Penicillium Amapaense sp. nov. Exilicaulis Section and New Records of Penicillium Labradorum in Brazil Isolated from Amazon River Sediments with Potential Applications in Agriculture and Biotechnology

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Abstract

The genus *Penicillium* is recognized for its ability to produce bioactive molecules with a wide range of biotechnological applications. Currently, the genus is distributed in 28 sections, with more than 50 species in the *Exilicaulis* section. Representative species of this section are responsible for the production of antimicrobial compounds, but they can also produce genotoxic compounds that affect commercial mushroom production or cause disease in immunosuppressed animals. In the present study, based on morphological characters such as the length of the conidia, phialides and stipes, as well as sequence analysis of the ITS region and partial sequence of *CAM*, *TUB2* and *RPB2* loci, we describe a new fungal species denominated *Penicillium amapaense* and report for the first time the occurrence of *Penicillium labrodorum* in Brazil, both of which were isolated from sediments of the Amazon River. The isolates obtained in this study for each species were submitted to antibiosis assays against 12 phytopathogenic fungi that affect important agricultural crops in Brazil and showed inhibition of 11 out of 12 of them. The production of amylase, cellulase and siderophore as well phosphate solubilization was also detected. Metabolomic analysis indicates the ability of *P. labrodorum* and *Penicillium amapaense* sp nov. to produce polyketides such as known curvularins and anthraquinones. In addition to these, unknown compounds were also detected. These results indicate the biotechnological and agricultural potential of *P. labrodorum* and *P. amapaense*.

Introduction

Species of the genus *Penicillium* (Ascomycota; *Trichocomaceae*) are present in different environments such as soils, water and air, and can be found living in plants as endophytes, while some species can also be plant pathogens and even cause disease in humans and other animals (Yadav et al. 2018). *Penicillium* is recognized for its wide range of biotechnological applications, due to the production of several secondary metabolites with antimicrobial, antioxidant, neuroprotective, insecticide, cytotoxic, anti-inflammatory, antioxidant and other properties (Yuan et al. 2017; Hawuas et al. 2019; Youssef et al. 2018; Kumar et al. 2018; Girich et al. 2020; Bai et al. 2019; Bai et al. 2021; Govindappa et al. 2021).

The genus *Penicillium* was first described by Link et al. (1809) and has since undergone several taxonomic revisions based on morphology (Thom 1930; Raper and Thom 1949: Pitt 1979; Ramírez 1982). A taxonomic review, based on ITS (Internal transcribed spacer) data, began in the 1990s with revisions by Pitt and Samson (1993). In 2011, a review based on the evaluation of its morphological characters and on multilocus phylogenetic analysis, using data from partial gene sequences: *RPB1* and *RPB2* (subunits of the catalytic core of RNA polymerase II), *TSR1* (ribosome biogenesis protein) and *CCT8* (component of the chaperonin complex) created a new classification system based on the 25 distinct monophyletic clades formed, which came to be treated as sections (Houbraken et al 2011). Visagie et al. (2014) provided the databases of the *Penicillium* type-species with partial sequence data for the *cam*, *TUB2* and *rpb2* genes and provided guidelines for the identification and nomenclature of the genus.
Recently, a new series classification system was proposed that was based on morphological data, secondary metabolism and molecular data of the ITS barcodes \textit{cam}, \textit{TUB2} and \textit{rpb2}. In this update, the genus \textit{Penicillium} now has 2 subgenera (\textit{Aspergilloides} and \textit{Penicillium}), 32 sections, 89 series and 483 valid species (Houbraken et al. 2020).

The \textit{Exilicaulis} section has 58 accepted species distributed in six series: \textit{Lapidosa, Corylophila, Restricta, Citreonigra, Alutacea} and \textit{Erubescentia}. Species of the \textit{Exilicaulis} section have monoverticillated and some biverticillated conidiophores with stipes without a terminal vesicular swelling (Pitt et al. 1980; Houbraken et al. 2011; Houbraken et al. 2020; Labuda et al. 2021). Although the species in this section are promising in the production of new bioactive metabolites against pathogenic strains of fungi and bacteria, they also deserve attention for the production of toxic and genotoxic compounds, such \textit{Penicillium canis} and the recently described species \textit{Penicillium labradorum} both of ser. \textit{Erubescentia} species, which can affect commercial mushroom cultures, and cause pathologies that are responsible for the death of immunosuppressed dogs, (Stewart et al. 2005; Langlois et al. 2014; Macmullin et al. 2014; Houbraken et al. 2019; Hothacker et al. 2020; Labuda et al. 2021; Zhuravleva et al. 2021).

During the isolation of fungi from sediments collected from the Amazon River in the state of Amapá in Brazil, four isolates stood out for their high production of exudates. These presented potential for biosynthesis of siderophores, phosphate solubilization, and production of compounds with antifungal activity and enzymes of industrial interesting. The phylogenetic analysis using the ITS regions \textit{CAM}, \textit{RPB2} and \textit{TUB2}, together with morphological characters, supports the proposition of a new species named \textit{Penicillium amapaense} that belongs to section \textit{Exilicaulis} ser. \textit{Erubescentia}. Furthermore, two of these isolates cluster with \textit{P. labradorum}, and is the first report of the occurrence of this species in Brazil.

\section*{Material And Methods}

\subsection*{Isolation and culture conditions}

For isolation, sediments from the Amazon River were collected in the city of Macapá in the state of Amapá, Brazil and kindly sent to Laboratory of Molecular Biology at Embrapa Western Amazon in Manaus by Cristóvão Tertuliano de Almeida Lins. The isolation was performed by serial dilution of 1 gram of sediments at a concentration of $10^3$ and inoculated using the spread plate technique in ISP2 (Yeast Extract-Malt Extract Agar) medium. All culture mediums are presented in SI 1.

