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Lower level relationships in the mushroom genus *Cortinarius* (Basidiomycota, Agaricales): A comparison of RPB1, RPB2, and ITS phylogenies

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Abstract

We sampled and analyzed approximately 2900 bp across the three loci from 54 taxa belonging to a taxonomically difficult group of *Cortinarius* subgenus *Phlegmacium*. The combined analyses of ITS and variable regions of RPB1 and RPB2 greatly increase the resolution and nodal support for phylogenies of these closely related species belonging to clades that until now have proven very difficult to resolve with the ribosomal markers, nLSU and ITS. We present the first study of the utility of variable regions of the genes encoding the two largest subunits of RNA polymerase II (RPB1 and RPB2) for inferring the phylogeny of mushroom-forming fungi in combination with and compared to the widely used ribosomal marker ITS. The studied region of RPB1 contains an intron of the size and variability of ITS along with many variable positions in coding regions. Though almost entirely coding, the studied region of RPB2 is more variable than ITS. Both RNA polymerase II genes were alignable across all taxa. Our results indicate that several sections of *Cortinarius* need redefinition, and that several taxa treated at subspecific and varietal level should be treated at specific level. We suggest a new section for the two species, *C. caesiocortinatus* and *C. prasinocyaneus*, which constitute a well-supported separate lineage. We speculate that sequence information from RNA polymerase II genes have the potential for resolving phylogenetic problems at several levels of the diverse and taxonomically very challenging genus *Cortinarius*.
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1. Introduction

1.1. RNA-polymerase genes for resolving phylogenetic relationships of Cortinarius

Until recently phylogenetic relationships of mushroom-forming fungi have been inferred almost entirely by sequence data from the nuclear (and mitochondrial) ribosomal RNA cistron. For species level analyses most studies have used information from the internal transcribed spacer region (ITS) (e.g., Aanen et al., 2000a; Hibbett et al., 1995; Hughes et al., 2001; Miller and Buyck, 2002). Studies based on ITS have often included the adjacent variable domains of nLSU (e.g., Geml et al., 2004; Vellinga, 2004), and some studies (e.g., Aanen et al., 2000a; Vellinga, 2001) have used information from the intergenic spacer region (IGS). Characteristic for these studies has been the relatively low amount of resolution and nodal support. ITS often strongly supports phylogenetic species but fails to provide robust resolution of the branching order among those species. This has among many other studies been evident in studies of the mushroom genera *Lentinula* (Hibbett et al., 1995),

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Hebeloma (Aanen et al., 2000b), *Lepiota* ss. lato (Vellinga, 2003; Vellinga et al., 2003) and *Sparassis* (Wang et al., 2004). In *Cortinarius* inference with traditional ribosomal genes (ITS and nLSU) has also provided very little phylogenetic resolution (Garnica et al., 2003; Høiland and Holst-Jensen, 2000; Liu et al., 1997; Peintner et al., 2001; Peintner et al., 2002, 2004; Seidl, 2000).

Though sequence data from nuclear single-copy protein-coding genes have shown promising results for several mushroom genera (Kretzer and Bruns, 1999; Thon and Royse, 1999), there are still remarkably few multigene phylogenetic studies of mushrooms incorporating information from these genes (Lutzoni et al., 2004). So far, phylogenetics of *Cortinarius* have been based exclusively on rDNA analyses, but multi-locus studies including protein-coding genes are underway (Peintner; personal communication). Sequences from RNA polymerase II genes have proven to be effective for inference of phylogenies in many different organisms (see references in Matheny, 2005). Sequence data from RPB2 have successfully been applied in several recent phylogenetic studies of fungal groups at different levels (Chaverri et al., 2003; Hansen et al., 2005; Liu and Hall, 2004; Liu et al., 1999; Lutzoni et al., 2004; Matheny, 2005; Matheny et al., 2002; Reeb et al., 2004; Tanabe et al., 2004; Wang et al., 2004), but the performance of RPB1 at species level is still relatively unknown (Kropp and Matheny, 2004; Matheny, 2005; Matheny and Ammirati, 2003).

Matheny (2005) showed that combined information from RPB1, RPB2, and nLSU sequences improved phylogenetic inference in *Inocybe*, a genus of ectomycorrhizal agarics believed to share evolutionary affinities with *Cortinarius* (Kühner, 1980; Singer, 1986). The study demonstrated the potential of RNA polymerase II genes for improving phylogenetic inference of relationships usually addressed with the ribosomal gene nLSU, and a potential for utility at even lower levels was hypothesized.

Here, we sampled variable regions of RPB1 between conserved domains A and C and RPB2 between conserved domains 6 and 7 in combination with ITS to infer the phylogeny of a group of closely related mushroom species (*Cortinarius* subgenus *Phlegmacium* pro parte)—a taxonomically and phylogenetically difficult group, where sequence data from nLSU and ITS have provided very little resolution and nodal support (Garnica et al., 2003; Peintner et al., 2004). This study represents the first comparison of RNA polymerase II genes and ITS for phylogenetic purposes.

1.2. The genus Cortinarius

Cortinarius is the largest of the mushroom-forming fungal genera (Agaricales ss. Singer (1986) ≈ the euagaric clade ss. Moncalvo et al. (2002)). At present there are more than 4125 published names in *Cortinarius* (Index fungorum, CABI Bioscience Databases, http://www.indexfungorum.org). Many of these have been shown to be synonyms, and more are yet to be listed as such. New taxa are, however, continuously being described, even from well-studied areas such as Europe (Antonini and Antonini, 2002; Brandrud, 1996; Brandrud et al., 1989–1998; Consiglio, 1996; Moser and Peintner, 2002; Moënne-Loccoz et al., 1991–2004) and 2000 may prove to be a conservative estimate of the actual number of species. The fruiting bodies of most species are typical mushrooms with a stipe and a pileus with a lamellate spore-producing layer. Most species have a cobweb-like partial veil protecting the young lamellae—the cortina, from which the generic name is derived. They have brown ornamented spores giving a cinnamon brown to rusty brown spore deposit. Apart from these unifying characters, a great inter- and intra-specific morphological variation is exhibited. All species form ectomycorrhizas (a form of mutualistic symbiosis between fungi and plants) with a variety of woody hosts mainly belonging to *Fagales*, *Pinaceae*, and *Salicaceae*, but species of *Cistaceae*, *Dipterocarpaceae*, *Eucalyptus*, and *Dryas* are also known as mycorrhizal hosts of *Cortinarius*. They are by far the dominant ectomycorrhizal fungal group in many northern temperate ectotrophic ecosystems both in terms of species diversity and above ground biomass (Brandrud et al., 1989–1998).

The infrageneric taxonomy of *Cortinarius* has largely been based upon macro-morphology, and is a matter of much debate. Several major classification schemes have been proposed (Kühner, 1980; Kühner and Romagnesi, 1953; Melot, 1990; Moser, 1983; Moënne-Loccoz et al., 1991–2004; Orton, 1958). In this paper, we follow the infrageneric taxonomy of Melot (1990) as in Brandrud et al. (1989–1998). Several "top-down" studies have attempted to address the phylogeny of the whole genus with traditional ribosomal markers (ITS, nLSU), but these have resulted in trees with very little resolution (especially at basal levels) and low nodal support (Garnica et al., 2003; Høiland and Holst-Jensen, 2000; Peintner et al., 2001, 2002, 2004). Many traditional taxonomic units have been shown to be artificial. Peintner et al. (2002) showed that some groups, formerly treated as separate genera, are derived within *Cortinarius* (i.e., *Cuphocybe*, *Rozites*, and *Rapacea*). Furthermore, some sequestrate (truffle- of puffball-like) genera (spore producing tissue not exposed) have been shown to be derived within *Cortinarius* (i.e., *Quadrispora*, *Thaxterogaster*, and *Hymenogaster pro parte*) (Peintner et al., 2001). The genus as such (including the derived groups) is well supported as monophyletic (Moncalvo et al., 2002; Peintner et al., 2001, 2004) belonging to the euagaric clade (≈Agaricales) of the fungal tree of life (Moncalvo et al., 2002). The classical major subgenera (i.e., *Cortinarius*, *Telamonia*, *Myxacium*, and *Phlegmacium*),

Fig. 1. Fruitbodies of *Cortinarius* subgenus *Phlegmacium*. (A) *C. magicus* from section *Glaucopodes*. (B) *C. elegantissimus* from section *Fulvi* subsection *Rufoolivacei*. (C) *C. splendens* from section *Fulvi* subsection *Splendentes*. (D) *C. barbarorum* from section *Calochroi*. (E) *Cortinarius caesiocortinatus* from section *Caesiocortinati*. (F) *C. atrovirens* from section *Fulvi* subsection *Atrovirentes*.

however, constitute polyphyletic and/or paraphyletic assemblages (Høiland and Holst-Jensen, 2000; Peintner et al., 2004). Peintner et al. (2004) provide a tentative phylogenetic classification based on ITS/nLSU data for future systematic and phylogenetic treatments of the genus. Despite this progress, it is clear that additional markers are necessary to resolve the relationships of *Cortinarius* at all levels.

