Biosystematic Studies in *Crepidotus* and the Crepidotaceae

(Basidiomycetes, Agaricales)

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(ABSTRACT)

Fungi of the Crepidotaceae are characterized by saprotrophic habit, filamentous cuticle, and brown-pigmented basidiospores that lack either a germ pore or plage. The majority of species belong to *Crepidotus*, distinguished by their pleurotoid basidiomata. Because of their diverse morphology, the presence of several conflicting classifications, and lack of data regarding the biology, phenotypic plasticity, or phylogeny of these fungi, the present study sought first to determine phylogenetic relationships among the different taxonomic groups as a basis for addressing other aspects of *Crepidotus* biology and evolution.

Sequencing analyses show the Crepidotaceae is not monophyletic, and the family concept is revised. *Crepidotus* and its sister genus *Simocybe* are found to be monophyletic. At least nine phylogenetic lineages within *Crepidotus* were uncovered, although relationships between them could not be resolved. However, none of the previously proposed infrageneric classifications are reflective of phylogeny.

Morphological, biological, and phylogenetic species concepts were compared within a single phylogenetic unit, termed the Sphaerula group, showing an unusual amount of phenotypic plasticity exists within species, and a taxonomic revision of these species proposed. Also reported are several unique or unusual aspects of *Crepidotus* biology, including presence of a prolonged latent period prior to basidiospore germination; spontaneous reversion of differentiated hymenial cells to vegetative growth; and the revelation that structures previously termed pleurocystidia are actually the expression of secondary growth from basidia. Results from mating system, culture, and type studies, reassessment of morphological characters traditionally applied to agaric taxonomy, and a revised life cycle for the *Crepidoti* are presented.

Acknowledgments

Many, many, people have been involved in the conduct of this project. Foremost are the members of my doctoral committee, Drs. Orson Miller, Joseph Falkinham, Duncan Porter, Stephen Scheckler, and Rytas Vilgalys. All have, at one time or another, graciously reviewed manuscripts, helped to focus my efforts, and provided much needed critiques and suggestions. Only by benefit of their combined wisdom, guidance, and expertise has this dissertation reached fruition.

The culture work could not have been completed during its final year without the assistance of two talented undergraduate researchers, Jessica Ball and Katrina Hochstein. Likewise, I owe an enormous debt to the members of the Mycology Laboratory at Duke University—in particular Drs. Rytas Vilgalys and Jean-Marc Moncalvo, who provided training and unconditional, unlimited access to their facilities during the first three years of the molecular systematic portion of this study. Thanks also to Shian-Ren Liou, Tim James, and Jean-Marc and Jasmine Moncalvo for providing their homes during my sometimes lengthy stays in Durham.

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For their assistance in collecting and/or the donation or loan of *Crepidotus* material from around the world, without which this study would have been significantly reduced, I thank Dr. Tim Baroni, Dr. Alan Bessette, Dr. Tsutomu Hattori, Terry Henkel, Josh Herr, Yoshinobu Hoashi, Dr. Egon Horak, Sonia Jancovicová, Tim James, Dr. Jim Johnson, Wade Leschyn, Brandon Matheny, Dr. Steve Miller, Dr. Eiji Nagasawa, Dr. Clark Overbo, Dr. Ursula Peintner, Allein Stanley, Dr. Rytas Vilgalys, Dr. Roy Watling, Dr. Kazumasa Yokoyama, the University of Michigan Herbarium, and, of course, Dr. Orson and Hope Miller.

Funding for travel and various components of this project was provided by: Mountain Lake Biological Research Station of the University of Virginia, The North American Mycological Association, Sigma Xi, The Mycological Society of America, The U.S. National Science Foundation (#SP-9800003) and Monbusho, The Explorer's Club Washington Group, The Graduate Student Assembly of Virginia Tech, The Virginia Museum of Natural History, and the Department of Biology of Virginia Tech. I also directly benefited from a grant to Dr. O.K. Miller from the Horton Foundation, and ASPIRES grant (#232095) to Drs. O.K. Miller, E.T. Nilsen, R.H. Jones, D.M. Porter, and H.L. Warren.

Finally, I wish to express my gratitude to Orson K. Miller, Jr., who, as he has done for so many before me and will no doubt continue to do, opened my eyes to the fascinating field of mycology and suggested, as a potentially interesting subject, the study of *Crepidotus*.

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Introduction

"This is a watershed period in fungal systematics."

—Linda Kohn 1992

That systematic studies should be based on all available data, from a variety of sources, has been a widely expressed concern, though a difficult if not impossible goal to achieve. Fungal systematics is especially prone to narrowness in scope, given the inherent difficulties in accumulating certain types of data for these organisms. Traditionally our recognition of species in the fungi has been rooted in morphology and a morphological species concept (MSC) still informs our classification for most of the fungi. Morphological data have been derived primarily from fruiting structures in the macrofungi, despite concerns that "the whole fungus," including vegetative structures should, ideally, be considered. The growing application of molecular tools in fungal systematics has revolutionized this field, but when morphological and phylogenetic data do not agree there are few guidelines for synthesis, and much current taxonomic revision is based entirely on a phylogenetic species concept (PSC), often limited to evidence from a single gene region. Biological species concepts (BSC) can provide a baseline for evaluating other data, but in so many cases, a BSC is difficult or impossible to obtain due to the difficulties in performing mating studies in the laboratory for many of the macrofungi.

In this study I have attempted to provide a systematic reassessment of the Crepidotaceae sensu Singer (Basidiomycetes, Agaricales) that incorporates data from as many diverse areas as possible. Ecological and morphological data have been collected from field notes, fresh descriptions, microscopy studies, scanning electron microscopy (SEM) studies of basidiospores, and culture studies of the vegetative thallus. Sequencing data from nuclear rDNA have been compiled and analyzed. Mating studies have been performed in culture within one species complex to infer the extent of phenotypic variation in fruiting body morphology within a single interbreeding species.

Detailed experiments on an unusual phenomenon of delayed spore germination in some *Crepidotus* (Fr.) Staude species have revealed phylogenetically-informative patterns. Examination of morphogenesis of hymenial cells in one species complex provides surprising insight into the mechanisms responsible for observed phenotypic plasticity. Type studies and a careful evaluation of the literature have also been important in forming the systematic reassessment proposed here. Although there have been many proposed classifications for the fungi, I have chosen that of Singer (1986) as my hypothetical base for study. Since the vast majority of species in the Crepidotaceae belong to the genus *Crepidotus*, my main focus is here. And likewise, in choosing a species complex for more detailed investigation, I have chosen Subgenus *Sphaerula* Hesler and Smith wherein approximately one-forth of described *Crepidotus* species appear to belong.

The Crepidotaceae (Imai) Sing., and *Crepidotus* in particular, are ideal targets for a systematic study because so little is known at present regarding their biology, distribution, phenotypic plasticity, or mating system(s). The morphological diversity within this group has

given rise to numerous conflicting classification systems for these fungi at the family, generic, and infrageneric level, and relationships at all taxonomic levels have yet to be resolved. European species of *Crepidotus* were the subject of two modern systematic studies (Nordstein 1990, Senn-Irlet 1994), but the North American flora has not been treated since the work of Hesler and Smith (1965). Data on the genus from a world-wide perspective have not been compiled since the keys of Pilát (1950), and generic and species concepts and taxonomy are quite variable between continents.

This work is the continuation of a prior study of *Crepidotus* (Aime 1999), and will be divided into three main sections, each covering a different phylogenetic level. Section I will discuss and revise our concept of the Crepidotaceae; section II concerns studies on key aspects of phylogeny, biology, morphology, and the generic concept in *Crepidotus*; section III will look at species concepts within one complex, the Sphaerula group (defined in Aime Chap. 7, and Table 0.1). Each level of inquiry necessitates evaluation of different types of data (for example, mating studies are nearly meaningless at the familial level), although cross-inference has been drawn between studies in certain sections. An introduction to relevant concepts and literature is provided at the beginning of each section or chapter. Much of what follows will explain, compare, and contrast data from *Crepidotus* with what is currently understood to be the typical agaric life cycle, presented in Figure 0.1. Clarification of some terms introduced in this dissertation is provided in Table 0.1.

Objectives of this dissertation:

1. To determine the phylogenetic components of the Crepidotaceae (Imai) Singer and evaluate the morphological characters traditionally applied for positing relationships between genera, as well those used for generic circumscription, in these fungi. (Chapters 1 and 2).
2. To determine the taxonomically correct names for some *Crepidoti* based on type studies and other data and propose taxonomic revisions where necessary (Chapters 1, 2, 5, and 8).
3. To determine the monophyly of the genera of the Crepidotaceae s.s. (Chapter 3).
4. To evaluate previously proposed infrageneric classifications for *Crepidotus* (Fr.) Stuade, and the morphological characters used to create them (Chapter 3).
5. To report several unusual biological/physiological phenomena in some *Crepidoti*, and how knowledge of these phenomena impact formation of generic (Chapter 4) and species (Chapters 5 and 8) concepts.
6. To determine the homogenic mating system of members of the Sphaerula group (Chapter 6).
7. To compare applications of a MSC, BSC, and PSC within members of the Sphaerula group (Chapters 7 and 8).
8. To outline the life cycle of a typical *Crepidotus* (Conclusions).

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Table 0.1. Terms introduced in this study.

TERM	DEFINITION
Crepidoti	Phylogenetic members of the genus *Crepidotus*.
crepidoti	Agarics belonging to the Crepidotaceae s. Singer, i.e., brown-spored agarics with some morphological/anatomical similarities to *Crepidotus*, but not necessarily related by phylogeny.
crepidotoid	Morphologically similar to *Crepidotus*.
Nyssicola group	A monophyletic clade of those *Crepidoti* in Stirps Sphaerula exemplified by *C. nyssicola*.
Sphaerula group	A monophyletic clade of those *Crepidoti* in Stirps Sphaerula exemplified by *C. crocophyllus*.
Stirps Sphaerula	A subset of Subgenus *Sphaerula* Hesler & Smith containing only those *Crepidoti* with globose spores and basidiospore ornamentation composed of truncate columns arising from the exosporium. Hypothetically of common ancestry, but relationships as yet unresolved between the two main lineages (Sphaerula group and Nyssicola group).

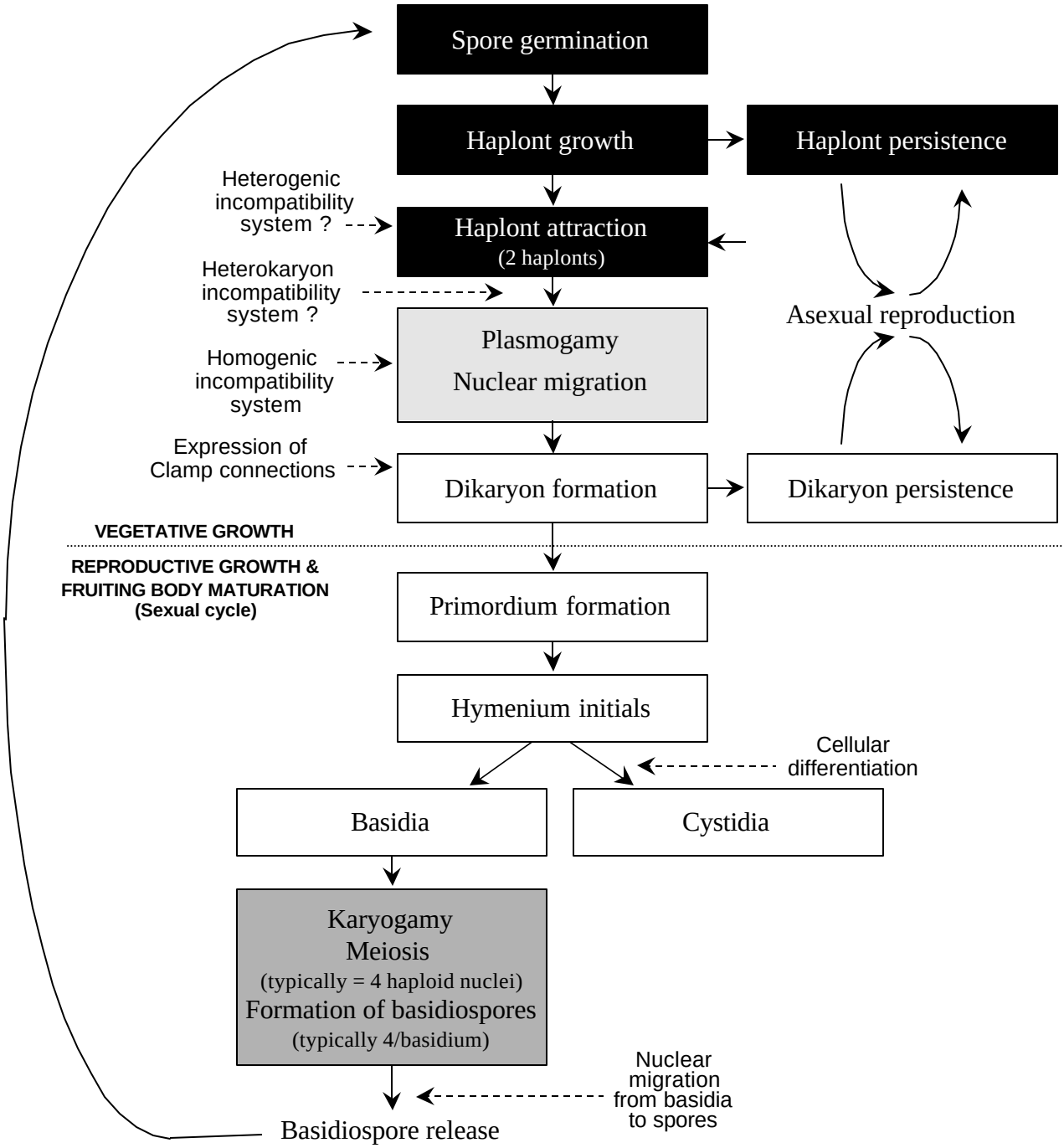

Fig. 0.1. Schematic representation of the life cycle of a typical agaric. Nuclear condition: Black box = haploid; White box = dikaryon; Gray box = diploid (fleeting); Gray/White stripes = intermediate between haploid and dikaryon. Vegetative growth occurs in steps indicated above the dotted line; sexual reproductive growth occurs in the steps indicated below the dotted line. A question mark denotes uncertainty about the exact stage where action occurs.

Part I

The Crepidotaceae (Imai) Singer

1

Crepidotus thermophilus comb. nov., a reassessment of *Melanomphalia thermophila*, a rarely collected tropical agaric.

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Manuscript.

Abstract

Melanomphalia thermophila (Sing.) Sing. is a rarely collected agaric previously known only from Florida and Brazil. This taxon was originally described as a species of *Tubaria* (W.G. Smith) Gill., and much of Singer's rationale for placing *Tubaria* within the Crepidotaceae (Imai) Sing. was based on anatomical similarities between *T. thermophila* and *Crepidotus* (Fr.) Staude. In later works, *T. thermophila* was transferred to *Melanomphalia* M.P. Christ., again forming the basis upon which *Melanomphalia* was placed within the Crepidotaceae by Singer. Based on examination of newly collected specimens from Puerto Rico and Panama, type studies, and nuclear large subunit rDNA analysis, we conclude that this taxon is, in fact, a *Crepidotus*. *Melanomphalia thermophila* is transferred to *Crepidotus*, fully described and illustrated.

Introduction

Tubaria thermophila Sing. was originally described from Florida (Singer 1948), where it held an isolated position within the genus as the only taxon with exosporial ornamentation. *Tubaria* Sect. *Thermophila* Sing. eventually came to include two species (Singer 1962), and it was largely based on similarities in spore ornamentation between this section and *Crepidotus* Sect. *Echinosporae* Pilát that led to Singer's transfer of *Tubaria* from the Cortinariaceae R. Heim ex Pouzar to the Crepidotaceae (Singer 1951, 1962).

Melanomphalia was a monotypic genus that Singer (1955) originally placed in the Cortinariaceae. Again, this decision was based on similarities in exosporial ornamentation, in this case between the type, *M. nigrescens*, and *Inocybe platensis* Speg., which he transferred to *Melanomphalia* (Singer 1955). In later works Singer reconsidered his treatment of these genera by transferring *Tubaria* Section *Thermophila* to *Melanomphalia* and placing *Melanomphalia* in the Crepidotaceae because of the stated anatomical similarities between *M. thermophila* and some *Crepidotus* species (Singer 1967, 1971). Thus the Crepidotaceae s. Singer came to include, among others, the pleurotoid genus *Crepidotus* (including both smooth- and ornamented-spored taxa), and the stipitate genera *Melanomphalia* (all members with ornamented basidiospores), *Tubaria*, and *Simocybe* Karst. (the latter two containing only smooth-spored taxa) (Singer 1986).

We have made several collections of a dark reddish brown stipitate agaric from the Luquillo Mountains of Puerto Rico in subtropical moist and wet forest between 70 and 110 m elevation. Anatomical features, including basidiospore ornamentation, are consistent with a diagnosis of *Melanomphalia thermophila*. In this paper we will present the results of

phylogenetic analysis of *M. thermophila*, propose a new combination, *Crepidotus thermophilus*, and fully describe and illustrate this taxon.

Materials and methods

Morphology—Color designations of basidiomata are given as general color terms, such as ochre, or in some cases color designations are from Kornerup and Wanscher (1978) and are indicated in the following manner: 7C5 - Brownish Orange (where "7C5" designates a plate, column and row, respectively). Methods used to prepare microscopic structures for data collection are those of Baroni (1981). All measurements of microscopic structures were made in mounts of 3% KOH or 10% NH_4OH. The measurement of basidiospores includes the hilar appendix or apiculus. The designations used for basidiospore measurements are those of Baroni and Horak (1994) with the exception that the symbol E (length/width of an individual spore) is herein designated as Q. All measurements were made with an Olympus BHS light microscope under Hoffman interference optics using a semi-automated image analysis system (a GTCO digitizer pad and Metrics5 software written by Dr. David Malloch). Descriptive statistical analysis of the measurements was obtained using EXCEL97 and/or SigmaStat 1.0. Scanning electron micrographs (SEMs) were produced with an ISI Supra IIIA scanning electron microscope generally run at 10 Kev. Methods for preparation of samples for SEM are those of Baroni (1981). Where noted, coordinates of collecting sites were obtained with a hand held GPS device and were referenced to map datum WGS84.

Sequence Analysis—To ascertain the natural affinities of *M. thermophila* DNA was extracted from two Puerto Rican collections made in consecutive years. Methods for extraction

of DNA, amplification, and sequencing follow Aime (1999). Primers LR0R, LR3R, LR5, and LR7 (Moncalvo et al. 2000) were used to sequence a portion from the 5'-end of the nuclear large subunit rDNA (nLSU). For sequence analysis, we assembled a data set of previously published sequences (Table 1.1), by selecting two generic exemplars from each family following the classification of Singer (1986) in order to approximate the range of diversity found within the dark-spored Agaricales. Three taxa of white-spored agarics were included for rooting purposes, with *Collybia dryophila* chosen as the outgroup. Taxa within *Crepidotus* were selected to include a broad cross-section of the phenotypic diversity inherent in the genus.

Sequences were manually aligned and analyzed in PAUP* 4.0b2 (Swofford 2001). The data matrix included a total of 1193 characters (including gaps), 139 of which were parsimony-informative. Parsimony analyses were performed using heuristic search algorithms with multiple (10) random sequence additions to generate starting trees, and tree-bisection-reconnection (TBR) branch-swapping. Bootstrapping frequencies (Hillis and Bull 1993) were calculated using TBR branch swapping with 1000 replicates; support of greater than 50% was considered significant. Jackknifing frequencies (Lanyon 1985) were calculated using TBR branch swapping with 1000 replicates; support of greater than 50% was considered significant. Decay values (Bremer 1988) were calculated with AutoDecay 4.0.1 (Eriksson 1998) in PAUP 3.1.1 (Swofford 1993).

Results

Type examinations show our Puerto Rico collections to be identical to *Melanomphalia thermophila* (Sing.) Sing. To infer the natural position of this taxon within the Crepidotaceae,

Table 1.1. Taxa selected for sequencing analysis.

Taxon[i]	Collection no.	GenBank accession no.	Source
Agaricaceae			
Agaricus bisporus (Lange) Sing.	SAR 88/411	U11911	Chapela et al., 1994
Leucocoprinus cepaestipes (Sow.: Fr.) Pat.	EFM 548	U85286	Johnson, 1997
Coprinaceae			
Coprinus atramentarius (Bull.: Fr.) Fr.	C 114 = VT 1131	AF041484	Hopple, 1994
Psathyrella candolleana (Fr.) Maire	J 181	AF041531	Hopple, 1994
Bolbitiaceae			
Bolbitius vitellinus (Pers.) Fr.	SAR 84/100	U11913	Chapela et al., 1994
Conocybe rickenii (Schaeff.) Kühner	J 183	AF041546	Hopple, 1994
Strophariaceae			
Pholiota squarrosoides Pk.	JJ 7	AF042568	Moncalvo et al., 2000
Stropharia rugosoannulata Farlow ex Murr.	D 258	AF041544	Hopple, 1994
Cortinariaceae			
Cortinarius iodes Berk. & Curt.	JM 96/23	AF042613	Moncalvo et al., 2000
Dermocybe marylandensis Ammirati & Smith	JM 96/24	AF042615	Moncalvo et al., 2000
Crepidotaceae			
Crepidotus cf. *amygdalosporus* Kühner	MCA 258	AF205675	Aime, 1999
Crepidotus aureus Horak	OKM 27300	AF205685	Aime, 1999
Crepidotus cinnabarinus Pk.	MCA 387	AF205686	Aime, 1999
Crepidotus fraxinicola Murr.	OKM 26748	AF205697	Aime, 1999
Crepidotus mollis (Fr.) Staude	OKM 26279	AF205677	Aime, 1999
Crepidotus thermophilus (Sing.) comb. nov. [ii]	TJB 8496	AF205691	Aime, 1999
Crepidotus thermophilus [ii]	OKM 27270	AF205669	Aime, 1999
Crepidotus versutus (Pk.) Sacc.	MCA 250	AF205695	Aime, 1999
Simocybe serrulata (Murr.) Sing.	OKM 27046	AF205688	Aime, 1999
Simocybe sp.	MCA 294	AF205699	Aime, 1999
Simocybe sp.	MCA 424	AF205687	Aime, 1999
Outgroups			
Collybia dryophila (Bull.:Fr.) Kumm.	RV 83/180	AF042595	Moncalvo et al., 2000
Omphalotus nidiformis Berk.	VTCC 1946.8	AF042621	Moncalvo et al., 2000
Crinipellis maxima Smith & Walker	DAOM 196019	AF042630	Moncalvo et al., 2000

[i] Taxa listed following the classification of Singer (1986).
[ii] Originally published as *Melanomphalia* sp.

DNA was isolated and sequenced from two Puerto Rican collections. Analysis of nLSU sequences, within both an extensive dataset of over 154 agaric taxa (Moncalvo et al. 2000, dataset available at http://www.botany.duke.edu/ fungi/mycolab), and within the pruned dataset presented (Fig. 1.11) show this taxon to be a component of *Crepidotus*. This species is described and illustrated, and a new combination proposed as follows.

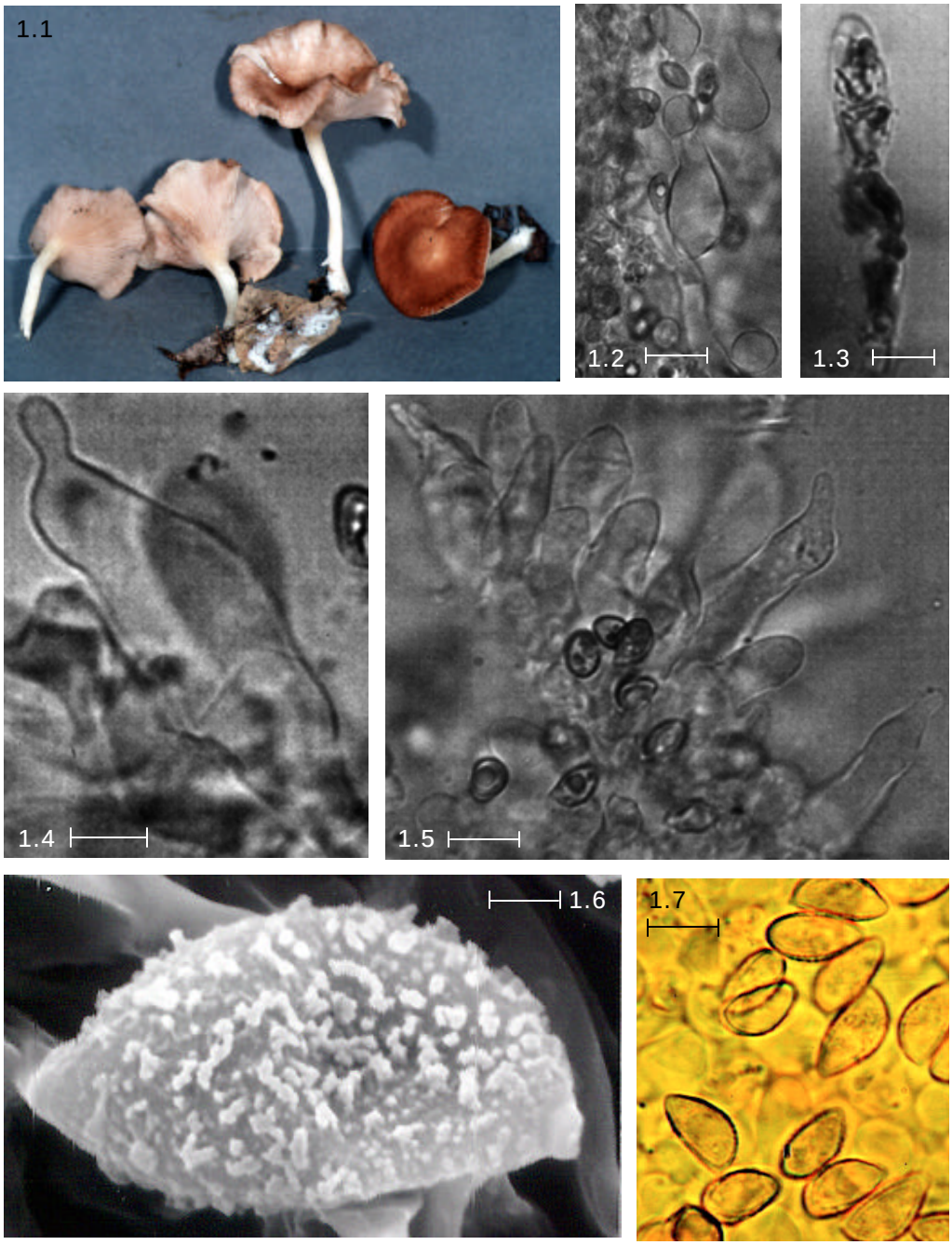
Taxonomy

Crepidotus thermophilus (Sing.) Aime, Baroni, et O.K. Miller, comb. nov. **Figs. 1-10**

= *Tubaria thermophila* Sing., Papers Mich. Acad. Sci., Arts & Letters 32:145. 1948.

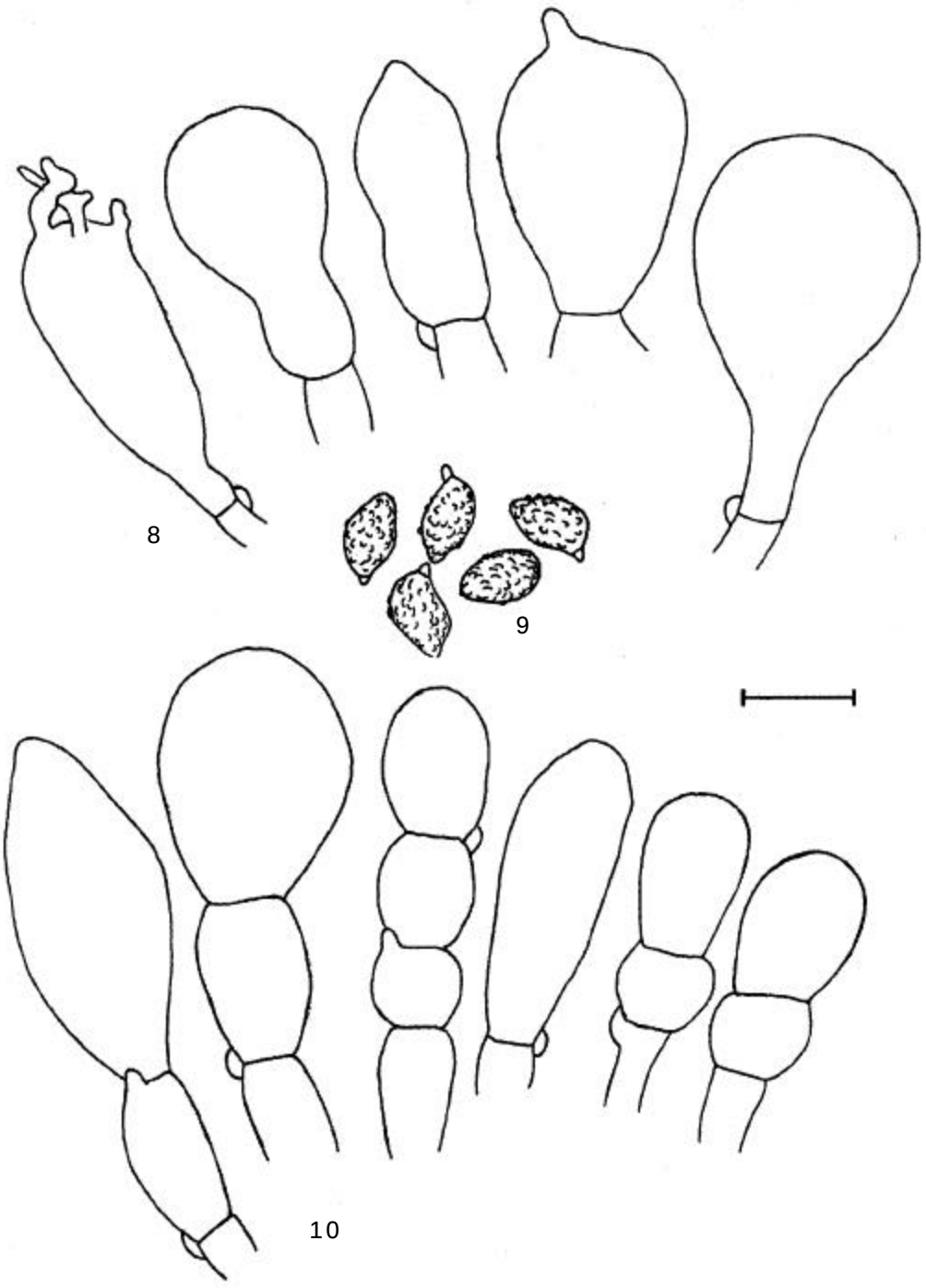
= *Melanomphalia thermophila* (Sing.) Sing., Atas Instituto de Micologia 5:481. 1967.

Pileus (Fig. 1.1) a deep rich reddish brown (6D5-7 to 7D8 – Sienna, Brick Red or Terra Cotta) slightly fading with age and expansion to Cinnamon Brown (6D6), 20-50 mm broad, convex becoming broadly convex, occasionally broadly umbonate, then plane, eventually uplifted, undulate and incised around the margin with age, moist or dry, becoming appressed fibrillose squamulose with age. *Lamellae* pale tan (5A2 to 5B3 – Orange White or Greyish Orange), short decurrent, close to crowded (2-3 tiers of lamellulae), narrow (up to 2 mm), edges concolorous or slightly paler and fimbriate. *Stipe* pale creamy white (4A2-3 – Yellowish White or Cream), 1.5-4.0 mm wide at apex, 20-35 mm long, equal, terete, often flexuous, central or very slightly eccentric, glabrous except for white fibrillose-pruinose apex, white mycelioid covering or strigose at base, solid and white context. *Odor* and *Taste* not distinctive. *Spore deposit* light reddish-brown.



Figs. 1.1-7. *Crepidotus thermophilus.* 1.1. Basidiomata TJB 8496. 1.2. Inflated tramal elements of the lamellae OKM 27270. Scale bar = 15 µm. 1.3. Hyphae of pileipellis with vacuolar pigments OKM 27270. Scale bar = 15 µm. 1.4. Cheilocystidia OKM 27270. Scale bar = 20 µm. 1.5. Cheilocystidia OKM 27270. Scale bar = 10 µm. 1.6. Scanning electron micrograph of basidiospore TJB 8496. Scale bar = 2 µm. 1.7. Basidiospores OKM 27270. Scale bar = 15 µm.

Basidiospores (Figs. 1.6-7, 1.9) 7.0-10.7 x (4-)4.8-5.9(-6.3) µm (n/6=136, L^m = 8.7 ± 0.83, W^m = 5.4 ± 0.38, Q = 1.29-2.12, Q^m = 1.62 ± 0.17; HOLOTYPE n = 21, 7.9-10.7 x 4.5-6, L^m = 9.6 ± 0.69, W^m = 5.3 ± 0.37, Q = 1.56-2.05, Q^m = 1.82 ± 0.14), amygdaliform in profile view, broadly fusiform-elliptical in face view, round in polar view, verrucose, yellow brown in 3% KOH. *Basidia* mostly 2-sterigmate, some 1- or 4- sterigmate, clavate, 17.8-24.3 x 6.4-8.0 µm. *Cheilocystidia* (Figs. 1.4-5, 1.8) abundant, hyaline, versiform, but mostly inflated, clavate, obpyriform, sphaeropedunculate, or broadly fusiform, some cells with apical digiform projections, 22-44 x 11-23 µm. *Pleurocystidia* absent. *Lamella trama* (Fig. 1.2) composed of a ± parallel, hyaline, cylindric or mostly inflated hyphae, (3.2) 8-17 µm in diam. *Pileus context* a hyaline layer of loosely interwoven, cylindric or slightly inflated, frequently branched hyphae, 4-14 µm in diam. *Pileipellis* a yellow brown layer of loosely entangled, cylindric or slightly inflated hyphae, 6-18 µm in diam., producing ascendant versiform pileocystidiate end cells (Fig. 1.3), cylindrical or slightly inflated or some tapered fusoid, 30-120 x 6-18 µm, all cells with dense dark yellow brown vacuolar pigments. *Stipitipellis* a dingy yellowish brown layer of repent, cylindric hyphae, 2.4-5.6 µm in diam, with abundant versiform *caulocystidia* (Fig. 1.10) at apex, quite variable, on young specimens mostly cylindrical-contorted, narrowly fusoid or narrowly clavate, on mature specimens mostly composed of clusters of inflated clavate or spherical end cells, 14.6-46 x 9.7-18 µm, sometimes with these spherical end cells sitting atop chains of 2-4 swollen spherical subtending cells. *Clamp connections* present in all tissues. *Habitat, habit, fruiting period:* Scattered in leaf litter, soil, sandy soil, or on well decomposed woody debris in subtropical moist and borderline



Figs. 1.8-10. *Crepidotus thermophilus* TJB 8496. Scale bar = 10 µm. 1.8 Cheilocystidia. 1.9. Basidiospores. 1.10. Caulocystidia.

subtropical wet forests at low elevations. May through August and January. Known from Puerto Rico, Florida, Brazil, and Panama.

Specimens examined. PANAMA: Province of Panama, Barro Colorado Island, Gatun Lake, W.M. Wheeler Trail, 14 May 2000, *C.L. Ovrebo 3808* (CSU). PUERTO RICO: Municipio Luquillo, between Luquillo and Sabana, off of Rt. 991, above Rio Sabana and a private chicken farm, approx. 70 m elev., N 18° 21' 3.4", W 65° 43' 50", 7 June 1997, *T.J. Baroni 8496* (CORT); same locality, 14 Nov. 1996, *T.J. Baroni 8309* (CORT); same locality, 15 January 1998, *O.K. Miller, Jr. 27270* (VPI); Municipio Luquillo, Luquillo Mts., Caribbean National Forest, near Sabana in a subtropical wet forest, approx. 110 m elev., N 18° 19' 34", W 65° 43' 22", 14 July 1998, collected by J. Mercado, *S. A. Cantrell PR4887* (NY). UNITED STATES: Florida, Highlands Co., near Seabring, Highlands Hammock State Park, 2 August 1942, *R. Singer F 20* (SYNTYPE, FH); same local, August 1942, *R. Singer F 20/III* ("authentic", FH).

Discussion

Crepidotus thermophilus is recognized in the field by the combination of its reddish brown subtomentose to fibrillose-punctate pileus surface, decurrent tan lamellae, pale creamy-white central stipe, and light reddish-brown spore deposit. Although the vast majority of described *Crepidotus* species are pleurotoid in form, the generic definition does not exclude taxa with a well-developed stipe. One other species of *Crepidotus* possess a prominent stipe, *C. nyssicola* (Murr.) Sing. (Hesler and Smith 1965, Bigelow 1980). *Crepidotus nyssicola* lacks pigmentation in the pileus, has globose, echinulate spores and is known from temperate

North America. *Crepidotus thermophilus* is differentiated by its amygdaliform, verrucose basidiospores and Neotropical distribution. The inflated cheilocystidia and two-sterigmate basidia of *C. thermophilus* are also diagnostic for this species.

Much of Singer's evolving concept of the Crepidotaceae was based on similarities in exosporial ornamentation between *Tubaria* (later *Melanomphalia*) *thermophila* and *Crepidotus* Sect. *Echinosporae*. Not surprisingly, basidiospore ornamentation in *C. thermophilus* as revealed by SEM (Fig. 1.6) falls well within the range of variation for *Crepidotus*, consisting of low, distinct, hemispherical verruculae, intermediate between those found in *C. variabilis* (Fr.) Kumm. (in Pegler and Young 1972) and *C. subverrucisporus* Pilát (in Senn-Irlet 1993). Previous work has suggested that the nature of exosporial ornamentation combined with the shape of basidiospores may be the best phenotypic indicator of phylogenetic relationships in the Crepidotaceae (Aime 1999, Chap. 3). Significantly, neither basidiospore ornamentation nor shape in *C. thermophilus* share similarities with those published for the generic type of *Tubaria*, *T. furfuracea* (Pers. ex Fr.) Gill. (in Clémençon 1977), or with *M. nigrescens* M.P. Christ. (in Horak 1968, Montag 1996), the type species of *Melanomphalia*.

This study has raised questions regarding the natural phylogenetic affinities of both *Tubaria* and *Melanomphalia* that we are currently working to address. In addition, we are re-examining other species currently placed in *Melanomphalia* to determine whether they also belong in *Crepidotus*. If these other taxa are transferred, it would increase the number of species with well-developed central stipes placed in *Crepidotus*.

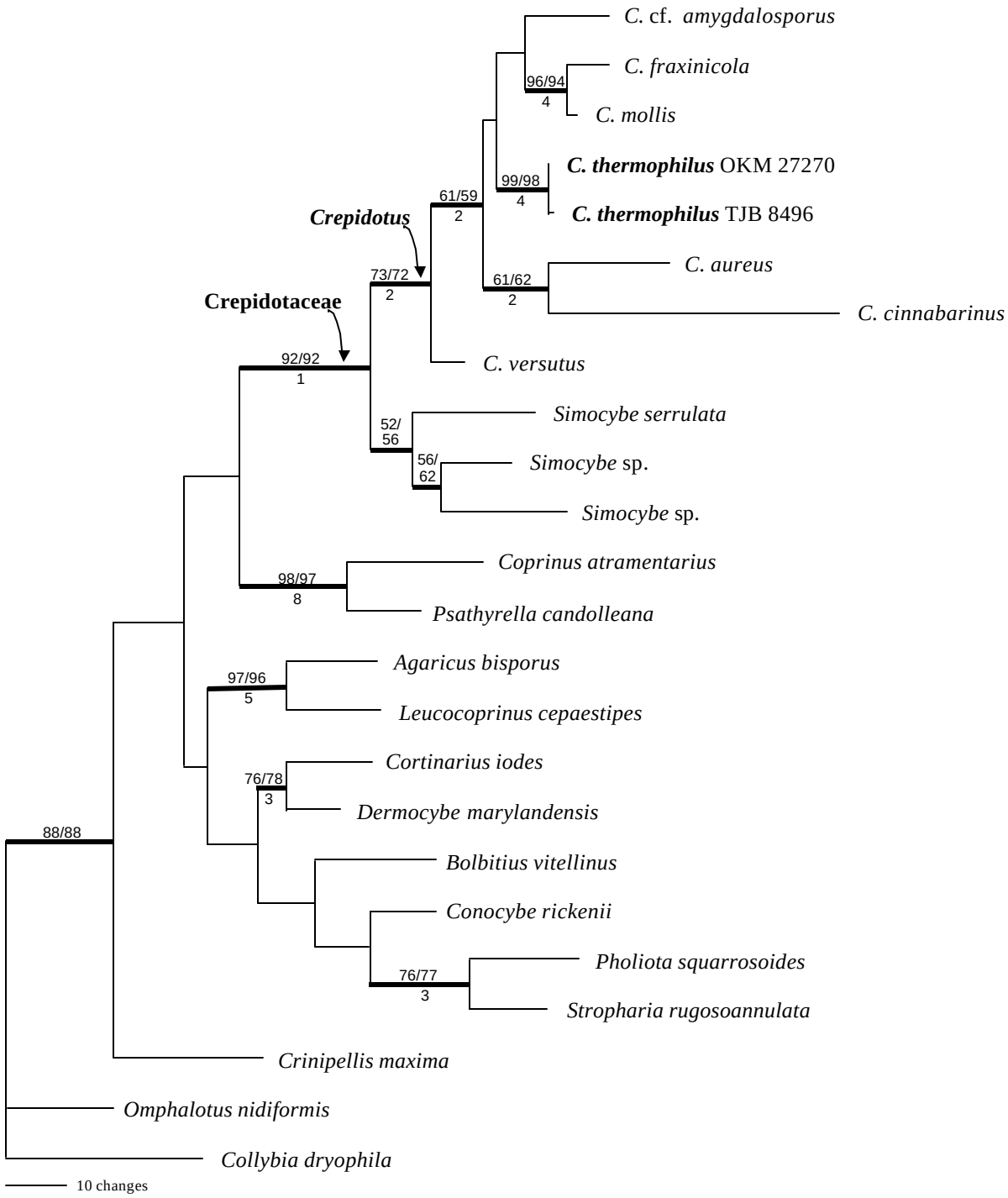


Fig. 1.11. Phylogenetic assignment of *Crepidotus thermophilus* within *Crepidotus* (Fr.) Staude. Analysis based on a portion from the 5'-end of the nuclear DNA encoding the large ribosomal subunit. The first of six most parsimonious trees is depicted (length = 587, RI = 0.54, CI = 0.50). Branches with strong statistical support are indicated by a bold line. Bootstrapping values (1000 replicates) are given as first number above supported branch; jackknifing values (1000 replicates) follow; decay values are indicated below branch.

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2

Reassessment of the Crepidotaceae (Imai) Singer based upon nuclear large subunit rDNA evidence.

Manuscript.

Abstract

Current classification of the dark-spored agaric genera of the Crepidotaceae s. Singer were evaluated using analyses of sequence data from the nuclear large subunit rDNA (nLSU). Taxa analyzed reflect a broad sampling of phenotypic diversity within the Crepidotaceae and include the type species for each agaric genus allied in the family: *Crepidotus*, *Simocybe*, *Pleurotellus*, *Tubaria*, and *Melanomphalia*. Phylogenetic relationships for these genera were evaluated within a dataset that samples representatives from one light-spored and all known dark-spored euagaric lineages. Results from parsimony analysis suggest that the crepidotoid fungi have three separate origins within the euagarics. The Crepidotaceae s.s includes *Crepidotus* and *Simocybe* and represents a new and separate lineage of dark-spored euagarics; relationships between this and other euagaric families remain unresolved. The generic name of *Pleurotellus* becomes a synonym for *Crepidotus* as the type species is a component of the latter genus. Results indicate the exclusion of both *Tubaria* and *Melanomphalia* from the Crepidotaceae s.s. *Tubaria* is most closely allied with the strophariaceous taxa *Phaeomarasmius* and *Flammulaster*, while the dark-spored genus *Melanomphalia* has arisen from within a lineage of light-spored omphalinoid euagarics. Given the broad sampling of

taxa, traditional methods of testing robustness of nodes via bootstrapping and jackknifing result in low values. However, statistical analysis of tree length values generated from monophyletic constraint analyses yield high confidence levels supporting the results from parsimony analyses.

