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Phylogenetic diversity of lichen-associated homobasidiomycetes

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Abstract

The vast majority of lichenicolous fungi are relatively host-specific, nonvirulent ascomycetes and heterobasidiomycetes. A few known lichenicolous homobasidiomycetes (mushroom-forming fungi) generally exhibit broad host ecologies and in some cases, high virulence. Many produce conspicuous sclerotia or bulbils, thought to be adaptive in dispersal and survival. To more clearly understand the evolution of these atypical behaviors in lichenicolous basidiomycetes, we isolated or acquired specimens or cultures of 23 lichenicolous homobasidiomycetes and their relatives, from which we obtained mainly nuclear and some mitochondrial rDNA sequences. Phylogenetic analyses in this study indicate that a lichenicolous habit arose in four major clades. In two of these clades the habit represents a major evolutionary theme linked to the origin of well-known basidiolichens. The phylogenetic diversity of these fungi indicates that the lichenicolous habit arose recently and independently in the mushroom-forming fungi.

Keywords: Basidiolichens; Bulbilliferous fungi; *Burgoa*; Evolution; Lichenicolous fungi; *Marchandiomyces*; Multi-gene analyses; Sclerotia; *Sistotrema*

1. Introduction

Lichenicolous fungi are a highly specialized and successful group of organisms that develop on lichens and form numerous ecological associations with them. There are around 1500 named species in nearly 300 genera, but estimates place the total number at over 3000 species (Lawrey and Diederich, 2003). More than 95% of the known species are ascomycetous, and of the lichenicolous basidiomycetes, most are heterobasidiomycetes (jelly fungi) in the genus *Tremella* (Diederich, 1996). The host-specificity of lichenicolous ascomycetes and heterobasidiomycetes appears to be high, with as many as 95% thought to be associated with a single lichen genus.

The lichen-associated fungi in the homobasidiomycetes (mushroom-forming fungi) are represented by far fewer species and they differ from most lichenicolous fungi by exhibiting broad host ecologies and in some cases high virulence. Since collected specimens rarely have sexual structures, the evolution and phylogenetic relationships of these fungi have not been reliably elucidated. Taxonomic concepts based on asexual morphology suggest they represent a wide assortment of phylogenetic groups, and this combined with their low numbers suggests that the lichenicolous habit has arisen recently and independently in the homobasidiomycetes. However, this hypothesis has never been tested.

A character common to many of these fungi is the production of sclerotia or bulbils (Fig. 1), and it has been suggested that both serve as specialized reproductive structures or resting stages. Sclerotia are of several types that differ anatomically, including true sclerotia, pseudosclerotia and

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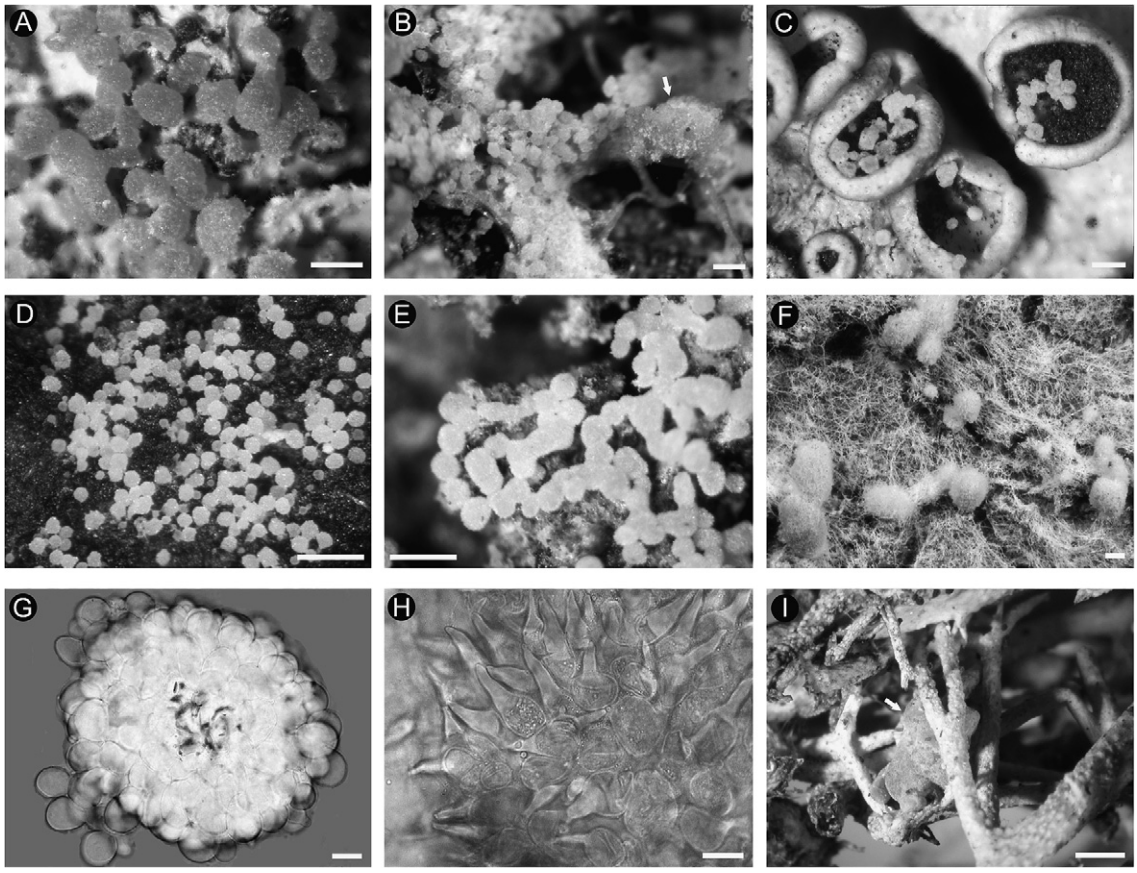

Fig. 1. Bulbils (A–E, G and H) and sclerotia (F, I) of a selection of lichenicolous and corticolous fungal species. (A) *Marchandiomyces corallinus*, (B) *Marchandiobasidium aurantiacum* (isotype; arrow: basidioma), (C) *Burgoa angulosa* (holotype), (D) *Burgoa moriformis* (holotype), (E) *Minimedusa pubescens*, (F) *Athelia arachnoidea*, (G) *Burgoa moriformis* (holotype), (H) *Minimedusa pubescens* (culture of holotype), (I) *Leucogyrophana lichenicola* (arrow: sclerotium). Scale bars: A–F = 200 μm; G and H = 10 μm; I = 1 mm.

bulbils (Clémençon, 2004). Bulbils are tightly coiled masses made from a single fungal hypha, and sclerotia are masses of interwoven hyphae that arise from several different sources and develop a center and a rind-like covering. A wide variety of sclerotial types are produced by lichenicolous homobasidiomycetes and their relatives. True sclerotia are known from the lichenicolous *Athelia arachnoidea* (Fig. 1F) and *Leucogyrophana lichenicola* (Fig. 1I), and bulbils from species of *Marchandiomyces* s.l. (Fig. 1A and B) and *Burgoa* s.l. (Fig. 1C–E, G and H), many of which are lichenicolous. It is not known for certain if or how the production of sclerotia or bulbils is adaptive in these fungi. Furthermore, since the phylogenetic position of these sclerotial fungi is not known, it is difficult to discuss when or how various sclerotial characters arose in the past.

