PHYLOGENY AND MORPHOLOGY OF *NEODIDYMELLIOPSIS* *IRANENSIS* SP. NOV., CAUSING DEAD BRANCHES OF CITRUS IN IRAN

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Abstract: Citrus plants, which are mostly cultivated in the southern tropical area of the country, are one of the important economic crops in Iran. Branch canker and dieback of citrus is an ongoing problem for citrus growers located in these areas and has imposed irreparable damage to the citrus production in this region in recent years. Disease symptoms consisted of the blight of vigorously growing shoots and dieback of the branches and rootstock trunks. This study aimed to characterize the citrus dieback pathogen morphologically and phylogenetically, and the species *Neodidymelliopsis iranensis* Soleimani & Goudarzi, sp. nov., is described and illustrated here. Isolates were derived from collected citrus samples with dieback symptoms. After preparing pure cultures from single spores on oatmeal agar and malt agar, the morphological features of the species were described and their pathogenicity was confirmed on lime (*Citrus aurantifolia*). Morphologically *N. iranensis* is easily separated from the other species of *Neodidymelliopsis* by the size of pycnidia, conidia septation, and NaOH test results. The morphological differences between our isolates and the other known species of *Neodidymelliopsis* were strongly supported by a multi-locus phylogenetic analysis based on the *ITS* region, and *LSU*, *RPB2*, and *TUB2* genes. In the reconstructed phylogenetic tree, *N. iranensis* formed a well-supported clade with other *Neodidymelliopsis* species in the *Didymellaceae* family, but was separated from all other *Neodidymelliopsis* species. The distinct phylogenetic position is supported by differences in morphological features. Consequently, the specificity of the morphological and phylogenetical features of the collected isolates has convinced us to describe *Neodidymelliopsis iranensis* as a new species.

Keywords: Citrus, *Didymellaceae*, Morphology, Pathogenicity, Phylogeny.

Introduction

Citrus are among the ten most important crops in terms of total fruit yield worldwide [10]. More than nine million hectares are planted with citrus throughout the world, a total production of c.146 million tonnes (FAO, 2016). Iran, with over 214,000 ha of citrus plantations and about 3,700,000 tonnes of citrus fruit production, has been ranked 10th in the world for this crop.

Citrus are affected by several diseases and pests. Dieback disease of citrus has caused increasing alarm to citrus growers located in the south of Iran, in that in many plantations the yield has been halved or reduced to nil. Canker and dieback diseases are common, destructive, and continue to be extensively studied in a wide range of woody plants [21]. Branch canker and dieback diseases of citrus have been reported since the early 1900s. Symptoms of these diseases generally include the presence of the dead, sunken bark around dark-colored cankers, twig and branch dieback, and gummosis. Many factors contribute to the occurrence of such symptoms. Fungi and fungi-like microorganisms have the greatest role in the occurrence of these symptoms [21]. Citrus have been threatened by parasitic pycnidial fungi for several decades. Several fungal taxa

belonging to the different families of *Botryosphaeriaceae* and *Didymellaceae* [12, 24] have been reported from the dead citrus branches: *Barriopsis fusca* [24], *Didymella microchlamydospora* [5], *Diplodia striatal* [24], *Lasiodiplodia citricola*, *L. iraniensis* [2], *L. mediterranea* [19], *L. pseudotheobromae* [2], *Neofusicoccum ribis* [13], *Phaeobotryosphaeria citrigena* [24], *Spencermartinsia citricola*, *S. plurivora* [1] and *Phaeobotryosphaeria citrigena* [24].

The new genus *Neodidymelliopsis* Q. Chen & L. Cai was recently proposed by Chen et al. (2015) with five species, including *Neodidymelliopsis longoicolla* L.W. Hou, Crous & L. Cai, *N. achlydis* L.W. Hou, Crous & L. Cai, *N. cannabis* (G. Winter) Q. Chen & L. Cai, *N. polemonii* Q. Chen & L. Cai, and *N. xanthina* (Sacc.) Q. Chen & L. Cai. These have been already isolated from soil in the desert and living or dead aerial parts of different plants such as *Achlys triphylla*, *Cannabis sativa*, *Urtica dioica*, *Polemonium acutiflorum* and *Delphinium* spp. [10, 12, 31]. Recently, *N. farrokhinejadii* was added to this genus [4] within the recently established family Didymellaceae [15]. The family embraces the species traditionally classified in the genera *Ascochyta*, *Didymella*, and *Phoma* [31]. Members of this family are pathogenic on a wide range of host plants, which mainly cause leaf and stem lesions and dieback disease [6, 11]. Molecular phylogenetic studies have shown that the family Didymellaceae includes most members of *Phoma* and related asexual genera, including *Didymella*, *Epicoccum*, *Allophoma*, *Heterophoma*, *Stagonosporopsis*, *Phoma*, *Boeremia*, *Paraboeremia*, *Ectophoma*, *Remotididymella*, *Similiphoma*, *Cumuliphoma*, *Juxtiphoma*, *Vacuiphoma*, *Briansuttonomyces*, *Pseudoascochyta*, *Neomicrosphaeropsis*, *Macroventuria*, *Nothophoma*, *Ascochyta*, *Calophoma*, *Leptosphaerulina*, *Phomatodes*, *Neascochyta*, *Xenodidymella*, and *Neodidymelliopsis* [11, 12, 31].

