BIODIVERSITY IN THE GENUS *PENICILLIUM* FROM COASTAL FYNBOS SOIL

Cobus M. Visagie

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Supervisor: Dr. Karin Jacobs

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SUMMARY

Penicillium is a well-known cosmopolitan genus with more than 225 accepted species. Species from this diverse genus, in general, are considered to primarily be soil fungi, with decomposition as its main function. Therefore, together with its ubiquitous nature, these species are of great importance in ecosystems, agriculture and biotechnology. However, in South Africa, very little research has been done on this complex genus, as species identification were often found to be problematic, even for experienced taxonomists. This lead to a number of South African studies only mentioning that a *Penicillium* spp. were isolated, without making any attempt of showing the extent of diversity within the genus from the unique habitats.

The present study set out to explore the extent of the species diversity in *Penicillium* isolated from the Cape Floristic Region, specifically focusing on coastal fynbos soil. Soil samples were collected from this region, at sites situated outside Malmesbury. Four hundred and thirty four *Penicillium* strains were isolated from soil-dilutions. The strains were characterized using morphological characters and subsequently placed into 24 morphological groups. There were, also, more or less 40 strains that could not be grouped with any other isolates. Groupings were made according to conidiophore branching patterns which divided the strains into their respective subgenera. Eight species from subgenus *Aspergilloides,* seven from subgenus *Furcatum*, eight from subgenus *Biverticillium* and one from subgenus *Penicillium* were isolated. The species were further characterized in subsequent chapters.

In the second chapter of this thesis, one of the taxonomic groups in subgenus *Biverticillium,* isolated from coastal fynbos soil, *Protea* infructescences and on moth-damaged Riesling grapes in Canada, was examined. This species was characterized using morphology and were found to have several unique characters, such as the very short synnema produced after prolonged incubation. These characters did not conform to descriptions of previously described species. Its novelty was confirmed by an ITS phylogeny and the strains were

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subsequently described as *Penicillium ramulosum* prov. nom. with *P. cecidicola* and *P. dendriticum* as its sister taxa.

In chapter three, a further seven groups belonging to *Penicillium* subgenus *Biverticillium* were characterized. These strains were identified as *P. minioluteum, P. verruculosum* and *P. rugulosum*-like, respectively. Four of the groups showed unique morphological characters, with the ITS phylogeny resolving the fynbos strains separate from all previously described species. The strains were, therefore, considered to be new to science and described as *P. solicola* prov. nom., *P. ptychoconidium* prov. nom., *P. occultum* prov. nom. and *P. chloroloma* prov. nom., respectively. A key to species from subgenus *Biverticillium* is also included.

Penicillium subgenus *Furcatum* was the subject of the fourth chapter of this thesis. Our survey found that although the species diversity in this group was not as high as for the other subgenera, it was the group most often isolated in this study. Species were identified as *P. janczewskii*, *P. canescens, P. melinii, P. corylophilum* and *P. citrinum* using morphological characters. One species belonging to subgenus *Penicillium*, *P. expansum*, was also isolated and compared to species recorded during a previous survey. Amongst the identified species, were two groups that could not be identified using published keys, with their novelty confirmed by an ITS phylogeny. They are, therefore, described here as *P. subturcoseum* prov. nom. and *P. hemitrachum* prov. nom. A key to species in this subgenus is also provided.

In Chapter 5 the presence of *Penicillium* subgenus *Aspergilloides*, which is characterized by monoverticillate conidiophores, were investigated. Species were identified as *P. roseopurpureum, P. restrictum, P. hirayamae* and *P. toxicarium*. Amongst the identified species, were four groups that did not conform to previously described species and are described here as *P. brachycaulon* prov. nom., *P. malacosphaerula* prov. nom., *P. cumulacinatum* prov. nom. and *P. vulgaris* prov. nom., respectively. The newly described species have been included in a key, together with closely related species and the other species of subgenus *Aspergilloides* from the fynbos soil.

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Species identifications in *Penicillium* is often problematic and South African taxonomists have often not attempt to identify strains down to species level. During this study, *Penicillium* was found to be well represented in the soil, with a large proportion being previously undescribed. For this reason, a dichotomous and synoptic key to species isolated during this study are provided in the final chapter. This study should thus serve as a basis for further explorations into the diversity and ecological role of this group of organisms in this ecologically important biome.

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OPSOMMING

Penicillium is 'n bekende kosmopolitaanse genus, met meer as 225 aanvaarde spesies. Spesies van hierdie diverse genus word in die algemeen beskou as grondswamme met die afbreek van organiese materiale as hooffunksie. Dit, en hul allomteenwoordige aard, maak individue baie belangrik in ekosisteme, landbou en biotegnologie. In Suid-Afrika is daar egter baie min navorsing op hierdie komplekse genus gedoen, aangesien identifikasies tot op 'n spesie vlak gereeld problematies is, selfs vir ervare taksonome. Dit het gelei tot 'n aantal Suid-Afrikaanse studies wat noem dat *Penicillium* geïsoleer is, met geen poging aangewend om die ware diverse natuur van die genus in ons unieke habitatte aan te dui.

Hierdie studie poog om die spesies diversiteit binne die genus *Penicillium* van die Kaapse Flora Streek, met spesifieke fokus op kusgebied fynbos grond, na te vors. Vier honderd vier en dertig *Penicillium* stamme is geïsoleer vanuit grond versamel net buite Malmesbury. Stamme is in 24 morfologies onderskeibare groepe verdeel, gebasseer op morfologiese karakters. Daar was ongeveer 40 stamme wat nie in groepe geplaas kon word nie. Die groepe is verder verdeel op grond van die aantal vertakkings wat plaasvind in hul konidiofore en verder gekarakteriseer in die onderskeie hoofstukke.

Hoofstuk twee fokus op 'n spesie van subgenus *Biverticillium*, wat vanuit fynbos grond, *Protea* koppe, asook mot-beskadigde Riesling druiwe in Kanada geïsoleer is. Die spesie is morfologies gekarakteriseer en het unieke eienskappe getoon, soos die kort synnema wat geproduseer is na verlengde inkubasie. Die ITS filogenie het stamme in 'n groep, apart van enige bekende spesies, geplaas en word dus hier beskryf as *P. ramulosum* prov. nom., met *P. cecidicola* en *P. dendriticum* as sy suster taxa.

In hoofstuk drie word 'n verdere sewe groepe van *Penicillium* subgenus *Biverticillium* gekarakteriseer. Groepe was as *P. minioluteum*, *P. verruculosum* en *P. rugulosum* geïdentifiseer. Vier van die groepe het unieke morfologiese eienskappe getoon, met die ITS filogenie, wat spesies in groepe, apart van enige bekende spesies geplaas het. Hierdie stamme word daarom beskou as nuut en is

beskryf as *P. solicola* prov. nom., *P. ptychoconidium* prov. nom., *P. occultum* prov. nom. en *P. chloroloma* prov. nom. ‘n Identifikasie-sleutel tot subgenus *Biverticillium* spesies, geïsoleer tydens hierdie studie, word ook verskaf.

Die fokus van hoofstuk vier is *Penicillium* subgenus *Furcatum.* In ons opname was die spesies diversiteit effens laer as gevind vir van die ander subgenera, maar subgenus *Furcatum* stamme het die meeste voorgekom. Spesies is geïdentifiseer as *P. janczewskii, P. canescens, P. melinii, P. corylophilum* en *P. citrinum*, met *P. expansum,* die enigste subgenus *Penicillium* spesie geïsoleer tydens hierdie studie. Twee groepe kon nie geïdentifiseer word met bestaande sleutels nie, met hul uniekheid wat bevestig is deur die ITS filogenie. Hulle word dus hier beskryf as *P. hemitrachum* prov. nom. en *P. subturcoseum* prov. nom. en word ingesluit in ‘n sleutel vir die identifikasie van subgenus *Furcatum* spesies, gevind in die fynbos grond.

Hoofstuk vyf fokus op *Penicillium* subgenus *Aspergilloides*. Spesies is geïdentifiseer as *P. roseopurpureum, P. restrictum, P. hirayamae* en *P. toxicarium*. Vier groepe het eienskappe getoon wat uniek is van enige voorheen beskryfde spesies en word dus hier onderskeidelik beskryf as *P. brachycaulon, P. malacosphaerula, P. cumulacinatum* en *P. vulgaris* prov. nom. Hierdie spesies word ingesluit in ‘n sleutel, saam met hul naasverwante, asook bekende spesies geïsoleer tydens hierdie studie.

Penicillium spesies identifikasie is gereeld problematies en Suid-Afrikaanse taksonome het dikwels nie stamme tot op spesie vlak geïdentifiseer nie. Tydens hierdie studie is daar gevind dat *Penicillium* volop in die grond was, met ‘n groot verhouding onbekende spesies. Daarom word ‘n digotome en sinoptiese identifikasie-sleutel tot spesies geïsoleer tydens hierdie studie, verskaf in hoofstuk ses. Hierdie studie sal dus as basis dien vir verdere eksplorasies in die diversiteit en ekologiese rol van hierdie groep organismes in hierdie ekologies belangrike bioom.

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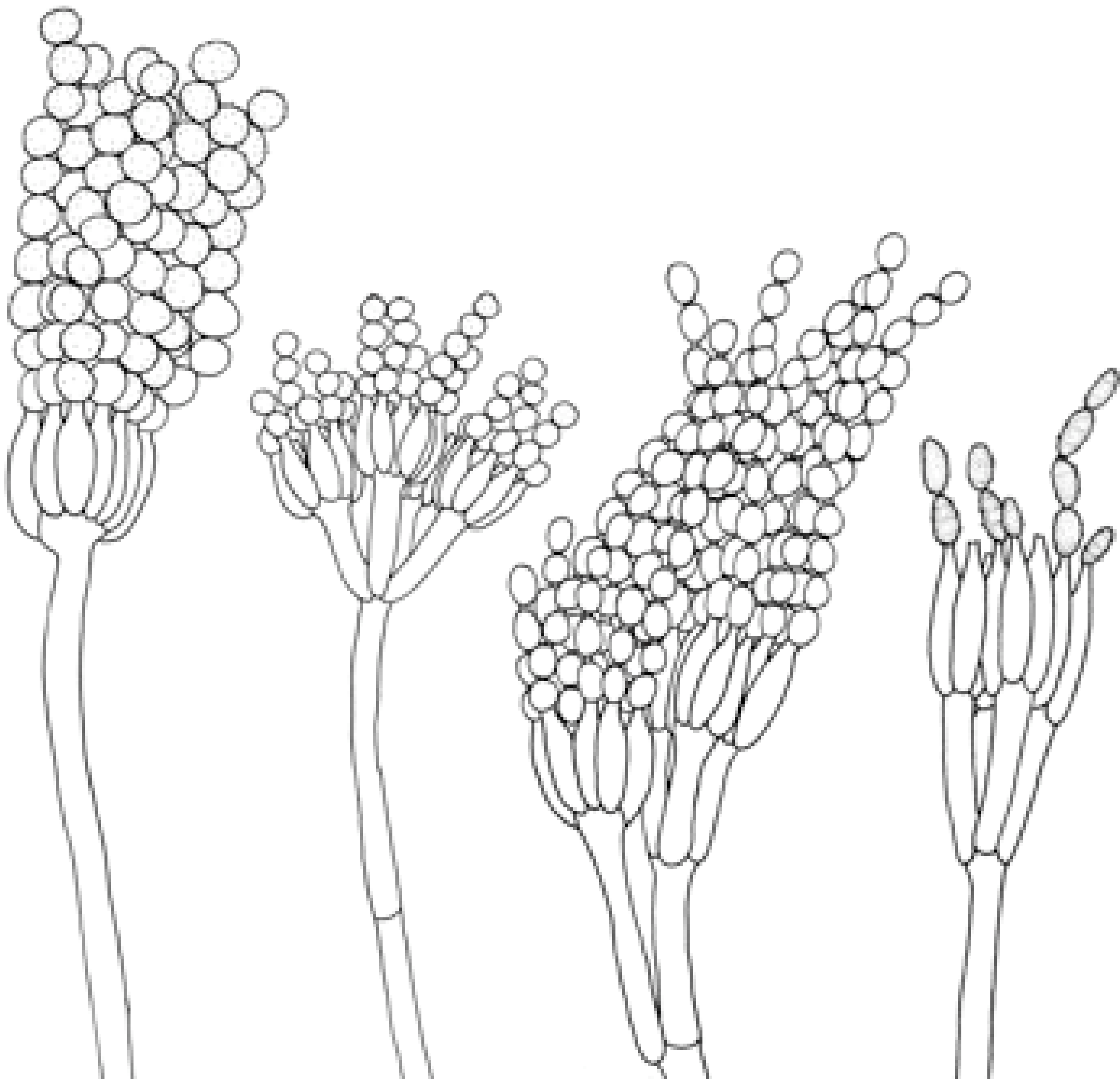
CHAPTER 1
Two-hundred years of *Penicillium* taxonomy



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1. TWO-HUNDRED YEARS OF *PENICILLIUM* TAXONOMY

1.1. Link (1809) and typification of the genus

The generic name, *Penicillium* Link (Latin: *penicillus,* meaning little brush), was first published in 1809 (Thom 1930, Raper and Thom 1949, Pitt 1979a, Schutte 1992). In his paper, Link described the three species, *Penicillium candidum* Link, *P. expansum* Link and *P. glaucum* Link, based on the morphological appearance of the brush-like structures (Thom 1930). In 1824, Link abandoned the names *P. expansum* and *P. candidum*, and placed all the green *Penicillium* spp. under *P. glaucum* (Saccardo 1886a, Thom 1930, Raper and Thom 1949, Pitt 1979a). This is unfortunate, because many workers did the same, which lead to *P. glaucum* referring to more than one species, making the identification of these earlier species problematic. This name has since been rejected as a *nomen confusum* (Pitt 1979a).

Much controversy arose during the middle 1900's surrounding the type species of the genus *Penicillium*. The debate was caused by article 13f in the International Code of Botanical Nomenclature (ICBN), which stated that a name can only validly be published after 1 January 1821. This implied that work published by Fries in "*Systema Mycologicum*" (1821-1832) was given priority over earlier names for the species (Pitt 1979a). Matters were further complicated by the fact that Fries listed *Mucor crustaceus* Linnaeus (1753), *P. expansum* and *P. glaucum* (1809) and his *P. crustaceum* as synonyms (Hawksworth et al. 1976). This effectively meant that he typified the genus by using *Mucor crustaceus* as reference, and not one of Link's species. This was clearly an error and Hawksworth et al. (1976) pointed out that *M. crustaceus* was most probably not a *Penicillium*, but a *Botrytis*-like fungus based upon the illustrations by Micheli (1729), tab 91, which were referred to by Linneaus in his publications (Hawksworth et al. 1976, Hawskworth 1985). *Mucor crustaceus* was, therefore, considered a *nomen dubium* and for maintenance of nomenclatural stability, another type specimen, *P. expansum* Link ex Gray was suggested as the lectotype by Hawksworth et al. (1976).
At the XIII International Botanical Congress (1981), changes made to the ICBN

resolved the typification issue, with the starting point date for the nomenclature of fungi changed from 1 January 1821 to 1 May 1753. This meant that the generic name published by Link in 1809 was valid (Hawksworth 1985) and that the type species had to be selected from one of Link's original species. *Penicillium expansum* was widely accepted as the lectotype based on Thom's (1930) works and was, therefore, retained as the type species for the genus.

1.2 Pre-Thom era (1830-1923)

A large number of *Penicillium* species were described from 1830 up to the 1900's. Because of a lack of culturing methods, species descriptions were mainly based on strains that grew on their natural substrates and since morphology is a response to a species environment, many of these species remained unrecognizable (Pitt 1979a, 1989). Brefeld (1874) was the first to emphasize the importance of standardized culturing techniques (Thom 1930, Raper 1949, Pitt 1979a). Although Brefeld never provided a species description for *P. glaucum*, he made illustrations of the different stages of its life-cycle, including the perfect state that is now recognizable as *Eupenicillium* Ludwig (Pitt 1979a).

Delacroix (1858–1907) recognized the importance of exchanging pure cultures, when in 1891 he described *P. duclauxii* Delacroix and distributed his live strains as type material (Pitt 1979a). About the same time, Biourge started work on a *Penicillium* monograph in 1897 after isolating a number of different strains from germinating barley, malt infusions, beer mort, hops, brewery water, cheese and fruit while doing research on the bacterial diseases of beer (Hennebert 1985). Work on the monograph progressed slowly and eventually Dierckx, one of his students took over all of Biourge's strains and in 1900 handed in his thesis, containing 25 new species descriptions and illustrations (Pitt 1979a, Hennebert 1985). This was published in 1901, but his species descriptions were considered to be inadequate, and identification using the published material was impossible (Thom 1930). He did, however, provide the first subgeneric classification by dividing it into subgenera *Aspergilloides* Dierckx (for monoverticillate species) and *Eupenicillium* Dierckx (for anamorphic species with complex branching conidiophores) (Hennebert 1985). Subsequently, Dierckx lost all of his

Penicillium strains during his travels in 1901 (Thom 1930, Raper 1949, Pitt 1979a, Hennebert 1985). After this setback, he tried to re-isolate and recover some of his species, but eventually abandoned the project. He did, however, lay the foundation for Biourges' monograph, later published in 1923 by donating all of his strains, color plates, drawings and even descriptions of unnamed species to Biourge (Hennebert 1985). The monograph described 125 species, with 60 being previously undescribed, cultured on thirteen growth media. He subdivided the genus, using similar concepts as proposed by Dierckx in 1901, into subgenera *Monoverticillium* Biourge and *Eupenicillium* Dierckx (Pitt 1979a, Hennebert 1985).

Many species belonging to *Coremium* Link (Saccardo 1886b) and *Citromyces* Wehmer (Saccardo 1895), today considered to be the only synonyms to *Penicillium* (Thom 1930, Raper and Thom 1949, Pitt 1979a, Seifert and Samson 1985), were described during this period. *Coremium* was described by Link in 1809, together with his original three *Penicillium* species, basing his descriptions on the fungus producing coremia on the rotting fruit. Thom (1930) and Raper and Thom (1949) did note that this is also a character common to *P. expansum* and, therefore, considered the genus to be a synonym to *Penicillium*. *Citromyces* was described by Wehmer in 1893, for monoverticillate penicilli producing citric acid. Morphologically these species do not vary from *Penicillium* and, therefore, Biourge in 1923 included these species in his subgenus *Monoverticillium* and this treatment was followed by later works by Thom (1930), Raper and Thom (1949) and Pitt (1979a).

1.3 Thom (1930)

Charles Thom (1872–1956) made significant contributions to our understanding of *Penicillium* today. He devoted most of his work on the taxonomy of the genera *Aspergillus* and *Penicillium*, with many of his concepts on speciation and subgeneric classification still in use today (Pitt 1979a). Recognizing the importance of using standardized media and the role of temperature control for describing species was just a few of his contributions. His first major work, published in 1910, described 13 new species and provided the first identification

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key to the genus (Pitt 1979a). Thom's most significant contribution to *Penicillium* taxonomy was, however, "The Penicillia" (1930) in which he gave descriptions of 300 species and provided an extensive key to these.

Thom brought much needed stability to the taxonomy of the genus by classifying it in an orderly fashion into four divisions (subgenera) and subdividing each division into sections and subsections (Pitt 1979a). Classification was done by using a wide range of characteristics from cultures grown on a number of standardized media in specific temperature ranges. Macromorphological characters used for classification included the color of conidial areas, mycelia, substratum and drops (exudate) as well as colony texture, zonation and odor. Micromorphologically he characterized species using branching patterns and textures of the conidiophore, arrangement and length of the metulae, shape and size of the sterigmata (phialides) and the shape, size, texture, germination, arrangement and connection bridges of the conidia and conidial chains when present.

Thom further divided the genus into its four divisions, *Monoverticillata*, *Asymmetrica*, *Biverticillata-symmetrica* and *Polyverticillata-symmetrica*, using the branching pattern of the penicillus as the distinguishing character. *Monoverticillata* was characterized by penicilli containing a single terminal verticil of sterigmata (phialides) at the tip of the conidiophore and included all or most of species previously designated to the genus *Citromyces*, subgenus *Aspergilloides* and subgenus *Monoverticillium*. Division *Assymetrica* was characterized by a penicillus with two or more branching stages, with the metulae or rami being arranged asymmetrically. Division *Biverticillata-symmetrica* on the other hand contained biverticillate species whose metulae are arranged symmetrically. Species divided into *Polyverticillata-symmetrica* were characterized by penicilli with symmetrical polyverticillate penicilli borne on short septate branches.

1.4 Raper and Thom (1949)

Thom had laid the foundations for Raper and Fennel to make the next big advance in *Penicillium* taxonomy when, in collaboration with Thom, they published their revision of the genus, "A Manual of the Penicillia" (1949). Thom's 40 years' experience and understanding of the genus was used as guide for compiling the monograph. It is thus not surprising that Raper (1949) used the same species concepts and subgeneric classification as proposed by Thom in his monograph (Thom 1930). They examined all known species that were in culture and reduced the 300 species accepted by Thom (1930), down to a 137, dividing them into four sections and 41 series (Raper and Thom, 1949; Pitt 1979a). Raper further investigated the effect of standard growth media and incubation temperatures and major advances were made into protocols used in the identification of *Penicillium* (Raper and Thom, 1949). This advance laid the foundation for Pitt's monograph published 30 years later.

1.5 Pitt (1979)

To date Pitt's monographic treatment of *Penicillium* is still the standard used for identification of species in this genus. Pitt (1973) again stressed the importance of standardized incubation conditions and times for morphological characterization of specimens. Because incubation time plays a major role in colony diameters and characters such as conidial color, Pitt recommended seven days of incubation at 25°C as a rule of thumb (Pitt 1973, 1979a). Although it was general knowledge that temperature plays an important role in colony characters, Pitt was the first to realize its taxonomic importance and further pioneered the use of media with reduced water activity as an important taxonomic criterion (Pitt, 1973).

Pitt (1979) accepted 97 species, divided into 4 subgenera, 10 sections and 21 series. He also based his subgeneric classification, similar to earlier monographs (Thom 1930, Raper and Thom 1949), on the branching pattern of the penicillus. However, Pitt replaced section *Monoverticillata* Raper and Thom with subgenus *Aspergilloides* Dierckx. Both contained monoverticillate species, but Raper and Thom (1949) included series *Ramigena* in section *Monoverticillata,* with species that can either have monoverticillate or biverticillate penicilli. These species,

according to Pitt (1979a), are more closely related to that of section *Assymetrica* Raper and Thom (1949) and, therefore, classified them into his subgenus *Furcatum* Pitt. Subgenus *Furcatum* in essence serves as a dumping ground for all species that do not belong to the other subgenera and was previously, by Raper and Thom (1949), classified in subsections *Divaricata* and *Velutina* of section *Assymetrica*, series *Ramigena* of section *Monoverticillata*, and series *Penicillium herqui* of section *Biverticillata-Symmetrica* (Pitt 1979a).

Penicillium species having biverticillate, with a proportion terverticillate, conidiophores and thin acerose phialides were placed into subgenus *Biverticillium* Dierckx, replacing section *Biverticillata-Symmetrica*. Pitt also replaced section *Assymetrica* with subgenus *Penicillium*, with this group defined by species being terminally terverticillate or more complex. In his monograph he also included species descriptions for the associated *Penicillium* teleomorphs, by accepting 37 *Eupenicillium* spp., dividing them into 8 series, and 16 *Talaromyces* spp. divided into 3 sections and 5 series.

Pitt placed great emphasize on the importance of rapid identification techniques, especially for industrial mycologists. To a large extent the standardization of incubation conditions and growth media, made identification of species easier. Pitt's understanding of the genus, led to his concepts and methods employed in his monograph, to be recommended as standard for working with the genus at the First International *Penicillium* and *Aspergillus* Workshop (Samson and Pitt 1985a).

1.6 Trends in **Penicillium** *taxonomy (the post Pitt era)*

Studies of the genus *Penicillium,* both its taxonomy as well as industrial application, has been active with many species described from new habitats (Ramirez and Martinez 1981, Quintanilla 1982, Udagawa and Ueda 1982, Quintanilla 1983, 1984, Ramirez and Muntañola-Cvetkovic 1984, Pitt and Hocking 1985a, 1985b, Quintanilla 1985, Ramirez 1985, Seifert and Samson 1985, Gochenaur and Cochrane 1986, Ramirez 1986, Frisvad et al. 1987, Manoch and Ramirez 1988, Firsvad and Filtenborg 1989, Vincent and Pitt 1989, Frisvad

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et al. 1990, Ramirez 1990, Tzean et al. 1992, Udugawa 1993, Lund and Frisvad 1994, Seifert et al. 1993, Ueda 1995, Boysen et al. 1996, Banke et al. 1997, Frisvad et al. 1997, Hocking et al. 1998, McRae et al. 1999, Peterson et al. 1999, Yaguchi et al. 1999, Wang and Kong 2000, Heredia et al. 2001, Muntañola-Cvetkovic et al. 2001, Chen et al. 2002, Peterson and Sigler 2002, Tuthill and Frisvad 2002, Kong and Liang 2003, Ovary and Frisvad 2003, Peterson et al. 2003, Frisvad and Samson 2004, Peterson et al. 2004, Seifert et al. 2004, Janso et al. 2005, Kwasna and Nirenberg 2005, Peterson et al. 2005, Wang and Zhuang 2005, Frisvad et al. 2006, Serra and Peterson 2007, Wang et al. 2007). This proliferation of species can be attributed to the solid foundation of the Pitt (1979a) monograph. Many more papers and treatments on the genus were published post 1979. It is important to take note of them since, although some more important than others, all of them aids in understanding the future of *Penicillium* systematics (Ramirez 1982, Stolk and Samson 1983, Samson and Pitt 1985b, Frisvad and Filtenborg 1989, Samson and Pitt 1990, LoBuglio et al. 1993, Lund and Frisvad 1994, Berbee et al. 1995, Skouboe et al. 1999, Samson and Pitt 2000, Heredia 2001, Frisvad and Samson 2004, Samson et al. 2004, Wang and Zhuang 2007, Seifert et al. 2007).

Ramirez published his "Manual and atlas of the Penicillia" in 1982. Although his work was not considered to be as important as Pitt's he emphasized the importance of using full color plates for species descriptions. The plates were considered by him, to be the first step in the identification stage for a non-expert *Penicillium* taxonomist. Unfortunately, a large number of his species descriptions were based on single specimen isolates (Christensen et al. 2000), incubated for two weeks, which was contrary to that proposed by Pitt (1979a), making comparisons with other studies difficult.

One of the main focus areas over the last few decades was to clarify species concepts and relationships within *Penicillium* subgenus *Penicillium* (Samson et al. 1976, Frisvad 1981, Frisvad and Filtenborg 1983, Frisvad 1985, Bridge et al. 1990, Frisvad and Filtenborg 1990a, Pitt and Cruickshank 1990, Stolk et al. 1990, Skouboe et al. 1999, Seifert and Louis-Seize 2000, Frisvad and Samson 2004,

Samson et al. 2004). This is an important group of species with major economic importance, not only as post-harvest pathogens, but also as producers of various toxins in these niches. Distinguishing between closely related species based on morphological differences is difficult.

Samson et al. (1976) addressed this difficulty when they revised Raper and Thom's (1949) subsection *Fasciculata* species within section *Assymetrica*. These species were characterized by their fasciculate (conidiophores partially or completely aggregated into loose or well-defined synnemata) colony textures. Problems arose because of intraspecies variation occurring and strains that share characteristic features from two different species, otherwise known as intergrading strains, as seen for some *P. crustosum* and *P. cyclopium* strains (Samson et al. 1976). Raper and Thom (1949) distinguished between species, based on characters such as colony colors and differences in colony textures. Samson et al. (1976) revised this by placing emphasis on micromorphological characters and reduced many of Rapers' species to synonymy. Pitt (1979a) on the other hand, with his reclassification of section *Assymetrica* (Raper and Thom 1949) into subgenus *Penicillium,* used concepts different from those employed by Samson et al. (1976), and this further complicated the issue of species delineation (Pitt and Cruickshank 1990).

To circumvent the problems experienced with morphological identifications, taxonomists started to explore other techniques for the characterization and separation of species. The first step towards a more stable taxonomy was the use of secondary metabolite profiles. Frisvad (1981, 1985, 1989), and Frisvad and Filtenborg (1983, 1989, 1990a), showed that by using separation techniques such as thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC), a secondary metabolite profile are created which is reproducible and taxonomically significant. Many of the species in subgenus *Penicillium* could be identified by using only these profiles (Frisvad 1985, Frisvad and Filtenborg 1990a). Using secondary metabolite profiles as the sole criterion is, however, not recommended since morphology is still a central part of species concepts within *Penicillium*. Secondary metabolite profiles have also

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been useful to successfully characterize and separate some species, previously considered to be synonyms based on morphology, within *Penicillium* subgenus *Furcatum* (Frisvad 1990b). Although this technique does not provide answers to all problems experienced in *Penicillium* taxonomy, it does provide good, reproducible taxonomic information on a species and is employed in a number of major laboratories working with *Penicillium*.

After the introduction of the polymerase chain reaction (PCR) during the late 1980's, a number of PCR-based typing techniques were developed, that included, to name a few, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single-strand conformation polymorphism (SSCP), microsatellites, and restriction fragment length polymorphism (RFLP) (Scott and Straus 2000). These techniques did have a number of problems, such as reproducibility, standardizing and containing limited taxonomic information (Scott and Straus 2000), associated with them. Since DNA sequencing became more accessible in the 1990's these PCR typing techniques were never really extensively used for solving taxonomic difficulties within *Penicillium*. They were, however, successfully used to create molecular markers for the easy identification of the human pathogen, *Penicillium marneffei* (Trewatcharegon et al. 2001, Fischer et al. 2004, Lasker and Ran 2004, Vanittanakom 2006).

Today, sequencing is probably the most powerful tool for answering some of the more complex taxonomic questions within *Penicillium*, *Eupenicillium*, *Talaromyces* and their closely related genera. The information age has also necessitated the need for taxonomic information such as databanking of names, DNA sequences, morphological data and electronic keys to be published on open access internet databases such Mycobank (Seifert and Seers 2000, Crous et al. 2004). These databases are extremely important for having a stable taxonomy and as was the case throughout history, taxonomists working with *Penicillium* are near the forefront of incorporating these techniques into taxonomic treatments (Seifert and Seers 2000). Frisvad and Samson (2004) published their monograph on the species from *Penicillium* subgenus *Penicillium*, extensively

explaining the characters and techniques used. They also took *Penicillium* taxonomy to the next level by publishing their electronic synoptic key, using morphology, chemotaxonomy and sequence data, online on the Mycobank website. Using this online classification system as base, we are already starting to move into the next step of *Penicillium* taxonomy, namely DNA barcoding for species identification (Seifert et al. 2007).

Correct identification of a species should never be underestimated, since it is the base from which all biological research are done. There is great meaning in correct species identification, since a single name contains a large amount of information that might prove vital in scientific studies (Seifert et al. 2007). Fungal identifications is, unfortunately, a daunting task, sometimes also for the experts. It is, therefore, no surprise that large international collaborative DNA barcoding initiatives have been started, since this would make species identification from a single DNA specimen theoretically possible (Blaxter 2003, Hebert et al. 2003a, Tautz et al. 2003, DeSalle et al. 2005, Min and Hickey 2007, Ratnasingham and Hebert 2007, Seifert et al. 2007). With the problems previously experienced in separating species from *Penicillium* subgenus *Penicillium*, these species serves as the perfect test organisms for barcoding possibilities.

At present, the problem with barcoding seems to be identifying one gene that contains enough taxonomic information. Seifert et al. (2007) recently published their findings with using the mitochondrial cytochrome c oxidase 1 (CO1) as a DNA barcode in *Penicillium* subgenus *Penicillium*. They chose to use this gene since it was successfully used in barcoding projects for the animal kingdom (Hebert et al. 2003a, 2003b, Smith et al. 2005, Ward et al. 2005, Hajibabaei et al. 2006, Costa et al. 2007, Smith et al. 2008a, 2008b) and, therefore, would provide a standard for comparing all living species. Also, other strong candidates such as ITS and β-tubulin, is often difficult to align and does not separate all species complexes within subgenus *Penicillium* (Skouboe et al. 1999, Seifert et al. 2007).
It is rather disappointing that CO1 were not able to separate the closely related species in subgenus *Penicillium*, but Seifert et al. (2007) did suggest that since

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CO1 sequences could be aligned over all fungal lineages, it might serve useful for studying fungal genetic systems such as rates of molecular evolution. They also proposed that ITS or CO1 could easily be used as an initial identification gene and when necessary, β-tubulin or other genes could serve for further identification purposes. Although we are far from having such an identification scheme, it is a very exciting idea that will one day have great implications on not only the way we approach research, but also the way that quarantine services for example deal with biological samples at border control stations (Armstrong and Ball 2005).

Penicillium has a rich history of almost 200 years. Taxonomists mentioned here, are just a few who have dedicated a life's work studying the complexities within the genus and who have laid the foundations for modern taxonomists to further its understanding of this arguably one of the most difficult fungal groups to work with. Although DNA sequencing is currently the most powerful tool available to taxonomists, the importance of good morphological data cannot be underestimated. The way in which we define a species will change, but it will almost certainly be a polyphasic concept which will include morphology, phylogeny and biochemical characters as is proposed by the Frisvad and Samson study (2004). It is difficult to predict what the future holds for *Penicillium* taxonomy, but it is clear that modern students of the genus are starting to lead fungal taxonomy to the next frontier.

2. TAXONOMIC CONCEPTS IN *PENICILLIUM*

2.1 Species concepts used in Penicillium *taxonomy*

Defining what constitutes a species of *Penicillium* is no simple task. In general, since the genus is asexual, a morphological species concept including macro- and micromorphology were used in all major treatments of the genus. Raper and Thom (1949), often separated species from each other based on minor differences in colony colors and textures, but this is not optimal since both of these characters are subject to the interpretation of the investigator (Samson and Gams 1984). In trying to resolve identification issues in Rapers' section

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Fasciculata, Samson et al. (1976) proposed a species concept mainly based on micromorphology. This does, however, represent the difficulty that often different species shows very similar microscopic characters, especially species from subgenus *Biverticillium* (Pitt 1979a), and that there are often considerable variation within a single strain. As Thom (1954) proposed, Pitt (1979a) based his concepts on the practicality and logic of a species or in other words, "a species is only valid if others can recognize them" (Pitt 1979a). He placed emphasis on colony growth rates at different temperatures and reduced water activity, under controlled conditions for separating many closely related species. His concepts in some instances were broad while in others very narrow (Samson and Gams 1984). This led to the synonymy of many species, with species identification often remaining problematic, especially in species from subgenus *Penicillium*. Frisvad and Samson (2004) and Samson et al. (2004) finally incorporated decades of research when publishing their monograph to *Penicillium* subgenus *Penicillium*, using morphological, physiological and molecular data for species identification and published an interactive polyphasic identification key on the world wide web (www.cbs.knaw.nl/penicillium.htm).

The pressure on taxonomists are, however, ever increasing to have molecular data play a larger role in our species concepts, since it would allow for faster and easier identification. Indeed we are starting to see this in the fact that most international journals do not accept new species descriptions when lacking a phylogenetic study. We are also starting to see species, previously thought to be synonyms, separated based on one- or multiple-gene phylogenies (Boysen et al. 1996, O'Donnell 2000, Peterson 2000a, 2000b, O'Donnell 2004, Peterson 2004, Serra et al. 2008). And as nice as it might seem, using only DNA sequences presents us with other problems such as how many base pair differences are needed to validate two different species, and can a species be validly accepted without any morphological data? The number of unknown sequences published on GenBank are ever increasing as molecular biodiversity studies becomes more advanced (Venter et al. 2004, Blaxter et al. 2005, Tringe and Rubin 2005, Edwards et al. 2006). If a species is accepted solely on the basis of these DNA sequences, then *Penicillium* taxonomy could possibly become more unstable and

dumped into chaos. It is, therefore, important for taxonomists to integrate new technology into their studies, in order to create a strong and stable taxonomy (Frisvad and Samson 2004, Samson et al. 2004), not only for use by taxonomists, but also for non-taxonomists.

2.2 *Morphological character interpretation*

Morphological data is still the most important taxonomic character used in *Penicillium* taxonomy. As mentioned earlier, Pitts' (1979) working methods and concepts is accepted as the standard for working with the genus and, accordingly, his methods will be followed in this study. The interpretation of some characters is often difficult and as such will be described below.

2.2.1 *Macromorphological characters*

Growth conditions — For studying the colony characters, it is important to use standardized conditions for incubation in order to reduce intraspecies variation as much as possible. Strains are grown on Czapek Yeast Autolysate agar (CYA), Malt Extract agar (MEA; after Blakeslee 1915) and Glycerol Nitrate agar (G25N) following the formulations of Pitt (1979a). Spore suspensions are prepared in a semi-solid solution (0.2% agar; 0.05% Tween80), with each suspension roughly containing equal spore concentrations. These suspensions then serve as inoculum, which are done in three-point style, equidistance from each other with a micropipetor. Since the volume of media used have an effect on colony characters (Okuda et al. 2000), media are dispensed in volumes of 20 ml per Petri-dish using a dispenser. Plates are then incubated for seven days in an incubator at 25°C (±1°C), in the dark (Pitt 1979a), with plates left unwrapped to allow for sufficient aeration (Okuda et al. 2000). Additional incubations are done on CYA at 37°C and at 5°C (Pitt 1979a). Some species are also known to produce synnema only in sunlight (Pitt 1979a, Seifert et al. 2004, Visagie et al. 2008) and additional CYA and MEA plates are, therefore, incubated on a laboratory bench where the temperature ranges between 20–25°C.

Growth rate — Pitt (1973, 1979) emphasized colony growth as an important

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taxonomic character. Growth is measured after exactly seven days, from the reverse side over the length of the colonies. When growth is not observed, the points of inoculation should be inspected underneath a stereomicroscope for possible germination (FIG. 1a) or formation of microcolonies (FIG. 1b).

Colors in colonies — Colony colors are largely subject to interpretation of the investigator, but it is still a relatively important character used. To standardize color descriptions, Kornerup and Wanscher (1966) is used as reference. Colors of conidia *en masse* are measured with an unaided eye in sunlight. Species from subgenus *Biverticillium* often have brightly colored mycelia which often serves as a good character for distinguishing between species (Pitt 1979a). In this study, the color of mycelia was determined underneath a stereomicroscope (FIG. 1c). Colonies often show a color without the mycelia having a definite color. Species showing this character are described here as having a "yellowish" color, as seen in FIG. 1d. The presence and colors of soluble pigments (FIG. 1d), reverse coloration (Fig. 1e) and exudates (FIG. 1f) are also often diagnostic in a species and are, therefore, of taxonomic value.

Sclerotia — A few *Penicillium* species produces sclerotia which often is a taxonomically important character. Sclerotia (FIG. 1g) are defined as hard structures that cannot be distinguished from immature cleistothecia, and do not produce asci (Pitt 1979a). When observing sclerotia, it is advisable to take note of its color and sizes, since Pitt (1979) considered this to be taxonomically informative.

Colony textures — Colony textures are determined by looking at conidiophore arrangements. The different textures are summarized in Fig. 1h–m and are based and adapted from the definitions given by Samson et al. (2000) and Frisvad and Samson (2004). Velutinous (FIG. 1h) is described as solitary conidiophores which are borne directly from the media, which gives the colonies a velvet look. When solitary conidiophores are borne on aerial mycelia, thereby giving colonies an almost cotton-wool appearance, it is termed as being floccose

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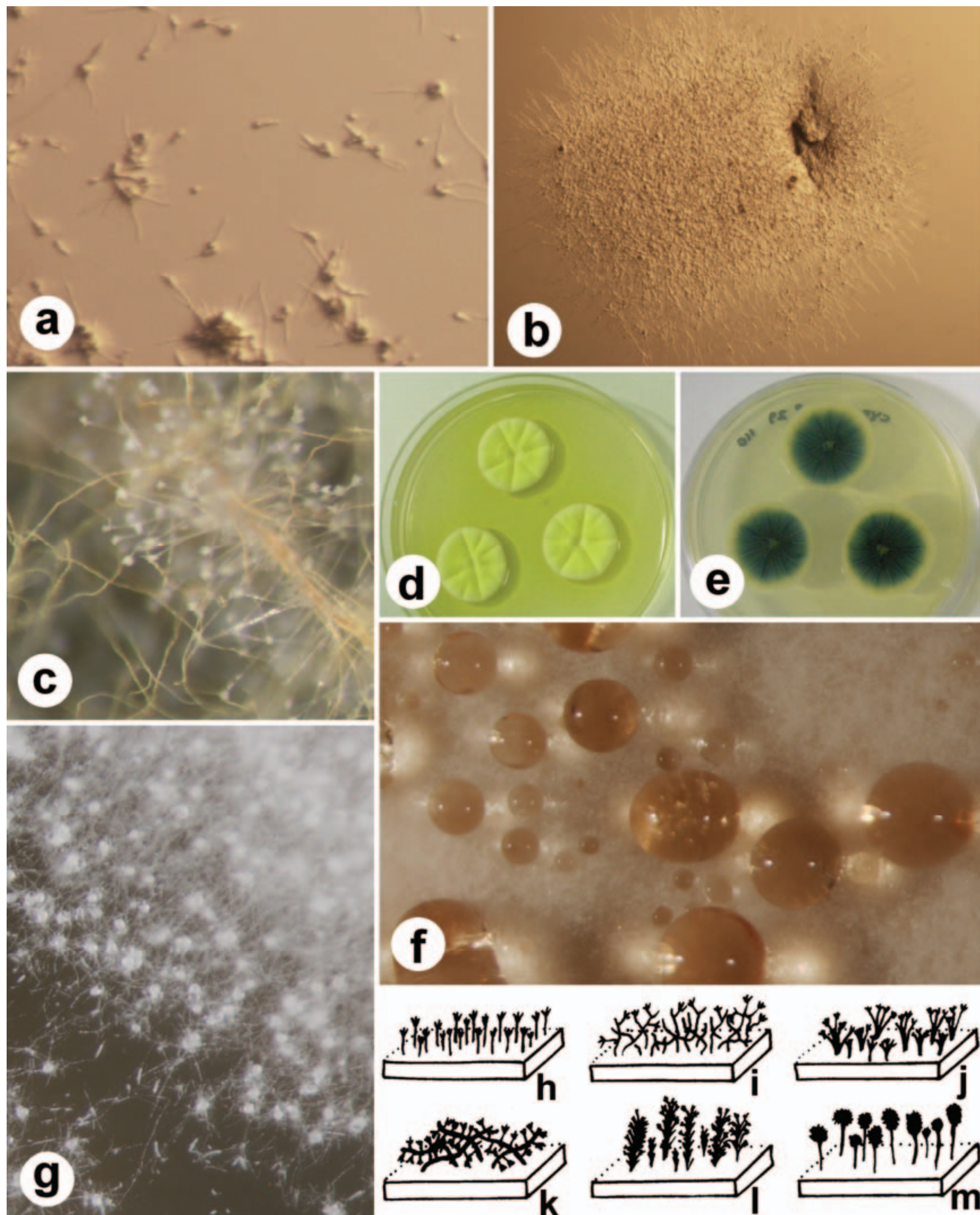

FIGURE 1. Macromorphological characters commonly used for describing *Penicillium* spp. a. Germinating spores viewed under stereomicroscope. b. Microcolony. c. Yellow mycelia produced by *P. verruculosum*. d. Yellow soluble pigments produced by *P. toxicarium*. e. Dark green to turquoise colony reverse produced by *P. subturcoseum* prov. nom. on CYA. f. Clear to orange exudates produced on CYA by *P. citrinum*. g. Sclerotia produced on CYA by *P. cumulacinatum* prov. nom. h–m. colony textures commonly produced by *Penicillium* spp. (adapted from Samson et al. 2000): h. velutinous i. floccose j. fasciculate k. funiculose l. indeterminate synnema m. determinate synnema.

(FIG. 1i). Funicles are described as a series of conidiophores that are borne on a single rope of fertile hyphae (FIG. 1k). Conidiophores can also occur in bundles which can be classified as fasciculate (conidiophores produced in tufts, FIG. 1j), indeterminate synnema (Pitt 1979a: named synnema) (conidiophores produced along the whole length of synnemata, FIG. 1l) or determinate synnema (Pitt 1979a: named coremia) (conidiophores bundled up at the top of synnemata, Fig. 1m) (Pitt 1979a, Samson et al. 2000, Frisvad and Samson 2004).

Medium buckling — Although not taxonomically an important character, medium buckling is still used in some species descriptions. A colony can either be plane, sulcate (having regular furrows) or plicate (furrows very close to each other) (Raper and Thom 1949, Pitt 1979a).

2.2.2. *Micromorphological characters*

Pitt (1979a) suggested that slides be prepared from CYA. However, conidiophores produced on CYA often show atypical structures (Constantinescu 1990) and, therefore, MEA is mostly used in laboratories working with *Penicillium* for studying the micromorphology (Okuda et al. 2000). A *Penicillium* sp. is defined by its conidiophores having a brush-like shape, which is created by the different branching stages of the penicillus (Thom 1930, Raper and Thom 1949, Pitt 1979a). FIGURE 2 is an example of one of the most complex branching patterns found in *Penicillium* and indicates the terms used for describing the different parts of the conidiophore [based on Pitt's (1979) terminology]. The arrangements, shapes, textures and sizes of all of these different parts are all of taxonomic importance and are often used for distinguishing between species (Thom 1930, Raper and Thom 1949, Samson et al. 1976, Pitt 1979a, Stolk and Samson 1983, Frisvad and Samson 2004).

Branching patterns — The different patterns found in the branching of conidiophores are used for subdividing the genus into its four subgenera (Pitt 1979a). Species with monoverticillate conidiophores are divided into subgenus *Aspergilloides* (FIG. 2a). Species with biverticillate conidiophores are divided into subgenus *Furcatum* (FIG. 3b), having ampulliform phialides, and subgenus

Biverticillium (FIG. 3d), having acerose phialides. *Penicillium* subgenus *Penicillium* contains the species which have the complex terverticillate conidiophores.

Conidia — Species of *Penicillium* produces conidia with a wide variety of shapes, which includes spheroidal (FIG. 4a; *P. melinii*), subspheroidal (FIG. 4b; *P. expansum*), ellipsoidal (FIG. 4c; *P. chloroloma* prov. nom.) and cylindrical (FIG. 4d; *P. digitatum*). The conidial walls can be smooth (FIG. 4e; *P. commune*), roughened (FIG. 4f; *P. janczewskii*), echinulate/spiny (FIG. 4g; *P. echinulatum*) or striated (FIG. 4h; *P. ptychoconidium* prov. nom.). The conidia is almost always pigmented and it is these pigments giving colonies their characteristic colors (Pitt 1979a, Frisvad and Samson 2004).

Phialide shapes — The phialides are the conidiogenous cells that gives rise to the millions of conidia in a *Penicillium* colony. Phialides that are broad and have a tenpin bowling pin shape are termed ampulliform (FIG. 4i, *P. janczewskii*) and are produced by most species outside of subgenus *Biverticillium*, which produces thin acerose phialides (FIG. 4j, *P. ptychoconidium*). Species such as *P. digitatum* and *P. italicum* produces a cylindrical phialide (FIG. 4k, *P. digitatum*) which makes their identification quite easy (Pitt 1979a, Frisvad and Samson 2004).

Stipes — Determining stipe lengths is often problematic when exceeding 100 µm (Pitt 1979a) and as such is often not such an important taxonomic character, but with the aid of digital cameras and software designed for use with specific microscopes, stipe lengths in general can today easily be determined. More important, however, is the texture of the stipe walls, which can be smooth (FIG. 4l; *P. olsonii*), rough (FIG. 4m; *P. crustosum*) or tuberculate/wart-like (FIG. 4n; *P. roqueforti*) (Pitt 1979a, Frisvad and Samson 2004). Stipes are also often swollen apically, a character Pitt (1979a) used for dividing subgenus *Aspergilloides* into its two sections. A stipe is considered to be vesiculate, when the apical swelling width is twice that of the stipes (Pitt 1979a).

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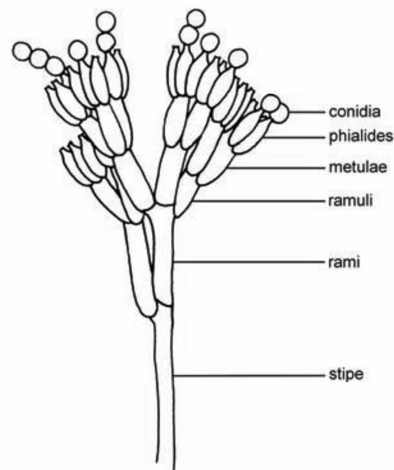

FIGURE 2. Terminology for describing the penicillus, as was used by Pitt (1979a).

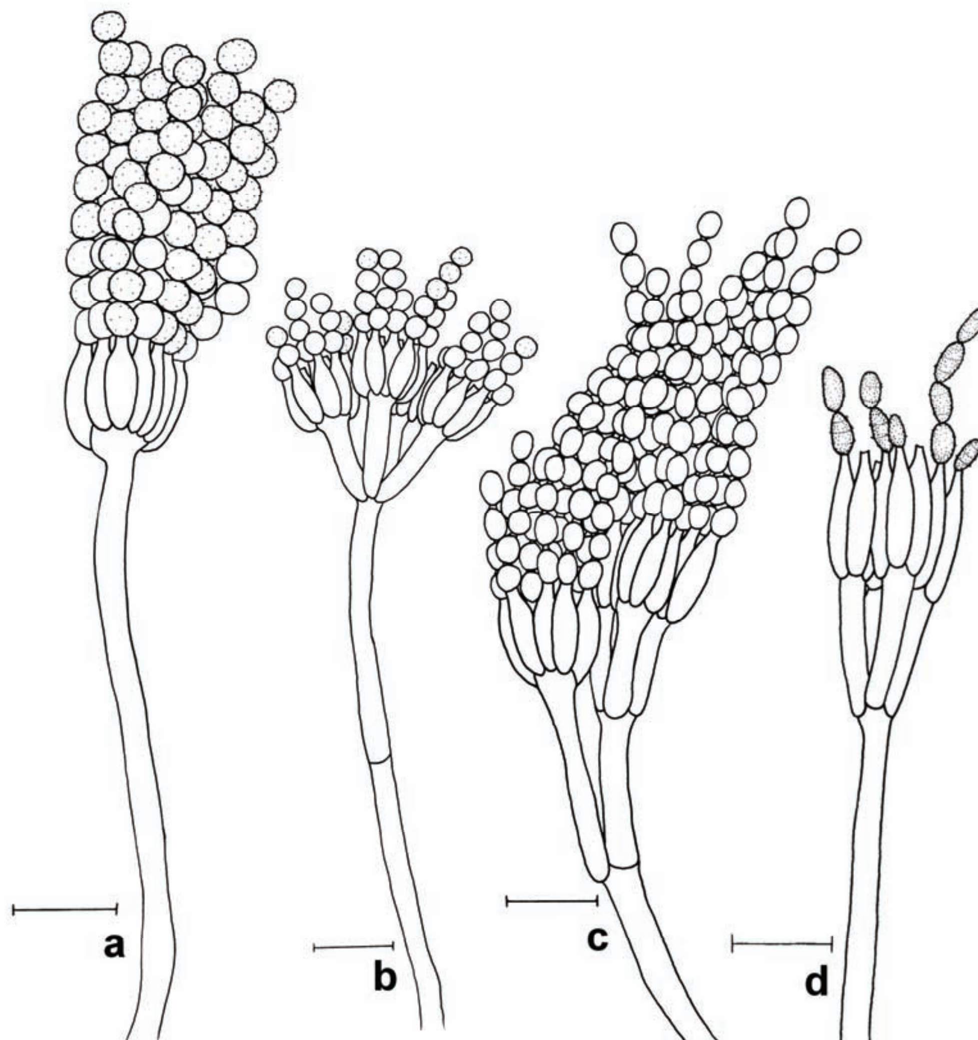

FIGURE 3. Different branching patterns used for subgeneric classification of *Penicillium*. a. *Penicillium cumulacinatum* prov. nom. belonging to subgenus *Aspergilloides* (monoverticillate). b. *Penicillium citrinum* belonging to subgenus *Furcatum* (biverticillate). c. *Penicillium expansum* belonging to subgenus *Penicillium* (terverticillate). d. *Penicillium ptychoconidium* prov. nom.

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belonging to subgenus *Biverticillium* (biverticillate).

