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ORIGINAL ARTICLE

Taxonomic revision of the biotechnologically important species *Penicillium oxalicum* with the description of two new species from acidic and saline soils

Alena Kubátová¹ · Martina Hujšlová² · Jens C. Frisvad³ · Milada Chudíčková⁴ · Miroslav Kolařík⁴Received: 20 February 2018 / Revised: 26 June 2018 / Accepted: 28 June 2018
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Abstract

Penicillium oxalicum is a frequently isolated fungus exhibiting a wide range of physiological activities that are of relevance in agriculture, biotechnology, food quality assessments, and medicine. Although widely studied, this fungus is usually identified on the basis of morphological characters but its taxonomy has never been systematically revised. In this study, we revised a set of *Penicillium* isolates from various sources including *P. oxalicum*-like strains obtained from acidic and saline soils in the Soos Nature Reserve (Czech Republic). Two phylogenetic sister species of *Penicillium oxalicum* are illustrated and described as *Penicillium diatomitis* sp. nov. (CCF 3904^T) and *Penicillium soosianum* sp. nov. (CCF 3778^T). Both species can be distinguished from *P. oxalicum* based on molecular data, morphological characters, and extrolite profiles. *Penicillium diatomitis* mimics *P. oxalicum* by its macro- and micromorphology but acid production on CREA distinguishes the two.

Keywords Soil fungi · Extreme habitats · Biotechnology · *Penicillium* subgenus *Aspergilloides* Dierckx · Section *Lanata-Dirvaricata* Thom

Introduction

The genus *Penicillium* represents an enormous range of medically, agriculturally, and industrially important and widely

distributed species that have a broad substrate spectrum and can act as saprotrophs, endophytes, or parasites. The genus exhibits a wide range of physiological activities that are important for the functioning of soil ecosystems and have actual and potential uses in agriculture, biotechnology, and medicine. *Penicillium oxalicum* is a well-known species described from moldy corn and soil in the USA and named after its production of oxalic acid (Currie and Thom 1915). It is especially common in soil and colonizes various organic materials (e.g., corn) mainly in tropical regions (Raper and Thom 1949; Pitt 1980; Domsch et al. 2007; Pitt and Hocking 2009). It has been isolated from extremely acid, alkaline or saline soils (Butinar et al. 2011), soils contaminated by pollutants (Zhou et al. 2016), and marine macroalgae (Park et al. 2016). Furthermore, *P. oxalicum* can occur in indoor air (Vesper et al. 2005), on dry sausages (Iacumin et al. 2009), as an endophyte of *Coffea arabica* (Vega et al. 2006) or as a pathogen of maize and cucumber (O'Neill et al. 1991; Tenuta 2006). Recently, it has also been recognized as a causal agent of invasive human mycoses (Chowdhary et al. 2014).

Penicillium oxalicum is a producer of natural products, including organic acids (Li et al. 2016), various enzymes (Kurakake et al. 2010; Cheng et al. 2016; Song et al. 2016;

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✉ Miroslav Kolařík
mkolarik@biomed.cas.cz

¹ Department of Botany, Faculty of Science, Charles University, Benátská 2, 128 01 Praha 2, Czech Republic

² Laboratory of Fungal Biology, Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Praha 4, Czech Republic

³ Department of Biotechnology and Biomedicine, Technical University of Denmark, Soltofts Plads, Building 221, 2800 Lyngby, Denmark

⁴ Laboratory of Fungal Genetics and Metabolism, Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Praha 4, Czech Republic

Xu et al. 2016; etc.), mycotoxins (e.g., secalonic acid D, Pitt 2002; Yassin et al. 2010), cytotoxic alkaloids (Yang et al. 2016), and even cytotoxic compounds with antitumor effects (Wang et al. 2016; Chen et al. 2015). It utilizes phosphate and contributes therefore to soil fertilization (Singh and Reddy 2011) and is known for its strong antibacterial (Santamarina et al. 2002), antifungal (Yang et al. 2008), and insecticidal effects (Fang and Tan 1986; Santamarina et al. 2002). Thus, it could be used as a biological control agent against agriculturally relevant pathogens or pests (De Cal et al. 1997, 2008, 2009; Sabuquillo et al. 2006, Sabuquillo and Szejnberg 2009; Sempere and Santamarina 2008, 2010). Several strains are patented as biofertilizers, commercial enzyme producers, or degraders of waste plant biomass and pollutants.

Penicillium oxalicum was morphologically characterized in several studies (Raper and Thom 1949; Pitt 1980; Ramirez 1982; Kozakiewicz 1992). Its subgeneric classification within the genus was revealed using a four-gene phylogeny by Houbraken and Samson (2011), who placed the species in subgenus *Aspergilloides*, section *Lanata-Divaricata*. However, a broader inventory of *P. oxalicum* and similar isolates using a modern polyphasic approach is lacking, which stands in great contrast to the large number of physiological and biochemical studies and its biotechnological applications.

In the present study, we used *P. oxalicum* isolates from the Culture Collection of Fungi – CCF (Prague, Czech Republic), including ex-type cultures, and strains isolated from acidic and saline soils by Hujšlová et al. (2010). Constant differences in morphology, extrolite (extracellular secondary metabolites) spectra, and DNA sequences suggest that the pool of isolates comprise three species, of which two are newly described.

