; knots of hyphae intercalary or terminal; aerial hyphae 2-5 μm , thin-walled, hyaline, clamped, loosely interwoven; crystals parallelogram-shaped, abundant. Submerged hyphae 1.5-4 μm diam, hyaline, dextrinoid, loosely interwoven, frequently branched, clamped, with no crystals. Gloeoplerous hyphae rare, up to 5 μm diam, with granular, dense contents. **Fruitbodies:** hyphae 3-8 μm , inflated, clamped, hyaline, thin- to slightly thick-walled (wall < 1 μm thick), densely packed, parallel; gloeoplerous hyphae abundant, 3-8 μm diam. **Phenoloxidase Reactions:** Week II: Laccase (+), Tyrosinase (-). Week VI:

Laccase (+), Tyrosinase (-). **Nobles Code:** 2a. 3c. 9. 22.
25. 26. 36. 38. 45. 48. 50. 60. **Stalpers Code:** 1. 9. 11.
13. 30. 36. 37a. 39. 45. 48. 53. 61. 65. 73. 80. 83. 94. 95.
97. 99.

Medium: PDA. **Mean growth rate:** (n = 3) 1 wk, 0.3 cm;
2 wks, 1.3 cm; 3 wks, 2.5 cm; 4 wks, 4.8 cm; 5 wks, 6.3 cm;
6 wks, plate covered. **Macromorphology:** Advancing zone
appressed; margin even to more or less fringed, silky;
aerial hyphae silky, pale orange (5A3), more or less zonate
and radial; reverse brown in center, light brown at margin;
odor like medicine. **Micromorphology:** Advancing zone hyphae
1.5-3 μm diam, hyaline, thin-walled, clamped; knots of
hyphae intercalary or terminal; gloeoplerous hyphae up to 5 μm
diam, yellowish; aerial hyphae 2-6 μm diam, inflated,
consisting of shortened cells, thin- to slight thick-walled
(wall < 1 μm thick), hyaline, clamped, densely packed,
textura epidermoidea; gloeoplerous hyphae 3-6 μm diam, with
spherical swellings up to 15 μm diam. Submerged hyphae 2-5
 μm diam, dextrinoid, infrequently branched, clamped, loosely
interwoven. Gloeoplerous hyphae as in aerial mycelium.

Phenoloxidase Reactions: Week II: Laccase (+), Tyrosinase
(+); Week VI: Laccase (+), Tyrosinase (+). **Nobles Code:** 2.
3c. 9. 22. 25. 26. 37. 39. 46. 48. 53. 60. **Stalpers Code:**
1. 9. 11. 13. 29. 35. 38. 39. 45. 48. 53. 61. 65. 73. 80.
83. 94. 95. 97. 99.

Medium: MYG. **Mean growth rate:** 1 wk, 0.2 cm; 2 wks, 1.0 cm 3 wks, 2.0 cm; 4 wks, 3.8 cm; 5 wks, 5.3 cm; 6 wks, plate covered. **Macromorphology:** Advancing zone appressed; margin more or less fringed, silky; aerial mycelium woolly, white; reverse brown in center to light brown at margin; odor not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam, as on PDA; aerial hyphae 3-6 μm diam, clamped, slightly thick-walled (wall < 1 μm thick), densely packed; spherical swollen hyphae up to 10 μm diam, intercalary or terminal; gloeoplerous hyphae 3-6 μm diam; thick-walled hyphae (wall > 1 μm thick), 2-5 μm diam, unbranched, elongate. Submerged hyphae 2-8 μm diam, dextrinoid, hyaline, infrequently branched, loosely interwoven, without crystals; gloeoplerous hyphae absent. **Phenoloxidase Reactions:** Week II: Laccase (+), Tyrosinase (+); Week VI: Laccase (+), Tyrosinase (+). **Nobles Code:** see PDA. **Stalpers Code:** 1. 9. 11. 13. 22. 29. 35. 38. 39. 45. 48. 53. 61. 65. 73. 80. 83. 94. 95. 97. 99.

Discussion: In *C. microspora*, fruitbodies were formed on MA. Gloeoplerous hyphae were abundant in fruitbodies but rare in aerial or submerged culture hyphae. The knots of hyphae were abundant at Week III, but rare at Week VI. Abundant crystals appeared at Week VI. On all three media submerged hyphae were dextrinoid, but not aerial hyphae.

Clavicornia pyxidata

Isolates examined: 24439, 1190, 10654, 1503, 1506, JPL479, 56655, 24439, 22501, 56667, 1449, 1541, GB1279, GB1280.

Medium: MA. Mean growth rate: (n = 14 X 3) 1 wk, 1.0 cm; 2 wks, 2.8 cm; 3 wks, 4.5 cm; 4 wks, 6.2 cm; 5 wks, plate covered. **Macromorphology:** Advancing zone appressed; margin even; silky; aerial mycelium poorly developed, silky, hyaline or with a few white radiating strands; reverse unchanged; odor like lemon or not distinctive.

Micromorphology: Advancing zone hyphae 1.5-4 μ m diam, hyaline, infrequently branched, non-amyloid, thin-walled, clamped; aerial hyphae rare, similar to advancing zone hyphae. Submerged hyphae 1.5-3 μ m diam, hyaline, dextrinoid, clamped, loosely interwoven, frequently branched, clamped. Gloeoplerous hyphae rare, with granular, dense contents. **Phenoloxidase Reactions:** Week II: Laccase (+), Tyrosinase (-). Week VI: Laccase (+; 1503, 1506 and 22501 -), Tyrosinase (-). **Nobles Code:** 2a. 3c. 11i. 15. 26. 36. 38. 45. 50. 54. 55. 60. **Stalpers Code:** 1. 8. 13. 14. 15. 20. 30. 36. 37a. 39. 45. 52. 73. 89. 90. 94. 95. 97. 99.

Medium: PDA. Mean growth rate: (n = 14 X 3) 1 wk, 1.0 cm; 2 wks, 2.8 cm; 3 wks, 5.0 cm; 4 wks, 6.5 cm; 5 wks, plate covered. **Macromorphology:** Advancing zone appressed; margin even; silky; aerial mycelium cottony at first, then matted to a felty layer, zonate, greyish yellow (4B4), with

white patches; reverse unchanged; odor like old lemon.

Micromorphology: Advancing zone hyphae 1.5-4 μm diam, hyaline, thin-walled, clamped, infrequently branched, non-amyloid. Aerial hyphae 3-5 μm diam, thin-walled (wall < 0.5 μm thick), hyaline, clamped, *textura epidermoidea*; spherical swollen hyphae up to 10 μm diam; gloeoplerous hyphae 4-8 μm diam, cylindrical or irregular shaped, slightly thick-walled (wall < 1 μm thick). Submerged hyphae 1.5-3 μm , thin-walled, infrequently branched, hyaline, dextrinoid, clamped, elongate, loosely interwoven or parallel. **Phenoloxidase Reactions:** Week II: Laccase (+), Tyrosinase (+; 10654, 1449 -); Week VI: Laccase (+; 1190, 1506, 1503, 1607, 10654 -), Tyrosinase (+; 1449, 1541, 22501 -). **Nobles Code:** 2. 3c. 11. 15. 26. 37. 38. 45. 50. 54. 55. 60. **Stalpers Code:** 1. 2. 8. 13. 14. 15. 25. 35. 36. 37a. 39. 45. 53. 80. 89. 90. 94. 95. 97. 99.

Medium: MYG. **Mean growth rate:** (14 X 3) 1 wk, 0.3 cm; 2 wks, 2.0 cm 3 wks, 3.8 cm; 4 wks, 6.0 cm; 5 wks, plate covered. **Macromorphology:** Advancing zone appressed; margin even; silky; aerial mycelium more or less felty, as on PDA, zonate, greyish yellow (4B4), with white patches; reverse unchanged; odor unpleasant. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam, hyaline, clamped, infrequently branched; aerial mycelium 2-4 μm diam, hyaline, clamped, thin-walled, *textura epidermoidea*, non-diverticulate, inamyloid; swollen hyphae not observed; gloeoplerous hyphae

up to 5 μm diam, cylindrical. Submerged hyphae 3-5 μm diam, dextrinoid, hyaline, thin-walled. Gloeoplerous hyphae 3-5 μm diam, typical. **Phenoloxidase Reactions:** Week II: Laccase (+), Tyrosinase (+; 1449, 1506, 1190 -); Week VI: Laccase (+; 1190, 1503, 1541, 10654, 22501, JPL479, 56655, 56667 -), Tyrosinase (+; 22501, 1449, 1506 -). **Nobles Code:** see under PDA. **Stalpers Codes:** see under PDA.

Discussion: Cultures of *C. pyxidata* were characterized by: 1) relatively high growth rate in contrast to other *Clavicornia* species; 2) yellow to brown coloration on PDA and MYG; 3) lack of crystals. Dodd (1972) reported that *C. pyxidata* produced chlamydospores in culture, but from his description and illustration, the so-called chlamydospores were really terminal swollen hyphae. Fourteen isolates of *C. pyxidata* were employed for culture studies. On MA, no distinct difference among these isolates were observed. On PDA and MYG, there was variation in culture mat coloration, greyish yellow to brown. Otherwise, culture morphology was very consistent in this species.

Clavicornia turgida

Isolates examined: 2651, 2682, 44103.

Medium: MA. **Mean growth rate:** ($n = 3 \times 3$) 1 wk, 0.5 cm; 2 wks, 1.7 cm; 3 wks, 2.5 cm; 4 wks, 3.8 cm; 5 wks, 5.5 cm; 6 wks, plate covered. **Macromorphology:** Advancing zone appressed; margin more or less fringed; silky; aerial

mycelium white, pellicular or subfelty in dark, prostrate in light, zonate; reverse unchanged. odor like lemon or not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam, hyaline, infrequently branched, non-amyloid, thin-walled, clamped; knots of hyphae mostly intercalary; aerial hyphae 1.5-3 μm diam, similar to advancing zone hyphae; gloeoplerous hyphae 1.5-3 μm diam; prostrate hyphae like submerged hyphae, dextrinoid, stained purple in Melzer's reagent. Submerged hyphae 2-6 μm diam, hyaline, dextrinoid, *textura intricata*, frequently branched, clamped. Gloeoplerous hyphae rare, 2-6 μm diam. Crystals parallelogram-shaped, few, 4-8 μm long. **Phenoloxidase**

Reactions: Week II: Laccase (+), Tyrosinase (-). Week VI: Laccase (+), Tyrosinase (-). **Nobles Code:** 2a. 3. 11i. 15. 22. 36. 38. 46. 50. 55. 60. **Stalpers Code:** 1. 9. 13. 24. 29. 30. 37a. 39. 45. 53. 61. 65. 73. 83. 89. 94. 95. 97. 99.

Medium: PDA. **Mean growth rate:** ($n = 3 \times 4$) 1 wk, 0.3 cm; 2 wks, 0.6 cm; 3 wks, 0.8 cm; 4 wks, 1.1 cm; 5 wks, 1.4 cm; 6 wks, 1.6 cm. **Macromorphology:** Advancing zone submerged; margin slightly waved; silky; aerial hyphae cottony at first, then packed to felty, crustose to radially lacunose, cream colored; reverse unchanged; odor not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-4 μm diam, hyaline, thin-walled, clamped, infrequently branched, non-amyloid; aerial hyphae 2-6 diam, thin-walled, hyaline, clamped, *textura angularis*. Submerged hyphae 1.5-3

μm , similar to advancing zone hyphae, dextrinoid.

Gloeoplerous hyphae 3-5 μm , thick-walled. Crystals abundant, needle-shaped or parallelogram-shaped.

Phenoloxidase Reactions: Week II: not tested. Week VI:

Laccase (+), Tyrosinase (+). **Nobles Code:** 2. 3. 11. 15.

22. 37. 38. 47. 50. 55. 60. **Stalpers Code:** 1. 2. 10. 13.

21. 25. 27. 28. 31. 37a. 39. 45. 53. 73. 83. 89. 94. 95. 97.

99.

Medium: MYG. **Mean growth rate:** 1 wk, 0.3 cm; 2 wks, 0.6 cm, 3 wks, 0.8 cm; 4 wks, 1.0 cm; 5 wks, 1.5 cm; 6 wks, 2.0 cm. **Macromorphology:** similar to that on PDA.

Micromorphology: similar to that on PDA. **Phenoloxidase**

Reactions: Week II: not tested. Week VI: Laccase (+),

Tyrosinase (+). **Nobles Code:** see under PDA. **Stalpers**

Codes: see under PDA.

Discussion: Growth rate of *C. turgida* varied on different media: on MA, mycelia covered the plates (six weeks), but on PDA and MYG, grew less than 2 cm. On MA, mycelia seemed to respond to exposure to light by forming distinct growth zones. This was not clearly observed in other species.

Clavicornia Taxon 1 (no. 1615)

Isolates examined: 1615.

Medium: MA. **Mean growth rate:** (n = 2) 1 wk, 0.2 cm; 2 wks, 0.5 cm; 3 wks, 1.0 cm; 4 wks, 1.5 cm; 5 wks, 2.0 cm; 6

wks, 2.5 cm. **Macromorphology:** Advancing zone submerged; margin fringed; aerial mycelium absent or poorly developed, silky, hyaline; reverse unchanged; odor not distinctive. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam, hyaline, infrequently branched, non-amyloid, thin-walled, clamped, non-diverticulate. aerial hyphae rare, similar to advancing zone hyphae. Submerged hyphae 1.5-3 μm diam, hyaline, dextrinoid, *textura intricata*, frequently branched, clamped. Gloeoplerous hyphae not observed. **Phenoloxidase Reactions:** Week II: not tested. Week VI: Laccase (+), Tyrosinase (-). **Nobles Code:** 2a. 3. 7. 11i. 36. 38. 54. **Stalpers Code:** 1. 10. 13. 20. 30. 37a. 39. 45. 52. 90.

Medium: PDA. **Mean growth rate:** (n = 2) 1 wk, 0.1 cm; 2 wks, 0.4 cm; 3 wks, 1.5 cm; 4 wks, 2.0 cm; 5 wks, 2.8 cm; 6 wks, 3.0 cm. **Macromorphology:** Advancing zone appressed; margin even, 1 mm wide, golden yellow (4A5), silky, dense; aerial hyphae poorly developed; surface mycelium felty to crustose, up to 1 mm thick, somalis brown (7E5) in center, fawn (7E4) outward, zonate, radially lacunose; reverse fawn (7E4) colored; odor earthy. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam, hyaline, thin-walled, clamped, frequently branched, non-amyloid, *textura epidermoidea*. Aerial hyphae rare, 1-2 μm diam (narrower than advancing zone hyphae), thin-walled, clamped, hyaline, *textura epidermoidea*; surface hyphae 1.5 -3 μm diam, hyaline, thin-walled, clamped, *textura epidermoidea*; gloeoplerous hyphae

3-5 μm diam, typical. Submerged hyphae 1.5-3 μm , thin-walled, clamped, *textura intricata*, dextrinoid.

Phenoloxidase Reactions: Week II: not tested. Week VI: Laccase (+), Tyrosinase (+). **Nobles Code:** 2a. 3. 11. 11i. 15. 37. 39. 47. 51. 54. **Stalpers Code:** 1. 2. 10. 13. 15. 25. 27. 28. 29. 34. 38. 39. 45. 52. 73. 90.

Medium: MYG. **Mean growth rate:** 1 wk, 0.2 cm; 2 wks, 0.5 cm, 3 wks, 1.0 cm; 4 wks, 2.0 cm; 5 wks, 3.5 cm; 6 wks, 4.0 cm. **Macromorphology:** Advancing zone appressed; margin even; silky; aerial mycelium absent; surface mycelium up to 0.5 mm thick, wet-looking, olive brown (4D6); reverse unchanged; odor as on PDA, but stronger. **Micromorphology:** Advancing zone hyphae 1.5-3 μm diam, hyaline, clamped, infrequently branched, *textura intricata*; aerial mycelium as on PDA; surface hyphae as on PDA, *textura epidermoidea*. Submerged hyphae as on PDA, dextrinoid. **Phenoloxidase Reactions:** Week II: not tested; Week VI: Laccase (+), Tyrosinase (+). **Nobles Code:** 2a. 3. 11. 11i. 16. 37a. 38. 47. 51. 54. **Stalpers Code:** 1. 2. 10. 13. 16. 25. 27. 28. 29. 34. 38. 39. 45. 52. 73. 90.

Discussion: Cultures of 1615 were characterized by : 1) fawn to olive-brown coloration on PDA and MYG; 2) felty to chamois surface on PDA and MYG; and 3) lack of knots and spherical swollen hyphae. Growth rate of this species was near the average for the genus.

C. RESULTS OF INCOMPATIBILITY TESTS

1. Self-crosses and Inter-collection Matings

Self-crosses were performed for each species to determine its mating system, and if more than one collection was available, inter-collection matings were carried out to test intercompatibility. The results are presented below under species names.

Clavicornia candelabrum

Mating Systems

Among the New Zealand collections, 2679, 2681, 2802 and 2807 were identified as *C. candelabrum*. Self-crosses within collection 2679 revealed a bifactorial (tetrapolar) mating system, although one of the four mating types was missing from the sample (Fig. 3). In Figure 3, isolates 1, 3, 4, 6, 8, 9 were compatible with isolates 2 and 10, representing two opposite mating types; isolates 5 and 7 were incompatible with any tested isolates, suggesting that these two isolates belonged to one mating type and the opposite mating type was missing from the sample. Self-crosses within collections 2681 and 2807, however, resulted in mostly claspless pairs with few compatible matings.

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| 1* | - | | | | | | | | | |
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| 4 | - | - | - | | | | | | | |
| 8 | - | - | - | - | | | | | | |
| 6 | nd | - | - | - | - | | | | | |
| 9 | nd | - | - | - | - | - | | | | |
| 2* | + | + | + | + | + | + | - | | | |
| 10 | + | + | nd | + | + | + | - | - | | |
| 5* | nd | - | - | - | - | - | - | - | - | |
| 7 | nd | nd | - | - | - | - | - | - | - | - |
| | 1* | 3 | 4 | 8 | 6 | 9 | 2* | 10 | 5* | 7 |
| | A_1B_1 | | | | | | A_2B_2 | | ? | |

Fig. 3. *Clavicornia candelabrum*: Self-crosses of collection 2679. + = compatible, clamp connections present; - = incompatible, clamp connections absent; nd = no data available; * = tester strains.

Mating Reaction Morphology

Monokaryotic hyphae were normally 2-5 μm diam, yellowish, elongated, thin-walled, with dense contents. Intercalary spherical swollen cells were abundant. Some inflated hyphae were full of multiguttulate and yellowish contents, apparently gloeoplerous hyphae. Three basic mating reaction types were identified although there were many ambiguous cases.

a) Flat reactions: Contact zones of most pairings seemed to exhibit flat reaction morphology. Donor monokaryon mycelia reduced growth when approaching each other, leaving a clear contact zone with sparse submerged hyphae. If no clamp connections were found, this type of reaction was interpreted as a flat.

b) Compatible matings: Contact zones were macromorphologically similar to a flat, with a clear contact zone consisting of sparse, submerged hyphae, but clamp connections, rare or abundant, were produced. In some cases, mycelia from both donors formed a clear contact zone in the center of mate plates, but were randomly intermingled at the periphery, and clamp connections were produced extensively in this area. Occasionally, a dense contact zone with fluffy mycelia was formed in compatible matings.

c) Incompatible with both A and B factors in common: hyphae from donor mycelia merely overgrow, with neither clear contact zone nor clamp connections.

Clavicornia colensoi

Mating Systems

Collections 2555, 2644, 2667 and 2685, from New Zealand, were identified as *C. colensoi*. Self-crosses within collection 2555 (Fig. 4) revealed a bifactorial (tetrapolar) mating system. Mating studies were carried out first on 1.5% MA and second on 0.5% MA, with up to 20 single-spore isolates of each collection employed. Pairings on 1.5% MA did not produce any compatible matings in 2644 and 2667 and produced very few compatible matings in 2555 and 2685 within 7 weeks. Moreover clamp connections were extremely rare in compatible matings. Pairing on 0.5% MA were incubated at room temperature and matings were read 2-3 months later. In self-crosses of collection 2555 (Fig. 4), clamp connections in compatible matings were observed after two months and the resultant mating pattern indicated a bifactorial mating system. In self-crosses of collection 2644, two mating types were found to be compatible with each other and the third mating type was incompatible with all isolates. It was deduced that the fourth mating type was missing from the sample. In self-crosses of collection 2685, only one compatible mating was recorded, while in self-crosses of collections 2667, clamp connections could not be found in any pairings.

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| 7* | - | + | - | + | - | | | | | | |
| 9 | - | + | - | + | - | - | | | | | |
| 11 | - | + | - | + | - | - | - | | | | |
| 12 | - | + | - | + | - | - | - | - | | | |
| 14 | - | + | - | + | - | - | - | - | - | | |
| 15* | - | - | - | - | - | - | - | - | - | | |
| 2* | - | - | - | - | - | - | - | - | + | - | |
| 17 | - | - | - | - | - | - | - | - | + | - | - |

$\underbrace{1 \quad 5^*}_{A_1 B_1} \quad \underbrace{7^* \quad 9 \quad 11 \quad 12 \quad 14}_{A_2 B_2} \quad \underbrace{15^*}_{A_2 B_1} \quad \underbrace{2^* \quad 17}_{A_1 B_2}$

Fig. 4. *Clavicornona colensoi*: Self-crosses of collection 2555. + = compatible, clamp connections are present only in contact zone; - = incompatible or no clamp connections; corner symbols (-) = no clamp connections at periphery.

Mating Reaction Morphology

Compared to *C. pyxidata*, mating interaction patterns in *C. colensoi* were very vague. Three basic mating reaction types were identified despite of many ambiguous cases.

a). Flat reaction: in all pairings it seemed that two paired mycelia stopped growing when approaching each other, leaving a clear contact zone. Under microscopy, this clear zone was devoid of hyphae or with very sparse, tortuous hyphae. If no clamp connections were observed, this type of reaction was considered as flat because no raised mycelium was found.

b). Compatible matings: uncommon-A and uncommon-B. Usually there was no clear contact zone as in the flat reaction, although hyphae were sparse in that area. Clamp connections were found only in the contact zone and paired nuclei were observed under epifluorescence microscopy. Some compatible matings were morphologically similar to flat reactions except that clamp connections were produced.

c). Incompatible matings: with both A and B factors in common. Pairings between two isolates of the same mating type did not produce the clear contact zone. Hyphae from donor mycelia merely overgrew.

