Mycol Progress (2012) 11:61–69
DOI 10.1007/s11557-010-0728-8

ORIGINAL ARTICLE

Chocolate spot disease of *Eucalyptus*

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Received: 30 July 2010 / Revised: 6 November 2010 / Accepted: 26 November 2010 / Published online: 24 December 2010

Abstract Chocolate Spot leaf disease of *Eucalyptus* is associated with several *Heteroconium*-like species of hyphomycetes that resemble *Heteroconium* s.str. in morphology. They differ, however, in their ecology, with the former being plant pathogenic, while *Heteroconium* s.str. is a genus of sooty moulds. Results of molecular analyses, inferred from DNA sequences of the large subunit (LSU) and internal transcribed spacers (ITS) region of nrDNA, delineated four *Heteroconium*-like species on *Eucalyptus*, namely *H. eucalypti*, *H. kleinziense*, *Alysidiella parasitica*, and one isolate resembling a novel species in a clade separate from the holotype of *Heteroconium*, *H. citharexyli*. Based on molecular phylogeny, morphology and ecology, the *Heteroconium*-like species associated with Chocolate Spot disease are reclassified in the genus *Alysidiella*, which is shown to have mycelium that is immersed in and superficial on the host tissue and conidiogenous cells that can have loci that are either inconspicuous or proliferating percurrently. Furthermore, conidiogenous cells can either occur solitary on hyphae, or be sporodochial, arranged on a weakly developed stroma, which further distinguishes *Alysidiella* from *Heteroconium*.

Keywords *Alysidiella* · *Aulographina* · *Capnodiales* · *Capnodiaceae* · *Heteroconium*

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Introduction

Over the past 20 years, several collections have been obtained of a foliar disease of *Eucalyptus* spp. that was commonly referred to as 'Chocolate Spot'. These lesions, which are typically circular to irregular in shape, dark brown and somewhat corky, frequently do not extend through the leaf lamina, and are always colonised by a hyphomycete with medium brown, transversely septate, catenulate conidia arising from conidiogenous cells that are solitary on superficial hyphae to somewhat aggregated on a weakly developed stroma, and have either indistinct scars, or apical, percurrent proliferation. Although this disease appears to be relatively unknown in literature (Park et al. 2000; Sankaran et al. 1995), it has been frequently collected by us on eucalypts in most parts of the world, namely Australasia (Australia, New Zealand), Africa (Madagascar, Robben Island, South Africa), South America (Argentina, Brazil, Colombia, Ecuador, Uruguay), Asia (China, Thailand) and Europe (Cyprus, Portugal, Spain).

The first attempt made at dealing with these taxa was the description of *H. eucalypti*, collected on *E. dunnii* leaves in Uruguay (Crous et al. 2006b). Although this species was associated with leaf spots, it was placed in the genus

Heteroconium, which in the strict sense is reserved for sooty moulds (Hughes 1976). *Heteroconium* s.lat., however, had become widely overextended, and presently includes several ecologically unrelated species of dematiaceous hyphomycetes, including those on eucalypts. This initial species description was followed by the description of *H. kleinziense*, which occurs on *Eucalyptus* leaves in the Northern Cape Province of South Africa (Crous et al. 2007a). Furthermore, the collection of a species associated with severe dark brown spots that occurred on leaves and petioles of a *Eucalyptus* species on Stellenbosch Mountain in South Africa, led to the introduction of a new genus *Alysidiella*, described on the basis that it had multi-septate, catenulate conidia, superficial mycelium, was plant pathogenic, and lacked setae and hyphopodia. The issue of the heterogeneity in '*Heteroconium*' was again raised in a treatment of the genus by Hughes (2007), who stated that a revision of *Heteroconium* had to be based on a better interpretation of the precise and obligate ecological niches, and different sequences of conidium septation of the various species compared to the type, *H. citharexyli*. The aim of the present study, therefore, was to clarify the phylogenetic position of the genus *Heteroconium* based on *H. citharexyli*, and to resolve the taxonomic position of those taxa occurring on *Eucalyptus* presently accommodated in *Heteroconium* and *Alysidiella*.

Materials and methods

Isolates

Single conidial cultures were established from sporulating colonies on symptomatic leaves using the methods of Crous et al. (1991). The colonies were then sub-cultured onto 2% potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), 2% malt extract agar (MEA), oatmeal agar (OA), and pine needle agar (PNA) (2% tap water agar, with sterile pine needles) (Crous et al. 2009b), and incubated at 25°C in the dark to promote sporulation. The nomenclatural novelty and description was deposited in MycoBank (www.MycoBank.org; Crous et al. 2004). Cultures obtained in this study are maintained in Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands, and in the working collection (CPC) of P.W. Crous.

