Chaenothecopsis in a molecular phylogeny based on nuclear rDNA ITS and LSU sequences

Leif Tibell[1] & Maria Vinuesa[2]

[1] *Department of Systematic Botany, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, S-75236 Uppsala, Sweden. leif.tibell@ebc.uu.se (author for correspondence)*
[2] *Department of Microbiology, Instituto Biomar S.A. C.E.E.I 24231 Onzonilla, León, Spain. hongos@institutobiomar.com*

Bayesian inference analyses of nuclear LSU rDNA sequences showed Verrucariales and Eurotiales to form the sistergroup of Mycocaliciales. A more detailed analysis of ITS rDNA sequences showed Sphinctrinaceae to be embedded in Mycocaliciaceae. Mycocaliciales contain two major lineages. Intron occurrence and phylogeny was partly congruent with the ITS-based phylogeny. *Chaenothecopsis* is paraphyletic with respect to *Phaeocalicium*, *Mycocalicium*, *Sphinctrina* and *Stenocybe*. Some morphological features traditionally used for characterising *Chaenothecopsis* and other genera in Mycocaliciales, such as ascus apex structure and stalk anatomy, were found to be homoplasious, while others, such as spore septation, intron distribution and nutritional biology, were consistent with major clades in the analysis. Within the two major clades of Mycocaliciales some subclades are strongly supported and well characterized by anatomical, nutritional and intron features.

KEYWORDS: Ascomycota, Bayesian analysis, *Chaenothecopsis*, classification, molecular phylogeny, Mycocaliciales, Mycocaliciaceae, nITS rDNA, nITS rLSU, Sphinctrinaceae.

INTRODUCTION

Historical background. — Calicioid lichens are often easily recognized. Many species are on Red Lists in different countries, and they have often been used for bioindication in old-growth forests. Calicioids (as "Caliciales") is an historic concept based on a 19th and 20th century tradition of considering these lichens and fungi to form a taxon and/or natural group, although we now know that they do not. Their similarities stem from convergent evolution in very diverse groups (Tibell, 1984; Wedin & Tibell, 1997).

When Vainio (1890) described *Mycocalicium*, he emphatically stated that no photobiont was present, and he also noted that the mazaedium—a loose spore-mass covering the ascoma surface—was very thin. Vainio already in the late 19th century advocated the integration of lichenized and non-lichenized fungi into a natural system, but these ideas did not gain general support until the middle of the 20th century. He also suggested ways of finding natural groups, and criticized the a priori recognition of cardinal characters in the classification (Tibell, 1998).

Vainio stressed the importance of the biology of calicioid lichens and fungi and clearly realized that many calicioids were not lichenized. In his *Lichenographia Fennica* (Vainio, 1927) he accepted the traditional families Caliciaceae, Sphaerophoraceae and Cypheliaceae as tribes, and described several new, non-lichenized genera in the tribe Calicieae. He stressed nutritional biology, recognising separate genera for lichenized species, lichen parasites and non-lichenized saprobes (Table 1). Thus *Chaenothecopsis* (type: *Chaenothecopsis rubescens* Vain.) and *Strongylopsis* were described for species that Vainio considered lichenized that have non-septate spores (in fact *Chaenothecopsis* species, including the type species of the genus, are not lichenized). Species occurring as parasites on lichens were included in other new genera such as *Caliciella* (type: *Calicium corynellum* Ach.), *Strongyleuma* (type not designated), and *Coniocybopsis* (type: *Cyphelium arenarium* Hampe ex A. Massal.); species with long stalks were incorporated in *Chaenothecopsis* and species with sessile ascomata in *Stronglylopsis*. The genera within these three groups were characterized by spore septation and stalk development (Table 1) and, in spite of his earlier views on "cardinal characters", Vainio seems to have had a strong urge to describe new genera to cover the different possible combinations of these characters. These ideas were published just after the appearance of the first volume of Zahlbruckner's *Catalogus* (1921–1922), and Vainio's generic concepts had little impact. They were, for example, not accepted in influential works like those of Keissler (1938) and Nádvorník (1942a, b, c). Only Räsänen (1943) followed Vainio's concepts.

Nádvorník (1942a, b, c) made important contribu-

Table 1. Vainio's generic concepts. Taxa in bold belong to Mycocaliciaceae. Genera in current use are underlined.

	Lichenized	Parasitic on lichens	Saprobes
Spores non-septate, ellipsoidal			
Ascomata sessile	***Strongylopsis***	***Strongyleuma***	*Microcalicium*
Ascomata stalked	***Chaenothecopsis***	*Coniocybopsis*	***Mycocalicium***
Spores non-septate, spherical			
	Chaenotheca, Coniocybe, Phacotiella	*Sphinctrina*	*Roesleria*
Spores septate			
Ascomata sessile	*Cyphelium*		
Ascomata stalked	*Calicium*	*Caliciella*, ***Stenocybella***	***Embolidium***, ***Stenocybe***

tions to the knowledge of calicioid lichens (Tibell & al., 2003), but he did not accept Vainio's view that non-lichenized species should be included in separate genera.

The description of Mycocaliciaceae. — Schmidt (1970) in an important paper dealt with the non-lichenized calicioids. He resurrected *Chaenothecopsis*, *Mycocalicium*, and *Strongyleuma*, accepted *Stenocybe*, and described *Phaeocalicium*. Schmidt emphasized that the species belonging to these genera are non-lichenized and have active spore dispersal insofar that their asci function in the ejaculation of the spores. He also suggested new delimitations of these genera. After thorough morphological investigations of the ascomata of selected species, Schmidt emphasized some features for the recognition of the genera he accepted, primarily stalk anatomy (Fig. 1), spore size and septation, ascus size and ascus apex structure (Fig. 1). Schmidt also concluded that these genera are closely related to each other, but not to lichenized calicoids. As a consequence he described Mycocaliciaceae as a new family to accommodate them.

Mycocaliciaceae were soon accepted, for example by Poelt (1973). Henssen & Jahns likewise (1973) accepted Mycocaliciaceae and also stressed the isolated position of the family. While Poelt (1973) still considered Caliciales to be a very uniform, natural order, Henssen & Jahns were less definite and questioned if perhaps a mazaedium might have developed repeatedly and independently as a result of convergence.

The classification of calicioid lichens and fungi was thoroughly revised by Tibell (1984) in a paper where it was concluded that Caliciales as previously construed were a very heterogeneous assemblage of genera forming a biological but not a natural group. Monophyletic entities were identified and a radically new classification was suggested. Eight families were recognized, among them Mycocaliciaceae. In a cladistic analysis of morphological features, Mycocaliciaceae formed a grade with Sphinctrinaceae and *Calicium* together forming the sister-group. The circumscription of Mycocaliciaceae by Schmidt was followed by Tibell, and some of the genera described by Vainio in 1927 were shown to be synonyms of other calicioid genera.

Results from molecular comparisons. — When SSU rDNA data became available it very early became evident that Mycocaliciaceae as represented by *Mycocalicium* were not closely related to *Calicium* (Gargas & Taylor, 1995; Gargas & al., 1995). It was later shown that Caliciaceae belong to Lecanorales (Wedin & Tibell, 1997; Wedin & al., 2000), whereas *Chaenothecopsis savonica*, *Mycocalicium albonigrum* and *Stenocybe pullatula*, representing Mycocaliciaceae, formed a monophyletic group, with *Sphinctrina turbinata*, representing Sphinctrinaceae, as sister-group (Wedin & Tibell, 1997). This relationship was also supported in another study based on SSU rDNA data focusing on Lichinales (Schultz & al., 2001). Here, as in the analysis of Gargas & al. (1995), Mycocaliciaceae (plus Sphinctrinaceae together with Mycocaliciaceae) quite surprisingly formed the sister group to Eurotiales. As a consequence of the monophyly of Mycocaliciaceae and Sphinctrinaceae and their isolated position from other calicioids, Mycocaliciales were described as a new order to include Mycocaliciaceae and Sphinctrinaceae (Tibell & Wedin, 2000). Mycocaliciales were characterized as having apothecia with at least in part sclerotized, blackish-brown hyphae, cylindrical asci, and dark-walled spores. It was noted that the order includes a variety of coelomycetous and hyphomycetous anamorphs.

Analyses of relationships between several species in Mycocaliciaceae were included in Vinuesa (2001), and these results form an important basis for the present paper.

Current generic circumscriptions in Mycocaliciaceae. — Mycocaliciaceae are still a poorly known family. Many species have been described during the past few decades, and many more will undoubtedly be described. The current generic delimitations are based on Schmidt (1970, Table 2), who emphasized ascus and ascoma stalk characteristics. When a larger number of species of Mycocaliciaceae are considered, the character state distributions do not agree with what Schmidt suggested from his investigation of comparatively few species. It thus became evident that some species did not easily fit into the genera as circumscribed by Schmidt. Tibell (1978) noted an ascus apex structure in *Chaenothecopsis fennica* different from the types described by Schmidt, and its asci are also much larger than indicated for *Chaenothecopsis*. It was also noted