In the present study, we collected *Phoma*-like fungi that caused dieback of citrus in the south of Iran. Citrus plants are one of the important economic crops in Iran, and thus taking appropriate and efficient strategies to manage their pathogens depends on a good knowledge of their taxonomy, pathogenicity, diversity, and ecology. Consequently, the objectives of this study were to (1) determine the taxonomy of collected isolates based on their morphological features and phylogenetic relationship with the other known taxa within the Didymellaceae (2) assess the pathogenicity and aggressiveness of these fungi on lime (*Citrus aurantifolia*).

Materials and Methods

Sample collection and pathogen isolation

Sample collection was performed as described by Adesemoye et al. (2014) from five citrus-growing provinces located in the south of Iran – Khuzestan, Kerman, Hormozgan, Fars and Kohkiluiye – from 2014 to 2016. Citrus branch samples with dieback, yellowing, defoliation and the symptoms of blight on vigorously growing shoots were collected from 10 randomly selected trees in each of 5 to 10 orchards per province, depending on orchard density. The samples were packed in paper bags and transferred to the laboratory. Pathogen isolation and propagation were performed according to the methods described by Taylor and Hyde [30]. The small pieces (0.5–1 cm) of infected tissue, taken from the junction of the diseased and healthy areas of the samples, were excised and surface-sterilized by dipping them in 0.5-1% NaOCl (2-4 minutes), followed by washing three times with sterile distilled water (2 min) and plated on potato dextrose agar medium (PDA, Difco, USA) amended with 0.01% tetracycline. The plates were incubated in the dark up to 5–15 days at $25 \pm 2^\circ\text{C}$. The isolates were purified by the single spore method and individual small colonies sub-cultured on PDA. Then, three isolates were selected as representatives, and their

morphological and molecular characteristics were evaluated carefully. The host and geographical location of these collected isolates were displayed in Table 1.

Mycological examination

The morphological properties of colonies were determined using the manual of Boerema et al. (2004). Isolates were cultivated on oatmeal agar (OA), potato dextrose agar (PDA) and malt extract agar (MEA). Plates were incubated in complete darkness at 20-22°C for 7 days. During a second week, the Petri dishes were placed at 13/11 h alternating NUV light and dark period to stimulate the pigmentation of the colonies and the formation of pycnidia. Colony diameters and morphologies were recorded 8 and 15 days after culture incubation. Rayners' colour chart [25] was used to rate the colours of each colony and detected the production of metabolite E+ using a droplet of 1N NaOH [8]. Finally, the cultured fungal colony preparations were mounted on glass slides with clear lactic acid for microscopic examination. The structure of the pycnidial wall and the shape of conidiogenous cells were analyzed in hand-cut sections of pycnidium. Conidia and pycnidia were measured in 30 rounds. An Olympus (DP25) digital camera installed on a BX50 Olympus light microscope was used for the illustration. The identification of fungi was based on morphological characteristics as described by Boerema et al. (2004), Chen et al. (2015, 2017) and Valenzuela-Lopez et al. (2018). Fungi isolates of this study were deposited at Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Center in the Netherlands and Iranian Fungal Culture Collection (IRAN.C) at the Iranian Research Institute of Plant Protection (Table 2).

