Basidioascus persicus sp. nov., a yeast-like species of the order Geminibasidiales isolated from soil

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A novel species of basidiomycetes was isolated from kitchen garden soil in Shahryar city, Tehran province, Iran. Molecular and conventional methods were employed to identify and classify this single isolate. Morphologically, the isolate was considered yeast-like with hyaline and oval cells reproducing by monopolar budding, forming ballistoconidia, hyphae, arthroconidia and didymospores. Basidia and basidiospores resembling those produced by Basidioascus species were observed. Sequencing and Bayesian phylogenetic analysis of rRNA genes and the internal transcribed spacer region revealed its sister relationship to described species of the genus Basidioascus. Assimilation and fermentation tests, cell-wall carbohydrate analysis and enzyme activity tests were performed to provide insight into the metabolism of the isolate. Based on morphology, physiology and phylogeny of rRNA gene sequences, the isolate was shown to represent a novel species of the genus Basidioascus, described as Basidioascus persicus sp. nov. (holotype IBRC P1010180T = ex-type IBRC M30078T = isotype CBS 12808T). The MycoBank number of the novel species is MB 804703. An emended description of the genus Basidioascus is also provided.

Introduction

Fungi, including yeasts, occur in a wide range of soil types (Anderson & Cairney, 2004; Jones & Pang, 2012; Carvalho et al., 2013) and abiotic factors such as temperature, humidity and chemical composition influence their diversity (Botha 2011; Yurkov et al., 2012). Soil yeasts are important; for example, they contribute to essential ecological processes such as the mineralization of organic material and dissipation of carbon and energy through the soil ecosystem (Botha, 2011). Many yeast genera have been isolated from soils, such as species of Kluyveromyces, Lipomyces, Schwanniomyces, Schizoblastosporium, Debaryomyces, Hansenula, Candida, Brettanomyces, Rhodotorula, Cryptococcus, Trichosporon and Torulopsis (Bresolin et al., 2010; Kurtzman et al., 2011; Carvalho et al., 2013). There are, at the time of writing, around 1500 yeast species described (Kurtzman, 2011). The doubling time of species discovery is about 10 years (Boekhout et al., 2012) so our knowledge of yeast biodiversity is rapidly advancing. However, even at the current rate of discovery, our knowledge of the fungal biodiversity on Earth, which includes the yeasts, is deficient with an estimated 5–10 % of fungi known to science currently (Blackwell 2011).

A fungal order called Geminibasidiales was taxonomically defined recently (Nguyen et al., 2013). The Geminibasidiales comprises two genera (Geminibasidium and Basidioascus). and together they form a sister group to the order Wallemiales based on phylogeny of rRNA gene sequences (Nguyen et al., 2013). These two orders are grouped under the class Wallemiomycetes and they represent a basal lineage to the major fungal subdivision Agaricomycotina. Species of the genera Basidioascus and Geminibasidium are hyphal,

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 28S rRNA gene (D1/D2 domains), ITS and 18S rRNA gene region sequences of Basidioascus persicus sp. nov. = ex-type IBRC M30078T = isotype CBS 12808T are KC751416, KC751417 and KC751418, respectively.

One supplementary table and two supplementary figures are available with the online version of this paper.
heat-resistant and xerotolerant, and they have been isolated from soil (Matsushima, 2001; Nguyen et al., 2013a). Only two species of the genus *Basidioascus*, *Basidioascus undulatus* and *Basidioascus magus*, have been described and the internal transcribed spacer (ITS) phylogeny with environmental sequences hinted at additional uncultured species in this genus (Nguyen et al., 2013a).

In the present study, an unknown fungal isolate was found from soil in Shahryar city, Tehran province, Iran. Based on morphology, physiology and phylogeny of rRNA gene sequences, this isolate is shown to represent a novel species of the genus *Basidioascus*.

**Methods**

**Isolation.** A kitchen garden soil sample from Shahryar city was transferred to the National Laboratory of Industrial Microbiology at Alzahr University. Dilutions of the sample were directly plated on Rose Bengal agar (Himedia) and incubated at 28 °C for 72 h. Fungal colonies were subcultured and purified on yeast peptone glucose agar [YPG; 5 g yeast extract, 10 g peptone, 20 g glucose and 20 g agar (all Merck), 1000 ml distilled water] at 28 °C. An unknown isolate designated IBRC M30078T was chosen for subsequent studies described below.

