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ORIGINAL ARTICLE

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Penicillium hermansii, a new species causing smoky mould in white button mushroom production

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Abstract
Competing fungi in white button mushroom (*Agaricus bisporus*, champignon) cultivation causes significant episodic losses. One of these competing fungi is known as "smoky mould". It owes its name to the production of high numbers of spores after the disturbance of compost, which resembles smoke. We investigated strains isolated from smoky mould cases in the Netherlands, UK and Canada and show that these outbreaks were caused by a new *Penicillium* species, named *P. hermansii* sp. nov. (type strain CBS 124296^{T}). Several *Penicillium* species are reported to cause smoky mould. However, we so far have no indications that smoky mould is caused by other *Penicillium* species than *P. hermansii*. This species belongs to section *Exilicaulis* and differs from other Penicillia by its slow growth rate on Czapek yeast agar (CYA) and malt extract agar (MEA) and its inability to grow on CYA supplemented with 5% salt and CYA and MEA incubated at 37 °C.

Keywords *Agaricus* · Taxonomy · Phylogeny · Mycobiota

Introduction

A critical step during mushroom production is the colonisation of the pasteurised phase II compost by *Agaricus bisporus*. Competitive moulds from spawn-run compost can have devastating effects on production levels. An important and well-known example is *Trichoderma aggressivum* and much research is performed on this species (Kosanovic et al. 2015; O'Brien et al. 2014; O'Brien et al. 2017; Radvanyi et al. 2016). Another less commonly and poorly documented competing contaminant is known as 'smoky mould', which is capable of wiping out a complete crop (Fletcher and Gaze 2008; Grogan and Harvey 1999; Grogan et al. 2000). In the last decade, several outbreaks occurred in the Netherlands (C. Hermans, pers. comm.). The first signal of infection by smoky mould is an increase of temperature of the compost during growth that is difficult to control even by lowering air temperature. Mushrooms grown on compost with a minor smoky mould infection are slightly paler, mature quicker and there is usually reduced pinning. In more severely infected compost, areas lacking growth and even complete empty mushroom beds occur. Digging into these infested areas releases large clouds of spores resembling smoke, hence the name smoky mould.

Various names, such as *Penicillium chermesinum*, *P. citreonigrum*, *P. implicatum* and *P. fellutanum* have been applied to smoky mould outbreaks (Baars et al. 2011; Beyer 2002; Fletcher and Gaze 2008; Grogan et al. 2001). These species are distantly related to each other and belong to different *Penicillium* sections (Houbraken and Samson 2011; Visagie et al. 2014). The use of these names in literature might suggest that smoky mould is caused by multiple species. However, in the past *Penicillium*, identification was primary based on phenotypic and physiological characters. It is probable that only one fungus is involved and that some or all of these identifications are incorrect.

In this study, strains isolated from smoky mould outbreaks in the Netherlands, Canada and UK were studied using a polyphasic approach. Physiological, macro- and

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microscopical characteristics combined with partial calmodulin (*CaM*), β-tubulin (*BenA*) and RNA polymerase II second largest subunit (regions 5–7) (*RPB2*) sequences demonstrate that this set of strains represents a new species in *Penicillium* section *Exilicaulis*.

Material and methods

Strains

An overview of examined strains is presented in Table 1. The 16 investigated strains were isolated from compost or the air of mushroom production farms, where *Agaricus bisporus* cultivations were infected with smoky mould. The strains that were isolated in different years were from different outbreaks and different production farms. No information is recorded at farm level and strains isolated in the same year could be obtained from more than one production location. Two of the strains are legacy strains from previous outbreaks, HRI 1043-D from the UK and the main strain mentioned in previous literature (Fletcher et al. 1989; Grogan et al. 2000), and VM-2 from Canada.

