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Original Article

New records of *Colletotrichum* species for the mycobiota of Iran

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Abstract: Sixteen isolates of *Colletotrichum* were collected from leaves with anthracnose symptoms or leaf spots of twelve wild, cultivated and ornamental plant species from the Guilan, Mazandaran, and Golestan provinces in Northern Iran. Five different species, including *C. aenigma, C. gigasporum, C. godetiae, C. karstii,* and *C. musae* were identified based on the DNA sequence data (*TUB2*, *GS*, *GAPDH*). Four of these species, termed as *C. aenigma, C. gigasporum, C. godetiae* and *C. karstii* represent new records for the mycobiota of Iran. Moreover, this study reports many plants as new hosts of *Colletotrichum* species. Comprehensive morphological descriptions and illustrations are provided for the species.

Key words: *Glomerellaceae*, phylogeny, systematics, taxonomy, plant pathogens

INTRODUCTION

Colletotrichum species are causal agents of anthracnose and other diseases on a wide range of plant species, and belong to the economically most important fungal pathogens (Sutton 1980; Hyde et al. 2009). Recently, the genus *Colletotrichum* was ranked in the top 10 fungal pathogens based on perceived scientific and economic importance (Dean et al. 2012). The genus has been recorded from approximately 2200 plant species (Farr & Rossman, 2016).

Precise identification has a crucial role in order to understand the epidemiology of *Colletotrichum* species and to develop effective control measures of the plant diseases. Traditional identification systems in *Colletotrichum* relied heavily on morphological and cultural characteristics (von Arx, 1957; Sutton, 1980, 1992; Bailey & Jeger, 1992; Freeman et al. 1998). However, Sutton (1992) mentioned that morphology does not provide sufficient and informative characters for an accurate identification. The systematics of *Colletotrichum* has been challenging due to the lack of reliable morphological characters making it difficult to delineate species boundaries (Cai et al. 2009). To resolve this deficiency, many researchers have used DNA sequence data, physiology, secondary metabolites and pathogenicity information, as part of a multifaceted approach (e.g. Than et al. 2008; Crouch et al. 2009; Prihastuti et al. 2009). Phylogenetic analyses based on DNA sequences have been led to significant progress in the systematics of the genus, and many species were differentiated within large species complexes, named as the Gloeosporioides species complex (Prihastuti et al. 2009; Phoulivong et al. 2010; Weir et al. 2012; Liu et al. 2013; Liu et al. 2015), the Acutatum species complex (Damm et al. 2012a), the Boninense species complex (Damm et al. 2012b), the Dematium species complex (Damm et al. 2009), the Orbiculare species complex (Damm et al. 2013) and the Destructivum species complex (Damm et al. 2014). Recently, two additional species complexes termed as the Caudatum species complex (Crouch, 2014) as well as the Gigasporum species complex (Liu et al. 2014) were introduced. The aim of this study was to characterize *Colletotrichum* species gained

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from the infected leaves of several plant species collected from the Northern Iran around the Caspian Sea, based on morphology, cultural characteristics and phylogenetic analyses of the DNA sequence data.

MATERIALS AND METHODS

Sample collection

Samplings were conducted during 2012–2013 in three provinces Guilan, Mazandaran and Golestan in Northern Iran. Samples were collected from hosts showing symptoms of anthracnose and spots on the leaves. Slices of plant samples (1 cm) were washed in tap water, surface-disinfected in 2% sodium hypochlorite solution for 1 min, rinsed in sterile distilled water and then moist incubated in glass petri dishes on paper towels soaked with sterile tap water. Petri dishes were kept at 20-25°C in the dark. Water agar (WA, 2%) plates supplemented with chloramphenicol (50 mg/L) were inoculated with a sterile needle dipped in conidial masses indicative of *Colletotrichum* that had formed on moist incubated plant tissue. Single spore or single hyphae were obtained on potato dextrose agar (PDA, Merck, Darmstadt, Germany) (Goh, 1999). Pure cultures were deposited at the Mycology Laboratory (UTFC ….) of College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. Additionally, the subcultures are preserved at Iranian Fungal Culture Collection (IRAN …C) at the Iranian Research Institute of Plant Protection, Tehran, Iran.

DNA extraction, PCR and sequencing

Fungal isolates were incubated on PDA at 25°C for 7–10 days. Genomic DNA was extracted using a standard phenol-chloroform extraction protocol (Sambrook, 2001). The primers T1 (O' Donnell & Cigelnik 1997) and Bt-2b (Glass & Donaldson 1995) were employed to amplify the partial β-tubulin (*TUB2*) gene. Polymerase chain reaction (PCR) was performed in a TProfessional Thermocycler (Biom-etra, Germany) in a total volume of 25 µL. The PCR mixture contained 1 µL genomic DNA, 0.2 µM of each primer, 1 × HF phusion PCR buffer (Thermo Scientific, Germany), 2 mM MgCl2, 20 µM of each dNTP, 0.75µ DMSO and 0.25 U Phusion High-Fidelity polymerase (Thermo Scientific, Germany). The PCR for *TUB2* was done as following; an initial step of 5 min at 98°C, 35 cycles of 10 s at 98°C, 20 s at 65°C and 20 s at 72 °C, followed by 10 min at 72 °C. Conditions for PCR amplification of the partial genes including glutamine synthetase (*GS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPD H*) followed by the protocols as previously described in Prihastuti et al. (2009) using the primers GSF1 & GSR1 (Stephenson et al. 1997) and GDF & GDR (Templeton et al. 1992), respectively. The PCR products were purified by Wizard Genomic DNA Purification Kit (Promega, USA) and sequenced by Macrogen Company (Amsterdam, the Netherlands) with the amplifying primers.

Morphology

Morphological experiments were performed according to Damm et al. (2012a). Both cultural and microscopic features were studied on synthetic nutrient agar (SNA) (Nirenberg 1976), and on oatmeal agar (OA) (Crous et al. 2009). Cultures were inoculated with 5-mm diameter plugs from 4–7 days old purified cultures. To enhance sporulation, isolates were transferred to SNA amended with autoclaved filter papers and double-autoclaved stems of *Anthriscus sylvestris*. SNA and OA cultures were incubated at 20°C under near-UV light with a 12 hours photoperiod for 10 days. Measurements and photomicrographs of fungal structures (conidia, conidiophores, conidiomata, setae, appressoria, sclerotia, perithecia, asci and ascospores) were made according to Damm et al. (2007). Appressoria were observed on the reverse side of SNA plates. Conidia were taken from acervuli. Microscopic preparations were made in clear lactic acid, with at least 30 measurements per structure and observed with a Nikon SMZ1000 dissecting microscope (DM) or with an Olympus BX51 microscope. Images were recorded with a Leica DFC320 camera on a Nikon SMZ1000 dissecting microscope (DM) or on an Olympus BX51 microscope. SNA and OA cultures were periodically examined for the development of perithecia. Ascospores were described from perithecia crushed in lactic acid. Colony characters and pigment production on SNA and OA cultures incubated for 14 days at 20°C under near-UV light with a 12 hours photoperiod were documented after 10 days. Colony color was determined according to the Rayner (1970). Growth rates were measured after 7 and 10 days.

Phylogenetic analysis

The programs Chromas Pro 1.34 and Edit Seq, parts of the DNA*Lasergene (DNAstar, Madison, WI) software package, were utilized for viewing, editing and assembling the sequences. The sequences were aligned by using MUSCLE, a multiple sequence alignment method (Edgar, 2004). The sequences of the 16 examined Iranian *Colletotrichum* isolates were compared with other fungal DNA sequences from NCBI GenBank database (www.ncbi.nlm.nih.gov/ genbank/) and the Q-bank Fungi database (http:// www.q-bank.eu/fungi/) through BLAST tool. Sixty sequences with high similarity were added to the alignment as reference strains (Table 1). Analyses were performed via the software MEGA v.6 (Tamura et al. 2013). Distance matrixes of the aligned sequences were calculated by the Kimura 2-parameter model (Kimura 1980), and analyzed with the maximum likelihood (ML) algorithm (Felsenstein 1985). The reliability of the inferred trees was estimated by bootstrap analyses with 1000 replicates.

In maximum parsimony (MP) analyses, the evolutionary distances were computed using the maximum composite likelihood method (Varin 2008). Gaps were treated as missing data in the pairwise sequence comparisons (Pairwise deletion option).

Table 1. A list of *Colletotrichum* isolates used in this study.

