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New records of apple endophytic fungi for the Funga of Iran

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Abstract: Endophytic fungi are microorganisms with the ability to colonize plant tissues without any symptoms, in whole or part of their life cycle. These fungi have been found in every plant species examined to date. In this study, 417 isolates of endophytic fungi were obtained from healthy and symptomless fruits, leaves and branches of 70 analyzed wild (*Malus orientalis*) and Iranian endemic (*Malus domestica*) apple cultivars trees in the north of Iran. Among the identified fungi, species *Coniochaeta endophytica* and *Curvularia hominis* were new for the Funga of Iran based on morphological features and molecular data. Furthermore, these species are reported for the first time as endophytic fungi of apple trees in the world.

Key words: Iranian endemic apple cultivars, phylogeny, symbiosis, taxonomy, wild apple

INTRODUCTION

Apple (*Malus* sp., *Rosacea*) is the most common and culturally important fruit crop worldwide and also in Iran due to its nutritional and export value (Ebrahimi et al. 2016). Wild (*Malus orientalis*) and Iranian endemic (*Malus domestica*) apple cultivars are mostly spread along the Caspian Sea coast in the north of Iran. Endophytic fungi are symptomless microbial organisms without causing negative effects to the host (Wilson 1995), establishing a plant-fungi association inside the living plant tissue, that may occur within roots, stems, leaves and/or fruits (Sherwood & Carroll 1974; Carroll 1988; Stone et al. 2004). These fungi are to be found in almost all plants including woody and herbaceous plants (Huang et al. 2001; Hyde & Soytong 2008). The fossil record indicates that plants have had associations with endophytic fungi for more than 400 million years, a relationship that has likely existed since the time when plants first colonized land, thus playing a long and important role in the driving force of evolution, and life on land (Krings et al. 2007).
The plant-associated habitat is a dynamic environment in which many factors affect the structure and composition of species that colonize different tissues. It has been previously shown that endophytic communities may vary spatially in many kinds of plants (Rivera-Orduña et al. 2011). Also, microorganisms' population can be different at natural forest from agro- ecosystem due to use of synthetic chemicals by farmers. Arrigoni et al. (2020) studies showed that fungal and bacterial diversity of apple is affected by bark age, orchard location and sampling time (Arrigoni et al. 2020). Afandhi et al. (2018) obtained more diverse endophytic fungi from apple mature leaves in comparison to young and old leaves. In Camatti-Sartori et al. (2005) study on apple endophytic fungi, genera *Colletotrichum* Corda, *Xylaria* Hill ex Schrank and *Botryosphaeria* Ces. & De Not., *Sporobolomyces* Kluyver & C.B. Niel, *Rhodotorula* F.C. Harrison, *Debaryomyces* Klöcker and *Cryptococcus* Kütz were the most frequent taxa. Liu et al. (2017) investigated biocontrol potential of 81 endophytic fungi (representing 33 fungal morphology groups) from apple shoots to protect apple trees against *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman infection. Among them, 15 selected fungal isolates were identified as *Epicoccum* Link, *Chaetomium* Kunze, *Biscogniauxia* Kuntze, *Neosetophoma* Gruyter, Aveskamp & Verkley, and *Penicillium* Link species. Study on apple tree endophytic fingi in 16 scion–rootstock combinations at two locations showed that endophyte diversity was primarily affected by the orchard location, followed by the scion genotype, whereas rootstock effects were small (Olivieri et al. 2021). In Iran, Alijani et al. (2016) purified about 350 isolates from shoots, leaves and barks of endemic and commercial apple trees in West Azerbaijan province. Based on the results, a total of 24

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species belong to 10 different genera of *Ascomycota* (*Alternaria* Nees, *Arthrinium* Kunze, *Aspergillus* P. Micheli ex Haller, *Chaetomium*, *Dicyma* Boulanger, *Doratomyces* Corda, *Paraconiothyrium* Verkley, *Stemphylium* Wallr., *Trichoderma* Pers. and *Trichothecium* Link) were identified.

