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Full paper

A new species of *Pithoascus* and first report of this genus as endophyte associated with *Ferula ovina*

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ABSTRACT

A newly described species of *Pithoascus* from roots of *Ferula ovina* differs from other *Pithoascus* species by producing larger ascomata than all described species except *P. exsertus*. The shape of its ascospores is similar to that of *P. lunatus*, but differs from it by having an asexual state. This new species differs from *P. ater* by having a sexual state. Phylogenetic analyses based on concatenated ITS rDNA, LSU rDNA and partial translation elongation factor 1 alpha gene datasets also confirmed the generic placement in *Pithoascus* and showed its close phylogenetic relationships to *P. ater* and *P. lunatus*.

1. Introduction

The family Microascaceae (Ascomycota) was introduced by Malloch (1970) covering five genera. Today it has forty-one genera recorded in MycoBank (http://www.mycobank.org). Anamorphic forms were traditionally placed under the genus *Scopulariopsis* (Abbott, Lumley, & Sigler, 2002; Barron, Cain, & Gilman, 1961).

Pithoascus has been proposed by von Arx (1973a) for some species previously classified in *Microascus*; he removed species without germ pores from *Microascus* and placed them (*P. nidicola* Massee & Salmon, *P. stoveri* Arx, *P. intermedius* Emmons & Dodge, *P. exsertus* (Skou) Arx, *P. schumacheri* Hansen, *P. platysporus* Arx & Veenbaas-Rijks) in a separate genus *Pithoascus*. The genus can be recognized by the very slow growth of the colonies and by dark, thick-walled, glabrous ascomata, which may be ostiolate or non-ostiolate (von Arx, 1973b). No conidial states were known for *Pithoascus* species until von Arx (1978) introduced *Pithoascus langeronii* Arx. After that, it was determined that *P. schumacheri* and *P. intermedius* produced anamorphic states included in the annellidic genera *Scopulariopsis* and *Doratomyces* (Valmaseda, Martinez, & Barrasa, 1987). *Pithoascus langeronii* was later transferred to the genus *Eremomyces* (Eremomycetaceae, Dothideomycetes) by Malloch and Sigler (1988) and more recently to *Arthrographis*, being renamed as *Arthrographis arxii* Guarro, Giraldo, Gené & Cano (Giraldo et al., 2014).

Benny and Kimbrough (1980) proposed the family Pithoascaceae to include the genera *Pithoascus* and *Faurelina*, distinguishing it from the Microascaceae by the presence of fusiform to navicular ascospores without germ pores and by anamorphs, when present, being arthroconidial. However, this proposal was rejected by Valmaseda et al. (1987).

Since morphology alone was not sufficient to establish species, several studies were conducted employing DNA sequences. The first phylogenetic study of *Scopulariopsis*-like species was based on LSU rDNA to assess potential relationships between asexual and sexual species (Issakainen et al., 2003). Recently, Sandoval-Denis et al. (2016) used a polyphasic approach based on the evaluation of molecular, physiological and morphological data in order to

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Table 1
Strains of *Pithoascus* and related fungi with their sources and GenBank accession numbers used for phylogenetic analyses. Strain characterized in this study and its newly generated sequences are highlighted in bold.

