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Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*; Agaricales)

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Abstract

Approximately 3000 bp across 84 taxa have been analyzed for variable regions of RPB1, RPB2, and nLSU-rDNA to infer phylogenetic relationships in the large ectomycorrhizal mushroom genus *Inocybe* (Agaricales; Basidiomycota). This study represents the first effort to combine variable regions of RPB1 and RPB2 with nLSU-rDNA for low-level phylogenetic studies in mushroom-forming fungi. Combination of the three loci increases non-parametric bootstrap support, Bayesian posterior probabilities, and resolution for numerous clades compared to separate gene analyses. These data suggest the evolution of at least five major lineages in *Inocybe*—the Inocybe clade, the Mallocybe clade, the Auritella clade, the Inosperma clade, and the Pseudosperma clade. Additionally, many clades nested within each major lineage are strongly supported. These results also suggest the family Crepiodataceae *sensu stricto* is sister to *Inocybe*. Recognition of *Inocybe* at the family level, the Inocybaceae, is recommended.

Keywords: Cortinariaceae; Fungi; Inocybaceae; nLSU-rDNA; RNA polymerase II; Systematics

1. Introduction

Nuclear genes that encode the two largest subunits of RNA polymerase II are proving useful to infer the phylogeny of organisms across many branches of the tree of life (Chaverri et al., 2003; Denton et al., 1998; Hirt et al., 1999; Klenk et al., 1995; Liu et al., 1999; Nickerson and Drouin, 2004; Pfeil et al., 2002; Sidow and Thomas, 1994; Stiller and Hall, 1999; Tanabe et al., 2004). Recently, an RNA polymerase II gene phylogeny using RPB1 (Matheny et al., 2002), the gene that encodes the largest subunit of the enzyme, was shown to improve phylogenetic inference among mushroom species in the genus *Inocybe* (Fr.) Fr. (Agaricales; Basidiomycota). Here, sampling across a representative set of 84 mushroom taxa in *Inocybe* and outgroups of the Agaricales, or euagarics clade, has been extended to include partial sequences of RPB1, RPB2, and nuclear large subunit ribosomal DNA (nLSU). Both RPB1 and RPB2 are demonstrated to offer variable regions at the nucleotide level to reconstruct the evolutionary history of mushroom-forming fungi.

Infrageneric studies of mushrooms have relied principally on nLSU and/or the internal transcribed spacer (ITS) regions of the nuclear rDNA tandem repeats to estimate evolutionary relationships (e.g., Aanen et al., 2000; Hopple and Vilgalys, 1999; Kretzer et al., 1996; Liu et al., 1997; Moncalvo et al., 1993; Weiß et al., 1998). Numerous recent studies continue their use. However, very few researchers have incorporated protein-coding loci to address lower-level systematic studies among mushrooms and their allies (Matheny and Ammirati, 2003; Matheny et al., 2002; Tabata et al., 2000; Thon and Royse, 1999; Wang et al., 2004). Matheny et al. (2002) showed that nucleotide sequences of exon regions near

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the beginning of the RPB1 gene were easily aligned, contributed a large amount of parsimony-informative sites, and increased confidence and resolution for many clades of *Inocybe*, including its monophyly, when analyzed separately and combined with nLSU sequence data. Liu et al. (1999) demonstrated that RPB2, the gene that encodes the second largest subunit of RNA polymerase II, contains a variable region (between conserved domains 6 and 7) that might be phylogenetically useful for studies among species at low taxonomic levels. Matheny and Ammirati (2003) used both RPB1 and RPB2 for such a purpose in a systematic study of *Cortinarius aureifolius* (Cortinariaceae; Agaricales).

Inocybe is exemplary among the many large genera of the Agaricales sensu Singer (1986) due to the combination of several morphological and anatomical characters. The core of the genus contains many species with specialized terminal cells (cystidia) that occur on the basidiomata (fruitbodies) and are often thick-walled and apically incrusted with precipitates of calcium-oxalate (Kuyper, 1986). A number of species exhibit protuberances that emerge from the basidiospore wall, a condition generally described as gibbous or nodulose. Biochemically, most species of *Inocybe* exhibit substantial amounts of muscarine, a quaternary ammonium compound that stimulates the parasympathetic nervous system of humans (Benjamin, 1995; Bresinsky and Besl, 1990; Brown, 1965). A few species that lack muscarine possess instead the hallucinogenic compounds psilocybin and baeocystin (Besl and Mack, 1985; Gartz and Drewitz, 1985; Stijve et al., 1985).

Species of *Inocybe* are generally recognized in the field by the combination of mundane colors, coarsely fibrillose texture to the basidiomata, brownish lamellae, occurrence on soil, and an unusual odor similar to piperidine (Heim, 1931), often described as spermatic. This odor is similar to the smell of Chestnut (*Castanea*) inflorescences. Ecological, anatomical, and molecular evidence suggest that *Inocybe* is ectomycorrhizal and symbiotic with numerous families of angiosperms and gymnosperms such as the Betulaceae, Casuarinaceae, Cistaceae, Dipterocarpaceae, Fabaceae, Fagaceae, Myrtaceae, Nothofagaceae, Pinaceae, Salicaceae, and Uapacaceae, among other families (Agerer, 1987–1998; Glen et al., 2001; Horak, 1977, 1980; Kuyper, 1986; Matheny and Watling, 2004; Matheny et al., 2003; Singer, 1986). Although most species of *Inocybe* occur in temperate forested areas, a number of taxa also occur in arctic–alpine settings with *Salix* and *Dryas* (Favre, 1955; Horak, 1987; Kühner, 1988); and in the tropics of Africa, southeast Asia, Australasia, and South America (Buyck and Eyssartier, 1999; De Meijer, 2001; Horak, 1979, 1980, 1981; Matheny et al., 2003; Watling, 2001). Unfortunately, species of *Inocybe* cannot be cultured successfully on standard agar plates (Boidin, 1986; Singer, 1986), which has led some workers, for instance, Fries (1982) to conclude that the genus is "… experimentally hopeless …"

It is difficult to estimate with accuracy the actual number of species of *Inocybe* and how these taxa should be classified within mostly European-based classifications. No world-wide monograph of *Inocybe* exists, and new groups (both species and higher-level ranks) of the *Inocybe* flora continue to be described (Kobayashi, 2002a; Matheny and Bougher, unpublished; Kropp and Matheny, 2004; Matheny et al., 2003; Villarreal et al., 1998; Watling, 2001). Kuyper (1986) estimated between 250 and 350 species of *Inocybe* world-wide, which is proving to be a conservative estimate. Within the past five years numerous new species continue to be described throughout the world (Buyck and Eyssartier, 1999; Esteve-Raventós, 2001; Esteve-Raventós and Villarreal, 2001; Esteve-Raventós et al., 2003; Kobayashi, 2002a,b, 2003; Kropp and Matheny, 2004; Matheny and Bougher, unpublished; Matheny et al., 2003; Matheny and Watling, 2004; Seok et al., 2000; Watling, 2001).

Historically, many taxonomic arrangements have been proposed for *Inocybe* (see Alessio and Rebaudengo, 1980; Bizio, 1997; Heim, 1931; Kuyper, 1986; Singer, 1986; Stuntz, 1940). Early classifications stressed gross morphological characters (Earle, 1909; Fries, 1821–1832, 1857–1863, 1874) to define higher-level taxa, but these soon yielded to systems that emphasized anatomical characters such as basidiospore morphology and placement and types of cystidia on the basidiomata (examples include Heim, 1931; Horak, 1967; Kauffman, 1924; Kühner, 1933; Kühner, 1980; Kühner and Romagnesi, 1953; Kuyper, 1986; Lange, 1917, 1938; Massee, 1904; Schroeter, 1889; Singer, 1986). Kuyper (1986), however, was the first to apply cladistic methods to test evolutionary hypotheses in *Inocybe*. Using morphological characters, the phylogeny of several groups was hypothesized, including the polyphyly of species with gibbous spores. Using nucleotide sequences, Matheny et al. (2002) affirmed several findings in Kuyper (1986) but drew upon a fairly small sample size. Furthermore, the sister group to *Inocybe* was not rigorously evaluated. Although nearly 2400 nucleotide sites were sequenced in that study, the addition of loci and an increase in taxon sample size is believed to raise overall confidence and resolution throughout a phylogenetic estimate (Pollock et al., 2002; Rosenberg and Kumar, 2001; Zwickl and Hillis, 2002).

The subgeneric classification of Kuyper (1986) is followed rather than Kühner (1980) and Singer (1986) in the discussion of subgeneric concepts for the following reasons. Singer's classification is artificial at the subgenus level (Kuyper, 1986; Matheny et al., 2002). These studies also point to the paraphyly of Kühner's subgenus *Inosperma*. Nevertheless, key sectional elements of both Kuyper and Singer are represented here, but since the publication of Matheny et al. (2002), sampling has increased to include: (1) denser selection within subgenus

Mallocybe and sections *Rimosae* and *Cervicolores* of subgenus *Inosperma*; (2) additional sampling in subgenus *Inocybe* including sections *"Geophyllinae"* (unnamed section 5), *Lacerae*, *Splendentes*, *Inocybe*, and *Marginatae* of Singer; and (3) the smooth-spored, thin-walled cystidiate *I. leptocystis*, over which debate exists concerning its classification (Kobayashi, 2002a,b; Kuyper, 1986; Singer, 1986). In Singer's system, only section 10 (*Rubellae*), which includes *I. bresadolae*, remains unsampled. *Inocybe alabamensis* is believed to represent section *Petiginosae*. The undescribed *I. "nothopes"* from New South Wales, Australia, shares some morphological affinities with the *Petiginosae* as well. Although no truffle-like (sequestrate) taxa with unambiguous affinities to *Inocybe* have yet been described (Francis and Bougher, 2002), one such putative truffle-like *Inocybe* with anatomical similarities to subgenus *Mallocybe* is sampled here.

Attempts were also made to represent tropical and southern hemisphere species (Horak, 1983; Matheny and Bougher, unpublished; Pirozynski, 1983; Rees et al., 2002). Of the 67 total species of *Inocybe* sampled, 17 originate from the neotropics or southern hemisphere—Guyana, Chile, Argentina, New Zealand, and Australia—with the majority of the 17 originating in Australia. Major gaps in geographic sampling still include temperate regions of Asia, tropical ectomycorrhizal forests of southeast Asia and Africa, and the *Nothofagus* zones of South America, New Zealand, and Australia—regions with very high endemism of *Inocybe* species.