But, there is not any study on endophytic fungi of wild and Iranian endemic apple cultivars which can comprise various groups of endophytes. Thus, the present study aims to evaluate the endophytic fungi associated with wild and Iranian endemic apple cultivars trees in the north of Iran. For this purpose, apple fruits, leaves, and branches were collected from the forests along the Caspian Sea coast. Endophytic fungi of the samples were isolated and identified based on morphological criteria and molecular data. Some taxa were new for the Funga of Iran which are reported in this study.

MATERIALS AND METHODS

Sample collection and endophytic fungi isolation

Healthy and symptomless fruits, leaves and 1- or 2-year-old branches of wild and endemic apple cultivars were collected from Guilan, Mazandaran and Golestan provinces of Iran, during the summer of 2019. The method modified by Strobel & Daisy (2003) was used for surface sterilization of plant samples. Plant materials were thoroughly washed in running tap water for 10 min before disinfection. The plant samples were surface disinfected with 70 % (v/v) ethanol for 45 s for fruits and leaves, and 60–90 s for twigs, then 2 % (v/v) sodium hypochlorite for 30 s, and 70 % (v/v) ethanol for 15 s and subsequently rinsed with sterile water and the outer tissue of the fruits and twigs were removed with a sterile scalpel. Disinfected plant materials were cut in small pieces (1 × 1 cm) and then placed in Petri dishes containing water agar (WA), corn meal agar (CMA) and potato dextrose agar (PDA). Petri dishes were kept in continuous dark conditions at 25 °C for one to four weeks. Then, the pure cultures of the grown fungi were obtained by transferring hyphal tips on PDA. Isolates were stored on PDA slant at 4 °C. All identified isolates were deposited in the Fungal Culture Collection (IRAN) of the Iranian Research Institute of Plant Protection, Tehran, Iran.

Morphological characterization

Colony colors were assessed on malt extract agar (MA), and PDA after 7 days or 2 weeks in the continuous dark condition or 12 h light / 12 h dark (due to the fungus) at 25 °C, using the color charts of Rayner (1970). In addition, *Coniochaeta* isolates were cultured on synthetic nutrient agar (SNA) with double-autoclaved pine needles to encourage perithecia formation (Damm et al. 2010). Microscopic slides were prepared in lacto-phenol or lacto-phenol cotton blue solutions after 7 and/or 14 days (due to the fungal species). Measurement (n = 30) and microphotographs of fungal features were taken from microscopic slides using an Olympus BH2 light microscope (Olympus, Japan).

Molecular analysis

For DNA extraction, fungal isolates were grown on PDA for one week in continuous dark condition at 25 °C. Fresh mycelia were collected and subjected to DNA extraction using the protocol of rapid simplified DNA extraction protocol provided by Cenis (1992). Extracted DNA was diluted in 50 µL distilled water and were kept at -20 °C for future use.

Molecular identification of the fungal isolates was performed based on Internal Transcribed Spacer (ITS)-rDNA and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) sequences that were amplified using the ITS1/ITS4 (White et al. 1990) and gpd1/gpd2 (Berbee et al. 1999) primer pairs, respectively. The reaction mixture and PCR conditions for ITS and *gapdh* were the same as described by Ebrahimi & Fotouhifar (2016) and Song et al. (2019), respectively. PCR products were purified and directly sequenced in one direction with ITS1 and gpd1 primers, respectively, by BGI Company, Denmark.

Phylogenetic analysis

Sequences were manually edited using BioEdit Sequence Alignment Editor ver. 7.2.5 software (Hall 1999). All obtained sequences of endophytic isolates in this study have been deposited in GenBank (NCBI). For phylogenetic analyses, sequences of genomic regions of ITS rDNA or *gapdh* from different species (Table 1) were aligned with the homologous reference sequences of the respective genomic regions of related species obtained from GenBank (Table 1) using ClustalW (Thompson et al. 1994). Maximum likelihood (ML) (Felsenstein 1981) analysis was done by heuristic search with MEGA software ver. 7 (Kumar et al. 2016). Models K2+G and TN93+G were recommended by MEGA as the optimal nucleotide substitution models for ITS and *gapdh* data, respectively. Characters were treated as un-weighted and unordered with gaps treated as missing data. Confidence of individual clades was assessed by ML bootstrap analysis (Felsenstein 1985) with 1000 replicates.