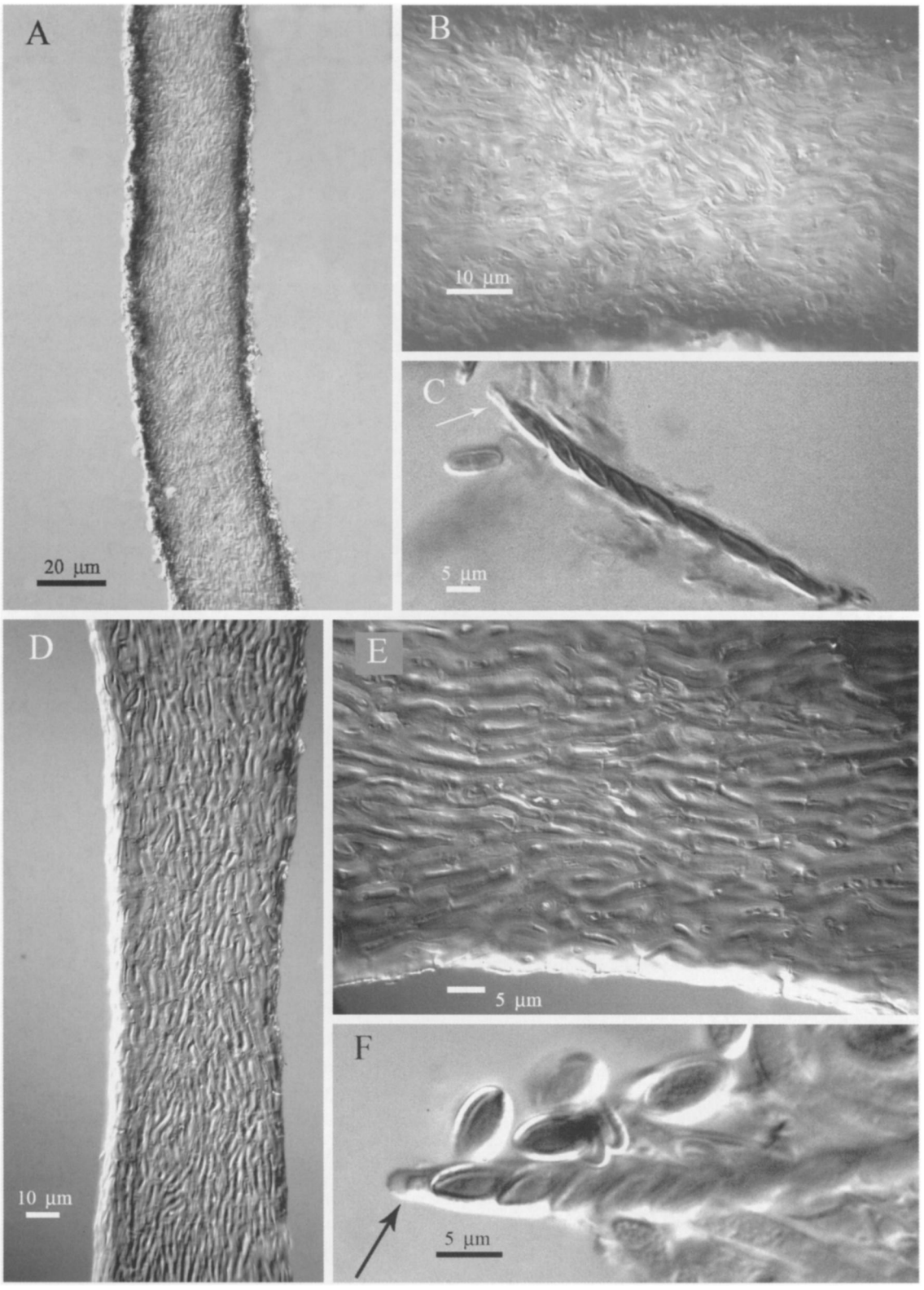

Fig. 1. Stalk and ascus features in Mycocaliciaceae. A–C, *Chaenothecopsis viridireagens*, representing the *Chaenothecopsis*-type of stalk anatomy and ascus apex; A, B, longitudinal section of stalk with pale interior consisting of irregularly intertwined hyphae; C, almost mature ascus with apex penetrated by a narrow canal (arrow). D, F, *Mycocalicium subtile*, representing the *Mycocalicium*-type of stalk anatomy and ascus apex; D, E, longitudinal section of stalk consisting of largely periclinally arranged, dark (sclerotized) hyphae; F, almost mature ascus with uniformly thickened apex without canal (arrow).

Table 2. A comparison of genera of Mycocaliciaceae as presently construed.

	Chaenothecopsis	*Mycocalicium*	*Phaeocalicium*	*Stenocybe*
Stalk	central part pale, consisting of irregularly intertwined hyphae (Fig. 1A, B)	dark throughout, consisting of periclinally arranged hyphae (Fig. 1D, E)	dark throughout, consisting of periclinally arranged hyphae	dark throughout, consisting of periclinally arranged hyphae
Spores	usually <10 µm, non-septate or 1-septate	usually <10 µm, non-septate	usually >10 µm, 0–1-septate	>10 µm, 4–7-septate
Asci	short (35–45 µm)	medium-sized (<65 µm)	long (>70 µm)	long (>70 µm)
Ascus apex	strongly thickened, penetrated by a narrow canal (Fig. 1C)	strongly thickened, without canal (Fig. 1F)	strongly thickened, without canal	strongly thickened, without canal
Ecology	parasites or commensals on lichens, on free-living algae, or as saprobes on wood or decaying vegetable matter, sometimes on exudate	saprobes on wood or on exudate	weak parasites or saprobes on bark of vascular plants	weak parasites or saprobes on bark of vascular plants, in one case on hepatics
Species	Schmidt included *C. consociata*, *C. faginea* Nádv., *C. gracilis* Nádv. (= *C. rubescens*), *C. pusilla*, *C. pusiola* (as *C. lignicola* Nádv.), *C. rubescens*, *C. viridialba* (Kremp.) A.F.W. Schmidt, and *C. viridireagens*. Numerous additional species have been described and several have been transferred to *Chaenothecopsis* from other genera. Presently c. 60 species are accepted in *Chaenothecopsis*.	Schmidt only included *Mycocalicium subtile*. Presently 10 species are accepted in *Mycocalicium*.	Schmidt included *P. compressulum* (Nyl. ex Vain.) A.F.W. Schmidt, *P. populneum*, and *P. praecedens* (Nyl.) A.F.W. Schmidt. Presently 17 species are accepted in *Phaeocalicium*.	Schmidt included *S. major* (Nyl.) Körb. and *S. pullatula*. Presently 9 species are accepted in *Stenocybe*.

(Tibell, 1981) that *Chaenothecopsis nana* is very similar to *Mycocalicium subtile* in ascoma anatomy and spore structure but has a very distinctive canal in its ascus apex. Other *Chaenothecopsis* species, such as *C. nigropedata* Tibell (Tibell, 1987), have periclinally arranged and dark hyphae in their stalks. On the other hand, *Mycocalicium albonigrum*, although morphologically very similar to *M. subtile*, differed in having a canal in its ascus apex. *Mycocalicium*, *Phaeocalicium* and *Stenocybe* are similar in many aspects, and for some species, e.g., *Phaeocalicium interruptum*, it is not at all obvious where they belong. The generic delimitations in Mycocaliciaceae thus seemed unsatisfactory. Further investigations of anamorph diversity in Mycocaliciaceae (Tibell, 1990, 1991, 1995, 1997; Tibell & Constantinescu, 1991) also contributed to the impression that the family is quite heterogeneous and anamorph features were often not congruent with current generic delimitations.

Insertions as a source of taxonomic information. — Insertions have long been known to occur in the rDNA of lichenized fungi (DePriest & Been, 1992). Most of these insertions have been identified as group I introns and are located in the SSU (DePriest, 1993a, b; Gargas & al., 1995; Stenroos & DePriest, 1998; Thell, 1999; Thell & Miao, 1999). Recently occurrences of spliceosomal introns have also been reported, most of them from the SSU (Zoller & al., 1999; Cubero & al., 2000; Bhattacharya & al., 2000) but some also from the LSU (Bhattacharya & al., 2000; Tibell, 2003).

Intron/insertion sequence potential for reconstructing phylogenies in ascomycetes has to some extent been investigated (e.g., Stenroos & DePriest, 1998; Thell, 1999; Thell & Miao, 1999; Myllys & al., 1999; Tibell, 2003).

Introns/insertions have proved to contribute phylogenetic resolution in phylogenies, but they are, however, problematic in many ways. Structural homology may be difficult to ascertain, and they may degrade and leave only short remnants. They may be transferred between organisms (Friedl & al., 2000), and they also may be transposed within the genome. A common assumption has been that introns at a certain insertion point are homologous, but this is not necessarily so since different introns may have moved to new locations and repeated insertions of non-homologous insertions may have taken place at the same position, particularly at locations not protected by the 3D structure of the DNA

Aim of the paper. — The principal aim of this paper is to compare the current, morphologically based generic delimitation of *Chaenothecopsis* with the results of analyses of sequence comparisons of LSU and ITS rDNA sequences of a reasonably wide taxon sampling of Mycocaliciaceae. Another aim is to investigate the relationship between Sphinctrinaceae and Mycocaliciaceae and to identify their closest relatives by using LSU data. For this purpose several major ascomycete lineages were included in the analysis.

MATERIALS AND METHODS

Sampling. — Sequences downloaded from GenBank are listed in the Appendix along with new sequences.

DNA extraction, amplification and sequencing. — For the new sequences total DNA was extracted from ascomata or axenic cultures using the Qiagen DNeasy Plant Mini Kit. Either 10–30 apothecia or about 0.5 cm^2 of an axenic culture of the mycobiont was extracted. Collections identified by UPSC numbers were isolated from cultivated material. Lichen sample vouchers of the new sequences are listed in the Appendix.

PCR amplification was conducted by using the primers ITS1-F (Gardes & Bruns, 1993), and ITS 4 (White & al., 1990) to specifically amplify the fungal ITS1-5.8S-ITS2, and by using the primers ITS1-F (Gardes & Bruns, 1993) or 5.8Sr and LR7 (both: http://www.biology.duke.edu/fungi/mycolab/primers.htm) to amplify the LSU. The PCR ran for 35 cycles (1 min at 94°C, 1 min at 54°C, 45 sec at 72°C with a 4 sec/cycle extension at 72°C) using ABI or Promega Taq. Part of the amplifications were carried out by using Ready To Go PCR Beads (Pharmacia Biotech, Piscataway, New Jersey) according to the protocol of the producer. Before sequencing, the PCR product was purified using the Qiaquick Spin kit and protocol by Qiagen or Millipore Cleanup Plates.

Sequencing reactions were carried out with the following primers: ITS1-F, ITS2, ITS3, ITS4, 5.8Sr, LR0R, LR3, LR5 and LR7 (White & al., 1990). The forward primer LSLTS1 (TTCTGACGTGCAAATCGATCGT) was designed to extend sequences with long introns downstream position 1019 in the LSU of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen. The sequencing reaction ran for 26 cycles (30 s at 96°C, 15 s at 50°C, 4 min at 60°C) for sequencing by the BigDye labelling method (Perkin-Elmer) and for 30 cycles (20 s at 95°C, 15 s at 50°C, 3 min at 60°C) for sequencing by the MegaBACE labelling method (Pharmacia-Amersham). Contigs were assembled manually.

The LSU sequences were aligned manually. The ITS1-5.8S-ITS2 sequences were aligned using ClustalW as implemented by the Bioedit software packet (http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html), and adjusted manually. Insertions were excluded from the LSU alignment. The alignments can be obtained from the first author on request.

Phylogenetic analyses. — *Bayesian analysis.* The dataset was subjected to Bayesian inference using the program MrBayes version 3.0 beta4 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Priors for the trees and other parameters of the model were used as implemented as default in MrBayes version 3.0beta 4. The models for nucleotide substitutions were selected for each analysis individually prior to the MCMC. The model of evolution was chosen with MrModeltest Version 1.1b (Nylander, 2002) in conjunction with PAUP* (Swofford, 2002). For all analyses the general time reversal model of evolution (GTR) with a gamma distribution of substitution rates (G) and a proportion of invariant sites (I) was suggested. The four Markov chains were run for 2,000,000 generation and every one hundred tree was sampled. The tree samples of the burn-in phase (2000) were discarded. Groups with posterior probabilities above 50 are shown in the trees.

Maximum parsimony analyses. The data matrices were processed by the computer software PAUP* 4.0 (Swofford, 2002). Analyses of LSU and ITS rDNA applied a heuristic search using 1000 random addition sequences, TBR branch swapping algorithm, collapse branches if maximum branch length is zero, save multiple trees, gaps treated as missing data, and characters given equal weight.

Jackknife support values were obtained from an heuristic search using 1,000 replicates with five random additional replicates, TBR branch swapping algorithm, save multiple trees, collapse zero length branches when maximum length is zero, gaps treated as missing data, characters given equal weight, nominal deletion of characters 36.79%.

Morchella esculenta in Pezizales is a conservative choice of outgroup for character polarization in the LSU-based analysis, since Pezizales has been shown to be well outside Eurotiales and Mycocaliciales in SSU-based phylogenies (Wedin & Tibell, 1997; Lutzoni & al., 2001). Based on the sister group relationship between Eurotiales/Verrucariales and Mycocaliciales, as demonstrated in the LSU analysis, a representative of Eurotiales, *Byssochlamys nivea*, was selected as outgroup for the ITS1-5.8S-ITS2-based analysis.

Spliceosomal introns (Bhattacharya & al., 2000; Tibell, 2003) were identified by sequence features (Table 3).