Materials and methods

Cultures and morphological observation

Altogether, 20 strains were used to study morphological and molecular characters (Table 1) including the ex-type strain of *Penicillium oxalicum*, CCF 2315 (= NRRL 787), CCF 2062 (ex-type of *P. asturianum*, obtained by O. Fassatiová from C. Ramirez), and 12 new isolates from acidic (pH 1.7–2.7) and saline soils from the Soos Nature Reserve in the Czech Republic (Hujšlová et al. 2010). The strains were cultivated for 7 days on Czapek yeast autolysate agar (CYA), malt extract agar (MEA, 2%) (both, Oxoid™), yeast extract sucrose agar (YES), and creatine sucrose agar (CREA) (both, Frisvad and Samson 2004) at 25 °C, and on CYA at 37 °C. Potato-carrot agar (PCA) was used for long-term cultivation (Fassatiová 1986). Micromorphology was observed on MEA after 7 days; microphotography and measurements were made using QuickPHOTO MICRO 3.0 software (Promicra). Conidia dimensions are presented as minimum, average, and

maximum from 50 conidia. Colony colors are given according to the NBS/ISCC Color System (2017) (<http://tx4.us/nbs-iscc.htm>). Fungal structures were mounted both in lactic acid with cotton blue and in Melzer's reagent (Leonard 2006) and observed under an Olympus BX51 optical microscope. Scanning electron microscopy (SEM) was performed on a 6380 LV SEM (JEOL) machine. Pieces of sporulating colonies growing on MEA (5 × 5 mm) were fixed in glutaraldehyde, dehydrated through descending series of ethanol, critical-point dried in a Bal-Tec CPD 030 drier, and gold coated using a Bal-Tec SCD 050 sputter coater. The specimens were observed using a spot size of 43 μm and accelerating voltage of 20 kV. The ex-type strains of new species were deposited in the CBS Collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). The ex-type strains and other representative isolates of newly described species were also deposited in IBT Culture Collection of Fungi (Lyngby, Technical University of Denmark) and in the CCF Culture Collection of Fungi (Prague, Charles University, Czech Republic). Dried herbarium specimens of the holotypes were deposited in the herbarium of the Mycological Department, National Museum in Prague, Czech Republic (PRM).

Extrolite analysis

Biochemical study was conducted on 16 representatives (Table 2), including *P. oxalicum* strain IBT 28206 (isolated from leaves, Columbia), IBT 3131 (aloe, South Africa), IBT 23552 (moldy leather), IBT 13309 (soil, USA), and IBT 32474 (wall in food factory, China). IBT 32474 was assigned to a newly described *P. soosianum* based on morphology and secondary metabolite profile. Isolates of *P. oxalicum* and the two new species were three-point inoculated on to CYA and YES agar and incubated for 1 week at 25 °C in darkness. Three plugs of CYA agar or YES agar were analyzed by HPLC-DAD (high-performance liquid chromatography with diode array detection) as described by Kildgaard et al. (2014) and based on Frisvad and Thrane (1987) and Nielsen et al. (2011). The extrolites were identified or partially characterized by their retention times and UV spectra measured in the range of 190–600 nm.

Molecular studies

Genomic DNA was isolated from 2 to 4 days old mycelium grown on MEA as described by Kolařík et al. (2017). A ribosomal DNA (rDNA) fragment containing the internal transcribed spacers (ITS1 and ITS2), the 5.8S subunit, and the D1/D2 region of the 28S subunit was amplified with the universal primers ITS1 and NL4 (White et al. 1990; O'Donnell 1993). A partial sequence of the beta-tubulin gene (*TUB2*, *benA*) was amplified with the primer set Bt2a/Bt2b (Glass and Donaldson 1995). The gene encoding the partial RNA

Table 1 *Penicillium* isolates used for phylogenetic analyses. Strains from the CCF and MH collection were used also for the morphological comparisons

