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# *Penicillium astrolabium* and *Penicillium neocrassum*, two new species isolated from grapes and their phylogenetic placement in the *P. olsonii* and *P. brevicompactum* clade

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***Abstract:*** We describe two new terverticillate *Penicillium* species isolated from grapes on the basis of phenotypic and phylogenetic differences from known species. The strains were isolated in the course of a study to establish the mycobiota of grapes in Portugal. *Penicillium astrolabium* is phenotypically similar to *P. olsonii* but differs from it by two cultural characters, growth rates and the colony reverse color. *P. neocrassum* is similar to *P. brevicompactum* but is readily distinguished by sclerotia production. Phylogenetically *P. astrolabium* and *P. neocrassum* are placed respectively in the *P. olsonii* and *P. brevicompactum* clade. Multilocus analysis confirmed the genetic distinctiveness of both species. The parsimony trees obtained for ITS-lsu rDNA region and two protein coding genes, calmodulin and β-tubulin, show congruence for all the species in the *Olsonii* series: *P. brevicompactum*, *P. bialowiezense*, *P. olsonii*, *P. astrolabium* and *P. neocrassum*, indicating that these taxa are genetically well isolated.

***Key words:*** DNA sequences, fungi, grapes, taxonomy

## INTRODUCTION

An extensive survey was conducted to determine the mycobiota colonizing grapes in Portugal with emphasis on *Aspergillus* and *Penicillium* species to establish the species responsible for ochratoxin A production in grapes and wine (Serra et al 2005). Nearly 1000 strains of *Aspergillus* and *Penicillium* were isolated. Grapes proved to be an interesting source of fungal biodiversity and a new black *Aspergillus* species isolated from berries, *A. ibericus*, was described for the first time (Serra et al 2006a).

A few isolates of *Penicillium* were not identified satisfactorily using the available monographs of Raper and Thom (1949), Pitt (1980, 1985) and Ramirez (1982). The strains were assigned on the basis of penicillus structure to the subgenus *Penicillium*, section *Coronata* Pitt series *Olsonii* Pitt, which contains three accepted species (Samson and Frisvad 2004): *P. bialowiezense* (synonym of *P. biourgeianum*), *P. brevicompacum* and *P. olsonii*. Peterson (2004) showed that these species are phylogenetically distinct but closely related. Nevertheless the isolates from grapes differed from the described species in evident cultural characters. To achieve an accurate identification, the strains were barcoded using the internal transcribed spacer region (ITS) and ca. 650 nucleotides of the large subunit rDNA (ID region) and compared to sequences from known species using BLAST searches of GenBank and unpublished sequences (Peterson unpublished). The strains were related to *P. brevicompactum* and *P. olsonii* but yet were distinctive at this locus.

We describe two new terverticillate species in the series *Olsonii* and compare them with the previously described species. The phylogenetic placement of the putative new species was assessed with multilocus DNA sequence analysis of sequences from the ID region and the protein coding loci calmodulin and β-tubulin.

## MATERIALS AND METHODS

*Fungal cultures.*—*P. astrolabium* and *P. neocrassum* together with a sclerotial strain of *P. olsonii* were isolated from Portuguese grape berries by plating methods without surface disinfection as described in detail by Serra et al (2005). The origin of these and all the strains used for comparison is provided (TABLE I). The strains are stored in the Agriculture Research Service (ARS) Culture Collection (NRRL) and in Micoteca da Universidade do Minho culture collection (MUM) and available on request.

*Media and growth conditions.*—Some isolates were examined phenotypically with the methodology of Pitt (1980). Czapek yeast extract (CYA), Blakeslee's malt extract (MEA) and G25N agar were formulated as described, and cultures were incubated under the conditions described by Pitt (1980).

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TABLE I. Origin of the *Penicillium* isolates included in this study sequenced for partial β-tubulin, calmodulin and ID region

| NRRL number | Origin | Source | Genbank Acession no.* |
|---|---|---|---|
| *Penicillium astrolabium* Serra & Peterson | | | |
| 35611 *ex-type* (= MUM 06.161) | Portugal | wine grapes | DQ645793 [BT]<br>DQ645808 [CF]<br>DQ645804 [ID] |
| *Penicillium biourgeianum* Zaleski | | | |
| 865 *ex-type* | Poland | forest soil | DQ645783 [BT] |
| 2013 | Germany | mushroom spawn | DQ645786 [BT] |
| 28149 | USA | dead agaric on logs | DQ645790 [BT] |
| 32205 | USA | fresh coconut | DQ645791 [BT] |
| 32207 | USA | Christmas fern | DQ645792 [BT] |
| *Penicillium brevicompactum* Dierckx | | | |
| 859 | USA | decaying mushroom | DQ645800 [BT] |
| 2011 *ex-neotype* | unknown | unknown | DQ645784 [BT] |
| 2012 | unknown | paprika | DQ645785 [BT] |
| 28120 | USA | wood decay fungus | DQ645789 [BT] |
| 28139 | USA | wood decay fungus | DQ645795 [BT] |
| *Penicillium canescens* | | | |
| 35656 | USA | cheek pouch of kangaroo rat | DQ658166 [BT]<br>DQ658167 [CF]<br>DQ658168 [ID] |
| *Penicillium neocrassum* Serra & Peterson | | | |
| 35639 *ex-type* (= MUM 06.160) | Madeira Isl., Portugal | wine grapes | DQ645794 [BT]<br>DQ645809 [CF]<br>DQ645805 [ID] |
| 35648 (= MUM 06.162) | Madeira Isl., Portugal | wine grapes | DQ645802 [BT]<br>DQ645810 [CF]<br>DQ645806 [ID] |
| *Penicillium olsonii* Bainier & Sartory | | | |
| 5267 | unknown | unknown | DQ645787 [BT] |
| 5916 | unknown | unknown | DQ645788 [BT] |
| 6446 | Russia | soil | DQ645796 [BT] |
| 13058 *ex-type* | Austria | roots in *Picea* | DQ645797 [BT]<br>DQ658165 [CF]<br>AF454076 [ID] |
| 31467 | Mexico | coffee berry borer | DQ645798 [BT] |
| 35612 | Portugal | wine grapes | DQ645801 [BT]<br>DQ645807 [CF]<br>DQ645803 [ID] |

* [BT] β- tubulin; [CF] calmodulin; [ID] ID region. Accession numbers for loci not explicitly listed here are found in Peterson (2004).