DNA isolation, amplification and phylogeny

Genomic DNA was isolated from fungal mycelium grown on MEA, using the UltraClean® Microbial DNA Isolation Kit (Mo-Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocols. Genomic DNA of *Heteroconium citharexyli* (holotype) was isolated directly from dried fungal conidia on the herbarium specimen as follows. Eighteen single conidia were removed, and examined under a stereo microscope (×80) to confirm their identity, and remove any contamination. Conidia were washed in 70% ethanol, and rinsed in sterile distilled water. The subsequent DNA extraction process was performed in two steps. Firstly, conidia were crushed to disrupt cells and release DNA. Each conidium was placed on a sterile glass slide with 5 μL TE buffer (0.01 M Tris/HCl, pH 8.0; 0.05 M EDTA, pH 8.0), and crushed with another glass slide until each conidial cell had been broken. The glass slides were then washed using TE buffer (approx. 100 μL) to flush the conidial cytoplasm into a sterile 1.5-mL microfuge tube. Secondly, 300 μL NaI was added, and incubated at room temperature for 5 min; the slurry was mixed well by shaking; 10 μL glass-milk was added, and the solution incubated on ice for 5–10 min with occasional shaking. The solution was then centrifuged at 12,000*g* for 5 s, and the supernatant was discarded. The residual pellet was washed twice with 300 μL ethanol, dried at room temperature, re-suspended in 15 μL TE buffer, incubated at 50°C for 10 min to elute the DNA, centrifuged at 12,000*g* for 1 min, and the DNA in the supernatant collected.

Primers V9G (de Hoog and Gerrits van den Ende 1998), LR3 (Cangelosi et al. 1997) and LR5 (Vilgalys and Hester 1990) were used to amplify part of the nuclear rDNA operon spanning the 3' end of the 18 S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8 S rRNA gene, the second ITS region (ITS2), and the first 900 bases at the 5' end of the 28 S rRNA gene (LSU). Primers ITS4 (White et al. 1990) and LR0R (Rehner and Samuels 1994) were used as internal sequence primers to ensure the quality of the consensus sequences. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous et al. (2006a). Sequences were compared with those available in NCBI's GenBank nucleotide (nr) database using a megablast search. Alignment gaps were treated as new character states. Sequence data and alignments were deposited in GenBank and in TreeBASE (www.treebase.org), respectively.

Morphology

Isolates were inoculated onto fresh MEA, OA, PDA and PNA plates, and subsequently incubated at 25°C in the dark, as well as under near-ultraviolet light to promote sporulation. Fungal structures were mounted on glass slides with lactic acid for microscopic examination. Measurements of all taxonomically relevant characters were made at ×1,000 magnification by Nikon NIS-Elements D3.0 Imaging Software, with 30 measurements per structure where possible. Colony colours on MEA (surface and reverse) were determined using the colour

charts of Rayner (1970) after 1 month of incubation at 25°C in the dark.

Results

Phylogenetic analysis

Approximately 1,700 bases, spanning the ITS and LSU regions, were obtained for three isolates, namely *Alysidiella suttonii*; CBS 124780 (LSU; GenBank HM628777, ITS; GenBank HM628774), *Aulographina eucalypti* CPC 12986 (LSU; GenBank HM535600, ITS; GenBank HM535599) and *Heteroconium citharexyli* (holotype) (LSU; GenBank HM628775, ITS; GenBank HM628776). It was not possible to obtain the complete LSU region for the type species of *Heteroconium*, *H. citharexyli*, and therefore only approximately 500 bp was used in the LSU phylogenetic analysis. The LSU region was used in the phylogenetic analysis for the generic placement, and the ITS to determine species-level relationships. The manually adjusted LSU alignment contained 46 taxa (including the two outgroup sequences, *Guignardia citricarpa* GenBank DQ377877 and *Botryosphaeria dothidea* GenBank DQ377852) and, of the 484 characters used in the phylogenetic analysis, 200 were parsimony-informative, 16 were variable and parsimony-uninformative and 268 were constant. Two equally most parsimonious trees were obtained from the heuristic search, the first of which is shown in Fig. 1 (TL=785, CI=0.457, RI = 0.796, RC=0.364). The present phylogenetic analyses based on LSU region reveal two strongly supported groups of *Heteroconium* species. They were split into two divergent clades within the *Dothidiomycetes*. *Heteroconium citharexyli* (holotype) formed a monophyletic clade and shared a lineage with *Capnodiaceae*. The *Capnodiales* comprised several sooty mould taxa including *Antennariella*, *Capnodium*, *Conidioxyphium*, *Leptoxyphium*, and *Microxyphium*. Another strongly supported monophyletic clade (incertae sedis) contained *Heteroconium*-like species occurring on *Eucalyptus*, including a new taxon.