Most systematists classify *Inocybe* in the Cortinariaceae (Horak, 1967; Kühner, 1980; Moser, 1983; Singer, 1986) and suggest the genus is most closely related to *Hebeloma* or *Cortinarius*. Jülich (1982), however, placed *Inocybe* in its own family, the Inocybaceae. Moncalvo et al. (2002) demonstrated that *Pleuroflammula* (Strophariaceae) is possibly sister to an inclusive clade of at least *Inocybe* and the Crepidotaceae, and that the Cortinariaceae and Strophariaceae are not monophyletic.

With these sampling issues in mind, the goals of this study have been to: (1) increase the taxon sample size of *Inocybe* and outgroups; (2) increase nucleotide sample size by incorporating a third locus, the variable 6–7 regions of RPB2; (3) compare the utility of RPB1, RPB2, and nLSU for lower-level phylogenetic studies of mushroom-forming fungi; and (4) present a phylogenetic framework for the discussion of evolution of *Inocybe*.

2. Materials and methods

2.1. Fungal accessions and taxon sampling

Sequences of RPB1, RPB2, and nLSU were obtained for 84 accessions with the exception of *Inocybe* sp. BK080299-1, for which the RPB2 locus was not sequenced (Table 1). Sequences are deposited in GenBank. Sampling includes 67 species of *Inocybe* plus several duplicates from different geographic areas, and 13 outgroups across five families—Amanitaceae, Cortinariaceae, Crepidotaceae, Entolomataceae, and Strophariaceae as recognized by Singer (1986). Accessions were selected for sequencing based on the infrageneric taxa proposed in the different classifications of Kuyper (1986) and Singer (1986), both of which represent modifications of a classification presented by Kühner (1933, 1980) and Kühner and Romagnesi (1953). To determine the sister group to *Inocybe*, exemplars from the above-mentioned families were sampled (see Table 1).

2.2. DNA extraction, PCR amplification, cloning, and sequencing

DNA was extracted from dried basidiomata as described by Matheny et al. (2002). PCR amplification and direct sequencing of RPB1 A to C conserved domains (about 1400 bp) and the 5′ end of nLSU (about 1400 bp) were described in Matheny et al. (2002). Primers gRPB1 A-for and fRPB1 C-rev were used for PCR with aRPB1 B-rev as an additional sequencing primer. nLSU products were amplified with 5.8SR and LR7 (Vilgalys and Hester, 1990) with LR0R, LR3R, LR16, and LR5 used as sequencing primers. The region between conserved domains 6 and 7 of RPB2 (Denton et al., 1998; Liu et al., 1999) was amplified and sequenced using degenerate basidiomycete specific primers bRPB2-6F and bRPB2-7.1R (Fig. 1). In a few cases, the fungal specific primer fRPB2-5F was paired with bRPB2-7R in order to obtain sequences of regions 6–7 when the former combination was unsuccessful. In such cases, bRPB2-6F was used as a nested sequencing primer. The *Inocybe* specific primer pair A-for-Ino and C-rev-Ino was used in a few instances to amplify and sequence regions of RPB1 A–C. These primer sequences are: A-for-Ino 5′-GTCCGGGWCATTTTGGTC-3′; C-rev-Ino 5′-TTGTCCATGTANGTRGCRACA-3′.

About 15 of the approximately 250 sequences generated for this study represent clones. Cloning was done when amplified products were either faint or displayed multiple bands when using the degenerate primers above or direct sequence quality was poor. PCR products were inserted into a pCR 2.1-TOPO plasmid vector (version P) and cloned using TOPO TA Cloning and TOPO One Shot kits (Invitrogen, Carlsbad, CA, USA). Colonies were screened for the presence of the desired products using primers M13F and M13R (sequences of which are available in the TOPO TA Cloning instruction manual) under the following PCR conditions: (1) 93.0 °C for 2:00 min; (2) 93.0 °C for 1:00 min; (3) annealing at 50.0 or 53.0 ° for 1:00 min; (4) extension at 72.0 °C for 1:00 min + 02 s/cyc or 1:30 min + 01 s/cyc; (5) 34 times to 2; and (6) a final extension of 72.0 °C for 10:00 min. Appropriate sized products were then sequenced.

Table 1
DNA sequences used in the phylogenetic analyses, their geographic origin, collection number, herbarium, and GenBank accession numbers

Species	Geographic origin[a]	Collection No. and herbarium[b]	Accession No. RPB1 A-C	Accession No. RPB2 6–7	Accession No. nLSU
AMANITACEAE					
Amanita phalloides (Vail.: Fr.) Link	Washington, USA	Ben Woo Oct 1986 (WTU)	AY485639	AY485609	AY380359
CORTINARIACEAE					
Cortinarius aureifolius Peck	New York, USA	Bessette 10705 (NYS)	AY333304	AY333319	AY380360
Cortinarius fibrillosus Cleland	Western Australia, AU	E6584 (CSIRO-PERTH)	AY333306	AY333317	AY380361
Galerina semilanceata (Peck) A. H. Sm. and Singer	Washington, USA	PBM 1398 (WTU)	AF389531	AY337357	AY038309
Gymnopilus sapineus (Fr.: Fr.) Maire	Wyoming, USA	PBM 1541 (WTU)	AY351789	AY337358	AY380362
Hebeloma olympianum A. H. Sm., Evenson, and Mitchell	Washington, USA	BK 211198-20 (UTC)	AF389532	AY337360	AY038310
Inocybe abietis Kühner	Washington, USA	PBM 1402 (WTU)	AF389533	AY337360	AY038311
Inocybe actinospora Matheny and Danielle ined., isotype	Argentina	D25 (WTU)	AY351790	AY337361	AY380363
Inocybe adaequata (Britz.) Sacc.	Finland	JV 16501F (WTU)	AY351791	AY333771	AY380364
Inocybe agardhii (Lund) P. D. Orton	Finland 1	JV 7485F (WTU)	AY351792	AY333772	AY380365
Inocybe agardhii	Finland 2	JV 13740 (WTU)	AY351830 AY351831	AY337362	AY380366
Inocybe agglutinata Peck	Washington, USA	PBM 1352 (WTU)	AF389534	AY509113	AY038312
Inocybe alabamensis Kauffman	Texas, USA	PBM 1883 (WTU)	AY536282	AY536281	AY536280
Inocybe armeniaca Huijsman	Washington, USA	PBM 1228 (WTU)	AY351793	AY337363	AY380367
Inocybe ayangannae Matheny, Aime, and Henkel, isotype	Guyana	MCA 1232 (WTU)	AY239028	AY337364	AY239018
Inocybe calamistrata (Fr.: Fr.) Gillet	Washington, USA	PBM 2351 (WTU)	AY351794	AY333764	AY380368
Inocybe calamistratoides Horak	New Zealand	ZT 96/30 (ZT)	AY351795	AY333765	AY380369
Inocybe calospora Quél.	Sweden	JFA 12539 (WTU)	AF389535	AY337365	AY038313
Inocybe candidipes Kropp and Matheny, Paratype	Arizona, USA	BK 240799-7 (UTC)	AY239029	AY337366	AY239019
Inocybe cerasphora Singer	Chile	BSI 01/184 (WTU)	AY351796	AY337367	AY380370
Inocybe chelanensis Stuntz	California, USA	PBM 491 (WTU)	AY239030	AY337368	AY239020
Inocybe chelanensis	Washington, USA	PBM 2314 (WTU)	AY239031	AY337369	AY239021
Inocybe corydalina Quél.	Belgium	TURA 6488 (WTU)	AF389536	AY337370	AY038314
Inocybe curvipes P. Karst.	Washington, USA	PBM 2401 (WTU)	AY239032	AY337414	AY239022
Inocybe dolichocystis Matheny and Trappe ined., isotype	New South Wales, AU	Trappe 24844 (WTU)	AY351797	AY337371	AY380371
Inocybe dulcamara (Pers.) P. Kumm.	Washington, USA 1	BK 030699-2 (UTC)	AF389537	AY337372	AY038315
Inocybe dulcamara	Washington, USA 2	PBM2296 (WTU)	AY351798	AY337373	AY380372
Inocybe dulcamara	New Mexico, USA	ST 9923301 (WTU)	AY351799	AY388644	AY380373
Inocybe fastigiella Atk.	Virginia, USA	JRH 408 (WTU)	AY351800	AY333770	AY380374
Inocybe flavicothurnata Stuntz ex Matheny ined., paratype	Washington	PBM 1615 (WTU)	AF389549	AY337374	AY038327
Inocybe flocculosa (Berk.) Sacc.	Norway	PBM 2392 (WTU)	AY351801	AY337375	AY380375
Inocybe fuscodisca (Peck) Massee	Washington, USA	PBM 1950 (WTU)	AY351802	AY337376	AY380376
Inocybe geophylla (Fr.: Fr.) P. Kumm.	Finland	JV6374 (WTU)	AY351803	AY333777	AY380377
Inocybe glabrodisca P. D. Orton	Washington, USA	PBM 2109 (WTU)	AY239033	AY337377	AY239023
Inocybe godeyi Gillet	Italy	JV14914F (WTU)	AF389538	AY337378	AY038316
Inocybe griseolilacina J. Lange	Washington, USA	PBM 2241 (WTU)	AY351832 AY351833	AY337379	AY380378
Inocybe heimii Bon	Italy	JV 14932F (WTU)	AY351804	AY337380	AY380379
Inocybe cf. *hirsuta* var. *maxima* A. H. Sm.	Washington, USA	PBM 1066 (WTU)	AF389539	AY333766	AY038317
Inocybe hystrix (Fr.) P. Karst.	Finland	RS 31493 (WTU)	AY351805	AY337381	AY380380
Inocybe jarrahae Matheny and Bougher ined., isotype	Western Australia, AU	PBM 2207 (WTU)	AY351806	AY337382	AY380381
Inocybe lacera (Fr.: Fr.) P. Kumm.	Washington, USA	PBM 1462 (WTU)	AF389540	AY337383	AY038318
Inocybe lanatodisca Kauffman	Colorado, USA	ST 9922901 (WTU)	AY351807	AY333769	AY380382
Inocybe lanuginosa (Bull.: Fr.) P. Kumm.	Washington, USA	PBM 956 (WTU)	AF389541	AY337384	AY038319
Inocybe leiocephala Stuntz	Finland	JV 9448 (WTU)	AY351808	AY337385	AY380383
Inocybe leptocystis Atk.	Finland	JV 10412 (WTU)	AY351813	AY337386	AY380384
Inocybe leptophylla Atk.	Utah, USA	BK 090797-19 (UTC)	AF389542	AY337387	AY038320
Inocybe lilacina (Boud.) Kauffman	Washington, USA	PBM 2039 (WTU)	AF390020 AY351835 AY351834	AY337388	AY380385