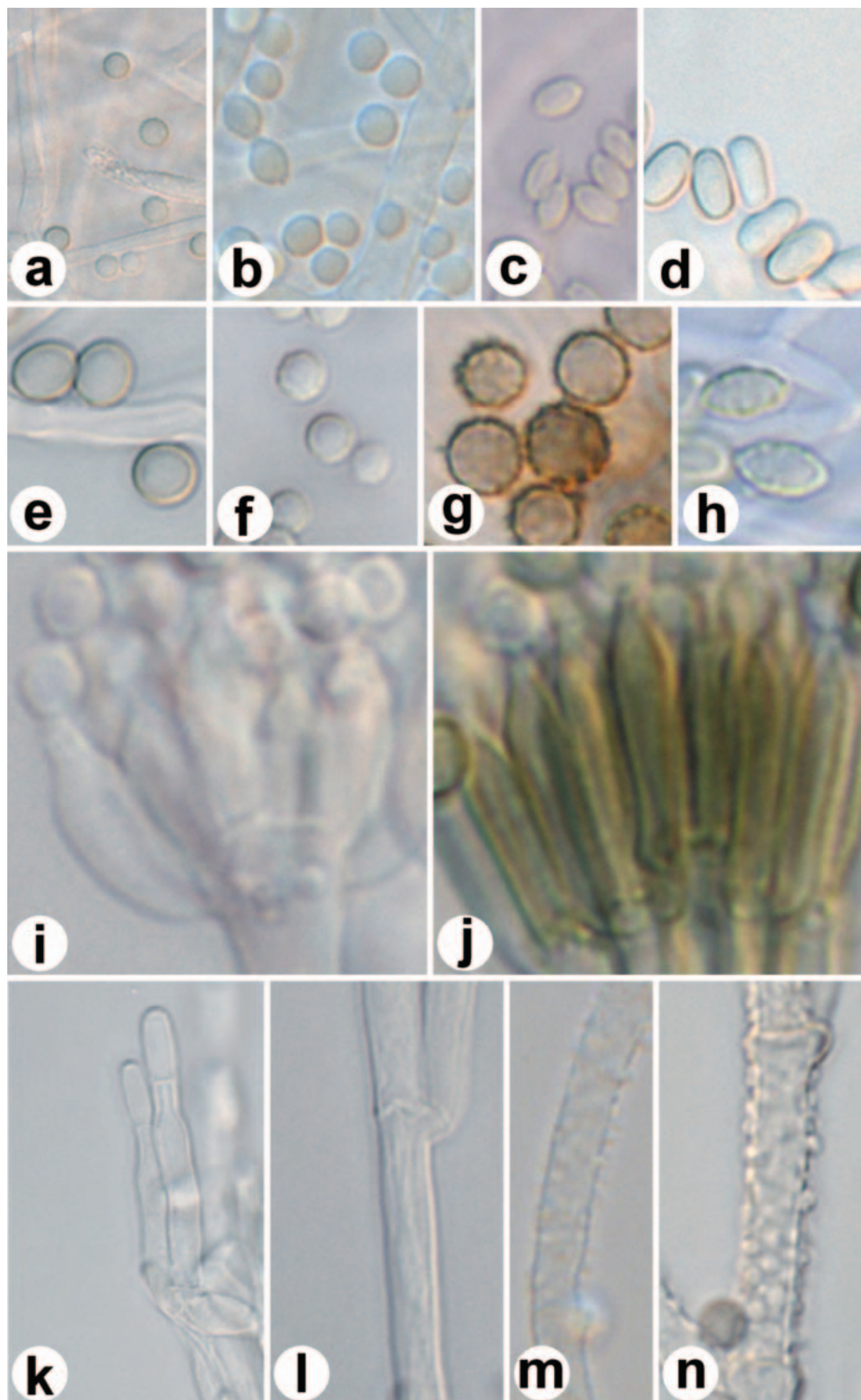

FIG. 4. Most important microscopic characters used for characterization. a–h. Different conidial shapes and textures of *Penicillium* spp*.* a. spheroidal (*P. melinii*) b. subspheroidal (*P. expansum*) c. ellipsoidal (*P. chloroloma* prov. nom.) d. cylindrical (*P. digitatum*) e. smooth-walled (*P. commune*) f. rough-walled (*P. janczewskii*) g. echinulate/spiny (*P. echinulatum*) h. striate (*P. ptychoconidium* prov. nom.) i–k. Different phialide shapes of *Penicillium* spp. i. ampulliform (*P. janczewskii*) j. acerose (*P. ptychoconidium* prov. nom.) k. cylindrical (*P. digitatum*) l–n. Different stipe wall textures of *Penicillium* spp. l. smooth-walled (*P. olsonii*) m. rough-walled (*P. crustosum*)

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n. tuberculate/wart-like (*P. roqueforti*).

2.3 Associated teleomorphic genera of **Penicillium**

The Trichocomaceae family was introduced by Fischer (1897) for the genus *Trichocoma,* which produces a biverticillate *Penicillium* anamorph (Geiser and LoBuglio 2001), and are characterized by fungi producing cleistothecial ascocarps (Malloch and Cain 1972, Fennel 1973, Benny and Kimbrough 1980, Malloch 1981, Malloch 1985a, Malloch 1985b, Berbee et al. 1995, Ogawa and Sugiyama 2000, Pitt et al. 2000, Tamura et al. 2000, Stchigel and Guarro 2007). Twenty nine genera, nine anamorphic and 20 holomorphic, has since been included in this family, with species from genera *Penicillium, Aspergillus* and *Paecilomyces* being most dominant in numbers (Ogawa and Sugiyama 2000, Pitt et al. 2000, Tamura et al. 2000). Studying relationships between Trichocomaceae genera have traditionally been based on the structure or development of the ascus and its ascospores, cleistothecial initials, centrum, peridium, as well as associated anamorphs (Benjamin 1955, Malloch and Cain 1972, Fennel 1973, Benny and Kimbrough 1980, Malloch 1981, Malloch 1985a, Malloch 1985b, Geiser and LoBuglio 2001).

Malloch (1985a, b) divided the family into subfamilies Trichocomoideae and Dichlaenoideae. Trichocomoideae were characterized by their asci being borne within tufts or layers of loose hyphae, the lack of pseudoparenchymatous or Hülle cells, rough-walled spherical or ellipsoidal ascospores which lack furrows, and included anamorphs of *Penicillium* and *Paecilomyces* (Malloch 1985a). Dichlaenoideae, on the other hand, were characterized by ascospores which can be produced inside cleistothecia, stromata or being surrounded by Hülle cells (Stchigel and Guarro 2007). In addition to this, ascospores are oblate to ellipsoidal, typically have furrows and are associated with a wide range of anamorphs, including *Penicillium, Aspergillus* and *Paecilomyces*. This division of Trichocomaceae is, however, at this stage not supported by recent phylogenetic conclusions (Berbee et al. 1995, Ogawa et al. 1997, Sugiyama 1998, Ogawa and Sugiyama 2000, Tamura et al. 2000, Stchigel and Guarro 2007), with morphological characters of the sexual structures, not resulting in phylogenetic groupings. The phylogenies also showed that *Trichocoma*, forming its

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biverticillate *Penicillium*, is resolved in a phylogenetic clade together with *Talaromyces* species (Ogawa and Sugiyama 2000). In addition to this, it is clear that the association between *Penicillium* and its other two teleomorphs, *Eupenicillium* and *Talaromyces,* have lead to the genus being polyphyletic (LoBuglio et al. 1993, Berbee et al. 1995, Heredia et al. 2001).

The first observation of *Penicillium* species producing a perfect state was made by Brefeld in 1874, when he described and illustrated the maturation of cleistothecial asci producing chains of ascospores in the species he identified as "*Penicillium crustaceum* (Link) Fries (*Penicillium glaucum*)" (Stolk and Scott 1967, Pitt 1979a, Stolk and Samson 1983). Winter, in 1887, then later used *Penicillium crustaceum* (Link) Fries for Brefeld's fungus, after which Ludwig in 1892, introduced the teleomorphic genus *Eupenicillium*, with *E. crustaceum* (Link) Fries (anamorph = *P. glaucum*) as type. The ICBN states that a teleomorphic fungus must have a perfect state name applied to it and, therefore, Langeron introduced the generic name *Carpenteles,* in 1922, for Brefeld's fungus, applying *P. glaucum* (Link) Brefeld as type (Shear 1934). He did this without observing any fungal material and, therefore, *Carpenteles* was never accepted as valid by his peers. Shear (1934), in his view, re-isolated Brefeld's *P. glaucum* and described it as *Carpenteles asperum,* because of the confusion surrounding the name *P. glaucum* (Raper and Thom 1949, Stolk and Scott 1967, Pitt 1979a, Stolk and Samson 1983). Thom (1930) and Raper and Thom (1949) characterized all species based on their conidiophores, ignoring the sexual structures, thereby rejecting both *Carpenteles* and *Eupenicillium* and synonymizing them with *Penicillium* for practical purposes. Benjamin (1955) reintroduced *Carpenteles* for *Penicillium* in its perfect state, using *C. asperum* Shear as type. In Stolk and Scotts' (1967) nomenclatural revision, however, *Eupenicillium* Ludwig was finally accepted as the correct genus name with *Eupenicillium crustaceum* Ludwig as its type species (Pitt 1979a, Stolk and Samson 1983).

Scott (1968b) published a monograph containing 26 *Eupenicillium* species descriptions and an identification key. This key was based on characters of mature asci, which prompted Pitt (1974) to publish his synoptic key on 36

Eupenicillium and 14 sclerotia producing anamorphic *Penicillium* spp. He used characters such as colony morphology and growth rates, together with conidiophore structures to separate species. Pitt (1979a) then introduced the first subgeneric classification for *Eupenicillium*, by dividing 37 species into eight series and once again providing a synoptic key to these and 14 sclerotigenic *Penicillium* species. Stolk and Samson (1983) then placed emphasis, similar to the Samson et al. (1976) revision of section *Fasciculata* (Raper and Thom 1949), on micromorphological characters such as the conidiophores and asci. They used these characters to divide the genus into four sections, also providing keys to each section.

In 1877, van Tieghem discovered a *Penicillium* strain producing an ascocarp without a wall and having a cotton texture (Pitt 1979a). Almost a century past until Benjamin (1955) introduced a genus name, *Talaromyces,* for species producing these type of ascocarps, with *Talaromyces vermiculatus* (Dangeard) C.R. Benjamin as designated type (Stolk and Samson 1972, Pitt 1979a). Stolk and Samson (1971) described the teleomorphic genus *Hamigera* for *Talaromyces* species bearing single asci. Stolk and Samson (1972) then provided the first *Talaromyces* generic classification dividing it into 4 sections, including the species having *Penicillium* or *Paecilomyces* anamorphs. Pitt (1979a) accepted the subgeneric classification of Stolk and Samson (1972), but did consider *Hamigera* as a synonym of *Talaromyces*. Pitt (1979b) and Pitt and Hocking (1979) then described the anamorphic genera, *Geosmithia* and *Merimbla* for *Talaromyces* species anamorphs not conforming to *Penicillium*.

In contrast to *Eupenicillium, Talaromyces* is associated with three anamorphic genera namely, *Penicillium, Paecilomyces* and *Geosmithia* (Pitt et al. 2000). *Eupenicillium* is characterized by species producing firm to sclerotioid cleistothecial ascocarps (Pitt 1979a, Stolk and Samson 1983) and *Talaromyces* by the production of soft gymnothecia, which does not have walls, and is covered by a hyphael network (Stolk and Samson 1972, Pitt 1979a). Interestingly, *Penicillium* subgenus *Biverticillium* species having a perfect state is always associated with *Talaromyces*, with the other three subgenera commonly

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associated with *Eupenicillium* (Pitt 1979a, LoBuglio et al. 1993, Berbee et al. 1995, Heredia et al. 2001, Peterson 2000, Seifert et al. 2004).

2.4 Phylogenetic studies on **Penicillium** *and its associated teleomorphic genera*

The advances made with DNA sequencing techniques during the 1990's gave taxonomists the opportunity to infer true evolutionary relationships between taxa. This has lead to numerous studies evaluating the classification schemes and relationships within *Penicillium*, *Talaromyces, Eupenicillium* and their morphologically related species (LoBuglio et al. 1993, Berbee et al. 1995, Boysen et al. 1996, Skouboe et al. 1999, Geiser et al. 2000, Ogawa and Sugiyama 2000, Peterson 2000, Seifert and Louis-Seize 2000, Skouboe et al. 2000, Heredia et al. 2001, Samson et al. 2004, Seifert et al. 2004, Peterson et al. 2005, Seifert et al. 2007, Wang and Zhuang 2007, Serra et al. 2008).

It is well documented that *Penicillium* is not a monophyletic group. *Penicillium* species associated with *Eupenicillium* teleomorphs, together with *Aspergillus* and its teleomorph *Eurotium*, forms a clade separate from those with *Talaromyces* states (Berbee et al. 1995, Ogawa and Sugiyama 2000). Other studies have also shown that *Talaromyces* spp. together with their *Penicillium* subgenus *Biverticillium* anamorphs form a distinct monophyletic clade separate from the other three *Penicillium* subgenera (LoBuglio et al. 1993, Berbee et al. 1995, Heredia et al 2001). This has lead to suggestions that *Penicillium* subgenus *Biverticillium* should probably be segregated into its own monophyletic genus (Seifert et al. 2004). This can, however, only be done after relationships within *Talaromyces* and its anamorphs have been determined.

Peterson (2000), in a phylogenetic study, mostly used sequences from ex-type strains and evaluated the subgeneric classification within *Eupenicillium* and *Penicillium* subgenera *Aspergilloides, Furcatum* and *Penicillium*. The ITS and 28S-rDNA phylogeny showed that the branching pattern of the penicillus cannot be used in defining subgenera since it is not a true evolutionary. From the phylogeny, Peterson (2000) also indicated that *Penicillium* strains form six

distinct clades and as such, with the addition of sequences from other genes, these clades will separately represent a monophyletic subgeneric classification. These studies used the ribosomal gene areas, such as the internal transcribed spacer (ITS) and large subunit ribosomal DNA (lsu-rDNA), for inferring evolutionary relationships. As was found with morphological studies, these genes were unable to separate species from *Penicillium* subgenus *Penicillium*, since these genes contains to little informative characters (Skouboe et al. 1999, 2000). The resolution power of other genes were assessed using subgenus *Penicillium* as model group. Samson et al. (2004) showed that β-tubulin works well as a marker for species identifications within subgenus *Penicillium,* although it was unable to distinguish between a few closely related species. Taxonomists also realized that using only one gene will not solve all taxonomic problems in *Penicillium* and, therefore, multi-gene phylogenies would be more useful (Skouboe et al. 1999, Geiser et al. 2000, Peterson 2000, Seifert and Louis-Seize 2000, Skouboe et al. 2000, Seifert et al. 2007). In addition to this, difficulties often experienced in aligning ITS and β-tubulin datasets (Seifert et al. 2007), have lead to exploration of genes such as the mitochondrial cytochrome c oxidase 1 (CO1) (Seifert et al. 2007), elongation factor 1-α (EF 1-α) (Peterson et al. 2005) and partial calmodulin gene (Wang and Zhuang 2007, Serra et al. 2008). Most of these studies are, however, only preliminary and the database for these genes are restricted, but slowly expanding.

3. *PENICILLIUM* TAXONOMY IN THE SOUTH AFRICAN CONTEXT

In the past, South Africa's contributions to *Penicillium* taxonomy have been limited. Research funding bodies traditionally do not fund environmental fungal studies (Crous et al. 2006). Mycologists were, therefore, forced to focus their studies on economically important habitats (Schutte 1992) such as maize (McLean and Berjak 1987, Wittaker et al. 1989, Rheeder et al. 1990), citrus (Pole Evans 1911, 1920, Martin 1960, Roth 1967), pome fruits (Matthee 1968, Combrink et al. 1985) litchi fruit (Roth 1963), bananas (Roth and Loest 1965), mangoes (Wehner et al. 1981), nuts and dried fruit (Wehner and Rabie 1970), dairy products (Lück et al. 1976, 1978, Lück and Wehner 1979), wine grapes (Le

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Roux et al. 1973) and cork used in the wine industry (Marais and Kruger 1975) to name but a few. These surveys all made mention of the presence of *Penicillium* species, but very few attempted identification themselves, rather sending the strains overseas (Schutte 1992). In addition to this, many articles only mentioned that members of *Penicillium* were present, but made no mention to the possible identity of these species (Le Roux 1973, Marasas and Bredell 1973, Marais and Kruger 1975, Eicker 1976, Lück et al. 1976, Holtzhausen 1978, Eicker et al. 1982, Dutton and Westlake 1985, Mycock and Berjak 1990, Schutte 1992).

In the few environmental studies done in South Africa, most only listed species that occurred in these samples, without attempting to describe any possible new species, with the exception of Scott (1968a), who described eight new *Eupenicillium* spp. that occurred in South African soils and Ramirez (1990) who described *Penicillium krugerii* isolated from soil in the Kruger National Park. Cohen (1950) conducted the first soil fungi survey in South Africa in which he recorded nine *Penicillium* spp., three of which the identity remained uncertain and two species which remained unidentified. In a microfungal survey from the Kwazulu Natal region, Eicker (1969, 1970) recovered 112 species, 20 of which were *Penicillium*. Eicker (1973) then studied the mycoflora in leaf litter of *Eucalyptus maculata* and recorded 14 *Penicillium* spp. from a total of 85 fungal species, after which he recovered a number of *Penicillium* isolates from *Panicum coloratum* phylloplane and litter, many which he never identified (Eicker 1976). Papendorf (1976), studying the soil mycoflora of *Acacia karroo,* isolated 25 *Penicillium* spp., five of which could not be identified. Further surveys done on *Eucalyptus* (Lundquist and Baxter 1985) and *Pinus* (Lundquist 1986, 1987) made mention of *Penicillium* species, without any additional work on the genus. One biodiversity study done in the diverse fynbos biome, isolated 14 *Penicillium* spp. out of 66, with two species which remained unidentified (Allsopp et al. 1987).

Schutte (1992) mentioned that, from previous South African literature, it is clear that we have a tendency to not identify species of *Penicillium* and to only include them in lists of other fungi. He also makes note of the fact that South African

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isolates often show variations from species descriptions given by Pitt (1979a) and raised the question whether this would be the case for all South African isolates. This variation from standard descriptions and the fact that most of the studies were done before Pitt's 1979 monograph might, therefore, imply that many South African isolates could have been misidentified, as Frisvad (1989) found for some South African studies (Schutte 1992).

Problems in *Penicillium* taxonomy in South Africa, therefore, seems significant, but since the incorporation of DNA sequencing into taxonomic studies, a basis for easier comparison of South African isolates to other strains are being created (Johnston and Korsten 2007, Visagie et al. 2008, Visagie and Jacobs 2008a, 2008b, 2008c). With the expectation that South Africa, having a number of unique and diverse floral areas, contains many undescribed species, we certainly in the future have the potential to make significant contributions to the better understanding of the genus, and its associated economic importance.

4. *PENICILLIUM* FROM TERRESTRIAL ENVIRONMENTS

Penicillium, in general, is thought to have primarily evolved from a soil environment and as such, a large proportion of known species have previously been isolated from this habitat (Raper and Thom 1949, Pitt 1979a). Even species such as *P. expansum*, normally isolated from rotting apples and pears (Pitt 1979a, Samson et al. 2000, Frisvad and Samson 2004), have been found to occur in soil (Pitt 1979a, Domsch et al. 1980, Frisvad and Samson 2004). This shows that in our quest to explore this genus in South Africa and their associated roles in life, soil is certainly a good base from which a better understanding can be build.

Penicillium is not only found in large numbers, but are also major contributors to the total microfungal diversity in soil. In a review of *Penicillium* species diversity in soil surveys from around the world, Christensen et al. (2000) found that, on average, *Penicillium* spp. account for 21% of the total fungi isolated, with the highest number found in Syria (reaching 49%) and the lowest in a legume crop from India (3%). They also found that *Penicillium* seems to be habitat selective on a subgenus level, but since subgeneric divisions in *Penicillium* is not based on

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true evolutionary lineages (Peterson 2000a), this might or might not be the case. Allsopp et al. (1987) completed the only microfungal survey previously done in the coastal fynbos region. In their survey, *Penicillium* spp. represented 21% (14 out of 66) of the total species isolated. Although this is only one survey, we have previously also found that *Penicillium* spp. were one of the major contributors to fungal biodiversity (Visagie and Jacobs, unpublished). Allsopp et al. (1987), only included species that were found in more than ten samples and since soil is not homogenous, they might have missed out on a large proportion of the actual number of species in this area. In our study, many single specimens of *Penicillium* spp. were recovered and, therefore, the estimated 21% should be seen as conservative.

The Cape Floristic Region, with its 9 030 vascular plant species, is one of the most botanically diverse on earth, when considering β-diversity which is a measure of diversity along environmental gradients or different ecological niches (Wilson and Shmida 1984, Kolef et al. 2003). In fact, plant life in the fynbos is so diverse, that it houses roughly 44% of the total floral species occurring in South Africa (Goldblatt and Manning 2002). Many hypotheses for this high diversity exist. Soils here are high in heterogeneity, have rugged landscapes, are acidic and typically low in nutrient levels (Kruger et al. 1983), are subjected to high summer temperatures and drought, combined with cold winter temperatures (Richards et al. 1997, Goldblatt and Manning 2002) and the frequent fires worsen living conditions for plants, animals and fungi (Goldblatt and Manning 2002). Although these factors contribute to the high diversity seen, it is not unique to the area when considering conditions in the southwest of Australia (Goldblatt and Manning 2002). The best explanation given by Goldblatt and Manning (2002) for the high diversity, is the fact that the climate of the Cape region did not change dramatically during the Pleistocene period, which was marked by great fluctuations in temperatures that brought along the ice age. Since the Cape climate was stable during this period, biological life had much more time to evolve and for speciation to take place.

The high plant diversity in the fynbos suggests that fungal life would be just as

diverse, considering the association between plants and fungi. Hawksworth (1991, 2001) estimated the total expected fungal species to be 1.5 million, without taking into account species associations with insects. He made this calculation by using a 1:6 ratio for plants *vs* fungi based on plant to fungi ratios from the well studied British islands. Crous et al. (2006) suggested that this ratio are more in the region of 1:7 for South Africa and estimated that 171 500 fungal species occur in South Africa. Extrapolating this to the fynbos biome, we can expect 63 210 fungal species of which 80% (Crous et al. 2006) might be endemic. South Africa has in the past greatly neglected studying fungi. With only 780 new fungal species described from South Africa (Crous et al. 2006) and expecting 50 000 endemic species, it means that mycologists working in South Africa have a unique opportunity and also responsibility to improve knowledge and understanding of the kingdom fungi. Indeed, the South African mycologists' work has only just begun.

5. OBJECTIVES OF THE STUDY

The fynbos is botanically one of the most diverse biome on earth, with almost 80% of its plant species that is endemic. We, therefore, hypothesize that species from *Penicillium*, one of the dominant genera in the soil, would be just as diverse and unique to the area. In order to test the hypothesis, the objectives of this study were to collect and characterize *Penicillium* strains from fynbos soil, using both morphological and genetic characters. These strains will be compared to previously described species and fynbos strains not conforming to known species, described as new. To aid in identification of fynbos *Penicillium* spp., keys to species and their close relatives are provided in chapters 3, 4 and 5, with both a synoptic and dichotomous key provided in chapter 6.

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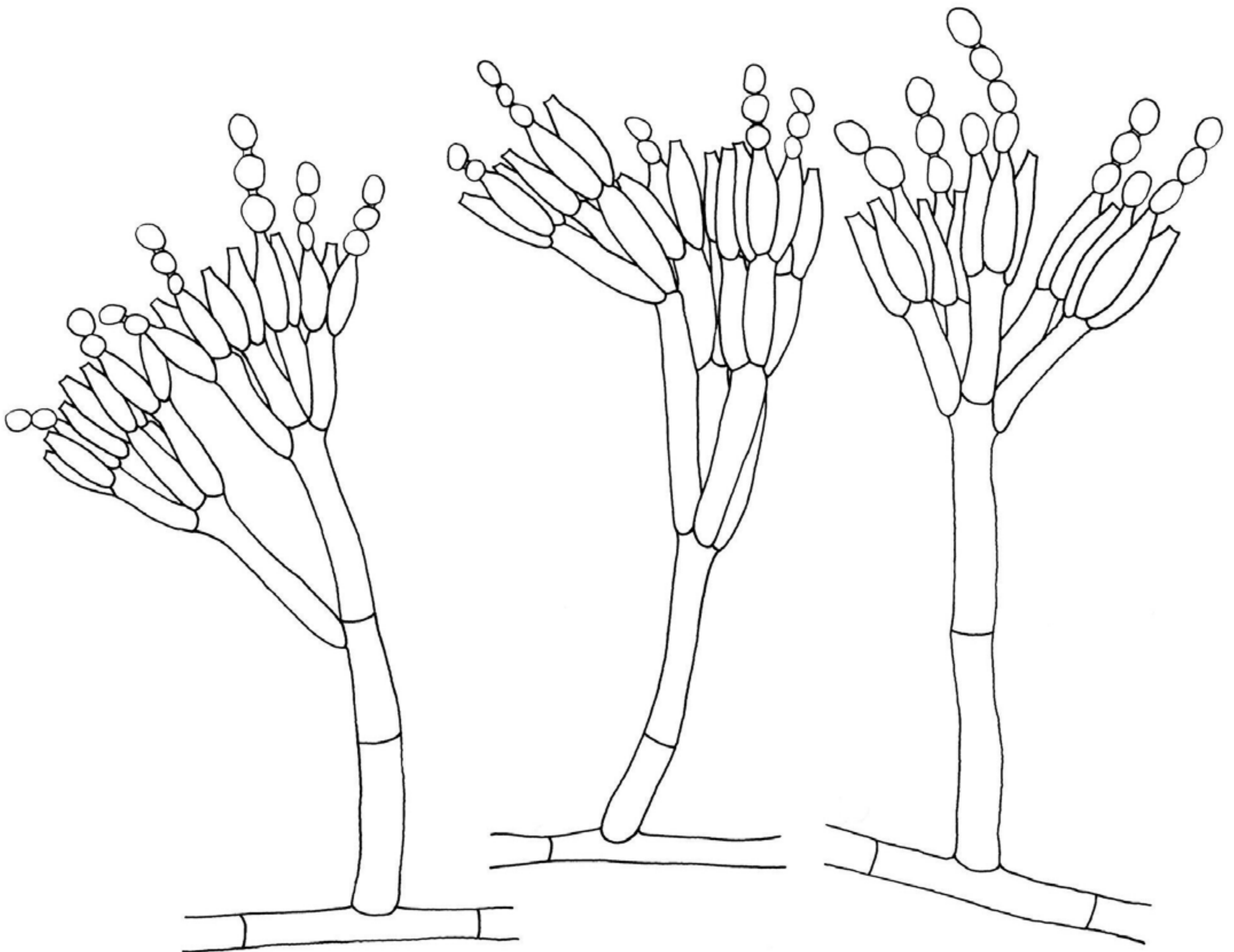
CHAPTER 2
A new species of *Penicillium, P. ramulosum* prov. nom., from the natural environment



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ABSTRACT

During a recent survey of *Penicillium* spp. from fynbos soils in the Western Cape Province of South Africa, several undescribed species were isolated. Similar isolates to one of these species were also collected in the Western Cape from *Protea* infructescences. These strains were compared morphologically to known species of *Penicillium*, but could not be identified using previously published keys. Morphologically, these strains belong to the subgenus *Biverticillium*. They are distinguished by strongly funiculose colonies covered by glutinous exudates and conidiophores having thin acerose phialides [8.5–10(–12) × 2–2.5 μm] giving rise to chains of subspheroid to ellipsoidal conidia (2.5–3 × 1.5–2.5 μm). Characteristically short [100–150(–250) μm] determinate synnema are produced in culture after prolonged incubation with much longer synnema produced in nature. Based on differences in morphology and molecular characters, the strains are described here as *Penicillium ramulosum* prov. nom.

The following chapter have been submitted for publication in Mycologia

INTRODUCTION

Penicillium is a well-known cosmopolitan genus of moulds. Its more than 225 species play various roles in natural ecosystems, agriculture and biotechnology. They function as decomposers of dead materials and are especially important as post-harvest organisms, where they spoil various food commodities (Janisiewicz 1987, Pitt and Hocking 1997, Holmes and Eckert 1999, Morales et al. 2007). *Penicillium* species are exploited for a wide range of industrial applications, such as in the cheese industry (Nelson 1970, Karahadian et al. 1985), production of antibiotics (Thom 1945, Raper and Thom 1949, Raper 1957, Okada et al. 1998, Rømer-Rassing and Gürtler 2000) and have emerged also as important producers of novel enzymes (Raper and Thom 1949, Law 2002, Adsul et al. 2007, Li et al. 2007).

Penicillium spp. are characterized by their branched or simple hyaline brush-like conidiophores that terminate in clusters of ampulliform or acerose phialides that give rise to long dry chains of conidia. The genus is subdivided into four subgenera based on the branching pattern of the penicilli. These subgenera are *Aspergilloides* (monoverticillate penicilli), *Penicillium* (terverticillate penicilli), *Furcatum* and *Biverticillium* (both have biverticillate penicilli) (Pitt 1979). Species of subgenus *Biverticillium* have acerose phialides, a metulae to phialide length ratio of 1–1.2 and generally poor growth at reduced water activity. Species from subgenus *Furcatum,* on the other hand, have ampulliform phialides, a metulae to phialide length ratio much greater than one and grow better at reduced water activity (Pitt 1997).

Penicillium is one of the dominant fungal genera in soil (Thom 1930, Christensen et al. 2000), where it is mainly responsible for decomposing organic matter and assists in the maintenance of soil nitrogen fertility in concert with other organisms (Seneviratne and Jayasinghearachchi 2005). *Penicillium* spp. may constitute up to 67% of the total fungal biomass of certain soil habitats (Christensen et al. 2000). Little is known about the diversity of *Penicillium* spp. in South African soils, as most diversity studies focused on economically important habitats such as maize (McLean and Berjak 1987, Rheeder et al. 1990),

citrus (Pole Evans 1911, 1920), pome fruits (Matthee 1968, Combrink et al. 1985) and a wide range of other crops (Roth 1963, Roth and Loest 1965, Wehner et al. 1981). The few environmental studies done over the years only briefly mentioned *Penicillium* spp. (Lundquist and Baxter 1985, Lundquist 1986, 1987, Allsopp et al. 1987, Schutte 1992), with the exception of Scott (1968) who described eight *Eupenicillium* species isolated from South African soils. One of these surveys conducted in the floristically diverse fynbos biome of the Western Cape indicated that these soils might contain new species in the rhizosphere and non-rhizosphere (Allsopp et al. 1987).

During various surveys in the Western Cape region of South Africa, a previously undescribed species of *Penicillium* belonging to subgenus *Biverticillium* Dierckx, section *Coremigenum* (Biourge) Pitt, series *Dendritica* Pitt (Pitt 1979), was isolated. The aim of this study was to compare these strains to known species in this group based on morphological and molecular characters.

MATERIALS AND METHODS

Isolations — Strains used in this study were collected from soil and the infructescences of *Protea burchellii* Stapf. Soil samples were collected at Kalbaskraal (S 33,57061°; E 18,62861°) and Riverlands (S 33, 49795°; E 18, 58931°), situated in the Sandveld Fynbos area near Malmesbury, in the Western Cape. Soil dilutions were plated onto potato dextrose agar (Biolab) amended with 50 ppm streptomycin (Applichem, South Africa) and 100 ppm chloramphenicol (Applichem, South Africa). The plates were incubated at 25°C for 6–7 days and examined for fungal growth. Colonies showing distinct *Penicillium* morphologies were transferred to Oatmeal Agar (OA) and incubated for a further 7 days. Infructescences of *Protea burchellii* were collected from the J.S. Marais Park, Stellenbosch, South Africa. Isolates of *Penicillium* spp. were collected from these by transferring fungal spores from infected tissues to Petri dishes containing Malt Extract Agar (MEA: after Blakeslee, 1915) with the aid of a sterile needle. A strain showing similarities to the undescribed strains was received from Dr. Keith Seifert. The strain was isolated on moth damaged Riesling grapes from a Niagara County vineyard, Ontario, Canada during

September 2005. The strains were isolated from the infected grapes, kept in a moist chamber for two days.

Morphology — Single spore cultures, in semi-solid 0.2% agar suspensions, were inoculated at three points on Czapek Yeast Agar (CYA), Malt Extract Agar (MEA: after Blakeslee, 1915) and 25% Glycerol Nitrate Agar (G25N) in 9 cm polystyrene Petri dishes containing 20 ml of media, and incubated at 25°C (CYA, MEA, G25N), 5°C and 37°C (CYA), respectively (Samson and Pitt 1985) in the dark, with plates left unwrapped to allow for sufficient aeration (Okuda et al. 2000). Additional inoculations were made on CYA and MEA, and incubated at room temperature in incidental light for 7–21 days of growth. Characterization and species descriptions followed methods described by Pitt (1979) and recently published descriptions (Samson and Pitt 1985, Pitt and Hocking 1997, Okuda et al. 2000). The "Methuen handbook of color" was used as reference for description of isolate color and codes (Kornerup and Wanscher 1966).

Phylogenetic analysis — DNA extractions were made from strains grown on MEA with the ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). The ITS1–5.8S–ITS2 rDNA region was amplified using PCR. Reaction mixtures (25 μl) consisted of 2.5 μl 10X Kapa Taq High Yield Buffer A, 2.5 U Kapa Taq (Kapa Biosystems, Woburn, USA), 250 μM dNTPs and 0.250 μM of primers ITS1 and ITS4 (White et al. 1990). PCR products were sequenced using a Big Dye terminator cycle sequencing premix kit (Applied Biosystems, California, USA) and sequenced with an ABI PRISM 310 genetic analyzer. Sequence contigs were assembled using SeqmanII (DNAstar), aligned in ClustalX (Thompson et al. 1997) and manually adjusted in Se-Al (Rambaut 2007).

ITS sequences of the unknown *Penicillium* strains (EU795704, EU795705, EU795706, EU795707, EU795708, EU835480) were compared to published sequences on GenBank of species from *Penicillium* subgenus *Biverticillium* and closely related *Talaromyces* spp., primarily based on the studies done by LoBuglio et al. (1993), Peterson (2000), Heredia et al. (2001) and Seifert et al. (2004). Species and their respective GenBank accession numbers are presented

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in TABLE 1. Ambiguously aligned regions were replaced by codes. Step matrices to assign different weights to these codes were computed using INAASE 2.3b (Lutzoni et al. 2000). Alignments of the dataset can be obtained from the compact disc, attached at the back of this thesis.

Sequence analysis was performed using PAUP* v4.0b10 (Swofford 2000) with gaps in the data set treated as missing data. A single tree for the data set was obtained using neighbour-joining analysis with an uncorrected P-distance and *Talaromyces thermophilus* chosen as outgroup based on the study done by Heredia et al. (2001). A bootstrap analysis (1000 replicates using the neighbour-joining option) was performed to determine confidence levels of the nodes.

RESULTS

Isolations from soil yielded 434 *Penicillium* strains, with six representing the species considered here. Two of 12 strains isolated from *P. burchellii* infructescences resembled the same species. Morphologically, these strains were characterized by the production of terminally biverticillate, sometimes terverticillate (or even more complex) conidiophores, terminating in thin, acerose phialides that bear chains of subspheroid to ellipsoidal conidia. Colonies on G25N (25°C) show restricted growth characteristic of subgenus *Biverticillium*, with colonies reaching 4–5 mm diam after 7 days. Synnema were produced after prolonged incubation in incidental light at 25°C. Various characters differentiate these isolates from previously described species of *Penicillium.* Micromorphological characters suggest that this set of isolates from the Western Cape are closely related to *P. cecidicola* Seifert, Hoekstra and Frisvad (Seifert et al. 2004) and *P. dendriticum* Pitt (Pitt 1979).

ITS amplifications resulted in amplicons ±600 bp in length. The aligned data set, including described species of *Penicillium* subgenus *Biverticillium* and *Talaromyces* species, was 544 base pairs long. Nine ambiguous sites were identified and replaced with weighted codes (Lutzoni et al. 2000).

All isolates of the presumed new *Penicillium* sp. formed a well-supported, monophyletic clade, distinct from any previously described species (FIG. 1). This clade also contained a strain from Canada, isolated from grapes. Consistent with the micromorphology, neighbour-joining analysis included the strains of this species in a larger clade containing other species of the subgenus *Biverticillium.* As suggested by morphological data, *Penicillium cecidicola* appears to be a sister group to these species.

TAXONOMY

Penicillium ramulosum C.M. Visagie & K. Jacobs, prov. nom.* **FIGS 2, 3**

Mycobank nr. MB 512023

Etymology. Latin, *ramulosum* = twiggy, referring to the appearance of synnema on colonies, which looks like little bushes.

Coloniae in CYA post 7 dies in 25°C 33–38 mm, in MEA 36–45 mm. Synnema determinatum post incubationem longum factum, 100–150(–250) × 130–190(–210) μm, stipite alba. Conidiophorae biverticillatae interdum quadriverticillatae, e funiculis definitis portatae; stipae cum parietibus laevis 10–62 × 3–4 μm, metulae plerumque appressae 8–11 × 2–3 μm, phialidae acerosae 8.5–10(–12) × 2–2.5 μm. Conidia subsphaeroidea vel ellipsoidea, laevia, 2.5–3 × 1.5–2.5 μm.

Colony morphology, CYA, 25°C, 7 days: Colonies 33–38 mm diam, plane to centrally umbonate, moderately dense; texture typically funiculose, occasionally almost velutinous and covered by white aerial mycelia at the centre; margins low to moderately deep, mycelia white; conidiogenesis moderate, in some isolates sparse, pink/rose (11A5) at centre, grayish green (27B6) elsewhere; clear slimy exudate produced, soluble pigment absent, reverse pale white at margins becoming centrally pink to Ruby (12D8). At 5°C, 7 days: No germination to some germination; 37°C, 7 days: Sometimes no growth, sometimes sparse growth up to 9–11 mm diam, of white mycelium. MEA, 25°C, 7 days: Colonies 36–45 mm diam, plane to centrally umbonate, dense; textured as on CYA but lacking aerial mycelia; margins low; mycelium white, Golden Brown (5D7) at the centre of

* Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere.

some isolates; conidiogenesis heavy, grayish green (27b6); clear slimy exudate produced, soluble pigment absent, reverse Yellowish Brown (5E8) at centre becoming greenish white elsewhere. G25N, 25°C, 7 days: Colonies 4–5 mm diam, plane, velutinous; mycelia white; conidiogenesis sparse, grayish yellow (2B4); exudate and soluble pigment absent; reverse pale.

Conidiophores borne from the agar surface, but mainly from well defined funicles, *stipes* smooth-walled, 10–62 × 3–4 μm when borne on funicles, and 100 μm or longer when borne in synnema, bearing terminal biverticillate penicilli, with terverticillate to quaterverticillate penicilli not uncommon; *branches*, when present single or sometimes in whorls of 2–4, 10–14(–18) × 3–4 μm; *metulae* cylindrical in whorls of 4–5, usually closely appressed, sometimes divergent, 8–11 × 2–3 μm, 14–19 μm across the apices; *phialides* 3–4 per metula, closely appressed, acerose 8.5–10(–12) × 2–2.5 μm, conidiogenous aperture 1–2 μm; *conidia* subspheroid to ellipsoidal 2.5–3 × 1.5–2.5 μm, smooth-walled, borne in closely packed chains, inconspicuous remnants of connectives sometimes visible. *Synnemata* produced on MEA after 14–21 days in incidental light, determinate, short unbranched stalk 100–150(–250) × 130–190(–210) μm, white, conidiophores bearing a powdery, dull green conidial mass at the apex. Within *Protea* infructescences, synnema are similar in shape and color to those produced in culture, but are usually considerably longer.

Specimens examined: South Africa, Western Cape Province, Malmesbury: (S 33, 49795°; E 18, 58931°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type culture CV299 (PREM 59945) (HOLOTYPE); *Additional specimens examined*: South Africa, Western Cape Province, Malmesbury: (S 33,57061°; E 18,62861° and S 33, 49795°; E 18, 58931°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV113 (PREM 59947); 317; 318; 330 (PREM 59946); 333 (PREM 59948); South Africa, Western Cape Province, Stellenbosch, J.S. Marais Park: Isolated from *Protea bruchelli,* Jun 2005, collected by F. Roets, FR4; Canada, Ontario, Niagara County vineyard: Isolated from moth damaged Riesling grapes, Sept 2005, collected by K. A. Seifert, KAS2792.

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DISCUSSION

Penicillium ramulosum, isolated from sandveld fynbos soil and *Protea burchellii* infructescences, displays all of the diagnostic characteristics of subgenus *Biverticillium*. It produces terminally biverticillate, sometimes terverticillate or even more complex, conidiophores with thin, acerose phialides bearing chains of subspheroid to ellipsoidal conidia. Synnemata are only produced after prolonged incubation in incidental light at 25°C. Morphologically, *Penicillium ramulosum* is very similar to *P. dendriticum* and *P. cecidicola*. All three species seems to be associated with specific hosts, have similar metula, phialide and conidial dimensions and produces characteristic synnema in nature and in culture after prolonged incubation.

Penicillium cecidicola produces shorter (250–1250 µm), determinate, synnematous conidiomata with white or creamish stipes, consisting of biverticillate to terverticillate conidiophores bearing dark green conidia (Seifert et al. 2004). This species was isolated from insect galls on twigs of oak trees (*Quercus pacifica*) (Seifert et al. 2004). In contrast, *P. dendriticum* is commonly associated with *Eucalyptus* spp., and has also been isolated from insect galls (Pitt 1979, Seifert et al. 2004). *Penicillium dendriticum* is morphologically similar to *P. cecidicola,* except for its longer synnema (2–4 mm) and sulphur-yellow synnema stipes. *Penicillium ramulosum* differ from *P. dendriticum* and *P. cecidicola* by its shorter synnema [100–150(–250) µm] on MEA after two to three weeks of growth. In addition, *P. dendriticum* has a yellowish to raw umber reverse on CYA, whilst *P. ramulosum* has a pink to dark ruby reverse coloration. The conidia of *P. ramulosum* are pink/rose at the centre of CYA colonies and grayish green elsewhere whereas those of *P. cecidicola* are grayish green to turquoise on this medium.

Penicillium ramulosum seems to have an interesting ecology. It was isolated from soil and *Protea* infructescences in the Western Cape fynbos region, as well as on Riesling grapes from the Niagara County, Ontario, in Canada that was damaged by moths. Although not much is known about possible interactions

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between species of *Penicillium* and insects, it has previously been shown that these interactions might be occurring (Peterson et al. 2003, Seifert et al. 2004). Species in the subgenus *Biverticillium* that form extended synemma may well have associations with arthropods, as these structures are considered to aid dispersal via arthropod vectors (Abbott 2000). Fungi associated with structurally closed environments (e.g. *P. ramulosum* from *Protea* infructescences) probably rely on vectored dispersal because other means of dispersal (i.e. wind or water) are less likely. Interestingly, *Penicillium* spp. are commonly isolated from mites in *Protea* infructescences (Roets, unpublished). This association may be merely incidental, because the mites seem to vector fungal spores without any obvious benefit from their association with the fungus (Roets et al. 2007).

The fynbos biome, with its 9 030 vascular plant species, is considered to be the most diverse on earth (Goldblatt and Manning 2002). Since a link between plant and fungal biodiversity exists (Hawksworth 1991, 2001), it can be assumed that the fungal populations in the fynbos are just as diverse and unique as its plant counterparts. An underestimate of 63 210 fungal species are expected to occur in the fynbos, deduced from the tentative estimate by Crous et al. (2006) of 1:7 for a plant to fungus ratio. As *Penicillium* appears to be contributing to a large portion of this fungal diversity in fynbos soil, it is expected that the current survey would reveal many more novel *Penicillium* species.

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TABLE 1: Species names and their respective culture collection and GenBank accession numbers used for phylogenetic comparisons.

Species	Culture collection number	GenBank number
P. aculeatum	NRRL2129	AF033397
P. cecidicola	NRRL35466	DQ123648
	DAOM233329	AY787844
P. dendriticum	DAOM233861	AY787843
	DAOM226674	AY787842
P. diversum	NRRL2122	DQ308554
P. duclauxii	FRR1031	L14534
P. funiculosum	FRR1823	L14503
P. isariiforme	NRRL2638	AF454077
P. islandicum	FRR1399	AY373919
	FRR2239	L14504
P. marneffei	FRR3841	L37406
P. minioluteum	FRR1714	L14505
P. piceum	ATCC10519	DQ666826
	95M102_CT18	AY787846
P. pinophilum	BCC14374	AY753344
		AF176660
P. purpurogenum	FRR1061	AY373926
P. ramulosum	CV113	EU795706
	CV299	EU795704
	CV318	EU795708
	CV330	EU795707
	CV333	EU795705
	KAS2792	EU835480
P. resedanum	NRRL578	AF033398
P. rugulosum	DAOM215361	AY787845
P. variabile	FRR1055	L14507
P. verruculosum	ATCC62396	AF510496
T. bacillisporus	NRRL1025	AF033388
T. flavus	FRR2386	U18354
T. gossypii	FRR3467	L14523
T. helicus	NRRL2106	AF033396
T. intermedius	FRR3526	L14524
T. panasenkoi	NBRC31758	AB176636
T. purpureus	FRR1731	L14527
T. rotundus	GB6125	AF285115
T. stipitatus	FRR2166	L14514
T. thermophilus	FRR1791	L14515
T. trachyspermus	FRR1792	L14516
T. wortmannii	FRR1795	L14532

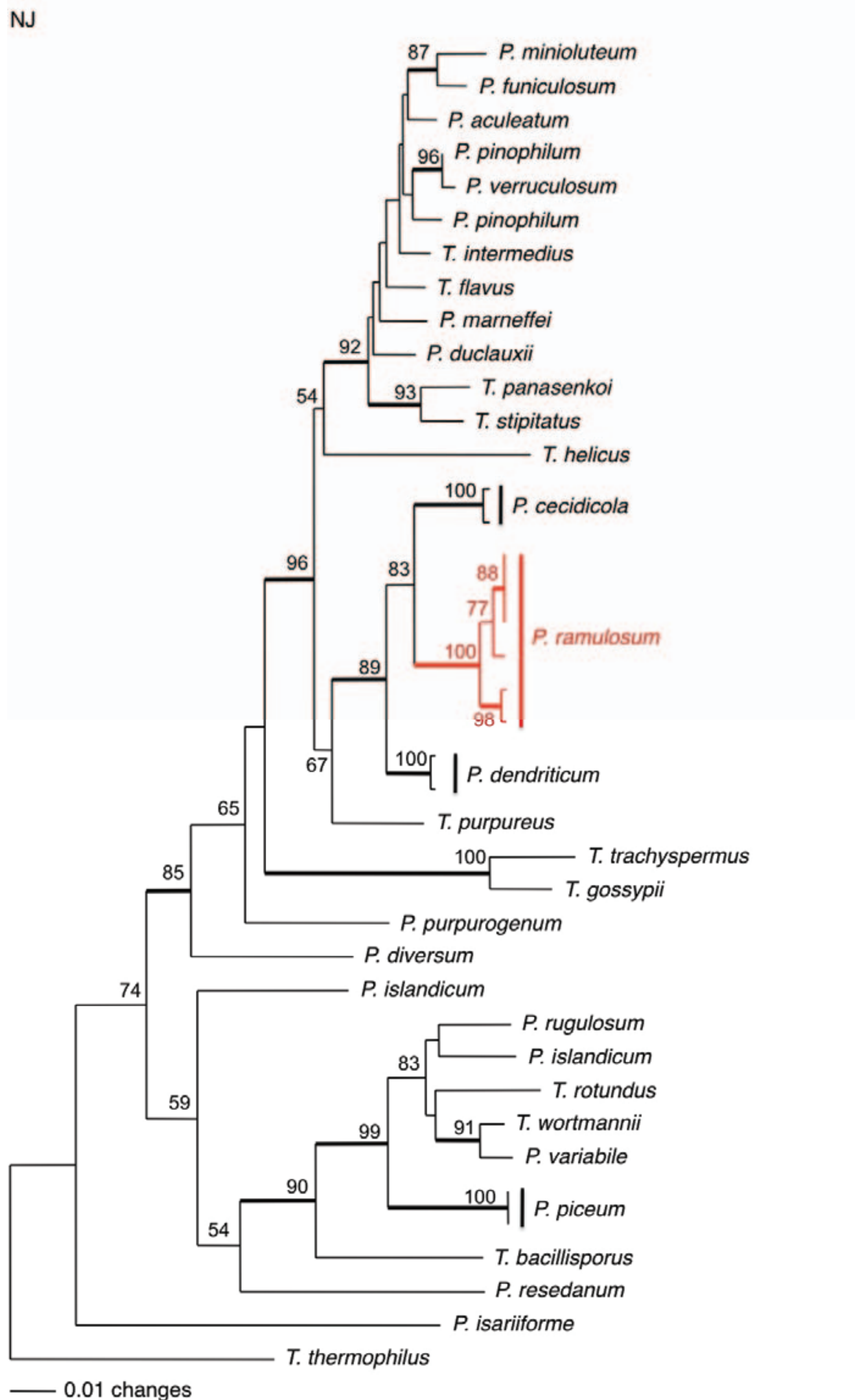

FIGURE 1. Neighbour-joining tree based on the phylogenetic analysis of the ITS1–5.8S–ITS4 rDNA sequences, showing relationships within *Penicillium* subgenus *Biverticillium* and the placement of the new species, *P. ramulosum,* as sister taxon to *P. dendriticum* and *P. cecidicola*. Numbers at branching nodes represents bootstrap values, with bold branches indicating bootstrap values higher than 85%. *Talaromyces thermophilus* was chosen as outgroup.

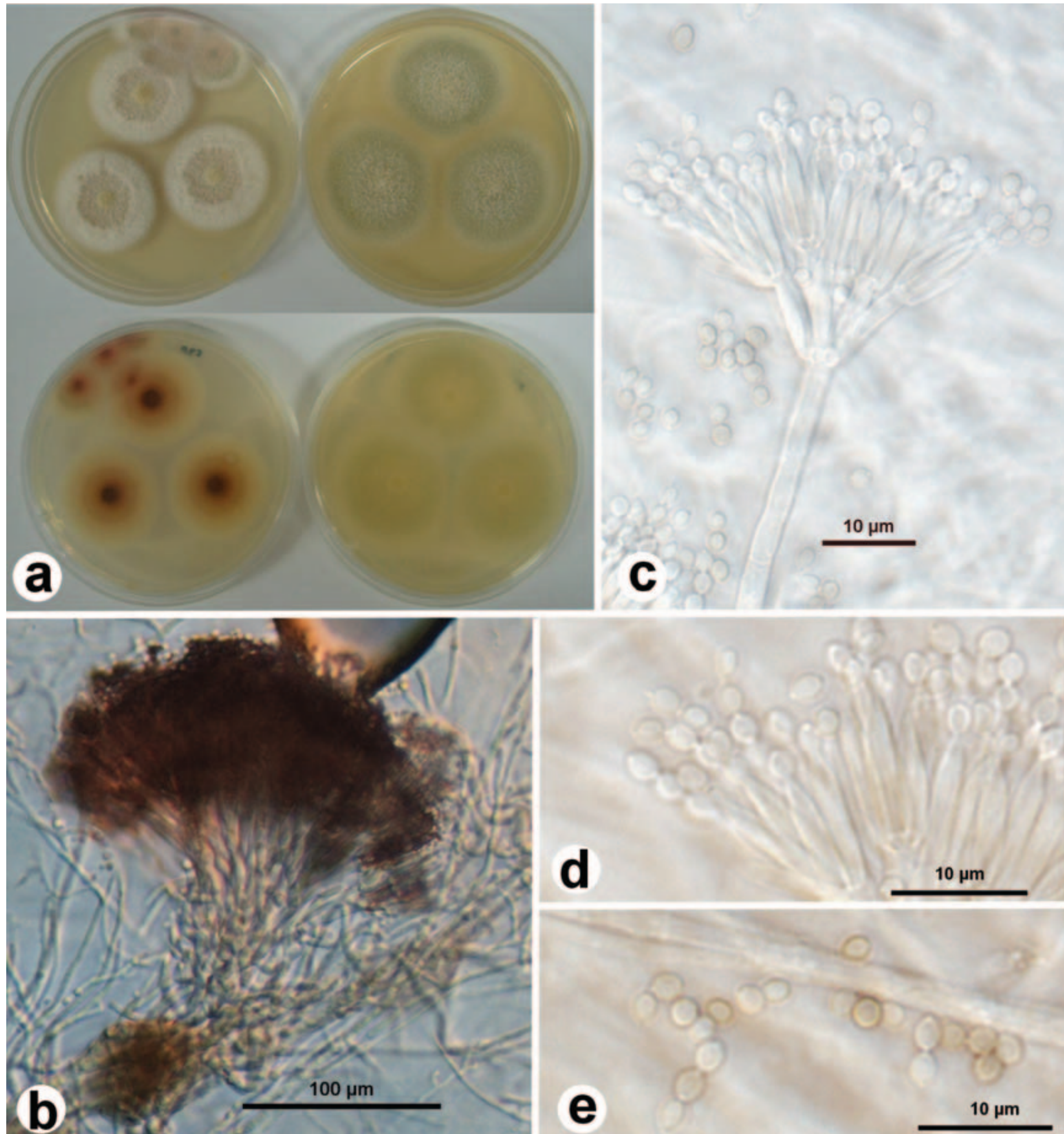

FIGURE 2. The most important taxonomic characters distinguishing *P. ramulosum* [PREM 59945 (holotype), 59946, 59947, 59948] from closely related species. a. *Penicillium ramulosum* grown on CYA (left) and MEA (right) for 7 days. b. Synnema produced on MEA, after 14 days of growth in incidental light. c. Conidiophores produced in culture grown on MEA. d. Conidiogenous cells showing acerose phialides with chains of conidia. e. Subspheroid, smooth, conidia.