Introduction

Recent advances in phylogenetic systematics of the Homobasidiomycetes have both reinforced, and encouraged reassessment of, traditional classification systems for these fungi. Large-scale molecular analyses have uncovered an evolutionary lineage of Homobasidiomycetes that is roughly equivalent to the Agaricales sensu Singer (1986) with several notable exceptions, now known as the "euagarics" (Hibbett et al. 1997, Moncalvo et al. 2000).

Phylogenetic systematics of select euagaric lineages has been the subject of many recent studies (e.g., Liu et al. 1997, Johnson and Vilgalys 1998, Drehmel et al. 1999, Hopple and Vilgalys 1999). Comprehensive molecular analyses of the euagarics as a whole was first undertaken by Moncalvo et al. (2000), providing a phylogenetic framework for examining evolutionary lineages in the agaric fungi, and their analysis included representative taxa from all of Singer's (1986) families with the exception of the Gomphidiaceae Maire ex Jülich and the Crepidotaceae (Imai) Singer. Phylogenetic study of the Gomphidiaceae has since been undertaken (Miller and Aime 2001), although these fungi are now considered to be gilled members of the suilloid (Boletales) lineage, and not true agarics. The present study provides the first such analysis of the agaricoid genera of the Crepidotaceae.

The earliest classification for the crepidoti was by Imai (1938) who erected the monogeneric tribe Crepidoteae to accommodate those species of agarics with eccentric, lateral,

or absent stipes, subdecurrent lamellae, and ochraceous or ferruginous spores. In the ensuing years a total of nine genera gradually came to be included in the Crepidotaceae (Singer 1951a, 1962, 1971, 1973). The last edition of *The Agaricales in Modern Taxonomy* (Singer 1986) lists the agaricoid genera *Tubaria* (W.G. Sm.) Gillet, *Melanomphalia* M.P. Christ., *Simocybe* Karst., *Crepidotus* (Fr.) Staude, and *Pleurotellus* Fayod, as well as four cyphelloid genera, *Episphaeria* Donk apud Sing. ex Donk, *Phaeosolenia* Speg., *Pellidiscus* Donk, and *Chromocyphella* de Toni and Levi, as the closely related members of this family.

Members of the family have little or no known economic importance, occur worldwide in a variety of habitats, and are phenotypically diverse. Singer's (1986) definition of the Crepidotaceae includes genera with pip-shaped, ellipsoid, or globose basidiospores usually without a germ pore, and with pale yellow to dark brown spore prints. The inamyloid spores may be either smooth walled or ornamented. Hyphae may or may not form clamp connections; cheilocystidia are nearly always present but pleurocystidia are present in only a few species. Species may be pleurotoid, collybioid, omphalinoid, or cyphelloid in habit, and are secondary decomposers, found on various types of organic debris and wood. Development is not known for all species, but believed to be gymnocarpic or hemiangiocarpic. As a family, Singer believes these fungi to be related to the Cortinariaceae R. Heim ex Pouzar, Entolomataceae Kotl. and Pouzar, and Paxillaceae R. Maire apud Maire, Dumée and Lutz.

Many modern authors follow Singer with minor modifications, while others have proposed major revisions to Singer's system, including the abolition of the Crepidotaceae. Most of these treatments include placing some or all of the Crepidotaceae genera within other families, especially the Cortinariaceae and/or the Strophariaceae Singer and A.H. Smith (e.g.,

Moser 1978, Kühner 1980, Jülich 1981, Bas 1988, Breitenbach and Kränzlin 1995, Hawksworth et al. 1995).

Morphological and biological studies of the crepidoti have provided further evidence that makes obvious the need for a critical reassessment of the current classification systems for these fungi. For example, morphological studies, especially of the pileipellis, have suggested that *Simocybe* might be more closely related to *Agrocybe* Fayod (Bolbitiaceae Singer) (Romagnesi 1962, Watling and Largent 1976). Basidiospore germination in members of *Crepidotus* is markedly different from that in species of *Tubaria*, suggesting a more distant common ancestry for them than proposed by Singer (Aime and Miller Chap. 4). Singer based his transfer of both *Tubaria* and, later, *Melanomphalia* into the Crepidotaceae on a single species (Singer 1951a, 1962, 1971); recent study of type and newly collected material has shown this taxon to be, in fact, a *Crepidotus* (Aime et al. Chap. 1). In addition, numerous studies have questioned the validity of delimiting genera based on single morphological characters (e.g., Smith 1967, Watling and Largent 1976), such as has been done in circumscribing *Pleurotellus* from *Crepidotus*.

Given the diverse phenotypes, contradictory proposed classifications, and biological differences, this study was undertaken to assess phylogenetic relationships among the genera of the Crepidotaceae s. Singer by analyzing nuclear DNA sequences encoding a portion of the large ribosomal subunit (nLSU). Any family concept for the Crepidotaceae must arise from interpretation of the type genus, *Crepidotus*, and its natural generic allies, also interpreted through the generic types. For this reason the type species from each of the five proposed agaric members of the Crepidotaceae were analyzed, as were other specific exemplars from each genus covering a broad range of the phenotypic diversity found within these fungi. Specific

questions addressed include: 1) is the Crepidotaceae, as proposed by Singer (1986), a monophyletic taxon; 2) what is the taxonomic status for each genus; and, 3) what morphological or biological characters are reliable predictors of evolutionary relationships between closely related crepidotoid genera?

Materials and Methods

Taxonomic sampling—A collection of nLSU sequences were selected from previous studies (Chapela et al., 1994, Lutzoni 1997, Johnson and Vilgalys 1998, Hopple and Vilgalys 1999, Moncalvo et al. 2000) to include three representatives from each lineage of dark-spored euagarics (designated Q through W) as identified in Moncalvo et al. (2000). Dark-spored euagarics were sampled extensively because all taxonomic treatments of the Crepidotaceae ally them within this group, and additional sampling of omphalinoid fungi (Moncalvo et al. 2000, lineage K) was included because preliminary analyses (not shown) suggested an affinity of *Melanomphalia* with this group. For rooting purposes, outgroup taxa were chosen from within the Boletales (Moncalvo et al. 2000, lineage AA) since prior phylogenetic analysis of both the nLSU (Moncalvo et al. 2000) and mitochondrial and nuclear small ribosomal subunit DNA sequences (Hibbett et al. 1997) suggest that the boletes and euagarics are sister lineages, and it has been shown that outgroup choice should, ideally, be confined to taxa sharing a sister group relationship to the ingroup under study (Hopple and Vilgalys 1999).

Singer's (1986) classification was used as the hypothetical basis for taxonomic sampling from within the Crepidotaceae. Sequences previously published by Aime (1999) and new sequences were obtained to represent the type species for each of the five agaric genera, and

Table 2.1. Origin of sequences used in this study, listed according to Singer's (1986) taxonomic system.

Taxa [a]	Lineage [b]	GenBank no.
Crepidotaceae		
Crepidotus aureus Horak (OKM 27300; Puerto Rico)		AF205685[f]
Crepidotus betulae Murr. (MCA 384; NC, USA)		AF205679[f]
Crepidotus brunswickianus (Speg.) Sacc. (MCA 580; Japan)		AF 367932
Crepidotus cinnabarinus Pk. (MCA 387; NY, USA[c])		AF205686[f]
Crepidotus fragilis Joss. (MCA 904; VA, USA)		AF367931
Crepidotus mollis (Fr.) Staude (OKM 26279; MT, USA)		AF205677[f]
Crepidotus thermophilus (Sing.) Aime, Baroni & O.K. Miller (OKM 27270; Puerto Rico)		AF205669[f]
Crepidotus versutus (Pk.) Sacc. (MCA 250; VA, USA)		AF205695[f]
Melanomphalia nigrescens M.P. Christ. (Kew 20553; U.K.[d])		AF367933
Pleurotellus hypnophilus (Pers.: Berk.) Fayod (IBNR 1997/0948; Russia[e])		AF367934
Simocybe amara (Murr.) Sing. (MCA 682; Japan)		AF205708
Simocybe centuncula (Fr.) Karst. (MCA 393; CA, USA)		AF205707
***Simocybe* sp.** (MCA 750; VA, USA)		AF205706
Tubaria furfuracea (Pers.: Fr.) Gill. (MCA 391; CA, USA)		AF205710
Tubaria pallidospora Lange (OKM 24351; ID, USA)		AF205711
Tubaria rufofulva (Clel.) Reid & Horak (OKM 24681; Australia)		AF205712
Tricholomataceae Subtribe Omphalinae		
Omphalina velutipes Orton	K	U66455[g]
Omphalina ericetorum (Fr.) Lange	K	U66445[g]
Agaricaceae		
Cystoderma granulosum (Batsch) Fayod	Q	U85299[h]
Lepiota cristata (Bolt.:Fr.) Kumm.	U	U85292[h]
Lepiota acutesquamosa (Weinm.) Sing.	U	U85293[h]
Leucocoprinus cepaestipes (Sow.:Fr.) Pat.	U	U85286[h]
Ripartitella brasiliensis (Speg.) Sing.	S	U85300[h]
Coprinaceae		
Coprinus atramentarius (Bull.:Fr.) Fr.	T	AF041484[i]
Lacrymaria velutina (Pers.:Fr.) Sing.	T	AF041534[i]
Panaeolus acuminatus (Schaeff.:Secr.) Quél.	R	AF041535[i]
Psathyrella candolleana (Fr.) Maire	T	AF041531[i]
Bolbitiaceae		
Bolbitius vitellinus (Pers.) Fr.	R	U11913[j]
Conocybe rickenii (Schaeff.) Kühner	R	AF041546[i]
Agrocybe praecox (Pers.:Fr.) Fayod	V	AF042644[k]
Agrocybe firma (Pk.) Sing. (MCA 948; VA, USA)		AF367935
Strophariaceae		
Pholiota squarrosoides Pk.	W	AF042568[k]
Psilocybe silvatica (Pk.) Sing. & A.H. Sm.	W	AF042618[k]
Stropharia rugosoannulata Farlow ex. Murr.	W	AF041544[i]

Table 2.1. Continued.

Taxa [a]	Lineage [b]	GenBank no.
Cortinariaceae		
Cortinarius iodes Berk. & Curt.	Q	AF042613[k]
***Cortinarius* sp.**	Q	AF042614[k]
Dermocybe marylandensis Ammirati	Q	AF042615[k]
Flammulaster rhombospora (Atk.) Watling		AF261493[l]
Hebeloma crustuliniforme (Bull.:Fr.) Quél.	V	U11918[j]
Inocybe geophylla* var. *lilacea (Sow.:Fr.) Kumm.	S	AF042616[k]
***Inocybe* sp.**	S	AF042617[k]
Phaeomarasmius erinaceus (Pk.) Sing. & Digilio		AF261492[l]
Boletaceae		
Boletus retipes Berk. & Curt.	AA	U11914[j]
Suillus luteus (L.:Fr.) S.F. Gray	AA	AF042622[k]

[a]Collection number and locality given for Crepidotaceae and novel sequences only. Voucher collections for Crepidotaceae and novel sequences housed at Virginia Tech Mycological Herbarium (VPI) unless otherwise noted.

[b]Phylogenetic lineage as reported in Moncalvo et al. (2000) for previously analyzed sequences. Lineages Q - W are comprised entirely or in part of dark-spored euagarics; lineage K consists of white-spored omphalinoid euagarics; lineage AA contains the boletes and is a sister lineage to the euagarics.

[c]Split collection (A. Bessette no. 10645).

[d]Voucher collection housed at the Royal Botanic Gardens, Kew (K).

[e]Voucher collection housed at Herbarium Universität Innsbruck (IB).

[f]Aime (1999).

[g]Lutzoni (1997).

[h]Johnson and Vilgalys (1998).

[i]Hopple and Vilgalys (1999).

[j]Chapela et al. (1994).

[k]Moncalvo et al. (2000).

[l] Moncalvo, J.M., S.A. Redhead, J.E. Johnson, M.C. Aime, V. Hofstetter, S. Verduin, T.J. Baroni, E. Larsen, R.G. Thorn, S. Jacobsson, H. Clémençon, O.K. Miller, and R. Vilgalys, in prep.

additional sampling was conducted to cover the range of phenotypic diversity found within each genus. The 44 taxa selected for analysis are listed in Table 2.1, along with the classification according to Singer (1986), phylogenetic lineage according to Moncalvo et al. (2000), and GenBank accession numbers for the nLSU sequences.

DNA extraction, amplification, and sequencing—Methods for DNA extraction follow the CTAB (hexadecyltrimethyl-ammonium bromide) extraction buffer protocol in Aime (1999). Amplification was achieved with primer pairs 5.8SR/LR7 (Vilgalys and Hester 1990) or LR5/LR0R (Moncalvo et al. 2000). Amplification parameters follow Aime (1999). PCR products were cleaned with Millipore Micron-PCR filters following the manufacturer's protocols. The 5'-end of the nLSU gene was targeted for sequencing and phylogenetic analysis as this region carries nearly 50% of the phylogenetic signal present within the entire nLSU molecule (Hopple and Vilgalys 1999). Primers LR0R, LR3R, LR5, and LR16 (Moncalvo et al. 2000) were used in sequencing reactions. Sequencing reactions were cleaned following Aime (1999) and sequences produced on an ABI373 (Perkin-Elmer) automated sequencer. The four sequence chromatograms generated per sample were compiled using SeqMan II v. 4.03 (DNAStar, Inc. 1997) to produce a single contiguous sequence for each sample.

Phylogenetic analysis—Sequences were manually aligned in PAUP* 4.0b8 (Swofford 2001). Gaps were introduced to maintain alignment through regions where indels occurred in one or more sequences. Regions with ambiguous alignment were excluded from the analyses. Analyses were performed on a Power Macintosh OS workstation. Methods for unweighted maximum parsimony analysis (UMP) follow Aime et al. (Chap. 1). Methods for weighted maximum parsimony analysis (WMP) follow Moncalvo et al. (2000) and apply a

stepmatrix based on dinucleotide frequencies and substitution rate estimates as observed in that work. Bootstrapping and jackknifing values were calculated by performing 1000 replicates of random addition sequence replicates with TBR (tree-bisection-reconnection) branch swapping.

Topological constraint trees were generated in the following manner. All members of *Crepidotus* and *Pleurotellus* were assigned to a single taxset; *Simocybe* taxa were assigned to a second taxset; *Tubaria* taxa assigned to a third taxset; all other ingroup taxa, with the exception of *M. nigrescens* and the two outgroup taxa were assigned to a fourth taxset. No topological constraints were enforced within any taxset. Separate analyses were then run with the following monophyletic constraints between taxsets: 1) no constraints; 2) taxset 1, 2, 3, and *M. nigrescens*; 3) taxset 1 and 2; 4) taxset 1, 2 and 3; 5) taxset 1, 2 and *M. nigrescens*; 6) taxset 1 and 3; 7) taxset 1 and *M. nigrescens*. For each of the above analyses, WMP, with weightings based on nucleotide frequencies as already described, using 20 random addition sequence replicates with TBR branch swapping, were conducted in PAUP*. Scores for each of the 20 most parsimonious trees uncovered were recorded for each constraint set. Tree scores were analyzed statistically in JMP v.3.2.1 (SAS Institute, Inc. 1997).

Results

In all, the data matrix assembled consisted of 44 taxa aligned across 1200 positions; 188 positions were excluded from analysis due to ambiguities in alignment, and by trimming of the extreme 5' and 3' ends; 231 of the remaining characters were parsimony-informative. A single shortest UMP tree was found of length 1193, CI = 0.450, RI = 0.527. A single shortest WMP tree of length 5057.4, CI = 0.456, RI = 0.535, was also found. Trees generated by both

weighted and unweighted parsimony analyses were not different in topology. Bootstrapping and jackknifing support of terminal clades is stronger than for internal nodes. Fig. 2.1 depicts the bootstrap 50% majority-rule consensus tree generated with these data, which is topologically congruent with both UMP and WMP trees.

Tree scores generated by 20 WMP random addition replicates enforcing six different topological constraints and without constraints are supplied in Table 2.2. Imposing monophyly in all combinations among the different genera of the Crepidotaceae resulted in trees that were significantly longer than the trees produced without constraint (Fig. 2.1) except when only *Crepidotus* and *Simocybe* are constrained together. Fig. 2.2 depicts the shortest tree obtained for each constraint set.

Ten lineages of brown-spored agarics were revealed, corresponding to previously reported lineages with two additions: The crepidotoid lineage of *Crepidotus* [type *C. mollis* (Fr.) Staude], *Pleurotellus* [type *P. hypnophilus* (Pers. ex Berk.) Fayod], and *Simocybe* [type *S. centuncula* (Fr. :Fr.) Karst.] (Fig. 2.1, clade I); and a lineage consisting of *Tubaria* [type *T. furfuracea* (Fr.) Gillet], *Phaeomarasmius* Scherff., and *Flammulaster* Earle (Fig. 2.1, clade II). Deeper relationships between these lineages could not be ascertained in this study. The genus *Melanomphalia* (type *M. nigrescens* M.P. Christ.) is strongly supported within a clade of white-spored omphalinoid fungi, in exclusion from the dark-spored euagarics (Fig. 2.1, clade III).

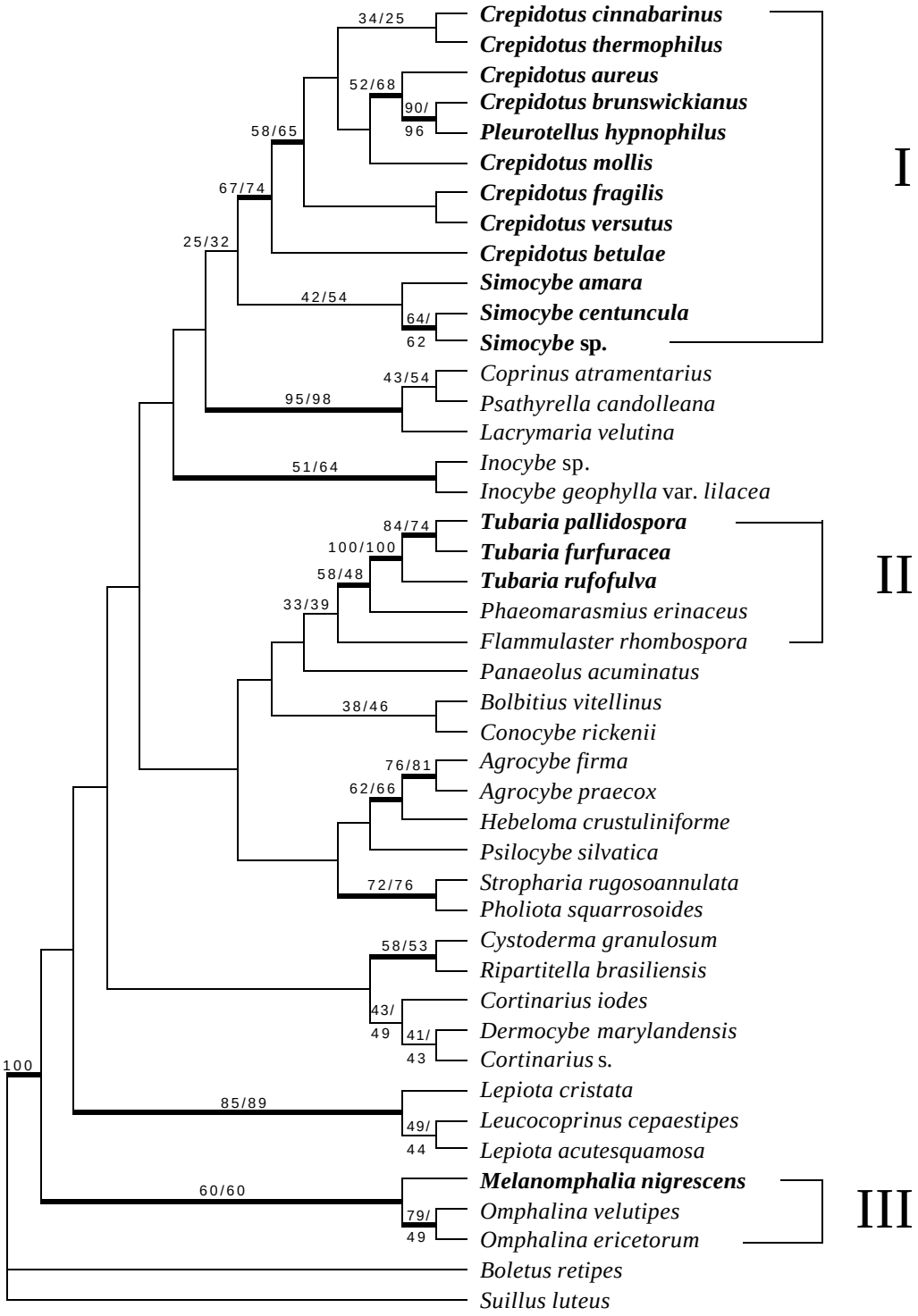

Fig. 2.1. Phylogenetic assignment of the genera of the Crepidotaceae s.l. based on a portion from the 5'-end of the nLSU. Bootstrapping 50% majority-rule consensus tree depicted. Bootstrapping followed by jackknifing values (1000 replicates of full MP analysis each) are indicated at branches supported by more than 25% of replicates. Bold lines indicate branches with bootstrapping support of >50%. Bold type indicates taxa formerly allied within the Crepidotaceae s. Singer. Roman numerals designate clades referred to in the text.

Discussion

In contrast to Singer's (1986) classification, sequence data from the first 1200 base region of the nLSU molecule indicate that the Crepidotaceae s.l. is polyphyletic, nor do these data reflect, *in toto*, any of the alternative classifications proposed for the genera examined. Both the genera *Tubaria* (Fig. 2.1, clade II) and *Melanomphalia* (Fig. 2.1, clade III) have originated independently from the core members of the Crepidotaceae s.s. (Fig. 2.1, clade I). Although strong statistical support via bootstrapping and jackknifing for some of these lineages is low, the conclusions reached by WMP are fully supported by: 1) analysis of trees produced by enforcing monophyletic constraints on the genera of the Crepidotaceae s.l. (Table 2.2); 2) phylogenetic analysis of the entire nLSU molecule and the nuclear DNA encoding the short ribosomal subunit for a cross-section of exemplar taxa from the euagarics including *Crepidotus*, *Simocybe*, and *Tubaria* (unpublished data); and, 3) strong bootstrapping support for each lineage after expansion of the current dataset to include a 300% increase in the number of taxa sampled from within the area of interest (dark-spored euagarics) (Appendix 1). A detailed discussion follows.

Phylogenetic inference—Several methods for evaluating support for phylogenetic clades have been debated and utilized. Decay indices (Bremer 1988) are a valuable method of detecting support for branches, but are impractical for large data sets (Moncalvo et al. 2000). Bootstrapping (Hillis and Bull 1993) and jackknifing (Lanyon 1985) values have gained wide acceptance in systematic literature, but in actuality provide an indication only of the degree of support for a particular clade or node given the specific technique and data matrix analyzed (Hillis and Bull 1993). In many instances, however, such as where rates of character change

are high enough to randomize some characters (i.e., saturation or high homoplasy), bootstrapping values can not be used as measures of accuracy, or the probability that a given tree or clade represents the true phylogeny (Hillis and Bull 1993).

In the present data matrix, >20% of the characters were found to be parsimony-informative. This is well beyond the optimal range of 5-15% (Olmstead and Palmer 1994), and given the broad phylogenetic range sampled and low consistency index, indicates that the variable positions within the matrix are probably saturated by change (Hillis and Huelsenbeck 1992). Phylogenetic resolution in parsimony analyses in such instances is likely to be improved by increasing taxon sampling, as the probability increases that more homoplasic characters will be correctly interpreted as such (Olmstead and Palmer 1994), although the debate between whether increasing sample size (Graybeal 1998, Hillis 1998) or increasing character numbers (Kim 1998) in phylogenetic analyses is still unresolved. However, in this instance, increased sampling of ingroup taxa (Appendix 1), and increased sampling of nucleotide characters from additional molecules (unpublished data) both resulted in WMP trees with high (>70%) bootstrap support for the same lineages of crepidotoid genera as uncovered in the pruned data set presented here.

An additional method for evaluating the probability that the three crepidotoid lineages uncovered by the parsimony analyses were accurate depictions of phylogeny was devised by comparing tree lengths derived from WMP analysis of the data matrix to tree lengths derived from the same method but with monophyletic constraints imposed on various combinations of crepidotoid genera (Table 2.2, Fig. 2.2). In all cases, trees derived when *Tubaria* and or *Melanomphalia* are constrained within the Crepidotaceae (as defined by the type genus

Crepidotus) were significantly (in most cases, $p < 0.001$) worse phylogenetic hypotheses than that of *Crepidotus* (including *Pleurotellus*) and *Simocybe* as the true familial components (Table 2.2). When *Melanomphalia* is constrained within the Crepidotaceae, *Omphalina* becomes part of the dark-spored euagarics, which is in contradiction to all previous classifications and nucleotide-based phylogenetic analyses (Fig. 2.2a, d, and f) . Likewise, when *Tubaria* is constrained within the Crepidotaceae, *Phaeomarasmius* and *Flammulaster* become basal to the family, contrary to previous phylogenetic hypotheses and analyses (Fig. 2.2a, c, and e).

A detailed taxonomic evaluation of each genus is beyond the scope of this paper where such work has been extensively addressed by other authors. Systematics of *Crepidotus* will be discussed in a separate paper. A discussion of the other genera of the Crepidotaceae s.l. within a phylogenetic context is provided below.

Clade I: The Crepidotaceae s.s.—*Crepidotus* and *Simocybe* are sister taxa representing the Crepidotaceae based on allegiance to the type *C. mollis* (Fig. 2.1, Appendix 1). The sister lineage to the Crepidotaceae cannot be resolved with these data. The nomenclatural debate surrounding the group of agarics now conserved and typified under *S. centuncula* (Greuter 1994) has been treated previously [Singer 1973, Redhead 1984 as *Naucoria* (Fr.) Kumm., Reid 1984 as *Naucoria*, Horak and Miller 1997]. Approximately 25 species are now allied in *Simocybe* (Hawksworth et al. 1995). All known members are saprotrophic. *Simocybe* is geographically distributed worldwide (Redhead and Cauchon 1989) and has been monographed from the Neotropics (Singer 1973), lower Pacific (Horak 1979a,

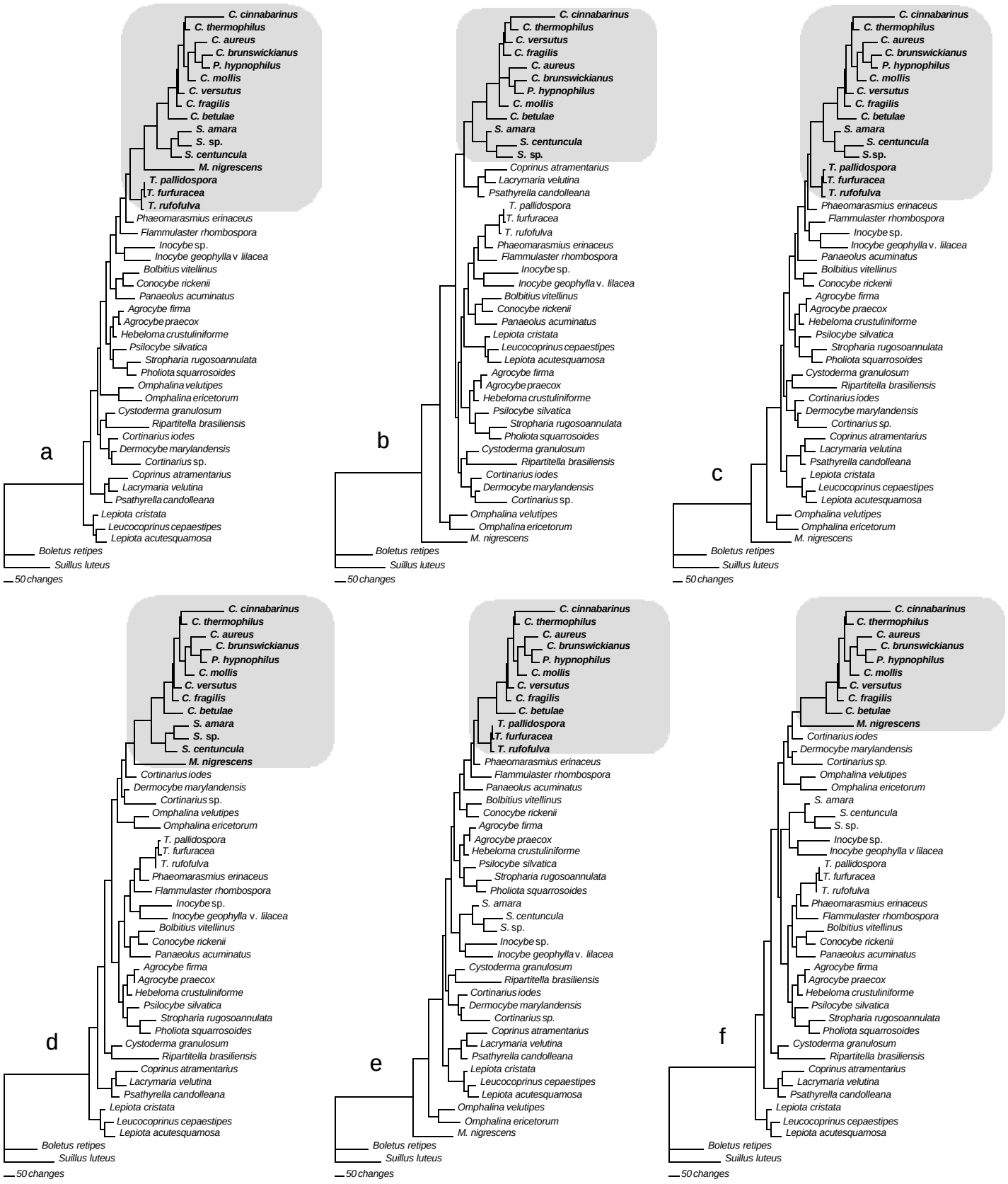

Fig. 2.2. Topological constraint trees generated with weighted parsimony analysis. The shortest tree of twenty WMP trees generated for each constraint set is presented. Taxa constrained to monophyly are indicated by a shaded ellipse; topological constraints of terminal taxa were not enforced within each monophyletically constrained set. Tree values = a. 5144.9 [*Simocybe, Crepidotus* (includes *Pleurotellus*), *Tubaria*, and *Melanomphalia*]; b. 5057.6 (*Crepidotus* and *Simocybe*); c. 5070.9 (*Crepidotus, Simocybe*, and *Tubaria*); d. 5109.5 (*Crepidotus, Simocybe* and *Melanomphalia*); e. 5063.7 (*Crepidotus* and *Tubaria*); f. 5105.8 (*Crepidotus* and *Melanomphalia*).

Table 2.2. Comparison of topological constraint trees generated with WMP analysis with 20 random addition replicates.

Constraint set[1]	Tree length	No. hits	Probability that trees are significantly worse than non-constrained trees[2]		
			Student's	Tukey-Kramer	Dunnett's
CM	5105.8	2	p<.001	p<.001	p<.001
	5108.6	5			
	5109.5	3			
	5111.8	1			
	5115.9	2			
	5116.7	1			
	5117.3	1			
	5118.2	1			
	5119.9	1			
	5123.3	1			
	5124.6	1			
	5124.6	1			
CS	5057.6	1	–	–	–
	5057.8	1			
	5058.9	2			
	5059.8	5			
	5060.0	1			
	5060.4	1			
	5063.2	5			
	5063.5	1			
	5064.3	1			
	5066.8	1			
	5067.1	1			
CST	5070.9	13	p<.001	p<.001	p<.001
	5076.7	6			
	5100.4	1			
CSM	5109.5	13	p<.001	p<.001	p<.001
	5114.8	3			
	5117.8	2			
	5129.5	1			
	5139.0	1			
CSTM	5144.9	10	p<.001	p<.001	p<.001
	5148.5	1			
	5148.8	4			
	5150.2	4			
	5164.4	1			
CT	5063.7	10	p<.001	p<.005	p<.005
	5067.3	2			
	5068.9	2			
	5070.9	1			
	5072.5	3			
	5072.8	1			
	5076.7	1			
None	5057.4	3	n/a	n/a	n/a
	5057.6	5			
	5057.9	2			
	5059.4	2			
	5060.0	1			
	5064.5	3			
	5065.6	1			
	5065.8	1			
	5066.4	1			
	5069.8	1			

[1]Taxa constrained to monophyly: C = *Crepidotus* and *Pleurotellus,* S = *Simocybe,* T = *Tubaria,* M = *Melanomphalia.*

[2]Statistical tests: Student's t test comparison of each pair; Tukey-Kramer comparison of all pairs; Dunnett's comparison with control (None).

1979b, 1980b), and the United Kingdom (Reid 1984, Watling and Gregory 1989 as *Ramicola* Velenovský).

Singer's (1973) transfer of *Simocybe* to the Crepidotaceae is by no means universally accepted. Most early classifications placed *S. centuncula* and its allies within the Cortinariaceae, and this system is still followed in most modern treatments (e.g. Jülich 1981, Bas 1988, Hawksworth et al. 1995, Grgurinovic 1997). Such positions can usually be traced to early nomenclatural difficulties in delimiting *Naucoria* (mycorrhizal, Cortinariaceae) from segregate genera including *Simocybe*.

Alternatively, *Simocybe* has been placed within the Strophariaceae (Kühner 1980) or Bolbitiaceae and synonymized with *Agrocybe* based on striking similarities in pileipellis construction between *Simocybe* and *A. firma* (Pk.) Sing. (Romagnesi 1962). The present study shows that *A. firma*, and *Agrocybe* in general, has no close affinities to the members of *Simocybe* (Fig. 2.1), which is in support of Watling and Largent's (1976) observation that given the fact that differences in construction also occur in the cuticle of the two taxa, the similarities that exist may be due to parallelism and not phylogeny.

Similarities in pileus structure between *Tubaria* and *Simocybe* have also been noted (Watling and Largent 1976, Vellinga 1986). True members of *Simocybe* all posses numerous pileocystidia terminal cells in the epicutis, which can resemble the velum-producing cells found in the pileipellis of tubarii. The *Simocybe* pileipellis is of simple construction, however, as in *Crepidotus* (Watling and Largent 1976), and the pileocystidia originate from the pileipellis as differentiated termini whose function is not analogous to that of the similar cells in *Tubaria*. Species of *Simocybe* are gymnocarpic (Horak 1979a, 1980b).

Pleurotellus was originally typified by Fayod (1889) based on Berkeley's interpretation of Persoon's description of *Agaricus hypnophilus*. Later, Fayod became doubtful as to whether he had correctly determined Persoon's taxon, and renamed his type collection *Pleurotellus graminicola* (Donk 1962, Nordstein 1990). Only two species are commonly accepted in the genus (Hawksworth et al. 1995), the identity of which are discussed in Nordstein (1990), yet the generic concept contains heterogeneous elements (Singer 1962, Watling 1988). While Orton (1960) interprets *Pleurotellus* as white-spored relatives of *Pleurotus* (Fr.) Kumm., of no affinity to *Crepidotus*, most consider the two closely related (e.g., Singer 1961, Hesler and Smith 1965, Watling and Gregory 1989, Nordstein 1990, Senn-Irlet 1995), although opinions vary as to whether *Pleurotellus* differs from other crepidoti on the generic level.

Proponents of segregating *Pleurotellus* and *Crepidotus* do so because of two morphological distinctions: the absence of clamp connections and the very pale yellow to sub-hyaline spore print color in *Pleurotellus* (e.g., Pilát 1948, Watling and Gregory 1989, Singer 1951a, 1962, 1986, Pegler and Young 1972). Clamp connections, while useful taxonomically for the circumscription of species, are not reliable indicators of phylogeny in many groups (Watling and Largent 1976), and *Crepidotus* contains several lineages that exhibit clamps and others that do not (Aime Chap. 3), so the character alone is not unique to *Pleurotellus*. Therefore, only the single character state of loss of spore pigmentation distinguishes the former from the latter. *Pleurotellus*, as represented here by the type, *P. hypnophilus*, is a component of *Crepidotus* (Fig. 2.1, Appendix 1). Since the use of single characters for circumscribing genera can introduce artificiality into classifications (Smith 1967), and *P. hypnophilus* is in all

other characters consistent with *Crepidotus*, *Pleurotellus* should no longer be considered a valid genus.

The taxon represented by *P. hypnophilus* has been reported from all over the world under a large number of synonyms, and the correct specific epithet for it has been the subject of much discussion (Hesler and Smith 1965, Nordstein 1990, Senn-Irlet 1995, Bandala et al. 1999). The first description that can be unambiguously applied to this taxon is that of Peck (1886) as *Agaricus herbarum*. However, the older name of *Agaricus hypnophilus* Pers.:Berk. takes precedent, if, has been noted by Singer (1961), Berkeley correctly interpreted Persoon's taxon, for which a type no longer exists. Berkeley's type does exist, and was the basis for Fayod's establishment of *Pleurotellus*, and several studies show it to be conspecific with the taxon analyzed in this paper (Singer 1961, Horak 1968, Donk 1962) therefore, the name *C. hypnophilus* (Pers.:Berk.) Nordstein should take precedence over *C. herbarum*.

Nonetheless, Senn-Irlet (1995) has investigated the possibility that an older Friesian name, *A. epibryus*, exists for this taxon. Since no type for *A. epibryus* exists, and the original description (Fries 1821) could be applied to many species of *Crepidotus* or even *Pleurotus* and allied genera, several differing concepts have been applied to this name in the past (Pilát 1950, Singer 1951b, Senn-Irlet 1995). Senn-Irlet's interpretation has been based on that of Quélet (1888), and a Neotype for *C. epibryus* (Fr.) Quél. established by her (Senn-Irlet 1995). Although the argument is convincing, a few problems exist with this interpretation. 1) Quélet's (1888) description of *C. epibryus* is as vague as Fries', and could equally fit other taxa within and even outside the confines of *Crepidotus*. 2) Quélet actually considers *A.*

epibryus as a *Crepidotus* in works as early as 1872 wherein the spores are described in more detail as being "pruniform", or plum-shaped, which is not the case with the taxon under question here (Quélet 1872). And, 3) The only substrate given for both Fries' (1821, 1836-1838) and Quélet's (1872, 1888) taxon is moss, whereas the taxon under question is almost exclusively confined to herbaceous and grassy substrates, occasionally on wood (Peck 1885, Watling & Gregory 1989, Nordstein 1990, Bandala et al. 1999), hence the name *C. herbarum* and Fayod's later change to *P. graminicola*.

Despite these concerns, the typification of the older Friesian name for the taxon that is most commonly known as *C. herbarum* or *P. hypnophilus* has already been accepted (Bandala et al. 1999), and the name *C. epibryus* (Fr.) Quél. sensu Senn-Irlet should be applied for it. This species has been previously described and illustrated by Senn-Irlet (1995), Bandala et al. (1999), Horak (1968, as *P. graminicola*), Nordstein (1990, as *C. hypnophilus*), and Hesler and Smith (1965, as *C. herbarum*).

Clade II:* Tubaria *and allies—Aime (1999) stated that the phylogenetic placement of *Tubaria* was unresolved. The data presented in the larger study here show this genus to be most closely allied with *Phaeomarasmius* and *Flammulaster*, and unrelated to the true members of the Crepidotaceae (Fig. 2.1, Appendix 1). Basidiospore germination and vegetative morphology of *T. furfuraceae* were studied by Ingold (1983) and shown to be considerably different from that of *Crepidotus* species (Aime and Miller Chap. 4), lending additional support to a proposed phylogenetic hiatus between the two genera. The sister group to the tubarioid clade cannot be determined within the confines of the present study. *Tubaria* contains approximately 15 saprotrophic species occurring worldwide in temperate climes (Hawksworth

et al. 1995), and only a few European species have been monographed (Lange 1938, Romagnesi 1940, 1943).

Singer (1951a) transferred *Tubaria* from the Cortinariaceae to the Crepidotaceae and the rationale behind this decision was discussed in Aime et al. (Chap. 1). Other authors still ally this genus with the Cortinariaceae s.l. (Romagnesi 1940, Vellinga 1986, Grgurinovic 1997) or place it within the Strophariaceae s.l. (Moser 1978). *Tubaria* has the distinction of being the only genus within Singer's Crepidotaceae in which some members undergo hemiangiocarpic development. Morphological delimitation and generic concepts between and within *Flammulaster*, *Phaeomarasmius*, and *Tubaria* have been repeatedly evaluated (Romagnesi 1940, Watling 1967, Kühner 1969, Harmaja 1978, Moser 1978, Horak 1980a, Vellinga 1986). The present study uncovers three lineages (Fig. 2.1) corresponding to three separate genera that most closely resemble the classification of Moser (1978).

Numerous characters and character suites have been hypothetical indicators of phylogeny in agarics. The alliance of *Tubaria, Flammulaster*, and *Phaeomarasmius* is best understood through the work of Watling and Largent (1976), which emphasizes pileipellis anatomy in drawing conclusions about higher-level relationships in the agarics. The members of all three genera possess an unusual pileipellis that contains inflated hyphae occurring in chains, usually encrusted with brown pigment (Watling and Largent 1976, Harmaja 1978, Vellinga 1986). These chains of hyphae appear to be the same elements that give rise to the partial veil present in these species, yet appear to originate from, and be an integral part of, the cutis rather than the stipe. The resulting partial veil is usually fugacious, and persistent in one species

(Watling 1967). All taxa tested so far are KOH+ on the pileus (Vellinga 1986, Watling 1967), which appears to be a secondary unifying character.

Clade III: **Melanomphalia *and its allies***—Perhaps the most interesting result of this study is the alliance of *Melanomphalia* with a subset of the genus *Omphalina* Quél. Generic affinities for *Melanomphalia* have never been certain (Lange 1940, Montag 1996), with the type itself seemingly holding a rather isolated position within the genus as expanded by Singer (Watling 1988). The genus was hypothetically allied with the Gomphidiaceae by Christiansen (1936) and early authors (Lange 1940) and with the Cortinariaceae (Singer 1955) until its transfer to the Crepidotaceae (Singer 1971) based on similarities between *M. thermophila* and some *Crepidoti* as previously discussed (Aime et al. Chap. 1).

Similarities between the type, *M. nigrescens* and some *Omphalina* have previously been noted (Montag 1996). Christiansen (1936) also, as the generic name implies, recognized similarities between *Omphalina* (as *Omphalia*, a synonym for *Omphalina*) and his genus, although the differences in spore pigmentation and ornamentation have always been viewed as too striking to consider other similarities as anything other than evidence of parallel evolution between these genera.

Taxonomically, the omphalinoid fungi have also been a difficult and heterogeneous group to delimit (Bigelow 1970, Redhead and Weresub 1978, Norvell et al. 1994), and recent phylogenetic analysis have uncovered several distinct lineages of polyphyletic origin (Lutzoni 1997, Moncalvo et al. 2000). What is extremely interesting is that *M. nigrescens* within the confines of this (Fig. 2.1) and more detailed analyses (Appendix 1) shows a sister relationship with a clade previously identified as the true *Omphalina*, which includes lichenized fungi. This

merits closer scrutiny of the phylogeny and ecology of *M. nigrescens*, which has only been reported on soil (Christiansen 1936, Montag 1996) or adventitiously on limestone or in association with herbaceous VAM plants (Watling 1988).

