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USING RPB1 SEQUENCES TO IMPROVE PHYLOGENETIC INFERENCE AMONG MUSHROOMS (*INOCYBE*, AGARICALES)[1]

P. BRANDON MATHENY,[2,4] YAJUAN J. LIU,[3] JOSEPH F. AMMIRATI,[2] AND BENJAMIN D. HALL[3]

[2]Department of Botany, Box 351330, Johnson 412, University of Washington, Seattle, Washington 98195 USA; and
[3]Departments of Botany and Genetics, Box 355325, Hitchcock 543, University of Washington, Seattle, Washington 98195 USA

An investigation of mushroom phylogeny using the largest subunit of RNA polymerase II gene sequences (RPB1) was conducted in comparison with nuclear ribosomal large subunit RNA gene sequences (nLSU) for the same set of taxa in the genus *Inocybe* (Agaricales, Basidiomycota). The two data sets, though not significantly incongruent, exhibit conflict among the placement of two taxa that exhibit long branches in the nLSU data set. In contrast, RPB1 terminal branch lengths are rather uniform. Bootstrap support is increased for clades in RPB1. Combined data sets increase the degree of confidence for several relationships. Overall, nLSU data do not yield a robust phylogeny when independently assessed by RPB1 sequences. This multigene study indicates that *Inocybe* is a monophyletic group composed of at least four distinct lineages—subgenus *Mallocybe*, section *Cervicolores*, section *Rimosae*, and subgenus *Inocybe* sensu Kühner, Kuyper, non Singer. Within subgenus *Inocybe*, two additional lineages, one composed of species with smooth basidiospores (clade I) and a second characterized by nodulose-spored species (clade II), are recovered by RPB1 and combined data. The nLSU data recover only clade I. The genera *Astrosporina* and *Inocybella* cannot be recognized phylogenetically. ''Supersections'' Cortinatae and Marginatae are not monophyletic groups.

Key words: Agaricales; *Inocybe*; molecular phylogeny; nuclear large subunit ribosomal DNA; RNA polymerase II; RPB1.

Although phylogenetic analyses of mushrooms and allied fungi using molecular-based methods at various taxonomic levels have blossomed in the last decade, these studies largely relied on a limited suite of nuclear and mitochondrial ribosomal RNA loci made accessible by universal primers. Indeed, protein-coding genes were deemed less tractable because of the lack of knowledge concerning suitable polymerase chain reaction (PCR) primer sequences and concerning gene copy numbers (Bruns, White, and Taylor, 1991). However, researchers more recently have begun to investigate alternative loci that may prove beneficial to phylogenetic reconstructions. For example, Mehmann, Brunner, and Braus (1994) demonstrated the taxonomic and phylogenetic utility of fungal-specific chitin synthase genes among ectomycorrhizal fungi; Thon and Royse (1999) studied the phylogenetic utility of partial ß-tubulin sequences among basidiomycetes; Kretzer and Bruns (1999) showed that *atp*6, a subunit of the mitochondrial ATPase, proved to be useful in the inference of Boletales (Basidiomycota) phylogeny; Hirt et al. (1999) demonstrated that microsporidia are related to fungi based on evidence from the largest subunit (RPB1) of RNA polymerase II and other proteins; Liu, Whelen, and Hall (1999) developed fungal specific primers among the Ascomycota for the second largest subunit (RPB2) of RNA polymerase II in their evaluation of phylogenetic relationships among ascomycetes; and O'Donnell et al. (2001) used translation elongation factor-1α exon sequences, among other nonprotein coding sequences, in their reconstruction of mucoralean (Zygomycota) phylogeny. Here we intend to demonstrate that partial sequence data of the single-copy RNA polymerase II largest subunit (RPB1) provide suitable alternatives to other markers to reconstruct lower level phylogeny among mushroom-forming fungi (Agaricales, Basidiomycota). Additionally, an RPB1 phylogeny can serve to assess evolutionary relationships inferred by sources such as rDNA. We present a nLSU phylogeny that is confounded in part by two long terminal branch lengths, a reconstruction in disagreement with those based on RPB1 sequences. In contrast, trees derived from RPB1 sequences exhibit increased resolution, greater confidence for several relationships, and relatively uniform terminal branch lengths.

RNA polymerase II is the enzyme responsible for transcription of protein-coding genes into pre-mRNA transcripts. The largest subunit of this protein, RPB1, is also of interest, ironically, since it possesses the active binding site for α-amanitin, a toxin produced by several agaric genera such as *Amanita*, *Pholiotina*, *Galerina*, and *Lepiota* (Horgen, Vaisius, and Ammirati, 1978; Wieland, 1986; Benjamin, 1995). Unlike previous studies that used RNA polymerase II sequence data to investigate higher level relationships among a broad array of eukaryotes (Nawrath, Schell, and Koncz, 1990; Iwabe et al., 1991; Sidow and Thomas, 1994; Klenk et al., 1995; Stiller and Hall, 1997; Denton, McConaughy, and Hall, 1998; Liu, Whelen, and Hall, 1999), this study evaluates taxonomically lower level relationships in a genus of mushroom-forming fungi, *Inocybe* (Fr.) Fr. (Cortinariaceae, Agaricales).

Inocybe is a species-rich group of higher basidiomycetes that is recognized morphologically by the fleshy fruit bodies (basidiomata) with a dry and often fibrillose, rimose, or squamulose pileus and often spermatic odor. The basidiospores are dull brown pigmented with a smooth spore wall, although in some species portions of the wall are projected outwards and resemble nodules to a varying degree, including spinose pro-

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[4] Author for reprint requests (e-mail: matheny@u.washington.edu).

tuberances, or may be angular in outline. The genus is considered ectomycorrhizal and exhibits poor mycelial growth on standard agar plates (Kuyper, 1986; Singer, 1986). Basidiospores of *Inocybe* fail to germinate under laboratory settings, and current techniques fail to establish successful cultures (Boidin, 1986). Additionally, many species possess muscarine, a toxin that stimulates the parasympathetic nervous system in humans (Bresinsky and Besl, 1990; Benjamin, 1995), and a few species even contain hallucinogenic compounds (Besl and Mack, 1985; Gartz and Drewitz, 1985; Stijve, Klan, and Kuyper, 1985). The genus is fairly large with a conservative estimate of between 250 and 350 species that occur worldwide in an ectomycorrhizal status with a wide range of angiosperms and gymnosperms (Kuyper, 1986), making it one of the most diverse genera in the Agaricales. Furthermore, new *Inocybe* species are still being described throughout the globe (e.g., Vauras, 1997; Buyck and Eyssartier, 1999; Kobayashi and Courtecuisse, 2000; Seok et al., 2000; Watling, 2001; P. B. Matheny, M. C. Aime, and T. W. Henkel, unpublished data; P. B. Matheny and Bougher, unpublished data; P. B. Matheny and N. L. Trappe, unpublished data).

Taxonomically, *Inocybe* has primarily been classified in the family Cortinariaceae where it occupies its own tribe, Inocybeae Fayod ex Singer (Kühner, 1980; Singer, 1986). Additionally, most systematists (Kühner, 1980; Moser, 1983; Kuyper, 1986; Singer, 1986) recognize one genus of inocyboid species in contrast to Horak (1968) and Jülich (1981), who prefer to treat the nodulose-spored species of *Inocybe* in the genus *Astrosporina*. Zerova (1974) even accomodated the spinose-spored (with spine-like protuberances) *I. calospora* in its own genus, *Inocybella*. Considerable debate exists as to the evolutionary importance of the topology of the basidiospore in *Inocybe*. Kuyper (1986), however, demonstrated that the nodulose-spored condition arose more than once using parsimony and character compatibility analyses of morphological characters. Furthermore, questions still surround the familial placement of *Inocybe* and just which taxa are sister to it. Jülich (1981), for example, elevated *Inocybe* to familial rank, and Kuyper (1986), in his morphological cladistic analyses, chose *Phaeomarasmius* (Strophariaceae), a saprobic smooth-spored genus, as the outgroup instead of a more traditional choice, *Hebeloma* (Cortinariaceae), an ectomycorrhizal warty-spored genus, long considered by many, beginning with Fries (1821–1832), to be closely related to *Inocybe*.