*Microscopy.*—SEM was performed on agar blocks of cultures grown in MEA fixed overnight in osmium tetroxide, dehydrated in a series of ethanol rinses of increasing concentrations, critical-point dried and sputter coated with gold-paladium in a JEOL scanning electron microscope following the procedure of Peterson (1992). Light microscopic measurements and examinations were made with a Zeiss axioscope and Kodak 420B camera on fungal material mounted in 0.5% low melting temperature agarose and water. Digital images from both sources were adjusted for brightness/contrast and fitted into composite plates using Adobe Photoshop 6.01.

*Molecular methods.*—DNA was isolated from mycelium using a variation of the method of Peterson et al (2005). Instead of vortexing mycelium and glass beads in 15 mL disposable tubes for 45–60 s, the process was scaled down so that the vortexing could be accomplished in a 2 mL screw-cap microcentrifuge tube. Using a 24-tube holder for the vortex mixer, up to 24 samples could be processed simultaneously and the period of cell breakage was increased to 10 min. Other conditions and processes were similarly scaled-down modifications of the basic protocol (Peterson et al 2005).

DNA from the β-tubulin locus was amplified with the

protocol and primers of Glass and Donaldson (1995) from calmodulin and the ITS and lsu-rDNA (ID) region with the methods of Peterson et al (2005). The amplified fragments were purified with the Multiscreen PCR system of Millipore (Billerica, Massachusetts) and sequenced with dye-labeled dideoxy terminator (v 3.1) and the ABI model 3730 DNA sequencer (both from Applied Biosystems Inc, Foster City, California).

Complementary DNA strands were compared and sequencing error corrected with Sequencher (Gene Codes, Ann Arbor, Michigan). Corrected sequences initially were aligned with Clustal W (Thompson et al 1994), examined and fine aligned with a text editor.

PAUP* (Swofford 2002) was used to analyze the DNA sequence data, with the parsimony criterion, random sequence addition, and with maximum trees set at 1000 in an heuristic search. Bootstrap analysis used the same criteria except that sequence addition was ''as-is''. Tree files were examined with Treeview (Page 1996) and redrawn for publication using CorelDraw. Datasets and results have been deposited in TreeBase (http://www.treebase.org) and sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov).

## TAXONOMY

Examination of morphological characters combined with the analysis of DNA sequences revealed that the terverticillate *Penicillium* strains related to *P. brevicompactum* and *P. olsonii* do not match any of the species described to date. Two new species, *P. astrolabium* and *P. neocrassum*, are proposed.

**Penicillium astrolabium** R. Serra & S.W. Peterson, sp. nov. Figs. 1–9

Coloniae crescentes post 7 dies in CYA ad 25 C 20–27 mm diam, radialiter et anulariter sulcatae, per mediam partem depressae, moderate profundae, densae, textura superficiei velutinosa ad granularis, margines profundae et curvatae, mycelium album vel interdum pallide-flavum; conidiogenesis magna per aream totam coloniae, celandino-viride (Ridgway Pl. XLVII), sudor moderatus per mediam partem coloniae productus est; primum perspicuus, deinde rubro-brunnea quando maturant, pigmentum brunneum et dissolubile adest, clarius quando maturant; pars reversa nigro-brunnea ad nigra, decolor solum in marginibus coloniae. Incubata ad 5 C et ad 37 C in CYA incrementem nullum post 7 dies. In G25N coloniae mediae 17–25 mm diam, irregulariter sulcatae et plicatae, cum propagine profunda mycelii albi a penicillis dispersis velata, textura superficiei velutinosa ad granularis, conidiogenesis moderata, piso-viridis ad celandino-viridis. Sudor et pigmentum dissolubile absunt, facies reversa pallida, obscure nigro-viridis per mediam partem (Ridgway, Pl. XLI). Conidiophora portata in hyphis sub superficie orientibus, maxima, stipites fere 500–2000 × 4.0-6.0 um, distincti et aliquando cristati in marginibus, tenuiter tunicati, terminantes in penicillis dense appressis et mulitramulatis, plerumque terverticillati sed interdum quaterverticillati, 2–5 rami per stipitem, 9–13(–20) × 4.0–5.0 µm cum penicillis distincte multiramulatis; metulae in verticillis 3–5 coninentibus, (8.0–)9–13 × 3.0–4.0 µm; phialides in verticillis 5–6 continentibus, ampulliformes, plerumque 8–10 × 2.5–3.0 µm, cum collulis brevibus; conidia ellipsoidea, 3.0–4.0 × 2.5–3.0 µm, tenuiter tunicata vel in extremis asperata, portata in catenis confusis.

Colonies grown 7 d on CYA (FIG. 1) at 25 C attain 20–27 mm diam, are radially and annularly sulcate, centrally depressed, moderately deep, dense, surface texture velutinous to granular, margins deep and wavy, mycelium white or light yellow in some areas; conidiogenesis heavy over the whole colony area, celandine green (Ridgway Pl. XLVII), limited amounts of exudate produced at the center of the colony, clear at first, then becoming reddish brown with age; brown soluble pigment produced becoming more evident with age; reverse blackish brown to black (FIG. 4), paler only at colony margins.

