



Characterization and pathogenicity of *Neoscytalidium novaehollandiae* causing dieback and sooty canker in Iran

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Received: 23 February 2023 / Accepted: 22 June 2023 / Published online: 11 July 2023
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Abstract

Members of the *Botryosphaeriaceae* commonly cause stem cankers and dieback of tropical and subtropical woody plants. A survey was conducted to isolate and identify the fungal causal agents of dieback and sooty canker of trees in some areas of Khuzestan province, southwestern Iran, 2015–2022. Following visual inspection, 63 trees belonging to 22 species were detected with typical signs of the disease in eight townships, and symptomatic material including branches and stems were sampled from those trees. Fungal isolation was performed on Potato Dextrose Agar (PDA), and the isolates obtained were induced into sporulation using pine needles in Water Agar (WA). All isolates were identified as *Neoscytalidium novaehollandiae*, based on multi-gene phylogenetic analyses of combined ITS, LSU, and *tef-1α* sequence data, in combination with morphological comparisons. In a pathogenicity trial, five representative strains of *N. novaehollandiae* were inoculated onto healthy stem fragments from their respective original host tree species, resulting in severe necrotic lesions in all cases. Field assessment of sooty canker and dieback of trees in areas under investigation showed that at least 5–30 percent of ornamental and fruit trees were affected by *N. novaehollandiae*, with disease severity of 11.1 to 28.2 percent. *Ficus benghalensis*, *Morus nigra*, *Juglans regia* and *Eucalyptus camaldulensis* trees were more frequently and severely affected, as compared to *Cassia fistula* and *Syzygium cumini*. This study reports 22 new hosts for *N. novaehollandiae* in association with serious disease symptoms in Iran.

Keywords Botryosphaeriaceae · Molecular identification · Ornamental trees · Woody plants

Introduction

Canker and dieback diseases are common, widespread, and destructive on a broad range of woody plants, including ornamental and fruit trees (Shurtleff 1997; Horst 2013). The damage of these diseases is aggravated by wounding and weakening of trees caused by frost, mechanical injuries,

mineral deficiencies and other abiotic factors because the canker pathogens mostly infect trees, which are low in vigor (Calavan and Wallace 1954; Whiteside 1988; Elshafie and Ba-Omar 2002). Among fungi, most members of the family Botryosphaeriaceae are well known as pathogens causing branch and stem canker, dieback, and gummosis of a wide range of fruit, ornamental, and forest trees (Mayorquin et al. 2016; Berraf-Tebbal et al. 2020). The Botryosphaeriaceae are widely distributed in most geographical and climatic areas throughout the world.

The genus *Neoscytalidium* was erected within the Botryosphaeriaceae to accommodate strains of *Hendersonula toruloidea* Nattrass (\equiv *Dothiorella mangiferae* Syd. & P. Syd., \equiv *Nattrassia mangiferae* (Syd. & P.Syd.) B.Sutton & Dyko, \equiv *Fusicoccum dimidiatum* (Penz.) D.F.Farr) that formed Scytalidium-like synanamorphs (Crous et al. 2006; Madrid et al. 2009; Phillips et al. 2013). These fungi are also known to produce multilocular or pycnidial conidiomata having holoblastic, thin-walled, cylindrical to fusiform, 1–3-euseptate conidia and the arthric conidia produced

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in dry powdery chains on aerial mycelia (Phillips et al. 2013). The number of authentic species currently recognized in *Neoscytalidium* might be a subject of debate. The global fungal nomenclator registry, Index Fungorum, still lists five species, including the type *N. dimidiatum* (Penz.) Crous & Slippers (Crous et al. 2006), *N. novaehollandiae* Pavlic, T.I. Burgess & M.J. Wingf. (Pavlic et al. 2008), *N. orchidacearum* S.K. Huang, Tangthir., J.C. Kang & K.D. Hyde (Huang et al. 2016), *N. oculi* R.P. Calvillo-Medina, J. Mena, Raymundo & V. Bautista-de-Lucio (Calvillo-Medina et al. 2018), and *N. hylocereum* Kheawheng, Intaraa-nun & Rodkaew (Wonglom et al. 2023). However, Zhang et al. (2021) proposed to retain only *N. dimidiatum* and *N. orchidacearum*, and to reduce all other species to synonyms to *N. dimidiatum*. However, *N. novaehollandiae* is distinguished from other species in producing muriform and dichomera-like conidia (Zhu and Liu 2012).