Taxonomy

Results from the molecular phylogenetic analyses as well as morphological and ecological habitat comparisons revealed that *Heteroconium*-like species occurring on *Eucalyptus* represent a different genus. As expected, *Heteroconium citharexyli* clustered with other sooty moulds in the *Capnodiaceae*. However, the *Heteroconium*-like taxa on *Eucalyptus* clustered with *Aulographina eucalypti*, *Alysidiella paracitica* and *Blastacervulus eucalypti* in the *Dothideomycetes* (*incertae sedis*), and one collection appeared to represent a new taxon. Based on these data, we concluded that these taxa should be reclassified in the genus *Alysidiella*, which shows a higher degree of similarity in both morphological characters and ecological habitat. Morphologically *Alysidiella* can be distinguished from *Heteroconium* by having immersed to superficial hyphae, and frequently have a weakly developed stroma. Ecologically, *Alysidiella* being plant pathogenic but *Heteroconium* being a sooty mould that grows superficially on plant surfaces (Summerell et al. 2006). These taxa are subsequently treated below.

Alysidiella eucalypti (Crous & M.J. Wingf.) Cheewangkoon & Crous, comb. nov. — MycoBank MB 518725.

Basionym. *Heteroconium eucalypti* Crous & M.J. Wingf., Fungal Planet 10: 1. 2006.

Alysidiella kleinziense (Crous & Z.A. Pretorius) Cheewangkoon & Crous, comb. nov. — MycoBank MB 518726.

Basionym. *Heteroconium kleinziense* Crous & Z.A. Pretorius, Fungal Diversity 25: 28. 2007.

Alysidiella suttonii Cheewangkoon & Crous, sp. nov. — MycoBank MB 518727; Fig. 2.

Conidiophora brunnea, verruculosa, crassitunicata, 1–2-septata, 5–15×5–7 μm. Cellulae conidiogenae integratae, terminales, percurrenter proliferentes, 5–7×4.5–7 μm. Conidia subcylindrica vel ellipsoidea, brunnea, verruculosa, 0–9-septata, (15–)20–30(–72)×(6–)8–10 μm.

Etymology. Named after Dr Brian C. Sutton, mycologist at the former Commonwealth Mycological Institute, Kew, Surrey, with whom these *Heteroconium*-like species occurring on eucalypts were discussed during a visit to the institute in 1990 after IMC4 by P.W.C.

Mycelium is internal and superficial, consisting of branched, smooth to verrucose hyphae, thick-walled, septate, medium brown, 3–5(–8) μm wide hyphae. *Conidiophores* arise as lateral branches from hyphae, or are somewhat aggregated on a weakly developed stroma; conidiophores erect, cylindrical to ellipsoidal, brown, thick-walled, 0–2-septate with visible terminal loci, (5–)8–12(–15)×5–7 μm. *Conidiogenous cells* are holoblastic, integrated, with terminal, percurrent proliferation, dark brown, 5–7×4.5–6(–7) μm. *Conidia* are subcylindrical to ellipsoidal, tapering towards both ends, catenate, dry, verruculose, with obtuse apex, and truncate base, 0–9 disto-euseptate, somewhat constricted at the septa, solitary or in chains, predominantly unbranched, (15–)20–30(–72)×(6–)8–10 μm.

Culture characteristics, Colonies on PDA at 25°C after 1 month erumpent, with sparse to moderate aerial mycelium, margins even but somewhat feathery; surface and reverse greenish to greyish black; colonies reaching 10 mm diam; colonies fertile on all media tested.

Specimen examined. CYPRUS, Larnaca, on *Eucalyptus* sp., 28 Mar. 2007, *A. van Iperen*, holotype CBS H-20305,

