Fungal Diversity (2010) 44:117–133
DOI 10.1007/s13225-010-0047-z

Taxonomy of *Penicillium citrinum* and related species

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Received: 5 July 2010 / Accepted: 7 July 2010 / Published online: 13 August 2010

Abstract *Penicillium citrinum* and related species have been examined using a combination of partial β-tubulin, calmodulin and ITS sequence data, extrolite patterns and phenotypic characters. It is concluded that seven species belong to the series *Citrina*. *Penicillium sizovae* and *Penicillium steckii* are related to *P. citrinum*, *P. gorlenkoanum* is revived, *Penicillium hetheringtonii* **sp. nov.** and *Penicillium tropicoides* **sp. nov.** are described here as new species, and the combination *Penicillium tropicum* is proposed. *Penicillium hetheringtonii* is closely related to *P. citrinum* and differs in having slightly broader stipes, metulae in verticils of four or more and the production of an uncharacterized metabolite, tentatively named PR1-x. *Penicillium tropicoides* resembles *P. tropicum*, but differs in the slow maturation of the cleistothecia, slower growth at 30°C and the production of isochromantoxins. The type strain of *P. hetheringtonii* is CBS 122392^T (=IBT 29057^T) and the type strain of *P. tropicoides* is CBS 122410^T (=IBT 29043^T).

Keywords *Penicillium* · Citrinin · *Steckii* · *Sizovae* · *Citrinum* · Taxonomy · Series *Citrina*

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Introduction

Penicillium citrinum is a commonly occurring filamentous fungus with a worldwide distribution and it may well be one of the most commonly occurring eukaryotic life forms on earth (Pitt 1979). This species has been isolated from various substrates such as soil, (tropical) cereals, spices and indoor environments (Samson et al. 2004). Citrinin, a nephrotoxin mycotoxin named after *P. citrinum* (Hetherington and Raistrick 1931), is consistently produced by *P. citrinum*. In addition, several other extrolites, such as tanzowaic acid A, quinolactacins, quinocitrinines, asteric acid and compactin are reported to be produced by this species (Kim et al. 2001; Kozlovskiĭ et al. 2003a, b, Malmstrøm et al. 2000; Turner and Aldridge 1983).

Raper and Thom (1949) placed *P. citrinum* in section *Asymmetrica*, subsection *Velutina* and introduced the "*Penicillium citrinum* series" for *P. steckii*, *P. corylophilum* and *P. citrinum*. Ramirez (1982) followed Raper and Thom's concept, and added *P. matritii* to this series. A classification system based on the branching pattern of the penicillus was introduced by Pitt (1979), and *P. citrinum* was placed in the subgenus *Furcatum*, section *Furcatum*, series *Citrina*. In this monograph, *P. citrinum* was used to typify the subgenus *Furcatum* and the series *Citrina*. Seven species were placed in the series *Citrina*, and members of this series share similar growth rates and have terminal verticils of metulae with small conidia. Several species were placed in synonymy with *P. citrinum*, namely *P. baradicum*, *P. gorlenkoanum*, *P. botryosum*, *P. sartoryi*, *P. steckii*, *P. aurifluum*, *P. subtile* and *Citromyces subtilis*. Peterson (2000) made a phylogenetic analysis of various *Penicillium* species belonging to the subgenera *Aspergilloides*, *Furcatum* and *Penicillium*. Based on his data, it was shown that *P. sartoryi* is distinct from *P. citrinum* and

should be revived. Furthermore, *P. matritii* and *P. corylophilum*, previously claimed to be related to *P. citrinum* (Raper and Thom 1949; Pitt 1979; Ramirez 1982), were positioned in phylogenetic distant clades.

In this study, *P. citrinum* and related species are examined using the ITS regions (intergenic spacer region and 5.8S rDNA gene) and parts of the β-tubulin and calmodulin gene, in combination with extrolite profiles, physiology and macro- and microscopical characters. A large set of isolates, including the type strains of various synonyms and freshly isolated strains are included in this study.

Material and methods

Isolates

The examined strains included type strains or representatives of species related to *P. citrinum*. Additional strains were isolated from various substrates, such as soils from different locations, food- and feedstuffs and air. An overview of strains used in this study is presented in Table 1. All strains are maintained in the CBS culture collection.

DNA isolation, amplification and analysis

The strains were grown on Malt Extract agar (MEA, Oxoid) for 4–7 days at 25°C. Genomic DNA was isolated using the Ultraclean™ Microbial DNA Isolation Kit (MoBio, Solana Beach, U.S.A.) according the manufacturer's instructions. Fragments, containing the ITS regions, a part of the β-tubulin or calmodulin gene, were amplified and subsequently sequenced according the procedure previously described (Houbraken et al. 2007). The alignments and analyses were preformed as described by Samson et al. (2009), with one modification: to prevent saturation of the computer's memory, the maximum number of saved trees for the ITS dataset was set to 5,000. *Penicillium corylophilum* CBS 330.79, was used as an outgroup in all analyses. Additional sequences of *P. sumatrense*, *P. manginii*, *P. decaturense*, *P. chrzaszcii*, *P. waksmanii*, *P. westlingii*, *P. miczynskii*, *P. paxilli*, *P. roseopurpureum*, *Penicillium shearii* and *P. anatolicum* were added to the ITS dataset to determine the phylogenetic relation with *P. citrinum*. The newly derived sequences used in this study were deposited in GenBank under accession numbers GU944519-GU944644, the alignments in TreeBASE (www.treebase.org/treebase-web/home.html), and taxonomic novelties in MycoBank (www.MycoBank.org; Crous et al. 2004).

Morphology and physiology

The strains were inoculated in a three point position on Czapek yeast autolysate agar (CYA), malt extract Agar (MEA), creatine agar (CREA) and yeast extract sucrose agar (YES). Growth characteristics were measured and determined after an incubation period of 7 days at 25°C in darkness. Light microscopes (Olympus BH2 and Zeiss Axiokop two Plus) were used for microscopic examination and a set 25 micromorphological dimensions was obtained for each characteristic. Ripening of the cleistothecia was checked for up to 3 months. Colours of cleistothecia were determined on Oatmeal agar (OAT) after seven and 14 days of incubation at 25°C. Temperature-growth data was studied on CYA plates, which were inoculated in a three-point position and incubated at 12°C, 15°C, 18°C, 21°C, 24°C, 27°C, 30°C, 36°C, 37°C and 40°C. The colony diameters were recorded after 7 days of incubation in darkness.

Extrolites

Culture extracts were made from the agar media CYA and YES according the method described by Smedsgaard (1997). The extracts were analysed by HPLC with diode array detection according the method described by Frisvad and Thrane (1987, 1993). The extrolites were identified by their retention times and UV spectra. Authentic analytical standards were employed for retention time and retention index comparison with the extrolites detected.

Results

Phylogenetic analysis

The ITS regions and parts of the β-tubulin and calmodulin gene were sequenced and analysed. The trees obtained from the maximum parsimony analysis are shown in Figs. 1, 2, 3. Molecular data revealed that six species are related to *P. citrinum*. Four of these species are strictly anamorphic, *P. hetheringtonii*, *P. sizovae*, *P. steckii* and *P. gorlenkoanum*, and two form a teleomorph, namely *P. tropicum* and *P. tropicoides*.