Table 1: The host and geographical location of collected isolates

Isolate number	Province	Region	Host	Height	Longitude	latitude
CBS 142208	Kerman	Anbar abad	<i>Citrus sinensis</i>	607	28.4683630	57.8514833
CBS 142211	Khuzestan	Dezful	<i>Citrus paradisi</i>	110	32.3268267	48.4169164
CBS 142210	Fars	Jahrom	<i>Citrus aurantium</i>	1098	28.5284902	53.5066273

Table 2: Isolates considered in this study. Newly generated sequences are indicated in bold

Species name	Strain number	Host, substrate	Country	GenBank accession numbers			
				LSU	ITS	RPB2	TUB
<i>Neoscochyta desmazieri</i>	CBS 247.79	<i>Graminea e</i>	Austria	KT389725	KT389507	—	KT389805
<i>Neoscochyta desmazieri</i>	CBS 297.69	<i>Lolium perenne</i>	Germany	KT389726	KT389508	KT389644	KT389806
<i>Neoscochyta desmazieri</i>	CBS 758.97	Hay	Norway	KT389727	KT389509	—	KT389807
<i>Neoa. europaea</i>	CBS 819.84	<i>Hordeum vulgare</i>	Germany	KT389808	KT389728	KT389510	KT389645
<i>Neoa. europaea</i>	CBS 820.84	<i>Hordeum vulgare</i>	Germany	KT389729	KT389511	KT389646	KT389809
<i>Neoa. graminicola</i>	CBS 301.69	<i>Lolium multiflorum</i>	Germany	KT389737	KT389519	KT389650	KT389817
	CBS 447.82	<i>Triticum aestivum</i>	Germany	KT389738	KT389520	—	KT389818
	CBS 586.79	<i>Hordeum vulgare</i>	Belgium	KT389739	KT389521	—	KT389819
	CBS 816.84	<i>Hordeum vulgare</i>	Germany	KT389741	KT389523	KT389651	KT389821
	CBS 102789	<i>Lolium perenne</i>	New Zealand	KT389736	KT389518	KT389649	KT389816
<i>Neoa. exitialis</i>	CBS 118.40	-	-	KT389732	KT389514	KT389647	KT389812

	CBS 389.86	<i>Triticum aestivum</i>	Switzerl and	KT389733	KT389515	KT389648	KT389813
	CBS 811.84	<i>Secale cereale</i>	Germany	KT389734	KT389516	—	KT389814
	CBS 812.84	<i>Hordeum vulgare</i>	Germany	KT389735	KT389517	—	KT389815
	CBS 110124	<i>Triticum sp.</i>	The Netherlands	KT389730	KT389512	—	KT389810
	CBS 113693; UPSC 1929	<i>Allium sp.</i>	Sweden	KT389731	KT389513	—	KT389811
<i>Neoa. paspali</i>	CBS 560.81; PD 92/1569Td	<i>Paspalum dilatatum</i>	New Zealand	GU238124	FJ427048	KP330426	FJ427158
<i>Neoscochyta sp.1</i>	CBS 112524	<i>Triticum aestivum</i>	Argentina	KT389742	KT389524	—	KT389822
<i>Neoscochyta sp.2</i>	CBS 516.81	<i>Oryza sativa</i>	Italy	KT389743	KT389525	KT389653	KT389823
<i>Neoscochyta sp.3</i>	CBS 689.97	Hay	Norway	KT389744	KT389526	KT389654	KT389824
<i>Neoscochyta sp.4</i>	CBS 544.74	<i>Triticum aestivum</i>	South Africa	EU754134	GU237887	KT389652	GU237488
<i>Neodidymellio psis cannabis</i>	CBS 121.75; ATCC 32164; IMI 194767; PD 73/584	<i>Urtica dioica</i>	The Netherlands	GU237972	GU237761	—	GU237535
	CBS 234.37	<i>Cannabis sativa</i>	—	GU237961	GU237804	KP330403	GU237523
	CBS 591.67	<i>Urtica dioica</i>	The Netherlands	KT389746	KT389528	—	KT389826
	CBS629.76	Packing material	The Netherlands	KT389747	KT389529	—	KT389827
IR26	CBS 142211; IRAN 16885F	<i>Citrus paradisi</i>	Iran	KY35507 4	KY29022 6	KY38628 5	KY407790
IR14	CBS 142210	<i>Citrus aurantium</i>	Iran	KY35507 3	KY29022 5	KY38628 4	KY407789
IR10	CBS 142208; IRAN 16886F	<i>Citrus sinensis</i>	Iran	KY35507 2	KY29022 4	KY38628 3	KY407788
<i>N. polemonii</i>	CBS 375.67	<i>Polemonium caeruleum</i>	The Netherlands	KT389748	KT389530	—	KT389828
	CBS 109181; PD 83/757	<i>Polemonium caeruleum</i>	The Netherlands	GU238133	GU237746	KP330427	GU237648
<i>N. achlydis</i>	CBS 256.77	<i>Achlys triphylla</i>	Canada	KT389749	KT389531	—	KT389829
<i>N. longicolla</i>	CBS 382.96	<i>Soil in desert</i>	Israel	KT389750	KT389532	—	KT389830
<i>N. xanthina</i>	CBS 168.70	<i>Delphinium sp.</i>	The Netherlands	KT389751	KT389533	—	KT389831
<i>Nothophoma anigozanthi</i>	CBS 381.91	<i>Anigozanthus maugeisii</i>	The Netherlands	KT389655	GU23758 0	GU238039	GU237852

