Pigment chemistry, taxonomy and phylogeny of the *Hypoxyloideae (Xylariaceae)*

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Summary

The *Hypoxyloideae* (*Xylariaceae* with *Nodulisporium*-like anamorphs) were evaluated by a morphological and HPLC-based chemotaxonomic survey of more than 2000 specimens and cultures. Conspecificity of recent records with ancient type specimens were established in many cases by HPLC, since their characteristic metabolites may remain stable for over 200 years. Most constitute novel natural products that were identified in the course of concurrent "mycochemical" studies. A comparison of HPLC profiles considering relationships within the *Hypoxyloideae* as inferred from the biogenesis of these pigments agreed fairly with concurrent molecular phylogenetic studies, based on sequences of actin, ß-tubulin, and 5.8S/ITS nrDNA genes. Anamorphic morphology and secondary metabolism of cultures agreed well at generic level and above. A combination of chemical and morphological traits is favored over PCR-based approaches for species discrimination, so long as only relatively few taxa of these diverse genera have been sequenced. An overview on the chemical structures and biological activities of the characteristic metabolites is provided, their ecological importance is discussed, and the utility of chemotaxonomy to support and predict phylogenetic relationships in the *Hypoxyloidae* is demonstrated. A polythetic approach is most useful to elucidate the phylogeny of the *Xylariaceae*. Chemotaxonomy to assess fungal biodiversity has considerable utility.

Key words

Chemotaxonomy, *Xylariales*, Azaphilone, Defense metabolites, Secondary metabolites

This review is intended to summarize recent results on the pigment chemistry, taxonomy and phylogeny of the *Hypoxyloideae*, an important group within the stromatal *Xylariaceae* (*Sordariomycetes*, *Xylariomycetidae*, *and Xylariales*). The teleomorphs of these fungi are associated preferentially with woody seed plants. They differ from the *Xylarioideae* and other small groups within the family in producing *Nodulisporium*-like anamorphs. Aside from *Biscogniauxia* and *Camillea*, most of the genera included in the *Hypoxyloideae* are known to biosynthesize manifold stromatal pigments. Their largest genus is *Hypoxylon*, which until recently comprised sect. *Hypoxylon* (ca. 170 spp.) and *Annulata* (ca. 40 spp.) [11,32]. The latter section is now regarded as *Annulohypoxylon* [8]. Other genera in the *Hypoxyloideae* include *Daldinia* (over 30 spp.), *Pyrenomyxa* (=*Pulveria*) [31], and the tropical *Entonaema*, *Phylacia*, *Rhopalostroma*, and *Thamnomyces* [11]. These genera are largely defined by deviations in stromatal anatomy, or the morphology of their perithecia, asci, and ascospores from *Hypoxylon* (Table 1), but the infrageneric classification in the *Hypoxyloideae* relies on a combination of holomorphic morphology with chemical traits.

The colors of subsurface pigment granules and KOH-extractable stromatal pigments are indispensable for species recognition in *Daldinia* and *Hypoxylon*/*Annulohypoxylon* [5,8-11,31,32]. These pigments are secondary metabolites ("extrolites" [27])[1]. As will be exemplified below, similar pigment colors may either be due to the presence of the same or similar metabolites in groups of related species, or result from different chemicals. Most were only isolated and identified in the past five years as novel natural products. Using these compounds as standards, a chemotaxonomic study based on HPLC profiles (employing diode array detection and mass spectrometry) of ca. 2000 specimens and cultures, revealed various chemotypes in the *Hypoxyloideae* [7,16,25,30,32,34]. Their HPLC profiles proved to be consistent, specific features in certain species or species groups. Frequently, the non-destructive methodology was suitable for ancient type specimens. Specific metabolite profiles related them to freshly collected, conspecific material [7,25,31], which may be further used as genuine representatives in morphological and molecular studies.

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[1] The term "extrolites" was recently introduced to describe all outward-directed signal molecules (including but not restricted to secondary metabolites) that appear essential for survival of the producers and are thus of taxonomic significance. The introduction of this term raises some controversial discussions. To clarify our standpoint: "Secondary metabolites" is preferred here because we do not deal with other extrolites

Table 1. Genera of *Hypoxyloideae*: Differences in a) stromatal morphology and anatomy, and b) morphology of asci and ascospores that are currently regarded as relevant for definition of generic boundaries. The anamorph of all genera included is known to be *Nodulisporium*-like, except for *Versiomyces.*

Genus	Ostioles	Ascal apical ring	Ascospore germ slit	Stromatal habit and anatomy	Metabolites in culture
Rhopalostroma	+	-	+	Stipitate or conspicuously constricted, homogenous	Naphthalenes/ Chromones
Entonaema	+	+	+	Stipitate or conspicuously constricted, hollow, filled with liquid when fresh	Naphthalenes/Chromones
Daldinia	+	+	+	Sessile or stipitate Internal concentric zones	Naphthalenes/Chromones
Versiomyces	+	+	+	Sessile, daldinioid stromata lacking concentric zones	unknown
Hypoxylon/ Annulohypoxylon	+	+	+	Sessile, essentially homogenous	Melleins only proven in *Hypoxylon*
Thamnomyces	+	-	+	Wiry, ascomata oriented horizontally, homogenous	unknown
Pyrenomyxa	-	-	+	Sessile, homogenous	Melleins
Phylacia	-	-	-	Stipitate or conspicuously constricted, homogenous	unknown

+ = present; - = absent.
Morphological features were compiled according to [8,10,11,31].

Whalley and Edwards [37], from whose results the current study has been inspired, demonstrated that various apparently genus-specific metabolites are produced in *Xylariaceae* cultures, but they did not use analytical HPLC methodology, and the procedures available at that time did not allow the study of stromatal constituents of inconspicuous species. Their results did reveal that *Hypoxyloidae* and *Xylarioideae* developed entirely different biogenetic strategies. Aside from mellein derivatives, which were reported from *Biscogniauxia*, *Hypoxylon*, and some *Xylaria* spp [1,37], most other genus-characteristic compounds of *Xylarioideae* were found absent in the genera that now belong to *Hypoxyloideae* - and vice versa [29,37].

In the present paper, we will assess a) the occurrence of characteristic pigments and the taxonomically significant metabolites from cultures, and b) their chemotaxonomic, biological, and chemoecological significance. For further compounds that are not stromatal pigments or have not proved taxonomically significant yet, we refer to [29]. We demonstrate that secondary metabolites or other phenetic features will, if present and proven to be taxonomically informative, facilitate species identification for specialists and non-specialists. For more information on the taxonomy and diversity of the family see: [*http://pyrenomycetes.free.fr*] and [*http://mycology.sinica.edu.tw/Xylariaceae*]. Fungal names are given as suggested in [*http://www.indexfungorum.org*]. If not indicated otherwise, all specimens depicted were collected in Southwestern France and are deposited in the herbarium of J. Fournier. There now follows a discussion on how the various chemotypes relate to the existing classification based on morphological traits.