Neoscytalidium species are capable of causing diseases in plants, humans, and animals (Ruíz-Cendoya et al. 2010; Bakhshizadeh et al. 2014; Dionne et al. 2015; Shokoohi et al. 2019). This genus is known as an opportunistic pathogen with the potential to cause severe invasive disease on a wide range of plants in tropical and sub-tropical regions including California, China, Egypt, Europe, India, Iran, Iraq, Jamaica, Niger, South Tunisia, Southern Thailand, Turkey, and West Africa (Natrass 1933; Calavan and Wallace 1954; Sommer 1955; Meredith 1963; Natour and El-Haideri 1967; Giha 1975; Reckhans and Adamou 1987; Cao and Wang 1989; Granata and Sidoti 1991; Harsh and Tiwari 1992; Matheron and Sigler 1993; Elliott et al. 1997; Msikita et al. 1997; Tsahouridou and Thanassouloupoulos 2000; Elshafie and Ba-Omar 2002; Heidarian and Alizadeh 1995; Namsi et al. 2010; Oksal and Özer 2021; Ören et al. 2020a, b; Wonglom et al. 2023). *Neoscytalidium* species can have a detrimental effect on various woody plants by causing cankers, dieback, gummosis, necrosis, wood rot and wilt (Phillips et al. 2013; Goudarzi and Moslehi 2020; Brito et al. 2020).

Neoscytalidium novaehollandiae was initially described as a new species for four isolates singularly producing Dichomera-like synanamorphs, but otherwise morphologically similar to *N. dimidiatum* (Pavlic et al. 2008). These isolates were obtained from asymptomatic branches of *Acacia synchronica* Maslin, *Adansonia gibbose* F. Muell., *Crotalaria medicaginea* Lam. and *Grevillia agrifolia* A. Cunn. ex R.Br. in north-western Australia (Pavlic et al. 2008). *N. novaehollandiae* was subsequently reported as a plant pathogen in Australia, in association with dieback of mango and common fig (Ray et al. 2010). Since then, accumulating reports have depicted this fungus as a destructive pathogen causing canker, decline, dieback, stem blight and shoot blight on various shrubs and trees in Australia, China, Iran and Turkey, including the species *Mangifera indica* (Ray et al. 2010; Sakalidis et al. 2011), *Ulmus densa* (Zhu and Liu

2012), *Pistacia vera* (Kurt et al. 2019), *Vitis vinifera* (Akgül et al. 2019), *Prunus dulcis* and *Diospyros kaki* (Ören et al. 2020a, b), *Solanum lycopersicum* L. (Derviş et al. 2020), *Pyrus communis* L. (Oksal and Özer 2021) and *Salvia officinalis* L. (Derviş et al. 2021). This species was also reported to cause wood canker and dieback symptoms on *Quercus brantii* (Sabernasab et al. 2019) and *Pinus eldarica* in Iran (Alizadeh et al. 2022).

Various fruit and ornamental trees are common in Khuzestan province in the southwest of Iran. These trees are affected by numerous plant diseases including leaf spot and blight (*Ascochyta* sp., *Pleospora* sp. and *Stemphylium* sp.), anthracnose (*Colletotrichum* spp.), stem canker and decay (*Inonotus rickii* (Pat.) D.A. Reid) and dieback and sooty canker (*Fusicoccum dimidiatum*) (Ershad 2009). In recent years, dieback and sooty canker diseases have become highly prevalent on a wide range of the ornamental and fruit trees in this province. Therefore, the purpose of this study was to identify the probable fungal pathogens responsible for canker and dieback on fruit and ornamental trees in Khuzestan province. To that end, fungi were isolated from symptomatic materials and characterized using morphological features and phylogenetic analyses of DNA sequence data. The pathogenic potential of representative isolates was also evaluated via artificial inoculation on healthy plant material. Furthermore, a field survey was undertaken to assess the incidence of the disease throughout the province.

Materials and methods

Sampling and fungal isolation

Samples were collected between 2015 and 2022 from eight townships, including Ahvaz, Andimeshk, Baghmalek, Behbahan, Dezful, Izeh, Masjedsoleyman and Molasani, in the Khuzestan province, southwestern Iran. The parks, boulevards, and gardens were evaluated, and in each place, the trees were visually inspected for the symptoms of dieback and sooty canker (Fig. 1). The diseased branches and trunks were collected from 63 trees belonging to 22 tree species (Table 1).

Small pieces (0.5–1 cm in size) were excised from healthy and discolored margins of symptomatic branches and surface-sterilised by dipping them in 2% NaOCl for 1–3 min. Pieces were then rinsed three times with sterile distilled water for 30 s and left to dried on sterile filter paper. Then, the fragments were plated on potato dextrose agar (PDA; Difco, USA) medium amended with streptomycin sulphate (30 mg/L). The plates were incubated in the dark at 28 °C for 5 to 7 days and individual colonies were transferred to fresh PDA. Purified cultures were obtained using a dilution method on quarter-strength



Fig. 1 Disease symptoms on specimens collected. (a–f). Lamination of the trunk bark and canker on, *Conocarpus erectus*, *Eucalyptus camaldulensis*, *Bauhinia purpurea* and *Syzygium cumini* respectively. (e–j). Sooty canker on *Morus nigra*, *Hibiscus rosa-sinensis*,

Albizia lebbek, *Prunus persica*, *Juglans regia* and *Nerium oleander* respectively. (k). Trunk cracking of *Ficus benghalensis*. (l–m). Die-back on *Ficus benghalensis* and *Ficus religiosa* respectively

PDA (Mehrabi-Koushki et al. 2018). A serial dilution of spore suspension was prepared for each isolate and 100 µl of each dilution was plated on quarter-strength PDA. The plates were incubated in the dark at 28 °C for 24–48 h and individual small colonies were transferred on new PDA plates as purified isolates. The representative cultures from the isolates tested in this study were deposited in the Iranian Fungal Culture Collection (Iranian Research Institute of Plant Protection, Tehran, Iran) and Collection of Fungal

Cultures, Department of Plant Protection, Shahid Chamran University of Ahvaz, Iran (SCUA).