The isolates obtained were submitted to the monosporic cultivation technique in order to obtain pure cultures in potato dextrose agar (PDA) medium and were then preserved in potato dextrose medium containing 20\% (v/v) glycerol at -80°C. The isolates were deposited in the biological collection at INPA (National Institute for Amazonian Research) under codes: INPA-AP07, INPA-AP25, INPA-AP10 and INPA-AP10a.

\subsection*{Morphological analysis}
Isolates were grown for 7 days at 25°C in PDA, MEA, YES, OA and CYA media. Additional plates of CYA at 30°C and 37°C were also observed (Supplementary information SI 1) (Visagie et al. 2014). The macromorphological characters evaluated were colony diameter, texture, mycelium color (front and back), presence or absence of soluble pigments and exudates. Colors were established according to the ISCC/NBS Color System.

Micromorphological characters, such as conidiophore ramifications, conidia texture and number of phialids per conidiophore were analyzed using scanning electron microscopy (JSM-IT500HR) at the Multiuser Center for the Analysis of Biomedical Phenomena at the Amazonas State University (CMABio). For this, the microculture was prepared in MEA at 28°C for 7 days, fixed with Karnovsky, and then dehydrated in increasing concentrations of alcohol (30%, 50%, 70%, 80%, 90%, 100%) for 15 min each. The step with absolute alcohol was repeated twice. Drying was performed in a critical point dryer (Leica EM CPD300) and submitted to sputter coating (DII-29010SCTR Smart Coater). The measurement of length and width of 30 conidia and phialides and the length of the stipes was performed using an optical microscope with a Carl ZEISS Axio Imager v2 camera.

**DNA extraction, PCR amplification, and sequencing**

The isolates were grown in PD medium at 28°C with rotation at 150 rpm for 7 days. The mycelial mass that was obtained was filtered and DNA extraction was performed with cationic detergent CTAB 2% according to Doyle and Doyle (1987).

PCR amplifications of the four loci (ITS, TUB2, CAM and RPB2) were carried out using an Easytaq® kit (Synapse Biotechnology) following the manufacturer's instructions. Thermocycling conditions were as follows: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 45 sec, annealing according to each primer-specific temperature (Supplementary information SI 2) for 45 sec, extension at 72°C for 1 min. Final extension at 72°C for 5 min.

The PCR products were resolved on a 1.5% (w/v) agarose gel stained with ethidium bromide and photodocumented under ultraviolet light using a molecular imaging system (Loccus Biotechnologic L-Pix. Chemi). The amplicon sizes were compared with the 1 kb plus marker (Invitrogen - Catalog number: 10787018). PCR products were purified enzymatically using the ExoSAP-IT reagent (USB, Cleveland, OH, USA) following the manufacturer's instructions. Sequencing reactions were carried out in a 10 µL reaction volume which contained 5 µL of the purified PCR product, 0.5 µL of BigDye terminator v3.1 (Thermo Fisher), 1.5 µL 5X buffer, 2 µL ultrapure water and 3.2 pmol of each primer. Thermocycling conditions were as follows: 96°C for 1 min, followed by 35 cycles at 96°C for 15 sec, 50°C for 15 sec, and 60°C for 4 min. Sequencing reactions were visualized in a genetic analyzer (ABI 3500, Thermo Fisher).

**Phylogeny**

Consensus sequences were obtained based on alignment of forward and reverse sequences using DNA baser assembly software (http://www.dnabaser.com/). The new sequences were deposited in GenBank (http://www.ncbi.nlm.nih) under accession numbers OL764382 to OL764385 (ITS), OL782584 to
OL782587 (CAM), OL782588 to OL782589 (RPB2), OL782590 to OL782593 (TUB2). Phylogenetic identification of the 4 isolates (INPA-AP07, INPA-AP25, INPA-AP10, INPA-AP10a) was performed using a dataset of 69 *Penicillium* sequences from the *Exilicaulis* section. The sequences were individually aligned using the MAFFT tool in the UGENE software (Okonechnikov et al. 2012). Alignments were plotted in IQ tree (Kumar et al. 2016) and a phylogenetic analysis, using maximum likelihood (ML), was performed from a concatenation of the ITS, *CAM*, *RPB2* and *TUB2* sequences. Bayesian inference (BI) was performed using CIPRES (www.phylo.org).

The ML analysis included 1,000 replicates (bootstrap), and used all sites, with the best model selected by IQtree. Bayesian inference (BI) was based on the model adopted in PAUP* 4.0 and MrModeltest v2 (Nylander 2004). All sites in the loci were considered and the analysis was performed for ten million generations, with the first 25% of the trees discarded and burned using the MrBayes v 3.7 tool available from CIPRES (https://www.phylo.org/). Posterior probability (PP) and tree topology were visualized with FigTree v1.1.2 (Rambaut 2009). The consensus tree of the ML and BI analyses was generated manually from the topology obtained using FigTree in BI analysis with the posterior probability values, plus the bootstrap values generated by the maximum likelihood analysis, using the CorelDraw editing package.
| Species                          | Isolate    | ITS          | CAM         | RPB2         | TUB2         |
|---------------------------------|------------|--------------|-------------|--------------|--------------|
| Penicillium amapaense T         | INPA-AP07  | OL764382     | OL782584    | -            | OL782590     |
| Penicillium amapaense           | INPA-AP25  | OL764385     | OL782587    | -            | OL782593     |
| Penicillium labradorum          | INPA-AP10  | OL764383     | OL782585    | OL782588     | OL782591     |
| Penicillium labradorum          | INPA-AP10a | OL764384     | OL782586    | OL782589     | OL782592     |
| Penicillium labradorum T        | DI19-20    | MK881918     | MK887899    | MK887900     | MK887898     |
| Penicillium melinii T           | CBS 218.30 | MH855124     | KP016792    | JN406613     | KJ834471     |
| Penicillium radulatum T         | CBS 280.58 | KC411727     | KP016801    | KP064658     | KP016763     |
| Penicillium lapidosum T         | CBS 343.48 | MH856379     | FJ530984    | JN121500     | KJ834465     |
| Penicillium hemitrachum T       | CBS:139134 | FJ231003     | JX157526    | KP064642     | JX141048     |
| Penicillium atrosanguineum T    | CBS 380.75 | NR_111670    | KP016771    | JN406557     | KJ834435     |
| Penicillium smithii T           | CBS 276.83 | NR_144839    | KP016806    | JN406589     | KJ834492     |
| Penicillium corynephorum T      | CBS 256.87 | NR_138321    | KP016781    | KP064635     | KP016755     |
| Penicillium sabulosum T         | CBS 261.87 | NR_138323    | KP016805    | KP064672     | KP016765     |
| Penicillium burgense T          | CBS 325.89 | NR_171581    | KP016772    | JN406572     | KJ834437     |
| Penicillium chalybeum T         | CBS 254.87 | NR_138320    | MG333472    | JN406596     | KJ834440     |
| Penicillium flavidostipitatum T | CBS 202.87 | KC411691     | KP016785    | KP064637     | KJ834451     |
| Penicillium maclenniae T        | CBS 198.81 | NR_138313    | KP016791    | KP064648     | KJ834468     |
| Penicillium namyslowskii T      | NRRL 1070  | NR_121244    | KP016795    | JF417430     | JX141067     |
| Penicillium raciborskii T       | NRRL 2150  | NR_121234    | KP016800    | JN406607     | JX141069     |
| Penicillium turrispainense T    | CBS 204.87 | KT155826     | KP016809    | KP064681     | KP016766     |
| Penicillium velutinum T         | CBS 250.32 | MH855309     | KP016810    | KP064682     | JX141170     |
| Penicillium luzonicum T         | CBS 622.72 | MH860599     | KP016790    | JN406544     | KP016759     |