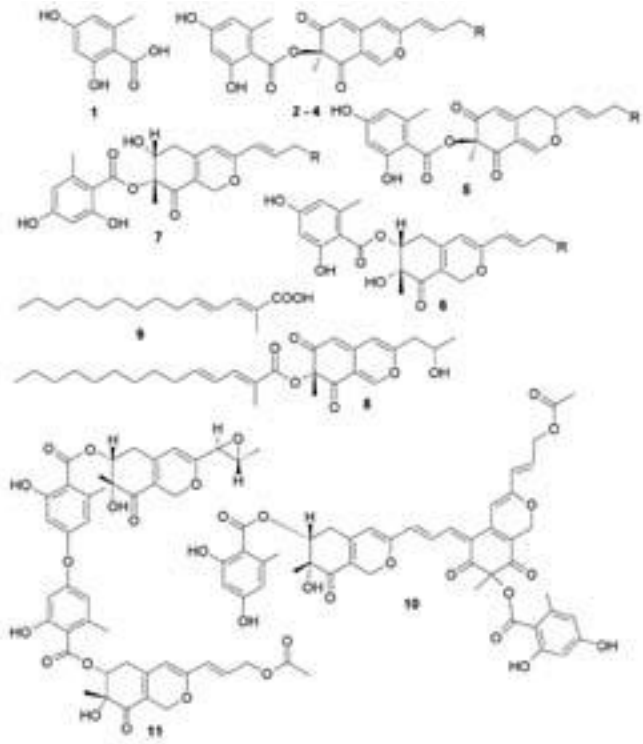

Figure 1. Azaphilones containing orsellinic acid and other secondary metabolites from the "red" *Hypoxylon* and *Entonaema* species. 1: Orsellinic acid; 2: Mitorubrin (R =H); 3: Mitorubrinol (R = OH); 4: Mitorubrinol acetate (R = $OCCH_3$). 5: Hypomiltin (R = $OCCH_3$); 6: Rubiginosin A (R = $OCCH_3$); 7: Rubiginosin B (R = $OCCH_3$); 8: Rubiginosin C; 9: Rubiginosic acid; 10: Rutilin; 11: Entonaemin C.

A. The "red Hypoxylons": Mitorubrin and related pigments

As amply summarized by Ju and Rogers [11], the definition of the genus *Hypoxylon* has undergone changes throughout the history of fungal nomenclature, especially in the past decades. The first described (i.e. type) species of *Hypoxylon* [2] is *Hypoxylon coccineum* Bull. Out of convenience, this name is synonymized with *Hypoxylon fragiforme* (Figure 2a), albeit Læssøe [13], stated that the data Bulliard provided on host plants of *H. coccineum* (*Juglans* and *Aesculus* rather than *Fagus* as typical for *H. fragiforme*) points toward *Hypoxylon howeanum* (Figure 2b). They also have a very similar secondary metabolism and mainly differ in their host range and ascospore size [11]. Consequently, both will continue to be members of the core group in the conserved genus *Hypoxylon*, regardless of any new taxonomic proposals. Their typical red stromatal pigments are the mitorubrins (**2-4** in Figure 1). Those are the first reported metabolites of the mitorubrin family, which include mitorubrins, rubiginosins, entonaemins, hypomiltin, and rutilins (Figure 1). They are azaphilones (i.e. "nitrogen-loving" bicyclic fungal pigments that share a highly reactive pyran ring moiety which spontaneously incorporates ammonium ions) that have an orsellinic acid (**1** in Figure 1) moiety attached by an ester bond to their bicyclic azaphilone carbon skeleton. They are characteristic features of most species in

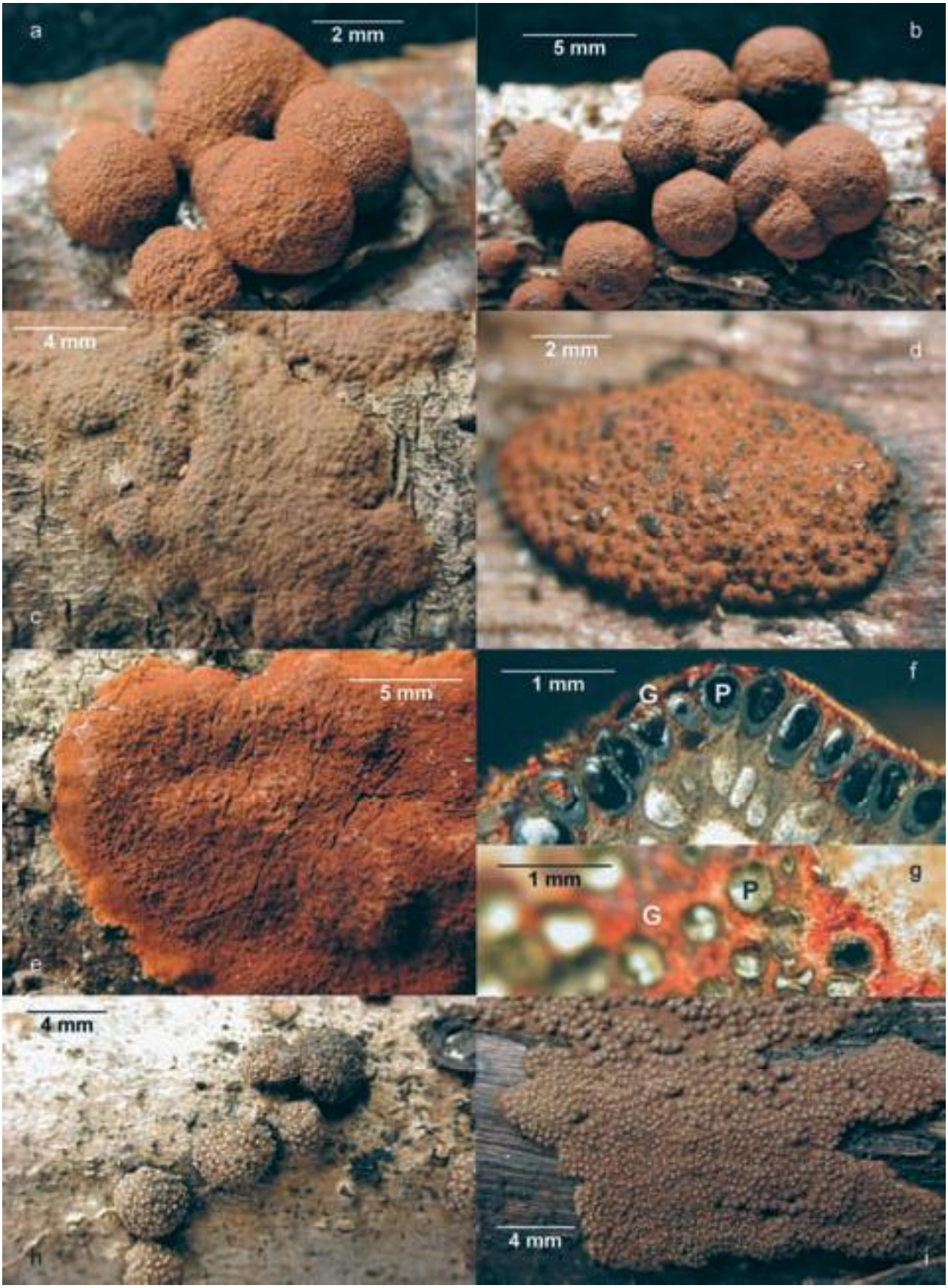

Figure 2. The core group of *Hypoxylon* spp., containing orsellinic acid and azaphilone esters thereof: a) *H. fragiforme* JF-04150 (mitorubrins). b) *H. howeanum* JF-96150 (mitorubrins). c) *H. rubiginosum* JF-99130 (mitorubrins and rubiginosins). e) *H. ticinense* JF-02012 (mitorubrins). d), f), g) *H. rutilum* JF-97105 (mitorubrins, rubiginosins, and rutilins); d) stromatal habit; f) section through a stroma showing perithecia (P) and pigment granules (G) containing rutilins; g) stromatal from above after partial removal of surface layer, revealing the subsurface granules. h) *H. intermedium* JF-00198 (hypomiltin). i) *H. perforatum* JF-98029 (hypomiltin). Scale is indicated by bars.

