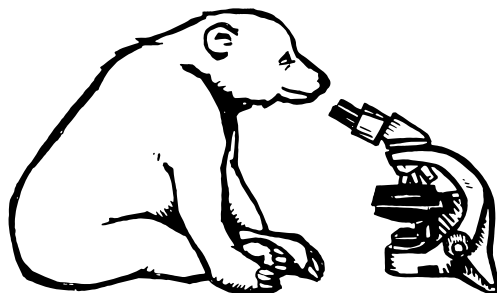


# **Sticky business – diversity and evolution of Mycocaliciales (Ascomycota) on plant exudates**

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**ACADEMIC DISSERTATION**

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kiitos että näytit Metsän.

*"There is nothing like looking,  
if you want to find something.  
You certainly usually find something,  
if you look, but it is not always  
quite the something you were after."*

– Thorin Oakenshield

# CONTENTS

<b>ABSTRACT</b> .....	<b>6</b>
<b>SUMMARY</b> .....	<b>7</b>
<b>1 Introduction</b> .....	<b>7</b>
Historical outline .....	7
Exudates of woody plants .....	10
Fungal fossils in amber .....	13
Species concept .....	16
<b>2 Aims of the thesis</b> .....	<b>20</b>
<b>3 Material and methods</b> .....	<b>20</b>
Biological material .....	20
Microscopy .....	21
DNA extraction and sequencing .....	22
Phylogenetic analyses .....	23
<b>4 Main results and discussion</b> .....	<b>25</b>
Phylogenetic relationships and substrate ecology .....	26
Diagnostic characters for species delimitation .....	33
<b>5 Concluding remarks</b> .....	<b>45</b>
<b>6 Acknowledgements</b> .....	<b>48</b>
<b>7 References</b> .....	<b>51</b>
<b>Supplementary tables 1, 2 &amp; 3</b>	

- I**     *Chaenothecopsis khayensis* – a new resinicolous calicioid from tropical Africa.
- II**    Three resinicolous North American species of mycocaliciales in Europe with a re-evaluation of *Chaenothecopsis oregana* Rikkinen.
- III**   Stuck in time – a new *Chaenothecopsis* species with proliferating ascomata from *Cunninghamia* resin and its fossil ancestors in European amber.
- IV**    New resinicolous mycocalicioid fungi from angiosperm exudates.

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- I** Tuovila, H., Cobbinah, J. & Rikkinen, J. 2011: *Chanothecopsis khayensis* – a new resinicolous calicioid from tropical Africa. – *Mycologia* 103 (3): 610–615.
- II** Tuovila, H., Larsson, P. & Rikkinen, J. 2011: Three resinicolous North American species of mycocaliciales in Europe with a re-evaluation of *Chanothecopsis oregana* Rikkinen. – *Karstenia* 51(2): 37–50.
- III** Tuovila, H., Beimforde, C., Schmidt, A. & Rikkinen, J. 2013: Stuck in time – a new *Chanothecopsis* species with proliferating ascomata from *Cunninghamia* resin and its fossil ancestors in European amber. – *Fungal Diversity* 58(1): 199–213.
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Authors' contributions to the articles:

- I** JR designed the study and collected the biological material. HT analyzed the material in detail, extracted the DNA and did the phylogenetic analysis, drew the line drawings and drafted the species description. HT wrote the paper as main author together with JR.
- II** JR and HT designed the study and collected the biological material. HT analyzed the material in detail, drew the line drawings, and drafted the species descriptions. HT wrote the paper as main author together with JR.
- III** JR, HT and AS designed the study. JR and HT collected the biological material of the extant species. HT analyzed the material in detail, extracted the DNA and did the phylogenetic analysis, drew the line drawings, and drafted the species description. AS produced the photomicrographs. HT wrote the paper as main author together with JR in correspondence with the other authors.
- IV** JR and HT designed the study and collected the biological material. HT analyzed the material in detail, extracted the DNA and did the phylogenetic analysis, drew the line drawings, and drafted the species descriptions. HT wrote the paper as main author together with JR, with contributions from MD and SH.

The Summary includes previously unpublished results not presented in Chapters I-IV.

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## **Sticky business – diversity and evolution of Mycocaliciales (Ascomycota) on plant exudates**

### **ABSTRACT**

Species of Mycocaliciales are a group of small ascomycetes with pin-like ascocarps. Among Fungi, many mycocalicioid species are rather unique in having specialized to live on the resinous exudates of vascular plants. Terpenoid and phenolic resins essentially are repellents for fungi, but some of these species even appear to use resins for their nutrition. This peculiar ecology has a long evolutionary history, judging from 20 to 40 Ma old amber fossils, in which the ascocarps growing on ancient resin bear remarkable resemblance to those of extant resinicolous species. In addition to resinicolous species, mycocalicioid fungi also include species that are saprotrophic on lignum or associated with lichens or non-symbiotic algal colonies.

The aim of this theses was to study the diversity, ecology and evolutionary relationships of mycocalicioid fungi, especially the resinicolous species. The study is based on specimens collected from different parts of the world, and analyzed using both molecular and morphological methods.

In this study seven new resinicolous *Chaenothecopsis* species are described from boreal North America and Europe, tropical Africa, and temperate China, four from angiosperm exudates and three from conifer resins. Two newly found fossils of *Chaenothecopsis* from Eocene and Oligocene ambers are described and their relations to extant species are elucidated. The phylogenetic relationships of several species and lineages, and the evolution of the resinicolous ecology are discussed in considerable detail. In addition, new morphological and chemical characters that can be used in future taxonomic studies are described.

# SUMMARY

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## 1 INTRODUCTION

### Historical outline

Mycocalicioid fungi, i. e. species in the order Mycocaliciales, are a group of small ascomycetes with pin-like ascomata. These hardy fungi live on all continents except Antarctica, in many different vegetation types and on many different substrates. Mycocalicioid fungi are best known from boreal and temperate forests of the northern hemisphere, but even here they seem to be undercollected and rather poorly known. This is despite the fact that Erik Acharius (1757 – 1819), the “father of lichenology”, began classifying calicioid lichens and fungi already at the beginning of 19<sup>th</sup> century (1815, 1816, 1817). He was later followed by several other well-known lichenologists, including William Nylander (1857, 1861), Edward August Vainio (1890, 1927), and Josef Nádvořník (1940, 1942). Due to superficial similarities in ascocarp morphology and shared habitats, non-lichenized mycocalicioid fungi were first classified into the lichenized genus *Calicium* Pers. (Lecanoromycetes). The oldest accepted species in Mycocaliciales is *Mycocalicium subtile* (Pers.) Szatala (initially described as *Calicium subtile* Pers. 1797).

For more than a century mycocalicioid fungi were thought to be lichen-symbiotic, although the apparent lack of a true thallus was often noted, and then described as being “obsolete”, “thin” or “membranaceus”. Differences in species concepts and generic delimitations of early lichenologists have been described and analyzed in several studies by Leif Tibell (e.g. Tibell 1984, 1991; Tibell et al. 2003) and in the monograph of Alexander

Titov (2006). Already Vainio (1927) highlighted many morphological and ecological differences between different groups in the Caliciales as delimited at the time. A further turning point was in 1970 when Alexander F. W. Schmidt presented a thorough discussion of ascocarp anatomy and excluded non-lichenized species without mazedia from Caliciaceae *sensu stricto* (s. s.).

Our current understanding of the classification and morphological variation of mycocalicioid fungi is largely based on Tibell's work on calicioid lichens and similar fungi (e.g. Tibell 1975, 1987, 1998). In those studies dealing with mycocalicioid species Tibell has mainly followed the generic concepts described by Schmidt and further added to the knowledge of the diversity of the group in different parts of the world. His treatments have in turn been followed by several other lichenologists, including Titov (1998, 2001). Titov's monograph (2006), "Mycocalicioid fungi (the order Mycocaliciales) of Holarctic", is a thorough review of the holarctic species in Russian, and the identification keys and new species descriptions of the monograph have since been translated into English (Stordeur et al. 2010). Jouko Rikkinen (e.g. 2003a, 2003b) and Steven Selva (e.g. Selva 1988; Selva & Tibell 1999) have also studied mycocalicioid fungi in considerable detail.

The order Mycocaliciales was established by Tibell and Wedin 30 years after Schmidt excluded non-lichenized species from the Caliciales s. s. (Tibell & Wedin 2000). The order was proposed to include two families, Mycocaliciaceae A.F.W. Schmidt and Sphinctrinaceae M. Choisy. Later, the isolated position of this group of fungi was underlined by their recent classification into Mycocaliciomycetidae Tibell, a distinct subclass within the Eurotiomycetes (Hibbett et al. 2007). Several DNA-based studies have shown that mycocalicioid fungi are related to the Eurotiomycetidae and Chaetothyriomycetidae, and that the many superficial similarities in habitat ecology and ascocarp shape with species of Caliciales and Coniocybaceae have resulted from convergence (Geiser et al. 2006; Schoch et al. 2009). Tibell and Vinuesa (2005) showed that while Sphinctrinaceae probably is monophyletic, the species are nested within Mycocaliciaceae and should thus be included in that family.



Roughly 150 legumite species in five different genera are currently recognized in Mycohaliciales. The genus *Sphinctrina* was described by Elias Fries (1825) with *Sphinctrina turbinata* (Pers.: Fr.) De Not. as the type species, and it thus is the oldest generic name in the order. *Stenocybe* was described by Nylander (Körber 1855) on the basis of *S. byssacea* (Fr.) Nyl. ex Körb. *Mycocalicium* was described by Vainio (1890) on the basis of *M. parietinum* (Ach.) Vain., which has later been synonymized with *M. subtile*. Also *Chaenothecopsis* was described by Vainio (1927) with *C. rubescens* Vain. as the type species. *Phaeocalicium* was established by Schmidt (1970) on the basis of *P. praecedens* (Nyl.) A.F.W. Schmidt. Schmidt differentiated species in the four genera of Mycohaliciaceae (excluding *Sphinctrina*) on the basis of ascus measurements, ascus tip characters, spore septation and hyphal structures of the stipe (Table 1). His generic concepts are still in use, with the exception of those concerning *Sphinctrina*, which Schmidt included in Caliciaceae on the basis of mazaedium formation.

**Table 1.** Distinguishing characters of *Chaenothecopsis*, *Mycocalicium*, *Phaeocalicium* and *Stenocybe* according to Schmidt (1970) and Tibell (1979, 1984, 1999). *Sphinctrina* according to Tibell (1999).

Genus	Ascus apex	Ascus length	Spores	Stipe	Excipulum
<i>Mycocalicium</i>	Evenly thickened, without canal	35–65 $\mu\text{m}$	aseptate	Periclinal, narrowly rectangular hyphae, in inner part also pigmented hyphae	Periclinal, isodiametric hyphae
<i>Phaeocalicium</i>	Evenly thickened, without canal	70–100 $\mu\text{m}$	aseptate or 1-septate	Periclinal, narrowly rectangular, swollen hyphae	Periclinal, narrow, rectangular hyphae
<i>Stenocybe</i>	Evenly thickened, without canal	70–250 $\mu\text{m}$	1–7 septate	Periclinal, narrowly rectangular, swollen hyphae	Periclinal, narrow, rectangular hyphae
<i>Chaenothecopsis</i>	Mature asci without thickening, narrow canal visible during maturation	25–55 $\mu\text{m}$ (35–45 $\mu\text{m}$ Schmidt)	aseptate or 1-septate	Periclinal or intricately interwoven, in inner part hyaline or only lightly pigmented hyphae	Periclinal hyphae
<i>Sphinctrina</i>	No apical structures	30–75 $\mu\text{m}$	aseptate or 1-septate	Periclinally oriented, in inner part hyaline or only lightly pigmented hyphae	Periclinal, intertwined, with constricted margin

Almost all data on the substrate and habitat requirements of mycocalicioid fungi have been gained through casual, direct observation in the field or from herbarium specimens. The apparent restrictions in the occurrences of several species to a single host organism and/or habitat or vegetation type indicates a high specificity in both substrate and habitat ecology. Tibell's regional monographs provide a wealth of valuable information on the ecology of many mycocalicioid fungi (e.g. Tibell 1975, 1987, 1998). Tibell (1992), Selva (1994), Kruys & Jonsson (1997), Rikkinen (2003c), and others have shown that some species of Mycocaliciales are confined to old-growth forests and their presence can even be used as an indicator of stand age and forest continuity. Tibell (1994) analyzed the global distribution patterns and dispersal strategies of calicioid and mycocalicioid fungi, and Rikkinen (1995) discussed many ecological factors that can influence their ecology in boreal forests.

Some mycocalicioid species are thought to be parasitic or sometimes parasymbiotic on lichens or parasitize non-lichenized green algae. Other species live as apparent saprotrophs on wood or bark, or on vascular plant exudates (e.g. Tibell 1999). Those species termed 'saxicolous' grow on leprose lichens or possibly on free-living algal colonies, not on the rock itself. Only a few species have been successfully cultured (Bonar 1971; Samuels & Buchanan 1983; Tibell 1997), and very little is known about the nutritional requirements of mycocalicioid species. Tibell's pioneering studies on the anamorphs of these fungi have confirmed that some are able to grow on different types of nutrient media (e.g. Tibell 1990, 1995). While close associations between *Chaenothecopsis* and algal cells have been observed (Tschermak-Woess 1980), definitive symbiosis and nutrient transfer has not been demonstrated.

### **Exudates of woody plants**

Woody plants have evolved to produce exudates containing a variety of primary and secondary metabolites, an adaptation against harmful microorganisms, potential herbivores, and adverse abiotic conditions. All true resins and some other exudates are primarily composed of terpene molecules (Bohlmann & Keeling 2008; Lambert et al. 2010). While the

purely terpenoid resins of conifers are chemically relatively uniform, there is a vast diversity of phenolic and other compounds that are also present in different angiosperm exudates (Langenheim 2003a; Courtois et al. 2009). The exudates of most angiosperms are not true terpenoid resins, but can be classified as gums, saps, and so on. Many plants produce and exude complex mixtures of many different types of secondary metabolites, and the term 'exudate' can be used to broadly refer to any such substance. In addition to resinous exudates, woody plants can for example exude many types of soluble metabolites, and microbiologically modified sap (Xu & Leininger 2001).

Langenheim (2003a) defined resin as "*--primarily a lipid-soluble mixture of volatile and nonvolatile terpenoid and/or phenolic secondary compounds that are (1) usually secreted in specialized structures located either internally or on the surface of the plant and (2) of potential significance in ecological interactions*". The volatile fraction of resin usually consists of mono- and/or sesquiterpene hydrocarbons with some oxygenated forms. The nonvolatile fraction constitutes of primarily di- or triterpene acids with some alcohols, aldehydes and esters and amorphous, neutral substances. In angiosperms the nonvolatile fractions usually also contain phenolic compounds (Langenheim, 2003a; Aguilar-Ortigoza & Sosa 2004; Lambert et al. 2010).