Isolate	Strain[a]	Country	Host	Accession number		
				ITS	LSU	TEF1
Cephalotrichum asperulum	CBS 582.71^{IT}	Argentina	Soil	KX923818	KX924027	KX924043
Microascus atrogriseus	CBS 295.52^{HT}	UK	Culture contaminant	LM652433	KX924030	KX924056
Microascus cirrosus	CBS 217.31^{HT}	Italy	Leaf of *Prunus* sp.	KX923838	KX924032	KX924064
Microascus croci	CBS 158.44^{HT}	Netherlands	*Crocus* sp.	KX923852	LM652508	KX924077
Microascus fusisporus	CBS 896.68^{HT}	Germany	Wheat-field soil	LM652432	LN850825	HG380372
Microascus gracilis	CBS 369.70^{NT}	Japan	Wheat flour	KX923861	HG380467	KX924086
Microascus longicollis	CBS 752.97	Brazil	Nut of *Anacardium occidentale*	KX923874	KX924035	KX924097
Microascus macrosporus	CBS 662.71	USA	Soil	LM652423	LM652517	LM652568
Microascus melanosporus	CBS 272.60^{HT}	USA	Milled *Oryza sativa*	KX923876	KX924036	LM652572
Microascus pyramidus	CBS 212.65^{HT}	USA	Desert soil	KX923925	KX924150	HG380435
Microascus senegalensis	CBS 277.74^{HT}	Senegal	Mangrove soil	KX923929	LM652523	KX924153
Microascus terreus	CBS 601.67^{HT}	Ukraine	Soil	LN850783	LN850832	LN850928
Pithoascus ater	CBS 400.34^{HT}		Human nail	MH855584	MH867092	LM652576
Pithoascus exsertus	CBS 819.70^{HT}	Denmark	*Megachile willoughbiella*,	LM652449	MH871757	LM652578
Pithoascus intermedius	CBS 217.32^{HT}	USA	Root of *Fragaria vesca*	LM652450	AF400872	LM652579
Pithoascus lunatus	CBS 103.85^{HT}	Germany	Skin showing Tinea plantaris	LN850784	LN850833	LN850929
Pithoascus nidicola	CBS 197.61^{HT}	USA	*Dipodomys merriami*	MH858021	MH869584	LM652451
Pithoascus persicus	**IRAN 3309C**	Iran	Root of *Ferula ovina*	**MF186873**	**MH400206**	**MK430530**
Pithoascus stoveri	CBS 176.71^{HT}	USA	Root of *Beta vulgaris*	NR132955	MH871835	KX924174
Pseudoscopulariopsis schumacheri	CBS 435.86^{NT}	Spain	Soil	KX923953	AF400874	LM652583

[a]Isotype, holotype, and neotype strains are indicated with IT, HT, and NT, respectively.

investigate taxonomic circumscription of this group. Their results showed that *Microascus* and *Scopulariopsis* constitute two phylogenetically distant lineages, which are clearly different from *Pithoascus*. The genus *Pithoascus* was reinstated and *P. schumacheri* was transferred to a new genus, *Pseudoscopulariopsis*. *Scopulariopsis atra* was also classified as *Pithoascus ater* (Zach) Sand.-Den., Cano & Guarro. *Pithoascus lunatus* Jagielski, Sand.-Den., Skóra & Gené, a new species from clinical samples, was introduced and described (Jagielski et al., 2016).

The aim of present study was to describe a new endophytic *Pithoascus* species from *Ferula ovina* Bioss (Apiaceae).

2. Materials and methods

2.1. Sample collection, fungal isolation and morphological identification

Ferula ovina samples were collected in May 2016 from the Zoshk highlands of Khorasan Razavi province, Iran (36°26′12.0″N 59°11′51.6″E).

Isolation of endophytic fungi followed Hallmann, Berg, and Schulz (2007) with minor modifications. Fresh and disease-free root samples were washed with running tap water and allowed to dry. Then, they were cut into pieces of 0.5–1 cm. Root pieces were placed in ethanol 75% for 1 min and in 1–4% sodium hypochlorite solution for 3 min (depending on the thickness of the tissue) and then 75% ethanol for 30 s, respectively. The samples were washed in distilled water after sterilization and placed on filter paper in sterile conditions for drying. After drying, the root parts were placed onto potato dextrose agar (PDA; Merck, Darmstadt, Germany) and malt extract agar (MEA; Merck) containing streptomycin (20 μg/mL) and chloramphenicol (30 μg/mL) and incubated at 25–30 °C for 7–14 d. A daily survey was conducted to ensure the absence of saprophytic contamination. Hyphal tips of fungi emerging out of the root tissues were grown on PDA, oatmeal agar (OA; 30 g boiled and filtered oat flakes, 15 g agar, 1 L distilled water), and potato carrot agar (PCA; 40 g of each boiled and filtered carrots and potatoes, 15 g agar, 1 L distilled water).