Intercollection Matings

Intercollection matings were carried out to determine the pattern of sexuality in this species and

intercompatibility relationship between collections. Since self-crosses had not been successful except in collection 2555, single-spore isolates were selected randomly. It was probable, therefore, that not all mating types were selected. Results of intercollection matings among collections 2555, 2644 and 2667 are given in Fig. 5.

Isolate 2667:1, 2 were compatible with 2555:3; 2667:4 was compatible with 2555:5 and 2644:7; 2555:3, 5 were compatible with 2644:2, 7, 8. Clamp connections were not found in the rest of the matings. The results cannot be interpreted as partially compatible because of lack of assigned mating types and difficulties of reading and probably unfavorable culture conditions, as in self-crosses. No further conclusions can be drawn from these intercollection matings, other than that these three collections are potentially intercompatible.

Clavicornia cristata

Mating Systems

Self-crosses of *C. cristata* revealed a heterothallic, bifactorial mating system. Single-spore isolates of collection DLL8974 from California were utilized to ascertain the mating system (Fig. 6). The Finnish collections, GB1700 and GB1701, were also of four mating types. Mating tests of DLL8974 were extenuated by its

| No.
Isolates | | 2667 | | | | 2555 | | | | 2644 | | | |
|-----------------|---|------|---|---|---|------|---|---|---|------|---|---|---|
| | | 1 | 2 | 3 | 4 | 2 | 3 | 5 | 7 | 2 | 4 | 7 | 8 |
| 2667 | 1 | - | - | - | - | - | + | - | - | - | - | - | - |
| | 2 | | - | - | - | - | + | - | - | - | - | - | - |
| | 3 | | | - | - | - | - | - | - | - | - | - | - |
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| 5* | - | - | | | | | | | | | | | | | | | |
| 19 | nd | - | - | | | | | | | | | | | | | | |
| 13 | - | - | nd | - | | | | | | | | | | | | | |
| 14 | - | - | nd | - | - | | | | | | | | | | | | |
| 2 | nd | - | + | + | - | - | | | | | | | | | | | |
| 4 | nd | + | + | + | nd | - | - | | | | | | | | | | |
| 10* | + | + | + | + | + | - | - | - | | | | | | | | | |
| 16 | nd | + | + | + | + | - | - | - | - | | | | | | | | |
| 7 | nd | + | nd | nd | nd | - | nd | nd | - | - | | | | | | | |
| 11 | + | + | + | nd | nd | nd | - | - | nd | - | - | | | | | | |
| 15* | - | - | - | - | - | - | - | - | - | - | - | - | | | | | |
| 18* | - | - | - | - | - | - | - | - | - | - | - | - | + | - | | | |
| | 1 | 5* | 19 | 13 | 14 | 2 | 4 | 10* | 16 | 7 | 11 | 15* | 18* | | | | |
| | A_1B_1 | | | | | A_2B_2 | | | | | | A_2B_1 | | A_1B_2 | | | |

extremely slow growth on agar medium. At first, mating tests were carried out on 1.5% MA (Appendix B), containing 20 g agar/liter, and resulted in a mostly negative grid even after a six-month incubation. Mycelia had not contacted yet in some pairings. Next, mating tests were carried out on modified MA (12.5 g malt extract; 10 g agar/liter). Mycelia grew faster on soft malt extract agar than on regular agar. More than 4 months were still needed to conduct a mating test. Self-crosses of DLL 8974 are shown in Fig. 6. All single-spore isolates segregated into one of four mating types. However, the incompatibility in the crosses 2 X 5 and 2 X 14 was hard to explain. In some matings, paired mycelia did not contact each other over a long period of incubation because of slow rate growth and occasional contamination, indicated by "o" in the grid. Nevertheless, the bifactorial mating system pattern was distinct. Relatively fast-growing isolates of each mating type were selected as tester strains.

Single-spore Isolate Morphology

Single-spore isolates obtained from spore germination of collection DLL8974 and incubated at room temperature were of two morphological types: 1) mycelium brownish, very dense, prostrate or submerged, growing very slowly (1 cm/4 months). Hyphae 2-8 μ m diam, silky, some hyphae slightly thick-walled, with spherical swollen cells up to 10 μ m diam.

Isolates 1, 2, 4, 7, 10, 14, 15 and 18 belonged to this type; 2) mycelium white, fluffy, growing relatively faster (2-3 cm/4 months). Hyphae 2-5 μ m diam, rarely thick-walled or swelled. Isolates 5, 11, 13, 16, 19 belonged to this type. Culture morphology of single-spore isolates was not related to the their mating types. After a certain storage time under refrigeration (4°C), most isolates became morphological type 2), i. e. white, spreading, healthy mycelium and higher growth rate. Lower temperature may be more favorable than room temperature for this species. Further physiological studies may reveal an optimum temperature range of *C. cristata*. Incubation of mating test plates under refrigeration may lead to better results.

Mating Reaction Morphology

As discussed above, the slow rate growth may result from unsuitable temperature, and mating reactions may also be affected in some ways. In pairings between isolates of type 1) single-spore morphology, spherical swollen hyphae were observed.

a) Compatible matings: uncommon-A and uncommon-B factors. Two isolates contacted smoothly. Clamp connections were present in the contact zone and occasionally present on one or both sides of the contact zone paired nuclei were observed under epifluorescence microscopy.

b) Flat reactions: common-A. Mycelia from both donors were

submerged and prostrate in contact zone. No clamp connections were found and hyphae were monokaryotic.

c) Barrage reactions: common-B. Mycelia of paired isolates were raised next to the contact zone, leaving a narrow clear contact zone. No clamp connections or false clamp connections were found and hyphae were monokaryotic.

d) Incompatible matings: common-A and common-B. These were morphologically similar to compatible matings except that no clamp connections were observed.

Intercollection Matings

Matings among tester strains of collection DLL8974 (California) and two Finnish collections, GB1700 and GB1701, are shown in Fig. 7.

| Coll. No.
Testers | | DLL8974
5 10 15 18 | | | | GB1700
2 3 7 1 | | | | GB1701
3 4 8 12 | | | | |
|----------------------|----|-----------------------|---|---|---|-------------------|---|----|----|--------------------|----|----|----|----|
| DLL8974 | 5 | - | + | - | - | - | + | + | nd | + | + | + | + | |
| | 10 | | - | - | - | - | - | - | nd | + | - | - | - | |
| | 15 | | | - | + | - | + | - | nd | + | - | nd | - | |
| | 18 | | | | - | - | + | nd | nd | + | - | - | - | |
| | | | | | | GB1700 | | | | 2 | + | + | + | + |
| | | | | | | | | | | 3 | - | + | - | + |
| | | | | | | | | | | 7 | + | + | + | + |
| | | | | | | | | | | 1 | nd | nd | nd | nd |

Fig. 7. *Clavicornia cristata*: Intercollection matings among DLL8974, GB1700 and GB1701, + = compatible; - = incompatible; nd = no data available.

Two conclusions could be drawn from Fig. 7: 1) the two Finish collections were intercompatible with each other and a common mating factor occurred in both collections. GB1701:3, 8 shared a common allele with GB1701:3 and probably 1; 2) DLL8974 was partially intercompatible with both Finish collections. In crosses between DLL8974 and GB1700, compatible matings were accompanied by incompatible matings without distinct patterns. GB1700:2 was incompatible with every testers of DLL8974, while GB1700:3 was compatible with three out of four tester strains of DLL8974. In pairings between DLL8974:5 and of GB1700:7, three types of hyphae were observed, i. e. monokaryotic hyphae with few binucleate cells accompanied by false clamp connections; dikaryotic hyphae with true clamp connections; and monokaryotic hyphae with spherical swollen cells. It was very interesting that both true and false clamp connections were present in a single mating. In false clamp connections, nuclear migration is prohibited and subsequent genetic exchange prohibited. Pairings between DLL8974 and GB1701 showed another pattern of partial intercompatibility. DLL8974:5 was compatible with all tester strains of GB1701, and GB1701:3 was compatible with all tester strains of DLL8974. The remaining pairings did not result in any compatible matings. In pairings between DLL8974:5 and GB1701:3, contact zone hyphae were well-developed, with paired nuclei and abundant clamp connections.

Although further mating studies are needed to confirm the above results by utilizing more single-spore isolates, and under physiologically determined conditions, these intercollection matings provided evidence that *C. cristata* (DLL8974) is in the process of speciation and adoption to various geographical and ecological environments.

Clavicornia microspora

Mating Systems

Single-spore isolates of two collections were used for mating system tests, one accompanied by a specimen (no. 2349), another from only spore print (KY5352). Self-crosses of these two collections all resulted in a bifactorial (tetrapolar) mating system pattern. All single spore isolates segregated into one of the four mating types. Self-crosses of collection 2349 are shown in Fig. 8.

Mating Reaction Morphology

a) Compatible matings: $A_1B_1 \times A_2B_2$, $A_1B_2 \times A_2B_1$. Mycelia from both donor were smoothly intermingled and formed an undifferentiated contact zone. Clamp connections first were formed in the contact zone, then were produced all over both mycelia within 4 weeks.

b) Flat reactions: $A_1B_1 \times A_1B_2$, $A_2B_1 \times A_2B_2$. Mycelia from both donors were intermingled, but hyphae became sparse in the

| | | | | | | | | | |
|-----|----------|---|----|---|----------|---|----------|---|----------|
| 1 | - | | | | | | | | |
| 4 | - | - | | | | | | | |
| 5* | - | - | - | | | | | | |
| 7 | - | - | - | - | | | | | |
| 3* | + | + | + | + | - | | | | |
| 6 | + | + | + | + | - | - | | | |
| 2* | - | - | - | - | - | - | - | | |
| 9 | - | - | - | - | - | - | - | - | |
| 10* | - | - | - | - | - | - | + | + | - |
| | 1 | 4 | 5* | 7 | 3* | 6 | 2* | 9 | 10* |
| | A_1B_1 | | | | A_2B_2 | | A_1B_2 | | A_2B_1 |

Fig. 8. *Clavicornia microspora*: Self-crosses of collection 2349. + = compatible, clamp connections present; - = incompatible, clamp connections absent; * = tester strains.

contact zone. Clamp connections were absent.

c) Barrage reactions: $A_1B_1 \times A_2B_1$, $A_1B_2 \times A_1B_2$. Hyphae from donor mycelia were raised and formed a clear contact zone. False clamp connections were not observed.

d) Incompatible matings: common-A and common-B factors. This type of reaction was morphologically similar to a compatible mating, but no clamp connections were observed.

Monokaryotic "Fruiting"

On some mating-carrying plates, including compatible matings and incompatible matings, small, branched sterile "fruitbodies" were formed in the contact zone or either sides of the inocula, the same as described in Chapter IIIB.

Intercollection Mating

Matings between tester strains of collection 2349 and KY5352 indicated that these two collections were fully compatible, as shown in Fig. 9.

In all pairings, clamp connections were formed all over the donor mycelia within four weeks. In some matings, fruitbodies were formed, which were small, crowded, up to 1 cm high, cream colored upward and tan downward. Basidiospores were shed to the top of plates and deposited in white spore prints. Spores collected from the spore prints germinated at nearly 100%. Subsequently, monokaryotic isolates were obtained. Self-crosses using

| Coll. No.
Testers | 2349 | | | |
|----------------------|------|---|---|----|
| | 5 | 3 | 2 | 10 |
| 1 | + | + | + | + |
| KY5352 3 | + | + | + | + |
| 6 | + | + | + | + |
| 10 | + | + | + | + |

Fig. 9. *Clavicornia microspora*: Matings between tester strains of collection 2349 and KY5352. + = compatible mating.

these isolates obtained from previous intercollection matings revealed a bifactorial mating system pattern. It indicated that these F_1 spores were normal, although subsequent fruitbodies were needed to prove that the two collections were "interfertile".

The full compatibility between the two Japanese collections indicated that monokaryotic strains of these collections were physiologically fertile, *i. e.* able to form dikaryotic hyphae when mated with genetically compatible isolates. This is a important clue when interpreting matings between isolates of *C. microspora* and *C. pyxidata*.

Clavicornia piperata

Single-spore isolates were obtained from collection 2822, which was identified as *C. piperata* in this study. Because of the slow growth rate of single-spore isolates, self-crosses within collection 2822 were carried out by

placing two monokaryotic isolate blocks together side by side. Matings were read after six weeks. No clamp connections were observed in any pairings. Mycelia were yellowish, consisting of four types of hyphae: a) normal hyphae, 1-4 μm diam, hyaline, infrequently branched, thin-walled; b) chlamydospores, up to 10 X 7 μm , with yellow-brownish contents, uninucleate, ellipsoid-fusoid, intercalary or terminal, thick-walled, without ornamentation; c) intercalary swollen cells; and 4) gloeoplerous hyphae, up to 5 μm diam, elongate, brownish, with dense, multiguttulate contents, staining black in SBZ.

Formation of chlamydospores usually indicates that culture conditions were poor for vegetative growth. This might be one of the reasons, if not the only one, for slow growth rate and lack of compatible matings. Physiological experiments concerning optimum culture medium and condition are necessary before performing further mating tests.

Clavicornia pyxidata

Mating Systems

Self-crosses of 28 collections of *Clavicornia pyxidata* revealed a heterothallic, bifactorial (tetrapolar) mating system. All single-spore isolates segregated into one of four mating types. Macroscopic and microscopic mating interactions were similar in all of the collections tested.

No recombination of incompatibility factors was observed. All possible combinations of four mating types were interpreted as shown in Table 3.

Table 3. All possible combinations of four mating types

| | A_xB_x | A_xB_y | A_yB_x | A_yB_y |
|----------|--------------|---------------------------|--|---|
| A_xB_x | Incompatible | Hemi-compatible
"Flat" | Hemi-compatible
barrage
false clamps | Compatible
with clamp
connections |
| A_xB_y | | Incompatible | Compatible
with clamp
connections | Hemi-compatible
"Barrage" |
| A_yB_x | | | Incompatible | Hemi-compatible
"Flat" |
| A_yB_y | | | | Incompatible |

Mating Reaction Morphology

a) Compatible matings: $A_xB_x \times A_yB_y$ or $A_xB_y \times A_yB_x$.

Monokaryotic donor colonies grew smoothly toward each other and formed an undifferentiated contact zone. Clamp connections formed first in the contact zone, and subsequently were found all over the mycelia within four weeks. Hyphae with clamp connections occasionally gave rise to primordia. Hyphae in primordia were more compact, thicker-walled, and wider.

b) Common-A hemicompatible matings: $A_xB_x \times A_xB_y$ or $A_yB_x \times A_yB_y$. Contact zone hyphae were sparse, often intermingling. After

a prolonged period, matings occasionally formed weak or strong "barrage" zones, but never formed false clamp connections.

c) Common-B hemicompatible matings: $A_xB_x \times A_yB_x$ or $A_yB_y \times A_xB_y$. Hyphae from both donor colonies were raised in the contact zone and showed a "barrage" reaction. False clamp connections were produced sporadically in the barrage zone, and occasionally yellow pigment was deposited along the contact zone.

d) Incompatible matings: Common-A, common-B. No distinct contact zone was formed, hyphae intermingled, and no false clamp connections were observed.

Figures 10 and 11 show two self-crosses of *C. pyxidata* no. 1190 from New York state and 1541 from China. Incompatibility factors in 1190 were assigned as $A_{1,2}$ and $B_{1,2}$. Assignment of mating types of subsequent collections of *C. pyxidata* was based on results of intercollection matings with, and the mating factors assigned to 1190.

Monokaryotic Fruiting

No fruitbodies were formed in compatible matings. This may be because nutritional requirements for fruiting were not satisfied by the growth medium (James, 1983). Haploid fruitbodies were occasionally produced by a few single-spore isolates. These haploid fruitbodies displayed abnormal branches, pale coloration and small size, and no

| | | | | | | | | | | | | |
|----|----------|---|---|----------|---|----------|----|----------|---|---|----|----|
| 1* | - | | | | | | | | | | | |
| 2 | - | - | | | | | | | | | | |
| 9 | - | - | - | | | | | | | | | |
| 4* | B | B | B | - | | | | | | | | |
| 8 | B | B | B | - | - | | | | | | | |
| 3* | - | - | - | + | + | - | | | | | | |
| 12 | - | - | - | + | + | - | - | | | | | |
| 5* | + | + | + | - | - | B | B | - | | | | |
| 6 | + | + | + | - | - | B | B | - | - | | | |
| 7 | + | + | + | - | - | B | B | - | - | - | | |
| 10 | + | + | + | - | - | B | B | - | - | - | - | |
| 11 | + | + | + | - | - | B | B | - | - | - | - | - |
| | 1 | 2 | 9 | 4 | 8 | 3 | 12 | 5 | 6 | 7 | 10 | 11 |
| | A_1B_1 | | | A_2B_1 | | A_1B_2 | | A_2B_2 | | | | |

Fig. 10. *Clavicorna pyxidata*: Self-crosses of collection 1190. + = compatible mating, clamp connections present; - = incompatible mating, no clamp connections observed; B = incompatible, barrage phenomenon observed.

| | | | | | | | | | |
|----|---|---|---|---|---|---|---|---|---|
| 1* | - | | | | | | | | |
| 8 | - | - | | | | | | | |
| 9 | - | - | - | | | | | | |
| 2* | + | + | + | - | | | | | |
| | + | + | + | | | | | | |
| 3 | + | + | + | - | - | | | | |
| | + | + | + | | | | | | |
| 4* | - | - | - | - | - | - | | | |
| | B | B | B | | | | | | |
| | - | - | - | | | | | | |
| 10 | - | - | - | - | - | - | - | | |
| | B | B | B | | | | | | |
| | - | - | - | | | | | | |
| 5* | - | - | - | - | - | + | + | + | - |
| | | | | B | B | + | + | + | |
| | | | | - | - | | | | |
| 6 | - | - | - | - | - | + | + | - | - |
| | F | | | B | B | + | + | | |
| | - | | | - | - | + | + | | |

1* 8 9

— A₂₃B₂₄

2* 3

— A₂₄B₂₅

4* 10

— A₂₄B₂₄

5* 6

— A₂₃B₂₅

Fig. 11. *Clavicornia pyxidata*: Self-crosses of collection 1541. + = compatible, with clamp connections; + = clamp connections were produced at periphery; - = incompatible, clamp connections absent; B = incompatible, barrages were observed; F = incompatible, flat reactions were observed; * = tester strains.

hymenium, basidia and basidiospores were produced. Some incompatible matings with common-A and/or common-B factors gave rise to similar sterile fruitbodies as found in monokaryons. Condensed mycelium was found in some compatible matings but not in incompatible matings. Such mycelium consisted of slightly thick-walled, inflated, densely interwoven, dikaryotic hyphae and was observed in tissue cultures of some collections. These phenomena were considered to be insignificant.

Intercollection Matings

a). Fruitbodies from the same log (Fig. 12)

Collections 1660, 1661, 1662 (North Carolina) were collected about 6-20 cm apart on the same rotten log. Intercollection matings between them resulted in the same pattern as self-crosses, indicating that these three collections were produced by one mycelial pad permeating much of the log.

b). Southern Appalachian Collections (Fig. 13)

Collections 56662, 56653, 56655 from North Carolina, 56667 and 56665 from Georgia and 1607 from Tennessee represented the southern Appalachian Mountain population. Matings among these collections showed intercompatibility and the presence of common alleles within this population. 56665 (GA) shared a common-B factor (B_{18}) with 1607 (TN). Pairings with B factor in common (1607:4, 6 X 56665:3, 9),

| No. | 1660 | | | | 1661 | | | | 1662 | | | |
|---------|------|---|---|---|------|---|---|---|------|---|---|----|
| Testers | 1 | 4 | 2 | 3 | 5 | 4 | 3 | 6 | 4 | 3 | 9 | 14 |
| 1660 1 | - | - | - | + | - | - | - | + | - | - | - | + |
| 4 | - | - | + | - | - | - | + | - | - | - | + | - |
| 2 | - | + | - | - | - | + | - | - | - | + | - | - |
| 3 | + | - | - | - | + | - | - | - | + | - | - | - |
| 1661 5 | | | | | - | - | - | + | - | - | - | + |
| 4 | | | | | - | - | + | - | - | - | + | - |
| 3 | | | | | - | + | - | - | - | + | - | - |
| 6 | | | | | + | - | - | - | + | - | - | - |
| 1662 4 | | | | | | | | | - | - | - | + |
| 3 | | | | | | | | | - | - | + | - |
| 9 | | | | | | | | | - | + | - | - |
| 14 | | | | | | | | | + | - | - | - |

Fig. 12. *Clavicornia pyxidata*: Matings among collections from the same log. + = compatible mating; - = incompatible mating.

| No. | 56662 | 56665 | 56667 | 56653 | 56655 | 1607 |
|---------|----------|---------|---------|---------|---------|---------|
| Testers | 1 2 10 3 | 1 2 9 3 | 2 3 4 9 | 2 5 8 4 | 1 3 4 7 | 4 6 7 5 |
| 56662 1 | - - - + | + + + + | + + + + | + + + + | + + + + | + + + + |
| 2 | - - + - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 10 | - + - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 3 | + - - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 56665 1 | - - - + | + + + + | + + + + | + + + + | + + + + | + + + + |
| 2 | - - + - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 9 | - + - - | + + + + | + + + + | + + + + | + + + + | - - + + |
| 3 | + - - - | + + + + | + + + + | + + + + | + + + + | - - + + |
| 56667 2 | - - - + | + + + + | + + + + | - + - + | + + + + | + + + + |
| 3 | - - + - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 4 | - + - - | + + + + | + + + + | - + - + | + + + + | + + + + |
| 9 | + - - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 56653 2 | - - - + | + + + + | + + + + | + + + + | + + + + | + + + + |
| 5 | - - + - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 8 | - + - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 4 | + - - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 56655 1 | - - - + | + + + + | + + + + | + + + + | + + + + | + + + + |
| 3 | - - + - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 4 | - + - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 7 | + - - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 1607 4 | - - - + | + + + + | + + + + | + + + + | + + + + | + + + + |
| 6 | - - + - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 7 | - + - - | + + + + | + + + + | + + + + | + + + + | + + + + |
| 5 | + - - - | + + + + | + + + + | + + + + | + + + + | + + + + |

Fig. 13. *Clavicornia pyxidata*: Matings among southern Appalachian Mountain collections. + = compatible mating; - = incompatible mating.

formed distinct barrage reactions and no clamp connections were observed. Isolates 56667:2, 4 shared a common-A factor (A_{11}) with 56655:1, 4, identified by flat contact zone morphology and absence of clamp connections. In all compatible matings, clamp connections formed extensively over all mycelia within 4 weeks.

c). Matings between northeastern United States and southern Appalachian Mountain population (Fig. 14)

Collection 1741, 1706, 1190 (New York), 239 (Minnesota) and 1506 (Wisconsin) were chosen to represent the northern United States population, while 56662, 56653, 56655, 56667 and 1607 represented the southern Appalachian Mountain population. Inter-collection matings between these two geographic populations are shown in Fig. 14.