All conifers produce resin and the terpenoid chemistries of different conifer resins are relatively uniform (Back 2002; Langenheim, 2003b; Lambert et al. 2010). Some genera and species only have small amounts of constitutive resin in their needles and other parts, while others can even exude abundant resin flows from the trunk, either constitutively or as an induced response (Nagy et al. 2000). In conifer resins, the volatile fraction is typically large (20–50%) and monoterpenoids tend to dominate over sesquiterpenes. The nonvolatile fraction usually consists mainly of diterpenes (Langenheim, 2003b).

While highly hydroxylated or sugar-substituted flavonoids are common in conifers, and some polyphenols can be present in trauma-induced resin (Nagy et al. 2000), only angiosperms produce true "phenolic resins" (Langenheim

2003c). The basic compounds of phenolic resin are phenylpropanoids and lipophilic flavonoids, and these compounds are usually mixed with different terpenes. In addition, mono-, sesqui- and triterpenes can react with different phenols to produce several types of terpenophenols (Kuzakov & Shmidt 2000; Aguilar-Ortigoza & Sosa 2004). Sesquiterpenes usually dominate over monoterpenes in the volatile fractions of phenolic resins, but in some groups of angiosperms monoterpenes dominate over sesquiterpenes. Typically triterpenes constitute the nonvolatile fraction, but diterpenes can also be the dominant fraction. While resin production in conifers seems to be a shared evolutionary trait, the production of resinous exudates in different angiosperm groups is clearly a result of convergent evolution (Farrel et al. 1991; Langenheim 2003a; Chen et al. 2011).

In plants, the differences in chemical compositions of resins between individuals, populations, and species arise from natural genetic diversity and the influence of local abiotic and biotic pressures such as drought or herbivores (Langenheim 2003b; Nikolić et al. 2011; Davis & Hofstetter 2012). The ecological significance of resins primarily arises from their defensive properties, partly due to their toxic compounds, and partly due to their ability to seal wounds and prevent the entrance of hostile organisms (Farrel et al. 1991; Langenheim 2003c; Trapp & Croteau 2001; Gershenzon & Dudareva 2007; Davis & Hofstetter 2012). Genetic variation in resin production can enhance the ability of plants to adapt by modifying the chemical composition of their resin (Huber & Ralph 2004; Sorensen et al. 2005; Nikolić et al. 2011). Resin compounds also play important roles in plant signaling (Gershenzon & Dudareva 2007), in pollination (Leonhardt et al. 2011), and provide a suitable substrate for some highly specialized organisms, including saprotrophic fungi.

Plant exudates are a chemically and physically challenging substrate for saprotrophic fungi. However, a relatively large number of fungi, mainly ascomycetes, live on these substrates, and this ecology is particularly well represented within the Mycocaliciales. In total 17 species in two different genera have been described so far from different types of plant exudates or growing on resin-impregnated wood and bark. While resinicolous species were already reviewed by Tibell and Titov in 1995, numerous new species

have since been described, bringing new insights into the morphology and interesting ecology of this well-defined group.

### **Fungal fossils in amber**

After exudation, viscous and soft resin begins to harden. The solidification involves two simultaneous processes: the mono- and sesquiterpenes start to volatilize and certain terpenoid compounds (tri-, and especially diterpenes) start to polymerize into more complex molecules. Sometimes, under highly specific conditions, the hardened resin may later fossilize into amber (Lambert & Poinar 2002; Langenheim 2003d; Vávra 2009; Penney & Green 2010).

Trace quantities of amber have been found from 320 million year old Carboniferous coal deposits. The minute droplets demonstrate that pre-conifer gymnosperms already had the ability to produce complex polyterpenoid resins similar to those of extant woody plants (Martínez-Delclòs et al. 2004; Bray & Anderson 2009). Triassic (~200 Ma) and Jurassic (~160 Ma) amber droplets have been found in several localities, and despite their small size, they can contain fossilized organisms as inclusions (Azar et al. 2010; Schmidt et al., 2012). The appearance of large amber pieces and deposits around the world in the early Cretaceous (~130 Ma) has been related to the coinciding emergence of Araucariacean conifers and the humid climates that prevailed on the shores of the Tethys Ocean (Martínez-Delclòs et al. 2004, Najarro et al. 2010). Many types of Tertiary (~40–50 Ma) amber have been found in different parts of the world. Undoubtedly the most famous deposits are those of Baltic amber (Schmidt & Dörfelt 2007; Wolfe et al. 2009). Amber has also been found from many Quaternary (~20–30 Ma) deposits such as those in the island of Hispaniola in the Caribbean (~20–30 Ma) (Lambert & Poinar 2002; Martínez-Delclòs et al. 2004; Schmidt & Dörfelt 2007).

While the chemical composition of resin is reflected in the chemical spectrum of fossilized amber, the plant source and age of amber is often difficult or impossible to determine on the basis of the amber piece itself (Wolfe et al. 2009). During the fossilization process the resins undergo major structural changes that are highly dependent on the prevailing

geological conditions. Thus, the maturation process of amber is relative, and not absolute within a given time frame (Anderson 1996; Bray & Anderson 2009). In addition, amber is semi-buoyant and may easily be transported from its place of origin even by weak streams. This clouds the origins and the age of amber, since it is usually not found in the sediment stratum and vicinity of plant remains it was originally buried with (Langenheim 2003d; Martínez-Delclòs et al. 2004). Nevertheless, the chemical and physical properties of amber can be compared with extant plant resins using several methods, including solid state nuclear resonance, infrared, and different mass spectrometry techniques. However, as these methods are also relative, the interpretation of the results are always dependent on the modern resins that can be used as reference material (Yamamoto et al. 2006; Otto et al. 2007; Vávra 2009; Wolfe et al. 2009; Lambert et al. 2010). Recently the comparisons of carbon isotopes of extant and fossil resins have proven to be useful for deducing botanical origin and even ecological conditions during exudation (Dal Corso et al. 2011; McKellar et al. 2011). In addition, inclusions, including plant fragments, arthropods and other micro-organisms, can often provide valuable clues of the paleoenvironment (e. g. Wolfe et al. 2009).

Being soft and ephemeral, fungi are not nearly as likely to become fossilized as the hard tissues of plants and animals. Often the proof of the presence of fungi in an ancient ecosystem is indirect and deduced from, for example, induced abnormalities in wood structure (Harper et al. 2012), structures needed for symbionts (Durringer et al. 2006), and changes in host structure related to pathogenicity (Hughes et al. 2011).

Silification is one of the few ways for soft fungal structures to fossilize. The Devonian (~400 Ma) Rhynie chert has produced wonderfully preserved fossils of many fungal organisms: saprotrophic, mycoparasitic, mycorrhizal, endophytic and lichenized zygomycetous and glomalean fungi, and ascomycetes (e.g. Hass et al. 1994; Berbee & Taylor 2007; Krings et al. 2007; Dotzler et al. 2008). One of the most striking organisms, *Paleopyrenomycites*, has well preserved perithecia with paraphyses and asci, in addition to a conidial stage (Taylor et al. 2005). Silificied fossils

from the Carboniferous (~300 Ma) include fungal endophytes from roots (Taylor et al. 2012), epiphyllous fungi on leaves (Krings et al. 2011), and dikaryotic hyphae with clamp connections, the oldest unequivocal fossil basidiomycete (Krings et al. 2010). Pre-Cretaceous fungal fossils also include glomalean endomycorrhizal fungi from the Permian (~255 Ma) (García Massini 2007), zygomycetous sporocarps from the Triassic (~245 Ma) (Krings & Taylor 2012), and arthropod coprolites containing remains of saprotrophic fungi from the Jurassic (~170 Ma) (García Massini et al. 2012). Silicified fungal fossils have also been found from more recent strata, these including, for example, epiphyllous fungi from the Cretaceous (Van der Ham & Dortangs 2005) and Oligocene (Shi et al. 2010), and a pleosporalean fungus from the Eocene (Mindell et al. 2007).

Entrapment in resin that eventually becomes fossilized as amber is the second important way for fungi to fossilize. The majority of fungal fossils in amber date from late Cretaceous or Tertiary sediments, although one anamorphic fungus has also been described from Triassic amber droplets (Schmidt et al. 2006). Cretaceous ambers have preserved a rather diverse assemblage of microfungi, including a predatory species (Schmidt et al. 2007, 2008), insect parasites (Sung et al. 2008), as well as several saprophytic and resinicolous species (Ascaso et al. 2005; Schmidt & al 2010; Saint Martin & al. 2012). Only a few fossilized basidiocarps have ever been found, the oldest of which is *Archaeomarasmius leggeti* from Cretaceous New Jersey amber (Hibbett et al. 1997). The other two fossilized basidiomycetes include *Coprinites dominicana* from the Eocene (Poinar & Singer 1990), and *Protomyцена electra* from the Miocene (Hibbett et al. 1997).

In addition to anamorphic ascomycetes (e.g. Sadowski et al. 2012), some well preserved lichens also have been found from Tertiary ambers (Poinar et al. 2000; Rikkinen & Poinar 2002, 2008). Ascocarps of the calicioid lichens *Calicium* and *Chaenotheca* have been found in Baltic amber (~35–55 Ma) (Rikkinen 2003d). Rikkinen and Poinar (2000) also described the first fossilized species of Mycohaliciales, *Chaenothecopsis bitterfeldensis*, from European Bitterfeld (22–24 Ma) amber.



## Species concept

The species concept is one of the most profound tenets of biology. The answer to "what is a species" is essential for biological studies from biodiversity to conservation and evolutionary theory. Mayden's *hierarchy of species concepts* (Mayden 2002) and de Queiroz's *unified concept of species* (de Queiroz 2005, 2007) both regard a separately evolving metapopulation lineage as the only necessary property of species. Both concepts are rooted in Mayr's (1942) and Dobzhansky's (1950) ideas on a *biological species concept*. They are independent from other post-Mayr species concepts, that are often more restricted to particular aspects in the biology of organisms, like the requirement of reproductive isolation (Naomi 2011).

According to de Queiroz (2005, 2007), most of the conflicts between different species concepts arise from the confusion between a theoretical, general species concept and the actual practice of delimiting species (de Queiroz 2005). In other words, de Queiroz distinguishes the conceptual problem of defining the species category (species conceptualization) from the methodological problem of inferring the boundaries between species (species delimitation). While this idea of two independent levels seems to be widely accepted, Naomi (2011) argues that Mayden's and de Queiroz's species concepts are in practice identical to Wiley's (1978) *evolutionary species concept*. Naomi proposed the *integrated framework of species concept*, a fusion of Mayden's, Wiley's and de Queiroz's ideas in which an *evolutionary species concept* refers to a theoretical species category, and the *secondary species concept* which is actually used to delimit the species.

Even if the idea of species as independently evolving lineages is easy to accept, one is still left with the problem of how to differentiate between species and thus delimit species in practice. In mycology the use of biological species concept has been particularly challenging, as discussed by Taylor et al. (2000) and others. Erehefsky (2010) pointed out that differences in the requirements (e.g. reproductive isolation, diagnosable features, monophyly, etc.) of different species concepts can easily lead to



the recognition of different sets of taxa, even if one only applies them in the context of secondary species concepts.

My own concepts regarding the delimitation of species are in line with de Queiroz's principle of delimiting species on the basis of accumulating evidence: as different lineages diverge, they accumulate genetic changes that will eventually also be manifested as distinctive phenotypic characters. Wiens and Servido (2000) pointed out that when using only one morphological trait, no study, regardless of specimen number, could give 100% identical results. De Queiroz also emphasizes the need to accumulate evidence from multiple, diagnosable traits. As the number of traits increases, it becomes more statistically likely that the species recognized are in fact distinct, even if some mistakes are made when categorizing the individual traits of single specimens.

The plasticity of fungal morphology is a true challenge for fungal systematics, as convergent and parallel evolution is more a rule than an exception in Fungi (e.g. Hibbett 2007; Rivas Plata & Lumbsch 2011). However, the molecular era has already revolutionized the taxonomy of many major groups (James et al. 2006; Hibbett et al. 2007; Schoch et al. 2009; Stajich et al. 2009), and rapid progress will undoubtedly continue in the future. The need for the re-examination of previously misunderstood morphological characters was recently emphasized by Schmitt (2011). Indeed, one recurring result of modern phylogenetic studies has been that many traditional morphological characters are in fact a result of convergent evolution and do not necessarily reflect evolutionary relationships (Liu & Hall 2004; Schmitt et al. 2005; Hibbett 2007; Schmitt et al. 2009). This general observation seems to apply to most lineages and all levels of diversity in Fungi (Redecker 2002; Blackwell 2011).

The concept of *cryptic species* refers to taxa that have only been identified with DNA analyses, i.e., those without any distinguishable morphological features (Bickford et al. 2007). The idea of cryptic species is in general agreement with the concept of species as separately evolving lineages. However, as Ereshefsky (2010) points out, the decision whether the lineages are only separate populations or two distinct species is quite

case specific and largely up to the researcher. While one can well expect to find some truly cryptic species especially among recently evolved fungi (De Queiroz 2005; Shaffer & Thomson 2007), a detailed analysis of the morphology, chemistry and ecology of proposed cryptic fungi has often revealed previously overlooked characters (e.g. Del Prado et al. 2007). Lately cryptic fungal species have received considerable attention (Grünig et al. 2009; Schneider et al. 2009; Druzhinina et al. 2010), especially in lichen-forming fungi (e.g. Grube & Kroken, 2000; Buschbom & Mueller, 2005; Argüello et al. 2007; Crespo & Pérez-Ortega 2010; Lumbsch & Leavitt 2011). Among the mycocalicioid fungi, Vinuesa et al. (2001) studied intraspecific variation in specimens identified as *Mycocalicium subtile* and concluded that they represent one morphologically variable species that also includes some cryptic taxa. Also Nilsson et al. (2008) found evidence of the presence of several cryptic species in the same DNA data.

Difficulties in defining species and genera among mycocalicioid fungi generally has been related to the existence of few stable morphological characters (Schmidt 1970; Tibell 1984; Vinuesa 2002). Many of the morphological characters that traditionally have been used are also quite ambiguous and difficult to observe. The earliest species descriptions in the group only included macroscopic characters, i.e., the size, shape and color of the ascocarp and capitulum, and stipe length. The microscopic features mainly included the shape and septation of ascospores and eventually also spore size. While Nylander (1865) already emphasized the importance of chemical color reactions in the study of lichens, he did not mention the use of potassium hydroxide (KOH), iodine solutions, or other chemical reagents in the context of calicioid lichens or mycocalicioid fungi. The use of KOH was mentioned by Vainio (1890), but distinct color reactions were only first described by Nádvorník (1940) half a century later. Iodine reactions of mycocalicioid fungi have been observed by many lichenologists, apparently first by Tuckerman (1872), and also Keissler (1938) and Lettau (1940) frequently mention iodine reactions of the hymenium. However, Schmidt (1970) did not recognize the value of iodine, probably because Lettau's confusing species concepts obscured the specificity of such reactions.