Dried specimens were preserved in the Fungarium of the Iranian Research Institute of Plant Protection, Tehran, Iran (IRAN).

For morphological identification, microscopic slides of the fungal isolate were prepared by staining with lactophenol cotton-blue (Vainio, Korhonen, & Hantula, 1998) and examined under a light microscope (BX43, Olympus, Tokyo, Japan). Primary identification of the genus was done using Ellis (1971) and species identification was performed according to keys provided in von Arx (1973b). Morphological characters were also compared with additional species of *Pithoascus* (Jagielski et al., 2016; Sandoval-Denis et al., 2016).

For field emission scanning electron microscopy (FESEM) micrographs, briefly, small pieces of fungal isolates, including mycelia, conidiophores and conidia, were placed on PELCO image tabs™ double-sided carbon adhesive discs (Ted Pella Inc., Redding, CA, USA), and coated with gold in a Q150R ES sputter coater (Quorum Technologies Ltd, East Sussex, United Kingdom) as previously described by Afanou et al. (2014). Samples were analyzed using FESEM (TESCAN BRNO-Mira3 LMU, 2014; Brno, Czech Republic) in the secondary electron imaging (SE) mode. The microscope was operated at 10 kV acceleration voltage, 1.8 kV extraction voltage and a working distance of 4.45 mm.

2.2. Genomic DNA extraction and PCR amplification

Genomic DNA of the fungal endophytes was isolated using DenaZist Asia fungal DNA isolation kit according to manufacturer's instructions. The DNA samples were stored at 4 °C for immediate use and stored at −20 °C for long-term storage. The rDNA-ITS region was amplified using the primers ITS5 and ITS4 (White, Bruns, Lee, & Taylor, 1990). Amplicon master mix was used to enhance amplification accuracy in a total reaction volume of 25 μL. PCR was performed in a Bio-Rad MyCycler™ Thermal Cycler (Hercules, California, USA) with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 20 s, and 72 °C for 30 s, with a final extension step of 72 °C for 10 min.

The primers LR0R and LR5 were used to amplify the LSU gene (Vilgalys & Hester, 1990). The partial translation elongation factor 1 alpha gene (TEF1) was amplified using the primers EF1-983F and Efgr (Rehner & Buckley, 2005). Primers melting temperature ™ were determined 53 °C and 62 °C for LSU and TEF1, respectively. The amplified regions were analyzed in 1.5% agarose gel electrophoresis in 1 × Tris-Boric acid-EDTA buffer (TBE) with a marker

ladder of 100-bp. PCR products were sent to Macrogen (Seoul, Korea) for sequencing. The obtained sequences were then analyzed using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and closely similar sequences were obtained from the National Centre of Biological Information (NCBI) database.

2.3. Phylogenetic analyses

Twenty-one reference strains of *Pithoascus* and *Microascus* including our isolate and *Cephalotrichum asperulum* (J.E. Wright & S. Marchand) Sand.-Den., Guarro & Gené and *Pseudoscopulariopsis schumacheri Pseudoscopulariopsis schumacheri* (E.C. Hansen) Sand.-Den., Gené & Guarro, as outgroup, were chosen for phylogenetic analyses. The strains and their GenBank (https://www.ncbi.nlm.nih.gov/) accession numbers are listed in Table 1.

Multiple sequence alignments made in MAFTT version 7 (https://mafft.cbrc.jp/alignment/server/). MrModeltest 2.3 (Nylander, 2004) was used to determine the best substitution models for each locus. MrBayes settings for the best-fit model (GTR + I + G), (rates = invgamma), (nst = 6) selected by AIC for the ITS and TEF1 partial gene, there (GTR + I), (rates = propinv), (nst = 6) selected by AIC for the LSU in MrModeltest. The alignments were submitted to TreeBASE (submission ID: S25569). For the multi-locus analysis, a phylogenetic analysis using a Markov chain Monte Carlo (MCMC) algorithm was followed with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). For the Bayesian

