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The phylogenetic position of *Rhopalostroma* as inferred from a polythetic approach

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Key words

Ascomycota
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Abstract The xylariaceous genus *Rhopalostroma* comprises a small conglomerate of stromatic, angiosperm-associated pyrenomycetes, which have so far exclusively been reported from the palaeotropics, above all from tropical Africa and South Asia. Morphological and chemotaxonomic studies had suggested their close relationship to the genera *Daldinia* and *Hypoxylon*. However, those results were mainly based on herbarium specimens, and no molecular phylogenetic data were available on *Rhopalostroma*. During a foray in Côte d'Ivoire, fresh material of *R. angolense* was collected, cultured and studied by microscopic methods and by secondary metabolite profiling using high performance liquid chromatography coupled with diode array and mass spectrometric detection. In addition, ITS nrDNA sequences of the cultures were generated and compared to those of representative *Xylariaceae* taxa, to evaluate the phylogenetic affinities of this fungus. The results showed that *R. angolense* is closely related to the daldinoid *Xylariaceae*, and in particular to the predominantly neotropical genera *Phylacia* and *Thamnomyces*.

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INTRODUCTION

The genus *Rhopalostroma* was erected by Hawksworth (1977) to accommodate a series of palaeotropical pyrenomycetes that are characterised by producing narrowly obconical, often stipitate stromata. Perithecia are embedded in a monostichous layer at the upper part of the stroma, below the convex surface of the semiglobose to subglobose stromatal head, surrounded by characteristic pigment granules. The asci are early deliquescent and can normally not be observed even in fresh material. The ascal tips lack the amyloid apical apparatus that is commonly encountered in most *Xylariaceae*. The ascospores of *Rhopalostroma* are unicellular, brown, ellipsoid to reniform and bear a germ slit. Ten species of *Rhopalostroma* are distinguished, based on the size and the morphological characteristics of ascospores and stromata (Whalley et al. 1998).

Rhopalostroma belongs to the hypoxyloid *Xylariaceae*, which have *Nodulisporium*-like anamorphic stages. Hawksworth (1977) had already observed such typical conidiophores on the stromata of the type specimen of *R. angolense*, and Hawksworth & Whalley (1985) later cultured another species from India, confirming these results. The morphologically highly similar, neotropical genus *Phylacia* mainly differs from *Rhopalostroma* in the lack of ascospore germ slits and perithecial ostioles, whereas *Thamnomyces*, which is distributed in tropical America and Africa, features wiry stromata. Hawksworth (1977) has already discussed the possible affinities of *Thamnomyces* and *Rhopalostroma*, and Ju et al. (1997) postulated affinities of the latter genus to *Daldinia*. Stadler et al. (2004a) reported that these genera and *Phylacia* have various stromatal secondary metabolites in common. Relationships of *Phylacia* and *Thamnomyces* to *Daldinia* were also established by employing molecular phylogenetic data (Bitzer et al. 2008, Stadler et al. 2010b). *Ruwenzoria*, a recently described tropical xylariaceous genus (Stadler et al. 2010a), also features early deliquescent asci that are devoid of an amyloid apical apparatus. However, its stromata are massive, showing an effused-pulvinate habit, and its closest relative as inferred from molecular phylogenetic data appeared to be *Entonaema liquescens*, a xylariaceous pyrenomycete featuring hollow, liquid-filled stromata.

As revealed by morphological, chemotaxonomic and molecular phylogenetic data, all the above genera appear to be allied to one another – although no molecular data have hitherto been recorded in a *Rhopalostroma* species. The only extant viable culture of *Rhopalostroma* was obtained from the perithecial contents of a specimen of *R. indicum*, the type species of the genus, that had been collected over 20 years ago (Stadler et al. 2004a). A *Nodulisporium*-like anamorph was observed in this culture. It had, however, been possible to obtain cultures from other *Xylariaceae* (e.g. *D. novaezelandiae*; cf. Stadler et al. 2004b) several years after collection, and we preferred to wait until fresh material became available to serve as a representative of the genus in our ongoing phylogenetic studies of xylariaceous fungi. Recently, *R. angolense*, one of the species originally included in the genus by Hawksworth (1977) was encountered in western Africa. The current study deals with the characteristics of this species and with its affinities to other members of the family as inferred from a polythetic approach.

MATERIALS AND METHODS

Fungal material studied

Various fungal sporocarps were collected by S.G. during a field trip to several countries in western Africa in the summer of 2009. The *Xylariaceae* amongst them were identified by using the microscopic methods described by Stadler et al. (2008), aided by a comparison of morphological and chemotaxonomic data with those previously obtained during studies of type and authentic specimens (cf. Stadler et al. 2004a for *Rhopalostroma*

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species). For preparation of cultures, the perithecial contents were plated on YMG agar (yeast extract 0.4 %; malt extract 1 %; dextrose 0.4 % (w/v); all ingredients supplied by Merck Darmstadt, Germany; see Stadler et al. 2008) and observed microscopically. The ascospores readily germinated after 2 d, and pure cultures were finally obtained by repetitive transfer of the mycelia onto new agar plates. Since the air-dried stromata had been infested by mites and psocids, they were frozen over night at –20 °C, prior to culturing. These invertebrate contaminants were successfully removed by an additional subculture on YMG agar supplied with 50 mg/L of the insecticidal and acaricidal agent Ivermectin (Sigma). The herbarium specimens are housed in M, and representative cultures were deposited with CBS and MUCL.

Microscopic observations of teleomorphic structures were made in water (for studying ascospore morphology), in Melzer's reagent (for testing the amyloidity of ascal apical structures), in Chlorazol black (for measurements of ascal stipes), and in 10 % KOH (for testing the dehiscence of perispore). In cases of apparent absence or lack of reaction of ascal apical structures in Melzer's reagent, an attempt was also made after pretreatment with 3 % KOH. Measurements of ascospores were made in water at 1 000 ×. Micrographs of ascospores were taken in water or 10 % KOH. KOH-extractable pigments were obtained as described in Ju & Rogers (1996). Inoculated plates or Erlenmeyer flasks, containing 100 mL of agar media or 20 g of cellulose pulp, respectively, were incubated for 12 h at 23 °C under fluorescent light. Microscopic observations of anamorphic structures from cultures on Difco Oatmeal agar (OA) or YMG media (Bitzer et al. 2008) were made in water at 400–1 000 ×, using phase contrast. For microscopic characteristics, c. 30 measurements were made to calculate the mean values. Colours were determined using the colour charts of Rayner (1970).

HPLC profiling

Secondary metabolite profiling was carried out using a dual HPLC system, comprising a diode array detector (DAD) and mass spectrometric (MS) detectors, the latter providing mass spectra in the positive and negative electrospray ionisation (ESI) mode, using HPLC-based dereplication library of *Xylariaceae* metabolites (Bitzer et al. 2007). The resulting chromatograms, MS and HPLC-DAD-spectra were used to identify the secondary metabolites in the crude extracts. Standards of numerous pure chemotaxonomic marker molecules obtained during previous studies of the *Xylariaceae* (in particular, those isolated previously from *Phylacia* and *Daldinia* species by Bitzer et al. (2008) and Stadler et al. (2004a), respectively; Fig. 1) were available, in order to provide unambiguous identifications of the compounds obtained from stromata and cultures of *R. angolense*. For the chemotaxonomic study, the cultures were grown on HLX (10 g Difco Bacto vitamin-free casamino acids (Becton Dickinson, Heidelberg), 1 g K_2HPO_4, 0.5 g $MgSO_4 \times 7\ H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \times 7\ H_2O$, 30 g sucrose, ad 1 L tap water, pH 6.3, supplemented with vitamin solution, as specified in Brewer et al. 1968) and YMG media as described by Bitzer et al. (2008). Fermentations were performed in 500 mL Erlenmeyer flasks containing 200 mL of culture media, employing the standardised conditions used in previous studies on *Xylariaceae*. The time course of metabolite production was followed by HPLC profiling of samples taken daily for up to 10 days of fermentation, and glucose and pH were determined concurrently. The rationale for using this standardised methodology is that in all *Xylariaceae* hitherto studied, secondary metabolite production