Many attempts have been made to find new characters for species delimitation in Mycocaliciales. These have included the analysis of spore

ontogeny with transmission and electron scanning microscopy (Tibell 1984) and the study of cultured anamorphs (Tibell 1997). Unfortunately, although SEM images are often very informative, the necessary equipment to generate them is typically available only in universities and herbaria. Vinuesa (2002) reported some species-specific differences in uncharacterized chemical compounds in ascocarps and anamorphs. However, this method also requires specific equipment and a precise identification method was not provided, making it unfeasible for practical, large-scale identification. While high substrate specificity is a widely accepted feature of many mycocalicioid fungi, differences in substrate preferences of species have mainly been emphasized in the delimitation of resinicolous and lichenicolous species. For example, Schmidt (1970) noted finding ecological and morphological differences between populations of *Mycocalicium subtile* in the Alps and in the lowlands, and between conifers and hardwood trees, but he did not give these observations any weight in species delimitation.

Currently the delimitation of mycocalicioid species is largely based on the morphology of the ascocarp and ascospores. The spore characters that are used at the species level include spore septation and size, and, to some degree, the ornamentation of the spore wall. Tibell (1982) introduced the use of the pigmentation of the septum as a new character. In the ascocarp, importance has been given to the structure of the excipulum and its hyphal layers, the organization of stipe hyphae, and KOH color reactions of the ascocarp. Many of these features are best visible in microtome thin sections. The use of thin sections was introduced by Schmidt and then followed by others. While sections can reveal many important features in the internal structure of the ascocarp, some characters are not easily observed from them. Especially paraphyses and croziers are often difficult to observe from thin sections alone. Also, the actual textura type is more reliably observed from squash mounts, in which a reasonably large area of the excipulum surface is in surface view.

Schmidt (1970) based his generic delimitation on a rather limited number of species and specimens, which apparently skewed some of his conclusions about the morphological variation of the group (Hawksworth, 1980). During the past few decades, many new species of Mycocaliciales

have been found and described especially by Tibell and Titov (e.g. Tibell 1987; Titov 2001; Titov & Tibell 1993), and the distinctions between the genera in Mycocaliciales have become more and more fuzzy. Several types of transitional forms of ascus tip and the hyphal structures of the excipulum and the stipe have been described and many species have only provisionally been placed in the different genera with doubts of their true affinities (e.g. Tibell 1982, 1998; Titov & Tibell 1993). As already noted by Samuels and Buchanan (1983), the four genera cannot be differentiated by any single character, and the current system has clearly become obsolete.

## **2 AIMS OF THE THESIS**

The aim of this thesis was to produce new information on the diversity, ecology and evolution of resinicolous species of the order Mycocaliciales. One important aim was to find new diagnostic characters that can be used for species delimitation and identification in this group of ascomycetes. The specific aim in Chapter I was to describe and study a new resinicolous *Chaenothecopsis* species from tropical Africa. In Chapter II the aim was to re-examine the type collection of one resinicolous *Chaenothecopsis* species from North America, and to study the diversity and distribution of related species in Europe. The aim of Chapter III was to describe a new resinicolous *Chaenothecopsis* species from China and two newly discovered *Chaenothecopsis* fossils in amber. In Chapter IV the aim was to describe several new resinicolous *Chaenothecopsis* species from China, and to analyze their phylogenetic relationships and substrate ecology.

## **3 MATERIAL AND METHODS**

### **Biological material**

The original papers (I–IV) of this study were largely based on field specimens collected by Jouko Rikkinen in USA, Ghana and China. I have also studied approximately 500 specimens I have collected myself

during 2006–2012 in Finland, Sweden (2009–2011), Norway (2009, 2012), Germany (2009, 2010), Austria (2009), France (2009), and USA (Oregon and Washington, 2006). These specimens were collected from many different forest vegetation types, with a focus on mature and old-growth coniferous forests. Herbarium material, including the type specimens of many species, was examined from H, TUR, OU, Oslo, and UPS. Additional mycocalicioid specimens were kindly given for analysis by several mycologists and lichenologists. In this study, the nomenclature of species described prior to 1980 follows Tibell (1999), as the oldest species descriptions typically are rather vague and lack micromorphological details and are frequently found in literature sources that are difficult to obtain. The nomenclature of species described since 1980 follow that presented in the original species descriptions.

## **Microscopy**

Macromorphological features of the specimens were observed by stereomicroscope, and micromorphological characters were observed and measured mainly from squash preparations in water using a compound microscope (Leica DMLS) with a 100× oil-immersion objective and additional 1.5× or 2.0× magnifications. Spores were measured from mature ascocarps, with 10 to 20 spores selected from each. Spore measurements are given in form (x–) a–b (–y), where “a–b” presents 95% of the measured variation, and “x” and “y” the smallest and largest observed value, respectively. If “x” and “y” have the same values as “a” and “b”, they are omitted. Both length and width were measured from the same spore to derive the Q-value (width/length). The Q-values are also given in the same form as spore measurements. Potassium hydroxide (10 %, KOH), Lugol’s reagent (IKI), Melzer’s reagent (MLZ), Congo Red (CR), Crezyl Blue (CRB) and nitric acid (N) were used to observe specific chemical reactions and for better contrast in some diagnostic morphological structures (Supplementary table 1). More details on the microscopic and chemical characters used are given in the Results.

For scanning electron microscopy, specimens were removed from the substrate, placed on a carbon-covered SEM-mount, sputter coated with

gold/palladium ( $2 \times 120$  seconds at 20 mA, 10 nm coat thickness) and examined under a Carl Zeiss LEO 1530 Gemini field emission scanning-electron microscope. Energy-dispersive X-ray spectroscopy (EDX) was performed on some ascomata using an INCA-EDX system (Oxford Instruments) and an excitation voltage of 15KV at this electron microscope (Chapter III).

The amber pieces were ground and polished manually with a series of wet silicon carbide abrasive papers [grit from FEPA P 600–4000 (25.8  $\mu\text{m}$  to 5  $\mu\text{m}$  particle size), Struers] to remove the weathered crusts and to minimize light scattering for the investigation (Chapter III). To reduce light scattering from fine surface scratches and to improve optical resolution, prepared specimens were placed on a glass microscope slide with a drop of water applied to the upper surface of the amber, covered with a glass coverslip. The inclusions were studied using a Carl Zeiss AxioScope A1 compound microscope, in most instances simultaneously with incident and transmitted light. In order to protect the amber from oxidation and breakage the polished Baltic amber piece was embedded using polyester resin (GTS cured with addition of MEKP hardener Vosschemie, Uetersen) as described by Hoffeins (2001).

To infer the morphological and ecological characters for Figure 1, the spore and textura types were examined with compound microscope. In addition, a piece of substrate attached to individual ascocarp was observed for the presence or absence of lichen thallus or algal colony.

### **DNA extraction and sequencing**

DNA was extracted from 5 to 10 fruiting bodies of each specimen with the NucleoSpin<sup>®</sup>Plant DNA extraction kit (Macherey-Nagel) with the following modification to the manufacturer's protocol: specimens were incubated for 12 or 2 hours (for the old and new version of the kit, respectively) to ensure lysis of the fruiting bodies. The nuclear large subunit ribosomal RNA (LSU) partial gene was amplified using the primers LROR and LR3 (Vilgalys & Hester 1990; Rehner & Samuels 1994). The ITS region of rDNA was amplified using the primers ITS4 and ITS5 (White et al. 1990) or alternatively ITS4 and ITS1F (Gardes & Bruns 1993). Protein coding genes

RNA polymerase II largest subunit (RPB1) were amplified and sequenced with primers RPB1-AFasc and RPB1-6R2 (Hofstetter et al. 2006) and the RNA polymerase II second largest subunit (RPB2) with RPB2-f7cF and RPB2-f11aR (Liu et. al. 1999).

PCR amplification was conducted using Phusion® High-Fidelity DNA Polymerase (Thermo scientific/Finnzymes) according to the manufacturer's specifications using a 1:4 – 1:1 dilution of template DNA. PCR products were purified with GeneJET™ PCR Purification Kit (Fermentas). Amplicons were sequenced by Macrogen Inc. (South Korea) in the forward and reverse directions using the same primers as during amplification. For samples that failed to amplify using the Phusion PCR method, amplification was conducted using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Piscataway NJ, USA) according to the manufacturer's instructions with the primers LROR & LR7 (Vilgalys and Hester, 1990) or ITS1F & ITS4, and 3 µl of template DNA in a total PCR reaction volume of 25 µl. These amplicons were then sequenced using an ABI 3100 automated sequencer (Applied Biosystems Inc., Foster City, CA, USA) with the primers ITS1F & ITS4, and LROR, LR3, LR5 (Vilgalys and Hester, 1990), and LR7.

### **Phylogenetic analyses**

Sequences for each gene region were assembled into contigs using Geneious v5.4 (Drummond et al. 2011) and the consensus sequences used for further analysis. If sequencing was successful only for one direction, the fate of the sequence was decided on the basis of the chromatograms. If the chromatogram was clean, with relatively high and even peaks, without noise, such a sequence was considered reliable and used for further analysis.

The dataset for Chapter I was aligned with Clustal W 1.8 (Thompson et al. 1994). Other datasets (Chapters III and IV) were aligned with MAFFT version 6 (Kato & Toh 2008), and adjusted manually in PhyDE® 0.9971 (Müller et al. 2010). Unequivocal short (1–10 nucleotides), uninformative insertions were first removed from the alignment, and the program Gblocks 0.91 (Castresana 2000) was then used with the most relaxed settings to remove ambiguously aligned regions. Models of evolution were



generated independently with jModeltest 1.1 (Posada 2008) (Chapters I and III) and jModeltest 2.0 (Darriba et al. 2012) (Chapter IV) for each of the gene regions (LSU, ITS1, 5.8S, ITS2). Alternatively, MrModeltest (Nylander 2004) was used in those occasions when jModeltest was not able to determine the model of the dataset (Chapters I and III).

To infer phylogenetic relationships, Bayesian analysis employing four Markov chain Monte Carlo (MCMC) chains were carried out with MrBayes versions 3.1.2 (Chapters I and II) and 3.2.1 (Chapter IV) (Ronquist & Huelsenbeck 2003) on the computational resource Bioportal at the University of Oslo (<http://www.bioportal.uio.no> Kumar et al. 2009). The four chains were run simultaneously for 10 million generations for the partitioned datasets with trees sampled every 100th generation. Average standard deviations of split frequency (ASDSF) values lower than 0.01 were taken as an indication that convergence had been achieved. A burn-in sample of 5000 trees was discarded for the run and the remaining trees were used to estimate branch lengths and posterior probabilities. Additional support values were estimated using the same model parameters in Garli 2.0 for maximum likelihood (Zwickl 2006) with 1000 bootstrap search-replicates (Chapters III and IV) and alternatively with parsimony analysis using DNAPenny of PHYLIP version 3.61 (Felsenstein 2005) with default settings (Chapter I).

The same molecular and phylogenetic methods as described above were also used to infer phylogenetic relationships for Figure 1. Sequences were aligned with Mafft version 6, and the alignments analyzed for phylogenetic relationships with MrBayes version 3.2.1 and Garli 2.0. The evolutionarily models selected with jModeltest 2.0 for ITS1, 5.8SR, ITS2, and partial LSU were: TPM2uf+G, TPM2+I, TPM2uf+G, TrNef+I+G, respectively. GenBank accession numbers and voucher information are given in Table 2 (Supplementary material). The alignment data has been submitted to TreeBase, accession no. S13948.



## 4 MAIN RESULTS AND DISCUSSION

The articles of this thesis show that resinicolous species are a species rich and phylogenetically diverse group within the order Mycohaliciales. Before this treatment seventeen species of resinicolous mycohalicioids were known from different parts of the world; twelve species from conifer resins and five from angiosperm exudates. Our studies have now increased the total number of known species to 24, and almost doubled the number of taxa known from angiosperm exudates. The new species are placed in *Chaenothecopsis* primarily based on current generic concepts, although we acknowledge that genetic data is beginning to amass that suggests future genus-level nomenclatural amendments will be needed in the Mycohaliciales.

In Chapter I we describe *Chaenothecopsis khayensis*, the first resinicolous mycohalicioid fungus from Africa and analyze its phylogenetic relationships. The fungus grows on the exudate of African mahoganies (*Khaya* sp.), which are the first known host tree of resinicolous mycohalicioids in the angiosperm family Meliaceae. We also discuss the possible association between the new fungus and wood-boring insects that damage the hosts.

In Chapter II we describe *Chaenothecopsis diabolica* from *Abies* resin in western North America, report new anatomical findings from two other conifer associated *Chaenothecopsis* species, and discuss the biogeography of these species. *Chaenothecopsis diabolica* and *C. oregana* are reported as new to Europe.<sup>1\*</sup>

In Chapter III we describe *Chaenothecopsis proliferatus* from China and compare the ecology and morphology of this and similar species with two newly discovered *Chaenothecopsis* fossils from European ambers, and propose an ecological role for proliferating ascocarps in resinicolous fungi.

In Chapter IV we describe four new resinicolous *Chaenothecopsis* species from temperate China, three of these from angiosperm exudates, and discuss

<sup>1\*</sup> The nomenclatural error in this Chapter is corrected in Tuovila et al. 2012. *Chaenothecopsis oregana* is reinstated with a new lectotype and *C. zebrina* is reduced to its synonymy.

their phylogenetic relationships and ecology. *Chaenothecopsis pallida* grows on *Ailanthus*, the first known host genus within the Simaroubaceae; *Chaenothecopsis perforata* grows on *Rhus*, the second known host genus within the Anacardiaceae; *Chaenothecopsis resinophila* grows on *Kalopanax*, the second known host genus within the Araliaceae; and *Chaenothecopsis hunanensis* grows on the resin of *Pinus massoniana*.

In this extended summary I place our new findings into a wider context and provide an overview of diagnostic characters that can be successfully used for the delimitation and identification of species within the Mycocaliciales.