Species	Strain no.[1]	Host	Locality	GenBank Accession number		
				***Tub2*[2]**	***GAPDH*[3]**	***GS*[4]**
C. aenigma	ICMP 18608*	*Persea americana*	Israel	JX010389	JX010044	JX010078
	ICMP 18686	*Pyrus pyrifolia*	Japan	JX010390	JX009913	JX010079
	UTFC 239, IRAN 2443 C	*Vigna unguiculata*	Langrood, Rasht, Guilan, Iran	KU726942	KU726946	KU726944
C. aeschynomenes	ICMP 17673*	*Aeschynomene virginica*	USA	JX010392	-	-
C. acutatum	CBS 112996*	*Carica papay*	Australia	JQ005860	-	-
C. alatae	ICMP 17919*	*Dioscorea alata*	India	JX010383	-	-
C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	JX010411	-	-
C. annellatum	CBS 129826*	*Hevea indica*	Colombia	JQ005656	-	-
C. aotearoa	ICMP 18537*	*Coprosma* sp.	New Zealand	JX010420	-	-
C. asianum	CBS 130418*	*Coffea arabica*	Thailand	JX010406	-	-
C. arxii	CBS 132511*	*Paphiopedilum* sp.	Germany	KF687881	-	-
C. boninense	CBS 123755*	*Crinum asiaticum* var. *sinicum*	Japan	JQ005588	-	-
C. camelliae	LC1364*	*Camellia sinensis*	China	KJ955230	-	-
C. clidemiae	ICMP 18658*	*Clidemia hirta*	USA, Hawaii	JX010438	-	-
C. cordylinicola	ICMP 18579*	*Cordyline fruticosa*	Thailand	JX010440	-	-
C. dianesei	MFLU 1300058*	*Mangifera indica*	Brazil	KC517254	-	-
C. fioriniae	CBS 128517*	*Fiorinia exter*	USA	JQ949992	-	-
C. fructicola	ICMP 18581*	*Coffea Arabica*	Thailand	JX010405	-	-
C. fructivorum	CBS 133125*	*Vaccinium macrocarpon*	USA	JX145196	-	-
C. gigasporum	CBS 133266*	*Centella asiatica*	Madagascar	KF687866	-	-
	CBS 124947	*Theobromae cacao*	Panama	KF687871	-	-
	UTFC 255, IRAN 2444 C	*Camellia sineasis*	Lahijan, Guilan Province	KR905717	-	-
	UTFC 256	*Euonymus japonicus*	Namak-Abrood, Mazandaran Province	KR905718	-	-
C. gloeosporioides	IMI 356878*	*Citrus sinensis*	Italy	JX010445	-	-
C. godetiae	CBS 133.44*	*Clarkia hybrida*, cv. *kelvon glory*	Denmark	JQ950053	-	-
	CBS 129913, CPC 15126	*Podocarpus*	South Africa	JQ950087	-	-
	UTFC 257	*Frangula alnus*	Kheiroodkenar forest, Noushahr, Mazandaran Province	KR905719	-	-
	UTFC 258, IRAN 2447 C	*Acer cappadocicum*	Kheiroodkenar forest, Noushahr, Mazandaran Province	KR905720	-	-
	UTFC 259	*Frangula alnus*	Kheiroodkenar forest, Noushahr, Mazandaran Province	KR905721	-	-
	ICMP 18539*	*Olea europaea*	Australia	JX010434	-	-
C. grevilleae	CBS 132879*	*Grevillea* sp.	Italy	KC297102	-	-
C. hebeiense	JZB330028	*Vitis vinifera*	China	KF288975	KF377495	
C. henanense	CGMCC 3.17354*	*Camellia sinensis*	China	KJ955257	-	-
C. hipeastri	CBS 125377	*Hippeastrum vittatum*	China	JQ005664	-	-
C. horii	ICMP 10492*	*Diospyros kaki*	Japan	JX010450	-	-
C. johnstonii	CBS 128532, ICMP 12926*	*Solanum lycopersicum*	New Zealand	JQ950095	-	-
C. karstii	CGMCC3.14194*	*Vanda* sp.	China	HM585428.1	-	-
	CBS 127536	*Eucalyptus grandis*	South Africa	JQ005635	-	-
	CBS 861.72	*Bombax aquaticum*	Brazil	JQ005618	-	-
	UTFC 242	*Euonymus japonicus* var. *aureo-marginata*	Amol, Mazandaran Province	KR867693	-	-
	UTFC 243	*Syringia reticulate*	Sari, Mazandaran Province	KR905710	-	-
	UTFC 244	*Euonymus japonicus*	Ramsar , Mazandaran Province	KR905711	-	-
	UTFC 245	*Lourus nobilis*	Ramsar , Mazandaran Province	-	-	-
	UTFC 246	*Ficus benjaminia*	Gorgan , Golestan Province	KR905712	-	-
	UTFC 247	*Cyperus* sp.	Jelin, Golestan Province	KR905713	-	-
	UTFC 248	*Euonymus japonicus*	Tonkabon, Mazandaran Province	KR905714	-	-
	UTFC 249, IRAN 2448 C	*Passiflora edulis*	Tonkabon, Mazandaran Province	KR905715	-	-
	UTFC 250	*Passiflora edulis*	Tonkabon, Mazandaran Province	KR905716	-	-
C. kahawae subsp. *ciggaro*	ICMP 18539*	*Olea europaea*	Australia	JX010434	-	-
C. kahawae subsp. *kahawae*	ICMP 17816*	*Coffea Arabica*	Kenya	JX010444	-	-
C. lupine	CBS 109225*	*Lupinus albus*	Ukraine	JQ949806	-	-
C. magnisporum	CBS 398.84*	Unknown	Unknown	KF687882	-	-
C. melanocaulon	CBS 133251*	*Vaccinium macrocarpon*	USA	JX145195	-	-
C. musae	ICMP 19119*	*Musa* sp.	USA	HQ596280	-	-
	ICMP 17817	*Musa sapientum*	Kenya	JX010395	-	-
	UTFC 233	*Musa sapientum*	Gorgan, Golestan Province	KU726940	-	-
	UTFC 234	*Citrus sinensis*	Gorgan, Golestan Province	KU726941	KU726945	KU726943
C. nupharicola	ICMP 18187*	*Nuphar lutea* subsp. *polysepala*	USA	JX010398	-	-
C. nymphaeae	CBS 515.78*	*Nymphaea alba*	Netherlands	JQ949848	-	-
C. orchidophilum	CBS 632.80*	*Dendrobium* sp.	USA	JQ949802	-	-
C. paxtonii	IMI 165753*	*Musa* sp.	Saint Lucia	JQ949936	-	-
C. phormii	CBS 118194*	*Phormium* sp.	Germany	JQ950097	-	-
C. phyllanthi	CBS 175.67*	*Phyllanthus acidus*	India	JQ005655	-	-
C. proteae	CBS 132882*	*Protea sp.*	South Africa	KC297101	-	-
C. pseudomajus	CBS 571.88*	*Camellia sinensis*	Taiwan	KF687883	-	-
C. psidii	ICMP 19120*	*Psidium sp.*	Italy	JX010443	-	-
C. pyricola	CBS 128531*	*Pyrus communis*	New Zealand	JQ950096	-	-
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	JX010414	-	-
C. radices	CBS 529.93*	Unknown	Costa Rica	KF687869	-	-

Table 1. Continued.

Species	Strain no.[1]	Host	Locality	GenBank Accession number		
				Tub2[2]	***GAPDH***[3]	***GS***[4]
C. rhexiae	CBS 133134*	*Rhexia virginica*	USA	JX145179	-	-
C. salicis	CBS 607.94*	*Salix* sp.	Netherlands	JQ950111	-	-
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	JX010403	-	-
C. siamense	CBS 130417*	*Coffea Arabica*	Thailand	JX010404	-	-
C. syzygicola	DNCL021	*Syzigium Samarangense*	Thailand	KF254880	-	-
C. temperatum	CBS 133122*	*Vaccinium macrocarpon*	USA	JX145211	-	-
C. theobromicola	ICMP 18649*	*Theobroma cacao*	Panama	JX010447	-	-
C. ti	ICMP 4832*	*Cordyline* sp.	New Zealand	JX010442	-	-
C. tropicale	ICMP 18653*	*Theobroma cacao*	Panama	JX010407	-	-
C. vietnamense	CBS 125478*	*Coffea* sp.	Vietnam	KF687877	-	-
C. viniferum	JZB330008	*Vitis vinifera*	China	KF288962	-	-
C. xanthorrhoeae	CBS 127831*	*Xanthorrhoea preissii*	Australia	JX010448	-	-
Colletotrichum sp.	CBS 159.50	*Derris* sp.	Indonesia	KF687867	-	-

[1] **CBS** Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; **CGMCC** China General Microbial Culture Collection Center; **ICMP** International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; **IRAN** Iranian Fungal Culture Collection at the Iranian Research Institute of Plant Protection, Tehran, Iran; **UTFC** University of Tehran, Fungal Culture Collection, at mycology laboratory, University College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. **MFLU** Mae Fah Luang University, Thailand
[2] β-tubulin
[3] Glutamine synthetase
[4] Glyceraldehyde-3-phosphate dehydrogenase
The isolated strains and newly generated sequences in this study are shown in bold
*indicates the ex-type cultures

The MP tree was obtained using the close-neighbor-interchange algorithm of Nei & Kumar (2000) with search level 1 (Felsenstein 1985, Nei & Kumar 2000) in which the initial trees were randomly obtained by the addition of sequences (100 replicates). Clade stability was assessed 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 unit of the number of changes over the whole sequence.