The ITS regions included 520 bp, of which 10% were parsimony-informative. The heuristic search generated more than 5,000 equally parsimonious trees, which were 129 steps long. Phylogenetic analysis of the ITS dataset resulted in low bootstrap supports of the clades and only the connection between *P. citrinum* and *P. hetheringtonii* was highly supported (100%). Both *P. sumatrense* and *P. gorlenkoanum* were basal to *P. citrinum* and related species. However, this is not supported by the β-tubulin and

Table 1 Details of isolates included in the morphological and/or molecular examination of this study

Species	CBS number	Substrate and locality
P. citrinum	139.45	Ex type of *P. citrinum* and *P. aurifluum*, unrecorded source
P. citrinum	252.55	Ex-type of *P.botryosum*, herbarium specimen, Recife, Brazil
P. citrinum	241.85	IMI 092267; ex type of *P. phaeojanthinellum*, unrecorded source
P. citrinum	122726	NRRL 783; representative of *P. sartoryi*, unrecorded source
P. citrinum	115992	Compost, the Netherlands
P. citrinum	122398	Peanut, Indonesia
P. citrinum	122397	Soil, Treasure Island, Florida, USA
P. citrinum	865.97	Patient with acute myeloid leukemia, Hong Kong, China
P. citrinum	122395	Coconut milk; produced in Indonesia, imported into the Netherlands
P. citrinum	122394	Soil, Merang, Malaysia
P. citrinum	232.38	Type of *P. implicatum*; original culture deposited by Thom (as Thom 4733.73), unknown source, Belgium
P. citrinum	117.64	Epoxy softener, the Netherlands
P. citrinum	122452	Coffee beans, Thailand; colour mutant
P. citrinum	122451	NRRL 2145; colour mutant;unrecorded source
P. citrinum	101275	Leaf, Panama
P. gorlenkoanum	408.69	Ex-type strain of *P. gorlenkoanum*; soil, Syria
P. gorlenkoanum	411.69	Ex-type strain of *P. damascenum*; soil, Syria
P. hetheringtonii	122392	Type; soil, Treasure Island, Florida, USA
P. hetheringtonii	124286	Soil, Lookout Kuranda, Queensland, Australia
P. hetheringtonii	DTO 30H7	Soil, Lookout Kuranda, Queensland, Australia
P. hetheringtonii	124287	Soil, Lake Easchem, Queensland, Australia
P. sizovae	413.69	Neotype of *P. sizovae*; soil, Syria
P. sizovae	122387	Margarine, the Netherlands
P. sizovae	139.65	Sea salt, Portugal
P. sizovae	122386	Glue, the Netherlands
P. sizovae	115968	Cropped soil, Italy
P. sizovae	117183	*Papaver somniferum*, the Netherlands
P. sizovae	117184	IBT 22812; salty water in saltern, Slovenia
P. steckii	325.59	Ex-type of *P. corylophiloides nom. inval.*;ex soil Japan
P. steckii	789.70	Unrecorded source
P. steckii	122391	Potting soil, the Netherlands
P. steckii	260.55	Ex-neotype of *P. steckii*; cotton fabric treated with copper naphthenate, Panama
P. steckii	122390	IBT 21096; *Caranx crysos* (blue runner, fish), sand bottoms with corals, surface water 23°C, dept 2–3 m at Cabruta, Mochima Bay, Venezuela
P. steckii	122389	IBT 19353 = IFO 6024; unrecorded source
P. steckii	122388	IBT 14691 = NRRL 6336; baled coastal grass hay, Bermuda
P. steckii	122418	IBT 6452; *Cynara scolymus* (Artichoke), Egypt
P. steckii	122417	IBT 20952; Ascidie (tunicate, urochordata), sand bottoms with corals, surface water 23°C, dept 2–3 m at Cabruta, Mochima Bay, Venezuela
P. tropicoides	122410	Type; soil of rainforest, near Hua-Hin, Thailand
P. tropicoides	122436	Soil of rainforest, near Hua-Hin, Thailand
P. tropicum	112584	Ex-type; soil between *Coffea arabica*, Karnataka, India

calmodulin datasets. *Penicillium gorlenkoanum* appeared to be related to *P. citrinum* in these datasets, and *P. sumatrense* formed a clade unrelated to *P. citrinum*, *P. westlingii*, *P. paxilli*, *P. roseopurpureum* or *P. shearii* (data not shown). A gap of 36–38 bp was observed in the ITS1 region of all *P. citrinum* and *P. hetheringtonii* isolates. However, analysis of other *Penicillium* strains showed that this feature is not species specific, since one isolate of *P. manginii* (CBS 327.79) also has this deletion, while another has not (CBS 253.31^{T}). The ITS dataset showed less resolution than the

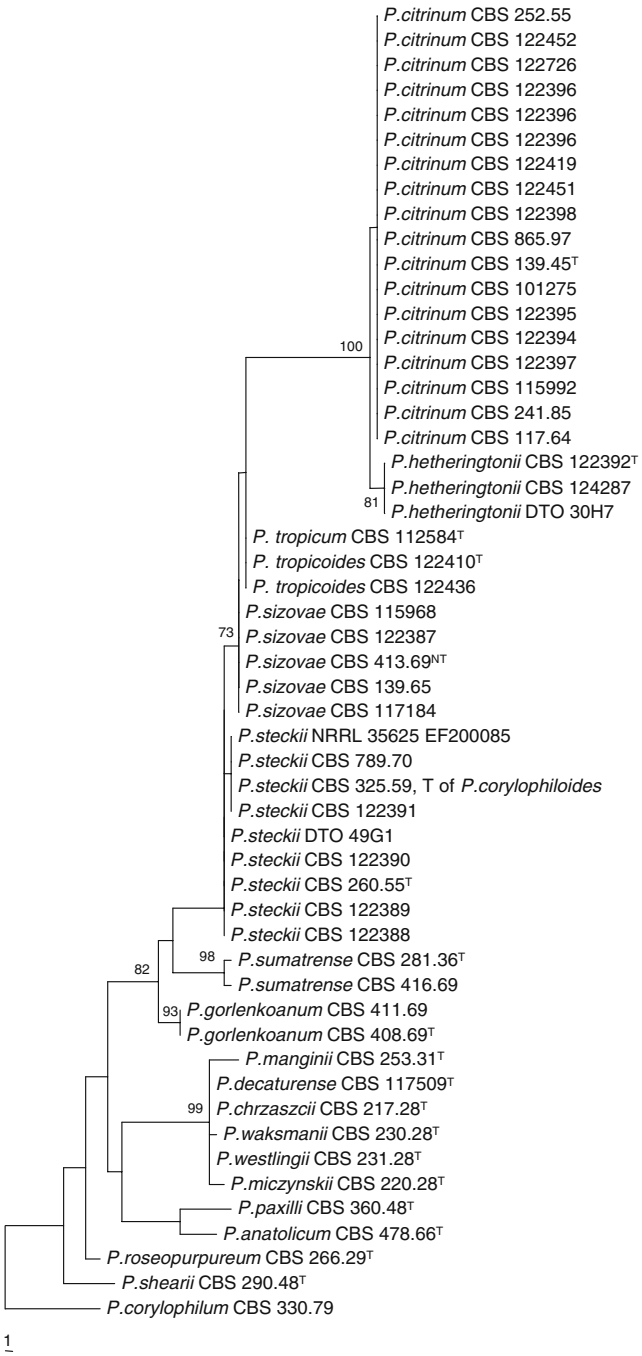


Fig. 1 One of the 128 equally most parsimonious trees of the analysed ITS region (55 of the 629 characters were parsimony informative; tree length=95, CI=0.652, RI=0.948, RC=0.653)

β-tubulin and calmodulin datasets, and *P. tropicum* and *P. tropicoides* had no differences in their ITS regions. The other five species could be differentiated based on their ITS sequence, and a subgroup in the *P. steckii* clade was observed. This subgroup, characterized by a single basepair difference on position 164 of the ITS2 region, included the type strain of *P. corylophiloides* nom. inval. (CBS 325.59).

The β-tubulin and calmodulin datasets were more variable than the ITS dataset. The β-tubulin dataset consisted of 473 bp, of which 15% was parsimony informative. The heuristic search yielded two equally parsimonious trees of 166 steps. 456 characters of the calmodulin dataset were analysed and 20% was parsimony informative. The analysis generated six equally most parsimonious trees of 171 steps long. Both phylograms only had high bootstrap support at the nodes. The basal nodes were different between the two datasets and they were in both cases not supported by high bootstrap values. *Penicillium steckii* was split, similar to the ITS dataset, into two groups with high bootstrap support. The grouping of the isolates was in all cases identical, suggesting absence of recombination between these clades. The calmodulin and ITS phylograms show a high bootstrap support (84% and 100% respectively) between *P. hetheringtonii* and *P. citrinum*. Also a high bootstrap support (89%) is present in the β-tubulin dataset between *P. sizovae* on the one hand and *P. tropicum* and *P. tropicoides* on the other.

