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Reconstructing the evolution of agarics from nuclear gene sequences and basidiospore ultrastructure

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ABSTRACT

Traditional classifications of agaric fungi involve gross morphology of their fruit bodies and meiospore print-colour. However, the phylogeny of these fungi and the evolution of their morphological and ecological traits are poorly understood. Phylogenetic analyses have already demonstrated that characters used in traditional classifications of basidiomycetes may be heavily affected by homoplasy, and that non-gilled taxa evolved within the agarics several times. By integrating molecular phylogenetic analyses including domains D1–D3 and D7–D8 of nucLSU rDNA and domains A–C of the *RPB1* gene with morphological and chemical data from representative species of 88 genera, we were able to resolve higher groups of agarics. We found that the species with thick-walled and pigmented basidiospores constitute a derived group, and hypothesize that this specific combination of characters represents an evolutionary advantage by increasing the tolerance of the basidiospores to dehydration and solar radiation and so opened up new ecological niches, e.g. the colonization of dung substrates by enabling basidiospores to survive gut passages through herbivores. Our results confirm the validity of basidiospore morphology as a phylogenetic marker in the agarics.

Introduction

Agarics comprise the most familiar fungal forms, and fossils exhibiting agaricoid features date back to the mid-Cretaceous (Hibbett *et al.* 1997a). Agarics have undergone an enormous adaptive radiation, with an extant described 8400 species, in the course of which they have occupied a broad range of ecological niches, including ectomycorrhizas, mutualisms with ants and termites, as decomposers of wood and other organic substrates, and as plant pathogens.

Molecular studies (e.g. Hibbett *et al.* 1997b; Larsson *et al.* 2004; Moncalvo *et al.* 2002; see also Hibbett 2004, 2007) have shown that: (1) the core group of agarics ('euagarics'; 'agarics' is used in this sense in this paper) does not contain all species of gilled mushrooms; and (2) this core group also includes non-gilled species. Thus, not only are there morphs representing gilled mushrooms, but also puffballs, bird's nest fungi, polypores, coral-shaped, cup-shaped, and crust-like agarics (Binder *et al.* 2005; Bodensteiner *et al.* 2004; Hibbett 2007; Hibbett *et al.* 1997b; Larsson *et al.* 2004; Moncalvo *et al.* 2002).

The traditional classifications of agarics characterize several higher groups, mostly families, on the basis of morphological traits (Singer 1986). These include the type of attachment of the gills to the stipe, and the colour of spore

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prints (Fig 1). However, although considerable progress has been made in recent years, there is still no consensus as to the natural delimitations within such groupings. We now know that fruit body gross architectures, as well as other morphological traits, evolved more than once in the agarics, but the often relatively uniform anatomy of the fruit bodies and also the scanty fossil record have hampered attempts to reconstruct the agaricalean branch of the tree of life with confidence. Molecular phylogenetic analyses, mostly based on partial sequences of the nucLSU rDNA gene, have revealed several monophyletic groups within the agarics (Moncalvo et al. 2000, 2002). Some of these are congruent with traditional groupings, whereas others are surprisingly different from morphologically-based classifications. However, these analyses could not resolve the deeper nodes of the agaricalean phylogeny.

The purpose of the present study was to derive supported hypotheses about phylogenetic relationships and trends in character evolution within the euagarics by sequencing nuclear genes and comparing macro- and micromorphology of the basidiomes as well as the ultrastructure of the basidiospores. In designing our molecular and morphological analyses, the spectrum of included genera was made as broad as possible. Each specimen used in the molecular analysis was also thoroughly studied using light microscopy.

Materials and methods

Taxon sampling

Ninety taxa, including representative species of 88 euagaric genera, were used in the molecular and morphological analyses to ensure a broad sampling of species covering the morphological variation and taxonomic diversity of this fungal group, both according to traditional treatments (e.g. Kühner 1980; Singer 1986) and to previous molecular studies (Hibbett et al. 1997b; Moncalvo et al. 2002). *Boletus edulis* and *Tapinella panuoides* (bolete clade) were used as outgroup species. Locations of the voucher specimens and GenBank accession numbers for the DNA sequences obtained for this study are listed in Table 1. Representative basidiome types of agarics are shown in Fig 2.

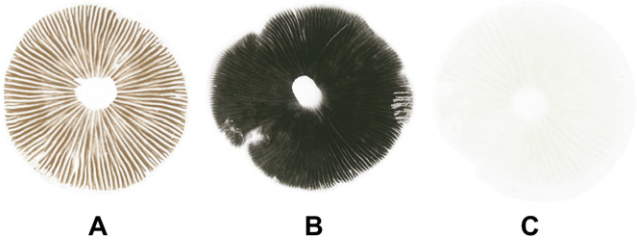

Fig 1 – Spore prints of gilled mushrooms (agarics) obtained from fresh fruiting bodies. (A) Brown spores from *Galerina marginata* TUB 012686. (B) Black spores from *Psathyrella candolleana* TUB 012687. (C) Light-coloured spores from *Laccaria bicolor* TUB 012688.

DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA was extracted from dried basidiomes or mycelial cultures using a DNeasy Plant Kit (QIAGEN, Hilden). The 5′ terminal domain of the nucLSU rDNA gene, including the variable regions D1–D8 (Hassouna et al. 1984) and the portion of the nuclear gene for RNA polymerase II coding for domains A–C of the largest subunit (*RPB1*) (Matheny et al. 2002) were amplified using PCR. The nucLSU sequences were amplified using the primer combinations LROR (5′-ACCCGCTGAACTTAAGC-3′; Moncalvo et al. 1995)/LR9 (5′-AGAGCACTGGGCAGAAA-3′; Hopple & Vilgalys 1999) or LROR/LR6 (5′-CGCCAGTTCTGC TTACC-3′; Vilgalys & Hester 1990) plus LR3R (5′-GTCTTGAAA CACGGACC-3′; Hopple & Vilgalys 1999)/LR9. PCR amplifications of *RPB1* A–C were made using the primers RPB1-A (5′-GARTGYCCDGGDCAYTTYGG-3′) and RPB1-C (5′-CCNGCDAT NTCRTTRTCCATRTA-3′), both described in Matheny et al. (2002). PCR products were purified with a QIAquick kit (QIAGEN). Products of the *RPB1* PCR reactions showing multiple bands (*Boletus edulis*, *Crucibulum leave*, *Hebeloma mesophaeum*, *Pseudoclitocybe cyathiformis*, *Rhodocollybia butyracea* f. *asema,* and *Psathyrella conopilus*) were inserted into a pCR 2.1-TOPO plasmid vector (version R) and cloned using a TOPO TA cloning kit (Invitrogen, Karlsruhe). Sequencing of regions D1–D3 and D7–D8 of the nucLSU and of domains A-C of *RPB1* was performed using an ABI PRISM BigDye cycle-sequencing kit and an automated sequencer ABI 3100 (Applied Biosystems, Foster City, CA). Additional sequencing primers used were LR8 (CACCTTGGA GACCTGCT-3′0; Hopple & Vilgalys 1999) for the nucLSU and RPB1-A SG forward (5′-YTSAARGCYGGTGAGT-3′) and RPB1-B SG reverse (5′-TCCGCRCCYTCYTTGG-3′) that were designed for this study. Forward and reverse sequences were assembled and edited using Sequencher, version 4.1 (Gene Codes, Ann Arbor, MI). The sequences reported in this paper have been deposited in the NCBI database (http://www.ncbi.nlm.nih.gov), and the alignment used for phylogenetic analysis can be obtained from TreeBASE (http://treebase.bio.buffalo.edu/treebase/).