**Morphological and physiological studies.** To assess colony morphology and sizes, isolate IBRC M30078T was grown on YPG agar, 2 % malt extract agar (MEA; Merck), dichloran 18 % glycerol agar (DG18; Merck) and malt yeast 40 % sucrose agar (M40Y; Nguyen et al., 2013a). To test for forcibly discharged basidia or basidiospores, the isolate was grown on corn meal agar (CMA; Merck). The dish was then inverted over another Petri dish of 5 % MEA containing a sterile slide, and incubated at 28 °C. After 21 days of incubation in the inverted position, the MEA dish was checked for structures shot down from the colony grown on CMA.

**Molecular studies.** The ITS region of the rRNA gene was amplified by PCR using the primer pair ITS1 (forward) (5′-TCCGGTATTGCTATCGGC-3′) and ITS4 (reverse) (5′-TCCGCTTATTGATATGC-3′) (White et al., 1990). The D1/D2 domain of the LSU was amplified by PCR with primers NL1 (forward) (5′-GCATATCATATACCGGAGGAGAAA-3′) and NL4 (reverse) (5′-GGTCCGTTTGTTCAAGGCGG-3′) (Lin et al., 1995; Kurtzman & Robnett, 1998). The SSU rRNA gene was amplified using the combination of primers NS1 (forward) (5′-GTAGCTATATGCTTGTCTC-3′) and NS8 (reverse) (5′-TCCGGAGGTCACTACCG-3′) (White et al., 1990). These genes were sequenced directly from PCR products using the same primer sequences. Alignments were performed using ClustalW (Thompson et al., 1994) and phylogenetic analysis was carried out with MrBayes version 3.2 (Ronquist & Huelsenbeck, 2003) following the method described by Nguyen et al. (2013a).

**Fermentation and assimilation.** The standard laboratory methods of Barnett et al. (2000) and Kurtzman et al. (2011) for identifying yeasts were used for determination of phenotypic and physiological characteristics of isolate IBRC M30078T. Fermentation ability was determined using Wickerham (1951) basal medium containing 4.5 g yeast extract 1−1 and 7.5 g peptone 1−1. Four millilitres of the stock solution (50 mg/75 ml) of Bromothymol blue was used as indicator and Durham tubes were used for detecting carbon dioxide production. The sugars used in fermentation tests were at 2 % (w/v), except for raffinose, which was at 4 % (w/v). The tubes were inspected at frequent intervals for accumulation of gas for up to 2 weeks. The assimilation ability of the isolate was determined by employing tubes containing 5 ml liquid nitrogen base medium (Sigma-Aldrich) and a particular carbon compound (50 mM) as a sole source of energy. The tubes were incubated at 28 °C for 4 weeks and the results from growth tests were read spectrophotometrically at 600 nm.

**Cell-wall carbohydrate analysis.** To analyse the carbohydrate composition of the cell wall, 50 mg dry cells cultivated from yeast extract peptone glucose broth [YPD: 1 % yeast extract, 2 % peptone and 2 % glucose (all w/v)], were hydrolysed with 1 ml of 1.0 M H2SO4 at 100 °C for 2 h, then cooled and the pH adjusted to 5.5 with a saturated solution of Ba(OH)2. The precipitate was removed by centrifugation and the supernatant was dried in a rotary evaporator and resolved in 50 μl distilled water. A 1 μl aliquot of this solution was applied to a cellulose TLC plate and developed with a solvent system containing n-butanol/water/piridine/toluene (10:6:6:1, by vol.) for 4 h. Sugars were visualized by an aniline phthalate solution and compared with a 1 μl aliquot of 1 % (w/v) standard solutions of high purity sugar mixtures (Komagata & Suzuki, 1987; Kurtzman et al., 2011).

**Coenzyme Q.** Intact cells were cultivated in YPD broth on a rotary shaker at 150 r.p.m., 28 °C for 72 h and were harvested by centrifugation. To identify coenzyme Q, these cells were washed three times with distilled water and freeze-dried. Purification of coenzyme Q was performed by preparative TLC (silica gel 60 F254, glass plates) using benzene for development. The dark band of coenzyme Q observed under UV light was scraped off and extracted with 2 ml acetone (Mikita & Yamada, 1999). Coenzyme Q isoprenologues were identified using HPLC as described previously (Limtong et al., 2007).

**Enzymic profiling.** To test for degradation of starch, protein (casein), lipids (Tweens 20 and 80), phospholipids (egg yolk), gelatin, DNA, xylan, CM-cellulose and guaiacol, the procedures from Brizzio et al. (2007) and Li et al. (2008) were employed. Petri dishes containing each substrate were incubated at 28 °C.

