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Research Article

New records of Eucalyptus endophytic fungi for the funga of Iran

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Abstract: Fungal endophytes are defined as microorganisms with the ability to colonize plants asymptomatically throughout or at least a significant part of their life cycle, thereby establishing a plant-fungal association. In the present study, 44 plant samples, including healthy and symptomless fruit, leaf and branch samples, were collected from Eucalyptus trees located in Tehran, Qom, Alborz, Esfahan, and Mazandaran provinces to isolate and identify the endophytic fungi. Among 170 fungal isolates from collected plant samples, two species were new based on morphological and molecular phylogeny of the ITS rDNA for the Funga of Iran, including *Phaeophleospora eucalypticola* and *Pseudosydowia eucalypti*. Furthermore, both species are reported for the first time as endophytic fungi of eucalyptus trees in the world.

Keywords: *Eucalyptus camaldulensis*, phylogeny, species, symbiosis, taxonomy

Introduction

The genus *Eucalyptus* (Myrtaceae), indigenous to the Australasian region, comprising more than 700 species, is cultivated worldwide due to its medicine, oil, gum, pulp, timber, paper, charcoal, etc. (Batish *et al.* 2008, Labate *et al.* 2009). The fast growth rate, large biomass production, and also the ability to grow in a wide range of environments and soils are some of several factors contributed to planting Eucalyptus in other regions (Labate *et al.* 2009). Eucalyptus was introduced to Iran more than a hundred years ago. Accordingly, 18 species such as *Eucalyptus camaldulensis* Dehnh, *Eucalyptus nitens* (Deane and Maiden) Maiden, *Eucalyptus striaticalyx* W. Fitzg. sens. lat., *Eucalyptus loxophleba* Benth., *Eucalyptus ovata* (Labill.), *Eucalyptus saligna* Smith, *Eucalyptus occidentalis* Endl., and *Eucalyptus intertexta* RT Baker, were imported from Australia (Haj Agha Mohammadi, 2003).

Fungal endophytes are defined as microorganisms with the ability to colonize the internal tissues of host plants without causing symptoms or signs of harm to the host (Anjum *et al.,* 2019). During long-term evolution, complicated interactions have been gradually formed between endophytes and plants, leading to symbiosis between them (Hassani *et al.* 2018). These microorganisms may not remain endophytes for their entire life cycle; thus, the definition of endophytes not only includes symbiotic species but also saprophytes and latent pathogens (Porras-Alfaro and Bayman 2011). Every plant harbors at least one

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endophytic fungal species, and many plants, especially woody plants, may contain hundreds or even thousands of different species (O'Hanlon *et al.,* 2012). Fungal endophytes have been isolated from a multitude of different plants, including trees, vegetables, fruits, cereal grains, and other crops. They primarily consist of the members of the Ascomycota and some taxa of the Basidiomycota, Zygomycota, and Oomycota (Mosaddeghi *et al.* 2021). In Fisher *et al.* (1993) study on eucalyptus leaves and branches endophytic fungi, *Botryosphaeria dothidea* (Moug.) Ces. and De Not., *Cytospora eucalypticola* Van der Westh. were the most frequent species. Lupo *et al.* (2001) isolated species *Cytospora chrysosperma* (Pers.) Fr., *Fusicoccum eucalypti* Sousa da Câmara, *Alternaria alternata* (Fr.) Keissl., *Fairmaniella leprosa* (Fairm.) Petr. and Syd., *Aureobasidium pullulans* (de Bary and Löwenthal) G. Arnaud, and *Cladosporium cladosporioides* (Fresen.) G. A. de Vries as endophytes from flowers, capsules and seeds of *E. globulus* Labill. Lacerda *et al.* (2018) studied the composition of fungal endophyte communities of *E. microcorys*. They reported *Castanediella eucalypticola* Crous and M. J. Wingf., and *Neophaeomoniella eucalypti* Roon.-Lath. and Crous, for the first time from Brazil. In an investigation of *E. globulus* twigs endophytes, 127 fungal isolates were obtained, and fungi *Pringsheimia smilacis* E. Müll., *Lophiostoma corticola* (Fuckel) E. C. Y. Liew, Aptroot and K. D. Hyde, *Hormonema* sp., *Neofusicoccum luteum* (Pennycook and Samuels) Crous, Slippers and A. J. L. Phillips, *Phaeomoniella effuse* Damm and Crous, *Ulocladium* sp. identified as laccase positive strains (Fillat *et al.* 2016). In another study, 80 endophytic fungal isolates were obtained from *E. exserta*, and 13 genera, namely, *Penicillium* Link, *Chaetomium* Kunze, *Cladosporium* Link, *Phyllosticta* Pers., *Eutypella* (Nitschke) Sacc., *Purpureocillium* Luangsa-ard, Hywel-Jones, Houbraken and Samson, *Gongronella* Ribaldi, *Talaromyces* C. R. Benj., *Pestalotiopsis* Steyaert, *Fusarium* Link, *Lophiostoma* Ces. and De Not., *Scedosporium* Sacc. ex Castell. and Chalm., and *Pseudallescheria* Negr. and I. Fisch. were identified (Mao *et al.* 2021).