<i>Xenodidymella applanata</i>	CBS 195.36	<i>Rubus idaeus</i>	The Netherlands	KT389764	KT389548	—	KT389852
	CBS 205.63	<i>Rubus idaeus</i>	The Netherlands	GU237998	GU237798	KP330402	GU237556
	CBS 115577	<i>Rubus idaeus</i>	Sweden	KT389762	KT389546	KT389688	KT389850
	CBS 115578	<i>Rubus arcticus nothosp. stellarticus</i>	Sweden	KT389763	KT389547	—	KT389851
<i>X. asphodeli</i>	CBS 375.62	<i>Asphodelus albus</i>	France	KT389765	KT389549	KT389689	—
	CBS 499.72	<i>Asphodelus ramosus</i>	Italy	KT389766	KT389550	—	KT389853
<i>X. catariae</i>	CBS 102635; PD 77/1131	<i>Nepeta catenaria</i>	The Netherlands	GU237962	GU237727	KP330404	GU237524
<i>X. humicola</i>	CBS 220.85; PD 71/1030	<i>Franseria sp</i>	USA	GU238086	GU237800	KP330422	GU237617

Pathogenicity test

Pathogenicity of isolates to lime (*Citrus aurantifolia*) was confirmed by inoculation of rootstocks with isolate mycelial plugs, in the middle of spring as described by Damm et al. (2007). The branches were wounded with a cork borer to remove the bark and mycelial plugs of a single-conidial isolate (5 mm diam) grown on PDA at 25°C were placed into the wound and the plants maintained in an open area. After inoculation, the branches were wrapped with parafilm. Three seedlings were maintained as a control treatment and were inoculated with sterile PDA, and the plants were assessed 2 months after inoculation. The pathogen was re-isolated from the infected plants and the morphological features of the resultant cultures were checked to confirm Koch's postulates.

DNA extraction, amplification, and sequencing

For molecular identifications, the procedure described by Doyle and Doyle (1987), was used to extract genomic DNA from fresh fungal mycelium cultivated on PDA media at 25–27°C for 4 weeks. The extracted DNA samples were diluted 10 × in distilled water and stored at 4 °C for further steps. The *LSU*, *ITS*, *RPB2* and *TUB* regions were amplified by polymerase chain reaction (PCR), respectively using LR0R/LR7 [26, 32], V9G/ ITS4 [16,33], RPB2-5F2/ fRPB2-7cR [28, 20], and Btub2Fd/Btub4Rd [34], primer pairs, as described by Chen et al. (2015). The conditions of PCR amplifications were as described by Chen et al. (2015). A total volume of 12.5 µl solution containing 0.5 µL 10 × diluted template DNA, 1×PCR buffer, 0.4 µM of each primer, 1 Unit Taq polymerase, 25 µM of each of the dNTP, 2 mM MgCl₂. After analyzing the PCR products by electrophoresis in a 1.0 % (w/v) agarose gel containing 0.1 ug/mL ethidium bromide in 1 × TAE buffer (0.4 M Tris, 0.05 M glacial acetic acid 0.01 M ethylenediaminetetraacetic acid [EDTA], pH 7.85), the amplicons were visualized under UV light and were sequenced by the Macrogen company in South Korea.

Phylogenetic analyses

Single gene and concatenated multi-locus dataset sequences of the 3 isolates recovered in

this study as well as related sequences, which were mostly published by Aveskamp et al. (2010), Chen et al. (2015, 2017) and Valenzuela-Lopez et al. (2018), retrieved from geneBank were aligned, using Clustal X2. The regions which were absent in more than 90% of the sequences were removed and gaps were treated as missing data. *Nothophoma anigozanthi* CBS 381.91 was used as the outgroup taxon in the analysis. We inferred the substitution model that best fits the model of DNA evolution using MrModeltest2 v. 2.3. [22]. Finally, the phylogenetic analysis was done by PAUP* v.4.0b10 [29], MrBayes v3.1.2 [18], and RAxML [27], which are based on maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML), respectively.

For maximum-parsimony (MP) analysis with PAUP*, we used the heuristic search option with 1,000 random sequences addition. Maxtrees were set up to 5000 with tree-bisection reconnection (TBR) as the branch-swapping algorithm. The robustness of the trees was evaluated by 1000 bootstrap replications. Moreover, tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC), were calculated.