Morphology and physiology

Various phenotypic differences were observed among the investigated species (see Table 2). Growth rates on CYA incubated at 30 and 37°C, and reverse colours and growth rates on CYA and YES at 25°C were useful characters for differentiation between *P. citrinum* and related species (Fig. 4). The examined *P. citrinum* strains consistently grew at 37°C. Some strains of *P. sizovae* (five of seven) and *P. hetheringtonii* (one of four) were able to grow at this temperature, though more restricted than *P. citrinum*. All species were able to grow at 30°C, though with different growth rates. This feature was also useful to differentiate between the members of the series *Citrina* and other related species such as *P. westlingii*, *P. waksmanii*, *P. miczynskii* and *P. manginii*, which were not able to grow at this temperature (data not shown). The reverse colours on YES varied from (pale) crème in *P. sizovae* and *P. steckii* to shades of orange in *P. citrinum* and *P. hetheringtonii*. The reverse colours on CYA were less pronounced and varied from pale to brownish yellow. Creatin agar, which is used for identification of species belonging to subgenus *Penicillium* (Frisvad 1985; Samson and Frisvad 2004) was also tested, but had little discriminatory power. Most species showed weak growth with no or weak acid production. The only exception was *P. steckii*, which grew weak to moderate on this medium.

Comparison of the micro-morphology showed differences in branching of the conidiophores, and shape and ornamentation of the conidia. All the species have smooth stipes, small conidia (2–3 μm) and share symmetric biverticillate conidiophores with occasionally an additional branch. Additional branching was most often seen in

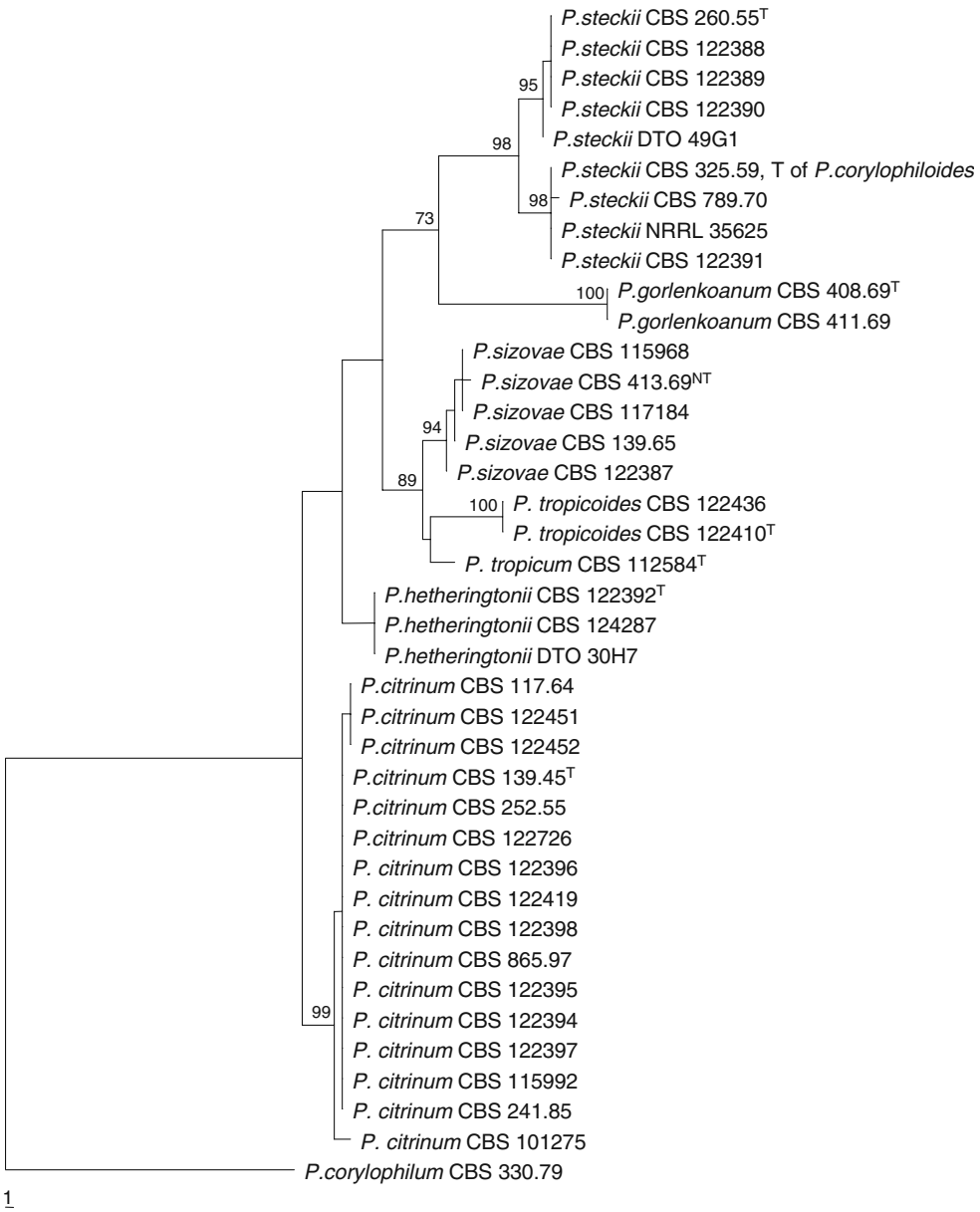


Fig. 2 One of the two equally most parsimonious trees of the analysed BenA region (71 of the 473 characters were parsimony informative; tree length=166, CI=0.898, RI=0.964, RC=0.865)

freshly isolated strains of *P. citrinum* and *P. hetheringtonii* and not or less in the other species. Most species had globose, smooth walled conidia. Exceptions were *P. steckii*, *P. tropicum* and *P. tropicoides*, which have (broadly) ellipsoidal conidia and *P. sizovae*, which has finely roughened conidia.

Extrolites

The mycotoxins and other extrolites produced by the examined species are listed in Table 3. Several extrolites, such as citrinin, quinolactacin, isochromantoxins and an unknown metabolite named PR1-x, were produced by more than one species. The examined species could be differentiated based on their characteristic pattern of extrolites.

Taxonomy

Penicillium citrinum Thom, Bulletin of the U.S. Department of Agriculture, Bureau Animal Industry 118: 61. 1910.

= *Citromyces subtilis* Bainier & Sartory, Saccardo's Syll. fung. XXV: 684. 1912.
= *Penicillium subtile* (Bainier & Sartory) Biourge, Cellule 33: 106, 1923 (nom. Illegit.,Art. 64; non Berk. 1841.
= *Penicillium aurifluum* Biourge, Cellule 33: 250. 1923.
= *Penicillium phaeojanthinellum* Biourge, Cellule 33: 289. 1923.
= *Penicillium implicatum* Biourge, La Cellule 33(1): 278. 1923.