Phenotypic and physiologic characterisation

All pure cultured strains were inoculated at three points on Czapek yeast autolysate agar (CYA), CYA supplemented with 5% NaCl (CYAS), malt extract agar (MEA, Oxoid), oatmeal agar (OA), creatine sucrose agar (CREA), dichloran 18% glycerol agar (DG18), yeast extract sucrose agar (YES) (Frisvad and Samson 2004). Additional CYA and MEA plates were incubated at 15, 30 and 37 °C. Colony diameters and other macroscopic colony characteristics (e.g. colour conidia, colony texture) were recorded after 7 days of incubation at 25 °C. Mounts were made from the MEA medium in lactic acid and microscopic features were studied by light microscopy using a Zeiss Axiokop 2 Plus. The temperature-growth response was investigated on CYA by recording diameters of three-point inoculated colonies of seven strains (CBS 122432, CBS 124296, CBS 124297, DTO 032-A2, DTO 032-A3, DTO 173-C9 and DTO 173-D1) after 14 days at 6–36 at 3 °C intervals and 40 °C. Alphanumeric codes for conidial and reverse colours refer to (Kornerup and Wanscher 1978). Isolates were also examined for the production of alkaloids with Ehrlich reagent using the filter paper method described by Lund (1995). The growth rate of two *P. hermansii* strains (CBS 124296 and DTO 173-D4) and four other *Penicillium* species (*P. brevicompactum* DTO 099-D1; *P. chrysogenum* CBS 306.48; *P. daleae* DTO 099-B8; *P. glabrum*, DTO 099-A6) was studied on an autoclaved compost based medium containing 250 g *Agaricus* grown compost (phase III) and 15 g agar per litre.

Table 1 Overview of *P. hermansii* isolates used in this study

Culture collection number	Substrate	Location	Year
DTO 031-A3 = CBS 122432 = DAOM 242544 = HRI 1043-D = DC-283	Mushroom compost (phase III) with smoky mould	UK	Around 1995
DTO 194-C7	Mushroom compost with smoky mould	The Netherlands	1997
DTO 194-C8 = CBS 132824	Mushroom compost with smoky mould	The Netherlands	1997
DAOM 242545 = VM-2	Mushroom compost	Nova Scotia, Canada	2000
DTO 032-A2	Mushroom compost with smoky mould	The Netherlands	2006
DTO 032-A3	Mushroom compost with smoky mould	The Netherlands	2006
DTO 079-D4 = IBT 29994	Mushroom compost with smoky mould	The Netherlands	2008
DTO 079-D5 = CBS 124296^{T}	Mushroom compost with smoky mould, type	The Netherlands	2008
DTO 079-D6 = CBS 124297	Air in mushroom growing room	The Netherlands	2008
DTO 173-C9	Mushroom compost with smoky mould, second harvesting period	The Netherlands	2011
DTO 173-D1	Mushroom compost with smoky mould, second harvesting period	The Netherlands	2011
DTO 173-D2	Mushroom compost with smoky mould	The Netherlands	2011
DTO 173-D3	Air in mushroom growing room	The Netherlands	2011
DTO 173-D4	Air in mushroom growing room	The Netherlands	2011
DTO 282-D3	Air in mushroom growing room	The Netherlands	2013
DTO 282-D6	Air in mushroom growing room	The Netherlands	2013

CBS, culture collection of the Westerdijk Institute; *DC*, disease collection at the Pennsylvania State University's Mushroom Spawn Lab, USA; *DTO*, internal culture collection of department Applied and Industrial Mycology, housed at the Westerdijk Institute; HRI, culture collection of Warwick HRI, UK

DNA extraction, sequencing and phylogenetic analysis

Strains were grown for 7–14 days at 25 °C on MEA prior to DNA extraction. Genomic DNA was extracted using the Ultraclean Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, USA) following the manufacturer's protocol. The ITS barcode and parts of the *BenA*, *CaM* and *RPB2* genes were amplified, sequenced and annotated according to the method described by Houbraken and Samson (2011) and Houbraken et al. (2012). Newly generated sequences are deposited in GenBank with accession numbers MG386210–MG386247 and MG333469–MG333479.

