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Multigene phylogeny reveals three new records of *Colletotrichum* spp. and several new host records for the mycobiota of Iran

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Abstract: The genus *Colletotrichum* comprises a number of plant pathogens of major importance which cause anthracnose diseases on a wide range of woody and herbaceous plants worldwide. With the advent of molecular studies, it has been shown that most of the previously known species e.g. *C. boninense*, *C. acutatum*, and *C. gloeosporioides* have been split into several species. In the present study, the identity of *Colletotrichum* isolates from the northern and northwestern zone of Iran were determined based on multi-gene phylogenetic analyses. Phylogenetic analysis based on a combination of internal transcribed spacer (ITS), beta tubulin (TUB), histone H3 (HIS), calmodulin (CAL), and actin (ACT) loci, clustered our isolates into three clades, including *C. salicis* on *Salix* sp., *Colletotrichum* sp. (*C. fuscum sensu lato*) within the *C. destructivum* species complex on *Viola* sp., and *C. fructicola* on *Citrus sinensis*, *Malus domestica*, *Gleditsia caspica*, and *Sambucus ebulus*. These three species are new for mycobiota of Iran. According to these results, *Viola* sp. from West Azerbaijan (Khoy-Firouragh) is a new host for *Colletotrichum* sp. in the *C. destructivum* species complex. Furthermore, *C. sinensis* from Mazandaran (Behshahr), and *G. caspica*, and *S. ebulus* from Guilan (Talesh), are new host records for *C. fructicola*.

Key words: anthracnose, *Colletotrichum*, host range, species complex, systematics

Introduction

The genus *Colletotrichum* is one of the most common and destructive fungal genera, comprising a number of devastating pathogens on economically important crops (Damm *et al.* 2010; Cannon *et al.* 2012). Species of the genus are mainly associated with anthracnose diseases on a wide range of woody and herbaceous plant hosts growing in tropical, subtropical, and temperate climates (Damm *et al.* 2010). Members of this genus mainly affect fruit production on economically important crops such as banana, mango, citrus, strawberry, avocado, cassava, coffee, and many other crops including cereals (e.g. maize, sugar cane, and sorghum), vegetables, and ornamentals (Cannon *et al.* 2012). However, other diseases e.g. coffee berry disease, crown rot of strawberry and banana, red rot of sugar cane, and brown blotch of cowpea as well as several post-harvest rots are caused by the *Colletotrichum* species (Cannon *et al.* 2012).

Colletotrichum species exhibit diverse nutritional styles. Many species are hemibiotrophic and/or necrotrophic invaders of plant hosts, while some are endophytes, living inside healthy plants without harming their host. In rare cases, some species have been reported as the causal agents of diseases on humans (keratitis and subcutaneous infections) or other animals (e.g. mycotic infection of a sea turtle) (Ritterband *et al.* 1997; Manire *et al.* 2002; Shiraishi *et al.* 2011; Shivaprakash *et al.* 2011).

The genus *Colletotrichum* was originally introduced by Corda (1831), based on *C. lineola* as the type species. Since the description of the genus, taxonomy of *Colletotrichum* has been clouded in controversy (Cannon *et al.* 2012; Damm *et al.* 2010, 2012; Weir *et al.* 2012). This problem is mainly due to the lack of reliable morphological features, which blurs the boundaries of the species. The host plant association has been the main criterion for species delineation in this genus. Considering that there are many species being host specific, the result has been the description of several hundred names in this genus (Sutton 1980, 1992; Cannon *et al.* 2012; Damm *et al.* 2012; Weir *et al.* 2012).

With the advent of molecular biology, diverse arrays of molecular techniques and sequence data from different genomic regions have been widely applied to delineate species boundaries in the genus *Colletotrichum* as well as other fungal groups (Bakhshi *et al.* 2014, 2015; Cannon *et al.* 2012; Arzanlou and Khodaie 2012a, b; Davari *et al.* 2012; Arzanlou *et al.* 2013; Arzanlou and Narmnai 2014, 2015). Phylogenetic analysis based on multigene sequence has revealed several cryptic species within species complexes such as *C. acutatum*, *C. gloeosporioides*, and several others (Damm *et al.* 2010, 2012, 2014; Weir *et al.* 2012).

The reports of *Colletotrichum* species from the mainland of Iran have been very limited. Up till now, 17 species of *Colletotrichum* have been reported from different

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plant species in Iran (Ershad 2009; Arzanlou *et al.* 2013; Alizadeh *et al.* 2013). Ershad (2009) has provided a comprehensive list of *Colletotrichum* spp. along with their host range in his book "Fungi of Iran". In the northwestern zone of Iran, severe epidemic of anthracnose disease has been reported by Arzanlou and Torbati (2013) on *Cornus mas*. The causal agent was characterised as *C. acutatum sensu lato* using Internal Transcribed Spacer-rDNA (ITS--rDNA) sequence data. However, many of these reports are based on herbarium specimens and there is no living culture available to practice modern taxonomy – in the light of recent molecular revisions of *Colletotrichum* spp. from other parts of the world. Accordingly, the aim of this study was to characterise *Colletotrichum* spp. in Northern Iran, based on a combination of morphology, culture characteristics, ecology, and DNA phylogeny (ACT, TUB, CAL, HIS, and ITS loci).

Materials and Methods

Isolates and morphology

Leaves of *Salix* sp., *Viola* sp., *Gleditsia caspica*, and *Sambucus ebulus* with evident anthracnose leaf spot symptoms, as well as infected fruits of *Malus domestica* and *Citrus cinensis*, were collected from the northern and northwestern zone of Iran. In the laboratory, the leaf and fruit spots were examined under a stereomicroscope, and acervuli were observed in the central parts of the lesions. Single-spore cultures were established as explained in Bakhshi *et al.* (2011). In brief, under a stereomicroscope, a mass of conidia was picked up from the centre of the lesion, using a sterile inoculation needle, and suspended on Potato Dextrose Agar (PDA) plates (supplemented with streptomycin sulphate, 100 mg · l^{-1}), containing 10 ml sterile water. The suspension was evenly spread on the surface of the culture. The plates were kept in a slanted position overnight. Then the plates were checked under the stereomicroscope and germinated conidia were transferred to new PDA plates. Cultural and microscopic features were studied on Malt Extract Agar (MEA; Fluka, Hamburg, Germany), Potato Dextrose Agar (PDA) (Crous *et al.* 2009), Synthetic Nutrient Agar (SNA), and Oatmeal Agar (OA) (Crous *et al.* 2009). The culture media were kept under near UV light with a 12 h photoperiod at 20°C for 10 days (Damm *et al.* 2009). After 7 days, colony morphology (including colour, shape, and growth rate) were determined on MEA, PDA, and OA. Microscopic characters were studied using a slide culture technique as explained by Arzanlou *et al.* (2007). Dimension of microscopic structures were calculated based on 30 measurements, where possible. For the measurements of each structure, 95th percentile intervals were derived at, with the extremes in parenthesis. Appressoria on hyphae were observed on the undersurface of the SNA and PDA cultures. Microscopic fungal structures were mounted in lactic acid and photographs were captured using a high resolution Leica camera system mounted on the Olympus BX41 light microscope. Single-spore cultures were preserved on Potato Carrot Agar (PCA) in 1.5 ml microtube slants at 4°C in the Culture Collection of Tabriz University (CCTU).

