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ORIGINAL PAPER

Aureobasidium mangrovei sp. nov., an ascomycetous species recovered from Hara protected forests in the Persian Gulf, Iran

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Abstract A new ascomycetous black yeast-like species was recovered from healthy plant (*Avicennia marina*) of Hara protected mangrove forests at Qeshm Island, Iran. Morphological, physiological analysis as well as a molecular analysis of the internal transcribed spacer (ITS) and partial large ribosomal subunit (D1/D2 domains) confirmed the placement of this strain in the genus *Aureobasidium* and based on considerable sequence divergence, distinguishable cardinal growth temperatures and salt tolerance a new species *Aureobasidium mangrovei* sp. nov. is proposed. However, the type strain micro-morphologically is not clearly distinguishable from other members of the genus. The type strain, *Aureobasidium mangrovei* was preserved in a metabolically inactive state at the Iranian Biological Resource Centre, Tehran, Iran as IBRC-M 30265^T and the ex-type culture is deposited in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands as CBS 142205^T. The GenBank accession numbers for the nucleotide sequences of the large subunit ribosomal DNA and ITS region are KY089084 and KY089085, respectively. The MycoBank number of the new species is MB 823444.

Keywords *Avicennia marina* · Biodiversity · Black yeast-like · Molecular phylogeny · 1 new taxon. Qeshm Island

Introduction

The ascomycetous genus *Aureobasidium* is a member of the family Saccotheciaceae within the class of the Dothideomycetes (Thambugala et al. 2014;

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Wijayawardene et al. 2014; Hymphries et al. 2017). In recent classification, *Aureobasidium* has synanamorphs named *Kabatiella* and *Selenophoma* (Schoch et al. 2006; Bills et al. 2012; Hymphries et al. 2017). Phenotypic varieties of the fungus were defined by Hermanides-Nijhof (1977) and then redefined using molecular methods by Zalar et al. (2008). Later on, the genome sequencing analysis by Gostinčar et al. (2014) revealed that the differences between the four varieties of *Aureobasidium pullulans* are significant enough to redefine them as separate species: *A. pullulans*, *Aureobasidium melanogenum*, *Aureobasidium subglaciale* and *Aureobasidium namibiae*. Members of this genus are known from their asexual reproduction and there in no teleomorph linked to this genus (Arzanlou 2014). While description of several distinguished clades based on multilocus DNA analyses was evident, yet nomination of separate taxa has not been performed (Manitchotpisit et al. 2009). The total number of classified *Aureobasidium* species currently varies in different official nomenclatural repositories and it is close to 38 species. The number of *Aureobasidium* species is increasing and the two most recent novel species (*A. iranianum* and *A. thailandense*) have been isolated from Bamboo stems, leaves and wooden surfaces (Arzanlou and Khodaei 2012; Peterson et al. 2013). Species in the genus *Aureobasidium* have been recovered from plant residues, soil, air, water, glacial ice and even the clinical laboratory as a contaminant or opportunistic infection (Loncaric et al. 2007; Zalar et al. 2008; Morais et al. 2011; Arfi et al. 2012; Santo et al. 2012). *Aureobasidium* spp. exhibit diverse life styles such as saprophytes, plant associated endophytes or opportunistic human pathogens (Arzanlou 2014). Occurrence, substrates and distribution of *Aureobasidium* spp. in Iran was investigated by Arzanlou (2014). Species of the genus *Aureobasidium* show significant biotechnological and commercial potentials due to their ability to produce extracellular polysaccharide (EPS), a variety of hydrolytic enzymes and a possible role as biocontrol agent against phytopathogenic fungi in fire blight disease (Manitchotpisit et al. 2009; Martini et al. 2009; Cheng et al. 2011; Rich et al. 2011). Furthermore, biofinishing, biosurfactant and single cell oil production recently has been introduced as a novel biotechnological application of some members of this genus (Wang et al. 2014; Kim et al. 2015; Nieuwenhuijzen et al. 2016).

