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Further characterization and pathogenicity of *Didymella microchlamydospora* causing stem necrosis of *Morus nigra* in Iran

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Abstract

In the last decade, canker and dieback diseases have caused disease of ornamental and fruit trees of Khuzestan Province in the southwest of Iran. Fourty-eight symptomatic branches and trunks were sampled and a survey was made to identify the probable pathogens, which led to the isolation of the recently established species, *Didymella microchlamydospora*. A multi-locus DNA sequence based phylogeny, in combination with morphology, was used to characterize seven isolates of this species. Two phylogenetic trees constructed based on the combined sequences of ITS/LSU/tub2 and ITS/LSU/tub2/rpb2 regions showed very little differences, and both trees presented generally consistent relationships among the strongly supported clades. In both of three- and four-locus based phylogenetic trees, our isolates and a reference strain, *D. microchlamydospora* CBS 105.95, formed supportive monophyletic clades with strong 99% and 100% BS support, respectively. In pathogenicity tests, the isolate of *D. microchlamydospora* SCUA 14_Dez_Mor formed the necrosis and wood discoloration on stem fragments of *Morus nigra*. To our knowledge, this is the first report of pathogenicity of *D. microchlamydospora* on *Morus nigra* and its association on plants of olive, bitter orange, oleander and bottlebrush worldwide. In addition, we gave a slightly amended description of this species.

Key words – die back – Khuzestan – multi-locus phylogeny

Introduction

Canker and dieback diseases are common, widespread, and destructive on a wide range of woody plants (Shurtleff 1997, Horst 2013). These diseases are caused by several fungal taxa belonging to the different families including *Botryosphaeriaceae* and *Didymellaceae* (Phillips et al. 2013, Chen et al. 2015) in *Dothideomycetes* and *Cytospora* and *Diaporthe* in *Sordariomycetes* (Sinclair et al. 1987, Lawrence et al. 2015). In the last decade, these diseases have threatened the ornamental and fruit trees of Khuzestan Province in the southwest of Iran. The potential pathogens infect all woody plants, especially those low in vigor. The disease causes stem necrosis and canker, wilting and dieback of twigs and branches (unpublished data).

The species *D. microchlamydospora* (Aveskamp & Verkley) Q. Chen & L. Cai (formerly known *Phoma microchlamydospora*) belongs to the recently established family *Didymellaceae* (de Gruyter et al. 2009, Hyde et al. 2013), which includes many taxa previously classified in the genus *Phoma* and their related taxa (Chen et al. 2015). This species has been isolated from leaves of *Eucalyptus* sp. and an unknown plant (Aveskamp et al. 2009). The genus *Didymella sensu lato* was

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established by Saccardo (1880) to accommodate *D. exigua* (Niessl) Sacc. (Holm 1975, Corlett 1981). This genus was originally placed in *Mycosphaerellaceae*, and then subsequently reclassified in the *Pleosporaceae, Phaeosphaeriaceae* and *Venturiaceae* (Hyde et al. 2013, Wijayawardene et al. 2014, Chen et al. 2015). In recent years, phylogenetic studies have resulted in the dramatic taxonomic changes in *Didymella* and other *Phoma*-like taxa (Aveskamp et al. 2009, 2010, de Gruyter et al. 2009, Woudenberg et al. 2009, Chen et al. 2015). In order to resolve phylogenetic relationships and improve the systematics of *Phoma* and allied genera, ITS, LSU, tub2 and rpb2 sequences were used for species demarcation (Aveskamp et al. 2009, 2010, Woudenberg et al. 2009, Chen et al. 2015). According to the most recent phylogenetic analysis of *Phoma*-like taxa (Chen et al. 2015, Hyde et al. 2016), *Didymella sensu stricto* (*Didymella* Sacc. ex Sacc., Syll. Fung. 1: 545. 1882. emend. Q. Chen & L. Cai.) was accommodated in the recently introduced family of *Didymellaceae* (de Gruyter et al. 2009). The molecular phylogenetic studies showed that the family *Didymellaceae* includes most members of *Phoma* and related asexual genera including the new emended and introduced genera of *Phoma*, *Ascochyta*, *Didymella*, *Epicoccum*, *Stagonosporopsis*, *Allophoma*, *Heterophoma*, *Boeremia*, *Paraboeremia*, *Macroventuria*, *Phomatodes*, *Calophoma*, *Leptosphaerulina*, *Neoascochyta*, *Xenodidymella*, *Nothophoma*, *Neodidymelliopsis*, *Neodidymella* and *Neomicrosphaeropsis. Didymella* had first been identified as paraphyletic taxon within the *Didymellaceae* (Aveskamp et al. 2010), then a comprehensive phylogenetic analysis of *Didymellaceae* was carried out (Chen et al. 2015), and in which *Didymella* was emended as monophyletic genus to accommodate 35 known and two unknown species. In the Chen et al. (2015) study, the genus *Didymella* was emended to accommodate the species of *Didymella exigua*, *D. microchlamydospora, D. acetosellae, D. aliena, D. americana, D. anserina, D. arachidicola, D. aurea, D. bellidis, D. boeremae, D. calidophila, D. chenopodii, D. coffeae-arabicae, D. dactylidis, D. dimorpha, D. eucalyptica, D. gardeniae, D. heteroderae, D. lethalis, D. longicolla, D. macrostoma, D. maydis, D. molleriana, D. musae, D. negriana, D. pedeiae, D. pinodes, D. pomorum, D. rhei, D. viburnicola, D. rumicicola, D. sancta, D. senecionicola, D. subglomerata, D. subherbarum, D. curtisii, D. glomerata, D. nigricans, D. pinodella, D. protuberans* and two unidentified species. Recently, *Didymella cirsii* was added (Liu et al. 2015).

The genus *Didymella* is widely distributed in field and ornamental crops as well as in wild plants (Chen et al. 2015). The species of this genus are mainly saprobes that are commonly found in living or dead aerial parts of herbaceous and wooden plants (Chen et al. 2015); some of them also act as mutualistic endophytes with some plant species (Rayner 1922). Very little is known about the pathogenicity of *Didymella sensu stricto*. However, a small number of species belonging to newly recombined genus of *Didymella* was reported as plant pathogen (Tivoli and Banniza 2007, Barilli et al. 2016). The species *Didymella pinodes* (formerly known *Mycosphaerella pinodes*) was reported as main causal agent of *Ascochyta* blight, one of the most important fungal diseases of pea worldwide (Tivoli and Banniza 2007, Barilli et al. 2016). In addition, *Didymella tanaceti* (Syn: *Microsphaeropsis tanaceti* haplotype I) and *D. rosea* (Syn: *M. tanaceti* haplotype II) were reported as plant pathogens, that caused tan spot of pyrethrum (Pearce et al. 2016).

According to the current literature (Aveskamp et al. 2009, Chen et al. 2015), two known strains of *Didymella microchlamydospora* (CBS 105.95 and CBS 491.90) were regarded as saprobes (Chen et al. 2015). In this study change it is evident that this species can cause dieback and necrosis. Here, seven isolates of *Didymella microchlamydospora* were identified using phylogenetic analysis based on ITS, LSU, tub2 and rpb2 sequence data. The morphology and pathogenicity of these isolates is also characterized.

