Mycol Progress (2015) 14: 96
DOI 10.1007/s11557-015-1118-z

ORIGINAL ARTICLE

Four new *Penicillium* species isolated from the fynbos biome in South Africa, including a multigene phylogeny of section *Lanata-Divaricata*

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Received: 4 May 2015 / Revised: 7 September 2015 / Accepted: 14 September 2015 / Published online: 28 September 2015

Abstract A survey of the fynbos biome of South Africa resulted in the isolation and characterization of 61 distinct *Penicillium* species. ITS barcodes place six of these in section *Lanata-Divaricata*. Based on morphology and multigene phylogenies, species were identified as *P. oxalicum*, *P. skrjabinii*, and four that were previously unknown. Multigene phylogenies resolve the new species into three consistent clades. Species from these clades were compared morphologically, and the new species are described here as *P. annulatum*, *P. curticaule*, *P. malacosphaerulum,* and *P. ortum. Penicillium annulatum* (CBS 135126^T) characteristically produces colonies on Czapek yeast autolysate (CYA) and yeast extract sucrose (YES) agar that sporulate in annular patterns and grow faster on CYA at 37 °C than those of closely related species, except for the faster growing *P. rolfsii. Penicillium curticaule* (CBS 135128^T) most closely resembles *P. raperi*, but grows more restrictedly on CYA at 37 °C and produces shorter phialides. *Penicillium ortum* (CBS135668^T) grows faster on CYA at 37 °C than *P. cremeogriseum*, its closest relative. *Penicillium malacosphaerulum* (CBS 135120^T) produces cleisthothecia that give colonies on most media a yellowish straw color, similar to those of *P. reticulisporum*. However, faster growth on CYA at 37 °C distinguishes the new species from *P. reticulisporum*.

Keywords *Penicillium janthinellum* · *Penicillium simplicissimum* · Iinternal transcribed spacer region · β-tubulin · Calmodulin · *RPB2*

Introduction

The use of multigene phylogenies to address complex taxonomic issues is the standard in mycology and currently carries

Section Editor: Roland Kirschner

Taxonomic novelties — *Penicillium annulatum* Visagie & Jacobs, *P. curticaule* Visagie & Jacobs, *P. malacosphaerulum* Visagie & Jacobs, *P. ortum* Visagie & Jacobs

Electronic supplementary material The online version of this article (doi:10.1007/s11557-015-1118-z) contains supplementary material, which is available to authorized users.

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the most weight in the polyphasic species concept used for *Penicillium*. This is clear from the work of Houbraken and Samson (2011), who reclassified *Penicillium* into 25 sections based on a four-gene phylogeny. It supersedes the subgeneric classifications of Thom (1930), Raper and Thom (1949), and Pitt (1979), which were based on conidiophore branching patterns. In the DNA barcoding age, which gives scientists in a broad range of disciplines the chance of correct identifications, a sectional classification based on sequence data was an important and invaluable step towards a user-friendly taxonomy for *Penicillium*. Following this sectional classification, Visagie et al. (2014a) updated the accepted species list for *Penicillium* and included reference sequences for ex-type strains of the internal transcribed spacer region (ITS), β-tubulin (*BenA*), calmodulin (*CaM*), and the DNA-dependent RNA polymerase II second largest subunit (*RPB2*) gene regions, where available. Also, *BenA* was proposed as a suitable secondary identification marker to supplement the official DNA barcode ITS (Schoch et al. 2012), which is often not diagnostic for closely related species of *Penicillium*.

Thom (1930) introduced section *Lanata-Divaricata* for species with biverticillate conidiophores that usually contain an elongation of the conidiophore's main axis and metulae that diverge from the axis to form an asymmetrical verticil. As a result, conidiophores can often be interpreted as monoverticillate, although they are in most cases divergently branched biverticilliate conidiophores (also termed divaricate). This group of species is commonly isolated from soil (Thom 1930; Raper and Thom 1949; Pitt 1979; Ramírez 1982; Christensen et al. 2000), but some are frequent on rotting leaf litter (Houbraken et al. 2011). Identifications in the section were traditionally very difficult using morphology. *Penicillium janthinellum*, the sectional type, and *P. simplicissimum*, are prime examples. Both were previously characterized by broad species concepts. Pitt (1979) synonymized nine species with *P. janthinellum* and ten with *P. simplicissimum*. In another study, Stolk and Samson (1983) synonymized *P. janthinellum* and *P. simplicissimum* and linked them and 24 other species to the teleomorph name *Eupenicillium javanicum* (≡ *P. javanicum*). However, phylogenetic data showed that *E. javanicum sensu lato* represents a complex including many distinct species (Peterson 2000; Tuthill et al. 2001; Houbraken et al. 2011; Houbraken and Samson 2011), even though consistent morphological differences are difficult to ascertain. Houbraken et al. (2011) accepted 36 species in the section based on ITS and *BenA* phylogenies. Unfortunately, Houbraken et al. (2011) did not include phylogenies for calmodulin (*CaM*) and DNA-dependent RNA polymerase II second largest subunit (*RPB2*) and we thus considered it advantageous to complete the four-gene data set commonly used in *Penicillium* for Genealogical Concordance Phylogenetic Species Recognition (GCPSR).

Recently, biodiversity surveys from unique habitats and underexplored countries resulted in description of several new species. One of these unique regions is the fynbos biome situated at the southwestern tip of South Africa. The biome is listed as a UNESCO world heritage site because of its immense biodiversity (Myers et al. 2000), and it boasts more than 9000 plant species, accounting for 44 % of the South African floral inventory (Goldblatt and Manning 2002; Mucina and Rutherford 2006). Over the last couple of years, biodiversity has become an important subject in South Africa, especially in relation to climate change (Midgley et al. 2002; Malcolm et al. 2006). However, knowledge on *Penicillium* species from South Africa is poor (Schutte 1992), but recent surveys show *Penicillium* to be a dominant genus in the fynbos region (Allsopp et al. 1987; Visagie et al. 2009, 2013, 2014b; Visagie and Jacobs 2012).

During our survey in the fynbos, 61 *Penicillium* species were isolated. Six belong to section *Lanata-Divaricata*, of which four represent previously undescribed species. The aim of this paper is to characterize these using morphology and multigene phylogenies. The new species are compared to their closest relatives and notes provided on their identification. Species names associated with section *Lanata-Divaricata* are considered based on phylogenies from four genes and suggestions made to modify the accepted species list of *Penicillium* for this section (Visagie et al. 2014a).

Materials and methods

Sampling and isolations

Penicillium strains were isolated from three fynbos sites that represent unique fynbos types (Musina Mucina and Rutherford 2006). Soil, air, and *Protea repens* infructescence samples were collected from Stellenbosch (33°56'47"S 18°52'49"), Riverlands Nature Reserve near Malmesbury (33°29'46"S 18°35'16") and Struisbaai (33°45'06"S 18°58'59"). Isolations were made in similar fashion to methods described by Visagie and Jacobs (2012) and Visagie et al. (2014b).

Reference and ex-type strains were obtained from the public collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands, the working collection of the Applied and Industrial Mycology department (DTO) of the same institute and from the collection of the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Peoria, IL, USA(NRRL). Strains used in this study are listed in Table 1.

DNA extraction, sequencing, and phylogenetic analysis

DNA was extracted from cultures grown on MEA for 7 days using the ZR Fungal/Bacterial DNA Kit (Zymo Research,

Table 1 Strains used for phylogenetic analyses of *Penicillium* section *Lanata-Divaricata* strains included in the study (n.a. = not available)

			GenBank accession no.			
Species	Strains	Substrate and locality	ITS	*BenA*	*CaM*	*RPB2*
P. abidjanum	CBS 246.67^T=ATCC 18385=FRR 1156=IMI 136244	Savannah soil, near Abidjan, Ivory Coast	GU981582	GU981650	KF296383	JN121469
P. annulatum	CBS 135123=CV 548=DTO 181-I1	Soil, Stellenbosch, South Africa	JX091423	JX091516	JX141547	KF296412
	CBS 135124=CV 1707=DTO 183-E9	Soil, Struisbaai, South Africa	JX091424	JX091517	JX141548	KF296413
	CBS 135125=CV 187=DTO 181-C3	*Protea repens* infructescence, Stellenbosch, South Africa	JX091425	JX091515	JX141546	KF296411
	CBS 135126^T=CV 37=DTO 180-G7	Soil, Stellenbosch, South Africa	JX091426	JX091514	JX141545	KF296410
P. araracuaraense	CBS 113149^T=IBT 23247	Forest leaf litter, Araracuara, Colombia	GU981597	GU981642	KF296373	KF296414
P. brasilianum	CBS 253.55^T=ATCC 12072=FRR 3466	Herbarium specimen, Recife, Brazil	GU981577	GU981629	AB667857	KF296420
P. brefeldianum	CBS 235.81^T=NRRL 710=FRR 710=IFO 31731=IMI 216896=MUCL 38762	Human alimentary tract, unknown country	AF033435	GU981623	AB667857	KF296421
P. caperatum	CBS 443.75^T=ATCC 28046=DSM2209=NHL 6465	Soil, Murrumbidgee Irrigation Area, NSW, Australia	KC411761	GU981660	KF296392	KF296422
P. cluniae	CBS 326.89^T	Soil, Burgos close to Clunia, Spain	KF296406	KF296471	KF296402	KF296424
P. coeruleum	CBS 141.45^T=NCTC 6595	Unknown source	GU981606	GU981655	KF296393	KF296425
P. cremeogriseum	CBS 223.66^T=ATCC 18320=ATCC 18323=FRR 1734=IJFM 5011=IMI 197492=NRRL 3389	Forest soil, Kiev, Ukraine	GU981586	GU981624	KF296403	KF296426
P. curticaule	CBS 135127^T=CV 2842=DTO 180-D3=DAOM 241159	Soil, Malmesbury, South Africa	FJ231021	JX091526	JX141536	KF296417
	CBS 135128=CV 2857=DTO 184-D1	Soil, Malmesbury, South Africa	FJ231022	JX091523	JX141537	KF296419
	CBS 135129=CV 2858=DTO 180-F2	Soil, Malmesbury, South Africa	FJ231023	JX091522	JX141538	KF296418
P. daleae	CBS 211.28^T=ATCC 10435=FRR 2025=IFO 6087=IFO 9072=IMI 034910=MUCL 29234=NRRL 2025	Soil under conifer, Poland	GU981583	GU981649	KF296385	KF296427
P. ehrlichii	CBS 324.48^T=ATCC 10442=IMI 039737=IMI 039737ii=NRRL 708	Substrate unknown, Poland	AF033432	KF296464	KF296395	KF296428
P. elleniae	CBS 118135^T=IBT 23229	Forest leaf litter, Araracuara, Colombia	GU981612	GU981663	KF296389	KF296429
P. glaucoroseum	CBS 138908=NRRL 908	Soil, Virginia, USA	KF296407	KF296469	KF296400	KF296430
P. griseopurpureum	CBS 406.65^T=ATCC 22353=FRR 3429=IFO 9147=IMI 096157	Soil under *Pinus*, Lancashire, England	KF296408	KF296467	KF296384	KF296431
P. janthinellum	CBS 340.48^T=ATCC 10455=IMI 040238=NRRL 2016=QM 6865	Soil, Nicaragua	GU981585	GU981625	KF296401	JN121497
P. javanicum	CBS 341.48^T=ATCC 9099=FRR 707=IFO 31735=IMI 039733=MUCL 29099=NRRL 707	Root of Camellia sinensis, Indonesia, Java	GU981613	GU981657	KF296387	JN121498
	CBS 349.51	Ex-type of *P. oligosporum*, Substrate unknown, Japan	KC411752	KF296466	KF296388	KF296446
P. levitum	CBS 345.48^T=ATCC 10464=IFO 6101=IFO 8849=IMI 039735=NRRL 705	Modeling clay, USA	GU981607	GU981654	KF296394	KF296432
P. limosum	CBS 339.97^T	Marine sediment, Nagasaki prefecture, Japan	GU981568	GU981621	KF296398	KF296433
P. lineolatum	CBS 188.77^T	Soil from copse, Japan	GU981579	GU981620	KF296397	KF296434
P. ludwigii	CBS 417.68^T=FRR 559	Polished rice, Japan	KF296409	KF296468	KF296404	KF296435
P. koreense	KACC 46682	Soil, Korea	KM048199	KM000844	n.a.	n.a.
	KACC 47720	Soil, Korea	KM048200	KM000845	n.a.	n.a.
	KACC 47721^T	Soil, Korea	KJ801939	KM000846	n.a.	n.a.
	KACC 47722	Soil, Korea	KM048201	KM000847	n.a.	n.a.
P.malacosphaerulum	CBS 135120^T=CV 2855=DTO 180-E6=DAOM 241161	Soil, Malmesbury, South Africa	FJ231026	JX091524	JX141542	KF296438
	CBS 135121=CV 2836=DTO 180-D1	Soil, Malmesbury, South Africa	FJ231024	JX091525	JX141540	KF296436
	CBS 135122=CV 2848=DTO 180-E1	Soil, Malmesbury, South Africa	FJ231025	JX091527	JX141541	KF296437

Table 1 (continued)

