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Phylogenetic Relationships in the Mushroom Genus *Coprinus* and Dark-Spored Allies Based on Sequence Data from the Nuclear Gene Coding for the Large Ribosomal Subunit RNA: Divergent Domains, Outgroups, and Monophyly

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Phylogenetic relationships were investigated in the mushroom genus *Coprinus* based on sequence data from the nuclear encoded large-subunit rDNA gene. Forty-seven species of *Coprinus* and 19 additional species from the families Coprinaceae, Strophariaceae, Bolbitiaceae, Agaricaceae, Podaxaceae, and Montagneaceae were studied. A total of 1360 sites was sequenced across seven divergent domains and intervening sequences. A total of 302 phylogenetically informative characters was found. Ninety-eight percent of the average divergence between taxa was located within the divergent domains, with domains D2 and D8 being most divergent and domains D7 and D10 the least divergent. An empirical test of phylogenetic signal among divergent domains also showed that domains D2 and D3 had the lowest levels of homoplasy. Two equally most parsimonious trees were resolved using Wagner parsimony. A character-state weighted analysis produced 12 equally most parsimonious trees similar to those generated by Wagner parsimony. Phylogenetic analyses employing topological constraints suggest that none of the major taxonomic systems proposed for subgeneric classification is able to completely reflect phylogenetic relationships in *Coprinus*. A strict consensus integration of the two Wagner trees demonstrates the problematic nature of choosing outgroups within dark-spored mushrooms. The genus *Coprinus* is found to be polyphyletic and is separated into three distinct clades. Most *Coprinus* taxa belong to the first two clades, which together form a larger monophyletic group with *Lacrymaria* and *Psathyrella* in basal positions. A third clade contains members of *Coprinus* section *Comati* as well as the genus *Leucocoprinus, Podaxis pistillaris, Montagnea arenaria,* and *Agaricus pocillator*. This third clade is separated from the other species of *Coprinus* by members of the families Strophariaceae and Bolbitiaceae and the genus *Panaeolus*. © 1999 Academic Press

INTRODUCTION

The genus *Coprinus* is generally regarded as a monophyletic genus by most fungal taxonomists. The genus is defined by its dark-spores possessing an apical germ pore and sequential development of basidia and spores, termed inaequihymeniiferous development (Buller, 1909). This developmental pattern is unique to *Coprinus* but is not found within all sections. In many *Coprinus* species this sequential development terminates in the autolysis of the lamellae and pileus, a characteristic that is responsible for the common name of the genus, the inky caps. Species of *Coprinus* display a wide array of morphological variation and, because they are saprobic, inhabit a great number of different substrates. Many taxa grow easily in culture and can be fruited on simple media, and several species are collected as edible mushrooms in different regions of the world (Chang and Hayes, 1978). Several species of *Coprinus,* notably *C. cinereus* have been useful as model systems for studies of mating compatibility (Bensaude, 1918), speciation (Kemp, 1975), molecular biology, and development (reviewed by Pukkila and Casselton, 1991). In this paper we discuss phylogenetic relationships within the genus *Coprinus* and among its allies in the families of dark-spored apically pored mushrooms.

Sectional taxonomy has varied within *Coprinus* since its first recognition as a distinct entity by S. F. Gray in 1821. Early sectional arrangements by Massee (1896) and Pennington (in Kauffman, 1918) were based solely on macroscopic characteristics. Lange (1939) was the first to contribute microscopic analysis toward defining a sectional nomenclature in *Coprinus,* which divided the genus into three sections based primarily on characteristics of the universal veil, the tissue that surrounds the developing fruit body primordia through its initial development. Sections were further subdivided according to the presence or absence of an annulus or ring around the stipe as well as microscopic evaluations of the veil and epidermal cells of the pileus.

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Subsequent taxonomic treatments have modified Lange's (1939) sectional nomenclature to varying degrees. Orton and Watling (1979) retained Lange's sectional system but used the informal *stirps* category to delimit the genus at a subsectional level (Table 1). Patrick (1977), Kühner and Romagnesi (1978), Van de Bogart (1979), Singer (1986), and others have retained various sectional taxonomies based on the third level of subdivisions of Lange's original system (Table 2). In contrast to the other taxonomic systems, Orton and Watling (1979) divided Lange's (1939) three sections into 21 stirps and contended that the great morphological and ecological diversity within the genus could not be easily organized at a higher taxonomic level. Species were placed into stirps based on ecological, microscopic, and macroscopic morphological characters of the fruit bodies with special emphasis on velar characteristics (Table 1). The taxonomy of Kühner and Romagnesi (1978) and of Orton and Watling (1979) are the two most commonly used systems within *Coprinus* and therefore will be the starting point for taxonomic comparisons within *Coprinus* in this paper.

The genus *Pseudocoprinus* was segregated out of *Coprinus* by Kühner (1929) to represent *Psathyrella disseminatus* (=*C. disseminatus*), a species of *Coprinus* with a lack of deliquescence and other psathyrelloid tendencies. More recently, *Pseudocoprinus* has been abandoned as a generic concept but remains in use as a sectional taxon by Orton and Watling (1979).

Several families of mushrooms, including Coprinaceae, Bolbitiaceae, Agaricaceae, and Strophariaceae, are taxonomically united by morphological and ecological characteristics (Singer, 1986). Individuals within these families are saprobes with thick-walled and pigmented spores. Spores may often have an apically positioned germ pore through which hyphae emerge upon spore germination. Although it has not been tested formally whether this assemblage of families is monophyletic, they represent a starting point for a phylogenetic study of dark-spored Agaricales. While there is a great diversity of morphological variation within the genera from these families, the distinctions among these families are still based largely on traditional taxonomy that emphasizes spore print color. The Agaricaceae are recognized by the chocolate brown color of the spores, free gills, and their habitat of grassy or manured soil, while the Strophariaceae have purple-brown spores, attached gills, and a habitat of wood, dung, or grassy soil. The Coprinaceae are recognized by the presence of black spores and free gills while the Bolbitiaceae have yellow-brown spores and free gills (Miller, 1981).

Although no formal superfamilial taxonomic groupings are recognized among different families, the Coprinaceae and the Bolbitiaceae have been suggested to form a natural group due to the shared presence of a cellular cuticle, a layer of spherical cells that forms the outermost tissue in the cap or pileus of the mushroom fruit bodies (Singer, 1986). On the other hand, the Strophariaceae and the Agaricaceae possess a filamentous cuticle wherein the outermost layer of cells is formed of filamentous hyphae that are oriented in a radial manner outward from the stalk or stipe.

Phylogenetic analyses of ribosomal DNA sequences are beginning to demonstrate relationships among fungi that had not previously been obtainable through morphological characterization alone (Bruns *et al.,* 1991; Hibbett and Thorne, 1998). Although much fungal taxonomy above the species level is still framed within a nonphylogenetic context, the advent of molecular phylogenetic methods for data analysis is contributing to a renaissance in fungal taxonomy. This renaissance has provided new ways of looking at relationships at all taxonomic levels and has served both to confirm supposed taxonomies and to suggest novel relationships.

Sequence data from ribosomal genes have been an important source of phylogenetic information for fungal systematics (Bruns *et al.,* 1991; Hibbett, 1992). Studies have made use of both small- and large-subunit sequences for inferring phylogeny above the species level (Spatafora and Blackwell, 1993; Swann and Taylor, 1993; Vilgalys and Sun, 1994). All of the rDNA genes, including the large-subunit rDNA, are known to be a mosaic of conserved and divergent regions (Hillis and Dixon, 1991; Larson, 1991; Kuzoff *et al.,* 1998). The conserved regions show great similarity in secondary structure even between prokaryotes and eukaryotes (Hassouna *et al.,* 1984). In contrast, the divergent regions (also known as divergent domains or expansion segments; Hassouna *et al.,* 1984; Clark *et al.,* 1984) vary greatly among themselves in their levels of heterogeneity (Hassouna *et al.,* 1984; Hillis and Davis, 1987; Allard and Honeycutt, 1991). These divergent domains occur in similar positions relative to the secondary structure of the large-subunit rRNA from different eukaryotes (Hassouna *et al.,* 1984). It is primarily within these divergent domains that phylogenetic information from large-subunit rDNA has been found (Michot and Bachellerie, 1990; Emberton *et al.,* 1990; Larson, 1991; Hillis and Davis, 1987; Kuzoff *et al.,* 1998). The rDNA sequenced in this study has focused on 7 of these 12 regions within the large-subunit rDNA.

The purpose of this study is to investigate relationships in the mushroom genus *Coprinus* and among its allies through phylogenetic analysis of sequence information from nuclear rDNA. In particular we address the following questions: (1) What are the phylogenetic limits of the genus *Coprinus?;* (2) What other groups of fungi are most closely related to *Coprinus?;* (3) Do the phylogenetic relationships determined within this study mirror other taxonomic systems of the genus, and if not, what new arrangement would be more appropriate?; (4) How do the results of this study bear on the

problem of outgroup selection?; and (5) What are the relative levels of divergence within the regions of sequence studied?

MATERIALS AND METHODS

Taxa

Taxa were sampled at three taxonomic levels to provide structure for assessing relationships within *Coprinus* and among its allies. Exemplar taxa were selected from within the four families of dark-spored apically pored mushrooms representing 12 different genera (Table 1). From within the Coprinaceae three species of the genus *Psathyrella* were selected. *Psathyrella* is generally believed to be closely related to *Coprinus* (Smith, 1972; Singer, 1986). One species each was chosen to represent the 4 other major genera in the Coprinaceae, *Lacrymaria, Annelaria, Panaeolus,* and *Panaeolina.*

Two secotioid taxa were also selected. While secotioid species bear a superficial resemblance to species within various genera of mushrooms, these species do not ballistically release their spores but instead release them passively as in gasteromycetes. One of these species, *Montagnea arenaria,* has been placed within the Coprinaceae by Moser (1983). The other secotioid taxon, *Podaxis pistillaris,* bears superficial resemblance to *Coprinus comatus,* the type species of the genus *Coprinus* (Miller and Miller, 1988).

Three species from different genera were selected to represent the Bolbitiaceae. Pegler and Young (1971) suggested that the Bolbitiaceae and Coprinaceae were closely related on the basis of shared spore characteristics. From within the Strophariaceae, *Stropharia rugosoannulata* and *Hypholoma fasiculare* were selected. *Agaricus pocillator* was selected to represent the genus *Agaricus* within the Agaricaceae.