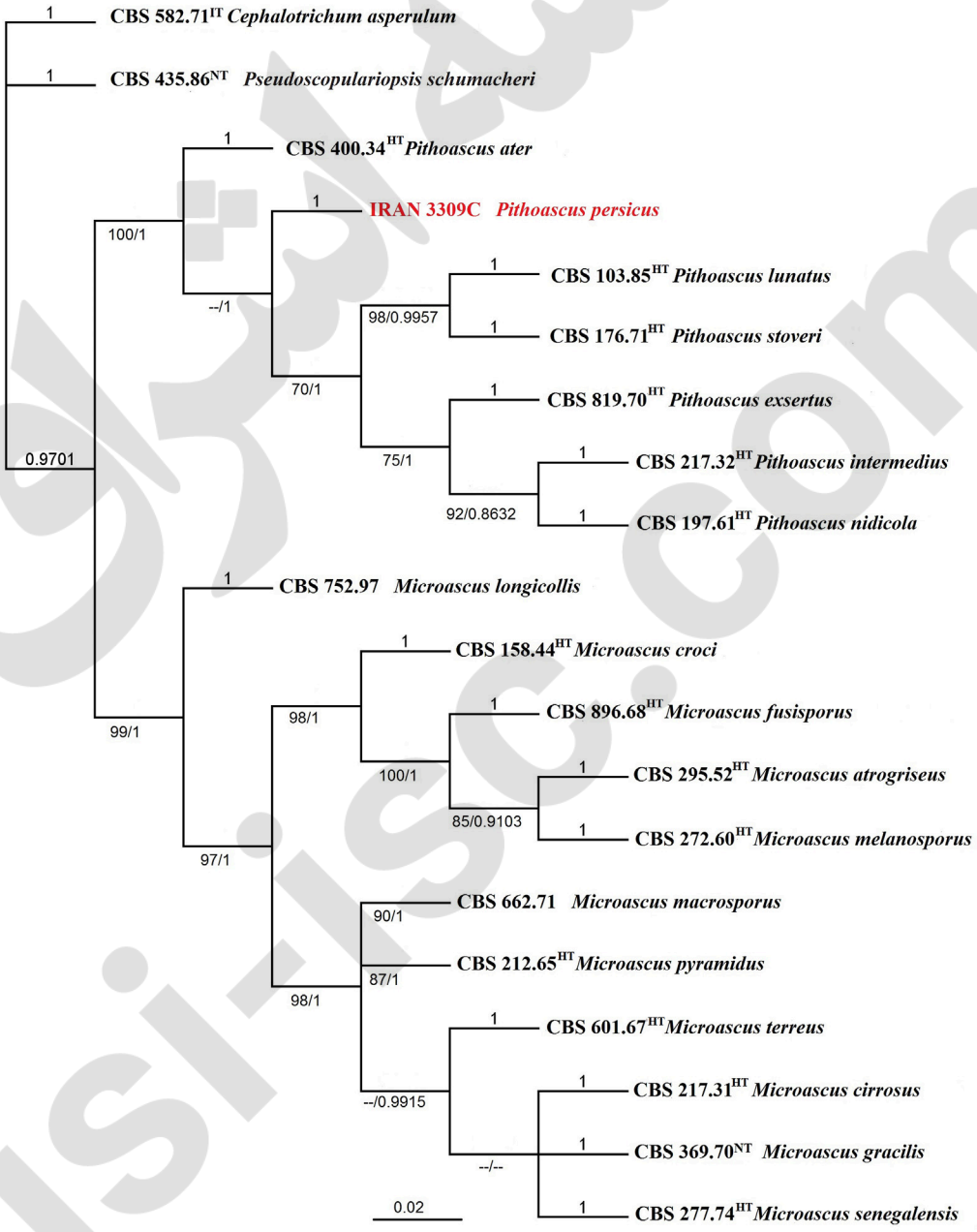

Fig. 1. Consensus phylogram resulting from a Bayesian inference analysis of the combined three-loci (ITS, LSU and TEF1) sequence alignment showing the phylogenetic position of the new species among the other *Pithoascus* species and some terricolous *Microascus* species. Numbers on the nodes are ML bootstrap values above 70% and BI posterior probabilities above 0.95. The scale bar indicates 0.02 expected changes per site. The tree was rooted to *Cephalotrichum asperulum* (CBS 582.71) and *Pseudoscopulariopsis schumacheri* (CBS 435.86). Isotype, holotype, and neotype strains are indicated with IT, HT, and NT, respectively.

