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First report of *Penicillium costaricense* Visagie, M. Urb & Seifert (Eurotiales, Ascomycota) in South America and a second report for the world

Laureana de Vasconcelos Sobral, Diogo Xavier Lima, Joenny Maria da Silveira de Lima, Renan do Nascimento Barbosa, Roberta Cruz, Cristina Maria de Souza-Motta

Programa de Pós-Graduação em Biologia de Fungos, Departamento de Micologia, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife, PE, Brazil
Corresponding author: Diogo Xavier Lima, diogo_xavier00@hotmail.com

Abstract
Penicillium Link is a cosmopolitan group of fungi able to colonize various substrates and habitats. Like other fungi, atmospheric air is one of the most common ways for dispersion. *Penicillium* species in indoor hospital air are an important risk for patients whom may develop infections. We isolated *Penicillium costaricense* Visagie, M. Urb & Seifert in air samples from a surgery center in a public hospital in Brazil. The isolate was identified by morphology together with the β-tubulin and calmodulin molecular markers. The only published data on the occurrence of this species is from the intestine of the caterpillar of *Rothschildia lebeau* (Guérin-Méneville, 1868) in Costa Rica. This report is a warning call to understand the pathogenicity of this species. To the best of our knowledge, this is the first report of *P. costaricense* in South American and only the second report in the world.

Keywords
Airborne fungi, Eurotiomycetes, indoor fungi, taxonomy

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Introduction

Penicillium Link is a generalist and cosmopolitan group of fungi which belongs to Ascomycetes and classified in the order Eurotiales, family Aspergillaceae (Visagie et al. 2020). Visagie et al. (2014) accepted 354 species, yet this number is rapidly increasing with the description of new species from several substrates and habitats (e.g. Houbraken et al. 2016a; Barbosa et al. 2018; Diao et al. 2018; George et al. 2019; Gonçalves et al. 2019; Visagie et al. 2020). Species of *Penicillium* are commonly found in soil, decaying organic materials, animal feed, and stored grains, as well as indoors (Cruz et al. 2013; Barbosa et al. 2016, 2018; Houbraken et al. 2016b; Yadav et al. 2018).

The typical morphological feature of *Penicillium* species is their dense, brush-like, spore-bearing structures called penicilli. The conidiophores are simple or branched and are terminated by clusters of flask-shaped phialides. The asexual spores (conidia) are produced in chains from the tips of the phialides, with the youngest spore at the base of the chain, and are nearly always green (Raper and Thom 1949; Onions and Brady 1987;

Pitt 1991). Even though the taxonomic structure of the genus *Penicillium* is well defined, the identification of species based only on morphology is still problematic (Visagie et al. 2014). Thus, multigene phylogenetics using β-tubulin (*BenA*) and calmodulin (*CaM*) is recommended to confirm species identification.

In order to present a natural classification, species of these genus are grouped in several sections based on morphology and phylogeny (Houbraken et al. 2020). The *Penicillium* section *Charlesia* Houbraken & Samson comprises rather rare species which exhibit monoverticillate or biverticillate conidiophores (Visagie et al. 2016). Currently, Houbraken et al. (2020) accepted nine species in this section: *P. charlesii* G. Sm., *P. chermesinum* Biourge, *P. coffeae* S.W. Peterson, F.E. Vega, Posada & Nagai, *P. costaricense* Visagie, M. Urb & Seifert, *P. cuddlyae* Visagie & I.H. Rong, *P. fellutanum* Biourge, *P. indicum* D.K. Sandhu & R.S. Sandhu, *P. lunae* Visagie & Yilmaz, and *P. phoeniceum* J.F.H. Beyma. Some of these species, such as *P. chermesinum*, *P. fellutanum*, and *P. phoeniceum*, were mainly reported as anemophilous in indoor hospital environments (Sarica et al. 2002; Okten and Asan 2012; Demirel et al. 2017; Cho et al. 2018).

Like other fungi, atmospheric air is one of the most common ways for *Penicillium* spores dispersion (Nascimento et al. 2019). Generally, these fungi are able to colonize various substrates (Lima et al. 2019) and are the main contaminants of indoor and artificially heated environments (Oliveira et al. 2020). Fungal spores can impact human health by simple allergic reactions or by spread of infections in immunocompromised individuals (Calumby et al. 2019; Souza et al. 2019). Thus, airborne fungi in indoor hospital environments are an important risk factor mainly for patients with immune system impairment, for whom the risk of developing infections caused by spores or fungal fragments present in the air increases substantially (Li et al. 2007; Nascimento et al. 2019).