Species	Strain number ¹	Origin	GenBank accession number ²				Reference
			ITS-LSU	TU/B2	RPB2	CAM	
<i>P. ditomitis</i>	CCF 3904 ^T = CBS 140107 ^T = IBT 30728 ^T CCF 3779 = IBT 30754 CCF 3780 = IBT 30747 CCF 3906 = IBT 30753 CCF 3907 = IBT 30746 CCF 4379 MH248 MH285 CBS 188.72 ^T CBS 340.48 ^T	Saline acidic soil, Soos NR near Františkovy Lázně, Czech R., M. Hujšlová: pH 1.7, 2003, MH53 pH 1.7, 2003, MH69 pH 2.1, 2005, MH490 pH 2.3, 2004, MH276 pH 2.3, 2004, MH272 pH 2.5, 2004, MH110 pH 2.3, 2004 pH 2.3, 2004 Arable soil, Alaska, USA Soil, Nicaragua	FJ430748	HE651133	LT797560	LT970912	Hujšlová et al. (2010), this study
			HE651147	HE651137	LT797558	–	This study
			HE651148	HE651138	LT797559	–	This study
			FJ430747	HE651136	LT797557	–	Hujšlová et al. (2010), this study
			FJ430749	FM865813	LT797555	–	Hujšlová et al. (2010), this study
			HE651151	HE651134	LT797556	–	This study
			HE651150	HE651135	–	–	This study
			HE651149	HE651139	–	–	This study
			JX012225	KM088702	KM089474	–	Houbraken et al. (2014)
			GU981585	GU981625	JN121497	–	Houbraken et al. (2011), Houbraken and Samson (2011)
<i>P. donkii</i>	CCF 2315 ^T = IJFM 19200 ^T = IBT 6566 ^T = CBS 219.30 ^T = NRRL787 ^T	T of <i>P. oxalicum</i> , soil, Connecticut, USA, 1914, C. Thom	HE651152	KF296462	JN121456	–	Visagie et al. (2015), this study
<i>P. janthinellum</i>	CCF 2062 ^T = IJFM 3871 ^T = CBS 173.81 ^T	T of <i>P. asturianum</i> ; air, Madrid, Spain, 1977, C. Ramirez	HE651146	LT934389	KF296416	–	Visagie et al. (2015), this study
	CCF 1659	Feed, Czech R., 1979, J. Blaha	LT797552	–	LT900000	–	This study
	CCF 1677	Imported feed, Czech R., 1979, J. Blaha	HE651145	–	LT900001	–	This study
	CCF 1959	Feed mixture for chickens, Czech R., 1984, V. Neumannová	HE651144	–	–	–	This study
	CCF 3009	Corn kernel, Illinois, USA, 1991, M. Christensen (RMF7976)	HE651142	HE651140	–	–	This study
	CCF 3438	Corn kernel after harvest, Prague-Ruzyně, Czech R., 2003, L. Slezáková	HE651143	HE651141	LT900002	–	This study
	CCF 4234	Indoor air, flat, Jihlava, Czech R., 2011, L. Hrubá	LT797553	–	–	–	This study
	PO13	Stored tobacco, Spain	EF103463	–	–	–	Larena and Melgarejo (2009)
	NRRL 790 = CBS 358.48 = IBT 21539	Danish blue cheese	AF034455	–	–	–	Peterson (2000)
	DAOM 213171	Cucumber canker, Canada	EF103455	–	–	–	Larena and Melgarejo (2009)
<i>P. rolsii</i>	NRRL 35183 = IBT 28206 CV 822 = DTO 182-B1	Leaves, Caldas, Chinchiná, Columbia Air, Malmesbury, South Africa	DQ123663	–	–	–	Vega et al. (2006)
	5648	Marine environment, Korea	JX091528	JX091431	KF296448	–	Visagie et al. (2016)
	CBS 368.48 ^T	Pineapple, Florida, USA	KJ527449	KJ527414	KJ527379	–	Park et al. (2014)
			NR_111669	GU981667	KF296455	–	Houbraken et al. (2011), Houbraken and Samson (2011)
<i>P. simplicissimum</i>	CBS 372.48 ^T	Flannel bag, South Africa	GU981588	GU981632	JN121507	–	Houbraken et al. (2011), Houbraken and Samson (2011)
<i>P. soosanum</i>							

Table 1 (continued)

Species	Strain number ¹	Origin	GenBank accession number ²				Reference
			ITS-LSU	TUB2	RPB2	CAM	
	CCF 3778 ^T = CBS 140106 ^T = IBT 30727 ^T	Saline acidic soil, Soos NR near Františkovy Lázně, Czech R., M. Hujšlová: pH 2.7, 2004, MH344	FJ430745	FM865811	LT9797561	LT970913	Hujšlová et al. (2010), this study
	CCF 3776 = IBT 30751	pH 1.7, 2003, MH73	LT979754	HE651132	LT9797563	-	This study
	CCF 3777 = IBT 30725	pH 2, 2005, MH583	-	-	-	-	This study
	CCF 3905 = IBT 30729	pH 1.9, 2005, MH792	FJ430746	FM865812	LT9797562	-	Hujšlová et al. (2010), this study

Sequences printed in bold were obtained during this study. Ex-type strains are marked by T

¹ CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CCF Culture Collection of Fungi, Charles University, Prague, Czech Republic; IBT IBT Culture Collection of Fungi, Technical University of Denmark, Lyngby, Denmark; LIFM Instituto 'Jaime Ferrán' de Microbiología Consejo Superior de Investigaciones Científicas, Madrid, Spain; MH Personal research collection of M. Hujšlová, Prague, Czech Republic; RMF Gungal collection (Rocky Mountain Fungi) of M. Christensen, Wyoming, USA

² CAM, calmodulin; ITS, internal transcribed spacer; TUB2, beta tubulin; RPB2, RNA polymerase second largest subunit

polymerase second largest subunit (*RPB2*) was amplified with the primer pair fRPB2-5F/fRPB2-7cR (Liu et al. 1999) and the partial calmodulin (*CAM*) gene was amplified with primer pair CF1M/CF4 (Peterson et al. 2008). PCR protocols, PCR product purification, and the both-strand sequencing procedure followed Sklenář et al. (2017) for *CAM* and Kolařík et al. (2017) for the other loci. Thirty-nine new sequences were deposited in the EMBL database under accession numbers HE651132–HE651152, LT9797552–LT9797563, LT900000–LT900002, LT934389, LT970912, and LT970913 (Table 1). Sequences were compared with data from the NCBI GenBank database using a BlastN similarity search including those published by Hujšlová et al. (2010). The species recognized as the phylogenetic sister of *P. oxalicum* (Visagie et al. 2015), and having the best BlastN similarity scores to *P. oxalicum*, were used for comparison (Table 1). *CAM* sequences were generated from the type strains only and were not included for phylogenetic analysis. The matrix containing separate ITS+LSU, *TUB2*, and *RPB2* sequence datasets were aligned in MAFFT 6 using the G-INS-i strategy (Katoh et al. 2009). Gblocks version 0.91b (Talavera and Castresana 2007) with less stringent selection allowing smaller final blocks and gap positions within the final blocks was used to remove gap and variable regions. Topologies of maximum likelihood (ML) phylogenetic trees generated from single-gene datasets were checked for incongruity and concatenated. The obtained alignment contained 29 sequences and 2412 characters (from the original 2830 positions), of which 519 were variable and 397 parsimony-informative. Bayesian phylogenetic (MB) analyses were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). A metropolis-coupled Markov chain Monte Carlo search algorithm with 2,000,000 generations was used. Trees were sampled every 1000 generations. Chain convergence was determined with Tracer 1.4 (<http://tree.bio.ed.ac.uk/software/tracer>), and the first 20% of trees were discarded as burn-in. Evolutionary models (K2 + G model for ITS and LSU, TN92+G for *TUB2*, K2+G+I for *RPB2* and T93 for the concatenated dataset) were determined for all datasets using MEGA 6.06 (Tamura et al. 2013). In MB analysis of the multigene dataset, four data partitions including ITS, LSU, *TUB2*, and *RPB2* were recognized. ML analyses were performed in PHYML (Guindon et al. 2010) using default settings and 500 bootstrap replicates with the T93 substitutions model. Maximum parsimony (MP) analyses were carried out using the dnaphars algorithm in PHYLIP (gaps treated as unknown states, 100 bootstrap replicates) (Felsenstein 1989) implemented in SeaView 4.0 (Galtier et al. 1996). The tree was rooted with *P. donkii* from the section *Stolkia*, which was used as an outgroup to the *Lanata-divaricata* section by Houbraken and Samson (2011). All alignments have been deposited in TreeBase (study accession number 22790, www.treebase.org/treebase/).

