Importance of secondary metabolites in the Xylariaceae as parameters for assessment of their taxonomy, phylogeny, and functional biodiversity

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This paper constitutes a synopsis of our polythetic studies of the Xylariaceae, which was originally compiled in the course of a Habilitation thesis. Based on several thousands of specimens and several hundreds of cultures, morphological studies of the teleomorphs and anamorphs of these fungi were combined with chemotaxonomic studies based on HPLC-DAD/MS profiling, as well as PCR fingerprinting and molecular phylogenetic analyses. Numerous novel pigments and other natural products, many of which were shown to have biological activities were also isolated and identified, and their production in the course of the life cycle of their producer organisms was followed by HPLC profiling and biological assays. Numerous new species and even new genera were recognised in the course of this work. Finally, secondary metabolite production in cultures of Xylariaceae was correlated with molecular data and the production of certain chemotaxonomic marker compounds was found to be strongly correlated with a phylogeny based on ITS nrDNA, demonstrating that secondary metabolite profiles are not only important species-specific characters but even have phylogenetic significance. The work on Xylariaceae is proposed as a model how interdisciplinary, international collaborations can help to increase our understanding of the phylogenetic and evolutionary relationships, as well as the biology of fungal organisms. Similar work on other groups of the Ascomycota and Basidiomycota would certainly be rewarding.

Key words – chemosystematics – Xylariales – extrolites – metabolomics – bioprospecting

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Introduction

This study originates from a pilot project at Bayer AG, when I had been responsible to build a culture collection of secondary metabolite producing fungi for pharmaceutical and agrochemical screening, which meanwhile comprises over 10.000 strains. The Xylariaceae were taken as model organisms to evaluate correlations between the diversity of morphotype-based taxonomy, secondary metabolite production and PCR-based molecular data. This approach, relating to the need for implementation of rigorous pre-selection procedures for samples to be included in High-Throughput Screening libraries, was outlined elsewhere in detail (Stadler & Hellwig 2004). Briefly, we aimed to find predictive parameters that could be effectively used to characterise the in-house fungal culture collection and screening libraries. Chemotaxonomic information was combined with classical taxonomy and PCR-based methods, to improve the efficacy of the screening process. The Xylariaceae were chosen as model organisms for the following reasons.

This family of Ascomycota represents one of the predominant groups of fungal

endophytes, which became of great interest as sources for novel lead compounds, but are particularly difficult to classify by morphological methods. As will be outlined below, Xylariaceae are "model endophytes".

Monographs of *Daldinia* (Ju et al. 1997) and *Hypoxylon* (Ju & Rogers 1996), relying in part on chemotaxonomic characters had just been published. Stromatal pigments were regarded as taxonomically significant, but their chemical structures remained widely unknown, leaving options for discovery of new bioactive molecules. On the other hand, Whalley & Edwards (1995) had demonstrated the chemotaxonomic value of metabolites from cultures.

The ubiquitous wood-decomposing Xylariaceae are also of interest in biodegradation of xenobiotics. Together with Heinz-Georg Wetzstein and Hans-Volker Tichy, we studied them to evaluate their capabilities for biodegradability of quinolone antibiotics, along with basidiomycetes (cf. Wetzstein et al. 1996). These studies included the characterisation of the fungi based on molecular methods. The Xylariaceae were at first studied by PCR fingerprinting; but DNA sequences were also generated later on.

Sophisticated analytical methods such as High performance liquid chromatography, hyphenated with diode array detection (HPLC-DAD) and mass spectrometry (HPLC-MS) had just become available in our laboratory in the search for novel drug candidates (Stadler et al. 1998). HPLC-DAD and direct inlet mass spectrometry were already used in the chemotaxonomy of *Penicillium* (Smedsgaard & Frisvad 1996), and HPLC-DAD was employed by Lumbsch (1998) for chemotaxonomy of lichenised ascomycetes. Inspired by these studies, we extended such techniques to the Xylariaceae (albeit HPLC-MS was to my knowledge used in fungal chemotaxonomy by us for the first time).

In retrospective, the project, baptised "*Molecular chemotaxonomy of the Xylariaceae*" (Stadler et al. 2001d), proved extremely helpful to increase the diversity of the screening libraries as the Xylariaceae are not the only group of filamentous fungi in which chemotaxonomic information is well-correlated with molecular and morphological data. The project soon developed its own dynamics, fuelled by the inestimable help of numerous other mycologists and natural product chemists from around the world. This thesis is intended to present the results in a historical and methodical context and give an outlook on what can be done in the future.

The model character of this study for interdisciplinary mycology and an integrative approach to biodiversity assessments in fungi will also be briefly addressed.

The Xylariaceae represent one of the largest and most important families of the Ascomycota and the Fungi. The exact number of currently accepted taxa varies between authors. For instance, sixty genera that potentially belong to the Xylariaceae were listed by Lumbsch & Huhndorf (2007). The Dictionary of the Fungi (Kirk et al. 2008) listed 85 accepted genera (including 71 synonyms) and 1343 accepted species. The vast majority of those are associated with plants, but some genera and species are known from herbivore dung or termite nests. As discussed below, further associations of Xylariaceae with other insects have recently been discovered.

The hypothesis by Hawksworth (1991), who postulated 1.5 million of fungal species to be extant (of which only 5% are hitherto known to Science), is largely based on an assumed ratio of five plant-associated fungal species per plant species, which can be assumed to be realistic from our current knowledge on the biodiversity in the well-investigated geographic areas of the world. Accordingly, as many as 10.000 additional Xylariaceae species may remain to be described. As outlined by Rogers (2000), their morphological diversity is particularly high in the tropics, and indeed, several genera have an exclusively tropical distribution. On the other hand, most taxonomic and chorological studies were carried out in the temperate regions of the Northern hemisphere, and biogeographic information on the global distribution of the Xylariaceae is still widely amiss.

Higher classification and teleomorphic stages of the Xylariaceae

The Xylariaceae belong to tribe Ascomycota, class Sordariomycetes, subclass Xylariomycetidae, Xylariales. In previous classi-

fications of ascomycetes they were included in class Pyrenomycetes, as their ascocarps are perithecia. In the Xylariaceae, the perithecia are frequently embedded in a conspicuous stroma. Morphological and anatomical features of the stroma have been traditionally used in generic classification of the Xylariaceae. The typical xylariaceous octosporous asci are tubular and stipitate, featuring an amyloid apical apparatus, which may be reduced in some genera and species. The spores are actively released from the perithecial ostioles in those species that have an apical apparatus. The ascospores (Fig. 2A) are arranged uniseriately in the asci, one-celled, mostly ellipsoid to ellipsoid-inequilateral and dark brown, and have a germ slit. They are designed to survive in the environment for a long time after discharge, and the germ slit allows a rapid germination under favourable conditions. The ecophysiological features of the Xylariaceae indicate a xerophilous lifestyle of their ancestors (Rogers 2000).

Infrageneric affinities and anamorphic stages

While stromata of many plant-associated Xylariaceae (Fig. 1) are frequently and ubiquitously encountered, others form stromata and sexual stages on the hosts only sparsely and occasionally. Their life cycle is apparently dominated by asexual propagation, and their mycelia remain dormant in the host plants for a long time. As in other groups of Ascomycota (e.g., in *Aspergillus* P. Micheli ex Haller and *Penicillium* Link), many Xylariaceae may have abandoned the production of the sexual stages altogether. In contrast to the aforementioned "moulds", the Xylariaceae, however, are not often encountered in soil (see Ecological aspects). Their asexual propagation is generally accomplished via the production of conidia. These propagules are borne on conidiophores of rather characteristic structures, which may arise either from the stromata or from the mycelia on the substrate. Many species will readily produce anamorphic structures in laboratory culture. Their production is not as dependent on the season as that of the sexual stages and may occur several times throughout the vegetation period under favourable conditions. They are relatively short-lived in contrast to the ascospores and do not appear to be ideally designed for anemochoric dispersal.

The anamorphic structures are most often referred to the genus *Nodulisporium* Preuss, which was already linked by Tulasne & Tulasne (1863) to xylariaceous teleomorphs. Nevertheless, it took another century until the significance of anamorphic morphology was fully appreciated in their classification and taxonomy. Greenhalgh & Chesters (1968), Martin (1969a, b, and further papers of his series), and Jong & Rogers (1972) gave extensive reference of anamorphic traits in several species. Petrini & Müller (1986) studied numerous European Xylariaceae for anamorphic and teleomorphic features. Today, the microscopic characters of the conidiophores and the macromorphology of mycelial cultures are regarded highly important at various taxonomic levels. New species are now often recognised, based on new combinations of teleomorphic and anamorphic characters, applying a holomorphic morphological species concept.

Jack D. Rogers, Y.-M. Ju and their coworkers provided numerous important monographs based on holomorphic morphology and published several papers on cultural and anamorphic characteristics of the known species. The term "*Nodulisporium*-like anamorph" was restricted by Ju & Rogers (1996) to the asexual stages of *Hypoxylon* Bull., and related genera. This definition now also embraces morphotypes such as *Xylocladium* P. Syd. ex Lindau, a characteristic anamorph of *Camillea* Fr., as well as the *Virgariella*-like, *Sporothrix*-like and *Periconiella*-like conidial stages that are found instead of or besides the common *Nodulisporium* type (Fig. 2B) in several species of *Hypoxylon* and its immediate allies (e.g., *Daldinia* Ces. & De Not.). These anamorph types mainly differ in the complexity of their conidiophores, whereas the morphology of conidiogenous cells appears rather uniform. The conidiogenesis occurs from apical regions of the terminal (or rarely, intercalary) swollen, denticulate, cylindrical to clavate conidiogenous cells. Most species produce conidia from sympodially proliferating conidiogenous cells in a holoblastic manner, albeit exceptions are known. For instance, some *Daldinia* spp. associated with Betulaceae, including *D. petriniae* Y.M. Ju, J.D. Rogers & San Martín and *D. decipiens* Wollw. & M. Stadler, predominantly

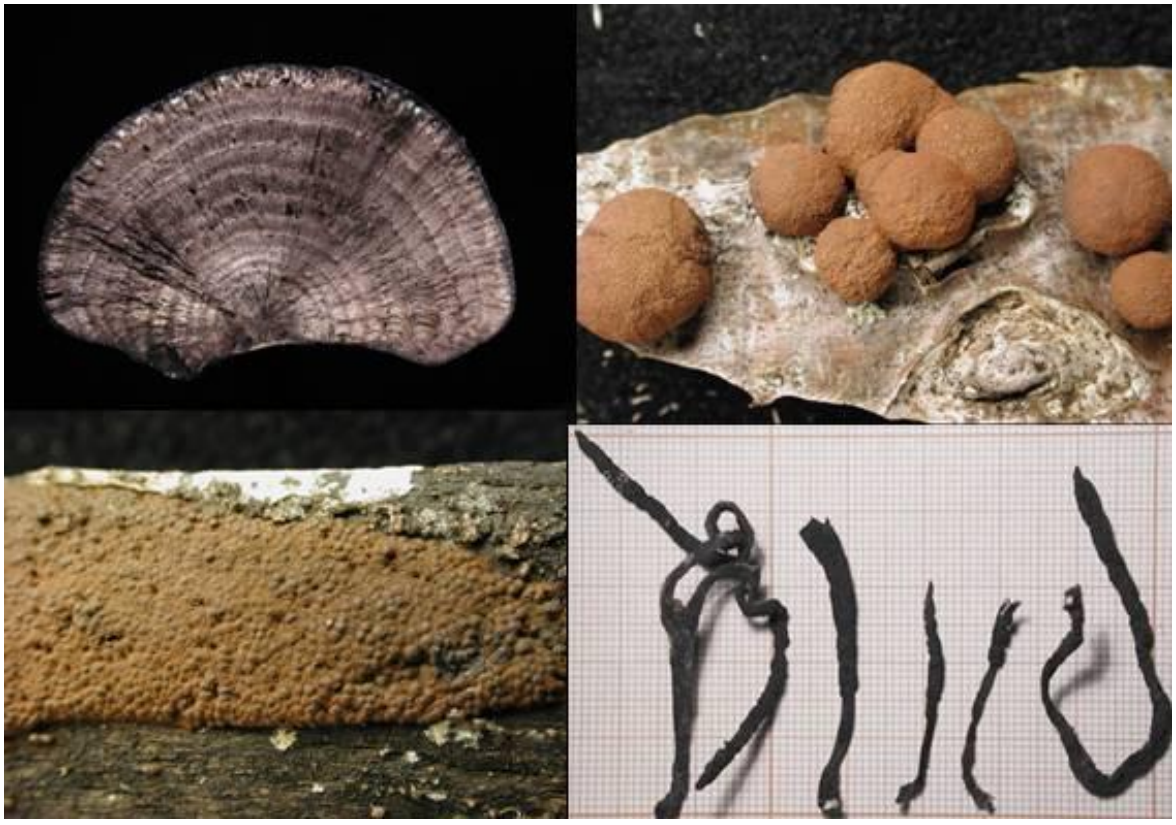


Fig. 1 – Stromata of Xylariaceae. **A** *Daldinia concentrica.* **B** *Hypoxylon fragiforme.* **C** *Hypoxylon rubiginosum.* **D** *Xylaria hypoxylon*. Images by Jacques Fournier

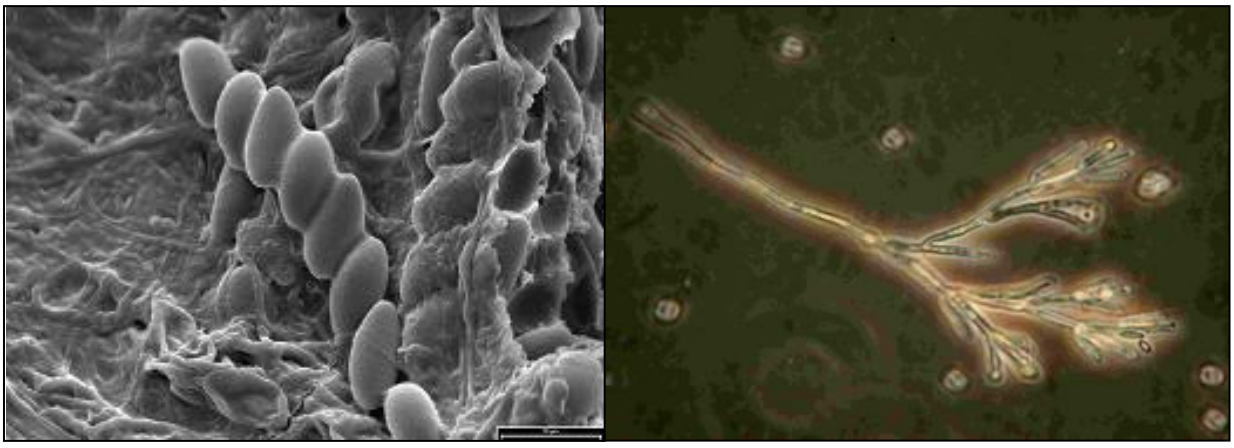


Fig. 2 – Characteristic microscopic features of the Xylariaceae, as exemplified by *Daldinia eschscholtzii* (Ehrenb.) Rehm. **A** Ascospores (SEM, 5000×). **B** *Nodulisporium* conidiophore (phase contrast micrograph, 400×).

show an enteroblastic conidiogenesis and produce conidia from percurrently proliferating conidiogenous cells (cf. Petrini & Müller 1986, sub *D. occidentalis* Child; Ju et al. 1997, sub *D. petriniae*; Stadler et al. 2001c).

The second, large group within the family is characterised by having "*Geniculosporium*-like" anamorphic stages *sensu* Ju & Rogers (1996), referring to *Geniculosporium* Greenh. & Chesters, the asexual stage first described in *Nemania serpens* (Chesters & Greenhalgh 1964, as *Hypoxylon serpens*). Aside from the genus *Nemania* S.F. Gray, this group comprises *Entoleuca* Sydow, *Euepixylon* Füisting, *Kretzschmaria* Fr., *Rosellinia* de Not., *Xylaria* Hill ex Schrank, and many other genera of the Xylariaceae. In contrast to the *Nodulisporium* type, their conidiogenous cells become geniculate after repetitive production of conidia from different areas on a relatively long rachis

as the production of conidiogenesis is not restricted to the apical portion of the conidiogenous cell. The *Geniculosporium* type of conidiophores (including the associated morphological structures) is more variable than the *Nodulisporium* type. Geniculate conidiophores may simply arise from vegetative hyphae, but they may also be arranged in palisades (as in the *Dematophora* R. Hartig stages of some *Rosellinia* spp.; cf. Petrini 1992, 2003. 2005). In *Xylaria hypoxylon* (L.) Greville, they can even be borne on coremia-like, conspicuous stromata. The genus *Collodiscula* I. Hino & Katum., which is now included in the Xylariaceae despite its two-celled ascospores, has an *Acanthodochium* Samuels et al. anamorph. This was also categorised by Ju & Rogers (1996) as *Geniculosporium*-like. The anamorphs of the known coprophilous Xylariaceae genera also belong to this group, but, as exemplified by the *Lindquistia* Subram. & Chandrash. stage of *Poronia* Willd., they show specific morphological adaptations to the different habitat. The conidia of most of the genera with *Geniculosporium*-like and *Nodulisporium*-like anamorphs are ellipsoid or ovoid. Some Xylariaceae genera cannot be assigned to either of the above groups and differ from them in producing anamorphic structures similar to those of the related family Diatrypaceae. They produce long, slender, scolecosporous conidia, similar to those of the coelomycete genus *Libertella* Desmaziéres, which is rather distantly related to the Xylariaceae. Genera with *Libertella*-like anamorphs [e.g., *Creosphaeria* Theiss., *Lopadostoma* (Nitschke) Traverso, *Jumillera* J.D. Rogers et al., *Whalleya* J.D. Rogers et al.] are maintained in the Xylariaceae because of their characteristic ascospore morphology. In case of *Jumillera*, the conidiophores are *Geniculosporium* like, while only the conidia differ in their morphology (Rogers et al. 1997). Both, *Libertella*-like and *Nodulisporium*-like anamorphs have been reported from the genus *Anthostomella* Saccardo.

To further complicate the taxonomy of the Xylariales, there are also genera with diatrypaceous teleomorphs and xylariaceous anamorphs, such as *Graphostroma* Pirozynski (Pirozynski 1974), for which an own family, Graphostromataceae, was erected (Barr et al. 1993). These examples show the difficulties to draw clear boundaries between the families of the Xylariales. The evolution of this fungal order obviously resulted in numerous convergent developmental lines, which remain to be evaluated by modern taxonomic techniques.

Nonetheless, the higher taxonomic classification of Xylariomycetidae is outside the major scope of this study, which concentrated on infrageneric and intergeneric relationships of the Xylariaceae. Only a few representatives of other families were included for comparison. This topic is therefore not further addressed here. A current overview on the organisation of the higher taxa in the Sordariomycetes and their phylogenetic relationships has been published by Zhang et al. (2006).

Taxonomic history of the Xylariaceae

The basionym of the type species of the family, *Clavaria hypoxylon* L. (syn. *Xylaria hypoxylon*) goes back to Linnaeus (1753). Persoon (1799) and Fries (1823) still preferred to use the generic name *Sphaeria* Persoon. Tulasne & Tulasne (1863) are often referred to as the mycologists who erected the family name. However, they used the term “Xylariei”, and it is not clear whether this was meant to related to a family concept as we would understand it today. Nevertheless, the Tulasne Brothers and Nitschke (1867) provided remarkable and valuable early contributions to their taxonomy. Many other famous mycologists of the 19th century, including M.J. Berkeley, M.C. Cooke, J.B. Ellis, J.F.C. Montagne, and L.D. von Schweinitz, also dealt with them and erected new taxa. In his *Sylloge fungorum*, P.A. Sac-cardo provided an account of all fungal species that were then known to Science (most of the Xylariaceae were treated in the first volume; Saccardo 1882) and listed several hundreds of Xylariaceae taxa. However, owing to the mo-numental task he had to accomplish, he mainly checked herbarium material and had to use rather broad taxonomic concepts, which must be kept in mind when interpreting his data.

A brilliant early study was published by Möller (1901) on neotropical Xylariaceae. He included meticulous field observations, microscopic studies on their anamorphs and stromatal development of the species he observed in

Brazil. In retrospective, his monograph appears visionary, as it relates to concepts that were only established several decades later. Another mycologist who undoubtedly strongly influenced our modern concept of the Xylariaceae was Julian H. Miller, who worked on these fungi for several decades. He provided several important papers on their taxonomy, including the first world monograph on *Hypoxylon* (Miller 1961). Meanwhile, Dennis (1957, 1959, 1961, 1963, 1964) revised Xylariaceae from around the tropics. His work heavily contributed to our present knowledge on the family in warmer climates and constituted an important starting point for our own work on their taxonomy.

However, Dennis and Miller both still used rather broad concepts. Only In the years following Miller's monograph, data on anamorphic morphology (see above), and details on ascal and ascospore morphology and ultrastructure became increasingly available. The work by Pouzar (1985a,b) was instrumental in segregating *Nemania* S. F. Gray from *Hypoxylon* sensu Miller (1961). In the following, this genus was segregated by applying a holomorphic concept. For instance, Læssøe et al. (1989) treated *Camillea* Fr. and revised some other taxa in *Hypoxylon* that have light-coloured ascospores. In the 1990s, various taxonomic revisions by J.D. Rogers, Y.-M. Ju and co-workers resulted in the recognition of additional genera of predominantly tropical distribution that had also been included in the "old" broad concept of *Hypoxylon*. However, as with the aforementioned *Nemania*, it was often possible to resurrect old generic names, such as *Creosphaeria* Theiss. (Ju et al. 1993), *Entoleuca* (Rogers & Ju 1996), and *Kretzschmariella* Viegas (Ju & Rogers 1994). The type species of these genera were all included as synonyms of *Hypoxylon* by Miller (1961), but proved to have anamorphs other than *Nodulisporium* and were therefore expelled from the large genus.

In Table 1 the above mentioned concept of "subfamilies" Hypoxyloideae and Xylarioideae) is adopted. Those names were proposed by Dennis (1961) in a different context, but will here be used for the two major groups of Xylariaceae *sensu* Ju & Rogers (1996) with *Nodulisporium*-like and *Geniculosporium*-like anamorphs, respectively. These "subfamilies" were not validly erected by *Dennis* (1961), and they remain to be emended and officially proposed in a taxonomic paper. Dennis did not yet consider anamorphic morphology to be significant. He could therefore not possibly have envisaged some major taxonomic changes relating to the resolution of *Hypoxylon* sensu Miller (1961). Rogers (1994) pointed out some problems that remain to be solved before final conclusions on higher classification of the Xylariaceae at suprageneric ranks can be drawn, and some of these still persist even today.

Strikingly, there are not many features relating to the teleomorph that can be universally applied to segregate the Hypoxyloideae from the Xylarioideae. Their ascospore and ascal morphology appear rather similar. The morphology of the ascal apical apparatus, which differs between the xylarioid and the hypoxyloid genera, may be taken as an additional diagnostic tool for differentiation of xylarioid and hypoxyloid teleomorphs (Rogers 1979). Several genera and species are characterised by having an aberrant ascal morphology. Cultures and anamorphs of numerous genera and species are as yet unknown. Many of our own studies (see below) were aimed at gaining further information on this matter, and were designed to employ complementary methodology to evaluate infrageneric and intergeneric affinities, above all in the Hypoxyloideae.

Ecological aspects

As pointed out by Whalley (1996), the Xylariaceae have long been considered to be wood-destroying or coprophilous saprobes, aside from a few facultative tree parasites. *Entoleuca mammata* (Wahlenberg) J.D. Rogers & Y.M. Ju, the causative agent of "*Hypoxylon* canker" of *Populus* spp. (Ostry & Anderson 2009) and *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin, a frequent parasite of beech, are among the few important xylariaceous plant parasites of the Northern Hemisphere. Certain species of *Rosellinia* are known to cause serious damage as pathogens of trees or agricultural plants (Edwards et al. 2003, ten Hoppen & Krauss 2006), but aside from *R. necatrix* Prillieux, those are mainly distributed in the tropics. These parasites all belong to the Xylarioideae as understood here. The only economically important pathogen among the

Table 1 Gross classification of the major Xylariaceae genera into "subfamilies".