During a study about the occurrence as anemophilous fungi in indoor hospital environments, one strain of *P. costaricense*, which belongs to the section *Charlesia*, was isolated. This species was described in 2016 from a single isolate obtained in 2001 from the intestines of *Rothschildia lebea* (Guérin-Méneville, 1868) feeding on *Spondias mombin* L. Seventeen years after the first isolation, this species is being reported for a second time in the world, however, now from the air of a surgery center in a public hospital in Caruaru, Pernambuco, Brazil. Using morphological and molecular analyses, we highlight the occurrence of this species for the first time in South America and the second time in the world.

Methods

Isolation and purification. Air samples from a surgery center in a public hospital in Caruaru, Pernambuco, Brazil (Fig. 1) were collected in August, October, and

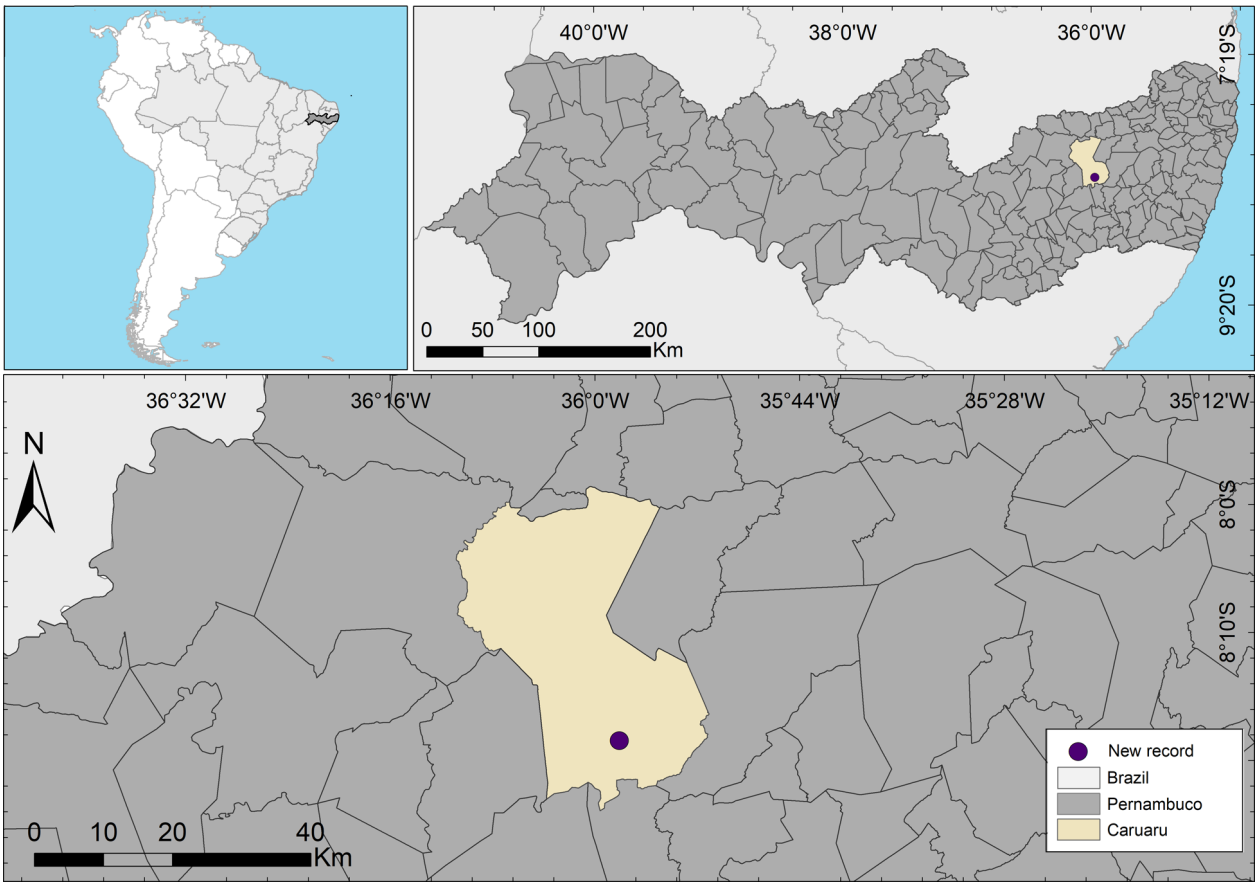

Figure 1. The location in South America (Caruaru, Pernambuco, Brazil; 08°18′27″S, 035°58′05″W) where *Penicillium costaricense* URM 8341 was found.

