



Phylogeny and taxonomy of four *Ramularia* species on trees and shrubs

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Abstract

Ramularia is a species-rich genus in the order *Mycosphaerellales* (*Dothideomycetes*, *Ascomycota*) that includes numerous phytopathogenic taxa which cause typical leaf spots, necrosis, or chlorosis on woody and herbaceous plants. During survey of ramularioid fungi causing leaf spot symptoms in northern Iran, several *Ramularia* isolates were identified from symptomatic leaves on woody plants. A polyphasic approach, combining morphological observations, cultural data, and multigene phylogeny (ITS, *ACT*, *TEF1-α*, *HIS*, and *GAPDH*), was used to classify these isolates. The isolates were grouped in four species clades including, *R. eucalypti* on *Rubus criticus* and *R. discolor*; *R. glennii* on *Acer palmatum*, *Alnus subcordata*, *Eriobotrya japonica*, *Eucalyptus* sp., *Ligustrum vulgare*, *Punica granatum*, and *Viburnum opulus*; *R. vizellae* on *Zelkova carpinifolia*; and a new species *Ramularia zelkovae* on *Zelkova carpinifolia*. Notably, *R. eucalypti* and *R. vizellae* are new records for the funga of Asia. Additionally, *Rubus criticus* and *R. discolor* are newly documented hosts for *R. eucalypti* while, *Zelkova carpinifolia* is a new host for *R. vizellae*. *Acer palmatum*, *Alnus subcordata*, *Eriobotrya japonica*, *Ligustrum vulgare*, *Punica granatum*, and *Viburnum opulus* are reported as new hosts of *R. glennii*.

Keywords Leaf spots · *Mycosphaerellaceae* · New taxon · Polyphasic approach · Ramularioid fungi · West Asia

Introduction

Species of *Ramularia* Unger are primarily recognized as phytopathogenic fungi affecting a wide variety of woody and herbaceous plants. Beyond their role as pathogens, *Ramularia* species can also be endophytic, saprobic, hyperparasitic, and occasionally pathogenic to humans (Braun 1995, 1998; Walters et al. 2008; Wieczorek et al. 2014; Videira et al. 2015a, b, 2016; Bakhshi et al. 2021a, b). Plant pathogenic taxa usually develop under conditions of high air humidity and low temperatures (Braun 1995, 1998; Videira et al. 2016) and cause leaf spots, necrosis, or chlorosis that may lead to early defoliation in diverse agricultural crops,

medicinal and ornamental plants, fruit and forest trees, etc. (Braun 1995, 1998; Videira et al. 2015a, b, 2016; Bakhshi et al. 2021b).

The genus *Ramularia* is classified under the family *Mycosphaerellaceae* (*Mycosphaerellales*, *Dothideomycetes*, *Ascomycota*). According to Braun (1995, 1998), *Ramularia* species are characterized by their hyaline conidiophores and conidia, which feature distinct, thickened, darkened, and refractive conidial scars and hila. Traditionally, species identification within this genus relied on host plant taxonomy and morphological traits, such as the shape, size, and septation of conidia as well as the type of conidiogenous loci and conidial hila (Braun 1995, 1998). However, due to broad host ranges of many *Ramularia* taxa, accurate species identification based solely on host data has proven challenging. The morphological overlap among species with similar dimensions has further complicated taxonomy (Videira et al. 2015a, b, 2016). Consequently, recent phylogenetic studies employing molecular markers such as LSU, ITS, *ACT*, *TEF1-α*, *GAPDH*, *HIS*, and *RPB2* have highlighted the necessity of molecular data for precise identification (Videira et al. 2015a, b, 2016; Bakhshi and Arzanlou 2017; Bakhshi et al. 2021a, b).

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In Iran, *Ramularia* species are found on a wide range of plant hosts, with a notable concentration in the northern regions due to favorable conditions of high humidity (Bakhshi et al. 2021a, b, 2022; Pirnia et al. 2012; Bicharanlou et al. 2014; Behrooz et al. 2015, 2017; Bakhshi and Arzanlou 2017; Bakhshi 2018; Ershad 2022). This study aims to identify *Ramularia* species associated with different woody hosts collected in the northern Iran by integrating both morphological and molecular data.

Materials and methods

Fungal collection and isolation

From 2017 to 2020, field surveys were conducted in the provinces Guilan, Golestan, and Mazandaran in the north of Iran. Leaf spot samples were collected from various woody hosts and brought to the laboratory for further processing. Single spore isolation described by Bakhshi et al. (2021b) was followed to obtain pure cultures of emerging fungi and maintained on malt-extract agar (MEA; Merck, Darmstadt, Germany). The specimens were deposited in the Fungarium of the Iranian Research Institute of Plant Protection (IRAN), Tehran, Iran, and the representative living fungal cultures are maintained in both the culture collection at IRAN and the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands.

Morphological observation and species descriptions

Microscopic fungal structures were examined from isolates sporulating both *in planta* and *in vitro*. For the morphological study in planta, measurements and descriptions of microscopic structures including stromata, conidiophores, conidiogenous cells and conidia, were taken from diseased leaf tissues and mounted on glass slides with Shear's mounting fluid. For the morphological study *in vitro*, slides were prepared using the inclined coverslip method (Nugenta et al. 2006) and also transparent adhesive tape (Bensch et al. 2012), from colonies sporulating on synthetic nutrient-poor agar plates (SNA; Crous et al. 2019), incubated at 21 °C with a day/night regime for 7–15 days (Videira et al. 2016; Bakhshi and Arzanlou 2017; Bakhshi et al. 2021b) to induce sporulation. Observations were made with an Olympus BX51 light microscope equipped with differential interference contrast (DIC) illumination and images were captured using an Olympus DP25 high-definition color camera. Colony characteristics were assessed by inoculating cultures in triplicate on MEA and PDA at 25 °C in the dark. The Rayner (1970) color chart was used to record colony colors. The name proposed for the newly identified species was registered at Index Fungorum (<https://indexfungorum.org/>).

Fungal DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted directly from fungal colonies growing on MEA following Möller et al. (1992). DNA samples were diluted to a range of 10–20 ng/μL prior to PCR amplification. Amplifications targeted the ITS and fragments of the *ACT* (actin), *TEF1-α* (translation elongation factor 1-α), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and *HIS* (histone H3) genes using protocols from Bakhshi and Braun (2022) for ITS; Bakhshi et al. (2015, 2021b) for *ACT*, *TEF1-α*, and *HIS*; and Bakhshi and Arzanlou (2017) for *GAPDH*. PCR products were sequenced in both directions by Microsynth (Balgach, Switzerland).