Materials & Methods

Collection of specimens

The specimens were collected from the township of Andimeshk, Ahvaz and Dezful Khuzestan Province in the southwest of Iran. This climate is hot semi-arid (Koppen climate classification BSh) with extremely hot summers and mild winters. These areas are generally very

hot and occasionally humid, while summertime temperatures routinely exceed 45C and in the winter, it can rarely drop below freezing. Rainfall is almost exclusively confined to the period from November to April. During 2015–2016, 48 symptomatic branches and trunks were sampled from the trees of olive (*Olea* spp), bitter orange (*Citrus aurantium*), blackberry (*Morus nigra*), oleander (*Nerium oleander*) and Bottlebrush (*Callistemon viminalis*), with the symptoms of dieback, yellowing and defoliation (Fig. 1). The samples were packed in paper bags and transferred to the lab.

Figure 1 – a The symptoms of die back and decline on *Citrus aurantium*. b The symptoms of stem canker and wood discoloration on *Morus nigra*. c Necrosis and discoloration of branches in *Callistemon viminalis*. d Pathogenecity test, necrosis symptom on a stem fragment of *Morus nigra* caused by pathogenic isolate of *D. microchlamydospora* SCUA 14_Dez_Mor (top) compared to a control fragment (bottom).

Isolation and purification

The small pieces (0.3–1 cm) from healthy and discolored margins of symptomatic branches and dead stems were excised and surface-sterilized by dipping them in 2% sodium hypochlorite (2–4 minutes), followed by washing three times with sterile distilled water (2 min). Then, the fragments were plated on petri plates containing potato dextrose agar (PDA, Difco, USA) supplemented with streptomycin (30 mg/L). The plates were incubated up to 5–15 days at 28 C, and individual colonies were cultured to PDA. The isolates were purified by single spore method. The spore suspension was prepared and 100 µL of which plated on a ¼-strenght PDA. The plates incubated in the dark at 28 C for 24–48 hours and individual small colonies sub-cultured on PDA as single-spore isolates. The living cultures of the isolates were deposited in the Collection of Fungal Cultures, Department of Plant Protection, Shahid Chamran University of Ahvaz, Iran (SCUA 11-SCUA 17).

Microscopy and growth indicators

The isolates of *Didymella* were grown on potato dextrose agar (PDA, Merck) and corn meal agar (CMA, Sigma Aldrich) at 28 C, with 12 hours fluorescent light and 12 hours darkness. The diameter of colonies was daily measured up to 10-day incubation. Morphological characters were made at 3–25 days post-inoculation and the colour rate was determined according to the Methuen handbook of color (Kornerup & Wanscher 1967). The microscopic preparations were made by

using the method of Riddle (1950) and Measurements were carried out with the 40× and 100× objective lens of a Leitz wetzlar (SM-LUX) Basic Biological Light Microscope. The sizes of characteristic structures were recorded with 50–70 measurements for each structure. The photomicrographs were made with an OLYMPUS BX51 microscope fitted with an OLYMPUS DP12 digital camera. Macroscopic and microscopic morphological characters were used to compare the isolated fungal taxa with the assistance of current mycological literature (Aveskamp et al. 2009, Chen et al. 2015). Then, for accurate identification, the isolates were subjected to DNA analysis.

Pathogenicity test

The stem fragments of each trees with similar height, diameter, and vigor were selected. After surface sterilizing the fragments with 2% sodium hypochlorite (2–4 min) and washing by sterilized distilled water, a 3-mm-diameter hole was made to the depth of the cambium at 2–3 cm from both sides of each stem using a scalper. A small quantity of inoculum taken from active-growing edge of the colonies *Didymella microchlamydospora* isolates was inoculated into each wound. Free culture media was placed into wounds as control. The replicates of each treatment were separately placed into water containing desiccators, sterilized as moist chamber. The desiccators were incubated at 28°C for 3 to 6 weeks after inoculation. Pathogenicity of each isolate were evaluated 3 to 6 weeks after inoculation by indicating: (i) the presence or absence of callus around the wound, (ii) the growth and sporulation of fungus in bark surrounding the inoculation point, (iii) the extent of external longitudinal spread of lesions and (iv) the internal longitudinal spread of discoloration in xylem.

DNA extraction and amplification

The mycelial biomass of *Didymella* isolates grown into flasks containing potato dextrose broth (PDB) was harvested by passing through sterilized filter papers. The mycelia were freeze-dried (Freeze-Dryer, Alpha 1-2LD Plus, Christ) and powdered in the mortar containing liquid nitrogen. The genomic DNA was isolated according to modified method established by Reader and Broda (1985). The mycelial powder was lysed with a lysis buffer and then extracted three times by Phenol:chloroform:isoamyl alcohol. The genomic DNA was recovered through ethanol-precipitation typical method. The DNAs were qualified and quantified using Spectrophotometer (Eppendorf BioPhotometer plus) and loading on the gel. The partial regions of ITS-LSU, rpb2 and tub2 were amplified using the primer pairs of ITS1/ NL4 (White et al. 1990, O'Donnell 1993), RPB2-5F2/ fRPB2-7cR (Liu et al. 1999, Sung et al. 2007) and Btub2Fd/ Btub4Rd (Woudenberg et al. 2009), respectively. PCR reactions were completed in 50 µL final volumes and consisted of 5 µL 10× prime Taq Reaction Buffer (GenBio, South Korea), 6 µL $MgCl_2$ (25mM), 0.6 µL Prime Taq DNA Polymerase (5U/ µ), 2 µL of each primer (10mM), 2 µL dNTP (10mM mix), 100–500ng DNA and miliqure water up to 50 µL. The amplification were performed in a thermocycler (MJ Mini^{TM} Gradient Thermal Cycler) and run with a temperature profile described in the following: the PCR cycling were for ITS-LSU amplification, initial melting at 94 C for 5 minutes, 35 cycles each of 30 seconds at 94 C, 30 seconds at 57 C, and 90 seconds at 72 C and followed with a final extension at 72 C for 10 minutes, for the tub2 amplification, initial melting at 94 C for 5 minutes, 35 cycles each of 30 seconds at 94 C, 30 seconds at 58 C, and 60 seconds at 72 C and followed with a final extension at 72 C for 10 minutes and for the rpb2 amplification, initial melting at 94 C for 5 minutes, 35 cycles each of 30 seconds at 94 C, 30 seconds at 57 C, and 60 seconds at 72 C and followed with a final extension at 72 C for 10 minutes.

Sequencing and phylogenetic analyses

PCR products were purified through ethanol-precipitation method (Crouse & Amorese 1987) and then sequenced using forward and reverse primers by Macrogen Company. The Sequences obtained from each primer pairs were assembled using DNA Baser Sequence Assembeler v4 programs (2013, Heracle BioSoft, www.DnaBaser.com). The phylogenetic analysis of *Didymella*

microchlamydospora isolates was carried out with including the reference sequences belonging to the known genera of *Didymellaceae* and species of *Didymella* (225 available sequences mostly from Aveskamp et al. 2010 and Chen et al. 2015 included) (Table 1). The species of *Pleospora betae* were used as outgroup taxon to root phylogenetic trees.

The sequences of ITS, LSU, tub2 and rpb2 were aligned individually using ClustalW in BioEdit v. 7.0.9.0 (Hall 1999), trimmed to the same starting position and then assembled. The combined ITS-LSU-tub2 and ITS-LSU-tub2-rpb2 datasets were multiple-aligned using ClustalW in BioEdit v. 7.0.9.0 (Hall 1999). Phylogenetic analysis was performed with maximum parsimony and maximum likelihood algorithm. Phylogenetic trees were constructed using MEGA version 6 (Tamura et al. 2013). Best-fitting ML nucleotide substitution model for each dataset was determined using the model test function in MEGA version 6. The phylogenetic trees were constructed with Subtree-Pruning-Regrafting (SPR) algorithm and following options: Gaps (insertion/deletions) were treated as missing data, Bootstrap (BP) analyses were done with 1000 replicates, Initial Trees for ML were made by NJ/BioNJ algorithm and Branch Swap Filter was set very strong. Two final alignments used for phylogenetic analyses were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S21100).