December 2018. For this purpose, the technique of passive sedimentation was used (Abbasi et al. 2020). Sabouraud dextrose agar with chloramphenicol (100 mg/L) (SDA), contained in Petri dishes, was used as the medium for isolation. Three plates were opened at 1m distance off the floor at different locations in the environment for 30 minutes for the deposition of the microorganisms. In the laboratory, the plates were kept at room temperature (T.A 28 °C ± 2 °C) for 10 days. All colonies grown on the dishes were transferred to tube slants with malt agar media until identification.

Morphology. Morphological analysis was performed according to the recommendations of Barbosa et al. (2018). In summary, the strain was three-point inoculated in 9-cm plastic Petri dishes using a dense conidial suspension in Czapek yeast extract agar (CYA), malt extract agar (MEA), and supplemented yeast extract agar (YES). All media were prepared according to Samson et al. (2010). For determination of cultural characteristics (growth rate, texture of colony, pigmentation, and exudates) plates were incubated in the dark at 25 °C for 7 days. Microscopic observations of the asexual stage were made from colonies grown on MEA. Lactic acid (60%) was used as a mounting fluid, and 96% ethanol was used to remove excess conidia. The cultural characteristics and microscopic observations were compared with previous descriptions by Visagie et al. (2016). Following the recommendations of Barbosa et al. (2020), the isolate was subsequently deposited in the Micoteca URM culture collection (Federal University of Pernambuco, Recife, Brazil).

DNA sequence analysis. Genomic DNA extractions were made from 7-day-old colonies grown on MEA using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). Polymerase chain reaction (PCR) amplifications of the *BenA* and *CaM* gene regions were performed using primers and conditions indicated by Visagie et al. (2014). The PCR products were sequenced in both directions with the same primers using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA, USA) and purified with NucleoSAP® (Exonuclease and Alkaline Phosphatase Mix), according to the manufacturers' recommendations. Contigs were assembled in the SeqMan (v. 10.0.1; Madison, WI, USA) program. Newly generated sequences were submitted to GenBank.

Phylogenetic analyses. The gene sequences generated were analyzed in the BLAST search and phylogenies were calculated for the section *Charlesia* with a reference sequence dataset from Visagie et al. (2016). The sequences were aligned using MAFFT v. 7 (Katoh and Standley 2013) with the G-INS-I algorithm and manually optimized using MEGA v. 6.06 (Tamura et al. 2013). Individual alignments were concatenated by using Mesquite v. 3.04 (Maddison and Maddison 2016). The most suitable substitution model was determined using jModelTest v. 2.1.7 (Posada 2008). Datasets were subsequently analyzed using a maximum likelihood (ML) analysis performed using RAxML v. 7.2.8 HPC BlackBox with the GTRGAMMA model for nucleotide substitution at the CIPRES science gateway (http://www.phylo.org/) (Miller et al. 2010). A Bayesian tree inference (BI) was performed in MrBayes v. 3.2.2 (Ronquist et al. 2012). In the BI analyses, every 1,000 generations were sampled and the first 25% of the samples were discarded. Trees were visualized in FigTree v. 1.1.2 (Rambaut 2009) and edited in Adobe Illustrator v. 5.1. BI, posterior probability (pp), and bootstrap (bs) values are labelled at the nodes. Values less than 0.95 pp and 70% bootstrap support are not shown. Branches with full support in Bayesian and RAxML analyses are thickened.

