

Three *Penicillium* species new for the mycobiota of Iran from soils of the National Park of Urmia Lake

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Abstract

In a survey on the biodiversity of *Penicillium* species in soils of the National Park of Urmia Lake (NW Iran), eight *Penicillium* isolates were isolated as members of the section *Citrina*. Based on a combination of cultural and morphological criteria, the isolates were identified as *Penicillium anatolicum*, *P. sanguifluum*, and *P. sizovae*. The identity of each species was further confirmed using sequence data of *-tubulin* gene (*BenA*). Three species are reported and described here as new records for the mycobiota of Iran.

Keywords: Hypersaline soils, National Park of Urmia Lake, *Penicillium*, section *citrina*, taxonomy

معرفی سه گونه جدید پنیسیلیوم برای مایکوبیوتای ایران از خاک‌های پارک ملی دریاچه ارومیه

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خلاصه

در مطالعه تنوع زیستی گونه‌های جنس پنیسیلیوم در خاک‌های پارک ملی دریاچه ارومیه، هشت جدایه پنیسیلیوم متعلق به بخش سیتیرینا جداسازی شدند. براساس ترکیبی از ویژگی‌های ریخت‌شناختی ماکروسکوپی و میکروسکوپی سه گونه *Penicillium anatolicum*، *P. sanguifluum* و *P. sizovae* از بخش سیتیرینا شناسایی شدند. هویت گونه‌های شناسایی شده با استفاده از توالی ژن بتا-توبولین تأیید گردید. هر سه گونه به عنوان گونه‌های جدید برای مایکوبیوتای ایران گزارش و توصیف می‌شوند.

واژه‌های کلیدی: بخش سیتیرینا، پارک ملی دریاچه ارومیه، پنیسیلیوم، تاکسونومی، خاک‌های فوق شور

Introduction

Penicillium is a well-known cosmopolitan genus of molds which phylogenetically belongs to the family *Aspergillaceae* (Houbraken & Samson, 2011). There are currently more than 360 recognized species in this genus, divided among 25 sections based on multigene phylogenetic analysis (Houbraken & Samson 2011, Visagie *et al.* 2009, 2014, Park *et al.* 2015, Peterson *et al.* 2015). Species of this genus are economically, ecologically, and medically important microorganisms (Asan 2004) with respect to their positive impact as fermentation agents in food industry, production of antibiotics and novel enzymes as well as their negative impact as degraders of agricultural products, their toxicity and pathogenicity (Visagie *et al.* 2009, Houbraken & Samson 2011).

Penicillium is one of the most common fungi occurring in a diverse range of habitats, including soil, vegetation, air, indoor environments and various food products (Visagie *et al.* 2014). Members of this genus are soil common saprotrophs, which might constitute up to 67% of the total fungal biomass of certain soil habitats (Christensen *et al.* 2000, Visagie *et al.* 2009, Houbraken & Samson 2011). *Penicillium* species occur in diverse ecological niches ranging from normal to extreme environments as they are not very selective in reaction to abiotic growth conditions (Krijgsheld *et al.* 2013). These species can grow in environments with high salt concentration, low oxygen concentrations, high or low temperatures, alkalinity, low nutrient availability, solar radiation or the presence of heavy metals and other toxic compounds (Houbraken & Samson 2011, Cardoso *et al.* 2007). Studies on the occurrence of fungi in salterns have indicated that *Aspergillus* and *Penicillium* species are among the predominant genera in these environments (Cantrell *et al.* 2011, Samadi *et al.* 2013).