Species traditionally treated in subgenus *Phlegmacium* are characterized by a slimy pileal surface in combination with a dry stipe. The subgenus is polyphyletic (Peintner et al., 2004), but contains some well-defined morphological groups that seem to constitute monophyletic entities. Most species with a marginate bulb at the base of the stipe (Fig. 1) seem to belong in the /Calochroi clade or the /Phlegmacium clade ss. Peintner et al. (2004). The first clade contains species belonging to the sections *Calochroi* and *Fulvi* and the other clade contains species from the sections *Coerulescentes*, *Glaucopodes*, *Phlegmacium*, *Phlegmacioides*, and *Fulvi* (Peintner et al., 2004).

We find many of the most strikingly colored species of *Cortinarius* within these sections of *Phlegmacium* (i.e., *Calochroi*, *Fulvi*, *Coerulescentes*, *Glaucopodes*, and their allies). Often they possess other striking characters: strong pleasant or unpleasant smells (i.e., pepper, banana, old cheese, metal, farinaceous, yeast, etc.),

peculiar tastes (farinaceous, bitter, etc), color reactions with alkaline substances (blood red, olivaceous, purple, pink, etc). Furthermore, a majority of these species are rare (Brandrud et al., 1989–1998). Most are calcifilous (growing only in calcareous soils) and have very narrow preferences in terms of habitat and mycorrhizal host. Though many species are widespread, they often exist in small, geographically isolated populations. Many are therefore listed on the various national red lists in Europe (Arnolds and Ommering, 1996; Bendiksen et al., 1997; Benkert et al., 1992; Gärdenfors, 2000; Stoltze and Pihl, 1998). The taxonomy of these groups is extremely difficult and much controversy exists between authors both relating to the number of species and the application of names, which is mainly due to the difficulty in assessing whether the great morphological variation in these groups is exhibited within or between species, and the existence of a host of rather ambiguously described names. Unfortunately, a strict Biological Species Concept (BSC) generally is difficult to apply to ectomycorrhizal fungi, as they often prove difficult to culture from single spores. Though successful mating studies have been carried out in the ectomycorrhizal genus *Hebeloma* (Aanen and Kuyper, 1999), species of *Cortinarius* have so far proven impossible to culture from spores (Brandrud et al., 1989–1998). Thus, it is important to get a better understanding of the low-level phylogenetic relationships of these groups to be able to appreciate the biological and genetical diversity of these rare taxa. Through the application of a Phylogenetic Species Concept (Taylor et al., 2000) we will arrive at a better delimitation of the biological units (species), enabling us to assess the conservation requirements of these rare taxa more appropriately. Furthermore, it is clear that the specialized ecology, rarity, and morphological diversity of this group, make them interesting and suitable for studies of speciation and evolution of host specificity and morphology. However, robust phylogenies based on dense taxon sampling that incorporate multiple genes are critical.

1.3. Objectives

A main objective of this study is to compare and examine the utility of single gene and combined analyses of RPB1, RPB2, and ITS for phylogenetic inference of mushrooms. Furthermore, we use the combined RPB1, RPB2, and ITS analyses to address some infrageneric taxonomic issues of *Cortinarius*: (1) What are the phylogenetic boundaries between the sections *Calochroi* and *Fulvi*, and between *Glaucopodes* and *Coerulescentes*? (2) Is the chemotaxonomical subdivision of *Fulvi* natural? (3) Is it possible to address the status of taxa in the *Cortinarius calochrous* ss lato "complex" with a multigene phylogeny? (4) Are the two globose-spored species *C. caesiocortinatus* and *C. prasinocyaneus* related to the other four sections with a broad marginate bulbous stipe?

2. Materials and methods

2.1. Taxon sampling

This study includes 54 samples representing 43 taxa of European species of *Cortinarius* subgenus *Phlegmacium* (Table 1). The selection of taxa for this study was based on a broader phylogenetic study of *Cortinarius* based on ITS sequences (results not shown) and on the findings of Peintner et al. (2004) and Garnica et al. (2003). We sampled taxa representative of the variation within the two sections *Fulvi* and *Calochroi*. Taxa from the morphologically similar sections *Glaucopodes*, *Coerulescentes*, and *Multiformes* were also sampled but with less density. Our sampling is biased towards species from north temperate deciduous forests. The section *Calochroi* as circumscribed here contains at least 30 taxa in Europe. Section *Fulvi* is slightly larger with at least 34 taxa, and sections *Glaucopodes* and *Coerulescentes* collectively encompass more than 35 taxa. These species-numbers reflect well-delimited groups of samples with identical or near identical ITS sequences and could be taken as a rather conservative estimate of the actual number of species in Europe.

The results of Peintner et al. (2004) and Garnica et al. (2003) based on ITS and nLSU indicated that *Fulvi* as traditionally circumscribed is polyphyletic with species assigned to subsection *Percomes* being unrelated to the rest of the section. We sampled species from both clades to address these findings with a multigene approach. The same studies indicated a close relationship of species of sections *Calochroi* and *Fulvi*, but internal relationships were unclear. Based on pigment chemistry section *Fulvi* can be divided into six subsections: *Elegantiores*, *Splendentes*, *Atrovirentes*, *Rufoolivacei*, *Sulfurini*, and *Percomes* (Brandrud, 1998). We sampled several species from the majority of these subsections to test the phylogenetic basis for this subdivision.

The taxonomic boundaries in section *Calochroi* are controversial. Some authors treat the taxa possessing a more or less strictly calochroid appearance (i.e., yellow pileus, lilac lamellae and a very broadly marginate bulb) as a host of subspecies and varieties (Brandrud et al., 1989–1998), whereas others acknowledge these taxa as separate species (Moënne-Loccoz et al., 1991–2004). We included several of these taxa to address their taxonomic status. We also included the morphologically divergent species, *C. caesiocortinatus* and *C. prasinocyaneus*, that have been treated in section *Calochroi* to test whether this could be phylogenetically justified. For other putative species we included several samples to examine the morphological species concept in a phylogenetic context.

Table 1
DNA sequences, their geographic origin, voucher number, and GenBank accession numbers

