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Molecular Characterization of *Aureobasidium* Species in Iran

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Abstract

Background: Members of this genus *Aureobasidium* are ubiquitous microorganisms which can be isolated from wide ranges of substrates such as plant materials (phyllosphere, plant debris, bark, roots, fruits and wood), soil, dead wood, air, and as rare etiologic agent of pheohyphomycosis, keratomycosis, septicemia, peritoneal sepsis, and dermatological infections in human. Very little is known on the identity, substrates and distribution of *Aureobasidium* spp. in Iran.
Materials and Methods: 14 *Aureobasidium* isolates were recovered from vascular tissues of pome and stone fruit trees displaying decline symptoms in orchards of West and East Azarbaijan provinces, Iran. Pure cultures were established by using a single spore technique. The identity of the isolates were determined using sequence data from ITS-rDNA region. Phylogenetic relationship among isolates was inferred based on sequence data from ITS-rDNA.
Results: A megablast search analysis of ITS sequence data at NCBI revealed the identity of *Aureobasidium* isolates as *A. pullulans*. A phylogeny inferred using sequence data from ITS region placed our isolates together with the other *A. pullulans* var. *pullulans* in GenBank. Morphological and cultural characteristics were in agreement with the description for *A. pullulans* var. *pullulans*.
Conclusion: Our results represent new report on the occurrence of *A. pullulans* var. *pullulans* in Iran. As *A. pullulans* is known as rare etiologic agent of pheohyphomycosis, keratomycosis, septicemia, peritoneal sepsis, and dermatological infections in human, possible occurrence and involvement of *A. pullulans* in human infections should be taken into account.

Keywords: ITS-rDNA; Endophytes; Black yeast; Pullulan; Human pathogen

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Introduction

The genus *Aureobasidium* accommodates species with one-celled conidia of various shapes which are produced synchronouly from hyaline and terminal, lateral or intercalary conidiogenous cells (Hermanides-Nijhof 1977; Zalar et al. 2008; Seifert et al. 2011). Members of the genus are ubiquitous and occur on different habitats such as plant materials (phyllosphere, plant debris, bark, roots, fruits, wood), water, marine sediments, marshland, soil, air, skin, nails, stone, glass and in the clinical laboratory as a contaminant or human pathogen (Hermanides-Nijhof 1977, de Hoog & Yurlova 1994, de Hoog et al. 1999, 2000, Urzı` et al. 1999). *Aureobasidium* spp. exhibit diverse life styles such as saprophytes, plant associated endophytes or pathogens and opportunistic human pathogens (Andrews et al. 2002, Loncaric et al. 2008, Martini et al. 2009). *Aureobasidium pullulans*, the type species of the genus, has become as one of the best-known and most studied species of this genus. This species is commonly associated with plant materials, found on the surface and in the tissues (as endophyte) of plant species. *Aureobasidium pullulans* is an important microorganism in industry, agriculture as well as medical. It is well known as a producer of pullulan, a biodegradable extracellular polysaccharide, with commercial significance (Singh et al. 2008, Zalar et al. 2008) and it also has been considered as a potential biocontrol agent of post harvest plant pathogens e.g., *Botrytis cinerea*, *Penicillium expansum* and *Fusarium* species.

A wide range of antagonistic strategies such as competing for nutrients and space and production of numerous compounds including pectolytic enzymes, antimicrobial metabolites and high-molecular-weight polysaccharides have been detected in the interaction of *A. pullulans* with plant pathogenic fungi (Castoria et al. 2001, Dugan et al. 2002, Schena et al. 1999, 2003, Seibold et al. 2004, Elmer & Reglinski 2006, Felice et al. 2008, Zalar et al. 2008, Martini et al. 2009). *Aureobasidium pullulans* is also known as ethological agent of phaeohyphomycosis, causing disseminated infection in humans (Revankar et al.2002). Members of this genus are known from their asexual morphs and there in no teleomorph linked to this genus. However, based on DNA sequence data members of this genus reside in the family Dothideaceae (order Dothideales, Pzizomycotina, Ascomycota) (Schoch et al. 2006).

There is a huge paucity of knowledge on the biodiversity of *Aureobasidium* spp. in the mainland of Iran. The aim of this study was to explore biodiversity of *Aureobasidium* spp. associated with woody hosts in Northwestern zone of Iran.