RESULT AND DISCUSSION

Phylogeny

The *TUB2* sequences of the 16 tested *Colletotrichum* isolates here were combined and aligned with the 60 reference sequences of 54 taxa from GenBank. The alignment consisted of 476 characters including alignment gaps, of which 244 were constant, 232 were variable and 178 were parsimony-uninformative. Maximum parsimony analysis of the parsimony-informative characters resulted in 10 most parsimonious trees with a consistency index of 0.53, the retention index of 0.908486 and composite index of 0.488597 ML analysis resulted in a tree with the same topology as the MP trees. The ML tree is shown with bootstrap values of both analyses at the nodes (Fig. 1). Based on the phylogenetic analyses, the 16 isolates from various plants were grouped in four species complexes and five different *Colletotrichum* species named as *C. gigasporum* belonging to the Gigasporum species complex, *C. godetiae* belonging to the Acutatum species complex, *C. aenigma* and *C. musae* belonging to the Gloeosporoides species complex and *C. karstii* belonging to the Boninense species complex were identified (Fig. 1, Table 1). Four of these species, *C. gigasporum, C. godetiae, C. karstii* and *C. aenigma* are new records for the mycobiota of Iran.

Characterizations of the species

All characterized species are similar by having straight conidia, but they can be distinguished from each other using different phenotypic and morphological traits such as colony size and shape, conidial size and shape as well as conidial mass color. To clarify the relationship and accurate identifications of *Colletotrichum* isolates, we conducted phylogenetic analyses using the partial sequence of *β-tubulin* gene combined with morphological features. The sequences of the identified isolates of *Colletotrichum* were aligned against those available in Q-bank (http://www.q-bank.eu/fungi) and GenBank through blast search (Altschul et al. 1990). The sequence of the *TUB2* gene could resolve the relationship of the *Colletotrichum* isolates, mostly in agreement with morphological characters. The five described species here including *C. karstii, C. gigasporum, C. godetiae, C. aenigma* and *C. musae* belonging to four *Colletotrichum* species complexes (Boninens, Gigasporum, Acutatum and Gloeosporioides complexes). According to Damm et al. (2012 a, b) and Liu et al. (2014), *C. gigasporum, C. godetiae, C. karstii* and *C. musae* can be readily distinguished from closely related species within the Boninense, Gigasporum, Acutatum and Gloeosporioides complexes, by using the *TUB2* sequence data. Similarly, *C. aenigma* can be distinguished from closely related taxa in the Gloeosporioides complex via the combined *TUB2*, *GS* and *GAPDH* sequences (Weir et al. 2012; Yan et al. 2015). Therefore, we performed the partial *TUB2*, *GS* and *GAPDH* genes sequencing for the isolates belonging to the Gloeosporioides complex.

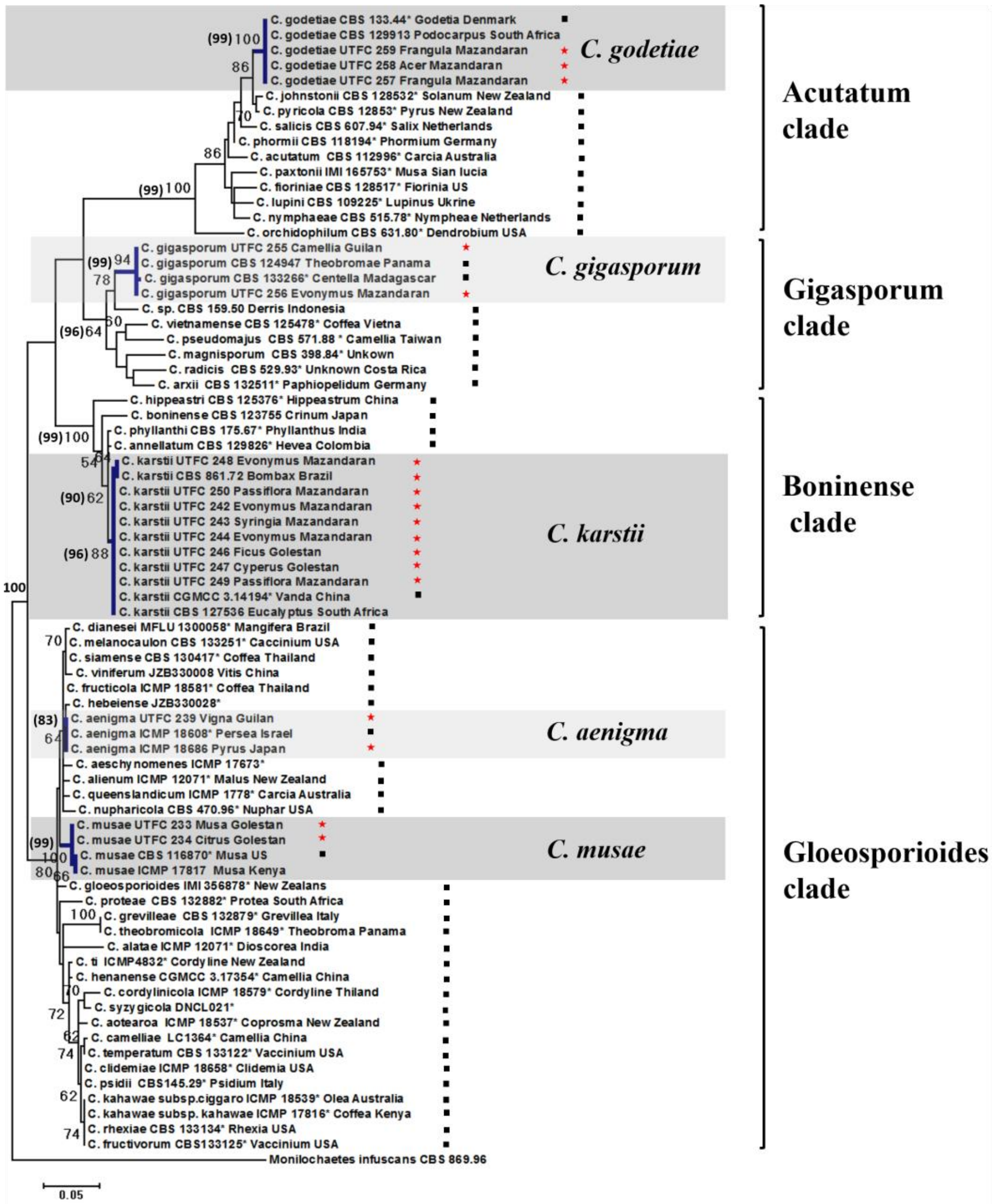

Fig. 1. The phylogenetic tree constructed from a maximum likelihood analysis based on the *TUB2* sequence data. Bootstrap values > 50% (1000 replicates) of ML analysis is exhibited above/below the branches and those of Maximum parsimony in brackets. Our isolates are marked with a red stars, and the strain number is followed by host and the country of origin. Black square indicates the ex-type strains. *Monilochaetes infuscans* was used as out-group taxon.

A BLASTN search of the *GS* and *GAPDH* sequences as well as phylogenetic analysis based on the combined *TUB2* and *GAPDH* sequences (Fig. 2) revealed that the *TUB2* sequence data is sufficient to precisely identify the examined species. Hence, the phylogenetic tree constructed for all tested isolates in this study is mainly based on the *TUB2* sequence alignment.

All identified species here are characterized as following:

Colletotrichum aenigma B. Weir & P.R. Johnst., Stud. Mycol. 73: 135 (2012) = *Colletotrichum populi* C.M. Tian & Z. Li, Mycotaxon 120: 283 (2012) (Fig. 3).

