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Expanding the geographical distribution of *Blastobotrys malaysiensis* (Saccharomycetales) beyond the Asian continent – a cave fungus first reported in the Americas

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Abstract

Background

Saccharomycetales are ascomycetic yeasts and among them the genus *Blastobotrys* has approximately 30 known species. *Blastobotrys malaysiensis* is a yeast species, described from cave samples, known until then only from Malaysia. In this study, we characterize a new strain and report the second occurrence record of this species. Here, *Blastobotrys malaysiensis* SXS675, was collected from soil samples from a cave in the Parque Estadual de Terra Ronca (PETER) in Goiás, Brazil. Phylogenetic analyzes revealed strong support with the sequence of the species type, as well as with other species of the clade. This new record contributes by providing new molecular data for the species and expanding the knowledge of its distribution beyond the Asian continent.

New information

First record of a yeast for the American continent and its second mention for the world.

Keywords

Saccharomycetales, geographic distribution, yeast, cave, phylogeny

Introduction

The order Saccharomycetales comprises the ascomycete yeasts, with about 1000 described species. They can be found in various niches, either as saprotrophs, in mutualistic associations with plants and animals, and even as pathogens (Suh et al. 2007).

Blastobotrys Klopotek (1967) is a genus of this order and has approximately 30 known species. The genus is characterized by the presence of setae, such as cell wall projections, micropores in the septa, central micropore, and formation of blastoconidia that form in denticles. Dimorphism is also observed in several species of the genus, and can be found either as a filamentous structure (mycelium) or in yeast-like growth (unicellular), with different dimorphic mechanisms for each species (Malak et al. 2016).

This genus forms a clade closely related to three other yeast genera (*Candida*, *Arxula* and *Sympodiomyces*), also presenting the genus *Trichomonascus* as an ascosporic state. Also, phylogenetic data showed that *Blastobotrys*, *Sympodiomyces*, *Arxula* and some *Candida* species correspond to a single genus, defined as *Blastobotrys*, and that species of *Arxula* and *Sympodiomyces* should be transferred to *Blastobotrys* (Kurtzman and Robnett 2007).

Blastobotrys has promising species for biotechnological applications, such as *B. adeninivorans* and *B. raffinosifermentans*, which are thermotolerant and xerotolerant, for producing and storing lipids at high temperatures (Thomas et al. 2019). *Blastobotrys malaysiensis* is a species, described by Kurtzman (2007) from a strain isolated from a cave in Malaysia in the yeast stage. Since its description, there are no more reports of the occurrence of this species, which results in little information about its ecology and distribution, available in the literature. This study is part of a broad survey of the mycobiota of karst caves in Central Brazil, and aims to characterize a new strain and report the second record of occurrence of this species, expanding the knowledge of its distribution beyond the Asian continent.

Materials and methods

Study area

The material studied was isolated from soil samples from the Angélica cave (-13.5173, -46.388077), located in the Parque Estadual de Terra Ronca (PETER), in the municipality of São Domingos, extreme east of the state of Goiás, border with the state from Bahia, Brazil Fig. 1. This cave has an extension of 14,100 m, being among the largest in the country (Matteucci 2001).

The PETER covers three Brazilian regions and its predominant biome is the Cerrado. The area comprises 57,000 hectares, with a climate of type AW (Tropical Savanna), with cold dry wets in winter and hot humid summers, and an average annual precipitation of 1500 mm (Koppen 1948). The PETER has an important speleological complex in South

America; in it lies part of the region known as the “Província Espeleológica de Bambuí ou Grupo Bambuí”, characterized by the outcropping of carbonate rocks, being the karstic region, among the 19 found in Brazil, with the largest number of known caves (CECAV (Centro Nacional de Pesquisa e Conservação de Cavernas) 2019).

Sampling and isolation

We sampled in the three zones of the cave (photic zone, intermediate/penumbra zone and aphotic zone), in each zone three sampling units were established and in each one soil samples were collected. The samples were collected by contacting the swab soaked in sterile saline solution (0.9%) and streaking on petri dishes containing Sabouraud Agar (Sa) medium, increased with chloramphenicol (15 mg L^{-1}), he plates were then incubated at 28°C in aerobiosis for 7 days.

Fungal growth was observed every day and each colony was purified, by repotting to another plate containing Potato-Dextrose-Agar (PDA) medium and incubated at 28°C. Microstructures were visualized using lactophenol cotton blue, and sterile water, in OLYMPUS CX31 light microscopy.