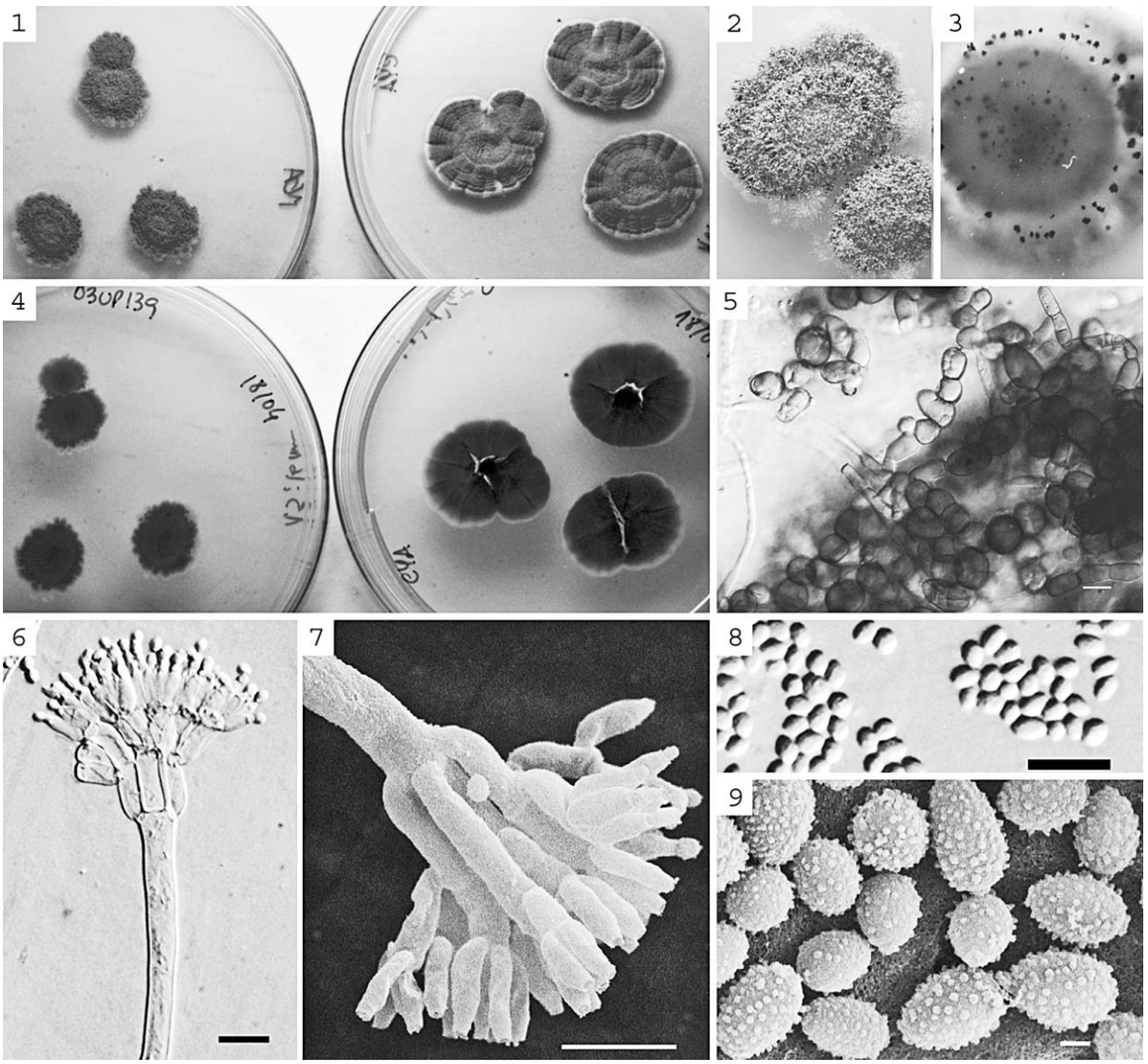
Colonies grown 7 d on MEA, at 25 C attain 15–16 mm diam, are plane sometimes centrally raised, moderately deep of granular texture (FIG. 2); margins narrow, irregular; mycelium inconspicuous, white; subsurface mycelium black (FIG. 5), visible on the obverse at punctuated areas surrounding colony center and at margin areas (FIG. 3), conidiogenesis heavy, celandine green, exudates and soluble pigment absent; reverse deep slate-olive to dull greenish black (Ridgway Pl. XLVII) (FIG. 4).

When incubated at 5 C and at 37 C in CYA, no growth was observed in 7 d. On G25N medium colonies attain 17–25 mm diam, are irregularly sulcate and plicate, composed of a deep layer of white mycelium covered with scattered penicilli, surface texture velutinous to granular; conidiogenesis light, pea to celandine green, no exudates and soluble pigment produced; reverse pale with a dull blackish green (Ridgway Pl. XLI) center.

Conidiophores borne from subsurface hyphae, large, with *stipes* typically 500–2000 × 4.0–6.0 µm, occurring as long tuffs at some marginal areas, smooth walled, terminating in closely appressed multiramulate penicilli (FIGS. 6–7), usually terverticillate but occasionally quaterverticillate; *rami* 2–5 per stipe, 9–13(–20) × 4.0–5.0 µm; *metulae* in verticils of 3–5, measuring (8.0–)9–13 × 3.0–4.0 µm; *phialides* in verticils of 5–6, ampulliform, commonly 8–10 × 2.5–3.0 µm, with short collula; *conidia* ellipsoidal, 3.0–4.0 × 2.5–3.0 µm, with walls smooth or finally roughened (FIGS. 8–9), borne in disordered chains.