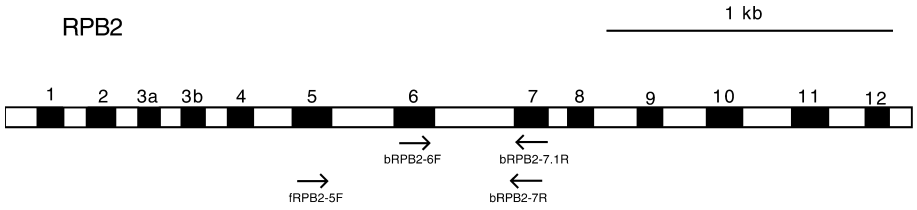
Table 1 (*continued*)

Species	Geographic origin[a]	Collection No. and herbarium[b]	Accession No. RPB1 A-C	Accession No. RPB2 6–7	Accession No. nLSU
Inocybe lilacinosquamosa Matheny, Aime, and Henkel, paratype	Guyana	MCA 1464 (BRG)	AY351836 AY351837	AY337389	AY380386
Inocybe cf. *maculata* Boud.	Washington, USA	PBM 525 (WTU)	AF389543	AY333775	AY038321
Inocybe mixtilis (Britz.) Sacc.	Washington, USA	PBM 1315 (WTU)	AY351814	AY337395	AY380387
Inocybe napipes J. Lange	Norway	PBM 2376 (WTU)	AY239034	AY337390	AY239024
Inocybe nothopes Matheny and Trappe, ined.	New South Wales, AU	Trappe 25060 (WTU)	AY351815	AY337391	AY380388
Inocybe praetervisa Quél.	Washington, USA	PBM 1021 (WTU)	AF389544	AY337392	AY038322
Inocybe pseudocystis Matheny and Trappe, ined.	New South Wales, AU	Trappe 24874 (WTU)	AY351809	AY337393	AY380389
Inocybe pudica Kühner	Washington, USA	PBM 1373 (WTU)	AF389545	AY337394	AY038323
Inocybe pusio P. Karsten	Washington, USA	PBM 2297 (WTU)	AY351810	AY337396	AY388643
Inocybe queletii Maire and Konrad	Washington, USA	PBM 935 (WTU)	AY351811	AY337397	AY380390
Inocybe relicina (Fr.) Ricken	Finland	JV 10258 (WTU)	AF389546	AY333778	AY038324
Inocybe serotina Peck	Ontario, CANADA	NI 210995 (WTU)	AY351812	AY337398	AY380391
Inocybe serpentinocystis Matheny and Trappe, ined., isotype	New South Wales, AU	Trappe 25080 (WTU)	AF389547	AY333773	AY038325
Inocybe cf. *serrata* Cleland	New South Wales, AU	Trappe 25079 (WTU)	AY351816	AY337399	AY380392
Inocybe sierraensis Kropp and Matheny, Holotype	California, USA	DED 6101 (SFSU)	AY239035	AY337400	AY239025
Inocybe sindonia (Fr.) P. Karsten	Washington, USA	PBM 2048 (WTU)	AF390021 AY351838 AY351839	AY337401	AY380393
Inocybe sp. BK 080299-1	Argentina	BK 080299-1 (UTC)	AF389548	—	AY038326
Inocybe sp. MCA 1882	Guyana	MCA 1882 (VPI)	AY509116	AY509114	AY509115
Inocybe sp. PBM 2355	Norway	PBM 2355 (WTU)	AY351826	AY333768	AY380402
Inocybe sp. PBM 2397	Norway	PBM 2397 (WTU)	AY351817	AY337402	AY380394
Inocybe sp. sequestrate H7344	Western Australia, AU	H7344 (WTU)	AY351818 AY351819	AY333774	AY380395
Inocybe stellatospora (Peck) Massee	Washington, USA	PBM 963 (WTU)	AF389550	AY337403	AY038328
Inocybe subflavospora Matheny and Bougher, ined., holotype	Western Australia, AU	E5880 (CSIRO-PERTH)	AY351820 AY351821	AY337404	AY380396
Inocybe subochracea (Peck) Peck	New Hampshire, USA	PBM 1143 (WTU)	AY351822	AY337405	AY380397
Inocybe subtilisior Matheny and Bougher, ined., isotype	Western Australia, AU	OKM 24631 (WTU)	AY351823	AY337406	AY380398
Inocybe tahquamenonensis Stuntz	New Hampshire, USA	PBM 1142 (WTU)	AY351824	AY337407	AY380399
Inocybe tanyosporota Stuntz ex Matheny, ined., paratype	Oregon, USA	BK 060697-24 (UTC)	AY351825	AY337408	AY380400
Inocybe terrigena (Fr.) Kuyper	Finland	JV 16431(WTU)	AY333301	AY333309	AY380401
Inocybe unicolor Peck	Missouri, USA	PBM 1481 (WTU)	AY351826	AY337409	AY380403
Inocybe violaceocaulis Matheny and Bougher, ined., paratype	Western Australia, AU	PBM 2198 (WTU)	AY351828	AY337410	AY380404
Naucoria escharoides (Fr.: Fr.) P. Kumm.	Washington, USA	PBM 1719 (WTU)	AY351840 AY351841	AY337411	AY380405
Phaeocollybia festiva (Fr.) Heim	Norway	PBM 2366 (WTU)	AY509117	AY509118	AY509119
CREPIDOTACEAE					
Crepidotus cf. *applanatus* (Pers.) P. Kumm.	Washington, USA	PBM 717 (WTU)	AY333303	AY333311	AY380406
ENTOLOMATACEAE					
Rhodocybe aureicystidiata Lennox ex Baroni	Washington, USA	PBM 1902 (WTU)	AY351842 AY351843	AY337412	AY380407
STROPHARIACEAE					
Flammulaster sp. PBM 1871	Washington, USA	PBM 1871 (WTU)	AY333308	AY333315	AY380408
Hypholoma fasciculare (Huds.: Fr.) P. Kumm.	Washington, USA	PBM 1844 (WTU)	AY351829	AY337413	AY380409
Phaeomarasmius proximans (A. H. Sm. and Hesler) Sing.	Vermont, USA	PBM 1936 (WTU)	AY333307	AY333314	AY380410

[a] If a state or country is represented by more than one collection (OTU) of the same species, it is distinguished by consecutive numbers.
[b] Herbarium abbreviations follow Holmgren et al. (1990).

2.3. Spliceosomal intron boundaries

Intron boundaries for RPB1 were determined by cDNA sequencing of two *Inocybe* species as outlined by Matheny et al. (2002). Only one intron within the 6–7 regions of RPB2 was inferred by insertion between conserved amino acid motifs and the canonical guanine–thymine (GT-) and adenosine–guanine (-AG) splice sites.



Basidiomycete and fungal specific RPB2 primers

bRPB2-6F	(WGMVCPA)	5'-TGG GGY ATG GTN TGY CCY GC-3'
bRPB2-7.1R	(AMGKQAMG)	5'-CC CAT RGC YTG YTT MCC CAT DGC-3'
bRPB2-7R	(PFPDHNQS)	5'-GA YTG RTT RTG RTC RGG GAA VGG-3'
fRPB2-5F	(DDRDHFG)	5'-GAY GAY MGW GAT CAY TTY GG-3'

Fig. 1. RPB2 primer sequences used in this study. Primer arrows are not drawn to scale.

2.4. Alignments

Alignments were done using Clustal X (Thompson et al., 1997) and manually adjusted using MacClade 4.0 (Maddison and Maddison, 2000). Exon regions of RPB1 and RPB2 were aligned easily. Other positions deemed ambiguous to align were excluded. Among intron regions, only intron 2 was included due to its large size (about 510–550 bp) and strongly conserved state at the 5′ end. The shorter introns of RPB1 (introns 1, 3, and 4) and a single but similarly sized intron of RPB2 (about 45–55 bp) were not obtained in full across all taxa and proved difficult to align across the breadth of taxa included in the study. These shorter introns were thus omitted prior to the final alignment. The alignment is available online at TreeBASE at <www.treebase.org> as matrix Accession No. M1793 (S1052).

2.5. Phylogenetic analyses

RPB1, RPB2, and nLSU sequences for 84 accessions were concatenated as a single file and partitioned for analysis in PAUP* 4.0 beta 10 (Swofford, 2003) and MrBayes 3.0 (Ronquist and Huelsenbeck, 2003). Maximum parsimony (MP) analyses entailed heuristic searches with a random addition sequence of 100 replicates, using the tree-bisection-reconnection (TBR) branch-swapping algorithm, holding one tree at each step during stepwise addition, and "MulTrees" option in effect. MaxTrees was set to 1000. Gaps were scored as missing. One thousand non-parametric bootstrap replicates (Felsenstein, 1985; Hillis and Bull, 1993) were performed under the MP criterion and involved various search strategies for most parsimonious trees. When the number of optimal trees was low, the same heuristic strategy was used as above except for fewer (10) random addition sequences; when the number of optimal trees was high, the bootstrap strategy was modified by turning "MulTrees" off (DeBry and Olmstead, 2000). *Amanita phalloides* was used to root all trees based on Moncalvo et al. (2002).

Maximum likelihood (ML) best-fit models were estimated using MODELTEST 3.0 (Posada and Crandall, 1998, 2001) to provide independent substitution models by locus, intron, and codon position for a heterogeneous Bayesian analysis in MrBayes 3.0. Models were estimated separately for partitions of nLSU, intron 2, RPB1 1st, 2nd, and 3rd codon positions, and RPB2 1st, 2nd, and 3rd codon positions. Bayesian analyses were conducted using six Metropolis-coupled Markov chain Monte Carlo (MCMC) chains with the temperature set to 0.2. Resulting likelihood scores were plotted against the number of generations to help determine the number of generations to run and burnin values, as well as to observe the frequency of state swapping among the six chains.