Results

Morphological characterization (Fig. 2)

Hyphae diameter in 14-day colonies 2.5–4 µm ($\overline{x}$ = 3.2 µm, n = 50). Conidiomata pycnidial, pycnidia mostly solitary or aggregated, superficial on or submerged into the agar, dark brown**,** with age becoming darker, variable in shape and size (macro-and micro-pycnidium). Macropycnidia globose, glabrous or covered with hyphal outgrows, 100–190 × 100–190 µm ($\overline{x}$ = 139 × 139 µm, n = 50) (Fig. 2). Ostioles 1–3, papillate, rarely on a distinct neck. Pycnidial wall pseudoparenchymatous, composed of oblong to isodiametric cells, 2–5 layers. Micropycnidia globose to subglobose, glabrous or covered with hyphal outgrows, 50–80 × (40–)49–70(–80) µm ($\overline{x}$ = 61 × 59 µm, n = 50). Conidia hyaline to pale brown, smooth- and thin-walled, subglobose to ellipsoidal, aseptate and guttulate, (2.5)3–5.5(6) × (1.5)2–3.2(3.8) µm ($\overline{x}$ = 4.3 × 2.4 µm, n = 70). Chlamydospores mostly unicellular, solitary or in chain, intercalary or terminal, smooth, brown, globose to subglobose, (3)4–7.5(10) × (2.5)3–7.5 µm ($\overline{x}$ = 5.9 × 4.6 µm, n = 50). Multicellular Chlamydospores (pseudosclerotioid and dictyosporous) variable in shape and size, brown, intercalary, sparse and solitary, smooth.

Colonies on PDA, 70–80 mm diameter after 10 days of incubation at 28±0.5 C, blackish-brown with whitish cream margins at early growth stage, with age becoming blackish green in the central and olivaceous green in the edge, staining the agar in pink collar due to the production of a diffusible pigment, floccose growth, the rings of sporulation containing black pycnidia becoming darker and compacter towards the center of the colony; reverse blackish green with creamy to orange edges, leaden black in zones with abundant pycnidia, darkening towards the center of the colony. Colonies on CMA, 65–75 mm diameter after 10 days of incubation at 28 ± 0.5 C, grey to brownish grey with lighter edge, smooth, the pycnidia appear as scattered small dots of brown to black or rings of sporulation; reverse grey to olivaceous green with lighter edge, leaden blackish brown in pycnidia containing zone.

Material examined – IRAN, Khuzestan Province, Andimeshk, on dead branch of *Olea europaea*, 11 August 2015, S.A. Ahmadpour (SCUA 11_And_ Ole); Ahvaz, on dead branch of *Olea europaea*, 12 Oceober 2015, S.A. Ahmadpour (SCUA 12_Ahw_Ole); on dead branch of *Citrus aurantium*, 12 Oceober 2015, S.A. Ahmadpour (SCUA 13_Ahw_Cit); on dead branch of *Olea* sp, 12 Oceober 2015, S.A. Ahmadpour (SCUA 16_Ahv_Ole); on dead branch of *Callistemon viminalis*, 12 Oceober 2015, S.A. Ahmadpour (SCUA 15_Ahv_Cal); on dead branch of *Nerium* sp, 12 Oceober 2015, S.A. Ahmadpour (SCUA 12_Ahv_Ner); Dezful, on dead branch of *Morus nigra*, 15 August 2015, S.A. Ahmadpour (SCUA 14_Dez_Mor).

Table 1 Strains used in this study and their GenBank accession numbers. Newly generated sequences are indicated in bold.

Species name	Isolate name or strain no.	Source	Origin	GenBank Accession number			
				ITS	LSU	rpb2	tub2
Didymella microchlamydospora	IRAN 2788C; SCUA 11_And_Ole	*Olea europaea*	Iran	**KX139019**	**KX139028**	**KY464923**	**KY449026**
D. microchlamydospora	IRAN 2789C; SCUA 12_Ahw_Ole	*Olea europaea*	Iran	**KX139018**	**KX139027**	**KX821250**	**KX821247**
D. microchlamydospora	IRAN 2790C; SCUA 13_Ahw_Cit	*Citrus aurantium*	Iran	**KX139014**	**KX139023**	**KX821249**	**KX821246**
D. microchlamydospora	SCUA 14_Dez_Mor	*Morus nigra*	Iran	**KX139012**	**KX139021**	**KX821248**	**KX821245**
D. microchlamydospora	IRAN 2791C; SCUA 16_Ahv_Ole	*Olea* sp.	Iran	**KY449004**	**KY449013**	**-**	**-**
D. microchlamydospora	SCUA 15_Ahv_Cal	*Callistemon viminalis*	Iran	**KY449005**	**KY449014**	**-**	**-**
D. microchlamydospora	IRAN 2792C; SCUA 12_Ahv_Ner	*Nerium* sp.	Iran	**KY449006**	**KY449015**	**-**	**-**
D. exigua	CBS 183.55	*Rumex arifolius*	France	GU237794	EU754155	EU874850	GU237525
D. acetosellae	CBS 179.97	*Rumex hydrolapathum*	The Netherlands	GU237793	GU238034	KP330415	GU237575
D. aliena	CBS 379.93	*Berberis* sp.	The Netherlands	GU237851	GU238037	KP330416	GU237578
D. americana	CBS 185.85	*Zea mays*	USA	FJ426972	GU237990	KT389594	FJ427088
D. anserina	CBS 253.80	-	Germany	KT389498	KT389715	KT389595	KT389795
D. arachidicola	CBS 333.75	*Arachis hypogaea*	South Africa	GU237833	GU237996	KT389598	GU237554
D. aurea	CBS 269.93	*Medicago polymorpha*	New Zealand	GU237818	GU237999	KT389599	GU237557
D. bellidis	CBS 714.85	*Bellis perennis*	The Netherlands	GU237904	GU238046	KP330417	GU237586
D. boeremae	CBS 109942	*Medicago littoralis*	Australia	FJ426982	GU238048	KT389600	FJ427097
D. chenopodii	CBS 128.93	*Chenopodium quinoa*	Peru	FJ427060	GU238053	-	GU237591
D. coffeae-arabicae	CBS 123380	*Coffea arabica*	Ethiopia	FJ426993	GU238005	KT389603	FJ427104
D. curtisii	PD 92/1460	*Sprekelia* sp.	The Netherlands	FJ427041	GU238012	KT389604	FJ427151
D. eucalyptica	CBS 377.91	*Eucalyptus* sp.	Australia	GU237846	GU238007	KT389605	GU237562
D. exigua	CBS 183.55	*Rumex arifolius*	France	GU237794	EU754155	EU874850	GU237525
D. microchlamydospora	CBS 105.95	*Eucalyptus* sp.	UK	FJ427028	GU238104	KP330424	FJ427138
D. rhei	CBS 109177	*Rheum rhaponticum*	New Zealand	GU237743	GU238139	KP330428	GU237653
D. rumicicola	CBS 683.79	*Rumex obtusifolius*	New Zealand	KT389503	KT389721	KT389622	KT389800
D. sancta	CBS 281.83	*Ailanthus altissima*	South Africa	FJ427063	GU238030	KT389623	FJ427170