Despite many studies that have been conducted on endophytic fungi of different plants, there has been no investigation on the endophytic fungi of eucalyptus trees in Iran, and there is no information about the presence or biodiversity of these fungi in this country. Therefore, the present study intended to isolate and identify *Eucalyptus camaldulensis* Dehnh. fungal endophytes in some of Iran's provinces.

Materials and Methods

Sample collection and endophytic fungi isolation

Plant samples, including healthy and symptomless leaves, 1- or 2-year-old branches, and fruits of eucalyptus trees (*E. camaldulensis*), were randomly collected in autumn from Tehran, Alborz, Qom, Isfahan, and Mazandaran provinces in Iran. To remove dust and debris, plant samples were washed thoroughly in running tap water for 10 minutes and surface sterilized using the method modified by Strobel and Daisy (2003). Cleaned samples were surface disinfected with 70% ethanol for about 45 s for fruits and leaves and 90 s for twigs, then 2% sodium hypochlorite for 30 s, and 70% ethanol for 15 s, and then rinsed with sterile water. The outer epidermal tissues and cuticle of the twigs were removed with a sterile scalpel. At the end, disinfected plant materials were cut under sterile conditions into small pieces (1 × 1 cm for leaves, and 1 cm length for twigs), and then placed on water agar (WA) medium, and kept in dark condition at 25 °C for one to four weeks. The hyphal tips of endophytic fungi growing from the plant tissue were transferred into potato dextrose agar (PDA). All isolates were stored on PDA slants at 4 °C. Identified isolates were deposited in the Fungal Culture Collection of the Agricultural Biotechnology Research Institute of Iran (ABRIICC), Alborz, Iran.

Morphological characterization

Colony shape and characters, colors, growth rate, and morphological characteristics of sexual and asexual reproductive structures (in case of

formation) were evaluated on PDA, MEA, OA, and PNA (due to the fungal genus) in the continuous dark condition at 25 °C for 7 days or 4 weeks (due to the fungal genus) (Crous *et al*. 2016, Thambugala *et al*. 2014). Microscopic slides of fungal endophytes were prepared in lacto-phenol or lacto-phenol cotton blue solutions. Measurement (n = 50) and microphotographs of fungal features were taken from microscopic slides using an ERMA microscope (ERMA, Japan).

Molecular analysis

Fungal isolates were grown on PDA medium for 10 days in continuous dark condition at 25 °C. Fresh mycelia were collected, and DNA extraction was done using CTAB DNA extraction protocol provided by Kärf (2009). Extracted DNA was diluted in 25 µL distilled water and kept at -20 °C for future use.

For molecular identification, Internal Transcribed Spacer (ITS)-rDNA region of fungal DNA was multiplied by using ITS1/ITS4 primer pairs (White *et al.* 1990). PCR conditions and the reaction mixture were the same as Ebrahimi and Fotouhifar (2016) description. The amplified genes were sequenced in one direction with ITS1 primers by Noor Genetics Center, Tehran, Iran.

