An emerging disease on young apple trees associated with crown and collar canker and necrosis caused by Cytospora balanejica sp. nov. in Iran

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Abstract

Apple is the most important fruit tree in West Azarbaijan province of Iran. However, a disease with crown and collar canker and necrosis was observed in three young apple orchards in Urmia, affected 15% and 1% of 'Red Delicious' and 'Golden Delicious' cultivars, respectively. A fungus with typical characteristics of the asexual morph of Cytospora was regularly isolated from the diseased tissues. Morphological characteristics and phylogenetic analysis inferred from the combined dataset of the ITS–rDNA, parts of LSU, tef1-α, rpb2 and act1 genes revealed that the isolates represent a new species of Cytospora, described herein as Cytospora balanejica sp. nov.. Pathogenicity of all isolates was confirmed on the cv. 'Red Delicious' based on Koch's postulates. Also, the reaction of 12 apple cultivars was assessed against five selected isolates with higher virulence. Results showed that except cv. 'Braeburn' which did not produce any symptoms of infection, the other 11 cultivars showed characteristic symptoms including sunken and discolored bark and wood. The mean length of discolored area was different among the 11 so-called susceptible cultivars, hence cvs. 'M4' and 'Golden delicious' had the highest and the lowest lesion length, respectively. Moreover, the aggressiveness of five tested isolates was varied and the isolates BA 2–4 and BA 3–1 had the highest and lowest aggressiveness, respectively. Based on our observations on the potential ability of the fungus in causing the disease on young and actively growing apple trees, it will be a serious threat to apple cultivation and industry.

Introduction

The domesticated apple (*Malus* × *domestica* Borkh.) is one of the oldest, most popular and widely grown temperate fruit crops in the world[1,2]. It is one of the most economically important fruit crops worldwide and is the 3rd most produced fruit crop[3]. The fruits are predominantly used for the fresh market, even though other uses are cider production and processing[4–6]. Apple is an ancient fruit crop in Iran, growing at different locations from the north to the west and central parts of the country[7,8]. A high level of genetic diversity is seen in cultivated apples in Iran and the results of a phylogenetic study showed that Iran could be a paramount center of diversity for domesticated apples and an important center for domestication and pass on from central Asia to the West via Silk Routes [8,9].

In Iran, West Azarbaijan province is the main apple growing region with 63,661 ha and total production of 1,118,285 metric ton in 2020, ranked first with 26.5% of the total production[10]. Apple trees have a long juvenile phase, often start bearing fruits after five years, so growers enterprise heavily before generating revenue and this led, they typically plant and grow a small number of well assessed and historically successful apple varieties[2]. Two apple cultivars, 'Red Delicious' and 'Golden Delicious' are the main commercially grown apples in this region which has contributed toward the genetic uniformity of the orchards. During the past two decades and largely due to climate change, apple orchards in West Azarbaijan province were under severe threat from both biotic and abiotic agents. Depending on the incidence and severity of the infection, impacts ranged from decreased yield with poor fruit quality and plant longevity to complete loss of fruit and trees, causing great economic losses to growers. Apple trees are affected by different fungal pathogens, among them, stem and trunk canker and dieback diseases are of great importance, causing progressive losses over the years[11–18]. It has been estimated that abiotic and biotic stresses reduce 12–25% of annual apple harvest[19].

Cytospora species are important plant pathogens associated with branch dieback and canker disease on a wide range of plants with worldwide distribution[20–23]. They are usually considered as wound pathogens, invade host tissues through cracks and wounds or other openings in the bark, leading to growth weakness and death of plants[21, 24–26]. The fungal hyphae invade host tissues, decompose the cambium and penetrate extensively into phloem and xylem of trunks, twigs and scaffold limbs, leading to perennial and latent infections providing a potential source of inoculum for the disease[27–30]. Thus far, about 24 species of *Cytospora* and their sexual allies have been reported from *Malus* spp. Worldwide[30–33], of which 14 species recognized in Iran[32,34,35]. However, taxonomic status of most of these species has not been approved through molecular approaches. Due to overlapping in morphological characteristics, poor condition of multi-locus phylogeny and insufficiency of fresh collected specimens, using multiphase approaches and multi-locus phylogeny have been suggested to elucidate accurate species boundaries among *Cytospora* isolates[21,26,36]. Incorrect diagnosis and treatment generally result in rapid spread of the diseases and small instances of diseases can quickly become a large and costlier problem under their rapid multiplication and conducive environmental conditions[37]. This occurred in West Azarbaijan province with a fungal trunk canker disease mainly caused by *Diplodia bulgarica*[18,38] which was erroneously identified as bacterial canker, so a large number of productive orchards of 'Golden Delicious' cv. (15–50 years old) were completely destroyed and replanted again with apple or other fruit crops (unpublished data).

During our investigations on apple diseases in West Azarbaijan province, Iran, we observed special disease symptoms including crown and collar canker and necrosis in three young apple orchards in Urmia, leading to relatively rapid tree decline and death. Plants of the cv. 'Red Delicious' were more susceptible than the cv. 'Golden delicious' in the same orchard environment as deduced from the percentage of affected plants. Culture of the plant samples resulted in permanent isolation of a fungus with *Cytospora* characteristics. The objective of this study was to (i) identify *Cytospora* species involved in the disease based on morphological characteristics and molecular multi-gene phylogeny, (ii) assessment of the pathogenicity of the isolates on apple cv. 'Red Delicious' and (iii) a preliminary evaluation of the reaction of 12 different apple cultivars to five selected isolates of the pathogen with higher aggressiveness.

Results

Disease symptoms, incidence and fungal isolations. Characteristic external disease symptoms including general decline, cankers and plant death were seen during the summer and early autumn in three young apple orchards, both on the cvs. 'Golden Delicious' and 'Red Delicious'. The leaves were pale yellow at first in some individual branches, then their margins became necrotic and in the late summer and early autumn, the color of the leaves changed to purple and finally died (Fig. 1). Shoot elongation was arrested in affected plants. Bark of the diseased plants was discolored and sunken at the soil line, longitudinal cracks and cankers were formed on the bark surface and discoloration was extended progressively both upward (up to 50 cm from the graft union) and

downward to the main roots and into the wood. A distinct margin separated healthy from infected bark tissue and trees were killed when the infected area girdled entire trunk base. In cross-sections, there was a light brown to brown discoloration and necrosis as V or U shape in the hard wood (Fig. 1). Based on these external symptoms in the surveyed orchards, the incidence of the disease on the cv. Red Delicious' (15%) was higher than the cv. Golden Delicious (1%).

In this study, 24 fungal isolates (19 from the cv. Red Delicious' and 5 from the cv. Golden Delicious') were obtained and purified. Based on the comparison of morphological characteristics of the purified isolates, three were selected for multi-gene phylogenetic analysis and accurate species identification.

Phylogenetic analysis

The phylogenetic analysis of combined dataset (ITS, LSU, *rpb2*, *act1* and *tef 1-a*) include 207 *Cytospora* ingroup strains representing 144 *Cytospora* species and *Diaporthe eres* CBS 145040 and *Diaporthe vaccinii* CBS 160.32 as outgroup strains with a total of 2676 characters including gaps (570 for ITS, 922 for LSU, 968 for *rpb2*, 238 for *act1* and 509 for *tef1-α*). The best scoring RaxML tree with the final ML optimization likelihood value of −36304.329463 (ln) is selected to denote and consider the phylogenetic relationships among the strains (Fig. 2). *Cytospora balanejica* represented a monophyletic clade with high support value (87%) (marked in pink in Fig. 2).

Taxonomy

Cytospora balanejica R. Azizi, Y. Ghosta & A. Ahmadpour sp. nov. (Fig. 3)

MycoBank No.: MB843116.

Etymology: Named after the locality, Balanej village, where the holotype was collected.

Typification: Iran, West Azarbaijan Province, Urmia City, Balanej Village, 37°23'50.4" N, 45°09'15.9" E., from crown of *Malus × domestica* cv. Red Delicious', 15 Oct. 2017, R. Azizi (Holotype: IRAN 18133F; ex-type living culture: IRAN 4419C).

Description: Asexual morph: Conidiomata labyrinthine cytosporoid, immersed in the bark, erumpent when mature through the surface of the bark, discoid to conical, pale luteous to luteous, with multiple locules, (800–)850–1,490(–1,700) µm in diam. Conceptacle conspicuous, black, circular, surrounded the stromata. Ectostromatic disk greenish black to black, circular to ovoid, (473–)563–802(–845) µm in diam., with a single ostiole per disk in center. Ostiole conspicuous, circular to ovoid, olivaceous grey, at the same level as the disk surface, (94–)101–215(–230) µm in diam. Locules numerous, arranged circularly with shared invaginated walls. Conidiophores borne along the locules, hyaline, smooth, thin walled, unbranched or occasionally branched at base. Conidiogenous cells entroblastic, phialidic, subcylindrical to cylindrical, tapering towards apices, (6.2–)9–17(–19) × (1–)1.2–2 µm. Conidia hyaline, smooth, elongate allantoid, mostly biguttulate, aseptate, 3–5 × 1–1.8 µm. Sexual morph: not observed.