Table 1 (continued)

Species name	Isolate name or strain no.	Source	Origin	GenBank Accession number			
				ITS	LSU	rpb2	tub2
Didymella sp. 1	CBS 379.96	*Pteris* sp.	The Netherlands	KT389504	KT389722	KT389624	KT389801
Didymella sp. 2	CBS 115.58	*Chrysanthemum roseum*	Germany	KT389505	KT389723	KT389625	KT389802
D. subglomerata	CBS 110.92	*Triticum* sp.	USA	FJ427080	GU238032	KT389626	FJ427186
D. viburnicola	CBS 523.73	*Viburnum cassioides*	The Netherlands	GU237879	GU238155	KP330430	GU237667
D. negriana	CBS 358.71	*Vitis vinifera*	Germany	GU237838	GU238116	KT389610	GU237635
D. nigricans	PD 77/919	*Actinidea chinensis*	New Zealand	GU237915	GU238001	KT389611	GU237559
D. pedeiae	CBS 124517	*Schefflera elegantissima*	The Netherlands	GU237770	GU238127	KT389612	GU237642
D. pinodella	CBS 531.66	*Trifolium pretense*	USA	FJ427052	GU238017	KT389613	FJ427162
D. pinodes	CBS 525.77	*Pisum sativum*	Belgium	GU237883	GU238023	KT389614	GU237572
D. protuberans	CBS 377.93	*Daucus carota*	The Netherlands	GU237847	GU238014	KT389619	GU237565
D. molleriana	CBS 229.79	*Digitalis purpurea*	New Zealand	GU237802	GU238067	KP330418	GU237605
D. exigua	CBS 183.55	*Rumex arifolius*	France	GU237794	EU754155	EU874850	GU237525
D. lethalis	CBS 103.25	-	-	GU237729	GU238010	KT389607	GU237564
D. mascrostoma	CBS 482.95	*Larix decidua*	Germany	GU237869	GU238099	KT389609	GU237626
D. maydis	CBS 588.69	*Zea mays*	USA	FJ427086	EU754192	GU371782	FJ427190
D. calidophila	CBS 448.83	Soil	Egypt	*FJ427059*	GU238052	-	FJ427097
D. dactylidis	CBS 124513	*Dactylis glomerata*	USA	GU237766	GU238061	-	GU237599
D. dimorpha	CBS 346.82	*Opuntiae* sp	Spain	GU237835	GU238068	-	GU237606
D. gardeniae	CBS 626.68	*Gardenia jasminoides*	India	FJ427003	GQ387595	KT389606	FJ427114
D. glomerata	CBS 528.66	*Chrysanthemum* sp.	The Netherlands	FJ427013	EU754184	FJ427013	FJ427124
D. heteroderae	CBS 109.92	Undefined material	The Netherlands	FJ426983	GU238002	KT389601	FJ427098
Neodidymelliopsis cannabis	CBS 234.37	*Cnnabis sativa*	-	GU237804	GU237961	KP330403	GU237523
Xenodidymella applanata	CBS 205.63	*Rubus idaeus*	The Netherlands	GU237798	GU237998	KP330402	GU237556
Paraboeremia adianticola	CBS 187.83	*Polystichum adiantiforme*	USA	GU237796	GU238035	KP330401	GU237576
Ascochyta pisi	CBS 122751	*Pisum sativum*	Canada	KP330432	KP330444	EU874867	KP330388
Phomatodes aubrietiae	CBS 627.97	*Aubrietia* sp.	The Netherlands	GU237895	GU238045	KT389665	GU237585
Calophoma clematidina	CBS 102.66	*Clematis* sp.	UK	FJ426988	FJ515630	KT389587	FJ427099
Phoma herbarum	CBS 377.92	Human leg	The Netherlands	KT389536	KT389756	KT389663	KT389837
Macroventuria anomochaeta	CBS 525.71	*Decayed canvas*	South Africa	GU237881	GU237984	GU456346	GU237544
Leptosphaerulina australis	CBS 317.83	*Eugenia aromatica*	Indonesia	GU237829	EU754166	GU371790	GU237540

Table 1 (continued)

Species name	Isolate name or strain no.	Source	Origin	GenBank Accession number			
				ITS	LSU	rpb2	tub2
Epicoccum nigrum	CBS 125.82	Human toenail	The Netherlands	FJ426995	GU237974	KT389631	FJ427106
Stagonosporopsis hortensis	CBS 104.42	-	The Netherlands	GU237730	GU238198	KT389680	GU237703
Allophoma tropica	CBS 436.75	*Saintpaulia ionantha*	Germany	GU237864	GU238149	KT389556	GU237663
Heterophoma adonidis	CBS 114309	*Adonis vernalis*	Sweden	KT389506	KT389724	KT389637	KT389803
Neoascochyta exitialis	CBS 118.40	-	-	KT389514	KT389732	KT389647	KT389812
Pleospora betae	CBS 523.66	*Beta vulgaris*	The Netherlands	FJ426981	EU754179	KT389670	KT389842

1 Abbreviation of culture collections: CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; PD: Plant Protection Service, Wageningen, the Netherlands; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; SCUA: the Collection of Fungal Cultures, Department of Plant Protection, Shahid Chamran University of Ahvaz, Iran.

DNA analysis and phylogenetic characterization

The sequences of ITS, LSU, tub2 and rpb2 belonging to the isolates under study were submitted to GenBank (table 1) under the generic name *Didymella microchlamydospora.* These isolates shared 98.8% sequence identity in the ITS region (430 bp) attributed to 2 SNPs and three bp insertion/deletion, 100% sequence identity in the LSU region (590 bp), 99.7% sequence identity in the *tub2* region (306 bp) attributed to one SNPs, and 99 % sequence identity in the rpb2 region (782 bp) attributed to eight SNPs. Using a BLASTn search, the ITS sequences of seven *D. microchlamydospora* isolates showed 99–100% sequence identity to reference strain *D. microchlamydospora* CBS 105.95.

Sixty-two and 55 taxa, including all described species of *Didymella* and a type species from all the known genera of *Didymellaceae*, were included in the three-locus and four-locus based phylogeny, respectively (Table 1). The composite sequence alignment was 1206 and 1809 characters in length, including alignment gaps (ITS: 420 bp, LSU: 500 bp, tub2: 286 bp, rpb2: 603 bp) for three and four regions, respectively. Of those characters 1233 bp (ITS: 324 bp, LSU: 450 bp, tub2: 176 bp, rpb2: 283 bp) were constant and 576 bp (ITS: 96 bp, LSU: 50 bp, tub2: 110 bp, rpb2: 320 bp) were variable. The best-fitting ML nucleotide substitution model for phylogenetic analysis of three-locus and four-locus combined datasets were selected Tamura-Nei (TN93+G+I) and General Time Reversible (GTR+G+I) models, respectively. The phylogenetic trees of the maximum likelihood analysis based on both combined datasets are shown in Figs 3 and 4. The topology of phylogenetic trees showed very little differences, and both trees presented generally consistent relationships among the strongly supported clades (Figs 3, 4). The trees topology of both three- and four-locus phylogenetic analysis provided the evidence that the isolates under study were associated with the species *Didymella microchlamydospora*. In both trees, our isolates and a reference strain from GenBank, *D. microchlamydospora* CBS 105.95, generated supportive monophyletic clades with strong BS 99% and 100% support. In both trees, the reference strain of *Neoascochyta exitialis* CBS 118.40 among the representative members of the family *Didymellaceae* positioned as a basal taxon. In addition, the trees obtained through maximum parsimony analysis supported the tree obtained from ML analysis (not shown).