RESULTS

Twenty-five new LSU rDNA and 29 new ITS1-5.8S-ITS2 sequences were produced in this study (Appendix).

Table 3. Location and sequence features of spliceosomal introns in the rDNA LSU sequences studied. Position refers to the homologous site before the intron in *Saccharomyces cerevisiae* GenBank Accession number J013551.

Position	Exon end	Donor site	Branch site	Acceptor site
1019	GTATAG	GTATG	CTAAC	CAG
	GCATAG	GTACG	TGAAC	
		GTAAG		

Phylogenetic analyses of the nLSU rDNA region. — The alignment of the rDNA LSU sequences included 49 sequences 1274 characters long representing 46 species. The tree resulting from the Bayesian inference is shown in Fig. 2. Posterior probability values (pp) are indicated. Using *Morchella esculenta* (Pezizales) as an outgroup, the analysis showed many major clades to be well supported such as Lichinales (pp = 1.00), Eurotiales (pp = 1.00), Arthoniales (pp = 1.00), Lecanoromycetes (excluding Lichinales; pp = 0.99), Verrucariales (pp = 1.00), and Coniocybaceae (pp = 1.00). Mycocaliciales are strongly supported (pp = 1.00). In the present taxon sampling the sister group of Mycocaliciales is formed by members of Eurotiales/Verrucariales (pp = 0.99). Mycocaliciales consist of two rather weakly supported clades, one containing *Mycocalicium subtile* and *M. albonigrum* (pp = 0.87), the other the rest of the species (pp = 0.63). *Sphinctrina*, representing Sphinctrinaceae, is embedded in a clade containing eight *Chaenothecopsis* species (Mycocaliciaceae), although this clade has only low support (pp = 0.74). It cannot, however, be excluded that Sphinctrinaceae are the sister group to Mycocaliciaceae, since the support for the consecutive lower nodes in the order is weak. Within Mycocaliciaceae one strongly supported node contains *Phaeocalicium populneum* and *Stenocybe pullatula* (pp = 1.00). One node (pp = 1.00) includes five *Chaenothecopsis* species (*C. dolichocephala*, *C. epithallina*, *C. fennica*, *C. pusiola*, and *C. viridireagens*).

Of the 1274 characters, 431 were parsimony informative. The heuristic search yielded 55 most parsimonious trees (CI = 0.36, RI = 0.53). For strongly supported groups the topology of the consensus tree (not shown) is very similar to that resulting from the Bayesian analysis. There is only one discrepancy in topology for nodes with a jackknife support >50%; all members of Mycocaliciales except for the two *Sphinctrina* species and *Chaenothecopsis savonica* in the maximum parsimony analysis form a very weakly supported clade (jackknife, j, = 52) not present in the Bayesian analysis. One further difference is that the clade containing the *Sphinctrina* species in the maximum parsimony consensus tree forms the sister group to the rest of Mycocaliciales, but there is no jackknife support for this relationship. Jackknife support as obtained in the analysis is indicated in italics in Fig. 2.

Insertions in the LSU. — Introns in the LSU of Mycocaliciales were only found in positions 1019 and 1042, whereas in one sequence from *Calicium adspersum* an intron was located at position 1314 (Table 4). The number of insertions in one species varies from none to two. Some insertions were recognized as spliceosomal introns (Bhattacharya & al., 2002). They were all located at position 1019 relative to the sequence of *Saccharomyces cerevisiae* and were found in *Chaenothecopsis consociata*, *C. fennica*, *C. pusiola* and *C. pusilla*. Three of them were of characteristic length (54–64 bp), whereas one insertion showing characteristics of the spliceosomal introns, in *Chaenothecopsis consociata*, was very long (378 bp). Length and donor and acceptor sequences of these spliceosomal insertions are included in Table 3.

Ten insertions (Table 4) of variable length (297–399) occurring in Mycocaliciales were not recognized as spliceosomal introns. They all were located at position 1042 and they were aligned. The Bayesian analysis of this alignment contained several strongly supported groups in the network obtained (Fig. 3). One strongly supported clade was formed by *Chaenothecopsis consociata*, *C. pusiola* and *C. pusilla* (pp = 0.99, j = 100). Another strongly supported clade was formed by *Mycocalicium subtile* and *Chaenothecopsis debilis* (pp = 1.00, j = 100). Further *Chaenothecopsis epithallina* and *C. fennica* formed a moderately supported clade (pp = 0.68, j = 88).

Table 4. Insertions in the nuclear rDNA LSU. Location refers to the homologous position before the intron in *Saccharomyces cerevisiae* GenBank Accession number J013551. S = spliceosomal intron, N = not classified insertion. Intron lengths within parentheses indicate incomplete introns. Clade designation refers to Fig. 4.

Species	GenBank accession number	Location	Length	Clade	Flanking sequences	Intron type
Chaenothecopsis consociata	DQ008999	1019	378	BF	GGGTATAG/GGGCGAAA	S
Chaenothecopsis fennica	AY795995	1019	64	B	GGGTATAG/GGGCGAAA	S
Chaenothecopsis pusilla	AY795998	1019	61	BF	GGGCATAG/GGGCGAAA	S
Chaenothecopsis pusiola	AY795999	1019	63	BF	GGGTATAG/GGGCGAAA	S
Sphinctrina turbinata T260	AY796007	1042	(225)	AC	GAACCAT/	N
Sphinctrina turbinata T307	DQ009001	1042	(254)	AC	GAACCAT/	N
Chaenothecopsis consociata	DQ008999	1042	309	BF	GAACCAT/CTAGTAGCT	N
Chaenothecopsis debilis VL2080	AY795991	1042	367	AD	GAACCAT/CTAGTAGCT	N
Chaenothecopsis epithallina	AY795994	1042	351	BF	GAACCAT/CTAGTAGCT	N
Chaenothecopsis fennica	AY795995	1042	399	B	GAACCAT/CTAGTAGCT	N
Chaenothecopsis pusilla VL2522	DQ009000	1042	297	BF	GAACCAT/CTAGTAGCT	N
Chaenothecopsis pusiola	AY795999	1042	308	BF	GAACCAT/CTAGTAGCT	N
Mycocalicium subtile VL2102	AY796003	1042	392	AE	GAACCAT/CTAGTAGCT	N
Mycocalicium subtile VL2504	AY796oo5	1042	399	AE	GAACCAT/CTAGTAGCT	N

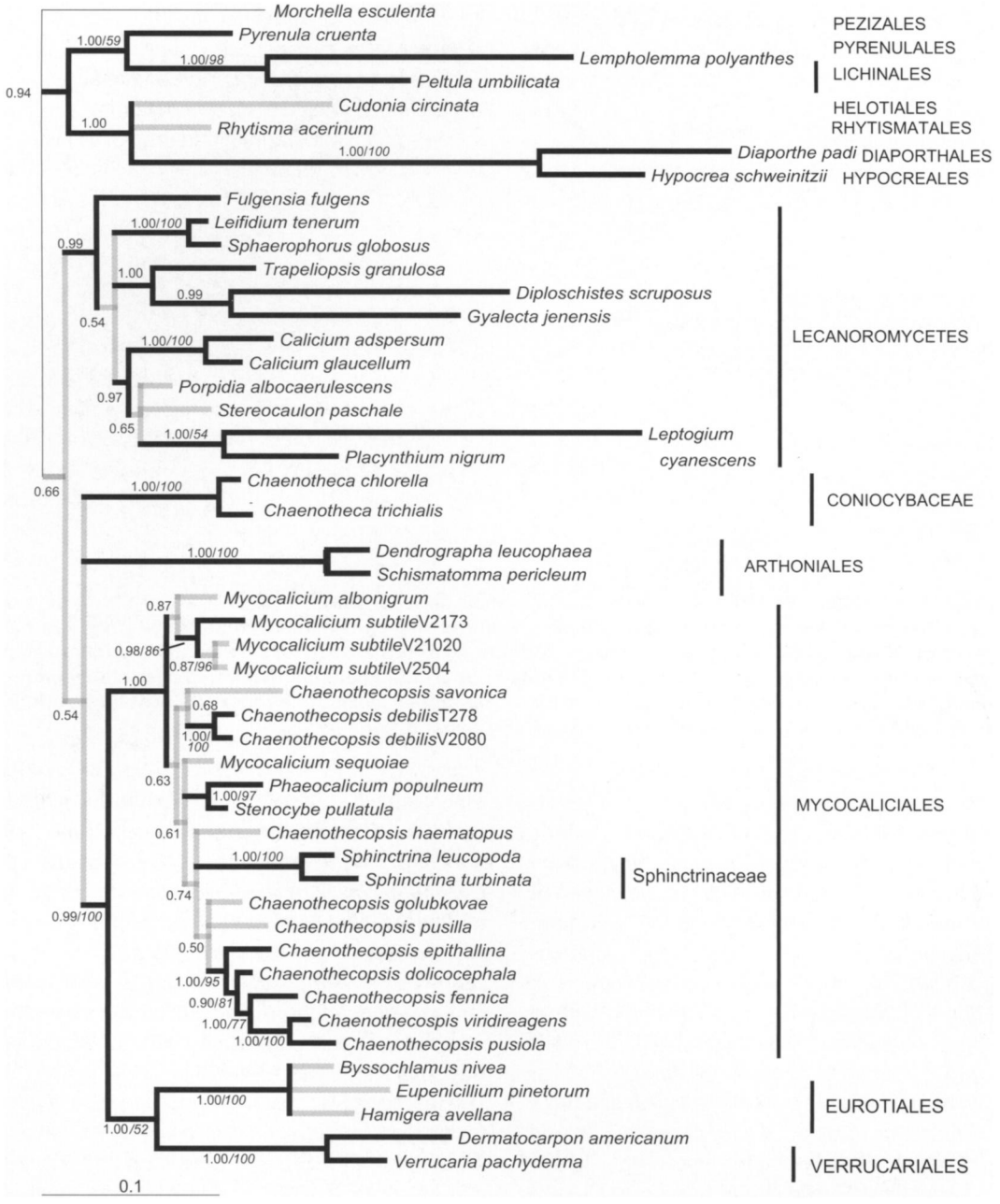

Fig. 2. LSU rDNA phylogeny of Mycocaliciales and selected ascomycetes using *Peziza* as outgroup. Majority-rule consensus tree of 18,000 trees sampled from a 2,000,000 generation MCMC Bayesian tree sampling under the GTR+G+I model. First value at branches indicates posterior probability; second value (italics) indicates jackknife support obtained in a maximum parsimony analysis of the same alignment. Strongly supported branches in black (pp and/or jackknife support at least 0.90 and 90, respectively); less strongly supported branches in grey. Scale bar = substitutions per site.

