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Paramicrosphaeropsis eriobotryae sp. nov., a new agent of loquat canker in Iran

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Abstract

Loquat (*Eriobotrya japonica*) is a subtropical evergreen plant, important in terms of both horticulture and green space in the urban environment. In recent years, symptoms of branch and trunk canker, dieback and decline have been observed in the main cultivation areas of this tree in Iran. To study the aetiology of loquat canker in Fars Province, branches and trunks of loquat trees with disease symptoms were sampled from 2018 to 2019. Several isolates of an unknown *Paramicrosphaeropsis* species (*Didymellaceae*, *Pleosporales*, *Dothideomycetes*) were recovered from the trunks and branches of infected loquat trees. Phylogenetic analysis of the isolates based on a multigene phylogeny of the ribosomal internal transcribed spacer (ITS), partial β-tubulin gene (*tub2*) and the second-largest subunit of RNA polymerase II (*rpb2*) loci revealed that the isolates belonged to a new taxon, *Paramicrosphaeropsis eriobotryae* sp. nov. Pathogenicity tests revealed that this species was able to cause canker, trunk flaking, dieback, decline, leaf yellowing, defoliation and growth reduction in loquat trees. A host range evaluation on detached branches of some important horticultural trees occurring adjacent to loquat tree orchards of Fars Province showed that *P. eriobotryae* might have a broader host range, as it was also able to infect almond and olive trees.

KEYWORDS
Didymellaceae, *Dothideomycetes*, loquat, multigene phylogenetic analysis, new taxon, trunk canker

1 | INTRODUCTION

Loquat, *Eriobotrya japonica*, is a subtropical evergreen tree of the *Rosaceae* family native to south-eastern China. It is an important fruit tree in horticulture and in green space in the urban environment and is adapted to the Mediterranean region, especially hot and humid climates. This tree is commercially cultivated for fruit production and grown as an ornamental plant in Asia (India, Japan and Pakistan), Australia, Mediterranean countries (Greece, Italy, Israel, Spain and Turkey), South America (Brazil, Venezuela) and the United States (Badenes et al., 2000; Chalak et al., 2014). In Iran, the northern provinces, such as Gilan, and Fars Province in the south are the central regions of loquat production (Gharaghani et al., 2015).

Loquat is susceptible to a wide range of fungal diseases (Ma et al., 2022), including postharvest rot (Palou et al., 2016), blossom blight (Sun et al., 2009), crown and root rot (Tian et al., 2011), anthracnose (Cao & Zheng, 2010), sunburn or purple spot (Caballero & Fernández, 2003), fungal black spot or scabies (Sánchez-Torres et al., 2009), decline (Agusti-Brisach et al., 2016; Gonzalez-Domínguez et al., 2009) and especially trunk and branch cankers (Gonzalez-Domínguez et al., 2016). Various families of ascomycetous fungi cause canker diseases in fruit trees (Agrios, 2005). Most of the fungal canker agents reported from loquat belong to different species of *Botryosphaeriaceae* in the genera *Diplodia*, *Dothiorella*, *Neofusicoccum* and *Spencermartinsia* (Besoain & Fuentes, 1988; Giambra et al., 2016; Gonzalez-Domínguez et al., 2016). In recent years, some symptoms of branch and trunk canker, dieback and decline have been observed in Iran's main loquat cultivation areas. A preliminary study on the aetiology of loquat canker in Fars Province showed that, unlike in previous reports, the fungal species associated with Iranian loquat canker resembles a coniothyrium-like fungus.

Coniothyrium-like fungi are coelomycetous asexual morphs of *Pleosporales* (*Dothideomycetes*, *Ascomycota*) (Verkley et al., 2014). These fungi are characterized by pycnidial or stromatic conidiomata, which are pale brown to brown, with small, one or two-celled conidia (Damm et al., 2008; Verkley et al., 2014). Most of their species are classified in *Coniothyrium* or *Microsphaeropsis* (Verkley et al., 2014). The taxonomy of coniothyrium-like fungi is complicated due to their simplicity and the variability of morphological features. Furthermore, attempts to define these genera based solely on features such as conidiomatal structure, conidiogenesis and conidial morphology have proved difficult (Sutton, 1980). Recent phylogenetic analyses including asexual and sexual morphs of genera in the *Pleosporales* showed that *Coniothyrium*, *Microsphaeropsis* and *Phoma* are polyphyletic. These genera have species that cluster in several clades of the order *Pleosporales* (Aveskamp et al., 2010; de Gruyter et al., 2010, 2012; Quaedvlieg et al., 2013; Verkley et al., 2004, 2013; Zhang et al., 2009, 2012). The genus *Paramicrosphaeropsis* (*Pleosporales*, *Didymellaceae*) was first introduced by Hou et al. (2020) with *Paramicrosphaeropsis ellipsoidea* as the type species, isolated from decayed twigs of *Quercus ilex* (*Fagaceae*) in Spain. The second species of *Paramicrosphaeropsis*, *P. iranica*, was described from *Quercus brantii* (*Fagaceae*) trees in Iran (Ahmadpour et al., 2022). *P. ellipsoidea* was previously referred to as a species of *Microsphaeropsis* but shown to cluster separately from *Microsphaeropsis* and *Neomicrosphaeropsis* (Hou et al., 2020). *Microsphaeropsis*, *Neomicrosphaeropsis* and *Paramicrosphaeropsis* are morphologically comparable in the initial production of hyaline conidia, which turn brown with age. The morphological distinction of *Paramicrosphaeropsis* from other genera of the *Didymellaceae* is the formation of pycnidial conidiomata with hyaline to pale brown, thin walls that do not turn dark brown at conidial maturity. The subglobose, ampulliform or doliiform conidiogenous cells of *Paramicrosphaeropsis* species morphologically differentiate them from their sister genus, *Neomicrosphaeropsis*, whose species tend to produce subcylindrical conidiogenous cells (Hou et al., 2020).

Our investigation on infected horticultural and ornamental loquat trees with canker symptoms in Fars Province revealed an unidentified species of *Didymellaceae* belonging to the genus *Paramicrosphaeropsis*.

This study aimed to distinguish this taxon from others in the genus using phylogenetic analyses based on a multigene genealogy and to evaluate its pathogenicity on loquat and other trees frequently cultivated in the vicinity of loquat orchards in Fars Province.

2 | MATERIALS AND METHODS

2.1 | Field survey and sampling

Field surveys were conducted during 2018 and 2019 in orchards of Fars Province in Iran (Shiraz, Khafr and Jahrom counties) for symptomatic branches and trunks of loquat trees. Wood samples were collected from trunks and branches of loquat with dieback symptoms, including dead shoots, cankers and internal wood necrosis. Additional samples of adjacent trees, including olive (*Olea europaea*), almond (*Prunus amygdalus*) and apple (*Malus domestica*), were also collected from selected orchards. In order to investigate the endophytic phase of fungal isolates, healthy loquat trees were also sampled (Table S1).

2.2 | Fungal isolation

Isolates were obtained from discoloured and necrotic wood tissue of infected branches and trunks. For each sample, 10–12 pieces of 5 × 5 mm were separated from the margin of infected and healthy tissues and placed under running water for 5 min. These wood tissue samples were surface sterilized for 60 s in a 70% ethanol and 2% sodium hypochlorite solution, washed twice with sterile distilled water for 60 s and placed on a sterile paper towel under a laminar flow hood to dry. Subsequently, surface-sterilized tissues were plated on potato dextrose agar (PDA; Crous et al., 2019) supplemented with 16 drops of 50% acetic acid per litre. Plates were stored at room temperature (25 ± 2°C) in the laboratory. After 6–8 days, the obtained colonies were subcultured on water agar (WA; Crous et al., 2019) and purified using a hyphal tip method (Damm et al., 2008).