Results

Penicillium costaricense **Visagie, M. Urb & Seifert**, Persoonia 36: 263. (Visagie et al. 2016)
Figure 2A–H

Identification. Colony morphology in CYA culture medium (1.9 cm in diameter after 7 days at 25 °C): low plane of radial sulcate, low margin, texture velutinous; dense sporulation, gray-turquoise to opaque green conidia on the surface (MP 114C) (Maerz and Paul 1950); soluble pigments absent; exudates absent; sclerotia absent; reverse yellow (MP 114D) and MEA (2.5 cm in diameter after 7 days at 25 °C): radially low sulcate plane, low margin, texture velutinous; sparse sporulation, opaque green conidia on the surface (MP 114C); absence of soluble pigments; exudates absent; sclerotia absent; reverse yellow (MP 114D). Microscopy: conidiophores monoverticillate 30–260 × 2–3 µm, stem smooth. Vesicles formed terminally on conidiophores, 2.4–4 µm long. Phialides ampulliform, 10–20 per stipe, 7.5–12 × 2–3.5 µm. Conidia subglobose, 2.5–3 × 2–3 µm, smooth-walled.

Our phylogenetic analyses of *P. costaricense* and related species were prepared for the *BenA* and *CaM* genes, as well as a concatenation of both. Results showed, with high support values, that the sequence from *P. costaricense* URM 8341 is placed in the same clade as the sequence of *P. costaricense* DAOMC250520 (Fig. 3). The BLASTn analysis of the *BenA* and *CaM* gene sequences showed 99.75% and 98.26% identity, respectively, between URM 8341 and the holotype.

Material examined. BRAZIL • Pernambuco, Caruaru; 08°18′27″S, 035°58′05″W; 27 Mar. 2020; Laureana de Vasconcelos Sobral URM 8341; habitat: as anemophile; GenBank accession no. *BenA*: LR796905; *CaM*: LR794231.

Distribution. Brazil and Costa Rica.

Discussion

Although *Penicillium costaricense* was first found inside the intestine of a caterpillar of *Rothschildia lebeau* in Costa Rica (Visagie et al. 2016), a specimen of the same