			GenBank accession no.			
P. mariae-crucis	CBS 271.83^{T}=IMI 256075	Secale cereale, Spain	GU981593	GU981630	KF296374	KF296439
P. meloforme	CBS 445.74^{T}=ATCC 28049=IMI 216903	Soil, Papua New Guinea	KC411762	GU981656	KF296396	KF296440
P. ochrochloron	CBS 357.48^{T}=ATCC 10540=IMI 039806=NRRL 926	Copper sulphate solution, Washington, USA	GU981604	GU981672	KF296378	KF296445
P. onobense	CBS 174.81^{T}=ATCC 42225=IJFM 3026	Soil, Navarra, Spain	GU981575	GU981627	KF296371	KF296447
P. ortum	CBS 135667=CV 95=DTO 180-I7	Soil, Stellenbosch, South Africa	JX091430	JX091519	JX141550	KF296442
	CBS 135668=CV 71=DTO 180-H7	Soil, Stellenbosch, South Africa	JX091429	JX091518	JX141549	KF296441
	CBS 135669^{T}=CV 102=DTO 180-I9	Soil, Stellenbosch, South Africa	JX091427	JX091520	JX141551	KF296443
	CBS 135670=CV 391=DTO 181-F5	*Protea repens* infructescence, Stellenbosch, South Africa	JX091428	JX091521	JX141552	KF296444
P. oxalicum	CBS 173.81^{T}=ATCC 42226=IJFM 3871=IMI 253788	Ex-type of *P. asturianum*, Air, Madrid, Spain	KF296405	KF296470	KF296366	KF296416
	CBS 219.30^{T}=ATCC 1126=FRR 787=IMI 192332=MUCL 29047=NRRL 787	Soil, Connecticut, USA	AF033438	KF296462	KF296367	JN121456
	CV 822=DTO 182-B1	Air, Malmesbury, South Africa	JX091431	JX091528	JX141543	KF296448
P. paraherquei	CBS 338.59^{T}=ATCC 22354=ATCC 46903=FRR 3454=IFO 6234=IMI 068220=NRRL 3454	Soil, Japan	AF178511	KF296465	KF296372	KF296449
P. penarojense	CBS 113178^{T}=IBT 23262	Forest leaf litter, Peña Roja, Colombia	GU981570	GU981646	KF296381	KF296450
P. piscarium	CBS 362.48^{T}=ATCC 10482=FRR 1075=IFO 8111=IMI 040032=NRRL 1075	Cod-liver oil emulsion, Norway	GU981600	GU981668	KF296379	KF296451
P. pulvillorum	CBS 275.83=IJFM 7673	Ex-type of *P. ciegleri*, Rye grain, Spain	AF178513	KF296463	KF296376	KF296423
	CBS 280.39^{T}=IFO 7763=NRRL 2026	Acidic soil, UK	AF178517	GU981670	KF296377	KF296452
P. raperi	CBS 281.58^{T}=ATCC 22355=IFO 8179=IMI 071625=NRRL 2674	Soil, Bedford, UK	AF033433	GU981622	KF296399	KF296453
P. reticulisporum	CBS 122.68^{T}=ATCC 18566=IFO 9024=IMI 136700=NHL 6105=NRRL 3447	Soil, Japan	AF033437	GU981665	KF296391	KF296454
	CBS 513.74=IFO 9712=NHL 6471	Ex-type of *P. arvense*, Soil, Japan	GU981618	GU981666	KF296390	KF296415
P. rolfsii	CBS 368.48^{T}=ATCC 10491=FRR 1078=IFO 7735=IMI 040029=MUCL 29229=NRRL 1078	Pineapple, Florida, USA	JN617705	GU981667	KF296375	KF296455
P. simplicissimum	CBS 372.48^{T}=ATCC 10495=FRR 902=IFO 5762=IMI 039816	Flannel bag, South Africa	GU981588	GU981632	KF296368	JN121507
P. singorense	CBS 138214^{T}=DTO 133-C6	House dust, Songkhla, Thailand	KJ775674	KJ775167	n.a.	n.a.
P. skrjabinii	CBS 439.75^{T}=NRRL 13055=FRR 1945=IMI 196528	Soil, Russia	GU981576	GU981626	KF296370	EU427252
	CV 85=DTO 180-I3	Soil, Stellenbosch, South Africa	JX091432	JX091529	JX141544	KF296456
P. subrubescens	CBS 129543=DTO 205-I2=IBT 13213	Soil, Minnesota, USA	KC797635	KC797610	KC797590	KC797600
	CBS 132785^{T}=DTO 188-D6=FBCC 1632	Field soil growing of Jerusalem artichoke (*Helianthus tuberosus*), Helsinki, Finland	KC346350	KC346327	KC346330	KC346306
P. svalbardense	CBS 122416^{T}=IBT 23856	Glacial ice, Svalbard, Greenland	GU981603	KC346325	KC346338	KF296457
P. vanderhammenii	CBS 126216^{T}=IBT 23203	Forest leaf litter, Araracuara, Colombia	GU981574	GU981647	KF296382	KF296458
P. vasconiae	CBS 339.79^{T}=ATCC 42224=IJFM 3008	Acid washed brown soil, Spain	GU981599	GU981653	KF296386	KF296459
P. wotroi	CBS 118171^{T}=IBT 23253	Forest leaf litter, Araracuara, Colombia	GU981591	GU981637	KF296369	KF296460
P. zonatum	CBS 992.72^{T}=ATCC 24353	Coastal marsh soil, North Carolina, USA	GU981581	GU981651	KF296380	KF296461

CA, USA). PCR amplification of the ITS, *BenA*, *CaM*, and *RPB2* gene regions and sequencing reactions followed the methods described by Visagie et al. (2014a).

Sequence contigs were assembled and adjusted in CodonCode Aligner v. 4.0.1 (CodonCode Corporation, USA). A sequence database for *Penicillium* section *Lanata-Divaricata* species was compiled from newly generated sequences and those previously published in GenBank. Reference sequences of ex-type cultures were obtained from Visagie et al. (2014a). Strains used for phylogenetic comparisons and their GenBank accession numbers are listed in Table 1. Alignments were done using MAFFT v. 7.037b (Katoh and Standley 2013) and aligned data sets analyzed using maximum likelihood (ML) and Bayesian tree inference (BI).

ML analysis was performed in MEGA v. 5.2 (Tamura et al. 2011). The most suitable substitution model for each analysis was chosen using the model test within MEGA, based on the lowest Bayesian information criterion (BIC) value. The initial tree was calculated with the BioNeighbour-Joining (BioNJ) option and subsequent Heuristic search done with the Nearest Neighbour Interchange (NNI) option. Statistical support in nodes was determined using bootstrap analysis with 1000 replicates.

BI analysis was performed in MrBayes v. 3.2.1 (Rondquist and Huelsenbeck 2003), with the most suitable substitution model selected using MrModeltest v. 2.3 (Nylander et al. 2004), based on the lowest Akaike information criterion (AIC) value. The analysis was performed with three sets of four chains. Each analysis was stopped at an average of standard deviation of split frequencies of 0.01. The sample frequency was set at 100, with 25 percent of trees removed as burn-in. ML phylograms were used for representing results with bootstrap (bs) values and posterior probabilities (pp) higher than 80 % bs and/or 0.95 pp represented above branch nodes. Alignments and trees were uploaded onto TreeBase (www.treebase.org) with submission ID 14419.

Morphology

Strains were characterized under standardized growth conditions (Visagie et al. 2014a). Culture media used for characterization include Czapek yeast autolysate agar (CYA), malt extract agar (MEA) (oxoid), yeast extract sucrose (YES) agar, dichloran 18 % glycerol (DG18) agar, CYA supplemented with 5 % NaCl (CYAS), oatmeal agar (OA), and creatine sucrose agar (CREA). Media preparation, inoculation, incubation, and microscope preparations were done as described in Visagie et al. (2014a). Color names and alphanumeric codes used in descriptions refer to the Methuen Handbook of Color (Kornerup and Wanscher 1967). To examine sexual reproductive states, preparations were made from OA.

Microscopic examinations were made using an Olympus BX50 light microscope and Olympus SZX12 stereomicroscope, equipped with an Evolution MP digital microscope camera. Pictures were captured with ImagePro v. 6.0 and microscopic measurements done in Nikon NIS-elements D v. 4.0. In species descriptions, average microscope measurements and their standard deviations are provided between brackets. Photographic plates were prepared in Adobe® Photoshop® CS6. For aesthetic reasons, backgrounds of images were cleaned up using the healing brush tool, without manipulating any of the scientifically relevant areas of images. Colony textures were captured using extended depth of view and processed in Helicon Focus v. 4.2. Line drawings were made using a Nikon Eclipse E800 light microscope with a drawing tube attachment.

Results

Isolations and identifications

Isolations from soil, air and *Protea repens* infructescence samples collected at three fynbos sites resulted in 1700 *Penicillium* strains, representing 61 species. Based on their ITS barcodes, six species belong in section *Lanata-Divaricata*. Two of the species were identified as *P. skrjabinii* and *P. oxalicum*. However, four species displayed unique phenotypic characters and grouped separately from known species in the multigene phylogenetic analyses. They are thus described in the taxonomy section as *P. annulatum*, *P. curticaule*, *P. malacosphaerulum*, and *P. ortum*.

Phylogeny

Fynbos strains were compared with other members of *Penicillium* section *Lanata-Divaricata* using four gene regions (Figs. 1 and 2). Aligned data sets for ITS, *BenA*, *CaM*, and *RPB2* were respectively 501, 442, 488, and 755 bp long when gaps are included. It should be noted that for the newly described *P. koreense* (You et al. 2014) and *P. singorense* (Visagie et al. 2014c), only ITS and *BenA* sequences were included in the analyses. Tree topologies did not differ between ML and BI analyses. For ML, the Kimura 2 parameter with Gamma distributed and Invariant sites (K2+G+I) was the best fit model for ITS, *CaM*, and *RPB2*, whereas the Kimura 2 parameter with Gamma distribution (K2+G) was the best fit model for *BenA*. For BI, the General Time Reversible with Gamma distributed and Invariant sites (GTR+G+I) was the best fit model for ITS, *CaM,* and *RPB2* phylogenies, whereas the Symmetrical with Gamma distribution (SYM+G) model was the best fit for the *BenA* phylogeny.

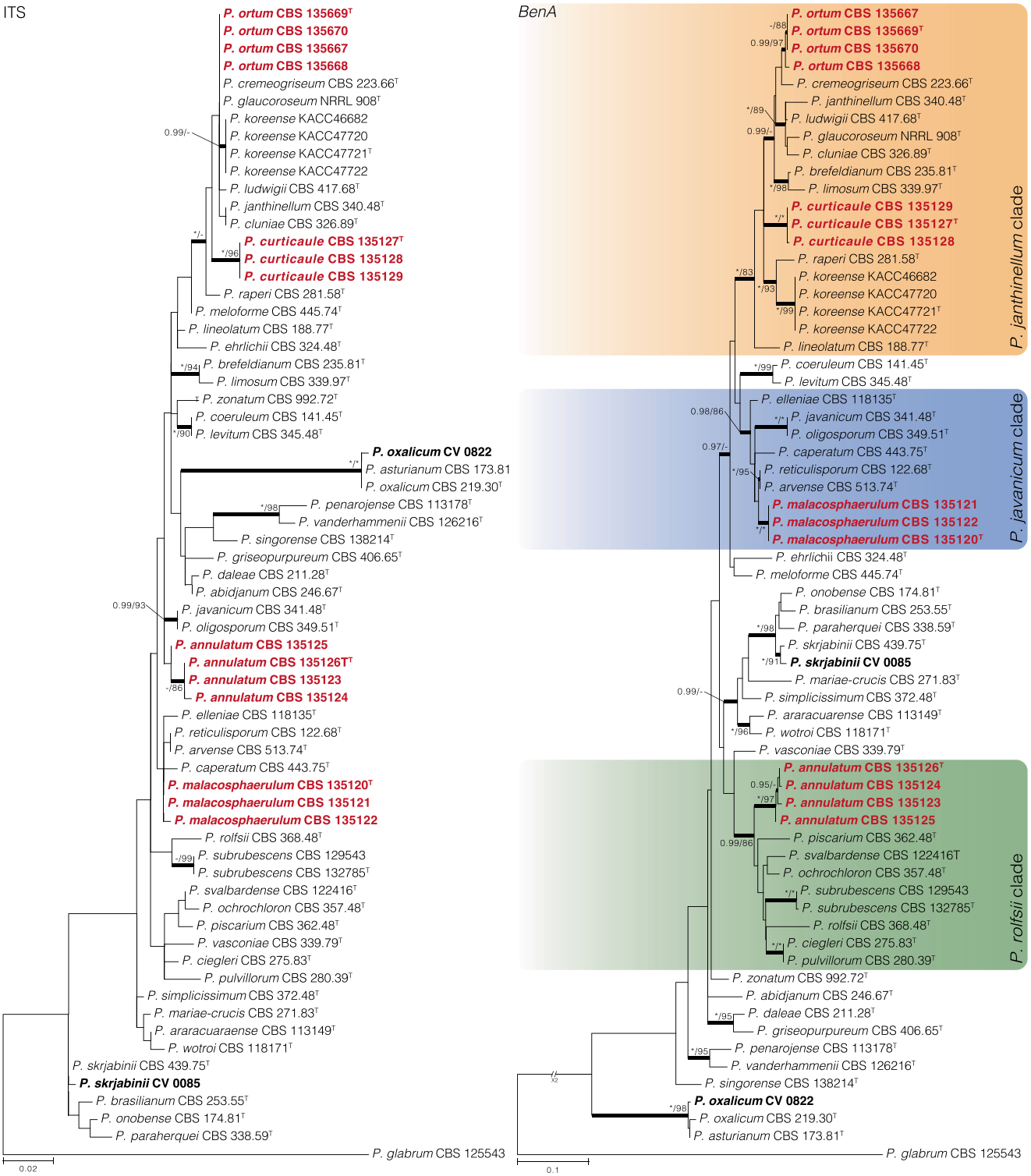


Fig. 1 Maximum likelihood trees based on ITS and *BenA*, showing the relationship of species in section *Lanata-Divaricata*. *Penicillium glabrum* was chosen as outgroup for both phylogenies. Thick branches represent good branch support with Bootstrap values above 80 % and Posterior Probabilities above 0.95 indicated above branches. (^{T}=ex-type; −= support lower than 80 % or 0.95 pp; *=support of 100 % or 1.00 pp). Bold names indicate strains isolated from fynbos. Names in reg indicate strains belonging to new species. Colored boxes indicate the clades to which new species belong (orange=*P. janthinellum* clade; blue=*P. javanicum* clade; green=*P. rolfsii* clade)