Isolates 1607:5, 7 were incompatible with tester 1706:1, 3. Mating reactions of these four pairs revealed a common-B pairing. Yellowish pigments and false clamp connections were found extensively in the contact zone. According to the order of assignment, this common designated as B_3 .

d). Matings between collections from northeastern United States and northeastern China (Fig. 15)

Collection 1190 was from New York and 1449 from northeast China. Matings between tester strains of these two collections resulted in partial compatibility.

| No. | | 56662 | 56665 | 56667 | 56653 | 56655 | 1607 | | | | |
|--------|---|---------|---------|---------|---------|---------|---------|---|---|---|---|
| Tester | | 1 2 3 0 | 1 2 3 9 | 2 3 4 9 | 2 4 5 8 | 1 3 4 7 | 4 5 6 7 | | | | |
| 1741 | 3 | + | + | + | + | + | + | + | + | + | + |
| | 4 | + | + | + | + | + | + | + | + | + | + |
| | 7 | + | + | + | + | + | + | + | + | + | + |
| | 8 | + | + | + | + | + | + | + | + | + | + |
| 1706 | 1 | + | + | + | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + | + | + | + |
| | 5 | + | + | + | + | + | + | + | + | + | + |
| | 9 | + | + | + | + | + | + | + | + | + | + |
| 1190 | 1 | + | + | + | + | + | + | + | + | + | + |
| | 4 | + | + | + | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + | + | + | + |
| | 5 | + | + | + | + | + | + | + | + | + | + |
| 239 | 1 | + | + | + | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + | + | + | + |
| | 8 | + | + | + | + | + | + | + | + | + | + |
| | 7 | + | + | + | + | + | + | + | + | + | + |
| 1506 | 2 | + | + | + | + | + | + | + | + | + | + |
| | 5 | + | + | + | + | + | + | + | + | + | + |
| | 7 | + | + | + | + | + | + | + | + | + | + |
| | 8 | + | + | + | + | + | + | + | + | + | + |

Fig. 14. *Clavicornia pyxidata*: Matings among collections from northeast and southeast United States. + = compatible mating; - = incompatible mating.

| 1190 | | A_1B_1 | | | A_1B_2 | | A_2B_1 | | | | A_2B_2 | | | | | |
|----------------|----|----------|---|---|----------|---|----------|---|----|---|----------|---|---|----|----|----|
| 1449 | | 1 | 2 | 9 | 3 | 2 | 4 | 8 | 13 | 1 | 5 | 6 | 7 | 10 | 11 | 15 |
| $A_{12}B_{23}$ | 1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 10 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 11 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 14 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| $A_{12}B_1$ | 7 | B | - | - | + | + | - | B | B | - | + | + | + | + | + | + |
| | 16 | B | - | - | + | + | - | - | B | - | + | + | + | + | + | + |
| $A_{22}B_{23}$ | 2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 4 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 6 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 9 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 12 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| $A_{22}B_1$ | 18 | - | - | - | + | + | - | - | - | - | + | + | + | + | + | + |

Fig. 15. *Clavicornia pyxidata*: Single-spore matings between collections from northeastern United States (1190) and northeastern China (1449). + = compatible mating; - = incompatible mating; B = incompatible with false clamp connections.

To determine mating relationship of the two collections, 14-15 single-spore isolates were employed and mated in all possible combinations. The larger strain sample, shown in Fig. 8, revealed the occurrence of common-B alleles, confirming the results obtained by tester matings, i. e. a collection from northeastern United States shared a common mating factor with a collection from northeastern China. Because of the assignment of mating types in collection 1190, the common-B factor in 1449 was also assigned as B₁.

e). Matings between European collections (Fig. 16)

Tester strains of two collections, GB1279 and GB1280 from Sweden, were contributed. Self-crosses and intercollection mating revealed a bifactorial mating system and presence of a common factor shared by these two collections.

| Coll. no.
Testers | | GB 1279 | | | | GB 1280 | | | |
|----------------------|----|---------|---|---|---|---------|---|----|----|
| | | 1 | 2 | 4 | 6 | 1 | 3 | 10 | 12 |
| GB 1279 | 1 | - | - | - | - | + | + | - | - |
| | 2 | | - | - | + | + | + | + | + |
| | 4 | | | - | + | + | + | + | + |
| | 6 | | | | - | + | + | - | + |
| GB 1280 | 2 | | | | | - | - | - | + |
| | 3 | | | | | | - | + | - |
| | 10 | | | | | | | - | - |
| | 12 | | | | | | | | - |

Fig. 16. *Clavicornia pyxidata*: Matings between European collections GB1279 and GB1280. + = compatible mating; - = incompatible mating.

In self-crosses of GB1279, single-spore isolates 2 and 4 were compatible with isolate 6, indicating that 2 and 4 belonged to the same mating type. Consequently, it was concluded that only three mating types were represented in GB1279. If isolate GB1279:1, 6 was incompatible with GB1280:10, 12, there would be a typical pattern for common alleles. It is difficult to understand the compatible mating between GB1279:6 and GB1280:12.

f). Matings among intercontinental collections (Fig. 17)

Collections 1190 and 1607 (eastern North America), and 1449 and 1541 (China) were selected to conduct matings with GB 1279 and GB 1280 (Sweden). These collections were compatible and shared common alleles as well. From Fig. 17, GB1279:1, 6 shared a common allele with GB1280:10, 12. As shown in Fig. 16, both GB1279 and GB1280 shared the same allele with 1541:2, 5. Apparently, the same mating factor occurred both in Sweden and China.

| No.
Testers | 1190 | | | | 1607 | | | | 1449 | | | | 1541 | | | |
|----------------|------|---|---|---|------|---|---|---|------|---|---|----|------|---|---|---|
| | 1 | 3 | 4 | 5 | 4 | 5 | 6 | 7 | 1 | 2 | 7 | 18 | 1 | 2 | 4 | 5 |
| GB 1279 | 1 | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - |
| | 2 | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + |
| | 4 | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + |
| | 6 | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - |
| GB 1280 | 2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 10 | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - |
| | 12 | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - |

Fig. 17. *Clavicornia pyxidata*: Matings among intercontinental collections. + = compatible mating; - = incompatible mating.

g). Overall intercollection mating results (Fig. 18)

Out of the 28 collections for which self-crosses were conducted, 16 collections were selected to represent various geographical areas. Results of intercollection matings in all possible combinations of the 16 collections are given in Fig. 18.

Clavicornia turgida

Mating System

Among collections identified as *C. turgida*, single-spore isolates of two collections, 2651 and 2682, were obtained. Self-crosses within the two collections revealed a bifactorial mating system. In self-crosses of 2651, ten single-spore isolates were used, out of which one was contaminated. The results are shown in Fig. 19.

In Fig. 19, it was unexplained why isolate 2 was compatible with 3, but incompatible with 9. Three and 9 belong to the same mating type because both were compatible with 4, 6 and 7. Isolate 1 was probably a foreign single-spore isolates because it was incompatible with any isolate of collection 2651.

Mating Reaction Morphology

Mating reaction morphologies in this species were very vague. It was not possible to identify mating types by

| Coll.
No. | 1
1
9
0 | 1
7
0
6 | 1
7
4
1 | 2
3
9 | 1
5
0
3 | 1
5
0
6 | 5
6
6
6
7 | 5
6
6
5
5 | 5
6
6
6
5 | 5
6
6
6
2 | 5
6
6
5
3 | 1
6
0
7 | 1
4
4
9 | 1
5
4
1 | 1
2
7
9 | 1
2
8
0 |
|--|------------------|------------------|------------------|-------------|------------------|------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------|------------------|------------------|------------------|------------------|
| 1190
A ₁ A ₂ B ₂ | | + | + | + | + | + | + | + | + | + | + | + | B
1 | + | B
2 | + |
| 1706
A ₂ A ₃ B ₂ | | | + | + | A
3 | A
4 | + | + | + | + | + | B
3 | + | + | + | + |
| 1741
A ₁ A ₂ B ₃ | | | | | + | + | + | + | + | + | + | + | + | + | + | + |
| 239
A ₂ A ₂ B ₃ | | | | | + | + | + | + | + | + | + | + | + | + | + | + |
| 1503
A ₂ A ₂ B ₁₀ | | | | | | A
3 | + | + | + | + | + | + | + | + | + | + |
| 1506
A ₂ A ₁₀ B ₁₁ B ₂ | | | | | | | + | + | + | + | + | + | + | + | + | + |
| 5667
A ₁₁ A ₁₂
B ₁₃ B ₁₄ | | | | | | | | A
11 | + | + | + | + | A
12 | + | + | + |
| 56655
A ₁₁ A ₁₃
B ₁₅ B ₁₆ | | | | | | | | | + | + | + | + | + | + | + | + |
| 56665
A ₁₄ A ₁₅
B ₁₇ B ₁₈ | | | | | | | | | | + | + | B
18 | + | + | + | + |
| 56662
A ₁₆ A ₁₇
B ₁₉ B ₂₀ | | | | | | | | | | | + | + | + | + | + | + |
| 56653
A ₁₉ A ₁₉
B ₂₁ B ₂₂ | | | | | | | | | | | | + | + | + | + | + |
| 1607
A ₂₀ A ₂₁ B ₂₃ B ₁₈ | | | | | | | | | | | | | + | + | + | + |
| 1449
A ₁₂ A ₁ B ₂₂ B ₂₃ | | | | | | | | | | | | | | + | + | + |
| 1541
A ₂₂ A ₂₄
B ₂₄ B ₂₅ | | | | | | | | | | | | | | | B
25 | B
25 |
| GB1279
A ₂₅ A ₂₆ B ₂₆ B ₂₅ | | | | | | | | | | | | | | | | B
25 |
| GB1280
A ₂₇ A ₂₈
B ₂₈ B ₂₉ | | | | | | | | | | | | | | | | |

Fig. 18. *Clavicornia pyxidata*: Matings among continental collections, with mating type assignment. + = all pairings are compatible; A = common A factor; B = common B factor.

| | | | | | | | | | |
|-----|---|---|---|---|---|---|---|---|---|
| 3* | - | | | | | | | | |
| 9 | - | - | | | | | | | |
| 4* | + | + | - | | | | | | |
| 6 | - | + | - | - | - | | | | |
| 7 | - | + | + | - | - | - | | | |
| 5* | - | - | - | - | - | - | | | |
| 10* | - | - | - | - | - | - | - | | |
| 2 | - | + | - | - | - | - | - | - | |
| 1 | - | - | - | - | - | - | - | - | - |

| | | | | | | | | |
|-------------------------------|---|-------------------------------|---|---|-------------------------------|-------------------------------|---|---|
| 3* | 9 | 4* | 6 | 7 | 5* | 10* | 2 | 1 |
| A ₁ B ₁ | | A ₂ B ₁ | | | A ₁ B ₂ | A ₂ B ₁ | | |

Fig. 19. *Clavicornia turgida*: Self-crosses of collection no. 2651, + = compatible, clamp connections present; - = incompatible, clamp connections absent; * = tester strains; + (in corner) = clamp connections present; - (in corner) = clamp connections absent.

macromorphology. A typical "flat" reaction might be found in a compatible mating and a "compatible"-like mating might be devoid of clamp connections. Moreover, clamp connections were rare in some compatible matings. To confirm a compatible mating, therefore, DAPI-fluorescence microscopic methods were employed.

a) Compatible matings: $A_1B_1 \times A_2B_2$, $A_1B_2 \times A_2B_1$. No clear morphological characters could be used to define a compatible mating, but paired nuclei and clamp connections on dikaryotic hyphae were reliable indicators. Clamp connections were rare in some matings and produced only in the contact zone, while in other compatible matings clamp connections were produced both in the contact zone and periphery, indicating that nuclear migration occurred.

b) Flat reactions: $A_1B_1 \times A_1B_2$, $A_2B_2 \times A_2B_1$. Hyphae from both donor colonies became sparse and formed a clear contact zone. Clamp connections were absent. Many matings were morphologically similar to flat reactions, but not all of them were true. In some flat-like matings, clamp connections were produced in the contact zone.

c) Barrage reactions: $A_1B_1 \times A_2B_1$, $A_1B_2 \times A_2B_2$. No distinct line could be drawn between barrage and flat reactions. No false clamp connections were observed in either. The mating types were assigned according solely as a result of compatible matings.

d) Incompatible matings: common-A and common-B. Two mycelia smoothly grew together, with no clear contact zone, and no clamp connections were produced.

Intercollection Matings

Intercollection matings were carried out among tester strains of collection 2651 and 2682, both from New Zealand. Mating results, as shown in Fig. 20, indicated that these two collections were fully compatible. Clamp connections were produced in all pairings, mostly restricted to the contact zone, but occasionally at the periphery. It seemed that more clamp connections were produced in intercollection matings than in intracollection matings.

| Coll. No.
Testers | 2651 | | | | 2682 | | | | |
|----------------------|------|---|---|----|--------|---|---|---|---|
| | 3 | 4 | 5 | 10 | 1 | 8 | 6 | 9 | |
| 2651 3 | - | + | - | - | + | + | + | + | |
| 2651 4 | | - | - | - | + | + | + | + | |
| 2651 5 | | | - | + | + | + | + | + | |
| 2651 10 | | | | - | + | + | + | + | |
| | | | | | | | | | |
| | | | | | 1 | - | + | - | - |
| | | | | | 2682 8 | | - | - | - |
| | | | | | 6 | | | - | + |
| | | | | | 9 | | | | - |

Fig. 20. *Clavicornia turgida*: Intercollection matings between tester strains of collection 2651 and 2682.
+ = compatible mating; - = incompatible mating.

***Clavicornia* Taxon 2 (collection 3456)**

Mating Systems

Collection 3456 was gathered from Puerto Rico very near the end of this study. Matings among 12 single-spore isolates of collection 3456 revealed a typical heterothallic, bifactorial (tetrapolar) mating system (Fig. 21). Compatibility test patterns were typical except that isolate 5 was compatible with isolate 10. In pairings 4 X 5, 4 X 9 and 9 X 10, typical barrage reaction morphology and false clamp connections were observed. In pairing 5 X 10, barrage morphology and false clamp connections were observed, but paired nuclei were also showed in some hyphae, which could be considered as an indication of a dikaryon, although such hyphae were rare. This particular mating might have resulted from contamination or recombination of suballeles.

Mating Reaction Morphology

Mating reaction morphology in collection 3456 was of a typical pattern, similar to that of *C. pyxidata*. It was noteworthy that some isolates of collection 3456 smelled like a fresh peach.

a) Compatible matings: $A_1B_1 \times A_2B_2$ or $A_1B_2 \times A_2B_1$. Two monokaryotic mycelia grew smoothly toward each other and formed an undifferentiated contact zone, or sometimes a

| | | | | | | | | | | | | |
|----|----------|----|---|----------|---|---|---|----------|----|----|----------|----|
| 1 | - | | | | | | | | | | | |
| 2* | - | - | | | | | | | | | | |
| 6 | - | - | - | | | | | | | | | |
| 3* | + | + | + | - | | | | | | | | |
| 8 | + | + | + | - | - | | | | | | | |
| 5 | + | + | + | - | - | - | | | | | | |
| 9 | + | + | + | - | - | - | - | | | | | |
| 7* | B | - | - | - | - | - | - | - | | | | |
| 11 | B | - | B | - | - | - | - | - | - | | | |
| 12 | B | - | B | - | - | - | - | - | - | - | | |
| 4* | F | F | - | - | - | B | B | + | + | + | - | |
| 10 | F | - | - | - | - | + | B | + | + | + | - | - |
| | 1 | 2* | 6 | 3* | 8 | 5 | 9 | 7* | 11 | 12 | 4* | 10 |
| | A_1B_1 | | | A_2B_2 | | | | A_2B_1 | | | A_1B_2 | |

Fig. 21. *Clavicornia* Taxon 2 (collection 3456): Self-crosses of collection 3456. + = compatible matings; - = incompatible matings; F = incompatible, with distinct flat morphology; B = incompatible with distinct barrage morphology and false clamp connections; * = tester strains.

clear contact zone with sparse hyphae. Clamp connections were produced abundantly in the center within 6 weeks, but rarely at the periphery.

b) Common-A incompatible matings: $A_1B_1 \times A_1B_2$ or $A_2B_1 \times A_2B_2$. Mycelium became sparse and intermingled in the center and left a clear contact zone. No clamp connections were produced.

c) Common-B incompatible matings: $A_1B_1 \times A_2B_1$ or $A_1B_2 \times A_2B_2$. Hyphae from both donor mycelia were raised in the contact zone and showed a "barrage" reaction. False clamp connections were formed sporadically in the contact zone.

d) Incompatible matings: common-A and common-B, $A_1B_1 \times A_1B_1$, $A_1B_2 \times A_1B_2$, $A_2B_1 \times A_2B_1$ or $A_2B_2 \times A_2B_2$. Hyphae from donor mycelia were intermingled and no distinct contact zone was formed. Mating morphology was occasionally similar to a compatible mating, but no clamp connections were produced.

2. Results of Interspecific Matings

a. Matings between *C. pyxidata* and *C. microspora* (Fig. 22)

Because Japanese collections 2349 and 5352 were collected and identified as *C. pyxidata* by Japanese mycologists, intercollection matings were carried out between the Japanese collections and some *C. pyxidata* collections. The results are shown in Fig. 22. All "interspecific" matings were incompatible and produced

C. pyxidata

| | | | | | | | | | | | |
|-------|---|---|---|---|---|---|---|---|---|---|---|
| Coll. | 1 | 1 | 2 | 1 | 1 | 5 | 5 | 5 | 1 | 1 | 1 |
| No. | 1 | 7 | 3 | 5 | 5 | 6 | 6 | 6 | 6 | 4 | 5 |
| | 9 | 0 | 9 | 0 | 0 | 6 | 6 | 6 | 0 | 4 | 4 |
| | 0 | 6 | | 3 | 6 | 6 | 5 | 6 | 7 | 9 | 1 |
| | | | | | | 7 | 5 | 5 | | | |
| 2349 | - | - | - | - | - | - | - | - | - | - | |
| 5352 | - | - | - | - | - | - | - | - | - | - | - |

Fig. 22. Matings between isolates of *C. pyxidata* and *C. microspora* (collections 2349 and 5352). - = All matings among tester strains were incompatible.

strong antagonism phenomena. Mycelia from both donors confronted and yellow to brownish pigments were deposited in the contact zone. Neither clamp connections nor false clamp connections were found. It was concluded that the Japanese collections and *C. pyxidata* collections belonged to two intersterility groups (i. e. the Japanese collections represented a different biological species from *C. pyxidata*).

b. Matings between *C. pyxidata* and *C. turgida* (Fig. 23)

Tester strains of collection 2682, representing *C. turgida*, were paired with tester strains of several collections representing *C. pyxidata*. All pairings between isolates of the two species were incompatible, as shown in Fig. 23, with strong antagonism phenomena.

| | | | | | | |
|-------------------|-----------|--------------------|------|------|--------|--------|
| | | <i>C. pyxidata</i> | | | | |
| <i>C. turgida</i> | Coll. No. | 1503 | 1506 | 1541 | GB1279 | GB1280 |
| | 2682 | - | - | - | - | - |

Fig. 23. Matings between isolates of *C. pyxidata* and *C. turgida*. - = All pairings between tester strains were incompatible.

c. Matings between *C. candelabrum* and *C. turgida* (Fig. 24)

Collection 2679, representing *C. candelabrum*, and 2651 and 2682, representing *C. turgida*, were all from New Zealand. Matings between tester strains of collection 2651 and 2682 indicated that these two collection were fully compatible. Matings between single spore isolates of 2679 and the two *C. turgida* collections, 2651 and 2682, were completely incompatible, as shown in Fig. 24, indicating that these two morphological species were also separate biological species.

| | | | |
|-----------------------|-----------|-------------------|------|
| | | <i>C. turgida</i> | |
| <i>C. candelabrum</i> | Coll. No. | 2651 | 2682 |
| | 2679 | - | - |

Fig. 24 Matings between isolates of *C. candelabrum* and *C. turgida*. - = All matings between tester strains were incompatible.

d. Matings between *Clavicornia* Taxon 2 and other species

(Fig. 25)

The collection 3456, representing *Clavicornia* Taxon 2, were paired with isolates of *C. microspora* (2349), *C. pyxidata* (56667, 1541) and *C. turgida* (2651), with those it shared certain morphological similarities. Mating results, as indicated in Fig. 25, showed that collection 3456 was incompatible with all the other species, indicating that collection 3456 belonged to a separate biological species. Two types of strong antagonism phenomena with difference in contact zone morphology were observed: 1) donor mycelia conflicted in the center or close to one donor mycelium and formed a yellowish brown contact zone, as in pairing between collection 3456 and *C. microspora*, *C. pyxidata* (1541) and *C. turgida*; 2) one donor mycelium lysed the other mycelium and subsequently formed a contact zone consisting of sparse, mycelium without contents in one side, as in pairings between collection 3456 and *C. pyxidata* (56667).

| <i>C. microspora</i> | | | | | <i>C. pyxidata</i> | | | | | <i>C. turgida</i> | | | | | | |
|----------------------|---|---|---|----|--------------------|---|---|---|---|-------------------|---|---|---|---|---|----|
| 2349 | | | | | 56667 | | | | | 1541 | | | | | | |
| | 2 | 3 | 5 | 10 | 2 | 3 | 4 | 9 | 1 | 3 | 4 | 5 | 3 | 4 | 5 | 10 |
| 3 | 2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | 3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | 4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 6 | 7 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Fig. 25. Matings between *Clavicornia* Taxon 2 (3456) and three other species, *C. microspora*, *C. pyxidata* and *C. turgida*. - = incompatible mating.