Hypoxyloideae	Xylarioideae	Others/Genera incertae sedis
Anthostomella Sacc. p.p.*	*Astrocystis* Berk. & Broome	*Anthostomella* Sacc. p.p.*
Areolospora S.C. Jong & E.E. Davis (= *Phaeosporis* Clem.)	*Ascotricha* Berk.	*Ascovirgaria* J. D. Rogers & Y.-M. Ju
***Biscogniauxia* Kuntze**	***Discoxylaria* Lindquist & J. Wright**	***Creosphaeria* Theiss.**
***Camillea* Fr.**	***Engleromyces* Henn.**	*Kretzschmariella* Viégas
***Chlorostroma* A.N. Miller et al.**	***Entoleuca* Syd.**	*Jumillera* J.D. Rogers et al. **
		***Lopadostoma* (Nitschke) Traverso**
***Daldinia* Ces. & de Not.**	***Euepixylon* Füisting**	***Whalleya* J.D. Rogers et al.**
***Entonaema* A. Möller**	*Halorosellinia* Whalley et al.	
***Hypoxylon* Bull.**	*Hypocopra* (Fr.) J. Kickx fil.	
Induratia Samuels et al.	*Jumillera* J.D. Rogers et al.	
***Obolarina* Pouzar**	***Kretzschmaria* Fr.**	
***Phylacia* Lév.**	***Leprieuria* Læssøe et al.**	
***Pyrenomyxa* Morgan**	***Nemania* S.F. Gray**	
***Rhopalostroma* D. Hawksworth**	***Podosordaria* Ellis & Holw.**	
***Thamnomyces* Ehrenb.**	***Poronia* Willd.**	
***Theissenia* Maubl.**	***Rosellinia* de Not.**	
***Thuemenella* Penz. & Sacc.**	***Sarcoxylon* Cooke**	
***Versiomyces* Whalley & Watl.**	***Stilbohypoxylon* Henn.**	
***Vivantia* J.D. Rogers et al.**	***Xylaria* Hill ex Schrank**	

Largely based on the overview by Ju & Rogers (1996), except that genera with *Libertella*-like anamorphs are listed here as "genera incertae sedis", and that recent taxonomic papers have been considered. The genera of which a significant number of representatives (in relation to the number of described species) were already included in our chemotaxonomic evaluation are printed in **bold**. Those taxa of which a significant number of representatives was also included in our molecular phylogenetic study are **highlighted in grey**. Some recently erected genera with unknown anamorphs, and non-stromatic genera that were placed in the Xylariaceae based on molecular phylogenetic evidence are not included. * *Anthostomella* species may have either *Libertella*-like or *Nodulisporium*-like anamorphs. **Several *Jumillera* species have *Libertella*-like anamorphs with *Geniculosporium*-like synanamorphs.

Hypoxyloideae in Europe is *Biscogniauxia mediterranea* (De Not.) Kuntze. This fungus seriously affects evergreen oaks in the Mediterranean (Collado et al. 2001, Nugent 2005). It has therefore been studied more intensively than most other Xylariaceae by plant pathologists.

The vast majority of the stromatic Xylariaceae colonise dead and decaying wood of angiospermous plants. Saprotrophic Xylariaceae are predominant in virtually all forested habitats of the world. They are considered to be white-rot fungi, owing to their ability to degrade lignin, but they even can degrade cellulose very effectively (Merrill et al. 1964, Wei et al. 1992). Some species (including *Hypoxylon fragiforme* and the "primary colonisers" of the genus *Daldinia*, which are discussed further below) appear in the succession of wood-decomposing fungi quite early. Their activities (or at least the formation of their stromata) apparently cease when the more competitive polyporaceous Basidiomycetes arrive. Other Xylariaceae including *Euepixylon udum* (Pers.) Füisting and many *Nemania* spp., exclusively produce stromata on damp, already highly decomposed wood (Laessøe & Spooner 1994).

The mechanisms of wood biodegradation have been studied extensively in some temperate (Sutherland & Crawford 1981, Boddy et al. 1985, Boddy & Rayner 1993) as well as the tropical Xylariaceae (Pointing et al. 2003, 2005), albeit most of the basic research with regard to this topic has been dedicated to their basidiomycete counterparts. For instance, *Phanerochaete chrysosporium* Burds. has been evaluated extensively as a "model wood-destroyer" for more than two decades, and even the sequencing of its genome has been accomplished. On the other hand, it remains widely obscure whether the biogenetic pathways that have evolved in the Xylariaceae for decomposition of wood and other organic substrates are analogous or homologous to those of the basidiomycetes. Shary et al. (2007) have recently demonstrated that wood-grown cultures of a fungus they named "*Daldinia concentrica*" degraded ^{14}C-labelled synthetic lignin in a similar manner as white-rot basidiomycetes. However, their conclusions were drawn from

chemical analyses of the biodegradation process, based on the end-products of the degradation process. It still remains unclear whether the molecular and biochemical prerequisites for this process have evolved in homology or in convergence in Xylariaceae and basidiomycetes.

The Xylariaceae have also received considerable attention because of their mutualistic relationships with plants. Carroll (1988), Petrini & Petrini (1985) and others showed by classical microbiological methodology and morphology-based identification techniques that xylariaceous anamorphs constitute one of the predominant groups of fungal endophytes in various habitats and plant taxa. Recent molecular ecology studies (Arnold et al. 2001, 2003, Guo et al. 2000, 2001) reinforced this view. Even fungal DNA extracted from the host plants can now be assigned to the Xylariaceae as inferred from similarity analyses of their rDNA genes. Recent reviews on fungal endophytes in general have been compiled by Schulz & Boyle (2005) and Hyde & Soytong (2008). The role of the Xylariaceae as endophytes was last reviewed by Petrini et al. (1995). Out of the vast amount of literature on this topic, only some recent studies dealing with Xylariaceae are treated here in detail.

a) Orchids are very well known as symbionts of mycorrhizal fungi, but Yuan et al. (2008) focused on their endophytic mycobiota, revealing that 14 out of 33 fungal "morphospecies" belonged to the Xylariaceae and verified their results by molecular data. The Xylariaceae were found in all organs of the plants.

b) Even liverworts are predominantly colonised by "xylarialean" endophytes in different regions of the world (Davis et al. 2003, Davis & Shaw 2008). Even though Davis & Shaw (2008) referred to the order Xylariales, most of the genera mentioned in the earlier paper apparently belong to the Xylarioideae. They comprised about two-thirds of the total endophyte populations, as revealed from 5.8S/ITS nrDNA sequences. Most of these liverwort-associated Xylariaceae have not been identified conclusively to species level for lack of reliable data on teleomorphic material for comparison, but in most cases, high similarities were observed to sequence data derived from stromatic taxa. Evidently, the fungi will not be able to form their massive stromata on such relatively small and fragile plants, even after decomposition. To my knowledge, anamorphic stages of Xylariaceae growing as saprobes on decaying liverwort thalli have likewise not been encountered. It is not clear whether the fungi are horizontally transmitted, and how the liverwort thalli are infected by fungal propagules.

c) A study in a national park of Thailand, using extensive culturing and similar methods for characterisation of genotypes (Okane et al. 2008), revealed over 270 endophytic Xylariaceae in different seed plants. Over 100 strains obtained from representative teleomorphic materials from the same site were examined for comparison. The authors recognised 21 different genotype clades that may represent species. Several of those did not correspond to ascospore isolates from the same site. As inferred from a comparison of DNA sequence data with those in public domain databases, some of these "endophyte" taxa may well correspond with the teleomorphic stages of fungi that have been studied in other parts of the world, since a high degree of homology to the corresponding published data in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) was observed.

Despite the general availability of sophisticated molecular methods, a problem still persists regarding the safe identification of the xylariaceous endophytes. While the keys by Petrini & Petrini (1985) and other detailed morphological descriptions of Xylariaceae anamorphs will often help in narrowing down the identification to the level of genus or species group, endophytes often remain entirely sterile in culture. In such cases, or when the identification is only based on "environmental" DNA sequences, the fungi can at present hardly be identified with certainty. An exception from this rule may be the fungal endophytes belonging to the genus *Nodulisporium*, which produce the antiparasitic agent nodulisporic acid and occur all over the tropics (Polishook et al. 2001). Notably, the detection of the antiparasitic secondary metabolite coincided with rather strong homologies of rDNA sequence data. Up to date, no xylariaceous teleomorph was associated to these endophytes. The DNA sequences derived from the producer strains are significantly distinct from those of all published

teleomorphic Xylariaceae. Possibly, the producers of nodulisporic acid constitute a highly specialised group within the Xylariaceae that has abandoned production of the teleomorph.

However, most xylariaceous endophytes appear to have close affinities to certain teleomorphic species or constitute the asexual stages of those fungi themselves. Not only are the ascospores of this family perfectly designed to withstand environmental hardships, but they may also have found a way to awake from dormancy at the right time. Chapela et al. (1990, 1991) studied in detail the process of host infection for *H. fragiforme*. For this species and *Fagus*, an intricate host/fungus recognition mechanism was assumed. Ascospore germination is mediated by mannolignol glucosides, present in the host, acting as specific recognition messengers. Ascospore eclosion and germination is induced by the messenger compound (Chapela et al. 1993), facilitating the colonisation of the host. A similar mechanism may be generally involved in the establishment of an endophytic relationship between other Xylariaceae and their host plants. This phenomenon deserves further study, using a broad range of host plants and xylariaceous model organisms.

Some other studies dealt with the role of xylariaceous and other fungal endophytes in the process of wood decay, in which these fungi turn from endophytes to saprotrophs (Boddy & Griffith 1989, Griffith & Boddy 1990). They examined the ecophysiological processes and the fungal succession in detail, highlighting the role of certain Xylariaceae in the decay community. For instance, it was established that the anamorph of *D. concentrica* (Bolt.) Ces. & de Not., which preferentially forms stromata on *Fraxinus*, is often present on other angiospermous hosts as an endophyte. This was later confirmed by Nugent (2003) using molecular identification methods.

The boundaries between the endophytic, parasitic, and saprotrophic lifestyles are still obscure. They may just reflect an equilibrium in the fungus-host relationship (Whalley 1996, Edwards et al. 2003). The host plants become more susceptible to their endophytic xylariaceous inhabitants after long periods of drought. When the host trees are subjected to water stress for a long time or in several consecutive vegetation periods, even living trees may become seriously affected by "saprobic" or "endophytic" Xylariaceae. Nevertheless, as compared to other groups of fungi, the damages caused in agriculture and forestry by parasitic Xylariaceae appear rather moderate.

Fungal endophytes were even postulated to have a positive effect on their host plants (Arnold et al. 2003). They might indeed act as antagonists to parasites, impeding colonisation by parasitic fungi. Evidently, a plant that is already inhabited by mutualistic organisms will not be as easily affected by parasites as an uncolonised plant. Possibly, the fungal endophytes dispose of their own armoury to defending their habitat, such as antifungal secondary metabolites. Such a phenomenon was proven conclusively in case of the systemic clavicipitaceous endophytes of ryegrass (Schardl et al. 2004), which produce alkaloids with antifeedant and other biological activities. Those compounds may substantially accumulate in the host plants and thus protect them against herbivore feeding.

The general availability of molecular identification techniques would be very helpful for studies of endophytic Xylariaceae. However, reliable reference sequence data in public databases are still amiss for numerous taxa of this family. During the last decade, several entries of new "unidentified" xylariaceous and xylarialean DNA sequences were made in the aforementioned databases from inventories of fungal endophytes.

Innovative methods, such as the design of specific primers (Johannesson et al. 2000a) or approaches based on microarrays or real time PCR have recently been developed to detect and study fungi in the environment. With regard to Xylariaceae, their application still remains largely restricted to economically important pathogens (see e.g., Schena et al. 2002 for *R. necatrix*; Luchi et al. 2005 for *Biscogniauxia mediterranea*). It would be a great benefit for studies aimed at elucidation of the ecology and the life cycle of the Xylariaceae if such methods became widely available. However, even the most sophisticated molecular identification technique will only produce inconclusive results in the lack of reliable reference data.

Much information has become available

on apparent host specificity of certain Xylariaceae, which also relates to ecological aspects. Such data are largely based on the apparent occurrence of the stromata. They need to be taken with caution in view of the results on the distribution of xylariaceous endophytes discussed above. Nevertheless, floristic and chorological studies on the prevailing host plants have already revealed interesting relationships among the Xylariaceae and associated woody angiosperms. Some examples for apparently entirely different strategies are compared below.

Stromata of some apparently host-specific *Hypoxylon* spp. (e.g., *H. laschii* Nitschke on *Populus*), are extremely rarely encountered. They have almost exclusively been found on dead branches that were still attached to the host tree and apparently stopped growing as the branches became detached.

In *Hypoxylon fuscum*, there are several predominant forms, which show apparent host-specificity for wood of different genera of Betulaceae (*Alnus*, *Betula*, *Carpinus*, *Corylus*). Certain morphological features such as ascospore sizes have been correlated to these host affinities (Petrini et al. 1987). This suggests the presence of a species complex that is about to evolve in co-evolution with the host plant family.

The ubiquitous *H. fragiforme* (Fig. 1A) is almost constantly associated with *Fagus* throughout the Northern hemisphere. Its corresponding anamorph is invariably obtained from living, apparently undamaged beech trees (Petrini & Petrini 1985). According to our unpublished results, *H. fragiforme* often will produce stromata already in the first season, only some weeks after the beech wood has been damaged or felled. The fungus may then persist on the substrate for up to a decade as saprotroph and regularly produce stromata.

Many other species which preferentially produce their stromata on dead, decomposing wood, appear to be less specialised and their stromata do not show apparent host specificity. Stromata of *Daldinia fissa* Lloyd and *D. loculata* (Lév.) Sacc. grow preferentially on freshly burnt wood. *Betula* is said to be the preferred host genus for the latter species, but we have also found the stromata of *D. loculata* on several other genera of woody angiosperms, inclu-ding Rosaceae and Salicaceae. From the view of a forest ecologist, they are "primary colo-nisers", and it was long assumed that the fungal spores only arrive shortly after the fire. In fact, the rapid production of stromata on the freshly burnt wood rather indicates that the fungi were already present as endobionts. The damage to the host had apparently induced the production of reproductive stages. While such evidence is still circumstantial in many cases, this phenomenon was studied intensively by molecular methods, including methods of population genetics, by Johannesson (2000, 2001) and Guidot et al. (2003), for *D. loculata* in Sweden. In the latter study it was postulated that pyrophilous insects associated with *D. loculata* are essential vectors for the realization of the sexual cycle of the fungus. By feeding on the conidia and flying between nearby trees inhabiting wood decay mycelia, these insects allow the opposite mating types to meet. Indeed, the Hypoxyloideae are well-known for their insect associations (see Hingley 1971, Wheeler & Wheeler Jr. 1994 and other references compiled by Johannesson 2001). Their stromata are inadvertently roamed by insects, and numerous insect species show host preferences for certain Xylariaceae. Recently, Srůtka et al. (2007) and Pažoutová et al. (2010) reported xylariaceous anamorphs which showed high homologies in their 5.8S/ITS nrDNA and β-tubulin DNA gene to *Daldinia decipiens* and *Entonaema cinnabarinum* (Cooke & Massee) Lloyd as apparently specific associates of woodwasps (genus *Xiphydria*). The putative anamorph of *D. decipiens* is constantly associated with a *Xiphydria* sp. that primarily colonises alder, whereas the other has a strong host preference for Salicaceae. The identity of the *D. decipiens* strains was confirmed by comparing its anamorphic morphology and HPLC profile (S. Pažoutová & M.S., unpublished results). Indeed, the stromata of *D. decipiens* and allies are constantly associated with betulaceous hosts, including *Alnus*. The woodwasps appear highly suitable as vectors, as postulated by Guidot et al. (2003) for *D. loculata* and other associated insects. Woodwasps and other insects are known to have "ambrosia fungi" for a long time (Mueller & Gerardo 2005). The role of Xylariaceae in these associations remains to be further

clarified. Probably, entomologists will need to become more strongly involved in order to obtain more information on the lifecycle and the ecology of the Xylariaceae. For instance, the role of the invertebrates in the transmission of endophytes should be studied further, considering the possible role of insect vectors. Visser et al. (2009) revealed an interesting phenomenon of co-speciation between termite-associated *Xylaria* spp. and their invertebrate hosts. Even though these fungi are morphologically highly variable, they appear to be derived from a monophyletic lineage that might have evolved parallel to the plant-inhabiting *Xylaria* species. These results point toward a close co-evolution between tropical insects and their fungal symbionts, similar to the relationship of the basidiomycete genus *Termitomyces* R. Heim, which has been studied more intensively than the *Xylaria* spp.

Finally, there are even mycophilic Xylariaceae. Those may colonise the surface, or grow inside the fruit-bodies of other fungi, including even the stromata of members of their own family. Some members of Xylariaceae, such as *Chlorostroma subcubisporum* A.N. Mill. et al. (Miller et al. 2007), apparently parasitize stromata of other Xylariaceae. We have repeatedly obtained "foreign" cultures of other xylariaceous genera from perithecial contents of herbarium specimens of the same family. These observations cannot be attributed to laboratory contaminations, as the isolation was reproducible in several instances. Little is known so far about the nature of these relationships. It is also interesting to note that *Hypoxylon* species often grow as consortia, and up to five species have been found to produce stromata simultaneously on the same woody substrate.

Undoubtedly, further interdisciplinary studies making use of molecular ecology will reveal further interesting relationships that highlight the co-evolution of the fungi and all organisms associated to them. However, as in case of the endophytes, the availability of a concise identification system that is independent from teleomorphic morphology will be an absolute prerequisite to accomplish such tasks.

Secondary metabolites and practical importance

The Xylariaceae have traditionally raised great interest because they can be easily cultivated in the laboratory and studied for biologically active and other secondary metabolites. Their massive, conspicuous stromata were also found to constitute a good source for hitherto unprecedented compounds. The first secondary metabolites were already isolated from members of the Xylariaceae prior to 1960. Meanwhile, several hundreds of metabolites have been obtained, as summarised in Stadler & Hellwig (2005). Since publication of this work, another 100 compounds have been isolated and published from this fungal family.

There has been much speculation about the natural functions of secondary metabolites in the past. But by now it is almost generally accepted that these compounds are not randomly produced, but have important functions for the biology of their producers.

Recent research has demonstrated that the biogenetic pathways for most of these compounds are encoded by gene clusters, such as polyketide synthases (PKS) and non-ribosomal polypeptide synthetases (NRPS), and that their production underlies a complex regulation (Hoffmeister & Keller 2007). Recent studies on the genome of filamentous fungi revealed a surprising diversity of secondary metabolite genes. In the genome of *Emericella nidulans* (Eidam) Vuill. (often referred to as "*Aspergillus*" despite its teleomorph association is known), each 27 NRPS and PKS gene clusters were already detected (von Döhren 2008). By far not all these gene clusters appear fully functional in wild type strains, which express only a small percentage of the corresponding secondary metabolite pathways. Numerous studies are ongoing, dedicated to their reactivation and to the evaluation of possible functions of these genes in the few strains of filamentous fungi that have already been chosen for sequencing of their genome. In the Xylariaceae, it is at present not possible to do similar studies, as not a single representative of the family has been subjected to genomic sequencing, and not even the order Xylariales has been tapped by genomic approaches. Recently, the molecular structure of the tryptophan synthetase gene of nodulisporic acid was accomplished (Ireland et al. 2008), which is to my knowledge the first study of this kind on a member of Xylariaceae.

However, the vast majority of the known Xylariaceae metabolites are polyketides. Some options for future exploitation of this matter will be discussed below in relation to the results of our "mycochemical" studies.

Natural functions of the secondary metabolites of the Xylariaceae become quite evident in case of the numerous antibiotics, which are produced by these fungi, or in other cases where the metabolites act against specifically associated organisms. For instance, the phytopathogenic Xylariaceae produce a large number of phytotoxins that are probably involved in parasitism and were discussed as important determinants of virulence (see Stadler & Hellwig 2005 for some examples). However, most phytotoxins of the Xylariaceae that were so far characterised exerted a rather broad-spectrum of bioactivities. They are not apparently specific, but their action may still facilitate the process of parasitism by weakening the defence of the host plants. In reality, the phytotoxins will likely act synergistically with lytic enzymes and other features that were developed by the parasites to colonise their host plants.

It even remains possible that the same compound or compound class has different functions in different producer organisms, or changes its function during the life cycle of its producer organism. For instance, *Rosellinia necatrix* produces cytochalasins which possess phytotoxic as well as antibiotic effects (ten Hoppen & Krauss 2006). The same class of compounds are overproduced in laboratory cultures by various other fungi, including members of the related genus *Xylaria* (Abate et al. 1997, Espada et al. 1997). Those *Xylaria* spp. are regarded as saprotrophic wood-destroyers and frequently occur as endophytes in healthy plants, but they have never been reported to cause disease symptoms. The cytochalasins would evidently serve well as means of defence for the saprobic stages of their xylariaceous producers, to combat other micro-organisms in the struggle for nutrient sources. However, it is most unlikely that they are overproduced *in planta* by the endophytic stages of the *Xylaria* spp., because the plants might suffer, too.

The lack of knowledge on the natural functions of secondary metabolites in the saprotrophic and endophytic Xylariaceae is easily explained by the fact that most of the hitherto known compounds were obtained in the course of screening projects or basic research on the mechanisms of fungal metabolism, including their pigment chemistry. So far, not much analytical work has been dedicated to their chemical ecology. Accordingly, it has not been proven conclusively at all whether the secondary metabolites are produced by endophytic Xylariaceae *in planta.* Even the most sophisticated methods of analytical chemistry now available have detection limits and may not work unless the respective metabolites accumulate considerably. It could eventually become an option to indirectly prove secondary metabolite production, using PCR-based detection methods *in planta* for the fungal secondary metabolite genes. However, for Xylariaceae, even methods to detect the fungal producer organisms in their hosts remain to be established at this time.

With respect to their effect on human civilisation, the Xylariaceae could even be regarded as beneficial. *Nodulisporium* spp. were occasionally detected as clinical isolates in patients suffering from cancer (Umabala et al. 2001). Otherwise these fungi can certainly not be regarded as pathogenic to humans or mammals. Some antibiotics and phytotoxins of the Xylariaceae including the cytochalasins, have previously been identified from other fungi that may colonise and poison food and are therefore regarded as "mycotoxins". However, there is no xylariaceous fungus among the most important food spoilage fungi.

But above all, the Xylariaceae have raised great interest in the pharmaceutical and agrochemical industry, since various interesting metabolites are already known from their cultures (see Stadler & Hellwig 2005 for comprehensive overview). No metabolite of a xylariaceous fungus has so far made it to the market. However, nodulisporic acid, PF-1022A and the sordarins (Fig. 3) reached developmental candidate status as antibiotics or antiparasitic agents. Numerous other compounds, such as hypoxyxylerone and the xyloketals (Fig. 3), were taken as templates for chemists, to develop mimetic or total syntheses based on the pharmacophores [the moiety of a given bioactive molecule that is important (or even essential) for a certain biological effect.] of the

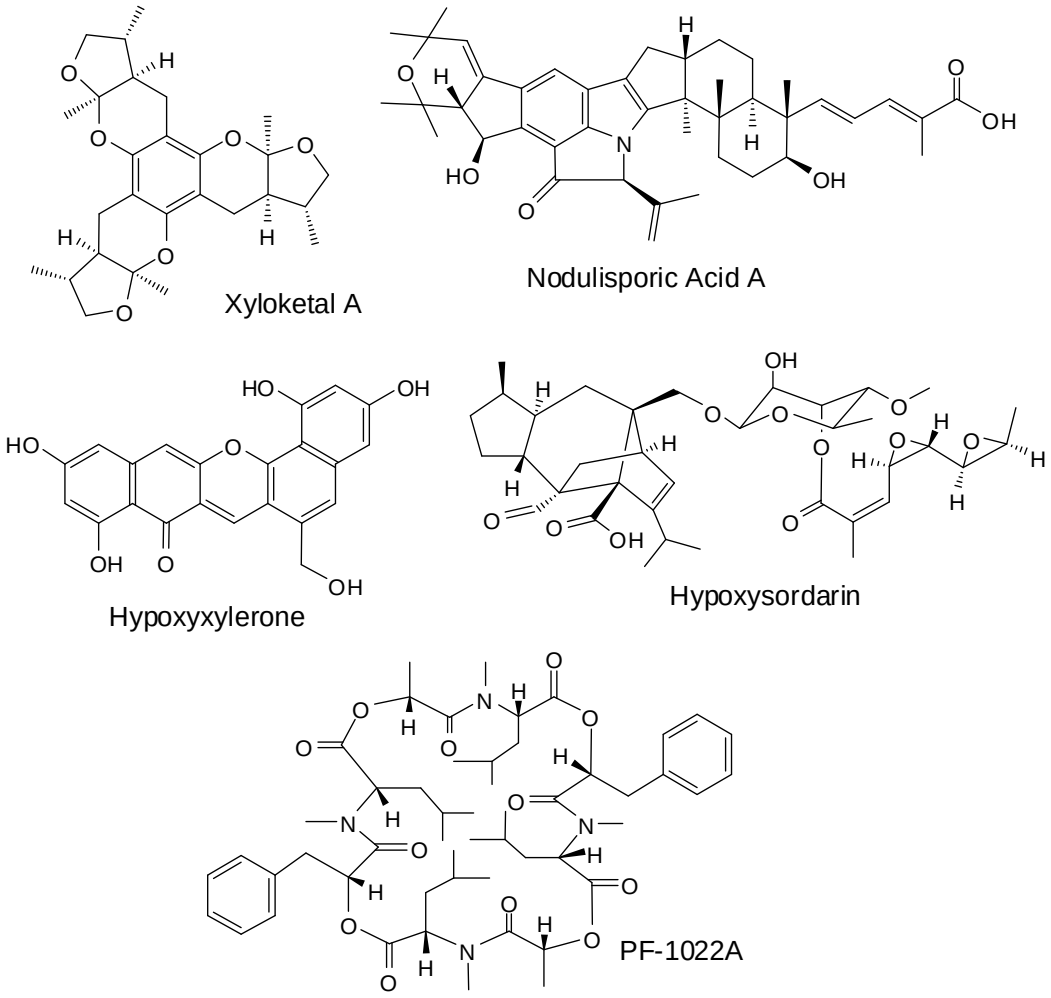


Fig. 3 – Some metabolites with interesting biological activities from Xylariaceae.