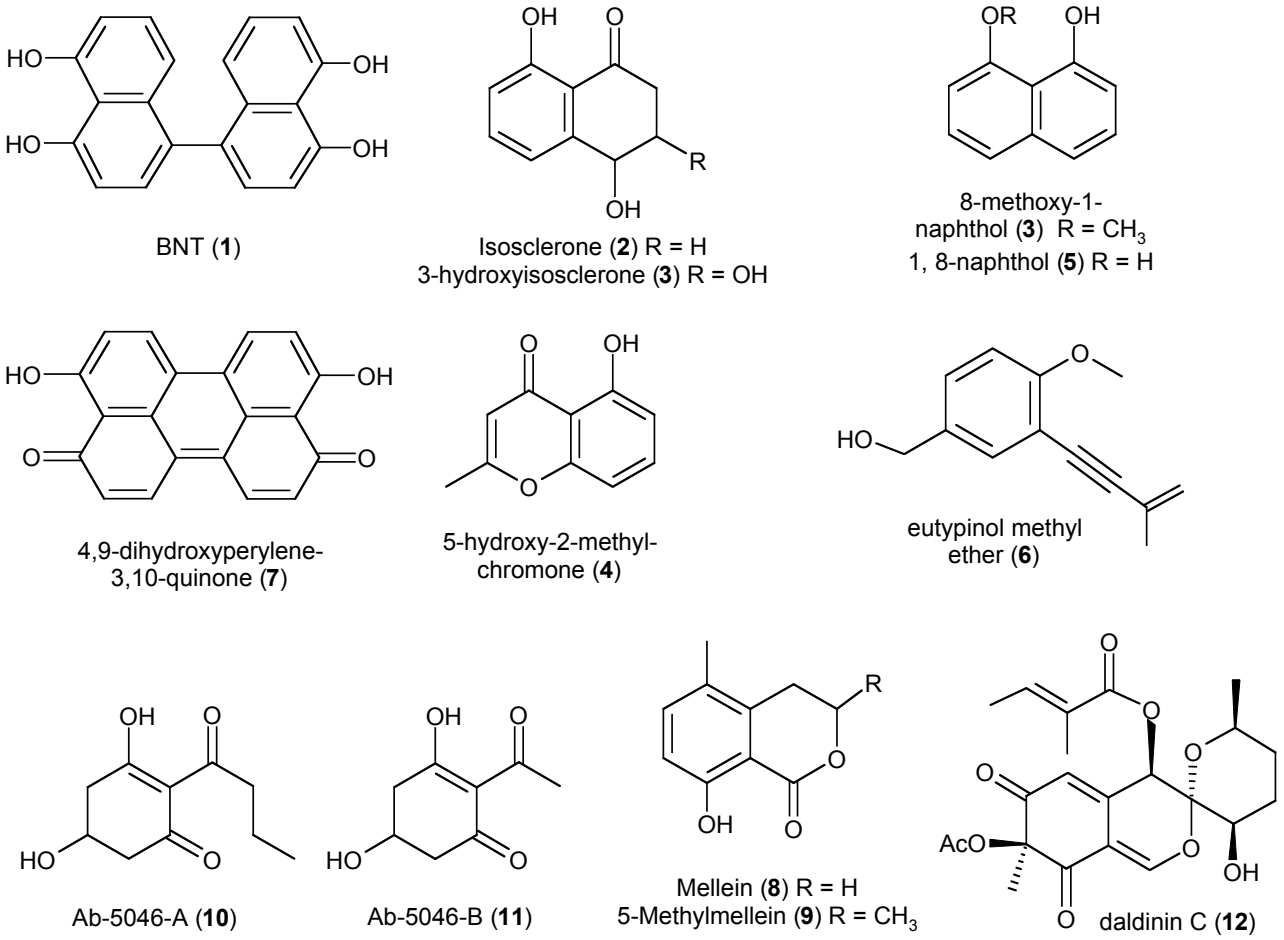

Fig. 1 Chemical structures of chemotaxonomically significant metabolites of the hypoxyloid *Xylariaceae*.

was previously found to reach its maximum intensity as the free glucose in the culture medium was depleted, accompanied by an increase of the pH value, and that such results were always highly reproducible in a given strain or species (cf. Bitzer et al. 2008).

Molecular-phylogenetic analysis

Total DNA was isolated from cultures of *Rhopalostroma angolense*, using the ChargeSwitch® gDNA Plant Kit (Invitrogen) as recommended by the manufacturer. The ITS region (ITS1, 5.8S, and ITS2 rRNA gene) was amplified using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Increments were added as recommended by the manufacturer of the Taq polymerase (Invitrogen) and sterile distilled water was added to obtain a final reaction volume of 25 µL. The PCR commenced by an initial denaturation step (95 °C, 3 min). Following 30 cycles of denaturation (94 °C, 0.5 min), primer annealing (52 °C, 1 min), and elongation (72 °C, 2 min), a final elongation (72 °C, 5 min) completed the PCR. Addition of 17.5 µL 100 % isopropanol and 2.5 µL 5 M NaCl served to precipitate the amplicons over night at room temperature. Following centrifugation and resuspension, the ITS region was sequenced through the sequencing service of the faculty of genetics (Ludwig-Maximilians-University, Munich), using an ABI 3730 capillary sequencer. A consensus sequence of the forward and reverse strands, obtained by applying the PCR primers, was created by using the Staden software package (Staden 1996).

The sequence was aligned by eye with selected sequences from well referenced strains/specimens. Only unambiguously