T - represents the type species
| Species                          | Isolate       | ITS            | CAM         | RPB2        | TUB2        |
|---------------------------------|---------------|----------------|-------------|-------------|-------------|
| Penicillium terrenum **T**      | CBS 313.67    | NR_163675      | KP016808    | JN406577    | KJ834496    |
| Penicillium xanthomelinii **T** | CBS 139163    | NR_144831      | JX157495    | KP064683    | JX141120    |
| Penicillium atrolazulinum **T** | CBS 139136    | NR_144830      | JX157416    | KP064575    | JX141077    |
| Penicillium fagi **T**          | CBS 689.77    | NR_163678      | KP016784    | JN406540    | KJ834449    |
| Penicillium rubefaciens **T**   | CBS 145.83    | MH861557       | KP016804    | JN406627    | KJ834487    |
| Penicillium momoii **T**        | CBS 139157    | JX140895       | JX157479    | KP064673    | MG386242    |
| Penicillium repensicola **T**   | CBS 139160    | NR_153209      | JX157490    | KP064660    | JX141150    |
| Penicillium pagulum **T**       | CBS 139166    | NR_153210      | JX157519    | KP064655    | JX141070    |
| Penicillium cravenianum **T**   | CBS 139138    | NR_144829      | JX157418    | KP064636    | JX141076    |
| Penicillium consobrinum **T**   | CBS 139144    | NR_144827      | JX157453    | KP064619    | JX141135    |
| Penicillium subturcoseum **T**  | CV110         | FJ231006       | JX157532    | KP064674    | JX141161    |
| Penicillium corylophilum **T**  | NRRL 802      | NR_121236      | KP016780    | KP064631    | JX141042    |
| Penicillium candidofulvum **T** | CBS 254.37    | KC411712       | KP016773    | KP064606    | KP016751    |
| Penicillium chloroleucon **T**  | CBS 127808    | KP016813       | KP016776    | KP064613    | KP016752    |
| Penicillium coeruleoviride **T**| CBS 259.67    | KC411717       | KP016779    | KP064617    | KP016754    |
| Penicillium humuli **T**        | CBS 231.38    | JN617696       | KP016787    | KP064645    | KP016756    |
| Penicillium obscurum **T**      | CBS 127807    | KP016815       | KP016797    | KP064654    | KP016761    |
| Penicillium kurssanovii **T**   | NRRL 3381     | EF422849       | KP016789    | KP064647    | KP016758    |
| Penicillium restrictum **T**    | NRRL 1748     | AF033457       | KP016803    | JN121506    | KJ834486    |
| Penicillium cinereoatrum **T**  | CBS 222.66    | KC411700       | KP125335    | JN406608    | KJ834442    |
| Penicillium katangense **T**    | NRRL 5182     | AF033458       | KP016788    | KP064646    | KP016757    |
| Penicillium meridianum **T**    | NRRL 5814     | AF033451       | KP016794    | JN406576    | KJ834472    |
| Penicillium heteromorphum **T** | CBS 226.89    | KC411702       | KP016786    | JN406605    | KJ834455    |