**Results and discussion**

**Morphological and physiological studies**

An unknown fungal isolate was found in the kitchen garden soil sample from Shahryar city. In an attempt to identify this unknown isolate, morphological, physiological and phylogenetic studies were performed initially.

The isolate produced cream-coloured colonies on MEA and M40Y (Fig. 1a, c), white hyphal colonies on YPG (Fig. 1b), and olive-coloured colonies on DG18 (Fig. 1d). Arthroconidia (Fig. 1e) and hyaline oval cells that multiplied by mono-polar budding (Fig. 1f) were observed. Additionally, the isolate produced didymospores on YPG and DG18 medium after 48 h of incubation at 28 °C (Fig. 1g) and ballistoconidia on PDA after at least 5 days of incubation at the same temperature (Fig. 1h–j). Based on our inverted Petri dish test for forcibly discharged spores, single colonies, shot from the top dish, were observed on the bottom dish, indicating that forcible discharge occurred. The ballistoconidium is produced on a short sterigma (Fig. 1h) attached to a dark oval basal cell and enlarged over the time. Their forcible discharge left a scar on the mother oval cell (Fig. 1k). Germination of ballistoconidia was observed (Fig. 1m). Sexual reproduction was
observed after 14 days of incubation at 28 °C on PDA and MEA (Fig. 1n–r). The probasidium was recognized by at least one basal lateral projection (Fig. 1n–p), a character that was previously observed in species of the genera *Geminibasidium* and *Basidioascus*, and a basidiospore was attached to it by a long tubular sterigma. The basal lateral projection is a small protrusion, resembling clamp connections, but not actually attached to the subtending cell, that is found near the base of the basidium in species of the genus *Basidioascus* (Nguyen et al., 2013a). Over time, the basidiospore became granular and verrucose with an orange to brown colour (Fig. 1p, q). Sometimes basidia lost their cytoplasm and collapsed (Fig. 1r). Only one mature basidiospore was observed per basidium. A similar maturation process was observed in existing species of the genus *Basidioascus*. The yeast cells produced both pseudohyphae (Fig. 1s) and septate hyphae (Fig. 1t). No clamp connections could be seen.

Species of the genus *Basidioascus* are known to be osmotolerant so physiological tests were performed on the isolate. The isolate grew in 5–10 % NaCl, in 50–60 % glucose and also on M40Y and DG18 agar, similarly to other species of the genus *Basidioascus*, particularly *Basidioascus undulatus* (Nguyen et al., 2013a). According to Nguyen et al. (2013), species of the genus *Basidioascus* are considered mesophilic and their optimum growth temperature is 25–30 °C; however, *Basidioascus undulatus* is able to grow at 37 °C. Isolate IBRC M30078T had a similar optimal growth temperature but it was not able to grow at 37 or 40 °C.

### Molecular studies

Amplification of the SSU, ITS and LSU loci followed by sequencing and phylogenetic analysis were performed to classify and identify the novel isolate. Amplicons of 1686, 651 and 600 bp were obtained for the SSU, ITS and D1/D2 region of the LSU, respectively. Searches via the BLAST program were performed with these sequences with the top five BLAST hits listed in Table S1 (available in the online Supplementary Material). All BLAST matches belonged to fungi isolated from soil. However, the closest identifiable match was *Basidioascus*, supporting the morphological and physiological observations described above.

Bayesian phylogenetic analyses were performed to assess the relationship of the novel isolate to fungi related to *Basidioascus*. The resulting rRNA gene phylogeny (Fig. 2) showed that the isolate grouped together with strains of species of the genus *Basidioascus* with a posterior probability of 1.00. The ITS phylogeny (Fig. 3) also confirmed this result and showed a sister group relationship to *Basidioascus undulatus* and *Basidioascus magus* with a posterior probability of 0.99. Strain MD76-1BY (GenBank accession nos. EU626069 and EU626068, otherwise not formally described), previously isolated from soil in China, represented the same species or a very closely related species to our isolate from Iran (Table S1 and Fig. 3) and was misidentified as representing *Cryptococcus* sp. Furthermore, another uncultured strain from soil in France (GenBank accession no. FN397319) was closely related to the new isolate. The ITS sequences from these fungi isolated in China and France form a strongly supported clade with strain IBRC M30078T from Iran, supporting the existence of a third but undescribed species of *Basidioascus*. The existence of these sequences suggests that this novel species of the genus *Basidioascus* was previously found, but not identified nor described previously.
Fermentation, carbohydrate analysis, coenzyme Q, enzymic profiling

While Basidioascus undulatus and Basidioascus magus are the only two known species of the genus Basidioascus, our study introduces a novel species of this genus with substantiating morphological and molecular phylogenetic evidence. Morphologically, the isolate behaved more like a yeast in our extensive observations. As the genus Basidioascus is composed of hyphal fungi and our isolate produced hyphal colonies only on YPG, this novel species should be treated as a yeast-like basidiomycete.

