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Morphological and molecular characterization of *Cytospora* species involved in apple decline in Iran

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Abstract: In a recent survey on apple orchards showing stem canker, dieback and decline symptoms in West Azerbayjan Province, Iran, several fungal isolates with typical characteristics of the genus *Cytospora* were obtained. Combination of morphological and cultural characteristics and phylogenetic analysis of internal transcribed spacer region of the nrDNA (ITS-rDNA) and parts of the large subunit of ribosomal DNA (LSU), actin (ACT) and RNA polymerase II (RPB2) genes were used to the accurate delimitation of fungal species. Four *Cytospora* species *viz*. *C. chrysosperma*, *C. germanica*, *C. paratranslucens* and *C. salicina* were identified. Pathogenicity tests were conducted on detached branches of 'Golden delicious' and 'Red delicious' apple cultivars. Isolates of *C. salicina* caused characteristic lesions on both 'Golden delicious' and 'Red delicious' apple cultivars, while isolates of *C. chrysosperma*, *C. germanica* and *C. paratranslucens* were only pathogenic on 'Red delicious' apple cultivar. Re-isolation and identification of the inoculated fungi confirmed Koch's postulates. This study indicated the presence of different *Cytospora* species causing apple canker, dieback and decline disease in the studied area. *Cytospora germanica* and *C. salicina* are reported for the first time as causal agents of apple canker disease. Moreover, these species are reported as new records for the mycobiota of Iran.

Keywords: Apple canker, *Cytosporaceae*, Dieback, Pathogenicity, Phylogeny

INTRODUCTION

Apple Cytospora canker which is commonly caused by different species of *Cytospora* Ehrenb., is one of the most important and destructive diseases of apples (*Malus* spp.) worldwide (Ashkan & Hedjaroude 1993, Lee et al. 2006, Abe et al. 2007, Suzaki 2008, Wang et al. 2011, Ma et al. 2018, Liu et al. 2020). The pathogen extensively invades host phloem and xylem tissues, causing the formation of cankers on the main stems, twigs and branches, affect the cultivation, productivity and longevity of trees and severely limits the development of apple industry (Tamura & Saito 1982, Fotouhifar et al. 2010, Meng et al. 2019). The genus *Cytospora* (*Cytosporaceae*, *Diaporthales*, *Sordariomycetes*, *Ascomycota*) was introduced by Ehrenberg (1818) with *C. chrysosperma* (Pers.) Fr. as the type species. It is considered as an asexual morph of several genera such as *Leucocytospora*, *Leucostoma*, *Valsa*, *Valsella* and *Valseutypella* (Fries 1823, Saccardo 1884, Spielman 1985, Adams et al. 2002, 2005, Castlebury et al. 2002), all of which were considered as synonyms of *Valsa* (Adams et al. 2005). According to one fungus one name (1F=1N), the generic name *Cytospora* has been recommended for use over *Leucocytospora*, *Leucostoma*, *Valsa*, *Valsella* and *Valseutypella* (Rossman et al. 2015). A search of MycoBank (October 2020, www.mycobank.org) revealed 634 legitimate names, while a search of Index Fungorum (October 2020, www.indexfungorum.org) lists 662 names. At present, 22 *Cytospora* species have been reported from apple trees as causal agents of apple canker and dieback disease worldwide (Anonymous 1960, Ershad 2009, Mehrabi et al. 2011, Wang et al. 2011, 2020, Azizi et al. 2018, Ma et al. 2018, Farr & Rossman 2021). Thus far, eleven species *viz. C. ambiens* Sacc., *C. chrysosperma* (Pers.) Fr., *C. cincta* Sacc., *C. leucostoma* (Pers.) Sacc., *C. paratranslucens* Norph., Bulgakov, T.C. Wen & K.D. Hyde, *C. rhodophila* Sacc., *C. rubescens* Fr., *C. schulzeri* Sacc. & P. Syd., *C. sorbicola* Norph., Bulgakov, T.C. Wen & K.D. Hyde, *Valsa malicola* Z. Urb. and *Leucostoma cinctum* (Fr.) Höhn. have been recorded from Iran (Ershad 2009, Fotouhifar et al.

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2010, Mehrabi et al. 2011, Azizi et al. 2018, Hanifeh et al. 2018).

West Azerbayjan Province is the main apple-growing area in Iran with 60470 ha and total production of 970714 tonnes (Ahmadi et al. 2018). Two apple cultivars, 'Golden Delicious' and 'Red Delicious' are the predominant commercial apple cultivars grown widely in this area. Recently, various types of disease symptoms were observed in apple orchards leading to tree decline and death. The aim of the present study was to identify *Cytospora* species involved in apple canker and dieback disease in West Azerbayjan Province, based on morphological characteristics and phylogenetic analysis using ITS, LSU, RPB2 and ACT sequences data.

MATERIALS AND METHODS

Sample collection and fungal isolation

Apple orchards in different locations of West Azerbayjan Province, Iran, were visited for suspected decline symptoms. Samples were collected from twigs and trunks showing cankered, bark discoloration and scaling and wood discoloration, placed in new paper bags and brought to the laboratory. The samples were washed thoroughly under running tap water, then small pieces (5 × 5 mm) from the interfaces of healthy and diseased tissue were cut, surface disinfested with 70% ethanol for 1 min, rinsed three times in sterile distilled water and blotted dry on sterilized paper towel. The plant segments were placed on potato dextrose agar (PDA, Merck, 39 g/L) amended with streptomycin sulfate and penicillin G (100 ppm each) in 90 mm diam. Petri dishes were incubated at 25 ± 1 °C in the dark. Hyphae growing out from the samples were transferred into fresh PDA and were purified using hyphal tip or single spore methods. All purified isolates were maintained on PDA slants and stored at 4 °C for further examination. In the cases that characteristic asexual conidiomata (pycnidia) were seen on the examined plant samples, the samples were washed gently with sterile distilled water, surface disinfested with 70% ethanol for 1 min, and kept under humid conditions for ooze formation. Then dilute spore suspension was prepared by suspending the oozing conidia in sterile distilled water and a drop of dilute spore suspension (≈25 µl) was spread out over PDA medium. Germinated solitary conidia were transferred into fresh PDA after 24–48 h. The growing colonies were purified again as described above. The isolates were deposited in the Fungal Culture Collection of the Iranian Research Institute of Plant Protection ("IRAN") and Fungal Culture Collections of Urmia University (FCCUU). Sequence data were deposited in GenBank dataset and their accession numbers are provided in the text (Table 1).