Culture characteristics: Colonies after 3 d at 25 °C on PDA average 57 mm and entirely covering the 9-cm diam. Petri dish after 7 days, margin entire, white to buff, with scattered aerial hyphae at center, the hyphae becoming very dense, pale luteous at center and honey at margins, forming abundant solitary or rarely aggregated pycnidia surrounded by off-white hyphae with age. Hyphae hyaline to light brown, septate, smooth walled and branched.

Habitat and distribution: Known only on *Malus × domestica* in Urmia, Iran.

Additional specimens examined: Iran, West Azarbaijan Province, Urmia City, Balanej Village, 37°24'26.1" N, 45°10'24.8" E., from the trunk of *Malus × domestica* cv. 'Red Delicious', 15 Oct. 2017, R. Azizi, (IRAN 4420C); West Azarbaijan Province, Urmia City, Kurane Village, 37°24'44.4" N 45°8'45.3" E., from the trunk of *Malus × domestica* cv. 'Golden Delicious', 12 Sept. 2018, R. Azizi, (FCCUU 350).

Notes: *Cytospora balanejica* was isolated from young, declining apple trees showing symptoms of crown and collar canker and necrosis. The phylogenetic inferences based on combined multi-gene phylogeny resolved this species as a separate lineage distinct from all other strains included in this study, although it shared a sister relationship with a clade containing *C. albodisca* M. Pan & X.L. Fan and *C. corylina* H. Gao & X.L. Fan (Fig. 2). However, *C. balanejica* differs from *C. albodisca* based on slower growth rate (57 mm vs. 70 mm after 3 days), colony color (pale luteous vs. dark herbage green to dull green in *C. albodisca*), absence of ascomata and smaller conidia (3–5 × 1–1.8 µm vs. 5–7 × 1–2 µm)[26]. Also, it differs from *C. corylina* based on slower growth rate on PDA medium (57 mm after 3 days vs. 90 mm after 2 days in *C. corylina*), colony color (pale luteous vs. fucous black), the formation of distinct conceptacle, larger conidiomata (800–1700 µm vs. 850–1280 µm) and shorter conidia (3–5 µm vs. 3.5–7.5 µm)[39]. Therefore, we describe *C. balanejica* here as a new species.

Pathogenicity trials

Results of pathogenicity tests of the isolates on shoots of the cv. 'Red Delicious' showed sunken discolored lesions around the inoculated sites 14 days post-inoculation. Bark and wood discoloration was extended progressively upward and downward the inoculation site and after 20 days, fungal pycnidia were formed on the discolored bark. Despite this, the mean length of necrotic lesions varied among the isolates (data not shown). Re-isolation of the inoculated fungus and re-identification based on morphological characteristics fulfilled Koch's postulates. All negative controls were symptomless and no colonies were obtained from samples taken from controls. The reaction of 12 tested cultivars against five selected isolates with higher virulence showed that the interaction between the factors isolates × cultivars was varied and significantly different at $P \leq 0.05$ (Fig. 5). Except for the cv. 'Braeburn' which did not produce any symptoms of infection similar to control treatment against all tested fungal isolates, the other cultivars showed symptoms of infection at least

against two fungal isolates (Fig. 4). The mean length of necrotic lesion ranged from 19.3 mm (the cv. 'Idared') to 188.3 mm (the cv. 'M4') for isolate BA 2–4 and from 63.3 mm (the cv. 'MM106') to 196.6 mm (the cv. 'M4') for isolate BA 1–1, both isolates were obtained from the cv. 'Red Delicious' (Fig. 5). The mean length of necrotic lesions ranged from 18.3 mm (the cv. 'MM106') to 193.3 mm (the cv. 'M4') for isolate KU 1–1 which was isolated from the cv. 'Golden Delicious', although the cvs. 'Granny Smith', 'MM109' and 'Idared' did not show any symptoms of infection against this isolate (Fig. 5). Also, the cvs. 'Delbard Estivale', 'MM109', 'Idared' and 'Golden Delicious' did not develop any symptoms of infection against BA 2–1 isolate and the mean length of necrotic lesion ranged from 101.6 mm (the cv. 'Red Delicious') to 190 mm (the cv. 'Granny Smith'). At last, only four cultivars including 'M4', 'M7', 'Golden Primrose' and 'Red Delicious' developed symptoms of infection against BA 3–1 isolate and the mean length of necrotic lesion ranged from 93.3 mm (the cv. 'Red Delicious') to 206.6 mm (the cv. 'M4') (Fig. 5). Moreover, the aggressiveness of five tested isolates was varied and the isolates BA 2–4 and BA 3–1 had the highest and lowest aggressiveness against 12 tested cultivars, respectively. Re-isolation of the inoculated fungus was done in all symptomatic shoots and re-identified by morphological characteristics, fulfilled Koch's postulates. Re-isolation of the fungus from asymptomatic shoots as well as negative controls was failed.

Discussion

In this study, we found a new species of *Cytospora*, *C. balanejica*, associated with crown and collar canker and decline symptoms in young apple trees. The incidence of the disease was greater on the cv. 'Red Delicious' than the cv. 'Golden Delicious', two commercial apple cultivars grown widely in the studied area, indicating more susceptibility of the first cultivar to this new *Cytospora* species. Our pathogenicity tests confirmed this, as all studied isolates were pathogenic on the shoots of the cv. 'Red Delicious' and had greater virulence (more the length of necrotic lesions) than the cv. 'Golden Delicious' (Fig. 5). Also, the results of our study showed that the cv. 'Braeburn' did not develop any symptoms of infection against all the tested fungal isolates, hence we considered it as resistant. Resistance of 53 accessions of diverse *Malus* species and their interspecific hybrids were tested against *Valsa ceratosperma* (syn.: *Cytospora ceratosperma*) using excised shoot assay and by measuring the length of necrotic lesion. Fourteen accessions were evaluated as resistant and the highest level of resistance was identified in *Malus sieboldii* Rehder, which was effective against different isolates of the tested fungus[40]. Similar results were also found in pathogenicity studies using different fungal species and host plants[41–46]. The virulence of the tested fungal isolates as measured by lesion length was varied and this could be attributed to the genetic diversity among the isolates. Variability in the lesion length has been reported in pathogenicity evaluation of the isolates of *Cytospora* spp. and other fungal pathogens[31,44,47–51]. In our study, disease symptoms differed from the previously reported symptoms of apple canker diseases caused by *Cytospora* species, as the disease starts from the crown and collar region of the tree (Fig. 1). Other apple diseases such as Neonectria canker, Phytophthora crown, collar and root rots, Rosellinia root rot and fire blight have been reported in the literature to cause similar symptoms on affected young apple trees[13,52]. *Cytospora* species cause canker, dieback and decline diseases with different symptoms on a wide range of woody perennials including fruit and nut trees, forest and urban trees and rarely on herbaceous plants with strong ecological adaptability[21,26,39,53,54]. The similarity in symptoms caused by this species and other diseases, especially in the early stages of disease development, make it difficult accurately identification of the causal agents without further laboratory examination.

Apple is one of the main hosts that suffer severe damage from the Cytospora canker disease[31,55,56]. At present, 24 *Cytospora* species have been reported as causal agents of canker disease in apple trees worldwide and 14 species have been recorded in Iran[30,32,33,35]. The results of our study indicated that more extensive pathogen surveys of apple production regions should be undertaken. Understanding the exact diversity of pathogenic fungi such as *Cytospora* spp. will be important in devising regional management strategies for each species, developing rapid diagnostic tools, screening for resistance and accomplishing regulatory control measurements[57]. Earlier species identification in *Cytospora* have relied on morphological characteristics and host association, however, these characteristics are not stable and informative causing confusion in species identification and delimitation[39,54,58]. Also, recent studies showed that ITS–rDNA, which has been accepted as a standard barcode marker for fungi, lacks enough resolution to delineate species in the genus *Cytospora*[21,36,59,60]. The results of our phylogenetic analysis using ITS–rDNA sequences placed *C. balanejica* together with *C. albodisca*, *C. corylina* and *C. olivacea* in a clade, confirmed poor utility of this genomic region in the differentiation of *Cytospora* species (Supplementary Fig. S1). Recent studies used polyphasic approach, morphology and multi-gene phylogeny, for species identification which has revealed hidden fungal diversity, separated closely related species into distinct monophyletic clades and led to the description of several new cryptic *Cytospora* species[21,23,26,36,39]. Based on our multi-gene phylogenetic analysis, *C. balanejica* formed a well-defined lineage distinct from all other strains used in this study and shared a sister relationship with *C. albodisca* and *C. corylina*, two recently described *Cytospora* species (Fig. 2). *Cytospora albodisca* and *C. corylina* were isolated from the branches of *Platycladus orientalis* (L.) Franco and *Corylus heterophylla* Fisch. ex. Trautv. in China showing canker and dieback symptoms, respectively[26,39]. Pairwise sequence comparisons of the genomic regions in *C. balanejica* strain IRAN 4419C showed considerable nucleotide differences from *C. albodisca* strain CFCC 53161 (including 2 out of 466 in ITS, 30 out of 726 in *rpb2*, 20 out of 160 in *act1* and 73 out of 516 in *tef1-α*) and *C. corylina* strain CFCC 54684 (including 2 out of 466 in ITS, 30 out of 726 in *rpb2*, 17 out of 161 in *act1* and 75 out of 513 in *tef1-α*).