Suitable models for the Bayesian analysis were first selected, using models of nucleotide substitution for each gene, as determined using MrModeltest2 v. 2.3. [22]. The Bayesian tree inference (BI) with a Markov Chain Monte Carlo (MCMC) in MrBayes was based on 5 M generations saving one tree per 1000 generations. Four MCMC chains were running simultaneously and automatically ended when the standard deviation of split frequencies was below 0.01. The first 25% of the saved trees, which represent the 'burn-in' phase, were discarded. RAxML analysis was performed on the XSEDE supercomputer at the CIPRES Gateway platform. Finally, the reconstructed trees were visualized in TreeView v. 1.6.6. [23]. New sequences were deposited in GenBank (Table 2).

Results

Pathogenicity test

Disease symptoms were assessed 2 months after inoculation. Symptoms were the typical pink to brown wood and vessel discoloration expanded rapidly in a longitudinal direction, leaf drop, and the twigs and branches dieback. The cultivation of the fungi derived from symptomatic rootstocks on PDA confirmed the pathogenicity of these isolates. These isolates were identified again as previously described. No symptoms were observed in the control plants (Fig. 1). The experiment was carried out using three replicates and was repeated three times with the same results.

Mycological examination

The colony characteristics of these three collected isolates (CBS 142208, CBS 142210, CBS 142211) differed from each other, so macroscopic characteristics of each separately mentioned as below. The microscopic characteristics of all three isolates are the same.

***Neodidymelliopsis* sp. CBS 142210** (MycoBank no.: MB 820336).

IRAN, Fars province, *Citrus aurantium*, 25 Jun 2013, Soleimani P.

Gene sequences: KY355073 (LSU), KY290225 (ITS), KY386284 (PRB2), KY407789 (TUB)

Culture characteristics

Colonies on MA, 5- 5.5 cm diam, pinkish-white, regular after 7 days, change to pale brown and completely filling the plate after 14 days (Fig 2. A,B).

Fig. 1: Symptoms of *Neodidymelliopsis* sp. on *Citrus aurantifolia* 2 months after inoculation
A, B, C, D, E, F, G, H, I, J, K, L, M & N inoculated rootstocks with *Neodidymelliopsis* sp. O control rootstock, inoculated with sterile PDA. Typical pink to brown wood and vessel discoloration A-E , H-J. The twigs dieback F, K-N. Control rootstock O.

Colonies on OA, 4.5-5.5 cm diam, colorless, regular after 7 days, black pycnidia are visible

near the centre, changing to black with concentric pycnidial rings, and completely filling the plate after 14 days (Fig2. C,D).

Colonies on PDA, 4.5- 5 cm diam, pinkish-brown near the centre and pale greyish-pink near the margin, with olivaceous grey aerial mycelia near the centre, regular after 7 days, changing to pale brown and completely filling the plate after 14 days (Fig2. E,F).

Fig. 2: *Neodidymelliopsis* sp. (CBS 142210)

A–B. Colony on MA (after one and two weeks). C–D, Colony on OA (after one and two weeks). E–F. Colony on PDA (after one and two weeks). G. Pycnidia forming on OA. H. I. Pycnidium. J. Ostiole. K. pycnidial wall. L. Conidia. M. Q. R. Section of pycnidial wall. N. O. P. Conidiogenous cell. Scale bars: H = 60 μ m ; I = 15 μ m ; J = 20 μ m; L = 7 μ m; M, ,O, Q = 7; N = 10 μ m ; P = 15 μ m, R = 70 μ m.

***Neodidymelliopsis* sp. CBS 142208** (MycoBank no.: MB 820339).

IRAN, Kerman province, *Citrus sinensis*, 24 Aug 2013, Soleimani P.

Gene sequences: KY355072 (LSU), KY290224 (ITS), KY386283 (PRB2), KY407788 (TUB)

Culture characteristics

Colonies on MA, 5-5.5 cm diam, pale pink, compact, covered by densely white-felty aerial mycelium, regular after 7 days, changing to pale brown and completely filling the plate after 14 days (Fig.3 A,B).

Colonies on OA, 5-5.5 cm diam, white near the margins; olivaceous grey near the centre,

aerial mycelium, white, change to olivaceous grey (Fig.3 C,D).

Colonies on PDA, 4.5-5 cm diam. After 7 days, margin regular, pale brown in the centre, pink near the colony margin, covered by white floccose aerial mycelia, changing to olivaceous grey after 14 days (Fig. 3 E,F).