Taxon	Origin	Voucher no#	ITS	RPB2	RPB1
Cortinarius aff. *calochrous* (1)	DK, Sjælland	TF2001-049	DQ083766	DQ083874	DQ083820
Cortinarius aff. *calochrous* (2)	DK, Møn	TF2001-103	DQ083767	DQ083875	DQ083821
Cortinarius alcalinophilus Rob.Henry (1)	DK, Møn	TF2001-086	DQ083768	DQ083876	DQ083822
Cortinarius alcalinophilus (2)	DK, Sjælland	TSJ2003-020	DQ083769	DQ083877	DQ083823
Cortinarius alcalinophilus (3)	DK, Sjælland	TSJ2003-097	DQ083770	DQ083878	DQ083824
Cortinarius atrovirens Kalchbr.	FR, Jura	TF2000-096	DQ083771	DQ083879	DQ083825
Cortinarius aurilicis Chevassut & Trescol	FR, Doubs	TSJ1998-101	DQ083772	DQ083880	DQ083826
Cortinarius barbarorum Bidaud, Moënne-Locc. & Reumaux = *C. calochrous* var. *coniferarum*	I, Kaltern	TSJ2000-069	DQ083773	DQ083881	DQ083827
Cortinarius caesiocortinatus Jul.Schaeff.	CR, Karlstejn	TSJ2002-028	DQ083774	DQ083882	DQ083828
Cortinarius cf. *calochrous* 1	FR, Hérault	TSJ2002-072	DQ083776	DQ083884	DQ083830
Cortinarius cf. *calochrous* 2	CR, Karlstejn	TF2002-025	DQ083775	DQ083883	DQ083831
Cortinarius cf. *natalis*	FR, Hérault	TF2002-039	DQ083777	DQ083885	DQ083832
Cortinarius cf. *parvus*	DK, Sjælland	TF2001-124	DQ083778	DQ083886	DQ083833
Cortinarius claroflavus Rob. Henry	FR, Porquerolles	TSJ2002-057	DQ083779	DQ083887	DQ083834
Cortinarius coerulescentium Rob.Henry (1)	DK	JV01-572	DQ083780	DQ083888	DQ083835
Cortinarius coerulescentium (2)	CR, Karlstejn	TF2002-002	DQ083781	DQ083889	DQ083836
Cortinarius dionysae Rob.Henry	D, Bayern	TSJ2000-102	DQ083782	DQ083890	DQ083837
Cortinarius elegantissimus Rob.Henry	DK, Sjælland	TF2000-048	DQ083783	DQ083891	DQ083838
Cortinarius flavovirens Rob.Henry	DK, Jylland	TSJ1999-076	DQ083784	DQ083892	DQ083839
Cortinarius fulvocitrinus Brandrud	DK, Sjælland	TF2001-045	DQ083785	DQ083893	DQ083840
Cortinarius gracilior (M.M. Moser) M.M. Moser	DK, Sjælland	TSJ2003-012	DQ083786	DQ083894	DQ083841
Cortinarius humolens Brandrud	CR, Karlstejn	TF2002-009	DQ083787	DQ083895	DQ083842
Cortinarius insignibulbus Bidaud & Moënne-Locc.	DK, Sjælland	TSJ2003-060	DQ083788	DQ083896	DQ083843
Cortinarius langei ss. lato (1)	DK, Jylland	TF1999-084	DQ083790	DQ083898	DQ083844
Cortinarius langei ss. lato (2)	DK, Fyn	JV01-642	DQ083789	DQ083897	DQ083845
Cortinarius lilacinovelatus Reumaux et Ramm (1)	DK, Jylland	TSJ1999-092	DQ083792	DQ083900	DQ083847
Cortinarius lilacinovelatus (2)	DK, Sjælland	TF2001-030	DQ083791	DQ083899	DQ083846
Cortinarius luhmannii Münzmay, Saar & Oertel	DK, Sjælland	TF2001-120	DQ083793	DQ083901	DQ083848
Cortinarius magicus Kalchbr.	DK, Sjælland	TSJ1999-050	DQ083794	DQ083902	DQ083849
Cortinarius molochinus Bidaud & Ramm (1)	CR, Karlstejn	TSJ2002-026	DQ083796	DQ083904	DQ083851
Cortinarius molochinus (2)	SZ, Neuchâtel	TSJ2000-086	DQ083795	DQ083903	DQ083850
Cortinarius natalis D. Antonini & M. Antonini	FR, Hérault	TSJ2002-070	DQ083797	DQ083905	DQ083852
Cortinarius neolangei ined. (1)	DK, Jylland	TF2000-012	DQ083798	DQ083906	DQ083853
Cortinarius neolangei (2)	S, Skåne	TSJ2003-078	DQ083800	DQ083908	DQ083855
Cortinarius neolangei (3)	DK, Sjælland	TSJ2003-057	DQ083799	DQ083907	DQ083854
Cortinarius ochraceopallescens Moënne-Locc. & Reumaux	SZ, Neuchâtel	TF2000-106	DQ083801	DQ083909	DQ083856
Cortinarius odorifer Britz.	S, Gotland	TSJ2000-024	DQ083802	DQ083910	DQ083857
Cortinarius olearioides Rob. Henry	S, Gotland	TF2000-036	DQ083803	DQ083911	DQ083858
Cortinarius parasuaveolens (Bon & Trescol) Bidaud	CR, Karlstejn	TSJ2002-015	DQ083804	DQ083912	DQ083859
Cortinarius percomis Fr.	FR, Hérault	TSJ2002-096	DQ083805	DQ083913	DQ083860
Cortinarius prasinocyaneus Rob.Henry	S, Öland	TSJ2003-033	DQ083806	DQ083914	DQ083861
Cortinarius prasinus (Schäff.: Fr.) Fr.	CR, Karlstejn	TSJ2002-034	DQ083807	DQ083915	DQ083862
Cortinarius quercilicis (Chevassut & Rob.Henry) Melot (1)	FR, Hérault	TSJ2002-075	DQ083808	DQ083916	DQ083864
Cortinarius quercilicis (2)	S, Öland	TSJ2003-050	DQ083809	DQ083917	DQ083863
Cortinarius rapaceus ss. lato	DK, Jylland	TSJ2003-100	DQ083810	DQ083918	DQ083865
Cortinarius saporatus Britzelm.	DK, Møn	TF2001-087	DQ083811	DQ083919	DQ083866
Cortinarius sodagnitus Rob.Henry	DK, Møn	TF2001-094	DQ083812	DQ083920	DQ083867
Cortinarius sp.	DK, Jylland	JV01-574	DQ083813	DQ083921	DQ083868
Cortinarius splendens Rob.Henry	DK, Sjælland	TF2001-122	DQ083814	DQ083922	DQ083869
Cortinarius splendificus Chevassut & Rob.Henry	FR, Hérault	TF2002-041	DQ083815	DQ083923	DQ083870
Cortinarius suaveolens Bataille & Joachim	DK, Jylland	TF2000-055	DQ083816	DQ083924	DQ083871
Cortinarius terpsichores Melot	CR, Karlstejn	TF2002-007	DQ083817	DQ083925	DQ083872
Cortinarius viridocoeruleus Chevassut & Rob. Henry	DK, Jylland	TF2000-056	DQ083818	DQ083926	DQ083829
Cortinarius xanthochlorus Rob. Henry	S, Öland	TSJ2003-027	DQ083819	DQ083927	DQ083873
Cortinarius aureifolius Peck (= *Inocybe angustispora*)	USA	B10705 (holotype, NYS)		AY333319	AY333304
Cortinarius aureifolius	S	SJ84127	AF268893		

Collection sites: DK, Denmark; FR, France; S, Sweden; SZ, Schwitzerland; CR, Czech Republic; I, Italy. All collections with initials TF or TSJ was collected by the first author and/or Thomas S. Jeppesen except TSJ 2003-097 collected by Thomas Læssøe. Collections with initials JV collected by Jan Vesterholt. All vouchered specimens (except for B10705 (NYS), and SJ84127 (Stig Jacobsson)) are deposited in C.

Most sampled collections have been collected by the first author and/or Thomas Stjernegaard Jeppesen and have been photographed and annotated and are deposited at the Mycological Herbarium of the Museums of Natural History at the University of Copenhagen (Herb C).

2.2. Molecular data

Genomic DNA was isolated from well-preserved herbarium specimens or fresh material with a standard CTAB procedure (CTAB with β-mercaptoethanol, pure formaldehyde, precipitation in isopropanol over night, one or two 70% ethanol washes, and eluation in 50–100 μL of 1% TE-buffer or water) or with DNeasy Plant Mini Kit (Qiagen) following the protocol of the manufacturer. Polymerase chain reactions (PCR) were performed on a MJ Research PTC-200 thermo-cycler to amplify ITS (the internal transcribed spacer region, ITS1-5.8S-ITS2), and the region 6–7 of RPB2 (RNA polymerase II second largest subunit) (Liu et al., 1999; Matheny, 2005), and the region A to C of RPB1 (RNA polymerase II largest subunit) (Matheny et al., 2002; Stiller and Hall, 1998). PCR-amplification and direct sequencing of ITS was done using primer ITS1F in combination with ITS4 (White et al., 1990). PCR-amplification and direct sequencing of RPB2 6–7 and RPB1 A to C followed Matheny et al. (2002), Matheny and Ammirati (2003), and Matheny (2005). Degenerate basidiomycete specific primers b6F and b7.1R were used to amplify the RPB2 region. For some samples cort6F (GCTTGTGGGCTTGTCAARAATC) was used in combination with b7.1R. PCR primers were used for sequencing, at times with cort6.3F (TTGGATAGGTGTRCATCG YGACC) and cort7R (ACTTGRTTGTGRTCKGGR AAHGG) as additional sequencing primers. For RPB1 primers fA-for and gC-rev were used for PCR. The following new sequencing primers were designed in the conserved region of intron 2 (Matheny et al., 2002): Int2f (TTMBTCTRCTCGTTTYGCAC), Int2.1f (GCTGAA CGAGSAGTGC), and Int2.1r (GCACTSCTCGYTC AGC). These intron primers have been successfully used as internal sequencing primers across a wide spectrum of Homobasidiomycetes (Matheny and Hibbett, unpublished). See Fig. 2 for primer maps and features of the genes. Sequencing was done on an ABI PRISM 377 DNA Sequencer or ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) or by MWG-BIOTECH AG, Ebersberg, Germany.

2.3. Phylogenetic analyses

2.3.1. Data sets, alignment, and variability

Sequence fragments were inspected and assembled using Sequencher 3.11 (Gene Codes Corporation) or Vector NTI (Invitrogen Life Science Software). Alignments of the three regions were done using the FFT-NS-i strategy as implemented in MAFFT v5.3 (Katoh et al., 2005), which constructs an initial alignment by the progressive method, and then refines it by the iterative refinement method (Katoh et al., 2005). Minor manual adjustments were carried out in Se-Al v2.11 (Rambaut, 1996–2002). Thereafter, the sequences were concatenated in a single nexus file containing the various relevant partitions for analyses. Bayesian analyses and equally weighted maximum parsimony (MP) analyses were carried out on single genes (ITS, RPB2, and RPB1) and on the combined data set (ITS+RPB2+RPB1). In the Bayesian analyses the ITS data set was analyzed using a single model of evolution. RPB2 and RPB1 were both assumed to have 4 partitions: 1st, 2nd, and 3rd base codon positions and intron positions. The combined data set was assumed to have all 9 partitions. The model of evolution of single partitions was estimated with the hierarchical likelihood test as implemented in MrModeltest (Nylander, 2004), which is a modification of Modeltest (Posada and Crandall, 1998) focusing on the 18 models implemented in MrBayes.