DNA extraction

For DNA extraction, fungal isolates were grown on PDA for 8 days in the dark, and fresh mycelia were harvested and subjected to DNA extraction by using the protocol of Moller *et al.* (1992). DNA samples were subsequently diluted 100 times in preparation for further DNA amplification reactions. Part of the nuclear rRNA operon spanning the 3′ end of 18S rRNA gene, the first internal transcribed spacer (ITS), the 5.8S rRNA gene, the second ITS region and the 5′ end of the 28S rRNA gene, and partial sequences of the actin (ACT), beta tubulin (TUB), histone H3 (HIS), and calmodulin (CAL) genes, were amplified and sequenced using the primer pairs V9G (de Hoog and Gerrits van den Ende 1998) + ITS4 (White *et al.* 1990), ACT-512F + ACT-783R (Carbone and Kohn 1999), T1 (O'Donnell & Cigelnik 1997) + Bt-2b (Glass and Donaldson 1995), CYLH3F + CYLH3R (Crous *et al.* 2004), and CAL 228F + CAL 737R (Carbone and Kohn 1999), respectively. Polymerase chain reaction (PCR) mixtures and conditions for ITS, ACT, HIS, and CAL were performed in a total volume of 12.5 µl as described by Bakhshi *et al.* (2015). The TUB mixture consisted of 5–10 ng genomic DNA, 1x PCR buffer (Bioline, London, UK), 2 mM $MgCl_2$ (Bioline), 40 µM each of deoxynucleotide (dNTP), 0.7 µl dimethylsulfoxide (DMSO), 0.2 µM of each primer, and 0.25 Unit GoTaq® Flexi DNA polymerase (Promega, Madison, Wisconsin, USA). For this gene, the PCR conditions constituted an initial denaturation step of 5 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 52°C and 30 sec at 72°C, and a final denaturation step of 5 min at 72°C. Following PCR amplification, amplicons were visualised on 1.2% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA) and viewed under UV light. The sizes of the amplicons were determined against a HyperLadder™ I molecular marker (Bioline, London, UK).

The ABI Prism BigDye® Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems™, Foster City, CA, USA) was used according to the manufacturer's instructions, for sequencing of PCR products in both directions using the same pairs of primers that were used for amplification. Sequencing products were purified through a 96--well multiscreen HV plate (Millipore) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO, USA) as outlined by the manufacturer, and analysed with an ABI Prism 3730XL Automated DNA analyser (Life Technologies Europe BV, Applied Biosystems™, Bleiswijk, The Netherlands) according to the manufacturer's instructions.

Sequence analysis

The raw trace files were edited using MEGA v. 5 (Tamura *et al.* 2011) and a consensus sequence was generated manually for each set of trace files from the forward and reverse sequences. The generated sequences were compared with other fungal DNA sequences from NCBIs GenBank sequence database using BLAST. Those sequences with high similarity were added to the alignments. The obtained sequences from GenBank, together with sequences generated in this study, were aligned using the multiple sequence alignment online interface

MAFFT (Katoh and Toh 2008), and if necessary, adjusted by eye in MEGA v. 5.

The best evolutionary model for each data partition was obtained using the software MrModelTest v. 2.3 (Nylander 2004). An initial Bayesian inference (BI) analysis was performed with MrBayes v. 3.2.1 (Ronquist and Huelsenbeck 2003) and was restricted to an individual alignment of ITS data to determine species complexes. Subsequently, separate BI analyses were run using a concatenated alignment of ITS, ACT, and CAL genes for the *C. gloeosporioides* species complex; ITS, ACT, HIS, and TUB genes for the *C. acutatum* species complex and ITS, ACT, HIS, and TUB genes for the *C. destructivum* species complex. The heating parameter was set at 0.15. The Markov Chain Monte Carlo (MCMC) analysis of four chains was started in parallel from a random tree topology and lasted until the average standard deviation of split frequencies came below 0.01. Trees were saved each 1,000 generations and the first 25% of saved trees were discarded as the 'burn-in' phase. Posterior probabilities (PP) were determined from the remaining trees. The resulting phylogenetic tree was printed with Geneious v. 5.6.7 (Drummond *et al.* 2012). Sequences derived from this study were lodged at NCBI's GenBank nucleotide database (http://www. ncbi.nlm.nih.gov; Table 1).

Results

DNA phylogeny

Seven isolates of *Colletotrichum* spp. isolated from seven host species (Table 1) were subjected to DNA sequence analyses.

ITS phylogeny

The final aligned ITS dataset contained 42 ingroup taxa with a total of 576 characters, containing 86 unique site patterns. *Monilochaetes infuscans* (isolate CBS 869.96) served as the outgroup taxon and a heating parameter set at 0.15. The results of MrModeltest recommended a general time reversible (GTR) substitution model with inverse gamma rates for ITS and dirichlet base frequencies. During the generation of the tree (Fig. 1), a total of 482 trees were saved. Consensus trees and posterior probabilities were calculated from the remaining 362 (75%) trees. Based on the results of the ITS sequence data, one isolate (CCTU 1243) resides in the *C. acutatum* species complex, one isolate (CCTU 1051) resides in the *C. destractivum* species complex, and five remaining isolates reside in the *C. gloeosporioides* species complex (Fig. 1).

Table 1. Strains included in this study and their corresponding GenBank accession numbers

Species	Culture accession number(s)[1]	Host name	Origin	GenBank accession numbers 2				
				ITS	TUB	ACT	CAL	HIS
Colletotrichum fructicola (*C. gloeosporioides* complex)	CCTU 1230	**Citrus sinensis* (L.) Osbeck	Iran, Mazandaran, Behshahr	KP716981	KP716988	KP716995	KP717002	KP717009
	CCTU 1232	*Malus domestica* Borkh	Iran, Guilan, Talesh, Jomakooh	KP716982	KP716989	KP716996	KP717003	KP717010
	CCTU 1014	**Gleditsia caspica* Desf.	Iran, Guilan, Talesh, Kishonben	KP716983	KP716990	KP716997	KP717004	KP717011
	CCTU 1229	**Sambucus ebulus* L.	Iran, Guilan, Talesh, Kishonben	KP716984	KP716991	KP716998	KP717005	KP717012
	CCTU 1221	unknown	Iran, Guilan, Astara	KP716985	KP716992	KP716999	KP717006	KP717013
Colletotrichum sp. (*C. fuscum sensu lato*, *C. destractivum* complex)	CCTU 1051	**Viola* sp.	Iran, West Azerbaijan, Khoy	KP716986	KP716993	KP717000	KP717007	KP717014
Colletotrichum salicis (*C. acutatum* complex)	CCTU 1243	*Salix* sp.	Iran, East Azerbaijan, Mianeh	KP716987	KP716994	KP717001	KP717008	KP717016

[1] CCTU – Culture Collection of Tabriz University, Tabriz, Iran; ITS – internal transcribed spacer, TUB – beta tubulin, ACT – actin, CAL – calmodulin, HIS – histone