Taxonomy of *Penicillium* has proven troublesome since the description of the genus (Houbraken *et al.* 2011); such as, several taxonomic schemes have been proposed for species delineation within this genus (Frisvad & Samson 2004). Based on a recent multi-gene phylogenetic analysis, *Penicillium sensu stricto* is

divided into two subgenera and 25 sections (Houbraken *et al.* 2011). Section *Citrina* comprises 40 species, which exhibit ubiquitous distribution in soil; also isolated from foodstuffs, leaf litter and indoor environments (Houbraken *et al.* 2010, 2011, Visagie *et al.* 2014). Members of this section can grow at low water activities and in substrates containing NaCl (Houbraken *et al.* 2011). The species in the section *Citrina* are morphologically characterized from other sections by restricted growth on Czapek's agar, production of symmetrically biverticillate conidiophores, flask-shaped phialides (7.0–9.0 µm long) and relatively small conidia (2.0–3.0 µm in diameter) (Houbraken *et al.* 2011, Visagie *et al.* 2014). Cultural and morphological features such as colony diameter, presence or absence of cleistothecia/sclerotia, maximum growth temperature, shape, ornamentation and size of conidia are useful for species delimitation in the section *Citrina* (Houbraken *et al.* 2011).

The National Park of Urmia Lake (NW Iran) is a protected area and has a unique ecosystem, owing to variable ranges of soil salinity and pH. The aim of the present study was to explore the biodiversity of *Penicillium* section *Citrina* in the soils of the National Park of Urmia Lake using morphological and cultural data.

Materials and Methods

- Fungal isolates

A total of 46 soil samples were collected at 5–15 cm depth from different locations of the islands in National Park of Urmia Lake and coastal areas of Urmia Lake (NW Iran), during 2011–12. Isolations were subsequently made using the soil dilution plate and Warcup soil plate methods on MEA (malt extract agar) (Merck, Germany), GPY (glucose pepton yeast extract agar) and PDA (potato dextrose agar) culture media containing 0–30% NaCl (Nagamani *et al.* 2006). Pure cultures were established using a single spore technique (Crous *et al.* 2009).

- Morphological analysis

Morphological identification was carried out according to Ramirez (1982) and Houbraken *et al.* (2011). For macro-morphological observations, isolates were grown on Czapek yeast autolysate agar (CYA; Visagie *et al.* 2014) and MEA. The isolates were inoculated at three points on each plate and incubated at 25° C in the dark for seven days. Colony growth characteristics were recorded on 7-days-old colonies (Frisvad & Samson 2004). Colony colours were described using colour charts of Rayner (1970).

For micro-morphology, characteristic structures such as conidiophores, stipe, phialides and conidia were mounted in 85% lactic acid and examined using a light microscope (Olympus-BX41). Thirty measurements were made for each relevant character. Photographs were captured using an Olympus digital camera system (DP 25) mounted on the microscope. Cultures were deposited in to the culture collection of University of Tabriz (CCTU) and culture collection of Applied and Industrial Mycology department (DTO) at CBS-KNAW Fungal Biodiversity Centre, the Netherlands.

- Molecular identification

Genomic DNA was extracted as described in Arzanlou *et al.* (2016).

A part of *-tubulin* gene (*BenA*) was amplified using primer sets T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass & Donaldson 1995). PCR was carried out in a final volume of 12.5 µL containing 10–15 ng genomic DNA, 1.25 µL of 10X reaction buffer, 60 µL of 1 mM dNTPs, 1.5 mM MgCl₂, 0.2 pM of each primer, 0.5 µL DMSO, and 0.5 U Taq Polymerase. The reaction was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with cycling

conditions consisting of 5 min at 96° C for primary denaturation, followed by 40 cycles of denaturation at 94° C for 30s, annealing at 56° C for 30s, extension at 72° C for 60s, with a final extension at 72° C for 7 min. PCR products were sequenced using BigDye Terminator v 3.1 (Applied Biosystems, Foster City, CA) Cycle Sequencing Kit according to the recommendation of the vendor and analyzed on an ABI Prism 3700 (Applied Biosystems, Foster City, CA). Raw sequence files were edited manually using SeqMan™II (DNASTAR, Madison, Wisconsin, USA) and a consensus sequence was generated for each of the sequences. Sequences were subjected to Megablast search analysis at NCBI's GenBank nucleotide database for sequence similarity.