RESULTS AND DISCUSSION

In the present study, we describe the new endophytic microbiota associated with wild and endemic apple cultivars in the north of Iran. From 70 analyzed wild and endemic apple trees (each includes twigs, leaves and fruits sample) in the north of Iran, 417 isolates of endophytic fungi were obtained, which some of them are described in this study. Among the identified fungi, two species were new for the Funga of Iran including *Coniochaeta endophytica* A.H. Harrington & A.E. Arnold (2 isolates – 0.48 %), and *Curvularia hominis* K.C. Cunha, Madrid, Gené & Cano (2 isolates – 0.48 %). Furthermore, both species are reported for the first time as endophytic fungi of apple trees in the world.

Phylogeny

ITS phylogeny: The phylogenetic analyses of *Coniochaeta* species performed using ITS rDNA nucleotide sequences of 18 isolates including one isolate of this study and 17 isolates from GenBank (including the out-group) (Table 1). DNA sequence

analysis revealed that our investigated isolate is placed in a clade with *Co. endophytica*, *Co. cephalothecoides* Kamiya, Uchiy. & Udagawa and *Co. prunicola* Damm & Crous species (Fig. 1) which are differentiated based on morphological features.

gapdh phylogeny: The *Curvularia* species were studied based on the *gapdh* gene sequences. Analyses included a total of 19 *gapdh* sequences (including one isolate from this study and 18 isolates from GenBank) (Table 1). Based on the Maximum Likelihood (ML) tree of the isolates, out examined isolate (IRAN 4400C) clustered with the other isolates of *Cu. hominis* from GenBank (Fig. 2).