The variability of the three genes was evaluated with MacClade (Maddison and Maddison, 2000) summarizing the number of steps in each character over one of the most-parsimonious trees (Fig. 2). The complete alignment is available on demand from the first author, and is deposited on TreeBASE (http://www.treebase.org/treebase/).

2.3.2. Incongruence

We used the conditional data combination approach before combining the three genes (De Quiroz, 1993; Huelsenbeck et al., 1996). For detecting topological incongruence between the three genes, we used a reciprocal 70% bootstrap criterion as in Reeb et al. (2004). Significant topological incongruence was assumed if two different relationships (one monophyletic and the other non-monophyletic) for any set of taxa were supported with bootstrap values above 70%. Bootstraps for these comparisons were generated using a neighbor-joining non-parametric bootstrap of 1000 replicates using maximum likelihood distances. Likelihood models for the three genes and the single partitions were selected as described above.

2.3.3. MP analyses

Heuristic maximum parsimony (MP) analyses were performed with 100 random addition sequences (RAS), tree bisection-reconnection (TBR) swapping, and "MulTrees" turned on. MaxTrees was set to 1000 and gaps treated as missing characters. Non-parametric bootstrap proportions were estimated with 1000 pseudo-replicates (PR) and 10 RAS per PR or with 100 RAS and MulTrees turned off and otherwise with settings as above. All MP analyses were conducted with PAUP*4b10 (Swofford, 2003).

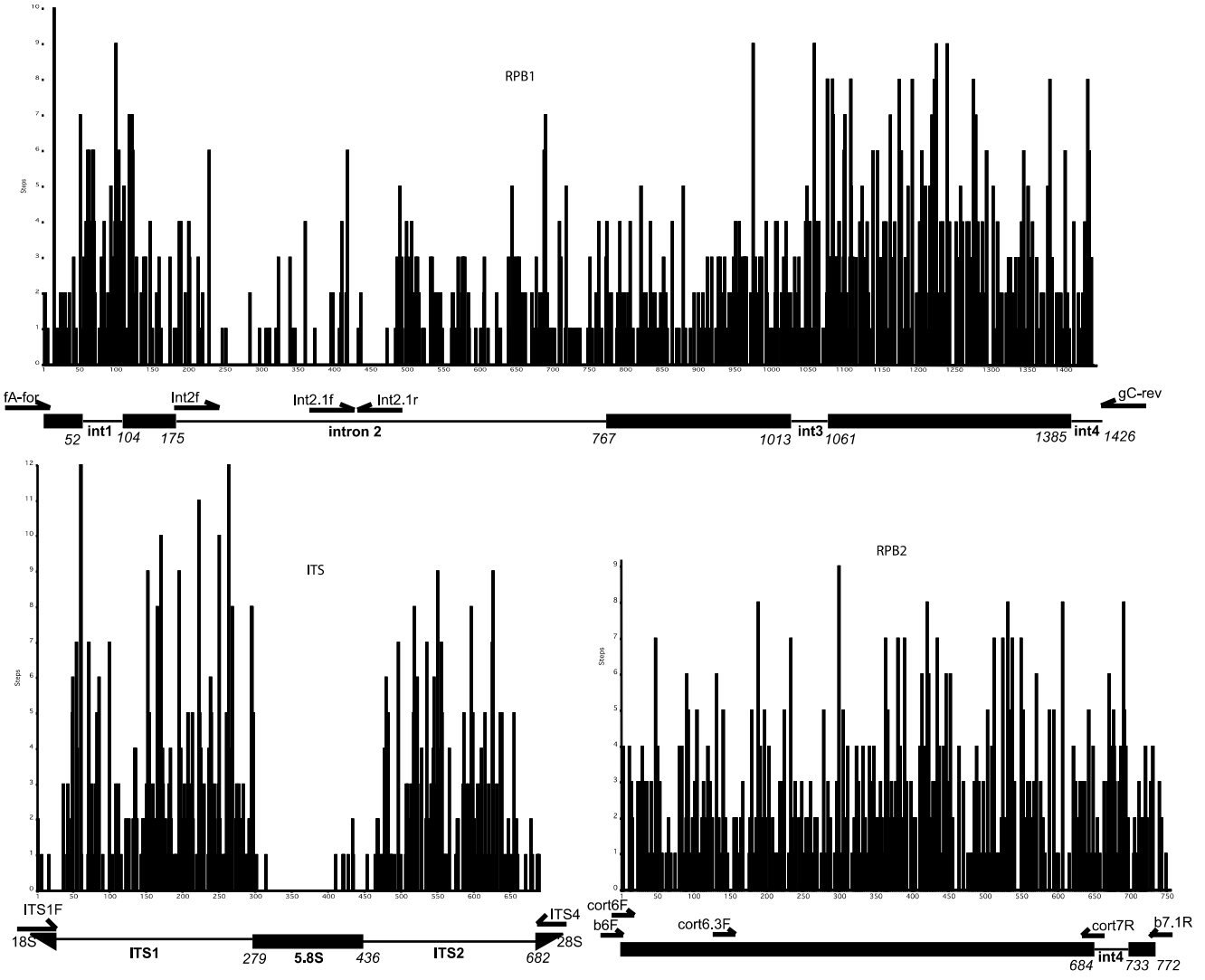

Fig. 2. Primer maps, features, and variability of ITS, RPB2, and RPB1. Priming sites indicated, but primers not drawn to scale. Bold areas indicate coding regions. ITS and RPB1 features based on sequences of *C. prasinocyaneus* (TSJ2003-033), and RPB2 based on *C. natalis* (TSJ2002-070).

2.3.4. Bayesian analyses

MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) was allowed to estimate the parameters of the models found with MrModeltest for all partitions. 2,000,000 generations were run for all single-gene analyses and 3,000,000 and 6,000,000 for the combined analysis with sampling every 100th generation. Six chains were run simultaneously with the "heat" set to 0.2. Branch lengths were saved, allowing us to construct a Bayesian majority-rule phylogram. To assure that each run had reached stationarity and that chains were mixing properly, we plotted the likelihood scores against the number of generations with the aid of Tracer 1.2 (Rambaut and Drummond (2003)). All trees sampled before stationarity were discarded using a very broad safety margin (a burn in of 10,000 trees (1,000,000 generations) was used in all single analyses and various higher burn ins were tried for the combined analyses). From the remaining samples, majority-rule consensus phylograms were constructed with MrBayes. We ran each analysis several times to assure that samples from single runs did not reflect sampling from local likelihood maxima. Tracer 1.2 was also used for calculating model parameters intervals for each run and comparing results between runs.

2.4. Measures of resolution and support

The resulting phylogenetic estimates from the different analyses of the four different data sets were compared in various ways. As in Matheny (2005), we calculated the resolution of strict consensus trees from MP analyses and of majority-rule consensus trees from Bayesian analyses following Colless (1980) and Thorley and Wilkinson (2000). The number of internal branches of each consensus tree was divided by the size of the tree ($n-2$) when rooted, resulting in a measure of resolution

Table 2
Alignment, resolution, and support of different data partitions

	ITS	RPB2	RPB1	Combined
Number of sites	689	759	1441	2889
Excluded positions	190–201	None	None	2391–2402
Number of invariable sites	422	469	927	1818
Number of parsimony-informative sites	155	233	358	746
Variable characters (proportion)	0.39	0.38	0.36	0.37
Parsimony-informative characters (proportion)	0.22	0.31	0.25	0.26
Contribution of informative characters to combined analyses	0.21	0.31	0.48	
Resolution strict consensus MP tree	0.62	0.65	0.75	0.73
Resolution Bayesian 50% majority-rule consensus tree	0.48	0.75	0.81	0.88
No. of clades >95% posterior probability	16	20	27	29
No. of clades >50% bootstrap	17	19	31	34
No. of clades >70% bootstrap	14	17	22	25
No. of clades >90% bootstrap	12	14	16	17

between 0 (not resolved) and 1 (fully resolved tree), allowing us to compare trees from different analyses. Furthermore, the numbers of nodes receiving more than 50, 70, and 90% non-parametric bootstrap support, respectively, and Bayesian posterior probabilities higher than 95% were counted. All these measures correspond to those in the study of *Inocybe* comparing the phylogenetic utility of RPB1 and RPB2 to nLSU at a higher (genus/family) taxonomic level, allowing us to compare the ability of RPB1 and RPB2 to improve phylogenetic inference in comparison to and combination with traditional ribosomal markers (ITS and nLSU) at two different levels in two separate clades of euagarics.

3. Results

3.1. Nucleotide sequences, alignment, and variability

Three gene regions from 54 taxa were sequenced for this study. They are deposited at GenBank (Accession Nos. DQ083766–DQ083927, see Table 1). All the RPB1 sequences contained four spliceosomal introns of which only the first intron deviates from a phase-zero insertion, being a phase-one insertion. This corresponds to the findings in *Inocybe* (Matheny et al., 2002). Intron 1 was 48–62 bp long. Intron 2 was the largest and was around 559–590 bp long. Intron 3 was around 47–51 bp long. Intron 4 was around 40 bp, but incompletely sequenced in most samples and was excluded from the analyses. The RPB2 region also contained a short (44–57 bp long) intron at the 3′ end, which has been identified as intron 4 in the Basidiomycota by Matheny and Hibbett (http://www.clarku.edu/faculty/dhibbett/rpb2%20primers.htm).