Molecular phylogenetic analysis

Sequences were aligned using CLUSTALX (Thompson et al. 1997) resulting in 2279 aligned nucleotide positions. Minor manual adjustments of the *RPB1* sequence alignment were made in SeAl v2.11 (Rambaut 2002). Ambiguous alignment regions were excluded for the phylogenetic analysis. The final alignment of 2047 characters was analysed using a Bayesian MCMC (BMCMC) approach as implemented in MrBayes, version 3.1 (Ronquist & Huelsenbeck 2003), with a partition of the dataset into subsets (Nylander et al. 2004) corresponding to the first, second, and third codon positions of the exon regions of the *RPB1* gene, and the nucLSU gene. Appropriate DNA substitution models for each subset were determined using MrModeltest (Nylander 2004) and the Akaike information criterion (Posada & Crandall 2001); the selected models were GTR+I+G for the first and second codon positions of the *RPB1* gene, as well as for the nucLSU gene, and GTR+G for the third codon position of the *RPB1* gene (Swofford et al. 1996). Two independent Metropolis-coupled (Geyer 1991) BMCMC processes were run, each over 10 M generations, and involving a cold and three incrementally heated chains, with each

100th tree of the cold chains sampled. Stationarity of the processes was assessed using Tracer (Rambaut & Drummond 2003). From the stored trees, a majority-rule consensus was computed, excluding the first 40 % of stored trees from each run. We also analysed the dataset using maximum parsimony (MP) bootstrapping (Felsenstein 1985) in PAUP (Swofford 2002), involving 1K resamplings of the original alignment, in each round performing a heuristic search with ten rounds of branch-swapping (tree bisection and reconnection) over starting trees obtained by consecutive addition of sequences in random order, with gaps treated as missing data.

Ancestral state reconstruction

We reconstructed ancestral states of morphological characters (thickness of basidiospore walls exceeding/not exceeding 200 nm; basidiospores pigmented/not or only slightly pigmented) using unweighted Wagner parsimony on the BMCMC consensus tree using PAUP. Ambiguous ancestral character states were resolved using the accelerated transformation (ACCTRAN) and the delayed transformation (DELTRAN) strategies (Swofford & Maddison 1987).

Morphological analyses

Anatomical and micromorphological analyses of all sequenced species were made from freshly collected and dried specimens by light microscopy (LM). Ultrastructure of the basidiospore walls was examined for selected species using transmission electron microscopy (TEM), with sample preparation as described in Bauer *et al.* (1997). Measurements of spore wall thickness were made from longitudinal serial and non-serial TEM sections. The following specimens were included to represent the morphological diversity of spore walls according to traditional concepts (e.g. Pegler & Young 1971; Singer 1986; Clémençon 1997): (1) thin-walled (= simple wall; *Hygrophorus eburneus* TUB 012681, *Cystoderma amianthinum* TUB 011551, *Crepidotus cesatii* TUB 014810, *Ripartites tricholoma* TUB 012683); (2) somewhat thickened, heterogeneous wall (Singer 1986; *Fayodia gracilis* TUB 011585); and (3) thick-walled (*Cortinarius elegantior* TUB 012684, *Cortinarius hercynicus* TUB 014801, *Hebeloma magnimamma* TUB 014806, *Hypholoma fasciculare* TUB 012682, *Laccaria amethystina* TUB 014802 and TUB 014853, *Laccaria tortilis* TUB 014808, and *Lacrymaria lacrymabunda* TUB 014804). Additionally, we compiled measurements of spore wall thickness from the literature (Bennell *et al.* 1985; Besson 1972; Capellano 1976; Capellano & Kühner 1975; Clémençon 1970, 1973, 1974, 1977a, 1977b, 1997; Kost 1981; Lingle *et al.* 1992; Pegler & Young 1986; Rast & Hollenstein 1977; Ruch & Motta 1987). Wall thickness measurements were always made excluding any ornamentation. We then integrated our original data (comparative LM from all sequenced species plus exact TEM measurements from the specimens detailed above) with literature reports to divide our set of sequenced species into two classes, corresponding to whether the spore wall thickness exceeded 200 nm or not. Our original observations concerning basidiospore colour, colour reactions with Melzer's reagent and Cotton-blue, morphology, and wall measurements, together with wall measurements compiled from literature, are summarized in Table 2.

Results and discussion

The results of our molecular phylogenetic analyses of the combined alignment of original nucLSU and *RPB1* sequences are shown in Fig 2. A comparison of the phylogenetic tree with the distribution of the ultrastructural character 'basidiospore wall thickness exceeding/not exceeding 200 nm' and the chemical character 'basidiospores pigmented/not or only slightly pigmented' in the species studied (Fig 2 and the supplementary Data appendix) shows that: (1) there are several distinct clades of agarics that include only species with unpigmented or only slightly pigmented and mostly thin-walled basidiospores; and (2) agarics with a combination of thick-walled and pigmented basidiospores (except for *Ripartites*) form a natural group (Fig 2, arrow). Alternative phylogenetic hypotheses assuming the monophyly of agarics with thick-walled basidiospores or those with pigmented basidiospores were rejected with posterior probabilities of zero in BMCMC analysis. MP reconstruction of ancestral states along the BMCMC consensus yielded the following results: (1) the evolution of complex basidiospore walls involved four gains and four losses (ACCTRAN) or five gains and three losses (DELTRAN); and (2) the evolution of pigmented basidiospores involved four gains and three losses (ACCTRAN) or seven gains and no loss (DELTRAN). Ancestral state reconstructions using the ACCTRAN and DELTRAN criteria are shown in Fig 2 and the Supplementary Data appendix, respectively. Both reconstruction variants suggest that complex basidiospore walls are a synapomorphy for the clade marked with an arrow in Fig 2.