**T** - represents the type species
| Species                  | Isolate | ITS      | CAM      | RPB2     | TUB2     |
|-------------------------|---------|----------|----------|----------|----------|
| *Penicillium philippinense* | CBS 623.72 | KC411770 | KP016799 | JN406543 | KJ834482 |
| *Penicillium chalabudae*  | CBS 219.66 | KP016811 | KP016767 | KP064572 | KP016748 |
| *Penicillium arabicum*    | CBS 414.69 | KC411758 | KP016770 | KP064574 | KP016750 |
| *Penicillium cinerascens* | NRRL 748  | AF033455 | JX157405 | KP064614 | JX141041 |
| *Penicillium citreonigrum* | NRRL 2046 | EF198647 | EF198625 | KP064616 | EF198623 |
| *Penicillium citreonigrum* | NRRL 761  | AF033456 | EF198628 | JN121474 | EF198621 |
| *Penicillium aeneum*      | CBS 321.59 | KP016812 | KP016769 | KP064573 | KP016749 |
| *Penicillium citroesulfuratum* | DTO 290-I4 | KP016814 | KP016777 | KP064615 | KP016753 |
| *Penicillium toxicarium*  | NRRL 31271 | EF198660 | EF198641 | EF198486 | EF198614 |
| *Penicillium alutaceum*   | NRRL 5812  | AF033454 | KP016768 | JN121489 | KJ834430 |
| *Penicillium decumbens*   | CBS 230.81 | AY157490 | KP016782 | JN406601 | KJ834446 |
| *Penicillium laeve*       | DTO270G8  | KF667369 | KF667367 | KF667371 | KF667365 |
| *Penicillium ovatum*      | DTO270G7  | KF667370 | KF667368 | KF667372 | KF667366 |
| *Penicillium griseolum*   | NRRL 2671 | EF422848 | EF506232 | KP064638 | EF506213 |
| *Penicillium dimorphosporum* | NRRL 5207 | AF081804 | KP016783 | JN121517 | KJ834448 |
| *Penicillium canis*       | NRRL 62798 | KJ511291 | KF900177 | KF900196 | KF900167 |
| *Penicillium menonorum*   | NRRL:50410 | HQ646591 | HQ646584 | KF900194 | HQ646573 |
| *Penicillium erubescens*  | NRRL 6223  | AF033464 | EU427281 | JN121490 | HQ646566 |
| *Penicillium striatisporum* | NRRL 26877 | AF038938 | KP016807 | JN406538 | JX141156 |
| *Penicillium nepalense*   | CBS 203.84 | KC411692 | KP016796 | JN121453 | KJ834474 |
| *Penicillium rubidurum*   | NRRL 6033  | AF033462 | HQ646585 | JN406545 | HQ646574 |
| *Penicillium parvum*      | CBS 570.73 | KC411767 | KP016798 | KP064657 | KP016762 |
| *Penicillium parvum*      | NRRL 2095  | AF033460 | KF900173 | JN406559 | HQ646568 |
| *Penicillium catenatum*   | CBS 352.67 | KC411754 | KP016774 | JN121504 | KJ834438 |
| *Penicillium vinaceum*    | NRRL 739   | AF033461 | HQ646586 | JN406555 | HQ646575 |

*T* - represents the type species
Species | Isolate | ITS | CAM | RPB2 | TUB2
--- | --- | --- | --- | --- | ---
*Penicillium guttulosum* T | NRRL 907 | HQ646592 | HQ646587 | KP064639 | HQ646576
*Penicillium pimiteouense* T | NRRL 25542 | AF037431 | HQ646580 | JN406650 | HQ646569
*Penicillium kiamaense* T | CBS 137947 | NR_137899 | KM089128 | KM089515 | KM088743
*Penicillium hermansii* T | DTO 079-D5 | MG333472 | MG386229 | MG386242 | MG386214

T - represents the type species

**Dual-culture antagonistic activity assay**

The antifungal activity was tested against the following phytopathogens from the microbiological collection of Embrapa Western Amazon: *Colletotrichum guaranicola* (INPA 2939), *Colletotrichum scovillei* (INPA 2910), *Colletotrichum siamense* (Coll 2N), *Colletotrichum* sp. (INPA 2973), *Colletotrichum spatulatum* (INPA 2908), *Corynespora cassiicola* (INPA 2671), *Fusarium decemcellulare* (Fdc 307), *Fusarium* sp. (MCT 10621), *Moniliophthora perniciosa* (MP01), *Neopestalotiopsis formicarum* (609), *Pseudopestalotiopsis gilvanii* (222) and *Rhizoctonia* sp. (INPA 2942). The host species from which the phytopathogens were isolated are presented in Supplementary Information SI 4.

Each antagonist (INPA-AP07, INPA-AP25, INPA-AP10 and INPA-AP10a) was tested against each of the aforementioned phytopathogenic fungi using petri dishes (9 cm) containing PDA medium at 28°C. Each inoculum was positioned at a distance of 5 cm from one another. The tests were carried out in triplicate and measurements were taken on the 14th day. The positive control consisted of the inoculum of each phytopathogenic fungus growing under the same conditions. The mycelial growth inhibition rate (IR) was calculated as follows:

$$IR = \frac{R \times 100}{R - r}$$

(R = control; r = treatment (LIANG et al. 2021). IR was analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s test with significance at p <0.05.

**Enzymatic activity and in vitro physiological traits for promotion of plant growth**

Qualitative analysis of the enzymatic activity was performed for the production of amylase, cellulase, chitinase, lipase, protease. We also performed *in vitro* evaluation of plant growth promoting (PGP) traits such as phosphate solubilization and siderophore production. The culture media used for each test are presented in Supplementary Information SI 3. Bioassays were performed in triplicate and incubation was at 28°C for 7 days, and the presence of halos around the colonies indicated a positive result for the selected enzymes. The colonies that showed a clear halo on NBRIP (National Botanical Research Institute’s phosphate growth) medium were marked as positive for phosphate solubilization and those with an orange halo on CAS agar medium were considered as positive for siderophore production.

**Chemical profile and compound dereplication analysis**
A 4 mL sample of resuspended crude extracts at 2 mg mL$^{-1}$ in acetonitrile (ACN) were injected in methanol and analysed via high resolution (5000) UPLC-MS analysis. The UPLC-MS analysis was performed using a UHPLC (Acquity Waters) coupled to a mass spectrometer (Q-ToF micro$^{\text{TM}}$ Micromass, Waters) using an electrospray ionization (ESI) source with a UPLC BEH C18 1.7 mm column (2.1x100 mm, Acquity Waters). The mobile phase A was milli-Q water with 0.1% of formic acid (FA), and mobile phase B was ACN with 0.1% of FA. The flow rate of 0.35 mL min$^{-1}$ was used following the gradient: 0-16 min, 20% B to 70% B; 16-20 min 70% B to 100% B; 20-23 min, 100% B (clean-up) and 2 min of post time at 20% B for column re-equilibration. The mass spectrometer voltages and temperatures were set as follows: capillary 4000 V; sample cone 40 V, extraction cone 2 V; gas source temperature at 150 $^\circ$C; desolvation temperature at 10 $^\circ$C; drying gas at 600 L hr$^{-1}$, and the fragmentation was performed using collision energy of 25 and 35 eV. Mass spectra were acquired in profile and positive or negative ion mode and the acquisition range was 100-1000 $m/z$. Data were treated using MassLynx V 4.1 and Mzmine 2.53. Compound dereplication was performed using the GNPS platform via FBMN and by manual interpretation of MS/MS spectra.