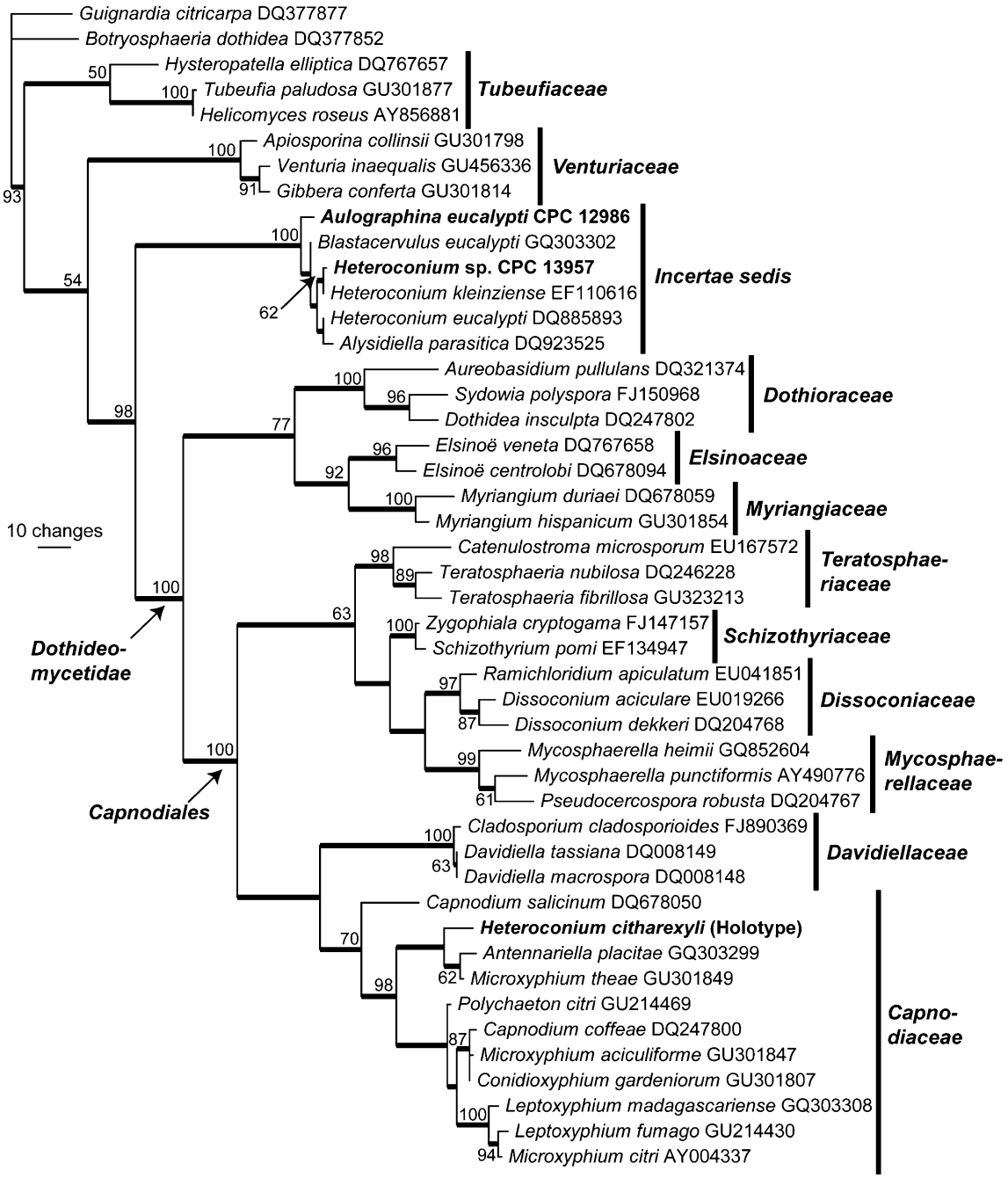

Fig. 1 The first of two equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The *scale bar* shows 10 changes, and bootstrap support values from 1,000 replicates are shown at the nodes. Novel and holotype species described in this study are *bold*. *Thickened lines* indicate those branches present in the strict consensus tree. Higher taxonomic rankings are indicated to the left and families to the right of the tree. The tree was rooted to a sequence of *Guignardia citricarpa* (GenBank accession DQ377877) and *Botryosphaeria dothidea* (GenBank accession DQ377852)

culture ex-type CPC 13957=CBS 124780, CPC 13958, 13959.

Notes Based on LSU sequence data (Fig. **1**), *A. suttonii* clustered with other *Alysidiella* species, *Aulographina eucalypti* and *Blastacervulus eucalypti* (100% bootstrap support). Although the ITS sequence data was similar to that of other *Alysidiella* species (*A. kleinziense*, EF110616, 99% identical; *A. parasitica*, DQ923525, 94% identical; *A. eucalypti*, DQ885893, 94% identical), the conidial range of *A. suttonii* (15–72×6–10 μm) is larger than that of the other three species; *A. eucalypti* (10–35×5–7 μm), *A. kleinziense* (10–60×7–8 μm), and *A. parasitica* (8–30×5–7 μm).