Morphology on SNA. Ascomata and perithecia are formed after 3-4 weeks, solitary, superficial or immersed in the agar medium, non-stromatic, globose to obpyriform, ostiolate, glabrous, brown, 190–250 × 150–200 µm. Asci unitunicate, 8-spored, cylindrical to clavate or fusiform, tapering to apex and base, smooth-walled, 42–68 × 10–11 µm. Ascospores uni or biseriately arranged, initially aseptate but often septate with age, hyaline, smooth-walled, variable in shape, fusiform to ovoid, slightly curved, (12–) 13–14 (–15) × (4.88–) 5.5–6 (–6.22) µm, mean ± SD = 13.5 ± 1.18 × 5.64 ± 0.62 µm, L/W ratio = 2.39. Conidiomata absent, the conidiophores are directly formed from vegetative hyphae (Fig. 3).

Morphology on Anthriscus stem. Conidiomata acervular, Conidiophores hyaline to pale brown, aseptate or septate, branched, Conidiogenous cells are haphazardly arised from dense, tangled hyphae across agar surface, short-cylindric with a poorly differentiated conidiogenous locus. Conidia often germinate soon after release, sometimes generate appressoria, thus create thin, compact, layers of germinated, septate conidia, germ tubes, and appressoria across the central part of the colony surface. Conidia (14.5–) 15.44–16 (–18.22) × (3.18–) 3.66–4 (–4.16) µm, mean ± SD = 16.1 ± 1.52 × 3.77 ± 0.44 µm, L/W ratio = 4.26, cylindric with broadly rounded ends. Appressoria 7–16 × 5–13 µm, mean ± SD = 11.46 ± 2.32 × 7.96 ± 1.69 µm, L/W ratio=1.43, subglobose or with a few broad lobes (Fig. 3).

Cultural characteristics. Colonies on OA flat with entire margin, aerial mycelium sparse, cottony, white, the surface of agar is uniformly pale orange towards center, more or less colorless towards edge, conidia are not well associated with acervuli and no masses of conidial ooze. In reverse pale orange towards center, 85 mm diameter after 10 days (Fig. 3).

Colletotrichum aenigma was described by Weir et al. (2012). Liu et al. (2013) considered *C. aenigma* similar to *C. populi*. *Colletotrichum aenigma* was isolated from *Pyrus pyrifolia* from Japan and *Persea americana* from Israel (Weir et al. 2012), *Vitis vinifera* from China (Yan et al. 2015) and *Populus* sp. from China (Liu et al. 2013). Weir et al. (2012) noted that the biology of the species is more or less unknown, but as it has been found in the widely separated regions, hence, it seems that to be a geographically widespread species.

Phylogenetically, *C. hebeiense* is a close species to *C. aenigma*, but the species are morphologically different. In *C. aenigma*, gattulates of the conidia are equally spread, while in *C. hebeiense* the granular contents are mostly present at the polar ends leaving a space in the middle. Appressoria of *C. hebeiense* are clavate to subglobose in shape while that of *C. aenigma* are subglobose, sometimes with few broad ends (Yan et al. 2015).

Phylogenetic analyses based on the partial *TUB2* gene (Fig. 1) grouped the studied isolate (UTFC 239) with ex-type strain ICMP 18608 from *Persea americana* from Israel, in a monophyletic clade with 62% bootstrap support. Additionally, in ML and MP trees, the ex-type strain of *C. hebeiense* (JZB330028) showed a close relationship to *C. aenigma* strains (Fig. 1). *Colletotrichum aenigma* differs from *C. hebeiense* by 1 base pair of ß-tubulin but they are distinguishable by the *GADPH* gene (Yan et al. 2015). Therefore, we employed the partial *TUB2*, *GS* and *GAPDH* genes to sequence the isolate UTFC 239. A BLASTN search was done by the *GS* and *GAPDH* sequences and the phylogenetic analysis was performed based on the combined *TUB2* and *GAPDH* sequences (Fig. 2) indicating the *TUB2* and *GAPDH* genes could act as suitable markers for the identification of *C. aenigma*.

Colletotrichum aenigma is a new record for the mycobiota of Iran. Furthermore, it is the first report of the species on *Vigna unquiculata*.

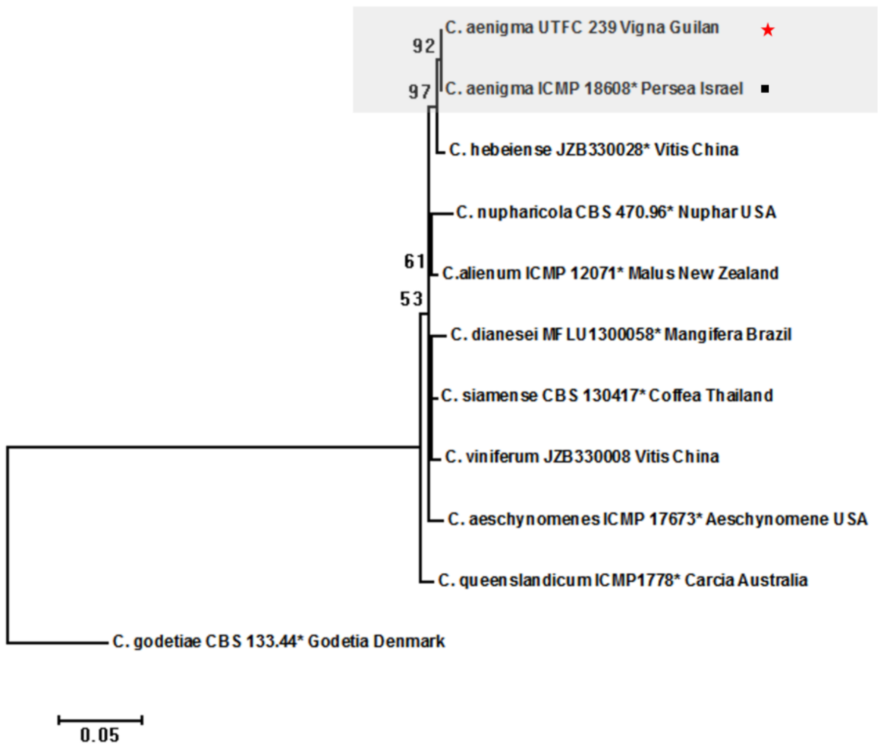

Fig. 2. The phylogenetic tree was generated by a maximum likelihood analysis based on the combined *TUB2* & GAPDH sequences data. Bootstrap values > 50% (1000 replicates) of ML analysis is shown above/below the branches. Our isolate is marked with a red star and a black square indicating the ex-type strain. *Colletotrichum godetiae* strain CBS 133.44 was used as out-group taxon.