Phylogenetic analysis

The obtained sequences were manually edited using Geneious Prime ver. 2019. 2.1 (Cooper and Walker, 2022). ITS sequences have been deposited in GenBank (NCBI). In order to achieve phylogenetic analyses, the homologous reference sequences of related species were received from GenBank (Table 1), and aligned with ITS rDNA sequences of our isolates using ClustalW (Thompson *et al.* 1994). MEGA ver. 10.2 (Takeuchi *et al.* 2018) was used to perform the maximum likelihood (ML) analysis (Felsenstein 1981). MEGA suggested K2 + G Model as the best nucleotide substitution model for ITS data. By the use of 1000 replicates of ML bootstrap analysis, the confidence of each clade was evaluated (Felsenstein 1985).

Results

In the present study, a report of new fungal endophytes associated with eucalyptus trees in Iran has been described. One hundred seventy fungal isolates were obtained from 44 plant samples (including leaves, branches, and fruits), two of them including *Phaeophleospora eucalypticola* Crous and M. J. Wingf., and *Pseudosydowia eucalypti* (Verwoerd and du Plessis) Thambug. and K. D. Hyde is described as a new species for the Funga of Iran in this study. Additionally, both species are reported for the first time as endophytic fungi of eucalyptus trees in the world.

Phylogeny

The phylogenetic analyses of *Phaeophleospora* and *Pseudosydowia* species were performed using ITS rDNA nucleotide sequences of 26 isolates, including one isolate of each obtained in this study and 23 isolates from GenBank (including the out-group) (Table 1). DNA sequences analysis indicates that our examined isolates *Phaeophleospora* XL4 and *Pseudosydowia* VL3 are each placed in the same clade with *Ph. eucalypticola* and *Ps. eucalypti,* respectively (Fig. 1).

Taxonomy

In this study, two species, including *Ps. eucalypti*, and *Ph. eucalypticola* were investigated and analyzed based on both morphological basis and molecular data.

Pseudosydowia eucalypti (Verwoerd & du Plessis) Thambug. & K. D. Hyde, Fungal Diversity 68: 140 (2014) (Fig. 2).

Specimen examined: IRAN, Mazandaran province, Babol, recovered from leaves of *Eucalyptus camaldulensis*, 27 October. 2022, P. Aleahmad, Culture ABRIICC 10385.

Colonies flat, spreading, with sparse aerial mycelium and smooth lobate margin, reaching 20 mm, 75 mm, 40 mm, and 22 mm on PDA, MEA, PNA, and OA, respectively, after 4 weeks at continuous dark conditions

and 25 °C. On PDA, the surface was pinkish-white with red streaks. In some parts, it had a velvety texture, and, in some parts, it had a pasty texture, sometimes umber, and sometimes greenish olivaceous. On MEA, surface and reverse umber have a slimy texture in some areas and a velvety texture in others. On PNA, the surface and reverse are pale brown. On OA, it was pink and had a slimy texture. On MEA, conidiophores reduced to conidiogenous cells, hyaline to pale brown, smooth, thick-walled, short-cylindrical to narrowly ampulliform, slightly tapered toward the apex, with 7.9-19.6 µm width. On PDA, PNA, and OA, conidiophores, thick-walled, branched, sometimes sympodial, with 10.6-15.8 (13.7) µm width. Conidia, oval to ellipsoid, colorless and unicellular (MEA, PDA, OA, and PNA) and bicellular (MEA culture), 4.30-9.59 (6.86) × 3.11-5.7 (4.53) µm. The sexual state was not observed.