Using the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept (Taylor et al. 2000), the results show that *Penicillium* section *Lanata-Divaricata* includes 43 species. From Fig. 1

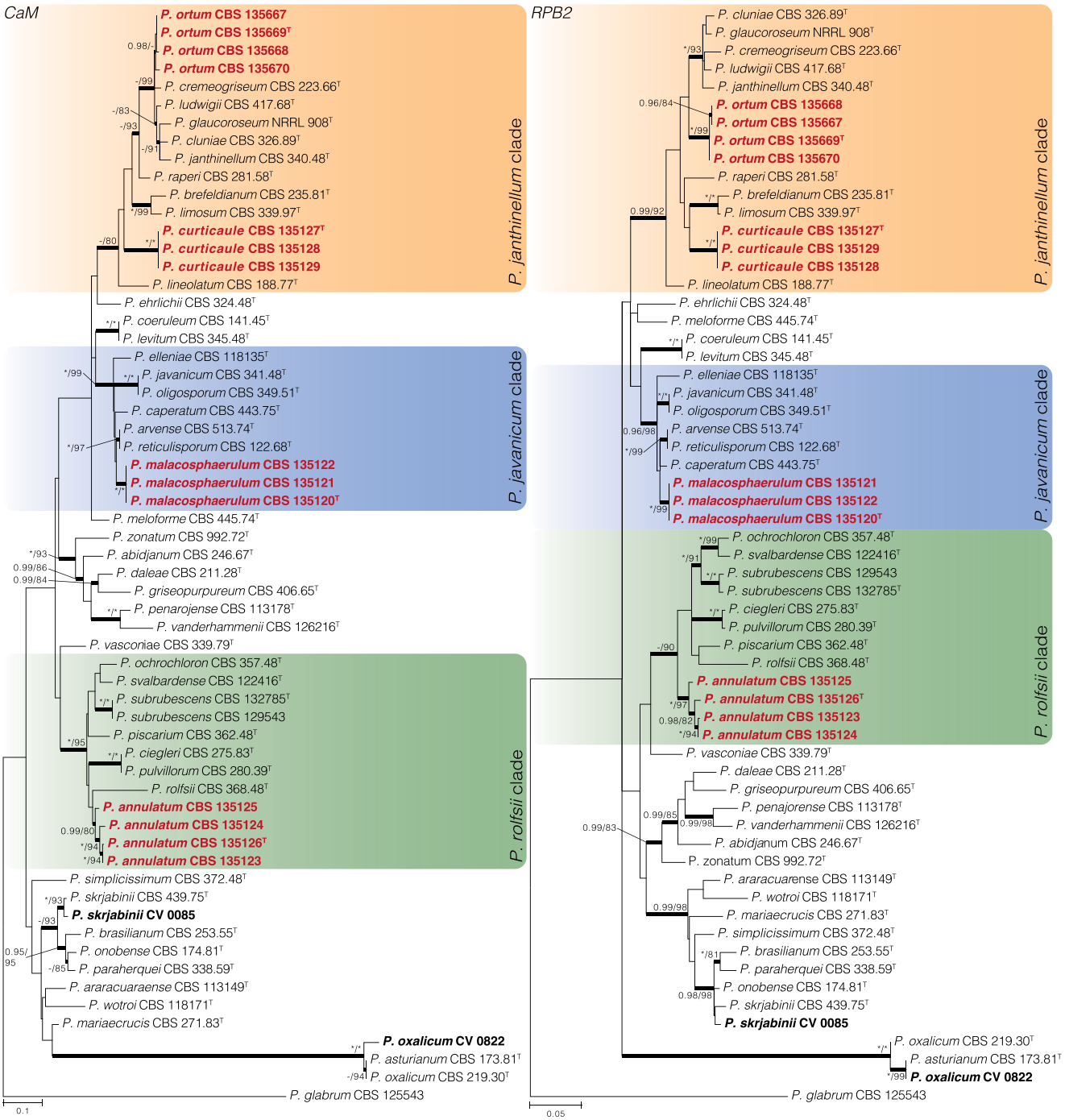


Fig. 2 Maximum likelihood trees based on CaM and *RPB2*, showing the relationship of species in section *Lanata-Divaricata*. *Penicillium glabrum* was chosen as outgroup for both phylogenies. Thick branches represent good branch support with bootstrap values above 80 % and posterior probabilities above 0.95 indicated above branches. (^{T}=ex-type; −= support lower than 80 % or 0.95 pp; *=support of 100 % or 1.00 pp). Bold names indicate strains isolated from fynbos. Names in reg indicate strains belonging to new species. Colored boxes indicate the clades to which new species belong (orange=*P. janthinellum* clade; blue=*P. javanicum* clade; green=*P. rolfsii* clade)

(left), it is clear that several species share identical ITS barcode sequences, in comparison to the other genes, which were able to consistently discriminate 43 species. Clades containing the new species were consistent and well defined in *BenA*, *CaM*, and *RPB2* phylogenies, and named here as the *P. janthinellum* (orange block), *P. javanicum* (blue block) and *P. rolfsii* (green block) clades. The phylogenies resolved two remaining fynbos strains with *P. oxalicum* (DTO 182-B1) and *P. skrjabinii* (DTO 180-I3).

Several species previously thought to belong to this section were also sequenced in this study. The phylogenies showed

Table 2 Summary of morphological features for distinguishing *P. curticaule* and *P. ortum* from its closest relatives

	Growth rates (diam after 7days, in mm)									Colony characters		Micromorphological characters			
Species	CYA	CYA 30 °C	CYA 37 °C	OA	MEA	YES	DG18	CYAS	CREA	Acid on CREA	Reverse CYA	Margin color on OA	Phialide length (μm)	Cleistothecia	Conidia
P. brefeldianum	36–38	45–50	35–37	48–51	45–46	44–45	25–26	15–16	9–10	absent	light brown	none	7.5–12	absent to present	smooth
P. cluniae	43–45	48–50	39–41	45–48	49–52	47–48	35–38	36–37	29–30	absent	pale yellow	none	8–10	absent	smooth to finely rough
P.cremeogriseum	28–30	30–32	25–30	40–42	45–46	38–40	20–21	28–29	22–23	weak	pale yellow to very light brown	none	7–11	absent	smooth to finely rough
P. curticaule	23–26	30–35	3–8	20–22	18–21	30–35	16–18	10–11	20–25	absent	olive yellow to olive to olive brown	olive yellow to olive brown	5–7.5	absent	finely rough
P. glaucoroseum	47–49	52–54	16–17	45–47	54–56	50–53	24–25	25–26	28–30	absent	dark brown	olive yellow to olive brown	7–10	absent	finely rough
P. janthinellum	32–33	40–42	20–30	38–41	43–44	44–46	25–27	26–27	18–20	absent	pale yellow to very light brown	none	7–11	absent	smooth to finely rough
P. koreense[1]	32–34	39–43	15–19	33–38	42–48	n.a.	n.a.	n.a.	32–35	absent	beige	none	8–11.5	absent	smooth to finely rough
P. limosum	29–31	40–45	35–37	38–40	37–38	38–40	17–18	17–18	22–23	absent	light brown near margin, brown elsewhere	brown	8–13	present, brownish	rough
P. lineolatum	28–29	38–40	27–32	35–42	38–39	40–42	21–22	18–20	13–14	moderate	pale yellow to very light brown	none	7–12 (−15)	present, yellow	smooth to finely rough
P. ludwigii	35–37	31–38	22–28	42–44	50–53	56–61	22–25	20–22	33–35	moderate to strong	brown at centre, pale elsewhere	light brown	7–12	present, white becoming brownish with age	smooth
P. ortum	28–40	36–50	35–40	40–45	48–53	40–50	20–27	30–36	25–30	moderate	olive to brownish orange in some isolates	none	6.5–10	absent	smooth
P. raperi	21–23	29–32	15–16	28–30	23–24	27–28	12–13	9–10	11–12	absent	olive yellow to deep olive	olive yellow to olive brown	7–9	absent	finely rough

[1] Description based on You et al. (2014)

Table 3 Summary of morphological features for distinguishing *P. malacosphaerulum* from its close relatives

	Growth rates (diam after 7days, in mm)									Colony characters		Micromorphological characters						
Species	CYA	CYA 30 °C	CYA 37 °C	OA	MEA	YES	DG18	CYAS	CREA	CYA soluble pigment	Acid on CREA, colony growth	Cleistothecia	Cleistothecia size (μm)	Asci size (μm)	Ascospore texture	Ascospore size (μm)	Stipe walls	Conidia
P. caperatum[1]	38–40	45–47	40–42	33–35	30–33	45–47	26–29	24–27	32–33	absent	moderate, sparse	creamish to yellowish brown	65–300	7.5–13×6–11	smooth walled, two longitudinal flanges	5–6×3–4	smooth	smooth
P. elleniae[2]	48–50	45–50	20–25	42–45	49–50	50–55	31–33	32–34	33–36	yellow	weak, dense	yellow-brown	100–200	8–12	spinose, traces of equatorial ridge present	3–3.5×2.5–3	smooth	spinose
P. javanicum	33–41	38–43	45–50	39–45	40–50	45–52	22–25	20–30	25–28	absent	absent, sparse	light brown	30–60	6.5–9×5–8.5	finely rough walled, flanges absent	3–3.5×2–3	rough	rough
P.malacosphaerulum	28–36	40–45	30–35	25–27	26–32	33–43	12–15	19–22	25–30	yellow	absent, sparse	dark yellow to brown	50–160	5.5–10×4.5–7	finely rough walled, two longitudinal flanges	2.5–3.5×2–3	smooth	smooth
P. reticulisporum	45–46	45–48	13–20	40–45	47–50	50–54	25–26	26–28	35–39	absent	absent, sparse	cinnamon to brown	50–200	8.5–12×5–8.5	rough walled, two longitudinal flanges	3.5–4.5×2.5–4	smooth	smooth

[1] Data for sexual reproductive state based on Stolk & Samson (1983). [2] Data for sexual reproductive state based on Houbraken et al. (2011)

Table 4 Summary of morphological features for distinguishing *P. annulatum* from its close relatives

	Growth rates (diam after 7days, in mm)									Colony characters	Micromorphological characters		
Species	CYA	CYA 30 °C	CYA 37 °C	OA	MEA	YES	DG18	CYAS	CREA	Acid produced on CREA	Stipe walls	Conidia walls	Conidia shape
P. annulatum	45–48	48–55	20–30	42–45	40–50	47–52	24–27	25–31	28–33	absent	rough	rough	globose to subglobose
P.ochrochloron	54–56	26–30	1–6	35–36	58–60	45–47	32–33	28–29	31–34	weak to moderate	smooth to rough	smooth	ellipsoid to fusiform
P. piscarium	42–44	45–48	no growth	45–50	45–47	45–46	8–10	21–22	24–25	absent	rough	echinulate	globose to subglobose
P. pulvillorum	37–40	42–45	0–2	42–45	42–45	42–43	20–22	23–25	20–21	absent	rough	smooth to finely roughened	globose to subglobose
P. rolfsii	52–60	65–70	43–45	45–50	56–60	65–70	34–35	40–41	30–31	absent	finely roughened	smooth	ellipsoid to fusiform
P.subrubescens	40–50	40–55	0–12	40–45	40–55	45–60	25–30	25–35	20–30	absent	rough	smooth to finely roughened	subglobose to broadly ellipsoid
P. svalbardense	34–37	43–46	no growth	38–40	40–42	39–42	19–20	20–21	24–26	absent	rough	smooth to finely roughened	globose to subglobose

that *P. glaucoroseum*, *P. cluniae*, and *P. griseopurpureum* are distinct species. The type and ex-type culture of *P. glaucoroseum* is unavailable, and we propose CBS H-22050 as epitype (ex-epitype CBS 138908=NRRL 908), considered by Raper and Thom (1949) as representative of the species, in the Names in Current Use (Online Resource 1). In addition, *P. asturianum*, *P. arvense*, *P. ciegleri*, and *P. oligosporum* are confirmed as synonyms of other species (Frisvad and Filtenborg 1990; Stolk et al. 1990; Houbraken and Samson 2011). No cultures are available for *P. es-suveidense*, *P. populi*, *P. aragonense*, and *P. vitale*, and we thus follow the synonomies proposed by Pitt (1979) and Stolk et al. (1990), but consider the synonymy of *P. aragonense* with *P. oxalicum*, proposed by Stolk et al. (1990), as doubtful because the original description and drawings (Ramírez and Martinez 1981) are not representative of the latter species in our opinion.

