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Penicillium persicinum, a new griseofulvin, chrysogine and roquefortine C producing species from Qinghai province, China

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Abstract

A unique *Penicillium* isolate from Chinese soil with terverticillate penicilli and ellipsoidal to cylindrical smooth-walled conidia, produces, in addition to the common metabolite ergosterol, copious amounts of an unknown peach-red pigment and the following secondary metabolites: griseofulvin, dechlorogriseofulvin, lichexanthone, roquefortine C, roquefortine D, chrysogine, 2-pyrovoylaminobenzamide, 2-acetyl-quinazolin-4(3H)-one. This isolate, CBS 111235, is described as *Penicillium persicinum* sp. nov., which belongs to subgenus *Penicillium* section *Chrysogena* but is morphologically similar to *P. italicum.* On the basis of the production of secondary metabolites it resembles *P. griseofulvum* and *P. coprophilum*. Sequence data using part of the β-tubulin gene showed that it is phylogenetically related to *P. chrysogenum* and *P. aethiopicum* in section *Chrysogena* with which it shares both secondary metabolites and ability to grow at 37 °C.

Introduction

The genus *Penicillium* contains approximately 225 species with 347 synonyms. Furthermore 45 species of the teleomorph *Eupenicillium* and 24 species of the genus *Talaromyces* have been accepted (Pitt et al. 2000). These species have a high diversity both regarding morphology and production of secondary metabolites. Most of the terverticillate *Penicillium* species are associated with foods, feeds or dung (Frisvad et al. 2000). In this paper we report here on a new terverticillate species from soil that produces several known bioactive secondary metabolites. Although this species is hitherto only represented by a single isolate, the combination of characters makes it unique and its description is therefore justified.

Materials and methods

CBS 111235 was obtained from a soil sample collected in Qinghai Province, China. The methods and media for isolation and identification followed Pitt (1979), Raper and Thom (1949) and Samson et al. (2002). The names of colours are based on Ridgway (1912). The cultures used for the molecular study were grown on Malt Peptone (MP) broth using 10% (v/v) of Malt Extract (Brix 10) and 0.1% (w/v) Bacto Peptone (Difco) in 2 ml of medium in 15 ml tubes. The cultures were incubated at 25 °C for 7 d in light/darkness.

Secondary metabolites were analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as described by Frisvad and Thrane (1987), with minor modifications by Smedsgaard (1997). Standards of griseofulvin, dechlorogriseoful-

vin, lichexanthone, roquefortine C, roquefortine D, chrysogine, 2-pyrovoylaminobenzamide, 2-acetylquinazolin-4(3H)-one and other secondary metabolites from the collection at Biocentrum-DTU (Kgs. Lyngby, Denmark) were used to compare with the secondary metabolites found in CBS 111235.

