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Species of *Colletotrichum* associated with citrus trees in Iran

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Abstract: *Colletotrichum* species are associated with citrus plants as pathogens, saprobes and endophytes. According to the most recent multigene phylogenetic analysis, a lot of changes were happened in the taxonomy and species delimitation in the genus *Colletotrichum*. In this investigation, 292 *Colletotrichum* isolates were obtained from leaves, fruits and stems of *Citrus* species at Golestan, Mazandaran, Guilan and Kerman provinces. After morphological studies, a multilocus molecular phylogenetic analysis (*TUB2*, *CHS*–1, *CAL*) of 13 isolates were carried out. Based on the morphological and molecular data, five species including *C. gloeosporioides* s. s., *C. fructicola* and *C. siamense* (from *C. gloeosporioides* s. l.); *C. karstii* and *C. novae zelandiae* (from *C. boninense* s. l.) were identified. According to our knowledge, this is the first report of *C. novae–zelandiae* from Iran and *C. siamense* and *C. karstii* from citrus plants in the country.

Key words: Biodiversity, morphology, pathogen, phylogeny, species complex

INTRODUCTION

Iran, with 276,000 hectares of cultivation area and annual production of 4.293 million tons of citrus fruit is among the top ten countries in the world in citrus production (Anonymous, 2014). Several *Colletotrichum* species cause anthracnose disease in many plants in tropical, subtropical and temperate areas (Jeffries et al. 1990; Bailey & Jeger 1992; Dodd et al. 1992; Freeman et al. 1998; Latunde–Dada, 2001; Wharton & Dieguez –Uribeondo 2004; Peres et al. 2005).

Colletotrichum gloeosporioides is one the most important species, which is regarded as the causal agent of anthracnose for many agriculturally important crops. However, for many years, *C. gloeosporioides* was considered as a complex species (Weir et al. 2012).

Colletotrichum acutatum (Damm et al. 2012 a) and *C. boninense* (Damm et al. 2012 b) are other important species complexes which cause plant diseases. For many years, identification and species delimitation were based on the morphological characters, such as shape and size of conidia and appressoria, color of colony, presence or absence of setae and teleomorph, host species and growth rate of hyphae on medium (Weir et al. 2012). These characters can vary in different media and cultural conditions, or be absent in subcultures (Weir et al. 2012). Recently, according to the molecular findings, especially the multi–gene phylogenetic analysis, classification, taxonomic placement and species concepts in *Colletotrichum* were changed (Cannon et al. 2012; Damm et al. 2012b; Weir et al., 2012).

Many genes and genomic regions such as actin (*ACT*), calmodulin (*CAL*), chitin synthase (*CHS*–1), glyceraldehyde–3–phosphate dehydrogenase (*GAPDH*), ribosomal internal transcribed spacer (*ITS*), glutamine synthase (*GS*), manganese superoxide dismutase (*SOD2*), β–tubulin 2 (*TUB2*) and histone3 (*HIS3*) have been used for revision and taxonomic treatment of some species complexes of *Colletotrichum* (Damm et al. 2012 a, b; Weir et al. 2012). Recent studies have led to the introduction of new species isolated from citrus. In Guizhou and Yunnan provinces in China, *C. boninense*, *C. brevispora*, *C. fructicola*, *C. gloeosporioides*, *C. karstii*, *C. simmondsii* and *C. murrayae* were isolated from citrus leaves (Peng et al. 2012). Also, *C. gloeospor–ioides, C. fructicola, C. karstii, C. citricola* and *C. citri* were reported from citrus in China (Huang et al. 2013).

Citrus anthracnose is a common disease of citrus in the north of Iran. Initially, *C. gloeosporioides* as the

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causal agent of disease was reported by Petrak & Esfandiari (1941) from coastal areas of Caspian Sea in the north, and in 1995 from citrus orchards in the south of Iran (Ershad 2009).

In Iran, based on multi–gene phylogenetic analyses of internal transcribed spacer (ITS), beta tubulin (TUB), histone H3 (HIS), calmodulin (CAL), and actin (ACT) loci, *C. fructicola* was reported from *Citrus sinensis* (Arzanlou et al. 2015).

During the recent years, several researchers have addressed some aspects of etiology and distribution (Babri et al. 2008), pathogenicity (Babri et al. 2009; Davarian et al. 2006) and vegetative compatibility groups of *C. gloeosporioides* (Khansari Atigh et al. 2010), relative susceptibility of some citrus cultivars to *C. gloeosporioides* (Babri et al. 2007), sexual fertility, mating type and genetic diversity of *Glomerella cingulata* by rep–PCR (Behnia et al. 2016) in Iran. However, identification of *Colletotrichum* species in these studies has been based on the morphological characteristics. Due to the high morphological similarity of these fungal isolates on citrus plants, identification of such species solely based on morphological characters have often been inadequate (Brown et al., 1996), and such species are considered as *C. gloeosporioides* complex species.

The main objective of this study was to reconstruct phylogenetic relationships and improve the current species concept of *Colletotrichum* on citrus plants based on morphological and molecular characteristics in Iran.

MATERIALS AND METHODS

Sampling, isolation and culture

During 2013–14, symptomatic tissues of different citrus varieties including leaves, fruits and stems were collected from Golestan, Mazandaran and Guilan provinces (north of Iran) and Kerman province (south of Iran). Samples were cultured on potato dextrose agar (PDA) medium. Plates were incubated in the dark at 25°C. Isolates were purified by single spore method (Ho & Ko 1997). From 428 samples, 292 *Colletotrichum* isolates were obtained.

ISSR fingerprinting for preliminary screening

Due to the huge number of isolates, we used ISSR fingerprinting for preliminary screening of isolates.

DNA extraction

DNA extraction was conducted according to the rapid minipreparation method (Liu et al. 2000) with a little modification. 50–100 mg mycelia of the seven–day–old monosporic colony were surface scraped by scalpel blade, placed in a 1.5 ml tube and ground by a close–ended tip in liquid nitrogen. 500 µL of extraction buffer (400 mM Tris, pH 8; 60 mM EDTA, pH 8; 15 mM NaCl; SDS 1%) was added to each tube. After 10 minutes at room temperature, 150 µL of potassium acetate buffer, pH 4.8 (60 mL potassium acetate 5 M, 11.5 mL acetic acid, 28.5 mL deionized water) was added to the tube and vortexed briefly. After two times of centrifugation with 13000 rpm for 1 minute with replacing the liquid phase in each time, an equal volume of chloroform was added, and centrifuged at 13000 rpm for 5 min. Supernatant was recovered carefully. Nucleic acid was precipitated by addition of the same volume of cold isopropyl alcohol and centrifuged at 13000 rpm for 2 min. The pellet was dried by using a vacuum pomp, and after adding 300 µL of cold ethanol 70% was recentrifuged at 13000 rpm for 1 min. Aquatic phase was removed and after drying, the pellet was dissolved in 50 µL distilled water.