Two white-spored species from within the genus *Leucocoprinus* were selected on the basis of the morphological similarity between them and members of *Coprinus.* These species possess thick-walled spores, although the spores lack the dark pigment characteristic of the other taxa in this study. Both leucocoprini have the characteristic plicate-striate pileal surface of species in *Coprinus,* although they do not possess inaequihymeniiferous development.

To root the study group, *Amanita citrina* was chosen from the Tricholomatales (Kühner, 1984) and *Russula virescens* from the Russulales (Moser, 1983; Kühner, 1984; Singer, 1986). Neither of these taxa are believed to have arisen from within the ingroup (Moncalvo *et al.,* submitted).

Species within *Coprinus* were selected to represent the eight sections of Kühner and Romagnesi (1978) and the three sections of Orton and Watling (1979) and within these sections the 21 stirps of Orton and Watling (1979). The sections of Kühner and Romagnesi (1978) were represented by a minimum of two species in the case of section *Comati* to a maximum of nine species from section *Impexi* and section *Vestiti* (Table 2), with an average of approximately six species per section. *Coprinus americanus,* which belongs to a species complex unknown to Kühner and Romagnesi and is found only in North America, has been placed in the *Impexi* based on velar characteristics. Twenty of the 21 stirps of Orton and Watling (1979) are represented (Table 2). Due to the uneven nature of species distribution and the rarity of some taxa, stirps tigrinellus was not represented. Neither *C. cf. Impexi* nor *C. americanus* could be placed within the stirps system. *C. cf. Impexi* has characteristics which suggest that it belongs in either stirps lagopus or friesii. *C. americanus* would be placed in either stirps atramentarius or stirps picaceus.

Sequencing

DNA for sequencing was extracted either from mycelia grown in culture or from fruit bodies. Mycelia were grown in liquid YPSS (15.0 g soluble starch, 4.0 g yeast extract, 1.0 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 15.0 g agarose in 1.0 L distilled water) for 2 weeks or until the mycelia covered the surface of the petri dish. Mycelia were harvested and washed with distilled water. Both culture-grown mycelia and fruit bodies were lyophilized and ground with glass beads in liquid nitrogen to aid cell disruption. DNA was extracted using CTAB buffers following the methods of Zolan and Pukkila (1986).

DNA for sequencing was enzymatically amplified using the polymerase chain reaction. Double-stranded DNA was amplified from genomic DNA using two sets of primers (5.8SR to LR7 and LR3R to LR11; Fig. 1, Table 2) using standard conditions (1 min denaturation at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, repeated for 30 cycles). DNA was purified following amplification by centrifugal microfiltration (Ultrafree-MC filters, Millipore). The region of DNA amplified using primers 5.8SR to LR7 was sequenced from asymmetrically amplified single-stranded DNA produced following the methods of Allard *et al.* (1991) by reamplification of the double-stranded DNA using a single primer (Fig. 1). After purification, single-stranded DNA was sequenced using three primers using standard dideoxynucleotide kits (U. S. Biochemical) or with cycle-sequencing kits (Gibco) using either [γ-^{33}P]ATP or [^{32}P-α]dATP (NEN or Amersham) as label. Sequenced reactions were separated on 6% polyacrylamide gels and later exposed to X-ray film for developing.

Divergent Domains

Sequences were read and assembled manually. GenBank accession numbers are given in Table 1. Alignment was performed using the computer programs ALIGN (1989) or ALIGN+ (1992) and the resultant alignments verified by eye. The final alignments and

TABLE 1

List of *Coprinus* Species and Outgroup Taxa Included in the Study Together with GenBank Accession Numbers for New rDNA Sequences

Collection[a]	Species	Section[b]	Stirps[c]	GenBank accession nos.
Agaricales, Coprinaceae				
C114	*Coprinus atramentarius*	Atramentarii	atramentarius	AF041484, AF041615, AF041681, AF041549
C229	*C. acuminatus*	Atramentarii	atramentarius	AF041485, AF041616, AF041682, AF041550
C124	*C. romagnesianus*	Atramentarii	atramentarius	AF041486, AF041617, AF041683, AF041551
C154	*C. cf. erythrocephalus*	Atramentarii	erythrocephalus	AF041496, AF041627, AF041693, AF041561
C262	*C. lagopides*	Lanatuli	lagopus	AF041488, AF041619, AF041685, AF041553
C432	*C. macrocephalus*	Lanatuli	lagopus	AF041489, AF041620, AF041686, AF041554
C28	*C. lagopus*	Lanatuli	lagopus	AF041490, AF041621, AF041687, AF041555
C4	*C. bilanatus*	Lanatuli	lagopus	AF041491, AF041622, AF041688, AF041556
C106	*C. pseudoochraceovelatus*	Lanatuli	lagopus	AF041492, AF041623, AF041689, AF041557
C18	*C. radiatus*	Lanatuli	lagopus	AF041493, AF041624, AF041690, AF041558
C13	*C. cinereus*	Lanatuli	lagopus	AF041494, AF041625, AF041691, AF041559
C192	*C. cf. Impexi*	Impexi	?[d]	AF041495, AF041626, AF041692, AF041560
C34	*C. americanus*	Impexi	?	AF041487, AF041618, AF041684, AF041552
C102	*C. xenobius*	Impexi	filamentifer	AF041498, AF041629, AF041695, AF041563
C163	*C. phlyctidosporus*	Impexi	echinosporus	AF041499, AF041630, AF041696, AF041564
C78	*C. kimurae*	Impexi	picaceus	AF041500, AF041631, AF041697, AF041565
C21	*C. dictyocalyptratus*	Impexi	picaceus	AF041497, AF041628, AF041694, AF041562
C182	*C. gonophyllus*	Impexi	friesii	AF041502, AF041633, AF041699, AF041567
C220	*C. friesii*	Impexi	friesii	AF041503, AF041634, AF041700, AF041568
C70	*C. utrifer*	Impexi	utrifer	AF041501, AF041632, AF041698, AF041566
C214	*C. flocculosus*	Vestiti	flocculosus	AF041515, AF041646, AF041712, AF041580
C151	*C. luteocephalus*	Vestiti	cortinatus	AF041505, AF041636, AF041702, AF041570
C181	*C. trisporus*	Vestiti	narcoticus	AF041504, AF041635, AF041701, AF041569
C249	*C. narcoticus*	Vestiti	narcoticus	AF041506, AF041637, AF041703, AF041571
C93	*C. semitalis*	Vestiti	narcoticus	AF041508, AF041639, AF041705, AF041573
C121	*C. sclerotiger*	Vestiti	narcoticus	AF041509, AF041640, AF041706, AF041574
C381	*C. cothurnatus*	Vestiti	niveus	AF041507, AF041638, AF041704, AF041572
C20	*C. latisporus*	Vestiti	niveus	AF041510, AF041641, AF041707, AF041575
C3	*C. cordisporus*	Vestiti	niveus	AF041511, AF041642, AF041708, AF041576
C12	*C. micaceus*	Micacei	micaceus	AF041513, AF041644, AF041710, AF041578
C143	*C. xanthothrix*	Micacei	domesticus	AF041512, AF041643, AF041709, AF041577
C62	*C. domesticus*	Micacei	domesticus	AF041514, AF041645, AF041711, AF041579
C91	*C. radians*	Micacei	domesticus	AF041516, AF041647, AF041713, AF041581
C159	*C. nudiceps*	Hemerobii	hemerobius	AF041517, AF041648, AF041714, AF041582
C170	*C. megaspermus*	Hemerobii	hemerobius	AF041518, AF041649, AF041715, AF041583
C153	*C. auricomus*	Hemerobii	auricomus	AF041519, AF041650, AF041716, AF041584
C95	*C. heterosetulosus*	Setulosi	ephemerus	AF041520, AF041651, AF041717, AF041585
C148	*C. bisporus*	Setulosi	ephemerus	AF041523, AF041654, AF041720, AF041588
C66	*C. aokii*	Setulosi	ephemerus	AF041526, AF041657, AF041723, AF041591
C294	*C. congregatus*	Setulosi	ephemerus	AF041528, AF041659, AF041725, AF041593
C276	*C. cf. sclerocystidiosus*	Setulosi	hiascens	AF041521, AF041652, AF041718, AF041586
C339	*C. callinus*	Setulosi	hiascens	AF041524, AF0416 55, AF041721, AF041589
C221	*C. disseminatus*	Setulosi	disseminatus	AF041525, AF041656, AF041722, AF041590
C299	*C. heptemerus*	Setulosi	disseminatus	AF041522, AF041653, AF041719, AF041587
C117	*C. curtus*	Setulosi	disseminatus	AF041527, AF041658, AF041724, AF041592
C116	*C. comatus*	Comati	comatus	AF041529, AF041660, AF041726, AF041594
C123	*C. sterquilinus*	Comati	comatus	AF041530, AF041661, AF041727, AF041595
J181	*Psathyrella candolleana*			AF041531, AF041667, AF041733, AF041601
J156	*P. delineata*			AF041532, AF041668, AF041734, AF041602
J130	*P. gracilis*			AF041533, AF041669, AF041735, AF041603
J100	*Lacrymaria velutina*			AF041534, AF041670, AF041736, AF041604
J129	*Panaeolus acuminatus*			AF041535, AF041671, AF041737, AF041605
SAR 85/162	*Annelaria semiovatus*			AF041536, AF041672, AF041738, AF041606
J152	*Panaeolina foensecii*			AF041537, AF041673, AF041739, AF041607
Agaricales, Montagneaceae				
J117	*Montagnea arenaria*			AF041538, AF041662, AF041728, AF041596
Podaxales, Podaxaceae				
J119	*Podaxis pistillaris*			AF041539, AF041663, AF041729, AF041597
Agaricales, Lepiotaceae				
J132	*Leucocoprinus fragilissimus*			AF041540, AF041664, AF041730, AF041598
J133	*L. birnbaumii*			AF041541, AF041665, AF041731, AF041599