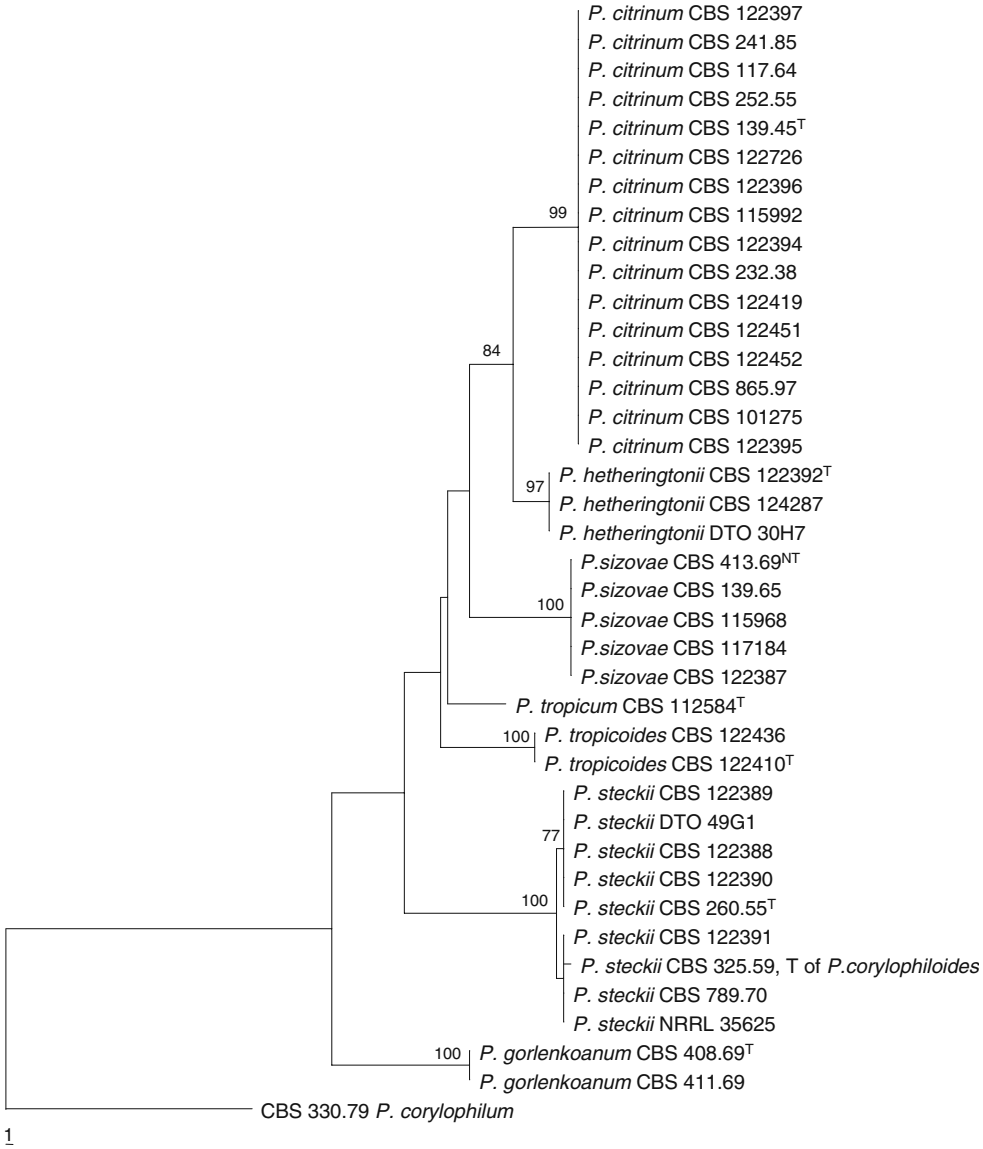


Fig. 3 One of the six equally most parsimonious trees of the analysed Cmd region (89 of the 456 characters were parsimony informative; tree length=171, CI=0.872, RI=0.959, RC=0.836)

Table 2 Overview of morphological and physiological characters to differentiate between *P. citrinum* and related species

Species	Colour conidia on MEA	Reverse colour on CYA	Reverse colour on YES	CYA 30°C (mm)	CYA 37°C (mm)	Shape and ornamentation conidia	Presence of cleistothecia
P. citrinum	Blue grey green	Brownish yellow	Yellow or orange-yellow	30–36 (−43)	2–11	Globose to subglobose, smooth	Absent
P. gorlenkoanum	Grey green	Crème-brown	Pale yellow	(20−) 25–30	No growth	Globose to subglobose, smooth	Absent
P. hetheringtonii	Dark blue green	Brownish yellow	Orange	29–35	0–5	Globose to subglobose, smooth	Absent
P. sizovae	Grey green	Pale	Yellowish crème to crème	30–35	0–5	Globose to subglobose, finely roughened	Absent
P. steckii	Grey or dull green	Crème-brown	Yellowish crème to crème	15–20 (−25)	No growth	Broadly ellipsoidal, in some strains slightly fusiform, smooth	Absent
P. tropicoides	Conidia sparely produced; blue grey green	Brown	Yellow	15–25	No growth	Broadly ellipsoidal, smooth	Present
P. tropicum	Conidia sparely produced; blue grey green	Brown	Crème yellow	25–30	No growth	Broadly ellipsoidal, smooth	Present

Fig. 4 Overview of *P. citrinum* and related anamorphic species on various agar media. Rows: CYA obverse, CYA reverse, YES obverse, YES reverse and CYA incubated 30°C. Columns, from left to right: *P. citrinum* CBS 232.38, *P. hetheringtonii* CBS 124287, *P. sizovae* CBS 122387, *P. steckii* CBS 122388, *P. steckii* (*"P. corylophiloides"*) CBS 122391 and *P. gorlenkoanum* CBS 408.69

= *Penicillium sartoryi* Thom [as *'sartorii'*], The Penicillia: 233. 1930.
= *Penicillium botryosum* Bat. & H. Maia, Anais Soc. Biol. Pernambuco 15(1): 157. 1957.

Type: IMI 92196ii^{NT} (*P. citrinum* and *P. aurifluum*); other ex-type: CBS 139.45 = Biourge 53 = Thom 4733.14 = ATCC 1109 = ATCC 36382 = CECT 2269 = FRR 1841 = IMI 091961 = IMI 092196 = LSHB P25 = LSHB P6 = LSHB Ad95 = MUCL 29781 = NRRL 1841 = NRRL 1842.

Description: Colony diameter, 7 days, in mm: CYA 27–33; CYA30°C 27–40; CYA37°C 2–12; MEA 18–25; YES 29–37; CYAS 29–36; creatine agar 10–19, poor growth, no or weak acid production.

Moderate sporulation on CYA with grey green or blueish grey green conidia, occasionally with small clear or pale yellow exudate droplets, reverse brownish-yellow, diffusible pigments yellow. Moderate to good sporulation on YES, conidial color variable: grey green to dark green, reverse yellow to orange yellow and strong yellow soluble pigment production. Colonies on MEA grey green with a strong blue element, velvety, occasionally with small pale yellow exudate droplets. No reaction with Ehrlich test.

Conidiophores arising from mycelium mat, predominant symmetrically biverticillate, terverticillate structures abundantly produced in fresh isolates; stipes smooth, width 2.0–3.0μm; metulae in whorls of 3–4(−6), $12-16 \times 2.0-2.7\mu$m; phialides ampulliform, 7.5–

Table 3 Mycotoxins and other extrolites produced by the examined species

Species	Extrolites
P. citrinum	Citrinadins, citrinin, quinolactacin, anthraquinone with emodin chromophore
P. gorlenkoanum	Chanoclavine-I, citrinin
P. hetheringtonii	Citrinin, quinolactacin, PR1-x[a]
P. sizovae	Agroclavine-I, epoxyagaroclavine-I and 1,1-bis(6,8-dimethyl-8,9-epoxy-5a,10e)-ergoline, quinolactacin
P. steckii	Isochromantoxins, quinolactacin, tanzawaic acids E and F
P. tropicoides	Isochromantoxins, PR1-x[a] and apolar indol alkaloids
P. tropicum	Apolar indol alkaloids and other uncharacterized extrolites

[a] PR1-x is an unknown extrolite with a characteristic UV spectrum.

$10 \times 2.0 - 2.5\mu$m; conidia smooth walled, globose to subglobose, $2.0 - 2.5 \times 1.8 - 2.5\mu$m.

Diagnostic features: Restricted growth on CYA37°C (2–12 mm), yellow reverse on CYA, globose, smooth walled conidia.

Extrolites: Citrinin, quinolactacins, citrinadins, several anthraquinones, the uncharacterized extrolites, tentatively named "CITY" and "shamix".

Distribution and ecology: Worldwide occurrence: predominant in (sub)tropical soils, but also isolated from indoor air, food and as an endophyte of root, stem and leaves of coffee plants (Posada et al. 2007) and roots of *Ixeris repens* (Khan et al. 2008; identity based on ITS sequences deposited on GenBank).

Notes: Thom (1910) did not designate a type, but a subculture from his original strain was sent, via Kral, to Biourge. Biourge believed that this strain was contaminated and a culture derived from this strain was described as *P. aurifluum*. Later, *P. aurifluum* was sent to Thom and he recognized it as *P. citrinum* and therefore this strain is accepted to be derived from the original isolate (Pitt 1979). Raper and Thom (1949) mentioned that their concept of *P. citrinum* is broad in scope and included forms which vary substantially in particular characteristics. It was noted that 75% of the strains fully comply with their species description, and for the remaining strains, six groups were introduced. Representatives of the first group are NRRL 1171 and NRRL 2143 and re-identification of these strains proved to be *P. citrinum* (Malmstrøm et al. 2000). Raper and Thom's group 2 is centered on strains NRRL 2144 and NRRL 1841, and the latter was later used to typify *P. citrinum*. Group 3 contains strains which are transitional towards *P. chrysogenum* and are claimed to produce both citrinin and penicillin. Examination of the representative of this group, NRRL 822, showed to be a *P. chrysogenum* (as *P. rubens*), and no citrinin was produced by this strain (Samson and Frisvad 2004). The *P. citrinum* isolates, which resemble typical *P. citrinum* strains in macromorphological characters, but have variously branched or monoverticillate conidiophores, were placed in group 4. NRRL 783 and NRRL 784 are representatives of this group and were described as *P. sartoryi* (Thom 1930). This species was placed in synonymy with *P. citrinum* (Pitt 1979; Pitt et al. 2000). However, Peterson (2000) suggested that *P. sartoryi* is a distinct species, based on ITS and partial 28S rDNA data. Re-analyses of the ITS regions of this species revealed a 2 bp difference with the sequence deposited in Genbank (AF033421). Our molecular data and the extrolite profiles show that this species is conspecific with *P. citrinum*. Group 5 contains colour mutants and examination of NRRL 2145, a representative of this group, and CBS 122452, a colour mutant isolated from Thai coffee beans, showed that these two strains are *P. citrinum*. Both strains have brown coloured conidia and share partial calmodulin and ITS sequences with CBS 139.48^T. In contrast, both strains differ one basepair with CBS 139.48^T in their partial BenA sequence. These colour mutants form a separate clade in the BenA phylogram, together with CBS 117.64, a green coloured *P. citrinum*, and therefore conidium colour is not an exclusive character for this subclade. Raper and Thom (1949) placed nutrient deficient mutants in group 6 and strains belonging to this group are characterized by sparse growth on Czapek's agar. The extrolite pattern of NRRL 2148, a representative of this group, was analyzed and this strain had a *P. citrinum* profile (Malmstrøm et al. 2000).