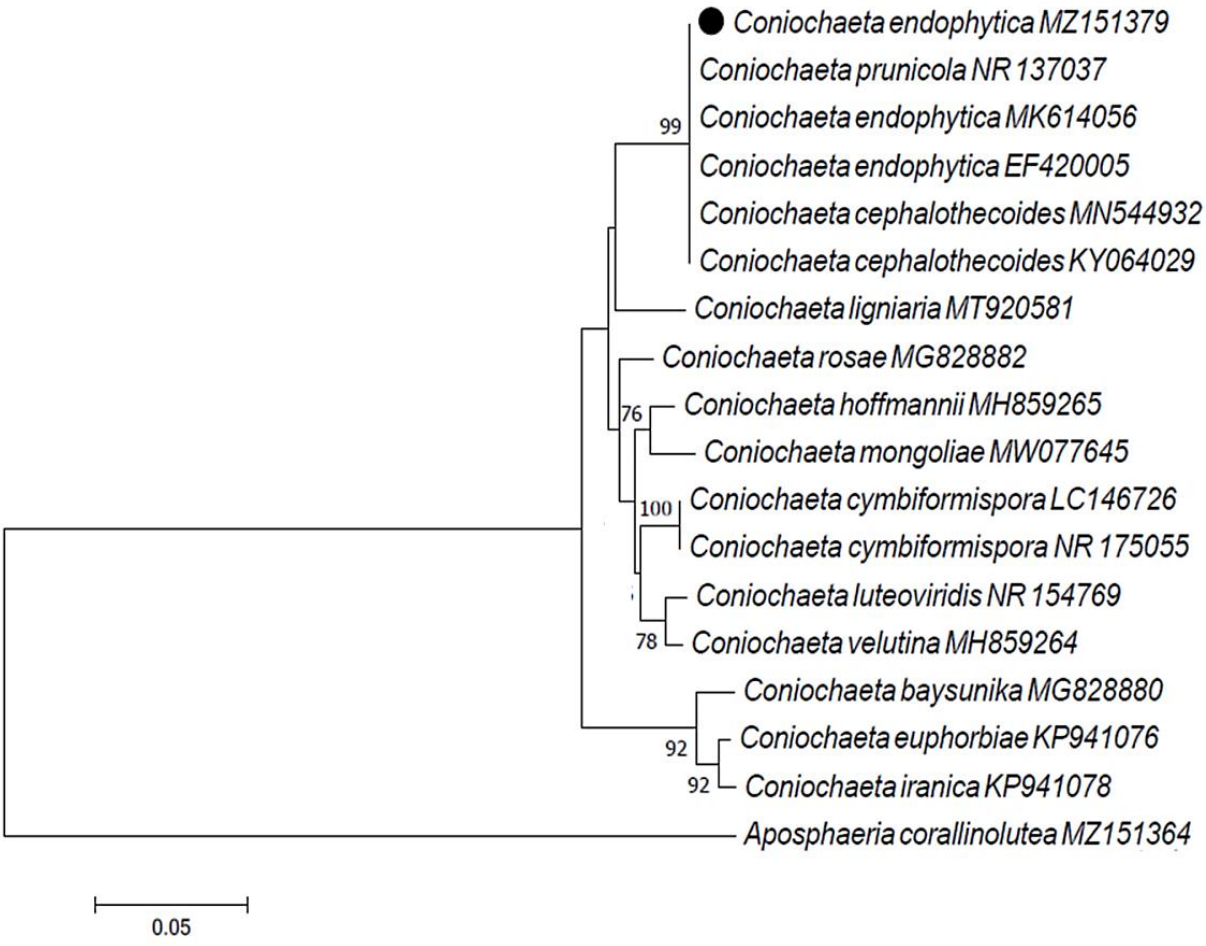

Fig. 1. Maximum Likelihood (ML) tree based on aligned sequences of ITS rDNA region of 18 isolates generated in MEGA 7. The tree was rooted to *Aposphaeria corallinolutea* (IRAN 4318C). Bootstrap values (1000 replicates) indicated at the nodes. The scale bar indicates nucleotide substitution in ML analysis, values ≥50 % are shown above/below the branches. The surveyed isolates in the current study are indicated in bold.

Table 1. Genbank accession numbers of the sequences used in the phylogenetic analysis.

Species	Culture accession number (s)	Source	Origin	GeneBank accession numbers	
				ITS	*gapdh*
Coniochaeta baysunika	MFLUCC 17-0830	*Rosa* sp.	Uzbekistan	MG828880	
Coniochaeta cymbiformispora	NBRC 32199	swamp soil	Japan	LC146726	
	Type	swamp soil	Japan	NR_175055	
Coniochaeta cephalothecoides	L821	*Trametes cinnabarina*	China	KY064029	
	TPYD-10	-	China	MN544932	
Coniochaeta endophytica	**IRAN 4366C**	***Malus domestica***	**Iran**	**MZ151379**	
	AEA 9094	*Platycladus orientalis*	USA	EF420005	
	AEA 9055	*Platycladus orientalis*	USA	MK614056	
Coniochaeta euphorbiae	1001	-	Iran	KP941076	
Coniochaeta hoffmannii	CBS 997.68	-	Austria	MH859265	
Coniochaeta iranica	0806	*Euphorbia polycaulis*	Iran	KP941078	
Coniochaeta ligniaria	TP131	*Cremastra appendiculata*	China	MT920581	
Coniochaeta luteoviridis	CBS 206.38	butter	Spain	NR_154769	