Morphological examination

Colony characteristics of the isolates were recorded from cultures grown on PDA in triplicate 90 mm diam. glass Petri dishes incubated in the dark at 25 ± 1°C after 3, 7 and 30 days. Radial growth was measured by taking two measurements perpendicular to each other in triplicates (Fan et al. 2015, Lawrence et al. 2018, Ma et al. 2018, Zhang et al. 2019). Colony colors were determined based on Rayner's color chart (Rayner 1970). Pycnidia formation were induced on pine needles embedded in 2% water agar (WA) medium or on one-year-old apple shoots embedded in PDA medium under near ultraviolet (NUV) light (12 h photoperiod). Both pine needles and apple shoots were autoclaved at 121 °C for 20 min. thrice, 24 h apart. Pycnidia formation was checked weekly for 30 days. Transverse and longitudinal sections of conidiomata were prepared by hand with a razor blade and microscopic slides were prepared in water or lactic acid as mounting solutions. Morphological characteristics including color, diameter, presence/absence of conceptacle; the color, shape and size of discs, number and size of ostioles per disc (n=20), arrangement of pycnidia locules and dimensions of conidia (n=40) and conidiogenous cells (n=20) were examined and measured at 1000 X magnification with an Olympus X61 microscope with differential interference contrast (DIC) illumination. Based on morphological grouping, 12 isolates were selected for molecular studies.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from fungal mycelium scraped with a sterile scalpel from the surface of 14–day–old PDA cultures using Exgene™ Cell SV mini kit (GeneAll Biotechnology Co., Korea) following the manufacturer's instructions. The extracted DNA was checked for quality on a 1% agarose gel. The internal transcribed spacer (ITS), the nuclear ribosomal large subunit (LSU), the partial actin (ACT) and the partial RNA polymerase II subunit (RPB2) regions were amplified using the primer sets ITS1/ITS4 (White et al. 1990), NL1/NL4 (O'Donnel 1993), ACT512F/ACT783R (Carbone & Kohn 1999) and RPB2–5F2/fRPB2–7cR (Liu et al. 1999, Sung et al. 2007), 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, 1.5 mM $MgCl_2$, Ampliqon, Denmark) in a total volume of 25 µl. Conditions for PCR of ITS, LSU and ACT consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 57°C and 1 min at 72°C, and a final extension step of 10 min at 72°C. The RPB2 gene was amplified using a touchdown PCR consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 45 s at 95°C, 45 s at 60–54°C (annealing temperature decreased 0.5°C per cycle in the first 10 cycles), 45 s at 72°C and a final extension step of 10 min at 72°C. The presence of expected amplicons was checked on a 1% agarose gel. PCR products were cleaned up and sequenced by Microsynth DNA Company (Balgach, Switzerland).

Table 1. *Cytospora* isolates used in molecular phylogenetic analysis in this study

Species	Strain	Apple cultivar	Location	Coordinates		GenBank accession numbers			
				Latitude	Longitude	ITS	*LSU*	*RPB2*	*ACT*
C. chrysosperma	IRAN 4240C	Red Delicious	Miyandoab	36°58'49.7"N	46°21'5"E	MW548189	MW548201	MW567945	MW556639
C. chrysosperma	IRAN 4241C	Red Delicious	Miyandoab	36°58'49.7"N	46°21'5"E	MW548190	MW548202	MW567946	MW556640
C. chrysosperma	FCCUU 333	Red Delicious	Miyandoab	36°58'49.7"N	46°21'5"E	MW548191	MW548203	MW567947	MW556641
C. chrysosperma	FCCUU 334	Red Delicious	Miyandoab	36°58'49.7"N	46°21'5"E	MW548192	MW548204	MW567948	MW556642
C. germanica	IRAN 4242C	Red Delicious	Oshnavieh	37°4'6"N	45°8'9.1"E	MW548193	MW548206	MW567954	MW567942
C. germanica	IRAN 4243C	Red Delicious	Oshnavieh	37°4' 6"N	45°8'9.1"E	MW548194	MW548205	MW567954	MW567943
C. germanica	FCCUU 337	Red Delicious	Oshnavieh	37°4'6"N	45°8'9.1"E	MW548195	MW548207	MW567954	MW567944
C. paratranslucens	IRAN 4245C	Red Delicious	Mahabad	36°48'53.7"N	45°44'1.5"E	MW548186	MW548208	MW567949	MW567937
C. paratranslucens	IRAN 4244C	Red Delicious	Mahabad	36°48'53.7"N	45°44'1.5"E	MW548187	MW548209	MW567950	MW567938
C. paratranslucens	FCCUU 340	Red Delicious	Salmas	38°8'16"N	44°43'49.3"E	MW548188	MW548210	MW567951	MW567939
C. salicina	IRAN 4246C	Golden Delicious	Miyandoab	36°56' 42"N	46°10'9"E	MW548197	MW548212	MW567953	MW567941
C. salicina	IRAN 4247C	Red Delicious	Nagadeh	37°0' 45.5"N	45°17'11.8"E	MW548196	MW548211	MW567952	MW567940

Phylogenetic analysis

The newly generated sequences were edited in BioEdit v.7.2.6 (Hall 1999), checked manually and deposited in GenBank (Table 1). Sequences of the homologous regions with high similarity from the type or representative *Cytospora* species were obtained from GenBank database according to Fan et al. 2020 and were included in phylogenetic analysis. Multiple alignments were generated with MAFFT online service (https://mafft.cbrc.jp/alignment/server/index.html) for each locus separately. The combined sequence dataset (ITS + LSU + RPB2 + ACT) was produced and proofed in Mesquite v. 2.74 (Maddison and Maddison 2010). Maximum likelihood analysis (ML) was performed on a concatenated dataset in RAxML-HPC BlackBox v. 8.2.12 (Stamatakis 2014) through the CIPRES Science Gateway v 3.3 (Miller et al. 2010) using the GTRGAMMA + I as substitution model. The resultant phylogenetic tree was observed in FigTree v. 1.4.4 (Rambaut 2019). *Diaporthe vaccinii* strain CBS 160.32 was used as an outgroup taxon.