The thick-walled and pigmented basidiospore syndrome

Our results support traditional classification systems in which agarics with thick-walled and pigmented basidiospores are treated as a derived group, and where ones with unpigmented and thin-walled basidiospores were considered as more basal taxa (Kühner 1980; Oberwinkler 1982; with the exception of the genera *Crucibulum*, *Cyathus*, *Laccaria*, and *Leucocortinarius*). Our ultrastructural analyses revealed that strongly pigmented basidiospores correlate with complex basidiospore wall architecture (Fig 3) in the vast majority of species, which is consistent with the results of Clémençon (1997), who analysed the ultrastructure of mature basidiospores of several species of *Agaricales*. Thus, the development of thick-walled and pigmented basidiospores (corresponding to the arrowed clade in Fig 2) was probably a key event in the evolution of the agarics.

Evolutionary advantages of thick-walled and pigmented basidiospores

The evolution of this novel syndrome of morphological and chemical traits can be interpreted as an adaptation to harsh environmental conditions on land. Spores of these fungi may be more resistant to dehydration and UV radiation than thin-walled and hyaline basidiospores. This agrees with the observations of Clémençon (1997), who reported that hyaline and thin-walled spores lose their capacity to germinate after a few days. Conversely, it has been demonstrated (Watling

Table 1 – List of specimens used in the morphological and molecular analyses

Species	Specimen voucher	GenBank accession nucLSU rDNA D1–D3	GenBank accession nucLSU rDNA D7–D8	GenBank accession *RPB1* A–C
Agaricaceae				
Agaricus bisporus	TUB 011586	DQ071710	DQ071783	DQ067962
Chamaemyces fracidus	GLM 45875	AY207148	DQ071787	DQ067957
Cystoderma amianthinum	TUB 011551	DQ071703	DQ071774	DQ067951
Cystolepiota sistrata	TUB 011552	DQ071711	DQ071786	DQ067958
Lepiota xanthophylla	TUB 011553	DQ071712	DQ071788	DQ067956
Macrolepiota procera	GLM 45957	AY207233	DQ071785	DQ067961
Phaeolepiota aurea	TUB 011557	DQ071704	DQ071775	DQ060817
Amanitaceae				
Amanita phalloides	TUB 011556	DQ071721	DQ071810	DQ067953
Limacella glioderma	HKAS ZLY D 72	DQ071728	DQ071818	DQ067952
Bolbitiaceae				
Agrocybe praecox	GLM 51238	DQ071692	DQ071758	DQ067985
Bolbitius vitellinus	GLM 45874	AY207147	DQ071761	DQ067989
Conocybe teneroides	GLM 45901	AY207171	DQ071760	DQ067981
Descolea antarctica	TUB 011558	DQ071693	DQ071762	DQ067982
Clavariaceae				
Macrotyphula fistulosa	TUB 011469	DQ071735	DQ071827	DQ067802
Coprinaceae				
Anellaria semiovata	GLM 51235	DQ071694	DQ071763	DQ067965
Coprinopsis lagopus	GLM 45907	AY207183	DQ071779	DQ067986
Coprinus comatus	GLM 45914	AY207179	DQ071784	DQ067959
Paneolina foenisecii	FO 46609	DQ071696	DQ071765	DQ067963
Paneolus acuminatus	GLM 46071	DQ071695	DQ071764	DQ067964
Psathyrella conopilus	TUB 011587	DQ071706	DQ071777	DQ067988
Cortinariaceae				
Cortinarius violaceus	TUB 011825	DQ071705	DQ071776	DQ067972
Galerina badipes	GLM 45922	AY207201	DQ071754	DQ067975
Gymnopilus penetrans	GLM 45929	AY207208	DQ071752	DQ060814
Hebeloma mesophaeum	TUB 011577	DQ071690	DQ071756	DQ067971
Inocybe fastigiata	FO 46800	DQ071697	DQ071766	DQ067980
Naucoria escharoides	FO 46893	DQ071691	DQ071757	DQ067970
Leucocortinarius bulbiger	TUB 011568	DQ071745	DQ071837	DQ060819
Phaeogalera oedipus	GLM 45993	AY207268	DQ071759	DQ067983
Crepidotaceae				
Crepidotus mollis	TUB 011566	DQ071698	DQ071767	DQ067977
Simocybe sumptuosa	TUB 011584	DQ071699	DQ071768	DQ067976
Tubaria hiemalis	GLM 46038	AY207311	DQ071769	DQ067979
Entolomataceae				
Nolanea sericea	GLM 45918	AY207197	DQ071794	DQ067948
Rhodocybe gemina	TUB 011578	DQ071715	DQ071793	DQ067944
Fistulinaceae				
Fistulina hepatica	TUB 011583	DQ071727	DQ071817	DQ060815
Gomphaceae				
Lentaria albovinacea	FO 46869	DQ071734	DQ071826	DQ060806
Hygrophoraceae				
Hygrocybe conica	FO 46714	DQ071739	DQ071831	DQ060810
Hygrophorus chrysodon	TUB 011570	DQ071733	DQ071825	DQ067995
Lentinaceae				
Phyllotopsis nidulans	TUB 011567	DQ071736	DQ071828	DQ060803
Lycoperdaceae				
Bovista nigrescens	FO 46606	DQ071709	DQ071782	DQ067960
Nidulariaceae				
Crucibulum laeve	TUB 011564	DQ071701	DQ071771	DQ067950
Cyathus striatus	TUB 011565	DQ071742	DQ071834	DQ060821
Omphalotaceae				
Omphalotus illudens	TUB 012155	DQ071741	DQ071833	DQ060813

Table 1 (*continued*)