Coniochaeta mongoliae	CS-09	-	China	MW077645	
Coniochaeta prunicola	CBS 120875	*Prunus armeniaca*	South Africa	NR_137037	
Coniochaeta rosae	MFLUCC 17-0806	*Rosa* sp.	Uzbekistan	MG828882	
Coniochaeta velutina	CBS 981.68	-	USA	MH859264	
Aposphaeria corallinolutea	IRAN 4381C	*Malus domestica*	Iran	MZ151364	
Curvularia buchloes	CBS 246.49	*Buchloe dactyloides*	USA		KM061789
Curvularia ellisii	CBS 193.62	culture from holotype	-		LT715811
	CBS 127083	*Dactyloctenium aegyptium*	Australia		MN688832
Curvularia muehlenbeckiae	UTHSC 08-2905	-	-		LT715807
Curvularia pisi	CBS 190.48	*Pisum sativum*	Canada		KY905690
Curvularia hominis	**IRAN 4400C**	***Malus orientalis***	**Iran**		**MZ339272**
	HNWB120	*Zea mays*	China		KX100868
	UTHSC 08-849	Clinical sample	-		HF565483
Curvularia lonarensis	type	-	India		KY007019
Curvularia mosaddeghii	IRAN 3131C	*Syzygium cumini*	Iran		MH392155
Curvularia platzii	BRIP27703b	*Cenchrus clandestinus*	Australia		MH433651
Curvularia rouhanii	CBS 144674	*Syngonium vellozianum*	Iran		MG428694
	CBS 144675	*Eucalyptus* sp.	Iran		MG428696
Curvularia spicifera	IRAN 4370C	*Malus domestica*	Iran		MZ339270
	IRAN 4371C	*Malus domestica*	Iran		MZ339271
Curvularia tribuli	CBS 126975	*Tribulus terrestris*	South Africa		MN688852
Curvularia variabilis	CPC 28813	*Digitaria ciliaris*	Thailand		MF490842
	CPC 28816	*Imperata cylindrica*	Thailand		MF490845
Alternaria tenuissima	IRAN 2428C	Quince	Iran		MN160228