Morphology

Fynbos strains produced the typical fast growing colonies of species belonging in *Penicillium* section *Lanata-Divaricata*. The strain identified as *P. oxalicum* (DTO 182-B1) produced large masses of ellipsoidal conidia that easily dislodge in crusts when disturbed. Its phialides are cylindrical and have very short necks. These characters are typical of *P. oxalicum*. The strain of *P. skrjabinii* (DTO 180-I3) produced fast growing colonies with variable growth at 37 °C. Its most striking characters are the heavily rough walled conidiophores and conidia. Phialides were observed to be rough walled, which was not previously reported (Pitt 1979; Ramírez 1982); perhaps this reflects the "wild type" of the species, no longer visible in the preserved cultures observed in earlier studies. The four new species were compared to their close relatives in, respectively, the *P. janthinellum*, *P. javanicum* and *P. rolfsii* clades. Colony growth rates and colors produced on different

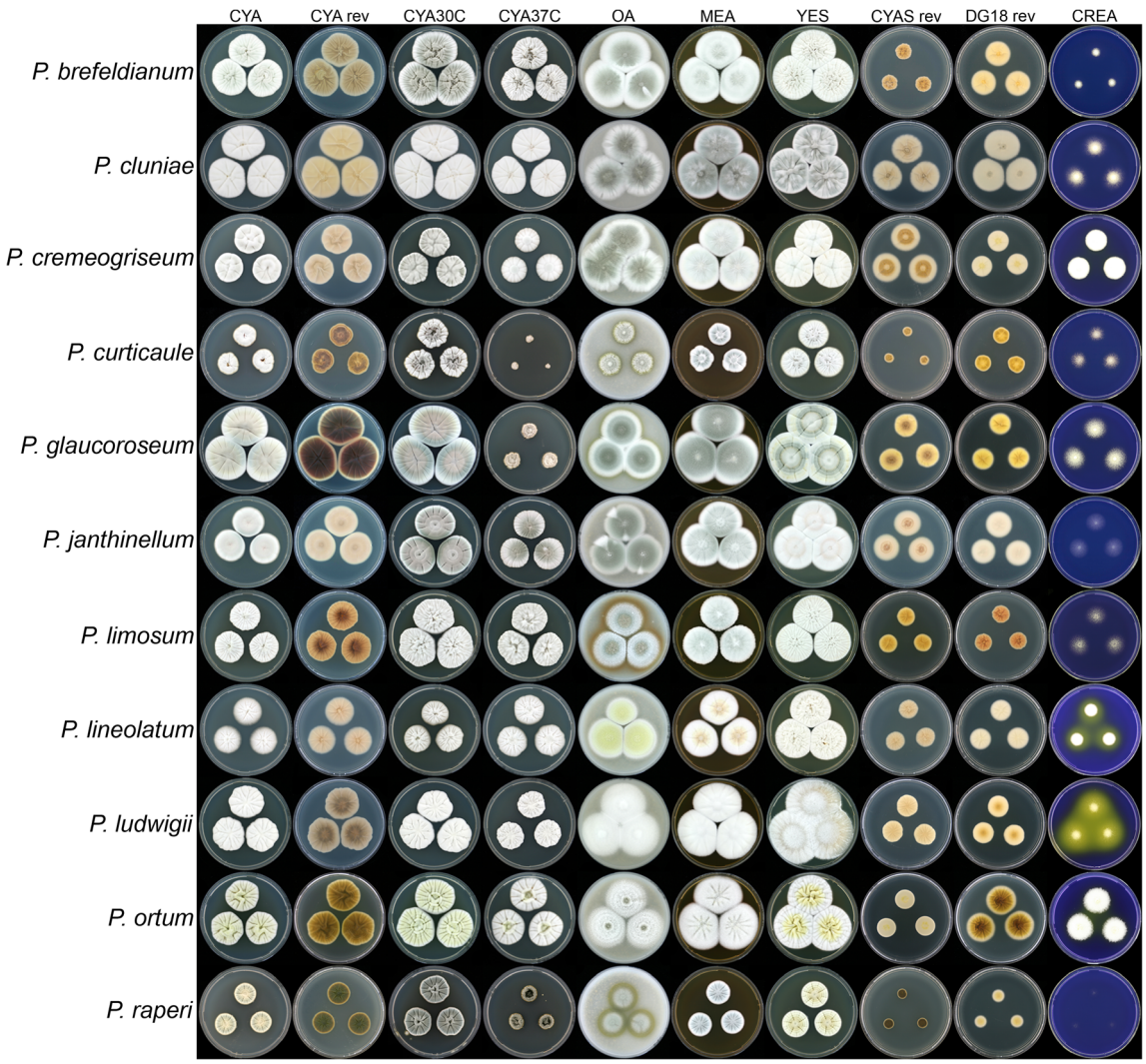


Fig. 3 Overview of colony characters in *Penicillium* section *Lanata-Divaricata* species in the *P. janthinellum* clade

media incubated at different temperatures were taxonomically informative for species identification. With regards to micromorphology, conidial shape and ornamentation were useful characters in the *P. janthinellum* and *P. rolfsii* clades, whereas the sexual reproductive states in the *P. javanicum* clade was most informative. A detailed discussion of these morphological differences is provided in the taxonomy section below.

Species identification

Multigene phylogenies (Figs. 1 and 2) distributed the newly described species among three well-defined clades, named here the *P. janthinellum*, *P. javanicum*, and *P. rolfsii* clades. An identification scheme for all species of these clades is provided here to aid morphological identifications among these close relatives. The scheme includes photoplates of

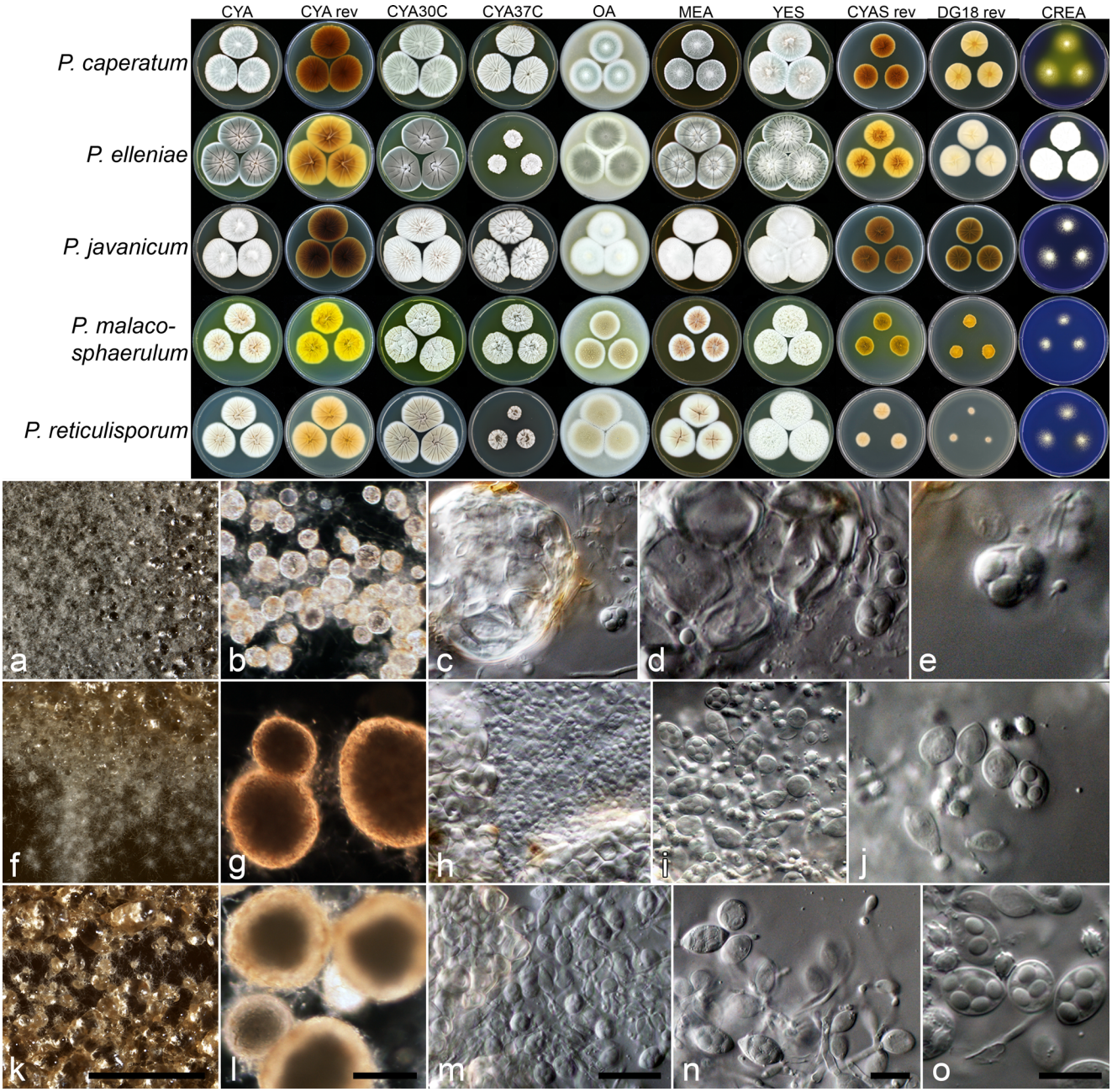


Fig. 4 Overview of colony characters in *Penicillium* section *Lanata-Divaricata* species in the *P. javanicum* clade. a–o Overview of sexual reproductive states of *P. malacosphaerulum* and its close relatives. Included from left to right are cleistothecia, asci and ascospores, a–e. *Penicillium javanicum* (CBS 341.48). f–j. *Penicillium malacosphaerulum* (CBS 135121). k–o. *Penicillium reticulisporum* (CBS 122.68) (— scale bar: in k=1 mm, applies to a, f, k; in l= 100 μm, applies to b, g, l; in m=25 μm, applies to c, h, m; in n= 10 μm, applies to d, i, n; in o=10 μm, applies to e, j, o)

colony characters as well as Tables 2, 3, and 4 summarizing diagnostic characters useful for species identification.

The P. janthinellum clade — Two of the new species, *P. curticaule* and *P. ortum*, belong to the *P. janthinellum* clade. The clade includes 12 species with the typical divaricate conidiophores observed for section *Lanata-Divaricata* as described by Thom (1930). Conidial wall texture and phialide length differed between some species (*P. raperi*, *P. curticaule*). However, species identification in this group using micromorphology remains very difficult. Colony morphology and growth rates, especially on CYA at 37 °C, and acid production on CREA, were the most important characters for identification (Table 2, Fig. 3). Furthermore, *P. lineolatum* and *P. ludwigii* were found readily to produce a sexual state.

Penicillium janthinellum, *P. glaucoroseum*, *P. ludwigii*, *P. curticaule*, *P. raperi*, and the newly described *P. koreense* (You et al. 2014), grow restrictedly on CYA 37 °C (<30 mm). Restricted growth and a brownish to olive reverse on CYA at 25 °C (<30 mm), distinguish *P. curticaule* and *P. raperi* from the others. A comparison of these two species reveals that *P. raperi* grows faster at 37 °C and produces longer phialides than *P. curticaule*. *Penicillium glaucoroseum* produces a dark red reverse on CYA and grows faster than *P. janthinellum*, *P. koreense*, and *P. ludwigii* on CYA at 25 and 30 °C. *Penicillium ludwigii* grows faster on MEA, YES, and CREA than *P. janthinellum*, and has moderate to strong acid production on CREA. *Penicillium koreense* shows similar fast growth on CREA to *P. ludwigii*. However, *P. koreense* lacks acid production on CREA, does not produce a sexual state and grows more restrictedly than *P. ludwigii* at 37 °C and on OA.

Fig. 6 Line drawings of the new *Penicillium* species isolated from the fynbos. A. *Penicillium annulatum* (CBS 135126). B. *Penicillium curticaule* (CBS 135127). C. *Penicillium malacosphaerulum* (CBS 135120). D. *Penicillium ortum* (CBS 135669). (a=cleistothecia with ascospores; cp=conidiophores and conidia; b=conidiophore branching pattern) (— scale bar: a, cp=10 μm; b=50 μm)

Penicillium limosum and *P. lineolatum* readily produce cleistothecia with ascospores, especially on OA. Bright yellow cleistothecia, acid production, and smooth conidia distinguish *P. lineolatum* from *P. limosum*. *Penicillium brefeldianum* has been reported to produce a sexual state (Pitt 1979; Stolk and Samson 1983), but we were unable to induce cleistothecia in this study. However, its colonies grow much faster than both *P. lineolatum* and *P. limosum*.