**Results**

**Phylogeny and nucleotide variation**

The alignment resulted in 2,649 characters, which included gaps in which the ITS locus contributed 580 characters and $\text{CAM}$, $\text{RPB2}$ and $\text{TUB2}$ contributed 559, 978 and 532, respectively. The best model adopted by PAUP*4.0 for BI data for all loci was GTR+I+G, while for the ML analyses the best fit model was TIM2e+I+G4 for ITS and TNe+I+G4 for $\text{CAM}$, $\text{TUB2}$ and $\text{rpb2}$. The ML and BI analyses revealed that all isolates in the present study were grouped in the ser. $\text{Erubescentia}$ species. The isolates INPA-AP10 and INPA-AP10a clustered with $\text{P. labradorum}$, while isolates INPA-AP07 and INPA-AP25 formed a sister group with $\text{P. rubidurum}$, with 87 bootstrap support in the ML analysis and 0.51 of PP in the BI analysis (Fig. 1).

The nucleotide variation between closely related species for INPA-AP07 and INPA-AP25 showed a total of 32 nucleotide differences (ND) in relation to $\text{P. rubidurum}$ ($22 = \text{CAM}, 7 = \text{TUB2}, 3 = \text{ITS}$); 39 ND in relation to $\text{P. guttulosum}$ ($25 = \text{CAM}, 12 = \text{TUB2}, 2 = \text{ITS}$); 38 ND in relation to $\text{P. pimiteouiense}$ ($29 = \text{CAM}, 8 = \text{TUB2}, 1 = \text{ITS}$) and 74 ND in relation to $\text{P. menorum}$ ($46 = \text{CAM}, 16 = \text{TUB2}, 12 = \text{ITS}$). Furthermore, INPA-AP07 and INPA-AP25 showed 15 single nucleotide polymorphisms (SNPs) ($9 = \text{CAM}, 5 = \text{TUB2}, 1 = \text{ITS}$) (Fig. 2). These data, together with the morphological analysis, indicate that INPA-07 and INPA-AP25 represent a new fungal species, named here as *Penicillium amapaense*.

**Taxonomy**

*Penicillium amapaense* I. J. S. Silva; T. F. Sousa and G. F. Silva, sp. nov. (Mycobank: MB842234); Figure 3; Genus: *Penicillium*, section: *Exilicaulis*, Series: *Erubescentia*.

Etymology: Refers to the Amapá state, Brazil, which is the location where the type species was isolated.
Type: BRAZIL. Amapa, sediments of Amazon River, I. J. S. Silva, T. F. Sousa and G. F. Silva, holotype: INPA-AP25. Sequences are deposited in GenBank under accession numbers: ITS = OL764382, CAM = OL782584, TUB2= OL782590.

Culture characteristics: Colonies grown at 25°C for 7 days on PDA medium have an average diameter of 14 mm. The edges have white aerial mycelium (ISCC-NBS 263) with a yellowish gray color in the center (ISCC-NBS 93), velvety, regular edges, presence of abundant exudate and bright yellow soluble pigment (ISCC-NBS 83), the reverse has a slightly grooved colony center, with bright yellow coloration (ISCC-NBS 82) and bright yellow edges. YES: 17 mm diameter; front: white aerial mycelium (ISCC-NBS 263), velvety with high center, highly grooved, irregular edges, absence of exudate and soluble pigment; reverse: highly grooved, dark brown (ISCC-NBS 56), edges bright greenish yellow (ISCC-NBS 97). MEA: 15 mm diameter; front: aerial mycelium white on the edges and yellowish gray on the center, velvety, smooth, regular edges, presence of bright greenish yellow exudate, absence of soluble pigment; reverse: slightly grooved and strong yellowish brown (ISCC-NBS 74) in the center and bright yellow around the edges. OA: 14 mm diameter; front: aerial mycelium white (color code) and cottony and elevated in the center of the colony, hyaline and regular borders, presence of colorless exudate and absence of soluble pigment; reverse: smooth, light yellow color (ISCC-NBS 86). CYA: 12 mm diameter; front: white, velvety mycelium, raised center and slightly grooved, irregular edges, absence of exudate, presence of soluble strong orange pigment (ISCC-NBS 50); reverse: smooth, strong yellowish brown center (Fig. 3a, 3b, 3c). In addition to the conditions described above, the strain was analyzed at temperatures of 30°C and 37°C, and showed the same characteristics mentioned above, with the exception of the size of the colony, which presented sizes of 15 mm and 5 mm, respectively.

Micromorphology: The conidiophores have stipes that measure 9.6-20.6 μm (M = 16.4 μm) and are strictly monoverticillate and vesiculate, containing solitary phialides (Fig. 3f) or 3-7 phialides per conidiophore that measure 2 – 8.5 μm (M = 5.8 μm) × 2.0 – 4.2 μm (M = 2.8 μm) (Fig. 3i). The conidia have a spiny ellipsoid shape, 1.3 – 2.0 (M = 1.5) × 1.1 – 11.8 (M = 1.3) (Fig. 3d).

Notes

*Penicillium amapaense* sp. nov. differs from *Penicillium rubidurum* by having a smaller stipe length and due to its ability to grow on CYA medium.

Dual-culture antagonistic activity of *P. labradorum* and *P. amapaense* isolates

Isolates INPA-AP10 (Supplementary Information SI 6) and INPA-AP10a (Supplementary information SI 7) were identified based on morphological and molecular characters as *P. labradorum*, and isolates INPA-AP25 (Fig. 3) and INPA-AP07 (Supplementary Information SI 5), herein characterized as *P. amapaense* sp. nov., were evaluated for antibiosis mechanisms against 12 phytopathogens.