Several samples contained polymorphic sites in either region (ITS, RPB2, and RPB1). The number of polymorphic sites ranges between 0 and 9, which is comparable for that reported in *Inocybe* (Matheny, 2005). Intron length heterogeneity was inferred for some RPB1 sequences. None of the sequences obtained with RNA polymerase II primers were assumed to represent pseudogenes, as we encountered no interruptions of reading frames or stop codons in exon regions. The low amount of polymorphic sites indicate that none of the RNA polymerase II sequences represent paralogous genes.

A summary of the features of the alignments can be seen in Table 2. Despite overall comparable levels of variability, the variability was not evenly distributed across the three genes. The 5.8 region of the ribosomal gene only had 5.6% variable positions, whereas ITS1 and ITS2 had 49.7 and 48.5%, respectively. The first half of intron 2 was the least variable of RPB1 with 13.5%, which is similar to that reported for *Inocybe* (Matheny et al., 2002). The four separate exon regions had between 28.2 and 37.4% variable sites with the regions between intron 2 and intron 3 and between intron 3 and intron 4 being the most variable. The second half of intron 2 was slightly more variable than coding regions with 41% variable positions. Intron 1 and intron 3 were the most variable with 70.5 and 73.1% variable positions, respectively.

3.2. Phylogenetic inference

3.2.1. Outgroup choice

Some initial analyses (Bayesian and MP, results not shown) were run with *Gymnopilus sapineus* as outgroup. These results unambiguously showed that *Cortinarius aureifolius* (from section *Dermocybe*) could be used as a closer outgroup for rooting purposes allowing us to make an unambiguous alignment of all characters (except a small portion of ITS).

3.2.2. Models of evolution

The Hierarchical Likelihood Ratio Test as implemented in MrModeltest selected a general time reversible (GTR) model including a gamma distribution parameter and a proportion of invariable sites for the following partitions: RPB1 1st positions and introns,

RPB2 1st and 3rd positions and ITS. A HKY model with a gamma distribution was selected for the RPB1 2nd and 3rd positions and RPB2 2nd position, and a HKY model with a equal rates was selected for the RPB2 intron.

3.2.3. Single-gene analyses

A total of 10,000 trees sampled in the Bayesian analyses was used to construct majority-rule consensus trees of the single genes (Figs. 3–5) with Bayesian posterior probabilities indicated above branches. Second runs yielded consensus tree with identical topologies and near identical posterior probabilities. Trees from the MP analyses are not shown, but tree statistics are given below and MP bootstrap supports are indicated on the branches of the Bayesian trees (Figs. 3–5).

3.2.3.1. ITS analyses. The Bayesian tree is shown in Fig. 3. All species of *Calochroi* and *Fulvi* (except subsection *Percomes* (Clade IV)) are supported as a monophyletic group (Clade I) containing two major resolved subclades—Ia and Ib—with Ia containing almost all calochroid taxa. The rest of the tree is largely unresolved with Clade III containing *C. caesiocortinatus* and *C. prasinocyaneus*. The MP analysis recovered 208 most parsimonious trees of 713 steps (CI = 0.502, RI = 0.643).

3.2.3.2. RPB2 analyses. The Bayesian tree is shown in Fig. 4. Clade I contained three resolved subclades a, b, and c. The position of the clade containing the majority of calochroid taxa (Ia) as derived from a clade with fulvoid taxa was indicated by the most parsimonious trees, but not supported by high posterior probabilities or bootstrap. The placement of *C. xanthochlorus* varied between Bayesian and MP analyses. The Bayesian analysis placed *C. xanthochlorus* in Clade Ib as a sister taxon to *C. odorifer*, whereas its placement was unresolved relating to Clade Ia and Ib in MP analyses. Species of section *Fulvi* subsection *Percomes* were supported as a separate lineage (Clade IV). Clade II containing species from sections *Glaucopus*, *Coerulescentes*, and *Multiformes* was resolved and received several internal nodal supports in contrast to the ITS analyses, but none was supported as separate lineages. The clade containing *C. caesiocortinatus* and *C. prasinocyaneus* (Clade III) was placed at an unresolved node with Clade II and Clade IV. The MP analysis recovered 101 most parsimonious trees of 846 steps (CI = 0.470, RI = 0.647).

3.2.3.3. RPB1 analyses. The Bayesian tree is shown in Fig. 5. Of the single genes analyzed, RPB1 contained the most informative characters, and produced the most robust gene tree with almost exactly the double amount of resolution and nodal support compared to ITS. Clade I was well supported and contained Clade Ia and Ib also found in ITS and RPB2 analyses. Species supported in Clade Ic by RPB2 did not form a clade but together with Ia formed a poorly supported sister group to Ib. The placement of Clade Ia (calochroid taxa) as a highly derived clade in Clade I was, however, well supported. Clades II–IV correspond to those from RPB2 but contained more well-supported nodes. The MP analysis recovered 243 most parsimonious trees of length 1382 (CI = 0.494, RI = 0.659).

3.2.4. Incongruence

Some minor intergenic incongruence was encountered based on the reciprocal 70% bootstrap threshold. There are major discrepancies between the indicated relationships in the clade containing *C. parasuaveolens*, *C.* cf. *calochrous* 1 and *C. insignibulbus* between analyses. RPB1 groups *C.* cf. *calochrous* 1 and *C. insignibulbus* with 93% bootstrap support and 97% posterior probability whereas ITS and RPB2 groups *C. parasuaveolens* and *C. insignibulbus* with 58% bootstrap support and 98% posterior probability and 96% bootstrap support and 100% posterior probability, respectively. In a more extensive sampling with ITS, we see a clear differentiation of these three taxa (results not shown), and their morphology does not suggest conspecificity. These incongruent genealogies might reflect lineage-sorting (Taylor et al., 2000). The exclusion of these taxa from combined analyses, however, did not affect other parts of the topology, which would not be expected as the three taxa cluster in a well-supported clade in all analyses. Thus, we did not exclude them from combined analyses.

3.2.5. Combined ITS+RPB2+RPB1 analyses

Several independent Bayesian analyses were carried out on the combined data set. In all analyses, the six chains failed to exchange states after stationarity was reached. However, the topology of the majority-rule consensus trees of four independent analyses of 3,000,000 and 6,000,000 generations showed the same topology with minor differences in posterior probabilities. A total of 20,000 trees sampled in the first Bayesian analysis was used to construct a majority-rule consensus tree (Fig. 6) with posterior probabilities indicated above branches. The MP analysis resulted in 134 most parsimonious trees of length 3033 (CI = 0.479, RI = 0.639) (trees not shown). Bootstrap support from the MP bootstrap analyses are indicated on branches in Fig. 6. The topology of the tree found in the combined Bayesian analysis is almost fully resolved (with a resolution of 0.88). New clade names are introduced in Fig. 6 with /Calochroi corresponding to Clade I in the single-gene trees and subclades /Calochroid, /Fulvi, and /Rufoolivacei corresponding more or less to Clades Ia, Ib, and Ic. The /Phlegmacium clade includes /Glaucopodes, /Caesiocortinati, and /Percomes corresponding to Clades II, III, and IV, respectively. The /Calochroid subclade is well supported as derived clade of /Calochroi with