Sequence alignment and phylogenetic analysis

Sequences were trimmed and edited using MEGA v. X (Molecular Evolutionary Genetics Analysis) software (Kumar et al. 2018). Phylogenetic analysis was performed on ITS, *ACT*, *TEF1-α*, *HIS*, and *GAPDH* sequences, incorporating both sequences generated in this study (Table 1) and those of ex-type and reference strains of *Ramularia* spp. from GenBank (Videira et al. 2015a, b, 2016; Bakhshi and Arzanlou 2017; Bakhshi et al. 2021b). *Xenoramularia neerlandica* Videira & Crous (isolate CBS 141101) was used as an outgroup. Each gene locus was aligned separately using MAFFT v. 7 online (<http://mafft.cbrc.jp/alignment/server/>) with default settings (Katoh and Standley 2013) followed by manual adjustment in MEGA v. X. The alignments of individual gene loci were concatenated in Mesquite v. 3.81 (Maddison and Maddison 2023). Bayesian inference (BI) and maximum parsimony (MP) methods were employed to assess the phylogenetic relationships within the aligned combined dataset. The BI analysis of the combined dataset was conducted using MrBayes v. 3.2.6 (Ronquist et al. 2012) under the best-fit nucleotide substitution model estimated by MrModeltest v. 2.3 (Nylander 2004). The heat parameter was set at 0.15 and the Markov chain Monte Carlo (MCMC) analysis of four chains started in parallel from a random tree topology, and lasted until the average standard deviation of split frequencies reached a value of 0.01. A burn-in period of 25% was applied, after which, posterior probabilities of the majority rule consensus tree were calculated based on remaining trees. The MP analysis was conducted using PAUP v. 4.0 (Swofford 2003), employing 10,000,000 replicates of fast stepwise search along with a tree-bisection-reconnection (TBR) branch-swapping algorithm. All characters were considered unordered and assigned equal weight, with

Table 1 List of fungal isolates included in this study and their corresponding GenBank accession numbers

Species	Culture accession number(s) ¹	Host	GenBank accession numbers ²				
			ITS	ACT	TEF1- α	HIS	GAPDH
<i>Ramularia eucalypti</i>	IRAN 4790C	<i>Rubus discolor</i>	PQ433380	PQ441769	PQ441758	PQ441783	PQ441794
<i>Ramularia eucalypti</i>	IRAN 5085C	<i>Rubus criticus</i>	–	PQ441770	PQ441759	PQ441784	PQ441795
<i>Ramularia glennii</i>	IRAN 4848C	<i>Acer palmatum</i>	PQ433381	PQ441771	PQ441760	PQ441785	PQ441796
<i>Ramularia glennii</i>	IRAN 4850C	<i>Alnus subcordata</i>	–	PQ441772	PQ441761	PQ441786	PQ441797
<i>Ramularia glennii</i>	IRAN 4849C	<i>Viburnum opulus</i>	PQ433382	PQ441773	PQ441762	PQ441787	PQ441798
<i>Ramularia glennii</i>	IRAN 4851C	<i>Punica granatum</i>	PQ433383	PQ441774	PQ441763	PQ441788	PQ441799
<i>Ramularia glennii</i>	IRAN 4852C	<i>Eucalyptus</i> sp.	PQ433384	PQ441775	–	PQ441789	PQ441800
<i>Ramularia glennii</i>	IRAN 4853C	<i>Eucalyptus</i> sp.	PQ433385	PQ441776	–	PQ441790	PQ441801
<i>Ramularia glennii</i>	IRAN 4854C	<i>Eucalyptus</i> sp.	PQ433386	PQ441777	–	PQ441791	PQ441802
<i>Ramularia glennii</i>	P 780 II	<i>Ligustrum vulgare</i>	PQ433387	PQ441778	PQ441764	PQ441792	–
<i>Ramularia glennii</i>	IRAN 4847C	<i>Eriobotrya japonica</i>	PQ433388	PQ441779	PQ441765	PQ441793	PQ441803
<i>Ramularia vizellae</i>	IRAN 4791C	<i>Zelkova carpinifolia</i>	PQ433389	PQ441780	PQ441766	–	–
<i>Ramularia zelkovae</i>	CBS 150863 = IRAN 3984C (T)	<i>Zelkova carpinifolia</i>	PQ433390	PQ441781	PQ441767	–	PQ441804
<i>Ramularia zelkovae</i>	IRAN 3976C	<i>Zelkova carpinifolia</i>	PQ433391	PQ441782	PQ441768	–	PQ441805

¹CBS culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; IRAN culture collection of the Iranian Research Institute of Plant Protection, Tehran, Iran

²ITS internal transcribed spacers and intervening 5.8S nrDNA, ACT actin, TEF1- α translation elongation factor 1- α , HIS histone H3, GAPDH partial glyceraldehyde-3-phosphate dehydrogenase gene

alignment gaps treated as a fifth character state. Branches with zero length were collapsed, and all multiple trees that were equally most parsimonious were saved. The robustness of the clades in the resulting trees was evaluated using 1000 bootstrap replications. Various tree metrics, including tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI), were computed. All resulting phylogenetic trees were visualized with Geneious 8.1.8 (Kearse et al. 2012) and edited in Adobe® Illustrator 2024 Artwork v. 28.0. All new sequences were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) (Table 1). Sequence alignments and data matrices used for phylogenetic analysis were deposited in figshare.com (<https://doi.org/10.6084/m9.figshare.27175959>).

Results

Phylogeny

A phylogenetic analysis was conducted on 118 taxa, including 117 strains of *Ramularia* spp. (103 strains from NCBI, and 14 strains from this study) and one outgroup, using a combined dataset of five loci with alignment gaps (2362 bp): ITS (542 bp), ACT (232 bp), TEF1- α (570 bp), GAPDH (644 bp), and HIS (374 bp). The length of the final concatenated alignment was 2362 positions and included

117 strains of *Ramularia* spp. and one outgroup taxon. The results of the MrModelTest analyses revealed that the ITS partition had fixed (equal) state frequency distribution, whereas all the other partitions employed a dirichlet (1, 1, 1, 1) state frequency distributions. The substitution models optimized for each locus were as follows: SYM with equal rate variation across sites for ITS, HKY + G with gamma-distributed rate variation for TEF1- α , and GTR + I + G with invariant-gamma-shaped rate variation for ACT, GAPDH, and HIS. The Bayesian analysis was performed over 11,510,000 generations and resulting in 23,022 trees. After discarding 5754 trees (25% burn-in), posterior probabilities (PP) were calculated from the remaining 17,268 trees, with the final consensus tree depicted in Fig. 1. The alignment contained a total of 1285 unique site patterns (208, 176, 364, 366, and 171 for ITS, ACT, TEF1- α , GAPDH, and HIS, respectively). The robustness of nodes based on the bootstrap support values (MP-BS) were indicated as the second value on the Bayesian tree (Fig. 1). Among the analysed characters, 924 were constant, 450 were variable and parsimony-uninformative, while 1020 were parsimony-informative. A parsimony consensus tree was generated from the equally most parsimonious trees, with branches represented by thicker lines on the Bayesian tree (length = 11,832, CI = 0.255, RI = 0.542, RC = 0.138, HI = 0.745). To test the robustness of the new species clade, individual Bayesian analyses were performed for each locus using their respective substitution models.