Table 2 *Penicillium diatomitis*, *P. oxalicum*, and *P. soosanum* extrolites detected after incubation at 25 °C in darkness for 1 week on CYA and YES

Species	Isolate number ¹	Extrolites produced								
		Asperflavin	Oxalicine	Oxaline	Secalonic acid	Terrecoumarin	'alko' ²	'fnot' ³	'oxyl' ⁴	'para' ⁵
<i>Penicillium oxalicum</i>	CBS 219.30 ^T = IBT 6566 ^T			+	D	+				
	CBS 460.67 = IBT 3131	+		+	D				2	
	ATCC 12556 = IBT 13309	+	A	+	D				2	
	ATCC 24784 = NRRL 2139 = IBT 23552			+	D				2	
	NRRL 35183 = IBT 28206	+	A, B	+	D, F	+			2	
<i>P. diatomitis</i>	CCF 3904 ^T = IBT 30728 ^T = CBS 140107 ^T								2	0, 1, 2, 3, 4
	CCF 3779 = IBT 30754						+	+	2	0, 1, 2, 3, 4, 5
	CCF 3780 = IBT 30747								1, 2	1, 2, 3, 5
	CCF 3906 = IBT 30753						+	+	2	1, 2, 3, 4
	CCF 3907 = IBT 30746							+	2	0, 1, 2, 3
<i>P. soosanum</i>	CCF 3778 ^T = IBT 30727 ^T = CBS 140106 ^T							+	1, 2	0, 1, 2
	CCF 3776 = IBT 30751								2	1, 2, 3, 4, 5
	CCF 3905 = IBT 30729								1, 2, 3	0, 1, 2
	CCF 3777 = IBT 30725								2	0, 1, 2, 3, 5
	IBT 32474								2	0, 1, 2, 3, 4

Ex-type strains are marked by T

¹ ATCC American Type Culture Collection, Virginia, USA; CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CCF Culture Collection of Fungi, Charles University, Prague, Czech Republic; IBT Culture Collection of Fungi, Technical University of Denmark, Lyngby, Denmark; NRRL Northern Regional Research Laboratory, Peoria, USA

² Retention time (RT) 4.46 min, UV maxima 204, 252 nm

³ RT 0.86 min, UV maxima 198, 240, 290 nm

⁴ OxyI1 - RT 0.81 min, UV maxima 190, 215, 283, 320sh nm; OxyI2 - RT 1.13, UV maxima 195, 214, 274; OxyI3 - RT 1.39, UV maxima 194, 213, 275 nm

⁵ Para0 - RT 1.32 min, UV maxima 205, 228, 275; Para1 - RT 1.78 min, UV maxima 205, 228, 275 nm; Para2 - RT 4.78 min, UV maxima 207, 228, 281 nm; Para3 - RT 3.63 min, UV maxima 201, 227, 275 nm; Para4 - RT 3.81 min, UV maxima 201, 227, 275 nm; Para5 - RT 4.31 min, UV maxima 194, 202sh, 226, 275 nm

202 The degree of cryptic diversity among published *P.*
 203 *oxalicum* sequences was assessed by analysis of *TUB2* se-
 204 quences deposited in the NCBI GenBank database.
 205 Sequences were obtained by blasting (BlastN tool in
 206 NCBI GenBank) the database using *TUB2* sequence
 207 KF296462 of *P. oxalicum* CBS 219.30^T and selecting en-
 208 tries that had a similarity of $\geq 82\%$. The final dataset of 67
 209 sequences was analyzed using the maximum likelihood
 210 method in PHYML 4.6.2 (Fig. S1).