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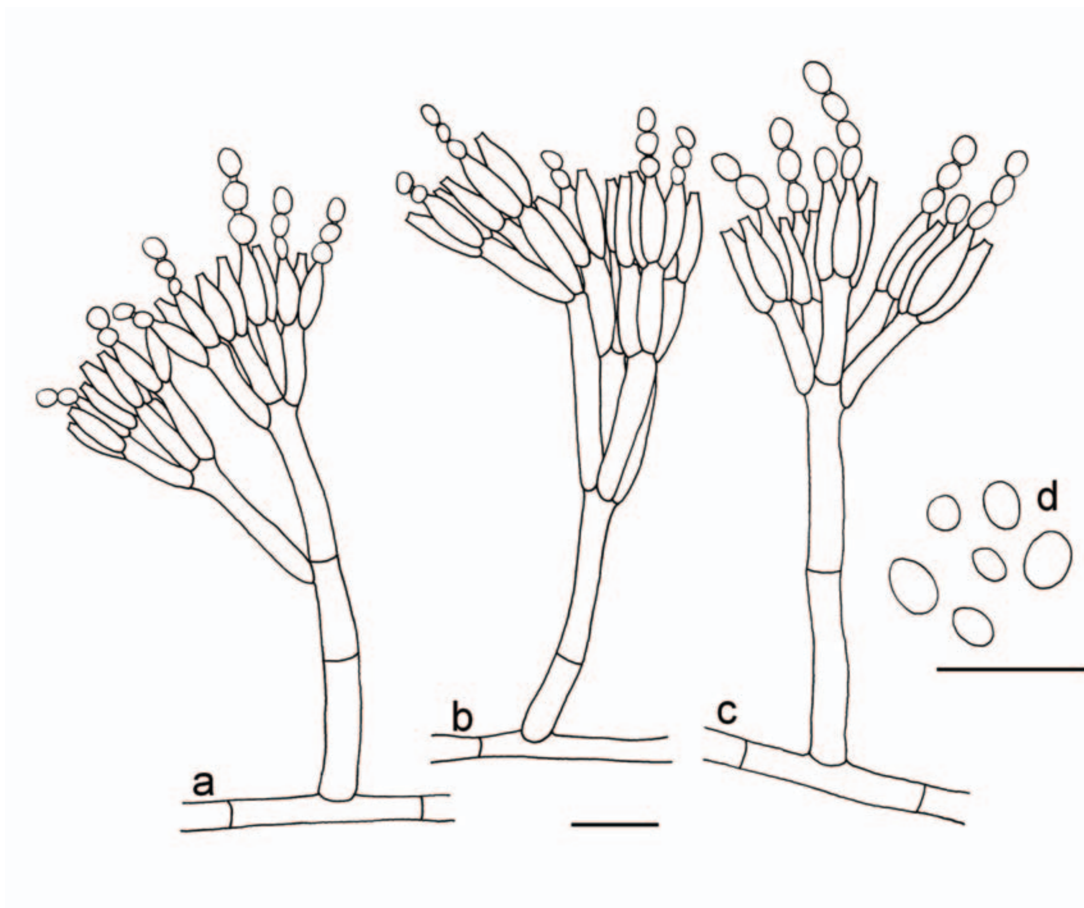

FIGURE 3. Line drawings showing the most prominent morphological features of *Penicillium ramulosum* (PREM 59945). a, b, c. Conidiophores (bar = 10 μm). d. Subspheroid to ellipsoidal conidia (bar = 10 μm).

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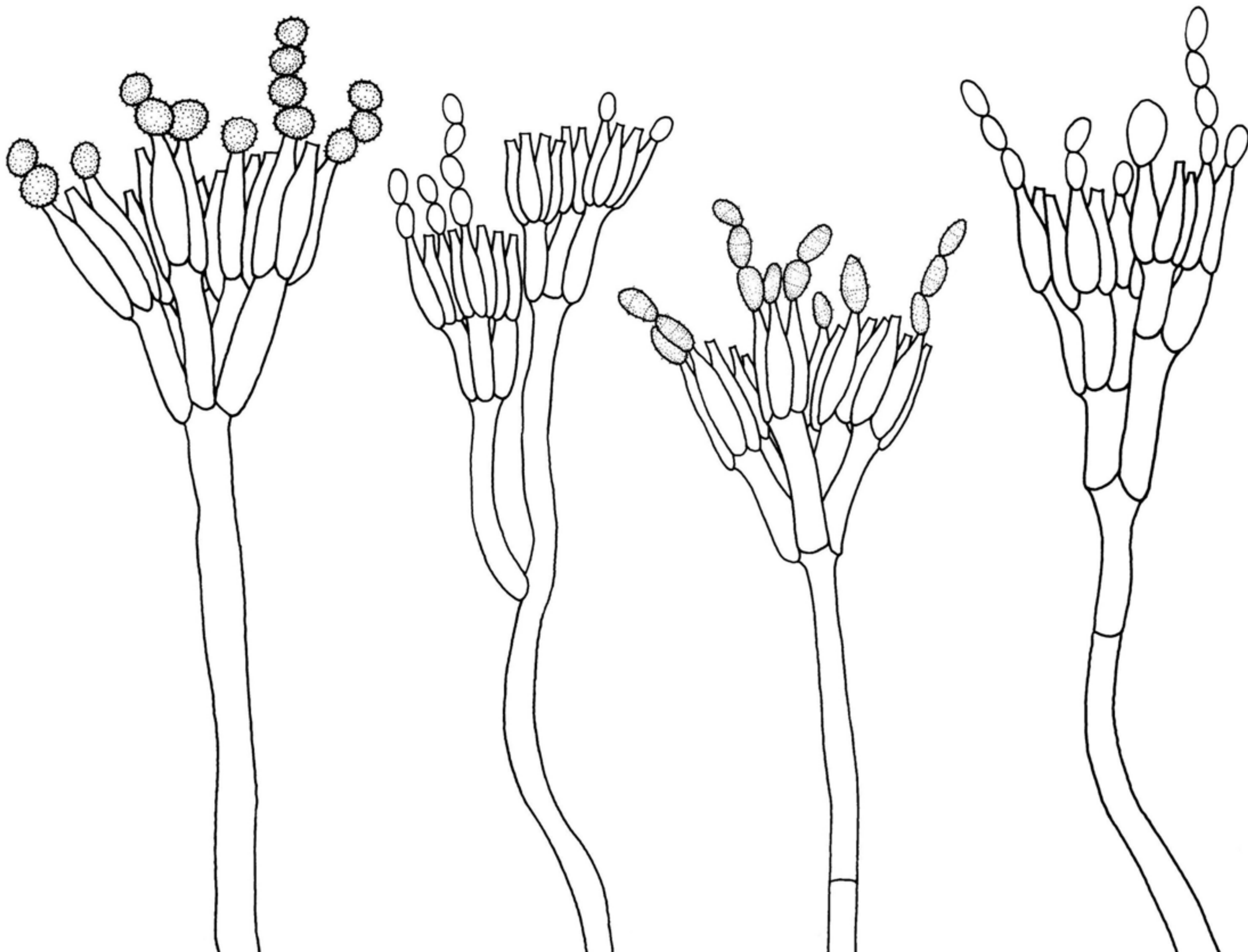
CHAPTER 3
New additions to *Penicillium* subgenus *Biverticillium* from coastal fynbos soils



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ABSTRACT

During a survey of *Penicillium* spp. occurring in soils from the diverse fynbos region in the Western Cape, South Africa, a number of previously undescribed species were isolated. Four of these species are classified in subgenus *Biverticillium* Dierckx, based on the branching patterns of the penicilli, phialide shapes and poor growth at reduced water activity. Morphological characters of the new species were compared to known species in subgenus *Biverticillium* as well as selected *Talaromyces* species. The ITS region were used for phylogenetic comparisons, which confirmed the novelty of the four soil fynbos species. The strains are, therefore, described here as *Penicillium solicola* prov. nom., *P. ptychoconidium* prov. nom., *P. occultum* prov. nom. and *P. chloroloma* prov. nom., respectively.

INTRODUCTION

The fynbos biome situated in the Western Cape, South Africa, is considered to be one of the most botanically diverse and unique habitats on earth with approximately 9 030 vascular plant species. This would account for 44% of the total floral inventory of South Africa (Goldblatt and Manning 2002). Based on the association between plants and fungi, Hawksworth (1991, 2001) estimated the amount of fungal diversity on earth to be 1.5 million species, without taking into account species associated with insects. This conservative number was calculated using a plant *vs* fungus ratio of 1:6. Crous et al. (2006) suggested this ratio to be 1:7 in South Africa, and estimated that a total of 171 500 fungal species should be present in this region. Extrapolating this ratio to the vascular plants from the fynbos area, 63 210 fungal species are expected to occur in the fynbos region of the Western Cape.

Similar to other global soil surveys (Christensen et al. 2000), *Penicillium* was found to be the dominant fungal genus in the fynbos soils, contributing to the majority of the species diversity in this niche. From the relative few mycological surveys done in this area, only one listed *Penicillium* spp. (Allsopp et al. 1987). Considering the inherent diverse and unique nature of the fynbos, one would expect that these soils harbor a large number of previously undescribed *Penicillium* species.

Subgenus *Biverticillium* is one of the better-defined subgenera of *Penicillium*, easily distinguished from species in the other subgenera based on a number of distinct morphological characters. Pitt (1979) assigned 23 species to *Penicillium* subgenus *Biverticillium*, which is characterized by biverticillate conidiophores bearing acerose phialides, a metulae to phialide length ratio of ±1–1.2 and poor growth at reduced water activity (Pitt 1979, Pitt and Hocking 1997). Penicillus dimensions and shapes within species of the subgenus *Biverticillium* are very similar and, therefore, colony morphology is often the criterion used to distinguish between closely related taxa. Differentiation between species based on colony appearance is possible, since colonies often have brightly colored

features such mycelia, soluble pigments, exudates and/or reverse colony color (Pitt 1979, Pitt and Hocking 1997).

Penicillium, together with *Geosmithia* and *Paecilomyces,* are the anamorph genera associated with the perfect states *Talaromyces* and *Eupenicillium*. (Pitt and Hocking 1997, Pitt et al. 2000). The *Penicillium* anamorphs of *Talaromyces* mostly conform to the characters defining subgenus *Biverticillium* (Pitt 1979, LoBuglio et al. 1993). Based on preliminary phylogenetic studies, *Talaromyces* spp. form a distinct clade, separate from *Eupenicillium* (Peterson 2000, Seifert et al. 2004). Species of *Penicillium* subgenus *Biverticillium* and their associated *Talaromyces* spp. cluster in a distinct monophyletic clade separate from *Penicillium* spp. from the other subgenera (LoBuglio et al. 1993, Berbee et al. 1995, Peterson 2000, Heredia et al. 2001, Seifert et al. 2004). This has lead to the suggestion that *Penicillium* subgenus *Biverticillium* species should probably be included into its own monophyletic genus (Seifert et al. 2004), but relationships within *Talaromyces* and its associated anamorphic genera first needs to be resolved.

During a soil survey in fynbos areas, eight taxa belonging to *Penicillium* subgenus *Biverticillium* were isolated from the coastal fynbos soil. Amongst these taxa were four previously undescribed *Penicillium* species. The aim of this study was, therefore, to compare these undescribed species to known *Penicillium* spp. by using morphological and phylogenetic characters, provide descriptions for the new species and compile a key for fynbos species and closely related sister taxa.

MATERIALS AND METHODS

Isolations — Strains used in this study were collected from coastal fynbos soil, situated near Malmesbury in the Western Cape, at Camphill Village (S 33,59787°; E 18,56433°), Kalbaskraal (S 33,57061°; E 18,62861°), Pella (S 33,51022°; E 18,55236°) and Riverlands (S 33,49066°; E 18,58388°). At each sampling site, random soil samples were collected from different plots. Five grams of each sample were added to a 100 ml dH_2O. A dilution series were prepared from this, with the 10^{-1} and 10^{-2} dilutions plated onto potato dextrose agar (PDA, Biolab,

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Johannesburg, South Africa), containing 50ppm Streptomycin (Applichem, South Africa) and 100ppm Chloramphenicol (Applichem, South Africa), incubated at 25°C for 6–7 days, after which colonies resembling those of *Penicillium* were transferred to Oatmeal agar (OA). Strains were incubated for a further 7 days at 25°C, after which single spore cultures were prepared from the strains.

Morphology — Spore suspensions of strains were prepared in a semi-solid agar (0.2% agar; 0.05% Tween80). Inoculations, from these spore suspensions, were done in three point style on Czapek Yeast Agar (CYA), Malt Extract Agar (MEA: after Blakeslee, 1915) and 25% Glycerol Nitrate Agar (G25N). The inoculated polystyrene Petri dishes (90 mm), containing 20 ml of media, were incubated at 25°C (CYA, MEA, G25N), 5°C (CYA) and 37°C (CYA) (Pitt 1979, Samson and Pitt 1985), in the dark with plates left unwrapped to allow for sufficient aeration (Okuda et al. 2000). Additional inoculated CYA and MEA plates were incubated in incidental light at room temperature (± 23°C) for 7–21 days. Strains were characterized and described following the methods of Pitt (1979), Samson and Pitt (1985) and Okuda et al. (2000). Color names and codes follows that of Kornerup and Wanscher (1966) as reference.

Phylogenetic analysis — DNA extractions were done from strains grown on MEA for 7 days using the ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). The subsequent PCR of the ITS1–5.8S–ITS2 rDNA region were prepared in 25 μl total volume reactions and consisted of 2.5 μl 10X Kapa Taq High Yield Buffer A, 2.5 U Kapa Taq (Kapa Biosystems, Woburn, USA), 250 μM dNTPs and 0.25 μM of primers ITS1 and ITS4, respectively (White et al. 1990). The thermal cycle profile had an initial denaturing step at 94 °C for 5 min, followed by 36 cycles at 94 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s, followed by a final elongation step at 72 °C for 10 min. PCR products were purified using the MSB®Spin PCRapace (Invitek, Berlin) kit. Sequencing reactions of the PCR products were set up using a Big Dye terminator cycle sequencing premix kit (Applied Biosystems, CA). The thermal cycle profile had an initial denaturing step at 94 °C for 5 min, followed by 25 cycles at 94 °C for 10 s, 55°C for 10 s and

60 °C for 4 min. Sequence reactions were analyzed with an ABI PRISM 310 genetic analyzer, with the subsequent sequence contigs assembled in SeqmanII (DNAstar).

ITS sequences of the fynbos soil strains were included in a dataset containing sequences, published on GenBank, of known *Penicillium* subgenus *Biverticillium* and *Talaromyces* species, mainly based on the studies done by LoBuglio et al. (1993), Peterson (2000), Heredia et al. (2001) and Seifert et al. (2004). Species and their GenBank accession numbers are represented in TABLE 1. The dataset was aligned in ClustalX (Thompson et al. 1997) and adjusted manually in Se-Al (Rambaut 2007). Ambiguously aligned regions typically contains numerous inserted gaps, which results in superficial branch lengths when computing phylogenetic trees. These regions were, therefore, replaced with weighted codes by computing step matrices using INAASE 2.3b (Lutzoni et al. 2000). The aligned dataset with step matrices can be obtained from the compact disc attached to the back of this thesis. Sequence analysis was done in PAUP* v4.0b10 (Swofford 2000) with gaps treated as missing data. The neighbour-joining option was used to calculate a single tree for the dataset, with the bootstrap analysis of a 1000 replicates performed to determine confidence levels of the nodes. *Talaromyces thermophilus* was chosen as outgroup based on the studies done by Heredia et al. (2001). Alignments of the ITS dataset can be obtained from the compact disc attached to the back of this thesis.

RESULTS

Isolations made from the soil dilutions yielded 434 *Penicillium* strains. The isolates were placed into their respective taxa, using colony characters on CYA and MEA. Sixty-six *Penicillium* strains, placed into eight distinct morphological taxa, had biverticillate conidiophores with acerose phialides and showed poor growth at reduced water activity, characteristic of species belonging to *Penicillium* subgenus *Biverticillium*. Three of the groups were identified, using Pitts' (1979) key, as *Penicillium minioluteum sensu* Pitt, *P. rugulosum, P. verruculosum* and one as *Penicillium ramulosum* (Visagie et al. 2008). *Penicillium minioluteum sensu* Pitt is distinguished from its close relatives based on slower

growth at 37°C, more appressed conidiophores and its smooth-walled conidia (Pitt 1979). The species characteristically also produces yellow mycelia and red colony reverse coloration on CYA and MEA. The fynbos strains did not vary from Pitt's (1979) species description, and were identified as such. Fynbos strains identified as *Penicillium verruculosum*, produced bright yellow mycelia on both CYA and MEA, as well as producing spheroidal verrucose conidia, which are definitive characters for the species (Pitt 1979). Four of the groups did, however, show unique morphological characters that did not conform to descriptions of any known species.

PCR reactions yielded amplicons of ±600 bp in length. The aligned dataset containing *Penicillium* subgenus *Biverticillium* and *Talaromyces* spp. was 532 base pairs long with nine ambiguous regions identified that were replaced by weighted codes. Morphological identifications were confirmed by neighbour-joining analysis, except in the case of *P. rugulosum*, and resolved the fynbos *Penicillium* spp. in the larger clade containing the subgenus *Biverticillium* and *Talaromyces* spp. (FIG. 1). Sequenced strains from the four, presumed to be new species, clustered together according to their respective morphological characters separate from previously described species. These are described as follow:

TAXONOMY

Penicillium solicola C.M. Visagie & K. Jacobs prov. nom.* **FIGS 2, 3**

Mycobank nr. MB 512403

Etymology. Latin, *solicola*: *solum* = soil; -*cola* = an inhabitant, an inhabitant of soil.

Coloniae in CYA post 7 dies in 25°C 3 mm, in MEA post 7 dies in 25°C 20–21 mm diametro, subtus brunneo-griseae. Conidiophora biverticillata in hyphis aeriis portata, stipa parietibus laevis (90–)160–230 × 2.5–3.5 µm; metulae appressae

* Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere.

8.5–10(–11) × 2.5–3.5 µm; phialides acerosae 9–11 × 2–2.5 µm; conidia subsphaeroidea verrucosa parietibus asperis (2–)2.5–3 × 2–2.5 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 3 mm diam, sometimes only germination, low, plane, loose; texture floccose; margins low, irregular, mycelia white; conidiogenesis absent to sparse, conidia *en masse* white when present; clear to orange exudate produced, soluble pigment absent, reverse pale white. At 5°C, 7 days: Germination. 37°C, 7 days: No germination. MEA, 25°C, 7 days: Colonies 20–21 mm diam, sometimes up to 25 mm, low, sulcate and sometimes slightly sunken at centre, loose, sometimes having a brownish orange color; texture floccose; margins low, 3–4 mm wide, regular, mycelia white; conidiogenesis medium, Dark Green (28f4); clear exudate produced, soluble pigment absent, reverse coloration Brownish Gray (6d2) at centre and Grayish Green (1c3) elsewhere. G25N, 25°C, 7 days: No germination or growth.

Conidiophores borne from aerial hyphae, *stipes* smooth-walled, (90–)160–230 × 2.5–3.5 µm, bearing strictly terminal biverticillate penicilli; *metulae* cylindrical in whorls of 5–8 sometimes up to 10, appressed, 8.5–10(–11) × 2.5–3.5 µm; *phialides* 3–4 per metula, closely appressed, acerose 9–11 × 2–2.5 µm, tapering to fine apical pore, 0.5–1 µm; *conidia* subspheroidal, verrucose, (2–)2.5–3.5 × 2–2.5 µm, rough-walled, borne in disordered chains.

*Specimens examined***:** South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type culture CV191 (PREM60037) (HOLOTYPE); *Additional specimens examined*: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV193 (PREM60038).

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Penicillium ptychoconidium C.M. Visagie & K. Jacobs prov. nom.* **FIGS 4, 5**
Mycobank nr. MB 512404
Etymology. Latin, *ptychoconidium*: *ptycho* = ridge. Refer to the ridges on the conidia that are distinctive in this species.

Coloniae in CYA post 7 dies in 25°C 8–10 mm, in MEA post 7 dies in 25°C 15–18 mm diametro, subtus crudo-umbrinae. Conidiophora biverticillata in hyphis aeriis et funiculis laxis portata, stipa parietibus laevis (38–)60–93 × 2.5–3.5 μm; metulae appressae 10–11.5 × 2–3 μm; phialides acerosae 10–12 × 2–2.5 μm; conidia ellipsoidea parietibus spiratim asperis 3–4.5(–5) × 2–3 μm.

Colony morphology, CYA, 25°C, 7 days: Colonies 8–10 mm diam, plane, loose to moderately dense; texture floccose; margins subsurface, 3–4 mm wide, regular, mycelia white; conidiogenesis absent to sparse, conidia *en masse* Grayish Green (1d4) when present; clear sticky exudate produced, soluble pigment absent, reverse pale to Grayish Yellow (1d4). At 5°C, 7 days: No germination; 37°C, 7 days: Colonies 8–9 mm diam, plane; texture velutinous; white mycelia; conidiogenesis sparse, Brownish Orange (6c3–6c5); exudate and soluble pigment absent, reverse pale (6b2). MEA, 25°C, 7 days: Colonies 15–21 mm diam, plane, loose, having a somewhat pinkish color; texture loosely funiculose; margins subsurface, narrow, irregular mycelia white; conidiogenesis sparse, Dark Green (28f4); clear to pale slimy exudate produced, soluble pigment absent, reverse Raw Umber (5f8). G25N, 25°C, 7 days: Colonies 4–5 mm diam, plane; margins low, white mycelia; conidiogenesis absent; clear exudate produced, soluble pigment absent, reverse white.

Conidiophores borne from aerial hyphae and loose funicles, *stipes* smooth-walled, (38–)60–93 × 2.5–3.5 μm when borne on aerial hyphae and funicles, bearing strictly terminal biverticillate penicilli; *metulae* cylindrical in whorls of (3–)5–7, appressed, 10–11.5(–12.5) × 2–3 μm; *phialides* 3–4 per metula, closely appressed, acerose, 10–12 × 2–2.5 μm, tapering to fine apical pore, 0.5–1 μm;

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conidia ellipsoidal, apiculate, 3–4.5(–5) × 2–3 µm, spirally rough-walled, borne in disordered chains;

Specimens examined: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type CV319 (PREM60041) (HOLOTYPE); *Additional specimens examined*: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV322 (PREM60043), CV323 (PREM60042).

Penicillium occultum C.M. Visagie & K. Jacobs prov. nom.* **FIGS 6, 7**

Mycobank nr. MB 512405

Etymology. Latin, *occultum*: meaning hidden. Refers to the hidden nature of the conidiophores that are produced underneath the sterile aerial hyphae.

Coloniae in CYA post 7 dies in 25°C 14–16 mm, subtus olivaceae; in MEA post 7 dies in 25°C 18–19 mm diametro, subtus olivaceo-brunneae. Conidiophora bis vel ter verticillata in hyphis aeriis, interdum funiculis debiliter evolutis, portata, stipa parietibus laevis 28–70 × 2.5–3 µm ramis appressis vel divergentibus; metulae appressae 7–9(–10) × 2–2.5 µm; phialides acerosae 7–9 × 1.5–2 µm; conidia ellipsoidea parietibus laevis 3–3.5 × 2–2.5 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 14–16 mm diam, low, convulate, moderately dense; texture velutinous to loosely funiculose, covered by sterile aerial hyphae hiding conidiophores produced; margins deep, narrow, entire, mycelia white; conidiogenesis sometimes only occurring after 2 weeks of growth, grayish green; clear exudate produced, soluble pigment absent, reverse coloration Olive Brown (4d5). At 5°C, 7 days: No germination; 37°C, 7 days: No growth MEA, 25°C, 7 days: Colonies 18–19 mm diam, low, convulate; texture velutinous to floccose, covered by sterile aerial hyphae; margins deep, 2–3 mm wide, entire, mycelia white; conidiogenesis occurring after 10 days of incubation,

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covered by sterile hyphae, conidia *en masse* grayish green; exudate yellowish white, soluble pigment absent, reverse coloration Olive Brown (3e7). G25N, 25°C, 7 days: Colonies 6–7 mm diam, plane; texture velutinous; mycelia conspicuously yellow; conidiogenesis sparse, conidia *en masse* grayish green at centre, yellowish elsewhere; exudate and soluble pigments absent, reverse coloration Poison Yellow (3e7).

Conidiophores borne from aerial hyphae and sometimes poorly developed funicles, *stipes* smooth-walled, 28–70 × 2.5–3 μm, bearing terminal biverticillate to terverticillate penicilli; *branches*, mostly 2, but sometimes 3, divergent, 10–16 × 2.5–3 μm; *metulae* cylindrical in whorls of 3–4, appressed, 7–9(–10) × 2–2.5 μm; *phialides* 3–4 per metula, acerose, 7–9 × 1.5–2 μm, tapering to fine apical pore, 0.5–1 μm; *conidia* ellipsoidal, smooth-walled, borne in single stranded chains, 3–3.5 × 2–2.5 μm.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Camphill Village: (S 33,59787°; E 18,56433°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type culture CV16 (PREM60039) (HOLOTYPE); *Additional specimens examined*: South Africa, Western Cape Province, Malmesbury, Camphill Village: (S 33,59787°; E 18,56433°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV69 (PREM60040).

Penicillium chloroloma C.M. Visagie & K. Jacobs prov. nom.[*] **FIGS 8, 9**

Mycobank nr. MB 512406

Etymology. Latin, *chloroloma*: *chloros-* = green + *-loma* = fringe. Indicating the green conidia *en masse* near fringes.

Coloniae in CYA post 7 dies in 25°C 34–37 mm, in MEA post 7 dies in 25°C 43–48 mm diametro, marginibus viridis. Synnema determinata post incubationem prolongatum in CYA casu in luce solis 700–1200 × 80–150 μm, stipe albo. Conidiophora bis vel ter verticillata in funiculis bene evolitis portata, stipa

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parietibus laevis 12–45 × 3–4 µm in funiculis, 700–1 200 µm in synnemate, ramis appressis vel divergentibus, 11–12(–15) × 3.0–3.5 µm; metulae appressae 7.5–9(–10.5) × 2–3 µm; phialides acerosae 7–8(–8.5) × 1.5–2 µm; conidia ellipsoidea parietibus laevis 2.5–3.5 × 1.5–2.0 µm, maioribus paucis 4.5–5(–6) × 2–2.5 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 34–37 mm diam, plane, moderately dense; texture floccose with funicles present, determinate synnema produced in incidental sunlight after prolonged incubation; margins subsurface, irregular, mycelia white, 4–5 mm wide, characteristic spiral growth at edge of colonies; conidiogenesis medium to heavy, Olive Brown (4e5–4e7) at centre, Pastel Green (27a4) at edge; exudate and soluble pigment absent, reverse Grayish Ruby (12c3–12c4) at centre, Greenish White (26a2) elsewhere. At 5°C, 7 days: Germination; 37°C, 7 days: Colonies 9–12 mm diam, plane, moderately dense; texture floccose to loosely funiculose; white mycelia; conidiogenesis sparse to medium, Dark Green (25f7); exudate and soluble pigment absent, Reverse Olive (1e6). MEA, 25°C, 7 days: Colonies 43–48 mm diam, plane, moderately dense; texture strongly funiculose to floccose; margins subsurface, 4–5 mm, irregular, mycelia white; conidiogenesis medium to dense, olive brown (27a4) at centre, Grayish Green (25c6–25d6) elsewhere; exudate and soluble pigment absent, reverse Grayish Green (29d5). G25N, 25°C, 7 days: Micro-colonies.

Conidiophores borne from well defined funicles, having a olive-like pigmentation, *stipes* smooth-walled, 12–45 × 3–4 µm when borne on funicles, and 700–1200 µm when borne in synnema, bearing terminal biverticillate penicilli, with terverticillate penicilli also present; *branches*, when present in appressed to almost parallel whorls of 2–4, but mostly 3 branches, 11–12(–15) × 3–3.5 µm; *metulae* cylindrical in whorls of 3–5, appressed, 7.5–9(–10.5) × 2–3 µm; *phialides* 4–5 per metula, closely appressed, acerose, 7–8(–8.5) × 1.5–2.5 µm, tapering to fine apical pore, 0.5–1 µm; *conidia* ellipsoidal, 2.5–3.5 × 1.5–2 µm, smooth-walled, borne in disordered chains, sparse larger conidia present 4.5–5(–6) × 2–2.5 µm; *Synnemata* on CYA produced after 14–21 days of growth in incidental sunlight determinate, unbranched white stalk 700–1 200 × 80–150 µm, 400–580

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μm across apex, conidiophores bearing a powdery, dark green conidial mass at the apex.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type culture CV389 (PREM60033) (HOLOTYPE); *Additional specimens examined*: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV390 (PREM60034).

Key to *Penicillium* subgenus *Biverticillium* incorporating the newly described fynbos species and its closely related sister species

1. Colonies on CYA <4 mm; red mycelia never present..**2**
1. Colonies on CYA >4 mm..**4**

2. Conidia distinctly rough-walled..***P. solicola***
2. Conidia smooth-walled...**3**

3. Growth only on acidified (pH 5/less) CYA; stipes <100 μm..................***P. lignorum***
3. Colonies on CYA 2–4 mm, sometimes only microcolony;
   stipes >100 μm..***P. diversum***

4. Conidia ornamented with transverse/spiral striations...**5**
4. Conidia without striations...**6**

5. Red mycelia on CYA and MEA; stipes vesiculate.....................................***T. purpureus***
5. Mycelia white on CYA and MEA;
   stipes non-vesiculate..***P. ptychoconidium***

6. Colonies on CYA <12 mm and MEA <20 mm...***P. rugulosum***
6. Colonies on CYA >12 mm or MEA >20 mm...**7**

7. Conidia spheroid..**8**
7. Conidia not spheroid..**10**

8. Conidia smooth to finely roughened...***P. pinophilum***
8. Conidia strongly rough-walled...**9**

9. Mycelia on CYA yellow; colonies at 37°C >20 mm...........................***P. verruculosum***
9. Mycelia on CYA white; colonies at 37°C <20 mm...................................***P. aculeatum***

10. Colonies on CYA and MEA <22 mm...**11**
10. Colonies on either CYA or MEA >22 mm..**12**

11. Conidial length 3–3.5 μm; white mycelia with exudate
    dominating colony appearance; stipes commonly <70 μm...............***P. occultum***
11. Conidial length 3.5–4 μm; yellow mycelia with abundant

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conidiogenesis dominating colony appearance; stipes often 100–200 μm..***P. variabile***

12. Colonies at 37°C >20 mm; mycelia on CYA salmon to peach..........................***P. funiculosum***
12. Colonies at 37°C <20 mm..**13**

13. Metulae divergent; mycelia bright yellow; dark red colony reverse on CYA; synnema not produced..........***P. minioluteum sensu*** **Pitt**
13. Metulae appressed; mycelia usually white; synnema produced after prolonged incubation..........................**14**

14. Synnema having yellow stalks..**15**
14. Synnema having white stalks..**16**

15. Synnema 2000–4000 μm tall..........................***P. dendriticum***
15. Synnema <400 μm..........................***P. aureocephalum***

16. Conidia *en masse* on CYA pink to grayish green; synnema 100–250 μm tall; abundant sticky exudates produced..........***P. ramulosum***
16. Synnema >250 μm..**17**

17. Colonies on CYA 34–37 mm and MEA 43–48 mm; conidia *en masse* olive brown, with light green edge..........***P. chloroloma***
17. Colonies on CYA 19–31 mm and MEA 32–40 mm; conidia *en masse* grayish green to grayish turquoise..........***P. cecidicola***

DISCUSSION

Penicillium subgenus *Biverticillium* are separated from the other three subgenera (subgenera *Aspergilloides, Furcatum* and *Penicillium*) based on their biverticillate, sometimes terverticillate, conidiophores bearing thin acerose phialides, with the metulae to phialide length ratio equal to ±1-1.2 (Pitt 1979, Pitt and Hocking 1997). Eight of the taxa isolated from coastal fynbos soil displayed all of these characters and subsequently were placed, according to Pitts' classification, into subgenus *Biverticillium*. Isolated species included *Penicillium minioluteum sensu* Pitt (FIGS 10, 11), *P. rugulosum*-like strains (FIGS 12, 13), *P. verruculosum* (FIGS 14, 15) and *P. ramulosum* (chapter 2, FIGS 2, 3), together with the four species described here. Allsopp et al. (1987) in a similar study also recorded *P. verruculosum*, as well as *P. purpurogenum* and *P. funiculosum*. In their survey they have, therefore, only isolated three species from subgenus *Biverticillium*, compared to the eight from our study. We did, however, found that species from this group often occurs in specific soil samples and in low numbers. Allsopp et al. (1987) only included species that occurred in ten or

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more samples, which explains the low species diversity of subgenus *Biverticillium* reported. The identity of the Allsopp et al. (1987) *P. funiculosum* strains identity are also questionable. *Penicillium ramulosum*, a newly described fynbos soil species (Visagie et al. 2008), have similar characters to that of *P. funiculosum*, for example strongly funiculose colonies on CYA and MEA, which can easily lead to confusion when using Pitt's (1979) key. This problem is addressed in the key provided in this paper.

One of the fynbos species isolated, showed no variance from Pitt's species description for *P. rugulosum*. The description given for *P. rugulosum* is, however, rather broad with the conidia for instance described as smooth to verrucose. This allows for a broad spectrum of morphological variation within this group. According to the ITS phylogeny, however, the fynbos strains shows the same amount of variance from *P. rugulosum* as the new species, *P. occultum* (FIG. 1), although it is clearly two distinct taxa based on morphology. In the absence of a more detailed dataset, this issue cannot be resolved and we consider *Penicillium rugulosum* to probably represent a species complex.

Penicillium occultum are characterized by colonies on CYA and MEA being covered by white sterile aerial hyphae, which generally hides the conidiophores which are borne on poorly developed funicles. Other species in subgenus *Biverticillium*, for instance *Penicillium aureocephalum* (Muntañola-Cvetkovic et al. 2001), are also known to produce these sterile overlaying aerial hyphae. The significance of this character is, however, not known since few have included it in their species descriptions. The new species shows a close affinity to *P. variabile* and *P. rugulosum*, but are readily distinguished from both. On CYA (25°C) it produces white mycelia and abundant exudate, compared to the yellow mycelia of *P. variabile* as well as the absence of exudates in the latter species. In addition to this, *P. variabile* conidiophores have longer stipes (100–200 μm) than *P. occultum* (28–70 μm). Phylogenetically, *P. occultum* are resolved in a clade distant to *P. variabile*, with *P. rugulosum* as a sister taxon. *Penicillium rugulosum* do, however, show restricted growth on CYA (25°C) and commonly germinates at 5°C, compared to *P. occultum* showing faster growth on CYA (25°C) and don't

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germinate at 5°C. In addition to the slower growth on CYA (25°C), *P. rugulosum* produces yellow mycelia compared to the white mycelia of *P. occultum*.

Penicillium solicola is distinguished by its characteristically poor growth on CYA, which would explain its phylogenetic placement as sister taxon to *P. diversum*. Another species, *P. lignorum*, only growing on acidified CYA, also displays this character. The new species is easily distinguished from both its close relatives based on its characteristic heavily rough-walled, subspheroidal conidia, compared to the smooth-walled conidia of its sister taxa. In addition to the conidial walls, *P. lignorum* produces shorter stipes [15–50(–100) μm] than *P. solicola*. *Penicillium diversum* often produce yellow colored mycelia at colony peripheral zones, which are absent in *P. solicola*. Phylogenetic analysis resolved *P. diversum* as the sister taxon of *P. solicola*, with no sequence data available for *P. lignorum*. Pitt (1979) did, however, mention that he does not expect *P. lignorum* to be closely related to other species in subgenus *Biverticillium*. This was based on the fact that *P. lignorum* require an acidic medium, produce complex penicilli and have the ability to grow on wood.

Penicillium ptychoconidium belongs to section *Simplicium*, series *Islandica* (Pitt, 1979). This species characteristically displays restricted growth on CYA, as well as on MEA. Its most striking feature is, however, its closely appressed conidiophores producing spirally rough-walled ellipsoidal conidia. This species, however, does not seem to have a close affinity to any of the species from this group. The phylogenetic analysis suggests *Talaromyces purpureus* (Stat. anam. *Penicillium purpureum*) as its sister taxon. Interestingly, both of these species produces spirally ornamented conidia. These two species are readily distinguishable from each other, based on the red mycelia produced by *T. purpureus* compared to the white mycelia of *P. ptychoconidium.* Microscopically *T. purpureus* have vesiculate stipes with both metulae (7–10 μm) and phialides (6–10 μm) shorter than *P. ptychoconidium.* Although belonging in subgenus *Furcatum*, based on microscopic features, *P. oxalicum* is very similar to the new species. *Penicillium oxalicum,* for instance, also produces conidia which sometimes is spirally rough-walled as well as having appressed conidiophores.

Penicillium ptychoconidium is, however, readily distinguishable from *P. oxalicum*, based on the latter species faster growth displayed on both CYA and MEA at 25 °C, as well as its longer metulae [15–25 (–30 μm] which is one of the reasons for its placement in subgenus *Furcatum*.

Penicillium chloroloma are distinguished by its strongly funiculose colonies producing olive colored conidia *en masse,* determinate synnema after prolonged incubation and appressed conidiophores, borne on short stipes, having an olive-like pigmentation. The new species are closely related to *Penicillium aureocephalum, P. ramulosum, P. cecidicola* and *P. dendriticum,* with the ITS phylogeny resolving the new species in a well developed clade with the latter three species. Interestingly, the species from this clade characteristically produces synnema after prolonged incubation. The new species are easily distinguished from *P. aureocephalum* based on faster growing colonies on CYA (34–37 mm) compared to the restricted colonies (9–10 mm) of its close relative. *Penicillium chloroloma* is distinguished from *P. cecidicola* by faster growth on CYA (34–37 *vs* 19–31 mm) and MEA (43–48 *vs* 32–40 mm) at 25°C and its ability to grow at 37°C on CYA. In addition, *P. chloroloma* conidiophores have shorter stipes (12–45 *vs* 20–80 μm), but generally longer synnema (700–1200 *vs* 250–1250 μm) compared to *P. cecidicola*. The longer synnema produced by *P. chloroloma* also distinguishes it from *P. ramulosum* which have very short synnema (110–150 μm) produced on MEA. Conidiogenesis in *P. chloroloma* are also much denser and have an olive-brown color compared to the pink to grayish green conidia of *P. ramulosum*. *Penicillium dendriticum* is easily distinguished by its production of long (2–4 mm) synnema with yellow stipes, compared to *P. chloroloma* which have shorter synnema with white stipes. The morphological relationship of these species are reflected in their phylogeny and are sister taxa with well supported branches in a neighbour-joining tree (FIG. 1).

Penicillium is generally regarded as having soil as its principle habitat, with a large proportion of species only known from soil (Pitt 1979). Fynbos soil is considered to be an especially harsh environment because of its acidity and poor nutrient levels (Kruger 1983), which is worsened by the low rainfall during

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summer and low temperatures during winter (Richards et al. 1997). Other external factors influencing organisms living in the fynbos is the heterogeneity of the soil and plants, as well as the constant fires associated in the fynbos (Goldblatt and Manning 2002). These are just some factors that affect and places constant evolutionary pressure on organisms actively living in these soils and may account for the high diversity seen in the area. Interestingly, the species found does not seem to be confined to the soil environment. *Penicillium ramulosum* have also been isolated from *Protea* infructescences (Visagie et al. 2008), with a number of other *Penicillium* spp. that were isolated from mites in these infructescences. *Penicillium ramulosum* and *P. chloroloma* were also isolated from apple orchards in the Western Cape (unpublished data). The occurrence and distribution patterns of the species isolated from fynbos soil in the Western Cape is still unknown. With more studies like these, uncovering and describing species from this unique area we might be surprised at the extent of its distribution and effect it plays in the environment.

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TABLE 1: Species names and their respective culture collection and GenBank accession numbers used for phylogenetic comparisons.

Species	Culture collection number	GenBank number
P. aculeatum	NRRL2129	AF033397
P. cecidicola	NRRL35466	DQ123648
	DAOM233329	AY787844
P. chloroloma	CV390	FJ160272
	CV389	FJ160273
P. dendriticum	DAOM233861	AY787843
P. diversum	NRRL2122	DQ308554
P. duclauxii	FRR1031	L14534
P. funiculosum	FRR1823	L14503
P. islandicum	FRR1399	L14504
	FRR2239	AY373919
P. marneffei	FRR3841	L37406
P. minioluteum	CV9	FJ160268
	CV284	FJ160269
	FRR1714	L14505
P. occultum	CV16	FJ160270
	CV69	FJ160269
P. piceum	ATCC10519	DQ666826
	95M102_CT18	AY787846
P. pinophilum	BCC14374	AY753344
		AF176660
P. ptychoconidium	CV322	FJ160266
	CV319	FJ160267
P. purpurogenum	FRR1061	AY373926
P. ramulosum	CV318	EU795708
	CV299	EU795704
P. resedanum	NRRL578	AF033398
P. rugulosum	CV68	FJ160275
	CV89	FJ160274
	96M111159*	
	KAS1864*	
	KAS1863*	
	DAOM215361	AY787845
P. solicola	CV191	FJ160265
	CV193	FJ160264
P. variabile	FRR1055	L14507
P. verruculosum	CV256	FJ160276
	CV121	FJ162077
	ATCC62396	AF510496
T. flavus	FRR2386	U18354
T. gossyppii	FRR3467	L14523

* Sequences provided by Dr. Keith Seifert, not available on GenBank

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T. helicus	NRRL2106	AF033396
T. intermedius	FRR3526	L14524
T. panasenkoi	NBRC31758	AB176636
T. purpureus	FRR1731	L14527
T. rotundus	GB6125	AF285115
T. stipitatus	FRR2166	L14514
T. thermophilus	FRR1791	L14515
T. trachyspermus	FRR1792	L14516
T. wortmannii	FRR1795	L14532

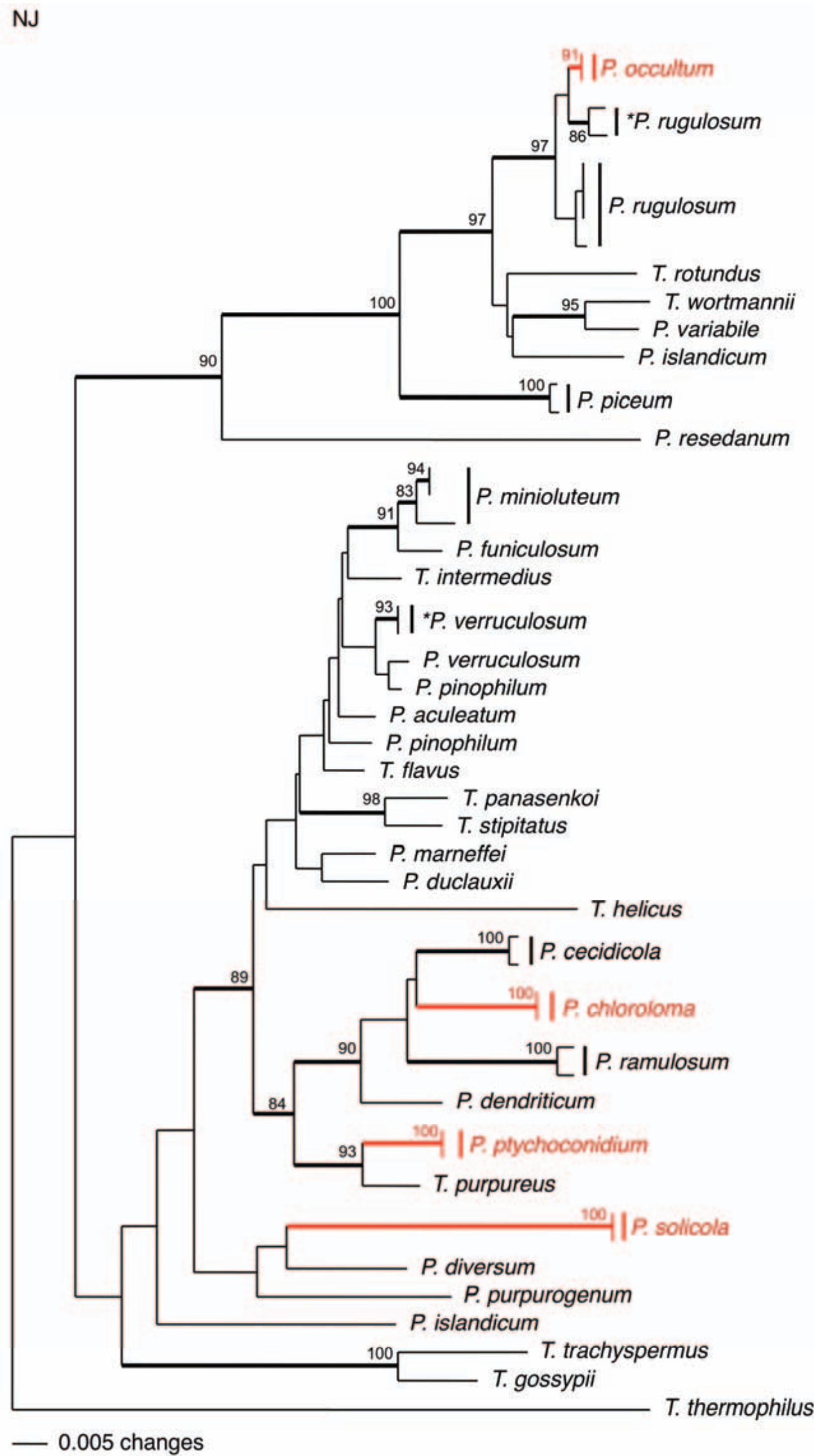

FIGURE 1. Neighbour-joining tree based on the ITS1–5.8S–ITS2 rDNA phylogeny, showing relationships within *Penicillium* subgenus *Biverticillium,* the genus *Talaromyces* and strains isolated from fynbos soil. Numbers at branching nodes represents bootstrap values (1000 replicates), with bold branches indicating bootstrap values higher than 80%. *Talaromyces thermophilus* was selected as outgroup. * = fynbos strains in the *P. rugulosum* and *P. verruculosum* clades respectively.

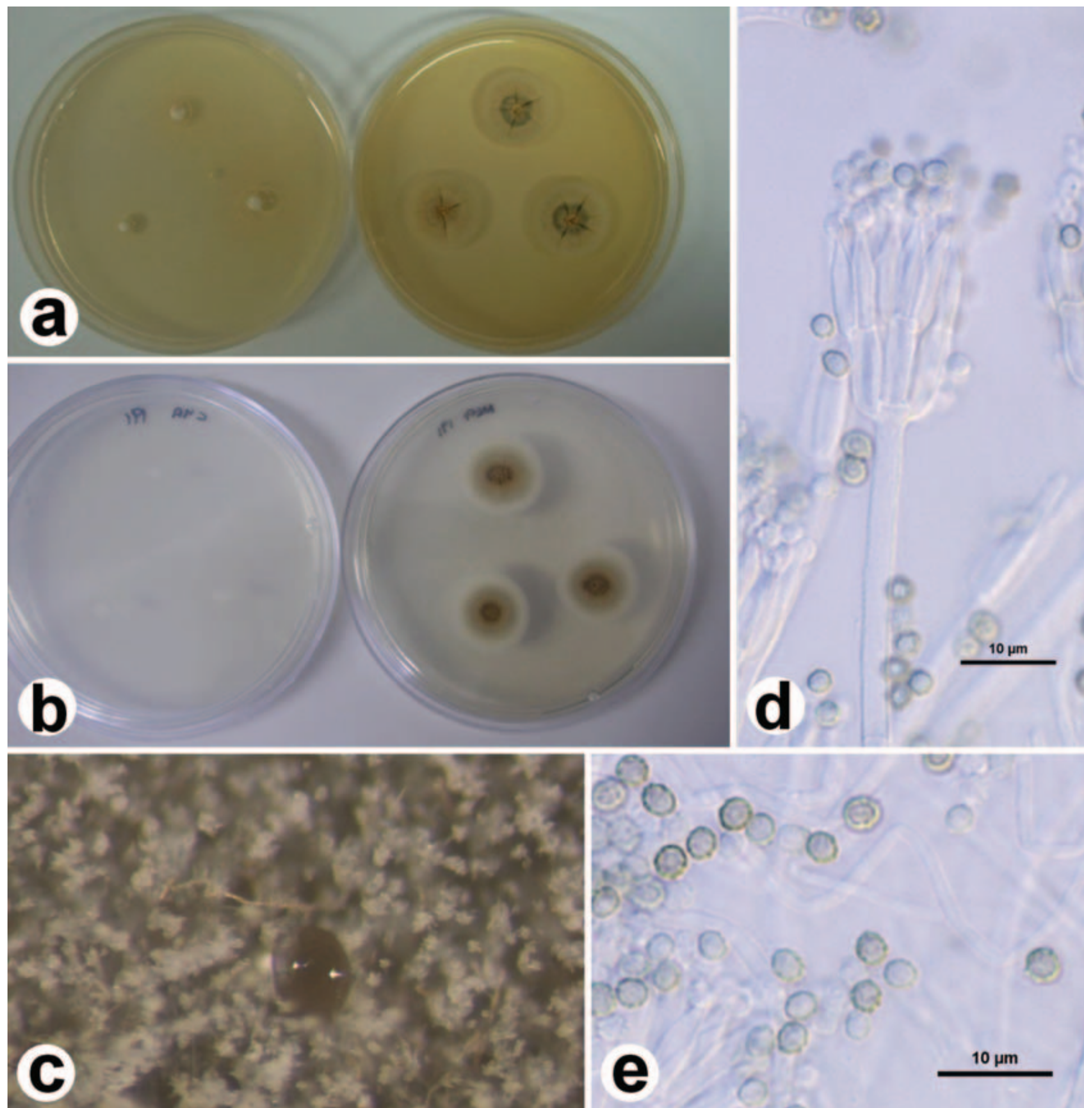

FIGURE 2. The most important taxonomic characters distinguishing *P. solicola*, holotype (PREM60037), from closely related species. a. *Penicillium solicola* grown on CYA (left) and MEA (right) for 7 days. b. Reverse of colonies showing the brownish gray coloration on MEA. c. Floccose texture of colonies grown on MEA, with the clear exudate produced. d. Biverticillate, appressed conidiophores produced in culture on MEA. e. Subspheroidal, rough-walled verrucose conidia.

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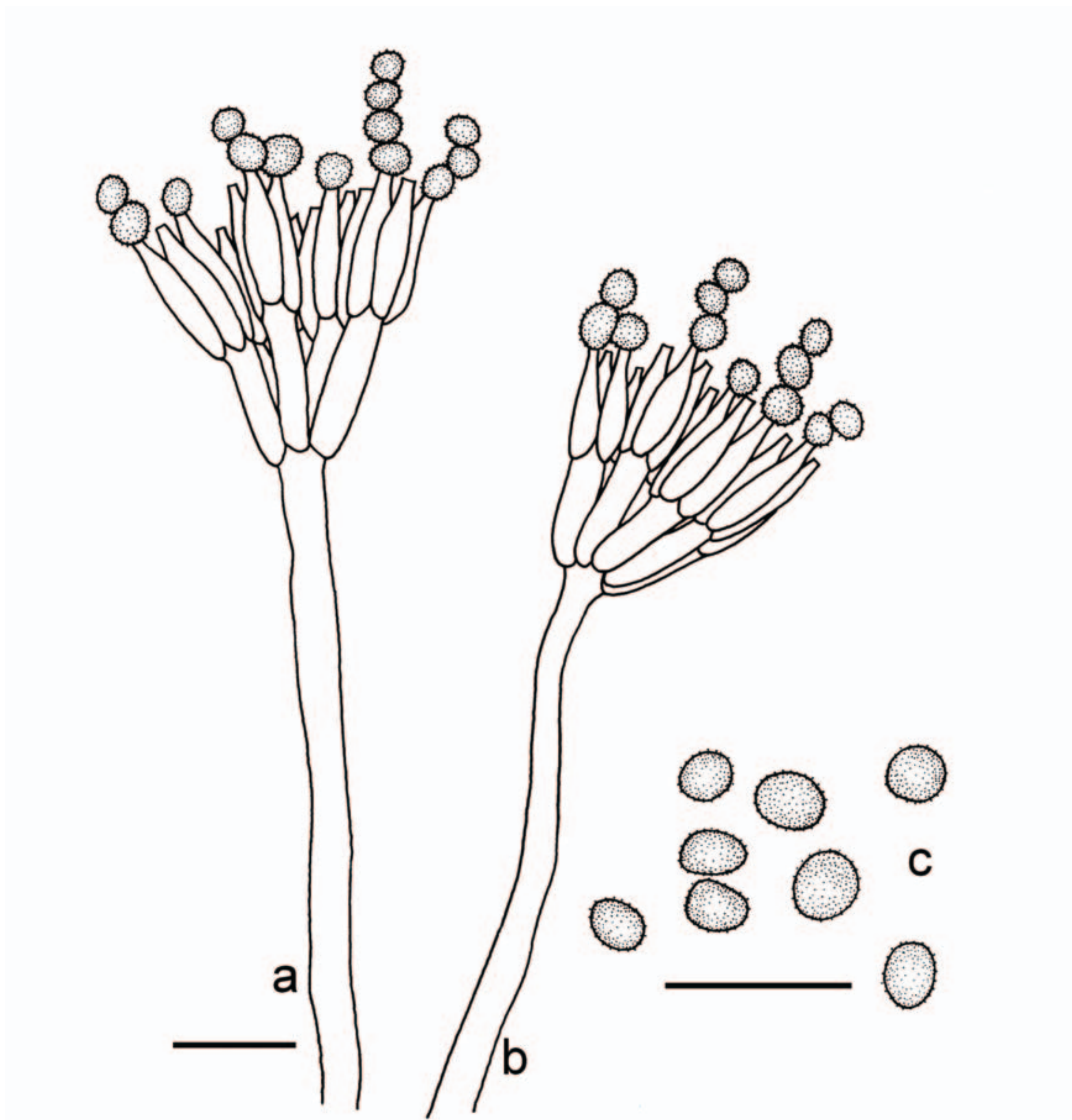

FIGURE 3. *Penicillium solicola* line drawings from holotype (PREM60037) material. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

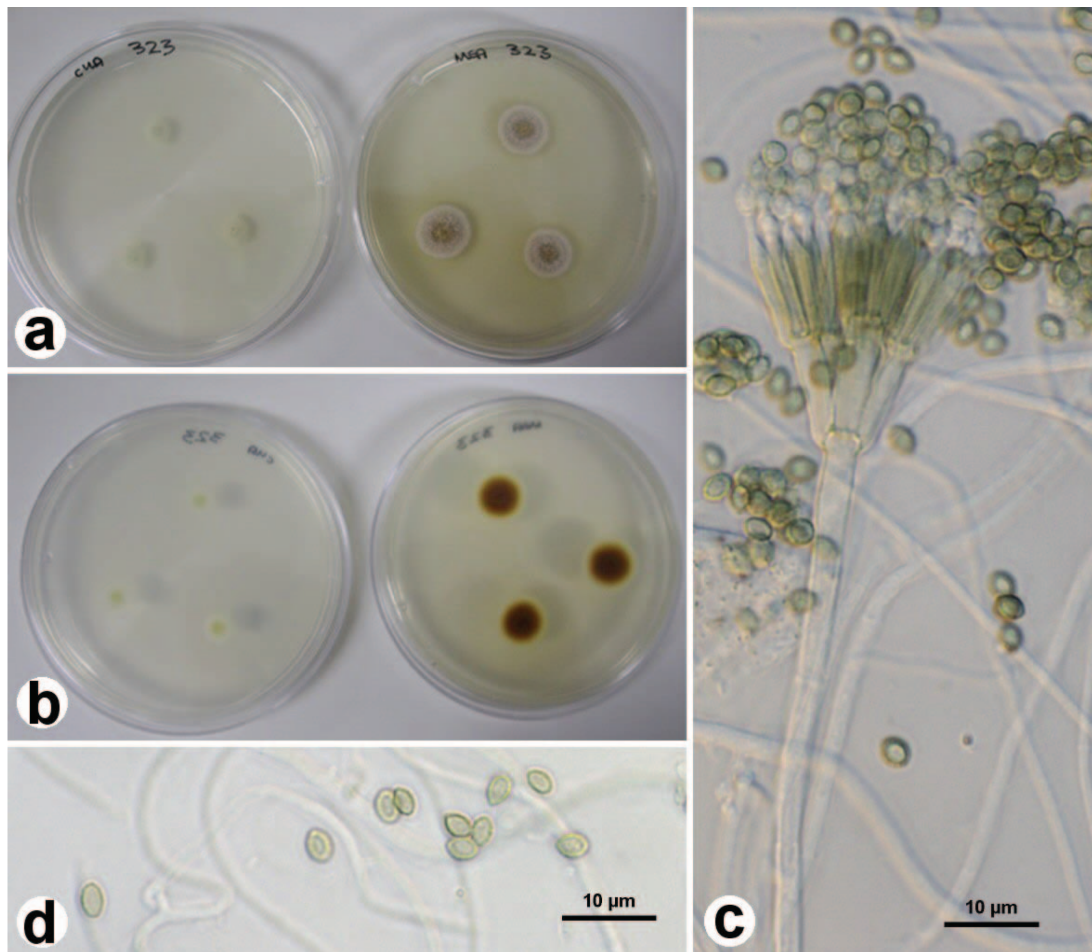

FIGURE 4. The most important taxonomic characters distinguishing *P. ptychoconidium*, holotype (PREM60041), from closely related species. a. Colonies of *Penicillium ptychoconidium* grown on CYA (left) and MEA (right) for 7 days. b. Reverse of colonies showing the characteristic raw umber coloration on MEA. c. Biverticillate conidiophores produced in culture. d. Ellipsoidal, fusiform, spirally roughened conidia.