Phylogenetic analyses of the nrITS-5.8S DNA region. — Results from the LSU-based analysis indicated that a close outgroup to Mycocaliciales would be found in Eurotiales, and *Byssochlamus nivea* was chosen for this purpose in the ITS analysis. A further Eurotiales species was also included in the analysis, *Eupenicillium pinetorum*. The ITS alignment included 37 sequences with 618 characters representing 26 species. The majority of the *Chaenothecopsis* species from Europe were included in the study along with several species from Eastern Asia and Australasia. For comparisons with other genera in Mycocaliciales, species of *Mycocalicium*, *Phaeocalicium*, *Sphinctrina* and *Stenocybe* were also included in the study. The tree resulting

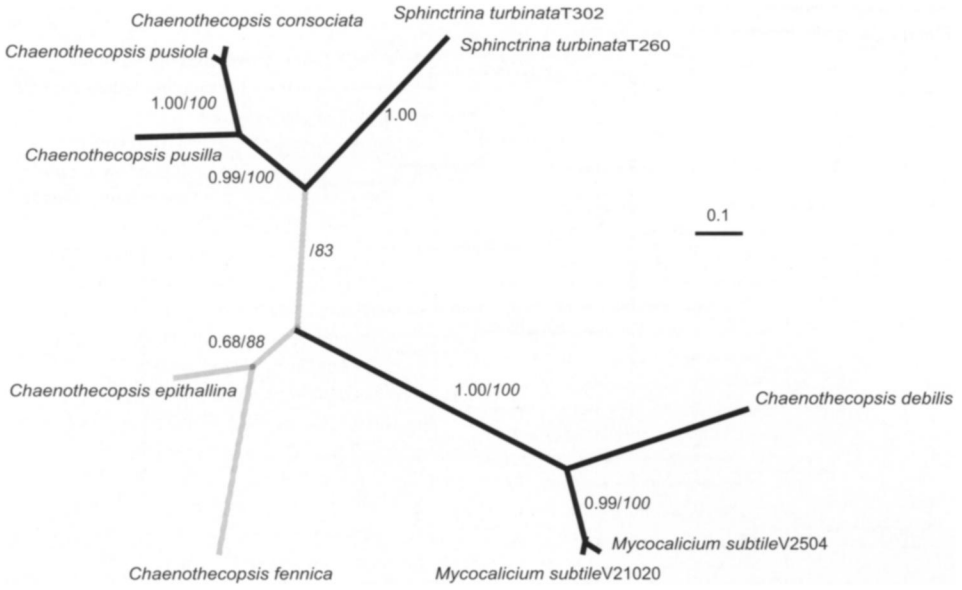

Fig. 3. Phylogenetic network of 10 introns from position 1042 derived from the rDNA LSU of six *Chaenothecopsis* species, *Mycocalicium subtile*, and *Sphinctrina turbinata*. Majority-rule consensus tree of 18,000 trees sampled from a 2,000,000 generation MCMC Bayesian tree sampling under the GTR+G+I model. First values at branches indicate posterior probability; second values (italics) indicate jackknife support obtained in a maximum parsimony analysis of the same alignment. Strongly supported branches are in black (pp and/or jackknife support at least 0.90 and 90, respectively); less strongly supported branches in grey. Scale bar = substitutions per site.

from the Bayesian inference is shown in Fig. 4. Mycocaliciales were resolved in two monophyletic groups (A and B). One group (A), containing fifteen species, was highly supported (pp = 99) in the Bayesian analysis, and had moderate support from the maximum parsimony analysis (j = 84). It includes representatives not only of *Chaenothecopsis* but also of *Mycocalicium*, *Phaeocalicium* and *Stenocybe*. One clade in this group (Clade C: pp = 0.98, j = 65) includes two species of *Sphinctrina* (*S. leucopoda* and *S. turbinata*), which together have high support (pp = 1.00, j = 100) and three species of *Chaenothecopsis* (*C. nana*, *C. resinicola* and *C. orientalis*), which also have high support (pp = 1.00, j = 99). *Chaenothecopsis haematopus* forms a well-supported clade with *C. savonica* (pp = 1.00, j = 100) and so does *Phaeocalicium interruptum* with *Chaenothecopsis tibellii* (Clade D: pp = 1.00, j = 100). There is also strong support for a clade including *Phaeocalicium populneum* and *Stenocybe pullatula* (pp = 1.00, j = 96). The *Mycocalicium* species form a group with moderate support in the Bayesian analysis (Clade E: pp = 0.95, j <50), with *Chaenothecopsis debilis*, *Phaeocalicium populneum* and *Stenocybe pullatula*, and a weakly supported (pp = 0.70) sister group relationship was found between Clades D and E. The clade consisting of D and E, Clade C and the clade consisting of *Chaenothecopsis haematopus* and *Chaenothecopsis savonica*, formed a basal trichotomy in Clade A. The other main clade of Mycocaliciales is well supported both in the Bayesian analysis and maximum parsimony analysis (Clade B: pp = 0.99, j = 99) and exclusively contains species of *Chaenothecopsis*. Here one well-supported group is formed by *Chaenothecopsis consociata*, *C. pusilla*, *C. pusiola* and *C. viridireagens* (pp = 1.00, j = 100), and another by *C. dolichocephala* and *C. fennica* (pp = 1.00, j = 99). Other relationships in this clade have only low or moderate support, excepting Clade F, which has a high Bayesian support (pp = 0.99, j = 51).

In the LSU analysis Verrucariales, together with Eurotiales, formed the sister group to Mycocaliciales. A sequence of a member of Verrucariales, *Dermatocarpon miniatum* (AF333144), was also tried as an outgroup in a Bayesian analysis of an alignment of the same sequences except for *Byssochlamys nivea* and *Eupenicillium pinetorum*. In this analysis (not shown) the tree was less resolved. Clade B of Fig. 4 was also here strongly supported (pp = 1.00). Further, several smaller clades, such as *Chaenothecopsis nana/C. resinicola/C. orientalis* (pp = 1.00), *C. consociata/C. pusiola/C. pusilla/C. viridireagens* (pp = 1.00), and *Sphinctrina leucopoda* and *S. turbinata* (pp = 1.00), obtained high support. There were, however, also conflicts in two parts of the trees. Compared to the analysis using *Byssochlamys nivea* as outgroup, there was a minor difference in Clade F insofar that *Chaenothecopsis epithallina* and *C. subparoica* were sister species and together with low support (pp =

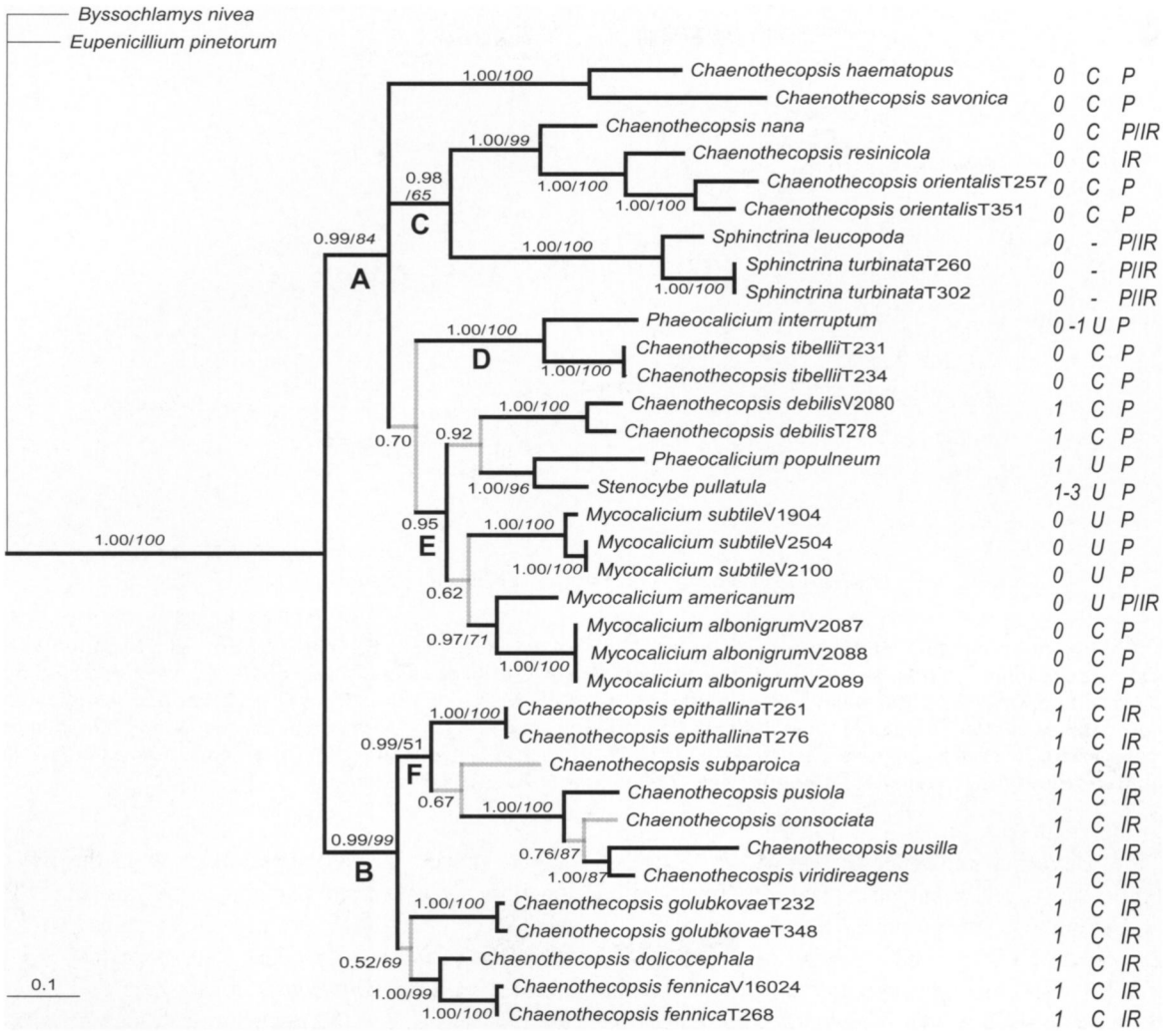

Fig. 4. ITS rDNA phylogeny of 16 *Chaenothecopsis* species and selected species from other genera in Mycocaliciales using *Byssochlamys nivea* as outgroup. Majority-rule consensus tree of 18,000 trees sampled from a 2,000,000 generation MCMC Bayesian tree sampling under the GTR+G+I model. First values at branches indicate posterior probability; second values (italics) indicate jackknife support obtained in a maximum parsimony analysis of the same alignment. Strongly supported branches in black (pp and/or jackknife support at least 0.90 and 90, respectively); less strongly supported branches in grey. After the species name the number of spore septa is indicated in the first column. In the second column the type of ascus apex is shown (*C* = presence of a canal, '*Chaenothecopsis*-type'; *U* = a uniformly thickened apex, '*Mycocalicium*-type'. The third column indicates the type of stalk anatomy (*P* = periclinally arranged, largely unbranched hyphae; *IR* = irregularly oriented and interwoven stalk hyphae in the inner part of the stalk; *P/IR* = variable or intermediate condition).

0.62) formed the sister group to *C. consociata*, *C. pusilla*, *C. pusiola* and *C. viridireagens*. Another difference was that in the latter analysis *C. nana*, *C. resinicola* and *C. orientalis* formed the sister group to *Phaeocalicium interruptum* and *Chaenothecopsis tibellii* (pp = 0.95), a relationship not supported in the first analysis, where these species were the sister group to the *Sphinctrina* species.

Of the 618 characters in the maximum parsimony analysis, 318 were informative. The heuristic search yielded two most parsimonious trees (CI = 0.43, RI = 0.66). For strongly supported groups, the topology of the strict consensus tree (not shown) is very similar to that resulting from the Bayesian analysis, but it is less resolved. There is only one discrepancy in topology. In the parsimony analysis *Chaenothecopsis epithallina* and *C. subparoica* are sister species with low support (j = 70), whereas in the Bayesian analysis a clade comprising *C. consociata*, *C. pusilla*, *C. pusiola* and *C. viridireagens* with low support (pp = 0.67) forms the sister group of *C. subparoica*.