Xylariaceae metabolites. Other compounds showed promising effects against biochemical targets or in cellular *in vitro* assays, but were apparently not further developed. As outlined by Larsen et al. (2005) and in agreement with the proceedings outlined in Stadler & Hellwig (2005), phenotypic taxonomy including chemotaxonomic methodology, aided by HPLC-profiling can be extremely helpful in the discovery of unprecedented compounds. The example of nodulisporic acid (Polishook et al. 2001) also demonstrates that molecular identification methods may be useful to find additional producers of a given compound and ultimately help to secure parent claims. Molecular identification techniques are now increasingly being employed for characterisation of fungal producers of novel compounds.

The majority of secondary metabolites known until 2000 from Xylariaceae had been derived from laboratory cultures, because relatively large amounts of material were then required for structure elucidation. Only a few compounds, including the mitorubrins from *Hypoxylon fragiforme* (Steglich et al. 1974) and the pigments and other metabolites of a Japanese *Daldinia* sp. (Hashimoto & Asakawa 1998) had been known from the stromata of Xylariaceae, prior to our studies (Fig. 4). Earlier attempts to characterise their stromatal pigment profiles by thin layer chromatography (TLC; Whalley & Whalley 1977) were hampered by the fact that no standard compounds and little information on the chemical structures of the detected pigments had been available at that time. The availability of standards and highly sensitive analytical HPLC-based methods constituted a great advantage over the TLC-based methodology.

PCR fingerprinting and molecular phylogenetic studies

Not too many studies using molecular

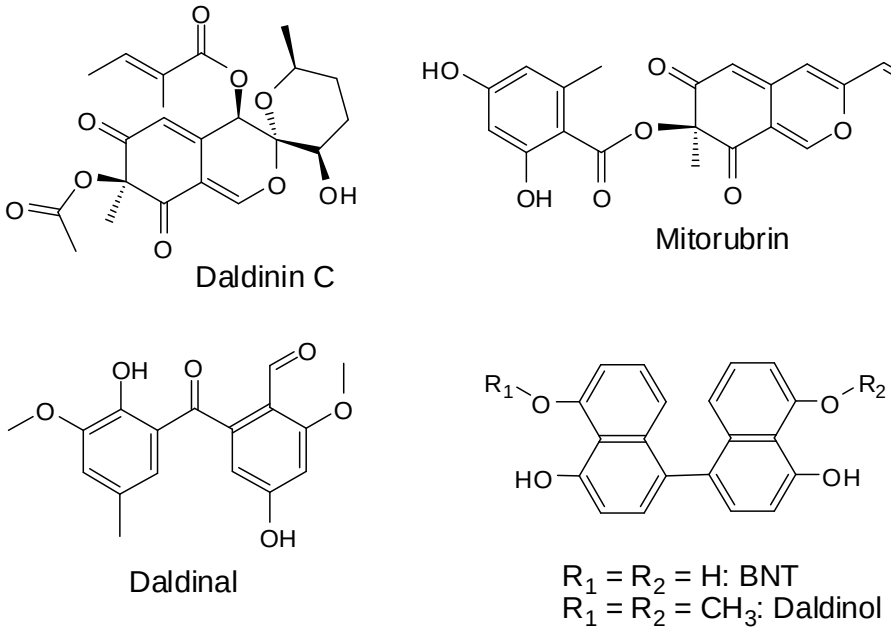


Fig. 4 – Selected secondary metabolites from stromata of the Xylariaceae that were known from the literature at the beginning of our studies.

methods based on PCR had been carried out on the Xylariaceae before our first papers dealing with such methodologies were published. For instance, Yoon & Glawe (1993) used random amplified polymorphic DNA (RAPD) to discriminate some isolates of *Hypoxylon truncatum* sensu Miller (1961). They recognised a taxon that is now regarded as *Annulohypoxylon annulatum* (Schweinitz) Y.M. Ju et al. (cf. Ju et al. 1996 as *Hypoxylon*; Hsieh et al. 2005). As sequencing costs rapidly declined and reference data became increasingly available in public databases, we also started to generate DNA sequences of Xylariaceae. Spatafora & Blackwell (1993) generated ribosomal DNA sequences of two representatives of Xylariaceae, *Daldinia concentrica* and *Xylaria hypoxylon*, in their early study on the molecular phylogeny of pyrenomycetes based on nrDNA sequence data. The sequence data derived from a "*X. hypoxylon*" strain, described first by Chacko & Rogers (1981) and deposited with ATCC were used in numerous phylogenetic studies as representative of Xylariales and Xylariaceae. We have only recently clarified that this fungus does not represent *X. hypoxylon* (see Peršoh et al. 2008).

Meanwhile, the availability of universal PCR primers for fungal rDNA (White et al. 1990) triggered numerous molecular phylogenetic studies. As compared to the Basidiomycota and other groups of ascomycetes, the Xylariaceae were neglected for a rather long time. The first molecular phylogenetic studies using a significant number of morphologically well-characterised xylariaceous species were only published several years later. Granmo et al. (1999) compared rDNA sequence data of a selection of *Nemania*, *Entoleuca*, and *Hypoxylon* species, but their sequences were unfortunately never deposited in a public database. Johannesson et al. (2000) and Sanchéz-Ballesteros (2000) focused on the hypoxyloid Xylariaceae, using morphologically verified specimens. Aside from some recent studies that relate to our own work in this field and will be treated below, the paper by Smith et al. (2003), in which the Xylariales were defined to be an order comprising seven families, deserves mentioning.

Synthesis of our own contributions

Morphological and chemotaxonomic studies

Several of the papers published in the course of this work deal with biodiversity inventories and taxonomic revisions of the genera *Daldinia* and *Hypoxylon*. It was regarded necessary to obtain sufficient data on herbarium specimens for chemotaxonomic studies and to collect and culture a significant number of representatives to evaluate the significance of chemotaxonomic data. Even today, the revi-

sions by Ju et al. (1997) and Ju & Rogers (1996) have still not been broadly applied, and the respective chorological data were based on outdated taxonomic schemes. Our strategy to obtain a global survey of the biodiversity, with emphasis on the chemical variability, is illustrated below to show the extent of these studies.

Aside from these herbarium specimens, considerable amounts of fresh material were supplied by J. Fournier (mostly from France, and in collaboration with C. Lechat and R. Courtecuisse, from the Caribbean). Numerous important specimens from different tropical regions were provided by C. Decock. M. Piepenbring (Panama), C. Douanla-Meli (Cameroon), T. Læssøe (Ecuador, Malaysia) and V. Kummer (South Africa) immediately after collection. Many other colleagues also sent material that could be cultured in our laboratory. My own field work encompassed forays in Germany, the Swiss and French Alps and the Spanish Pyrenees, Wales, Scandinavia, the Macaronesian Islands, mainland Spain and Portugal, Hawaii and different regions of mainland USA, and Japan. About 30% of the specimens hitherto studied belong to the genus *Daldinia*, 40% to the genera *Hypoxylon* and *Annulohypoxylon*, and the remainder to other Xylariaceae. Over 300 cultures resulting from this work were already characterised and deposited in public collections (CBS, MUCL).

The objectives of these studies can be circumscribed as follows:

- Establish a methodology based on analytical HPLC that works at high throughput to study a significant number of specimens of Xylariaceae for secondary metabolites and allows for concise identification of these compounds using sophisticated analytical methodology.
- Correlate chemotaxonomic data to morphological concepts and identify the prominent stromatal pigments of the Hypoxyloideae that are deemed taxonomically significant.
- Establish correlations between chemical types of the secondary metabolites encountered, based on a comparison of their chemical structures and current knowledge on the biosynthesis of the corresponding metabolite families.
- Verify current taxonomic concepts and evolutionary hypotheses in the Xylariaceae by means of chemotaxonomic data.

The genus *Daldinia*

The genus *Daldinia* was erected by Cesati & De Notaris (1863) to accommodate *Sphaeria concentrica* Bolt. and *S. vernicosa* Schwein. (= *D. fissa* in current nomenclature). It comprises stromatic Xylariaceae that may form rather large, conspicuous stromata on dead wood of angiosperms, which are easily recognisable, based on their internal concentric zones. The generic status of *Daldinia* had been disputed by various taxonomists in the course of the past century, owing to the fact that the internal concentric zones are the only morphological feature to discriminate *Daldinia* from *Hypoxylon*. The morphology of the asci, ascospores, and conidiophores is indeed highly similar among these genera. Læssøe (1994) therefore also proposed to unite them. Ju et al. (1997), however, chose to accept *Daldinia* as a genus, arguing that the peculiar stromatal anatomy relates to the biology of the genus. They pointed out that *Daldinia* has also affinities to other genera of Xylariaceae that Læssøe chose to accept. Those genera [i.e., *Entonaema* Möller, *Phylacia* (Lév.) Sacc., *Rhopalostroma* D. Hawksw., and *Thamnomyces* Ehrenb.] will be treated in detail further below. Furthermore, Whalley & Edwards (1995) had just reported that *Daldinia* spp. differed from *Hypoxylon* by the presence of naphthalenes in their cultures, whereas various types of dihydroisocoumarins prevailed in cultures of *Hypoxylon* and other Xylariaceae they studied concurrently. It therefore appeared practical to verify the generic organisation of the Hypoxyloideae by complementary methodology.

In addition, various questions related to the taxonomy of *Daldinia* remained to be resolved at the infrageneric level. The monograph by Child (1932), who had confused the type specimens of various important taxa (cf. Ju et al. 1997) had been in use for over 50 years. The generic concept proposed by Child (1932) had changed drastically due to the recent revision, and virtually all specimens identified using the latter monograph needed to be revised.

Concurrently, Frisvad and co-workers

had published exciting data on the chemotaxonomy of *Penicillium* (Samson & Frisvad 2005, Frisvad et al. 2008, and references therein). Our work was strongly inspired by their conclusive results, and we decided to use a similar methodology to study the Xylariaceae. We decided to start with *Daldinia* as it constitutes a relatively small but morphologically well-defined genus, which was treated controversially in the literature nevertheless with respect to its taxonomic affinities. For our project, we focused on stromata as well as on cultures; therefore this study is the first one in fungal chemotaxonomy where all developmental stages of a certain taxonomic group have been considered.

A pilot study, originally aimed at the revision of *Daldinia* in Germany and Europe according to Ju et al. (1997), was initiated in collaboration with H. Wollweber (Wollweber & Stadler 2001). However, while the type species, *D. concentrica*, was reported in the recent monograph to have yellow-brown stromatal pigments, all European material studied by us corresponding to the morphological description by Ju et al. (1997) had purple pigments in KOH. We therefore established contact to Jack D. Rogers, Thomas Læssøe, and Anthony J.S. Whalley, to clarify the situation and also discussed our chemotaxonomic data based in HPLC profiling with them. Based on the rediscovery of a specimen that was originally studied by Bolton (1789), and on material from the herbarium of Anthony J.S. Whalley, Rogers et al. (1999) revised the status of *D. concentrica*. They erected *D. childiae* J.D. Rogers & Y.M. Ju for the species that is most frequently encountered in America and selected an epitype for *D. concentrica*, based on a specimen from UK. For our own work, we used numerous reference materials treated by Ju et al. (1997). The stromatal pigment colours in 10% KOH by comparing them with a botanical chart (Rayner 1970) was correlated to HPLC profiles (Fig. 5), allowing for a better resolution. Hundreds of additional specimens were revised according to the new taxonomic concepts, verifying that HPLC profiling in mycology constitutes an additional important tool to generate reliable and highly reproducible data for chemotaxonomic evaluation of Macromycetes at rather high throughput. Some of the prevailing stromatal pigments of *Daldinia* were identified in the stromata by using standards, which had been isolated de-novo and characterised by NMR spectroscopy and mass spectrometry. This study provided the basis for various subsequently published papers, which are discussed in a broader context further below in the chemota-xonomic part. A monograph employing the new concept in German language (Wollweber & Stadler 2001), included various field observations and illustrations of several *Daldinia* spp., which were then recorded for Germany or other European countries for the first time. Material from America (Ju et al. 1997) and Papua New Guinea (van der Gucht 1994, 1995) was also studied for comparison. Another paper (Stadler et al. 2001e) introduced the HPLC profiling technique, and also dealt with a preliminary characterisation of *Daldinia* spp. by PCR fingerprinting methodology, using the ARDRA technique based on restriction fragment analysis of SSU rDNA. This technique provided specific results only for part of the species studied and revealed identical fragment patterns even among representatives of different genera, even if fragment patterns obtained by using several different restriction polymerases were combined. These results were complemented by minisatellite PCR fingerprinting, which proved much more specific for species discrimination (Stadler et al. 2001e). Concurrent morphological studies resulted in two papers (Stadler et al. 2001b,c) dealing with new species descriptions (*D. decipiens*, *D. albofibrosa* M. Stadler et al., *D. pyrenaica* M. Stadler & Wollw., *D. steglichii* M. Stadler et al.) and the characterisation of the anamorphic stage of *D. loculata*, which had not been cultured before. Due to this work, the novelty of some species previously treated tentatively by van der Gucht (1994) and Petrini & Müller (1986), respectively, under known species *sensu* Child (1932), was established. The morphological data were backed up by HPLC profiling.

Van der Gucht (1993) had compared ascospores of *Daldinia* spp. by scanning electron microscopy (SEM) and demonstrated the utility of this technique for segregation of morphologically similar taxa. This was confirmed by us, based on a large number of representatives for the first time (Stadler et al. 2002). Strong correlations were found between

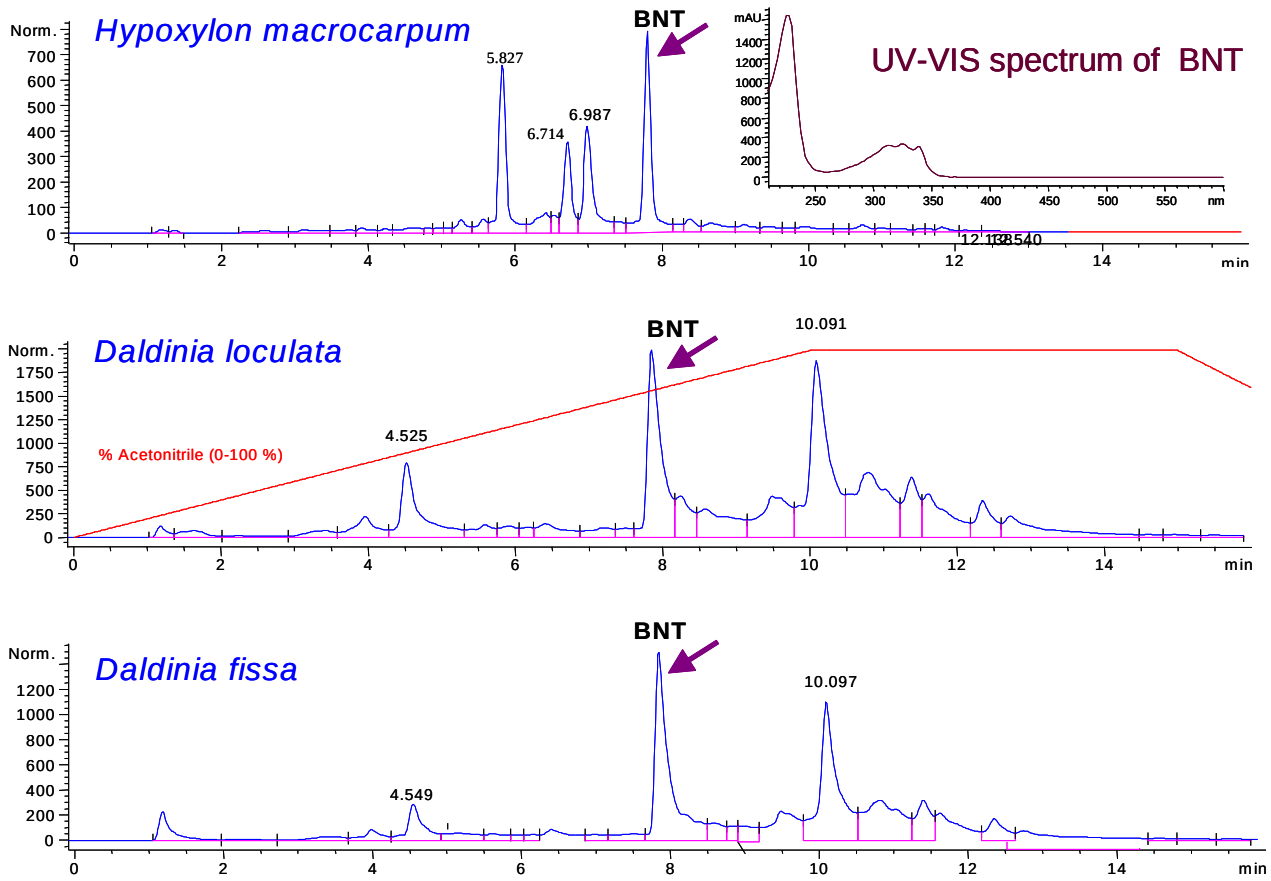


Fig. 5 – Illustration of the HPLC profiling methodology for chemotaxonomic studies on the Xylariaceae. Comparison of three HPLC chromatograms from stromatal extracts of different Xylariaceae spp. containing binaphthalene tetrol (BNT; see arrow), chemical structure and DAD spectrum of the corresponding peak (cf. Stadler et al. 2001d). The compound was safely identified by matching its Identical DAD spectra and retention times with data from standard compounds stored in a spectral library. Mass spectrometric data as revealed by Electrospray HPLC-MS, were also recorded concurrently. This unpublished figure is taken from a lecture presented at the IMC8, Oslo, 2002).

the ascospore ultrastructure as inferred from SEM and the secondary metabolite production in the stromata of the investigated species. Furthermore, the results gave rise to study the teleomorphic and anamorphic morphology in some specimens from the isle of Jersey, the Canary Islands, and Sicily. Five new cryptic species (*D. macarone-sica* M. Stadler et al., *D. martinii* M. Stadler et al., *D. palmensis* M. Stadler et al., *D. raimundi* M. Stadler et al., and *D. vanderguchtiae* M. Stadler et al.), all appearing closely allied to *D. concentrica* s. str., were described. They all deviate from the type species in their anamorphic morphology and/or in the morphology and ultrastructure of their asci and ascospores Stadler et al. 2004d).

Another study, mainly based on comparative morphology and ultrastructure of ascospores (Stadler et al. 2004d), dealt with *Daldinia grandis* Child and related taxa, focusing on the *Daldinia* species of the Southern hemisphere and other species featuring relatively large ascospores. Once again, it became evident that the concept established by Child (1932) had apparently caused much confusion, which was not completely resolved by Ju et al. (1997) for lack of fresh material. *Daldinia grandis* was restricted to a taxon that has up to date not been found outside the Americas. *Daldinia loculatoides* Wollw. & M. Stadler and *D. novae-zelandiae* Wollw. & M. Stadler were recognised from new combinations of teleomorphic and anamorphic characters. Another species with close relationships to *D. concentrica* was recognised as *D. dennisii* Wollw. & M. Stadler, of which two varieties were

erected.

The species composition in the temperate Southern hemisphere appears to be entirely different from that of the Northern hemisphere. Even though the predominant taxa of both hemispheres are apparently related to one another (e.g., both varieties of *D. dennisii* show high morphological similarities to *D. concentrica*; *D. novae-zelandiae* and *D. bakeri* have clear affinities to *D. fissa*) the deviations are significant enough to assume a long independent evolution of these species groups in the respective geographic areas. For instance, *D. dennisii* is the only taxon in the *D. concentrica* complex in which concentricols are apparently absent, and *D. fissa* has much smaller ascospores and also significantly differs in its anamorphic morphology from *D. novae-zelandiae*. In general, the only Hypoxyloideae that were so far encountered in Europe as well as in Australia and New Zealand were found on introduced host plants in the latter country. Out of the tropical *Daldinia* species, *D. caldariorum* Henn. and *D. eschscholtzii* have made it also to tropical Australia. The *D. decipiens* group, comprising species with enteroblastic conidiogenesis, which are closely associated with betulaceous hosts, remains to be found in the Southern hemisphere. On the other hand, the Southern hemisphere *Daldinia* spp. are associated with *Nothofagus* and other endemic plants of those geographic areas.

The above examples only give some hints on what can be accomplished with our data. In the past years, numerous further specimens were examined, and a new world monograph is now in preparation. However, this work was not yet finished, because it took quite a long time to obtain some important type materials, and the tropical species of *Daldinia* appeared rather complicated. Recently, three additional species of the genus (*D. barkalovii* Lar.N. Vassiljeva & M. Stadler, *D. carpinicola* Lar.N. Vassiljeva & M. Stadler, and *D. govorovae* Lar.N. Vassiljeva & M. Stadler) were described from Fareastern Russia (Vasilyeva & Stadler 2008), a geographic region which had already shown earlier on to harbour highly interesting Xylariaceae (cf. Ju et al. 1999). They all show an enteroblastic conidiogenesis and appear closely related to *D. decipiens* and *D. petriniae*.

In conclusion, the concept proposed by Ju et al. (1997) was further elaborated due to these studies. Most of their species concepts were validated using a combination of chemotaxonomy and PCR-based methods, and numerous additional taxa of *Daldinia* were already encountered and described. Fourteen new species were already erected, and there is evidence that numerous additional ones remain to be described, especially from the tropics. With a single exception (*D. bakeri* Lloyd, see below and Stadler et al. 2004b), the results reported by Ju et al. (1997) on stromatal pigment colours were correlated to genuine stromatal constituents, which were unambiguously identified using HPLC profiling. It was also found that, at least in mature stromata of the same species, the secondary metabolite profiles are surprisingly consistent with respect to the presence of major metabolites. No dependence from the substrate or the host plant was noted, aside from those cases where deviations in the secondary metabolites were also accompanied by consistent morphological differences and apparent host affinities, as in case of *D. petriniae* from different betulaceous hosts (Stadler et al. 2001c, d). This chemotaxonomic significance was further emphasised by the fact that the characteristic marker compounds were still found intact in specimens that had been collected up to 200 years previously. This is important to note, because as shown for the type specimen of *D. bakeri* (Stadler et al. 2004b) the pigment colours detected by using KOH in old herbarium specimens may occasionally be falsified by the presence of obvious artifacts, such as preservatives that were eventually added to the specimens to prevent insect and mite contamination.

It was found that similar pigment colours may be due to the presence of entirely different metabolites. In some cases (e.g., concentricols, cytochalasins), even compounds that are not pigments but only exhibit UV absorption, were revealed to be taxonomically significant, which demonstrates the advantage of HPLC profiling over conventional colour reactions. The specificity of secondary metabolite production in cultures of *Daldinia* proposed by Whalley & Edwards (1995), who had not given details on the numbers of examined representatives and species involved, were confirmed based on a

significant number of representative cultures. Several types of metabolites were meanwhile found that do not occur in *Hypoxylon* cultures while being omnipresent in *Daldinia* and related genera (see also Bitzer et al. 2008). This also reinforced the generic status of *Daldinia*. Under standardised fermentation conditions, the secondary metabolite profiles observed in cultures were highly specific for certain species groups or even genera, but the prevailing stromatal metabolites were rarely detected in the mycelial cultures and vice versa.

A panel of ca. 300 cultures of *Daldinia* from around the world is now available to further study the distribution and evolution of morphological and chemotaxonomic traits in this group of ascomycetes. Along with ongoing phylogenetic studies, the data available may ultimately also facilitate to draw further conclusions on their biogeography.

Hypoxylon* and *Annulohypoxylon

Like *Daldinia*, the genus *Hypoxylon* had just been thoroughly revised by Ju & Rogers (1996). Even after removal of the taxa with *Geniculosporium*-like anamorphs (see Introduction), it then still constituted a very large conglomerate of taxa. Their common features include stromata with an essentially homogeneous context, the presence of stromatal pigments and *Nodulisporium*-like anamorphs in all species that had been cultured. Ju & Rogers (1996) divided the genus into sections *Hypoxylon* and *Annulata.* The latter section was delimited by the presence of ostioles that are encircled with a truncate area, carbonized stromatal tissue that discretely encloses each individual perithecium, and a thickening on the ascospore perispore that is absent in most species of sect. *Hypoxylon.* European representatives of *Hypoxylon* were already included for comparison in our studies focusing on the chemotaxonomy of *Daldinia* (Stadler et al. 2001d,e). Several specific metabolites including the binaphthalene tetrol BNT and the mitorubrins were isolated to purity and their structures confirmed by mass spectrometry and NMR spectroscopy. HPLC profiling revealed a considerable metabolic diversity within the genus *Hypoxylon*. In some species, such as *H. fuscum* (Pers.) Fr. similar pigment profiles as in *Daldinia childiae* were encountered, and the mitorubrins were found to be widely distributed. However, many other species of *Hypoxylon* contained apparently specific major metabolites. The interpretation of these HPLC profiling data (Mühlbauer et al. 2002) was rather difficult. This changed as the chemical work described below was accomplished and the chemical structures of the metabolites detected in the crude extracts were elucidated. In addition to a HPLC profiling study, Mühlbauer et al. (2002) included the isolation and structure elucidation of the macrocarpones.