2.3 | Morphological, morphometric and physiological studies

Morphological studies of living cultures were conducted following the methods described by Chen et al. (2015, 2017) and Hou et al. (2020). Mycelial plugs of a 5-day-old fungal culture was transferred onto PDA, malt extract agar (MEA; Crous et al., 2019) or oatmeal agar (OA; Crous et al., 2019) and incubated at 25°C under fluorescent white light (12 h light/12 h dark). Colony morphology and diameter were determined after 14 days of incubation on these culture media, and colour (upper surface and reverse) rated using the colour chart of Rayner (1970) (Chen et al., 2015; Hou et al., 2020). In order to induce sporulation, a 0.5 mm mycelial plug of the fungus was placed on pine needle agar (PNA; 2% WA with pine needle pieces; Smith et al., 1996) and incubated at 25°C under fluorescent white light (12 h light/12 h dark; Damm et al., 2008). Isolates were examined weekly for the formation of pycnidia and conidia. Slide preparations were mounted in distilled water and lactic acid to study the micromorphological structures of mature conidia, and conidia-producing structures such as pycnidia and conidiogenous cells were examined with an Axio Imager 2 compound microscope (Zeiss) using differential interference contrast (DIC) illumination; images were recorded on a DS-Ri2 camera (Nikon) with associated software. In isolates selected for morphological study, the length and width of 30 conidia, pycnidia and conidiogenous cells were measured (Chen et al., 2015).

In order to measure radial growth rate at different temperatures, 5-mm diameter mycelial plugs from the margins of 5-day-old colonies were transferred to Petri dishes containing MEA. Petri dishes were incubated in darkness from 5 to 35°C, at 5°C intervals. Colony

diameters were measured daily for 10 days and the average radial growth rate of each isolate was calculated in mm/day for three replicates at each temperature.

2.4 | DNA extraction, PCR amplification and sequencing

DNA extraction followed the protocol of Mirsoleimani and Mostowfizadeh-Ghalamfarsa (2013). Colonized agar pieces from the margins of the young colonies of the isolates were transferred into 250-mL Erlenmeyer flasks containing 50 mL sterile potato extract broth (extract of 300 g/L boiled potato in distilled water) containing 5 g/L of malt extract and 20 g/L of dextrose at 25°C. After 7–10 days, mycelia were harvested, freeze dried and DNA was extracted with a DNG-PLUS extraction kit (Sinagene). DNA quality was examined with an MD-1000 spectrophotometer (NanoDrop). Three loci were amplified and sequenced, namely the internal transcribed spacer regions 1 and 2, and 5.8S nuclear ribosomal RNA gene (ITS), partial DNA-directed RNA polymerase II second largest subunit (*rpb2*) and partial β-tubulin gene (*tub2*) (Chen et al., 2015; Hou et al., 2020) using the primer pairs ITS1 and ITS4 (White et al., 1990), RPB2-5F2 and fRPB2-7cR (Liu et al., 1999; Sung et al., 2007), and Btub2Fd and Btub4Rd (Woudenberg et al., 2009), respectively. The thermal cycling programme for PCR amplification for the ITS region was 95°C, 2 min; 30 cycles of 95°C, 20 s, 55°C, 25 s, 72°C, 50 s; then 72°C, 10 min. For *rpb2*, the PCR programme was 94°C, 5 min; 35 cycles of 95°C, 45 s, 56°C, 80 s, 72°C, 2 min; then 72°C, 10 min. For *tub2*, the PCR programme was 95°C, 2 min; 35 cycles of 95°C, 90 s, 62°C, 60 s, 72°C, 45 s; then 72°C, 10 min. PCR products were sequenced with the primers used for amplification by a dye terminator cycle (Cardiogenetic Research Center, Tehran, Iran). Novel sequence data from this study were deposited into GenBank (http://www.ncbi.nlm.nih.gov/genbank).

2.5 | Phylogenetic analysis

Sequences resulting from the PCR amplifications described above were edited with BioEdit v. 7.0.9.0 (Hall, 1999). The consensus sequences of ITS, *rpb2* and *tub2* loci were assembled from the forward and reverse sequences with MEGA v. 7.0 (Kumar et al., 2016). Partition homogeneity tests were conducted on the combined nuclear gene alignment with PAUP v. 4.0a136 (Swofford, 2002) using 100 replicates and the heuristic general search option. Sequences generated from this study are listed in Table 1, as well as reference sequences of *Microsphaeropsis*, *Neomicrosphaeropsis* and *Paramicrosphaeropsis* species, chosen from Hou et al. (2020) and Ahmadpour et al. (2022), and downloaded from GenBank. The sequence alignment was conducted with ClustalX (Thompson et al., 1997) with subsequent visual adjustment. *Nothophoma brennandiae* was chosen as phylogenetic outgroup. In order to investigate the phylogenetic relation and identity of isolates at species level, both Bayesian inference (BI) and maximum-likelihood (ML) methods were used for phylogenetic analyses on individual and concatenated sequence alignments of ITS, *rpb2* and *tub2* loci. BI analyses were carried out with MrBayes v. 3.1 (Ronquist & Huelsenbeck, 2003), imposing a symmetrical substitution model (SYM) with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Both analyses were conducted with the same data set according to Hou et al. (2020) and Ahmadpour et al. (2022). The best nucleotide substitution model was determined with MrModelTest v. 2.3 (Nylander, 2004). Two independent runs of Markov chain Monte Carlo (MCMC) using four chains were run over 1,000,000 generations. Trees were saved each 1000 generations, resulting in 10,001 trees. Burn-in was set at 25% generations (Salmaninezhad & Mostowfizadeh-Ghalamfarsa, 2019). In order to conduct phylogenetic comparisons, ML estimations were carried out using PHYLIP DNAML (Felsenstein, 1993) with TrN equal frequencies substitution model (TrNef). The robustness of the ML trees was estimated by 1000 bootstraps. Phylogenetic trees were edited and displayed with TreeGraph (Stover & Muller, 2010). Alignments derived in this study were deposited in Figshare (www.figshare.com; doi identifier https://doi.org/10.6084/m9.figshare.21586584).

2.6 | Pathogenicity tests

2.6.1 | Inoculation of detached shoots

Inoculations were conducted on detached woody shoots of loquat trees to determine the pathogenicity of selected isolates. Fresh shoots of loquat trees were collected and cut into c.35 cm pieces of 5–7 mm diameter. The outer bark at the inoculation areas was surface sterilized with 70% ethanol and flame sterilized. A mycelial plug was taken from the margin of a 1-week-old fungal colony on PDA and placed into a 1 cm hole in the branch. The inoculated areas were covered with Parafilm (Pechiney Plastic Packaging). Three shoots were inoculated per treatment with two mycelial plugs per shoot. Shoots were also inoculated with noncolonized PDA plugs to serve as negative controls. One end of each inoculated shoot was covered with melted paraffin, and the other end placed into an Erlenmeyer flask containing 300 mL sterile distilled water. Inoculated shoots were arranged in a complete randomized design in a germinator with a light period of 12 h light/12 h dark and a temperature of 25 ± 2°C. After 14 days, the stems were sectioned longitudinally and transversely and the bark surrounding each wound site was stripped off to evaluate the discolouration of the bark, wood and vessels upward and downward from the point of inoculation. Small pieces of necrotic tissues from the margins of lesions were plated onto PDA amended with lactic acid to fulfil Koch's postulates (Ghasemkhani et al., 2015; Soltaninejad et al., 2017).