Species	Specimen voucher	GenBank accession nucLSU rDNA D1–D3	GenBank accession nucLSU rDNA D7–D8	GenBank accession *RPB1* A–C
Pleurotaceae				
Pleurotus ostreatus	TUB 011571	DQ071722	DQ071811	DQ060804
Pluteaceae				
Pluteus cervinus	FO 46619	DQ071729	DQ071819	DQ067955
Volvariella caesiocincta	FO 46681	DQ071726	DQ071816	DQ068009
Schizophyllaceae				
Schizophyllum commune	TUB 012156	DQ071725	DQ071814	DQ068011
Strophariaceae				
Flammula alnicola	GLM 45994	AY207269	DQ071755	DQ067974
Flammulaster muricata	TUB 012150	DQ071740	DQ071832	DQ060812
Hypholoma radicosum	TUB 011572	DQ071685	DQ071748	DQ067968
Phaeomarasmius rimulincola	FO 46666	DQ071700	DQ071770	DQ067978
Pholiota flammans	TUB 011573	DQ071688	DQ071751	DQ067973
Psilocybe inquilina	GLM 51242	DQ071689	DQ071753	DQ067969
Stropharia aeruginosa	TUB 012151	DQ071686	DQ071749	DQ067967
S. coronilla	GLM 46074	DQ071687	DQ071750	DQ067966
Tricholomataceae				
Armillaria ostoyae	GLM 45872	AY207145	DQ071808	DQ067932
Arrhenia auriscalpium	TUB 011588	DQ071732	DQ071824	DQ068008
Calocybe gambosa	TUB 011576	DQ071716	DQ071795	DQ067945
Catathelasma imperiale	TUB 011562	DQ071743	DQ071835	DQ060820
Clitocybe connata	TUB 011574	DQ071714	DQ071792	DQ067946
C. geotropa	GLM 45881	AY207154	DQ071821	DQ067938
C. vibecina	GLM 45888	AY207160	DQ071791	DQ067947
Crinipellis stipitaria	GLM 45915	AY207194	DQ071800	DQ067933
Collybia marasmioides	GLM 45932	AY207167	DQ071804	DQ060816
Fayodia gracilis	TUB 011585	DQ071744	DQ071836	DQ060818
Flammulina velutipes	GLM 45921	AY207200	DQ071806	DQ067998
Hohenbuehelia petaloides	TUB 011579	DQ071723	DQ071812	DQ060805
Laccaria bicolor	TUB 011581	DQ071702	DQ071773	DQ067942
Lachnella villosa	CCJ 1547	DQ071724	DQ071813	DQ060807
Lentinula edodes	TUB 012149	DQ071718	DQ071802	DQ067997
Lepista nuda	TUB 012152	DQ071713	DQ071790	DQ067954
Lyophyllum decastes	GLM 45952	AY207228	DQ071789	DQ067949
Marasmiellus ramealis	GLM 45958	AY207236	DQ071801	DQ067996
Marasmius rotula	GLM 45962	AY207238	DQ071799	DQ067934
Megacollybia platyphylla	GLM 45963	AY207239	DQ071798	DQ067935
Melanoleuca melaleuca	FO 46845	DQ071730	DQ071820	DQ067941
Mycena polygramma	TUB 011575	DQ071707	DQ071780	DQ060800
Mycenella bryophila	KR 7435	DQ071720	DQ071809	DQ067937
Oudemansiella radicata	FO 46757	DQ071719	DQ071807	DQ067930
Panellus stipticus	GLM 51801	DQ071708	DQ071781	DQ060801
Physalacria sp.	HKAS 32011	DQ071717	DQ071797	DQ067990
Pleurocybella porrigens	TUB 012154	DQ071737	DQ071829	DQ067994
Pseudoclitocybe cyathiformis	GLM 46020	AY207296	DQ071772	DQ067939
Rhodocollybia butyracea f. *asema*	GLM 46024	AY207163	DQ071803	DQ067999
Ripartites tricholoma	GLM 46025	AY207297	DQ071822	DQ067936
Sarcomyxa serotina	TUB 012153	DQ071731	DQ071823	DQ067993
Strobilurus esculentus	GLM 46027	AY207299	DQ071805	DQ067931
Tricholoma vaccinum	GLM 46037	AY207307	DQ071796	DQ067943
Tricholomopsis rutilans	TUB 011582	DQ071738	DQ071830	DQ067984
Xeromphalina campanella	GLM 46039	AY207312	DQ071815	DQ067940
***Boletales* (outgroup)**				
Boletus edulis	FO 46874	DQ071747	DQ071839	DQ067991
Tapinella panuoides	GLM 45992	DQ071746	DQ071838	DQ067992

Classification is according to Singer (1986), Index Fungorum (http://www.indexfungorum.org), and the NCBI taxonomy homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/).

Herbarium acronyms: CCJ, private herbarium C.-J. Chen; FO, private herbarium F. Oberwinkler; KR, private herbarium K.-H. Rexer; GLM, Staatliches Museum für Naturkunde Görlitz; HKAS, Cryptogamic Herbarium, Kunming Institute of Botany, Academia Sinica; TUB, Herbarium, University of Tübingen.