D. SPOT TESTS FOR LACCASE AND TYROSINASE ACTIVITY

1. Laccase Activity in *Clavicornona*

Isolates tested for laccase and tyrosinase were the same as those utilized in culture studies (Appendix A). Tests were performed on MA, PDA and MYG at Week II and Week VI. Syringaldazine [N, N'-bis-(3,5-dimethoxy-4-hydroxybenzylidene) hydrazine] was selected as the spot test reagent for laccase activity because it had been proven to be a highly sensitive and laccase-specific substrate for the rapid detection of laccase activity (Marr, 1986; Desjardin, 1989). A positive test was indicated by a fuschia or purple coloration. No color change after added syringaldazine for 1 hour was considered as a negative reaction. In a few cases, the color of the culture plug changed to bright yellow instead of fuschia, but this reaction was also considered to be negative.

Results of spot tests for laccase activity in cultures of *Clavicornona* species are shown in Table 4. In all isolates tested, positive reactions for laccase were observed on at least one medium at Week II and/or Week VI. Isolates of *C. colensoi* showed strong laccase-positive reactions on MA, relatively weaker positive reactions on PDA, and negative reactions on MYG.

Table 4. Results of spot tests for laccase activity in cultures of *Clavicornia* species.

| Epithets & isolates | | MA | | PDA | | MYG | |
|---------------------|---------|-----|-----|-----|-----|-----|-----|
| | | II | VI | II | VI | II | VI |
| <i>colensoi</i> | 2555 | +++ | +++ | + | + | - | - |
| | 2644 | +++ | +++ | + | + | - | - |
| | 44117 | +++ | ++ | + | + | - | - |
| | 44120 | +++ | +++ | ++ | + | - | - |
| <i>cristata</i> | DLL8974 | | +++ | | - | | + |
| | GB1700 | | +++ | | - | | + |
| <i>microspora</i> | 2349 | ++ | + | ++ | + | + | + |
| <i>pyxidata</i> | 1190 | + | ++ | +++ | - | ++ | - |
| | 10654 | +++ | +++ | +++ | - | + | - |
| | 1503 | +++ | - | ++ | - | ++ | - |
| | 1506 | + | - | ++ | - | +++ | + |
| | JPL479 | ++ | ++ | +++ | + | - | + |
| | 56655 | ++ | +++ | ++ | +++ | ++ | - |
| | 24439 | +++ | + | +++ | + | +++ | - |
| | 22501 | + | - | +++ | + | +++ | - |
| | 56667 | +++ | ++ | +++ | + | + | - |
| | 1607 | +++ | + | +++ | - | +++ | +++ |
| | 1449 | +++ | +++ | +++ | +++ | +++ | +++ |
| | 1541 | + | +++ | + | +++ | + | - |
| | GB1279 | ++ | + | ++ | ++ | + | - |
| | GB1280 | ++ | + | ++ | ++ | + | - |
| <i>turgida</i> | 2651 | +++ | +++ | | +++ | | ++ |
| | 2682 | +++ | +++ | | +++ | | +++ |
| | 44103 | +++ | +++ | | +++ | | +++ |
| Taxon 1 | 1615 | | +++ | | ++ | | + |

Isolates of *C. cristata* exhibited strong laccase activity on MA, weaker on MYG and no laccase activity on PDA. Isolates of *C. turgida* showed strong positive reactions for laccase on all three media.

In the fourteen isolates of *C. pyxidata*, strong laccase activity was recorded at Week II, with weaker or no laccase activity at Week VI, especially on MYG.

In positive reactions for laccase, the fuschia reaction appeared very quickly, within 1-10 minutes after the reagent was added, and started to fade after 15 minutes. In some cases, the strongest coloration appeared in the older part of the culture plug, but in other cases the strongest coloration appeared near the advancing zone. This varied for different isolates within a single species or for single isolates on different media.

In summary, all isolates of *Clavicornia* showed laccase activity on at least one medium during development in culture. Laccase activity was not detected from isolates of *C. colensoi* grown on MYG and isolates of *C. cristata* grown on PDA. The lack of variation indicated that laccase spot tests can only be of very limited value for taxonomic separation at the species level. On the other hand, this also proved that laccase is widely distributed in *Clavicornia*, which showed the potential for further analysis on laccase.

2. Tyrosinase Activity in *Clavicornona*

In the spot tests for tyrosinase activity, *p*-cresol was employed because it is substrate-specific for tyrosinase and gave a more intense and faster reaction than the other established reagent, L-tyrosine (Marr, 1984; Desjardin, 1989). A positive reaction for tyrosinase was indicated by a brown coloration which appeared 30 minutes to after the reagent had been added.

Most isolates of *Clavicornona* showed tyrosinase activity on PDA and MYG (Table 5). No tested isolates of *Clavicornona* showed tyrosinase activity on MA. All isolates of *C. colensoi*, *C. cristata*, *C. microspora* and *C. turgida* exhibited tyrosinase activity on PDA and MYG at Week II and Week VI. There was variation within *C. pyxidata*: isolate 1506 showed tyrosinase activity on PDA, but not on MYG, and isolate 1449 showed no tyrosinase activity on all three media.

The results for tyrosinase activity (Table 5) indicate that the presence or absence of tyrosinase may be insignificant in the taxonomy of *Clavicornona* at the species level.

Table 5. Results of spot tests for tyrosinase activity in cultures of *Clavicornia* species

| Epithets & isolates | | MA | | PDA | | MYG | |
|---------------------|---------|----|----|-----|-----|-----|-----|
| | | II | VI | II | VI | II | VI |
| <i>colensoi</i> | 2555 | - | - | + | ++ | + | ++ |
| | 2644 | - | - | + | ++ | + | ++ |
| | 44117 | - | - | ++ | ++ | + | + |
| | 44120 | - | - | ++ | + | + | ++ |
| <i>cristata</i> | DLL8974 | | - | | + | | + |
| | GB1700 | | - | | + | | + |
| <i>microspora</i> | 2349 | - | - | +++ | +++ | +++ | +++ |
| <i>pyxidata</i> | 1190 | - | - | + | +++ | - | ++ |
| | 10654 | - | - | - | ++ | - | ++ |
| | 1503 | - | - | ++ | ++ | +++ | ++ |
| | 1506 | - | - | +++ | ++ | - | - |
| | JPL479 | - | - | +++ | +++ | +++ | +++ |
| | 56655 | - | - | ++ | ++ | ++ | +++ |
| | 24439 | - | - | + | ++ | ++ | +++ |
| | 22501 | - | - | + | - | + | - |
| | 56667 | - | - | +++ | +++ | +++ | +++ |
| | 1607 | - | - | ++ | +++ | + | + |
| | 1449 | - | - | - | - | - | - |
| | 1541 | - | - | + | - | + | +++ |
| | GB1279 | - | - | + | ++ | + | + |
| | GB1280 | - | - | + | ++ | + | + |
| <i>turgida</i> | 2651 | - | - | | +++ | | +++ |
| | 2682 | - | - | | +++ | | +++ |
| | 44103 | - | - | | +++ | | +++ |
| <i>Taxon 1</i> | 1615 | | - | | +++ | | +++ |

E. ISOZYME ANALYSES

Laccase and tyrosinase are two copper-containing enzymes involved in lignin degradation in white-rot fungi, as discussed in Chapter I. Electrophoresis was performed to determine the variation of enzyme banding patterns of laccase and tyrosinase with respect to: 1) age of the cultures (for laccase only); 2) isolates of a given species; and 3) isolates of different species, and consequently, to determine the potential value of laccase and tyrosinase electrophoretic profiles in the systematics of *Clavicornia*.

Thirty-eight dikaryotic isolates representing all species in this study were selected for laccase isozyme electrophoretic comparison, as listed in Appendix A. Electrophoretic methods for laccase followed Kerrigan and Ross (1988), with *o*-toluidine as substrate. Twenty-eight dikaryotic isolates were selected for tyrosinase analysis. Electrophoretic methods for tyrosinase followed Laemmli (1970) and Ingebritsen et al. (1989), with L-dopa as a substrate. One isolate from the representative specimen, *C. pyxidata*, was selected as the standard sample for the purpose of internal mobility comparison.

1. Laccase Electrophoresis

Variation in R_f values between Gels

Variation between gels of same concentration in the R_f value of the enzyme bands was observed in preliminary experiments. For example, R_f value of 24439 was 0.65 in one gel and 0.59 in another. In contrast, mobility of any band relative to other bands was quite constant. A standard sample (*C. pyxidata*, 24439), therefore, was used for comparison, and the internal mobility, R_i , instead of relative mobility, was calculated for each band ($R_i = R_f/R_s$; R_f : relative mobility of tested sample; R_s : relative mobility of standard sample). The R_i value of the standard sample (24439) was determined as 1.0 in every gel. The R_i value varied from 0.8% to 2.0% of its mean value. Electrophoresis was carried out at constant voltage of 50 V.

Developmental Variation

Isolates of *C. colensoi*, *C. cristata*, *C. microspora*, *C. pyxidata*, *C. turgida* and Taxon 1 (1615) were selected to examine developmental variation in laccase production (isolates listed under each species). Cultural media of these isolates were sampled weekly starting at Week III following inoculation, through Week IX or occasionally Week XII. Isolates of the remaining species, namely *C. candelabrum*, *C. piperata*, and Taxon 2, were either slow-

growing or were collected too late for inclusion, so their culture media were sampled only two or three times.

The majority of tested isolates began laccase activity at Week III or IV and declined after the twelve week incubation. The banding patterns of most isolates did not change during the incubating periods, except for isolates of *C. microspora* and Taxon 1, as reported below in detail.

Clavicornia pyxidata (Fig. 26)

Isolate 1541 (China) and 24439 (North Carolina) were selected to represent *C. pyxidata*. Samples were collected from broth cultures at Weeks IV through Week X. A single band indicating laccase activity was detected from Week IV through Week X, increasing in width with age of the culture. The band pattern did not change during mycelium mat development in both isolates. These two isolates, however, produced laccases with different mobility. R_f value of isolate 1541 was 1.06. This result indicated that there was no variation in laccase banding pattern of collection 24439. This proves its validity of being a standard.

Clavicornia colensoi (Fig. 27)

Samples of the broth culture of two isolates of *C. colensoi*, 44120 and 2555, aged 3-9 weeks were collected and electrophoresed on a common gel. The results showed that no laccase was produced at Week III and/or IV by isolate 44120,

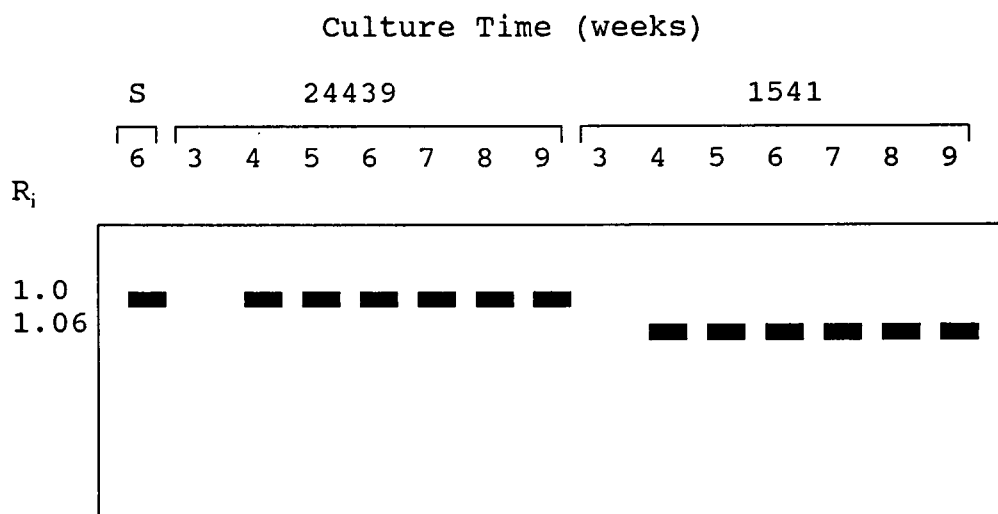


Fig. 26. *Clavicornia pyxidata* (24439, 1541): electropherogram of laccase from liquid media of cultures after 3 to 9 week incubation. S = Standard sample, 24439. R_i = internal mobility.

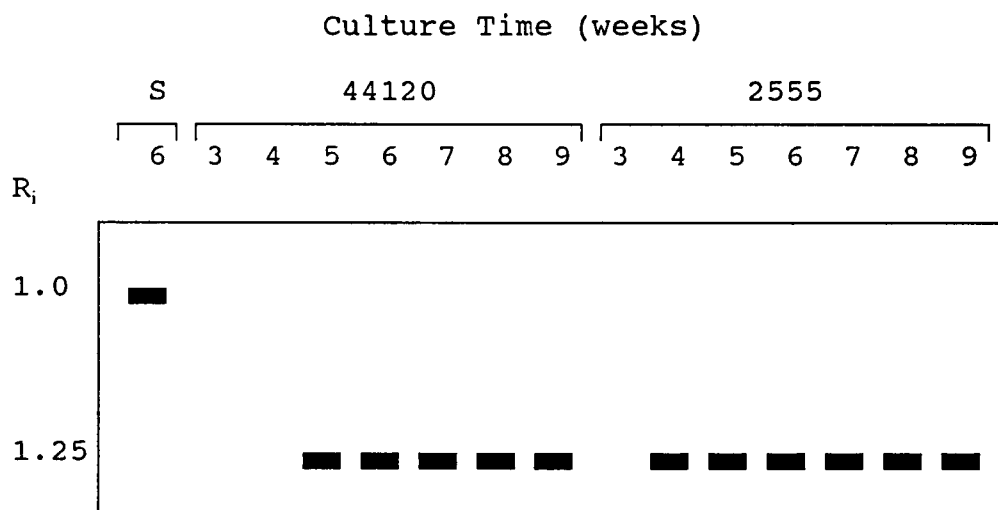


Fig. 27. *Clavicornia colensoi* (44120, 2555): electropherogram of laccase from liquid media of cultures after 3 to 9 week incubation. S = Standard sample, 24439. R_i = internal mobility.

and little laccase was produced in three week-old cultures of isolate 2555. The remaining samples showed a single band. Relative mobility (R_f) of the band exhibited by *C. colensoi* was 0.85, that of the *C. pyxidata* standard sample was 0.68, so the internal mobility (R_i) of *C. colensoi* was 1.25. In both isolates, no variation in the banding pattern among different samples of the same isolate was observed. This indicated that laccase production in *C. colensoi* was stable and consistent during mycelial development.

Clavicornia cristata (Fig. 28)

Clavicornia cristata was characterized by low growth rate. Inoculum plugs were divided into several pieces. The mycelium mat grew up to 3 cm diam/9 weeks. A single band was produced by this isolate at Week IV and the band stained darker at Week IX. The R_i of *C. cristata* was 1.27. No variation in mobility was detected in samples from different culture times.

Clavicornia microspora (Fig. 29)

Broth cultures of isolates 2349 and 5352 were sampled weekly and co-electrophoresed on a single gel. Laccase was not detected in cultures incubated for less than 6 weeks. Two separate bands were produced by samples incubated for 6-9 weeks. Developmental variation in laccase banding patterns of isolate 2349 was observed.

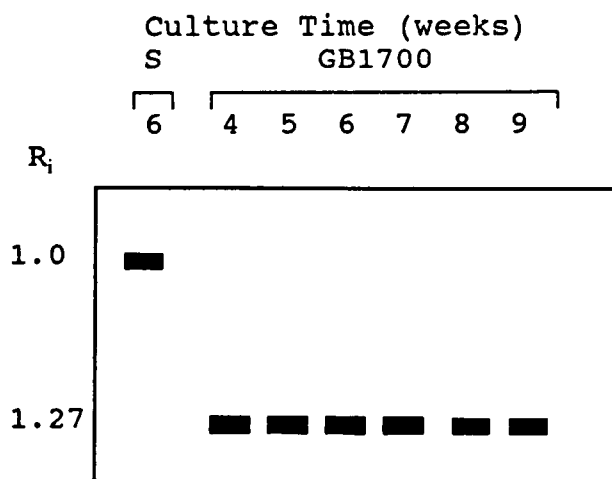


Fig. 28. *Clavicornia cristata* (GB1700): electropherogram of laccase from liquid media of cultures after 4 to 9 week incubation. S = standard sample, 24439; R_i = internal mobility.

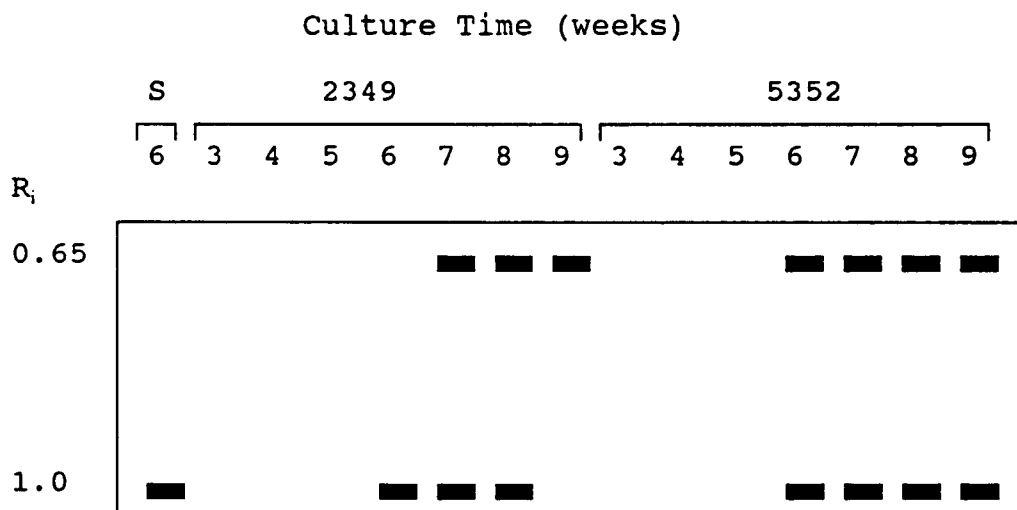


Fig. 29. *Clavicornia microspora* (2349, 5352): electropherogram of laccase from liquid media of cultures after 3 to 9 week incubation. S = Standard sample, 24439. R_i = internal mobility.

The fast band was produced at Week VI, VII and VIII, but did not show at Week IX. The slow band appeared at week VII and VIII, and showed higher intensity at Week IX. The positions of these two bands, however, did not change in any sample during mycelium development. The fast band had the same relative mobility as *C. pyxidata* standard sample ($R_f = 1.0$), while the R_f of the slow band was 0.65.

***Clavicornia turgida* (Fig. 30)**

Cultures of isolates 2682 and 44103 were sampled from Week III through IX. A single band was produced by samples of these two isolates. No variation in laccase mobility was detected. The R_f value of *C. turgida* was 1.14.

***Clavicornia* Taxon 1 (1615) (Fig. 31)**

Cultures of 1615 were sampled weekly, starting at Week III through Week IX and co-electrophoresed with one isolate of *C. microspora* (2349). A complex banding pattern was produced by all samples. A fast band and a complex of slow bands were produced by isolate 1615, with variation over time. The fast band was thin and had almost the same mobility as the band produced by the standard isolate ($R_f \approx 1.0$). The mobility difference between the fast band produced by 1615 and those by *C. microspora* (2349) is showed in Fig. 31. The complex of slow bands varied in different mycelial developmental stages. Samples obtained at Week III

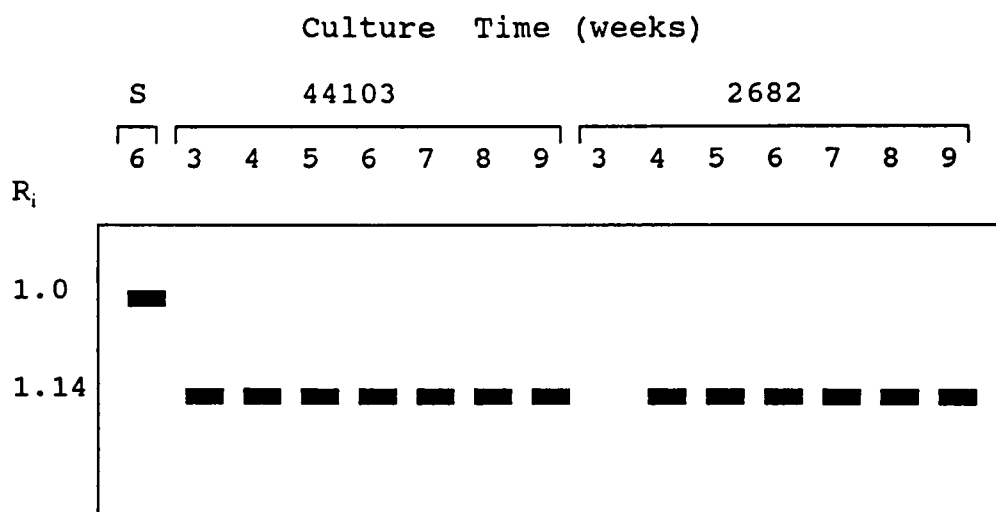


Fig. 30. *Clavicornona turgida* (44103, 2682): electropherogram of laccase from liquid media of cultures after 3 to 9 week incubation. S = Standard sample, 24439. R_i = internal mobility.

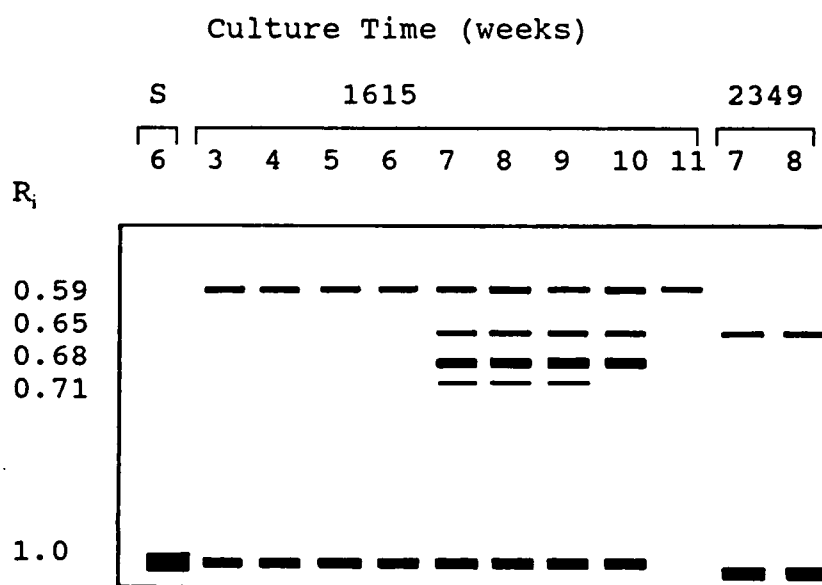


Fig. 31. *Clavicornona taxon 1* (1615): electropherogram of laccase from liquid media of cultures after 3 to 11 week incubation, with comparison to *C. microspora* (2349). S = Standard sample, 24439. R_i = internal mobility.

to V produced a simple slow band, with R_f value as 0.59. Samples collected at Week VI and VII produced a faster band, with the R_f value as 0.68. Samples at Week VIII and IX produced a complex consisting of four diffused slow bands, with R_f value ranged from 0.59 to 0.72. The slow band produced by isolate 2349 of *C. microspora* had an internal mobility as 0.68, but mobility of the fast band in the two species was slightly different.