Morphological characterization

Microscopic features, colony shape, and growth speed, were evaluated on PDA, at 28 °C, under photoperiod of 12 h. The color rate was determined according to the Methuen handbook of color (Kornerup and Wanscher 1967) and the

Table 1 Tree species identity and characteristics of samples collected for fungal isolation

Tree species	No. of trees sampled	Observed symptoms	No. of isolates obtained
<i>Albizia lebbek</i>	2	Sooty canker	4
<i>Bauhinia purpurea</i>	1	Lamination of the trunk bark	3
<i>Callistemon viminalis</i>	2	Canker	2
<i>Cassia fistula</i>	1	Canker	1
<i>Cassia floribunda</i>	1	Canker	1
<i>Citrus aurantifolia</i>	3	Canker and dieback	3
<i>Citrus limetta</i>	2	Dieback	2
<i>Citrus sinensis</i>	1	Dieback	1
<i>Conocarpus erectus</i> var <i>erectus</i>	7	Lamination of the trunk bark	8
<i>Cordia myxa</i>	2	Canker	4
<i>Cupressus sempervirens</i>	2	Canker and dieback	2
<i>Eucalyptus camaldulensis</i>	6	Dieback, lamination of the trunk bark and Sooty canker	5
<i>Ficus benghalensis</i>	5	Sooty canker and dieback	5
<i>Ficus religiosa</i>	5	Sooty canker and dieback	5
<i>Hibiscus rosa-sinensis</i>	2	Canker and dieback	2
<i>Juglans regia</i>	4	Sooty canker	6
<i>Morus nigra</i>	6	Sooty canker	9
<i>Nerium oleander</i>	2	Sooty canker	4
<i>Prunus persica</i>	3	Sooty canker	5
<i>Punica granatum</i>	1	Sooty canker	3
<i>Syzygium cumini</i>	3	Lamination of the trunk bark	3
<i>Ziziphus spina-christi</i>	2	Canker	2

diameter of colonies was measured at 24-h intervals up to 7 days of incubation. Fungal structures were mounted in a drop of lactophenol or lactophenol cotton blue on microscopic slides. In order to induce sporulation and formation of the fruiting bodies (pycnidia), two ways were performed: **i**) double-autoclaved pine needles were placed into 2% water agar (WA, 2% agar; Merck) plates, and a mycelial disk from the pure culture of each isolate was placed in the center of each petri plate. The plates were incubated at 25 °C, with 12-hour light–dark regime for six weeks. **ii**) the isolates were grown on oatmeal agar (OA; oatmeal 30–60 g L⁻¹, agar 12 g L⁻¹) at 25 °C under 12 h photoperiod for 7–14 days. Photomicrographs were taken with an OLYMPUS DP12 digital camera fixed on OLYMPUS BX51 microscope. At least, 50 measurements of each fungal structure were made using a Leitz Wetzlar (SM-LUX) Basic Biological Light Microscope at 400× and 1000× magnification. A maximum and minimum range, 95% confidence intervals, means and standard deviations were calculated for the size of each structure.

DNA extraction and amplification

The isolates were grown on PDA at 25 °C in darkness for 1–3 weeks. Mycelial biomass was scraped off from the

surface of each culture using a sterile glass slide. Total genomic DNA was extracted using the method described by Raeder and Broda (1985), with some modifications (Ahmadpour et al. 2017). Briefly, the ground mycelia were lysed with a SDS-based lysis buffer and then extracted three times by Phenol: chloroform: isoamyl alcohol (25:24:1). The first extract was treated with RNase a and then subjected to two following extractions. The genomic DNA was recovered through an ethanol-precipitation method. DNA extracts were qualified and quantified using a spectrophotometer (Eppendorf BioPhotometer plus). Nuclear genomic regions, including the first and second internal transcribed spacers and intervening 5.8S subunit (ITS) and the D1/D2 domain of the 28S subunit (LSU) of the nuclear ribosomal RNA, as well as partial translation elongation factor 1- alpha (*tef-1α*) were amplified in polymerase chain reactions (PCR). The primer combinations utilized were ITS1/NL4 (White et al. 1990; O'Donnell 1993) and EF1- 688F/ EF2-R (Alves et al. 2008; O'Donnell et al. 1998) for the ITS-LSU and *tef-1α*, respectively. Each 50 μL reaction mixture contained 5 μL of 10× prime Taq Reaction Buffer (GenBio, South Korea), 6 μL of MgCl₂ (25 mM), 0.6 μL of Prime Taq DNA Polymerase (5U/μL), 2 μL of each primer (10 mM), 2 μL of dNTP (10 mM mix), 100–500 ng of DNA and milliquore water up to final volume. PCR amplification was performed

