

A taxonomic and phylogenetic revision of the *Penicillium sclerotiorum* complex

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Abstract: The morphological concept of *Penicillium sclerotiorum* (subgenus *Aspergilloides*) includes strains with monoverticillate, vesiculate conidiophores, and vivid orange to red colony colours, with colourful sclerotia sometimes produced. Multigene phylogenetic analyses with the nuclear ribosomal internal transcribed spacer (ITS) region, cytochrome c oxidase subunit 1 (*cox1*), β -tubulin (*benA*), translation elongation factor 1- α (*tef1-a*), and calmodulin (*cmd*), reveal that the *P. sclerotiorum* morphospecies is a complex of seven phylogenetically distinct species, three of which were recently described, namely *P. guanacastense*, *P. mallochii*, and *P. viticola*. Three previously unidentified species are described here as *P. cainii*, *P. jacksonii*, and *P. johnkrugii*. The phylogenetic species are morphologically similar, but differ in combinations of colony characters, sclerotium production, conidiophore stipe roughening and branching, and conidial shape. Ecological characters and differences in geographical distribution further characterise some of the species, but increased sampling is necessary to confirm these differences. The fungal DNA barcode, the ITS, and the animal DNA barcode, *cox1*, have lower species resolving ability in our phylogenetic analyses, but still allow identification of all the species. *Tef1-a* and *cmd* were superior in providing fully resolved, statistically well-supported phylogenetic trees for this species complex, whereas *benA* resolved all species but had some issues with paraphyly. *Penicillium admetzioides* and *P. multicolor*, considered synonyms of *P. sclerotiorum* by some previous authors, do not belong to the *P. sclerotiorum* complex.

Key words: DNA barcoding, multigene phylogeny, sclerotia, soil-borne hyphomycetes.

Taxonomic novelties: New species: *Penicillium cainii* K.G. Rivera, Malloch & Seifert, *P. jacksonii* K.G. Rivera, Houbraken & Seifert, *P. johnkrugii* K.G. Rivera, Houbraken & Seifert.

INTRODUCTION

Penicillium sclerotiorum was first isolated from air in Java, Indonesia, by K.B. Boedijn, and then described by van Beyma (1937). The species has monoverticillate, vesiculate conidiophores and vivid orange to red colony colours, and some strains produce orange sclerotia that give the species its name. Cultures identified as *P. sclerotiorum* have been isolated from many countries in Africa, Asia, and North America, suggesting a cosmopolitan distribution, but it has been reported infrequently (Pitt 1980, Ramirez 1982). Strains commonly originate from soil, and occasionally textiles, but are also isolated from house dust (Vesper *et al.* 2005), diseased grape fruit and stems (De Lucca *et al.* 2008), and as a potential endophyte of *Coffea arabica* berries (Vega *et al.* 2006). Air sampling revealed higher concentrations of *P. sclerotiorum* outdoors than in indoor environments in India (Sawane & Saoji 2004).

Penicillium sclerotiorum is now classified in *Penicillium* subgenus *Aspergilloides*, section *Sclerotiora* by Houbraken & Samson (2011). As noted by Peterson (2000) and discussed at length by Houbraken & Samson (2011), the monoverticillate conidiophore, although a useful phenotypic character for identification, is phylogenetically uninformative and the concept of subgenus *Aspergilloides* promoted by Pitt (1980) has been substantially revised. Species with such conidiophores are phylogenetically intermingled with species with divaricate or symmetrically biverticillate conidiophores. Pitt (1980) classified *P. sclerotiorum* in his broadly circumscribed subgenus *Aspergilloides* series *Glabra*, which Peterson's phylogenetic studies distributed among his Clades 2, 3, and 5 (Peterson 2000). Peterson's 'Clade 3' of *Penicillium* included the predominantly monoverticillate species *P. admetzii*, *P. admetzioides*, *P. bilaiae*, and the biverticillate

P. herquei, all now classified in section *Sclerotiora* by Houbraken & Samson (2011). Three biverticillate species were included by Houbraken & Samson (2011) in section *Sclerotiora*, namely *P. herquei*, classified by Pitt (1980) in *Penicillium* subgenus *Furcatum* section *Furcatum*, and the subsequently described *P. malachiteum* and *P. nodositatum*. Sclerotia are sporadically produced by species across many sections of *Penicillium*, with the orange sclerotia of *P. thomii* (section *Aspergilloides*) perhaps the most conspicuous and commonly encountered example.

Our recent studies of *Penicillium* strains isolated from the guts of tropical leaf-eating caterpillars in Costa Rica (Rivera *et al.* 2011) led to the description of two phylogenetically distinct species, *P. mallochii* and *P. guanacastense*, which although they do not produce sclerotia, otherwise conform to the morphological concepts of *P. sclerotiorum* of Pitt (1980) and Stolk & Samson (1983). This led to the realisation that the morphological concept of *P. sclerotiorum* includes a complex of phylogenetically distinct species, and to the taxonomic revision presented in this paper. Among the modern revisions of this taxon by Raper & Thom (1949), Pitt (1980), Ramirez (1982), and Stolk & Samson (1983), only the latter proposed synonyms for *P. sclerotiorum*; we reconsidered the status of *P. admetzioides* and *P. multicolor* as possible names for some of the phylogenetic lineages we observed. This paper provides a polyphasic taxonomic revision of 29 strains from this complex, based on morphological and microscopic characters, and phylogenetic analyses of five genes: β -tubulin (*benA*), cytochrome c oxidase subunit 1 (*cox1*), the internal transcriber spacer (ITS) region, translation elongation factor 1- α (*tef1-a*), and calmodulin (*cmd*). The ITS and *cox1* genes are of particular relevance as DNA barcodes, with ITS now functioning as the sanctioned DNA barcode for fungi (Schoch *et al.*, in prep.), and *cox1* as the DNA barcode for animals (Hebert *et al.* 2003).

Table 1. Accession numbers of cultures, isolation details and GenBank accession numbers for the five genes used for phylogenetic analysis of the *Penicillium sclerotiorum* complex.

Species	Accession number	Location	Host or substrate	GenBank Accession numbers				
				ITS	<i>benA</i>	<i>tefl-α</i>	<i>cmd</i>	<i>cox1</i>
<i>P. adamezii</i>	CBS 209.28 (T)	Poland, Poznan	Soil under conifers	JN714929	JN625957	JN626136	-	JN626049
	KAS 3463	Malaysia, Kedah	Forest soil	JN714930	JN625958	JN626137	-	JN626050
	KAS 3464	Malaysia, Kedah	Forest soil	JN714931	JN625959	JN626138	-	JN626051
	KAS 3465	Malaysia, Kedah	Forest soil	JN714932	JN625960	JN626139	-	JN626052
	KAS 3466	Malaysia, Kedah	Forest soil	JN714933	JN625961	JN626140	-	JN626053
	DAOM 239916	Canada, Ontario, Vmeland	Riesling grapes	JN686434	JN799643	-	JN686387	JN686410
<i>P. bilaiae</i>	CBS 313.59 (T)	Japan	Soil	JN686433	JN799642	-	JN686388	JN686411
	NRRL 3391 (T)	Ukraine, Kiev	Soil	JN714937	JN625966	JN626145	JN626009	JN626058
	ATCC 20851	Canada, Alberta, Lethbridge	Soil	JN714934	JN625964	JN626141	JN626005	JN626054
	ATCC 22348	Ukraine, Kiev	Soil	JN714935	JN625962	JN626143	JN626007	JN626056
	DAOM 197974	Canada, Alberta	Soil	JN714936	JN625965	JN626144	JN626008	JN626057
	DAOM 239914 (T)	Canada, Ontario, Niagara, Niagara Falls, Fireman's Park	Nuts of <i>Juglans nigra</i>	JN686435	JN686366	JN686456	JN686989	JN686412
<i>P. cainii</i>	DAOM 239915	Canada, Ontario, Niagara, Niagara Falls, Fireman's Park	Nuts of <i>Carya ovata</i>	JN686436	JN686367	JN686457	JN686390	JN686413
	DAOM 239912(T)	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Gut of the caterpillar <i>Eutelia</i> sp. reared on leaves of <i>Spondias mombin</i>	JN626098	JN625967	JN626146	JN626010	JN626059
	DAOM 239913	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Gut of the caterpillar <i>Eutelia</i> sp. reared on leaves of <i>Spondias mombin</i>	JN626099	JN625968	JN626147	JN626011	JN626060
	CBS 336.48 (T)	France	Leaf of <i>Agauria piriifolia</i>	JN626101	JN625970	JN626149	JN626013	JN626062
	CBS 136.22	France	Wakamoto corn and rice cake	JN626100	JN625969	JN626148	JN626012	JN626061
	CBS 347. 51	Japan, Nehira	Forest soil	JN626102	JN625971	JN626150	JN626014	JN626063
<i>P. hiryamae</i>	CBS 110644	USA, Wisconsin	Milled rice	JN626103	JN625972	JN626151	JN626015	JN626064
	CBS 229.60 (T)	Thailand	Corn meal	JN626095	JN625955	JN626135	JN626003	JN626046
	CBS 238.65	South Africa	Forest soil	JN626096	JN625956	JN626134	JN626004	JN626047
	DAOM 239937 (T)	Australia, Queensland, Barrine Lake	Forest soil	JN686437	JN686368	JN686458	JN686391	JN686414
	DAOM 239938	Australia, Queensland, Barrine Lake	Forest soil	JN686438	JN686369	JN686459	JN686392	JN686415
	DAOM 239939	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Rainforest soil	JN686443	JN686374	JN792190	JN686397	JN686420
<i>P. johnkrugii</i>	DAOM 239940	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686444	JN686375	JN792191	JN686398	JN686421
	DAOM 239941	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686445	JN686376	JN792192	JN686399	JN686422
	DAOM 239942	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686446	JN686377	JN792193	JN686400	JN686423
	DAOM 239943 (T)	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686447	JN686378	JN792194	JN686401	JN686424

Table 1. (Continued)..

Species	Accession number	Location	Host or substrate	GenBank Accession numbers				
				ITS	benA	tefl-α	cmd	cox1
	DAOM 239944	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686448	JN686779	JN792195	JN686402	JN686425
	DAOM 239945	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686449	JN686780	JN792196	JN686403	JN686426
	DAOM 239946	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686450	JN686781	JN792197	JN686404	JN686427
	KAS 3479	Malaysia, Kedah, Langkawi, Gunung Raya Rainforest	Forest soil	JN686451	JN686782	JN792198	JN686405	JN686428
<i>P. levitum</i>	NRRL 705 (T)	USA, New York	Modeling clay	JN626097	JN714938	JN714928	JN714939	JN626048
<i>P. mallochii</i>	DAOM 239917 (T)	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Caterpillar on <i>Spondias mombin</i>	JN626104	JN625973	JN626152	JN626016	JN626065
	DAOM 239919	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Midgut of the caterpillar <i>Citheronia lobesis</i> feeding on <i>Spondias mombin</i>	JN626106	JN625975	JN626154	JN626018	JN626067
	DAOM 239922	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Hindgut of the caterpillar <i>Rothschildia lebeau</i> reared on leaves of <i>Spondias mombin</i>	JN626109	JN625978	JN626157	JN626020	JN626070
	DAOM 239925	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Guts of the caterpillar <i>Citheronia lobesis</i> reared on leaves of <i>Cochlospermum vitifolium</i>	JN626112	JN625980	JN626159	JN626023	JN626072
	DAOM 239926	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Frass of the caterpillar <i>Rothschildia lebeau</i> reared on leaves of <i>Spondias mombin</i>	JN626111	JN625981	JN626160	JN626024	JN626073
	DAOM 239927	Costa Rica, Santa Rosa, Área de Conservación Guanacaste	Gut of the caterpillar <i>Rothschildia lebeau</i> reared on leaves of <i>Spondias mombin</i>	JN626113	JN625982	JN626161	JN626025	JN626074
<i>P. multicolor</i>	CBS 501.73 (T)	USSR	Soil	JN799647	JN799645	JN799648	JN799646	JN799644
<i>P. sclerotiorum</i>	NRRL 2074 (T)	Indonesia, Java, Buitenzorg	Air	JN626132	JN626001	JN626180	JN626044	JN626093
	NRRL 32583	USA, Hawaii, Kuauai	Coffee seeding crown	JN626133	JN626002	JN626181	JN626045	JN626094
	DAOM 239930	Thailand, Hua Hin	Forest soil	JN626129	JN625998	JN626177	JN626041	JN626090
	DAOM 239931	Australia, Queensland, Barron Falls	Forest soil	JN626130	JN625999	JN626178	JN626042	JN626091
	DAOM 239932	Australia, Queensland, Barron Falls	Forest soil	JN626131	JN626000	JN626179	JN626043	JN626092
	CBS 128.65	Zaire, Leopoldville, Nsang-Nginga River	Forest litter	JN686452	JN686783	JN686464	JN686406	JN686429
	CBS 258.55	Turkey, Istanbul	Culture contaminant	JN686453	JN686784	JN686465	JN686407	JN686430
	CBS 118889	Korea	Soil	JN686454	JN686785	JN686466	JN686408	JN686431
<i>P. viticola</i>	DAOM 239933	Australia, Queensland, Barron Falls	Forest soil	JN686439	JN686370	JN686460	JN686393	JN686416
	DAOM 239934	Australia, Queensland, Atherton	Forest soil	JN686440	JN686371	JN686461	JN686394	JN686417
	DAOM 239935	Australia, Queensland, Atherton	Rainforest soil	JN686441	JN686372	JN686462	JN686395	JN686418
	DAOM 239936	Australia, Queensland, Atherton	Rainforest soil	JN686442	JN686373	JN686463	JN686396	JN686419
<i>Penicillium</i> sp.	CBS 248.65	South Africa	Corn meal	JN686455	JN686786	JN686467	JN686409	JN686432

Abbreviations: CBS - CBS-KNAW Biodiversity Centre culture collection, Utrecht, the Netherlands. DAOM - culture collection and herbarium of the National Mycological Collections, Agriculture & Agri-Food Canada, Ottawa. NRRL - culture collection of USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL, USA. ATCC - American Type Culture Collection, Manassas, VA, USA. KAS - personal culture collection of Keith A. Seifert.