TABLE 1—*Continued*

Collection[a]	Species	Section[b]	Stirps[c]	GenBank accession nos.
Agaricales, Agaricaceae				
J173	*Agaricus pocillator*			AF041542, AF041666, AF041732, AF041600
Agaricales, Bolbitiaceae				
SAR 84/100	*Bolbitius vitellinus*			AF041543, AF041674, AF041740, AF041608
J123	*Agrocybe praecox*			AF041545, AF041676, AF041742, AF041610
J183	*Conocybe rickenii*			AF041546, AF041677, AF041743, AF041611
Agaricales, Strophariaceae				
D258	*Stropharia rugosoannulata*			AF041544, AF041675, AF041741, AF041609
J143	*Hypholoma fasiculare*			AF041678, AF041744, AF041612
Agaricales, Amanitaceae				
J187	*Amanita citrina*			AF041547, AF041679, AF041745, AF041613
Russulales, Russulaceae				
DHN1045	*Russula virescens*			AF041548, AF041680, AF041746, AF041614

[a] Voucher collections and cultures deposited at DUKE.
[b] Sections within *Coprinus* according to Kühner and Romagnesi (1978); stirps within *Coprinus* based on Orton and Watling (1979).
[c] *C. cf. Impexi* has definite affinities with the *Impexi* but its stirps affiliation is unclear. *Coprinus americanus* also belongs in section *Impexi* but cannot be placed in a stirps within the present scheme of Orton and Watling (1979).

trees associated with phylogenetic analysis are deposited within TreeBase (http://www.herbaria.harvard.edu/treebase/index.html). Divergent domains (Hassouna *et al.,* 1984) were mapped onto the alignments based on their position on *Saccharomyces carlsbergensis* (Veldman *et al.,* 1981). Pairwise sequence divergence was estimated using the Dnadist algorithm within PHYLIP (Felsenstein, 1988) for aligned sequences in all pairwise combinations of taxa according to the two-parameter model of Kimura. Relative rates of divergence among different divergent domains were compared by linear regression using SYSTAT 5.2 (1990–1992) to contrast relative amounts of divergence between the divergent domains.

Phylogenetic Analysis

Parsimony analysis was performed using PAUP 3.1 (Swofford, 1993). Ambiguous (undeterminable) nucleotides were coded as missing. Single-position gaps were coded as characters using the GAPMODE = NEWSTATE command. All characters for the data sets were coded as unordered, and only phylogenetically informative characters were included in the parsimony analysis. The heuristic algorithm in PAUP employing tree bisection–reconnection (TBR) branch swapping was used after the removal of regions where reasonable alignment was not possible. Ten replicates were performed within each heuristic search using random taxon addition. A strict consensus integration of resultant equally most parsimonious trees was produced. Both equally weighted (Wagner parsimony) and unequally weighted (character-state weighted) approaches were used. For unequally weighted parsimony a stepmatrix was utilized, making transversions carry twice the value of transitions. (See Albert and Mishler, 1992 for discussion on weighting sequence data.)

TABLE 2

Large-Subunit rDNA Primers Used for DNA Amplification and Sequencing

Primer	Sequence (5′ to 3′)	Position within *S. cereviseae* rRNA
5.8SR	TCGATGAAGAACGCAGCG	34–51 (5.8S RNA)
LR3R	GTCTTGAAACACGGACC	638–654
LR5	TCCTGAGGGAAACTTCG	964–948
LR7	TACTACCACCAAGATCT	1448–1432
LR8	CACCTTGGAGACCTGCT	1861–1845
LR9	AGAGCACTGGGCAGAAA	2204–2188
LR11	GCCAGTTATCCCTGTGGTAA	2821–2802
LR14	AGCCAAACTCCCCACCTG	2616–2599
LR16	TTCCACCCAAACACTCG	1081–1065
LR21	ACTTCAAGCGTTTCCCTTT	424–393

Five indicators of clade robustness were calculated for the Wagner parsimony analysis. These included the number of characters on a branch, the number of unambiguous characters on a branch, the number of unambiguous transversions on a branch, the decay index (Bremer, 1994), and bootstrap proportions (Felsenstein, 1985; Hillis and Bull, 1993) for each branch. Only bootstrap proportions were calculated for the character-state weighted approach. All indices were calculated using PAUP 3.1.

Agreement among alternative taxonomic systems was explored using PAUP 3.1 and MacClade 3.01 (Maddison and Maddison, 1992). Phylogenetic trees containing various taxonomic groupings were created using MacClade. These tree topologies were then submitted to PAUP as constraint trees. The lengths of most parsimonious trees fitting constraints of previous classifications were then found using PAUP under different topological constraints. Tree length differences between constrained vs unconstrained trees were tested

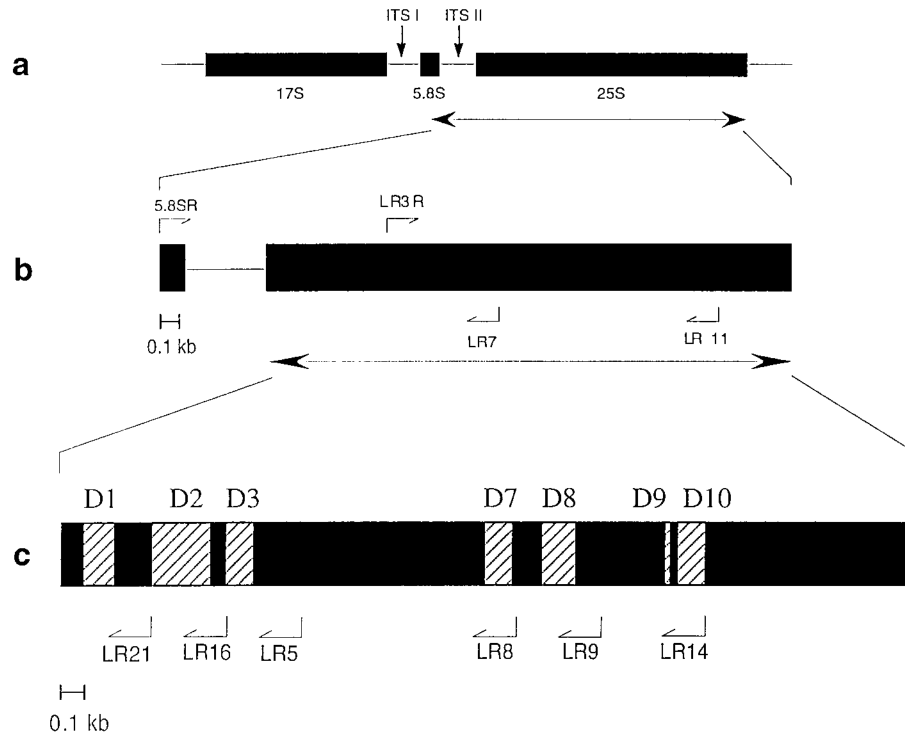

FIG. 1. Ribosomal DNA regions included in the study. (a) General diagram of ribosomal subunit showing area amplified (top arrow). (b) Sections of 5.8S, ITS II, and the large ribosomal subunit. Primer pairs 5.8SR-LR7 and LR3R-LR11 were used for sequencing template preparation using the PCR. (c) Positions of primers used in sequencing large ribosomal subunit rDNA. Hatched areas represent seven divergent domains which were sequenced.

using the nonparametric rank-sum test of Templeton (1983).

RESULTS

Sequencing

A total of 1360 positions were sequenced for each of 66 taxa (Table 1). Sequences obtained with primers LR21, LR16, and LR5 were assembled to yield continuous sequence corresponding to positions 74–866 of the yeast large-subunit rDNA (Hassouna *et al.,* 1984). Shorter sequences determined using primers LR8, LR9, and LR14 did not overlap; these correspond to positions 1645–1830, 1909–2114, and 2448–2587 in yeast, respectively (Hassouna *et al.,* 1984). Alignment problems were encountered between positions 177–180, 471–473, 548–560, and 1171–1175 within the large-subunit RNA. These regions along with buffer zones of two bases to either side of the unalignable regions were excluded from phylogenetic analysis.

Three hundred and two potentially phylogenetically informative characters were coded from the aligned sequence data. Twenty-four of these characters (8%) were located outside of divergent domains and 16 of these 24 were found in the D7 app. region. Five percent of the positions in the intervening sequence yielded informative characters when including the D7 app. region or 1.5% of the intervening sequence without the D7 app. region. Thirty-two percent of the positions in the divergent-domain sequence yielded informative characters.

Divergent Domains

An examination of sequence diversity for the regions studied illustrates the wide range of variability found within the large-subunit rDNA (Table 3). Comparison of mean sequence divergence among taxa shows D8 to be the most variable divergent domain followed in order of decreasing divergence by regions D2, D3, D1, D10, and D7. A short region of rDNA 5′ to D7 (labeled as "D7 appendix" in Table 3) also shows variation similar to that seen within the divergent domains. The D7 app. region had a mean divergence of 0.057, making it more divergent, on average, than either region D7 or D9–D10. At least within the dark-spored fungi studied here, our study suggests a boundary for region D7 that was more extended than was previously assumed.

A comparison of divergence between each of the divergent domains and the entire large-subunit sequence provides information about the relative rate of evolution within different regions of large-subunit rDNA (Fig. 2). Based on slopes of linear regressions for pairwise comparisons of divergences among taxa between different regions, region D8 is 2.6 times as

TABLE 3

Pairwise Sequence Divergence (Minimum–Maximum) among Divergent Domains within the Large-Subunit rDNA

DNA region	Length (bp)	Pairwise sequence divergence
D1	149	0–0.255
D2	261	0.004–0.377
D3	119	0–0.288
D7	100	0–0.202
D7 appendix	84	0–0.258
D8	144	0–0.435
D9–D10	104	0–0.141
Intervening sequence	399	0–0.041
Entire sequence	1360	0.002–0.187

Note. Sequence divergence was calculated based on Kimura 2-parameter model in PHYLIP (Felsenstein, 1988).

divergent as the entire sequence studied and 1.4 times as divergent as D2, the next most divergent region (Fig. 2). The least divergent of the divergent domains was the D9–D10 region, which actually showed less divergence than the entire sequence studied (slope y = 0.681). The D7 app. region showed amounts of divergence similar to both the D7 and the D9–10 regions (data not shown). Divergent domains ranged from 5.0 to 18.5 times more divergent than the intervening sequences without the D7 app. region (data not shown).

