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Cephalotrichum asperulum and *C. gorgonifer*, two synnematous species from pistachio in Iran

A. Alizadeh ✉
S. Akbarzadeh
A. Shirzad
Department of Plant Protection, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran

Abstract: In March 2019, a number of synnematous fungal specimens were isolated from the dead tissues of *Pistacia vera* (pistachio) seedlings in a greenhouse at the Azarbaijan Shahid Madani University of Tabriz, Iran. Preliminary identification based on morphological characteristics showed that the fungal isolates belong to the genus *Cephalotrichum*. Two species namely *C. asperulum* and *C. gorgonifer* were identified by combining morphology and phylogeny inferred from ITS-rDNA sequence. To our knowledge, *C. gorgonifer* is a new record for the Iranian Funga. Moreover, pistachio is reported as a new host for *C. asperulum and C. gorgonifer*.

Key words: Funga, phylogeny, *Pistacia*, morphology, Taxonomy.

INTRODUCTION

Fungi are one of the most important groups of terrestrial organisms that play vital roles in the biosphere (Kendrick 2011). They exist in all possible habitats on the Earth's surface (Peay et al. 2016). Fungi as the main components of natural ecosystems are of great importance in terms of their ability to cause important diseases in humans, animals and plants, high bioremediation capacity, establishing a mutualistic symbiosis with a wide range of organisms (angiosperms, bryophytes, coleopteran, cyanobacteria, dipteran, green algae, gymnosperms, homopteran, hymenopteran, isopteran insects and pteridophytes) and their potential as important resources of medicines, antibiotics and secondary metabolites (Alfen 2001, Dighton 2016, Nicolás et al. 2019, Salimi et al. 2019, Ayofemi Olalekan Adeyeye 2020, Kalra 2020, Kalsoom 2020, Pietro-Souza 2020, Litwin 2020, Mantzoukas et al. 2020, Rodrigues & Nosanchuk 2020, Schweiggert-Weisz 2020, Singh 2020, Srivastava et al. 2020, Srivastava 2021). Also, these organisms are considered as the main decomposers of plant and animal debris and therefore play a very vital role in recycling nutrients in nature (McClaugherty 2001, Cosgrove et al. 2007, Peay et al. 2016, Lin et al. 2019, Nicolás et al. 2019). The growth of plants in many habitats largely depends on the decomposition activity of organic matter by saprobic fungi, because without this activity, undecayed debrides accumulate on the surface and it is not possible for plants to benefit from these organic materials (Kendrick 2011).

So far, many fungi have been isolated and reported from decayed plant tissues or plants that show tissue rot symptoms (Kodsueb et al. 2008). These fungi are either the main causal agent of the diseases or act as saprobic agents on decayed tissues (Alizadeh et al. 2010, Alizadeh et al. 2017, da Silva et al. 2021). The study of fungi associated with rot diseases in plants provides useful information about their possible role as causal agents of diseases or as saprobic agents in decomposition of dead tissues.

The genus *Cephalotrichum* (Microascaceae, Microascacales) comprises synnematous saprobic fungal species with a worldwide distribution which have been mostly reported from air and soil, additionally from the indoor or built environment (Woudenberg et al. 2017) as well as decaying plant materials, straw, dung, wood (Domsch et al. 2007), so far. In recent years, significant changes have been made in the taxonomy of the genus using the polyphasic approach combining morphological, physiological and multigenic phylogenetic analyses (ITS, *LSU*, *tef1* and *β-tubulin*) to delimiting generic boundaries within the Microascaceae (Sandoval-Denis et al. 2016). As a result, it turned out that three microascaceous genera *Cephalotrichum*, *Doratomyces* and *Trichurus* are congeneric and *Cephalotrichum* accepted as synonymy of the mentioned genera. After that, more new species within the genus has been described and the number of discovered and accepted species of this genus is

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✉ Corresponding Author: E-mail: alireza.alizadeh@azaruniv.ac.ir
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increasing. It appears that there are still many unknown *Cephalotrichum* species are waiting to be discovered and described in the genus.