Description of a new *Chaenothecopsis* species. — ITS sequences of two collections of *Chaenothecopsis* grouped together (Fig. 4) and were dif-

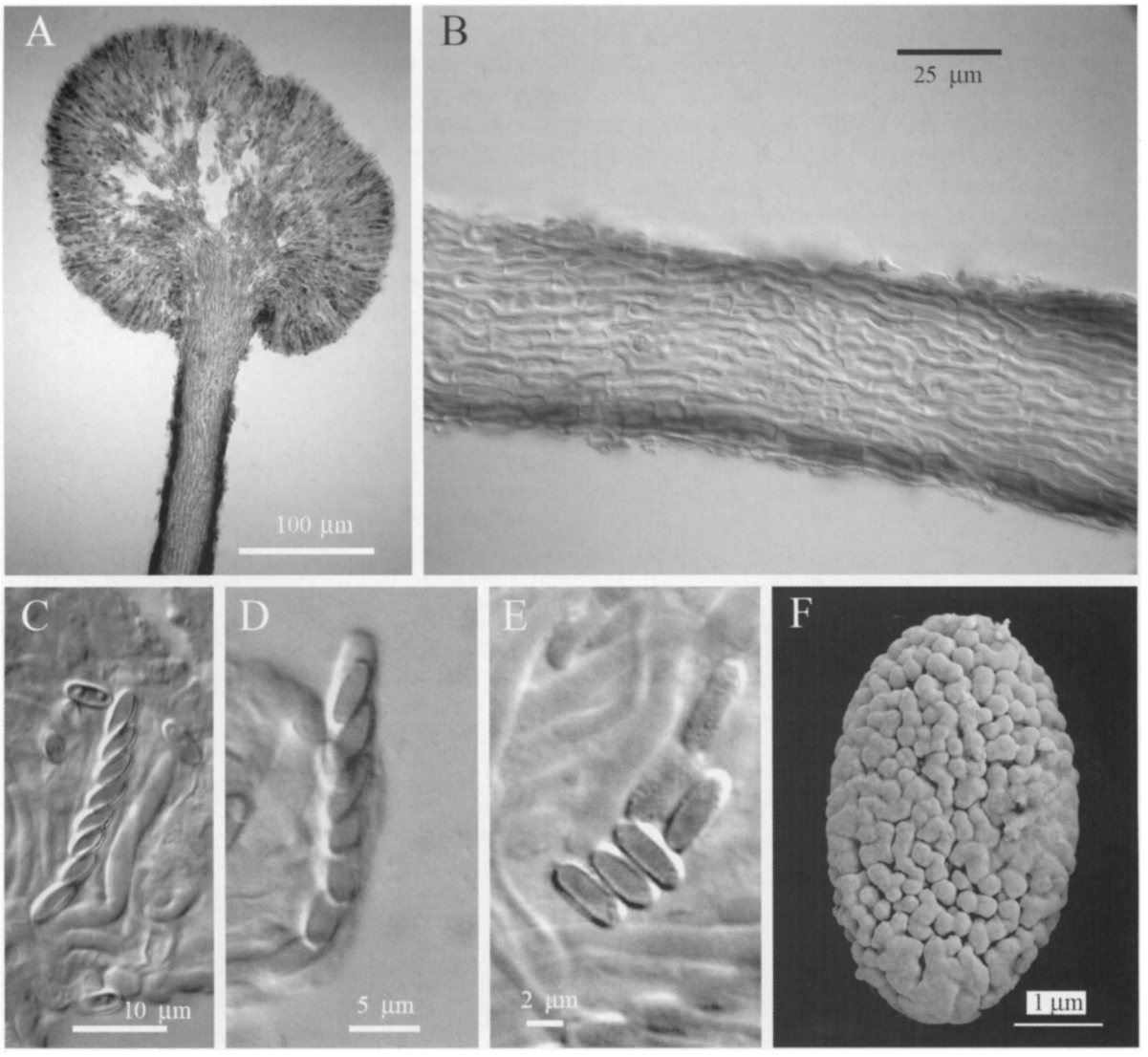

Fig. 5. *Chaenothecopsis orientalis*. Holotype. A, longitudinal section of mature, stalked ascoma; B, longitudinal section of ascoma stalk consisting of largely periclinally arranged hyphae; C, mature ascus containing eight non-septate spores; D, mature ascus with uniformly thickened ascus apex; E, mature spores with minute ornamentation; F, mature spore with ornamentation consisting of small, strongly convex, subspherical to slightly elongated or irregular projections.

ferent from those of the other species. A careful morphological investigation revealed that these specimens had been misidentified and indeed represented an undescribed species.

Chaenothecopsis orientalis Tibell, **sp. nov.** – Fig. 5. Holotype: INDIA. Uttaranchal: 26 km NNE of Ghuttu, Kharsoli, just N of the campsite, 30°44'N, 78°54'E, 2950 m, on trunk of *Quercus semecarpifolia* in shaded situation in mixed oak-spruce-hemlock forest, 18 May 2003, *Tibell 23216* (UPS).

Parasiticus ad parasymbioticum in algis vel saprobicum. Apothecia 0.7–1.1 mm alta. Capitulum subsphaericum, nigrum, non nitidum, fuscum vel paulo aeruginosum. Epithecium tenuissimum, debiliter evolutum, pallide olivaceobrunneum, hypothecium pallidum, excipulum debilissime evolutum. Stipes rectus vel paulo flexuosus, 0.04–0.06 mm diametro, fuscus ad nigrum, pars interiora pallida, e hyphis periclinaliter ordinatis et solum paulo irregulariter intertextis constans, parietes hypharum tenues. Pars extima stipitis olivaceobrunnea. Omnes partes apotheicorum KOH± rufescentes et HNO_3–. Asci 28–31 × 3–3.5 µm, cylindrici. Apex asci incrassatus, in ascis juvenibus canale angustissimo perforatus, in ascis maturis dilatato. Sporae in ascis uniseri-

ate ordinatae, periclinaliter vel oblique positae, partim imbricatae. Sporae non-septatae, 4.5–6.0 × 2.0–2.5 µm, ellipsoidales, pallidofuscae, laeves.

Parasitic-parasymbiotic among colonies of *Stichococcus* infested with fungal hyphae, but possibly saprobic on the underlaying bark. Apothecia 0.7–1.1 mm high. Capitulum subspherical, 0.14–0.28 mm in diam., black, not glossy, blackish-brown to slightly aeruginose. Epithecium very thin, poorly developed, pale olivaceous brown. Hypothecium pale, consisting of radiating, somewhat intertwined hyphae. Excipulum very poorly developed, forming a minute collar at the top of the stalk. Stalk straight or somewhat flexuous, 0.04–0.06 mm in diam., dark-brown to black, but sometimes partially pale brown. The interior of the stalk pale, consisting of pale, periclinally arranged, only slightly interwoven hyphae with thin walls. The outermost part of the stalk consists of 2–3 layers of periclinally arranged, brownish hyphae forming a 5.5–8.5 µm thick pigmented layer. Outermost stalk hyphae 3–4 µm wide. All parts of the capitulum KOH+ weakly to distinctly reddish-brown, HNO_3–. Stalk KOH+ distinctly reddish-brown, swelling strongly. Asci 28–31 × 3–3.5 µm, cylindrical. Ascus apex thickened, about 1.5 µm thick and in young asci for a time penetrated by a very fine canal, which, however, is not seen in mature asci. Spores uniseriately arranged in the asci, periclinally or obliquely oriented and partly overlapping. Spores non-septate, 4.5–6.0 × 2.0–2.5 µm, ellipsoidal, sometimes with a slight constriction at the middle, pale brown, smooth or with a very minute ornamentation barely visible under a light microscope. As viewed by SEM, the spores have a distinctive ornamentation consisting of small, strongly convex, subspherical to slightly elongated or irregular projections

Further material investigated: RUSSIA. Primorskij Territory, Lazovskij Region, 29 km NW of Lazo, side valley along road from Lazo to Sergejevka, 43:11N, 133:38E, c. 630 m, on decorticated stump of *Picea ajanensis* in mixed *P. ajanensis-Abies nephrolepis* forest on N-facing slope, 1991, *Tibell 19371* (UPS).

Chaenothecopsis orientalis may easily be mistaken for *C. savonica*. Both these species have non-septate spores, the surface of the capitulum sometimes has a slight aeruginose tinge, and they occur in association with colonies of free-living algae or as saprobes. *Chaenothecopsis orientalis* differs from *C. savonica* in having paler, brownish stalks, a much paler interior of the stalk, and by the KOH+ reddish-brown reaction of the stalk accompanied by a strong swelling. The outermost hyphae of the stalk are 3–4 µm diam. In *C. savonica* the interior of the black stalks consists of dark, periclinally arranged hyphae with a few pale hyphae in the centre, the hyphae at the surface are 1–2 µm diam., the stalk only swells moderately in KOH, and a yellowish-brown reaction is obtained. In water under the light microscope the unsectioned stalk of *C. orientalis* is medium-brown and in *C. savonica* black or very dark greenish-black. Pending a clarification of the generic relationships in Mycocaliciaceae, this species has been described in *Chaenothecopsis*. In the ITS-based phylogeny it forms a strongly supported group with *C. nana* and *C. resinicola*, which both also have a canal penetrating the ascus apex and non-septate spores. They possibly represent a distinct genus in Mycocaliciaceae that according to present molecular evidence is closely related to *Sphinctrina*.

DISCUSSION

Mycocaliciales. — In the Bayesian inference analysis of LSU sequences (Fig. 2) with *Morchella esculenta* (Pezizales) as an outgroup, several clades, such as Lichinales, Eurotiales, Arthoniales, Lecanoromycetes (excluding Lichinales), Verrucariales, and Coniocybaceae, were well supported. Mycocaliciales received high support. As in other LSU or partly LSU-based analyses (Bhattacharya & al., 2000; Lutzoni & al., 2001; Kauff & Lutzoni, 2002), some of the lower nodes adjacent to Pezizales are poorly resolved and/or supported. The taxon sampling, however, was not designed to reveal these relationships, since the focus was on identifying the sistergroup to Mycocaliciales. It is interesting, however, to note that Pyrenulales and Lichinales form a strongly supported group, although Pyrenulales were represented by one species only. Pyrenulales were not included in the analyses either by Schulz & al. (2001) or by Kauff & Lutzoni (2002). In the present taxon sampling the sistergroup of Mycocaliciales is formed by Eurotiales and Verrucariales jointly. In earlier investigations (Gargas & al., 1995; Wedin & Tibell, 1997) Eurotiales were identified as the sister group of Mycocaliciales.