Hara Protected Forests is the common name for mangrove forests on the southern coast of Iran, in the Hormozgan province, Persian Gulf (Ghasemi et al. 2012). It is considered a unique ecosystem that plays a multi-purpose role for biodiversity protection and biotechnology by protecting the shore line, providing food sources and safe habitat for fish, wild bird and also domestic animals, participating in the biogeochemical cycle, honey production and production of medicinal materials, such as saponin, flavonoid and tannins (Ali Zahed et al. 2010; Faridah-Hanum et al. 2014). Unfortunately, such ecosystems have been facing destructive threats in Iran and also around the globe. Over the past 50 years, approximately one-third of the world's mangrove forests have been lost. Natural and anthropogenic threats including growing coastal populations, global climate change, increasing pollution and industrialization are considered the main threats (Parvaresh 2011; Faridah-Hanum et al. 2014).

During a research project aimed at identifying biodiversity of yeasts species related to plant flora of Hara forests at Qeshm Island, Iran, two conspecific unknown ascomycetous black yeast-like strains were isolated. We used a combination of morphological and physiological characteristics, and DNA sequence analyses to characterize the two strains and describe them as novel species of the genus *Aureobasidium*.

Materials and methods

Sample collection and fungal isolation

Healthy leaves were collected from hara mangrove trees (*Avicennia marina*) growing in the marsh zone (approximately coordinates: 26°47′N, 55°45′E), cut into small pieces (2 × 2 cm) aseptically and macerated in 90 ml saline. Serial dilutions in saline (0.9% NaCl) from healthy plant leaves were directly plated on Rose Bengal agar (Himedia) supplemented with 0.1 g chloramphenicol l^{-1} and incubated at 28 °C for 48 h.

Phenotypical and physiological characterization

Developing colonies were grouped into morphotypes based on their cultural characteristics and representatives of each colony type per plate purified by streaking on yeast peptone glucose (YPG) agar medium at 28 °C. Morphological characters of the isolates were studied on potato dextrose agar (PDA,

Merck), malt extract (MEA) agar (MEA: 20 g malt extract (Merck), 1 g peptone (Merck), 20 g glucose (Merck) and 20 g agar (Merck), 1000 ml distilled water, pH 5.5), Harrold's M40Y and M60Y media as described (Raper and Fennell 1965; Klich 2002). Colony diameters and appearance were recorded. Photographs were taken from 7 days old culture plates incubated at 28 °C unless otherwise mentioned. For micromorphological observations, temporary mounts were made in lactic acid. The standard methods of identification described by Barnett et al. (2000) and the key of Kurtzman et al. (2011) were used to explore the physiological and biochemical characteristics of the isolates.

DNA extraction and phylogenetic analysis of marker genes

For DNA isolation, the strains were grown on YPG broth at 28 °C for 24 h. The DNA was extracted according to Hanna and Xiao (2006). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified by PCR using the primer pair ITS1 and ITS4 (White et al. 1990). The D1/D2 domain of the large ribosomal subunit (LSU) was amplified by PCR with NL1 and NL4 (Lin et al. 1995; Kurtzman and Robnett 1997). The nucleotide sequences of the large ribosomal subunit and ITS region of IBRC-M 30265^T and IBRC-M 30266 were deposited in GenBank as KY089084, KY089085 and KY089086, KY089087, respectively.

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 proposed new species was conducted using the MEGA version 7 software package from the evolutionary distance data method using the two-parameter model (Kimura 1980) The robustness of the clades was assessed using bootstrap analysis with 1000 replicates (Felsenstein 1985).

Results and discussion

During our survey of yeast biodiversity on plant residues at Qeshm Island, Iran the following fungal and yeast isolates were found: *Sympodiomycopsis kandeliae* (two), *Quambalaria simpsonii* (one), *Hortaea werneckii* (two), *Rhodotorula mucilaginosa* (one); *Aureobasidium pullulans, Exophiala* spp. (three) and a newly described species (Nasr et al. 2017) named as *Jaminaea pallidilutea* (two). In addition, we isolated two unknown and potentially conspecific ascomycetous black yeast-like strains (IBRC-M 30265^T and IBRC-M 30266). We successfully PCR amplified and sequenced the D1/D2 domains of the LSU and ITS rDNA region. An initial megablast similarity search in the NCBI nr database using the LSU sequence showed the highest similarity to *Aureobasidium* sp. strain RBF-17A2 (FN665420; 98% identity). Megablast similarity search using the ITS sequence showed the highest similarity to *Kabatiella* sp. strain SQU-MA24 (KU945924; 99%) and uncultured fungus clone CMH306 (KF800397; 97%). Phylogenetic analyses of LSU and ITS rRNA regions based on both the neighbor-joining (NJ) and the maximum-likelihood (ML) method were performed to assess the relationship of isolates IBRC-M 30265^T and IBRC-M 30266 to related *Aureobasidium* species. The ML tree (see Online Resource: Figs. 1 and 2) showed essentially the same topography as the NJ tree (Figs. 1, 2) however, the bootstrapping is lower by using ML method. Therefore, we considered both of these strains we isolated to be putative novel species of *Aureobasidium* and proceeded to perform morphological, physiological analysis.