HOLOTYPE: BPI 872160 here designated, deposited in the U.S. National Fungus Collection, is a dried culture of NRRL 35611 on MEA and CYA. Culture extype: NRRL 35611.

*Etymology. astrolabium* from astrolabio, the navigation instrument used by Portuguese sailors to cross the




FIGS. 1–9. *Penicillium astrolabium*, NRRL 35611. 1. Colonies on MEA and CYA after 7 d. 2. Detail of colony obverse on MEA where black hyphae masses are visible. 3. Colony reverse on MEA after 12 d incubation with black hyphae masses on agar subsurface. 4. Dark pigmentation in colonies reverse visible in MEA and CYA. 5. Dark hyphae. 6–7. Penicilli. 8–9. Conidia. Bar in 5–8 = 10 μm, FIG. 9. = 1 μm.

seas and discover the New World, in honor of the transatlantic cooperation that resulted in describing the new species.

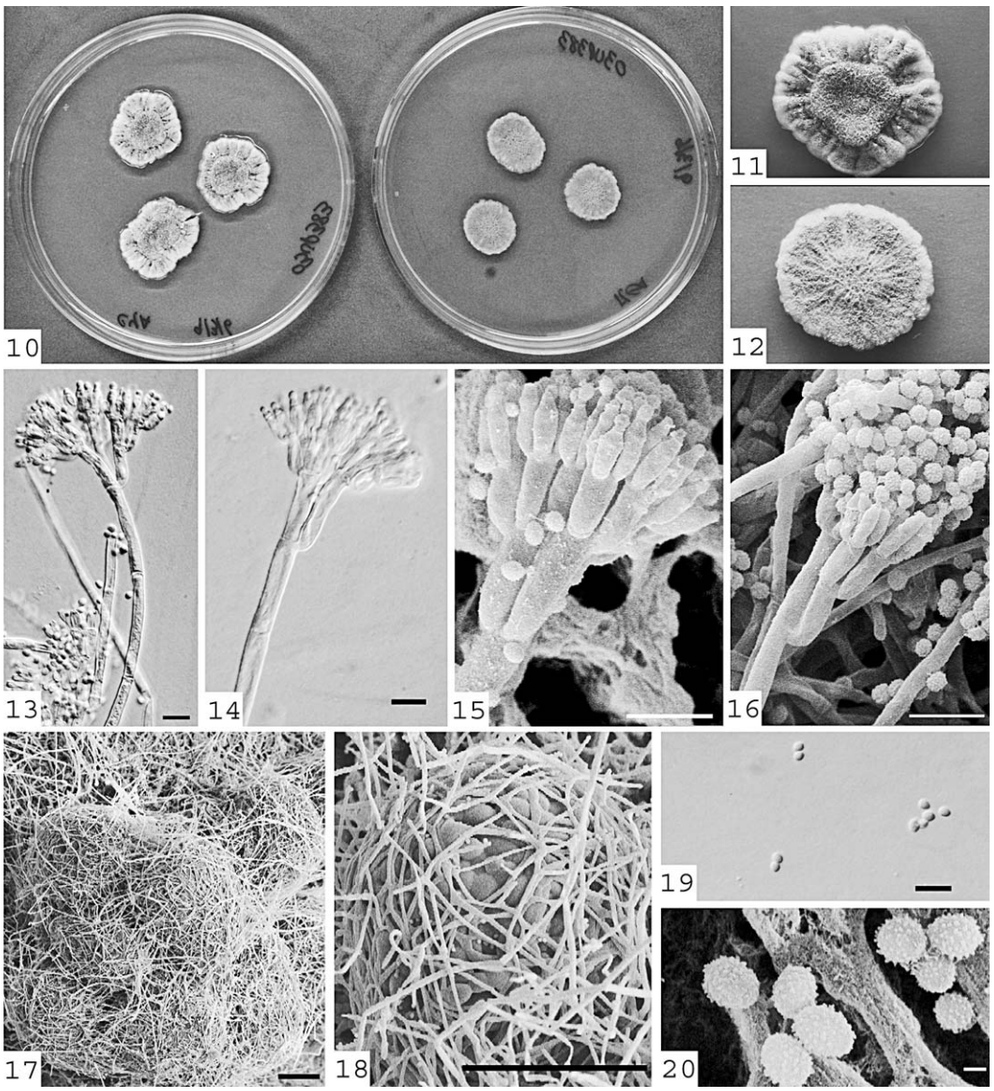
*Origin of strains.* HOLOTYPE. PORTUGAL. RIBATEJO, Almeirim. Vineyard located 39°12′34″N and 8°37′46″W, on healthy grapes, 4 Oct 2001, *Rita Serra 03UP139*. Isotype deposited in the MUM culture collection, Braga, Portugal.

*Additional specimens examined.* This species is known from the type strain only.

*Commentary.* Microscopically this species resembles *P. olsonii* but differs from it in cultural characters. The growth rates of *P. astrolabium* in CYA and MEA are slower than *P. olsonii*, and the colony reverse is dark olive or brown to black. Black hyphae masses are clearly visible on the reverse with age.