There is little information about *Cephalotrichum* species in Iran and the knowledge is limited to only a few reports (*C. asperulum* from leaf debris of apple and sycamore and stem debris of reed plants, *C. nanum* from shoots of apple trees and leaf debris of apple and ash (Ghosta et al. 2020), *C. microsporum* as endophyte from apple trees (Alijani et al. 2016) and *C. oligotriphicum* from *Robinia pseudoacacia* (Paripour et al. 2019). So, there is a high interest to identify more species of the genus and also to obtain more information about their distribution and new substrates in Iran. Thus, this study aimed to identify obtained *Cepahotrichum* isolates from dead tissues of pistachio seedlings in the greenhouse of Azarbaijan Shahid Madani University of Tabriz in Iran by combining of morphology and phylogeny inferred from ITS-rDNA sequence.

MATERIALS AND METHODS

Isolation and purification of fungi

In the spring of 2019, sampling was conducted from the two-months-old pistachio seedlings (*Pistacia vera*) showing root and crown rot symptoms in a greenhouse at Azarbaijan Shahid Madani University of Tabriz, Iran. Crown samples (approximately 1 cm diam) were washed in tap water, surface-disinfected in 0.5 % sodium hypochlorite for 1 min, rinsed in sterile distilled water and moist incubated in glass petri dishes on autoclaved paper towels soaked with sterile tap water. Petri dishes were kept at 20–25 °C in the darkness for two weeks. Synnematous fungi that grew directly on the pistachio tissues were transferred directly to the 2% water agar (WA, 2 %) culture medium supplemented with chloramphenicol (50 mg/L). Single spore or single hyphae isolates were obtained on potato dextrose agar (PDA, Merck, Darmstadt, Germany). Purified isolates were stored at Fungal Culture Collection, Mycology Laboratory of the Azarbaijan Shahid Madani University of Tabriz, Iran (AZFC).

Morphological characterization

The isolates were incubated on potato carrot agar (PCA) (20 gr potato, 20 gr carrot and 20 gr agar per liter of distilled water), potato dextrose agar (PDA) (20 gr potato, 20 gr dextrose and 20 gr agar per liter of distilled water) and malt extract agar (MEA) (30 gr malt extract, 5 gr peptone and 15 gr agar per liter of distilled water) at 25°C in the dark. Colony characters on cultures incubated at 25°C under near-UV light with a 12 hours photoperiod were documented after 7 and 14 days. Growth rates were measured after 7 and 10 days. Microscopic preparations were made in clear lactic acid, with at least 30 measurements per structure and observed with an OLYMPUS BX53 microscope. Comprehensive morphological description and illustrations are provided for the species.

Phylogenetic analysis

Genomic DNA was extracted from colonies grown on PDA (25 °C for 7–10 days) using a standard phenol-chloroform extraction protocol (Sambrook & Russell, 2001). Amplification of the internal transcribed spacer (ITS) region of rDNA was performed using the ITS1 (5´TCCGTAGGTGAACCTGCGG3´) and ITS4 (5´TCCTCCGCTTATTGATATG3´) primers (White et al. 1990). The Polymerase chain reaction was performed in a total volume of 25 µL. The PCR mixture contained 10–15 ng genomic DNA, 0.2 µM of each primer, 1X Taq PCR buffer, 2 mM $MgCl_2$, 20 µM of each dNTP, 0.75 µL DMSO and 0.25 U Smart Taq DNA Polymerase (Pishgam Co, Iran). The PCR program was run as following: an initial step of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 45 s at 58 °C and 45 s at 72 °C, followed by 10 min at 72°C. The PCR products were sequenced by Biomagic Gene Company (BMG, China) with the amplifying primers.

The programs Chromas ver. 2.6.6 and Edit Seq, part of the DNA*Lasergene (DNAstar, Madison, WI) software package, were used for editing and assembling the sequences. The sequences were aligned using MUSCLE, a multiple sequence alignment method (Edgar, 2004). The sequences of the examined Iranian *Cephalotrichum* isolates were compared with other fungal DNA sequences from NCBI GenBank database (www.ncbi.nlm.nih.gov/genbank/) through BLAST tool. Twenty-seven sequences with the highest similarity were added to the alignment as reference strains (Table 1). Analyses were performed via the software MEGA v.6 (Tamura et al. 2013). Gaps were treated as missing data in the pairwise sequence comparisons (Pairwise deletion option). The evolutionary distances between the sequences were computed using the maximum composite likelihood method (Varin 2008). The MP tree was obtained using the close-neighbor-interchange algorithm of Nei & Kumar (2000) with search level 1 (Felsenstein 1985, Nei & Kumar 2000) wherein the initial trees were obtained by random addition of sequences (100 replicates). Clade stability was calculated in a bootstrap analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa. The tree branches were drawn to scale, with lengths calculated using the average pathway method (Nei & Kumar 2000), as the units of the number of changes over the whole sequence. Tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for the resulting tree. The reliability of the inferred tree was estimated by bootstrap analyses with 1000 replicates. Sequence data were deposited in GenBank with the following accession numbers (AZFC P1-1: MZ506741; AZFC P2: MZ506742).