211 Results

212 Phylogenetic analyses

213 Our phylogenetic analysis of the concatenated dataset re-
 214 vealed two fully supported main clades (Fig. 1). The first clade
 215 was formed by isolates morphologically assigned to *P.*
 216 *oxalicum*, including the ex-type strains of *P. oxalicum* and *P.*
 217 *asturianum*. The second clade is further divided into two well-

supported lineages, whose separation is supported by morpho-
 logical characters and which are described here as the new
 species *P. diatomitis* and *P. soosanum*. The two main clades
 formed a monophyletic group representing the *P. oxalicum*
 species complex and this group clustered separate from any
 of the other related species. The newly described species dif-
 fered from each other consistently in two substitutions located
 in the ITS2 spacer, but have identical LSU sequences. All *P.*
oxalicum isolates showed 100% sequence similarity and ex-
 hibited a difference of 1.7% in the ITS region (9 of 521 bp)
 and 1.1% in the LSU region (7/642 bp) when compared with
P. soosanum CCF 3778. The difference from *P. diatomitis*
 CCF 3904 was 1.3% (7/521 bp) for the ITS region and 0.9%
 (6/ 642 bp) for LSU region. With respect to *RPB2* (1082 bp,
 single exon, 359 amino acids), *P. soosanum* was represented
 by one and *P. diatomitis* by two haplotypes, and the two spe-
 cies differed in 1.7% of nucleotide positions and had identical
 protein sequences. The ex-type strain of *P. oxalicum* and *P.*
soosanum, respective *P. diatomitis*, differed in 12.8%, respec-
 tive 16.3% of positions, corresponding to four amino acid

Q5

Fig. 1 Bayesian phylogenetic tree
 based on concatenated ITS, LSU,
TUB2, and *RPB2* sequences
 showing the relatedness of
Penicillium oxalicum and related
 taxa. Bayesian PP followed by
 bootstrap support values (BS)
 from maximum likelihood and
 maximum parsimony analyses are
 shown near nodes. Branches with
 PP = 1.00 and BS = 100 are
 shown in bold. *Penicillium donkii*
 was used as outgroup

positions. *TUB2* sequences (520 bp, including four exons corresponding to 83 amino acid residues) of *P. diatomitis* differed from *P. soosanum* in 1.3% of nucleotide positions, and in a single amino acid position. The ex-type strains of *P. oxalicum* and *P. soosanum*, respective *P. diatomitis*, differed in 20.2%, respective 24.2% of nucleotide positions and a single amino acid position. The *CAM* gene (760 bp, including five exons corresponding to 126 amino acid residues) of the ex-type strain of *P. diatomitis* differed from *P. soosanum* in 4.5% of nucleotide positions and showed identical amino acid sequences. The ex-type strain of *P. oxalicum* differed from *P. soosanum*, respective *P. diatomitis* in 34.78%, respective 31.5% of positions but had identical amino acid sequences. The ex-type strain of *Penicillium asturianum* is identical in ITS and *RPB2* (e.g., KF296448 and KJ527379) and nearly identical (e.g., KP329982, 395/397 pb) in *TUB2* with *P. oxalicum* strains.

Analysis of the most variable region, *TUB2*, downloaded from the NCBI GenBank database, resulted in the same topology as our multigene analysis (Fig. 1) with the presence of a main clade containing sequences identified in our study as *P. oxalicum* (Fig. S1). *Penicillium diatomitis* and *P. soosanum* formed sister clades of the *P. oxalicum* clade.

Extrolite analysis

Detected compounds are listed in Table 2. The two new species, *P. diatomitis* and *P. soosanum*, produced several extrolites, whose structure is yet to be elucidated. The two new species could not be separated chemotaxonically. They share the “oxyl” type of chromophore compounds with *P. oxalicum*. The two new species did not produce the other compounds known from *P. oxalicum*, including secalonic acids, oxaline, oxalazines, asperflavin, and terrecoumarins.

Taxonomy

A full description of *P. oxalicum* sensu stricto is provided for comparison with newly described species. *Penicillium oxalicum* is characterized by heavy sporulation, dark green colonies with a shiny or silky appearance, and large ellipsoidal conidia. All these characters are shared with *P. diatomitis*, but the latter does not produce acid on CREA, distinguishing it from *P. oxalicum*. *Penicillium soosanum* forms more globose conidia (L/W ratio 1.1–1.2) and does not form acid on CREA. In all species, fresh isolates sporulated strongly, while repeated subculturing resulted in colonies with nonsporulating white sectors.

Penicillium oxalicum Currie & Thom, Journal of Biological Chemistry 22: 289 (1915)
MycoBank: MB121033
Fig. 2