Hypoxylon that possess orange, red or yellow pigments in KOH *fide* Ju and Rogers [11]. Interestingly, this group in *Hypoxylon* is regarded as the most evolutionary advanced one, i.e. particular morphological traits are accompanied by the possession of red or orange pigments. Indeed, all species that contain metabolites of the mitorubrin family also possess extensive waxy stromatal tissue, in which the perithecia are embedded, and many of them show other characters that are regarded as derived [11], such as stromata with conspicuous perithecial mounds and papillate ostiola, a reduced *Sporothrix*- or *Virgariella*-like anamorph, a highly reduced ascal apical ring, and an indehiscent ascospore perispore.

In most cases, azaphilone patterns (i.e. the distribution and ratio of single components of the HPLC profiles) - proved to be highly specific. For example, extracts and even preparative HPLC fractions of *H. fragiforme* and *H. howeanum* do not reveal any rubiginosins (**6 – 8** in Figure 1), which prevail in *Hypoxylon rubiginosum* and allies [7,32] as another variant of orange-red azaphilones that may co-occur with mitorubrins and prevail in many species of *H. rubiginosum* ss. Miller [14], including *H. rubiginosum* ss. str. (Figure 2c). Despite their different stromatal pigments in KOH, species containing hypomiltin (**5** in Figure 1, contained in *Hypoxylon perforatum* and *Hypoxylon intermedium*; Figures 2h, 2i) appear chemotaxonomically related to the mitorubrin-producing ones, as hypomiltin only lacks one double bond compared to mitorubrinol acetate [7].

Aside from the *H. rubiginosum* complex, rubiginosin-like metabolites also occur in *Entonaema*, but were not yet detected in *Daldinia* [33,34]. *H. rubiginosum* and allies also contain rubiginosin C (**8**), which is absent in *Entonaema*. In this molecule, rubiginosic acid (**9**), rather than orsellinic acid (**1**), is connected by an ester bond to the azaphilone ring system [20]. Despite these differences, azaphilones remain stable in the waxy subsurface layers of herbarium material for centuries. However, such metabolites are highly unstable and difficult to isolate once they have been removed from the stromatal matrix. The recently identified rutilins (e.g. **10**) are the scarlet pigments in the subsurface granules of *Hypoxylon rutilum*, *Hypoxylon julianii*, and the tropical *Hypoxylon erythrostroma*. These dimeric azaphilones are derived from biogenic condensations of two moieties of mitorubrin- and rubiginosin-like monomers [21]. They are not major detectable components in other members of the *H. rubiginosum* complex. Entonaemin C (**11**) was isolated previously from an *Entonaema* sp. [6]. It contains two independent moieties of mitorubrin-like monomers that are not connected by a C-C bond as in the rutilins, but rather an ester bond. Compounds with similar mass spectra to that of entonaemin C remain to be found in *Hypoxylon*. Likewise, the apparent lack of rubiginosin C-like components in *Entonaema* (see below) indicates that the pigment chemistry of that genus and the *H. rubiginosum* complex is different and could have arisen convergently. The xylariaceous genus *Pyrenomxya* (syn. *Pulveria*), which was recently evaluated by a combination of morphological and chemotaxonomic methodology [31], also shows strong affinities to this group in *Hypoxylon*. All of the three currently accepted species contain orsellinic acid and either, rubiginosins, hypomiltin or other related azaphilones. One of them was shown to produce mellein derivatives in culture. Regardless of the peculiar ascospore morphology and the lack of ostioles, this genus may soon be revealed as a cleistocarpous *Hypoxylon*. From a chemotaxonomic viewpoint, it appears more closely related to the core group of the genus than all other fungi treated below [31].

B. The *Hypoxylon fuscum* chemotype and other *Hypoxylon* spp. that do not contain mitorubrin-like azaphilones as major components

Type and recently collected specimens of *H. fuscum* and relatives (*Hypoxylon anthochroum* p.p., *Hypoxylon fuscopurpureum*, *Hypoxylon macrosporum*, *Hypoxylon notatum* and *Hypoxylon undulatum*) contain daldinin type azaphilones (e.g. daldinin C, **14** in Figure 3) in their stromatal granules, which are frequently accompanied by daldinal A (**13** in Figure 3). All of them lack pigments of the mitorubrin family as major stromatal components (Figure 1). Daldinin C (**14**) may be a "plesiomorphic" molecule, since it was still encountered in traces in *H. rubiginosum* after removal of the prevailing rubiginosins by preparative HPLC [20]. Along with daldinal A (**13**), it was isolated first from *Daldinia* [6,34]. Meanwhile, they were also found, e.g., in *Phylacia surinamensis* [26] and *Hypoxylon symphyon* (see below). The daldinin containing species represent a separate chemotype in *Hypoxylon*, but at least in *H. fuscum*, HPLC revealed host-specific chemical races that may eventually be considered as different species. The Persoon type (L) of *Sphaeria fusca* shows the same morphological and chemical features as 40 recent collections from *Corylus* in various European countries (Figure 4a, c). They contain daldinal A (**13**), 1,1´-binaphthalene-4,4'-5,5'-tetrol (BNT, **15**), and daldinins C (**14**), E and F and yield yellowish to olivaceous stromatal pigments in KOH. Some specimens keying out as *H. fuscum* from *Alnus*, *Betula*, and *Salix* have pale purple gray granules and KOH-extractable pigments and smaller ascospores (Figure 4b) and deviate in their HPLC profiles from the typical form on *Corylus*. We recently confirmed the results of Granmo [5], who separated *H. porphyreum* from *H. fuscum* ss. Ju & Rogers [11]. The new species is consistently associated with *Quercus* and has now been encountered in France aside from Scandinavia (Figure 4d). It shows morphological similarities to *H. fuscum*, but its major pigments are not the daldinins (Granmo JFA and Fournier J, unpublished). Granmo et al. [4] had reported *H. porphyreum* to differ from *H. fuscum*, which is associated with *Betulaceae* rather than *Quercus*, in its ITS nrDNA. An extensive HPLC-based study on the *H. fuscum* chemotype, accompanied by morphological studies on a broad range of specimens from different hosts and localities, in a similar manner to Granmo [5] and Petrini et al. [17], may further resolve such species complexes.