PCR amplification

PCRs were performed in an MJ research thermal cycler using five ISSR primers (table 1). PCR was carried out in 12 µL reactions, containing 6 µL of 2X PCR Master Mix (Topaz Gene Research), 1 µL (10 pM) of each forward and reverse primers (table 2) and 75 ng DNA, using cycling parameters as: initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and extension at 72 °C for 2 min, followed by final extension at 72 °C for 7 min.

Products were loaded on 1% (w/v) agarose gel containing 1×TBE (45 mM Tris–borate, 1 mM EDTA) and 0.5 µg/ml aqueous solution of ethidium bromide. PCR products were separated through electrophoresis at constant voltage of 110 V for 2 h. DNA fragments were visualized and documented with UV doc system (EBOX VX5/20, Vilber Loumat, France), and size of the PCR products was determined by comparison with the migration of GeneRuler 100 bp DNA Ladder (Viogene).

Data analysis

Amplified fragments were scored as present (1) or absent (0). The ISSR–PCR fragment profiles were subjected to cluster analysis to produce a dendrogram based on Jaccard's similarity coefficient by Un–weighted Pair Group Method (UPGMA) using NTSYS ver2.02 software (Rohlf, 1998).

Table 1. Primers were used for preliminary screening of *Colletotrichum* isolates.

ISSR Primers	Sequence (5′–3′)*	References
ISSR3	HVHTGTGTGTGTGTGTGT	Fang.and Roose, 1997
ISSR4	ACACACACACACACACYA	Nourollahi and Shahbazi, 2015
ISSR5	GAGAGAGAGAGAGAGAYG	Malekzadeh *et al.*, 2011
ISSR7	AGAGAGAGAGAGAGAGYC	Wang *et al.*, 2005
ISSR9	AGAGAGAGAGAGAGAGC	Bagherabadi *et al.*, 2015

* Y= pyrimidine, H= non–G, V= non–T

Phylogenetic study

A part of β–tubulin 2 (*TUB2*), chitin synthase (*CHS*–1) and calmodulin (*CAL*) genes were amplified by using primers shown in table 2. Samples were run in 25 μL reactions using 12 μL of 2X PCR Master Mix (Topaz Gene Research), 1 μL (10 pM) of each forward and reverse primers (table 3) and 75 ng DNA. Cycling program conditions were as follows: initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for *TUB,* 58 °C for *CHS*–1 and *CAL* genes for 30 s and extension at 72 °C for 45 s, followed by final extension at 72 °C for 7 min. Single DNA bands of the expected size of *TUB2* gene and PCR products of *CHS*–1 and *CAL* genes were purified with the QIAquick PCR purification kit (QIAGEN) and sequenced in both forward and reverse directions by Bioneer company (SK, Seol).

Sequences of the *TUB2*, *CHS*–1 and *CAL* genes were compared by BLAST search against related sequences retrieved from GenBank and were aligned using MEGA 5 (Tamura et al. 2011). Gaps were treated as missing data. The data were analyzed using Maximum Likelihood (ML) method with Tamura-Nei distance model and bootstrap values of 1000 replicates in MEGA 5.0 software. The sequence of *Colletotrichum truncatum* OOC72 (obtained from GenBank) was used as outgroup.

Morphological study

Morpholgical characteristics such as color of the colony, mycelial growth and presence or absence of setae in each isolate were investigated. Slide culture technique was used to produce appressoria. Spores were placed on drops of sterile water on a microscope slide in Petri dishes and incubated at 25°C in the dark or light condition. After 24–48 hours, the shape and color of appressoria were studied (Johnston and Jones 1997). Size of 50, seven–day–old conidia on PDA were measured and their shape were studied (Sutton 1992) by light microscopy (Nikon Eclipse E600 FN, Nikon, Japan).

RESULTS

Phylogenetic relationships

Based on the preliminary screening and cluster analysis dendrogram (data not shown) and morphological characters, 13 out of 292 isolates were selected for molecular studies.

According to a partial sequence of *CAL*, *TUB2* and *CHS* genes, there was 98.33– 100 percent sequence identity between the studied isolates and the ex–type or ex–epitype species including *C. gloeosporioides* isolate IMI 356878 (Accession No: JX009818.1 for *CHS*, JX010445.1 for *TUB2,* JX009731.1 for *CAL* genes), *C. fructicola* isolate C1315.3 (Accession No: JX009866.1 for *CHS*, JX010405.1 for *TUB2,* JX009676.1 for *CAL* genes), *C. siamense* isolate C1315.2 (Accession No: JX009865.1 for *CHS*, JX010404.1 for *TUB2,* JX009714.1 for *CAL* genes), *C. novae zelandiae* CBS:128505 (Accession No: JQ005402.1 for *CHS*, JQ005662.1 for *TUB2,* JQ005749.1 for *CAL* genes) and *C. karstii* isolate CORCG6 (Accession No: HM582023.1 for *CHS*, HM585428.1 for *TUB2,* HM582013.1 for *CAL* genes) (table 3).

Some authentic fungal DNA sequences which had high similarity with our isolates and ex–type or ex–epitype species of *C. gloeosporioides*, *C. fructicola*, *C. siamense*, *C. novae–zelandiae* and *C. karstii*, available from GenBank were aligned with our isolates and used for phylogenetic study (Table 3).

The isolates formed a monophyletic clade with the reliable reference relatives of their species from GenBank isolates (Fig. 1–3).

According to the trees derived from *CHS I* and *Tub 2* genes, eight out of 13 isolates investigated in this study, belonging to the *C. gloeosporioides* complex were clustered together in a big clade (Fig. 1 and 2). On the other hand, based on the tree inferred from *CAL* gene, isolates 296, 382, 472, RMN and TABN were clustered with *C. gloeosporioides* members, supported by a bootstrap value of 99% (Fig. 3).

Isolate 283 was clustered with *C. novae zelandiae* clade, while isolates 288 and 339s were grouped with members of *C. fructicola* and *C. siamense* groups, respectively. The phylogenetic trees inferred from *CHS I* and *Tub 2* genes showed that the isolates 283, 288 and 339s are *C. novae–zelandiae, C. fructicola* and *C. siamense* respectively (Fig 1, 2).

Phylogenetic analysis of the isolates 272, 296, 382, 472, RMN and TABN along with reference isolates of *C. gloeosporioides* allowed these isolates to be ascribed to the *C. gloeosporioides* s. str. (Fig. 1–3) and finally, the isolates 298, 357, 445 and 468 were grouped with members of *C. karstii.* Cluster analysis and phylogenetic results confirmed the BLAST data.

Table 2. Primers were used for phylogenetic analysis of *Colletotrichum* isolates.