Results

Several *Penicillium* isolates were recovered from soils of the National Park of Urmia Lake, of those eight isolates were identified as members of the section *Citrina* based on cultural and morphological characteristics (Table 1). The isolates were identified as *P. anaticum*, *P. sanguifluum*, and *P. sizovae* based on the present literature (Houbraken *et al.* 2011). Sequence data of *BenA* gene for each of the species showed substantial homology to representative reference strains. DTO 203-D5=DTO 204-C2=DTO 204-B9=98.9% with CBS 127032 (type of *P. sanguifluum*), DTO 204-C1=DTO 204-C2=98.6% with *P. sanguifluum* CBS 127032, DTO 203-F2=100% identical with *P. sanguifluum* CBS 127032; DTO 203-G1=99.8% with *P. sizovae* CBS 41.69, and DTO 204-A5=99.8% with *P. anaticum* CBS 479.66. The sequences generated in this study were submitted in to GenBank (Table 1). The identified species are described alphabetically.

Table 1. List of *Penicillium* species belonging to section *Citrina* isolated from the National Park of Urmia Lake (NW Iran)

Taxon	CCTU* No.	Other collection numbers	Substrate	Location	Isolated by	Date of isolation	BenA
<i>Penicillium anaticum</i>	CCTU808	DTO **204-A5	Soil	Kabodan Island, Urmia	Ghosta & Samadi	2011	KY124549
<i>P. sanguifluum</i>	CCTU754	DTO 203-D5	Soil	Kabodan Island, Urmia	Ghosta & Samadi	2011	KY124543
<i>P. sanguifluum</i>	CCTU769	DTO 203-F2	Soil	Asper Island, Urmia	Ghosta & Samadi	2011	KY124544
<i>P. sanguifluum</i>	CCTU821	DTO 204-B9	Soil	Kabodan Island, Urmia	Ghosta & Samadi	2011	KY124545
<i>P. sanguifluum</i>	CCTU822	DTO 204-C1	Soil	Kabodan Island, Urmia	Ghosta & Samadi	2011	KY124546
<i>P. sanguifluum</i>	CCTU823	DTO 204-C2	Soil	Kabodan Island, Urmia	Ghosta & Samadi	2011	KY124547
<i>P. sanguifluum</i>	CCTU827	DTO 204-C6	Soil	Kabodan Island, Urmia	Ghosta & Samadi	2011	KY124548
<i>P. sizovae</i>	CCTU777	DTO 203-G1	Soil	Jadeh Darya (seaside), Urmia	Ghosta & Samadi	2011	KY124550

* CCTU: Culture Collection of University of Tabriz, Department of Plant Protection

** DTO: Culture Collection of Applied and Industrial Mycology Department, CBS-KNAW Fungal Biodiversity Centre, the Netherlands

1. *Penicillium anaticum* Stolk, Antonie van Leeuwenhoek 34(1): 46 (1968)

- Macroscopic characteristics:

On MEA: colony diameter after seven days at 25° C, 18 mm; mycelium white; pure yellow, pigment diffusing into the surrounding agar; exudate absent; limited sporulation throughout in greyish blue-green shades; colony floccose; sulcate with centrally umbonate; reverse luteous to pale luteous; margin regularly and entire (Fig. 1).

On CYA: colony diameter after seven days at 25° C, 40 mm; mycelium white; yellow pigment diffusing into the surrounding agar; exudate yellow, produced in small droplets; sporulation throughout in grey-green shades; colony velvety to floccose; sulcate with centrally umbonate; reverse amber; margin regularly and entire (Fig. 1).

- Microscopic characteristics:

Mycelium with smooth walled hyphae, conidiophores arising from aerial hyphae, consisting of variously asymmetrical to symmetrical biverticillate, sometime monoverticillate, rarely terverticillate; forming smooth stipe, 2–3 µm in width; verticils of 2–3 metulae, divaricate, usually (7–)13–17(–35) × 2–3.5 µm, cylindrical, apically inflated to slightly inflated; bearing clusters of phialides of 2–11 elements in the verticil, mostly (6–)7.5–9.0(–14) × (2–)2.5(–5) µm, flask-shaped with distinct collula, smooth walled; conidial chains tending to adhere in well-defined loose, divergent short columns (h); conidia citrine, globose, finely roughened (1–)2(–3) µm in diameter. After 10 days on PDA, honey cleistothecia formed on the centre of colony, cleistothecia becoming brown at age, (160–)230–310(–500) × (5–)8–10.5 (–17) µm (Fig. 1).