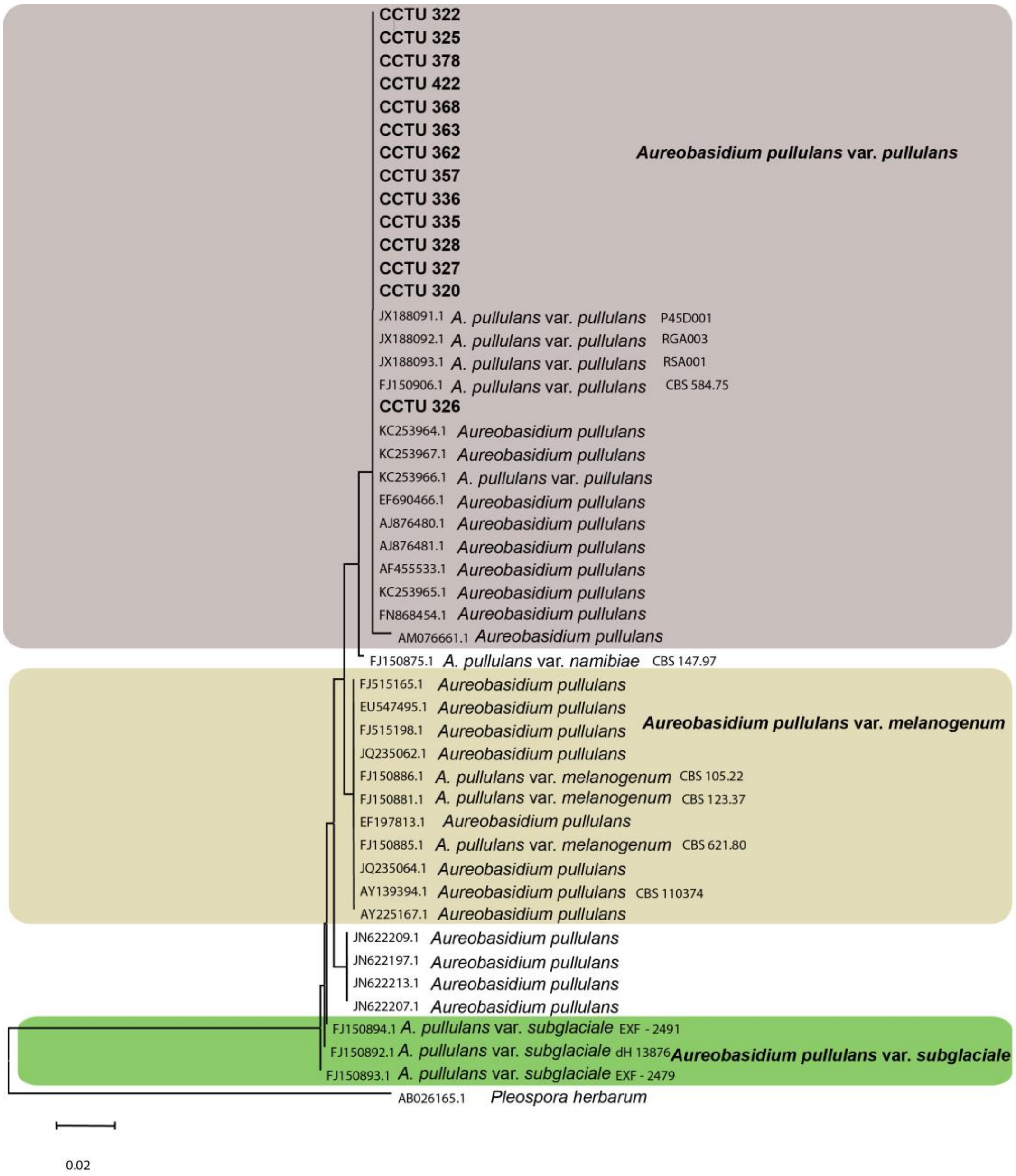

Figure 1. A neighbor-joining phylogenetic tree obtained from the ITS region and 5.8S rDNA sequence data. Bootstrap support values from 1000 replicates are indicated at the nodes. The tree was rooted to *Pleospora herbarum*. The scale bar indicates 0.02 substitutions per site.