Gene	Product	Primer	Direction	Sequence (5'–3')	Reference
TUB2	β–Tubulin 2	T1	Foward	AACATGCGTGAGATTGTAAGT	O'Donnell & Cigelnik 1997
TUB2	β–Tubulin 2	Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	O'Donnell & Cigelnik 1997
CAL	Calmodulin	CL1	Foward	GARTWCAAGGAGGCCTTCTC	O'Donnell et al. 2000
CAL	Calmodulin	CL2A	Reverse	TTTTTGCATCATGAGTTGGAC	O'Donnell et al. 2000
CHS–1	Chitin synthase	CHS–79F	Foward	TGGGGCAAGGATGCTTGGAAGAAG	Carbone & Kohn 1999
CHS–1	Chitin synthase	CHS–345R	Reverse	TGGAAGAACCATCTGTGAGAGTTG	Carbone & Kohn 1999

Table 3: The percent of sequence identity between a partial sequence of *CAL*, *TUB2* and *CHS* genes of *Colletotrichum* isolates obtained from citrus in this study and ex–type or ex-epitypes of the same *Colletotrichum* species.

Isolate	Accession No.	***Genes***		
		CHS	***TUB***	***CAL***
283 (*C. novae–zelandiae*)	IRAN 2575 C	98.93	99.68	
288 (*C. fructicola*)	IRAN 2576 C	100	99.56	
339s (*C. siamense*)	IRAN 2579 C	98.66	98.99	
298 (*C. karstii*)	IRAN 2578 C	99.19	100	
357 (*C. karstii*)	IRAN 2580 C	100	99.7	
445 (*C. karstii*)	-	99.19	99.7	
468 (*C. karstii*)	IRAN 2582 C	99.19	100	
272 (*C. gloeosporioides*)	IRAN 2574 C	99.33	100	100
296 (*C. gloeosporioides*)	IRAN 2577 C	98.66	99.68	100
382 (*C. gloeosporioides*)	IRAN 2581 C	98.99	99.63	99.59
472 (*C. gloeosporioides*)	IRAN 2583 C	98.33	99.85	100
RMN (*C. gloeosporioides)*	IRAN 2584 C	100	99.13	99.59
TABN (*C. gloeosporioides*)	IRAN 2585 C	98.33	100	99.59

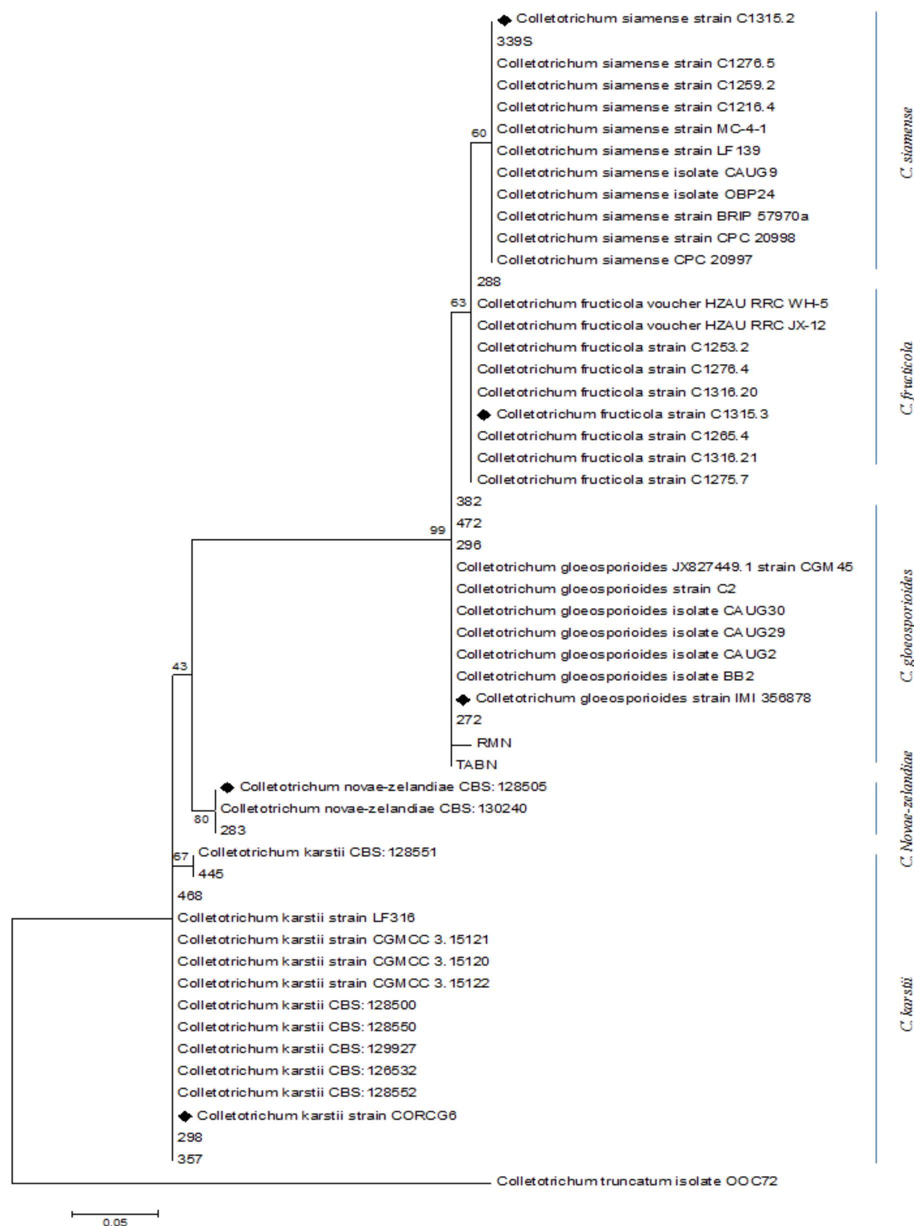

Fig. 1. Maximum likelihood phylogram inferred from partial *Tub 2* sequence data, showing phylogenetic relationships of *Colletotrichum* species isolated from citrus and selected sequences of *Colletotrichum* species. The numbers above the branches represent branch support using1000 bootstrap replications. *Colletotrichum truncatum* isolate OOC72 is used as outgroup. ◆: ex–type or ex–epitype.