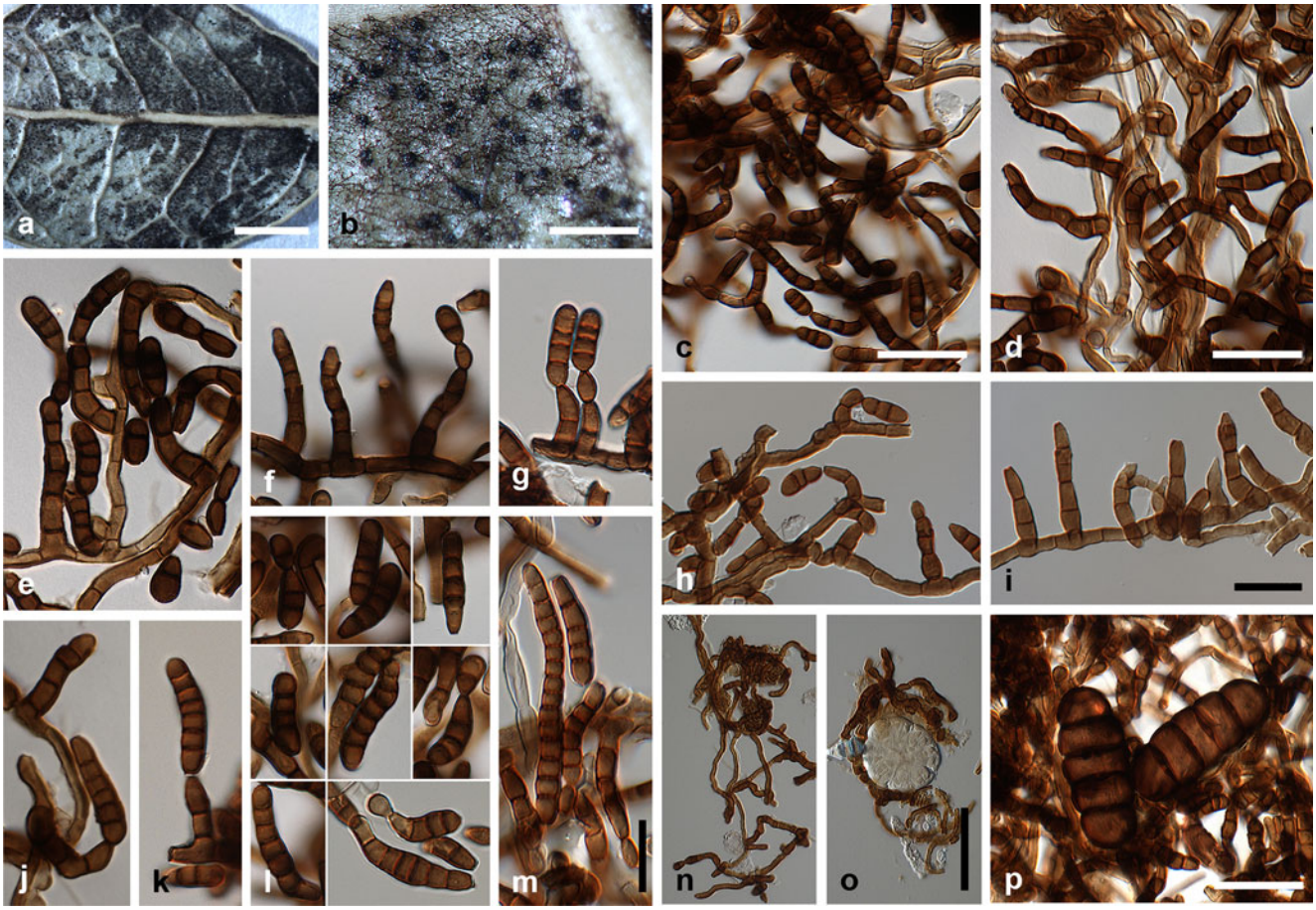

Fig. 2 *Heteroconium citharexyli* Petrak. **a**, **b** Colony on leaves. **c**–**k** Mycelium bearing conidiophores and conidia. **l**, **m** Conidia. **n**, **o** Hyphal aggregation around trichome. **p** Ascospores of *Maiola* sp. *Scale bars* (**a**) 5 mm, (**b**) 1 mm, (**c, d, h, i, p**) 30 μm, (**e**–**m**) 20 μm, (**n, o**) 200 μm; (**i**) applies to (**h, i**), (**m**) applies to (**e**–**m**), (**o**) applies to (**n**–**o**)

Heteroconium citharexyli Petrak, Sydowia 3: 265. 1949. Fig. 3.

The holotype has been examined in the present study, and is depicted in Fig. 3. Morphologically, however, all characters confer with the description provided by Hughes (2007).

Morphology. Colonies effuse, dark brown. *Hyphae* medium to dark brown, hypophyllous, straight to flexuous, thick walled, (3.5–)4.5(–5.5) μm wide, particularly aggregated around trichomes, lacking immersed hyphae. *Trichomes* globose, multi-celled, hyaline, somewhat pale brown, 8-cells dividing radially (upper view), with two ellipsoidal vacuole-like structures in each cell, frequently constricted at the celled septum, 150–180 μm. *Conidiophores* cylindrical, straight to flexuous, erect, lateral or terminal on hyphae, scattered, simple, branching not observed, thick-walled, somewhat constricted at septum, with up to 7 percurrent proliferations, 4.5–6.5 μm wide, up to 38 μm high. *Conidiogenous cells* obconical to subcylindrical, tapering to a flattened end, conidiogenous loci 2.5–4 μm wide, unbranched. *Conidia* ellipsoidal to cylindrical, acropetal chain, seceding schizolytically, flattened at both ends in intercalary conidia, but one end rounded in terminal conidia; hila 2.5–4 μm wide. Range of conidium measurements and septation: 7–9×4.5–5.5(–7) μm (0-septate); (5.5–)11–13(–15.5)×4.5–7.2(–9.5) μm (1-septate); (12–)14–17(–20)×5.3–7 μm (2-septate); (15.5–)16–19(–21.5) × 5.5–8 μm (3-septate); (16.5–)18–22(–25.5) × 5–7.5 μm (4-septate); (21.5–)23–26×5–7 μm (5-septate); (24.5–)27–32(–37.5)×5–6.5 μm (6-septate); (24.5–)27–30×5.5–6.5 μm (7-septate); 34–38×5.5–6.5 μm (8-septate); 50–56× 5.5 μm (14-septate).