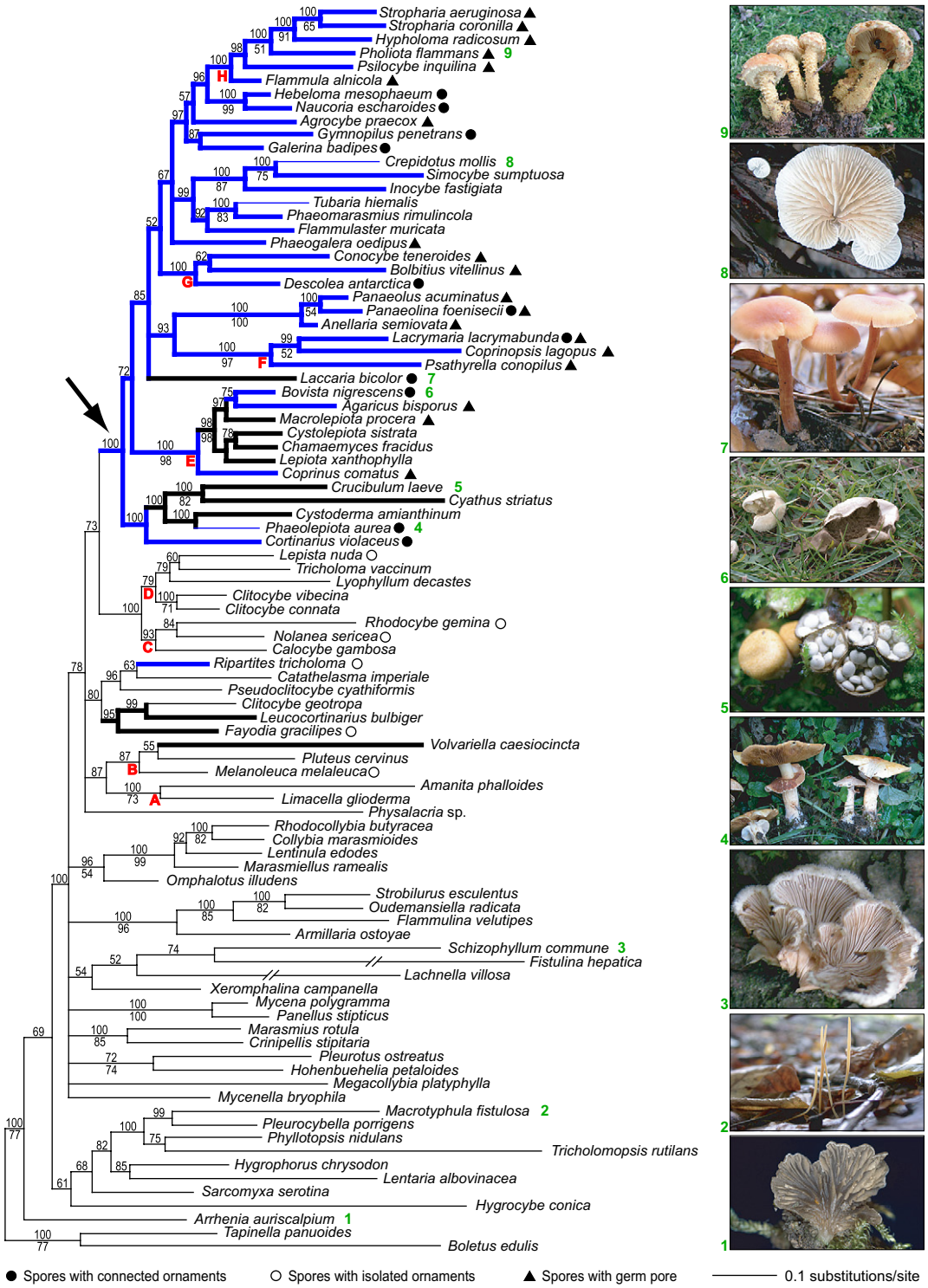

Fig 2 – Phylogeny of agaric mushrooms with *Boletus edulis* and *Tapinella panuoides* as outgroup species. The phylogram was inferred from 2047 concatenated nucleotides of nuclear genes covering domains D1–D3 and D7–D8 of the nucLSU and exon regions of the domains A–C of the largest subunit of RNA polymerase II, *RPB1*, using a BMCMC approach. Branch support is given in terms of posterior probabilities as inferred from the BMCMC analysis (numbers above branches) and MP bootstrap values (numbers below branches). Values below 50 % are omitted. The terminal branches of *Fistulina hepatica* and *Lachnella villosa* were reduced to half for graphical reasons. Blue lines indicate dark-spored species. Spore walls exceeding 200 nm in width are designated with bold lines. Ancestral character states were reconstructed using MP; ambiguous character states are resolved according to the accelerated transformation (ACCTRAN) criterion. The arrow marks the clade showing complex basidiospore walls as a synapomorphy. Red letters indicate branches that correspond to the following families: A, *Amanitaceae*; B, *Pluteaceae*; C, *Entolomataceae*; D, *Tricholomataceae s. str.*; E, *Agaricaceae*; F, *Psathyrellaceae*; G, *Bolbitiaceae*; H, *Strophariaceae*.

Table 2 – Basidiospore features for the agarics used in the molecular phylogenetic analysis