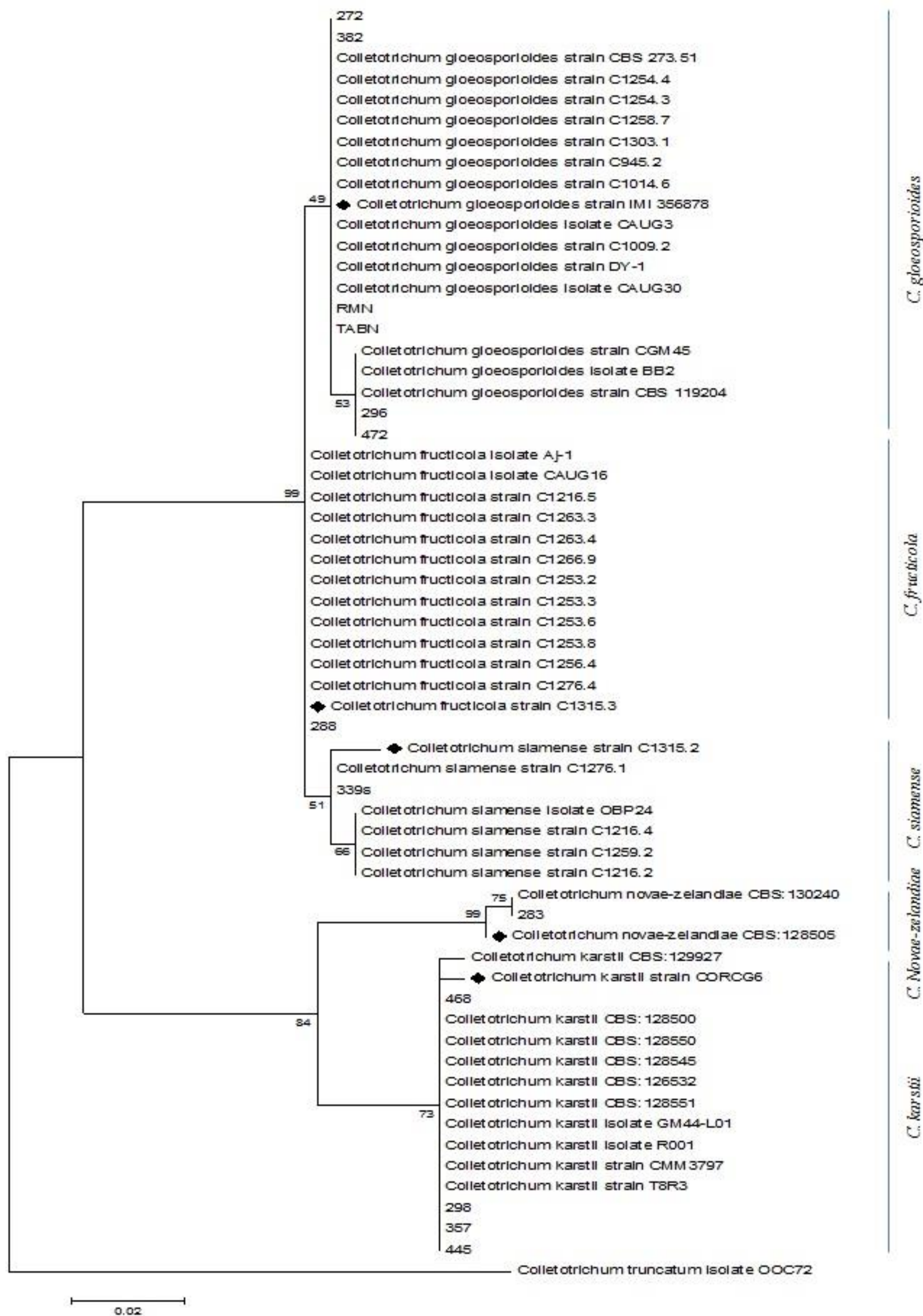

Fig. 2. Maximum likelihood phylograms inferred from partial *CHS I* sequence data, showing phylogenetic relationships of *Colletotrichum* species isolated from citrus and selected sequences of *Colletotrichum* species. The numbers above the branches represent branch support using1000 bootstrap replications. *Colletotrichum truncatum* isolate OOC72 is used as outgroup. ◆: ex–type or ex–epitype.

Morphological study

Colletotrichum fructicola Prihastuti, L. Cai & K.D. Hyde, Fungal Diversity 39: 158. 2009.

Colonies on PDA reaching 6–7 mm average growth per day at 25°C. Mycelia white and then turning to gray at the center over time. Seldom, conidia masses are seen at the center, with filter paper. Pale gray aerial mycelia abundant. Sclerotia, acervuli and setae absent. Conidia hyaline, cylindrical with obtuse to rounded ends, aseptate and smooth. 4.5–6.2 × 12.5–15 µm. appressoria ovoid or irregular. 5.3–6.8 × 8.4–11 µm. (Fig. 4)

Specimens examined. IRAN, Mazandaran Province, Babol, isolated from stem of *C. sinensis*, 15 June 2014, *H. Taheri*, (IRAN 2576 C, isolate 288).