This study found that apple trees are hosts of a new pathogenic species of *Cytospora*, which should be considered as a potentially important causal agent of crown and collar canker on this plant in the studied area. Although the disease incidence was significantly lower in the cv. 'Golden Delicious' than the cv. 'Red Delicious', it is important to note that the infected plants can provide an inoculum reservoir for the pathogen. *Cytospora* species are generally considered as wound pathogens, infecting plants through cracks and wounds caused by freezing injuries, leaf scars, sunburn, oil injuries, shade weakened twigs and pruning wounds[21,51,61]. Despite the fact that this study extends our knowledge about the role of *Cytospora* species in crown and collar canker disease on young apple trees, more studies are needed to reveal its biology and ecology, assess the susceptibility/resistance of apple cultivars under field conditions and its host range and epidemiology in order to development of effective management strategies.

Material And Methods

Collection of samples and fungi isolation

Young apple trees (2–6 years old) showing symptoms of retarded growth, decline and dying from three orchards in Urmia, West Azarbaijan province, Iran, were evaluated and samples from their crown, collar and trunk base showing bark and wood discoloration and canker (Fig. 1) were collected, placed separately in clean paper bags and transferred to the laboratory for further study. The samples were washed gently under running tap water, then smaller pieces from the interfaces of the healthy and diseased tissues were cut and surface disinfested in 3% sodium hypochlorite solution for 2 min, washed again three times with sterile distilled water and blotted dry on autoclave sterilized filter paper. The pieces were plated onto potato-dextrose-agar (PDA; Merck, Darmstadt, Germany) medium in 90 mm diam. glass Petri dishes supplemented with streptomycin sulfate and penicillin G (150 ppm each) to inhibit bacterial growth. Petri dishes were incubated at 25±1 °C in darkness, examined at 24 h intervals and hyphae growing out from the plant tissues were transferred to fresh PDA. Pure cultures were obtained using hyphal tip method. The purified isolates were maintained on PDA slants containing a piece of filter paper and stored at 4 ºC. The isolates were deposited in the Fungal Culture Collection of the Iranian Research Institute of Plant Protection ("IRAN") and Fungal Culture Collection of Urmia University (FCCUU). Sequence data were deposited in GenBank dataset and their accession numbers are provided in the text (Table 1).

Table 1 Fungal isolates used in the molecular analysis in this study and GenBank accession numbers.

[1] CBS: Westerdijk Fungal Biodiversity Institute (CBS-KNAW Fungal Biodiversity Centre), Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Centre, Beijing, China; MFLUCC: Mae Fah Luang University Culture Collection, Thailand; NE: Gerard Adams collections, University of Nebraska, Lincoln NE, USA; PPRI: Culture collection of the Plant Protection Research Institute, Agriculture Research Center, Pretoria, South Africa. All type materials are marked with T.

Plant materials

It is notified that plant materials used in this study were legitimate samples from apple orchards and all methods comprising plant studies were performed in accordance with the relevant guidelines, regulations and legislation. Required permission to collect samples of apple trees from various orchards in Urmia, West Azarbaijan province was obtained.

DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Total genomic DNA was extracted from the mycelial mass of fungal isolates cultured in potato dextrose broth (PDB) for 7–10 days using the Exgene^{TM} Cell SV mini kit (GeneAll Biotechnology Co, South Korea) following the manufacturer's instruction. Amplification of the internal transcribed spacer region of nuclear ribosomal DNA (ITS1–5.8S–ITS2), parts of nuclear ribosomal large subunit (LSU), translation elongation factor 1-α (*tef1-α*), RNA polymerase II (*rpb2*) and actin (*act1*) genes was done using the primer pairs ITS5/ITS4[62], LR0R/LR7[63], EF1-728F/EF-2[64,65], RPB2-5F2/fRPB2-7cR[66,67] and ACT512F/ACT783R[65], respectively. The PCR mixtures for all reactions consisted of about 10 ng/µl of genomic DNA, 0.4 µM of each primer and 12.5 µl of 2X ready-to-use reaction mix (Taq DNA polymerase 2X Master Mix Red, 2 mM $MgCl_2$, Ampliqon, Denmark) in a total volume of 25 µl. Thermal conditions for PCR amplification of ITS, LSU, *act1* and *tef1-α* consisted of an initial denaturation step of 5 min at 95 °C, followed by 35 cycle of 30 s at 95 °C, 30 s at 57 °C and 60 s at 72 °C, and a final extension step of 5 min at 72 °C. The part of *rpb2* gene was amplified using touch down PCR consisted of an initial denaturation step of 5 min at 95 °C, followed by 40 cycle of 45 s at 95 °C, 45 s at 60–55 °C (annealing temperature decreased 0.5 °C in the first 10 cycles), 45 s at 72 °C and a final extension step of 10 min at 72 °C. PCR products were visualized on a 1.5% Agarose gel (100 V for 30 min) stained with CyberSafe (Safe DNA Stain, 6X Pishgam, Iran), following the manufacturer's instruction to confirm the amplicon presence and size. Amplification products were purified and sequenced by Macrogen Inc. (Seoul, South Korea).

The newly generated sequences were checked and trimmed manually in BioEdit v. 7.2.6[68] and deposited in GenBank (Table 1). Sequences based on the combined dataset (ITS–rDNA, LSU, *act1*, *rpb2* and *tef1-α*) were aligned using the MAFFT v. 7 online service (https://mafft.cbrc.jp/alignment/server/)[69] for each locus separately by including the sequences of ex-type and representative *Cytospora* strains available in literature and adjusted where necessary. The concatenated sequence dataset (ITS–rDNA, LSU, *act1*, *rpb2* and *tef1-α*) were produced in Mesquite v. 2.74[70] and used for phylogenetic analysis. Maximum likelihood analysis (ML) was conducted in RAxML-HPC BlackBox v. 8.2.12[71] provided by the CIPRES Science Gateway v 3.3[72]. Substitution model was set as GTRGAMMA+I and branch stability was estimated by 1000 bootstrap replications to produce a cladogram with nodal support values. Sequences of *Diaporthe eres* CBS 145040 and *Diaporthe vaccinii* CBS 160.32 were used as outgroups. Resultant phylogenetic tree was visualized in FigTree v. 1.4.4[73] and edited in graphic design software, Adobe Illustrator CC 2018 (Adobe Inc., San Jose, California). The ultimate concatenated alignment and ML generated tree file were submitted to TreeBASE (https://www.treebase.org) under the accession number 29113.

Morphological characterization

Purified cultures were grown on PDA medium in 90 mm diameter Petri dishes, incubated in the dark at 25 ± 1 °C and examined after three, seven and 30 days. Radial growth was measured by taking two measurements perpendicular to each other in triplicates[21,55,58,74]. Colony color was determined based on Rayner's color charts[75]. Pycnidia formation was induced on pine needles embedded in 2% water agar (WA) medium or on one-year-old apple shoots embedded in PDA medium and incubated under near ultraviolet (NUV) light (12 h photoperiod) at room temperature. Both pine needles and apple shoots were autoclave sterilized at 121 °C for 20 min. thrice, 24 h apart. Pycnidia formation was checked weekly for 30 days. Hand sections of the conidiomata (both transverse and longitudinal) were prepared and mounted in water or lactic acid and examined for morphological details. Macro-morphological characters including size and arrangement of stromata, presence or absence of conceptacle, number and diameter of ostioles per ectostromatic disk, arrangement of locules and color, shape and size of discs were examined using an Olympus SZX-ILLB200 dissecting microscope. Micro-morphological characters including shape and size of conidia (n=50) and conidiophores/conidiogenous cells (n=25) were determined at 1000X magnification under an Olympus AX70 compound microscope with