The preliminary results appeared rather promising, and by studying numerous additional taxa, including over 100 type specimens, it was meanwhile established that the specificity of stromatal HPLC profiles is as high in *Hypoxylon* as in *Daldinia*. Based on a revision of the *H. rubiginosum* complex in Europe, a new species (*H. petriniae* M. Stadler & J. Fournier) was described, and HPLC profiles of several other *Hypoxylon* spp. were reported and compared, revealing further species-consistent and specific features that relate to the production of certain types of orange azaphilone pigments in the stromata (Stadler et al. 2004c). In addition, this paper included a newly elaborated key to *Hypoxylon* in Europe, taking the taxonomic changes proposed by Ju & Rogers (1996) and Granmo (1999, 2001), as well as our own observations into account. Most of these data, including the above mentioned chemotaxonomic results as well as illustrations of the diagnostic characteristics of *Hypoxylon* and other Xylariaceae and determination keys, are available on http://pyrenomycetes.free.fr. The chemotaxonomic evaluation of *Hypoxylon* continued, based on a set of characteristic specimens kindly provided by Y.-M Ju and J.D. Rogers. Results were published along with the chemical structure of hypomltin (Hellwig et al. 2005). Meanwhile, Hsieh et al. (2005) had published a molecular phylogenetic study of Xylariaceae that resulted in the segregation of the new genus *Annulohypoxylon* Y.M. Ju et al., being identical with the former sect. *Annulata* of *Hypoxylon* sensu Ju & Rogers (1996). The erection of the new genus was based on the above mentioned morphological differences in conjunction with a comparison of α-actin and β-tubulin DNA sequence data. Concurrently, numerous taxa of *Annulohypoxylon* were stu-

died for stromatal HPLC profiles, and the results (Quang et al. 2005c) backed up the new taxonomic concept. Aside from the binaphthalene BNT, which is present in numerous genera of Xylariaceae, no common pigments were encountered in the two sections. Azaphilones were also detected in *Annulohypoxylon*, but those were shown to possess entirely different carbon skeletons from those of the mitorubrins and other pigments of (sect.) *Hypoxylon* (see below for cohaerins and multiformins).

Finally, in the paper by Stadler et al. (2008b), a part of the results on HPLC profiling of *Hypoxylon* spp. from around the world, focussing in the species of the subtropical and temperate Northern hemisphere, were published. Results on HPLC profiling and morphological studies of a rather large number of ancient specimens, including authentic and type material from the herbaria of C. H. Persoon, F. Nitschke, and other mycologists of the 18th and 19th century were compared with fresh, conspecific material. Once again, the results were quite conclusive. Especially the specimens of the *H. rubiginosum* complex appeared to be well suited for HPLC analyses. They still revealed their characteristic metabolites even if they had been collected 200 years previously. Aside from a survey of *Hypoxylon* in the Canary Islands and the description of two new species (*H. canariense* J. Fournier et al., *H. urriesii* J. Fournier & M. Stadler), this paper (Stadler et al. 2008b) also included some preliminary data on some putatively new taxa. Those were, however, not yet officially described because they are so far only known from old specimens. In other cases, certain species in the concept by Ju & Rogers (1996) showed heterogeneous HPLC profiles, along with matching morphological deviations, suggesting the involvement of species complexes. Data on some representatives of *Hypoxylon* that occur in tropical as well as subtropical regions were also included for comparison. However, the bulk of HPLC profiling data we have obtained from *Hypoxylon* spp. from tropical Africa, America, and Asia still remains unpublished.

Revision of *Pyrenomyxa*

The genus *Pyrenomyxa* Morgan was discussed to be a synonym of *Pulveria* Malloch & C.T. Rogerson by Laessoe (1994). Both taxa are characterised by a hypoxyloid stromatal habit, with effused-pulvinate stromata and granules below the stromatal surface. However, their asci are globose, lacking an amyloid apical apparatus, and their ascospores are phaseoliform. Speer (1980) even excluded *Pulveria* from the Xylariaceae, lumped it with *Phylacia* Lév. in an own family Phylaciaceae. Our studies of the type material of both genera and some recently collected specimens revealed the following facts:

- *Pyrenomyxa* and *Pulveria* (Malloch & Rogerson 1977) are indeed synonyms. *Pulveria porrecta* Malloch & C.T. Rogerson is a later synonym of *Hypoxylon piceum* Ellis, for which the new combination *Pyrenomyxa picea* (Ellis) M. Stadler et al. was made.
- *Pyrenomyxa invocans* Morgan is indeed highly similar to the above taxa but differs in its HPLC profile and microscopic characters.
- A third species, *P. morganii* M. Stadler et al., was found from Eastern Russia. Its culture produced a *Virgariella*-like anamorph and 5-methlymellein, the characteristic marker metabolite of the *H. rubiginosum* complex.
- All three accepted *Pyrenomyxa* spp. produced orsellinic acid and azaphilones of the mitorubrin type in their stromata. *Pyrenomyxa picea* contained in addition macrocarpone derivatives.

The genus *Pyrenomyxa* was therefore emended (Stadler et al. 2005). A case could be made to include it in *Hypoxylon*, as molecular data also point toward its close relationships to the *H. rubiginosum* complex. However, the type species and *P. picea* remain to be studied further to assure that *Pyrenomyxa* is actually monophyletic before further taxonomic conclusions can be drawn. It still remains possible that *Pyrenomyxa*-like fungi have evolved more than once from *Hypoxylon*, because the tendency to lose the amyloid apical apparatus and other morphological features that point toward cleistocarpy are known from several, apparently unrelated taxa in *Hypoxylon*.

Revision of *Entonaema*

The genus *Entonaema* A. Möller was

erected to accommodate two tropical Xylariaceae spp. with hollow, gelatinous, liquid-filled stromata (Möller 1901). Rogers (1981) reviewed the taxonomic history of this genus, and subsequently (Rogers 1982) described a typical *Nodulisporium* anamorph for *E. liquescens*, the type species. Our paper (Stadler et al. 2004a) dealt with a comparison of HPLC profiles in stromata and cultures of various taxa of *Entonaema* and other Xylariaceae that were previously discussed to be related to this genus, such as *Sarcoxylon* (Jungh.) Cooke. Interestingly, their stromatal pigments were of the mitorubrin and rubiginosin types, which are widespread in the genus *Hypoxylon*, while the secondary metabolites of their cultures were reminiscent of *Daldinia*. The presence of naphthol and chromone derivatives instead of mellein dihydroisocoumarins in *E. liquescens* and *E. cinnabarinum* also corresponded with molecular phylogenetic data, suggesting that the affinities of the genus are with *Daldinia* rather than with *Hypoxylon*.

These data were complemented (Stadler et al. 2008a) by the recovery of authentic material of *Entonaema mesentericum* A. Möller, the second species erected by Möller (1901). Even though this type specimen was stored in ethanol for over 100 years, it was shown to correspond with *Entonaema pallidum* G.W. Martin. The major stromatal constituent was identified as xylaral, a metabolite known from *Xylaria polymorpha* (Pers.) Grev. (Gunawan et al. 1990). This compound was detected by us in several species of *Xylaria*, and even in the concentrated spirit in which the type specimen of *E. mesentericum* had been preserved. Cultures (Fig. 6) of *E. pallidum* were obtained for the first time. They resembled those of *Xylaria*, substantially differing from other *Entonaema* spp., in their morphology, 5.8S/ITS nrDNA sequences, and HPLC profiles. *Entonaema pallidum* is thus regarded as a later synonym of *E. mesentericum*, and the latter name was transferred to *Xylaria* [as *Xylaria mesenterica* (Möller) M. Stadler et al.].

A phylogenetic tree (Fig. 7) illustrates the affinities of *X. mesenterica* to other Xylariaceae inferred from 5.8S/ITS nrDNA data, as compared to some representatives used for the phylogenetic tree published earlier on (Triebel et al. 2005). Other *Xylaria* spp. including *X. telfairii* in which xylaral was concurrently detected did not appear particularly closely related to *X. mesenterica* as inferred from the molecular phylogeny. Xylaral was also detected in some taxa of *Nemania* and *Stilbohypoxylon*, and is therefore not of taxonomic significance beyond species rank.

The status of some other *Entonaema* spp. sensu Rogers (1981), as well as affinities between this genus and the presumably related genus *Sarcoxylon*, remain to be established. Stromata of these taxa neither contain xylaral nor any of the known metabolites of the Hypoxyloideae (Stadler et al. 2004a, 2008a). A culture in CBS, which did not produce conidiophores when studied by us, was deposited by P. Martin as *Penzigia compuncta* (Jungh.) Sacc. & Paol., which is a synonym of *Sarcoxylon compunctum*. The 5.8S/ITS nrDNA sequence of this strain clustered in the Xylarioideae (Fig. 7), but the corresponding specimen is not extant, the culture did not produce an anamorph when studied in our laboratory, and Martin apparently never published details on the material. Fresh material of *S. compunctum* remains to be found. Owing to the lack of "hypoxyloid" stromatal metabolites, and for the xylarioid morphology of its ascal apical apparatus, this genus was included tentatively in the Xylarioideae (Table 2).

The genus *Rostrohypoxylon*

During a recent foray in Northern Thailand, an interesting fungus was found, for which the erection of a new genus appeared justified. At first glimpse, *Rostrohypoxylon terebratum* J. Fournier & M. Stadler (Fig. 8) appears morphologically rather similar to *Biscogniauxia*-like taxa because of its erumpent carbonaceous stromata. However, stromata are unipartite, and show various characters that are unknown from other Xylariaceae. The most characteristic feature that does not occur in any other described taxon of the family is the strongly uneven stromatal surface, owing to the presence of stout ostiolar necks and cylindrical holes. A detailed study showed that these "superficial" features are accompanied by the lack of an amyloid ascal apical apparatus, and the ability

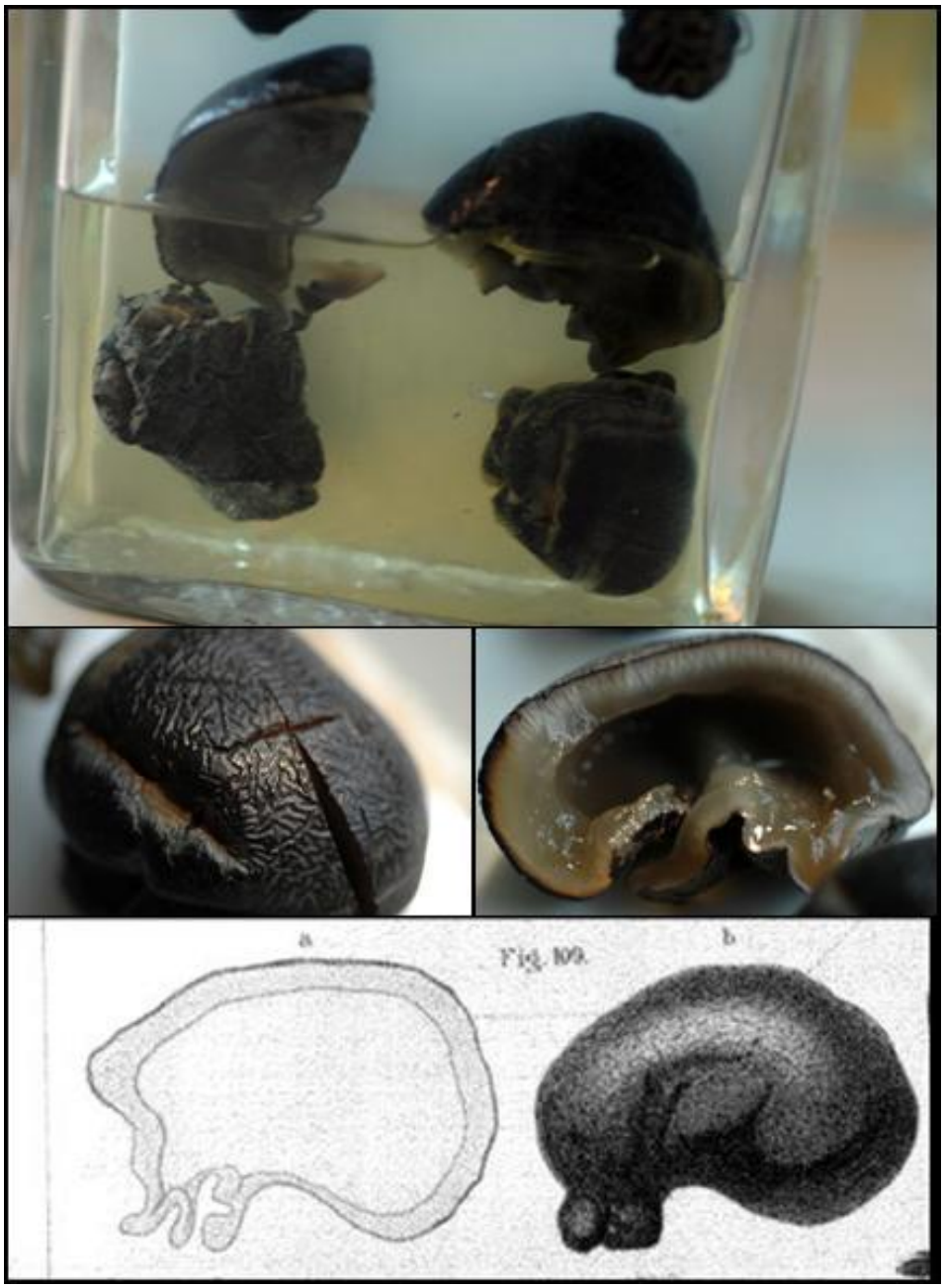
to produce conidia directly from vegetative hyphae besides the presence of a typical hypoxyloid anamorph of the reduced *Nodulisporium* type. The type species was included in a molecular study (Tang et al. 2009, as "*Xylariaceae* sp."), revealing phylogenetic affinities of the new taxon with three representatives of *Annulohypoxylon*. However, despite stromatal pigments were detected in the type specimen, the HPLC profile of *R. terebratum* did not reveal any known metabolites of the Hypoxyloideae. The ex-type culture was also studied by HPLC profiling, revealing 5-methylmellein aside from some unknown metabolites.

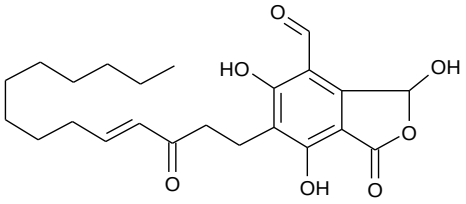
In conclusion. *R. terebratum* appears to represent a lineage of the Xylariaceae that has further evolved from *Annulohypoxylon*, characterised by various reductions of morphological structures that are present in the latter genus, and by significant differences of its secondary metabolism to representatives of *Annulohypoxylon*, which remain to be further explored by identification of the specific metabolites. The fungus shows numerous features, suggesting its being derived from the lineage comprising Xylariaceae with bipartite stromata, like *Biscogniauxia*.

In the course of this work, two additional species of the related genus *Annulohypoxylon*, *A. bahnphadengense* J. Fournier & M. Stadler and *A. maeteangense* J. Fournier & M. Stadler, were also recognised and formally described. This paper only constitutes the first of a series which will deal which will deal with the description of new taxa of tropical Xylariaceae that we found in the past years.

Table 2: Overview on the provenience of the specimens studied

Geographic origin	**Number of specimens studied**	**Herbaria (Important collections and publications)**
Europe	ca. 3,500	**B** (Nitschke, Möller, Hennings); **C** (T. Læssøe); **CWU** (A. Akulov); **L** (Persoon); **PAD** (Saccardo); A.J.S. Whalley (personal herbarium; **E**, Whalley & Watling 1981); **PRM** (Pouzar); **ST** (G.J. Krieglsteiner); **S, UPS** (Nannfeldt); **TROM** (Granmo 1999, 2001); **ZT** (Petrini & Müller 1986); Herbarium of H. Wollweber (Wuppertal); **GLM**, **KR**, **M**, **MA**, **TFC**
North America	ca. 1,500	**BPI** (C. G. Lloyd herbarium; material studied by Child 1932); **GAM** (Miller 1961); **NY** (Martin 1961; collections by G. Samuels; J.D. Rogers (personal herbarium; **WSP** (Ju & Rogers 1996, Ju et al. 1997); numerous specimens from **F** and **FH**.
South & Central America	ca. 1,000	**C** (T. Læssøe); **LPS** (Spegazzini herbarium); **LIP** (fresh material collected by C. Lechat and J. Fournier); **S** (Starbäck 1901, Möller (1901)
Africa	ca. 400	**BR** (Dennis 1961); **CABI** (Martin 1969) **K** (Deighton); Pers. herbarium of C. Douanla-Meli
Asia	ca. 700	**K** (Dargan & Thind 1984, Petch 1924; Y.-M. Ju (personal herbarium; **HAST**); VLA (L. Vasilyeva)
Australia & New Zealand	ca. 200	**PDD** (Ju & Rogers 1996, Ju et el. 1997)
Additional material from around the world, including numerous type specimens, was obtained from BPI, GAM, K, M, NY, S, and WSP.		





Xylaral

Fig. 6 – Type specimen of *Entonaema mesentericum* in herb. B, from spirit collection, and corresponding drawings by Möller (1901). Below: chemical structure of xylaral.

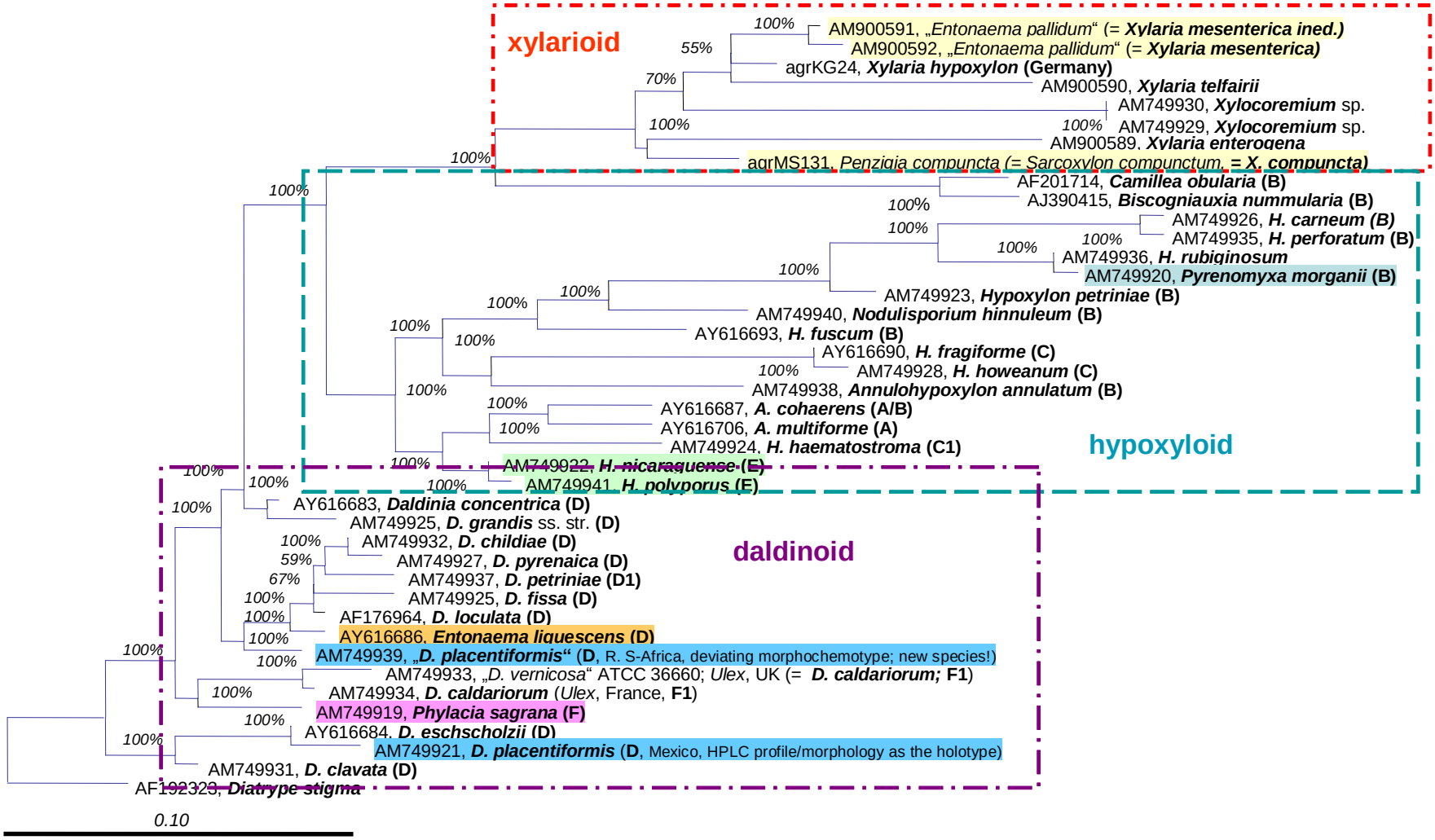


Fig. 7 – Preliminary molecular phylogeny based on 5.8S/ITS nrDNA sequences of selected Xylariaceae representing different morphotypes and chemotypes, resulting from a Parsimony Ratchet analysis (20 replicates a 800 iterations). Majority consensus tree of the 15258 most parsimonious trees (395 steps) found. From a plenary lecture at the AMC 2007, Penang, Malaysia, summarising the results of Bitzer et al. (2008) and Stadler et al. (2008a, 2010a)

Phylacia*, *Rhopalostroma* and *Thamnomyces

Type and authentic material of *Phylacia*, *Rhopalostroma* and *Thamnomyces* (Fig. 9) were also studied concurrently with the *Entonaema* spp. (Stadler et al. 2004a). These genera show characteristic features such as reductions of their ascal apparatus and ascospore germ slit, suggesting their being phylogenetically derived (cf. Table 3).

Representatives of all three genera contained BNT and other compounds that are prevalent in the stromata of *Daldinia* and *Hypoxylon*. This was in accordance with the *Nodulisporium*-like anamorphs reported earlier on by Hawksworth & Whalley (1985), Rodrigues & Samuels (1989), Samuels & Müller (1980). Affinities between these genera had already been discussed by Hawksworth (1977), Ju et al. (1997), and Medel et al. 2006) Our chemotaxonomic study, in conjunction with molecular data discussed further below, provided strong evidence that these genera are very closely related to *Daldinia*. In case of *Rhopalostroma*, the data presented by Stadler et al. (2004a[1]) will need to be confirmed based on a freshly isolated culture as the only culture available as now was derived from an old herbarium specimen this has meanwhile been realised by Stadler et al. (2010b), and Whalley & Edwards (1995) reported griseofulvin derivatives from another species of that genus. The cultures used by the latter authors, however, are apparently not extant anymore in any public collection.

Preliminary chemotaxonomic studies of the Xylarioideae

Aside from *Daldinia*, *Hypoxylon*, and their allies, numerous representatives of other genera were also studied for characteristic secondary metabolites. So far, about 200 cultures of ca. 90 taxa of Xylariaceae genera with anamorphs other than *Nodulisporium* (among those, the majority of cultures deposited with ATCC, CBS and MUCL) were studied for comparison. Some additional cultures were obtained by ourselves in the course of the phylogenetic evaluation of *Entonaema* (Stadler et al. 2008a) and the *X. hypoxylon* complex (Peršoh et al. 2008), respectively. These data served as prerequisite to evaluate the specificity of the compounds encountered in the hypoxyloid genera and allowed for some additional conclusions on chemotaxonomic differences between the large groups of Xylariaceae. In contrast to the hypoxyloid Xylariaceae, no large-scale fermentations resulting in the isolation of the characteristic major metabolites were carried out. Our ongoing studies were so far restricted to a screening of these cultures by HPLC profiling for the presence of known compounds. This helped to establish that the Xylarioideae are entirely different from the Hypoxyloideae with respect to their secondary metabolome. Various apparently specific, taxonomically significant compounds were only detected in one of these subfamilies by HPLC profiling. Some compound classes, like the cytochalasins, occur throughout the family, but most of the characteristic metabolites detected in the Xylarioideae remain to be isolated to purity and identified.