This association is also extraordinary in that it suggests another independent origin of dark spore pigmentation in the euagarics. Interestingly, loss of spore wall pigmentation has now been shown through molecular studies to have occurred in several agaric cohorts, such as has occurred in the Agaricaceae (Johnson and Vilgalys 1998), the Crepidotaceae with *C. epibryus* sensu Senn-Irlet, and in the possible derivation of all pale-spored lineages from dark-spored ancestors (Moncalvo et al. 2000), but the acquisition of pigmented spores from an unpigmented ancestor appears to be a very rare phenomenon.

Prior work has shown that at least one species previously placed within *Melanomphalia* is, in fact, a *Crepidotus* (Aime et al. Chap. 1), and preliminary study of other taxa currently placed in the genus also suggests that most are more naturally allied within *Crepidotus* or other genera (unpublished data). At present, *Melanomphalia* (Fig. 2.1) appears to be a monotypic genus, sister to *Omphalina*, and unrelated to the true members of the Crepidotaceae. The taxonomic disposition of other taxa currently allied in *Melanomphalia* and detailed phylogeny of the type will be the focus of an upcoming paper.

Redefining the Crepidotaceae—Initially, the macroscopic character of habit delineated *Simocybe* from *Crepidotus*. Transfers of some pleurotoid taxa from *Crepidotus* to *Simocybe* based on microanatomy (Singer 1973, Watling 1988, Horak and Miller 1997), and of some stipitate taxa to *Crepidotus* (Aime et al. Chap. 1) have blurred the macroscopic distinctions between the two, but result in a more natural classification based on microscopic

characters. Chiefly *Simocybe* can be diagnosed from *Crepidotus* by: 1) basidiospores that are always smooth and differ from smooth-spored *Crepidoti* in that the adaxial side is applanate to depressed (Appendix 2); and, 2) abundant pileo-, caulo-, and cheilocystidia (the latter two most typically subcapitate), lending a pruinose appearance to these structures macroscopically. Additionally, many *Simocybe* members have an olivaceous tint to the pileus, lacking in the *Crepidoti.*

The Crepidotaceae s.s. are differentiated from all other euagaric lineages by the following suite of characters: saprotrophic on woody or herbaceous matter; gymnocarpic; spore prints within the pale yellow to brown range, not pink, purple-brown, orange, or black; simple cuticle, although differentiated termini in the form of pileocystidia may be present; cheilocystidia always present; pleurocystidia absent in most taxa and never thick-walled or originating from the lamellar trama; basidiospores entire, with neither a true germ pore nor plage, smooth or ornamented but never angular or reticulate.

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Part II

Crepidotus (Fr.) Staude

3

Generic concepts in *Crepidotus* (Fr.) Staude based upon large subunit rDNA sequences.

Introduction

In 1821 Fries established Tribus *Crepidotus* within genus *Agaricus*. The 14 agarics (i.e., gilled mushrooms) in this assemblage were described as variable in form with a fibrillose veil, eccentric or lateral stipe, and rust, pale clay, or reddish-tinged spores. Recognizing the heterogeneity in this tribe, Fries (1836-1838) later refined his concept to include only species that lacked a veil, were primarily resupinate in form (but also laterally or eccentrically stipitate), and had rust, brown-rust, or brownish spore deposits. Thirty-six years after its inception, Staude (1857) elevated Tribus *Crepidotus* to generic rank[1], with *Agaricus mollis* (Schaeff.) Fr. as the monotypic representative.

Early treatments of the genus were based almost exclusively on macroscopic features, and considered between six and forty-six taxa in *Crepidotus* (Peck 1886, Quélet 1888, Murrill 1913, 1917, Kauffman 1918, Imai 1938, Lange 1938). At this point, the concept of *Crepidotus* was evolving to include only pleurotoid (i.e., sessile, or with greatly reduced lateral or eccentric stipe) agarics, usually with non-hyaline spore deposits.

[1] There is some doubt as to whether Staude intended to elevate many Friesian tribes to genera, or whether he simply misunderstood the taxonomic difference between the two ranks (Singer 1955). This has caused historical debate between accepting Staude as generic authority (e.g., Donk 1962), or designating as authority those authors who made their intentions clear in later-dated works. Hence, there is Singer's insistence on *Crepidotus* Kummer (1871), and not *Crepidotus* (Fr.) Staude (1857).

Singer (1947) was the first to monograph the genus, providing a detailed discussion of microscopic features, and an infrageneric classification. Singer's work is also important for delimiting *Crepidotus* primarily by microscopic anatomy and de-emphasizing macroscopic characters. In doing so, Singer expanded the generic concept to include centrally stipitate as well as pleurotoid forms (by transferring *Pleuropus nyssicola* Murr., a centrally stipitate fungus, to *Crepidotus*), while restricting the genus to include only brown-spored species that lack a germ pore and have a filamentous cuticle. After excluding 16 pleurotoid species using the criteria described above, a more homogenous (although still phenotypically diverse) group of 30 species remained in Singer's *Crepidotus*.

Originally unaware of Singer's work, Pilát (1948) provided a monograph of the European species of *Crepidotus*. Like Singer, Pilát used microscopic analyses in both redefining the genus and in proposing subgenera. Pilát's work however, relies more heavily on pleurotoid form than microscopic anatomy in drawing generic boundaries. In 1950, Pilát, expanding on both his and Singer's monographs, provided a key to a total of 75 species. Although Pilát's works remain extremely valuable for the extensive type examinations and compilation of synonyms, most researchers consider his generic concepts too broad. Many of his *Crepidotus* taxa have been transferred to other genera such as *Paxillus* Fr., *Pyrrhoglossum* Sing., *Melanotus* Pat. and *Simocybe* Karst.

To date, at least 425 names have been applied in *Crepidotus*, of which approximately 150 (Singer 1986, Hawksworth et al. 1995) are widely accepted, and despite refinement of the generic concept, the members display an unusual amount of phenotypic diversity. *Tremellopsis* Pat. (type, *T. antillarum* Pat.) was introduced for those *Crepidoti* with smooth spores and

clamp connections, and *Dochmiopus* Pat. (type, *Agaricus variabilis* Fr.) (*Tremellopsis*) for those with elongated, ornamented spores. However, both are considered congeneric with *Crepidotus* by modern researchers (Singer 1947, Horak 1968, Hawksworth et al. 1995).

While Singer's (1947, 1951, 1986) concept of the genus has remained virtually unchanged, his infrageneric classification was challenged by Hesler and Smith (1965), who differed from Singer in the morphological characters emphasized as systematically important (Table 3.1). Singer recognized a few morphologically-coherent sections from Hesler and Smith's at the level of stirps that fit within his pre-existing classification. However, he did not impart "any taxonomic-nomenclatorial status" to them (Singer 1973). The two systems are, however, on the whole incompatible, and modern researchers are divided in their adherence to either Singer (e.g., Pegler 1986) or Hesler and Smith (e.g., Pegler and Young 1972) or various combinations of the two (e.g., Nordstein 1990). Others have devised alternate subclassifications ranging from a sound grouping of morphologically similar taxa into stirps (Watling and Gregory 1989) to Senn-Irlet's (1995) phenetic analysis based on 16 characters and 15 taxa.

In addition to the lack of an agreed upon (and ideally phylogenetically-based) system for the infrageneric classification of *Crepidotus*, species concepts have floundered. In part this problem can be traced to the loss of many important early holotypes, the paucity of microscopic data recorded in descriptions prior to the late 1940s, and to the fact that concepts for individual taxa have quite often evolved separately on different continents. Compounding this difficulty, no data exist regarding the natural phenotypic range or plasticity within single species of

Table 3.1. Infrageneric Classifications of *Crepidotus* (Fr.) Staude

Hesler and Smith (1965)	**Defining characters**	**Notes**
I. Subgenus *Crepidotus*	- clamp connections	
1. Section *Cinnabarini* Hes. & Sm.	+ dissolved red pigments	monotypic
2. Section *Tubariopsis* Hes. & Sm.	fusoid to subfusoid spores	monotypic; synonym for *C. herbarum* (Sect. *Versuti*)
3. Section *Stratosi* Hes. & Sm.	pileus structure a duplex	synonym for C. *versutus* (Sect. *Versuti*)
4. Section *Parvuli* Hes. & Sm.	spores ornamented and globose	synonym for *C. applanatus* (Sect. *Sphaerula*)
5. Section *Crepidotus*	+ gelatinization	
6. Section *Versuti* Hes. & Sm.	spores ellipsoid	
II. Subgenus *Sphaerula* Hes. & Sm.	+ clamp connections, spores globose	
7. Section *Nyssicolae* (Sing.) Hes. & Sm.	fruiting body stipitate	
8. Section *Sphaerula*	fruiting body pleurotoid	
a. Subsection *Sphaeruli*		
b. Subsection *Colorantes* Hes. & Sm.		
c. Subsection *Fulvifibrillosi* Hes. & Sm.		
III. Subgenus *Dochmiopus* (Pat.) Pilát	+ clamp connections, spores elongated	
9. Section *Cystidiosi* Hes. & Sm.	+ pleurocystidia	
10. Section *Fulvidi* Hes. & Sm.	pileus and/or fibrils colored	
11. Section *Phaseoli* Hes. & Sm.	spores phaseoliform	monotypic
12. Section *Fusisporae* Hes. & Sm.	spores fusoid	monotypic
13. Section *Betulae* Hes. & Sm.	spores ellipsoid, smooth	
14. Section *Dochmiopus* (Pat.) Pilát	spores ornamented, small	
15. Section *Crepidotellae* Hes. & Sm.	spores ornamented, large	
Singer (1986)	**Defining characters**	
I. Section *Echinospori* Pilát	ornamented spores	
1. Subsection *Porpophorini* Sing.	+ clamp connections	
2. Subsection *Aporpini* Sing.	– clamp connections	
II. Section *Crepidotus*	smooth spores	
3. Subsection *Fibulatini* Sing.	+ clamp connections	
4. Subsection *Crepidotus* (= *Defibulatini* Sing. 1947)	– clamp connections	

Crepidotus, and many species and generic subdivisions are circumscribed on the basis of single characters. While application of mating studies has been a reliable tool for addressing morphological variation within species for other agarics (Peterson and Hughes 1999), the unique nature of basidiospore dormancy and germination in *Crepidotus* (Aime and Miller Chap. 4) have limited the practicality of this approach on a large-scale basis, and no such studies have been reported for these fungi to date.

World distributional information is patchy and entirely lacking in many areas. The *Crepidotus* flora has been relatively well documented from the Northern temperate (Imai 1938, Ito 1959, Orton 1960, Hansen and Knudson 1992, Watling and Gregory 1989, Ortega and Buendia 1989, Nordstein 1990, Senn-Irlet 1991, Stangl et al. 1991, Senn-Irlet 1995), and South American (Singer and Diglio 1951, Horak 1964, Singer and Moser 1965, Senn-Irlet and De Meijer 1998) regions, with few new species described from these in the last 25 years (Hesler 1975, Hausknecht and Krisai 1988, Pöder and Ferrari 1994). However, many new *Crepidotus* species, and agarics in general, are being discovered from previously underexplored regions such as the tropics and the Pacific rim. Singer (1973) provided a monograph to *Crepidotus* in the Neotropics, describing 62 species, of which 27 taxa were new to science. Several of these new taxa possess basidiospore or cheilocystidial forms that find no counterpart in the temperate flora. Horak (1977) described four new species of *Crepidotus* from the Southern hemisphere that cannot confidently be assigned to any previously described section. As biologists strive to record the biodiversity of vanishing ecosystems, the need for a phylogenetically-based classification system that can accommodate exotic as well as temperate

taxa becomes even more obvious and necessary, as does the need for establishing sound species concepts, in order to accurately record and understand this diversity.

Despite the large number of described species, very little is known of the biology, phylogeny, or evolution of *Crepidotus* species, and the relationships between morphology and phylogeny, at the generic, infrageneric, and species levels, remain conjectural. All known species of *Crepidotus* are secondary decomposers of plant matter. Most species are saprotrophic on wood; a few [such as *C. epibryus* s. Senn-Irlet and *C. versutus* (Pk.) Sacc.] are also decomposers of herbaceous litter. There are no known parasites in the genus, although one species has been reported to form stephanocysts in culture (Senn-Irlet and Scheidegger 1994), and one study suggests that these structures, in *Hyphoderma* (Basidiomycetes, Stereales) species, are formed to attack nematodes for a nitrogen source (Liou and Tzean 1992). Interestingly, some Japanese studies showed that a water soluble polysaccharide, isolated from a *Crepidotus* culture, has antitumor properties (Nakayoshi 1967, 1968; Nakayoshi et al. 1968).

Crepidotus species are small agarics, many reaching less than one or two millimeters in diameter, although a few species have individuals which may reach three to five centimeters in length. All species are believed to develop gymnocarpically (Aime Chap. 2). In many species, primordia are centrally stipitate, but at an early stage of development the stipe "aborts" and the resulting mature sporophyte becomes pleurotoid in form. Cultures are slow-growing in comparison to other saprotrophic agarics despite the experimental manipulation of numerous abiotic factors (Senn-Irlet 1994 and unpublished data). Since nitrogen is often a limiting resource for wood decomposers, it is possible that these fungi meet this requirement by

parasitizing other microorganisms in the natural environment, such as nematodes, or possibly other fungi (Roy Watling, pers. comm.).

Various taxonomic treatments have allied *Crepidotus* with, for example, the Strophariaceae Singer and A.H. Smith (Kühner 1980) or the Cortinariaceae Heim ex Pouzar (Bas 1988), but molecular analysis, based on an approximately 1000 base pair portion from the 5'-end of the nuclear DNA encoding the nuclear large ribosomal subunit (nLSU), supports the recognition of the Crepidotaceae (Imai) Singer as a separate family within the euagarics with *Simocybe* Karsten as the sister genus to *Crepidotus* (Aime Chap. 2). This chapter will examine infrageneric phylogeny in *Crepidotus* through nLSU sequence analysis, and examine species morphology within this framework for phenotypic indicators of phylogeny. Subsequent chapters will explore aspects of *Crepidotus* biology and morphology, and species concepts. Specific questions addressed in the present study are: 1) Is *Crepidotus* a monophyletic genus? 2) What are the monophyletic groups within *Crepidotus*, and do they correspond to previously proposed subgenera or sections? 3) Are the phenotypic characters traditionally applied in *Crepidotus* infrageneric classification good indicators of phylogeny? A final goal of this study is to uncover a monophyletic section of *Crepidotus* for which species concepts can be addressed.

Materials and Methods

Taxonomic sampling—*Crepidotus* collections selected for sequencing analysis covered a broad geographic range including samples from North America, Central America, the Caribbean, Asia, Africa, Europe, and Australia. In addition, to approximate the gamut of phenotypic expression, representatives from all four subsections proposed by Singer (1986)

Table 3.2. Taxa selected for analysis. Taxa listed following the classification of Singer (1986).

Taxon	H&S[1]	Collection No.	Locality	GenBank No.
Crepidotus section *Echinospori* subsection *Porpophorini*				
C. cf. *amygdalosporus* Kühner	15	MCA 258	Montgomery Co., VA	AF205675[2]
C. appalachianensis Hesler & Smith	8c	OKM 27048	Wintergreen, VA	AF205672[2]
C. applanatus v. *applanatus* s. Joss.	8a	MCA 170	Montgomery Co., VA	AF205694[2]
C. applanatus v. *globigera* (Berk.) Sacc.	8a	MCA 188	Giles Co., VA	AF205673[2]
C. aureus Horak	8b	OKM 27300	Bisley Exp. Site, Puerto Rico	AF205685[2]
C. brunnescens Hesler & Smith	8a	MCA 864	Giles Co., VA	AF367936
C. brunswickianus (Speg.) Sacc.	15	MCA 580	Kyoto-ken, Japan	AF367932
C. cesatii (Rab.) Sacc.	15	OKM 26976	Baker Co., OR	AF205681[2]
C. croceitinctus Pk.	14	IBNr 1997/0947[3]	Kamtchatka, Russia	AF367937
C. crocophyllus(Berk.) Sacc.	8c	OKM 25806	Giles Co., VA	AF367938
C. crocophyllus	8c	SJ1	Bratislava, Slovakia	AF367939
C. distortus Hesler & Smith	8c	MCA 386	Durham, NC	AF205671[2]
C. fragilis Joss.	9	MCA 904	Giles Co., VA	AF367931
C. inhonestus complex	15	MCA 163	Giles Co., VA	AF205705
C. inhonestus complex	15	MCA 638	Nagano-ken, Japan	AF205704
C. cf. *lanuginosus* Hesler and Smith	15	OKM 27331	Valley Co., ID	AF367940
C. lundellii Pilát	13	IBNr 1997/0946[3]	Kamtchatka, Russia	AF367941
C. lundellii	13	SJ6[4]	Bratislava, Slovakia	AF367942
C. malachius (Berk. & Curt.) Sacc.	8a	MCA719	Nagano-ken, Japan	AF367943
C. martini Sing.	15	MCA 640	Nagano-ken, Japan	AF367944
C. nephrodes (Berk. & Curt.) Sacc.	8c	MCA 189	Giles Co., VA	AF205670[2]
C. nyssicola (Murr.) Sing.	7	TJB 8699[5]	Falmouth, ME	AF205690[2]
C. sinuosus Hesler & Smith	8b	OKM 26290	MT	AF367945
C. sphaerosporus (Pat.) Lange	15	OKM 27013	Copper Mountain, CO	AF205682[2]
C. submollis Murr.	15	MCA 920	Giles Co., VA	AF367946
C. cf. *subsphaerosporus* (Lange) Kühn. & Rom.	14	OKM 24649	West Australia	AF367947
C. subverrucisporus Pilát	15	MCA 774	Durham, NC	AF367948
C. thermophilus (Sing.) Aime, Baroni & Miller	10	OKM 27270	near Sabana, Puerto Rico	AF205669[2]
C. cf. *variabilis* (Fr.) Kumm.	14	MCA 633	Ibaraki-ken, Japan	AF367949
C. sp.	8b	MCA 442	Bisley Exp. Site, Puerto Rico	AF367950
C. sp.	8b	MCA 499	Rio Abajo, Puerto Rico	AF367951
C. sp.	8a	MCA 679	Tottori-ken, Japan	AF367952
C. sp.	8b	MCA 717	Nagano-ken, Japan	AF367953
C. sp.	8a	MCA 941	Shiga-ken, Japan	AF367954
C. sp.	8a	OKM 27503	Portland Parish, Jamaica	AF367955
C. sp.	8c	OKM 27540	MO	AF367956
C. sp.	10	Overbo 3075[6]	Costa Rica	AF367957
C. sp.	15	OKM 26899	Chantaburi Prov., Thailand	AF205684[2]
Crepidotus section *Echinospori* subsection *Aporpini*				
C. cinnabarinus Pk.	1	MCA 387[7]	Oneida Co., NY	AF205686[2]
C. epibryus s. Senn-Irlet	6	IBNr 1997/0948[3]	Kamtchatka, Russia	AF367934
C. versutus (Pk.) Sacc.	6	MCA 250	Montgomery Co., VA	AF205695[2]
C. versutus	6	MCA 381	Macon Co., NC	AF205683[2]
C. versutus	6	IBNr 1997/0962[3]	Kamtchatka, Russia	AF367958

Table 3.2. Continued.

Taxon	H&S[1]	Collection No.	Locality	GenBank No.
Crepidotus section *Crepidotus* subsection *Fibulatini*				
C. cf. *albissimus* Murr.	13	MCA 697	Nagano-ken, Japan	AF367959
C. antillarum (Pat. apud Duss) Sing.	13	OKM 26827	La Vega Prov., Dominican Rep.	
C. betulae Murr.	13	MCA 384	Durham, NC	AF205679[2]
C. occidentalis Hesler & Smith	13	OKM 26740	Pierce Co., WA	AF205678[2]
C. podocarpi Sing.	13	OKM 27303	Bisley Exp. Site, Puerto Rico	AF205696[2]
Crepidotus section *Crepidotus* subsection *Crepidotus*				
C. alabamensis Murr.	5	MCA 778	Durham, NC	AF367960
C. fraxinicola Murr.	5	OKM 26739	Pierce Co., WA	AF205676[2]
C. melleus (Berk. & Br.) Petch	5	MCA 672	Tottori-ken, Japan	AF205702
C. mollis (Fr.) Staude	5	OKM 26279	MT	AF205677[2]
C. subaffinis Pilát	6	MCA 604	Hokkaido, Japan	AF205703
C. uber (Berk. & Curt.) Sacc.	5	MCA 1403	Madagascar	AF367961
Simocybe P. Karsten				
S. amara (Murr.) Sing.		MCA 682	Tottori-ken, Japan	AF205708
S. americana Horak & Miller		VTMH 3760	Alberta, Canada	AF205709
S. centuncula (Fr.) Karst.		MCA 393	Asilomar, CA	AF205707
S. cf. *laevigata* (Favre) Orton		MCA 294	Giles Co., VA	AF205698[2]
S. cf. *laevigata*		MCA 750	Giles Co., VA	AF205706
S. serrulata (Murr.) Sing.		OKM 27046	Giles Co., VA	AF205688[2]
S. sp.		MCA 424	Floyd Co., VA	AF205687[2]
Outgroup				
Pleuroflammula flammea (Murr.) Sing.		MCA 339	Giles Co., VA	AF367962
Pleuroflammula sp.		OKM 24609	Perth, Australia	AF208533
Pleuroflammula sp.		OKM 27686	Santiago Prov., Dominican Rep.	AF367963

[1] Hesler and Smith (1965) infrageneric classification of *Crepidotus*; number corresponds to section no. given in Table 3.1.
[2]Aime 1999.
[3]Voucher collections housed at Herbarium Universität Innsbruck (IB).
[4]Voucher collection housed at Herbarium Instituti Botanici Universitatis Comeniane, Bratislava (SLO).
[5]Voucher collection housed at SUNY Cortland Herbarium (CORT).
[6]Collection loaned by Clark Overbo (CSU).
[7]Split collection, Alan Bessette #10645.

and all three subgenera along with 12 of the 17 sections and subsections, proposed by Hesler and Smith (1965) were included. Seven *Simocybe* sequences, representing both stipitate and astipitate (*S. amara*, *S. americana*) taxa were included to test the monophyly of *Crepidotus*. For rooting purposes, outgroup taxa were chosen from the genus *Pleuroflammula*, which is one of several equally likely sister groups to the Crepidotaceae identified in prior studies. Origins of the 64 taxa analyzed are listed in Table 3.2, along with classification according to Singer (1986) and Hesler and Smith (1965), and GenBank accession number. Voucher materials for all sequences are housed in the Virginia Tech Mycological Herbarium (VPI) unless otherwise noted.

DNA extraction, amplification, sequencing, and phylogenetic analysis— Methods for DNA extraction follow the CTAB (hexadecyltrimethyl-ammonium bromide) extraction buffer protocol in Aime (1999). Amplification and sequencing protocols for the target region, the first three divergent domains of the 5'-end of the nLSU gene, were given in Aime (Chap. 2). Sequences were manually aligned in PAUP* 4.0b8 (Swofford 2001) and analyzed with weighted maximum parsimony (WMP) as described in Aime (Chap. 2). Decay values (Bremer 1988) were calculated with AutoDecay 4.0.1 (Eriksson 1998) in PAUP* 4.0b8 (Swofford 2001).

Results

In all, the data matrix assembled consisted of 64 taxa aligned across 1187 positions; 187 positions were excluded from analysis due to ambiguities in alignment, and by trimming of the extreme 5' and 3' ends; 161 of the remaining characters were parsimony-informative. A

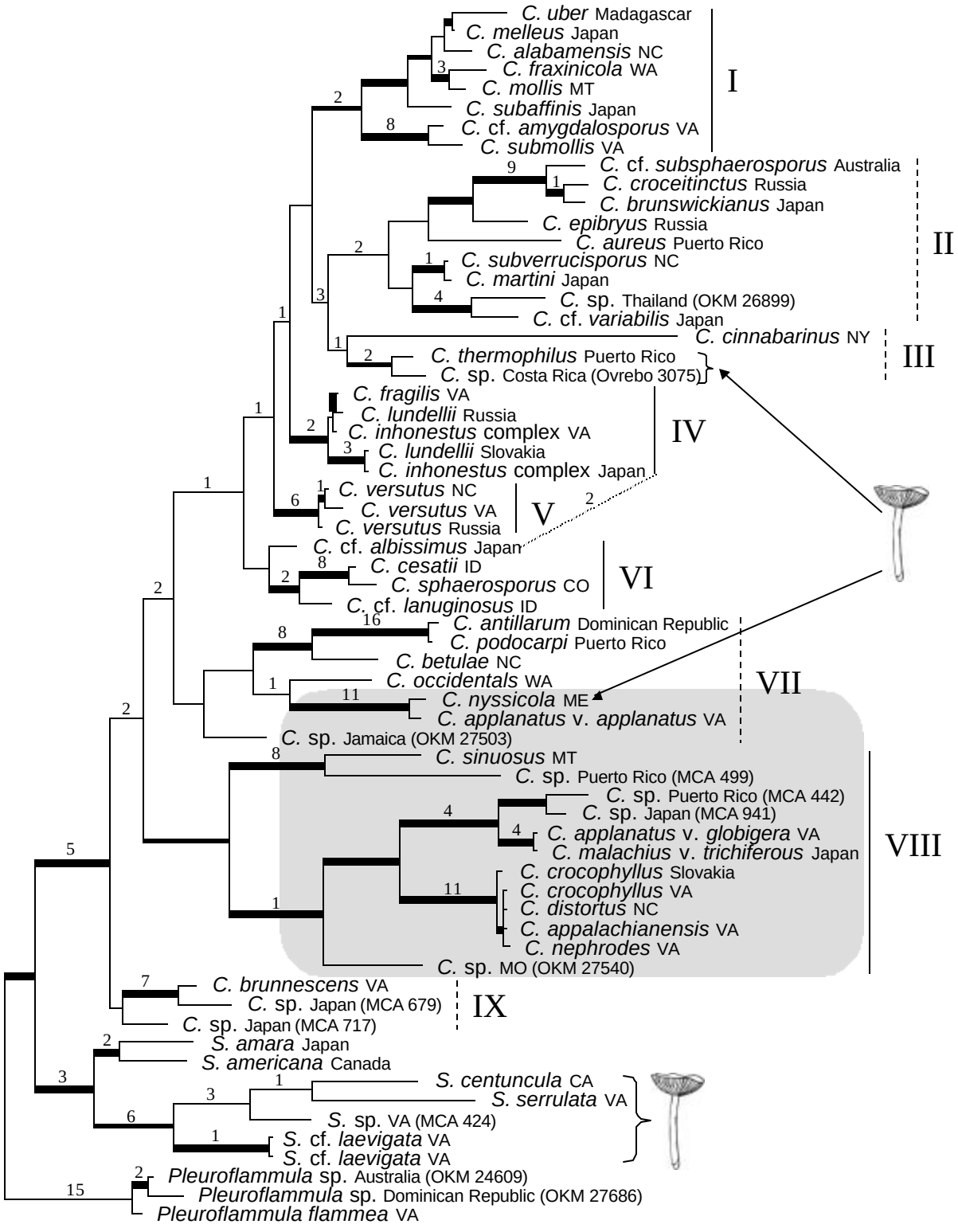

Fig. 3.1. Phylogeny of *Crepidotus* using nLSU rDNA sequences. Single most parsimonious tree (WMP) depicted as a phylogram showing relative branch lengths (tree length = 3228.1, CI = 0.347, RI = 0.688). A thickened line (▬) indicates branch with bootstrapping and jackknifing support of 40-50%; blackened line (▬) indicates branch with bootstrapping and jackknifing support >50%. Decay indices are indicated above supported branch. A fine dotted line indicates a node supported by decay analysis not present in WMP or bootstrap and jackknife analyses. Clade numbers indicated in roman numerals; clades without support indicated by dashed line. The gray shaded ellipse indicates taxa in Stirps Sphaerula. Mushroom illustration indicates stipitate taxa; all others are pleurotoid.

single shortest WMP tree of length 3228.6, CI = 0.347, RI = 0.688 was found. Figure 3.1 depicts the WMP tree generated with these data; the tree is presented as a phylogram to show the number of character state changes per branch. Branches with low (40-50%) and high (>50%) bootstrap and jackknife support are noted. Nodes supported by decay values are also indicated. A few nodes supported by decay indices are unsupported by bootstrapping and jackknifing, for example, that joining clade II and clade III. Conversely, some nodes with strong bootstrap and jackknife support are unsupported by decay indices, for example, the node joining *C. subaffinis* with the *C. mollis* clade (clade I). A single node joining *C. albissimus* (clade VI) with clade IV is revealed by decay analyses that is not reflected in the most parsimonious tree.

Two distinct nodes can be defined supporting the monophyly of the sister-genera *Simocybe* and *Crepidotus*. Two sister-lineages within *Simocybe* are supported, one consisting only of pleurotoid taxa [*S. amara* (Murr.) Sing., and *S. americana* Horak and Miller], the other of stipitate taxa. Within *Crepidotus*, five supported groups (clades I, IV, V, VI, and VIII) appear monophyletic. Four other assemblages without support (clades II, III, VII, and IX) may not be monophyletic. There is limited resolution of relationships within *Crepidotus* and no bootstrap support internally between clades.

Neither the infrageneric classification of Hesler and Smith nor Singer (Figs. 3.2-3) described monophyletic groups as described with nLSU sequence analysis. None of the characters emphasized in *Crepidotus* classification (Table 3.1) are synapomorphic: 1) clamp connections are absent in some or all of the members of clades I, II, III, and V; 2) globose basidiospores occur in some or all of the members of clades II, VII, VIII, and IX; 3) smooth

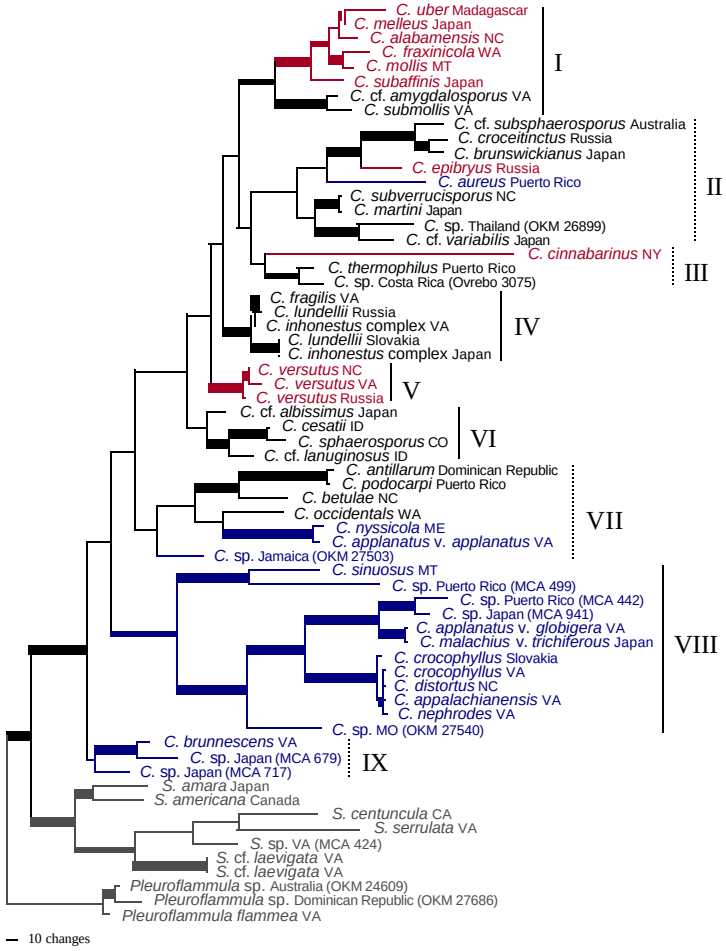

Fig. 3.2. Phylogeny of *Crepidotus* using nLSU rDNA sequences, showing infrageneric classification of Hesler and Smith (1965). Subgenus *Crepidotus* shown in red; Subgenus *Sphaerula* shown in blue; Subgenus *Dochmiopus* shown in black; outgroups shown in gray.

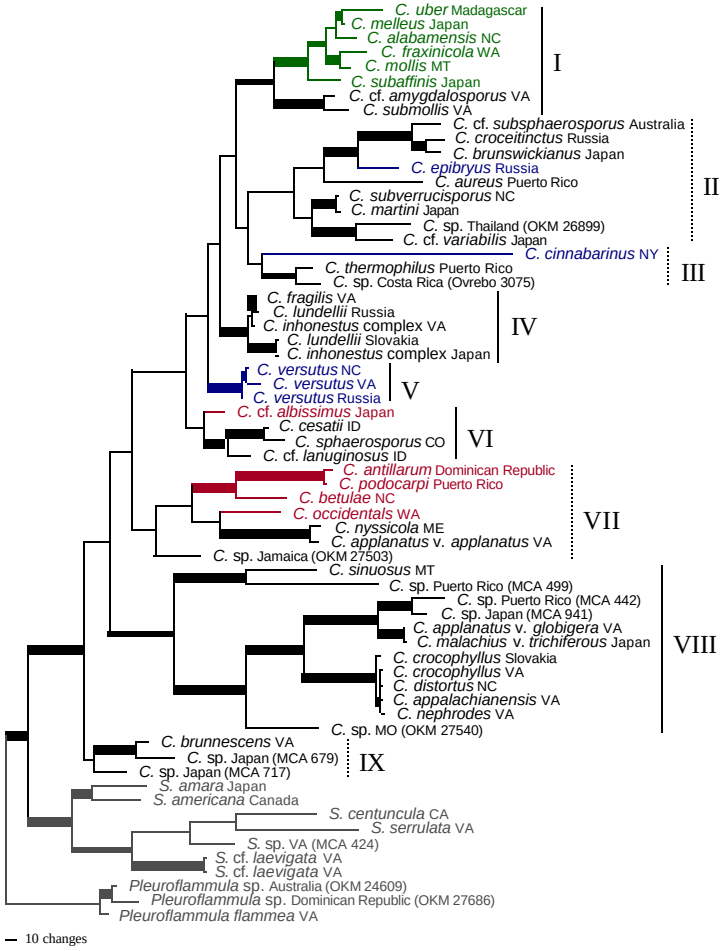

Fig. 3.3. Phylogeny of *Crepidotus* using nLSU rDNA sequences, showing infrageneric classification of Singer (1986). Subsection *Echinospori* shown in black; Subsection *Aporpini* shown in blue; Subsection *Fibulatini* shown in red; Subsection *Crepidotus* shown in green; outgroups shown in gray.

basidiospores occur in some or all of the members of clades I, VI, and VII. Other homoplasious characters include the presence of a gelatinous layer (some members of clades I and VII) and presence of a well-developed, central stipe (some members of clades III and VII).

Discussion

***Monophyly of* Crepidotus**—Historically, the circumscription of *Crepidotus* has been problematic, and, due to the wide range of phenotypic diversity in this group, several segregate genera have been proposed in the past (Patouillard 1887, 1903, Donk 1962). Sequence data from the nLSU gene region provide strong support for recognizing the monophyly of this genus, with no evidence supporting segregation. In fact, these data indicate that Singer's (1947, 1986) concept of the genus may actually be too narrowly conceived. When Singer's generic concept is expanded to include pale-spored elements, i.e., *Pleurotellus* Fayod, (Fig. 3.1, clade II, as *C. epibryus* s. Senn-Irlet) (Aime Chap. 2) and several stipitate taxa previously allied under *Melanomphalia* M.P. Christ. (Fig. 3.1, clade III, *C. thermophilus* and *C.* sp.) (Aime et al. Chap 1), a natural lineage results.

Infrageneric classification—Nuclear LSU sequence data have been successfully applied for resolving generic-level problems in many euagarics (Moncalvo et al. 1993, Vilgalys et al. 1996, Drehmel et al. 1999, Hopple and Vilgalys 1999). However, as was also reported for the genus *Lepiota* (Pers.) Gray (Johnson and Vilgalys 1998), nLSU resolving power was sufficient for defining the deepest nodes (i.e., those supporting *Crepidotus* and *Simocybe*), and most terminal nodes, but insufficient to determine the relationship between terminal clades. Only

five clades of *Crepidotus* receive strong support with these data (Fig. 3.1). Nonetheless, even without backbone resolution, neither the infrageneric classification of Singer (1986) nor Hesler and Smith (1965) are supported because some supported clades in this analysis (e.g., clade I) contain heterogeneous elements from either system (Figs. 3.2-3).

Character evolution— The absence of backbone resolution within this study precludes the definitive assignment of character polarity, or potential identification of morphological synapomorphies for clades at this stage. However, some inferences can be drawn. Singer placed great emphasis on spore ornamentation in this genus, and believed that all ornamentation in *Crepidotus* was caused by imbedded, short cylindrical columns (Singer 1951, 1962, 1973). Scanning electron microscopy (SEM) studies have confirmed that this type of ornamentation is present in some of the species found in clade VII and all of those in clade VIII, but that a variety of other types exist in this genus (e.g., Pegler and Young 1972, Clémençon 1977, Senn-Irlet 1992, 1993). Evaluation of basidiospore wall SEMs conducted for exemplars for each clade uncovered in this study (Appendix 2) supports the contention that the nature of exosporial ornamentation in *Crepidotus* may be consistent and diagnostically valuable at the level of phylogenetic section or subsection (Aime 1999). Since all species of *Simocybe* have smooth basidiospores (Appendix 2, Figs. A.2.41-42), and several lineages of *Crepidotus* also contain members with smooth spores (Fig. 3.3, shown in red and green type), it is assumed that the ancestor to *Crepidotus* also contained smooth spores, and that basidiospore ornamentation has arisen more than once in the genus.

Of the characters traditionally applied in infrageneric classification of *Crepidotus* (Table 3.1), the majority appear to be the result of homoplasy. For example, species lacking clamp

connections are present in well-supported clades in conjunction with species that exhibit clamps [Fig. 3.1, clade I (*mollis* clade), clade II (*C. epibryus*)], and the character has probably been lost, rather than gained, repeatedly. Plasticity of cheilocystidia will be discussed in Chap. 5, and the potential origins of pleurocystidia in *Crepidotus* will be discussed in Chap. 8.

Whether the ancestor to *Crepidotus* was stipitate or pleurotoid remains conjectural. Pleurotoid members of *Simocybe* appear confined to a single lineage, but stipitate members of *Crepidotus*, thus far, appear in two lineages (Fig. 3.1), and examination of the extra-generic elements now placed in *Melanomphalia* is expected to uncover additional lineages of stipitate *Crepidoti* (Aime et al. Chap. 1). As already stated, relationships between clades and within some clades defined in this study are unresolved. However, clade IX (Fig. 3.1), which appears basal to the other *Crepidoti* in this study, contains two undescribed species that are unique in that their morphology is intermediate between *Crepidotus* and *Simocybe*. For these collections, basidiospores are globose and ornamented, like many *Crepidoti*, but the abundant pileo- and cheilocystidia, the latter usually subcapitate, are reminiscent of *Simocybe*. These morphological findings support a basal-most position for this clade, and would suggest that the ancestor of *Crepidotus* was similarly pleurotoid. If so, this raises interesting questions regarding the reversibility of character loss in some agarics, since the stipitate *Crepidoti* would have to have arisen from these astipitate ancestors.

Phylogenetic analysis—Whether phylogenetic resolution in parsimony analyses can be improved by increasing taxon sampling (Olmstead and Palmer 1994, Graybeal 1998, Hillis 1998) versus increasing the number of molecular characters (Kim 1998) is still debated. Adding taxa was found to increase robustness in the analysis of a broad sampling of euagaric

families (Aime Chap. 2, Appendix 1). In the present study, however, an increase in ingroup taxa of >200% did not significantly improve the infrageneric resolution in *Crepidotus* from that of Aime (1999), although most of the known phenotypic diversity within this group has now been sampled (Hillis 1998). The combination of ribosomal DNA with mitochondrial gene sequences has been shown to result in more robustly supported trees with higher resolution than were recoverable with either data set alone by other investigators (Sullivan 1996, Soltis et al. 1998), and future strategies will apply this approach toward recovering a robust phylogeny for *Crepidotus*.

A final goal of the present study was to identify an exemplar phylogenetic clade for further studies on species concepts in *Crepidotus*. While Subgenus *Sphaerula* Hesler and Smith, as shown in Fig. 3.2 (taxa indicated in blue type) is polyphyletic, a core group of species with exosporial ornamentation consisting of truncate columns (Appendix 2, Figs. A.2.1-8), containing the lower branches of clade VII (*C. nyssicola* (Murr.) Sing., *C. applanatus* s. Joss., and *C.* sp. OKM 27503) and all of clade VIII, has been uncovered (Fig. 3.1). The proposed additional phylogenetic analyses will resolve whether the species with this particular spore type, herein termed Stirps Sphaerula, evolved from a single evolutionary event. Regardless, the members of the monophyletic clade VIII, herein termed the Sphaerula group, will be the subject of the final section of this thesis. The members of clade VII that share a hypothetical common-ancestry with the Sphaerula group, herein termed the Nyssicola group, will be discussed separately (Aime Chap. 5).

Acknowledgments

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4

Delayed germination of basidiospores in temperate species of *Crepidotus* (Fr.) Staude.

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Submitted.

Abstract

Related here for the first time is the delayed germination (endogenous dormancy) within the Agaricales of the basidiospores within a cluster of temperate species (13 total) of *Crepidotus* (Crepidotaceae). Ninety percent of recovered monokaryotic isolates germinated between 18 and 36 weeks after collection; average germination was 25 weeks after collection. The period in which 90% of germinations occurred was found to be between February 17 and April 19, with 50% of recovered isolates germinating in March. Many abiotic factors were experimentally manipulated in an effort to reduce or alter the necessary incubation period without effect. The latent period was consistent for a given collection, with the majority of recovered isolates from fall-fruiting collections germinating during early spring, regardless of whether spores were plated immediately after harvesting, or stored for one to several months prior to plating. The identity of the cultures derived from delayed germination was confirmed by DNA sequencing.

Introduction

The genus *Crepidotus* is comprised of dark-spored, saprophytic agarics, cosmopolitan in distribution, and most frequently inhabiting woody substrates (Singer 1986). There are approximately 150 described species (Hawksworth et al. 1995) that are a common component of forest ecosystems in temperate climes.

Obtaining monokaryotic isolates from germinating basidiospores is an important step in performing mating studies in basidiomycetes (Raper 1966; Petersen and Hughes 1999). Most saprophytic basidiomycetes germinate readily in culture (Lamoure 1989; Petersen 1997), although a few documented exceptions exist, most notably for species of *Pluteus* Fries (Banerjee and Sundberg 1993). The mechanism behind failure to germinate *in vitro* in these instances has never been understood. Mating studies and the recovery of single spore isolates (SSIs) from any species of *Crepidotus* remain unreported.

Accomplishing spore germination *in vitro* of mycorrhizal basidiomycetes is more difficult, in general, than for saprophytes, and much research has been conducted toward understanding spore dormancy in these cases. Fries and others have worked to elucidate the effects of either supplying or removing external growth factors, produced by basidiospores or growing hyphae, on the basidiospore germination of mycorrhizal agarics (Bulmer and Beneke 1964, Fries 1977, 1978, 1979, 1983, Macko et al. 1974, Nguyen et al. 1987). Other researchers have found that cold regimens will break dormancy in the spores of some basidiomycetes (Bromfield 1964, Booth 1971, Gemma and Koske 1988, Griffen 1994). In addition, spore germination in some parasitic basidiomycetes as well as a few saprophytic

phycomycetes appears to be activated by exposure to light (Sussman 1966). Nutritional supplements such as thiamin, biotin, and nitrogen have also been shown to be important for germination and subsequent growth of recalcitrant basidiomycetes (Palmer 1969).

In an attempt to recover single spore isolates from species of *Crepidotus* it was discovered that those basidiospores that germinated did so only after incubation on malt agar plates for several months. Sequencing of randomly selected SSI cultures and the basidiocarps from which they were derived confirmed the identity of these isolates. Further experimentation was designed in an attempt to break this period of spore dormancy by cold treating, varying spore concentration per plate, altering the supplied nutritional component, varying the time interval between harvesting and plating spores, and by varying the amount of light exposure received by spores. This paper reports the recovery of SSIs from 13 species of the genus *Crepidotus*; provides data on the effect of known abiotic factors on basidiospore germination in this genus; and presents data suggesting that the spores of temperate species of *Crepidotus* undergo a period of endogenous dormancy. We will also discuss how differences in basidiospore germination behavior within the proposed generic components of the Crepidotaceae may relate to phylogeny.