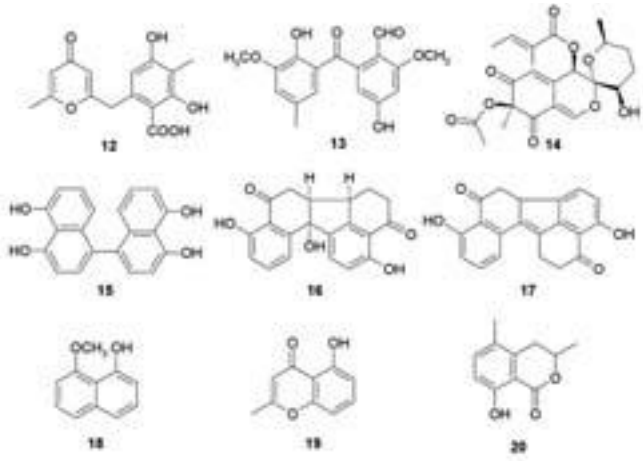

Figure 3. Naphthalene pigments and other characteristic metabolites from stromata and cultures of the *Hypoxyloideae*. 12: Macrocarpon A.
13: Daldinal A. 14: Daldinin C. 15: BNT. 16: Daldinone A. 17: Truncatone.
18: 8-Methoxy-1-naphthol. 19. 2-Hydroxy-2-methylchromone.
20: 5-Methylmellein.

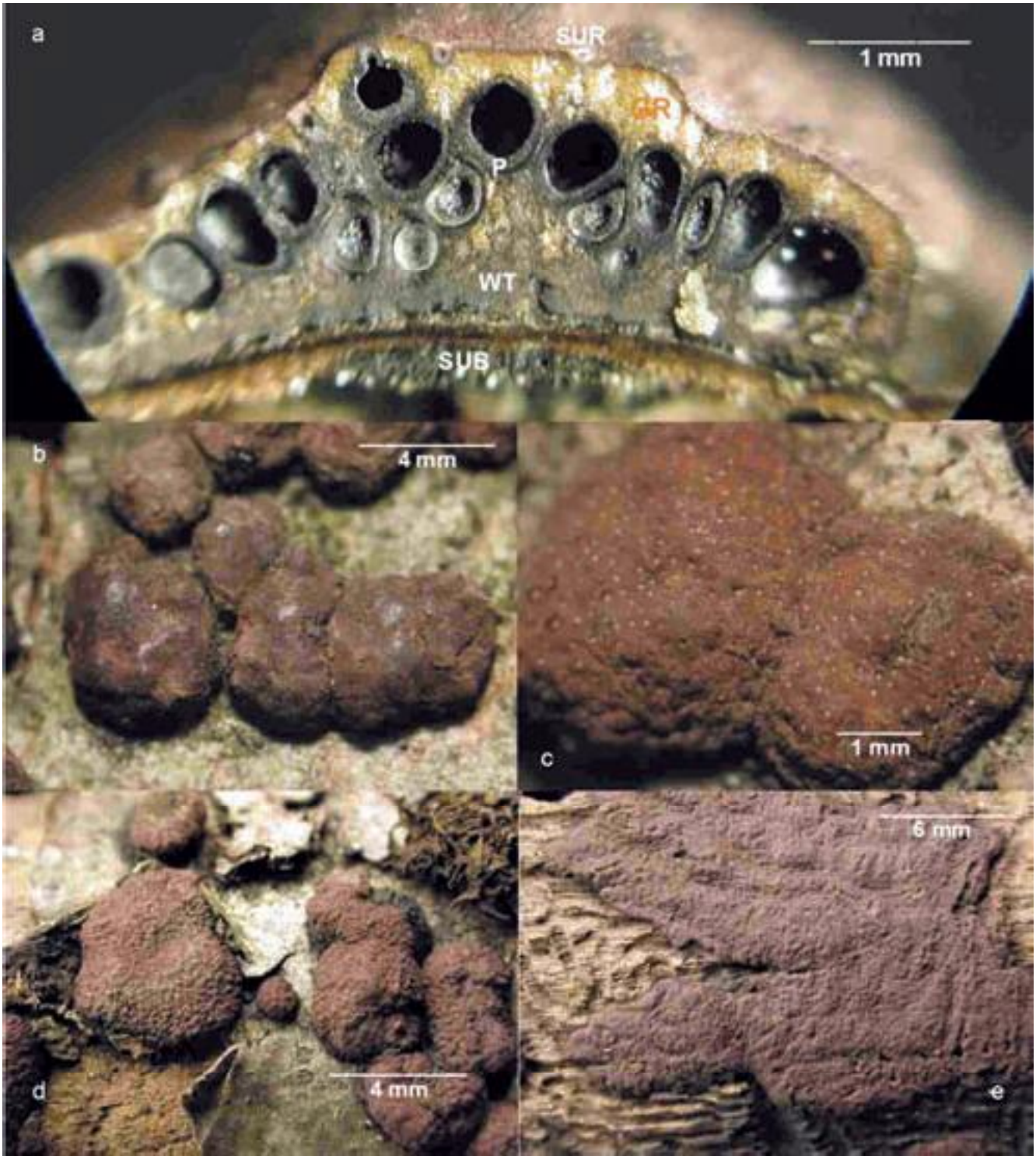

Figure 4. *Hypoxylon* spp. that are devoid of azaphilones that constitute esters of orsellinic acid and contain BNT and/or other pigments instead: a-c) *H. fuscum*; a) JF-97004 from *Corylus*, section through stroma, revealing perithecia (P), waxy tissue with orange brown granules containing daldinins embedded (G), stromatal surface containing BNT (SUR), woody stromatal tissue (WT), and substrate (SUB). b) stromata of deviating morphochemotype of *H. fuscum* from *Alnus*. c) stromata of JF-97004. d) *H. porphyreum* JF-03167 (devoid of daldinins, containing BNT and specific unidentified pigments). e) *H. macrocarpum* JF-97095 (containing BNT and macrocarpones).

This also applies to their tropical relatives, such as *H. anthochroum*. Unlike previous generic concepts [14], the current one [11] relies on anamorphic morphological characters. However, conidiogenous structures are known for only a few of this and other "pantropical" species. For instance, the holotype of *H. anthochroum* from Sri Lanka contains BNT and daldinin C, but its anamorphic description was based on two cultures obtained from Mexico [11]. We did not detect any daldinins in American and African specimens of this species and found that they contained different major components than the holotype (Stadler M and Fournier J, unpublished), which indicates the presence of another species complex.

Interestingly, the yellow KOH-extractable stromatal pigments in the holotype of *Daldinia bakeri* and other ancient specimens in the herbaria BPI, K, and M were shown to correspond with non-xylariaceous compounds [30], regardless of taxonomy. These "pigments" were probably preservatives added to the herbarium material to prevent destruction by insects or molds. Therefore, the detection of apparently specific metabolites in type specimens needs verification by their occurrence in recently collected, and untreated material

H. macrocarpum (Figure 4e), shows a similar morphology to *H. fuscum*. Its major constituents are orsellinic acid (**1**) and macrocarpones (e.g. **12** in Figure 3), rather than daldinins. Various other *Hypoxylon* spp. do not contain mitorubrin- or daldinin-like prevailing stromatal constituents, since only BNT or as yet unidentified compounds metabolites were detected in their HPLC profiles [7]. Additional investigations will be necessary to establish their chemotaxonomic affinities. Since most of the recently identified pigments obtained from previously unexplored *Hypoxyloideae* were new to science, they appear very promising in the search for novel bioactive compounds [29].