approach with MrBayes, two parallel runs of 100,000,000 generations were conducted with a sampling frequency of 1000 trees. Tree reconstruction and visualization were produced with Geneious 5.1.7 (https://www.geneious.com). Distance matrix between *Pithoascus* species estimated by Geneious using a concatenated dataset including ribosomal gene regions (ITS and LSU) and partial TEF1 gene. Phylogenetic reconstructions by maximum likelihood (ML) with Tamura–Nei and G + I as the best model with 1000 bootstrap replicates were carried out using MEGA v. 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

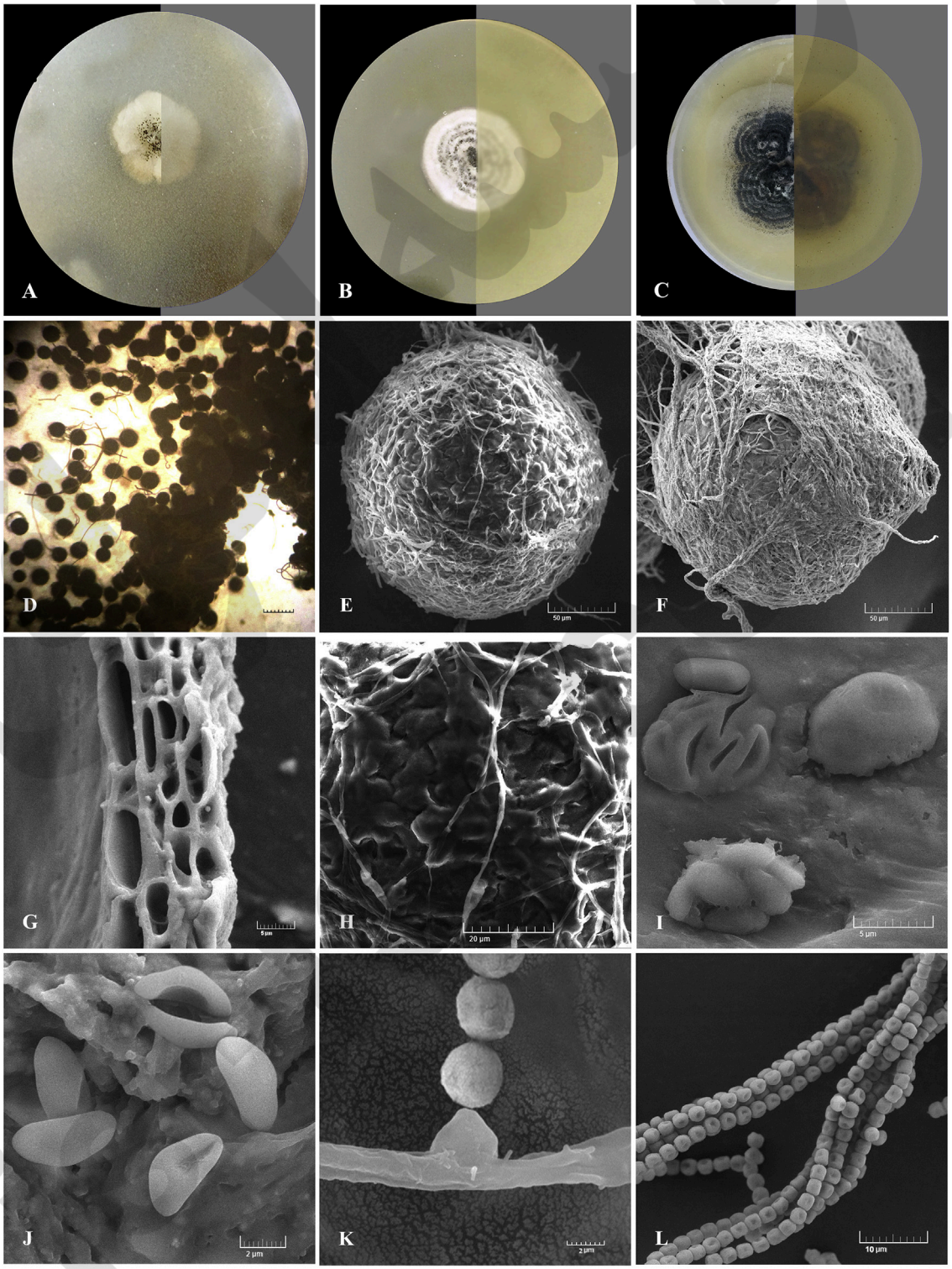

Fig. 2. *Pithoascus persicus* (IRAN 3309C). A: Colony morphology after 14 d at 25 °C on OA (surface and reverse). B, C: Colony on PCA (14 and 30 d). D: Accumulation of ascomata on OA. FESEM showing details of E, F: non–ostiolate and ostiolate ascomata. G: Wall of ascoma. H: Peridium. I: Asci. J: Ascospores. K: Conidiogenous cell and conidia. L: Group of conidia. *Bars*: D 500 µm; E, F 50 µm; H 20 µm; L 10 µm; G, I 5 µm; J, K 2 µm.

Table 2
Distance matrix between *Pithoascus* species estimated by Geneious using a concatenated dataset including ribosomal gene regions (ITS and LSU) and partial TEF1 gene.

	P. persicus	*P. ater*	*P. stoveri*	*P. lunatus*	*P. intermedius*	*P. nidicola*	*P. exsertus*
P. persicus	0.0000	0.0508	0.0528	0.0561	0.0844	0.0876	0.0978
P. ater	0.0508	0.0000	0.0407	0.0440	0.0723	0.0755	0.0857
P. stoveri	0.0528	0.0407	0.0000	0.0210	0.0620	0.0652	0.0754
P. lunatus	0.0561	0.0440	0.0210	0.0000	0.0653	0.0685	0.0787