Materials and methods

Materials—Fresh material was collected during three field seasons from North America, the Greater Antilles, and Japan. Collection data for all materials studied are given in Table 4.1. Voucher collections are housed in the Massey Herbarium of Virginia

Table 4.1. *Crepidotus* collections used in this study.

Species[a]	Collection No./ Culture No.[b]	Collection date	Collection site	Plates[c]
C. cf. *amygdalosporus* Kühner	MCA 258	10/10/96	Montgomery Co., VA	3 (8)
C. antillarum (Pat. apud Duss) Sing.	MCA 443	6/4/98	Caribbean National Forest, Puerto Rico	0 (6)
C. antillarum	MCA 492	6/10/98	Caribbean National Forest, Puerto Rico	0 (4)
C. antillarum	OKM 26827	1/14/97	La Vega Prov., Dominican Republic	0 (9)
C. antillarum	OKM 27248	1/13/98	Caribbean National Forest, Puerto Rico	0 (5)
C. appalachianensis Hesler & Smith	OKM 27048	9/19/97	Wintergreen, VA	0 (7)
C. appalachianensis	MCA 749A/ VTCC 3667	10/3/98	Giles Co., VA	3 (5)
C. appalachianensis	MCA 322	7/23/97	Macon Co., NC	0 (4)
C. applanatus (Pers. ex Fr.) Kumm. v. *applanatus* [s. Josserand]	MCA 366	9/18/97	Giles Co., VA	0 (22)
C. applanatus v. *applanatus*	MCA 317	7/22/97	Macon Co., NC	0 (18)
C. applanatus v. *applanatus*	MCA 348	7/31/97	Weston, VT	0 (9)
C. applanatus v. *globigera* (Berk.) Sacc. [s. Hesler & Smith]	MCA 188/ VTCC 3587	9/14/96	Giles Co., VA	1 (7)
C. applanatus v. *globigera*	MCA 351	8/28/97	Giles Co., VA	2 (12)
C. aureifolius Hesler & Smith	MCA 373/ VTCC 3600	9/17/97	Iredell Co., NC	8 (21)
C. aureus Horak	OKM 27300	1/19/98	Caribbean National Forest, Puerto Rico	5 (19)
C. betulae Murr.	MCA 384/ VTCC 3595	10/31/97	Durham, NC	2 (7)
C. brunswickianus (Speg.) Sacc.	MCA 580	7/19/98	Kyoto-ken, Japan	0 (4)
C. cesatii (Rab.) Sacc.	OKM 26976	7/2/97	Baker Co., OR	0 (9)
C. distortus Hesler & Smith	MCA 386/ VTCC 3603	10/31/97	Durham, NC	7 (9)
C. fraxinicola Murr.	OKM 26739/ VTCC 3586	11/16/96	Pierce Co., WA	5 (7)
C. fraxinicola	OKM 26741/ VTCC 3584	11/16/96	Pierce Co., WA	2 (7)
C. fraxinicola	OKM 26748/ VTCC 3583	11/16/96	Pierce Co., WA	2 (4)
C. inhonestus complex	MCA 163/ VTCC 3596	9/5/96	Giles Co, VA	2 (7)
C. inhonestus complex	MCA 597/ VTCC 3669	7/25/98	Hokkaido, Japan	2 (5)

Table 4.1. Continued.

Species[a]	Collection No./ Culture No.[b]	Collection date	Collection site	Plates[c]
C. inhonestus complex	MCA 663/ VTCC 3678	8/13/98	Nagano-ken, Japan	2 (5)
C. inhonestus complex	MCA 664/ VTCC 3668	8/13/98	Nagano-ken, Japan	1 (4)
C. inhonestus complex	MCA 718	8/24/98	Nagano-ken, Japan	0 (4)
C. cf. *lanuginosus* Hesler & Smith	OKM 27331	5/20/98	Valley Co., ID	0 (4)
C. latifolius Pk.	OKM 27051	9/20/97	Wintergreen, VA	0 (7)
C. malachius (Berk. & Curt.) Sacc.	MCA 343/ VTCC 3605	8/21/97	Giles Co., VA	4 (13)
C. malachius	MCA 335	9/23/95	Montgomery Co., VA	0 (3)
C. malachius	MCA 740	8/12/98	Aichi-ken, Japan	0 (4)
C. mollis (Fr.) Staude	MCA 394/ VTCC 3606	2/14/98	Monterey, CA	1 (3)
C. nephrodes (Berk. & Curt.) Sacc.	MCA 189/ VTCC 3588	9/14/96	Giles Co., VA	5 (7)
C. nyssicola (Murr.) Sing.	TJB 8699 (CORT)	1/16/98	Falmouth, ME	0 (7)
C. occidentalis Hesler & Smith	OKM 26740	11/16/96	Pierce Co., WA	0 (4)
C. sphaerosporus (Pat.) Lange	OKM 27013	8/13/97	Copper Mountain, CO	1 (12)
C. cf. *sphaerosporus*	OKM 27334	5/24/98	Valley Co., ID	0 (4)
C. subapplanatus Hesler & Smith	MCA 331	8/10/97	Montgomery Co., VA	0 (12)
C. cf. *sublevisporus* Sing.	OKM 26826	1/14/97	La Vega Prov., Dominican Republic	0 (3)
C. versutus (Pk.) Sacc.	MCA 250	10/10/96	Montgomery Co., VA	0 (5)
C. versutus	MCA 267	10/12/96	Giles Co., VA	0 (5)
C. versutus	MCA 289	6/18/97	Montgomery Co., VA	0 (12)
C. versutus	MCA 382	10/18/97	Macon Co., NC	0 (5)
C. versutus	MCA 383	10/18/97	Macon Co., NC	0 (2)
C. sp.	MCA 596	7/25/98	Hokkaido, Japan	0 (5)
C. sp.	MCA 500	6/9/98	Rio Abajo, Puerto Rico	0 (5)
C. sp.	MCA 717	8/24/98	Nagano-ken, Japan	0 (4)

[a] Species designations are based on morphology only.
[b] Voucher collections are housed at the Virginia Tech Mycological Herbarium (VPI) unless otherwise noted. Voucher cultures are housed in the Virginia Tech Culture Collection (VTCC).
[c] Plate statistics are given as the total number of plates on which at least one spore germination was recorded; total number of plates on which basidiospores were incubated is given in parentheses.

Tech (VPI) and voucher cultures deposited in the Virginia Tech Culture Collection (VTCC).

Single spore isolates—Fresh basidiocarps were placed on sterile white bond paper and covered with a glass Petri dish until spores were cast. The resulting spore prints were labeled, kept moisture free, and stored in individual manila envelopes at room temperature. Spore prints were harvested on the day of collection, therefore, spore release date (the assumed time of spore maturation) is identical to collection date.

The methods for recovering SSIs were described in detail in Aime (1999). In brief, basidiospores from a spore print were aseptically transferred into a test tube of sterile distilled water and agitated. Three to four serial dilutions were made for each collection and plated on agar media. Plates were incubated and checked approximately twice a week for germinations. The germination date was recorded as the time when mycelial growth was first visible to the unaided eye.

Germinating isolates were aseptically transferred onto malt agar and allowed to grow out at 26 C in the dark. Once sufficient growth was achieved, a squash mount of a piece of the growing mycelium was stained in congo red and examined under an oil-immersion objective to confirm that the growth was consistent with that of a *Crepidotus*. Monokaryotic status of isolates was determined by screening for the absence of clamps or by nuclear staining with DAPI (4,6-diamidino-s-phenylindole) (Williamson and Trezzi 1979).

Experimental conditions—Standard experimental control conditions consisted of basidiospore dilutions plated on malt agar (15 g Sigma malt extract, 15 g Sigma agar, 1 L dH_20) on 100-mm Petri plates and incubated in the dark at 26 C for the duration of the experiments. Plates were wrapped tightly in parafilm and re-wrapped whenever the original parafilm

appeared to be loosing integrity. Controls were exposed to only brief intervals of light during regular screenings.

In addition to platings on malt agar, the same basidiospore suspensions from many collections were also plated on one or more of the following media: water agar (15 g Sigma agar in 1 L dh_20); yeast extract agar (15 g Sigma yeast extract, 15 g Sigma agar in 1 L dh_20); potato dextrose agar (39 g DIFCO potato dextrose agar in 1 L dh_20); nitrogen-enriched agar (15 g Sigma malt extract, 1.5 g ammonium hydroxide, 15 g Sigma agar in 1 L dh_20); and biotin- and thiamin-enriched agar (15 g Sigma malt extract, 5 µg biotin, 100 µg thiamin, 15 g Sigma agar in 1 L dh_20). Plates were subsequently treated as controls.

The effect of cold treatment was gauged by duplicating series plates from the same serial dilution tubes onto malt agar. One series was incubated under standard conditions, and the other series was first incubated at 4 C for two weeks and then incubated at 26 C for the remainder of the study. The effect of regular light exposure was tested by replicate plating of spores from the same suspension onto malt agar and incubating one set of replicates under standard conditions while the duplicate set was incubated at 26 C under alternating periods of 12 h of light and 12 h of dark.

Collections with sufficiently strong spore deposits were also subjected to additional dilution platings, made from the original spore prints, at monthly intervals. These plates were treated under the same incubation conditions as control plates.

Statistical analyses of germination data were conducted in JMP v. 3.2 (SAS Institute Inc. 1997).

Table 4.2. Taxa selected for sequencing analysis.

Taxon [a]	Collection no.	GenBank accession no.	Source
Agaricaceae			
Agaricus bisporus (Lange) Sing.	SAR 88/411	U11911	Chapela et al., 1994
Leucocoprinus cepaestipes (Sow.: Fr.) pat.	EFM 548	U85286	Johnson, 1997
Coprinaceae			
Coprinus atramentarius (Bull.: Fr.) Fr.	C 114 = VT 1131	AF041484	Hopple, 1994
Psathyrella candolleana (Fr.) Maire	J 181	AF041531	Hopple, 1994
Bolbitiaceae			
Bolbitius vitellinus (Pers.) Fr.	SAR 84/100	U11913	Chapela et al., 1994
Conocybe rickenii (Schaeff.) Kühner	J 183	AF041546	Hopple, 1994
Strophariaceae			
Pholiota squarrosoides Pk.	JJ 7	AF042568	Moncalvo et al., 2000
Stropharia rugosoannulata Farlow ex Murr.	D 258	AF041544	Hopple, 1994
Cortinariaceae			
Cortinarius iodes Berk. & Curt.	JM 96/23	AF042613	Moncalvo et al., 2000
Dermocybe marylandensis Ammirati & Smith	JM 96/24	AF042615	Moncalvo et al., 2000
Crepidotaceae			
Crepidotus antillarum (Pat. apud Duss) Sing.	OKM 26827	AF205680	Aime, 1999
C. applanatus (Pers. ex Fr.) Kumm.	MCA 188	AF205673	Aime, 1999
C. applanatus [culture]	VTCC 3587.8	AF205700	Aime, 1999
C. aureus Horak	OKM 27300	AF205685	Aime, 1999
C. betulae Murr.	MCA 384	AF205679	Aime, 1999
C. cesatii (Rab.) Sacc.	OKM 26976	AF205681	Aime, 1999
C. fraxinicola Murr.	OKM 26739	AF205676	Aime, 1999
C. fraxinicola [culture]	VTCC 3586.5	AF205699	Aime, 1999
C. fraxinicola	OKM 26748	AF205697	Aime, 1999
C. fraxinicola [culture]	VTCC 3583.2	AF205701	Aime, 1999
C. nephrodes (Berk. & Curt.) Sacc.	MCA 189	AF205670	Aime, 1999
C. sphaerosporus (Pat.) Lange	OKM 27013	AF205682	Aime, 1999
C. versutus (Peck) Sacc.	MCA 250	AF205695	Aime, 1999
Outgroups			
Collybia dryophila (Bull.:Fr.) Kumm.	RV 83/180	AF042595	Moncalvo et al., 2000
Omphalotus nidiformis Berk.	VTCC 1946.8	AF042621	Moncalvo et al., 2000
Crinipellis maxima Smith & Walker	DAOM 196019	AF042630	Moncalvo et al., 2000

[a] Taxa listed following the classification of Singer (1986).

DNA sequencing and analysis—The extreme delayed germination prompted the necessity to confirm the identity of the isolates. To carry this out we sequenced a portion from the 5'-end of the nuclear large subunit (LSU) rDNA of three randomly selected cultures from the first growing season (1996-1997), and compared these within a data matrix that included sequences from the parental basidiocarp tissue and other exemplar *Crepidotus* and Agaricales taxa (Table 4.2). The cultures selected were: VTCC 3587.8, derived from MCA 188, plated three days after collection, germinating 163 days after collection; VTCC 3586.5, derived from OKM 26739, plated 105 days after collection, germinating 190 days after collection; and VTCC 3583.2, derived from OKM 26748, plated five days after collection, germinating 258 days after collection. Sequencing primers used were: LROR (5' –ACCCGCTGAACT TAAGC), LR3R (5' -GTCTTGAAACACGGACC), LR5 (5' -TCCTGAGGGAAACTTCG), and LR16 (5' -TTCCACCCAAACACTCG) (Moncalvo et al. 2000). Methods for DNA extraction, PCR amplification, and sequencing were described in Aime (1999).

Sequences were manually aligned and analyzed in test version 4.0b2 of PAUP* (written by David Swofford, Smithsonian Institution, Washington, DC). The data matrix included a total of 656 characters, 113 of which were parsimony-informative. Parsimony analyses were performed using heuristic search algorithms with multiple (10) random sequence additions to generate starting trees, and tree-bisection-reconnection (TBR) branch swapping. Bootstrapping frequencies (Hillis and Bull 1993) were calculated using TBR branch swapping with 1000 replicates; support of greater than 50% was considered significant. Jackknifing frequencies (Lanyon 1985) were calculated using TBR branch swapping with 1000 replicates; support of greater than 50% was considered significant.

Table 4.3. Basidiospore germination in *Crepidotus*: Length of pre-germination period for all observed germinations.

Collection name and no.	No. of germs.[a]	Date of plating	Date of germ.	No. days coll.-germ.[b]	No. days plate-germ.[c]
C. cf. *amygdalosporus* MCA 258	2	10/11/96	2/25/97	138	137
	1	10/11/96	3/4/97	145	144
	2	10/11/96	3/20/97	161	160
	3	2/27/97	4/28/97	200	60
	3	2/27/97	5/20/97	222	82
	6	2/27/97	5/25/97	227	87
	++	2/27/97	6/4/97	237	97
	++	2/27/97	6/11/97	244	104
	2	2/27/97	6/25/97	258	118
	+	2/27/97	7/25/97	288	148
C. appalachianensis MCA 749A	1	1/22/99	3/2/99	150	39
	2	1/22/99	3/4/99	152	41
	10	1/22/99	3/15/99	163	52
	7+	1/22/99	3/22/99	170	59
C. applanatus v. *globigera* MCA 188	5	9/17/96	2/17/97	156	153
	3	9/17/96	2/24/97	163	160
	1	9/17/96	2/25/97	164	161
	5	9/17/96	3/4/97	171	168
	2	9/17/96	3/10/97	177	174
	3	9/17/96	3/13/97	180	177
	+	9/17/96	3/31/97	198	195
C. applanatus v. *globigera* MCA 351	1	11/15/97	1/29/98	154	75
	2	11/15/97	3/9/98	193	114
	1	11/15/97	3/17/98	201	122
	1	11/15/97	3/20/98	204	125
	2	11/15/97	3/23/98	207	128
	1	11/15/97	4/2/98	217	138
	1	9/8/97	5/19/98	264	253
C. aureifolius MCA 373	2	10/18/97	1/6/98	111	80
	3	10/18/97	1/21/98	126	95
	2	10/18/97	2/2/98	138	107
	1	11/15/97	2/5/98	141	82
	1	1/13/98	2/5/98	141	23
	2	11/15/97	2/10/98	146	87
	2	11/15/97	2/18/98	154	95
	3	11/15/97	2/24/98	160	101
	3	11/15/97	3/1/98	165	106
	3	11/15/97	3/5/98	169	110
	1	1/13/98	3/5/98	169	51
	6	11/15/97	3/9/98	173	114
	1	11/15/97	3/13/98	177	118
	1	1/13/98	3/13/98	177	59
	1	1/13/98	3/20/98	184	66

Table 4.3. Continued.

Collection name and no.	No. of germs.[a]	Date of plating	Date of germ.	No. days coll.-germ.[b]	No. days plate-germ.[c]
C. aureifolius MCA 373. Continued.	2+	1/13/98	3/23/98	187	69
	2	11/15/97	3/26/98	190	131
	3	1/13/98	3/26/98	190	72
	1	11/15/97	3/30/98	194	135
	3	1/13/98	3/30/98	194	76
	3	1/13/98	4/2/98	197	79
	2	11/15/97	4/6/98	201	142
	1	1/13/98	4/6/98	201	83
	1	1/13/98	4/10/98	205	87
	1	2/24/98	4/21/98	216	56
	2	2/24/98	4/28/98	223	63
	2	2/24/98	5/1/98	226	66
	4	2/24/98	5/4/98	229	69
C. aureus OKM 27300	1	1/26/98	3/17/98	57	50
	1	1/26/98	3/20/98	60	53
	sdc	1/19/98	3/30/98	70	70
	1	1/26/98	5/19/98	120	113
	1	2/18/98	5/26/98	127	97
	2	4/28/98	6/27/98	159	60
C. betulae MCA 384	1	11/15/97	2/24/98	116	101
	2	11/15/97	3/1/98	121	106
	4	11/15/97	3/5/98	125	110
	4	11/15/97	3/9/98	129	114
	3	11/15/97	3/13/98	133	118
	2	11/15/97	3/17/98	137	122
	2	11/15/97	3/20/98	140	125
	4+	11/15/97	3/26/98	146	131
	+	11/15/97	3/30/98	150	135
	+	11/15/97	4/6/98	157	142
	2	11/15/97	5/26/98	207	192
	1	11/15/97	5/29/98	210	195
	+	11/15/97	6/27/98	239	224
C. distortus MCA 386	1	11/15/97	2/24/98	116	101
	1	1/13/98	2/24/98	116	42
	2	11/15/97	3/1/98	121	106
	4	11/15/97	3/5/98	125	110
	4	11/15/97	3/9/98	129	114
	1	1/13/98	3/9/98	129	55
	5	11/15/97	3/13/98	133	118
	1	11/15/97	3/17/98	137	122
	2	11/15/97	3/26/98	146	131
	1	11/15/97	4/2/98	153	138
	1	11/15/97	4/6/98	157	142
	3	1/13/98	4/10/98	161	87
	4	1/13/98	4/13/98	164	90
	2	1/13/98	4/21/98	172	98
	3	11/15/97	5/1/98	182	167

Table 4.3. Continued.

Collection name and no.	No. of germs.[a]	Date of plating	Date of germ.	No. days coll.-germ.[b]	No. days plate-germ.[c]
C. fraxinicola OKM 26739	3	11/25/96	1/6/97	51	42
	1	11/25/96	5/7/97	172	163
	1	2/27/97	5/25/97	190	87
	1	2/27/97	6/4/97	200	97
	1	11/25/96	9/3/97	291	282
C. fraxinicola OKM 26741	1	11/25/96	1/6/97	51	42
	1	2/27/97	4/28/97	163	60
C. fraxinicola OKM 26748	1	11/21/96	7/25/97	251	246
	1	11/21/96	8/1/97	258	253
C. inhonestus complex MCA 163	sdc	9/5/96	10/31/96	56	56
C. inhonestus complex MCA 597	2	1/22/99	4/26/99	275	94
	1	1/22/99	5/31/99	310	129
	1	1/22/99	6/8/99	318	137
C. inhonestus complex MCA 663	3	1/22/99	4/26/99	256	94
	1	1/22/99	5/11/99	271	109
C. inhonestus complex MCA 664	1	1/22/99	4/26/99	256	94
C. malachius MCA 343	4	10/18/97	2/10/98	173	115
	3	10/18/97	2/18/98	181	123
	3	10/18/97	2/24/98	187	129
	4	10/18/97	3/1/98	192	134
	3	9/8/97	3/5/98	196	178
	1	9/8/97	3/9/98	200	182
	2	1/13/98	3/9/98	200	55
	1+	10/18/97	3/9/98	200	142
	1+	9/8/97	3/13/98	204	186
	2+	10/18/97	3/13/98	204	146
	1	1/13/98	3/13/98	204	59
	1	10/18/97	3/17/98	208	150
	1+	10/18/97	3/20/98	211	153
	2+	10/18/97	3/28/98	219	161
	3	1/13/98	4/2/98	224	79
	1	9/8/97	4/6/98	228	210
	+	9/8/97	4/21/98	243	225
	+	9/8/97	5/1/98	253	235
	2	9/8/97	6/1/98	284	266
C. mollis MCA 394	1	2/24/98	6/27/98	133	123
C. nephrodes MCA 189	3	9/17/96	2/17/97	156	153
	2	9/17/96	3/4/97	171	168
	2	9/17/96	3/7/97	174	171
	1	9/17/96	3/20/97	187	184

Table 4.3. Continued.

Collection name and no.	No. of germs.[a]	Date of plating	Date of germ.	No. days coll.-germ. [b]	No. days plate-germ. [c]
C. nephrodes MCA 189. Continued	1	9/17/96	4/10/97	208	205
	10	2/27/97	4/10/97	208	42
	5+	2/27/97	4/14/97	212	46
	1	2/27/97	5/20/97	248	82
	1	2/27/97	5/25/97	253	87
C. sphaerosporus OKM 27013	1	9/8/97	1/6/98	146	120
	1	9/8/97	3/17/98	216	190

[a] Number of observed basidiospore germinations per date. A '+' indicates that additional germinations were observed but not harvested. A 'sdc' indicates a spore drop culture (composed of an indeterminate number of individual germinating spores) germinated.
[b]Number of days between date of collection (i.e., harvest of spore print) and date of spore germination.
[c]Number of days from date of spore print plating and date of spore germination.

Decay values (Bremer 1988) were calculated with Autodecay 4.0.1 (Eriksson 1998) in PAUP 3.1.1 (Swofford 1993).

Results

A total of 49 collections of *Crepidotus* representing 27 taxa were plated during three collecting seasons in an attempt to obtain single spore isolates (Table 4.1). Of the 354 SSI plates made, basidiospore germination was recorded on only 58, representing 19 collections and 13 taxa (Table 4.3). Germinations were scattered and erratic, both temporally and spatially. Single spores germinated, on average, 25 weeks after collection, with 50% of germinations occurring between 21 and 30 weeks post-harvest (Fig. 4.1). Examination of a subset of these data that includes only those collections that fruited during the fall season (late August to early November) shows that 50% of these SSIs germinated during the month of March, and 90% of germinations occurred between Feb. 17th and April 19th (Fig. 4.2).

Abiotic factors—A number of abiotic factors were tested for their effect on both timing and frequency of spore germination in *Crepidotus*. The number of successful germinations for each experiment was too small to provide statistically meaningful comparisons in most cases, and a large number of experimental plates succumbed to contamination or drying rendering direct comparison of treatment types impossible. Nonetheless, a few overall trends were observed. No difference was found between germinations occurring on malt, potato dextrose, biotin and thiamin enriched, nitrogen enriched, and yeast extract agar medias. However, no germinations were observed on any of the 40 plates made on water agar, even though some matched controls did germinate on malt agar.

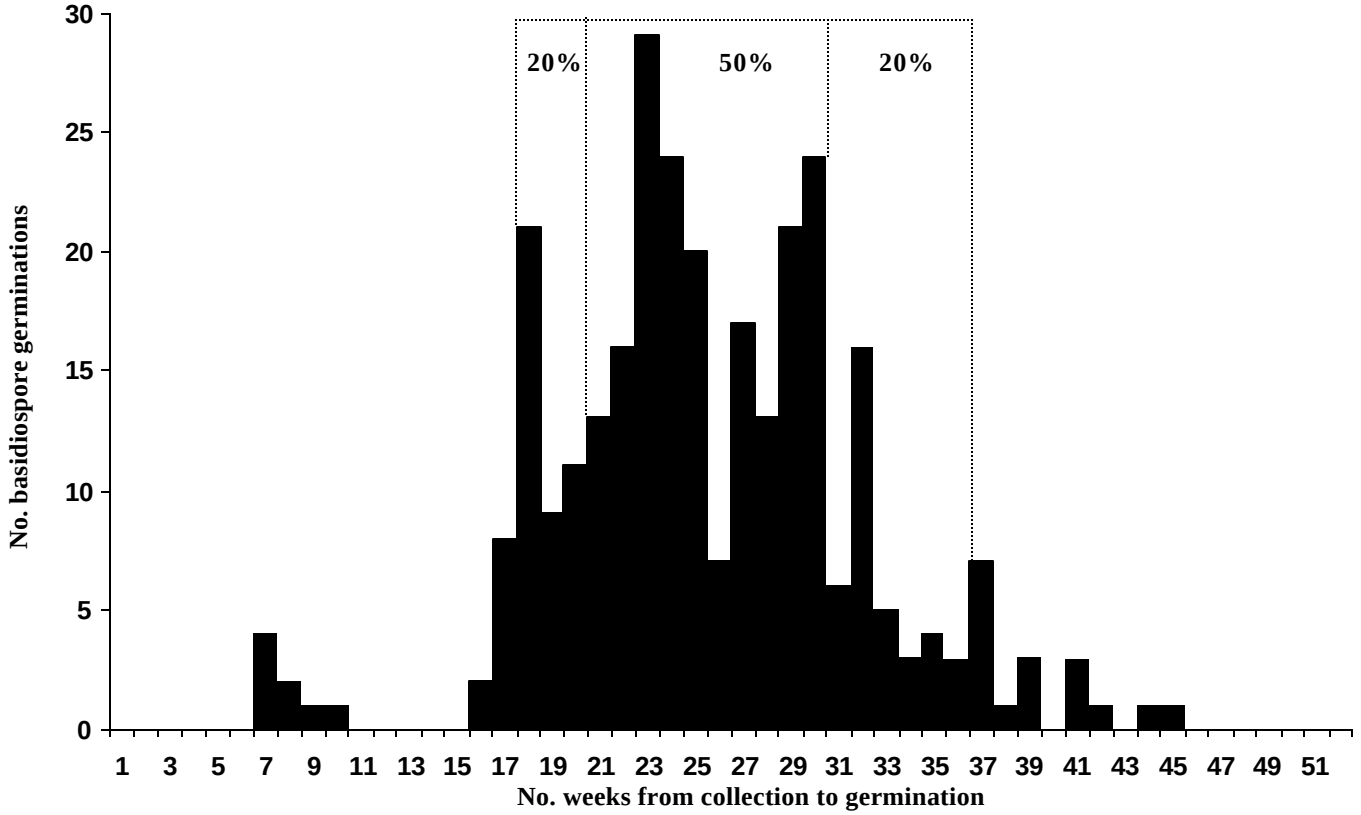

Fig. 4.1. Number of weeks between collection of spore deposit and basidiospore germination for all collections of *Crepidotus.* Data are given for all *Crepidotus* collections and treatments for which germination was recorded; germination data are shown as the number of weeks elapsed between spore print collection and individual basidiospore germination, for all experimental treatments. 50% of all spore germinations occurred between week 21 and week 30; 90% of all spore germinations occurred between week 17 and week 36. Mean and median number of weeks to germination = 26. Distribution is not significantly different from normal (Shapiro-Wilk test for normality, W = 0.29).

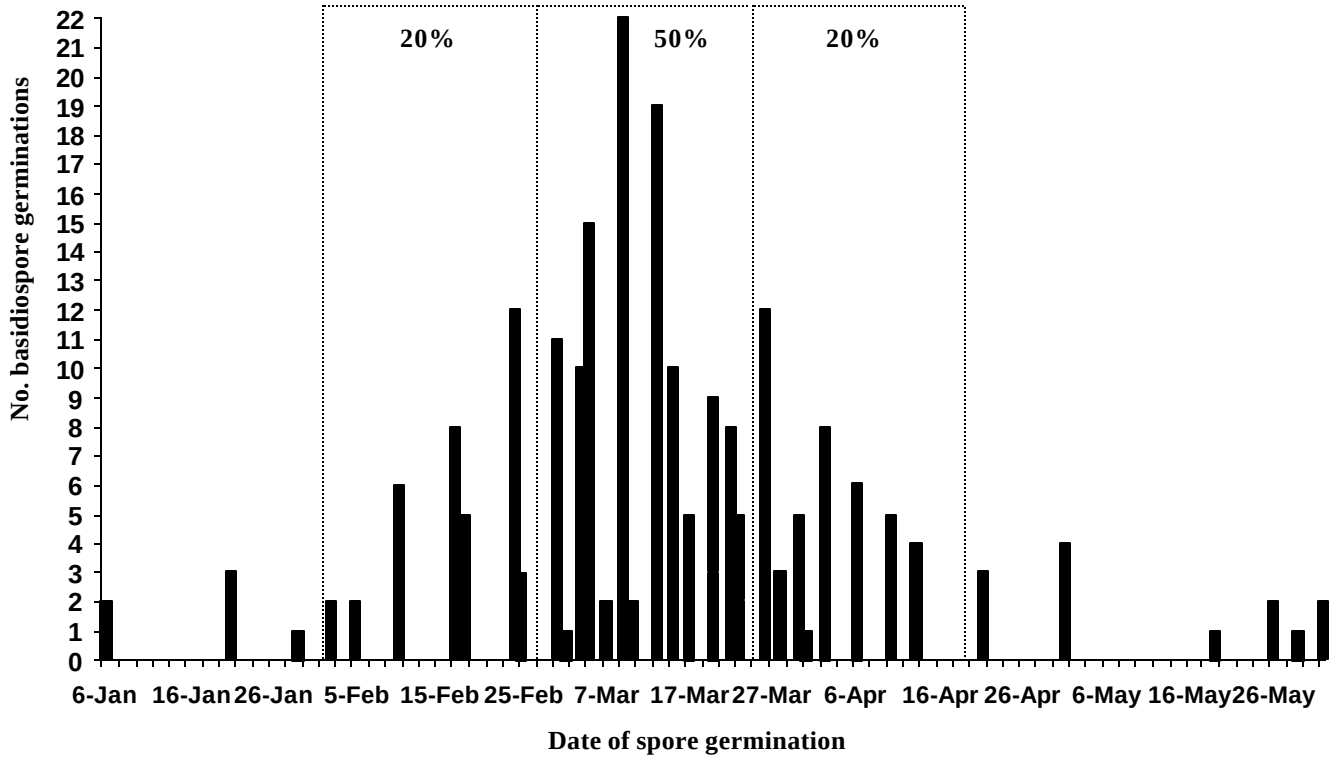

Fig. 4.2. Spore germination of fall-fruiting collections. Dates shown for individual basidiospore germinations for all fall-fruiting collections (three-month period between the 3rd week of August and the third week of November) and all plates made within 165 days of collection during the three year study period (1996-1998). 50% and 90% of all germinations are shown within dotted-outline boxes. Average germination date = March 12; roughly 50% of germinations occurred during the month of March.

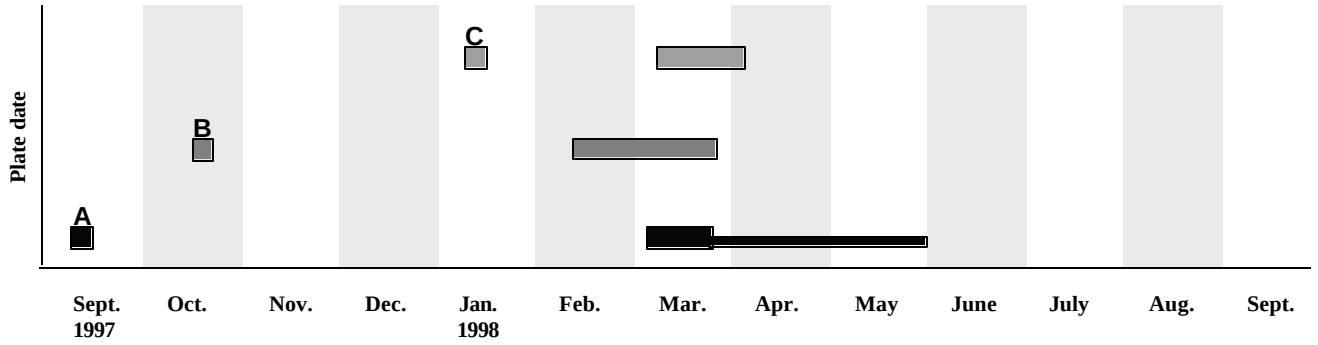

Fig. 4.3. Spore germination in *Crepidotus malachius* following delayed spore plating. A = 18 day-old spore print; B = 58 day old spore print; C = 145 day old spore print. Square indicates date when spores were plated; rectangle shows temporal span of spore germinations for each plating; a narrowed rectangle used when sporadic germinations continued in very reduced numbers. Collection number MCA 343, collected 8/21/97, spores plated 9/8/97, 10/18/97, and 1/13/98. Average germination date = March 13. No significant difference in germination time was found between the three plate dates (Analysis of variance, F = 0.41).

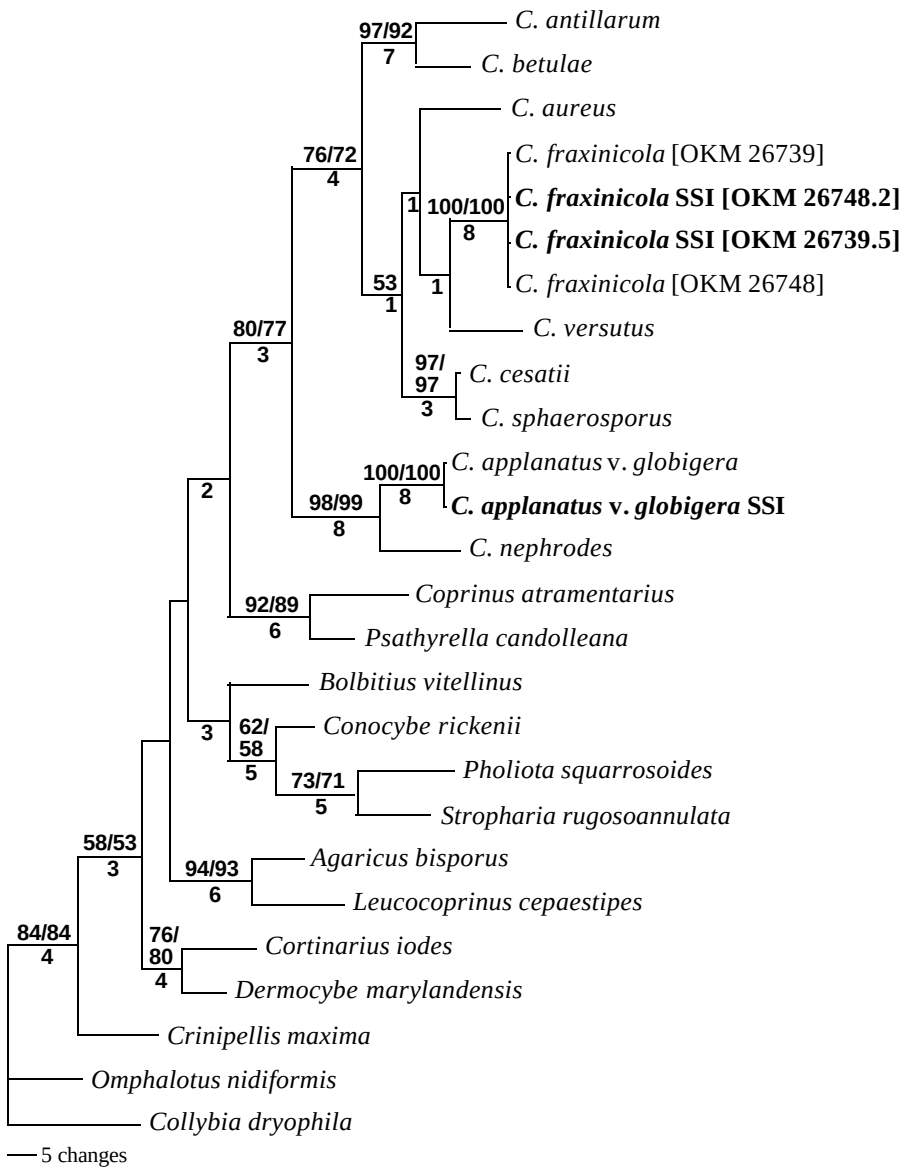

Figure 4.4. Identification of single spore isolates (SSIs) by DNA sequencing. Analysis based on a portion from the 5'-end of the nuclear DNA encoding the large ribosomal subunit. The first of two most parsimonious trees is depicted (length = 437, RI = 0.64, CI = 0.50). Bootstrapping values (1000 replicates) are given as first number above supported branch; jackknifing values (1000 replicates) follow; decay values are indicated below branch. Culture-derived sequences of SSIs shown in bold type.

Basidiospores first treated to a cold regimen germinated within the same two-week period as controls. Likewise, regular light exposure did not affect the timing of basidiospore germination when compared to controls. No differences were found in the timing of germination between spores plated in high concentrations compared to low concentrations of the serial dilutions. However, as expected, more germinations were observed on plates with higher spore concentrations.

The spore prints from 23 of the collections of *Crepidotus* were subjected to multiple monthly platings spanning the period between collection and first observed germination for that collection. Plates from nine of these collections showed germination in more than one trial (see Table 4.3, Date of Plating). Data from one of these collections are graphically presented in Fig. 4.3. Germinations were found to be equally likely on all plates, regardless of when the spores were plated, provided that the plates were made at least one month before any germinations occurred for that collection. No SSIs were observed to germinate without incubation of at least 39 days.

Isolate identification—Due to the lengthy period between spore plating and spore germination, we sequenced the LSU of three SSIs to confirm their identity as *Crepidotus* isolates. Sequences were identical to those obtained from the parental basidiocarp tissue, with the exception that a single base insertion was present in the culture isolate sequence of *C. applanatus* v. *globigera* that was not present in the sporocarp sequence of this collection. Phylogenetic analysis of these data is presented in Fig. 4.4.

Discussion

As previously described in species of *Pluteus* (Banerjee and Sundberg 1993) we found basidiospore germination in *Crepidotus* to be scanty and unaffected by different nutritional media, cold-shocking, or spore concentration in dilution. We did find that SSI recovery was possible for many *Crepidotus* taxa when incubation periods were increased well beyond the norm of one to several weeks. Additionally, we found that no SSI germinations occurred on non-nutritive water agar.

Isolates were only recovered from 13 of the 27 taxa for which spore prints were obtained. A few species, most notably *C. versutus*, and *C. antillarum*, were never induced to germinate in culture despite numerous attempts, while other taxa, such as those allied in Hesler and Smith's (1965) section Sphaerula (i.e., *C. appalachianensis, C. applanatus*, *C. distortus*, *C. malachius*, *C. nephrodes*, and *C. aureifolius*), comprised the majority of recovered isolates. On-going phylogenetic studies of the genus show several distinct evolutionary lineages (Aime Chap. 3), and we might expect some physiological basis for differences in spore germination behavior between these.

We have also examined basidiospore germination in two other members of the Crepidotaceae s. Singer (1986). Recent phylogenetic analysis shows *Simocybe* Karsten as a sister genus to *Crepidotus* (Aime Chap. 2). Interestingly, while the necessary incubation period demonstrated in four collections of *S. serrulata* (Murr.) Sing. was somewhat extended, ranging between 28 and 69 days from plating (data not shown), it is nowhere near as lengthy as that observed for species of *Crepidotus*. In contrast, the spores of eight *Tubaria* (W.G. Sm.) Gillet collections representing three taxa displayed synchronized germination within as few as eight

days (data not shown). Phylogenetic analysis of sequencing data does not support a monophyletic relationship between *Tubaria* and the Crepidotaceae s.s. (Aime Chap. 2), and the marked differences in basidiospore germination behavior tend to support the inference of a distant relationship between these taxa.

Two different types of dormancy have been widely applied to fungal spores. Exogenous dormancy is the condition of delayed germination due to the lack of favorable environmental conditions and is only broken when such external requirements are met (Bromfield 1964, Gottlieb 1978, Griffin 1994, Sussman 1966). Endogenous (sometimes termed constitutional) dormancy cannot be broken by environmental factors, but rather, is an innate property of the spore (Gottlieb 1978). Endogenous spore dormancy has been demonstrated in VAM spores (Gemma and Koske 1988) and in the teliospores of some rusts (Macko et al. 1974), and has been implicated in the work of Miller et al. (1993) on select basidiospores.

An intriguing finding in our study was that the SSIs of most fall-fruiting, temperate *Crepidotus* collections germinated in early spring. That this pattern was unaffected by time of plating or any manipulated abiotic factors suggests that an innate latent period is present in the spores of some *Crepidotus* species that is not broken by external factors. However, given the low numbers of recovered germinating spores per plate, and the low overall number of SSIs recovered in this study, some other requirement for growth is probably not being satisfactorily met. Some researchers have implicated more complex patterns of dormancy in other fungi, wherein spores from a single species may exhibit both exogenous and endogenous dormancy (Gemma and Koske 1988, Miller et al. 1993, d'Enfert 1997). In the taxa examined here, it is

possible that a spore-dependent threshold period of maturation, during which the spore is impervious to environmental influence, is followed by an environmentally activated stage.

The factors required for activation at the end of the maturation period would certainly include water and oxygen (Šubíková and Šubík 1974, d'Enfert 1977, Gottlieb 1978), and as the water content and humidity on these plates is gradually depleted over the long incubation time, this may account, in part, for the relatively low number of germinations. There also appears to be a minimal nutritional requirement, as no germinations were ever encountered on non-nutritive water agar.

In summary, these experiments demonstrate that obtaining single spore isolates from germinating basidiospores in species of *Crepidotus* is dependent on several factors. First, in those species sampled, basidiospore dormancy appears to be under endogenous control, and is not affected by such external factors as light, nutrition type, temperature, or spore concentration. Second, once the endogenous period has expired, subsequent germination of the basidiospores appears to be optimized by supplying a minimal nutritive source, and by maintaining moisture content. There appears to be a seasonal orientation, under endogenous control, to basidiospore germination within the temperate species of *Crepidotus*.

Acknowledgements

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5

Crepidotus applanatus s. Josserand: Secondary growth of cheilocystidia and taxonomic and biological implications.

Manuscript.

Abstract

Cheilocystidia size and shape have been applied diagnostically at the species level for many agaric taxa. In *Crepidotus* (Agaricales, Crepidotaceae) this is especially evident, where, for example, 26 new taxa were described based primarily on cheilocystidial characters by Hesler and Smith (1965). However, recent studies have shown that cheilocystidia morphology can be variable within single species of *Crepidotus* and may be dependent on basidiocarp age and maturity, or a result of natural variation between populations. Moreover, as reported here, environmental factors, most likely relating to high humidity, can dramatically alter cheilocystidial form and even induce secondary growth and production of multi-septate cheilocystidia in *Crepidotus applanatus*. Morphogenesis and phenotypic plasticity of cystidia are discussed. Type studies of *C. applanatus* v. *phragmocystidiosus* Hesler and Smith and *C. applanatus* v. *diversus* Hesler and Smith show these to be conspecific with *C. applanatus* v. *applanatus* s. Josserand; taxonomy of the *C. applanatus* complex is discussed.

Introduction

Cheilocystidia (or marginal cystidia) are sterile differentiated cells that line the lamellar edges in many agarics, existing in a tremendous variety of sizes, shapes, and forms that are often diagnostic for given genera or species (Romagnesi 1944). Cheilocystidia morphology has been especially emphasized in the taxonomy of *Crepidotus* (Fr.) Staude, as exemplified in the work of Hesler and Smith (1965). All species of *Crepidotus* thus far known possess cheilocystidia (Singer 1986), and form ranges from thick-walled encrusted cells (Horak 1977) to thin-walled and clavate, branching, contorted, or multi-septate cells (Hesler and Smith 1965), to name a few. In fact, cheilocystidial form is so variable in this genus that Hesler and Smith (1965) delimited eight new taxa on this basis alone, and an additional 18 taxa on cheilocystidial characters combined with pileus morphology (such as color, shape, or cuticular structures).