Penicillium cremeogriseum and *P. ortum* produce acid on CREA, but lacks cleistothecia of *P. limosum* and *P. lineolatum*. They have similar conidiophore morphologies and grow strongly on CREA. However, *P. ortum* consistently grows faster on CYA at 30 and 37 °C, CYAS, YES, and MEA. The remaining species include *P. brefeldianum* and *P. cluniae*. *Penicillium cluniae* displays faster growth on CYA, DG18, and CYAS, making the species readily identifiable, compared to the more restricted growth of *P. brefeldianum*.

The P. javanicum clade — *Penicillium malacosphaerulum* belongs to the *P. javanicum* clade. Colony growth rates and characters of their sexual states were taxonomically most informative (Table 3, Fig. 4). All species in the clade are reported to reproduce sexually, although we did not observe cleistothecia in *P. caperatum* or *P. elleniae*. The large dense

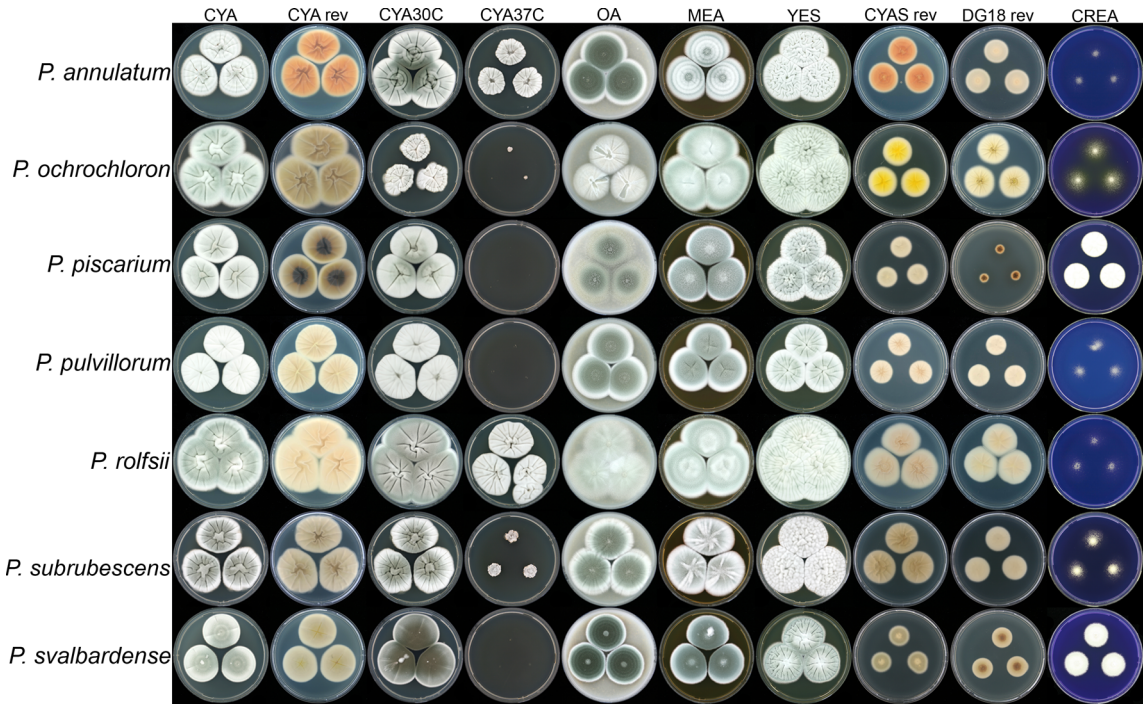
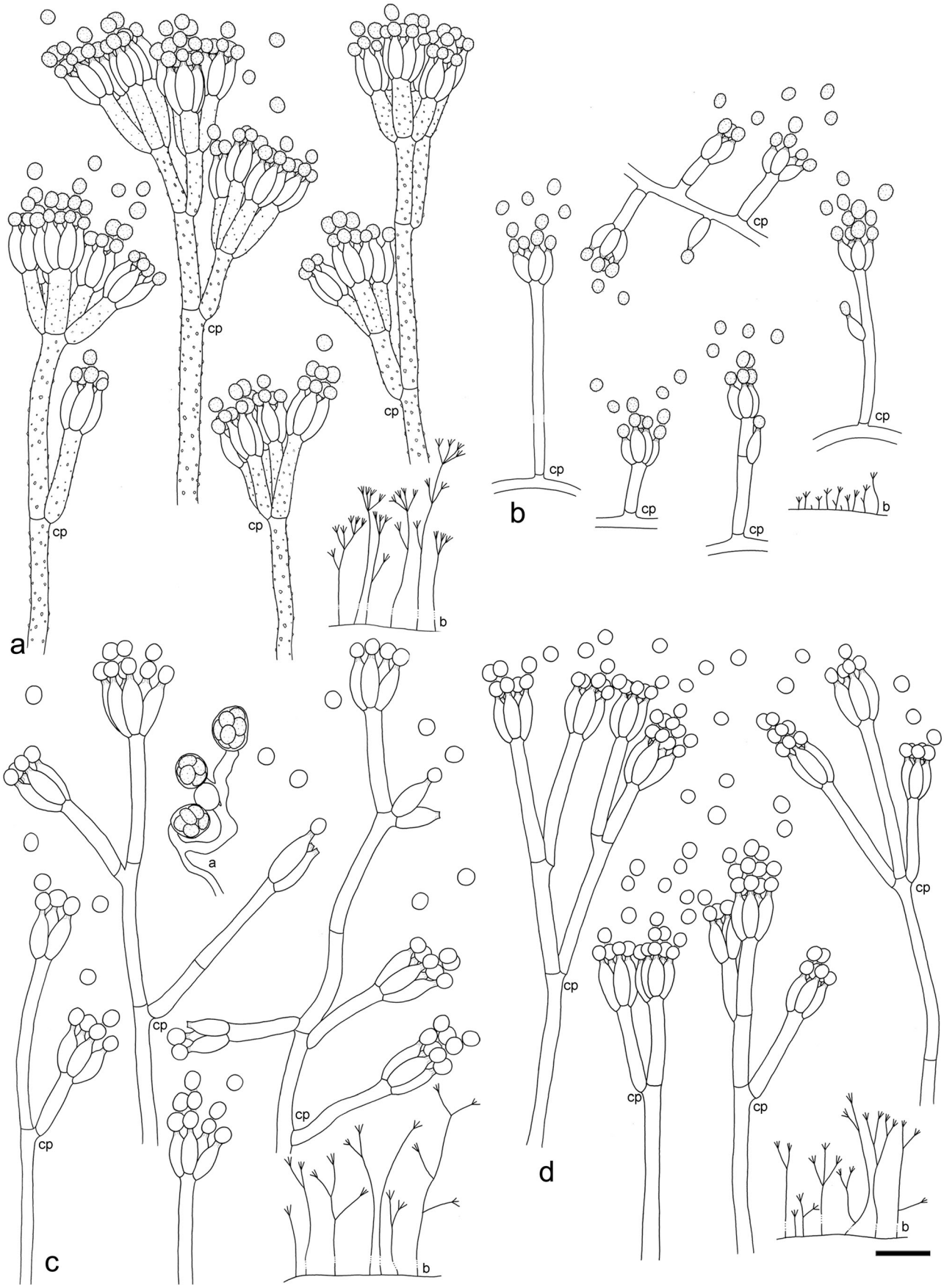


Fig. 5 Overview of colony characters in *Penicillium* section *Lanata-Divaricata* species in the *P. rolfsii* clade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colonies of *P. elleniae* on CREA distinguish it from other members in this clade. Also, this species produces smooth walled stipes and spinose conidia, unique for the clade. *Penicillium javanicum* is the only species in the clade that produces roughened stipes. Ascospores of *P. caperatum*, *P. malacosphaerulum*, and *P. reticulisporum* have two longitudinal flanges, which are absent on *P. javanicum* ascospores. *Penicillium caperatum* is reported to produce larger ascospores (5–6×3–4 μm) with smooth walls (Stolk and Samson 1983), in comparison to the roughened ascospores of *P. javanicum*, *P. malacospaerulum*, and *P. reticulisporum*. In addition, *P. caperatum* has moderate acid production on CREA, absent in the other species. *Penicillium malacosphaerulum* produces smaller ascospores compared to *P. reticulisporum*. The new species also grows slower on CYA at 25 °C, but faster at 37 °C than *P. reticulisporum*.

The P. rolfsii clade — Penicillium annulatum belong to the *P. rolfsii* clade, which contains species producing terminally biverticillate conidiophores with rough walled stipes. Colony growth rates and conidial shapes are most useful for distinguishing among the species in this clade (Table 4, Fig. 5).

Penicillium annulatum and *P. rolfsii* grow well on CYA at 37 °C, compared to the generally poor growth observed for other species in the clade. *Penicillium rolfsii* displays much faster growth on all media and has ellipsoidal conidia, compared to slower growth and globose to subglobose conidia of *P. annulatum*. Dense white colonies on CREA are characteristic of both *P. piscarium* and *P. svalbardense*, but *P. piscarium* grows faster on CYA, MEA, YES, and OA, and *P. svalbardense* grows faster on DG18.

The remaining three species, *P. ochrochloron*, *P. pulvillorum,* and *P. subrubescens*, can also be distinguished based on colony growth rates. On CYA, *P. ochrochloron* grows faster than *P. subrubescens*. In turn, *P. subrubescens* grows faster than *P. pulvillorum*. However, *P. ochrochloron* grows slower than both species on CYA at 30 °C. In addition, *P. ochrochloron* and *P. rolfsii* produce ellipsoidal to fusiform conidia, in contrast with *P. pulvillorum*'s globose to subglobose conidia. *Penicillium pulvillorum* generally grows more restrictedly compared to *P. subrubescens*.

Taxonomy

Penicillium annulatum **Visagie & K. Jacobs, sp. nov. Figs. 6A, 7**

MycoBank: MB809817

ITS barcode: JX091426. The species has unique ITS sequences.

Alternative markers: *BenA*=JX091514; *CaM*=JX141545; *RPB2*=KF296410.

Etymology: Latin, *annulatum* meaning surrounded by rings; referring to the rings of sporulation observed in colonies.

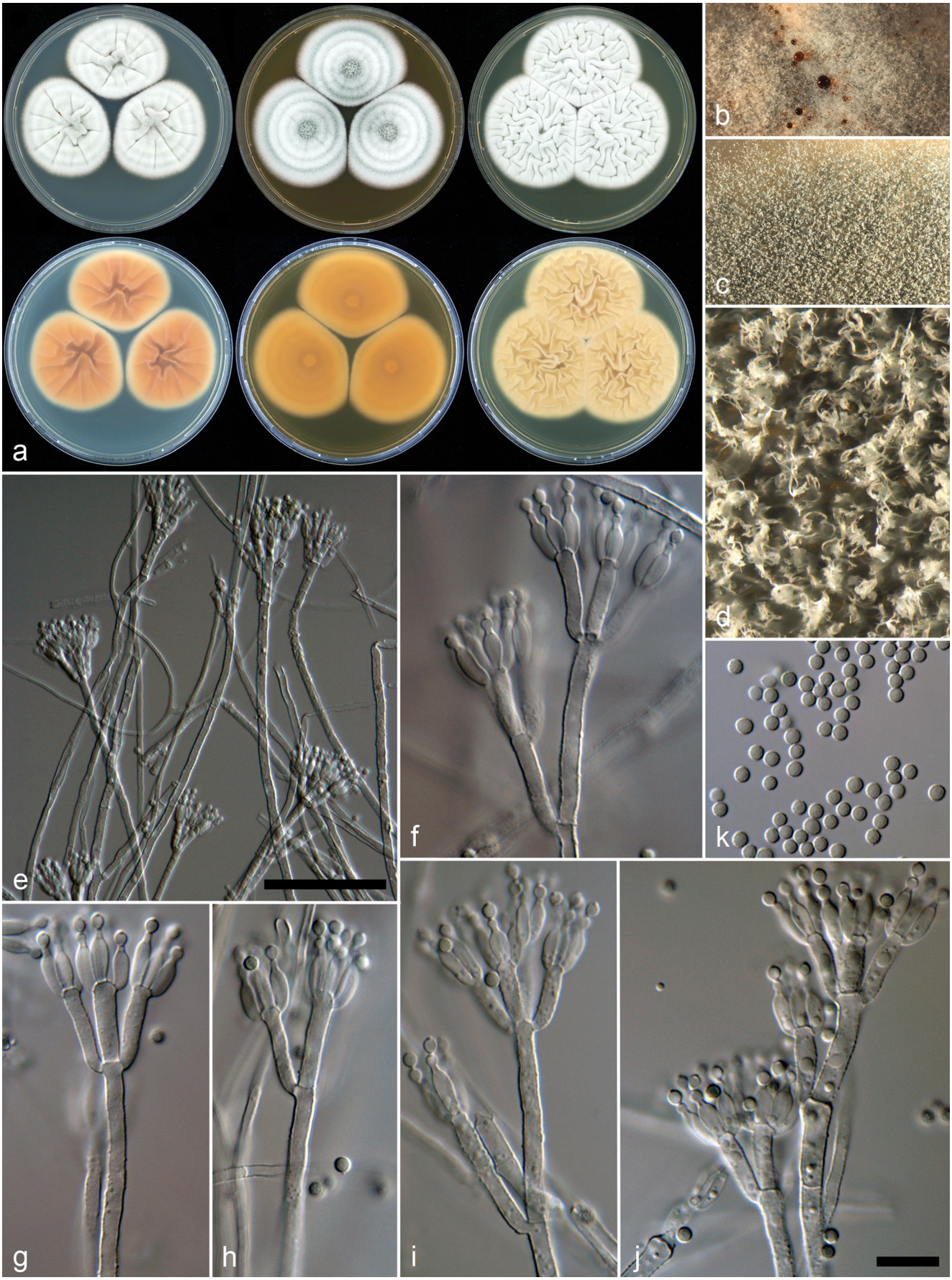
Fig. 7 *Penicillium annulatum* (CBS 135126). a. colonies on CYA, MEA, and YES from left to right (top=obverse, bottom=reverse). b. texture on CYA. c, d. texture on MEA. e–j. conidiophores. k. conidia (— scale bar in e=50 μm; — scale bar in j=10 μm, applies to f–k)

Diagnosis — Relatively fast growing colonies on CYA at 25 °C (45–48 mm) and 37 °C (20–30 mm). Stipes rough walled, conidia globose to subglobose and rough walled.