Phylogenetic Analysis

Wagner parsimony analysis resulted in two most parsimonious trees of 1461 steps (Fig. 3). Both trees had a consistency index of 0.313 and a rescaled consistency index (RCI) of 0.212. These trees differ by the placement of three clades marked a, b, and c in Fig. 3. In the one topology presented in Fig. 3, clades a and b are shown together. In the other topology, clades b and c form a monophyletic group with clade a as a sister group.

Weighted parsimony resulted in 12 equally most parsimonious trees of 1799 steps each (Fig. 4), which

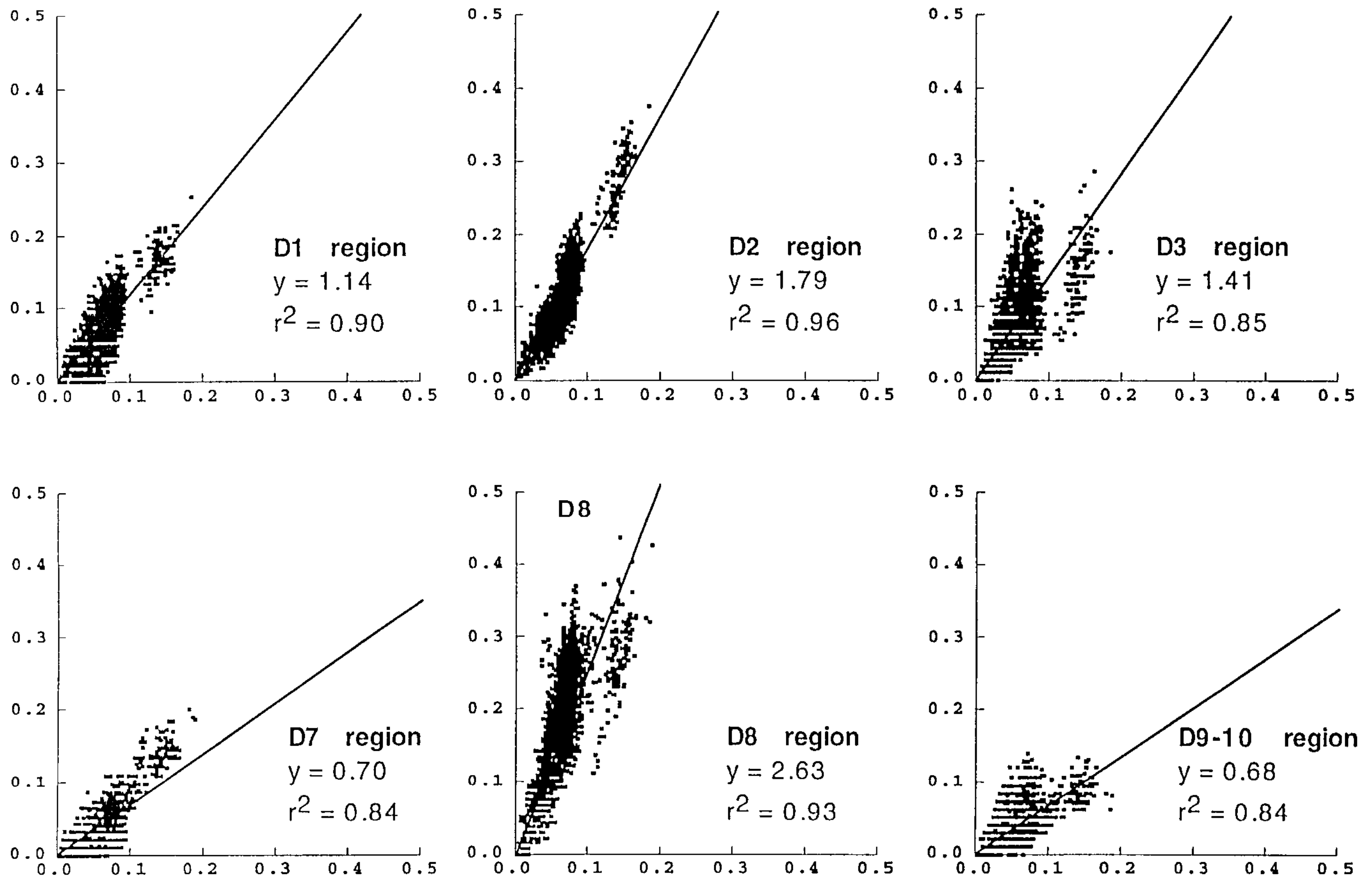

FIG. 2. Sequence divergence among different divergent domains within the large-subunit rDNA from *Coprinus.* Pair-wise distance estimates for each divergent domain are plotted against distances calculated over the entire large-subunit sequence. Distances were estimated based on the two-parameter model of Kimura implemented using PHYLIP (Felsenstein, 1988).

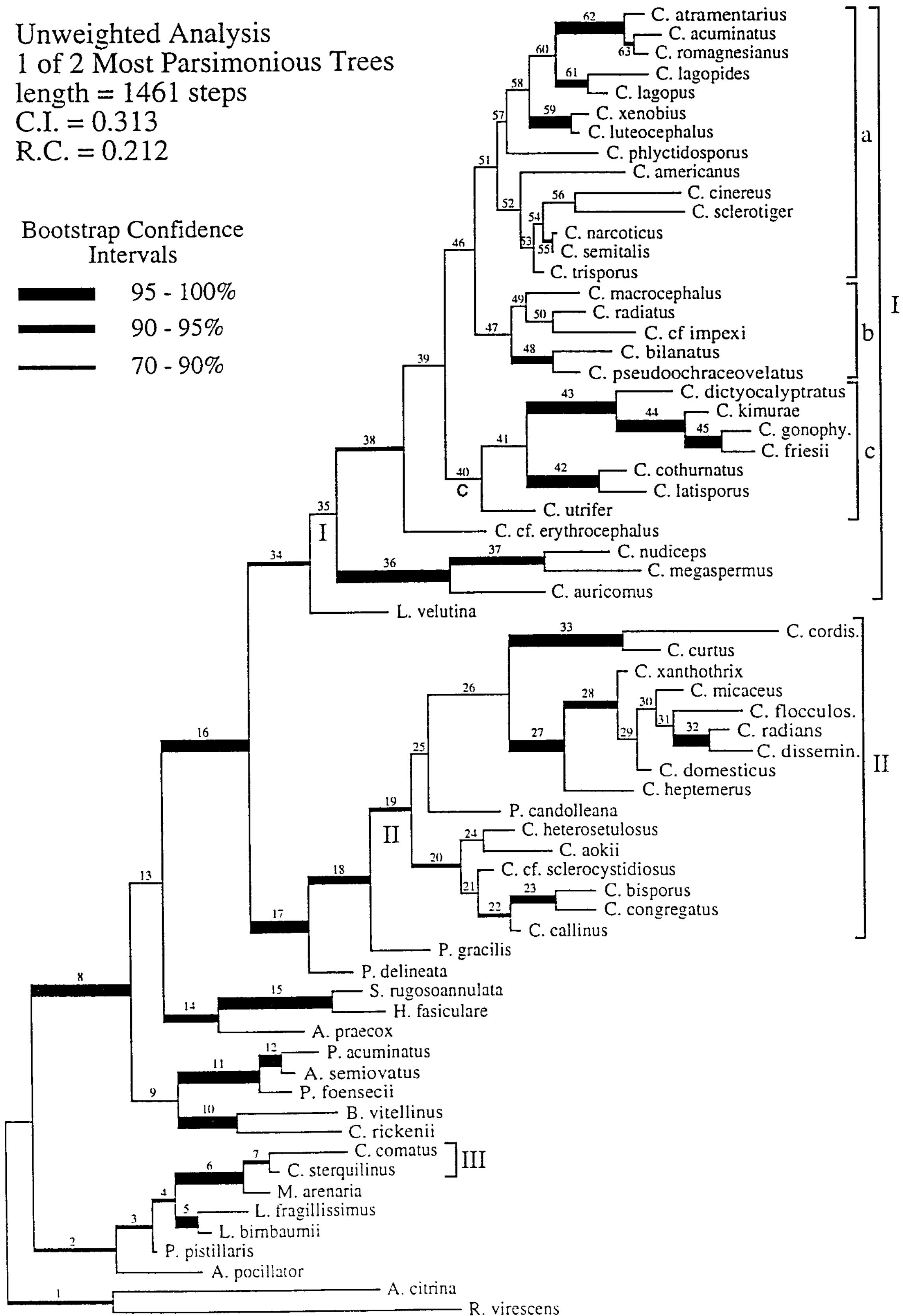

FIG. 3. Phylogram showing one of two equally most parsimonious trees obtained using (Wagner) parsimony (characters unweighted and unordered). Branch nodes are labeled 1–63 for reference with Table 4. Line widths indicate different levels of bootstrap support above 70%.

