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Pestalotiopsis biciliata, a new record for funga of Iran

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Abstract: In spring 2023, during a survey of Eucalyptus trees (*Eucalyptus camaldulensis* Dehn.) in Jiroft, Kerman Province, Iran, leaves with leaf spot symptoms were collected. Fungal colonies with similar growth patterns were isolated from symptomatic leaves. The recovered isolates were identified based on the combination of morphological features and sequence data of the ITS-rDNA and part of the translation elongation factor 1-alpha (*tef-1*α) gene as *Pestalotiopsis biciliata*. Pathogenicity tests were carried out by using detached leaves and seedlings of Eucalyptus, fulfilling the Koch's postulates. To our knowledge, this is the first report of *P. biciliata* for the Iranian Funga and also the first report of the pathogenicity of this species on *E. camaldulensis* in Iran.

Keywords: Foliar disease, pathogenicity, *Pestalotiopsis*, phylogeny, *Pestalotiopsidaceae*.

INTRODUCTION

Eucalyptus (*Eucalyptus camaldulensis* Dehn) trees, although not native to Iran, have been introduced and cultivated in specific regions, holding significance for various reasons. Their rapid growth and adaptability make them valuable for afforestation efforts, combating soil erosion and desertification. Eucalyptus wood is prized for its versatility, contributing to the construction, furniture, and paper industries, thereby supporting the local timber sector sustainably. Additionally, Eucalyptus trees serve as a source of essential oils with medicinal properties, playing a role in pharmaceuticals, cosmetics, and aromatherapy (Dale et al. 2013). Managed properly, Eucalyptus plantations in Iran offer benefits such as biodiversity support and habitat creation for wildlife, while their aesthetic appeal enhances landscapes and provides recreational spaces in urban areas (Pairo et al. 2020). It is emphasized that the introduction of non-native species necessitates careful management to prevent adverse ecological impacts, requiring oversight from local authorities and environmental organizations.

The genus *Pestalotiopsis* Steyaert, belonging to the *Pestalotiopsidaceae* (*Amphisphaeriales*, *Ascomycota*) was first described in 1949 (Maharachchikumbura et al. 2014). The genus demonstrates a wide range of host plants and diverse lifestyles, including endophytic, plant pathogenic, and saprobic forms. The classification of the genus *Pestalotiopsis* has undergone numerous revisions, incorporating both morphological and molecular data. Initially, *Pestalotiopsis* was classified based on the cell count in its conidia, resulting in the establishment of three genera: *Pestalotia* (six-celled conidia), *Pestalotiopsis* (five-celled conidia), and *Truncatella* (four-celled conidia) (Guba, 1956, 1961). Molecular phylogenetic analysis has played a crucial role in refining the classification of *Pestalotiopsis*, revealing the organization of its species into distinct clades based on their DNA sequences. Recently, *Pestalotiopsis* is placed within the family *Sporocadaceae*, encompassing various genera like *Pestalotiopsis sensu stricto*, *Neopestalotiopsis*, and *Pseudopestalotiopsis* (Maharachchikumbura et al. 2014). These genera are distinguished by their morphology, phylogeny, and other characteristics.

Based on current knowledge, several *Pestalotiopsis* species, including *P. funereal* (Borhani & Mousazadeh 2004), *P. neglecta* (Arzanlou et al. 2012), *P. acacia* (Khodaparast et al. 2012), *Pestalotia disseminate* (Naeimi et al. 2015), *Neopestalotiopsis asiatica* (Ayoubi & Soleimani 2016a), *N. iranensis* (Ayoubi & Soleimani 2016b), *N. mesopotamica* (Ayoubi & Soleimani 2016c), *P. vismiae* (Radi & Hamedi 2017), *P. trachycarpicola* (Atashi Khalilabad & Fotouhifar 2022) *P. longiseta*, *P. longisetula*, *P.

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uvicola (Bakhshi et al. 2022), *Pseudopestalotiopsis theae*, and *Pestalotiopsis nattrassii* (Khodaparast et al. 2022), have been reported from Iran. Additionally, in Iran, the fungal species including *Kirramyces epicoccoides* and *Pseudocercospora eucalyptorum* have been identified as the causal agents of Eucalyptus leaf spot in the northern provinces of the Caspian Sea (Aghapour et al. 2012; Khodaparast et al. 2012). Thus, the current investigation aimed to isolate and identify fungal species linked to leaf spot symptoms observed on Eucalyptus trees in the southern area of Iran.