P. intermedius	0.0844	0.0723	0.0620	0.0653	0.0000	0.0488	0.0752
P. nidicola	0.0876	0.0755	0.0652	0.0685	0.0488	0.0000	0.0784
P. exsertus	0.0978	0.0857	0.0754	0.0787	0.0752	0.0784	0.0000

3. Results

3.1. Phylogenetic analyses

The BLAST search of the ITS sequence showed 96.73% similarity with the type species *P. stoveri* strain CBS 176.71 and 96.22% similarity with *P. ater* strain CBS 400.34. The LSU region showed 98.36% similarity with *P. stoveri* strain CBS 176.71 and 98.11% with *P. ater* strain CBS 400.34. When we compared TEF1, our isolate showed 95% similarity with both of these species. In the multi-locus phylogenetic analyses, the new species was placed in a clade which includes *P. ater*, *P. exsertus*, *P. intermedius*, *P. nidicola*, *P. lunatus* and the type species *P. stoveri* (see Fig. 1).

3.2. Taxonomy

Pithoascus persicus, Tazik & Rahnama, sp. nov. Fig. 2.
MycoBank no.: MB 831300.

Diagnosis: *Pithoascus persicus* differs from other *Pithoascus* species by producing larger ascomata than all described species except *P. exsertus*. *Pithoascus persicus* also has the thickest peridium. The shape of the ascospores is similar to that of *P. lunatus*, but *P. persicus* differs from it by having an asexual state. *Pithoascus persicus* differs from *P. ater* by having a sexual state.

Etymology: Referring to Persia.

Holotype: IRAN, Khorasan Razavi province, Zoshk highlands (36°26′12.0″N 59°11′51.6″E), on root of *Ferula ovina*, May 26, 2016, (Holotype IRAN 17623F; ex-type strain, AT01 (IRAN 3309C)). GenBank accession numbers: MF186873 (ITS), MH400206 (LSU), MK430530 (TEF1).