C. Naphthalenes in *Annulohypoxylon* Group A and *Hypoxylon* spp. with purple or olivaceous pigments

In general, the species of the *H. fuscum* chemotype have a purple surface, which can be attributed to the presence of BNT (**15** in Figure 3), a compound that is omnipresent in stromata of all members of *Annulohypoxylon*, *Daldinia*, *Hypoxylon*, *Thamnomyces*, and *Phylacia* [7,25,30,31,34]. The azaphilones are usually concentrated in the subsurface granules (Figure 4a), whereas BNT and other components [e.g. daldinone A (**16**), daldinin A (**13**)] are located on the stromatal surface [7,21]. Even in a few species of the *H. rubiginosum* complex (e.g. *Hypoxylon petriniae* [32]), this binaphthalene co-occurs with rubiginosins (**6-8**) or mitorubrins (**2-4**), resulting in a specific HPLC profile. BNT may be converted by biosynthetic oxidation to benzo(*j*)fluoranthenes (daldinone A, truncatone; **16-17**), which appear greenish olivaceous in KOH. This

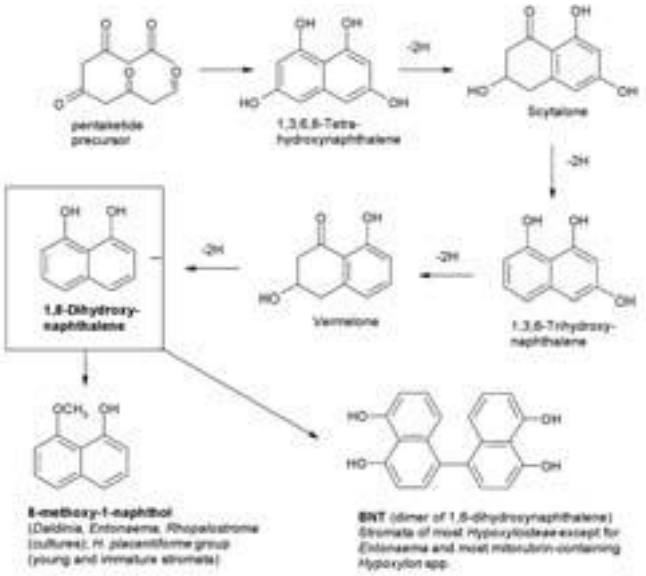

Figure 5. Scheme for the ubiquitous pentaketide 1,8-dihydroxymelanin (1,8-DHN) melanin biogenesis, slightly modified from [39].

phenomenon occurs in many species of *Annulohypoxylon* and in some members of *Hypoxylon*. Only truncatone (**17**) is restricted to *Annulohypoxylon* [25].

BNT is also characteristic of *Hypoxylon monticulosum* (Figure 7a), a species that is regarded as basal in *Hypoxylon* as inferred from morphological traits [11]. *Hypoxylon investiens* (Figure 7b) differs from *H. monticulosum* predominantly by its green pigments in KOH and ascospore morphology. BNT (**15**) is hardly detectable, while daldinone A (**16**) may comprise up to 90% of its stromatal methanol extract. The latter compound was originally found as a minor component in *Daldinia* [24], suggesting that the biogenesis of such modified binaphthalenes may have arisen independently several times in *Hypoxyloideae* containing BNT. In *Annulohypoxylon*, BNT and congeners (including daldinone A and truncatone) prevail in taxa with ostiolar disks. Those are regarded as primitive [11], and further referred to as group A. Species that appear more evolutionarily advanced (ostiolar disks gradually reduced, and finally replaced by papillate ostioles), are referred to as *Annulohypoxylon* group B. As compared to group A, they have developed additional biosynthetic genes for specific azaphilone pigments (see section D).

Surprisingly, all species in the "*Hypoxylon placentiforme* line" [11] with massive peltate, discoid or pulvinate stromata and tubular perithecia (e.g. *H. placentiforme*; Figure 7g) were found to contain 8-methoxy-1-naphthol (**18** in Figure 3). This molecule is present in cultures of *Daldinia* [34], *Entonaema* and *Rhopalostroma* [30] so far studied, but was not yet detected in other *Xylariaceae*. This observation supports a century-old hypothesis as to the evolutionary roots of *Daldinia* [15,35]. The origin of 8-methoxy-1-naphthol (**18**) and BNT (**15**) as side metabolites of the 1,8-DHN melanin biogenesis is illustrated in Figure 5. This melanin pathway is a common feature of filamentous ascomycetes [39], yet only the *Xylariaceae* and *Bulgaria inquinans* (*Helotiales*) were proven to contain BNT [34], and the naphthol (**18**) is only known to accumulate in the above *Hypoxyloideae*. The core group of *Hypoxylon* (including all species treated in parts A and B) differs from *Daldinia* and relatives in producing dihydroisocoumarins (e.g. 5-methylmellein, **20** in Figure 3) in culture. Preparative HPLC studies and HPLC profiling of several hundreds of specimens and cultures revealed that melleins were not detected in *Daldinia* and immediate allies [30,33,34]. Accordingly, chemotaxonomy agrees with Hsieh et al. [8] who referred to *H. placentiforme* as its synonym, *Daldinia placentiformis* (Berk. & Curt.) Theiss 1909, since their molecular study revealed this fungus to be included in their *Daldinia* clade (see below). The holotype of *H. placentiforme* and various conspecific specimens that we have studied concurrently, indeed contained BNT, daldinone A (**15-16**) and the naphthol (**18**), with (**16**) being the major pigment. *Hypoxylon symphyon*, another member of the *H. placentiforme* line, even contains the naphthol (**18**) besides daldinal A (**12**) and further unidentified components that have so far only been encountered in stromata of *Daldinia childiae* (Figure 7h) and allies. A revision of their status to unite them in one genus is feasible as inferred from the above mentioned differences to *Hypoxylon*.

D. Pigments of *Annulohypoxylon* group B start

As discussed above, the members of *Annulohypoxylon* Group B, were recently found to contain specific azaphilones (Figure 6) that were not detected in *Hypoxylon*

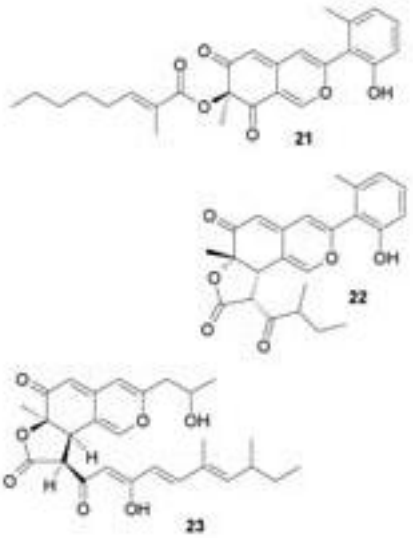

Figure 6. Azaphilones of *Annulohypoxylon* and *Creosphaeria* species. 21: Cohaerin A. 22: Multiformin A. 23. Sassafrin D.