Taxa	Basidiospore features	Basidiospore wall thickness (nm)
Stropharia aeruginosa	Ovoid–ellipsoid with a small germ pore, smooth, brownish, inamyloid	>200
S. coronilla	Ellipsoid with a truncated germ pore, brownish, smooth, inamyloid	540[2]
Hypholoma radicosum	Ellipsoid with a small germ pore, smooth, yellowish brown, inamyloid	>200 [303–750][2,*]
Pholiota flammans	Ellipsoid with a small germ pore, smooth, yellowish, inamyloid	>200 [280–795][5,7]
Psilocybe inquilina	Lentiform (hexagonal in outline) with a truncated germ pore, smooth, brownish, inamyloid	>200 [700–800][15]
Flammula alnicola	Oval with a germ pore, smooth, yellowish brown, inamyloid	>200
Hebeloma mesophaeum	Ellipsoid minutely punctate, yellowish brown, inamyloid	>200 [357–392]*
Naucoria escharoides	Almond-shaped to subcitriform, warty-rough, yellowish brown, inamyloid	>200 [296–370][2]
Agrocybe praecox	Ellipsoid with a truncated germ pore, smooth, yellowish brown, inamyloid	>200 [400–1000][5]
Gymnopilus penetrans	Almond-shaped, warty-rough, rust brown, inamyloid	420[7]
Galerina badipes	Ellipsoid to almond-shaped, warty-rough with a plage, rust brown, dextrinoid	500–590[7,10] [327–770][5,10]
Crepidotus mollis	Ellipsoid to subalmond-shaped, smooth, yellowish brown, inamyloid	<200 [110–131][8,10,*]
Simocybe sumptuosa	Ellipsoid to reniform, smooth, brownish, inamyloid	>200
Inocybe fastigiata	Phaseoliform, smooth, yellowish brown, inamyloid	>200 [161–656][3]
Tubaria hiemalis	Ellipsoid, smooth, pale brownish, inamyloid	<200 [77–145][9,10]
Phaeomarasmius rimulincola	Ellipsoid, smooth, rust brown, inamyloid	>200 [281][10]
Flammulaster muricata	Ellipsoid to reniform, smooth, yellowish brown, inamyloid	>200
Phaeogalera oedipus	Ellipsoid with a small germ pore, smooth, brownish, inamyloid	>200 [491][10]
Conocybe teneroides	Ellipsoid with a germ pore, smooth, intense rust brown, inamyloid	>200 [842][2]
Bolbitius vitellinus	Ellipsoid with a truncated germ pore, smooth, red brown, inamyloid	>200
Descolea antarctica	Citriform, apically mucronate, warty-rough, rust brown, inamyloid	>200
Panaeolus acuminatus	Almond-shaped to citriform with a broad germ pore, smooth, reddish brown, inamyloid	>200 [1000–1094][2]
Paneolina foenisecii	Almond-shaped to citriform with a truncated germ pore, warty-rough, brown, inamyloid	>200
Anellaria semiovata	Ellipsoid with a broad germ pore, smooth, dark reddish brown, inamyloid	1750–2375[2]
Lacrymaria lacrymabunda	Citriform with a protracted germ pore, coarsely warty, black brown, inamyloid	357–482*
Coprinopsis lagopus	Ellipsoid with a distinctive germ pore, smooth, dark red–brown, inamyloid	>200 [576–667][10]
Psathyrella conopilus	Ellipsoid with a slightly truncated germ pore, smooth, dark brown, inamyloid	>200
Laccaria bicolor	Broadly ellipsoid to subglobose, spiny, hyaline, inamyloid, weakly cyanophilous	>200 [295–714][2,10,*]
Bovista nigricans	Subglobose with a pedicel, finely warty, brownish, inamyloid	>200
Agaricus bisporus	Ovate to ellipsoid with a germ pore, smooth, brown to dark brown, inamyloid	580[14] [636][10]
Macrolepiota procera	Ellipsoid with a distinctive germ pore, smooth, hyaline, dextrinoid, cyanophilic	1019–1154[2]
Cystolepiota sistrata	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	>200
Chamaemyces fracidus	Ovoid to ellipsoid, smooth, hyaline, inamyloid, cyanophilic[16]	>200
Lepiota xanthophylla	Sub almond-shaped, smooth, hyaline, dextrinoid, cyanophilic	>200 [518][2]
Coprinus comatus	Ellipsoid to almond-shaped with a truncated germ pore, dark brown, inamyloid	>200
Crucibulum laeve	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	>200
Cyathus striatus	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	>200
Cystoderma amianthinum	Subcylindric–ellipsoid, smooth, amyloid, hyaline, weakly cyanophilic	202*
Phaeolepiota aurea	Ellipsoid–fusoid to elongate almond-shaped, finely warty-rough with a suprahilar plage, rust brown, inamyloid	[152–177][10]
Cortinarius violaceus	Almond-shaped, warty-rough with a distinctive plage, rust brown, inamyloid	285–410* [285–920][5,6,*]
Lepista nuda	Ellipsoid minutely spiny, hyaline, inamyloid, cyanophilic	<200 [58–140][5,10]
Tricholoma vaccinum	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200 [81–150][11]
Lyophyllum decastes	Subglobose, smooth, hyaline, inamyloid, cyanophilic	<200 [81–159][10]
Clitocybe vibecina	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200
C. connata	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200
Rhodocybe gemina	Ellipsoid to broadly ellipsoid, warty rough (angular in polar view), yellowish, inamyloid, cyanophilic	<200 [152–214][2]
Nolanea sericea	Angular subglobose with angular outline, pale yellow, inamyloid, cyanophilic	91[2]
Calocybe gambosa	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200 [125–208][2,10]
Ripartites tricholoma	Subglobose to round, warty, pale yellowish brown, inamyloid, cyanophilic	224–265* [262][2]
Catathelasma imperiale	Ellipsoid to cylindric, smooth, hyaline, amyloid, acyanophilic	<200
Pseudoclitocybe cyathiformis	Ellipsoid, smooth, hyaline, amyloid, cyanophilic	<200
Clitocybe geotropa	Globose, smooth, hyaline, inamyloid, acyanophilic	<200
Leucocortinarius bulbiger	Ellipsoid to subamygdaliform, smooth, hyaline, inamyloid, cyanophilic	312[10]
Fayodia gracilipes	Globose, warty, hyaline, amyloid, cyanophilic	202–281[10,*]
Volvariella caesiocincta	Broadly ellipsoid, smooth, slightly yellowish, inamyloid, cyanophilic	>200 [500–525][4]
Pluteus cervinus	Broadly ellipsoid, smooth, slightly yellowish, inamyloid, cyanophilic	187–211[4] [150–325][4]
Melanoleuca melaleuca	Broadly ellipsoid, minutely warty-rough, hyaline, amyloid, cyanophilic	<200 [170–200][5]
Amanita phalloides	Broadly ellipsoid to subglobose, smooth, hyaline, amyloid, acyanophilic	<200 [50–105][2,10]
Limacella glioderma	Subglobose, smooth, hyaline, inamyloid, acyanophilic	<200
Physalacria sp.	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200

(*continued on next page*)

Table 2 (continued)

Taxa	Basidiospore features	Basidiospore wall thickness (nm)
Rhodocollybia butyracea	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200
Collybia marasmioides	Ellipsoid, smooth, hyaline, inamyloid, acyanophilic[16]	<200
Lentinula edodes	Ellipsoid to cylindric, smooth, hyaline, inamyloid	<200
Marasmiellus ramealis	Elongate ellipsoid, smooth, hyaline, inamyloid, weakly cyanophilic	<200
Omphalotus illudens	Round, smooth, hyaline to yellowish, inamyloid, weakly cyanophilic	<200
Strobilurus esculentus	Cylindric, smooth, hyaline, inamyloid, cyanophilic	<200
Oudemansiella radicata	Broadly ellipsoid to subglobose, smooth, hyaline, inamyloid, weakly cyanophilic	<200 [194–1500][10, 13]
Flammulina velutipes	Cylindric, hyaline, smooth, inamyloid, weakly cyanophilic	<200
Armillaria ostoyae	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	146[10] [200–450][1]
Schizophyllum commune	Cylindric, often curved, smooth, hyaline, inamyloid	<200
Fistulina hepatica	Ellipsoid to subglobose, smooth, yellowish, inamyloid, acyanophilic	<200
Lachnella villosa	Ellipsoid, smooth, hyaline, inamyloid	<200
Xeromphalina campanella	Ellipsoid, smooth, hyaline, amyloid, acyanophilic	<200
Mycena polygramma	Ellipsoid, smooth, hyaline, amyloid, acyanophilic	<200 [65–75][5]
Panellus stipticus	Cylindric–ellipsoid, smooth, hyaline, amyloid, acyanophilic	~40[12]
Marasmius rotula	Elongate elliptical, smooth, hyaline, inamyloid, cyanophilic	<200
Crinipellis stipitaria	Ellipsoid, smooth, hyaline, inamyloid, cyanophilic	<200
Pleurotus ostreatus	Cylindric, smooth, hyaline, inamyloid, acyanophilic[16]	<200
Hohenbuehelia petaloides	Ellipsoid, smooth, hyaline, inamyloid, acyanophilic	<200
Megacollybia platyphylla	Subglobose, smooth, hyaline, inamyloid, acyanophilic	<200
Mycenella bryophila	Subglobose, distinctively spiny, hyaline, inamyloid, cyanophilic	<200
Macrotyphula fistulosa	Almond-shaped, smooth, hyaline, inamyloid, acyanophilic	<200
Pleurocybella porrigens	Broadly ellipsoid to subglobose, smooth, hyaline, inamyloid, acyanophilic	<200
Phyllotopsis nidulans	Reniform to arcuate-cylindric, smooth, hyaline, inamyloid, acyanophilic	<200
Tricholomopsis rutilans	Ellipsoid, smooth, hyaline, inamyloid, acyanophilic	<200
Hygrophorus chrysodon	Ellipsoid, smooth, hyaline, inamyloid, acyanophilic	<200 [50]*
Lentaria albovinacea	Ellipsoid to cylindric, smooth, hyaline, inamyloid	<200
Sarcomyxa serotina	Curved cylindrical, smooth, hyaline, amyloid, acyanophilic	<200
Hygrocybe conica	Broadly ellipsoid, smooth, hyaline, inamyloid, acyanophilic	<200
Arrhenia auriscalpium	Ellipsoid to guttiform, smooth, hyaline, inamyloid, cyanophilic	<200