Aureobasidium species are not distinguished from each other using micro-morphological characters. Rather, they are characterized by cardinal growth temperatures, salt tolerance and production of reddish brown hyphal pigmentation in PDA cultures (Zalar et al. 2008; Peterson et al. 2013; Hymphries et al. 2017). Under the microscope, isolates IBRC-M 30265^T and IBRC-M 30266 proliferate by polar budding and we also observed synchronous budding (Fig. 3a). They produced chlamydospores which are one and two celled, subglobose to ovoid, hyaline and dark brown, and of variable size (Fig. 3b). Hyaline hyphae were observed (Fig. 3c). Production of hyphae with intercalary chlamydospores were also observed (Fig. 3d, e). According to Zalar et al. (2008) the main difference observed among members of *Aureobasidium* spp. are pigmentation of cultures, halo- and thermotolerance. Isolates IBRC-M 30265^T and IBRC-M 30266 both produce pinkish colonies on YPG, SDA

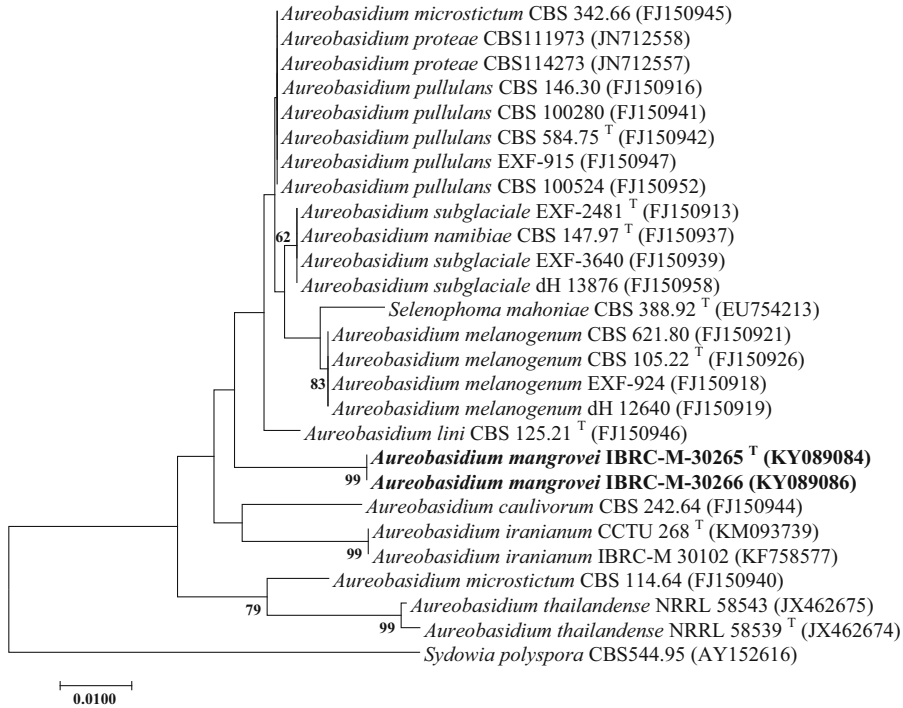

Fig. 1 Neighbour-joining phylogenic analysis of the LSU D1/D2 region showing the relationship of *Aureobasidium mangrovei* IBRC-M 30265^T and related taxa. The numbers given on the branches are the frequencies with which a given branch appeared in 1000 bootstrap replications. Bar, 0.0100 substitutions per nucleotide position

