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 root of *Ferula ovina* differs from other *Pithoascus* species by producing larger ascomata than all described species except *P. exsertus*. The shape of the ascospores is similar to that of *P. lunatus*, but larger in length and width. It differ from *P. ater* by having a sexual state. Phylogenetic analyses based on concatenated ITS rDNA, LSU rDNA and partial EF1-α gene datasets also confirmed the generic placement in *Pithoascus* and showed its close phylogenetic relationships to *P. ater* and *P. lunatus*. *P. stoveri* and *P. intermedius* have already been isolated from the roots of plants (*Beta vulgaris* and *Fragaria vesca*) but this is first report of the genus as an endophyte associated with roots of a medicinal plant.

Keywords: Microascaceae, Fungal endophyte, Multi-locus Phylogeny, *Pithoascus persica*

1. Introduction

Microascaceae (Ascomycota) was a family introduced by Malloch in 1970 to include five genera [1]. Today it has forty-one genera recorded in Mycobank (http://www.mycobank.org). Anamorphic forms were traditionally placed under the genus *Scopulariopsis* [2, 3].

Pithoascus has been proposed by von Arx for some species previously classified in *Microascus* [4]; he removed species without germ pores from *Microascus* and placed them (*P. nidicola*, *P. stoveri*, *P. intermedius*, *P. exsertus, P. schumacheri*, *P. platysporus*) in a separate genus *Pithoascus*. The genus can be recognized by the very slow growth of the colonies and by glabrous ascomata, which may be ostiolate or non-ostiolate. No conidial states are known in *Pithoascus* species [5]. *Pithoascus langeronii* was the first species of the genus with an anamorph and could also be distinguished by its small and only slightly pigmented ascospores and the dark ascomata wall which is nearly amorphous with age [6]; its anamorph described as hyphomycete *Arthrographis langeroni* [7]. After that, it was determined that *P. schumacheri*, *P. intermedius*, and *P. stysanophorus* produce anamorphic states, included in the annellidic form-genera *Scopulariopsis* and *Doratomyces* [8].

Benny and Kimbrough [9] proposed the family Pithoascaceae to include the genera *Pithoascus* and *Faurelina* Locquin-Linard, distinguishing it from the Microascaceae Luttrell ex Malloch by the presence of fusiform to navicular ascospores without germ pores and by anamorphs, when present, arthroconidial. But this proposal was rejected by Valmaseda et al [8].

So far, the taxonomy of this genus, based on morphological characteristics, remained incomplete. The first phylogenetic study of *scopulariopsis*-like species was based on the large subunit 28S rDNA, which assessed potential relationship between asexual and sexually reproducing species [10]. Sandoval et al. used a polyphasic approach based on the evaluation of molecular, physiological and morphological data in order to 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 renamed as *Pithoascus ater* [11]. *P. lunatus*, new species from clinical samples, was introduced and described [12].

Since comprehensive taxonomic studies have not been carried out on Microascaceae, and there is no comprehensive and focused reference to the taxonomy of this group of fungi, in this study we tried to review the taxonomy of this genus, and introduce a new species.

2. Materials and methods

2.1. Sample collection and fungal isolation

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

Isolation of endophytic fungi was fallowed according to the method described by Hallmann et al. [13] with minor modifications. Fresh and disease-free root samples were washed with running tap water and dried. Then, cut into pieces of 0.5-1 cm. Root pieces were placed in ethanol 75% for 1 minute and in sodium hypochlorite solution 1-4% for 3 minutes (depending on the thickness of the tissue) and then 75% ethanol for 30 seconds, respectively. The samples were washed in distilled water after sterilization and were placed on filter paper in sterile conditions for drying. After drying, the root parts were placed in Potato Dextrose Agar (PDA) and malt extract agar (MEA) media containing streptomycin (20 µg / ml) and chloramphenicol (30 µg / ml) and incubated at 25-30° C for 7-14 days. A daily survey was conducted to ensure the absence of saprophytic contamination. Hyphal tips of fungi, emerging out of the root tissues, were picked and grown on potato dextrose agar in pure culture.