Based on traditional taxonomic concepts established for these fungi, lichenicolous basidiomycetes appear to have relatives representing all possible nutritional groups, including host-specific parasites, broad-spectrum pathogens, saprotrophs, mycorrhiza-formers, and even lichen-formers. However, the relationships of lichen-associated fungi to these other nutritional types are not known with any certainty. Nor is it known how frequently homobasidiomycetes made the nutritional transition to a lichenicolous habit. We attempted to answer some of these questions by establishing the phylogenetic placement of presently described lichen-associated fungi among the major clades of the homobasidiomycetes analyzing two datasets. We used a 'core' dataset of 142 species, each of which is represented by four rDNA regions (mitochondrial and nuclear large and small subunits) from a recent comprehensive phylogenetic study of the group (Binder et al., 2005) and added rDNA sequences of 126 species, mostly nuclear large subunit data. The second dataset was limited to large subunit rDNA sequences and extensively sampled the cantharelloid clade or Cantharellales using 93 taxa. Our objectives were: (i) to determine the extent to which lichenicolous specimens are members of natural groups, (ii) to investigate the possible role of sclerotia/bulbil production in the evolution of a lichenicolous habit, (iii) and to consider possible nutritional transitions that took place in lineages containing lichenicolous homobasidiomycetes.

2. Materials and methods

2.1. Isolation of fungal cultures

Cultures of 22 sclerotial or bulbilliferous lichen-associated homobasidiomycetes or their close relatives were

obtained by us for sequencing (Table 1). In the case of one specimen, *Burgoa moriformis*, no culture was obtained and a sequence was obtained directly. The cultures of sclerotial or bulbilliferous fungi were either isolated from freshly collected material or obtained from culture collections. Isolation techniques are discussed in Lawrey (2002). All fungi grew on either potato dextrose agar (PDA) or Sabouraud's dextrose agar (SDA).

2.2. Molecular techniques

Genomic DNA was extracted from fungal tissue using the Bio 101 Fast DNA Spin Kit for tissue (Qbiogen) according to the manufacturer's protocol with slight modifications. About 10 ng of extracted DNA were subjected to a standard PCR in a 50 μL reaction volume. Amplified products included the nuclear large (nuc-lsu) and small (nuc-ssu) rDNA subunits and the mitochondrial small (mt-ssu) rDNA subunit. We initially attempted to amplify the complete nuc-lsu gene (3800–4000 bp) using an array of primers (LR0R, LR3R, LR17R, LR8R, LR16, LR5, LR7, LR9, LR12) available from the Vilgalys laboratory web site (http://www.biology.duke.edu/fungi/mycolab/primers.htm) for PCR and sequencing. However, limited amounts of genomic DNA prohibited this approach and for most of the species listed in Table 1, at least 1500 bp (bounded by LR0R and LR7) were sequenced. The nuc-ssu with an approximate length of 1750 bp for most species was completely sequenced using NS17UCB, NS19UCB, NS3, NS21UCB, NS23UCB, NS24UCB, NS22UCB, NS20UCB, NS2, and CNS26 (Gargas and Taylor, 1992; White et al., 1990). The amplification of the mt-ssu product using primers MS1 and MS2 (White et al., 1990) proved to be difficult due to insertions at the primer hybridization sites and only three sequences were obtained. After confirming the PCR product by running on a 1% agarose gel using ethidium bromide, the products were purified with magnetic beads (Agencourt Biosciences).

The purified PCR products were used in standard sequencing reactions with BigDye Terminator Ready Reaction Mix (Applied Biosystems). The sequencing reactions were then purified using Sephadex G-50 (Sigma–Aldrich), dried in a speed vac, denatured in HiDi Formamide (Applied BioSystems) and run on a SCE-9610 capillary machine (SpectruMedix LLC). The data collected were analyzed using BaseSpectrum software (SpectruMedix LLC) and about 600 bases were collected for each primer used. These sequences were then transferred to Sequencher (GeneCodes Corporation) for manual base calling and to make contiguous alignments of overlapping fragments.

The nuclear large subunit rDNA sequence of *Burgoa moriformis* was obtained from the herbarium type specimen by the method of direct PCR modified from Wolinski et al. (1999). Thirty bulbils were detached with sterile tweezers, transferred to a drop of 15 μL of sterile water on a clean microscope glass slide. A cover slip (18 mm × 18 mm) was placed over the material. The bulbils were crushed by exerting pressure on the cover slip. Ten microlitres of that solution was added to 40 μL of PCR master mix and used directly for PCR-amplification with the following program: 94 °C (10 min), 34 cycles of 94° (30 s), 54° (30 s), 72° (1 min 30 s), and a final extension of 72° (10 min). Amplification products were cloned using a TOPO Cloning Kit (Invitrogen).

2.3. Taxon sampling and alignment

The core dataset includes nuclear small and large subunit (nuc-ssu and nuc-lsu) and mitochondrial small and large subunit (mt-ssu and mt-lsu) rDNA sequences from 142 species (see the supplement in Binder et al., 2005 for strain information and GenBank accession numbers). We used a supermatrix approach and added sequences from two sources: those representing our cultures (Table 1) and another set representing lichen-forming or sclerotial species of interest to us in the study, sequences of which were obtained from GenBank. The second dataset focusing on the cantharelloid clade was assembled to sample the large proportion of nuc-lsu sequences that were left out of the core dataset to minimize the amount of missing data. In addition, some groups in the cantharelloid clade, such as *Tulasnella* and *Cantharellus*, are well known for inherently divergent sequences that are prone to produce long branch artifacts in larger phylogenies (Pine et al., 1999). Both datasets were aligned by eye in MacClade 4.08 (Maddison and Maddison, 2005) and submitted to TreeBASE (SN3180, 13478 and 13479).