Three measures were used to compare trees derived from different data sets: non-parametric bootstrap support greater than 50, 70, and 90% (Felsenstein, 1985; Hillis and Bull, 1993), Bayesian posterior probabilities greater than 95% (Huelsenbeck et al., 2002), and the amount of resolution a tree contains. Resolution is defined following Colless (1980) and Thorley and Wilkinson (2000), in which the maximum number of internal branches of a consensus tree is divided by the size of the tree ($n-2$) when rooted. This procedure normalizes the measure between 0 and 1 (where 0 is an unresolved polytomy and 1 fully resolved) and allows a direct comparison despite differences in tree size.

The conditional data combination approach was invoked before proceeding to combine the three gene regions (Huelsenbeck et al., 1996). This method aims to combine data from different sources as long as a measure of partition heterogeneity is not detected. To assess heterogeneity between data sets, strongly supported clades were inspected for conflict with >70% bootstrap support (De Quiroz, 1993).

3. Results

3.1. Introns, pseudogenes, and paralogy

RPB2 sequences from conserved domains 6–7 reveal a single phase-zero intron (Endo et al., 2002) that ranges from 43 to 61 bp in length and is conserved by presence and position across all taxa. This intron corresponds to *Arabidopsis* intron 16 (Liu et al., 1999). The RPB1 A–C region in *Inocybe* and related agaric fungi includes four spliceosomal introns (Matheny et al., 2002). Of the four introns, only intron 2 deviates from a phase-zero insertion, with a phase-one insertion between a first and second codon position. Intron 1 exhibits length variation ranging from 45 to 82 bp. Intron 2 ranges from 430 to 573 bp. Intron 3 ranges from 46 to 57 bp, and intron 4 ranges from 46 to 69 bp in length.

A pseudogene of RPB1 was identified in the species *I. fastigiella*. Sequences of multiple clones confirmed 50 substitutions in protein-coding regions. Of these substitutions 10 occur at first positions, 7 at second positions, and 33 at third positions. Twenty-five substitutions represent CT transitions, 14 AG transitions, and 11 transversions. A single base pair insertion interrupts the reading frame in the exon just downstream from intron 2. Several small in-frame deletions occur upstream as well. In addition, a major portion of the intron 2 sequence is deleted. Comparatively, the intact copy contains 512 positions for intron 2 versus 430 for the pseudogene.

A duplication of RBP1, but not of RPB2, was found in the outgroup taxon, *Hypholoma fasciculare*. The two copies differ at 15 of the 169 (8.9%) amino acid positions sequenced. A phylogenetic analysis (data not shown) supports the two sequences in a clade, however, with 100% bootstrap support exclusive of other genera sampled. The proportional or "*p*" distance between the two copies is 0.04, four times as great compared, for example, to the divergence between two RPB1 alleles of *I. "subflavospora"* ("*p*" = 0.01).

3.2. Polymorphic sites

Polymorphic sites of *Inocybe* and related groups numbered mostly from zero to nine as inferred by direct and cloned sequences. RPB2 fragments of *I. fastigiella* and *Crepidotus versutus*, however, exhibited 16 and 26 polymorphic sites, respectively. Most polymorphisms were observed within spliceosomal introns or at synonymous sites within codons. These differences probably represent alleles, which have also been reported in the Chytridiomycota (Liu, Hodson, and Hall, unpublished). The presence of frameshifts and deletions in coding and intron regions of an additional RPB1 fragment of *I. fastigiella* suggests it is a pseudogene. Only the intact copy is included in this study. Introns of *I.* "*subflavospora*," *I. unicolor*, and *Inocybe* sp. sequestrate H7344 were found to be polymorphic in length due to small insertions or deletions.

The length of introns does not vary within dikaryotic individuals except for few exceptions. For example, *I. unicolor* contains two alleles of intron 4 inferred by cloning experiments. One version of the intron exhibits seven CA repeats, whereas the other version has eight CA repeats, thus representing a microsatellite marker in this species. One putative monokaryon of *I.* "*subflavospora*" is inferred to have an allele of intron 1 that contains a 3 bp deletion.

3.3. Likelihood models

A general time reversible (GTR) model (Felsenstein, 2004), including a proportion of invariable sites and a gamma distribution parameter, was selected as the best-fit model for the following partitions: nLSU, RPB2 2nd positions and 3rd positions, and RPB1 1st, 2nd, and 3rd positions. A GTR model plus a gamma distribution was best-fit for the RPB2 1st position partition. A HKY model (Hasegawa et al., 1985), including a proportion of invariable sites and a gamma distribution parameter, was selected for the intron 2 partition.

3.4. Phylogenetic inference of nLSU

The nLSU data set is composed of 1355 sites, of which 72 were excluded prior to analysis. The first 47 positions in the data set represent the end of the ITS2 region and were excluded due to lack of taxonomic overlap. Twenty-five positions were too ambiguous to align elsewhere and were also excluded. In the Bayesian analysis two million generations were run sampling trees every 100 generations. However, the "cold" chain failed to swap states after stationarity. This could indicate the process was fixed at the top of a suboptimal hill of trees. A total of 12,247 trees were used to build a 50% majority-rule consensus tree, the posterior probabilities of which are shown in Fig. 2. The MP analysis (trees not shown) produced 209 MP trees (CI = 0.313), for which there are 273 parsimony-informative sites. One hundred six sites are variable but parsimony-uniformative.

Inocybe is supported as monophyletic with 98% posterior probability but below 70% bootstrap support. The sister group to *Inocybe* is not resolved by the Bayesian method and is not strongly supported by MP. Subgenus *Inocybe* receives 91% posterior probability but is poorly supported as paraphyletic due to the inclusion of *I.* cf. *maculata* of subgenus *Inosperma*. All species that have pleurocystidia (sterile terminal cells that occur on the sides of lamellae) occur in this clade regardless of their basidiospore morphology. Taxa with gibbous spores (terminals in bold text) fail to form a monophyletic group. Few branches within the clade are strongly

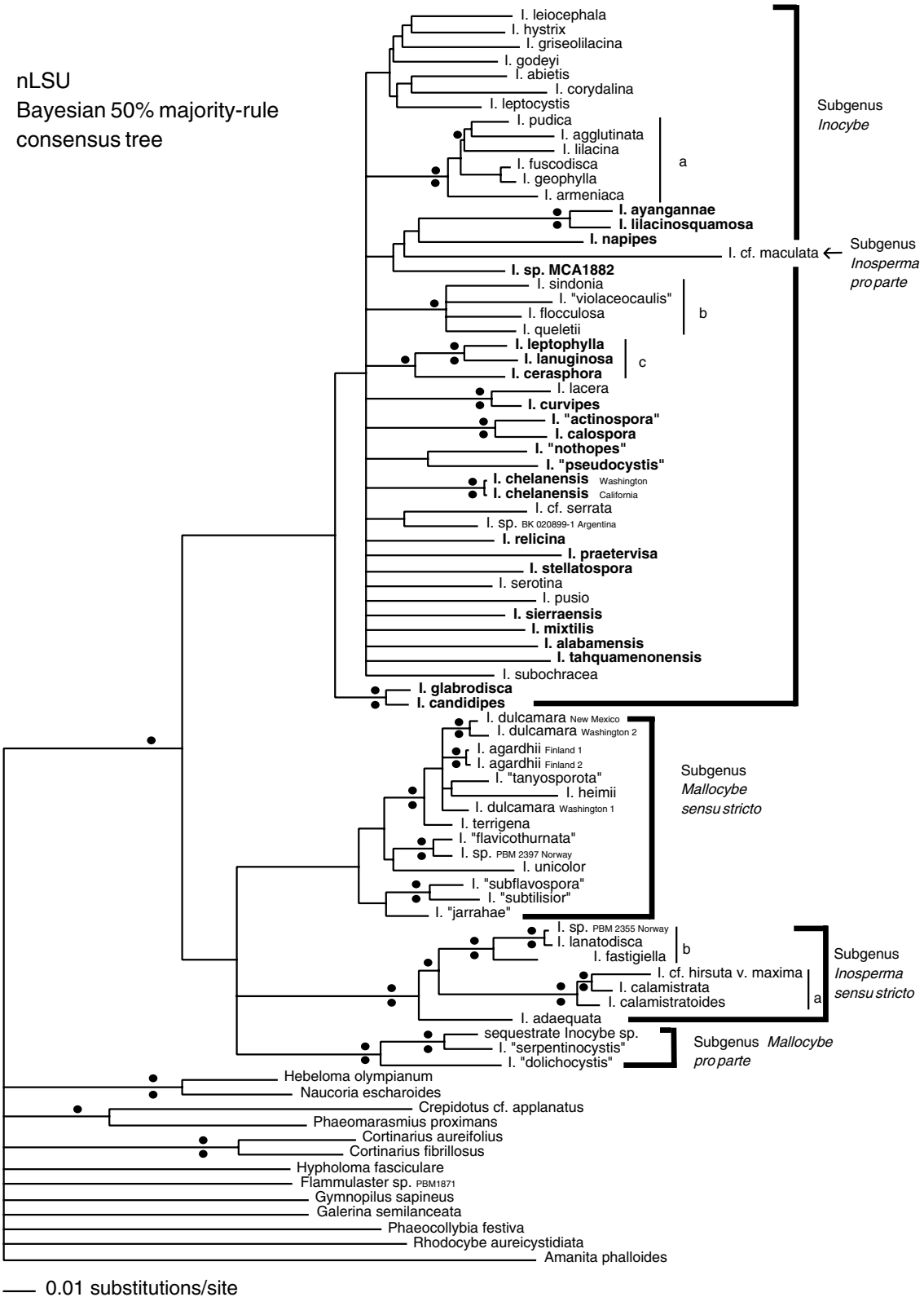


Fig. 2. The Bayesian 50% majority-rule consensus tree inferred from nLSU. Posterior probabilities >95% and bootstrap values >70% are indicated as black filled circles above and below branches, respectively. Taxa with gibbous spores are in bold text. Subgeneric names are from Kuyper (1986).

supported. Notable exceptions include: clade "a" or section "*Geophyllinae*" (99% posterior probability/73% bootstrap) (Bresinsky and Besl, 1990), a group of smooth-spored, cortinate species; clade "b," a second smooth-spored group of four species with a cortina (98% posterior probability); and clade "c," *I. lanuginosa* and

allies (100% posterior probability only). Several additional well-supported clades are recovered but are limited to pairs of terminal taxa.