Recent type studies aimed at clarifying species concepts for some *Crepidotus* taxa have demonstrated that intercollection variation in some microscopic characters has often been underestimated when applied to species delimitation (Bigelow 1980, Luther and Redhead 1981, Senn-Irlet 1992, 1993, Bandala et al. 1999, Bandala and Montoya 2000a, 2000b). In particular, the need for baseline data regarding the natural range of morphological variation within taxa before making inferences about species boundaries has been emphasized (Nordstein 1990, Senn-Irlet 1995, Bandala and Montoya 2000a). Factors that might have an effect on cheilocystidia morphology within a single biological species could potentially include fruiting body age, naturally occurring variation between individuals or populations, or environmental influence.

In an extensive examination of collections in two species complexes, Bandala and Montoya (2000a) found a natural gradation in cheilocystidial shape between collections that was consistent with expected variation within a single taxon, not indicative of separate species as previously interpreted. Senn-Irlet (1995) found that cheilocystidia could be quite variable between populations of a single species, or even between basidiomata from a single collection in some taxa. However, the same study concluded that within the *Crepidotus applanatus* (Pers.) Kumm. complex, cheilocystidial forms did not overlap between some collections, and this was interpreted as being consistent with a diagnosis of discreet taxa at the varietal level (Senn-Irlet 1995).

Detailed examination of *Crepidotus applanatus* s. Josserand (1937) has revealed that the cheilocystidia in this taxon are capable of secondary growth under conditions of high-humidity that dramatically alters the length, shape, and number of septa per cystidium. Examination of additional collections of *C. applanatus*, including the types of *C. applanatus* v. *phragmocystidiosus* Hesler and Smith, and *C. applanatus* v. *diversus* Hesler and Smith, show that cheilocystidia form and shape is naturally variable within and between collections. The purpose of this paper is to: 1) describe, illustrate, and discuss secondary growth of cheilocystidia in *C. applanatus*; 2) describe and illustrate the normal range of cheilocystidial variation in this taxon; and, 3) discuss the taxonomy of this taxon and synonymize *C. applanatus* v. *phragmocystidiosus* and *C. applanatus* v. *diversus* with *C. applanatus* s. Josserand.

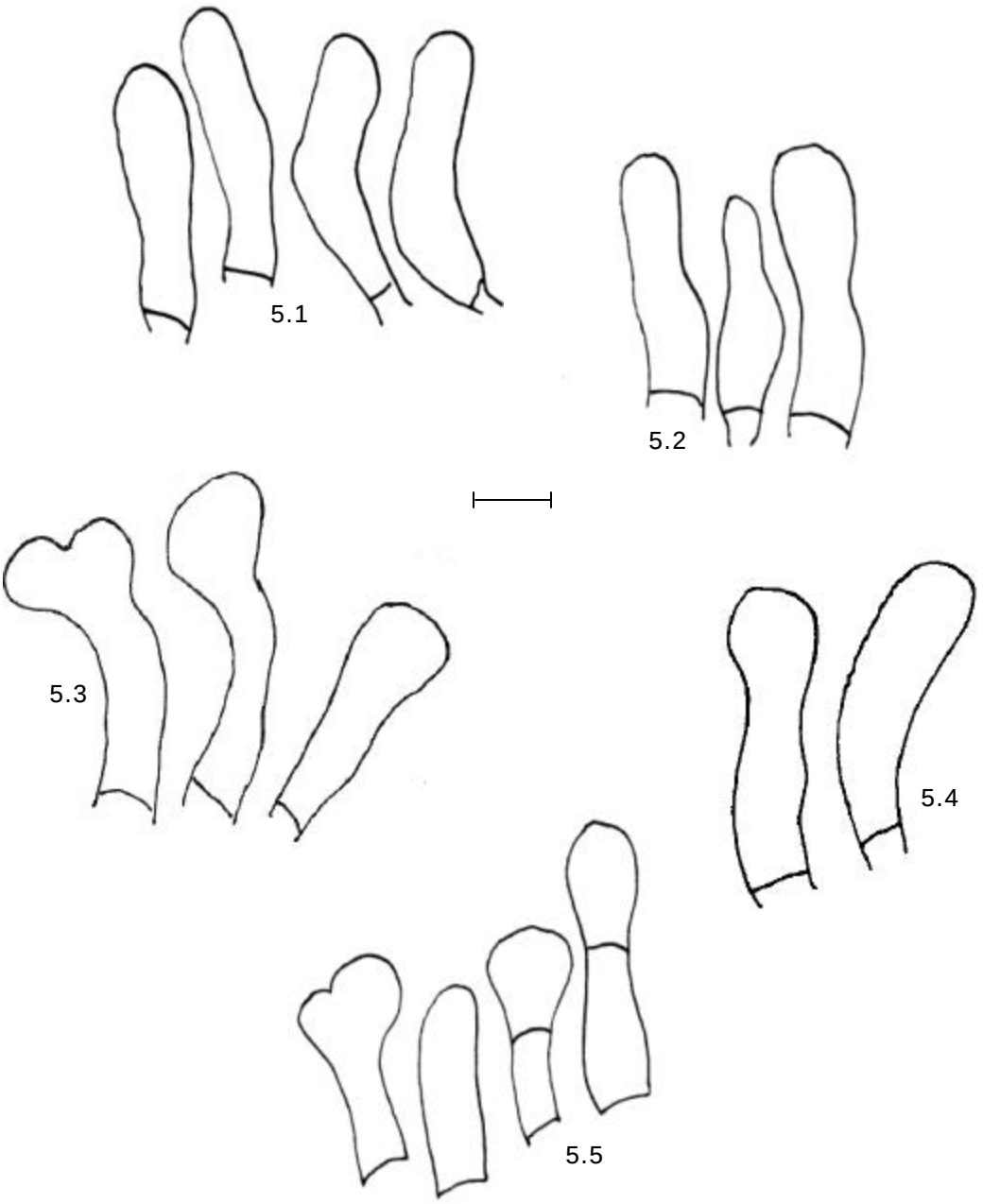
Materials and methods

Materials examined—USA: VIRGINIA, Montgomery Co., Jefferson National Forest, Pandapas Pond, decorticated hardwood, 11 Sept. 1996, MCA 170; same location, on decorticated hardwood stump, 24 Aug. 2000, MCA 1417; Giles Co., Mountain Lake Biological Station, 3800' elev., decorticated hardwood, 18 Sept. 1997, MCA 366; same location, on cut *Quercus* log under bark, 14 Sept. 2000, MCA 1433; Giles Co., Jefferson National Forest, Cascades Recreational Area, on decorticated *Quercus*, 16 Sept. 1999, MCA 838. GEORGIA, Macon Co., Nantahala National Forest, Highlands Research Center, Rte. 64, on decorticated hardwood, 22 July 1997, MCA 317. NORTH CAROLINA, near Little Switzerland, near Armstrong Creek, 29 Sept. 1999, OKM 27606. MICHIGAN, Leelanau Co., North Manitou Island, on hardwood, 3 Aug. 1957, *C. applanatus* v. *phragmocystidiosus*, HOLOTYPE, AHS 57475 (MICH); Chippewa Co., Emerson, 18 Aug. 1959, *C. applanatus* v. *diversus*, PARATYPE, AHS 61486 (MICH). VERMONT, near Weston, Green Mountain National Forest Campground, 31 July 1997, coll. Steven L. Miller, MCA 348. WASHINGTON, King Co., Hazel Wolf Wetland, scattered on rotten *Alnus*, coll. P.B. Matheny, PBM 1183 (WTU). CANADA: QUEBEC, Laurentide Provincial Park, Mercier, 31 Aug. 1959, *C. applanatus* v. *diversus*, PARATYPE, coll. A.H. Smith, AHS 61703 (MICH). All collections are housed in the Virginia Tech Herbarium (VPI) unless otherwise indicated.

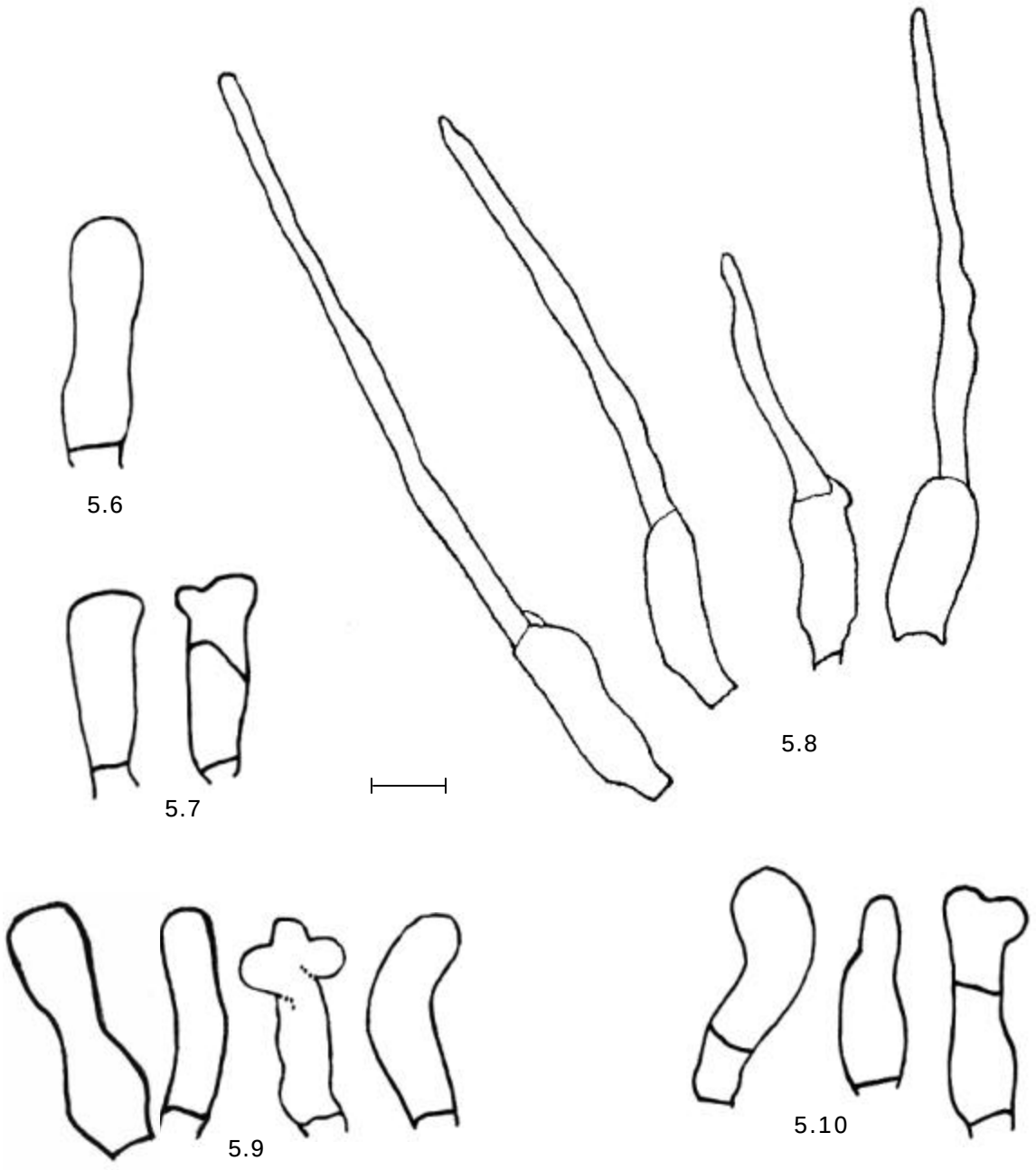
Dried material was revived in 80% EtOH and water. Thin sections of fresh and revived material were mounted in 10% NH_4OH and viewed under oil immersion. Drawings were made with the aid of a drawing tube or a thermal video graphic printer (Sony) attached to a Leitz microscope. Photomicrographs were taken with a 35mm camera attachment on a Leitz

microscope. Measurements are based on five to ten cheilocystidia per collection and two to three basidiomata from each collection were examined; basidiospore measurements are based on at least 30 measurements per collection. Fresh collections were preserved by low convection heat immediately after recording fresh data with the exception of a single or partial basidiomata per collection that was retained for spore printing. In most cases, spore prints were attempted by placing a fresh pileus directly on sterile bond paper and covering with a glass Petri dish for 2-24 hours, or until pilei were too dry to shed additional spores. For collection OKM 27606, an approximately 2 mm wide wedge from a single basidiocarp was mounted with Vaseline, lamellar side down, onto a 25mm plastic Petri lid, and then suspended about 1mm over a malt agar (15g Sigma malt extract, 15 g Sigma agar, 1 L water) plate, parafilmed, and left undisturbed for 48 hours at room temperature to obtain a spore drop culture. High humidity on the plate was evidenced by thick condensation on the inside of the lid. The still fresh pileus wedge was then sectioned and examined as described above.

Nuclei were visualized by staining with DAPI (4,6-diamidino-s-phenylindole), following the methods of Williamson and Trezzi (1979) except that the final DAPI concentration was 1.0 microgram/mL, and glass slides were first frozen at -20 C before staining and mounting of sections. Mounts were then examined by fluorescence microscopy and photographed on thermal paper as described above.



Figs. 5.1-5. *Crepidotus applanatus* s. Josserand cheilocystidia. Scale bar = 10 µm. 5.1. MCA 366. 5.2 MCA 838. 5.3. OKM 27606. 5.4. MCA 317. 5.5. AHS 57473 (*Crepidotus applanatus* v. *phragmocystidiosus*, HOLOTYPE).

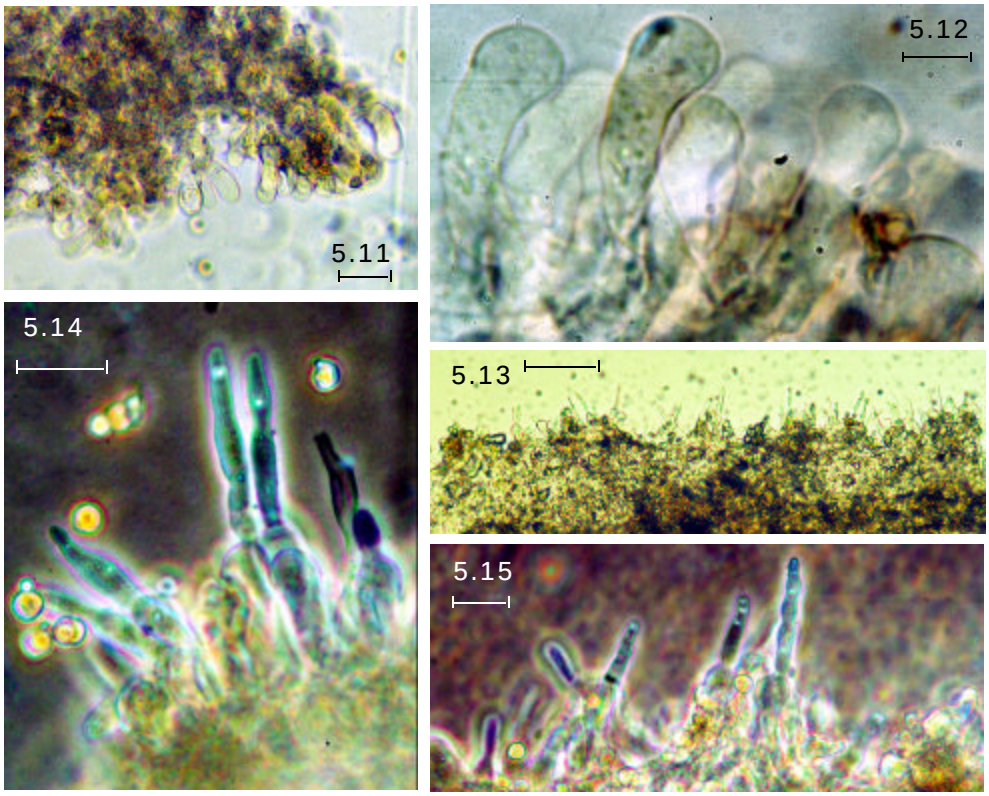


Figs. 5.6-5.10. *Crepidotus applanatus* s. Josserand cheilocystidia. Scale bar = 10 µm. 5.6. OKM 27606. 5.7. MCA 348. 5.8. Cheilocystidia with secondary growth OKM 27606. 5.9. AHS 61703 (*Crepidotus applanatus* v. *diversus*, PARATYPE). 5.10. AHS 61486 (*Crepidotus applanatus* v. *diversus*, PARATYPE).

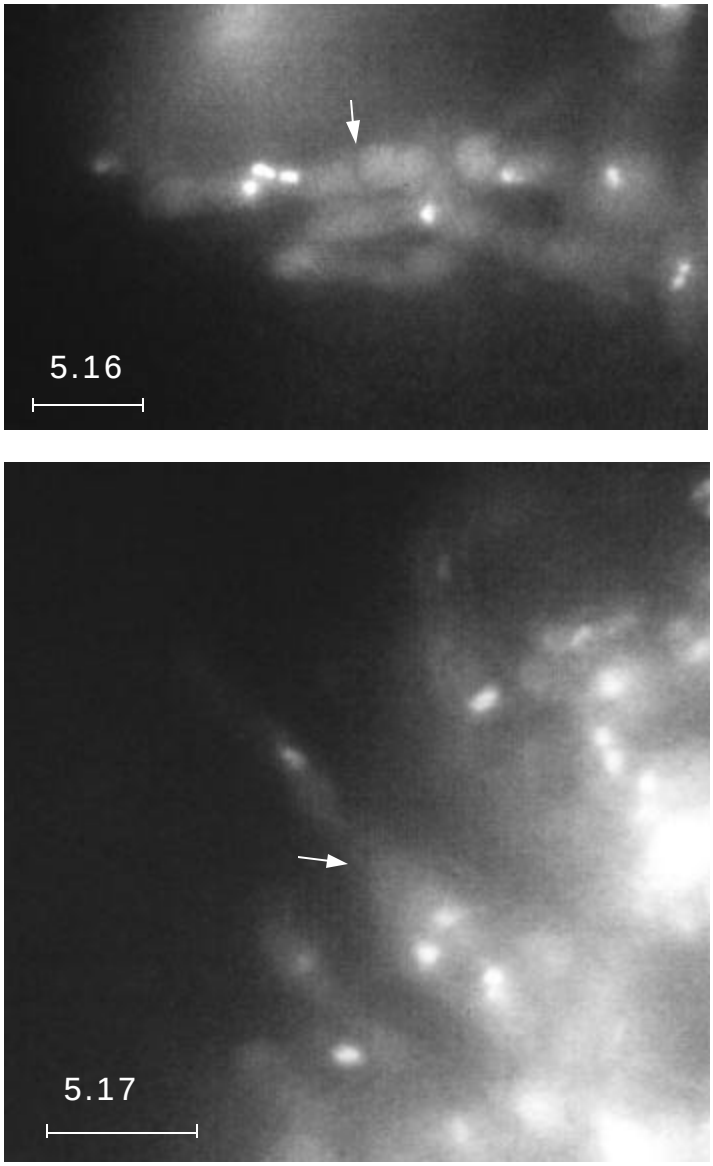
Results

Microscopic analysis of cheilocystidia from dried basidiomata for all collections showed variation within the normal expected range for this taxon as interpreted by Josserand (1937). Shape ranged from clavate to subcapitate to truncate or knobbed, with a few cheilocystidia from several collections being two-celled with simple septa (Figs. 5.1-7, 5.9-12). Cystidia were thin-walled, often inflated and/or collapsed against the lamellar edge, and measure (18) 25-36 (42) x 7-12 (16) μm (avg. = 31.2 x 9.4 μm).

A single basidiocarp from collection OKM 27606 was divided into two portions, one of which was immediately dried, the other was left in a humid Petri plate for 48 hours. When examined, the two portions from the same pileus contained cheilocystidia of very different proportions and shapes. Cheilocystidia from the dried pileus portion were normal and comparable to those in other collections of the same species (Figs. 5.3, 5.6, 5.11-12). However, cheilocystidia from the portion of the same basidiocarp that was placed in a humid environment for 48 hours were markedly different (Figs. 5.8, 5.13-15). These cystidia are comprised of two cells with a clamp at the septum; the basal cell is clavate, thin-walled, and similar in appearance to normal cheilocystidia, whereas the uppermost cell is longer and narrower, lanceolate with a broader base, and slightly flexuous. Overall measurements for these cheilocystidia are 80-124 x 8 μm (avg. = 96.4 x 8 μm); the basal cell alone measures 26-34 x 8 μm (avg. = 29.8 x 8 μm), and the lanceolate extension measures 50-95 x 3-4 μm (avg. = 66.6 x 3.6 μm). Staining with DAPI revealed multiple nuclei within both the basal and terminal cells of the growing cheilocystidia (Figs. 5.16-17).



Figs 5.11-15. *Crepidotus applanatus* s. Josserand OKM 27606. Fig. 5.11. Normal cheilocystidia. Scale bar = 20 µm. 5.12. Normal cheilocystidia. Scale bar = 10 µm. Fig. 5.13. Cheilocystidia with secondary growth. Scale bar = 40 µm. Fig. 5.14. Cheilocystidia with secondary growth, phase contrast. Scale bar = 15 µm. Fig. 5.15. Cheilocystidia with secondary growth, phase contrast. Scale bar = 15 µm.



Figs. 5.16-17. *Crepidotus applanatus* s. Josserand OKM 27606 fluorescent microscopy showing multinucleate cheilocystidia with secondary growth. Scale bar = 10 µm. 5.3. Secondary cell with three nuclei. Arrow indicates septum. 5.4. Basal cell with four nuclei, one nucleus showing in secondary cell. Arrow indicates septum.

Discussion

Crepidotus applanatus (Pers.:Fr.) Kumm. is one of the many species of *Crepidotus* for which the original type specimen no longer exists, and subsequently this taxon has been interpreted in many different ways, although the concept of Josserand (1937) is that most frequently followed (Singer 1947, Nordstein 1990). Singer's (1947) recommendation that this name be typified with Josserand's collection housed in Lyon, France, was never accomplished.

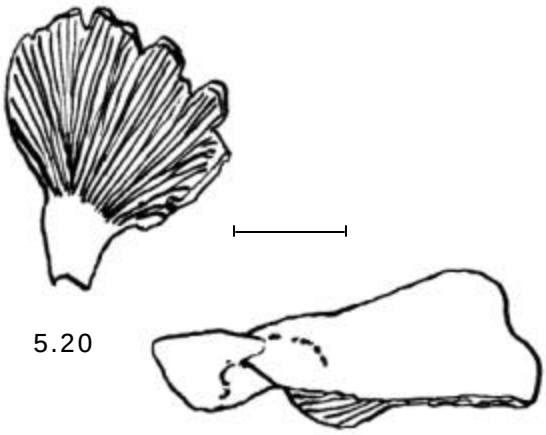
Hesler and Smith (1965) split *C. applanatus* into four varieties: *C. applanatus* v. *phragmocystidiosus* and *C. applanatus* v. *diversus* were described as new and were delimited by septate or branched and knobbed cheilocystidia, respectively; *C. globigera* (Berk.) Sacc. was reduced to *C. applanatus* v. *globigera*, which was actually done previously by Pilát (1948); and *C. applanatus* v. *applanatus* is Josserand's taxon.

Phylogenetic analysis of DNA sequencing data have shown that *C. applanatus* v. *applanatus* and *C. applanatus* v. *globigera* (both sensu Hesler and Smith) are actually two distinct and unrelated species (Aime Chap. 3). The clade to which var. *applanatus* belongs has been named the Nyssicola group (Aime Chap. 3, clades VII and VIII). The consistently correlated phenotypic characters that can be used to distinguish between these two species are: 1) absence of clamp formation in dikaryotic cultures of *C. applanatus* v. *applanatus*, while dikaryons of *C. applanatus* v. *globigera* form clamps at at least 25% of septa (unpublished data); and, 2) spore diameter in *C. applanatus* v. *applanatus* ranges from 4-5.5 µm while spore size in *C. applanatus* v. *globigera* ranges from 5-7 µm, so, while there is some overlap in spore size as previously noted (Nordstein 1990, Senn-Irlet 1995), no spores in var. *applanatus* exceed the upper size limit of 5.5 µm, while no spores in var. *globigera* have been

found smaller than 5 µm (unpublished data based on 39 collections from three continents). There is also a tendency for the basidiocarps of *C. applanatus* v. *applanatus* to form a lateral pseudostipe (Figs. 5.18-20), whereas such a structure is never formed by the mature fruiting bodies of *C. applanatus* v. *globigera*.

Recently, a Neotype from Scotland was designated for *Crepidotus applanatus* (Senn-Irlet 1995). In this modern taxonomic treatment of the *C. applanatus* complex, Senn-Irlet recognizes two varieties of *C. applanatus* (var. *applanatus*, and var. *subglobiger* Sing.), distinguished on the basis of cheilocystidia morphology. Descriptions for both taxa, based on numerous collections (apparently combining data from collections of both *C. applanatus* v. *applanatus* and var. *globigera* as defined above) encompass spores ranging in size from 4.5-7 µm. Basidiospores from the Neotype range in length from 5-6.3 µm (B. Senn-Irlet, pers. comm.), and therefore the name *C. applanatus* now applies to the larger-spored Berkeley (1872) species *C. globigera*. The species discussed in this paper is the smaller-spored taxon described in detail by Josserand (1937); the taxonomically correct specific epithet for this species will be the subject of another paper.

Crepidotus applanatus var. *phragmocystidiosus* is described with spores of 4-5 µm in diameter and reportedly distinguished by the bottle-shaped or subcylindric, septate cheilocystidia, and the name is supported by a single collection from Michigan (Hesler and Smith 1965). *Crepidotus applanatus* v. *diversus* Hesler and Smith is distinguished by the branched or knobbed cheilocystidia (Hesler and Smith 1965), also with basidiospores of 4-5 µm in diameter. Examination of type collections for both taxa reveal a mixture of



Figs. 5.18-20. *Crepidotus applanatus* s. Josserand basidiomata. 5.18. MCA 366. 5.19. MCA 1433. 5.20. OKM 27606. Scale bar = 10 mm.

clavate, simple septate, and knobbed cheilocystidia (Figs. 5.5, 5.9-10) that are within the normal range of variation found in other collections examined (Figs. 5.1-4, 5.6-7). In the absence of any unique defining phenotypic characters, both varieties are considered to be conspecific with *C. applanatus* s. Josserand. The value of delimiting taxa based on single, potentially variable characters has been questioned (Smith 1967, Watling and Largent 1976), and certainly this study reveals an unsuspected plasticity in cheilocystidial form within a single taxon and even within individual collections that suggest that taxonomic delineation based on cystidial morphology, at least in *Crepidotus*, should be applied with extreme caution.

Since the basal cells of the abnormal cystidia were in all respects identical to the normally formed cystidia, while the terminal cell was exceptionally long and nearly filamentous, it would appear that these cheilocystidia are capable of meristemmatic growth in response to some stimulus that was present in the Petri plate. This view is supported by the finding of multiple nuclei in the growing cells (Figs. 5.16-17), which is the hallmark of actively growing and differentiating primordial cells of the lamellar trama (Moore 1987, Moore et al. 1998). Time may be a factor, but other pilei, left for extended periods of time to shed spores on bond paper under drier conditions did not exhibit any such growth. It is also possible that the fungus was responding to some growth stimulus induced by the nutrient-containing agar although the pileus was suspended such that no portion of it came into direct contact with the medium below. Equally tenable is the hypothesis that the high humidity, present in the Petri plate and absent in all other treatments, created a favorable environment for secondary cystidial growth once the basidiospores were shed.

Secondarily septate, lageniform cheilocystidia, of the type shown in Figs. 5.8 and 5.13-15, have been noted in other agarics, such as certain species of *Melanoleuca* Pat. (Gillman and Miller 1977). Interestingly, in these species, the lageniform cystidia are most numerous in the short lamellae, closest to the pileus, which are presumed to be microenvironments of higher humidity (O.K. Miller, pers. comm.). Much remains to be learned of morphogenesis and cellular differentiation of basidiomata. However, it does appear that cell differentiation can be flexible and influenced by microenvironment (Moore et al. 1998). Gross morphology, pleurocystidial differentiation, and basidial form were found to be plastic under certain conditions *in vitro* for *Psilocybe merdaria* (Fr.) Ricken (Watling 1971), and karyogamy (which normally occurs only in the sexual basidial cells) has been found in presumably sterile pleurocystidia of *Coprinus cinereus* (Schaeff.:Fr.) Gray (Chiu and Moore 1993).

Together, the above reports suggest that hymenial cells of the lamellar face (basidia and pleurocystidia) can be morphogenetically flexible (Moore et al. 1998, Chiu and Moore 1993). The present is the first study to show that entry into the cheilocystidial developmental pathway, as well, does not preclude further differentiation or vegetative growth. There is evidence that developmental pathways leading to cystidium formation may be activated by microenvironment, probably humidity (Horner and Moore 1987). It seems likely that the same environmental cues can also promote secondary growth of differentiated cystidial cells, once mature basidiospores have been cast, in a reversion from the fruiting stage to the vegetative stage.

Acknowledgements

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Part III

The Sphaerula group

"(W)ithin species with highly conserved compatibility mechanisms, considerable variation can be found in other characters."

—Ron Petersen 1995

6

The mating system in two species of *Crepidotus.*

M. Catherine Aime and Jessica Ball.

Submitted.

Abstract

Crepidotus aureifolius Hesler and Smith and *C. nephrodes* (Berk. & Curt.) Sacc. (Agaricales, Crepidotaceae) exhibit a unifactorial (bipolar) mating system. This is the first known report of mating studies conducted in this genus.

Introduction

This study is one component of a larger study that will attempt to apply morphological, biological, and phylogenetic species concepts toward a systematic revision of the genus *Crepidotus* (Fr.) Staude. *Crepidotus* is comprised of mostly small, pleurotoid, saprophytic agarics, with an estimated 150 species in the genus (Singer 1986). Section Sphaerula Hesler and Smith contains 42 taxa, including *C. aureifolius* and *C. nephrodes*, many of which are distinguished on the basis of single morphological character differences that may or may not be true indicators of non-interbreeding species. In order to apply a biological species concept within this complex the recovery of single spore isolates and determination of mating types is of paramount importance (Petersen 1995). This paper will report the mating system in two morphological species of *Crepidotus* in Section Sphaerula, based on intracollection pairings.

Materials and Methods

Materials—USA: NORTH CAROLINA, Iredell County, West of Bridge 766, 17-Sept. 1997, coll. A. Stanley, MCA 373 (VPI); VIRGINIA, Giles County, Jefferson National Forest, Cherokee Flats, 14-Sept. 1996, MCA 189 (VPI).

All cultures and mating crosses were grown on malt extract agar (15g Sigma malt extract, 15g Sigma agar, 1 L distilled H_2O) and incubated in the dark at 24-26 C. Methods for dilution plating and isolation of monkaryotic isolates follow Aime (1999). Isolates were stored at 4 C for two-three years prior to use. Isolates were then grown out and examined microscopically for the absence of clamp connections before being used in crosses. Confirmed monokaryons were crossed, in all possible pair-wise combinations, on an individual malt agar plate for each cross, and incubated for approximately four weeks. Hyphae from the contact zone on each cross were then mounted in Congo red and examined under oil immersion; compatible matings were inferred by the presence of clamp connections; the absence of clamp connections was interpreted as an incompatible mating.

The stability of dikaryons was tested by transferring a small portion of the interface from a compatible cross onto a new malt agar plate and incubating for two months or until growth had covered the plate. A portion of the outermost region of the culture was then examined for clamps as above. In addition, all crosses that initially appeared negative at the four-week examination were re-examined after two to eight weeks of additional incubation at 24-26 C.

A total of eight monokaryotic isolates for MCA 373 and six for MCA 189 were recovered which maintained viability throughout the study. These cultures are maintained in the Virginia Tech Culture Collection as VTCC 3600.2, VTCC 3600.4, VTCC 3600.8, VTCC

3600.12, VTCC 3600.14, VTCC 3600.15, VTCC 3600.18, and VTCC 3600.39 (MCA 373) and VTCC 3588.1, VTCC 3588.3, VTCC 3588.5, VTCC 3588.8, VTCC 3588.13, and VTCC 3588.18 (MCA 189).

Results and Discussion

Spore prints from a total of 49 *Crepidotus* collections representing 27 morphological taxa were plated during a three year period in an attempt to obtain monokaryotic single spore isolates (SSIs) (Aime 1999, Aime and Miller Chap. 4). The same studies found that the recovery of SSIs in *Crepidotus* was complicated by a dormant period in the temperate North American species and by a very low rate of germinations per plate. Ultimately, only nine of the collections plated in those studies yielded more than ten SSIs; the largest number of isolates (39) was obtained for collection MCA 373, *C. aureifolius*, which was repeatedly plated to gauge the effect of various abiotic factors on spore dormancy and germination. Furthermore, it was discovered that the viability of monokaryotic isolates was reduced after long term storage at 4 C. As a result, only two collections of *Crepidotus*, MCA 373 and MCA 189, yielded an adequate number of SSIs for inferring mating types.

Figure 6.1 shows the results from intracollection matings of *Crepidotus aureifolius* (MCA 373). Two mating types were identified indicating the presence of a unifactorial (bipolar) mating system. Assignment of mating types for this collection are shown in Table 6.1. Figure 6.2 shows the results from intracollection matings of *Crepidotus nephrodes* (MCA 189), which also indicate a unifactorial system; assignment of mating types are given in Table 6.2.

	3600.4	3600.8	3600.12	3600.14	3600.15	3600.18	3600.39
3600.2	—	—	—	+	+	—	+
3600.4		—	—	+	+	—	+
3600.8			—	+	+	—	+
3600.12				+	+	—	+
3600.14					—	+	—
3600.15						+	—
3600.18							+

Fig. 6.1. Intracollection mating of single spore isolates of *Crepidotus aureifolius* (MCA 373). + = clamp connections formed; — = no clamp connections formed.

Table 6.1. Assignment of mating types to single spore isolates of *Crepidotus aureifolius* (MCA 373), based on intracollection crosses.

Mating type	Isolate number
A1	.2, .4, .8, .12, .18
A2	.14, .15, .39

	3588.3	3588.5	3588.8	3588.13	3588.18
3588.1	+	+	+	—	—
3588.3		—	—	+	—
3588.5			—	+	—
3588.8				+	—
3588.13					+

Fig. 6.2. Intracollection mating of single spore isolates of *Crepidotus nephrodes* (MCA 189). + = clamp connections formed; — = no clamp connections formed.

Table 6.2. Assignment of mating types to single spore isolates of *Crepidotus nephrodes* (MCA 189), based on intracollection crosses.

Mating type	Isolate number
A1	.1, .13,
A2	.3, .5, .8, .18

Contact zone morphology varied among crosses from almost no observable interaction to a dense hyaline line of mycelium built up at the interaction zone. No correlation could be made between the contact zone morphology and the type of mating interaction. However, there

was some tendency for isolates to form certain types of interactions, as has been found in the genus *Melanotus* (Petersen 1992). Specifically, a nodulose interaction zone was only formed in some of the crosses with isolate 3600.18, while a dense build-up of hyaline mycelium forming a line between isolates was found in three of the seven crosses with isolate 3600.4.

The morphology of clamp connections was also diverse and clamp morphology appears to correlate with isolate strain. The majority of dikaryons produced clamps at about 20-25% of septa. In contrast, one strain (3600.15), when crossed with compatible isolates, produced fewer clamps of reduced size, while compatible crosses formed with isolate 3600.39 produced an abundance of larger clamps.

In some compatible pairings, clamp connections were not observed until the cross had been allowed to incubate for an additional two to eight weeks after a contact zone had been established. However, all dikaryons obtained from the interface of compatible pairings and grown out separately remained stable and continued to exhibit clamp connections at the growing margin after incubation for several months.

Since it has been demonstrated that, statistically, a minimum of 11 SSIs from a single collection should be self-crossed to ensure retrieval of all four mating types in a tetrapolar mating system (Vilgalys 1982), it is possible but unlikely that, had more isolates been available for crosses, two additional compatible mating types would have been uncovered, resulting in a bifactorial system for this fungus. However, the same statistics show that at least three mating types are likely to be uncovered when either six or eight SSIs are crossed pair-wise. We therefore conclude that these fungi possess a unifactorial mating system.

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7

Intercompatibility tests and phylogenetic analysis in the *Crepidotus* Sphaerula group complex: Concordance between ICGs and nuclear rDNA sequences highlight phenotypic plasticity within species.

Manuscript.

Abstract

Sexual intercompatibility was tested within a group of morphological species of *Crepidotus* (Fr.) Kumm. belonging to subgenus *Sphaerula*, section *Sphaerula* Hesler and Smith. Intercompatibility tests were applied to 15 collections representing nine morphological species in this complex for which monokaryotic isolates could be obtained. These experiments indicated the existence of only five intercompatibility groups (ICGs). The ICGs uncovered correlate with phylogenetic species found with sequencing analysis of the nuclear DNA coding for the large ribosomal subunit (nLSU). Phenotypic diversity within ICGs was found to be much greater than previously suspected, especially in ICG4 where five distinct morphological species were found to be intercompatible. Sources of interincompatibility in the group are discussed.

Introduction

Crepidotus is comprised of mostly small, pleurotoid, saprophytic agarics, with an estimated 150 species in the genus (Singer 1986). Subgenus *Sphaerula* Hesler and Smith

(1965) contains 42 taxa, many of which are distinguished on the basis of single morphological character differences that may or may not be true indicators of non-interbreeding species. Section *Sphaerula* unites those pleurotoid species of *Crepidotus* that possess globose basidiospores (Hesler and Smith 1965). Phylogenetic studies based on nLSU sequencing data have shown that this section is not monophyletic and members show several evolutionary origins within *Crepidotus* (Aime Chap. 3, clades II, VII, VIII, and IX). These different phylogenetic lineages of Hesler and Smith's subgenus can be distinguished by analyzing the nature of the exosporial ornamentation by scanning electron microscopy (SEM). The microscopic punctate appearance of basidiospores in clades VIII and the *C. nyssicola* (Murr.) Sing. lineage in clade VII (Chap 3, Fig. 3.1) arises from numerous truncate columns embedded in the exosporium (Appendix 2, Figs. A.2.1-8). The punctate appearance of spores in clades II (*C. aureus* Horak) and clade IX (Chap. 3, Fig. 3.1) is due to fused columns or warts (Appendix 2, Figs. A.2.13-14). Of those species with truncate columnar exosporial ornamentation, those in clade VII can be distinguished from clade VIII by a combination of vegetative and microscopic characters (Aime Chap. 5). The *C. nyssicola* lineage, termed the Nyssicola group (Aime Chap. 3), as exemplified by *C. applanatus* s. Josserand, was previously discussed (Aime Chap. 5). This paper will focus on those species belonging to clade VIII, hereinafter referred to as the Sphaerula group.

While sequencing studies identified lineages with different evolutionary origins within Subgenus *Sphaerula* Hesler and Smith, they also showed little or no sequence divergence between several morphological species in the Sphaerula group (Aime Chap. 3). Such results may be interpreted as either evidence of recent speciation within this complex with insufficient

time for nucleotide substitution to be observable (Murphy 1997), or, conversely, as a single biological species with an unsuspected amount of phenotypic plasticity. Intercompatibility experiments in the fungi are often employed for delimiting species within taxonomically problematic groups (Anderson and Ullrich 1979, Galland et al. 1979, Boidin 1986, Petersen 1995a, Petersen 1995b, Petersen and Hughes 1999). Intercompatibility testing has uncovered evidence of recent or incipient biological speciation where other methods could not (Galland et al. 1979, Murphy 1997, Wilson 1990, Miller and Pearce 1996). These studies have helped resolve questions regarding the degree of morphological variability within diverse agaric species complexes (Vilgalys and Miller 1987, Lamoure 1989). Finally, intercompatibility tests, when combined with molecular phylogenetic analyses, can provide a powerful framework for the application of species concepts and determining patterns of speciation within taxonomically difficult genera or complexes (Vilgalys 1991, Vilgalys et al. 1996, Aanen 1999).

Two distinct mating compatibility regulatory systems are present in agarics. Homogenic incompatibility refers to the mating system present within a given species; homogenic incompatibility tends to reduce inbreeding and mating will not occur when the same mating allele(s) is shared between two haplonts of the same species (Raper 1966, Kemp 1995). Two types of homogenic mating systems occur in agarics, unifactorial and bifactorial, differentiated by the number of mating loci involved in mating system interactions (Raper 1966). Species whose homogenic incompatibility is determined by two loci are termed bifactorial (tetrapolar). However, *Crepidotus* species in the Sphaerula group have been identified as unifactorial (bipolar), i.e., only one locus is involved in mating-system interactions (Aime and Ball Chap. 6). Because potentially hundreds of different alleles for each locus (loci) can exist within an

interbreeding species, most haplont pairings within a species are compatible, while haplont pairings from a single basidiocarp are compatible in only 25% (tetrapolar) or 50% (bipolar) of crosses, which is the probability that any two haplonts will share the same mating-system allele.

Heterogenic incompatibility (also termed intersterility) is involved in species recognition and restricts gene flow between sufficiently incompatible populations (Kemp 1980), and presumably leads to speciation (Kemp 1995). In the case of heterogenic incompatibility, two haplonts will mate if they share a single allele at any one of the different heterogenic loci, but pairings of haplonts from different interbreeding groups will fail to undergo dikaryon formation even if the homogenic alleles are different [Chase and Ullrich 1990a, (although the term "heterokaryon incompatibility" is applied here, the authors are clearly discussing the heterogenic, not heterokaryon, as defined below, incompatibility system)]. Regulation of heterogenic incompatibility is not well characterized, but interactions appear to be under the control of between two (Kemp 1995) to at least four to five loci in some species (Chase and Ullrich 1990b).

A third type of mating incompatibility system has more recently been identified in one agaric genus and may exist in others. Heterokaryon incompatibility occurs when two otherwise compatible haplonts do not form a dikaryon because they have different alleles at one or more of the loci regulated by this system (Kemp 1980). Heterokaryon incompatibility appears to function in reducing heterozygosity by controlling fusion of vegetative hyphae between strains (Kemp 1995); it is not known whether all species or strains carry heterokaryon-type alleles, nor how many loci or alleles per locus exist (Kemp 1980), although at least ten loci have thus far

been identified (Kemp 1995). The typical agaric life cycle, including stages where different incompatibility systems are presumed to take effect, is illustrated in Fig. 0.1.

Formation of a stable dikaryon can only result from pairing of haplonts compatible across all three systems described above. Failure to form a stable dikaryon can clearly result from incompatibility in any one, or combination of, the above systems, only one of which (heterogenic) results from intersterility between biological species. Since uncovering true intersterility groups is the goal of most mating studies (Boidin 1986, Petersen 1995a, Petersen 1999b), caution must be exercised in interpreting data less erroneous or misleading results are reported (Kemp 1980, Wilson 1990). Intersterility groups are usually equated with biological species in mycological studies (Petersen and Hughes 1999) although this requires the assumption that all dikaryons formed by two compatible haplonts can form fertile basidiocarps (Boidin 1986, Aanen and Kuyper 1999). Where demonstration of fertility is not possible, use of the term intercompatibility group (ICG) is preferred (Boidin 1986).

In this study, monokaryotic isolates from different morphological species in the Sphaerula group were mated in culture to identify ICGs. Compatible crosses were subcultured and monitored for several months to determine the dikaryon stability in lieu of fruiting. Results of intercompatability tests are compared with results from nLSU sequencing analysis and with vegetative morphology; taxonomy and gross morphology of this group will be addressed in a separate paper (Aime Chap. 8). Possible mechanisms of interincompatibility between haplonts from the same ICG are discussed. Because obtaining monkaryotic isolates in *Crepidotus* is difficult (Aime and Miller Chap. 4, Aime and Ball Chap. 6) and cultures slow-growing, a final

objective was to produce a biological yardstick for evaluating phylogenetic data in the study of other groups of *Crepidotus* where intercompatibility testing is impractical or impossible.