MATERIALS AND METHODS

Fungal isolates and herbarium specimens

We focused on cultures identified as *P. sclerotiorum*, and the related species in Clade 3 from the phylogenetic study of Peterson (2000). They were obtained from the CBS Fungal Biodiversity Centre (CBS) and USDA-ARS, National Center for Agricultural Utilization Research (NRRL) culture collections, and the personal research collections of K.A. Seifert and C. André Lévesque (Ottawa, ON, Canada), D. Malloch (formerly University of Toronto, ON, Canada), and J. Houbraken and R.A. Samson (CBS). Representative strains are deposited in the Canadian Collection of Fungal Cultures (DAOM) and CBS. Table 1 includes the metadata for the 29 strains used in this study. We note that cultures of this complex do not preserve well in sterile water at 4 °C, often dying within one year. Some *P. sclerotiorum* strains (DAOM 239931, 239932) and all *P. johnkrugii* strains were isolated from ethanol treated soils from Australia and Malaysia (Houbraken, pers. comm.).

Morphological analysis

All strains were inoculated at three points onto Blakeslee's Malt Extract Agar (MEA, for microscopic analysis and colony characters), Czapek Yeast Agar (CYA, for colony characters; Pitt 1973), Czapek Agar (CZ, for ability to grow and sporulate in the absence of ammonia; Raper & Thom 1949), Yeast Extract Sucrose Agar (YES to stimulate colony pigmentation by enhancing secondary metabolite production; Filtenborg *et al.* 1990), Oatmeal Agar (OA, to stimulate sclerotial or ascomatal development) and Creatine Sucrose Agar (CREA, to test for acid production; Frisvad 1993). All measurements and observations were performed in duplicate from cultures inoculated at different times. Plates were incubated in the dark at 25 °C for 7 days.

Microscopic observations employed a BX 50 light microscope (Olympus Canada, Richmond Hill, ON), using tissue removed from 7 d old colonies grown on MEA, and mounted in 85 % lactic acid. Microphotographs were taken and characters measured with an Evolution MP Camera using Image-Pro Plus v. 6 (both from Media Cybernetics, MD, USA). For each strain, automated measurements of 25 conidia were made from phase contrast images using the count/size algorithm of Image-Pro, and manual measurements were made for ten phialides, stipes, branches, vesicles, sclerotia and sclerotial cells. Variations in microscopic dimensions are presented as mean \pm standard error. Alphanumeric colony colour codes are based on Kornerup & Wanscher (1978). Colony photographs were taken using a copy stand and a Coolpix P5000 camera (Nikon Canada, Mississauga, ON) under incandescent light.

DNA extractions, PCR and DNA sequencing

Genomic DNA was extracted from strains grown on MEA or CYA using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Montreal, Canada) following the manufacturer's protocol. *BenA* was amplified and sequenced with primers Bt2a and Bt2b (Glass & Donaldson 1995); ITS with primers ITS1 and ITS4 (White *et al.* 1990); *cox1* with primers PF and AR (Seifert *et al.* 2007); *tef1-a* with primers EF1c and EF6 (Peterson *et al.* 2004); and *cmd* with primers CMD5 and CMD6 (Hong *et al.* 2006).

PCR reactions were performed in 10 μ l reaction mixtures containing 1 μ l genomic DNA, 1X PCR Buffer, 0.1 mm of dNTPs,

0.08 μ M of each primer, and 0.5X Taq polymerase. Amplifications were performed in a TGradient (Biometra, Montreal, Canada) or a Genius (Techne, Duxford, Cambridge, UK) thermocycler. The PCR parameters for ITS, *cox1*, *benA* were denaturation at 95 °C for 1 min, followed by primer annealing at 56 °C for 45 s, and primer extension at 72 °C for 90 s for 35 cycles, plus a final 10 min elongation step at 72 °C. The profile for *tef1-a* and *cmd* was denaturation at 94 °C for 1 min, annealing at 62 °C for 30 s, primer extension at 72 °C for 90 s for 42 cycles, then a final elongation step at 72 °C for 10 min. PCR products were visualised by gel electrophoresis in a 1 % agarose gel containing ethidium bromide (0.05 μ g/mL).

Sequencing reactions were performed directly on PCR amplicons using forward and reverse primers. Reactions with a total volume of 10 μ l contained 1 μ l amplicon, 0.5 μ l of ready-made BigDye and terminator mix v. 7 and 0.125X BigDye buffer (Applied Biosystems, Foster City, CA, USA), and 0.161 μ M of primer. Reactions were performed in the thermocyclers noted above, programmed for denaturation at 95 °C for 1 min, followed by primer annealing at 56 °C for 30 s and primer extension at 72 °C for 1 min for 30 cycles, plus a final 10 min elongation step at 72 °C. Sequence reaction mixtures were precipitated using ethanol/EDTA/sodium acetate precipitation. Samples were analysed on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Two strains of *P. herquei* (CBS 336.48^T, 110644) produced two amplicons (*ca.* 360 bp and 470 bp long) for *benA*. PCR products with multiple bands were cloned using Promega's pGEM[®]-T Vector Kit and JM109 High Efficiency Competent Cells (Madison, WI, USA) following the manufacturer's protocol. Fifteen transformed colonies were selected for PCR and sequencing.

Consensus sequences were assembled using Sequencher v. 4.8 (Genes Codes Corporation, Ann Arbor, MI, USA) and SeqMan in the LASERGENE package v. 8 (DNASTAR Inc., Madison, WI, USA). Alignments were constructed using the online version of MAFFT v. 6 (Katoch *et al.* 2009), and adjusted to optimise homology using BioEdit 7.0.9 (Hall 1999).

Maximum parsimony (MP) analyses were performed using heuristic searches in PAUP v. 4 (Swofford 2002) with the tree bisection-reconnection (TBR) branch swapping algorithm. Uninformative characters were removed for all analyses, gaps were treated as missing data, and maxtrees were set to 5000. Consensus trees were calculated, and the robustness of the gene trees was tested using a full heuristic search, saving 10 trees per replicate (1000 replicates).

Maximum likelihood (ML) analyses were performed using Phylogenies for Maximum Likelihood (PhyML) v. 2.4.4 (Guindon *et al.* 2005). Tree searches for each alignment were run under the nucleotide substitution models obtained from ModelTest 3.7, namely GTR+G (*benA*, *cox1*, ITS, *tef1-a*), and GTR+I+G (*cmd*), using the Akaike Information Criteria (AIC) (Posada & Crandall 1998). The starting tree for branch swapping was obtained using the modified neighbour joining algorithm BIONJ (Gascuel 1997), as implemented in PhyML. The robustness for each tree was tested by performing 1000 bootstrap replicates.

Bayesian inference (BI) analyses were performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), using the same models noted for ML above. All Bayesian analyses were performed using random starting trees, and were run for four chains with one million generations for all genes, sampling every 100 generations, generating 10,001 trees, with the first 2,500 discarded as 'burn-in' for each chain.

Table 2. Comparative summary of colony and microscopic characters of species in the *P. sclerotiorum* complex.

Species	Colony characters on CYA			Morphological characters				Substrate/host	Geographical distribution	
	Conidium colours	Diam (mm)	Sclerotia	Conidiophores		Conidia				
				Roughening	Length > 135 µm	Branching	Roughening			Shape
<i>P. sclerotiorum</i>	Greenish grey/orange	18–40	Present	Smooth to finely rough	All	Unbranched	Finely rough	Subglobose to ellipsoidal	Soil, litter, coffee beans, air	Pantropical and subtropical
<i>P. cainii</i>	Deep blue to deep green	23–29	Absent	Rough	No	Unbranched or ~10% branched	Finely rough	Globose	Nuts of <i>Juglandaceae</i>	Canada
<i>P. guanacastense</i>	Greenish grey	25–33	Absent	Finely rough	No	~10% branched	Finely rough	Globose	Caterpillars feeding on leaves	Costa Rica
<i>P. jacksonii</i>	Deep green	30–33	Absent	Smooth to rough	No	Unbranched or < 65% branched	Finely rough	Globose	Soil	Australia
<i>P. johnkrugii</i>	White	30–38	Present	Smooth to finely rough	Most	Unbranched	Finely rough	Globose to subglobose	Soil	Malaysia
<i>P. mallochii</i>	Turquoise grey/greenish grey	29–39	Absent	Smooth to finely rough	Most	~10% branched	Finely rough	Globose to subglobose	Caterpillars feeding on leaves	Costa Rica
<i>P. viticola</i>	Greenish grey	26–36	Absent	Rough	No	Unbranched or ~10% branched	Smooth	Globose	Grape vines, soil	Australia, Japan

Individual gene trees and a combined data set with all five genes were analysed by MP, ML and BI individually. A partition homogeneity test (PHT) was performed in PAUP v. 4 (Swofford 2002) using all genes to determine whether combining the genes was advisable. Missing *cmd* sequences for *P. adametzii* strains and missing *tef1-a* sequences for DAOM 239916 were replaced by N's to indicate missing data. Parsimony parameters were set to TBR, 1000 replicates, 10 trees saved per replicate, and maxtrees were set to 5000.

All new sequences used for our analyses are deposited in GenBank under accession numbers JN625955–JN799648 (Table 1). Alignments are in TreeBase under study S12031.

RESULTS

Analysis of morphological characters

Morphological species descriptions for *P. cainii*, *P. jacksonii*, *P. johnkrugii*, *P. viticola*, and the revised species description for *P. sclerotiorum* are provided in the Taxonomy section. Together with the descriptions of *P. guanacastense* and *P. mallochii* in Rivera *et al.* (2011), this constitutes a monographic revision of the *P. sclerotiorum* species complex. Most species can be identified by subtle phenotypic characters with the aid of Table 2 and the dichotomous key at the end of the paper. Several species seem to be ecologically distinct, but the apparent geographical disjunctions are preliminary and more sampling is needed to confirm these patterns.

In general, the species of this complex grow 20–40 mm diam in 7 d on CYA, and slightly slower, about 15–35 mm, on MEA. The conidial colours are green, often with grey or turquoise tinges; some species can be distinguished by subtle colour differences. Reverse colours are typically orange or red on CYA, and variations from this can be useful for species identification. Conidiophores are usually monoverticillate, but in several species up to about 10% of conidiophores may have a single branch, and in some strains of *P. jacksonii* more than half of the conidiophores may be branched. Stipe roughening is usually slight, except for the conspicuous roughening in *P. cainii* and *P. viticola*. Stipe length varies considerably among the species; although ranges overlap, there seems to be a division between species with most conidiophores shorter or longer than 135 µm. We evaluated the width of the vesicles of monoverticillate conidiophores; they vary between 3–6 µm wide, and although there are minor statistical differences, we do not think this is a useful character for species recognition. Similarly, the number of phialides per vesicle, a highly variable character that is difficult to count confidently, is not useful as a species character. The conidia of most species in the complex are globose or subglobose and about 2–3 µm diam (L/W ratio 1:1 to about 1.2:1). Conidia of *P. sclerotiorum sensu stricto* are the most ellipsoidal in the complex. The conidia of most species are slightly roughened, although this is rather inconspicuous, and does not seem to be a useful character for species recognition, at least as observed with the light microscope.