* new host record

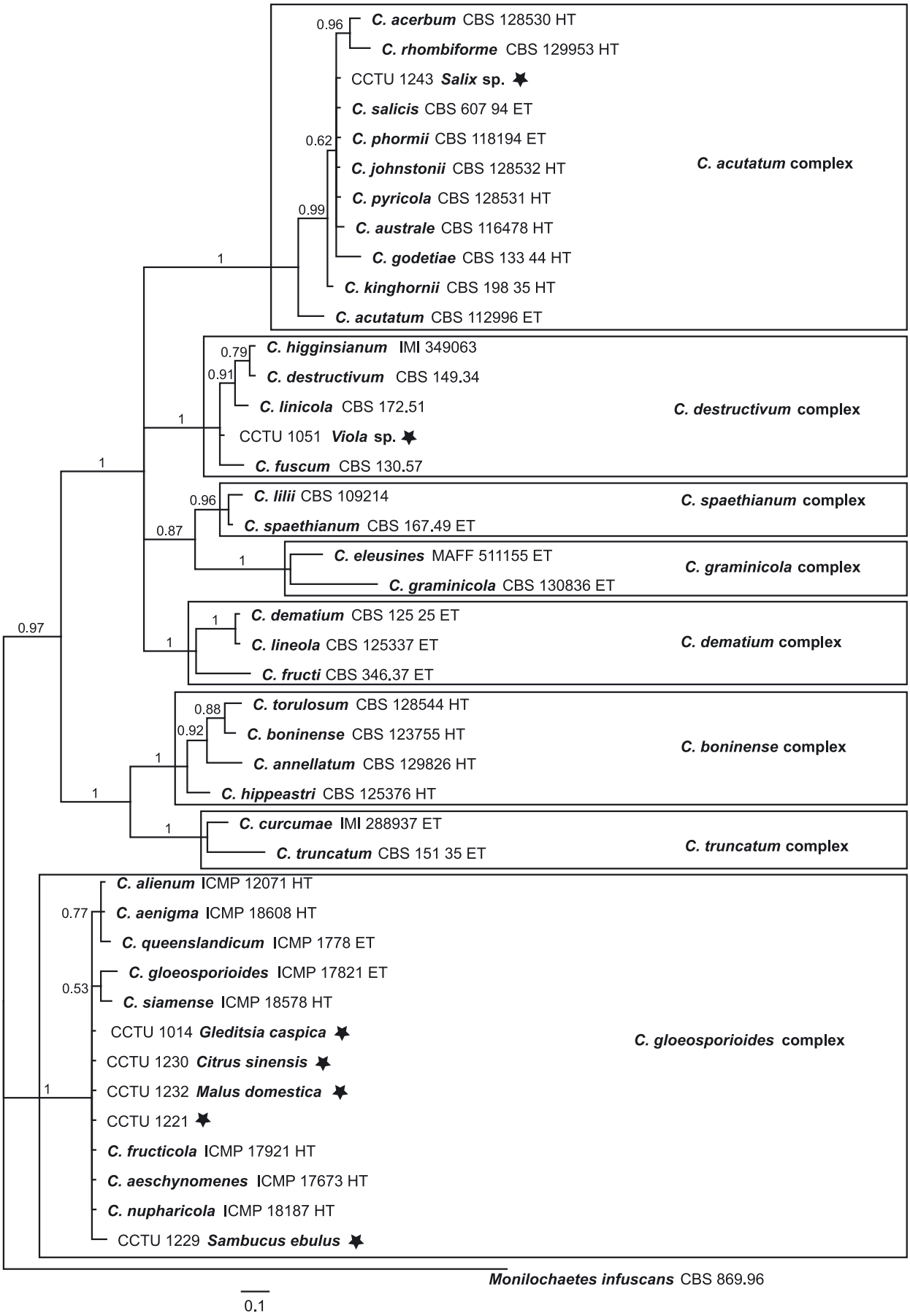

Fig. 1. Consensus phylogram (50% majority rule) of 362 trees resulting from a Bayesian analysis of ITS sequence alignment using MrBayes v. 3.2.1. Based on the results of the ITS sequence data, one isolate (CCTU 1243) resides in the *Colletotrichum acutatum* species complex, one isolate (CCTU 1051) resides in the *C. destructivum* species complex, and five remaining isolates reside in the *C. gloeosporioides* species complex (marked with asterisk). The scale bar indicates 0.1 expected changes per site. The tree was rooted to *Monilochaetes infuscans* (CBS 869.96)

Multi-gene phylogeny for the *C. acutatum* species complex

In the multi-gene analyses (gene boundaries of ITS: 1–542, ACT: 553–800, TUB: 811–1302 and HIS: 1313–1698) of 34 isolates of different species belonging to the *C. acutatum* species complex (including 33 taxa from NCBI, and one taxon from this study), 1,668 characters – including the alignment gaps, were used. These characters contained 315 unique site patterns (45, 78, 99, and 93 for ITS, ACT, TUB, and HIS, respectively). *Colletotrichum orchidophilum* (CBS 632.80) was used as outgroup. GenBank numbers of strains obtained from NCBI can be found in Damm *et al.* (2012). The results of MrModeltest recommended a GTR+I+G with inverse gamma distributed rate variation for ITS; HKY+I+G with inverse gamma distributed rate variation for HIS; and a GTR+G with gamma-distributed rate variation for ACT and TUB. All partitions had dirichlet base frequencies. The Bayesian analysis lasted 180,000 generations and saved a total of 362 trees. After discarding the first 25% of sampled trees for burn-in, the consensus trees and posterior probabilities were calculated from the remaining 272 trees. Based on the results of the combined sequence data for *C. acutatum* species complex, our isolate (CCTU 1243) was recognised as *C. salicis* (Fig. 2).

Multi-gene phylogeny for the *C. destructivum* species complex

In the multi-gene analyses (gene boundaries of ITS: 1–571, HIS: 572–970, ACT: 971–1243, and TUB: 1244–1757) of 58 isolates from different species belonging to the *C. destructivum* species complex (including 57 taxa from NCBI, and one taxon from this study), 1,727 characters including the alignment gaps were used. These characters contained 237 unique site patterns (52, 66, 46, and 73 for ITS, HIS, ACT, and TUB, respectively). *Colletotrichum pisicola* (CBS 724.97) was used as outgroup. GenBank numbers of strains obtained from NCBI can be found in Damm *et al.* (2014). The results of MrModeltest recommended a GTR+I+G with inverse gamma distributed rate variation for ITS and HIS; HKY+G with gamma distributed rate variation for ACT; and a GTR+G with gamma-distributed rate variation for TUB. All partitions had dirichlet base frequencies. The Bayesian analysis lasted 220,000 generations and saved a total of 442 trees. After discarding the first 25% of the sampled trees for burn-in, the consensus trees and posterior probabilities were calculated from the remaining 332 trees. Based on the results of the combined sequence data for *C. destructivum* species complex, our isolate (CCTU 1051) clustered with three unnamed isolates from *Heracleum* sp. (Damm *et al.* 2014) basal to *C. fuscum*, *C. antirrhinicola*, *C. bryoniicola*, and *C. vignae* (*C. fuscum sensu lato*) (Fig. 3).

Multi-gene phylogeny for the *C. gloeosporioides* species complex

In the multi-gene analyses (gene boundaries of ITS: 1–552, ACT: 563–846, and CAL: 857–1494) of 37 isolates of different species belonging to the *C. gloeosporioides* species complex (including 33 taxa from NCBI, and five taxa from this study), 1,474 characters including the alignment gaps were used. These characters contained 287 unique site patterns (53, 78, and 156 for ITS, ACT, and CAL, respectively). *Colletotrichum hippeastri* (ICMP 17920) was used as outgroup. GenBank numbers of strains obtained from NCBI can be found in Weir *et al.* (2012). The results of the MrModeltest recommended a GTR+G with gamma distributed rate variation for ITS, ACT, and CAL. All partitions had dirichlet base frequencies. The Bayesian analysis lasted 350,000 generations and saved a total of 702 trees. The first 25% of sampled trees were discarded for burn-in. The consensus trees and posterior probabilities were calculated from the remaining 528 trees. Based on the results of the combined sequence data for the *C. gloeosporioides* species complex, five isolates in this study from different host plants, were recognised as *C. fructicola* (Fig. 4).