DNA extraction, sequencing and analysis

The total fungal genomic DNA was isolated using FastDNA® Kit (Bio 101, Carlsbad, USA) according to the manufacturer's instructions. Amplification of beta-tubulin gene was performed using the primers Bt2a and Bt2b (Glass 1995). PCR was performed in a 50 µl reaction mixture containing 1 µl of genomic DNA (10 ng/µl), 5 µl of PCR buffer, 30 µl of ultra pure sterile water, 10 µl dNTP (1 mM), 1 µl of each primer (50 pmol/µl) and 1 µl Taq polymerase (2.5 U/µl DNA) (SpaeroQ, Leiden, The Netherlands). Amplification was performed in a GeneAmp PCR system 9700 (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands); programmed for 5 cycles of 1 min. denaturation at 94 °C followed by primer annealing 1 min.30 s at 68 °C and primer extension 2 min. at 72 °C with a decrease of the annealing temperature 1 °C /cycle followed by 25 cycles of 1 min. denaturation at 94 °C followed by primer annealing 1 min.30 s at 64 °C and primer extension 2 min. at 72 °C and a final 10 min. elongation step at 72 °C. After amplification of the beta-tubulin gene, excess primers and dNTP's were removed from the reaction mixture using a commercial GFX column, PCR DNA Purification kit (Amersham Bioscience, Roosendaal, The Netherlands). The purified PCR fragments were resuspended in 50 µl of TE buffer. The PCR fragments were directly sequenced (White et al. 1990) in both directions with the primers Bt2a and Bt2b using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Roosendaal, The Netherlands). The sequence PCR reaction mixture, total reaction mix is 10 µl, contained 2 µl of template DNA (15-45 ng/µl), 4 µl Dye terminator RR mix, 3 µl ultra pure sterile water and 1 µl primer Bt2a or Bt2b (4 pmol/µl). The reaction was performed in a GeneAmp PCR system 9700 run in 9600 mode (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands); programmed for 25 cycles of 10 s denaturation at 96 °C followed by primer annealing 5 s at 50 °C and primer extension 4 min. at 60°C. Sequencing products were purified according to the manufacturer's recommendations with Sephadex G-50 superfine column (Amersham Bioscience, Roosendaal, The Netherlands) in a multiscreen HV plate (Millipore, Amsterdam, The Netherlands) and with MicroAmp Optical 96-well reaction plate (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). The samples were analyzed on an ABI Prism 3700 Genetic Analyzer (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). A concensus was computed from the forward and reverse sequences with software package Seqman and Editseq from the lasergene package (DNAStar Inc., Madison, WI). The alignments of the partial beta-tubulin gene sequence data were performed using the software package BioNumerics from Applied Maths and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, missing data were identified by '?', uninformative characters were excluded and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also calculated. Sequences of strains obtained in this study and shown in Figure 1 were deposited at GenBank (CBS 306.48 = AY 495981; CBS 111235 = AY 495982; CBS 484.84 = AY 495983; CBS 270.97 = AY 495984; CBS 287.97 = AY 495985; CBS 776.95 = AY 495986; CBS 775.95 = AY 495987; CBS 478.84 = AY 495988; CBS 170.87 = AY 495989; CBS 110413 = AY 495990; CBS 110412 = AY 495991; CBS 112578 = AY 495992; CBS 419.89 = AY 495993; CBS 110406 = AY 495994; CBS 110407 = AY 495995; CBS 412.69 = AY 495996; CBS 172.87 = AY 495997; CBS 112104 = AY 495998; CBS 352.48 = AY 495999; CBS 318.92 = AY 496000; CBS 653.82 = AY 496001).

Results and discussion

CBS 111235 is characterized by its terverticillate penicilli and cylindrical to ellipsoidal smooth-walled conidia, and the abundant typical peach-red soluble pigment on CYA and YES. The morphology of the conidiophores and conidia resemble those of *P. itali-*