Publically available *BenA*, *CaM* and *RPB2* sequences of the type strains of species belonging to section *Exilicaulis* were retrieved from GenBank and supplemented with the newly generated sequences. A concatenated dataset with *BenA*, *CaM* and *RPB2* sequences including nine smoky mould strains was generated and used to determine the phylogenetic relationship of the smoky mould strains with other members of *Penicillium* section *Exilicaulis*. Single gene phylogenies were made by combining the generated sequences from smoky mould isolates with sequences of strains belonging to the *P. parvum*-clade (Visagie et al. 2016) of sect. *Exilicaulis*. The sequence data sets were aligned using MAFFT (Katoh and Standley 2013). Prior to analysis, the optimal substitution model was determined in MEGA v.6.06. (Tamura et al. 2013), utilising the Akaike information criterion (AIC). Maximum likelihood (ML) analysis of the single gene datasets were analysed in MEGA 6.06 and the combined dataset in RAxML-VI-HPC v. 7.0.3 (Stamatakis 2006) using the GTRGAMMA substitution model. The robustness of the phylograms was evaluated by 1000 bootstrap (bs) runs. Bayesian inference (BI) was performed in MrBayes v.3.2.1 (Ronquist et al. 2012) using Markov chain Monte Carlo (MCMC) algorithm. The analysis stopped when the average standard deviation of split frequencies reached 0.01. The sample frequency was set to 100; the first 25% of trees were removed as burnin. Convergence and ESS values of the runs were examined by Tracer 1.6 (Rambaut et al. 2014).

Results

Phylogeny

The total length of the combined data set was 1729 characters (individual datasets: *BenA* 421; *CaM*, 518; *RPB2*, 788 characters) and the proportion of gaps was 5.7%. The phylogram was drawn to scale and the results of this analysis are shown in Fig. 1. Nine smoky mould isolates were included in this analysis and those isolates form a unique clade within section *Exilicaulis*. Two well-supported clades are present in this section and the smoky mould strains group in a cluster previously named the *Penicillium parvum*-clade (Visagie et al. 2016). *Penicillium canis* and *P. striatisporum* are basal to the clade containing the smoky mould isolates; however, statistical support is lacking. With the data available, it was not possible to conclusively identify the phylogenetic sister species of the smoky mould isolates.

BenA, *CaM* and *RPB2* gene sequences were generated to confirm the phylogenetic coherence of the smoky mould strains at the species level, and their relationship with other species in the *P. parvum*-clade. The Tamura Nei (TN93) model using the gamma distribution (+G) was found to be optimal for the *BenA* data set (length 372 sites); and the TN93 model using a discrete gamma distribution with invariant sites (G+I) was optimal for the *CaM* (length 518 sites) and *RPB2* (length 789 sites) datasets. The best-scoring ML trees are presented in Fig. 2 and show that the smoky mould strains form a coherent species level clade, distinct from other members of the *P. parvum*-clade. The smoky mould strains cluster together in trees based on all loci on a strongly supported branch (> 95% bs, 1.00 pp). Most bootstrap percentages and posterior probability (pp) values were low (< 70% bs, < 0.95 pp) in these gene trees.

Morphology and physiology

Phenotypic characters of the smoky mould strains were recorded and compared with each other. All strains displayed similar macro- and microscopical characters. Most strikingly, the isolates grew slow on CYA and MEA, even at an optimum growth temperature (27–30 °C). Furthermore, the conidiophores had short stipes (10–40 (− 120) μm) and the strains did not grow on CYAS, and CYA and MEA incubated at 37 °C. The optimum growth temperature on CYA was between 27 and 30 °C, with a colony diameter of 15–18 mm after 14 days. The minimum growth temperature was between 12 and 15 °C and the maximum growth temperature between 33 and 36 °C. Strains incubated at 33 °C reached 11–14 mm and no growth was observed at 36 °C. The colony diameters of the smoky mould strains on autoclaved compost agar (CA) were similar to those on MEA (CA 9–12 vs MEA 9–12 mm). Also the growth density was similar on both media. The other examined *Penicillium* species grew well on CA and their colony diameters were similar or slightly smaller than on MEA (*P. brevicompactum*, CA 18–22 vs MEA 20–24 mm; *P. chrysogenum*, CA 40–43 vs MEA 30–35 mm; *P. daleae*, CA 24–27 vs MEA 34–37 mm and *P. glabrum*, CA 38–40 vs MEA 41–45 mm).