Table 1: *Cephalotrichum* isolates used in this study along with reference sequences obtained from GenBank.

Species	Strain	Substrate/host	Location	GenBank accession number
				ITS
C. asperulum	CBS 127.22	Seed	Netherlands	LN850959
	CBS 215.49	Unknown	Indonesia	KY249250
	CBS 582.71^{IT}	Soil	Argentina	LN850960
	UTHSCSA DI14-62	BAL	USA	LN850961
	UTHSCSA DI14-65	BAL	USA	LN850962
	AZFC P2	***Pistacia vera***	**IRAN**	**MZ506742**
C. brevistipitatum	CBS 157.57^{T}	*Solanum tuberosum*	Netherlands	LN850984
C. cylindricum	UAMH 1348^{ET}	*Sorghum* seed	USA	LN850965
C. dendrocephalum	CBS 528.85I^{T}	Cultivated soil	Iraq	LN850966
C. domesticum	CBS 142035^{T}	Indoor air, house	Netherlands	KY249280
C. gorgonifer	CBS 368.53	Treated wood	South Africa	LN850976
	CBS 120011	Soil	South Africa	KY249257
	CBS 124434	Human foot	Denmark	KY249258
	CBS 125064	Mouldarray fungi	Denmark	KY249259
	CBS 496.62	Compost ground domestic waste	Italy	KY249255
	CBS 104.15	Unknown	UK	KY249254
	DTO 240-B2	Indoor swab, archive	Netherlands	KY249268
	AZFC P1-1	***Pistacia vera***	**IRAN**	**MZ506741**
C. hinnuleum	CBS 289.66^{T}	Dung of deer	Australia	LN850985
C. lignatile	CBS 209.63^{T}	Timber in cave	Belgium	KY249269
C. microsporum	CBS 523.63^{ET}	Wheat field soil	Germany	LN850967
	UAMH 9365^{T}	Indoor air	Canada	LN850968
C. nanum	CBS 191.61^{ET}	Dung of deer	UK	LN850969
C. purpureofuscum	CBS 174.68	*Zea mays*, grain	Unknown	KY249281
C. stemonitis	CBS 103.19^{NT}	Seed	Netherlands	LN850951
C. telluricum	CBS 336.32^{T}	Soil	Cyprus	KY249287
C. tenuissimum	CBS 127792^{T}	Soil	USA	KY249286
C. transvaalense	CBS 448.51^{T}	Timber	South Africa	LN850964
C. verrucisporum	CBS 512.72	Agricultural soil	Netherlands	KY249289
Wardomyces inflatus	CBS 367.62^{NT}	Greenhouse soil	Belgium	LN850994

The obtained strains and newly generated sequences in this study are shown in bold.

AZFC: Fungal Culture Collection, Mycology Laboratory of the Azarbaijan Shahid Madani University of Tabriz, Iran; CBS: Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands; DTO: Working Collection of the Applied and Industrial Mycology Group of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; UAMH: University of Toronto, UAMH Centre for Global Microfungal Biodiversity, Toronto, Canada.
$^{T, ET, IT, NT}$ indicates the type strains.

RESULTS

Characterizations of the species

Seven fungal isolates with different colony types were isolated from dead root and crown tissues of two-months-old pistachio seedlings. Preliminary identification based on morphological characteristics showed that four of them belong to the genus *Cephalotrichum*. The isolates were nominated for further analyses. A BLAST search of the resulting ITS-rDNA sequences of AZFC P2 and AZFC P1-1 isolates showed 99.64% and 100% identity to *C. asperulum* strain CBS 127.22 (LN850959) and *C. gorgonifer* strain HHAUF170572 (MG886296), respectively. A combining of morphology and phylogeny inferred from ITS-rDNA sequences showed that they belong to *C. asperulum* and *C. gorgonifer*. To our knowledge, *C. gorgonifer* is a new record for the Iranian Funga. Furthermore, *Pistacia vera* is a new host (*matrix nova*) for *C. asperulum* and *C. gorgonifer*.