Stadler et al. (2008a) applied colour reactions (NH_3, KOH) of the ectostroma to a limited number of *Xylaria* spp. and other Xylarioideae, and especially those species that contained xylaral derivatives showed such colour reactions. According to my knowledge, this study provided first evidence that certain species of the Xylarioideae may also contain pigments extractable in KOH. As xylaral was also found in specimens of *Nemania* and *Stilbohypoxylon* Henn. while being apparently absent in *Hypoxylon* and allied genera, it may be a chemotaxonomic marker for certain taxa of Xylarioideae. However, over 90% of the xylarioid taxa so far studied were found devoid of stromatal pigments. Therefore, HPLC profiles of cultures appear more promising to reveal useful chemotaxonomic traits to segregate them. Whalley & Edwards (1995) and their co-workers, as well as other research groups, had previously studied various cultures of xylarioid Xylariaceae and reported rather interesting metabolites (see overview by Stadler & Hellwig 2005). Only few of these strains are deposited in public collections and could so far be re-examined (Bitzer et al.

[1] A recent study in comparison with the culture of a *Rhopalostroma* sp. collected in fresh state revealed that the culture described by Stadler et al. (2004a) was derived from *Daldinia eschscholtzii*, which had apparently colonised the stromata of the *Rhopalostroma* specimen, cf. Stadler et al. (2010b).

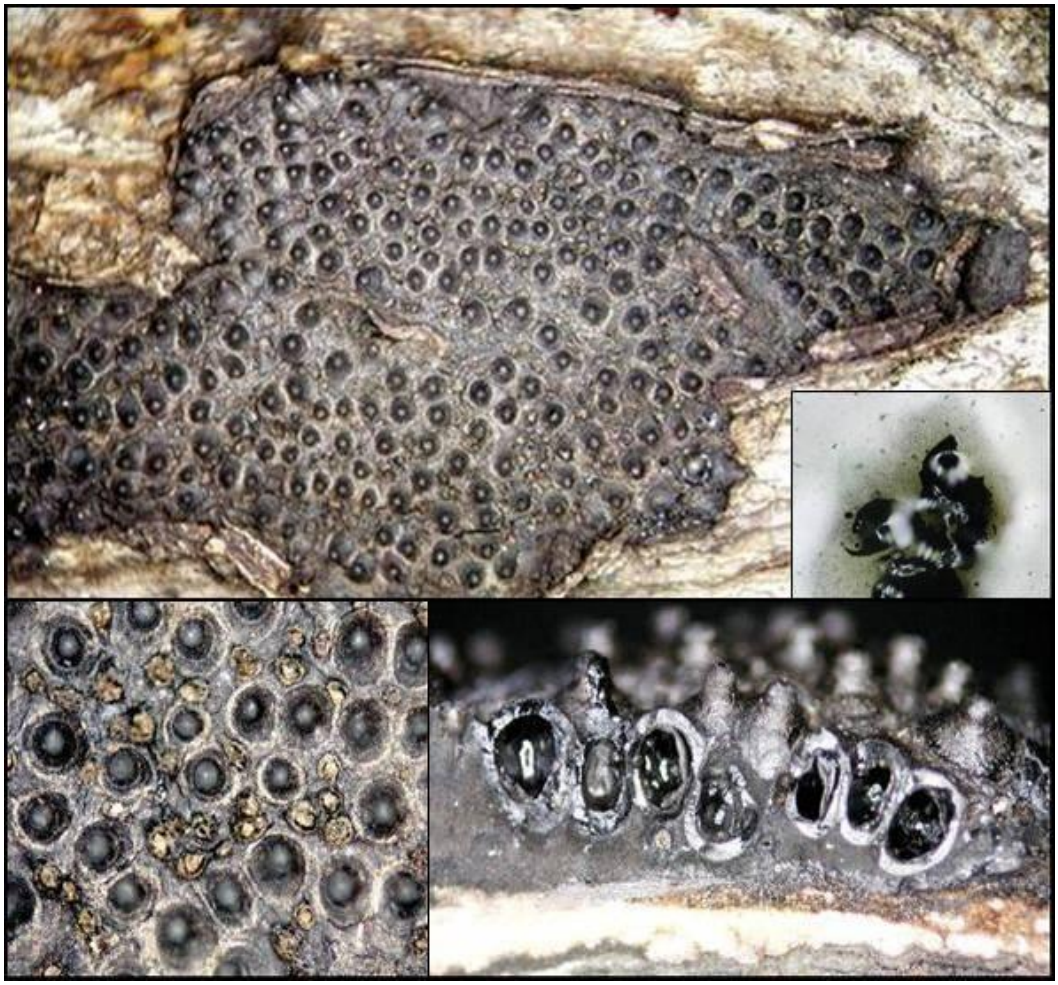


Fig. 8 – Stromatal habit of *Rostrohypoxylon terebratum*. (ex holotype, images by J. Fournier).

2008). Attempts to isolate and identify these and other metabolites are currently pending, to extend our chemotaxonomic work to the Xylarioideae. The numerous metabolites reported by Whalley & Edwards (1995) from *Xylaria* and its allies would clearly be helpful as standards for these tasks. However, most of them are probably not available anymore and will need to be re-isolated from the respective organisms.

Application of PCR-based methods

As already mentioned, our study relied from the beginning also on PCR-based data for characterisation of the Xylariaceae. Two of our early papers (Stadler et al. 2001d,e) dealt with the utility of PCR fingerprinting for discrimination of Xylariaceae, based on amplified ribosomal DNA restriction fragment analysis (ARDRA) and minisatellite PCR, respectively. Especially the latter technique apparently provided specific results when a significant number of isolates were studied. It soon became evident that PCR fingerprinting would not remain the method of choice for molecular characterisation of fungi. A collaboration with Dagmar Triebel (Botanische Staatsammlung München) and Derek Peršoh (University of Bayreuth) was initiated to explore the utility of rDNA sequences. The objectives of these studies can be circumscribed as follows:

- Use DNA sequence data are to verify morphological concepts in the Xylariaceae and obtain additional information on their phylogenetic relationships.
- Evaluate the potential utility of rDNA sequence data to reflect the evolutionary history of the Xylariaceae at generic and species level.
- Compare molecular phylogenies based on DNA sequence data to chemotaxonomic results.

Fig. 9 – Stromata of *Thamnomyces dendroidea* and *Phylacia sagrana*. Image by J. Fournier.

- Provide the basis for safe molecular identification techniques for endophytic and insect-associated xylariaceous anamorphs.

Our first publication (Triebel et al. 2005) appeared parallel to the study by Hsieh et al. (2005). In both studies, a significant number of the hypoxyloid Xylariaceae were compared on similarities of their DNA sequence data. Whereas Hsieh et al. (2005) used α-actin and β-tubulin genes, our own work focused on the 5.8S/ITS region of the rDNA, taking the previously published sequence data then available from GenBank into account.

The phylogenetic tree based on 5.8S/ITS nrDNA data of *Hypoxylon* spp. was not suited well to resolve the genus according to the current morphological concepts. The matrix for this study was derived from the corresponding DNA sequence data that had by then been published in GenBank. We supposed that the poor resolution was due to the fact that too few representatives of this large genus had been sequenced at that time, while certain taxa were over-represented. In retrospective, the apparent incongruities of these phylogenetic studies were also due to the fact that about 30% of the sequences we had retrieved as background from GenBank were either obviously derived from misidentified isolates, or it is not possible to verify their taxonomic status as no voucher specimens are available.

By contrast, Hsieh et al. (2005) found monophyletic clades for the sections *Annulata* and *Hypoxylon* of *Hypoxylon* sensu Ju & Rogers (1996), which gave rise to erect the genus *Annulohypoxylon* Y.M. Ju et al. As confirmed from recent phylogenetic studies (Suwanassai et al. 2005, Pelaez et al. 2008, Tang et al. 2009), it was so far not possible to verify this taxonomy by rDNA sequence analyses, despite numerous additional data meanwhile became available. Representatives of *Hypoxylon* and *Annulohypoxylon* still became intermingled when their rDNA sequence data were compared using different calculation methods. The same applies to other genera of Xylariaceae, which can be segregated conclusively by using morphological data [e.g., *Biscogniauxia* Kuntze and *Camillea* Fr. (Laessøe et al. 1989, Ju et al. 1998)].

The genus *Daldinia* was fairly resolved from *Hypoxylon* by using nrDNA as well as α-actin and β-tubulin genes. All members of the genus studied by Hsieh et al. (2005) clustered in a monophyletic clade, derived from within *Hypoxylon*. The same was found based on 5.8S/ITS rDNA phylogeny (Triebel et al. 2005), however, members of *Entonaema* also appeared in the clade comprising otherwise only *Daldinia* species. Representatives of other genera that have similar metabolites in culture as *Daldinia*, i.e., *Phylacia* and *Thamnomyces*, were included in the molecular phylogeny (Bitzer et al. 2008**,** Stadler et al. 2010a). The

results suggest that they are closely related to the latter genus, while the genera containing mellein derivatives in their cultures are more closely related to *Hypoxylon*. The current status of these attempts to reconstruct the phylogenetic affinities within the Xylariaceae is still rather preliminary because many of the known taxa have not been included, and different portions of the DNA were used in independent studies, sometimes with contradictory results. The data presently available appear insufficient to challenge the current generic organisation in the Xylariaceae.

Further clarifications could be obtained by the

- inclusion of additional taxa, especially of those that show phenotypic traits that are interpreted as either basal or advanced;
- a concise multigene genealogy using a significant numbers of representatives of all known morphological lineages and chemotypes;
- clarification of the relationship of genera with *Geniculosporium*-like anamorphs to the large, heterogeneous genus *Xylaria*, and inclusion of other representatives of those genera currently included in the Xylariales showing intermediate morphological features between the Xylariaceae and other families.

A thorough revision of the Xylariaceae using molecular phylogenetic data does not appear feasible at this time, because no representatives of important taxa, including ca. 80% of the described species and about one third of the accepted genera remain to be sequenced. Not even the type species of important genera have been clearly defined by molecular data. This problem was addressed in another molecular study with emphasis on xylarioid taxa (Peršoh et al. 2008). It revealed that *Xylaria hypoxylon*, the type species of *Xylaria*, was ill-defined. This species has always been regarded as complicated and complex, and cosmopolitan. Teleomorphic as well as anamorphic features were reported independently by various researchers, but the results are contradictory to some extent. A concise study of the holomorphic morphology of various specimens from around the world was never carried out before. As mentioned in the Introduction, the first description of this species was based on material from Europe, which was most probably collected in Sweden. Our comparison of molecular data based on 5.8S/ITS nrDNA revealed that the data published in GenBank on *X. hypoxylon* obviously correspond with many different species. Little variations were observed among the 5.8S/ITS nrDNA sequences derived from the most common European morphotype of this species complex, which were newly generated by us based on material from France, Germany and Sweden. However, only a small percentage of the published DNA sequence data was found similar to the European genotype. Moreover, various cultures in public collections that had been sequenced in the past were found to correspond with different species, or to be degenerate. This especially concerns the strains used in the study on the molecular phylogeny of *Xylaria* by Lee et al. (2000). The genotype most frequently referred to in the literature corresponds to a culture which was deposited by Chacko & Rogers (1981) and only tentatively referred to as *X. hypoxylon* by the authors. DNA sequences derived from this material were published by Spatafora & Blackwell (1993) and since then used as references (and representative of Xylariaceae and Xylariales) in numerous molecular phylogenetic studies. In fact, this fungus most probably belongs to *X. longiana* Rehm, which remains to be recorded from Europe. The results of Peršoh et al. (2008) gave impetus to attempt a concise re-typification of *X. hypoxylon*, based on representative European material, which is currently pending. Our intention is to select a fresh representative specimen that can be cultured, studied for anamorphic morphology and HPLC profiles, sequenced, and used in modern taxonomy and phylogeny. This might facilitate the future revision of the genus *Xylaria*, once a stable concept of the type species has become available. However, a morphological comparison of specimens from the collections of Linnaeus, E. Fries, and J. Dillenius, and a critical review of the respective taxonomic literature needs to be carried out Such work is presently on going in preparation of a subsequent publication. Three additional European species of *Xylaria*, two of which are similar to *X. hypoxylon*, have meanwhile been described by Fournier et al. (2011).

Applications of analytical chemistry and biological assays

From the results of the HPLC profiling study, it soon became apparent that it was not possible to cope with the wealth of information provided by the analytical HPLC-DAD and HPLC-MS data, unless we would be able to get further information based on chemical structures. Various apparently specific metabolites were detected in the stromata of these fungi, which could not be identified for lack of standards. Therefore, we started to collect larger amounts of the stromata on sites where we had previously found them in abundance. Observations throughout the vegetation periods to analyse the succession of metabolite production in the stromata (see below) aided to determine the optimal time for collection of material for preparative studies. From collections of several "common" species of Europe and Taiwan, sufficient quantities of stromatal extracts to allow for preparative studies were finally obtained. The results are summarised briefly for each single species; extensive overviews on most of these results were published in the reviews by Stadler & Hellwig (2005) and Stadler & Fournier (2006). Only representative (and preferentially, novel) chemical structures are shown here, out of a total of ca. 100 secondary metabolites that were isolated from the targeted species.

Daldinia concentrica was collected in large amounts from a site in the Neanderthal for preparative chromatographic work. In the course of a diploma thesis, Baumgartner (2001) isolated an unprecedented linear polyhydroxylated squalene, named concentricol (Stadler et al. 2001a). This terpenoid (Fig. 14) is prevalent in the stromata of *D. concentrica* and a group of related species (i.e., the *D. concentrica* complex). Subsequently, three further congeners of this type were identified from the stromata of *D. concentrica*, along with ten further metabolites, five of which were new to Science at the time of publication (Quang et al. 2002a,b). One of the most interesting compounds among the stromatal constituents of *D. concentrica* is concentricol D, which bears a rather unique cyclisation in its ring system. The concentricols are not produced in cultures of *Daldinia* spp. They did not exhibit any significant activities in the biological test systems which we have so far employed. Their natural function, and the reason why they specifically accumulate in stromata of *D. concentrica* and related species remains obscure.

Hypoxylon fuscum is one of the most frequently encountered ascomycetes in Europe (cf. Petrini & Müller 1986, Granmo 1999). Fide Ju & Rogers (1996), it occurs throughout the Northern hemisphere. Stromata collected in Germany from *Corylus avellana* were extracted and subjected to analytical studies (Quang et al. 2004b). They yielded BNT, daldinal A and daldinin C, and two new azaphilones related to daldinin C, which we named daldinins E and F (Fig. 11). Only the latter two compounds are apparently specific for *H. fuscum* and some of its allies, whereas Daldinin C also occurs in *H. rubiginosum*, *Daldinia childiae*, and a series of other daldinoid Xylariaceae. The daldinins represent the prevailing pigments in a broad range of other *Hypoxylon* spp. as will be outlined elsewhere. Collections of *H. fuscum* from *Alnus* and *Betula*, as well as some specimens from *Corylus* in USA, however, contain smaller amounts of the daldinins and have additional pigments which have so far not been identified. These specimens with deviating HPLC profiles also differ in their ascospore size range from the "chemical race" on *Corylus* found in Europe. It was so far not possible to obtain a conclusive segregation of the *H. fuscum* complex, aside from the recognition of *H. porphyreum* Granmo and other specimens, which are associated with Fagaceae, rather than Betulaceae (Granmo 1999, Stadler et al. 2008b).

The characteristic stromatal pigments of ***Hypoxylon rubiginosum***, which has effused-pulvinate stromata featuring orange stromatal pigments and is widely distributed in the Northern hemisphere, were isolated and identified. The species was targeted because HPLC profiling revealed chromophores of the major components, which were different from those of the known mitorubrins. The major constituents were identified as novel natural products, for which the trivial names, rubiginosin A–C (Fig. 11), were proposed (Quang et al. 2004a**)**. Another congeneric pigment of this type, entonaemin A, appears chemically related to them. It was previously

obtained from an *Entonaema* sp. (Hashimoto & Asakawa 1998). The azaphilone backbone of the rubiginosins is identical to that of the mitorubrins, while the substitution by orsellinic acid via an ester bond occurs at a different carbon atom. As shown in Fig. 10, DAD-based HPLC profiling can easily discriminate the two resulting types of mitorubrin vs. rubiginosin chromophores, despite both types of azaphilones give orange colours in 10% KOH. As such HPLC profiling data are species-consistent, the identification of the rubiginosins was an important prerequisite for the above described chemotaxonomic evaluation of the *H. rubiginosum* complex. Daldinin C (see above) was also found in *H. rubiginosum* in small amounts. Rubiginosin A turned out to be widely distributed in the genera *Hypoxylon* and *Entonaema* and proved to be an important chemotaxonomic marker.

The relatively rare species ***Hypoxylon rutilum*** Tul. & C. Tul. is characterised by scarlet pigment granules beneath its stromatal surface. The scarlet pigments are the rutilins, which were also detected in other *Hypoxylon* spp. which have similar features (Quang et al. 2005b). The rutilins (Fig. 11) are dimeric azaphilones composed of monomers, reminiscent of mitorubrinol acetate and rubiginosin A, which were also observed in rather large quantities as co-metabolites in the stromata. Interestingly the dimeric azaphilones were found devoid of antimicrobial and cytotoxic effects, but Quang et al. (2006c) tested them positively as inhibitors of nitric oxide synthesis. Hence, they apparently have pronounced antioxidant activities and may eventually become useful as templates for new cardiovascular drugs.

Hypoxylon perforatum (Schwein.) Fr. is one of the species which was synonymised with *H. rubiginosum* by Miller (1961). It was revealed by Ju & Rogers (1996) to have greenish yellow stromatal pigments (rather than the orange pigments of *H. rubiginosum*). From this species, as well as from a Taiwanese collection of *H. hypomiltum* Mont., a major pigment named hypomiltin (Hellwig et al. 2005) was obtained. This azaphilone (Fig. 11) has a very similar molecular structure to mitorubrinol acetate. The only difference between these molecules is a reduced double bond in the azaphilone ring system. The chromophore is thus altered as compared to the "orange" rubiginosin and mitorubrin chromophore, which explains the different colours of this pigment (for DAD spectrum of hypomiltin, see Fig. 10). *Hypoxylon trugodes* and several other species of this genus, but even *Pyrenomyxa picea*, also contain hypomiltin, and a number of further derivatives with the same chromophore, but deviating molecular masses were observed by HPLC profiling in those species, which probably also correspond with novel hypomiltins.

Hypoxylon macrocarpum Pouzar, only segregated by Pouzar (1976) as a separate taxon of the aggregate species "*H. rubiginosum"* sensu Miller (1961), is actually quite frequent in Europe and America but probably its stromata are still being frequently overlooked. The species was found to contain BNT and three specific compounds, which were revealed to possess an unprecedented polyketide carbon skeleton with an orsellinic acid-like moiety attached (Mühlbauer et al. 2002). The specificity of the macrocarpones chemical structures see, e.g. macrocarpon (Fig. 11). *H. macrocarpum* was meanwhile largely confirmed. They do apparently not occur in most other species of the *H. rubiginosum* complex. We only found them in a specimen of *Hypoxylon* cf. *anthochroum* from tropical Africa (Stadler et al 2008b), and in *Pyrenomyxa picea* (Stadler et al. 2004a, as *Pulveria porrecta*). Interestingly, *H. macrocarpum* did not contain any rubiginosin or mitorubrin type azaphilone pigments, as meanwhile established from HPLC profiling of more than 1000 specimens.

Hypoxylon carneum Petch is another species of the *H. rubiginosum* complex which contains exceptionally small amounts of rubiginosins, which can only be detected by HPLC-MS in the crude extracts. The species was studied by HPLC and found to contain BNT and a number of highly specific compounds, which are not pigments, in large amounts. Two of these compounds were identified as carneic acids (Figs 11A, B), two antibiotics chemically related to phomopsidine (Quang et al. 2006a). At least ten further putative novel carneic acid derivatives were detected in the stromatal extract by HPLC-MS, but we have yet to observe carneic acid in

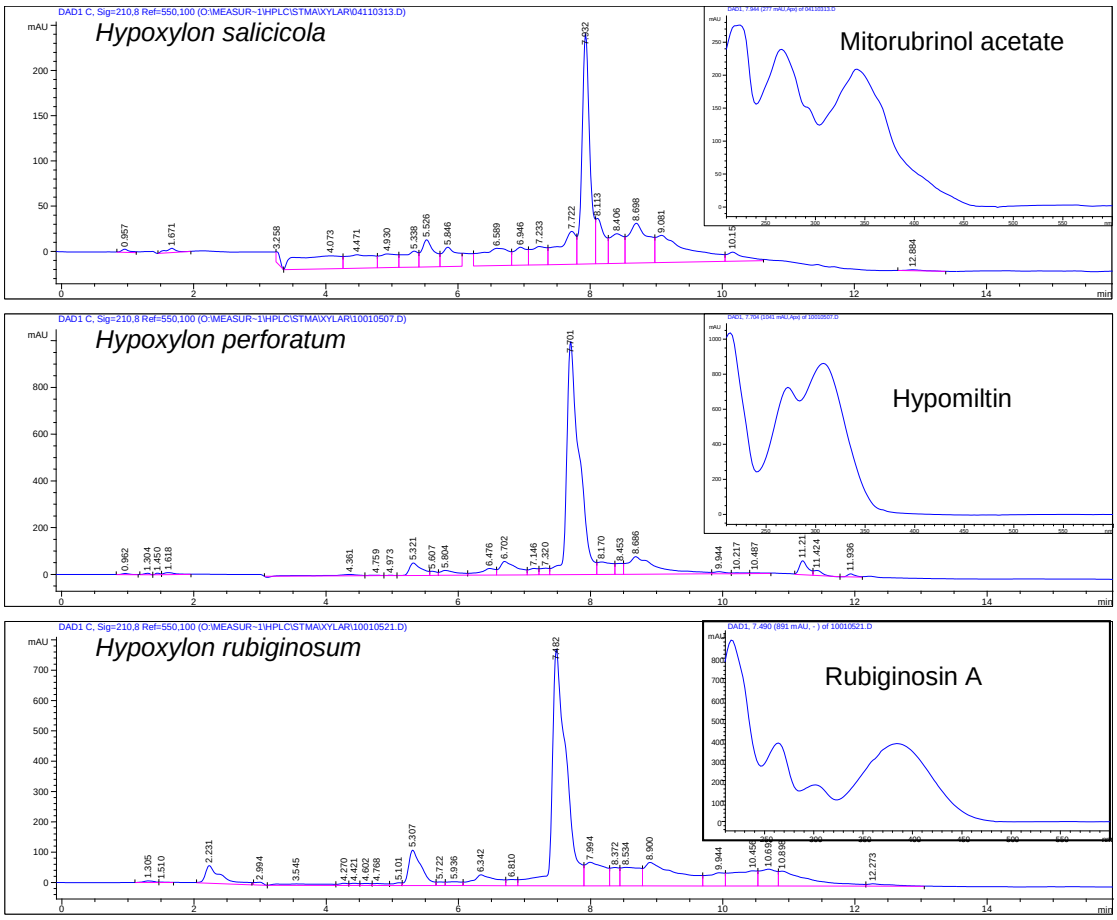


Fig. 10 – Comparison of the stromatal HPLC-UV chromatograms (210 nm) of three *Hypoxylon* species belonging to the *H. rubiginosum* complex, including characteristic UV-visible spectra of characteristic azaphilone pigments. The stromata of these species show a rather similar morphology, whereas their major extractable pigments are rather easy to discriminate by diode array analyses.

other species of *Hypoxylon*.

Annulohypoxylon cohaerens (Pers.) Y.M. Ju et al. and ***A. multiforme*** (Fr.) Y.M. Ju et al. were also subjected to preparative studies, as the pigments of these species were found by HPLC profiling to differ very much from those of the above described species, except that BNT was also present in their stromata. A series of azaphilones named multiformins or cohaerins (Fig. 12) after the respective producer species, were identified (Quang et al. 2005c,e, 2006b).

Creosphaeria sassafras (Schwein.) Y.M. Ju et al. was included in the genus *Hypoxylon*, until Ju et al. (1993) noted its *Libertella*-like anamorph. Indeed the hypoxyloid stromata even contain pigment granula below the stromatal surface. These pigments were isolated and identified as sassafrins A-D (Quang et al. 2006d). Interestingly, the carbon skeleton of these compounds is quite different from those of the pigments of *Hypoxylon* and *Annulohypoxylon*, and only the azaphilone chromophore is similar. Sassafrin D even represents an unprecedented carbon skeleton.