Pathogenicity tests and statistical analysis

In order to assess the pathogenicity of fungal isolates, in vitro inoculation test was performed (Ma et al. 2018). One– to two-year-old healthy shoots of both 'Red Delicious' and 'Golden Delicious' cultivars were taken from an apple orchard, cut into 20 cm fragments in length, washed thoroughly under running tap water, then surface disinfested in 75% ethyl alcohol for 2 minutes and were kept under aseptic conditions for 30 minutes. The bark was removed with the aid of a sterilized 5 mm cork borer and immediately inoculated with a mycelium plug (5 mm in diameter) cut from the margin of a 7–day–old actively growing fungal culture. The inoculated site was covered with a moist cotton ball and wrapped with Parafilm to maintain high humidity (Azizi et al., 2020, Tang et al., 2012). In the control treatments, PDA plugs without fungal mycelium were used. One to two isolates from each species were used in the pathogenicity tests, three shoots were inoculated with each isolate and all the tests were replicated twice. The inoculated shoots were kept in clean plastic boxes lined with moistened sterile paper towels and kept at 25 ºC in darkness for 21 days. Shoots were examined for the presence or absence of necrotic lesions around the inoculated sites, and while present, the bark was removed carefully and the length of necrotic lesion was measured. To complete Koch's postulates, fungi re-isolation was conducted from the diseased parts of inoculated shoots in the same way as described earlier. The identity of the isolates was confirmed again based on morphological characteristics. The experiments were conducted based on a completely randomized design (CRD) with three replications. After analysis of variance (ANOVA), lesion length means were compared with Duncan's test ($P = 0.05$) using SAS (9.1) software.

RESULTS

Disease symptoms

In total, 68 isolates with the typical characteristics of the genus *Cytospora* were obtained from 45 samples collected in different parts of West Azerbaijan Province. Symptoms associated with *Cytospora* cankers include depressed lesions with bark splitting, cankers, discoloration of xylem and wood necrosis and dieback (Fig. 1).

Morphological identification

Morphological comparisons of the examined isolates with the species described in the literatures grouped our isolates with the isolates of four previously described *Cytospora* species. Two species, *C. parasitica* and *C. paratranslucens* have been reported previously from apple trees, but *C. germanica* and *C. salicina* are described as apple pathogens for the first time in the world and are reported here for the first time to mycobiota of Iran.

Molecular phylogenetic analysis

PCR amplifications of the selected 12 *Cytospora* isolates produced DNA fragments approximately 563 bp for ITS, 516 bp for LSU, 893 bp for RPB2 and 282 for ACT. Sequence combination of four loci for a total of 75 fungal strains, including ingroup and outgroup taxa, contained 1943 characters. The best scoring RaxML tree with the final ML optimization likelihood value of -10074.977337 (ln) is selected to demonstrate and discuss the phylogenetic relationships among the studies strains (Fig. 2). Maximum likelihood analysis of the combined dataset revealed that our strains clustered well with representative strains of four previously described species *viz*. *C. chrysosperma*, *C. germanica*, *C. paratranslucens* and *C. salicina* with 88–100% ML bootstrap values (Fig. 2) and confirmed morphological identification. All the identified species are illustrated and described.

Fig. 1. Disease symptoms associated with *Cytospora* spp. on apple trees. a, b. Apple trees with dieback symptoms. c, d. Bark discoloration, splitting, and canker formation on main branches and twigs. e. Asexual fruiting bodies formed on the diseased bark of apple tree. f, g, h. Wood discoloration.