To assess the phylogenetic utility of RPB1, partial exon sequences are compared with partial nLSU sequences for the same set of 21 taxa—18 species of *Inocybe* and three outgroup representatives, *Hebeloma olympianum*, *Galerina semilanceata*, and *Phaeomarasmius curcuma*. Internal transcribed spacer (ITS) regions, which are phylogenetically useful for assessment of recently diverged fungal groups (Bruns, White, and Taylor, 1991; Hibbett, 1992; Kohn, 1992; Vilgalys, Hopple, and Hibbett, 1994) were not explored in our study due to an ambiguous alignment that resulted during a trial survey of several *Inocybe* species (B. Kropp, Utah State University, personal communication). The nLSU marker has been used to assess phylogenetic relationships at the generic and/or infrageneric taxonomic level for a few mushroom groups, for example, *Amanita*, *Coprinus* sensu lato, and *Craterellus* (Weiss, Yang, and Oberwinkler, 1998; Drehmel, Vilgalys, and Moncalvo, 1999; Hopple and Vilgalys, 1999; Dahlman, Danell, and Spatafora, 2000; Moncalvo, Drehmel, and Vilgalys, 2000). The nLSU sequence data also have proven useful to address relationships at the family or ordinal level among mushroom-forming fungi (e.g., Binder, Besl, and Bresinsky, 1997; Hughey et al., 2000; Miller et al., 2000; Moncalvo et al., 2000; Thorn et al., 2000; Grubisha et al., 2001). Additional studies among mushroom families and genera that rely on nLSU sequences are anticipated because this locus is easily amplified and phylogenetically useful. However, aside from comparisons with morphological or mitochondrial ribosomal RNA sequences, very few studies offer comparisons with protein-coding sequences and at times are marked by interior nodes with short branches that lack high (>70%) bootstrap support.

The goals of the present study are twofold: (1) to compare the utility of partial nLSU sequences to those of partial RPB1 sequences in agaric phylogeny and (2) to reconstruct the evolutionary history of *Inocybe*. RPB1 provides flexibility in addressing multiple-level systematic questions given its amino acid or exon content. Moreover, the 5′ proximal sequences of the RPB1 coding region (conserved domains A–C) contain a large intron often >500 base pairs (bp) in length and guanine-cytosine rich. With these goals in mind, we ask the following questions: (1) Is RPB1 useful to determine agaric phylogeny, and how does this data set compare to trees derived from nLSU sequences? (2) Do combined data sets offer greater resolution and/or bootstrap support? (3) Is *Inocybe* monophyletic, and what are monophyletic groups within *Inocybe*?

MATERIALS AND METHODS

Fungal accessions—Twenty taxa of the Cortinariaceae, including 18 species of *Inocybe* and one each of *Hebeloma* and *Galerina*, and one taxon from the Strophariaceae, *Phaeomarasmius*, were sampled for two genes: partial sequences of RPB1 (A–C) and partial sequences of nuclear large subunit ribosomal DNA (nLSU). Exsiccata used as DNA sources and GenBank accession numbers (GBAN-AY038309 to GBAN-AY038329 and GBAN-AF389531 to GBAN-389551) are archived on the Botanical Society of America web site (http://ajbsupp.botany.org/v89/matheny.pdf). Herbarium abbreviations are listed according to Holmgren, Holmgren, and Barnett (1990).

Taxon sampling—A number of infrageneric classifications have been proposed for *Inocybe* (Massee, 1904; Lange, 1917; Heim, 1931; Kühner and Romagnesi, 1953; Horak, 1968; Kühner, 1980; Kuyper, 1986; Singer, 1986; Bon, 1997a, b, 1998) and some modifications suggested by others (e.g., Jülich, 1981; Kobayashi, 1993). Our data set contains a sample from each of the three subgenera and ''supersections'' proposed by Kuyper (1986) in addition to samples from all but three of Singer's 14 sections that he recognized in the genus (Singer, 1986). One of the unsampled sections of Singer is monospecific (section *Rubellae* Kühner & Romagnesi) and another contains only two species (section *Hystrices* Stangl & Veselsky); the last unsampled section is *Petiginosae* R. Heim. However, we included two noteworthy species associated with unique hosts, one smooth-spored cortinate species associated with *Nothofagus* from Argentina, South America (*Inocybe* sp. BK 8-Feb-99-1), and one mallocyboid species with necropigmented basidia associated with *Eucalyptus* from southeast Australia (*I.* ''*serpitocystis*,'' a provisional name for an undescribed species). The taxon sampling scheme is presented in Table 1, which presents two major competing classifications (Kuyper, 1986; Singer, 1986). To gauge the phylogenetic breadth of RPB1, we deliberately sampled among putatively closely related species as well, such as *I. lanuginosa*, *I. leptophylla*, and *I. stellatospora* (Matheny and Kropp, 2001) and *I. pudica* and *I. agglutinata*.

DNA extraction, PCR amplification, cloning, and sequencing—Genomic DNA was extracted from dried basidiome tissue using a modified cetyltrimethylammonium bromide (CTAB) method with liquid nitrogen followed by chloroform extraction based on Rogers and Bendich (1985, 1994). Primers were designed to amplify regions from conserved domain A to conserved

TABLE 1. Taxonomic sampling of ingroup.

Kuyper (1986)	Singer (1986)
Subgenus *Mallocybe*	**Subgenus *Inosperma***
Inocybe dulcamara	**Section 1 *Depauperatae***
Inocybe sp. PBM 1615	*Inocybe dulcamara*
Inocybe "serpitocystis"	*Inocybe* sp. PBM 1615
Subgenus *Inosperma*	*Inocybe "serpitocystis"*
Section *Cervicolores*	**Section 2 *Rimosae***
Inocybe hirsuta var. *maxima*	*Inocybe maculata*
Section *Rimosae*	**Section 3 *Cervicolores***
Inocybe maculata	*Inocybe hirsuta* var. *maxima*
Subgenus *Inocybe*	**Subgenus *Inocibium***
Supersection *"Cortinatae"*	**Section 4 *Lactiferae***
Inocybe agglutinata	*Inocybe corydalina*
Inocybe corydalina	**Section 5 or *"Geophyllinae"***
Inocybe lacera	*Inocybe agglutinata*
Inocybe lanuginosa	*Inocybe godeyi*
Inocybe leptophylla	*Inocybe pudica*
Inocybe pudica	**Section 6 *Lacerae***
Inocybe relicina	*Inocybe lacera*[a]
Inocybe sp. BK 8-Feb-99-1	*Inocybe* sp. BK 8-Feb-99-1
Inocybe stellatospora	**Section 8 Splendentes**
Supersection *"Marginatae"*	*Inocybe abietis*
Inocybe abietis	**Subgenus *Inocybe***
Inocybe calospora	**Section 9 (subg. *Leptocybe*)**
Inocybe godeyi	*Inocybe leptophylla*
Inocybe praetervisa	**Section 11 *Inocybe***
	Inocybe lanuginosa
	Inocybe relicina[b]
	Inocybe stellatospora
	Section 12 *Calosporae*
	Inocybe calospora[c]
	Section 14 *Marginatae*
	Inocybe praetervisa[d]

[a] Holotype of the genus *Inocibium* Earle.
[b] Lectotype of the genus *Inocybe* (Fr.) Fr.
[c] Holotype of the genus *Inocybella* Zerova.
[d] Lectotype of the genus *Astrosporina* Schröter.