**Penicillium neocrassum** R. Serra & S.W. Peterson, sp. nov. FIGS. 10–20

Coloniae crescentes post 7 dies in CYA ad 25 C 23–26 mm diam, radialiter sulcatae cum sulco anulario circum mediam partem elevatam coloniae, interdum crateriformes, moderate profundae, compositae de propagine scleroticorum brunneorum et confinium in superficie agaris quae dominant aspectum coloniae, cum penicillis dispersis et superimpositis, textura superficiei velutinosa, margines albae, irregulares, angustae, mycelium album; conidiogenesis moderata, gnaphalium viride ad theae-viride (Ridgway




FIGS. 10–20. *Penicillium neocrassum* NRRL 35639. 10. Colonies on MEA and CYA after 7 d. 11. Detail of colony on CYA. 12. Detail of colony on MEA. 13–16. Penicillus. 17–18. Sclerotia. 19–20. Conidia. Bar in FIGS 13–16 = 10 μm, FIG 17–18 = 100 μm, FIG 19 = 10 μm, FIG 20 = 1 μm.

Pl. XLVII), sudor moderatus et perspicuus in margine coloniae productus est; pigmentum dissolubile abest; pars reversa fusce-rava ad nigro-brunnea (Ridgway Pl. XLV), pallida in marginibus coloniarum. Incubata ad 5 C et ad 37 C in CYA incrementem nullum post 7 dies. In G25N coloniae 22–24 mm diam, irregulariter sulcatae et centraliter convolutae, moderate profundae, textura superficiei velutinosa, margines extendentes 2–3 mm sub superficie, mycelium album et sclerotia alba ad brunneo-alba circumfundens, conidiogenesis moderata, theae-viridis. Sudor et pigmentum absunt, facies reversa brunnea per mediam partem.

Sclerotia ellipsoidea ad forma irregularia, fere 200–360 μm per axim longiorem, rapide sclerotioidea, primum pallida, deinde brunnea, fusce-rava ad nigro-brunnea (Ridgway Pl. XLV) in CYA, Kaiser-brunnea ad castaneo-brunnea in MEA (Ridgway Pl. XIV), pallide brunneo-alba in G25N. Status teleomorphosis ignotus.

Conidiophora portata in hyphis sub superficie orientibus, stipes fere longus et latus, 500–800 × 4.5–6.0 μm, tenuiter tunicatus, portans penicillos confertos et late terverticillatos, fere minus quam 40 μm longitudine et 35–53 μm latitudine, interdum cum penicillis quaterverticillatis et biverticillatis, rami singuli, breves et lati, 11–18 × 4.0–5.0 μm, aliquando in apice inflati, appressi prope basim et saepe ab axi averti, metulae portatae in uvis manifeste diversis 5–7 continentibus, breves et latae, 9–12 × 3.5–4.0 μm, plerumque in apice inflatae; phialides in verticillis diversis 6–8 continentibus, ampulliformes, plerumque 7–10 × 2.2–3.0 μm, cum collulis brevibus; conidia ellipsoidea,

2.5–3.5 × 2.0–2.5 µm, tenuiter tunicata vel in extremis asperata, portata in catenis confusis.

Colonies grown 7 d on CYA at 25 C (FIGS. 10–11) attain 23–26 mm diam, are radially sulcate with an annular furrow around the raised colony central area, occasionally crateriform, moderately deep, composed of a layer of contiguous brown sclerotia on the agar surface that dominate the colony appearance surmounted by scattered penicilli, surface texture velutinous, margins white, irregular, narrow; mycelium white; conidiogenesis moderate, gnaphalium green to tea green (Ridgway Pl. XLVII), limited amounts of clear exudate produced at the margins of the colony; soluble pigment absent; reverse dusky drab to blackish brown (Ridgway Pl. XLV), pale at colony margins.

Colonies grown 7 d on MEA at 25 C (FIGS. 10 and 12) attain 15–17 mm diam, are plane but centrally raised, moderately deep of velutinous texture with abundant clusters of brown sclerotia (FIG. 17) produced on the agar surface surmounted by scattered penicilli; margins narrow, irregular; mycelium inconspicuous, white; conidiogenesis moderate in colors similar to those on CYA, heavier between clusters of sclerotia formed in radial lines and at colony margins, exudates and soluble pigment absent; reverse pale brown darker at the center.

When incubated at 5 C and 37 C in CYA, no growth was observed in 7 d. On G25N colonies attain 22–24 mm diam, irregularly sulcate and centrally convolute, moderately deep, surface texture velutinous, margins extending 2–3 mm subsurface; mycelium white enveloping white to cream sclerotia, conidiogenesis moderate, tea green, no exudates and soluble pigment produced; reverse pale brownish at the center.

Sclerotia (FIGS. 17–18) ellipsoidal to irregular in shape, usually 200–360 µm in long axis, rapidly becoming sclerotioid, pale at first, then brown, dusky drab to blackish brown (Ridgway Pl. XLV) on CYA and Kaiser Brown to Chestnut Brown on MEA (Ridgway XIV), but pale cream on G25N. Teleomorphic state not known.

Conidiophores (FIGS. 13–16) borne from subsurface hyphae, *stipes* usually long and broad, 500–800 × 4.5–6.0 µm, smooth walled, bearing compact, broad terverticillate penicilli, usually less than 40 µm long and 35–53 µm broad, with quaterverticillate and biverticillate penicilli usually evident also; *rami* usually borne singly, short and broad, 11–18 × 4.0–5.0 µm, sometimes apically inflated, appressed at the base and often bent away from the axis, with penicilli distinctively multirramulate also; *metulae* borne in markedly divergent clusters of 5–7, short and broad, 9–12 × 3.5–4.0 µm, typically apically inflated; *phialides* in divergent verticils of 6–8, ampulliform, commonly 7–10 × 2.2–3.0 µm, with short collula; *conidia* ellipsoidal, 2.5–3.5 × 2.0–2.5 µm, with walls smooth or finally roughened (FIGS. 19–20), borne in disordered chains.

HOLOTYPE: BPI 872161 here designated, deposited at the U.S. National Fungus Collection is a dried culture of NRRL 35639 on MEA and CYA. Culture extype: NRRL 35639.