or other *Xylariaceae*, supporting the status of *Annulohypoxylon* as a separate genus. The cohaerins (**21, 22**) are the azaphilone pigments of *Annulohypoxylon cohaerens* (Figure 7f). Like the previously treated daldinone A and truncatone (**16, 17**) of *Annulohypoxylon* group A, they are greenish olivaceous in 10% KOH. While being apparently absent in *Annulohypoxylon multiforme* (Figure 7e), they were still detected by HPLC in stromatal granules of the type specimens of *Sphaeria cohaerens* (Persoon herbarium in L), which had been collected prior to 1800 [25]. The multiformins (**23-24**) are the reddish-brown pigments in the stromata of *A. multiforme*. They appear to be chemically related to the cohaerins and also occur in other *Annulohypoxylon* spp. of group B (including *Annulohypoxylon michelianum*, Figure 7d, a species with ostiolar disks), but were not yet found in *A. cohaerens* itself. Like other azaphilone pigments, they show strong, non-selective antifungal and antibacterial activities [22]. Daldinone A (**16**) co-occurs with cohaerin-like pigments in the tropical *Annulohypoxylon moriforme*, while the morphologically similar *Annulohypoxylon nitens* contains derivatives of truncatone (**17**) [25]. Possibly, *H. moriforme* and its relatives constitute transitional forms between groups A and B of *Annulohypoxylon.* Morphological trends as proposed in the evolution of *Annulohypoxylon*, i.e., *Hypoxylon* sect. *Annulata* ss. Ju & Rogers [11], such as the successive reduction of the ostiolar disks, demonstrated by some representatives (Figures 7c-f) are accompanied by the evolution of stromatal pigments. "Primitive" *Annulohypoxylon* spp. of group A, such as *Annulohypoxylon bovei*, contain mainly BNT (**15**). Its oxidized derivatives (**16, 17**) are found in the group that is regarded as more advanced, and the structurally more complicated azaphilones (e.g. **21-22** in Figure 6) were mainly detected in those members of the genus that are regarded as most advanced (*A. cohaerens*, *A. multiforme*) [11].

From a comparison of morphological and anatomic features, it has been postulated that *Biscogniauxia* represents a lineage in the *Hypoxyloideae* that retained various ancestral characters [11]. Accordingly, the ostiolar disks in species of *Annulohypoxylon* group A relate them to particular species in *Biscogniauxia*. Ostiolar disks and the carbonaceous tissue between perithecia of these *Annulohypoxylon* spp. are considered to be remnants of the bipartite stromata in *Biscogniauxia* [11]. The latter genus does not even contain BNT, while the occurrence of melleins in their stromata [38] relates it to other *Hypoxyloideae*. Hence, the division of the two groups in *Annulohypoxylon* by Hsieh et al. [8], with *Biscogniauxia* appearing ancestral, and their theory on evolution of *Hypoxylon* and *Annulohypoxylon* from *Biscogniauxia*-like fungi, widely agree with chemotaxonomic data (Figure 8).

In one instance, (elevation of *"H. cohaerens var. microspora"* to specific rank as *Annulohypoxylon minutellum*), the new subgeneric classification proposed in [8] is also supported by the fact that *A. minutellum* contains multiformins, while *A. cohaerens* (formerly known as *H. cohaerens* var. *cohaerens*) contains cohaerins. Quang et al. [25] also revealed that both varieties each of *A. moriforme* and *A. bovei* (Figure 7c) differ substantially in their HPLC profiles [25], which might soon be reflected by further changes in their classification.

E. Sassafrins – biological activities and possible chemoecological functions of Xylariaceae pigments

The sassafrins (e.g. **23** in Figure 6) are the red azaphilone pigments of *Creosphaeria sassafras*. Like other azaphilones, they possess broad spectrum antibiotic activities [19]. This fungus was still included in *Hypoxylon* by Miller [14] as *Hypoxylon sassafras*, but it has a xylariaceous teleomorph and a "diatrypaceous" *Libertella*-like anamorph [11]. Accordingly, the chemical structures of the sassafrins are entirely different from those of the pigments of *Hypoxyloideae*. Sassafrin D (**23**) even has an unprecedented carbon skeleton. All the above features, along with molecular data [36], suggest that *Creosphaeria* represents a distinct evolutionary lineage of the *Xylariales* that may soon be expelled from the *Xylariaceae* altogether, once detailed molecular studies on a larger number of their taxa are available. Aside from the sassafrins, the multiformins (**23, 24**) were also reported to exhibited broad spectrum activities in biological systems [22]. Preliminary results also indicate that this applies to all other azaphilone pigments of the *Hypoxyloideae*.

These findings are not surprising, since various further azaphilones that have previously been characterized on their effects in biological systems, were all found to show a rather unspecific mode of action [22]. The generic name of this pigment class is derived from their ability to form spontaneously ammonia adducts. However, even cysteine or terminal amino groups in the active centers of important proteins are conceivable biochemical targets when biological systems are exposed to such azaphilones. The fact that the pigments are located in extremely high concentrations in granules directly beneath the stromatal surface (see Figures 2f, 2g, and 4a), particularly in young stromata, suggests that their natural role is an outward-directed chemical defense to protect the maturing teleomorphs. We think they are essential for survival of their producers and co-evolved with morphological and biological features, hence their taxonomic significance. Hence, the definition "extrolite" [27] is fully justified for these secondary metabolites of *Xylariaceae*.

F. Molecular (nucleic acid) vs. phenetic (morphological and secondary metabolite) data

The above results are summarized in figure 8, demonstrating that various chemotaxonomic features from the *Hypoxyloideae* help to verify or modify the current theories on evolution of hypoxyloid taxa. In general the