Taxonomy

The Consolidated Species Concept (Quaedvlieg *et al.* 2014) was employed in this study to distinguish the *Colletotrichum* species from Iran. Three species including *C. salicis*, *Colletotrichum* sp. (*C. fuscum sensu lato*) in the *C. destructivum* species complex, and *C. fructicola* were recognised as new species for mycobiota of Iran. In addition, *Viola* sp. is reported as a new host for *Colletotrichum* sp. (*C. fuscum sensu lato*) in the *C. destructivum* species complex and *C. sinensis*, *G. caspica*, and *S. ebulus* are new host records for *C. fructicola* all over the world. Novel host records are shown with an asterisk, in table 1. The descriptions of these species are presented in a list-form below:

Colletotrichum sp. (*C. fuscum sensu lato*)

Colonies on PDA reaching 65–67 mm diam. after 7 days at 26°C in the dark; circular, flat, without aerial mycelium and with even margins. Colony colour grey to brown at the centre, becoming pale to bright yellowish towards the margin. On MEA colonies reaching 57 mm in diam. after 7 days at 26°C in the dark; circular, flat, without aerial mycelium except at the center, with even margins. Colony colour black or grey at center, pale yellow towards the margin. On PDA, the mycelia are septate, branched, thin-walled, hyaline to pale brown, smooth, and 2–7 µm in diam. Conidiomata formed in the centre of the colony, acervular. Setae straight, or slightly curved at the base, brown to pale brown, smooth, 3–5 cross walls, unbranched. Conidiogenous cells monophialidic, smooth, hyaline, ampulliform to subcylindrical, slightly undulatory. Conidia unicellular, smooth and hyaline, cylindrical to oblong, slightly curved, not in a chain, rounded at one end, truncated at the other end, sometimes with a hilum-like low protuberance at the base, (12–)17–18.5(––21) × 3–4(–5) µm in size. Various big and small guttules observed inside conidia. Appressorium in slide culture mostly formed in terminals of hyphal branches and also from conidia as sessile appressorium, globose, subglobose, ovoid, ellipsoids, undulate and other shapes, rarely as small groups in irregular and short chains, brown to

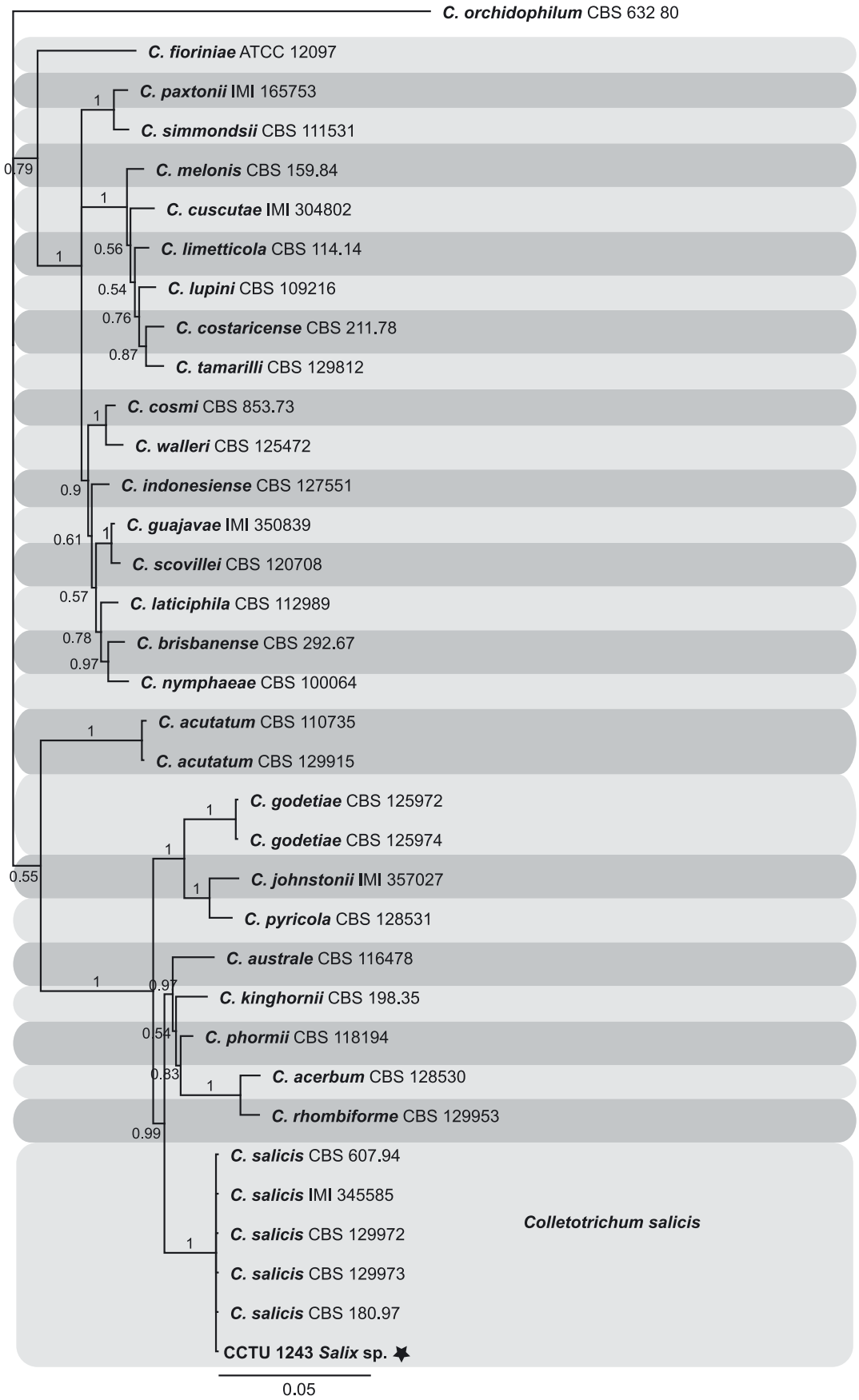

Fig. 2. A Bayesian inference phylogenetic tree of the *Colletotrichum acutatum* species complex. The tree was built using concatenated sequences of the ITS (internal transcribed spacer), ACT (actin), TUB (beta tubulin) and HIS (histone H3) each with a separate model of DNA evolution. Based on the results of the combined sequence data for *C. acutatum* species complex, our isolate (CCTU 1243) was identified as *C. salicis* (marked with asterisk). The tree was rooted to *C. orchidophilum* (CBS 632.80). The scale bar indicates 0.1 expected changes per site