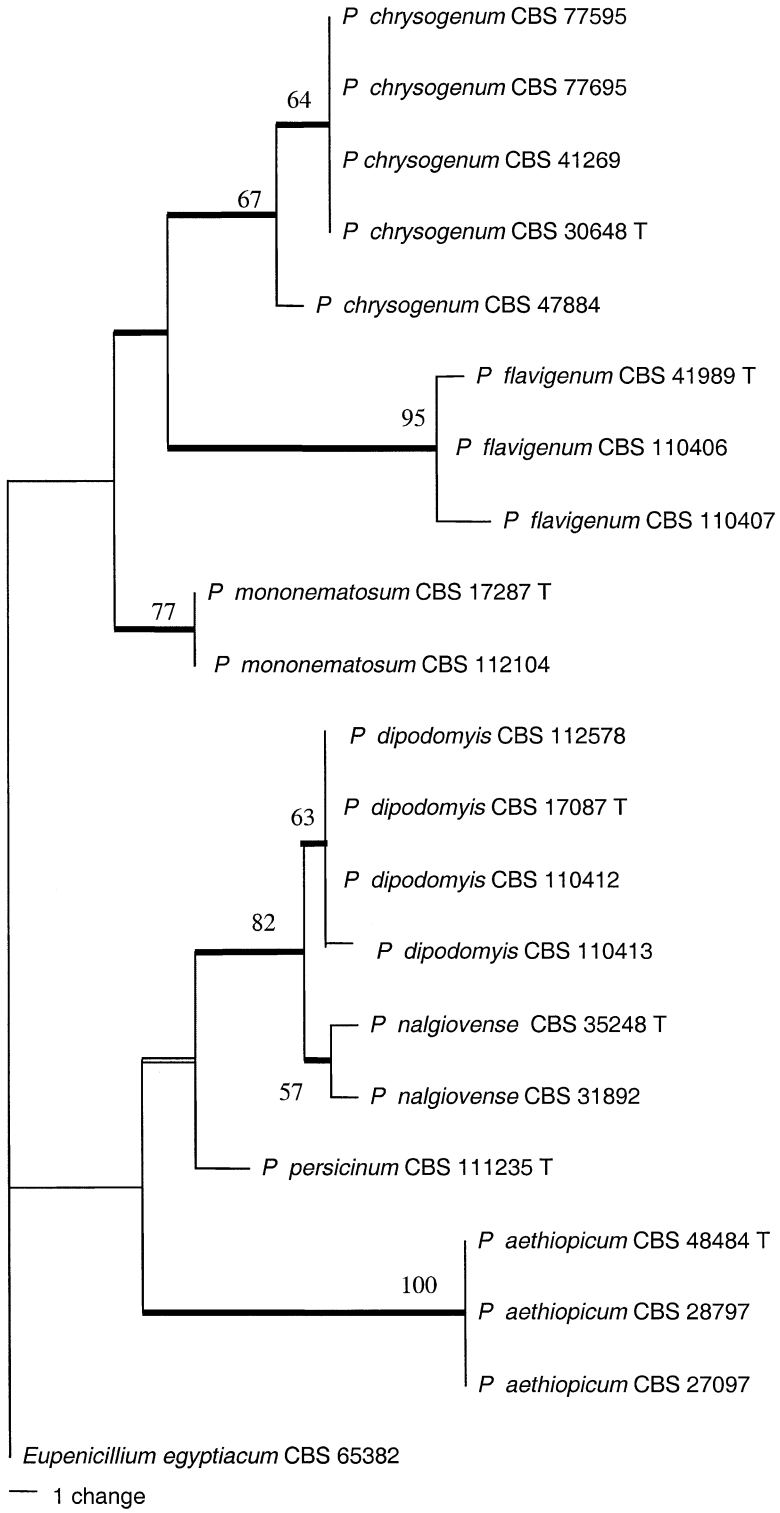

Figure 1. Phylogram inferred from the partial β-tubulin DNA sequence data, showing phylogenetic relationships of *Penicillium persicinum*, compared with relevant taxa of subgenus *Penicillium.* T = type culture; other strains are typical representatives of the taxon based on phenotypical and biochemical characters. One of the eight trees of 48 steps based on heuristic search partial β-tubulin sequences.The branches in bold are 100% and in the 70% majority-rule consensus of equally parsimonious trees. The numbers represent bootstrap percentages > 50% (CI = 0,771, RI = 0,905, RC = 0,698, HI = 0,229).

Table 1. Production of secondary metabolites by P. persicinum relative to ergosterol on CYA and YES agar*

Medium	metabolite	Amount of secondary metabolite relative to ergosterol**
CYA	Griseofulvin	12.88
	Roquefortine C	6.69
	Chrysogine	1.75
	2-acetyl-4(3H) quinazolone	4.37
YES	Griseofulvin	1.03
	Roquefortine C	3.71
	Chrysogine	2.75
	2-acetyl-4(3H) quinazolone	8.24

*Secondary metabolites produced in small amounts: Lichexanthone, roquefortine D, 2-pyrovoylaminobenzamide; ** Results are an average of two HPLC runs.

cum Wehmer and *P. ulaiense* Hsieh, Su and Tzean which are, however, both strongly fasciculate and adapted to citrus fruits. Moreover, CBS 111235 produces the combination of secondary metabolites listed above (Table 1) which has not been found in any other *Penicillium* species or any other fungi described so far.

The new species produced, in addition to the unknown red pigment, metabolites of the griseofulvin biosynthetic family: griseofulvin, dechlorogriseofulvin and lichexanthone; metabolites of the oxaline biosynthetic family: roquefortine C and D; and those of the chrysogine biosynthetic family: 2-pyrovoylaminobenzamide, 2-acetyl-quinazolin-4(3H)-one and chrysogine. The combined production of griseofulvin, dechlorogriseofulvin and lichexanthone with roquefortine C and D is also found in *P. griseofulvum* Dierckx (series *Urticicolae*) and *P. coprophilum* (Berk and M. A. Curtis) Seifert and Samson (series *Claviformia*)(Frisvad and Filtenborg 1989; Frisvad et al. 2000). CBS 111235 shares the smooth conidiophore stipe walls and the smooth ellipsoidal to smooth-walled conidia with *P. coprophilum* and *P. griseofulvum* but it differs from these species by the production of chrysogine, the striking red pigment and the cylindrical conidia that are more akin to those of *P. italicum* (series *Italica*) as emended by Frisvad et al. (2000). In addition *P. coprophilum,* has synnematous conidiophores with both rough- and smooth-walled stipes and ellipsoidal conidia and produces the metabolites meleagrin and oxaline. *P. griseofulvum* has quaterverticillate fasciculate conidiophores with short phialides.