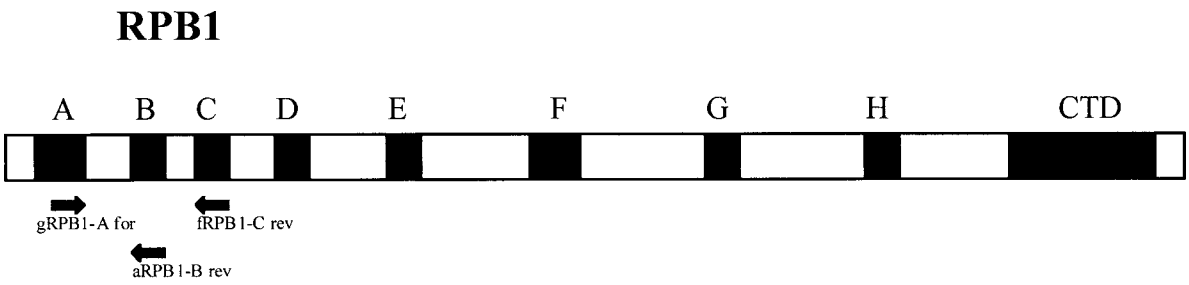
domain C in RPB1 (about 1400 bases) (Fig. 1). RPB1 amplification primers included the general gRPB1-A forward (Stiller and Hall, 1997) and fungal-specific fRPB1-C reverse. The C reverse primer was designed from sequence comparisons of the basidiomycete *Cryptococcus neoformans* and several ascomycetes that included *Saccharomyces cerevisiae*, *Schizosaccahoromyces pombe*, *Candida krusei*, and *Exophiala jeanselmei*. RPB1 sequencing primers included gRPB1-A forward, fRPB1-C reverse, and an additional primer, aRPB1-B reverse, designed from sequence comparisons of *Inocybe* species, *Hebeloma*, and *Galerina*. RPB1 sequencing primers specific to *Inocybe hirsuta* var. *maxima* A. H. Smith of section *Cervicolores* included B forward (5′-TTGTCCCTTGGCACCCGAG-3′) and B.5 reverse (5′-CCGCTTAGTTTCCTCATTATCG-3′). Primers used to amplify the 5′ end of the nLSU gene included 5.8SR and LR7; these primers, along with sequencing primers LR0R, LR3R, LR16, and LR5, were obtained from http://www.botany.duke.edu/fungi/mycolab/primers.htm.

Polymerase chain reaction amplification entailed the use of Taq DNA polymerase and the following conditions: (1) hot start with 94°–95°C for 4–5 min, (2) 1 min at 94°–95°C, (3) 1–1.5 min with various annealing temperatures between 50° and 57°C, (4) an increase of 1°C per 5 sec to 72°C, (5) 1.5–2 min at 72°C, (6) 34 times (cycles) to step 2, (7) 10 min at 72°C, and (8) 15°C up to 18 h. Then PCR amplified fragments were purified and sequenced automatically (ABI PRISM Dey Terminator Cycle Sequencing and ABI PRISM Sequencer model 377; Perkin-Elmer, Boston, Massachusetts, USA) except for *Hebeloma olympianum* (RPB1), which was first cloned by pCR 2.1 plasmid vector, Version F (Invitrogen, Carlsbad, California, USA).

RNA extraction, RNA amplification, and sequencing—RNA was extracted from two fresh basidiomes, *Inocybe sindonia* (Fr.) P. Karsten (PBM 2048, WTU) and *I. geophylla* var. *lilacina* (Peck) Gillet (PBM 2039, WTU), using an RNeasy Plant Mini Kit, catalogue number 74904 (Qiagen, Valencia, California, USA) to confirm RPB1 spliceosomal intron boundaries. The cDNA was synthesized using RNA template and reverse transcription PCR following "Protocol 2" of the displayTHERMO-RT Kit Version 1.4, catalogue number 570-100 (Display Systems Biotech, Vista, California, USA). The cNDA was then amplified using the degenerate RPB1 primers discussed above. The resulting PCR product was sequenced as discussed above. Exon (DNA) sequences of both samples are available from GenBank (GBAN-AF390019 and GBAN-AF390020).

Phylogenetic analyses—Sequence data were aligned using CLUSTAL W (Thompson, Higgins, and Gibson, 1994), adjusted by eye, if necessary, and analyzed with PAUP 4.0b4 (PAUP*) (Swofford, 2000). Alignments are available from the authors by request or on TreeBASE (study accession number S686). *Hebeloma olympianum* was designated as outgroup in PAUP* for rooting purposes based on a more inclusive study of agaric phylogeny (Moncalvo et al., 2000).

Sequences were examined using both equally weighted maximum parsimony (MP) and differentially weighted maximum parsimony (WP). In weight-



General RPB1 primer
gRPB1-A for (ECPGHFG) 5'–GA(G/T)TGTCC(T/G)GG(A/T)CATTTTGG–3'

Fungal specific RPB1 primer
fRPB1-C rev (YMDND/EIAG) 5'–C(A/C/G/T)GC(A/T/G)AT(A/C/G/T)TC(A/G)TT(A/G)TCCAT(A/G)TA–3'

Agaricales specific RPB1 primer
aRPB1-B rev (PLED/EGG/A/VD) 5'–TCCGC(A/G)CC(C/T)TCTTC(C/T)TTGG–3'

Fig. 1. RPB1 amplification and sequencing primers used in this study. Shaded boxes represent conserved amino acid motifs among eukaryotes.

TABLE 2. Maximum parsimony (MP) and weighted parsimony (WP) character status summaries for the nLSU and RPB1 data sets.

Data set	Total characters	Parsimony-informative characters	Length (steps)	No. of optimal trees via heuristic search	CI	No. of clades >70% bootstrap support
nLSU-MP	1292	132	520	6	0.569	4
nLSU-WP[a]	1292	135	933	2	0.595	6
RPB1-MP	642	233	1041	8	0.458	8
RPB1-WP[b]	642	233	1630	1	0.527	8
Intron 2	462	116	485	27	0.606	4
nLSU + RPB1-MP	1934	365	1579	2	0.490	9
nLSU + RPB1-WP[a,b]	1934	368	2590	1	0.546	9
nLSU + RPB1 + int2-MP	2396	481	2079	4	0.513	8
nLSU + RPB1 + int2-WP[a,b]	2396	484	3092	4	0.552	9

[a] WP on nLSU data set employing a user-defined step matrix with a weight of three steps for all transversions, a weight of two steps for A–G transitions, and a weight of one step for C–T transitions.

[b] WP on RPB1 data set using a weight of four steps for first-position codon substitutions, five steps for second-position codon substitutions, and one step for third-position codon substitutions.

ed analyses of the nLSU data sets, transitions and transversions were estimated using maximum likelihood. Simulation studies indicate that only a slight weighting of transversions is needed to reach accuracy (Hillis, Huelsenbeck, and Swofford, 1994), so these estimates served to generate a symmetric step matrix for the nLSU data in which we weighted transversions three steps, AG transitions two steps, and CT transitions one step. This step matrix is also similar to that employed by Moncalvo, Drehmel, and Vilgalys (2000) in their weighted parsimony analyses of *Amanita*. For a brief consideration of transversion weighting of fungal rDNA data, see Hibbett (1992). In weighted analyses of the RPB1 data set, first codon positions were weighted four steps, second positions five steps, and third positions one step to reflect the relative costs of transformations at these three positions. In the nLSU and exon data, gaps were rescored as separate presence/absence characters, a method coined as ''simple indel coding'' (Simmons and Ochoterena, 2000). The rescored indels were appended to the data matrix and partitioned for optional inclusion in parsimony analyses.