Species	Strain1	Host	Origin	GenBank accession numbers				
				ITS	*LSU*	*act1*	*rpb2*	*tef1-α*
Cytospora ailanthicola	CFCC 89970T	*Ailanthus altissima*	Ningxia, China	MH933618	MH933653	MH933526	MH933592	MH933494
C. abyssinica	CMW 10181T	*Eucalyptus globulus*	Ethiopia	AY347353	-	-	-	-
C. abyssinica	CMW 10178	*Eucalyptus globulus*	Ethiopia	AY347354	-	-	-	-
C. acaciae	CBS 468.69	*Ceratonia siliqua*	Spain	DQ243804	-	-	-	-
C. albodisca	CFCC 53161T	*Platycladus orientalis*	Beijing, China	MW418406	MW418418	MW422899	MW422909	MW422921
C. albodisca	CFCC 54373	*Platycladus orientalis*	Beijing, China	MW418407	MW418419	MW422900	MW422910	MW422922
C. ampulliformis	MFLUCC 16-0583T	*Sorbus intermedia*	Russia	KY417726	KY417760	KY417692	KY417794	-
C. ampulliformis	MFLUCC 16-0629	*Acer platanoides*	Russia	KY417727	KY417761	KY417693	KY417795	-
C. amygdali	CBS 144233T	*Prunus dulcis*	California, USA	MG971853	-	MG972002	-	MG971659
C. atrocirrhata	CFCC 89615	*Juglans regia*	Qinghai, China	KR045618	KR045700	KF498673	KU710946	KP310858
C. atrocirrhata	CFCC 89616	*Juglans regia*	Qinghai, China	KR045619	KR045701	KF498674	KU710947	KP310859
C. austromontana	CMW 6735T	*Eucalyptus pauciflora*	Australia	AY347361	-	-	-	-
C. balanejica	**IRAN 4419C**T	***Malus* × *domestica***	**Urmia, Iran**	**MZ948960**	**MZ948957**	**MZ997842**	**MZ997845**	**MZ997848**
C. balanejica	**IRAN 4420C**	***Malus* × *domestica***	**Urmia, Iran**	**MZ948961**	**MZ948958**	**MZ997843**	**MZ997846**	**MZ997849**
C. balanejica	**FCCUU 350**	***Malus* × *domestica***	**Urmia, Iran**	**MZ948962**	**MZ948959**	**MZ997844**	**MZ997847**	**MZ997850**
C. beilinensis	CFCC 50493T	*Pinus armandii*	Beijing, China	MH933619	MH933654	MH933527	-	MH933495
C. beilinensis	CFCC 50494	*Pinus armandii*	Beijing, China	MH933620	MH933655	MH933528	-	MH933496
C. berberidis	CFCC 89927T	*Berberis dasystachya*	Qinghai, China	KR045620	KR045702	KU710990	KU710948	KU710913
C. berberidis	CFCC 89933	*Berberis dasystachya*	Qinghai, China	KR045621	KR045703	KU710991	KU710949	KU710914
C. berkeleyi	StanfordT3T	*Eucalyptus globulus*	USA	AY347350	-	-	-	-
C. berkeleyi	UCBTwig3	*Eucalyptus globulus*	USA	AY347349	-	-	-	-
C. brevispora	CBS 116811T	*Eucalyptus grandis* × *tereticornis*	Congo	AF192315	-	-	-	-
C. bungeana	CFCC 50495T	*Pinus bungeana*	Shanxi, China	MH933621	MH933656	MH933529	MH933593	MH933497
C. bungeana	CFCC 50496	*Pinus bungeana*	Shanxi, China	MH933622	MH933657	MH933530	MH933594	MH933498
C. californica	CBS 144234T	*Juglans regia*	California, USA	MG971935	-	MG972083	-	MG971645
C. carbonacea	CFCC 89947	*Ulmus pumila*	Qinghai, China	KR045622	KP310812	KP310842	KU710950	KP310855
C. carpobroti	CMW 48981T	*Carpobrotus edulis*	South Africa	MH382812	MH411216	-	-	-
C. cedri	CBS 196.50	-	Italy	AF192311	-	-	-	-
C. celtidicola	CFCC 50497T	*Celtis sinensis*	Anhui, China	MH933623	MH933658	MH933531	MH933595	MH933499
C. celtidicola	CFCC 50498	*Celtis sinensis*	Anhui, China	MH933624	MH933659	MH933532	MH933596	MH933500
C. centrivillosa	MFLUCC 16-	*Sorbus domestica*	Italy	MF190122	MF190068	-	MF377600	-

	1206T							
C. centrivillosa	MFLUCC 17-1660	*Sorbus domestica*	Italy	MF190123	MF190069	-	MF377601	-
C. ceratosperma	CBS 192.42	*Taxus baccata*	Switzerland	AY347333	-	-	-	-
C. ceratospermopsis	CFCC 89626T	*Juglans regia*	Shaanxi, China	KR045647	KR045726	KU711011	KU710978	KU710934
C. ceratospermopsis	CFCC 89627	*Juglans regia*	Shaanxi, China	KR045648	KR045727	KU711012	KU710979	KU710935
C. chrysosperma	CFCC 89981	Populus alba subsp. pyramidalis	Gansu, China	MH933625	MH933660	MH933533	MH933597	MH933501
C. chrysosperma	CFCC 89982	*Ulmus pumila*	Tibet, China	KP281261	KP310805	KP310835	-	KP310848
C. cinerostroma	CMW 5700T	*Eucalyptus globulus*	Chile	AY347377	-	-	-	-
C. cinnamomea	CFCC 53178T	*Prunus armeniaca*	Xinjiang, China	MK673054	MK673084	MK673024	-	-
C. coryli	CFCC 53162T	*Corylus mandshurica*	Beijing, China	MN854450	MN854661	-	MN850751	MN850758
C. corylina	CFCC 54684 T	*Corylus heterophylla*	Beijing, China	MW839861	-	MW815951	MW815937	MW815886
C. corylina	CFCC 54685	*Corylus heterophylla*	Beijing, China	MW839862	-	MW815952	MW815938	MW815887
C. cotoneastricola	CF 20197030	Cotoneaster sp.	Tibet, China	MK673074	MK673104	MK673044	MK673014	MK672960
C. cotoneastricola	CF 20197031T	Cotoneaster sp.	Tibet, China	MK673075	MK673105	MK673045	MK673015	MK672961
C. cotini	MFLUCC 14-1050T	*Cotinus coggygria*	Russia	KX430142	KX430143	-	KX430144	-
C. davidiana	CXY 1350T	*Populus davidiana*	Inner Mongolia, China	KM034870	-	-	-	-
C. diatrypelloidea	CMW 8549T	*Eucalyptus globulus*	Australia	AY347368	-	-	-	-
C. diopuiensis	MFLUCC 18-1419T	Undefined wood	Chiang Mai, Thailand	MK912137	MK571765	MN685819	-	-
C. disciformis	CMW 6509T	*Eucalyptus grandis*	Uruguay	AY347374	-	-	-	-
C. discostoma	CFCC 53137T	*Platycladus orientalis*	Beijing, China	MW418404	MW418416	MW422897	MW422907	MW422919
C. discostoma	CFCC 54368	*Platycladus orientalis*	Beijing, China	MW418405	MW418417	MW422898	MW422908	MW422920
C. donglingensis	CFCC 53159T	*Platycladus orientalis*	Beijing, China	MW418412	MW418424	MW422903	MW422915	MW422927
C. donglingensis	CFCC 54371	*Platycladus orientalis*	Beijing, China	MW418413	MW418425	MW422904	MW422916	MW422928
C. elaeagni	CFCC 89632	*Elaeagnus angustifolia*	Ningxia, China	KR045626	KR045706	KU710995	KU710955	KU710918
C. elaeagni	CFCC 89633	*Elaeagnus angustifolia*	Ningxia, China	KF765677	KF765693	KU710996	KU710956	KU710919
C. elaeagnicola	CFCC 52882T	*Elaeagnus angustifolia*	China	MK732341	MK732338	MK732344	MK732347	-
C. elaeagnicola	CFCC 52883	*Elaeagnus angustifolia*	China	MK732342	MK732339	MK732345	MK732348	-
C. eriobotryae	IMI 136523T	*Eriobotrya japonica*	India	AY347327	-	-	-	-
C. erumpens	CFCC 50022	*Prunus padus*	Shanxi, China	MH933627	MH933661	MH933534	-	MH933502
C. erumpens	MFLUCC 16-0580T	Salix × fragilis	Russia	KY417733	KY417767	KY417699	KY417801	-
C. eucalypti	CBS 144241	*Eucalyptus globulus*	California, USA	MG971907	-	MG972056	-	MG971617
C. eucalypticola	ATCC 96150T	*Eucalyptus nitens*	Australia	AY347358	-	-	-	-