in a thermocycler (MJ Mini™ Gradient Thermal Cycler) using the following parameters: initial melting at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C (ITS-LSU) or 56 °C (*tefl-α*) and 90 s at 72 °C, followed a final extension at 72 °C for 10 min. PCR products were evaluated by agarose gel electrophoresis in 1.0×Tris–acetic acid-EDTA (TAE) buffer for amplicon size. The amplicons of the expected size were purified with the GF-1 AmbiClean Kit (Vivantis, Malaysia) and sequenced in the forward and reverse directions (Codon Genetics, Tehran, Iran). The nucleotide sequences were edited in BioEdit v. 7.0.9.0 (Hall 1999) and assembled using DNA Baser Sequence Assembler v4 programs (2013, Heracle BioSoft, www.DnaBaser.com). All DNA sequences generated in this study are deposited in GenBank (Table 2).

Phylogenetic analyses

To identify taxa most closely related to our isolates based on sequence similarity, our newly generated sequences were queried against the nucleotide sequence database of the NCBI's GenBank using the BLASTn algorithm. This enabled us to select sequences of authentic strains of *Neoscytalidium* and other genera of Botryosphaeriaceae that were included in phylogenetic analyses (Table 2). The phylogenetic analyses were performed with a combined sequence data of ITS, LSU, and *tefl-α* regions. The alignments of ITS, LSU, and *tefl-α* were generated separately using Clustal W in BioEdit v. 7.0.9.0 (Hall 1999) and manually adjusted where necessary. The combined ITS-LSU-*tefl-α* dataset was created by concatenation of all individual alignments.

Phylogenetic analyses were conducted using maximum likelihood (ML) in the raxmlGUI 2.0 beta (Edler et al. 2019), maximum parsimony (MP) in MEGA7 (Tamura et al. 2013) and Bayesian analysis (BI) in MrBayes v.3.2.6 (Ronquist et al. 2012). The phylogenetic tree was rooted with *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. strain CBS 164.96. ML analysis was done using general time-reversible model with gamma distributed and invariant sites (GTR + G + I). Bootstrap values in MP (MPBS) and ML (MLBS) analyses were evaluated from 1000 replicates. The best fit evolutionary models for each single gene partition were estimated based on the lowest BIC score (Bayesian information criterion) in jModelTest 2 (Darriba et al. 2012) for the Bayesian phylogenetic analysis. As a result, GTR + G was used for ITS, GTR + I was used for LSU and HKY + G was used for *tefl-α*. The Bayesian inference posterior probabilities (PP) distribution was estimated by Markov chain Monte Carlo (MCMC) algorithm with the following options: four MCMC chains were sampled over 10,000,000 generations, sampling every 1000 generations, the standard deviation below 0.01 and removing the first 25% of trees for calculating posterior probability values (BPP). Data

matrixes used for analysis were deposited in TreeBase under the accession number of S30423 (<http://purl.org/phylo/treebase/phyloids/study/TB2:S30423>).

To investigate the genetic diversity and detect a possible structure in the global population of *N. novaehollandiae*, we used the unweighted pair group method with arithmetic average (UPGMA), as implemented in MEGA7 (Tamura et al. 2013), to build a dendrogram of *N. novaehollandiae* isolates from various locations and hosts based *tefl-α* sequence data. All strains represented in the GenBank in that respect were included. We also included three sequences from Khuzestan newly generated in this study. The *tefl-α* gene locus was selected for this analysis because of its greater variability as detected in sequences alignments.

Field disease assessment

During 2021–2022, the health status of trees in the sampling areas as indicated earlier was evaluated. To assess disease incidence (DI) and severity (DS), 50 trees were randomly selected for each tree in each area (township). The disease incidence was calculated using the following formula (1):

$$DI = \frac{A}{B} \times 100 \quad (1)$$

where: A = Number of trees showing dieback and sooty canker and B = Number of trees examined.

Disease severity was also evaluated using an infection category scoring from 0 to 9, based on the sooty canker area and percentage of dryness of the branches (Table 3). Then, disease severity was calculated with the following formula (2):

$$DS = \frac{\sum(n.v)}{N.Z} \times 100 \quad (2)$$

where: n = The number of trees in each severity category, v = Score for disease severity, N = Number of trees examined, Z = The score for the highest category.