Frisvad et al. (1990) noted that the type of *P. implicatum* is a synonym of *P. citrinum*. Pitt (1979) was unaware of the existence of the type material and designated IMI 190235 as a neotype. CBS 232.38, the type culture of *P. implicatum*, resembles *P. citrinum* in having typical *P. citrinum* colonies and conidiophores and shares identical BenA sequences with the type of *P. citrinum*. Therefore Frisvad et al. (1990) is followed and the neotype proposed by Pitt (1979) is rejected. *Penicillium phaeojanthinellum* and *P. fellutanum* were also proposed by Frisvad et al. (1990) as synonyms for *P. citrinum* and Pitt (1979) placed *P. botryosum* in synonomy with *P. citrinum*. The placement of *P. phaeojanthinellum* and *P. botryosum* in synonymy with *P. citrinum* is confirmed here. No type material of *P. fellutanum* could be obtained and therefore the placement of this species remains unknown.

Penicillium gorlenkoanum Baghdadi, Nov. sist. Niz. Rast., 1968: 97. 1968.

= *Penicillium damascenum* Baghdadi, Nov. sist. Niz. Rast., 1968: 101. 1968.

Type: CBS 408.69^{NT} (designated here); other cultures ex-type: FRR 511 = IMI 140339 = VKM F–1079

Description: Colony diameter, 7 days, in mm: CYA 26–31; CYA30°C 20–30; CYA37°C no growth; MEA 20–27; YES 26–30; CYAS 27–33; creatine agar 13–19, weak growth and no or weak acid production.

Moderate or good sporulation on CYA with grey, dull green or dark green conidia, small clear or weak yellow coloured exudate droplets, soluble pigments absent, reverse pale yellow or crème-brown. Degree of sporulation on YES variable: weak (CBS 409.69) to strong (CBS 408.69), soluble pigment absent, grey green conidia, reverse pale yellow. Colonies on MEA grey green, velvety to floccose. No reaction with Ehrlich test.

Conidiophores from aerial hyphae, predominantly irregularly biverticillate, stipes smooth, width 2.0–2.7μm; metulae terminal in whorls of 2–3, $12-17\times2.2-3.0\mu m$; phialides ampulliform, $7.5-9.0\times2.0-3.0\mu m$; conidia smooth to finely rough walled, globose to subglobose, variable in size, predominantly 2.0–2.5 μm, smaller portion of conidia larger, 2.5–3.0 μm.

Diagnostic features: No growth at 37°C, production of chanoclavine-I.

Extrolites: Citrinin, costaclavin, chanoclavine-I (Kozlovskiĭ et al. 1981a, b), and uncharacterized extrolites, tentatively named "KUSK", "WK", "WS", "WT" and "WØ".

Distribution and ecology: Soil, Syria.

Notes: *Penicillium gorlenkoanum* was placed in synonymy with *P. citrinum*, while *P. damascenum* was claimed to be conspecific with *P. melinii* (Pitt et al. 2000). Molecular data and extrolite patterns showed that *P. gorlenkoanum* and *P. damascenum* were conspecific. Both species are described in the same publication, and the name *P. gorlenkoanum* has been chosen above *P. damascenum*. Only two strains of this species were available for examination (CBS 408.69 and CBS 409.69) and both strains did not show typical terminal metulae in whorls of 5–8, as reported and shown in the original descriptions (Baghdadi 1968). This might be due to degeneration of these cultures during preservation. The conidial size and the original drawings of the conidiophores indicate that this species belongs to the series *Citrina*.

Penicillium hetheringtonii Houbraken, Frisvad and Samson, **sp. nov.**—MycoBank MB518292; Fig. 5.

Etymology. Named after A.C. Hetherington, who first isolated citrinin (together with H. Raistrick).

Penicillio citrino affine, sed metullis 4–8(−12) verticillatis, revero eburneo-brunneo coloniae in agaro YES, sine pigmentis diffluentibus, solutabilibus, metabolito obscuro (PR 1-x) producenti.

Holotype: CBS 122392^{T} is designated here as the holotype of *Penicillium hetheringtonii*, isolated from soil of beach, Land's end Garden, Treasure Island, Florida, USA.

Description: Colony diameter, 7 days, in mm: CYA 26–32; CYA30°C 26–34; CYA37°C 0–2; MEA 17–23; YES 27–35; CYAS 21–31; creatine agar 13–17, poor growth on creatine agar, no acid production.

Moderate to good sporulation on CYA with dull green or dark green conidia, small hyaline exudate droplets, diffusible pigments absent, reverse colour crème-brown. Moderate to good sporulation on YES, dark green conidia, reverse orange, soluble pigments absent. Colonies on MEA dark grey green, velvety, floccose in centre. No reaction with Ehrlich test.

Conidiophores borne from surface hyphae, predominant symmetrically biverticillate, terverticillate occasionally present; stipes smooth, 2.5–3.5 μm in width; metulae in whorls of 4–8 (−12), $11-15\times2.5-3.5\mu m$; phialides ampulliform, $7.0-9.2\times2.0-3.0\mu m$; conidia smooth to finely roughened, globose to subglobose, $2.1-2.6\times1.9-2.5\mu m$.

Extrolites: Citrinin, quinolactacin, two anthraquinones, a compound with a chromophore like shamixanthone ("SHAMIX") and the uncharacterized extrolite PR1-x.

Diagnostic features: Metulae in verticils of 4–8 (−12), crème-brown reverse on YES without diffusible soluble pigments, production of uncharacterized metabolite PR1-x.

Ecology and distribution: Soil; Florida, USA and Queensland, Australia.