2.6.2 | Inoculation of 1-year-old seedlings

Pathogenicity of isolates was also evaluated on 1-year-old loquat seedlings. The main stems were wounded at 10 cm above the crown

TABLE 1 Details of isolates included in the phylogenetic analysis.

Species	Old name	Isolate	Host	Host family	Country	GenBank accession number[a]		
						ITS	*rpb2*	*tub2*
Microsphaeropsis fusca	*M. olivacea*	CBS 139603	*Sarothamnus scoparius*	*Fabaceae*	Germany	MN973571	MT018218	MT005674
M. olivacea		CBS 233.77	*Pinus laricio*	*Pinaceae*	France	GU237803	MT018217	GU237549
M. taxicola	*M. olivacea*	CBS 469.80	*Rhus typhina*	*Anacardiaceae*	Netherlands	MN973565	MT018210	MT005668
M. viridis	*M. olivacea*	CBS 763.73	*Populus tremula*	*Salicaceae*	France	MN973561	MT018205	MT005664
M. proteae		CBS 111303^{T}	*Protea nitida*	*Proteaceae*	Not known	JN712495	MT018221	MT005677
Neomicrosphaeropsis alhagi-pseudalhagi		MFLUCC17-0825^{T}	*Alhagi maurorum*	*Fabaceae*	Uzbekistan	MH069664	MH069682	MH069689
N. elaeagni		MFLUCC17-0740^{T}	*Elaeagnus angustifolia*	*Elaeagnaceae*	Russia	MH069666	MH069684	MH069691
N. italica		MFLUCC 16–0284	*Tamarix* sp.	*Tamaricaceae*	Italy	KU900321	KU714604	KX453299
Nothophoma brennandiae	*Phoma* sp.	CBS 125539	*Amygdalus* sp.	*Rosaceae*	Greece	MN973553	MT018197	MT005656
Paramicrosphaeropsis ellipsoidea	*Microsphaeropsis* sp.	CBS 197.97	*Quercus ilex*	*Fagaceae*	Spain	MN973574	MT018224	MT005680
P. eriobotryae sp. nov.		EG076-4, CBS 149853	*Eriobotrya japonica*	*Rosaceae*	Iran	**OP740722**	**OP856725**	**OP856722**
P. eriobotryae sp. nov.		Gh095-4, CBS 149854	*E. japonica*	*Rosaceae*	Iran	**OP871278**	**OP856724**	**OP740723**
P. eriobotryae sp. nov.		KhA173-4, CBS 149855	*E. japonica*	*Rosaceae*	Iran	**OP871279**	**OP893803**	**OP740724**
P. eriobotryae sp. nov.		KhR155-4, CBS 148866^{T}	*E. japonica*	*Rosaceae*	Iran	**OP871280**	**OP856723**	**OP740725**
P. eriobotryae sp. nov.		KhR158-4, CBS 149856	*Prunus amygdalus*	*Rosaceae*	Iran	**OP871281**	**OP893804**	**OP740726**
P. iranica		IRAN 2929C	*Quercus brantii*	*Fagaceae*	Iran	OK247737	OK257016	OK247743

Note: GenBank accession numbers in bold were newly generated in this study. ^{T}, type isolate.

[a]ITS, internal transcribed spacers 1 and 2 and 5.8S rRNA gene of rDNA; *rpb2*, partial DNA-directed RNA polymerase II second largest subunit; *tub2*, partial β-tubulin gene.

with a 4 mm cork-borer, and a 4 mm mycelial plug (from a 1-week-old culture) was placed into the wound and the wounds were covered with Parafilm (Gonzalez-Domínguez et al., 2016). Three 1-year-old plants were inoculated per isolate and three mycelial plugs were inoculated into each plant. Noncolonized PDA plugs were used to inoculate three additional plants to serve as negative controls. Inoculated seedlings were kept in the greenhouse at 20–35°C in a complete randomized design and watered as needed up to field capacity. After 5–7 months, the stems were sectioned longitudinally and the bark surrounding each wound site was stripped off to evaluate discolouration of the bark, wood and vessels upward and downward from the point of inoculation. Reisolation of fungal isolates was made from the margins of necrotic lesions onto PDA amended with lactic acid to fulfil Koch's postulates (Gonzalez-Domínguez et al., 2016).

2.6.3 | Host range survey

During field surveys in the loquat orchards of Fars Province, other trees were found in the vicinity of loquat trees, including olive, pomegranate (*Punica granatum*), apple, quince (*Cydonia oblonga*) and almond. Fresh vegetative shoots of these neighbouring trees were collected and inoculated using the same method for inoculation of detached twigs described above.

3 | RESULTS

3.1 | Field survey and disease symptoms

During investigations of loquat orchards and landscape loquat trees of Shiraz, Khafr and Jahrom counties, symptoms of branch and trunk canker, dieback and decline were observed in most orchards and ornamental trees. Distribution of the symptoms was more common in less cared for, old orchards, parks and scattered trees along the streets than well cared for, old and young orchards. External and internal canker symptoms on loquat trees were observed in both commercial orchards and landscape trees, which eventually led to the trees' gradual death. External symptoms included decline, leaf yellowing, defoliation, dieback, cankers on trunks and branches and trunk flaking (Figure 1a–f). Internal symptoms included brown to dark brown discolouration of vascular tissues and internal wood necrosis (Figure 1g). A pale brown gum was also observed oozing out of the wound area on the trunks of some infected trees, which ultimately dried and changed into dark brown gum after several days (Figure 1h). Cross-sections of thick branches with dieback symptoms revealed internal wood necrosis (Figure 1g). Sampling was conducted from branches and trunks of 58 loquat trees, two olive trees, one apple tree and one almond tree located in the loquat orchards of Fars Province. Almond and apple trees had canker symptoms on their trunks, but the olive trees looked healthy.

From loquat orchards, 43 isolates were recovered and morphologically identified as a new fungal species of *Paramicrophaeropsis* (Table S1). One of the isolates was obtained from an almond tree with canker and trunk flaking symptoms (KhR158-4, Oct 2019, Jahrom-Tadvan 28°51.276′ N, 53°09.187′ E). Of the three sampled healthy-looking loquat trees, two were uninfected, but the branches of one tree had internal signs of discolouration and necrosis, from which a pathogenic isolate (KhA173-4) of the same *Paramicrosphaeropsis* species was recovered (Table S1). In general, out of 140 ascomycete isolates recovered, eight belonged to a newly described species, *Stilbocrea banihashemiana* (Bolboli et al., 2022), 18 isolates were identified as *Neosetophoma iranianum* and 71 isolates belonged to endophytic taxa including *Acremonium* spp., *Alternaria* spp., *Fusarium* spp., *Leptosphaeria* spp., *Neoscytalidium* spp., *Neosetophoma* spp., *Rhizoctonia* spp., *Seimatosporium* spp., *Stemphylium* spp. and *Ulocladium* spp.