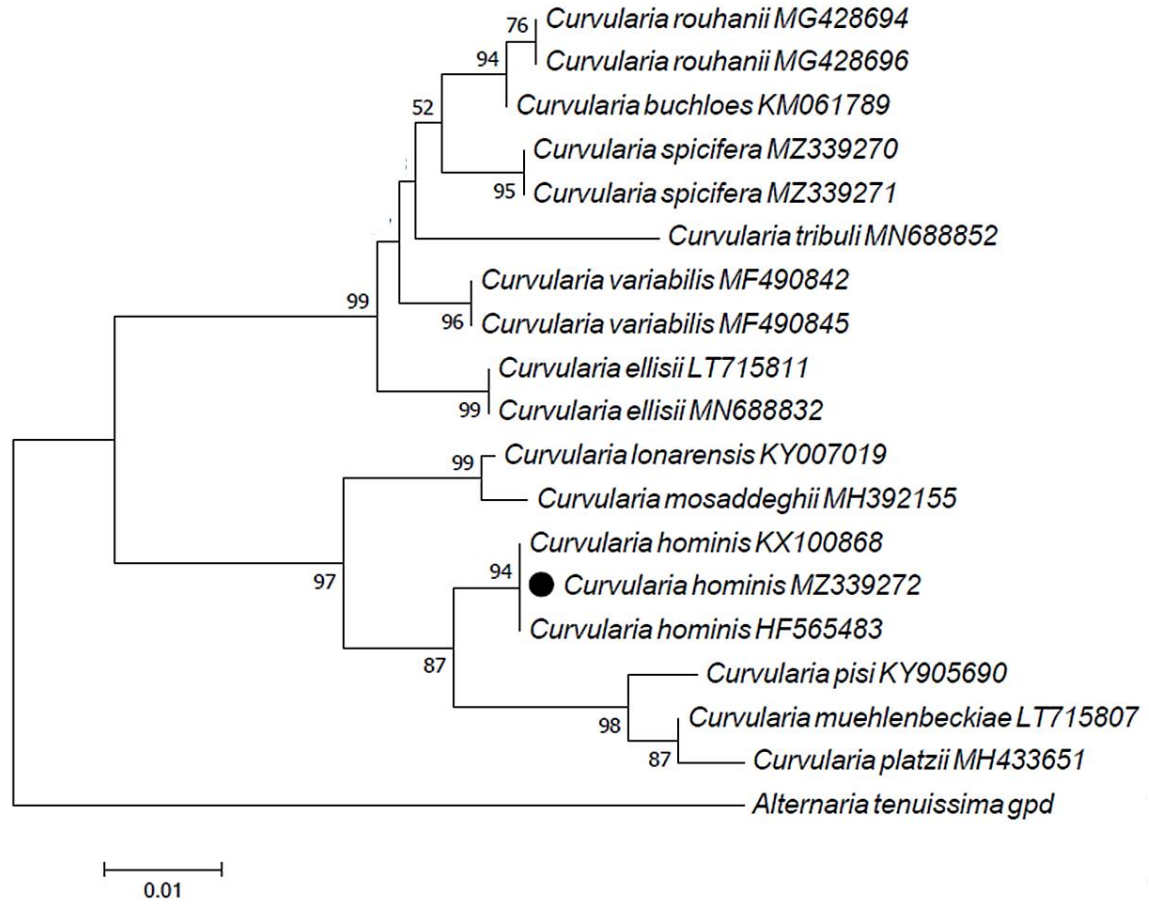

Fig. 2. Maximum Likelihood (ML) tree based on aligned sequences of *gapdh* gene of 19 isolates generated in MEGA 7. The tree was rooted to *Alternaria tenuissima* (IRAN 2428C). Bootstrap values (1000 replicates) indicated at the nodes. The scale bar indicates nucleotide substitution in ML analysis, values ≥50 % are shown above/below the branches.

Taxonomy

In this study, two species including *Co. endophytica*, and *Cu. hominis* were identified and described based on both morphological criteria and molecular data.

Coniochaeta endophytica A.H. Harrington & A.E. Arnold, in Harrington, del Olmo-Ruiz, U'Ren, Garcia, Pignatta, Wespe, Sandberg, Huang, Hoffman & Arnold, Plant and Fungal Systematics 64 (1): 65. 2019. Fig. 3.

Colonies on PDA orange with white margin from above and below, reaching 41 mm diam after 2 weeks at 12 h light / 12 h dark condition and 25 °C; on MEA white with regular margins, reaching 45 mm diam after 2 weeks at 12 h light / 12 h dark condition and 25 °C. Vegetative hyphae hyaline, 1–3 µm wide, lacking chlamydospores. Conidiophores undifferentiated from vegetative hyphae, reduced to condidoigenus cell. Conidiogenous cells phialidic (mono), hyaline and ampulliform, 5–10 × 2–3 µm. Conidia aggregated in heads, hyaline, single-celled, and ellipsoid to fusiform or allantoid (at the apex), 3–5 ($\overline{x}$ = 4.2) × 1–2 µm (Fig. 3). No perithecia formed on autoclaved pine needles after three months.

Specimen examined: IRAN, Guilan province, Paresar, Dinachal, recovered from branch of *Malus domestica*, 19 August. 2019, L. Ebrahimi, culture IRAN 4366C.

Notes: Morphological characteristics of the investigated isolate are similar to the description of *Co. endophytica* provided by Harrington et al. (2019). Our isolate (GenBank accession No. MZ151379) showed 99 % similarity to other isolates of this species in GenBank (EF420005) in BLAST search and grouped with *Co. cephalothecoides* and *Co. prunicola* in the same clade. However, *Co. endophytica* can be differentiated from other species based on morphological features; conidia in *Co. endophytica* are typically more linear and less curved than those of *Co. prunicola* (Damm et al. 2010) and occasionally more

spherical or ovoid, which is not recorded for *C. prunicola*.

In our survey, no mature perithecia formed on autoclaved pine needles, but both *Co. cephalothecoides* and *Co. prunicola* form perithecia based on the Kamiya et al. (1995) and Harrington et al. (2019) studies. Several species of *Coniochaeta* have been reported as endophytes i.e. *Co. ligniaria* (Grev.) Cooke on *Baeckea frutescens* L. (Kokaew et al. 2011), *Co. velutina* (Fuckel) Cooke on *Tsuga heterophylla* (Raf.) Sarg. (Xie et al. 2015), *Co. endophytica* on *Platycladus orientalis* (L.) Franco (Harrington et al. 2019), and *Co. tritici* M. Mehrabi, Asgari & Zare on stem of *Triticum aestivum* in Iran (Mehrabi et al. 2022). This is the first record of *Co. endophytica* as apple endophytic fungus in the world and new record for the Funga of Iran.