C. eucalyptina	CMW 5882	*Eucalyptus grandis*	Columbia	AY347375	-	-	-	-
C. euonymicola	CFCC 50499T	*Euonymus kiautschovicus*	Shaanxi, China	MH933628	MH933662	MH933535	MH933598	MH933503
C. euonymicola	CFCC 50500	*Euonymus kiautschovicus*	Shaanxi, China	MH933629	MH933663	MH933536	MH933599	MH933504
C. euonymina	CFCC 89993T	*Euonymus kiautschovicus*	Shanxi, China	MH933630	MH933664	MH933537	MH933600	MH933505
C. fraxiicola	MFLU 17-2392	dead branches	Russia	-	MN764356	MN995562	-	-
C. fraxinigena	MFLUCC 14-0868T	*Fraxinus ornus*	Italy	MF190133	MF190078	-	-	-
C. friesii	CBS 194.42	*Abies alba*	Switzerland	AY347328	-	-	-	-
C. fugax	CBS 203.42	Salix sp.	Switzerland	AY347323	-	-	-	-
C. galegicola	MFLUCC 18-1199T	*Galega officinalis*	Forlì-Cesena, Italy	MK912128	MK571756	MN685810	MN685820	-
C. gelida	MFLUCC 16-0634 T	*Cotinus coggygria*	Russia	KY563245	KY563247	KY563241	KY563243	-
C. germanica	CXY 1322	*Elaeagnus oxycarpa*	China	JQ086563	JX524617	-	-	-
C. gigalocus	CFCC 89620T	*Juglans regia*	Qinghai, China	KR045628	KR045708	KU710997	KU710957	KU710920
C. gigalocus	CFCC 89621	*Juglans regia*	Qinghai, China	KR045629	KR045709	KU710998	KU710958	KU710921
C. gigaspora	CFCC 50014	*Juniperus procumbens*	Shanxi, China	KR045630	KR045710	KU710999.	KU710959	KU710922
C. gigaspora	CFCC 89634T	*Salix psammophila*	Shaanxi, China	KF765671	KF765687	KU711000	KU710960	KU710923
3. *globosa*	MFLU 16-2054^{T}	*Abies alba*	Italy	MT177935	MT177962	-	MT432212	-
C. granati	CBS 144237T	*Punica granatum*	California, USA	MG971799	-	MG971949	-	MG971514
C. hippophaës	CFCC 89639	*Hippophaë rhamnoides*	Gansu, China	KR045632	KR045712	KU711001	KU710961	KU710924
C. hippophaës	CFCC 89640	*Hippophaë rhamnoides*	Gansu, China	KF765682	KF765698	KF765730	KU710962	KP310865
C. japonica	CBS 375.29	*Prunus persica*	Japan	AF191185	-	-	-	-
C. joaquinensis	CBS 144235T	*Populus deltoides*	California, USA	MG971895	-	MG972044	-	MG971605
C. junipericola	BBH 42444	*Juniperus communis*	Italy	MF190126	MF190071	-	-	-
C. junipericola	MFLU 17-0882T	*Juniperus communis*	Italy	MF190125	MF190072	-	-	-
C. juniperina	CFCC 50501T	*Juniperus przewalskii*	Sichuan, China	MH933632	MH933666	MH933539	MH933602	MH933507
C. juniperina	CFCC 50502	*Juniperus przewalskii*	Sichuan, China	MH933633	MH933667	MH933540	MH933603	MH933508
C. kantschavelii	CXY 1386	*Populus maximowiczii*	Chongqing, China	KM034867	-	-	-	-
C. kuanchengensis	CFCC 52464T	*Castanea mollissima*	China	MK432616	MK429886	MK442940	MK578076	-
C. kuanchengensis	CFCC 52465	*Castanea mollissima*	China	MK432617	MK429887	MK442941	MK578077	-
C. kunzei	CBS 118556	*Pinus radiata*	South Africa	DQ243791	-	-	-	-
C. longiostiolata	MFLUCC 16-0628T	Salix × fragilis	Russia	KY417734	KY417768	KY417700	KY417802	-
C. longispora	CBS 144236T	*Prunus domestica*	California, USA	MG971905	-	MG972054	-	MG971615

C. leucosperma	CFCC 89622	*Pyrus bretschneideri*	Gansu, China	KR045616	KR045698	KU710988	KU710944	KU710911
C. leucosperma	CFCC 89894	*Pyrus bretschneideri*	Qinghai, China	KR045617	KR045699	KU710989	KU710945	KU710912
C. leucostoma	CFCC 53140	*Prunus sibirica*	Beijing, China	MN854445	MN854656	MN850760	MN850746	MN850753
C. leucostoma	CFCC 53141	*Prunus sibirica*	Beijing, China	MN854446	MN854657	MN850761	MN850747	MN850754
C. lumnitzericola	MFLUCC 17-0508T	*diozera racernosa*	Tailand	MG975778	MH253461	MH253457	MH253453	-
C. mali	CFCC 50030	*Malus pumila*	Shaanxi, China	MH933643	MH933677	MH933550	MH933608	MH933524
C. mali	CFCC 50031	*Crataegus* sp.	Shanxi, China	KR045636	KR045716	KU711004	KU710965	KU710927
C. mali-spectabilis	CFCC 53181T	*Malus spectabilis* 'Royalty'	Xinjiang, China	MK673066	MK673096	MK673036	MK673006	MK672953
C mali-sylvestris	MFLUCC 16-0638	*Malus sylvestris*	Russia	KY885017	KY885018	KY885019	KY885020	-
C. melastoma	A 846	*Malus domestica*	USA	AF191184	-	-	-	-
C. melnikii	MFLUCC 15-0851T	*Malus domestica*	Russia	KY417735	KY417769	KY417701	KY417803	-
C. melnikii	MFLUCC 16-0635	Populus nigra var. italica	Russia	KY417736	KY417770	KY417702	KY417804	-
C. mougeotii	ATCC 44994	*Picea abies*	Norway	AY347329	-	-	-	-
C. multicollis	CBS 105.89T	Quercus ilex subsp. rotundifolia	Spain	DQ243803	-	-	-	-
C. nitschkii	CMW 10180T	*Eucalyptus globulus*	Ethiopia	AY347356	-	-	-	-
C. nivea	MFLUCC 15-0860	*Salix acutifolia*	Russia	KY417737	KY417771	KY417703	KY417805	-
C. nivea	CFCC 89641	*Elaeagnus angustifolia*	Ningxia, China	KF765683	KF765699	KU711006	KU710967	KU710929
C. notastroma	NE_TFR5	*Populus tremuloides*	USA	JX438632	-	-	-	-
C. notastroma	NE_TFR8	*Populus tremuloides*	USA	JX438633	-	-	-	-
C. ochracea	CFCC 53164T	Cotoneaster sp.	Xinjiang, China	MK673060	MK673090	MK673030	MK673001	MK672949
C. oleicola	CBS 144248T	*Olea europaea*	California, USA	MG971944	-	MG972098	-	MG971660
C. olivacea	CFCC 53176T	*Sorbus tianschanica*	Xinjiang, China	MK673068	MK673098	MK673038	MK673008	MK672955
C. olivacea	CFCC 53177	*Prunus virginiana*	Xinjiang, China	MK673071	MK673101	MK673041	MK673011	-
C. palm	CXY 1276	*Cotinus coggygria*	Beijing, China	JN402990	-	-	-	-
C. palm	CXY 1280T	*Cotinus coggygria*	Beijing, China	JN411939	-	-	-	-
C. parakantschavelii	MFLUCC 15-0857T	Populus × sibirica	Russia	KY417738	KY417772	KY417704	KY417806	-
C. parapersoonii	T28.1T	*Prunus persica*	USA	AF191181	-	-	-	-
C. parapistaciae	CBS 144506T	*Pistacia vera*	California, USA	MG971804	-	MG971954	-	MG971519
C. parasitica	MFLUCC 15-0507T	*Malus domestica*	Russia	KY417740	KY417774	KY417706	KY417808	-
C. parasitica	XJAU 2542-1	Malus sp.	Xinjiang, China	MH798884	MH798897	-	-	MH813452
C. paratranslucens	MFLUCC 15-0506T	Populus alba var. bolleana	Russia	KY417741	KY417775	KY417707	KY417809	-