Bitzer et al. (2007) have illustrated the methodology now available for effective dereplication of natural products by analytical HPLC-DAD and HPLC-MS. "Dereplication" is equivalent to the early stage identification of secondary metabolites in crude extracts from natural sources, aided by spectral libraries and special software programs. As the methodology was validated and further developed in part through our chemotaxonomic studies, the examples used for illustration of the dereplication process were taken from the Xylariaceae project. Aside from some metabolites of *H. fragiforme*, the evaluation of the tropical species ***Annulohypoxylon urceolatum*** (Rehm) Y.M. Ju et al. was also described. This species had shown a rather specific HPLC profile

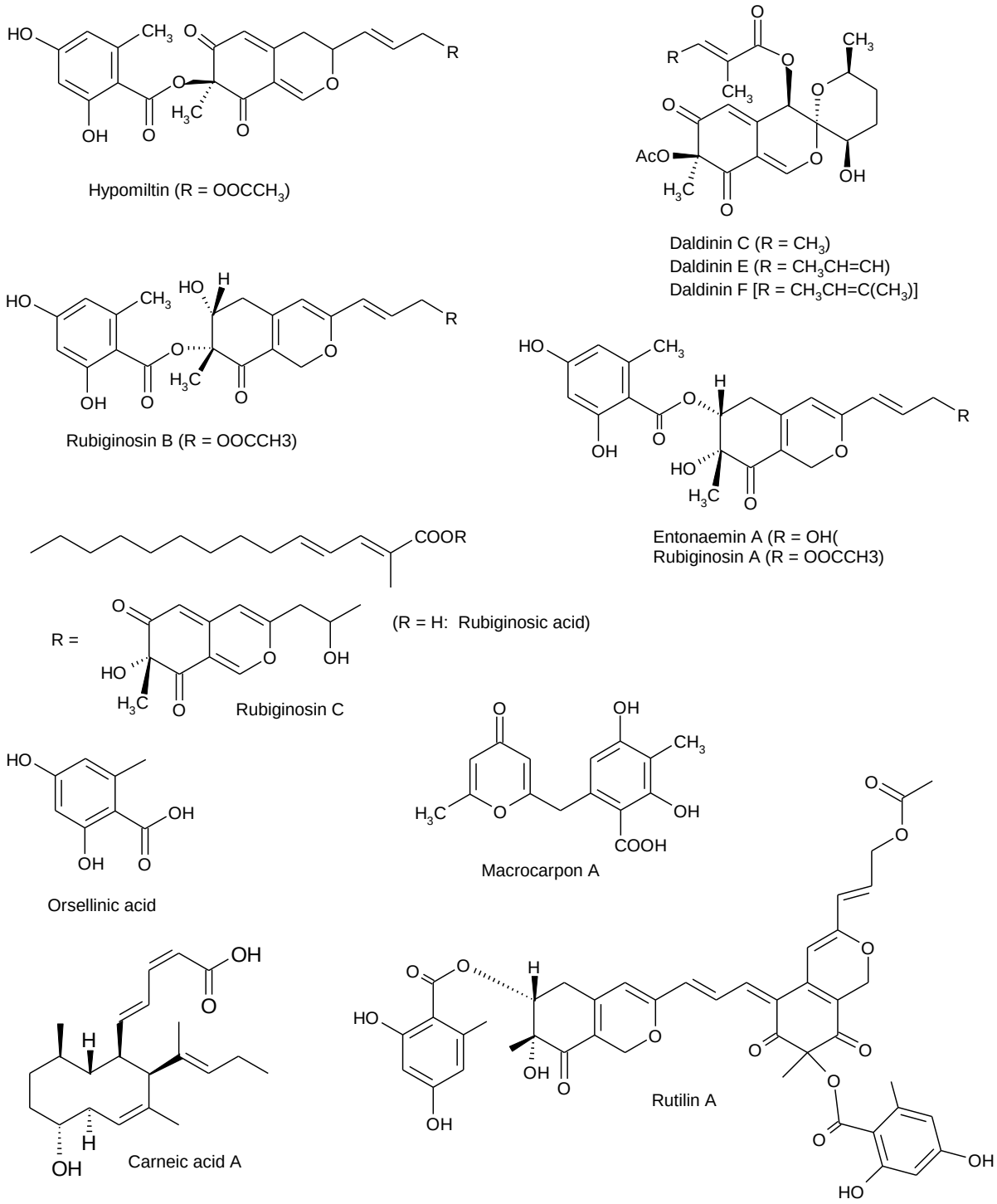


Fig. 11 – Chemical structures of metabolites from stromata of *Hypoxylon species*.

(Hellwig et al. 2005). Aside from the ubiquitous BNT and the naphthoquiinone derivative hypoxylone, a novel binaphthalene was obtained and named urceolone (Fig. 14), after the producer organism. These compounds are now available as standards for concise identification of hitherto unknown metabolites in the crude extracts of tropical Xylariaceae in the course of our ongoing chemotaxonomic work. Interestingly, compounds similar to urceolone are also present in *Hypoxylon monticulosum* Mont. and *H. submonticulosum* Y.M. Ju & J.D. Rogers, two species that were regarded basal in *Hypoxylon* by Ju & Rogers (1996). Hypoxylone was reported by Bodo et al. (1983) from stromata of *Hypoxylon sclerophaeum* Berk. & M.A. Curtis sensu Miller (1961), which was resolved into several species by Ju & Rogers (1996). This compound constitutes a naphthoquinone, which is, like

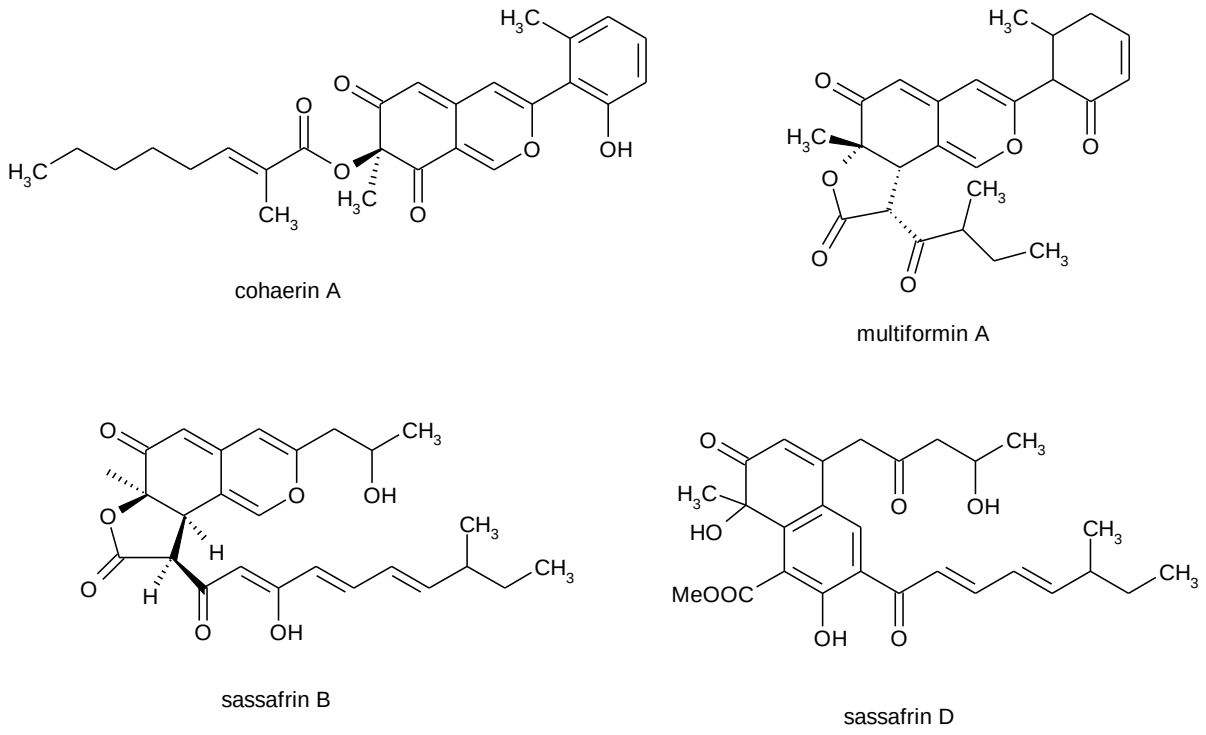
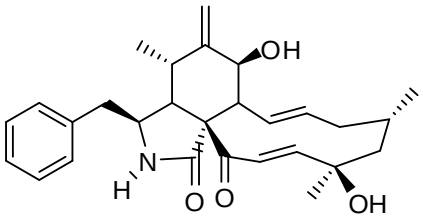


Fig. 12 – Chemical structures of stromatal pigments of *Annulohypoxylon* and *Creosphaeria* spp.

urceolone, conceivably derived from the same biogenetic pathway as BNT. The chemotaxonomic significance of these compounds is not yet fully understood, but remarkably they both constitute naphthoquinone derivatives. The biogenesis of such naphthoquinones from the 1,8-DHN (pentaketide) pathway of melanin biosynthesis is conceivable. Their biosynthesis could be accomplished via dimerisation of naphthol precursors in a similar manner as that of BNT and its oxidised derivatives and subsequent oxidations, as has been postulated for the perylene quinones, daldinone A and truncatone. Interestingly, compounds with HPLC-MS and HPLC-DAD spectra similar to those of hypoxylone occur in the stromata of some Xylariaceae, which are devoid of daldinones and truncatones but still show greenish pigments with 10% KOH. Identification of this unknown putative naphthoquinones will be important in the further chemotaxonomic evaluation of those Xylariaceae taxa that preferentially produce naphthalene-derived compounds in their stromata. Those include, for instance, *Daldinia placentiformis* (Berk. & M.A. Curtis) Theissen, *Hypoxylon nicaraguense* Ellis & Everh., and morphologically similar species.

Antimicrobial and other biological activities were already included in some of the above cited papers dealing with the isolation and structure elucidation of the stromatal metabolites of Xylariaceae. An overview of a bioassay of several representatives against human pathogenic microbes (Quang et al. 2005a) revealed significant biological activities for most tested azaphilones. However, these effects were not selective against fungi or bacteria.

Two papers (Stadler et al. 2006, 2007) deal with functional aspects of the secondary metabolism in Xylariaceae, which are most remarkable in view of the possible ecological role and the natural functions of these compounds. These studies were initiated when HPLC profiling of stromata in different developmental stages had revealed inconsistencies that could at first not be easily explained by morphological data. Only when the stage of development was correlated to the respective HPLC profile, it became obvious that immature stromata and stromata bearing the anamorph had different HPLC profiles than the mature stromata. We therefore decided on monitoring the secondary metabolism of certain species in their natural environment throughout several consecutive vegetation periods and to pay more



Fragiformin B

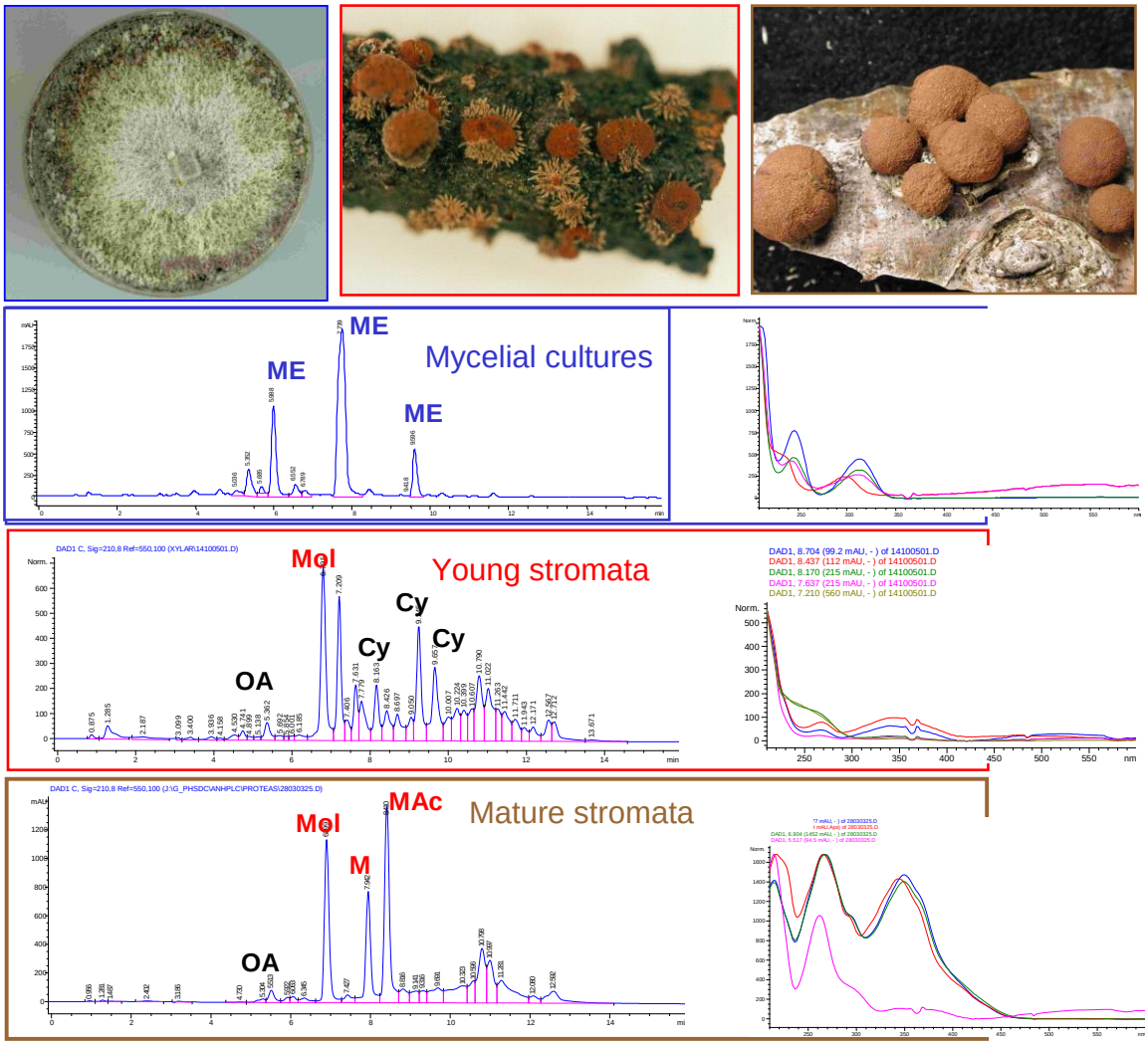


Fig. 13 – HPLC-UV chromatograms of mycelial cultures, young, and mature stromata of *Hypoxylon howeanum*, illustrating the succession of secondary metabolite production in different developmental stages of the fungus. Major metabolites (as identified by HPLC-MS): Cytochalasins (Cy; as exemplified by the chemical structure of fragiformin B, depicted above the young stromata)), mellein dihydroisocoumarins (ME), mitorubrinol (Mol), mitorubrin (M), mitorubrinol acetate (MAc), and orsellinic acid (OA). Notably, numerous yet unidentified metabolites were detected in the young, maturing stromata of the fungus, which also exhibited the strongest antimicrobial activities. Image of mature stromata by J. Fournier. From a lecture presented at the IMC 9, Cairns, Australia (2006).

attention to the stage of development when interpreting stromatal HPLC profiles.

Stromata of *Hypoxylon fragiforme* growing on different sites were studied for three consecutive vegetation periods by HPLC profiling, revealing changes in the composition during stromatal development (Stadler et al. 2006). Three cytochalasins (among those the new natural products, fragiformins A–B, see chemical structure of a representative in Fig.

13) were identified as major constituents of the young, maturing stromata, whereas mature, ascogenous material yielded large amounts of mitorubrins. The above compounds, further cytochalasins from Xylariaceae and other fungi, and additional azaphilones of the mitorubrin type were compared on their nematicidal effects against *Caenorhabditis elegans* and their antimicrobial activities against *Bacillus subtilis*, and various fungi. The results confirmed data in the literature on broad-spectrum non-selective activities of azaphilones and cytochalasins in biological systems.

Interestingly, laboratory cultures of the above *Hypoxylon* spp. mainly produced dihydroisocoumarin derivatives and were found devoid of mitorubrins and cytochalasins. A comparison of the HPLC chromatograms including illustrations of the different developmental stages, is provided in Fig. 13. The extracts from the young, growing stromata also yielded a series of further unknown components that were neither detected in the cultures nor in the mature stromata (see HPLC-UV chromatogram in the centre of Fig. 13). The strong antimicrobial activities detected in such samples provide some evidence on the natural function of this onset of secondary metabolism during stromatal ontogeny, which is also associated with the rapid colonisation of the woody substrate. Certainly, these results were also rather interesting for applied mycology, in view of future options to fully explore the repertoire of fungi to produce unprecedented, useful secondary metabolites and discover novel lead compounds by targeting these organisms at their most productive stage of development.

The production of bioactive metabolites in young stromata is not restricted to the above mentioned species pair, since similar results were obtained on a broad range of other species of the Hypoxyloideae. Over fifty representatives of Xylariaceae and fifty of the above mentioned secondary metabolites were tested for antimicrobial and nematicidal activities.

The major constituents in the stromata were also quantified by HPLC-DAD, revealing yields of up to 10% of a single component in the dry fruiting body biomass. *Hypoxylon fuscum* may even contain up to 20% of secondary metabolites in its stromatal biomass. In several species, significant antimicrobial effects were noted upon direct incubation of the fruiting bodies in an agar diffusion assay, i.e., without any need for extraction and concentration of active constituents. From these studies, we concluded that most of the characteristic constituents of subfamily Hypoxyloideae are involved in non-specific defence reactions that underwent specific permutations in the course of evolutionary processes, resulting in a broad diversity of unique polyketides and other secondary metabolites.

In contrast, the fruiting bodies of representative species of *Biscogniauxia* and subfamily Xylarioideae generally contained no significant activities. Only cultures of most Xylarioideae exhibited antimicrobial effects, due to the presence of cytochalasins and other toxins, while extracts from cultures of most Hypoxyloideae were only weakly active. Usually, the highest yields of secondary metabolites and the strongest activities were observed in the growing stromata of Hypoxyloideae.

Phylogenetic significance of secondary metabolite production in cultures of Xylariaceae

Some of our recent papers dealt with an extensive polyphasic evaluation of specimens and cultures which had been studied over the past years. The paper by Bitzer et al. (2008) resulted from chemotaxonomic studies of over 150 cultures selected from 600 strains of Xylariaceae. The fermentation of the fungi was optimised in several different culture media, and samples were taken during the time course of fermentations to monitor the onset of secondary metabolite production. Two new chemotypes were observed in cultures of *Phylacia sagrana* Mont. (Fig. 16), and *Hypoxylon nicaraguense* (Fig. 15). and *H. polyporus* (Starbäck) Y.M. Ju & J.D. Rogers, respectively. While the *Phylacia* culture appeared similar to *Daldinia*, the other two species strongly deviated from over 50 taxa studied concurrently of the genus *Hypoxylon*. This gave impetus to scale-up of fermentation, isolate and identify the characteristic, apparently specific metabolites.

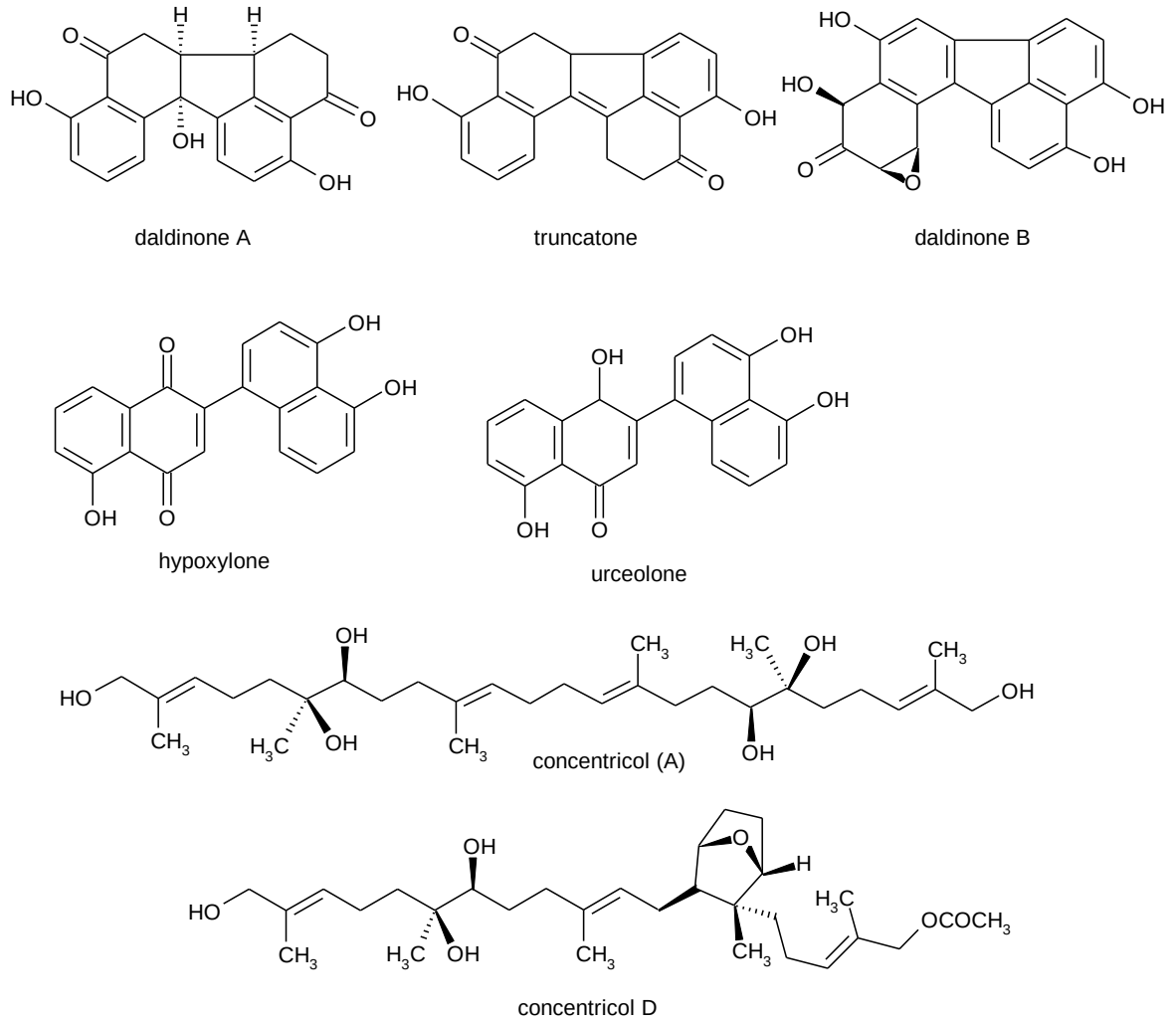


Fig. 14 – Chemical structures of characteristic stromatal pigments of the Xylariaceae derived from 1,8-dihydroxynaphathalene biosynthesis, and of the concentricol sesquiterpenes from *D. concentrica*.

The *Phylacia* culture was found to contain several major metabolites that also prevail in *Daldinia.* In addition, it also produced eutypinol derivatives and was devoid of 8-methoxy-1-naphthol as major differences to *Daldinia*. A concurrent molecular study based on 5.8S/ITS nrDNA sequence data demonstrated that these differences in the secondary metabolite profiles were reflected by similarity analyses of molecular data. Interestingly, the *Daldinia* sp. that appeared most closely related to *P. sagrana* (i.e., *D. caldariorum* Henn.*)* as inferred from the molecular phylogeny also produced eutypinols under the chosen, standardised fermentation conditions. As meanwhile revealed from a subsequent study (Stadler et al. 2010a), in which an additional *Phylacia* sp. and three *Thamnomyces* spp. were cultured and studied in a similar manner, the genus *Thamnomyces* is also closely allied to *Phylacia* and *Daldinia*, despite its aberrant stromatal morphology. The cultures of *Phylacia* were found to have a highly similar morphology and secondary metabolism as those of *Thamnomyces*. The relationships postulated by the previous comparison of stromatal HPLC profiles (Stadler et al. 2004a) were thus confirmed. Interestingly, representatives of the genus *Daldinia* became split into two different clades in a phylogenetic tree included in Stadler et al. (2010a**)**. One of these clades comprised representatives of *Phylacia* and *Thamnomyces*, along with some tropical representatives,

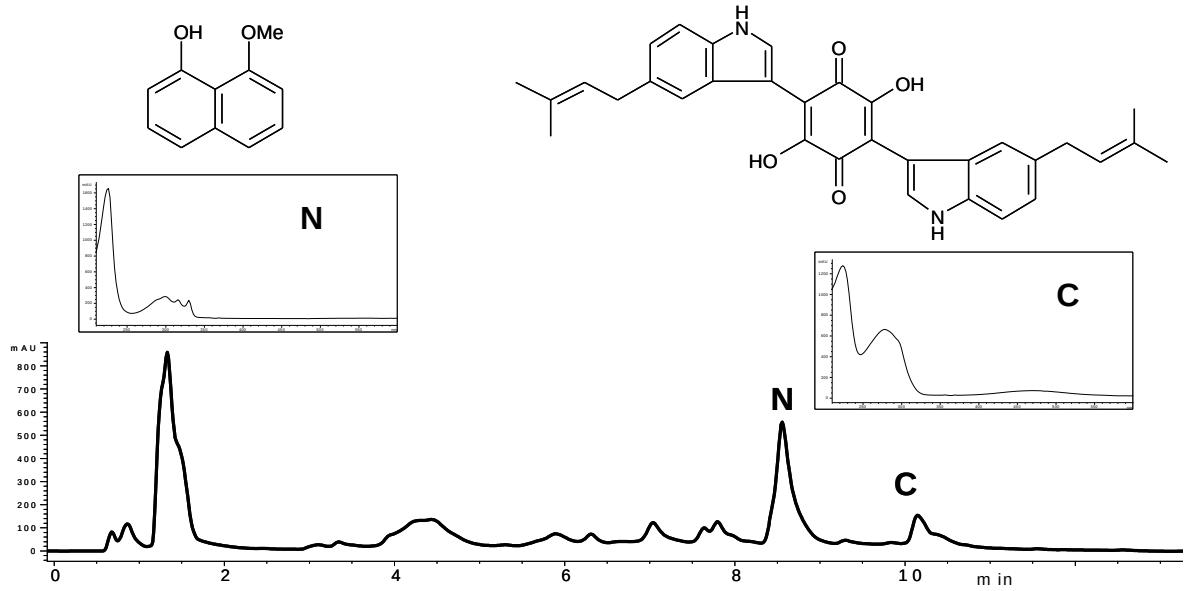


Fig. 15 – HPLC-UV chromatogram of an ethyl acetate extract from a culture of *Hypoxylon nicaraguense* (HLX medium, 168h, revealing cochliodinol (**C**) and 8-methoxy-1-naphthol (N). Mellein derivatives were not detected. Modified from Bitzer et al. (2008).