MATERIALS AND METHODS

Sampling and fungal isolation

In May 2023, leaf spots were observed on the foliage of Eucalyptus (*Eucalyptus camaldulensis* Dehn), an ornamental plant specimen located in Jiroft, Kerman Province, Iran. Specimens exhibiting characteristic leaf spot symptoms, including brown, oval, or irregularly shaped lesions on the leaf margins, center or tips, and small black pustules on necrotized tissues, were collected and transported to the laboratory for further examination. Leaf fragments showing distinct spots were subjected to surface decontamination by immersing them in a 2% sodium hypochlorite (NaClO) solution for 30 seconds, followed by exposure to 70% ethanol for the same duration and then rinsed twice with sterile distilled water. The sanitized plant segments were then dried with a sterile paper towel and positioned onto a 2% water agar (WA) medium in Petri dishes. These dishes were maintained at a temperature of 25ºC under alternating near-UV light conditions (12 hours light/12 hours dark) for five days. After the incubation period, fungal colonies with similar growth patterns emerged around the plant tissues. Isolates were subsequently purified using both hyphal tip and single spore techniques following the methods outlined by Ho & Ko (1997).

Morphological characterization

Colonies were cultured on potato dextrose agar (PDA) medium to evaluate the colony morphology and microscopic characteristics. The plates were maintained at a temperature range of 23–25 °C under a 12-hour dark/12-hour near-ultraviolet light regimen, leading to sporulation occurring within five to seven days. Data collection and image acquisition were conducted through slide mounts prepared in lactophenol and lactophenol cotton blue, with measurements derived from 20 conidiophores and 50 conidia, then the preparations were studied under a BH2 Olympus light microscope (Japan). Specimens examined and pure cultured were deposited in the Agricultural Biotechnology Research Institute of Iran Culture Collection (ABRIICC10387).

DNA extraction, and phylogenetic analysis

In order to enhance our understanding of species relationships, we conducted a phylogenetic analysis by combining data from both the ITS regions and a partial sequence of the translation elongation factor 1-alpha (*tef-1α* gene. The genomic DNA of isolate ZIZQ2023 was extracted from freshly grown mycelium on PDA (seven days old) using a method adapted from Zhong and Steffenson (2001). Amplification of the complete internal transcribed spacer (ITS1-5.8S-ITS2) region of rDNA and the partial sequence of the translation elongation factor 1-alpha (*tef-1α*) gene was carried out using the ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCC TCGCTTATTGATATGC) primers (White et al., 1990), and EF1-728F (CATCGAGAAGTTCGAG AAGG) and EF-2 (GGARGTACCAGTSATCA TGTT) primer pairs, respectively (O'Donnell et al. 1998, Carbone & Kohn, 1999). The PCR programs were set as follows: For ITS amplification, the protocol began with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds. The program concluded with a final extension step at 72°C for 10 minutes. For amplifying *tef-1α*, the process started with an initial denaturation at 94 °C for 8 min, followed by 35 cycles consisting of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s and extension at 72 °C for 1 min, and the program ended with a final extension at 72 °C for 5 min. The PCR products were analyzed on 1.5% agarose gels using electrophoresis and PCR products were sent to Codon Genetic Group Lab (IRAN) for sequencing. Following sequencing, the sequences were manually edited utilizing Chromas 2.6.6 software (Technelysium, Australia), and saved in FASTA format. The final ITS sequence (600 bp) and *tef1* sequence (460 bp) were then submitted to the National Centre of Biotechnology Information (NCBI) for similar sequences search using BLAST (Altschul et al., 1990). For phylogenetic analyses, twenty-five reference sequences of ITS and *tef1* from *Pestalotiopsis*, *Pseudopestalotiopsis*, and *Neopestalotiopsis* species, along with *Broomella vitalbae* as the outgroup taxon, were selected (Table 1). Subsequently, the alignment of sequences utilized Clustal W (Thompson et al., 1994), and Maximum Likelihood (ML) analysis (Felsenstein, 1973) was performed via heuristic search using MEGA X (Kumar et al., 2018). Bootstrap analysis (Felsenstein, 1985) of the ML tree was carried out with 1000 replicates (Fig. 2).

Pathogenicity test

The evaluation of pathogenicity for the obtained isolate mainly entailed performing tests on Eucalyptus leaves within a controlled setting. To accomplish this, spores in bulk were gathered from colonies aged seven days cultured on PDA medium at 25 °C with a 12-hour light/dark cycle. Following that, the concentration of the inoculum suspension was

Table 1. The sequences utilized for phylogenetic analyses (The newly generated sequence highlighted in bold).