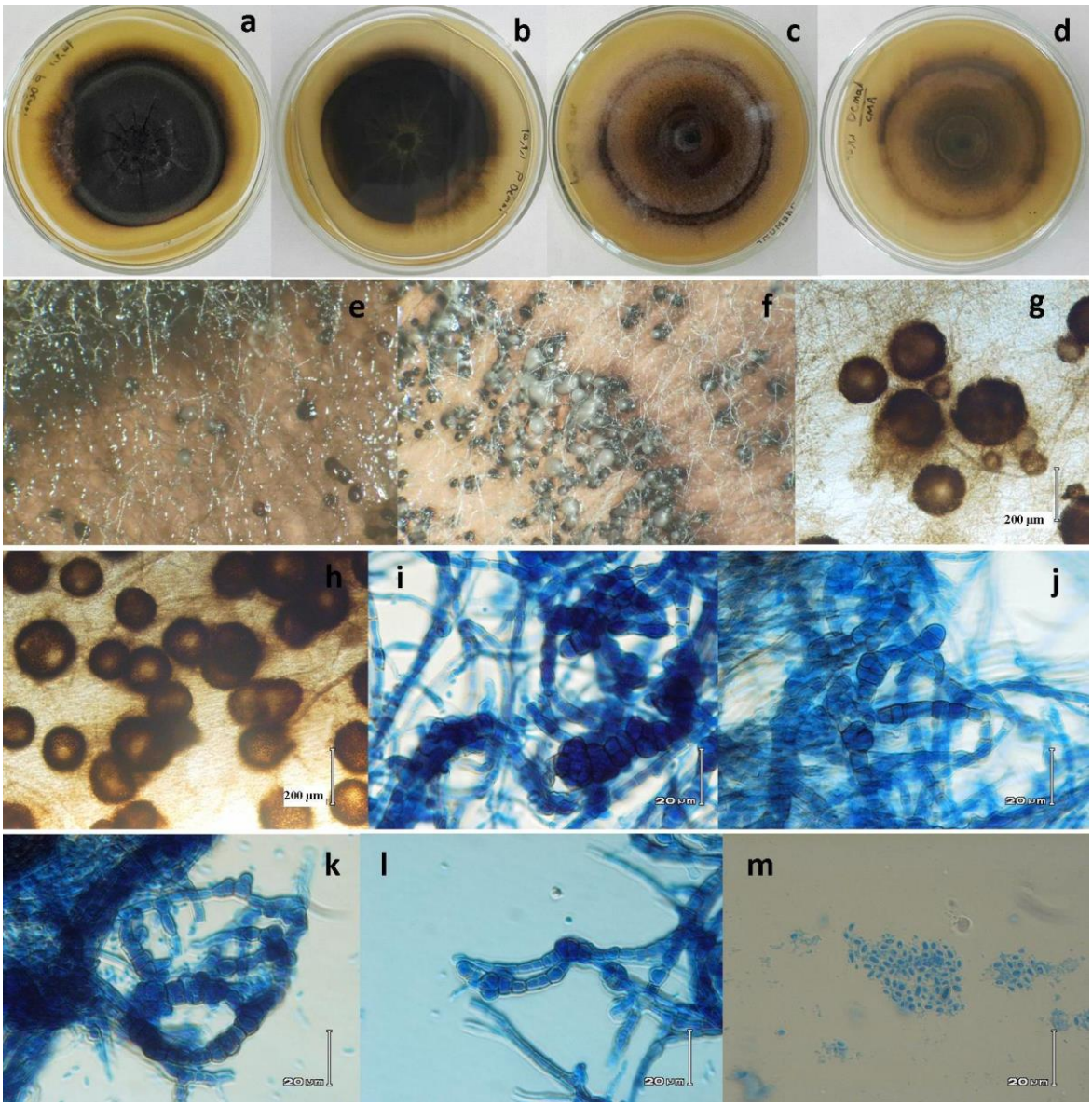

Figure 2 – *Didymella microchlamydospora* isolate SCUA 14_Dez_Mor. a, b Colony on PDA (front and reverse). c, d Colony on CMA (front and reverse). e, f, g, h Pycnidia formed on PDA and CMA. i, j, k, l Chlamydospores. m Conidia.

Ecology and distribution

In the last decade, some of the decline symptoms including; yellowing, wilting, defoliation, dieback and canker were observed on various tree species in whole area of investigation. This disease affected about 5% of the various ornamental and fruit trees such as *Citrus* spp., *Eucalyptus* spp., *Morus* spp., *Conocarpus erectus*, *Ziziphus nummularia*, *Nerium oleander*, *Juglans regia*, *Prosopis spicigera*, *Cupressus semperviren*, *Punica grenatum*, *Prosopis stephaniana*, *Olea europaea*, *Callistemon viminalis*, *Bauhinina purpurea*, *Albizia lebbeck* and *Cordia mixa*. The first observed signs in affected trees were dieback, and in which the dead of infected tissues resulted in the girdling of shoots and branches. Following, the causal fungus developed internally and destroyed the growth rings, which is the characteristic of other stem canker causing agents. Death of branches throughout the crown led to gradual tree decline or the tree was dying starting from the top of the crown. An attempt was made to identify the potential canker pathogens and other associated agents. In all, 48 samples were surveyed, 49 isolates of potential pathogenic fungi were detected (unduplicated data), and seven isolates were identified as *Didymella microchlamydospora*. The isolates of *Didymella microchlamydospora* SCUA 11-And_Ole and SCUA 12-Ahv_Ole were