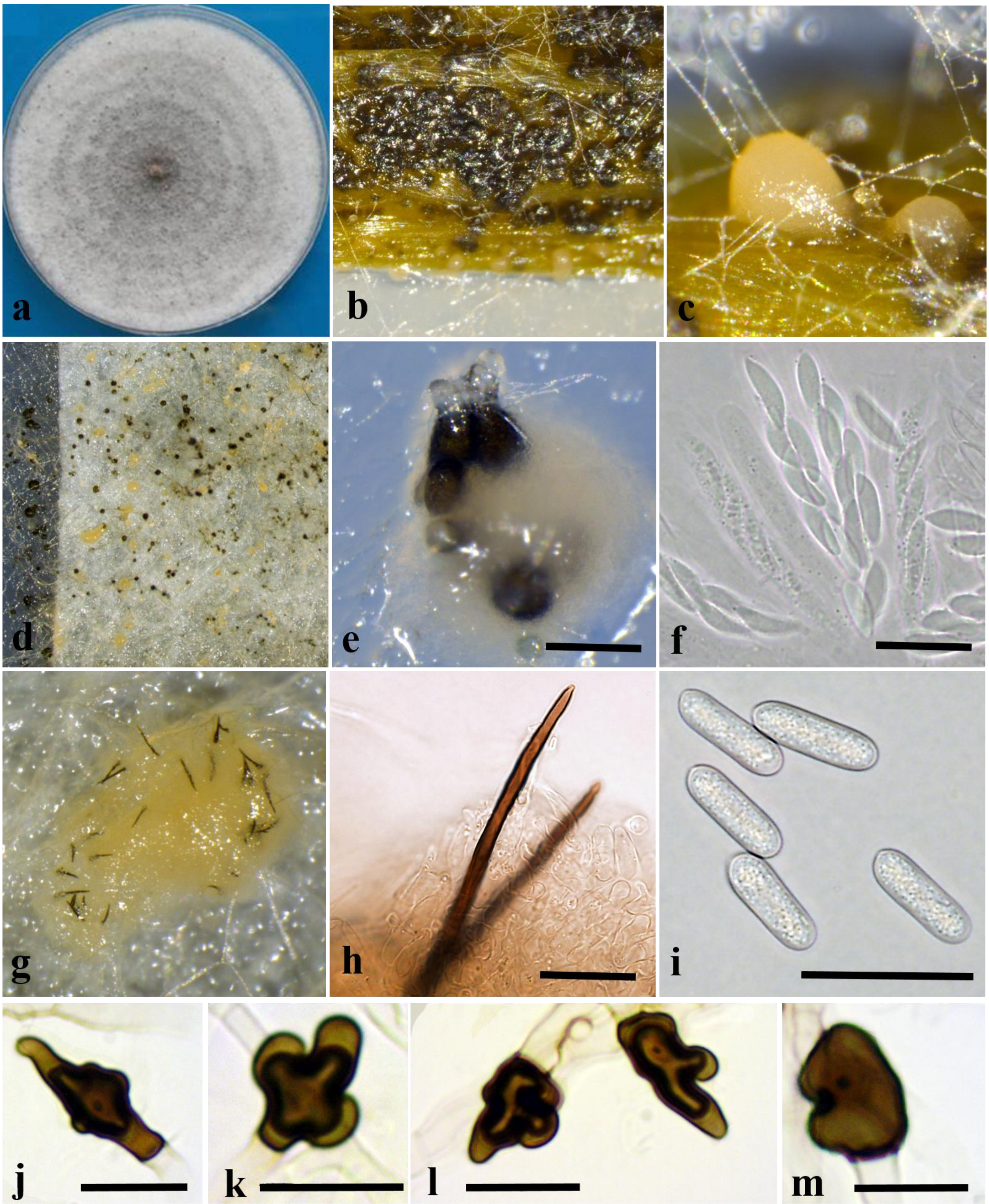

Fig. 3. *Colletotrichum aenigma* (UTFC 239), (IRAN 2443 C). **a.** Culture on OA, 10 days growth (upper); **b.** Ascomata and conidiomata on *Anthryscus* stem; **c.** Conidiomata on *Anthryscus* stem; **d.** Conidiomata on filter paper; **e.** Ascomata on SNA; **f.** Asci; **g.** Conidiomata on filter paper; **h.** Setae; **i.** Conidia; **j–m.** Appressoria. Scale bars — **c, d, f, g** = 200 µm; **e**= 100 µm; **h–i** = 20 µm; **j–m** =10 µm.

Colletotrichum gigasporum E.F. Rakotoniriana & Munaut, Mycol. Progr. 12: 407 (2013) (Fig. 4).

Morphology on Anthriscus stem. Conidiomata acervular, conidiophores and setae were formed on a cushion of angular brown cells. Setae medium to dark brown, smooth-walled to verruculose, 2–3-septate, 40–136.5 µm long, base cylindrical to inflated, 4.65–8.1 µm diameter, tip acute to obtuse. Conidiophores hyaline to brown, septate sometimes branched 27–34 × 5.5–10 µm. Conidiogenous cells hyaline to medium brown, cylindrical or clavate. Conidia hyaline, aseptate, smooth-walled, cylindrical with rounded ends (19–) 20.5–22(–23.7) × (6.65–) 7.15–7.85(–

8.30) µm, mean ± SD = 21.4 ± 1.83 × 7.45 ± 0. 67 µm, L/W ratio = 2.87 (Fig. 4).

Morphology on SNA. Conidiomata acervular. Appressoria pale brown, aseptate, solitary, with a ellipsoidal to irregular outline and a crenate or lobed margin, 6–23 × 4.5–13.5 µm, mean ± SD = 13 ± 4.5 × 8.5 ± 2.17 µm, L/W ratio = 1.52 (Fig. 4).

Cultural characteristics. Colonies on OA flat with entire margin, surface iron-grey with a white margin, aerial mycelium lacking; reverse grey to iron-grey; colonial diameter 6.6 mm in 7 days (> 90 mm in 10 days). Colonies on SNA flat with entire margin, medium hyaline, buff around *Anthryscus* stem, aerial mycelium lacking; colonial diameter 6.2 mm in 7 days (> 90 mm in 10 days) (Fig. 4).

Colletotrichum gigasporum is characterized by the large conidia (22–)25–29(–32) × (6–)7–9 µm. The phylogenetic analyses based on the ITS and *TUB2* sequences placed some species with large conidia in a distinct clade, which was far from the other accepted *Colletotrichum* species (Liu et al. 2014). Therefore, Rakotoniriana et al. (2013) considered a new complex and positioned these species in *C. gigasporum* species complex. Species of the *C. gigasporum* species complex can be easily distinguished from each other using all individual genes used by Liu et al. (2014): ITS, *ACT*, *TUB2*, *CHS-1* and *GAPDH* (Liu et al. 2014).

A BLASTN analysis for ITS sequences of the 22 previously misidentified strains of *C. crassipes, C. gloeosporioides, C. incarnatum, C. orbiculare* and *C. taiwanense* (*Glomerella septospora*) originating from GenBank clustered them with the ex-type strain of *C. gigasporum*. Thus, Liu et al. (2014) re-identified them as *C. gigasporum*. The size of ascospores and conidia of *C. gigasporum* is similar to those of *C. taiwanense*, but *C. gigasporum* forms aseptate conidia and 0–1-septate ascospores (Rakotoniriana et al. 2013, Liu et al. 2014), whereas the conidia of *C. taiwanense* may become 1–5 septate with age and ascospores are mostly 3 septate and may become up to 6 or 8 septate when getting old (Sivanesan & Hsieh 1993). Likewise, *C. thailandicum* was morphologically similar to *C. gigasporum*. Phylogenetic analyses of multilocus data demonstrated that the ex-type strains of the two species group together in one strongly supported clade. Therefore, Liu et al. (2014) synonymized *C. thailandicum* with *C. gigasporum*. Moreover, *C. gigasporum* differs from *C. incarnatum* (Zimmermann 1901), the species, which was initially described from *Coffea liberica* in Java, by generating larger conidia (20.5–25.5 × 6–9 µm vs 14–19 × 5 µm).

Colletotrichum gigasporum enables to infect the wide range of hosts and is geographically distributed (Africa, Central and South America, Asia and New Zealand). Rakotoniriana et al. (2013) reported that *C. gigasporum* can ecologically occur as either endophyte or pathogen, even were isolated from human tissues (Liu et al. 2014). The studied strains by Liu et al. (2014) was isolated from *Diospyros kaki*, *Solanum betaceum*, *Theobroma cacao*, *Virola surinamensis*, *Coffea* sp., *Trichilia tuberculata*, *Acacia auriculiformis*, *Musa* sp., *Centella asiatica*, *Homo sapiens*, air and stored grains, indicating it is not a host-specific isolate. *C. gigasporum* obtained from *Camellia sinensis* and *Euonymus japonicus* in Northern Iran. Therefore, we report *C. sinensis* and *E. japonica* as new hosts for *C. gigasporum*.

Colletotrichum godetiae Neerg., Friesia 4: 72 (1950) (Fig. 5).

Morphology on Anthryscus stem. Conidiomata acervular, Conidiophores hyaline, smooth-walled, simple, up to 15 µm long. Conidiogenous cells hyaline, smooth-walled, cylindrical, 5–12 × 3–5 µm, opening 1–2 µm diameter. Conidia hyaline, smooth-walled, aseptate, straight, cylindrical to fusiform with both ends acute or one end round and one end slightly acute, (8–) 10–14(–14.5) × (3.5–) 4–4.5(–5) µm, mean ± SD = 12.6 ± 4.0 × 4.4 ± 0.5 µm, L/W ratio = 2.85 (Fig. 5).

Morphology on SNA. Sexual state, chlamydospores and conidiomata absent, conidiophores are directly formed on hyphae. Setae absent. Appressoria solitary, medium brown, smooth-walled, clavate to elliptical, the edge entire or undulate 6.5–15.5 × 4.5–9 µm, mean ± SD = 9.5 ± 1.8 × 6 ± 1.1 µm, L/W ratio = 1.58 (Fig. 5).

Cultural characteristics. Colonies on SNA flat with entire margin, hyaline, with little low white aerial mycelium, SNA growth rate 5.2 mm in 7 days (70 mm in 10 days). Colonies on OA flat with entire margin; surface salmon to olive-gray, dark gray in center, no aerial mycelium, reverse salmon to gray; growth rate 40 mm in 7 days (6.2 mm in 10 days) (Fig. 5).