The remainder of the *Inocybe* topology is composed of three additional major clades composed of species that lack pleurocystidia: subgenus *Mallocybe sensu stricto*, subgenus *Inosperma sensu stricto*, and subgenus *Mallocybe pro parte*. High posterior probabilities and bootstrap values support the monophyly of subgenus *Inosperma sensu stricto*, as well as branches in this clade. Two internal groups are recognized as clades "a" and "b." However, three taxa with necropigmented basidia from Australia (subgenus *Mallocybe pro parte*) fail to form a monophyletic group with three other Australian species and north temperate representatives of subgenus *Mallocybe*. Subgenus *Mallocybe pro parte* also includes an undescribed truffle-like or sequestrate species (H7344 from Western Australia) with 100% posterior probability and 100% bootstrap support.

The MP analysis (trees not shown) differs in a few respects from the Bayesian tree. For instance, *I.* cf. *maculata* is suggested as sister to subgenus *Inocybe* (<50% bootstrap), thereby rendering the latter monophyletic instead of paraphyletic as indicated in Fig. 2. Also, species with necropigmented basidia (subgenus *Mallocybe*) are suggested (<50% bootstrap) as paraphyletic giving rise to subgenus *Mallocybe pro parte* and *Inosperma sensu stricto*, instead of unresolved by the Bayesian analysis.

3.5. Phylogenetic inference of RPB1

The RPB1 data set contains a total of 1360 positions, of which 1053 were included after alignment. Intron 2 sequences are missing entirely for *I. griseolilacina*, *I. dulcamara* BK 030699-2, *I.* "*dolichocystis*," *Naucoria escharoides*, *Flammulaster* sp. PBM 1871, and *Rhodocybe aureicystidiata*. The intron 2 sequence is partially missing for *I. lilacinosquamosa*. Three hundred seven sites are excluded from the analysis. Thirty seven of the excluded sites occur at the 5′ end of the amplified RPB1 products, for which taxonomic representation is uneven. The remaining 270 excluded sites are distributed towards the 3′ end of intron 2, a region too difficult to align given the broad array of included taxa. The Bayesian process revealed a frequent mixing of chains. Thus, one million generations were run sampling trees every 100 generations. A total of 5092 trees was used to construct a 50% majority-rule consensus tree. Fig. 3 illustrates the Bayesian tree including branch lengths. The MP analysis (trees not shown) produced 80 MP trees (CI = 0.249), for which 454 sites are parsimony-informative. One hundred twenty-four sites are variable but parsimony-uniformative.

RPB1 data provide the best resolved phylogeny and the highest number of strongly supported branches among the three loci used in this study (Table 2). *Inocybe* is supported as monophyletic with high posterior probability (96%) and bootstrap support (88%). *Crepidotus* is suggested as the sister group to *Inocybe* but with poor support. Subgenus *Inocybe*, which includes all taxa with pleurocystidia, forms a strongly supported monophyletic group (96% posterior probability/100% bootstrap). Taxa with gibbous spores (in bold text) do not form a monophyletic group. In contrast to the nLSU data (Fig. 2), *I.* cf. *maculata* is sister with high support to subgenus *Inocybe* (96% posterior probability/73% bootstrap). Like the nLSU data, clade 2 receives strong support (100% posterior probability/92% bootstrap). However, the smooth-spored clade 3, including *I. pusio*, gets strong support (100% posterior probability/93% bootstrap) and is sister to clade 2 (100% posterior probability/85% bootstrap). Together, clades 2 and 3, composed entirely of smooth-spored, cortinate species, receive strong support and are labeled as clade 1 (Fig. 3). A fourth smooth-spored group, clade 4, is identified with 98% posterior probability but less than 70% bootstrap. High posterior probabilities and bootstrap also support two gibbous-spored, cortinate groups: clades 6 and 7. A high posterior probability unites two spinose-spored species and the stellate-spored *I. praetervisa* into clade 5. The three members of clade 5 lack a cortina.

Elsewhere on the topology, both subgenus *Mallocybe sensu stricto* and *pro parte* clades are strongly united as an inclusive clade by posterior probabilities (100%) but with low bootstrap support (<70%). Most branches within subgenus *Mallocybe sensu stricto* are strongly supported by both posterior probabilities and bootstrapping, including the north temperate *I. dulcamara* group (clade 1). A cluster of three undescribed Australian species (clade 2) is supported as monophyletic.

Similarly, strong support values occur on all branches within subgenus *Inosperma sensu stricto* with the exception of *I. adaequata*. Bayesian posterior probabilities strongly support (98%) the sister position of *I. adaequata* (a species with reddening flesh that lacks muscarine) with additional reddening species of the *I. calamistrata* group, clade 1. These species are also thought to lack muscarine (Kuyper, 1986). However, MP bootstrapping places *I. adaequata* sister to *I. fastigiella* and allies (clade 2). Among the outgroups, *Galerina semilanceata*, *Phaecollybia festiva*, and *Gymnopilus sapineus* are supported as monophyletic with 95% posterior probability but less than 70% bootstrap. The MP analysis (tree not shown) is very similar in topology and support values as the Bayesian analysis with the exception of the position of *I. adaequata* reported above.

3.6. Phylogenetic inference of RPB2

The RPB2 data set includes 83 accessions due to the inability to obtain a sequence for *Inocybe* sp. BK080299-1 from Argentina. RPB2 contains 705 included charac-

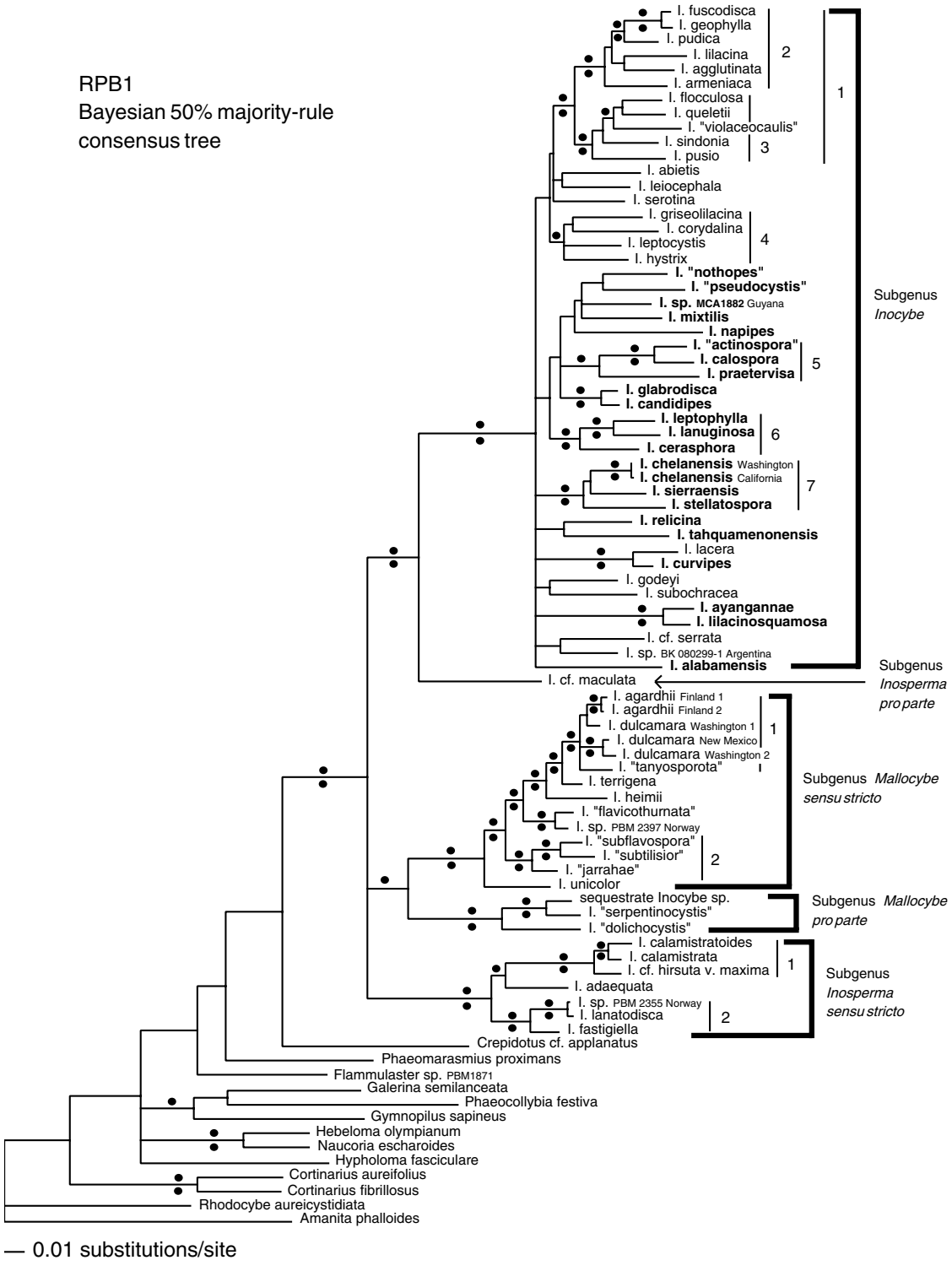


Fig. 3. The Bayesian 50% majority-rule consensus tree inferred from RPB1. Posterior probabilities >95% and bootstrap values >70% are indicated as black filled circles above and below branches, respectively. Taxa with gibbous spores are in bold text. Subgeneric names are from Kuyper (1986).

ters. Fourteen sites at the 5′ end of the amplified RPB2 products, for which taxonomic representation was uneven, were excluded. Similar to the RPB1 data set, the Bayesian process showed frequent swapping of states between chains. One million generations were run, and trees were sampled every 100 generations. A total of 6575 trees were used to reconstuct a 50% majority-rule consensus tree. Fig. 4 illustrates the Bayesian tree including branch lengths. The MP analysis (trees not shown) produced 48 MP trees (CI = 0.212), for which 326 sites

Table 2
Resolution and branch support values under maximum parsimony (MP) and Bayesian criteria for single locus and combined analyses

Data set	Resolution[a] strict consensus MP tree	Resolution Bayesian 50% majority-rule consensus tree	No. of clades >95% posterior probability	No. of clades >50% bootstrap	No. of clades >70% bootstrap	No. of clades >90% bootstrap
nLSU	0.90	0.59	27	30	21	13
RPB1	0.91	0.78	42	42	37	28
RPB2	0.86	0.65	36	45	35	26
Combined	0.91	0.87	46	51	42	36

[a] To calculate resolution divide the maximum number of internal branches of the consensus tree by the size of the tree ($n-2$) when rooted.

are parsimony-informative. Sixty-four variable sites are parsimony-uniformative.