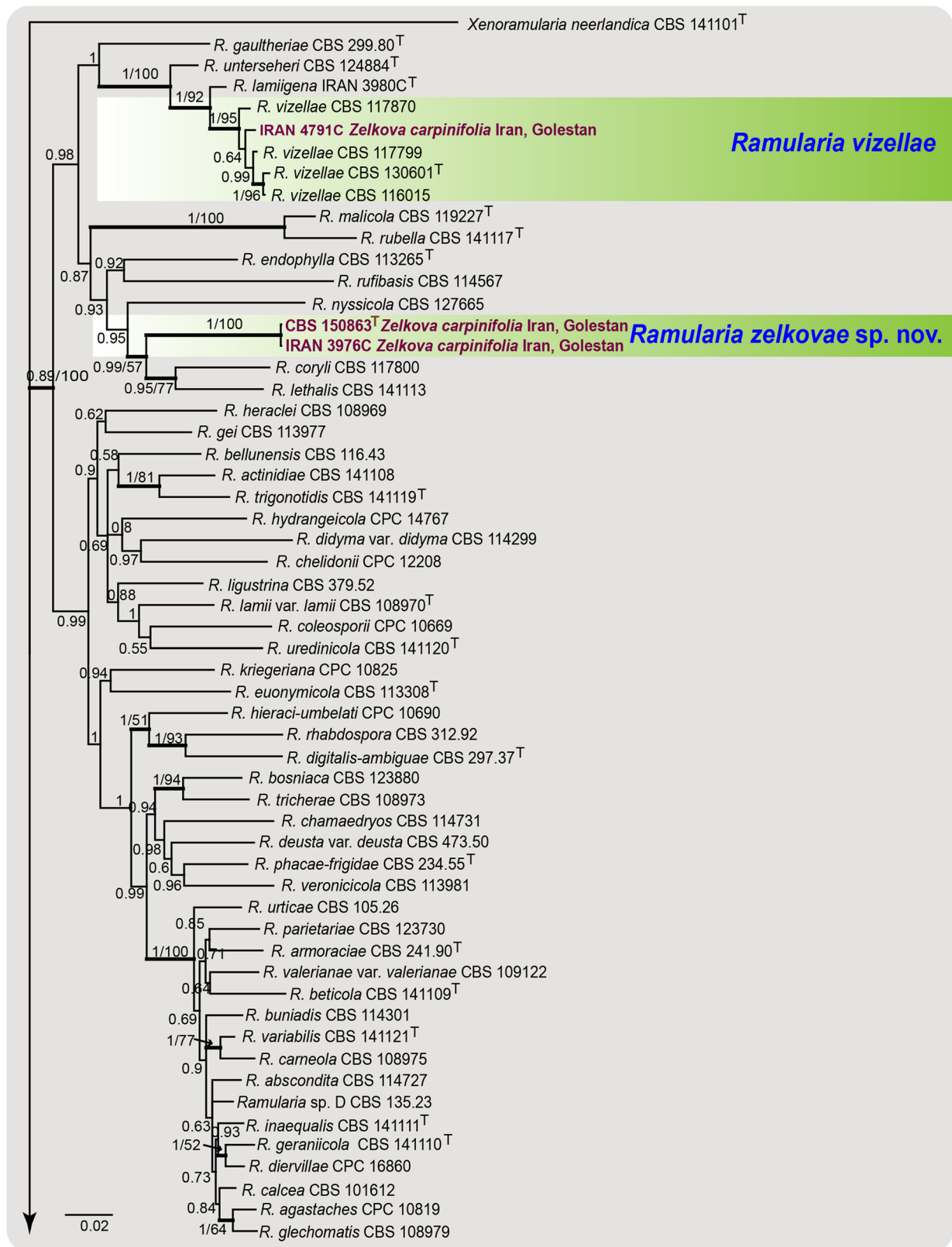


Fig. 1 Consensus phylogram (50% majority rule) resulting from a Bayesian analysis of the combined five-gene (ITS, *ACT*, *TEF1- α* , *HIS* and *GAPDH*) sequence alignment using MrBayes v. 3.2.6. The Bayesian posterior probabilities (PP) and maximum parsimony boot-

strap support values (MP-BS) are given at the nodes (PP/MP-BS), and the bar indicates 0.02 expected changes per site. Type strains are indicated by the letter “T”. The tree was rooted to *Xenoramularia neerlandica* (CBS 141101)