Phylogeny

The ITS-rDNA sequences of the two examined *Cephalotrichum* isolates here were combined and aligned with the 27 reference sequences of 16 taxa from GenBank. The alignment consisted of 591 characters including alignment gaps, of which 528 were constant, 58 were variable and 49 were parsimony-informative. Maximum parsimony

analysis resulted in a most parsimonious tree with a consistency index of 0.756098, the retention index of 0.888889 and composite index of 0.693529. Phylogenetic analyses based on ITS-rDNA sequence (Fig. 1) placed the examined isolates AZFC P2 and AZFC P1-1 with reference strains of *C. asperulum* CBS 582.71 and *C. gorgonifer* CBS 635.78, respectively in strongly supported monophyletic clades. Furthermore, *C. asperulum* and *C. gorgonifer* clades well separated from the close *Cephalotrichum* species. Based on the phylogenetic analyses and drawn MP tree the two *Cephalotrichum* isolates were belonged to *C. asperulum* and *C. gorgonifer*.

TAXONOMY

Cephalotrichum asperulum (Wright JE & Marchand S) Sandoval-Denis, Guarro & Gene, Studies in Mycology 83: 201. 2016. Fig. 2.

Colonies on PDA flat, white, gray to greenish gray at the center with age, reaching 33 mm diam in 14 d at 25 °C, sporulation rare; on PCA flat with regular margins, white, forming pale brown to brown concentric circles on the colony, reaching 38 mm diam in 14 d at 25 °C, synnemata forming on the surface of colony, sporulation initiates in the center and extends to the margins; on MEA, flat, white, turning pale brown to brown at the center with age, reaching 33 mm diam in 14 d at 25 °C. Mycelium composed of hyaline to pale brown, septate, smooth and thin-walled, 1.5–3.5 µm wide hyphae. Mononematous conidiophores pale brown to brown with a short stipe, abundantly seen among synnemata, regularly mono-verticillate or bi-verticillate, sometimes 2-level verticillate. Synnemata 610–1900 µm × 15–30 µm, stipes distinct, unbranched, pale brown to brown, conidial heads hyaline to pale brown, ellipsoidal or cylindrical. Hyphae of stipes 1.5–2.5 µm in width, pale-brown to brown, slightly thick-walled. Setae absent. Conidiophores in synnemata erratically mono-verticillate, bi-verticillate and rarely 3-level verticillate, metulae 6–9 × 2.5–3 µm. Conidiogenous cells ampulliform, hyaline to pale brown, 5–7 × 2.5–3 µm, smooth-walled, wide at the broadest part, tapered slowly to a cylindrical zone. Conidia ellipsoidal to obovoid, base mostly truncate and apices slightly pointed, 4.6–6.5 × 2.9–4 µm, pale brown to brown, smooth and thick-walled, forming basipetal chains (Fig. 2. d–g). Sexual morph not observed.

Specimen examined: IRAN, East Azarbaijan, Azarbaijan Shahid Madani university (37.812881, 45.953120), from decaying root and crown of pistachio seedlings, March 2019, Alireza Alizadeh, culture AZFC P2.

Notes: Our examined AZFC P2 isolate confidently clustered with the authentic reference strains and the type strain of *C. asperulum* (CBS 582.71) in the MP phylogram. *Cepahlotrichum cylindricum* and *C. transvaalense* have the closest relationship with *C. asperulum* in the tree, but the ITS-rDNA could well distinguish this species from *C. cylindricum* and *C. transvaalense* and all accepted taxa in the genus *Cepahlotrichum*. *Cepahlotrichum cylindricum* and *C. transvaalense* are two closely related taxa which can be differentiated also morphologically from *C. asperulum* by forming straight setae arising from the conidial head and lack of setae in the *C. asperulum*. Morphologically, *C. asperulum* resembles *C. spirale* in forming distinct verrucose conidia but *C. asperulum* can be discriminate from *C. spirale* generally by the conidial shape. *C. spirale* forming conidia with rounded apices whereas conidia in *C. asperulum* have pointed apices. Moreover, *C. asperulum* differs from *C. spirale* in terms of the conidial roughness, which the degree of this feature is not noticeable in *C. asperulum* which often form smooth conidia (Woudenberg et al. 2017).