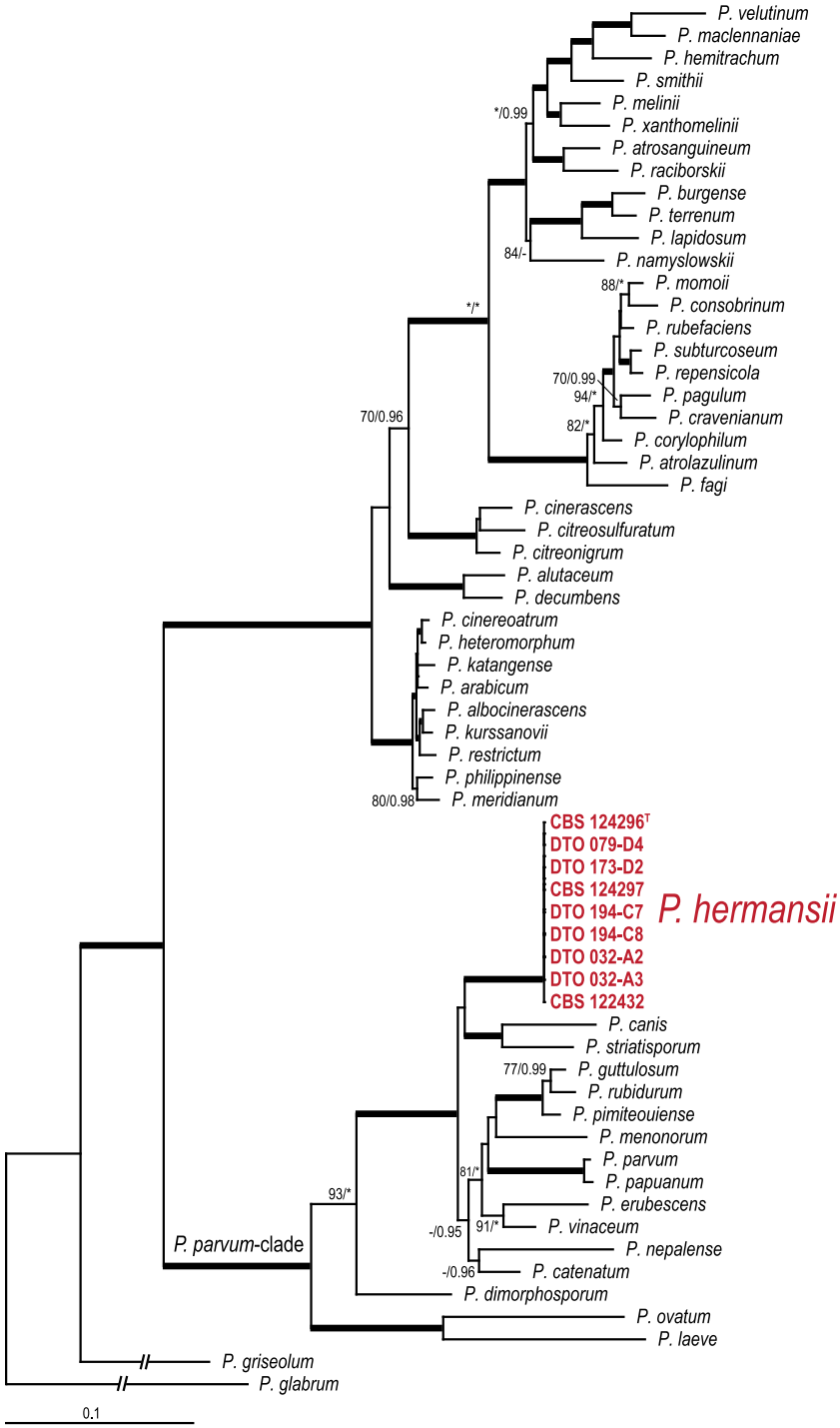


Fig. 1 Best-scoring maximum likelihood tree using RAxML based on a combined dataset with partial *BenA*, *CaM* and *RPB2* sequences, showing the relationship of species accommodated in section *Exilicaulis*. The phylogram is rooted with *P. glabrum* CBS 125543. Bootstrap percentages and posterior probability values are presented at the nodes. Values less than 70% bs or 0.95 pp are not shown and branches with more than 95% bs and 1.00 pp are thickened. The bar indicates the number of substitutions per site

Taxonomy

Based on phylogenetic coherence of the smoky mould causing isolates and their unique phenotypic and physiologic characters, we describe them here as a new species named *Penicillium hermansii* sp. nov.

Penicillium hermansii Houbraken, Seifert & Samson, sp. nov. Mycobank MB823949 (Fig. 3).

In: *Penicillium* subgenus *Aspergilloides* section *Exilicaulis*.

Etymology: Named after Con Hermans, who studied the occurrence of smoky mould and other competing fungi in the Netherlands.

Diagnosis: Slow growth on CYA (5–10 mm) and MEA (8–15 mm) at 25 °C after 7 days incubation; no growth on CYAS; no growth on MEA and CYA incubated at 37 °C; conidiophores biverticillate and short-stiped, 10–40 (−120) μm.