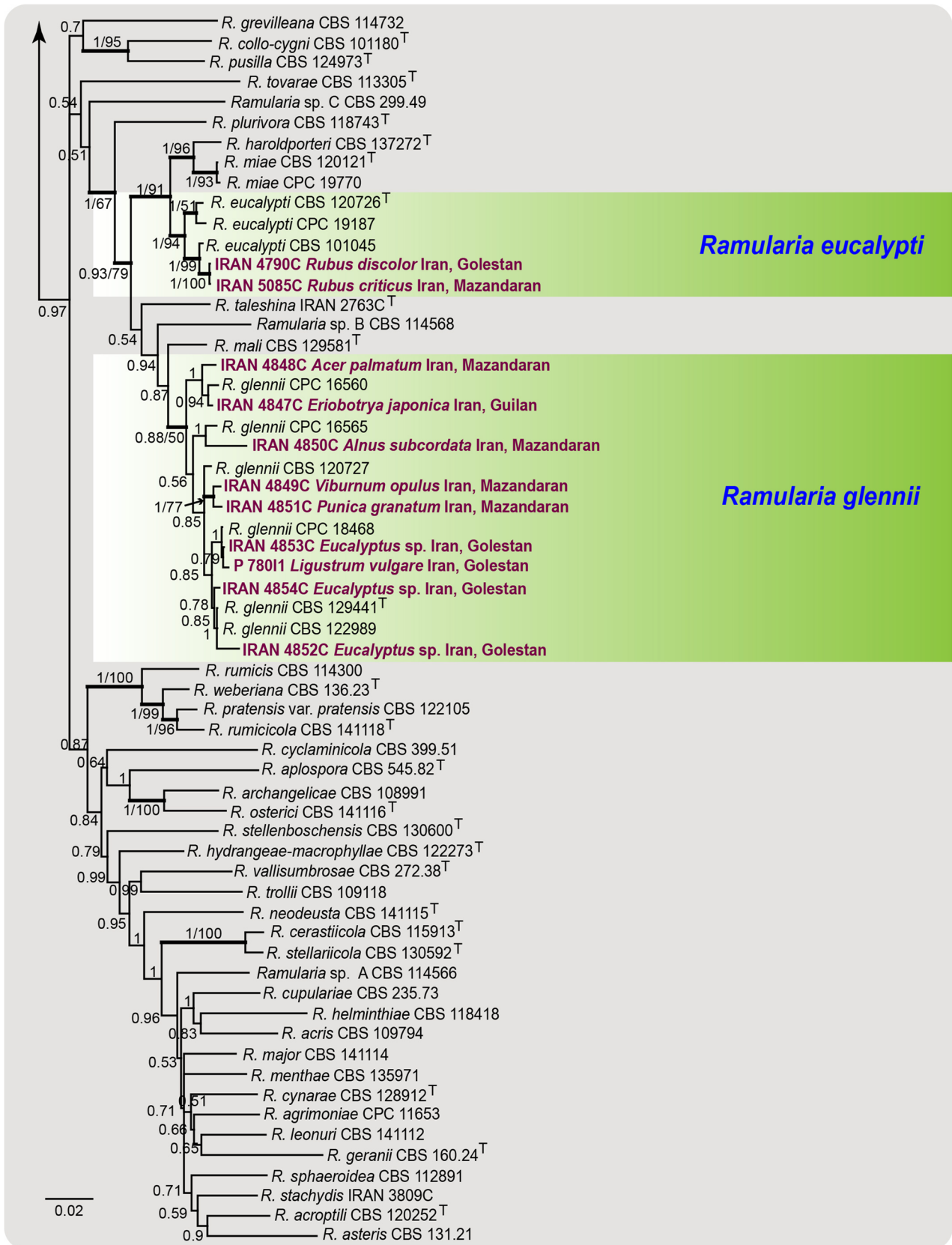


Fig. 1 (continued)

Taxonomy

Based on phylogenetic analyses, the isolates collected in this study from various trees and shrubs are grouped in four clades (Fig. 1) that are assignable to the following species:

Ramularia eucalypti Crous, Fungal Diversity 26: 174 (2007) Fig. 2.

Description *in vitro*: on SNA: Mycelium consisting of smooth to slightly verruculose, hyaline, septate, branched hyphae, 1–2.5 μm wide. Conidiophores reduced to conidiogenous cells. Conidiogenous cells thin-walled, hyaline, smooth, terminal and lateral on hyphae, (5–)9–16(–22) $\times$ 1(–2.5) μm , sympodial proliferation with 1–3(–4) apical loci, flattened or short cylindrical; loci thickened, darkened, refractive, 0.5–1 μm diam. Conidia hyaline, thin-walled, smooth to slightly verruculose. Ramoconidia subcylindrical to fusiform, 0(–1)-septate, (6–)10–13 $\times$ (1.5–)2–3 μm , with 1–2 apical hila. Intercalary conidia, oval to fusiform, aseptate, (4.5–)7–8(–10) $\times$ 1.5–2(–2.5) μm , in branched chains of up to 9. Terminal conidia aseptate, obovoid, (3–)4–5(–6) $\times$ 1–2 μm ; hila thickened, darkened, 0.5–1 μm diam.

Cultural characteristics: On MEA, surface folded, smoke-grey, radially striated, with sparse fluffy aerial mycelium, with lobate, concave margins, reverse iron-grey, reaching 26 mm after 20 days at 25 °C.

Material examined: Iran: Mazandaran Province, Sangdeh, 36° 08' 05" N, 53° 12' 49" E, alt. 1400 m, on

Rubus creticus Tourn. ex L. (*Rosaceae*), 4 Nov. 2017, M. Bakhshi, IRAN 18448F, culture IRAN 5085C; Golestan Province, Aliabad Katul, Afratakhteh, 36° 49' 32" N, 55° 00' 26" E, alt. 540 m, on *Rubus discolor* Weihe & Nees (*Rosaceae*), 10 Nov. 2019, M. Bakhshi, IRAN 18449F, culture IRAN 4790C.

Ramularia glennii Videira & Crous, Persoonia 34: 57 (2014).

Description *in vitro*: Bakhshi and Arzanlou (2017).

Material examined: Iran: Mazandaran Province, Nowshahr, Botanic Garden, 36° 39' 00" N, 51° 30' 50" E, alt. 1 m, on *Acer palmatum* Thunb. (*Sapindaceae*), 3 Aug. 2019, M. Bakhshi, IRAN 18451F, culture IRAN 4848C; Mazandaran Province, Nowshahr, Botanic Garden, 36° 39' 00" N, 51° 30' 50" E, alt. 1 m, on *Alnus subcordata* C.A.Mey. (*Betulaceae*), 3 Aug. 2019, M. Bakhshi, IRAN 18453F, culture IRAN 4850C; Mazandaran Province, Nowshahr, Botanic Garden, 36° 39' 00" N, 51° 30' 50" E, alt. 1 m, on *Viburnum opulus* L. (*Viburnaceae*), 3 Aug. 2019, M. Bakhshi, IRAN 18452F, culture IRAN 4849C; Mazandaran Province, Amol, 36° 28' 31" N, 52° 27' 56" E, on *Punica granatum* L. (*Lythraceae*), 11 Nov. 2019, M. Bakhshi, IRAN 18454F, culture IRAN 4851C; Golestan Province, Azadshahr-Aliabad Katoul, Khanbebin, Sadabad Fenderesk, 36° 59' 02" N, 54° 58' 21" E, alt. 663 m, on *Eucalyptus* sp. (*Myrtaceae*), 30 April 2020, M. Bakhshi, IRAN 18455F, cultures IRAN 4852C, IRAN 4853C, IRAN 4854C. Golestan Province, Aghghala, 36° 59' 02" N, 54° 58' 21" E, alt. 663 m, on *Ligustrum vulgare* L.