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

Species	Culture accession number	Source	Origin	GeneBank accession numbers	References
Phaeophleospora eucalypticola	ABRIICC 10384	leaves of *E. camaldulensis*	Iran	PP320330	Present study
Ph. eucalypticola	NTOU:4394	-	Taiwan	MK448260	Pang *et al.* (2019)
Ph. eucalypticola	CPC 26523	leaves of *Eucalyptus robusta*	Netherlands	KX228267	Crous *et al.* (2013)
Ph. eucalypticola	FKII_L3_CM_PAB3	-	USA	MT704916	Blachowicz *et al.* (2020)
Ph. hymenocallidis	CPC 25018	leaves of epiphyte	Thailand	KR476740	Crous *et al.* (2015)
Ph. eugeniae	CPC 15143	*Eugenia uniflora*	Brazil	FJ493188	Crous *et al.* (2009)
Ph. eugeniae	CPC 15159	*Eugenia uniflora*	Brazil	FJ493189	Crous *et al.* (2008)
Ph. pteridivora	COAD:1182	*Serpocaulon triseriale*	Brazil	KT037547	Guatimosim *et al.* (2016)
Ph. pteridivora	CPC:24683	*Serpocaulon triseriale*	Brazil	NR_155664	Guatimosim *et al.* (2016)
Ph. eugeniicola	CPC 2557	-	Netherlands	FJ493190	Crous *et al.* (2009)
Ph. eugeniicola	CPC 2558	-	Netherlands	FJ493191	Crous *et al.* (2009)
Ph. scytalidii	CPC 10988	*Eucalyptus* sp.	Colombia	DQ303015	Crous *et al.* (2006)
Ph.scytalidii	CPC 10998	*Eucalyptus* sp.	Colombia	DQ303016	Crous *et al.* (2006)
Ph. concentrica	CPC 3615	-	Kenya	FJ493187	Crous *et al.* (2009)
Ph. epicoccoides	MUCC430	*Eucalyptus grandis*	Indonesia	DQ632708	Andjic *et al.* (2007)
Ph. epicoccoide	CMW22483	*Eucalyptus grandis*	Indonesia	DQ632709	Andjic *et al.* (2007)
Pseudosydowia eucalypti	ABRIICC 10385	leaves of *E. camaldulensis*	Iran	PP320331	Present study
Ps. phantasmae	CPC 38883	*Moringa ovalifolia*	Namibia	MW175364	Crous *et al.* (2020)
Ps. phantasmae	CBS 146830	*Moringa ovalifolia*	Namibia	NR_171999	Crous *et al.* (2020)
Ps. phantasmae	CBS:146982	*Moringa ovalifolia*	Namibia	ON811504	Crous *et al.* (2022)
Ps.eucalyptorum	CBS 145546	*Eucalyptus* sp.	Australia	NR_165231	Crous *et al.* (2020)
Ps.eucalyptorum	CBS:145546	*Eucalyptus* sp.	Australia	MK876406	Crous *et al.* (2019)
Ps. eucalypti	CPC:14028	*Eucalyptus* sp.	Australia	GQ303296	Cheewangkoon *et al.* (2009)
Ps.eucalypti	CPC:14927	*Eucalyptus* sp.	Portugal	GQ303297	Cheewangkoon *et al.* (2009)
Gloeophyllum sepiarium	Wilcox-3BB	-	USA	NR_119869	Schoch *et al.* (2014)