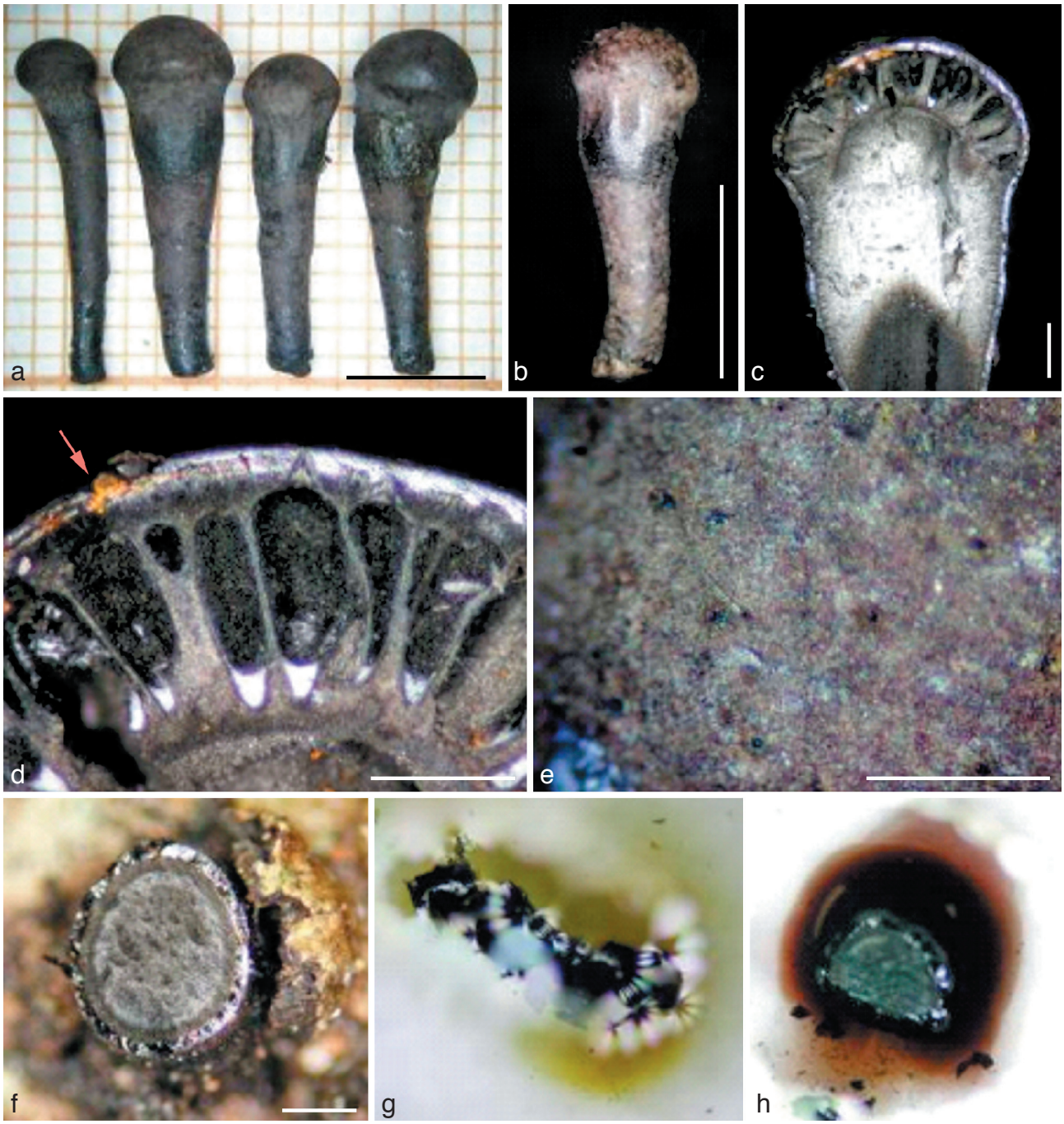

Fig. 2 Characteristics of *Rhopalostroma angolense*, specimen S. Gardt n° 284. a. Four stromata; b. stroma; c. dissected stroma, showing perithecial regions in the head and white stromatal context; d. vertical section of the fertile part of the stroma showing the crust composed of orange yellow granules visible in a bruised part (arrow) and the perithecial layer; e. stromatal surface with darker ostiolar openings; f. transverse section through the stipe; g. olivaceous pigments released in KOH, from the crust in the upper part; h. brown pigments released in KOH, from the crust of stipe. — Scale bars: a, b = 5 mm; c = 1 mm; d–f = 0.5 mm.

alignable positions were used for the phylogenetic analysis (positions 31–58, 122–143, 154–355, 360–380, 390–440, and 448–473, according to AM993138, *Xylaria hypoxylon*). The most likely molecular-phylogenetic tree was reconstructed using RAxML v7.0.3 (Stamatakis 2006), as implemented in ARB (Ludwig et al. 2004). The program was also used to test the robustness of the tree topology by calculating 500 bootstrap replicates. Default parameters and the GTRCAT model of nucleotide substitution were applied for both analyses, with all free model parameters having been estimated by RAxML.

RESULTS

Extended description of Rhopalostroma angolense

Specimens examined. Angola, Golungo Alto, Quibolo, on rotten wood, Mar. 1856, *Welwitsch* 103 p.p., K(M) 110674 – holotype of *R. angolense*, fide Hawksworth (1977). – Côte d'Ivoire, Région Sud, Station Ecologique de Lamto, N 06° 13' W 005 0', dense forest, on dead bark of *Ceiba pentandra*, 4 Aug. 2009, leg. *S. Gardt* 284 (M, culture in CBS 126414 and MUCL 52664, GenBank acc. no of ITS nrDNA sequence FN821965), used in the morphological, chemical and molecular studies). – Sierra Leone, Yoabu, Bari, *Ceiba pentandra*, 25 Nov. 1949, *F.C. Deighton* (IMI 40343).

Stromata (Fig. 2a, b) narrowly obconical with a rounded apex, erect, gregarious, unbranched, 0.8–1.3 cm high × 2.6–4 mm diam at apex, 1.5–1.8 mm diam at base, dark brown with purplish tinge, hard-textured; fertile head hemispherical. Surface (Fig. 2e) finely pruinose, matt, without visible perithecial mounds, dotted with minute black ostioles flush with the surface; outer crust 80–100 µm thick, brittle, at base composed of dull red brown granules yielding dark brick (60) pigments in 10 % KOH (Fig. 2h), at apex composed of dull yellow granules (arrow in Fig. 2d) yielding olivaceous (48) pigments in 10 % KOH (Fig. 2g). Stromatal interior (Fig. 2c, f) soft-textured, pithy-fibrous, dark grey between the perithecia, delimited from the pale grey underlying tissue by a thin black line, blackish below down to the base of the stipe. *Perithecia* (Fig. 2d) 0.65–0.75 mm high × 0.2–0.25 mm diam, lanceolate, monostichous, crowded, in contact. *Asci* (Fig. 3a–c) cylindro-clavate to cylindrical, stipitate, containing 8 obliquely uniseriate overlapping ascospores in unilaterally spicate arrangement on elongated ascogenous hyphae, 130–150 µm total length, the spore bearing pars 80–100 µm long × 7.5–8.5 µm broad, the stipes 40–50 µm long with a bulbous base, very thin–walled and readily deliquescent, without apical apparatus nor reaction in Melzer's reagent. *Paraphyses* 5–6 µm at base, progressively tapering above, deliquescent. *Ascospores* (Fig. 3d, e) 13–17 × 6–7 µm (M = 14.7 × 6.3 µm), ellipsoid-inequilateral with broadly rounded ends to reniform, dark brown, with small guttules clustered at both ends, smooth, with a straight germ slit 3/4 to 4/5 spore-length on flattened or concave side; lacking a dehiscent perispore in KOH.