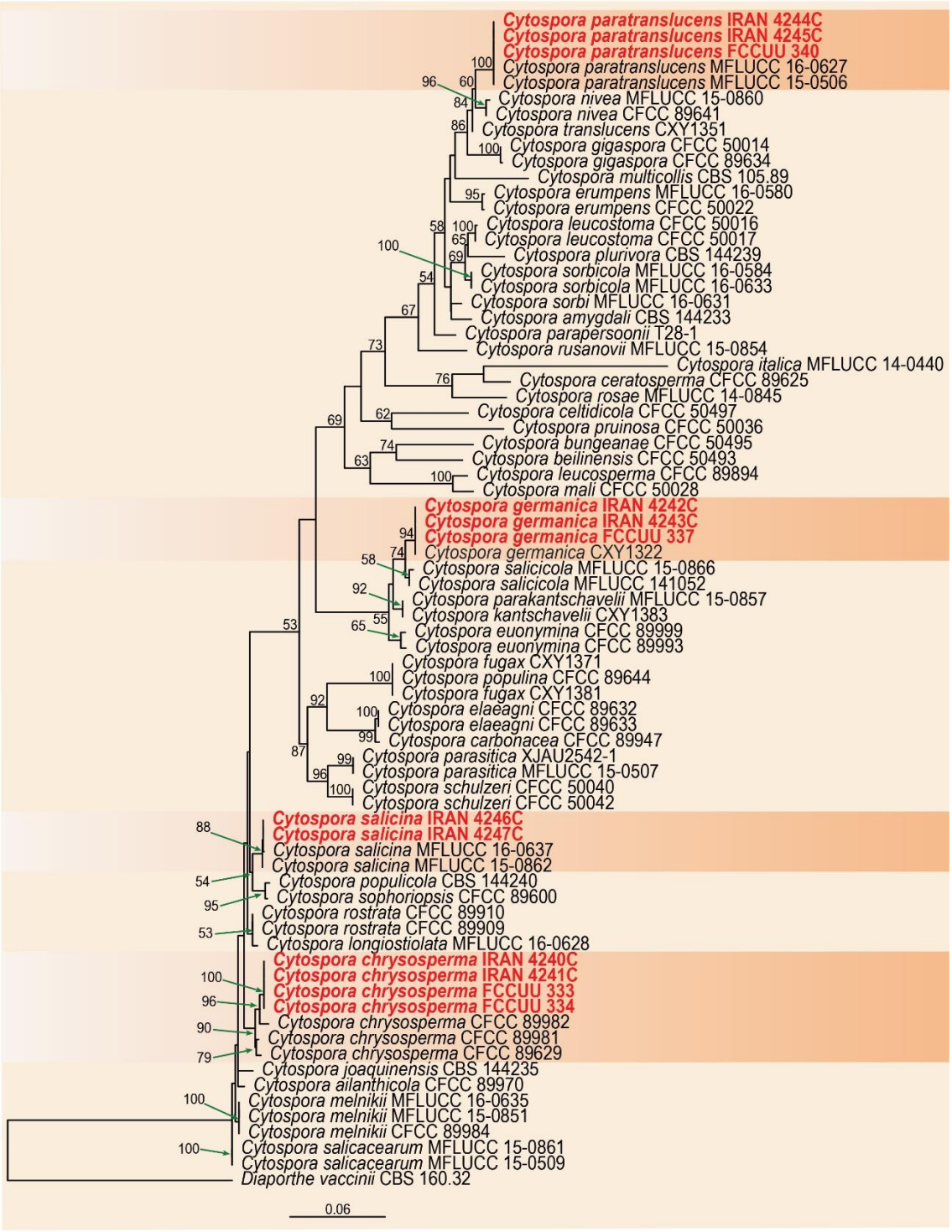

Fig. 2. Phylogenetic tree of *Cytospora* spp. inferred from Maximum Likelihood (ML) analysis of a combined dataset of ITS, LSU, RPB2 and ACT sequences. The Maximum Likelihood bootstrap support (BS) values >50% are given at the nodes. The tree was rooted to *Diaporthe vaccinii* strain CBS 160.32 and newly identified strains are in red. The scale bar indicates the number of nucleotide substitutions.

Taxonomy

Cytospora chrysosperma (Pers.) Fr., Systema Mycologicum (Lundae) 2: 542 (1823) Fig. 3

Conidiomata pycnidial, scattered, semi-immersed in host tissue, erumpent through the bark surface, without conceptacle, with multiple locules, circular, (1066–)1128–2000(–2325) µm diam (n=20). Disk white to dilute brown, with one ostiole. Ostioles circular to ellipsoid and 145–500 µm diam. Conidiophores borne along the locules, hyaline, unbranched, thin-walled, reduced to conidiogenous cells. Conidiogenous cells entroblastic, phialidic, hyaline, smooth-walled, (6.5–)6.9–9.6(–10.7) × (1.2–)1.3–1.8 µm ($\bar{x}$= 8.7 × 1.6 µm, n= 20), tapering toward

the apex. Conidia unicellular, hyaline, allantoid, thin-walled, eguttulate, (3.4–)3.6–4.8(–5) × 1–1.3 µm ($\bar{x}$= 4.1 × 1.1 µm, n= 40) (Fig. 3). Sexual morph was not observed.

Culture characteristics. Colonies on PDA at 25±1ºC and in the dark reaches 40 mm diameter after 3 days and entirely covering the 9 cm Petri dishes after 7 days. Colonies at first white, becoming off-white to cream after 30 d, with short aerial hyphae giving a cottony appearance to colony surface, margin irregular. Pycnidia irregularly distributed on the PDA surface.

Specimens examined. IRAN, West Azerbayjan Province, Miyandoab, from apple trees with canker, decline and dieback symptoms, 5 Sept. 2017, R. Azizi, IRAN 4240C, IRAN 4241C, FCCUU 333, FCCUU 334.

Hosts and distribution. *Cytospora chrysosperma* has been reported from more than 260 hosts in Africa, Asia, Australia, New Zealand, Europe and North and South America. In Iran, it has been reported from *Armeniaca vulgaris*, *Crataegus azarolus*, *Ficus carica*, *Fraxinus excelsior*, *Fraxinus* sp., *Juglans regia*, *Ligustrum latifolium*, *Malus pumila*, *Morus alba*, *Olea sativa*, *Persica vulgaris*, *Platanus orientalis*, *Populus alba*, *Populus deltoides*, *Populus nigra*, *Prunus domestica*, *Prunus persicae*, *Robinia pseudoacacia*, *Salix aegyptiaca*, *Salix excelsa*, *Salix* sp., *Tamarix* sp., *Thuja orientalis* and *Vitis vinifera* (Fotouhifar et al. 2010, Arzanlou & Narmani 2015, Bagherabadi et al. 2017).

Fig. 3. *Cytospora chrysosperma* (IRAN 4240C) on *Malus domestica*. a–b. Habit of conidiomata on a branch with pale luteous to luteous tendril, c–d. Longitudinal section through conidiomata, e–f. Cross-section through conidiomata, g. Conidiogenous cells with attached conidia, h. Conidia, i. Colony on PDA after 3 days (half part, left) and after 30 days (half part, right). Scale bars: b, c, e =250 µm; f, d=100 µm and g, h= 10 µm.

***Cytospora germanica* Sacc., Sylloge Fungorum 3:262 (1884) Fig. 4**

Conidiomata pycnidial, scattered, semi-immersed in host tissue, erumpent through the bark surface, with multiple locules, circular, (800–) 850–1100 µm diameter (n=20). Disk white, circular, grey at the center and with one ostiole. Ostioles circular to ellipsoid and 100–250 µm diam. Conidiophores borne along the locules, hyaline, branched at the base or unbranched, thin-walled. Conidiogenous cells entroblastic, phialidic, hyaline, smooth-walled, (5.7–)6.3–14.3(–15.1) × (1.8–)1.9–3.4(–3.5) µm ($\bar{x}$= 10 × 2.4 µm, n= 20), tapering toward the apex. Conidia unicellular, hyaline, allantoid, thin-walled, eguttulate, (5.9–)6.2–9.4(–10.3) × (1.2–)1.5–1.9(–2) µm ($\bar{x}$= 7.2 × 1.7 µm, n= 40) (Fig. 4). Sexual morph was not observed.

Culture characteristics. Colonies on PDA at 25±1 ºC and in the dark reaches 20 mm diameter after 3 days and 53 mm diameter after 7 days. Colonies at first white, becoming amber after 7 days and buff after 30 d, with short aerial hyphae giving a cottony to felt like appearance to colony surface, margin irregular.

Specimens examined. IRAN, West Azerbayjan Province, Oshnavieh, from apple trees with canker, decline and dieback symptoms, 24 Oct. 2017, R. Azizi, IRAN 4242C, IRAN 4243C, FCCUU 333.

Hosts and distribution. *Cytospora germanica* has been reported from 15 hosts, mostly plants in the *Salicaceae* family in Asia, Europe and South Africa (Farr & Rossman 2021).

Fig. 4. *Cytospora germanica* (IRAN 4243C) on *Malus domestica*. a–b. Habit of conidiomata on branch; c–d. Longitudinal section through conidiomata; e–f. Cross-section through conidiomata; g. Conidiogenous cells with attached conidia; h. Conidia; i. Colony on PDA after 3 days (half part, left) and after 30 days (half part, right). — Scale bars: b, c, e = 250 µm; f, d = 100 µm and g, h = 10 µm.