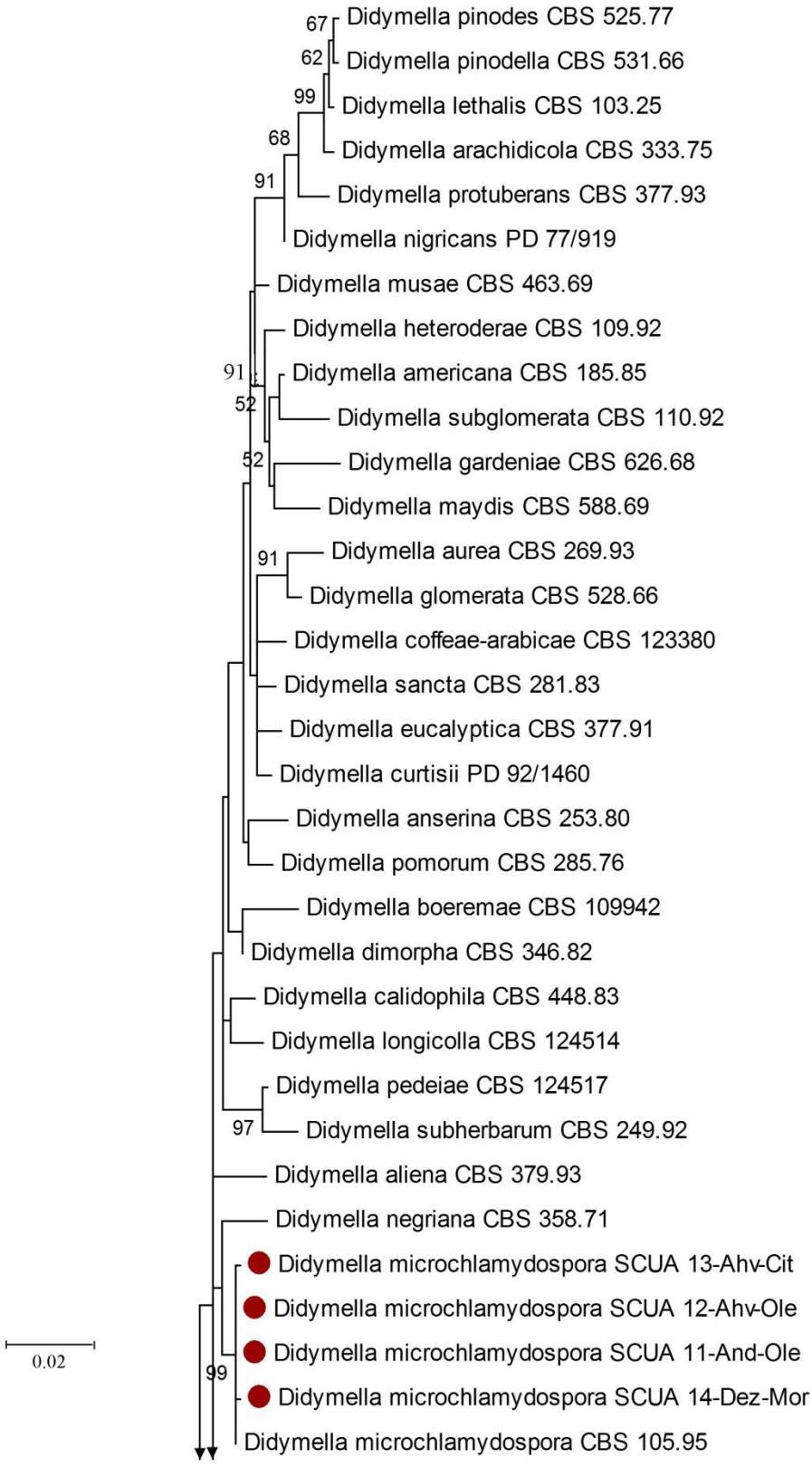

Figure 3 – Phylogenetic tree constructed from a maximum likelihood analysis based on a concatenated alignment of ITS, LSU and tub2 sequences of four *Didymella microchlamydospora* isolates under study and 16 type strains representing a type species of each described genus of *Didymellaceae* and 42 described species of genus *Didymella* downloaded from GenBank. Bootstrap values greater than 50% (expressed as percentages of 1000 replications) are shown at the nodes. The tree was rooted with *Pleospora betae* CBS 523.66.

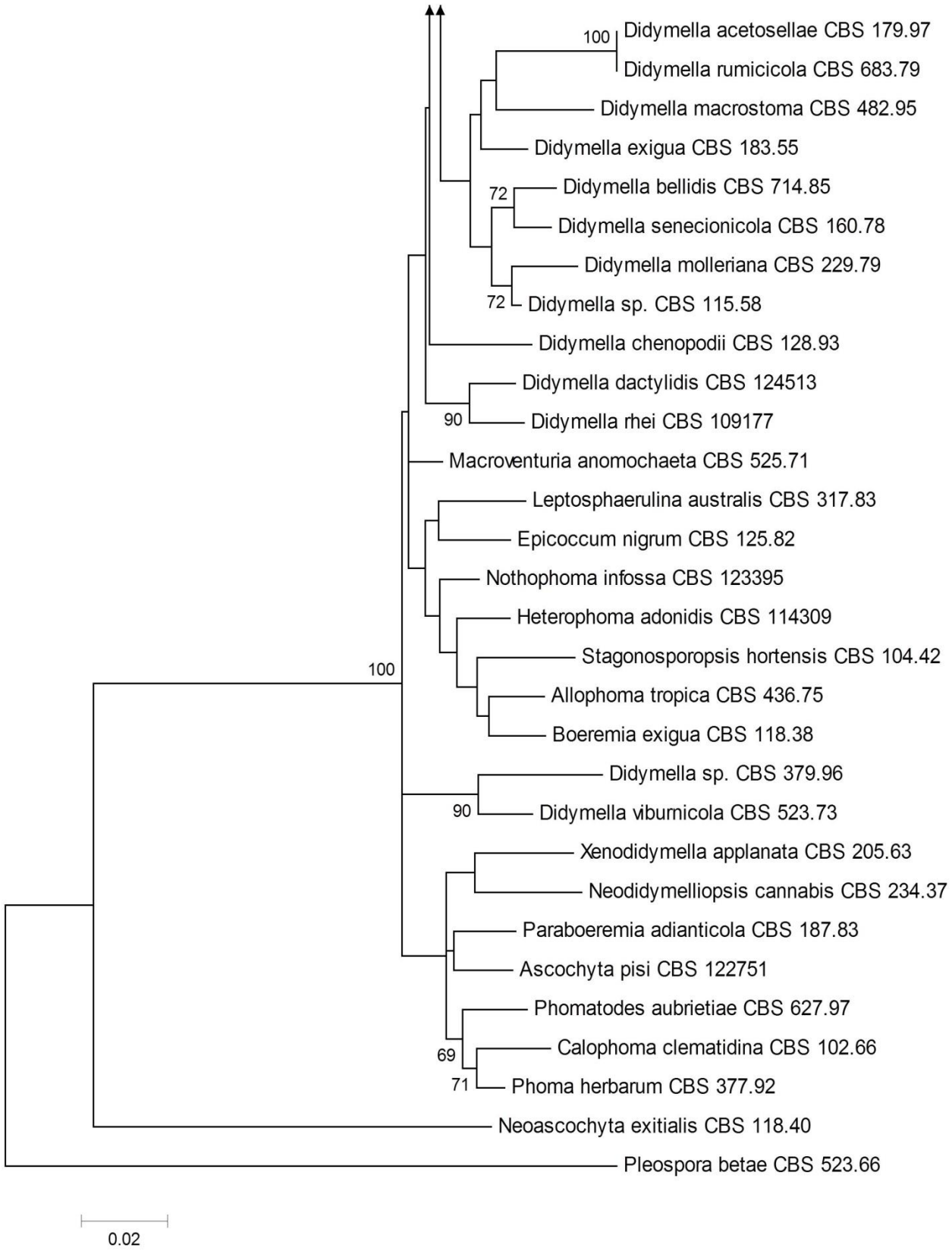

Figure 3 (continued)