Pathogenicity test

Pathogenicity tests of five representative isolates were performed on the same hosts from which the isolates were obtained, including *Bauhinia purpurea*, *Conocarpus erectus*, *Eucalyptus camaldulensis*, *Ficus religiosa* and *Morus nigra*. Pathogenicity trials were performed on detached stems of the host trees (8–10 years old) with similar height (~15–20 cm), diameter (~2 cm) and healthy under a controlled condition. The fragments were disinfected with 2% sodium hypochlorite (2–5 min), then washed three times with sterile distilled water. After drying, two wounds with a diameter of 5 mm were created on the stem fragments by

Table 2 Strains used in this study and their GenBank accession numbers. Newly generated sequences are indicated in bold

Species name	Isolate name	Host	GenBank Accession number		
			ITS	LSU	EF1- α
<i>Botryosphaeria dothidea</i>	CBS 115476=	<i>Prunus</i> sp.	AY236949	AY928047	AY236898
	CMW 8000 ^T				
<i>Cophinforma atrovirens</i>	CBS 110302	<i>Vitis vinifera</i>	AY259092	EU673243	AY573218
	MFLUCC 11-0425 ^T	<i>Eucalyptus</i> sp.	JX646800	JX646817	JX646865
	MFLUCC 11-0655	<i>Eucalyptus</i> sp.	JX646801	JX646818	JX646866
<i>Lasiodiplodia theobromae</i>	CBS 164.96	unidentified fruit along coral reef coast	AY640255	EU673253	AY640258
<i>Macrophomina phaseolina</i>	CBS 162.25	<i>Eucalyptus</i> sp.	KF531826	DQ377905	KF531803
	CBS 227.33	<i>Zea mays</i>	KF531825	DQ377906	KF531804
<i>Neofusicoccum parvum</i>	ATCC 58191 ^T	<i>Populus nigra</i>	AY236943	AY928045	AY236888
	CBS 110301	<i>Vitis vinifera</i>	AY259098	AY928046	AY573221
<i>Neoscytalidium dimidiatum</i>	CBS 499.66	<i>Mangifera indica</i>	KF531820	DQ377925	KF531798
	CBS 251.49	<i>Juglans regia</i>	KF531819	DQ377923	KF531797
	CBS 145.78	Homo sapiens	KF531816	DQ377922	KF531795
<i>N. hylocereum</i>	TSU-HP01	<i>Selenicereus undatus</i>	LC590860	–	LC590863
	PSU-HP01	<i>Selenicereus undatus</i>	LC590859	–	LC590862
<i>Neoscytalidium novaehollandiae</i>	CBS 122071 ^T	<i>Crotalaria medicaginea</i>	EF585540	–	EF585580
	WAC 13286	<i>Mangifera indica</i>	GU172393	–	GU172425
	CBS 122610	<i>Acacia synchronicia</i>	EF585536	–	EF585578
	IRAN 4173C; SCUA-Y6	<i>Ziziphus spina-christi</i>	OQ306627	OQ309005	OQ329984
	IRAN 4176C; SCUA-Y9	<i>Citrus aurantifolia</i>	OQ306631	OQ309009	OQ329989
	SCUA-Ahv-Bau	<i>Bauhinia purpurea</i>	KY449010	KY449019	OQ329995
	IRAN 4172C; SCUA-Y5	<i>Conocarpus erectus</i> var <i>erectus</i>	OQ306634	OQ309012	OQ329993
	IRAN 4171C; SCUA-Y4	<i>Cupressus sempervirens</i>	OQ306633	OQ309011	OQ329992
	IRAN 4174C; SCUA-Y7	<i>Callistemon viminalis</i>	OQ306635	OQ309013	OQ329994
	IRAN 4179C; SCUA-And-Mor	<i>Morus nigra</i>	KY449012	KY449021	OQ329991
	IRAN 4181C; SCUA-Y16	<i>Eucalyptus camaldulensis</i>	OQ306628	OQ309006	OQ329985
	IRAN 4175C; SCUA-Y8	<i>Albizia lebbek</i>	OQ306636	OQ309014	OQ329996
	IRAN 4169C; SCUA-Y2	<i>Cupressus sempervirens</i>	OQ306632	OQ309010	OQ329990
	IRAN 4177C; SCUA-Ez-Mor	<i>Morus nigra</i>	KY449011	KY449020	OQ329988
	SCUA-Y27	<i>Prunus persica</i>	OQ306629	OQ309007	OQ329986
SCUA-Y26	<i>Juglans regia</i>	OQ306630	OQ309008	OQ329987	
<i>N. oculi</i>	IOM325287	Homo sapiens	MG764431	MG764432	–
<i>Neoscytalidium orchidacearum</i>	MFLUCC 12-0533	on dead leaves of orchid	KU179865	KU179864	–
	CMU 287	<i>Cattleya</i> sp.	KY933091	KY933092	–

^a Abbreviation of culture collections: *CBS* Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, *IRAN* Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Tehran, Iran, *MFLUCC*, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; Others are not registered abbreviations. Newly generated sequences are in bold. T ex-type strains

a metal cork borer at a distance of 50 mm from each end. A mycelial PDA plug from the actively growing margin of each 3-day-old colony was inoculated into each wound. For each isolate, four stem fragments were used and an equal number was also inoculated with sterile PDA plugs as negative controls. Inoculated and control stems of each isolate were separately placed on a plastic mesh into water-containing

desiccators, sterilised as a moist chamber. Then, the desiccators were kept at 28 °C under a 12-h photoperiod. The severity of the lesions induced by isolates were estimated in terms of percentage of wood discoloration (PWD) 10 days after inoculation when discoloration reached about 85% in at least one of the stem fragments. The PWD values obtained on each host was summarized as mean \pm standard deviation.