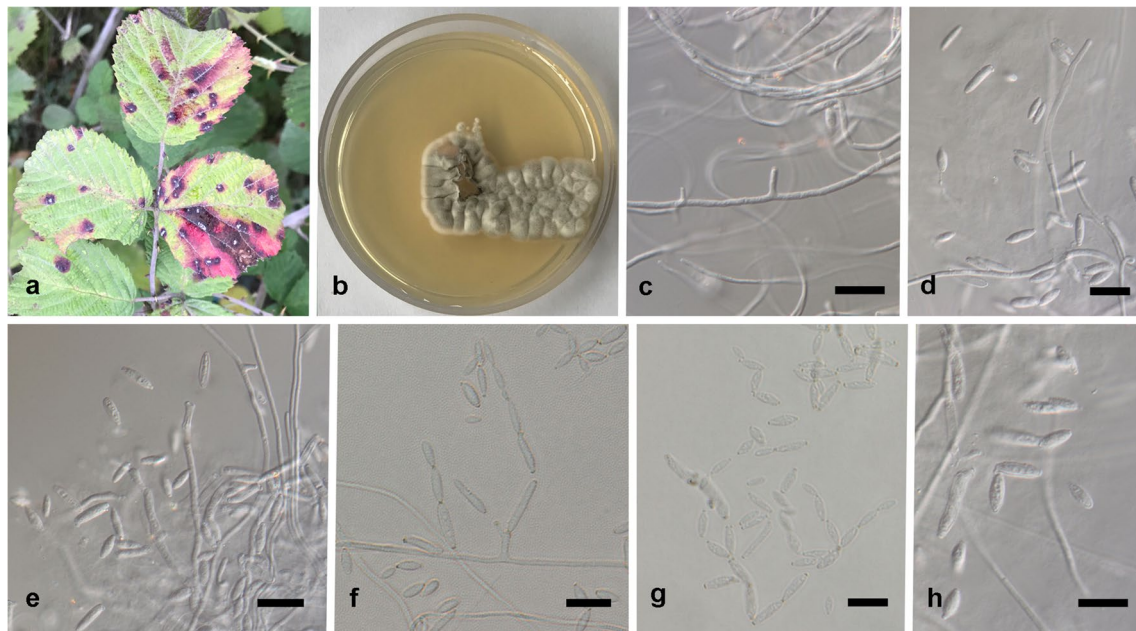


Fig. 2 *Ramularia eucalypti* (IRAN 4790C). **a** Leaf spot symptoms on the host. **b** Culture on MEA. **c, d, e, f, g, h** Hyphae, conidiophores and conidia. Bars = 10 μm

(*Oleaceae*), Oct. 2018, M. Bakhshi, culture P 780 I1. Guilan Province, Jowkandan, on *Eriobotrya japonica* (Thunb.) Lindl. (*Rosaceae*), Oct. 2018, S. Moradian, IRAN 18450F, culture IRAN 4847C.

Ramularia vizellae Crous, Persoonia 27: 37 (2011) Fig. 3.

Description in vitro: On SNA: *Mycelium* consisting of smooth to slightly verruculose, hyaline, septate, branched hyphae, 1–2.5 μm wide. *Conidiophores* solitary, terminal or lateral on hyphae, smooth, hyaline, cylindrical, straight to curved, (4–)18–45(–78) $\times$ 1.5–2(–2.5) μm or reduced to conidiogenous cells. *Conidiogenous cells* thin-walled, hyaline, smooth, terminal and lateral on conidiophores, 4–8(–23) $\times$ 1.5–2 μm , sympodial proliferation with 1–3 apical loci; loci thickened, darkened, refractive, 1–1.5 μm diam. *Conidia* hyaline, thin-walled, smooth to finely verruculose. *Ramoconidia* subcylindrical to obclavate or ellipsoid, 0–1(–3)-septate, (6.5–)8.5–14(–19) $\times$ 3–4(–5) μm , with 1–3 apical hila. *Intercalary conidia*, aseptate or rarely 1-septate, ellipsoid, (5–)7–11 $\times$ 2.5–3.5 μm , in branched chains of up to 6. *Terminal conidia* aseptate, ellipsoid, 3–5(–6.5) $\times$ 2–3(–3.5) μm ; hila thickened, darkened, 0.5–1 μm wide.

Cultural characteristics: On MEA, surface erumpent, folded, smoke-grey to dirty cream, with moderate aerial mycelium, smooth, lobate margins, reverse grey, reaching 11 mm after 20 days at 25 $^{\circ}\text{C}$. On PDA, surface raised, folded, dirty cream, with entire lobate margins, reverse dirty cream, reaching 12 mm after 20 days at 25 $^{\circ}\text{C}$.

Material examined: Iran: Golestan Province, Golestan National Park, Tangerang, 37 $^{\circ}$ 23' 57" N, 55 $^{\circ}$ 47' 53" E, alt. 300 m, on *Zelkova carpinifolia* (Pall.) K. Koch (*Ulmaceae*), 1 May 2020, M. Bakhshi, IRAN 18458F, culture IRAN 4791C.

Ramularia zelkovae Bakhshi, Zare & Druzhin., *sp. nov.* Figs. 4 and 5.

Index Fungorum IF 902830.

Etymology. Named after the host genus on which it was collected, *Zelkova*.

Holotype. Iran: Golestan Province, Golestan National Park, Tangebol, 37 $^{\circ}$ 22' 15.82" N, 55 $^{\circ}$ 47' 53" E, alt. 700 m, on leaves of *Zelkova carpinifolia* (*Ulmaceae*), 4 Nov. 2017, M. Bakhshi, IRAN 18456F.

Ex-type strain. CBS 150863 = IRAN 3984C.

Description in planta: *Leaf spots* amphigenous, sub-circular to circular, 2–6 mm diam., brown to grey, with definite dark brown border and yellow halo. *Caespituli* mostly hypophyllous, occasionally amphigenous, white. *Mycelium* internal and external. *Conidiophores* arising from internal hyphae, emerging through stomata in solitary or in small loose fascicles or from smooth to slightly verruculose, hyaline, septate, branched, 1–2 μm wide superficial hyphae, simple, hyaline, thin-walled, smooth to slightly verruculose, subcylindrical, straight to flexuous or moderately geniculous-sinuuous, (7.5–)17–26(–55) $\times$ 1.5–2(–3) μm , septate or reduced to conidiogenous cells. *Conidiogenous cells* hyaline, smooth