2.4. Phylogenetic analyses

The core dataset consisting of 268 terminals was tested for positive conflict by bootstrapping nuclear small and large subunit rDNA (nuc-ssu and nuc-lsu) and mitochondrial small and large subunit rDNA (mt-ssu and mt-lsu) partitions separately in PAUP*4.0b10 (Swofford, 2002) using maximum parsimony employing 500 replicates (Binder and Hibbett, 2002; Binder et al., 2005). No conflict between partitions >70% was detected and the data were combined. The combined core dataset consisted of 3709 characters, of which 214 ambiguous characters were excluded. The general time reversible model (GTR) using a proportion of invariant sites and distribution of rates at variable sites modeled on a discrete gamma distribution with four rate classes was estimated with MODELTEST 3.06 (Posada and Crandall, 2001) as best-fit likelihood model for both nuclear and mitochondrial partitions; however, model parameters varied considerably. To implement the optimal model in Bayesian analyses, the GTR model was specified as prior for both nuc- and mt-rDNA partitions. The substitution rate matrix, transition/transversion rate ratio, character state frequencies, gamma shape parameter α, and proportion of invariant sites were unlinked across nuclear and mitochondrial partitions. Bayesian phylogenetic analyses were carried out using the Metropolis-coupled

Table 1
Cultures of sclerotial and bulbilliferous lichenicolous fungi from which sequences were obtained in this study

Species name	Location	Collector	Substrate	GMU isolate number	Strain	Sclerotia or bulbils produced in culture	GenBank Accession Nos.
Aegerita candida	USA	C. Shearer	Freshwater		CS 989	—	mt-ssu: DQ915482 mt-lsu: DQ915479
Athelia arachnoidea	Catoctin Mt. Park, MD, USA	J. Lawrey 3300	*Punctelia rudecta*	JL267-04	ATCC MYA 3672	sclerotia	nu-ssu: DQ915454
Burgella flavoparmeliae	Sequoyah Co., OK, USA	W. Buck 38682	*Flavoparmelia baltimorensis*	JL192-01	ATCC MYA 2157	bulbils	nu-ssu: DQ915455 nu-lsu: DQ915469
Burgoa-like sp. of Ceratobasidiales	Baden, Oberrhein, Germany	V. Wirth 32360	Tree bark	JL134-99	ATCC 208870	bulbils	nu-ssu: AF289662 nu-lsu: DQ915470
Burgoa angulosa	Huesca, Spain	J. Etayo 16256	*Physcia aipolia*	JL146-00	ATCC MYA 1121	bulbils	mt-ssu: DQ915480 nu-ssu: DQ915456 nu-lsu: DQ915471
Burgoa moriformis	Ireland	B. Coppins 15829 & A. O'Dare	*Salix* bark		No culture	—	nu-lsu: DQ915477
Burgoa turficola	Nordrhein-Westfalen, Germany	dep. P. Hoffmann	Turf containing potting soil in green house		DSMZ 12882	bulbils	nu-lsu: DQ915467
Burgoa verzuoliana	Italy	dep. P. LeClair	Timber of *Populus* sp.		ATCC 24040	bulbils	nu-lsu: DQ915475
Cytidia salicina	USA		*Salix*		CBS 727.85	—	nu-lsu: DQ915478
Leucogyrophana lichenicola	Ontario, Canada	R. Thorn 871024/01 & R. Teunissen	*Cladonia* sp., under mats		DAOM 212444	neither	nu-lsu: DQ915468
Marchandiobasidium aurantiacum	San Diego Co., CA, USA	M. Cole 8457	*Physcia* sp.	JL219-01	ATCC MYA 2502	neither	nu-ssu: DQ915460
Marchandiomyces buckii	Carteret Co., NC, USA	W. Buck 43835	*Bacidia heterochroa*	JL244-03	ATCC MYA 2992	neither	nu-ssu: DQ915462 nu-lsu: DQ915472
Marchandiomyces corallinus	Reynolds Co., MO, USA	M. Cole 7500	*Flavoparmelia baltimorensis*	JL128-98	Isolate lost	bulbils	nu-lsu: AY583331
Marchandiomyces corallinus	Pendleton Co., WV, USA	M. Cole 8628	*Imshaugia aleurites*	JL214-01	ATCC MYA 2500	bulbils	nu-ssu: DQ915459
Marchandiomyces corallinus	Grant Co., WV, USA	J. Lawrey 1744	*Flavoparmelia baltimorensis*	JL196-01	ATCC MYA 2346	bulbils	nu-ssu: DQ915457
Marchandiomyces corallinus	Isle of Skye, Scotland	P. Diederich 15630	*Parmelia sulcata*	JL248-03	ATCC MYA 3182	bulbils	nu-ssu: DQ915464
Marchandiomyces lignicola	Rappahannock Co., VA, USA	J. Lawrey 1716	Decorticated *Quercus* branch	JL152-00	ATCC MYA 835	neither	nu-ssu: AY583333
Marchandiomyces lignicola	Prince William Co., VA, USA	J. Lawrey 1797	Decorticated *Quercus* branch	JL258-03	ATCC MYA 3674	neither	nu-ssu: DQ915465
Marchandiomyces lignicola	Grant Co., WV, USA	J. Lawrey 1746	Decorticated *Quercus* branch	JL198-01	ATCC MYA 2347	neither	nu-ssu: DQ915458
Marchandiomyces lignicola	Tolland Co., CT, USA	J. Lawrey 1776	decorticated *Quercus* branch	JL239-02	ATCC MYA 2831	neither	nu-ssu: DQ915461
Marchandiomyces nothofagicola	Isla Navarino, Chile	W. Buck 45976	Decorticated *Nothofagus* wood	JL261-04	Isolate lost	neither	nu-ssu: DQ915466 nu-lsu: DQ915474
Minimedusa polyspora	USA	dep. P. LeClair			ATCC 24041	bulbils	nu-lsu: DQ915476
Minimedusa pubescens	Booischot, Belgium	O. Heylen L03/117	*Scoliciosporum chlorococcum*	JL247-03	ATCC MYA 2993	bulbils	mt-ssu: DQ915481 nu-ssu: DQ915463 nu-lsu: DQ915473

Markov chain Monte Carlo method (MCMCMC) in MrBayes v3.0b4 (Ronquist and Huelsenbeck, 2003), performing two runs each using four chains and 5,000,000 generations. Trees were sampled every 100th generation. The proportion of burn-in trees sampled before reaching equilibrium was estimated by plotting likelihood scores as a function of the number of generations. Posterior probabilities (PP) were determined by calculating a 50% majority-rule consensus tree in PAUP* with the proportion of trees gathered after convergence of likelihood scores was reached, and clades with PP $\geqslant 0.95$ were considered to be significantly supported. In addition to Bayesian analyses, branch robustness was estimated by maximum parsimony bootstrap proportions (BP), using 1000 replicates, each consisting of a single heuristic search with 10 random taxon addition sequences, MAXTREES set to auto increase, and TBR branch swapping in PAUP*.

The cantharelloid clade dataset consisted of 93 terminals including 12 outgroup sequences that represent the remaining major lineages of Basidiomycota. The data were analyzed in PAUP* using maximum likelihood under the GTR + Γ + I model (estimated with MODELTEST 3.06) with nucleotide frequencies estimated (A = 0.22, C = 0.23, G = 0.30, T = 0.24), a rate matrix of substitutions (A–C = 1.04, A–G = 3.18, A–T = 1.48, C–G = 0.77, C–T = 5.75, G–T = 1.0), proportion of invariable sites = 0.13, and $\alpha = 0.73$. A maximum likelihood bootstrap analysis was performed under the same settings using 1000 replicates with MAXTREES set to 1000. In addition, Bayesian analyses were run under the GTR model using gamma shape distribution and proportion of invariable sites. Two parallel MCMCMC runs were performed each using four chains and 5,000,000 generations, sampling trees every 100th generation. Posterior probabilities were determined as stated for the core dataset.