Mycocaliciaceae and Sphinctrinaceae. — Support for clades within Mycocaliciales is not strong, with many nodes having quite low support, and the clades having short branch-lengths. Mycocaliciales consist of two moderately to weakly supported clades, one containing *Mycocalicium subtile* and *M. albonigrum*, the other the rest of the species. *Sphinctrina*, representing Sphinctrinaceae, is embedded in a clade containing species of *Chaenothecopsis* (Mycocaliciaceae), although this clade has low support. It cannot in this analysis, however, be excluded that Sphinctrinaceae are sister to Mycocaliciaceae, since support for the consecutive lower nodes in the order is weak. This LSU-based analysis indicates that Sphinctrinaceae are possibly embedded in Mycocaliciaceae, whereas Sphinctrinaceae and Mycocaliciaceae formed sister groups in the analysis of Wedin

& Tibell (1997) based on SSU sequences. It was evident from these results that a more fast-evolving locus was needed to elucidate relationships within Mycocaliciaceae and that members of Sphinctrinaceae should not be used as an outgroup in the analysis. A close outgroup may thus be chosen from Eurotiales or Verrucariales.

In the analysis based on ITS1-5.8S-ITS2 sequences using *Byssochlamys nivea* as an outgroup, *Sphinctrina* is embedded in the strongly supported Clade A along with species in *Chaenothecopsis*, *Mycocalicium*, *Phaeocalicium* and *Stenocybe*. This makes the present delimitation of *Chaenothecopsis* versus *Sphinctrina* non-monophyletic. Further it gives strong evidence that Sphinctrinaceae and Mycocaliciaceae cannot be maintained in their present circumscription. According to our phylogeny, the formation of a mazaedium is a synapomorphy for *Sphinctrina* in Mycocaliciales.

Two major clades in Mycocaliciales. — In the analysis based on ITS1-5.8S-ITS2 sequences using *Byssochlamys nivea* as an outgroup and otherwise only including one other species of Eurotiales (*Eupenicillium pinetorum*) and species of Mycocaliciaceae and Sphinctrinaceae, two major clades were formed (Fig. 4). The first of these, Clade A, is strongly supported in the Bayesian analysis (pp = 0.99, j = 84) and includes species in *Chaenothecopsis*, *Mycocalicium*, *Phaeocalicium*, *Sphinctrina* and *Stenocybe*.

In clade A, *Phaeocalicium populneum* forms a strongly supported clade with *Stenocybe pullatula*, whereas *Phaeocalicium interruptum* forms a strongly supported group with *Chaenothecopsis tibellii*. This illustrates that current generic delimitations do not correspond to monophyletic groupings. Furthermore, several *Chaenothecopsis* species in Clade A group with species in *Mycocalicium*, *Phaeocalicium*, *Sphinctrina* and *Stenocybe*, and are more distantly related to the species of *Chaenothecopsis* of Clade B. The close relationship between *Phaeocalicium populneum* and *Stenocybe pullatula* is perhaps not surprising. The distinction between *Phaeocalicium* and *Stenocybe* was based on spore septation and capitulum shape only, and Schmidt (1970) assumed a close relationship. Members of both genera occur as saprobes or weak parasites on trees and shrubs, mainly on thin twigs. There are also differences between the *Chaenothecopsis* species in Clade A and Clade B. Those in the former are all saprobes, with only one species, *Chaenothecopsis resinicola*, occurring on resin. All species of *Chaenothecopsis* in Clade A, except for *C. debilis*, have non-septate spores in contrast to the *Chaenothecopsis* species in Clade B, which all have 1-septate spores.

The other main clade, Clade B, is also well supported (pp = 0.99, j = 99). It exclusively contains *Chaenothecopsis* species that all have 1-septate spores. Clade F is remarkable insofar that it contains species that all live as commensals or parasites on lichens or sometimes possibly on free-living algae. The species in the sister clade of Clade F are saprobes, *C. fennica* growing on wood and the other species on resin. Resinicolous species thus do not form a monophyletic group.

Abberrant sequences. — Some of the ITS1-5.8S-ITS2 sequences obtained were quite aberrant in comparison to the majority of the sequences, and aligned very poorly with the majority of sequences. Such sequences were obtained for *Chaenothecopsis amurensis* Titov, *C. sanguinea* Tibell, and *C. rubescens*. They all, however, contained a 5.8S part almost identical with that of the other species investigated, i.e., this part of the genome may well be fully functional. Aberrant sequences may be caused by contaminations, but searches in GenBank did not reveal any similar sequences. Two specimens of *C. rubescens* were sequenced, one from Estonia and one from India. Their sequences were almost identical. These sequences thus did not seem homologous to the other ones studied and included in the alignment. There is also a possibility that lineage sorting has eliminated different gene copies in different lineages and the different genes are then not homologous. A possibility that still remains, of course, is that the anomalous sequences were obtained from an alien organism. Sequences of *Mycocalicium victoriae* available from GenBank (AF243135, AJ312123, AY128701, and AY128702) do not seem to be derived from an organism in Mycocaliciales.

Taxonomic information from introns in the LSU. — Although introns were only found in a limited number of species, they still provided useful phylogenetic signal. Four spliceosomal introns at position 1019 were all found in *Chaenothecopsis* species of Clade B, viz., in *C. fennica*, *C. consociata*, *C. pusiola* and *C. pusilla*, and the three latter species belong to Clade F. No spliceosomal introns were found in Clade A.

An analysis of ten unidentified introns from eight species provided phylogenetic information that was largely congruent with that of the ITS-based phylogeny. The analysis was restricted to introns occurring at the same position (1042), but they may still not be homologous since intron movement within genes (Bhattacharya & al., 2002)—and even between organisms—has been demonstrated. A further limitation of the analysis is that introns occurred in a few species only, in accordance with an hypothesis of rare intron gain and frequent loss (Bhattacharya & al., 2002). Congruence between the ITS and intron phylogenies were noted in some instances. Thus *C. consociata*, *C. pusilla*, and *C. pusiola* form a well-supported clade in the intron phylogeny and also form a strongly supported subclade of Clade B. Introns of *Mycocalicium subtile* and *Chaenothecopsis debilis*

also form a strongly supported clade and both belong to Subclade E. *Chaenothecopsis epithallina* and *C. fennica* form a moderately supported clade and both belong to Clade B, but to different subclades. Their separation from the *C. consociata*, *C. pusilla*, and *C. pusiola* intron clade, like the isolation of *Sphinctrina turbinata* from the other members of Clade A, may be a result of different origin of the introns, or other processes such as lineage sorting.

Although very incomplete, it is obvious that several of the LSU intron sequences studied here, as with some other rDNA introns (e.g., Thell & Miao, 1999; Lohtander & al., 2000; Bhattacharya & al., 2002; Tibell, 2003) contain phylogenetic signal congruent with that of exon sequences, but also that introns in the same position may have different evolutionary histories. Intron phylogenies may, however, often be poorly resolved because of saturation and a pattern of rare origin, intragenic spread and frequent loss (Bhattacharya & al., 2002).

Ascus apex structure. — The structure of the ascus apex was considered a very important feature for the recognition of *Chaenothecopsis* by Schmidt (1970). A thickened ascus apex (Fig. 1) penetrated by a narrow canal is found in most species of *Chaenothecopsis*, but not in all, as was noted in *C. fennica* by Tibell (1978). Ascus apex structure is indicated in relation to the phylogeny in Fig. 4. A wide canal also occurs in *C. dolichocephala* and *C. golubkovae*, which together with *C. fennica* form a poorly supported clade. The other species in Clade B have a more typical *Chaenothecopsis*-type ascus apex with a narrow canal. *Mycocalicium* was described by Schmidt as having a uniformly thickened ascus apex. This is, however, not true for *M. albonigrum*, which has a moderately widened canal in its ascus apex. The *Chaenothecopsis* species in Clade A all have a *Chaenothecopsis*-type of ascus apex. In Clade D *Phaeocalicium interruptum* has a uniformly thickened ascus apex and in Clade D *Sphinctrina* has evanescent asci. In conclusion, ascus apex structure is largely correlated with current generic delimitations in Mycocaliciceae and is indeed consistent in several of the monophyletic groups.

Stalk anatomy. — Another feature that was stressed by Schmidt (1970) was the anatomy of the stalk. In *Chaenothecopsis* (Fig. 1A, B) the stalk hyphae were described as pale and intertwined in the central part and dark and more or less periclinally arranged at the surface of the stalk. In *Mycocalicium* (Fig. 1D, E), *Phaeocalicium*, and *Stenocybe*, the stalk hyphae were described as dark and periclinally arranged throughout the stalk. Stalk anatomy structure is indicated in relation to the phylogeny in Fig. 4. The *Chaenothecopsis* species in Clade B consistently have a pale stalk interior with more or less irregularly arranged hyphae. In Clade A there is considerable variation in stalk anatomy. *Mycocalicium albonigrum* and *M. subtile* have periclinally arranged, dark hyphae throughout the stalk, but in the central parts of the stalk of *M. americanum* the hyphae are pale and have somewhat intertwined and irregularly arranged hyphae. *Stenocybe pullatula* and *Phaeocalicium populneum* have dark, largely periclinally arranged hyphae in accordance with Schmidt's description. In Clade C, *Chaenothecopsis nana* has largely periclinally arranged stalk hyphae that are dark at the surface but paler in the centre of the stalk, whereas in *C. resinicola* the hyphae in the central part are pale and irregularly arranged, and in *C. orientalis* they are largely periclinally arranged and pale. In *Sphinctrina* the stalk hyphae are irregularly to weakly periclinally arranged. In *Phaeocalicium interruptum* and *Chaenothecopsis tibellii*, *C. haematopus* and *C. savonica*, the stalk hyphae are largely periclinally arranged, but not black. In conclusion, stalk anatomy is not consistent in *Chaenothecopsis* and *Mycocalicium* as currently circumscribed, but is indeed consistent in several of the monophyletic groups.

Anamorphs. — A remarkable variety of anamorphs are known from Mycocaliciaceae (Tibell, 1990, 1991, 1993, 1995, 1997; Tibell & Constantinescu, 1991). Both coelomycetous and hyphomycetous anamorphs occur, sometimes as synanamorphs in the same species (Tibell, 1991, 1993). In Clade A (Fig. 4) *Chaenothecopsis savonica* produces a coelomycetous anamorph in culture (Tibell, 1991) with conidiomata having a very simple wall with the conidiogenous cells also forming the conidioma wall. Conidiophores are of Vobis' Type I and the ontogeny of *Lecanactis*-type (Vobis, 1980). In addition to this, *C. savonica* also produces a hyphomycetous synanamorph of poorly differentiated hyphae. Of the other species in Clade A known to produce anamorphs, *Chaenothecopsis debilis* (Tibell, 1995) has a conidioma wall consisting of several layers of cells lined on the inner surface by the conidiogenous cells. The conidiogenous cells belong to Vobis Type I or II (Vobis, 1980). The conidia are hyaline. *Mycocalicium albonigrum* and *M. subtile*, species which like *C. debilis* belong to Clade E, have similar conidiomata, but the conidia are brown. A spectacular hyphomycetous anamorph is produced by *Chaenothecopsis haematopus*, in our analysis the sister species of *C. savonica*, in which thick-walled conidia are formed in long and sometimes branched chains originating from the tip of a stalk (Tibell & Constantinescu, 1991). In Clade B only *Chaenothecopsis viridireagens* is known to produce anamorphs (Tibell, 1993). In this species the coelomycetous conidiomata are stalked and often situated on branched stalks. Although these pycnidia are distinctive in many details, they are generally similar to those of *C. savonica* in the structure of conidiogenous cells and pycnidium and conidium

ontogeny. A simple hyphomycetous anamorph is also present in *C. viridireagens*. *Chaenothecopsis* has been considered to show extreme diversity of anamorphs (Tibell, 1997; Table 1), and this may well reflect the paraphyletic nature of our present circumscription of *Chaenothecopsis*. Anamorph features may be more uniform in monophyletic groups, as they are in Clade E, and additional information of anamorph features may help to elucidate the phylogeny of Mycocalicaceae.