and PDA medium that become darkly pigmented in center areas after one week from the production of melanized conidia (Fig. 4a–c). Isolates IBRC-M 30265^T and IBRC-M 30266 both produce pinkish colonies with irregular margins on M40Y (Fig. 4d); cream coloured colonies on M60Y (Fig. 4e); reddish brown colonies with dark brown pigmentation in the center on MEA (Fig. 4f). In comparison, *A. pullulans* produces colonies that are white to pink for at least a week, usually 2–3 weeks (Peterson et al. 2013). *A. melanogenum* colonies are smooth and slimy due to abundant sporulation and extracellular polysaccharide (EPS) formation, but after 14 days the entire colonies turn green to black. *A. subglaciale* isolates remain pinkish at 7 days but are melanized by 14 days. *A. namibiae* isolates become melanized in the first week producing an olive–brown colour (Zalar et al. 2008; Peterson et al. 2013). *A. thailandense* resembles *A. pullulans* in producing initially pale yeast-like colonies with EPS. After 7 days of growth, the center of the *A. thailandense* colony is reddish brown colored (Peterson et al. 2013). *A. iranianum* produce white to pink colony on YPG medium (Fig. 4g). The level of pigmentation on SDA (Fig. 4h) and PDA (Fig. 4i) are much lower in comparison with IBRC-M 30265^T and IBRC-M 30266.

The growth diameters, sugar and salt tolerance and different cardinal growth temperature of *A. iranianum*, *A. thailandense* and isolates IBRC-M 30265^T and IBRC-M 30266, were compared and the results are shown in Table 1. All *Aureobasidium* species failed to grow at 4 °C, while according to Peterson et al. (2013) weak growth is observed in the strain Y-12974 of *A. pullulans* at this temperature. Isolates IBRC-M 30265^T, cannot tolerate NaCl concentration of 20% and above. No growth was observed either for *A. pullulans* CBS 621.80 or *A. thailandense*. There are some strains of *A. pullulans* previously reported to grow on media with more than 15% NaCl concentration (Zalar et al. 2008; Peterson et al. 2013). The optimum growth temperature for both species is 25–28 °C. The isolates IBRC-M 30265^T and IBRC-

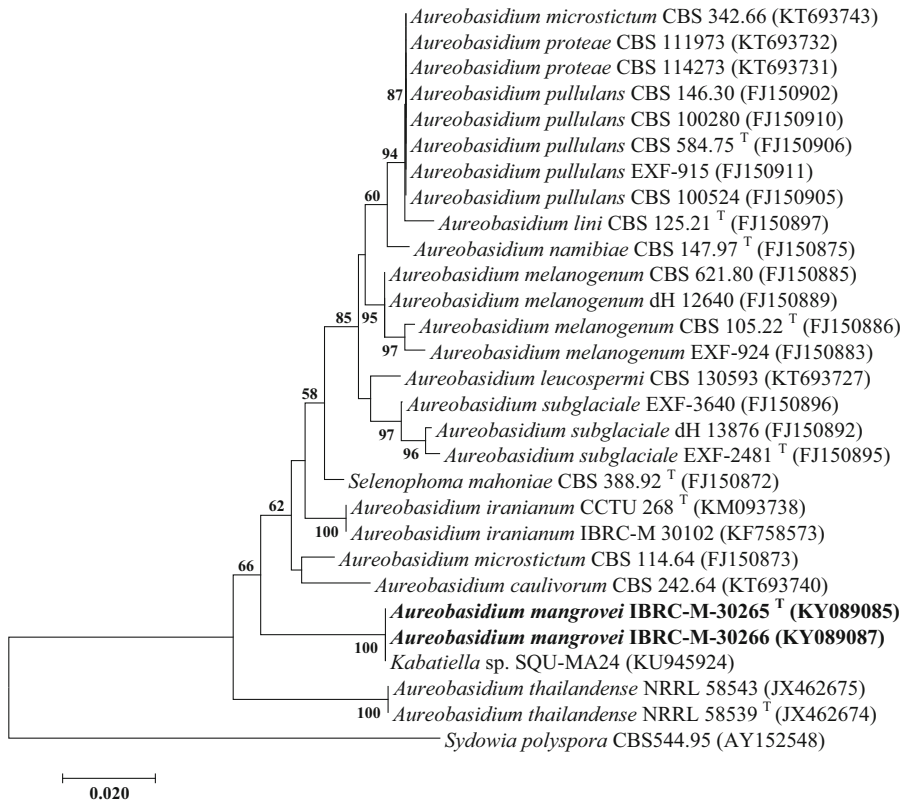

Fig. 2 Neighbour-joining phylogenic analysis of ITS region showing the relationship of *Aureobasidium mangrovei* IBRC-M 30265^T to related taxa. The numbers given on the branches are the frequencies with which a given branch appeared in 1000 bootstrap replications. Bar, 0.020 substitutions per nucleotide position

M 30266 weakly grow at 37 °C (Table 1). In comparison with *A. thailandense*, isolates IBRC-M 30265^T and IBRC-M 30266 grew weaker on moderate sugar level media (PDA) and on both M40Y and M60Y. In comparison with *A. iranianum,* isolates IBRC-M 30265^T and IBRC-M 30266 grew stronger on both M40Y and M60Y.