Species	Isolate	Host/Source	Country	GenBank Accession no.		References
				ITS	*tef-1α*	
Pestalotiopsis biciliata	CBS 124463	*Platanus* x *hispanica*	Slovakia	KM199308	KM199505	Maharachchikumbura et al. 2014
P. biciliata	**ZIZQ2023**	***Eucalyptus camaldulensis***	**Iran (Kerman)**	**OR758863**	**OR768874**	**This study**
P. australasiae	CBS 114141	*Protea* sp.	Australia	KM199298	KM199501.	Maharachchikumbura et al. 2014
P. australasiae	CBS 114126	*Knightia* sp.	New Zealand	NR_147546	KM199499	Maharachchikumbura et al. 2014
P. rhizophorae	MFLUCC 17-0416	*Rhizophora apiculata*	Thailand	MK764283	MK764327	Norphanphoun et al. 2019
P. parva	CBS 114972	leaf	Hong Kong	MH553980	MH554397	Liu et al. 2019
P. parva	CBS 278.35	*Leucothoe fontanesiana*	Thailand	KM199313	KM199509	Maharachchikumbura et al. 2014
P. humicola	CBS 115450	*Ilex cinerea*	Hong Kong	KM199319	KM199487	Maharachchikumbura et al. 2014
P. humicola	CBS 336.97	soil in tropical forest	New Guinea	KM199317	KM199484	Maharachchikumbura et al. 2014
P. adusta	CBS 263.33	*Rhododendron ponticum*	Netherlands	KM199316	KM199489	Maharachchikumbura et al. 2014
P. adusta	ICMP6088	refrigerator door PVC gasket	Fiji	JX399006	JX399070	Maharachchikumbura et al. 2014
P. pini	MEAN 1095	*Pinus pinea*	Portugal	MT374682	MT374695	Silva et al. 2020
P. pini	MEAN 1094	*Pinus pinea*	Portugal	MT374681	MT374694	Silva et al. 2020
P. rhododendri	OP086	*Rhododendron sinogrande*	China	KC537804	KC537811	Maharachchikumbura et al. 2014
P. chamaeropis	CBS 186.71	*Chamaerops humilis*	Italy	KM199326	KM199473	Maharachchikumbura et al. 2014
P. chamaeropis	CBS 113607	Unknown	Unknown	KM199325	KM199472	Maharachchikumbura et al. 2014
P. australis	MEAN 1096	*Pinus pinea*	Portugal	MT374684	MT374696	Silva et al. 2020
P. australis	MEAN 1109	*Pinus pinea*	Portugal	MT374679	MT374692	Silva et al. 2020
P. portugallica	CBS 684.85	*Camellia japonica*	New Zealand	MH554065	MH554501	Liu et al. 2019
P. portugallica	CBS 393.48	Unknown	Portugal	KM199335	KM199510	Maharachchikumbura et al. 2014
P. camelliae	CBS 443.62	*Camellia sinensis*	Turkey	KM199336	KM199512	Maharachchikumbura et al. 2014
P. camelliae	MFLUCC 12-0277	*Camellia japonica*	China	JX399010	JX399074	Maharachchikumbura et al. 2014
Pseudopestalotiopsis theae	MFLUCC12-0055	*Phoenix dactylifera*	China	JQ683727	JQ683743	Maharachchikumbura et al. 2014
Neopestalotiopsis aotearoa	HNPeHNLD2002	*Heveabrasiliensis brasiliensis*	China	MT764948	MT800517	Yang et al. 2021
Neopestalotiopsis aotearoa	HNPeHNLD2001	*Heveabrasiliensis brasiliensis*	China	MT764947	MT800516	Yang et al. 2021
Broomella vitalbae	HPC 1154	Unknown	Unknown	MH554173	MH554608	Liu et al. 2019

fine-tuned to reach 10^6 spores/mL. Subsequently, Eucalyptus leaves were exposed to a 10 ml conidia suspension spray, and control leaves received a 10 ml spray of sterile distilled water. The leaves affected by the infection remained moist in the substrate for seven days, as detailed in the study conducted by Shi et al. (2012). The pathogenicity test was repeated three times.

RESULTS

Morphology and phylogeny

Pestalotiopsis biciliata Maharachch., K.D. Hyde & Crous, Stud. Mycol. 79: 156 (2014) Fig 1.