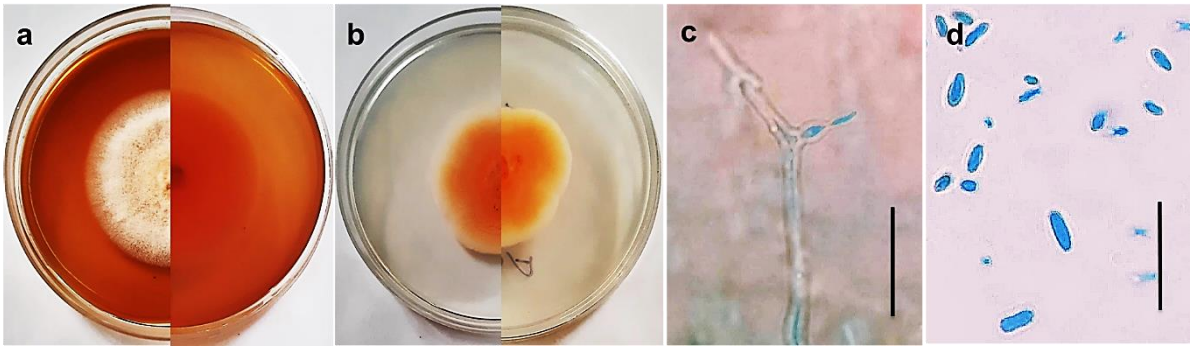

Fig. 3. *Coniochaeta endophytica*: a. Colony on MEA and, b. PDA after 2 weeks at 12 h light / 12 h dark condition and 25 °C; c. conidiogenous cell; d. conidia. Scale bars = 10 µm

Curvularia hominis K.C. Cunha, Madrid, Gené & Cano, in Madrid, da Cunha, Gené, Cano, Sutton, Guarro & Crous, Persoonia 33: 55. 2014. Fig. 4.

Colony on PDA attaining 65 mm diam after 7 days at continuous dark condition and 25 °C, funiculose and dark green at the center, floccose and olive towards the periphery, with a fimbriate margin; reverse black at center and olive to brown towards the margin (Fig. 4a). Vegetative hyphae septate, subhyaline to pale brown, branched, smooth to slightly asperulate, 2–5 µm in wide. Conidiophores semi- to macronematous, mononematous, septate, simple or branched, geniculate towards the apex, subhyaline to dark brown, smooth to asperulate, with cell walls often thicker than those of the vegetative hyphae, 40–210 ($\overline{x}$ = 149.4) × 3–5 ($\overline{x}$ = 4.1) µm. Conidiogenous cells terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to irregularly shape. Conidia 4-celled, slightly curved, 19–32 ($\overline{x}$ = 23.8) × 6–15 ($\overline{x}$ = 10.7) µm in the broadest part, with the third cell from the base often larger and unequal sided; verruculose and darker than the others, brown, end cells subhyaline to pale brown and smooth-walled; hilum non-protruding, flat, darkened and thickened, 2 µm wide (Fig. 4 b-c). Microconidiation and chlamydospores were not observed.