Specimen examined. ECUADOR, near Quito, Pichincha Slopes, on leaves of *Citharexylum ilicifolium*, Sept. 1937, H. Sydow, type material in S.

Notes. Empty ascomata-like structures were found on the type specimen. These were globose, up to 170 μm diam, superficial, occurring on a mass of brown mycelium, dark brown to black; no complete setae seen. Brown ascospores were also found nearby (Fig. 3), which are ellipsoid with

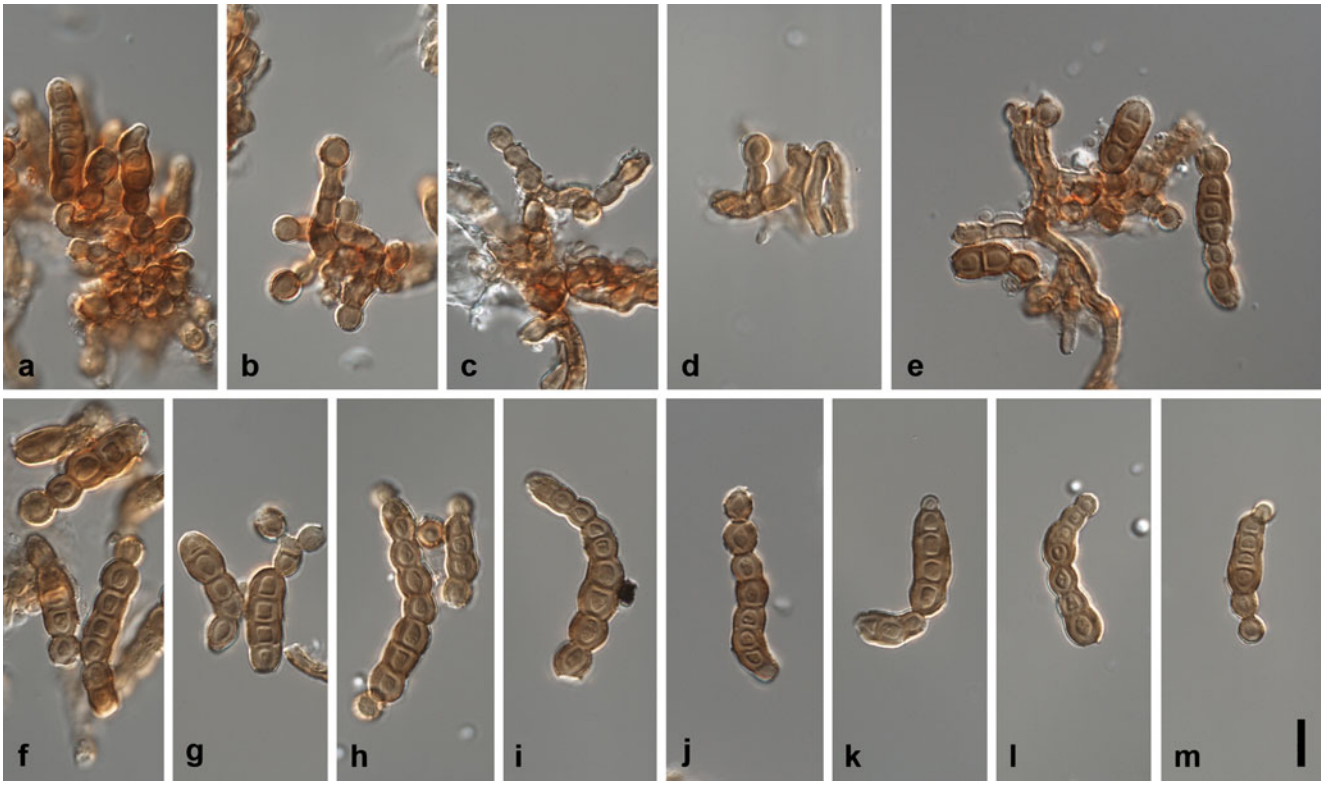

Fig. 3 *Alysidiella suttonii*. **a**–**e** Conidiophores, conidiogenous cells, percurrent proloferations. **f**–**m** Conidia. *Scale bars* (**a**–**m**) 10 μm; (**m**) applies to (**a**–**m**)

rounded ends, 4-septate, 20–23×52–57 μm, constricted at the septum, occurring in groups of two, presumably discarded from the ascomata-like structure mentioned above. Ascomata and ascopores resemble that of the genus *Meliola*, but there was insufficient information to facilitate identification to species level. Although this teleomorph occurred on the same leaf, the connection of the telemorph-anomorph relationship with *H. citharexyli* was not evident.