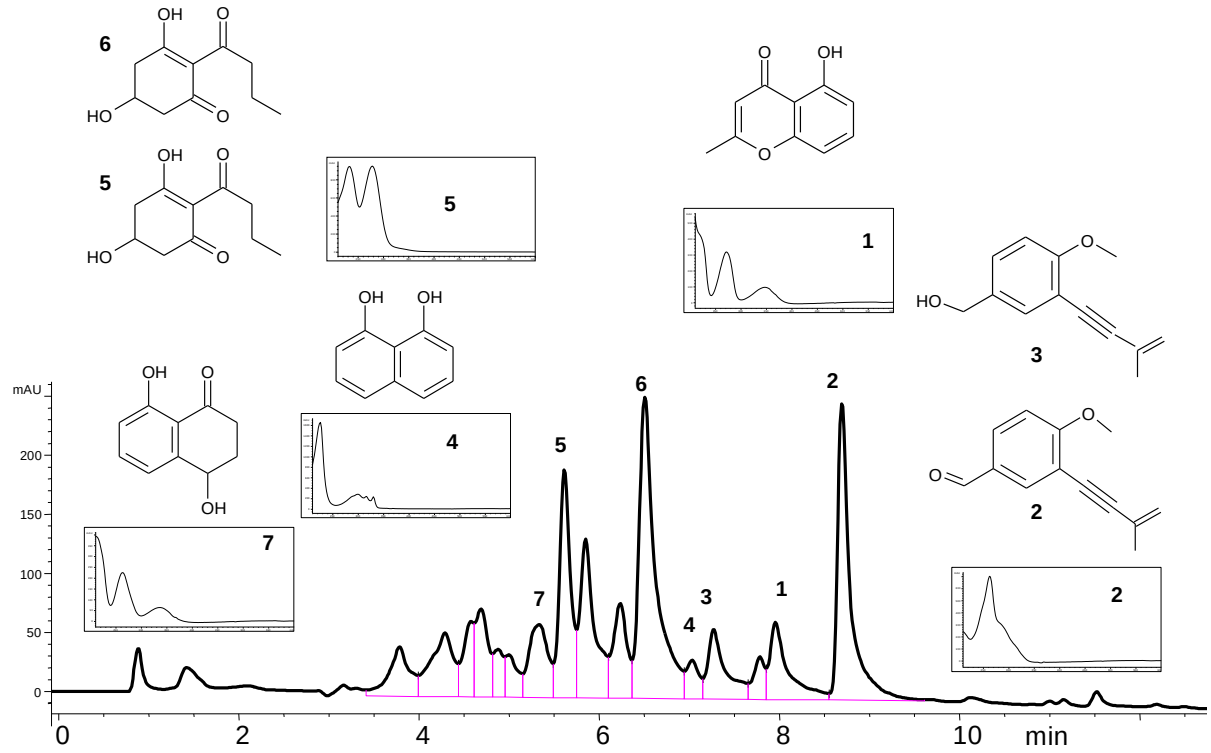


Fig. 16 – HPLC-UV chromatogram (210 nm) of ethyl acetate extract prepared from a culture of *Phylacia sagrana*, including DAD spectra and corresponding chemical structures of some of the major components. Modified from Bitzer et al. (2008).

whereas the other comprised clade comprised the temperate *Daldinia* spp. and *Entonaema liquescens*. Furthermore, these two clades did not appear nested in the clade containing representatives of *Hypoxylon* and *Annulohypoxylon*. These results disagree with the Parsimony Ratchet analysis (Fig. 7) and with other phylogenetic studies based on 5.8/ITS nrDNA sequence data. They need to be confirmed through the inclusion of further taxa, hence they are not discussed here at length.

The two above mentioned *Hypoxylon* species shared with *Daldinia* and *Phylacia* the production of 8-methoxy-1-naphthol but

additionally produced aromatic alkaloids of the cochliodinol type. Similar compounds had previously only been obtained from the unrelated genus *Cochliobolus* (Pleosporaceae) and from a *Nodulisporium* sp. with unknown teleomorph connections. In the preliminary molecular phylogeny, their 5.8S/ITS nrDNA sequences formed a cluster that appeared derived from the clades representing other members of *Hypoxylon*.

Two different specimens that matched the concept of *Hypoxylon placentiforme sensu* Ju & Rogers (1996) and their corresponding cultures were studied for comparison, revealing several interesting facts: a) in accordance with their deviating stromatal metabolite profiles and ascospore size ranges, they clustered far apart from one another in the molecular phylogeny; b) in agreement with the recent revision by Hsieh et al. (2005), who transferred *H. placentiforme* to *Daldinia*, resurrecting the name *D. placentiformis*, they showed similar HPLC profiles as all *Daldinia* spp. studied concurrently. The observed affinities of *Daldinia*, *Phylacia*, and *Thamnomyces* as revealed by chemotaxonomic and molecular data gave rise to reject the concept of a family "Phylaciaceae" (Speer 1980) for cleistocarpous genera with affinities to the Hypoxyloideae, as the affinities of *Pyrenomyxa* to the *H. rubiginosum* complex (see above) were confirmed. Cleistocarpous stromata have obviously evolved at least twice in separate lineages of the Hypoxyloideae. In Table 3, morphological and chemotaxonomic traits of the daldinoid and hypoxyloid Hypoxyloideae have been summarised.

Outlook

The above results have helped to settle various questions regarding the taxonomy of the Xylariaceae, but there still remains a lot of work to do. The steadily increasing matrix of molecular, morphological and chemotaxonomic data will facilitate further monographic studies. The most important topics to be addressed in the near future have been summarised below in relation to the objectives of our polyphasic studies.

Many of the characteristic pigments and other chemotaxonomically significant secondary metabolites of the genera *Daldinia*, *Hypoxylon*, and their closest allies have been identified and made available for HPLC profiling studies. However, additional metabolites remain to be identified, e.g., from cultures of the Xylarioideae, and from stromata of certain species of *Annulohypoxylon*, *Hypoxylon*, and other genera. For instance, the blue-green pigments of *Chlorostroma subcubisporum* and *Hypoxylon aeruginosum* J.H. Miller and the pigments of *Daldinia cuprea* Starbäck are rather specific according to unpublished HPLC profiling results, and no close matches were found when their spectra and retention times were matched with the database of Xylariaceae metabolites. However, these species appear to be extremely rare and/or have relatively small stromata. The option to culture them in the laboratory and isolate their constituents from artificially produced stromata should be further explored in future.

Chemotaxonomic data on several thousands of specimens and several hundreds of cultures of Hypoxyloideae have been correlated to the morphological concepts. This work has also led to the recognition of further, undescribed taxa by resolution of species complexes. Some genera and species groups, however, still remain to be evaluated in-depth. One taxonomic problem to be addressed in the near future is the redefinition of the boundaries between *Hypoxylon* and *Daldinia*. The inclusion of *D. placentiformis* in *Daldinia* as inferred from molecular data and chemotaxonomic results is problematic because this species (which actually appears to constitute a species complex as deduced from the high variability of morphological traits, HPLC profiles, and DNA sequence data) does not match the generic concept of *Daldinia*, which was recognised and segregated from *Hypoxylon* by Cesati & Notaris (1863) based on the presence of concentric zones. Therefore, a revision and amendment of the generic description of *Daldinia* will also be envisaged in the near future. The genus *Rhopalostroma* also remains to be studied further based on fresh material (Stadler et al. 2010b), and the same holds true for certain tropical taxa of *Hypoxylon*, where the available morphological and chemotaxonomic data point toward the presence of additional species complexes. As demonstrated above by the revisions of

Entonaema and *Pyrenomyxa*, a taxonomic revision of such "problem taxa" makes more sense, once cultures and molecular data have become available. However, this will afford the availability of fresh material. Additional field work, especially in tropical countries, will therefore be indispensable.

The concurrent molecular phylogenetic studies have also proved most useful to verify morphological concepts in the Xylariaceae. Ribosomal DNA sequence data were shown to reflect the evolutionary history of the Xylariaceae at generic and species level in many instances, and they were even found to be in accordance with chemotaxonomic results. The results on the molecular phylogeny already published constitute only a small part of the molecular work that has already been accomplished, but remains to be disclosed. Ribosomal DNA sequences of most of the strains belonging to the Hypoxyloideae studied for metabolite production in culture (Bitzer et al. 2008) were already obtained. Representatives of some additional, small genera such as *Lopadostoma* Pouzar, *Thuemenella* Penz. & Sacc., and *Vivantia* J.D. Rogers et al. were recently made available. Respective data for a large number of representatives of xylarioid genera, such as *Rosellinia* and *Nemania*, that had so far not been represented sufficiently, were also recorded from strains available in public collections as well as via our collaboration network. The DNA sequence data will also serve to detect the respective taxa in plant material and other environmental samples, using phylochips, microarrays and related PCR-based methodologies to facilitate further studies aimed at clarification of the molecular ecology of these fungi. Another option for future work could be to include data from additional genes, such as the large subunit of the ribosomal gene, the IGS region and non-ribosomal genes to create a multi-gene genealogy of Xylariaceae.

Various correlations could already be established between the observed chemotypes and the current knowledge on the biosynthesis of the corresponding metabolite families that are specifically produced by the Xylariaceae. One task for the future could be to confirm these phenotype-based correlations by studying the genetic background of their biosynthesis. In this context it is important to keep in mind that the biosynthesis of these secondary metabolites is mediated by modular gene clusters, such as PKS and NRPS, the former being apparently more widely distributed in the species we have so far studied. These gene clusters contain preserved moieties, which can be used as templates for primers to screen the genomic DNA of their producer organisms. For evaluation of the diversity of PKSs in a *Xylaria* sp. (Amnuaykanjanasin et al. 2005), two degenerate primer sets were designed, to amplify reducing PKS and PKS-NRP synthetase hybrid genes, respectively. Seven genes predicted to encode proteins homologous to highly reduced PKSs were identified, and additional four PKS gene fragments were found using different, previously published primers. Using these PKS fragments as probes to identify PKS genes from the genomic library of the *Xylaria* sp. led to full-length sequences for five PKS genes. The authors discussed the utility of their approach to further reveal the genetic potential of fungi with regard to the production of polyketides. Although they did not finally correlate the function of any of the PKS genes to the production of particular metabolites, the outcome of their study appears promising. The lack of detection of a certain compound may relate to the absence of respective gene clusters, but it remains possible that the respective genes are present but down-regulated or the gene cluster has become non-functional. It remains to be proven whether the differences seen by HPLC profiling can be correlated to differences at the genetic level. For instance, members of the genus *Hypoxylon* that produce mellein in culture could as well have genes for naphthol biosynthesis, as many of them produce the binaphthyl BNT in their stromata.

Differences in these "phenochemotypes" of various Xylariaceae become even more evident when the carbon skeletons of the prevailing metabolites from their cultures (Fig. 17) and their stromatal pigments (Fig. 18) are compared. The chemical structures of selected compounds have been reduced to the corresponding carbon skeletons in these figures a similar manner as accomplished previously by Turner (1970), who illustrated different types of polyketides and interpreted the putative

biogenesis of many fungal polyketides, based on biosynthetic studies using radioactively labelled precursors. As such studies yet remain to be done on the majority of Xylariaceae metabolites, the discussion presented is highly hypothetical. In Fig. 17 it is clarified that the melleins from cultures of *Hypoxylon* and other genera are derived from entirely different folding types than those of the prevailing metabolites in cultures of *Daldinia*. In Fig. 17, some possible origins of the polyketide-derived pigments from one or two PKS gene clusters have been postulated. Thus, BNT is assumed to be derived from condensation of two monomeric naphthalene-like molecules, resulting in the formation of new C-C bonds. For daldinin C and daldinal A, which co-occur in several species of *Daldinia* and *Hypoxylon*, an octaketide origin is assumed. The mitorubrins are probably hexaketides, esterified with the tetraketide, orsellinic acid, hence their biogenesis would depend on two different PKS gene clusters. It should be highly interesting to elucidate the genetic background of this phenomenon, using methods of comparative functional genomics to elucidate the structures and functions of the PKS genes and study their regulation. Notably, the production of azaphilone pigments appears to be correlated with formation of stromatal primordia and precedes the production of the teleomorph in those species that have been studied by HPLC profiling in the natural environment.

Discussion

The work summarised in the present thesis relates to two major topics, which are closely related to one another:

- Employment of modern analytical chemistry to evaluate fungal chemotaxonomy.
- Establishment of an integrative approach to characterise and assess the functional biodiversity of fungi.

Based on the Xylariaceae example, these topics will now be discussed in a broader context.

As mentioned in the Introduction, there is accumulating evidence that fungi show a very high diversity and have an immense importance for the functions of all ecosystems. They contribute substantially to the global biological diversity, but their cryptic lifestyle may often render their detection rather difficult. Mueller et al. (2004) have summarised the current state of the art on identification methodology, and provided numerous protocols and suggestions for conduction of mycological biodiversity inventories, from various kinds of habitats. This manual demonstrates the great need of interdisciplinary teamwork for comprehensive monitoring of the mycobiota in a given habitat or geographic region. The use of standardised methodologies and the concise documentation of specimens and data are strongly encouraged, to facilitate the comparison of results from different areas. Special emphasis is given on how to isolate fungal cultures from various environments and their preservation.

As fungi are associated with virtually all plants and animals, and even add substantially to the microbial communities in soil, freshwater and marine habitats, "all taxa mycodiversity inventories" are certainly not easy to perform. Depending on the situation, close collaborations of mycologists with other biologists are indispensable, since host affinities and other aspects of ecology can otherwise hardly be addressed. Where direct collaborations are not feasible, the Internet comes in handy to summarise data from different categories, interlink them and thus make specialist knowledge available to non-specialists. The use of analytical chemistry in mycology also relates to specialist knowledge. Fungal pigments, volatiles and toxins are traditionally important in taxonomy, and diagnostic chemicals like Melzer's reagent and KOH are widely used in fungal identification. Nevertheless, modern analytical methods like HPLC are only available in a few mycological laboratories. No chapter on the utility of chemical profiling techniques is included in the above cited manual by Mueller et al. (2004).

Even in the contemporary literature, the occurrence of specific secondary metabolites is still often being attributed to certain "*formae specialis*", "*host-specific varieties*", or "*strain-specific*" properties, and not regarded as taxonomically significant. Our own studies confirmed that strain-specific metabolite

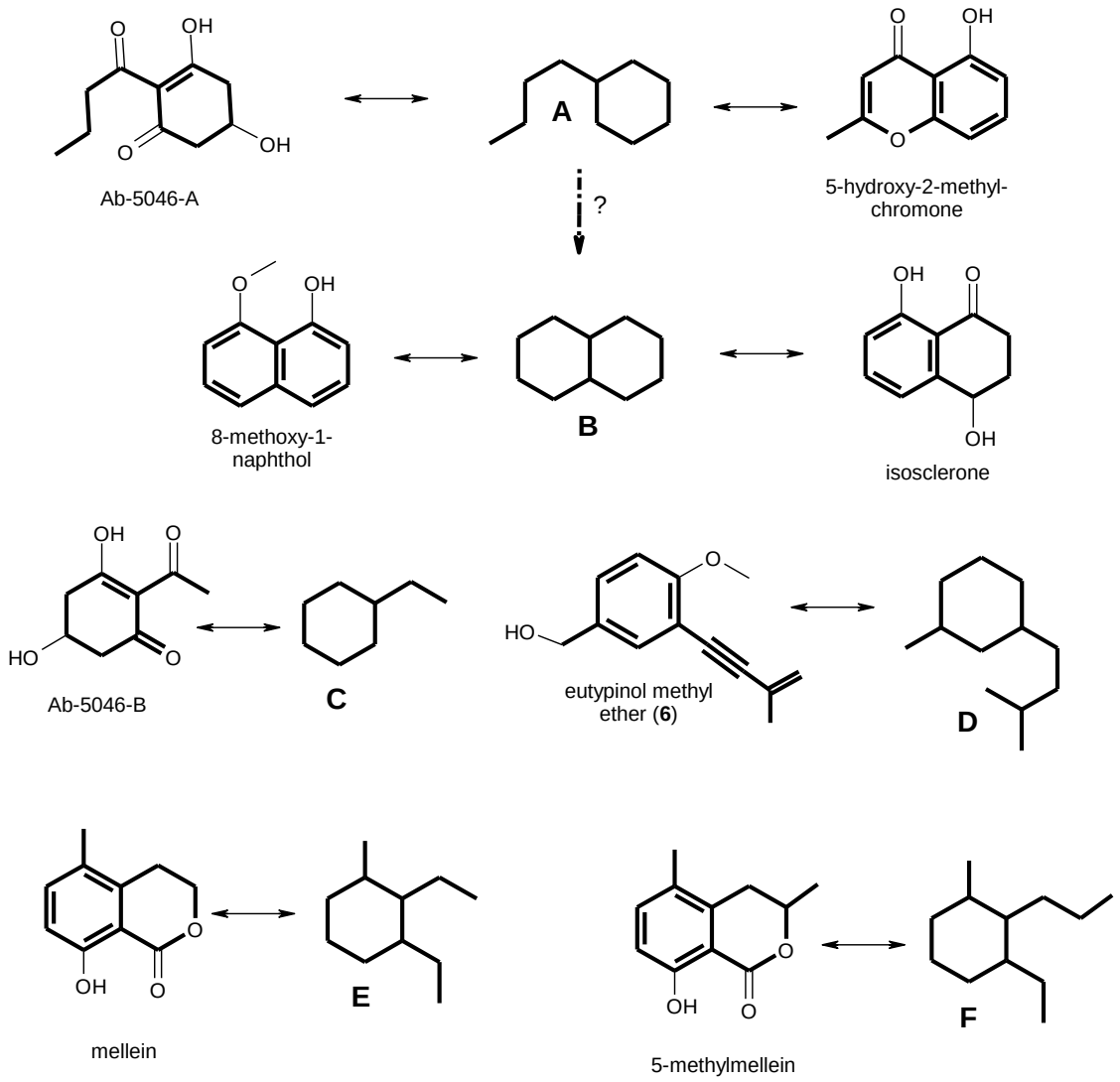


Fig. 17 – Differences in the carbon skeletons among the putative polyketides, indicated by bold letters, that are prevalent in cultures of the genera *Daldinia*, *Entonaema*, *Phylacia*, and *Thamnomyces* (**A**-**D**) and *Annulohypoxylon*, *Biscogniauxia*, *Camillea*, *Hypoxylon and Pyrenomyxa* (**E**, **F**) respectively). One representative metabolite is depicted and connected by an arrow to the respective carbon backbones. For classification of these molecules and theoretical considerations, see Turner (1971). The fact that the occurrence of the carbon skeletons A-D is apparently restricted to those taxa that are devoid of the skeletons and folding types E-F is remarkable in view of the concurrently obtained molecular phylogenetic data. It is to be assumed that specific PKS gene clusters encode the biosynthesis of each carbon skeleton; whereas variations in the side chains may be mediated by different genes that are located outside these gene clusters.

production may occasionally occur, besides the production of the chemotaxonomically significant marker compounds. For instance, if biosynthetic gene clusters are disrupted by mutation, this may lead to accumulation of intermediate products of the original biosynthesis. The degeneration of cultures upon frequent subculturing may result in the loss of production of the characteristic compounds and was also occasionally observed in the Xylariaceae. In fact, the reliability of chemotaxonomic data will always be hampered by the fact that they relate to a dynamic, physiological process and is therefore highly dependent on standardised methodology. On the other hand, the same holds true for the classical morphology-based taxonomic concepts. Even molecular identification techniques will inadvertently have to rely on the availability of data from well-characterised "standard" organisms that

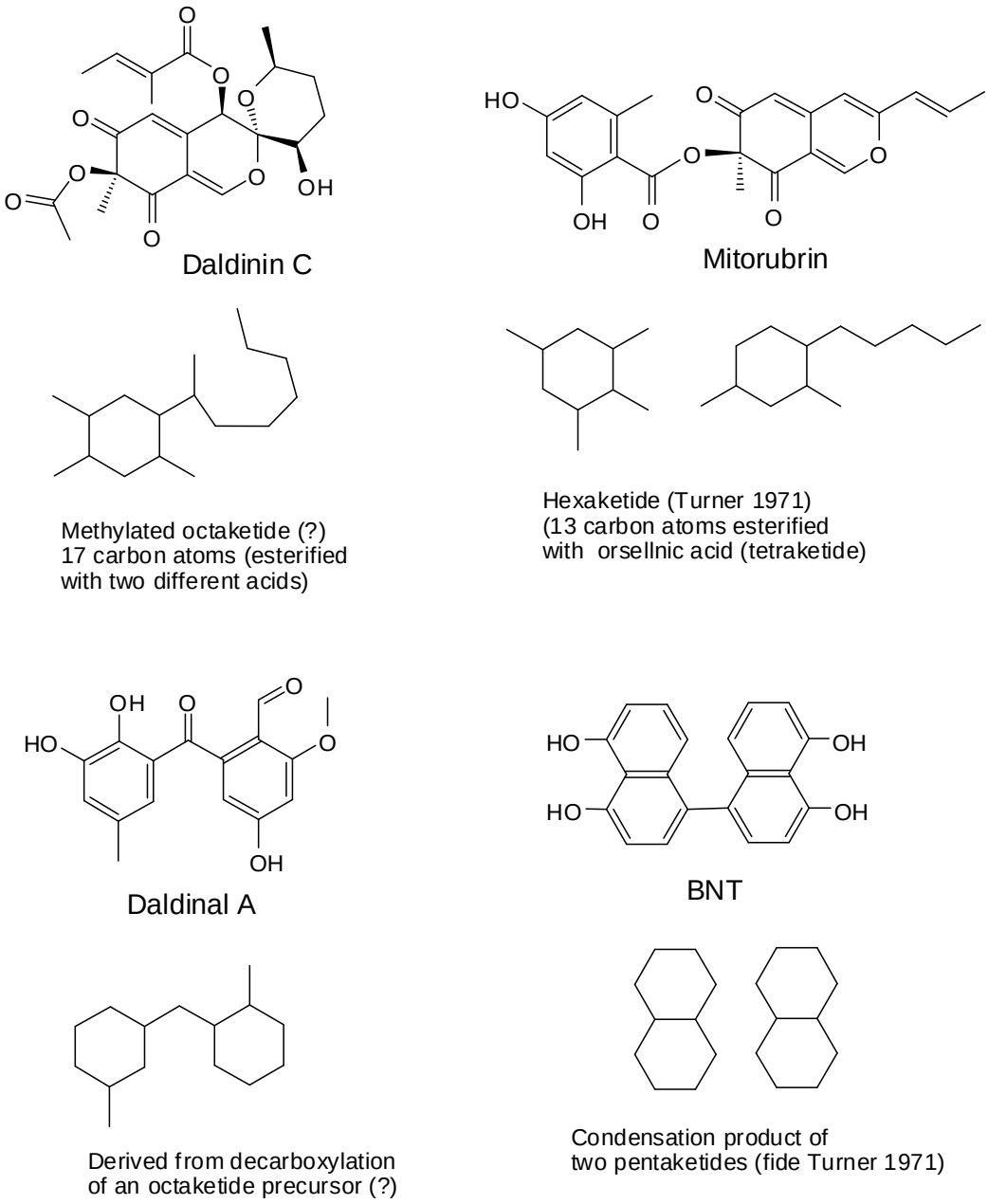


Fig. 18 – Chemical structures of four compounds that specifically occur in the stromata of certain Xylariaceae and their corresponding carbon skeletons, drawn after reduction of the chemical structures by omission of oxygen atoms and double bonds in a similar manner as proposed by Turner (1971). Accordingly, the mitorubrins are esters of the tetraketide, orsellinic acid, with a hexaketide-derived azaphilone carbon backbone. Daldinin C and Daldinal A could be octaketides. BNT and other binaphthyls are probably derived from condensation of two pentaketide units derived from biogenetic pathways related to that of the dhydroxynaphthalene melanin biosynthesis. These highly hypothetical assumptions remain to be proven by experimental data, including methods of functional genomics as well as classical labelling studies.

need to be chosen first by using complementary data.