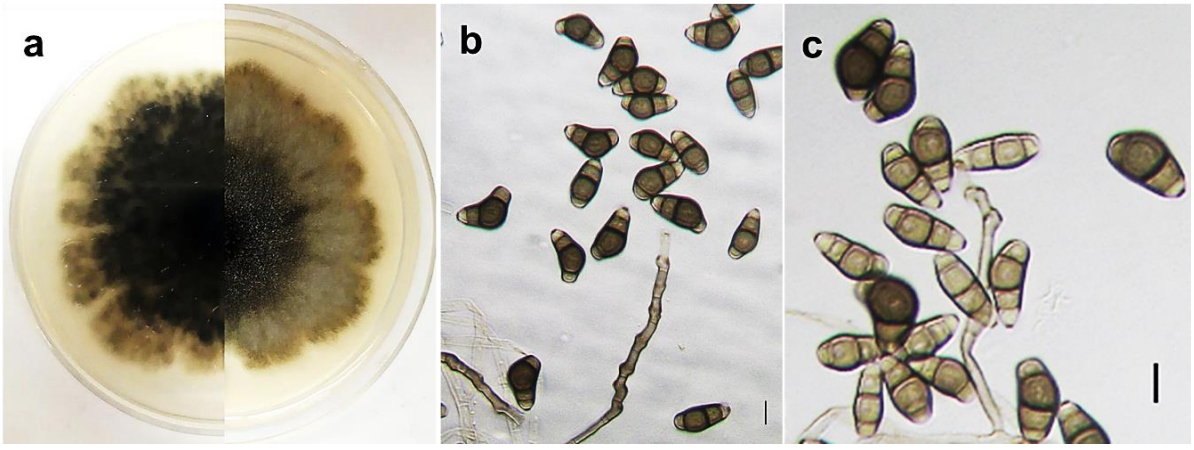

Fig. 4. *Curvularia hominis*: a. Colony on PDA after 7 days at continuous dark condition and 25 ºC; b-c. Conidiophores and conidia. Scale bars = 10 µm.

Specimen examined: IRAN, Guilan province, Mehrbon, recovered from leaf of *Malus orientalis*, 17 August. 2019, L. Ebrahimi, culture IRAN 4400C.

Notes: Morphological features of the isolate IRAN 4400C were according to the description of *Cu. hominis* provided by Madrid et al. (2014). The examined isolate (GenBank accission No. MZ339272) showed 100 % similarity to other isolates of *Cu. hominis* in GenBank and in ML tree placed with other isolates of *Cu. hominis* from GenBank in the same clade (Fig. 2). This species belongs to *Pleosporaceae* family of *Pleosporales*, taxonomically resembling other species of the genus with 4-celled conidia and an asymmetrically swollen, dark third cell, such as *Cu. aeria* (Bat., J.A. Lima & C.T. Vasconc.) Tsuda, *Cu. lunata* (Wakker) Boedijn and *Cu. prasadii* R.L. Mathur & B.L. Mathur, but differs from them in producing conidia with verruculose intermediate cells (Fig. 4 b-c) (Madrid et al. 2014). Madrid et al. described this species from clinical samples from the USA in 2014. Also, it was isolated from the leaf of *Acmella ciliate* (Kunth) Cass. as an endophytic fungus in Brazil (Ortiz-Ojeda et al. 2020). This research is the first isolation of *Cu. hominis* as endophytic fungus of apple in the world and new record for the Funga of Iran.

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گزارش جدید از قارچ‌های اندوفیت سیب برای فونگای ایران

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چکیده: قارچ‌های اندوفیت میکروارگانیسم‌هایی هستند که در کل یا بخشی از چرخه زندگی خود، گیاهان را بدون ایجاد هیچگونه علائمی کلونیزه می‌کنند. این قارچ‌ها در هر گونه گیاهی که تا کنون مورد بررسی قرار گرفته یافت شده‌اند. در این پژوهش، ۴۱۷ جدایه قارچ اندوفیت از نمونه‌های سالم و بدون علائم میوه، برگ و شاخه ۷۰ درخت بومی (*Malus domestica*) و وحشی (*Malus orientais*) سیب مورد بررسی در شمال ایران به دست آمد. در این مطالعه، از بین قارچ‌های شناسایی شده، گونه‌های *Coniochaeta endophytica* و *Curvularia hominis* که بر اساس ویژگی‌های ریخت‌شناختی و اطلاعات مولکولی برای فونگای ایران جدید بودند ارائه می‌شوند. همچنین، این گونه‌ها برای اولین بار به عنوان قارچ‌های اندوفیت درختان سیب در دنیا معرفی می‌شوند.

کلمات کلیدی: ارقام سیب بومی ایران، فیلوژنی، همزیستی، رده‌بندی، سیب وحشی

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