For morphological identification, microscopic slides of fungal isolate were prepared by staining with lactophenol cotton-blue [14] and were examined under light microscope (Olympus, USA). Primary identification of the genus was done using Ellis key for Dematiaceous Hyphomycetes [15] and species identification was performed according to key provided by Von Arx [5]. Morphological characters were also compared with other 4 new reported species of *Pithoascus* which were not available in the Von Arx key.

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^{o}C for immediate use and stored at -20^{o}C for long-term storage. Fungal rDNA-ITS region was amplified using the fungal domain specific ITS5 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS4 (5′-TCC GTA GGT GAA CCT GCG G-3′) [16]. 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^{TM} Thermal Cycler (Hercules, California, USA) with an initial denaturation step at 94^{o}C for 5 min, followed by 30 cycles of 94^{o}C for 30 sec, 58^{o}C for 20 sec, and 72^{o}C for 30 sec, with a final extension step of 72^{o}C for 10 min.

LROR (5′-ACC CGC TGA ACT TAA GC-3′) and LR5 (5′-TCC TGA GGG AAA CTT CG-3′) were used to amplify nuclear LSU region [17]. The gene fragment translation elongation factor (TEF1) were amplified using EF1-983F (5′-GCY CCY GGH CAY CGT GAY TTY AT-3′) and Efgr (5′-GCA ATG TGG GCR GTR TGR CAR TC-3′) primers [18]. The amplified regions were analysed in 1.5% agarose gel electrophoresis in 1X Tris-Boric acid-EDTA buffer (TBE) with a marker ladder of 100-bp. PCR products were sent to Macrogen Korea for sequencing. The obtained sequences were then analyzed using the BLAST

algorithm and closely related phylogenetic sequences obtained from the National Centre of Biological Information (NCBI) database.

2.3. Phylogenetic analysis

Twenty-one reference strains of *Pithoascus* and *Microascus* including our isolate and *Cephalotrichum asperulum*, as outgroup, were chosen for phylogenetic analyses. List of the strains and their GenBank accession numbers is available in Table 1.

Multiple sequence alignments were made in MAFTT version 7 (https://mafft.cbrc.jp/alignment/server/). MrModeltest 2.3 [19] 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 ITS and ef1-α partial gene, there (GTR+I), (rates= propinv), (nst=6) selected by AIC for LSU regions in MrModeltest. For the multi-locus analysis, a phylogenetic analysis using a Markov chain Monte Carlo (MCMC) algorithm was followed with MrBayes version 3.1.2 [20]. For the Bayesian approach with MrBayes, two parallel runs of 100,000,000 generations were conducted with a sampling frequency of 1,000 trees. Tree reconstruction and visualization were produced with Geneious 5.1.7 [21].

3. Results:

3.1. Taxonomy

Pithoascus persica, Tazik Z. & Rahnama K. *sp. nov.* (Fig 2)

MycoBank: MB 831300

Etymology: Name refers to the old name of country (Persia) where the fungus was collected.

Type: Iran, Khorasan Razavi province, Zoshk highlands (36°26′12.0″N 59°11′51.6″E), from root of *Ferula ovina*, May 2016, IRAN 3309C (Holotype in WDCM 939). NCBI accession numbers: MF186873 (ITS), MH400206 (nLSU), MK430530 (EF1α).

