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Blastobotrys persicus sp. nov., an ascomycetous yeast species isolated from cave soil

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Abstract Two strains (AHD129-1^T and AHD129-2) of a new anamorphic yeast species were isolated from Mejare cave soil samples of Abdanan, Ilam, Iran. Nucleotide divergence in the D1/D2 domain of the large subunit (LSU) rRNA, and internal transcribed spacer (ITS) genes suggest that the two strains can be assigned to the *Trichomonascus/Blastobotrys* clade. A maximum likelihood tree based on sequences of the D1/D2 domain revealed that the new species is closely related to the species *Trichomonascus ciferrii*, *Candida allociferrii,* and *Candida mucifera.* The new species could be distinguished from the closely related species by its ability to grow at 42 °C and the inability to assimilate D-arabinose and D-mannitol. The name *B. persicus* sp. nov. is proposed for the new anamorphic species. The type strain of *B. persicus* is AHD129-1^T = IBRC-M30238^T = CBS 14259^T, and the Mycobank number is MB 819148.

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Abbreviation

LSU	Large subunit
ITS	Internal transcribed spacer
SSU	Small subunit

Introduction

The family *Trichomonascaceae* was proposed by Kurtzman and Robnett (2007) through multi-gene phylogenetic analysis of the teleomorphic genera *Trichomonascus*, *Wickerhamiella, Sugiyamaella,* and *Zygoascus* (Lachance et al. 2000; Kurtzman and Robnett 2007; Smith et al. 2011; Péter et al. 2011). The *Trichomonascus/Blastobotrys* clade includes anamorphic and teleomorphic members and comprises species from the polyphyletic genus *Candida* (Lachance et al. 2011) and the genus *Blastobotrys* (Kurtzman and Robnett 2007) as anamorphic members. The genus *Blastobotrys* was described in 1967 by von Klopotek, as a hyphomycete (von Klopotek 1967), but phylogenetic analysis of D1/D2 of the large-subunit (LSU) rDNA demonstrated that assigned species are actually anamorphic members of the *Saccharomycetales* (Kurtzman and Robnett 1998). The multigene analysis of LSU rDNA, the cytochrome

oxidase II (COXII) and small-subunit (SSU) rDNA gene demonstrated *Blastobotrys*, *Arxula*, *Sympodiomyces* and several *Candida* species to be members of the same clade, which was interpreted as a single genus. Kurtzman and Robnett in 2007 transferred the anamorphic species of the *Trichomonascus* clade to the genus *Blastobotrys* (Kurtzman and Robnett 2007; de Hoog et al. 1985), which has taxonomic priority over *Sympodiomyces* (Fell and Statzell 1971) and *Arxula* (van der Walt et al. 1990), and several species of *Candida*. Although *Trichomonascus* (Jackson 1947) has taxonomic priority over *Blastobotrys*, the use of *Blastobotrys* as generic name for the *Trichomonascus/Blastobotrys* clade was favoured by participants of a workshop on yeast taxonomy (Utrecht, The Netherlands 2015, pers. information H. M. Daniel). However, this proposal still needs to be formalized. Members of the *Trichomonascus/Blastobotrys* clade are heterothallic which raises the possibility that anamorphic species in the clade may represent mating types (Kurtzman 2007).

In cave and cave-like habitats (i.e. mines) microbiota are related to changes in organic compounds from the outside environment, and fungal growth is generally nutrient-limited due to the rarity of organic materials in caves (Chelius et al. 2009; Jurado et al. 2009). There are some reports about the biodiversity of fungi in caves. Rutherford and Huang (1994) detected 127 species in 59 genera of fungi found in caves habitats. Vanderwolf et al. (2013) reviewed 1029 species in 518 genera of fungi, slime molds, and yeasts from different caves (Rutherford and Huang 1994; Vanderwolf et al. 2013). Most of the fungal taxa commonly studied from caves are associated with insects, soils, or plant materials and they generally function as decomposers or parasites in the cave environment (Yoder et al. 2009). Of course, it should be noted that many fungi found in caves could be carried by water, air currents, or animals in the cave environment and are present as spores. In 2004, Grishkan reported that many fungal species in the cave environment were isolated with low abundance and had low frequency of occurrence (Grishkan et al. 2004).