*Etymology*. *neocrassum* related to *P. crassum* Sopp, the only species that was ever described to produce sclerotia in the *P. brevicompactum* series but not typified.

*Origin of strains*. HOLOTYPE: PORTUGAL. MADEIRA ISLAND, Câmara de Lobos. Vineyard at 32°38′N and 16°56′W, on healthy grapes, 8 Sep 2003, *Rita Serra 03UP383*. Isotype deposited in the MUM culture collection, Braga, Portugal.

*Additional specimens examined:* NRRL 35648 (MUM 06.162), PORTUGAL. MADEIRA ISLAND, Câmara de Lobos, from healthy grapes, 8 Aug 2003, *Rita Serra 03UP426* (isolated from a different *Vitis vinifera* plant than the ex-type strain). The growth rates were slightly slower on all culture media achieving 19–21 mm diam on CYA. Poor germination was observed at 5 C. Colony texture on CYA is floccose and the conidial color is paler than the type strain. Sclerotia were observed after 7 d growth on MEA only but were clearly visible on CYA and G25N after 12 d. A higher proportion of multirramulate penicilli was observed than in the type strain.

*Commentary*. Microscopically this species resembles *P. brevicompactum* but penicilli typical of *P. olsonii* are evident also. The colonies resemble those of *P. brevicompactum* and differ from it mainly by the production of sclerotia visible at least on MEA.

## RESULTS

The total length of the ID region DNA sequence of *P. astrolabium* NRRL 35611 is 1148 base pairs. In BLAST search *P. astrolabium* was a 98–99% match to the nucleotide sequences of *P. olsonii* strains, differing from *P. olsonii* at 11–13 nucleotide positions. The total length of the ID region DNA sequence of *P. neocrassum* NRRL 35639 and 35648 was respectively 1149 and 1150 base pairs. The *P. neocrassum* strains differed in one single base position. In BLAST searches *P. neocrassum* showed 99% similarity to the nucleotide sequences from *P. brevicompactum* strains, differing from *P. brevicompactum* at 5–7 nucleotide positions and had 98% similarity to *P. bialowiezense* and *P. olsonii* strains. The sclerotial strain of *P. olsonii* matched 100% with the ID sequence of the ex-type *P. olsonii* strain and differed at up to two base pairs with other *P. olsonii* sequences deposited in GenBank. The polymorphic sites between *P. astrolabium*/*P. olsonii* and *P. neocrassum*/*P. brevicompactum* are indicated (TABLE II).

TABLE II. Polymorphisms in the ID region for *P. astrolabium* and *P. neocrassum* comparatively to *P. olsonii* and *P. brevicompactum*, respectively

| | Nucleotide position | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *75* | *91* | *94* | *104* | *105* | *108* | *362* | *443* | *456* | *457* | *572* | *575* | *597* | *913* | *938* | *940* | *979* | *1017* | *1018* |
| *P. astrolabium* 35611 | | | | A | T | | C | | A | C | A | T | T | | T | C | A | T | C |
| *P. olsonii* 35612 | | | | | | | T | | | | | | | | | | | | T |
| *P. olsonii* | | | | T | C | | T | | C | T | G | C | C | | C | T | G | C | C |
| | | | | | | | C | | | | | | | | | | | | T |
| *P. neocrassum* 35639 | C | A | T | | | T | | T | T | | | | | A | | | | | C |
| *P. neocrassum* 35648 | C | A | T | | | T | | T | T | | | | | G | | | | | C |
| *P. brevicompactum* | T | G | T | | | C | | C | C | | | | | G | | | | | T |
| | | T | C | | | | | | | | | | | | | | | | |

Nucleotide changes at the β-tubulin locus were found in both the introns and coding regions and resulted in distinct amino acid sequences even between strains of the same species. *P. brevicompactum* and *P. olsonii* strains had a single amino acid substitution each while *P. bialowiezense* strains had four amino acid changes. *P. neocrassum* strains did not differ in the amino acid sequence. Seven amino acid changes were observed between *P. astrolabium* and *P. olsonii* type strain and two amino acid substitutions were found between *P. neocrassum* and *P. brevicompactum* type strain. On the contrary, most of the changes detected in calmodulin DNA sequences were in the introns. No amino acid substitution was observed between *P. astrolabium*, *P. neocrassum*, *P. olsonii* and *P. brevicompactum* type strains.

Parsimony analysis of the ID region dataset produced a single most parsimonious (MP) tree. *P. astrolabium* and *P. olsonii* were placed in the same group, and *P. bialowiezense* and *P. brevicompactum* were clustered in a distinct group. *P. neocrassum* was in the same clade as *P. bialowiezense*. The bootstrap support for the *P. astrolabium* and *P. olsonii* branch was strong (87%), but the support for *P. bialowiezense* and *P. brevicompactum* was lower (56%). Nevertheless the support for *P. neocrassum* in a distinct clade from *P. bialowiezense* was high (88%).

Parsimony analysis of the protein coding DNA sequence datasets produced six equally most parsimonious trees (β-tubulin) and 25 equally most parsimonious trees (calmodulin). The protein coding DNA sequences produced phylogenetic trees better supported in boostrap analysis. The aligned β-tubulin data and calmodulin datasets both placed *P. astrolabium* and *P. olsonii* in one clade and *P. neocrassum* and *P. brevicompactum* in a distinct clade. Because trees from the three loci were congruent or at least noncontradictory, sequences were combined into a single data set. The single most parsimonious tree obtained and bootstrap values from the combined dataset are shown (FIG. 21).

## DISCUSSION

*P. astrolabium* and *P. neocrassum* are well supported by phylogenetic analysis of each locus and the combined data, which clearly indicate that these species are related to *P. olsonii* and *P. brevicompactum*, respectively, but are phenotypically and genetically distinct.