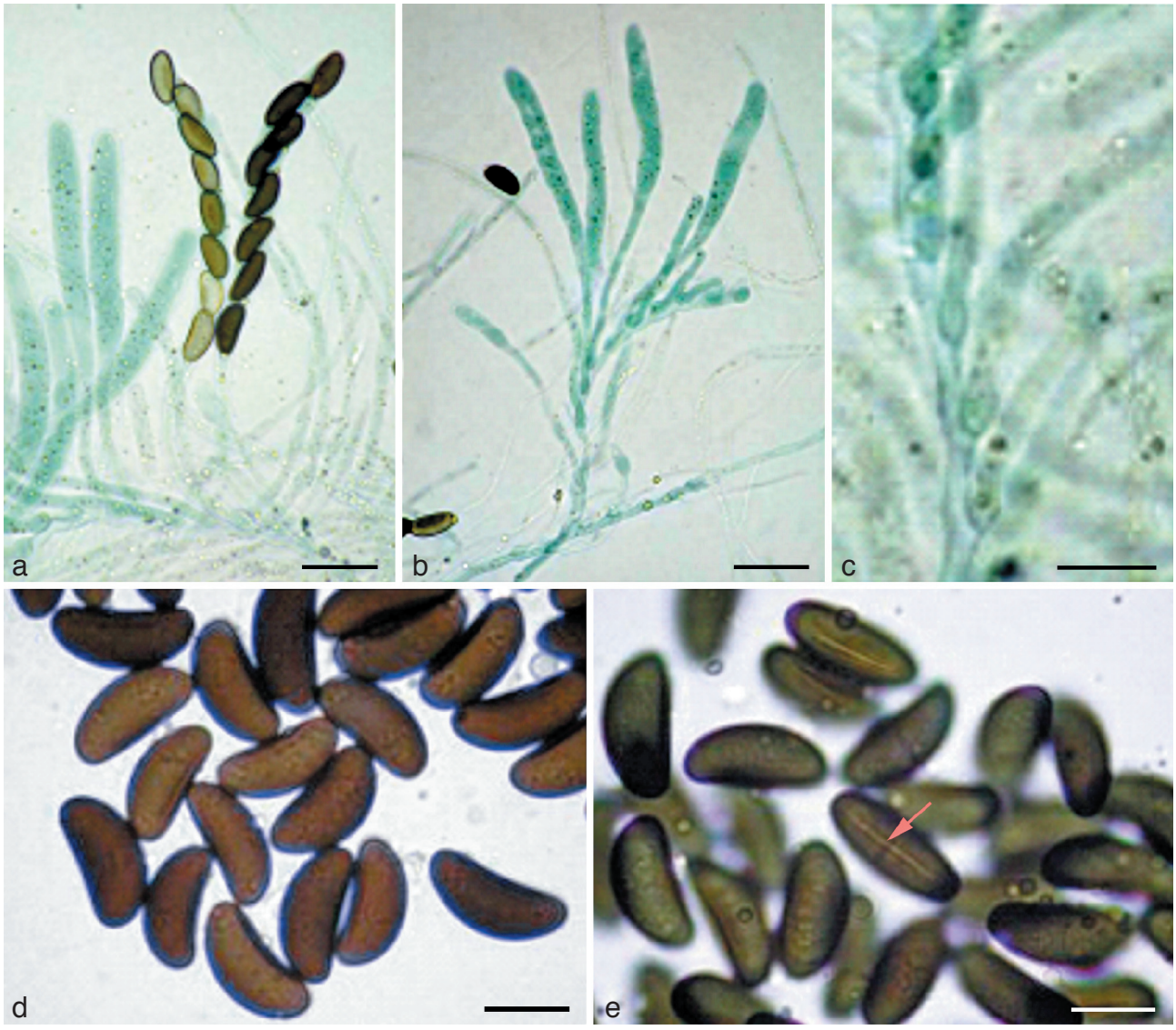

Fig. 3 Asci and ascospores of *Rhopalostroma angolense*, specimen S. Gardt n° 284. a. Asci, ascospores and paraphyses; b. immature asci; c. close up on attachment of asci to ascogenous hypha; d. ascospores in water; e. ascospores in KOH, showing the germ slits (arrow). a, b and c stained in chlorazol black — Scale bars: a = 20 µm; b = 30 µm; c–e = 10 µm.

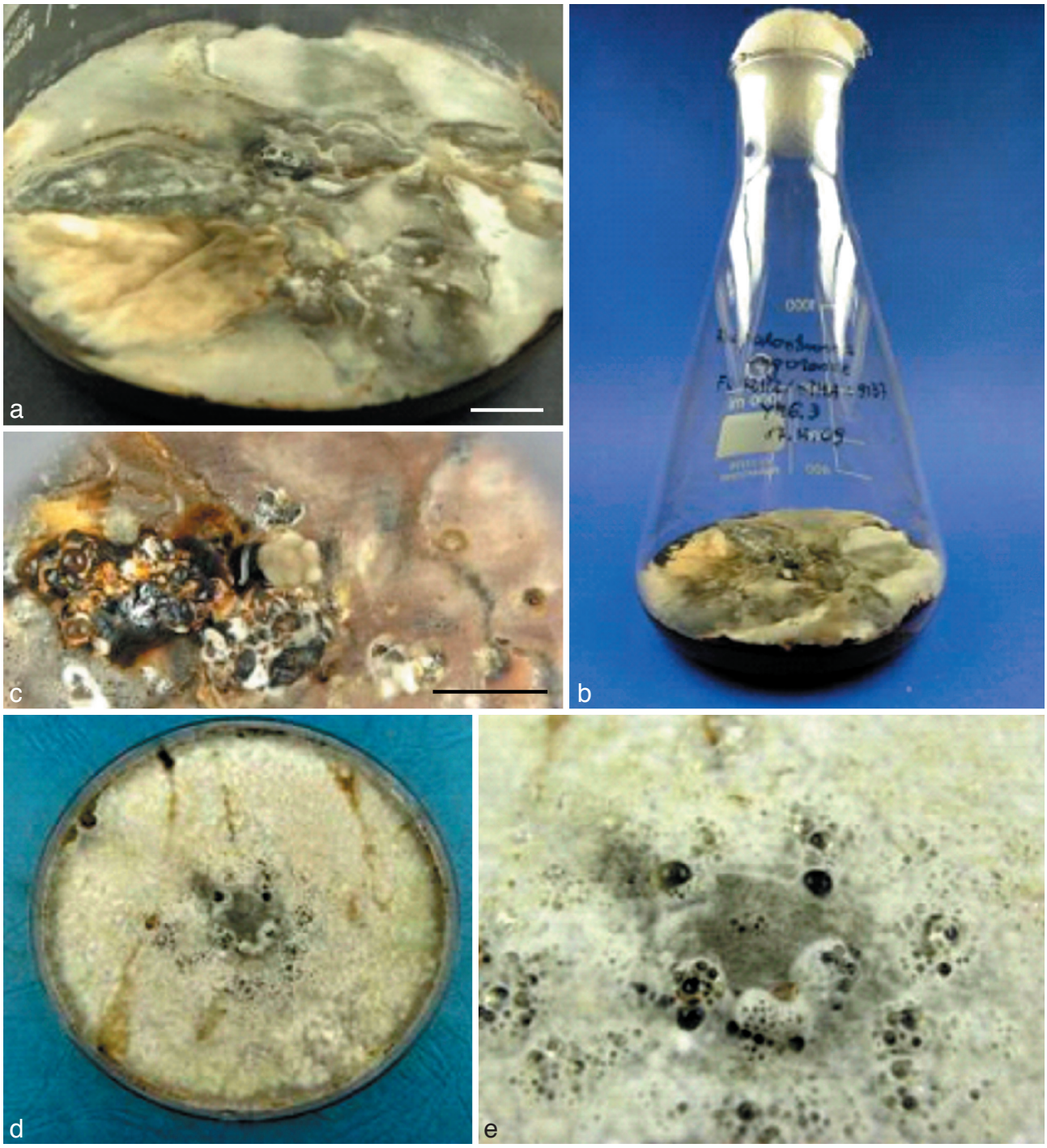

Fig. 4 Cultures of *Rhopalostroma angolense* in 500 mL Erlenmeyer flasks supplied with 100 mL of Difco OA after 5 wk (a, b) and YMG medium after 6 wk (c), respectively, showing stromatal primordia (c); d, e. culture of *R. angolense* on a Difco OA plate (9 cm diam); e showing augmented section of d, revealing stromatal primordia and oily droplets in the centre of the colony. — Scale bars: a, b = 10 µm; c = 1 cm.