= *Penicillium asturianum* C. Ramírez & A.T. Martínez, Mycopathologia 74 (1): 42 (1981)
MycoBank: MB112518
Fig. S2
Type: USA, Connecticut, isolated from soil, 2014, C. Thom, IMI 192332 [neotype (Pitt 1980), dried culture]; ex-neotype cultures: CBS 219.30 (= ATCC 1126, FRR 787, IBT 6566, IMI 192332, MUCL 29047, NRRL 787, QM 7606, CCF 2315).
ITS barcode: HE651152, AF033438. Alternative identification markers, *TUB2* (KF296462), *CAM* (KF296367), *RPB2* (JN121456).
Asexual state: *Conidiophores* terminally branched, typically bi-verticillate with 2–3 adpressed metulae or monoverticillate; terverticillate conidiophores rarely occurring in some strains; *stipes* smooth, most commonly 75–400(– 550) × 2.5–3.0 µm; *metulae* of unequal length, 11–25 × 2.5–4.5 µm; *phialides* cylindrical with distinct collula of various sizes, 2–6 per metula, 8.5–15(– 19) × 2.0–4.0 µm; *conidia* finely roughened, ellipsoidal, (3.5–)3.9–5.3(– 6.5) × (2.5–)2.7–3.5(– 4) µm, L/W ratio 1.26–1.96 Sexual state not observed.
Culture characters: Colony diam, 7 d (mm): CYA, (36–)40–55(– 62) CYA, 37 °C 10–38; MEA, (25–)30–55(– 62); YES, (37–)40–58(– 63); CREA, 12–19. Colony characters: CYA, 25 °C, 7 d: Colonies slightly radially sulcate or plane; margins whitish; texture velutinous to floccose; sporulation dense, conidia *en masse* moderate yellowish green (ISCC-NBS No. 136) to grayish olive green (No. 127); exudate and soluble pigment absent; reverse pale or moderate yellow (No. 87) to dark grayish yellow (No. 91). CYA, 37 °C, 7 d: Colonies irregularly sulcate, velutinous, whitish to light green (No. 144); reverse pale, moderate brown (No. 58) to strong brown (No. 55) or grayish brown (No. 61). MEA, 25 °C, 7 d: Colonies plane; marginal hyphae submerged; texture velutinous to lanose (in old isolates); conidia *en masse* light green (No. 144) to dark yellowish green (No. 137); exudate and soluble pigment absent; reverse pale, pale greenish yellow (No. 104) to light greenish yellow (No. 101). YES, 25 °C, 7 d: Colonies plane, slightly radially sulcate; texture velutinous; conidia *en masse* dark yellowish green (No. 137) to grayish olive green (No. 127); reverse strong yellow (No. 84) to deep yellow (No. 85) or deep yellowish brown (No. 75). CREA, 25 °C, 7 d: Acid production poor, only in colony periphery.
Material examined: Living cultures CCF 1659, 1677, 1959, 2062^T, 3009, 3438, and 4234 (Table 1 specifying strain data, Table 2 listing extrolites of studied strains).
Extrolites: Production of 43 natural compounds is reported in literature, including anthglutin (Kinoshita and Minato 1978; Minato 1979), secalonic acid A (= ergochrome AA = entothien = YC3), secalonic acid D (= ergochrome EE = itabashilin), secalonic acid F and related compounds such as

Fig. 2 *Penicillium oxalicum* (CCF 2315^T). **a** Colonies on CYA after 7 days at 25 °C. **b** Colonies on MEA after 7 days at 25 °C. **c** Colonies on CYA after 7 days at 37 °C. **d** Colonies on CREA after 7 days at 25 °C.

e, f Conidiophores. **g** Conidia. **h** Conidia, SEM. Scale bars: **e–f** = 10 µm; **g** = 5 µm; **h** = 2 µm

340 endocrocin and 6,8-dihydroxy-3-methyl-9-oxo-9H-xanthene-
 341 1-carboxylic acid (Steyn 1970; Li et al. 2010; Chen et al.
 342 2015; Wang et al. 2013a), oxaline and the related meleagrins,
 343 glandicolin A and B, and precursors of roquefortine C and D
 344 (Nagel et al. 1974, 1976; Steyn and Vleggaar 1983; Kim et al.
 345 2012), oxalazines such as oxalazine A and B, and decaturin A,
 346 C, D, E, F (Ubillas et al. 1989; Wang et al. 2013b),
 347 oxilacumone A-E and related compounds, including
 348 coniochaetone A-B, coniothienol A and α - and β -
 349 diversonolic ester (Sun et al. 2013; Bao et al. 2014; Wang et

al. 2016; Shi et al. 2017), hamisonine (Seetharaman et al. 350
 2017), terrecoumarins (Li et al. 2015), ergosterol peroxide 351
 (Kuo et al. 2005), 5-hydroxy-2-methoxybenzoic acid and 2- 352
 phenyl acetic acid (Li et al. 2010), 5H-pyrrolo[3,4-d]-2- 353
 piperidinecarboxylic acid, (E)- and (Z)-N-(4- 354
 hydroxystyryl)formamide, 2-(4-hydroxybenzyl)quinazolin- 355
 4(3H)-one, penipanoid A, asperazine, penicilliumthiamine A 356
 and B (Xu et al. 2014; Yang et al. 2016), vermiculidol (Kim et 357
 al. 2012) and 2,2',4,4'-tetrahydroxy-8'-methyl-6-methoxy-ac- 358
 yl-ethyl-diphenylmethanone (Liu et al. 2015). 359

Notes: The description presents a consensus concept from all observed strains. Conidia size of CCF 2315^T was (4.0–)4.5(– 5.5) × (2.5–)3.0(– 4.0) μm (L/W 1.41) and colony diam (7 d, mm) was: CYA, 36–53; CYA, 37 °C, 10–36; MEA, 58–62; YES, 60–63; and CREA, 15–16. Morphometrical dimensions were rather uniform in most of the examined strains. The ex-type strain of *P. asturianum*, CCF 2062, formed unusually long and slender conidia (5.3 × 2.7 μm, L/W ratio = 1.96) (Fig. S2, Table S3), while other characteristics allow its recognition as *P. oxalicum*. Colonies were variable in size, which was observed by Pitt 1980, too. The ex-type strain of *P. oxalicum* (CCF 2315) produces mainly monoverticillate conidiophores; the ex-type strain of *P. asturianum* (CCF 2062) produces biverticillate conidiophores; strain CCF 3009 sporulates very strongly and forms conidial crusts, not seen in other strains.

Penicillium diatomitis Kubátová, Hujšlová, M. Kolařík & Frisvad, **sp. nov.**

MycoBank: MB824352

Fig. 3

Etymology: The name derives from the term *diatomite*, a lake sediment containing fossilized remains of *diatoms* that commonly occur in the Soos Nature Reserve.

Type: **Czech Republic**, western Bohemia, Soos Nature Reserve near Františkovy Lázně, 50.1499 N, 12.4047 E, isolated from saline acidic soil (pH 1.7), 2003, isol. M. Hujšlová (**holotype**, dried culture, PRM 861476; isotype, dried culture, PRM 861477; ex-type cultures, CCF 3904 (= MH 53, CBS 140107, IBT 30728).