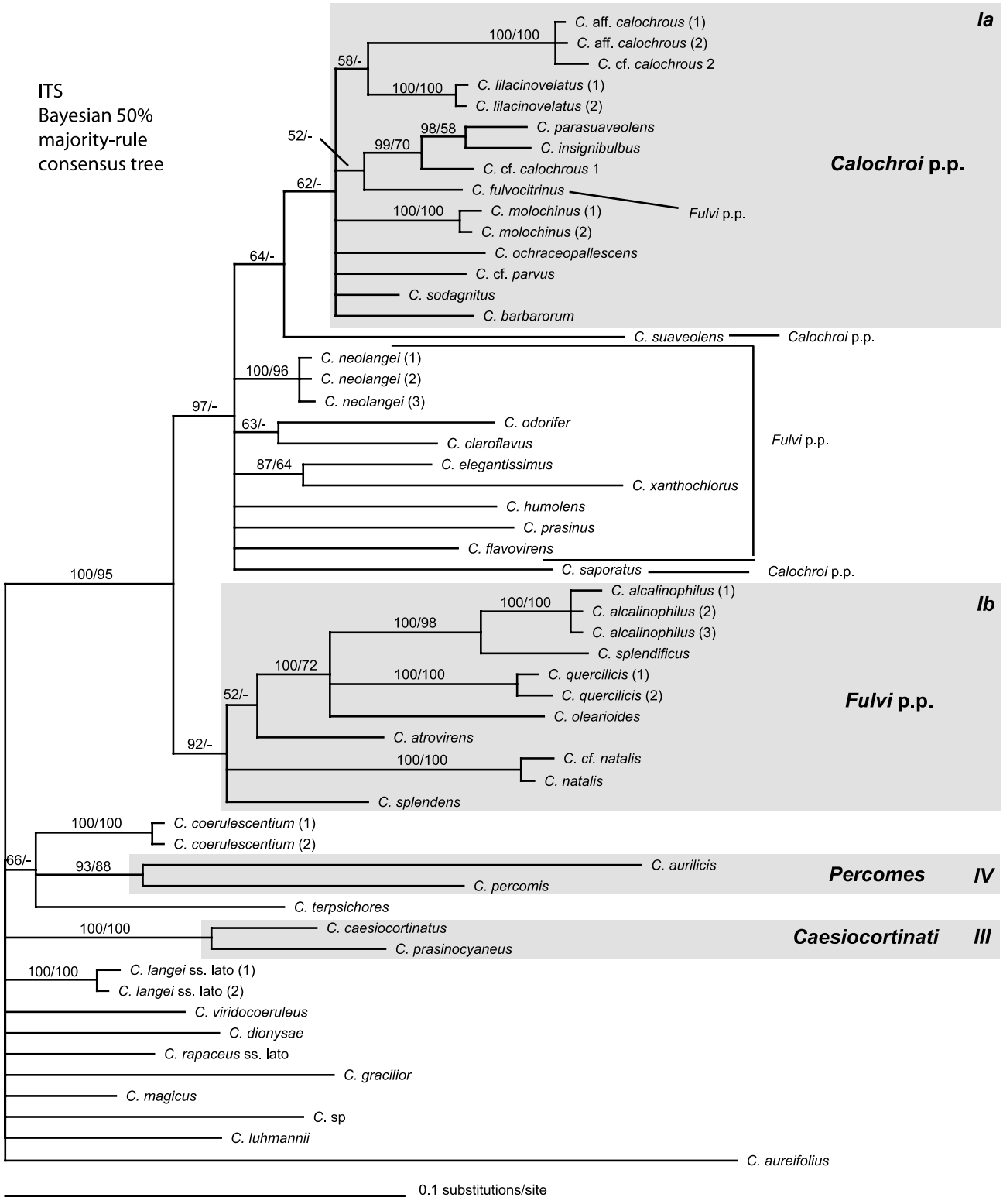

Fig. 3. The Bayesian 50% majority-rule consensus tree inferred from ITS. Posterior probabilities and bootstrap values above 50% are indicated above branches (PP/BP). Major clades corresponding with minor modifications to taxonomical groups are indicated.

C. flavovirens as a sister. The subclades /Calochroid and /Rufoolivacei are collectively supported as a sister clade to the /Fulvi subclade. The support for subclades /Caesiocortinati and /Percomes is strong, but the support for /Glaucopodes is weak. The /Calochroi clade was also strongly supported in Peintner et al. (2004) and Garnica et al. (2003). The /Phlegmacium clade was supported with 70% posterior probability in Peintner et al. (2004),

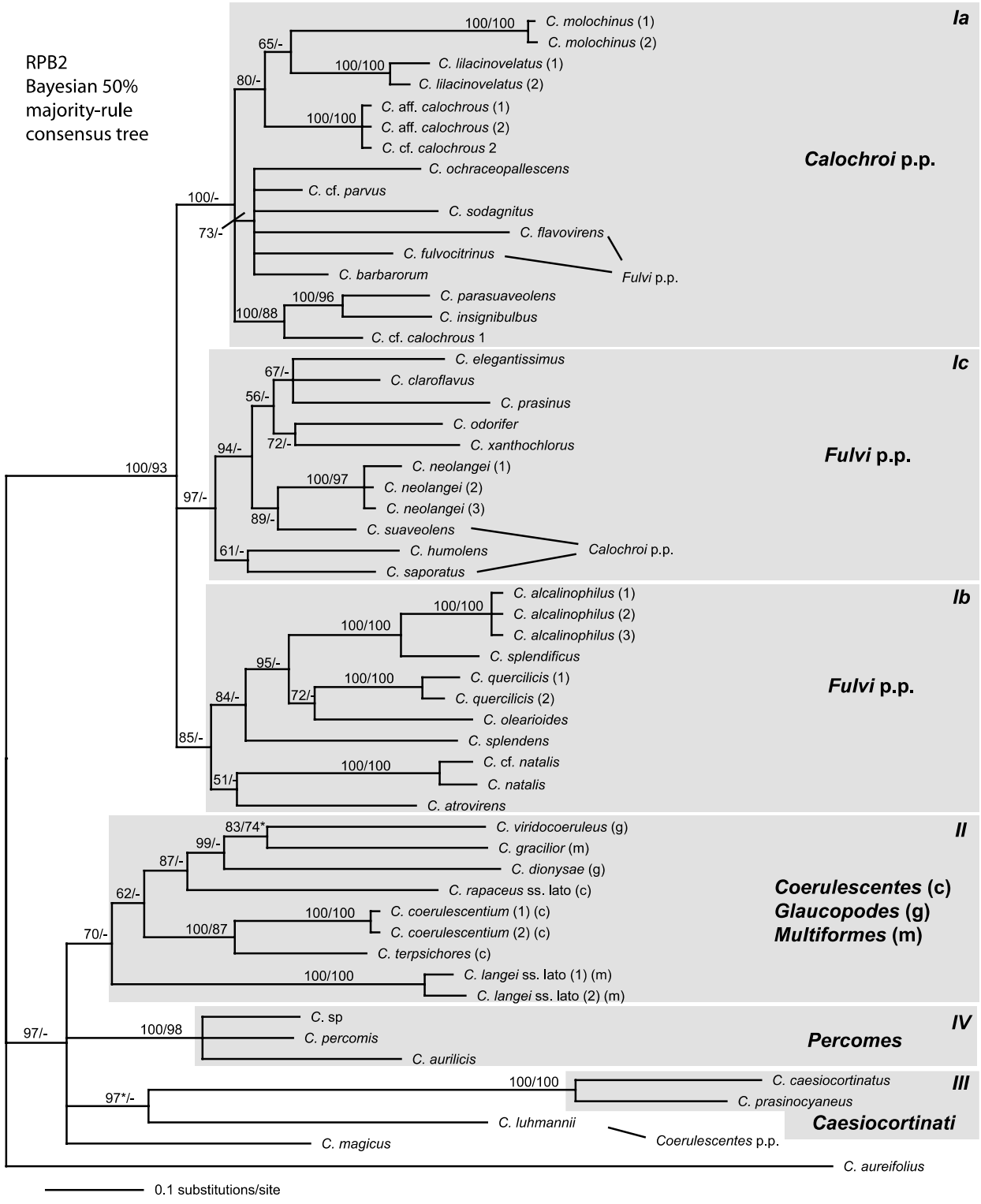

Fig. 4. The Bayesian 50% majority-rule consensus tree inferred from RPB2. Posterior probabilities and bootstrap values above 50% are indicated above branches (PP/BP). Bayesian posterior probabilities above 95% and bootstraps above 65% marked with an asterisk support clades not supported in combined analyses. Major clades corresponding with minor modifications to taxonomical groups are indicated.

but was paraphyletic with respect to /Calochroi in Garnica et al. (2003). The subclades /Calochroid, /Fulvi, and /Glaucopodes were also supported in Garnica et al. (2003).

3.2.6. Comparison of analyses

The amount of resolution and supported nodes (see Table 2) is lowest in ITS analyses and highest in the combined analyses. Single analyses of RPB1 and RPB2