Culture characteristics — *Colonies* on Difco OA plates (Fig. 4d, e) at 23 °C reaching the edge of 9 cm Petri dish in 6–8 d, at first whitish, felty, azonate, with diffuse margins, becoming smoke-grey (105) with an olivaceous tone; reverse turning citrine (13) or remaining uncoloured. Small stromatal primordia and oily droplets of exudates occurring on the agar plates after 7–10 d, and cultures developing a characteristic odour, reminiscent to that of *Daldinia* species. Mycelium composed of thick-walled hyphae (Fig. 5a), becoming melanised, irregularly swollen between the septa, and up to 6 µm thick. Production of conidiophores sparse, starting in zones near the centre of colonies as the mycelium becomes melanised. *Conidiophores* macronematous, simple, hyaline, slightly roughened, up to 60 µm long and 3–3.5 µm diam, referable to the *Sporothrix*-like branching pattern of Ju & Rogers (1996). *Conidiogenous cells* terminal, cylindrical, 12–20 × 3.5 µm, producing conidia holoblastically. *Conidia* (Fig. 5f–h) hyaline, finely roughened, aseptate, pyriform to ellipsoid, often truncate at the base, 5–7 × 2.5–4 µm.

Cultures in 500 mL — *Erlenmeyer flasks* (Fig. 4a–c) containing 20 g cellulose pulp or 100 mL Difco OA showed a similar morphology as those on agar plates, but produced semiglobose stromatal primordia to 2.5 cm diam after 4–6 wk. Conidiogenous structures arising as a greyish pruina, covering the primordia, showing the following characteristics: *Conidiophores* (Fig. 5b–e) up to 150 µm long, simple or dichotomously branched, more differentiated than the conidiophores in the mycelia, developing a *Virgariella*- to *Nodulisporium*-like branching pattern as defined by Ju & Rogers (1996). The conidia formed on these stromatal primordia had essentially the same dimensions as

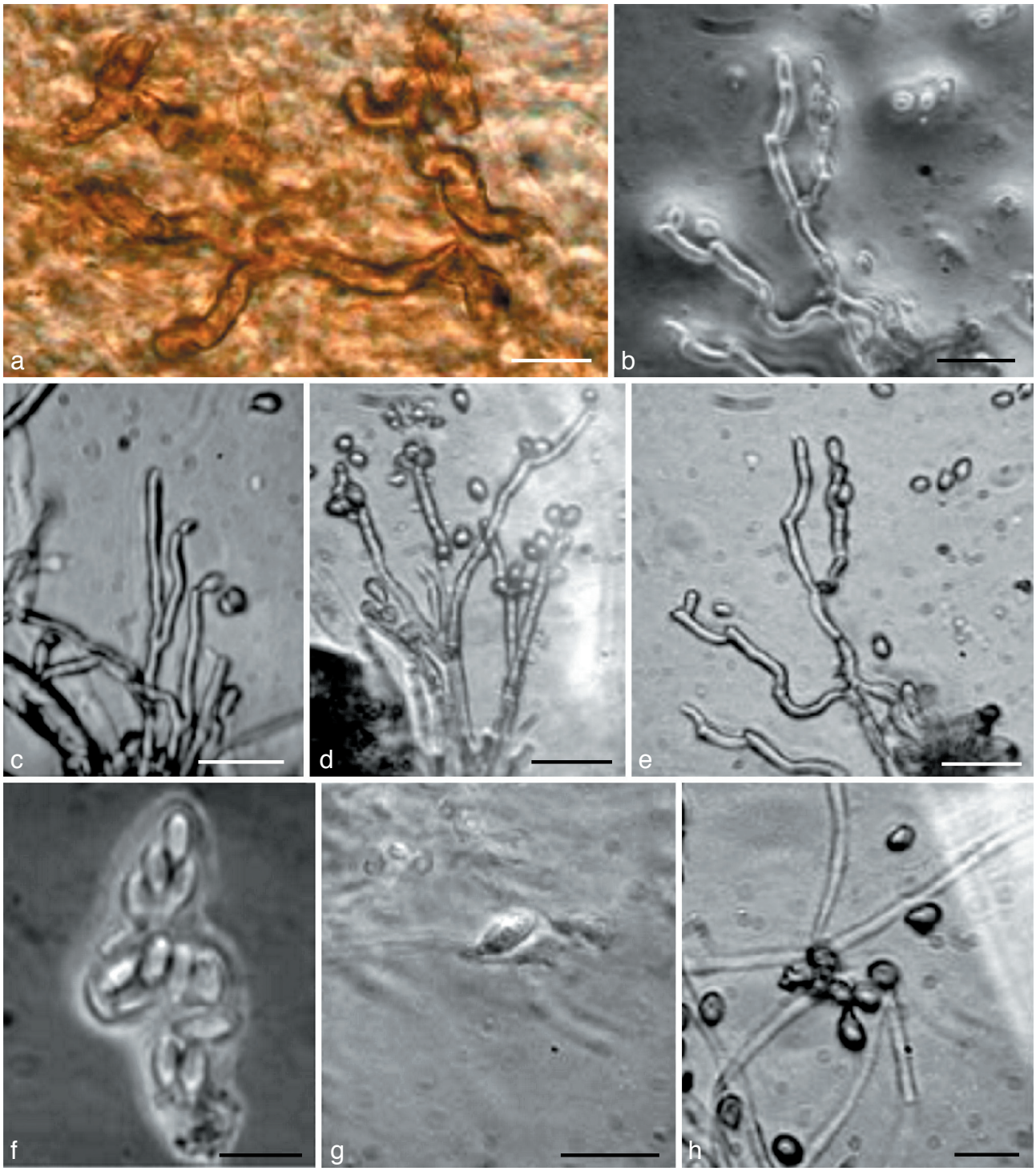

Fig. 5. Microscopic characteristics of *Rhopalostroma angolense*, from Difco OA plates (a, f, g) and 500 mL Erlenmeyer cultures, each after 5 wk of incubation. a. Thick-walled hyphae observed in melanising cultures; b–e. conidiophores, ranging from the simple *Sporothrix* to the more complex *Virgariella* types sensu Ju & Rogers (1996); f–h. conidia; g. conidium arising laterally from a simple conidiogenous cell of the *Sporothrix* type sensu Ju & Rogers (1996). — Scale bars: a, d–h = 10 µm; b–e = 20 µm.

those observed in the agar cultures. No fertile stromata were obtained even at prolonged incubation times (9 wk).

Secondary metabolites and chemotaxonomy (Fig. 6) — In accordance with the study of Stadler et al. (2004a), the fresh stromata of *R. angolense* contained BNT (binaphthalene tetrol; **1**) and a series of yet unknown metabolites. Some of them appeared similar to the major components of certain *Thamnomyces* and *Phylacia* species, and especially the unidentified compound **U1** appears to be a marker for the genus, because it was previously also detected in four other *Rhopalostroma* species. The secondary metabolite profiles of the cultures were very similar to those obtained from representatives of the genera *Phylacia* (Bitzer et al. 2008), *Ruwenzoria* (Stadler et al. 2010b), and *Thamnomyces* (Stadler et al. 2010a), in that they were apparently devoid of 1-methoxy-8-naphthol (**3**) and contained compounds **4**, **6**, **10**, and **11** as major components. In contrast to *Daldinia* and the aforementioned genera, the isosclerones **2** and **3** were not observed in the cultures of *R. angolense*. Neither were mellein derivatives (**8**, **9**), which are characteristic of *Hypoxylon* (Bitzer et al. 2008).