We performed physiological analysis. The isolates IBRC-M 30265^T and IBRC-M 30266 utilized a broad spectrum of carbon sources except methanol, ethanol, n-hexadecane, citrate DL-lactic acid. Urease activity was negative. However, *A. iranianum* had urease activity. The pattern of carbon utilization was very similar in both species. Lactose assimilation which was negative for *A. iranianum* and it was weakly assimilated by IBRC-M 30265^T and IBRC-M 30266. Assimilation of starch was positive (weak) in *A. iranianum* and it was negative by IBRC-M 30265^T and IBRC-M 30266.

Description of *Aureobasidium mangrovei* sp. nov., S. Nasr

Mycobank number: MB 823444

Type strain: The type strain, *Aureobasidium mangrovei* was isolated from healthy plant leaves of *Avicennia marina* collected at Qeshm Island, Iran, on 1 January 2016 and preserved in a metabolically inactive state at the Iranian Biological Resource Centre, Tehran, Iran as IBRC-M 30265^T and the ex-type culture is deposited in the CBS yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands as CBS 142205^T.

Etymology: referring to the isolation of this microorganism from the mangrove ecosystem of Qeshm Island, Iran.

Description

Aureobasidium mangrovei sp. nov. initially grows as pinkish colonies with glistening moist surfaces on YPG medium. After 7 days of incubation at 28 °C, colonies are darkly pigmented. On MEA and PDA after 7 days of incubation at 28 °C colonies attain diameter of 28 and 12 mm, respectively. Microscopically, cells measure 7–13.5 × 5.0–11 (mean = 10 × 7 μm), proliferating by polar budding. Synchronous budding observed (Fig. 3a). Chlamydospores separate or intercalary, one or two celled (Fig. 3b–e), subglobose to ovoid, of variable size, hyaline and dark brown.

Fig. 4 Colony morphology of *Aureobasidium mangrovei* ▶ IBRC-M 30265^T incubated at 28 °C. **a** YPG medium. **b** SDA medium. **c** PDA medium. **d** M40Y medium. **e** M60Y medium. **f** MEA medium. Colony morphology of *Aureobasidium iranianum* CCTU 268^T. **g** YPG medium. **h** SDA medium. **i** PDA medium. **j** M40Y medium. **k** M60Y medium. **l** MEA medium

Fermentation ability is weakly positive for D-glucose, maltose, cellobiose, trehalose, raffinose, sucrose, melibiose, D-galactose. Fermentation of inulin, lactose, soluble starch and D-xylose is negative.

The following carbon compounds are assimilated: D-glucose, sucrose, maltose, cellobiose, trehalose, raffinose, melibiose, melezitose, D-xylose, L-arabinose, L-rhamnose, ribitol, D-mannitol, myo-Inositol. Weak assimilation of following compounds is observed: succinic acid, L-galactose, lactose, L-arabinose, D-ribose and glycerol. No growth occurs on soluble starch, ethanol, citric acid, DL-lactic acid,