Colonies on OA, PCA and MEA attaining a diameter of 12, 21 and 23 mm, respectively after 2 wk at 25 °C in darkness. Colonies creamy white, somewhat velvety. Mycelium septate, hyaline, smooth-walled hyphae 1–1.5 μm wide. Dark ascomata abundantly seen on the surface of two wk-old colonies, on PCA in concentric circles (Fig. 2B and C). Ascomata often forming dense crusts which ripen within 5–7 wk, dark brown to black, glabrous, with a diameter of 160–240 μm, non–ostiolate, rarely ostiolate with an ostiolar neck up to 15–20 μm long and 25 μm wide, peridium with a textura angularis, 10–12 μm wide. Asci thin-walled ellipsoidal, 8.5–9 × 6–7 μm. Ascospores abundant, one-celled, flat, boat-shaped, nearly lunate, yellowish, smooth-walled and without germ pores, 4–6.5 × 2–2.5 μm. Asexual morph observed in 4–mo–old cultures on MEA when gray masses of conidia filled the agar surface. Conidiogenous cells borne singly and laterally on the vegetative hyphae, annellidic, short, ampulliform, hyaline, and smooth-walled. Conidia 1–celled, globose, 2.5 μm in diameter and smooth.

4. Discussion

Various studies have been conducted to determine the best molecular markers for phylogenetic relationships of Microascaceae over the last decade. Using LSU sequences as a marker provided an initial insight into the generic relationships of *Scopulariopsis* species and allied fungi. A study of clinical isolates in Poland confirmed that the LSU sequence alone is insufficient for species delimitation in *Scopulariopsis* (Jagielski, Kosim, Skora, Macura, & Bielecki, 2013).

In taxonomic studies using the beta-tubulin gene (TUB2), TEF1 and LSU to identify *Scopulariopsis* species (Ropars, Cruaud, Lacoste, & Dupont, 2012), TEF1proved to be the most phylogenetically informative genomic region and was proposed for identifying *Scopulariopsis* species (Jagielski et al., 2016; Sandoval-Denis et al., 2013).

In this study, a combination of ITS, LSU and TEF1 data confirmed that our isolate belongs to *Pithoascus*. According to the latest taxonomic changes in Microascaceae, *Pithoascus* includes six valid species. Our isolate showed significant phylogenetic distance from the other species of the genus (i.e., *P. ater*, *P. exsertus*, *P. intermedious*, *P. lunatus*, *P. nidicola*, and *P. stoveri*) (Table 2).

The morphology of ascospores, asci, ascomata and the cultural characteristics of our isolate also matched the description of *Pithoascus* species. *Pithoascus persicus* differs morphologically from *P. ater*, the closest species phylogenetically, in having a sexual morph and differs from *P. lunatus* in having an asexual morph. Its ascomata are larger (160–240 μm) than both *P. lunatus* and *P. stoveri*. *P. stoveri* produces ascomata measuring 50–110 μm diam (von Arx, 1973b; Abbott et al., 2002) and *P. lunatus* ascomata are 111–143 μm diam (Jagielski et al., 2016).

In terms of asexual morph, *P. persicus* is one of the species that produces conidia in old cultures. Its conidia are the smallest among all *Pithoascus* species (2.5 μm diam).

Conidia of *P. stoveri* and *P. intermedius* are rarely seen in culture and, when present, are hyaline, obovate to pyriform (5–8 × 3–4 μm) or globose to subglobose (4–8 × 4.5–7.5 μm), respectively. *Pithoascus ater* is the only species of the genus for which a sexual morph is unknown and by contrast shows abundant conidial production (4–9 × 4.5–8.5 μm). Other species, i.e. *P. nidicola*, *P. exsertus* and *P. lunatus* produce only sexual morphs in culture (Jagielski et al., 2016; Sandoval-Denis et al., 2016).

Pithoascus species have various habitats and are isolated from plants, soil, human skin and nails. *Pithoascus stoveri* and *P. intermedius* have previously been isolated from the root of plants (*Beta vulgaris* L. and *Fragaria vesca* L.) (von Arx, 1973b), but this is the first report of this genus as an endophyte associated with a member of Apiaceae (*F. ovina*).

Declaration of competing interest

The authors declare no conflict of interest.

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