Interestingly, the stromatal KOH reaction, which was not studied by Hawksworth (1997), varied from brown at the base to olivaceous at the top in the freshly collected material from Côte d'Ivoire. According to concurrent HPLC analyses of both

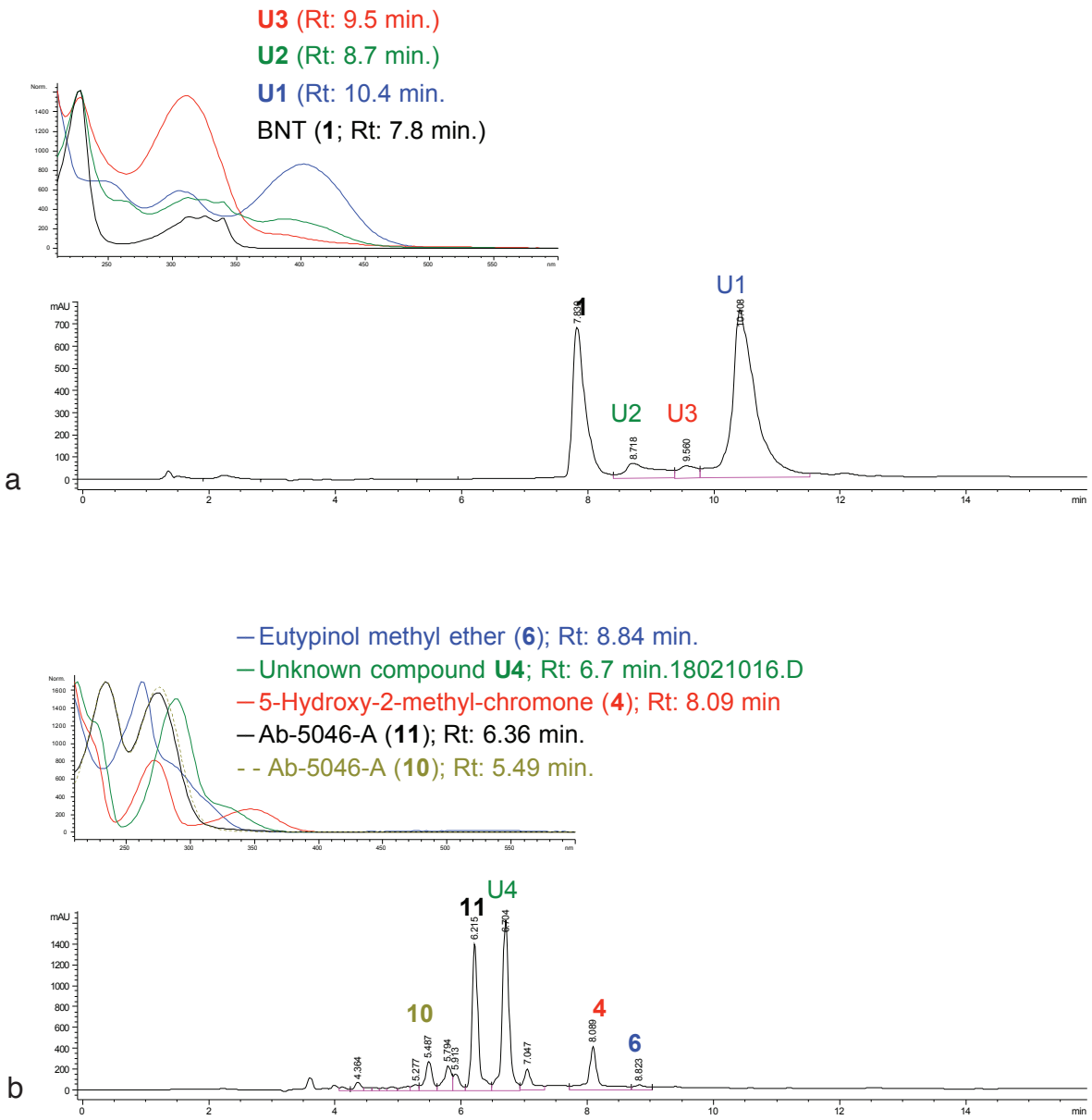

Fig. 6 HPLC-UV chromatograms (210 nm) of *Rhopalostroma angolense* and DAD spectra of major components. a. Stromatal MeOH extract, revealing BNT (**1**) and three other, yet unknown, major components; b. culture in YMG after 120 h of fermentation, revealing 5-hydroxy-2-methyl-chromone (**4**), eutypinol methyl ether (**6**), the phytotoxins of the 'Ab-5046' type (**10**, **11**) and a yet unknown component **U4**.

parts, this unusual phenomenon is merely due to different concentrations of essentially the same compounds, rather than to different molecules. Larger amounts of BNT were observed in the extracts from the base, whereas the unknown components that might be azaphilones prevailed in extracts from the perithecial region.

Notes — The specimen from Côte d'Ivoire agreed well with the description of *R. angolense* by Hawksworth (1977), with regard to both its teleomorphic and anamorphic characters. Affinities with *Daldinia*, *Phylacia*, and *Thamnomyces* are obvious, based on the presence of a continuous outer layer of granules enclosing the perithecia and the internal sterile tissues, as well as by the macro- and micromorphology of the cultures. The anamorph observed is rather similar to that described by Hawksworth (1977), who had observed the conidiogenous structures on young stromata of herbarium specimens. Hawksworth (1977) had also observed asci, but gave slightly deviating dimensions. To our knowledge, this study is the first report including photographic illustrations of the ascal structures of a *Rhopalostroma* species. The specimens examined for comparison with the fresh material were rather similar with respect to their morphology and HPLC profiles, except that slightly larger ascospores (13–19 × 6–8 µm; M = 15.9 × 6.7 µm) were found in IMI 40343, and the holotype specimen deviated from the other two in containing additional compounds that are presumably perylene quinones (cf. Stadler et al. 2004a and next paragraph).

KOH-extractable pigments of other Rhopalostroma species

In this context, it appeared practical to report unpublished morphological and chemotaxonomic data on *Rhopalostroma* spp. studied previously by Stadler et al. (2004a) and more recently, during a revision of the specimens in IMI, since only the HPLC profiling data, but not the KOH-extractable pigments of these materials had been studied earlier on.

Three specimens of *R. kanyae* (India, Rajasthan, Mt Abu, *Euphorbia caducifolia*, 14 Sept. 1974, *K.S. Panwar*, (IMI 188030; see Hawksworth 1977 as *Rhopalostroma* sp.); Mayda Pradesh, Shabdol, amerkantak, on *Bauhinia retusa*, 5 Mar. 1993, *U.S. Patel* (IMI 361432 as *Rhopalostroma* sp.). Thailand, Chiangmai Prov. Doi Inthanon NP, Mae Klang Waterfall, 23 Sept. 1993,