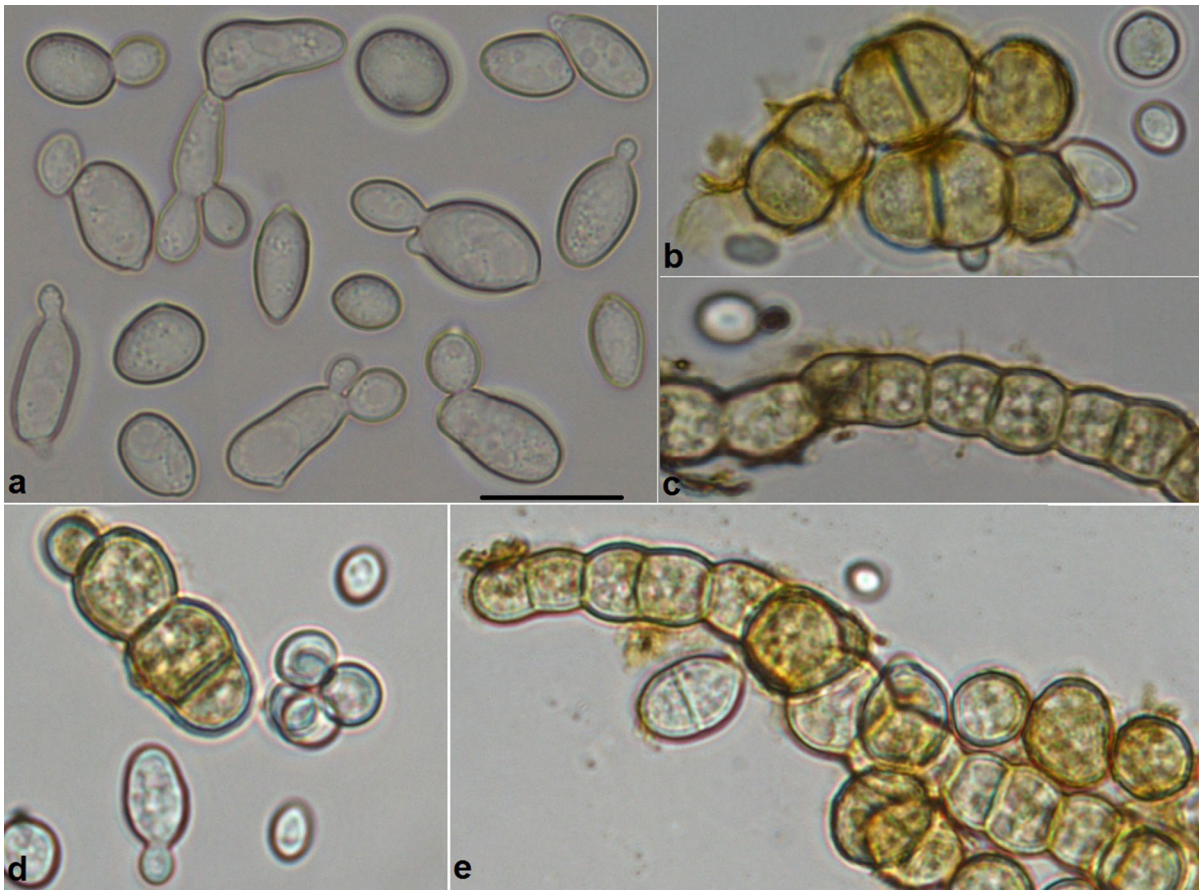

Fig. 3 Morphology of *Aureobasidium mangrovei* IBRC-M 30265^T incubated at 28 °C. **a** Synchronous budding on yeast cells. **b** One and two celled chlamydospores. **c–e** Hypha which contains intercalary chlamydospores Scale bar: 10 μm, for figures **a–e**