The species mentioned here were obtained from soil samples in the aphotic zone at a temperature of 27°C, relative humidity of 75.5% and luminosity of 0 Lux (lumens/m2). Physical parameters were measured using a multi-parameter environmental meter, Thermo-Hygro-Anemo-Luximeter (Pyromed-PY 875).

After obtaining the pure colony, a 5 × 5 mm inoculum was removed and inoculated into an erlenmeyer flask containing Yeast-Peptone-Dextrose (YPD) broth, and incubated under constant agitation (130 RPMs) at a temperature of ± 28°C, in order to be used in the assimilation and fermentation experiments. In addition, inocula from the pure colony were subjected to growth at different temperatures (25, 28, 30, 37, and 40ºC), and in different culture media, such as PDA, Malt Extract Agar (MEA), and Mycosel Agar, the latter being used to verify resistance to cycloheximide. The purified colonies were stored in triplicates using the method Castellani and deposited in the culture collection of the Laboratório de Micologia Básica, Aplicada e Divulgação Científica (FungiLab), da Universidade Estadual de Goiás, campus Central, under voucher SXS675.

Assimilation and fermentation test

The assimilation and fermentation tests were performed with five sugars: xylose, glucose, maltose, lactose, galactose. The isolate of *B. malaysiensis* was inoculated in 5 mL of basal medium (Peptone and Yeast extract) increased with 2% of each sugar (carbon source) and incubated at 27 and 30°C for 8 days.

The assimilation of the carbon sources was considered positive when the presence of cell mass was observed, verified according to the concentration of cells, through the optical density spectrophotometric method (OD 600). For the fermentation test the Durham tube

technique was used, being considered positive fermentation when half of the tube was filled with gas.

DNA extraction, PCR amplification and Sequencing

For taxonomic identification, a 0.5 ml of cell mass was collected from the culture in YPD broth and submitted to DNA extraction using the CTAB method (Goés-Neto 2005, Hosaka 2009). After genomic DNA was obtained, the ITS (Internal Transcribed Spacer) ribosomal nuclear region was amplified from primers ITS5 / ITS4 (White et al. 1990), using DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD), with initial denaturation at 90ºC for 5 minutes, and then 35 cycles of denaturation at 95ºC for 30 seconds, the annealing occurred at 55ºC for 30 seconds, extension at 72ºC for 1 minute; the reaction ended with a final extension of 7 minute at 72°C and storage to 4°C. The amplification product was purified using the Multiscreen filter plate (Millipore Corp.). Sequencing was performed from the same primers used in amplification, performed by Macrogen Inc. (Seoul, South Korea).

Phylogenetic analysis

The sequences obtained, as well as the sequences retrieved from GenBank (NCBI), shown in Table 1, were combined and aligned in MAFFT 7 (Katoh et al. 2017). The alignments were analyzed and minor adjustments were performed manually with MEGA 6 (Tamura et al. 2011). The sequences used in this analysis correspond to species and genera closely related to *Blastobotrys* (Kurtzman 2007). The new sequence obtained here has been deposited in GenBank under accession number MZ702867. *Schizosaccharomyces japonicus* was used as an outgroup for phylogenetic inferences.

We used two different analyses, Maximum Likelihood (ML) and Bayesian Inference (BI). ML was performed from TOPALi v2 (Milne and Lindner 2009) determined by 1,000 bootstrap replications, resulting in the branching support value (BS), whereas BI was conducted using MrBayes 3. 2.7 (Ronquist 2012), with runs performed with 2,000,000 generations, the convergence and stability of the runs were evaluated from the average standard deviation (>0.01) in Tracer v.1 .6, as well as the calculation of the Baysesian posterior probability (BPP).

Materials

Enter subsection text

Taxon treatment

Blastobotrys malaysiensis Kurtzman, 2007

Material

a. order: Saccharomycetales; scientificNameAuthorship: Blastobotrys malaysiensis Kurtzman, 2007; higherGeography: South America: Brazil: Goiás: Parque Estadual de

Terra Ronca; continent: South America; country: Brazil; countryCode: Brazil/BR; stateProvince: Goiás; municipality: São Domingos de Goiás; locality: Cave Lapa do Angélica; decimalLatitude: -13.5173; decimalLongitude: 46.388077; occurrenceDetails: Isolated from soil in cave; recordNumber: SXS 675; associatedSequences: MZ702867; identifiedBy: Sá-Ferreira A.S., Leonardo-Silva, L. Xavier-Santos, S.