Materials and methods

Intercompatibility tests—Methods for obtaining single spore isolates (SSIs) were described in Aime and Miller (Chap. 4). Monokaryotic strains were stored at 4 C for six months to three years prior to use in this study. Viable isolates were available from a total of 15 collections of *Crepidotus* in the Sphaerula group (Table 7.1). All cultures are maintained in the Virginia Tech Culture Collection (VTCC). After removal from storage, isolates were grown out on individual malt agar (15 g Sigma malt extract, 15 g Sigma agar, 1 L distilled water) plates at 24-26 C, and screened for the absence of clamp connections before use in mating experiments.

Isolates chosen for study were selected at random from each collection. Initially, one, or where available, two, haplonts from each of ten collections were paired in all possible combinations following the methods in Aime and Ball (Chap. 6). An additional five collections were screened by crossing one or two isolates from each with each other and with at least two randomly chosen testers from each ICG identified in the initial experiments, in the manner of Vilgalys and Miller (1987). A compatible reaction was inferred by the presence of clamp connections in the interface between two paired haplonts. Since sample size was relatively small, stringent procedures for scoring data were adopted; cultures were incubated for up to four months with periodic screening, and the interface from compatible crosses was subcultured for three to four months to determine dikaryon stability (Boidin 1986). Stable dikaryons were

Table 7.1. Collections of the *Crepidotus* Sphaerula group complex used in this study.[1]

Culture no.	Locality, Collection no.	Date	Morphological species	Phylogenetic clade	ICG
3666.4	Puerto Rico, Bisley Exp. Plots, MCA 442	3-June-98	*C.* sp.	I	1
3696.1	Japan, Shiga-ken, MCA 941	21-Sept.-99	*C.* sp.	II	2
3587.12	VA, Cherokee Flats, MCA 188	14-Sept.-96	*C. applanatus* v. *globigera* (Berk.) Sacc.	III	3
3587.16	"	"	"	III	3
3605.20	VA, Cherokee Flats, MCA 343	21-Aug.-97	*C. malachius* v. *malachius* (Berk. & Curt.) Sacc.	III	3
3685.2	NC, Durham, MCA 775	22-June-99	*C. malachius* v. *malachius*	III	3
3685.3	"	"	"	III	3
	Japan, Nagano-ken, MCA 719	24-Aug. 98	*C. malachius* v. *trichiferus* Hesler & Smith	III	
3588.3	VA, Cherokee Flats, MCA 189	14-Sept.-96	*C. nephrodes* (Berk. & Curt.) Sacc.	IV	4
3588.13	"	"	"	IV	4
3600.4	NC, Iredell Co., MCA 373	17-Sept.-97	*C. aureifolius* Hesler & Smith	IV	4
3600.18	"	"	"	IV	4
3603.15	NC, Durham, MCA 386	31-Oct.-97	*C. distortus* Hesler & Smith	IV	4
3667.19	VA, Cascades Rec. Area, MCA 749A	3-Oct.-98	*C. appalachianensis* Hesler & Smith	IV	4
3676.1	NC, Durham, MCA 781	22-June-99	*C. nephrodes*	IV	4
3676.2	"	"	"	IV	4
3677.11	NC, Durham, MCA 783	22-June-99	*C. nephrodes*	IV	4
3677.18	"	"	"	IV	4
3680.13	VA, Cascades Rec. Area, MCA 836	16-Sept.-99	*C. nephrodes*	IV	4
3680.19	"	"	"	IV	4
3687.7	NC, Durham, MCA 784	22-June-99	*C. nephrodes*	IV	4
3687.12	"	"	"	IV	4
3688.17	VA, Cascades Rec. Area, MCA 841	16-Sept.-99	*C. aureifolius*	IV	4
	VA, Wintergreen, OKM 27048	19-Sept. 97	*C. appalachianensis*	IV	
	VA, Mountain Lake, OKM 25806	9-Sept.-93	*C. crocophyllus* (Berk.) Sacc.	IV	
	MO, Jefferson Co., OKM 26173	1-Oct.-94	*C. crocophyllus*	IV	
	VA, Pandapas Pond, OKM 25896	26-Sept.-93	*C. nephrodes*	IV	
3700.5	MO, Duck Creek Wildlife Ref., OKM 27540	12-Aug.-99	*C.* sp.	V	5
3700.7	"	"	"	V	5

[1] Collections used in the mating study are designated by a culture number; collections without a culture number were used in the phylogenetic study only.

inferred by the presence of abundant clamp connections at the growing margin of subcultures. Paired isolates that produced stable dikaryons were considered to belong to the same ICG.

Morphological data were recorded for all original isolates from each collection prior to storage; morphological data were recorded a second time for each retrieved isolate used in this study. Data on vegetative morphology were pooled for each SSI (including those not used in this study) within each collection assigned to each ICG.

Molecular phylogenetic analysis—DNA was extracted from fresh or dried basidiocarps; collections used are given in Table 7.1. Voucher collections from which all cultures and DNA sequences were derived are housed at Virginia Tech (VPI). Methods for extraction, amplification, and sequencing of the 5'-end of the nuclear large subunit rDNA (nLSU) follow Aime (Chap. 2). Sphaerula group sequences used for phylogenetic analysis were published in Aime (Chap. 3), with the addition of one sequence from GenBank (*C. crocophyllus* AF139946) and four additional collections: *C. malachius* v. *malachius* (MCA 775), GenBank no. AY029706; *C. malachius* v. *malachius* (MCA 343), GenBank no. AF205674; *C. nephrodes* (OKM 25896), GenBank no. AF205693; and *C. crocophyllus* (OKM 26173), GenBank no. AY029707. Two *Crepidotus* taxa from outside the Sphaerula group, *C. mollis* (Fr.) Staude and *C. brunnescens* Hesler and Smith, were included for rooting purposes.

Sequences were manually aligned and analyzed in PAUP* 4.0b5 for Macintosh (D. Swofford 2001). The data matrix included a total of 1176 characters (including gaps), 63 of which were parsimony-informative. Parsimony analyses were performed using heuristic search algorithms with multiple (10) random sequence additions to generate starting trees, and tree-

bisection-reconnection (TBR) branch-swapping. Bootstrapping frequencies (Hillis and Bull 1993) were calculated using TBR branch swapping with 1000 replicates; support of greater than 50% was considered significant. Jackknifing frequencies (Lanyon 1985) were calculated using TBR branch swapping with 1000 replicates; support of greater than 50% was considered significant.

Results

Phylogenetic analysis—The eleven morphological taxa examined within the Sphaerula group belong to only five distinct clades or lineages (Fig. 7.1). Clade III is represented by four collections from three morphological taxa; sequences were identical across all 1176 bases with the exception of a single base deletion at position #708 for *C. malachius* v. *trichiferus* from Japan. Clade IV is comprised of seven collections representing four species; sequences are identical except that a collection of *C. nephrodes* from Virginia (MCA 189) shows a single base deletion at position #684. Clades I, II, and V are each composed of a single undescribed species. Clades differ from each other at a minimum of ten positions.

Intercompatibility tests—Results from intercollection pairings are shown in Table 7.2. All isolates tested can be unambiguously assigned to one of five identified ICGs that correspond to the five phylogenetic clades as shown in Table 7.1. Vegetative morphology of haplonts is variable between groups; diagnosable ICG-specific phenotypes can be identified and are summarized in Table 7.3.

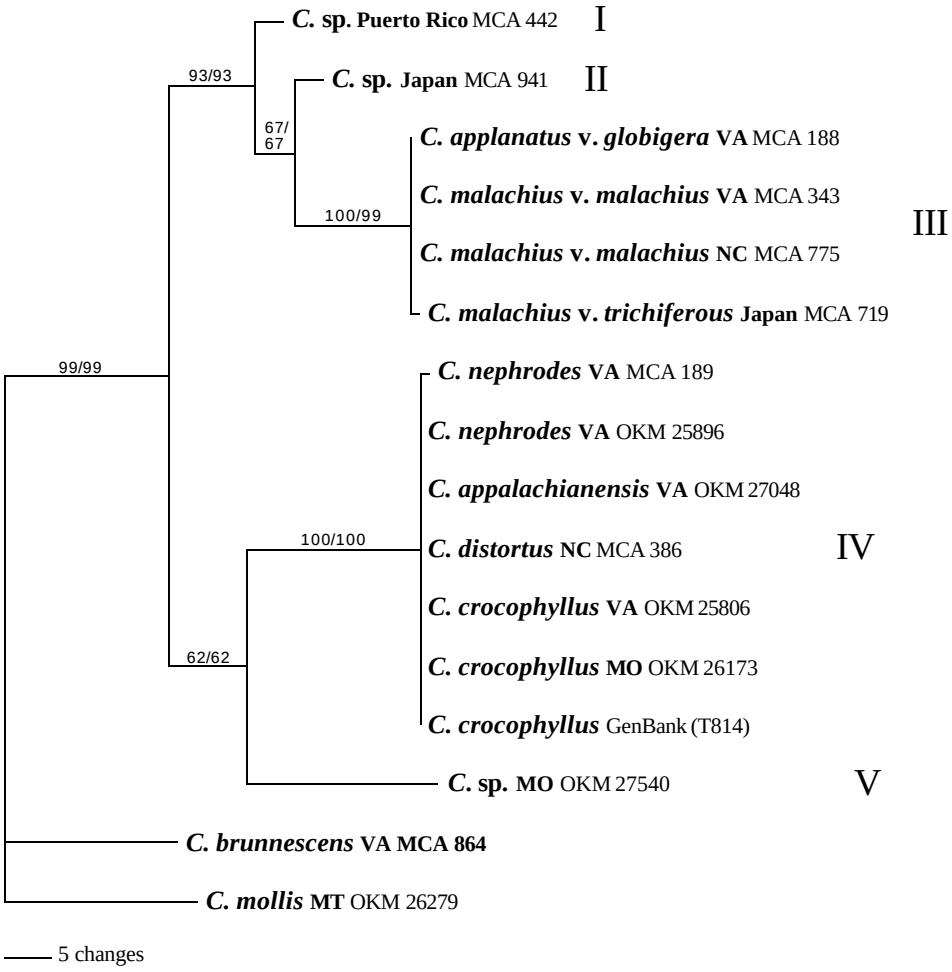

Fig. 7.1. Phylogenetic relationships of morphological species in the *Crepidotus* Sphaerula group complex (clade VIII, Aime Chap. 5) based on nLSU rDNA sequences. Bootstrapping and jackknifing values (respectively) are indicated above supported branches. Clades are designated by roman numerals and correspond to phylogenetic lineages discussed in text.

Table 7.2. Intercompatibility test results within the Sphaerula group complex

ICG	Isolate	5		4														3					2
		3700.7	3700.5	3688.17	3677.18	3677.11	3667.19	3603.15	3600.18	3600.4	3687.12	3687.7	3680.19	3680.13	3676.2	3676.1	3588.13	3588.3	3587.16	3587.12	3685.3	3685.2	3605.20	3696.1
1	3666.4	–^{2}						–^{2}			–^{2}												–^{2}	–^{2}
2	3696.1	–^{2}	–^{2}				–^{2}	–^{2}	–^{4}	–	–	–	–	–	–	–^{2}	–	–^{4}	–^{2}	p	–^{2}	p^{2}	–	
3	3605.20	–^{2}	–^{3}				–^{2}	–^{2}	–^{3}	–^{3}	–^{3}	–	–^{2}	–^{2}	–^{2}	–^{2}	–^{2}	–	–^{4}	+	+^{2}	–^{2}	–		
	3685.2			–																+^{1}	+^{1}				
	3685.3			–																+	+				
	3587.12						–^{2}	–^{2}	–^{4}	–	–^{2}	–^{2}	–^{2}	–^{2}	–^{2}	–^{2}	–^{2}	–^{5}							
	3587.16			–^{2}	–^{2}		–^{2}	–^{5}	–^{2}	–^{2}	–^{5}	–	–	–^{5}	–^{5}	–	–^{5}	–							
4	3588.3					+	+^{2}	+	+	+	+	+	+^{2}	+	+^{2}	+									
	3588.13						+^{2}	+^{3}	+	+^{1}	+^{2}	+^{2}	+	+	+^{1}	+									
	3676.1						+^{2}	+	+	+^{3}	+	+	+	+											
	3676.2			+^{2}		+	+	+^{1}	+	+	+	+^{1}	+												
	3680.13			+^{1}	+	+	–^{2}	+	+	+	+	+^{1}												
	3680.19						–^{2}	+	+	+	+	+												
	3687.7				+		+^{2}	+	+	+^{3}														
	3687.12	–	–^{2}	+^{2}		+^{1}	+	+	+	+^{3}														
	3600.4			+			+^{2}	+																
	3600.18					+^{1}	+^{2}	+																
	3603.15	–	–^{5}	+^{2}	+	+	+^{1}																	
	3667.19			+^{2}	+	+																		
	3677.11			+																				

Compatibility Scoring:

+ = clamps formed at interface; dikaryons remain stable.

– = no clamp formation at interface.

p = very few pseudoclamps formed at interface only after 3.5 months incubation; no stable dikaryon.

Interface morphology:

^{1} = submerged nodulose clusters of hyphae within scantier superficial growth, hyaline.

^{2} = colonies flush with no distinct interface morphology.

^{3} = hyaline wall of hyphae built up between colonies, denser than adjacent growth.

^{4} = pigmented wall of hyphae built up between colonies, denser than adjacent growth (barrier reaction).

^{5} = mutual repulsion of colonies (barrage reaction).

No superscript = interwoven, appressed, and hyaline, scantier growth than adjacent colonies.

Table 7.3. Culture morphology of single spore isolates within ICGs[1].

ICG	Growth rate[2]	Colony color/texture	New growth color/texture	Diffusible pigments in media	Inoculation plug	Reverse
1	fast	white/cottony	same	brownish-orange	brown diffusible pigments	brownish-orange
2	medium	white/silky	same	none	brown diffusible pigments	Hyaline
3	medium	hyaline to white/silky	same	none	brown diffusible pigments	Hyaline
4[3]	slow	orange/creamy	same	none	white crustose	orange
	medium	yellow-orange/fibrillose	cream/nodulose	"	"	yellow
	"	yellow/fibrillose	"	"	"	"
	"	cream/silky	"	"	"	cream
5	medium	orange-brown/creamy	cream to hyaline/nodulose	none	no differentiation	same as surface

[1] Data are pooled and summarized for all SSIs recovered from each collection known to belong to each ICG.
[2]Relative growth rates. Fastest isolates covered a centrally inoculated 55 mm culture plate within about two weeks; isolates with a medium growth rate covered a centrally inoculated 55 mm plate within about six weeks; slowest isolates reached a maximum colony diameter of about 20-25 mm after three months.
[3]ICG4 showed four intergrading SSI morphologies, indicated by four separate entry lines.

Establishment of an interface between haplonts usually occurred within about three to four weeks; clamp connections in compatible pairings were usually observed in the interface about a week after establishment and were present on at least 25% of septa. Isolates with rich pigmentation (e.g., 3667.19, 3688.17) exhibited greatly retarded growth and crosses with these strains required incubation periods of at least two weeks post-interface establishment before clamp connections were observed. Pairings between isolates from ICG3 were also slow to develop clamp connections after interface establishment. However, in all cases, initial positive interactions remained stable after subculturing and subsequent colony growth.

Six types of macroscopic interactions were observed between paired haplonts (Table 7.2). The most commonly formed interaction consisted of a zone of less dense, superficial, slightly interwoven or overlapping hyphae from both colonies, and occurred in both compatible and incompatible crosses. Colonies were also observed growing flush against each other at the point of contact, with no thinning at the interface, in both compatible and incompatible crosses. A third type of interaction, here termed nodulose, was frequently observed only in compatible crosses. In the nodulose interaction, the interface zone consisted of dense knots of submerged hyaline hyphae within a band of more interwoven, superficial hyphae from both colonies. A fourth type of interaction is characterized by the production of a thin, submerged uniform wall of hyaline hyphae between colonies that was observed in a few pairings. This type of interaction appeared strain-specific, limited almost entirely to a few crossings with either isolate 3605.20 or 3600.4.

The final two types of macroscopic interactions were only observed in incompatible crosses. Interaction zones consisting of a dense buildup of brown or orange-brown pigmented

hyphae transecting the interface (classic "barrier" reaction) were rare and also appeared to be isolate-specific, only occurring in some incompatible crosses with either isolate 3600.18 or 3588.3. The final type of interaction consisted of mutual repulsion and a clear zone of no growth between colonies (classic "barrage" interaction), even after several months' incubation. The barrage type interaction occurred most frequently in crosses between ICG3 and ICG4. Fruiting body initials were never observed, and, despite numerous experiments, fruiting of dikaryons was never achieved in culture (data not presented).

All ICGs identified were completely isolated with no cases of partial intercompatibility found. However, although no clamp-like structures were found in earlier screenings, after three and one-half months' incubation, pseudoclamps (or false clamps) had formed on a few septa in the interface between the isolate from ICG2 and two strains of ICG3. Subcultures from the interface between these pairings exhibited neither clamps nor false clamps.

Two cases of interincompatibility were found. Within ICG3, strain 3605.20 (Virginia) did not form dikaryons with either strain of 3685 (North Carolina). All haplont pairings within ICG4 were completely intercompatible except between isolate 3667.19 (Virginia, Cascades Recreational Area) and 3680.13 and 3680.19 (Virginia, Cascades Recreational Area).

Discussion

Sample size in these experiments has been limited due to poor recovery of haplont isolates in *Crepidotus*. Because obtaining large samples for mating studies is not always feasible, Boidin (1986) has provided a series of criteria for evaluating such data, which have been adhered to in interpreting the results from this study. Murphy and Miller (1997)

demonstrated that a larger portion of homogenic mating alleles were shared *between* populations of the bipolar fungus *Collybia subnuda* (Ellis ex Pk.) Gilliam than *within* a population, a finding that suggests sampling from within a few populations may provide as much allelic diversity as larger and broader sampling from across many populations.

Macroscopic interactions between haplonts have been predictable indicators of compatibility for some agarics (Raper 1966, Anderson and Ullrich 1979, Murphy and Miller 1997), but are not consistent within this group. Antagonistic barrage (Raper 1966, Boidin 1986, Wilson 1990,) and barrier (Wilson 1990) reactions were recorded only in incompatible matings, but appeared to be strain- or ICG-specific rather than universal, as has also reported by Petersen (1995a) and Aime and Ball (Chap. 6).

Fertility barriers between ICGs appear to be absolute, with no instances of inter-ICG compatibility observed. A very slight degree of mating "recognition", in the form of pseudoclamps, however, was observed in a few haplont pairings of ICG2 with ICG3. Pseudoclamps are normally formed in tetrapolar mating systems between isolates that share the same B-locus mating allele (Raper 1966). Since species of *Crepidotus* in the Sphaerula group have been shown to posses a bipolar mating system (Aime and Ball Chap. 6), such interactions are difficult to interpret in this instance.

Limited formation of pseudoclamps in a few inter-ICG pairings has also been observed in the *Collybia dryophila* complex (Vilgalys and Miller 1987). Although this group has a tetrapolar mating system, false clamp formation could not be attributed to a common-B interaction (Vilgalys and Miller 1987). Very prolonged incubation periods were found to induce dikaryon formation in a few inter-ICG pairings that were otherwise completely intersterile

(Murphy 1997). Finally, false clamps were also observed in intra-ICG matings where common-B interactions could again be ruled out (Vilgalys 1991). While the above results remain inexplicable, the homogenic incompatibility system does not appear to be involved. More likely, if, as has been proposed (Chase and Ullrich 1990a, 1990b, Vilgalys 1991), the evolution of loci in different incompatibility systems is not codependent or linked, then some form of inter-ICG recognition may still exist within one incompatibility system, whereas incompatibility in the others still form a barrier to interfertility. Such mating "recognition" would be most expected in cases of recent or incipient speciation events, e.g., between sister species as was the case in this study and Murphy (1997), and might require long incubations prior to manifestation.

Two intra-ICG pairings within ICG4 failed to produce a dikaryon (3680.13 and 3680.19 X 3667.19). Both of these collections were obtained from the same locality in consecutive years, and both are completely compatible with all other strains from the same ICG. It is quite possible that sampling occurred from within the same or two closely related genets and that incompatibility between these collections is due to a shared mating allele from the homogenic system, which would indicate that these collections are members of the same species.

A possible case of restricted gene flow is represented by the pattern of interincompatibility found in ICG3. Two Virginia collections are intercompatible, but only one (3587) is compatible with a North Carolina collection. A recent argument has been made for interpreting such cases of triangular partial intercompatibility (known as A-B-C type interactions), as the result of heterokaryon incompatibility (Kemp 1980), which would indicate

that all members belong to the same species. However, the presence of shared mating alleles (homogenic incompatibility) cannot be dismissed (same species), nor can the possibility of heterogenic incompatibility. Interestingly, Petersen (1995a) reported a similar pattern of cryptic speciation within a morphological species of the saprotrophic genus *Marasmius* Fr. wherein one ICG appeared to be widespread throughout North America and Europe while the other was confined to the Southern Appalachian Mountains. More mating intercompatibility testing is needed within this ICG before any satisfactory conclusions can be drawn.

The failure to produce fertile fruiting bodies in culture precludes equating the five ICGs in this study with true biological species. Nevertheless, the formation of stable dikaryons between compatible haplonts certainly indicates a close intercompatibility relationship and the potential to interbreed between members of the same ICG (Boidin 1986, Lamoure 1989, Wilson 1990). When intercompatibility data (Table 7.2) are combined with the phylogenetic data (Fig. 7.1) and vegetative macromorphology (Table 7.3), an interpretation of five distinct species is fully supported, despite the large number of described morphotaxa included within some of these species. Morphology and taxonomy of ICG4 will be treated in a separate paper.

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8

Crepidotus crocophyllus: Phenotypic plasticity and secondary growth of basidia with concomitant spore production.

Manuscript.

Abstract

Previous mating intercompatibility studies and phylogenetic analyses based on nuclear large subunit ribosomal DNA sequences (nLSU) have shown that several morphological taxa allied in *Crepidotus* Subgenus *Sphaerula* Section *Sphaerula* Subsection *Fulvifibrillosi* Hesler and Smith represent a single species. This has led to an examination of type collections, and a re-evaluation of the phenotypic characters used to define taxa in this complex. *Crepidotus distortus* Hesler and Smith, *C. appalachianensis* Hesler and Smith, *C. subaureifolius* Hesler and Smith, *C. aureifolius* Hesler and Smith, and *C. nephrodes* (Berk. and Curt.) Sacc. are conspecific with *C. crocophyllus* (Berk.) Sacc. *Crepidotus subfibrillosus* Hesler and Smith is confirmed as distinct. Culture studies of vegetative growth show that differences in pigmentation may be based, at least in part, on Mendelian determinants or to physiological changes accompanying maturation. Most revealing, however, is the finding that hymenial structures, previously described as pleurocystidia, arise as secondary growth from basidia in mature basidiocarps of *C. crocophyllus*. The taxa, microscopic features, and basidium life cycle are described, illustrated and discussed.

Introduction

Crepidotus (Fr.) Staude is comprised of mostly small, pleurotoid, saprotrophic agarics, with an estimated 150 species in the genus (Singer 1986, Hawksworth et al. 1995). Subgenus *Sphaerula* Hesler and Smith (1965) contains at least 42 taxa, divided into two Sections, and three Subsections, which are distinguished as those species of *Crepidotus* containing clamp connections on the basidiomata and possessing globose basidiospores. The artificiality of this classification has previously been demonstrated (Aime Chap. 3). Nonetheless, a core group of species, roughly corresponding to Section *Sphaerula* (Hesler and Smith 1965), termed Stirps Sphaerula has been recognized (Aime Chap. 3, Chap. 7). Scanning electron microscopy (SEM) of some of the members of this group has revealed exosporial basidiospore ornamentation composed of truncate columns in *C. applanatus* (Pers.:Fr.) Kumm. in Pegler and Young (1972), Clémençon (1977), and Ortega and Buendia (1989) and *C. nyssicola* (Murr.) Sing. in Bigelow (1980). Additional SEM studies of exemplars of the other lineages in the complex show this type of ornamentation exists within the members of Stirps Sphaerula to the exclusion of all other phylogenetic sections of the genus *Crepidotus* (Appendix 2).

Two different lineages have been uncovered within Stirps Sphaerula (Aime Chap. 3). One lineage, loosely corresponding to Hesler and Smith's (1965) Section *Nyssicola*, and termed the Nyssicola group (Aime Chap. 5), consists of *C. nyssicola*, *C. applanatus* sensu Joss., and allies, and can be defined by the consistently smaller basidiospores, vegetative characters, and the tendency of the basidiomata to form a true or pseudostipe (Aime Chap. 5). Members of the second lineage, termed the Sphaerula group (Aime Chap. 7), include *C. applanatus* sensu Senn-Irlet (1995), *C. nephrodes*, and *C. crocophyllus*, among others, and

are distinguished from the Nyssicola group by the consistently larger basidiospores (5-7 µm in diameter), clamped dikaryotic mycelium, and basidiomata that have broad lamellae and do not form a pseudostipe at maturity (Aime Chap. 5, Chap. 7). Additional analyses of nLSU sequences within the Sphaerula group, combined with mating intercompatibility tests, revealed that many morphological species placed in Subsection *Fulvifibrillosi* Hesler and Smith (1965) were conspecific (Aime Chap. 7), warranting a re-evaluation of the phenotypic characters previously applied to species circumscription in this complex. Taxonomy and morphology of the members of Subsection *Fulvifibrillosi* Hesler and Smith will be the subject of this paper.

Hesler and Smith (1965) define Subsection *Fulvifibrillosi* as containing those species of *Crepidotus* Section *Sphaerula* in which the pileus may be colored or white, but bears brown fibrils on the epicutis. Eight species are placed in *Fulvifibrillosi*, distinguished by the presence or absence and shape of pleurocystidia (sterile hymenial cells borne on the lamellar sides, also termed facial cystidia), shape of cheilocystidia (sterile hymenial cells borne on the lamellar edge, also termed marginal cystidia), color of lamellae, color of pileus, and basidiospore ornamentation. Members belonging to this Subsection are known throughout North America (Murrill 1917, Hesler 1937, 1943, Hesler and Smith 1965), South America (Singer 1954, Horak 1964, 1979, Senn-Irlet and de Miejer1998), Europe (Pilát 1948, Lazebnícek 1970, Toma 1972, Dermek 1987, Nordstein 1990, Stangl et al. 1991), Japan (Imai 1939, Ito 1959), Australia and New Zealand (Hood 1992, Grgurinovic 1997) and East Africa (Pegler 1977).

Type collections of the new taxa described by Hesler and Smith in Subsection *Fulvifibrillosi* have been examined, as have additional collections from North America and Europe. This paper provides: 1) a taxonomic revision of the species in Subsection

Fulvifibrillosi; 2) discussion, illustrations, and description of *C. crocophyllus*; 3) discussion of the morphological characters previously used to segregate species in this complex, including pigmentation and cystidial characteristics; 4) description, illustrations, and discussion of the origins of "pleurocystidia" within this complex.

Materials and Methods

Methods for gathering morphological data of basidiomata follow Aime et al. (Chap. 1). Color designations are given as descriptive terms; for basidiomata some color designations are from Kornerup and Wanscher (1978) and are indicated in the following manner: brownish orange (7C5), where "7C5" designates a plate, column, and row, respectively. Microscopic mounts were illustrated and photographed and nuclei stained as described in Aime (Chap. 5). Dikaryotic cultures were obtained by sterile tissue isolation (Watling no date), polysporus deposit (Aime 1999), or subculturing of compatible haplont pairings (Aime and Ball Chap. 6). Monokaryotic cultures were obtained by the single spore isolation method in Aime and Miller (Chap. 4). Data on vegetative culture characteristics were gathered following the methodology of Nobles (1948). Designations for herbaria are from Holmgren et al. (1990).

Taxonomy

***Crepidotus crocophyllus* (Berk.) Sacc**. Syll. Fung. 5:886. 1887. **Figs. 8.1-31, 8.38-42**

= *Agaricus crocophyllus* Berk., London Jour. Bot. 6:313. 1847.

= *Crepidotus applanatus* v. *crocophyllus* (Berk.) Pilát, Atl. Champ. Eur. 6:35. 1948.

= *Agaricus* (*Crepidotus*) *nephrodes* Berk. & Curt., Ann. Mag. Nat. Hist. II 12:442. 1853.

= *Crepidotus nephrodes* (Berk. & Curt.) Sacc., Syll. Fung. 5:882. 1887.

= *Agaricus dorsalis* Pk., N.Y. State Mus. Ann. Rep. 24:69. 1872.

= *Crepidotus dorsalis* (Pk.) Sacc., Syll. Fung. 5:883. 1887.

= *Crepidotus fulvifibrillosus* Murr., N. Amer. Flora 10:153. 1917.

= *Crepidotus applanatus* v. *fulvifibrillosus* (Murr.) Pilát, Atlas Champ. Eur. 6:35. 1948.

= *Crepidotus distortus* Hesler & Smith, N. Am. Spp. *Crepidotus*, p. 72. 1965.

= *Crepidotus appalachianensis* Hesler & Smith, N. Am. Spp. *Crepidotus*, p. 72. 1965.

= *Crepidotus subaureifolius* Hesler & Smith, N. Am. Spp. *Crepidotus*, p. 74. 1965.

= *Crepidotus aureifolius* Hesler & Smith, N. Am. Spp. *Crepidotus*, p. 75. 1965.

= *Crepidotus applanatus* s. Horak, Flora Cripto. Tierra del Fuego 11:325. 1979.

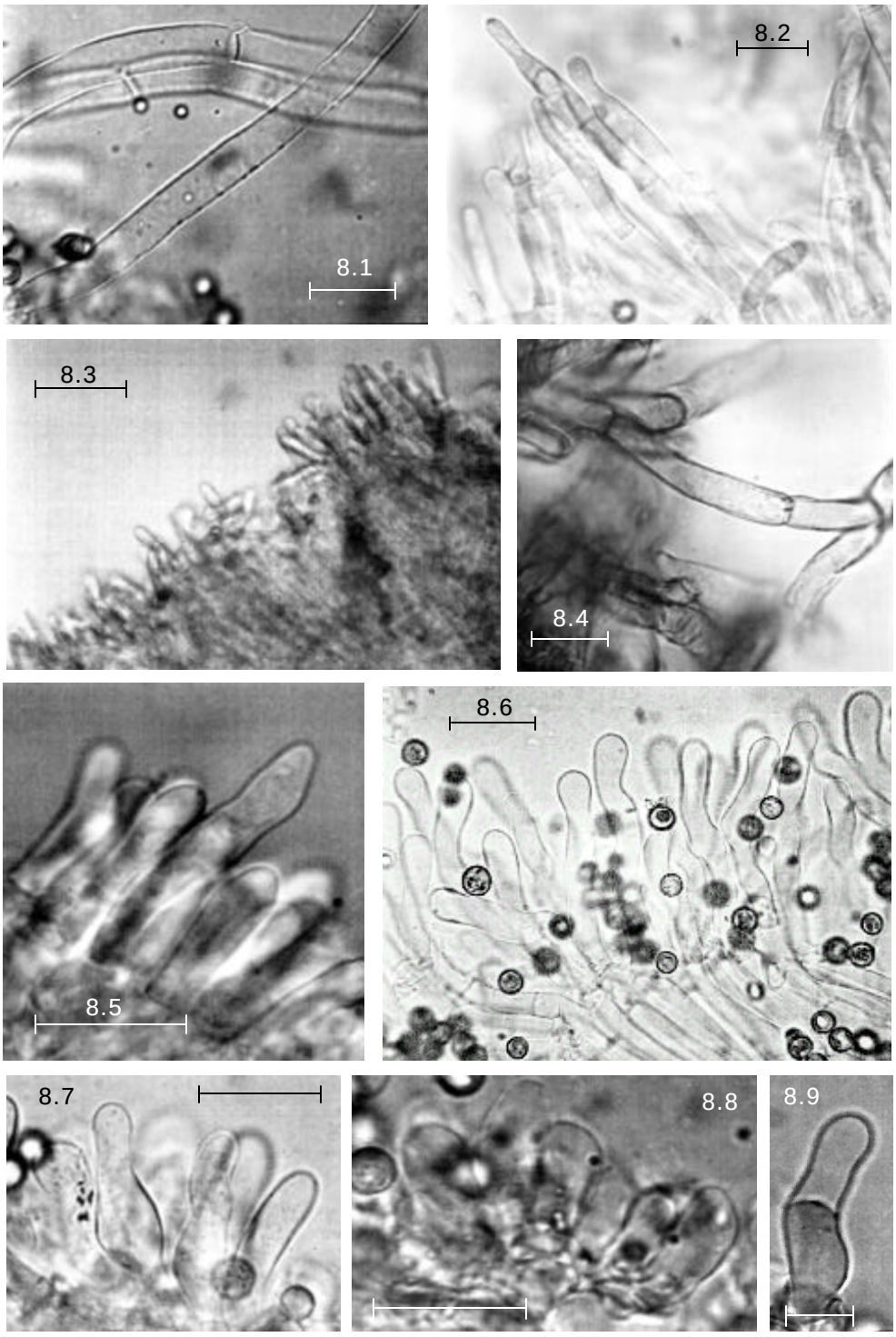
= *Crepidotus applanatus* s. Nordstein, in parte, Gen. *Crepidotus* in Norway, p. 88. 1990.

cf. *Crepidotus badiofloccosus* Imai, Stud. Agaricacearum Jap. I. p. 399. 1939.

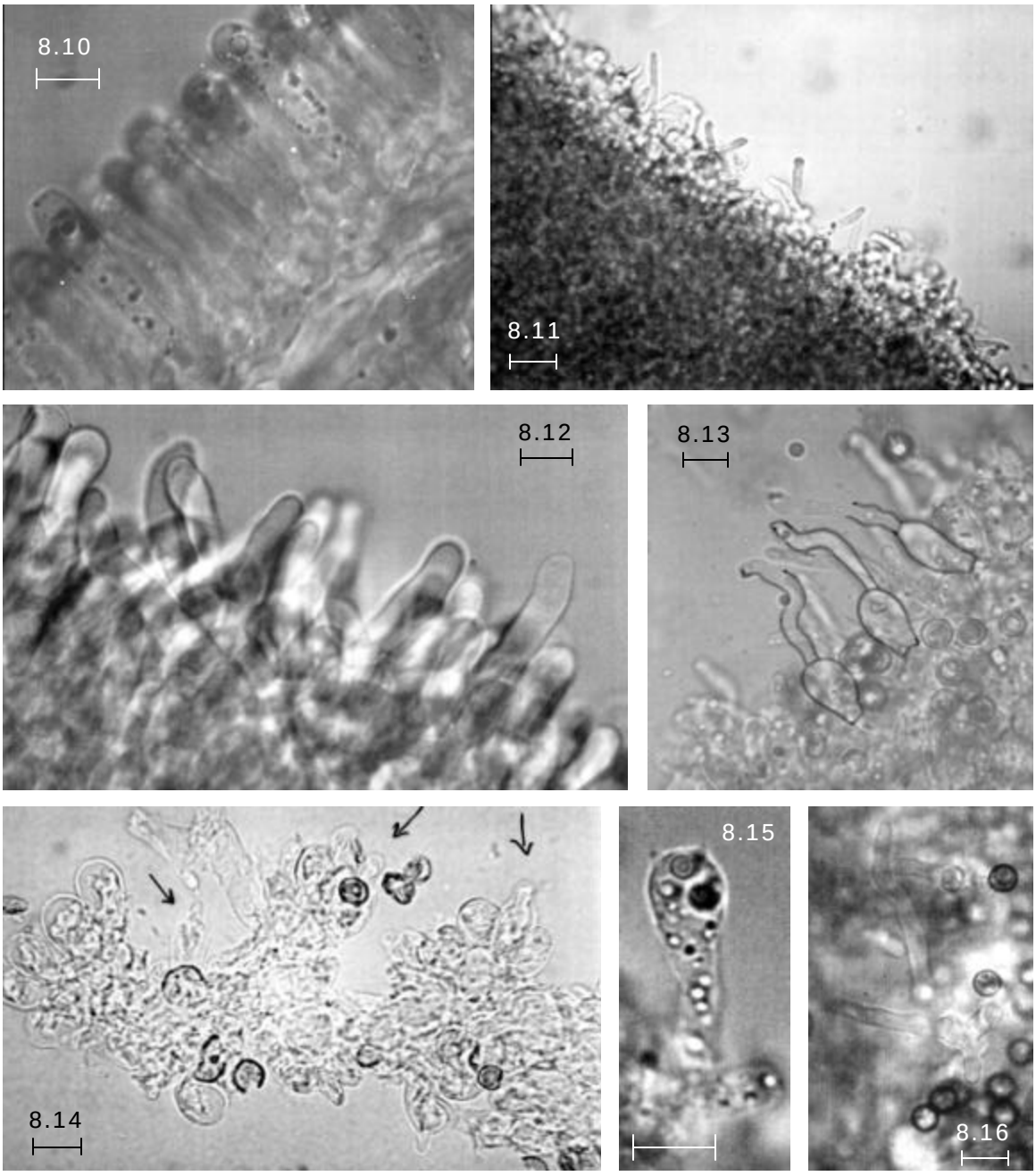
non *Crepidotus applanatus* s. Josserand, Bull. Soc. Myc. Fr. 53:219. 1937.

non *Crepidotus nephrodes* s. Singer, Nova Hedwig., p. 379. 1973.

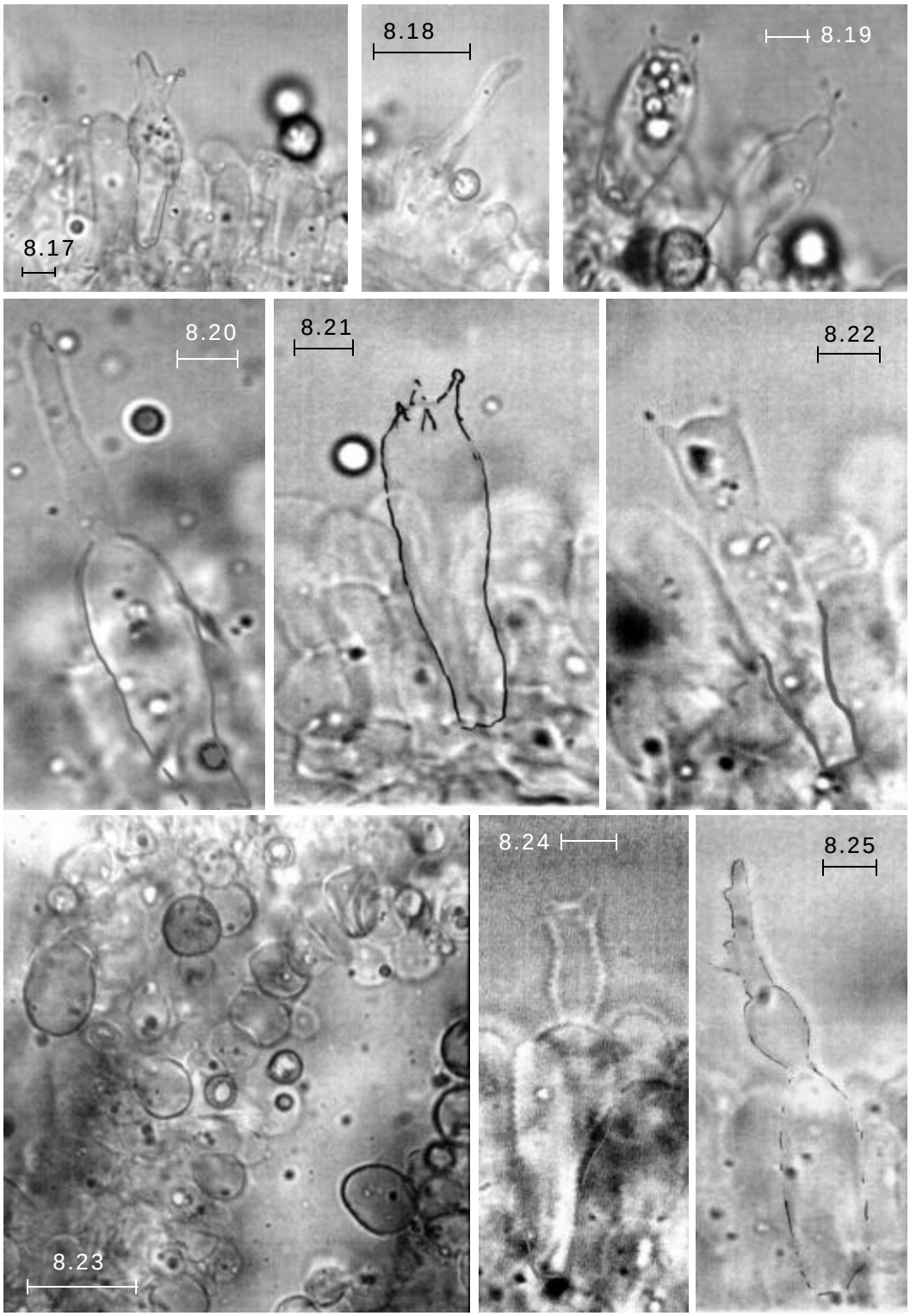
Pileus (Figs. 8.38, 8.40-42) 5-60 mm broad, sessile, semiorbicular, reniform, flabelliform, or dimidiate, convex to subconchate, white, off-white, pale yellow, yellow-ochre, pinkish orange (6A2) or bright orange-tawny (6C5), hygrophanous at times, dry, glabrous or more commonly with scattered to numerous brownish or cinnamon (7C4) fibrils or fibrillose-tomentose to densely squamulose with brownish scales, margin even, inrolled when young, usually becoming glabrous or translucent striate. *Lamellae* (Figs. 8.39-42) adnate, radiating from point of attachment, close to crowded (1-3 tiers of lamellulae), broad (up to 6 mm), at first off-white or pale yellow (4A4), yellow-orange, or rich orange (7C4), becoming tan, yellow-tan,



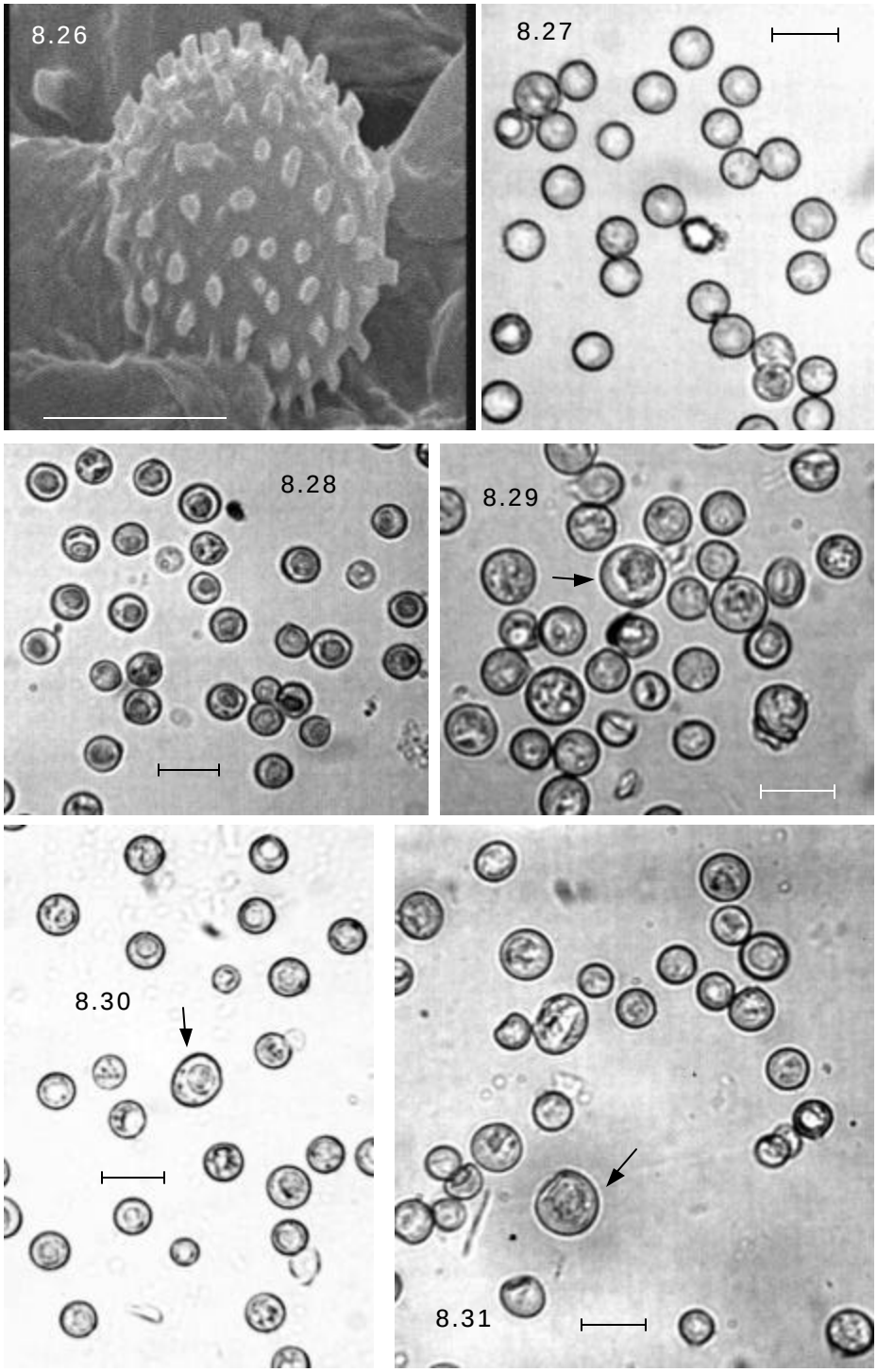
Figs. 8.1-9. *Crepidotus crocophyllus.* 8.1. Encrusted epicuticular hyphae MCA 781 (*C. nephrodes*). Scale bar = 20 µm. 8.2. Pileocystidia OKM 27048 (*C. appalachianensis*). Scale bar = 20 µm. 8.3. Pileocystidia OKM 27048 (*C. appalachianensis*). Scale bar = 45 µm. 8.4. Encrusted epicuticular hyphae AHS 67333 (*C. aureifolius* HOLOTYPE). Scale bar = 20 µm. . 8.5. Cheilocystidia OKM 27048 (*C. appalachianensis*). Scale bar = 20 µm. 8.6. Cheilocystidia MCA 373 (*C. aureifolius*). Scale bar = 20 µm. 8.7. Cheilocystidia MCA 841 (*C. appalachianensis*). Scale bar = 20 µm. 8.8. Cheilocystidia AHS 67160 (*C. distortus* HOLOTYPE). Scale bar = 20 µm. 8.9. Cheilocystidia OKM 25896. (*C. nephrodes*). Scale bar = 10 µm.