In vitro, the colony exhibited rapid growth, reaching a diameter of 56 mm on PDA after seven days of incubation at 25°C under alternating near-UV light conditions (12 hours light/12 hours darkness) in a light incubator. The colony on PDA was white fluffy, with sparse green-brown aerial mycelium on the surface and a lobate edge. Its reverse side appeared pale honey-coloured. Mycelia were hyaline, smooth, septate, and measured 2–4 µm in diameter. Conidiomata pycnidial on PDA, globose, scattered and less aggregated, semi–immersed, appeared gradually, black, conidial masses globose and black. Conidiophores septate, branched or unbranched, up to 45 µm long, or reduced to conidiogenous cells, cylindrical to subcylindrical, discrete, smooth, thin neck, hyaline, with 2–5 proliferations. 11–42 × 2.5–4.5 µm ($\bar{x}$= 23 × 3.5 µm, n = 20). Conidia fusoid, straight to slightly curved, 4-septate, 20–27 × 6–7.5 µm ($\bar{x}$= 25.5 × 6.5 µm, n = 50), thin-walled, smooth to minutely verruculose, Basal cell obconic with truncate base, thin-walled, hyaline, 4–6 µm long ($\bar{x}$= 5 µm); Three median cells dolioform, with thick versicolor walls, 13–15.5 µm long ($\bar{x}$= 15.2 µm), constricted at the septa, concolorous, olivaceous to golden brown, septa darker; Apical cell conical, hyaline, smooth wall, 3–4 µm long ($\bar{x}$= 3.6 µm), with 2–4 concurrent tubular apical appendages (mostly 3), filiform, unbranched, arising from the apical crest, 6–25 µm long ($\bar{x}$= 18 µm); single to two basal appendages is straight, centric to eccentric, tubular, 3–9 µm long ($\bar{x}$= 6.4 µm). Sexual morph was not observed.

Specimen examined. IRAN, Kerman Province, Jiroft, isolated from living leaves of *Eucalyptus camaldulensis* (*Myrtaceae*, *Myrtales*), May. 12, 2023, A. Amirmijani and A. Atashi, ABRIICC10387 (Isolate ZIZQ2023).

Note: These characteristics of the recovered isolate matched well with the description of *Pestalotiopsis biciliata* provided by Maharachchikumbura et al. (2014). The combination of the sequence data obtained from ITS and *tef-1α* revealed that this isolate, with a bootstrap value of 99%, was clustered in a clade containing type species of *P. biciliata* CBS 124463 (Fig. 2). Both morphological and molecular data, confirmed that this species corresponds to *P. biciliata.* According to the best of our knowledge, this is the first report of this species as new taxon for the Iranian funga.

Pathogenicity test

Symptoms appeared on the third day following pathogen inoculation, initially manifesting as leaf discoloration at the site of inoculation and the emergence of small brown spots with well-defined borders. Over the following ten days, these spots progressed into a necrotic state, culminating in the emergence of conidia and conidiophores within conidiomata. In contrast, the control plants exhibited no discernible symptoms, as illustrated in Figure 3. Importantly, the fungi were successfully re-isolated from the inoculated plants, confirming compliance with Koch's postulates and validating the established causal link between the identified fungi and the observed symptoms.

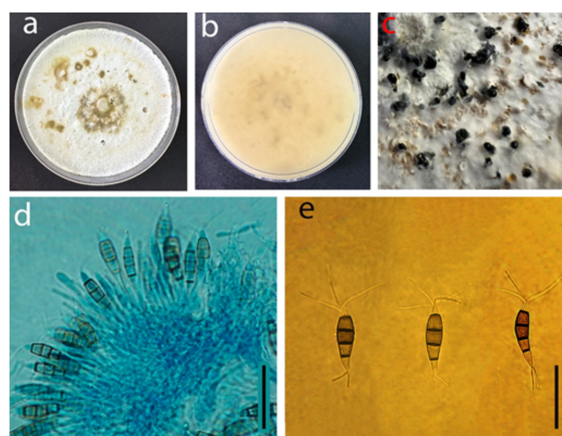

Fig. 1. Culture characteristics and the morphology of *Pestalotiopsis biciliata*, strain ZIZQ2023: (A) Surface (top) and (B) reverse (bottom) of the colony on PDA after 14 days. (C) Conidiomata, (D) Conidiogenous cells with conidia. (E) Conidia with three to four apical and two basal appendages. Scale bars = 30 µm.