Fungal taxa reported from caves belong mostly to Ascomycota (69%), Basidiomycota (20%), Zygomycota (7), and fungus-like microorganisms (3.6%). The main reason for the relative rarity of Basidiomycota versus Ascomycota can be attributed to the lack of large nutrient-rich substrates in caves such as spoilable plant materials and dung. The most frequent yeast and yeast-like fungi genera reported from caves all around the world are *Histoplasma, Candida, Aureobasidium, Microascus, Cryptococcus, Saccharomyces, Rhodotorula, Trichosporon, Sporobolomyces* and *Monascus* (Vanderwolf et al. 2013). In this study, *Blastobotrys* was reported from cave soil for the first time. Many studies reported that the diversity in microbiota and biomass decrease from the entrance to deep zones in caves (Hsu and Agoramoorthy 2001; Urzi et al. 2010; Kuzmina et al. 2012; Mulec et al. 2002). Unexpectedly, in a South African gold mine, Pohl et al. (2007) reported that the biodiversity of filamentous fungal genera increased from the outside to deeper zones of cave and the opposite trend was reported for yeast genera. Direct air supply from the surface was considered as a reason for the increase in the number of filamentous fungi in the air. Moreover, the presence of yeast isolates related to human and animal activities and the biodiversity of yeast genera decreased from the outside to the inside (Pohl et al. 2007). Vaughan-Martini et al. (2000) studied the yeast communities of three caves of the Frasassi complex in the Marche region of Italy. They reported that the higher counts of yeast isolates were directly related to the frequencies of human or animal visitation. The number of yeast isolates in the Grotta del Vento cave with mass tourism and human visitors was 3.25 times of those found in the Grotta del Fiume cave (Vaughan-Martini et al. 2000).

In this study, we report the isolation of two yeast strains from cave soil samples in Ilam, Iran, based on comparisons of rRNA gene sequences and propose them as new anamorphic species with the name *Blastobotrys persicus* sp. nov.

Materials and methods

Yeast isolation

Soil samples (200 g) were collected from different locations in December 2015 by digging 5–10 cm into the ground from the Mejare cave, Abdanan, Ilam, Iran (N32°35′24″, E47°31′12″), 1100 m above sea level. The feces and wool of domestic animals, such as goats and sheep, were seen at the cave entrance. Standing or running water was not observed in the sampling areas.

The samples were obtained about 3–10 m into the dark zones in different areas (about 20–30 m from the entrance). The temperature of the cave was 25 °C. Spiders and ants were seen as the dominant animals of the cave. In total, 10 soil samples were placed in sterile plastic bags (10 × 10 mL) and were transported to the laboratory over a period of no more than 24 h. Afterward, 1 g of each soil sample was homogenized in sodium chloride solution (0.9% w/v) and then serially diluted in the same solution (10^{-1}–10^{-3}), and 100 µL from each dilution was spread over the yeast–peptone–glucose (YPG) agar (0.5% yeast extract, 1.0% peptone, 2.0% glucose, and 2.0% agar) supplemented with 0.01% chloramphenicol and 0.005% rose bengal. The plates were then incubated at 28 °C in the dark and monitored daily up to 5 days until yeast colonies developed. The colonies showing different morphotypes, mostly from dilution 10^{-2}, were picked and purified on YPG agar as soon as they grew on the plates. The yeast colonies' stock cultures were stored on the YPG broth with 20% (v/v) glycerol at − 80 °C until further analysis.

Phenotypic and physiologic characterization

Morphological characteristics were observed by light microscopy after culture on YPG, potato dextrose agar (PDA) (Merck), and cornmeal agar (Fluka). The phenotypic and physiological characteristics of the strains IBRC-M 30238^{T} and IBRC-M 30239 were examined in accordance with the standard methods (Barnett et al. 2000; Kurtzman et al. 2011). Carbon assimilation tests were carried out with the auxanographic method at 28 °C in triplicates, and the results were recorded after 2 weeks of incubation.