Frisvad et al. (2008) have highlighted the importance of chemotaxonomy for the exploration of fungal biodiversity, but their overview also shows that many groups of fungi remain to be studied. Except for the Trichocomaceae (including *Aspergillus* and *Penicillium*), no other fungal family has so far been characterised by chemotaxonomic methodology as intensively as the Xylariaceae. The model character of this work may also be illustrated by the following facts:

In contrast to other chemotaxonomic

studies of filamentous fungi, we have examined several genera concurrently, used HPLC profiling techniques to study teleomorphic and anamorphic stages (i.e., stromata and cultures, in case of the Xylariaceae) in concordance. Quantitative HPLC profiling, was so far not used to correlate metabolite production during the ontogeny of sexual and asexual propagation stages.

For the chemotaxonomic work on *Aspergillus* and *Penicillium*, numerous mycotoxins and antibiotics were readily available as standards, due to their importance in agricultural and pharmaceutical research. In contrast, our own work involved the *de novo* isolation and structure elucidation of the chemotaxonomically significant metabolites. Among those were numerous novel natural products.

Our extensive studies of type material and other herbarium specimens by HPLC-MS are also quite unprecedented in fungal chemotaxonomy. It could not be expected that the characteristic marker metabolites may remain stable in herbarium material for such a long time.

An attempt to correlate molecular phylogenetic data with metabolomic studies to identify phylogenetically significant "marker molecules" is also quite unparalleled in mycology.

Despite the innovative character of this metabolomic approach, the chemical data would have little practical value, were it not possible to interpret them in a framework that was elaborated by other mycologists throughout the past centuries. The HPLC profiles were merely used as a vehicle, e.g., to link morphological evidence obtained in the past to molecular data from the present, to verify and refine taxonomic concepts, and to allow for a more concise revision of herbarium specimens. Along with concurrent studies on the biological activities of the respective secondary metabolites, studies on the distribution of the chemotaxonomic marker compounds allowed for preliminary conclusions on their biological functions. Other metabolites, which are specifically overexpressed, such as extracellular enzymes or volatiles, might have a similar importance for the biology of different groups of fungi which do not show such a high diversity of medium polar low molecular secondary metabolites (cf. discussion on "extrolites" by Samson & Frisvad 2005). However, they need to be characterised by methods other than HPLC.

The data presented here on the Xylariaceae should be viewed in a broader context, which will be briefly explored at this point. Not only for Xylariaceae, but for fungi in general, it now appears feasible to attain unprecedented amounts of information that is useful for both basic and applied research in mycology. An integrative assessment of biodiversity, considering both functional and non-functional aspects, now appears feasible for the first time for the entire fungal kingdom. Some important resources providing information on fungi available on the Internet have become available in the past decade. They have already been interlinked by certain Internet portals (e.g., *http://www.gbif.org* for global information on biodiversity of all organisms; *http://www.mycology.net/* for mycological resources). The scheme in Fig. 19 illustrates how synergistic work on different aspects of fungal biology (including microbiology, biotechnology, physiology, ecology, genetics and phytopathology) can ideally be accomplished by utilising these resources.

Specific websites provide comprehensive information on certain groups of fungi (see for the Xylariaceae e.g. *http://mycology.sinica.edu.tw/Xylariaceae*, or *http://pyrenomycetes.free.fr*). They contain online identification keys and illustrations of macroscopic and microscopic morphological features which will often allow for concise identification of specimens. Other websites provide information on biogeography and chorology. For instance, a good overview on Xylariaceae from Ecuador, including illustrations and detailed morphological descriptions of recently collected specimens, is provided at (*http://www.mycokey.com/Ecuador.html*). The websites dedicated to global biogeographic distribution patterns of fungi might reflect the state of the art with respect to flowering plants and certain groups of basidiomycetes. However, they have apparently not been updated, according to the current knowledge on taxonomy and chorology of Xylariaceae and many other groups of

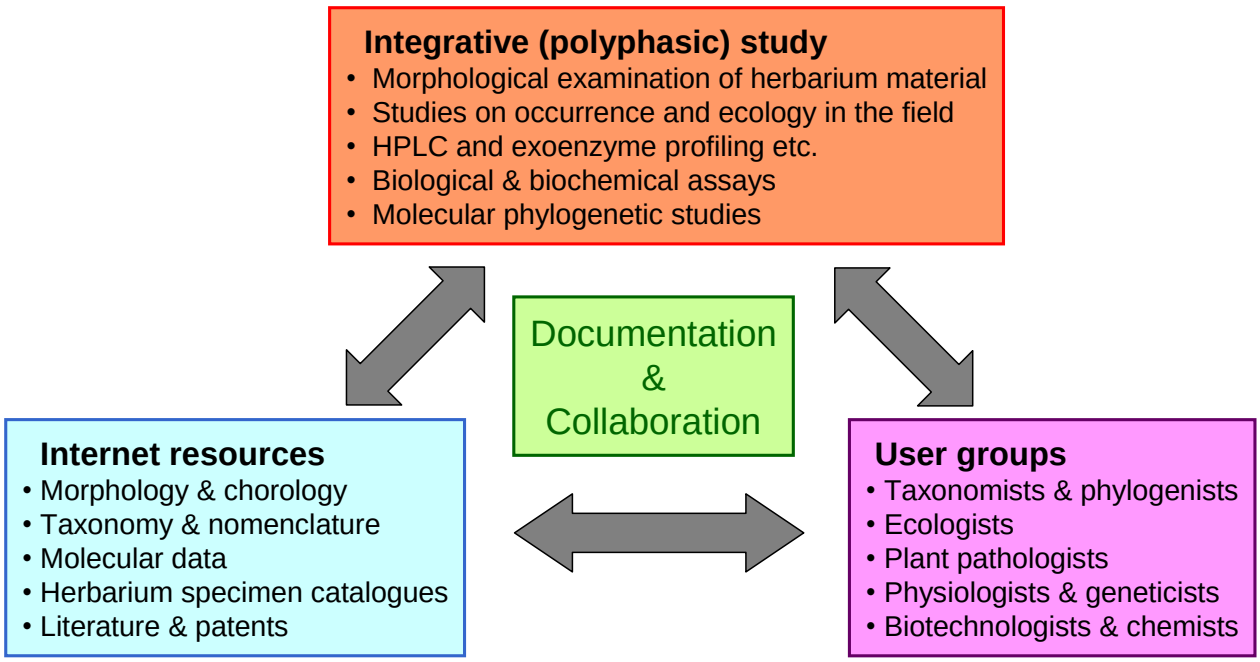


Fig. 19 – Importance of an integrative approach to fungal taxonomy and biodiversity, to be accomplished by interdisciplinary research: Mycological resources on the Internet and potential user groups for the resulting data.

Ascomycota. Other databases (*http://www.indexfungorum.org*; *http://www.mycobank.org*) provide a comprehensive overview of data on fungal taxonomy and nomenclature. Most mycological journals now request authors of new taxa to register them in these databases. Mycobank even provides options to include morphological descriptions, pathogenicity, host-affinities, biogeographic, molecular, and other relevant data. The global portal, Index herbariorum (*http://sciweb.nybg.org/science2/IndexHerbariorum.asp*) contains links to the websites of most herbaria, culture collections and universities, which are relevant for mycologists. Some of their inventories are available for on-line in search; some have even progressed to add digital images and/or morphological descriptions of their specimens. Molecular data, ranging from the results of genome sequencing projects to phylogenetically informative rDNA and other sequence data, are available on the Internet in GenBank and EMBL (*http://www.embl-heidelberg.de*). Other Internet websites, like Treebase (*http://www.treebase.org*) contain molecular phylogenetic trees. Even information relating to beneficial and detrimental properties (e.g., production of secondary metabolites, extracellular enzymes; pathogenicity, etc.) is only occasionally included, e.g. in the online strain catalogues of the international culture collections.

Evidently, most of the user groups listed in Fig. 19, and especially the taxonomists and phylogenists among them, are also potential contributors to the above resources. We have contributed ourselves by adding specimens, cultures, and data, which can now be utilised by other mycologists. A generally accepted taxonomic system, including well-defined species concepts, will provide the prerequisite for communication between the different user groups. Those who are interested in the physiological functions and products of the respective fungal organisms, or who do not dispose of molecular identification methods, would evidently prefer species concepts, in which practical aspects are strongly considered. On the other hand, the availability of molecular data has certain advantages, and phylogenetic aspects should ideally also be taken into account.

As outlined in the Introduction, the current concepts in the taxonomy of the Xylariaceae are based on holomorphic morphology. Chemotaxonomic features, including stromatal pigment colours and HPLC profiles, also constitute characters of the phenotype. As alternative, Taylor et al. (2000) compared a theoretical, phylogenetic approach to recognise

fungal species based on concordance of multiple gene genealogies to the classical, practical concepts based on morphology and reproductive behaviour. Indeed, molecular phylogenetic data are becoming increasingly important at different levels of fungal taxonomy. Multi-gene genealogies, including information from genes encoding for ubiquitously available proteins (including, e.g., α-actin and β-tubulin), have recently been favoured over the evaluation of single genes (e. g, comparison of rDNA sequence data). Notably, these alternative genes belong to the "housekeeping" category. They can from experience only be evaluated in cultures or by destroying relatively large amounts of specimens. Despite their analyses may in some instances provide more conclusive information than that of the rDNA, the broad application of such genes as informative characters is out of the question for phylogenetic studies involving historical type specimens, which do often not yield any DNA suitable for PCR-based investigations at all. For the same reason, molecular studies of environmental samples, e.g., to detect endophytic fungi *in planta*, should also best be accomplished using rDNA primers, since the rDNA is normally available in larger amounts than the alternative genes. This poses less of a problem if recent material that has been cultured can be linked conclusively to the ancient type specimens; an epitype can then be designated and the ex-type culture used for the more extensive molecular studies.

Our rationale was therefore to follow a rather pragmatic approach, realising that practical and theoretical species concepts do not necessarily exclude each other. Grant (2003) commented on the historical struggle between cladists and taxonomists, based on examples from plant systematics. He concluded that molecular evidence should be combined with phenetic evidence, "*in order to bring more characters to bear on the question*". Evidently, this was realised in our study on Xylariaceae. All species concepts hitherto applied to the Xylariaceae are clearly practical, as they are based on phenotypic recognition. However, in the course of our work, molecular data became increasingly available and were interpreted in conjunction with the phenotype-based evidence. The availability of fresh cultures was regarded as indispensable for the eventual generation of more comprehensive molecular data (including multi-gene genealogies), as well as for studies on their physiology and the metabolism of the respective species. By culturing as many representatives and taxa as possible, we therefore were able to hit two birds with a single stone. The availability of concise molecular data, along with corresponding cultures, will facilitate future studies on molecular ecology, e.g., to further elucidate the life cycle and host affinities of endophytic Xylariaceae. Now it appears feasible to compare endophytic cultures of Xylariaceae with their ascospore-derived counterparts that have similar rDNA sequences for possible differences in their physiology and morphology.

In Fig. 20, some data were compiled to give an overview on the current state of the art on biodiversity research of the Xylariaceae, according to the above proposed workflow. Using the numbers of accepted species fide Kirk et al. (2008), the status has been illustrated for some important genera of the major subfamilies, as for the Hypoxyloideae (excluding the non-stromatic genus *Anthostomella*, in which a *Nodulisporium* type anamorph has only been identified for some species, and molecular and chemical data are widely amiss), vs. the non-hypoxyloid Xylariaceae, and the entire family. It becomes evident immediately that the hypoxyloid genera have been rather well evaluated, whereas the other genera need further studies.

Whereas the morphological diversity of *Rosellinia* is quite well-studied (Petrini 2003, Petrini & Petrini 2005), only some of its pathogenic species have been studied for secondary metabolites and by molecular methodology. For *Xylaria*, even molecular data are now becoming increasingly available, especially on Northern temperate representatives. However, the genus is rather large and extremely diverse, above all in the tropics. Many uncertain synonymies have been postulated for names in *Xylaria* that need to be verified by modern methodology, and even the status of the type species remains to be settled. Likewise, most other genera of Xylarioideae remain to be studied in depth by molecular and

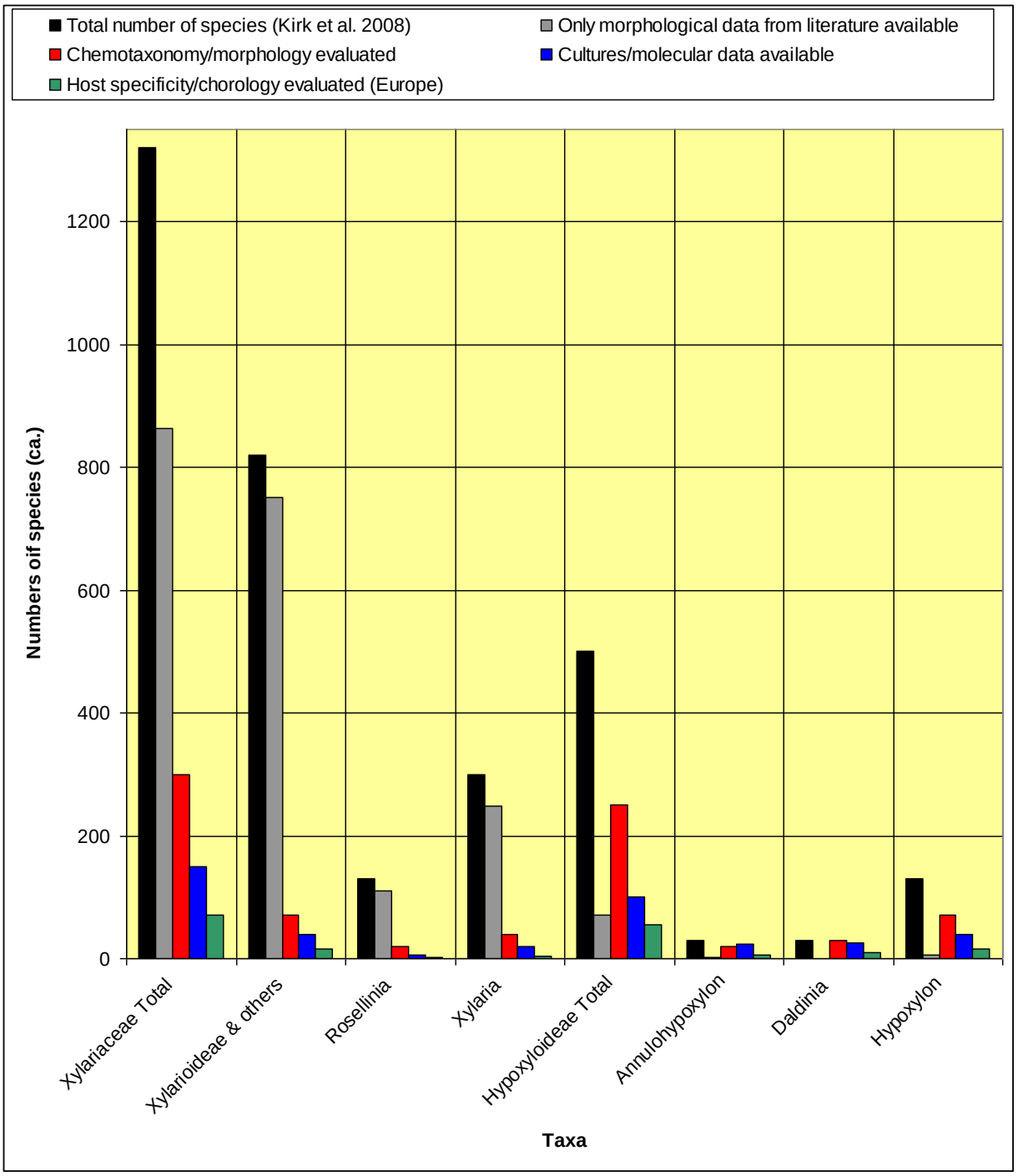


Fig. 20 – Overview of state of the art on selected genera of the Hypoxyloideae and Xylarioideae in comparison to the entire family Xylariaceae. Species numbers are given according to Kirk et al. (2008). Species numbers for Hypoxyloideae exclude the genus *Anthostomella*.

chemotaxonomic methods. Only a few representatives of the Xylarioideae have already been integrated into our HPLC profiling study, and even those largely remain to be evaluated by molecular methods. In summary, the lack of data on the Xylarioideae strongly accounts for the fact that an estimated two thirds of the currently accepted Xylariaceae species remain to be studied by our polythetic approach.

In the (stromatic) Hypoxyloideae, however, the situation is much different. The majority of their known species have already been evaluated using the above proposed approach. The taxa insufficiently studied from this subfamily are mostly of tropical origin. Many of them belong to genera like *Camillea* and *Biscogniauxia* that have no stromatal pigments. As in the Xylarioideae, progress in

their further study will be highly dependent on the availability of fresh material and cultures. Due to the work by Y.-M. Ju and J.D. Rogers and our own efforts, the number of well-characterised Xylariaceae strains available in public collections has at least doubled in the past decade. However, about 80% of these cultures belong to *Annulohypoxylon*, *Hypoxylon*, and *Daldinia*. Morphological, chemotaxonomic, molecular, and chorological data are available for many of the representatives of the above genera. A significant number of specimens cited in the last monographs (e.g., Ju & Rogers 1996, Ju et al. 1997, Granmo 1999) were already re-examined; several species were cultured for the first time and studied by molecular methodology. Extensive chorological data, backed up by morphological, molecular and chemical studies are so far available for over 40 taxa of Xylariaceae, most of which are frequently encountered in their respective areas of distribution. For an additional 30 taxa (including ubiquitous species, plant parasites, or "model" species such as *D. loculata*, which were already studied in-depth by other working groups and provided to us only for chemotaxonomic work or as reference material), it can be safely assumed from our corroborating data that their chorology, morphology, chemotaxonomy and host specificity is well established by now. Most of those are distributed in Europe or the Northern hemisphere.

Many of the other currently accepted species so far studied were not yet cultured at all, or only one or a few representatives are so far available. Some of them are known to only rarely produce stromata; with some luck they may soon be shown by molecular methods to occur among the endophytes. Others are only known from remote areas of the tropics, including regions that were once heavily forested and were last collected in the 19th century but are now used for housing or agriculture. Their natural habitats may have meanwhile been destroyed. Even in Central Europe, there are species like *Hypoxylon commutatum* Nitschke, which are only known from the type specimen despite they were erected over 150 years ago.

Numerous morphological lineages of the Xylariaceae are still insufficiently represented in the panel of cultures available. In all likelihood, their inclusion will in future gradually allow for the phenotype-based species concepts to become more congruent to the theoretical concept. HPLC profiles of newly obtained material will continue to be valuable in establishing its identity with herbarium specimens, and resolve the remaining species complexes. The revision of the herbarium inventories will also have to continue, especially if they have last been revised using the outdated taxonomic concepts, such as that by Miller (1961) for *Hypoxylon* species. For resolution of the species complexes that were tentatively identified by HPLC profiling of herbarium specimens, intensive field work and morphological studies will have to go on, above all in the tropics.

The bulk of the chemotaxonomic work, along with a thorough revision of morphological data, has been accomplished either in our laboratory or in collaboration with other mycologists who are the co-authors of the cited papers. The amount of molecular data contributed by us in the course of this work presently accounts for less than 10% of the currently published DNA sequences, but this will substantially increase once the unpublished data have been released.

As pointed out by Wheeler (2004), who reviewed the situation in entomology, it is crucial not to neglect the classical morphological-based taxonomy, even in the era of molecular phylogeny. This certainly also holds true for fungal biology. Well-trained fungal morphologists, who are acquainted also with microbiological, molecular, and ideally even chemical techniques, are desperately needed even in the future. They are needed to fill the above gaps, since morphological studies are especially important to generate data on the xylarioid taxa, where chemical traits have so far not proved significant. Hence, it can only be hoped that HPLC profiling of mycelial cultures will eventually provide additional valuable information. Chemotaxonomic data could certainly also become important for characterisation of numerous other groups of Ascomycota and Basidiomycota which have proved to be creative secondary metabolite producers.

The data matrix obtained and the methodology developed have meanwhile also

become most useful for applied mycology, in particular regarding the design of a library of samples prepared from fermentation of fungal cultures and fruiting body extracts for high throughput screening. Of course, it will never be possible to do an in-depth characterisation of all biological sources, prior to their inclusion in a screening program aimed at the discovery of novel natural products, but some important improvements associated with the progress of the pilot study on Xylariaceae that substantially helped to increase the probability of success became evident as the work proceeded. Three of those are mentioned further below.

As HPLC profiling results also agreed with molecular phylogenies in other groups of filamentous fungi (e.g., Stadler et al. 2003) and sequencing costs have substantially decreased in the past decade, it now appears worthwhile to characterise the strains used for screening by molecular techniques prior to the screening over their random selection. Generation of their 5.8S/ITS nrDNA sequences will give good correlations with known taxonomic groups. This allow to assess the potential pathogenicity of unknown strains, and the early identification of duplicates, which often occur among populations of endophytic or insect pathogenic fungi, can also be recognised easily prior to the screening. Even desired redundancies can be revealed. It is possible to find additional producers of a certain metabolite (or even producers of additional, new compunds of a certain secondary metabolite family), by comparing its DNA sequence data with those of strains available in public domain databases as well as in the in-house repositories. This may be helpful to gain further intellectual property.

The concentrations of secondary metabolites in stromata of most Hypoxyloideae were found extremely high. In addition, over 90% of the metabolites of the Hypoxyloideae isolated in the course of our work were found to constitute novel natural products. These observations gave rise to study additional species with apparently inconspicuous stromata or other types of fruiting bodies, which would otherwise not have been regarded worthwhile to study because of their small size and therefore constitute hitherto untapped potential sources for novel lead compounds.

The complementarity of secondary metabolite production in stromata vs. cultures and the high dependence of metabolite production on the developmental stages also gave strong impetus for the future design of the natural product-derived screening libraries. The fact that the onset of metabolite production is associated with the morphogenesis of the anamorph was meanwhile also observed in other ascomycetes according to unpublished results. Despite it has been known for a long time that mycelial cultures often show different secondary metabolite profiles than the fruiting structures, this has never before been demonstrated for an entire family. It could certainly not have been anticipated that no single Xylariaceae species contains the same major metabolites in cultures and its stromata.

As mentioned in the introduction, this thesis was inspired by the work of Samson & Frisvad (2005), and continued the chemotaxonomic evaluation by Whalley & Edwards (1995) of the Xylariaceae, which had commenced at a time when no HPLC profiling was possible at all and molecular techniques were still about to be developed. The prerequisites to use a similar approach for characterisation of fungi have certainly improved since then. Numerous other groups of fungi, known to be rich in secondary metabolites, remain to be explored. I therefore hope the work presented here will also be suitable to inspire others, and it will in future be possible to employ additional innovative methodologies, including genomics and proteomics for broad characterisation of many fungal organisms to further explore their biodiversity.

Conclusion

Since this thesis, which constitutes a review of our previous work on the Xylariaceae, was presented for review to the habilitation committee in 2009, I have been encouraged by several colleagues to publish it in a public domain journal. In view of the recent discussions in the mycological community about the value of traditional vs. molecular data in fungal taxonomy, and especially of the changes in mycological nomenclature and taxonomy that have been brought about at the International Botanical Congress, Melbourne, 2011, it now appears all

the more practical to make this thesis available. After all, it provides an example on how much work still needs to be done in order to understand the taxonomic and phylogenetic relationships in one of the most diverse and important groups of ascomycota, above all among the taxa from warmer climates and the southern hemisphere. Even after over 15 years of intensive studies, which have meanwhile included several thousands of Xylariaceae specimens, we are still far away from reaching conclusions on several important matters, relating to the taxonomy and biology of these fungi.

I hope that this paper will encourage others to study other groups of tropical fungi, using a polythetic approach. Not only in the Xylariaceae, thousands of species may remain to be found and described; hundreds of previously published names need to be epi- or neotypified based on comparisons of type specimens with fresh material that can be used, e.g., for molecular phylogenetic studies and to establish anamorph-teleomorph relationships. Even many of the known and frequently reported taxa do in fact constitute heterogeneous species complexes, which remain to be resolved by a combination of genotype- and phenotype-derived data. It appears all the more urgent to stop the current trend to undertake premature name changes and erect unnecessary genera based on rather preliminary molecular data and in the absence of corroborating phenotype-derived evidence.

Hence, I am very grateful to Kevin D. Hyde, Editor-in-Chief of CREAM for allowing me to publish this work in an open access mycological journal, for their help in transferring the contents of the thesis into an adequate format for publication.

At the time when this thesis was presented, the papers by Fournier et al. (2009) and Stadler et al. (2010a) had still not been accepted, and the more recent studies on *Ruwenzoria* and *Rhopalostroma* (Stadler et al. 2010b,c) as well as the papers by Fournier et al. (2010, 2011) on *Hypoxylon* and *Xylaria*, and the paper by Læssøe et al. (2010) had not even been submitted.

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