Discussion

In the present paper, we managed to isolate DNA from the type specimen of *H. citharexyli*, which is the type of the genus *Heteroconium*. To achieve this goal, an improved protocol was established for DNA isolation from dried fungal herbarium material using a direct glass–milk extraction protocol. During DNA isolation from dried fungal specimens, the cleaning of fungal structures with sterile water and preventing the loss of fungal DNA during the extraction protocol are essential (see also Simon et al. 2009). Other extraction methods employed which do not involve these steps resulted in substantial degeneration and loss of DNA.

Heteroconium citharexyli is a sooty mould with mycelium growing superficially on leaves of *Citharexylum ilicifolium* in Ecuador (Petrak 1949). The genus is characterised by foliicolous hyphomycetes which possess short chains of cylindrical, septate, thick-walled, macronematous and mononematous conidiophores, and brown, percurrently proliferating conidiogenous cells which form as lateral branches on hyphae that are closely appressed to leaf surfaces (Castañeda Ruíz et al. 1999; Morgan-Jones 1975, 1976; Petrak 1949; Taylor et al. 2001). Although a number of species have previously been classified under *Heteroconium*, these classifications were based on morphological characters without the benefit of a phylogenetic assessment, which was further problematic in that the type species has never before been subjected to molecular analysis.

In a recent treatment of the genus, Hughes (2007) re-examined 16 species. Of these, he suggested that only three species were congeneric with the type species, namely *H. asiaticum*, *H. glutinosum* and *H. neriifoliae*. A further 10 species (*H. arundicum*, *H. chaetospira*, *H. decorosum*, *H. eucalypti*, *H. indicum*, *H. lignicola*, *H. ponapense*, *H. queenslandicum*, *H. triticicola* and *H. tropicale*) do not appear to be congeneric with *Heteroconium*, as they were described from decaying leaves, twigs, wood and bark, with immersed or partly immersed hyphae. Two other species

(*H. solaninum* and *H. tetracoilum*) were transferred as *Pirozynskiella solaninum* and *Lylea tetracoila*, respectively. He also commented that the obligate ecological niches and sequences of conidium septation are significant evidence which should be emphasized, and used for the identification of *Heteroconium* species.

Several recent studies have suggested that *Heteroconium* has affinities to diverse orders, and is polyphyletic (Crous et al. 2007a; Kwasna and Bateman 2007). *Heteroconium triticicola*, which was isolated from roots of wheat in the U. K., was found to be phylogenetically similar to the mycorrhizal ascomycete family *Hyaloscyphaceae* (Kwasna and Bateman 2007). Results from molecular studies on *Herpotrichiellaceae* and *Venturiaceae* fungi indicated that *H. chaetospira*, which is commonly found on rotting wood in Europe (Ellis 1976), showed a much higher phylogenetic similarity to *Chaetothyriales*, and was thus placed in *Cladophialophora* (Crous et al. 2007b). Similarly, the *Heteroconium*-like species associated with Chocolate Spot disease of *Eucalyptus* was shown to cluster apart from the *Capnodiales* (Crous et al. 2006b, 2007a). Furthermore, the species occurring on *Eucalyptus* are monophyletic, and belong to a well supported group (100% bootstrap support) including *Aulographina eucalypti* and *Blastacervulus eucalypti*, the latter having acropetal conidial chains with brown, thick walls. Morphologically, *Alysidiella* has similar morphological features to *Heteroconium*, such as brown, monoblastic conidiophores, terminal conidiogenous cells with schizolytic conidial succession and percurrent proliferation. Furthermore, conidia are brown, acropetal, cylindrical to ellipsoid, euseptate, solitary or catenulate. However, species of *Alysidiella* have immersed to superficial hyphae, and frequently have a weakly developed stroma. Furthermore, ecologically they are also distinct, with *Alysidiella* being plant pathogenic (Fig. 4), and *Heteroconium* being a sooty mould that grows superficially on plant surfaces.