We employed heuristic tree searches under the maximum parsimony criterion using 30 random addition sequence replicates with one tree held at each step during stepwise addition with the tree-bisection-reconnection (TBR) branch-swapping algorithm and ''MulTrees'' option in effect. Bootstrapping (Felsenstein, 1985) was performed on each parsimony data set using a simple addition sequence but was otherwise the same as in the heuristic tree searches, and 1000 bootstrap replicates were performed for each maximum parsimony analysis.

We chose to analyze each data set individually, examine combined data sets for incongruence, and then combine the data. Data sets were assessed for their topological congruence using the partition homogeneity test (ILD test; Farris et al., 1994). Results with a P value <0.05 indicate an incongruence between topologies derived from two different sources of data. However, whether to combine the data despite a significant incongruence remains debatable (Hibbett and Donoghue, 2001).

RPB1 intron sequences were analyzed using maximum parsimony separately and in combination with exon sequences. A large number of indels, many overlapping, occurred in the intron 2 alignment that include distantly related taxa. These indels were scored as missing.

Maximum likelihood analyses relied on models determined as best-fits to each data set by comparing different nested models of DNA substitution in a hierarchical hypothesis-testing framework following the likelihood ratio test as implemented by MODELTEST version 3.04 (Posada and Crandall, 1998).

TABLE 3. Maximum likelihood best-fit evolutionary models and parameters determined by hierarchical likelihood ratio tests.

	nLSU	RPB1	Combined
Number of sites	1292	642	1934
Model	TN + I + G[a]	TN + I + G	TN + I + G
−L ln	4471.97351	5229.42616	9908.63239
Base frequencies			
A	0.2709	0.2500	0.2500
C	0.1880	0.2500	0.2500
G	0.2820	0.2500	0.2500
T	0.2591	0.2500	0.2500
Substitution model: rate matrix			
[A–C]	1.0000	1.0000	1.0000
[A–G]	5.0218	4.8557	4.9523
[A–T]	1.0000	1.0000	1.0000
[C–G]	1.0000	1.0000	1.0000
[C–T]	11.0282	5.7121	6.6412
[G–T]	1.00000	1.0000	1.0000
Among-site rate variation			
I[b]	0.6652	0.4904	0.5894
G[c]	0.7447	1.4335	0.7096

[a] Tamura-Nei model with an estimated proportion of invariable sites and an estimated gamma distribution shape parameter.

[b] Proportion of invariable sites.

[c] Variable sites gamma distribution shape parameter.

RESULTS

Nucleotide sequences—RPB1 exon sequences were easily aligned and contained a single autapomorphic indel, an amino acid deletion in *Hebeloma*, that was scored as missing in separate parsimony analyses. After removal of ambiguously aligned regions in the nLSU data set, 37 indels, 14 of these parsimony informative, were identified and rescored as separate presence/absence characters (Simmons and Ochoterena, 2000). Table 2 presents a parsimony character status summary of the data sets with gaps scored as missing. This table contains the number of characters, number of parsimony informative characters, tree length, the number of most parsimonious trees, the consistency index (CI), and the number of clades with >70% bootstrap support per data set. Table 3 includes the maximum likelihood models evaluated as best fits by MODELTEST (Posada and Crandall, 1998) and their concomitant parameters.

Four spliceosomal introns within the RPB1 region were recognized by sequence comparisons and confirmed by cDNA sequencing (Fig. 2). Three of the introns—intron 1, intron 3, and intron 4—are consistent in size with most introns that characterize filamentous fungal genomes (Gurr, Unkles, and Kinghorn, 1988; Mehmann, Brunner, and Braus, 1994). These three introns range in length from 46 to 74 nucleotides. Intron 1 occupies a phase 1 insertion with respect to the reading frame, while the other three have phase 0 insertions. Intron 2

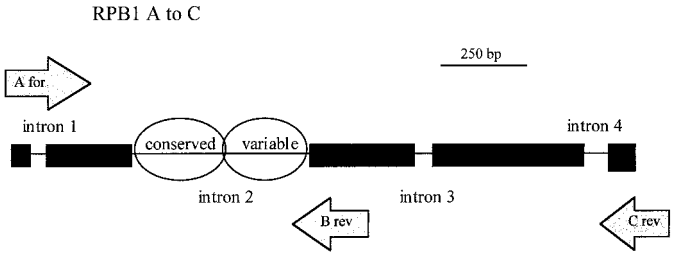


Fig. 2. Schematic diagram of exon and intron regions within RPB1 from conserved domains A to C. Larger shaded boxes are exons; intervening lines represent introns. Amplification and sequencing primers are indicated as thick arrows. The conserved and variable regions of intron 2 are indicated by ellipses.

ranges in length from 459 to 548 nucleotides and contains a mean GC content across taxa at 52.6%. Interestingly, a sample of *Cryptococcus neoformans* (Sanfelice) Vuillemin (Y. J. Liu and B. D. Hall, unpublished data), a heterobasidiomycetous yeast, shares an insertion at the intron 2 splice site with the agarics, which are homobasidiomycetes. However, intron 2 of *C. neoformans* is 60 nucleotides long and has a GC content of only 38.3%. Table 4 shows a summary of proportional pairwise distances between *I. pudica* and increasingly distant relatives across nLSU, RPB1 exon, intron 2, and intron 3 sequences.

No extreme nucleotide compositional bias was evident across taxa for any of the data sets (chi-square tests of homogeneity of base frequencies across taxa produce nonsignificant results in PAUP* under the ''Base Frequencies'' option), unlike comparisons between distantly related taxa where nucleotide composition bias (Klenk et al., 1995; Foster and Hickey, 1999) or sequences of paralogous loci among closely related taxa (Mayol and Rosselló, 2001) can mislead phylogenetic reconstructions. The nLSU data set showed a slight overall composition bias at 52% AT, whereas the RPB1 exon data set demonstrated an overall (% AT) of 50%. However, RPB1 showed % adenine-thymine composition bias per codon position. First positions demonstrated a range of 40–45%, second positions 63–65%, while third positions were more balanced with 48–49% AT content.

Phylogenetic inference from nLSU sequences—Our MP (maximum parsimony) data set resulted in six equally parsimonious trees produced from 1292 total sites, of which 132 are parsimony informative. One of the six trees is shown in Fig. 3a. Although the deeper topology is resolved in a strict consensus of the six most parsimonious trees, only four clades receive >70% boostrap support. Support for the monophyly of the genus *Inocybe* (99%) and the sister position of the genus *Phaeomarasmius* (93%) is high, but confidence elsewhere is restricted to the two sets of closely related species pairs, *pudica-agglutinata* and *lanuginosa-leptophylla*. However, the two longest branches on the tree, *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*'' form a moderately supported clade with 63% bootstrap support. This result is surprising because *I.* ''*serpitocystis*'' shares a morphological synapomorphy, necropigmented basidia, with *dulcamara-Inocybe* sp. PBM 1615, two members of subgenus *Mallocybe*. Clade I, composed of five smooth-spored species with hymenial metuloid cystidia, is more clearly resolved by WP and maximum likelihood (ML) analyses (Fig. 3a–c). Additionally, weak monophyletic relationships are suggested for stirps Lanuginosa sensu R. Heim (*lanuginosa-leptophylla-stellatospora*) (Heim, 1931; Matheny and Kropp, 2001) and section 5 or the ''*Geophyllinae*'' (*agglutinata-pudica-godeyi*) of Singer (1986), neither of which is monophyletic in a strict consensus of the most parsimonious trees nor by any other data set, whether using MP, WP, or ML. A separate analysis included 37 gaps, which contributed 14 additional parsimony informative sites and reduced the number of MP optimal trees to four without significantly affecting bootstrap support (data not shown).