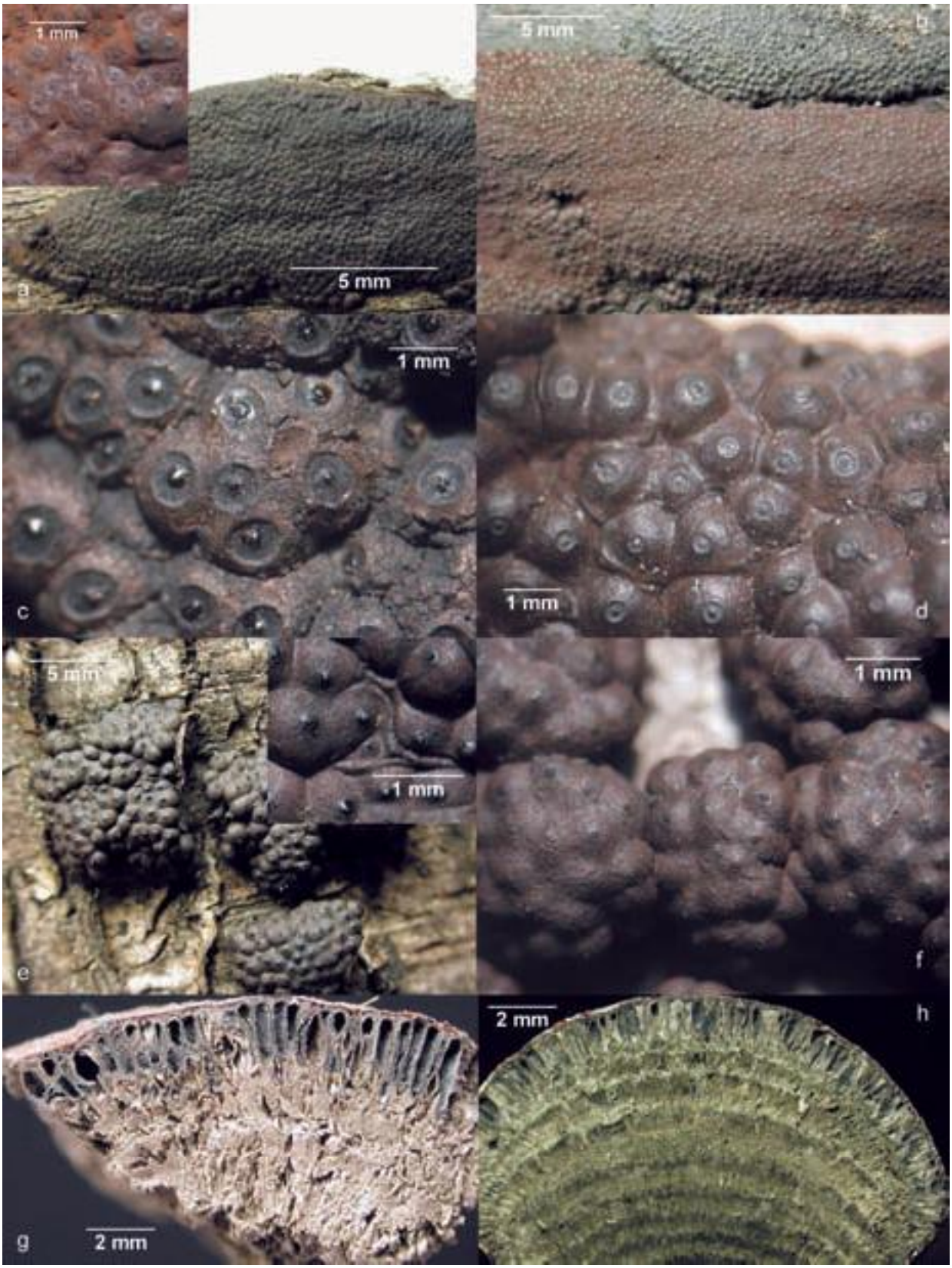

Figure 7. Further *Hypoxylon*, *Annulohypoxylon*, and *Daldinia* spp. a) *H. monticulosum* CL-0982 (Guadeloupe, leg. C. Lechat), large pictures showing mature melanized stromata and small picture showing augmented section with perithecial mounds and purple coating containing BNT. b) *H. investiens* (Taiwan, ex herb. Yu-Ming Ju), melanized and less melanized stromata containing BNT and large amounts of daldinone A. c) *A. bovei* LB 1090 (Argentina, leg. L. Beenken), perithecial mounds of young stromata showing large annulate ostiolar disks and purple coating by BNT; d) *A. michelianum* FC-5285-4 (Spain, leg. F. Candoussau), perithecial mounds of stromata showing annulate disks; e) *A. multiforme* JF-01075, stromatal habit and augmented section of perithecial mounds showing papillate ostioles. f) *A. cohaerens* JF-99236, perithecial mounds showing papillate ostioles. g) *H. placentiforme* (Dem. Rep. Congo, herbarium BR), section through stromata showing long tubular perithecia and essentially homogenous context beneath. h) *D. childiae*, showing tubular perithecia and stromatal context with horizontally orientated concentric zones. Scale is indicated by bars.

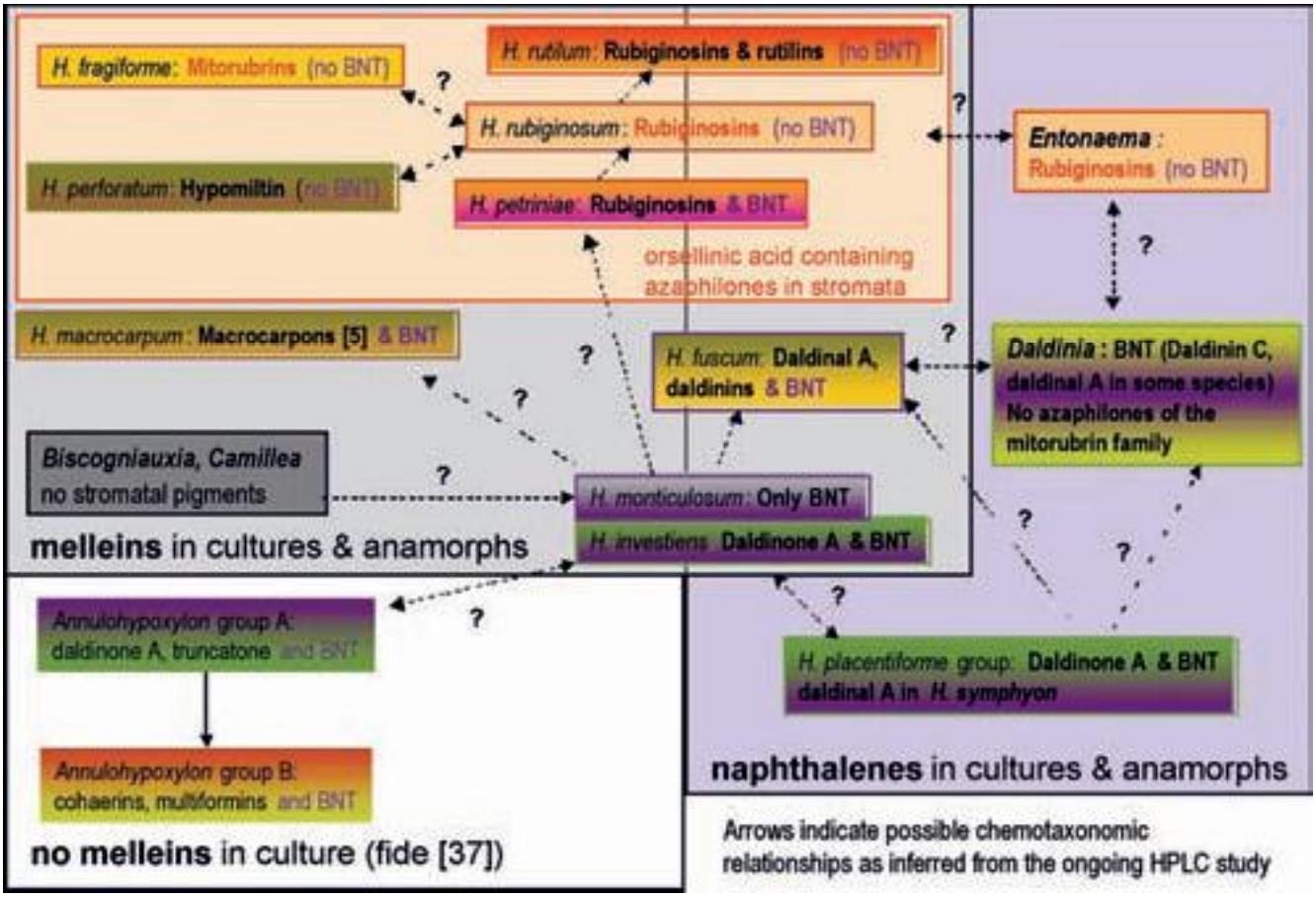

Figure 8. Chemotaxonomy and evolution of the *Hypoxyloideae* as inferred from the theory of evolution in *Hypoxylon* and allies [8,11,37], considering the data obtained in the current chemotaxonomic survey (summary of the cited publications). Connections indicated by question marks require verification, best by employing a polythetic approach to taxonomy and phylogeny of these fungi.

scheme agrees with preliminary results on molecular phylogeny of these fungi [8,9,28,36]. Nonetheless, due to insufficient taxon sampling, the "molecular picture" is not yet clear, and even contradictory results were obtained when, e.g., actin, ß-tubulin and ribosomal DNA gene phylogenies were compared [8,36]. However, some putative chemotaxonomic relationships (indicated by question marks in Figure 8) should best be verified by the inclusion of molecular data. It might be a challenge to assess the template provided by the framework of HPLC profiles and morphological data, using additional molecular phylogenetic approaches. A vast amount of work remains to be done until all taxa of the *Hypoxyloideae* have eventually been included in a similar scheme. Only then will it be feasible to assess the true value of chemotaxonomic data for the phylogeny of *Xylariales*.