3.2 | Molecular identification and phylogenetic analysis

Paramicrosphaeropsis eriobotryae sp. nov. differed from its sister taxa, *P. ellipsoidea* and *P. iranica*, at 1, 7, 4, and 1, 15, 6 variable nucleotide sites in the ITS (785 bp), *rpb2* (970 bp) and *tub2* (398 bp) gene regions, respectively (Table S2). Furthermore, our phylogenetic results recovered low diversity within the new lineage, so that of the 2144 nucleotides included in the three aligned genes (ITS, *tub2* and *rpb2*), only one variable nucleotide site was observed. The single-locus phylogenies of ITS and *tub2* displayed low resolution at generic and species level, respectively, but the *rpb2* phylogeny was able to distinguish all three generic clades with good resolution. The ITS sequences of selected isolates of this study (EG076-4, Gh095-4 and KhR155-4) with 514 bp length showed 100% overlap and 100% similarity with *Microsphaeropsis olivacea* (MK179294), *Ascochyta* sp. (MG065764), and 100% overlap and 99.59% similarity with *Phoma herbarum* (KF309191) and *Coniothyrium glomerulatum* (MH854688). The *tub2* sequences of selected isolates with 333 bp length showed 100% overlap and 97.20% similarity with *Nothophoma quercina* (MW302313) and 90% overlap and 98.85% similarity with *Paramicrosphaeropsis ellipsoidea* (MT005680). The *rpb2* sequences of selected isolates with 1000 bp length showed 94% overlap and 96.62% similarity with *Microsphaeropsis* sp. (KU714603).

Although it was previously claimed that the amplification of the *rpb2* region is challenging (Chen et al., 2015), further studies using a reformed protocol using bovine serum albumin (BSA) could enhance the amplification of the *rpb2* region up to 95% (Hou et al., 2020). In this study, we also encountered the problem of multiple bands of *rpb2*, and adding the recommended amount of BSA did not solve the problem. We amplified a single band of the gene by increasing the annealing temperature to 62°C and decreasing the extension time to 90 s.

The results of phylogenetic analysis of ITS, *tub2* and *rpb2* genetic regions, as well as their concatenated sequences (Figures 2 and 3, respectively), showed that these isolates formed a monophyletic group apart from previously described taxa of *Microsphaeropsis*

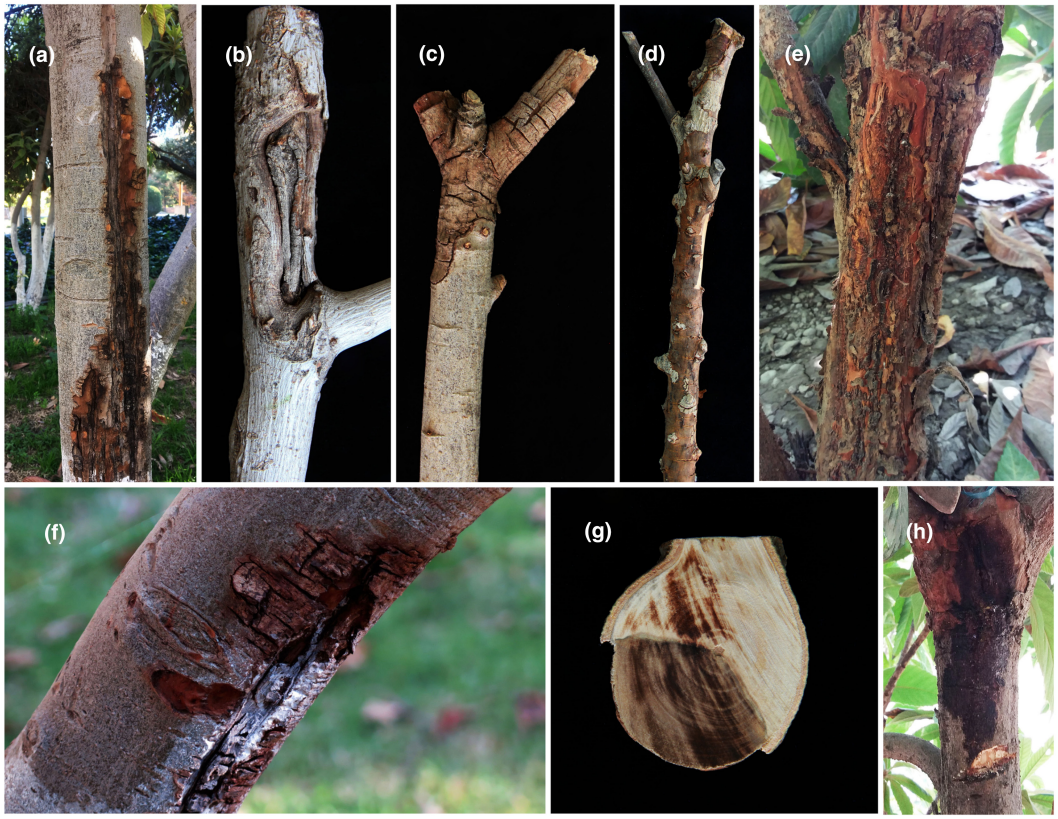

FIGURE 1 Symptoms of loquat canker in orchards in Fars Province. (a, b, f) Trunk canker; (c, d) dieback; (e) trunk flaking; (g) discolouration of vascular tissues and internal wood necrosis in the cross-section; (h) pale brown gum exuding from the wound area.

(Hou et al., 2020). Based on the phylogenies and morphology data, the new low-diversity lineage is proposed here as a new species, *Paramicrosphaeropsis eriobotryae* sp. nov.

Bayesian trees are shown with Bayesian posterior probability and ML bootstrap values (ML-BS) in Figures 2 and 3. The Bayesian posterior probability was 1.00 (ML-BS = 100) in the ITS, *tub2* and *rpb2* combined tree. Phylogenetic analysis of the three nuclear genes showed that the *P. eriobotryae* sp. nov. isolates clustered in a highly supported lineage in all phylogenetic trees and shared a common ancestor with *P. ellipsoidea* (Figures 2 and 3). Both species were located among the members of clade 24 in Hou et al. (2020). The final alignment length for the nuclear genes (ITS, *tub2* and *rpb2*) consisted of 785, 389 and 970 characters, respectively. Across the concatenated 2144 bp sequence alignment of all three genetic regions, *P. eriobotryae* sp. nov. showed 12 unique polymorphisms compared to *P. ellipsoidea* (Table S2).

3.3 | Taxonomy

Paramicrosphaeropsis eriobotryae B. Tavakolian & Mostowf. sp. nov. MycoBank MB 846538. Figures 4 and 5.

Typification: Iran, Fars Province, Jahrom County, Tadvan (29°38.251′ N, 52°32.275′ E), on trunk of *Eriobotrya japonica*, 1 October 2019, B. Tavakolian (holotype CBS 148866, preserved in a metabolically inactive state, Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands).

Etymology: Name reflects the host genus, *Eriobotrya japonica* (loquat).

Description: Conidiomata pycnidial, 242.4–1117.6 × 130.1–944.4 μm (av. 513.4 ± 182.2 × 363.8 ± 143.1 μm), globose to subglobose, mostly solitary, sometimes confluent, pale brown and semi-immersed (Figure 4a–d). Ostiole erumpent and somewhat papillate (Figure 4e). Pycnidial wall pseudoparenchymatous, multi-layered, thin, of *textura angularis*, hyaline at the beginning, pale brown at maturity (Figure 4f). Chlamydospores in chains, 5.4–14.3 × 3.9–14.3 μm (av. 8.7 ± 1.6 × 7 ± 1.5 μm), brown, ellipsoid to globose, smooth, thick-walled (Figure 4i,j). Conidiogenous cells 7.2–20.2 × 5.3–15.4 μm (av. 11.8 ± 2.8 × 8.6 ± 2.1 μm), hyaline, phialidic, smooth, subglobose, ampulliform or lageniform to doliiform (Figure 4g,h). Conidia 3.5–11.2 × 2.8–7.9 μm (av. 6.7 ± 0.5 × 4.3 ± 0.5 μm), solitary, variable in shape, ellipsoid, subcylindrical, obpyriform, straight to slightly curved, aseptate, hyaline when young, becoming pale brown, yellowish brown or greenish brown with age (Figure 4k).