Colony diam, *7 days*, *in mm* — CYA 45–48; CYA 5 °C no germination; CYA 30 °C 48–55; CYA 37 °C 20–30; MEA 40–50; YES 47–52; DG18 24–27; CYAS 25–31; OA 42–45; CREA 28–33.

Macromorphology — CYA, 25 °C, 7days: Colonies low to moderately deep, radially and concentrically sulcate, having a ring-like appearance because of sporulating and non-sporulating areas; margins low, narrow (2 mm), entire; mycelia white; texture floccose; sporulation sparse to moderately dense, conidia dull green (25E4–26E4); exudate dark red, soluble pigment absent, reverse in some isolates dark ruby (12 F8), mostly light to greyish orange (5A5–B5–6). CYA 30 °C, 7 days: Colonies low, lightly radially sulcate, having a ring-like appearance because of sporulating and non-sporulating areas; margin low, narrow (2 mm), entire; mycelia white; texture floccose; sporulation moderately dense, conidia dull to greyish green (25E4–6), sometimes (26E4–6); exudate sometimes present as dark red droplets, soluble pigment absent, reverse dark ruby (12 F8) in central areas for some isolates, others greyish orange (5B4–6) fading into pale yellow (4A3) margin. CYA 37 °C, 7 days: Colonies deep, radially sulcate, concave; margins moderately deep, narrow, irregular; mycelia white; sporulation absent; exudate absent, soluble pigment absent, reverse brownish orange (5C4–6). MEA, 25 °C, 7 days: Colonies 29–38 mm, low to moderately deep, plane; margins low, narrow (2 mm), irregular; mycelia white; texture floccose; sporulation moderately dense to dense, conidia greyish green (25E6–7); exudate absent, soluble pigment absent, reverse greyish yellow (2B3–4). YES, 25 °C, 7 days: Colonies low, lightly radially sulcate, having a ring-like appearance because of sporulating and non-sporulating areas, but less obvious than those on CYA; margins low, narrow (2 mm), entire; mycelia white; texture floccose; sporulation sparse to moderate, conidia greyish green (25B4–5–30D5); exudate absent, soluble pigment absent, reverse dull yellow to yellowish white (3A3–B3–B4). CREA 25 °C, 7 days: Colonies not producing acid.

Micromorphology — Conidiophores bi- and terverticillate, often with subterminal branches produced; stipes rough walled, 180–750×3–4.5 μm; rami/branches divergent, 6–40×3–4.5 μm (19.8±6.73); metulae 3–6 per stipe/branch, appressed to divergent, angle 28–80° (52±11.3°), great variation in length in the same conidiophore, 8–20×2.5–4.5 μm (11.5±1.89×3.5±0.43), vesicle 3–4.5 μm (3.38±0.41); phialides ampulliform, 4–5 per metula, 6–8×2–3.5 μm (7.1±0.5×2.8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±0.25); conidia rough walled, globose to some subglobose, 2.5–3×2–3 μm (2.5±0.12×2.4±0.13), average width/length ±stdev=0.93±0.04, n=116.

Holotypus — South Africa, Stellenbosch, (33°56'47"S 18°52'49"), from air sample, 14 March 2009, collected and isolated by CM Visagie, CBS H-21333 (ex-typus: CBS 135126=CV 37=DTO 180-G7).

Additional cultures examined — South Africa, Stellenbosch, (33°56'47"S 18°52'49"), from mite in *Protea repens* infructescence, 14 March 2009, collected and isolated by CM Visagie, CBS 135125=CV 187=DTO 181-C3; South Africa, Stellenbosch, (33°56'47"S 18°52'49"), from soil, 14 March 2009, collected and isolated by CM Visagie, CBS 135123=CV 548=DTO 181-I1; South Africa, Struisbaai, (33°45'06"S 18°58'59"), from soil, 14 August 2009, collected and isolated by CM Visagie, CBS 135124=CV 1707=DTO 183-E9.

Penicillium curticaule **Visagie & K. Jacobs, sp. nov. Figs.** 6B & 8

MycoBank: MB809818

ITS barcode: FJ231021. The species has unique ITS sequences.

Alternative markers: *BenA*=JX091526; *CaM*=JX141536; *RPB2*=KF296417.

Etymology: Latin, *curticaule*: *Curtus* meaning short, *caulis* meaning stemmed; referring to the short stipes produced by the species.

Diagnosis — Relatively slow growing colonies on CYA at 25 °C (23–26 mm) and 37 °C (3–8 mm). Colonies on OA have olive yellow to olive to olive brown color at margins. Conidiophores with very short stipes (9–20 μm) and phialides (5–7.5 μm).

Colony diam, 7 days, in mm — CYA 23–26; CYA 5 °C germination; CYA 30 °C 30–35; CYA 37 °C 3–8; MEA 18–21; YES 30–35; DG18 16–18; CYAS 10–11; OA 20–22; CREA 20–25.

Macromorphology — CYA, 25 °C, 7 d: Colonies low to moderately deep, radially sulcate, random furrows also present; margins low, narrow (2 mm), yellowish olive; mycelia white; texture floccose; sporulation sparse to moderate, conidia dull green (25D3–4); exudate absent, soluble pigment absent, reverse olive yellow to olive to olive brown (3D7–8–4D7–8). CYA, 30 °C, 7 days: Colonies low to moderately deep, irregular furrows present; margins low, narrow (1–2 mm), having a yellowish olive color, entire; mycelia white; texture floccose; sporulation sparse, conidia greyish turquoise (25C3); exudate absent, soluble pigment absent, reverse Brown (6E7–8) at centre, olive brown (4D8) and greenish yellow (1A7) elsewhere. CYA, 37 °C, 7 days: Microcolonies of white mycelia present. MEA, 25 °C, 7 days: Colonies low, plane, slightly raised at centre; margins low, narrow (2–3 mm); mycelia white; texture floccose, loosely funiculose; sporulation moderately dense, conidia greyish to Dull Green (25C3–D4–5); exudate absent, soluble pigment absent, reverse olive to Yellowish Brown (4E4–5E4) at centre, margin greyish yellow (3B6). YES, 25 °C, 7d: Colonies low, radially sulcate, random furrows also present; margins low, narrow (1–2 mm), entire, yellowish olive color; mycelia white; texture floccose; sporulation sparse to moderate, conidia dull green (25D3–4); exudate absent, soluble pigment absent, reverse olive yellow to olive to olive brown (3D7–8–4D7–8). CREA, 25 °C, 7 days: Colonies not producing acid.

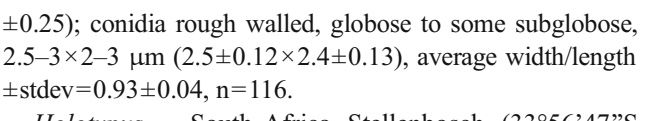


Fig. 8 *Penicillium curticaule* (CBS 135127). a. colonies on CYA, MEA, and YES from left to right (top=obverse, bottom=reverse). b, c. texture on MEA. d–j. conidiophores. k. conidia (— scale bar in d=10 μm; — scale bar in j=10 μm, applies to e–k)

Micromorphology — Conidiophores mostly very short and monoverticillate, although these might be considered side branches of divaricate type conidiophores, very few true biverticillate conidiophores were observed; coiling of mycelia often observed; stipes/metulae smooth walled, 9–20×2–3.5 μm (13.3±3.5×2.6±0.33), vesicle 2.5–3.5 μm (2.8±0.28), biverticillate conidiophore stipes 50–200 μm, only 2 metulae present, often a solitary phialide borne on same level as metula; phialides 1–5 per stipe/metula, ampulliform, 5–7.5×2–3 μm (6.4±0.78×2.8±0.28); Conidia rough walled, broadly ellipsoidal, 2–3×2–2.5 μm (2.6±0.17×2.2±0.14), average width/length=0.86±0.05, n=77.

Holotypus — South Africa, Malmesbury, (33°29'46"S 18°35'16"), from soil, 21 February 2007, collected and isolated by CM Visagie, CBS H-21334 (ex-typus: CBS 135127=CV 2842=DTO 180-D3=DAOM 241159).

Additional cultures examined — South Africa, Malmesbury, (33°29'46"S 18°35'16"), from soil, 21 February 2007, collected and isolated by CM Visagie, CBS 135128=CV 2857=DTO 184-D1, CBS 135129=CV 2858=DTO 180-F2.

Penicillium glaucoroseum **Demelius, Verh. Zool.-Bot. Ges. Wien 72: 72, 1923 (1922)**

MycoBank: MB158423

ITS barcode: KF296407. The species share identical ITS sequences with *P. ortum* and *P. cremeogriseum*.

Alternative markers: *BenA*=KF296469; *CaM*=KF296400; *RPB2*=KF296430.

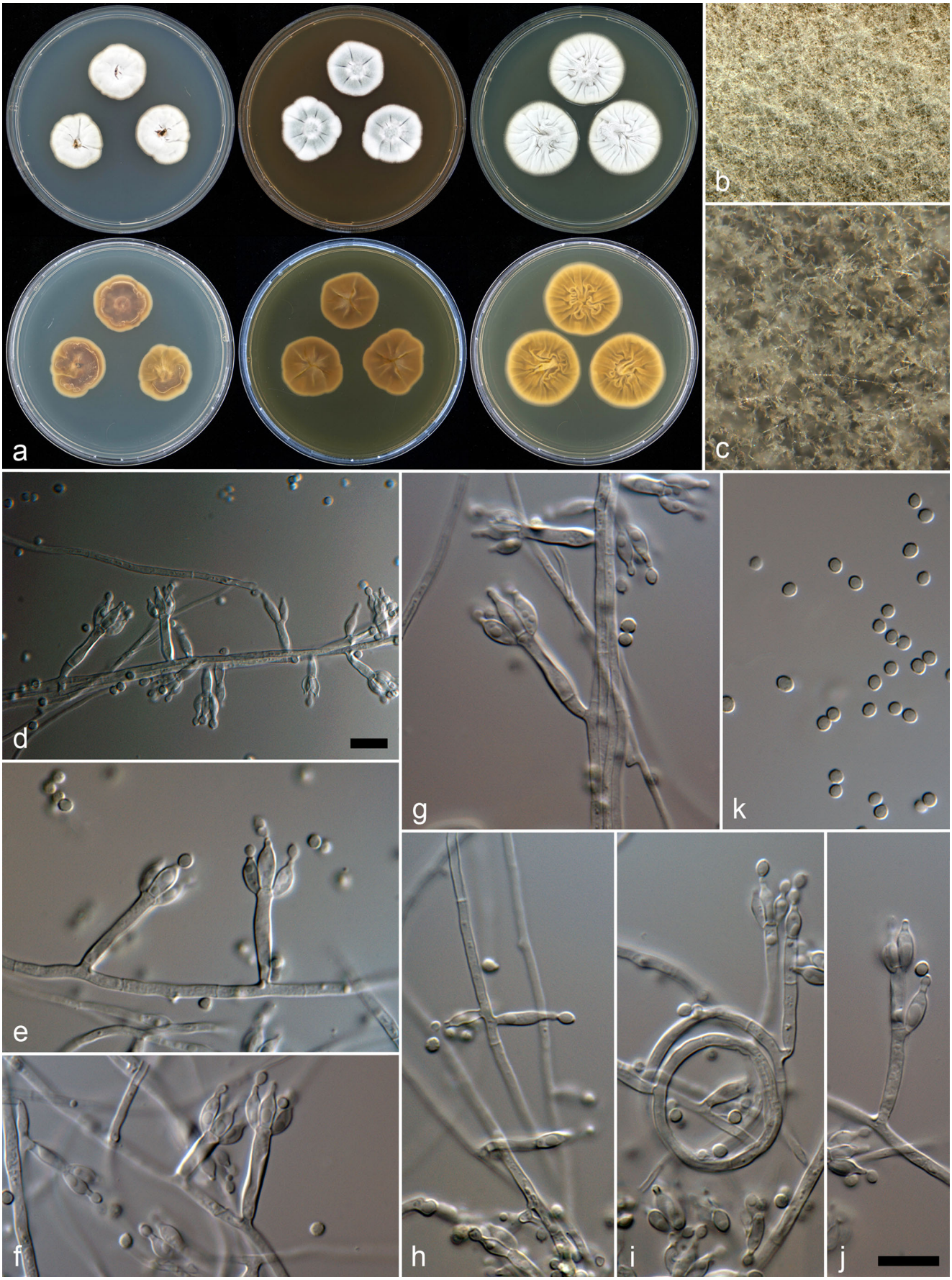
Lectotypus — Fig. 3. (Demelius, Verh. Zool.-Bot. Ges. Wien 72: 73, 1923 (1922)), hic designatus.