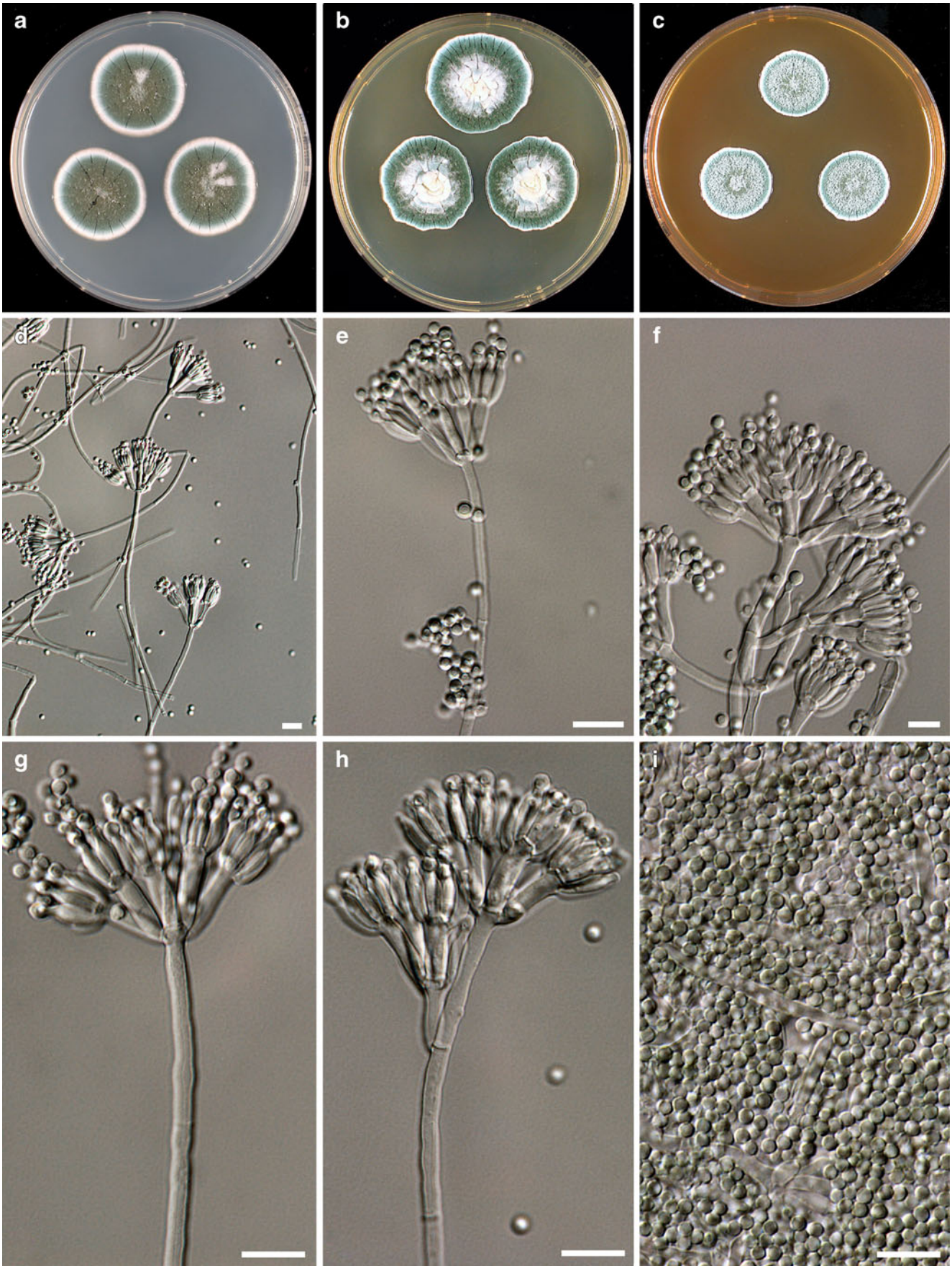
Notes: *Penicillium hetheringtonii* resembles *P. citrinum* in having similar growth rates on agar media and orange reverse on YES, but differs from *P. citrinum* in having broader stipes and 4–8 closely appressed metulae. Superficially, *P. hetheringtonii* resembles *P. paxilli*, though *P. paxilli* produces paxilline while *P. hetheringtonii* does not produce this compound. *Penicillium hetheringtonii* infrequently produces rami and might resemble *P. brevicompactum* (see Fig. 5h). Isolates of *P. brevicompactum* consistently produce rami which are more appressed, do not or grow restrictedly at 30°C and produce the extrolites brevianamide A, mycophenolic acid, pebrolides and Raistrick phenols (Samson and Frisvad 2004).

Penicillium sizovae Baghdadi, Nov. sist. Niz. Rast., 1968: 103. 1968.

Type: CBS 413.69^{NT}; other cultures ex-type are FRR 518 = IMI 140344 = VKM F-1073

Description: Colony diameter, 7 days, in mm: CYA 28–39; CYA30°C 28–34; CYA37°C 0–4; MEA 27–35; YES 40–50; CYAS 29–40; creatine agar 15–23, poor growth, weak acid production.

Good sporulation on CYA with grey green conidia, small clear exudate droplets, soluble pigments absent, reverse pale and occasionally pale crème-brown, often with concentric sulcations. Moderate to good sporulation on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◀ **Fig. 5** *Penicillium hetheringtonii*. **a-c** Colonies grown at 25°C for 7 days, **a** CYA, **b** YES, **c** MEA; **d-h** conidiophores; **i** conidia.—*scale bar*=10 μm

YES, dark green conidia, reverse pale or pale yellow-crème, soluble pigments absent. Colonies on MEA grey green, floccose. No reaction with Ehrlich test.

Conidiophores from aerial hyphae and mycelium mat, symmetrically biverticillate, stipes smooth, width 2.5–3.2 μm; metulae in whorls of 2–5, $11-16\times2.5-3.2\mu$m; phialides ampulliform, $7.0-9.4\times2.0-3.0\mu$m; conidia finely rough walled, globose to subglobose, 2.0–2.5 μm.

Diagnostic features: Fast growing on MEA and YES (in comparision with other related species), pale reverse on CYA, finely roughened conidia.

Extrolites: Quinolactacin, and uncharacterized extrolites, tentatively named "AFSI" and "PNUF".

Distribution and ecology: This species has been isolated from soil, margarine, sea salt, salty water in saltern, glue and *Papaver somniferum* in The Netherlands, Portugal, Syria, Italy, Slovenia.

Notes: Pitt (1979) placed *P. sizovae* in synonymy with *P. fellutanum*, but the former species was later accepted and reinstated by Pitt and Samson (1993). CBS 413.69^{NT} is degenerated and shows both conidiophores with terminal metulae, as well as subterminal and intercalary metulae. This could explain the placement in *P. fellutanum*. Fresh isolates of *P. sizovae* have similar growth rates on CYA as *P. citrinum* and form terminal metulae, which indicates that this species is related to *P. citrinum*.

Penicillium steckii K.M. Zalessky, Bulletin Acad. Polonaise Sci., Math. et Nat., Sér. B: 469. 1927.

= *Penicillium corylophiloides* S. Abe, J. gen. appl. Microbiol, Tokyo 2: 89. 1956. (nom. inval, Art. 36)

Type: IMI 40583^{NT}; other cultures ex-type: CBS 260.55 = ATCC 10499 = CECT 2268 = DSM 1252 = NRRL 2140 = QM 6413 = NDRC 52B4C

Description: Colony diameter, 7 days, in mm: CYA 24–32; CYA30°C 15–23; CYA37°C no growth; MEA 21–30; YES 29–40; CYAS 26–36; creatine agar 12–18, weak to moderate growth, no or weak acid production.

Moderate or good sporulation on CYA with grey green conidia, small clear or weak yellow exudate droplets, soluble pigments absent, reverse in shades of crème (crème, pale crème, yellow-crème or brown crème). Moderate to good sporulation on YES, grey or dull green conidia, reverse light yellow, some strains yellow or light yellow with a yellow-brown center, soluble pigment absent. Colonies on MEA grey green or dull green, velvety. No reaction with Ehrlich test, with exception of CBS 122391.

Conidiophores from surface hyphae, symmetrically biverticillate, stipes smooth, width 2.2–3.0; metulae in whorls of 3–6, $13-18\times2.5-3.3\mu$m; phialides ampulliform, $7.0-10\times2.2-3.0\mu$m; conidia smooth walled, broadly ellipsoidal, in some strains slightly fusiform, $2.3-3.1\times2.0-2.6\mu$m.

Diagnostic features: No growth at 37°C, reverse colours on CYA in shades of crème, broadly ellipsoidal conidia.

Extrolites: Isochromantoxins (Cox et al. 1979; Malmstrøm et al. 2000), quinolactacin, tanzawaic acid E and uncharacterized extrolites tentatively named "FON", "FOS", "phoe" and "STOK".

Distribution and ecology: This species has a worldwide distribution and has been isolated in Japan, the Netherlands, Panama, Venezuela, Bermuda, Egypt, Venezuela, Indonesia and Slovenia. *Penicillium steckii* has been isolated from cotton fabric treated with copper naphthenate, (potting) soil, hypersaline water, blue runner fish, baled coastal grass hay, artichoke, Ascidie (tunicate, urochordata), endophyte of root of coffee plant (Posada et al. 2007).