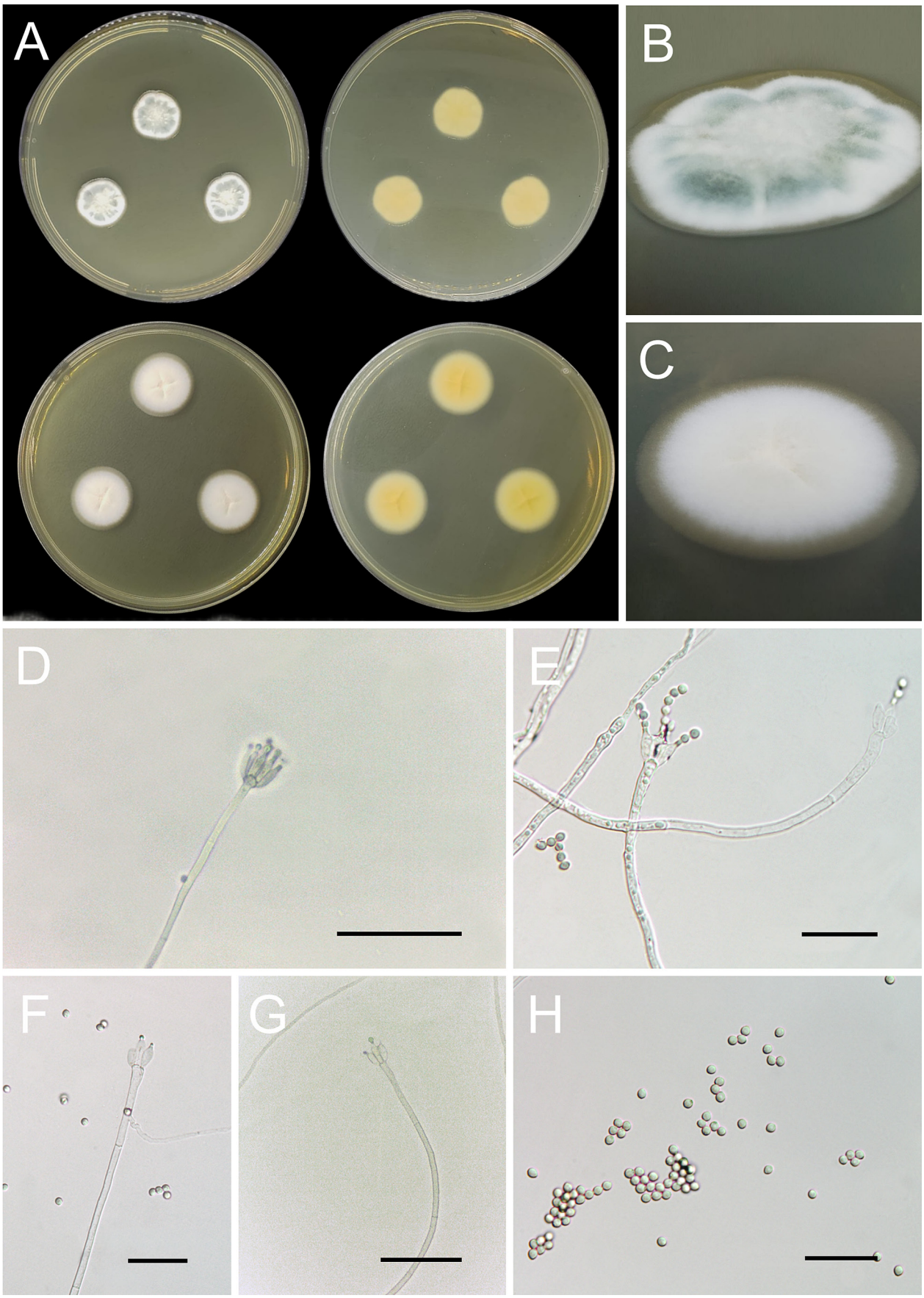

Figure 2. *Penicillium costaricense* URM 8341. **A.** Colonies in the top row grown on CYA, and reverse (from left to right), in the bottom row grown on MEA, and reverse (from left to right). **B.** Colony texture on CYA **C.** Colony texture on MEA. **D, E, F, G.** Conidiophores. **H.** Conidia. Scale bars: E, G, H = 30 µm; D = 50 µm; F = 20 µm.