Sphinctrina. — *S. leucopoda* and *S. turbinata* formed a monophyletic group both in the LSU-based and ITS1-5.8S-ITS2-based phylogeny. This group was, however, embedded in Mycocaliciaceae, and Sphinctrinaceae may thus be included in Mycocaliciaceae. The independent origin of evanescent asci and a mazaedium in *Sphinctrina* as suggested by Wedin & Tibell (1997) is supported.

Chaenothecopsis. — *Chaenothecopsis* did not form a monophyletic group either in the LSU-based, or in the ITS1-5.8S-ITS2-based, phylogeny. Species with the *Chaenothecopsis*-type of ascus apex did not form a monophyletic group and neither did species with the interior of the stalk consisting of pale, intertwined hyphae. Consequently Schmidt's circumscription of *Chaenothecopsis* is incongruent with our phylogenies. Some monophyletic groups from the molecular phylogenies receive morphological and/or ecological support. Thus Clade F (containing species being commensals on lichens or possibly parasites on free-living algae) and its sister group (containing saprobes) together form a clade with strong support with species that all have 1-septate spores and a similar stalk anatomy. Only species in Clade B (*Chaenothecopsis consociata*, *C. fennica*, *C. pusiola* and *C. pusilla*) were found to have spliceosomal introns. These introns were all located at position 1019. *Chaenothecopsis consociata*, *C. epithallina*, *C. fennica*, *C. pusilla* and *C. pusiola* from this clade also contained unclassified introns at position 1042.

All *Chaenothecopsis* species in Clade A, except for *C. debilis*, have non-septate spores and a *Chaenothecopsis*-type of ascus apex, but they do not form a monophyletic group, although the relationships between *C. debilis*, *C. tibellii*, and species in *Mycocalicium*, *Phaeocalicium*, *Sphinctrina* and *Stenocybe* in the phylogeny only have moderate support. Few introns were encountered in Clade A; no spliceosomal introns were noted, and unclassified introns were only found in *Mycocalicium subtile* and *Chaenothecopsis debilis* at position 1042. Unfortunately the type species of *Chaenothecopsis*, *C. rubescens*, yielded aberrant sequences that could not in the ITS regions be aligned with those of the other species.

The recognition of *Chaenothecopsis*, *Mycocalicium*, *Phaeocalicium*, and *Stenocybe* has been based on a few morphological cardinal characters, and as shown here they only partly reflect the phylogenetic relationships of the species in Mycocaliciaceae as inferred from ITS sequences. *Chaenothecopsis* and *Phaeocalicium* cannot be retained with current circumscriptions. For an evaluation of the delineation of *Mycocalicium* and *Stenocybe* in relation to *Mycocalicium* and *Phaeocalicium,* a much more extensive taxon sampling directed towards that problem is needed. This will hopefully lead to the identification of well-supported monophyletic groups that can be assigned generic rank. This may necessitate not only inclusion of more taxa, but also study of additional genes.

ACKNOWLEDGEMENTS

The fellow members of the Lichen Group in Uppsala have supplied cordial encouragement and taken an interest in the investigations. Nahid Heidari has offered valuable support in laboratory work in the Department of Plant Systematics. Several colleagues have kindly supplied fresh material for DNA isolations, and we particularly thank K. Kalb, Neumarkt/Obf.; Joukko Rikkinen, Helsinki; A. Titov, St. Petersburg; and M. Tretiach, Trieste. This investigation has been supported by the Olsson-Borgh Foundation of Uppsala University and the Magnus Bergvall Foundation, Stockholm.

LITERATURE CITED

Bhattacharya, D., Friedl, T. & Helms, G. 2002. Vertical evolution of and intragenic spread of lichen-fungal group I introns. *J. Molec. Evol.* 55: 74–84.

Bhattacharya, D., Lutzoni, F., Reeb, V., Simon, D., Nason, J. & Fernandez, F. 2000. Widespread occurrence of spliceosomal introns in the rDNA genes of ascomycetes. *Molec. Biol. Evol.* 17: 1971–1984.

Cubero, O., Bridge, P. & Crespo, A. 2000. Terminal-sequence conservation identifies spliceosomal introns in ascomycete 18S RNA genes. *Molec. Biol. Evol.* 17: 751–756.

DePriest, P. 1993a. Molecular innovations in lichen systematics: the use of ribosomal and intron nucleotide sequences in the *Cladonia chlorophaea* complex. *The Bryologist* 96: 314–325.

DePriest, P. 1993b. Small subunit rDNA variation in a population of lichen fungi due to optional group I introns. *Gene* 134: 67–71.

DePriest, P. & Been, M. 1992. Numerous group I introns with variable distributions in the ribosomal DNA of a lichen fungus. *J. Molec. Biol.* 228: 315–321.

Friedl, T., Besendahl, A., Pfeiffer, P. & Bhattacharya, D. 2000. The distribution of group I introns in lichen algae suggest that lichenization facilitates intron lateral transfer. *Molec. Phylog. Evol.* 14: 342–352.

Gardes, M. & Bruns, T. D. 1993. ITS primers with enhanced

specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Molec. Ecol.* 2: 113–118.

Gargas, A., DePriest, P. & Taylor, J. W. 1995. Positions of multiple insertions in SSU rDNA of lichen-forming fungi. *Molec. Biol. Evol.* 12: 208–218.

Gargas, A., Taylor, J. W. 1995. Phylogeny of discomycetes and early radiations of the apothecial Ascomycotina inferred from SSU rDNA sequence data. *Exp. Mycol.* 19: 7–15.

Henssen, A. & Jahns, H. M. 1973 ["1974"]. *Lichenes. Eine Einführung in die Flechtenkunde.* Thieme, Stuttgart.

Huelsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

Kauff, F. & Lutzoni, F. 2002: Phylogeny of the Gyalectales and Ostropales (Ascomycota, Fungi): among and within order relationships. *Molec. Phylog. Evol.* 25: 138–156.

Keissler, K. 1938. *Pyrenulaceae bis Mycoporaceae. Coniocarpinae.* In Rabenhorst's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz 9(1:2): 1–846. Akademische Verlagsgesellschaft, Leipzig.

Lohtander, K., Myllys, L., Moberg, R., Källersjö, M. & Tehler, A. 2000. The genus *Physcia* (Physciaceae): phylogeny inferred from ITS and intron sequences. P. 95 in: Xavier, L., Hladun, N. L., Barbero, M., Navarro-Rosinés, R. P., Gómez-Bolea, A. & Giralt, M. (eds.), *The Fourth IAL Symposium. Progress and Problems in Lichenology at the Turn of the Millennium*. Univ. Barcelona, Barcelona. [Abstr.]

Lutzoni, F., Pagel, M. & Reeb, V. 2001. Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 411: 937–940.

Myllys, L., Lohtander, K., Källersjö, M. & Tehler, A. 1999. Sequence insertions and ITS data provide congruent information on *Roccella canariensis* and *R. tuberculata* (Arthoniales, Euascomycetes) phylogeny. *Molec. Phyl. Evol.* 12: 295–309.

Nádvorník, J. 1942a. Kurze Übersicht der Flechtenfamilie Caliciaceae. *Stud. Bot. Cech.* 5: 121–128.

Nádvorník, J. 1942b. Systematische Übersicht der mitteleuropäischen Arten der Flechtenfamilie Caliciaceae. *Stud. Bot. Cech.* 5: 6–46.

Nádvorník, J. 1942c. Beiträge zur Kenntnis der aussereuropäischen coniocarpen Flechten. *Ann. Mycol.* 40: 133–139.

Nylander, J. 2002. *MrModeltest v10b.* Department of Systematic Zoology, Uppsala University. http://www.ebc.uu.se/systzoo/staff/nylander.html.

Poelt, J. 1973. Classification. Pp. 599–632 in: Ahmadjian, V. & Hale, M. E. (eds.), *The Lichens.* Academic Press, New York.

Räsänen, V. 1943. Das System der Flechten. *Acta Bot. Fenn.* 33: 1–82.

Ronquist, F. & Huelsenbeck, J. P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Schmidt, A. 1970. Anatomisch-taxonomische Untersuchungen an europäischen Arten der Flechtenfamilie Caliciaceae. *Mitt. Staatsinst. Allg. Bot. Hamburg* 13: 111–166.

Schultz, M., Arendholz, W.-R. & Büdel, B. 2001. Origin and evolution of the lichenized ascomycete order Lichinales: monophyly and systematic relationships inferred from ascus, fruiting body and SSU rDNA evolution. *Pl. Biol.* 3: 116–123.

Stenroos, S. & DePriest, P. 1998. Small insertions at a shared position in the SSU rDNA of Lecanorales (lichen-forming Ascomycetes). *Curr. Gen.* 33: 124–130.

Swofford, D. L. 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4.0. Sinuaer Associates, Sunderland, Massachusetts.

Thell, A. 1999. Group I intron versus ITS sequences in phylogeny of cetrarioid lichens. *Lichenologist* 31: 441–449.

Thell, A. & Miao, V. 1999. Phylogenetic analysis of ITS and group I intron sequences from European and North American samples of cetrarioid lichens. *Ann. Bot. Fenn.* 35: 275–286.

Tibell, L. 1978. Comments on Caliciales exsiccatae 1. *Lichenologist* 10: 171–178.

Tibell, L. 1981. Comments on Caliciales exsiccatae II. *Lichenologist* 13: 51–64.

Tibell, L. 1984. A reappraisal of the taxonomy of Caliciales. *Beih. Nova Hedw.* 79: 597–714.

Tibell, L. 1987. Australasian Caliciales. *Symb. Bot. Uppsal.* 27: 1–279.

Tibell, L. 1990. Anamorphs in *Mycocalicium albonigrum* and *M. subtile*. *Nord. J. Bot.* 10: 221–242.

Tibell, L. 1991. The *Asterophoma* anamorph of *Chaenothecopsis savonica* and its hyphomycetous synanamorph. *Can. J. Bot.* 69: 2427–2433.

Tibell, L. 1993. The anamorphs of *Chaenothecopsis viridireagens*. *Nord. J. Bot.* 13: 331–335.

Tibell, L. 1995. The anamorph of *Chaenothecopsis debilis*. *Mycologia* 87: 245–252.

Tibell, L. 1997. Anamorphs in mazaediate lichenized fungi and the Mycocaliciaceae ("Caliciales s. lat."). *Symb. Bot. Ups.* 32: 291–322.

Tibell, L. 1998. Practice and prejudice in lichen classification. *Lichenologist* 30: 439–453.

Tibell, L. 2003. *Tholurna dissimilis* and generic delimitations in Caliciaceae inferred from nuclear ITS and LSU rDNA phylogenies (Lecanorales, lichenized ascomycetes). *Mycol. Res.* 107: 1403–1418.

Tibell, L. & Constantinescu, O. 1991. *Catenomycopsis rosea* gen and sp. nov. (Hyphomycetes), the anamorph of *Chaenothecopsis haematopus*. *Mycol. Res.* 95: 556–560.

Tibell, L., Titov, A. & Lisická, E. 2003. Calicioid lichens and fungi described by J. Nádvorník. *Mycotaxon* 87: 3–24.