The results of maximum parsimony analysis based on partial β-tubulin sequences of selected species belonging to subgenus *Penicillium* resulted in 8 equally most parsimonious trees (TL = 48 steps, CI = 0,771, RI = 0,905, RC = 0,698), one of which is shown in Figure 1. The tree was rooted to *Eupenicillium egyptiacum* CBS 65382. Bootstrap support from 1000 replicates is shown at the nodes. CBS 111235 appears phylogenetically most closely related to species in series *Chrysogena*, series *Mononematosa* and *P. aethiopicum,* all from section *Chrysogena* of subgenus *Penicillium.*

The red pigment produced by CBS 111235 has not been structurally elucidated, but is rather uncommon in *Penicillium*. Such a pigment has been seen in *Penicillium pulvillorum* (on MEA), *P. matriti*, *P. fennelliae, Eupenicillum baarnense* and *E. bovifimosum*, but these fungi are not closely related to *P. persicinum*. The conidia and conidiophores of CBS 111235 may resemble those of *P. fennelliae*, but *P. fennelliae* has rough-walled conidia and produces penicillic acid. The above distinctions and its unique secondary metabolite profile indicate that CBS 111235 should be regarded as a new species.

Penicillium persicinum L. Wang, H. Zhou, Frisvad et Samson, sp. nov.

In agaro Czapek Yeast (CYA) 25 °C post 7 die: coloniae 22-30 mm diametro, tenues, radialiter et quoque leniter concentrice sulcatae; leniter umbonatae in medio; velutinae; sporulatio sparsa, glauca; mycelium aerium album ad dilute persicinum vergens; exsudatum absens; pigmentum solubile copiosum, persicinum; reversum roseum.

Conidiophora ex hyphis aeriis, stipites glabri, (200-)300-500(-600) × 3.5-4.5 μm; penicilli praecipue terverticillati, interdum biverticillati; rami 2-3 per stipitem, 18-36 × 3.5-4.5 μm; metulae 2-5 in quoque ramo, 10-20 × 3.5-4.5(-5.4) μm, clavatae, sursum vesiculosae, ad 10 μm diam.; phialides 4-8 verticillatae, ampulliformes, 7-11 × (2.0-)2.5-3.5(-4.0) μm, collula distincta; conidia cylindrica vel ellipsoidea, 3.5-4.5 × (1.5-)2.0-3.0(-3.5) μm, levia, in catenis inordinatis adhaerentia.

Holotypus: HMAS 80638-1-4 (cultura viva AS 3.5891 = IBT 24565 = CBS 111235), isolatus ex solo, Provincia Qinghai Sinica, in Instituto Microbiologico Academiae Sinicae, Beijing, conservatur.