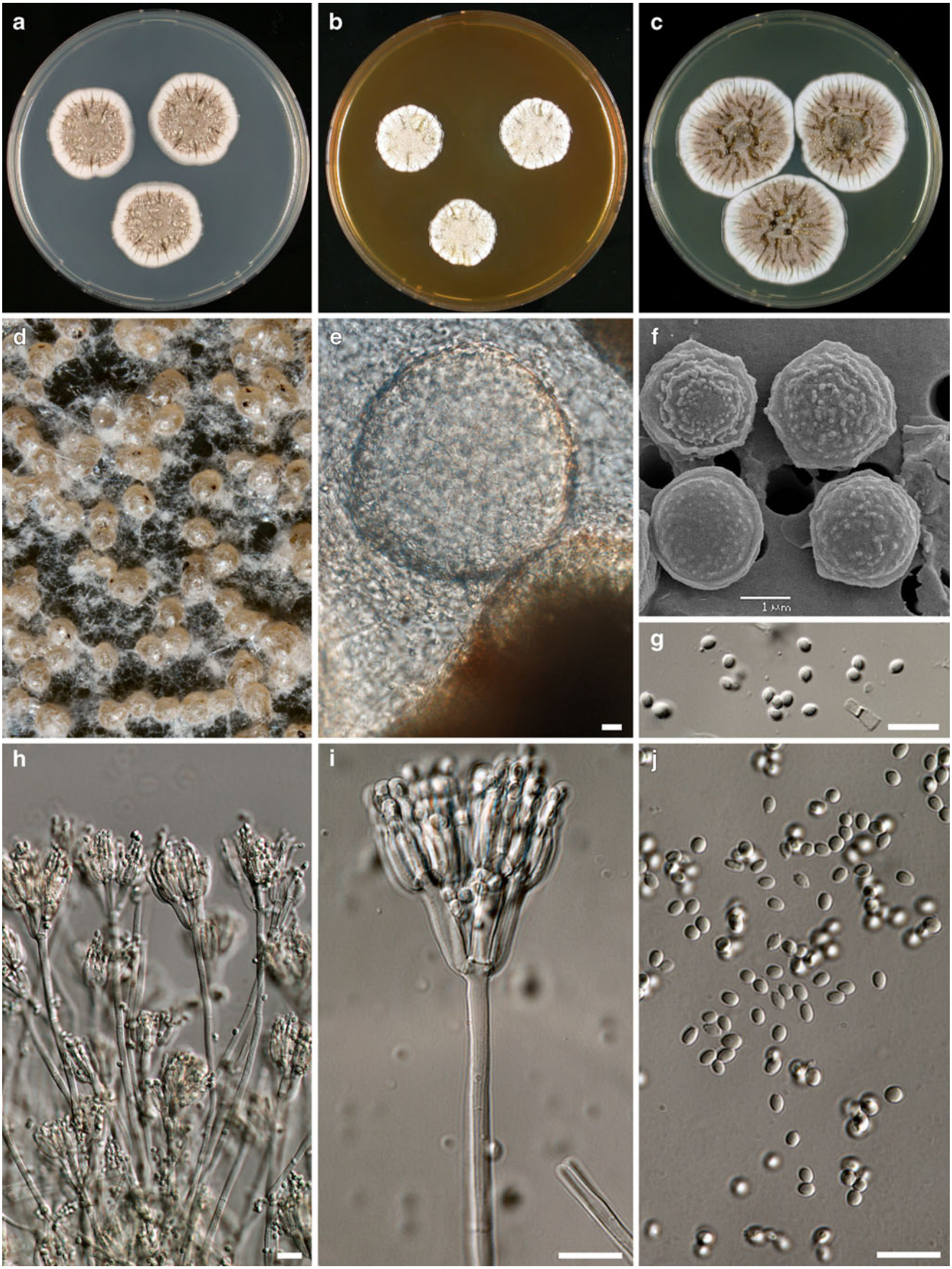
Notes: *Penicillium steckii* was described by Zaleski (1927) and accepted by Raper and Thom (1949) and Ramirez (1982), but was placed by Pitt (1979) in synonymy with *P. citrinum*. Pitt (1979) broadened the concept of *P. citrinum* for *P. steckii* and noted that strains of this species do not produce citrinin and are not able to grow at 37°C. This study shows that this is sufficient to raise these isolates to species level. *Penicillium corylophiloides* was described without a Latin diagnosis and designation of a holotype specimen (Abe 1956). After its description, this species was placed in synonymy with *P. corylophilum* by Smith (1963), while Pitt (1979, 2000) placed this species in synonymy with *P. jensenii*. Abe (1956) noted that *P. corylophiloides* formed typically elliptically formed conidia, in contrast with *P. citrinum* and *P. steckii*. However, our analysis showed that *P. steckii* also forms broadly ellipsoidal conidia. Following the phylogenetic species concept, *P. steckii* and *P. corylophiloides* are separated species; however, no differences in morphology, physiology or extrolites patterns could be observed between these species and are therefore placed in synonymy. Further work should show if these are two distinct species.

Penicillium tropicoides Houbraken, Frisvad and Samson, **sp. nov.**—MycoBank MB518293; Fig. 6.

Etymology: The new species is related to *P. tropicum*.

Eupenicillio tropico affine, sed coloniis 30°C tarde et 38°C haud crescentibus, cleistotheciis griseo-brunneis abundantibus, maturescentibus post tres menses; isochromantoxina formantur.

Holotype: CBS 122410^{T} is designated here as the holotype of *Penicillium tropicoides*, isolated from soil of a rainforest, near Hua-Hin, Thailand.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◀ **Fig. 6** *Penicillium tropicoides*. **a-c** Colonies grown at 25°C for 7 days, **a** CYA, **b** MEA, **c** YES, **d-e** sclerotia, **f-g** ascospores, **h-i** conidiophores, **j** conidia.—*scale bar*=10 μm, except f.=1 μm

Description: Colony diameter, 7 days, in mm: CYA 24–30; CYA30°C 12–18; CYA37°C no growth; MEA 18–23; YES 36–43; CYAS 31–39; creatine agar 13–16, poor to moderate growth and weak acid production (under colony).

Cleistothecia abundantly produced on CYA and drab grey coloured; conidia sparsely produced, blue grey green, colonies typical with large hyaline exudate droplets, reverse on CYA crème-brown, soluble pigments absent. Weak sporulation on YES, cleistothecia abundant present and drab-grey in colour, soluble pigment absent. Colonies on MEA ascomatal, in shades of grey. No reaction with Ehrlich test.

Cleistothecia sclerotioid, 200–300 μm in diameter, ripening slowly and mature after 3 months on MEA and Oatmeal agar. Ascospores ellipsoidal, $2.4-3.2\times1.7-2.4\mu$m, with two narrow, closely appressed equatorial ridges, valves smooth by light microscopy, warted with anastomosing ribs by SEM. Conidiophores arising from mycelium mat, symmetrically biverticillate, stipes smooth, width 2.5–3.5; metulae in whorls of 2–5, $13-17\times3.0-3.8\mu$m; phialides ampulliform, $8.5-10.5\times2.0-3.0\mu$m; conidia smooth walled, broadly ellipsoidal, $2.3-2.8\times1.9--2.4\mu$m.

Diagnostic features: Slow growth at 30°C and no growth at 37°C, abundant production of drab-grey cleistothecia, maturing after prolonged incubation, over 3 months.

Extrolites: Isochromantoxins, several apolar indol-alkaloids, and uncharacterized extrolites tentatively named "CITY", "HOLOX", "PR1-x" and "RAIMO".

Distribution and ecology: Soil in rainforest, Thailand.

Notes: *Penicillium tropicoides* morphologically resembles *P. tropicum*, but also has similarities with *P. saturniforme* and *P. shearii*. All these four species form lenticular ascospores with two closely appressed equatorial flanges and biverticillate conidiophores. The differences between *P. tropicoides* and *P. tropicum* are the slower maturation of the cleistothecia, slower growth rate at 30°C and the production of isochromantoxins by *P. tropicoides*. *Penicillium shearii* has a higher maximum growth temperature than *P. tropicoides*, and *P. saturniforme* has mostly smooth walled ascospores (Wang and Zhuang 2009; Stolk and Samson 1983).

Penicillium tropicoides and *P. tropicum* form ascospores, and in accordance with the "International Code of Botanical Nomenclature", the genus name *Eupenicillium* should be used. However, as shown in the phylograms (Figs. 1, 2, 3), these species are a homogeneous monophyletic group with other Penicillia. The assignment of the Penicillia to *Eupenicillium* (and *Carpenteles*) was rejected by Thom (1930) and Raper and Thom (1949). They adopted a classification with the emphasis on the Penicillium stage and treated all species, including the teleomorphic genera, as members of this genus. Using this approach and applying the concept of one name for one fungus (Reynolds and Taylor 1991), we have chosen to describe these two species under its anamorphic name.

Penicillium tropicum Houbraken, Frisvad and Samson, **comb. nov.**—MycoBank MB518294.

= *Eupenicillium tropicum* Tuthill and Frisvad, Mycological Progress 3(1): 14. 2004.

Type: SC42-1; other cultures ex-type: CBS 112584 = IBT 24580.