Weighted parsimony recovered three additional parsimony informative sites (for a total of 135) and reduced the number of optimal parsimony trees to two. One of the two WP trees is shown (Fig. 3b). Resolution is increased, for example, for clade I. Furthermore, the long-branch attraction between *I.* ''*serpitocystis*'' and *I. hirsuta* var. *maxima* is broken; however the former joins a weakly supported clade with *I. maculata*, a clade that collapses in the strict consensus of the two most parsimonious trees. Likewise, *I. hirstua* var. *maxima* forms a weakly supported clade with *dulcamara-Inocybe* sp. PBM 1615 but with the former collapsing in the strict consensus tree.

A Tamura-Nei model with unequal base frequencies, an estimated proportion of invariable sites, and an estimated gamma distribution shape parameter was determined as the best-fit model to the data by MODELTEST (Table 4). The ML tree (Fig. 3c) is largely congruent with the WP tree shown except for a few rearrangements within subgenus *Inocybe* that receive poor bootstrap support in both the MP and WP treatments. Unlike the MP analysis, the ML and WP results do not support a clade of *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*'' (with 63% bootstrap support under MP). Furthermore, both WP and ML support a basal position for *lanuginosa-leptophylla* within

TABLE 4. Percentage differences in pairwise distance comparisons between *Inocybe pudica* and increasingly distant relatives[a] across loci evaluated in this study.

Data set	*pudica/ agglutinata* (*Inocybe*)	*pudica/ relicina* (*Inocybe*)	*pudica/ maculata* (*Inosperma*)	*pudica/ hirsuta* var. *maxima* (*Inosperma*)	*pudica/ dulcamara* (*Mallocybe*)	*pudica/ Galerina* Outgroup	*pudica/ Hebeloma* Outgroup
nLSU	2.23	2.48	4.55	7.18	3.51	4.71	3.59
RPB1[b]	7.36	10.80	18.47	20.19	20.81	21.13	21.58
Intron 2	3.09	7.01	11.08	13.64	14.42	18.50	19.42
Intron 3	6.25	36.00	44.68	n/a[d]	n/a	n/a	n/a

[a] Subgenus is in parentheses.
[b] Exon sequences.
[c] *Inocybe* sp. PBM 1615, closely related to *I. dulcamara*, was used in lieu of *I. dulcamara*.
[d] Not alignable.

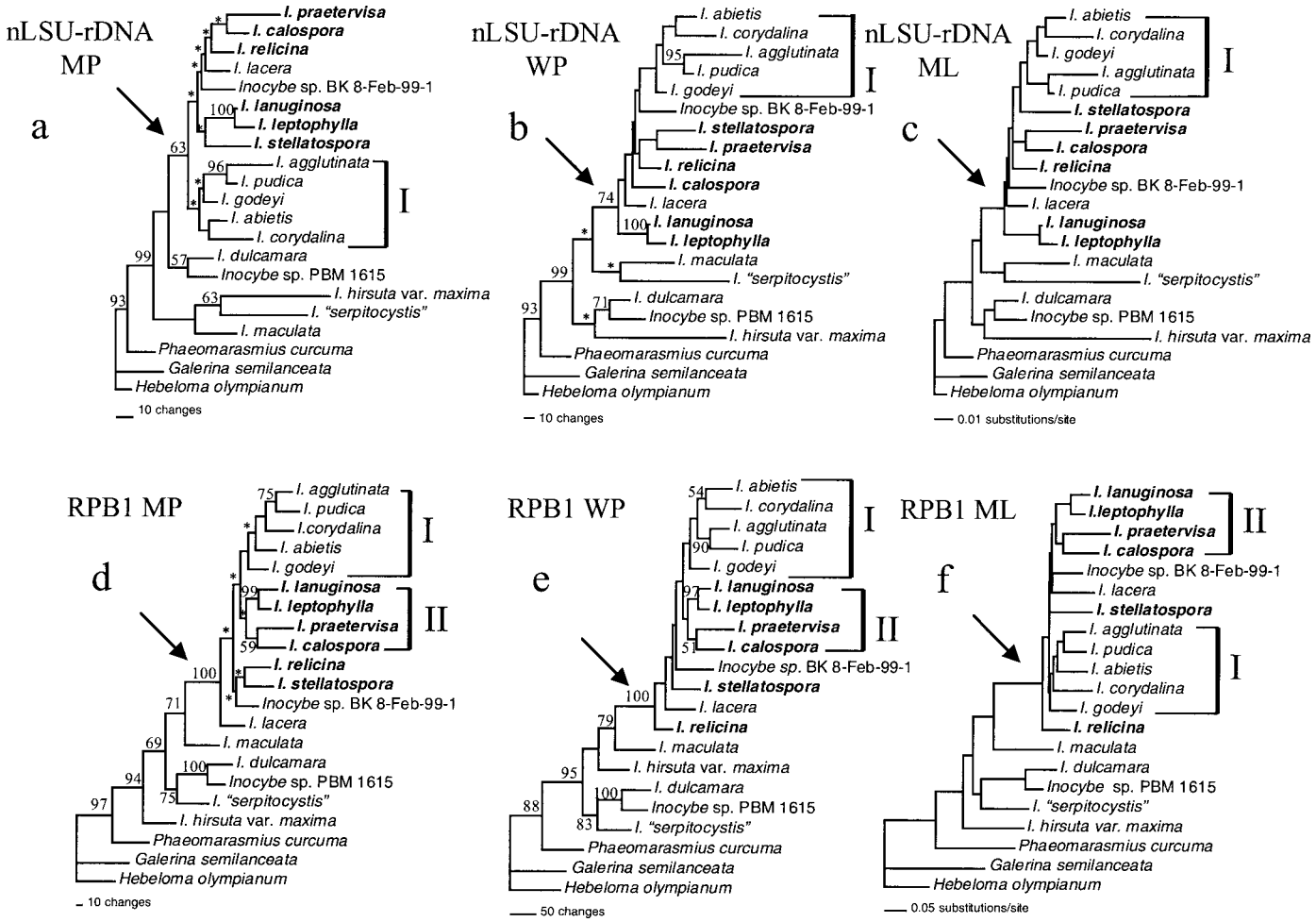


Fig. 3. (a) One of six equally weighted maximum parsimony (MP) trees derived from partial nLSU sequences. (b) One of two weighted parsimony (WP) trees based on partial nLSU sequences. (c) The maximum likelihood tree generated with the best-fit model. (d) One of eight MP trees derived from partial RPB1 exon sequences. (e) The single WP tree based on partial RPB1 exon sequences. (f) The maximum likelihood tree based on the best-fit model. Gaps are treated as missing data. Heuristic searches were used to find most parsimonious trees. One thousand bootstrap replicates were performed for each analysis. nLSU WP analyses employed a user-defined step matrix that weighted transversions more heavily than transitions; RPB1 WP analyses employed differential weights by codon position. Bootstrap values >50% are indicated above or below the branches. Asterisks indicate the collapse of a branch in the strict consensus tree. Species with nodulose basidiospores are indicated in boldface type. An arrow indicates the branch giving rise to the clade containing members of subgenus *Inocybe*. Clade I contains five smooth-spored species. Clade II contains four nodulose-spored species.

subgenus *Inocybe*. Overall, WP records six clades with >70% bootstrap support, and WP plus gaps recovers a single most parsimonious tree. The inclusion of gaps, however, reduces the number of clades with >70% bootstrap support to four (data not shown).