The two strains IBRC-M 30238^{T} and IBRC-M 30239 were examined (individually and crossed in mating experiments) for sporulation after growth on Gorodkowa (Merck), cornmeal (Fluka), malt 5% (Merck), and YM agar (Merck) (Kurtzman et al. 2011). In brief, to test for sexual compatibility, IBRC-M 30238^{T} and IBRC-M 30239 were separately grown overnight in 5 mL YPG broth at 28 °C. The cells were collected by centrifugation at 14,000 rpm for 15 min and washed with sterile normal saline. The pellet was then re-suspended in 100 µL distilled normal saline and each cell suspension (10 µL) was spread in the above-mentioned sporulation media and incubated at 15 and 28 °C for 6 weeks.

Phylogenetic analysis

The small subunit (SSU), the internal transcribed spacer (ITS) region, and the D1/D2 domain of the large subunit (LSU) of ribosomal DNA (rDNA) gene were amplified by PCR using primers nu-SSU-0817 (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536 (ATTGCAATGCYCTATCCCCA) (Moghimi et al. 2017), ITS1(TCCGTAGGTGAA CCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990), NL1 (GCATATCAATAAGCGGAGGAAAAG), and NL4 (GGTCCGTGTTTCAAGACGG) (Kamyabi et al. 2017) respectively. The PCR products were checked by agarose gel electrophoresis and purified by using the Bioneer purification kit (Bioneer, South Korea). The purified PCR product was submitted to the Bioneer Corporation (South Korea) for sequencing with the same primers used for gene amplification.

BlastN searches for sequences deposited in the NCBI GenBank were carried out. The sequences of related species retrieved from the GenBank were aligned iteratively by using the multiple alignment program CLUSTAL X, version 1.81 (Thompson et al. 1997).

The phylogenetic placement of the new species was conducted using the MEGA version 6 software package from the evolutionary distance data using the two-parameter model (Kimura 1980) and the maximum likelihood method. The robustness of the clades was assessed using bootstrap analysis with 1000 replicates (Felsenstein 1985). The GenBank/EMBL/DDBJ accession numbers of the strain IBRC-M 30238^{T} for the sequences of the ITS, LSU and the SSU genes are KU659140, KU659141, and KU659142 respectively. The accession numbers for the sequences of the ITS and the LSU genes of IBRC-M30239 are KY352042 and KY352043 respectively.

Results and discussion

Species delineation and phylogenetic placement

A total of 31 yeast strains were isolated from 10 different soil samples during an investigation on the biodiversity of yeasts associated with the soil samples from caves. We isolated IBRC-M 30238^{T} and IBRC-M 30239 from two separate cave soil samples, which

were 5 m from each other. These two strains were representatives of a new species based on the sequence similarity in the D1/D2 and ITS regions, and their physiological, morphological, and phylogenetic properties were studied. From the soil samples, the following additional fungal and yeast isolates were found: *Naganishia adeliensis* (two), *Naganishia uzbekistanensis* (one), *Basidioascus* sp. (two), *Candida albicans* (two), *Candida powellii* (three), *Candida restingae* (one), *Candida tropicalis* (two), *Sarocladium kiliense* (six), *Sarocladium strictum* (three), *Sarocladium oryzae* (one), *Rhodotorula mucilaginosa* (one), *Rhodotorula lactosa* (two), *Exophiala jeanselmei* (two), and *Exophiala exophialae* (one).

The two strains, IBRC-M 30238^{T} and IBRC-M 30239, share identical D1/D2 sequences and differ sufficiently from the genetically closely related species in the *Trichomonascus/Blastobotrys* clade, so they were considered to be a separate species (Fig. 1). The comparison of the D1/D2 large subunit rRNA gene sequence of the new species with entries in the dataset revealed the closest match in terms of the pairwise sequence identity to be the type strains of *Trichomonascus ciferrii* NRRL Y-10943^{IT} (DQ442681) by 1.5% divergence (9 nt substitutions in 583 nt), *Candida allociferrii* IFO 10194^{T} (AB041003) by 2% divergence (12 nt substitutions in 577 nt) and *Candida mucifera* IFO 10918^{T} (AB041006) by 1.75% divergence (10 nt substitutions in 577 nt) (Fig. 1).