In all, eleven out of the twelve phytopathogenic fungi tested presented inhibition of mycelial growth for at least one of the four *Penicillium* isolates obtained in this study (Fig. 4a). Among the phytopathogenic
fungi tested, the isolates of *P. amapaense* and *P. labradorum* showed potential for inhibiting mycelial growth of up to 49 and 58%, respectively. Among the phytopathogenic fungi inhibited by all the *Penicillium* isolates, *C. guaranicola* presented a mycelial growth inhibition rate (IR) that ranged from 34.28 to 58.53%, *Ps. gilvanii* ranged from 44.10 to 58.09%, and *Fusarium* sp. ranged from 21.42 to 48.88%, while *F. decemcellulare* ranged from 32.22 to 47.77% (Fig. 4a). Some phytopathogenic fungi were not inhibited by all the *Penicillium* isolates; *C. spathulatum* and *Colletotrichum* sp. were not inhibited by the *P. amapaense* isolate INPA-AP07, *C. siamense* was not inhibited by the *P. amapaense* isolate INPA-AP25, and *C. scovillei* was not inhibited by the *P. labradorum* isolate INPA-AP10a. Conversely, *N. formicarum* was inhibited only by the *P. labradorum* isolate INPA-AP10, while none of the four *Penicillium* isolates showed inhibition of mycelial growth against *Rhizoctonia* sp. (Fig. 4a).

### Analysis of secondary metabolites

The investigation of the secondary metabolites obtained from the different strains was carried out manually via the analysis of accurate mass, fragmentation profile and specific annotations in the databases used. In the process of chemical dereplication of extracts, the isolate *P. labradorum* INPA-AP10 stood out for producing the largest number of compounds previously identified (Fig. 5). In total, fourteen compounds were detected, which corresponded to the polyketide subclasses of the curvularins (compounds 1a, 1b, 1c, 1d and 2) and presented as accurate mass m/z 307.1190, m/z 307.1183, m/z 307.1169, m/z 307.1181, m/z 305.1497 and anthraquinones (compounds 3, 4 and 5) with m/z 299.0198, m/z 318.9999, m/z 332.9801 (Table 2).

In general, the *P. labradorum* strains presented a similar metabolic profile, but with the absence of anthraquinones in the extract obtained from the isolate INPA-AP10a. The specimens of the species *P. amapaense* (INPA-AP25 and INPA-AP07) also showed high similarity in the compounds produced by the two strains. Coincidentally, the production of anthraquinones was detected only in the *P. amapaense* isolate INPA-AP25, with the most significant distinction found between the compounds produced by this strain and *P. amapaense* INPA-AP07. Interestingly, when comparing the metabolic profiles of *P. labradorum* and *P. amapaense* species, the main metabolic difference occurred in the production of curvularins. As seen in Fig. 5, none of the isomers referring to the accurate mass of m/z 307 (1a-1d isomers) were identified in the *P amapaense* strains, while the production of compound 2 was significantly observed (Fig. 5).

The analysis of the LC-MS data using the GNPS platform indicated that peak 1 is the curvularin, 5,13,15-trihydroxy-9-methyl-10-oxabicyclo[10.4.0]hexadeca-1(12),13,15-triene-3,11-dione (Table 2). The cosine value of 0.92 was obtained when comparing the MS/MS spectra for compound 1a and for curvularin, which was indicated by the platform, thus suggesting high similarity in the fragmentation profile. This data is corroborated by the comparison of the spectra (Supplementary Information SI 8). The molecular formula C_{16}H_{20}O_{6}, obtained from the accurate mass of compound 1a (Supplementary Information SI 9), is also in accordance with the curvularin indicated on the GNPS platform. Furthermore, for the remaining peaks referring to the possible curvularin isomers (1b-1d), lower values of cosine were obtained, suggesting other possible identities for them; all are found in the Supplementary Information SI 14. The
query of the molecular formula C\textsubscript{16}H\textsubscript{20}O\textsubscript{6} was performed in other databases (see Table 1) and indicated possible hits that correspond to other known curvularins. The hits obtained can be seen in Fig. 5b.

The molecular formula C\textsubscript{16}H\textsubscript{22}N\textsubscript{2}O\textsubscript{4}, suggested for the accurate mass \textit{m/z} 305.1490 (2) (Supplementary Information SI 10), was consulted in the databases used and no hit was found, thus suggesting that it is a metabolite that has not yet been reported in the literature. Although there are two nitrogen units in the molecule, there is evidence that this compound has a biosynthetic origin that is related to the class of curvularins, due to the similarity in retention time and the observed hydrogen deficiency index (see Table 1) and, mainly, to the MS/MS spectrum when compared to that of compound 1a (Supplementary Information SI 11).

Regarding the anthraquinones observed in the extracts of \textit{P. labradorum} INPA-AP10 and \textit{P. amapaense} INPA-AP07, data processing on the GNPS platform and database consultation allowed the identification of emodic acid for compound 3, 5-chloro-ω-hydroxyemodin for compound 4 and 2-chloroemodic acid for compound 5. In all cases, the molecular formulas were obtained with low values of deviation from the respective exact masses (see Table 2), as well as the observed isotopic profile for 4 and 5 (Supplementary Information SI 12 and SI 13), which corroborated in confirming the identity of the compounds (Figs. 5a and 5b).