Materials and Methods

Sampling and isolation

Wood samples were collected from branches and trunks of stone fruit trees (almond, apricot, cherry and peach) showing decline and dieback symptoms in West and East Azerbaijan provinces during growing seasons of 2009–2010. In transverse sections through wood various degrees and forms of wood discoloration and necrosis were evident. Isolation was made using the protocol explained by Arzanlou and Dokhanchi 2013a, b; Arzanlou and Torbati 2013; Arzanlou et al. 2013a, b, in brief, small pieces, approximately 5 × 5 ×5 mm were cut from just below the surface, around and in the darkened vascular tissues and submerged in 1 percent sodium hypochlorite for 30 sec, subsequently rinsed with sterile water and dried on sterile filter paper. Five pieces were transferred on to 2 % malt extract agar (MEA, Fluka, Germany) amended with 2 ml of 25 percent lactic acid per litre of medium and incubated in the dark at 25 °C. Pure cultures were established by using a single-spore technique. Fungal cultures were deposited in to the culture collection of Tabriz University (CCTU) for further characterizations.

DNA extraction

Fungal isolates were grown on MEA for 15 days at 25 ˚C in dark. Approximately 300 mg of fungal mycelia was scrapped from cultures and genomic DNA was extracted following the protocol of Moller et al. (1992).

Sequence analysis

The sequence data from ITS-rDNA was used for phylogenetic analysis. The primer set V9G (de Hoog and Gerrits van den Ende, 1998) and ITS4 (White et al. 1990) was used to amplify the nuclear rRNA operon spanning the 3’ end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5’ end of the 28S rRNA gene from *Aureobasidium* isolates. The reaction mixture and PCR conditions were the same as Arzanlou et al. 2013a, b. PCR products were sequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) Cycle Sequencing Kit according to the recommendation of the seller and analyzed on an ABI Prism 3700 (Applied Biosystems, Foster City, CA). Raw sequence files were edited manually by using SeqMan™II (DNASTAR, Madison, Wisconsin, USA) and a consensus sequence was generated for each of the sequences. The sequences were subjected to a nucleotide Blast search at NCBI’s GenBank nucleotide database and sequences with high similarity were obtained and aligned together with the sequence obtained in this study. Sequences were compared with the sequences available in NCBI’s Gen Bank nucleotide (nr) database using a megablast search (Table 1). Sequence alignment was carried out by using the Clustal W algorithm implemented in MEGA 5 (Tamura et al., 2011). Phylogenetic analysis was performed using maximum likelihood method with program default settings in MEGA 5. Bootstrap analysis was performed with 1000 replicates. The phylogenetic tree was rooted to *Pleospora herbarum* (GenBank accession number AB026165.1).

Results

Morphology

The morphological characteristics of the isolates were in agreement with the description for *Aureobasidium pullulans* (Zalar et al. 2008). Colony attained a diameter about 40 mm after one week of incubation at 25 ^{0}C in dark on MEA; colonies smooth and slimy with no aerial mycelium, pinkish to yellowish in surface and yellowish to light in reverse. With age, black sectors of dark pigmented hyphae or conidia developed in some isolates. On MEA, hyphae hyaline, septate, smooth, thin-walled, occasionally dark-brown hyphae developed in older cultures. Conidiogenous cells not differentiated from vegetative hyphae, terminal, intercalary or lateral on hyaline hyphae. Conidia hyaline to dark brown, generally produced synchronously on dense clusters of small denticles, sometimes formed percurrently on short lateral denticles. Conidia variable in shape and size, hyaline conidia amero, smooth, ellipsoidal, often with an inconspicuous hilum, 7–15 × 3–6.5 μm. Dark brown conidia 0-1 septate, observed in some of the isolates. Yeast-like budding of hyaline and dark brown conidia frequently observed.

DNA phylogeny

The alignment file included 37 sequences (14 generated in this study and 23 obtained from GenBank) (Table 1). The phylogeny inferred using the sequence data obtained in this study together with the sequence data from GenBank, clustered our isolates with *Aureobasidium pullulans* from different substrates in a monophyletic group; however, several sub-clades were identified within the monophyletic clade (Figure 1). The bootstrap supports for the sub-clades were below 50. Three major groups corresponding to the varieties of *Aureobasidium pullulans* were identified in phylogenetic tree. Our isolates clustered in *Aureobasidium pullulans* var. *pullulans* clade.

Discussion

Species in the genus *Aureobasidium* exhibit diverse life styles such as saprophytes, plant associated endophytes or pathogens and opportunistic human pathogens (Andrews et al. 2002, Loncaric et al. 2008, Martini et al. 2009). *A. pullulans*, the type species of the genus, is the most well know species in this genus with industrial, agricultural as well as medical importance. It is well known for its commercial product, pullulan, a biodegradable extracellular polysaccharide (Singh et al. 2008, Zalar et al. 2008) and a potential biocontrol agent of post harvest plant pathogens.