Description: Colony diameter, 7 days, in mm: CYA 24–30; CYA30°C 20–30; CYA37°C no growth; MEA 23–27; YES 33–37; CYAS 29–33; creatine agar 16–20, poor growth and weak acid production.

Colony appearance similar to *P. tropicoides*. Cleistothecia abundantly produced on CYA, orange-tan, becoming in warm shades of grey (brownish-grey) in age, conidia sparsely produced, blue grey green, exudate copious, large and hyaline, soluble pigments absent, reverse crème coloured. Weak sporulation on YES, cleistothecia abundantly produced deep dull grey in colour, soluble pigment absent. Colonies on MEA ascomatal, in shades of grey. No reaction with Ehrlich test.

Cleistothecia sclerotioid, 200–300 μm in diameter, ripening within 3–6 weeks on MEA and Oatmeal agar. Ascospores ellipsoidal, $2.5-3\times2-2.5\mu$m, with two narrow, closely appressed equatorial flanges and slightly roughened valves. Conidiophores arising from mycelium mat, symmetrically biverticillate, stipes smooth, width 2.5–3.3 μm; metulae in whorls of 2–5(−8), 12–16×2.5–3.5 μm; phialides ampulliform, $8.0-10.5\times2.0-3.0\mu$m; conidia smooth walled, broadly ellipsoidal, $2.3-3.0\times2.0-2.5\mu$m.

Diagnostic features: No growth at 37°C, abundant production of cleistothecia in warm shade of grey (brownish grey), maturing within 2–5 weeks.

Extrolites: Several apolar indol-alkaloids and the uncharacterized extrolites tentatively named "CITY", "EMON", "HOLOX" and "RAIMO" (Tuthill and Frisvad 2004).

Distribution and ecology: *Penicillium tropicum* has been isolated from (sub)tropical soils (e.g. India, Costa Rica, Ecuador and Galapagos Islands).

Notes: See *P. tropicoides*.

Discussion

Extrolite analyses showed that all species have a unique profile of metabolites (see Table 3). In general, the extrolite profiles, phenotypes and phylogeny were congruent. The only discrepancy is that *P. steckii* and *P. corylophiloides* have identical extrolite profiles, while these two species are

phylogenetically distinct. The most well known mycotoxin produced in this group of species is citrinin. This study shows that this extrolite is produced by *P. citrinum*, *P. gorlenkoanum* and *P. hetheringtonii* and not by *P. steckii*, even though citrinin production is claimed by Jabbar and Rahim (1962). Citrinin appears to be a commonly occurring extrolite in the *Citrina* series and it is also produced by, for example, the closely related species *P. chrzaszczii*, *P. westlingii*, and several other related (undescribed) species (Pollock 1947; Frisvad 1989; Frisvad et al. 2004; Houbraken et al. unpublished results). Following the assumption that biosynthetic gene clusters, once acquired, for example by horizontal gene transfer, are only maintained if natural selection favours their presence (Zhang et al. 2005), it can be speculated that this biosynthetic gene cluster has been acquired once and maintained during evolution in series *Citrina*. In this assumption, the fungus should benefit by the production of citrinin and the biological function of this extrolite should have an important purpose. Important functions of citrinin include inhibition of bacteria (Raistrick and Smith 1941; Oxford 1942; Kiser and Zellert 1945; Michaelis and Thatcher 1945; Kavanagh 1947; Taira and Yamatodani 1947), protozoa (Hamada et al. 1952), fungi (Haraguchi et al. 1987, 1989), human cell lines (causing apoptosis) (Huang et al. 2008), cholesterol synthesis (Endo and Kuroda 1976), aldose reductase (DeRuiter et al. 1992), and UV protection (Størmer et al. 1998). Furthermore, citrinin is a strong nephrotoxin, and it is immunosuppresive, teratogenic and mutagenic and causes hemolysis of human erythrocytes (Ambrose and Deeds 1945; Lurá et al. 2004). The relative small size (20 kb) of this biosynthetic cluster of citrinin (Sakai et al. 2008) might also be beneficial for maintaining it in the genome during evolution. Another scenario is that horizontal gene transfer of the citrinin biosynthetic gene cluster occurred several times during the evolution of the series *Citrina*. The evolution of these biosynthetic genes remains unknown and more research is needed.

Besides citrinin and a series of derivates or precursors of citrinin (Clark et al. 2006; Wakana et al. 2006; Lu et al. 2008; Zhu et al. 2009), several other metabolites are also claimed to be produced by *P. citrinum*, including compactins (Endo et al. 1976), agroclavine-1 and epoxyagroclavine-1 (Kozlovskiĭ et al. 2003a, 2005), asterric acid (Turner 1971; Turner and Aldridge 1983), cathestatins (Woo et al. 1995), citrinadin A (Tsuda et al. 2004; Mugishima et al. 2005), quinocitrinines and ergot alkaloids (Kozlovskiĭ et al. 2005), quinolactacins (Kakinuma et al. 2000; Takahashi et al. 2000; Kim et al. 2001), quinolactacide (Abe et al. 2005), tanzawaic acids (Kuramoto et al. 1997), scalusamides A-C (Tsuda et al. 2005), perinadine A (Sasaki et al. 2005), cyclocitrinols (Kozlovskiĭ et al. 2000a; Amagata et al. 2003), ergosta-4,6,8 (14),22-tetraen-3-one (Price and Worth 1974), 2,3,4-trimethyl-5,7-dihydroxybenzofuran (Chen et al. 2002) and gibberellins (Khan et al. 2008). Of these metabolites, we have confirmed the production of citrinin and some of its derivatives, quinolactacins (= quinocitrinins), and citrinadins. Compactins have been incorrectly linked to "*P. citrinum*" NRRL 8082 and re-examination of this isolate showed it was a *P. solitum* (Frisvad and Filtenborg 1983). Clavine ergot alkaloids and citrinin have been linked to *P. citrinum*, VKM F-1079 (Kozlovskiĭ et al. 2000b), but the strain that was used has been re-identified as *P. gorlenkoanum*. *Penicillium sizovae* was claimed to produce agroclavine-I and epoxyagroclavine-I and 1,1-bis(6,8-dimethyl-8,9-epoxy-5a,10e)-ergoline, a dimer of epoxyagroclavine-I (Kozlovskiĭ et al. 1986). The *P. citrinum* strain VKM FW-800 was isolated from 1.8 to 3 million years old Arctic permafrost sediments. This strain produces quinolactacin (= quinocitrinin) and the ergot alkaloids agroclavine-I and epoxyagroclavine-I, which indicates that this isolate is not *P. citrinum*, and if it is not a contaminant, then it maybe a ancestor of the group of fungi treated here.

Of the investigated group of species, *P. citrinum* is most commonly occurring. This species has a worldwide distribution and has been isolated from various sources, such as soil, indoor environments and foodstuffs. In our study we found that *P. citrinum* is commonly occurring in (sub)tropical soils, and only present in low numbers in soils from temperate regions (the Netherlands, Poland, Canada), where *P. westlingii* and related species predominate. This is also reflected in the maximum and optimal growth temperature: *P. citrinum* grows up to 37°C, while *P. westlingii* and related species have a maximum growth temperature of 30°C. Besides commonly occurring in soil, *P. citrinum* is also reported to be an endophyte of various plants. It was the most frequently isolated species in the stem and roots of coffee plants (Posada et al. 2007), roots of *Ixeris repenes* (Khan et al. 2008), and from leaves of qat (*Catha edulis*) (Mahmoud 2000). Endophytic fungi form mutualistic interactions with their host, the relationship therefore being beneficial for both partners (Tejesvi et al. 2007; Hyde and Soytong 2008; Giordano et al. 2009). The beneficial interaction for the plant could be the production of gibberellins, which enhances stem growth, and which are claimed to be produced by *P. citrinum* (Khan et al. 2008). But also other plant growth regulators, citrinolactones A and sclerotinin C, were isolated from *P. citrinum* (Kuramata et al. 2007) and it is reported that citrinin induces swarming motility of *Paenibacillus polymyxa*, a growth promoting rhizobacterium (Park et al. 2008). The production of these metabolites by *P. citrinum* in culture and/or in plants remains largely unknown and the role of this species may deserve further investigations.

Acknowledgements The authors are extremely grateful for the technical assistance of Martin Meijer and Ellen Kirstine Lyhne. Mr. Dae-Hoo Kim is thanked for the preparation of the SEM photos and prof. Uwe Braun is acknowledged for providing the Latin diagnoses.

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