Cephalotrichum gorgonifer (Bainier) Sandoval-Denis, Gene & Guarro, Studies in Mycology 83: 201. 2016. Fig. 3.

Colonies on PDA white, becoming gray to dark gray at the center with age, margins irregular, reaching 50 mm diam in 14 d at 25 °C; on PCA, flat, grey olivaceous to brownish grey with regular margins, reaching 38 mm diam in 14 d at 25 °C; on MEA, flat, gray, with white outer ring, reaching 38 mm diam in 14 d at 25 °C. sporulation initiates in the center and extends to the margins. Sporulation in PDA is much less than PCA and MEA. Mononematous conidiophores sparse among synnemata, hyaline, simple, mono-verticillate or irregularly bi-verticillate. Synnemata 1000-1990 × 8.5–12 µm, stipes brown to dark brown, conidial heads gray to brown, obclavate, cylindrical and elongated. Hyphae of stipe pale-brown, slightly thick-walled, irregular in width, the widest hyphae in the center of the stipe near the base. Setae present, coiled, unbranched, septate, pale brown, up to 200 µm long, apex rounded or acute. Conidiophores in synnemata solitary, monoverticillate, metulae 7–8 × 2.5–3 µm. Conidiogenous cells hyaline, ampulliform, 5.5–9 × 3–4 µm, wide at the broadest part, tapered slowly to a cylindrical annellated zone (1–2 µm wide), smooth-walled. Conidia ovoid to fusiform, gray to grayish brown, 4.8–5.8 × 2.8–3.8 µm, smooth, thick-walled, forming basipetal chains. Sexual morph not observed.

Specimen examined: IRAN, East Azarbaijan, Azarbaijan Shahid Madani university (37.812881, 45.953120), from decaying root and crown of pistachio seedlings, March 2019, Alireza Alizadeh, culture AZFC P1-1.

Notes: Our examined AZFC 1-1 isolate placed in a well-supported clade with the authentic reference strains of *C. gorgonifer* in the MP phylogram. So, ITS-rDNA could easily separate *C. gorgonifer* distinctly from all accepted *Cephalotrichum* species. *Cephalotrichum telluricum* is closely related to *C. gorgonifer* both morphologically and phylogenetically.

Two species resembles in developing spirally coiled setae arising from the conidial head, but can be

discriminate by height of synnemata (<500 µm in *C. telluricum* vs. >500 µm in *C. gorgonifer*). *Cephalotrichum gorgonifer* and *C. telluricum* can be easily distinguished from each other by ITS sequence so that ex-epitype isolate of C. *gorgonifer*, CBS 635.78 having 5 nt differences with *C. telluricum*. *Cephalotrichum gorgonifer* resembles morphologically *C. dendrocephalus* and *C. cylindricus*, but can be easily recognized from them in developing spirally coiled setae. Strains that do not form setae can be confused with *C. purpureofuscum*, but two species differ in term of conidial shape and color. The conidia of *C. gorgonifer* are gray to grayish brown in color, ovoid to fusiform with rounded apices while those of *C. purpureofuscum* are brown with slightly pointed apices (Sandoval-Denis et al. 2016; Woudenberg et al. 2017).