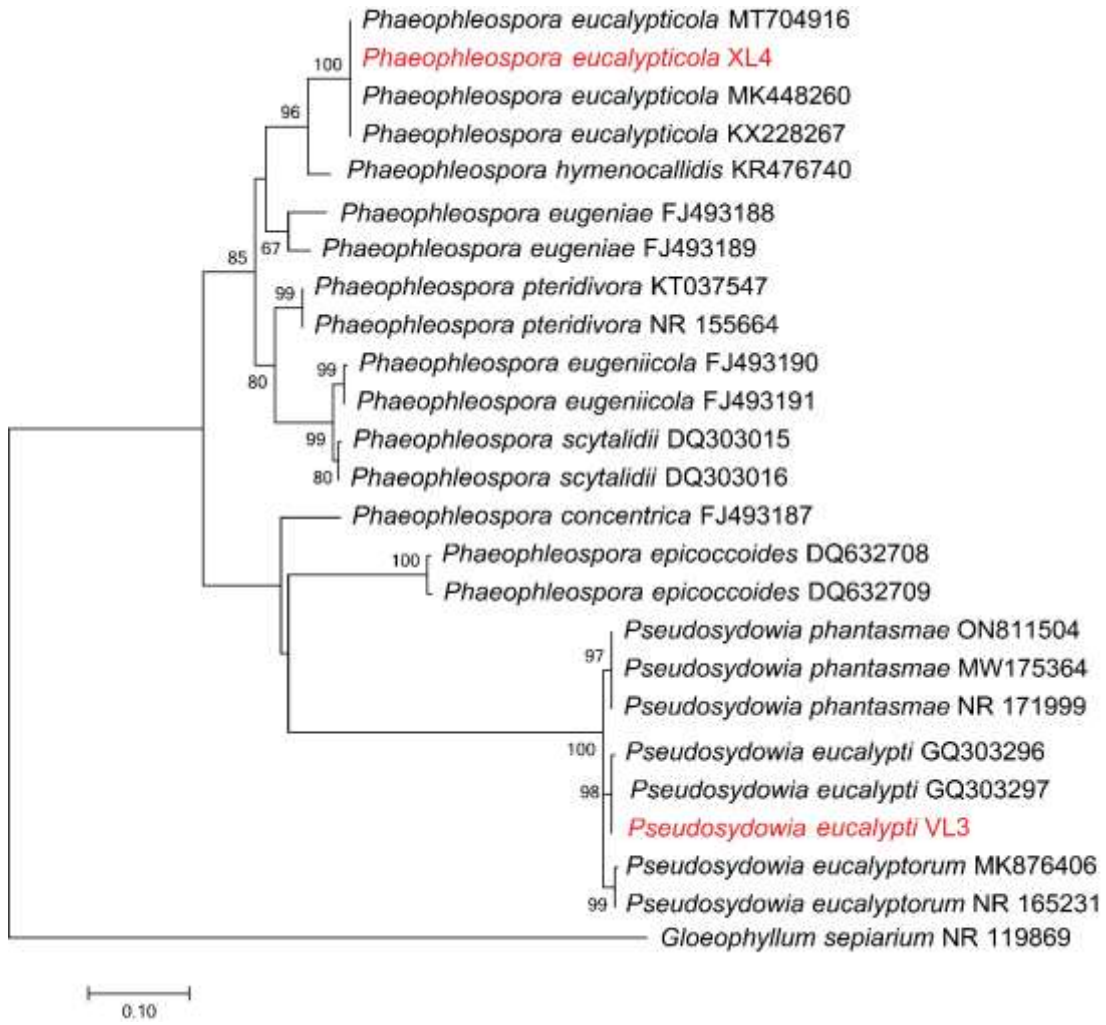

Figure 1 Maximum Likelihood (ML) tree based on aligned sequences of ITS rDNA region of 26 isolates generated in MEGA 10. The tree was rooted to *Gloeophyllum sepiarium*. Bootstrap values (1000 replicates) are indicated at the nodes, values ≥50 % are shown above/below the branches. The scale bar indicates nucleotide substitution in ML analysis. The surveyed isolates in the current study are indicated in red.

Phaeophleospora eucalypticola Crous & M. J. Wingf., Persoonia 36: 351 (2016) Crous and M. J. Wingf., Crous et al., Persoonia 36: 351 (2016). (Fig. 3).

Specimen examined: IRAN, Mazandaran province, Mahmudabad, recovered from leaves of *Eucalyptus camaldulensis*, 28 October. 2022, P. Aleahmad, Culture ABRIICC 10384.

Colonies reaching up to 20 mm and 30 mm diam after two weeks at continuous dark condition and 25 °C on PDA and MEA, respectively, with spreading, erumpent surface; margins smooth, lobate, and moderate aerial mycelium. On PDA and MEA, surface and reverse mouse-grey. Conidiophores, hyaline, smooth, reduced to conidiogenous cells, or with a supporting cell, branched at the base or not, ampulliform to subcylindrical, 5–10 × 2.5–3.5 µm. Pycnidia spherical and brown, in single or multiple groups, 220.6–240.5 × 250–296 µm, the conidial mass was abundantly oozed out of the pycnidia in pink drops, conidia unicellular, with smooth surface, ellipsoid, 4.5–6 × 2(–2.5) µm; abundance crystals were produced on PDA (Fig. 3).