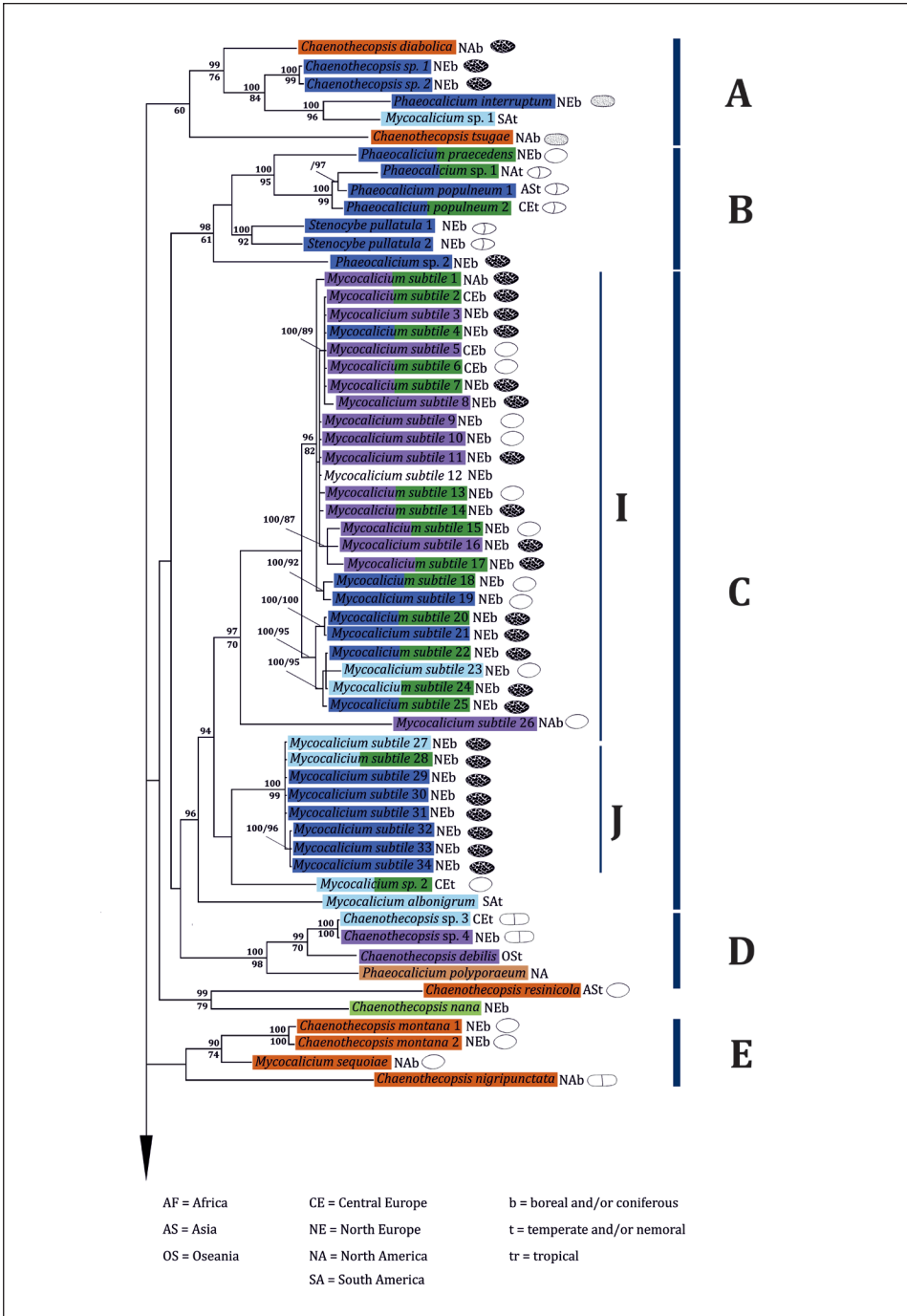
### **Phylogenetic relationships and substrate ecology**

Before this treatment, only Tibell and Vinuesa (2005) had studied the phylogenetic relationships of mycocalicioid species in any detail. The new results offer some valuable insights into the inter- and intrageneric relationships within the order (Chapters I, III, IV). The generic concepts that are currently in use are mainly based on the monograph of Schmidt (1970), but their limitations have been well known for quite some time (e.g. Tibell 1984; Tibell & Titov 1995). Our results confirm that the genus *Chaenothecopsis* is not monophyletic, and also indicate that *Phaeocalicium* is more diverse than previously thought (IV). The results also confirm that the family Sphinctrinaceae is superfluous, as the species included in our study were placed within Mycocaliciaceae with strong support in all analyses (I, III, IV). This relationship was first recovered by Tibell and Vinuesa (2005), but was not statistically strongly supported. In our analyses the crown clades tended to be well supported and individual species were consistently nested within specific crown clades. Unfortunately the deeper nodes remained unresolved or with poor support values. One must hope that worldwide sampling and the analysis of additional gene regions will enhance the resolution in future studies.

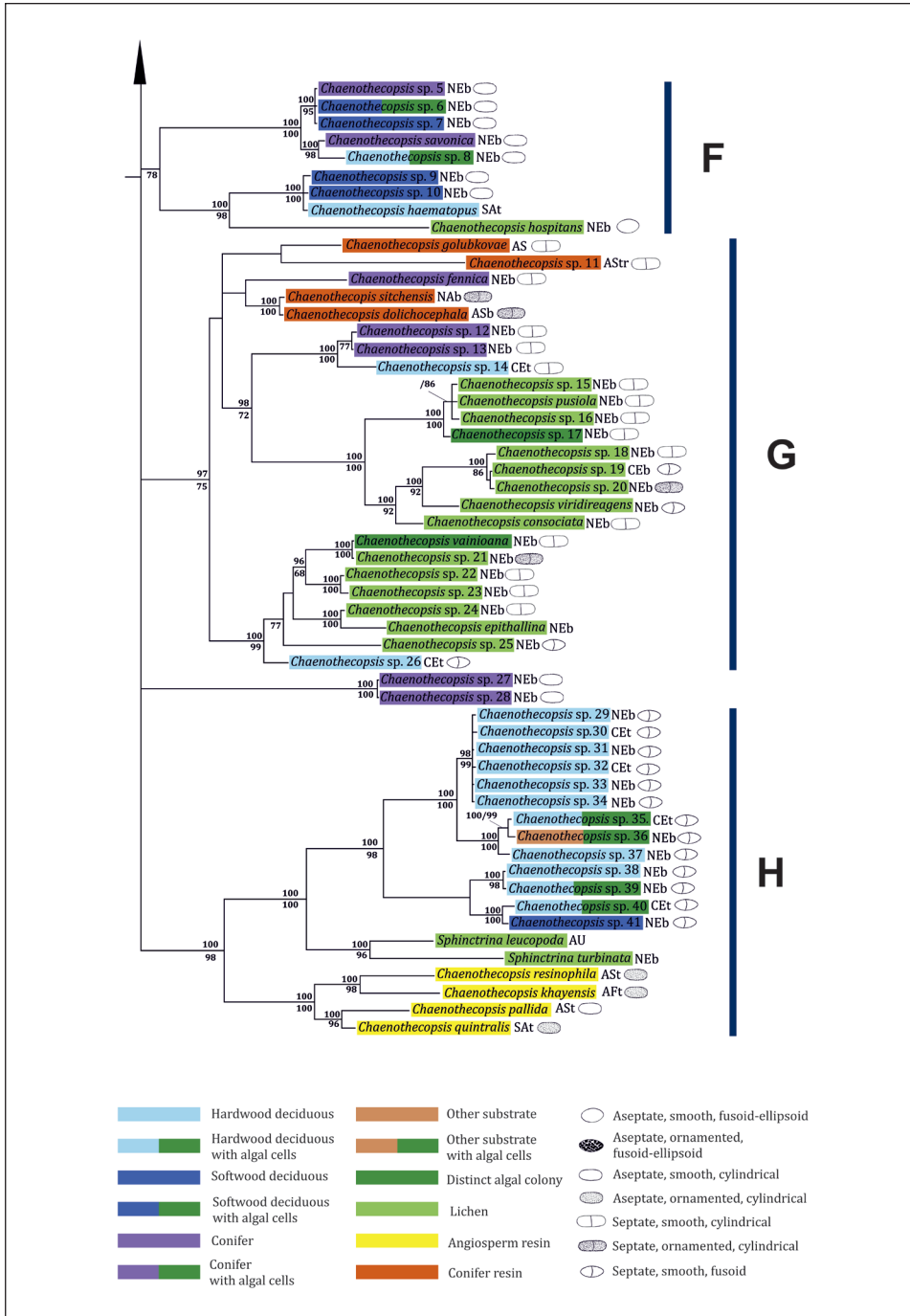
Some features in ascocarp morphology, spore septation, and substrate ecology correlated with the clades delimited on the basis of DNA data (III, IV). To further confirm some of these findings, a larger dataset was analyzed and the results are shown in Figure 1. The features of this tree are generally

congruent with those seen in the smaller phylogenetic trees of Chapters I, III and IV. Several well-supported clades appear in all the larger phylogenetic trees (Fig. 1, III, IV): clade C (Fig. 1) including only *Mycocalicium* species with aseptate spores; clade H including a well-supported group of resinicolous species from angiosperm exudates with aseptate spores, two *Sphinctrina* species with aseptate spores, and a group of lignicolous *Chaenothecopsis* specimens with septate spores; and finally clade G with parasitic and conifer-associated resinicolous *Chaenothecopsis* specimens with septate spores. Also several smaller clades in the phylogenetic trees receive good statistical support, but their exact positions in relation to the aforementioned larger clades are not resolved.

Without adequate resolution or support of deeper nodes in the phylogeny, I refrain from making any formal suggestions regarding generic delimitations within Mycocaliciales. However, if the overall grouping of taxa shown in Figure 1 is also supported by the results of future studies, one feasible action could be to assign all taxa in clade C to *Mycocalicium* (this lineage is likely to also contain the type specimen *Mycocalicium parietinum*), and those of clade H to *Sphinctrina*. If the close relationship between clades C and B, with the latter group also including species currently assigned to *Phaeocalicium* and *Stenocybe*, reflects the true relationship between the groups, these two genera could be included in *Mycocalicium*. Alternatively, *Stenocybe* could be retained in clade B and its generic name conserved. Clade D poses a taxonomic challenge, as its position in our analysis was never well supported. Based on a morphological analysis of the voucher material of Figure 1, there appear to be some consistent differences between the *Chaenothecopsis* specimens grouped into clades G and H, respectively, and specimens in both of these groups differ from those in clade D. For example, most specimens in clade H have *textura prismatica* in the stipe, while all specimens in clade G have *textura intricata*. From this perspective the last mentioned group better fits the original description of the genus *Chaenothecopsis* (Schmidt 1970). However, on the basis of stipe and ascospore morphology, and the KOH reaction, the type species *Chaenothecopsis rubescens* most likely belongs to clade F. To confirm this relationship, a vouchered, verified specimen of this species has to be sequenced. The problem remains that the type specimens of most



**Figure 1.** Phylogenetic relationships among Mycocaliciales. Bayesian tree inferred from ITS and 28S (partial) sequences (445 and 521 nucleotides, respectively). Posterior probabilities  $\geq 90\%$  for Bayesian inference (above node or first value) and bootstrap values  $> 60\%$  for maximum likelihood (below node or latter value) are given for



supported nodes. Taxon names are followed by referral number, abbreviations indicating type of forest and geographic origin, and a small schematic representation of general spore type. The ecology is further indicated by color. More information on the species, substrates, voucher specimens and GenBank accession numbers is given in Table S2.

mycocalicioid species are sparse and with current methods, the likelihood of obtaining sufficient DNA for phylogenetic studies from old specimens is very low, even with destructive sampling. During the course of this study the sequencing was usually only successful from relatively fresh specimens (aged five years or less), and the maximum age of specimens from which DNA could be successfully amplified was approximately ten years (III, IV). My repeated attempts to also sequence older material were never successful.

It appears that in evolutionary terms some mycocalicioid species are very old and in certain cases their morphology and ecology has remained unchanged for at least tens of millions of years (III). Specimens of *Chaenothecopsis montana* Rikkinen are quite similar in North-West America and Scandinavia, and the distinctive morphology of this species only resembles those of its closest relatives *Chaenothecopsis nigripunctata* Rikkinen and *Mycocalicium sequoiae* Bonar (II). However, there are slight genetic differences between specimens collected from Oregon, Finland and Sweden (data not shown), and these differences may well be related to the population dynamics of *C. montana* during and after the last glacial maximum (II). Some other groups of mycocalicioid fungi may have diversified relatively recently. One such case is the almost cosmopolitan but variable *Mycocalicium subtile* group (clade C in Fig. 1). The pronounced morphological, ecological and genetic variation seen even among specimens collected from limited geographic regions such as Scandinavia indicates that the group is in fact “hyperdiverse”. In the future we may well see several of Nylander’s and Vainio’s old species resurrected, including the original type species, *Mycocalicium parietinum*.

The results of the phylogenetic analyses clearly show that the resinicolous species do not form a monophyletic group within the Mycocaliciales (Fig. 1, III, IV). However, those resinicolous species included in well-supported clades share some morphological and ecological characters. For example, all the newly described species from angiosperm resins have aseptate spores and they belong to the same, well supported group (clade H). Similarly, all the resinicolous species with septate spores on conifers belong to another well supported group (clade G). Interestingly, species of the latter clade

share a similar morphology with all the fossil specimens from European ambers (III). Three species from conifer resins, *Chaenothecopsis montana*, *C. nigripunctata*, and *Mycocalicium sequoiae*, always form a group of their own (clade E). These three species also share some morphological features which distinguish them from all other mycocalicioid species (II). *Chaenothecopsis diabolica* and *C. tsugae* Rikkinen group together in all analyses (clade A), but with variable support. Also *Chaenothecopsis resinicola* Tibell & Titov may be related to these species (IV), which grow on conifer resins and have aseptate spores.

The *Phaeocalicium* and *Stenocybe* species of clade B live on temperate and boreal angiosperms, especially on the thin branches of *Alnus*, *Betula* and *Populus*. They are generally thought to be saprotrophs or weak parasites (Tibell 1996). Interestingly, Bryant (1981) found that all the above-mentioned host trees produce and accumulate resins in their growing shoots, especially in the internodes of young branches. Thus, the occurrence of several species of *Phaeocalicium* and *Stenocybe* on branch internodes may not be a mere coincidence, but an indication that also these mycocalicioid fungi have some association with plant resins – while they may not necessarily be truly resinicolous, they likely can tolerate resin compounds.

For most saprotrophic or parasitic fungi, the complex terpene and phenol compounds typically found in resins and other plant exudates are toxic or at least difficult to degrade. The sporadic distribution of resinicolous species within the order Mycocaliciales indicates that there have been multiple losses or gains of this ecology during evolution. Due to the low support values of the deeper nodes within our phylogeny, ancestral state reconstruction analysis could not reliably be applied to our data set. However, some patterns are clear: 1) The ability to tolerate resin compounds is strikingly common in the Mycocaliciales. No comparable group of fungi has a similar number or proportion of resinicolous species. 2) *Mycocalicium sequoiae* only grows on the resinous exudates of two “living fossils”, *Sequoiadendron giganteum* and *Sequoia sempervirens*, in their natural, residual range. The early evolution of this association has been proposed to date back to the Mesozoic, when trees of the host lineage



(Sequoioideae) had a much wider distribution than today (Rikkinen & Poinar 2000). 3) The three presently known amber fossils of resinicolous *Chaenothecopsis* species show that some species in the genus were already well adapted to a resinicolous ecology in the Eocene, and that relevant morphological adaptations have since remained more or less unchanged (III).

The phylogenetic analyses revealed that lichenicolous species occur in at least three different lineages of mycocalicioid fungi. The species of *Sphinctrina* are lichenicolous, and placed with high support in clade H, together with the group of species from angiosperm exudates (I, III, IV). The lichen-associated *Chaenothecopsis* species fall into two clades: *C. hospitans* (Th. Fr.) Tibell is in clade F, while *C. epithallina* Tibell, *C. consociata* (Nádv.) A.F.W. Schmidt and *C. pusiola* (Ach.) Vain. are nested in clade G, together with resinicolous species from conifer resins (III, IV). Also the single *C. nana* Tibell specimen used in the analysis had apparently grown on a lichen. It grouped with strong support with *C. resinicola*, a morphologically similar species from conifer resin (IV). These results indicate that there may have been multiple switches between a resinicolous and lichenicolous ecology during the evolution of mycocalicioid fungi. Gueidan et al. (2008) proposed that the common ancestor of Eurotiomycetes would have been non-lichenized, and Geiser et al. (2006) interpreted the sporadic occurrence of lichen parasites in both Mycocaliciomycetidae and Chaetothyriomycetidae as evidence of evolutionary plasticity towards the lichenicolous ecology, rather than an indication of ancestral state. Neither of these interpretations contradicts the assumption that saprotrophy (lignicolous or resinicolous) is the ancestral state for Mycocaliciales. Several *Chaenothecopsis* specimens in clade G typically associate with different species of *Calicium* and *Chaenotheca*. As fossils of both lichen genera have been found from Eocene Baltic amber (Rikkinen 2003d), the parasitic interactions between *Chaenothecopsis* species and their calicioid hosts have clearly had the potential to evolve already tens of millions of years ago.