Fig. 4 depicts a greater number of strongly supported clades than nLSU (Fig. 2). *Inocybe* receives a high posterior probability (98%) but less than 70% bootstrap for its monophyly including *Crepidotus* cf. *applanatus*. The strongly supported (100% posterior probability only) outgroup pair *Flammulaster* and *Phaeomarasmius* is poorly supported as sister to an inclusive clade of *Inocybe* and *Crepidotus*.

Subgenus *Inocybe* is strongly supported by both Bayesian and MP analyses (98% posterior probability/70% bootstrap), similar to RPB1 (Fig. 3). All taxa sampled with pleurocystidia occur in this clade. However, like nLSU and RPB1, taxa with gibbous spores fail to form a monophyletic group. Strongly supported clades of Fig. 3 within subgenus *Inocybe* likewise receive strong support from RPB2 with the exception of smooth-spored clade 4 in Fig. 3. Additionally, a group of caulocystidiate species and the bulbous-stiped *I. napipes*, clade 8, is strongly supported by Bayesian analysis (98% posterior probability but less than 70% bootstrap).

The inclusive monophyly of taxa with necropigmented basidia of subgenus *Mallocybe sensu stricto* and subgenus *Mallocybe pro parte* is not supported in opposition to RPB1 results (Fig. 3). Both posterior probabilities and bootstrap values are high for branches within subgenus *Mallocybe sensu stricto*, as they are for RPB1. Similar high support values are obtained for branches in subgenus *Inosperma sensu stricto*. In this group clades 1 and 2 are recovered consistent with nLSU and RPB1 data. The position of *I.* cf. *maculata* is unresolved, though it is nested outside subgenus *Inocybe* contrary to nLSU data.

The MP topology is very similar to that generated by the Bayesian analysis with exception to the following branches: *Crepidotus* is resolved (<70% bootstrap) in an inclusive clade with *Phaeomarasmius* and *Flammulaster* that is sister to *Inocybe*. *Inocybe* cf. *maculata* is sister (<70% bootstrap) to the remainder of subgenus *Inosperma sensu stricto*.

3.7. Measures of heterogeneity among loci

One strongly supported (>70% MP bootstrap) conflicting clade is observed across the gene partitions of optimal MP bootstrap trees. The nLSU (Fig. 2) New Zealand sample *I. calamistratoides* (in subgenus *Inosperma sensu stricto*) is sister to a north temperate clade of *I. calamistrata* and *I.* cf. *hirsuta* var. *maxima* with 77% bootstrap support (DNA was extracted a second time for the *I.* cf. *hirsuta* var. *maxima* accession and its nLSU sequence confirmed). In contrast, both RPB1 (Fig. 3) and RPB2 (Fig. 4) place *I. calamistratoides* sister to *I. calamistrata* with 98 and 80% bootstrap support, respectively. Posterior probabilities >95% also support these gene histories. However, extended nLSU taxon sampling within this clade reduces this strongly supported conflict below 70% bootstrap (Matheny, unpublished). Because these species are considered very closely related (Horak, 1977; Smith, 1939) and do not impact the phylogeny of *Inocybe* at large, they were not excluded from the combined analysis.

3.8. Phylogenetic inference of combined nLSU, RPB1, and RPB2

The combination of nLSU, RPB1, and RPB2 sequences produces the best resolved phylogeny and the highest number of strongly supported clades (Fig. 5; Table 2). Forty-six clades receive more than 95% Bayesian posterior probabilities and 42 receive more than 70% bootstrap support. The Bayesian 50% majority-rule consensus tree is shown. One million generations were run sampling trees every 100 generations. Swapping of chains occurred frequently, and 2016 trees were used to build the 50% majority-rule consensus tree. MP analysis (trees not shown) produced 26 MP trees (CI = 0.243). This combined data set comprises 3041 included characters, of which 1053 are parsimony-informative. Two hundred ninety-four characters are variable but parsimony-uniformative. Clade names of major monophyletic groups, which are not italicized to distinguish them from taxonomic names, are labeled in Fig. 5.

A 95% posterior probability (but below 70% bootstrap) unites all brown-spored taxa on the tree to the exclusion of *Rhodocybe*, a pink-spored group of the Entolomataceae. The pair, *Phaeomarasmius* and *Flammulaster*, a clade supported by 100% posterior probability but <70% bootstrap, is sister to an inclusive clade of *Crepidotus* (Crepidotaceae) and *Inocybe* but with poor

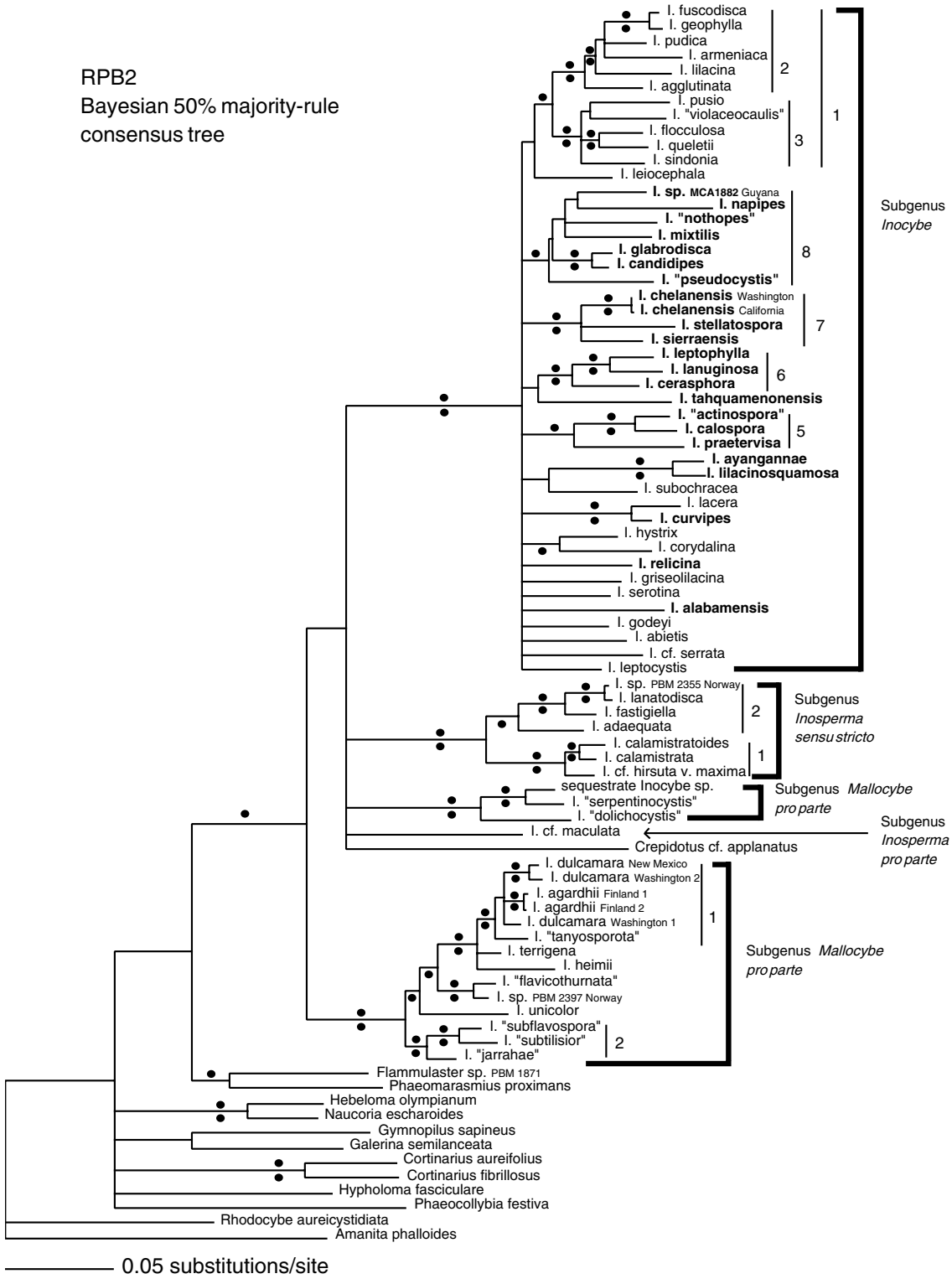


Fig. 4. The Bayesian 50% majority-rule consensus tree inferred from RPB2. Posterior probabilities >95% and bootstrap values >70% are indicated as black filled circles above and below branches, respectively. Taxa with gibbous spores are in bold text. Subgeneric names are from Kuyper (1986).

support. Both *Phaeomarasmius* and *Flammulaster* have been classified in the Strophariaceae (Moser, 1983). *Crepidotus* is sister to *Inocybe* with 83% bootstrap support (higher than any single locus study) and 89% posterior probability. This result appears to support a sister-level relationship between the Crepidotaceae *sensu stricto* (Aime, 2001) and *Inocybe* (Cortinariaceae *pro parte*).