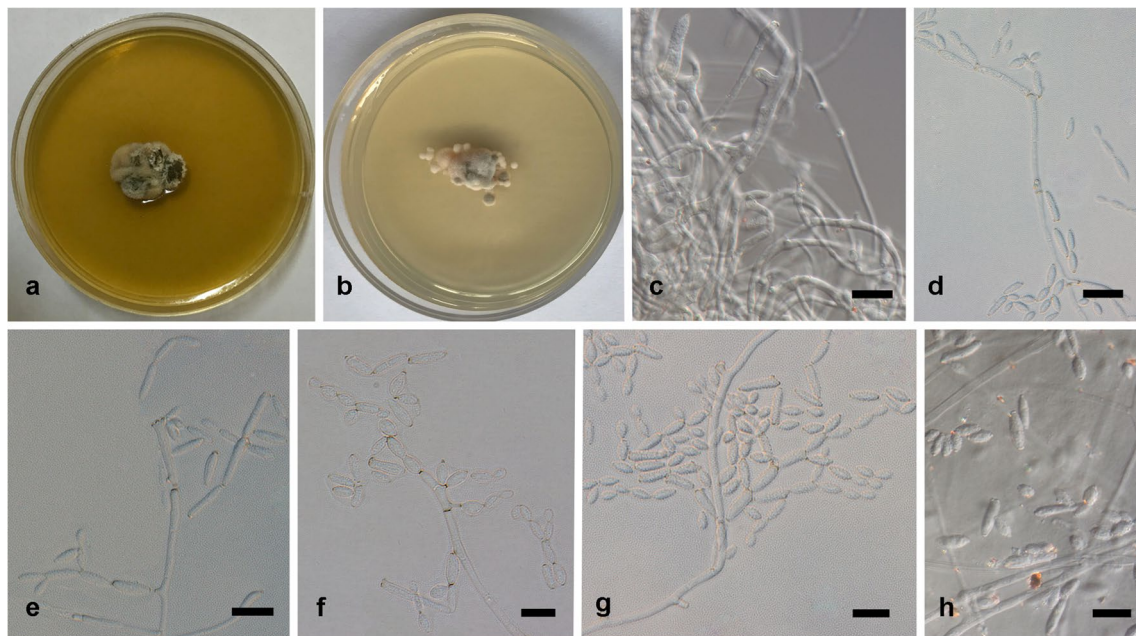


Fig. 3 *Ramularia vizellae* (IRAN 4791C). **a** Culture on MEA. **b** Culture on PDA. **c, d, e, f, g, h** Hyphae, conidiophores and conidia. Bars = 10 μm

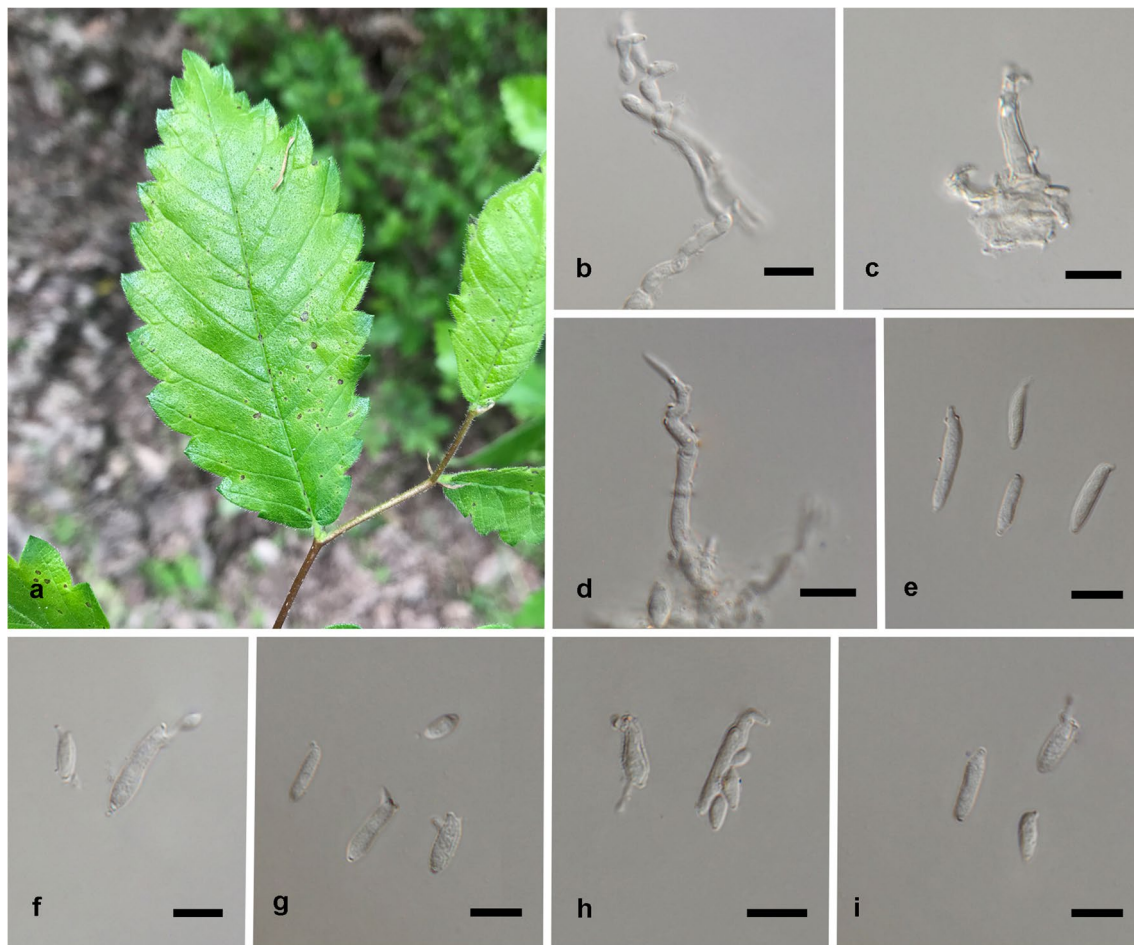


Fig. 4 *Ramularia zelkovae* (IRAN 18456F). **a** Leaf spot symptoms on the host. **b c, d, e, f, g, h i** Hyphae, conidiophores and Conidia. Bars = 10 μ m

to slightly verruculose, terminal or intercalary on conidiophore or superficial hyphae, proliferating sympodially, conidiogenous loci 1 or more, apical or formed on shoulders caused by geniculation, thickened, darkened, refractive, 1–2 μ m diam. *Conidia* hyaline, thin-walled, smooth to finely verruculose, solitary or catenate, in simple or branched chains, subcylindrical, to fusiform or obclavate, 0–1(–2)-septate, apex subobtuse and base subtruncated, (3–)5.5–13(–22) $\times$ (1.5–)2–3(–4) μ m; hila, thickened and darkened, 1–2 μ m wide.