Culture characteristics: Colonies on PDA reaching 6.9 mm after 14 days, aerial mycelium flat, white at the beginning and darkened over time, dark orange at the centre, margin regular, some black pycnidia visible after 20–25 days; reverse, white at the beginning and brown to dark brown with age (Figure 5a,b). Colonies on MEA reaching 6.8 mm after 14 days, aerial mycelium floccose, white at

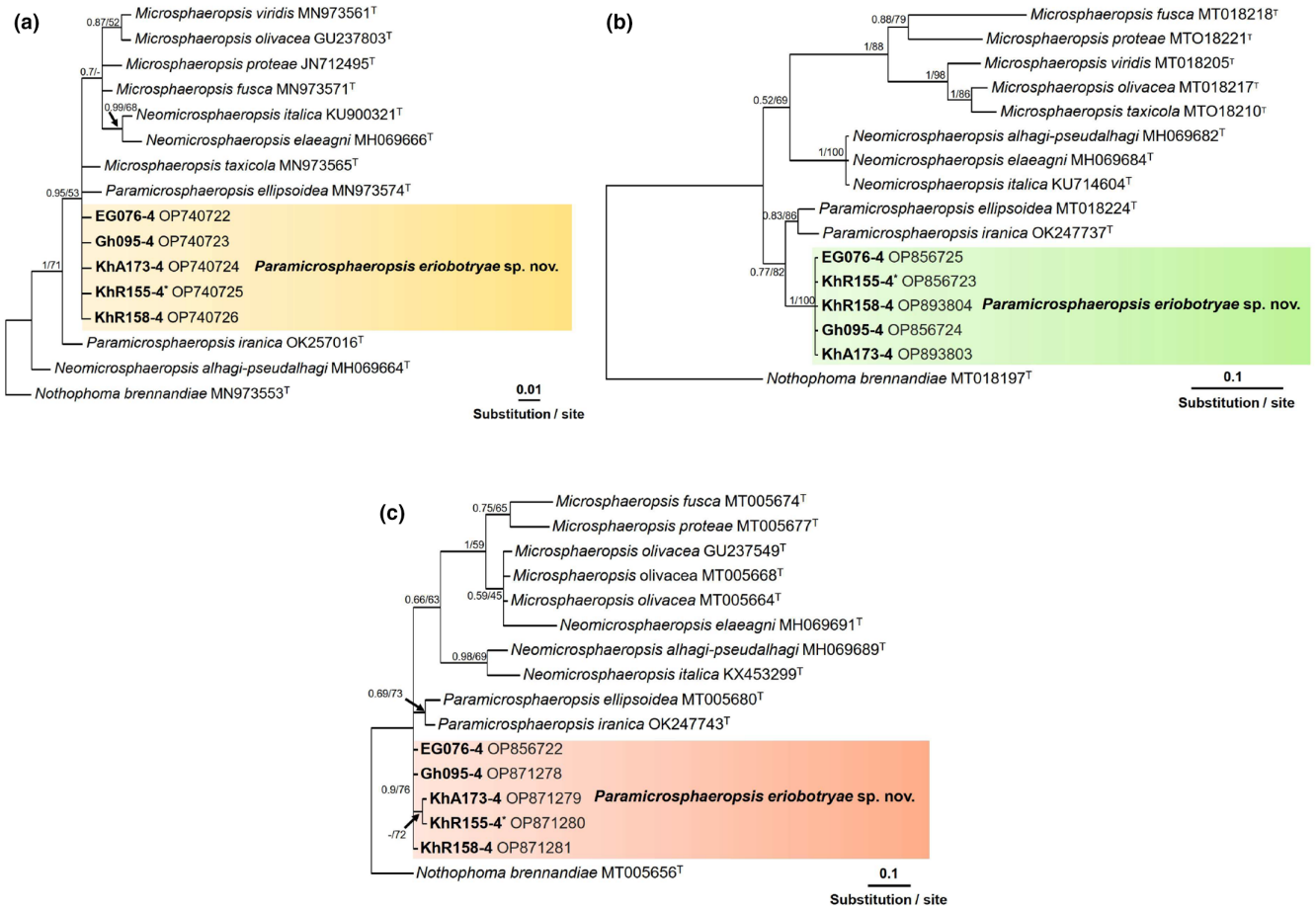

FIGURE 2 Phylogenetic relationships of *Paramicrosphaeropsis eriobotryae* sp. nov. from loquat orchards of Fars Province: relationships among five *Microsphaeropsis* species, three *Neomicrosphaeropsis* species and two *Paramicrosphaeropsis* species based on Bayesian inference analysis of (a) internal transcribed spacers 1 and 2 and 5.8S gene of rDNA (ITS); (b) the second largest subunit of RNA polymerase II (*rpb2*); and (c) partial β-tubulin gene (*tub2*). Numbers on the nodes are Bayesian inference posterior probability values followed by maximum-likelihood bootstrap values. The tree was rooted to *Nothophoma brennandiae*. Ex-type isolates are indicated with ^{T}. *=ex-type of *P. eriobotryae* sp. nov.

the beginning, brown with age, margin smooth, pycnidia visible after 1 month if formed; reverse, dark orange at the centre and dark brown with age (Figure 5c,d). Colonies on OA 6.7 mm after 14 days, immersed mycelium flat, white and pale olivaceous at the beginning, dark olivaceous with age, forming growth circles and sector, margin wavy, pycnidia visible after 14–20 days at 25°C under fluorescent light (12 h light/12 h dark), like small black dots; reverse dark olivaceous (Figure 5e,f).

Additional specimens examined: Isolate EG076-4 (CBS 149853), Iran, Fars Province: Shiraz-Eram Botanical Garden (29°38.115′ N, 52°31.503′ E) from the trunk of *E. japonica*, 11 December 2018, B. Tavakolian, GenBank: ITS, OP740722; *rpb2*, OP856725; *tub2*, OP856722. Isolate Gh095-4 (CBS 149854), Iran, Fars Province: Shiraz (29°39.403′ N, 52°28.777′ E) from the trunk of *E. japonica*, 22 April 2019, B. Tavakolian, GenBank: ITS, OP740723; *rpb2*, OP856724; *tub2*, OP871278. Isolate KhA173-4 (CBS 149855), Iran, Fars Province: Jahrom County, Khafr (28°58.825′ N, 53°12.185′ E) from the trunk of *E. japonica*, 1 October 2019, B. Tavakolian, GenBank: ITS, OP740724; *rpb2*, OP893803; *tub2*, OP871279. Isolate KhR158-4 (CBS 149856), Iran, Fars Province: Jahrom County, Tadvan (28°51.276′ N, 53°09.187′ E) from the trunk of *Prunus amygdalus*, 1 October 2019, B. Tavakolian, GenBank: ITS, OP740726; *rpb2*, OP893804; *tub2*, OP871281.