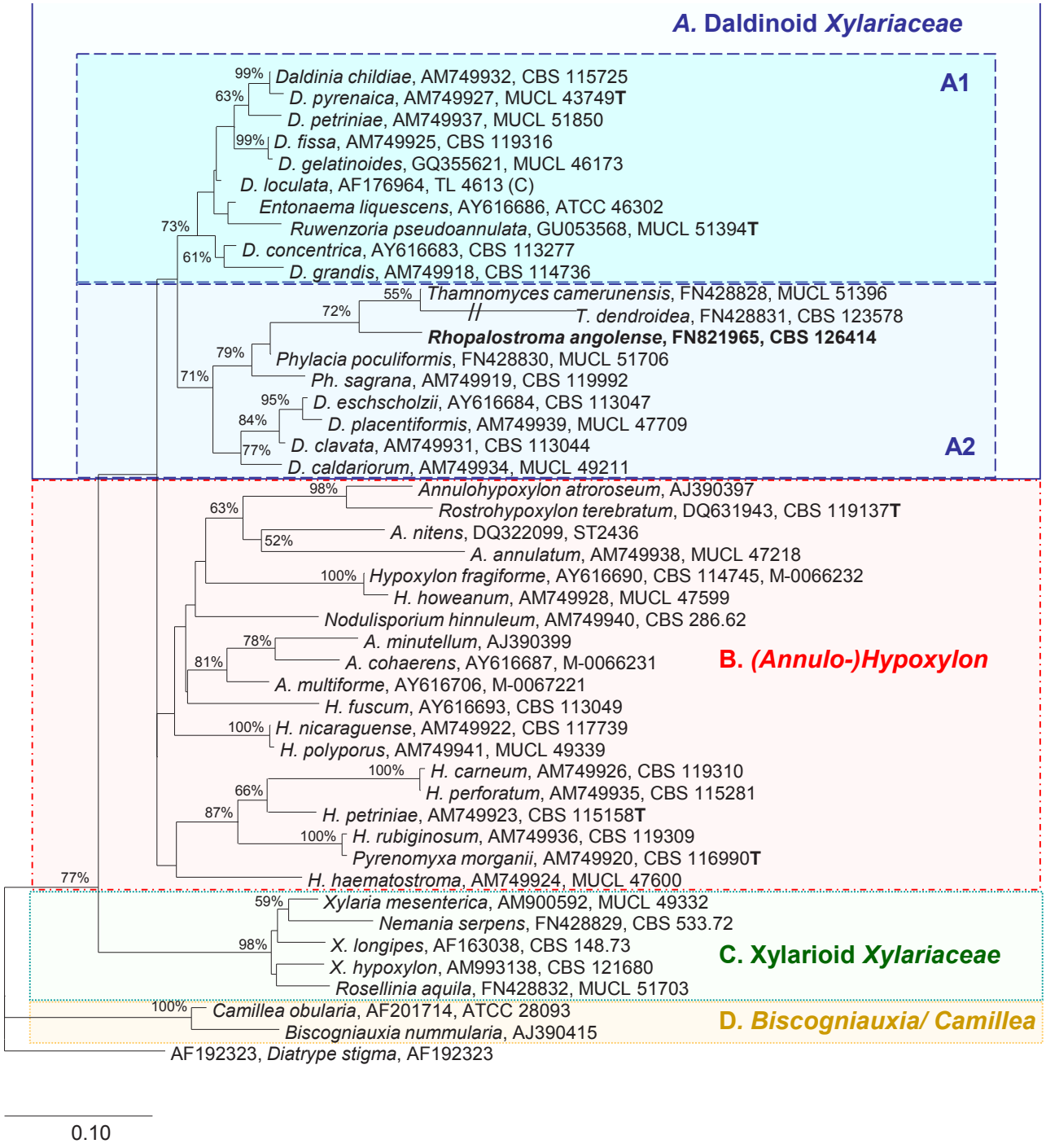

Fig. 7 Phylogenetic relationships among *Xylariaceae* as inferred from ITS nrDNA sequence data. Bootstrap support values exceeding 50 %, from 500 RAxML replicates, are assigned to the tree topology of the most likely tree found by RAxML. Taxon names are followed by the GenBank Acc. No. of the sequences and the culture collection and herbarium access numbers, if available (a 'T' indicates type strains). Selected long branches were bisected in length (*//*).

K. Auncam (IMI 368200, holotype of *R. kanyae*) were identified among the collections in IMI. Specimen IMI 188030 had been described by Hawksworth (1977) as '*Rhopalostroma* sp.', but he did not formally name it as a new taxon, because the specimen was in rather poor condition. We found that it corresponded well with the type of *R. kanyae*, a species subsequently erected by Whalley & Thienhirun (1996). Another specimen in IMI, originating from India, was also identified as *R. kanyae*, which is to our knowledge here reported for the first time from outside Thailand. In all three specimens, HPLC revealed BNT, overlaid by other components that are also present in, e.g., *Daldinia petriniae* and *D. lloydii*, further binaphthalenes were detected in traces by HPLC-MS. According to recent results on *Thamnomyces* (Stadler et al. 2010a), these pigments are most probably perylene quinones (e.g., compound **7** in Fig. 1), arising from oxidation of BNT (**1**). The KOH-extractable pigments of this species ranged from olivaceous-grey (121) to isabelline (65) or fawn (87). The type specimen of *R. africanum* (Uganda, Mt Elgon, on dead wood, Dec. 1814, Small 137.1, K(M) 110622, holotype) showed similar pigments in KOH. However, stromatal fragments from the type specimen of *R. angolense* (see above) also showed an isabelline tinge in KOH some minutes after incubation. Moreover, the putative perylene quinones were also detected in traces in the ancient material, despite being absent in fresh collections. Therefore, this phenomenon could be due to autoxidation of BNT during storage to perylene quinones. In this context, it has been discussed that KOH-extractable pigments of ancient type material should be confirmed by studies in fresh specimens, wherever possible (Stadler et al. 2004a, 2010a).

The type specimen of *R. gracile* (Thailand, Kanchanaburi, Cassia, Jan. 1982, *L. Manoch* (IMI 62895, isotype)) showed weakly purple pigments in KOH, and BNT (**1**) was detected in traces by HPLC. The holotype specimen in K (cf. Stadler et al. 2004a) was in better condition and yielded vinaceous-purple (101) to vinaceous-grey (116) pigments in KOH. The same was found for the type specimen of *R. indicum* (India, Karnataka St., Belehonnur, *Ficus retusa*, 25 Nov. 1974, *B.N. Muthappa* (BPI 586811, isotype). The isotype specimen of *R. dennisii* (India, Kerala, Calicut University, *Artocarpus integrifolia*, 29 June 1978, *S. Zachariah* (IMI 227748, isotype)) also revealed BNT (**1**) and other binaphthyls; as well as daldinin C (**12**) and other azaphilones, which are presumably daldinin derivatives. The KOH-extractable pigments were isabelline (65) to honey (64). These preliminary results appear rather promising as additional characters to include in a future key to identify *Rhopalostroma* species. However, the type specimens of several species were not yet available for comparison, and fresh material is also unavailable of some taxa that were described first in the 19th century. Additional field work in the Asian and African tropics will be indispensable to study such characters in detail, based on fresh material.

Molecular-phylogenetic analysis

The likelihood of the most likely tree found is -3727.46. Relative to a substitution rate of 1.0 for transversions between guanine and thymine (G↔T), the nucleotide substitution rates estimated by RAxML ranged from 0.9 (A↔C and C↔G) to 1.2 (A↔T) for transversions and from 3.0 (A↔G) to 4.4 (C↔T) for transitions. The topology of the most likely tree reveals four major groupings (clades A–D), two of which (clades A and B) are, however not supported by the bootstrap analysis (Fig. 7).

Representatives of the genera *Camillea* and *Biscogniauxia* (clade D in Fig. 7: *Xylariaceae* featuring *Nodulisporium*-like anamorphs and, bipartite stromata, but lacking apparent KOH extractable pigments) cluster as sister group to the other *Xylariaceae* in the phylogenetic analysis. Aside from these, four major groupings are revealed in the most likely tree. With 98 % bootstrap support (BS), the monophyletic origin of the xylarioid *Xylariaceae* (clade C in Fig. 7, i.e., species of *Nemania*, *Rosellinia*, and *Xylaria* with *Geniculosporium*-like anamorphs) is supported best. All representatives of the hypoxyloid genera, *Annulohypoxylon*, *Hypoxylon*, *Pyrenomyxa*, and *Rostrohypoxylon*, featuring stromatal pigments and unipartite stromata, are found in two weakly supported clades. While species of *Annulohypoxylon* and *Hypoxylon* become intermingled according to the most likely tree, the results are not contradictory to the separation of both genera (Hsieh et al. 2005), due to the low support (40 % BS at most) of the respective branches. Despite that, both genera appear paraphyletic in their current circumscription for other reasons, as already shown (Hsieh et al. 2005, Tang et al. 2007) using different methodologies to assess molecular phylogeny: *Pyrenomyxa morganii* clusters within *Hypoxylon* (100 % BS) and *Rostrohypoxylon terebratum* within *Annulohypoxylon* (98 % BS). What we refer to as the daldinoid *Xylariaceae* here, forms a monophyletic group (clade A in Fig. 7) according to the most likely tree, which is, however, not supported by the bootstrap analysis (31 % BS). Nevertheless, the two subclades A1 and A2, are reasonably well supported (71 % and 73 % BS, respectively). One comprises the majority of *Daldinia* spp. from the temperate climate zones, along with the tropical *D. grandis*, *Entonaema liquescens* and *Ruwenzoria pseudoannulata*. Within the second subclade, the predominantly tropical *Daldinia* spp., *D. eschscholzii*, *D. caldariorum*, and *D. placentiformis* (77 % BS) cluster as sister group to a reasonably well-supported clade (79 % BS) including the species of *Phylacia* and *Thamnomyces* as well as *Rhopalostroma angolense*. The monophyletic origin of *Thamnomyces* and *R. angolense* is supported with 72 % BS.