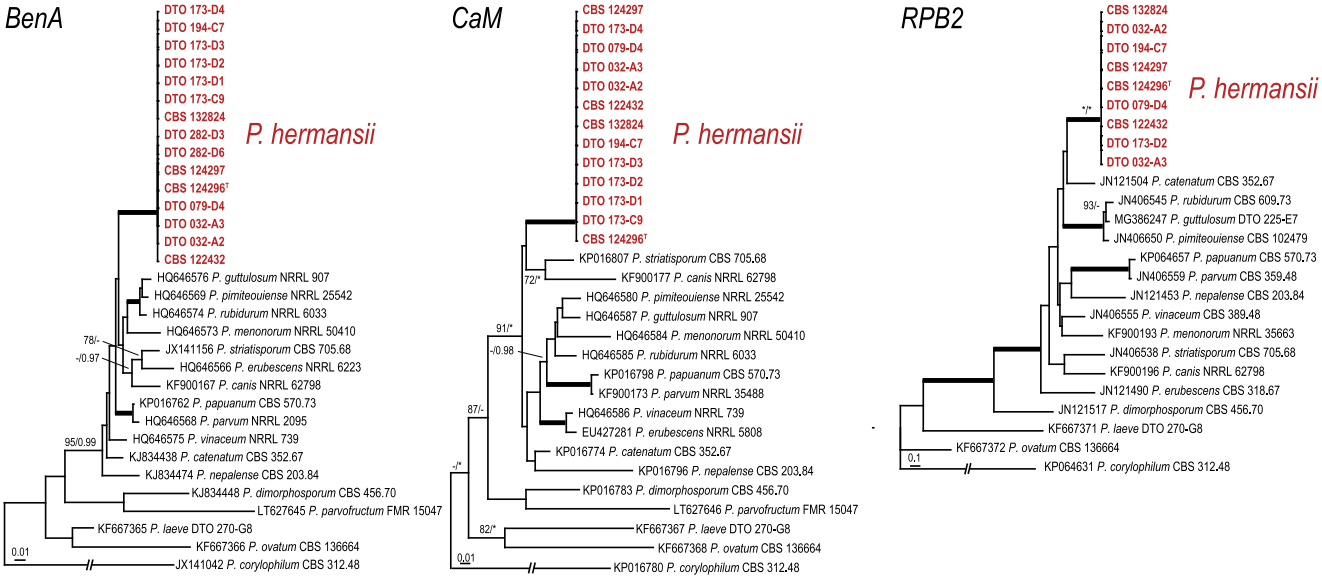


Fig. 2 Maximum likelihood (ML) trees generated for *BenA*, *CaM* and *RPB2* with MEGA6. The phylograms are rooted with *P. corylophilum* CBS 312.48. Bootstrap percentages and posterior probability values are presented at the nodes. Values less than 70% bs or 0.95 pp are not shown and branches with more than 95% bs and 1.00 pp are thickened. The bar indicates the number of substitutions per site

Typus: the Netherlands, ex compost with *Agaricus bisporus*, 2008, collected by *C. Hermans*, isolated by *J. Houbraken* (holotype CBS H-21028, culture ex-type CBS 124296 = DTO 079-D5).