Phylogenetic inference from RPB1 exon sequences—A total of 642 sites, 233 of them parsimony informative, were obtained from RPB1 exon sequence data. Thirty-four (15%) informative sites occur at first-codon positions, 14 (6%) at second-codon positions, and 185 (79%) at third-codon positions. Eight equally most parsimonious trees were recovered, one of which is presented (Fig. 3d). The outer backbone of this tree is resolved with moderate to strong bootstrap support unlike the nLSU trees. *Inocybe* ''*serpitocystis*'' joins a resolved and moderately supported clade with *dulcamara*-*Inocybe* sp. PBM 1615, two northern hemispheric members of subgenus *Mallocybe*. In fact, this relationship is supported by a high bootstrap value (75%) and anatomical similarities (P. B. Matheny, unpublished data) and conflicts with the MP nLSU tree. *Inocybe hirsuta* var. *maxima* is basal in our sample with moderate bootstrap support (69%). Additionally, *I. maculata* is supported as sister to subgenus *Inocybe* with moderate confidence (bootstrap = 71%). In contrast, none of the nLSU analyses provided any confidence for this sister relationship. Subgenus *Inocybe*, indicated by an arrow, receives strong boostrap support compared to the nLSU-MP results (63% → 100%). *Inocybe lacera* is indicated as basal in subgenus *Inocybe*, but this branch collapses in the strict consensus of the eight most parsimonious trees. *Inocybe praetervisa* and *I. calospora*, which form a poorly resolved and weakly supported clade in nLSU-MP, receive moderate boostrap support (59%) and do not collapse in the RPB1-MP strict consensus tree. Overall, eight clades receive >70% bootstrap support compared to four for the nLSU-MP data.

Differentially weighting the RPB1 data set results in a single most parsimonious tree (Fig. 3e). The higher level branching order outside subgenus *Inocybe* remains the same in the RPB1-

MP data set except for the placement of *I. hirsuta* var. *maxima*, which now occupies a weakly supported branch basal to *I. maculata*. Resolution and high confidence are maintained for a clade of the three samples with necropigmented basidia, *I.* ''*serpitocystis*'' and *dulcamara-Inocybe* sp. PBM 1615, again in contrast to the poor resolution and moderate to weak bootstrap support from the nLSU data. Within subgenus *Inocybe*, two principal clades, one of five smooth-spored species (clade I), the other of four nodulose-spored species (clade II), are resolved but lack bootstrap support. *Inocybe relicina* is supported weakly as basal in this subgenus. The positions of *I. lacera*, *I. stellatospora*, and *Inocybe* sp. BK 8-Feb-99-1 are resolved but poorly supported by bootstrapping. These three taxa stem from very short internodes and their positions in other trees are just as poorly supported or not resolved in consensus trees. The bootstrap support for *agglutinata-pudica* increases in the WP analysis (75 → 90%).

The model bestfit to the RPB1 data is a Tamura-Nei model with equal base frequencies, an estimated proportion of invariable sites, and an estimated gamma distribution shape parameter (Table 3). The C–T transitions occur at a slightly higher rate than the A–G transitions. The ML tree agrees with the MP tree in the branching order of those taxa basal to *I. relicina*. However, as in the WP tree, *I. relicina* is basal within subgenus *Inocybe*. Clades I and II in subgenus *Inocybe* are maintained by the ML tree. The positions of *I. stellatospora*, *I. lacera*, and *Inocybe* sp. BK 8-Feb-99-1 are not in agreement with any other analysis.

Phylogenetic inference based upon intron 2 sequences—Sequences of intron 2 across the RPB1 data set contributed 116 parsimony-informative sites out of 462 sites that could be aligned. The 5′ half of this intron, approximately the first 260 bp, is remarkably invariant while the latter half of the intron is highly variable. Over 50 indels were recorded, many of which overlap and thus would contribute missing data to the matrix if rescored under simple indel coding (Simmons and Ochoterena, 2000). Thus, the indels in this region were scored as missing. The complete intron sequence of *I. dulcamara* was not obtained, so it was omitted from the parsimony analysis. An MP analysis of this data set produced 27 equally most parsimonious trees with a resolved backbone but little resolution within subgenus *Inocybe* (trees not shown). Only two highly supported relationships within subgenus *Inocybe* are recovered by the intronic sequence, relationships equally well recovered by previous analyses (*lanuginosa-leptophylla* and *agglutinata-pudica*). The deeper topology is similar to other trees with the exception that *Inocybe* sp. PBM 1615, *I.* ''*serpitocystis*,'' *I. hirusuta* var. *maxima*, and *I. maculata* form a paraphyletic grade. The intron 2 data, like the RPB1 exon sequences, do not support a clade of *I.* ''*serpitocystis*'' and *I. hirsuta* var. *maxima*.

Partition homogeneity tests between RPB1 exon sequences and intron 2, on the one hand, and nLSU and intron 2, on the other, yielded nonsignificant results ($P = 0.320$ and $P = 0.410$, respectively). However, the addition of intron 2 sequences to the RPB1 and nLSU data failed to improve resolution. One of the four MP trees produced the exact same topology without the intron sequences and with similar bootstrap values (trees not shown). An analysis including weighted nLSU and exon sequences plus equally weighted intron 2 sequences produced four equally parsimonious trees, all of which supported the basal position of *I. relicina* in subgenus *Inocybe* in contrast to the WP analysis without the intron, which places *I. stellataspora* basal to *I. relicina*. Again, bootstrap support values were very similar to the WP analysis without the intron.

Given the higher level of our taxonomic questions, the accelerated rate of change in intron 3 prohibited its employment in our phylogenetic analyses. However, this small intron might be useful in addition to exon sequences and intron 2 sequences to infer relationships of closely related species or populations within a species.

The nLSU and RPB1 exon data combined—Because moderately supported conflict exists over the placement of *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*,'' we partitioned the two data sets and performed a test of congruence using the partition homogeneity test in PAUP*. The result of this test ($P = 0.080$) suggests that the conflict between the two data sets does not statistically merit incongruence. As suggested by Wiens (1998), we chose to combine the two data sets since major portions of the trees indicate a shared evolutionary history for the two genes. The combined data set results in 365 parsimony informative sites from a total of 1934 sites and two equally most parsimonious trees under the MP criterion. One of these trees is shown as Fig. 4a. Nine clades with >70% bootstrap support are recovered compared to eight for the RPB1 data and four for the nLSU data alone. The combined data indicate for the first time moderate bootstrap support (71%) for clade I, composed of five smooth-spored species. Clade II, however, is weakly supported and not well resolved because subtending branches collapse in the strict consensus of the two most parsimonious trees. However, within clade II *I. praetervisa* and *I. calospora* form a strong monophyletic group (90% bootstrap support), which at best is weakly supported by the RPB1 or nLSU data alone. Basal to clade II, a nodulose-spored clade, are the poorly resolved *I. lacera* and *Inocybe* sp. BK 8-Feb-99-1. The basal position of *I. stellatospora* in subgenus *Inocybe* is not entirely resolved across MP, WP, and ML analyses; and although *I. hirsuta* var. *maxima* is inserted into a clade with *I. dulcamara*, *Inocybe* sp. PBM 1615, and *I.* ''*serpitocystis*'' under MP and ML criteria, this relationship is not supported by the WP analysis.

Weighted parsimony analysis of the combined data (Fig. 4b) produces a single most parsimonious tree with some increased resolution for clades I and II of the MP tree shown in Fig. 4a. Bootstrap support for clade I remains moderate (66%). However, high bootstrap support (76%) is indicated for the first time for a clade of *I. abietis* and *I. corydalina*. The WP tree, unlike that for MP, suggests that *I. hirsuta* var. *maxima* does not form a clade with *I.* ''*serpitocystis*,'' which is in agreement with all individual RPB1 analyses. *Inocybe stellatospora* occupies a poorly supported basal position to *I. relicina*. If one includes 14 parsimony-informative indels in the WP analysis, then ten clades with >70% bootstrap support are recovered, more than any other analysis of individual or combined data sets. Inclusion of these indels, however, raised bootstrap support for clade I (66% to 73%) by only a minor degree (data not shown).

The ML tree (Fig. 4c), generated by a Tamura-Nei model (Table 4), supports clades I and II but places *I. relicina* in the basal position in subgenus *Inocybe*. Unlike the WP analysis, *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*'' do form a clade.