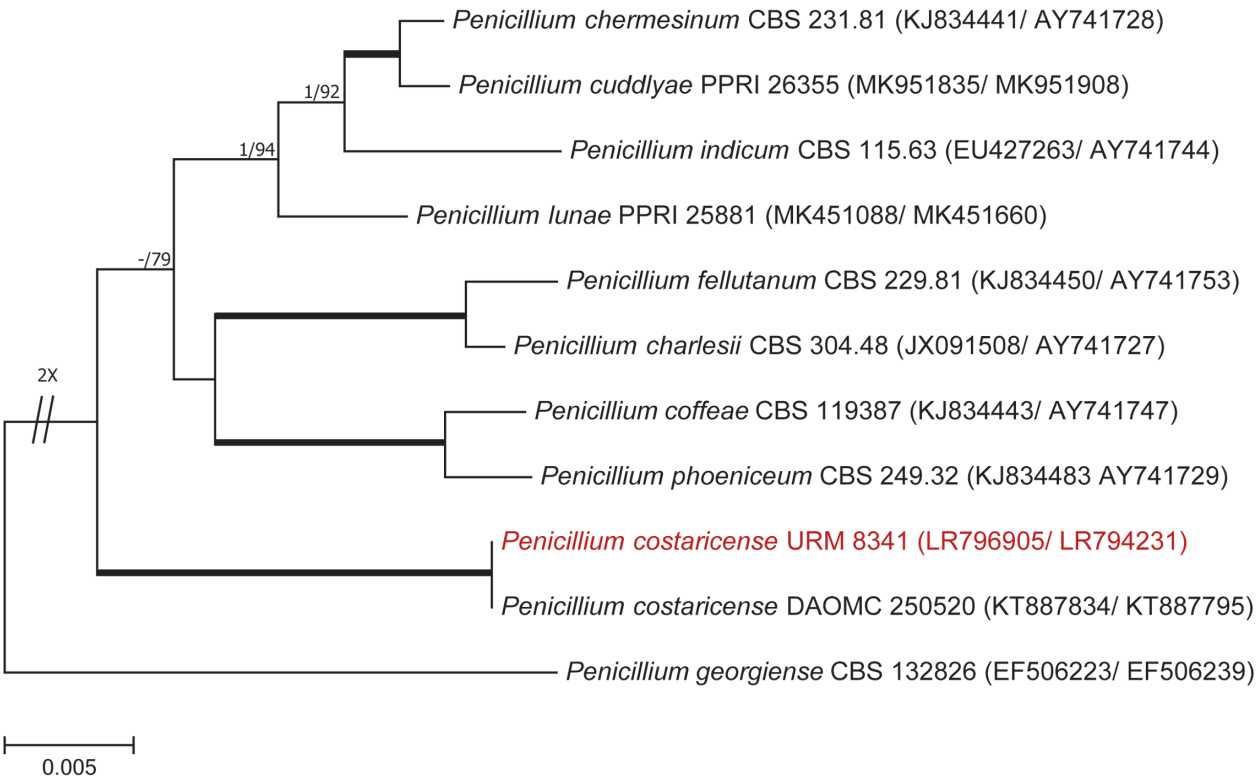

Figure 3. Phylogenetic tree of *Penicillium* section *Charlesia* and *P. costaricense* URM 8341, constructed using the *BenA* and *CaM* genes sequences. *Penicillium georgiense* was used as the outgroup. Sequences are labeled with their database accession numbers. Support values were obtained from Bayesian and Maximum likelihood analysis. Values below 0.95 pp and 70 % are not shown and indicated with a hyphen. Branches with posterior probability values of 1.00 and >95 % are thickened.

species was isolated as an anemophile in a surgery center at a public hospital in the state of Pernambuco, Brazil. It is not the first time that species of *Penicillium* section *Charlesia* have been found in critical areas of hospital environments (Sarica et al. 2002; Okten and Asan 2012; Demirel et al. 2017; Cho et al. 2018), but nevertheless, it is important that the second record of this *Penicillium* species has been found occurring in such a sensitive hospital environment, especially as information about pathogenicity of this species remains unknown. Additionally, in this study we contribute to the knowledge of the geographic distribution of *P. costaricense* in the world. The morphological characteristics of *P. costaricense* URM 8341 show close similarity to the description by Visagie et al. (2016), although we observed a few variations in relation to the size of conidiophore and phialides, which are shorter than those of the holotype (30–260 µm vs 50–280 µm and 7.5–12 × 2–3.5 µm vs 8.5–11.5 × 2–3.5 µm, respectively). The colonies of *P. costaricense* described by Visagie et al. (2016) in CYA are slightly different, as their conidia were found in a grayish turquoise mass, whereas our strain has grayish-turquoise to opaque green conidia on the back. *Penicillium costaricense* URM 8341 also has sparse sporulation on MEA, different from the moderately dense sporulation in the holotype. The phylogenetic analysis based on the *BenA* and *CaM* genes indicated that *P. costaricense* URM 8341 is closely related to species belonging to the section *Charlesia* and was compared with close relatives. *Penicillium charlesii*, *P. fellutanum*, and *P. georgiense* produce biverticillate to divaricate conidiophores, whereas *P. costaricense* has monoverticillate conidiophores. The conidiophores of *P. coffeae*, *P. indicum*, and *P. phoeniceum* are also monoverticillate. However, *P. coffeae* could be distinguished on the basis of smaller colony diameter on MEA (1.3 cm) and CYA (1.7 cm) at 25 °C. Unlike *P. costaricense*, *P. indicum*, and *P. phoeniceum*, it can be easy distinguished by producing intensely pigmented exudate and abundant sclerotia on MEA, respectively (Raper and Thom 1949; Sandhu and Sandhu 1963; Peterson et al. 2005; Visagie et al. 2016).

We report the first occurrence of *P. costaricense* to South America, its first report as an anemophile, and only the second record worldwide; we isolated this species from air in a surgical center in a public hospital in Brazil, which contributes to the knowledge of the geographic distribution of a eurotialean fungi. Although *P. costaricense* URM 8341 was isolated from a hospital environment, its pathogenicity is unknown, and therefore, further studies are needed to determine its medical importance and understand the possible ecological relationships of this species.

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Authors' Contributions

CMSM, RC, and LVS prepared the project and supervised the experiment; LVS and JMLS collected the material and executed DNA sequence analysis; LVS and DXL wrote the text; DXL and RC identified the species; RNB made the phylogenetic trees; CMSM, RC, DXL, RNB, and JMLS revised the text.

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