Diagnosis: *ITS barcode:* FJ430748. Alternative identification markers, *TUB2* (HE651133), *CAM* (LT970912), *RPB2* (LT797560). *Penicillium diatomitis* differs from *P. oxalicum* by its inability to produce acids on CREA and by a quite different profile of extrolites. While *P. diatomitis* forms ellipsoidal conidia, those of *P. soosanum* are subglobose.

Asexual state: *Conidiophores* terminally branched, with 2–4 adpressed metulae (bi-verticillate); *stipes* smooth, most commonly 75–180(– 300) × 2.5–3.1 μm; *metulae* of unequal length, 13.5–24 × 3–5 μm; *phialides* cylindrical with distinct collula, 3–7 per metula, 12.4–17 × 3.0–4.8 μm; *conidia* finely roughened, ellipsoidal, (3.5–)3.8–5.5(– 6.6) × (2.6–)2.9–3.5(– 4.6) μm, L/W ratio 1.26–1.57. Sexual state not observed.

Culture characters: *Colony diam, 7 d (mm):* CYA, (45–)50–60(– 65); CYA, 37 °C, 5–25; MEA, (48–)50–60(– 62); YES, 59–67; CREA, 7–13. *Colony characters:* CYA, 25 °C, 7 d: Colonies radially sulcate; margins whitish; texture velutinous; sporulation dense, conidia *en masse* pale green (ISCC-NBS No. 149) to grayish green (No. 150); exudate and soluble pigment lacking; reverse light olive (No. 106), moderate olive (No. 107) or moderate greenish yellow (No. 102). CYA, 37 °C, 7 d: Colonies faintly irregularly sulcate; velutinous; whitish to pale green (No. 149) or greenish gray

(No. 155), reverse strong brown (No. 55) to deep brown (No. 56). MEA, 25 °C, 7 d: Colonies plane; marginal hyphae submerged; texture velutinous; sporulation heavy, conidia *en masse* pale green (No. 149) to grayish green (No. 150); exudate and soluble pigment lacking; reverse pale. YES, 25 °C, 7 d: Colonies plane, faintly radially sulcate; texture velutinous; conidia *en masse* light green (No. 144); reverse deep yellow (No. 85) to dark orange yellow (No. 72). CREA, 25 °C, 7 d: Acid not produced.

Extrolites: Ten compounds of unknown structure (‘alko,’ ‘fnot,’ ‘oxyl1, 2,’ ‘para0-5). *P. diatomitis* produces the same extrolites as *P. soosanum*, but both only shared ‘oxyl2’ metabolite with *P. oxalicum* (Table 2).

Material examined: Living cultures CCF 3779, 3780, 3904^T, 3906, 3907 and 4379 (Table 1 specifying strain data, Table 2 listing extrolites of studied strains).

Notes: The description is a consensus among all observed strains. Conidia size of CCF 3904^T was (3.5–)3.9(– 4.5) × (2.5–)3.0(– 3.5) μm (L/W 1.26) and colony diam (7 d, mm) was: CYA, 44–54; CYA, 37 °C, 7–14; MEA, 50–62; YES, 64–67; CREA, 12. Colony morphology was homogeneous across the strains. After prolonged cultivation, a few isolates formed small bodies resembling *sclerotia*, especially on PCA (see Fig. 3j). Conidial size fully overlaps with the ranges seen in *P. oxalicum*. Absence of acid production on CREA allows its distinction from *P. oxalicum*.

Penicillium soosanum Kubátová, Hujšlová, M. Kolařík & Frisvad, **sp. nov.**

MycoBank: MB824353

Fig. 4

Etymology: The name derives from the name of its type locality (Soos).

Type: **Czech Republic:** western Bohemia, Soos Nature Reserve near the town of Františkovy Lázně, 50.1499 N, 12.4047 E, isolated from saline acidic soil (pH 2.7), 2004, isol. M. Hujšlová (**holotype**, dried culture, PRM 861478; isotype, dried culture, PRM 861479; ex-type culture, CCF 3778 (= MH 344, CBS 140106, IBT 30727).

Diagnosis: *ITS barcode:* FJ430745. Alternative identification markers, *TUB2* (FM865811), *CAM* (LT970913), *RPB2* (LT797561). *Penicillium soosanum* differs from *P. oxalicum* by its subglobose conidia, inability to produce acids on CREA and a quite different profile of extrolites. It differs from *P. diatomitis* by subglobose conidia.

Asexual state: *Conidiophores* terminally branched, with 2–4 adpressed metulae (bi-verticillate), rarely with a subterminal metula; *stipes* smooth, most commonly 60–250 × 2.5–3.1 μm; *metulae* of unequal length, 12.4–24 × 2.5–4.0 μm; *phialides* cylindrical with distinct collula, 3–7 per metula, 9–14.5 × 2.8–4.3 μm; *conidia* roughened, broadly ellipsoidal to subglobose, (3.3–)3.4–4.4(– 5.0) × (2.8–)3.2–3.8(– 4.3) μm, L/W ratio 1.06–1.18. Sexual state not observed.