Table 3 Category score for evaluation of disease severity of each tree

Scoring	The percentage of the infected part of the stems and branches*
0	No visible symptoms
1	2% of the surface of stems and branches of tree covered with sooty canker
2	4% of the surface of stems and branches of tree covered with sooty canker
3	6% of the surface of stems and branches of tree covered with sooty canker
4	8% of the surface of stems and branches of tree covered with sooty canker
5	10% of the surface of stems and branches of tree covered with sooty canker
6	15% of the surface of stems and branches of tree covered with sooty canker and 5% dryness of the shoots
7	> 15% of the surface of stems and branches of tree covered with sooty canker and 20% dryness of the shoots
8	> 15% of the surface of stems and branches of tree covered with sooty canker and 30% dryness of the branches
9	Sooty canker and dryness of most branches, tree death

* The percentage of the surface of stems and branches of a tree that are covered with sooty canker or dried

Stem fragments from each treatment and control were randomly selected for re-isolation to fulfill Koch's postulates. All pathogenicity tests were repeated once.

Results

Two hundred symptomatic branches and stems from 22 tree species, showing dieback and sooty canker, were collected. In total, 80 fungal isolates with identical culture characteristics were isolated from sample materials (Table 1). Of those, 13 isolates were characterized based on PCR amplification and sequencing of the ITS, LSU and *tefl-α* gene loci, generating 1200 bp and 500 bp DNA sequence data, respectively. Aligning the newly generated sequences for comparison revealed that all the isolates share 100% sequence similarity with one another at all three loci, respectively. Evaluation of our sequences against the GenBank using BLASTn revealed that our isolates were 100% identical to the type strain of *N. novohollandiae* (CBS 122071) at the ITS and LSU. They showed a 0.7% difference with CBS 122071 at the *tefl-α*. This difference rested on 2/293 bp variation detected when the two sequences were aligned.

Phylogenetic analyses

Combined sequence data (ITS, LSU, and *tefl-α*) used in phylogeny consisted of 88 sequences from 32 ingroup taxa (Table 2), with *Lasiodiplodia theobromae* strain CBS 164.96 as the outgroup. The resulting concatenated aligned dataset contained a total of 1198 bp (410 bp for ITS, 505 bp for LSU and 283 bp for *tefl-α*) including alignment gaps. Among those sites, 953 bp were constant and 233 bp were variable (180 bp were parsimony informative and 53 bp were parsimony-uninformative). Single-locus trees did not show any conflicts in their topologies for the 70% reciprocal trees, which allowed to combine the three loci for a multi-locus

phylogenetic analysis. The ML tree showed a similar topology to those obtained in BI and MP analyses in major clades (Fig. 2). In the three *loci* phylogenetic tree (Fig. 2), the 13 surveyed strains were grouped in a moderately-supported clade (MLBS 75%, MPBS 77%, BPP 0.91) with an internal subclade containing three reference strains of *Neoscytalidium novaehollandiae*, including CBS 122071, CBS 122610 and WAC 13286 (MLBS 85%, MPBS 90%, BPP 0.99). Hence, we identified our strains as *N. novaehollandiae*.