Peterson (2004) showed using ID, calmodulin and elongation factor 1-α loci that *P. bialowiezense*, *P. brevicompactum* and *P. olsonii* are distinct but closely related species using the genealogical concordance phylogenetic species concept (Taylor et al 1999). Samson and Frisvad (2004) produced a β-tubulin tree showing the same relationships as Peterson (2004) and formed their taxonomic scheme on the basis of the phylogeny. They recognize three species in the *Olsonii* series and supplement the DNA sequence data with secondary metabolite information also.

Most differences in the trees produced with DNA sequences of the three distinct loci were at the tips of the branches. However one branch point in the ID tree differed from the two protein coding gene trees in showing *P. neocrassum* as a sibling of *P. bialowiezense* rather than *P. brevicompactum*. Three phylogenetically informative sites in the ID dataset supported the *P. neocrassum/P. brevicompactum* branching while two informative sites supported the *P.neocrassum/P. bialowiezense* branching. Bootstrap support for this branch was 56% of the bootstrap samples. With the low number of informative sites and low bootstrap support we view the data as being insufficient to correctly reconstruct the branching order at this node and thus the tree is noncontradictory to the trees based on protein coding loci.

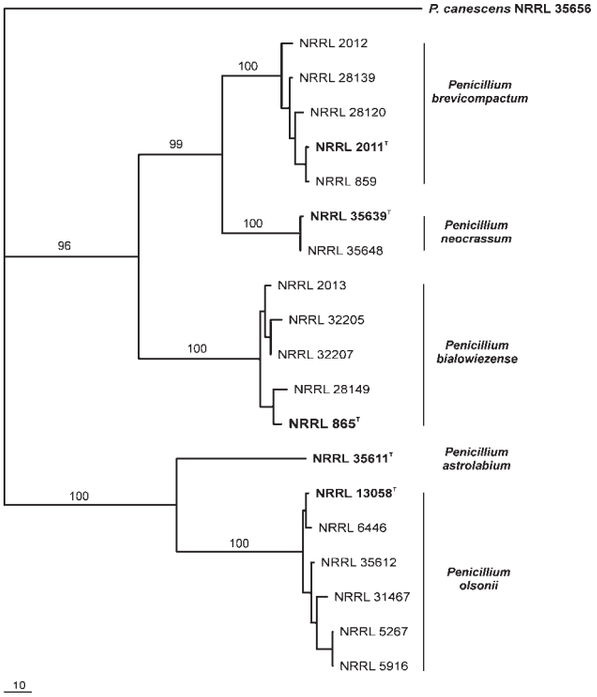



FIG. 21. Phylogenetic tree generated in a heuristic search of the combined sequence of ID locus and partial calmodulin and β-tubulin sequences. Bootstrap values based on 1000 replicates.

Although Peterson (2004) could not resolve the branching order of *P. olsonii*, *P. brevicompactum* and *P. bialowiezense*, the combined dataset analyzed (FIG. 21) are strongly supportive of *P. brevicompactum* and *P. bialowiezense* as members of the same clade, with *P. neocrassum*, *Penicillium olsonii* and *P. astrolabium* forming a distinct clade.

The phylogenetic analysis of the five species shows that they are genetically well isolated with the multilocus approach. According to Taylor and co-workers (1999), it is expected that genetically isolated species will exhibit morphological differences with time by genetic drift. Phenotypically all the *Penicillium* species in this group share unique terverticillate penicillin, broad and compact, with *P. brevicompactum* in one extreme, characteristically with only one rami and *P. olsonii* at the other extreme with complex multirramulate penicilli. The phenotypic differences between *P. brevicompactum* and *P. olsonii* are clear enough to recognize the species but intermediate forms are observed. Pitt (1980) described the presence of multiramulate penicilli in *P. brevicompactum* strains such as NRRL 5276 and synonymized *P. volgaense* with *P. brevicompactum*. Peterson (2004) confirmed with multilocus analysis that the strain NRRL 5276 fits perfectly in the *P. brevicompactum* clade but *P. volgaense* fits into and was synonymized with *P. olsonii*, demonstrating the affinities in the penicilli of both species that we clearly observed in *P. neocrassum* also.

The effects of genetic drift can be recognized in *P. bialowiezense* by the differing phenotypes among the isolates. However no obvious, fixed synapomorphies have been found that can be used reliably to identify *P. bialowiezense* isolates. The morphological recognition of *P. astrolabium* and *P. neocrassum* from its allies *P. olsonii* and *P. brevicompactum* is possible based on evident cultural characters. *P. astrolabium* produces a characteristic dark pigmentation on reverse and unusual enlarged and heavily pigmented hypha that resembles subsurface sclerotia (FIGS. 3, 5). Thom's notes on the culture 4725.1021 (Thom and Raper 1949) with penicilli typical of *P. olsonii* mention the production of ''masses of hyphae with sporadically at least the development of sclerotia or perithecia, black, more or less submerged, brittle but so far not producing asci.'' This is the only mention of black hypha structures on a culture with conidiophores typical of *P. olsonii*, but it is not possible to assign Thom's culture to *P. astrolabium* because the culture was lost. The production of sclerotia by terverticillate species is uncommon. In the past few years Samson and Frisvad (2004) described some isolates of *P. olsonii* from tropical soil that produced sclerotia. We also isolated a sclerotial *P. olsonii* strain from Portuguese grapes. The presence of sclerotia in *P. olsonii* is rare but no differences in DNA sequences were found with the *P. olsonii* type strain. The sclerotia present in *P. olsonii* do not indicate a genetic isolation of the strains.