Description in vitro: On SNA: *Mycelium* consisting of smooth to slightly verruculose, hyaline, septate, branched 1–2 μ m wide hyphae. *Conidiophores* solitary, terminal or lateral on hyphae, smooth to slightly verruculose, hyaline, simple or branched, cylindrical, straight to sometimes geniculate-sinuous, (3.5–)17–29(–65) $\times$ (1–)2–2.5(–3) μ m or reduced to conidiogenous cells. *Conidiogenous cells* thin-walled, hyaline, smooth to slightly verruculose, terminal and lateral on conidiophores, (3.5–)7.5–10.5(–17) $\times$ (1–)2–2.5(–3) μ m, sympodial proliferation with 1–4 apical

loci; loci almost flat to cylindrical-protuberant, thickened, darkened, refractive, 0.5–1.5 μ m diam. *Conidia* hyaline, thin-walled, smooth to finely verruculose. *Ramoconidia* subcylindrical to fusiform or obclavate, 0–1(–3)-septate, (6–)11.5–15(–20) $\times$ (1.5–)2–3(–4) μ m, with 1–4 apical or lateral, flat to cylindrical-protuberant hila, occasionally showing microcyclic conidiation. *Intercalary conidia*, 0–1(–2)-septate, subcylindrical to fusiform, (5.5–)6–10 $\times$ 1–1.5(–2) μ m, in branched chains of up to 9. *Terminal conidia* aseptate, subcylindrical or obclavate, 3.5–5.5(–8.5) $\times$ 1–1.5(–2) μ m; hila thickened, darkened, 0.5–1 μ m wide.

Cultural characteristics: On MEA, surface erumpent, folded, mostly dirty cream but with smoke-grey tones, with sparse fluffy aerial mycelium, and concave, lobate margin, reverse isabelline, reaching 14 mm after 20 days at 25 $^{\circ}$ C.

Additional material examined: Iran: Golestan Province, Gorgan, on *Zelkova carpinifolia*, 10 Nov. 2019, M. Bakhshi, IRAN 18457F, culture IRAN 3976C.

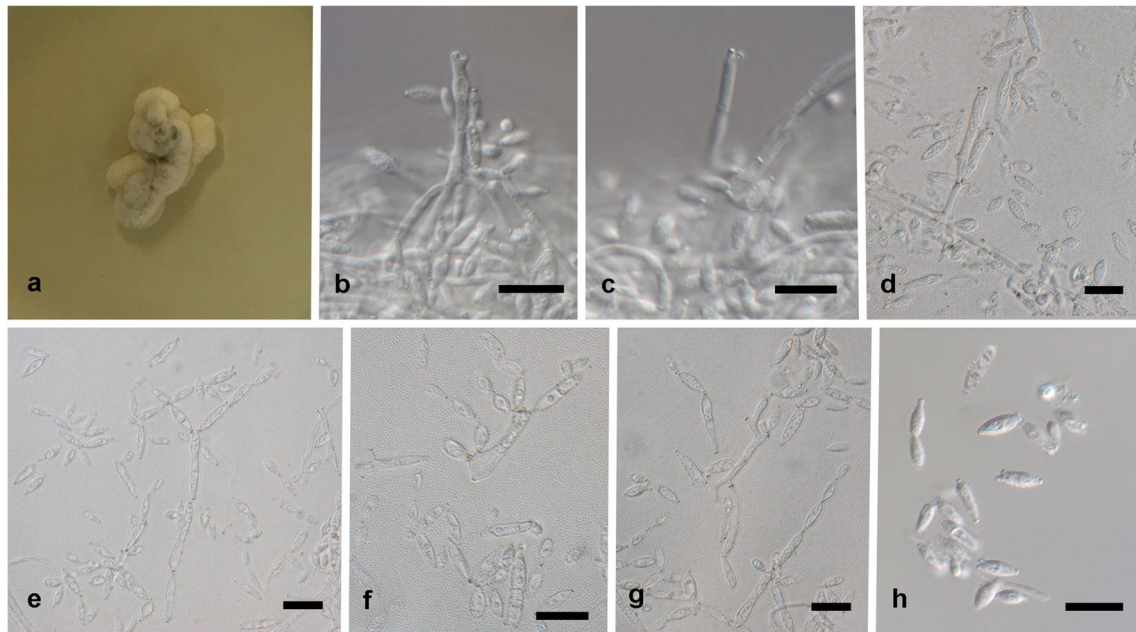


Fig. 5 *Ramularia zelkovae* (CBS 150863 = IRAN 3984C). **a** Culture on MEA. **b c, d, e, f, g, h** Hyphae, conidiophores and conidia. Bars = 10 μ m

Discussion

The current study aimed to distinguish *Ramularia* species discovered on some woody plants in the northern zones of Iran based on multi-locus DNA sequence analyses together with host data and morphological characteristics. Members of the genus *Ramularia* are mostly phytopathogenic and associated with leaf spots, necrosis, or chlorosis, also on economically important woody and herbaceous plants, but some species can be endophytic, saprobic, or even human pathogenic (Braun 1995, 1998; Videira et al. 2015a, b, 2016; Bakhshi and Arzanlou 2017; Bakhshi et al. 2021a, b). Videira et al. (2015a, b, 2016) revised the delimitation of *Ramularia* and related genera by integrating morphological and molecular data. Their research underscored the necessity of employing multi-gene phylogenetic markers for precise species identification within *Ramularia* and allied genera. This approach addresses the complexities in distinguishing species and enhances the accuracy of taxonomic classifications in these fungi.

This study investigated *Ramularia* isolates associated with leaf spot symptoms across various tree and shrub species from the families *Betulaceae*, *Lythraceae*, *Myrtaceae*, *Oleaceae*, *Rosaceae*, *Sapindaceae*, *Ulmaceae*, and *Viburnaceae*. A multi-gene phylogenetic approach utilizing ITS, *ACT*, *TEFI- α* , *GAPDH*, and *HIS* sequences was employed. The findings revealed that these isolates grouped into four distinct species clades: *R. eucalypti*, *R. glennii*, *R. vizellae*, and the new species *R. zelkovae*. Consistent with previous studies (Videira et al. 2015a; Hernandez-Restrepo et al.