Sclerotium production is inconsistent within some species, particularly *P. sclerotiorum*, where only four of eight strains produced them. The ex-type strain is very unpredictable, sometimes producing colonies completely covered with sclerotia, and in other transfers producing none at all. Sclerotia are normally visible after 7 d in fresh strains, but production is sometimes delayed to 10–14 d in some transfers of older strains. All strains of *P. johnkrugii*

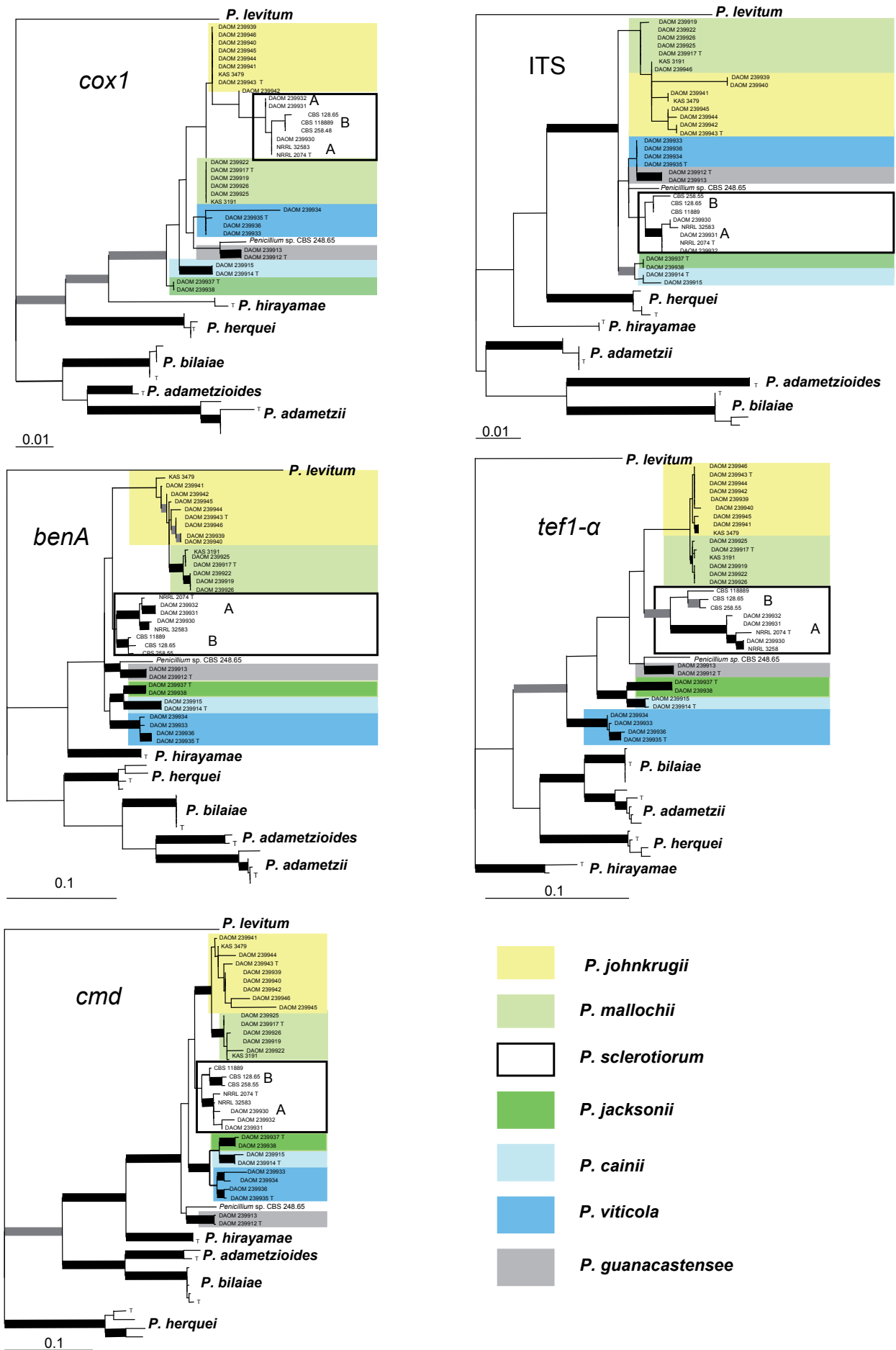


Fig. 1. Maximum likelihood (ML) trees generated for *cox1*, ITS, *benA*, *tef1-α* and *cmd* with PhyML using the GRT+G model (except *cmd*, GTR+I+G model), showing the clades representing the seven species of the *P. sclerotiorum* complex. Bold black branches have ML support > 0.90, MP bootstrap support > 90 %, and BI > 0.95; bold grey branches have ML support > 0.700, MP bootstrap support > 70 %, and BI > 0.95 (see Table 3 for details).

Table 3. Statistical support for species clades for individual gene trees using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI).

		<i>P. cainii</i>	<i>P. guanacastense</i>	<i>P. jacksonii</i>	<i>P. johnkrugii</i>	<i>P. mallochii</i>	<i>P. sclerotiorum</i>		<i>P. viticola</i>
							A	B	
ITS	MP bootstrap (%)	< 70	92.00	< 70	< 70 *	< 70 *	85.00	< 70	< 70
	ML probability	0.82	0.99	0.93	0.718*	0.772*	0.19	0.76	0.49
	BI posterior probability	0.63	1.00	0.99	0.00	0.00	1.00	0.89	0.00
cox1	MP bootstrap (%)	98.00	96.00	92.00	0.00	< 70	< 70	< 70	72.00
	ML probability	1.00	1.00	1.00	0.237*	0.502*	0.00	0.00	0.79
	BI posterior probability	1.00	1.00	0.94	0.68*	0.99*	1.00	0.99	0.94
tef1-α	MP bootstrap (%)	100.00	100.00	100.00	<70	99.00	100.00	84.00	100.00
	ML probability	1.00	1.00	1.00	0.66	0.83	0.99	0.85	0.98
	BI posterior probability	1.00	1.00	1.00	0.55	0.95	1.00	0.86	1.00
benA	MP bootstrap (%)	100.00	100.00	100.00	< 70 *	< 70 *	99.00	100.00	100.00
	ML probability	1.00	1.00	1.00	1.00*	0.975*	1.00	1.00	1.00
	BI posterior probability	1.00	1.00	1.00	1.00*	1.00*	1.00	1.00	1.00
cmd	MP bootstrap (%)	100.00	100.00	100.00	<70	100.00	77.00	87.00	82.00
	ML probability	1.00	1.00	1.00	0.68	1.00	0.94	1.00	0.85
	BI posterior probability	1.00	1.00	1.00	0.92	1.00	1.00	1.00	0.99

* indicates analyses where *P. johnkrugii* and *P. mallochii* were paraphyletic.

produced abundant sclerotia on all media, visible within 7 d. The colourful sclerotia often dramatically affect colony appearance, particularly, as occurs in *P. sclerotiorum*, when sclerotial colonies have much reduced, or absent, conidiation. *Penicillium cainii*, *P. guanacastense*, *P. jacksonii*, *P. mallochii*, and *P. viticola* strains produced no sclerotia on the media tested. All strains of all species were left for eight months in the dark at 25 °C on OA; ascospores were not produced in any of the sclerotial strains.

On MEA, several species, notably *P. guanacastense* and *P. mallochii*, have a tendency towards crustose colonies, with planar sheets of conidia dislodging *en masse* onto the Petri dish lid or slipping sideways across the agar. The phenomenon is less dramatic than in the terverticillate species *P. crustosum* and similar to that seen in monoverticillate species related to *P. glabrum*.

We included CREA to determine whether it had any diagnostic value for this group, but most strains of all species produced abundant acid, turning the entire plate yellow within 1 wk, and they did not produce any base to restore the original purple medium. All strains sporulated poorly and grew weakly or moderately. Only the slightly different but overlapping growth rates, and the presence of sclerotia, are recorded in the species descriptions. We do not consider this medium helpful for diagnosis in this group.

Phylogeny

Multigene phylogenetic analyses (ITS, *cox1*, *benA*, *cmd*, and *tef1- α*) revealed that strains previously identified as *P. sclerotiorum* comprise a complex of phylogenetically distinct species. The complex includes *P. sclerotiorum*, the newly described species in this paper, *P. cainii*, *P. jacksonii*, and *P. johnkrugii*, and the recently described *P. guanacastense*, *P. mallochii* (Rivera *et al.* 2011), and *P. viticola* (Nomura *et al.* 2011). Representative ML trees for each gene are presented in Fig. 1; the log likelihood values for these trees are *benA* -3725.72122, *cmd* -4537.96193, *cox1* -1919.56534, ITS -1846.917238, and *tef1- α* -4663.651570. The MP and BI trees are not shown, but differences in topology are discussed and

support values for each analysis are mapped onto the ML trees and included in Table 3. Sequences were obtained for all genes and strains, except no *cmd* or *tef1- α* PCR products were obtained for the outgroup species *P. adametzii* and *P. adametzioides*. We experimented with denaturation temperatures as low as 40°C for 1 min, and for *cmd* with the primer combinations CF4 and CF5, CF4 and CMD6, and CMD5 and CF5 (Peterson *et al.* 2004, Hong *et al.* 2006), without success. Two strains of the outgroup species *P. herquei*, CBS 336.48^T and CBS 110644, yielded multiple bands after *benA* amplification; the most similar copy to that of the other species was selected for alignment.

In general, the trees of the two DNA barcode markers, ITS and *cox1*, were less resolved and although most species formed monophyletic groups in the strict consensus trees, bootstrap support was low for several (Table 3). Paraphyly was a problem with *P. johnkrugii* and *P. mallochii* in all analyses of these genes. Although both species formed coherent groups that could successfully be identified using these DNA barcodes, *P. mallochii* generally arose from within *P. johnkrugii*. However, these two species are easily distinguished morphologically by the presence or absence of sclerotia, and are ecologically distinct and geographically disjunct. All of the recognised species received strong support as distinct monophyletic clades in all *cmd* and *tef1- α* analyses. *BenA* was intermediate in providing better support for the species than ITS or *cox1*, but the issue of paraphyly of *P. johnkrugii* and *P. mallochii* remained. As noted below, *P. sclerotiorum* resolves into two clades in most analyses (except *cox1*), but we elected not to describe clade B as a distinct species because we did not have sporulating strains. Strain CBS 248.65, identified as *P. sclerotiorum*, did not group with the *P. sclerotiorum* ex-type group or any of the newly described species, and appears to represent a distinct species in the *P. sclerotiorum* complex. The strain did not produce the typical vivid orange to red reverse colony colours and sporulated poorly, and we elected to leave it undescribed pending the isolation of more vigorously sporulating strains. The PHT value for the combined data set for five genes was 0.0001, suggesting

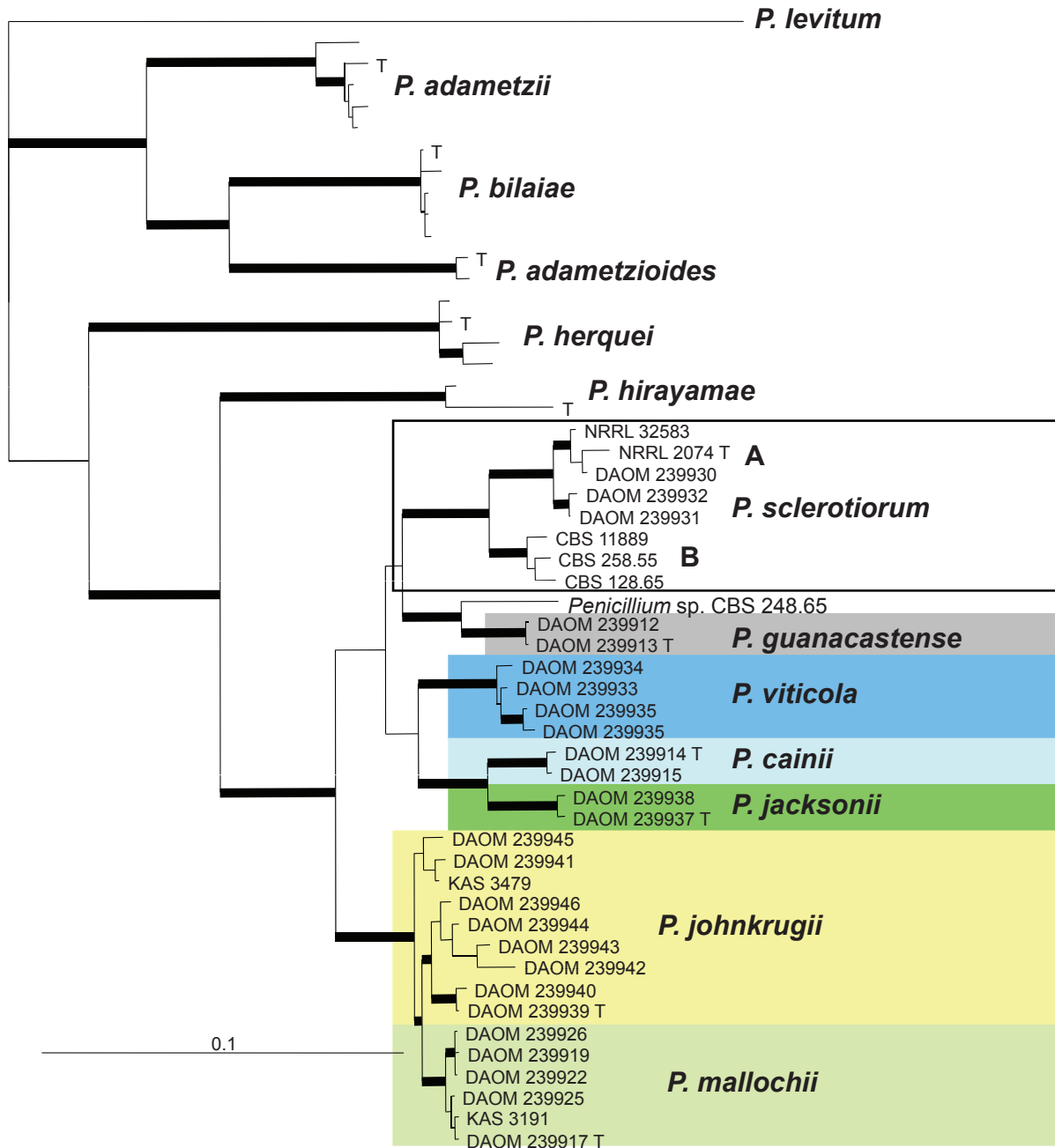


Fig. 2. Bayesian inference (BI) tree for the combined *cox1*, ITS, *BenA*, and *tef1- α* data set generated using MrBayes using the GTR model (except *cmd*, GTR+I+G model), showing the relationships among the seven species of the *P. sclerotiorum* complex. Bold black branches have ML support > 0.90, MP bootstrap support > 90 %, and BI > 0.95 (see Table 3 for details).