Figure 5a \textit{Penicillium labradorum} and \textit{Penicillium amapaense} extracted ion chromatograms of \textit{m/z} 307.1181; \textit{m/z} 305.1501; \textit{m/z} 299.0191; \textit{m/z} 319.0009, \textit{m/z} 333.9880, with deviation of ± 0.005 u obtained for the INPA-AP25, INPA-AP07, INPA-AP10 and INPA-10a strains. Peaks comprising substances that could not be annotated were referred to as (*) unknown compounds. b Chemical structures of annotated polyketides: curvularin (1a), emodic acid (3), 5-chloro-ω-hydroxyemodin (4) and 2-chloroemodic acid (5).
Table 2
Chemical annotation of the LC-MS analysis of *Penicillium labradorum* and *Penicillium amapaense* sp. nov.

| Peak ID | rt (min) | Accurate mass (m/z) | Exact mass | Molecular formula | Deviation (ppm) | IDH | Putative hit                |
|---------|----------|---------------------|------------|-------------------|-----------------|-----|---------------------------|
| 1a      | 7.5      | 307.1190            | 307.1181   | C$_{16}$H$_{20}$O$_6$ | 2.6             | 7   | Curvularin                |
| 1b      | 8.5      | 307.1183            | 307.1181   | C$_{16}$H$_{20}$O$_6$ | 0.6             | 7   | (1a) isomer               |
| 1c      | 9.5      | 307.1169            | 307.1181   | C$_{16}$H$_{20}$O$_6$ | 3.9             | 7   | (1a) isomer               |
| 1d      | 10.6     | 307.1181            | 307.1181   | C$_{16}$H$_{20}$O$_6$ | -0.1            | 7   | (1a) isomer               |
| 2       | 7.5      | 305.1490            | 305.1501   | C$_{16}$H$_{22}$O$_4$N$_2$ | -3.6           | 7   | no hit                    |
| 3       | 10.1     | 299.0198            | 299.0191   | C$_{15}$H$_{8}$O$_7$ | -0.3            | 12  | Emodic acid               |
| 4       | 10.7     | 318.9999            | 319.0009   | C$_{15}$H$_{9}$O$_6$Cl | -3.1           | 11  | 5-Chloro-ω-hydroxyemodin  |
| 5       | 11.3     | 332.9801            | 333.9880   | C$_{15}$H$_{7}$O$_7$Cl | -0.3            | 12  | 2-Chloroemodic acid       |

**Enzymatic activity**

The production of amylase, cellulase and siderophores were detected in all isolates of *P. amapaense* and *P. labradorum*. Only in *Penicillium amapaense* (INPA-AP07 and INPA-AP25) was it able to solubilize phosphate. No enzymatic activity was observed for chitinase, lipase and protease for all isolates analyzed (Table 3, Fig. 6).
Table 3
Enzymatic activity and in vitro physiological traits for plant growth promotion of *Penicillium amapaense* sp. nov. and *Penicillium labradorum*.

|                      | *P. amapaense* INPA-AP07 | *P. amapaense* INPA-AP25 | *P. labradorum* INPA-AP10 | *P. labradorum* INPA-AP10a |
|----------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Amylase              | +                         | +                         | +                         | +                         |
| Cellulase            | +                         | +                         | +                         | +                         |
| Chitinase            | -                         | -                         | -                         | -                         |
| Lipase               | -                         | -                         | -                         | -                         |
| Protease             | -                         | -                         | -                         | -                         |
| Siderophore          | +                         | +                         | +                         | +                         |
| Phosphate solubilization | +                     | +                         | -                         | -                         |

+ Halo formation, - No halo formation.

**Discussion**

A total of four isolates of the genus *Penicillium* were recovered from sediments of the Amazon River, which is a rich but still underexplored source of microorganisms. These isolates were subject to morphological characterization as well as sequence analyses of ITS, *CAM*, TUB2 and RPB2 loci. The isolates INPA-AP25 and INPA-AP07 were characterized as a new species and named here as *P. amapaense* sp. nov, while the other two isolates INPA-AP10 and INPA-AP10a were identified as *P. labradorum*. These data also support that both species are members of the sect. *Exilicaulis* and ser. *Erubescentia*.

The phylogenetic inference of *P. amapaense* sp. nov, based on four loci (ITS, *CAM*, TUB2 and RPB2), had low bootstrap support (ML = 87) and posterior probability from Bayesian inference (PP = 0.51). Despite the relatively low support achieved by bootstrap and PP, *P. amapaense* sp. nov. could be differentiated from the four most phylogenetically related species (*P. rubidurm*, *P. menorum*, *P. guttulosum*, and *P. pimiteouiense*) due to the presence of 15 SNPs identified in ITS, *CAM* and TUB2 loci. By narrowing the comparisons to the sister clade, *P. rubidurum*, 22 SNPs were identified only in the partial sequence of the calmodulin (*CAM*), thus evidencing the molecular differences between the two species (Fig. 2). In addition, *P. amapaense* differs from *P. rubidurum* by having smaller stipe length (9.6 – 20.6 µm vs. 15 – 60 µm) and also by growing on CYA medium (Peterson et al. 1999).

Phylogenetic analysis of *Penicillium* sect. *Exilicaulis* ser. *Erubescentia* evidenced the lower resolution achieved by the current recommended barcode, which, for most of branches, produced values lower than
80 and 0.95 for bootstrap and posterior probability from Bayesian inference, respectively (Visagie et al. 2016). Recently, a phylogenetic tree of *Penicillium* species was also constructed with bootstrap values below 70 and posterior probability from Bayesian inference below 0.95, but still allow the characterization of *P. hermansii* as a new species (Houbraken et al. 2019). Taken together, our results and the literature indicate the need to incorporate new loci, or even the development of new algorithms for a more accurate taxonomic analysis to better resolve the evolutionary relationships of fungal species (Choi & Kim 2017).

In the future, we believe that unambiguous and highly supported phylogenetic analysis will be obtained for *Penicillium* species based on whole genome sequencing (WGS) drafts. Similar analyses have already been carried out for species of the genus *Fusarium* for which 59.1 Kb was obtained based on 19 housekeeping genes, thus allowing high support that included highly related members of the *F. solani* species complex (FSSC) (Geiser et al. 2021). In the near future, the lower cost of obtaining fungal genomes will allow us to perform WGS-based phylogenetic analysis, as well as the creation of an automated high-throughput platform for taxonomic analysis of fungal species, as well as the Type Strain Genome server (TYGS) platform, which is now available for taxonomy of bacteria based on bacterial genomes (Meier-Kolthoff & Göker 2019).