3. Results

3.1. Taxonomic conclusions

The taxonomical findings of this study are published in a separate paper by Diederich and Lawrey (in press). Taxonomical novelties include the new genera *Burgella* (*B. flavoparmeliae*) and *Marchandiomphalina* (*M. foliacea*). Several new species are described in the genera *Burgella*, *Burgoa*, *Marchandiomyces*, and *Minimedusa*.

3.2. Analyses of the core dataset

The phylogenetic distribution of lichenicolous forms among mushroom-forming fungi and their relationships to other fungal groups were investigated using a multi-gene approach. The Bayesian analyses converged to stable likelihood values after 2×10^6 generations and a total of 59,568 trees were used to calculate posterior probabilities. Of the 12 independent lineages of homobasidiomycetes identified by Binder et al. (2005), five clades (Agaricales, Atheliales, Boletales, Corticiales, Cantharellales) contain lichenicolous homobasidiomycetes (Fig. 2). Our sequences represent four of these clades; the lichenicolous members of the Agaricales have not yet been sequenced and are therefore not included in our trees. Four of these five clades (Agaricales, Atheliales, Corticiales, Cantharellales) also contain obligate lichen-forming fungi; however, no sequences of the known lichen-forming *Athelia* species are available and they are not included in the tree. In four of the five clades (Atheliales, Boletales, Corticiales, Cantharellales) the lichen-associated fungi are either sclerotial or bulbilliferous (Fig. 2). Given our current knowledge, it appears that the Russulales, Gloeophyllales, Thelephorales and the gomphoid-phalloid clade (or Phallomycetidae) are all lacking lichenicolous, lichenized, sclerotial or bulbilliferous species.

The major result of this study is that *Marchandiomyces* s.l. (incl. *Marchandiobasidium*) and *Burgoa* s.l. (incl. *Burgella* and *Minimedusa*) do not form monophyletic groups and are not closely related. The species previously considered *Marchandiomyces* are all placed in the Corticiales, as has been shown in the study of DePriest et al. (2005). With the addition of new isolates, *Marchandiomyces* species form three distinct groups (Fig. 2) that are supported by BP and PP, one of them now called *Marchandiobasidium* (Diederich and Lawrey, in press). Our results also suggest affinities between the lichenicolous *Marchandiomyces* s.l. and the lichen-forming *Marchandiomphalina* (*Omphalina*) *foliacea* in the Corticiales, a result strongly supported in our analysis by PP values.

Isolates of the *Burgoa* species we studied are distributed among the Agaricales and Cantharellales. We did not study all known species of *Burgoa* (cf. *Burgoa anomala*, *B. aurantiaca*, *B. nigra*, *B. pisi*), but included as many as we could of those for which we had cultures or new collections (*Burgoa turficola*), those that were lichen-associated (*Burgoa angulosa*, *Burgella flavoparmeliae*, *Minimedusa pubescens*), and others representing generic types (*Burgoa verzuoliana*, *Minimedusa polyspora*). In the Agaricales, which is the largest clade of homobasidiomycetes, *Burgoa turficola* surprisingly groups with the ectomycorrhizal gasteromycete *Stephanospora caroticolor*, and there is no morphological or ecological basis to explain this relationship. The entire clade may be labeled as Pterulaceae and is strongly supported by PP and moderately supported by BP (74%). A possible affiliation of *Burgoa turficola* to *Athelia* species in the Atheliales suggested by Schlechte and Hoffmann (2000) can consequently be rejected.

Sequences of the remaining *Burgoa* species we studied are distributed in at least four lineages in the Cantharellales. Nevertheless, we observed long branch attraction caused by *Cantharellus tubaeformis*, forming an unresolved sister-group to Atheliales, and *Tulasnella pruinosa* that groups with the bulbilliferous species *Aegerita candida* in the Polyporales. As expected, both artifacts are not supported by PP and BP values, but warrant the analysis of the Cantharellales in a separate dataset, discussed in the next section.

3.3. Phylogenetic resolution of the cantharelloid clade

The maximum likelihood analysis resulted in one optimal tree (Fig. 3) with a score of $-\ln L = -12161.886$). Bayesian runs converged after 500,000 generations and 89,300 trees were used to compute posterior probability values. The Cantharellales are strongly supported by PP values; bootstrap support, however, decreases from 86% in