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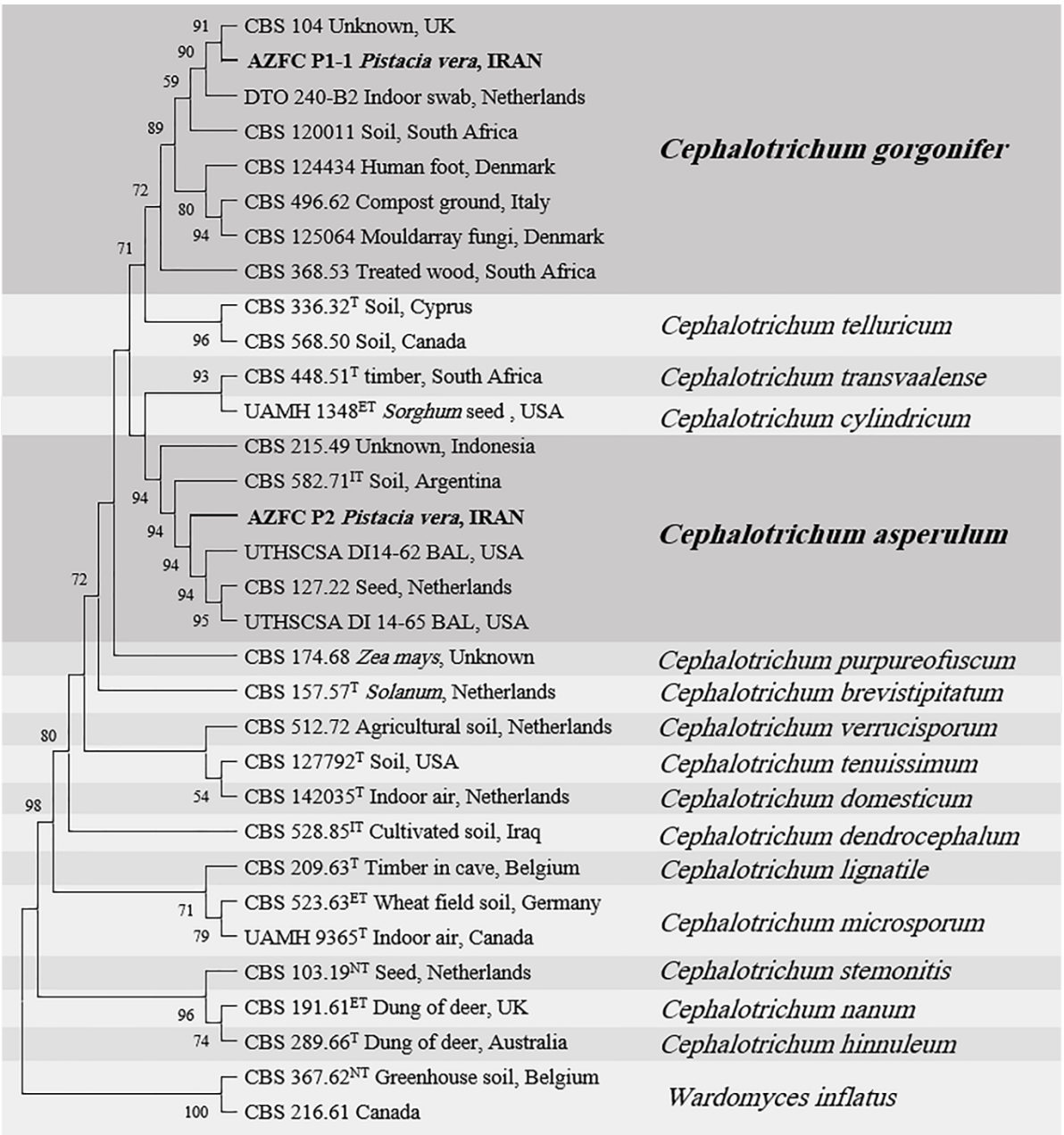

Fig. 1. The phylogenetic tree constructed from a maximum parsimony analysis for *Cephalotrichum* isolates based on the ITS-rDNA sequence data. Bootstrap values > 50% (1000 replicates) of MP analysis is shown on the branches. Obtained isolates AZFC P1-1 and AZFC P2 in this study are shown in bold. *Wardomyces inflatus* (CBS 367.62 and CBS 216.61) is used as out-group taxon.