Epitypus — USA, Virginia, from soil, unknown date and collector, CBS H-22050 (ex-epitypus: CBS 138908=NRRL 908), hic designatus.

Penicillium malacosphaerulum **Visagie & K. Jacobs, sp. nov. Figs.** 6C & 9

MycoBank: MB809819

ITS barcode: FJ231026. The species has unique ITS sequences, but they are very similar to ITS barcodes of *P. caperatum* and *P. reticulisporum*.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Alternative markers: *BenA*=JX091524; *CaM*=JX141542; *RPB2*=KF296438.

Etymology: Latin, *malacosphaerulum*: *malacos* meaning soft, *sphaerula* meaning small ball; referring to the soft yellow ascocarps produced by this species.

Diagnosis — Relatively slow growth on CYA at 25 °C (28–36 mm), but fast growth at 30 °C (40–45 mm) and 37 °C (30–35 mm). Colonies produce yellow soluble pigment. Cleistothecia dark yellow to brown, ascospores finely roughened with two longitudinal flanges present and relatively small (2.5–3.5×2–3 μm).

Colony diam, *7 days*, *in mm* — CYA 28–36; CYA 5 °C sometimes germination; CYA 30 °C 40–45; CYA 37 °C 30–35; MEA 26–32; YES 33–43; DG18 12–15; CYAS 19–22; OA 25–27; CREA 25–30.

Macromorphology — CYA, 25 °C, 7 days: Colonies low to moderately deep, radially, and concentrically sulcate; margins low, wide (3 mm), entire; mycelia white and inconspicuously yellow; texture floccose; sclerotia produced especially near colony centre, giving colony a greyish yellow (4C4) color, after 2–4 weeks developing into cleistothecia, sporulation absent; exudate dark brown (8 F8), soluble pigment bright yellow, reverse yellowish brown (5D7–E7) at centre, fading into greyish yellow (4B5) into a yellow (2A7–8) margin. CYA, 30 °C, 7 days: Colonies similar to that of CYA at 25 °C. CYA, 37 °C, 7 days: Colonies low to moderately deep, radially, and concentrically sulcate; margins low, narrow (1 mm), somewhat irregular; mycelia white; texture floccose; sclerotia abundantly produced, giving a greyish yellow (4C4) color to colonies; sporulation moderate, conidia dull green (26D3); exudate a few clear droplets produced, soluble pigment bright yellow, greyish green (1C5) at centre, elsewhere greenish to greyish yellow (1A7–B7), in some isolates brownish orange (5C5) at centre. MEA, 25 °C, 7 days: Colonies low, plane; margins low, narrow (1–2 mm), irregular; mycelia white, inconspicuously yellow; texture floccose; sclerotia abundantly produced, which develop into mature cleistothecia after 2–4 weeks, color ranging from light yellow to greyish orange (2A4–5B3); sporulation absent, conidiophores developing only after 14 days of incubation, conidia indeterminable; exudate absent, soluble pigment absent, reverse yellow (3A8) near centre fading into yellowish white (3A2) margin. YES, 25 °C, 7 days: Colonies low, radially and concentrically sulcate, randomly furrowed as well, sunken in at centre; margins low, narrow (1–2 mm), entire; mycelia white, inconspicuously yellow; texture floccose; sclerotia abundantly produced, giving a greyish yellow (4C4) color; sporulation absent to sparse, conidia indeterminable; exudate absent, soluble pigment yellow, reverse mostly light yellow (2A5–3A5) with yellow (3A6–7) areas present. CREA, 25 °C, 7 days: Colonies not producing acid.

Micromorphology — Conidiophores irregularly biverticillate and divaricate; stipes smooth walled, 100–500×2–3 μm, sometimes shorter, then 30–80 μm; metulae mostly 2 with occasionally 3 per stipe, verticils appressed to divergent, angle 22–41° (32±6.7°), 6.5–22×2–3.5 μm (14.1±4.4×2.6±0.33), vesicle 2.5–4 μm (3.3±0.34); phialides ampulliform, 3–5 per metula/stipe, (6)7–10×2–3(3.5) μm (7.8±0.84×2.77±0.27); conidia smooth walled, globose to subglobose, 2.5–3×2.5–3 μm (2.6±0.14×2.4±0.16), average width/length=0.92±0.04, n=81; cleistothecia abundantly produced on most media, brownish to dark yellowish, 50–140×50–130 μm (83±19.61×73±17.6); asci borne singly, 5.5–10×4.5–7 μm; ascospores finely rough walled, with two longitudinal flanges, subglobose to broadly ellipsoidal, 2.5–3.5×2–3 μm (3±0.18×2.5±0.17), average width/length=0.82±0.04, n=83.

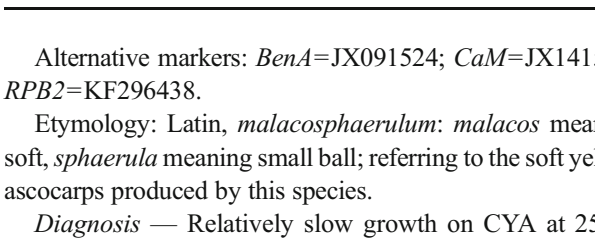


Fig. 9 *Penicillium malacosphaerulum* (CBS 135121). a. colonies on CYA, MEA, and YES from left to right (top=obverse, bottom=reverse). b, c. texture on MEA. d. cleistothecia. e–i. conidiophores. j. conidia. k. asci and ascospores (— scale bar in d=50 μm; — scale bar in i=10 μm, applies to e–i; sScale bar in k=10 μm)

Holotypus — South Africa, Malmesbury, (33°29'46"S 18°35'16"), from soil, 21 February 2007, collected and isolated by CM Visagie, CBS H-21332 (ex-typus: CBS 135121=CV 2855=DTO 180-E6=DAOM 241161).

Additional cultures examined — South Africa, Malmesbury, (33°29'46"S 18°35'16"), from soil, 21 February 2007, collected and isolated by CM Visagie, CBS 135120=CV 2836=DTO 180-D1, CBS 135122=CV 2848=DTO 180-E1.

Penicillium ortum **Visagie & K. Jacobs, sp. nov. Figs. 6D & 10**

MycoBank: MB809820.

ITS barcode: JX091427. This species shares identical barcodes with *P. cremeogriseum*, and has very similar barcodes to *P. glaucoroseum*, *P. ludwigii*, *P. janthinellum,* and *P. cluniae*.

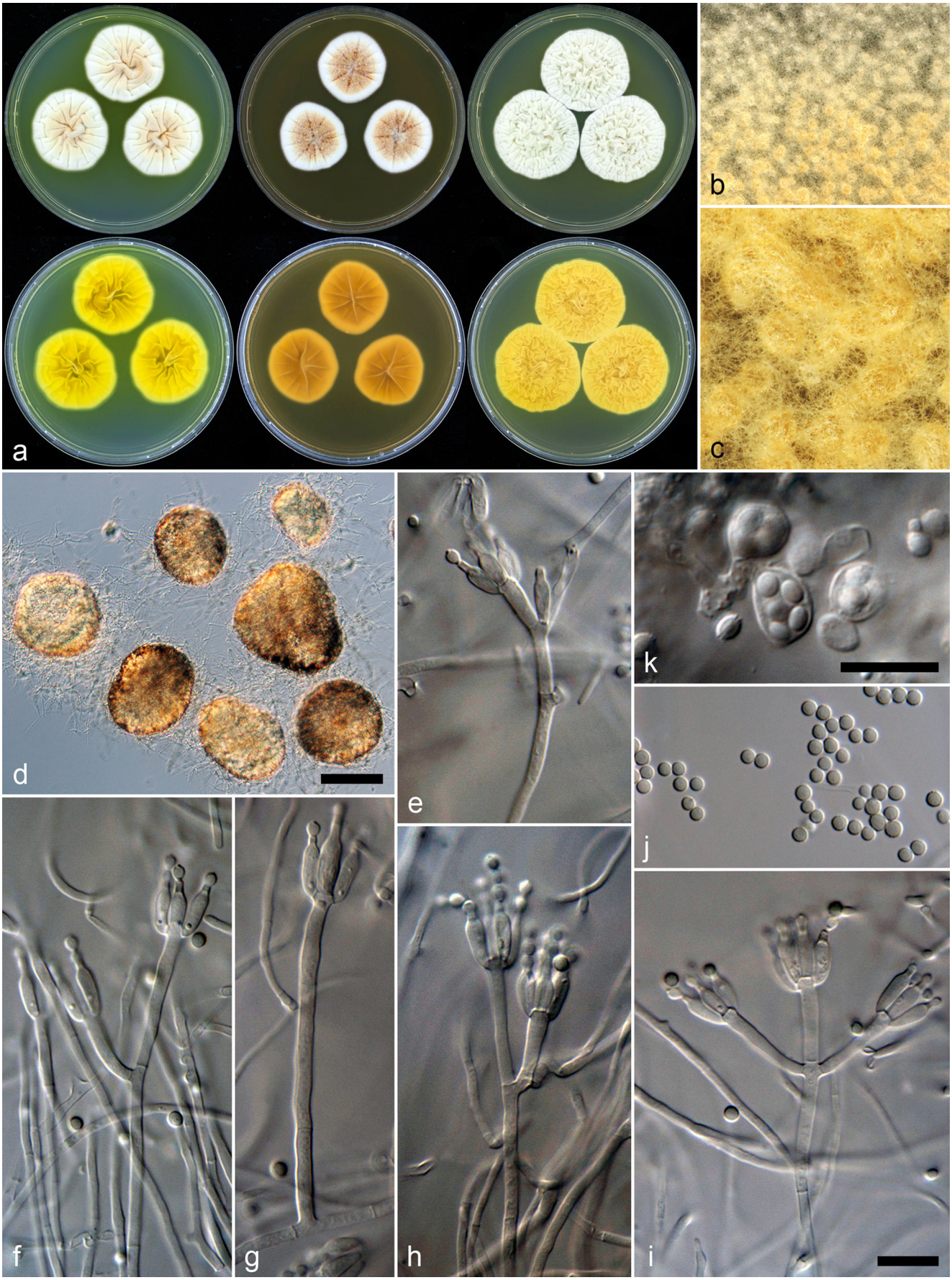
Alternative markers: *BenA*=JX091520; *CaM*=JX141551; *RPB2*=KF296443.

Etymology: Latin, *ortum* meaning sunrise; referring to the colony appearance on YES resembling that of a "sunny side up egg".

Diagnosis — Relatively fast growth on CYA at 30 °C (36–50 mm) and 37 °C (35–40 mm). Strong growth on CREA with moderate acid production. Reverse on CYA olive to brownish orange. Conidia smooth walled.

Colony diam, *7 days*, *in mm* — CYA 28–40; CYA 5 °C germination; CYA 30 °C 36–50; CYA 37 °C 35–40; MEA 48–53; YES 40–50; DG18 20–27; CYAS 30–36; OA 40–45; CREA 25–30.

Macromorphology — CYA, 25 °C, 7 days: Colonies moderately deep, lightly radially sulcate; margins low, narrow (1–2 mm), entire; mycelia white at margin, light yellow to yellowish grey elsewhere; texture floccose; sporulation absent to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sparse, conidia dull green (26D3); exudate mostly absent, but sometimes clear, soluble pigment yellow, reverse olive (3E8) becoming lemon yellow (3B8) near margins, brownish orange (7C6) areas also present. CYA 30 °C, 7 days: Colonies moderately deep, radially sulcate, having a yellowish colour; margins low, narrow 2–3 mm, entire; mycelia white; sporulation absent; exudate yellow, soluble pigment absent, reverse brown (5 F7–8) at colony centre, elsewhere olive to dark yellow (3C6–8–4C6–8), with yellow (2A6) margin. CYA 37 °C, 7 days: Colonies moderately deep, radially and concentrically sulcate, having a pale yellow (1A3) colour; margins low, narrow (1–2 mm), entire; mycelia white; texture floccose; sporulation sparse, only near colony margins, conidia greenish grey (29C2–30B2); exudate yellow, soluble pigment yellow, reverse olive brown (4E8) at centre, fading into olive to greyish yellow (3C6–4C6). MEA, 25 °C, 7 days: Colonies moderately deep, plane; margins low, narrow, entire; mycelia white at margin, light yellow elsewhere; texture floccose; sporulation sparse to moderate, conidia dull green (26D3); exudate absent, soluble pigment absent, reverse olive yellow (3C8) becoming yellow (3A7) near margins. YES, 25 °C, 7 days: Colonies moderately deep, radially sulcate, also randomly furrowed; margins low, very narrow (1–2 mm), entire; mycelia white at margin, light yellow elsewhere; texture floccose; sporulation sparse to sometimes absent, conidia dull green (26D3); exudate absent, soluble pigment absent, reverse areas of brown to dark brown (5 F8–6 F8), elsewhere varying from greyish yellow to orange yellow (4B6–8), margins light yellow (4A4). CREA 25 °C, 7 days: Colonies producing moderate acid.