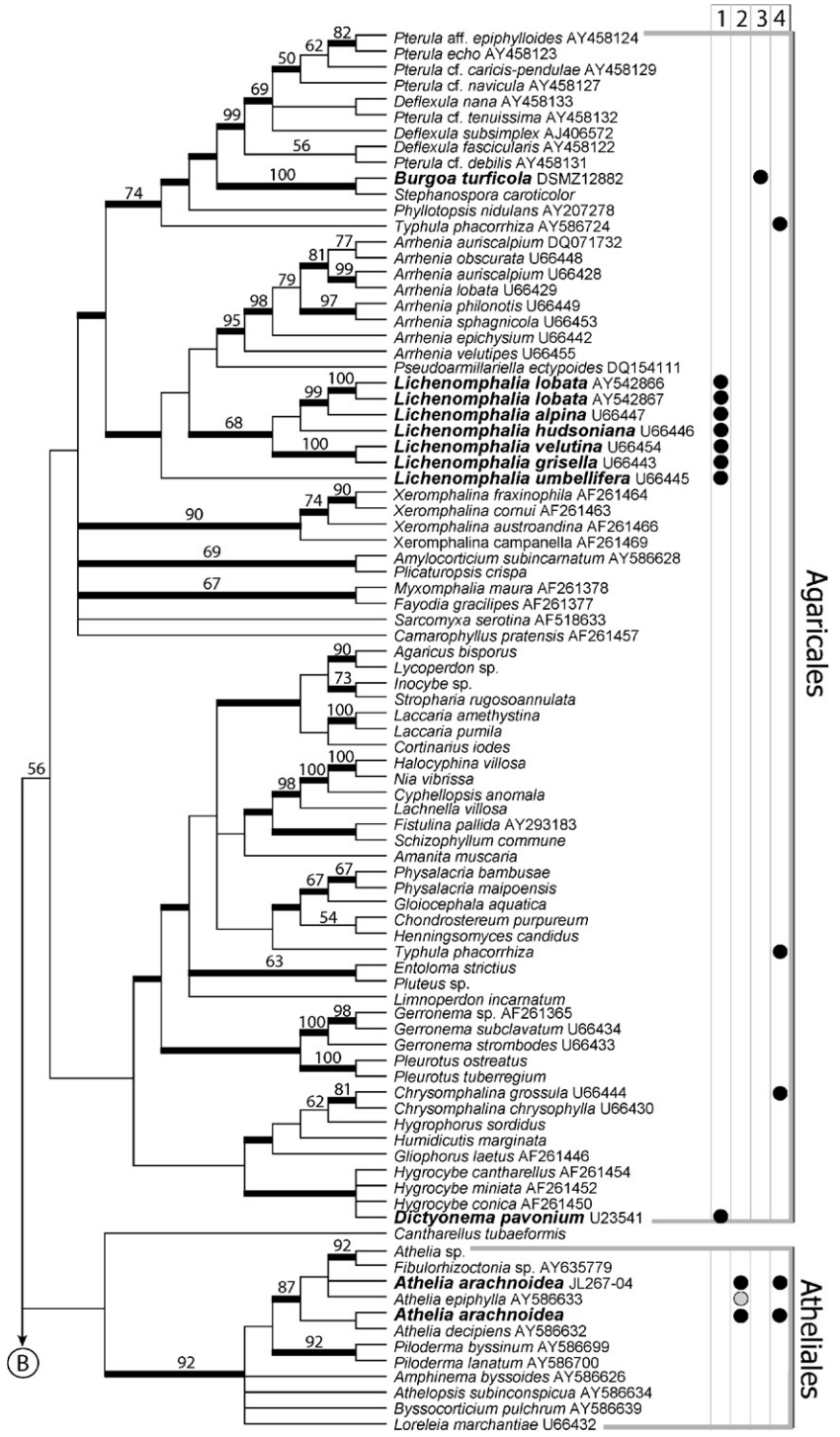

Fig. 2. Phylogenetic relationships of lichenized, lichenicolous, bulbilliferous and sclerotia-producing fungi in homobasidiomycetes inferred from multigene data using Bayesian MCMCMC analyses, 50% majority-rule tree. The labeling of the major clades of homobasidiomycetes follows the AFTOL initiative (http://www.clarku.edu/faculty/dhibbett/AFTOL/AFTOL.htm) to provide a unifying classification for the kingdom Fungi. Branches in boldface indicate posterior probability values >0.95, bootstrap support values (in %) are provided along nodes. Published single sequences are labeled with GenBank accession numbers and newly generated sequences are labeled with strain numbers. Species that are not marked with strain numbers or GenBank numbers form the multi-gene core dataset. Focal species are highlighted in boldface type and the distribution of morphological and ecological traits is indicated to the right of the tree.

the core dataset analyses to 66%. *Burgoa*-like fungi are not monophyletic and group with resupinate fungi in the clade. All these relationships are supported byPP = 1.0 while BP values varied in range. One unidentified *Burgoa*-like isolate (JL134-99) forms a clade with *Uthatobasidium fusisporum* (BP = 62%) in the Ceratobasidiaceae. Most of the other *Burgoa* species show strong affiliations with *Sistotrema* species, a polyphyletic group of morphologically diverse fungi

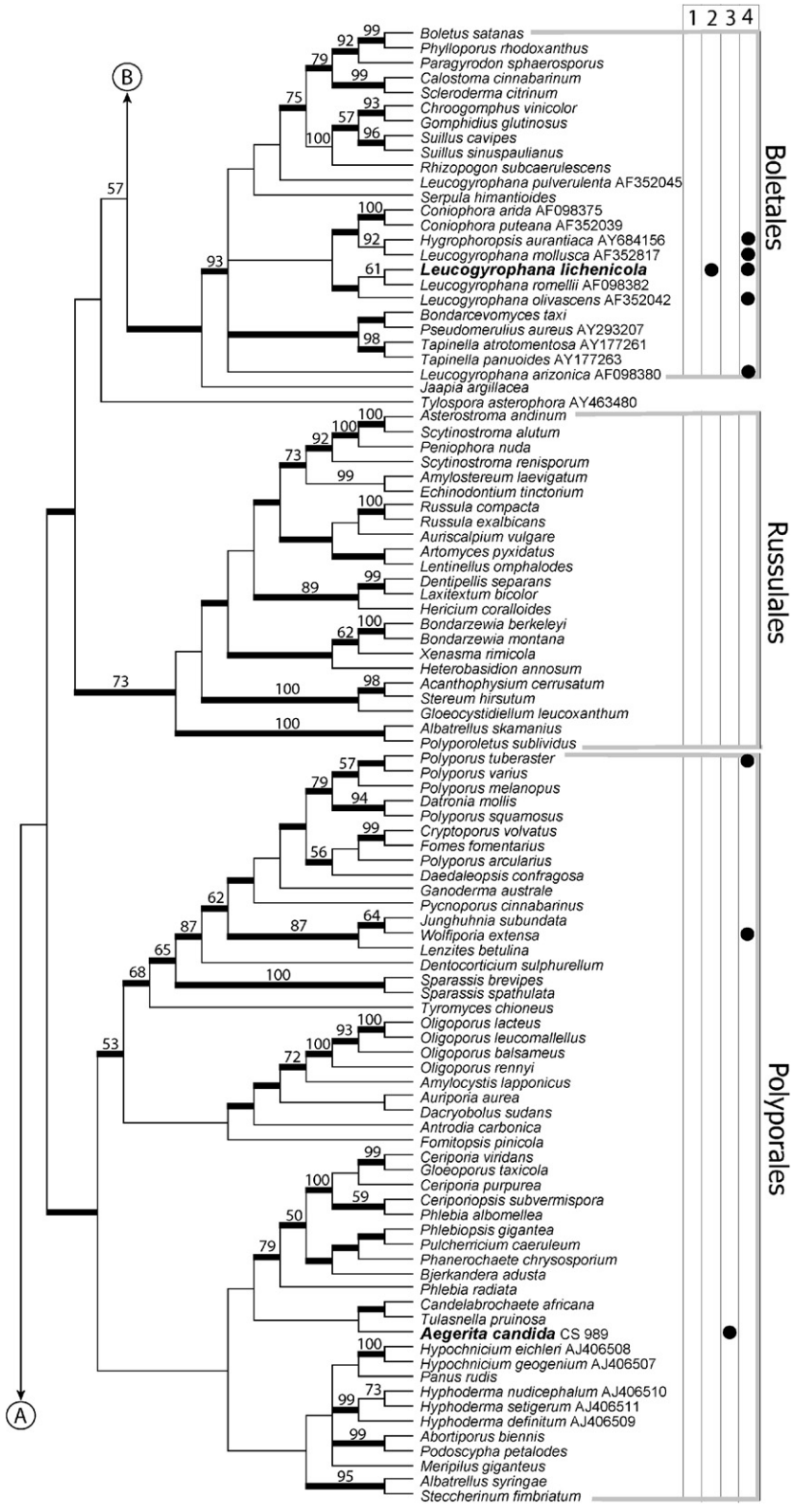

Fig. 2 (*continued*)