Cytospora paratranslucens **Norph., Bulgakov, T.C. Wen & K.D. Hyde, in Norphanphoun, Doilom, Daranagama, Phookamsak, Wen, Bulgakov & Hyde, Mycosphere 8(1): 75 (2017) Fig. 5**

Conidiomata pycnidial, scattered, semi-immersed in host tissue, erumpent through the bark surface, with multiple locules, circular, 1000–1375 µm diameter (n=20). Disk olivaceous dark green with 1-2 ostioles. Ostioles circular to ellipsoid and 105–205 µm diam. Conidiophores borne along the locules, hyaline, unbranched, reduced to conidiogenous cells, thin-walled. Conidiogenous cells entroblastic, phialidic, hyaline, smooth-walled, (9.3–)9.5–14.2(–17.4) × (0.9–)1.3–2.1(–2.8) µm ($\bar{x}$= 11.65 × 1.75 µm, n= 20), tapering toward the apex. Conidia unicellular, hyaline, allantoid, thin-walled, eguttulate, (5.6–)5.8–8.4(–8.7) × (1.2–)1.3–1.9 µm ($\bar{x}$= 6.9 × 1.6 µm, n= 40) (Fig. 5). Sexual morph was not observed.

Culture characteristics. Colonies on PDA at 25 ± 1ºC and in the dark reaches 41 mm diameter after 3 days and entirely covering the 8-cm Petri dishes after 7 days. Colonies at first white, then becoming grey at the margin and olivaceous grey at center after 30 d, margin regular.

Specimens examined. IRAN, West Azerbayjan Province, Mahabad, from apple trees with canker, decline and dieback symptoms, 31 Oct. 2017, R. Azizi, FCCUU 339, IRAN 4245C; West Azerbayjan Province, Salmas, from apple trees with canker, decline and dieback symptoms, 30 Aug. 2017, R. Azizi, IRAN 4244C.

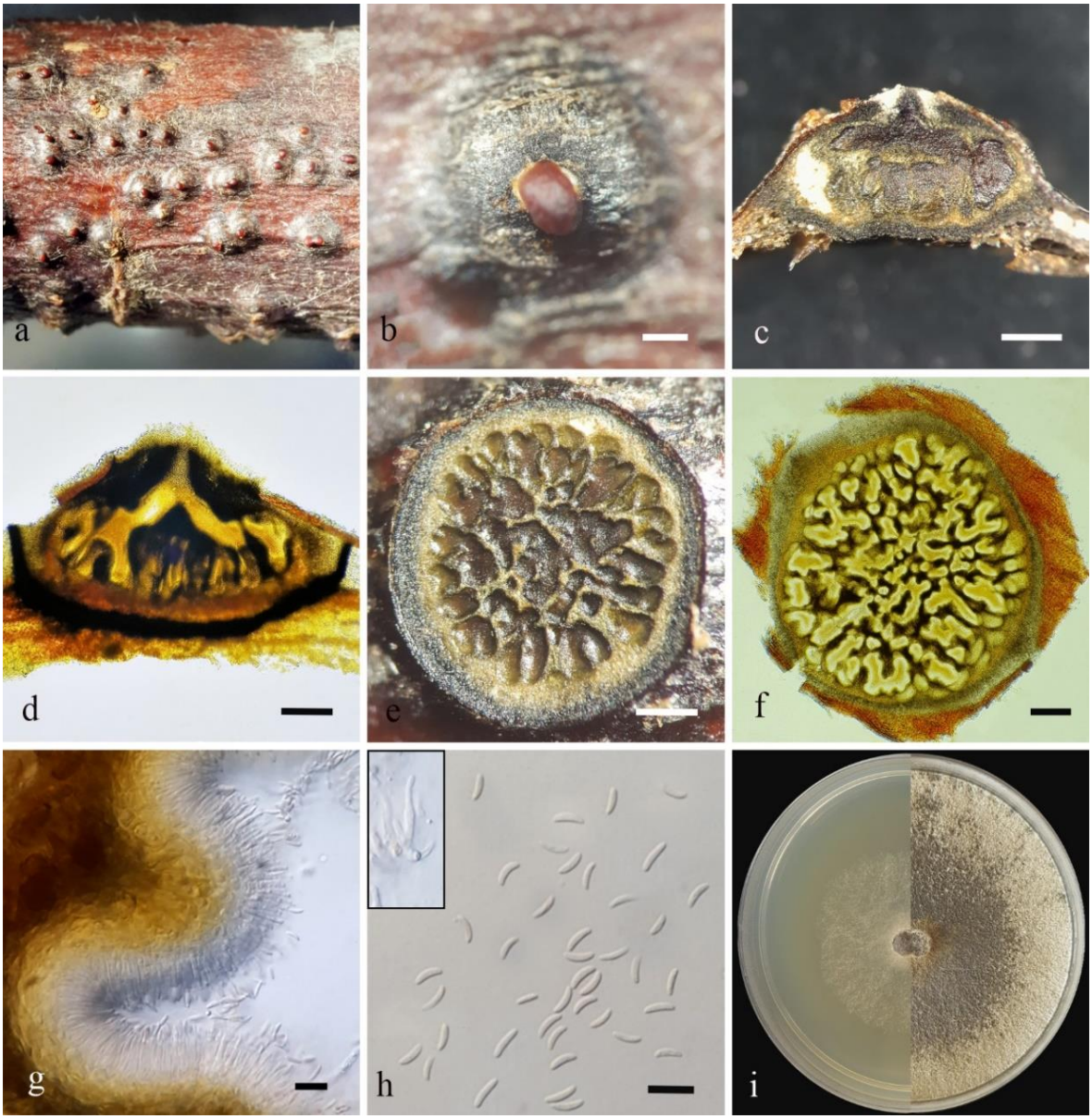

Fig. 5. *Cytospora paratranslucens* (IRAN 4245C) on *Malus domestica*. a–b. Habit of conidiomata on a branch with short, bay to rust color tendril; c–d. Longitudinal section through conidiomata; e–f. Cross-section through conidiomata; g. Conidiogenous cells with attached conidia; h. Conidia; i. Colony on PDA after 3 days (half part, left) and after 30 days (half part, right). — Scale bars: b, c, e = 250 µm; f, d = 200 µm and g, h = 10 µm.