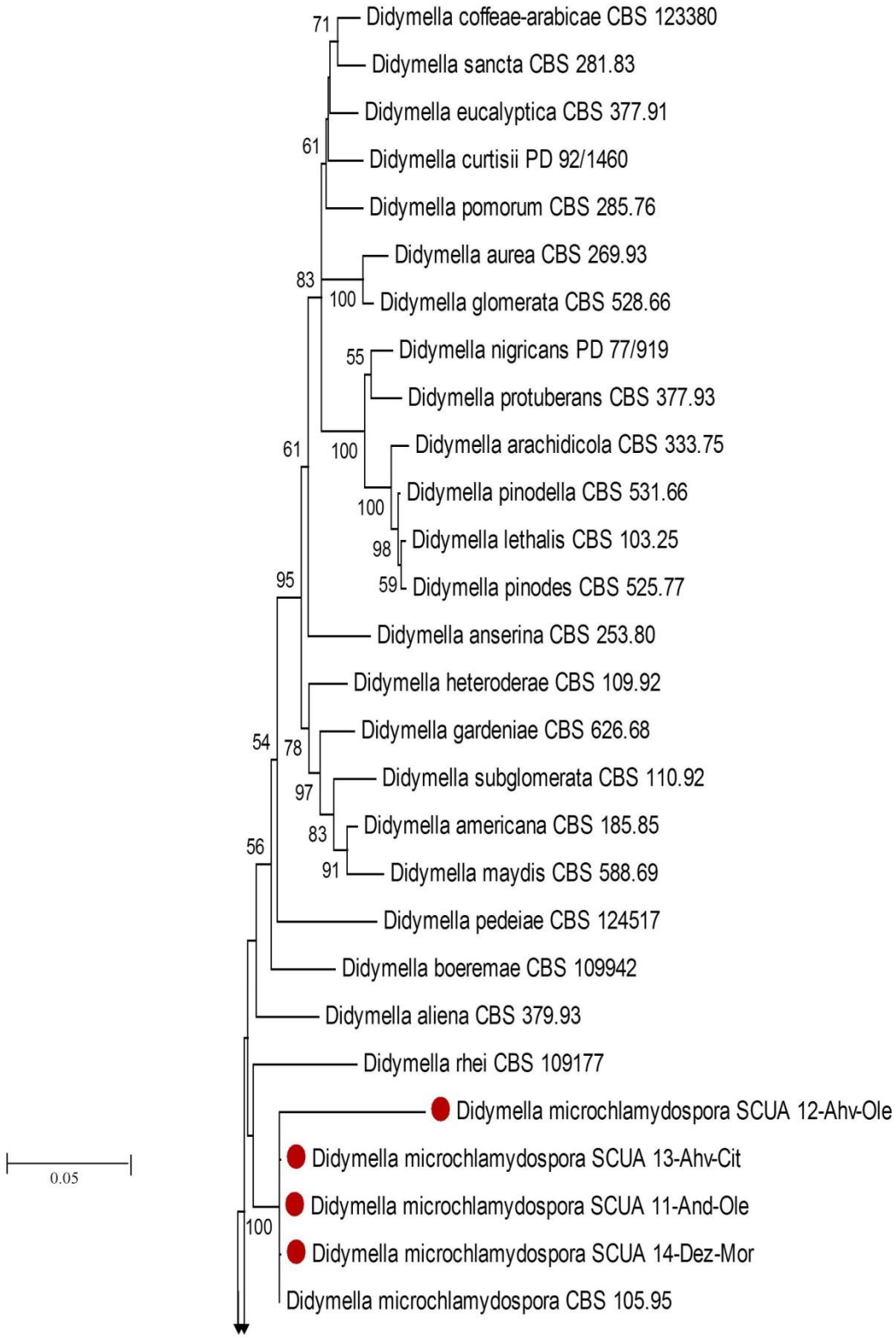

Figure 4 – Phylogenetic tree constructed from a maximum likelihood analysis based on a concatenated alignment of ITS, LSU, tub2 and rpb2 sequences of four *Didymella microchlamydospora* isolates under study and 16 type strains representing a type species of each described genus of *Didymellaceae* and 35 described species of genus *Didymella* downloaded from GenBank. Bootstrap values greater than 50% (expressed as percentages of 1000 replications) are shown at the nodes. The tree was rooted with *Pleospora betae* CBS 523.66.

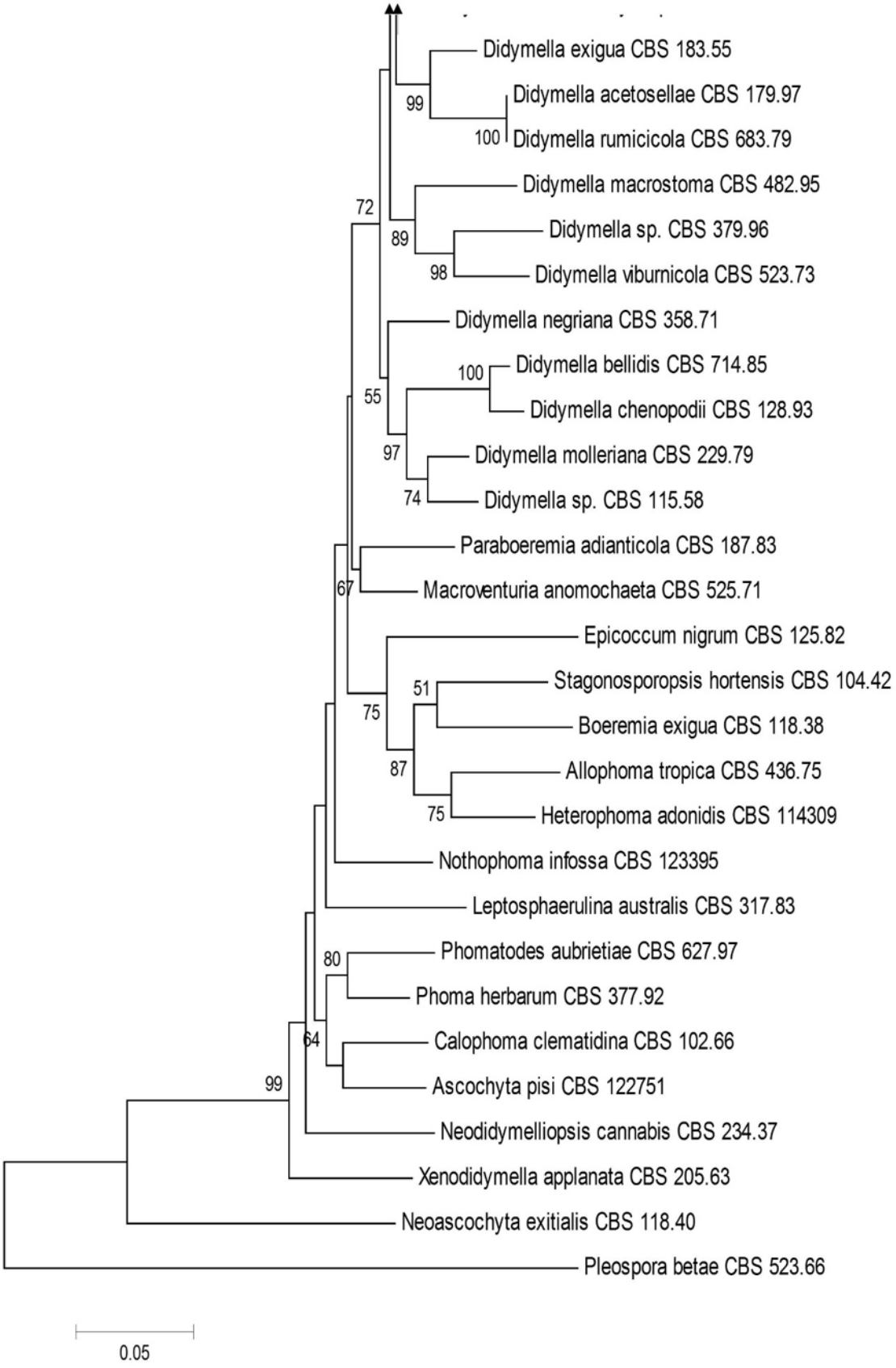

Figure 4 (continued)

firstly isolated from dead branches of olive (*Olea europaea, Oleaceae*) in Andimeshk and Ahvaz, and then subsequently, *D. microchlamydospora* SCUA 13-Ahv_Cit from Bitter orange (*Citrus aurantium, Rutaceae*) in Ahvaz, *D. microchlamydospora* SCUA 14-Dez_Mor from blackberry (*Morus nigra, Moraceae*) in Dezful, *D. microchlamydospora* SCUA 15- Ahv_Ner from oleander *(Nerium oleander, Apocynaceae*) in Ahvaz and *D. microchlamydospora* SCUA 16-Ahv_Cal from weeping bottlebrush (*Callistemon viminalis, Myrtaceae*) in Ahvaz.