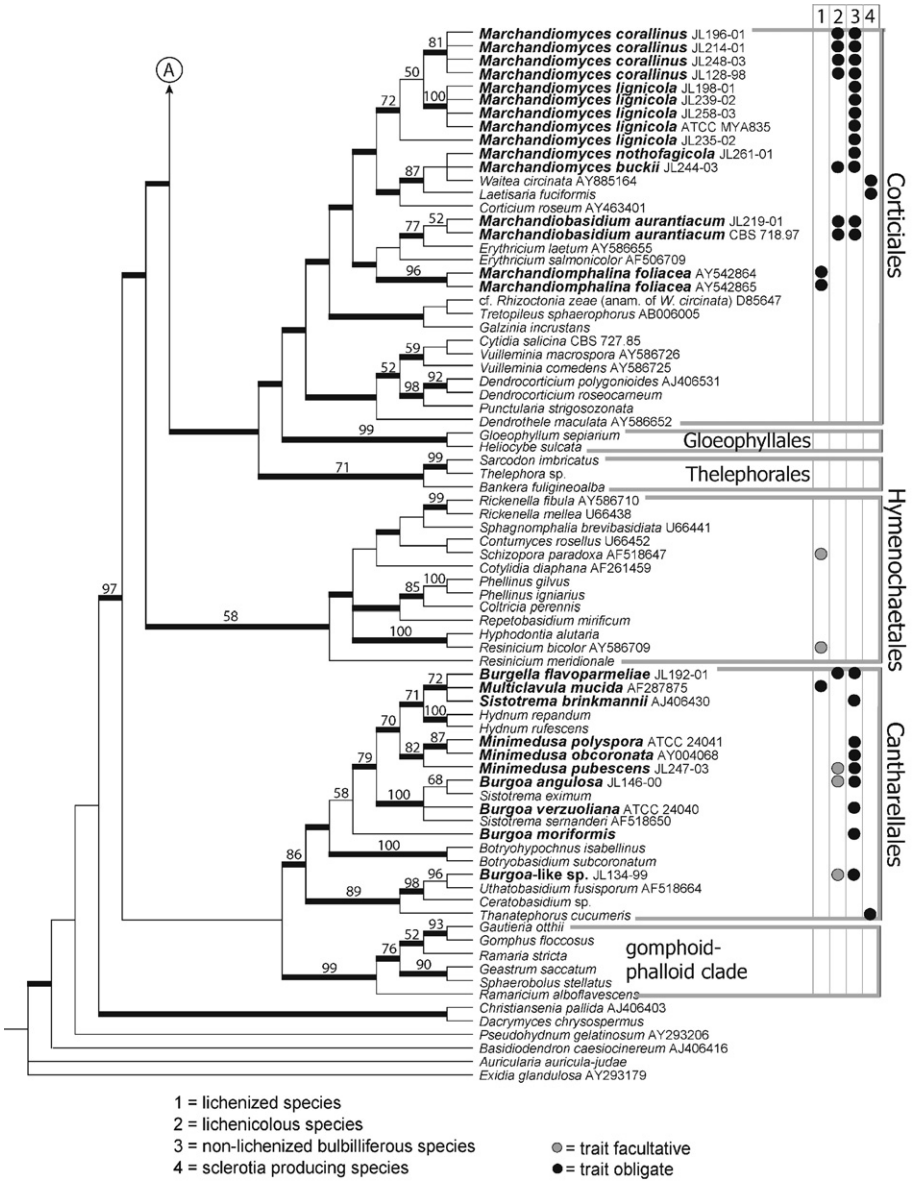

Fig. 2 (*continued*)

(Larsson et al., 2004). For example, *B. verzuoliana* (type of *Burgoa*) and *B. angulosa* form a clade with *S. sernanderi* and *S. eximum* (BP = 99%). *Burgella flavoparmeliae* is sister to *S. oblongisporum* (BP = 94%), and is also closely related to the basidiolichen genus *Multiclavula*. In addition, the bulbilliferous species in the genus *Minimedusa* form a well supported group with *Sistotrema coronilla* (BP = 86%). *Burgoa moriformis* forms an independent lineage and does not cluster with any of the other species in the clade.

4. Discussion

4.1. Overall phylogenetic distribution of lichen-associated homobasidiomycetes

Homobasidiomycetes include the familiar gilled mushrooms, polypores, coral fungi and gasteromycetes, but also resupinate forms that form flattened, crust-like bodies (Larsson et al., 2004; Binder et al., 2005). The resupinate forms have long been considered polyphyletic, but placement of these among the better known mushroom-forming groups has been uncertain until recently when molecular phylogenetic studies began to elucidate their relationships. Most lichenicolous homobasidiomycetes are distributed among the resupinate forms that exhibit little morphological complexity but represent important sources of information about the evolution of nutritional modes in the basidiomycetes.

Despite the fact that lichenicolous homobasidiomycetes are not represented by large numbers of species, they can be found in five of the twelve major clades recognized by Binder et al. (2005). Lichenicolous members of these clades are generally asexual, sclerotial or bulbilliferous, and sometimes virulent pathogens. Close relatives