Figs. 8.10-16. *Crepidotus crocophyllus*. 8.10. Basidia OKM 27048 (*C. appalachianensis*). Scale bar = 20 µm. 8.11. Pleurocystidiate projections on side of lamellae MCA 749A (*C. appalachianensis*). Scale bar = 45 µm. 8.12. Pleurocystidiate projections on side of lamellae OKM 27048 (*C. appalachianensis*). Scale bar = 20 µm. 8.13. Appendiculate pleurocystidiate cells on lamellae side AHS 67160 (*C. distortus* HOLOTYPE). Outline enhanced. Scale bar = 20 µm. 8.14. Short-necked pleurocystidiate cells on lamellar face MCA 373 (*C. aureifolius*). Arrows indicate exemplar cells. Scale bar = 20 µm. 8.15. Basidium MCA 781 (*C. nephrodes*). Scale bar = 20 µm. 8.16. Long outgrowths from pleurocystidiate cells on lamellar face MCA 386 (*C. distortus*). Scale bar = 20 µm.



Figs. 8.17-25. *Crepidotus crocophyllus* basidia. Scale bar = 20 µm. 8.17. Two-sterigmate AHS 67333 (*C. aureifolius* HOLOTYPE). Outline enhanced. 8.18. Appendiculate pleurocystidiate AHS 66273 (*C. subaureifolius* PARATYPE). 8.19. One- and two-sterigmate with developing spores attached AHS 67333 (*C. aureifolius* HOLOTYPE). Outline enhanced. 8.20. One-sterigmate with attached spore AHS 67333 (*C. aureifolius* HOLOTYPE). Outline enhanced. 8.21. Four-sterigmate AHS 67333 (*C. aureifolius* HOLOTYPE). Outline enhanced. 8.22. Two-sterigmate with spores attached AHS 67333 (*C. aureifolius* HOLOTYPE). Outline enhanced. 8.23. Inflated basidioles on lamellar face AHS 66273 (*C. subaureifolius* PARATYPE). 8.24. Two-sterigmate adventitious basidium AHS 67333 (*C. aureifolius* HOLOTYPE). 8.25. Pleurocystidiate AHS 67333 (*C. aureifolius* HOLOTYPE). Outline enhanced.



Figs. 8.26. *Crepidotus crocophyllus* basidiospores. 8.26. Scanning electron micrograph MCA 896 (*C. badiofloccosus*). Scale bar = 2 µm. Figs. 8.27-31. Scale bar = 10 µm. 8.27. Uniform size MCA 836 (*C. nephrodes*). 8.28. Uniform size MCA 781 (*C. nephrodes*). 8.29. Dimorphic (arrow) AHS 67333 (*C. aureifolius* HOLOTYPE). 8.30. Dimorphic (arrow) MCA 841 (*C. aureifolius*). 8.31. Dimorphic (arrow) AHS 67333 (*C. aureifolius* HOLOTYPE).

or orange-tan with age, edges concolorous or tinted ochre or creamy orange. *Stipe* absent at maturity, point of attachment a white, yellow (4A4), yellow-orange (5B4), or orange (5B8), villose or glabrous tubercle. *Context* 1-1.5 mm, thin, white. *Odor* and *Taste* not distinctive. *Spore deposit* brown (5D6-7, 5E7).

Pileipellis a loosely interwoven layer of repent hyaline hyphae, 6-12 µ in diam., producing a few to numerous ascendant clavate or subcylindric pileocystidiate end cells (Figs. 8.2-3), bearing a few scattered to catenate chains to dense aggregates of epicuticular hyphae with thick hyphal walls and irregularly deposited brown or rusty in 3%KOH encrustations (Figs. 8.1, 8.4). *Pileitrama* a hyaline to melleus layer of loosely to densely interwoven, cylindric or slightly inflated, thin-walled hyphae, 6-12 µm in diam. *Lamella trama* composed of subparallel, hyaline to melleus, cylindric, thin-walled hyphae, (3) 6-15 µm in diam. *Cheilocystidia* (Figs. 8.5-9) 23-60 X 4-10 (12) µm, versiform, mostly clavate, but also subcylindric, appendiculate, at times with pronounced filamentous neck, constricted, subcapitate, septate, or forked and branched, abundant, hyaline, thin-walled. *Pleurocystidia* absent. *Basidia* normal (Figs. 8.10, 8.15, 8.19, 8.21) (20) 30-40 X 5-8 (9) µm, typically clavate, mostly 4-sterigmate, but also 1- or 2- sterigmate, in older basidiocarps (Figs. 8.17-18, 8.20, 8.22, 8.24-25) 20-63 X 3-15 µm, versiform, elongate-constricted, cylindric, subfusoid, more rarely forked or branched, appendiculate from secondary hyphal outgrowths, outgrowths short or more commonly projecting beyond hymenium, quiescent basidia (Fig. 8.23) up to 30 (40) X 15 (25) µm, clavate becoming inflated and globose or obpyriform in very mature basidiomata. *Basidiospores* (Figs. 8.26-31) primarily 5-7 µm in diam., however in older basidiomata distinctly dimorphic with secondary basidiospores produced reaching up to 7-9 µm in diam., globose, punctate due to

embedded truncate columns of the exosporium (Fig. 8.26), rusty-brown in 3% KOH and 1-10% NH_4OH, no apical thinning or discontinuity, apiculus indistinct. *Clamp connections* present in all tissues. *Habit, habitat,* and *distribution*: Scattered to gregarious on decorticated hardwoods or woody hardwood litter, May-October. Reported from North America, Europe, South America, Australia, New Zealand, Japan, and East Africa.

Specimens of C. crocophyllus *examined*. USA. CALIFORNIA: Arcada, on wood, 17 Nov. 1986, coll. D. Largent, O.K. Miller 22721 (*C. crocophyllus*, VPI). MICHIGAN: Haven Hill, Highland State Rec. Area, Oakland Co., on hardwood, 1 Sept. 1963, A.H. Smith 67333 (HOLOTYPE, *C. aureifolius*, MICH); Emerson, Chippewa Co., 12 Aug. 1963, A.H. Smith 67160 (HOLOTYPE, *C. distortus*, MICH); Mackinac Co., 7 Aug. 1953, A.H. Smith 66273 (PARATYPE, *C. subaureifolius*, MICH); Univ. of Michigan Biol. St., Grapevine Pt., Cheboygan Co., on hardwood, 1949, A.H. Smith 66275 (PARATYPE, *C. appalachianensis*, MICH). MINNESOTA: Bemijdi, on hardwood sticks, 26 Aug. 1995, O.K. Miller 26403 (*C. crocophyllus*, VPI). MISSOURI: Jefferson Co., 1 Oct. 1994, O.K. Miller 26173 (*C. crocophyllus*, VPI). NORTH CAROLINA: Duke Forest along New Hope Creek, Orange Co., decorticated stick, 22 June 1999, M.C. Aime 781, 783, 784 (*C. nephrodes*, VPI); same locale, 31 Oct. 1997, coll. R. Vilgalys & S. Miller, M.C. Aime 386 (*C. distortus*, VPI); same locale, 19 June 1998, coll. T. James, M.C. Aime 507 (*C. crocophyllus*, VPI); Iredell Co., West of Bridge 766, Greenbriar Road, decorticated log, 17 Sept. 1997, coll. A. Stanley, M.C. Aime 373 (*C. aureifolius*, VPI); Macon Co., Nantahala National Forest, Road to Standing Indian, 23 July 1997, coll. S. Miller, M.C. Aime 322 (*C. appalachianensis*, VPI). VERMONT: Indian Brook Rec. Area, Chittenden Co., on hardwood limb, O.K. Miller 27759 (*C.*

nephrodes, VPI). VIRGINIA: Pandapas Pond Rec. Area, Jefferson National Forest, Montgomery Co., on decorticated oak?, 26 Sept. 1993, O.K. Miller 25896 (*C. nephrodes*, VPI); Cascades Rec. Area, Jefferson National Forest, Giles Co., 16 Sept. 1999, coll. J. Herr, M.C. Aime 836 (*C. nephrodes*, VPI); same locale and date, M.C. Aime 841 (*C. aureifolius*, VPI); same locale, decorticated hardwood logs under bark, 3 Oct. 1998, M.C. Aime 749A (*C. appalachianensis*, VPI); Cherokee Flats, Jefferson National Forest, Giles Co., decorticated hardwood stump, M.C. Aime 189 (*C. nephrodes*, VPI); Mountain Lake Biological Station, Giles Co., well rotted hardwood, 9 Sept. 1993, O.K. Miller 25806 (*C. crocophyllus*, VPI); Wintergreen, on hardwood oak? log, 19 Sept. 1997, O.K. Miller 27048 (*C. appalachianensis*, VPI); same locale, on hardwood branch, 20 Sept. 1997, O.K. Miller 27052 (*C. crocophyllus*, VPI). EUROPE. SLOVAKIA: Bratislava, Danube Island Sihot, floodplain forest, on fallen dead trunk of *Acer negundo*, 22 Sept. 1998, S. Jancovicová 3 (*C. crocophyllus*, SLO); same locale, 7 Aug. 1997, S. Jancovicová 1 (*C. crocophyllus*, VPI); same locale, Oct. 1998, S. Jancovicová 2 (*C. crocophyllus*, VPI). JAPAN. SHIGA PREFECTURE: Shiga University Guest House Forest, Otsu, on decayed *Quercus serrata*, 9 June 1997, coll. Y. Hoashi, M.C. Aime 551 (*C. badiofloccosus*, VPI); KAGOSHIMA PREFECTURE: Takakuma, 28 Oct. 1998, coll. T. Hattori, M.C. Aime 896 (*C. badiofloccosus*, VPI).

Specimens of closely related taxa examined for comparison. USA. MICHIGAN: Wolf Bog, Cheboygan Co., on Ash, 29 June 1949, A.H. Smith 32367 (HOLOTYPE, *C. subfibrillosis*, MICH). MISSOURI: Duck Creek Wildlife Refuge, on rotten wood, 12 Aug.

Table 8.1. Morphotypes of *Crepidotus crocophyllus* discussed in this study.

Morphotaxon	Pileus color	Lamellar color	Cheilocystidia	"Pleurocystidia"	Distribution
appalachianensis	pallid to yellowish	pallid	clavate to subcylindric	versiform, appendiculate	Eastern and Southern North America
aureifolius	yellowish	orange	clavate to subcylindric	versiform, appendiculate	Eastern North America
crocophyllus	yellow-orange to orange	yellow orange to orange	clavate, subcapitate, to subcylindric	none	North America, Eastern & Central Europe
distortus	pallid	yellowish	versiform, distorted	versiform, appendiculate	Eastern North America
nephrodes	pallid	pallid	clavate, appendiculate, to subcapitate	none	N. America, Europe, S. America, Australia & New Zealand, Japan, East Africa
subaureifolius	yellowish ocher to tawny	orange	clavate to subcylindric	versiform, broad	Eastern North America

1999, O.K. Miller 27540, 27542 (*Crepidotus* sp., VPI). NORTH CAROLINA: Duke Forest along New Hope Creek, Orange Co., 22 June 1999, coll. J.E. Johnson, M.C. Aime 775 (*C. malachius*, VPI). VIRGINIA: Cherokee Flats, Jefferson National Forest, Giles Co., decorticated hardwood, 14 Sept. 1996, M.C. Aime 188 (*C. applanatus* v. *globigera*, VPI); same locale, 21 Aug. 1997, M.C. Aime 343 (*C. malachius*, VPI). PUERTO RICO: Bisley Experimental Plots, on small hardwood log, some on bark, 3 June 1998, M.C. Aime 442 (*Crepidotus* sp., VPI). JAPAN. SHIGA PREFECTURE: Imodani Shrine, on wood in mixed forest, 21 Sept. 1999, coll. Y. Hoashi & T. Nakamori, M.C. Aime 941 (*Crepidotus* sp., VPI); NAGANO PREFECTURE: Experimental Forest of the Agricultural College of Yatsugatake, on hardwood twigs, 24 Aug. 1998, M.C. Aime 719 (*C. malachius* v. *trichiferous*, VPI).

Remarks. The members of Stirps Sphaerula, to which *C. crocophyllus* belongs, are distinguished from all other *Crepidoti* with globose to subglobose basidiospores by the ornamentation, which consists of truncate columns as depicted in Fig. 8.26. The Sphaerula group, to which *C. crocophyllus* belongs (Aime Chap. 7) is differentiated from the other group in Stirps Sphaerula, the Nyssicola group (Aime Chap. 5), by having basidiospores in each collection measuring greater than 5 μm in diameter, by always exhibiting clamp connections in the dikaryotic vegetative stage, and by the broad lamellae (up to 6 mm in the Sphaerula group, up to 1 mm in the Nyssicola group) and sessile growth without a true or pseudostipe at maturity. *Crepidotus crocophyllus* differs from all other members of the Sphaerula group thus far examined by a single consistently correlated character: the presence of brown or cinnamon pigments on some of the epicuticular hyphae which result from irregular encrusted deposits on the hyphal wall (Figs. 8.1, 8.4). Species closely related to *C. crocophyllus* include the *C.*

malachius (Berk. & Curt.) Sacc./*C. applanatus* s. Senn-Irlet (1995) complex (Fig. 8.43), which lack pigmented hyphae, and *C. subfibrillosus* Hesler and Smith, in which some of the epicuticular hyphae contain brown pigments, but these are non-encrusted, evenly distributed, and integral components of the cell wall.

Mating and phylogenetic studies of the different morphotypes have revealed that *Crepidotus crocophyllus* is a single species with a high degree of phenotypic plasticity (Aime Chap. 7). Pileus and lamellar pigmentation ranges from pallid to yellow or ochre to orange. Macroscopically, the pigment-encrusted epicuticular hyphae may be barely expressed, only visible with a hand lens as a few scattered brown fibrils, to abundantly present, forming a brown fibrillose covering of the pileus, or breaking into dense brown squamules (Figs. 8.38-42). Microscopically, cheilocystidia are always present but variable in density and form (Figs. 8.5-9), and structures previously described as pleurocystidia, when present, are also versiform (Figs. 8.11-14, 8.16, 8.18, 8.20, 8.24-25). A detailed discussion of morphology follows.

Results and Discussion

Of the eight taxa placed in Section *Fulvifibrillosi*, six were found to be conspecific, one, *C. subfibrillosus*, is found to be distinct from the others, and one, *C. subnidulans* (Overh.) Hesler and Smith, which possesses smooth basidiospores, was not studied as it does not belong in *Crepidotus*, the correct name being *Phyllotopsis subnidulans* (Overh.) Sing. For purposes of this discussion, the terms morphotypes or forms are used, without imparting any taxonomic value, for previously described taxa now known to be conspecific with *C. crocophyllus*. Table 8.1 presents the key diagnosable characters and known distribution for

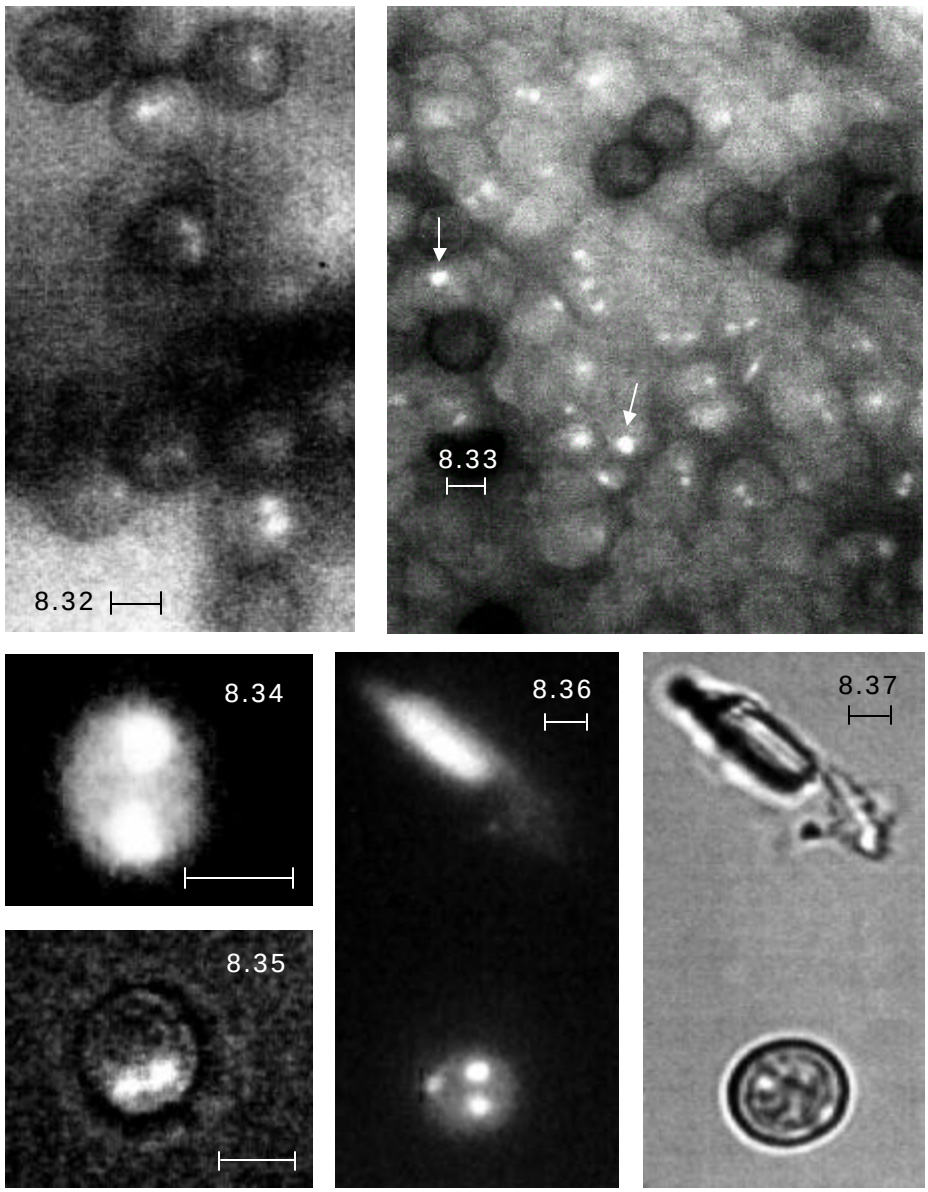
each morphotype discussed. Morphotypes will be referred to by only their specific epithet (e.g., *nephrodes*, *crocophyllus*), while *C. crocophyllus* refers to the taxon as described above.

Pigmentation of basidiomata—Many commonly collected color forms of *C. crocophyllus* exist, resulting in several published names for this species. At least one other species, *Pleurotus djamor* (Fr.) Boedijn, for which numerous color variants (white, tan, olive, pink, and gray, glabrous or fibrillose) with different, sometimes overlapping, distributions, described as separate taxa, is now known to be an intercompatible, globally distributed single taxon (Petersen 1995b, Nicholl 2000). *Pleurotus djamor* has been collected from Neotropical forests in Guyana with both the white and pink forms growing intermixed. Interestingly, white variants gave white spore prints, and pink variants gave pink spore prints, although all recovered single spore isolates (SSIs) from both variants exhibited white mycelial growth in culture (unpublished data).

The reverse phenomenon has been found in *C. crocophyllus*, where spore deposits from all variants were similarly pigmented, yet recovered SSIs were quite variable in color, ranging from hyaline (most frequent) to yellow to orange (least frequent), with some intergradation between (Aime Chap. 7). Basidiomata color was not a predictor of SSI color for these collections. In some collections of the *nephrodes* morphotype, only hyaline isolates were recovered, others produced SSIs of all pigmentations. Collections of the *crocophyllus* morphotype produced hyaline or occasional yellow SSIs, and collections of the *aureifolius*, *appalachianensis*, and *distortus* morphotypes produced mostly orange SSIs. Dikaryotic cultures produced by subculturing of compatible pairings between SSIs of different color variants displayed pigmentation intermediate between the parental types. Vilgalys (1991) also

found two cultural color variants in *Collybia dryophila* (Bull.:Fr.) Kumm., which produced hybrid cultures of intermediate pigmentation when mated.

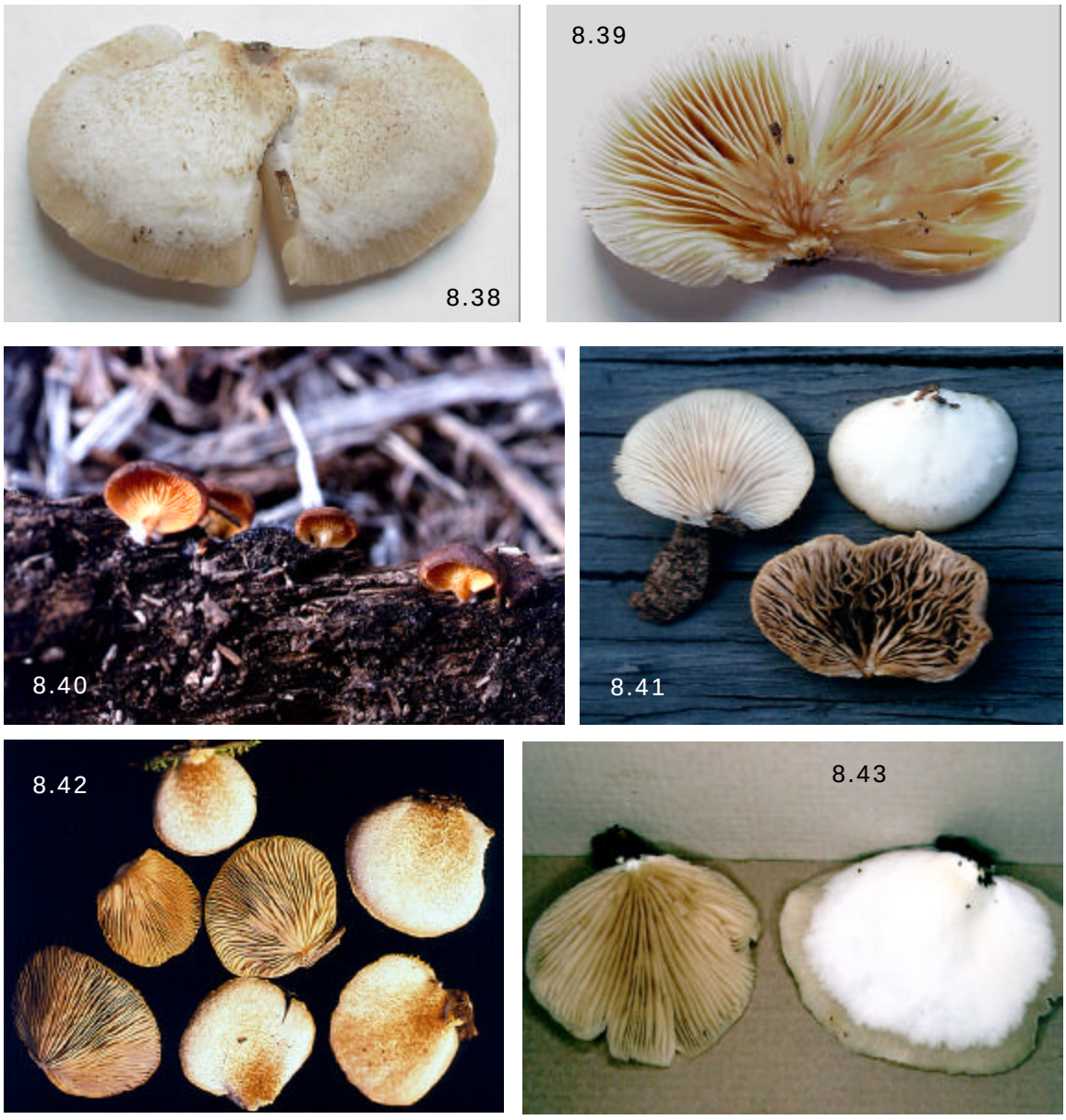
The inability, at present, to fruit this species in culture precludes deriving any satisfactory conclusions regarding the inheritable *versus* environmental/physiological component to pigmentation in this species. Nonetheless, three different mechanisms can be posited to explain the naturally occurring variation observed. 1) Pigmentation results from inheritance of Mendelian determinants. Other factors, such as mating incompatibility loci, are now known to be determined by the regulation of Mendelian determinants in the agarics (Chase and Ullrich 1990). Assuming that hyaline and orange pigmentation are co-dominant, with a yellow intermediate, and that alleles for orange pigmentation are less frequently distributed, and confined to European and North American populations, would explain some of the morphotype distributional patterns and culture data. 2) Pigmentation of the basidiomata changes during maturation. Coloration of fruiting bodies in the agaric *Hygrocybe psittacina* (Schaeff.:Fr.) Kumm. are remarkable for the striking change in coloration as they age (O.K. Miller, pers. comm.). Almost certainly, a difference in the quantity of encrusted epicuticular hyphae present in different collections of *C. crocophyllus* is due to a combination of pileus expansion and loss of fibrils correlated with age of basidiocarp (personal observation). Almost all collections of the *crocophyllus* morphotype are limited to a few, relatively small, fruiting bodies (Fig. 8.40), whereas collections of the *nephrodes* morphotype usually consist of numerous, large individuals (personal observation). This observation could be explained if orange pigments were lost during maturation, but it does not fully explain the data from SSIs, or the fact that both



Figs. 8.32-36. *Crepidotus crocophyllus* fluorescent micrographs of nuclei stained with DAPI. Scale bar = 5 µm. Fig. 8.32. Binucleate basidiospores MCA 836 (*C. nephrodes*). 8.33. Fusion nuclei (arrows) in pleurocystidiate cells of lamellar face AHS 67333 (*C. aureifolius* HOLOTYPE). 8.34. Binucleate basidiospore AHS 67333 (*C. aureifolius* HOLOTYPE). 8.35. Binucleate basidiospore MCA 781 (*C. nephrodes*). 8.36. Basidiospore with three nuclei showing AHS 67333 (*C. aureifolius* HOLOTYPE). 8.37. Same basidiospore without fluorescence.

morphotypes have not, as yet, been found growing from a single genet. 3) The type of basidium that produced the basidiospore determines pigmentation of the resultant SSIs. This hypothesis is based on the observation that the majority of orange SSIs were produced by collections that had so-called "pleurocystidia" (*aureifolius*, *distortus*, and *appalachianensis*) (Table 8.1). These pleurocystidia will be discussed below. However, this study has shown that they are actually abnormal basidia with the capability of producing basidiospores. In this view, abnormal basidia produce unusually large basidiospores, and the resulting isolates exhibit orange colonies with exceptionally slow growth (Aime Chap. 7). Dimorphic basidiospores (Figs. 8.29-31) were noted in all collections from which some orange SSIs were recovered, and from none of the collections for which only hyaline or yellow SSIs were recovered. If this is the case, then the pleurocystidiate morphotypes that so far are unreported from Europe (Table 8.1) would be expected to geographically co-exist with the orange (*crocophyllus*) morphotype, which has so far only been reported from Central and Eastern Europe as rare (Lazebnícek 1970, Toma 1972, Moser 1978, Dermek 1987, Stangl 1991, Senn-Irlet 1995).

Cystidia—Cheilocystidia are versiform in *C. crocophyllus* (Figs. 8.5-9), and cheilocystidial form has been the basis of delimiting *C. distortus* from the other pleurocystidiate forms. Several studies have since found cheilocystidia to be of limited value for delimiting *Crepidoti* taxa (Senn-Irlet 1995, Bandala and Montoya 2000), and within the closely related Nyssicola group, secondary growth resulting in morphological alterations in size and form of cheilocystidia has been reported (Aime Chap. 5).



Figs. 8.38-42. *Crepidotus crocophyllus* basidiomata. 8.38. OKM 27759 (*C. nephrodes*). Scattered fibrils on pileus. 8.39. OKM 27759 (*C. nephrodes*). Lamellae. 8.40. OKM 26173 (*C. crocophyllus*). Dense felty covering of pileus. 8.41. OKM 27048 (*C. appalachianensis*). No macroscopically visible fibrils. 8.42. MCA 836 (*C. nephrodes*). Squamulose pileus. 8.43. *Crepidotus malachius* OKM 25882. A closely related species that has no macro- or microscopic brown fibrils on the pileus.

During microscopic examination of the "pleurocystidiate" morphotypes of *C. crocophyllus*, several observations of note were recorded. 1) While basidiospores in non-pleurocystidiate forms consistently measured 5-7 µm, 5-25% of basidiospores in the pleurocystidiate forms ranged from 7-9 µm in diameter. "Normal" basidiospores were found to be binucleate (Figs. 8.32, 8.34-35), as is the case for most *Crepidoti* thus far examined (unpublished data); "abnormal" basidiospores contained from three to four nuclei per spore (Figs. 8.36-37). 2) Very few normal, clavate basidia were seen in pleurocystidiate forms, the hymenium being composed almost entirely of quiescent basidioles and "pleurocystidia", whereas non-pleurocystidiate forms contained numerous normal clavate, four-sterigmate, basidia. A few basidia in the pleurocystidiate forms were greatly elongated, subcylindric, and two-sterigmate (Fig. 8.22); no such basidia exist in the non-pleurocystidiate forms. 3) While "pleurocystidia" were versiform both within single basidiomata, between collections, and between morphotypes, most were composed of a clavate basal cell, identical to the basidioles, subtended by various outgrowths and divided by a simple septum (Fig. 8.25). 4) "Pleurocystidial" secondary cells, when appendiculate, resembled exaggerated 1-sterigmate basidia (Figs. 8.13, 8.18); other secondary cells resembled reduced 2-sterigmate basidia perched upon basidioles (Fig. 8.24). 5) Quiescent basidioles in aged basidiomata were greatly inflated and/or collapsed (Fig. 8.23). 6) Some pleurocystidia were found to contain a single large fusion nucleus, characteristic of karyogamy which occurs in basidia (Fig. 8.33). 7) Several "pleurocystidial" outgrowths were observed with developing or mature basidiospores attached at the apex (Figs. 8.17, 8.20).

True pleurocystidia are sterile cells in the hymenium, oriented along the gill face, and may originate from within the trama (often termed lamprocystidia) or from the hymenial layer (often termed leptocystidia) (Romagnesi 1944). In several model agarics, leptocystidia initials were found to be indistinguishable from probasidia in the prohymenium; during the course of maturation, certain prohymenial cells become differentiated and committed as pleurocystidia, others as basidia (Horner and Moore 1987, Chiu and Moore 1993, Moore et al. 1998). While pleurocystidia function is poorly understood, lamprocystidia most likely play a role in eliminating secondary byproducts of cellular metabolism (Romagnesi 1944, Lentz 1954) while leptocystidia probably act as buttresses in separating the lamellae in *Coprinus* species, and possibly help to regulate microhumidity (Lentz 1954, Moore et al. 1998). While certain aspects of the cystidial developmental pathway are believed to be flexible, including reversion to vegetative growth of leptocystidia (Moore et al. 1998) and cheilocystidia (Aime Chap. 5) no known cases of spontaneous secondary growth of basidia *in vivo* have been reported.

Lamprocystidia are not produced by any recorded species of *Crepidotus*. Leptocystidia have been recorded from only a few species, and their presence is usually considered significant enough for species circumscription. Nonetheless, Singer (1973) expressed doubts as to the taxonomic value of the presence of pleurocystidia, noting that cystidioles (here used to signify either leptocystidia or proleptocystidia) and basidioles (an imprecise term used to connote either probasidia or quiescent basidia that have cast their spores) were indistinguishable in those *Crepidoti* that possessed both. Senn-Irlet and de Meijer (1998) noted that pleurocystidia in *C. cystidiosus* Hesler and Smith bore a resemblance to one-sterigmate basidia. The present study leaves little doubt that so-

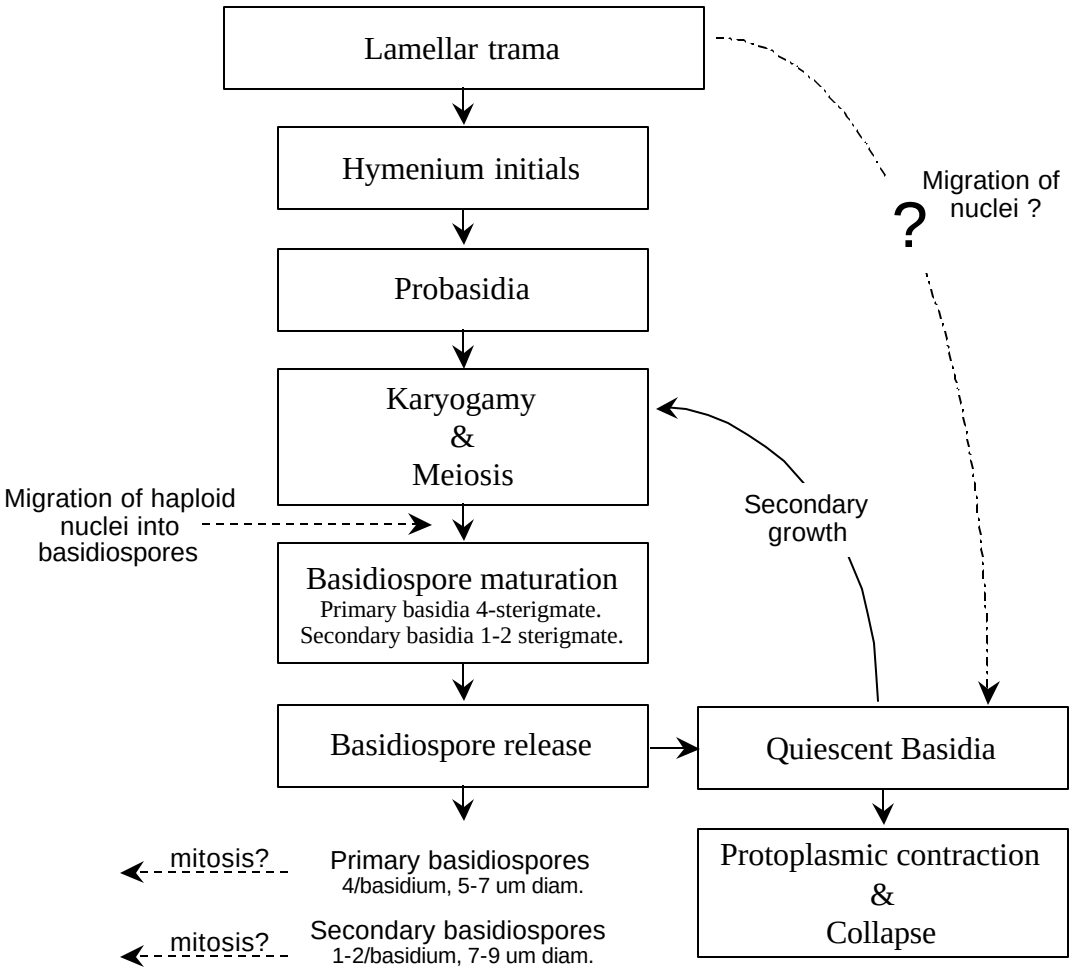


Fig. 8.44. Schematic outline of hypothetical basidium cycle in *C. crocophyllus.* Primary ("normal") basidia differentiate from hymeneal initials on the lamellar face and undergo normal growth and maturation with concomitant release of "normal" basidiospores. After a period of quiescence, of unknown length, basidia undergo a second stage of growth, characterized by the production of only 1- to 2-sterigmate outgrowths, versiform in size/shape depending on temporal (?) or environmental (?) factors, with nuclei potentially donated from the lamellar trama. Secondary basidia then re-enter the karyogamy/meiotic pathway (?) to produce secondary basidiospores. After two (or more?) repetitions and repletion of resources, the extended basidia retract into an expanded isodiametric "basidiole" and collapse.

called pleurocystidia, at least when present in *C. crocophyllus*, are actually basidia that have undergone a second phase of growth and spore production post-quiescence.

As previously stated, no analog to the *C. crocophyllus* phenomenon of secondary basidial growth with subsequent production of spores has been recorded from nature. However, vegetative outgrowth of basidia from the sterigmata, without production of basidiospores, has been induced in *Coprinus cinereus* (Schaeff.:Fr.) Gray under certain experimental conditions, such as bathing of hymenial cells in inorganic salts (Chiu and Moore 1990). Schwalb (1978) also reports the production of elongated sterigmata from the basidia of a temperature-sensitive mutant of *Schizophyllum commune* Fr. at the restrictive temperature. Vegetative-like growth of sterigmata were discovered in a few basidia produced by a sterile (sporeless) mutant of *Lentinus edodes* (Berk.) Sing. (Hasebe et al. 1991). Finally, in an exhaustive study of a spontaneous mutant of *Psilocybe merdaria* (Fr.) Ricken, produced in culture, fruiting bodies with gasteroid forms were found with a tremendous variety of basidial shapes, including two- to six- sterigmate forms, simple septa, or exaggerated growth of sterigma, with concomitant basidiospore production (Watling 1971).

All of the above examples of abnormal basidial growth were recorded from mutant strains or cells exposed to potential mutagens, and spore production from abnormal basidia was only observed in the study of Watling (1971). If secondary growth and spore production of basidia in *C. crocophyllus* is due to spontaneous mutation, then it does not appear too have hindered the success, in terms of geographic distribution and frequency, of this species. More likely, such secondary activity may be a successful strategy for recycling any remaining resources in an otherwise "spent" fruiting body into a final production of propagules. Fig. 8.44

presents a proposed outline of the basidium life cycle in this species. Petersen (1995a) observes that "within species with highly conserved compatibility mechanisms, considerable variation can be found in other characters." Certainly *C. crocophyllus* is just such a species.

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Summary and Conclusions

"It will be a long time before a sufficient number of agarics have been analysed in such a way as to give a complete picture of development in the Agaricales."

—Roy Watling 1975

This work deals with the biosystematics, including taxonomy, biology, and phylogeny, of crepidotoid fungi, especially those in *Crepidotus*. Traditional classifications for these fungi have been based on morphology of the fruiting body, and their taxonomy, at all categories below that of order, has been controversial. The overriding goal of this dissertation was to determine the extent of phenotypic plasticity within single interbreeding species of *Crepidotus* for herein lies the root of most taxonomic disagreement—what, if any, morphological characters are reliable indicators of discreet phylogenetic units in the *Crepidoti*?

In order to answer this question at the species level, taxa examined for comparison should be members of a single evolutionary lineage. However, since the previously proposed infrageneric classifications for the *Crepidoti* are in conflict, where two taxa may be closely related under one system and very distantly related in another, there exists a great potential for introducing error, in the form of unrecognized homoplasy, if taxa are selected from the "wrong" classification. Therefore, molecular systematics was chosen as the tool for identifying a complex of closely related species for further study. It became obvious during the course of this project that, in order to identify a single species complex for study, the entire genus had to be examined,

and, in order to correctly root and interpret a phylogeny for *Crepidotus*, the sister to the genus must be found. Eventually, both familial and generic concepts for the crepidoti were re-examined, as well as species concepts within a single identified clade, termed the Sphaerula group.

Analysis of nLSU sequences for exemplar taxa from each of the agaric genera of the Crepidotaceae sensu Singer revealed a polyphyletic assemblage that had several origins from within the euagarics (Chap. 2). However, a new and unique lineage of euagarics was uncovered, corresponding to the Crepidotaceae s.s., with *Simocybe* and *Crepidotus* supported as monophyletic sister genera (Chap. 2, Chap. 3). Examination of types and newly collected specimens, within this phylogenetic framework, provided insight into how, at least for the Crepidotaceae s.l., the original family concept was derived, and show that the Friesian characters of spore print color and pleurotoid habit do not always unite members of the same family or genus (Chap. 1, Chap. 2). Family and generic concepts were revised based upon these new data (Chap. 2, Chap. 3). Future efforts at this level will focus on a taxonomic reassignment of the non-generic members of *Melanomphalia*, many of which are now believed to be *Crepidoti* (Chap. 1) by combining sequencing and type studies. Similar examinations of the proposed cyphelloid members of the family should also be performed to gain a complete understanding of the range of phenotypic variation within the Crepidotaceae.

While *Crepidotus* was shown to be monophyletic, based on sampling designed to include as much geographic and phenotypic diversity as possible, and several monophyletic clades were uncovered within the genus, infrageneric relationships between clades could not be resolved (Chap. 3). Even with this limitation, however, it appears that none of the infrageneric

classifications previously proposed for *Crepidotus* reflect the natural history of the genus, leading to a reassessment of basidiomata characters conventionally applied at this level (Chap. 3). Again, what becomes obvious is that fruiting body characters believed to be robust are, in fact, quite plastic. Clamp connections of the basidiomata can, and have, been lost several times in the genus, and spore ornamentation has arisen several times, albeit with some evidence that the ultrastructure of the ornaments may have arisen differently each time (Chap. 3).