A comparison of the ITS sequences revealed that the new species differed from the type strains *Trichomonascus ciferrii* NRRL Y-10943^{IT} (KY105703.1), *Candida allociferrii* IFO 10194^{T} (LC158134.1), and *Candida mucifera* IFO 10918^{T} (EF568082.1) by 3.82% (17 nt substitutions out of 445 nt), 5% (25 nt substitutions out of 500 nt), and 4% (20 nt substitutions out of 494 nt) nucleotide substitutions respectively.

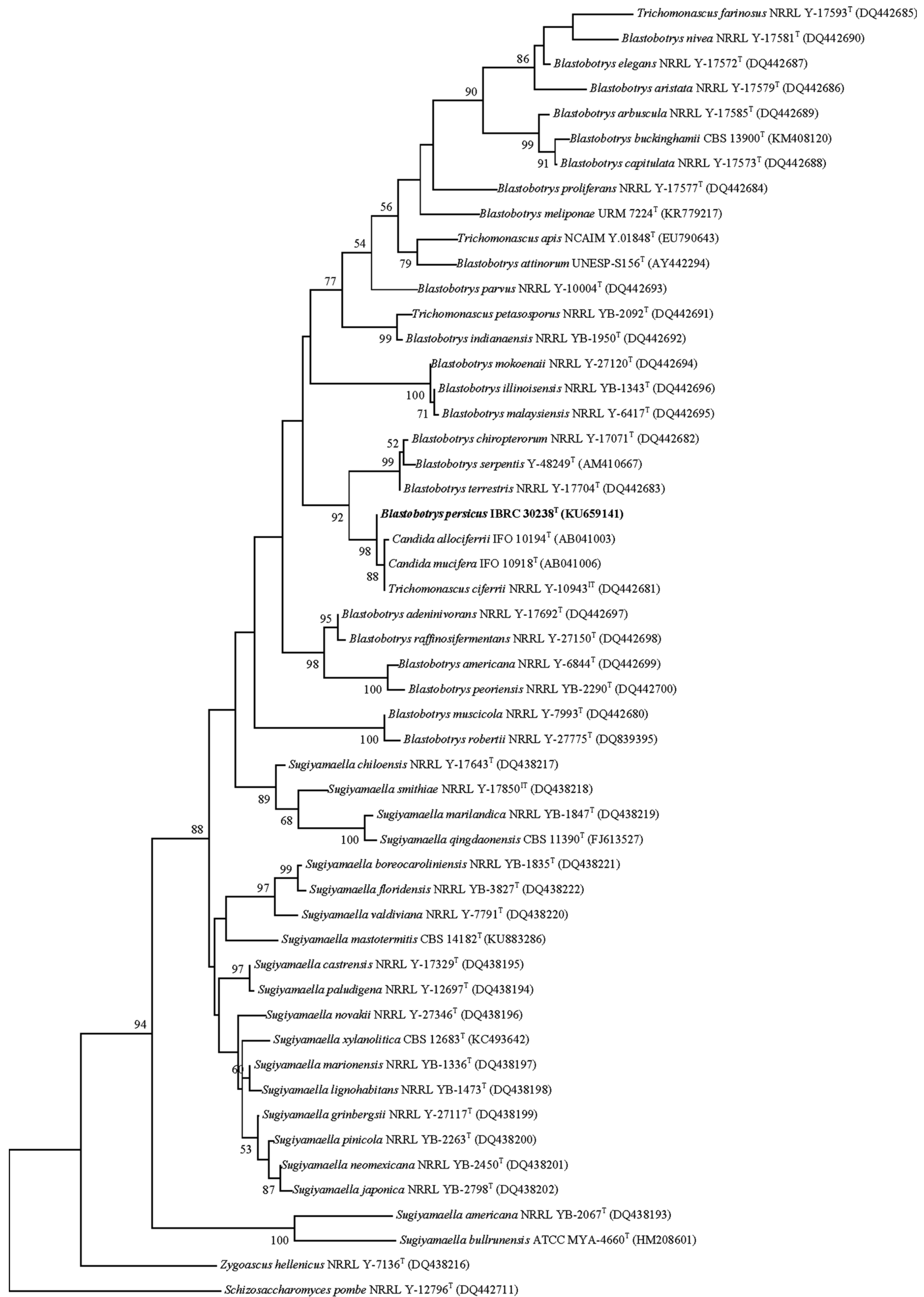
The sequence data and phylogenetic analysis of the D1/D2 large subunit rRNA suggested that the new species belongs to the subclade that contains *T. ciferrii, C. allociferrii, C. mucifera, Blastobotrys chiropterorum,* and *Blastobotrys terrestris* (bootstrap value, 97%) (Fig. 1). The presented phylogenetic tree demonstrated an arrangement of the species that is similar to the multigene sequence tree introduced by Kurtzman and Robnett (2007).