Fig. 1. *Penicillium anatolicum*: a, b. Colony on MEA, c, d. Colony on CYA, e-g. Cleistothecia, h. Conidial head, i-k. Conidiophore, l. Conidia (Bar = 10 µm).

2. *Penicillium sanguifluum* (Sopp) Biourge, La Cellule 33: 105 (1923)

- Macroscopic characteristics:

On MEA: colony diameter after seven days at 25° C, 17 mm; mycelium white; yellow pigment diffusing into the surrounding agar; exudate abundantly, orange; fairly abundant sporulation throughout in malachite green shades in centre and margin surrounded by a narrow white fringe of trailing hyphae; colony floccose; sulcate; reverse luteous; margin regularly and entire (Fig. 2).

On CYA: colony diameter after ten days at 25° C 28 mm; mycelium white; orange soluble pigment diffusing into the surrounding agar; exudate abundant, orange; sporulation throughout in pale luteous shades; colony

lanose; sulcate; reverse luteous; margin regularly and entire (Fig. 2).

- Microscopic characteristics:

Mycelium with smooth-walled hyphae 1–1.5 µm wide, conidiophores variable in size, arising from the trailing hyphae, monoverticillate; forming smooth stipe, (9–)21–40(–95) × 1–2 µm; apically vesiculate; bearing clusters of phialides of 2–10 elements in the vertical, terminally and subterminally, mostly (6–)7–8(–10) × 2–2.5 µm, flask-shaped with distinct collula; conidial chains tending to adhere in well-defined columns; conidia globose, 2 µm, smooth or finely roughened, citrine (Fig. 2).

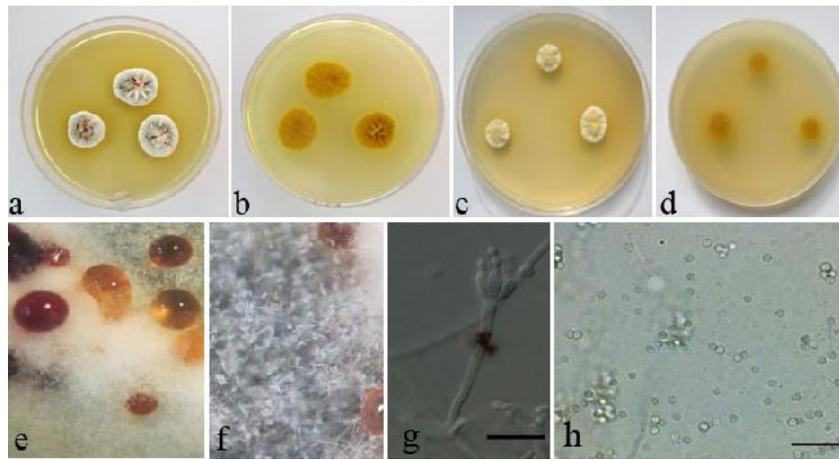


Fig. 2. *Penicillium sanguifluum*: a, b. Colony on MEA, c,d. Colony on CYA, e. Exudate, f. Conidial head, g. Conidiophore, h. Conidia (Bar = 10 µm).

3. *Penicillium sizovae* Baghd., Novosti Sistematiki Nizshikh Rastenii 5: 103 (1968)

- Macroscopic characteristics:

On MEA: colony diameter after seven days at 25° C, 30 mm; mycelium white; coloured pigment diffusing into the surrounding agar not produced; exudate colourless in the margin of colony; fairly abundant sporulation throughout in leek green shades in centre and margin surrounded by a narrow white fringe of submerged hyphae; colony velvety to floccose; sulcate with centrally umbonate; reverse buff; margin regularly and entire (Fig. 3).