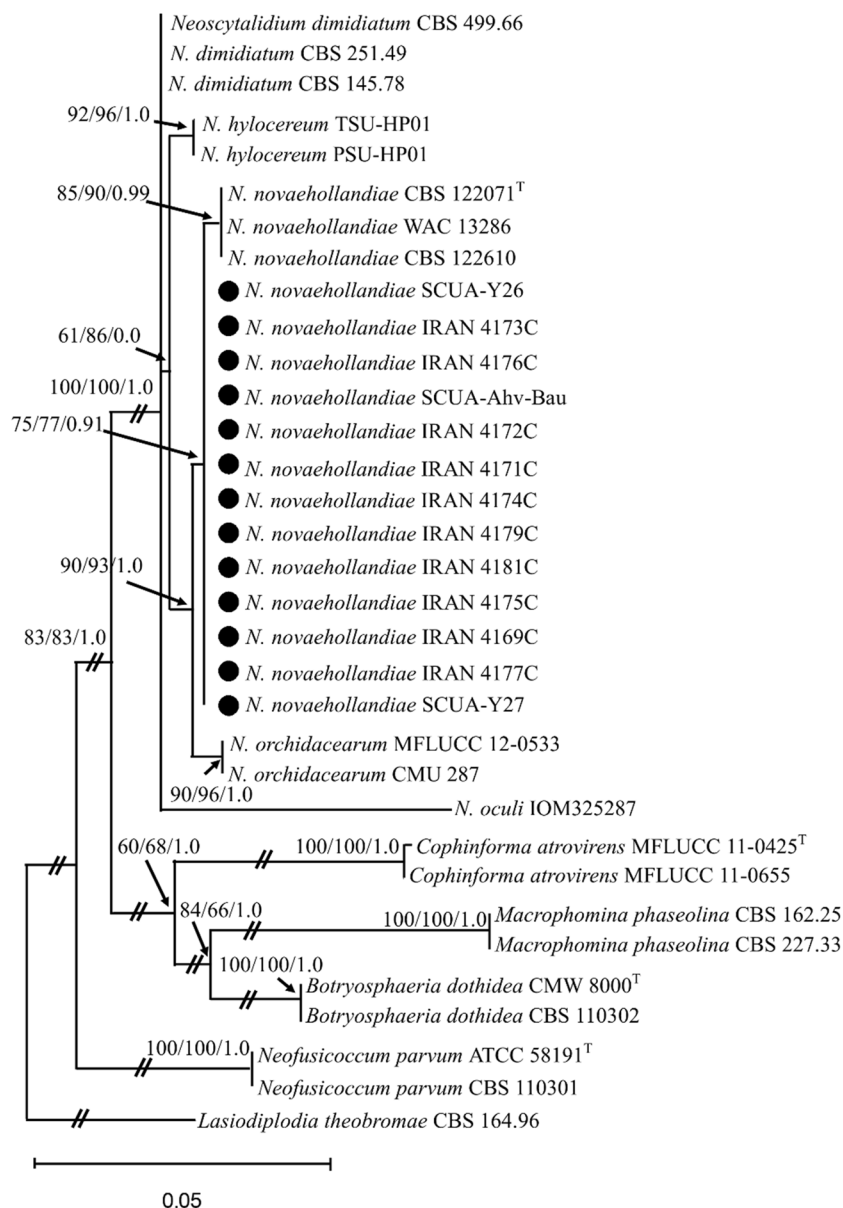
In the UPGMA dendrogram (Fig. 3), the strains of *N. novaehollandiae* clustered in three low-supported groups including most strains of Turkey, China, the present study (Khuzestan Province, Iran) and one other strain from Iran (Tehran Province) in group 1, two other strains from Turkey in group 2, and strains of Australia and two other strains from Iran (Kermanshah Province) and Turkey in group 3.

Morphological characteristics

Neoscytalidium novaehollandiae Pavlic, T.I. Burgess & M.J. Wingf., 2008 Fig. 4.

Conidiomata pycnidial or multilocular stromata, semi-immersed or superficial, black, globose. **Conidia** of the coelomycetous state formed in two distinct types: **i**) ellipsoid to ovoid, with rounded apex, at first hyaline, aseptate, becoming cinnamon to sepia, 0–1-septate or 2-septate with darker central cell, (4.2–)5–9.8(–10.3) × (2–)2.5–6.1(–7.9) μm, **ii**) variable in shape and size, globose, subglobose to obpyriform with muriform septa, at first hyaline with age becoming cinnamon to sepia, (5–)5.2–11(–15) × (3–)3.5–8.8(–10) μm. **Aerial mycelium** produced chains of one-celled spores (arthroconidia). **Arthroconidia** dry powdery to the touch, occurring in disarticulating aerial mycelium, cylindrical, oblong–obtuse to doliiform, thick-walled, initially hyaline, with age becoming cinnamon to sepia, 0–1-septate, (5–)6–10(–11.2) × 2.5–3.8(–4) μm.

Fig. 2 Phylogenetic tree constructed from a maximum likelihood analysis based on the combined ITS, LSU and *tef1- α* sequences. Bootstrap values obtained in maximum likelihood (MLBS) and maximum parsimony (MPBS) analyses $\geq 50\%$ and Bayesian posterior probability values (BYPP) ≥ 0.5 are shown at the nodes, respectively. The scale bar shows the expected number of changes per site. The tree is rooted in *Lasiodiplodia theobromae* strain CBS 164.96. Letter T indicates the ex-type strains. Taxa under study are shown with black-color filled circles



Culture characteristics: Colonies on PDA, 90 mm diameter after three days of incubation at 28 ± 0.5 C, white to olivaceous, buff with regular margins at early growth stage, becoming blackish green in the central and olivaceous green at the edge within seven days, and black with age; reverse blackish green to black.

Pathogenicity test

Five representative isolates of *Neoscytalidium novaehollandiae* were examined on the trees from where they were originally isolated, including the strain SCUA-Ahv-Bau on *Bauhinia purpurea*, IRAN 4172C on *Conocarpus erectus*, IRAN 4181C on *Eucalyptus camaldulensis*, SCUA-Y29 on *Ficus religiosa*, and IRAN 4179C on *Morus nigra*. These

strains were able to grow and sporulate in the bark surrounding the inoculation point on stem fragments of the mentioned plants. Results of the pathogenicity tests showed that all isolates were pathogenic on inoculated detached stems (Table 4). No lesions were produced on control shoots. The colonies of *N. novaehollandiae* developed on the inoculation points on the stem fragments two weeks after inoculation which was associated with wood necrosis and discoloration in the xylem (Fig. 5). The severity of induced lesions in inoculated material was in the following order: *Eucalyptus camaldulensis* and *Morus nigra* > *Conocarpus erectus* > *Ficus religiosa* > *Bauhinia purpurea*. Accordingly, the rate of wood necrosis in *E. camaldulensis* was higher than others and *B. purpurea* was the lowest. Masses of black fungal spores appeared under the bark and the canker surface on the