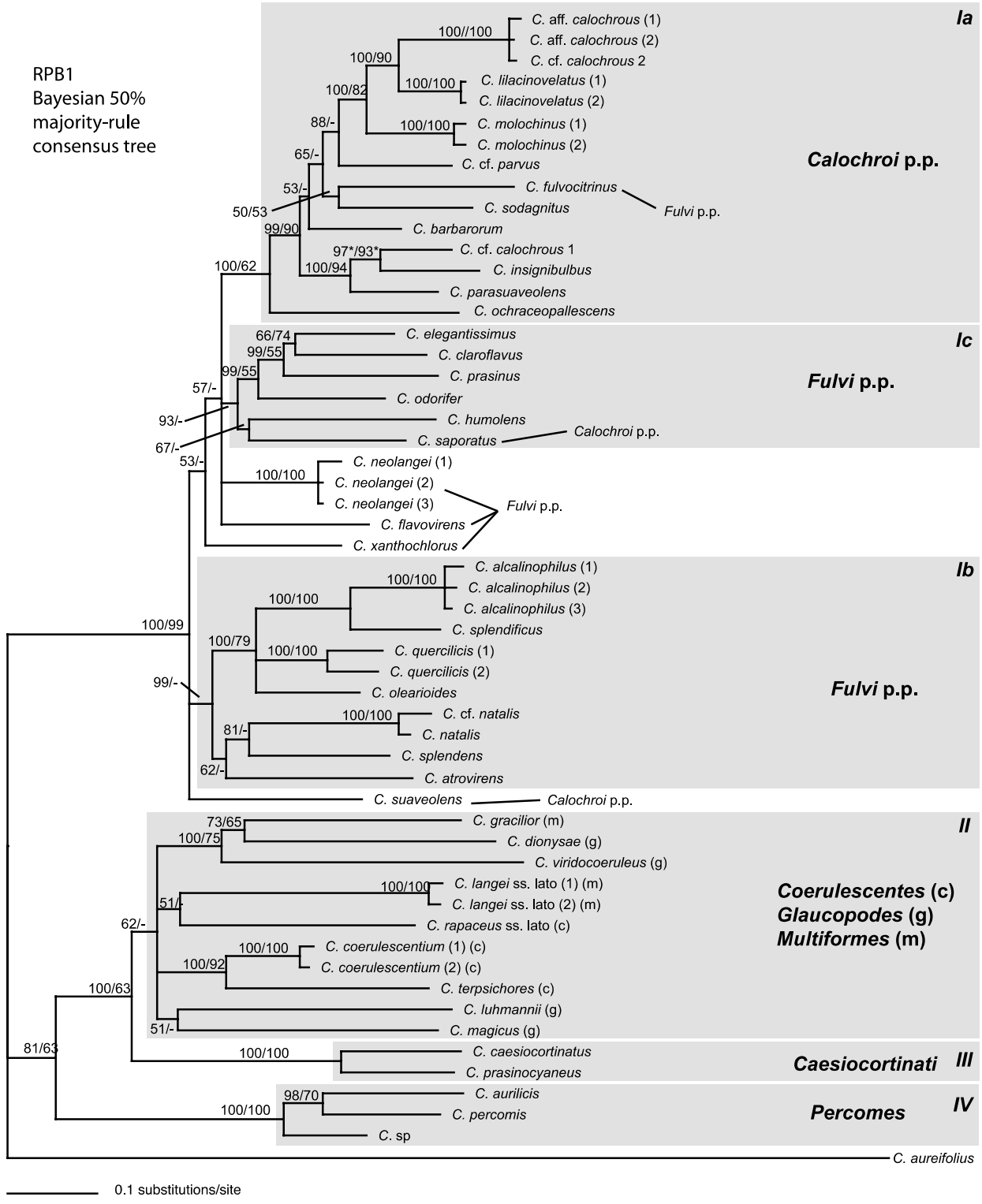

Fig. 5. The Bayesian 50% majority-rule consensus tree inferred from RPB1. Posterior probabilities and bootstrap values above 50% are indicated above branches (PP/BP). Bayesian posterior probabilities above 95% and bootstraps above 65% marked with an asterisk support clades not supported in combined analyses. Major clades corresponding with minor modifications to taxonomical groups are indicated.

give the second and third most resolved and supported topologies, respectively. As would be intuitively expected, the performances of the single genes is related to the amount of parsimony-informative sites. However, the single-gene phylogenies show resolving power for different parts of the overall phylogeny, and the combination of all three genes results in an inference that is not contradicting any of the single-gene analyses markedly,

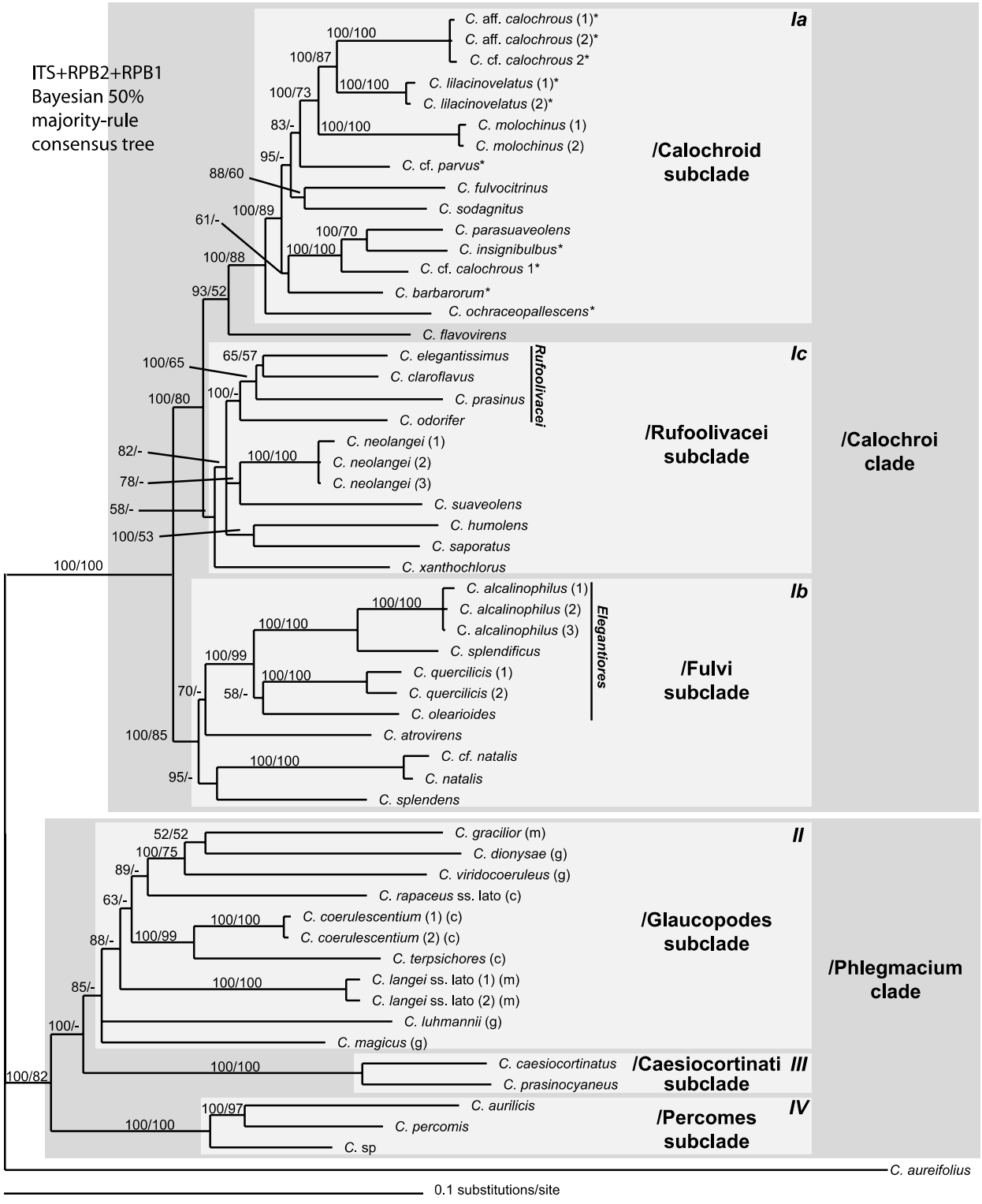

Fig. 6. The Bayesian 50% majority-rule consensus tree inferred from RPB1+RPB2+ITS. Posterior probabilities and bootstrap values above 50% are indicated above branches (PP/BP). New clade names introduced (see text for discussion). Species of /Calochroid matching a broad definition of *C. calochrous* ss. lato are marked with an asterisk.

but contains more resolution and support. The combined Bayesian analyses showed the greatest amount of resolution and nodal support of all data sets. Some authors argue that this can be taken as a proof of the combinability of the information from separate genes (Cunningham, 1997a,b; Hoffstetter et al., 2002; Moncalvo et al., 2000). Analyses of RPB2 show more resolution than ITS analyses, and recover all the major clades

found in the analyses of RPB1 and the combined RPB1+RPB2+ITS data set. The amount of support and resolution of the single RPB1 analysis is only slightly less than the combined Bayesian analysis. These patterns are very similar to those found in the comparison of support and resolution for nLSU and RNA polymerase II genes in another clade of mushroom-forming fungi (Matheny, 2005).

Two of the 63 nodes supported by more than 95% posterior probability and two of the 56 nodes receiving a bootstrap value above 65% in the single-gene analyses are not supported in the combined analyses (one in both categories being the node discussed in section 3.3.5). These are indicated in the single-gene trees. Of the 42 nodes supported by 50–95% posterior probability 20 were not supported by combined analyses, and 2 of the 8 receiving 50–65% bootstrap support were not supported in combined analyses. This indicates that posterior probabilities of more than 95% and bootstrap values of more than 65% are good indicators of robust single-gene branches in these analyses.

4. Discussion

4.1. Variable regions of RPB1 and RPB2 produce more phylogenetic signal than ITS

Sequence data from the two largest subunits of RNA polymerase II increase resolution and nodal support of closely related species of *Cortinarius*.