Taxa are ordered according to their appearance in the phylogenetic tree (Fig 2)
Colour reactions of basidiospores were observed in 3 % potassium hydroxide, Melzer's reagent (Singer 1986), and cotton-blue (for light-spored agarics; Singer 1972) using a light microscope.
<200, >200, spore wall thickness below or exceeding 200 nm, extrapolated as described in Materials and Methods from own light and electron microscopical observations. Numbers in brackets designate spore wall measurements from other species in the genus, compiled from literature as detailed with superscript numbers. Spore wall measurements from osmium tetroxide fixed specimens are indicated with an asterisk; other measurements are from potassium permanganate fixed material (Besson 1972; Capellano 1976; Capellano & Kühner 1975; Clémençon 1970, 1973, 1974, 1977a, 1977b, 1997). Comparative analyses have shown that spore wall measurements of a specimen fixed with potassium permanganate may result in values exceeding those made from osmium tetroxide-fixed specimens of the same species by up to 30 %.
References: [1]Bennell *et al.* (1985), [2]Besson (1972), [3]Capellano (1976), [4]Capellano & Kühner (1975), [5]Clémençon (1970), [6]Clémençon (1973), [7]Clémençon (1974b), [8]Clémençon (1977a), [9]Clémençon (1977b), [10]Clémençon (1997), [11]Kost (1981), [12]Lingle *et al.* (1992), [13]Pegler & Young (1986), [14]Rast & Hollenstein (1977), [15]Ruch & Motta (1987), [16]Singer (1972). Other measurements are from this study.

1963) that thick-walled and pigmented basidiospores of some *Bolbitiaceae* remain viable for up to three years in herbarium collections. In the present study, collapsed basidiospores were observed in the microscopical mounts much more frequently for the basal taxa in the phylogenetic tree (Fig 2) than in species with thick-walled and pigmented basidiospores. However, representative comparative studies on the germination capability of basidiospores after long dormancy periods are still lacking for agarics.

A higher complexity of the basidiospore wall involves the differentiation of the primary wall (the layer continuous with the wall of the sterigma, from which the mature spore is released), and/or the development of a secondary wall. As a consequence of their thickened cell walls, basidiospores of many dark-spored agarics possess germ pores, i.e. apical regions with a reduced cell wall width, to facilitate germination (Fig 3C–D).

Complex basidiospore walls may have opened up new ecological niches for many derived agaric lineages, which in turn may have led to a radiation of species exploring these new habitats. Thus, dung-inhabiting basidiomycetes, such as species of *Bolbitius*, *Conocybe*, *Coprinellus*, *Coprinopsis*, *Panaeolus*, *Psathyrella*, *Psilocybe*, or *Stropharia* (Dix & Webster 1995) have thick-walled and pigmented basidiospores, which may be the reason why spores of these species can survive gut passages through herbivores to induce an effective primary colonization of such substrates (Larsen 1971).

Convergent evolution of partially thick-walled or pigmented basidiospores

Unpigmented or only slightly pigmented basidiospores with a wall thickness exceeding 200 nm have evolved also in few genera on more basal branches of the agarics, such as *Fayodia*,

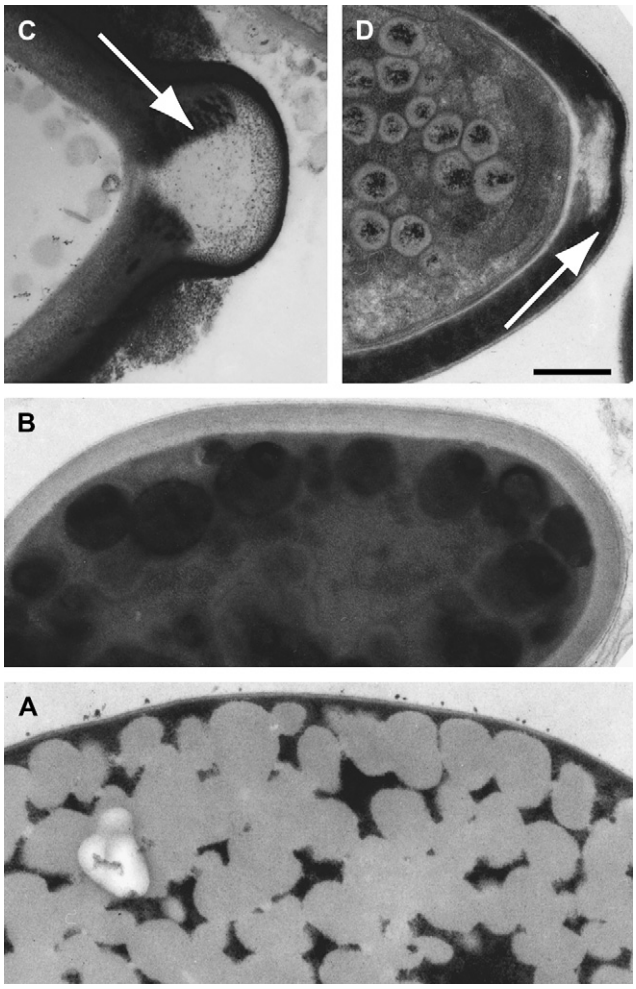

Fig 3 – Transmission electron micrographs of basidiospore walls of agarics. (A) Thin-walled and smooth basidiospore of *Hygrophorus eburneus* TUB 012681. (B) Thick-walled and smooth basidiospore of *Cystoderma amianthinum* TUB 011551. (C) Thick-walled and ornamented basidiospore with a germ pore (indicated with an arrow) of *Lacrymaria lacrymabunda* TUB 014804. D. Thick-walled and smooth basidiospore with a germ-pore (indicated with an arrow) of *Hypholoma fasciculare* TUB 012682. Bar = 500 nm.