Notes: *P. eriobotryae* shares a common ancestor with *P. ellipsoidea* (Hou et al., 2020) and *P. iranica* (Ahmadpour et al., 2022). These three species have some morphological differences. Pycnidia in *P. iranica* are thick-walled and produce few conidia (Ahmadpour et al., 2022), while pycnidia in *P. ellipsoidea* and *P. eriobotryae* sp. nov. are thin-walled and produce a large number of conidia. Although pycnidia are globose to subglobose in *P. ellipsoidea* (Hou et al., 2020), they tend to be more papillate in *P. eriobotryae* sp. nov. and *P. iranica* (Ahmadpour et al., 2022). Isolates of *P. iranica* have smaller pycnidia and shorter conidia than *P. ellipsoidea* and *P. eriobotryae* sp. nov. Furthermore, they are distinguished by their unique DNA sequences, especially for *rpb2*. Radial growth rate of *P. eriobotryae* sp. nov. on MEA at different temperatures is shown in Figure S1. All isolates of this species have a minimum growth temperature of 5°C, an optimum of 25°C and a maximum of 30°C on MEA in the dark.

3.4 | Pathogenicity tests

Pathogenicity tests on detached branches and 1-year-old loquat seedlings showed that the isolates could induce disease symptoms, including canker, trunk flaking, dieback, decline, leaf yellowing, defoliation and growth reduction in loquat trees. Symptoms in inoculated detached twigs of loquat appeared in the form of brown blotches around the fungal plug 7 days after inoculation, and discolouration of vascular tissues and internal wood necrosis were observed in the cross-section of the infected twigs after 14–20 days; symptoms covered the entire twig over time (Figure 6, Table S3).

In inoculated seedlings, symptoms included the appearance of brown blotches on the sides of the fungal plug on the main trunk, expansion of the blotches along the trunk, leaves drying from the margins, defoliation, growth reduction and finally decline and drying of whole seedlings. After 20–30 days, black pycnidia and brown blotches were observed at the inoculation area, expanding along the trunk. At the lower parts of the inoculation area, canker and trunk flaking were observed after about 1 year (Figures 7 and 8, Table S4). Apart from inoculation scars, none of the described symptoms were observed on the control seedlings and detached branches.

Host range tests of *P. eriobotryae* sp. nov., on detached twigs of almond, olive, apple, quince and pomegranate trees, resulted in symptoms being induced on almond and olive in the form of brown blotches at the inoculation site, which expanded along the twigs (Figure S2).

4 | DISCUSSION

Canker is one of the most important fruit tree diseases, causing significant economic losses. In a field survey in Eram Botanical Garden, Shiraz, symptoms of a novel canker, decline and dieback were observed on trunks and branches of loquat trees. The prevalence of this type of canker in commercial orchards of Fars Province prompted us to conduct a survey for the disease on loquat trees. In areas where loquat is frequently grown, such as Spain and Italy, some members of the *Botryosphaeriaceae* (e.g., *Diplodia malorum*, *D. seriata*, *D. olivarum*, *D. alatafructa*, *D. pseudoseriata*, *Diplodia* sp., *Neofusicoccum mediterraneum*, *N. parvum*, *Dothiorella sarmentorum*, *Spencermartinsia plurivora* and *S. viticola*) are recognized as the primary agents of loquat canker, with the most common species being *D. seriata* (Giambra et al., 2016; Gonzalez-Domínguez et al., 2016). In Iran, another member of *Botryosphaeriaceae*, *Fusicoccum dimidiatum* (now *Neoscytalidium dimidiatum*), is commonly isolated from loquat trees in Shiraz (Jamali & Banihashemi, 2012). However, the canker-causing isolates from loquat encountered in the present study belong to another family of *Ascomycota*, that is, the coniothyrium-like fungi of *Didymellaceae*. Morphological and phylogenetic analysis of representative isolates confirmed the existence of a new lineage in this family, which we describe here as *Paramicrosphaeropsis eriobotryae* sp. nov.

Based on the analysis of three genetic regions (ITS, *tub2* and *rpb2*) and their concatenated phylogeny, *P. eriobotryae* sp. nov. isolates obtained from Iranian loquat canker clustered in a monophyletic lineage in all phylogenetic trees and shared a common ancestor with the two described species of *Paramicrosphaeropsis*, *P. ellipsoidea* and *P. iranica*. Because the morphological features of *Microsphaeropsis*, *Neomicrosphaeropsis* and *Paramicrosphaeropsis* are very similar, phylogenetic studies are required to ensure their accurate identification. However, our results showed that the ITS regions cannot differentiate these genera, and that housekeeping genes such as *rpb2* and *tub2* are required in addition. According to Hou et al. (2020), the ITS region is also unsuccessful in distinguishing 13 other genera from *Didymellaceae*. Phylogenetic analyses of the *rpb2* and *tub2* region in the present study revealed that our isolates clustered adjacent to *P. ellipsoidea* and *P. iranica*, as members of clade 24 sensu Hou et al. (2020).

Previous studies reported two *Paramicrosphaeropsis* species, *P. ellipsoidea*, isolated from decayed twigs of *Q. ilex* in Spain (Hou et al., 2020) and *P. iranica*, isolated from *Q. brantii* in Iran (Ahmadpour et al., 2022). However, no pathogenicity assessments of these species have been conducted to date and this study is the first to confirm the pathogenicity of a species of *Paramicrosphaeropsis*, namely *P. eriobotryae*, on loquat.

The present study is also the first report of loquat Paramicrosphaeropsis canker globally. This species was the most abundant taxon isolated from loquat orchards during our field survey. Only *P. eriobotryae* sp. nov. was found to be associated with trunks of loquat trees exhibiting symptoms of canker, trunk flaking and internal wood necrosis. Some of these infected trees with severe canker

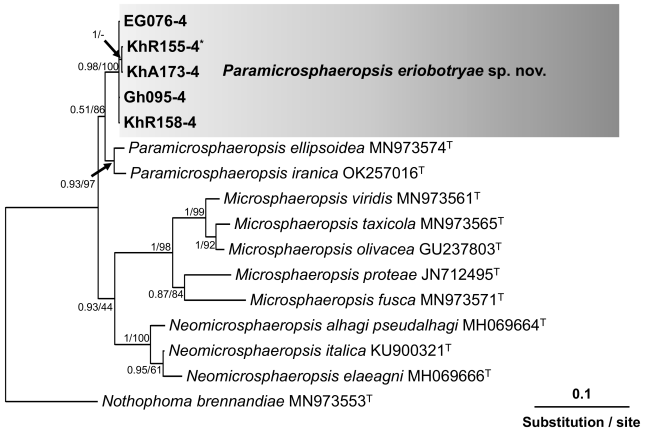

FIGURE 3 Phylogenetic relationships of *Paramicrosphaeropsis eriobotryae* sp. nov. from loquat orchards in Fars Province: relationships among five *Microsphaeropsis* species, three *Neomicrosphaeropsis* species and two *Paramicrosphaeropsis* species based on Bayesian inference analysis of multigene genealogies of ITS (internal transcribed spacers 1 and 2 and 5.8S gene of rDNA), *tub2* (partial β-tubulin gene) and *rpb2* (the second largest subunit of RNA polymerase II). Numbers on the nodes are Bayesian inference posterior probability values followed by maximum-likelihood bootstrap values. The tree was rooted to *Nothophoma brennandiae*. Ex-type isolates are indicated with ^{T}. *=ex-type of *P. eriobotryae* sp. nov.