C. paratranslucens	MFLUCC 16-0627	*Populus alba*	Russia	KY417742	KY417776	KY417708	KY417810	-
C. pavettae	CBS 145562^{T}	*Pavetta revoluta*	South Africa	MK876386	MK876427	MK876457	MK876483	MK876497
C. piceae	CFCC 52841T	*Picea crassifolia*	Xinjiang, China	MH820398	MH820391	MH820406	MH820395	MH820402
C. piceae	CFCC 52842	*Picea crassifolia*	Xinjiang, China	MH820399	MH820392	MH820407	MH820396	MH820403
C. pingbianensis	MFLUCC 18-1204T	Undefined wood	Yunnan, China	MK912135	MK571763	MN685817	-	-
C. pini	CBS 197.42	*Pinus sylvestris*	Switzerland	AY347332	-	-	-	-
C. pini	CBS 224.52T	*Pinus strobus*	USA	AY347316	-	-	-	-
C. pistaciae	CBS 144238T	*Pistacia vera*	California, USA	MG971802	-	MG971952	-	MG971517
C. platanicola	MFLU 17-0327	*Platanus hybrida*	Italy	MH253451	MH253452	MH253449	MH253450	-
C. platycladi	CFCC 50504T	*Platycladus orientalis*	Yunnan, China	MH933645	MH933679	MH933552	MH933610	MH933516
C. platycladi	CFCC 50505	*Platycladus orientalis*	Yunnan, China	MH933646	MH933680	MH933553	MH933611	MH933517
C. platycladicola	CFCC 50038T	*Platycladus orientalis*	Gansu, China	KT222840	MH933682	MH933555	MH933613	MH933519
C. platycladicola	CFCC 50039	*Platycladus orientalis*	Gansu, China	KR045642	KR045721	KU711008	KU710973	KU710931
C. plurivora	CBS 144239T	*Olea europaea*	California, USA	MG971861	-	MG972010	-	MG971572
C. phialidica	MFLU 16-2442^{T}	*Alnus glutinosa*	Italy	MT177932	MT177959	-	MT432209	-
C. populicola	CBS 144240T	*Populus deltoides*	California, USA	MG971891	-	MG972040	-	MG971601
C. populina	CFCC 89644T	*Salix psammophila*	Shaanxi, China	KF765686	KF765702	KU711007	KU710969	KU710930
C. populinopsis	CFCC 50032T	*Sorbus aucuparia*	Ningxia, China	MH933648	MH933683	MH933556	MH933614	MH933520
C. predappioensis	MFLUCC 17-2458T	*Platanus hybrida*	Italy	MG873484	MG873480	-	-	-
C. prunicola	MFLU 17-0995T	Prunus sp.	Italy	MG742350	MG742351	MG742353	MG742352	-
C. pruni-mume	CFCC 53179	*Prunus armeniaca*	Xinjiang, China	MK673057	MK673087	MK673027	-	MK672947
C. pruni-mume	CFCC 53180T	*Prunus mume*	Xinjiang, China	MK673067	MK673097	MK673037	MK673007	MK672954
C. pruinopsis	CFCC 50034T	*Ulmus pumila*	Shaanxi, China	KP281259	KP310806	KP310836	KU710970	KP310849
C. pruinopsis	CFCC 50035	*Ulmus pumila*	Jilin, China	KP281260	KP310807	KP310837	KU710971	KP310850
C. pruinosa	CBS 201.42T	Syringa sp.	Switzerland	DQ243801	-	-	-	-
C. pruinosa	CFCC 50036	*Syringa oblata*	Qinghai, China	KP310800	KP310802	KP310832	-	KP310845
C. pubescentis	MFLUCC 18-1201T	*Quercus pubescens*	Forlì-Cesena, Italy	MK912130	MK571758	MN685812	-	-
C. punicae	CBS 144244	*Punica granatum*	California, USA	MG971943	-	MG972091	-	MG971654
C. quercicola	MFLU 17–0881	Quercus sp.	Italy	MF190128	MF190074	-	-	-
C. quercicola	MFLUCC 14-0867T	Quercus sp.	Italy	MF190129	MF190073	-	-	-

C. rhizophorae	MUCC302	*Eucalyptus grandis*	Australia	EU301057	-	-	-	-
C. ribis	CFCC 50026	*Ulmus pumila*	Qinghai, China	KP281267	KP310813	KP310843	KU710972	KP310856
C. ribis	CFCC 50027	*Ulmus pumila*	Qinghai, China	KP281268	KP310814	KP310844	-	KP310857
C. rosae	MFLU 17-0885	*Rosa canina*	Italy	MF190131	MF190076	-	-	-
C. rosicola	CF 20197024T	Rosa sp.	Tibet, China	MK673079	MK673109	MK673049	MK673019	MK672965
C. rosigena	MFLUCC 18-0921^{T}	Rosa sp.	Russia	MN879872	MN879873	-	-	-
C. rostrata	CFCC 89909T	*Salix cupularis*	Gansu, China	KR045643	KR045722	KU711009	KU710974	KU710932
C. rostrata	CFCC 89910	*Salix cupularis*	Gansu, China	KR045644	KR045723	KU711010	KU710975	KU710933
C. rusanovii	MFLUCC 15-0854T	*Salix babylonica*	Russia	KY417744	KY417778	KY417710	KY417812	-
C. salicacearum	MFLUCC 15-0861	Salix × fragilis	Russia	KY417745	KY417779	KY417711	KY417813	-
C. salicacearum	MFLUCC 15-0509T	*Salix alba*	Russia	KY417746	KY417780	KY417712	KY417814	-
C. salicicola	MFLUCC 14-1052T	*Salix alba*	Russia	KU982636	KU982635	KU982637	-	-
C. salicina	MFLUCC 15-0862T	*Salix alba*	Russia	KY417750	KY417784	KY417716	KY417818	-
C. salicina	MFLUCC 16-0637	Salix × fragilis	Russia	KY417751	KY417785	KY417717	KY417819	-
C. schulzeri	CFCC 50040	*Malus domestica*	Ningxia, China	KR045649	KR045728	KU711013	KU710980	KU710936
C. schulzeri	CFCC 50042	*Malus pumila*	Gansu, China	KR045650	KR045729	KU711014	KU710981	KU710937
C. sibiraeae	CFCC 50045T	*Sibiraea angustata*	Gansu, China	KR045651	KR045730	KU711015	KU710982	KU710938
C. sibiraeae	CFCC 50046	*Sibiraea angustata*	Gansu, China	KR045652	KR045731	KU711016	KU710983	KU710939
C. sophorae	CFCC 50048	*Magnolia grandiflora*	Shanxi, China	MH820401	MH820394	MH820409	MH820397	MH820405
C. sophorae	CFCC 89598	*Styphnolobium japonicum*	Gansu, China	KR045654	KR045733	KU711018	KU710985	KU710941
C. sophoricola	CFCC 89596	*Styphnolobium japonicum* var. *pendula*	Gansu, China	KR045656	KR045735	KU711020	KU710987	KU710943
C. sophoricola	CFCC 89595T	*Styphnolobium japonicum* var. *pendula*	Gansu, China	KR045655	KR045734	KU711019	KU710986	KU710942
C. sophoriopsis	CFCC 89600T	*Styphnolobium japonicum*	Gansu, China	KR045623	KP310804	KU710992	KU710951	KU710915
C. sorbi	MFLUCC 16-0631T	*Sorbus aucuparia*	Russia	KY417752	KY417786	KY417718	KY417820	-
C. sorbicola	MFLUCC 16-0584T	*Acer pseudoplatanus*	Russia	KY417755	KY417789	KY417721	KY417823	-
C. sorbicola	MFLUCC 16-0633	*Cotoneaster melanocarpus*	Russia	KY417758	KY417792	KY417724	KY417826	-
C. sorbina	CF 20197660T	*Sorbus tianschanica*	Xinjiang, China	MK673052	MK673082	MK673022	-	MK672943
C. spiraeae	CFCC 50049T	*Spiraea salicifolia*	Gansu, China	MG707859	MG707643	MG708196	MG708199	-
C. spiraeae	CFCC 50050	*Spiraea salicifolia*	Gansu, China	MG707860	MG707644	MG708197	MG708200	-
C. spiraeicola	CFCC	*Spiraea salicifolia*	Beijing,	MN854448	MN854659	-	MN850749	MN850756

	53138T		China					
C. spiraeicola	CFCC 53139	*Tilia nobilis*	Beijing, China	MN854449	MN854660	-	MN850750	MN850757
C. tamaricicola	CFCC 50508T	*Tamarix chinensis*	Yunnan, China	MH933652	MH933687	MH933560	MH933617	MH933523
C. tanaitica	MFLUCC 14-1057T	*Betula pubescens*	Russia	KT459411	KT459412	KT459413	-	-
C. thailandica	MFLUCC 17-0262T	*Xylocarpus moluccensis*	Thailand	MG975776	MH253463	MH253459	MH253455	-
C. thailandica	MFLUCC 17-0263T	*Xylocarpus moluccensis*	Thailand	MG975777	MH253464	MH253460	MH253456	-
C. tibetensis	CF 20197026	Cotoneaster sp.	Tibet, China	MK673076	MK673106	MK673046	MK673016	MK672962
C. tibetensis	CF 20197032T	Cotoneaster sp.	Tibet, China	MK673078	MK673108	MK673048	MK673018	MK672964
C. translucens	CXY 1351	*Populus davidiana*	Inner Mongolia, China	KM034874	-	-	-	-
C. ulmi	MFLUCC 15-0863T	*Ulmus minor*	Russia	KY417759	-	-	-	-
3. *ulmicola*	MFLUCC 18-1227T	*Ulmus pumila*	Russia	MH940220	MH940218	MH940216	-	-
C. valsoidea	CMW 4309T	*Eucalyptus grandis*	Indonesia	AF192312	-	-	-	-
C. verrucosa	CFCC 53157T	*Platycladus orientalis*	Beijing, China	MW418408	MW418420	-	MW422911	MW422923
C. verrucosa	CFCC 53158	*Platycladus orientalis*	Beijing, China	MW418410	MW418422	MW422901	MW422913	MW422925
C. vinacea	CBS 141585T	*Vitis interspecific* hybrid 'Vidal'	USA	KX256256	-	-	-	-
C. viridistroma	CBS 202.36^{T}	*Cercis canadensis* Castigl.	USA	MN172408	MN172388	-	-	MN271853
C. viticola	Cyt2	*Vitis interspecific* hybrid 'Frontenac'	USA	KX256238	-	-	-	-
C. viticola	CBS 141586T	*Vitis vinifera* 'Cabernet Franc'	USA	KX256239	-	-	-	-
C. xinjiangensis	CFCC 53182	Rosa sp.	Xinjiang, China	MK673064	MK673094	MK673034	MK673004	MK672951
C. xinjiangensis	CFCC 53183T	Rosa sp.	Xinjiang, China	MK673065	MK673095	MK673035	MK673005	MK672952
C xinglongensis	CFCC 52458	*Castanea mollissima*	China	MK432622	MK429892	MK442946	MK578082	-
C xinglongensis	CFCC 52459	*Castanea mollissima*	China	MK432623	MK429893	MK442947	MK578083	-
C. xylocarpi	MFLUCC 17-0251T	*Xylocarpus granatum*	Thailand	MG975775	MH253462	MH253458	-	-
Diaporthe eres	CBS 145040	*Lactuca satia*	Netherlands	MK442579	MK442521	MK442634	MK442663	-
Diaporthe vaccinii	CBS 160.32	*Vaccinium macrocarpon*	USA	KC343228	-	JQ807297	-	-