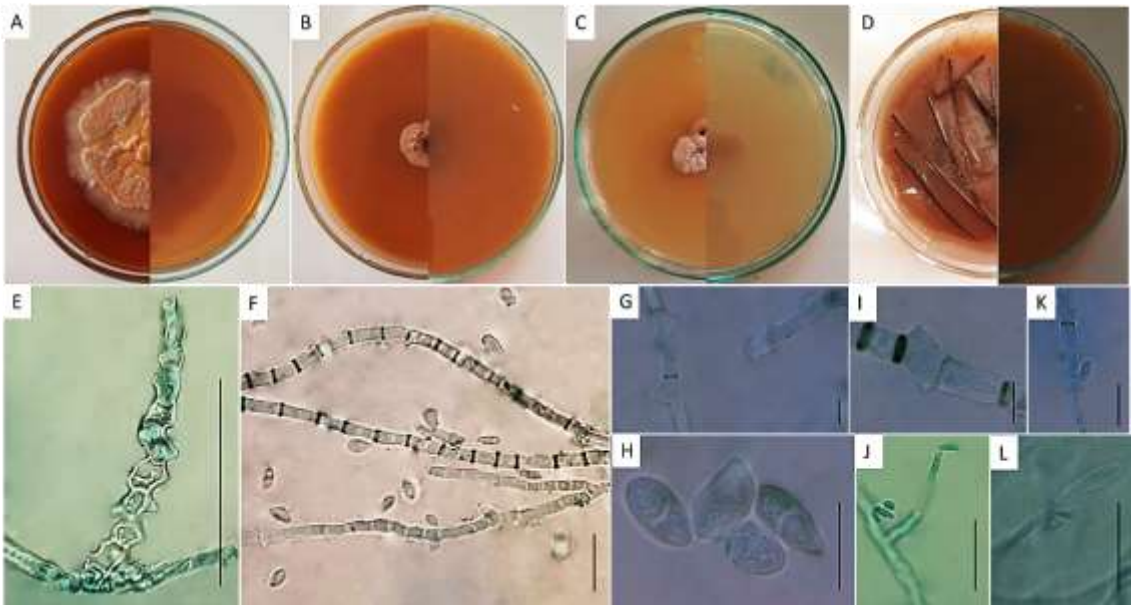

Figure 2 *Pseudosydowia eucalypti*, isolate VL3. **A)** Colonies on MEA, **B)** OA, **C)** PDA, and **D)** PNA, after four weeks at 25 °C and continuous darkness; **E)** Conidiophore; **F)** Conidia and conidiogenous cell; **G-I)** Conidiogenous cell; **K-J-L)** Conidia and conidiophores; **H)** Unicellular and bicellular conidia; Scale bars = 10 µm.

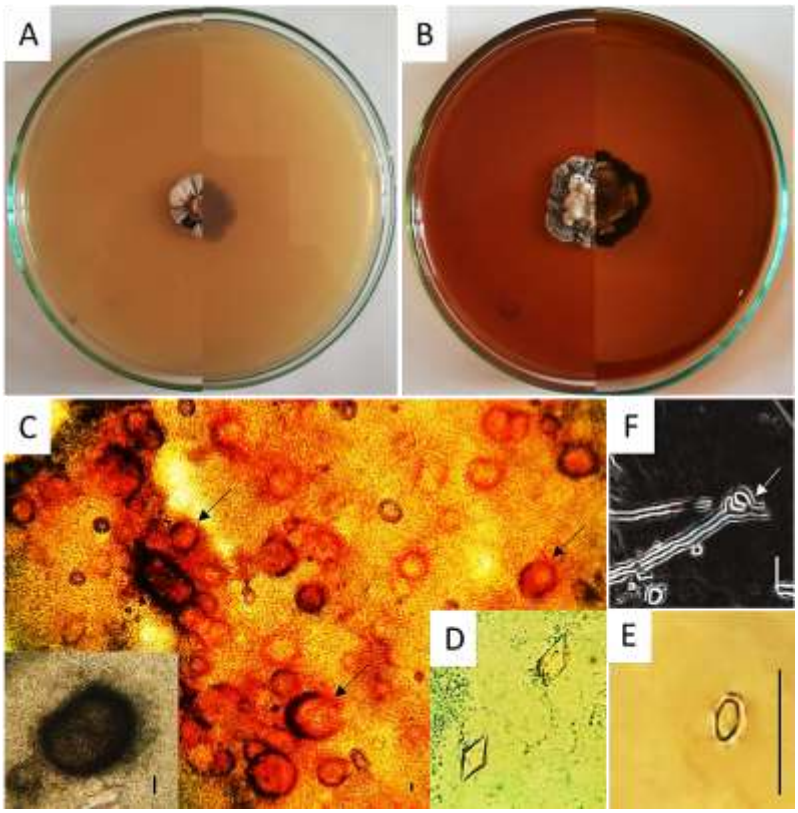

Figure 3 *Phaeophleospora eucalypticola*, isolate XL4. **A)** Colonies on PDA, and **B** MEA, after two weeks at 25 °C and continuous darkness; **C)** Pycnidia; **D)** Crystals; **E)** Conidium; **F)** conidiophore; Scale bars = 10 µm.