Micromorphology — Conidiophores biverticillate with a large number of subterminal side branches formed that may be mono- or biverticillate; stipes smooth walled, 150–550×2–3 μm, side branched "conidiophore" stipes 18–90 μm; metulae 2 to 4 per stipe, mostly divergent, angle 19–50° (34±9.2°), 10–30×2–3 μm (18.1±4.91×2.7±0.27), vesicle 2.5–4 μm (3.1±0.33); phialides ampulliform, 3 to 7 per metula, 6.5–9(10)×2–3 μm (7.6±0.75×2.8±0.21); conidia smooth walled, globose to subglobose, 2.5–3.5×2.5–3.5 μm (2.9±0.2×2.7±0.2), average width/length=0.92±0.04, n=76.

Holotypus — South Africa, Stellenbosch, (33°56'47"S 18°52'49"), from soil sample, 14 March 2009, collected and isolated by CM Visagie, CBS H-21602 (ex-typus: CBS 135669=CV 102=DTO 180-I9).

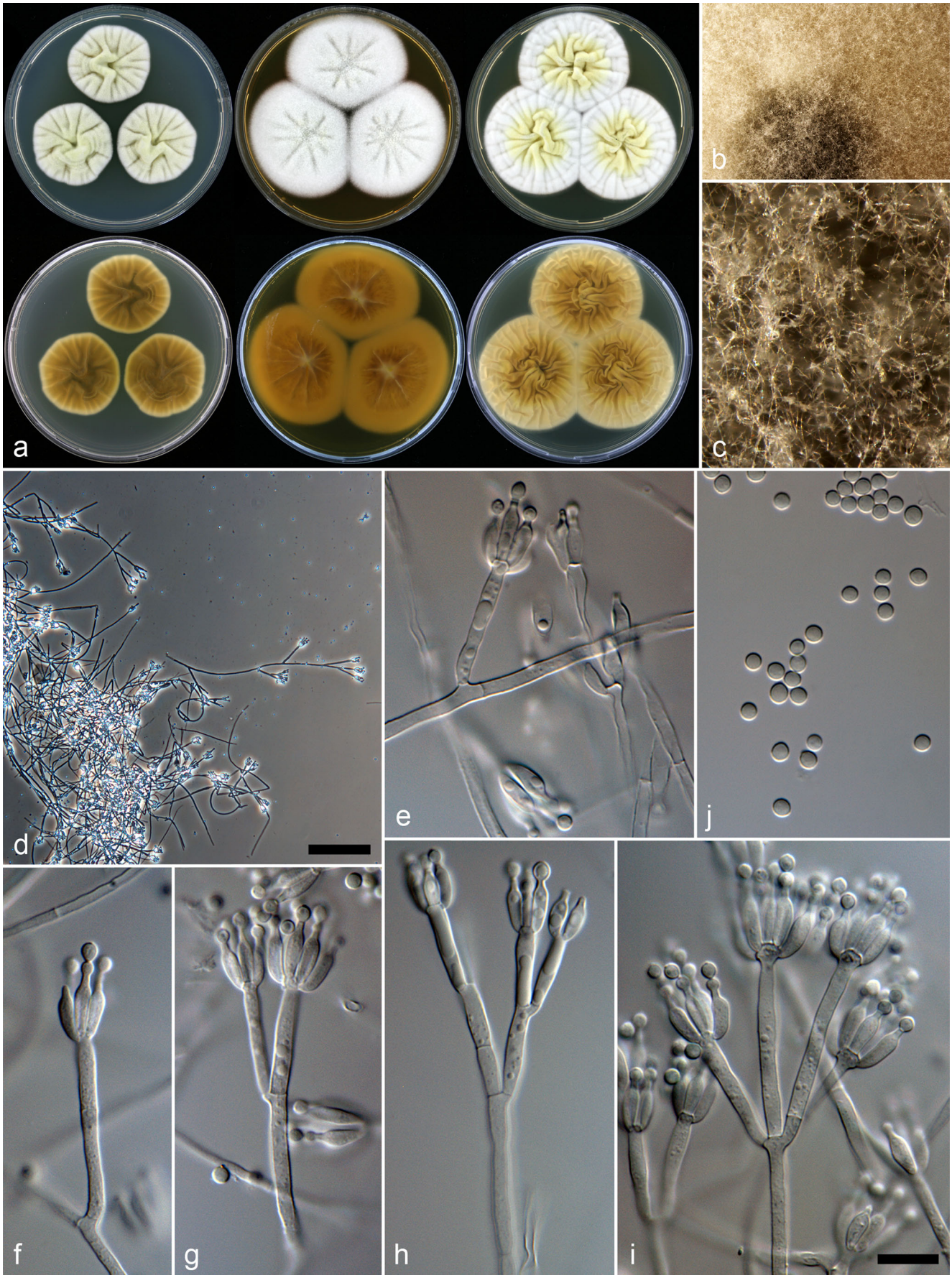
Additional cultures examined — South Africa, Stellenbosch, (33°56'47"S 18°52'49"), from soil sample, 14 March 2009, collected and isolated by CM Visagie, CBS 135668=CV 71=DTO 180-H7, CBS 135667=CV 95=DTO 180-I7; South Africa, Stellenbosch, (33°56'47"S 18°52'49"), from *Protea repens* infructescence bract, 14 March 2009, collected and isolated by CM Visagie, CBS 135670=CV 391=DTO 181-F5.

Fig. 10 *Penicillium ortum* (CBS 135669). a. colonies on CYA, MEA, and YES from left to right (top=obverse, bottom=reverse). b, c. texture on MEA. d–i. conidiophores. j. conidia (— scale bar in d=50 μm; — scale bar in i=10 μm, applies to e–j)

Discussion

Traditionally, *Penicillium* section *Lanata-Divaricata* is taxonomically challenging because of morphological similarities between species and variation within a species. This resulted in Pitt (1979) synonomising a large number of species with *P. janthinellum* and *P. simplicissimum* citing the roughened stipes and more regular branching pattern of the latter diagnostic in the latter in comparison with the smooth walled stipes and divaricate branching of *P. janthinellum*. Stolk and Samson (1983) on the other hand considered both *P. janthinellum* and *P. simplicissimum* as the anamorph state of *Eupenicillium javanicum* and thus lumped 24 species, irrespective of conidiophore morphologies. Even though these authors attempted to simplify identifications in this difficult group, it always remained problematic. One problem associated with the group is the variation observed from strain to strain, wether it be slightly different colors in colonies or growth rates. This creates the difficulty of not knowing wether strains with morphological variation represented a previously described species or not. Only with the incorporation of DNA sequence data into the species concept used for *Penicillium* were we able to satisfactorily resolve these problems and make identifications easier. This is because of the clearer species boundaries inferred from phylogenies that makes morphological comparisons easier.

The study presented here compared section *Lanata-Divaricata* species based on phylogenies from four genes (ITS, *BenA*, *CaM*, and *RPB2*) and morphology, applying GCPSR to the phylogenetic data. The phylogenies presented here supported the acceptance of 36 species by Houbraken et al. (2011), and demonstrated distinctiveness of *P. cluniae*, *P. glaucoroseum*, and *P. griseopurpureum*. We introduced four new species isolated from the fynbos biome in South Africa as *P. annulatum*, *P. curticaule*, *P. malacosphaerulum*, and *P. ortum*, which are phylogenetically resolved in consistent clades with their respective close relatives. These clades were designated here as the *P. janthinellum*, *P. javanicum*, and *P. rolfsii* clades. All species from these clades were characterized to more precisely define them morphologically and aid future identifications. Generally, colony growth rates on different media incubated at different temperatures were informative characters. This is especially true for growth on CYA at 37 °C. Also, acid production and colony characters on CREA were valuable for distinguishing among closely related species. For the *P. javanicum* clade, characters of the sexual reproductive structures distinguish among species, whereas conidiophore morphologies were informative in the *P. rolfsii*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clade. This includes the size of asci and ascospores, as well as ascospore ornamentation, similar to the conclusions of Udagawa and Horie (1973). Generally, conidiophore morphology was very difficult to consistently interpret; mainly because of conidiophore branching that was found to be so diverse and variable within a species that it becomes uninformative for species identification. This was evident in especially the *P. janthinellum* and *P. javanicum* clades. In the *P. janthinellum* clade, only phialide length and conidial wall texture was informative. Pitt (1979) emphasized the long slender phialide necks and divaricate branching in *P. janthinellum*. However, the long phialides were also observed in *P. lineolatum* and *P. ludwigii* and while divaricate branching is characteristic of all species in this clade, it is not restricted to it. In the *P. javanicum* clade only stipe and conidial wall texture was informative.

Dodge (1933) described *P. brefeldianum* based on CBS 235.81. This particular strain lost the ability to reproduce sexually, and Pitt (1979) neotypified the species with CBS 233.81. Houbraken and Samson (2011) showed that CBS 233.81 is phylogenetically identical to *P. caperatum* (CBS 443.75) and reinstated CBS 235.81 as type for *P. brefeldianum*. In our study, none of these strains were able to reproduce sexually. Phylogenetically, *P. brefeldianum* and *P. caperatum* are respectively resolved in the *P. janthinellum* and *P. javanicum* clades, even though morphologically they are very similar. The main difference is that *P. caperatum* typically produces acid on CREA and grows slightly slower on MEA than *P. brefeldianum*.

Previous phylogenetic studies focused on section *Lanata-Divaricata* did not include *P. glaucoroseum*, *P. cluniae*, or *P. griseopurpureum* (Houbraken et al. 2011; Houbraken and Samson 2011). Houbraken and Samson (2011), however, suggested that unpublished data showed that they are distinct species that are appropriately classified in the section. Here we confirm this, with *P. glaucoroseum* and *P. cluniae* resolved in the *P. janthinellum* clade and *P. griseopurpureum* closely related to *P. daleae*. Demelius (1922) described *P. glaucoroseum* and emphasized the production of rosy crystals and granules in colonies. Based on the original description, Thom (1930) placed the species in his *P. janthinellum* series and considered strain NRRL 908 a good representative for *P. glaucoroseum*. Unfortunately, none of Demelius' authentic material is obtainable. In common with Thom (1930) and Raper and Thom (1949), we consider NRRL 908 to be in good condition, with the culture producing the dark reddish to purple reverse and irregular conidiophores as described by Demelius (1922). As such, we lectotypified *P. glaucoroseum* based on Demelius' Fig. 3 (1922) and epitypified it with the dried specimen CBS H-22050 (ex-epitype NRRL 908=CBS 138908) above.

Ramírez and Martinez (1981) described *Penicillium aragonense*, with the ex-type strain submitted and preserved in the CBS-KNAW culture collection under accession number CBS 171.81. However, this culture is badly contaminated with *P. glabrum*. Based on the original description, Stolk et al. (1990) synonymized the species with *P. oxalicum*. In contrast, our opinion is that the drawing and original description of *P. aragonense* (Ramírez and Martinez 1981) do not match *P. oxalicum*. Even though the large, ellipsoidal conidia (4–6×3.2–5 μm) seem similar to those of *P. oxalicum*, the description does not resemble the conidiophore shapes or the large masses of conidia in colonies typical of *P. oxalicum*. Based on this and the absence of additional material, we have to consider its identity uncertain. In the same paper, Ramírez and Martinez (1981) described *P. asturianum*. Although their description and drawings resemble *P. oxalicum*, they considered it a close relative of *P. janthinellum*. Subsequently, Stolk et al. (1990) reduced *P. asturianum* to synonymy with *P. oxalicum*. Morphological characters combined with the multigene phylogeny presented here confirm this species as a synonym of *P. oxalicum*.

This study provided phylogenies to species belonging in *Penicillium* section *Lanata-Divaricata* and reported on four new species. The paper follows previous publications reporting new species from the diverse fynbos biome situated in South Africa (Visagie et al. 2013, 2014b; Houbraken et al. 2014), and represents a small proportion of undescribed *Penicillium* species isolated during a survey of the genus in the fynbos, which will be described in future publications.

Acknowledgments This research was supported by grants from the South African Biosystematics Initiative (SABI) of the National Research Foundation (NRF). The Western Cape Nature Conservation Board issued permits for collection trips in the fynbos. We are grateful to Hugh Glen for providing nomenclatural assistance on the manuscript.

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