The *Xylarioideae* have few stromatal metabolites. However, numerous unique bioactive compounds, including many compounds derived from industrial screening, have already been obtained from their cultures [29,37]. Most pigments of *Hypoxyloidae* are either pentaketides from the 1,8-DHN melanin biosynthesis [39] or hexaketides from other variants of the acetate-malonate pathway [3], and may be conjugated with the tetraketide, orsellinic acid, or with fatty acids. These findings suggest that specific polyketide synthases (PKS) are involved in their biogenesis. Hence, the differences observed in this "metabolomic" approach may eventually be reflected at the molecular level. Since stromata of *Biscogniauxia*, *Camillea*, and most genera in the *Xylarioideae* are poor in secondary metabolites, only a comparison of secondary metabolites from their cultures will result in additional chemotaxonomic evidence, as previously shown for a limited number of taxa [1,37]. Notably, many of the *Hypoxylon* spp. so far checked by HPLC were found to contain unidentified major components as prevailing stromatal constituents. Further unprecedented bioactive molecules, as well as additional chemotaxonomic evidence, may be revealed from them in the near future. As discussed more intensively elsewhere [29], such an approach can be combined with industrial natural products screening programs, increasing the probability of success in finding novel bioactive agents by assessing redundant and unique metabolites and focussing on the latter for preparative "mycochemical" studies.

The occurrence of stromatal pigments in *Hypoxyloidae* does not correlate as well with the current generic classification as secondary metabolite production in cultures and anamorphs. The latter (i.e. distribution of melleins and naphthalenes, respectively), emphasizes the differences between *Annulohypoxylon*, *Daldinia*, *Entonaema*, and *Hypoxylon* - aside from the *Hypoxylon placentiforme* line belonging to the *Daldinia* chemotype.

Clearly, chemotaxonomy alone will not provide the ultimate solution to establish a stable phylogeny. Recent studies on the molecular phylogeny of *Xylariales* [8,28,36] revealed the *Hypoxyloideae* as a largely monophyletic sister clade to the *Xylarioideae*. Interestingly, *Creosphaeria*, *Whalleya*, the *Diatrypaceae*, and other families of *Xylariales* appeared as further sister clades to the latter two major lineages in the *Xylariaceae*. This study revealed *Daldinia* and *Entonaema* to be members of a separate sub-

clade that appeared as a sister group to various other clades comprising *Hypoxylon* (and *Annulohypoxylon* ss. [8]). Triebel et al. [36] used several sequences from Genbank that could not be verified, since no herbarium material or cultures were apparently deposited in public institutions, and/or they were not published anywhere. If such results are disregarded and the unreliable sequences expelled from the phylogenetic trees, there still remain some problems with the use of this gene portion for taxonomic and phylogenetic purposes. Due to high similarities of 5.8S/ITS nrDNA sequences in *Hypoxyloideae* on the one hand, and varying degrees of infraspecific variability in certain species on the other hand, such data are not well-suited to reflect boundaries in morphologically and chemically homogenous populations – at least so long as an insufficient number of representatives have been evaluated by a polyphasic methodology. Molecular data suggest a high degree of infraspecific variability, e.g., in *H. fuscum* [28,36], whereas in other species such as *Daldinia loculata* [9] the 5.8S/ITS nrDNA is obviously a good marker for this particular morphological species. The isolates of *Daldinia concentrica* studied by Triebel et al. [36], a good "morphochemical" species showing consistent HPLC-profiles, morphological characters, and even minisatellite PCR fingerprints [33,34] also showed substantial divergences in their 5.8S/ITS nrDNA. It therefore may not be possible to use this portion of the ribosomal gene for general taxonomic purposes, let alone species identification.

The results by Hsieh et al. [9] suggest that genes such as actin and ß-tubulin may in the long run appear more informative and therefore come closer to a natural classification, but still their phylogeny was not in full agreement with species diversity as inferred from concurrent morphological and chemotaxonomic traits and reflected a "big picture" on generic affinities. For instance, species of the *H. fuscum* chemotype that have very similar anamorphs, teleomorphs, and HPLC profiles appeared in two different major branches of their *Hypoxylon* subclade. After all, no selective evolutionary pressure appears to be directed toward or against a certain motif in the ribosomal gene (or in non-coding parts of other genes). In *Xylaria hypoxylon*, it has even been found that other "alien" genes may be incorporated into the ITS-1 region [18], rendering species recognition based on ribosomal DNA sequences very difficult. Molecular taxonomy and phylogeny of ascomycetes have as of recently switched to multi-gene genealogies that appear to reflect morphological and chemical traits much more closely. However, the various tropical taxa in the *Hypoxyloideae* may not be made available readily for molecular studies. Even if this were the case, and for reasons discussed above, it would still be necessary to rely on a substantial number of representatives, rather than a single isolate of each taxon.

In fact, all current evidence suggests that the roots of the *Xylariaceae* are to be found in the tropics [38]. If it holds true that - as discussed by Rogers [26] - tropical rainforests may be "*museum-like refuges*" for *Xylariaceae* that eventually developed in dry areas, then it becomes all the more important to study those materials that are only extant from the types that were collected in tropical countries about a century ago. Those taxa showing important phylogenetic characters, such as "non-missing links" between different evolutionary lineages are more likely to be encountered there than in the relatively well-explored temperate areas of the world. For the time being, it appears practical to provide a template by using a combination of morphological and chemical traits by which fresh culturable material can be more easily identified and subsequently used for phylogenetic studies.

Conclusions

The results discussed above support the approach by Samson and Frisvad [27], since they demonstrate the feasibility of polythetic taxonomic approaches not only at the infrageneric level but even point toward the utility of polyphasic taxonomic studies for evaluation of phylogeny and taxonomy of higher taxa in the Ascomycota. In addition, secondary metabolites are expressions of the genome and ultimately will reveal true relatedness. The proposed scheme (Figure 8) offers several hypotheses that may be important to prove by future molecular studies. Even before their chemistry had been elucidated, many of the informative chemical characters in *Hypoxyloideae* and other macromycetes could be readily recognized in the field, or evaluated by using conventional microscopy. Such information will be invaluable in the discovery of novel natural products that may have utility in the pharmaceutical and agricultural industry. Aiding such identification work, user-friendly keys should be provided to facilitate finding the missing taxa that need to be studied to obtain conclusive results on molecular phylogeny. Only then will a polyphasic taxonomy and phylogeny of *Xylariaceae* be feasible and practical.

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