Fig. 1 Phylogenetic placement of *Blastobotrys persicus* IBRC-M 30238^{T} and some related species based on the maximum likelihood analysis of D1/D2 LSU rRNA gene sequences. *Schizosaccharomyces pombe* and *Zygoascus hellenicus* were designated as the out-group species for this analysis. GenBank accession numbers are shown in parentheses. Numbers at branch points are bootstrap percentages derived from 1000 replicates

Ueda-Nishimura and Mikata (2002) investigated nine strains of the *T. ciferrii* complex and separated them into three groups in accordance with their sequences and the presence of Group I intron in SSU gene. The DNA similarity values showed that each group represented a separate species. Based on this classification, Group A was designated as *C. allociferrii.* In this group, the SSU sequences had no introns and the strains could not form asci by themselves. Group B comprised *T. ciferrii, C. ciferrii,* and *Sporothrix catenata,* all containing one Group I intron, Sc1506-1, at position 1506 in the SSU gene. Group C included strains of *C. mucifera* and they contain two Group I introns, Sc943 at position 943, and Sc1506-2 at position 1506 (Ueda-Nishimura and Mikata 2002). The SSU sequence analysis of the here introduced new *Blastobotrys* species showed no intron at positions 943 and 1506 (KY510967). Considering the difference of the LSU and ITS sequence analysis between the new *Blastobotrys* species and *C. allociferrii,* and according to the intron group classification by Ueda-Nishimura and Mikata, this new species should be considered as a new member of Group A along with *C. allociferrii.*

Phenotypic and growth characteristics

The strains IBRC-M 30238^{T} and IBRC-M 30239 were examined for sporulation. No signs of conjugation and the formation of asci or ascospores in any of the tested media were observed. In both strains, yeast colonies are white and somewhat raised. Budding cells were formed by multilateral budding, and the production of true hyphae was not observed (Fig. 2). Different physiological properties, such as sugar consumption and growth at 42 °C, were investigated. The results for *T. ciferrii* NRRL Y-10943^{IT} (DQ442681), *C. mucifera* IFO 10918^{T} (AB041006), and *C. allociferrii* IFO