RPB1 was the largest fragment (≈1400 bp) and also contributed with the highest proportion of informative characters (0.48) to the combined analyses, and the results of single-gene RPB1 analyses approach the combined analyses. ITS (≈680 bp) and the RPB2 fragment (≈750 bp) were of comparable length, but RPB2—though almost entirely protein-coding—contributed with a higher proportion (0.31) of parsimony-informative characters than ITS (0.21), and recovered more of the clades found in combined analyses. These results indicate that, with these markers and an increased taxon sampling at this low level, we will be able to produce robust phylogenies allowing us to trace the evolution of morphology and ecology for species in *Cortinarius*.

Cortinarius has proven very difficult to resolve phylogenetically. As RPB1 and RPB2 have been shown to be very effective at higher taxonomic levels (genus/family level) for *Inocybe*, it is our prediction that these markers might resolve more basal relationships of *Cortinarius* as well, allowing us to address the major infrageneric relationships of this large genus, and test biogeographic and major evolutionary hypotheses.

4.2. Concerns about ITS as a single marker for mushroom phylogenetic studies

It is clear from these findings that phylogenies of *Cortinarius* based on information from ITS alone should be interpreted with caution. For very closely related species pairs, ITS produces comparable results to RPB1 and RPB2 and combined analyses. It clusters "species" and closely related groups with high nodal support and also supports very distinct evolutionary lineages (e.g., the /Caesiocortinati and /Calochroi clades). However, of the few remaining relationships recovered by ITS, only nodes receiving high support (>65% bootstrap support and >95% Bayesian posterior probability) are consistently supported in combined analyses. All other nodes (of strict consensus MP trees or Bayesian consensus trees) are not supported by RPB1, RPB2 or combined analyses. This indicates that nodes receiving support below this level are not robust and do not reflect evolution as it is inferred from RPB1 and RPB2 and combined analyses.

4.3. Phylogenetic relationships of Cortinarius subgenus Phlegmacium p.p. and taxonomic implications

The following discussion will be based on the findings of the combined analyses except where major discrepancies between analyses exist. The nomenclature refers to the names introduced in Fig. 6.

Our analyses show two major well-supported major clades, which correspond to clades /Calochroi and /Phlegmacium in Peintner et al. (2004). However, we find several additional well-supported clades of which several correspond with some morphological characters and/or traditional taxonomic groups. The /Calochroi clade contains two well-supported subclades (/Calochroid and /Fulvi) and a third weakly supported clade (/Rufoolivacei). The /Phlegmacium clade contains two well supported subclades (/Caesiocortinati and /Percomes) and a major clade receiving less support (/Glaucopodes).

The /Calochroi clade was recovered with strong support in all analyses. All species of the clade have a simplex cap cuticle and a very broad marginate bulb and a coarse net-like spore ornamentation. The simplex cap cuticle, which is synapomorphic for species of /Calochroi, is, however, also found in *C. langei* ss. lato in the /Glaucopodes clade and *C. caesiocortinatus* and *C. prasinocyaneus* of the /Caesiocortinati clade. The subclades /Calochroid and /Fulvi + /Rufoolivacei correspond with minor modifications to sections *Calochroi* and *Fulvi*, respectively. In ITS, RPB1 and combined analyses the /Calochroid subclade is strongly supported as a derived clade making section *Fulvi* paraphyletic. Subclades /Fulvi and /Rufoolivacei contain almost all taxa with anthraquinoid pigments and only two non-anthraquinoid species, *C. saporatus* and *C. suaveolens*. The

/Calochroid subclade contains only one taxon with anthraquinoid pigments (*C. fulvocitrinus*). *Cortinarius flavovirens* (also containing anthraquinoid pigments) is supported as a sister group to the /Calochroid clade with 93% posterior probability, a relationship only strongly supported by RPB2. Some of the subsections of *Fulvi* defined on the type of anthraquinoid pigment present (Brandrud, 1998) are found with minor modifications. Most species of subsection *Elegantiores* are supported as a monophyletic group in /Fulvi receiving 100% posterior probability. *C. humolens* also classified in *Elegantiores* is, however, unrelated. The three species of subsection *Splendentes* (*C. splendens*, *C. fulvocitrinus*, and *C. xanthochlorus)* are unrelated, with one species in each major sub-clade of /Calochroi. Subsection *Rufoolivacei* represented by *C. elegantissimus*, *C. claroflavus*, *C. prasinus*, and *C. odorifer* formed a highly supported (100% posterior probability) monophyletic group. Furthermore, our results confirm the results of Garnica et al. (2003) showing that the lineage (/Percomes) of species containing anthraquinoid pigments, treated as subsection *Percomes* of section *Fulvi* by Brandrud (1998) is unrelated to the other anthraquinone containing species.

Many calochroid taxa (yellow cap and lilac lamellae) have been treated as subspecies and varieties of *C. calochrous*. Several of the included samples (marked with an asterisk in Fig. 6) could within a broad morphological species concept be classified as *C. calochrous*: *C.* aff. *calochrous*, *C.* cf. *calochrous*, *C. barbarorum*, *C. insignibulbus*, *C. lilacinovelatus*, *C. ochraceopallescens*, and *C.* cf. *parvus* (1, 2). These results, however, clearly support the existence of many separate species within this morphological complex. First of all, there is congruency between single-gene analyses satisfying a Genealogical Concordance Phylogenetic Species Concept ss Taylor et al. (2000). Furthermore, the group of *C. calochrous* ss lato is paraphyletic with respect to several morphologically (and phylogenetically) well-delimited species (*C. molochinus*, *C. sodagnitus*, *C. parasuaveolens*, and *C. fulvocitrinus*).

The /Phlegmacium clade is only well-supported by RPB1 and RPB2 and combined analyses. All sampled species of sections *Coerulescentes*, *Glaucopodes*, and *Multiformes* are recovered in the /Glaucopodes subclade. The internal branching of /Glaucopodes is not robust, but the presence of (often weak) bluish colors on the pileus, the character used to delimit section *Coerulescentes* (species marked with c) from *Glaucopodes* (species marked with g) does not seem to be phylogenetically informative. The sampling of /Glaucopodes is relatively sparse and internal branching is also relatively weak. Further sampling of the /Glaucopodes clade is urgently needed to elucidate the phylogenetic relationships of these taxa.

All analyses support the /Caesiocortinati clade. The two contained species have a very similar habit. They both have a slightly deviating type of marginate bulb (Fig. 1E). It is not as wide in other groups, has a slightly arrowhead shaped (sagittiform) and is more rooting. The margin of the pileus is often draped with remnants of the cortina (the partial veil) colored from mature spores. In most other species the partial veil is only seen as a zone on the stipe. The most distinguishing character, however, is the globose spores with a conspicuous ornament distinguishing it from all other phlegmacioid species. *C. caesiocortinatus* has traditionally been placed in section *Calochroi* due to the simplex structure of the cap cuticle and absence of anthraquinoid pigments. *C. caesiocortinatus* and *C. prasinocyaneus* are however not related to this or other formally defined groups of *Cortinarius* species. We therefore suggest a new section for these two species that so far are the only taxa known with the mentioned combination of characters.

Cortinarius Section *Caesiocortinati* T.G. Frøslev and T. S. Jeppesen sect. nov.

Pileo 5–10 cm lato, hemisphaerico, dein plano-convexo, glutinoso. Lamellis emarginatis. Velo universale albido vel violaceo. Stipite bulboso, bulbo marginato. Sporis subgloboseis, grosse verrucosis. In silvis frondosis. In solo calcareo. Typus sectionis: *Cortinarius caesiocortinatus* Jul. Schäff.

Pileus 5–10 cm wide, hemispheric, then plano-convex, glutinous. Lamellae emarginate, often serrulate. Stipe bulbose, bulb marginate, but relatively narrow and slightly radicating. Spores (sub-)globose, with coarse ornament. In deciduous forest on calcareous ground.

5. Conclusions

Sequence data from the most variable regions of the two largest subunits of RNA polymerase II (RPB1 and RPB2) greatly increase resolution and nodal support in phylogenetic analyses of *Cortinarius*. Phylogenetic relationships based on analysis of ITS alone are only reliable for nodes receiving high bootstrap support or posterior probability. Phylogenetic inferences based on RPB1 data alone result in almost the same topology and amount of resolution and nodal support as in combination with RPB2 and ITS. RPB1 might therefore provide a choice marker for single-gene (and multigene) analyses at several levels in *Cortinarius* and other mushroom groups. Allowing for minor adjustments our findings support some classical views of *Cortinarius* relationships and refute others. Species of section *Calochroi* have been derived several times from fulvoid species with most species belonging to an apical subclade of the /Calochroi clade. The sections *Coerulescentes*, *Glaucopodes*, and *Multiformes* cannot be separated phylogenetically. Some of the chemotaxonomically defined subsections of section *Fulvi* form natural units and others not. Our results support more species than accepted in some morpholog-

ically based taxonomies in the *C. calochrous* "complex." A clade containing *C. caesiocortinatus* and *C. prasinocyaneus* is supported as a separate evolutionary lineage, which we describe as *Cortinarius* subgenus *Phlegmacium* section *Caesiocortinati*.

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