DISCUSSION

Morphological features alone are often insufficient to distinguish between different *Pestalotiopsis* species. Studies have shown that relying solely on conidial morphology can lead to misclassification. Utilizing multiple gene regions (e.g., ITS, *tub2*, *tef-1α*) provides a more robust approach for *Pestalotiopsis* species identification and phylogenetic analysis (Maharachchikumbura et al. 2012, 2014). The study identified the fungal species *Pestalotiopsis biciliata*, which is characterized by the presence of two basal appendages. *Pestalotiopsis biciliata* morphologically overlaps with *P. trachicarpicola*. However, phylogenetic analyses revealed that it forms a distinct lineage separate from both *P. kenyana* (which has wider conidia) and *P. trachicarpicola* (Maharachchikumbura et al. 2014). As evident in the phylogenetic tree (Fig 2), this species is

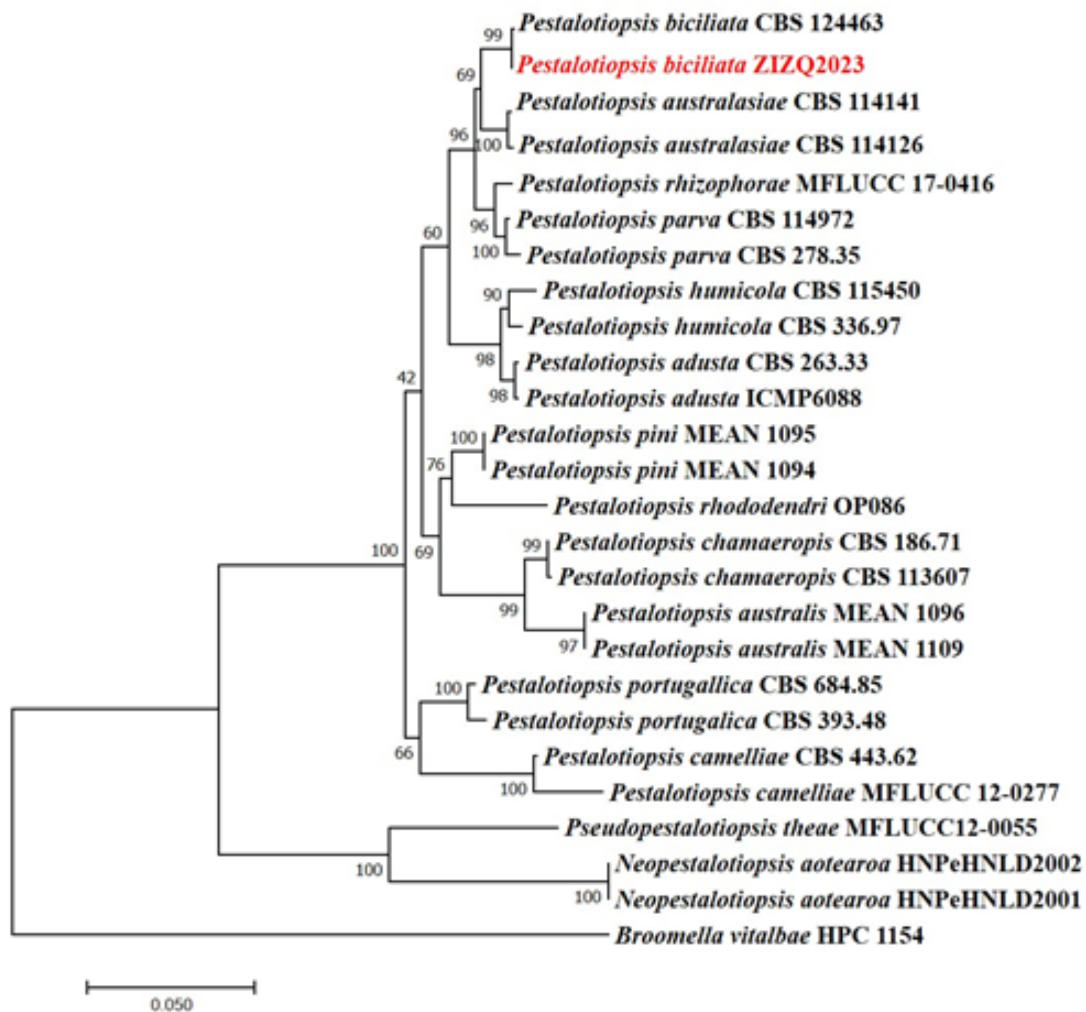

Fig. 2. The Maximum Likelihood (ML) tree obtained from of the combined of ITS and *tef-1α* sequences alignment of 25 isolates of *Pestalotiopsis*, *Pseudopestalotiopsis*, and *Neopestalotiopsis*, with *Broomella vitalbae* HPC 1154 serving as the outgroup taxon. values from 1000 replicates are displayed at the nodes. The strain highlighted in red represents the specimen recovered in this study.