Description was written based on CBS 119644 at 25 °C after 2 weeks in darkness. Colonies on OA, PCA and MEA attaining a diameter of 12, 21 and 23 mm, respectively after 2 weeks. Colonies were creamy white, somewhat velvety and with very slow-growth. Dark ascocarps were abundantly seen on the colony surface of two week-old colonies. Ascocarps formed concentric circles on PCA medium (Fig 2- C,D). Mycelium with septate, hyaline, smooth-walled hyphae 1 to1.5 µm wide. Ascomata often formed dense crusts, dark brown to black, glabrous, non-ostiolate with a diameter of 200-260 µm, peridium with a textura angularis and asci were thin-walled. Single-celled ascospores were abundant, and were flat, boat-shaped, nearly lunate, yellowish, 6.5-8 × 3.5-3.8 µm, smooth-walled and without germ pores. The asexual morph was observed in 4-month-old cultures when gray masses of conidia filled the petri dish containing MEA medium. Conidiogenous cells were annellidic, borne singly and laterally on the vegetative hyphae, short, ampulliform, hyaline, and smooth-walled. Conidia were 1-celled, globose, 4 -5 µm in diameter and smooth.

3.2. Phylogenetic analyses

The blast search of ITS regions showed 96.73% similarity with type species *P. stoveri* and 96.22% similarity with *P. ater*. The LSU region had 98.36% similarity with *P.stoveri* and 98.11% with *P. ater.* When we compared partial sequence of protein coding gene EF1-α, our isolate showed 95% similarity with both of these species. In multi-locus phylogenetic analysis, the new species placed as a sister species between these two clades bearing *P. ater*, *P.stoveri* and *P. lunatus.*

4. Discussion:

Various studies have been conducted to determine the best molecular markers for phylogenetic relationships of Microascaceae, over the last decade. Using LSU rDNA sequences as a marker, provided an initial insight into the genetic composition 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* [22].

A taxonomic study of cheese fungi used the beta-tubulin (tub2) and translation elongation factor 1-alpha (tef1) gene regions next to LSU to identify their *Scopulariopsis* species [23]. Tef1 showed to be the most phylogenetically informative genomic region and was proposed for identifying *Scopulariopsis* species [12, 24].

In this study, a combination of ITS rDNA, LSU rDNA and partial EF1-α gene data was used for phylogenetic analyses of relationships among *Pithoascus* species. Molecular phylogenetic analysis using these three regions confirmed that our isolate belongs to genus *Pithoascus*. According to the latest taxonomic changes in family Microascaceae, *Pithoascus* includes six valid species. This isolate showed significant phylogenetic distance from the other species of the genus (i.e., *Pithoascus ater*, *Pithoascus exsertus*, *Pithoascus intermedious*, *Pithoascus lunatus*, *Pithoascus nidicola*, and *Pithoascus stoveri*) (table 2).

The morphology of ascospores, asci, ascomata and the cultural characteristics of our isolate also matched the description of *Pithoascus* species. *Pithoascus persica* differs morphologically from *P. ater*, the closest species phylogenetically, in having sexual morph and differs from *P. lunaus* in having an asexual morph. Its ascomata and ascospores are larger than both *P. lunaus* and *P. stoveri.*

In terms of asexual morph, it is one of the species which produces conidia in old cultures.

Pithoascus ater is the only species of the genus for which a sexual morph is unknown and by contrast shows abundant conidial production. 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. Other species, i.e. *P. nidicola*, *P. exsertus* and *P. lunatus* produce only sexual morphs in culture [11, 12].

Pithoascus species have various habitats and are isolated from plants, soil, human skin and nails. *P. stoveri* and *P. intermedius* have previously been isolated from the root of plants (*Beta vulgaris* and *Fragaria vesca*) but this is the first report of this genus as an endophyte associated with a medicinal plant.

Based on morphological and molecular data described here, this endophytic isolate is introduced as a new taxon.

Table 1. Strains of *Pithoascus* and *Microascus* with their sources and GenBank accession numbers used for phylogenetic analysis.