In summary, laccase activity was stable and reproducible in most tested species. Electrophoregrams of laccase in most species were consistent in mycelium developmental stage and in different samples of the same isolates. Exceptions were detected in the isolates of *C. microspora* and Taxon 1 which produced slightly different banding patterns in different developmental stages. These results provided a basis for the application of laccase profiles in the systematics of *Clavicornia*.

Laccase Comparison Within and Among Species (Fig. 32)

Laccase electrophoresis of thirty-eight isolates representing nine taxa in *Clavicornia* resulted in a distinct pattern as shown in Fig. 32. The bands were arranged based on Internal mobility value (R_f), with reference to the R_f value of the standard culture (24439; 1.0).

Twelve electropherogram types (ET) of extracellular laccase were recognized, as shown in Table 6.

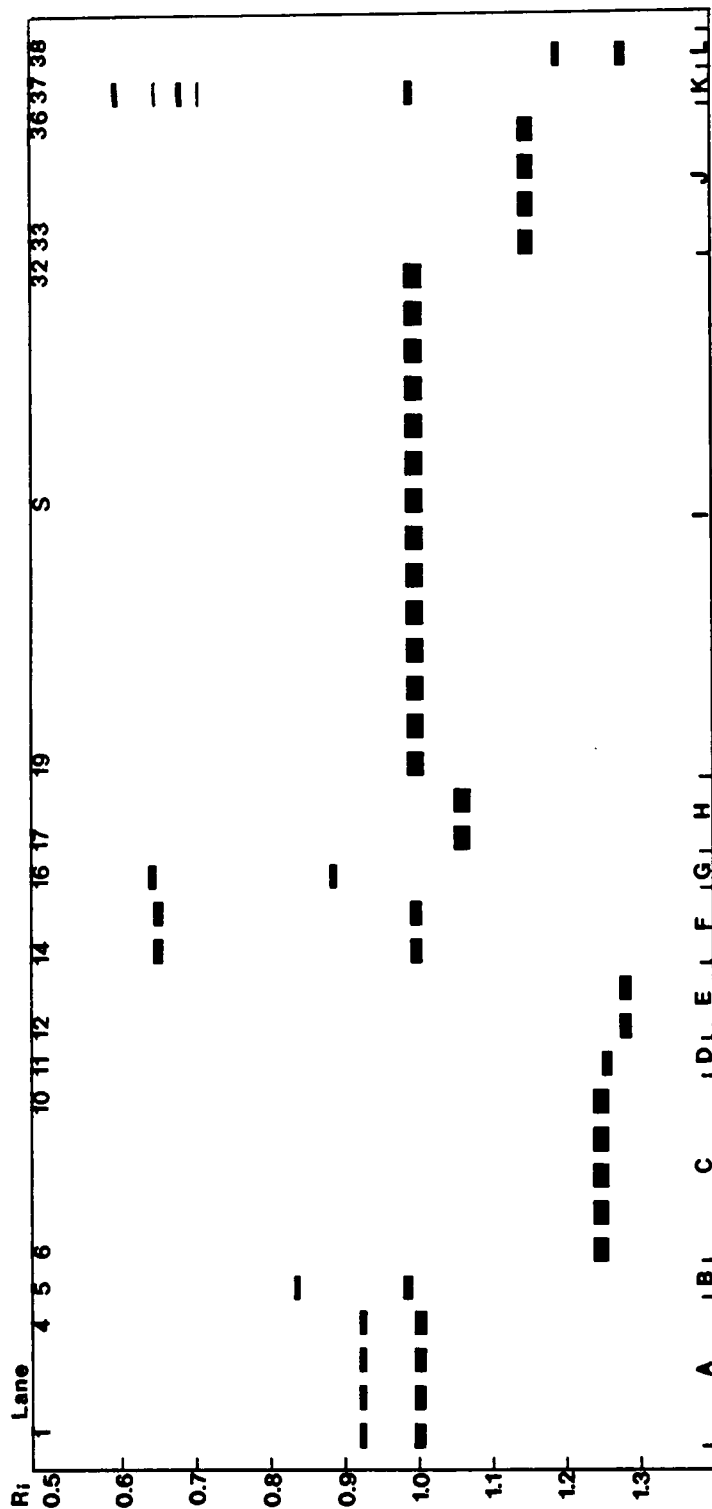


Fig. 32. Laccase electropherogram of *Clavicornora*. Bands are displayed according to their internal mobility value (R_i; percent mobility relative to *C. pyxidata* collection 24439), which are showed on the left. S: standard sample. Lane 1-4 (A): *C. candelabrum*; lane 5 (B): *C. colensoi* collection 2685; lane 6-10 (C): *C. colensoi*; lane 11 (D): *C. cristata* collection DLL8974; lane 12-13 (E): *C. cristata*; lane 14-15 (F): *C. microspora*; lane 16 (G): *C. piperata*; lane 17-18 (H): *C. pyxidata* collections from China; lane 19-32 (I): *C. pyxidata*; lane 33-36 (J): *C. turgida*; lane 37 (K): taxon 1 (1615); lane 38 (L): taxon 2.

Table 6. Electrophoretic types (ET) of extracellular laccase in *Clavicornona*.

| ET Lane | R _f | Epithet | Isolates |
|---------|------------------------------|--------------------|---|
| A 1-4 | 0.93; 1.01 | <i>candelabrum</i> | 2679, 2681, 2802, 2807 |
| B 5 | 0.84; 0.98 | <i>colensoi</i> | 2685 |
| C 6-10 | 1.25 | <i>colensoi</i> | 2555, 2644, 2667, 2637, 44120 |
| D 11 | 1.26 | <i>cristata</i> | DLL8974 |
| E 12-13 | 1.27 | <i>cristata</i> | GB1700, GB1701 |
| F 14-15 | 0.65; 1.0 | <i>microspora</i> | 2349, 5352 |
| G 16 | 0.64; 0.88 | <i>piperata</i> | 2822 |
| H 17-18 | 1.06 | <i>pyxidata</i> | 1449, 1541 |
| I 19-32 | 1.0 | <i>pyxidata</i> | 56667, 1503, 1506, 1607, 1190, 24439, 56655, GB1702, JPL479, 22501, 10654, GB1279, GB1280, GB1137 |
| J 33-36 | 1.14 | <i>turgida</i> | 2672, 2682, 2651, 44103 |
| K 37 | 0.59; 0.65; 0.68; 0.71; 0.98 | Taxon 1 | 1615 |
| L 38 | 1.19; 1.27 | Taxon 2 | 3456 |

In most cases, an electropherogram type (ET) specifically corresponded to one morphological species, and was invariable within the species. Exceptions occurred in three species: 1) in *C. pyxidata* there were two ETs, H, occurring only in Chinese isolates; and I, distributed in all other tested isolates from North America and Europe; 2) in *C. colensoi*, five out of six isolates showed the same ET, C, but one isolate, 2685, displayed a different ET, B, composed of two bands); 3) in *C. cristata*, all isolates produced single-banded electropherogram, but the isolate from California displayed a slower band than that of Europe isolates.

In summary, the banding patterns of laccases were simple and reproducible. Extracellular laccase is, therefore, useful for systematics in *Clavicornia*.

2. Tyrosinase Electrophoresis

Clavicornia pyxidata (Fig. 33)

Sixteen isolates of *C. pyxidata* obtained worldwide were cultured for seven weeks, and mycelial extracts were co-electrophoresed on a single gel following the methods described in Chapter II. Results showed that there was great variation within species in tyrosinase electrophoretic banding patterns within the species. Isolates 1503, 1506 (Wisconsin), 22501 (North Carolina) and 1541 (China) had identical banding patterns, consisting of three bands, two fast bands of which were of the same mobility as that of 1607 (Tennessee), and one fast band was of the same mobility as that of JPL479 (Colorado). The remaining isolates produced bands with different mobilities. The relative mobility value ranged from 0.37-0.77.

New Zealand isolates (Fig. 34)

Nine isolates from New Zealand representing *C. candelabrum* (2679), *C. colensoi* (44120, 2555, 2644, 2685), and *C. turgida* (2637, 2651, 2682, 44103), were co-electrophoresed on a single gel. The results showed that

Lane

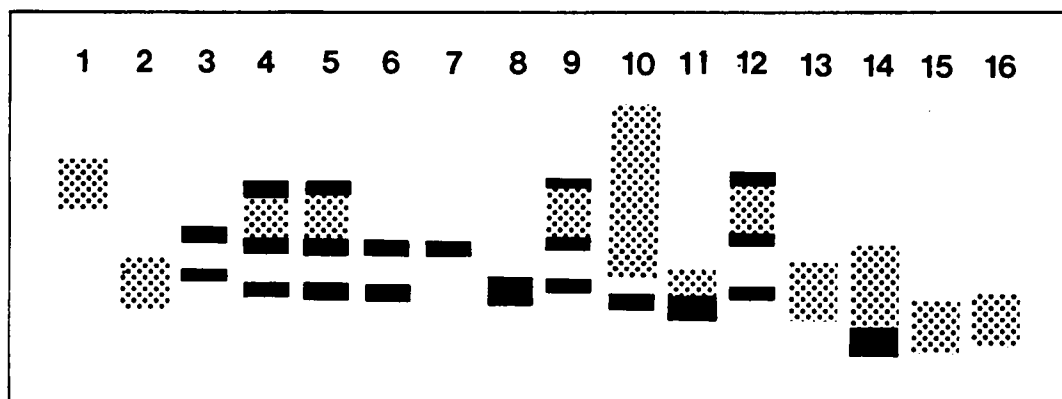


Fig. 33. Electropherogram of tyrosinase from mycelia of liquid cultures of *Clavicornia pyxidata* isolates obtained worldwide. From lane 1-16: GB1137, 56667, 1449, 1503, 1506, 1607, 56655, JPL479, 22501, 1190, 24439, 1541, 10654, GB1279, GB1280, GB1702.

Lane

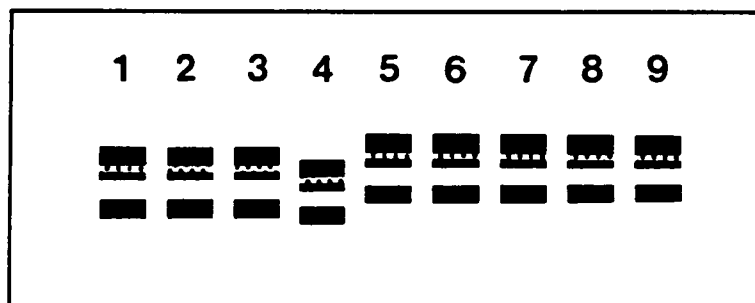


Fig. 34. Electropherogram of tyrosinase from mycelium of liquid cultures of *Clavicornia* spp. from New Zealand. Lane 1-3: *C. turgida*; lane 4-8: *C. colensoi*; lane 9: *C. candelabrum*.

the tyrosinase banding patterns of isolates from these different species were similar. Typically three bands were produced: one fast band, joint with middle band and slow band.

Isolates 2644, 2555, 2637, 2685 and 2679 produced an identical three band pattern. The relative mobilities of these bands were 0.55, 0.58, and 0.6. Isolates 44103, 2682, 2651 of *C. turgida* produced three bands with a slightly higher mobilities of 0.57, 0.6, and 0.64. Isolate 44120 also produced three-bands with even higher mobilities, 0.58, 0.62, and 0.65. In summary, New Zealand isolates of three different species all produced three-band tyrosinase electrophoretic patterns, with slight difference in band mobility.

Tyrosinase electropherogram of other species (Fig. 35)

Isolates representing several species of *Clavicornia* were sampled and co-electrophoresed on a common gel. The results showed that taxon 1 (1615) produced two distinct bands with much higher mobility. The isolate (2349) of *C. microspora* showed a single band pattern.

Isolate GB1700, representing *C. cristata*, showed a single band with relative mobility as 0.62, identical to the medium band produced by isolate 44120 (*C. colensoi*, Fig. 34). Different species produced different tyrosinase electrophoretic profiles, but tyrosinase profiles of

Lane

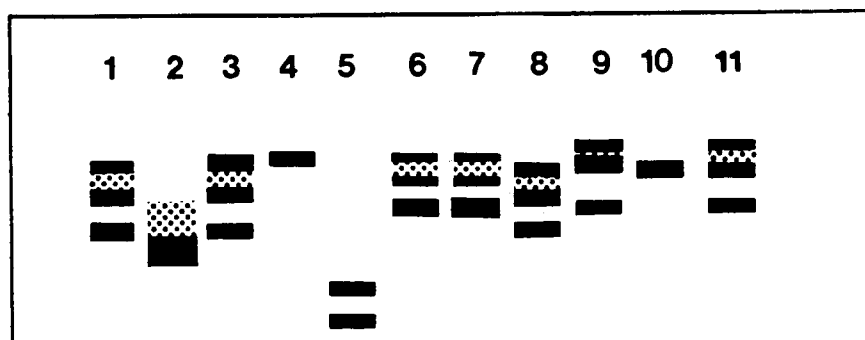


Fig. 35. Electropherogram of tyrosinase from mycelia of liquid cultures of *Clavicornia*. Lane 1-3: *C. pyxidata*; lane 4: *C. microspora*; lane 5: Taxon 1 (1615); lane 6-7: *C. turgida*; lane 8-9: *C. colensoi*; lane 10: *C. cristata*; lane 11: *C. candelabrum*.

different isolates within a single species also varied. No specific banding patterns can be attributed to such species.

Variations between gels

Changes in tyrosinase banding patterns from a single isolate were observed. For example, isolates 24439 did not produce any band in one gel, but produced a diffuse long band in another gel. This could be caused by inappropriate preparation of samples or may be because tyrosinase was unstable. Nonetheless, the tyrosinase electropherograms were less reproducible than that of laccase.

In summary, tyrosinase electrophoretic data are incongruent with other systematic information in *Clavicornia*.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

In Chapter III, results of studies on *Clavicornona* fruitbody and culture mat morphology, mating systems, spot test reactions, and isozyme electrophoresis were described. In this chapter, the implication of these results to systematics in *Clavicornona* are discussed in two ways: 1) comparison and summary of the resolution of these methods at the species level; and 2) discussion of the roles and validity of each method in *Clavicornona* systematics.

A. Summary of Methods at Species Level

Clavicornona candelabrum

This species is probably the most morphologically distinct of the taxa of *Clavicornona* from New Zealand. All collections identified as *C. candelabrum* were characterized by very densely gregarious habitat (easily noted in the field; Petersen, field notes), small, milk-chocolate brown colored fruitbodies, occasionally thick and roughened hyphae, and spore measurements. Dried specimens are usually black, which is quite distinct in the genus. The presence of thick-walled hyphae in the fruitbody also seems characteristic of this species. Spore measurements in various collections are surprisingly uniform, with Em values

ranging 1.53-1.71, and Lm ranged 4.3-4.4 μm .

The culture mat of *C. candelabrum* on MA was characterized by dull yellowish pigmentation, presence of gloeoplerous hyphae and spherical swollen hyphae, all similar to the culture mat of *C. colensoi*. The pigments produced by *C. candelabrum*, however, were darker than those of *C. colensoi*. Laccase and tyrosinase spot test reactions were also similar to *C. colensoi*.

Mating tests indicated that *C. candelabrum* was governed by a homogenic bifactorial incompatibility system, although for most collections, the mating tests were unsuccessful. The fact that most pairings were incompatible indicated that there are probably other factors controlling mating behavior, as discussed in Chapter III and later in this chapter.

Extracellular laccase electropherograms of all collections of *C. candelabrum* were identical, exhibiting two bands. The fast band had the same mobility as for the single band of *C. pyxidata*. The two-band pattern of *C. candelabrum* is different from other *Clavicornia* species.

Tyrosinase electropherograms of collections of *C. candelabrum* were identical with those of most isolates of *C. colensoi*.

In summary, *C. candelabrum* represents a well-defined morphological species, with diagnostic laccase electropherograms. The morphospecies is expected to be a

single biological species. Mating tests, however, were not possible in this study. The physiological studies of this species are needed in order to conduct productive mating tests and to confirm intercompatibility among collections.

Clavicornia colensoi

This species was originally described from New Zealand. It is easily confused with *C. piperata* and *C. turgida*. Corner (1950) suggested that *C. colensoi* and *C. piperata* were synonymous. Petersen (1988) reported that fruitbody size, spore measurements and shape in *C. colensoi* were different from those of *C. piperata*. My study provided further evidence that *C. colensoi* is morphologically distinct from *C. piperata*. Collections identified as *C. colensoi* were characterized by small, pale ochraceous buff fruitbodies, and spore measurements ($E_m = 1.48$; $L_m = 5.2 \mu m$). In contrast, *C. piperata* was characterized by large fruitbodies and long, stout spores ($L_m = 1.19$; $L_m = 5.8 \mu m$). There are, however, some exceptions. Collection 2637, for instance, showed similar microscopic characters to *C. colensoi*, but produced large, *piperata*-like fruitbodies. Collection 2685 has *colensoi*-like fruitbodies, but smaller, narrower spores ($E_m = 1.65$; $L_m = 4.8 \mu m$). The reasons for including these two collections in *C. colensoi* is based on congruence of most macromorphological and micromorphological characters.

Extracellular laccase electropherograms showed that collection 2637 had an identical pattern as other *C. colensoi* collections. Collection 2685, however, produced an unique laccase banding pattern. It seems that spore measurements correspond more closely to laccase banding pattern than fruitbody size in this species.

The culture mat of *C. colensoi* was characterized by light yellow coloration, different from any other species in the genus. The growth rate of *C. colensoi* on MA, PDA and MYG was about average for the genus. Furthermore, laccase activity was not detected in any *C. colensoi* isolates grown on MYG. The unique culture morphology and spot test results suggested that *C. colensoi* was a distinct species.

Mating tests show that *C. colensoi* is governed by a homogenic bifactorial incompatibility system. Matings among three available collections showed partial compatibility. Because of the failure of most self-crosses, however, it cannot be concluded that these three collections are truly "partially" compatible. More physiological studies are needed here.

Laccase electropherograms of *C. colensoi* were of two types: a single-band pattern, produced by all collections except 2685, and a double-band pattern, produced by collection 2685. This agreed with the result of morphological studies discussed above. Collection 2685 was divergent from *C. colensoi* as indicated by spore

measurements and laccase electropherogram.

Tyrosinase electropherograms of *C. colensoi* were all of a three-band pattern, with difference in mobility. Four isolates out of the five tested, including 2685 and 2637, had identical lower relative mobility than the single isolate 44120. No significant morphological difference could be found in 44120.

In conclusion, *C. colensoi* is a distinct species as determined by fruitbody and culture mat morphology, laccase and tyrosinase banding pattern. This species, however, is also internally divergent, with genetic variation occurring with or without morphological differentiation.

Clavicornia cristata

As previously noted, cultures of three collections of *C. cristata* were available for this research, one from California (DLL8974), and two from Finland without specimens (GB1700, GB1701). *Clavicornia cristata* can be immediately distinguished from other species by its elongated spores.

The culture mat of *C. cristata* on MA, PDA, and MYG was characterized by extremely slow growth. Collection DLL8974 produced dark ruby coloration on PDA and MYG, but GB1700 and GB1701 did not produce such pigments.

Self-crosses of *C. colensoi* isolates revealed a homogenic bifactorial incompatibility system. Inter-collection mating results showed that the two Finnish

collections were intercompatible and shared a common mating factor, and that the California collection was partially compatible with both Finnish collections. These intercollection mating results indicated that *C. cristata* is in the process of speciation.

All three collections exhibited a single band pattern from laccase, but the identical bands produced by the two Finnish collections showed higher mobility than that produced by the California collection. These results supported the mating test results, and confirmed that there is genetic diversity within *C. cristata*.

Corner (1950, 1970) suggested that *C. cristata* might be a juvenile state of *C. pyxidata* or *C. colensoi*. Dodd (1972) accepted *C. cristata* as a distinct species with close affinities to *C. piperata* and *C. divaricata*. In this study, the laccase banding pattern of the California collection of *C. cristata* was nearly identical to that of *C. colensoi*, and only different in band intensity. This may or may not imply a certain genetic similarity between these two species.

Clavicornia microspora

Fruitbodies of *C. microspora* were characterized by crowded, pyxidate, fine branches with light orange colored apices; fragility of dried fruitbodies; extremely abundant gloeoplerous hyphae; short basidia; and very small spores. Based on morphological characters alone, this species has

been proposed as distinct. *Clavicornia microspora* and *C. pyxidata* are very similar in fruitbody appearance. Spores of *C. microspora* are shorter and wider than those of *C. pyxidata*, and probably are the smallest in the genus.

The culture mat of *C. microspora* was characterized by fruitbody formation on MA within five weeks, and abundant crystal formation. *Clavicornia pyxidata* had similar growth rate, but never formed fertile fruitbodies on MA.

Self-crosses in *C. microspora* revealed a homogenic bifactorial mating system. Inter-collection matings of *C. microspora* show intercompatibility within this intersterility group, but collections of *C. microspora* were completely incompatible with all tested collections of *C. pyxidata*. These results agreed with morphological studies. Genetic exchange is inhibited by interincompatibility, and reproductive isolation has been accompanied by morphological divergence.

Laccase electropherograms produced by *C. microspora* were characterized by a two-band pattern; one of the two bands had a close or identical mobility to that of the standard band produced by *C. pyxidata* collections.

Tyrosinase banding patterns of *C. microspora* were different from any of those of *C. pyxidata*.