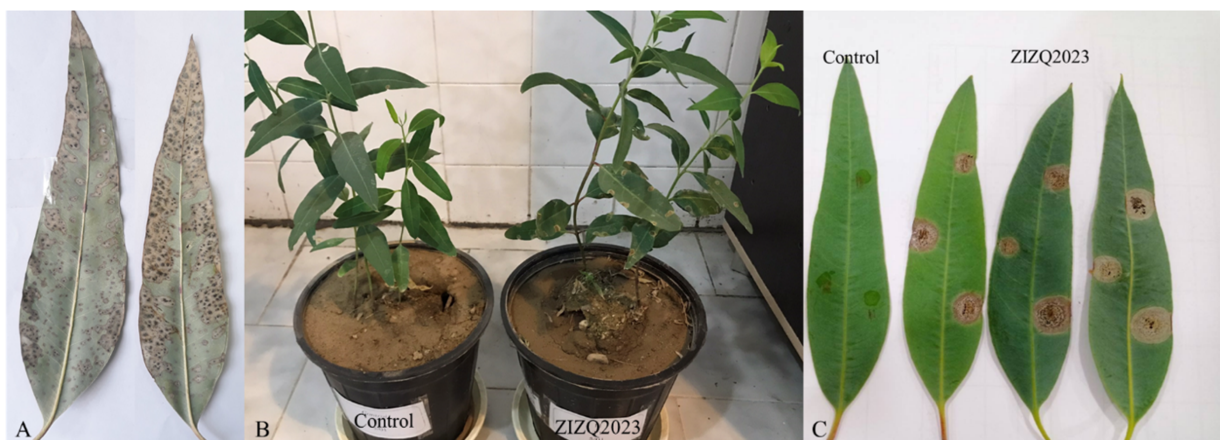

Fig. 3. Leaf spot symptoms caused by *Pestalotiopsis biciliata* A: *in vivo*, B-C*:* On seedlings and detached leaf of Eucalyptus, 10 days after inoculation in pathogenicity test.

phylogenetically close to *P. australasiae*. Various studies conducted globally have documented the

presence of *P. biciliata* on grapes, *Taxus* spp. and *Platanus* spp. (Maharachchikumbura et al. 2014). Additionally, instances of necrotic leaf spot caused by this species have been reported on Grape and Eucalyptus in Italy, as well as on *Ceratonia siliqua* in Algeria (Lorenzini & Zapparoli 2018, Morales-Rodríguez et al. 2019, Louanchi et al. 2021). Although several *Pestalotiopsis* species have been recorded in Iran previously, this investigation signifies the first report of *P. biciliata*, enhancing our knowledge of fungal diversity impacting Eucalyptus trees in the locality. Pathogenicity trials affirmed the capability of *P. biciliata* to prompt leaf spot disease in *E. camaldulensis*, with symptoms aligning with those typically associated with this fungus.

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نخستین گزارش از *Pestalotiopsis biciliata* برای قارچ‌های ایران

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چکیده : طی بررسی درختان اکالیپتوس (*Eucalyptus camaldulensis* Dehn.) شهرستان جیرفت، که در بهار ۱۴۰۲ انجام شد، برگ‌های دارای علائم لکه‌برگی جمع‌آوری شدند. پرگنه‌های قارچی با الگوهای رشد مشابه از برگ‌های دارای علایم جدا شدند. جدایه‌های به دست آمده بر اساس ویژگی‌های ریخت‌شناسی و داده‌های حاصل از توالی ناحیه ITS-rDNA و بخشی از ژن فاکتور طویل¬سازی ترجمه ۱-آلفا (*tef-1α*) به‌عنوان *Pestalotiopsis biciliata* شناسایی شدند. آزمون بیماری‌زایی جدایه‌ها روی برگ‌های جدا شده و نهال اکالیپتوس انجام شد. طبق اطلاعات ما، این اولین گزارش از گونه *Pestalotiopsis biciliata* برای مجموعه قارچ‌های ایران و اولین گزارش بیماری‌زایی این گونه بر روی *Eucalyptus camaldulensis* در ایران است.

کلمات کلیدی: بیماریزایی، فیلوژنی، *Pestalotiopsis*،*Pestalotiopsidaceae*

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