Hosts and distribution. *Cytospora paratranslucens* have been reported from *Populus alba* (Salicaceae) in Europe (Russia) and *Malus domestica* in Asia (Iran) (Azizi et al. 2018, Farr & Rossman 2021).

***Cytospora salicina* Norph., Bulgakov, T.C. Wen & K.D. Hyde, in Norphanphoun, Doilom, Daranagama, Phookamsak, Wen, Bulgakov & Hyde, Mycosphere 8(1): 80 (2017) Fig. 6**

Conidiomata pycnidial, scattered, semi-immersed in host tissue, erumpent through the bark surface, with multiple locules, circular to ovoid, (1000–)1076–1650(–1775) µm diameter (n=20). Disk creamy-white, grey at the center and with one ostiole. Ostioles circular to ellipsoid and 125–185 µm diam. Conidiophores borne along the locules, hyaline, unbranched, very rarely branched at base, thin-walled, reduced to conidiogenous cells. Conidiogenous cells entroblastic, phialidic, hyaline, smooth walled, (8.1–)8.6–13.5(–15) × 1.5–2.3(–2.5) µm ($\bar{x}$= 10.5 × 1.8 µm, n= 20), tapering toward the apex. Conidia unicellular, hyaline, allantoid, thin-walled, eguttulate, (3.8–)4–5.6(–5.8) × (1–)1.1–1.5(–1.6) µm ($\bar{x}$= 4.8 × 1.8 µm, n= 40) (Fig. 6). Sexual morph was not observed.

Culture characteristics. Colonies on PDA, at 25 ± 1ºC and in the dark reaches 12 mm diameter after 3 days and 29 mm diameter after 7 days, at first white, then change to olivaceous buff at the margin, greenish olivaceous at middle and off-white to cream at the center, margin irregular, without aerial mycelium and colony appearance felt like.

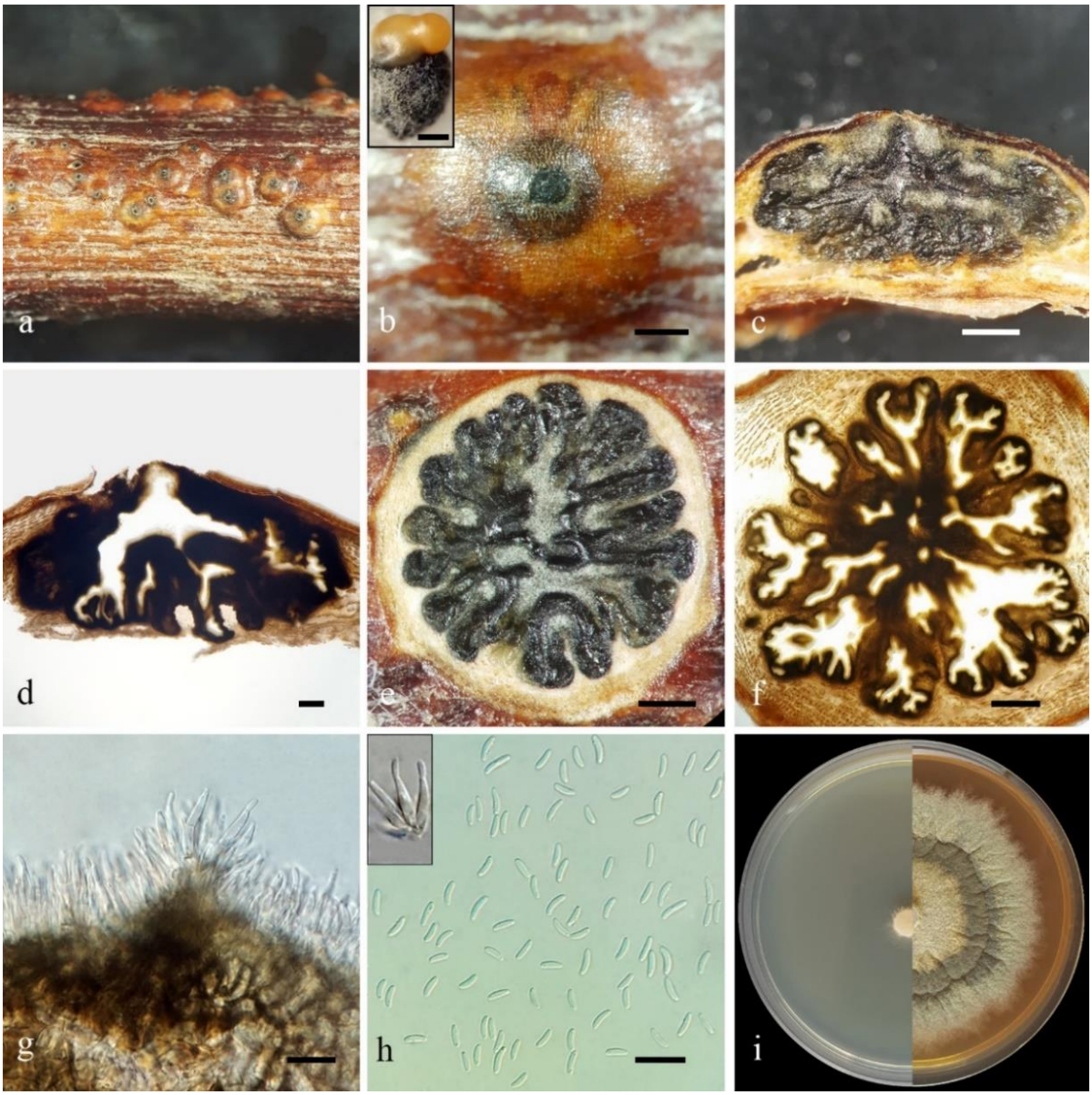

Fig. 6. *Cytospora salicina* (IRAN 4247C) on *Malus domestica*. a–b. Habit of conidiomata on a branch with pale luteous to luteous tendril; c-d. Longitudinal section through conidiomata; e–f. Cross section through conidiomata; g. Conidiogenous cells with attached conidia; h. Conidia; i. Colony on PDA after 3 days (half part, left) and after 30 days (half part, right). — Scale bars: b, c, e = 250 µm; f, d = 100 µm and g, h = 10 µm.

Specimens examined. IRAN, West Azerbayjan Province, Miyandoab, from apple trees with canker, decline and dieback symptoms, 5 Sept. 2017, R. Azizi, IRAN 4246C; West Azerbayjan Province, Nagadeh, from apple trees with canker, decline and dieback symptoms, 24 Oct. 2017, R. Azizi, IRAN 4247C.