Pluteus, *Ripartites*, and *Volvariella*. Parsimony reconstruction of ancestral states (Fig 2; Supplementary Data appendix) suggests that several convergent evolutionary processes are responsible for this. Basidiospores of these species all lack germ pores. Convergent evolution of complex spore wall architecture is also likely for some members of *Oudemansiella* and *Armillaria* (Bennell *et al.* 1985; Pegler & Young 1986) that could not be included in our study.

'Non-agaric' agarics

Our phylogenetic reconstruction confirms that the light-spored agarics include a number of species with non-agaric morphology that have been interpreted as derived from the agaricoid Bauplan (Bodensteiner *et al.* 2004; Hibbett 2004; Moncalvo *et al.* 2002), such as pileate morphs with reduced stipes (e.g. *Panellus*, *Phyllotopsis*, *Pleurocybella*); pileate forms lacking gills and stipes (e.g. *Arrhenia*); and coralloid (*Lentaria*), clavarioid (*Macrotyphula*), cup-shaped (*Lachnella*) or even complex tubular morphs (*Fistulina*). Although these taxa strongly deviate from other agarics with respect to their fruit bodies, similarities in anatomy (e.g. the lack of pigmentation in the trama and partly the presence of sarcomitic tissues) (Redhead 1987) and micromorphology (e.g. the presence of basidia of a similar form with basal loop-like clamps, collapsing and thin-walled spores; Oberwinkler 1985; Pegler & Young 1971) are consistent with their phylogenetic placement within the light-spored agarics.

Phylogenetic relationships at the familial level

Our analyses yielded a number of interesting results concerning phylogenetic relationships at the familial level, some of which will affect future classification systems. Consistent with previous molecular studies (Hofstetter *et al.* 2002; Matheny *et al.* 2007; Moncalvo *et al.* 2000, 2002; Vellinga 2004), *Tricholomataceae* were split into separate groups distributed over several clades of light-spored agarics. Also *Hygrophoraceae* are polyphyletic, consisting of at least two separate branches that occupy more basal positions within the agarics. Our study also provides evidence for the monophyly of *Entolomataceae*, and suggests that this family is the sister group to a taxon corresponding to *Tricholomataceae* in a new, restricted concept (Fig 2, clade D). Though members of *Entolomataceae* and *Tricholomataceae* differ substantially in their ecological adaptations, they are similar in basidiospore wall ultrastructure and share a siderophilous granulation in the basidia, supporting a closer relationship. Within the dark-spored agarics, *Agaricaceae* was supported as a monophyletic group. This family shows a huge diversity in basidiome morphologies, ranging from agaricoid to gastroid (puffballs), and also a wide variation of basidiospore ultrastructure and veil anatomy. Other families supported in our analyses are *Bolbitiaceae* and *Psathyrellaceae*, which is in agreement with classifications based on basidiome morphology (Singer 1986), as well as with basidiospore morphology (Pegler & Young 1971), similarities in asexual stages (Walther *et al.* 2005; Walther & Weiß 2006), and previous molecular phylogenetic analyses (Hopple & Vilgalys 1999; Moncalvo *et al.* 2000, 2002).

Phylogenetic placement of Cortinarius

An interesting well-supported group in the agarics with thick-walled basidiospores, which has not been detected in molecular analyses before, clarifies the phylogenetic position of the largest agaric genus, *Cortinarius*, in which more than 2000 species have been described. Our analysis shows that *Cortinarius* is phylogenetically close to *Phaeolepiota*, *Cystoderma*, and the bird's nest fungi *Crucibulum* and *Cyathus*. There are indications that similar anthraquinone pigments occur in the taxa grouped here together with *Cortinarius* (Ayer & Taylor 1976; Gill & Steglich 1987). Other members of *Cortinariaceae* as currently conceived, such as *Naucoria*, *Galerina*, *Gymnopilus* and *Hebeloma*, appear to be closely related to the *Strophariaceae*

(excluding *Phaeomarasmius* and *Flammulaster*), a relationship supported by shared styrylpyrone pigments of the fruit bodies (Gill & Steglich 1987), as well as by molecular phylogenetic analyses (Gulden *et al.* 2005). This analysis is the first to significantly support a close relationship between *Phaeolepiota*, *Cystoderma*, *Crucibulum*, and *Cyathus*. We suspect that similarities in pigments located in the velar hyphae might further support this grouping. *Phaeolepiota–Cystoderma* and *Crucibulum–Cyathus*, respectively, appear as sister taxa in our phylogenetic tree with 100 % BMCMC support. Members of *Phaeolepiota* and *Cystoderma* share features of veil anatomy, whereas *Crucibulum* and *Cyathus* are similar in hymenial organization and basidiome shape.

Laccaria *is closely related to dark-spored agarics*

This is the first study that demonstrates that the light-spored genus *Laccaria* evolved within the dark-spored agarics (Fig 2). In previous molecular studies (Hibbett & Binder 2002) based on nu- and mt-rDNA sequences, *Laccaria* species appeared close to members of the dark-spored genus *Cortinarius*, but without significant support. *Laccaria* species, which are currently classified in *Tricholomataceae*, have thick-walled basidiospores bearing spectacular ornamentation. The ornamentation is connected by a continuous cell wall layer, and this architecture was also found in dark-spored agarics with thick-walled basidiospores. In contrast, spore ornamentation in light-spored agarics is isolated, except for species of *Laccaria*. This is another example for the importance of basidiospore-related characters in a natural classification of the agarics.

In conclusion, our study demonstrates that higher-level phylogenetic relationships in agarics can be resolved using sequence analysis of appropriate genetic markers, and forms the basis for a more natural classification. We conclude from new molecular and morphological data that the development of complex architecture of the basidiospore walls was a key event in the evolution of the euagarics, and suggest that this probably increased ability to survive in harsh ecological conditions and enabled subsequent adaptive radiations to new ecological niches. Future molecular analyses involving more genes and an extended sampling of species, together with careful morphological and chemical (re-)examinations, will provide more insight into the evolution of the fascinating agaric branch of the fungal tree of life.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.mycres.2007.03.019

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