In summary, *C. microspora* represents a differentiated species from *C. pyxidata*. Bresinsky et al. (1987) suggested, in *Pleurotus*, that reproductive isolation

occurred at a primary stage of speciation. Theoretically there is a significant probability for the occurrence of *C. pyxidata* in Japan, although collections used in this study did not confirm this. There are many reports in higher basidiomycetes showing that a morphospecies can consist of several reproductively isolated biological species: for example, *Armillaria mellea* (Anderson et al., 1980) and the *Collybia dryophila* complex (Vilgalys and Miller, 1983). Morphological differences between the two *Clavicornia* biological species from Asia, however, should not be ignored. Further studies using more isolates will be desirable to determine whether *C. pyxidata* occurs in Japan and to assess the incompatibility relationships between these two possibly sympatric taxa.

Clavicornia piperata

This species has been reported from Idaho, Washington, British Columbia, Australia, and New Zealand (Kauffman, 1928; Leathers and Smith, 1967; Dodd, 1972; Petersen, 1988), but only two collections from New Zealand are identified as *C. piperata*, characterized by relatively large fruitbodies and broad spores. These two collections were basically identical, with very similar spore measurements (2672: Em = 1.24, Lm = 5.6 μ m; 2822: Em = 1.19, Lm = 5.8 μ m). Morphologically, the species was similar to *C. colensoi*, and *C. turgida*.

The culture mat of *C. piperata* is dull yellowish. Single-spore isolates of *C. piperata* form ellipsoid-fusoid chlamydospores, peculiar to this species. Maas Geesteranus (1973) reported a new species, *Clavicornia dryophila*, characterized by trumpet-like fruitbodies and chlamydospore production. Later, this species was believed to be an immature state of a *Lentinellus* (Petersen, pers. comm.). The formation of chlamydospores on agar medium provides evidence for a close intergeneric relationship between *Clavicornia* and *Lentinellus*.

Mating test data were not available.

Laccase produced by *C. piperata* exhibited two different patterns: a two-band pattern, with mobilities different from any other bands produced by *Clavicornia* species, and a single-band pattern, with the same mobility as that of *C. turgida*. These results were in disagreement with morphological studies. The two morphologically very similar collections within a single morphospecies were divergent in isozyme loci. Collection 2672 may represent an intermediate state between *C. piperata* and *C. turgida*.

Clavicornia pyxidata

This species showed a wide range of variation in macroscopic morphology, especially fruitbody coloration, as discussed in Chapter III. A. One of the important characters in identifying *C. pyxidata* is narrow

basidiospores, with average length/width ratio of 1.8-2.0. The two collections from China (no. 1449 and 1541) could be easily identified as *C. pyxidata* on this basis, although there were certain differences in fruitbody stature (1449, 1541) and hymenium texture (1541).

Culture mat morphology of *C. pyxidata* was characterized by relatively rapid growth on all three media, and yellow to brown coloration on PDA and MYG. Culture morphology of fourteen collections, including the two from China, was very consistent, with no significant differences observed among these isolates.

Self-crosses of 28 collections of *C. pyxidata* revealed a uniformly homogenic bifactorial mating system. Among these, 16 collections representing various geographical areas were paired in all possible combinations. Results of intercollection matings show that they were either fully compatible or shared common mating factors.

Collections of *C. pyxidata* were all intercompatible and, therefore, revealed a multiple-allelism phenomenon. Out of a possible 32 A alleles and 32 B alleles from the 16 collections, 28 A alleles and 26 B alleles are identified. The number of alleles in the natural population was estimated by applying Whitehouse's (1949) formula to the obtained data. In the natural population of *C. pyxidata*, the estimated number of A-alleles is 104, and B-alleles is 70. James (1983) used the formula of Dobzhansky and Wright

(1941) in a 15-collection sample and estimated approximately 87 A-alleles and 73 B-alleles. In *Schizophyllum commune*, estimates calculated by Dobzhansky and Wright (1941), and Whitehouse's (1949) method were slightly different (Raper, 1966). Both estimated numbers of incompatibility factors in *C. pyxidata* were close to others calculated for higher basidiomycetes (Raper, 1966; Ullrich and Raper, 1974).

Laccase electropherograms of *Clavicornia pyxidata* were of two types: a single-band pattern produced by all collections from North America and Europe, and a different single-band pattern with higher mobility produced only by collections from China. Although the Chinese collections are intercompatible with other collections, there was genetic variation as indicated by the difference in the laccase locus. Prillinger and Molitoris (1979) found the same situation in *Pleurotus ostreatus*, in which two geographical races were fully intercompatible, but genetically diverse as detected by presence of a specific secretory mechanism for extracellular laccase. In both cases, genetic divergence seems to occur before reproductive isolation.

Tyrosinase electropherograms of *C. pyxidata* were variable among collections. Tyrosinase is involved in the formation of pigments in fungi (Mayer, 1987), and variation in tyrosinase banding patterns in *C. pyxidata* might be related to variation in coloration of fruitbodies.

Clavicornona turgida

This species has been reported worldwide. From New Zealand, this species can be confused with *C. piperata* and *C. colensoi*. According to Dodd (1972), one of the important characters for *C. turgida* is the gelatinous subhymenium and hymenium.

On MA, isolates of *C. turgida* seemed to respond to exposure to light by forming distinct growth zones.

Self-crosses of *C. turgida* isolates revealed a homogenic bifactorial mating system, congruent with other tested species in the genus. Matings among tester strains of two available collections of *C. turgida* showed that they were fully intercompatible. Interspecific matings between *C. turgida* and *C. candelabrum*, as well as *C. turgida* and *C. pyxidata*, showed that they were all incompatible. *Clavicornona turgida* is, therefore, genetically isolated from these other two morphospecies.

Laccase produced by *C. turgida* exhibited a single-band pattern, with mobility lying between that of the band produced by *C. colensoi* and that by *C. pyxidata*. The electropherograms of *C. turgida* were invariant, and different from all other species except collection 2672, which was identified as *C. piperata* on morphological characters.

Tyrosinase produced by isolates of *C. turgida* was

uniformly three-banded. Mobilities of these three bands were different from those of *C. colensoi* and *C. candelabrum*, although their mobility was close.

In summary, *C. turgida* represents a distinct species among other taxa from New Zealand, as reflected by morphological characters, mating behavior and isozyme electropherograms. *Clavicornia turgida* has also been reported from North America, South America, and Asia. Dodd (1972) stated that South American and North American specimens of *C. turgida* were different from most of the Asia-New Zealand taxa in that the former produced more robust, non-cartilaginous fruitbodies. There was no distinct difference in microscopic morphology. If cultures of *C. turgida* can be established from North America and South America, it would be interesting to conduct intercollection matings and laccase electrophoresis for comparison to the results gathered from New Zealand collections.

***Clavicornia* Taxon 1 (collection 1615)**

This is a single collection from North Carolina, which cannot be recognized as an established species. It occurs within the distribution range of *C. pyxidata*, but easily can be distinguished from the latter by the stout and uniformly brown fruitbodies, and spore germination failure. Microscopically, collection 1615 had gelatinous hyphae and

broader spores than those of *C. pyxidata*. The robust, brownish fruitbodies of collection 1615 were similar to those of *C. turgida*. This collection may be a link between *C. pyxidata* and New Zealand species.

The culture mat of *Clavicornia* Taxon 1 is characterized by fawn to olive-brown coloration on PDA and MYG, not found in any other observed species.

Electrophoresis of *Clavicornia* Taxon 1 showed production of multiple forms of extracellular laccase. The fast band had similar mobility to the standard type of *C. pyxidata*, but different in band intensity. This result may indicate that Taxon 1 and *C. pyxidata* have some degree of genetic identity.

Tyrosinase produced by *Clavicornia* Taxon 1 exhibited a two-band pattern, with the mobilities of these bands much higher than those produced by other species.

Results of fruitbody and culture mat morphology studies, and laccase and tyrosinase electrophoresis are congruent in suggestion that collection 1615 probably represents a distinct species, and a new species name may be needed for this taxon.

***Clavicornia* Taxon 2 (collection 3456)**

This single specimen from Puerto Rico is in the same situation as Taxon 1, i. e. it is premature to describe it as new. This collection was characterized by its pure white

fruitbodies which are quite distinct in the genus.

The culture mat morphology of this taxon was similar to that of *C. pyxidata*, although with a lower growth rate.

Self-crosses of *Clavicornia* Taxon 2 revealed a typical homogenic bifactorial mating system. Inter-collection matings between Taxon 2 and collections of *C. microspora*, *C. pyxidata*, and *C. turgida* showed that Taxon 2 was genetically isolated from the other tested species.

Extracellular laccase produced by Taxon 2 exhibited a two-band pattern. Slow band mobility was different from any other taxon, but the mobility of the fast band was identical to the band produced by European collections of *C. cristata*. It is doubtful that these two species are closely related, although morphological examination is needed for European collections of *C. cristata*.

In summary, morphological characters, inter-collection mating results and extracellular laccase banding patterns suggested that *Clavicornia* Taxon 2 (3456) represents a distinct species. Its relationships with other species are not clear. More cultures from the neotropics may be helpful for further studies on intercompatibility within this species and its genetic diversity from other species in the genus.

B. Roles and Validity of Utilized Methods in *Clavicornia* Systematics

Fruitbody Morphology

Morphological characters have been used in the taxonomy of almost all organisms for hundreds of years, and will always provide the very basic view of organisms, although there are more and more criticisms. Any morphological characters can be used for purposes of classification as long as they distinguish taxa, and they do not necessarily reflect genetic diversity or phylogenetic events. Some morphological characters, however, may be more important than others for a specific taxon.

Presence of gloeoplerous hyphae; small, amyloid and slightly roughened or smooth spores; clavarioid, usually pyxidately branched fruitbodies; and lignicolous habit are the characters that group species in *Clavicornia*.

Coloration of fruitbodies has been considered one of the valuable characters in *Clavicornia*, as in other higher basidiomycetes. In contrast, in many species of *Clavicornia*, the colors of fruitbodies vary for different individuals and/or different stages of fruitbody maturation. Dodd (1972) reported, however, that fruitbody colors were variable in *C. pyxidata* with age and weather conditions during fruitbody formation. It was confirmed through this study that collections of *C. pyxidata* from various

geographic areas are variable in coloration, from cream to very bright yellow, to tan. This color variation does not exhibit any patterns, and it may have something to do with tyrosinase-catalyzed metabolism related to pigmentation during fruitbody formation (Robb, 1984). Petersen (1988) noted color changes in *C. colensoi* and *C. piperata* during fruitbody development. It was noted in this study that *C. candelabrum* growing very densely on a piece of wood consists of individuals that were variously cream colored and wood brown. Subsequent examination of microscopic characters and laccase electrophoresis show that no significant differences could be found: they all belong to the same species. Similar situations can also be found in *C. turgida*. It must be pointed out, however, that although there are developmental variations, colors of mature fruitbodies are still useful in identifying species in *Clavicornia*.

Clavicornia cristata seems to be an intermediate between unbranched species(*i. e. C. taxophila*), and branched species (*i. e. C. colensoi*). No cultures of unbranched species were available for this study. Extracellular laccase produced by *C. cristata* and *C. colensoi* collections exhibited a similar (if not identical) banding pattern with the same mobility and different only in band intensity. Further comparison of these species using isozyme electrophoresis and/or DNA techniques may provide insight into whether *Clavicornia* should be a discrete genus or

divided into two genera as proposed by Jülich (1981).

The size of fruitbodies should be combined with other characters in identifying species. In *C. pyxidata*, fruitbodies range from 3-10 cm high. Basically *C. colensoi* can be distinguished from *C. piperata* and *C. turgida* by the small fruitbodies, but intermediates can also be found.

The taste of *Clavicornia* fruitbodies is characteristically acrid. This acidity is suspected to be related with the presence of gloeoplerous hyphae. Two chemicals isolated from *C. pyxidata* have been found to be responsible for the acrid taste, stearoylvelutinal and isovellereal (Steglich and Sterner, 1988). According to Steglich (1989), these two compounds possess strong antibiotic, cytotoxic and mutagenic activities and are efficient antifeedants. These results may explain why the fruitbodies of *C. pyxidata* are rarely inhabited by insects and consequently rarely contaminated by bacteria, making tissue culture work quite easy.

The presence of thick-walled hyphae in *C. candelabrum* is among other characters that distinguish this species. All examined species of *Clavicornia* produce clamped generative hyphae and similar gloeoplerous hyphae which are not sufficiently useful for delimitating species. Formation of leptocystidia is common in most species, but shape of leptocystidia is sometimes inconsistent within species, and therefore is not a very useful character.

A gelatinous subhymenium and hymenium may an important character for *C. turgida*, although one exception has been found in a Chinese collection of *C. pyxidata*.

The size of basidia seems correlated to the size of basidiospores and may be used as an affiliate criterion. Spore shape, ornamentation and measurement are important characters. Statistics of a large number of spores may provide information to species boundaries. Dodd (1972) and Petersen (1988) offered good discussions on this matter. The shapes of gloeocystidia were considered to be an important taxonomic character (Dodd, 1972), but because they are inconsistent within species, they may be only of very limited value.

Culture mat morphology

Cultural characters are important in taxonomy of wood-rotting basidiomycetes. Nobles (1965) and Stalpers (1978) developed a series of key code designations with which to identify wood-inhabiting Aphyllophorales. The medium they employed, basically malt extract agar (MA), was used as a standard medium to analyze many species from a wide range of genera. In most cases diagnostic characters for identifying species have been obtained. This medium, however, is not necessarily sufficient for classifying species within a given genus. Because congeneric species may be closely related and share some common physiological features, and

they might not be differentiated on the medium useful for delimitation of species from other genera or families. Utilization of different kinds of media may be valuable for intrageneric taxonomy. Desjardin (1990) reported in *Marasmius* that very little culture morphology variation was observed in isolates grown on MA, whereas distinct morphologies were observed isolates grown on PDA. Observations on culture mat morphology of *Clavicornia* are in agreement with the results obtained in *Marasmius*.

Cultural characters in *Clavicornia* have been discussed in Chapter III B and earlier in this chapter. Briefly, growth rate on various media is distinct for different species and consistent among isolates of the same species. Color, texture and odor of culture mats, especially on PDA and MYG, are valuable macromorphological characters in identifying species. In contrast, presence of clamped, inflated generative hyphae, spherical swollen hyphae, gloeoplerous hyphae, and crystal formation are common in all species. Each species, however, may be characterized by certain peculiar features. The culture mat of *C. colensoi*, for example, is characterized by bright yellow coloration; that of *C. turgida* by formation of growth zones on MA; that of *C. microspora* by rapid fruiting on agar media; that of *C. cristata* by the extremely low growth rate on all tested media; and that of *Clavicornia* Taxon 1 by fawn coloration on PDA and MYG.

Dikaryotic hyphae of *C. cristata* exhibit normally paired nuclei separated by septa with typical clamp connections. A few verticillate and sprouting clamp connections, however, were observed. The sprouting clamp connections may be a valuable character. The significance of formation of a few verticillate clamp connections is not understood. According to Boidin (1971), verticillate clamp connections are a sign of holocenocytic condition. The extremely slow growth of *C. cristata* may reflect an unfavorable physiological condition, and occasional abnormal phenomena may be related to poor growing conditions.

Incompatibility tests

All known *Clavicornia* species are governed by two mating factors, so called bifactorial incompatibility, as found in many basidiomycetes. Bifactorial incompatibility is the most complicated pattern of sexuality known among fungi. Raper and Flexer (1971) discussed the possible mechanisms by which bipolar and homothallic systems may be derived from the tetrapolar system. Mutation may be one of them.

In most matings of *Clavicornia*, barrage reactions can be identified by raised mycelia in the contact zone and usually can be used as an indication of common-B pairing. Sometimes the occurrence of barrage, however, is variable

and unpredictable in different pairings of different collections. Some common-B pairings did not form typical barrage morphology and barrage was observed occasionally in compatible matings or common-A matings. This is in agreement with James' (1983) observation in *C. pyxidata* and many other studies in basidiomycetes (Raper, 1966; Esser and Hoffman, 1977).

The morphology of barrage reactions is also variable. Some barrages were characterized by raised hyphae from both donor mycelia and a clear, fine line in the contact zone, while other barrages comprised two raised zones of hyphae and a much wider clear zone in the middle. Some barrages produced yellowish pigments, while others produced false clamp connections. Variability of barrage reactions seems to be a common phenomenon in higher basidiomycetes, and may confirm the instability of heterokaryotization resulting from common-B matings. In matings of *C. microspora*, barrage reactions were similar to flat reactions, except that hyphae in the contact zone were more fluffy in barrage reactions than in flat reactions, making it even more difficult to define.

In all matings, dikaryotization followed by formation of clamp connections is the only reliable evidence of compatibility. In species such as *C. microspora*, *C. pyxidata* and *Clavicornia* Taxon 2, clamp connections were abundant and usually could be observed all over in compatible

matings, whereas in other species such as *C. cristata*, *C. colensoi* and *C. candelabrum*, clamp connections were few in compatible matings and could be found only in contact zones.

False clamp connections usually occurred on heterokaryotic hyphae resulting from common-B matings. They were incomplete clamp connections, with septa which inhibit nuclear migration. False clamp connections can be easily identified under epifluorescence microscopy. James (1983) reported that false clamp connections were produced sporadically and in small numbers in common-B matings of *C. pyxidata*. My study confirms his observations. Exceptions were found, however, in some common-B intercollection matings of *C. pyxidata* which produced large numbers of false clamp connections.

It is interesting that in intercollection matings of *C. cristata*, both false and true clamp connections were observed in pairings between isolates from different geographic areas. The true clamp connections indicated different mating types and consequently compatible mating, whereas the false clamp connections indicated that nuclear migration was blocked due to genetic or biochemical factors other than mating type alleles. If this is true, then inhibition of nuclear migration may be an indication of reproductive isolation, and subsequently allopatric speciation may be in progress between these two populations.

Some factors may affect formation of false clamp

connections, as previously discussed (Raper, 1966; James, 1983), with culture growth time as one. In matings between isolates of *C. microspora*, no false clamp connections were observed. This may merely be a reflection of the rarity of false clamp connections, and the small number of samples tested.

In most collections of certain *Clavicornia* species (*i. e. C. candelabrum*, *C. colensoi*, and *C. piperata*), self-crosses were not productive (*i. e.* pairings were dominated by incompatible matings), even on media of different concentration and allowing mating for long periods of time.

There are several possible reasons for failure to produce compatible matings: 1) mating types of spores may be correlated to spore germination, so among early-germinating spores there may be only one, two or three mating types. Most of the mating reactions appeared as flat, without the usual distinct three types of mating reactions; 2) the medium used for mating tests may not be suitable for the physiological requirements of the species, and matings, therefore, may be inhibited. Growth rate of these species on MA was not very slow, although slower than *C. pyxidata*; 3) in nature, these species may have developed ways of reproduction and dispersal not dependent on sexual reproduction, so mating ability may be lost or impaired. This may be reflected in rarity of clamp connections in compatible matings; 4) because of the rarity of clamp

connections, mating results may have been misread and clamp connections overlooked; 5) reproduction in these species may require out-crossing (*i. e.* spores from one fruitbody may not be compatible with spores from the same fruitbody, but only with spores from different fruitbodies); and 6) there may be separate genes controlling "fertility" (in the sense of Chase and Ullrich, 1990). This may be true because a few collections showed the typical bifactorial mating system.

Multiple allelism phenomena of mating factors have been observed in *Clavicornia* species. Multiple allelism favors outbreeding. Mating results among collections of *C. pyxidata* from various geographical areas show that mating factors are randomly distributed in the range of species distribution.

Matings between collections of different species were all incompatible, and often exhibited strong antagonism phenomena. Antagonism was characterized by mycelial confrontation and usually yellow to brownish pigmentation. Antagonism can be easily distinguished from barrage phenomena by the formation of a protruding condensed conflict zone, presence of pigments deposited along this zone, and lack of false clamp connections. Ainsworth and Rayner (1989) observed self and non-self response prior to and at hyphal fusion. Pairings of homokaryons from different taxonomic species of *Stereum* gave rise to

unilaterally extensive degenerative reactions in plate culture, sometimes accompanied by production of pigments and crystals. Similar phenomena were also observed in *Clavicornia*.

The concept of biological species has been adopted from Esser and Hoffmann (1977): 'Populations belong to different species when the failure to interbreed and to produce viable offspring in nature is not caused by genetic parameters operating in the completion of the sexual cycle'. In higher basidiomycetes, certain assumptions are needed when applying mating results to the concept of biological species : 1) two paired mycelia which are compatible on cultural media will be also compatible and form dikaryons in nature, and vice versa; 2) in nature, dikaryotic mycelia resulting from compatible matings can produce fertile fruitbodies under suitable physiological conditions.

In known *Clavicornia* species, biological species are basically congruent with morphological species, i. e. collections from the same morphospecies are intercompatible, whereas collections from different morphospecies are interincompatible.

Morphologic or genetic difference may be present between two intercompatible collections. In *C. pyxidata*, for example, collections from China were fully compatible with collections from North America and Europe. Extracellular laccase banding patterns of collections from

China were different from those of collections from North America and Europe. Genetic diversities have been found within a biological species. Conversely the partial compatibility between collections of *C. cristata* from North America and Europe may be considered as a sign of incomplete reproductive barriers. The difference between these two populations is also reflected by laccase electrophoresis banding patterns. The same situation has been found in *Pleurotus* species (Bresinsky, et al., 1987).

In discussing the events in fungal speciation, Kemp (1975) proposed that genetic isolation can be prior to morphological differentiation. This was in agreement with observations in *Pleurotus* (Bresinsky, et al., 1987). Genetic isolation can also be preceded by genetic divergence. This seems the case in *Clavicornia*.

Laccase and tyrosinase spot tests

Two effective spot test reagents, syringaldazine and p-cresol, were used to detect laccase and tyrosinase activities in culture mats of *Clavicornia*. All tested species showed laccase activity on all three media, except that collections of *C. colensoi* exhibited laccase activity on MA and PDA, but not on MYG, and two tested collections of *C. cristata*, did not show laccase activity on PDA. In contrast, all tested collections of *Clavicornia* showed tyrosinase production on PDA and MYG, but not on MA.