Isolates	Strain numbers	Country	Host	Accession numbers		
				ITS	LSU	EF1α
Pithoascus persica	IRAN 3309C	Iran	Root of *Ferula ovina*	MF186873	MH400206	MK430530
Pithoascus stoveri	CBS 176.71	USA	*Beta vulgaris*, root	NR132955	MH871835	KX924174
Pithoascus ater	CBS 400.34		ex-type, human nail	MH855584	MH867092	LM652576
Pithoascus exsertus	CBS 819.70	Denmark	*Megachile willoughbiella*,	LM652449	MH871757	LM652578
Pithoascus intermedius	CBS 217.32	USA	Root of *Fragaria vesca*	LM652450	AF400872	LM652579
Pithoascus lunatus	CBS 103.85	Germany	Skin(*Tinea plantaris*)	LN850784	LN850833	LN850929
Pithoascus nidicola	CBS 197.61	USA	*Dipodomys merriami*	MH858021	MH869584	LM652451
Pithoascus platysporus	CBS 419.73	Netherlands	Agricultural soil	MH860726	MH872437	-
Pseudoscopulariopsis schumacheri	CBS 435.86	Spain	Soil, ex-neotype	KX923953	AF400874	LM652583
Microascus atrogriseus	CBS 295.52	UK	Culture contaminant	LM652433	KX924030	KX924056
Microascus cirrosus	CBS 217.31	Italy	*Prunus* sp., leaf	KX923838	KX924032	KX924064
Microascus croci	CBS 158.44	Netherlands	*Crocus* sp.	KX923852	LM652508	KX924077
Microascus fusisporus.	CBS 896.68	Germany	Wheat-field soil	LM652432	LN850825	HG380372
Microascus gracilis	CBS 369.70	Japan	Wheat flour	KX923861	HG380467	KX924086
Microascus longicollis	CBS 752.97	Brazil	*Anacardium occidentale*, nut	KX923874	KX924035	KX924097
Microascus macrosporus	CBS 662.71	USA	Soil	LM652423	LM652517	LM652568
Microascus melanosporus	CBS 272.60	USA	*Oryza sativa*, milled	KX923876	KX924036	LM652572
Microascus pyramidus	CBS 212.65	USA	Desert soil	KX923925	KX924150	HG380435
Microascus senegalensis	CBS 277.74	Senegal	Mangrove soil	KX923929	LM652523	KX924153
Microascus terreus	CBS 601.67	Ukraine	Soil	LN850783	LN850832	LN850928
Cephalotrichum asperulum	CBS 582.71	Argentina	Soil	KX923818	KX924027	KX924043

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

	AT01	*P.ater*	*P.stoveri*	*P.lunatus*	*P.intermedius*	*P.nidicola*	*P.exsertus*	*P.schumacheri*
AT01	0.0000	0.0508	0.0528	0.0561	0.0844	0.0876	0.0978	0.1263
P.ater	0.0508	0.0000	0.0407	0.0440	0.0723	0.0755	0.0857	0.1036
P.stoveri	0.0528	0.0407	0.0000	0.0210	0.0620	0.0652	0.0754	0.1163
P.lunatus	0.0561	0.0440	0.0210	0.0000	0.0653	0.0685	0.0787	0.1195
P.intermedius	0.0844	0.0723	0.0620	0.0653	0.0000	0.0488	0.0752	0.1478
P.nidicola	0.0876	0.0755	0.0652	0.0685	0.0488	0.0000	0.0784	0.1510
P.exsertus	0.0978	0.0857	0.0754	0.0787	0.0752	0.0784	0.0000	0.1612
Ps.schumacheri	0.1263	0.1036	0.1163	0.1195	0.1478	0.1510	0.1612	0.0000