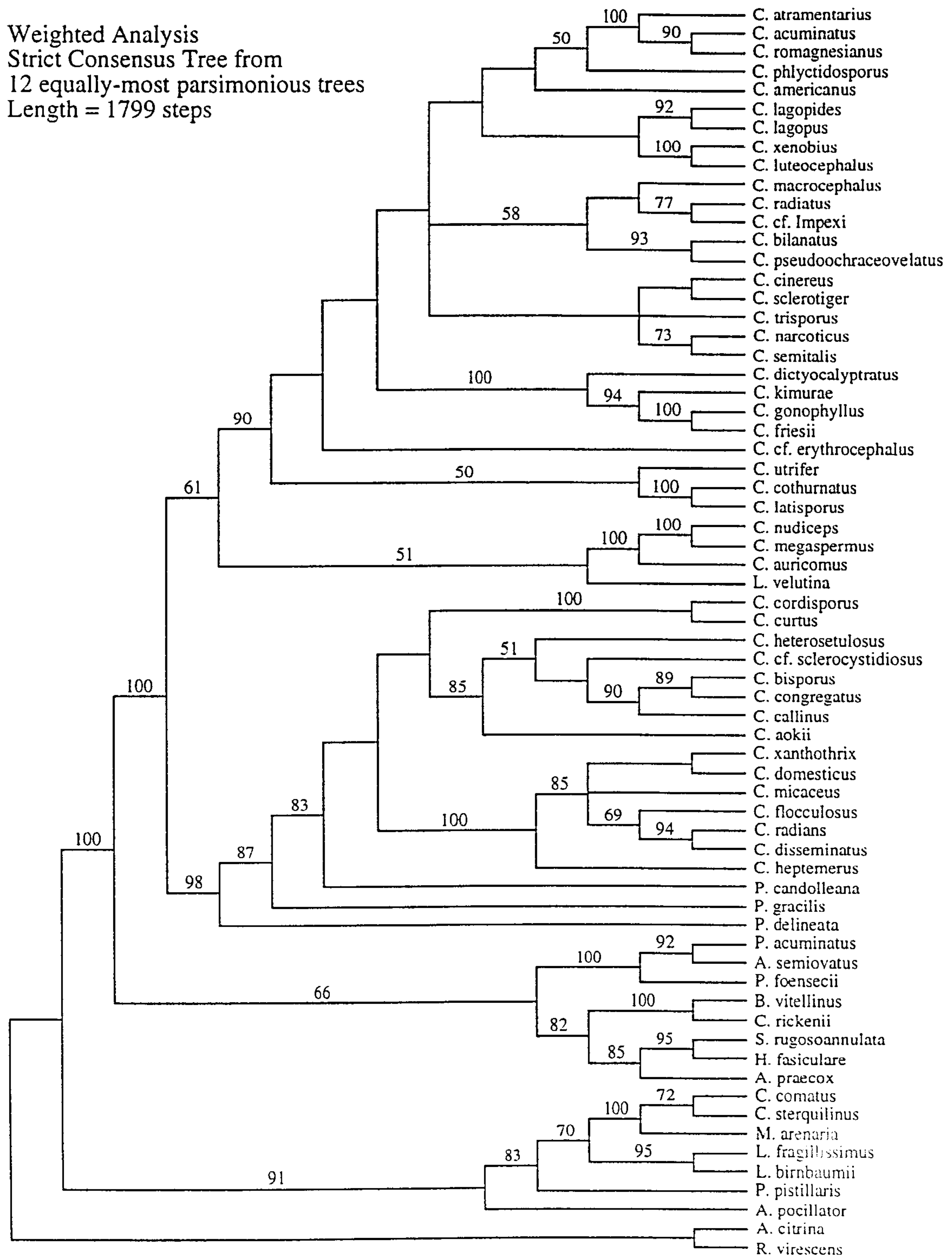

FIG. 4. Strict consensus cladogram of 12 equally most parsimonious trees obtained using stepmatrix weighted parsimony analysis (transversions weighted twice over transitions). Numbers above branches indicate bootstrap values (based on 40 replicates).

were also similar to trees produced using Wagner parsimony. Differences found in the character-state weighted topology include the grouping of the *Stropharia*/*Hypholoma*/*Agrocybe* clade with the Bolbitiaceae and *Panaeolus*/*Annelaria*/*Panaeolina* clades. *Coprinus cordisporus* and *C. curtus* from section *Setulosi* are grouped with the rest of this section under character-state weighted parsimony as opposed to being grouped with section *Micacei*. *Lacrymaria* is grouped with section *Hemerobii* in the character-state weighted

topology instead of as a basal clade to this section. Other rearrangements occur in the clades containing sections *Atramentarii, Lanatuli, Vestiti,* and *Impexi.* All of the rearrangements occurred on branches that were not well supported by robustness indices.

As a heuristic test for how much phylogenetic signal is contained within each divergent domain, we performed a phylogenetic analysis (Table 4) for nine taxa representing the major lineages in Fig. 3. These nine taxa were selected based on the high level of support which they received in the phylogenetic analysis. BRANCH and BOUND searches of the nine exemplar sequences yielded one phylogenetic tree of 246 steps, with four terminal clades supported by bootstrap proportions greater than 95% (Fig. 5, Table 5). When individual analyses were performed using each divergent domain as a separate data set, region D2 had the highest RCI and region D7 (including D7 app.) the lowest (Table 5). Since different trees were resolved by each divergent domain, these analyses were also repeated by constraining the searches to match the tree obtained for the total nine-taxon data set (Fig. 5). With this constraint in place, region D3 was found to have the highest RCI while regions D9–D10 had the lowest. Regions D2 and D8 both had average bootstrap proportions of 99% for the four terminal clades. These values were the highest among the divergent domains while regions D7 (including D7 app.) and D9–D10 had the lowest with 66% and 63%, respectively.

The distribution of phylogenetic signal in the data set was explored by plotting the number of informative changes inferred for each character as a histogram along the length of the entire sequence (Fig. 6). Virtually all phylogenetically informative sites (>98%) were found to occur within the divergent domains of the large subunit rDNA gene, with most changes observed within divergent domains D2 and D8. Half of the total phylogenetic signal (50%) was found to occur within the divergent domains D1–D3, with an additional 23% occuring within the D7–D8 region.

DISCUSSION

Divergent Domains

Amounts and rates of sequence evolution vary greatly across the large-subunit rDNA gene (Figs. 2 and 6). Regions D2 and D8 appear to be most divergent while D7 and D10 are the least divergent (Fig. 2). Based on the distribution of informative nucleotide sites in the data set, the seven divergent domains investigated accounted for 98% of the average divergence between taxa while the intervening sequence supplied less than 2% of this divergence (Fig. 6). The high level of divergence in D2 and D8 has been recognized in a number of other eukaryotes including fungi (Hassouna *et al.,* 1984; Larson, 1991; Hibbett, 1991; Hillis and Dixon, 1991; Kurzoff *et al.,* 1998).

The striking differences in divergence seen for different divergent domains and their intervening sequences suggest that not all regions of DNA sequence have the same utility for phylogenetic analyses (Emberton *et al.,* 1990; Hillis and Dixon, 1991, Hibbett, 1992). It has been suggested that divergent domains should have more utility for phylogenetic inference below the ordinal level, while intervening sequences have variation that is more useful at higher taxonomic levels (Hillis and Dixon, 1991). Although large-subunit rDNA sequences are now being increasingly used for phylogenetic studies in many fungi, there has been little attention given to the question of utility of various regions within this gene. Based on our study, which is aimed at the level of genus/family, intervening sequences within the large-subunit rDNA gene showed little or no variation for phylogenetic analysis, while some individual divergent domains (e.g., D2) were found to be capable of resolving most major lineages. These results suggest that, where sequencing efforts are constrained by limited resources, shorter regions of DNA containing divergent domains might still provide adequate signal for phylogenetic analysis. This observation might also explain how some recent analyses of short large-subunit rDNA sequences were able to resolve phylogenetic relationships across broad taxonomic groups of yeasts and other fungi using relatively short segments (500 bp) of DNA sequence (Kurtzman and Robnett, 1998).

The utility of sequence data for phylogenetic analysis is dependent on both the amount of variation and the quality of phylogenetic signal that is present. Slowly evolving sequences that show too little variation may not provide enough characters for resolving phylogenetic relationships, while the presence of multiple substitutions in sequences with too much variation would result in phylogenetic signal being overwhelmed. For this reason, we explored the potential of our data to obtain a heuristic estimate of phylogenetic signal within different portions of the large-subunit rDNA using an abbreviated taxon set (Fig. 5, Table 5). Although this analysis should ideally be performed on the complete data set in order to observe the behavior of character sets across all taxa, only nine taxa were used to allow for the computationally expensive process of generating statistically significant bootstrap proportions (see Hedges, 1992, for discussion). Two criteria were used to evaluate phylogenetic signal: (1) comparison of rescaled consistency indices for trees produced using the phylogenetically informative characters from each divergent domain and (2) comparison of bootstrap proportions for each of four robust branches supported by each data set (Table 5). For this analysis, the true phylogeny is taken to be the most parsimonious tree generated by the full data set for these nine taxa.

One most parsimonious tree was produced for the nine-taxon data set (Fig. 5); however, only one of the

TABLE 4

Character Analysis for Branch Nodes within Most Parsimonious Tree (Fig. 3)

Branch	Branch length	Unambiguous character changes	Unambiguous transversions	Decay index[a]	Bootstrap value[b]
1	29	15	2	—	89
2	18	10	4	>2+	87
3	8	4	1	2	79
4	5	3	2	1	71
5	5	5	1	>2+	99
6	15	11	2	>2+	100
7	6	5	2	2+	82
8	22	13	3	>2+	100
9	10	7	2	1	
10	13	10	2	>2+	99
11	18	10	4	>2+	100
12	5	4	3	2+	95
13	7	2	0	1	
14	12	11	3	>2+	93
15	25	5	2	>2+	95
16	19	16	5	>2+	100
17	13	11	3	>2+	98
18	14	13	2	>2+	91
19	9	4	2	2	72
20	11	10	6	2+	75
21	4	1	0	1	50
22	7	7	3	2+	74
23	10	8	3	>2+	92
24	5	3	0	1	
25	4	4	2	1	
26	18	5	0	1	
27	12	11	6	>2+	100
28	12	7	1	>2+	89
29	4	2	0	1	
30	4	2	2	1	57
31	4	2	0	1	65
32	8	6	4	>2+	97
33	25	23	9	>2+	100
34	14	9	1	>2+	85
35	6	3	1	1	
36	25	17	4	>2+	100
37	21	18	6	>2+	94
38	15	8	1	>2+	87
39	9	4	1	1	
40	8	3	1	1	50
41	10	6	0	1	50
42	16	10	3	>2+	100
43	20	12	7	>2+	100
44	15	11	1	>2+	98
45	8	6	4	>2+	96
46	7	2	0	0	
47	8	7	4	>2+	58
48	9	7	3	>2+	91
49	3	1	1	1	
50	6	6	1	2	63
51	5	2	0	1	
52	5	4	2	1	
53	3	2	1	1	
54	2	2	1	1	
55	2	2	1	2	82
56	7	5	0	1	
57	2	2	1	1	
58	5	4	3	1	
59	9	9	2	>2+	95
60	6	4	0	1	
61	7	6	1	>2+	92
62	15	12	6	>2+	100
63	2	2	0	2	88

Note. Support indices were calculated using Paup 3.1 (Swofford, 1993). Characters, unambiguous characters, and unambiguous transversions were identified using the LIST APOMORPHIES command. Decay index of >2+ and bootstrap confidence intervals were generated using Wagner pasimony.

[a] Because of the large number of alternative trees, decay indices could not be calculated above 2.