Trichomonascus farinosus NRRL Y-17593^{T} (DQ442685)
Blastobotrys nivea NRRL Y-17581^{T} (DQ442690)
86
Blastobotrys elegans NRRL Y-17572^{T} (DQ442687)
90
Blastobotrys aristata NRRL Y-17579^{T} (DQ442686)
Blastobotrys arbuscula NRRL Y-17585^{T} (DQ442689)
99
Blastobotrys buckinghamii CBS 13900^{T} (KM408120)
91
Blastobotrys capitulata NRRL Y-17573^{T} (DQ442688)
Blastobotrys proliferans NRRL Y-17577^{T} (DQ442684)
56
Blastobotrys meliponae URM 7224^{T} (KR779217)
54
Trichomonascus apis NCAIM Y.01848^{T} (EU790643)
79
Blastobotrys attinorum UNESP-S156^{T} (AY442294)
77
Blastobotrys parvus NRRL Y-10004^{T} (DQ442693)
Trichomonascus petasosporus NRRL YB-2092^{T} (DQ442691)
99
Blastobotrys indianaensis NRRL YB-1950^{T} (DQ442692)
Blastobotrys mokoenaii NRRL Y-27120^{T} (DQ442694)
100
Blastobotrys illinoisensis NRRL YB-1343^{T} (DQ442696)
71
Blastobotrys malaysiensis NRRL Y-6417^{T} (DQ442695)
52
Blastobotrys chiropterorum NRRL Y-17071^{T} (DQ442682)
99
Blastobotrys serpentis Y-48249^{T} (AM410667)
Blastobotrys terrestris NRRL Y-17704^{T} (DQ442683)
92
***Blastobotrys persicus* IBRC 30238^{T} (KU659141)**
Candida allociferrii IFO 10194^{T} (AB041003)
98
Candida mucifera IFO 10918^{T} (AB041006)
88
Trichomonascus ciferrii NRRL Y-10943^{IT} (DQ442681)
95
Blastobotrys adeninivorans NRRL Y-17692^{T} (DQ442697)
Blastobotrys raffinosifermentans NRRL Y-27150^{T} (DQ442698)
98
Blastobotrys americana NRRL Y-6844^{T} (DQ442699)
100
Blastobotrys peoriensis NRRL YB-2290^{T} (DQ442700)
Blastobotrys muscicola NRRL Y-7993^{T} (DQ442680)
100
Blastobotrys robertii NRRL Y-27775^{T} (DQ839395)
Sugiyamaella chiloensis NRRL Y-17643^{T} (DQ438217)
89
Sugiyamaella smithiae NRRL Y-17850^{IT} (DQ438218)
68
Sugiyamaella marilandica NRRL YB-1847^{T} (DQ438219)
88
100
Sugiyamaella qingdaonensis CBS 11390^{T} (FJ613527)
99
Sugiyamaella boreocaroliniensis NRRL YB-1835^{T} (DQ438221)
97
Sugiyamaella floridensis NRRL YB-3827^{T} (DQ438222)
Sugiyamaella valdiviana NRRL Y-7791^{T} (DQ438220)
Sugiyamaella mastotermitis CBS 14182^{T} (KU883286)
97
Sugiyamaella castrensis NRRL Y-17329^{T} (DQ438195)
Sugiyamaella paludigena NRRL Y-12697^{T} (DQ438194)
Sugiyamaella novakii NRRL Y-27346^{T} (DQ438196)
94
Sugiyamaella xylanolitica CBS 12683^{T} (KC493642)
60
Sugiyamaella marionensis NRRL YB-1336^{T} (DQ438197)
Sugiyamaella lignohabitans NRRL YB-1473^{T} (DQ438198)
Sugiyamaella grinbergsii NRRL Y-27117^{T} (DQ438199)
53
Sugiyamaella pinicola NRRL YB-2263^{T} (DQ438200)
Sugiyamaella neomexicana NRRL YB-2450^{T} (DQ438201)
87
Sugiyamaella japonica NRRL YB-2798^{T} (DQ438202)
Sugiyamaella americana NRRL YB-2067^{T} (DQ438193)
100
Sugiyamaella bullrunensis ATCC MYA-4660^{T} (HM208601)
Zygoascus hellenicus NRRL Y-7136^{T} (DQ438216)
Schizosaccharomyces pombe NRRL Y-12796^{T} (DQ442711)
0.02