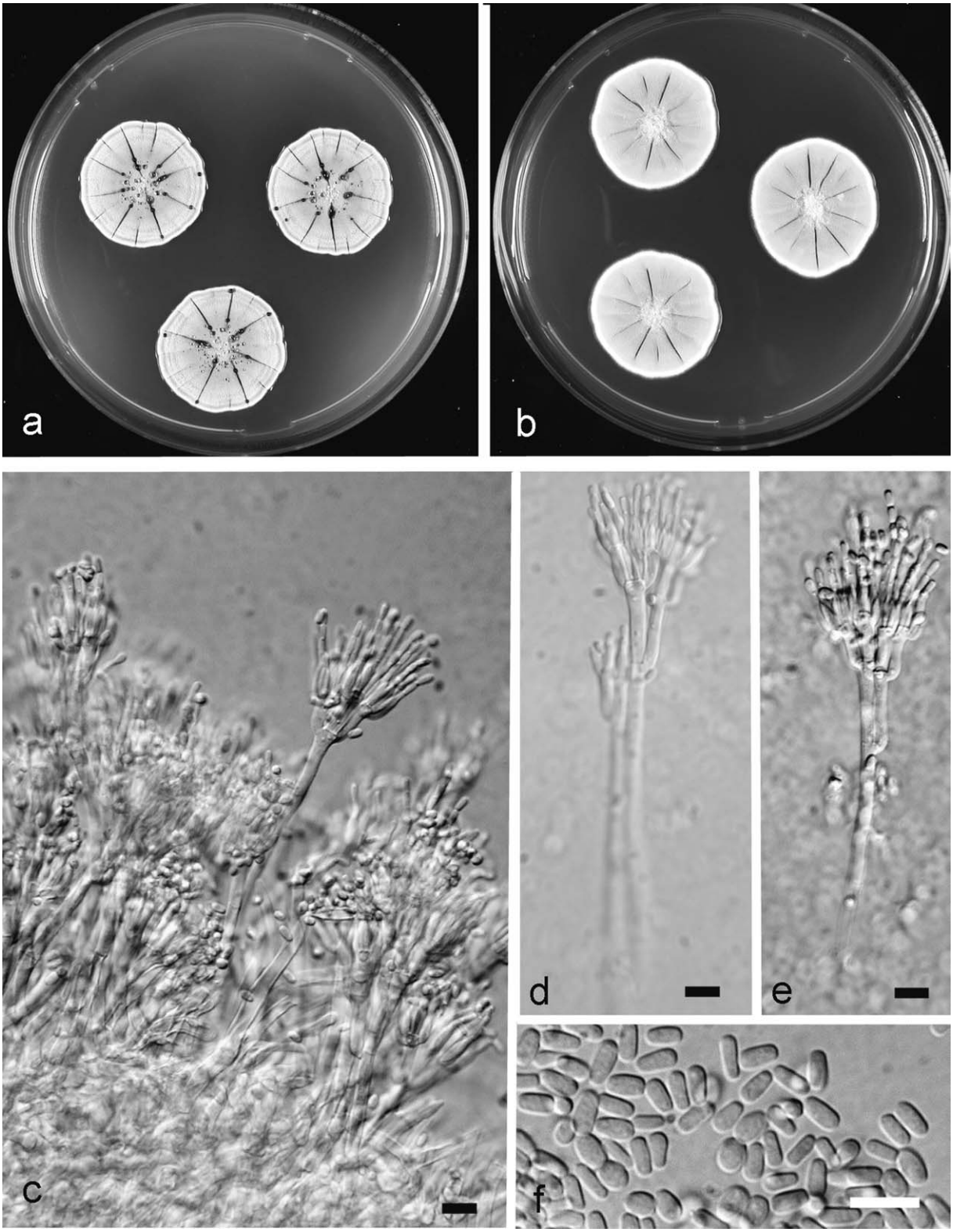

Figure 2. P. persicinum: a, b. colonies on CYA and MEA at 25 °C, 7days; c-f. penicilli and conidia on MEA (white and black bars = 10 μm).