Especially revealing was the discovery that cheilocystidial morphology, the basis for much taxonomic circumscription in this genus, can be influenced by microenvironment (Chap. 5). The findings in Chapter 5 also suggest that commitment to the sexual stage of reproduction and fruiting body formation may not preclude a reversion to the vegetative cycle by previously differentiated cells of the hymenium, which would impact our understanding of agaric morphogenesis and even mycosociology. Additional work at the generic level is presently focused on obtaining a robustly resolved infrageneric phylogeny for the *Crepidoti*, so that synapomorphies and character polarity can be mapped, along with ecological and geographical data to gain a perspective on biogeography and substrate specificity in these fungi. This portion of the study has raised several questions regarding fungal physiology that have yet to be satisfactorily addressed, such as that of the role of clamp formation, which is believed to be integral for proper nuclear division and maintenance of the dikaryotic state yet its loss has been repeatedly tolerated by these fungi, and the extent and nature of vegetative growth in hymenial cells

In order to derive a biological species concept (BSC) for these fungi, it was necessary to first recover SSIs for performing mating compatibility tests (Chap. 4), and to discern the

homogenic mating system for species in this complex (Chap. 6). In so doing, it was discovered that basidiospore germination in the species tested appears to be under endogenous control, for reasons that remain unclear (Chap. 4). However, if reversion to vegetative growth from cheilocystidial cells is a common phenomenon in this genus, as is suspected given the large number of species described with appendiculate or lageniform cystidia, then perhaps such a mechanism of delayed spore germination reduces substrate competition between germinating spores and parental thalli. However, during the course of this study it was discovered that haplont cultures were especially sensitive to cold, and many isolates did not survive standard storage at 4 C (Chap. 6). Combined with the finding that most basidiospores of temperate *Crepidoti* were shed in the fall but germinated the following spring (Chap. 4), a strategy of endogenous control of spore germination may simply have arisen as an overwintering mechanism in these fungi.

Finally, comparison of a morphological species concept (MSC), BSC, and phylogenetic species concept (PSC) were undertaken within the Sphaerula group. What was found is that nLSU sequence data correlated with intercompatibility groups (ICGs), and that neither were correlated well with morphotaxa in this complex (Chap. 7). Examination of numerous collections and types in this complex showed that a MSC had been too stringently applied in the past to this group, and led to a taxonomic revision of most of the taxa in Subsection *Fulvifibrillosi* Hesler and Smith (Chap. 8). The findings within the Sphaerula group have helped to identify other phylogenetic complexes of *Crepidotus* in need of future study. For example, if the correlations within the Sphaerula group can be extrapolated to other groups, then within clade IV (Fig. 3.1), there are two distinct nLSU species for which at least four

morphotaxa (*fragilis*, *lundellii*, *inhonestus*, and *subtilis*, the last not shown) have been described based on geography and morphology, yet neither of these criteria reflect the pattern of speciation in this clade. To make one other example, clade V (Fig. 3.1) contains a single morphotaxon, *C. versutus*, that may contain at least two cryptic species, based on differences in nLSU sequences.

More importantly, the data presented on the Sphaerula group showed that a single species, *C. crocophyllus*, displays a tremendous amount of phenotypic plasticity, especially in the arena of pigmentation and morphology of hymenial cells (Chap. 8). A more detailed hypothesis than that proposed in Chap. 8 is proposed here to explain these findings: 1) newly matured basidia produce binucleate basidiospores, the two nuclei probably, but not yet conclusively, resulting from a mitotic division of a single haploid nucleus that has migrated from the basidium to the spore (corresponding to the *nephrodes* morphotype); 2) spores are shed, and the basidium undergoes a quiescent period, of undetermined length, wherein it is usually identified as a basidiole, and no nuclei are visible within the cell; 3) quiescent basidioles begin secondary growth, at first appearing as a few pleurocystidia with short appendiculate outgrowths (*appalachianensis* morphotype), the outgrowths becoming longer and more noticeable while normal clavate basidia become less obvious as more basidia enter this phase (*distortus* and *subaureifolius* morphotypes); 4) spores are produced by these secondary basidia, with new nuclei most likely donated by the gradually senescing trama. Only one or two sterigma are produced by the secondary basidia and resulting basidiospores are much larger than the initial crop, perhaps as a result of the increased protoplasmic content produced from the volumetrically increased basidia, combined with decrease in number of spores produced per

basidium (*aureifolius* morphotype); 5) either more than one nucleus migrates into each basidiospore, or more than one mitotic event takes place within the basidiospore to compensate for the increase in protoplasmic volume, however, the resulting basidiospores are multinucleate; 6) basidia are eventually exhausted, and without pressure on the apex from growth and spore production, collapse into inflated globose "sacs"; 7) intense orange pigmentation and slow growth of colonies derived from a single germinating spore is due to over-expression of some nuclear-encoded protein in the multinucleate spores. Whether vegetative pigmentation in the dikaryon correlates with fruiting body pigmentation has yet to be ascertained.

A hypothetical life cycle for the *Crepidoti* is presented in Fig. 0.2, mapping new pathways described in this dissertation, although, obviously, much remains to be learned of the biology of *Crepidoti.* For example, simply to test the above hypothesis detailed data on basidial and basidiospore cytology need to be compiled, and distribution maps for all morphotypes need to be drawn and analyzed, which would entail anatomical examination of collections from around the globe. A simplified method for the isolation of haplonts would allow extensive mating studies to be performed, and a method for fruiting of dikaryons needs to be found. Characterization and function of the compounds responsible for membranal pigmentation would also provide important data. It is clear, however, that future systematic efforts need to involve all aspects of crepidotoid biology and morphology, in order to produce meaningful phylogenies and simply to further our understanding of the agarics as a whole.

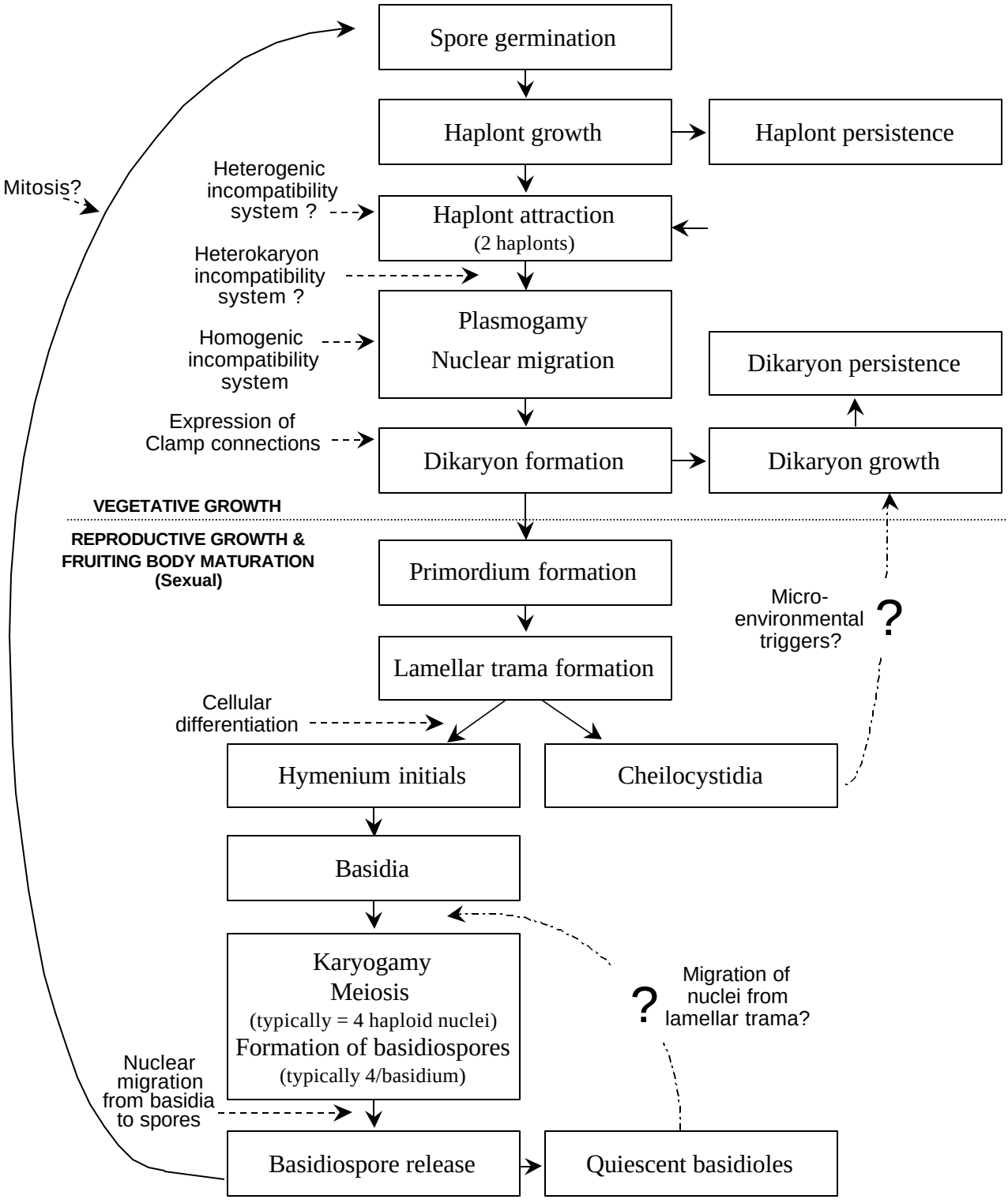


Fig. 0.2. Life cycle of *Crepidotus* (Fr.) Stuade, based on evidence presented in this dissertation. Asexual reproduction by propagules has not been observed in any species. Question mark denotes uncertainty. The reversion to vegetative growth by cheilocystidia, and recycling of basidia are potential new pathways.

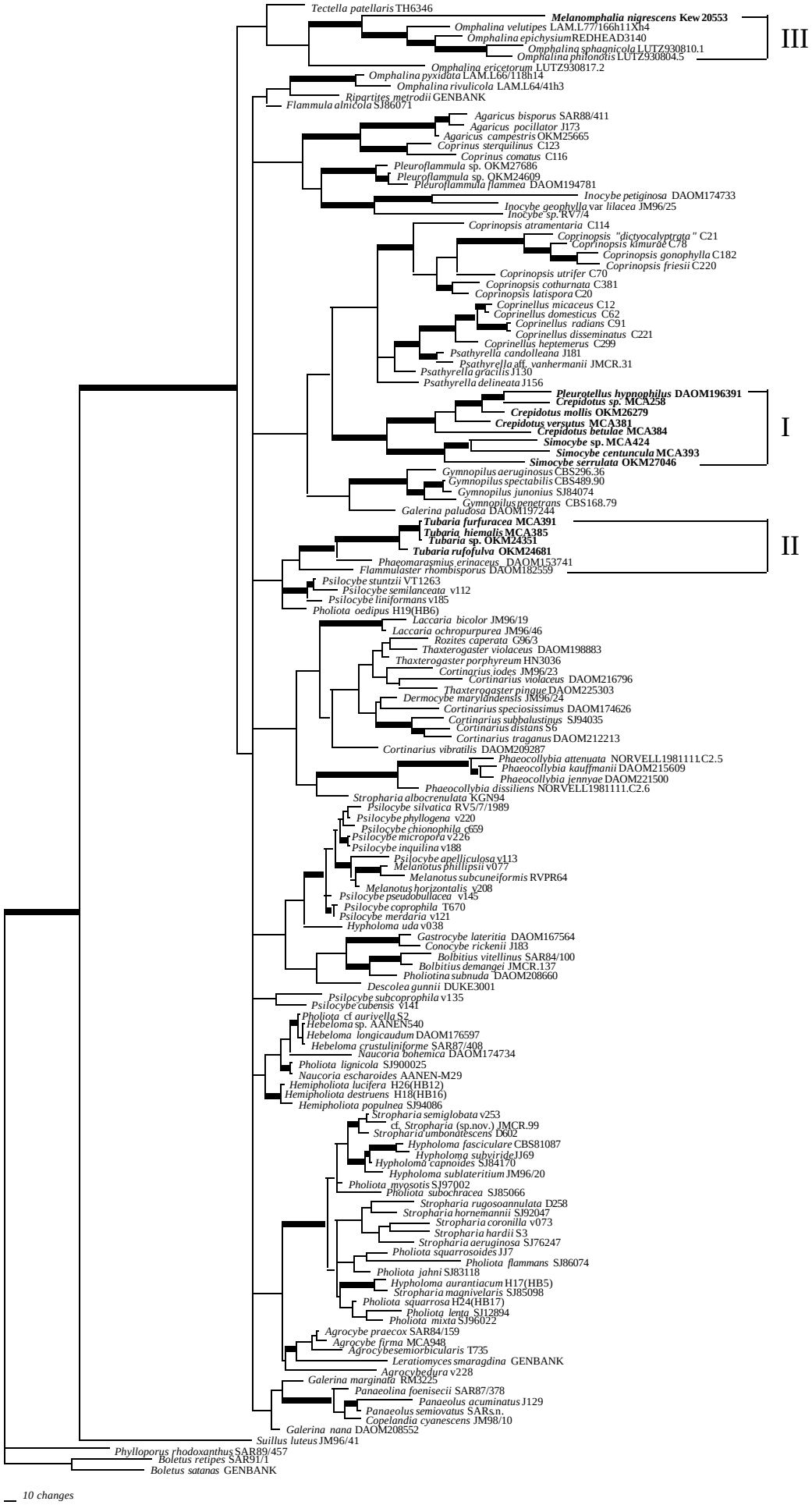
Appendix 1

Phylogenetic analysis of the genera of the Crepidotaceae s.l.—Extended analysis within the euagarics.

Fig. A.1.1 (following page). Phylogenetic analysis of the Crepidotaceae s.l. based on WMP analysis of large nuclear subunit rDNA sequences. Data matrix has 149 taxa, representing: 1) all known phylogenetic lineages of dark-spored euagarics; 2) white-spored omphalinoid fungi in the euagarics; 3) representatives, including generic types, of each agaric genus in the Crepidotaceae s. Singer; and, 4) four bolete taxa included as outgroups for rooting purposes. Data matrix has 1573 characters per taxon; 792 characters excluded due to ambiguities in alignment; 231 characters are parsimony-informative. Bootstrap 50% majority-rule consensus tree produced from 100 bootstrapping replicates of WMP with 10 random addition sequences to obtain starting trees per replicate. Bold lines indicate branches with >50% bootstrapping support. Bold type indicates taxa placed in the Crepidotaceae s. Singer. Clades I, II, and III indicate three separate origins of the crepidotoid fungi (corresponding to those discussed in Chapter 2).

This matrix includes unpublished sequences selected from a pre-released nLSU data set of 800 agaric taxa produced by the Mycology Lab, Duke University, Durham, NC. The entire data set is analyzed and discussed in:

Moncalvo, J.M., R. Vilgalys, S.A. Redhead, J.E. Johnson, T.Y. James, M.C. Aime, V. Hofstetter, S. Verduin, E. Larsen, T.J. Baroni, R.G. Thorn, S. Jacobsson, H. Clémençon, and O.K. Miller, in prep.

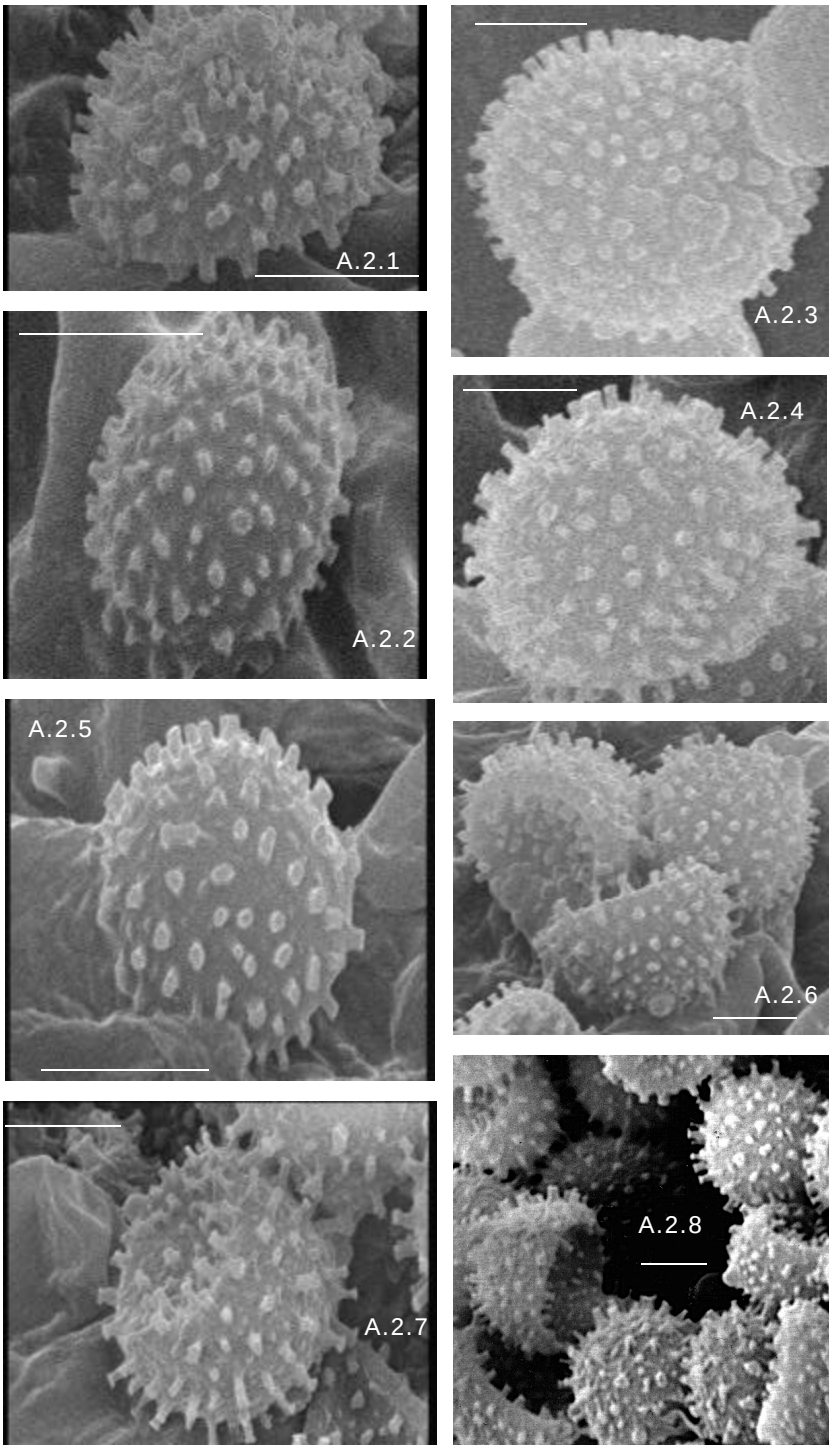

Tectella patellaris TH6346
Melanomphalia nigrescens Kew 20553
Omphalina velutipes LAM.L77/166h11Xh4
Omphalina epichysium REDHEAD3140
Omphalina sphagnicola LUTZ930810.1
Omphalina philonotis LUTZ930804.5
III
Omphalina ericetorum LUTZ930817.2
Omphalina pyxidata LAM.L66/118h14
Omphalina rivulicola LAM.L64/41h3
Ripartites metrodii GENBANK
Flammula alnicola SJ86071
Agaricus bisporus SAR88/411
Agaricus pocillator J173
Agaricus campestris OKM25665
Coprinus sterquilinus C123
Coprinus comatus C116
Pleuroflammula sp. OKM27686
Pleuroflammula sp. OKM24609
Pleuroflammula flammea DAOM194781
Inocybe petiginosa DAOM174733
Inocybe geophylla var lilacea JM96/25
Inocybe sp. RV7/4
Coprinopsis atramentaria C114
Coprinopsis "dictyocalyptrata" C21
Coprinopsis kimurae C78
Coprinopsis gonophylla C182
Coprinopsis friesii C220
Coprinopsis utrifer C70
Coprinopsis cothurnata C381
Coprinopsis latispora C20
Coprinellus micaceus C12
Coprinellus domesticus C62
Coprinellus radians C91
Coprinellus disseminatus C221
Coprinellus heptemerus C299
Psathyrella candolleana J181
Psathyrella aff. vanhermanii JMCR.31
Psathyrella gracilis J130
Psathyrella delineata J156
Pleurotellus hypnophilus DAOM196391
Crepidotus sp. MCA258
Crepidotus mollis OKM26279
Crepidotus versutus MCA381
Crepidotus betulae MCA384
Simocybe sp. MCA424
Simocybe centuncula MCA393
Simocybe serrulata OKM27046
I
Gymnopilus aeruginosus CBS296.36
Gymnopilus spectabilis CBS489.90
Gymnopilus junonius SJ84074
Gymnopilus penetrans CBS168.79
Galerina paludosa DAOM197244
Tubaria furfuracea MCA391
Tubaria hiemalis MCA385
Tubaria sp. OKM24351
Tubaria rufofulva OKM24681
II
Phaeomarasmius erinaceus DAOM153741
Flammulaster rhombisporus DAOM182559
Psilocybe stuntzii VT1263
Psilocybe semilanceata v112
Psilocybe luniformans v185
Pholiota oedipus H19(HB6)
Laccaria bicolor JM96/19
Laccaria ochropurpurea JM96/46
Rozites caperata G96/3
Thaxterogaster violaceus DAOM198883
Thaxterogaster porphyreum HN3036
Cortinarius iodes JM96/23
Cortinarius violaceus DAOM216796
Thaxterogaster pinque DAOM225303
Dermocybe maryldensis JM96/24
Cortinarius speciosissimus DAOM174626
Cortinarius subbalustinus SJ94035
Cortinarius distans S6
Cortinarius traganus DAOM212213
Cortinarius vibratilis DAOM209287
Phaeocollybia attenuata NORVELL1981111.C2.5
Phaeocollybia kauffmanii DAOM215609
Phaeocollybia jennyae DAOM221500
Phaeocollybia dissiliens NORVELL1981111.C2.6
Stropharia albocrenulata KGN94
Psilocybe silvatica RV5/7/1989
Psilocybe phyllogena v220
Psilocybe chionophila c659
Psilocybe micropora v226
Psilocybe inquilina v188
Psilocybe apelliculosa v113
Melanotus phillipsii v077
Melanotus subcuneiformis RVPR64
Melanotus horizontalis v208
Psilocybe pseudobullacea v145
Psilocybe coprophila T670
Psilocybe merdaria v121
Hypholoma uda v038
Gastrocybe lateritia DAOM167564
Conocybe rickenii J183
Bolbitius vitellinus SAR84/100
Bolbitius demangei JMCR.137
Pholiotina subnuda DAOM208660
Descolea gunnii DUKE3001
Psilocybe subcoprophila v135
Psilocybe cubensis v141
Pholiota cf aurivella S2
Hebeloma sp. AANEN540
Hebeloma longicaudum DAOM176597
Hebeloma crustuliniforme SAR87/408
Naucoria bohemica DAOM174734
Pholiota lignicola SJ900025
Naucoria escharoides AANEN-M29
Hemipholiota lucifera H26(HB12)
Hemipholiota destruens H18(HB16)
Hemipholiota populnea SJ94086
Stropharia semiglobata v253
cf. Stropharia (sp.nov.) JMCR.99
Stropharia umbonatescens D602
Hypholoma fasciculare CBS81087
Hypholoma subviride JJ69
Hypholoma capnoides SJ84170
Hypholoma sublateritium JM96/20
Pholiota myosotis SJ97002
Pholiota subochracea SJ85066
Stropharia rugosoannulata D258
Stropharia hornemannii SJ92047
Stropharia coronilla v073
Stropharia hardii S3
Stropharia aeruginosa SJ76247
Pholiota squarrosoides JJ7
Pholiota flammans SJ86074
Pholiota jahni SJ83118
Hypholoma aurantiacum H17(HB5)
Stropharia magnivelaris SJ85098
Pholiota squarrosa H24(HB17)
Pholiota lenta SJ12894
Pholiota mixta SJ96022
Agrocybe praecox SAR84/159
Agrocybe firma MCA948
Agrocybe semiorbicularis T735
Leratiomyces smaragdina GENBANK
Agrocybe dura v228
Galerina marginata RM3225
Panaeolina foenisecii SAR87/378
Panaeolus acuminatus J129
Panaeolus semiovatus SARs.n.
Copelandia cyanescens JM98/10
Galerina nana DAOM208552
Suillus luteus JM96/41
Phylloporus rhodoxanthus SAR89/457
Boletus retipes SAR91/1
Boletus satanas GENBANK
10 changes

Appendix 2

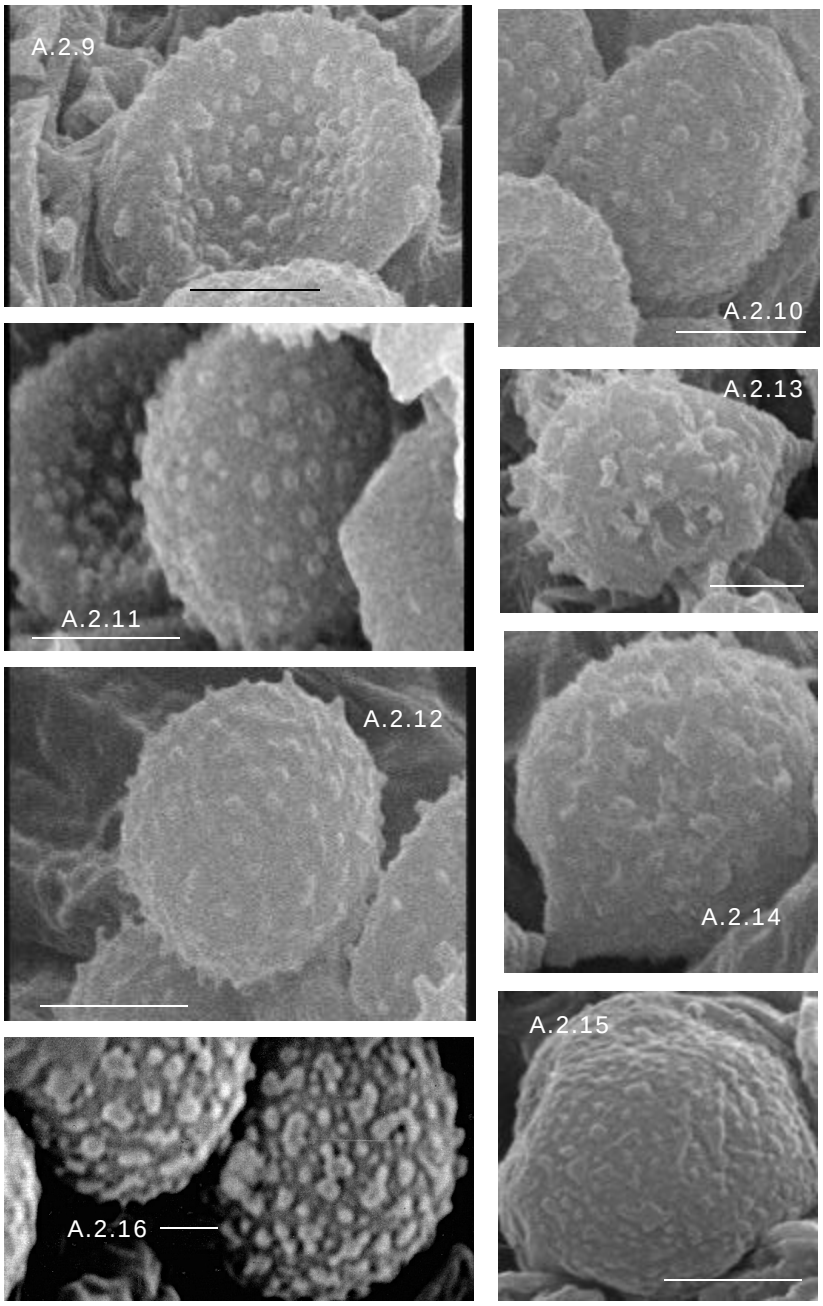
Scanning electron micrographs (SEMs) of basidiospores of the Crepidotaceae s.s.

The following micrographs are grouped by ornamentation type. Exosporial ornamentation in *Crepidotus* will be analyzed and discussed in:

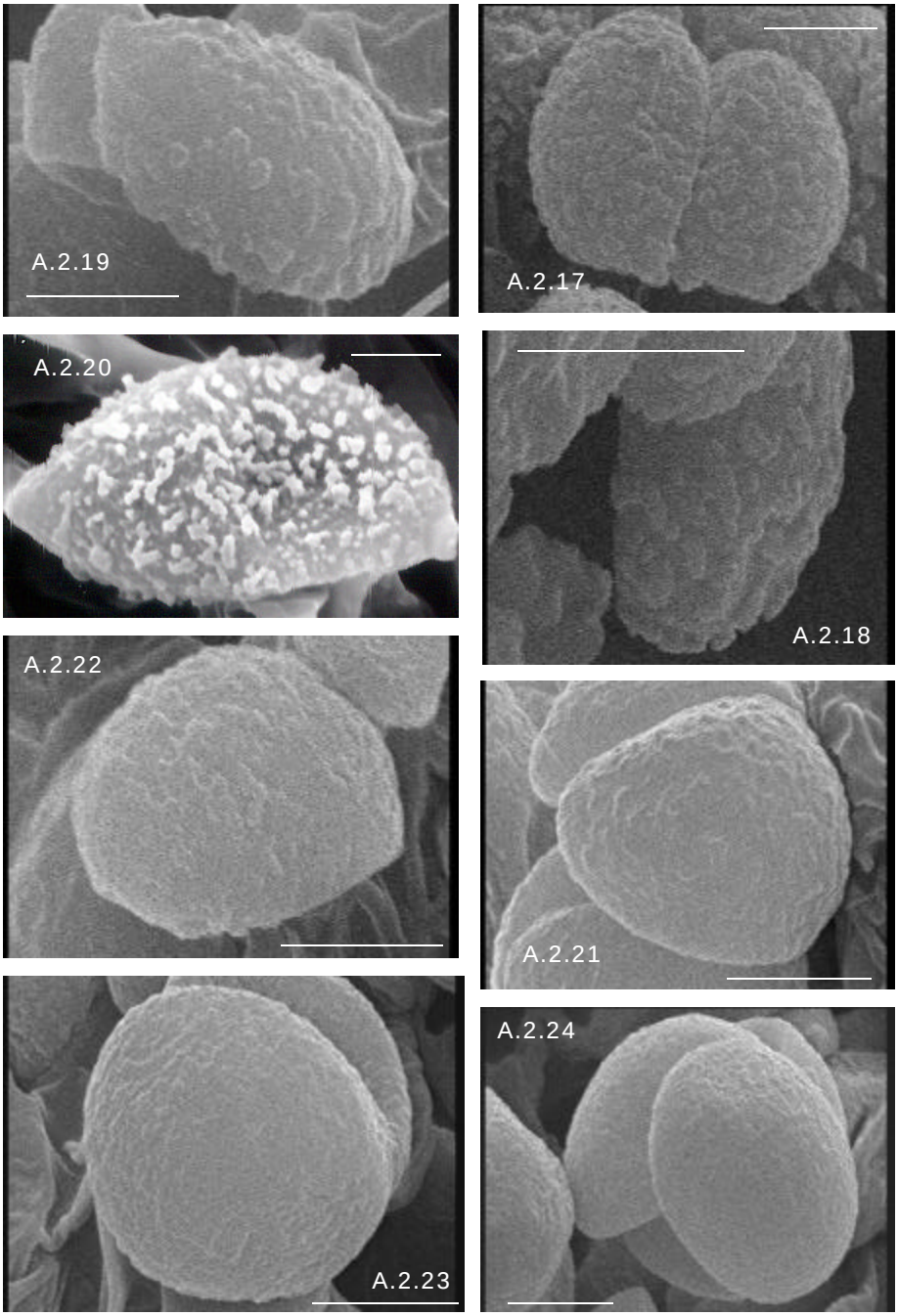
Aime, M.C., and C. Neinhuis. In prep.



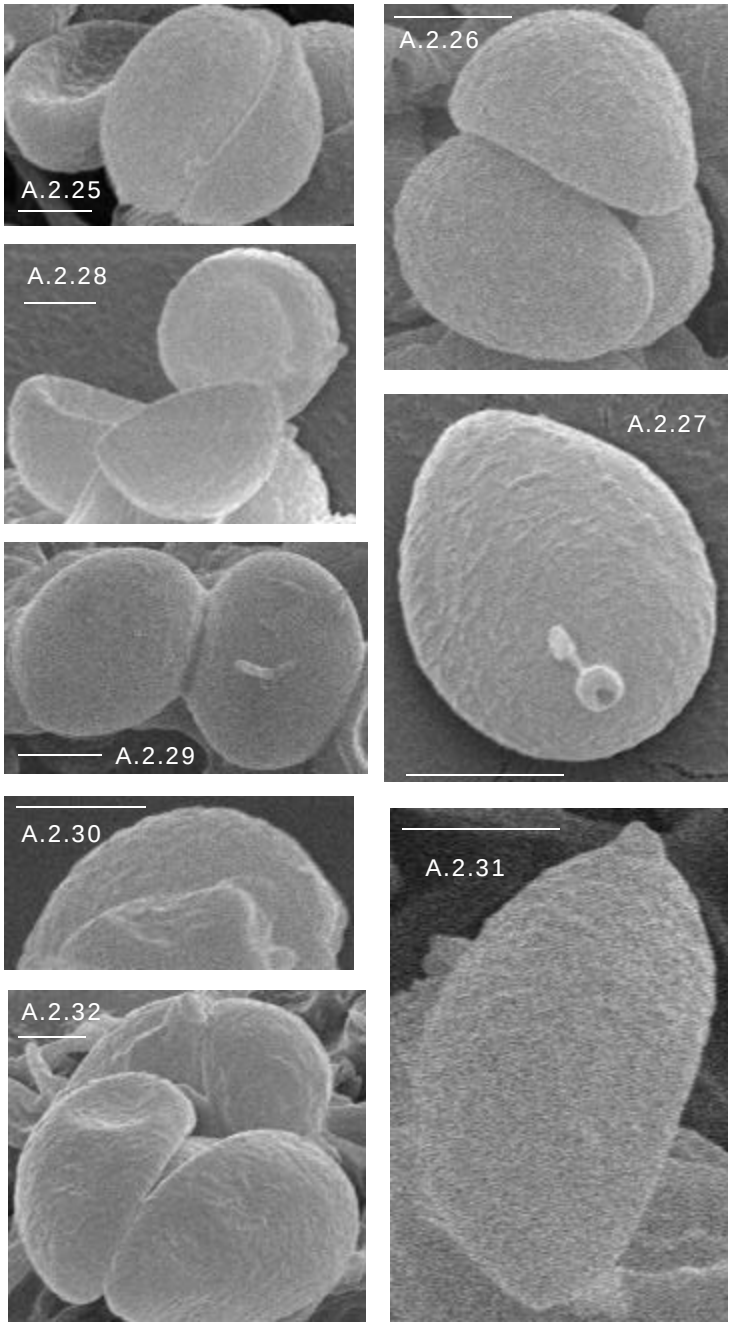
Figs. A.2.1-8. Scanning electron microscopy (SEM) of *Crepidotus* basidiospores: punctate spores due to exosporial columns. Bar = 2 µm. A.2.1. *C. subapplanatus* MCA 331. A.2.2. *C. applanatus* v. *applanatus* s. Josserand MCA 170. A.2.3. *C. malachius* MCA 343. A.2.4. *C. applanatus* v. *globigera* MCA 188. A.2.5. *C. badiofloccosus* MCA 896. A.2.6-7. *C.* sp. MCA 499. A.2.8. *C.* sp. MCA 680.



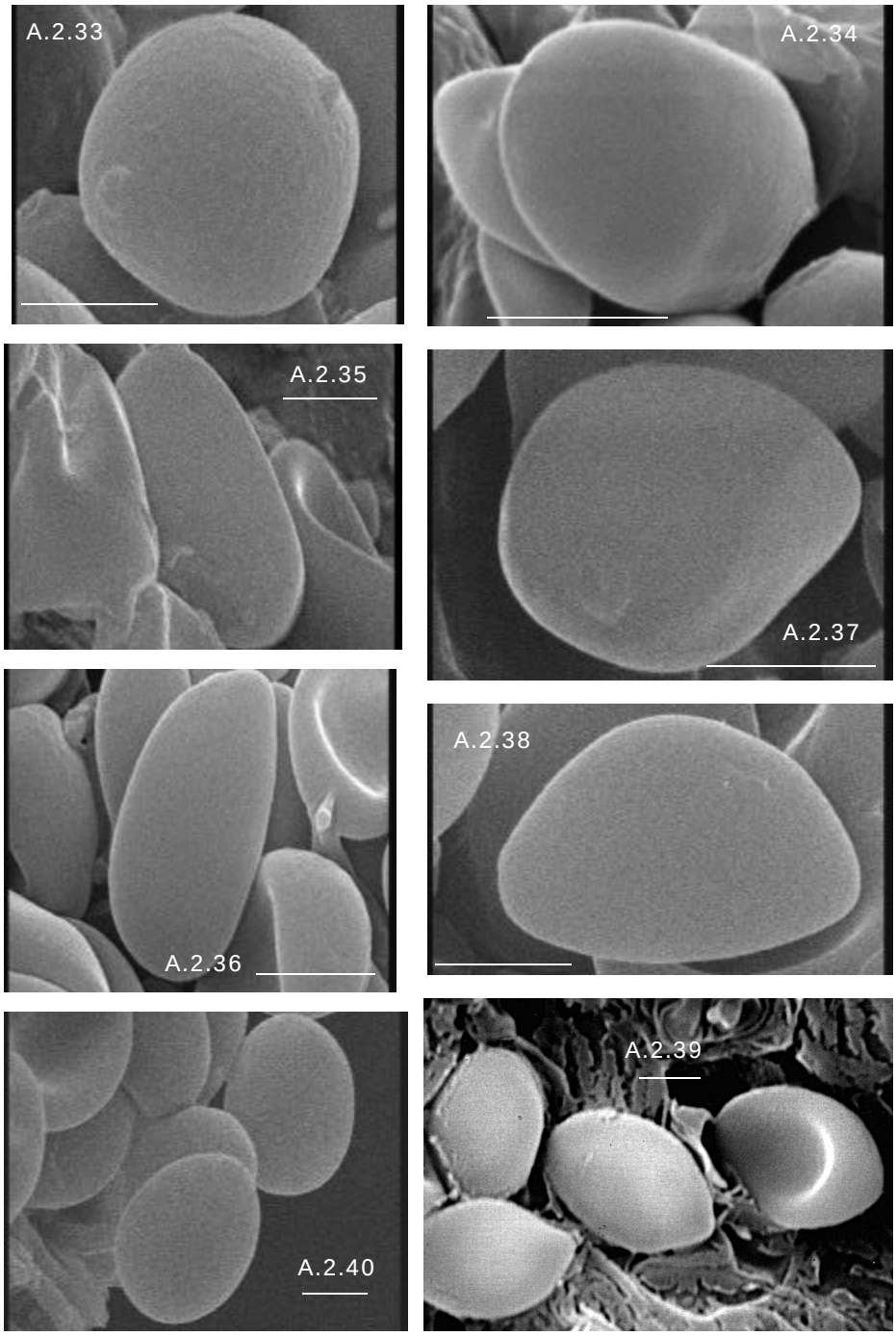
Figs. A.2.9-16. SEMs of *Crepidotus* basidiospores. Bar = 2µm. Embedded hemispherical warts (knobby): A.2.9-10. *C. cesatii* OKM 26976. A.2.11. *C. latifolius* OKM 27051. Short spinulose: A.2.12. *C.* sp. OKM 27503. Fused columns: A.2.13-14. *C. aureus* OKM 27300. Low warts: A.2.15. *C. brunnescens* MCA 864. Coarse warts: A.2.16. *C.* sp. MCA 717.



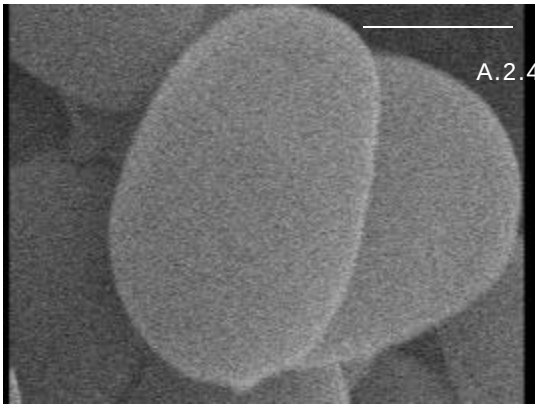
Figs. A.2.17-24. SEMs of *Crepidotus* basidiospores. Bar = 2 µm. Coarse warts: A.2.17-18. *C.* sp. OKM 24649. A.2.19. *C. martini* MCA 640. Verrucose: A.2.20. *C. thermophilus* TJB 8496. Coarsely rugose: A.2.21. *C.* cf. *amygdalosporus* MCA 258. A.2.22. *C.* sp. MCA 757. Finely rugose: A.2.23. *C. inhonestus* complex MCA 638. A.2.24. *C. inhonestus complex* MCA 718.

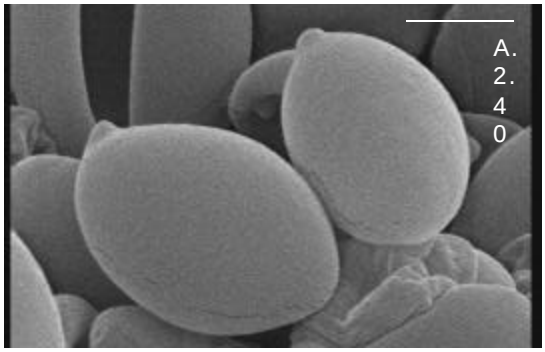


Figs. A.2.25-32. SEMs of *Crepidotus* basidiospores. Bar = 2µm. Rugulose: A.2.25. *C. inhonestus* complex MCA 163. A.2.26. *C. submollis* MCA 920. A.2.27-28. *C.* sp. MCA 717. A.2.29. *C. variabilis* MCA 633. A.2.30. *C.* sp. OKM 26899. A.2.31. *C.* sp. OKM 25016. A.2.32. *C. versutus* MCA 250.



Figs. A.2.33-40. SEMs of *Crepidotus* basidiospores. Bar = 2 µm. Faintly rugulose: A.2.33. *C. subaffinis* MCA 604. Smooth: A.2.34. *C. betulae* MCA 384. A.2.35. *C. antillarum* OKM 26827. A.2.36. *C.* cf. *albissimus* MCA 697. A.2.37. *C. alabamensis* MCA 840. A.2.38. *C. alabamensis* MCA 778. A.2.39. *C. uber* MCA 672. A.2.40. *C. occidentalis* OKM 26740.





Figs. A.2.41-42. SEMs of *Simocybe* basidiospores. Bar = 2µm. A.2.4. *S. serrulata* OKM 27047. A.2.42. *S. haustellaris* MCA 674.

VITA

Mary Catherine Aime was born May 2, 1965 to David and Carolyn Aime in Winchester, Virginia. She was raised in Springfield, Virginia, where she graduated from Robert E. Lee High School in 1983.

Cathie began work towards a B.S. in Biology at Virginia Tech in 1983, leaving in 1986, and returning to complete her degree in 1995. During the interval, she lived, worked, and/or performed in bands in Louisiana, California, Italy, Virginia, New York, and Washington, DC. Cathie is a classical pianist, and plays bass and acoustic guitars. She has been employed as a graphic artist, scientific meetings coordinator, journal reporter, exhibition manager, substitute teacher of special education, collections curator, and managing editor, among other things, and is an amateur entomologist. Cathie has traveled extensively throughout North and Central America, Eastern, Central and Western Europe, Japan, Morocco, the Caribbean, and Guyana.

While completing her undergraduate degree, Cathie had the fortune of enrolling in the Introductory Mycology class taught at Virginia Tech by Orson K. Miller Jr. She began graduate studies in his laboratory during the Fall of 1996. She is a member of the Mycological Society of America, the North American Mycological Association, the British Mycological Society, the Entomological Society of America, the Virginia Academy of Sciences, the Honor Society of Phi Kappa Phi, Phi Sigma Biological Honor Society, and Sigma Xi, The Scientific Research Society. Cathie has received several distinctions including a Graduate Research Award from the Mycological Society of America and an Award of Recognition for Graduate Teaching Assistance Excellence from Virginia Polytechnic Institute and State University.