Table 1. List of *Aureobasidium* isolates subjected to DNA sequence analyses.

Collection Code	Host/ substrate	Origin
CCTU 320	Peach	Iran
CCTU 322	Peach	Iran
CCTU 325	Peach	Iran
CCTU 326	Apricot	Iran
CCTU 327	Cherry	Iran
CCTU 328	Almond	Iran
CCTU 335	Peach	Iran
CCTU 336	Apricot	Iran
CCTU 357	Apricot	Iran
CCTU 362	Peach	Iran
CCTU 363	Apricot	Iran
CCTU 368	Apricot	Iran
CCTU 378	Apricot	Iran
CCTU 422	Soil	Iran
UOA11686: KC253964.1	Cerebrospinal fluid	Greece
UOA12768B: KC253967.1	Otitis	Greece
UOA12688A: KC253966.1	Skin	Greece
C202: AM076661.1	*Plantago lanceolata*, roots	Germany
TJY13b: EF690466.1	Marine	China
MT 7: AJ876480.1	*Steganacarus magnus* (oribatid mite)	Germany
MT 5: AJ876481.1	*Steganacarus magnus* (oribatid mite)	Germany
wb149: AF455533.1	Nasal mucus	Austria
UOA12626: KC253965.1	Pre-cooked pasta meal	Greece
BLE6: FN868454.1	*Pinus halepensis*	Spain
Z-28: JN622213.1	*Populus euphratica*	China
Z-20: JN622207.1	*Populus euphratica*	China
Z-3: JN622197.1	*Populus euphratica*	China
Z-23: JN622209.1	*Populus euphratica*	China
LB3: AY225167.	*Saraca indica*	Thailand

Collection Code	Host/ substrate	Origin
CO-4: EU547495.1	Fermented tea	Korea
UM16: FJ515198.1	Sea surface	Taiwan
P-19: JQ235062.1	*Populus euphratica*	China
HK58-3(1): EF197813.1	Marine	China
P-21: JQ235064.1	*Populus euphratica*	China
CBS 110374: AY139394.1	Air sample	Thailand
P45D001: JX188091.1	*Vitis vinifera*	USA
RGA003: JX188092.1	*Vitis vinifera*	USA
RSA001: JX188093.1	*Vitis vinifera*	USA
CBS 584.75: FJ150906.1	*Vitis vinifera*	France
CBS 147.97: FJ150875.1	Dolomitic marble	Namibia
CBS 105.22: FJ150886.1	-	-
CBS 123.37: FJ150881.1	-	-
CBS 621.80: FJ150885	Deteriorated army supplies	Russia
P-21: JQ235064.1	*Populus euphratica*	China
EXF-2491: FJ150894.1	Subglacial ice	Norway
dH 13876: FJ150892.1	Coastal ponds of melted	Norway
EXF-2479: FJ150893.1	Glacial ice from sea water	Norway

Based on cultural and morphological differences among the isolates several varieties have been distinguished: var. *pullulans*, occurring particularly in (occur on the phyllosphere and slightly osmotic substrates); var. *melanogenum*, (occur in watery habitats); var. *subglaciale* (known from subglacial ice); var. *namibiae* (a single strain known from dolomitic marble) and var. *aubasidani* (Zalar et al. 2008). These varieties have been described based on the variation in physiological, cultural and micromorphological features.

A wide range of molecular markers (rDNA RFLP and UP-PCR/hybridisation) have been used to explore intraspecies variations in *A. pullulans* (Yurlova et al. 1996). In their study Yurlova et al. (1996) identified four groups among the *Aureobasidium* strains; however, there was no correlation with morphological differences. Zalar et al. 2008 applied sequence data from rDNA (internal transcribed spacers, partial 28 S rDNA), and partial introns and exons of genes encoding *β-tubulin* (TUB), translation elongation factor (EF1α) and elongase (ELO) to characterise the genetic variability among *A. pullulans* from diverse substrates. We used sequence data from ITS region to characterize the isolates obtained from stone fruit trees. Our isolates clustered in var. *pullulans* clade (Figure 1).

This study represents new report on the occurrence of *A. pullulans* var. *pullulans* in Iran. To the best of our knowledge, stone fruit trees (peach, cherry, almond and apricot) are reported as new hosts for *A. pullulans* var. *pullulans.* The antagonistic potential of these isolates against plant pathogenic species as well as pullulan production remain to be tested.

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