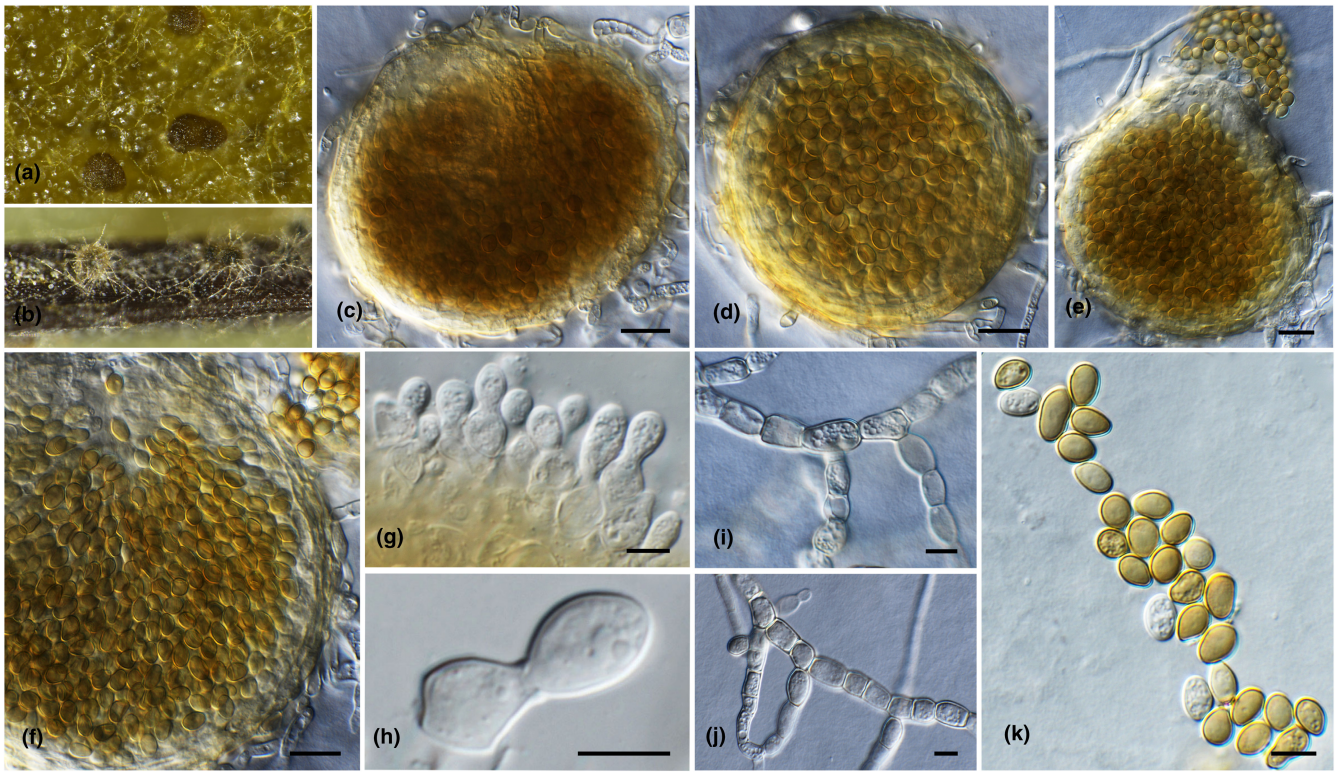

FIGURE 4 Morphological structures of *Paramicrosphaeropsis eriobotryae* sp. nov. (a) Pycnidia forming on oatmeal agar; (b) pycnidia forming on pine-needle agar; (c, d) pycnidia; (e) pycnidium with papillate ostiole; (f) section through pycnidial wall; (g, h) conidiogenous cells; (i, j) chlamydospores; (k) conidia. Scale bars: c–f = 20 μm; g–k = 10 μm.

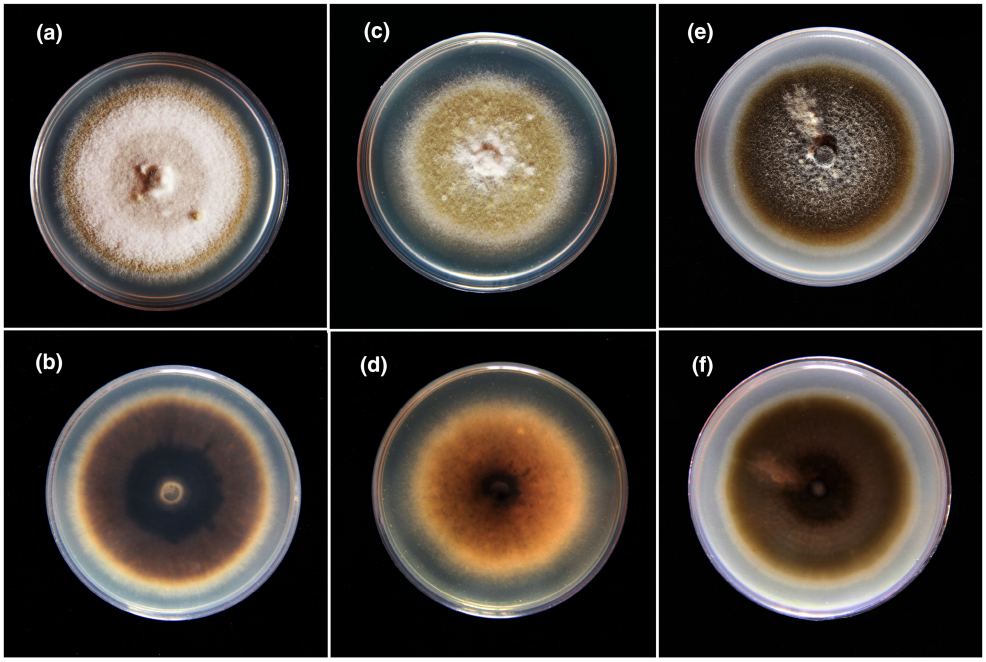

FIGURE 5 Colony morphology of *Paramicrosphaeropsis eriobotryae* sp. nov. on various media at 25°C, under fluorescent white light (12 h light/12 h dark) after 14 days. (a, b) Colony on potato dextrose agar (front and reverse); (c, d) colony on malt extract agar (front and reverse); (e, f) colony on oatmeal agar (front and reverse).

symptoms on their trunk also showed dieback and canker symptoms on their branches, but these symptoms could not be linked to *P. eriobotryae* sp. nov. Recently, new ascomycetous agents belonging to *Bionectriaceae* have also been obtained from the cross-sections of loquat branches with dieback, canker and internal wood necrosis symptoms in Iran (Bolboli et al., 2022). Our pathogenicity test

FIGURE 6 Pathogenicity test results of *Paramicrosphaeropsis eriobotryae* sp. nov. on a detached twigs of loquat after 14–20 days. (a) Healthy controls; (b) longitudinal sections of twigs with symptoms of brown blotches around the fungal blocks at the inoculation areas, and expansion of the blotches along the twigs. (c) Cross-sections of twigs with symptoms of discolouration of vascular tissues and internal wood necrosis.