ITS barcode: ITS = MG333472 (alternative markers: *BenA* = MG386214, *CaM* = MG386229, *RPB2* = MG386242). All examined *P. hermansii* strains share identical ITS, *BenA*, *CaM* and *RPB2* sequences. The species can be differentiated from other known *Penicillium* species by ITS sequences.

Distribution and ecology: The Netherlands, United Kingdom, Canada. Isolated from compost colonised by *Agaricus bisporus* and from the air of white button mushroom production farms.

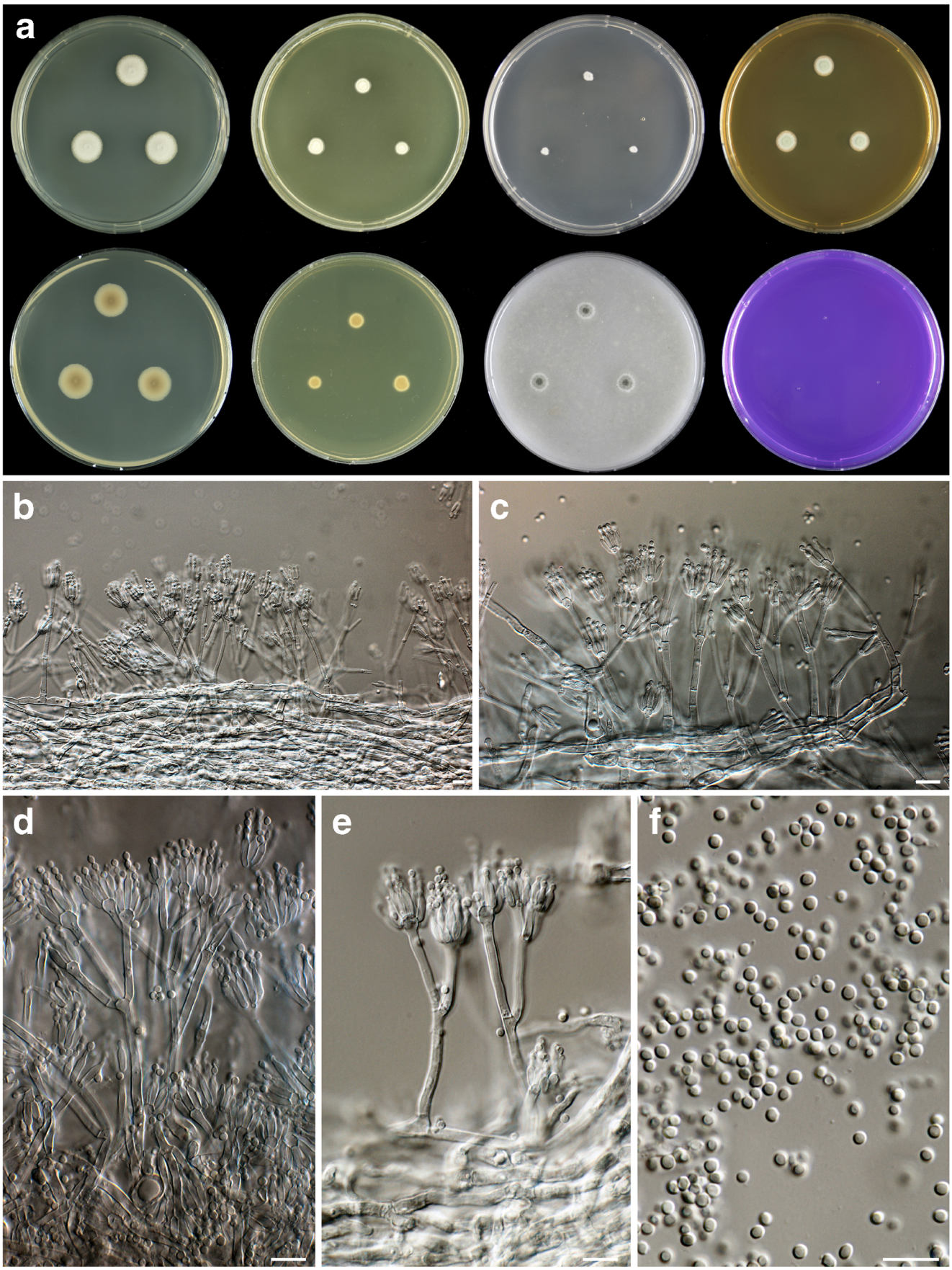
Colony diameter: 7 days, in mm, 25 °C (unless stated otherwise): CYA 5–10; CYA15°C no growth or spore germination; CYA 30 °C 7–12; CYA 37 °C: no growth; MEA 8–15; YES 5–9; DG18 3–7; CYAS no growth; OA 10–15; creatine agar no growth or spore germination. Optimum growth temperature on CYA 27–30 °C.