Tibell, L. & Wedin, M. 2000. Mycocaliciales — a new order for non-lichenized calicioid fungi. *Mycologia* 92: 577–581.

Vainio, E. 1890. Étude sur la classification naturelle et la morphologie des lichens du Brésil. Acta Soc. *Fauna Fl. Fenn.* 7: 1–256.

Vainio, E. 1927. Lichenographia fennica. III. *Acta Soc. Fauna Fl. Fenn.* 57, 1: 1–138.

Vinuesa, M. 2001. *Estudios Filogenéticos en la Familia Mycocaliciaceae (Mycocaliciales, Ascomycota).* Univ. Complutense, Madrid.

Vobis, G. 1980. Bau und Entwicklung der Flechten-Pycnidien und ihrer Conidien. *Bibl. Lichenol.* 14: 1–149.

Wedin, M. & Tibell, L. 1997. Phylogeny and evolution of Caliciaceae, Mycocaliciaceae and Sphinctrinaceae (Ascomycetes), with notes on the evolution of the prototunicate ascus. *Can. J. Bot.* 75: 1236–1242.

Wedin, M., Döring, H., Nordin, A. & Tibell, L. 2000. SSU rDNA phylogeny shows the lichen families Caliciaceae and Physciaceae (Lecanorales, Ascomycotina) to form a monophyletic group. *Can. J. Bot.* 78: 246–254.

White, T. J., Bruns, T. D., Lee, S. & Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. Pp. 315–322 in: Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (eds.), *PCR Protocols: a Guide to Methods and Applications*. Academic Press, San Diego.

Zahlbruckner, A. 1921–1922. *Catalogus Lichenum Universalis* I. Gebrüder Borntraeger, Leipzig.

Zoller, S., Lutzoni, F. & Scheidegger, C. 1999. Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. *Molec. Ecol.* 8: 2049–2059.

Appendix. Sequences downloaded from GenBank and newly produced sequences (in bold).

Species, Code, Origin, Voucher, DNA, GenBank accession no.

Byssochlamys nivea Westling, -, -, -, ITS, LSU, U18361, AY176750; *Calicium adspersum* Pers., V2299, New Zealand, *Tibell 16902* (UPS), LSU, AY452504; *Calicium glaucellum* Pers., T139, Sweden, *Tibell 22319* (UPS), LSU, AY453646; *Chaenotheca chlorella* (Ach.) Müll. Arg., V2301, Sweden, *Tibell 16867* (UPS), LSU, **AY804191**; *Chaenotheca trichialis* (Ach.) Th. Fr., T251, Argentina, *Tibell 17577* (UPS), LSU, **AY795990**; *Chaenothecopsis consociata* (Nádv.) A.F.W. Schmidt, T241, Sweden, *Tibell 224729* (UPS), ITS, LSU **AY795851, DQ008999**; *Chaenothecopsis debilis* (Turner & Borrer ex Sm.) Tibell, V2080, New Zealand, *Tibell 16643* (UPS), ITS, LSU, **AY795852, AY795991**; *Chaenothecopsis debilis* (Turner & Borrer ex Sm.) Tibell, T278, Sweden, *Tibell 22746* (UPS), ITS, LSU, **AY795853, AY795992;** *Chaenothecopsis dolichocephala* Titov, T227, Russia, *Tibell 19281* (UPS), ITS, LSU, **AY795854, AY795993**; *Chaenothecopsis epithallina* Tibell, T261, Sweden, *Tibell 22704* (UPS), ITS, **AY795855**; *Chaenothecopsis epithallina* Tibell, T276, Sweden, *Tibell 22793* (UPS), ITS, **AY795856**; *Chaenothecopsis epithallina* Tibell, T294, Sweden, *Tibell 22716* (UPS), LSU, **AY795994**; *Chaenothecopsis fennica* (Laurila) Tibell, V16024, Sweden, *Tibell 16024* (UPS), ITS, LSU, **AY795857, AY795995**; *Chaenothecopsis fennica* (Laurila) Tibell, T268, Sweden, *Tibell 22718* (UPS), ITS, **AY795858**; *Chaenothecopsis golubkovae* Titov, T232, China, *Titov 6707* (UPS), ITS, LSU, **AY795859, AY795996**; *Chaenothecopsis golubkovae* Titov, T348, India, *Tibell 23211* (UPS), ITS, **AY795860**; *Chaenothecopsis haematopus* Tibell, V2082, New Zealand, *Tibell 16625* (UPS), ITS, LSU, **AY795861, AY795997**; *Chaenothecopsis nana* Tibell, T242, Sweden, *Tibell 22473* (UPS), ITS, **AY795862**; *Chaenothecopsis orientalis* Tibell, T257, Russia, *Tibell 19371* (UPS), ITS, **AY795863**; *Chaenothecopsis orientalis* Tibell, T351, India, *Tibell 23216* (UPS), ITS, **AY795864**; *Chaenothecopsis pusilla* (Flörke) A.F.W. Schmidt, T280, Sweden, *Tibell 22804* (UPS), ITS, **AY795866**; *Chaenothecopsis pusilla* (Flörke) A.F.W. Schmidt, T207, New Zealand, *Tibell 16603* (UPS), LSU, **AY795998**; *Chaenothecopsis pusilla* (Flörke) A.F.W. Schmidt, New Zealand, *Tibell 16580* (UPS), LSU, **DQ009000**; *Chaenothecopsis pusiola* (Ach.) Vain., T254, Sweden, *Tibell 15884* (UPS), ITS, **AY795865**; *Chaenothecopsis pusiola* (Ach.) Vain., V19258, Russia, *Tibell 19258* (UPS), LSU, **AY795999**; *Chaenothecopsis resinicola* Tibell & Titov, T255, Russia, *Tibell 19234* (UPS), ITS, **AY795867**; *Chaenothecopsis savonica* (Räsänen) Tibell, V2200, Sweden, *Tibell 15876* (UPS), ITS. LSU, **AY795868, AY796000**; *Chaenothecopsis subparoica* (Nyl.) Tibell, T329, Italy, *Tretiach* (hb. Tretiach), ITS, **AY795869**; *Chaenothecopsis tibellii* Titov, T231, China, *Titov 6655* (LE), ITS, **AY795870**; *Chaenothecopsis tibellii* Titov, T234, China, *Titov 6662* (UPS), ITS, **AY795871**; *Chaenothecopsis viridireagens* (Nádv.) A.F.W. Schmidt, T279, Sweden, *Tibell 22803* (UPS), ITS, LSU, **AY795872, DQ013257**; *Cudonia circinans* (Pers.) Fr., -, -, -, LSU, AF107553; *Dendrographa leucophaea* (Tuck.) Darb., -, -, -, LSU, AF279382; *Dermatocarpon americanum* Vainio, -, -, -, LSU, AF279384; *Diaporthe padi* G.H. Otth, -, -, -, LSU, AF408354; *Diploschistes scruposus* (Schreb.) Norman, -, -, -, LSU, AF279389; *Eupenicillium pinetorum* Stolk, -, -, -, LSU, AF033411; *Eupenicillium pinetorum* Stolk, -, -, -, ITS, AF033411; *Fulgensia fulgens* (Sw.) Elenkin, -, -, -, LSU, AF278883; *Gyalecta jenensis* (Batsch) Zahlbr., -, -, -, LSU, AF465450; *Hamigera avellanea* Stolk & Samson, -, -, -, LSU, AB105350; *Hypocrea schweinitzii* (Fr.) Sacc., -, -, -, LSU, AY283549; *Leifidium tenerum* (Laurer) Wedin, V2091, New Zealand, *Tibell 16587* (UPS), LSU, AY453643; *Lempholemma polyanthes* (Bernh.) Malme, -, -, -, LSU, AF356691; *Leptogium cyanescens* (Pers.) Körb., -, -, -, LSU, AF356672; *Morchella esculenta* (L.) Pers., -, -, -, LSU, AF279398; *Mycocalicium albonigrum* (Nyl.) Tibell, V19038, New Zealand, *Tibell 19038* (UPS), LSU, **AY796001**; *Mycocalicium albonigrum* (Nyl.) Tibell, -, -, -, ITS, AF223967; *Mycocalicium albonigrum* (Nyl.) Tibell, -, -, -, ITS, AF223968; *Mycocalicium albonigrum* (Nyl.) Tibell, -, -, -, ITS, AF223969; *Mycocalicium americanum* (R. Sant.) Tibell, T330, Mexico, *Kalb & Nash* (UPS), ITS, **AY795879**; *Mycocalicium sequoiae* Bonar, -, U.S.A., *Rikkinen* (H), LSU, **AY796002**; *Mycocalicium subtile* (Pers.) Szatala, V21020, Sweden, *Tibell 21020* (UPS), LSU, **AY796003**; *Mycocalicium subtile* (Pers.) Szatala, V2173, New Zealand, *Tibell 16744* (UPS), LSU, **AY796004**; *Mycocalicium subtile* (Pers.) Szatala, V2504, Sweden, *Tibell 17164* (UPS), LSU, **AY796005**; *Mycocalicium subtile* (Pers.) Szatala, -, -, -, ITS, AF225431; *Mycocalicium subtile* (Pers.) Szatala, -, -, -, ITS, AF225433; *Mycocalicium subtile* (Pers.) Szatala, -, -, -, ITS, AF225444; *Mycocalicium victoriae* (C. Knight) Nádv., -, -, -, ITS, AF213135; *Peltula umbilicata* (Vain.) Swinscow & Krog, -, -, -, LSU, AF356689; *Phaeocalicium interruptum* (Nyl.) Tibell, T317, Sweden, *Tibell 23044* (UPS), ITS, **AY795873**; *Phaeocalicium populneum* (Brond. ex Duby) A.F.W. Schmidt, V19286, Sweden, *Tibell l9286* (UPS), ITS, LSU, **AY795874, AY796009;** *Placynthium nigrum* (Huds.) Gray, -, -, -, -, LSU, AF356674; *Porpidia albocaerulescens* (Wulfen) Hertel & Knoph, -, -, -, LSU, AF356675; *Pyrenula cruenta* (Mont.) Vain., -, -, -, LSU, AF279407; *Rhytisma acerinum* (Pers.) Fr., -, -, -, LSU, AF356696; *Schismatomma pericleum* (Ach.) Branth & Rostr., -, -, -, LSU, AF279408; *Sphaerophorus globosus* (Huds.) Vain., -, -, -, LSU, AF356680; *Sphinctrina leucopoda* Nyl., T312, Australia, *Kalb 33829* (hb. Kalb), ITS, LSU, **AY795875, AY796006**; *Sphinctrina turbinata* (Pers.) De Not., T260, Sweden, *Tibell 22478* (UPS), ITS, LSU, **AY795876, AY796007**; *Sphinctrina turbinata* (Pers.) De Not., T302, Sweden, *Tibell 23093* (UPS), ITS, LSU **AY795877, DQ009001**; *Stenocybe pullatula* (Ach.) Stein, V2448, Sweden, *Tibell 17117* (UPS), ITS, LSU, **AY795878, AY796008**; *Stereocaulon paschale* (L.) Hoffm., -, -, -, LSU, AF279413; *Trapeliopsis granulosa* (Hoffm.) Lumbsch, -, -, -, LSU, AF279415; *Verrucaria pachyderma* Arnold, -, -, -, LSU, AF356668.