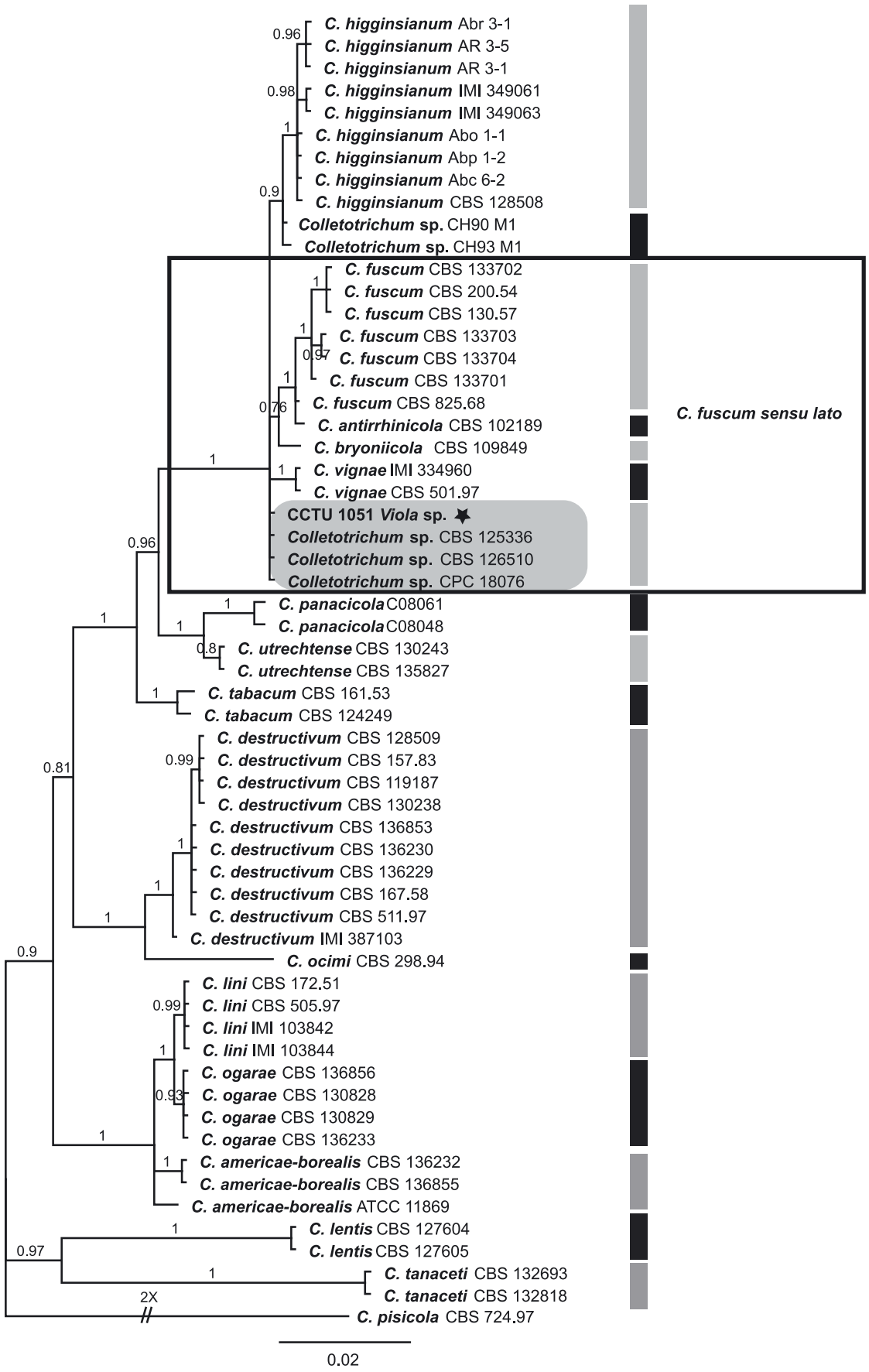

Fig. 3. A Bayesian inference phylogenetic tree of *Colletotrichum destructivum* species complex. The tree was built using concatenated sequences of the ITS (internal transcribed spacer), ACT (actin), HIS (histone H3), and TUB (beta tubulin) each with a separate model of DNA evolution. Based on the results of the combined sequence data for *C. destructivum* species complex, our isolate (CCTU 1051) identified as *Colletotrichum* sp. (*C. fuscum sensu lato*) (marked with asterisks). The tree was rooted to *C. pisicola* (CBS 724.97). The scale bar indicates the number of expected changes per site

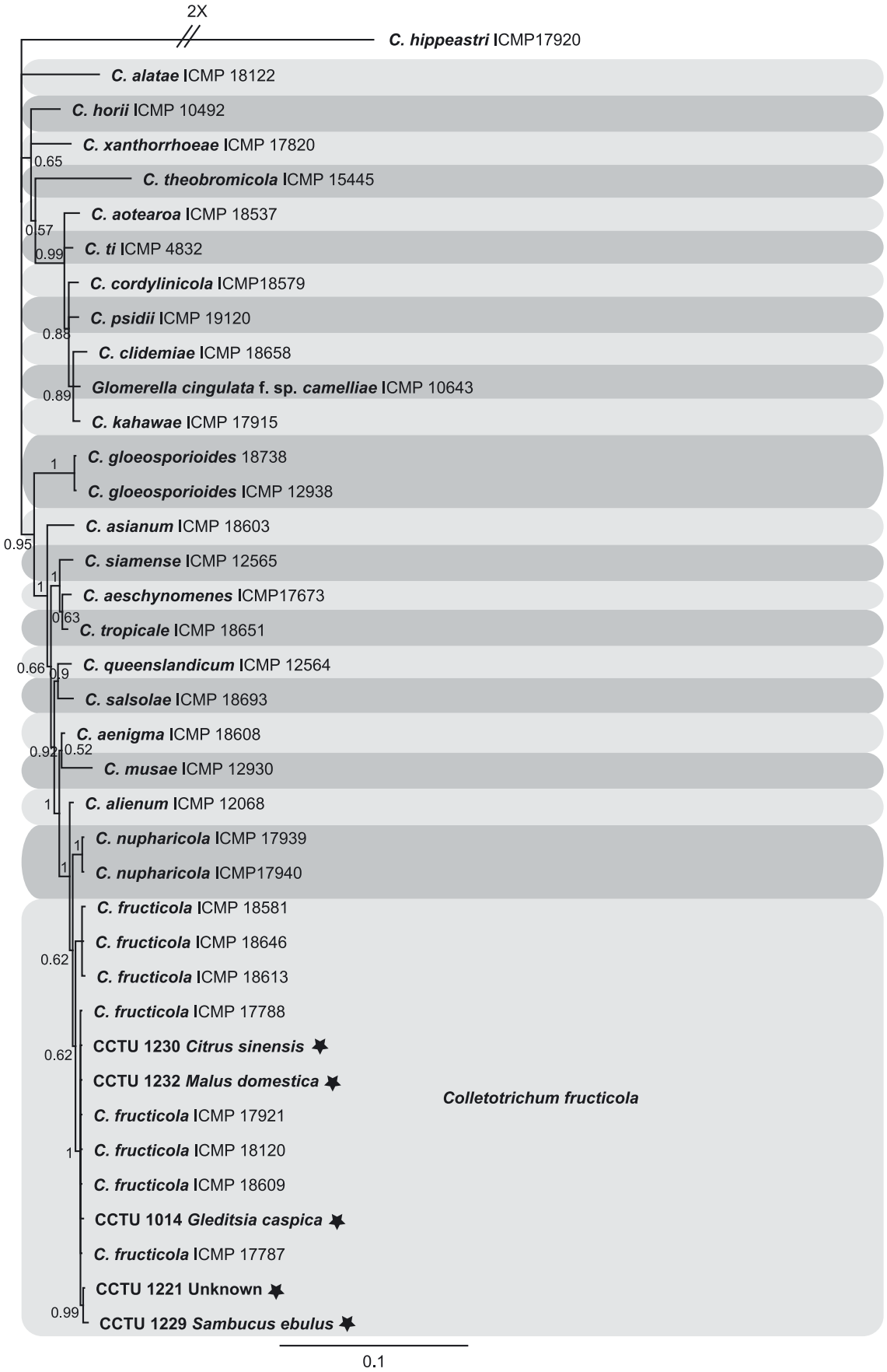

Fig. 4. A Bayesian inference phylogenetic tree of *Colletotrichum gloeosporioides* species complex. The tree was built using concatenated sequences of the ITS (internal transcribed spacer), ACT (actin), and CAL (calmodulin), each with a separate model of DNA evolution. Based on the results of the combined sequence data for *C. gloeosporioides* species complex, five isolates in this study from different host plants were noted as *C. fructicola* (marked with asterisks). The tree was rooted to *C. hippeastri* (ICMP 17920). The scale bar indicates the number of expected changes per site