The distinction between lichenicolous, algicolous and lignicolous species can often be obscure, and there may even be a continuum between strictly saprotrophic and parasitic species. Some species of *Chaenothecopsis* (e.g.



*C. pusiola*) are almost always found growing together with alga, and dozens of living or dying algal cells can be seen at the base of their ascocarps. At the other end of the spectrum there are the species (e.g. *C. fennica* [Laurila] Tibell) which apparently never associate with green algae. However, in many cases the exact relation of the fungi and algal cells within the substrate is very difficult to elucidate.

Lignicolous *Chaenothecopsis* specimens from conifer and angiosperm substrates mix widely with both resinicolous species from conifer exudates (clades A and G) and lichenicolous species (clades F and G). However, one group of lignicolous taxa has only been collected from deciduous trees of Fagales and Apiales. In the phylogenetic analysis these fungi group together with the lichenicolous *Sphinctrina* species and the group of *Chaenothecopsis* species from angiosperm exudates (clade H). The latter association is particularly interesting as it implies that a restriction to exclusively angiosperm hosts has occurred in both the resinicolous and lignicolous members of a single lineage, despite the fact that the two groups have disparate morphological characters. This type of connection between lignicolous and resinicolous ecologies would not be all that surprising considering that similar or even identical secondary metabolites are often present in both substrate types (e.g. Kundu & Laskar 2009; Zhang et al. 2009).

The *Mycocalicium subtile* specimens in Figure 1 grouped into two strongly supported clades. The specimens of clade I formed two further groups: one consisting of specimens from conifer lignum and the second mainly including specimens from deciduous angiosperms with soft lignum, such as *Alnus*, *Sorbus*, and *Salix*. All the specimens in clade J came from deciduous angiosperms, including *Betula* species, but also hardwoods such as *Quercus* and *Ulmus*. While many specimens of clade I could often have some algal cells near the base of their ascocarps, the specimens of clade J were never associated with algal cells.

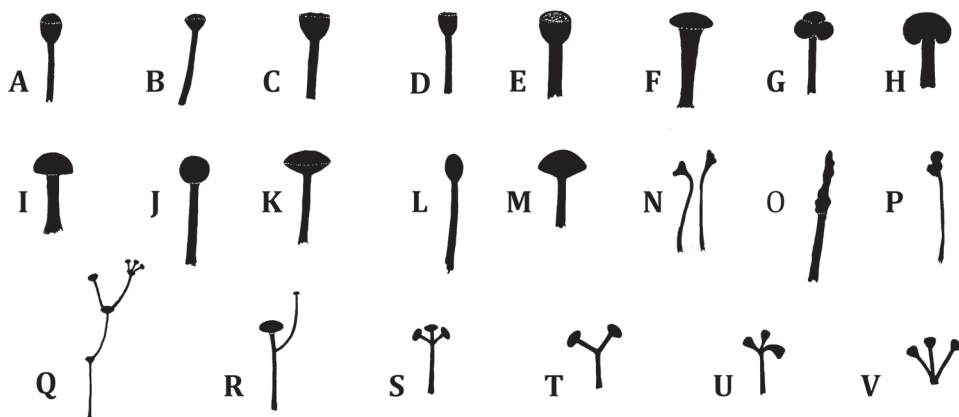
### **Diagnostic characters for species delimitation**

While DNA-phylogenies and substrate preferences give much important insights into specific problems of species delimitation in different groups of mycocalicioid fungi, the practical process of species identification continues

to be based on morphological and chemical characters. The new results in Chapters I–IV demonstrate the usefulness of many well tested characters, but also reveal several new features that can be used in species delimitation and identification. My overall observation is that mycocalicioid species can only rarely be distinguished by a single character, but have unique combinations of several different chemical and morphological features. The number of characters needed varies and no universal set of characters can be given that would reliably distinguish between all species. In the following section the most relevant morphological and chemical characters are described in detail. My hope is that this treatment will help interested readers to accurately identify and describe resinicolous *Chaenothecopsis* species and other mycocalicioid fungi.

**Ascocarp size and color.** The size of the ascocarps is an important diagnostic feature in all mycocalicioid fungi (I–IV). Their populations typically consist of several age cohorts of mature ascocarps that have developed during consecutive growing seasons. Young, immature ascocarps are typically smaller than mature ones. In addition, the size of mature ascocarps can vary both between and within populations – often such size differences can even be twofold. The size differences within populations are likely to mainly reflect micro variation in environmental conditions during ascocarp development. Thus, the size of mature ascocarps tends to vary only within certain limits, and based on maximum ascocarp size the species can be divided into four size classes: tiny (height < 5 mm), small (6–10 mm), large (11–15 mm) and huge (> 15 mm). Most of the newly described species (I, II, IV) belong to the second and third groups.

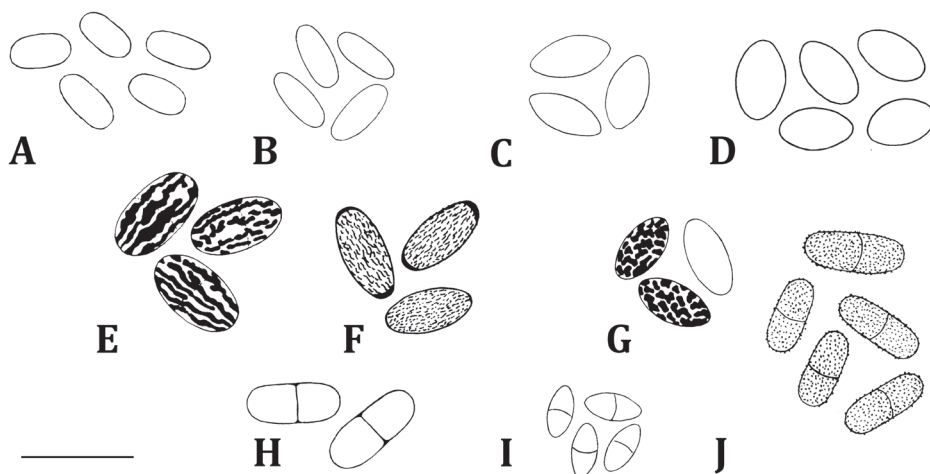
The ascocarps of most mycocalicioid fungi appear more or less black under incident light. However, there are also some colour differences that can be observed under a good stereomicroscope (I, IV). Frequently the color of young and mature ascocarps of the same population can be quite different. Besides black, the ascocarps may be dark brown (IV), green (I), pale yellowish or light brown (IV), or have a distinct reddish, greenish or bluish tinge (III). In addition, the surface of the stipe and/or capitulum may be shiny or matte, and some species have a colored pruina on their stipe and sometimes also on the excipulum. This pruina may



**Figure 2.** Diagrammatic representations of typical ascocarp shapes (A–P) and types of branching (Q–V) in the Mycocaliciales. Cupulate (A, C–E), conical (B), hemispherical (F, I), lobed or multiheaded (G), emarginate (H), spherical (J), lentiform (K), ellipsoid (L), flabelliform (M, N), and irregular (N–P). The shape of irregular capitula can vary a lot between and within species, but the overall form (e.g. elongated in O, flat in N) is usually constant. The variation of capitulum width (C vs. E), in the presence/lack of epithecium (E), and epithecium height (e.g. raised in A, flat in D) are only seen in the cupulate type. Note the difference between long and slender stipes (N, P–Q), and thick stipes (C, E, F, H–I). In some cases the ascocarps can proliferate through the capitulum (Q), or from the stipe (R), or branch from the stipe (from the upper part in S, middle in T, from the base in V); or form whorls (U). Picture H has been sketched from cross-section, others from profile. The species and voucher information of the illustrated specimens are given in Table S3. Drawings by HT.

originate from senescent, fracturing hyphae (Tibell & Titov 1995), or from extracellular pigment crystals (Bonar 1971; Selva & Tibell 1999). In some species mature ascospores tend to accumulate on the top of the epithecium and this may have a significant effect on the color and overall appearance of the capitulum (III, IV).

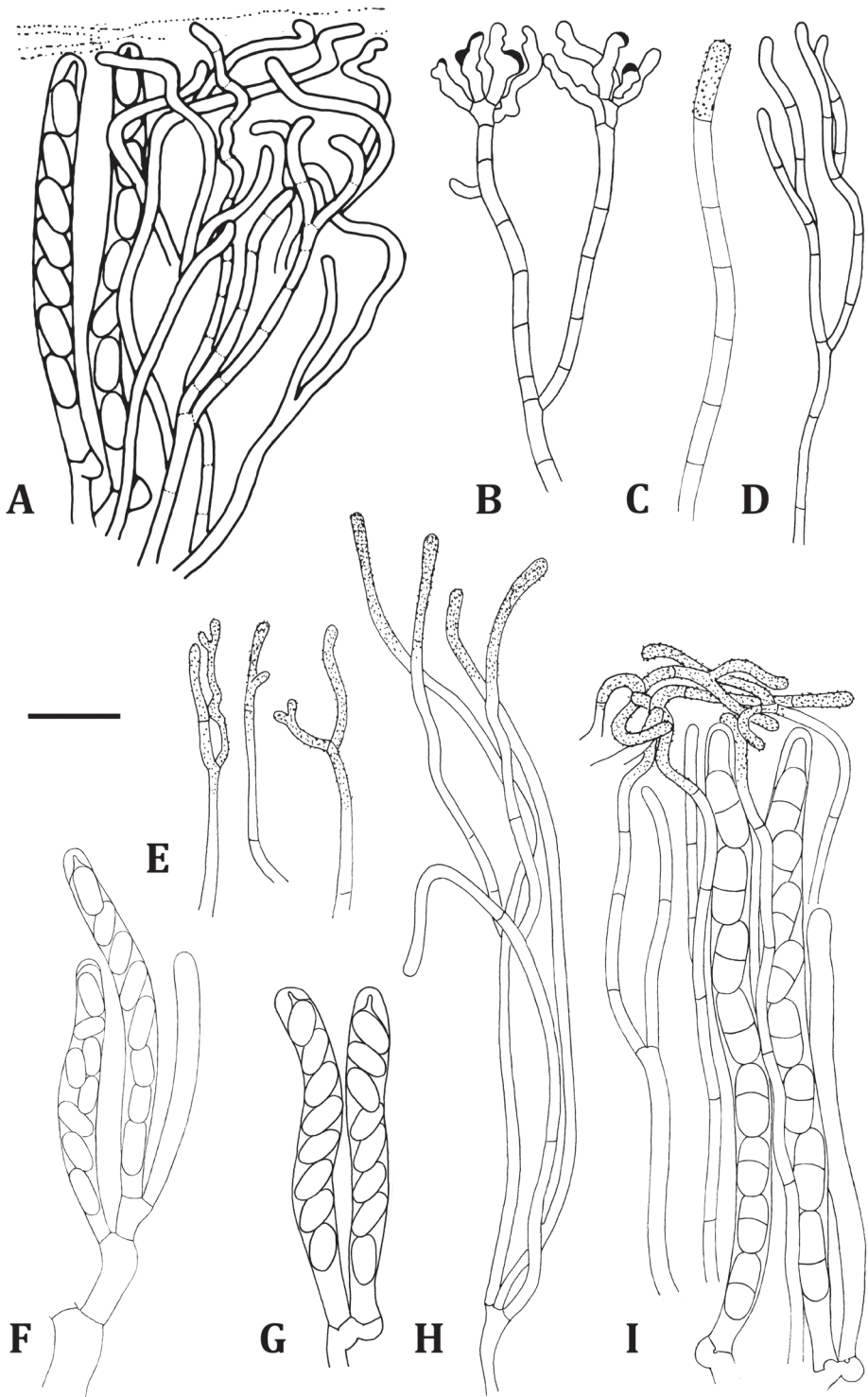
**Branching.** While a pronounced tendency to produce branched ascocarps and type of ascocarp branching are both rather species specific features (II, III), all branching species can also produce non-branched ascocarps (II, III), and most predominately non-branching taxa will occasionally produce a branched ascocarp (I, IV). Examples of common branching types are illustrated in Figure 2. The frequent branching and proliferation of certain resinicolous species seems to be an adaptation to living near active resin flows, as well timed proliferation would prevent burial in resin and enable the release of spores above the new resin surface (III).

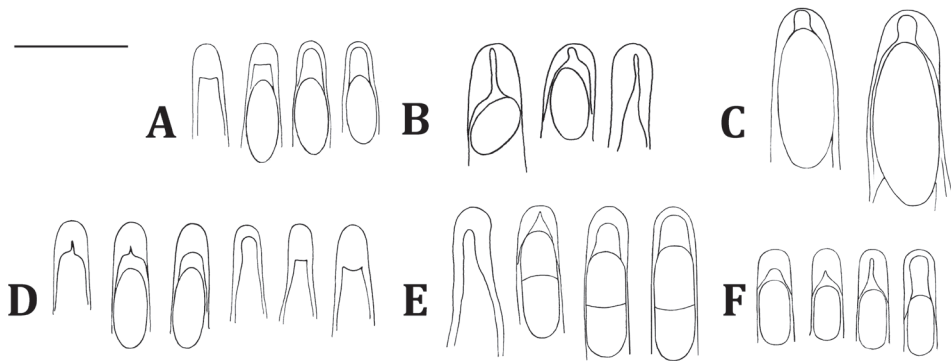


**Figure 3.** Diagrammatic representations of some aseptate (A–G) and septate (H–J) spore types in the Mycocaliciales. Cylindrical (A, H, J), ellipsoid (B, E, F), fusoid (C, G, I), and ovoid (D; can be more distinct than presented here). Note the thickened walls at the spore apices (F) and septum thickened at the junction between septum and spore wall (H). Some typical ornamentation types as seen in water with compound scope: smooth (A–D, H–I), finely spinulose to verrucose (J), ridged (E–F), and panther-like (G). Scale bar 10  $\mu\text{m}$ . The species and voucher information of the illustrated specimens are given in Table S3. Drawings by HT.

**Stipe.** The stipe length and width vary between species, and both features can have value in species delimitation. However, the length of the stipe seems particularly dependent on ecological conditions (Rikkinen 1999). Of the newly described species, *Chaenothecopsis proliferatus* and especially *C. humanensis* have relatively long and slender stipes (III, IV), whereas in all the other new species the stipe is relatively wide in relation to the capitulum (I, II, IV). Species with very short or practically non-existent stipes were reviewed by Tibell and Ryman (1995). Some examples of the overall variation in stipe length and width can be seen in Figure 2.