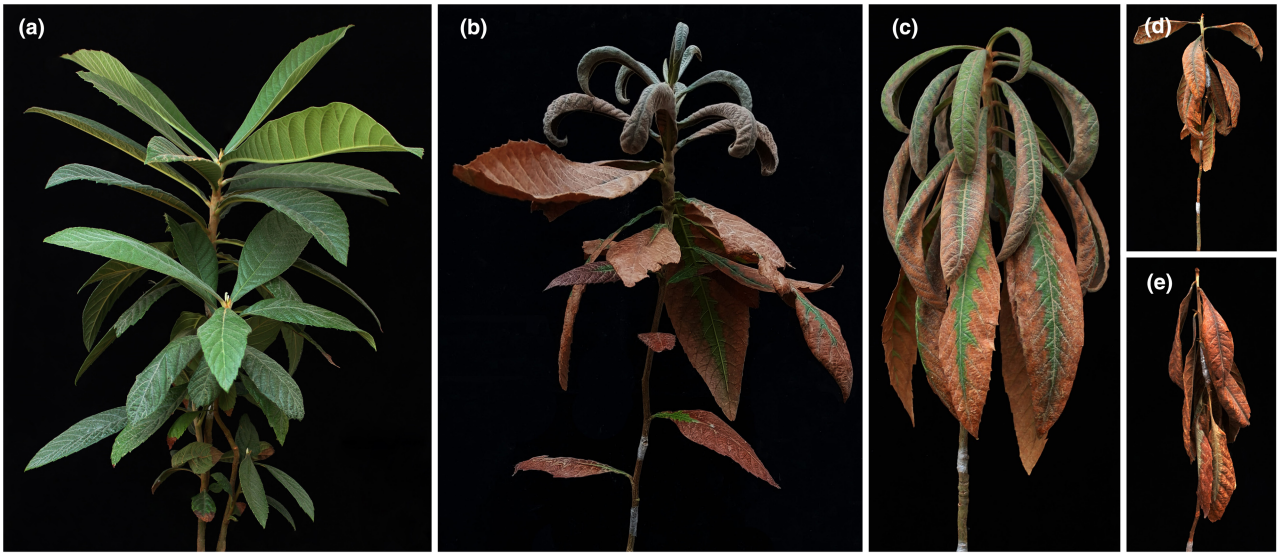

FIGURE 7 Pathogenicity test results of *Paramicrosphaeropsis eriobotryae* sp. nov. on 1-year-old loquat seedlings. (a) Healthy control. (b–d) Seedlings with symptoms of decline, growth reduction, drying leaves (margins) and defoliation. (e) Dying seedling.

on the detached twigs and 1-year-old loquat seedlings showed that *P. eriobotryae* sp. nov. could cause canker, trunk flaking, decline, leaf yellowing, defoliation and growth reduction in loquat trees. Disease symptoms, such as decline and cankers, were observed 10 months after inoculation, whereas Botryosphaeria canker only caused wounds at the inoculation site (Gonzalez-Domínguez et al., 2016). Because some 40-year-old loquat trees showed signs of canker and the disease was more prevalent in less cared for, old orchards, it can be concluded that predisposition to poor irrigation, drought stress and nutrient deficiencies can act as a stimulant for disease development.

Unexpectedly, one of the healthy-looking loquat trees sampled had internal wood necrosis in its branches, and an isolate of *P. eriobotryae* sp. nov. was obtained from this tree. This finding indicated that *P. eriobotryae* sp. nov. could be a latent pathogen, attacking plants due to changes in environmental conditions, such as drought. Moreover, the evaluation of host range on the detached branches

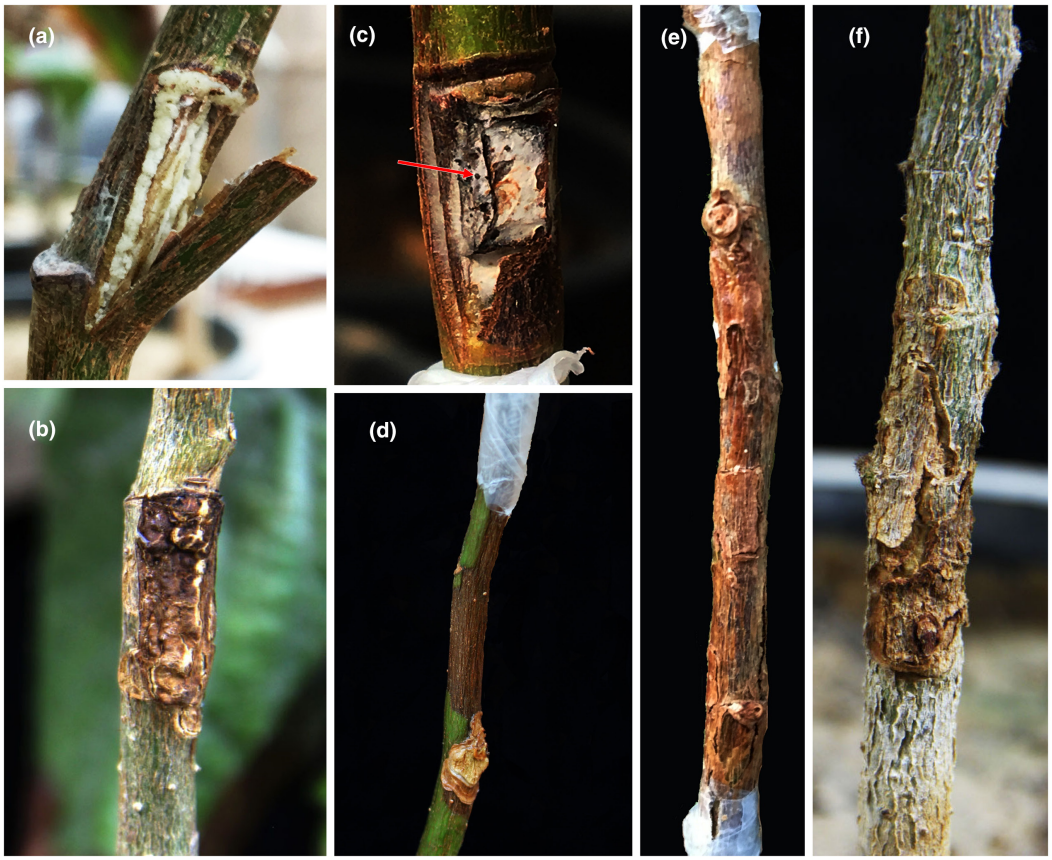

FIGURE 8 Reactions of seedlings to inoculation with *Paramicrosphaeropsis eriobotryae* sp. nov. (a, b) Plant reaction to pathogen (isolate KhB181-2) by creating callus tissue 3 weeks (a) and 1 year (b) after inoculation; (c) formation of pycnidia at the inoculation area (arrow) after 20–30 days (isolate EG076-4); (d) expansion of brown blotches outside the inoculation area. (e, f) Trunk canker and flaking about 1 year after inoculation (isolate KhH168-3).

of some horticulturally important trees adjacent to the loquat trees showed that isolates of *P. eriobotryae* sp. nov. were pathogenic on almonds and olives. Furthermore, in sampling such trees, another isolate of *P. eriobotryae* sp. nov. was also recovered from an almond tree with canker and dieback symptoms. Although *P. eriobotryae* sp. nov. could threaten loquat orchards and green spaces in semi-arid parts of southern Iran, the pathogen may also have a bridgehead effect to other fruit orchards in this region. The isolation of *P. eriobotryae* sp. nov. from an almond tree and its pathogenicity to other hosts indicate that this species might have a broader host range. Therefore, further surveys are required to determine its host range on other economically important trees.

This study is the first report of *P. eriobotryae* sp. nov. as an agent of loquat canker. Thus, it is important to survey for this pathogen in other economically significant regions of loquat production in Iran and worldwide. Furthermore, developing a molecular method to detect the pathogen in asymptomatic trees could be part of the disease management protocols.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets generated during and analysed during the current study are in supplementary tables or available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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