Yeast vary in their ability to ferment sugars. In contrast to ascomycetes, basidiomycete yeasts are generally less fermentative and less copiotrophic (Kurtzman et al., 2011).

Fig. 2. Bayesian ribosomal (SSU + 5.8S + LSU) phylogeny showing the relationship of isolate IBRC M30078\textsuperscript{T} to other fungi in the Basidiomycota and the Wallemiomycetes. Posterior probabilities are displayed at nodes of the tree. Asterisks indicate SSU and LSU sequences that were combined belong to different strains of the same species. Bar, 0.05 expected changes per site.
Xanthophyllomyces ferment glucose weakly (Kurtzman et al., 2011; Valente et al., 2012). The fermentation ability of the novel isolate was studied according the standard methods of Kurtzman et al. (2011) and Barnett et al. (2000). The fermentation result is based on the time and the amount of gas accumulated. Based on the indicator changes from green to yellow, consumption of sugars was observed after 24 h but the accumulation of gas was seen after 6 days. However, further incubation for up to 2 weeks did not result in further gas production except for sucrose (Fig. S1). Fermentation is considered weakly positive when less than one-third of the insert Durham tubes is filled with gas, whereas it is considered positive when more than one-third is filled with gas. Therefore, the fermentation of D-glucose, maltose, melezitose, trehalose and raffinose was recorded as weakly positive and the fermentation of sucrose was considered positive.

The carbohydrate components of the cell wall were studied. The TLC chromatogram from the whole-cell hydrolysate of sugars (Fig. S2) indicated a Tremella type with glucose being predominant, and with xylose, mannose and galactose present in trace amounts (Dörfler, 1990). Extracellular amyloid compounds were not found in either solid or liquid medium. These results suggested placement of the novel species near the Tremellales, consistent with phylogenetic analysis.

The yeasts and yeast-like fungi have coenzyme Q, which is a naturally occurring coenzyme formed from the conjugation of a benzoquinone ring with a hydrophobic isoprenoid chain of varying chain length, depending on the species (Kurtzman et al., 2011). The number of isoprene units in the side chains of coenzyme Q differs from Q5 to Q10 among fungal taxa (Olsen, 1990; Kurtzman et al., 2011). In general, species of a single genus have the same Q-system,
but exceptions exist in both the Ascomycota and the Basidiomycota (Olsen, 1990). The major coenzyme Q in isolate IBRC M30078\textsuperscript{T} was Q6.

Determination of enzymic activity profiles in novel microorganisms can reveal their potential value for biotechnological application as enzyme producers. In that regard, enzymic activity of isolate IBRC M30078\textsuperscript{T} was studied, and the preliminary screening revealed DNase and laccase activity.

**Novel yeast-like species of *Basidioascus***

Morphological, physiological and phylogenetic evidence suggests that isolate IBRC M30078\textsuperscript{T} represents a novel species of the genus *Basidioascus*. The substrate assimilation and fermentation results, cell-wall carbohydrate analysis and coenzyme Q determination, as well as enzyme activity tests gave further insight into the metabolism of this novel species and these data could serve as a starting point for comparison studies of other species of *Basidioascus*. The results supported the identification of isolate IBRC M30078 as representing a novel species of the genus *Basidioascus*, described as *Basidioascus persicus* sp. nov. Furthermore, the genus description of *Basidioascus* was emended slightly to accommodate *Basidioascus persicus*.

**Description of *Basidioascus persicus* S. Nasr, M. R. Soudi & H. D. T. Nguyen sp. nov.**

*Basidioascus persicus* (per.si.cus. L. masc. adj. persicus of Persia, present Iran, referring to the location where this species was isolated).