**Figure 4.** (on the facing page) Diagrammatic representations of paraphysis types and asci in the Mycocaliciales. Asci with croziers (A, G, I) and asci without croziers (F). paraphysis unbranched, as long as the asci or only slightly exceeding their length (C); paraphysis branched, as long as the asci or only slightly exceeding their length (D, H); paraphysis branched, longer than the asci and covering their apices (A–B, E, I). paraphysis may branch in their lower parts (A, D, H) or only or mainly in the apical part (A, E, I). paraphysis may be smooth (A–B, D) or covered with usually hyaline crystals, warts or amorphous material (C, E, H–I). Epithecium consisting only paraphysis (I) and epithecium consisting of paraphysis and a net of excipular hyphae (A). Scale bar 10  $\mu\text{m}$ . The species names and voucher information of the illustrated specimens are given in Table S3. Drawings by HT.

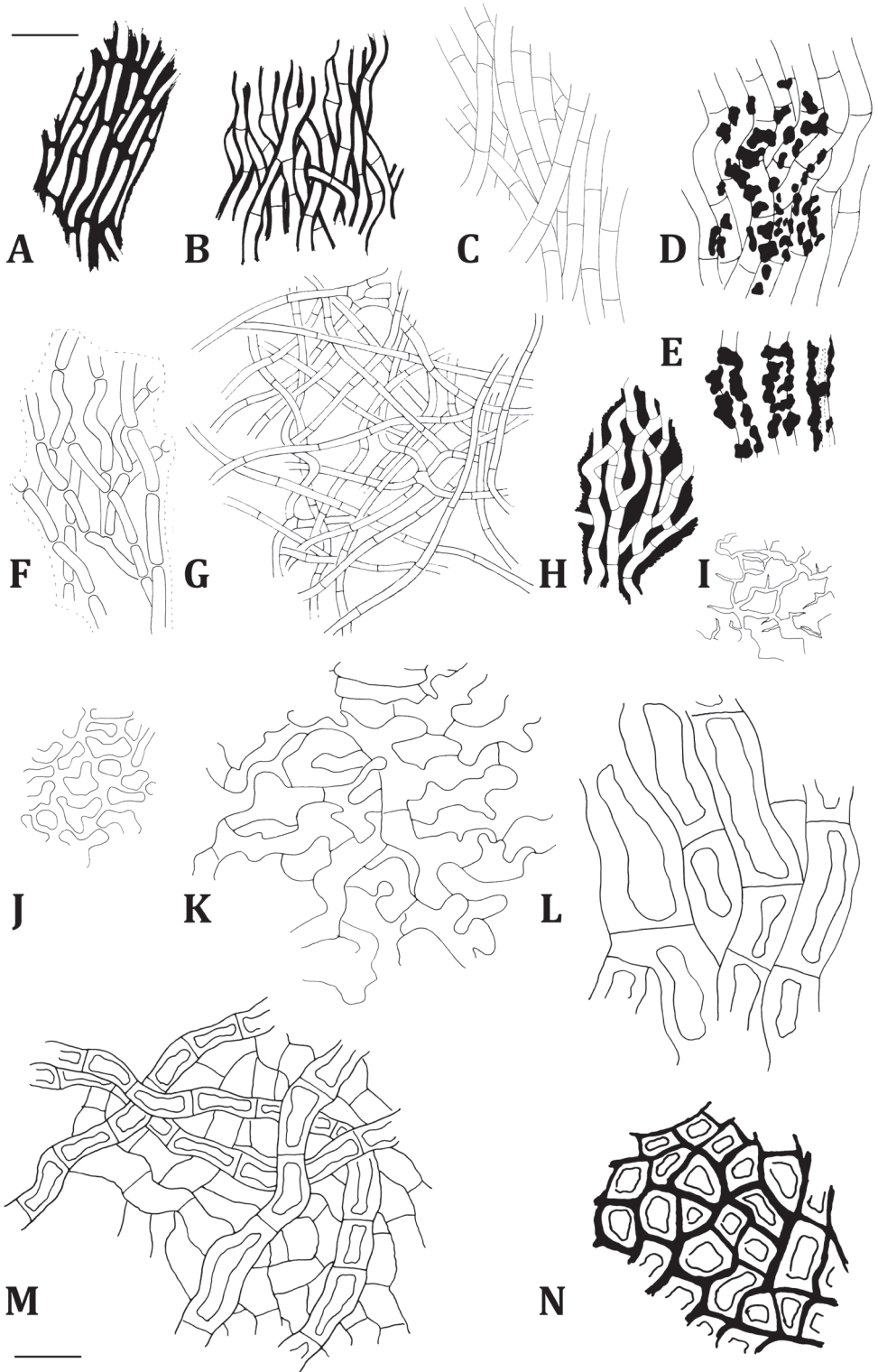




**Figure 5.** Diagrammatic representations of ascus tip variation in the Mycocaliciales. Type with channel in ascus tip (B–F), the tip is more or less distinctively thickened in young, immature asci, and the channel is present at some stages of development, but mature asci are usually without a pronounced apical thickening. Type with thickened ascus tip (the rightmost ascus in A), in which the tip is more or less evenly thickened at all stages of ascus development. Vanishing type (A), in which the tip is strongly and evenly thickened in very young to semi-mature stages, but the thickening disappears gradually without the formation of a channel, and mature asci are without thickening. The developmental phase with a channel can either be distinct (B, C) or barely noticeable (D, E), in which case it is usually only observable with cytoplasm staining (e. g. CRB) and high magnification. Scale bar 10  $\mu\text{m}$ . The species names and voucher information of the illustrated specimens are given in Table S3. Drawings by HT.

When studied with transmitted light, pigments can often be seen in the cell walls of outermost hyphae of the stipe and excipulum. The color range of such pigments tends to be species specific, and old ascocarps tend to be much darker than young ones (III). Different shades of green and brown are commonly seen, but also shades of gray, yellow, and brownish red occur in some species (I–IV). The stipe and excipulum may share the same color or have different combinations of colors. In some species the colors

**Figure 6.** (on the facing page) Diagrammatic representations of pigmentation types (A–F, H–I, N) and textura types in excipulum and stipe of the Mycocaliciales (A–H, J–N). *Textura prismatica*, elongated cells more or less periclinal (A, D, L); *textura intricata*, hyphae intertwined (B, C); *textura angularis*, cells angular or isodiametric (M, N); *textura epidermoidea*, intertwined hyphae with fused cell walls and/or irregularly shaped cells (J, K); and *textura oblita*, fused cell walls periclinally or loosely intricated (F–H). A strictly prismatic textura seems to be rare in mycocalicioid species, and their hyphae tend to be at least slightly intertwined (D). In all textura types the septa of hyphae may either be thin (B–D, G–H, M) or thick (A, F, J, L, N). Also the hyphal walls may be thin (C) or thick (B, L, N). In some cases a net of branching hyphae covers the excipulum (M), and pigment can accumulate as irregular lumps and pieces on the the hyphal walls of the outer layer of the excipulum (D–E). Scale bar 10  $\mu\text{m}$ . The species names and voucher information of the illustrated specimens are given in Table S3. Drawings by HT.

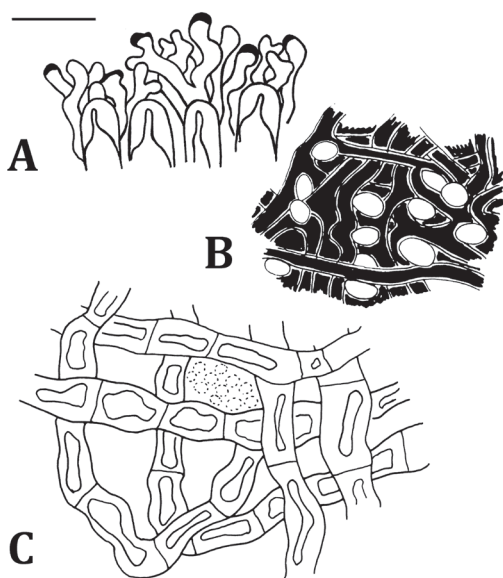




are very pale, and this is usually (but not always) also reflected in the color observable under incident light (IV).

**Capitulum.** The young, emerging ascocarps of mycocalicioid fungi tend to have very small capitula, or, at very young stages, no capitula at all. Before the development of the capitulum, the developing ascocarp often has a sharp apex (III). The size and shape of the capitulum typically changes quite a bit during maturation. Thus, when using the shape of the capitulum as a morphological character one should always compare ascocarps of the same age, since the shape of juvenile ascocarps is often quite variable. Some typical capitulum shapes of mycocalicioid fungi are illustrated in Figure 2.

**Excipulum.** The textura type of the excipulum varies widely, and is often useful for species delimitation (II–IV). For example, the ascospores of *C. perforata*, *C. pallida* and *C. resinophila* are almost identical, but the excipulum is completely different in each species (IV). Some typical textura types are illustrated in Figure 6. The textura type of the excipulum and stipe of a single ascocarp may be the same or different, but even in the former case the hyphae and cell walls are usually somewhat wider in the



**Figure 7.** Diagrammatic representations of epithecium types in the Mycocaliciales. Epithecium consisting only of paraphysis, apices branched (A), epithecium consisting of very tight, even surface of excipular hyphae (B) epithecium consisting only of a net of excipular hyphae (C). Scale bar 10  $\mu\text{m}$ . The species names and voucher information of the illustrated specimens are given in Table S3. Drawings by HT.



stipe (II, IV). The typical width of the hyphae of the stipe and excipulum varies considerably between species, but is usually between 2.0 and 6.0  $\mu\text{m}$ . The hyphal walls are typically at least 0.5  $\mu\text{m}$  and usually around 1.5  $\mu\text{m}$  thick (I–IV). The surface of the excipulum and stipe is sometimes covered by a sparse or dense net of arching or even anticlinal hyphae (I–IV). This structure is usually clearly distinguishable from the excipulum and stipe and seems to be a good character for distinguishing certain species. The hyphal net can sometimes cover the whole ascocarp but is more commonly restricted to the upper part of the stipe and lower part of the excipulum.

**Epithecium.** The structure of the epithecium varies less than that of the stipe and excipulum. Nonetheless, differences in the epithecium also have value in the identification of several species (II–IV). The epithecium can consist of paraphyses (II), paraphyses and excipular hyphae (II–IV), or exclusively excipular hyphae (II, IV). Some epithecial types are illustrated in Figures 4 and 7. The epithecia consisting of both paraphyses and excipular hyphae are extremely resistant and very difficult to break in squash preparations. In such cases the asci and paraphyses typically detach from the hypothecium and the ascus tips stay tightly inside the dense and structurally complex epithecial layer.

**Paraphyses.** The paraphyses of mycocalicioid fungi are hyaline, septate with varying septal intervals, long and slender, and usually 1.0–2.0  $\mu\text{m}$  wide. While distinct pigments or colors were not observed in the paraphyses of any species, some other features, like the presence or absence of surface incrustations (III, IV), and the way in which paraphyses contribute to the epithecium are species specific. Also the branching of paraphyses was found to be a phylogenetically informative character (II–IV). Some characteristic types of paraphyses are illustrated in Figure 4.

**Asci.** The asci of all mycocalicioid species have been thought to arise from croziers. However, three of the newly described species from angiosperm exudates, *C. khayensis*, *C. pallida* and *C. resinophila* produce asci without croziers (I, IV). This is thus a new character state for mycocalicioid fungi. Many mycocalicioid species live in periodically dry habitats. This may explain why there seems to be very little variation in their asci between fresh

material and dry herbarium specimens. This is in contrast to some other groups of ascomycetes in which the living asci have a completely different form and often also a different ascospore orientation (Baral 1992).

**Ascus apex.** All the newly described species have a thickened ascus apex with a distinct canal at least at some point of the ascus development (I–IV), and they were thus described into the genus *Chaenothecopsis* (Schmidt 1970; Tibell 1999). It appears that the distinctive canal reported in most species of *Chaenothecopsis* is best seen in herbarium specimens. In fresh material the thickening of the ascus tip is often less pronounced and the characteristic canal appears only after a killing mounting medium (e. g. KOH or MLZ) has been added (Baral, pers. comm. in 2010). In any case, the type of ascus apex in dried material seems to be a species-specific character. Some examples of typical ascus apices are illustrated in Figure 5.

**Ascospores.** Ascospore type is definitely an important character (Fig. 1). Some common ascospore types are illustrated in Figure 3. Not only spore shape and size (II, IV), and ornamentation (I, II, IV), but also the Q-value (IV) was found to be useful for distinguishing between morphologically similar species. While variation of spore size is quite large within the Mycocaliciales, in many cases the measured spore lengths can be almost identical in two or more species (IV). In such cases, the Q-value (spore length/spore width measured from same individual spores) can differ between species, and help to distinguish between species (IV). Tibell (1982, 1984) first brought to attention differences in the appearance of the septum. In some species the septum is thin and translucent, and this character is also species specific (IV). In other cases the septum is thicker than the outer wall or thickened only at the junction of the septum and outer wall (III, IV).

**Crystals.** Many mycocalicioid species have embedded crystals in their ascocarps. They may only be found in certain parts of the ascocarp (e.g. only in the stipe or covering the paraphysis tips) or occur scattered throughout many parts of the ascocarp (III, IV). The crystals can be colored (usually yellow or red) or hyaline, and often have a distinct color reaction to some reagents. The presence and position of some crystals is species

specific, although their occurrence seems to vary somewhat depending on the maturity of the ascocarp, with very young ascocarps seeming to be devoid of the typical crystals. *Chaenothecopsis pallida* has an interesting, almost glass-like, amorphous material that covers the stipe or sometimes even the whole ascocarp, especially the young ascocarps (IV). In squash preparations the material breaks down like ice (Fig. 6I), and makes the hyphal structure below difficult to observe. The material dissolves in KOH and CR.

**Notes on chemical reactions and reagents.** Color reactions to potassium hydroxide (KOH) and nitric acid (N) have traditionally been used in the identification of mycocalicioid species. Well known reactions to KOH include the dramatic color reactions of several species with unidentified, crystalline pigments. So far at least the following reactions have been described (e.g. Schmidt 1970, Tibell 1999): red pigment turning green (e.g. *C. viridireagens* (Nádv.) A.F.W. Schmidt, *C. viridialba* (Kremp.) A.F.W. Schmidt), yellow/red pigment turning red (*C. consociata*, *C. rubescens*) and yellow turning aniline/violet red (*C. hospitans*, *C. pusiola*). KOH is also a bleaching reagent, and most of the color reactions reported for different species involve the removal of the pigments from the cell walls, sometimes leaking in the medium, and eventually fading. Thus, if a species (e.g. *C. oregana*) has a distinctive pigment in the cell walls (red in this case), the pigment usually leaks with the addition of KOH (II). Additionally, the ascocarp tissues of most mycocalicioid fungi swell in KOH, and the swelling is especially pronounced in species with *textura oblita* (IV). For this reason, spores and other structures should not be measured in KOH. A color reaction to nitric acid (N) is seen in some *Chaenothecopsis* species including *C. debilis* (Sm.) Tibell. However, none of the newly described species of this study (I–IV) showed any distinct reaction, at most the natural ascocarp colors were slightly intensified.