that the total combined data set was incongruent. Subsequent PHT analysis of gene pairs revealed that the *cmd* partition was incongruent with the other genes, and thus it was excluded from the construction of the Bayesian multigene phylogeny shown in Fig. 2. In all trees, *P. johnkrugii* and *P. mallochii* were siblings with strong statistical support. In the MP and BI trees, *P. sclerotiorum* and *P. guanacastense* were siblings, lacking strong statistical support (not shown), but in the ML tree (log likelihood -18058.213184), *P. sclerotiorum* was sibling to a clade including *P. cainii*, *P. jacksonii*, and *P. viticola*, again lacking strong statistical support. *Penicillium cainii* and *P. jacksonii* were consistently grouped together, with *P. viticola* as a sister group, all with strong statistical support.

We examined two of the species proposed as synonyms of *P. sclerotiorum* by Stolk & Samson (1983) and found that they did not belong to the revised complex. The ex-type strain of *P. adametzioides*, CBS 313.59, is sterile, and individual gene trees (Fig. 1) and the combined gene trees (Fig. 2) reveals that

this is a distinct species, not part of the *P. sclerotiorum* complex, but still part of section *Sclerotiora* as defined by Houbraken & Samson (2011). Preliminary phylogenetic analyses with all five genes excluded the ex-type strain of *P. multicolor*, CBS 501.73, from the *P. sclerotiorum* complex, and BLAST results with *benA*, ITS, *tef1- α* , and *cmd* sequences had 100 % sequence similarity to *P. fellutanum*, classified by Houbraken & Samson (2011) in subgenus *Aspergilloides* section *Charlesii*. Morphologically, this strain grew slower, 13–15 mm after 7 d on all media, and lacked the vivid red to orange colony colours and vesiculate conidiophores usually observed in the *P. sclerotiorum* complex. We observed only monoverticillate conidiophores in the culture, not the metulate conidiophores emphasised in the description of *P. fellutanum* by Pitt (1980), but our strain of the ex-type was degenerated and sporulated poorly. *Penicillium multicolor* is not accepted as a synonym for *P. sclerotiorum* here, and it is probably a synonym of *P. fellutanum*.

TAXONOMY

Penicillium sclerotiorum J.F.H. Beyma, Zentralbl. Bakteriolog., 2 Abt. 96: 416. 1937. MycoBank MB277708. Figs 3A, 4.

Colonies on CYA after 7 d at 25 °C: 18–40 mm diam, low and velutinous, ca. 1 mm deep, with 8–11 sulcae and 2–3 wrinkles in some strains, sporulation dense in absence of sclerotia, very sparse or absent when orange sclerotia produced, conidia Greenish Grey (25–27E2), aerial mycelium sparse or absent, exudate moderately produced and light yellow in some strains, margin entire, reverse Orange to Reddish Yellow (3A7–4A6), Brown (7E4–8) or pale, some strains with darker colours turning orange towards the centre, vivid orange soluble pigments present in some strains. Colonies on CZ resembling those on CYA, 15–30 mm diam, with fewer sulcae, the sclerotia tending to be darker or reddish orange when present, exudate droplets light yellow to red when present, reverse less pigmented in some strains and then Yellowish White (1–4A2–3).

Colonies on MEA after 7 d at 25 °C: 15–32 mm diam, planar, strictly velutinous when sporulating, orange sclerotia produced by some strains, in some strains with yellow sclerotia towards the centre, conidia of medium abundance, Grey (25–28D–F1) or Greyish Green (25–28C2), aerial mycelium sparse or absent, exudates not produced, margin entire, reverse Orange (6B7–8), Reddish Orange (7A–B7–8), Reddish Yellow (4A6) or Pale (1A2), soluble pigments not produced.

Colonies on YES after 7 d at 25 °C: (20–) 33–38 (–44) mm diam, dense and with 16–20 sulcae and 6–8 wrinkles present in some strains, light orange and orange sclerotia present in some strains, conidia Turquoise Grey (24B–F2), Grey (27B1), Greenish Grey (25–27C–D2), with raised concentric rings of aerial mycelium, exudates not produced, margin entire, reverse Reddish Yellow (4A6–7), Orange (7C8) turning Pastel Yellow (3A4) near the margins or Pale Yellow (4A3), soluble pigments not produced.

Colonies on CREA after 7 d at 25 °C: (13–) 20–28 mm diam, sclerotia present in some strains.

Conidiophores monoverticillate on MEA, borne directly from agar surface, stipes smooth to finely roughened, septate, (88–) 190–400 µm × 2–3 µm, unbranched, vesicles 4–7 µm wide (mean for different strains 4.6–5.9 ± 0.6). Phialides ampulliform to cylindrical, 8–11 × 2.5–3 µm, with short to long necks, periclinal thickenings not obvious. Conidia produced in columns, ellipsoidal, finely roughened, 2–3 µm diam (means for different strains = 2.5–3.0 ± 0.1 × 2–2.5 ± 0.1 µm, n = 25), mean L/W ratio 1.3:1. Sclerotia yellow, orange or reddish orange, subglobose to ellipsoidal, 180–320 µm diam; sclerotial cells 7–11 × 5–8 µm.

Habitat: Air, forest soil, berries of *Coffea arabica*.

Distribution: Asia (Indonesia), Australia, Pacifica (Hawai'i), but see notes below.

Typification: **Indonesia**, Java, Buitenzorg, isol. ex air, K.B. Boedijn, **holotype** IMI 40569 (not seen) **ex-type** NRRL 2074*, with equivalent culture collection numbers CBS 287.36, ATCC 10494. DNA barcodes: ITS JN626132, *cox1* JN626093.

Other cultures examined (Clade A): NRRL 32583, DAOM 239930, 239931*, 239932*. (Clade B): CBS 258.55, 126.65, 118889 (see Table 1).

* indicates sclerotium producing strains.

Notes: *Penicillium sclerotiorum* is the only species in the complex with clearly ellipsoidal conidia. About half the strains we examined

produced orange sclerotia, and as noted above in the analysis of morphological characters, the ex-type strain produces abundant sclerotia in some transfers, and few or none in others. When sclerotia are observed, strains need only be compared with *P. johnkrugii*, which differs in colony colours on CYA and CZ, and by its subglobose conidia. Because of the revision of the complex here, it is difficult to evaluate the geographical distribution of *P. sclerotiorum* as reported in the literature. However, even from our limited sampling it is clear that this species is relatively widely distributed in tropical and subtropical areas, and that it is usually associated with soil. Similarly, as reviewed in more detail in the Discussion, the metabolites attributed to this morphological species need to be reevaluated in light of the revised species concept.

A phylogenetic division within *P. sclerotiorum* was suggested by all gene trees, the first comprising the ex-type group (marked A in Figs 1, 2), and group B comprising strains that did not produce conidia or sclerotia. We excluded the latter from our morphological description of *P. sclerotiorum* above, which thus describes Clade A. Description of Clade B as a new species may be warranted if sporulating strains can be isolated.

Penicillium cainii K.G. Rivera, Malloch & Seifert, **sp. nov.** MycoBank MB563159. Figs 3D, 5.

Etymology: Named for Roy F. Cain, a faculty member of the University of Toronto, a world authority on coprophilous Ascomycota, who was the Ph.D. supervisor of David Malloch and John Krug.

Coloniae in agaro CYA post 7 dies ad 25 °C 23–29 mm diam, conidia veneta vel viridia, exudatum flavum, in reverso flaviscentes vel flavi-brunneum. Coloniae in agaro MEA post 7 dies ad 25 °C 28–35 mm diam. Conidiophora monoverticillata vel raro metula singula, stipites 69–79 µm × 2.5–3 µm, parietibus asperulatis, ad apicem in vesiculam 4–6 µm latam inflati; metulae adsunt 31–34 µm longae. Cellulae conidiogena phialidicae, ampulliformes, 7.5–10 × 2–3 µm. Conidia globosa vel subglobosa, levia vel plusminusve asperulata, 2.0–2.5 µm diam.

Colonies on CYA after 7 d at 25 °C: 23–29 mm diam, dense and velutinous, ca. 1 mm deep, with 14–18 sulcae and 1–2 radial wrinkles in some strains, sclerotia absent, conidia medium in abundance, Deep Blue to Deep Green (23–27E2), 1–2 mm of white mycelia at the margin, clear yellow exudates produced moderately by some strains, margin entire, reverse Golden Yellow (5B7) and Brownish Yellow (5C8–9), Yellowish White (2–3A2) towards the edges, soluble pigments not produced, margin entire. Colonies on CZ after 7 d at 25 °C: 18–21 mm diam, similar to colonies on CYA but lacking sulcae and radial wrinkles, conidia Turquoise Grey (24B–F2), with a concentric ring of paler shades of this colour towards the centre, margin entire, reverse Brownish Yellow (5–6C8), turning Yellowish Grey (3–4B2) towards the edges.

Colonies on MEA after 7 d at 25 °C: 28–35 mm diam, planar, dense and velutinous, sclerotia not produced, conidia Greenish Grey (28E2) and Dull Green (27C–D2–4), aerial mycelium not present, exudate not produced, margin entire, reverse Orange to Dark Orange (5A–B8), Yellowish White (2–3A2) towards the edges, and soluble pigments not produced.

Colonies on YES after 7 d at 25 °C: 31–34 mm diam, dense and velutinous, with 21–23 sulcae and 8–10 radial wrinkles, sclerotia not produced, conidia Greenish Grey (27D–F2), 2 mm of white mycelia at the margin, clear dark orange exudates droplets produced in negligible amounts in some strains, margin entire, reverse Brownish Orange to Reddish Orange (7B–D8), Greyish Brown (2B3) towards the edges, wrinkled towards the centre, soluble pigments not produced.