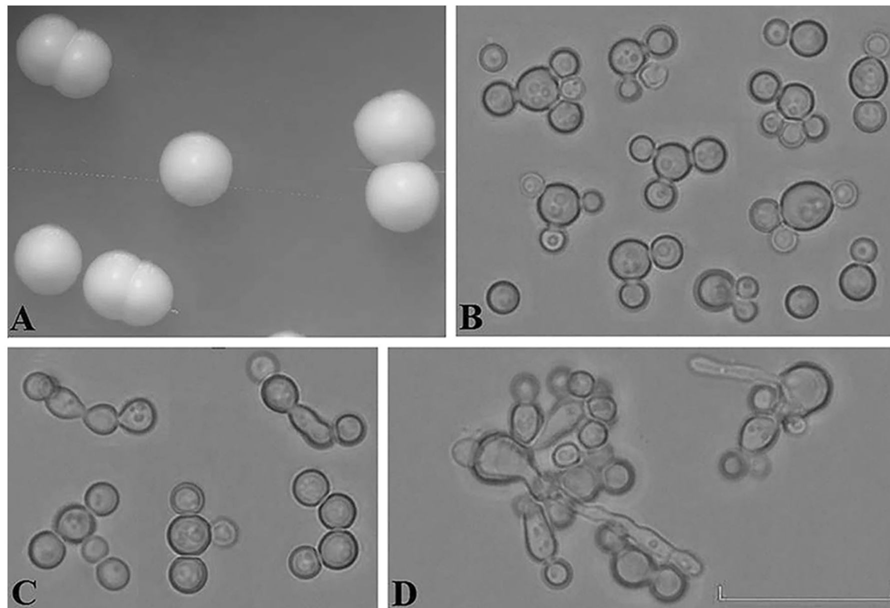


Fig. 2 Photomicrographs of *Blastobotrys persicus* IBRC-M 30238^{T}. **a** Butyrous, glistening, white-colored colony on YPG medium at 28 °C after 72 h of incubation. **b** Budding cells. **c** Short chains of blastoconidia. **d** Formation of protuberances, indicating the early stage of germ-tube production on YPG agar after 7 days' incubation at 25 °C. Bars = 10 μm and valid for figures **a–d**

10194^{T} (AB041003) were compared with that for *Blastobotrys persicus* IBRC-M 30238^{T} and summarized in Table 1.

Description of *Blastobotrys persicus* H. Nouri, S. Nasr and H. Moghimi, sp. nov

Blastobotrys persicus (per' si.cus. L. masc. adj. *persicus* of Persia, present Iran, refer to the location where the type strain of the species was isolated). Growth on YPG agar: after 72 h at 28 °C, the streak culture is butyrous, glistening, and white-colored with a smooth surface (Fig. 2a). Colony margins are entire (Fig. 2a). The cells range from being spherical to ovoid (3–4 × 2.5–4 μm); they occur singly, in pairs, or in small clusters. Budding is multilateral (Fig. 2b). Pseudohyphae are rare on YPG, PDA and cornmeal agar. Protuberances as potential early stage of germ-tube production (Fig. 2d) and short chains of blastoconidia (Fig. 2c) are formed. Production of ascospores was not observed on Gorodkowa, cornmeal, malt 5%, and YM agar after 6 weeks neither at 15 °C nor at 28 °C. Fermentation is absent. Glucose, galactose, sucrose, maltose, α,α-trehalose, D-xylose, cellobiose, L-arabinose, L-rhamnose (weak), ribitol, melibiose (slow or weak), raffinose, melezitose (weak), salicin

Table 1 Physiological and growth characters that can distinguish *Blastobotrys persicus* from its closest relatives

Species	Growth								
	Citrate	Inulin	Melezitose	D-ribose	D-mannitol	D-arabinose	Soluble starch	Succinate	42 °C
T. ciferrii	v	–	–	v	+	+	v	v	–
C. allociferrii	–	w	–	+	+	+	+	+	–
C. mucifera	+	–	–	+	+	+	+	+	–
B. persicus	–	–	w	–	–	–	–	–	+

Delayed positive: d/weak: w/positive: +/negative: –

(weak), arbutin, *n*-hexadecane (weak), myo-inositol, glycerol, DL-lactate (weak), and ethanol (weak) are assimilated; no growth occurs on D-ribose, D-mannitol, D-arabinose, starch, lactose, methanol, succinate, and citrate.

Ethylamine hydrochloride, L-lysine, cadaverine dihydrochloride, creatinine (weak), and glucosamine (weak) are assimilated as nitrogen sources, while potassium nitrate, sodium nitrite, creatine, and imidazole are not assimilated. The formation of amyloid material is negative. Growth is weak at 15 °C. Growth occurs at 25, 30, 34, 37, 40, and 42 °C. Growth at 4, 10, and 45 °C is negative.

Growth with 0.01 and 0.1% cycloheximide is negative. Growth is present on 50% (w/w) glucose–yeast extract agar. Growth is weakly positive on 60% (w/w) glucose–yeast extract agar. No growth occurs on 1% acetic acid. Growth is present on 5 and 10% NaCl. No growth occurs on 16% NaCl. Urea hydrolysis and color reaction with diazonium blue B are negative.

The holotype strain is IBRC-M 30238^T isolated from Abdanan, Ilam, Iran. The holotype is permanently preserved in a metabolically inactive state at the Iranian Biological Resource Center (IBRC), Iran, as IBRC-M 30238^T and an ex-type culture is deposited in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands, as CBS 14259^T.

Strains examined

IBRC-M 30238^T (CBS 14259^T); IBRC-M 30239.

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Conflict of interest The authors declare no conflicts of interest.

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