Fig. 3: *Neodidymelliopsis* sp. (CBS 142208)

A–B, Colony on MA (after one and two weeks). C–D, Colony on OA (after one and two weeks). E–F, Colony on PDA (after one and two weeks). G. Pycnidia forming on OA. H. J, Pycnidium. I, Ostiole. K, pycnidial wall. L, Conidia. M. N, Section of pycnidial wall. O, Conidiogenous cell. Scale bars: H = 70 µm ; I = 30µm ; J = 20 µm; L = 3; M,O = 10 µm, N = 25 µm.

Neodidymelliopsis sp. CBS 142211 (MycoBank no.: MB 820338)

IRAN, Khuzestan province, *Citrus paradisi*, 4 Sep 2014, Soleimani P.

Gene sequences: KY355074 (LSU), KY290226 (ITS), KY386285 (PRB2), KY407790 (TUB)

Culture characteristics Colonies on MA, 4.5–5 cm diam, after 7 days, margin crenate, pink with honey and light brown zone, with pinkish aerial mycelium, changing to pale brown and completely filling the plate after 14 days (Fig.4 A,B).

Colonies on OA, 3.5-4 cm diam, after 7 days, without aerial mycelium, with abundant scattered dark brown pycnidia, margin regular, changing to black with concentric pycnidial rings, and completely filling the plate after 14 days (Fig.4 C,D).

Fig. 4: *Neodidymelliopsis* sp. (CBS 142211)

A–B, Colony on MA (after one and two weeks). C–D, Colony on OA (after one and two weeks). E–F, Colony on PDA (after one and two weeks). G, Pycnidia forming on OA. H–I, Pycnidium. J, L, Section of pycnidial wall. K, pycnidial wall. M, Conidiogenous cell. N, Conidia. Scale bars: H = 100 μ m ; I = 50 μ m ; J = 20 μ m; K = 7 μ m; L = 10 μ m; M–N = 6 μ m.

Colonies on PDA, 4–4.5 cm diam, after 7 days, dark brown with pinkish margin, covered by densely olivaceous brown aerial mycelia, margin regular, black pycnidia are visible near the centre after 7 days, changing to black with concentric pycnidial rings, and completely filling the plate after 14 days (Fig. 4 E,F).

Conidiomata pycnidial, variable in shape and size, scattered or in concentric rings, solitary or confluent, superficial on or immersed into the agar, globose or sub-globose or flask-shaped, often elongated or irregular, with some hyphal outgrows, variable in dimensions, mostly $55.082\text{--}283.98 \times 28.046\text{--}221.53$ μ m diam (Fig. 2 G–J) (Fig. 3 H–J) (Fig. 4 H,I).

Ostioles, 1–4, on elongated neck sometimes on a short neck. *Pycnidial wall*, pseudoparenchymatous, 4–6 layered, 3.8–13.19 μ m thick., outer wall pigmented (Fig. 2 K,M,Q) (Fig. 3 K,M,N) (Fig. 4 J–L).

Conidiogenous cells, phialidic, hyaline, smooth, ampulliform to doliiform, $3.5\text{--}7.8 \times 4.8\text{--}8.7$ μ m (Fig. 2 O,P) (Fig. 3 O) (Fig. 4 M). *Conidia*, ovoid, ellipsoid to cylindrical, 0–1 septate, $4.9\text{--}9.29 \times 2.3\text{--}3.48$ μ m, smooth- and thin-walled, hyaline to pale brown (Fig. 2 L) (Fig. 3 L) (Fig. 4 N). Application of NaOH resulted in a reddish discoloration.

Phylogenetic analyses

Three different MP, Bayesian inference and ML methods were provided with the concatenation of the alignments of the *LSU*, *ITS*, *RPB₂* and *TUB* genetic regions in 46 selected taxa (Table 2). The combined *LSU*, *ITS*, *RPB₂* and *TUB* alignments had 2,384 characters including the alignment gaps. The reconstructed trees drawn in TreeView were highly similar in topology, and the selected outgroup taxon, *Nothophoma anigozanthi* CBS 381.91 was basal to the major clade in all the reconstructed trees.