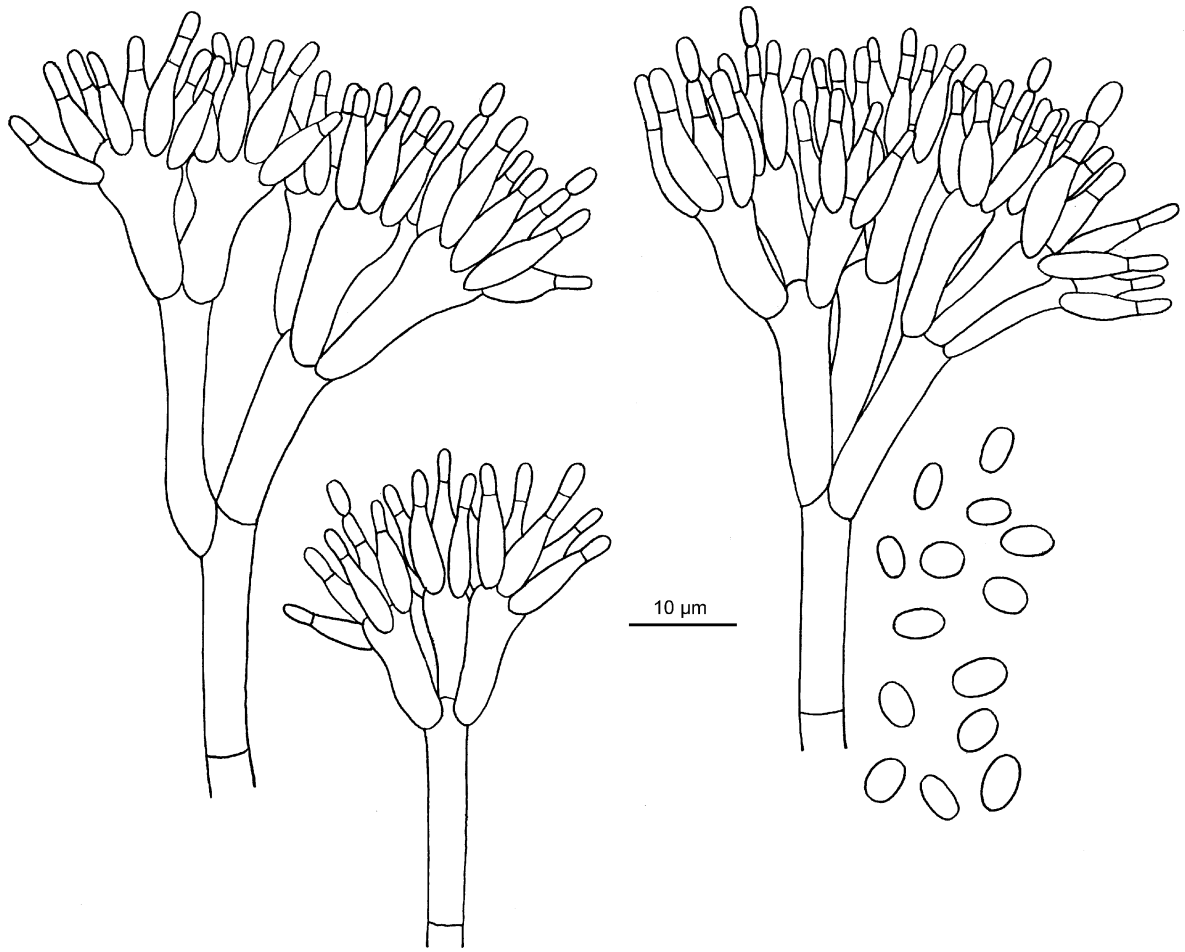

Figure 3. Camera lucida drawings of conidiophores and conidia of *P. persicinum* on Czapek Yeast agar.

Etymology: *persicinum* = peach-red, referring to the pigment produced on CYA and YES.

On Czapek agar (CA) at 25 °C in 7days: Colonies 27-29 mm in diam., low, plane, umbonate in centres; velutinous and slightly floccose; conidiogenesis light, mainly in central areas, near Sage Green (R. Pl. XLVII); mycelium white at margins, but Pinkish Buff (R. Pl. XXIX) to Shell Pink (R. Pl. XXVIII) near centres; no exudate and soluble pigment; reverse yellow white.

On CYA at 25 °C in 7days (Figure 2a): Colonies 22-30 mm in diam., slightly deep, with few light radial and annular zones, slightly umbonate in centres; velvety; conidiogenesis light to moderate, near Gnaphalium Green (R. Pl. XLVII); mycelium white with a tint of pinkish red, near Japan Rose (R. Pl. XXVIII) in central areas; no exudate or few; soluble pigment typically produced, abundant, near Peach Red (R. Pl. I) or Coral Red (R. Pl. XVIII); reverse near Old Rose (R. Pl. XVIII).

On MEA (2% Malt extract agar) at 25 °C in 7days (Figure 2b): Colonies 22-26 mm in diam., low, plane, centrally umbonate; velutinous; conidiogenesis abundant, near Gnaphalium Green (R. Pl. XLVII); no exudate and soluble pigment; reverse uncoloured to light creamish.

On YES (yeast extract-sucrose agar) at 25 °C in 7days: Colonies 24-33 mm in diam.; conidiogenesis weak; pinkish red soluble pigment abundant; reverse pinkish red.

On CREA (creatine-sucrose agar) at 25 °C in 7days: growth weak and no acid production.

On OA (oatmeal agar) at 25 °C in 7days: Colonies 25-30 mm in diam.; strong conidium production; reverse cream coloured.