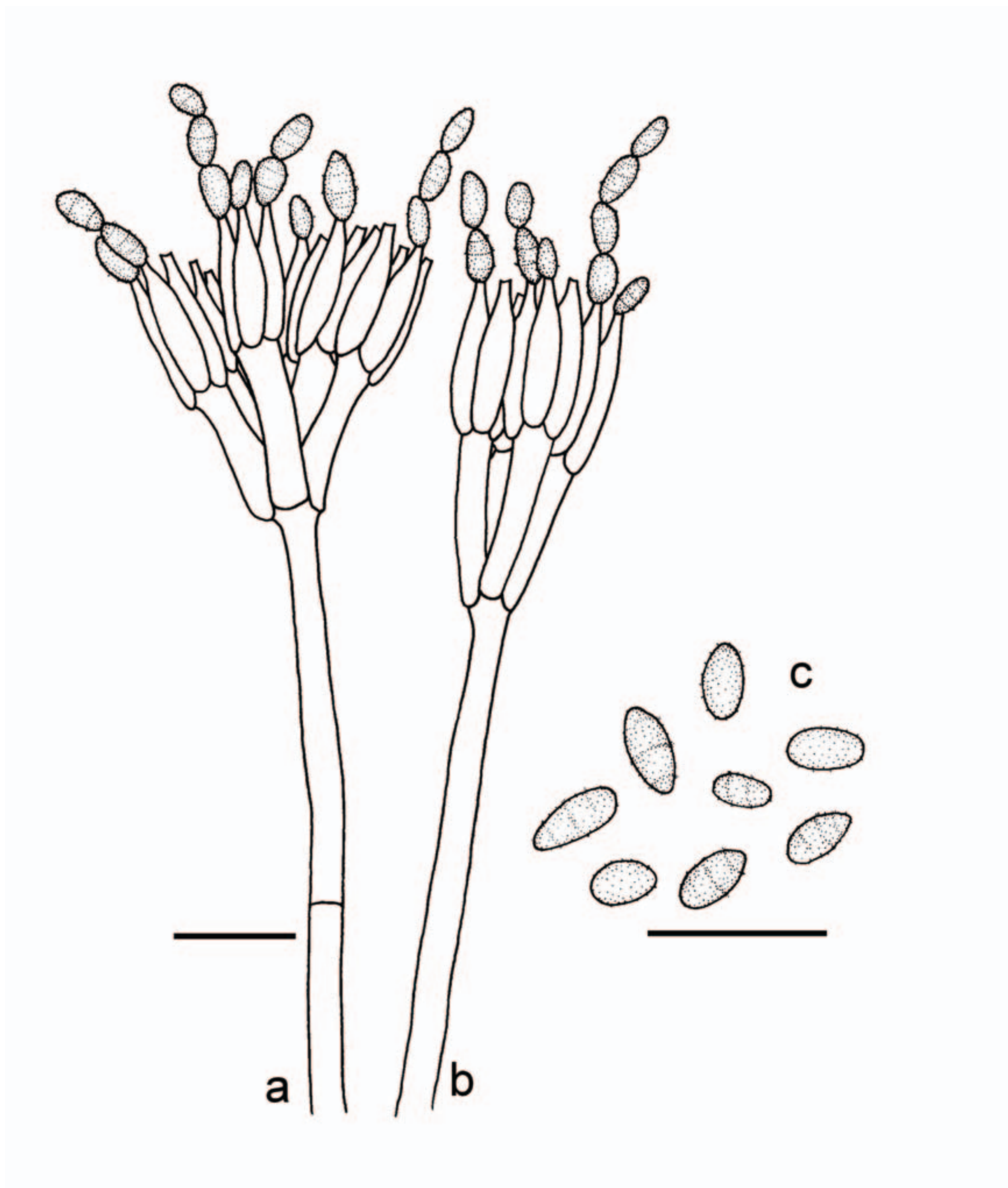

FIGURE 5. *Penicillium ptychoconidium* line drawings from holotype (PREM60041) material. a, b. Conidiophores (bar = 10μm). c. Conidia (bar = 10μm).

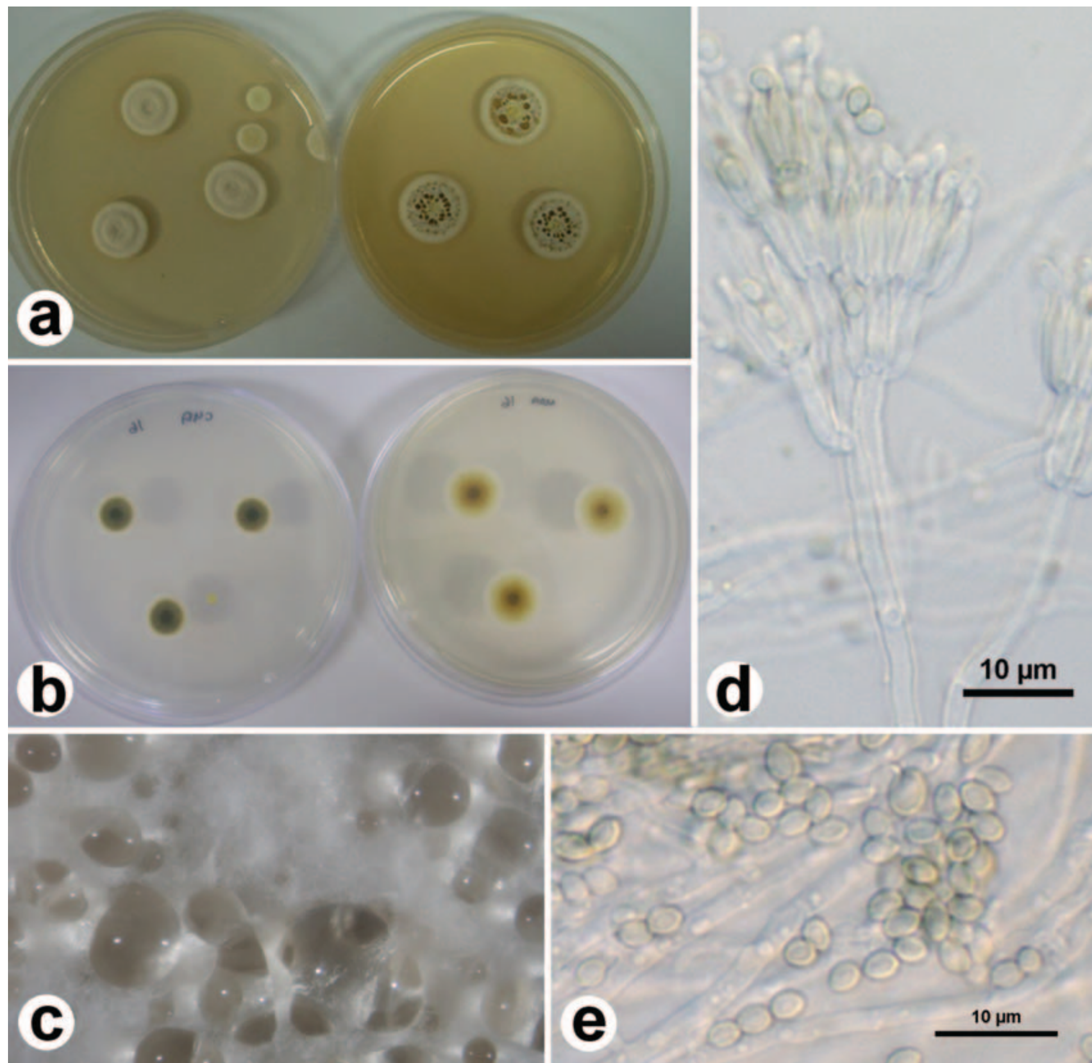

FIGURE 6. Most important taxonomic characters distinguishing *P. occultum*, holotype (PREM60039), from closely related species. a. Colonies produced by *P. occultum*, on CYA and MEA after 7 days. b. Characteristic olive-brown reverse coloration on both CYA and MEA. c. Clear exudate and sterile aerial hyphae dominating colony appearance on CYA. d. Biverticillate to terverticillate conidiophores produced by *P. occultum*. e. Ellipsoidal, smooth-walled conidia.

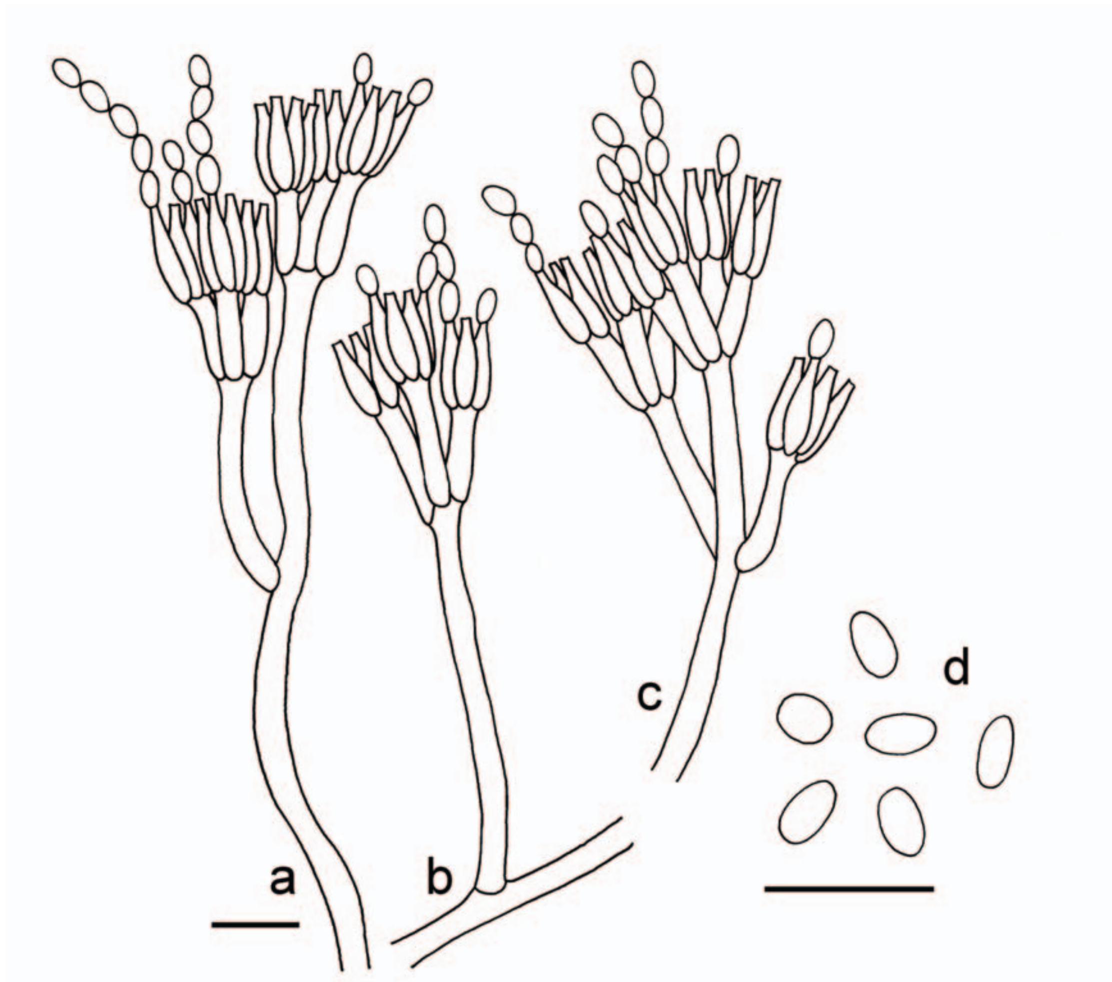

FIGURE 7. *Penicillium occultum* line drawings from holotype (PREM60039) material. a, b, c. Conidiophores (bar = 10μm). d. Conidia (bar = 10μm).

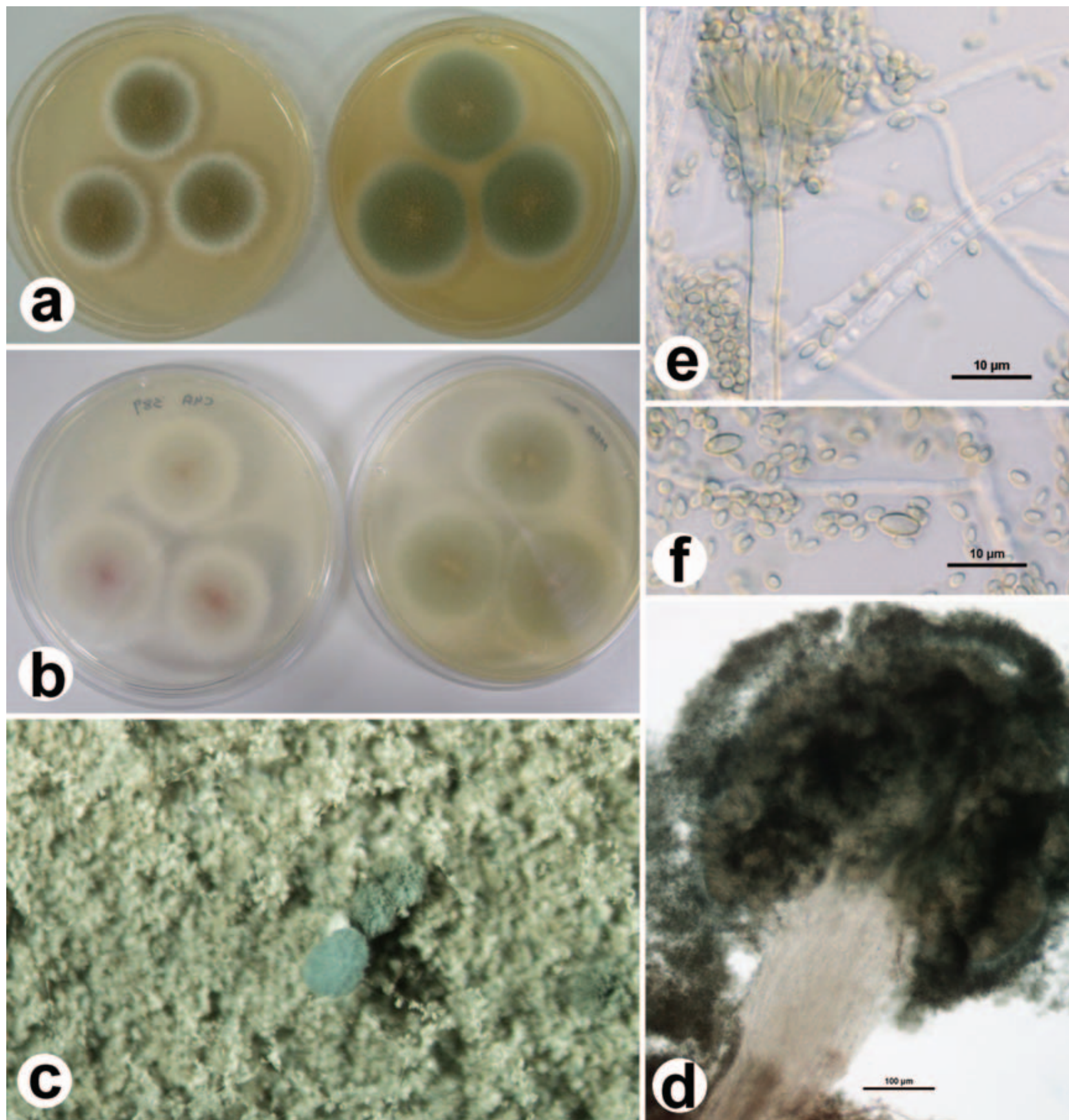

FIGURE 8. Most important taxonomic characters of *P. chloroloma*, holotype (PREM60033), distinguishing it from closely related species. a. Colonies of *P. chloroloma* incubated on CYA (left) and MEA (right) for 7 days. b. Reverse colonies showing pinkish reverse on CYA (left). c, d. Synnema produced on CYA after prolonged incubation. e. Conidiophore produced in culture. f. Ellipsoidal, smooth-walled conidia.

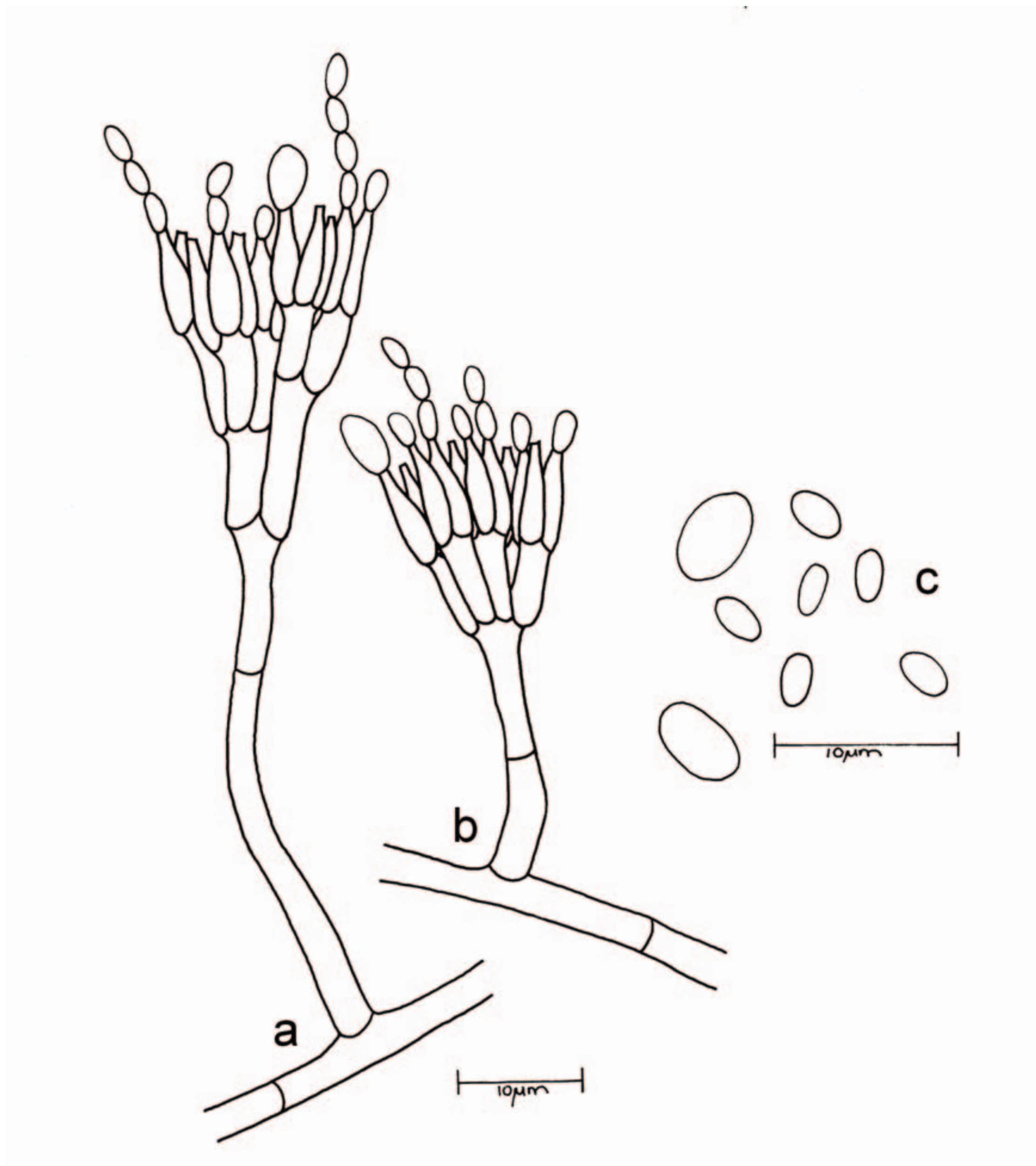

FIGURE 9. *Penicillium chloroloma* line drawings from holotype (PREM60033) material. a, b. Conidiophores (bar = 10μm). c. Conidia (bar = 10μm).

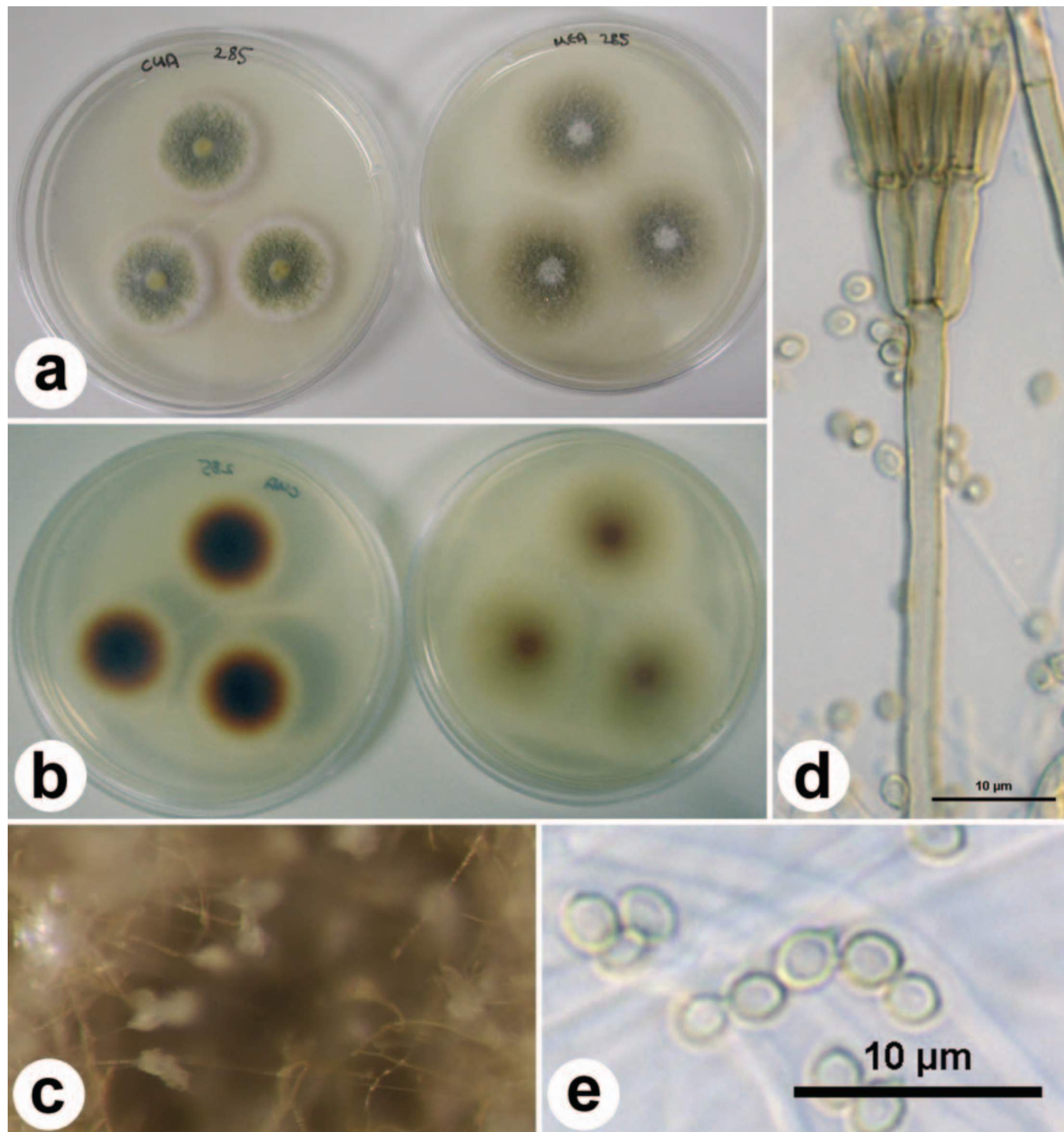

FIGURE 10. Most important taxonomic characters of *P. minioluteum sensu* Pitt (CV285) distinguishing it from closely related species. a. Colonies of *P. minioluteum* incubated on CYA (left) and MEA (right) for 7 days. b. Reverse colonies showing dark red coloration on CYA and MEA. c. Yellow mycelia produced by colonies. d. Biverticillate, appressed conidiophore typically produced in culture. e. Subspheroid to ellipsoid, smooth-walled conidia.

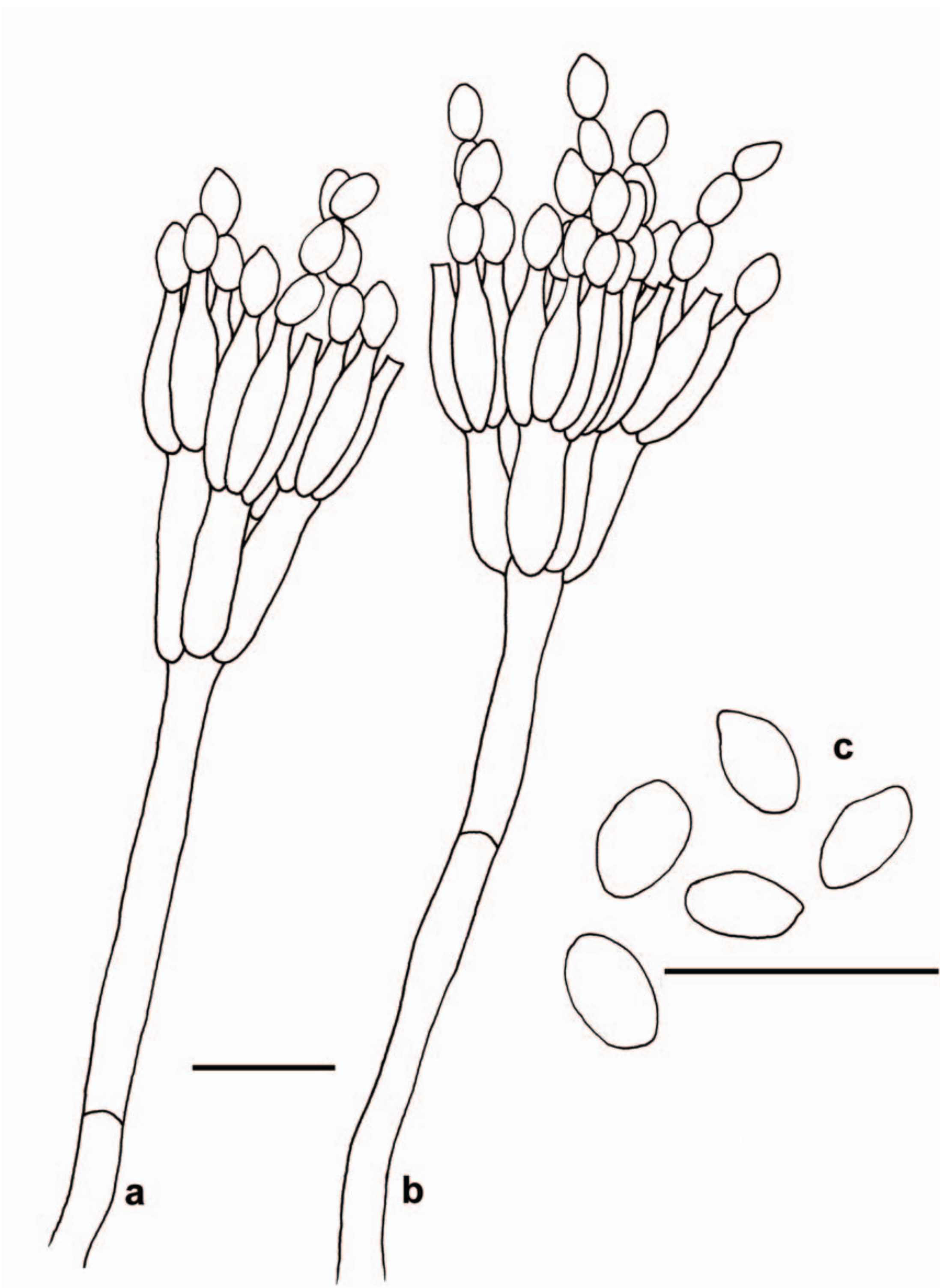

FIGURE 11. *Penicillium minioluteum sensu* Pitt line drawings from strain CV284. a, b. Conidiophores (bar = 10μm). c. Conidia (bar = 10μm).

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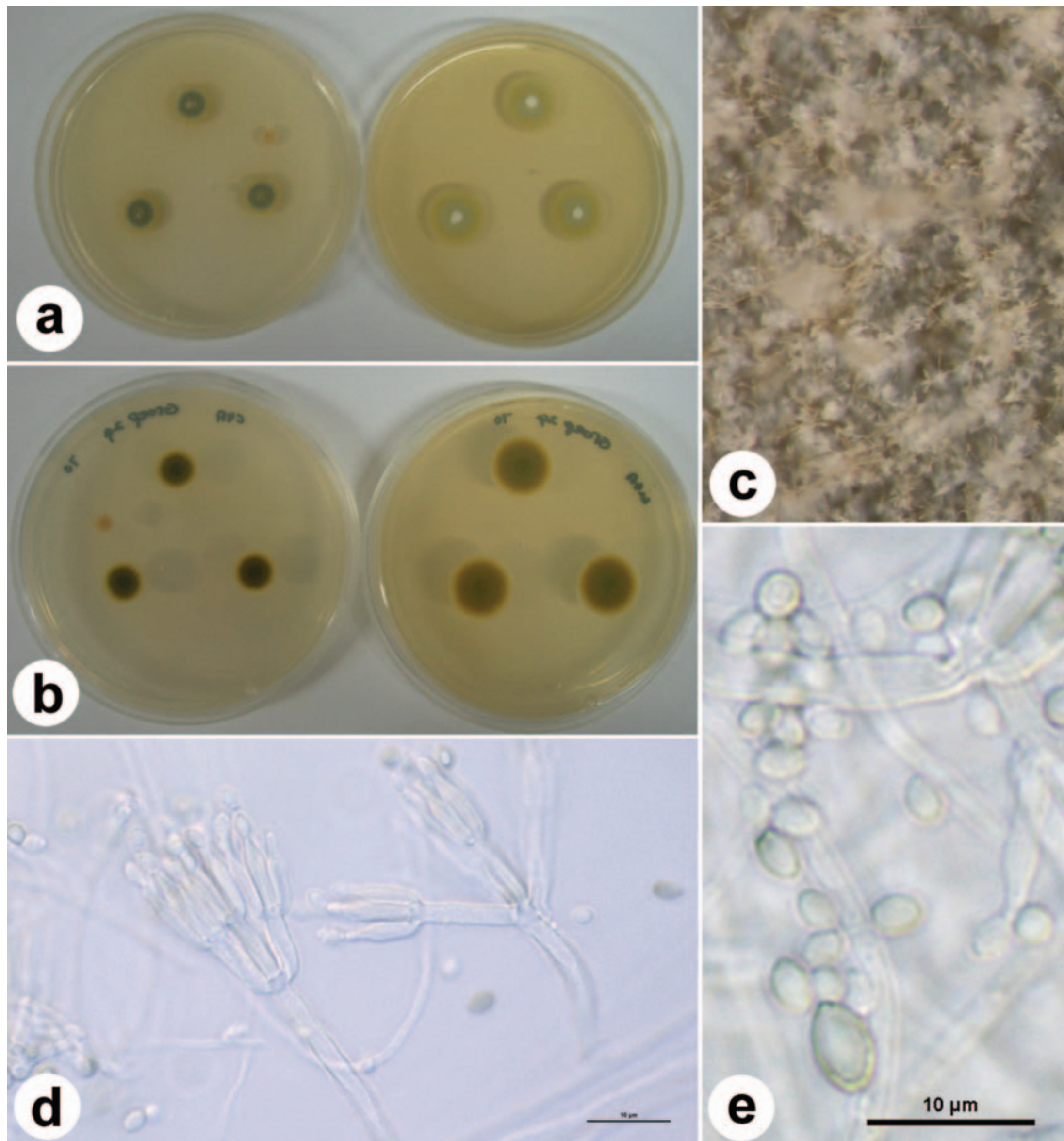

FIGURE 12. Most important taxonomic characters of *P. rugulosum* (CV89) distinguishing it from closely related species. a. Colonies of *P. rugulosum* incubated on CYA (left) and MEA (right) for 7 days. b. Reverse colonies showing olive coloration. c. Yellow mycelia produced on MEA. d. Conidiophores produced in culture, showing its either appressed or divergent character. e. Ellipsoidal, smooth-walled conidia, with enlarged conidia sometimes present.

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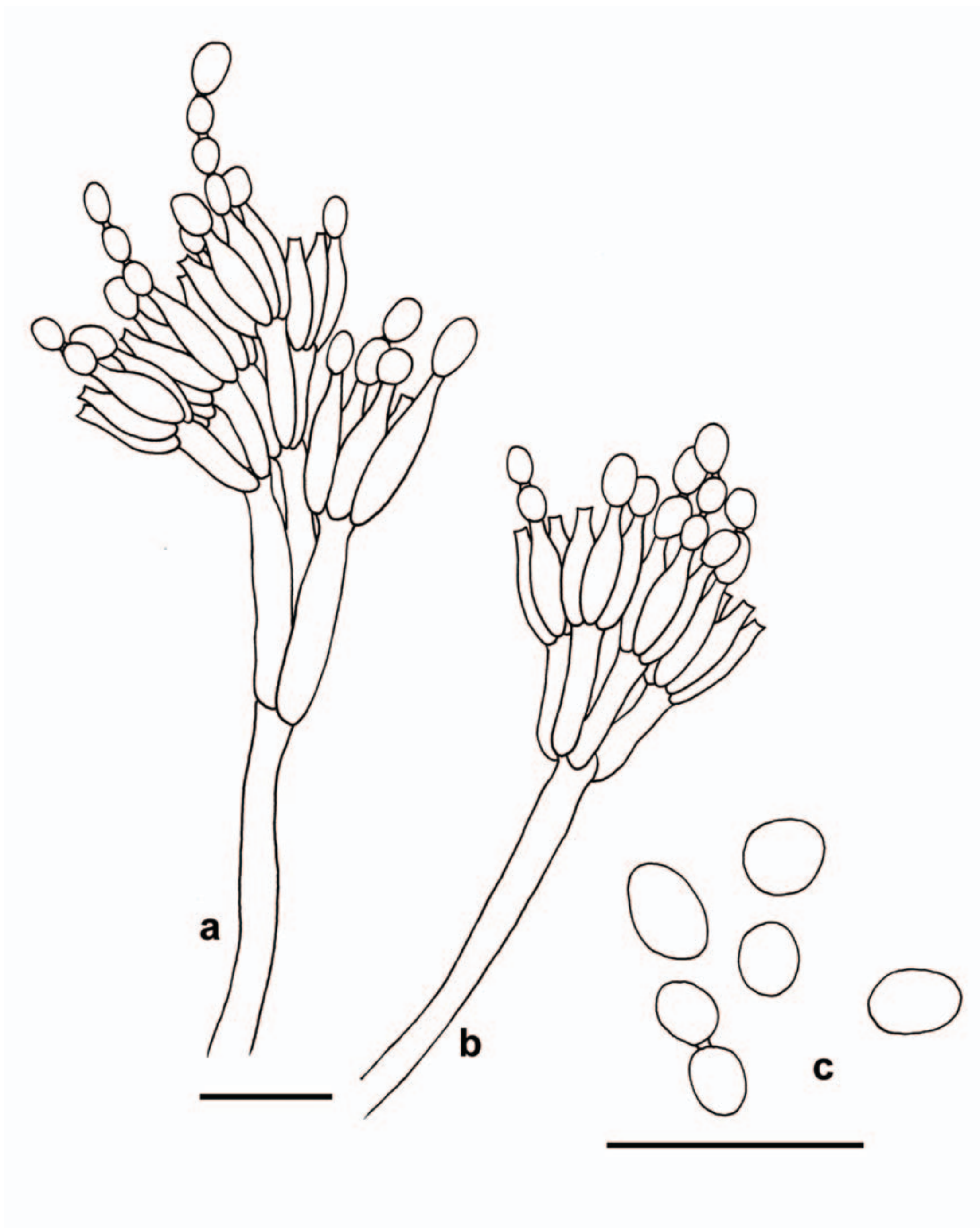

FIGURE 13. *Penicillium rugulosum* line drawings from strain CV89. a, b. Conidiophores (bar = 10μm). c. Conidia (bar = 10μm).

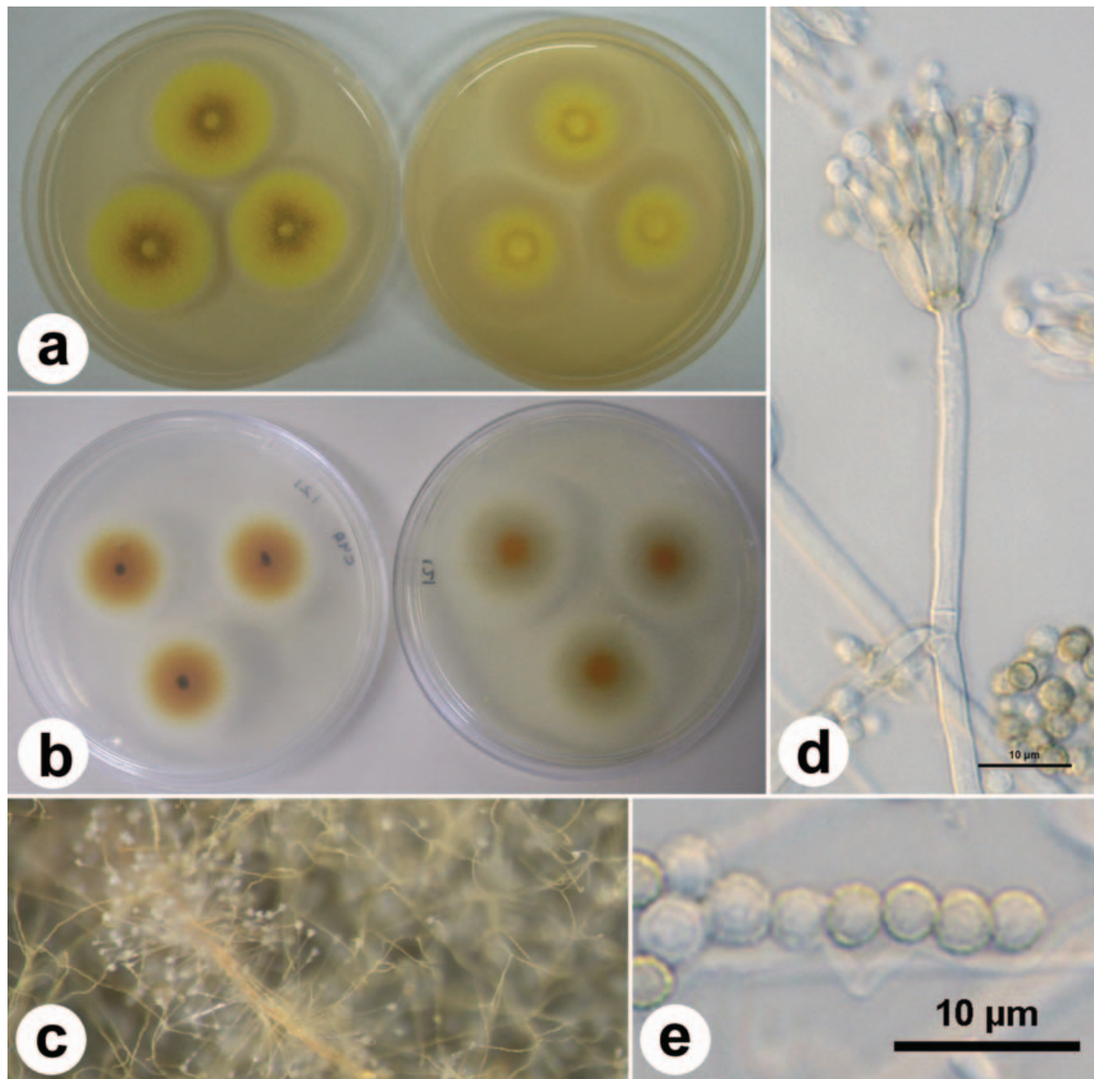

FIGURE 14. Most important taxonomic characters of *P. verruculosum* (CV121) distinguishing it from closely related species. a. Colonies of *P. verruculosum* incubated on CYA (left) and MEA (right) for 7 days. b. Reverse colonies showing orange brown coloration. c. Yellow mycelia, with conidiophores oftern borne on funicles. d. Biverticillate conidiophore produced in culture. e. Spheroidal, heavy rough-walled conidia.

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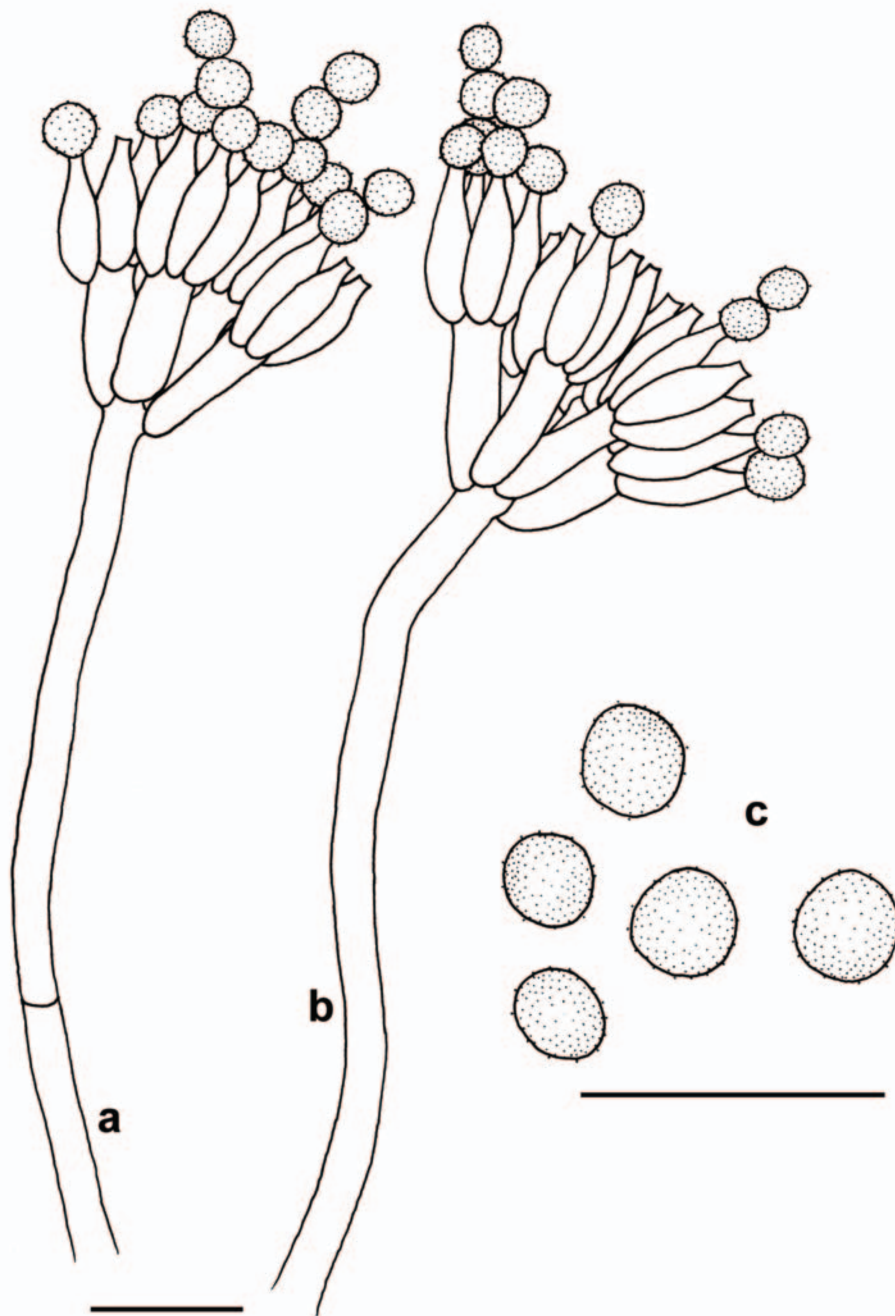

FIGURE 15. *Penicillium verruculosum* line drawings from strain CV121. a, b. Conidiophores (bar = 10μm). c. Conidia (bar = 10μm).

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Chapter 4
Penicillium subgenus *Furcatum* and subgenus *Penicillium*, isolated from coastal fynbos soil, including two new species

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ABSTRACT

As part of an ongoing survey on the biodiversity of *Penicillium* spp. in soil from the Cape Floristic Region in South Africa, several species of *Penicillium* subgenus *Furcatum* were isolated and identified. These include *Penicillium canescens, P. citrinum*, *P. corylophilum*, *P. janczewskii* and *P. melinii*. Amongst these strains were two groups that could not be positively identified using current taxonomic keys. The first of these is phylogenetically related to *Eupenicillium terrenum* and *P. raciborskii*, but differs in colony morphology, growth rates and micromorphology. The second group is phylogenetically closely related to *P. corylophilum*, but can be differentiated based on colony morphology and penicillus ornamentation. Based on molecular and morphological differences, these strains were described as *Penicillium subturcoseum* prov. nom and *P. hemitrachum* prov. nom., respectively.

INTRODUCTION

Pitt (1979) erected subgenus *Furcatum* as an intermediate between the subgenera *Aspergilloides* and *Penicillium.* It served to accommodate all species that cannot be placed in one of the other three subgenera (Pitt 1979). This group, thus, consists of species producing both biverticillate and monoverticillate conidiophores, with ampulliform phialides.

The current classification scheme, however, makes placement and identification of species in this group very difficult (Frisvad and Filtenborg 1990a). Morphologically, species in subgenus *Furcatum* are very similar and are often separated from one another based on minor differences in cultural or microscopic features. In addition, although there are obvious taxonomic problems in this group, with studies focusing on them limited. This may be attributed to the fact that this group mainly consist of soil-borne species (Raper and Thom 1949, Pitt 1979). They are generally saprophytes in this habitat and economically less important than the terverticillate species. This led to the general neglect of the group by taxonomists.

A limited number of studies have been done to investigate some of the synonymies and species complexes within this subgenus (Fassatiová and Kubatová 1990, Frisvad and Filtenborg 1990a, Christensen 1999). Fassatiová and Kubatová (1990) clearly delineated the species *P. simplicissimum, P. janczewskii, P. daleae, P. canescens* and *P. melinii* in section *Divaricatum* and highlighted the most important diagnostic features of these species. Similar to previous studies (Frisvad and Filtenborg 1983, 1989, 1990b), Frisvad and Filtenborg (1990a), used secondary metabolites for their revision of the group and showed that species produce profiles helpful for species delineation. Christensen et al. (1999) revised the *P. miczynskii*-complex and other sclerotia-producing species by using morphological and secondary metabolites, publishing this in a synoptic key. These studies, although important, form only the base from which to start unraveling the complexities within the subgenus. With focus starting to shift to the conservation of biodiversity, more ecological studies are being done, which in turn would uncover more strains and species belonging to

this group. The need for resolving species within this group should, therefore, be considered to be very important.

Previous microfungal surveys have shown that the soils from the Cape Floristic Region, South Africa, contain a large number of *Penicillium* species, many of these unique to the area (Allsopp et al. 1987, Visagie et al. 2008, Visagie and Jacobs 2008). This particular study focus on species belonging to *Penicillium* subgenus *Furcatum* occurring in these diverse fynbos soils. The aim of this study was, therefore, to compare isolates and identify species from *Penicillium* subgenus *Furcatum* in fynbos soil, based on morphological and phylogenetic characters. A dichotomous key are also provided for these species and their closest relatives.

MATERIALS AND METHODS

Isolations — Strains used in this study were collected from soil sampled at four sites, Camphill Village (S 33,59787°; E 18,56433°), Kalbaskraal (S 33,57061°; E 18,62861°), Pella (S 33,51022°; E 18,55236°) and Riverlands (S 33, 49066°; E 18, 58388°), which are situated in the coastal fynbos region, Western Cape, South Africa. Random sampling were done at these sites and a subsequent dilution series of each soil sample prepared by adding 5 g soil to 100 ml dH_2O and diluting this to 10^{-2}. These dilutions were plated onto Potato Dextrose agar (Biolab), containing 50ppm Streptomycin (Applichem, South Africa) and 100ppm Chloramphenicol (Applichem, South Africa), and plates incubated at 25°C. After 6–7 days colonies showing *Penicillium*-like characters were transferred to Oatmeal agar (OA), incubated at 25°C for 7 days from which single spore cultures were prepared.

Morphology — Spore suspensions of strains, prepared in a semi-solid agar (0.2%) and Tween80 (0.05%) solution (Pitt 1979), were used for three point inoculations on Czapek Yeast Agar (CYA), Malt Extract Agar (MEA: after Blakeslee, 1915) and 25% Glycerol Nitrate Agar (G25N). The inoculated polystyrene Petri dishes (90mm), containing 20 ml of media, were incubated at 25°C (CYA, MEA, G25N), 5°C (CYA) and 37°C (CYA) (Pitt 1979, Samson and Pitt

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1985), in the dark with plates left unwrapped to allow for sufficient aeration (Okuda et al. 2000). Characterization and species descriptions followed the methods as described by Pitt (1979), Samson and Pitt (1985) and Okuda et al. (2000). Capitalized color names and codes refer to the "Methuen handbook of colour" (Kornerup and Wanscher 1966)

Phylogenetic analysis — DNA extractions were done from strains grown on MEA for 7 days using a modified Möller et al. (1992) technique. The subsequent PCR and cycle sequencing of the ITS1–5.8S–ITS2 rDNA region, using primers ITS1 and ITS4 (White et al. 1990) were done using the methods described by Visagie and Jacobs (2008). An ITS dataset were constructed using the sequences from the strains isolated from fynbos soil and compared to other species from this group, mainly done by Peterson (2000). Sequence accession numbers to species used in the analysis are represented in TABLE 1. The dataset was aligned in ClustalX (Thompson et al. 1997), with manual adjustments to alignments done in Se-Al (Rambaut 2007). Ambiguously aligned regions are characterized by having numerous inserted gaps, which results in branch lengths of phylogenetic trees being superficial. This is prevented by replacing these ambiguous regions with codes, by computing step matrices to assign different weights to these codes using INAASE 2.3b (Lutzoni et al. 2000). Sequence analysis was done in PAUP* v4.0b10 (Swofford 2000) with gaps treated as missing data. The neighbour-joining option was used to calculate a single tree for the dataset, with *Talaromyces trachyspermus* chosen as a suitable outgroup. Confidence levels in the nodes were determined by a bootstrap analysis using a 1000 replicates. Alignments used for phylogenetic comparisons can be obtained from the compact disc attached to the back of this thesis.