From the 961 characters of the *LSU* alignment, 911 were constant, 19 were informative non-parsimony, and 31 were informative parsimony. These numbers for *ITS* alignment with 490 characters were, respectively, 385, 14 and 91; for the *RPB₂* alignment with 596 characters were, 389, 25 and 182; and finally for the *TUB* alignment with 337 characters were, 209, 20 and 108 characters. The concatenated alignment of the four genes with 2,384 total characters consisted of 1,894 constant, 78 informative non-parsimony and 412 informative parsimony characters. A MP heuristic search produced a single most parsimonious tree with a length of 1187 steps (CI = 0.561, RI = 0.867, RC = 0.486, HI = 0.439). Dirichlet base frequencies and the GTR+I+G model with inverse gamma rates were determined as the best for the combined and individual gene alignments to be set in MrBayes. The topology and support values of the MP trees were in congruence with BI and ML trees (Fig. 5). Analysis of the combined dataset from the *ITS* region and *LSU*, *RPB₂* and *TUB* genes successfully resolved the phylogeny of the three isolates of *Neodidymelliopsis*-like fungus of this study (CBS 142208, CBS 142210, CBS 142211) with high bootstrap support. These isolates formed a clade with the species of *Neodidymelliopsis* with strong support (1.00 PP / 100% ML / 100% MP) and separated from *Neodidymelliopsis longicolla* CBS 382.96 and *N. farrokhinejadii* CBS 142850 with a high support (1.00 PP / 100% ML / 99% MP) and were not conspecific with any other species in the NCBI.

Discussion

The dieback and decline of citrus trees have recently been recorded in the orchards at the centre of citrus cultivation in the south of Iran. Thus far, several pathogenic genera like *Didymella*, *Dothiorella*, and *Neoscytalidium* fungi have been isolated from symptomatic plants. This study reports the *Neodidymelliopsis* as responsible for dieback symptoms in citrus orchards of Iran and describes the taxonomic features of the fungus.

Comparing the morphological features of the present study isolates showed that they share *Didymellaceae* properties. These isolates, like other species in the *Didymellaceae*, have phialidic conidiogenesis. Other characterization of conidiomata (such as pycnidial, pycnidial wall pseudoparenchymatous, 2–7-layered, outer wall pigmented), conidiogenous cells (phialidic and thin-walled), conidia (aseptate or septate, smooth-walled, and hyaline to brown in a later stage) also fit the description of *Neodidymelliopsis* Chen et al. (2015).

Neodidymelliopsis was recently proposed by Chen et al. (2015) and is characterized by producing globose to flask-shaped, glabrous or pycnidia with hyphal outgrowths. The most characteristic features include its elongated neck, the conidia that are initially hyaline and aseptate but became pale-brown and septate with age [31]. Morphological features of these new fungal isolates collected from citrus in the south of Iran were confirmed that they share *Neodidymelliopsis* properties by having possessed pycnidial conidiomata and phialidic conidiogenesis, with hyaline, ampulliform and thin-walled conidiogenous cells, and none or 1-septate, smooth-walled, hyaline to pale brown conidia. Characterization of conidiomata, conidia and conidiogenous cells of the

isolates were in consistent with the description of *Neodidymelliopsis* reported by Chen et al. (2015).

Fig. 5: Phylogenetic trees generated by Bayesian inference (BI), Maximum likelihood (ML) and maximum parsimony (MP) analysis of the combined dataset of *ITS* region and *LSU*, *RPB2* and *TUB* genes

The Bayesian posterior probabilities (BPP), RAxML bootstrap support values (MLBS) and maximum parsimony bootstrap support (MPB) are given at the nodes (BPP/MLBS/MPB). New isolates are in red and the tree is rooted with *Nothophoma anigozanthi* CBS 381.91.

In the present study isolates differed morphologically from the six previously described species of *Neodidymelliopsis*, viz. *N. achlydis*, *N. cannabis*, *N. farrokhinejadii*, *N. longoicolla*, *N. polemonii* and *N. xanthine* [4, 11, 12, 31]. We therefore propose that our isolates comprise a new species, *Neodidymelliopsis iranensis* Soleimani & Goudarzi, sp. nov.

N. iranensis differs from *N. polemonii* and *N. xanthina* in producing pycnidia with 1-4 ostioles vs. a single ostiole and 0-1 septate conidia vs. aseptate conidia [12] as well as reddish-brown discoloration in *N. iranensis* vs. negative NaOH reaction in *N. polemonii* [12]; from *N. achlydis* in its septate conidia (0-1-septate vs. aseptate) as well as in NaOH discoloration (reddish-brown vs. dull green with a reddish brown margin [11]; from *N. cannabis* in the number of pycnidial ostioles (1-4 vs. 1) [9]; from *N. longoicolla* in producing smaller pycnidia ($55\text{--}283 \times 28\text{--}221 \mu\text{m}$ vs. $200\text{--}490 \times 150\text{--}360 \mu\text{m}$) [11] and smaller conidia ($4.9\text{--}9.29 \times 2.3\text{--}3.48 \mu\text{m}$ vs. $12\text{--}16.5 \times 4\text{--}7 \mu\text{m}$) [11]; from *N. farrokhinejadii* in pycnidia size ($55\text{--}283 \times 28\text{--}221 \mu\text{m}$ vs. $120\text{--}400 \times 50\text{--}290 \mu\text{m}$) [4], and conidia size ($4.9\text{--}9.29 \times 2.3\text{--}3.48 \mu\text{m}$ vs. $5\text{--}8(10) \times 2.5\text{--}3(5) \mu\text{m}$ (1-cellular conidia), $7\text{--}10.25(12.5) \times 2.5\text{--}5 \mu\text{m}$ (2-cellular conidia) [4].