*P. neocrassum*'s most evident cultural trait that distinguishes it from *P. brevicompactum* is the production of sclerotia. The presence of sclerotia production by a *P. brevicompactum*-like strain isolated from a rotten apple was reported by Sopp after growth on rice. This was the only time that sclerotia were reported on a culture with *P. brevicompactum* conidiophores. Sopp considered this to be sufficient to define a new species, *P. crassum*, but failed to typify the species. Thom (1930) accepted the species and Raper and Thom (1949) and Ramirez (1982) synonymized it with *P. brevicompactum*. Because no type or type culture of *P. crassum* exists we cannot be certain whether it would match *P. neocrassum*.

The phenotypic differences that allow the clear identification of *P. astrolabium* and *P. neocrassum* probably reflect selective pressures found in each species' niche that led to fixation of particular survival-critical features. Wicklow and associates (Belofsky et al 1998, Wang et al 1995, Wicklow et al 1994) have speculated on the survival value of sclerotia and secondary metabolites in different habitats. Sclero-

tium production appears to be a variable trait but nevertheless can be a valuable indicator of new species. However due to strain variability sclerotia per se cannot be used alone for new taxon definition, as the discovery of sclerogenic *P. olsonii* demonstrates with phylogenetic analysis. The *olsonii* series lacks known teleomorphs. With time yellow clusters of mycelium are formed in *P. astrolabium* vaguely reminiscent of cleistothecia, but no teleomorph was found after 2 mo incubation.

Species in this tree branch are capable of growing at low water activities and are common as decaying agents. *P. astrolabium*, *P. olsonii*, *P. brevicompactum* and *P. neocrassum* were all isolated from grapes. The presence of *P. astrolabium* was rare, being isolated from one grape sample only and the presence of *P. olsonii* in grapes was also uncommon. On the contrary *P. brevicompactum* was one of the most abundant species found in berries with and without surface disinfection. *P. bialowiezense* was not morphologically recognized and therefore no information exists concerning its presence or absence in grapes. *P. neocrassum* was isolated from two out of 10 samples from a vineyard on Madeira Island that had a mycobiota distinct from the continental grapes. *Penicillium* species, in particular *P. brevicompactum*, were the dominant fungi isolated from Madeira Island samples (Serra et al 2006b). Some rare *Penicillium* species were isolated from those samples and recently were identified with the DNA barcoding methodology described in this paper (data not shown) such as *P. adametzioides* previously known from type isolate only and some undescribed sclerogenic strains related to *P. adametzioides* that will be described separately.

At this time the ecological role of *P. astrolabium* and *P. neocrassum* in vineyards is not known. The ultimate aim of the authors in describing these new taxa is to contribute to the knowledge of biodiversity in a genus useful in biotechnology and to attract attention to the existence of these easily recognizable species in future field studies and surveys. More information on the ecology and biogeographical distribution of these rare species will result.

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## LITERATURE CITED

Belofsky GN, Gloer KB, Gloer JB, Wicklow DT, Dowd PF. 1998. New p-terphenyl and polyketide metabolites from the sclerotia of *Penicillium raistrickii*. J Nat Prod 61: 1115–1119.

Page RDM. 1996. Treeview: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12:357–358.

Peterson SW, Vega FE, Posada F, Nagai C. 2005. *Penicillium coffeae* a new endophytic species isolated from a coffee plant and its phylogenetic relationship to *P. fellutanum, P. thiersii* and *P. brocae* based on parsimony analysis of multilocus DNA sequences. Mycologia 97: 659–666.

———. 1992. *Neosartorya pseudofischeri sp.* nov. and its relationship to other species in *Aspergillus* section *Fumigati*. Mycol Res 96:547–554.

———. 2004. Multilocus DNA sequence analysis shows that *Penicillium biourgeianum* is a distinct species closely related to *Penicillium brevicompactum* and *P. olsonii*. Mycol Res 108:434–440.

Pitt JI. 1980. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. New York: Academic Press. 634 p.

———. 1985. A laboratory guide to common *Penicillium* species. North Ryde: CSIRO Division of Food Processing.

Ramirez C. 1982. Manual and atlas of the Penicillia. New York: Elsevier Biomedical Press. 874 p.

Raper KC, Thom C. 1949. A manual of the Penicillia. Baltimore, Maryland: Williams & Wilkins Co. 875 p.

Ridgway R. 1912. Color standards and color nomenclature. Washington, D.C.: Published by the author. 43 p. 53 plates.

Samson RA, Frisvad JC. 2004. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Stud Mycol 49:1–260.

Serra R, Braga A, Venâncio A. 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res Microbiol 156:515–521.

———, Cabañes FJ, Perrone G, Castellá G, Venâncio A, Mulè G, Kozakiewicz Z. 2006a. *Aspergillus ibericus*: a new species of section *Nigri* isolated from grapes. Mycologia 98:295–306.

———, Mendonça C, Venâncio A. 2006b. Fungi and ochratoxin A detected in healthy grapes for wine production. Lett Appl Microbiol 42:42–47.

Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fun genet biol 31:21–32.

Thom C. 1930. The Penicillia. Baltimore, Maryland: Williams & Wilkins Co. 644 p.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal V:

improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acid Res 22:4673–4680.

Wang H, Gloer JB, Wicklow DT, Dowd PF. 1995. Aflavinines and other antiinsectan metabolites from the ascostromata of *Eupenicillium crustaceum* and related species. Appl Environment Microbiol 61:4429–4435.

Wicklow DT, Dowd PF, Gloer JB. 1994. Antiinsectan effects of *Aspergillus* metabolites. In: Powell KA, Renwick A, Peberdy JF, eds. The genus Aspergillus. New York: Plenum Press. p 313–320.