Colletotrichum godetiae was initially described from the seed of *Clarkia* (syn. *Godetia*) *hybrida* cv. *Kelvedon Glory* by Neergard (1943). The species happens on a wide host range such as *Fragaria*, *Malus*, *Prunus*, *Rhododendron*, *Aeschynomene*, *Agrimonia*, *Bonzai*, *Ceanothus*, *Citrus*, *Fragaria*, *Juglans*, *Laurus*, *Mahonia*, *Olea*, *Parthenocissus*, *Podocarpus*, *Prunus*, *Rubus*, *Sambucus*, *Vitis*, *Schinus*, *Solanum* and *Ugni*, the species that is mainly present in the Europe and the Near East causing fruit, leaf, stem, cane and twig diseases (Damm et al. 2012). *C. godetiae* (UTFC 257, UTFC 258 and UTFC 259) obtained from *Frangula alnus* and *Acer cappadocicum*. Therefore, we report these plants as new hosts for *C. godetiae*. Furthermore, *C. godetiae* is a new record for the mycobiota of Iran.

Colletotrichum godetiae is separated from other species in the Acutatum species complex by the ITS, *ACT*, *TUB2*, *CHS*-1, *GAPDH* genes. The *TUB2*, *ACT* and *HIS3* provide more robust and precise phylogeny (Dam et al. 2012). In *TUB2* ML tree UTFC 257, UTFC 258 and UTFC 259 isolates clustered with holotype strain CBS133.44 from *godetia* and CBS 129913 from *Podocarpus* that in a clade with 100% bootstrap support (Fig. 1).

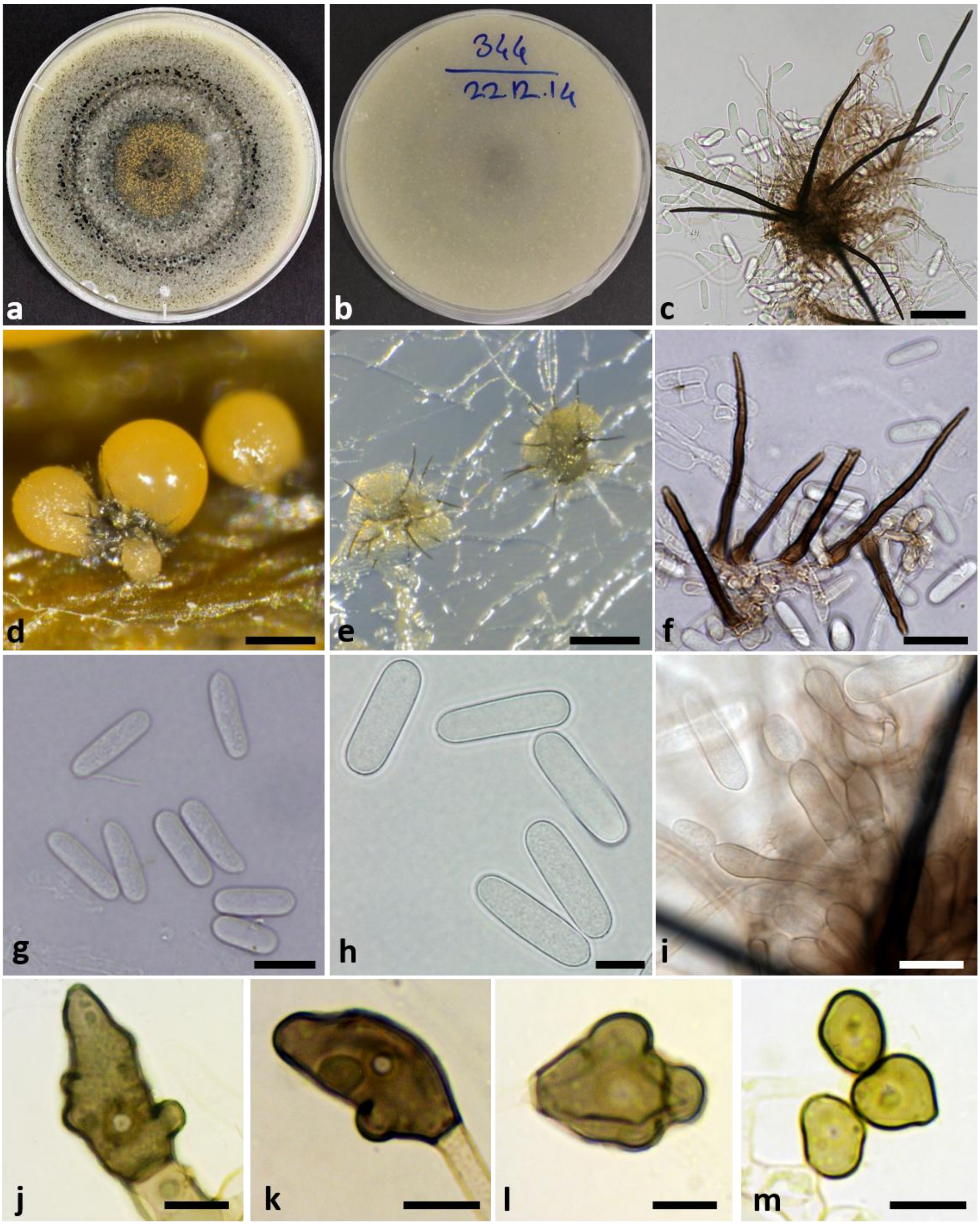

Fig. 4. *Colletotrichum gigasporum* (UTFC 255), (IRAN 2444 C). **a–b**. Cultures on OA, 10 days growth, upper (**a**) and reverse (**b**); **c.** Setae; **d.** Conidiomata on *Anthryscus* stem; **e–f**. Conidiomata on SNA; **g–h.** Conidia; **i.** Conidiophores; **j–m.** Appressoria. Scale bars — **c** = 20 µm; **d–f** = 50 µm; **g–m** = 10 µm.

Colletotrichum karstii Y.L. Yang, Z.Y. Liu, K.D. Hyde & L. Cai, Cryptogamie Mycologie 32: 241 (2011) (Fig. 6).

Morphology on Anthriscus stem. Conidiomata acervular, conidiophores hyaline to pale brown, aseptate or septate, branched, 30-80 µm long. Conidiogenous cells hyaline to pale brown, smooth-walled, cylindrical to ampulliform, sometimes extending to form new conidiogenous loci, 4.55–9.7 × 3–4.5 µm, opening 1–2 µm diameter. Conidia hyaline, smooth-walled, aseptate, straight, cylindrical, apex round, base round with a prominent hillum, the contents appearing granular, (12.85–) 13.90–14.2(–14.65) × (4.8–) 4.85–5(–5.35) µm, mean ± SD = 13.90 ± 0.76 × 5.0 ± 0.32 µm, L/W ratio = 2.75 (Fig. 6).

Morphology on SNA. Ascomata and perithecia are formed after 3-4 weeks, solitary, superficial or immersed in the agar medium, non-stromatic, globose to obpyriform, ostiolate, glabrous, brown, 96–122 × 91–111 µm. Asci unitunicate, 8-spored, cylindrical to clavate or fusiform, tapering to apex and base, smooth-walled, 39–54 × 10–12 µm. Ascospores are arranged either uni or biseriately, initially aseptate but often generate septate with age, hyaline, smooth-walled, variable in shape, fusiform to ovoid, slightly curved, (14.95–)16.8–17.2(–18.3) × (5.3–)5.7–6.15(–7.1) µm, mean ± SD = 16.8 ± 1.45 × 6.1 ± 0.75 µm, L/W ratio = 2.75. Chlamydospores and conidiomata absent, the conidiophores are directly formed from vegetative hyphae. Setae absent (Fig. 6).

Cultural characteristics. Colonies on SNA flat with entire margin, hyaline, with filter paper and *Anthryscus* stem covered with orange conidiomata and partly with white mycelium; reverse hyaline with grey flecks mainly under the filter paper, 59 mm in 7 days (80 mm in 10 days). Colonies on OA flat with entire margin, pale brown or buff to rosy buff to pale salmon, covered with orange to grey conidiomata in center; reverse brown to pale brown, buff, rosy buff to honey, 50 mm in 7 days (72 mm in 10 days) (Fig. 6).

Colletotrichum karstii described by Yang et al. (2011), and is known as pathogen and endophyte from the leaf and root of some plants such as *Vanda* sp., *Calanthe argenteo-striata*, *Eria coronaria*, *Pleione bulbocodioides* (*Orchidaceae*) (Yang et al. 2011), *Bletilla ochracea* (*Orchidaceae*) (Tao 2013) and *Passiflora edulis* in Brazil (Tozze et al. 2010). It occurs on many host plants and is the most common and geographically diverse species in the *C. boninense* complex (Damm et al. 2012).