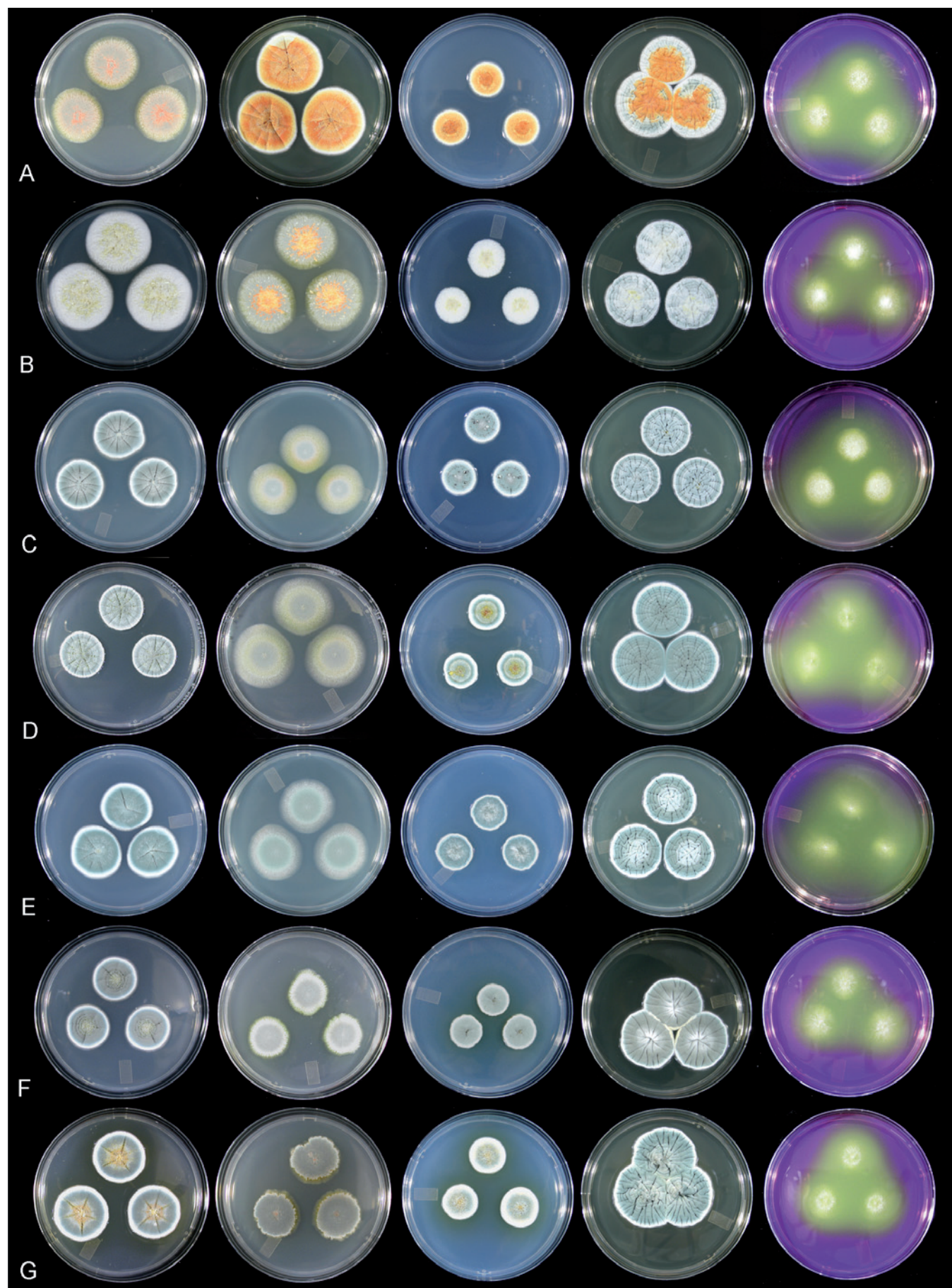


Fig. 3. Colonies of the seven species of the *P. sclerotiorum* complex grown for 7 d at 25 °C on five media, right to left CYA, MEA, CZ, YES and CREA. A. *P. sclerotiorum*. B. *P. johnkrugii*. C. *P. viticola*. D. *P. cainii*. E. *P. jacksonii*. F. *P. mallochii*. G. *P. guanacastense*. All ex-type strains except A DAOM 239931, C DAOM 239935.

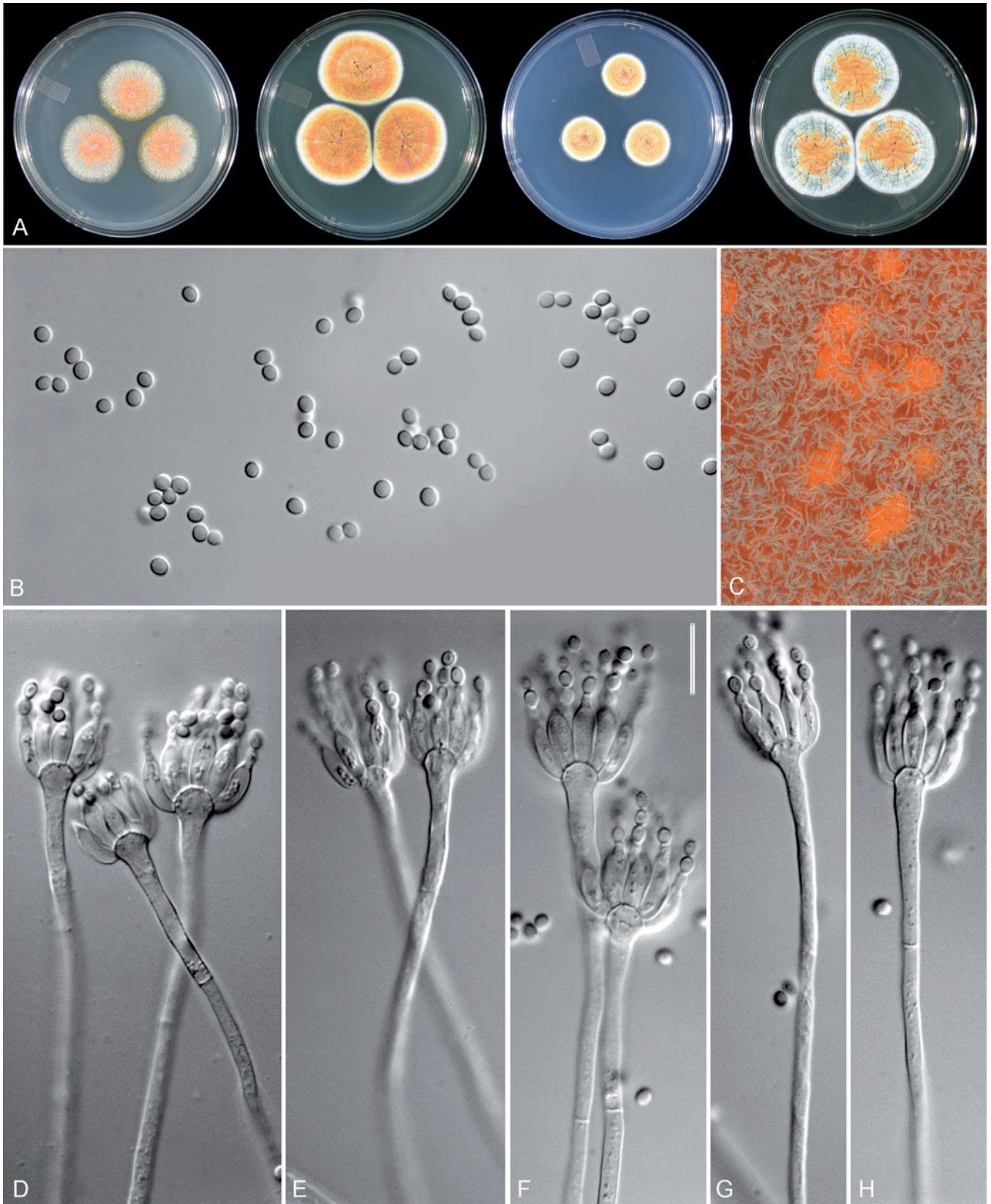


Fig. 4. *Penicillium sclerotiorum*, DAOM 239931. A. Colonies grown for 7 d on at 25 °C CYA, MEA, CZ, YES. B. Conidia. C. Close-up of colony on MEA, showing conidial columns and orange hyphae around sclerotia. D–H. Conidiophores. Scale bar in F = 10 µm for all micrographs.

Colonies on CREA after 7 d at 25 °C: (13–) 25–31 mm diam.

Conidiophores mostly monoverticillate on MEA, borne from agar surface, stipes rough-walled, septate, 70–80 µm × 2.5–3 µm, vesicle 3.5–5.0 µm wide, unbranched or in some strains with ca. 10 % of conidiophores with a single branch 31–34 µm long. Phialides ampulliform, 7.5–10 × 2–3 µm, with a distinguishable neck and inconspicuous periclinal thickening. Conidia globose,

finely roughened, 2.0–2.5 µm diam (mean for different strains = 2.3–2.4 × 2.1–2.2 ± 0.035 µm), mean L/W ratio 1.07:1.

Habitat: Nuts of *Juglans nigra* and *Carya ovata*.

Distribution: North America (Canada: Ontario).

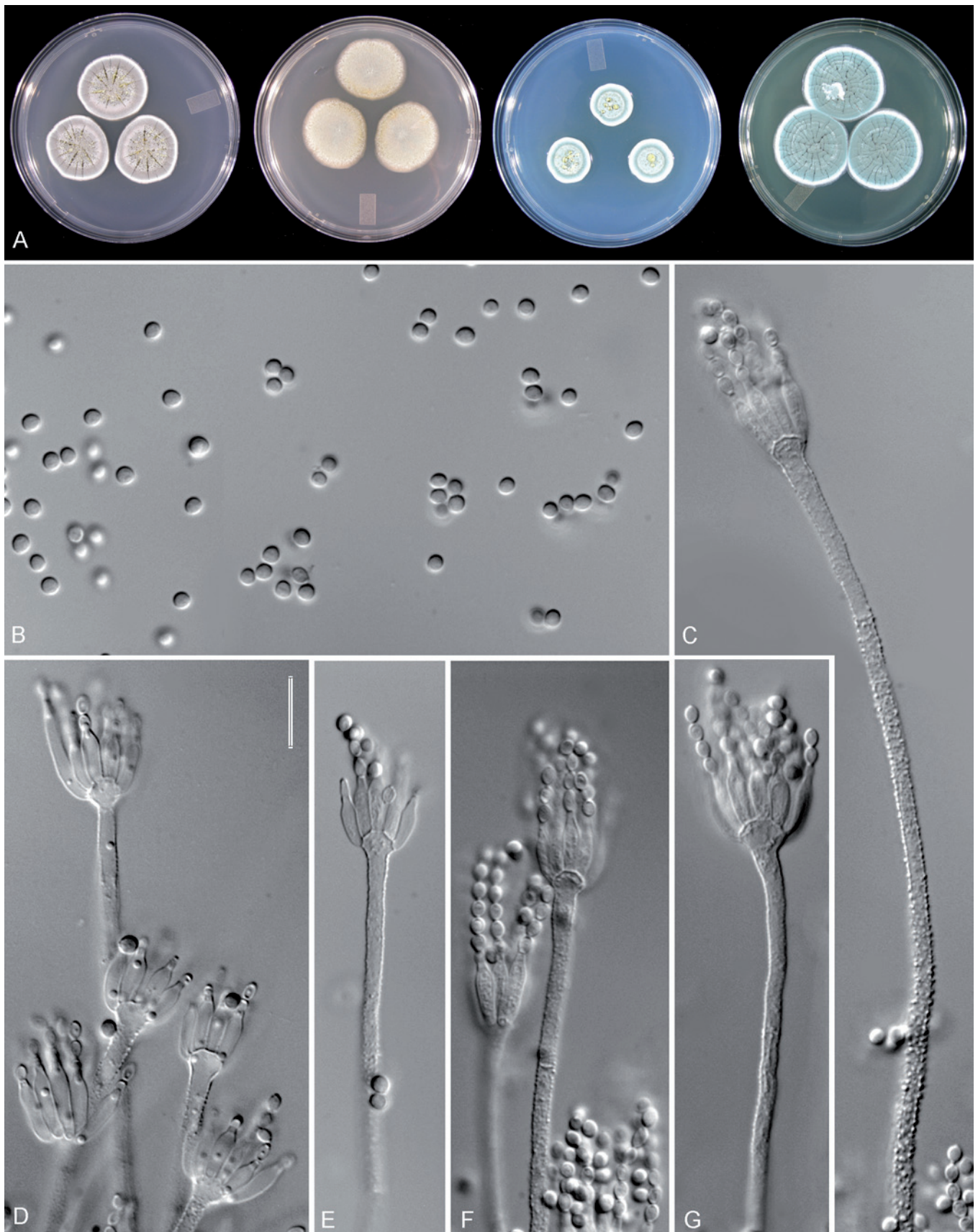


Fig. 5. *Penicillium cainii*. A. Colonies of DAOM 239915 grown for 7 d on at 25 °C CYA, MEA, CZ, YES. B–G. Microphotographs of ex-type strain. B. Conidia. C–G. Conidiophores. Scale bar in D = 10 µm for all micrographs.

Typification: **Canada**, Ontario, Niagara, Niagara Falls, Fireman's Park, N43° 08' 49" W79° 07' 04", isol. ex nuts of black walnut, *Juglans nigra*, D. Malloch W-10, May 1996, **holotype** DAOM 239914 (dried culture). The **ex-type** strain has the same accession number and is maintained in the Canadian Collection of Fungal Cultures. DNA barcodes: ITS JN686435, *cox1* JN686412.

Other culture examined: Same location and date as type, isol. ex nuts of shagbark hickory, *Carya ovata*, D. Malloch W-15, DAOM 239915.

Notes: *Penicillium cainii* produces short conidiophores with conspicuously roughened stipes, and these microscopic characters combined with the substrate of nuts of various species of the family

Juglandaceae make the species easily recognisable. Neither strain produced sclerotia. Colonies resemble those of *P. sclerotiorum*, *P. mallochii*, *P. guanacastense*, and *P. viticola* on CYA, MEA, and YES, but colonies on CZ differ by the production of a concentric ring of a light shade of turquoise grey and clear yellow exudate droplets in the centre of the colony. The vesicles terminating the monoverticillate conidiophores tend to be more clavate than swollen, but this is a difficult character to interpret.