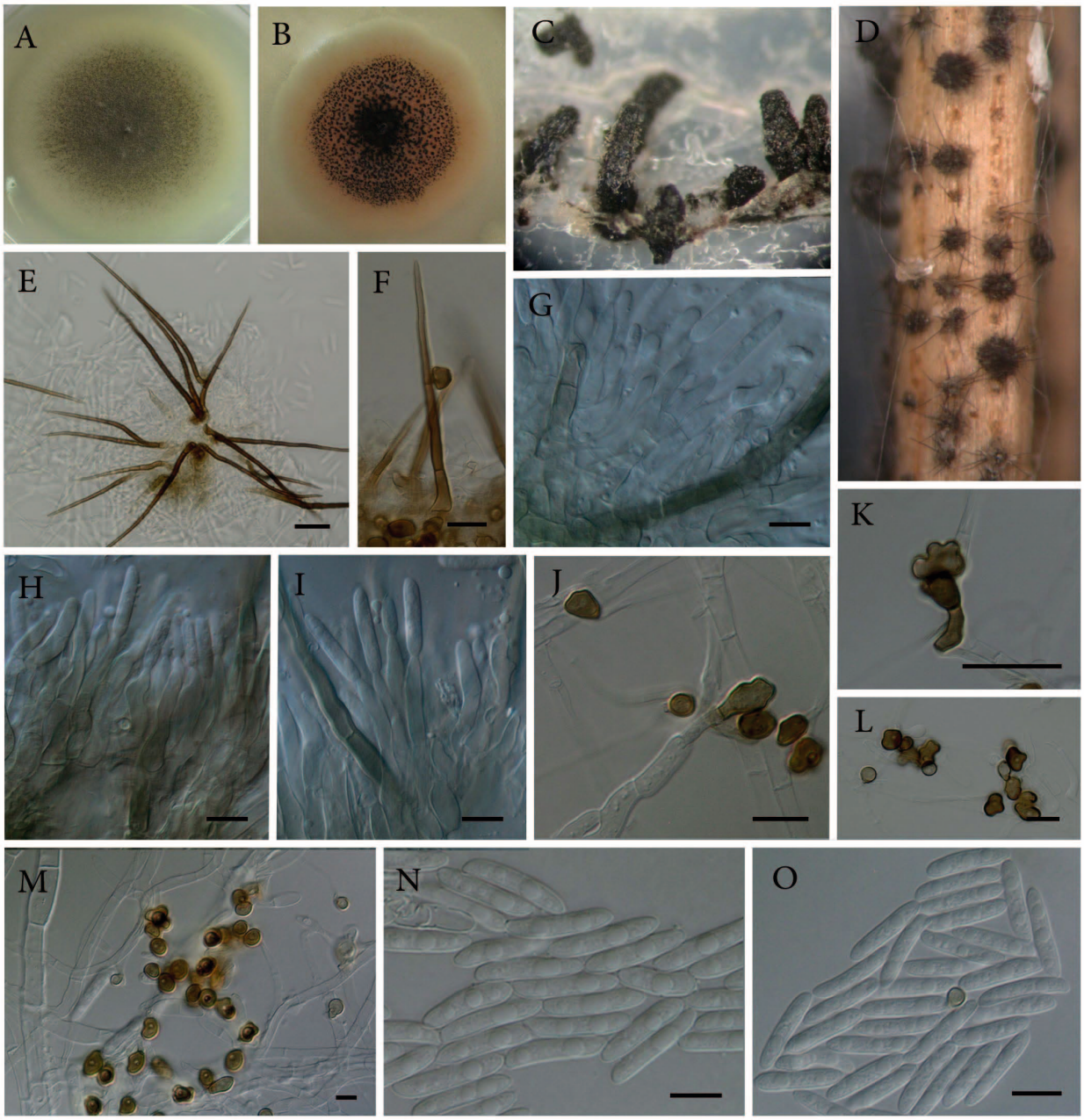

Fig. 5. *Colletotrichum* sp. (*C. fuscum sensu lato*): A, B – colonies on PDA and MEA media; C – sclerotia; D – acervulus on pine leaf; E, F – conidiomata and setae; G, H, I – conidiophores; J, K, M – appressoria; N, O – conidia. Scale bars: E = 20 µm, F–O = 10 µm

pale brown, smooth, and (4–)7.17–9.73(–18) × (4–)5.8–7.15(–11) µm in size. Clamydospores formed as intercalary, smooth, brown, globose to fusiform. Sclerotia formed on PDA with age on the surface of conidiomata and on pine needles on SNA, in various shapes, club-shaped or conic and lengthy or in irregular shapes, black. Teleomorph was not observed (Fig. 5).

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

Colonies on PDA were fast growing, reaching 82 mm diam. after 7 days at 26°C in the dark; circular, abundant aerial mycelium, wooly to felt in the centre, flat towards the margins. Colony colour dark grey at the centre, pale grey to pale yellow towards the margin. On MEA, colonies reach 81 mm diam. after 7 days at 26°C in the dark; circular, flat, without aerial mycelium except at the centre and with even margins. Colony colour white to grey at the centre, whitish at the margins including black sectors.

On PDA, mycelia septate, hyaline to pale brown, smooth, branched, thin-walled, 3–7 µm wide. Conidiomata was absent in the culture. Setae absent. Sclerotia absent. Conidiogenous cells in aerial mycelia cylindrical, subcylindrical to elongate ampulliform, terminal or intercalary, smooth-walled, periclinal thickening visible. Conidia unicellular, smooth, hyaline, mostly with two visible guttules, cylindrical, rarely oblong. Obtuse to slightly rounded ends, and (10–)11.5–12(–13) × 5–5.5(–6) µm in size. In slide culture, appressoria mostly formed from mycelia, brown to dark brown, smooth, globose, subglobose, ovoid, clavate, undulate and other shapes, in small groups as irregular and short chains, and (5–)8.8–11.8 (–24) × (3–)5.5–6.4(–8) µm in size. Perithecia dark brown,

brown, to pale brown, globose, subglobose to pyriform, with hairs around. Completely immersed or semi-immersed in the PDA medium, distributed on the medium surface, covered under aerial mycelia; peridium texture angularis and thick-walled. Asci clavate to cylindrical, 6–8 spores, unitunicate, thin-wall. Ascospores mostly arranged biseriately, and (43–)49.5–52.5(–60) × (8–)9–9.5 (–11) µm in size. Paraphyses were present, hyaline, septate, wide at the base and narrower towards the end. Ascospores (10–)14–15.5(–19) × (4–)4–4.5(–6) µm, unicellular, hyaline, smooth, slightly curved to very slightly curved. Both ends round or one side obtuse and another side round, rarely were guttules visible on ascospores (Fig. 6).

Colletotrichum salicis (Fuckel) Damm, P.F. Cannon & Crous, Studies in Mycology 73: 97 (2012)

Colonies on PDA reaching 74–80 mm in diam. after 7 days at 26°C in the dark. Circular, flat, with abundant aerial mycelium at the centre and with even margins. Colony colour grey at the centre, whitish towards the margin. On MEA colonies reached 82 mm in diam. after 7 days at 26°C in the dark. Circular, flat, with abundant aerial mycelium at the centre and with even margins. Colony colour whitish at center, grey towards the margin. Hyphae 2–9 µm in diam., smooth-walled, hyaline to pale brown, septate and branched. Conidia masses were visible around the center and margin as sporadic – especially