Pathogenicity tests

Both isolates of *Didymella microchlamydospora* SCUA 11_And_Ole and *D. microchlamydospora* SCUA 14_Dez_Mor were able to grow and sporulate in the bark surrounding the inoculation point on stem fragments of *Olea europaea* and *Morus nigra*, respectively. The isolate of *D. microchlamydospora* SCUA 14_Dez_Mor developed the external longitudinal lesion on the inoculation point three weeks after inoculation, which was associated with wood necrosis and discoloration in xylem (Fig. 1), while the isolate of *D. microchlamydospora* SCUA 11_And_Ole did not. In both test plants, the callus was not formed around the inoculation wound. This pathogenic fungus was re-isolated from necrosis-like areas formed on stem fragments of *M. nigra*, and the identity as *D. microchlamydospora* species was confirmed by morphological characterization.

Discussion

In our study, seven *Didymella microchlamydospora* isolates were recovered from 48 plant species. This is the first report of *D. microchlamydospora* in Iran. *Phoma microchlamydospora* Aveskamp & Verkley, was described by Aveskamp et al. (2009), and then, recombined into *Didymella microchlamydospora* by Chen et al. (2015). Here, further morphological and molecular characterization, pathogenicity on *Morus nigra*, and a phylogenetic analysis between the isolates under study and other species within the *Didymellaceae* was evaluated.

In morphology, our isolates are slightly different from reference strain of D. *microchlamydospora* CBS 105.95. The diameter of macropycnidia was less than to the strain *D. microchlamydospora* CBS 105.95 (100–190 vs. 150–260 μm) (Aveskamp et al. 2009). In similar to the reference strain (Aveskamp et al. 2009), our isolates produced ostiolate and papillate pycnidia, but rarely on a distinct neck as described for *D. microchlamydospora* CBS 105.95. The width and length of conidia and unicellular chlamydospores are somewhat different but it cannot be used to distinguish the species from each other. The numerous measurements in this study and previous observations (McPartland 1994, Chen et al. 2015) demonstrated that, in general, the conidial length is much more variable, and the conidial size mostly depends on the location of pycnidia. Furthermore, Conidia in pycnidia produced on culture have been usually observed somewhat larger than those from living host (McPartland 1994).

In the current study, the identification of *Didymella microchlamydospora* isolates based on morphological characterization and BLAST search algorithm is strongly supported in multi-locus phylogeny based on the combined regions of ITS, LSU, tub2 and rpb2. Four isolates of *D. microchlamydospora* were used in the phylogenetic analyses for constructing two phylograms based on three-locus (ITS-LSU-tub2) and four-locus (ITS-LSU-tub2-rpb2) based combined datasets. In both three- and four- locus based phylogenetic trees, sequence dataset worked well to distinguish closely related species in *Didymella* and our isolates clustered with reference strain *D. microchlamydospora* CBS 105.95, distinct from the other *Didymella* species (Figs 3, 4). Analysis of congruence between the ITS, LSU, tub2 and rpb2 loci used in the phylogenetic analysis showed that the LSU region had the lowest correlation scores with 10% sequence diversity and rpb2 region had the highest correlation scores with 53% sequence diversity. This was most probably due to the low resolution provided by the LSU, which was expected due to the nature of its evolution within species. The LSU locus shared 90% sequence identify between the species of *Didymellaceae*, indicating their close phylogenetic relationship. However, the LSU locus of filamentous fungi is often not sufficient to delimit taxa at the species level (Lumbsch et al. 2000, Eberhardt 2010). Due

to the abundant homoplasy in phenotypic characteristics and difficulties in the morphological identification, it is difficult to distinguish *Phoma*-like taxa including, the species of *Didymella* (Chen et al. 2015). Genealogical concordance analysis using several unlinked DNA loci have been already resulted in the dramatic taxonomic changes in *Phoma* and *Phoma*-like genera (de Gruyter et al. 2009, 2010, 2012, Aveskamp et al. 2010, Ariyawansa et al. 2015, Chen et al. 2015, Liu et al. 2015, Hyde et al. 2016, Li et al. 2016, Tibpromma et al. 2017) as well as other fungi such as in *Alternaria* (Woudenberg et al. 2013), *Bipolaris* (Manamgoda et al. 2011, 2012), *Colletotrichum* (Cannon et al. 2012, Jayawardena et al. 2016), *Fusarium* (Short et al. 2013, Laurence et al. 2014), *Phyllosticta* (Wikee et al. 2011, Hyde et al. 2014), *Trichoderma* (Druzhinina et al. 2010) and other taxa in Kingdom *Myceteae* (Ariyawansa et al. 2015, Crous et al. 2015, Liu et al. 2015, Hyde et al. 2016, Li et al. 2016, Tibpromma et al. 2017). Chen et al. (2015) have indicated, that the combined sequence of ITS, LSU, tub2 and rpb2 work well in demarcating *Didymella* species. The results of these phylogenetic analyses validate the species delimitation of our isolates as *D. microchlamydospora*.

In pathogenicity tests, of the four tested isolates, *D. microchlamydospora* SCUA 14_Dez_Mor formed the necrosis symptom on stem fragments of *Morus nigra* (Fig. 1). Dark brown to black discoloration expanded rapidly in a longitudinal direction. Previous studies have shown that *Didymella pinodes* on *Pisum sativum* (Tivoli & Banniza 2007, Barilli et al. 2016) and *Didymella tanaceti* and *Didymella rosea* on pyrethrum plant (Pearce et al. 2016) acts as a phytopathogen in the UK and Australia, respectively. Chen et al. (2015) and Pearce et al. (2016) supported the placement of these phytopathogenic species in *Didymella sensu stricto*. In our study, one isolate of *Didymella microchlamydospora* infected plant hosts and developed necrosis symptoms. To the best of our knowledge, this is the first phyto-pathogenicity report for *Didymella microchlamydospora* worldwide.

Observational assessment of areas sampled showed, the disease index and tree mortality positively correlates with environmental stress. Since drought and extremely hot summers became more common in Khuzestan during the last decade, higher than usual incidence of die back diseases may be due to drought stresses and higher annual temperatures that made trees more susceptible to the disease. Observational assessment showed that there was a clear increase in decline symptoms in the zones with low fertility soils, deficiency of water, prolonged exposure to extremely high temperatures, summer sunscald, nutritional imbalances, soil compaction, changes in the soil grade and mechanical injuries. Previous studies showed that environmental stress, such as high temperatures and drought periods could play a role in increasing the virulence and expansion of the *Didymellaceae*, *Botryosphaeriaceae* and other decline pathogens (Smith et al. 1996, Kim et al. 2001, Arnold & Herre 2003, Desprez-Loustau et al. 2007, Slippers & Wingfield 2007, Botella et al. 2010, Dissanayake et al. 2015, Fan et al. 2016, Anonym 2017, Delgado-Cerrone 2017).

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