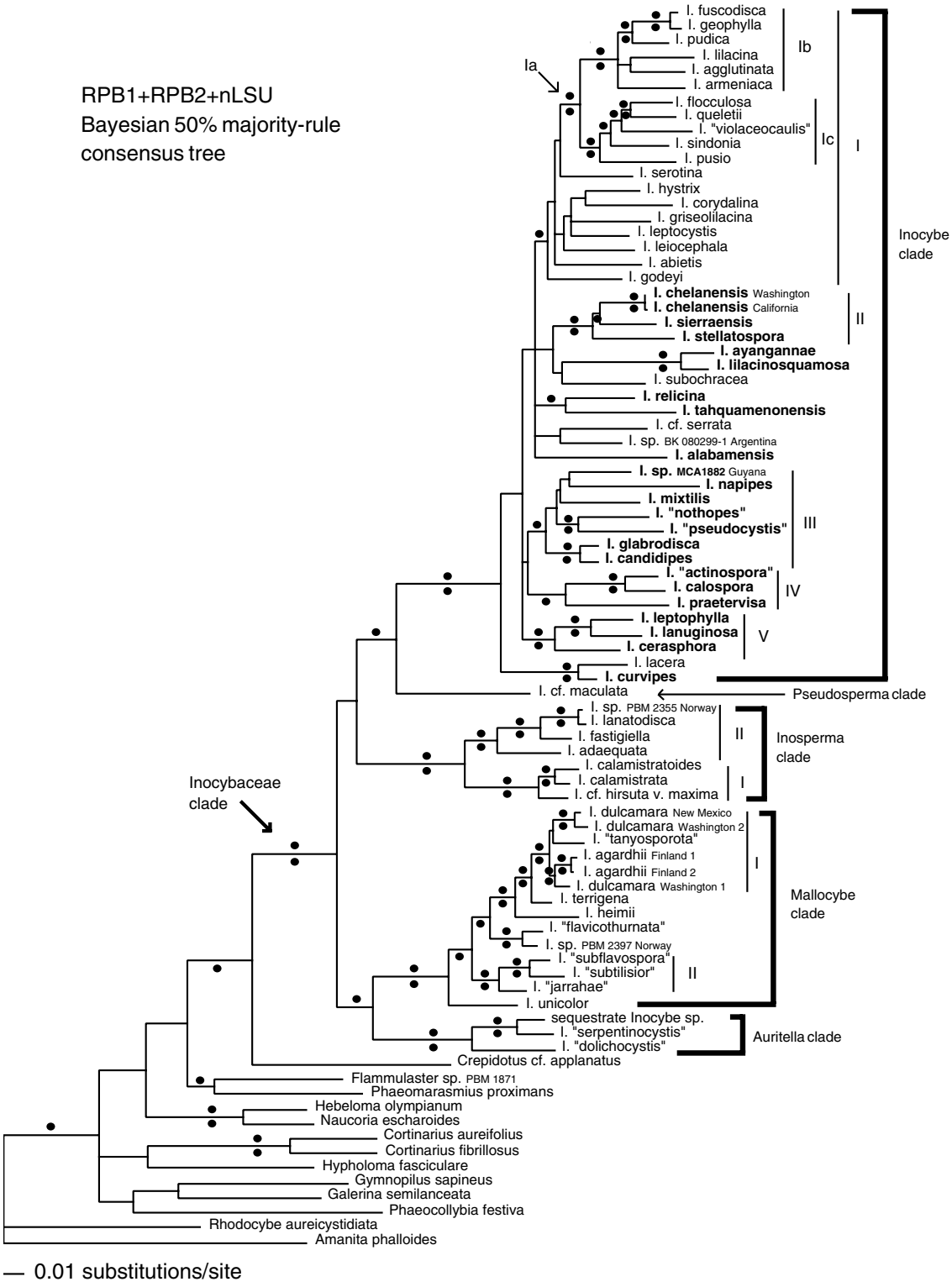


Fig. 5. The Bayesian 50% majority-rule consensus tree inferred from combined nLSU, RPB1, and RPB2. Posterior probabilities >95% and bootstrap values >70% are indicated as black filled circles above and below branches, respectively. Taxa with gibbous spores are in bold text. New clade names are presented.

Two taxa previously classified in *Inocybe*—*I. angustispora* (Bessette and Fatto, 1998) and *I. fibrillosa* (Grgurinovic, 1997)—actually represent species of *Cortinarius*, *C. aureifolius* and *C. fibrillosus*, respectively. Both species form strongly supported clades with the addition of *C. croceus* and a species of *Protoglossum* (Matheny

and Ammirati, 2003). Fig. 5 shows both *C. aureifolius* and *C. fibrillosus* as monophyletic with strong support (100% posterior probability/89% bootstrap) and unrelated to *Inocybe*.

Support values greater than 95% are achieved for the first time in support of the monophyly of *Inocybe*, here referred to as the Inocybaceae clade (Jülich, 1982). Within the Inocybaceae clade, five major clades are identified that receive more than 95% posterior probabilities and higher than 70% MP bootstrap. *Inocybe* cf. *maculata*, the Pseudosperma clade, is strongly supported as sister to the Inocybe clade by posterior probability only. The Inosperma clade is composed of two strongly supported groups. Clade I appears to conform to section *Cervicolores*, whereas clade II appears to represent section *Rimosae* (Kuyper, 1986). Both the Mallocybe and Auritella clades are united by a high posterior probability (but bootstrap less than 70%). All taxa within these two clades contain taxa with necropigmented basidia. One undescribed species is sequestrate (Matheny and Bougher, unpublished). Well-supported subclades are marked by Roman numerals and discussed in more detail below.

4. Discussion

4.1. Nucleotide sequences of nLSU, RPB1, and RPB2

About 3000 bp of nucleotide sequence data from three genes, including roughly 1750 bp of protein-coding loci, have been analyzed to infer the phylogeny of *Inocybe*. The 5′ end of nLSU-rDNA in mushroom and allied genera is characterized by regions of strongly conserved sequences interspersed with shorter regions prone to higher rates of substitution (Hopple and Vilgalys, 1999; Moncalvo, Drehmel, and Vilgalys (2000).). Lower values of the gamma distribution and higher proportion of invariable sites in nLSU indicate the nLSU locus exhibits more extreme rate heterogeneity than RPB1 and RPB2 (data not shown), and that large portions of this gene are more conserved than RPB1 and RPB2.

Exon-coding sequences of RPB1 and RPB2 can be aligned easily across representatives of at least five families of the Agaricales *sensu* Singer (1986). Amino acid indels are few and generally autapomorphic within the protein-coding regions. A homoplastic amino acid insertion is shared between outgroup genera *Galerina* and *Crepidotus*. Polymorphic sites in coding regions are generally low in number (zero to three) and occur at first or third codon silent positions. On rare occasions, however, RPB2 exhibited numerous (16) polymorphisms at synonymous sites in *Crepidotus versutus* (data not shown). Overall, the amount of divergence between alleles of RPB1 and RPB2 is routinely quite small overall (up to 9 bp). The only instance of a viable gene duplication occurs in an outgroup taxon, *Hypholoma fasciculare*. The RPB1 putative paralog has diverged up to 9% of its amino acid sequence. Both copies form a strongly supported monophyletic group (data not shown) that suggests the duplication is topologically local.

Several mono- and dinucleotide insertions characterize all members of the Auritella clade (Fig. 5) in generally conserved regions of nLSU. A mononucleotide insert also occurs in all members of the Inosperma and Pseudosperma clades (Fig. 5). *Inocybe lacera* and *I. curvipes* share a "CTT" insert that is synapomorphic for this clade.

Phylogenetic signal also stems from intervening sequences (introns) in RPB1 and RPB2. About 300 bp from the approximately 500–575 bp of intron 2 of RPB1 are alignable and are a rich source of parsimony-informative sites. Much of the 5′ region is remarkably conserved and may be functional though it is not expressed in cDNA (Matheny et al., 2002). Previous patterns of unexpected intron conservation have been reported (Mattick, 1994), however, regions of the 3′ end of the intron were too variable to align unambiguously across all taxa included in this study. Application of the entire sequence of intron 2 for phylogenetic purposes is probably more suitable for groups of closely related species. Although the short intron (ca. 50–60 bp) of the RPB2 region was sequenced for many taxa, alignment of the region was ambiguous across the taxonomic breadth in this study. However, this intron starts with "GC" for all members of the Inosperma clade. Overall, spliceosomal intron sequences of RPB1 and RPB2 will probably prove to be of more phylogenetic value among closely related species. More detailed studies in less inclusive clades should be able to utilize the entire sequences, thereby preserving more sequence data for analysis. These introns could provide useful population level markers because of the presence of polymorphic sites and indels that characterize alleles of several *Inocybe* species such as *I. unicolor*, *I.* "*subflavospora*," and *Crepidotus versutus*.

4.2. Phylogenetic inference of single gene trees is not as robust as combined data

The nLSU data set generates the least robust phylogenetic estimate of *Inocybe* and outgroup taxa of the three genes were investigated (Table 2). It contains the fewest moderately and strongly supported branches and the lowest resolution across the Bayesian tree (Table 2). These results caution against the sole reliance upon nLSU data at this taxonomic level for phylogenetic inference of mushrooms. Moreover, it is not possible to ascertain whether clades that are poorly supported on the nLSU estimate (Fig. 2) represent real relationships or not without the addition of more sequence data for those samples (Olmstead and Sweere, 1994). Thus, adding more nLSU taxa to the estimate is not the best strat-

egy to uncover the gross phylogeny of *Inocybe* (Rosenberg and Kumar, 2001). Rather, the addition of nLSU to more rapidly evolving loci like the variable regions of RPB1 and RPB2 generates a more robust phylogeny. Similar caveats apply to separate RPB1 and RPB2 analyses but with much less concern. Many branches, however, in the Inocybe clade remain poorly supported despite the combination of nearly 3000 bp of sequence data.

4.3. The Inocybe clade

This clade includes the type of *Inocybe*, *I. relicina* (Kuyper, 1986; Moser, 1978; Singer, 1986). Although many branches remain poorly supported, the combined analysis suggests 12 strongly supported clades that contain at least 3 species, more than any single locus analysis. Clade I (95% posterior probability/bootstrap below 70%) comprises 19 smooth-spored species with pleurocystidia, and several with lilac pigmentation. Not all smooth-spored species with pleurocystidia or all species with lilac pigmentation occur in clade I, however. Nevertheless, species with and without caulocystidiate stipes (e.g., *I. godeyi* and *I. griseolilacina*, respectively) are distributed in this clade. Clade Ia is composed of cortinate species only and receives 100% posterior probability and 98% bootstrap and contains many well-supported interior branches (greater than 95% posterior probabilities). Clade Ib is represented by members of the *I. geophylla* group or section "*Geophyllinae*" (Bresinsky and Besl, 1990), all of which possess elliptic spores with rounded apices. Clade Ic is composed of four north temperate species and the undescribed Australian *I.* "*violaceocaulis*". Clade II is composed of *I. stellatospora* (Matheny and Kropp, 2001) and members of the *I. chelanensis* group as reported by Kropp and Matheny (2004) using RPB1 and nLSU sequences only. The four species in clade II possess gibbous spores to some degree and a cortina. Clade III (95% posterior probability only) comprises six species with gibbous spores and caulocystidiate stipes (thus lacking a cortina) and the cortinate *I. napipes*. Species in clade III are widely distributed occurring in Guyana, Australia, Norway, and the United States. Two species with spinose spores (*I. calospora* and an ally) plus the stellate-spored *I. praetervisa* are monophyletic with 95% bootstrap only. Clade V is strongly supported by both Bayesian posterior probability and MP bootstrap and is composed of species of the *I. lanuginosa* group (Matheny and Kropp, 2001) and the *Nothofagus*-associate, *I. cerasphora* from Chile (Singer, 1953). Members of this group share a similar dark umbrinous coloration, a scaly pileus, presence of a cortina, and tendency to occur on rotten wood.