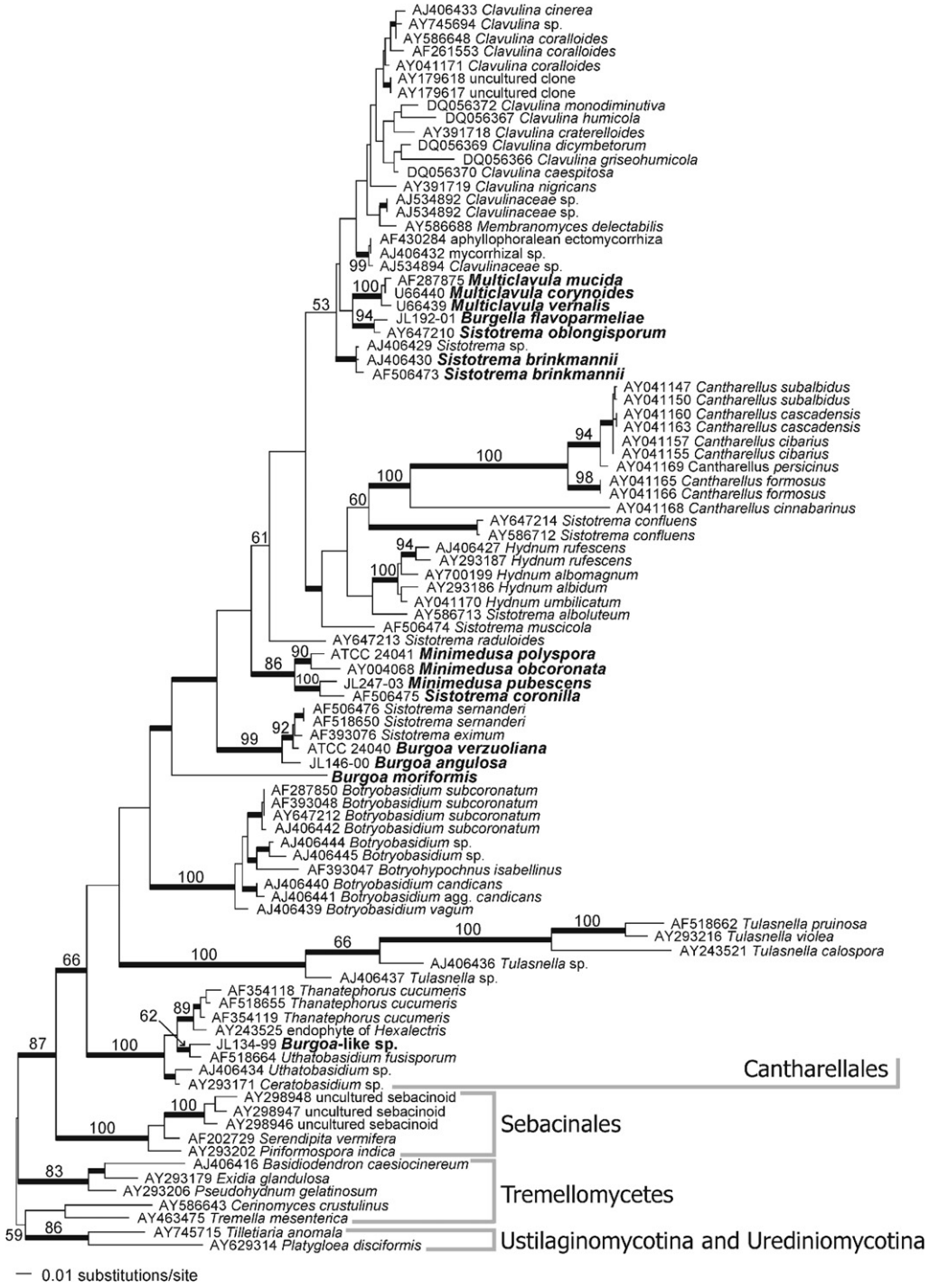

Fig. 3. Phylogenetic relationships of lichenized, lichenicolous, bulbilliferous, and sclerotia-producing fungi in the Cantharellales inferred from nuclear large subunit rDNA sequences using maximum likelihood. Published sequences that were downloaded from GenBank are marked with accession numbers, newly generated sequences are marked with strain numbers. Branches in boldface indicate posterior probability values >0.95, bootstrap support values (in %) are provided along nodes. Species names highlighted in boldface type are lichenized, lichenicolous, or bulbilliferous (see Section 4).

represent a wide assortment of nutritional modes, including plant pathogens, saprobes, mycorrhiza-formers, and lichen-formers. The wide phylogenetic distribution of a relatively small number of lichenicolous homobasidiomycetes indicates a recent and opportunistic origin of the lichenicolous habit from nutritionally diverse ancestors.

In two clades (Cantharellales and Corticiales), the lichenicolous habit represents a major evolutionary theme. In addition, the lichenicolous fungi in Cantharellales and Corticiales are all bulbilliferous and some are phylogenetically close to basidiolichens. This last result was entirely unanticipated and led us to focus attention specifically on these two clades. In each case, we noted the nutritional modes of

fungi representing possible relatives of lichen-associated species, and we also investigated possible phylogenetic relationships among lichen-associated and lichen-forming taxa.

4.2. Phylogenetic relationships of lichen-associated fungi within the cantharelloid clade

The monophyly and basal position of this clade in the mushroom-forming fungi have been discussed previously (Binder et al., 2005). Fungi in the Cantharellales exhibit a mixture of homobasidiomycete/heterobasidiomycete morphological and anatomical characters. The members of this clade vary widely in mode of nutrition, with plant pathogens, saprobes, and mutualists common throughout. Our results indicate that both lichenicolous and lichen-forming fungi are important evolutionary aspects of the clade (Fig. 3).

Lichen-associated fungi in the Cantharellales are anatomically similar to the genus *Burgoa*, usually considered to be the anamorphic state of *Sistotrema* (Weresub and LeClair, 1971; Eriksson et al., 1984; Hallenberg, 1984; Clémençon, 2004; Diederich and Lawrey, in press). *Burgoa*-like fungi are widely distributed in the cantharelloid clade (Fig. 3), suggesting that production of bulbils and association with lichens are both common themes.

One of these *Burgoa*-like fungi, *Burgoa angulosa* Diederich, Lawrey and Etayo (Fig. 1C), is sister to a group containing the type species *Burgoa verzuoliana* and two *Sistotrema* species (*S. sernanderi* and *S. eximum*). Another was named *Minimedusa pubescens* Diederich, Lawrey and Heylen (Fig. 1E and H), because of its close association with a group containing *Minimedusa polyspora*, *Minimedusa* (= *Pneumatospora*) *obcoronata*, and *Sistotrema coronilla*. Each of these species is commonly lichenicolous. *Burgoa* and *Minimedusa* species are all bulbilliferous, some of them are facultatively lichenicolous or muscicolous, never host specific, and rarely immersed in the host thallus (Diederich and Lawrey, in press).

In the clade containing various members of the Clavulinaceae is an obligately lichenicolous species, *Burgella flavoparmeliae* Diederich and Lawrey, which is closely related to *Sistotrema oblongisporum*, and sister to a group of basidiolichens in the genus *Multiclavula*. The fungi of this clade exhibit a wide range of nutritional modes, including saprophytes, parasites, mycorrhizae, and lichens. The basal group includes *Sistotrema brinkmannii*, a soil-borne saprophyte that has been reported to parasitize green algae in the laboratory (Oberwinkler, 1970). Bulbils of *B. flavoparmeliae* are clearly lichenicolous, possibly host specific on species of the foliose lichen genus *Flavoparmelia*, often slightly immersed in the host thallus, leaving distinct scars when removed (Diederich and Lawrey, in press).

Two additional *Burgoa*-like fungi could not be positioned reliably in the tree. One of these, *Burgoa moriformis* (Fig. 1D and G), is clearly cantharelloid but did not associate with any of the sequences of *Burgoa* s.str., *Minimedusa* or *Sistotrema* used in the analysis. Another, referred to as *Burgoa*-like sp., associates with species of *Uthatobasidium*, *Thanatephorus* and *Ceratobasidium*. These two *Burgoa*-like species develop bulbils on bark near lichen thalli, but are probably not lichenicolous (Diederich and Lawrey, in press).

The close association of many of these bulbilliferous *Burgoa*-like fungi with species of *Sistotrema* lends support to the hypothesis that *Burgoa* s.l. is a common anamorph of named species of *Sistotrema*. The extent to which this is true will depend on a more detailed phylogenetic analysis of *Sistotrema*, a genus obviously in need of revision. None of the lichen-associated fungi we studied associated closely with the type species *Sistotrema confluens* in our analyses.