[b] Values given only for bootstrap values above 50%.

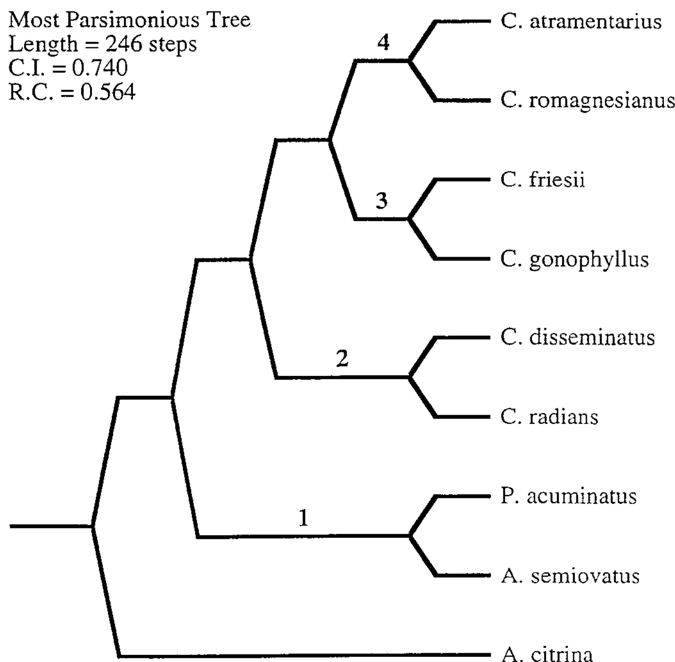

FIG. 5. Single most parsimonious tree from analysis of nine taxa based on sequence data from the entire data set. Branch nodes are labeled for reference with Table 5.

divergent domains (D3) contained enough signal to produce the same topology as the combined data. As a consequence, region D3 also had the highest RCI when the phylogenetic search within each divergent domain was constrained by the "true" topology based on total evidence (Table 5). Based on RCI as a criterion, region D1 had the least amount of homoplasy with an RCI of 0.728, with region D3 having a slightly lower RCI of 0.701. Based on bootstrap values as criterion, regions D2 and D8 had the best support for the four clades, even though both had lower RCI values than region D3. Region D3 had the second highest average bootstrap values for the four robust clades. Region D9–D10 had the lowest RCI for the constrained topology and the lowest bootstrap average.

From an empirical standpoint, and within the taxonomic range of the dark-spored apically pored fungi studied here, regions D2 and D8 have both high levels of divergence and high relative levels of signal. Region D3, which has a high relative level of signal, is not as divergent as either D2 or D8. This region would not be expected to produce as many phylogenetically informative characters as either D2 or D8 even though the signal in these characters might be more useful. The size of the taxon set may therefore be of primary importance in determining which divergent domain(s) would be most useful for phylogenetic analysis.

Outgroup Rooting

There has been a great deal of discussion concerning the choice of outgroups and their effect on polarity (Lundberg, 1972; Watrous and Wheeler, 1981; Donoghue and Cantino, 1984; Maddison *et al.,* 1984). Less attention has been devoted to the proper choice of outgroup for the purpose of rooting. When phylogenetic relationships are unclear it is possible to incorrectly select an outgroup taxon that may even be derived from within the ingroup. For higher level taxonomic studies that include representative samples from the ingroup, the choice of outgroup is greatly simplified. For every group that has yet to be included in a higher taxonomic level study, however, the selection of an outgroup remains problematic.

The mushrooms and their allies represent several good examples of the problem of identifying a proper outgroup. Relationships of most major groups of mushrooms are still not well known, although recent molecu-

TABLE 5

Statistics for Phylogenetic Signal Analysis of Divergent Domains with Reference to Fig. 5

	Divergent domain						Entire sequence
	D1	D2	D3	D7	D8	D9–D10	
Sequence length	149	261	119	184	144	104	1360
RCI unconstrained[a]	0.728	0.609	0.701	0.520	0.615	0.587	0.564
RCI constrained	0.510	0.555	0.701	0.399	0.558	0.387	0.564
Bootstrap support[b] for							
Node 1	100	100	99	77	98	58	100
Node 2	99	100	89	78	100	70	100
Node 3	79	98	100	25	99	33	100
Node 4	72	99	99	84	99	89	100
Nodes 1–4 (average)	87	99	97	66	99	63	100

Note. See text for additional explanation.

[a] Rescaled Consistency Indices given for most parsimonious trees based on partial sequence (unconstrained analysis) and for complete sequence (Fig. 5).

[b] Bootstrap proportions based on 1825 replicates.

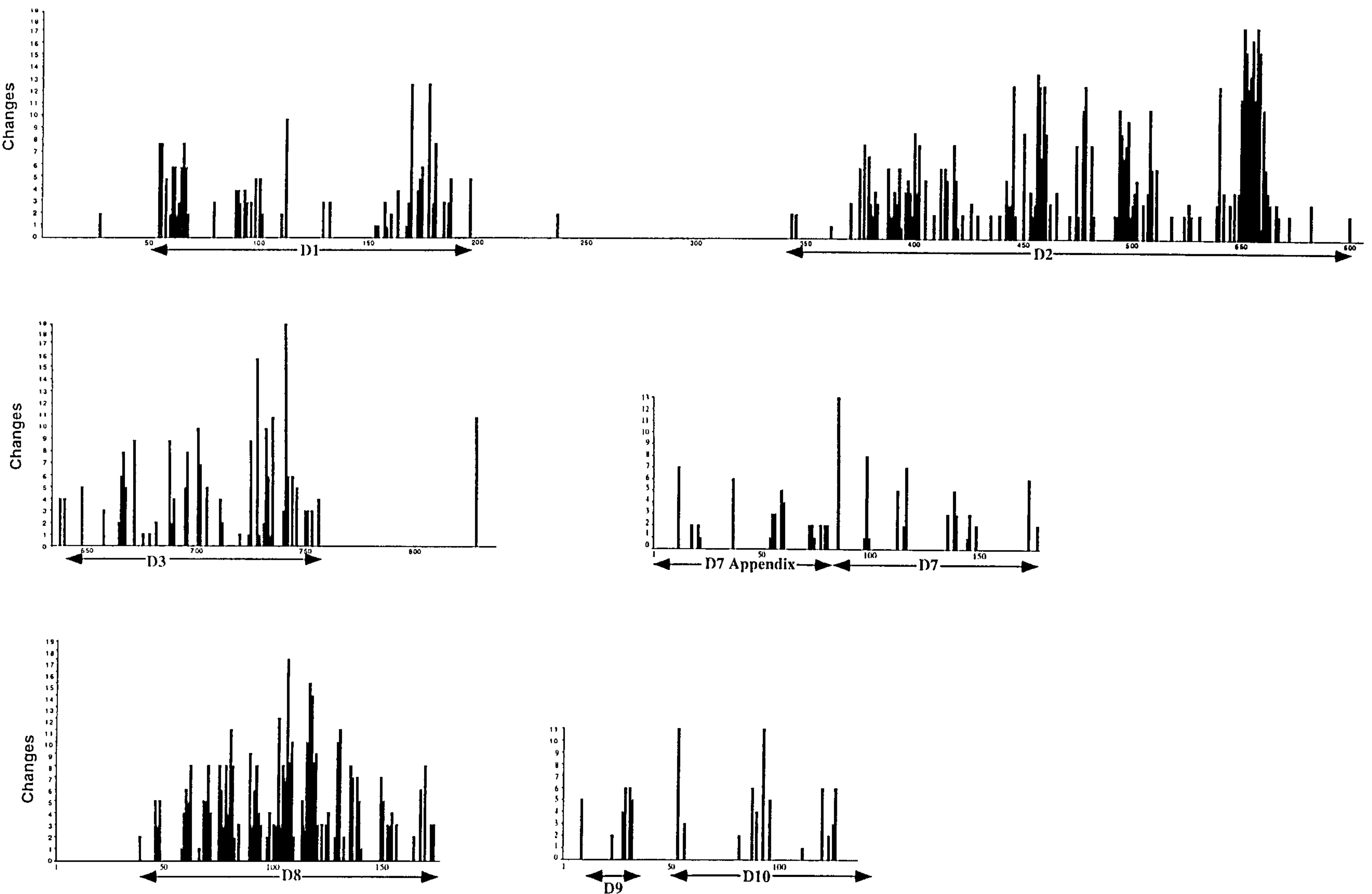

FIG. 6. Histogram showing inferred character changes along the large-subunit rDNA gene. Divergent domains are indicated by labeled arrows beneath the base pair positions.

lar studies have suggested the Agaricales as a sister group to the Boletales (Bruns *et al.,* 1992; Hibbett *et al.,* 1997; Moncalvo *et al.,* submitted). Morphological analysis of major groups has also led most authors to consider the Russulales and the Boletales as orders taxonomically equivalent to the Agaricales (Moser, 1983; Kühner, 1984; Singer, 1986).

For phylogenetic analysis one would ideally choose the sister group to the study group as the outgroup for polarization of characters within the study group. This requires either prior knowledge or assumptions of phylogenetic relationships between the study and the sister groups: the study group is known or assumed to be monophyletic and the taxon chosen to represent the outgroup is not derived from within the study group. When these assumptions cannot be made, the analysis is at risk of giving false results regarding both determination of polarity and indications of monophyly within the study group. If these assumptions cannot be made, then the choice of outgroup taxa must proceed to a higher taxonomic level (more distantly related taxa) until the assumptions are met. This search continues to ever higher taxonomic levels until a taxon is located that meets the above conditions with regard to the study group. For example, within the context of the present study, if it cannot be assumed that *Coprinus* is monophyletic and that members of the genera *Panaeolus, Panaeolina, Annelaria, Psathyrella,* or *Lacrymaria,* all taxa within the Coprinaceae, are not derived from within *Coprinus,* then the selection of outgroup taxa must move from the generic level within the Coprinaceae to some taxon within another family. Since these conditions may still not be met, then the selection must move to some taxon within another order.