Colletotrichum karstii is a polymorphic isolate in terms of sequence and is morphologically diverse isolate as well. Moreover, strains of the species show significant differences in conidium size and conidiomatal structures ranging from sporodochial to acervular to closed. Therefore, it is difficult or impossible to identify the species based on morphology. The conidia of *C. karstii* are broader than those of *C. phyllanthi* and smaller than those of *C. hippeastri* and *C. dracaenae*. Similarly, the asci are shorter than those of *C. brassicicola* and *C. dracaenae*.

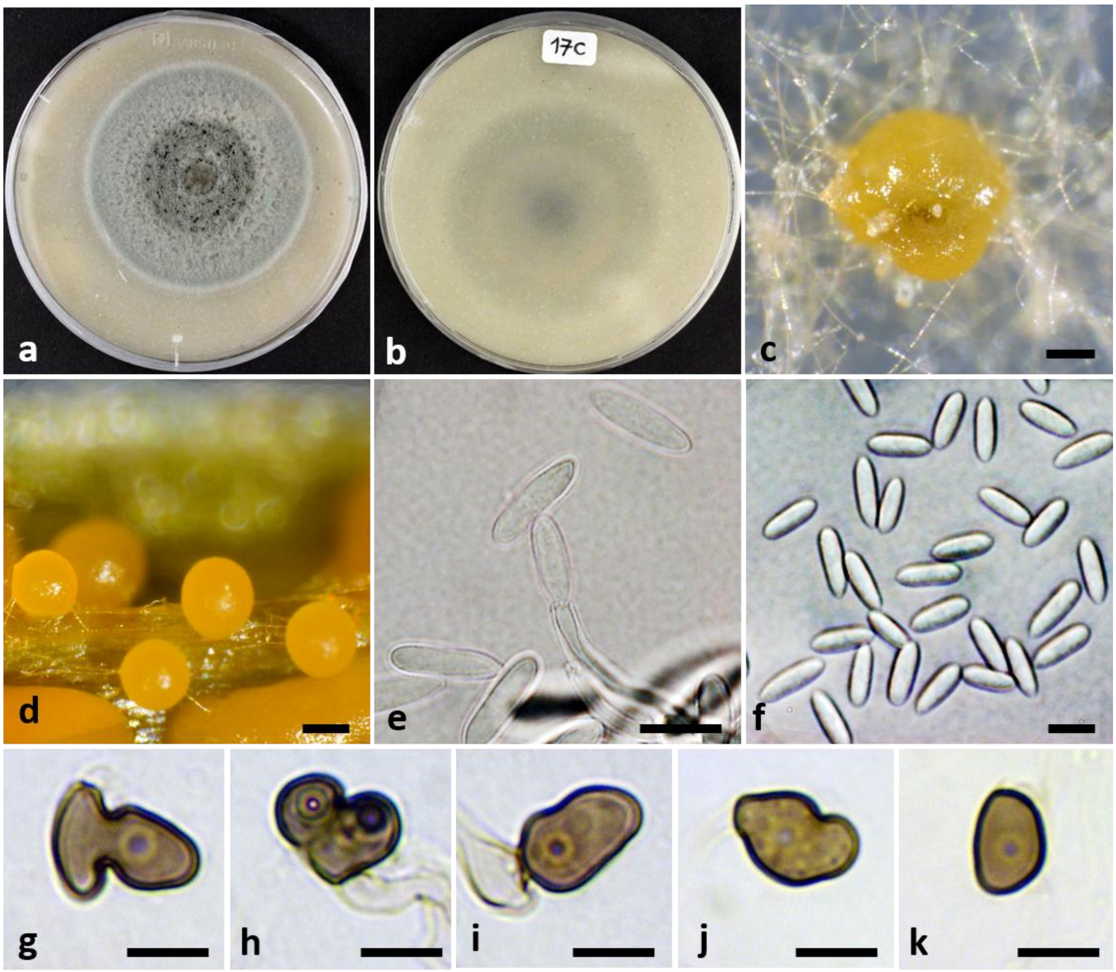

Fig. 5. *Colletotrichum godetiae* (UTFC 258), (IRAN 2447 C). **a–b.** Cultures on OA, 10 days growth, upper (**a**) and reverse (**b**); **c.** Conidiomata on SNA; **d.** Conidiomata on *Anthryscus* stem; **e.** Conidiophores; **f.** Conidia; **g–k.** Appressoria. Scale bars — **c–d** = 50 µm; **e–f** = 10 µm; **g–k** = 5 µm.

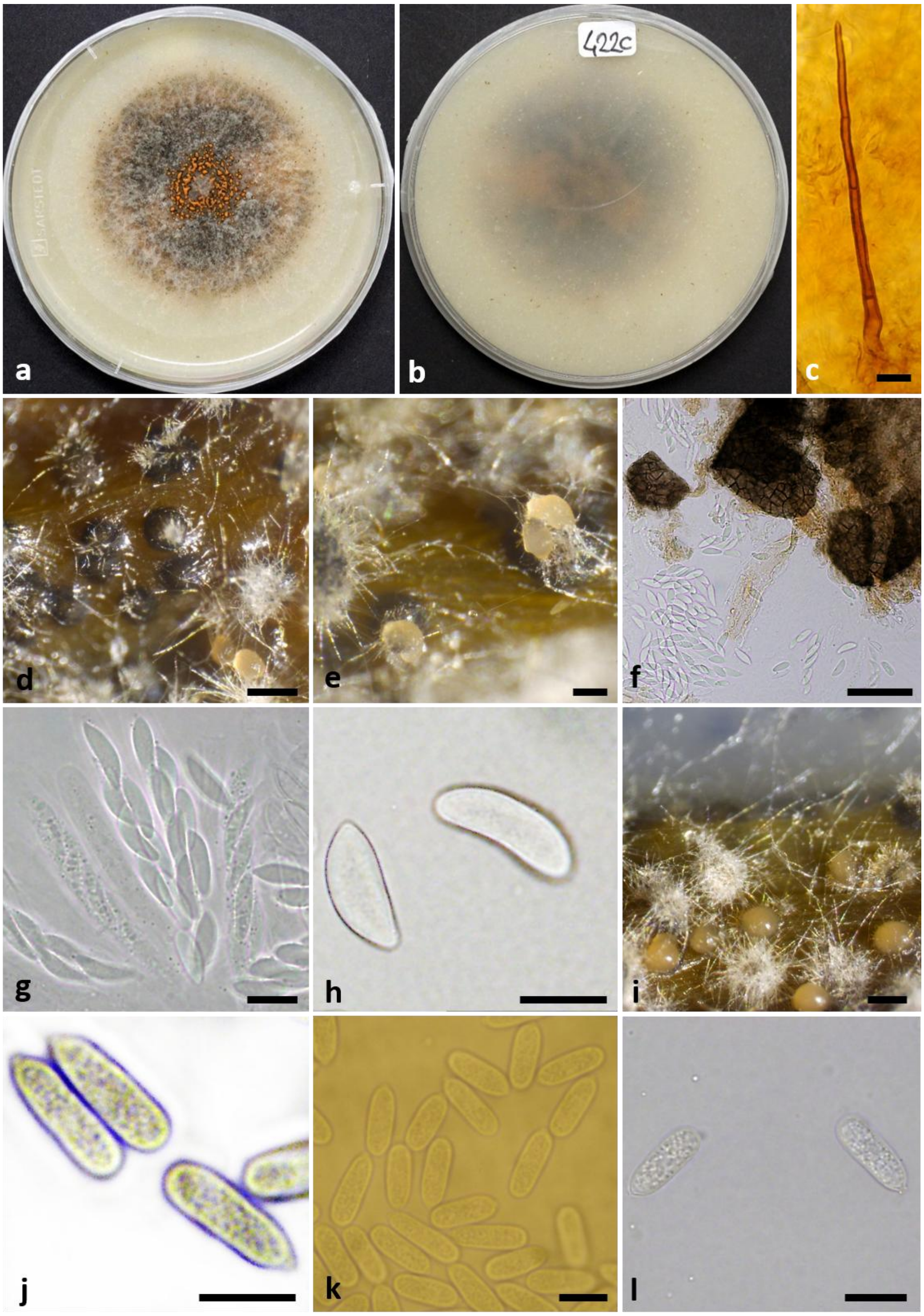

Fig. 6. *Colletotrichum karstii* (UTFC 242), (IRAN 2448 C). **a–b.** Cultures on OA, 10 days growth, upper (**a**) and reverse (**b**). **c.** Setae. **d–f.** Ascomata on *Anthryscus* stem. **g.** Asci. **h**. Ascospores**. i.** Conidiomata on *Anthryscus* stem. **j–l.** Conidia. Scale bars — **c, h, j-l** = 10 µm; **e–f, i** = 50 µm; **d** = 100 µm.