On CYA: Colony diameter after seven days at 25° C, 25 mm; mycelium white; coloured pigment diffusing into the surrounding agar not produced; exudate colourless in the centre of colony; fairly abundantly sporulation throughout in greyish blue-green shades in centre and margin surrounded by a narrow white fringe of

submerged hyphae; colony velvety; sulcate; reverse primomose; margin regularly and entire (Fig. 3).

- Microscopic characteristics:

Mycelium (1–)3–3.5(–4) µm with smooth walled, penicilli variable in size, borne either upon conidiophores arising directly from the mycelial mat or sometime from aerial hyphae, varying in length, consisting of variously symmetrical to rarely irregular divergent structures, biverticillate occasionally with an additional branch, rarely monoverticillate; forming smooth stip, 2–3 µm in width; apically flat; verticils of 1–5 metulae, usually (11–)13–14.5(–20) × (2–)3(–5) µm; cylindrical; apically inflated; bearing clusters of phialides of (1–)3–4(–7) elements in the verticil, mostly (6–)7–9(–14) × (2–)3(–4) µm, flask-shaped with short collula; conidial chains in more or less long, tending to adhere in well-defined divergent columns (i); conidia globose, with smooth to finely roughened wall, citrine, (1.8–)2–2.5(–5) µm in diameter (Fig. 3).

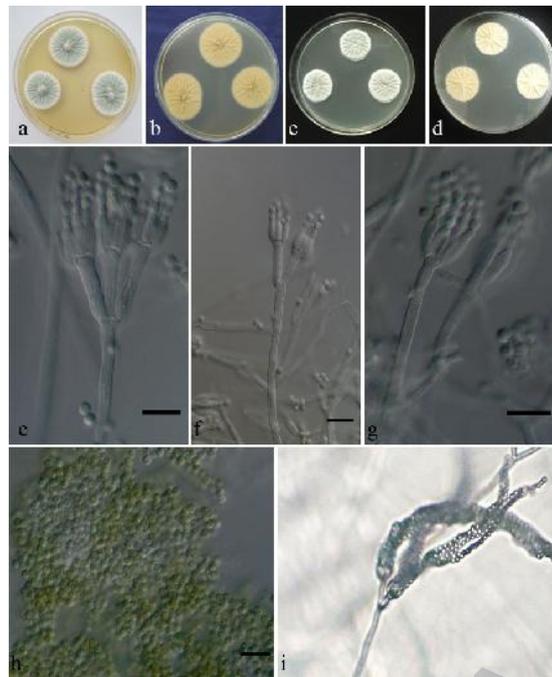


Fig. 3. *Penicillium sizovae*: a, b. Colony on MEA, c,d. Colony on CYA, e-g. Conidiophore, h. Conidia, i. Conidial head (Bar = 10 μ m).

Discussion

The present study was aimed to explore biodiversity of *Penicillium* section *Citrina* in soils of the National Park of Urmia Lake. Several isolates were recovered from the soils surrounding Urmia Lake with salinity ranging from 0.0003-72 ds/m salinity; of those eight isolates were identified in this study as members of the section *Citrina*. Species in section *Citrina* commonly occur in soil and exhibit worldwide distributions. Members of this section can grow at low water activities and in substrates containing various concentrations of NaCl (Houbraken *et al.* 2011). In the present study, members of this section were recovered from soils with salinity range of 0.001-35ds/m.

Species in *Penicillium* section *Citrina* are characterised based on a combination of cultural and morphological features including restricted growth on Czapek's agar, production of symmetrically biverticillate conidiophores, flask-shaped phialides (7.0–9.0 μ m long) and relatively small conidia (2.0–3.0 μ m in diameter) (Houbraken *et al.* 2011). However, identification of *Penicillium* species based on morphological features alone has proven troublesome, which is mainly due to overlap in micromorphology and lack of distinguishing

morphological features among different species. In the last two decades, taxonomy of *Penicillium* and allied genera has largely been influenced by DNA sequence data; hence, sequence data from different genomic regions have been used to resolve taxonomy and phylogeny of this genus at different levels of interest. Sequence data from different genomic regions including ITS-rDNA region, *-tubulin (BenA)*, *Cytochrome C oxidase I (COXI)*, *Calmodulin (CAL)*, *RNA polymerase II largest subunit (rpb1)* and *Translation elongation factor 1-alpha (EF-1a)* genes have been widely used for species identification in *Penicillium* (Peterson 2000, Seifert *et al.* 2007, Visagie *et al.* 2014).