Colony characters: Sporulation on CYA absent to moderate; colonies entire, plane, velvety; mycelium white, sporulation grey-green to greyish-brown green (~5D3); exudate absent; soluble pigments absent; colony reverse brown (5C3–E3). Soluble pigments on YES absent, sporulation light, (pale) grey-green or inconspicuous; exudate absent, reverse brown. Colonies on MEA velvety, dull green or grey-green (25–26E3), reverse brown (5D3). Ehrlich reaction negative.

Micromorphology: Conidiophores borne from surface; slightly vesiculate; stipes short, 10–40 (− 120) × 2–3.5 μm, with walls smooth or very finely roughened; conidiophores of freshly isolated strains bearing terminal verticils of 2–4 metulae, and sometimes subterminal or intercalary metulae present; in degenerated strains monoverticillate conidiophores predominantly present. Terminal and subterminal metulae unequal in length, 10–20 (− 25) × 2.0–3.5 μm, often with inflated apex, 3–5 μm wide. Phialides ampulliform, in verticils of 4–10, closely packed, 7–9 × 2–3.5 μm. Conidia globose to subglobose, smooth-walled, 2.0–2.5 μm. Ascomata and sclerotia not observed.

Discussion

Grogan et al. (2001) expressed uncertainty about the identity of the smoky mould fungus in mushroom cultivation because taxonomic authorities that they consulted did not agree on a common identification. Most identifications of smoky mould isolates were performed before the era of DNA sequence-based identification. Grogan et al. (2000) studied the identity of four smoky mould isolates (including isolate HRI 1043-D) using ITS sequences, but the exact identification of the species involved in smoky mould problems remained uncertain. As demonstrated in this paper, the smoky mould isolates also turn out be a previously undescribed species. Although several *Penicillium* species (e.g. *P. chermesinum*, *P. citreonigrum*, *P. implicatum* and *P. fellutanum*) were named as causal agents of smoky mould, we suggest that these outbreaks were probably all caused by *P. hermansii* alone. Strains referred to as smoky mould in the literature share a slow growth rate on agar media and most were reported to be monoverticillate (Baars et al. 2011; Beyer 2002; Fletcher and Gaze 2008). *Penicillium hermansii* also grows slowly, but fresh isolates have terminal verticils of 2–4 metulae.