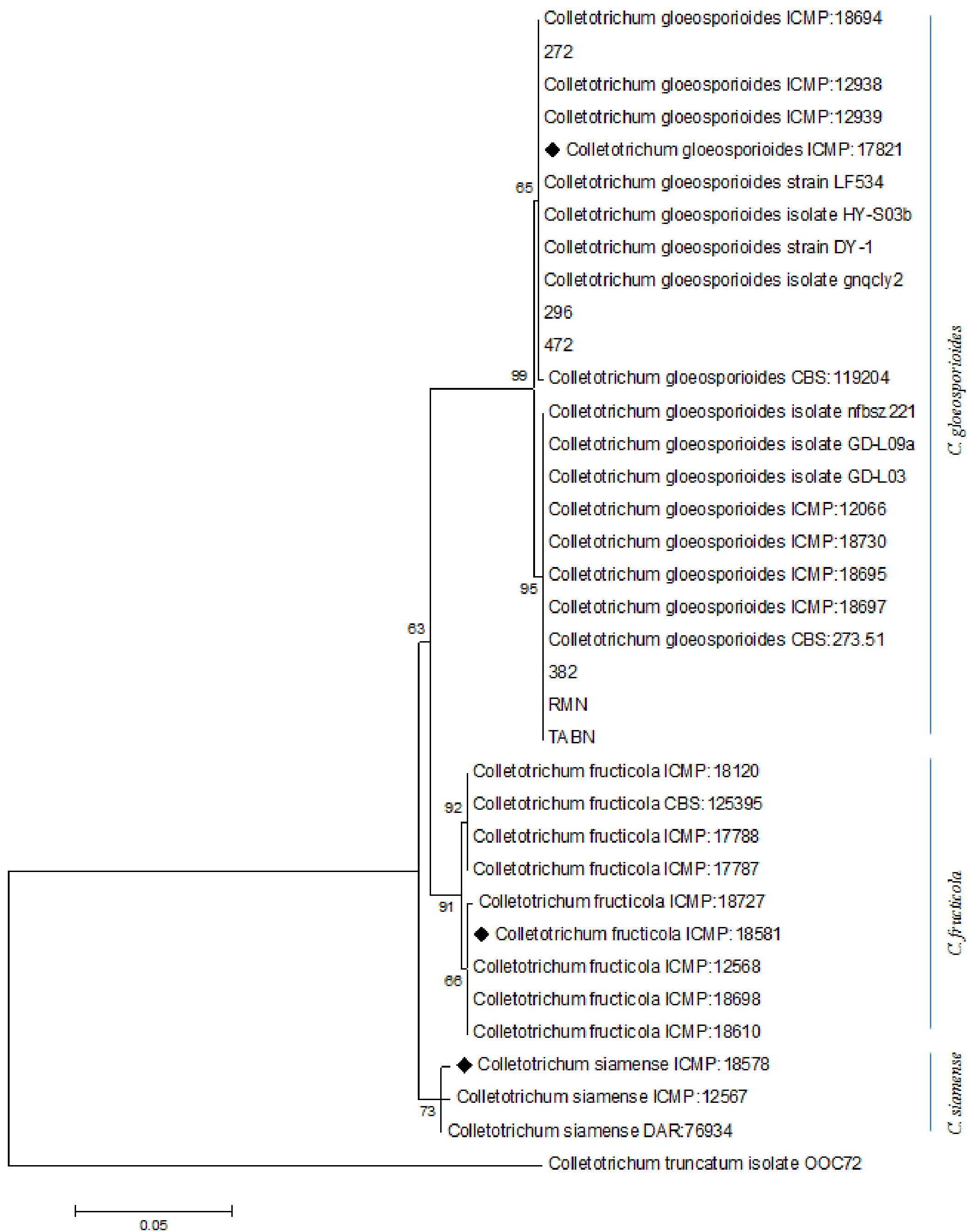

Fig. 3. Maximum likelihood phylograms inferred from partial *CAL* sequence data, showing phylogenetic relationships of *Colletotrichum* species isolated from citrus and selected sequences of *Colletotrichum* species. The numbers above the branches represent branch support using1000 bootstrap replications. *Colletotrichum truncatum* isolate OOC72 is used as outgroup. ♦: ex–type or ex–epitype.

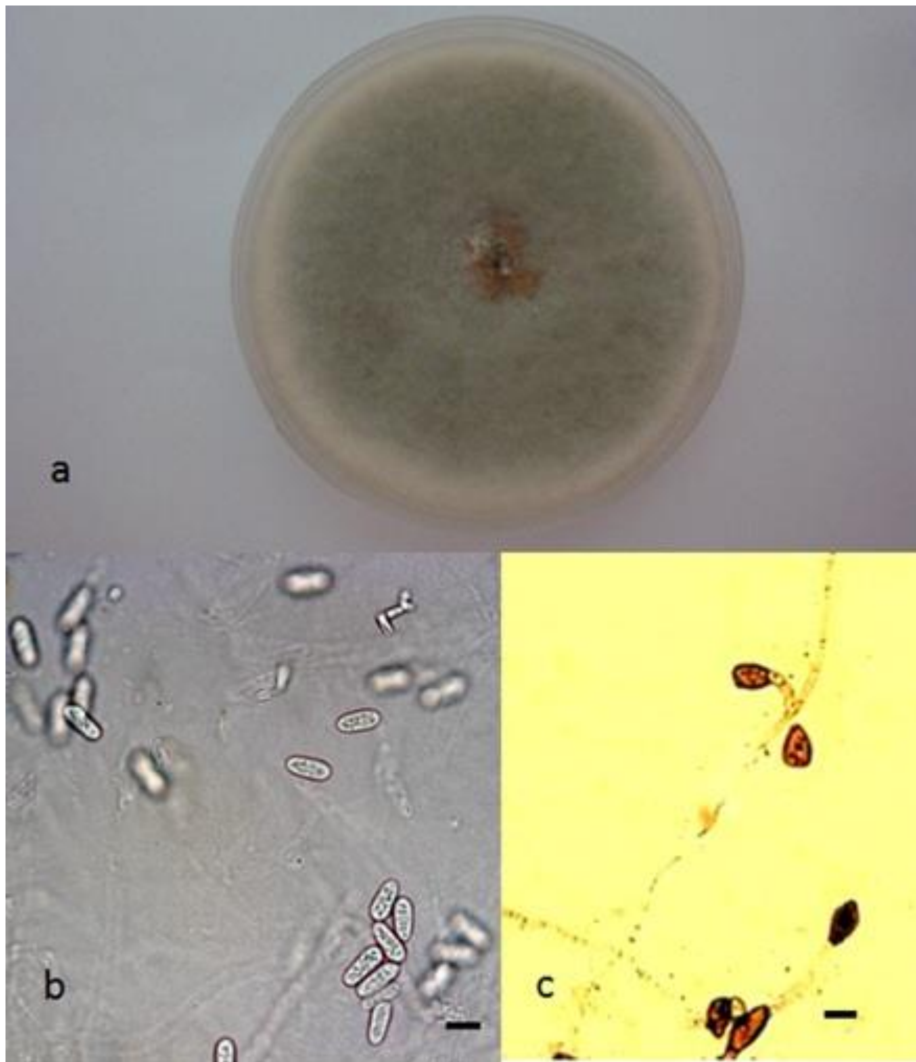

Fig. 4. *Colletotrichum fructicola.* a. Colony on PDA; b. Conidia; c. Appressoria. — Scale bars = 10 µm.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., Atti Reale Ist. Veneto Sci. Lett. Arti., Serie 6, 2: 670. 1884.

Colonies on PDA reaching 6.5–8.7 mm average growth per day at 25°C. Mycelia, initially white and turning to pale brownish with a few orange conidial masses. Aerial mycelia white to gray in color. Conidia hyaline, cylindrical to fusiform, aseptate, smooth, 5–7.5 × 15–17.5 µm. Appressoria brown, ovoid and sometimes clavate, 4.7–5.8 × 7.9–8.4 µm. (Fig. 5).

Specimens examined. IRAN, Mazandaran Province, Sari, isolated from stem of *C. sinensis*, 15 June 2014, *H. Taheri*, (IRAN 2581 C, isolate 382); Mazandaran Province, Ramsar, isolated from fruit of *C. sinensis*, 15 June 2014, *H. Taheri*, (IRAN 2584 C, isolate RMN).

Colletotrichum karstii Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, Cryptogamie Mycologie 32: 241. 2011.

Four out of 13 selected isolates were identified as *C. karstii*. Colonies on PDA reaching 5–6.5 mm. average growth per day at 25°C. The surface of colony varies from white to gray with orange conidia mass with entire margin, the reverse is pale orange. Orange conidiomata are seen on PDA with filter paper. Conidia hyaline, cylindrical and aseptate. Apex usaully rounded with a hilum at the base, 4–6.5 × 10–16.5 µm. Setae absent. Appresorium is pale brown in color and naviculate to ovoid shaped, 3.7–5.8 × 5.8– 11.59 µm. (Fig. 6).