Description

At five days of growth at 25, 28 and 30°C, in PDA medium, the colony of showed opaque white coloration, with mycelial fringe and lobed margin; when growing in MEA, at 27ºC, yellowish colony, with cottony aerial mycelium in the center and dense and opaque margin was observed. In both media, growth of septate hyphae and pseudohyphae was noted. In MEA medium, in samples from the margin of the colony, abundant spherical cells (2.74 - 4.50 µm) with multilateral budding were observed (Fig. 3A- B); blastoconidia were also observed, formed from small pedicels (Fig. 3C- D). In our cultures, ascospore production was not observed. At 37ºC strain SXS675 showed good growth on MEA and Mycosel agar, with yellowish-white colony of dense aspect, after seven days more abundant yeast cells were observed, with few pseudohyphae and setae. At 40ºC, in the same culture media, after 10 days of incubation the colony grew less than 0.5 cm beyond the inoculum, presenting a yellowish color, with a wrinkled aspect.

Habitat and distribution: Isolated from soil in cave environments, the current distribution reveals that the species is restricted to tropical environments, with only two records: Malaysia (Kurtzman 2007) and Brazil (this study).

Note: *B. Malaysiensis* showed extensive growth at 37ºC, with discrete development at 40ºC. Besides to growing on medium supplemented with cyloheximide. We observed that temperature did not affect the fermentative capacity of *B. malaysiensis*, as the results were the same regardless of the temperature (27 or 30ºC) (Table 2).

Analysis

Molecular phylogeny

The dataset included sequences from 20 yeast species that are related to the *B. malaysiensis* clade, according to Kurtzman (2007). The two analyses resulted in similar topology, however only the Bayesian topology is shown (Fig. 2) and the statistical values (BS/ BPP), respectively, are indicated for each node. The evolutionary model used in the ML and IB analyses was TVM+G, based on the AIC (Akaike Information Criterion) criteria. *Blastobotrys malaysiensis* (SXS675) showed strong support (BS = 100%, BPP = 0.98) clustering close to the type species (CBS10336), with 100% similarity.

Discussion

As verified by Kurtzman (2007) in the Asian strain, we found that the South American strain of *B. malaysiensis* (SXS675) also showed resistance to cycloheximide, as well as growth at 37°C and 40°C. This thermotolerant characteristic is well understood and observed in several species of the genus, which makes it considered biotechnologically promising (Sanya et al. 2021). The fermentative characteristics also coincide with those found in the description of this species, and in addition to data from Kurtzman (2007), we tested the ability of *B. malaysiensis* to ferment glucose. We observed that the strain fermented little of this sugar under the conditions presented, corresponding to less than half of the gas occupying the Durham tube.

The strain reported here was isolated in the cave's resurgence, an area that is not open to tourists, as it is difficult to access. This access is made either externally, through a 10 km trail in a dense forest, or internally, through the river inside the cave, a route that presents great obstacles, considered very dangerous by regional guides and the speleological community. For this reason, it is an environment that has suffered little impact from human visitation.

Some hypotheses may explain how this yeast was dispersed to this specific environment, since until now it was only reported occurring in a cave environment in Malaysia. Zhang et al. (2018) state that fungal species diverged long before the formation of karst caves, which refutes the hypothesis that these species are troglobic.

Thus, we cannot assume that *B. malaysiensis* is a troglobic yeast, despite only being known in cave environments, but we emphasize the importance of further research efforts involving this species, in order to elucidate its current distribution. Whether it is a species restricted to subterranean environments or if this current distribution is only due to the lack of sampling and precise taxonomic identification. The present study reports the second worldwide occurrence of *B. malaysiensis*, expanding its distribution beyond the Asian continent.

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Author contributions

SXS and ASFS contributed to the study conception and design. Material preparation, data collection, and data analysis were performed by ASFS and LLS. The first draft of the manuscript was written by ASFS. All authors commented on previous versions of the

manuscript and approved the final version. SXS provided funds and supervised this research.

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Figure 1.

A Location of the studied area, Parque Estadual de Terra Ronca (PETER), Goiás, Brazi **B** Entrance of Lapa do Angélica cave **C** Internal part of cave (aphotic zone) **D** Resurgence.