The results of the present phylogenetic analysis demonstrate several problems in outgroup selection. Based on conventional taxonomy, a logical source for outgroup taxa for *Coprinus* would be the other genera within the Coprinaceae. Due to the suite of unique characters found within *Coprinus,* it would seem that *Coprinus* would be monophyletic and that taxa from any of these other genera would be derived from taxa outside *Coprinus,* in short that the assumptions for proper outgroup selection are met. Results from our phylogenetic analyses (Figs. 3 and 4) suggest that neither of these assumptions is met for either the study group (*Coprinus*) or any of the other genera within the Coprinaceae. The consequences of selecting any of these genera as an outgroup for the study would be significant. Moving to a higher taxonomic level, it can be seen that no taxa from any of the other families within the dark-spored allies to *Coprinus* can satisfy the conditions for a proper outgroup. It is only when moving to the next highest taxonomic level, that of order, that these assumptions are likely to be met.

Taxonomic Relationships

The relationship between current classification and phylogeny was explored by plotting alternative taxonomic systems onto the Wagner tree (Fig. 3) using MacClade 3.01 (Maddison and Maddison, 1992). Taxonomic monophyly was explored through this process at many different levels, including family, genus, section, and stirps. Although Wagner and character-state weighted trees are very similar, the Wagner tree has been selected for the purpose of analyzing taxonomies for operational considerations. (Generating the character-state weighted tree required prohibitively long computer times for both heuristic and bootstrap operations. Additionally, the number of transitions to transversions on branches is not meaningful under the character-state weighting since transformations were weighted in favor of minimizing transversions.)

The level of support for branches in a phylogenetic tree provides a useful criterion for accepting or rejecting a phylogenetic relationship. In this discussion branch support has been investigated through comparison of bootstrap proportions, the decay index, and the number of characters on a branch. There has been much discussion about the acceptability of bootstrap proportions as a measure of phylogenetic support (Sanderson, 1989; Bremer, 1994). Although Felsenstein (1985) advocated using traditional P values ($P \geq 0.95$ and $P \geq 0.90$) to indicate clade robustness based on bootstrapping, Hillis and Bull (1993) later suggested that much lower levels (to 0.70) may be acceptable. All three levels have been indicated on the Wagner tree from our study (Fig. 3, Table 4). The stability of individual branches in this tree was further explored by testing branch movement at different stepmatrix-facilitated weightings, which might indicate a lack of stability. It is difficult to draw conclusions about which of these indices is better or more reliable as they are all relative indicators and as such may not be compared in an absolute fashion. Based on our study, there appears to be an association between branches with high bootstrap proportions and decay indices of two steps or greater. Thirty-six of the 37 branches with bootstrap proportions over 0.70 (Fig. 3) also had decay indices of two steps or greater (Table 4; all comparisons were performed excluding branch 1, which was constrained by the analysis to contain the two outgroup taxa). Conversely, 36 of 38 branches with decay indices over two had bootstrap value greater than 0.70. All of the branches supported by greater than 10 unambiguous characters had bootstrap values over 0.90 and decay indices of greater than two steps (because of computational limitations, decay indices could not be calculated beyond two steps). Branches supported by four or more transversions were also supported by bootstrap values over 0.85 in 11 of 13 cases and decay indices of greater than two steps in all cases. In two cases, however,

branches supported by six and four transversions had bootstrap values of 0.75 and 0.58, respectively, with decay indices of greater than two steps.

Sectional-level relationships. At the sectional level within the genus *Coprinus,* neither the taxonomy of Kühner and Romagnesi (1978) nor the less-narrowly conceived taxonomy of Orton and Watling (1979) is appropriate when viewed within the context of the Wagner tree (Fig. 3). Among the eight sections of Kühner and Romagnesi, only sections *Comati* and *Hemerobii* (Table 2) are monophyletic. Monophyly of both sections is well supported by Wagner analysis (*Comati* 6:5:2:2+:0.82, *Hemerobii* 25:17:4:>2+:100). The character-state weighted analysis also supports both groups (bootstrap value = 1.00 for the *Hemerobii* and 0.72 for the *Comati*).

A narrower sectional concept for the *Atramentarii* would make it monophyletic by excluding *C. cf. erythrocephalus* (This taxon is similar in all respects to *C. erythrocephalus* but is intersterile from that species (Roger Kemp, pers. comm.)). As the section is conceived by Kühner and Romagnesi (1978), it would require 10 additional steps for monophyly and is rejected by the Templeton test ($P < 0.05$). Section *Lanatuli* also requires an additional 15 steps to make it monophyletic (Templeton test, $P < 0.01$) if *C. cf. Impexi* is excluded but only 9 steps (Templeton test not significant) if this taxon is maintained within the section. *Coprinus cf. Impexi* is unique in that it has two types of veil cells. Taken separately, each type of veil cell would position this species in a different section. Taken together, however, the presence of diverticulate branching veil cells places this species in the *Impexi.* Based on rDNA evidence, *C. cf. Impexi* falls in with the *Lanatuli,* although with only moderate support. Nonmonophyly of the *Lanatuli* is surprising in light of the morphological similarities among species in this section. Further studies using additional sequence evidence and other data may help to resolve the question of monophyly of the *Lanatuli.*

The *Impexi* are a heterogeneous assemblage of taxa united by cylindrical veil cells with side branches or diverticulae. There are many forms that these side branches take and it is not clear that all are homologous. This section appears as a nonmonophyletic group of disparate taxa, supporting the lack of homology in the key character for this section. It requires 17 additional steps to unite the species in the *Impexi,* which is significantly longer than the most parsimonious trees (Templeton test, $P < 0.01$).

Sections *Vestiti* and *Setulosi* are also nonmonophyletic. To make these sections monophyletic requires an additional 66 and 48 steps, respectively (both significantly longer, $P < 0.001$), in order to break apart the clade of *C. cordisporus* and *C. curtus,* which is strongly supported (25:23:9:>2+:1.00). Section *Vestiti* is much the same as section *Impexi* in being defined on the basis of what appear to be nonhomologous structures (in this case globose veil cells on top of a radially filamentous cap). Section *Setulosi* is based on the presence of setules or hair-like cells that project out of the mushroom cap and stalk. This group however may be further subdivided into taxa that also contain globose veil cells in addition to setules. This latter group of *Setulosi* appears with section *Micacei* in the Wagner tree. With the exclusion of *C. curtus,* species in *Setulosi* and section *Micacei* are grouped together in a robustly supported group in the character-state weighted tree with a bootstrap value = 1.00. Because taxa in *Setulosi* are found in both basal and terminal positions around the *Micacei,* the placement of *C. flocculosus* within the *Micacei* also prevents the *Micacei* from being monophyletic. It requires an additional 7 steps to achieve monophyly in this group, although a Templeton test was not significant. It would appear that the *Micacei,* along with *C. flocculosus,* were derived from within the globose veil-celled *Setulosi.* Remnants of the relationship can be found in the setules found along the stipe in *C. domesticus* and *C. xanthothrix.*

The sectional taxonomy of Orton and Watling (1979) has similar problems in accounting for relationships within *Coprinus.* None of the three sections in this taxonomy is monophyletic. Section *Coprinus,* which essentially incorporates sections *Comati, Atramentarii, Lanatuli,* and *Impexi* of Kühner and Romagnesi requires an additional 85 steps to become monophyletic (Templeton test $P < 0.001$). Section *Micaceus* (incorporating sections *Micacei* and *Vestiti* of Kühner and Romagnesi) requires an additional 101 steps and section *Pseudocoprinus* (incorporating sections *Setulosi* and *Hemerobii*) an additional 74 steps to become monophyletic (also significantly longer, $P < 0.001$)). The greater length of constrained topologies in this sectional taxonomy appears to be the result of two different causes. The first is the larger number of taxa per section in the three-section taxonomy (as opposed to the eight sections of Kühner and Romagnesi (1978)). Larger numbers of taxa result in longer trees as sampling density increases. The second reason for having longer constraint trees is that taxa must be moved across at least three well-supported branches in Fig. 3. Section *Coprinus* unites the *Comati* form clade III with the *Atramentarii, Lanatuli,* and *Impexi* of clade I. Section *Micaceus* attempts to bring together sections *Micacei* from clade II with section *Vestiti* from clade I. Last, section *Pseudocoprinus* unites sections *Setulosi* of clade II with section *Hemerobii* of clade I.

In contrast to Kühner's and Romagnesi's sectional taxonomy, Orton and Watling's system of stirps seems to fit our data best in terms of representing monophyletic relationships within *Coprinus.* This is primarily a result of there being a larger number of stirps compared to either of the sectional taxonomies. Twelve of the 20 stirps represented within this study are mono-

phyletic. Eight of these 12 stirps are monophyletic by virtue of being represented by only one taxon in this study. Stirps atramentarius is well supported (15:12:6: >2+:1.00) as is stirps friesii (8:6:4:>2+:0.96) and stirps hemerobius (21:18:6:>2+:0.94). However, stirps comatus is less well supported in both the Wagner (6:5:2:>2+:82) and the character-state weighted (bootstrap value = 0.72) trees.

Six of Orton's and Watling's stirps are not monophyletic. Stirps lagopus is identical to section *Lanatuli* of Kühner and Romagnesi and presents the same difficulties in terms of monophyly described above. Stirps picaceus is paraphyletic, giving rise to stirps friesii in both analyses. It requires 7 additional steps to make this stirps monophyletic, but the results are not significant. Stirps narcoticus would be monophyletic but for the position of *C. cinereus* of stirps lagopus. The grouping of *C. cinereus* is problematic as this species is so similar morphologically to those in the clade containing *C. radiatus.* The grouping of *C. cinereus* with *C. sclerotiger* is weak (7:5:0:1:≤0.50), as is the support for the stirps as a whole (3:2:1:1:≤0.50). Stirps niveus requires an additional 49 steps to become monophyletic (significantly longer, $P < 0.001$). Much of this additional length results from bringing *C. cordisporus* together with the well-supported group of *C. cothurnatus* and *C. latisporus.* Stirps disseminatus requires an additional 40 steps to become monophyletic (also significant, $P < 0.001$). Again, as in section *Setulosi,* much of this length is required to break apart the *C. cordisporus/C. curtus* clade. Stirps ephemerus and hiascens require only an additional 3 and 4 steps, respectively, to become monophyletic. Stirps ephemerus is paraphyletic with stirps hiascens arising from within it. These two stirps are separated taxonomically by habitat, with species in stirps ephemerus growing on dung and species in stirps hiascens on soil. With the tremendous variation in habitat found throughout *Coprinus* and in light of this phylogeny, it can be seen that this habitat difference is not taxonomically important.