On G25N at 25 °C in 7days: Colonies 22-25mm in diam., low, plane, slightly umbonate in centres; velutinous; conidiogenesis moderate, near Pea Green to Gnaphalium Green (R. Pl. XLVII); mycelium white at margins, Pale Pinkish Cinnamon (R. Pl. XXIX) in central areas; no exudate and soluble pigment; reverse uncoloured.

On CYA at 37 °C in 7days: Colonies 8-9 mm.

On CYA at 5 °C in 7days: Germination only.

Conidiophores (Figure 3) from surface hyphae, stipes (200-) 300-500 (-600) × 3.5-4.5 μm, smooth-walled; penicilli mainly terverticillate, occasionally biverticillate with 2-5 metulae; rami 2-3 per stipe, 18-36 × 3.5-4.5 μm; metulae 2-5 per ramus, 10-20 × 3.5-4.5 (-5.4) μm, clavate to apically vesiculate up to 10 μm; phialides 4-8 per metula, ampulliform with distinguishable collula, 7-11 × (2.0-)2.5-3.5(-4.0) μm; conidia cylindrical to ellipsoidal, 3.5-4.5 × (1.5–) 2.0-3.0(-3.5) μm, smooth-walled, born in long tangled chains. On CYA conidia are more ellipsoidal than cylindrical, whereas on MEA conidia are predominantly cylindrical.

According to the classification of Pitt (1979), *P. persicinum* could be placed in subgenus *Penicillium*, section *Cylindrosporum*, series *Italica*; but in accordance with Raper and Thom (1949) or Ramiréz (1982), it would belong to section *Asymmetrica-Velutina*, subsection *Velutina*.

P. persicinum is phylogenetically most closely related to species in series *Chrysogena*, series *Mononematosa* and *P. aethiopicum,* all from section *Chrysogena* of subgenus *Penicillium.* Like most species in section *Chrysogena, P. persicinum* can grow at 37 °C and it produces the same metabolites chrysogine and roquefortine C as *P. chrysogenum* and griseofulvin with *P. aethiopicum*. However as stated above the ellipsoidal to cylindrical conidia, and the production of roquefortine C and griseofulvin are shared with *P. griseofulvum* and *P. coprophilum*, so the features most indicative of the molecular phylogenetic placement are the ability to grow at 37 °C and the production of chrysogine.

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References

Frisvad J.C. and Filtenborg O. 1989. Terverticillate penicillia: chemotaxonomy and mycotoxin production. Mycologia 81: 837–861.

Frisvad J.C. and Thrane U. 1987. Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone indices and UV-VIS spectra (diode array detection). J. Chromatogr. 404: 195–214.

Frisvad J.C., Filtenborg O., Lund F. and Samson R.A. 2000. The homogeneous species and series in subgenus *Penicillium* are related to mammal nutrition and excretion.. In: Samson R.A. and Pitt J.I. (eds), Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Harwood Academic Publishers, Amsterdam, The Netherlands, pp. 265-283.

Glass N.L. and Donaldson G.C. 1995. Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes. Applied and Environmental Microbiology 61(4): p. 1323–1330.

Hillis D.M. and Bull J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182–192.

Pitt J.I. 1979. The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Academic Press, London, UK.

Pitt J.I., Samson R.A. and Frisvad J.C. 2000. List of accepted species and their synonyms in the family Trichocomaceae.. In: Samson R.A. and Pitt J.I. (eds), Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Harwood Academic Publishers, Amsterdam, The Netherlands, pp. 9-49.

Ramiréz C. 1982. Manual and Atlas of the Penicillia. Elsevier Biomedical Press, Amsterdam, The Netherlands.

Raper K.B. and Thom C. 1949. A Manual of the Penicillia. Williams and Wilkins, Baltimore, USA.

Ridgway R. 1912. Color Standards and Color Nomenclature. Published by the author, Washington, D.C., USA.

Samson R.A., Hoekstra E.H., Frisvad J.C. and Filtenborg O. 2002. Introduction to Food- and Airborne Fungi. 6th ed. second print. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Smedsgaard J. 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J. Chromatogr. A 760: 264–270.

Swofford D.L. 2000. PAUP* 4.0: phylogenetic analysis using parsimony. Sinauer Associates. Sunderland, Massachusetts, USA.

White T.J., Bruns T., Lee S. and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.. In: Innis M.S. and Gelfand D.H. (eds), PCR protocols: a guide to methods and applications. (pp. 315-322) Academic Press, New York, New York, USA.