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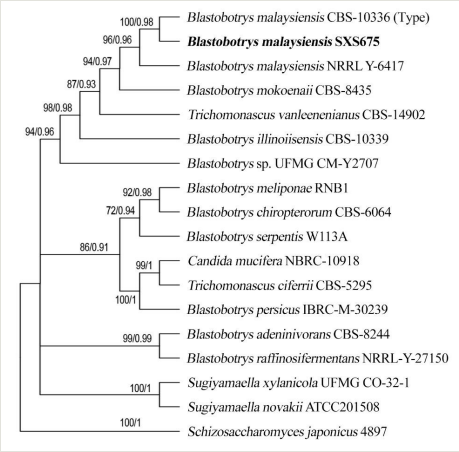

Figure 2.

Phylogenetic relationships between *Blastobotrys malaysiensis* SXS 675 (in bold) and other *Blastobotrys* species and corresponding clade, based on rDNA of the ITS (Internal Transcribed Spacer) region. Values at nodes indicate bootstrap from Maximum Likelihood/Bayesian posterior probability analysis. *Schizoaccharomyces japonicus* was included as an outgroup.

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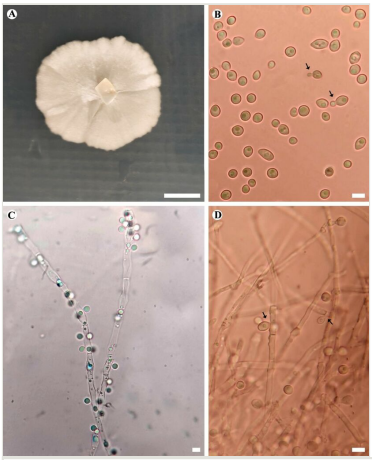

Figure 3.

Morphology of *Blastobotrys malaysiensis* (SXS 675). **A** Colony of *B. malaysiensis* **B** Yeast cells, with multilateral budding (arrows) **C** Blastoconidia stained with lactophenol blue **D** Blastoconidia attached to hyphae from pedicels (arrows). A-B grown on PDA (Potato Dextrose Agar) after eight days of growth at 27ºC, and C-D grown on MEA (Malt Extract Agar) at 27ºC for five days. Scale bar: 10 mm (A), 10 µm (B, C, D).

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Table 1.

List of species, strains, and GenBank accession code for ITS sequences used in phylogenetic analyses.

Species	**Strain/ Specimen No.**	**Country**	**GenBank accesion N° (ITS)**	**Reference**
Blastobotrys malasyensis	CBS: 10336-Type	Malaysia	NR_165958.1	Vu 2016
Blastobotrys illinoisensis	CBS: 10339	EUA	NR-165957.1	Vu 2016
Blastobotrys adeninivorans	CBS: 8244	Netherlands	EU343811.1	GenBank
Blastobotrys chiropterorum	CBS: 6064	Colombia	KY101750.1	Vu 2016
Blastobotrys malasyensis	NRRL Y-6417	-	DQ898170.1	Kurtzman 2007
Blastobotrys malaysiensis	SXS 675	Brazil	MZ702867	This study
Blastobotrys meliponae	RNB1	Brazil	KT448719	Crous 2016
Blastobotrys mokoenaii	CBS: 8435	South Africa	KY101754.1	Vu 2016
Blastobotrys persicus	IBRC-M 30239	Iran	KY352042.1	Nouri et al. 2017
Blastobotrys raffinosifermentans	NRRL Y-27150	-		Kurtzman 2007
Blastobotrys serpentis	W113A	India	AM410670	Bhadra 2008
Blastobotrys sp. E4	UFMG-CM-Y2707	Brazil	KT377031.1	GenBank
Candida mucifera	NBRC 10918	Brazil	LC158135.1	Tsang 2017
Schizosaccharomyces japonicus	4897	Japan	AB243296	GenBank
Sugiyamaella novakii	ATCC201508	-	LC120357	Tanahashi and Hawes 2016
Sugiyamaella xylanicola	UFMG-CO-32.1	Brazil	KC493642	Morais et al. 2013
Trichomonascus ciferrii	CBS: 5295	-	NR_111160	Schoch et al. 2014
Trichomonascus vanleenenianus	CBS: 14902	Netherlands	NR_168170.1	Groenewald 2018

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Table 2.

Fermentative and assimilative characteristics of *Blastobotrys malaysiensis* for five carbon sources. (+) Positive, (*) Poor result.

Substrate	Assimilation	Fermentation
Glucose	+	*
Lactose	+	+
Maltose	+	+
Galactose	+	+
Xylose	+	*