Discussion

Morphological characteristics of *Ps. eucalypti* VL3 are similar to the description of *Ps. eucalypti* by Crous *et al.* (2013). Our isolate (GenBank accession No. PP320331) showed 99.28% similarity to other isolates of this species in GenBank (GQ303296) in BLAST search and was grouped with *Ps. eucalypti* in the same clade. Moreover, our isolate showed 97.12% identity to *Ps. eucalyptorum,* and 89% similarity to *Ps. phantasmae*. The species *Ps. eucalypti*, which has not been extensively studied, was first introduced in 1931 as *Sphaerulina eucalypti*, causing eucalyptus leaf spots in Africa (Verwoerd, 1931). In research conducted to determine the fungal causes of eucalyptus tree dieback in California, this fungus was reported as the most frequent pathogen of eucalyptus plants with symptoms (Garbelotto *et al.* 2021). In a study, *Ps. eucalypti* were isolated with several other species from soil collected in a peat swamp forest (PSF) area in eastern Thailand (Khunnamwong *et al.* 2020). This is the first record of *Ps. eucalypti* as eucalyptus endophytic fungus in the world and a new record for the Funga of Iran.

The morphological features of *Ph. eucalypticola* XL4 were according to the description of *Ph. eucalypticola* provided by Crous *et al.* (2016). The examined isolate (GenBank accession No. PP320330) showed 100% similarity to other isolates of *Ph. eucalypticola* in GenBank (MK448260). In ML tree placed with different isolates of *Ph. eucalypticola* from GenBank in the same clade (Fig. 1). Moreover, in NCBI BLAST search, our isolate exhibits 91.76% similarity to *Ph. hymenocallidicola* and 92.22% similarity to *Ph. eugeniae*. However, *Ph. eugeniae* is morphologically distinct, with subcylindrical, curved conidia measuring 70–107 × 2–3 µm (Guatimosim *et al.* 2016). The species *Ph. eucalypticola* was first reported as a pathogen of eucalyptus in Australia in 2016 (Crous *et al.* 2016). Also, in 2022, this fungus was reported as an endophyte of two pine species *Pinus thunbergii*, and *Chamaecyparis obtusa,* from South Korea (Choi *et al.* 2022). In the Kim *et al.* (2022) study, *Ph. eucalypticola* was isolated from polyethylene terephthalate (PET) waste on Korean seacoasts and showed the strongest PCL degradation ability among 262 evaluated strains. This is the first record of *Ph. eucalypticola* as eucalyptus endophytic fungus worldwide and a new record for the Funga of Iran.

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گزارش قارچ‌های اندوفیت جدید از درختان اکالیپتوس برای فونگای ایران

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چکیده: قارچ‌های اندوفیت میکروارگانیسم‌هایی هستند که می‌توانند در بخشی یا تمام چرخه زندگی خود بافت‌های گیاه میزبان را بدون بروز علائم آشکار به‌صورت داخلی کلنیزه و یک ارتباط هم‌زیستی ایجاد کنند. در تحقیق حاضر، به‌منظور جداسازی و شناسایی قارچ‌های اندوفیت اکالیپتوس، ۴۴ نمونه گیاهی شامل میوه، برگ و شاخه‌های سالم و بدون علائم درختان اکالیپتوس از استان‌های تهران، قم، البرز، اصفهان و مازندران در فصل پاییز جمع‌آوری شدند. در نهایت ۱۷۰ جدایه قارچی اندوفیت از نمونه‌های جمع‌آوری شده به‌دست آمد؛ که از این میان، براساس ویژگی‌های ریخت‌شناسی و اطلاعات مولکولی ناحیه ITS rDNA، دو گونه *Phaeophleospora eucalypticola* و *Pseudosydowia eucalypti* برای فونگای ایران جدید بودند. علاوه‌براین، هر دو گونه برای اولین‌بار به‌عنوان قارچ اندوفیت درختان اکالیپتوس در دنیا گزارش می‌شوند.

واژگان کلیدی: فیلوژنی، هم‌زیستی، تاکسونومی، گونه، *Eucalyptus camaldulensis*