More collections must be utilized for certain species. There are some other reagents which may occasionally provide supplementary detection on laccase and tyrosinase activity (Marr, 1983; Desjardin, 1990). Growth medium and culture age affect the results of spot tests as indicated in Table 4 and Table 5 (on page 128 and 131).

Nevertheless, from present results it can be concluded that presence of laccase and tyrosinase is quite consistent within the genus, and not variable enough at the specific level.

Laccase electrophoresis

In general, extracellular laccase electrophoresis patterns were congruent with results of morphological examination and mating tests. All tested species produced characteristic laccase banding patterns consistent within species and different from other species. Laccase banding patterns can be used as systematic criteria at the species level, and sometimes can also be used to detect genetic diversity among populations.

Clavicornia candelabrum, *C. microspora*, *C. turgida* and two unnamed taxa each produce characteristic laccase banding patterns and which are stable within the species. Analysis of laccase in other basidiomycetes also showed that intraspecific enzyme patterns were highly congruent (Blaich and Esser, 1975).

Collections of a single species which are morphologically slightly divergent can be separated by laccase banding patterns. All but one collection of *C. colensoi* produced a distinctive, consistent laccase banding pattern, with the exceptional collection producing a different banding pattern. Differences in spore measurements between this latter collection and the other *C. colensoi* collections have been found as well.

Collections which are partially intercompatible can also be separated by laccase banding pattern. In *C. cristata*, the banding pattern produced by the California collection was slightly different in band mobility from European collections, and these collections were partially intercompatible.

Collections of one species which are fully intercompatible but from different geographical areas can be separated by laccase banding patterns. In *C. pyxidata*, collections from China are fully compatible with those from North America and Europe, but they produce bands with different mobility.

Multiple forms of laccase have been reported from other higher basidiomycete fungi, for example, *Trametes versicolor*, *Coprinus plicatilis* and *Panus stipticus* (Blaich & Esser, 1975). Laccase electropherograms in those fungi were unsuitable for systematics purposes because of the complexity of the enzyme patterns. In contrast, most

Clavicornia species produced relatively simple laccase patterns, except *Clavicornia* Taxon 1 which produced multiple forms of laccase. This property makes the comparison of laccase electrophoretic banding pattern useful for taxonomy. The same property has been found in some other species, for example, *Polyporus brumalis*, *Leptoporus litschaueri* and *Agaricus* (Blaich & Esser, 1975; Kerrigan and Ross, 1988).

Some separate species of *Clavicornia* produce laccase bands with close or identical mobility; for example, the fast band of *C. microspora* has the same mobility as the standard band produced by *C. pyxidata*. This may or may not indicate some degree of genetic similarity. Blaich and Esser (1975) stated that the high resolving power of isoelectric focusing may show that interspecies congruence of laccase electropherograms does not exist.

In summary, species of *Clavicornia* produce simple and stable extracellular laccase, consistent within species and varied between species. The electrophoresis of laccase is a valuable tool for systematics in *Clavicornia* at the species level, occasionally showing genetic diversity among populations.

Tyrosinase electrophoresis

Tyrosinase electrophoretic banding pattern is consistent in some species and variable in others. Despite the variable pattern in some species, most of the tested

species seem to produce characteristic tyrosinase banding pattern.

The tyrosinase banding pattern is variable within *C. pyxidata*. One banding pattern is shared by four collections from the same and different geographic areas. Tyrosinase from other collections did not result in comparable patterns.

The tyrosinase banding patterns in *C. colensoi* are basically consistent, except for one collection which produced a different pattern. *Clavicornia candelabrum*, however, produced the same tyrosinase banding pattern as most collections of *C. colensoi*.

The tyrosinase banding patterns in *C. turgida* were consistent within the species. *Clavicornia* Taxon 1 produced tyrosinase with peculiar banding patterns, distinguishing the taxon from all other species.

Variation in tyrosinase banding pattern between gels has been found, with one *Clavicornia pyxidata* collection as an example. Variation in tyrosinase banding patterns in mycelia of different developmental stages was observed. Ingebrigtsen et al (1989) reported that tyrosinase activity and isozyme patterns varied in developing fruitbodies of *Agaricus bisporus*. This may also apply to the situation in *Clavicornia* species. All these variations in tyrosinase banding patterns reduces reproducibility of tyrosinase electrophoresis results.

In summary, electrophoretic banding pattern of tyrosinase is consistent in certain species of *Clavicornona* and varies in other species. Some peculiar banding patterns are characteristic for certain species. Different species may have identical tyrosinase banding patterns. Nevertheless, tyrosinase banding patterns may be of some significance to systematics in *Clavicornona*.

LITERATURE CITED

Ainsworth, A. M. and A. D. Rayner. 1989. Hyphal and mycelial response associated with genetic exchange within and between species of the Basidiomycete genus Stereum. J. Gen. Microbi. 135: 1643-1659.

Ander, P. & K. -E. Eriksson. 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Arch. Microbi. 109: 1-8.

Ander, P & K. -E. Eriksson. 1977. Selective degradation of wood components by white-rot fungi. Physiol. Plant. 41:239-248.

Anderson, J. B., K. Korhonen & R. C. Ullrich. 1980. Relationships between European and North American biological species of Armillaria mellea Exper. Mycol. 4:87-95.

Anderson, J., R. C. Petsche, and M. L. Smith. 1987. Restriction fragment polymorphisms in biological species of Armillaria mellea. Mycologia 79: 69-76.

Anderson, J. and R. Ullrich. 1979. Biological species of Armillaria mellea in North America. Mycologia 71: 402-414.

Angleton, E. L. & W. H. Flurkey. 1984. Activation and alteration of plant and fungal polyphenoloxidase isoenzymes in sodium dodecylsulfate electrophoresis. Phytochem. 23: 2723-2725.

Arita, I. and K. Mimura. 1969. The mating systems in some hymenomycetes. II. The mating systems in Fayolus arcularius (Botsch ex Fr.) Ames, F. mikawai (Lloyd) Imaz., Pholiota adiposa (Fr.) Quel, and Pleurotus cornucopiae (Paul. ex Pers.). Roll. Rep. Tott. Mycol. Inst. 7: 51-18.

Arnold, J. 1935. A comparative studies of certain species of Marasmius and Collybia in culture. Mycologia 27: 388-417.

Bandoni, R. J. 1963. Conjugation in Tremella messenterica. Can. J. Bot. 41: 467-474.

Bandoni, R. J. 1965. Secondary control of conjugation in Tremella mesentterica. Can. J. Bot. 43: 627-630.

Bauch, R. 1930. Über Multipolare Sexualität bei Ustilago longissima. Arch. Protist. 70: 417-466.

Barnett, H. L. 1937. Studies on the sexuality of the Heterobasidia. Mycologia 29 : 626-649.

Bensaude, M. 1918. Recherches sur le cycle évolutif et la sexualité chez les Basidiomycètes. Thesis, Neumours. 156pp.

Berthier, J. 1974. Reproduction et comportements uncléaires chez les Typhula Fr. (Clavariacées) et genres affines. C. R. Acad. Sc. Paris, ser. D, 278: 3307-3310.

Bertrand, G. 1896. Sur une nouvelle oxydase, ou ferment soluble oxydant, d'origine végétale, C. R. Acad. Sci. Paris, 122: 1215.

Blaich, R & K. Esser. 1975. Function of enzymes in wood destroying fungi. Arch. Microbi. 103: 271-277.

Blakeslee, A. F. 1904. Sexual reproduction in the Mucorineae. Proc. Amer. Acad. Sci., 40: 206-319.

Boidin, J. 1971. Nuclear behavior in the mycelium and the evolution of the Basidiomycetes, in Petersen, R. H. (ed.): Evolution of Higher Basidiomycetes, an International Symposium. University of Tennessee Press, 124-148.

Boidin, J. 1977. Intéret des cultures dans la délimitation des espèces chez les Aphyllophorales et les Auriculariales, in Clemençon, H.: The species concept in Hymenomycetes. Bibl. Mycol. 61: 277-321.

Boidin, J. 1986. Intercompatibility and the species concept in the saprobic basidiomycotina. Mycotaxon 26: 319-336.

Boidin, J. and P. Lanquetin. 1965. Hétérobasidiomycètes saprophytes et Homobasidiomycètes résupinés. X: Nouvelles données sur la polarité dite sexuelle. Rev. Mycol. Paris. 30: 3-16.

Boidin, J. and P. Lanquetin. 1973. Podoscypha involuta (Klotzsch) Imaz. est une espèce composite (Basidiomycetes, Podoscyphaceae). Persoonia 7: 141-150.

Boidin, J. and P. Lanquetin. 1984. Répertoire des données utiles pour effectuer les tests d'intercompatibilité chez les Basidiomycetes. II. Phragmobasidiomycètes saprophytes. Cryptog. Mycol. 5: 47-50.

Boidin, J. 1986. Intercompatibility and the species concept in the saprobic Basidiomycotina. Mycotaxon 26: 319-336.

Bresinsky, A., M. et al. 1977. The genus Pleurotus as an aid for understanding the concept of species in Basidiomycetes. pp. 229-258. In H. Clemençon (ed.): The species concept in Hymenomycetes. Cramer.

Bresinsky, A. M. et al. 1987. Speciation in Pleurotus. Mycologia 79: 234-245.

- Bruehl, G. W. et al. 1975. Taxonomic relationships among Typhula species as revealed by mating experiments. *Phytopathol.* **65**: 1108-1114.
- Buller, A. H. R. 1909. *Researches on Fungi*. Longmans, Green, and Co., London; Hafner Publishing Co., New York.
- Burgeff, H. 1920. Sexualität und Parasitismus bei den Mucorineen. *Ber. deutsch. bot. Ges.* **38**: 318-327.
- Burnett, J. H. 1965. The natural history of recombination systems. p. 98-112. In: *Incompatibility in fungi*. Eds., K. Esser and J. R. Raper. New York: Springer-Verlag.
- Burnett, J. H. 1983. Speciation in fungi. *Trans. Br. Mycol. Soc.* **81**: 1-14.
- Burnett, J. H. and Partington. 1957. Spatial distribution of fungal mating type factors. *Proc. Roy. Phys. soc. Edinb.* **26**: 61-68.
- Campbell, M. P. & R. H. Petersen. 1975. Cultural characters of Amanita taxa. *Mycotaxon* **1**(3): 239-258.
- Chase, T., and R. Ullrich. 1985. Fruiting and genetic analysis of Heterobasidion annosum. *Mycologia* **77**: 464-471.
- Chase, T. and R. Ullrich. 1990a. Genetic basis of biological species in Heterobasidion annosum: Mendelian determinants. *Mycologia* **82**(1): 67-72.
- Chase, T. and R. Ullrich. 1990b. Five genes determining intersterility in Heterobasidion annosum. *Mycologia* **82**(1): 73-81.
- Clutterbuck, A. J. 1972. *J. Gen. Microbi.* **70**: 423.
- Coates, D., A. D. M. Rayner and N. K. Todd. 1981. Mating behaviour, mycelial antagonism and the establishment of individuals in Stereum hirsutum. *Trans. Br. mycol. Soc.* **76**(1): 41-51.
- Corner, E. J. H. 1950. A monograph of Clavaria and allied genera. *Ann. Bot. Mem.* **1**. 740 pp.
- Corner, E. J. H. 1970. Supplement to "A monograph of Clavaria and allied genera." *Beih. Nova Hedwigia* **33**: 1-299.
- Desjardin, D. E. 1989. The genus Marasmius from the southern Appalachian Mountains. Ph. D. Dissertation. The University of Tennessee. 837 pp.

- Desjardin, D. E. 1990. Culture morphology of Marasmius species. *Sydowia* **42**: 17-87.
- Devries, O. M. H. et. al. 1986. Formation of an extracellular laccase by a Schizophyllum commune dikaryon. *J. Gen. Microbi.* **132**: 2817-2826.
- Dodd, J. L. 1970. The genus Clavicornia. with emphasis on north American species. Ph. D. Dissertation. The University of Tennessee. 71 pp.
- Dodd, J. L. 1972. The genus Clavicornia. *Mycologia* **64**: 737-773.
- Doty, M. S. 1947. Clavicornia, a new genus among the clavarioid fungi. *Lloydia* **10**: 38-44.
- Duncan, E. G. 1972. Microevolution in Auricularia polytricha. *Mycologia* **64**: 394-404.
- Esser, K. 1962. Die Genetik der sexuellen Fortpflanzung bei den Pilzen. *Biol. Zentralbl.* **81**: 161-172.
- Esser, K. 1966. Incompatibility. In *The Fungi* Vol. II. Academic Press. Inc.
- Esser, K. and R. Keunen. 1976. *Genetics of Fungi*. Springer-Verlag, NY. 500 pp.
- Esser, K. and P. Hoffmann. 1976. Genetic basis for speciation in higher basidiomycetes with special reference to the genus Polyporus. In Clemencon, H. (ed.): *The Species Concept in Hymenomycetes*. J. Cramer Press.
- Flurkey, W. H. & Joseph J. Jen. 1978. Peroxidase and polyphenol oxidase activities in developing peaches. *J. Food Sci.* **43**: 1826-1831.
- Fries, N. 1985. Intersterility groups in Paxillus involutus. *Mycotaxon* **24**: 403-409.
- Fries, N. and G. Mueller. 1985. Incompatibility systems, cultural features and species circumscriptions in the ectomycorrhizal genus Laccaria (Agaricales). *Mycologia* **76**: 633-642.
- Galland, M. C. et al. 1979. The species problem in the Psathyrella candolleana complex. *Mycotaxon* **8**: 329-332.
- Giasson, L. et al. 1989. Cloning and comparison of Aa mating-type alleles of the Basidiomycete Schizophyllum commune. *Mol. Gen. Genet.* **218**: 72-77.

- Ginns, J. 1974. Secondly homothallic hymenomycetes: several examples of bipolarity are reinterpreted as tetrapolar. *Can. J. Bot.* **52**: 2097-2110.
- Ginns, J. 1985. Hericium in North America: cultural characteristics and mating behavior. *Can. J. Bot.* **63**: 1551-1563.
- Goodenough, P. W. 1978. The o-diphenol-oxygen-oxidoreductase of *agaricus bisporus*: activity and multiple forms during ageing. *Phytochem.* **17**: 633-636.
- Hallenberg, N. 1984. Compatibility between species of Corticiaceae s. l. (Basidiomycetes) from Europe and North America. *Mycotaxon* **21**: 335-388.
- Hallenberg, N. 1985. Compatibility between species of Corticiaceae s. l. (Basidiomycetes) from Europe and North America. II. *Mycotaxon* **24**: 437-443.
- Hallenberg, N. 1986. On speciation and species delimitation in Peniophora cinerea-group (Corticiaceae, Basidiomycetes). *Windahlia* **16**: 73-80.
- Hallenberg, N. 1988. Species delimitation in Corticiaceae (Basidiomycetes). *Mycotaxon* **31**(2): 445-465.
- Hillis, D. M. & C. Moritz. 1990. Molecular systematics. Sinauer Associates, Inc. Sunderland, Massachusetts, 588 pp. U. S. A.
- Ingebrigtsen, J et. al. 1989. Tyrosinase activity and isoenzymes in developing mushrooms. *J. Food Sci.* **54**(1): 128-131.
- Imazeki, R., Y. Otani, & T. Hongo (Eds.) 1988. Mushrooms of Japan. Yama-Lo- Keikoku-Sha Co. 623 pp.
- Iwasaki, H. et al. 1967. A fungal laccase, its properties and reconstitution from its protein and copper. *J. Biochem.* **61**: 814.
- James, S. W. 1983. Physiology of fruitbody development and genetics of sexuality and incompatibility in Clavicornia pyxidata (Fr.) Doty. University of Minnesota (M. A. Thesis).
- Jülich, W. 1981. Higher Taxa of Basidiomycetes. Vaduz: J.Cramer.

- Kanagy, C et. al. 1988. Luminescence from the carbon monoxide derivative of *Agaricus bispora* tyrosinase. Arch. of Biochem. and Biophys. **267(2)**: 668-675.
- Kemp, R. F. O. 1974. Bifactorial incompatibility in the spores basidiomycetes Coprinus sassii and C. bilanatus. Trans. Br. Mycol. Soc. **62**: 547-555.
- Kemp, R. F. O. 1975. Breeding biology of Coprinus species in the section Lanatuli. Trans. Br. Mycol. Soc. **65**: 375-378.
- Kerrigan, R. W. & I. K. Ross. 1988. Extracellular laccases: biochemical markers for agaricus systematics. Mycologia. **80(5)**: 689-695.
- Khan, I. A. & R. Ali. 1986. Antigenicity, Catalytic activity and conformation of *Agaricus bisporus* tyrosinase: interaction of conformation-directed antibodies with the native and irradiated enzyme. J. Biochem. **99**: 445-452.
- Kirk, T. K. 1971. Effect of microorganisms on lignin. Ann. Rev. Phytopathol. **9**: 185.
- Kirk, T. K. 1987a. Enzymatic combustion: the degradation of lignin by white-rot fungi. Ed. INRA, Paris.
- Kirk, T. K. 1987b. Lignin-degrading enzymes. Phil. Trans. R. Soc. Lond. A. **321**: 461-474.
- Kniep, H. 1919. Untersuchungen über den Antherenbrand (Ustilago violacea Pers.). Z. Bot. **11**: 257-284.
- Kniep, H. 1920. Über morphologische und physiologische Geschlechts-differenzierung (Untersuchungen an Basidiomyceten). Verh. Physik. Mediz Ges. Würzburg **46**: 1-28.
- Korhonen, K. 1978. Interfertility and clonal size in the Armillariella mellea complex. In Karstenia **18**: 31-42.
- Kornerup A. & J. H. Wanscher. 1978. Methuen Handbook of Colour. Methuen Ltd. 252 pp.
- Kuyper, Th. W. 1988. Specific and infraspecific delimitation. In Flora Agaricina Neerlandica. ed. A. A. Balkema Press.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**: 680-686.
- Leathers, C. R. & A. H. Smith. 1967. Two new species of clavarioid fungi. Mycologia **59**: 456-462.

- Lemke, P. A. 1969. A reevaluation of homothallism, heterothallism and the species concept in Sistotrema brinkmanni. Mycologia 61: 57-76.
- Leonard, T. J. 1971. Phenoloxidase activity and fruiting body formation in Schizophyllum commune. J. of Bacter. 106: 162-167.
- Leonard, T. J. & L. E. Phillips. 1973. Study of phenoloxidase activity during the reproductive cycle of Schizophyllum commune. J. Bacter. 114: 7-10.
- Leslie, J. F. & T. J. Leonard. 1979. Monokaryotic fruiting in Schizophyllum commune: phenoloxidases. Mycologia 71: 1082-1085.
- Lewis, D. 1954. Comparative incompatibility in Angiosperms and funig. Adv. Genet. 6: 235-285.
- Lewontin, R. C. and T. Prout. 1956. Estimation of the number of different classes in a population. Biometrics 12: 211-223.
- Maas Geesteranus, R. A. 1973. A new Clavicornia. Persoonia 8(2): 213-226.
- Macrae, R. 1942. Interfertility studies and inherence of luminosity in Panus stypticus. Canad. J. Res. C. 20: 411-434.
- Macrae, R. 1967. Pairing incompatibility and other distinctions among Hirschioporus [Polyporus] abietinus, H. fusco-violaceus, and H. laricinus. Can. J. Bot. 45: 1371-1398.
- Maekawa, N., M. Fukuda, I. Arita, & M. Komatsu. 1988. Cryopreservation of edible Basidiomycetous fungi in liquid nitrogen. Rep. Tott. Mycol. Inst. (Japan) 26: 15-28.
- Markert, C. L. and F. Moller. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species-specific patterns. Proc. Natl. Acad. Sci. U. S. A. 45: 753-763.
- Marr, C. D. 1979. Laccase and tyrosinase oxidation of spot test reagents. Mycotaxon IX: 244-276.
- Marr, C. D. 1984. Spot tests for detection of tyrosinase. Mycotaxon XIX: 299-305.
- Marr, C. D. 1986. The taxonomic potential of laccase and tyrosinase spot tests. Mycologia 78(2): 169-184.

- May, B. & D. J. Royse. 1982a. Confirmation of crosses between lines of Agaricus brunnescens by isozyme analysis. *Exp. Mycol.* **6**: 283-292.
- May, B. & D. J. Royse. 1982b. Genetic variation and joint segregation of biochemical loci in the common meadow mushroom Agaricus campestris. *Biochem. Genet.* **20**: 1165-1173.
- Mayer, A. M. 1987. Polyphenol oxidases in plants-recent progress. *Phytochem.* **26**: 11-20.
- Mayer, A. M. & Eitan Harel. 1979. Polyphenol oxidases in plants. *Phytochem.* **18**: 193-215.
- McKeen, C. G. 1952. Studies of Canadian Thelephoraceae. IX. A cultural and taxonomic study of three species of Peniophora. *Can. J. Bot.* **30**: 764-787.
- Micales, J. A. et al. 1986. The use of isozyme analysis in fungal taxonomy and genetics. *Mycotaxon* **XXVII**: 405-449.
- Miller, R. F. 1971. Evidence of sexuality in the cultivated mushroom Agaricus bisporus. *Mycologia* **63**: 630-634.
- Molitoris, H. P. et al. 1972. The phenoloxidases of the ascomycete Podospora anserina x. electron microscopic on the structure of laccases i, ii and iii. *Biochem. Biophys. Acta.* **271**: 286-291.
- Moore, B. M. & W. H. Flurkey. 1989. Tyrosinase activities and isoenzymes in three strains of mushrooms. *J. Food Sci.* **5**: 1377-1378.
- Moore, B. M. et al. 1989. Histochemical localization of mushroom tyrosinase in whole tissue sections on nitrocellulose. *Histochem.* **90**: 379-381.
- Mounce, I. 1922. Homothallism and heterothallism in the genus Coprinus. *Trans. Br. Mycol. Soc.* **7**: 256-269.
- Nobles, M. K. 1948. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. *Can. J. Research, C.* **26**: 281-431.
- Nobles, M. K. 1958. A rapid test for extracellular oxidase in cultures of wood-inhabiting hymenomycetes. *Can. J. Bot.* **36**: 91-99

Nobles, M. K. 1958. Cultural characters as a guide to the taxonomy and phylogeny of the polyporaceae. Can. J. Bot. **36**: 883-926.

Nobles, M. K. 1965. Identification of Cultures of wood-inhibiting Hymenomycetes. Can. J. Bot. **43**: 1097-1139.