In the present study, *Penicillium* section *Citrina* isolates were assigned to three species, namely, *P. anatolicum*, *P. sanguifluum*, and *P. sizoviae*, based on an integration of cultural and morphological traits, and partial sequences of the *BenA* gene.

Penicillium anatolicum, *P. sanguifluum*, and *P. sizoviae* can be identified from each other and the other species in this section based on cultural and morphological characteristics. *Penicillium anatolicum* has been originally described from soil in Turkey (Houbraken *et al.* 2010, 2011); since then this species has

been isolated from soil in Florida and South Africa. In the present study, a single isolate of *P. anaticum* was isolated from soils with 7 ds/m salinity in Kabodan Island. The important morphological features of *P. anaticum* are production of yellow soluble pigments, metulae of unequal length with inflated tip (Houbraken *et al.* 2011). This species is phylogenetically related to *P. euglaucum*, and *P. argentinense*. However, *P. anaticum* can be distinguished from *P. argentinense* by the lack of yellow soluble pigments in the later species (Houbraken *et al.* 2011). *Penicillium anaticum* can be differentiated from *P. euglaucum* based on ascospores size, which are larger in the later species (Houbraken *et al.* 2011). *Penicillium gallaicum* is another species which produces yellow soluble pigments (citroviridins); but, it differs from *P. anaticum* by the presence of predominantly monoverticillate conidiophores, production of sclerotia and lack of ascoma (Houbraken *et al.* 2011).

Penicillium sanguifluum was isolated from soils of the National Park of Urmia Lake with 0.001-32 ds/m salinity. Six isolates from *P. sanguifluum* were isolated from Asper (one isolates) and Kabodan (five isolates) Islands. This species has been isolated from soil in (Wyoming, USA) and indoor air (the Netherlands) (Houbraken *et al.* 2011). The main morphological features of this species include monoverticillate conidiophores, luteous colour in reverse on CYA with the production of orange soluble pigment and growth on CYA at 30° C (Houbraken *et al.* 2011). *Penicillium sanguifluum* is phylogenetically and phenotypically similar to *P. roseopurpureum*; but, can be differentiated from *P. roseopurpureum* by its high growth rate and

ability to grow on CYA at 30° C (Houbraken *et al.* 2011).

Penicillium sizovae was recovered from soils of the National Park of Urmia Lake with 36 ds/m solitary. A single isolate from this species was isolated from a soil sample taken from Jade-darya (seaside). The main characteristics of this species include fast growth on MEA and yeast extract-sucrose (YES) and finely roughened conidia (Houbraken *et al.* 2011). The isolates attained 30 mm in diameter on MEA, and the reverse on CYA was primomose colored. This species was originally described from soil in Syria and since then has been isolated from a number of substrates such as cropped soil (Italy), margarine, glue and *Papaver somniferum* (The Netherlands), sea salt (Portugal) and salty water in saltern (Slovenia) (Houbraken *et al.* 2011). *Penicillium sizovae* is phylogenetically related to *P. citrinum*, *P. hetheringtonii*, *P. steckii*, and *P. gorlenkoanum*; and can be differentiated from those species based on fast growth rate on MEA and formation of finely roughened conidia.

In the present study we characterized three *Penicillium* in the section *Citrina* from hypersaline soils of the Urmia Lake basin, considering the rich diversity of *Penicillium* species in soils of the National Park of Urmia Lake, we aim to further explore the biodiversity of this economically, ecologically, and medically important genus in this region.

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