While iodine reactions can be highly species specific and are widely used for identifying and delimiting species in many fungal groups, they do not seem to have been systematically used in the characterization of mycocalicioid fungi. For example, the amorphous material surrounding the asci of *Chaenothecopsis montana* is euamyloid, and this characteristic feature can

only be seen by applying iodine reagent (II). Reactions to iodine are observed also in the stipe (I, III, IV), hymenium (II, III, IV), and the excipulum and epithecium (IV). Lugol's and Melzer's reagents, the two commonly used iodine solutions, yield different reactions and should not be confused with each other (Baral 1987). The reactions induced by Melzer's reagent (MLZ) can also be observed by adding the reagent to a squash preparation after Lugol's reagent (IKI) has first been applied, but not vice versa. Two types of reactions are present only in Lugol's reagent (hemiamyloid, i.e. color disappearing when MLZ is added). The first reaction, *persisting red*, may be present in hyphae of stipe, excipulum and epithecium. The red IKI reaction appears to be most common in the stipe, either in the whole stipe or just at the base (I, IV). If present in the excipulum, it usually is also present in stipe, and if present in the epithecium, it is also present in the excipulum and stipe. The observed color varies from faint grayish pink to strong violet red (IV). The second reaction, *disappearing blue*, is present only in the hymenium and often also in the hypotechium. In the specimens examined during this study (I–IV) this color reaction was never observed in the excipulum, stipe or the epithecium. The observed color is usually light blue, but can vary from faint bluish gray to intense ink blue. The speed of the reaction varies from very fast and transient to nearly persisting, and in some cases the reaction seems to persist almost indefinitely. This reaction is easily overlooked, but can be best observed by first mounting the specimen in water, and after the preparation has first partly dried (air has penetrated under the coverslip), a drop of freshly made 1% Lugol's reagent is added to the edge of the coverslip while simultaneously observing the specimen at 40 × magnification. The reaction occurs when the reagent penetrates the squashed ascocarp. Sometimes it only appears as a rapidly moving blue ring and may only last a few seconds or less (III). One should emphasize that the hemiamyloid reactions can usually only be observed with freshly made (maximum one day old) Lugol's reagent.

The euamyloid blue reactions can be induced with both Melzer's and Lugol's reagents. Thus it is important to test both reagents to know whether the blue in Lugol's reagent is hemiamyloid or euamyloid. In the specimens examined during this study (I–IV) the euamyloid reaction was always observed only in hymenium, and usually in all parts of it. The euamyloid reaction is quite persisting, and the observed color varies from light to

dark ink blue. A dextrinoid reaction in Melzer's reagent (Baral 1987) was not observed in any described species.

Finally, standard staining reagents, such as Crezyl Blue or Congo Red help to observe especially thin and hyaline structures, e.g. paraphyses, ascus tip characters and croziers. Structures that are difficult to observe in water (e.g. septal intervals), can be relatively reliably measured in these reagents, as the relevant structures do not markedly swell. Congo Red often dissolves glass-like surface layers, but does not induce swelling in hyphal structures, thus making the textura type more easily observable (IV). Some hyaline crystals and lightly colored amorphous materials of the epithecium are also easier to observe in this stain (III, IV).

## 5 CONCLUDING REMARKS

Fungi are notorious for their evolutionary flexibility, which has resulted in convergent evolution at all taxonomic levels and in almost all imaginable characters. The multiple origins of many ecological and morphological features have often obscured and hindered our understanding of phylogenetic relationships between taxa. Only recently, with the help of DNA-based molecular methods, have we really become to appreciate the true diversity of Fungi. In this thesis I have studied and described a number of new resinicolous *Chaenothecopsis* species, and while doing so, shed new light on the diversity and ecology of these peculiar ascomycetes.

In this thesis, I have confirmed that many lignicolous, lichenicolous and resinicolous mycocalicioid species are highly substrate specific. This specificity appears to be especially pronounced in the species that grow on angiosperm exudates. In light of my results it also seems highly likely that many new mycocalicioid species will be found in detailed studies of angiosperm exudates. Although definitive evidence is lacking, it remains an interesting possibility that the common ancestor of all extant mycocalicioids might have been a resinicolous fungus. The ecological requirements of fungi are still a relatively unexplored area, and a combined ecological approach should receive more emphasis in future taxonomic studies (Raxworthy et al. 200; Rissler & Apodaca 2007).

Different mixtures of chemical compounds can create strong selective pressures and highly directional evolutionary development (Giraud et al. 2006). The huge chemical variation seen in vascular plant exudates may give one explanation for the unrivaled diversity of resinicolous species found in Mycocaliciales. Within this group, there appear to be highly specific relationships between individual mycocalicialean species and their hosts. Current estimates place the Eurotiomycete divergence at 270–350 Ma (Early Carboniferous to Early Permian; Lücking et al. 2009), at a time when many conifer groups had already diversified. One can speculate that some species of Mycocaliciales may have begun to colonize conifers already at this early stage and have since co-evolved with them. The Sapindales, which includes the hosts of several resinicolous mycocalicioid species that live on angiosperm exudates, diverged around the Cretaceous-Palaeogene boundary (Appelhans et al. 2012). While the ability to degrade angiosperm exudates may be of more recent origin than that of conifer resins, this ecology also may be quite ancient. In our analysis, the East Asian *Chaenothecopsis pallida* was found to be the sister species of *C. quintralis* which was recently described from South America (Messuti et al. 2012). Similarly, the East Asian *C. resinophila* was the sister species of *C. khayensis* from West Africa. Thus, neither the two Chinese species nor the two species that live on Sapindalean hosts (*C. khayensis* and *C. pallida*) grouped together. This suggests that with further study, resinicolous mycocalicioids may prove to be interesting examples of long-distance dispersal and vicariance in fungal evolution.

One of our studies has also confirmed that mycocalicioid fungi were morphologically and ecologically well adapted to their resinous exudates already tens of millions of years ago. Their tendency to grow in close proximity to fresh resinous exudates and their perennial ascocarps have obviously enhanced their probability to preserve within amber, and hopefully new mycocalicioid fossils will be discovered in the future. I have added to the knowledge of the distribution and ecology of resinicolous mycocalicioids in Europe, and discussed their possible range during and after the last glacial maximum.

Finally, I have demonstrated that even during the molecular era of phylogenetics, subtle differences in morphological characters can still be

successfully used in the delimitation of many superficially similar fungi. I found several exciting new morphological and chemical characters that should now be analyzed from more mycocalicioid species, as it is possible that many of the presently “cryptic” mycocalicioid species can actually be accurately identified with a basic compound microscope and a short list of classical chemical reagents. This is an especially promising prospect with regards to the thousands of valuable herbarium specimens that are now kept in scientific collections, but which may never be studied with standard DNA-methods due to DNA degradation.

Concepts crucial for species delimitation will undoubtedly continue to be debated in modern systematics. Most systematists agree that species are real and important, even though they sometimes may be extremely difficult to conceptualize. They are the fundamental units in biogeography, ecology, and conservation. Thus, being able to effectively and reliably delimit species is one of the most crucial aspects of biology. While next-generation sequencing, barcoding, and other molecular initiatives will produce great amounts of new information about diversity at the molecular level, there is there is an increasing need for correctly identified, specimen-based sequences in reference databases like GenBank. In addition, the non-academic world has a continuous need to identify environmental samples without relying on DNA methods that are often expensive and impractical for large-scale survey work. In the times when we should be able to somehow “control” or even slow down the sixth mass extinction, we cannot overlook the importance of the work in biodiversity documentation done by students, non-specialists and nature enthusiasts. Birdwatching is one excellent example of importance of a non-scientific community in generating major scientific results. The same could potentially happen with many groups of fungi – if precise species descriptions, and good floras and keys are widely available.

In my mind, the true future challenge in the scientific world is not in outer space or the search for eternal life. The challenge is to balance limited available financial resources between applied studies, modeling, experimental studies and the slow processing, observing studies that are needed for conquering the unknown. As my thesis and many similar studies show, there are still many areas and fungal groups unexplored – even



literally in our own backyards. In well-known areas of science, one can ask very specific questions and formulate detailed hypotheses, but there has to be room also for simple questions, asking only "What are these, what do they do, and why are they here?". Without such questions, we will really be "stuck", only reorganizing what we already know.

## 6 ACKNOWLEDGEMENTS

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Someone once said that you have to be a bit freakish to be a biologist. I then replied that well, you also have to be twisted to become a mycologist.



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## SUPPLEMENTARY TABLE 1.

### CHEMICAL REAGENTS USED IN THE STUDY

#### **Lugol's reagent (IKI)**

10% stock solution:

- Potassium iodide 2 g
- Iodine 1 g
- Distilled H<sub>2</sub>O 90 ml

1% solution for examining specimens:

- stock solution 1 ml
- Distilled H<sub>2</sub>O 9 ml

#### **Melzer's reagent (MLZ)**

- Potassium iodide 1.5 g
- Iodine 0.5 g
- Chloral hydrate 22 g
- Distilled H<sub>2</sub>O 20 ml

#### **Congo Red (CR)**

congo red dye

25% ammonium solution

used as saturated solution

#### **Potassium hydroxide (KOH)**

used as 10% w/v aqueous solution

#### **Nitric acid (N)**

used as 10% v/v aqueous solution

## SUPPLEMENTARY TABLE 2.

### Specimen and voucher information for Figure 1.

AT = Alexander Titov, HR = Heini Rämä, HT = Hanna Tuovila, JP = Juha Pykälä, JR = Jouko Rikkinen, K = Kalb, L = Oslo herbarium number, LT = Leif Tibell, MP = Marja Pennanen, PL = Per Larsson. Sequences generated in Chapters I, III and IV are in bold.

Taxon	Country	Substrate	Collection number	GenBank ITS/LSU	References
<i>Chaenothecopsis consociata</i>	Sweden New	<i>Chaenotheca</i> <i>Podocarpus</i>	LT 22472 (UPS)	AY795851/DQ008999	Tibell & Vinuesa 2005
<i>Chaenothecopsis debilis</i>	Zealand	<i>dacrydioides</i>	LT 16643 (UPS)	AY795852/AY795991	Tibell & Vinuesa 2005
<i>Chaenothecopsis diabolica</i>	USA, OR	Conifer resin	HT 06-035 (TUR)	<b>JX119109/JX119114</b>	Chapter III
<i>Chaenothecopsis dolichocephala</i>	Russia	Conifer resin	LT 19281 (UPS)	AY795854/AY795993	Tibell & Vinuesa 2005
<i>Chaenothecopsis epithallina</i>	Sweden	<i>Chaenotheca</i>	LT 22705 (UPS)	AY795855/-	Tibell & Vinuesa 2005
<i>Chaenothecopsis fennica</i>	Sweden	<i>Picea abies</i>	LT 16024 (UPS)	AY795857/AY795995	Tibell & Vinuesa 2005
<i>Chaenothecopsis golubkovae</i>	China New	Conifer resin	AT 6707 (H)	AY795859/AY795996	Tibell & Vinuesa 2005
<i>Chaenothecopsis haematopus</i>	Zealand	<i>Nothofagus</i>	LT 16625 (UPS)	AY795861/AY795997	Tibell & Vinuesa 2005
<i>Chaenothecopsis hospitans</i>	Finland	Lichen	HT 09-086 (H)	To be submitted	This study
<i>Chaenothecopsis khayensis</i>	Ghana	<i>Khaya</i> resin	JR 04G058 (H)	<b>JX122785/HQ17289</b> 5	Chapter III, Chapter I
<i>Chaenothecopsis montana 1</i>	Finland	Picea resin	HT 07-086 (H)	<b>JX119105/JX119114</b>	Chapter III
<i>Chaenothecopsis montana 2</i>	Sweden	<i>Picea</i> resin	PL 2009 (pers.)	To be submitted	This study
<i>Chaenothecopsis nana</i>	Sweden	<i>Chaenotheca</i>	LT 22473 (UPS)	AY795862/-	Tibell & Vinuesa 2005
<i>Chaenothecopsis nigripunctata</i>	USA, OR	<i>Abies</i> resin	HT 06-013 (TUR)	<b>JX119103/JX119112</b>	Chapter III
<i>Chaenothecopsis pallida</i>	China	<i>Ailanthus</i> resin	JR 10652 (H)	<b>JX122779/JX122781</b>	Chapter III
<i>Chaenothecopsis pusiola</i>	Finland	<i>Pinus sylvestris</i>	HT 09-047 (H)	<b>JX119106/JX119115</b>	Chapter III Messuti, Vidal-Russell,

(continued on the next page)

(Supplementary Table 2, continued)

<b>Taxon</b>	<b>Country</b>	<b>Substrate</b>	<b>Collection number</b>	<b>GenBank ITS/LSU</b>	<b>References</b>
<i>Chaenothecopsis quintralis</i>	Argentina	<i>Tristerix viscin</i>	BCRU 05233	-/JQ267741	Amico & Lorenzo 2012
<i>Chaenothecopsis resinicola</i>	Russia	<i>Pinus resin</i>	LT 19234 (UPS)	AY795867/-	Tibell & Vinuesa 2005
<i>Chaenothecopsis resinophila</i>	China	<i>Kalopanax resin</i>	JR 000424 (H)	<b>JX122780/JX122782</b>	Chapter III
<i>Chaenothecopsis savonica</i>	Sweden	Lignum	LT 15876 (UPS)	AY795868/AY796000	Tibell & Vinuesa 2005
<i>Chaenothecopsis sitchensis</i>	USA, OR	<i>Tsuga resin</i>	HT 06-033 (TUR)	<b>JX119102/JX119111</b>	Chapter III
<i>Chaenothecopsis tsugae</i>	USA, OR	<i>Tsuga heterophylla</i>	JR 07005B (H)	<b>JX119104/JX119113</b>	Chapter III
<i>Chaenothecopsis vainioana</i>	Finland	<i>Alnus glutinosa</i>	HT 09-066 (H)	<b>JX119107/JX119116</b>	Chapter III
<i>Chaenothecopsis viridireagens</i>	Finland	Conifer lignum	HT 09-068 (H)	<b>JX119108/JX119117</b>	Chapter III
<i>Chaenothecopsis</i> sp. 1	Sweden	<i>Betula</i> sp.	HT 09-155 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 2	Finland	<i>Betula</i> sp.	HT 08-004 (TUR)	<b>KC590480/KC590485</b>	Chapter IV
<i>Chaenothecopsis</i> sp. 3	France	<i>Olea europea</i>	HT 09-134 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 4	Finland	<i>Betula</i> sp.	HT 09-010(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 5	Finland	<i>Juniperus communis</i>	MP1000317	To be submitted	This study
<i>Chaenothecopsis</i> sp. 6	Finland	<i>Betula</i> sp.	HT 09-041 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 7	Norway	<i>Alnus incana</i>	HT 09-201 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 8	Norway	<i>Fagus sylvatica</i>	HT 09-260 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 9	Finland	<i>Betula</i> sp.	HR 762 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 10	Sweden New	<i>Betula pubescens</i>	HT 10-025 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 11	Caledonia	<i>Agathis resin</i>	JR (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 12	Finland	<i>Pinus sylvestris</i>	HT 09-018(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 13	Finland	<i>Pinus sylvestris</i>	HT 09-234(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 14	Germany	<i>Quercus robur</i>	HT 10-011 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 15	Finland	<i>Quercus robur</i>	HT 09-097 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 16	Finland	<i>Salix caprea</i>	HR 893 (TUR)	To be submitted	This study