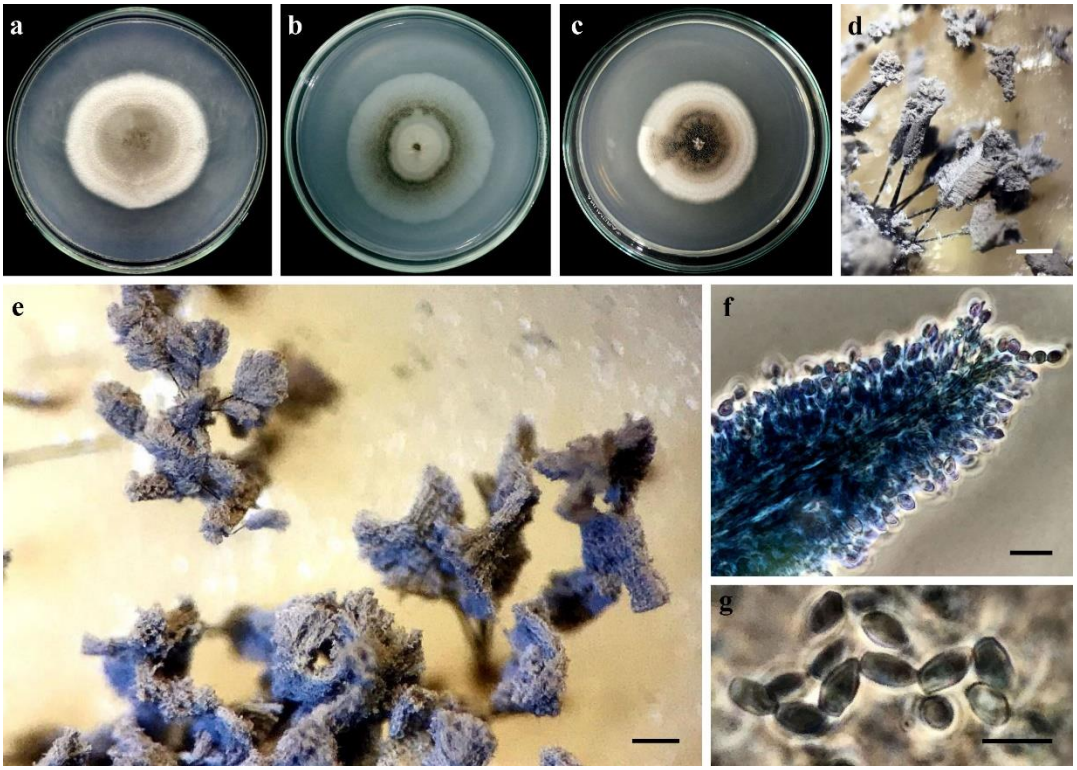

Fig 2- *Cephalotrichum asperulum* (isolate AZFC P2). Colonies on **a:** PDA, **b:** PCA and **c:** MEA. **d-e.** Synnemata. **f.** Apical portion of a synnema **g.** Conidia. Scale bars: **d-e** = 200 µm; **f-g** = 10 µm.