Penicillium jacksonii K.G. Rivera, Houbraken & Seifert, **sp. nov.** MycoBank MB563160. Figs 3E, 6.

Etymology: Named for H.S. Jackson, faculty member at the University of Toronto, an authority on rusts, but an avid collector of all fungi, and the Ph.D. advisor of R.F. Cain.

Coloniae in agar CYA post 7 dies ad 25 °C 30–33 mm diam, conidia viridia, exudatum flavum, in reverso flaviscentes. Coloniae in agar MEA post 7 dies ad 25 °C 31–37 mm diam. Conidiophora monoverticillata vel modice metula singula, stipites 83–134 × 2–3 µm, parietibus plusminusve asperulatis, apicem in vesiculam 3–6 µm latam inflati; metulae adsunt 28–48 µm longae. Cellulae conidiogenae phialidicae, ampulliformes, 6–5 (–13) × 2–3 µm. Conidia globosa vel subglobosa, levia vel plusminusve asperulata, 2.5–3 µm diam.

Colonies on CYA after 7 d at 25 °C: 30–33 mm diam, dense and velutinous, ca. 1 mm deep, with 10–11 sulcae but no radial wrinkles, conidia produced abundantly, Deep Green (25–26E2), with concentric rings of paler shades of these colours, aerial mycelium present towards the centre, clear yellow exudate produced sparsely by some strains, with a margin 1–3 mm of white mycelia, margin entire, reverse Yellow (3A6–7), Vivid Yellow (2–3A8), or near the edges Yellowish White (2A2), soluble pigments not produced. Colonies on CZ after 7 d at 25 °C: 19–23 (–30) mm diam, similar to colonies on CYA but lacking sulcae, conidia Greenish Grey (26–27E2), aerial mycelium present throughout the colony, one strain floccose near the centre, with 1–2 mm of white mycelia near the margin, reverse Brownish Yellow (5C8–9), Deep Yellow (4A8), Yellowish White towards the edges (1A2), soluble pigments not produced

Colonies on MEA after 7 d at 25 °C: 31–37 mm diam, planar and velutinous, moderately dense, sporulation dense, conidia Dull Green (26–27E3), with concentric ring near the edges of paler shades of these colours, margin entire, reverse pale.

Colonies on YES after 7 d at 25 °C: 30–32 mm diam, dense, velutinous, with 8–10 sulcae in the centre and 19–23 sulcae at the margin, and 6–9 radial wrinkles, sporulation good, conidia Greenish Grey (25E2), with concentric rings of paler shades of this colour present in some strains, aerial mycelia absent or white patches present occasionally, margin entire, reverse Yellowish White (3A2) or Reddish Yellow (4A6), soluble pigments not produced.

Colonies on CREA after 7 d at 25 °C: 26–33 mm diam.

Conidiophores monoverticillate and/or once-branched on MEA, borne from agar surface, stipes smooth to rough, septate, 80–135 × 2–3 µm, vesicle 3–6 µm wide (mean for different strains 4.4–4.7 ± 0.4 µm), in some strains all unbranched, in others with ca. 65 % of conidiophores with a single branch 28–48 µm long. Phialides ampulliform, 6–5 (–13) × 2–3 µm wide, with distinguishable collarette and inconspicuous periclinal thickening. Conidia globose, walls finely roughened, 2.5–3 µm diam (mean for different strains 2.8 × 2.5–2.6 ± 0.035 µm), mean L/W ratio 1.1:1.

Habitat: Forest soil.

Distribution: Queensland, Australia.

Typification: **Australia**, Queensland, Barrine Lake, S17° 15' 1" E145° 38' 7" E, isol. from soil pretreated with ethanol, Sept. 2006, leg. J. Houbraken, L. Janson, **holotype** DAOM 239937 (dried culture). The **ex-type** strain has the same accession number and is maintained in the Canadian Collection of Fungal Cultures. DNA barcodes: ITS JN686437, *cox1* JN686414.

Other culture examined: Same data as type, DAOM 239938.

Notes: *Penicillium jacksonii* produces short conidiophores, but otherwise has few microscopic characters that clearly distinguish it from related species. It is unusual in the *P. sclerotiorum* complex for the production of a high proportion of conidiophores with a single branch, but this is an inconsistent character and some transfers are strictly monoverticillate. Colonies of *P. jacksonii* colonies are similar to those of *P. mallochii*, *P. guanacastense*, *P. viticola*, and *P. cainii* on CYA, MEA, and on YES, but the conidia are perhaps the darkest green of the species. The most closely related species, *P. cainii* (Fig. 2), has conspicuously roughened conidiophores.

Penicillium johnkrugii K.G. Rivera, Houbraken & Seifert, **sp. nov.** MycoBank MB563161. Figs 3B, 7, 8.

Etymology: Named for John Krug, faculty member at the University of Toronto and a research associate at the Royal Ontario Museum (ROM). Like his PhD supervisor, RF Cain, he was a specialist on coprophilous fungi, but also an avid lichen collector. He introduced KGR to the world of fungal taxonomy.

Coloniae in agar CYA post 7 dies ad 25 °C 30–38 mm diam, alba, conidia sparsa, sclerotia abundans, grisea vel aurantia, ca. 135–550 × 130–430 µm, exudatum plusminusve flavum, in reverso aurantiae vel flaviscentes. Coloniae in agar MEA post 7 dies ad 25 °C 26–36 mm diam. Conidiophora monoverticillata, stipites 88–229 µm × 2–2.5 µm, parietibus plusminusve asperulatis, apicem in vesiculam 4–6 µm latam inflati. Cellulae conidiogenae phialidicae, ampulliformes, 7–11 × 2–3 (–5) µm. Conidia globosa vel subglobosa, levia vel plusminusve asperulata, 2–3 µm diam.

Colonies on CYA after 7 d at 25 °C: 30–38 mm diam, planar, surface mycelia White (1–3A1), ca. 1 mm deep, with 5–9 sulcae and 2–3 (–9) radial wrinkles, sclerotia white, conidia not produced, light yellow exudate droplets produced in low to moderate amounts, margin entire, reverse Reddish Yellow to Orange (4–6AB7–8) and Yellowish Grey (3–4B2) towards the edges, stellate with radial wrinkles, soluble pigments absent. Colonies on CZ after 7 d at 25 °C: 15–23 mm diam, occasionally 10–19 mm diam, velutinous and dense, 4–6 sulcae and 1–2 radial wrinkles present in some strains, sclerotia white, with sparse to negligible sporulation, conidia Greenish Grey (25–27D2), light yellow exudates droplets sparsely produced, reverse Yellow to Deep Yellow (3A6–8) and Orange (5–6B8), some strains Yellowish White (3A3) or Pastel Yellow (3A4) towards the centre, soluble pigments absent.

Colonies on MEA after 7 d at 25 °C: 26–36 mm diam, planar, low and velutinous, sclerotia orange, yellow towards the centre, and white towards the margin, conidia Greenish Grey (24–27DE 3–5), conidia and sclerotia sectoring in some strains, aerial mycelium not present, exudates not produced, margin entire, reverse Orange (5–6B8), Vivid Yellow (3A8), Yellowish Grey (3–4B2) towards the centre, soluble pigment not produced.

Colonies on YES after 7 d at 25 °C: 28–38 mm diam, velutinous, with 11–17 sulcae and 6–10 radial wrinkles, sclerotia white, sporulation poor, conidia Greenish Grey (25D2), yellow exudate droplets produced sparsely by some strains, white mycelia

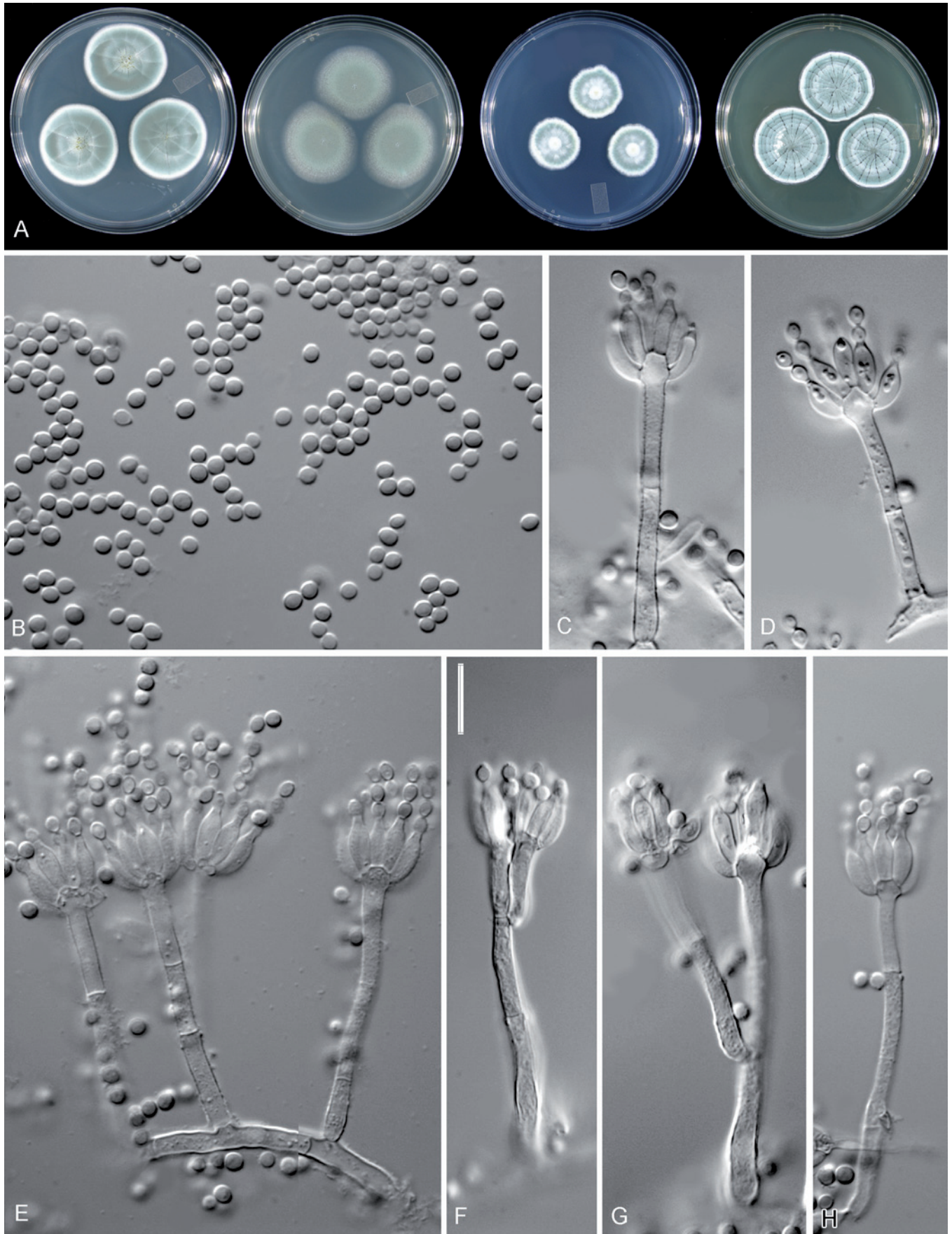


Fig. 6. *Penicillium jacksonii*. A. Colonies of DAOM 239938 grown for 7 d on at 25 °C CYA, MEA, CZ, YES. B–H. Microphotographs of ex-type strain. B. Conidia. C–H. Conidiophores. Scale bar in F = 10 µm for all micrographs.

at the marginal 1 mm, margin entire, reverse Yellow (3A3–4) and Orange (5–6B8), some strains Pastel Yellow (3A3–4) towards the edges, stellate with radial wrinkles or wrinkled, soluble pigments not produced.

Colonies on CREA after 7 d at 25 °C: 14–22 mm diam, sclerotia present.