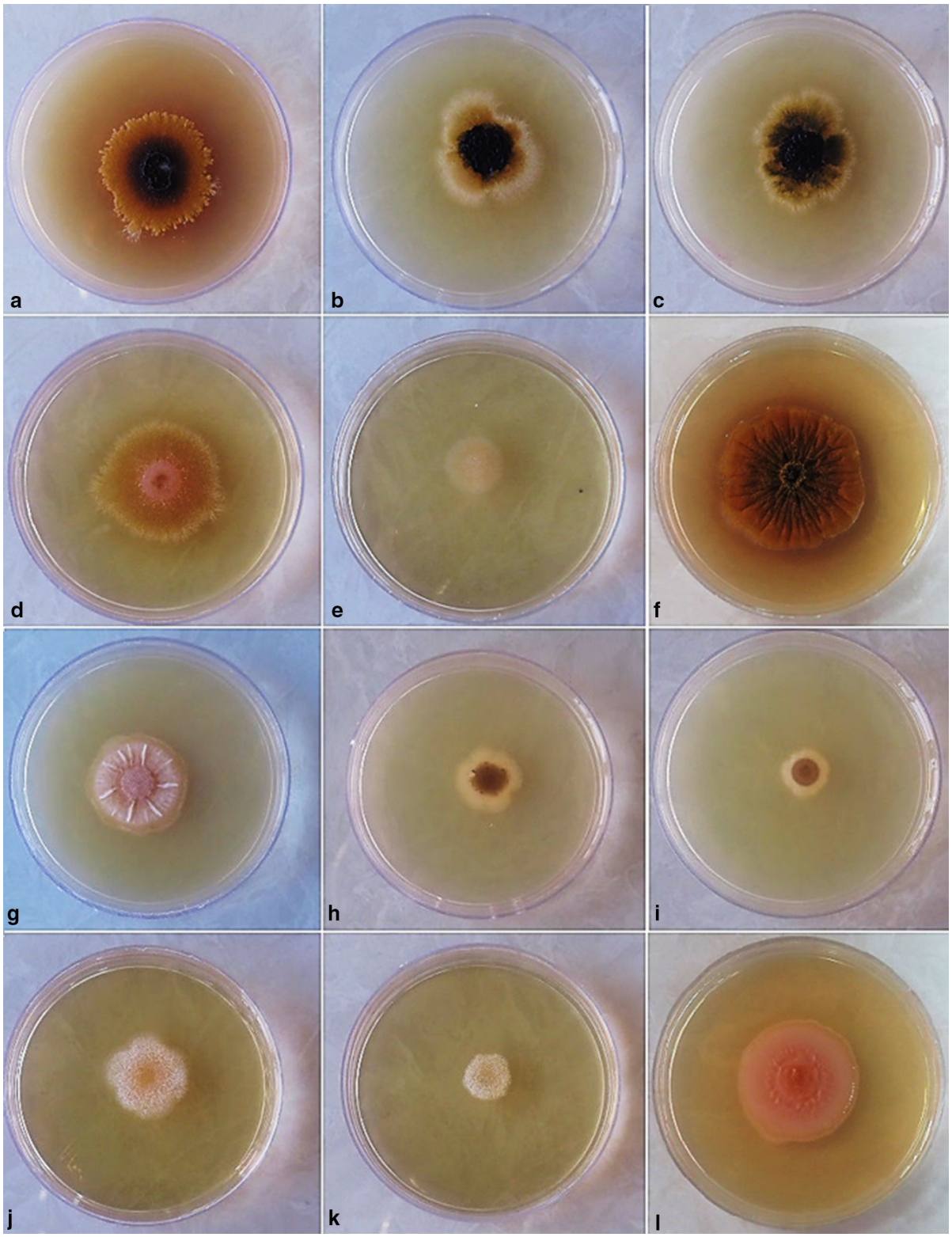
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Table 1 Colony diameters (mm) on PDA, M40Y, M60Y and MEA of *A. thailandense* NRRL58039^T, *A. iranianum* CCTU 268^T and *A. mangrovei* IBRC-M 30265^T after 7 days of incubation at 28 °C

Growth condition	*A. thailandense* NRRL58039^T	*A. iranianum* CCTU 268^T	*A. mangrovei* IBRC-M 30265^T	*A. mangrovei* IBRC-M 30265
YPG	ND	22	25	25
SDA	ND	15	22	22
PDA	25	12	12	12
M40Y	28	20	25	26
M60Y	23	10	15	15
MEA + 5% NaCl	15	10	31	30
MEA + 10% NaCl	8	5	16	16
MEA + 15% NaCl	0	0	5	5
MEA + 20% NaCl	0	0	0	0
MEA + 25% NaCl	0	0	0	0
MEA at 4 °C	0	0	0	0
MEA at 15 °C	17	22	21	21
MEA at 25 °C	27	35	39	40
MEA at 28 °C	ND	25	28	26
MEA at 34 °C	ND	16	11	11
MEA at 37 °C	0	0	6	6
MEA at 40 °C	0	0	0	0

Cardinal growth temperatures and salt tolerance were tested on MEA

methanol, *n*-hexadecane. The nitrogen compounds imidazole and glucosamine are assimilated. Growth occurs at 34 and 37 °C but not at 40 °C. Growth occurs in vitamin free medium, and media containing 50 and 60% glucose. Growth occurs in the presence of 15 % sodium chloride. No starch-like substances are produced. Diazonium blue B reaction is negative. Urease activity is negative.

Additional strain examined: IBRC-M 30266. The strain was isolated from healthy plant leaves of *Avicennia marina* collected at Qeshm Island, Iran, on 1 January 2016, and preserved in a metabolically inactive state at the Iranian Biological Resource Centre, Tehran, Iran.

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Conflict of interest The authors declare that they have no conflict of interest.

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