DISCUSSION

The molecular and chemotaxonomic data generated in this study further confirmed the affinities of *Rhopalostroma* as postulated previously (Hawksworth 1977, Ju et al. 1997, Stadler et al. 2004a, 2010a; see summary in Table 1). The closest relatives of *Rhopalostroma* are *Daldinia*, *Phylacia*, and in particular, *Thamnomyces*. Along with *Entonaema* and the recently erected genus *Ruwenzoria*, these genera comprise a lineage in the hypoxyloid *Xylariaceae*, for which even the erection of a new higher taxon might eventually be appropriate. Interestingly, the phylogenetic tree is congruent with chemotaxonomic data. For instance, all species included in clade B have been shown previously to produce mellein type isocoumarins in their culture, a feature that is also encountered in *Biscogniauxia* and *Camillea*, whereas all taxa included in clades A and C produce different compounds instead (cf. Bitzer et al. 2008, Fournier et al. 2010, Stadler et al. 2010a, b).

Despite their aberrant ascal and stromatal morphology, *Phylacia* and *Thamnomyces* were linked to the *Xylariaceae* several years ago. While R.W.G. Dennis had still been unsure as to their affinities in his taxonomic studies of tropical *Xylariaceae* in the 1950s and 1960s (cf. discussion in Stadler et al. 2010a), he later proposed that these genera might be cleistocarpous relatives of the *Xylariaceae* (Dennis 1970). Subsequently, this hypothesis was reinforced by studies of their anamorphic morphology: *Nodulisporium*-like anamorphic structures were observed in *Phylacia* (Rodrigues & Samuels 1989), as well as in *Thamnomyces* (Samuels & Müller 1979 for *Thamnomyces*) and even in *Rhopalostroma* (cf. Hawksworth & Whalley 1985). All these genera seem to have gradually lost the ability to actively discharge ascospores in the course of their evolution and adaptation to plants (and possibly insect vectors, which are always found in abundance in the overmature stromata of the *Xylariaceae*). The striking resemblance of the HPLC profiles in stromata as well as cultures of these genera is in complete accordance with the outcome of the molecular phylogeny. As previously discussed for *Phylacia* (Bitzer et al. 2008) and *Thamnomyces* (Stadler et al. 2010a), these relationships are also reflected by certain common characters of the phenotypes that are not currently regarded as taxonomically significant. For instance, the macromorphology of cultures of these taxa is quite similar, they tend to develop thick inflated hyphae or stromatic structures in the ageing mycelia, and they all develop a characteristic odour, owing to sweet to acrid-smelling volatile components that remain to be identified. These characters are rarely observed in cultures of *Hypoxylon* and *Annulohypoxylon*.

Asci arising from elongated ascogenous hyphae are present in all the aforementioned daldinoid and hypoxyloid genera, except for *Phylacia*. They are known with certainty from some species of *Hypoxylon* and *Daldinia*, but were to our knowledge never observed in other hypoxyloid *Xylariaceae* (*Biscogniauxia*, *Camillea*), nor in the xylarioid *Xylariaceae* (i.e. the genera with *Geniculosporium*-like anamorphs as defined in Ju & Rogers 1996). Out of the hypoxyloid *Xylariaceae*, *Pyrenomyxa* and *Phylacia* appear most derived with respect to having abandoned active discharge of ascospores. However, as already shown by Stadler et al. (2005) and Bitzer et al. (2008), the cleistocarpous features of these genera arose independently from one another, with *Pyrenomyxa* being closely related to the *H. rubiginosum* complex (cf. phylogenetic position of *Py. morganii* in Fig. 7.). From a morphological point of view, *Rhopalostroma* spp. are reminiscent of small stipitate *Daldinia* spp. lacking internal

Table 1 Comparison of *Xylariaceae* with regard to characters relating to stromatal morphology and anatomy and morphology of asci and ascospores that are currently used for delineation of generic boundaries, and characteristic metabolite types produced by representatives of these genera in culture. Legends: **+** = present; – = absent.

Genus	Ostioles	Ascal apical ring	Ascospore germ slit	Stromatal anatomy	Metabolites in culture
Phylacia	–	–	–	Essentially homogeneous (occasionally with white interior, but azonate)	Ab-5046 lactones, Naphthalenes, Chromones and Eutypins present
Rhopalostroma	+	–	+		AB5054 lactones, Chromones, Eutypins present; Naphthols not detected
Thamnomyces	+	–	+	Wiry, homogeneous	
Ruwenzoria	+	–	+	Essentially homogeneous	Naphthols and Eutypinol methyl ether present; Ab-5046 lactones and chromones not detected
Entonaema	+	+	+	Hollow, liquid-filled	Ab-5046 lactones, Naphthalenes, an d Chromones present; Eutypinols only detected in *D. caldariorum* Mellein derivatives apparently absent
Daldinia	+	+	+	Internal concentric zones*	
Annulohypoxylon	+	+	+	Essentially homogeneous	Melleins (dihydroisocoumarin derivatives) present All other compound classes apparently absent
Rostrohypoxylon	+	–	+		
Hypoxylon	+	+	+		
Pyrenomyxa	–	–	+		
Biscogniauxia/Camillea	+	+	+	Homogeneous, becoming bipartite during development	

* Except for *D. placentiformis* (stromatal context essentially homogenous) and *D. gelatinoides* (stromata hollow and liquid-filled when fresh).

zonation and with asci that have lost their apical ring (notably, *R. angolense* was first described as a member of *Daldinia*). Ascospores of *Rhopalostroma* never have a dehiscent perispore and the germ slit can be located either on the flattened side (e.g. *R. angolense*) or on the convex side (e.g. SEM illustrations of *R. kanyae* by Whalley & Thienhirun 1996). In the majority of *Daldinia* species, except for *D. caldariorum* (cf. Ju et al. 1997), the germ slit is always located on the convex side of the inequilateral ascospores.

The culture previously obtained from the herbarium specimen of *R. indicum* (BPI 586811; Stadler et al. 2004a) is probably not of that fungus. The 5.8S/ITSnrDNA data (not shown), as well as the morphology of the anamorph, suggest that it represents *D. eschscholzii* or a closely related taxon. It remains unclear whether it was derived from a laboratory contamination or whether the stromata were already contaminated by spores of the *Daldinia*. In this connection it may be pertinent to note that we have recently obtained the anamorph of *D. eschscholzii* from a series of other *Xylariaceae* stromata, including species of *Kretzschmaria* and *Xylaria* (M. Stadler, unpubl. data). Bitzer et al. (2008) also reported that stromata of *Phylacia* may be inhabited by xylariaceous *Xylocoremium* spp. In all these three genera, the isolations were reproducible and so cannot be attributed to a superficial contamination; interestingly, the 'mycophilic' contaminants are species that are widely known to be common as ubiquitous endophytes in the tropics.

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