Conidiophores strictly monoverticillate on MEA, borne from agar surface, stipes smooth to finely roughened, septate, 85–230

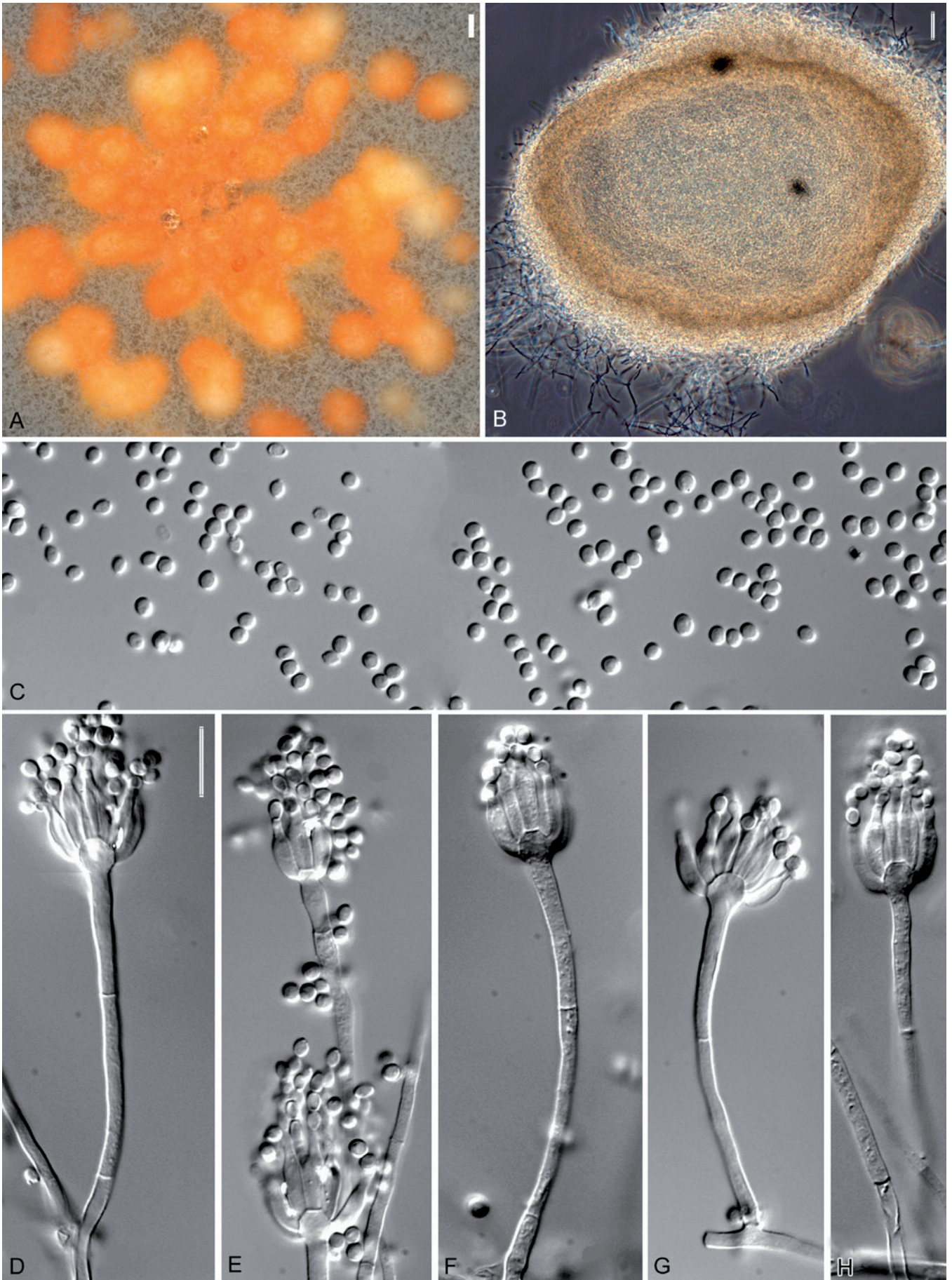


Fig. 7. *Penicillium johnkrugii*, ex-type strain A. Sclerotia on MEA. B. Micrograph of sclerotium. C. Conidia. D–H. Conidiophores. Scale bars A = 200 μm , B = 20 μm , in D = 10 μm for micrographs (C–H).

$\times 2\text{--}2.5$ μm , vesiculate, 4–6 μm wide (means for different strains 4.6–5.4 ± 0.3). Phialides ampulliform to cylindrical, 7–11 $\times 2\text{--}3$ (–5)

μm , with short to distinguishable necks, periclinal thickenings not obvious. Conidia globose to subglobose, finely roughened, 2–3 μm

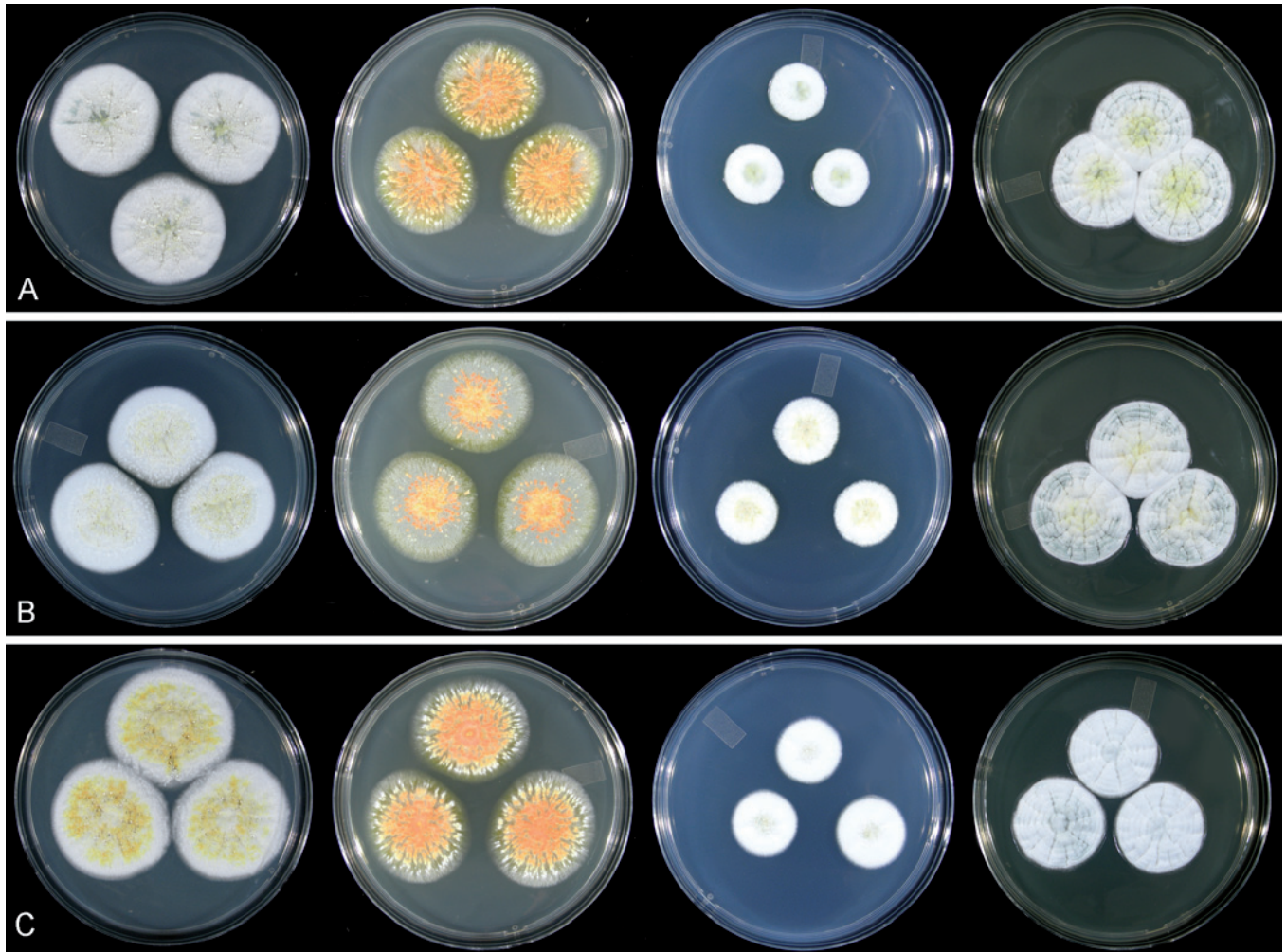


Fig. 8. *Penicillium johnkrugii*, three strains grown for 7 d on at 25 °C CYA, MEA, CZ, YES. A. DAOM 239942. B. DAOM 239943 (T). C. DAOM 239944.

diam (means for different strains $2.7\text{--}2.9 \pm 0.01 \times 2.3\text{--}2.5 \pm 0.01$ μm , $n = 25$), mean L/W ratio 1.1:1. Sclerotia produced on all media, subglobose to ellipsoidal, at first white, becoming orange or yellow, $136\text{--}552 \times 131\text{--}433$ μm , sclerotial cells $5\text{--}8 \times 3\text{--}6$ μm .

Habitat: Forest soil.

Distribution: Langkawi, Malaysia.

Typification: **Malaysia**, Kedah, Langkawi, N 6° 19' 24" E 99° 51' 45", isol. ex soil after ethanol treatment, Nov. 2007, leg. R.A. Samson, isol. J. Houbraeken, **holotype** DAOM 239943 (dried culture). The **ex-type** strain has the same accession number and is maintained in the Canadian Collection of Fungal Cultures. DNA barcodes: ITS JN686447, *cox1* JN686424.

Other cultures examined: Same data as type and presumably from the same soil sample, DAOM 239939, DAOM 239940, DAOM 239941, DAOM 239942, DAOM 239944, DAOM 239945, DAOM 239946.

Notes: *Penicillium johnkrugii* is distinct within the *P. sclerotiorum* complex for its production of white colonies and abundant grayish sclerotia on CYA and CZ; on other media, the sclerotia tend to be yellow or orange. The species is morphologically similar to *P. sclerotiorum*. Both have vivid yellow to orange reverse colony colours although *P. johnkrugii* differs by subglobose conidia and more conspicuously vesiculate conidiophores.

Penicillium johnkrugii and *P. mallochii* were paraphyletic in some analyses of *benA*, *cox1*, and ITS. Monophyletic recognition of the species occurred in all *tef1-a* analyses, and in the MP and

ML trees for ITS, and ML and BI trees for *cmd*. Despite these phylogenetic issues, all genes provided diagnostic sequences for *P. johnkrugii*, and the species is morphologically distinct.

The strain DAOM 239944 did not cause any colour changes when grown on CREA.

Penicillium viticola Nonaka & Masuma, *Mycoscience* 52: 339. 2011. MycoBank MB516048. Figs 3C, 9.

Colonies on CYA after 7 d at 25 °C: 26–30 (–36) mm diam, dense and velutinous, ca. 1 mm deep, with 6–13(–17) sulcae and 2–3 radial wrinkles, sclerotia absent, conidia Greenish Grey (25–27E2), with Bluish Grey (22D–F2) concentric rings, pale yellow exudate droplets produced sparsely by some strains, with white aerial mycelium in the central 2–3 mm and marginal 2–3 mm, margin entire, reverse Light Yellow (5C8), Yellowish Grey (3–4B2), Orange Grey (6B7–8), sometime Yellowish Grey (3–4B2) or Yellowish White (1A2–3) near the margin, stellate with some radial wrinkles, soluble pigments not produced. Colonies on CZ after 7 d at 25 °C: 17–25 mm diam, similar to those on CYA, but some strains floccose at the centre, with 5–6 sulcae and 2–3 wrinkles in some strains, mycelia at margin 1–2 mm, reverse Reddish Yellow (4A7) and Brownish Yellow (5C8) in some strains.

Colonies on MEA after 7 d at 25 °C: 23–35 (–40) mm diam, planar and moderately dense, velutinous, sclerotia not observed, conidia Greenish Grey (25–27E2), aerial mycelium not observed, exudates not produced, margin entire, reverse Orange Yellow to Orange (4–6B7–8), Brownish Orange (7C7–8), Pastel Yellow