a
b
c
d
e
f

◀ **Fig. 3** *Penicillium hermansii*, **a** 7 day-old cultures, 25 °C, left to right; first row, all obverse, CYA, YES, DG18, MEA; second row, CYA reverse, YES reverse, OA obverse and CREA obverse. **b**–**e** Conidiophores. **f** Conidia. Scale bars = 10 μm

Phylogenetically, *P. hermansii* is most closely related to *P. canis*, *P. catenatum*, *P. erubescens*, *P. guttulosum*, *P. menonorum*, *P. nepalense*, *P. papuanum, P. parvum*, *P. pimiteouiense*, *P. rubidurum*, *P. striatisporum* and *P. vinaceum* that all align to the *P. parvum*-clade of section *Exilicaulis*. These species share a slow growth rate on CYA and MEA and produce short-stiped, monoverticillate conidiophores. Like the other members of this clade, *P. hermansii* also grows slowly on agar media and along with *P. nepalense* and *P. catenatum* is among the slowest growing species of the clade. Morphologically, it differs from all other species of this clade by the production of biverticillate conidiophores, with monoverticillate conidiophores only seen in older, presumably degenerated strains. *Penicillium hermansii* also differs from most of the phylogenetically related species by its inability to grow at 37 °C, a character shared only with *P. nepalense* (Pitt 1980 ['1979']; Takada and Udagawa 1983). Using the dichotomous key published in the *Penicillium* monograph of Pitt (1980 ['1979']), *P. hermansii* keys out as *P. fellutanum*. That species most obviously differs from *P. hermansii* by its faster growth rate on CYA (5–10 vs 17–25 mm).

In addition to its distinctive phenotype, *P. hermansii* is also unique in *Penicillium* for its association with *Agaricus*-colonised compost. Until now, this species has never been isolated from other habitats, despite extensive surveys of soil, food, indoor air and other substrates from all over the world. Strong associations between *Penicillium* species and particular habitats have been known for a long time (Westerdijk 1949). For example, *P. italicum*, *P. ulaiense* and *P. digitatum* are associated with rot of citrus fruits and *P. allii* is strongly associated with rot of garlic. Generally, *Penicillium* species with a specific association to certain substrates grow well on standard laboratory media and do not require special compounds from its associated natural source, e.g. citrus peels or garlic (Frisvad and Samson 2004). In contrast, *P. hermansii* grows slowly on general purpose agar media CYA, MEA and DG18, but observations in practice show that the species thrives well on *Agaricus* grown compost. All *Penicillium* strains used in this study grew well on CA. The components of this autoclaved medium cannot explain the profuse growth of *P. hermansii* in *Agaricus* colonised compost. The compost composition might therefore not be the defining parameter for growth of *P. hermansii*. Actually, many *Penicillium* species occur in compost and seem to have little or no effect on *Agaricus* growth (Grogan et al. 2001).

Very little work has been carried out on competition with *Agaricus* by *P. hermansii* and most research on competing fungi in *Agaricus* production has focused on *Trichoderma aggressivum*. This species grows quickly on agar media and seems to compete with *Agaricus* for space and nutrients effectively. The association of *T. aggressivum* with *Agaricus* production is probably first based on chemical communication via extrolites, including volatiles, and/or extracellular enzymes. For example, the mycelium of *A. bisporus* is required for induction of intensive sporulation in *T. aggressivum* (Krupke et al. 2003; Mamoun et al. 2000; Mumpuni et al. 1998; Seaby 1987; Seaby 1996). Similarly, Beyer (2002) indicated an interaction between the mycelium of *Agaricus* and smoky mould and suggested that the *Agaricus* is either parasitised or repressed. More research is needed to understand the biology of smoky mould in button mushroom cultivation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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