RESULTS

The isolations from coastal fynbos soil resulted in 434 *Penicillium* strains, which were placed into 24 distinct morphological groups. Seven of these groups, representing 186 isolates in total, showed morphological characters distinctive of species belonging to *Penicillium* subgenus *Furcatum* and one which belong to *Penicillium* subgenus *Penicillium*. Species were identified as *Penicillium*

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canescens, P. citrinum, P. corylophilum, P. melinii, *P. janczewskii,* and *P. expansum,* respectively. Strains identified as *P. citrinum,* produced a yellowish-orange exudate on CYA, together with the terminal verticil of 3–5 metulae of uniform length, characteristic of the species (Pitt 1979). The strains were resolved in a clade (FIG. 1) with *P. citrinum, P. westlingii* and *P. sartoryi*. The latter species is a synonym of *P. citrinum*, but *P. westlingii* is considered to be a distinct species (Pitt et al. 2000). The fynbos strains did, however, fit the description of *P. citrinum* and were identified as such. Colonies of *P. expansum* was typically coremial and very deep, but with regards to conidial color on CYA and MEA were generally more olive, with only one strain (CV432) displaying the dull green color as described by Pitt (1979) and Frisvad and Samson (2004). ITS and β-tubulin (not published) sequences, however, was able to confirm the strains' identity as *P. expansum*.

Penicillium corylophilum colonies were typically centrally depressed on CYA, and had a brownish conidial color at centre of colonies. Its unmistakable identity could be confirmed by the smooth-walled conidiophores having a terminal verticil of metulae of unequal lengths, typical of the species (Pitt 1979). The fynbos strains identified as *P. melinii*, showed colonies typical of the species, but did vary in its micromorphology. Contrary to descriptions by Pitt (1979) and Fassatiová and Kubatová (1990), the fynbos strains generally showed more monoverticillate, with very short stipes, than biverticillate conidiophores. Pitt (1979) does make mention of these short conidiophores being present amongst the commonly occurring biverticillate conidiophores. Other characters, however, conform to descriptions for *P. melinii,* with its identity confirmed by the ITS phylogeny. Two groups of strains showed unique morphological characters which did not conform to any previously described species.

The aligned ITS dataset was 512 bp long, with seven ambiguously aligned regions which were replaced by weighted codes. The phylogeny confirmed the morphological identifications, except for species from the *Penicillium canescens* clade (FIG. 1). Sequenced strains from the two morphologically unique groups resolved into separate clades, according to their respective morphological

characters. Based on the morphological and phylogenetic data, these were described as follow:

TAXONOMY

Penicillium hemitrachum C.M. Visagie & K. Jacobs prov. nom.[*] **FIGS 2, 3**

Mycobank nr. MB 512437

Etymology. Latin, *hemitrachum*: Refers to half of the branches being rough-walled.

Coloniae in CYA post 7 dies in 25°C 54–56 mm pigmento dissolubili virescente-vel toxico-flavidae. Coloniae in MEA post 7 dies in 25°C 51–60 mm. Conidiophorae biverticillatae, in superficie portatae; stipa laevis vel interdum cellulis singulis subtiliter exasperatis, (80–)100–300 × 2.5–3 µm; metulae in verticillo terminali divergente, verticillo uno cum metulis laevis et parietibus asperis interdum subtiliter exasperatis (9–)10–12 × 3–4 µm; phialides ampulliformae (5–)6–7 × 2–3 µm; conidia sphaeroidea laevia vel subtiliter exasperata 2.5–3.5 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 54–56 mm diam, sulcate, moderately dense; texture floccose at centre of colonies and velutinous elsewhere; margins low, irregular, mycelia white; conidiogenesis dense, Beige (4c3) becoming gray green (25b3–4); exudate yellow, soluble pigment Greenish to Poison Yellow (1a8), reverse Rust Brown (6e8) to brown (6e8–6E6). At 5°C, 7 days: Germination occurring; 37°C, 7 days: Colonies 11–12 mm diam, plane; white mycelia; conidiogenesis sparse to absent, grayish green; soluble pigment and exudate absent, reverse yellow orange to khaki. MEA, 25°C, 7 days: Colonies 51–60 mm diam, plane, moderately dense; texture floccose at centre and velutinous elsewhere; margins subsurface, irregular, mycelia white; conidiogenesis moderate, grayish green (26d5–6); exudate absent, soluble pigment pale yellow (2a3), reverse yellowish green (30b7–8). G25N, 25°C, 7 days: Colonies 16–18 mm diam, plane; mycelia white; conidiogenesis moderate, grayish green at margins, orange white (6a2) elsewhere; exudate and soluble

[*] Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere.

pigment absent, reverse yellowish white (4a2) at margins and Spinach Green (29e6) at centre.

Conidiophores borne from surface, pigmented in some shade of brown, *stipes* smooth-walled, sometimes single cells finely rough-walled, (80–)100–300 × 2.5–3 μm, bearing terminal biverticillate penicilli; *metulae* in whorls of 2–4, verticil having finely roughened and smooth walls, divergent, (9–)10–12 × 3–4 μm; *phialides* 4–6 per metula, closely appressed, ampulliform, (5–)6–7 × 2–3 μm; *conidia* spheroid 2.5–3.5 μm, smooth to finely rough-walled.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type culture CV219 (PREM60048) (HOLOTYPE); *Additional specimens examined:* South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV207 (PREM60049).

Penicillium subturcoseum C.M. Visagie and K. Jacobs, prov. nom.* **FIGS 4, 5**

Mycobank nr. MB 512438

Etymology. Latin, *subturcoseum*: *sub-* = below; *turcoseus* = turquoise, referring to colonies on CYA having a dark turquoise reverse coloration.

Coloniae in CYA post 7 dies in 25°C 29–39 mm infra atroturcosae. Coloniae in MEA post 7 dies in 25°C 34–43 mm, infra interdum atrovirides. Conidiophorae plerumque biverticillatae, rarius univerticillatae; stipa parietibus asperis, interdum subtuberculata, 250–350 × 2.5–3.5 μm; metulae in verticillo terminali divergente, vesiculatae parietibus asperis 12–15(–18) × 2.5–3.5 μm; phialides ampulliformae 6.5–8.5 × 2.5–3.5 μm; conidia sphaeroidea parietibus asperis 2.5–3.0 μm.

Colony morphology, CYA, 25°C, 7 days: Colonies 29–39 mm diam, sulcate, dense; texture velutinous, sometimes floccose; margins moderately wide, low, mycelia white; conidiogenesis moderate, grayish green (25e6); exudate and soluble

* Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere.

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pigment mostly absent, some isolates producing a pinkish soluble pigment, reverse coloration Dark Turquoise (24f7) to almost blue, but less pronounced in some isolates. At 5°C, 7 days: Microcolony; 37°C, 7 days: Microcolony. MEA, 25°C, 7 days: Colonies 34–43 mm diam, plane, moderately dense; texture velutinous; margins moderately wide, low, mycelia white; conidiogenesis heavy, grayish green (25e5) to dark green (25f8); exudate and soluble pigment absent, reverse coloration greenish gray (29b2), sometimes dark green (25f8). G25N, 25°C, 7 days: Colonies 18–22 mm diam, plane, velutinous; margins moderately wide, mycelia white; conidiogenesis similar to that on CYA; exudate and soluble pigment absent, reverse Pastel Green (27a4) to Light Green (27a5).

Conidiophores borne from surface, *stipes* rough-walled, sometimes almost tuberculate 250–350 × 2.5–3.5 μm, bearing terminal biverticillate, with monoverticillate penicilli present; *metulae* vesiculate, in whorls of mostly 3–4, but sometimes 2, divergent, metulae often having unequal lengths 12–15(–18) × 2.5–3.5 μm; *phialides* 10–12 per metula, appressed, ampulliform 6.5–8.5 × 2.5–3.5 μm; *conidia* spheroid 2.5–3.0 μm, rough-walled, borne in short columns.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Camphill Village: (S 33,59878°; E 18,56433°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type culture CV110 (PREM60050) (HOLOTYPE); *Additional specimens examined:* South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°), Camphill Village: (S 33,59878°; E 18,56433°), Kalbaskraal: (S 33,57061°; E 18,62861°), Pella: (S 33,51022°; E 18,55236°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV11, 15 (PREM60052), 18 (PREM60053), 20, 24, 26, 27, 32, 41, 42, 47, 48, 51 (PREM60051), 54, 58, 64, 66, 77, 81, 84, 90, 97, 100, 102, 159, 192, 210, 234, 239, 243, 251, 252, 258, 260, 283, 287, 332, 342, 384, 391, 395, 422.

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Key to fynbos species and their close relatives in *Penicillium* subgenus *Furcatum*

1. Penicillus mainly produced as terminal verticil.......................................**2**
1. Penicillus irregular...**8**

2. Metulae appressed...**3**
2. Metulae divergent...**4**

3. Colonies on MEA >20 mm; black sclerotia produced
 on both CYA and MEA; stipe rough-walled..............................***P. novae-zeelandiae***
3. Colonies on MEA <20 mm; smooth-walled stipes ....***P. madriti***

4. Colonies on MEA >50 mm...**5**
4. Colonies on MEA <50 mm...**6**

5. Colonies on CYA >50 mm; phialides 5–7 μm;
 conidia 2.5–3.5 μm..***P. hemitrachum***
5. Colonies on CYA <50 mm; phialides 7–10 μm;
 conidia 2–2.5 μm…..***Eup. terrenum***

6. Stipes and conidia rough-walled; CYA colony reverse
 commonly dark turquoise to blue...***P. subturcoseum***
6. Stipes and conidia smooth-walled; CYA colony reverse not blue.............................**7**

7. Colonies on MEA >25 mm; metulae of equal length..................................***P. citrinum***
7. Colonies on MEA <25 mm; metulae of unequal length..................***P. corylophilum***

8. Stipes rough-walled...**9**
8. Stipes smooth-walled...**10**

9. Conidia spinose...***P. melinii***
9. Conidia smooth-walled...***P. canescens***

10. Conidia spinose >2.5 μm..***P. janczewskii***
10. Conidia smooth <2.5 μm..***P. raciborskii***

DISCUSSION

Penicillium subgenus *Furcatum* is characterized by species with biverticillate and a few monoverticillate conidiophores, producing ampulliform phialides (Pitt 1979). Allsopp et al. (1987) in a microfungal survey, isolated 13 *Penicillium* species occurring in the root systems and surrounding soils of the fynbos region, only reporting species which occurred in more than ten environmental samples. Six of these, *P. novae-zeelandiae, P. raistrickii, P. janczewskii, P. melinii, P. citrinum* and *P. miczynskii*, belongs to subgenus *Furcatum*. In the present study, *P. citrinum* (FIGS 6, 7)*, P. janczewskii* (FIGS 8, 9) and *P. melinii* (FIGS 10, 11) were again recovered from the soil. Although the other three species isolated by

Allsopp et al. (1987) were not found during our survey, we did additionally isolate *P. canescens* (FIGS 12, 13)*, P. corylophilum* (FIGS 14, 15) and *P. expansum* (FIGS 16, 17), the latter the only species representing subgenus *Penicillium* in our study thus far.

Penicillium canescens, together with *P. janczewskii* were the most commonly isolated species during the current study and we might, therefore, consider that it is most probable that Allsopp et al. (1987), by mistake, might have placed *P. canescens* strains in his *P. janczewskii* group, since they produce similar colony morphologies and is only distinguishable from each other based on the ornamentation of their conidiophores. *Penicillium corylophilum,* and *P. expansum* were found to be confined to specific soil samples and their presence in these soils could, therefore, not be compared to that of the Allsopp et al. (1987) study.

Two previously undescribed species in this group were also isolated. *Penicillium hemitrachum* typically produces rapidly growing colonies and biverticillate conidiophores that have a brownish pigmentation, with metulae and conidia that can be rough or smooth-walled. Interestingly, the new species often produces intermixed rough or smooth-walled metulae on a single conidiophore. Morphologically the new species is most similar to *P. madriti* and *P. raistrickii,* but is distinguished from both based on its faster growing colonies on CYA and MEA. Phylogenetically, however, *P. hemitrachum* have *Eupenicillium terrenum* (stat. anam. *P. terrenum*) and *P. raciborski* as its sister taxa. Once again, its faster growing colonies on CYA and MEA easily distinguish it from its close relatives. In addition to the faster growing colonies, *P. hemitrachum* have shorter phialides and metulae, with bigger conidia produced than *Eup. terrenum* [Pitt 1979: metulae 10–15 (–20) μm, phialides 7–8 (–10) μm, conidia 2–2.5 μm].

Penicillium subturcoseum are distinguished by its colonies on CYA having a dark turquoise reverse color, together with its characteristic heavily rough-walled conidiophores. Based on morphological characters, *P. subturcoseum* are closely related to *P. corylophilum,* which was confirmed by the ITS phylogeny. The ITS region from both these species are very similar and bootstrap support is low for

separating the two. Morphologically they share similar colony characters, as well as their metulae being of unequal lengths. The strongly rough-walled stipes and conidia, together with the dark turquoise CYA colony reverse does, however, distinguish *P. subturcoseum* from *P. corylophilum*, which has smooth-walled conidiophores and a pale, brown or dark gray CYA colony reverse. *Penicillium corylophilum* has also been isolated from fynbos soil and can clearly be separated from *P. subturcoseum,* based on morphology.

From the ITS phylogeny it is clear that this gene region was not able to resolve all species used for the study, as is also evident from Peterson (2000). There were no support for separation of species within the *P. corylophilum*-, *P. melinii*- and *P. canescens* complexes, indicated on FIG. 1 as A, B and C, respectively. This is more pronounced in the *P. canescens* clade containing *P. jensenii* and *P. janczewskii*, which has identical ITS gene regions, but are morphologically distinct. Pitt (1979) made mention of the close relationship between *P. janczewskii* and *P. canescens*. These two are separated from each other based on rough-walled stipes of *P. canescens* and generally smooth-walled conidia compared to *P. janczewskii* having smooth-walled stipes and rough-walled conidia (Pitt 1979, Fassatiová and Kubatová 1990). Pitt (1979) also makes mention of the existence of morphotypes intermediate between these two species, specifically looking at two strains, *P. janczewskii* (IMI149218) and *P. canescens* (FRR97). Identification of both may, therefore, often be problematic and subject to the investigator's interpretation of characters. This was particularly evident from one of the taxa isolated from the fynbos soil. The strains showed no morphological differences from the descriptions provided by Pitt (1979) and Fassatiová and Kubatová (1990) for *P. janczewskii* and, therefore, were tentatively identified as such. Although there was good bootstrap support for the separation from the main clade, the one available ITS sequence for *P. janczewskii* (AY157487, Weber et al. 2003) was not of an ex-type strain, as was the case for many of the other sequences (Peterson 2000) used during our study. This clade is, therefore, currently problematic and as such, additional sequencing of genes such as β-tubulin or calmodulin, of the type-strains would prove valuable in the identification of these more commonly found soil species.

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The other mentioned clades (A, B), although having low bootstrap support, currently does not have the problems as seen for the *P. canescens* clade, since the groups suggested by the ITS phylogeny are supported by good morphological data. With taxonomists becoming more pressurized to provide an easier method of identification, as is proposed by DNA barcoding (Blaxter 2003, Hebert et al. 2003, Min and Hickey 2007, Seifert et al. 2007), it is clear that genes giving a better species resolution would be needed. β-tubulin have been successfully used to resolve most species in subgenus *Penicillium* (Samson et al. 2004) Studies on this are currently limited, but calmodulin at the moment seems like an attractive option, based on the Wang and Zhuang (2007) study, being able to resolve closely related species.

This particular study forms part of a bigger survey, exploring the biodiversity of *Penicillium* spp. in the Cape Floristic Region. Previous papers listed eight species belonging to subgenus *Biverticillium*, five of which were newly described (Visagie et al. 2008, Visagie and Jacobs 2008). Looking at representatives from both groups, subgenus *Biverticillium* were isolated in low numbers and were often confined to specific soil sampling sites, whereas species from subgenus *Furcatum* were isolated from almost all the soil samples. This might, therefore, suggest that species from the latter group are actively growing in the soil as its principle habitat, as was suggested by Raper and Thom (1949) and Pitt (1979), whereas species from subgenus *Biverticillium* might not have soil as its only habitat. This certainly seems to be the case for at least *P. ramulosum*, that was isolated during two other independent surveys from *Protea burchellii* infructescences in the Western Cape fynbos region and from moth-damaged Riesling grapes in Ontario, Canada (Visagie et al. 2008). The abundance of species from *Penicillium* subgenus *Furcatum* also suggest that they play a very important ecological role in the soil and since the major function of *Penicillium* is that of decomposition (Thom 1930, Raper and Thom 1949, Pitt 1979), these species might well be prolific enzyme producers important to biotechnology, as is the case for *P. janczewskii* (Weber et al. 2003), *P. janthinellum* (Adsul et al. 2007), *P. oxalicum* (Li et al. 2007) and other species (Law 2002).

Species of *Penicillium* subgenus *Furcatum* have often been neglected in the past. Their shear abundance in soil and the biotechnological potential of these species surely would validate a taxonomic revision of this group, using both morphology and multigene phylogenies. This would greatly aid in the identification of these species, something that has been problematic in the past.

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TABLE 1. Species names and their respective culture collection and GenBank accession numbers used for phylogenetic comparisons.

Species	Culture collection number	GenBank number
Eup. terrenum	NRRL5824	AF033446
Eup. tropicum	SC42-1	AY232277
	SS133-17	AY232280
P. atrovenetum	NRRL2571	AF033492
P. canescens	CV13	FJ231000
	CV135	FJ230999
	CV194	FJ231001
	NRRL910	AF033493
P. citrinum	CV10	FJ230991
	CV14	FJ230992
	NRRL1841	AF033422
P. coralligerum	IBT17894	AJ010484
P. corylophilum	CV298	FJ230998
	CV300	FJ230997
	CV304	FJ230996
	NRRL802	AF033450
P. expansum	ATCC7861	AY373912
	CV407	FJ230989
	CV432	FJ230990
	KRCF699	AB298711
P. fagi	NRRL28201	AF481124
P. hemitrachum	CV207	FJ231002
	CV219	FJ231003
P. jameslonlandense	IBT22005	DQ267911
	IBT24411	DQ267912
P. janczewskii	CBS221.28	AY157487
	CV164	FJ230987
	CV433	FJ230988
P. jensenii	NRRL909	AY443470
P. madriti	NRRL3452	AF033482
P. megasporum	NRRL2232	AF033494
P. melinii	CV221	FJ230994
	CV29	FJ230993
	CV320	FJ230995
	FRR2041	AY373923
	NRRL2041	AF033449
P. novae-zeelandiae	NRRL35618	EF200078
P. raciborskii	NRRL2150	AF033447
P. raistrickii	NRRL2039	AF033491
P. ribeum	IBT16537	DQ267916
	IBT18924	DQ267917
P. sartoryi	NRRL783	AF033421
P. scabrosum	DAOM214786	DQ267906
P. steckii	NRRL35463	EF634431
P. subturcoseum	CV110	FJ231006

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P. subturcoseum	CV15	FJ231004
	CV18	FJ231005
	CV51	FJ231007
P. swiecickii	NRRL918	AF033490
P. velutinum	NRRL2069	AF033448
P. westlingii	NRRL800	AF033423
T. trachyspermus	FRR1792	L14516

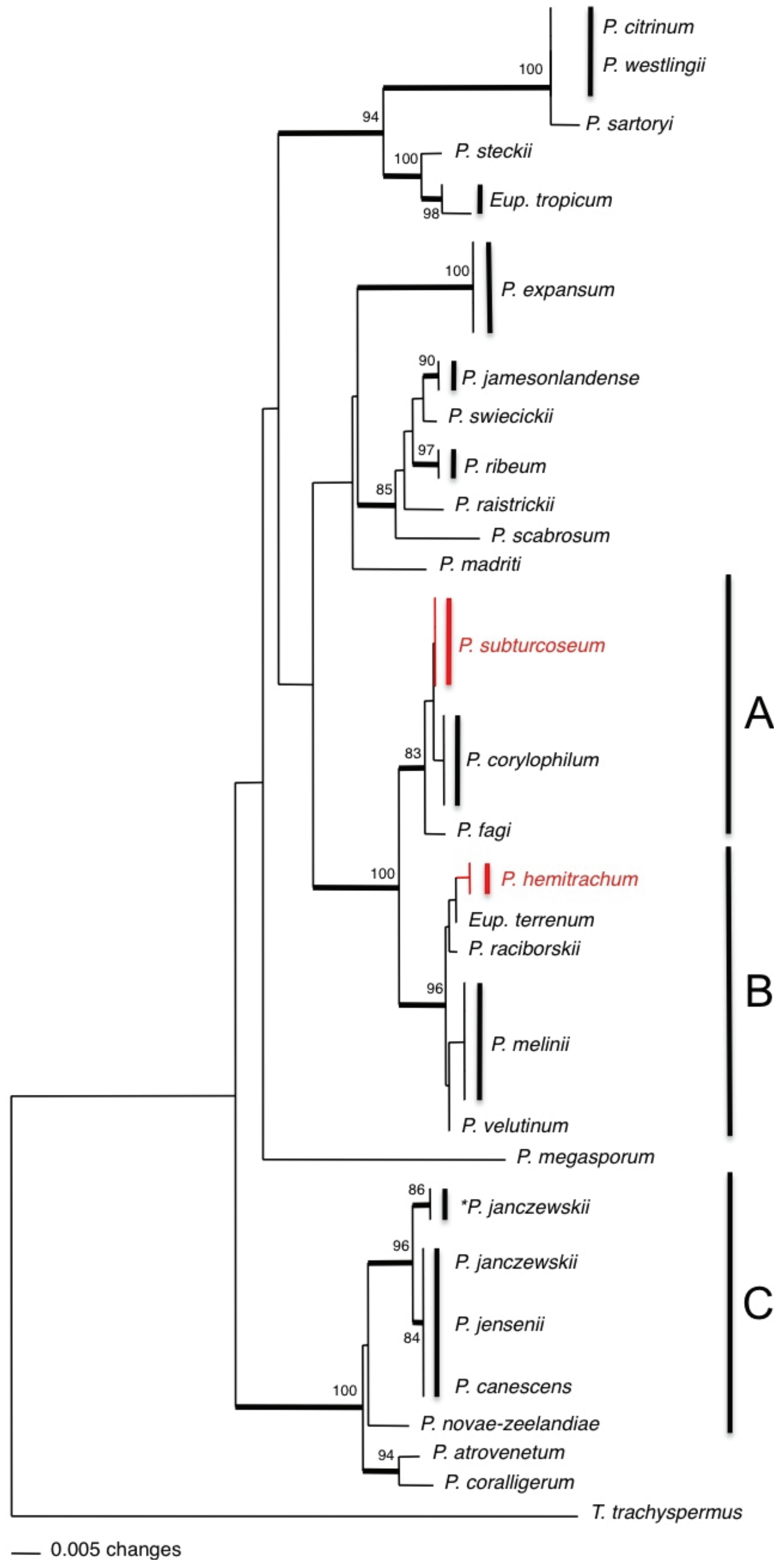

FIGURE 1. Neighbour-joining tree showing species isolated from fynbos soil and their closest related species based on an ITS phylogeny. Bootstrap values higher than 80 are indicated above the thicker branches. *Talaromyces trachyspermus* were chosen as the outgroup. * = fynbos *P. janczewskii* strains.

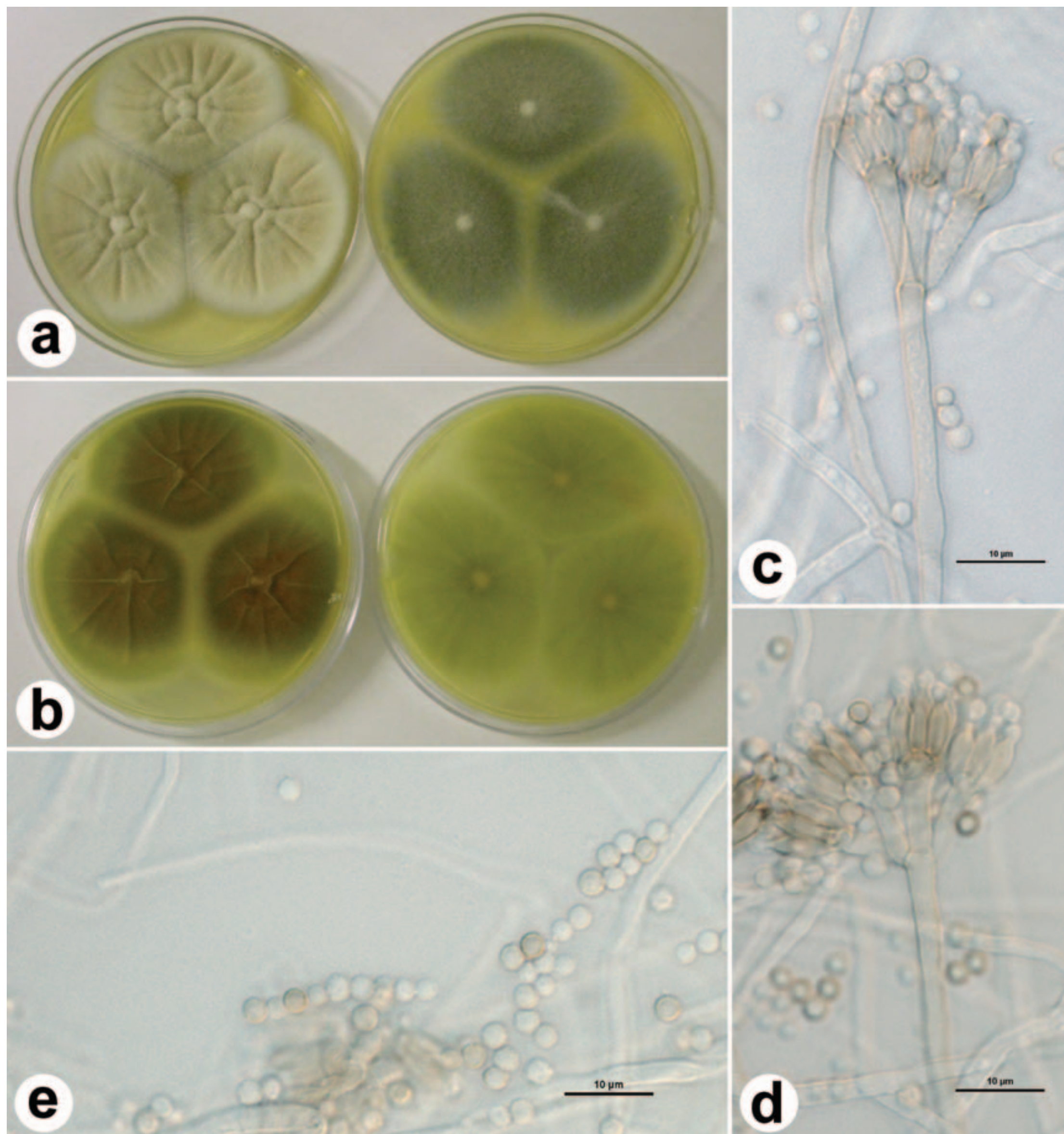

FIGURE 2. Morphological features characteristic of *P. hemitrachum,* holotype (PREM60048). a. Colonies of *P. hemitrachum* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies, showing the brown color on CYA. c, d. Biverticillate conidiophores produced in culture. e. Smooth to finely rough-walled conidia.

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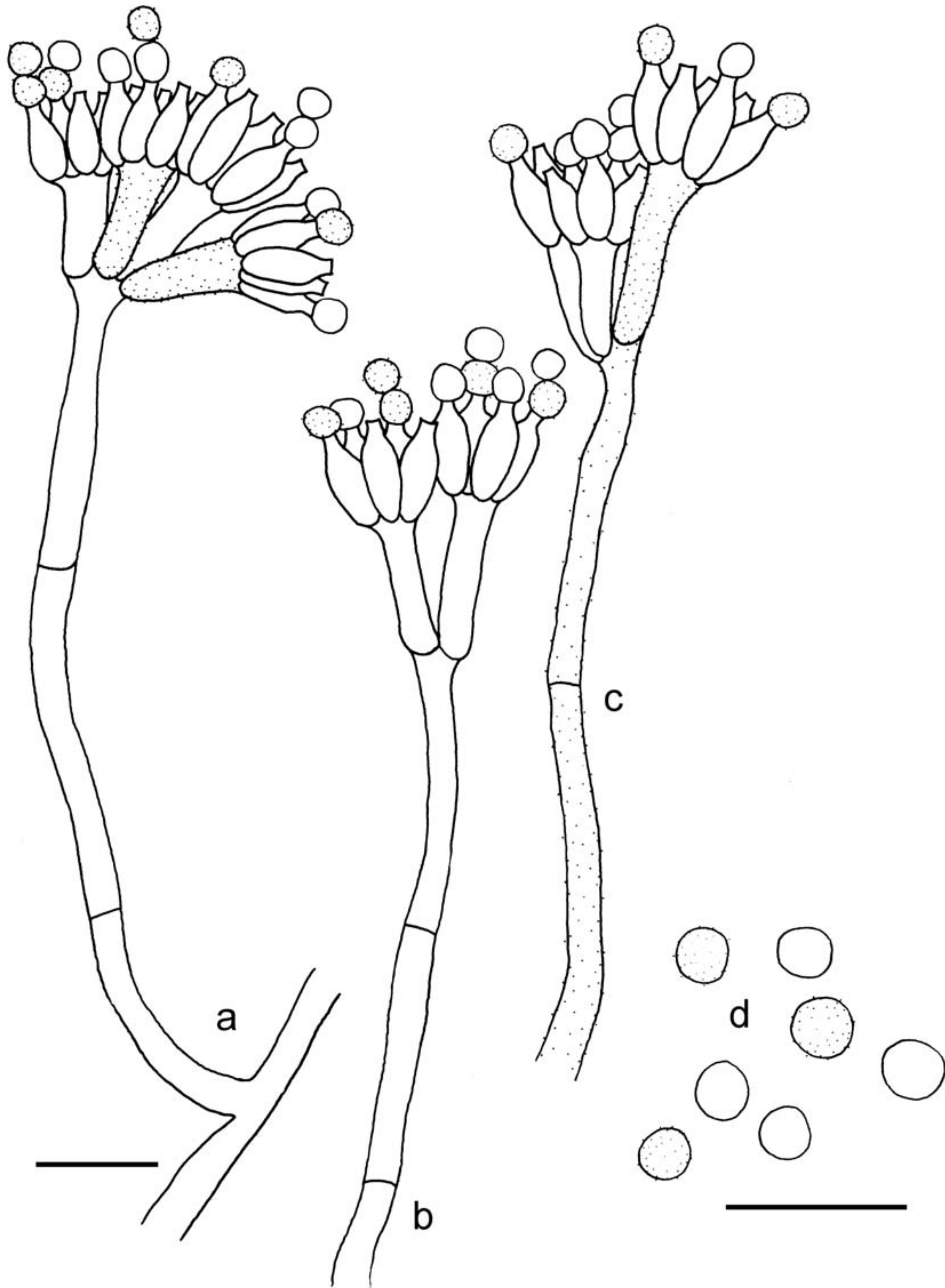

FIGURE 3. *Penicillium hemitrachum* line drawings from holotype (PREM60048) material. a, b, c. Conidiophores (bar = 10 μm). d. Conidia (bar = 10 μm).

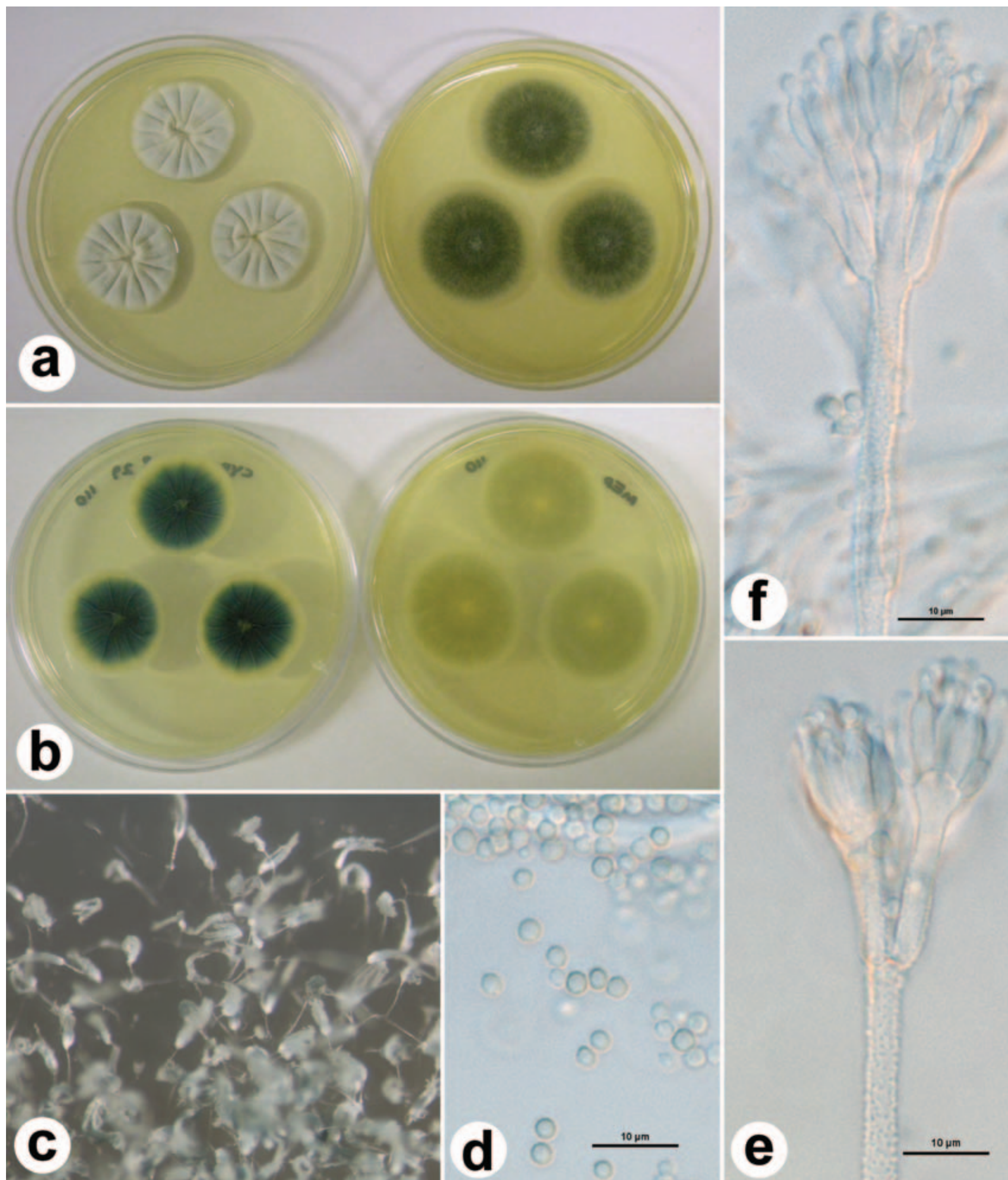

FIGURE 4. Morphological features characteristic of *P. subturcoseum*, holotype (PREM60050). a. Colonies of *P. subturcoseum* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies, showing the characteristic dark turquoise reverse on CYA. c. Velutinous colony texture on MEA. d. Rough-walled, spheroid conidia. e, f. Biverticillate conidiophores produced in culture.

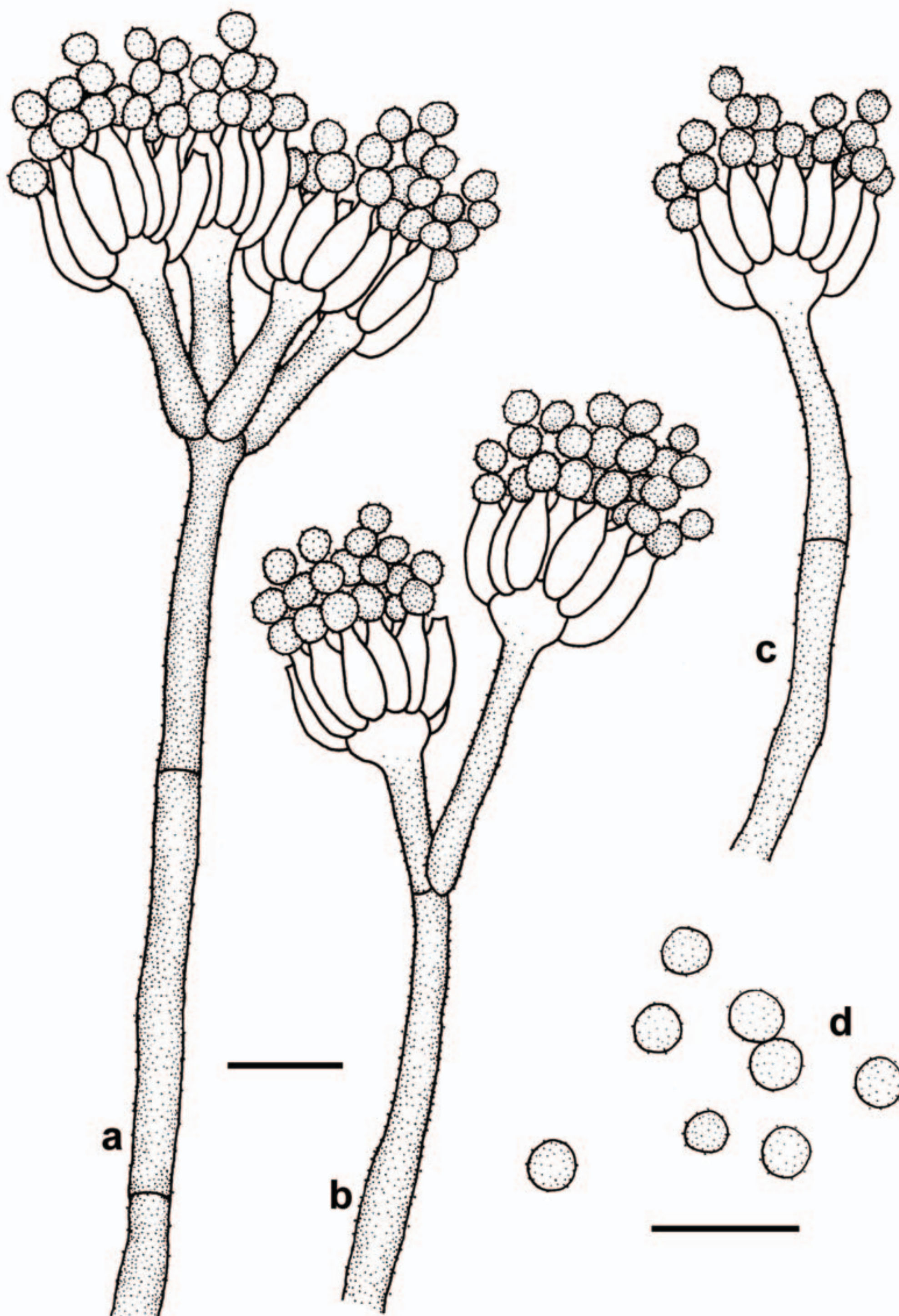

FIGURE 5. *Penicillium subturcoseum* line drawings from holotype (PREM60050) material. a, b, c. Conidiophores (bar = 10 μm). d. Conidia (bar = 10 μm).

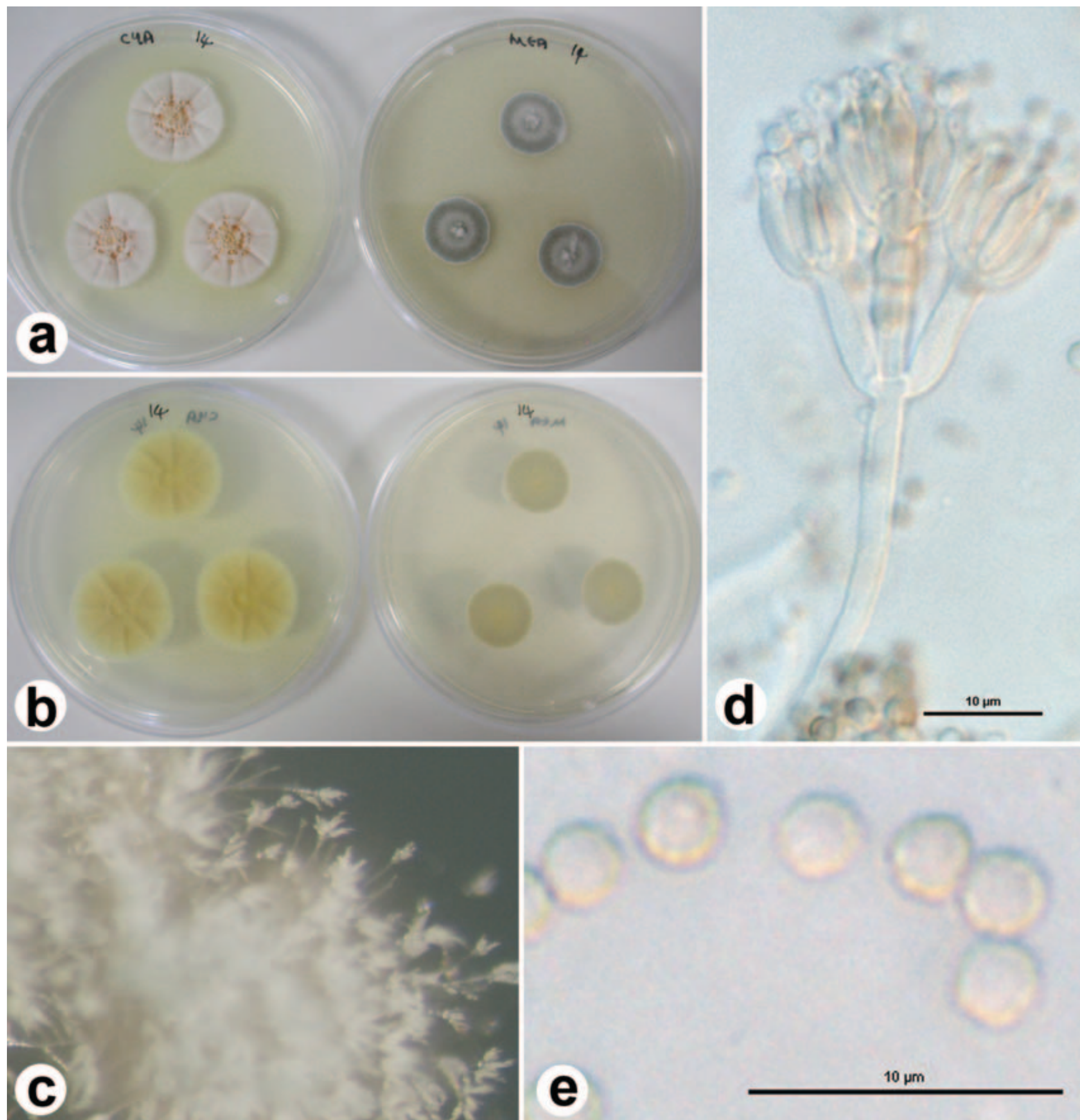

FIGURE 6. Morphological features characteristic of *P. citrinum* (CV14). a. Colonies of *P. citrinum* incubated on CYA (left) and MEA (right) after 7 days, showing the characteristic orange exudates produced on CYA. b. Reverse of the same colonies c. Floccose colony texture on MEA. d. Biverticillate conidiophores produced in culture. e. Smooth to finely rough-walled conidia.

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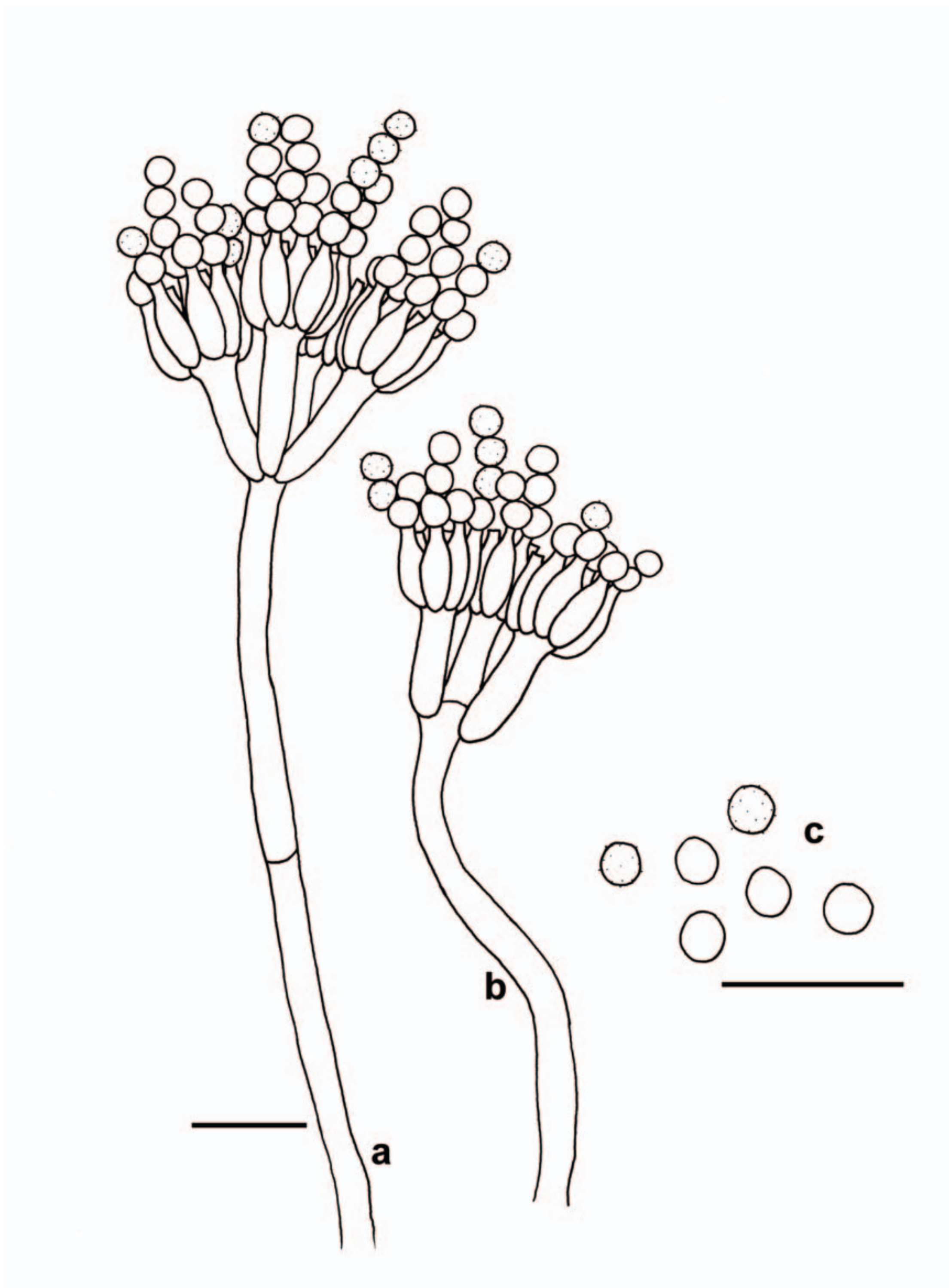

FIGURE 7. *Penicillium citrinum* line drawings from strain CV14. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

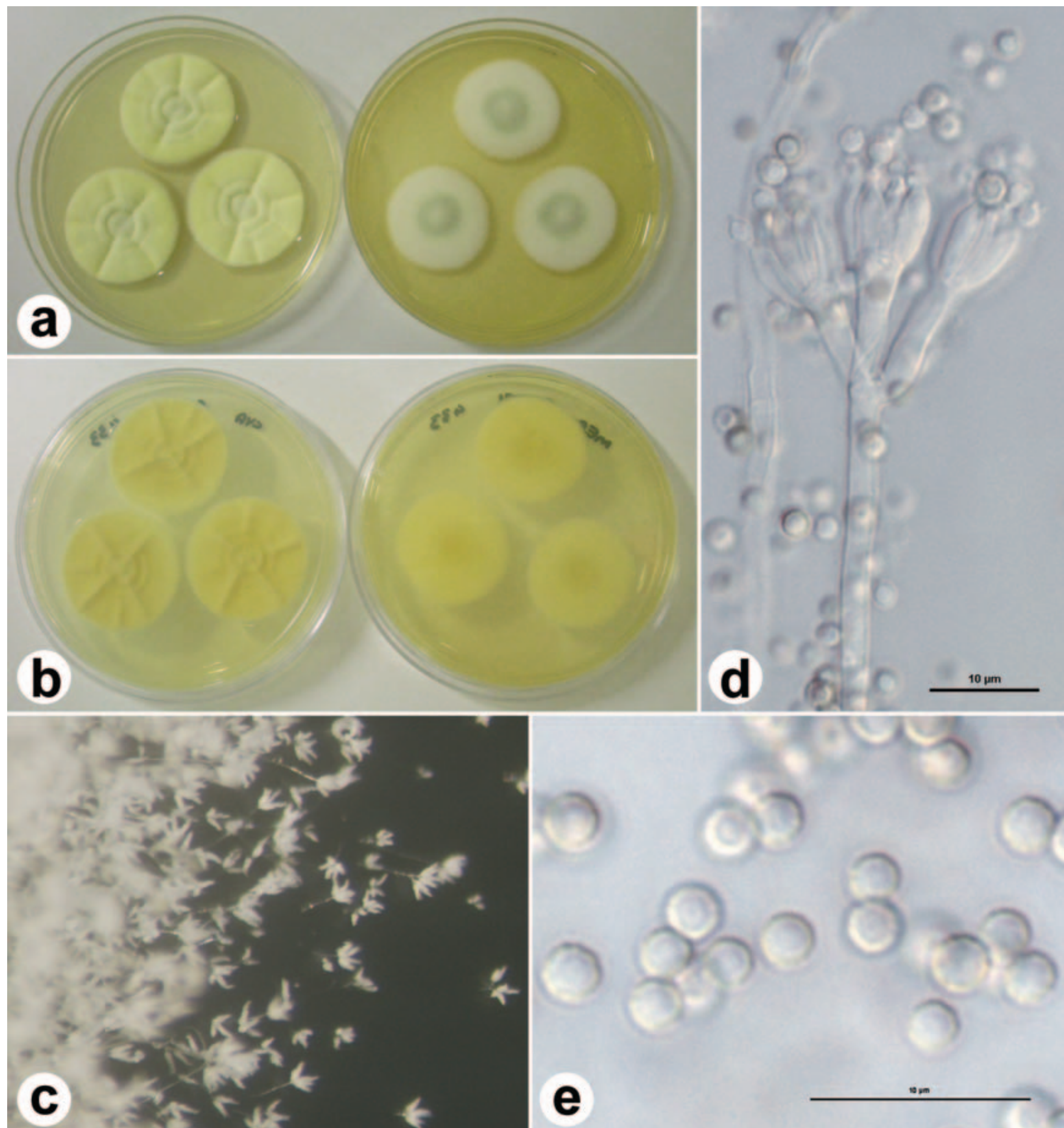

FIGURE 8. Morphological features characteristic of *P. janczewskii* (CV433). a. Colonies of *P. janczewskii* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c. Conidiophores borne from surface at colony edge. d. Biverticillate conidiophore, with smooth-walled stipe produced in culture. e. Rough-walled spheroid conidia.