In addition, *C. boninense* has slightly wider and less tapered ascospores in comparation with *C. karstii* (Damm et al. 2012b).

Phylogenetic analyses based on the partial *TUB2* gene (Fig. 1) put together the examined isolates in this study (UTFC 242, UTFC 243, UTFC 244, UTFC 246, UTFC 247, UTFC 248, UTFC 249, UTFC 250) with holotype strain CGMCC 3.14194 from *Vanda* sp. in a monophyletic clade with 100% bootstrap support. Furthermore, the UTFC 248 exhibited a close relationship in a sub clade with strain CBS 861.72, which was isolated from Bombax in Brazil (Fig. 1).

Colletotrichum karstii is a new record for the mycobiota of Iran. In addition, it is first report of the species on *Euonymus japonicus* var. *aureo-marginata*, *Euonymus japonicus*, *Syringa* *reticulata*, *Laurus nobilis*, *Ficus benjamina* and *Cyperus* sp. Moreover, this is the first report indicating the occurrence of *C. karstii* on *Passiflora edulis* in Iran.

Colletotrichum musae (Berk. & M.A. Curtis) Arx, Verh. Kon. Ned. Akad. Wetensch., Afd. Natuurk., Sect. 2 51(3): 107. 1957 (Fig. 7).

Morpholgy on Anthryscus stem. Conidiophores cylindrical, tapered toward the apex, hyaline, subhyaline toward he base, up to 30 µm long. Conidia abundant, hyaline, aseptate, guttulate, oval, elliptical or cylindrical, often with a flattened base, apex obtuse, 9.5–15 × 4.35–6, mean ± SD = 13.5 ± 2.1 × 5.15 ± 1.1 µm, L/W ratio=2.62. Appressoria common, medium to dark brown, irregular in shape, often with large or deep lobes, 9-13 × 9-11.5µm (Fig. 7).

Cultural characteristics. Colonies on OA circular, with sparse to abundant, white to grey floccose aerial mycelium, conidial masses well developed, orange to cinnamon conidial mass; reverse grey-yellowish, 75 mm in 7 days. Sclerotia and Setae absent (Fig. 7).

Colletotrichum musae was originally described from the North Carolina (Berkeley 1874), and the name was epitypified by Su et al. (2011). The species is known as the pathogen of *Musa* sp. worldwide (Sutton 1980, Su et al. 2011, Weir et al. 2012). The *TUB2* sequence separates *C. musae* from all other species in the Gloeosporioides complex.

Phylogenetic analyses based on the partial *TUB2* gene (Fig. 1) assigned the examined isolates in this study (UTFC 233 and UTFC 234) with holotype strain ICMP 19119 from *Musa* sp. in a monophyletic clade with 98% bootstrap support.

Colletotrichum musae isolates (UTFC 233 and UTFC 234) obtained from *Musa sapientum* and *Citrus sinensis*. Consequently, this is the first report of *C. musae* on *C. sinensis*.

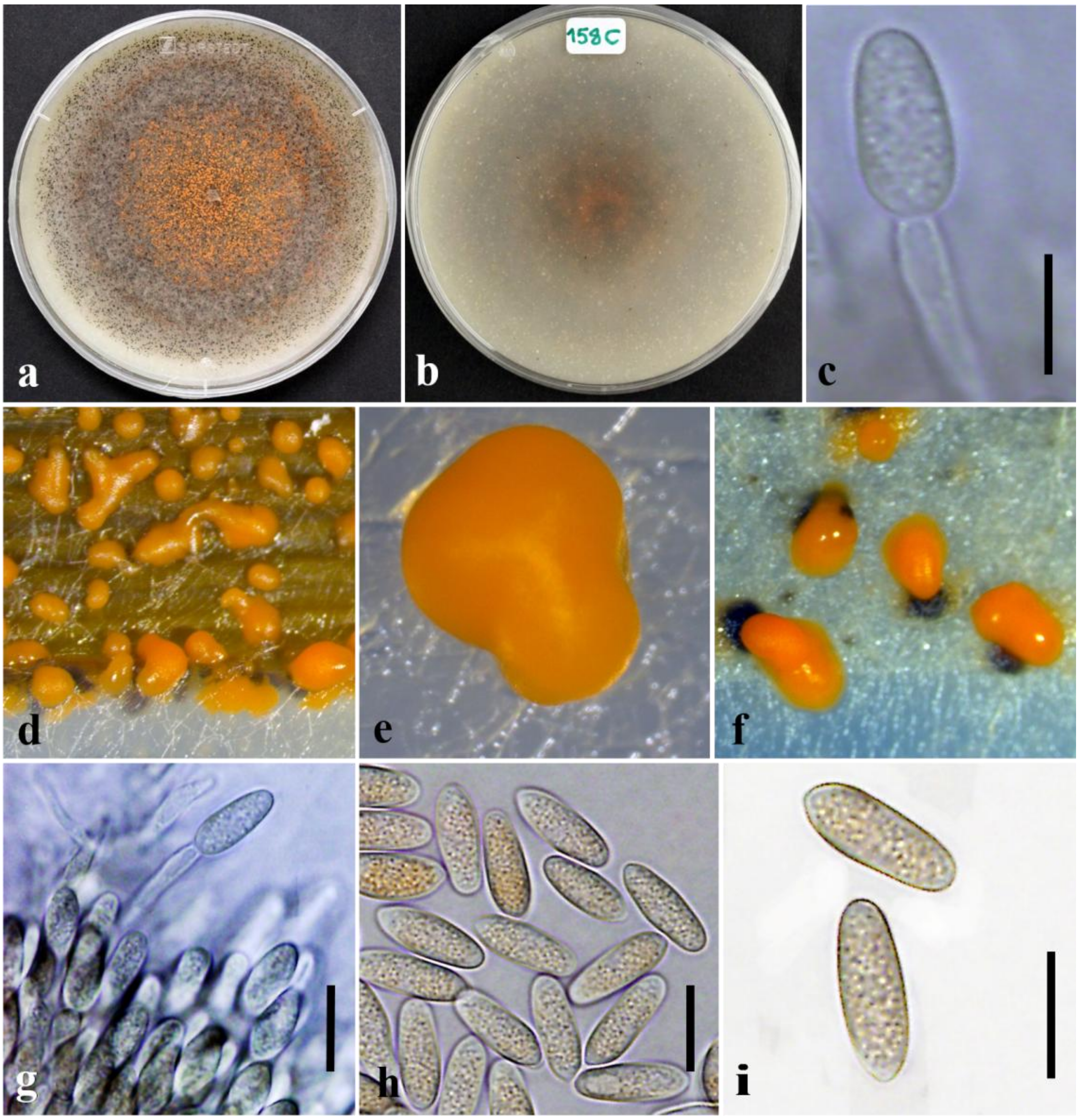

Fig. 7. *Colletotrichum musae* (UTFC 234). **a–b.** Cultures on OA, 10 days growth, upper (**a**) and reverse (**b**); **c.** Conidiophore; **d.** Conidiomata on Anthryscus stem; **e.** Conidiomata on SNA; **f.** Conidiomata on filter paper; **g.** Conidiophore; **h–i.** Conidia. Scale bars — **c** = 20 µm; **d, f** = 200 µm; **e** = 50 µm; **g–i** = 20 µm.

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گزارش جدید از گونه‌های *Colletotrichum* برای میکوبیوتای ایران

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چکیده: تعداد شانزده جدایه از جنس *Colletotrichum* از دوازده گونه از گیاهان وحشی، اهلی و زینتی از سه استان گیلان، مازندران و گلستان در شمال ایران که علایم آنتراکنوز و لکه برگی در اندام برگ نشان می‌دادند، جداسازی گردید. پنج گونه شامل گونه‌های *C. aenigma*، *C. gigasporum*، *C. godetiae*، *C. karstii* و *C. musae* از طریق تعیین توالی نواحی ژنی DNA (*TUB2*، *GS* و *GAPDH*) و بررسی‌های ریخت شناختی شناسایی گردید. از این میان، چهار گونه *C. aenigma*، *C. gigasporum*، *C. godetiae* و *C. karstii* برای اولین بار از ایران گزارش می‌شوند. همچنین در این مطالعه تعداد زیادی از گیاهان به عنوان میزبان جدید برای گونه‌های جنس *Colletotrichum* معرفی می شوند. توصیف جامع ریخت شناختی و تشریح مصور برای گونه‌ها ارایه شده است.

واژه‌های کلیدی: *Glomerellaceae*، فیلوژنی، سیستماتیک، تاکسونومی، بیمارگر گیاهی

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