Herein, we examined the biotechnological and agricultural potential of *P. amapaense* sp. nov.. The two isolates of this species (INPA-AP25 and INPA-AP07) showed the ability to produce compounds of biotechnological interest, as well as possible metabolites (Fig. 5). Moreover, the *in vitro* antibiosis assays demonstrated the ability to control phytopathogenic fungi that affect important agricultural crops in Brazil. The results showed that isolates could produce compounds that inhibited up to 58% of mycelial growth of 11 out of the 12 phytopathogenic fungi tested. In addition, the isolates demonstrated the ability to produce enzymes such as amylase and cellulase, as well as the ability to produce functional traits related to plant growth promotion such as siderophores and phosphate solubilization. These results are the first indication that this new species has both biotechnological and agricultural potential that needs to be further investigated.

The LC-MS data allowed the annotation of nine compounds, which were all of a polyketide nature. Among the species studied, there is a metabolic proximity that is corroborated by the proximity in the phylogenetic analysis. The differences observed refer to the presence or absence of anthraquinones, especially chlorinated ones. In general, curvularins and anthraquinones produced by *Penicillium* species are bioactive (Bladt et al. 2013). Anthraquinones have shown themselves to be strong phytotoxic, antibacterial, antiviral, anticancer and antifungal agents (Masi & Evidente 2020). Curvularins have also shown interesting biological activities, especially the ability of curvularin (1) to inhibit nitric oxide synthase (NOS) expression and, therefore, a desirable characteristic for anti-inflammatory drugs (Elzner et al. 2008). The observance of peaks that could not be annotated indicates the potential of the section *Exilicaulis* to provide new substances of biotechnological interest (Visagie et al. 2014; Labuda et al. 2021).

*P. labradorum* has recently been described as causing disease in an immunosuppressed dog in Florida/USA (Rothachker et al. 2020) and, since this event, only one report in Genbank was found in
November, 2020, in Los Angeles, USA, which was isolated from the mushroom *Panaeolus cinctulus* and identified by ITS region (accession number: MW241166.1). These two reports are an indication that this species should be further studied as to its ability to cause diseases in other animals or even in humans, as well as its ability to contaminate edible mushroom species. *Penicillium hermansii*, which is also a member of the ser. *Erubescentia*, has been reported to cause problems in mushroom cultivation (Houbraken et al. 2019).

In this work, *P. labradorum* was isolated for the first time as a saprophyte, and this is the first study that reports its ability to control phytopathogenic fungi, to produce enzymes of industrial interest and to possess traits related to plant growth promotion, in addition to the first report of the identification of extrolites produced by this species. The results reveal that *P. labradorum* showed the best performance against the 12 phytopathogens evaluated when compared to *P. amapaense*. The isolate INPA-AP10 showed the best performance in growth inhibition rates (IR) for most of the tested phytopathogens, which were mostly species of the *Colletotrichum* genus, in addition to being the only one with the ability to inhibit *N. formicarum* (Fig. 4a). This fact is possibly due to the greater diversity of compounds produced by this isolate in relation to the others (Fig. 5).

Our report presented two physiologically interesting *Penicillium* species with potential for production of enzymes of industrial interest, but which are also promising from a chemical point of view due to the production of compounds with antifungal activity against different phytopathogens. Furthermore, the ability to produce siderophores and phosphate solubilization are interesting characteristics to be further investigated for these isolates. New fungal species, such as *P. amapaense* or yet unexplored species such as *P. labradorum*, are a potential source for identification and characterization of new molecules and for developing new products and biotechnological processes that together are the key to the new bio-economy and a new form of ecologically sustainable agricultural development.

**Declarations**

**Author contributions**

All the authors contributed equally to the study conception and design. All the authors commented on the previous versions of the manuscript. All the authors read and approved the final manuscript.

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**Conflict of interest** The authors declare no competing interests.

**Legal authorizations** Ministry of Environment - Council for the Management of Genetic Patrimony - (CGEN)

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**Data availability** Datasets generated and analyzed during the current study are available either in GenBank at NCBI (National Center for Biotechnology Information) or included in this published article (and its supplementary information files).

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**Figures**

**Figure 1**

Phylogram based on the concatenation of ITS, *TUB2, CAM* and *RPB2* sequences of species of *Penicillium* sect. *Exilicaulis*. The numbers indicate the number of branches supported (ML/PP). The name of the new species is highlighted in pink.
Figure 2

Polymorphism identified in ITS CAM and TUB2 loci in *Penicillium amapaense* sp. nov. and in closely related species; Asterisk indicates single nucleotide polymorphism (SNP) present only in *P. amapaense* species.

Figure 3

*Penicillium amapaense* INPA-AP25. a and b Colonies grown on PDA, YES, MEA, OA and CYA medium at 25 °C for 7 days. c Production of exudates in PDA and MEA. D Mature conidia. e Solitary phialide f: Early-stage conidiophore. g-i Monoverticillate conidiophores with conidial chains.

Figure 4

Antifungal activity of *Penicillium amapaense* sp. nov. (INPA-AP07 and INPA-AP25) and *Penicillium labradorum* (INPA-AP10 and INPA-AP10a) against different phytopathogenic fungal co-cultivated on PDA medium for 14 days. a Percentage of mycelial growth inhibition rate (IR). b Dual-culture antagonistic activity.
Figure 5

a Penicillium labradorum and Penicillium amapaense extracted ion chromatograms of m/z 307.1181; m/z 305.1501; m/z 299.0191; m/z 319.0009, m/z 333.9880, with deviation of ± 0.005 u obtained for the INPA-AP25, INPA-AP07, INPA-AP10 and INPA-10a strains. Peaks comprising substances that could not be annotated were referred to as (*) unknown compounds. b Chemical structures of annotated polyketides: curvularin (1a), emodic acid (3), 5-chloro-ω-hydroxyemodin (4) and 2-chloroemodic acid (5).
Figure 6

*Penicillium amapaense* and *Penicillium labradorum* showed halo formation for amylase, cellulase, siderophores production and phosphate solubilization.

**Supplementary Files**

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