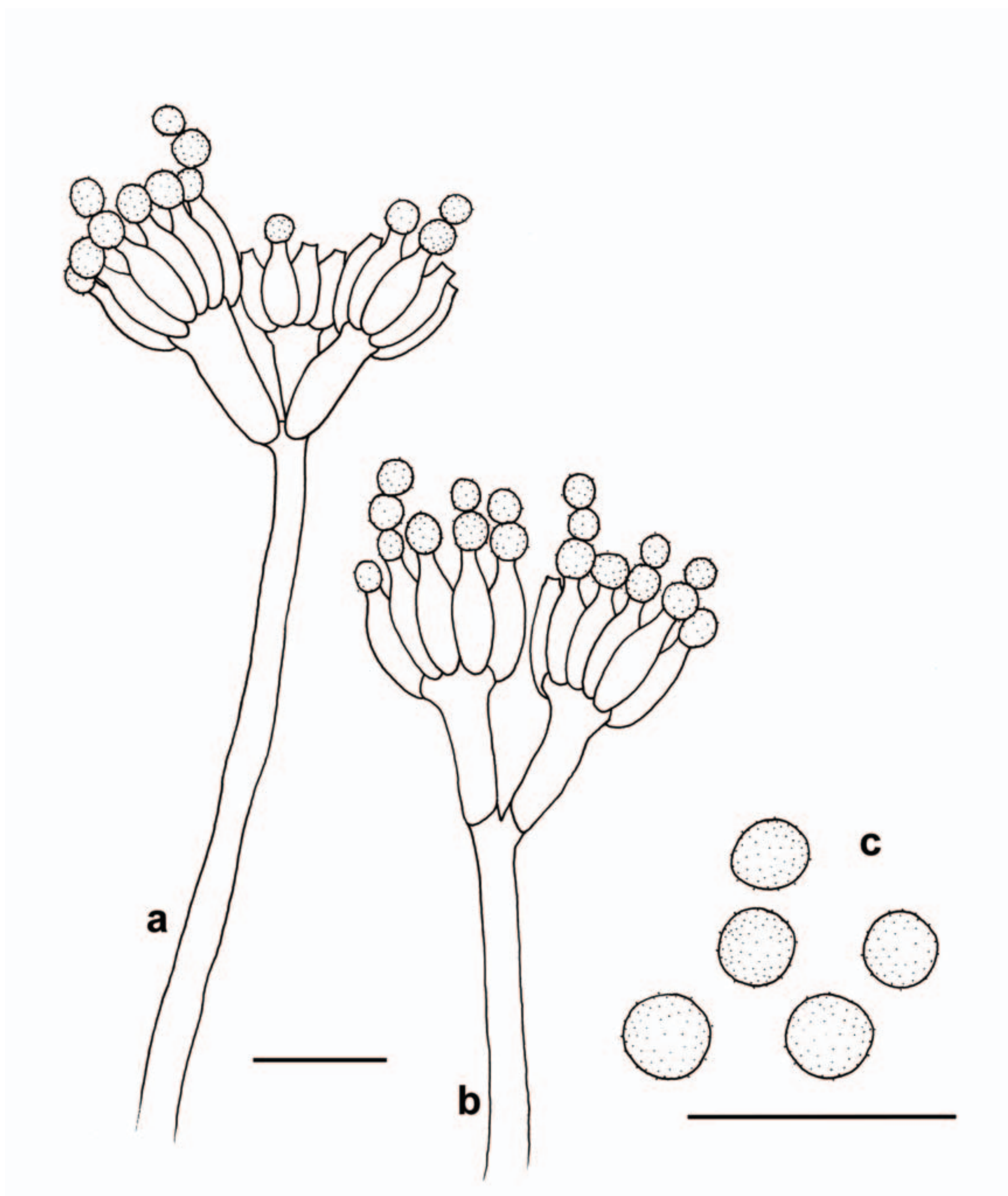

FIGURE 9. *Penicillium janczewskii* line drawings from strain CV433. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

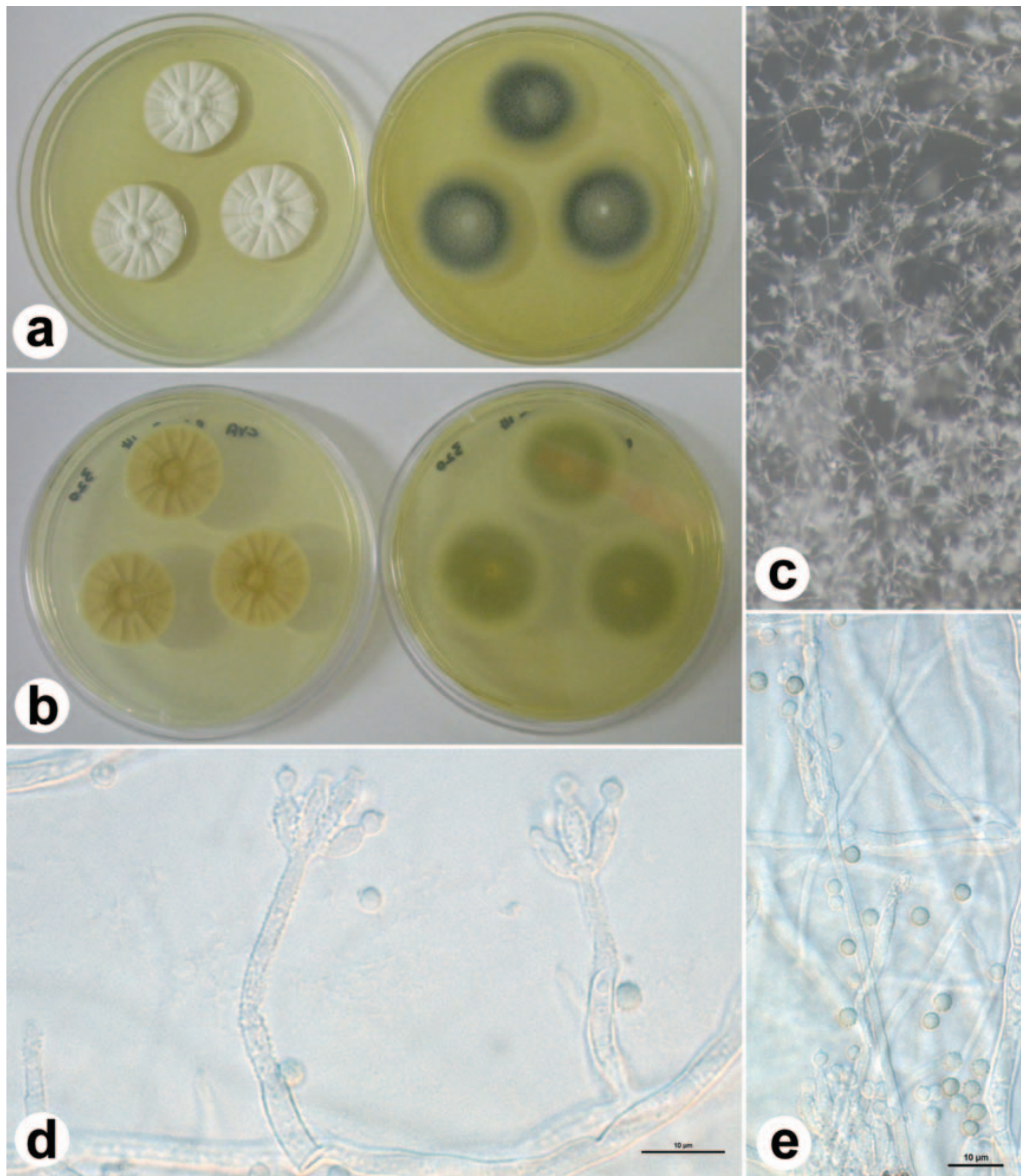

FIGURE 10. Morphological features characteristic of *P. melinii* (CV320). a. Colonies of *P. melinii* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c. Conidiophores borne from aerial hyphae on MEA. d. Monoverticillate conidiophores, having heavy rough-walled stipes and phialides, most commonly produced by the fynbos strains in culture. e. Heavy rough-walled conidia.

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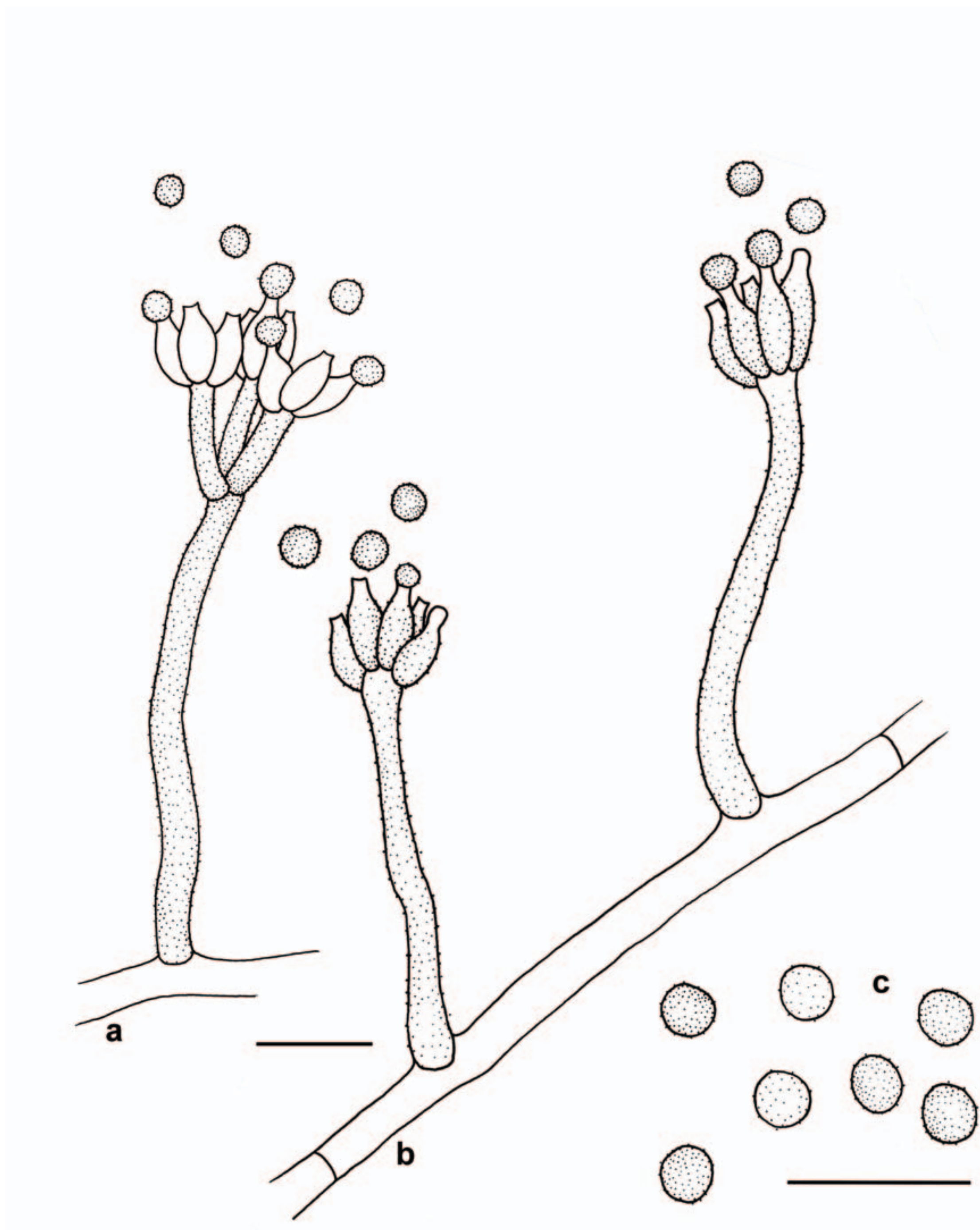

FIGURE 11. *Penicillium melinii* line drawings from strain CV320. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

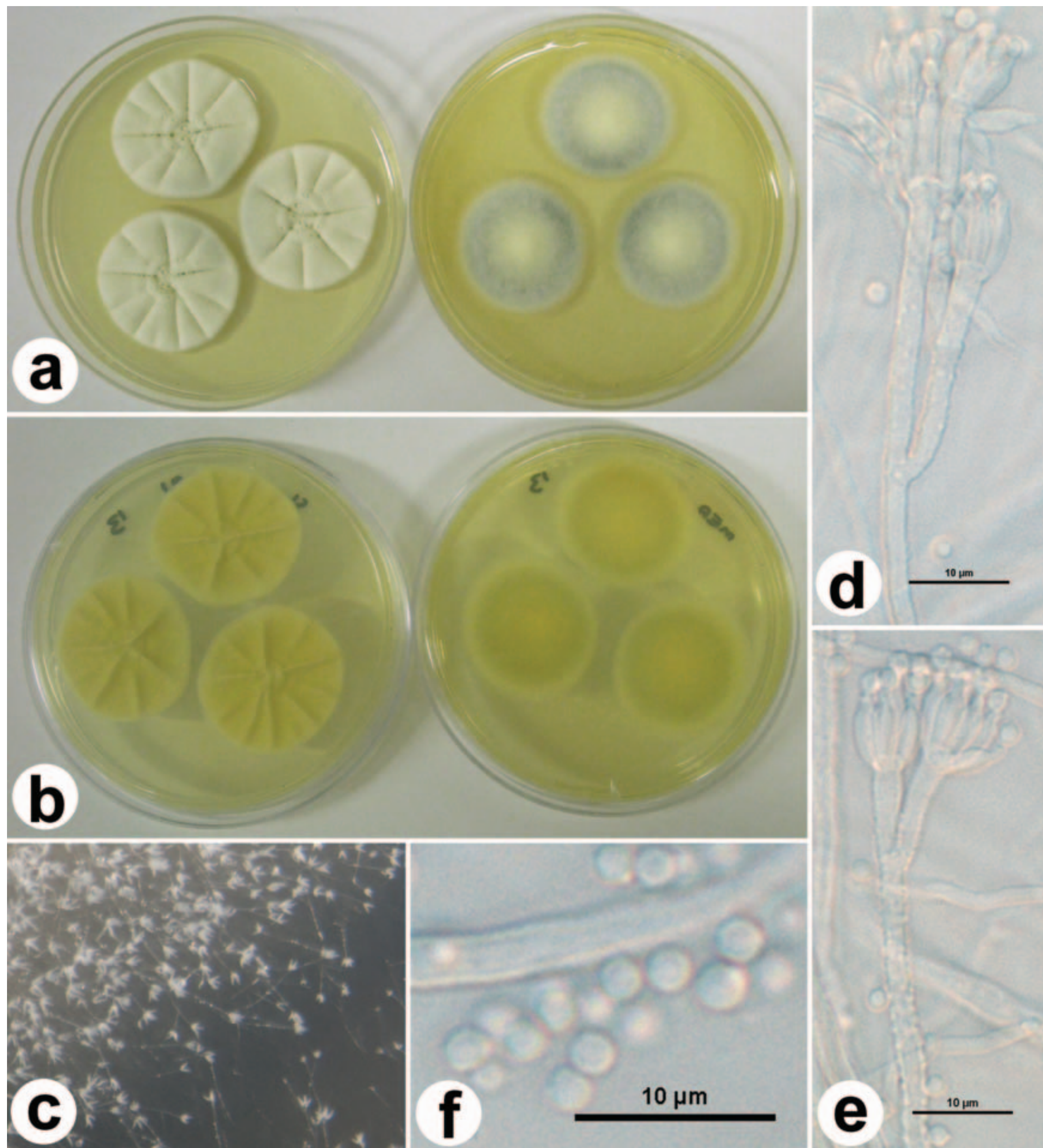

FIGURE 12. Morphological features characteristic of *P. canescens* (CV13). a. Colonies of *P. canescens* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c. Conidiophores often borne from surface at edge of colonies. d, e. Biverticillate conidiophores, with characteristic rough-walled stipes produced in culture. f. Smooth spheroid conidia.

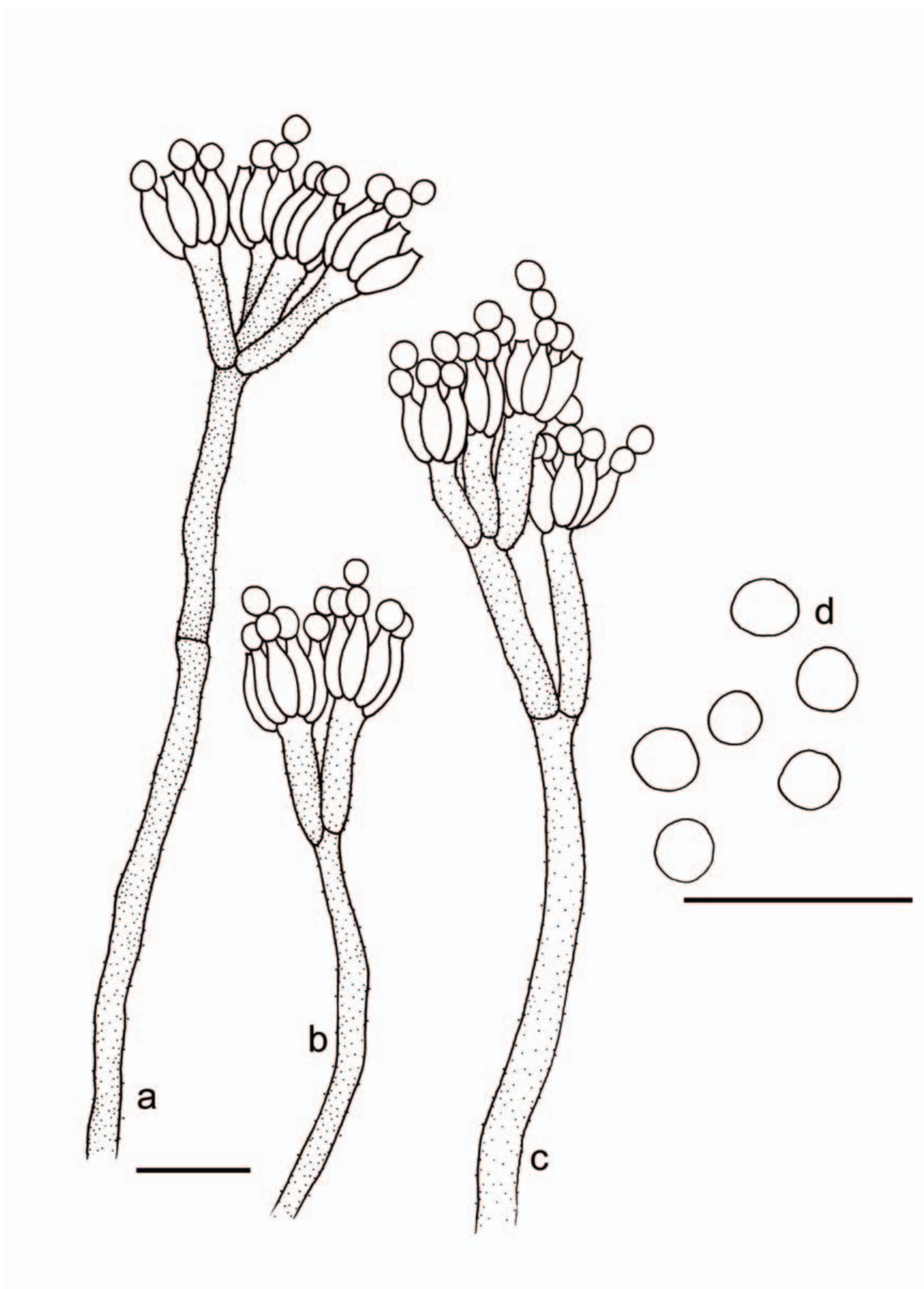

FIGURE 13. *Penicillium canescens* line drawings from strain CV13. a, b, c. Conidiophores (bar = 10 μm). d. Conidia (bar = 10 μm).

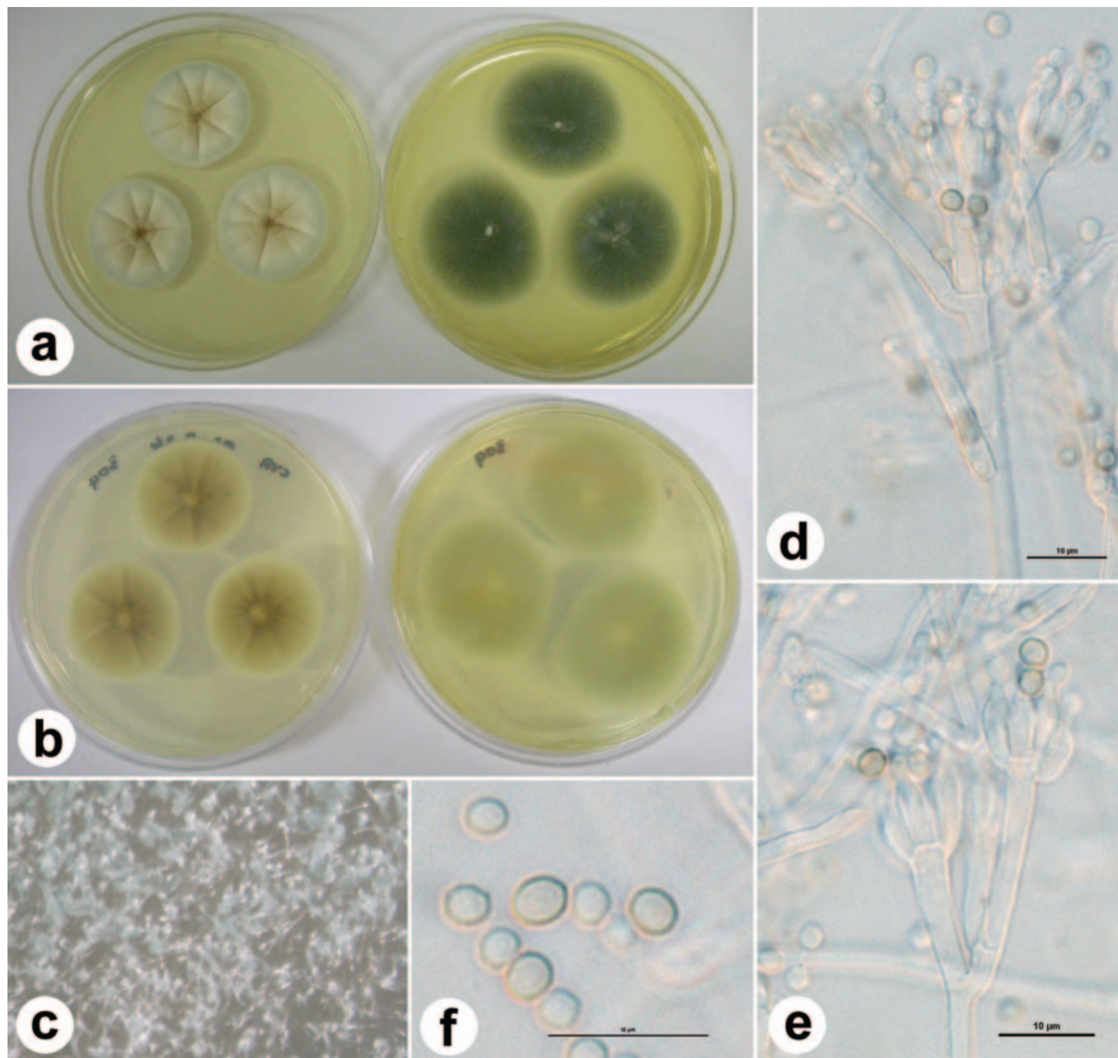

FIGURE 14. Morphological features characteristic of *P. corylophilum* (CV304). a. Colonies of *P. corylophilum* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies, showing the brown color on CYA. c. Velutinous colony texture on MEA. d, e. Biverticillate conidiophores, with metulae of unequal lengths produced in culture. f. Smooth-walled, spheroid to subshperoid conidia.

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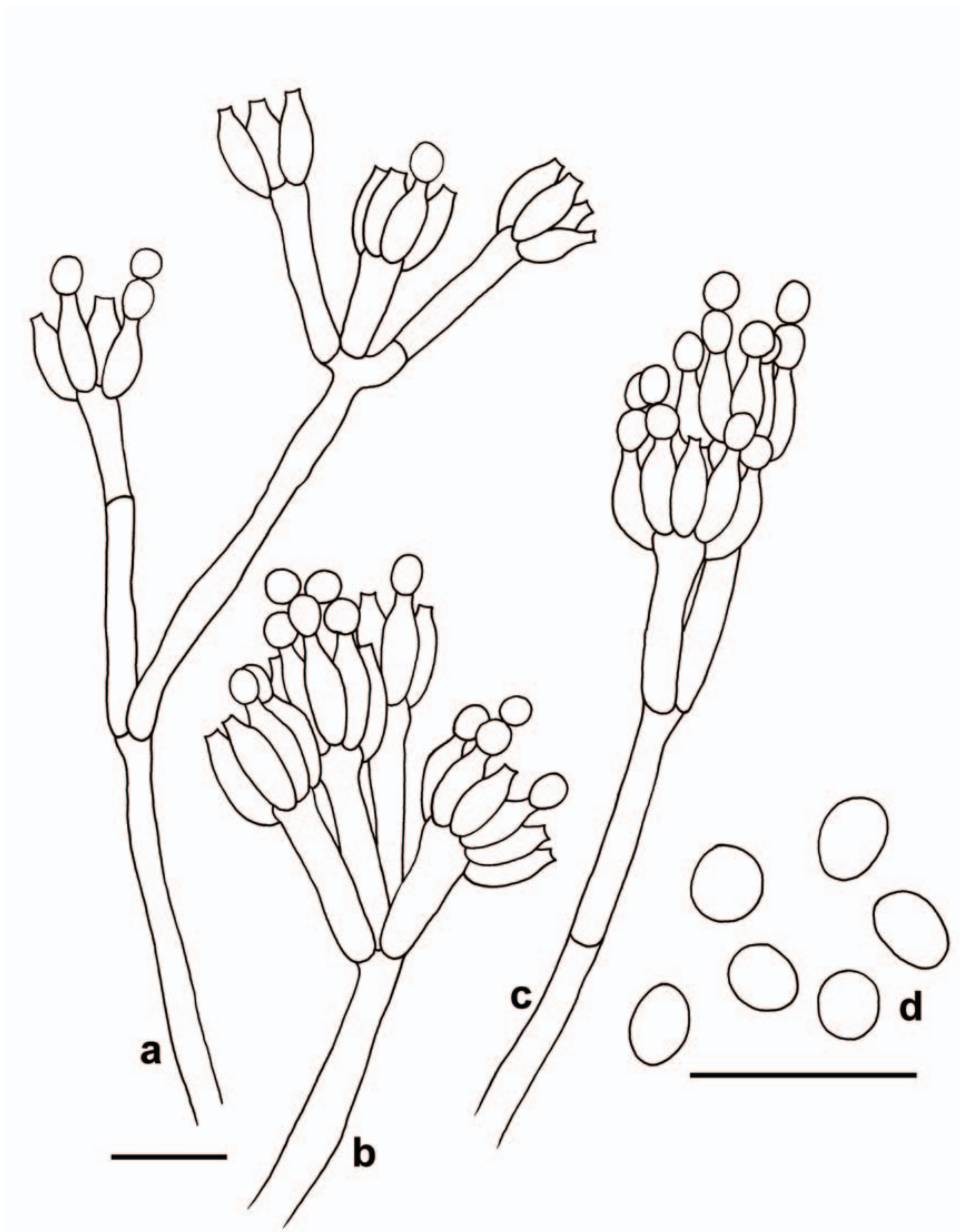

FIGURE 15. *Penicillium corylophilum* line drawings from strain CV304. a, b, c. Conidiophores (bar = 10 μm). d. Conidia (bar = 10 μm).

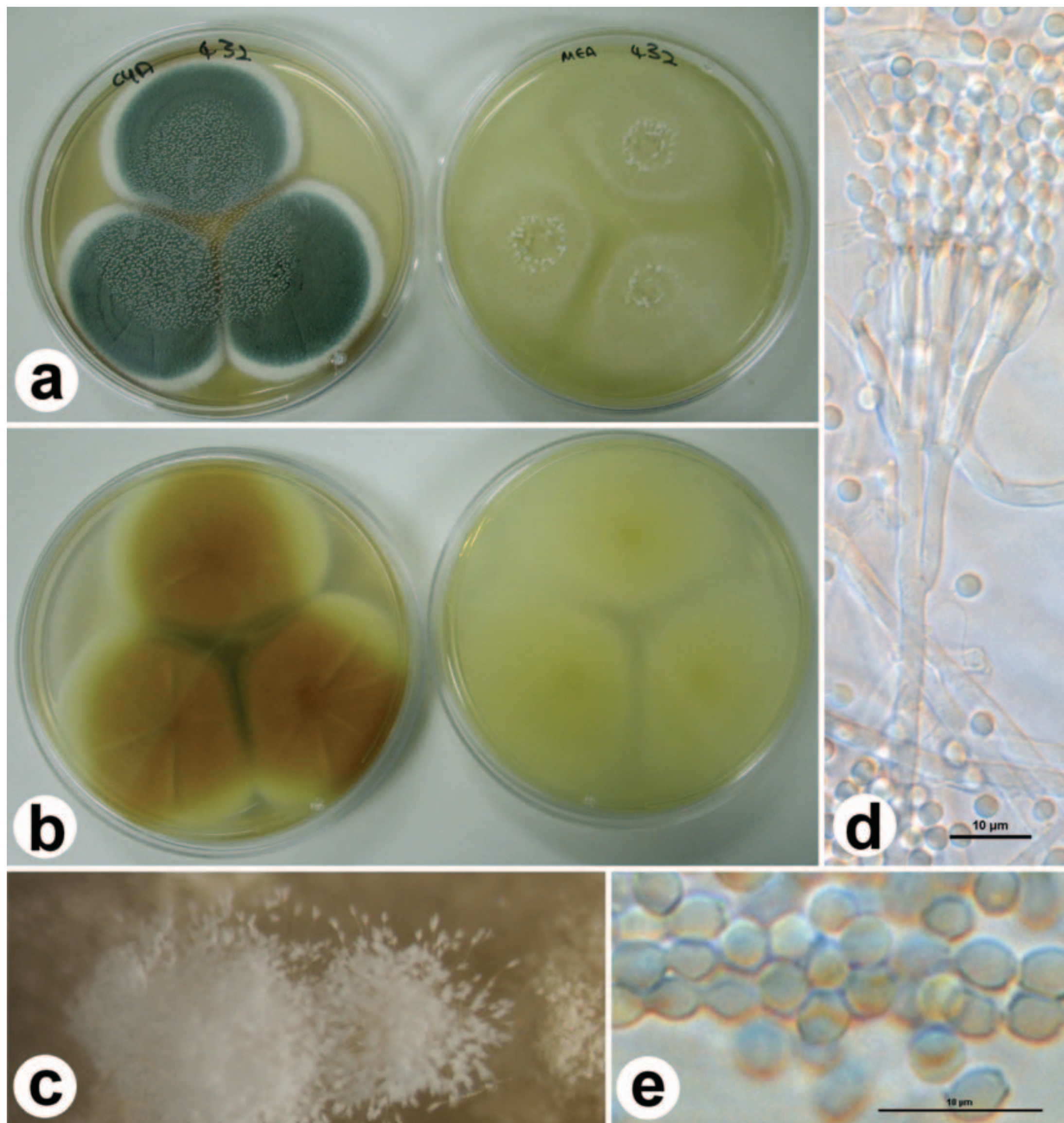

FIGURE 16. Morphological features characteristic of *P. expansum* (CV432). a. Colonies of *P. expansum* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies, showing the brownish orange color on CYA. c. Fascicles present on MEA. d. Terverticillate conidiophores produced in culture. e. Smooth subspheroid to ellipsoid conidia.

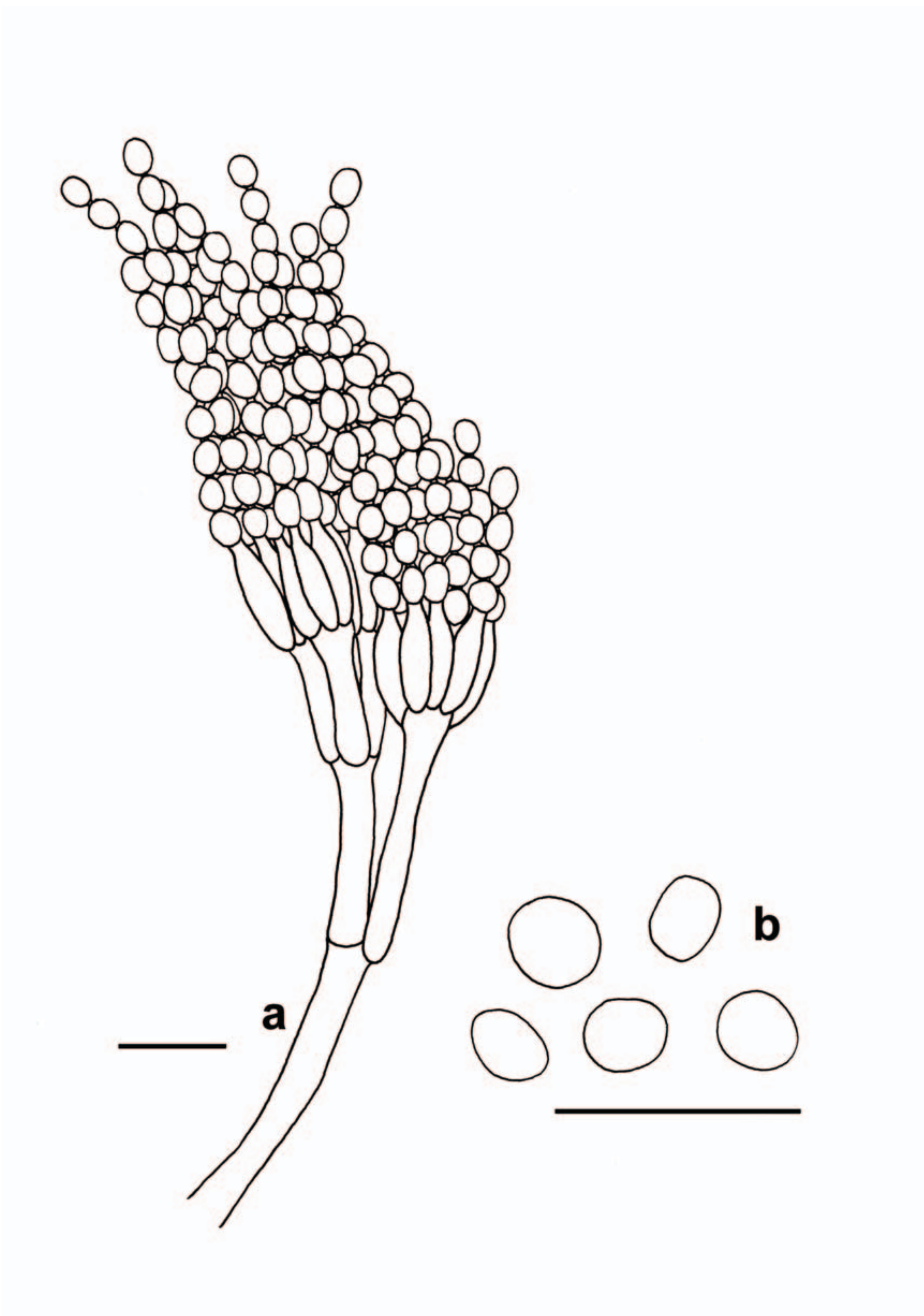

FIGURE 17. *Penicillium expansum* line drawings from strain CV432. a. Conidiophore (bar = 10 μm). b. Conidia (bar = 10 μm).

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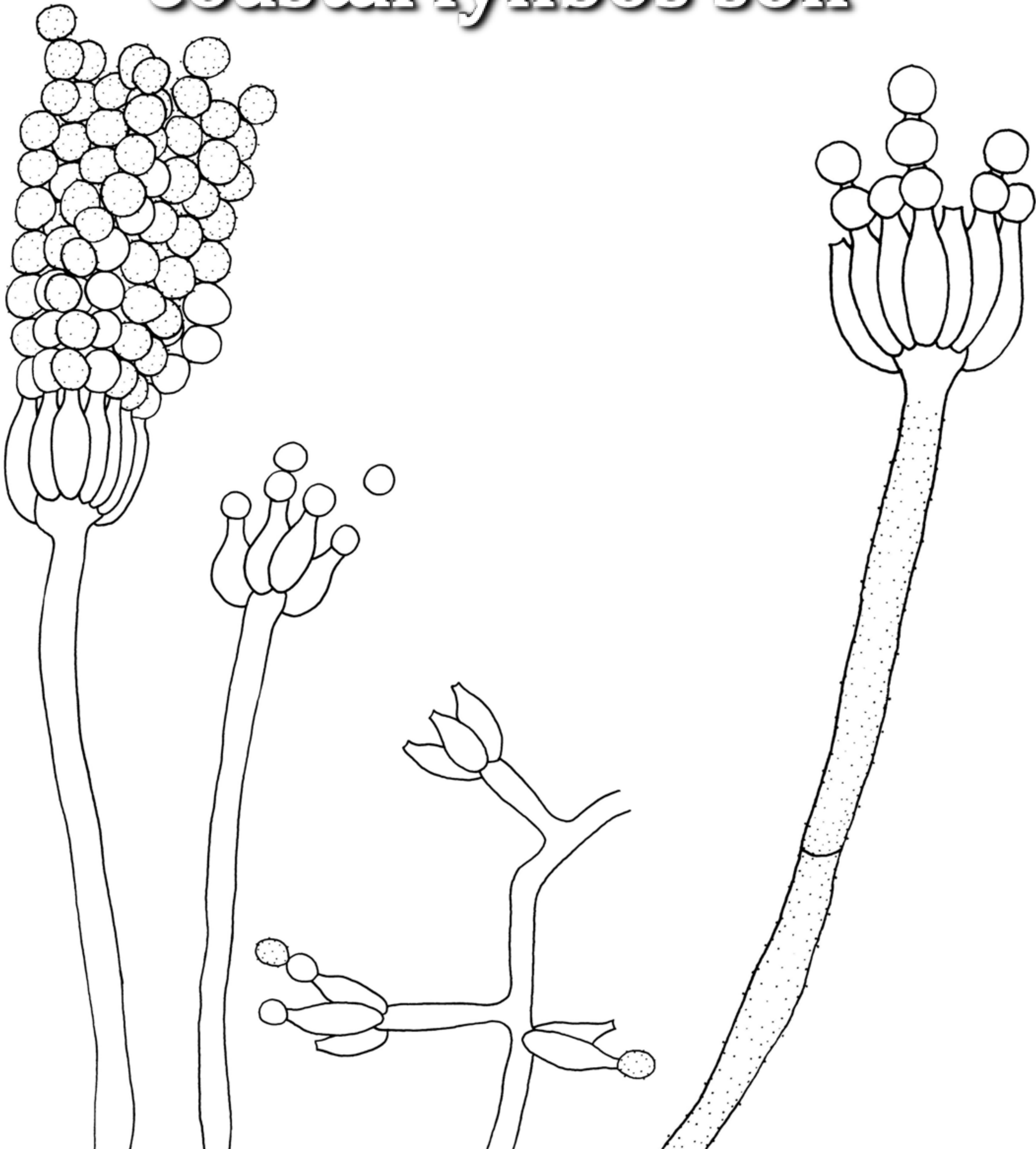
CHAPTER 5

Diversity of *Penicillium* subgenus *Aspergilloides* in coastal fynbos soil



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ABSTRACT

During a survey on *Penicillium* spp. occurring in coastal fynbos soil, Western Cape of South Africa, eight species with monoverticillate conidiophores, characteristic of species belonging to *Penicillium* subgenus *Aspergilloides*, were isolated. Species were characterized using morphological and genetic characters and identified as *Penicillium hirayamae, P. restrictum, P. roseopurpureum* and *P. toxicarium*. Four species, however, did not conform to characters of known species and are, therefore, described here as *P. brachycaulon* prov. nom., *P. cumulacinatum* prov. nom., *P. malacosphaerula* prov. nom. and *P. vulgaris* prov. nom.

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INTRODUCTION

Previous studies from coastal fynbos soil have shown an high species diversity in the genus *Penicillium*, with seven species described as new to science (Visagie et al. 2008, Visagie and Jacobs 2008a, 2008b). This high proportion of novel species are to be expected based on the 63 210 fungal species, associated with the 80% endemic plant species, estimated to occur in the Cape Floristic Region (CFR) (Crous et al. 2006). The uniqueness of the area should, therefore, be manifested in the fungal flora, of which *Penicillium* seems to be dominant, at least in the soil.

This particular chapter focused on species belonging to *Penicillium* subgenus *Aspergilloides*. Species from this group characteristically produces monoverticillate, with a minor proportion biverticillate, conidiophores (Pitt 1979), which in general makes them easily distinguishable from the other subgenera. During this survey, eight species with characters resembling subgenus *Aspergilloides* were isolated and compared to previously described species. Both morphological characters and phylogenetic analysis of the internal transcribed spacer (ITS) region were used in the comparisons. Four of the species displayed unique characters and the aim of this study was, therefore, to provide descriptions for the new species, as well as providing an extensive key to these species and their closely related sister taxa.

MATERIALS AND METHODS

Isolations — Soil were collected at different plots (21 February 2007) from sampling sites at Camphill Village (S 33,59787°; E 18,56433°), Kalbaskraal (S 33,57061°; E 18,62861°), Pella (S 33,51022°; E 18,55236°) and Riverlands (S 33, 49066°; E 18, 58388°). These sites are situated in the coastal fynbos region near Malmesbury in the Western Cape. A dilution series of each sample were prepared by adding 5 g soil to 100 ml dH_2O and diluting this to 10^{-2}. These dilutions were plated out onto Potato Dextrose agar (Biolab), supplemented with 50 ppm Streptomycin (Applichem, South Africa) and 100 ppm Chloramphenicol (Applichem, South Africa) and incubated at 25°C for 6–7 days. Colonies showing

the typical brush-like characters of *Penicillium* were then transferred to Oatmeal Agar, from which single spore cultures were prepared.

Morphology — Three point inoculations were made from spore suspensions in a semi-solid agar (0.2%) and tween80 (0.05%) solution on Czapek Yeast agar (CYA), Malt Extract agar (MEA: after Blakeslee, 1915) and 25% Glycerol Nitrate agar (G25N) (Pitt, 1979). Inoculated 90 mm polystyrene Petri dishes contained 20 ml of media and were incubated at 25°C (CYA, MEA, G25N), 5°C (CYA) and 37°C (CYA) (Pitt 1979, Samson and Pitt 1985), in the dark with plates at 25°C and 5°C left unwrapped to allow for sufficient aeration (Okuda et al. 2000). Strains were characterized and described following the concepts of Pitt (1979) and Samson and Pitt (1985). Capitalized color names and codes refer to the "Methuen handbook of colour" (Kornerup and Wanscher 1966).

Phylogenetic analysis — Strains used for phylogenetic comparisons were incubated on MEA for 7 days and the DNA extractions done using a modified Möller et al. (1992) technique. The PCR and cycle sequencing of the ITS1–5.8S–ITS2 rDNA region, using primers ITS1 and ITS4 (White et al. 1990), followed methods described by Visagie and Jacobs (2008a). ITS sequences of the coastal fynbos soil species and closely related species, obtained from GenBank, were included in a dataset mainly based on the study by Peterson (2000). *Talaromyces trachyspermus* were chosen as a suitable outgroup. *Penicillium* species and their accession numbers used for comparisons are included in TABLE 1. The dataset was aligned in ClustalX (Thompson et al. 1997) and manually adjusted in Se-Al (Rambaut 2007). Ambiguously aligned regions typically contain numerous inserted gaps, which results in superficial branch lengths in the phylogenetic trees. These regions were, therefore, replaced with codes, by computing step matrices to assign different weights to the codes using INAASE 2.3b (Lutzoni et al. 2000). Sequence analysis was done in PAUP* v4.0b10 (Swofford 2000) with gaps treated as missing data. A single tree for the dataset was calculated using the neighbour-joining option, with confidence levels in the nodes determined by a bootstrap analysis of a 1000 replicates. Alignments of the ITS dataset can be obtained from the compact disc attached at the back of the thesis.

RESULTS

In total, 434 *Penicillium* strains were isolated from coastal fynbos soil and placed into 24 distinct morphological groups. Eight of these groups, containing 73 isolates in total, conformed to characters distinctive of *Penicillium* subgenus *Aspergilloides*. Three of these groups could be positively identified using morphology as *Penicillium restrictum, P. roseopurpureum* and *P. hirayamae* (teleomorph = *Eupenicillium hirayamae*) (Pitt, 1979), as well as one group identified as *P. toxicarium*, using phylogenetic characters (Serra et al. 2008).

Penicillium restrictum characteristically produces restricted colonies on CYA and MEA, with stipes commonly shorter than 60 µm and with conidia being smooth, spinose or echinulate (Pitt 1979). Similar to the findings of Pitt (1979), strains showed considerable variation, although still fitting within the broader description of this species. *Penicillium roseopurpureum* have slow growing colonies producing characteristic reddish pigmentations and conidiophores with short stipes (Pitt 1979). The fynbos strains showed characters similar to this species, except for slightly faster growth rates and smooth-walled conidia in contrast to the very finely roughened conidia observed by Pitt (1979). The fynbos strains identified as *P. hirayamae*, produced bright orange to yellowish colonies and colony reverse coloration, which is characteristic of the species (Pitt 1979). The restricted conidiophores, borne on loosely developed funicles, further made the identity of these strains unmistakable. In addition, strains produced protocleistothecia after one week of incubation on both CYA and MEA, at 25°C in the dark, but did not develop into asci. The fynbos *P. toxicarium* strains are morphologically similar to *P. citreonigrum*, which Pitt (1979) considered as synonymous with *P. toxicarium*. Based on morphological characters, it is difficult to distinguish between these two species, although they are phylogenetically distinct (Serra et al. 2008). The fynbos strains were found to be similar to *P. toxicarium*. Four groups showed unique morphological characters, which distinguishes it from all previously described species.

The aligned ITS dataset was 524 bp long, with six ambiguously aligned regions that were replaced by weighted codes. The ITS phylogeny (FIG. 1) was able to confirm morphological identifications and resolve new species in clades separate

from previously described species. Bootstrap support was low for clades A, B, and C respectively, but morphological characters for the fynbos soil species in clades A and C, are deemed sufficient for describing these as new species.

TAXONOMY

Penicillium brachycaulon C.M. Visagie & K. Jacobs prov. nom.[*] **FIGS 2, 3**

Mycobank nr. MB 512439

Etymology. Latin, *brachycaulon*: *brachys* = short; *caulon* = stem, referring to the short stipes, characteristic of the species.

Coloniae in CYA post 7 dies in 25°C 23–27 mm medio depressae infra olivaceo-flavidae. Coloniae in MEA post 7 dies in 25°C 30–33 mm, infra olivaceo-flavidae. Conidiophorae semper univerticillatae, in hyphis aeriis portatae; stipa laevis, 8–20(–30) × 2–3 µm; phialides ampulliformae rare proxime in hyphis fertilis portatae 4–6(–7) × 2–2.5 µm; conidia subsphaeroidea vel elliptica laevia vel subtiliter exasperata 2.5–3 × 2–2.5 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 23–27 mm diam, sulcate and centrally depressed, dense, floccose; margins narrow, low, irregular, commonly yellowish olive underneath substrate, mycelia white; conidiogenesis sparse, grayish green (25b5), orange grey at centre of colonies in some isolates; exudate absent, reddish brown soluble pigment inconspicuously produced, reverse sometimes Rose Wood (9d5) at centre and Olive Yellow (2c6) elsewhere; At 5°C, 7 days: Germination with microcolonies occasionally formed; 37°C, 7 days: Colonies 15–20 mm, but sometimes as large as 25 mm, sulcate to plicate; margins medium, irregular, mycelia white, conidiogenesis sparse, exudate and soluble pigment absent, reverse grayish yellow (4b4–4b5) to grayish orange (5b5). MEA, 25°C, 7 days: Colonies 30–33 mm diam, plane, with floccose texture; margins moderately wide, subsurface, regular, mycelia white; conidiogenesis moderate to sparse in some isolates, conidia grayish green; exudate and soluble pigment absent, reverse Olive Yellow (2c6). G25N, 25°C, 7 days: Colonies 10–17

[*] Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere.

mm, centrally sunken in; mycelia white; conidiogenesis mostly sparse, but moderate in some isolates, grayish green (26d6); exudate and soluble pigment absent, reverse pale yellow.

Conidiophores borne from aerial hyphae, stipes smooth-walled, non–vesiculate, 8–20(–30) × 2–3 μm, bearing monoverticillate penicilli; phialides 3–4(–5), slightly divergent, solitary phialide borne directly on mycelia not uncommon, ampulliform 4–6(–7) × 2–2.5(–3) μm; conidia sub-spheroid to ellipsoid 2.5–3 × 2–2.5 μm, smooth to finely roughened, disordered.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Kalbaskraal: (S 33,57061°; E 18,62861°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type CV188 (PREM60045) (HOLOTYPE); *Additional specimens examined:* South Africa, Western Cape Province, Malmesbury, Kalbaskraal and Pella: (S 33,57061°; E 18,62861° and S 33,51022°; E 18,55236°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV162, 173, 354 (PREM60046), 357, 361 (PREM60047), 362, 392, 399.

Penicillium malacosphaerula C.M. Visagie & K. Jacobs prov. nom.* **FIGS 4, 5**

Mycobank nr. MB 512440

Etymology. Latin, *malacosphaerula*: *malacos* = soft; *shpaerula* = small ball, referring to the soft sclerotia produced in culture.

Coloniae in CYA post 7 dies in 25°C 34–37 mm sclerotia mollia abundantia facientes. Coloniae in MEA post 7 dies in 25°C (24–)34–40 mm, sclerotia similia facientes. Conidiophorae semper univerticillatae, in hyphis aeriis portatae; stipa laevis, 100–220 × 2–3 μm; phialides ampulliformae vel subacerosae 5.5–8(–9) × 2–3(–3.5) μm; conidia sphaeroidea laevia 2.5–3 μm.

Colony morphology, CYA, 25°C, 7 days: Colonies 34–37 mm diam, sulcate, moderately dense, floccose texture, abundant soft white sclerotia with loose almost wooly texture produced, ascospores not present; margins low, narrow,

* Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere

regular, mycelia white; conidiogenesis sparse, yellowish white; brownish red to clear exudate, soluble pigment Greenish Yellow (1a8), reverse Wine Yellow (3b3) to Ivory (4b3); At 5°C, 7 days: Germination occurring; 37°C, 7 days: Colonies 33–38 mm, plicate; mycelia white; conidiogenesis absent, but sparse in some isolates, floccose; exudate absent, soluble pigment yellow, reverse Vivid Yellow (2a8). MEA, 25°C, 7 days: Colonies (24–)34–40 mm diam, plane, floccose texture; margins wide 3–4 mm, low, regular, sometimes irregular in some isolates, mycelia white; conidiogenesis sparse, yellowish white (27e4), becoming a darkish green; exudate absent, soluble pigment absent but sometimes a very light yellow, reverse a pale yellow. G25N, 25°C, 7 days: Colonies 13–18 mm, sulcate, with floccose texture; mycelia white; conidiogenesis sparse, yellowish white; exudate absent, soluble pigment yellow, reverse Vivid Yellow (3a8).
Conidiophores borne from aerial hyphae, stipes smooth-walled, non-vesiculate, 100–220 × 2–3 µm, bearing monoverticillate penicilli, with very few biverticillate; phialides 3–5, appressed, ampulliform to almost acerose 5.5–8(–9) × 2–3(–3.5) µm, some having a elongated neck of up to 2 µm in length; conidia spheroid 2.5–3 µm, smooth-walled, disordered.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type CV311 (PREM60054) (HOLOTYPE); *Additional specimens examined:* South Africa, Western Cape Province, Malmesbury, Kalbaskraal and Riverlands (S 33,57061°; E 18,62861° and S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV112 (PREM60055), 271.

Penicillium cumulacinatum C.M. Visagie & K. Jacobs prov. nom.* **FIGS 6, 7**
Mycobank nr. MB 512441
Etymology. Latin, *cumulacinatum*: *cumulus* = pile; *acinus* = berry, referring to the long chains of conidia produced in culture.

* Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere

Coloniae in CYA post 7 dies in 25°C 37–48 mm sclerotia dura abundantia facientes. Coloniae in MEA post 7 dies in 25°C 35–50 mm, rarius minores, sclerotia similia facientes. Conidiophorae semper univerticillatae, in superficie portatae; stipa laevis, vesiculata, 250–400(–490) × 2–3 µm; phialides ampulliformae 7–9 × 2–3 µm; conidia sphaeroidea subtiliter exasperata vel laevia 2.5–3 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 37–48 mm diam, sulcate, moderately dense to loose in some isolated, mostly velutinous, but somewhat floccose at centre, brown very hard sclerotia produced on CYA and MEA; margins low, regular, mycelia white; conidiogenesis sparse to moderate, grayish green to Pale Green (27a3) and Deep Turquoise (24d8) in isolates sporulating more readily; exudate clear to brownish, soluble pigment Poison Yellow (1a8) to Vivid Yellow (2a8), sometimes more brownish, reverse pale to Poison Yellow (1a8); At 5°C, 7 days: Germination occurring; At 37°C, 7 days: No growth; MEA, 25°C, 7 days: Colonies 35–50 mm diam, sometimes slightly smaller, plane, moderately dense, velutinous; margins low, regular, mycelia white; conidiogenesis moderate, similarly colored as on CYA; exudate and soluble pigment commonly not produced, but in some isolates clear exudate, with yellowish soluble pigment, reverse pale. G25N, 25°C, 7 days: Colonies 15–18 mm, sulcate, velutinous to floccose; mycelia white; conidiogenesis moderate, similarly colored as on CYA; exudate absent, soluble pigment Poison Yellow (1a8) to more brownish in some isolates, reverse Poison Yellow (1a8) to brownish.

Conidiophores borne from surface, stipes smooth-walled, 250–400(–490) × 2–3 µm, commonly vesiculate, 4–6 µm, bearing strictly monoverticillate penicilli; phialides 9–13, appressed, ampulliform, 7–9 × 2–3 µm; conidia spheroid, 2.5–3 µm, commonly finely roughened, but smooth present, produced in long compact chains.

Specimens examined: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type CV237 (PREM60056) (HOLOTYPE); *Additional specimens examined:* South Africa, Western Cape Province, Malmesbury, Kalbaskraal, Camphill Village and Riverlands: (S 33,57061°; E 18,62861°, S 33,59787°; E 18,56433° and S 33,49066°; E 18,58388°). Isolated from soil, 21

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Feb 2007, collected by C.M. Visagie, CV80 (PREM60057), 155 (PREM60058), 197 (PREM60060), 222, 229, 397 (PREM60059).

Penicillium vulgaris C.M. Visagie & K. Jacobs prov. nom.* **FIGS 8, 9**

Mycobank nr. MB 512442

Etymology. Latin, *vulgaris*: meaning ordinary.

Coloniae in CYA post 7 dies in 25°C 42–45 mm sclerotia dura facientes. Coloniae in MEA post 7 dies in 25°C 57–60 mm. Conidiophorae semper univerticillatae, in hyphis aeriis portatae; stipa parietibus asperis, plerumque vesiculata, (90–)120–345 × 2–3 µm; phialides ampulliformae vel subacerosae 7–9 × 2–3 µm; conidia sphaeroidea vel subsphaeroidea laevia 2–3 µm.

Colony morphology, CYA, 25°C, 7 days: Colonies 42–45 mm diam, sulcate, moderately dense, floccose texture, hard sclerotia present; margins subsurface, wide, regular, mycelia mostly white, but inconspicuously salmon, giving colonies a pinkish/salmon color; conidiogenesis moderate, grayish green (25b3–25c3); clear exudate produced, soluble pigment absent, reverse light yellow (4a4); At 5°C, 7 days: Microcolonies; 37°C, 7 days: No growth. MEA, 25°C, 7 days: Colonies 57–60 mm diam, plane, texture velutinous; margins narrow, low, regular, mycelia white; conidiogenesis heavy, dark green to grayish green (25d7–25e7); exudate and soluble pigment absent, reverse a pale whitish green. G25N, 25°C, 7 days: Colonies 20–24 mm, plane, velutinous; mycelia white; moderate conidiogenesis, grayish green at centre and Absinth Green (30D5); exudate absent and soluble pigment absent, reverse grayish yellow to Chartreuse (2c6).