<b>Taxon</b>	<b>Country</b>	<b>Substrate</b>	<b>Collection number</b>	<b>GenBank ITS/LSU</b>	<b>References</b>
<i>Chaenothecopsis</i> sp. 17	Norway	Deciduous lignum	L155006 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 18	Finland	<i>Betula</i> sp.	HR 2990 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 19	Austria	Conifer lignum	HT 09-121(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 20	Finland	<i>Salix caprea</i>	HR 1655 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 21	Finland	<i>Quercus robur</i>	HR 1264 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 22	Finland	Conifer resin	HT 09-227(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 23	Finland	<i>Picea abies</i>	HR 2911 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 24	Finland	Lichen	HR 1208 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 25	Finland	<i>Quercus robur</i>	HT 09-093(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 26	Germany	<i>Quercus robur</i>	HT 10-003 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 27	Finland	<i>Picea abies</i>	JP 24105(H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 28	Finland	Conifer lignum	HT 09-074 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 29	Finland	<i>Betula</i> sp.	HT 08-009 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 30	Germany	<i>Quercus robur</i>	HT 10-010 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 31	Norway	<i>Salix caprea</i>	HT 09-190 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 32	Germany	<i>Fagus sylvatica</i>	HT 09-146 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 33	Finland	<i>Acer platanoides</i>	HT 09-052 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 34	Finland	<i>Salix caprea</i>	HT 09-002 (TUR)	To be submitted	Chapter III
<i>Chaenothecopsis</i> sp. 35	Germany	<i>Fagus sylvatica</i>	HT 10-008 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 36	Finland	Cane	HT 09-007 (TUR)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 37	Finland	<i>Acer platanoides</i>	HT 09-103 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 38	Finland	<i>Quercus robur</i>	HT 09-245 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 39	Finland	<i>Quercus robur</i>	JP 34399 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 40	France	<i>Olea europaea</i>	HT 09-129 (H)	To be submitted	This study
<i>Chaenothecopsis</i> sp. 41	Finland	<i>Betula</i> sp.	HR 760 (TUR)	To be submitted	This study

(continued on the next page)

(Supplementary Table 2, continued)

<b>Taxon</b>	<b>Country</b>	<b>Substrate</b>	<b>Collection number</b>	<b>GenBank ITS/LSU</b>	<b>References</b>
	New Zealand	<i>Nothofagus</i>	LT 19038 (UPS)	AF223966/AY796001	Vinuesa et al. 2001
<i>Mycocallicium albonigrum</i>	USA, CA	<i>Sequoiadendron</i>	JR 92055 (H)	- /AY796002	Tibell & Vinuesa 2005
<i>Mycocallicium sequoiae</i>	USA, OR	Conifer lignum	HT 06-018 (TUR)	To be submitted	Tibell & Vinuesa 2005
<i>Mycocallicium subtile</i> 1	Germany	<i>Picea abies</i>	HT 09-147 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 2	Finland	<i>Pinus sylvestris</i>	HT 08-018 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 3	Finland	<i>Alnus incana</i>	JP 34351 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 4	Finland	<i>Pinus sylvestris</i>	HT 09-145 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 5	Switzerland	<i>Pinus sylvestris</i>	HT 09-125 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 6	Austria	<i>Picea abies</i>	HT 09-158 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 7	Sweden	<i>Picea abies</i>	HT 09-251 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 8	Norway	<i>Picea abies</i>	HT 09-197 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 9	Norway	<i>Juniperus communis</i>	HT 09-153 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 10	Sweden	<i>Pinus sylvestris</i>	HT 07-068 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 11	Finland	<i>Pinus sylvestris</i>	LT 21020 (UPS)	AF225445/AY796003	Vinuesa et al. 2001
<i>Mycocallicium subtile</i> 12	Sweden	Lignum	HT 09-081 (H)	To be submitted	Tibell & Vinuesa 2005
<i>Mycocallicium subtile</i> 13	Finland	<i>Picea abies</i>	HT 07-074 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 14	Finland	<i>Pinus sylvestris</i>	HT 09-163 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 15	Finland	<i>Picea abies</i>	HT 07-092 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 16	Finland	<i>Pinus sylvestris</i>	HT 07-067 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 17	Finland	<i>Pinus sylvestris</i>	HT 09-060 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 18	Finland	<i>Alnus glutinosa</i>	HT 09-031 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 19	Finland	<i>Salix</i> sp.	HR 1499a (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 20	Finland	<i>Alnus incana</i>	HR 1630 (TUR)	To be submitted	This study
<i>Mycocallicium subtile</i> 21	Finland	<i>Salix caprea</i>	HT 09-099 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 22	Finland	<i>Alnus glutinosa</i>	HT 09-100 (H)	To be submitted	This study
<i>Mycocallicium subtile</i> 23	Finland	<i>Quercus robur</i>			This study

Taxon	Country	Substrate	Collection number	GenBank ITS/LSU	References
<i>Mycocalicium subtile</i> 24	Norway	<i>Quercus robur</i>	HT 09-253 (TUR)	To be submitted	This study
<i>Mycocalicium subtile</i> 25	Finland	<i>Alnus glutinosa</i>	JP 34374 (H)	To be submitted	This study
<i>Mycocalicium subtile</i> 26	USA, OR	<i>Pinus albicaulis</i>	JR 07017 (H)	To be submitted	This study
<i>Mycocalicium subtile</i> 27	Norway	<i>Ulmus</i> sp.	HT 09-185 (TUR)	To be submitted	This study
<i>Mycocalicium subtile</i> 28	Finland	<i>Quercus robur</i>	HT 09-006 (TUR)	To be submitted	This study
<i>Mycocalicium subtile</i> 29	Finland	<i>Salix caprea</i>	HT 09-054 (H)	To be submitted	This study
<i>Mycocalicium subtile</i> 30	Finland	<i>Populus tremula</i>	HT 09-051 (H)	To be submitted	This study
<i>Mycocalicium subtile</i> 31	Finland	<i>Sorbus aucuparia</i>	HT 08-007 (TUR)	To be submitted	This study
<i>Mycocalicium subtile</i> 32	Finland	<i>Betula pendula</i>	HT 09-167 (H)	To be submitted	This study
<i>Mycocalicium subtile</i> 33	Finland	<i>Betula</i> sp.	HT 09-095 (TUR)	To be submitted	This study
<i>Mycocalicium subtile</i> 34	Finland	<i>Betula</i> sp.	HT 09-116 (H)	To be submitted	This study
<i>Mycocalicium</i> sp. 1	Argentina	<i>Nothofagus</i> sp.	LT 17604 (UPS)	AF243133/- <b>KC590482/KC59048</b>	Vinuesa et al. 2001
<i>Mycocalicium</i> sp. 2	France	<i>Olea europea</i>	HT 09-131 (TUR)	<b>7</b>	Chapter IV
<i>Phaeocalicium interruptum</i>	Sweden	<i>Salix myrsinifolia</i>	LT 23044 (UPS)	AY795873/-	Tibell & Vinuesa 2005
<i>Phaeocalicium polyporaenum</i>	USA, MA	Polypore		AY789363/AY789362	Wang et al. 2005
<i>Phaeocalicium populneum</i> 1	Russia	<i>Populus</i> sp.	LT 19286 (UPS)	AY795874/AY796009	Tibell & Vinuesa 2005
<i>Phaeocalicium populneum</i> 2	France	<i>Populus nigra</i>	HT 09-135 (TUR)	To be submitted	This study
<i>Phaeocalicium praecedens</i>	Finland	<i>Populus tremula</i>	HT 09-240 (H)	<b>KC590481/KC59048</b>	
<i>Phaeocalicium</i> sp. 1	USA, OR	<i>Populus latifolia</i>	JR 07016 (H)	To be submitted	Chapter IV
<i>Phaeocalicium</i> sp. 2	Sweden	<i>Betula pubescens</i>	HT 10-017 (TUR)	<b>KC590483/KC59048</b>	This study
<i>Sphinctrina leucopoda</i>	Australia	Lichen	K33829 (UPS)	<b>4</b> AY795875/AY796006	Chapter IV
<i>Sphinctrina turbinata</i>	Sweden	Lichen	LT23093 (UPS)	AY795877/DQ009001	Tibell & Vinuesa 2005
<i>Stenocybe pullatula</i> 1	Finland	<i>Alnus incana</i>	HT 09-071 (TUR)	To be submitted	Tibell & Vinuesa 2005
<i>Stenocybe pullatula</i> 2	Sweden	<i>Alnus incana</i>	LT 17117 (UPS)	AY795878/AY796008	This study
					Tibell & Vinuesa 2005



## SUPPLEMENTARY TABLE 3.

### Specimen and voucher information used for drawings in Figures 2–6.

HT = Hanna Tuovila, JR = Jouko Rikkinen, AT = Alexander Titov.

Fig.	Species	Voucher	Reference
2A	<i>Chaenothecopsis</i> sp.	HT 10-037 (TUR)	
2B	<i>Mycocalicium subtile</i> coll.	HT 08-004 (TUR)	
2C	<i>Chaenothecopsis</i> sp.	HT 09-008 (TUR)	
2D	<i>Mycocalicium subtile</i> coll.	HT 09-178 (TUR)	
2E	<i>Mycocalicium calicioides</i>	Thulin 2623 (UPS)	
2F	<i>Chaenothecopsis</i> sp.	Wetmore 71837 (UPS)	
2G	<i>Chaenothecopsis tasmanica</i>	LT 17877 (UPS)	
2H	<i>Chaenothecopsis vainioana</i>	HT09-066 (H)	
2I	<i>Chaenothecopsis montana</i>	HT 07-086 (H)	Chapter II
2J	<i>Chaenothecopsis pallida</i>	JR 001390 (H)	Chapter IV
2K	<i>Chaenothecopsis proliferatus</i>	JR 990312 (H)	Chapter III
2L	<i>Chaenothecopsis khayensis</i>	JR 04G058 (H)	Chapter I
2M	<i>Phaeocalicium cf. compressulum</i>	HT 10-017 (TUR)	
2N	<i>Chaenothecopsis hunanensis</i>	JR 990059 (H)	Chapter IV
2O	<i>Chaenothecopsis rubina</i>	LT 8293 (UPS)	
2P	<i>Chaenothecopsis consociata</i>	HT 09-237 (TUR)	
2Q	<i>Chaenothecopsis proliferatus</i>	JR 990312 (H)	Chapter III
2R	<i>Chaenothecopsis hunanensis</i>	JR 990059 (H)	Chapter IV
2S	<i>Chaenothecopsis diabolica</i>	JR 98418 (H)	Chapter II
2T	<i>Chaenothecopsis resinophila</i>	JR 00424 (H)	Chapter IV
2U	<i>Stenocybe pullatula</i>	HT 09-071 (TUR)	
2V	<i>Chaenothecopsis</i> sp.	HT 08-004 (TUR)	
3A	<i>Chaenothecopsis resinophila</i>	JR 00424 (H)	Chapter IV
3B	<i>Chaenothecopsis</i> sp.	Muhr 11228 (UPS)	
3C	<i>Mycocalicium subtile</i> coll.	HT 09-105 (TUR)	
3D	<i>Chaenothecopsis montana</i>	HT 07-086 (H)	Chapter II
3E	<i>Chaenothecopsis kalbi</i>	Moberg 8895 (UPS)	
3F	<i>Chaenothecopsis diabolica</i>	JR 98418 (H)	Chapter II
3G	<i>Mycocalicium subtile</i> coll.	HT 09-104 (TUR)	
3H	<i>Chaenothecopsis proliferatus</i>	JR 990312 (H)	Chapter III
3I	<i>Chaenothecopsis</i> sp.	AT 6707 (H)	
3J	<i>Chaenothecopsis hunanensis</i>	JR 990059 (H)	Chapter IV
4A	<i>Chaenothecopsis oregana</i>	Gröner 2783 (Gröner)	Chapter II
4B	<i>Chaenothecopsis montana</i>	JR 98008 (H)	Chapter II
4C	<i>Chaenothecopsis resinophila</i>	JR 00424 (H)	Chapter IV
4D	<i>Chaenothecopsis diabolica</i>	JR 9363 (H)	Chapter II
4E	<i>Chaenothecopsis proliferatus</i>	JR 990346 (H)	Chapter III
4F	<i>Chaenothecopsis resinophila</i>	JR 00424 (H)	Chapter IV
4G	<i>Chaenothecopsis montana</i>	HT 07-086 (H)	Chapter II
4H	<i>Chaenothecopsis proliferatus</i>	JR 000595 (H)	Chapter III
4I	<i>Chaenothecopsis hunanensis</i>	JR 990059, 990055 (H)	Chapter IV

<b>Fig.</b>	<b>Species</b>	<b>Voucher</b>	<b>Reference</b>
5A	<i>Mycocalicium subtile</i> coll.	HT 09-104 (H)	
5B	<i>Chaenothecopsis montana</i>	HT 07-086 (H)	Chapter II
5C	<i>Chaenothecopsis nigripunctata</i>	JR 07001 (H)	
5D	<i>Chaenothecopsis</i> sp.	Wetmore 71837 (UPS)	
5E	<i>Chaenothecopsis proliferatus</i>	JR 990061, JR 000595	Chapter III
5F	<i>Chaenothecopsis pallida</i>	JR 001390 (H)	Chapter IV
6A	<i>Chaenothecopsis hunanensis</i>	JR 990167 (H)	Chapter IV
6B	<i>Chaenothecopsis proliferatus</i>	JR 990048 (H)	Chapter III
6C	<i>Chaenothecopsis resinophila</i>	JR 00424 (H)	Chapter IV
6D	<i>Mycocalicium subtile</i> coll.	HT 09-105 (TUR)	
6E	<i>Mycocalicium subtile</i> coll.	HT 09-105 (TUR)	
6F	<i>Chaenothecopsis pallida</i>	JR 001390 (H)	Chapter IV
6G	<i>Chaenothecopsis</i> sp.	Wetmore 71837 (UPS)	
6H	<i>Chaenothecopsis perforata</i>	JR 01538 (H)	Chapter IV
6I	<i>Mycocalicium subtile</i> coll.	HT 09-104 (TUR)	
6J	<i>Mycocalicium subtile</i> coll.	HT 09-104 (TUR)	
6K	<i>Chaenothecopsis golubkovae</i>	AT 303 (H)	
6L	<i>Chaenothecopsis diabolica</i>	JR 98418 (H)	Chapter II
6M	<i>Chaenothecopsis diabolica</i>	JR 98418 (H)	Chapter II
6N	<i>Chaenothecopsis montana</i>	HT 07-086 (H)	Chapter II
7A	<i>Chaenothecopsis montana</i>	HT 07-086 (H)	Chapter II
7B	<i>Chaenothecopsis</i> sp.	Wetmore 71837 (UPS)	
7C	<i>Chaenothecopsis diabolica</i>	JR 98418 (H)	Chapter II