Phylogenetic analyses trees, planted with maximum parsimony, Bayesian and maximum likelihood methods using combination sequence of the *ITS* region and *LSU*, *RPB2* and *TUB* genes, showed that our isolates were clustered with *Neodidymelliopsis* with strong support (1.00 PP / 100% ML / 100% MP) and separated from *Neodidymelliopsis longoicolla* CBS 382.96 and *N. farrokhinejadii* CBS 142850 with a high support (1.00 PP / 100% ML / 99% MP) as our isolates formed a distinct subclade (1.00 PP / 100% ML / 87% MP).

According to our results, the differences observed in this study, including the size of pycnidia and conidia, the number of conidia septa, as well as differential reaction to NaOH test, have indicated that our isolates are different from those of the other species of *Neodidymelliopsis*. This separation was confirmed by phylogenetical studies. Finally, based on phylogenetic analyses and morphological properties, we have proposed that our isolates comprise a species of the genus *Neodidymelliopsis* that is new to science. Hence, we have described our isolates as *Neodidymelliopsis iranensis*.

Conclusion

In this study, we have introduced one new species and provided reference (type) specimens for this species. The effects of pathogenicity, host specificity, and biology of the characterized species on disease severity remain obscure and require further studies in the future. In order to develop effective integrated control strategies, the epidemiology of this pathogen needs to be studied further, as well to understand the ecology and the distribution patterns of the pathogen. The most effective means to control disease in plants on a large scale would be to use tolerant or resistant cultivars. Therefore, the citrus species resistant to *Neodidymelliopsis* should be studied for appropriate management of disease and prevent yield losses.

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**FILOGENIA ȘI MORFOLOGIA SPECIEI *NEODIDYMELLIOPSIS IRANENSIS* SP. NOV.
CE CAUZEAZĂ NECROZA RAMURILOR DE CITRUS ÎN IRAN**

(Rezumat)

Citricele, care sunt cel mai mult cultivate în partea sudică, tropicală a țării, reprezintă una dintre cele mai importante culturi din Iran. Cancerul ramurilor și necroza citricelor este o problemă permanentă pentru cultivatorii de citrice din aceste zone și a produs pagube totale pentru producția de citrice din ultimii ani. Simptomele bolii constau în afectarea lăstarilor cu creștere viguroasă, cancerul ramurilor și a trunchiului portaloiului. În acest studiu ne-am propus caracterizarea patogenului din punct de vedere morfologic și filogenetic. Specia nouă *Neodidymelliopsis iranensis*, sp. nov. este descrisă și ilustrată în acest studiu. Izolatele au provenit de la mostre de ramuri cu simptome specifice. După obținerea culturilor pure din spori unici pe medii de cultură cu ovăz și malț-agar, au fost descrise caracterele morfologice ale speciei și s-a testat patogenicitatea acestora pe lămâi (*Citrus aurantifolia*). Din punct de vedere morfologic, *N. iranensis* se separă ușor de alte specii de *Neodidymelliopsis* în funcție de mărimea picnidiilor, septele conidiilor și rezultatul testelor NaOH. Diferențele de morfologie dintre izolatele obținute și alte specii cunoscute de *Neodidymelliopsis* au fost confirmate și de analiza filogenetică multi-locus pe baza regiunii ITS și a genelor LSU, RPB2 și TUB2. În arborele filogenetic reconstruit, *N. iranensis* formează un grup distinct cu alte specii de *Neodidymelliopsis* din familia *Didymellaceae*, însă este separat de alte specii de *Neodidymelliopsis*. Poziția filogenetică distinctă este confirmată și de diferențele morfologice. În consecință, specificitatea caracterelor morfologice și filogenetice ale izolatelor obținute ne-au determinat să le descriem ca fiind *Neodidymelliopsis iranensis*, sp. nov.

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