Conidiophores borne from aerial hyphae, stipes rough-walled, generally vesiculate with apical swelling of up to 5 µm, (90–)120–345 × 2–3 µm, bearing strictly monoverticillate penicilli; phialides 12–14, appressed, ampulliform to almost acerose 7–9.5 × 2–3 µm; conidia spheroid to somewhat subspheroidal 2–3 µm, smooth-walled, in short columns, connectives visible.

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* Please note that the following species description should not be cited and are printed here in preliminary form. These will be formally printed elsewhere

Specimens examined: South Africa, Western Cape Province, Malmesbury, Riverlands: (S 33,49066°; E 18,58388°). Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, ex-type CV282 (PREM60061) (HOLOTYPE); *Additional specimens examined:* South Africa, Western Cape Province, Malmesbury, Kalbaskraal (S 33,57061°; E 18,62861°), Riverlands (S 33,49066°; E 18,58388°), Isolated from soil, 21 Feb 2007, collected by C.M. Visagie, CV286 (PREM60062).

Key incorporating the new species from *Penicillium* subgenus *Aspergilloides* and their closely related species

1. Stipe vesiculate.......................................**2**
1. Stipe non-vesiculate.......................................**8**

2. Conidia ellipsoidal.......................................**3**
2. Conidia spheroid to subspheroidal.......................................**4**

3. Conidia smooth-walled 2.5–3 µm in length.......................................***Eup. lapidosum***
3. Conidia finely rough to conspicuously rough-walled
   3.5–4 µm in length.......................................***P. thomii***

4. Conidia spinose or rugulose.......................................**5**
4. Conidia smooth to finely roughened.......................................**6**

5. Conidia rugulose.......................................***P. purpurescens***
5. Conidia spinose.......................................***P. spinulosum***

6. Brown hard sclerotia produced on CYA and MEA.......................................***P. cumulacinatum***
6. Brown hard sclerotia absent on CYA or MEA.......................................**7**

7. Stipes conspicuously roughened; colonies on MEA >55 mm.......................................***P. vulgaris***
7. Stipes smooth to finely roughened; colonies on MEA <55 mm.......................................***P. glabrum***

8. Colonies on CYA and MEA <25 mm and stipes <60 µm.......................................**9**
8. Colonies on CYA or MEA >25 mm or stipes >60 µm.......................................**11**

9. Colonies on CYA and MEA reddish brown soluble pigment and
   deep reddish brown reverse coloration.......................................***P. roseopurpureum***
9. Soluble pigment absent and reverse, when
   colored, lightly so.......................................**10**

10. Conidia rough to echinulate, sometimes smooth;
    colonies on G25N >10 mm.......................................***P. restrictum***
10. Conidia smooth; colonies on G25N <10 mm.......................................***Eup. meridianum***

11. Stipes commonly <60 µm.......................................**12**
11. Stipes commonly >60 µm.......................................**16**

12. On CYA and MEA having brightly orange colored
    colonies and colony reverses.......................................***P. hirayamae* (=*Eup. hirayamae*)**
12. Colonies not having orange features.......................................**13**

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13. Conidia >4.5 μm in long axis..**14**
13. Conidia <4.5 μm in long axis..**15**

14. Colonies on G25N >6 mm and CYA (37°C) >35 mm.............................***Eup. levitum***
14. Colonies on G25N <6 mm and CYA (37°C) <35 mm...........................***Eup. ehrlichii***

15. Colonies on MEA <30 mm; phialide length 7–9 μm;
conidia 3–3.5 μm..***P. raperi***
15. Colonies on MEA >30 mm; phialide length 4–7 μm;
conidia 2.5–3..***P. brachycaulon***

16. Colonies on CYA and MEA <30 mm; colonies on both
having bright yellow features...............................***P. toxicarium/P. citreonigrum***[*]
16. Colonies on CYA and MEA >30 mm; colonies lacking
bright yellow features..**18**

18. Penicillus commonly biverticillate...***P. janthinellum***
18. Penicillus commonly monoverticillate..**19**

19. Conidia spheroidal; colonies on CYA (37°C) >25 mm.........***P. malacosphaerula***
19. Conidia ellipsoidal; colonies on CYA (37°C) <25 mm..........***Eup. reticulisporum***

DISCUSSION

During this study, eight species belonging to *Penicillium* subgenus *Aspergilloides* were isolated. In a similar study in the CFR, Allsopp et al. (1987) isolated four monoverticillate species from roots and surrounding soils, which included *P. restrictum, P. glabrum, Eup. hirayamae* and *Eup. pinetorum*. During our survey, *P. restrictum* (FIGS 10, 11) and the anamorphic *P. hirayamae* (FIGS 12, 13) were also isolated, as well as *P. roseopurpureum* (FIGS 14, 15) and *P. toxicarium* (FIGS 16, 17).

Penicillium restrictum strains from fynbos soil, clustered with *Eupenicillium meridianum*, close to *P. restrictum* (ex-neotype, AF033459) (FIG. 1). Morphologically, the fynbos strains are easily distinguished from *Eup. meridianum*, but it did fit Pitt's (1979) *P. restrictum* description, with the exception of slightly faster growing colonies. Pitt (1979) found large intraspecies variations for *P. restrictum*, as is evident in his description of the conidia which can be spheroid, ellipsoid or pyriform with smooth, finely

[*] Species distinguished on the basis of their genetic characters

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roughened or echinulate walls. This variation, especially in conidial ornamentation, was also seen in the fynbos strains. The difficulty in delineating this species is also evident in its phylogenetic characters. Sequences of the ITS gene region revealed that the fynbos strains, some having smooth-walled and others echinulate conidia, were identical. When these strains β-tubulin regions were compared, they varied by much as five base pairs, but did not group according conidial ornamentation (data not shown). From this, it is evident that much more work regarding this particular species is needed.

Strains identified as *Penicillium roseopurpureum* and *P. hirayamae,* respectively, exhibited characters similar to that previously reported by Pitt (1979). In addition, these strains clustered together with the ex-type strain of *P. roseopurpureum* (AF033415) and the non-type *Eup. hirayamae* (AF033415) in the phylogenetic tree. The fynbos *P. toxicarium* strains was originally identified, using morphology, as *P. citreonigrum*, which Pitt (1979) considered to be synonymous with *P. toxicarium*. The fynbos strains does, however, differ slightly from previous descriptions for *P. toxicarium* in that they have smooth-walled conidia, as Pitt (1979) described for *P. citreonigrum*, compared to the slightly rough-walled conidia for *P. toxicarium* proposed by Ramirez (1982). From a multi-gene phylogeny, done by Serra et al. (2006), it was clear that these are two separate species. The fynbos strains clustered into a clade together with *P. toxicarium*, and were, therefore, identified as such. From our study, *P. toxicarium* did show more restricted growth to that suggested by Pitt for *P. citreonigrum*. We did, however, not examine the latter's type strains and currently cannot distinguish between these two species using morphological characters and as such, the key provided in this thesis, does not attempt this.

Penicillium brachycaulon characteristically produces olive reverse colored colonies, as well as the colonies often being centrally sunken in. Micromorphologically, the new species produces monoverticillate conidiophores borne on very short stipes, with solitary phialides borne directly on fertile mycelia not uncommon, and conidia that is typically lightly rough-walled. *Penicillium brachycaulon* is phylogenetically closely related to *P. janthinellum*

and *P. raperi*, which Pitt (1979) considered to be synonyms and belonging in subgenus *Furcatum*. *Penicillium brachycaulon,* however, clearly belong to subgenus *Aspergilloides,* since it strictly produces monoverticillate conidiophores. In addition to penicillus orientation, the new species are easily distinguished from *P. janthinellum* on the basis of its much shorter stipes and finely roughened conidia compared to the latter's longer stipes and smooth-walled conidia, as well as growing much more restricted. *Penicillium raperi* seems to be, at least morphologically, more similar to *P. brachycaulon. Penicillium raperi* does, however, often produce irregular biverticillate conidiophores and have longer phialides and conidia (Smith 1957), when compared to the new species.

Penicillium malacosphaerula are distinguished by the yellow nature of its rapidly growing colonies, as well as numerous protocleistothecia produced. In addition to this, the new species produces monoverticillate conidiophores with phialides that is variable in its shape and, therefore, dimensions. The new species are resolved in a clade consisting exclusively of species that produce teleomorph states, and includes *Eupenicillium reticulisporum, E. levitum* and *E. ehrlichii*. Although the new species are placed amongst *Eupenicillium* spp., it produces only protocleistothecia, without ascospores, despite all *Eupenicillium* spp. being homothallic (Scott 1968). The reason for the new species not producing asci are unclear. Since it may take weeks for maturation to occur (Pitt 1979), the fact that it is not producing a sexual state is not considered to be essential for identification purposes. *Penicillium malacosphaerula* is easily distinguished from the other species in this clade, based on its much smaller conidia (2.5–3 μm), when compared to *E. levitum* and *E. ehrlichii*. Compared to *E. reticulisporum*, *P. malacosphaerula* grows much faster on CYA at 37°C and produces spheroid conidia compared to its sister taxons' ellipsoidal conidia. Although there was little bootstrap support for branches in clade A (FIG. 1) the clear morphological differences easily distinguishes *P. malacosphaerula* as distinct.

Penicillium cumulacinatum are characterized by colonies producing abundant hard sclerotia on CYA and MEA, as well as its monoverticillate vesiculate

conidiophores which bears long chains of smooth to finely rough-walled conidia. *Penicillium cumulacinatum* is resolved in a clade separate of all previously described species, with species from clade C (FIG. 1) as its closest relatives. This is also reflected in its morphological characters, which places *P. glabrum* and *P. spinulosum* as its closest relatives. *Penicillium cumulacinatum* is distinguished from both these species based on its smooth stipes compared to the often rough-walled stipes of *P. glabrum, P. vulgaris* and *P. spinulosum*. In addition to the ornamentation of the stipes, it is in general also much longer than other species from clade C. *Penicillium cumulacinatum* also produces abundant hard brown sclerotia on both CYA and MEA which is absent in both *P. glabrum* and *P. spinulosum*.

Penicillium vulgaris are characterized by its colonies on CYA having a salmon/pink color and producing whitish hard sclerotia. Micromorphologically, the new species produce monoverticillate vesiculate conidiophores having rough-walled stipes and smooth-walled conidia. *Penicillium vulgaris* has *P. spinulosum* as its closest related sister taxon, as well as being closely related to species resolved in clade C (FIG. 1), based on morphology and the ITS phylogeny. Species from this clade are considered to be distinct, although difficulties are experienced in separating them (Pitt et al. 1990). *Penicillium vulgaris* are distinguished from *P. spinulosum* by its faster growing colonies on MEA and its smooth-walled spheroid conidia compared to the finely rough-walled to spinose conidia of *P. spinulosum*. The new species are distinguished from *P. glabrum*, based on its colonies on CYA having a definite floccose texture. The species included in clade C (FIG. 1), although they are morphological considered distinct, are known to have a slow evolving ITS gene region showing very little variability. However, using the calmodulin gene region, Wang and Zhuang (2007) could successfully distinguish between these species, but as there is not a comprehensive database for the calmodulin gene sequences of *Penicillium,* the ITS data for this group has been used for characterization in this study.

From this and previous studies (Visagie et al. 2008, Visagie and Jacobs 2008a, 2008b) it is clear that much more work remains to be done on *Penicillium* from

the fynbos habitat. Although many new species were described and incorporated into identification keys, isolations resulted in roughly forty single specimen isolates that could not be placed into groups, based on their unique morphological characters. In addition to these, the strains examined were isolated from only one sampling date and considering the considerable difference in the summer and winter climate of the area, we expect to find a shift in species populations over the seasons. The number of species that have to date been identified from the coastal fynbos soil is, therefore, considered to be less than half of the total number of species expected to occur in this diverse region. Isolations made from soil collected in April and July 2007, resulted in a further 800 strains and when characterization of these commence, we do expect to find more unique species. In addition to the proportion of species thus far characterized, the coastal fynbos region represents only a small portion of the fynbos biome. The species diversity, and number of unique *Penicillium* spp. in the CFR could, therefore, be staggering. This, together with previous studies (Visagie et al. 2008, Visagie and Jacobs 2008a, 2008b) is, therefore, considered to only serve as a base from which we can start exploring the extent of diversity in the fynbos biome and a better understanding of this complex genus.

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TABLE 1. Species names and their respective culture collection and GenBank accession numbers used for phylogenetic comparisons.

Species	**Culture collection number**	**GenBank number**
Eup. brefeldianum	NRRL710	AF033435
Eup. ehrlichii	NRRL708	AF033432
Eup. hirayamae	NRRL143	AF033418
Eup. katangense	NRRL5182	AF033458
Eup. lapidosum	NRRL718	AF033409
Eup. levitum	NRRL705	AF033436
Eup. meridianum	NRRL5814	AF033451
Eup. reticulisporum	NRRL3447	AF033437
Eup. shearii	NRRL715	AF033420
	SS133-1	AY232278
Eup. stolkiae	NRRL5816	AF033444
P. adametzii	NRRL25744	AF034459
	NRRL737	AF033401
P. brachycaulon	CV188	FJ231021
	CV354	FJ231022
	CV361	FJ231023
P. citreonigrum	NRRL2046	EF198647
	NRRL761	AF033456
P. cumulacinatum	CV155	FJ231031
	CV197	FJ231028
	CV237	FJ231027
	CV397	FJ231030
	CV80	FJ231029
P. daleae	NRRL708	AF033442
P. donkii	NRRL5562	AF033445
P. dravuni	F01V25	AY494856
P. glabrum	NRRL766	AF033407
P. hirayamae	CV161	FJ231014
	CV21	FJ231013
P. janthinellum	NRRL2016	AF033434
P. lividum	NRRL754	AF033406
P. malacosphaerula	CV112	FJ231024
	CV271	FJ231025
	CV311	FJ231026
P. purpurescens	NRRL720	AF033408
P. quercetorum	NRRL3758	AY443471
P. raperi	NRRL2674	AF033433
P. restrcitum-like	CV136	FJ231011
	CV301	FJ231012
	CV316	FJ231010
	CV387	FJ231008
	CV419	FJ231009
P. restrictum	NRRL1748	AF033457
	NRRL25744	AF033459
P. roseopurpureum	CV186	FJ231020

P. roseopurpureum	CV36	FJ231018
	CV86	FJ231019
	NRRL2064	AF033415
P. sclerotiorum	NRRL2074	AF033404
P. spinulosum	FRR1750	AY373933
	MTFC02	DQ132828
	NRRL1750	AF033410
	NRRL728	AF034461
		AM176710
P. subarcticum	NRRL31108	AF481120
P. thomii	NRRL2077	AF034448
P. toxicarium	CV200	FJ231015
	CV204	FJ231016
	CV232	FJ231017
	IBL03172	DQ682595
	NRRL2047	EF198648
P. vulgaris	CV282	FJ231033
	CV286	FJ231032
T. trachyspermus	FRR1792	L14516

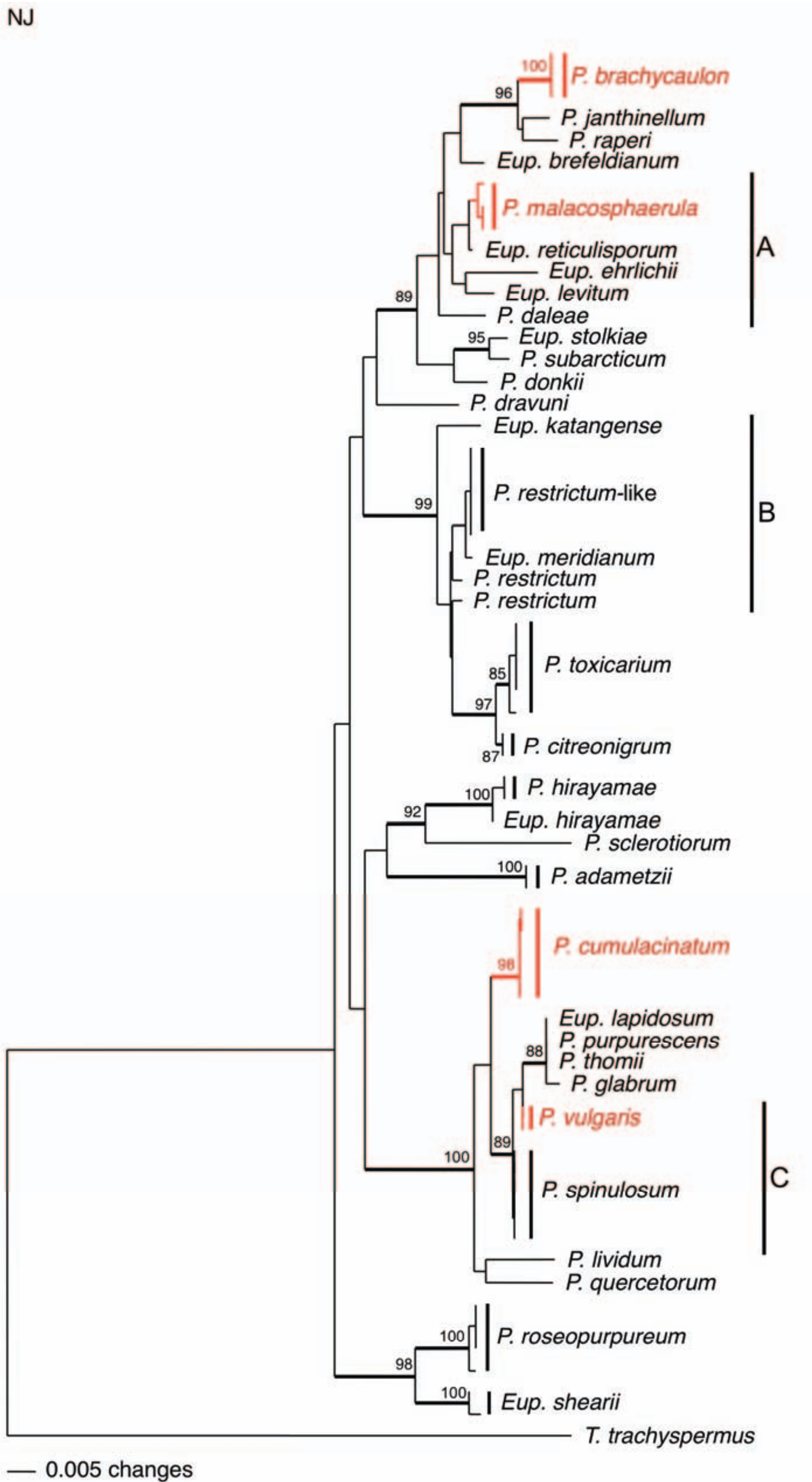

FIGURE 1. Neighbour-joining tree showing subgenus *Aspergilloides* species isolated from fynbos soil and their closest relatives, based on an ITS phylogeny. Bootstrap values higher than 80 are indicated above the thicker branches. *Talaromyces trachyspermus* were chosen as the outgroup.

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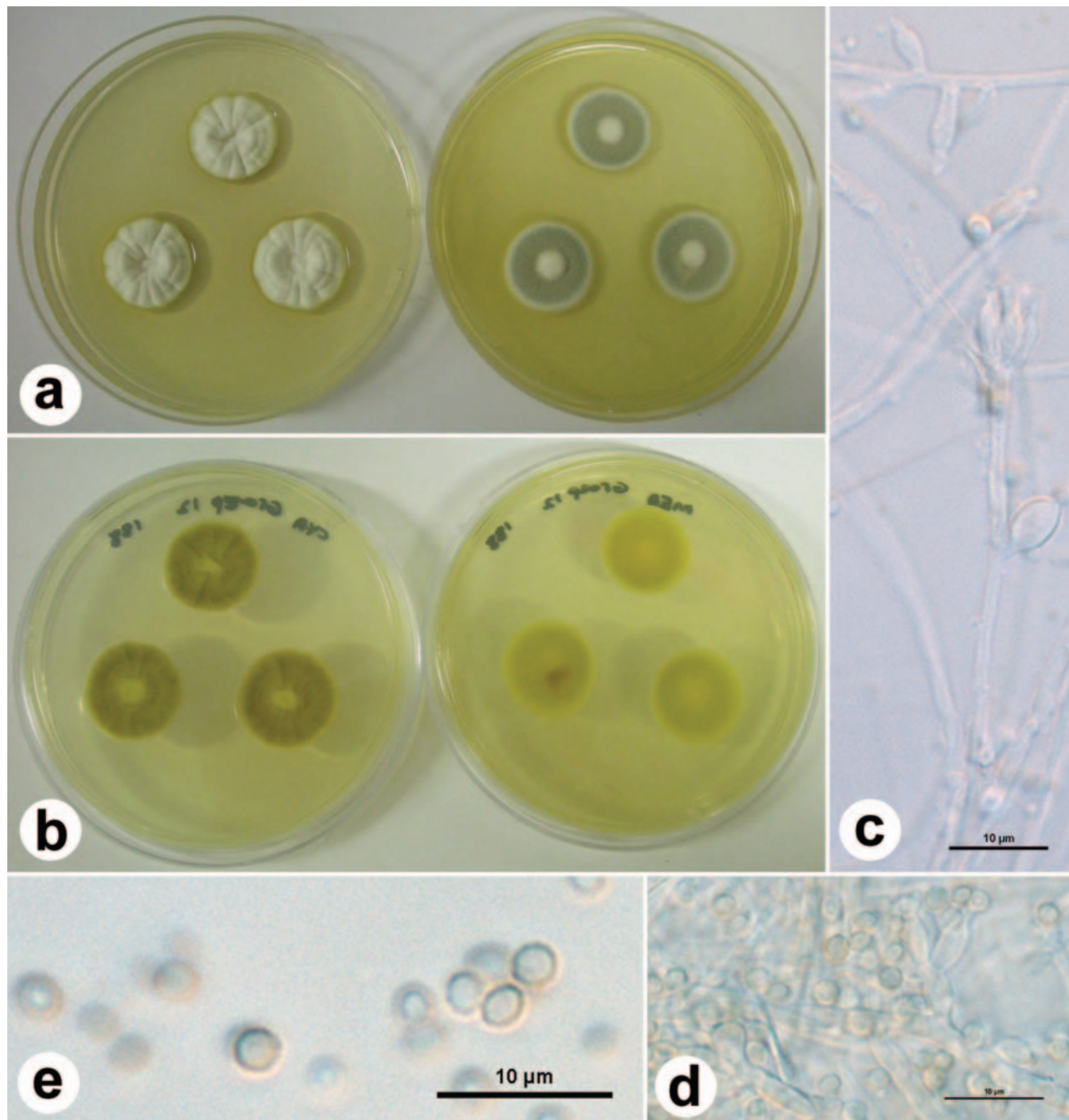

FIGURE 2. Morphological features characteristic of *P. brachycaulon*, holotype (PREM60045). a. Colonies of *P. brachycaulon* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies showing the characteristic olive colored reverses. c, d. Conidiophores typically produced in culture, sometimes with very short stipes, and solitary phialides borne directly on vegetative hyphae. e. Smooth to finely roughened subspheroidal to ellipsoidal conidia.

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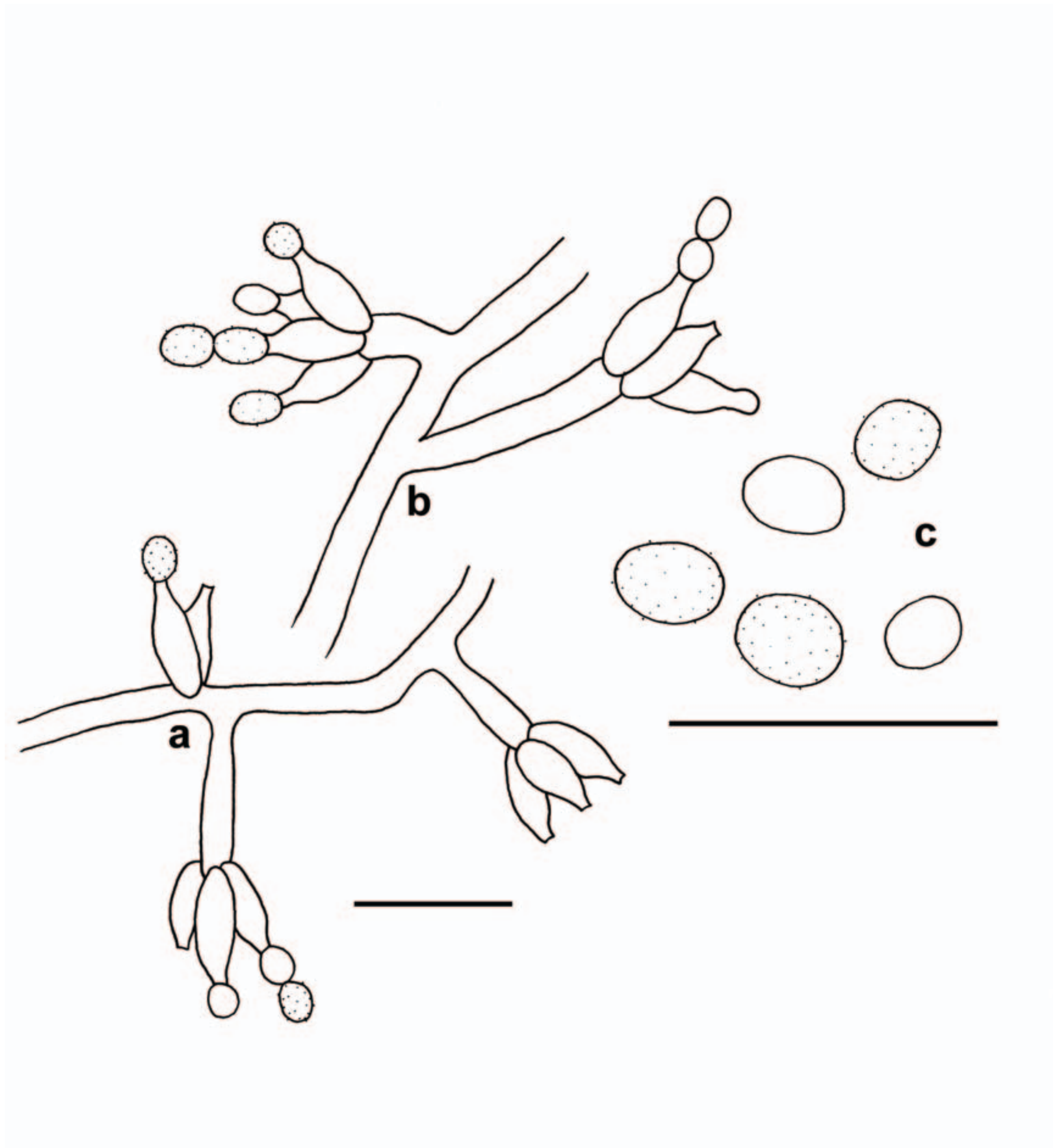

FIGURE 3. *Penicillium brachycaulon* line drawings from holotype (PREM60045) material. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

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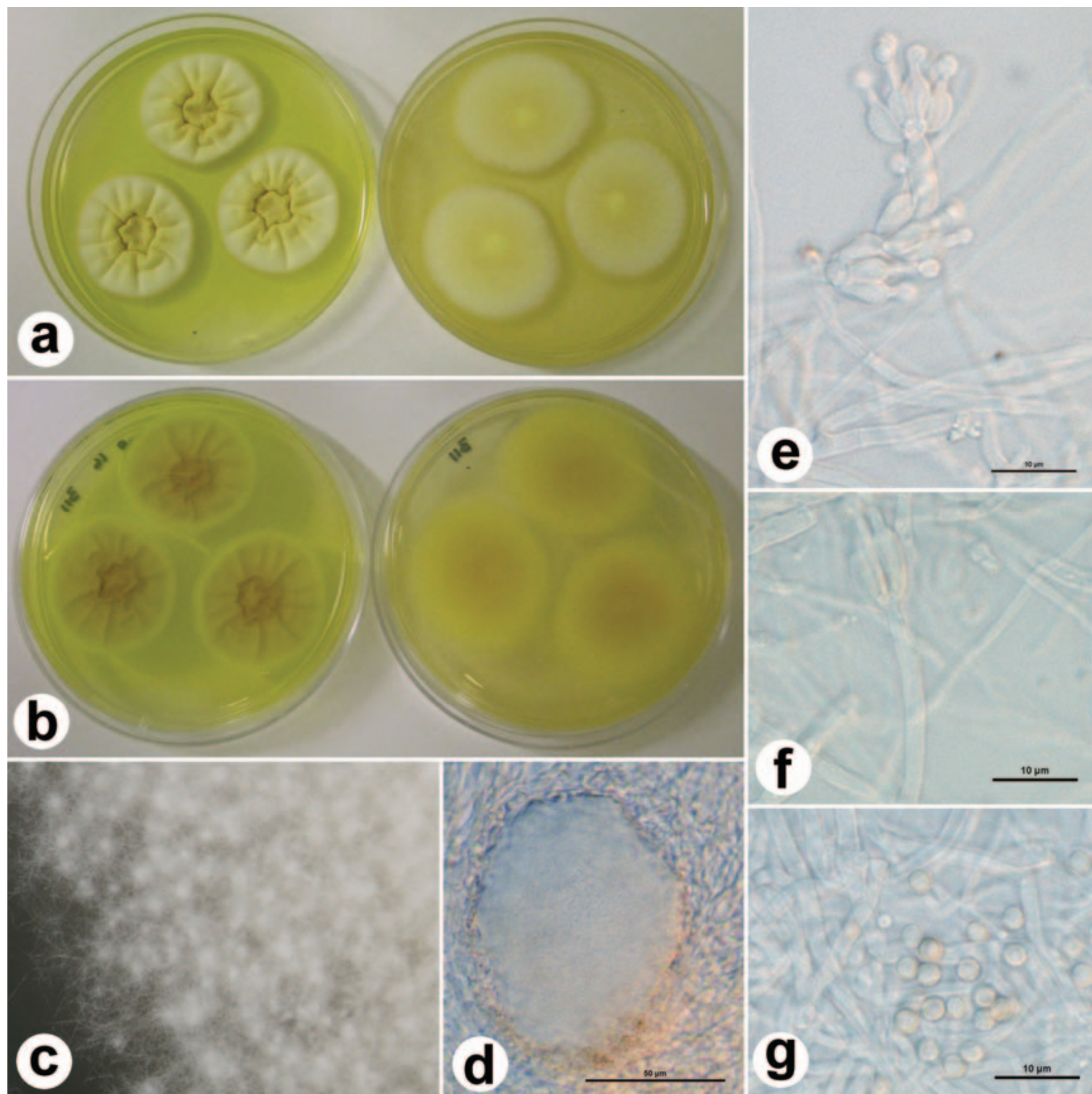

FIGURE 4. Morphological features characteristic of *P. malacosphaerula*, holotype (PREM60054). a. Colonies of *P. malacosphaerula* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c, d. Soft sclerotia produced on CYA. e, f. Conidiophores typically produced in culture. g. Smooth spheroid conidia.

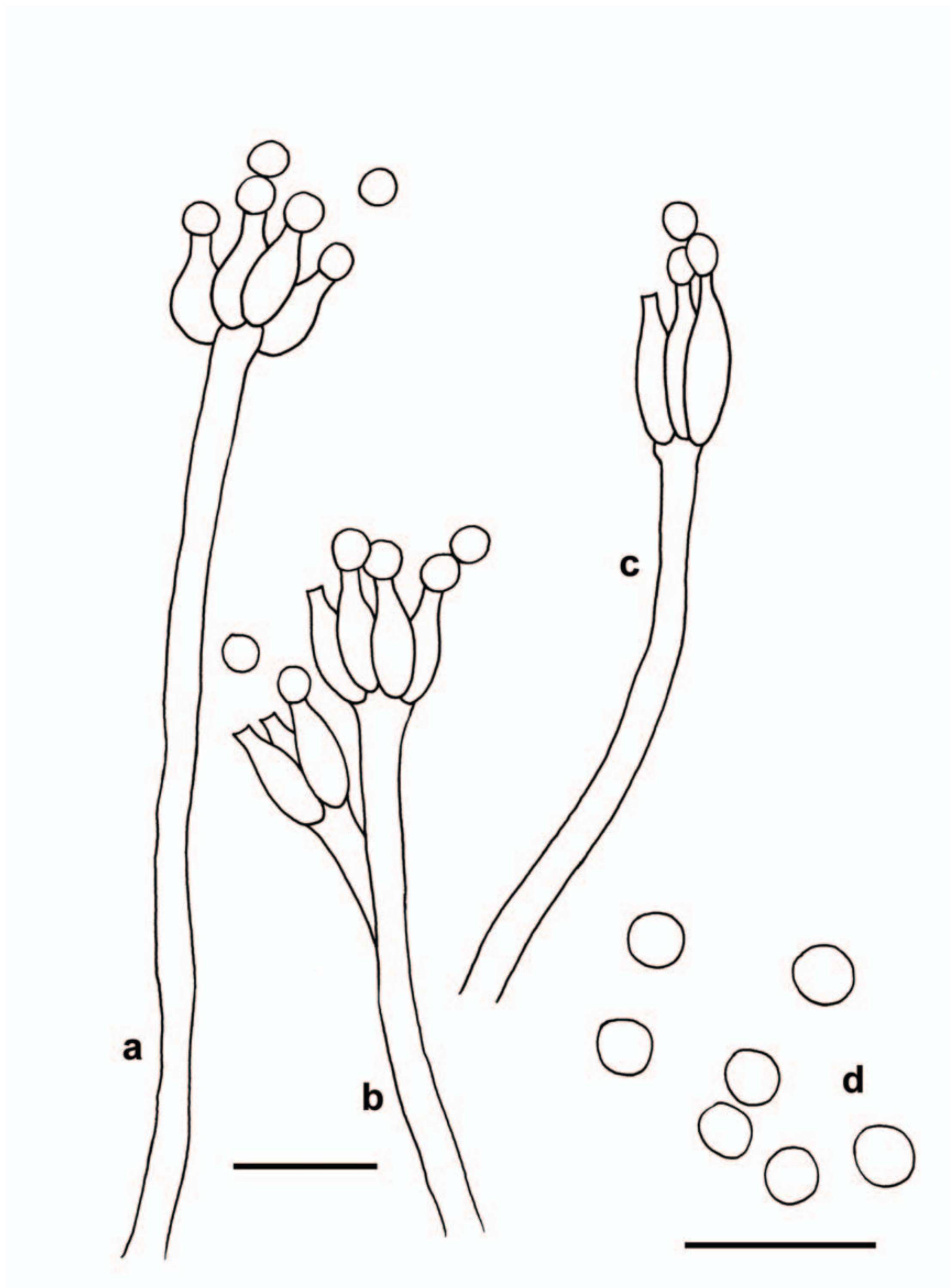

FIGURE 5. *Penicillium malacosphaerula* line drawings from holotype (PREM60054) material. a, b, c. Conidiophores (bar = 10 μm). d. Conidia (bar = 10 μm).

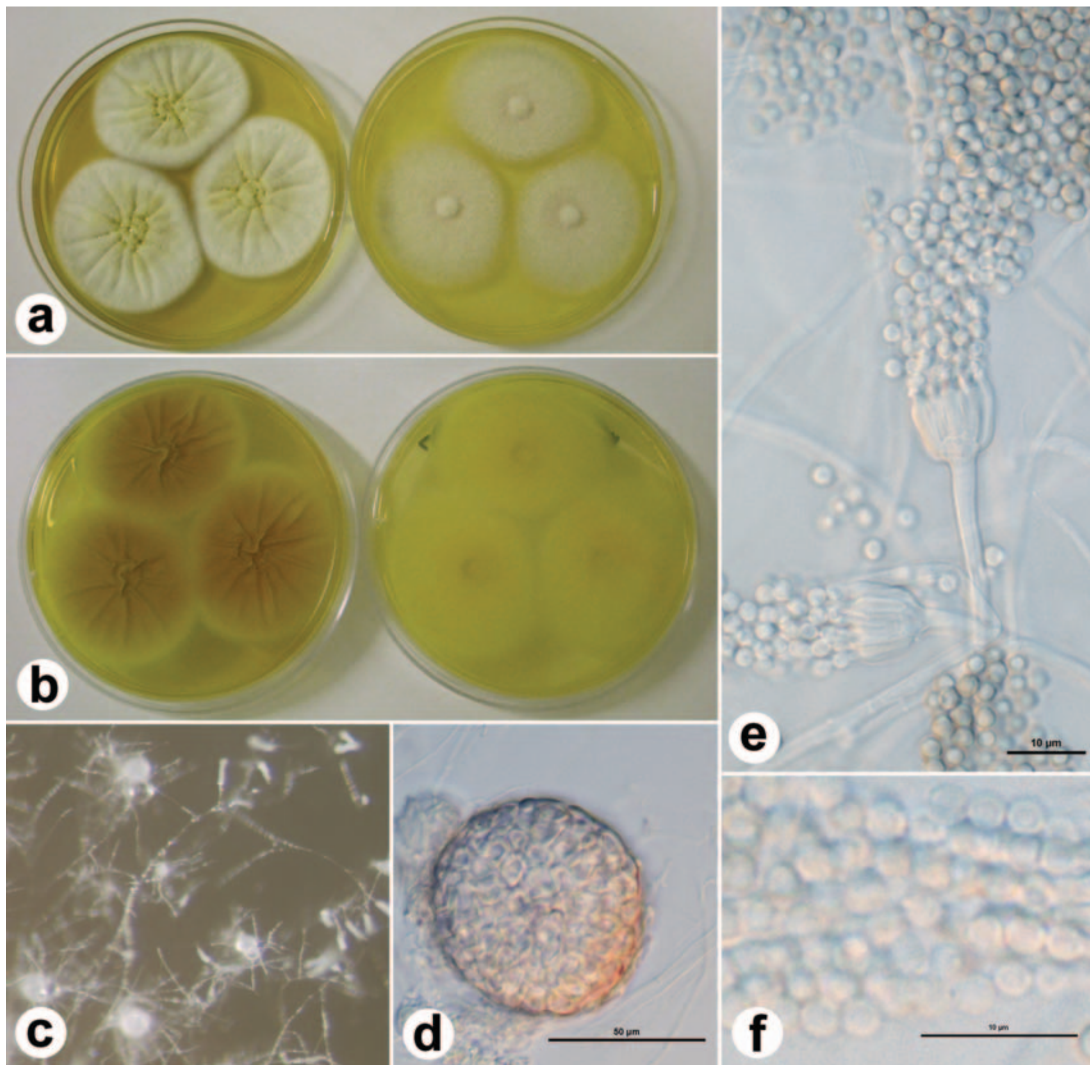

FIGURE 6. Morphological features characteristic of *P. cumulacinatum*, holotype (PREM60057). a. Colonies of *P. cumulacinatum* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c, d. Hard sclerotia produced. e. Conidiophores typically produced in culture. f. Smooth to finely roughened spheroid conidia produced in long chains.

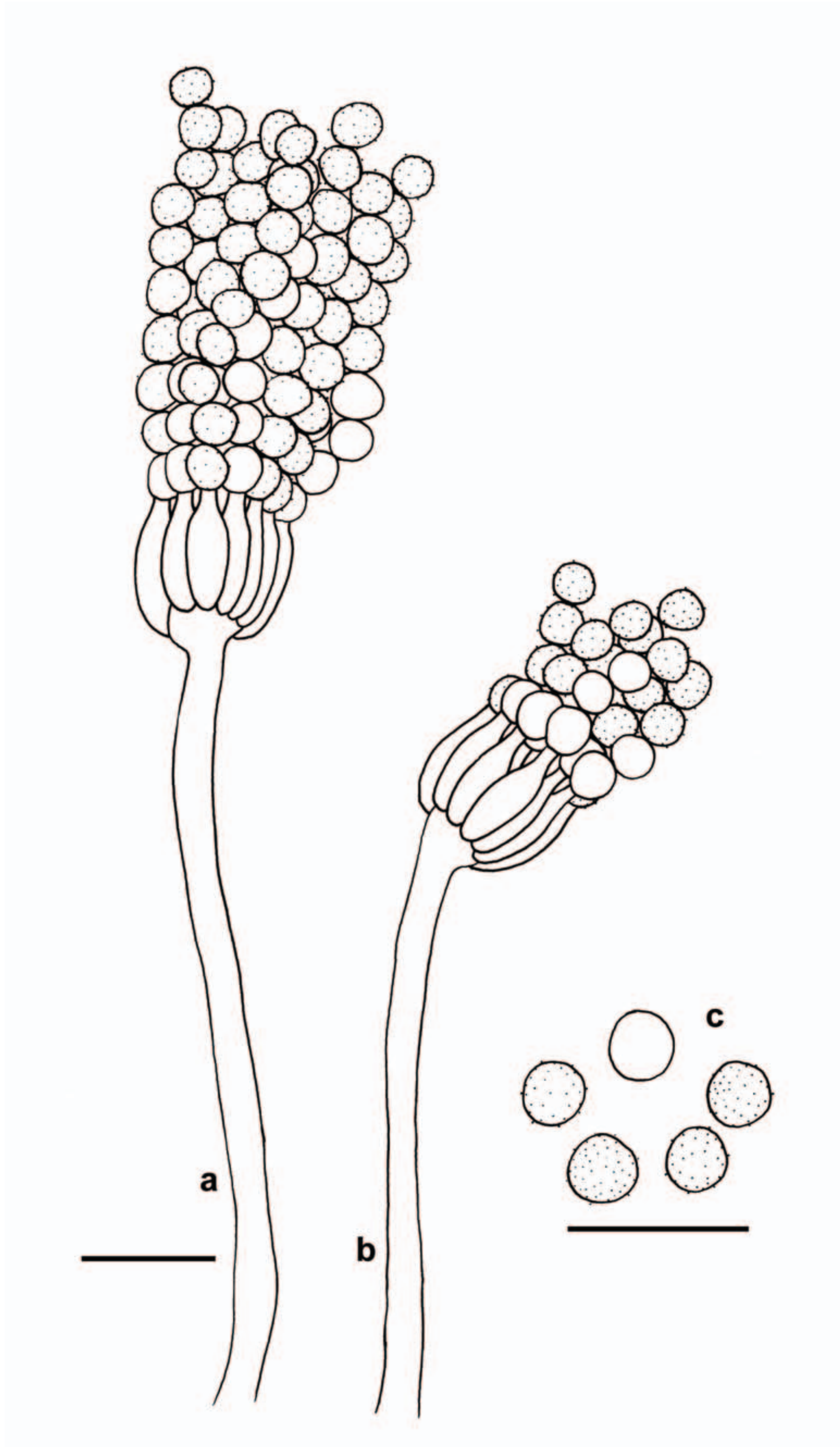

FIGURE 7. *Penicillium cumulacinatum* line drawings from holotype (PREM60056) material. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

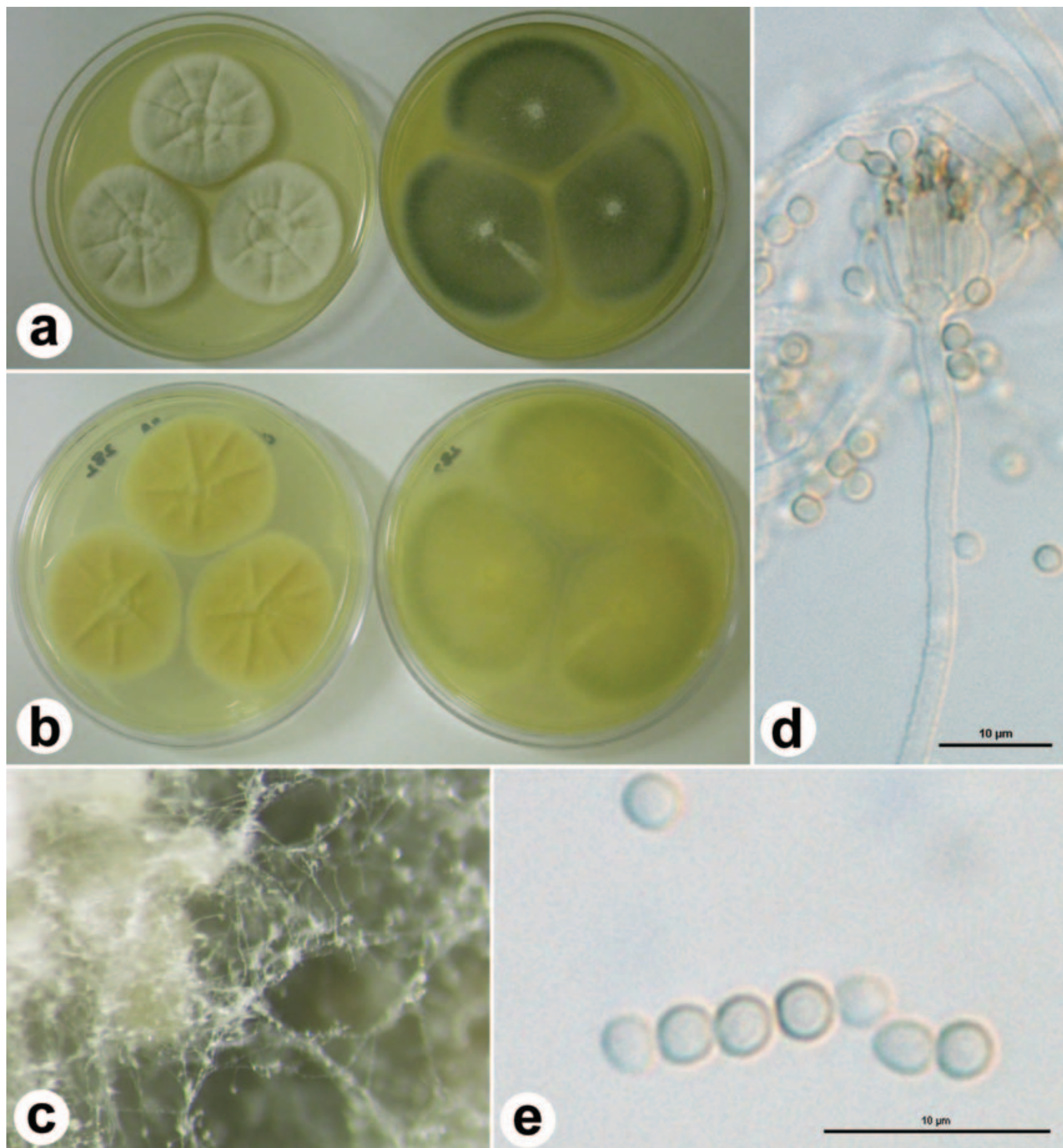

FIGURE 8. Morphological features characteristic of *P. vulgaris*, holotype (PREM60061). a. Colonies of *P. vulgaris* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c. Sclerotia produced, covered by mycelia. d. Conidiophores, typically with roughened stipes, produced in culture. e. Smooth spheroid, to occasionally subspheroidal, conidia produced.

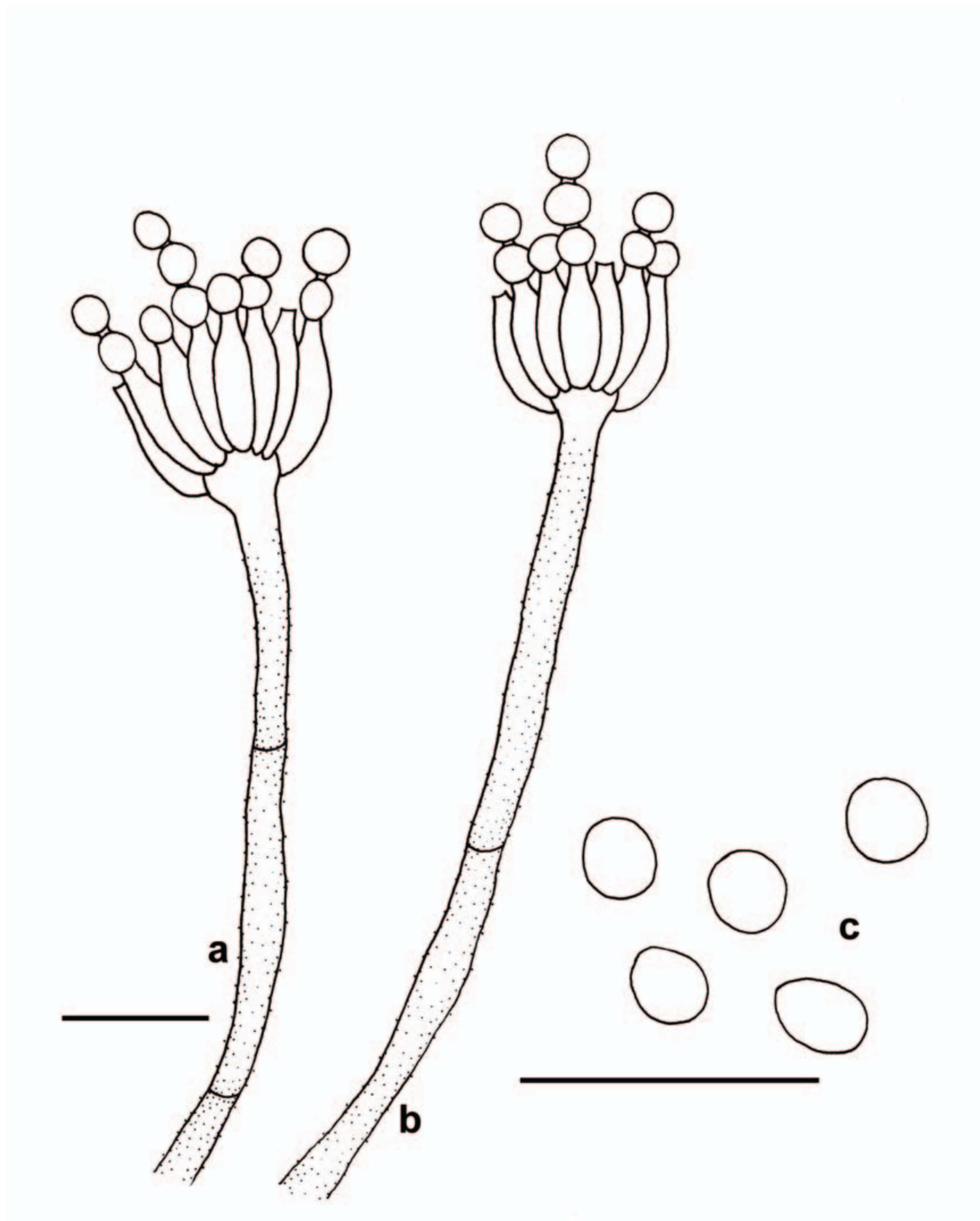

FIGURE 9. *Penicillium vulgaris* line drawings from holotype (PREM60061) material. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

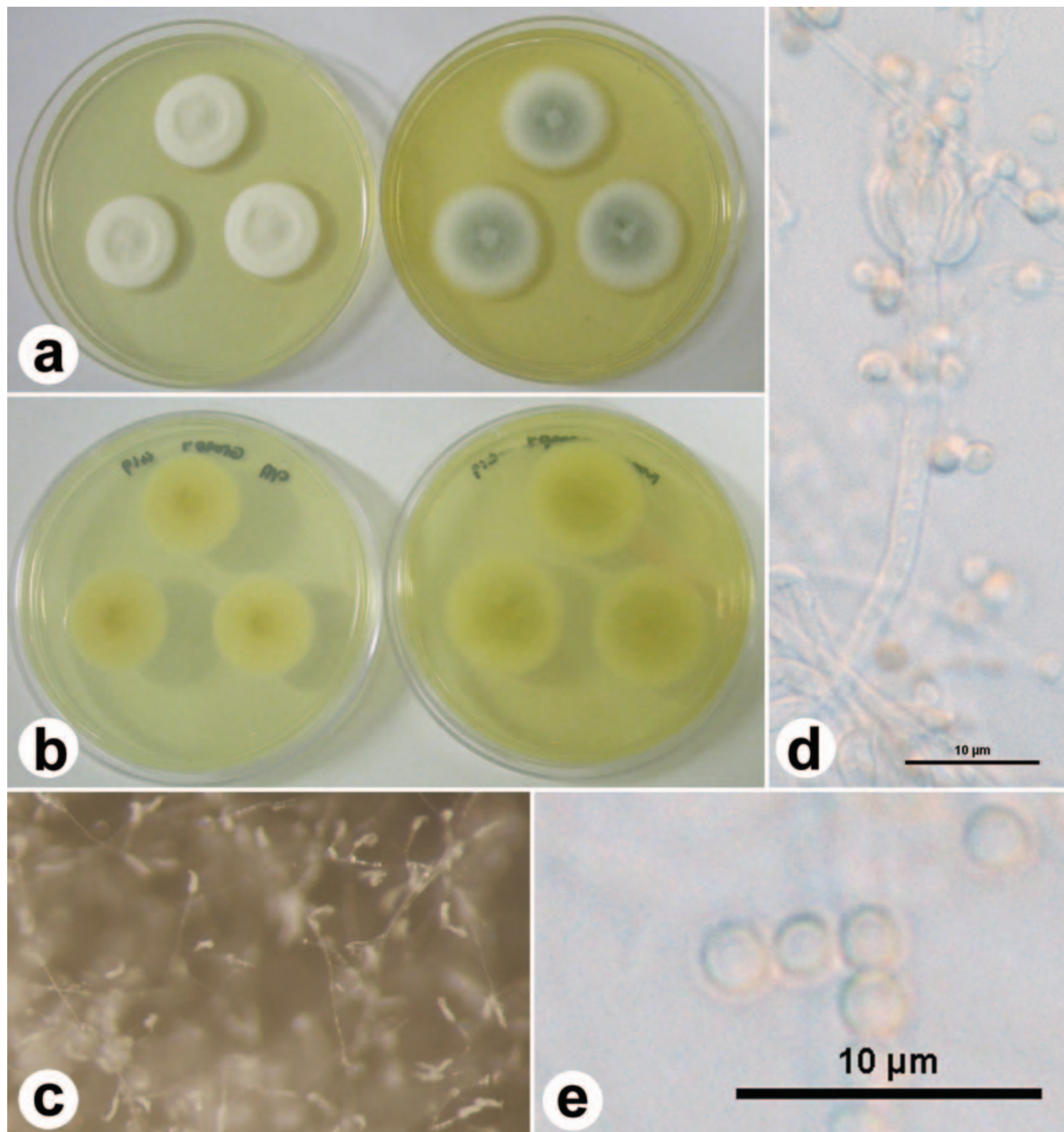

FIGURE 10. Morphological features characteristic of *P. restrictum* (CV419). a. Colonies of *P. restrictum* incubated on CYA (left) and MEA (right) after 7 days. b. Reverse of the same colonies. c. Floccose texture with conidiophores with short stipes borne on aerial hyphae. d. Conidiophores produced in culture. e. Smooth spheroid conidia produced by strain CV387.