Hosts and distribution. *Cytospora salicina* has been reported from *Salix alba*, *Salix fragilis* and Salix × *rubens* (Salicaceae) and *Cornus sanguinea* (Cornaceae) in Europe (Russia) (Farr & Rossman 2021).

Pathogenicity tests

Seven days after inoculation of shoots with the fungal isolates, bark tissue around the inoculated site showed brown discoloration and shrinkage. The lesions were expanded to girdle the shoots, and after 14 days, small raised, black spots reminiscent of pycnidial initials were observed on discolored bark. Two tested isolates (IRAN 4246C and IRAN 4247C) of *C. salicina* caused characteristic lesions on detached branches of both 'Golden Delicious' and 'Red Delicious' cultivars (Fig. 7), but the tested isolates of *C. chrysosperma*, *C. germanica* and *C. paratranslucens* were only pathogenic on 'Red Delicious' cultivar and caused characteristic symptoms on inoculated shoots, while no symptoms were produced on 'Golden Delicious' cultivar as well as control treatments (Fig. 7). The fungal isolates were re-isolated from inoculated shoots and confirmed Koch's postulates. The mean lesion length showed significant differences (P<0.05) among four species, five isolates and the two cultivars, 'Red Delicious' and 'Golden Delicious' (Fig. 8). The highest and lowest pathogenicity were observed in the IRAN 4245C (*C. paratranslucens*) and IRAN 4243C (*C. germanica*) isolates on 'Red Delicious' cultivar, respectively.

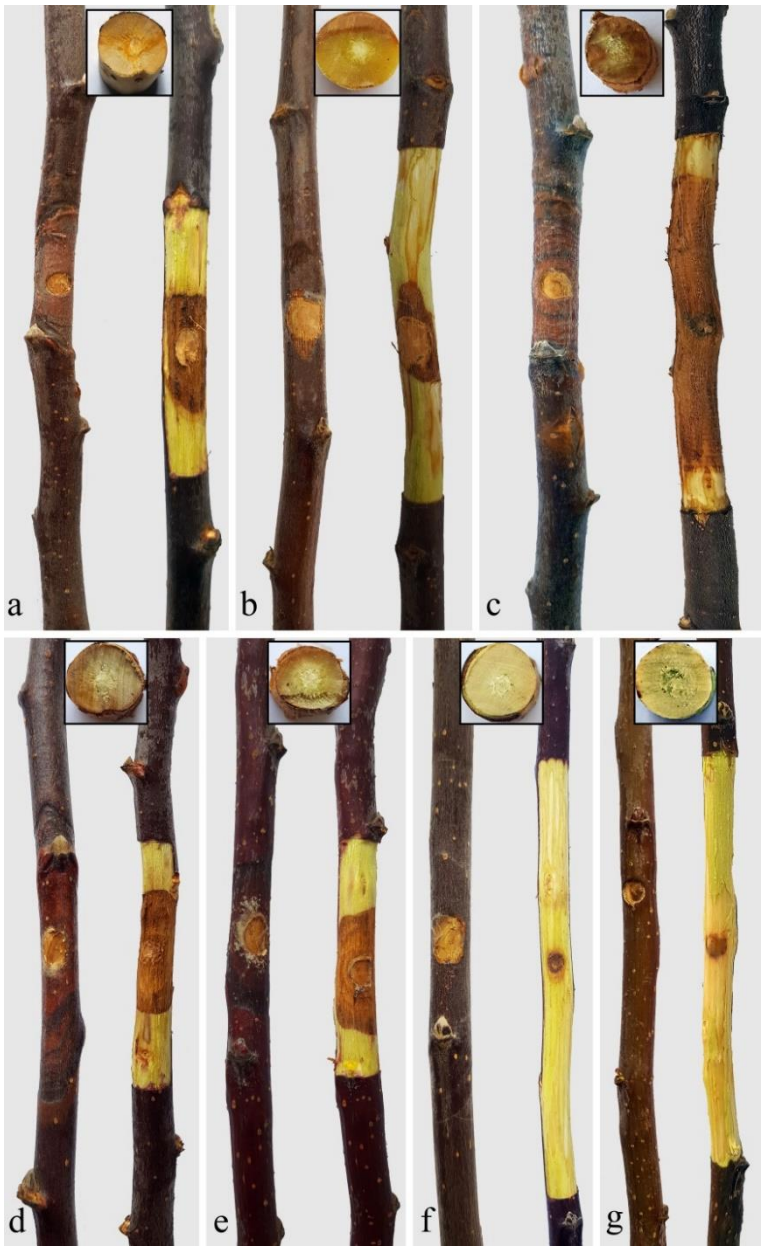

Fig. 7. Pathogenicity tests on detached apple shoots. a, b and c: apple 'Red Delicious' cultivar inoculated with *Cytospora chrysosperma* (isolate IRAN 4240C), *C. germanica* (isolate IRAN 4243C) and *C. paratranslucens* (isolate IRAN 4245C), respectively. d and e: apple 'Red Delicious' and 'Golden Delicious' cultivars inoculated with the same isolate of *C. salicina* (isolate IRAN 4246C). f: apple 'Red Delicious' and g: 'Golden Delicious' control treatments.