Specimens examined. IRAN, Mazandaran Province, Savadkuh, isolated from stem of *C. aurantifolia*, 6 Sept. 2013, *H. Taheri*, (IRAN 2582 C, isolate 468); Mazandaran Province, Galugah, isolated from stem of *C. sinensis*, 16 June 2014, *H. Taheri*, (IRAN 2578 C, isolate 298).

This is the first report of *C. karstii* from citrus plants in Iran.

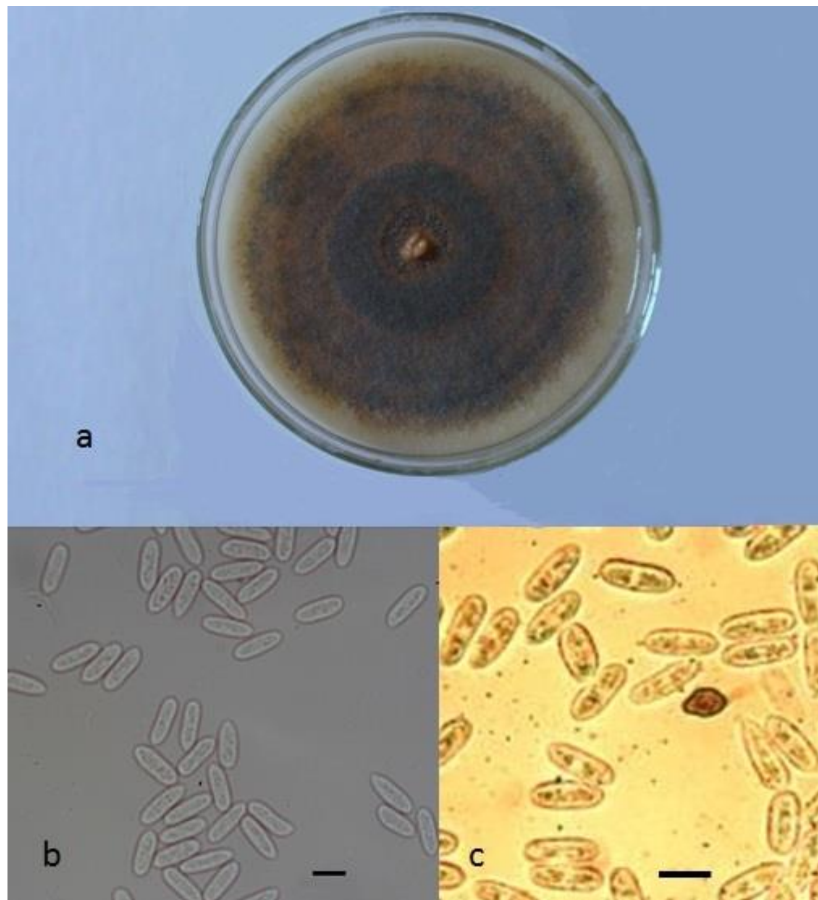

Fig. 5. *Colletotrichum gloeosporioides.* a.Colony on PDA; b. Conidia; c. Appressoria. — Scale bars = 10 µm.

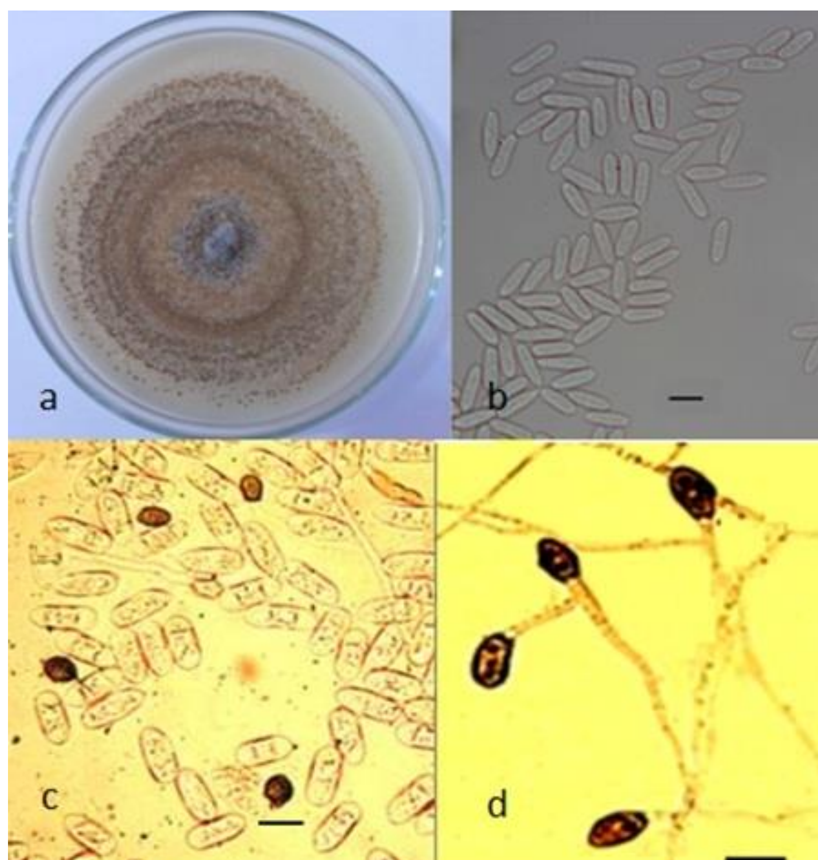

Fig. 6. *Colletotrichum karstii.* a.Colony on PDA; b. Conidia; c, d. Appressoria. — Scale bars = 10 µm.

Colletotrichum novae–zelandiae Damm, P.F. Cannon, Crous, P.R. Johnst. & B. Weir, Studies in Mycology 73: 25 (2012) [MB560742].

Colonies on PDA reaching 3.8–5.2 mm average growth per day at 25°C. Flat, with white margins, orange in color with dark gray flecks. Conidia hyaline, cylindrical, aseptate, rounded at the both ends with a hilum at the base, 5–6.5 × 13–15 µm. Conidiomata absent. Appressoria are irregular rounded and brown in color, 3.7–5.3 × 5.3–8.4 µm. Acervuli are present on PDA plates with dark brown setae. (Fig. 7).

Specimens examined. IRAN, Mazandaran Province, Babol, isolated from stem of *C. sinensis*, 15 June 2014, *H. Taheri*, (IRAN 2575 C, isolate 283).

According to our knowledge, *C. novae–zelandiae* is a new record for mycobiota of Iran.

Colletotrichum siamense Prihastuti, L. Cai & K.D. Hyde, Fungal Diversity 39: 98. 2009.

Colonies on PDA reaching 7–8.5 mm average growth per day at 25 °C. At first, they are white and then become pale pinkish with white to pale gray. Dense and cottony aerial mycelia and orange conidia mass at the center. Brown acervuli with setae and dark brown to black sclerotia on PDA plates. Conidia hyaline, cylindrical, aseptate with obtuse apex and round ended base, 6–7.5 × 13.5–15.5 µm. Appressoria ovoid to fusiform and brownish, 3.7–4.7 × 4.2–7.9 µm. (Fig. 8).