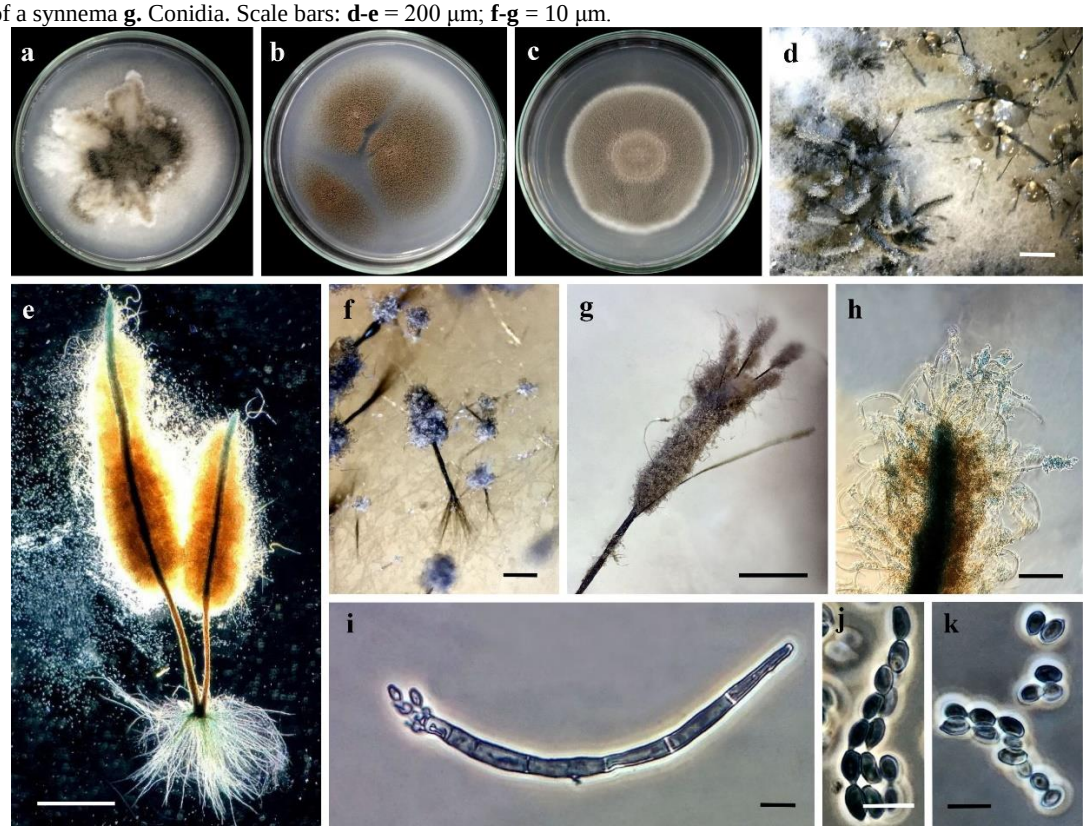

Fig 3- *Cephalotrichum gorgonifer* (isolate AZFC P1-1). Colonies on **a:** PDA, **b:** PCA and **c:** MEA. **d-g.** Synnemata. **h.** Apical portion of a synnema. **i.** Conidiophore and Conidiogenous cells. **j-k.** Conidia. Scale bars: **d-h** = 200 µm; **i-k** = 10 µm.

DISCUSSION

The genus *Cephalotrichum* is a synnematous hyphomycete that comprises several species that have been isolated and identified from very different substrates such as human foot, human hair, indoor air, grassland soil, forest soil, wheat field soil, rice field soil, mountain soil, sand dune soil, timber in cellar, manure, dung of hare, dung of deer, compost ground, mushroom compost, timber, tunnel wall and from a number of plant species (*Ligustrum vulgare*, *Zea mays*, *Eucalyptus saligna*, *Sorghum* seed) (Sandoval-Denis et al. 2016; Woudenberg et al. 2017). The taxonomy of the genus and the probable synonymy of *Cephalotrichum* and *Doratomyces* has been a subject of debate for many years. Sandoval-Denis et al. (2016), have been accepted *Doratomyces* as a synonym of *Cephalotrichum* based on analyses of the *LSU* and ITS rDNA genomic regions.

In this study, two synnematous species namely *C. asperulum* and *C. gorgonifer* were identified from decaying root and crown of pistachio based on a combination of morphological features and phylogeny inferred from ITS-rDNA sequence. According to Woudenberg et al. (2017), all phylogenetic species of *Cephalotrichum* recognized can be identified with ITS, except *C. cylindricum* and *C. transvaalense* which cannot be distinguished based on ITS alone, but morphology and *tub2* and *tef1* sequences clearly differentiate them from each other. The sequence of the ITS-rDNA could resolve the relationship of the examined *Cephalotrichum* isolates well, in concordance of morphological characters.

To our best of knowledge, this is the first report of *C. gorgonifer* for the mycobiota of Iran. *Pistacia vera* is also a new host (*matrix nova*) for the *C. asperulum* and *C. gorgonifer*. *Cephalotrichum gorgonifer* had been reported from different continents except Asia, so far. Isolating this species from decaying root and crown of pistachio in Iran propose that distribution of this species might be wider and further studies are needed to understand the geographical distribution of the species. This study provided new insights into the distribution and substrate range of the identified species.

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Cephalotrichum asperulum و *C. gorgonifer* دو آرایه قارچی سینمادار از پسته در ایران

علیرضا علیزاده✉، سمیه اکبرزاده، اکبر شیرزاد
گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه شهید مدنی آذربایجان، تبریز، ایران.

چکیده: در اسفند سال ۱۳۹۷، تعدادی از جدایه‌های قارچی سینمادار از بافت‌های مرده گیاهچه‌های پسته در گلخانه دانشگاه شهید مدنی آذربایجان، تبریز، ایران جداسازی شد. شناسایی مقدماتی بر اساس ویژگی‌های ریخت شناختی نشان داد جدایه‌های قارچی به جنس *Cephalotrichum* تعلق دارند. دو گونه *C. asperulum* و *C. gorgonifer* پس از ادغام داده‌های ریخت‌شناختی و تجزیه و تحلیل تبارزایی بر اساس توالی ناحیه ژنومی ITS-rDNA شناسایی شدند. بر اساس اطلاعات موجود *C. gorgonifer* آرایه جدیدی برای میکوبیوتای ایران محسوب می‌شود. همچنین پسته به عنوان میزبان جدید برای آرایه‌های *C. asperulum* و *C. gorgonifer* معرفی می‌شود.

کلمات کلیدی: پسته، تاکسونومی، ریخت‌شناسی، فیلوژنی، میکوبیوتا.

مکاتبه کننده: علیرضا علیزاده Email: alireza.alizadeh@azaruniv.ac.ir
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