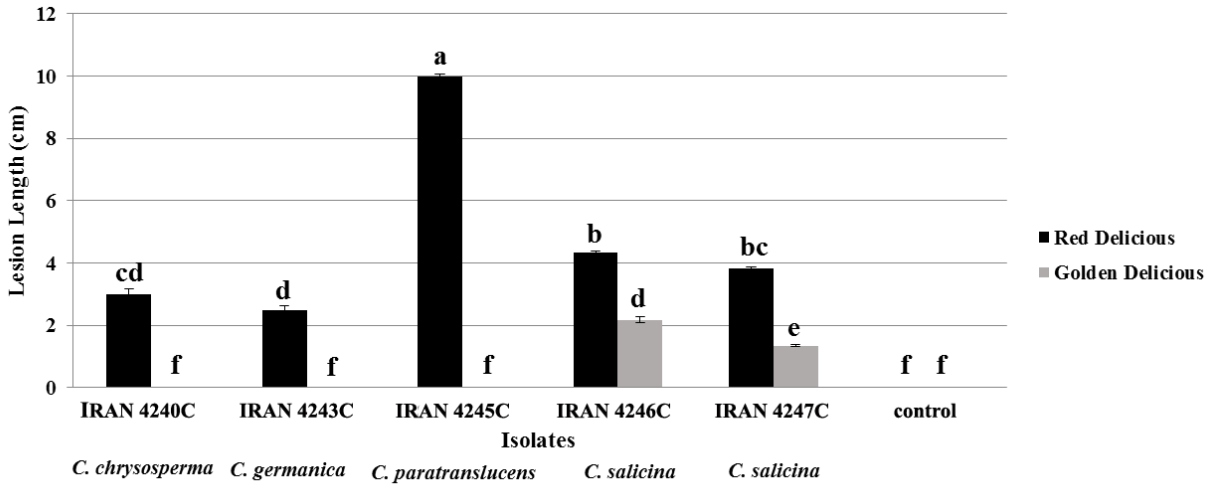

Fig. 8. Mean lesion length (cm) on detached branches of apple cultivars inoculated with *Cytospra* spp. based on Duncan's test. Different letters show significant difference at $P = 0.05$.

DISCUSSION

Apples are important economic trees in West Azerbayjan Province, Iran. However, in recent years and due to climatic changes, severe canker and decline diseases were observed throughout the apple orchards, caused significant economic losses to growers. *Cytospora* species are ubiquitous, important pathogens of many economically important woody plant hosts, which are associated with canker, dieback, decline and mortality of many fruit crops, forest and urban trees (Adams 2006, Fan et al. 2015, Lawrence et al. 2018, Pan et al. 2020). Traditionally, identification of *Cytospora* species was based on their host affiliation and morphological characteristics of asexual morph or rarely on the sexual morph. The utility of these characters was questioned in several studies so that more than a single *Cytospora* species was isolated from a single host and a single *Cytospora* species was isolated from different hosts (Fotouhifar et al. 2011, Mehrabi et al. 2011, Fan et al. 2015, Pan et al. 2020, Zhu et al. 2020). Also, many *Cytospora* species have overlapping morphological characteristics, so complicating the morphological separation of the species (Adams et al. 2005, Wang et al. 2011, Lawrence et al. 2018). Recent studies have proposed the use of multiphase approaches to accurate delimitation and identification of *Cytospora* species and emphasized using multilocus phylogeny instead of ITS phylogeny to test the relationships among species (Lawrence et al. 2017, 2018, Norphanphoun et al. 2017, Pan et al. 2018, 2020). In our study, four species of *Cytospora* were identified from a single host (*Malus domestica*) based on morphological characteristics and multigene phylogenetic analysis. Of these, *C. germanica* and *C. salicina* are reported for the first time from apple trees, expands our knowledge about their geographic distribution and host range. Also, *C. paratranslucens* which was reported from apple trees in Iran (Azizi et al. 2018), and there is no detailed description and illustration associated with this record, was illustrated and described. Morphological characteristics such as dimensions of conidiomata, conidiophores and conidia were slightly different from those reported in the literature, which might be influenced by factors such as host, geographic origin, culture conditions or climate (Ma et al. 2018). In a recent report on fungal agents damaging shoot and trunk of apple trees in Iran (Hanifeh et al. 2018), two species of *Cytospora viz. C. rhodophila* and *C. sorbicola* were reported based on morphological characteristics and sequence data of ITS region. While ITS is accepted as the primary marker for fungal barcoding, but in some fungal groups, it has insufficient power for robust species delimitation, so using protein-coding genes may be more informative for species identification (Úrbez-Torres 2011, 2020, Cabral et al. 2012, Wang et al. 2014, 2020, Lawrence et al. 2018). Therefore, we recommend using multigene sequence data for accurate identification of *Cytospora* species. The current study implies that additional undiscovered *Cytospora* species may exist in Iran and further studies are needed to discover species diversity of *Cytospora* associated with dieback, canker and decline diseases of apple trees and to understand their roles in causing plant diseases. Accurate identification of major pathogenic species will help studies involving ecological, epidemiological pathogenicity and host range of the species and aid focusing management strategies against the most aggressive species.

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شناسایی ریخت‌شناختی و مولکولی گونه‌های *Cytospora* عامل زوال درختان سیب در ایران

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چکیده: در بررسی‌های اخیر باغات سیب با نشانه‌های شانکر، سرخشکیدگی و زوال در استان آذربایجان‌غربی، چندین جدایه قارچی با ویژگی‌های شاخص جنس *Cytospora* به دست آمد. ترکیب ویژگی‌های ریخت‌شناختی و تجزیه و تحلیل‌های تبارشناختی بر اساس ناحیه فاصله‌انداز رونویسی‌شونده داخلی از دی‌ان‌ای ریبوزومی (ITS-rDNA) و بخش‌هایی از زیرواحد بزرگ دی‌ان‌ای ریبوزومی (LSU)، ژن‌های اکتین (ACT) و آران‌ای پلیمراز *II* (RPB2) برای شناسایی دقیق گونه‌های قارچی استفاده شد. چهار گونه از جنس *Cytospora* شامل *Cytospora chrysosperma*، *C. germanica*، *C. paratranslucens* و *C. salicina* شناسایی شدند. آزمون‌های بیماریزایی روی شاخه‌های ارقام سیب 'رد دلیشیوز' و 'گلدن دلیشیوز' انجام گرفت. جدایه‌های گونه *C. salicina* لکه‌های مشخصی را روی شاخه‌های مایه‌زنی شده هر دو رقم سیب ایجاد کردند، اما جدایه‌های گونه‌های *C. chrysosperma*، *C. germanica*، و *C. paratranslucens* تنها روی شاخه‌های سیب رقم 'گلدن دلیشیوز' بیماریزا بودند. جداسازی و شناسایی مجدد قارچ‌های مایه‌زنی شده، اصول کخ را تایید نمود. این مطالعه نمایانگر وجود گونه‌های مختلفی از جنس سیتوسپورا در منطقه مورد مطالعه است که سبب بیماری شانکر، سرخشکیدگی و زوال در درختان سیب می‌شوند. دو گونه *C. germanica* و *C. salicina* برای اولین بار به عنوان عوامل ایجاد کننده شانکر در درختان سیب معرفی می‌شوند. همچنین، این دو گونه به عنوان ثبت‌های جدید برای بیوتای قارچی ایران گزارش می‌شوند.

کلمات کلیدی: شانکر سیب، *Cytosporaceae*، سرخشکیدگی، بیماریزایی، تبارشناسی

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