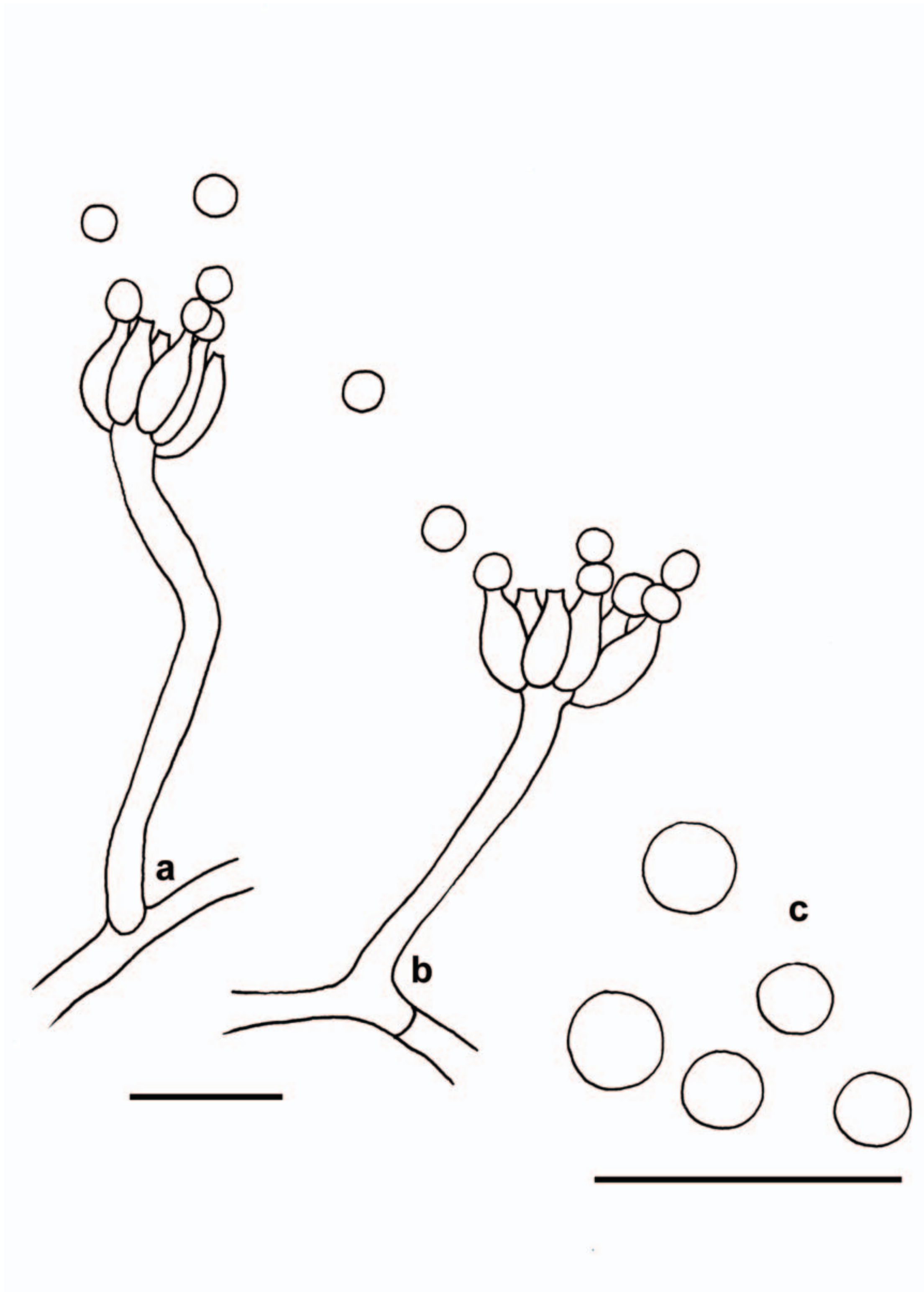

FIGURE 11. *Penicillium restrictum* line drawings from strain CV419. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

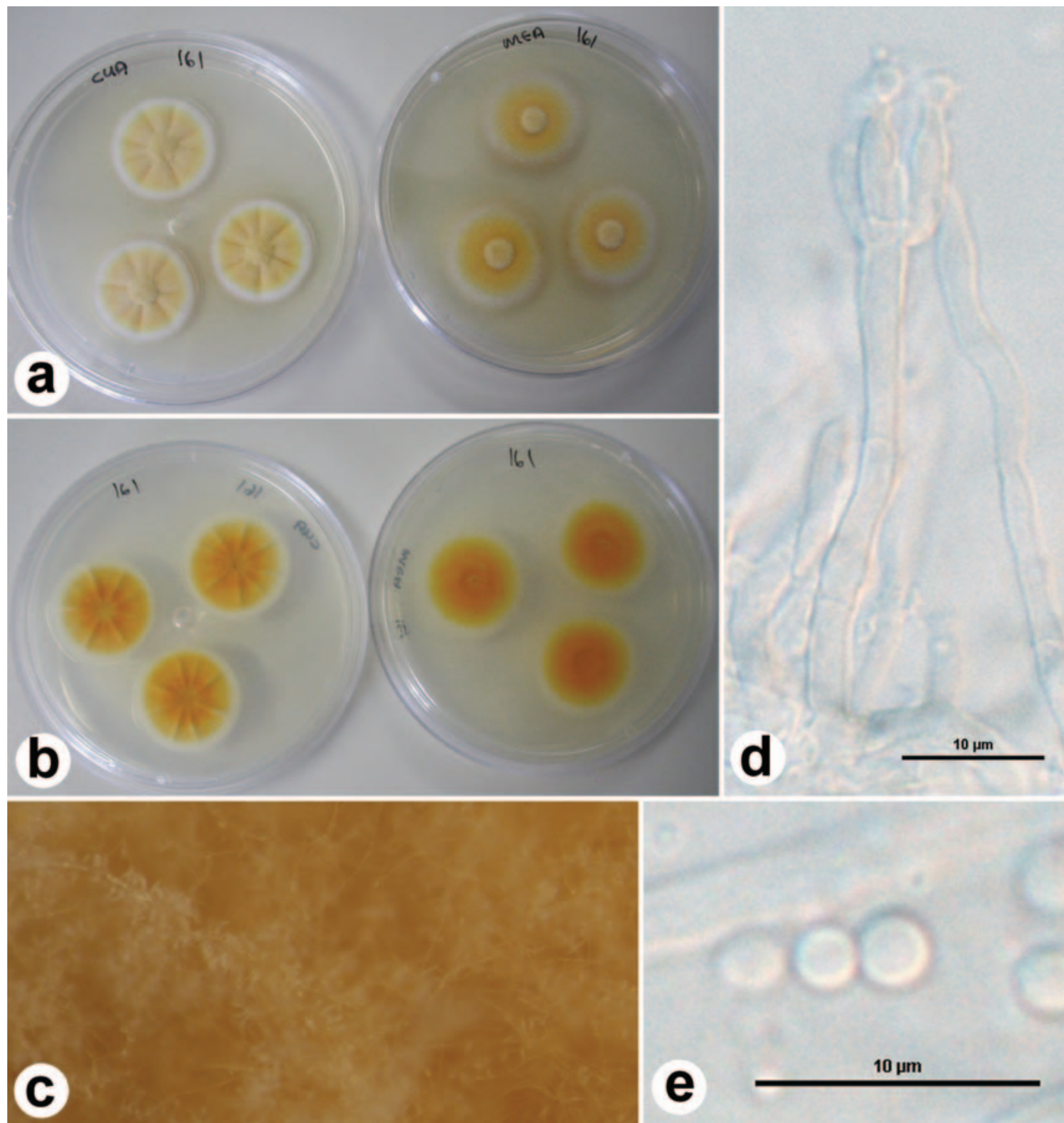

FIGURE 12. Morphological features characteristic of *P. hirayamae* (teleo = *Eupenicillium hirayamae*) (CV161). a. Colonies of *P. hirayamae* incubated on CYA (left) and MEA (right) after 7 days, showing the bright orange colors. b. Reverse of colonies, showing the characteristic deep orange colors. c. Orange mycelia producing the funicles from which conidiophores are borne. d. Conidiophores typically produced in culture. e. Smooth spheroid conidia produced.

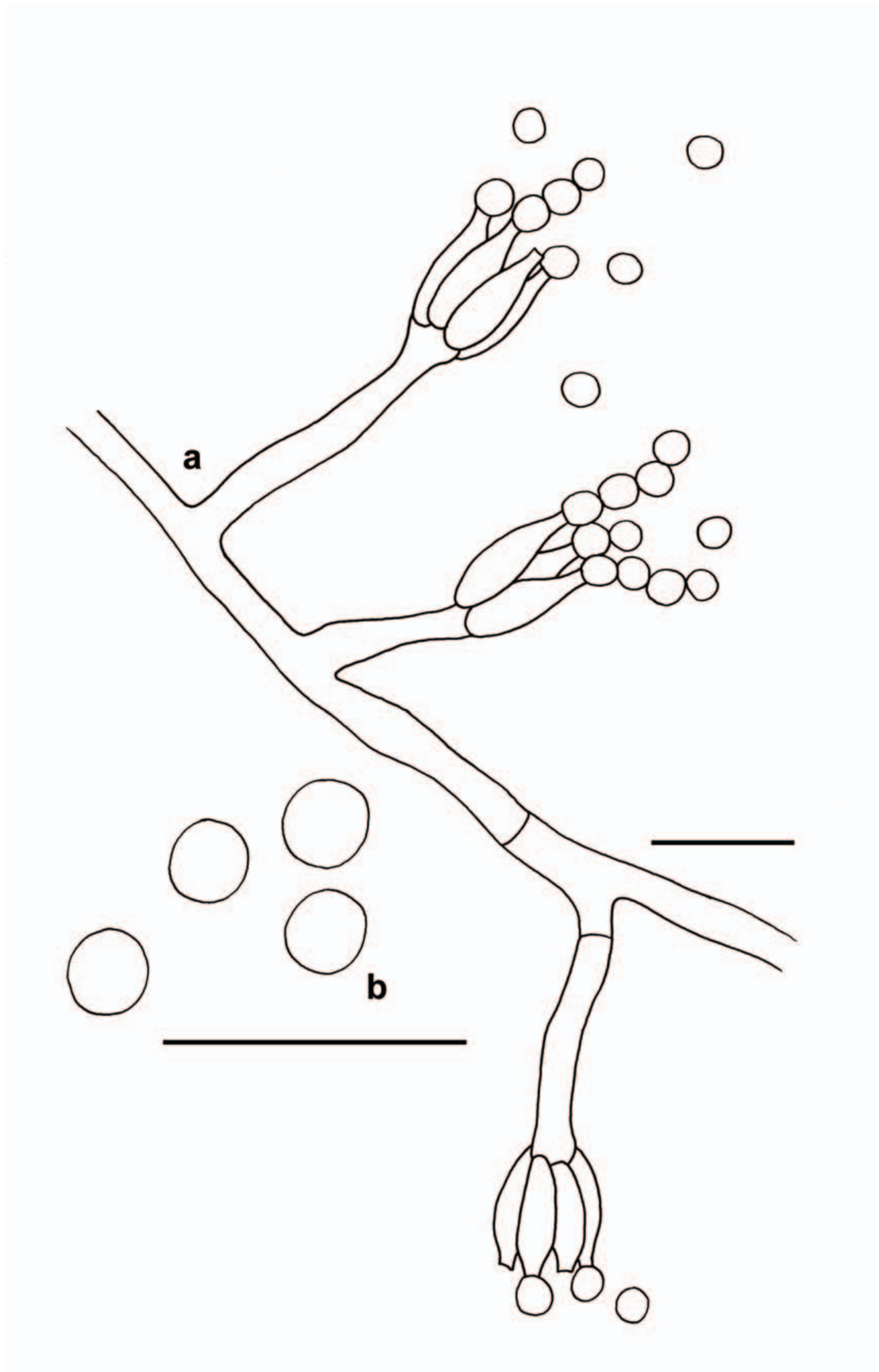

FIGURE 13. *Penicillium hirayamae* (teleo = *Eupenicillium hirayamae*) line drawings from strain CV161. a. Conidiophores (bar = 10 μm). b. Conidia (bar = 10 μm).

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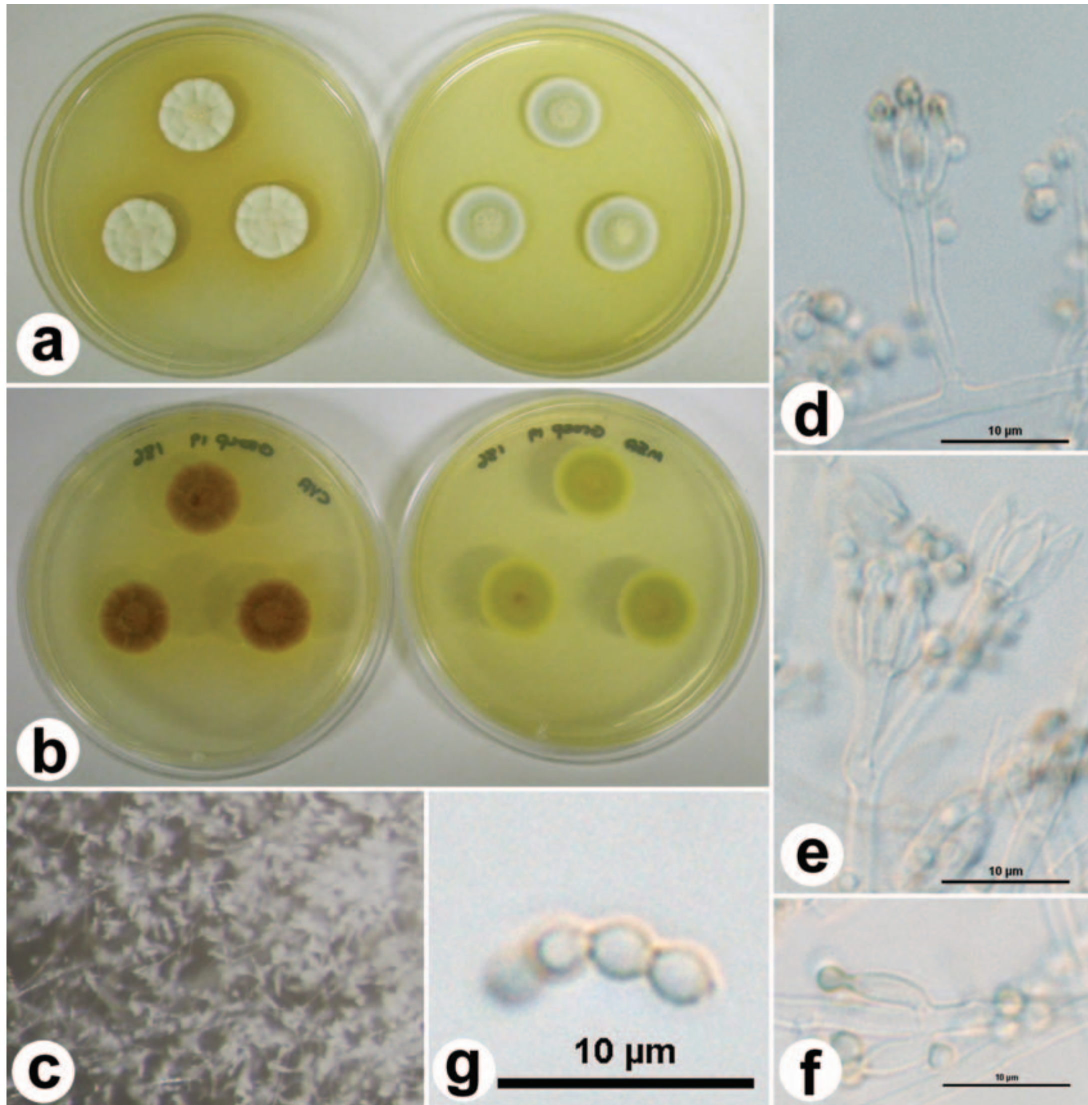

FIGURE 14. Morphological features characteristic of *P. roseopurpureum* (CV186). a. Colonies of *P. roseopurpureum* incubated on CYA (left) and MEA (right) after 7 days. b. Colonies showing the characteristic red reverse color on CYA. c. Moderate conidiogenesis occurring on MEA. d, e. Conidiophores typically produced in culture. f. Phialides occasionally borne directly on vegetative hyphae. g. Smooth spheroid conidia produced.

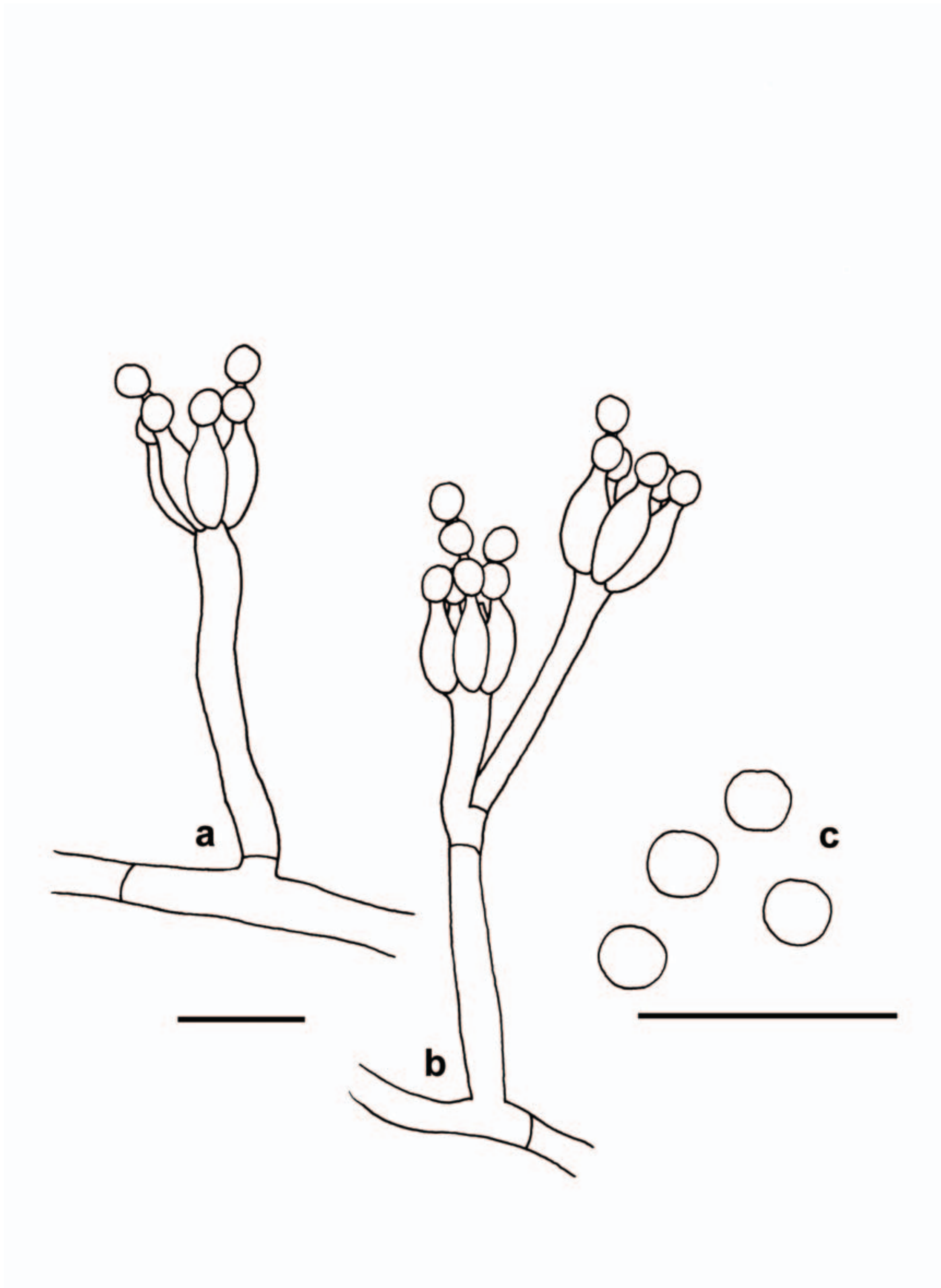

FIGURE 15. *Penicillium roseopurpureum* line drawings from strain CV186. a, b. Conidiophores (bar = 10 μm). c. Conidia (bar = 10 μm).

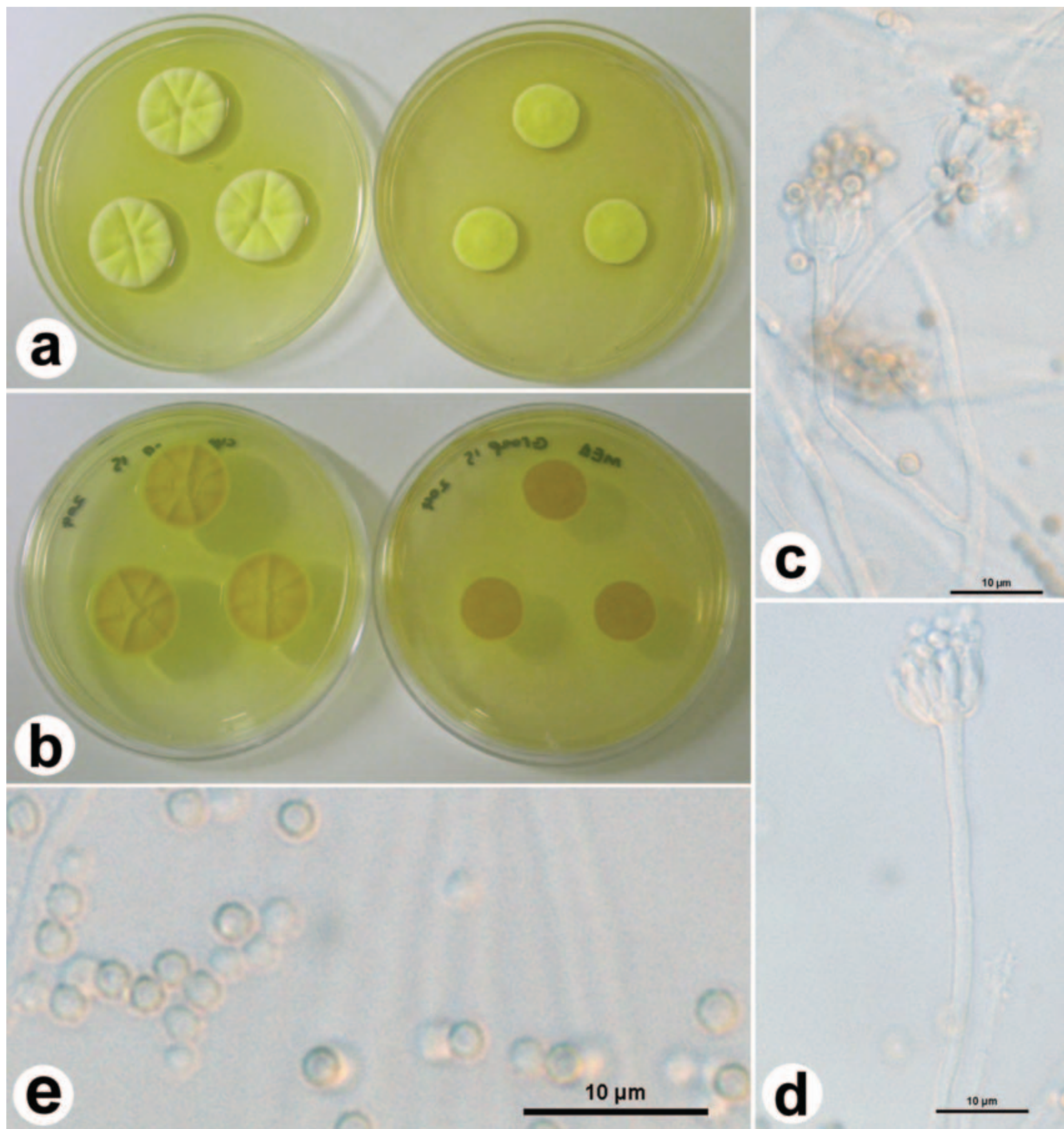

FIGURE 16. Morphological features characteristic of *P. toxicarium* (CV204). a. Colonies of *P. toxicarium* incubated on CYA (left) and MEA (right) after 7 days, showing the characteristic bright yellow features. b. Reverse of colonies showing the deeper yellow colors. c, d. Conidiophores typically produced in culture. e. Smooth spheroid conidia produced.

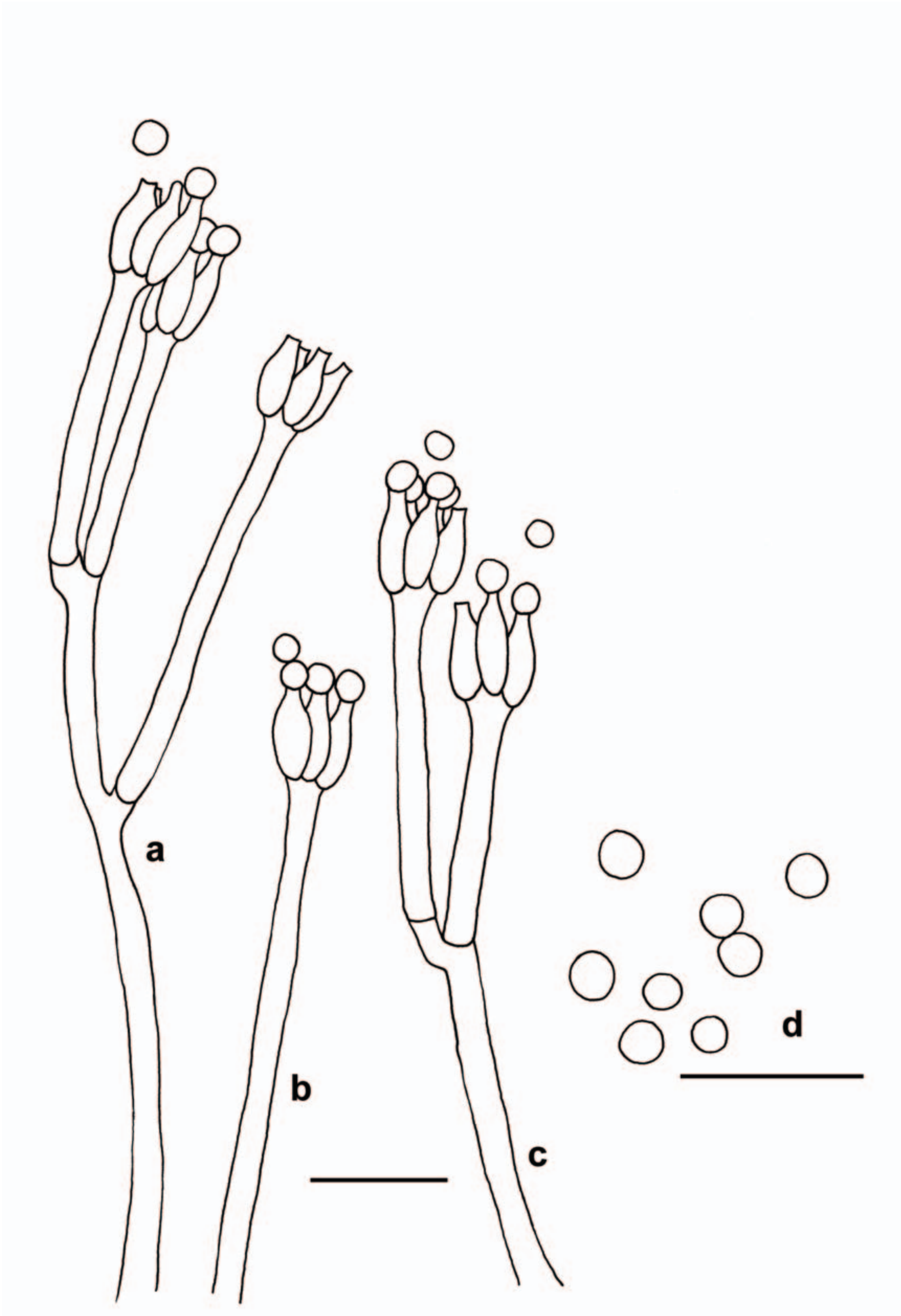

FIGURE 17. *Penicillium toxicarium* line drawings from strain CV204. a, b, c. Conidiophores (bar = 10 μm). d. Conidia (bar = 10 μm).

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CHAPTER 6
Dichotomous and synoptic keys to *Penicillium* species from coastal fynbos soil

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ABSTRACT

During a study on the biodiversity of *Penicillium* spp. occurring in coastal fynbos soil, 24 distinct species were isolated. Amongst these species, 11 were new to science. The difficulties associated with species identifications, together with the large number unique species from the fynbos area, have necessitated the construction of keys to aid in the identification of these species.

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INTRODUCTION

Penicillium is one of the most commonly encountered fungal genera in the world (Thom 1930, Raper and Thom 1949, Pitt 1979). It is, however, also one of the more complex fungal groups to work with and species identification is often problematic (Thom 1930, Raper and Thom 1949, Pitt 1973, Pitt 1979, Samson and Gams 1984, Pitt and Samson 1990, Dörge et al. 2000, Frisvad et al. 2006, Clemmensen et al. 2007). Identification of South African strains is especially difficult, because of the limited knowledge on the species occurring here. Strains isolated from South African habitats often shown variations from Pitt's (1979) species descriptions (Schutte 1992), which further complicates morphological identifications. In the past, South African researchers have had a tendency of not identifying strains from this genus the species level (Le Roux 1973, Marasas and Bredell 1973, Marais and Kruger 1975, Eicker 1976, Lück et al. 1976, Holtzhausen 1978, Eicker et al. 1982, Dutton and Westlake 1985, Mycock and Berjak 1990, Schutte 1992). This makes biodiversity surveys and comparative studies extremely difficult. From previous studies, it is clear that South Africa, at least for fynbos soil, have unique *Penicillium* populations, with eleven species described as new to science (Visagie et al. 2008, Visagie and Jacobs 2008a, 2008b, 2008c). We are, however, only starting to uncover the extent of *Penicillium* diversity from this area. For future surveys, a key to species from this region would be of great aid. The aim of this study was, therefore, to provide both a dichotomous and synoptic key to the 24 fynbos species, characterized thus far.

Working methods, techniques, characterization and species descriptions are based on that of Pitt (1979, 1985), Samson and Pitt (1985) and Okuda et al. (2000), and have been described in previous chapters. Color plates and line drawings to species are provided in their respective chapters, with page numbers to these plates indicated next to species names in the synoptic key. Often a specific morphological character is not commonly seen in a species, but is only present in a few isolates. This was taken into account when the synoptic key was constructed, with these characters indicated in brackets.

DICHOTOMOUS KEY TO *PENICILLIUM* SPP. ISOLATED FROM COASTAL FYNBOS SOIL AND THEIR CLOSELY RELATED SPECIES

1. Phialides acerose; penicilli commonly biverticillate, sometimes more complex; colonies on G25N <10 mm……..........**subgenus *Biverticillium* A**
1. Phialides ampulliform; colonies on G25N >9 mm..**2**

2. Penicilli commonly terverticillate; colonies on CYA strongly coremial to fasciculate..***P. expansum***
2. Penicilli not

terverticillate..**4**

3. Penicilli predominately biverticillate, with a proportion monoverticillate...**subgenus *Furcatum* B**
3. Penicilli predominately monoverticillate...................**subgenus *Aspergilloides* C**

A. *Penicillium* subgenus *Biverticillium*

1. Colonies on CYA <4 mm; red mycelia never present...**2**
1. Colonies on CYA >4 mm..**4**

2. Conidia distinctly rough-walled...***P. solicola***
2. Conidia smooth-walled...**3**

3. Growth only on acidified (pH 5/less) CYA; stipes <100 μm..................***P. lignorum***
3. Colonies on CYA 2–4 mm, sometimes only microcolony; stipes >100 μm...***P. diversum***

4. Conidia ornamented with transverse/spiral striations..**5**
4. Conidia without striations..**6**

5. Red mycelia on CYA and MEA; stipes vesiculate....................................***T. purpureus***
5. Mycelia white on CYA and MEA; stipes non-vesiculate...***P. ptychoconidium***

6. Colonies on CYA <12 mm and MEA <20 mm...***P. rugulosum***
6. Colonies on CYA >12 mm or MEA >20 mm..**7**

7. Conidia spheroid..**8**
7. Conidia not spheroid..**10**

8. Conidia smooth to finely roughened...***P. pinophilum***
8. Conidia strongly rough-walled...**9**

9. Mycelia on CYA yellow; colonies at 37°C >20 mm..........................***P. verruculosum***
9. Mycelia on CYA white; colonies at 37°C <20 mm..................................***P. aculeatum***

10. Colonies on CYA and MEA <22 mm..**11**
10. Colonies on either CYA or MEA >22 mm...**12**

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11. Conidial length 3–3.5 µm; white mycelia with exudate dominating colony appearance; stipes commonly <70 µm............... ***P. occultum***
11. Conidial length 3.5–4 µm; yellow mycelia with abundant conidiogenesis dominating colony appearance; stipes often 100–200 µm.. ***P. variabile***

12. Colonies at 37°C >20 mm; mycelia on CYA salmon to peach.. ***P. funiculosum***
12. Colonies at 37°C <20 mm... **13**

13. Metulae divergent; mycelia bright yellow; dark red colony reverse on CYA; synnema not produced..................... ***P. minioluteum sensu*** **Pitt**
13. Metulae appressed; mycelia usually white; synnema produced after prolonged incubation.. **14**

14. Synnema having yellow stalks... **15**
14. Synnema having white stalks... **16**

15. Synnema 2000–4000 µm tall... ***P. dendriticum***
15. Synnema <400 µm.. ***P. aureocephalum***

16. Conidia *en masse* on CYA pink to grayish green; synnema 100–250 µm tall; abundant sticky exudates produced.................. ***P. ramulosum***
16. Synnema >250 µm.. **17**

17. Colonies on CYA 34–37 mm and MEA 43–48 mm; conidia *en masse* olive brown, with light green edge....................... ***P. chloroloma***
17. Colonies on CYA 19–31 mm and MEA 32–40 mm; conidia *en masse* grayish green to grayish turquoise......................... ***P. cecidicola***

B. *Penicillium* subgenus *Furcatum*

1. Penicillus mainly produced as terminal verticil.. **2**
1. Penicillus irregular.. **8**

2. Metulae appressed.. **3**
2. Metulae divergent... **4**

3. Colonies on MEA >20 mm; black sclerotia produced on both CYA and MEA; stipe rough-walled............................... ***P. novae-zeelandiae***
3. Colonies on MEA <20 mm; smooth-walled stipes .... ***P. madriti***

4. Colonies on MEA >50 mm.. **5**
4. Colonies on MEA <50 mm.. **6**

5. Colonies on CYA >50 mm; phialides 5–7 µm; conidia 2.5–3.5 µm... ***P. hemitrachum***
5. Colonies on CYA <50 mm; phialides 7–10 µm; conidia 2–2.5 µm…... ***Eup. terrenum***

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6. Stipes and conidia rough-walled; CYA colony reverse
   commonly dark turquoise to blue..***P. subturcoseum***
6. Stipes and conidia smooth-walled; CYA colony reverse not blue.............................**7**

7. Colonies on MEA >25 mm; metulae of equal length..................................***P. citrinum***
7. Colonies on MEA <25 mm; metulae of unequal length..................***P. corylophilum***

8. Stipes rough-walled..**9**
8. Stipes smooth-walled..**10**

9. Conidia spinose..***P. melinii***
9. Conidia smooth-walled..***P. canescens***

10. Conidia spinose >2.5 μm...***P. janczewskii***
10. Conidia smooth <2.5 μm...***P. raciborskii***

C. *Penicillium* subgenus *Aspergilloides*

1. Stipe vesiculate..**2**
1. Stipe non-vesiculate..**8**

2. Conidia ellipsoidal...**3**
2. Conidia spheroid to subspheroidal..**4**

3. Conidia smooth-walled 2.5–3 μm in length...***Eup. lapidosum***
3. Conidia finely rough to conspicuously rough-walled
   3.5–4 μm in length...***P. thomii***

4. Conidia spinose or rugulose...**5**
4. Conidia smooth to finely roughened...**6**

5. Conidia rugulose...***P. purpurescens***
5. Conidia spinose..***P. spinulosum***

6. Brown hard sclerotia produced on CYA and MEA.......................***P. cumulacinatum***
6. Brown hard sclerotia absent on CYA or MEA..**7**

7. Stipes conspicuously roughened; colonies on MEA >55 mm..................***P. vulgaris***
7. Stipes smooth to finely roughened; colonies on MEA <55 mm.............***P. glabrum***

8. Colonies on CYA and MEA <25 mm and stipes <60 μm..**9**
8. Colonies on CYA or MEA >25 mm or stipes >60 μm..**11**

9. Colonies on CYA and MEA reddish brown soluble pigment and
   deep reddish brown reverse coloration.......................................***P. roseopurpureum***
9. Soluble pigment absent and reverse, when
   colored, lightly so..**10**

10. Conidia rough to echinulate, sometimes smooth;
    colonies on G25N >10 mm..***P. restrictum***
10. Conidia smooth; colonies on G25N <10 mm.................................***Eup. meridianum***

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11. Stipes commonly <60 μm..**12**
11. Stipes commonly >60 μm..**16**

12. On CYA and MEA having brightly orange colored
    colonies and colony reverses............................***P. hirayamae* (=*Eup. hirayamae*)**
12. Colonies not having orange features...**13**

13. Conidia >4.5 μm in long axis..**14**
13. Conidia <4.5 μm in long axis..**15**

14. Colonies on G25N >6 mm and CYA (37°C) >35 mm.............................***Eup. levitum***
14. Colonies on G25N <6 mm and CYA (37°C) <35 mm...........................***Eup. ehrlichii***

15. Colonies on MEA <30 mm; phialide length 7–9 μm;
    conidia 3–3.5 μm...***P. raperi***
15. Colonies on MEA >30 mm; phialide length 4–7 μm;
    conidia 2.5–3...***P. brachycaulon***

16. Colonies on CYA and MEA <30 mm; colonies on both
    having bright yellow features................................***P. toxicarium/P. citreonigrum***[*]
16. Colonies on CYA and MEA >30 mm; colonies lacking
    bright yellow features...**18**

18. Penicillus commonly biverticillate...***P. janthinellum***
18. Penicillus commonly monoverticillate..**19**

19. Conidia spheroidal; colonies on CYA (37°C) >25 mm..........***P. malacosphaerula***
19. Conidia ellipsoidal; colonies on CYA (37°C) <25 mm..........***Eup. reticulisporum***

SYNOPTIC KEY TO *PENICILLIUM* SPP. ISOLATED FROM COASTAL FYNBOS SOIL

SPECIES LIST

1.	*Eup. hirayamae*	p. 165, 166
2.	*P. brachycaulon*	p. 155, 156
3.	*P. canescens*	p. 129, 130
4.	*P. chloroloma*	p. 92, 93
5.	*P. citrinum*	p. 123, 124
6.	*P. corylophilum*	p. 131, 132
7.	*P. cumulacinatum*	p. 159, 160
8.	*P. expansum*	p. 133, 134
9.	*P. hemitrachum*	p. 119, 120
10.	*P. janczewskii*	p. 125, 126
11.	*P. malacosphaerula*	p. 157, 158
12.	*P. melinii*	p. 127, 128

[*] Species distinguished on the basis of their genetic characters

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13. *P. minioluteum* p. 94, 95
14. *P. occultum* p. 90, 91
15. *P. ptychoconidium* p. 88, 89
16. *P. ramulosum* p. 62, 63
17. *P. restrictum* p. 163, 164
18. *P. roseopurpureum* p. 167, 168
19. *P. rugulosum* p. 96, 97
20. *P. solicola* p. 86, 87
21. *P. subturcoseum* p. 121, 122
22. *P. toxicarium* p. 169, 170
23. *P. verruculosum* p. 98, 99
24. *P. vulgaris* p. 161, 162

COLONY CHARACTERS ON CYA:

Growth rate:
a. germination: (20)
b. <15 mm: 14, 15, 18, 19, 20
c. 15–19 mm: 14, 17, 18, 22
d. 20–24 mm: 1, 2, 17, 18, 22, 23
e. 25–29 mm: 1, 2, 3, 5, 6, 10, 12, 13, 17, 21, 23
f. 30–34 mm: (1), 3, 4, 5, 6, 8, 10, 11, 12, 13, 16, 21, 23
g. 35–39 mm: (1), 3, 4, 6, 7, 8, 11, (12), 13, 16, 21
h. 40–44 mm: (1), 7, 8, 24
i. 45–49 mm: 7, 8, 24
j. >50 mm: 9

Colony texture:
a. determinate synnema (7d): 8
b. determinate synnema (7d+): 4
c. floccose: 2, 3, 4, 5, 8, (7), 9, 10, 11, (12), 13, 15, 17, 18, 20, (21), 22, 23, 24
d. funiculose: 1, 4, 13, 14, 16, 23
e. velutinous: 5, 6, 7, 8, 9, 12, 13, 14, (16), 18, 19, 21, 23

Mycelia colors:
a. orange: 1, (3), 5, (18), (23)
b. pinkish: (24)
c. red: (23)
d. yellow: 1, 3, (10), (12), 13, 19, 22, 23,
e. white: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, (13), 14, 15, 16, 17, 18, 19, 20, 21, 22, 24

Conidia en masse color:
a. blue green: (6), (7), 12, 13
b. brown: 4, (6)
c. dark green: 12, 19, 23
d. dull green: 1, 6, 8, 10, 12, 13, 19
e. greenish grey: 1, 3, 10, 12, 17
f. grey green: 2, 3, 4, 6, 7, 9, 10, 14, 15, 16, 21, 24
g. olive: 4, 8

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h. pink: 16
i. shade of yellow: 9, 11, 22
j. turquoise: 5, 17, 19
k. white to grey: 17, 18, 20

Exudate:
a. absent: 2, 4, (6), (12), 16, (17), (18), 19, 21, 23
b. brown: 3, 5, (7), 8, 12, 18
c. clear: 1, 3, 5, 6, 7, 8, (10), 11, (13), 14, 15, (17), 20, 22, (23), 24
d. orange: 5, 8, 12, 18, 20
e. red: 11, (23)
f. yellow: 1, 5, 9

Soluble pigment:
a. absent: 1, (3), 4, 5, 6, 13, 14, 15, 16, 17, 19, 20, 21, 23, 24
b. brown: (2), 3, (7), 8, (10), 12, 18
c. orange: 8, 12, 18
d. pink: (21)
e. yellow: 3, 5, 7, 9, 11, 12, 22

Reverse coloration:
a. blue to turquoise: 21
b. brown: 2, 3, 5, 6, 8, 9, 12, 14, 17, 18, 19, 23
c. olive: 2, 5, 12, 14, 19
d. orange: 1, 8, 23
e. pale: (12), 15, 16, 17, 20
f. pink: 4, 16
g. red: 13, 18, 23
h. yellow: 5, 7, 11, (12), 15, 22, 24

COLONY CHARACTERS ON MEA:

Growth rate:
a. <15 mm: 5, 18, 19
b. 15–19 mm: 1, 3, 5, 10, 12, 14, 15, 17, 18, 19, 22
c. 20–24 mm: 1, 3, 8, 10, (11), 12, 15, 17, 18, 19, 20, 22
d. 25–29 mm: (1), 3, 8, (10), (11), (17), (20)
e. 30–34 mm: (1), 2, (3), 6, 8, 11, (17), 21, 23
f. 35–39 mm: (3), 6, 7, 8, 11, 13, 16, 21, 23
g. 40–44 mm: 4, 6, 7, 8, 11, 13, 16, 21, 23
h. 45–49 mm: 4, 6, 7, 8, 13, 16, 23
i. 50–55 mm: 7, 8, 9
j. >55 mm: 9, 24

Colony texture:
a. determinate synnema (7d): 8
b. determinate synnema (7d+): 16
c. floccose: 2, 3, 5, 9, 10, 11, (12), 13, 14, 17, 18, 19, 20, 22, 23

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d. funiculose: 1, 4, 13, 15, 16, 23
e. velutinous: 6, 7, 8, 9, 12, 13, 14, (16), 18, 19, 21, 23, 24

Mycelia colors:
a. brown: (16)
b. orange: 1, (5), (10), (23)
c. red: (23)
d. yellow: 1, 3, 10, 13, 19, 23
e. white: 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 20, 21, 22, 24

Conidia en masse color:
a. blue green: 5, (7), 13
b. dark green: 11, 12, 15, 19, 20, 21, 23, 24
c. dull green: 1, 5, 6, 8, 12, 13, 19
d. greenish grey: 1, 3, 10, 17, 18
e. grey green: 2, 3, 4, 6, 7, 9, 10, 14, 16, 17, 19, 21, 24
f. olive: 4, (8)
g. shade of yellow: 11, 22

Exudate:
a. absent: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 19, 21, 22, 23, 24
b. clear: (6), (7), (12), 15, 16, (17), 20, (23)
c. red: (23)
d. yellow: 1, (12), 14

Soluble pigment:
a. absent: 1, 2, 3, 4, 5, 6, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 23, 24
b. brown: (8), 12
c. orange: (8), (10)
d. yellow: 7, 9, (10), (11), 12, 22

Reverse coloration:
a. brown: 3, 5, (8), 12, 14, 15, 16, 17, 18, 19, 20, 23
b. dark green: 6, (21)
c. olive: 2, 14, 19
d. orange: 1, (8), 10
e. pale: 5, 7, 8, 13, 17, 23, 24
f. red: (13), 15
g. shade of green: 4, 6, 9, 16
h. yellow: 2, 3, 5, 9, 11, 12, 22, 23

SCLEROTIA OR CLEISTOTHECIA PRODUCED ON CYA/MEA
a. yes: 1, 7, 11, (13), 24
b. no: 2, 3, 4, 5, 6, 8, 9, 10, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23

GROWTH ON G25N:
a. no germination: 20,
b. <6 mm: 4, 13, 14, 15, 19, 23,
c. 6–14 mm: 1, 2, 5, 6, 12, 14, 17, 18, 19, 22

d. >14 mm: 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 18, 21, 22, 24

GROWTH ON CYA AT 37°C:

a. no germination: 3, (5), 6, 7, 8, 10, 12, 14, 16, 18, 19, 20, 24
b. germination–20 mm: 1, 2, (3), 4, 5, 9, (10), (12), 13, 15, 16, 17, (19), 21, 22
c. >20 mm: 1, 11, 23

GROWTH ON CYA AT 5°C:

a no germination: 1, 5, 13, 14, 15, 16, 17, 19, 23
b. germination: 2, 3, 4, 6, 9, 10, 12, 16, (17), 18, 19, 20, 22
c. microcolonies: (2), 7, (8), (10), 11, 21, 24
d. >2 mm: 3, (6), 8, (12), (22)

MICROMOPRHOLOGY

Conidiophore branching:

a. monoverticillate: 1, 2, 6, 7, 11, 12, 17, 18, 21, 22, 24
b. biverticillate: 3, 4, 5, 6, (8), 9, 10, (11), 12, 13, 14, 15, 16, 19, 20, 21, (22), 23
c. terverticillate or more complex: 4, 8, 14, 16, 19

Conidial shape:

c. spheroidal: 1, 3, 5, 6, 7, 9, 10, 11, 12, 17, 18, 21, 22, 23, 24
b. subspheroidal: 1, 2, 5, 6, 16, 17, 19, 20, (23), (24)
a. ellipsoidal: 2, 4, 8, 13, 14, 15, 16, 17, 19

Conidial ornamentation:

a. smooth: 1, 2, 3, 5, 6, 4, 7, 8, 9, 11, 13, 14, 16, 17, 18, 19, 22, 24
b. finely roughened: 2, 3, 5, 7, 9, 17, 18
c. rough: 10, 12, 15, 17, 19, 20, 21, 23
d. spirally rough: 15

Conidial length:

a. <3 µm: 1, 2, 3, 4, 5, 7, 9, 10, 11, 13, 16, 17, 18, 19, 20, 21, 22, 24
b. 3–4 µm: 4, 6, 8, 9, 10, 12, 13, 14, 15, 19, 20, 23
c. >4 µm: (4), 15

Conidial width:

a. <3 µm: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24
b. 3–4 µm: 9, 10, 12, 23

Phialide shape:

a. acerose: 4, 11, 13, 14, 15, 16, 19, 20, 23
b. ampulliform: 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18, 21, 22, 23, 24

Penicillus divergence (when biverticillate or more complex):

a. appressed: 4, 8, 11, (13), 14, 15, 16, 19, 20
b. divergent: 3, 5, 6, 9, 10, 12, (13), (16), 21, 22, 23

Stipe Length:

a. <60 µm: 1, 4, 10, 12, 14, 15, 16, 17, 18, 22

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b. >60 µm: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 20, 21, 22, 23, 24

Stipe ornamentation:

a. smooth: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23
b. rough: 3, 12, 21, 24
c. tuberculate: 12, (21)

Stipe vesiculate (for monoverticillate species):

a. yes: 7, 24

DISCUSSION AND FUTURE RESEARCH

During this survey, 434 *Penicillium* strains were isolated and characterized using morphology. The strains were placed into 24 morphologically well defined groups, with representatives from each group used for phylogenetic comparisons, based on the internal transcribed spacer (ITS) region. Groups were subsequently identified using both morphological and genetic characters, and lead to 11 new species being described. These, together with the previously known species, was incorporated into both a dichotomous and synoptic key, with color photo plates and line drawings as supplements, which will greatly aid in the identification of species from the unique fynbos biome.

Our exploration into the diversity of *Penicillium* occurring in the fynbos have, however, only just begun. In addition to the 24 identified species, roughly 40 isolates represented single specimen groups and still awaits characterization and identification. This would almost triple the total number of species found from this small area within the larger fynbos biome. Since fynbos soil is known to be high in heterogeneity (Goldblatt and Manning 2002), we expect to see a shift in species populations when surveying other areas within the biome. This initial study would, therefore, only serve as a basis from which future surveys can start exploring the extent of *Penicillium* diversity, not only in the fynbos, but also in the rest of southern Africa. Amongst identified strains, were species of which the morphological identifications did not fully correlate with the phylogenetic data. This was most notable in strains identified as *P. rugulosum* (chapter3), *P. janczewskii* (chapter4) and *P. restrictum* (chapter5), and this was discussed in the respective chapters. In addition to this, similar to previous studies, the ITS gene region was unable to resolve all species within *Penicillium*

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subgenera *Aspergilloides, Furcatum* and *Biverticillium* (Skouboe et al. 1999, Peterson 2000). Many taxonomic questions, therefore, still needs answering. Datasets for sequences other than ITS are, however, limited for these subgenera. The β-tubulin gene was able to resolve most closely related species of subgenus *Penicillium* (Samson et al. 2004), with calmodulin also seeming like a good alternative gene (Wang and Zhuang 2007). In future studies it would, therefore, be advantageous to sequence these genes. This will not only help to solve problems found during this study, but also deeper taxonomic problems such as easier species identifications. With large sequencing initiatives, such as the Barcoding of Life projects, South Africans also have the opportunity to, with our unique *Penicillium* populations, help achieve the goals set by these initiatives.

An interesting fungal genus, *Torulomyces*, of which 31 strains were isolated during this study, was not reported, but will serve as an interesting future study. Species from this genus produces solitary phialides on short stipes (Barron 1967, Pitt and Hocking 1985, Stolk and Samson 1985, Ando et al. 1998). The relationship of this genus to *Penicillium* has been questioned in the past, with Stolk and Samson (1983, 1985) suggesting synonymy between these two. This was, however, rejected by Pitt and Hocking (1985) based on morphological characters. A study of this group will benefit from sequence information for inferring the true evolutionary relationship of *Torulomyces* and *Penicillium.* Preliminary data suggests that Stolk and Samson (1983, 1985) might have been correct in their placement of *Torulomyces* as a synonym to *Penicillium*.

Future studies will also focus on the ecology of *Penicillium* strains from the fynbos. *Penicillium* subgenus *Biverticillium* species were found to occur at specific sites in the sample areas. It appears from the data that species from this group do not have soil as its only habitat. *Penicillium ramulosum* has been isolated from *Protea burchelli* infructescences, apples in the Western Cape, as well as from moth-damaged grapes in Canada. *Penicillium ramulosum* and *P. chloroloma* commonly forms synnema in culture, with *P. ramulosum* also found to produce these structures inside *Protea* infructescences. Within these enclosed structures, species most probably rely on vectored dispersal different from air or water. These extended structures might, therefore, aid in a species chances of

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being dispersed via arthropod vectors. *Penicillium* spp. have been frequently isolated from mites (Roets, unpublished) and an association between these two would, therefore, not be unexpected. Insect-fungus associations have been suggested in the past (Peterson et al. 2003, Seifert et al. 2004), but have never been proved. A study of this nature would, therefore, not only be novel but may give us insights into alternative spore dispersal methods of *Penicillium* species.

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