Several novel or noteworthy species pairs are strongly supported as well. *Inocybe relicina*, an apparent endemic to Fennoscandia (Moser, 1978) is sister to *I. tahquamenonensis*, an endemic to eastern North America (Matheny and Kropp, 2001) with 99% posterior probability. Both species share small gibbous spores that at times are cruciform in outline. *Inocybe ayangannae* and *I. lilacinosquamosa* are two gibbous-spored, cortinate species recently described from Guyana that are ectomycorrhizal with the caesalpinioid legume, *Dicymbe* (Matheny et al., 2003). This pair is monophyletic with 100% posterior probability and 100% bootstrap. Strong support values are also obtained for *I. lacera* and *I. curvipes*, previously reported by Kropp and Matheny (2004) using RPB1 and nLSU data. Phenotypic similarities between the two species include the darkening of the stipe base and mucronate pleurocystidia (Kuyper, 1986). The spores of *I. lacera* are at most minimally angular, whereas those of *I. curvipes* exhibit few to several low nodules.

It is clear that gibbous-spored taxa fail to form a monophyletic group (Kuyper, 1986; Matheny et al., 2002). Results here affirm these earlier studies that point to the polyphyly of the genus *Astrosporina*, a group of nodulose-spored species of *Inocybe* only. However, a clade of at least 19 species with smooth spores is strongly supported by Bayesian posterior probability >95% (Fig. 5, clade I). This suggests basidiospore topology is stable for some groups.

The very short internodes along the backbone of the Inocybe clade might suggest a rapid radiation in this group (Fig. 5). Neither MP nor Bayesian analysis resolves the backbone of the Inocybe clade. The evolution of pleurocystidia, especially of the metuloid type (Singer, 1986), may have permitted the Inocybe clade some selective advantage perhaps in combination with the evolution of novel habitats and hosts. About 85% of *Inocybe* species occur in this clade (Stangl, 1989), which is consistent with a rapid radiation. Additional sampling within the clade will not appear to increase support along the backbone of this large heterogeneous group. Branch support has its limits (Fishbein et al., 2001), and the challenge remains to find characters (loci) that evolve appropriately to resolve the polytomy.

4.4. The Pseudosperma clade

Combined nLSU, RPB1, and RPB2 sequences of *I.* cf. *maculata* support its placement sister to the Inocybe clade with 95% posterior probability but less than 70% bootstrap. The general morphology of this species (viz., rimose pileus, small subphaseoliform spores, absence of pleurocystidia) would suggest a relationship with the Inosperma clade. Additional taxon sampling (Matheny, unpublished) supports the position of additional taxa with phenotypic affinities to section *Rimosae* in the Pseudosperma clade. The combined Bayesian tree and strict consensus MP tree (MP tree not shown) fail to support Kühner's hypothesis (1980) of the separation of *Inocybe* into two subgenera, *Inocybe* and *Inosperma*.

4.5. The Inosperma clade

This clade is composed of two strongly supported interior clades, I and II, that appear to conform to sections *Cervicolores* and *Rimosae*, respectively (Kuyper, 1986; Singer, 1986). The position of *I. adaequata* is resolved by both Bayesian and MP analyses with strong support (95% posterior probability and 99% bootstrap) in contrast to RPB1 and RPB2 results (Figs. 3 and 4). Reddening flesh, therefore, might be a shared ancestral state for the Inosperma clade, but more taxa remain to be sampled. Additionally, *I. calamistrata* and *I. calamistratoides* (clade I) receive strong support (100% posterior probability/80% bootstrap). However, nLSU analysis (Fig. 2) strongly suggests a different relationship. Because no paralogy has been found in RPB1 and RPB2 sequences of *Inocybe*, other potential explanations of heterogeneity might explain the conflicting gene phylogenies in this group. Such processes that might account for this discrepancy include incomplete lineage sorting or hybridization (Maddison, 1997; Sang and Zhong, 2000). Nevertheless, it appears reasonable to defer phylogenetic conclusions for the taxa in question until a majority of data sets are able to resolve the problem (Wiens, 1998).

A molecular synapomorphy for the Inosperma clade appears to be a unique 5′ splice junction in an RPB2 intron. Instead of the canonical "GT" starting sites (Tomita et al., 1996), members of this clade all share "GC" starting sites. *Inocybe* cf. *maculata*, however, possesses the "GT" canonical splice site.

4.6. The Mallocybe clade

High posterior probabilities (more than 95%) and/or bootstrap values (higher than 70%) characterize most branches in this clade when nLSU, RPB1, and RPB2 data are combined. The Mallocybe clade contains species with a woolly, fibrillose or tomentose to scaly pileus; often similarly textured stipe; generally adnate to subdecurrent lamellae; often short or squat stature; and necropigmented basidia. This clade also contains the type of subgenus *Mallocybe—I. terrigena.* The *I. dulcamara* group (clade I) is monophyletic with 100% posterior probability and 99% bootstrap and contains *I. agardhii* and an undescribed species, *I.* "*tanyosporota*", that is similar to *I. malenconii* Heim but with much longer basidiospores. The limits of *I. dulcamara* and other species in the *I. dulcamara* group are called into question by this study, but separate gene phylogenies (Figs. 2–4) fail to contradict the monophyly of the three strongly supported subgroups within the clade (Fig. 5). The basal branch of the Mallocybe clade is occupied by a species that appears endemic to eastern North America, *I. unicolor*. This species is characterized in part by elongated cheilocystidia and association with oak-hickory forests. The position of the branch is not strongly supported by posterior probabilities, however. Three undescribed Australian species, clade II (Matheny and Bougher, unpublished), branch next in the tree and are strongly supported as monophyletic (100% posterior probability and 100% bootstrap). These three species share the presence of short clavate cheilocystidia (or their absence) and association with *Eucalyptus* (Myrtaceae) forests in temperate regions of Australia. This Australian group is sister to a north temperate group also typically characterized by possession of short clavate cheilocystidia (or their absence) that associate with conifers (Pinaceae) or willows and poplars (Salicaceae).

Two species that lack pleurocystidia and were suspected to belong in *Inocybe* (*I. angustispora* and *I. fibrillosa* (Cleland) Grg., *non* Peck) (Bessette and Fatto, 1998; Grgurinovic, 1997) actually represent species of *Cortinarius*. *Inocybe angustispora* is a later synonym of *Cortinarius aureifolius* Peck (Ammirati and Gilliam, 1975; Keller and Ammirati, 1995; Matheny and Ammirati, 2003). *Inocybe fibrillosa non* Peck is better regarded in *Cortinarius* where it was originally placed as *C. fibrillosus* (Cleland, 1928). RPB1 and RPB2 gene phylogenies support both species in *Cortinarius* in addition to *C. croceus* (Matheny and Ammirati, 2003).

4.7. The Auritella clade

Three undescribed Australian species (Matheny and Bougher, unpublished) comprise a second major clade of taxa with necropigmented basidia. Unlike the Mallocybe clade, the agaricoid representatives are characterized by tough basidiomata and elongated cheilocystidia. Bayesian posterior probabilities support the inclusive monophyly of the Mallocybe and Auritella clades as predicted by the shared presence of necropigmented basidia. However, bootstrap support for an inclusive clade is below 70%, and the possession of necropigmented basidia may be a symplesiomorphic character, or has evolved independently several times. *Inocybe calamistratoides* (Inosperma clade) possesses necropigmented basidia in addition to *I. misakaensis* (Matheny and Watling, 2004), recently described from Zambia. All species of the Auritella clade sampled to date originate from Australia and Africa (Matheny and Bougher, unpublished).

4.8. The Crepidotaceae appears sister to Inocybe

The mostly pleurotoid, brown-spored, saprophytic genus, *Crepidotus*, appears sister to *Inocybe* with strong bootstrap support (83%) but with a posterior probability of only 89% when the three loci are combined (Fig. 5). This is a novel and unanticipated relationship. Separate MP analyses of each gene provides at most 60% support (RPB2) for this topology. The addition of nLSU data boosts the bootstrap support of a Crepidotaceae–*Inocybe* clade up to 83% but does not generate a significant Bayesian posterior probability. Previous studies

suggested *Phaeomarasmius* (Strophariaceae) as sister to *Inocybe* (Matheny et al., 2002) but with poor bootstrap support and inadequate taxon sampling. The relationship between a saprophytic family and a large ectomycorrhizal genus affirms the relative plasticity of the mycorrhizal symbiosis at higher taxonomic levels within the Basidiomycota (Hibbett et al., 2000).

Aime (2001) was unable to resolve the sister lineage to the Crepidotaceae *sensu stricto*, which, among non-reduced agarics, is now restricted to the stipitate genus, *Simocybe*, and *Crepidotus* based on nLSU sequences. A broad survey of the euagarics clade at the nLSU locus by Moncalvo et al. (2002) also suggests a close relationship between the Crepidotaceae *sensu stricto* and *Inocybe*. *Pleuroflammula*, another brown-spored saprophytic genus often with a reduced or absent stipe (Singer, 1986), appears sister to an inclusive clade of *Inocybe*, *Pholiota tuberculosa*, *Simocybe*, and *Crepidotus* (Moncalvo et al., 2002). Affinities between *P. tuberculosa* and *Inocybe* have been raised previously (Kühner and Romagnesi, 1953; Vellinga, 1986; Watling, 2001). The study presented here suggests a sister relationship between *Inocybe* and the Crepidotaceae *sensu stricto*.

4.9. The Inocybaceae

It is clear that the Cortinariaceae is not a monophyletic group (Moncalvo et al., 2000a,b, 2002). Evidence is accumulating that shows *Inocybe* not only consists of at least five distinct lineages but that it is related to, and possibly derived from, saprophytic ancestors of the Strophariaceae and Crepidotaceae (Singer, 1986). Thus, it is recommended here to follow Jülich (1982) and separate Singer's tribe Inocybeae from the Cortinariaceae and recognize the tribe at the family level—the Inocybaceae.

Several proposed ideas about evolution in *Inocybe* are not supported by this study, namely the separation of gibbous-spored taxa from smooth-spored taxa (Horak, 1967; Kobayashi, 2002a,b; Singer, 1986). Elements of Kühner (1980) and Kuyper (1986) are supported but adjustments to their classifications will prove necessary. Recommendations will be put forward elsewhere to propose an evolutionary-based classification of the group. Convergent gross morphologies, homoplastic anatomical characters, ambiguous species boundaries, and taxon and gene sampling present many challenges for future research.

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