4.3. Lichen-associated fungi in the corticioid clade

The corticioid clade is an ecologically diverse group of homobasidiomycetes containing saprobes, plant pathogens, and mutualists, in addition to those that commonly associate with lichens (Fig. 2). Fungi with a lichenicolous habit are all similar in appearance to *Marchandiomyces corallinus* (Fig. 1A), a lichen pathogen widely distributed in the eastern United States and Europe that forms coral-colored bulbils on a wide variety of lichens. Our analyses indicate that this species and the closely related lignicolous *M. lignicola*, which forms smaller coral-colored bulbils, together are sister to a diverse group that includes the lichenicolous *Marchandiomyces buckii* Diederich and Lawrey, its sister species, the lignicolous *M. nothofagicola* Diederich and Lawrey, various plant pathogens (*Laetisaria fuciformis*, *Waitea circinata*), and saprobes (*Corticium roseum*).

These results indicate that there are two sister groups that each contain named *Marchandiomyces* species in addition to other fungi. It is interesting that in each of these groups the *Marchandiomyces* species form closely related pairs, and in each pair one of the species is lichenicolous (*M. corallinus* and *M. buckii*) and the other is lignicolous (*M. lignicola* and *M. nothofagicola*). DePriest et al. (2005) suggested that numerous recent transitions to a lichenicolous habit had probably taken place in the corticioid fungi, and our results are further evidence of this flexibility. At the present time it is difficult to reconstruct the evolution of nutritional modes in these species pairs, but the frequent close association of lichenicolous and lignicolous species indicates a possible tendency for transitions between these particular ecological habits.

Another common lichenicolous member of the corticioid clade is the phylogenetically more distant *Marchandiobasidium* (= *Marchandiomyces*) *aurantiacum* (Fig. 1B). It is a common European fungus (also collected in the United States) that associates mainly with *Physcia* species, exhibits high levels of virulence, and forms orange bulbils. It appears to group closely with *Erythricium laetum*, a salmon-colored lignicolous fungus that grows on living mosses, and *E. salmonicolor*, a fungus causing pink disease in citrus, coffee and rubber trees.

This nutritionally diverse group is sister to the enigmatic lichenized basidiomycete *Marchandiomphalina* (*Omphalina*) *foliacea*, a species unlike any of the known basidiolichens in its foliose thallus structure and production of asexual soredia-like goniocysts. No sexual material has ever been observed. A recent study of its phylogenetic position in the basidiomycetes (Palice et al., 2005) suggested that it belongs in the hymenochaetoid clade, but our data indicate that its closest relatives are corticioid. If this is true it is the only known basidiolichen in the corticioid clade.

Formation of bulbils (or sclerotia) is relatively common among the corticioid fungi, and is a characteristic of all of the lichenicolous species in the clade. Bulbils of *Marchandiobasidium aurantiacum* are lichenicolous, develop superficially over lichen thalli that they almost entirely degrade, leaving only the cortical layers after invasion by the fungus. Bulbils of *Marchandiomyces* are either lignicolous or lichenicolous, and when lichenicolous they develop inside the lichen thallus, then break through the upper cortex before becoming superficial, and never degrade the thalli.

4.4. A hypothesis concerning the evolution of lichenized structures within bulbilliferous fungi

Among the lichen-associated bulbilliferous fungi, two species appear to be obligately lichenicolous and host-specific, and may therefore serve as primary examples to illustrate the evolution of these fungi: the cantharelloid *Burgella flavoparmeliae*, known so far only from *Flavoparmelia baltimorensis*, and the corticioid *Marchandiobasidium aurantiacum*, which is confined to *Physcia* species. Surprisingly, our results indicate that both species are closely related to lichenized basidiomycetes. *Burgella flavoparmeliae* together with *Sistotrema oblongisporum* are the sister group of the lichen-forming genus *Multiclavula* (Fig. 3), and *Marchandiobasidium aurantiacum* together with *Erythricium* are the sister group of the lichen-forming *Marchandiomphalina* (Fig. 2). We therefore hypothesize that in both cases the lichenized species evolved from lichenicolous ancestors. Our data also suggest that the production of bulbils may promote the evolution of the lichenized habit. A similar link can be seen in the Agaricales, in which some lichenized *Lichenomphalia* species, e.g., *L. umbellifera*, form a thallus composed of small green bulbils (formerly referred to as '*Botrydium*'), and other species, such as *L. hudsoniana*, have a more evolved, squamulose thallus (formerly referred to as '*Coriscium*').

Does the production of bulbils in these fungi somehow predispose them to form lichens? Our phylogenetic results indicate that this may be true in some cases. In the Cantharellales, most of the lichen-forming *Multiclavula* species are themselves bulbilliferous, and they have numerous non-lichenized bulbilliferous relatives. The three *Multiclavula* species included in Fig. 3 all develop thalli composed of lichenized bulbils; one species, *M. calocera*, forms lobate squamules. Although *M. calocera* has not yet been sequenced, it is reasonable to assume that it is closely related to the other *Multiclavula* species and that both bulbilliferous and squamulose species have the same ancestor. In the Corticiales, the lichenized *Marchandiomphalina* is itself squamulose, but has a large number of bulbilliferous, nonlichen-forming relatives. Further evidence for our hypothesis can be found in the Agaricales, in which the bulbilliferous *Lichenomphalia umbellifera* appears to be basal to most species of *Omphalina* s.l. (incl. *Lichenomphalia*), a result obtained also by Redhead et al. (2002).

In three clades of homobasidiomycetes, then, there is evidence that production of lichenized bulbils represents an important ancestral state in the evolution of basidiolichens. Tests of this hypothesis will require further elucidation of the homobasidiomycete clades containing lichens. It may also be possible to experimentally synthesize basidiolichens using cultures of the bulbil-forming fungi and photobionts to see the extent to which bulbil formation leads to formation of basidiomycete thallus structures.

5. Conclusions

Lichen-associated fungi among homobasidiomycetes are distributed across at least five major clades and have therefore evolved independently. This finding is rather surprising, because despite their phylogenetic diversity, lichenicolous homobasidiomycetes are remarkably similar to each other and generally unlike all other known groups of lichen-associated fungi. The overall evolutionary impact of lichenicolous forms in homobasidiomycetes may not have been as fundamental as it appears to be in Ascomycetes, but they play an important role in the evolution of Cantharellales and Corticiales. Clarifying their phylogenetic distribution therefore provides a broader understanding of the diversification of mushroom-forming fungi. In their ecologies, many of the better known lichenicolous species have wide host amplitudes and some are among the most virulent of lichen pathogens. Some are phylogenetically linked with lichen-forming basidiomycetes, and many are either sclerotial or bulbilliferous. Lichen-associated fungi with alternative life histories (ecologically specialized, persistent, nonvirulent, nonsclerotial), found frequently among the ascomycetes and certain groups of heterobasidiomycetes, appear to be rare among the homobasidiomycetes. We suggest this may be explained by the relatively recent origin of the lichenicolous habit among some of the major groups of the homobasidiomycetes.

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