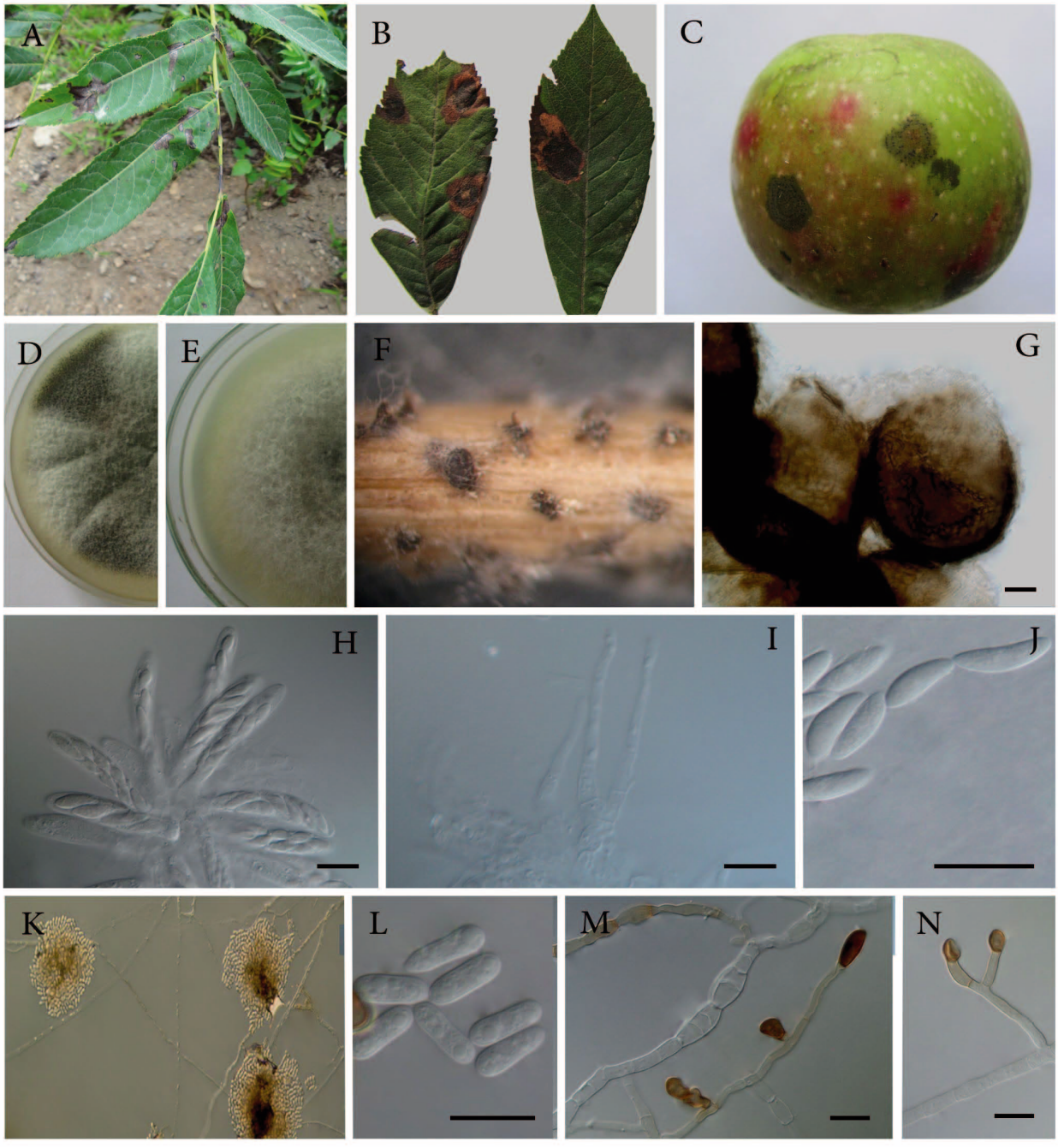

Fig. 6. *Colletotrichum fructicola*: A, B, C – naturally infected plant materials including fruit and leaves; D, E – colonies on MEA and PDA; F, G – perithecia on pine leaf, and perithecium shape; H – asci; I – paraphyses; J – ascospores; K – conidia common in mycelium; L – conidia; M, N – appressoria. Scale bars: G = 20 µm, H–J = 10 µm, K = 50 µm, L–N = 10 µm

with increasing age. Conidiomata acervular. Setae present, brown to pale brown, widest at base and narrower towards the end, 1–3 cross walls, smooth. Sclerotia and chlamydospores absent. Conidiophores directly formed in acervuli and on mycelia; hyaline, smooth-walled, simple or branched and septate. Conidiogenous cells hyaline, smooth-walled, cylindrical or elongate ampulliform. Periclinal thickening visible. Conidia hyaline. Unicellular, smooth-walled, cylindrical, guttules were observed on some conidia as different designs, mostly two ends obtuse or one end obtused and one end tapered, and (4–)4.5–5(–6) × (12–)14–14.5(–16) µm in size. In slide culture, appressoria were formed terminally on hyphae as single or small groups, pale brown to brown, globose, subglobose, clavate, ellipsoid, ovoid and other irregular shapes, entire or undulate in the margin, (7–)11–13.5(–20) × × (4–)5.5–6.5(–9) µm in diam. Sexual structure (perithecia) on PDA: ascomata mostly globose and slightly pyriform, ostiolate, brown to darker brown, predium texture as angularis. Ascogenous hyphae hyaline, smooth-walled. Paraphyses septate, hyaline, smooth-walled, wide at base and round at end, asci clavate to cymbiform, 8-spored, uniseriate or biseriate, 54–85 × 9–11.76 µm in size. Ascospores unicellular, hyaline, smooth-walled, fusiform, ellipsoid or ovoid, rounded at both ends or tapered at one, sometimes having a very slight curve, (14–)15.5–16.5(–22) × × (4–)4.5–5 µm in diam. (Fig. 7).

Discussion

The present study aimed to characterise *Colletotrichum* species from the northern and northwestern zones of Iran based on a combination of DNA phylogeny and morphological characteristics. Members of this genus