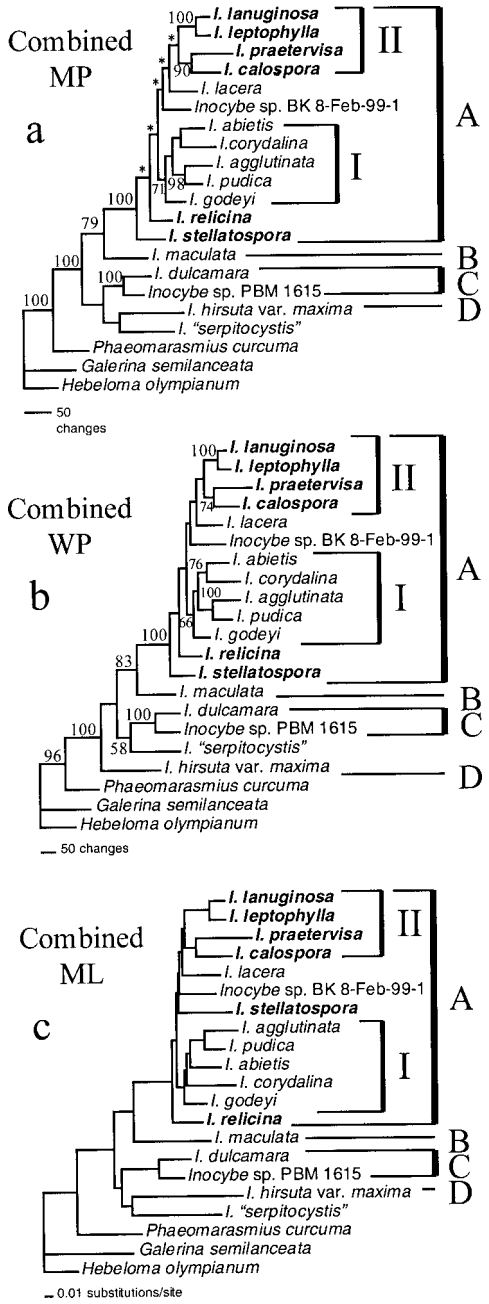


Fig. 4. (a) One of two equally weighted maximum parsimony (MP) trees obtained from a combined analysis of partial nLSU and partial RPB1 nucleotide sequences. (b) The single weighted parsimony (WP) tree derived from a combined partial nLSU and partial RPB1 nucleotide sequences. (c) The maximum likelihood tree based on a best-fit model for the combined partial nLSU-rDNA and partial RPB1 nucleotide sequences. Gaps are treated as missing data. Heuristic searches were used to find most parsimonious trees, and 1000 boostrap replicates were performed for each analysis. The WP analysis employed a user-defined step matrix that weighted transversions more heavily than transitions in the nLSU-rDNA partition; the RPB1 partition was weighted with differential weights per codon position. Bootstrap values >50% are indicated above or below the branches. Asterisks indicate the collapse of a branch in a strict consensus tree. Species with nodulose basidiospores are indicated in boldface type. Clade A represents sugenus *Inocybe*. Clade I contains five smooth-spored species, and clade II contains four nodulose-spored species within clade A. Clade B represents section *Rimosae*. Clade C is subgenus *Mallocybe*. Clade D represents section *Cervicolores*.

DISCUSSION

Usefulness of RPB1 in agaric phylogeny—Because the questions addressed in this study concern generic- and subgeneric-level relationships, variable regions of RNA polymerase II sequences were targeted for phylogenetic analysis and comparison with nuclear large subunit ribosomal DNA sequences (nLSU). As indicated earlier, ITS sequences appear to be too variable to address our higher level questions. The RNA polymerase II sequences analyzed are from the 5′ proximal region of the gene encoding RPB1 that includes conserved domains A–C (Allison et al., 1985). We sequenced 642 bases within three exons and 505–603 bases of intron sequence. For the nLSU sequences, 1292–1329 sites are included in the data matrix, depending upon whether indels were rescored.

RPB1 contributes more parsimony-informative sites than the nLSU data set (Table 2). Furthermore, only one single amino acid indel occurs in the set of RNA polymerase II exon sequences, including the outgroups. Thus, alignment ambiguities are not encountered at our taxonomic level. Spliceosomal intron boundaries were confirmed by cDNA sequences, which enable the intronic regions to be identified easily.

Intron 2 sequences were alignable across all taxa, including three outgroup representatives. The first 260 bp and a downstream 50-bp region are remarkably conserved across our samples. Intervening regions, however, are highly variable and difficult to align except among closely related taxa. *Inocybe stellatospora* and *I. relicina*, for example, are characterized by two nonhomologous and partially overlapping large deletions. Additionally, both *I. corydalina* and *I. stellatospora* share a 6-bp deletion, but this gap must be viewed as a homoplasious event because these two taxa do not share an immediate common ancestor. In contrast to the extraordinarily conserved 5′ proximal region of intron 2, the last 200 bp would appear promising for resolving very closely related species. The large number of gaps and distantly related taxa complicate alignments of this region in our data set. Unexpected patterns of intron conservation have been reported previously (Mattick, 1994), but we are unaware of the function of this intronic sequence.

Several topological distinctions are noted between the trees inferred for RPB1 and those for nLSU. RPB1, for example, demonstrates fairly uniform terminal branch lengths across taxa and exhibits a highly resolved outer backbone with moderate to strong support among higher level taxa (Fig. 3d–e). These results stand in contrast to the nLSU data sets that are characterized by heterogeneous terminal branch lengths, viz., *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*,'' and poor resolution along the outer backbone portion of the trees (Fig. 3a–b). The positions of *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*'' are tenuous because the long branches of these two species shuffle within the lower part of the tree with varying resolution and moderate to weak bootstrap support depending on the phylogenetic inference method. An RPB1 analysis of the same taxa produces a highly resolved and well-supported relationship that suggests *I.* ''*serpitocystis*'' is sister to a clade of north temperate members of subgenus *Mallocybe*, with which the sample shares a number of anatomical similarities (Fig. 3d–f). Moreover, the branch lengths of *I. hirsuta* var. *maxima* and *I.* ''*serpitocystis*'' are consistent with those of other RPB1 samples and are not plagued by long-branch attraction. Additionally, the well-resolved but poorly supported

clade II, discussed in detail below, is recovered by RPB1 sequences under WP and ML but not by our nLSU data.

Combined data sets increase resolution and confidence: clades I and II—Protein-coding sequences not only achieve independent assessments of ribosomal RNA phylogeny but also can be used in combination to raise the number of informative sites and amplify signal from either data set that otherwise is weak. For example, two clades are discussed below that are characterized by improved resolution, bootstrap support, or both when the data are combined.

Clade I: smooth-spored Inocybes with metuloid hymenial cystidia—A clade of five species (*pudica-abietis*) with smooth basidiospores and presence of metuloid hymenial cystidia was recovered across all data sets, whether examined individually or combined using either maximum parsimony or maximum likelihood (Figs. 3a–f, 4a–c). Boostrap support among individual data sets is weak for clade I (<50%). However, when combined it receives 66–71% bootstrap support. The nLSU-MP data set (Fig. 3a) suggests that section 5 or ''*Geophyllinae*'' (Singer, 1986)—including our *I. pudica*, *I. agglutinata*, and *I. godeyi*—is monophyletic, however, the position of *I. godeyi* collapses in a strict consensus of this tree. Other analyses point to a nonmonophyletic result for this section. The interior clade of *I. abietis* and *I. corydalina* is recovered across all analyses with the exception of RPB1-MP and combined-ML. Only combined-WP suggests any bootstrap support for the clade (76%), which is composed of two different sections in Singer (1986) (Fig. 4b). Smooth-spored species with metuloid hymenial cystidia excluded from clade I include *I. lacera*, a species with a boletoid to minimally angular spore outline, and the unidentified *Nothofagus*-associate from Argentina.