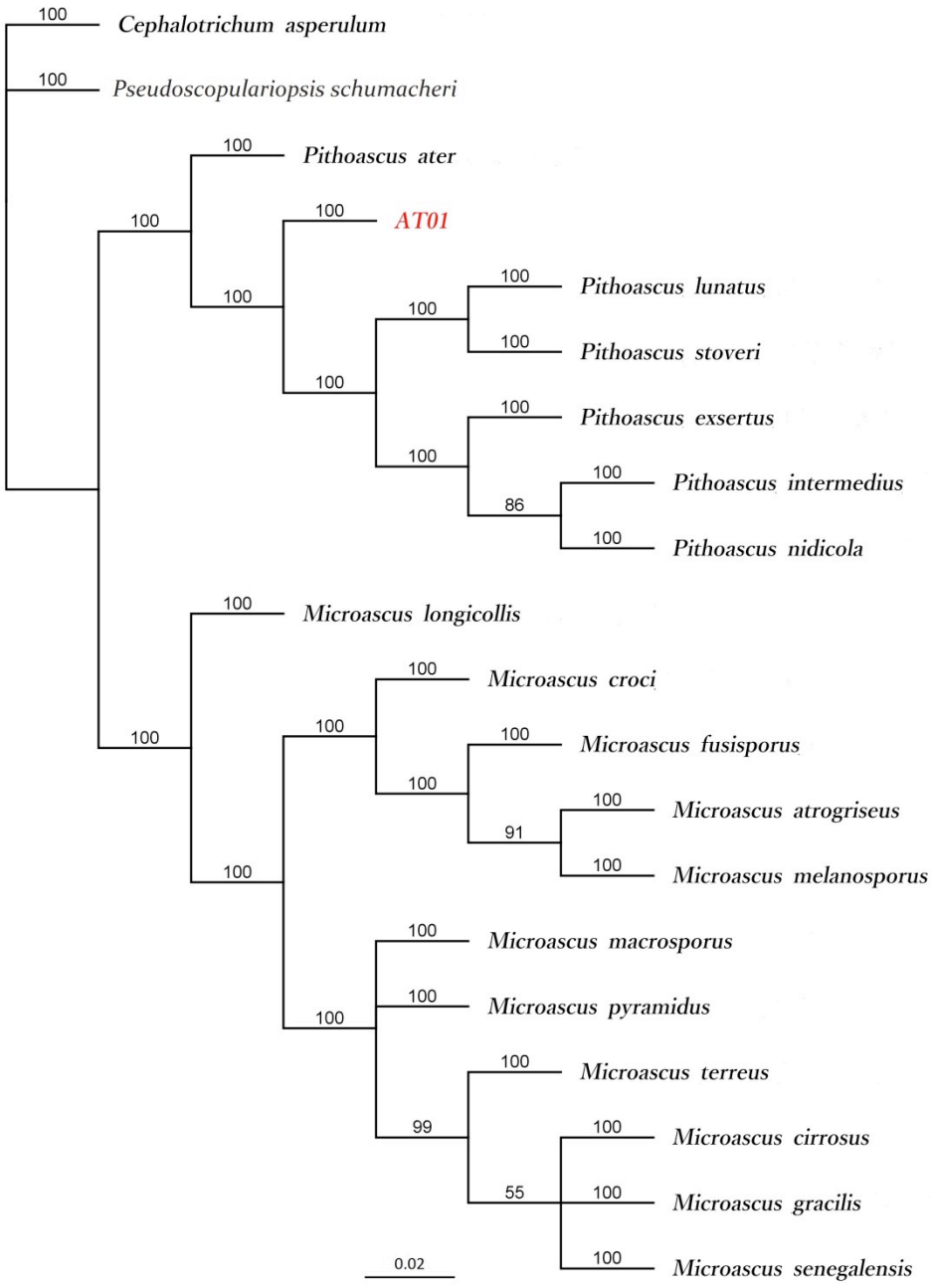

Figure 1. Consensus phylogram resulting from a Bayesian inference analysis of the combined three-loci (ITS, LSU and ef1-α) sequence alignment showing the phylogenetic position of the new species among the other *Pithoascus* species and some terricolous *Microascus* species. The scale bar indicates 0.02 expected changes per site. The tree was rooted to *Cephalotrichum asperulum*