differential interference contrast (DIC) illumination. Adobe Photoshop 2020 v. 2.10.8 software (Adobe Inc., San Jose, California) was used for manual editing.

Pathogenicity trials

Pathogenicity trials were done based on the standard and routine method described by different researchers[31,40,42,55,76-79]. Detached, dormant, one– or two–years–old, 25 × 1.5–2 cm apple shoots of the cv. Red Delicious' were collected from healthy trees in the apple cultivar collection farm of Urmia University. The shoots were washed under running tap water, surface disinfested with 75% ethanol for 4 min., washed again in sterile distilled water and blotted dry on sterile paper towel. The bark of the shoots was removed in the center with a 5-mm-diameter flame sterilized cork borer and inoculated with a 5 mm diam. mycelial plug of actively growing fungal isolates (7–days–old on PDA). Each inoculated site was covered with a sterile moistened cotton ball and wrapped

with Parafilm™ (Bemis™, pm996, USA) to maintain the moisture. Sterile PDA plugs were used as the controls. Six shoots were used for each fungal isolate and control treatments. Inoculated shoots were placed in clean plastic containers containing three layered moistened sterile paper towels and incubated under laboratory conditions (diurnal light, 25 ± 2 °C, 80% relative humidity) for 21 days. All the experiments were repeated once under similar conditions. Length of bark and wood discoloration around the inoculated sites were measured 21 days post-inoculation.

Also, five fungal isolates (BA 1–1, BA 2–1, BA 2–4, KU 1–1 and BA 3–1 isolates) which had the higher virulence in the above trials (data not shown) were chosen for the evaluation of reaction of 12 apple cultivars including Braeburn', Delbard Estivale', Fuji', Granny Smith', Golden Delicious', Golden Primrose', Idared', Red Delicious', M4', M7', MM106' and MM109' against these isolates. Healthy shoots were collected from the apple cultivar collection farm of Urmia University and used for pathogenicity tests as described above and the length of bark and wood discoloration around the inoculated sites was measured 21 days post-inoculation. Experiments were laid down following a completely randomized design (CRD). Data were subjected to analysis of variance (ANOVA) using SAS v.9.1 software (SAS Institute, Inc., USA), transformed by square-root (because of the existence of zero values in data) before analyses and lesion length means were compared with Duncan's multiple range test ($P \leq 0.05$). In order to confirm Koch's postulates, fungal re-isolation was carried out from the margins of the developed lesions in all symptomatic samples and isolates were re-identified morphologically as described previously.

Declarations

Data availability

All sequence data generated in this study are available in NCBI GenBank (https:// www. ncbi. nlm. nih. gov/ genbank/) following the accession numbers MZ948960–MZ948962 (ITS); MZ948957–MZ948959 (LSU); MZ997842–MZ997844 (*act1*); MZ997845–MZ997847 (*rpb2*) and MZ997848–MZ997850 (*tef1α*). Also, the ultimate concatenated alignment and ML generated tree file were submitted to TreeBASE (https://www.treebase.org) under the accession number 29113. All data analyzed during this study are included in this manuscript.

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Author contributions

Y.G. and A.A. designed and supervised the project. R.A. and Y.G. performed sampling, fungal isolation, experiments and photography. R.A. and A.A. carried out statistical and phylogenetic analyses. All authors contributed to the preparation of the manuscript and reviewed the manuscript.

Competing interest

The authors declare no competing interests.

Additional information

Supplementary Information The following supplementary information can be downloaded at:

Correspondence and requests for materials should be addressed to Y.G.

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Figures

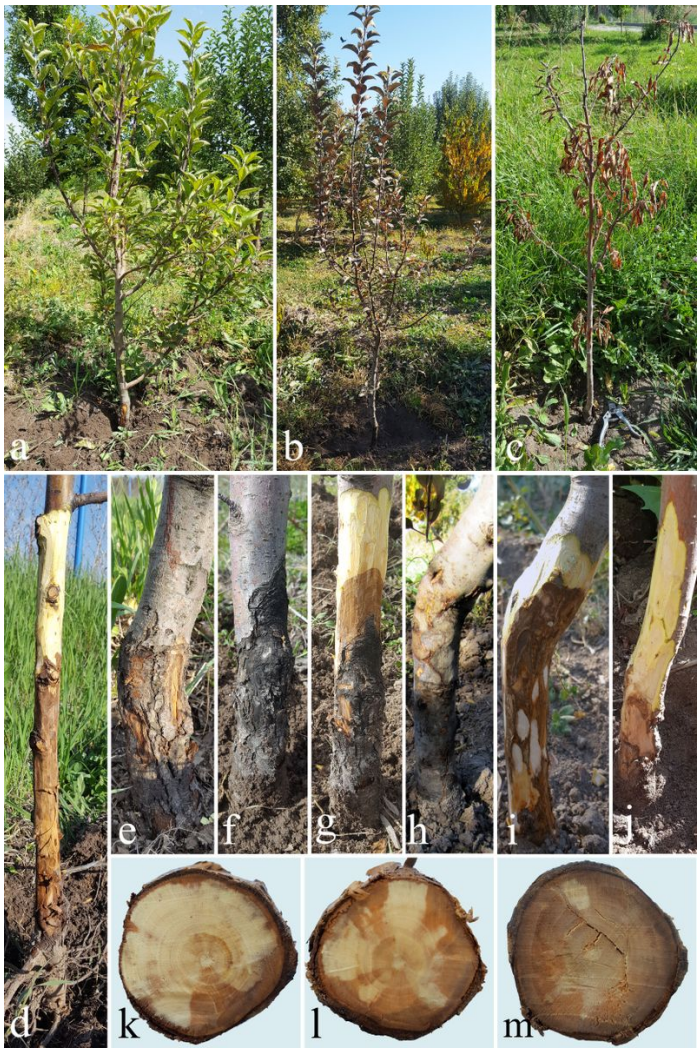

Figure 1

Typical symptoms of crown and collar canker and necrosis on naturally infected young apple trees cvs. ‘Red Delicious’ and ‘Golden Delicious’. a: cv. ‘Red Delicious’, 7 July, 2017. b: cv. ‘Red Delicious’, 20 October, 2017. c: cv. ‘Golden Delicious’, 1 September, 2018. d-i. Disease symptoms on the crown, collar and trunk of the cvs. ‘Red Delicious’ and j. ‘Golden Delicious’. k-m: Cross sections showing disease progress in the infected trunks of the cv. ‘Red Delicious’.