Specimens examined. IRAN, Kerman Province, Jiroft, isolated from stem of *C. sinensis*, 6 Nov. 2013, *H. Taheri*, (IRAN 2579 C, isolate 339s).

This is the first report of *C. siamense* isolated from citrus in Iran.

DISCUSSION

In this study, some samples from citrus orchards were collected in Golestan, Mazandaran, Guilan and Kerman provinces. According to morphological characters and ISSR–PCR test (data not shown), 13 isolates were selected for molecular studies. A part of *TUB2* (about 700bp) and *CHS–1* (about 305bp) genes were amplified in all isolates, but *CAL* (about 750 bp) gene was amplified just in *C. gloeosporioides* isolates. According to the morphological characters and molecular data, five species including *C. gloeosporioides*, *C. fructicola* and *C. siamense* (*C. gloeosporioides* complex) and *C. karstii* and *C. novae zelandiae* (*C. boninense* complex) were identified.

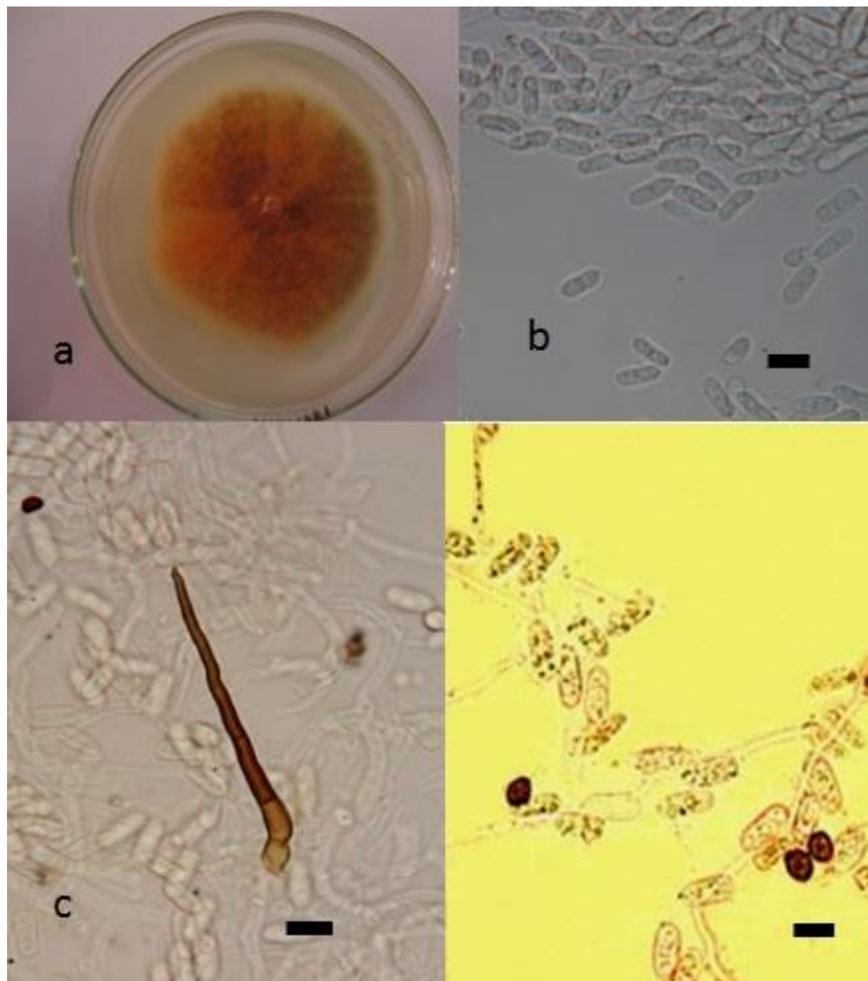

Fig. 7. *Colletotrichum novae—zelandiae.* a.Colony on PDA; b. Conidia; c. Setae; d. Appressoria. — Scale bars = 10 µm.

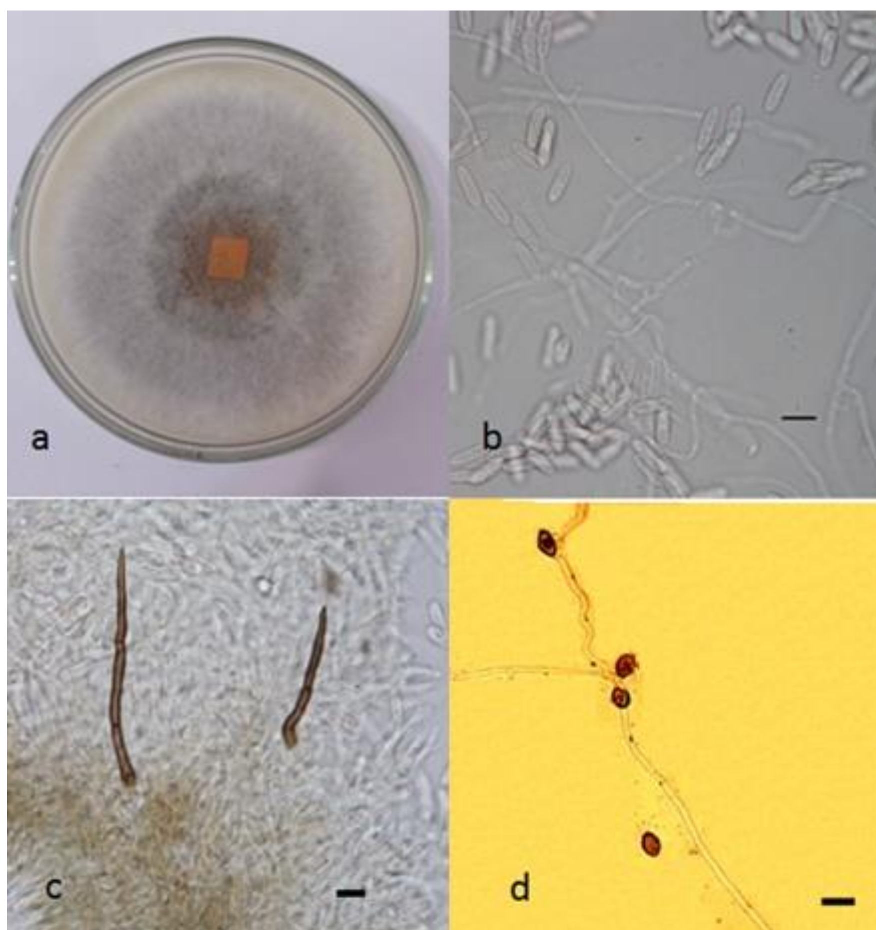

Fig. 8. *Colletotrichum* siamense. a.Colony on PDA; b. Conidia; c. Setae; d. Appressoria. — Scale bars = 10 µm.