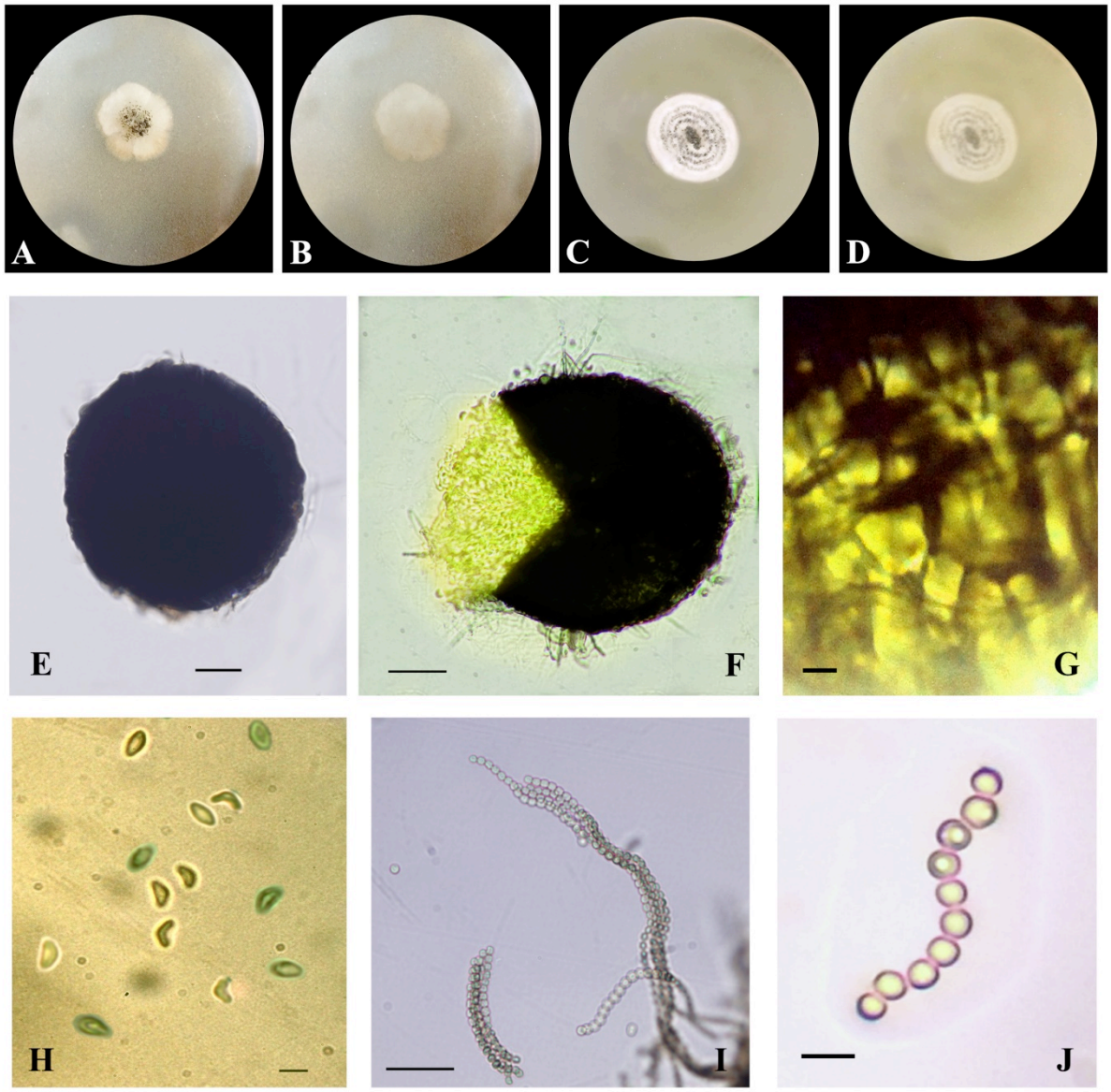

Figure 2. *Pithoascus persica* (IRAN 3309C). Colony morphology after 14 d at 25 °C on OA (A, B-reverse) and PCA (C, D - reverse). Ascomata (E, F). Detail of the peridium (G). Ascospores (H). Conidia (I, J). Scale bars: E, F, I = 50 µm; G,H,J = 10 µm

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Conflict of Interest

The authors declare no conflict of interest.

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