MycoBank number: MB 804703

Colony diameters (mm) after 7 days at 28 °C: MEA, 11–17 (mean = 14.0, n = 9); YPG, 18 (mean = 17.8, n = 9); M40Y, 13–15 (mean = 14.0, n = 9); DG18, 10 (mean = 10.2, n = 9). The isolate produces cream-coloured yeast colonies on MEA (Fig. 1a), white hyphal colonies with a beige centre on YPG at 28 °C (Fig. 1b), cream-coloured yeast colonies with a yellow centre on M40Y (Fig. 1c) and olive-coloured yeast colonies on DG18 (Fig. 1d). Forms arthroconidia 7.0–13.0 × 2.5–4.5 μm (mean ± SE = 10.0 ± 0.4 × 3.4 ± 0.1 μm) (Fig. 1e), cells that are hyaline and oval, 5.5–12.0 × 3.5–5.5 μm (mean ± SE = 8.3 ± 0.4 × 4.7 ± 0.1 μm) (Fig. 1f) and didymosporae 10.0–17.5 × 4.5–7.5 μm (mean ± SE = 13.3 ± 0.4 × 5.8 ± 0.2 μm) (Fig. 1g). Oval cells reproduce by monopolar budding and sometimes produce ballistoconidia 3.5–7.5 × 3.0–7.0 μm (mean ± SE = 5.1 ± 0.3 × 4.7 ± 0.3 μm) attached by short sterigmata (Fig. 1h) that may be forcibly ejected leaving a scar on the mother oval cell (Fig. 1k). Basidia are 7.0–14.0 × 3.0–6.0 μm (mean ± SE = 8.7 ± 0.4 × 4.8 ± 0.1 μm) (Fig. 1n–r), with one or sometimes more than one basal lateral projection (Fig. 1n–p), and are seen with one basidiospore attached by a long sterigma. Basidiospores are globose, orange to brown, verrucose (Fig. 1q) and 2.0–6.0 μm in diameter (mean ± SE = 3.8 ± 0.2 μm). Basidia lose their cytoplasm and collapse as the attached basidiospore matures (Fig. 1r). Pseudohyphae (Fig. 1s) and septate hyphae (Fig. 1t) are also observed. No clamp connections are seen. Growth is observed in vitamin-free medium, in 0.1 and 0.01 % cycloheximide, in 5 and 10 % NaCl, and in 50 and 60 % glucose. Growth is positive at 28, 30 and 35 °C, but negative at 37 and 40 °C in YEP medium. No growth is recorded in 16 % NaCl or in the presence of 1 % acetic acid. Urease activity and diazonium blue B reaction are positive. Starch-like compounds are not produced. The major coenzyme Q is Q6. Laccase and DNase enzyme activity are detected. Negative enzyme activity for amylase, protease, lipase, lecinthinase, gelatinase, xylanase and cellulase. Positive for fermentation of sucrose and weakly positive for fermentation of D-glucose, maltose, melezitose, trehalose and raffinose. D-Galactose, D-arabinose, cellobiose, myoinositol, D-xyllose, D-ribose, L-arabinose, D-rhamnose, D-arabinitol, L-arabinitol, lactose, succinate, citrate, DL-lactate, gluconic acid, starch, inulin, D-mannitol, salicin, methanol, ethanol and n-hexadecane are not fermented. Positive result in tests for assimilation of D-glucose, sucrose, maltose, melezitose, trehalose, raffinose, inulin, ethanol, D-ribose (weak), D-xyllose (weak), cellobiose, citrate, gluconic acid, mannitol, D-arabinitol, L-arabinose (weak) and arbutin, but negative result for assimilation of D-galactose, D-arabinose, L-rhamnose, lactose, myo-inositol, starch, succinate, DL-lactate, salicin, methanol and n-hexadecane. Ethylamine hydrochloride, creatine, L-lysine, cadaverine and potassium nitrate are assimilated, but glucosamine, sodium nitrite, creatinine and imidazole are not.

A dried holotype (IBRC P1010180\textsuperscript{T}) and an ex-type (IBRC M30078\textsuperscript{T}) culture were deposited at the Iranian Biological Resource Center, Tehran, Iran. The dried holotype was created from a culture of the ex-type grown on YPG dried under the biological safety cabinet for 4 days in January 2014. An isotype strain was deposited at the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, the Netherlands, as CBS 12808\textsuperscript{T}. The type strain was isolated from the soil of a kitchen garden in Shahryar city, Tehran province, Iran.


MycoBank number: MB28558

Basidia are produced singly or in clusters, and have granular cellular contents and one or more basal lateral projections. One sterigma, and in one species more than one sterigma, arises randomly over the apical two-thirds of the surface of the basidium. Only one mature basidiospore develops per basidium. Basidiospores are symmetrical on sterigma, hyaline or brown when mature. Basidia lose their cytoplasm and sometimes collapse as basidiospores mature. Arthroconidia are produced in two species and didymosporae are observed in one species. Species are xerotolerant and two species are heat-resistant. Analyses of SSU,
5.8S and LSU rRNA genes suggest a sister relationship to *Geminibasidium*.

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**References**