2016), *R. eucalypti* was shown to have a broad host range such as *Geraniaceae* (*Geranium pusillum* L.), *Myrtaceae* [*Corymbia grandifolia* (R.Br. ex Benth.) K.D. Hill & L.A.S. Johnson, *Eucalyptus grandifolia* R. Br. ex Benth., *Eucalyptus* sp.], *Pinaceae* (*Pinus wallichiana* A.B. Jacks.), *Poaceae* (*Phragmites* sp.), *Rosaceae* [*Malus sylvestris* (L.) Mill.], and *Rutaceae* [*Citrus maxima* (Burm.) Merr.]. Notably, this study represents the first global report of *R. eucalypti* on *Rubus* spp. and its first record in Asia.

Ramularia glennii, first described by Videira et al. (2015a), is a versatile fungal species known for its wide substrate range. Its taxonomy is based on morphological characteristics and multi-gene phylogenetic analysis, confirming its plurivorous nature. This species has been recorded on various substrates, including rubber, human bronchial alveolar lavage, and human skin tissue. It has also known from a wide range of plant associations, including those in the *Euphorbiaceae* (*Acalypha australis* L.), *Moraceae* (*Ficus carica* L.), *Myrtaceae* (*Corymbia grandifolia*, *Eucalyptus camaldulensis* Dehnh.), and *Platanaceae* (*Platanus* sp.) families (Videira et al. 2015a, b; Bakhshi and Arzanlou 2017). In the present investigation, *R. glennii* was discovered on six additional plant families. Therefore, these findings extend its host range to *Betulaceae* (*Alnus subcordata*), *Lythraceae* (*Punica granatum*), *Oleaceae* (*Ligustrum vulgare*), *Rosaceae* (*Eriobotrya japonica*), *Sapindaceae* (*Acer palmatum*), and *Viburnaceae* (*Viburnum opulus*). This expanded host range highlights the fungus's broad adaptability and potential impact on diverse plant species.

The discovery of *R. vizellae* and the newly described species *R. zelkovae*, both associated with *Zelkova* trees (*Zelkova carpinifolia*), represents a significant contribution to fungal diversity knowledge within Iran's Hyrcanian forests. These trees are known for their high-quality wood and picturesque canopy, with *Z. carpinifolia* being the sole *Zelkova* species native to this region (Ahmadi et al. 2014) out of the ten species recognized globally (Chen and Huang 1999; Jin et al. 2009).

Ramularia vizellae is a well-documented species with a broad host range, encompassing multiple plant families. It has been reported on species from the families *Betulaceae* (*Carpinus betulus* L., *Carpinus* sp., *Corylus americana* Walter), *Brassicaceae* (*Brassica* sp.), *Fabaceae* (*Lotus uliginosus* Schkuhr, *Phaseolus* sp.), *Fagaceae* (*Fagus sylvatica* L., *Fagus* sp., *Quercus robur* L., *Q. rubra* L.), *Malvaceae* (*Tilia* sp.), *Proteaceae* (*Protea* sp.), *Rosaceae* (*Amelanchier lamarckii* F.G. Schroed., *Malus sylvestris*, *Sorbus aucuparia* L.), *Sapindaceae* (*Acer campestre* L., *Acer pseudoplatanus* L., *Aesculus hippocastanum* L.), and *Viburnaceae* (*Sambucus nigra* L.), across various European countries and South Africa. The observation of *R. vizellae* on *Z. carpinifolia* represents a novel host record, expanding its known range to include the *Ulmaceae* family. This is also the first documented occurrence of *R. vizellae* in Asia, adding a new dimension to its distribution and ecological role.

In contrast, *R. zelkovae* is described here for the first time as a new species. Phylogenetic analysis places it distinctly within a well-supported clade in the concatenated multi-locus phylogram, separating it from other *Ramularia* species (Fig. 1). This uniqueness is corroborated by individual gene tree analyses for loci available for this species: ITS, *ACT*, *TEF1-α*, and *GAPDH*. The closest match in GenBank for the ITS sequence was *R. coryli* Chevassut (isolate CBS 117800, GenBank KX287391), with 97% identity (475/490) and no gaps. For *ACT*, the nearest hit was *R. acroptili* Bremer (isolate CBS 120253, GenBank KX287596), showing 92% identity (115/125) and no gaps. The *TEF1-α* sequence matched *R. trigonotidis* Videira, H.D. Shin & Crous (isolate CPC 14766, GenBank KX288081) with 85% identity (251/295) and 19 gaps (6%). The *GAPDH* sequence aligned most closely with *R. lethalis* Ellis & Everh. (isolate CPC 25910, GenBank KX288312), with 89% identity (424/477) and 7 gaps (1%). In the combined tree, *R. zelkovae*, while closely related to *R. coryli* and *R. lethalis*, stands out as a distinct species. It maintains a unique position separate from other related species like *R. acroptili* and *R. trigonotidis*.

Ramularia zelkovae can be distinguished from *R. coryli* and *R. lethalis* by several key morphological traits (Braun 1998). Unlike *R. coryli*, which has smoother and broader conidiophores (3–4 μm), *R. zelkovae* features slightly verruculose and narrower conidiophores, measuring 1.5 to 3 μm in diameter. Additionally, the conidia

of *R. zelkovae* vary from smooth to finally verruculose, whereas the conidia of *R. coryli* are consistently smooth. *Ramularia zelkovae* also possesses verruculose and longer conidiophores, ranging from 7.5 to 55 μm, compared to the smoother and shorter (5–15 μm) conidiophores of *R. lethalis*. These distinguishing characteristics highlight the unique morphological profile of *R. zelkovae* and its specific association with *Zelkova* trees, setting it apart from its relatives in the genus. It is worth noting that the correlation of *Mycosphaerella zelkovae* Syd., described on dead leaves of *Zelkova acuminata* (Lindl.) Planch. in Japan (Sydow and Sydow 1913), as a possible sexual morph of *R. zelkovae* is unlikely, particularly given the difference in host species. However, this correlation remains to be determined and would require further investigation, with the availability of living cultures of the holotype or epitype of *M. zelkovae*.

In recent years, much interest has been dedicated to study the *Ramularia* species obtained from various plants in north Iran by employing the Consolidated Species Concept (Bakhshi and Arzanlou 2017; Bakhshi et al. 2021a, b; Bakhshi 2018). Nevertheless, most *Ramularia* species introduced from Iran (Bakhshi 2018) have been identified based only on morphological characters and host range data, but living cultures and DNA data are still lacking. Therefore, future studies should be directed towards collecting, cultivating, and examining ramularioid plant pathogens from diverse host plants in various geographical regions of Iran.

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Data availability The datasets generated during the current study can be found in online repositories or available from the corresponding author on reasonable request. The names of the repositories and accession numbers can be found in the article.

Declarations

Ethics approval Not applicable.

Competing interests The authors declare no competing interests.

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