Using multi genes to identify *Colletotrichum* species is necessary. Protein–coding genes are more useful due to more variation than ITS. For instance, ITS sequences cannot reliably separate *C. siamense* from *C. alienum* or *C. fructicola*. These species are best distinguished using *CAL* or *TUB2* (Weir et al. 2012). *Colletotrichum karstii* can be distinguished from closely related species within the Boninense, Gigasporum, Acutatum and Gloeosporioides complexes, by using the *TUB2* sequence data (Damm et al. 2012a, b). *TUB2* gene sequence data could resolve the relationship of the *Colletotrichum* isolates, specially, in agreement with morphological characters (Alizadeh et al. 2015). We used *TUB2, CAL* and *CHS*–1 genes in this study. There is some incongruence between gene trees of *C. gloeosporioides* complex (Fig. 1, 2). It is due to the low levels of genetic divergence within this species complex and imply that species within the *C. gloeosporioides* complex are recently evolved (Silva et al. 2012; Weir et al. 2012).

Morphological identification of *Colletotrichum* spp. and species complexes is not a reliable method (Damm et al. 2012; Weir et al. 2012), so both morphological and molecular data were used in this study.

The first report of *C. fructicola* is from coffee berries from Thailand (Prihastuti et al. 2009). The next reports are from *Coffea* sp*., Pyrus pyrifolia*, *Limonium* sp*.*, *Malus domestica*, *Ficus* sp*.*, *Theobroma* sp. (Weir et al. 2012) , *Citrus* sp. (Huang et al. 2013), *Citrus sinensis, Malus domestica, Gleditsia caspica, Sambucus ebulus* (Arzanlou et al. 2015), *Phaseolus vulgaris* and *Vigna unguiculata* from Iran (Atghia et al. 2015). Morphological characteristics of *C. fructicola* such as presence of pale grey aerial mycelia, absence of Sclerotia, Setae and.acervuli in culture, conidium and appressorium shape were in agreement with the description provided by Prihastuti et al. (2009). *Colletotrichum gloeosporioides* is mainly associated with *Citrus*, but it has been isolated from different hosts (Weir et al. 2012). It is a species complex comprising morphologically indistinguish-able, but genetically and biologically isolated species (Cai et al. 2009; Phoulivong et al. 2010).

Colletotrichum karstii is a polymorphic species in terms of sequence and is a morphologically diverse species as well (Alizadeh et al. 2015). In *C. Karstii*, size of the conidium has a different range: (11.5–14.5) × (5–6.5) µm (Damm et al. 2012b) or (13.5–15) × (4–5) µm (Aiello et al. 2015). Color of the upper surface of the colony varies from white to gray and pink in reverse (Velho et al. 2014) or white to gray, usually with pink conidial masses, reverse yellow to dark brown (Yang et al. 2011). These variations have been seen in other *Colletotrichum* species, too. During the storage period, even freshly isolated cultures may lose

the ability to produce pigments or form well–differentiated acervuli, conidia or perithecia. Often, the aerial mycelia become very dense and felted (Weir et al. 2012). *Colletotrichum karstii* is a common and geographically diverse species, occurring on various host plants (Liu et al. 2015). It was reported from *Orchidaceae* hosts (Yang et al. 2011), *Passiflora edulis* (Alizadeh et al. 2015), Citrus plants in South Africa, New Zealand (Damm et al. 2012 b) and China (Peng et al. 2012).

Colletotrichum novae–zelandiae is only reported from New Zealand from grapefruit and *Capsicum*. It is morphologically indistinguishable from other species of the *C. boninense* species complex. (Damm et al. 2012b). According to studies by Damm et al. (2012b) on *C. novae–zelandiae*, teleomorph, conidiomata and chlamidospre are not observed on SNA. We also did not observe them on PDA. In phylogenetic studies with single gene, it forms a separate cluster as a sister group to a group including *C. karstii*, *C. petchii*, *C. annellatum* and *C. phyllanthi* (Damm et al. 2012b). In our study, *C. novae–zelandiae* is phylogenetically close to *C. karstii* based on *TUB2* and *CHS*–1 genes sequence data.

A distinctive character between *C. boninense* and *C. gloeosporioides* species complexes is the presence of a prominent hilum or scar on the conidium of the first complex (Damm et al. 2012b), which was obsereved in *C. karstii* and *C. novae–zelandiae* especies.

Colletotrichum siamense was described by Prihastuti et al. (2009), Yang et al. (2009) and Wikee et al. (2011). It was originally described from coffee in Thailand, but it has a wide host range (Weir et al. 2012). Also, it was reported from *Citrus reticulata* Blanco cv. Shiyue Ju (Cheng et al. 2013). Morphological characteristics of *C. siamense*, such as presence of conidial mass at the inoculation point, sclerotia and acervuli in culture, colony, conidium and appressorium shape were in agreement with the description provided by Prihastuti et al. (2009) and Wikee et al. (2011).

According to this research, *C. novae–zelandiae* is a new record for mycobiota of Iran. *Citrus* sp. is a new host recorded for *C. siamense* and *C. karstii* in Iran. Results indicate that the combination of morphological characters with the multigene sequence studies is necessary for an accurate identification of the *Colletotrichum* species.

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گونه‌های *Colletotrichum* همراه درختان مرکبات در ایران

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چکیده: گونه‌های *Colletotrichum* به عنوان بیمارگر، گندروی و اندوفیت از مرکبات گزارش شده‌اند. در سال‌های اخیر بر اساس بررسی‌های فیلوژنی چند ژنی تغییرات زیادی در رده‌بندی جنس *Colletotrichum* بوجود آمده است. در این تحقیق ۲۹۲ جدایه *Colletotrichum* از برگ، میوه و شاخه مرکبات استان‌های گلستان، مازندران، گیلان و کرمان جداسازی شدند. پس ازمطالعات ریخت‌شناسی، بررسی فیلوژنی چند ژنی با استفاده از ژن‌های *CAL*، *CHS–1* و *TUB2* برای ۱۳ جدایه انتخابی صورت گرفت. بر اساس داده‌های ریخت‌شناسی و مولکولی پنج گونه *Colletotrichum gloeosporioides* s.str.، *C. fructicola* و *C. siamense* از *C. gloeosporioides* complex و *C. karstii* و *C. novae–zelandiae* از *C. boninense* complex شناسایی شدند. با توجه به دانسته‌های ما این اولین گزارش *C. novae–zelandiae* برای میکوبیوتای ایران و *C. siamense* و *C. karstii* از مرکبات ایران می‌باشد.

کلمات کلیدی: تنوع زیستی، ریخت‌شناسی، بیمارگر، تبارشناسی، گونه مرکب

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