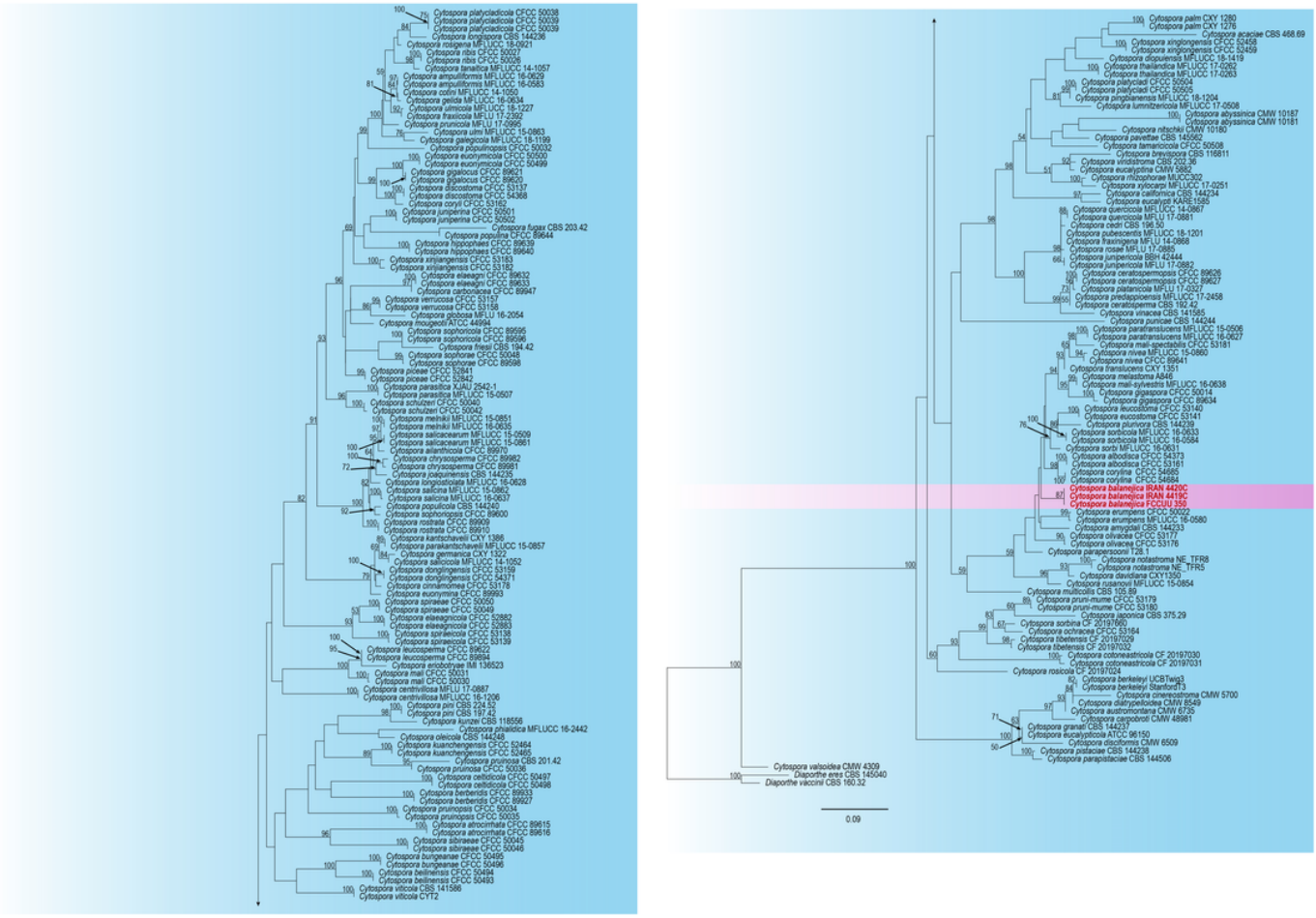

Figure 2

Maximum Likelihood tree based on combined ITS, LSU, *rpb2*, *act1* and *tef 1-a* sequences matrix in different *Cytospora* species. Bootstrap support values of ≥50% are given above the nodes. The tree was rooted to *Diaporthe eres* CBS 145040 and *Diaporthe vaccinii* CBS 160.32. The scale bar indicates the number of nucleotide substitutions.

Figure 3

Morphology of *Cytospora balanejica* (IRAN 4419C). a–b: Habit of conidiomata on twig. c–d: Longitudinal section through conidiomata. e–f: Transverse section of conidiomata. g: Conidiophores and conidiogenous cells. h: Conidia. i: Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: b, c and e = 250 µm; f, d = 100 µm and g, h = 10 µm.

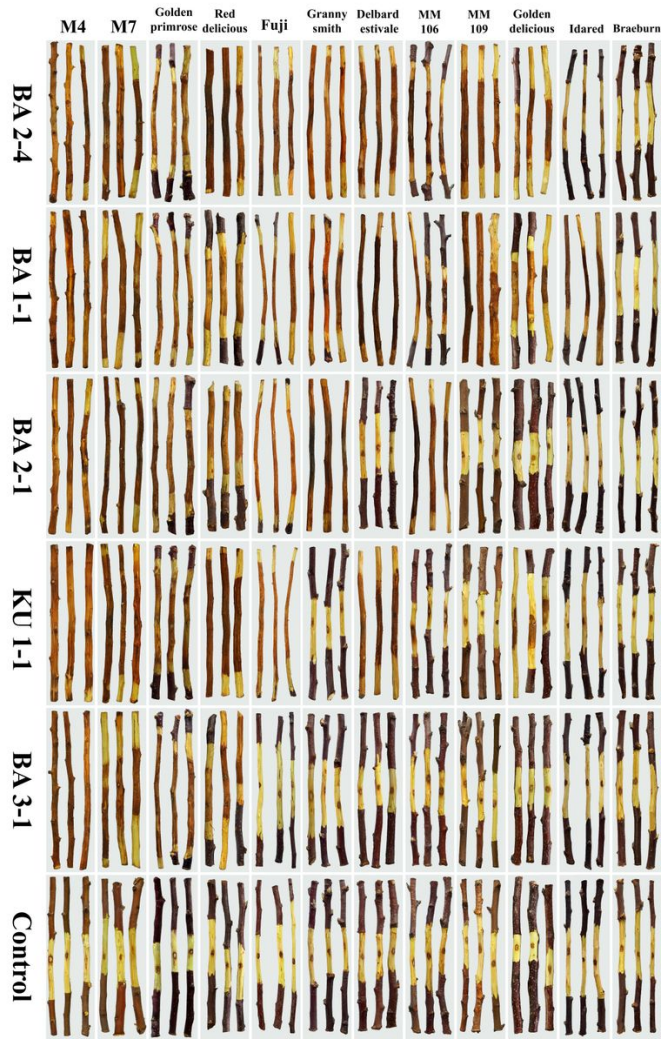

Figure 4

Pathogenicity tests of five selected isolates (BA 1–1, BA 1–2, BA 2–4, BA 3–1 and KU 1–1) of *Cytospra balanejica* against 12 apple cultivars.

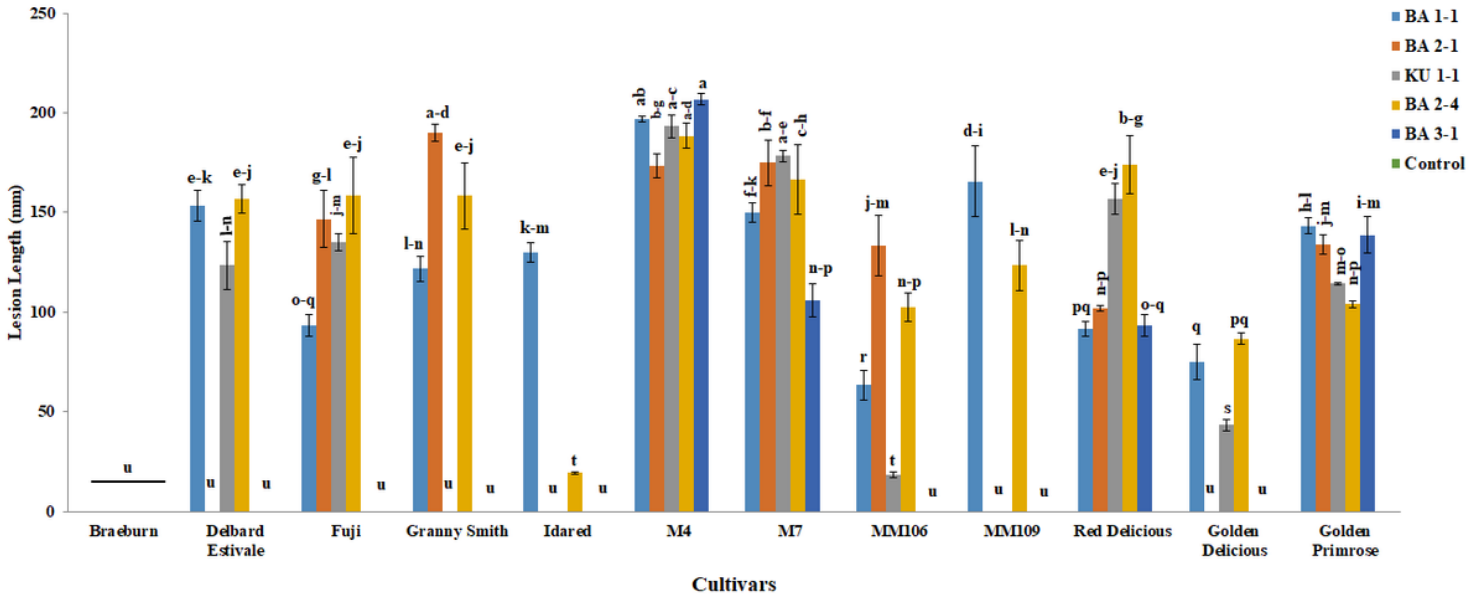

Figure 5

Mean lesion lengths (mm) of 12 apple cultivars inoculated with five selected isolates of *Cytospora balanejica* based on Duncan's multiple range test. Different letters show significant difference at $P \leq 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplement1continued.jpg
- Supplement1.jpg