The three alternative taxonomic systems were compared against the Wagner tree using phylogenetic constraints implemented using PAUP. *Coprinus americanus* was removed from this analysis due to the uncertainty of its position in two of the three systems. To achieve monophyly across the three sections of Orton and Watling required an additional 180 steps. For Kühner and Romagnesi's taxonomy an additional 148 steps were required to create eight monophyletic sections. Orton and Watling's stirps system best mirrored monophyletic groupings, requiring only 106 additional steps to achieve total monophyly in the 20 stirps studied here. However, constrained topologies under all three taxonomic systems were always significantly worse than the best Wagner trees (Templeton test, $P < 0.001$), suggesting that further work is still necessary to revise and update subgeneric classification within *Coprinus* to reflect phylogenetic relationships.

Phylogenetic placement of model species. Several species of *Coprinus* are widely employed as model systems for studies of mushroom development and mating genes, including *C. cinereus* and *C. bilanatus* (Pukilla and Casselton, 1991). In addition, several species of *Coprinus* are known to be important as edible mushrooms which can be cultivated as food, mostly notably *C. comatus, C. atramentarius,* and *C. micaceus* (Chang and Hayes, 1978). It was therefore of interest to determine how these species are related with other *Coprinus* species. Phylogenetic analysis placed these species into several groups as follows: *C. cinereus, C. bilanatus,* and *C. atramentarius* within Group I; *C. micaceus* in Group II; and *C. comatus* within group III (Fig. 3). Of these species, *C. comatus* and its sister *C. sterquilinus* were found to be very distantly related to other species in *Coprinus,* which might warrant its exclusion from the genus. Ironically, since *C. comatus* is currently recognized as the type species for the genus *Coprinus,* under the rules of the International Code of Botanical Nomenclature, the bulk of species in *Coprinus* (including several model species mentioned above) might therefore need to be transferred into another genus (Vilgalys *et al.,* 1994; Johnson and Vilgalys, 1998). We are currently examining alternative nomenclatural alternatives to address this issue.

Phylogenetic limits of the genus Coprinus. The genus *Coprinus* is not monophyletic based on our cladistic analysis of the large-subunit rDNA (Fig. 3). While clades I and III are monophyletic for the species of *Coprinus* within them, clade II has *P. candolleana* intermixed with species from sections *Setulosi* and *Micacei* and is thus paraphyletic. All three clades are well supported and each has members of other genera basal to them. To bring these three clades together into a monophyletic *Coprinus* requires 77 additional steps beyond the 1461 steps in the most parsimonious trees and is statistically rejected by the Templeton test ($P < 0.001$). Even less-constrained phylogenetic searches, in which *Lacrymaria, Psathyrella,* and the basal counterparts of clade III are retained within a paraphyletic *Coprinus,* resulted in trees that were still significantly longer (+46 steps, $P < 0.001$) than the most parsimonious Wagner tree. In a similar manner, constraint trees in which only clades I and II were considered (that is, only excluding *Psathyrella* and *Lacrymaria*) were also significantly longer than the most parsimonious Wagner tree (+23 steps, $P < 0.01$).

The actual phylogenetic limits of *Coprinus* are difficult to determine due to differing interpretations regarding its nonmonophyletic nature. Two interpretations are possible. One is that *Coprinus* is paraphyletic and that at least several other genera have arisen from within it. This assumes a single origin for the genus as

currently circumscribed. The second interpretation is that *Coprinus* is polyphyletic and that morphological characters associated with *Coprinus* have arisen two (Fig. 3: in clades I and II, and again in clade III) or possibly three times (Fig. 3: within clades I, II, and III).

The difficulty in accepting a paraphyletic *Coprinus* lies in accepting that so many different genera (including numerous additional genera from the Bolbitiaceae, Strophariaceae, Agaricaceae, and Coprinaceae) would have arisen by the independent loss of coprinoid features. While it is generally accepted that it is simpler to lose complex characteristics than to gain them, it is unlikely that the entire suite of coprinoid characteristics, including deliquescence, inaequihymeniiferous development, pleated cap surfaces, and hymenial supporting cystidia, would be lost as a group independently in these other genera. For this reason we reject the hypothesis of paraphyly.

Taxonomic problems associated with accepting a polyphyletic *Coprinus* also concern the unique characters possessed by many species of the genus in all three clades. These characters have provided the basis for many agaricologists to presume *Coprinus* to be a natural group (Smith, 1971; Singer, 1986). The most parsimonious explanation that takes into account the morphological anomalies of the genus while still heeding the phylogenetic hypothesis based on molecular characters is that both *Psathyrella* and *Lacrymaria* have arisen from within *Coprinus* (clades I and II). This requires only 16 additional steps compared to the Wagner tree. This hypothesis also allows for the independent loss of coprinoid characteristics in both *Lacrymaria* and *Psathyrella* but only once for each genus. It does presuppose the independent evolution of many coprinoid features, including deliquescence, hymenial supporting cystidia, and inaequihymeniiferous lamellar development once in clades I and II and once in clade III. There are also a number of other morphological characters that are present in clade III but not in the rest of *Coprinus* (Hopple, unpub.) that could be interpreted to suggest the independent origin of this clade. A combined analysis of morphology and molecules is needed to help resolve this issue.

Aside from *Coprinus* and *Psathyrella,* only the genus *Leucocoprinus* has multiple members among the study group. This genus is monophyletic and strongly supported in both the Wagner analysis (five characters, five unambiguous characters, one unambiguous transversion, a decay index of greater than 2+ steps, and bootstrap proportion of 0.99) and a bootstrap proportion of 0.95 in the character-state weighted tree. (To shorten the reporting of branch-support indices they will be reported in the same order as above, separated by colons, e.g., 5:5:1>:0.99).

Familial relationships. At the family level, only the Strophariaceae and Lepiotaceae are monophyletic based on rDNA evidence (Fig. 3). The Coprinaceae is paraphyletic if all other families from the study group are derived from within it. Constraint trees with the Coprinaceae monophyletic were significantly longer (+42 additional steps, $P < 0.001$). Much of this added length results when separating clade III from those taxa basal to it (*Montagnea, Leucocoprinus,* and *Podaxis*). If clade III and these taxa are brought into the Coprinaceae, only 13 additional steps are required, though this additional length is still significantly longer than the most parsimonious trees ($P < 0.05$). Eleven additional steps are required to make the Bolbitiaceae monophyletic, which is also significantly worse than the most parsimonious trees (Templeton test, $P < 0.05$). Thus, morphological characters by which these last two families are delimited, spore color and shape of cap surface cells, are apparently not well correlated with the molecular characters.

Sister groups to Coprinus. The three independent clades of *Coprinus* each have different sister taxa. Based on the Wagner tree, *Lacrymaria* is the sister taxon to clade I, *Psathyrella* is the sister taxon to clade II, and *Montagnea* is the sister taxon to clade III. In the second sister group relationship, *Psathyrella* is both basal to and derived from within clade II. The placement of *P. candolleana* within clade II has only moderate support, however (9:4:2:0.72). Within the character-state weighted tree the three *Psathyrella* species appear paraphyletic to clade II.

Together with *Lacrymaria* and *Psathyrella,* Wagner trees show that clades I and II form a monophyletic group that is sister group to the Strophariaceae/ *Agrocybe* clade. This latter clade contains all members of the Strophariaceae studied and *A. praecox* from the Bolbitiaceae. This set of relationships is not strongly supported, however, since character-state weighting groups the same *Coprinus* species together with the *Panaeolus sensu lato* (*Panaeolus, Panaeolina,* and *Annelaria*) and the *Bolbitius*/*Conocybe* clades (although with a bootstrap value of only 0.66). In the Wagner tree there is low support for branch 13 separating these two clades (7:2:0:1:<0.50). Within this context the sister group to clades I and II could come also from members of the *Panaeolus sensu lato*/*Conocybe*/*Bolbitius* clade. Members of this second clade seem more likely candidates for sister group to *Coprinus* (groups I and II) on a morphological basis.

Perhaps the most interesting grouping in the tree contains clade III and those taxa basal to it. This seemingly heterogeneous group contains members from no less than five taxonomically different genera spanning five families. The grouping of these taxa is well supported in the Wagner tree (18:10:4:>2+:0.87) and by a bootstrap value of 0.91 in the character-state weighted tree. A close relationship among *Coprinus, Montagnea,* and *Podaxis* has been suggested by numerous authors (Morse, 1933; Miller and Miller, 1988; Moser, 1983; Singer, 1986) and is also supported by

molecular evidence based on restriction analysis of the same large-subunit rDNA region (Hopple and Vilgalys, 1994). Also, similarities can clearly be seen between *Leucocoprinus* (traditionally classified in the Lepiotaceae) and section *Comati,* including the presence of a plicate- striate cap, a universal veil, an annulus, and thick-walled apically pored spores. It is likely that other members of the Agaricaceae *sensu lato* fall into this clade, which is represented by only a limited sampling in this study. While some taxonomic treatments still maintained a separation of the Lepiotaceae from the Agariceae (Smith *et al.,* 1979; Miller, 1981; Largent and Baroni, 1988), most modern treatments group these taxa into a single family Agaricaeae (Singer, 1986; Moser, 1983). Recent molecular systematic studies also suggest that both *Lepiota* and *Agaricus* belong within this clade (Chapella *et al.,* 1994; Johnson and Vilgalys, 1998). Additional research is necessary to further establish the limits of this "Agaricaceae" clade.

In summary, evidence from large-subunit rDNA genes strongly suggests that the genus *Coprinus* is not monophyletic as once widely assumed by mycologists. Given the uniqueness of many unusual characteristics within this family, a reappraisal of morphological and developmental characters supporting taxonomic grouping in the Coprinaceae is warranted.

Study of the large-subunit rDNA gene itself has also served to further our understanding of the levels of variation and their phylogenetic utility in rDNA genes. It is clear that the divergent domains in this gene contain the greatest amount of phylogenetic information. Patterns of variation between divergent domains are also considerable. Our analysis indicates that regions D2 and D8 are the most divergent but not necessarily the most useful for phylogenetic analysis. Analyses of additional taxonomic groups should add to this characterization of the large-subunit rDNA.

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