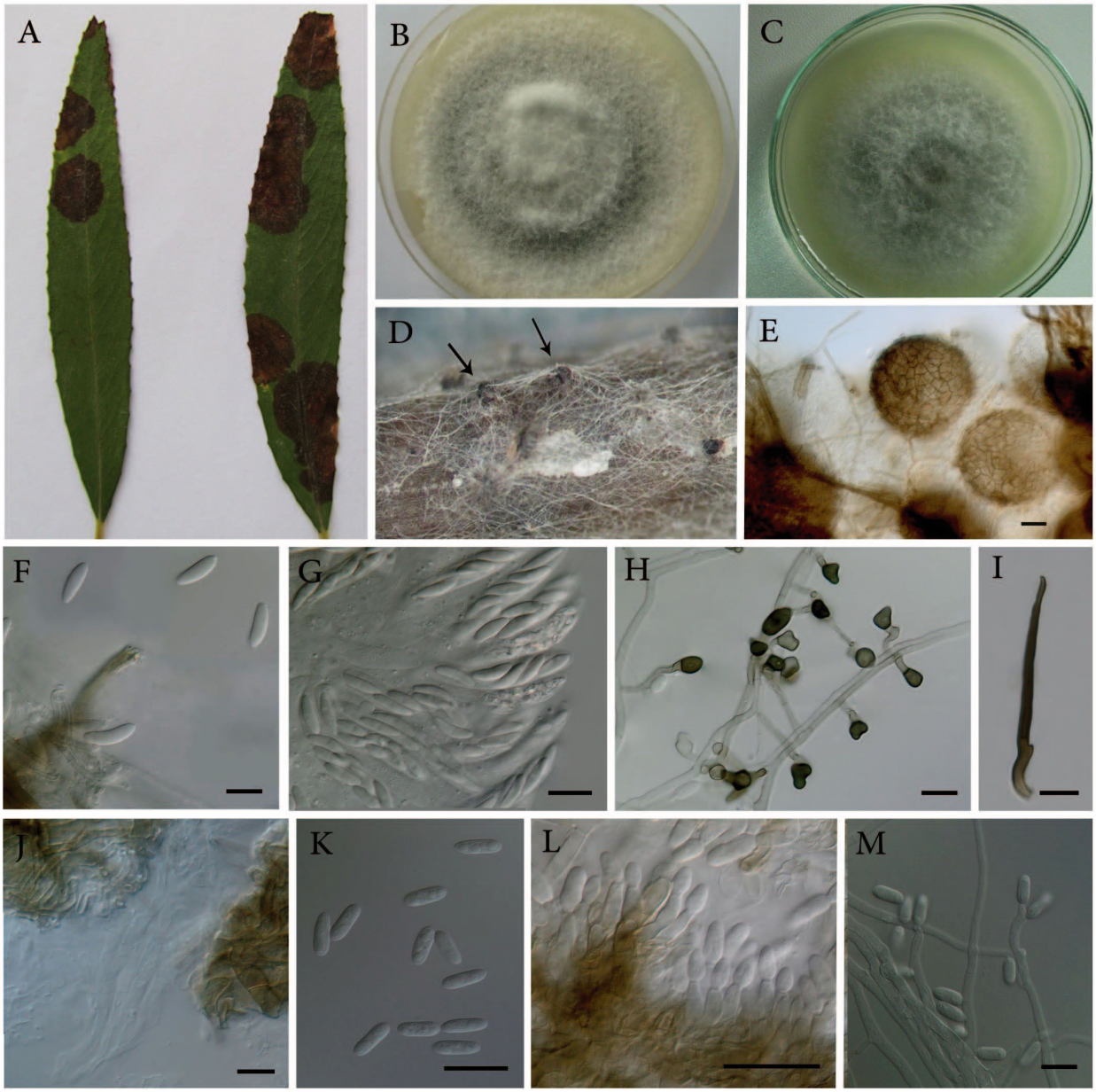

Fig. 7. *Colletotrichum salicis*: A – naturally infected leaves; B, C – colonies on MEA and PDA; D, E – perithecia on pine leaf, and perithecium shape; F – ascospores; G – asci; H – appressoria; I – setae; J – paraphyses; K – conidia; L – conidiophores; M – conidiogenous cells on aerial mycelia. Scale bars: E = 20 µm, F–M = 10 µm

are devastating plant pathogens on a wide range of economically important agricultural crops all over the world as well as Iran (Damm *et al.* 2010; Cannon *et al.* 2012). In the past, several *Colletotrichum* species have been reported from diverse plant species in Iran. However, the majority of *Colletotrichum* species known from Iran, have been identified based on morphological characteristics and host range. There is no culture available to confirm this species identity using molecular data (Ershad 2009). Species identification in *Colletotrichum* based solely on morphological features has proven troublesome (Cannon *et al.* 2012; Damm *et al.* 2010, 2012; Weir *et al.* 2012). Therefore, application of sequence data for precise identification of the species in this group of fungi, is crucial.

In this study, a combination of multigene phylogenetic sequence data and morphological characteristics enabled us to identify *Colletotrichum* species from different host species in Iran. As shown in figure 1, a single gene phylogeny based on ITS-rDNA sequence could only resolve species complexes. Our isolates clustered in three species clades including the *C. acutatum* species complex, *C. destructivum* species complex, and *C. gloeosporioides* species complex. Recently, multigene phylogenetic analyses have distinguished several cryptic species in each of these clades (Cannon *et al.* 2012; Damm *et al.* 2010, 2012, 2014; Weir *et al.* 2012). Sequence data from additional genes (ACT, HIS, TUB, and CAL) could then be used to further ascertain the identity of each species. A phylogenetic inference using combined data set of the ITS, ACT, and CAL sequences resolved our isolates residing in the *C. gloeosporioides* species complex as *C. fructicola* (Fig. 4) and a phylogenenetic inference using a combined data set of the ITS, ACT, TUB, and HIS sequences resolved our isolate residing in *C. acutatum* as *C. salicis* (Fig. 2). Furthermore, multigene phylogenetic inference using sequence data from the ITS, HIS, ACT, and TUB sequences confirmed the identity of our isolate in the *C. destructivum* species complex as *Colletotrichum* sp. (*C. fuscum sensu lato*) (Fig. 3).

These three species can be identified from each other and other *Colletotrichum* species based on cultural and morphological characteristics. Morphological characteristics of *C. fructicola* were in full agreement with the description provided by Prihastuti *et al.* (2009). *Colletotrichum fructicola* was first described by Prihastuti *et al.* (2009) on *Coffea arabica* and later additional hosts such as *Persea americana*, *Malus domestica*, *Fragaria ananassa*, *Ficus edulis*, *Camellia sinensis*, *Limonium sinuatum*, *Limonium* spp., *Pyrus pyrifolia*, *Dioscorea* spp., *Tetragastris panamensis*, *Theobroma cacao* (Weir *et al.* 2012), *Pyrus bretschneideri* (Li *et al.* 2013), and *Capsicum* spp. (Sharma and Shenoy 2014) were recorded for this species. In the present study, *C. sinensis*, *G. caspica* and *S. ebulus* are introduced as new hosts for this species.

Colletotrichum salicis was originally described as *Sphaeria salicis* by Fuckel (1870). Since then, several synonyms have been regarded for this species. A new combination has recently been proposed for this species as *C. salicis* by Damm *et al.* (2012). *Colletotrichum salicis* isolates have been reported to occur on a diverse range of plants including *Salix* spp., *Solanum lycopersicum*, *Araucaria excels*, *Acer platanoides*, *Fragaria ananassa*, *Populus* spp., *Pyrus pyrifolia* and *Rhododendron* sp. (Damm *et al.* 2012). In the present study, *C. salicis* was recovered from leaves of *Salix* sp. The leaves had severe anthracnose symptoms. Morphological descriptions of our isolates show little discrepancy with the description provided by Damm *et al.* (2102). The presence of seta and visible conidia on SNA in our isolates were the main differences with pervious descriptions provided for *C. salicis*. The size and shape of conidia and ascospores slightly differed from pervious descriptions. However, sequence data of ITS, ACT, TUB, and HIS in our isolate, showed a 100 percent similarity with the sequence data for the other isolates of this species in GenBank. The problem, that morphological features of *Colletotrichum* spp. change under different growth conditions, or can be lost or changed with repeated sub-culturing was also previously stated by Weir *et al.* (2012). Hence, these data further illustrate that morphological characters alone are insufficient to delineate the species of the genus *Colletotrichum.*

Colletotrichum isolate (CCTU 1051) from *Viola* sp. (Family: Violaceae) clustered with three unnamed isolates from *Heracleum* sp. (Family: Apiaceae) in the *C. fuscum* clade (Damm *et al.* 2014). There is no description available for *Colletotrichum* isolates from *Heracleum* sp. in the scientific research literature. However, our isolate is morphologically different from *C. fuscum* and the other species described in this clade, namely *C. antirrhinicola, C. vignae*, and *C. bryoniicola.* Our isolate developed chlamydospore, appressorium, and sclerotium in culture (Fig. 5), which makes it distinct from the other species in the clade. None of the species described in this clade develops chlamydospore, appressorium, and sclerotium all together, in culture. This isolate together with three unnamed isolates from *Heracleum* sp. probably represents a new species. We have postponed our final decision until additional collections become available.

These three species are new records for mycobiota of Iran. This study provides the first report on the occurrence of *C. salicis* on *Salix* in Iran. According to these results, *Viola* sp. from West Azerbaijan is a new host for the *Colletotrichum* sp. within the *C. destructivum* clade. Furthermore, *C. sinensis* from Mazandaran, *G. caspica* and *S. ebulus* from Guilan, are new host records for *C. fructicola*. However, the economic impact, distribution, and host ranges for these species remain to be studied.

The data presented in this study further emphasises the significance of multigene sequence in *Colletotrichum* taxonomy and the combination of such data with the morphology, ecology, and hosts range for accurate identification of the *Colletotrichum* species.

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