The third genus in this clade is *Aulographina eucalypti*, which is the causal agent of target spot on *Eucalyptus*. Although the leaf spots appear somewhat similar to those of *Alysidiella* species (circular to irregular, corky, not extending to the opposite side of the leaf), spots are usually colonised by prominent black hysterothecia of *Aulographina*, or superficial pycnidia of its *Thyrinula* anamorph. Target spot of corky spot as it is also referred to, is one of the most common leaf diseases in southern Australia (Swart 1988; Wall and Keane 1984), and has been reported from most continents where eucalypts are cultivated, such as New Zealand (Dick 1982), Africa (South Africa; Crous et al. 1989), South America (Brazil; Ferreira 1989), Europe (U.K.; Spooner 1981) and Asia (Vietnam; Old and Yuan 1994). The genus *Aulographina* is based on *A. pinorum*, a fungus which commonly occurs on needles of various *Pinus* species in Europe (von Arx and Müller 1960). However, when Crous et al. (2009a) obtained sequence data of two strains deposited in CBS under this number (CBS 174.90, 302.71), they clustered in the *Teratosphaeriaceae*, and produced the anamorphic fungus *Catenulostroma abietis* in culture. The latter species occurs commonly on pine needles, and has brown acervuli (Butin et al. 1996), and thus could be mistaken for hysterothecia of *A. pinorum*. One species in this complex, *C. microsporum*, was described from a *Teratos-*

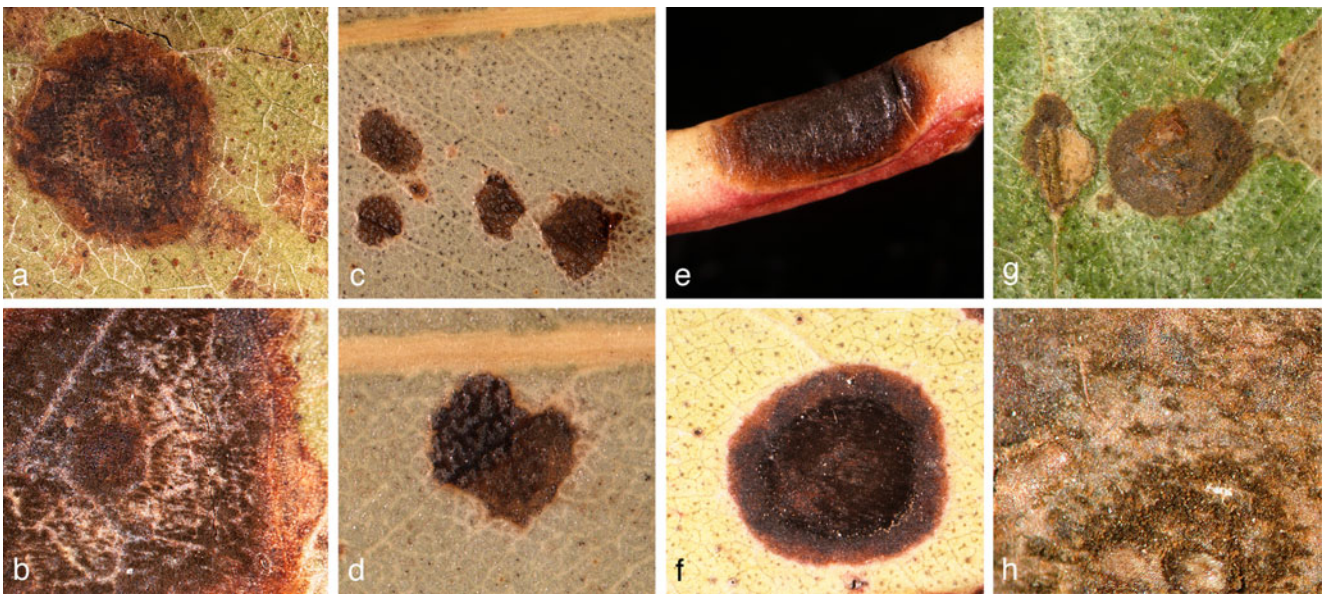

Fig. 4 Leaf and petiole lesions associated with *Alysidiella* spp. **a**, **b** *A. eucalypti*. **b**, **c** *A. kleinziense*. **e**, **f** *A. parasitica*. **g**, **h** *A. suttonii*

phaeria-like teleomorph on *Protea* (Taylor and Crous 2000). We therefore regard it highly unlikely that these strains are authentic for the name *A. pinorum*, and fresh material will have to be collected to resolve its status, and to determine if it is related to species of *Alysidiella* and *Aulographina eucalypti*. Further collection of more taxa would also be required to elucidate the relationships with other families in the *Dothideomycetes*, as presently this clade appears to represent an unknown family in this order (Schoch et al. 2009).

Acknowledgements We thank Dr Angus Carnegie (Forest Resources Research, DPI, Australia) for the culture of *Aulographina eucalypti*, isolated from *Eucalyptus cloeziana* in Australia. The curator of S is also thanked for the lending us the holotype specimen of *Heteroconium citharexyli*, and for granting us permission to isolate DNA for this study. This study was funded in part by the Thailand Research Fund (DBG5380011). The first author gratefully acknowledges Chiang Mai University Graduate School for partially support to this doctoral study.

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