Papazian, H. P. 1951. The incompatibility factors and a related gene in Schizophyllum commune. Genetics **36**: 441-459.

Papazian, H. P. 1958. The genetics of Basidiomycetes. Adv. Genet., **9**: 41-69.

Petersen, R. H. 1985. Type studies in the clavarioid fungi - IX Miscellaneous taxa, with a section on Tremellodendropsis. Persoonia **12**: 401-413.

Petersen, R. H. 1988. The clavarioid fungi of New Zealand. 170 pp. Science Information Publishing Centre, Wellington 1988.

Pomerantz, S. H. 1964. Tyrosine hydroxylation catalyzed by mammalian tyrosinase: An improved method of assay. Biochem. Biophys. Res. Comm. **16**: 188-194.

Prillinger, H & H. Peter Molitoris. 1979. Genetic Analysis in Wood-Decaying Fungi. Physiol. **46**: 265-277

Prillinger, H. J. and H. P. Molitoris. 1979. Genetic analysis in wood-decaying fungi. I. Genetic variation and evidence for allopatric speciation in Pleurotus ostreatus using phenoloxidase zymograms and morphological criteria. Physiol. Plant. **46**: 256-277.

Raper, C. A. 1976. Sexuality and life-cycle of the edible wild Agaricus bisporus. J. Gen. Microbi. **95**: 54-66.

Raper, J. R. 1954. Life cycles, sexuality and sexual mechanisms in the fungi. Pp. 42-81. Sex in Microorganisms. Amer. Assoc. Adv. Sci., Washington.

Raper, J. R. 1960. Control of sex in fungi. Amer. J. Bot. **47**: 794-808.

Raper, J. R. 1963. Device for the isolation of spores. J. Bacter. **86**: 342-344.

Raper, J. R. 1966. Genetics of sexuality in higher fungi. New York: the Ronald Press Co. 283 pp.

Raper, J. R. and C. A. Raper. 1968. Genetic regulation of sexual morphogenesis in Schizophyllum commune. J. Elisha Mitchell Sci. Soc. **84**: 267-273.

Raper, J. R. and K. Esser. 1964. The fungi. The cell (J. Brachet and A. E. Mirsky eds.) **6**: 139-245.

Redhead, S. A., J. Ginns, and R. A. Shosemaker. 1987. The Xerula (Collybia, Oudemansiella) redicata complex in Canada. Mycotaxon **30**: 357-450.

Reinhammar, B. 1984. Laccase Ch. 1. In Lontie R. (ed.): Copper Proteins and Copper Enzymes. p. 1-35. CRC Press. Boca Raton, FL.

Ridgway, R. 1912. Color standard and color nomenclature. Publ. by the author. Washington, D. C. 43 pp.

Robb, D. A. 1984. Tyrosinase. Ch.7. In Lontie R. (ed.): Copper Proteins and Copper Enzymes. p.207-240. CRC Press, Boca Raton, FL.

Robb, D. A. & S. Gutteridge. 1981. Polypeptide composition of two fungal tyrosinases. Phytochem. **7**: 1481-1485.

Royce, D. J. & B. May, 1982a. Use of isozyme variation to identify genotypic classes of Agaricus brunnescens. Mycologia **74**: 93-102.

Royce, D. J. & B. May 1982b. Genetic relatedness and its application in selective breeding of Agaricus brunnescens. Mycologica **74**: 569-575.

Sass, J. E. 1929. The cytological basis for homothallism and heterothallism in the Agaricaceae. Amer. J. Bot. **16**: 663-701.

Sharma, R. C. & R. Ali. 1981. Hydrodynamic properties of mushroom tyrosinase. Phytochem. **20**:399-401.

Stalpers, J. A. 1978. Identification of wood-inhibiting fungi in pure culture. Studies in Mycol. **16**.

Steglich, W. 1989. Some chemical phenomena of mushrooms and toadstools. Biologically Active Molecules U. P. Schlunegger.

Steglich W. & O. Sterner. 1988. Isolierung von sesquiterpenoiden aus der Becherkoralle , Artomyces pyxidatus (Clavicornaceae). Zeitschrift für Mykologie **54**(2): 175-177.

Strothkamp, K. G. et. al. 1976. Quaternary structure of mushroom tyrosinase. Biochem. Biophys. Res. Comm. **70**: 519-524.

Ullrich, R. 1973. Sexuality, incompatibility, and intersterility in the biology of the Sistotrema brinkmanni aggregate. Mycologia **65**: 1234-1249.

Ullrich, R. 1978. On the regulation of gene expression: incompatibility in Schizophyllum. Genetics **88**: 709-722.

Ullrich, R. C. & J. R. Raper. 1974. Number and distribution of bipolar incompatibility factors in Sistotrema brinkmannii. Amer. Nat. **108**: 507-518.

Vandendries, R. 1933. De la valeur de barrage sexuel, come criterium dans l'analyse d'une sporee tetrapolaire de basidiomycete: Pleurotus ostreatus. Genetics **15**: 202-212.

Vilgalys, R. & O.K. Miller. 1983. Biological species in the Collybia dryophila group in North America. Mycologia **75**: 707-722.

Vilgalys, R. & O. K. Miller. 1987. Mating relationships within the Collybia dryophila group in Europe. Trans. Br. Mycol. Soc. **89**: 295-300.

Wells, K. 1985. Inter- and intracollection matings of several species of Exidiopsis (Tremellales). Mycol. Soc. Amer. Newsl. **36**: 44.

Wells, K. and G. Wong. 1989. Partial intersterility and evidence of allopatric speciation in Exidiopsis plumbescens (Exidiaceae). Mycologia **81**(4): 567-586.

Whitehouse, H.L.K. 1949. Multiple-allelomorph heterothallism in the fungi. New Phytol. **48**: 212-244.

Wong, G.J. 1985. Interfertility between Auricularia polytricha and Auricularia tenuis. Mycol. Soc. Amer. Newsl. **36**: 44-45.

Wong, G. J. and K. Wells. 1985. Modified bifactorial incompatibility in Tremella mesenterica. Trans. Br. Mycol. Soc. **84**: 95-100.

Wong, G. J. and K. Wells. 1987. Comparative morphology, compatibility, and interfertility of Auricularia cornea. Mycologia **79**: 847-856.

- Wong, G. J., K. Wells, and R. J. Bandoni. 1985. Interfertility and comparative morphological studies of Tremella mesenterica. *Mycologia* 77: 36-49.
- Wood, D. A. 1980. Production, purification and properties of extracellular laccase of Agaricus bisporus. *J. Gen. Microbi.* 117: 327-338.
- Wood, D. A. 1985. Production and roles of extracellular enzymes during morphogenesis of basidiomycete fungi. In Moore, D. et. al., eds.): *Developmental Biology of Higher Fungi*. pp. 375-387. Cambridge: Cambridge University Press.
- Wright, S. 1941. (in T. Dobzhansky and Wright, T.) *Genetics of natural populations. V. Relations between mutation rates and accumulation of lethal in population of Drosophila pseudoobscura*. *Genetics* 26: 23-52.
- Wu, Q. X. and R. H. Petersen. 1991. Morphology and mating studies on Asian Clavicornia. *Mycosystema* 4: (in press).
- Yoshida, H. 1883., *Zur Chemie des Urushi-Firnis* *J. Chem. Soc. (Tokyo)* 43: 472.

APPENDICES

Appendix A

CULTURE LIST OF CLAVICORONA

| Species
epithet | Coll.
No. | Location | Single
Spores | Dikary-
otic | Culture
study | Mating
test | Enzyme
study |
|--------------------|--------------|----------------|------------------|-----------------|------------------|----------------|-----------------|
| candela-
brum | 2681 | New Zealand | + | - | - | + | + |
| | 2679 | " | + | - | - | + | + |
| | 2802 | " | - | + | - | - | + |
| | 2807 | " | + | + | - | + | + |
| colensoi | 2555 | New Zealand | + | + | + | + | + |
| | 2637 | " | + | + | - | - | + |
| | 2644 | " | + | + | + | + | + |
| | 2667 | " | + | - | - | + | + |
| | 2685 | " | + | + | - | + | + |
| | 44117 | " | - | + | + | - | - |
| | 44120 | " | - | + | + | - | + |
| cristata | 8974 | California | + | + | + | + | + |
| | GB1700 | Finland | + | + | + | + | + |
| | GB1701 | Finland | + | + | - | + | + |
| micro-
spora | 2349 | Japan | + | + | + | + | + |
| | 5352 | Japan | + | - | - | + | + |
| piperata | 2672 | New Zealand | - | + | - | - | + |
| | 2822 | " | + | + | - | + | + |
| pyxidata | 1706 | New York | + | - | - | + | - |
| | 1190 | " | + | + | + | + | + |
| | 239 | Minnesota | + | - | - | + | - |
| | 10654 | Virginia | - | + | + | - | + |
| | 1503 | Wisconsin | + | + | + | + | + |
| | 1506 | " | + | + | + | + | + |
| | JPL479 | Arizona | - | + | + | - | + |
| | 56655 | North Carolina | + | + | + | + | + |
| | 56662 | " | + | + | - | + | - |
| | 56653 | " | + | + | - | + | - |
| | 56681 | " | + | + | - | + | - |
| | 1660 | " | + | - | - | + | - |
| | 1661 | " | + | + | - | + | - |
| | 1662 | " | + | - | - | + | - |
| | 1616 | " | + | - | - | + | - |
| | 1650 | " | + | - | - | + | - |
| | 1834 | " | + | + | - | + | - |
| | 2092 | " | + | - | - | + | - |
| | 24439 | " | - | + | + | - | + |
| | 22501 | " | - | + | + | - | + |

Appendix A CULTURE LIST OF *CLAVICORONA* (Continued)

| Species
epithet | Coll.
No. | Location | Single
Spores | Dikary-
otic | Culture
study | Mating
test | Enzyme
study |
|--------------------|--------------|----------------|------------------|-----------------|------------------|----------------|-----------------|
| pyxidata | 1607 | Tennessee | + | + | + | + | + |
| | 1606 | " | + | + | - | + | - |
| | 56665 | Georgia | + | + | - | + | + |
| | 56667 | " | + | + | + | + | + |
| | 56668 | " | + | + | - | + | - |
| | 1741 | New York | + | - | - | + | - |
| | 1449 | China, Yunnan | + | + | + | + | + |
| | 1541 | China, Jilin | + | + | + | + | + |
| | GB1137 | Sweden | + | + | - | + | + |
| | GB1279 | Sweden | + | + | + | + | + |
| | GB1280 | Sweden | + | + | + | + | + |
| | GB1702 | Finland | + | + | - | + | + |
| turgida | 2682 | New Zealand | + | + | + | + | + |
| | 2651 | " | + | + | + | + | + |
| | 44103 | " | - | + | + | - | + |
| Taxon 1 | 1615 | North Carolina | - | + | + | - | + |
| Taxon 2 | 3456 | Puerto Rico | + | + | - | + | + |

Appendix B

SPECIMENS EXAMINED

1. Clavicornia candelabrum

New Zealand

Auckland: Waitahere Ranges, Farley track, 22. v. 90, Petersen, 2679 (TENN 48923), 2681 (TENN 48922); Piha Valley track, 26. v. 90, Petersen & Hughes, 2802 (TENN 48924), 2807 (TENN 48925).

2. Clavicornia colensoi

New Zealand

Auckland: Waitakere Ranges, Farley track, 22. v. 90, Petersen, 2685 (TENN 48919). Canterbury: vic. Lewis Pass, Nina River Swring bridge, 11. v. 90, Petersen, 2555 (TENN 48930). Fjordland: vic. Te Anau Keplar track, 17. v. 90, Petersen, 2637 (TENN 48917), P. Johnston, 2644 (TENN 48918); Monowai, Borland Lodge track, 19. v. 90, Petersen, 2667 (TENN 48920). Waipoua Kauri Reserve: Yakas Tree track, 12. v. 89, Petersen, 44117 (TENN). Titirangi: Mill Bay, 3. v. 83, Petersen, 44120 (TENN). North Canterbury: Arthur's Pass National Park, Old Coach Road, 5. v. 89, Buchanan, 55203 (TENN 48932).

3. Clavicornona cristata

North America

California Trinidad Co: south side of Highway 299 across from Grays Fall Camp Ground, 22. xii. 89, Pam Laegeut, DLL8974 (TENN 48928).

4. Clavicornona microspora

Japan

Mashiko town: 29. ix. 89, Petersen, 2349 (TENN 48856).

5. Clavicornona piperata

New Zealand

Fjordland: Monowai, Borland Lodge track, 19. v. 90, Petersen, 2672 (TENN 48915). North Island: Urewera National Park, 29. v. 90, Petersen & Hughes, 2822 (TENN 48916). Westland: Lake Kaniere Scenic Reserve Lake, Kaniere Road, 6. v. 89, Buchanan, 55204 (TENN 48931).

6. Clavicornona pyxidata

China

Jilin Province. Anto Co.: Baihe, Forest behind fire tower, 14. viii. 88, Petersen, 1449 (TENN 48279).

Yunnan Province. Simao Co.: Mounai Mountains, 5. viii. 90, Wu, 1541 (TENN 48855).

North America

Arizona. Graham Co., Colorado National Forest, 24. vii. 76, Lindsay, JPL479.

Georgia. Rabun Co.: Warwoman Road., 24. v. 89, Petersen, 56665 (TENN 48853), 56666 (TENN 48887), 56667 (TENN 48854), 56668 (TENN 48888); Double Bridge Road., 26. vii. 89, Petersen, 1834 (TENN 48899).

Maryland. Prince Georges Co.: Laurel, Beltsville Expt. Forest, 7. viii. 69, Lindsay & Swanm, AES 515. Beech Island Patuxent Wildlife Ref., Laurel, 9. vii. 68, Burdsall & Lindsay, HHB 810.

New York. Tompkins Co.: Dryden, Ringwood forest, 30. viii. 88, Petersen, 1704 (TENN 48902), 1706 (TENN 48841), 1707 (TENN 48905), McGowan's Woods, 29. viii. 88, Petersen, 1493 (TENN 48911), 1498 (TENN 48908), 1499 (TENN 48910); vic. Ithaca, Coy Glen, 31 viii. 88, Petersen, 1724 (TENN 48906); Robert Treman State Park, Finger Lakes trail, 31. viii. 88, Petersen, 1732 (TENN 48907), 1733 (TENN 48901), 1739 (TENN 48903), 1741 (TENN 48840). St. Lawrence Co.: Cranberry Lake, Wanakena, 27. viii. 88, Wu, 1190 (TENN 48842).

North Carolina. Haywood Co: GSMNP, Cataloochee Cove, Caldwell Creek, 30. vi. 89, Wu, 1616 (TENN 48895), 1617 (TENN 48900). Macon Co.: Highlands, Bull Pen Road., 22. v. 89, Petersen, 56653 (TENN 48846), 56655 (TENN 48847), 56662 (TENN 48848), 1. vii. 89, Wu, 1621 (TENN 48898); 17. viii. 89, Bandoni, 1660 (48849), 1661 (TENN 48850), 1662 (TENN

48851); Blue Valley, 8. vi. 89, Wu, 1614 (TENN 48896); Chattooga Loop trail, 26. viii. 89, Petersen, 2092 (TENN 48933); vic. Highland Biological Station, 12. vii. 88, Kay, 1135 (TENN 48914), 21. vi. 69, Dodd, Dodd 397 (TENN 37734). Swain Co.: GSMNP, Kephart Prong trail, 25. v. 89, Petersen, 56680 (TENN 48891).

Tennessee. Sevier Co.: GSMNP, 8. vi. 89, Wu, 1606 (TENN 48894), 1607 (TENN 48852), 1608 (TENN 48897).

Wisconsin. vic. Madison, Leopold Memorial Reserve, 23. vi. 90, Wu, 1501 (TENN 48843), 1502 (TENN 48893), 1503 (TENN 48844), 1504 (TENN 48879), 1505 (TENN 48874), 1506 (TENN 48845), 1507 (TENN 48880), 1508 (TENN 48892), 1509 (TENN 48885), 1510 (TENN 48883), 1511 (TENN 48872), 1512 (TENN 48886). Sauk Co.: Natural Bridge State Park, 24. vi. 90, Wu, 1513 (TENN 48875), 1514 (TENN 48872), 1515 (TENN 48876), 1516 (TENN 48878), 1517 (TENN 48881), 1518 (TENN 48884), 1519 (TENN 48877), 1520 (TENN 48882).

Virginia. Giles Co.: Mt. Lake Biological Station, 28. vii. 79, Burdsall, 10654.

7. Clavicornia turqida

New Zealand.

Auckland: Waitakere Ranges, Farley track, 22. v. 90, Petersen, 2682 (TENN 48926). Fjordland: vic. Te Anau, Keplar track, 17. v. 90, Petersen, 2651 (TENN 48934). Cascade Valley, Beech forest, 11. iv. 83, Petersen, 55137

(TENN 44103).

8. Clavicornia Taxon 1

North Carolina. Haywood Co.: GSMNP, Cataloochee Cove,
Caldwell Creek Trail Road., 30. vi. 89, Petersen, 1615 (TENN
48890).

9. Clavicornia Taxon 2

Puerto Rico. Caribbean National Forest, El Junque, Big Tree
Trail, 4. xii. 90, Petersen & Gorden, 3456 (TENN 48929).

Appendix C
CULTURE MEDIA

Malt Extract Agar (MA)

| | |
|----------------------|---------|
| Malt Extract (Difco) | 15 g |
| Agar (Difco) | 20 g |
| Distilled Water | 1000 ml |

Malt-Yeast-Glucose (MYG)

| | |
|-----------------------|---------|
| Malt Extract (Difco) | 10 g |
| Yeast Extract (Difco) | 4 g |
| Glucose | 4 g |
| Agar (Difco) | 10 g |
| Distilled Water | 1000 ml |

Medium 597 (M597; ATCC)

| | |
|-----------------------|---------|
| Malt Extract (Difco) | 7.5 g |
| Yeast Extract (Difco) | 0.5 g |
| Peptone | 1.0 g |
| Agar (Difco) | 15.0 g |
| Distilled Water | 1000 ml |

Potato-Dextrose Agar (PDA)

| | |
|----------------------------------|---------|
| Difco Bacto Potato Dextrose Agar | 39.0 g |
| Distilled Water | 1000 ml |

Potato-Dextrose-Yeast-Mineral Broth Medium (PDYB)

| | |
|--|---------|
| Potato Dextrose Broth (Difco) | 24 g |
| Yeast Extract (Difco) | 2 g |
| MgSO ₄ | 0.25 g |
| KH ₂ PO ₄ | 0.46 g |
| K ₂ HPO ₄ •3H ₂ O | 1.25 g |
| Distilled Water | 1000 ml |

YpSs Agar (Emerson)

| | |
|---------------------------------|---------|
| Soluble Starch | 15 g |
| Yeast Extract (Difco) | 4 g |
| Streptomycin | 0.8 g |
| K ₂ HPO ₄ | 1.0 g |
| MgSO ₄ | 0.5 g |
| Agar (Difco) | 20 g |
| Distilled Water | 1000 ml |

Appendix D

STOCK SOLUTIONS

FOR LACCASE AND TYROSINASE ELECTROPHORESIS

- 1) Acrylamide - 30%, Volume 50 ml

| | |
|----------------|--------|
| Acrylamide | 15.0 g |
| Bis-acrylamide | 0.4 g |

Add to 25 ml water, dissolve and bring to 50 ml total volume. Filter through 0.45 μ m membrane using vacuum.

- 2) Stacking gel buffer - 4X (0.5 M) Vol 100 ml

| | |
|----------|--------|
| Tris HCl | 6.05 g |
| Water | 50 ml |

Dissolve tris and add concentrated HCl until pH=6.8. Bring to 100 ml with distilled water.

- 3) Separating gel buffer - 4X (1.5 M) Vol 100 ml

| | |
|----------|--------|
| Tris HCl | 2.42 g |
| Water | 50 ml |

Dissolve tris and add concentrated HCl to pH=8.8. Bring volume to 100 ml with distilled water.

- 4) Upper electrode buffer - 5X Vol 500 ml pH=8.3

| | |
|---------|-------------------|
| Tris | 15.1 g (0.25 M) |
| Glycine | 71.87 g (1.915 M) |

When using at 1 X, pH should be at 8.3

- 5) Lower electrode buffer - 5X Vol 500 ml pH=7.5

| | |
|-------|-------------------|
| Tris | 18.89 g (0.315 M) |
| water | 300 ml |

Dissolve tris and add concentrated HCl to pH=7.5, then bring volume to 500 ml with distilled water

- 6) Sucrose sample buffer (for laccase), 10%

| | |
|---------------------------|-------|
| 1X upper electrode buffer | 10 ml |
| Sucrose | 1 g |

7) Sodium phosphate buffer, 25 mM, pH=6.0

| | |
|------------------|---------|
| Sodium Phosphate | 0.34 g |
| Water | 100 ml. |

8) 10% Ammonium persulfate 1 ml

| | |
|---------------------|-------|
| Ammonium persulfate | 0.1 g |
| Water | 1 ml |

Prepare immediately before use

9) Staining solution for laccase activity

| | |
|---------------------|---------|
| O-tolidine | 1 g |
| 100% ETOH | 100 ml |
| Na Acetate | 16.4 g |
| Glacial Acetic Acid | 11.5 ml |

Water added to 1000 ml

pH should be 4.7

Dissolve O-tolidine first in 100% ETOH.

10) Staining solution for tyrosinase activity
(0.01 M L-Dopa, pH=6.0)

| | |
|------------------|---------|
| L-Dopa | 2 g |
| Catalase | 1 g |
| Sodium Phosphate | 13.79 g |
| Water | 1000 ml |

VITA

Qiuxin Wu was born on 23 March, 1963, in Huadian, Jilin Province, P. R. China. She received elementary and high school education in that city. In September, 1979, she was enrolled into Northeast Forestry University, Harbin, and majored in forest pathology, where she began to study mycology. After she graduated with Bachelor of Agriculture degree from that university in 1983, she entered the Institute of Microbiology, Academia Sinica, Beijing, to study systematics of higher basidiomycetes. She received a Master of Science degree in mycology in August, 1986. In September, 1987, Ms. Wu accepted a graduate assistantship and enrolled at the University of Tennessee, Knoxville, to pursue a Doctor of Philosophy degree in botany. Under the supervision of Dr. Ronald H. Petersen, Ms. Wu studied the systematics of a basidiomycete genus using various methods.