Fig. 3 *Penicillium diatomitis* (CCF 3904^T). **a** Colonies on CYA after 7 days at 25 °C. **b** Colonies on MEA after 7 days at 25 °C. **c** Colonies on CYA after 7 days at 37 °C. **d** Colonies on CREA after 7 days at 25 °C.

e, f Conidiophores. **g** Conidiophores, SEM. **h** Conidia. **i** Conidia, SEM. **j** *Penicillium diatomitis* (CCF 3779). Sclerotium-like bodies, PCA after 7 weeks. Scale bars: **e-f**, **h** = 10 µm; **g** = 5 µm; **i** = 2 µm; **j** = 100 µm

Culture characters: Colony diam, 7 d (mm): CYA, 45–65; CYA, 37 °C, 5–20; MEA, 55–65; YES, 60–69; CREA, 13–17. Colony characters: CYA, 25 °C, 7 d: Colonies faintly radially sulcate; margins whitish; texture velutinous;

sporulation dense, conidia en masse grayish yellow green (No. 122), light bluish green (No. 163) to moderate green (No. 145); exudate and soluble pigment lacking; reverse light greenish yellow (No. 101) to dark greenish yellow (No. 103).

Fig. 4 *Penicillium soosanum* (CCF 3778^T). **a** Colonies on CYA after 7 days at 25 °C. **b** Colonies on MEA after 7 days at 25 °C. **c** Colonies on CYA after 7 days at 37 °C. **d** Colonies on CREA after 7 days at 25 °C.

e, f, g Conidiophores. **h** Conidia. **i** Conidiophores, SEM. **j, k** Conidia, SEM. Scale bars: **e–i** = 10 µm; **j–k** = 2 µm

474 CYA, 37 °C, 7 d: Colonies faintly irregularly sulcate,
 475 velutinous, whitish to pale green (No. 149) or greenish gray
 476 (No. 155), reverse strong brown (No. 55) to deep brown (No.
 477 56). MEA, 25 °C, 7 d: Colonies plane; marginal hyphae sub-
 478 merged; texture velutinous; sporulation heavy, conidia *en*
 479 *masse* light green (No. 144), moderate green (No. 145) to dark
 480 yellowish green (No. 137); exudate and soluble pigment lack-
 481 ing; reverse pale. YES, 25 °C, 7 d: Colonies plane, faintly
 482 radially sulcate or without sulci; texture velutinous; sporula-
 483 tion heavy, conidia *en masse* light bluish green (No. 163),
 484 moderate yellowish green (No. 136) to moderate green (No.
 485 145); reverse deep yellow (No. 85) to dark orange yellow (No.
 486 72). CREA, 25 °C, 7 d: Acid not produced.

487 *Extrolites*: See *P. diatomitis*.

488 *Material examined*: Living cultures CCF 3776, 3777, 3778
 489 and 3905 were used for morphological study (Table 1 specifying
 490 strain data, Table 2 listing extrolites of studied strains).

491 *Notes*: The description is a consensus among all observed
 492 strains. Conidia size of CCF 3778^T was (3.5–)3.8(–
 493 4.5) × (2.5–)3.4(– 4.0) μm (L/W 1.12) and colony diam (7 d,
 494 mm) was: CYA, 53–60; CYA, 37 °C, 5; MEA, 54–57; YES,
 495 60–65; CREA, 16–17. Colony morphology was homogenous
 496 across the strains. Strain IBT 32474, isolated from a wall in a
 497 food factory from China, is identified as *P. soosanum* based on
 498 its extrolite profile and subglobose conidia.

499 Discussion

500 In this study, we revised a set of isolates that respond morpho-
 501 logically to the concept of *P. oxalicum*. Two names, *P.*
 502 *aragonense* (nom. inval., Frisvad et al. 1990) and *P.*
 503 *asturianum*, were previously considered synonyms of *P.*
 504 *oxalicum* (Stolk et al. 1990; Pitt et al. 2000). *Penicillium*
 505 *asturianum* was described based on a single strain, which
 506 was also used in our study. This strain differed from others in
 507 having somewhat more slender conidia (Fig. S2), but its other
 508 morphological features are indistinguishable from *P. oxalicum*
 509 and *P. diatomitis*. Molecular data confirm that *P. asturianum* is
 510 a taxonomic synonym of *P. oxalicum* sensu stricto as previous-
 511 ly noted (Stolk et al. 1990; Pitt et al. 2000, Visagie et al. 2015).

512 Both new species were isolated from acid soil with a pH
 513 below 3 (Table 1) and can be considered as acidotolerant.
 514 Cultivation tests on *P. diatomitis* CCF 3405 showed that it is
 515 able to tolerate pH 2 (optimum at pH 5) and high salinity
 516 (0.5 M Na₂SO₄), confirming its halo- and acidotolerance
 517 (Hujšlová et al. 2010).

518 Our revision and mining of the public sequence database (Fig.
 519 S1) suggests that *P. oxalicum* is a well-defined species with rel-
 520 atively minor intraspecies variability and no undescribed cryptic
 521 diversity. This corresponds with the finding of Rivera (2009)
 522 who studied 38 *P. oxalicum* isolates using five genetic loci and
 523 did not find support for the presence of any cryptic speciation.

Penicillium oxalicum is extremely attractive for all kinds of
 biotechnological applications. On the list of 2837 patents deal-
 ing at least marginally with *Penicillium* (obtained by
 searching for the string “penicillium” in the text of patent
 abstracts on the Google Patents website on 17 July 2017),
 79 used strains of *P. oxalicum*. Only *P. chrysogenum* and *P.*
citrinum were more frequently encountered. The close phylo-
 genetic relatedness of newly described species with *P.*
oxalicum, and the uniqueness of their secondary metabolite
 spectrum, suggests that the newly described species could be
 biotechnologically relevant as well. It is also possible that
 some isolates identified previously as *P. oxalicum* could be-
 long to the morphologically similar *P. diatomiti*.

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