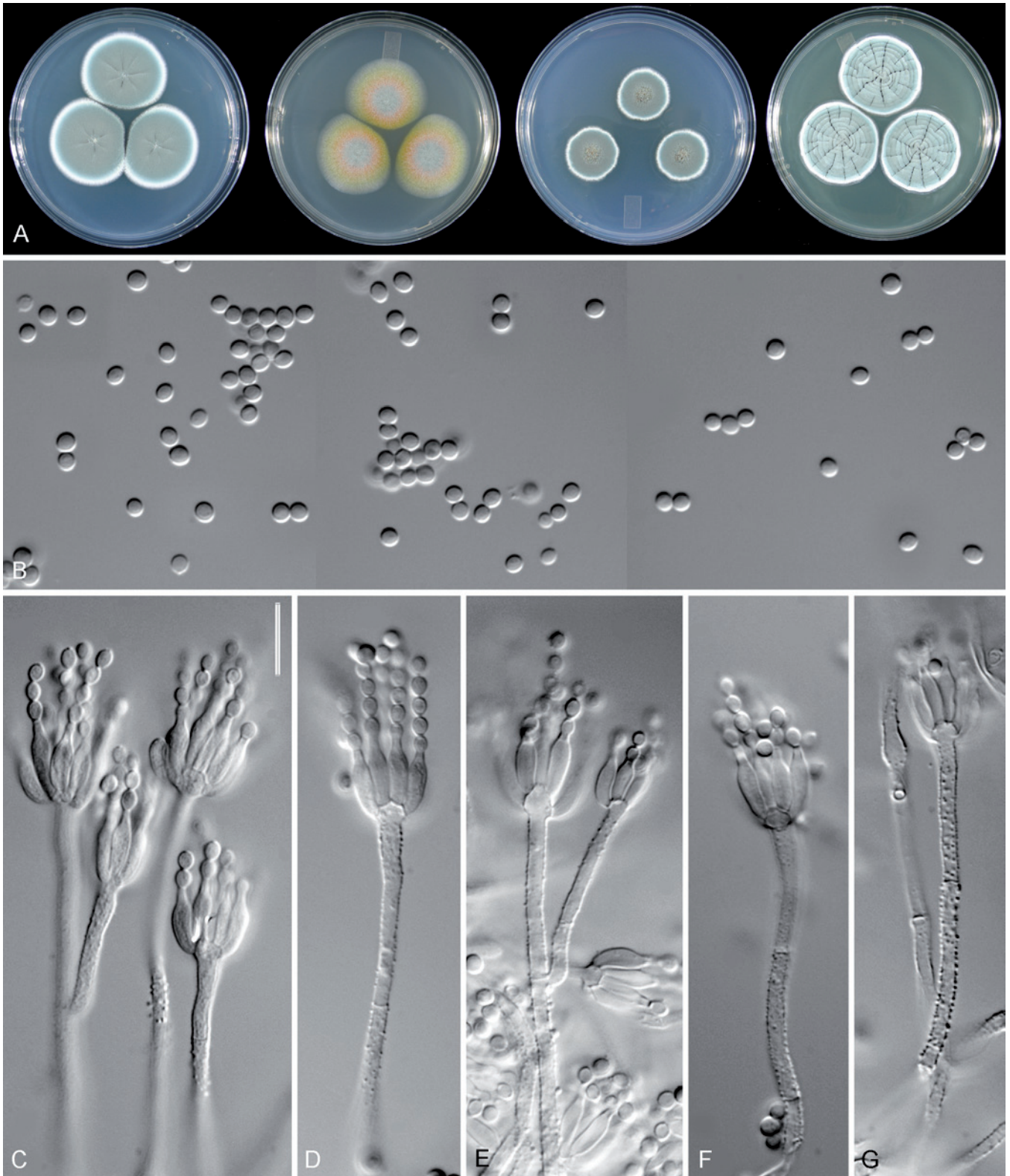


Fig. 9. *Penicillium viticola*. A. Colonies of DAOM 239933 grown for 7 d on at 25 °C CYA, MEA, CZ, YES. B–G. Microphotographs of DAOM 239935. B. Conidia. C–G. Conidiophores. Scale bar in C 10 μ m for all micrographs.

or Reddish Yellow (4A6–7) near the edges, soluble pigment not produced.

Colonies on YES after 7 d at 25 °C: 29–33 mm diam, less commonly 34–36 mm diam, dense and velutinous, densely sulcate, especially near the edges (with 15–21 sulcae), with 7–9 radial wrinkles, sclerotia not produced, conidia Greenish Grey (25–26D–E2) and Turquoise Grey (24D2), concentric rings of different shades of turquoise grey present in some strains, white mycelia 2–3 mm at margins, clear vivid yellow exudate droplets produced sparsely by some strains, margin entire, reverse Reddish Yellow (7B8), Orange

(6B8), Greyish Yellow (2–4B3), some strains Yellowish Grey (2B–C3) or Dull Yellow (3B3–4) towards the edges, wrinkled towards the centre with visible sulcae and radial wrinkles towards the margin, soluble pigment not observed.

Colonies on CREA after 7 d at 25 °C: 23–29 mm diam.

Conidiophores predominantly monovercillate on MEA, borne from agar surface, stipes rough, septate, 30–135 \times 2–3 μ m wide, moderately vesiculate, vesicles 4–6 μ m wide (means for different strains 4.6–5.0 \pm 0.3 μ m), mostly unbranched, sometimes with about 10 % of conidiophores with a single branch 22–55 \times 2–3 μ m

wide. Phialides ampulliform, 7–10 × 2–3 µm, with a distinguishable collarette. Conidia borne in columns, globose, smooth, 2–3 µm diam (means for different strains 2.6–2.7 × 2.3–2.6 ± 0.01 µm), mean L/W ratio 1.1:1.

Typification: Japan, Yamanashi, isol. ex grape (*Vitis* sp.), 11 Jul 2006, K. Nonaka, **holotype** TNS-F-38702, **ex-type** culture JCM 17636 = FKI-4410 (not seen). DNA barcode: ITS AB606414 (Nonaka *et al.* 2011).

Other cultures examined: DAOM 239933, DAOM 239934, DAOM 239935, DAOM 239936, see Table 1 for details.

Habitat: Rainforest soil, and vines of *Vitis* sp.

Distribution: Asia (Japan), Australia (Queensland).

Notes: *Penicillium viticola* produces short, rough-walled conidiophores, slightly less roughened than those of the closely related species *P. cainii*, but rougher than all other species of the complex. The colony characters of *P. viticola* colony morphology resemble those of *P. mallochii*, *P. guanacastense*, *P. cainii*, and *P. sclerotiorum* on CYA and MEA. On CZ, it resembles *P. mallochii* and *P. guanacastense*. Microscopically it lacks the more vesiculate conidiophore apices observed in *P. mallochii*, *P. guanacastense*, and *P. sclerotiorum*.

Penicillium viticola was described by Nonaka *et al.* (2011) when our own revision was completed, and we were unable to examine the type strain. Their description matches ours in most respects, with only minor differences in growth rates, colony colours and exudate production that may be attributed to subtle variations in media. The published calmodulin tree in Nonaka *et al.* (2011) inadvertently included a mislabeled sequence for *P. viticola* that indicated a relationship with *P. angulare*; the sequence deposited by the authors (AB540173) is correct (R. Masuma, pers. comm.). With the *benA* sequence for the type strain (AB540174), this species is firmly established as a member of the *P. sclerotiorum* complex, and conspecific with our Australian strains, as it is treated here.

Iwatsuki *et al.* (2010) reported the production of tropolone compounds by the ex-type strain of *P. viticola* (in the publication named only as *Penicillium* sp.), including the anti-malarial compound puberulic acid, stipitatic acid and their novel analogues, viticolins A–C.

DISCUSSION

The *Penicillium sclerotiorum* complex now includes seven phylogenetically distinct but morphologically similar species. In this paper, three species in the *P. sclerotiorum* complex were described, added to the two species recently described from Costa Rica (*P. guanacastense*, *P. mallochii*; Rivera *et al.* 2011) and one recently described from Japan (*P. viticola*, Nonaka *et al.* 2011). Members of the complex can be recognised by the general suite of characters used by previous authors to delineate the species, namely moderately fast growing colonies on CYA and MEA, with a tendency to produce orange or reddish colony reverses, monoverticillate conidiophores with some conidiophores having a single branch, vesiculate conidiophore apices, and globose to ellipsoidal conidia in greyish green, dull green, or turquoise green colours. Careful examination of colony and microscopic characters revealed subtle morphological differences among species. The species differ by some conidial colours, variation in the roughening of the stipe, stipe

lengths, and inconspicuously in the extent of vesiculation of the conidiophore apex and in conidial shape. Sclerotia are produced abundantly and consistently by *P. johnkrugii*, in most but not all fresh strains of *P. sclerotiorum*, and have not been seen in the other species. Sclerotium production is a difficult taxonomic character, because they may be constant or inconstant within a species or even a strain. Physiological experiments with *Aspergillus caelatus* show that a pH of 6–10 and temperatures of 28–30 °C are optimal for sclerotia formation, production peaks at a C:N ratio of 8.6, and the sugar used in CYA (sucrose) suppresses production by 12 % (McAlpin 2004). Similar results have been seen in other *Aspergillus* species (Rudolph 1962), *Sclerotinia rolfii* (Wheeler & Sharan 1965), and *Verticillium* species (Wyllie & DeVay 1970).

Five of the species so far have restricted geographical distributions, but sampling is still very meagre. *Penicillium johnkrugii* was isolated from soil from Langkawi, Malaysia, *P. jacksonii* from soil from Queensland, Australia, *P. cainii* from nuts collected in Niagara Falls, Ontario, while *P. mallochii* and *P. guanacastense* were isolated from the guts of two different caterpillar families reared in the Área de Conservación Guanacaste, Costa Rica. *Penicillium sclerotiorum* has the broadest known distribution (Table 1), while *P. viticola* has a disjunct distribution (soil from Australia, grapes from Japan) that hints at a broader distribution and a so-far undefined ecology.

Extrolite profiling is a common practice for characterising *Penicillium* species and contributed significantly to developing polyphasic species concepts in *Penicillium* subgenus *Penicillium* (Frisvad & Samson 2004). We were unable to study extrolites in this study, but there are indications that the *P. sclerotiorum* complex may be metabolically diverse, providing further characters for delimiting these morphologically similar species. Secondary metabolites with antibacterial and antifungal activities were reported for some strains identified as *P. sclerotiorum sensu lato*, including the antimicrobial compounds sclerotin (Curtin & Reilly 1940), isochromphilone VI and pencolide (De Lucca *et al.* 2008). Pairet *et al.* (1995) reported two azapholines from *P. sclerotiorum* as antagonists of endothelin-A and endothelin-B receptors, vasoconstriction peptides implicated in hypertension, heart and renal failure, ischemia, and cerebral vasospasms. We could not trace strains from these studies and therefore it is unclear what phylogenetic species produce these compounds. As noted above, only the production of puberulic acid, stipitatic acid and viticolins A–C can be attributed with any certainty to the phylogenetically defined *P. viticola*, but only the ex-type has been examined for these metabolites (Iwatsuki *et al.* 2010).

Phylogenetic analyses using maximum parsimony, maximum likelihood, and Bayesian inference algorithms, and DNA sequence data *benA*, ITS, *cox1*, *tef1-α*, and *cmd*, gave consistent results. The less variable genes did not provide robust statistical support for all species of the complex, but the results did not conflict with the other gene trees. The recognised species generally conform to the Genealogical Concordance Phylogenetic Species Recognition (Taylor *et al.* 2000) concept, although some of the species are only represented by two strains. There were problems with paraphyly of *P. johnkrugii* and *P. mallochii* in some *benA*, *cox1*, and ITS analyses, but all five genes yielded species specific sequences for all species, and each species formed a cohesive (if not strictly monophyletic) group that reaffirmed the species concepts proposed here. We have listed accession numbers of ITS and *cox1* barcodes in the paragraphs on Typification above, although all tested genes would work as barcodes in this complex.

The partition homogeneity test (PHT) indicated that the *cmd* was incongruent with the other genes, and this gene was not included

in combined phylogenetic analyses. Incongruency in phylogenetic signal between genes suggests different evolutionary histories for these genes (Scott *et al.* 2006). Visual comparisons of the *cmd* results suggest that the variable position of *P. guanacastense* within the ingroup, and a generally discordant arrangement of outgroups, probably led to the failure of the PHT. From the perspective of recognising phylogenetic species, the species groupings remained constant for all genes. From the perspective of accurately determining sister group relationships among species, the rejection of *cmd* is unfortunate.

The designation of *P. adametzioides* and *P. multicolor* as synonyms of *P. sclerotiorum*, as proposed by Stolk & Samson (1983), was not accepted here. *Penicillium adametzioides* is a distinct phylogenetic species, also noted by Peterson (2000) and accepted by Houbraken & Samson (2011). According to our molecular data, *P. multicolor* is a synonym of *P. fellutanum*, but the degenerated morphology of the ex-type strain we examined leaves this conclusion tentative.

KEY TO SPECIES

1. Conidiophore stipes distinctly roughened 2
 1. Conidiophore stipes at most finely roughened 3
 2. On CYA conidial colours with a blue tinge, colonies usually less than 30 mm diam; on nuts *P. cainii*
 2. On CYA, conidia colours lacking blue tinge, colonies 26–36 mm diam; on grape vines or in soil *P. viticola*
 3. Sclerotia produced 4
 3. Sclerotia not produced 5
 4. Colonies on CYA white, sclerotia white or grey, turning orange on MEA; conidia globose or subglobose *P. johnkrugii*
 4. Colonies on CYA with obvious green areas, sclerotia orange; conidia subglobose to ellipsoidal *P. sclerotiorum*
 5. Associated with caterpillars feeding on leaves in the neotropics; colonies on MEA crustose 6
 5. Soilborne; colonies on MEA not crustose 7
 6. Associated with Saturnid caterpillars; conidiophores 50–380 µm long *P. mallochii*
 6. Associated with Noctuid caterpillars; conidiophores 85–100 µm long *P. guanacastense*
- Note: Colony photographs of *P. guanacastense* and *P. mallochii* on CYA, MEA, CZ, YES and CREA are included in Fig. 3; the species are described in Rivera *et al.* (2011).
7. Colony reverse colours on CYA and MEA pale; some strains with > 50 % of conidiophores with a single branch, other strains all monoverticillate; conidia globose *P. jacksonii*
 7. Colony reverse colours on CYA and MEA yellow, orange or in vivid red colours; conidiophores strictly monoverticillate; conidia subglobose to ellipsoidal *P. sclerotiorum* (non-sclerotial strains)

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