Clade II: nodulose-spored Inocybes of stirps Lanuginosa, section Marginatae, and section Calosporae—Clade II, composed of four species with nodulose basidiospores, is recovered by RPB1 data but not by the individual nLSU analyses. Combined data maintain the clade but with weak support (Fig. 4a–b). Moreover, within clade II a confident relationship (74–90% bootstrap support) is inferred by combined-MP and WP analyses for *praetervisa-calospora*, support for which is marginal based on the RPB1 data alone and weakly contradicted by nLSU-WP. This relationships is interesting because *I. calospora* was placed by Zerova (1974) in a monotypic genus due to the unusual basidiospores that feature radial spinose nodules; additionally, *I. praetervisa* is the lectotype of the genus *Astrosporina* (Horak, 1968). Both species, however, share entirely pruinose stipes and lack a cortina. Our results suggest *praetervisa-calospora* form a strongly supported clade inclusive within a larger clade that we have identified as subgenus *Inocybe*. Two nodulose-spored species are not members of clade II: *I. stellatospora* and *I. relicina*, the latter the type of *Inocybe*.

Current assessment of Inocybe phylogeny—*Inocybe*, as currently circumscribed by most taxonomists, is a large genus of agarics. Between 250 and 350 species, a conservative estimate, are represented throughout the world among a wide array of ectomycorrhizal hosts (Kuyper, 1986). This number is likely an underestimate given that ectotrophic regions such as Australia (P. B. Matheny and N. L. Bougher, unpublished data) contain incompletely documented agaric floras. Our findings indicate that *Inocybe* is a monophyletic group although our sampling among outgroups is rather limited based on the data sets presented here. A higher-level study by Moncalvo et al. (2000) also indicates the monophyly of *Inocybe*. Phylogenetic analyis of expanded nLSU data sets (P. B. Matheny, unpublished data) suggest that none of the following genera, *Conocybe*, *Simocybe*, *Tubaria*, *Crepidotus*, *Agrocybe*, *Naucoria*, *Hypholoma*, *Flammulaster*, *Gymnopilus*, *Cortinarius*, nor an Australian sample of *Hymenogaster* (in addition to *Hebeloma*, *Phaeomarasmius*, and *Galerina*) are derived within *Inocybe*.

Several monophyletic groups are recovered within *Inocybe*, some of which are clearly distinguished by their nLSU nucleotide sequences and RPB1 exon sequences. Combined sequences of RPB1 and nLSU regions suggest *Inocybe* is at least composed of four principal monophyletic groups: subgenus *Inocybe* (clade A), section *Rimosae* (clade B), subgenus *Mallocybe* (clade C), and section *Cervicolores* (clade D) (Fig. 4a–c). These findings support a cladistic study of European representatives of the genus based on morphological characters (Kuyper, 1986). Indeed, Kuyper suspected that subgenus *Inosperma*, containing sections *Rimosae* and *Cervicolores* (clades B and D, respectively), might not be monophyletic. However, the two loci sampled here support conflicting placement of the mallocyboid Australian sample *I.* ''*serpitocystis*,'' and the *Cervicolores* sample, *I. hirsuta* var. *maxima*, which raises questions regarding their phylogeny. Their relationships must be interpreted as dubious in the nLSU analyses given the long branches they exhibit (Felsenstein, 1978) (Fig. 3a–c). RPB1 data suggest *I.* ''*serpitocystis*'' is sister to *dulcamara-Inocybe* sp. PBM 1615 with high bootstrap support (75–83%), a relationship also supported by several morphological characters (P. B. Matheny, unpublished data). Combined data, however, suggest a unique result (Fig. 4a–c) that itself is not consistent depending on the inference method used. Based on this evidence it is likely that a clade of *I. hirsuta* var. *maxima* and *I.* ''*serpitocytsis*'' is an artifact. Additional sources of data and continued sampling among similar taxa could alleviate their equivocal status. Nucleotide compositional bias appears not to be the source of the nLSU long-branch attraction.

Within subgenus *Inocybe*, which in combined analyses receives 100% bootstrap support, nodulose-spored species do not form a monophyletic group. Therefore, recognition of the genus *Astrosporina*, circumscribed by nodulose-spored taxa and typified by *I. praetervisa*, is not warranted. The name, however, could be applied to clade II, which contains *I. praetervisa*, but at a lower rank. Basal to clade II are *I. lacera* and *Inocybe* sp. BK 8-Feb-99-1, the latter a *Nothofagus* associate from Argentina with a cortina and amydaliform spore outline. Although this branching order lacks strong bootstrap support, it might suggest the derivation of the nodulose-spored condition from a minimally angular or an amydaliform-shaped state. Clade I in subgenus *Inocybe* consists entirely of species with amygdaliform to elliptic spore outlines. This clade receives moderate boostrap support (66–71%) in combined parsimony analyses and represents three different sections in Singer (1986). One of these, section 5 or ''*Geophyllinae*'' is likely not monophyletic unless *I. godeyi* is excluded. Clade I also contains a species, *I. corydalina*, which is remarkable for its lack of muscarine and possession of psilocybin, an hallucinogenic compound (Besl and Mack, 1985; Stijve, Klan, and Kuyper, 1985). Distributed among these two clades are mem-

bers of ''supersection'' Marginatae sensu Kuyper (1986), a monophyletic group inferred by morphological characters, viz. lack of a cortina. The monophyly of the ''supersection'' is not supported here because *I. praetervisa*, *I. abietis*, and *I. godeyi* never form a clade in any analysis.

Resolution of the sister group to subgenus *Inocybe* is improved when combining data sets or treating RPB1 data alone. Section *Rimosae* (clade B) is supported as sister to subgenus *Inocybe* (clade A) no matter the method and with moderate to high bootstrap support (71% MP; 83% WP) in these analyses (Fig. 4a–c).

Although this study does not rigorously evaluate a large number of outgroups to determine a sister-level relationship to *Inocybe*, it suggests that the genus *Phaeomarasmius* (Strophariaceae) is more closely related to *Inocybe* (Cortinariaceae) than is *Galerina* (Cortinariaceae) in contrast to a morphological numerical analysis of these relationships (Machol and Singer, 1971). Moreover, Moncalvo et al. (2000) have shown that the Cortinariaceae are likely not a monophyletic group. However, their analyses fail to indicate any well-supported sister group to *Inocybe*. Compounding this lack of resolution is the problem that neighboring genera such as *Phaeomarasmius*, *Flammulaster*, *Tubaria*, and *Simocybe* are incompletely known (Horak, 1979). Furthermore, future research should consider the systematic placement of *Fissolimbus* and *Asproinocybe*, two genera with various inocyboid features (Singer, 1986).

Concluding remarks—Taxon sampling has been demonstrated to be problematic in our nLSU data set due to the presence of two long branches and lack of resolution and support in the remainder of the topology when independently evaluated by protein-coding sequences. Other sources of data are needed not only to assess the nLSU estimate, but to infer the phylogeny where nLSU data suggest doubt. In contrast, most regions of our RPB1 phylogeny are stable despite the inclusion of the taxa that are problematic in our nLSU estimates. Moreover, when assessed by different methods of phylogenetic inference, the RPB1 data tend to yield more consistent results.

Classification is an additional problematic issue since the four (or five) principal lineages within *Inocybe* are almost as distinct from each other as from the outgroup representatives. Yet, these lineages are classified at different ranks. Alternatives include recognition of the lineages as sections, subgenera, or genera. Due to the high amount of pairwise distance dissimilarities among the inocyboid lineages and outgroups, it would appear justified to relegate the lineages to generic rank and accept Jülich's intuitive proposal for the family Inocybeaceae. Additional sampling from multiple genes across sections *Rimosae*, *Cervicolores*, subgenus *Mallocybe*, southern hemispheric relatives, and additional outgroups are envisioned prior to any reclassification.

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