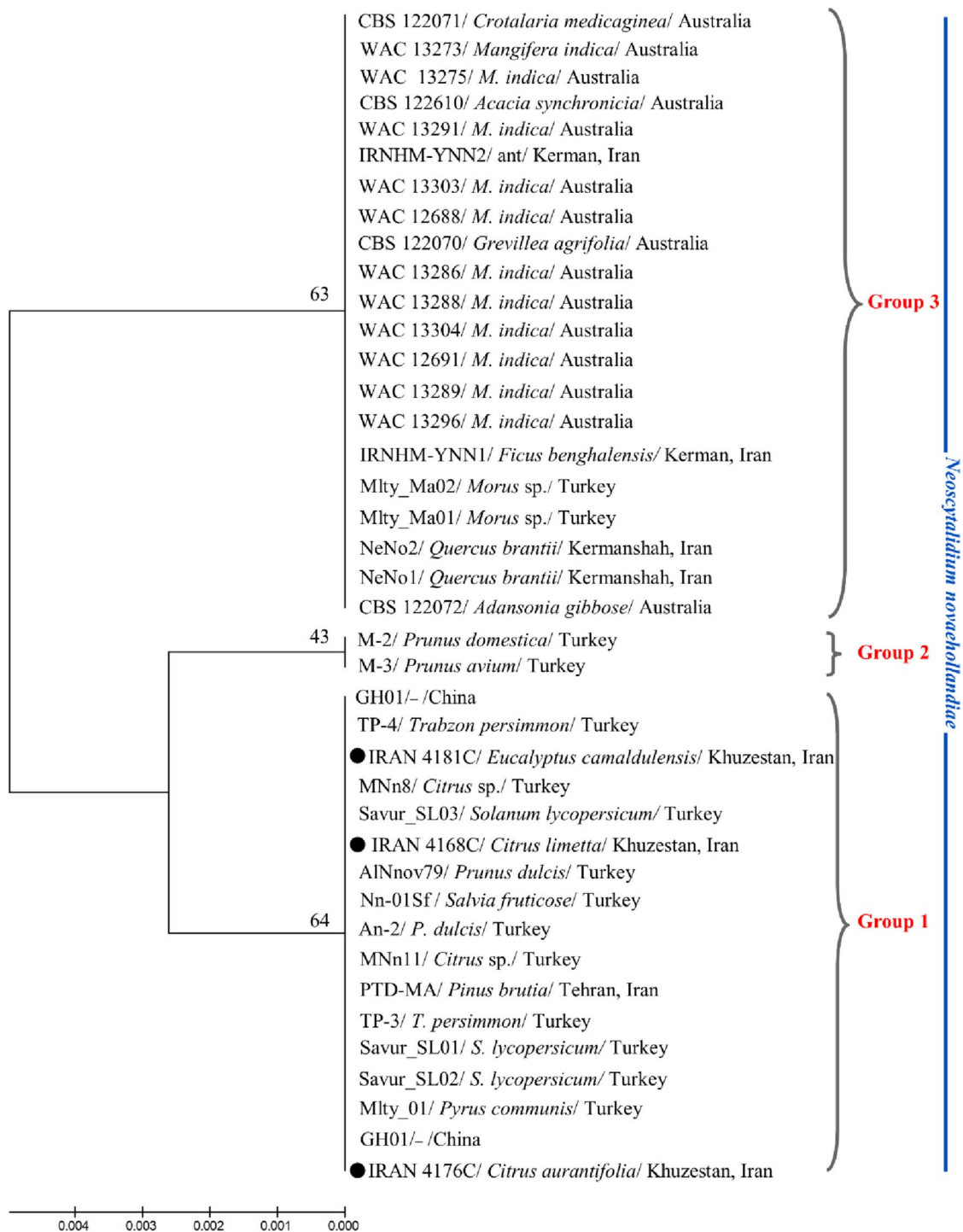


Fig. 3 UPGMA method phylogenetic tree based on *tef1-α* sequences of *Neoscytalidium novaehollandiae* strains obtained in this study and all existing strains in GenBank. The percentage of replicate in which

the associated taxa clustered together in the bootstrap test (1000) is shown next to the branches. Taxa under study are shown with black-color filled circles

stem fragments of *C. erectus*, *E. camaldulensis*, and *M. nigra* after three weeks of inoculation (Fig. 5). This pathogenic fungus was re-isolated from the necrosis-like areas formed

on stem fragments and re-identified as *N. novaehollandiae* species based on morphological characterization. None of the fungal isolates were re-isolated from the control shoots.

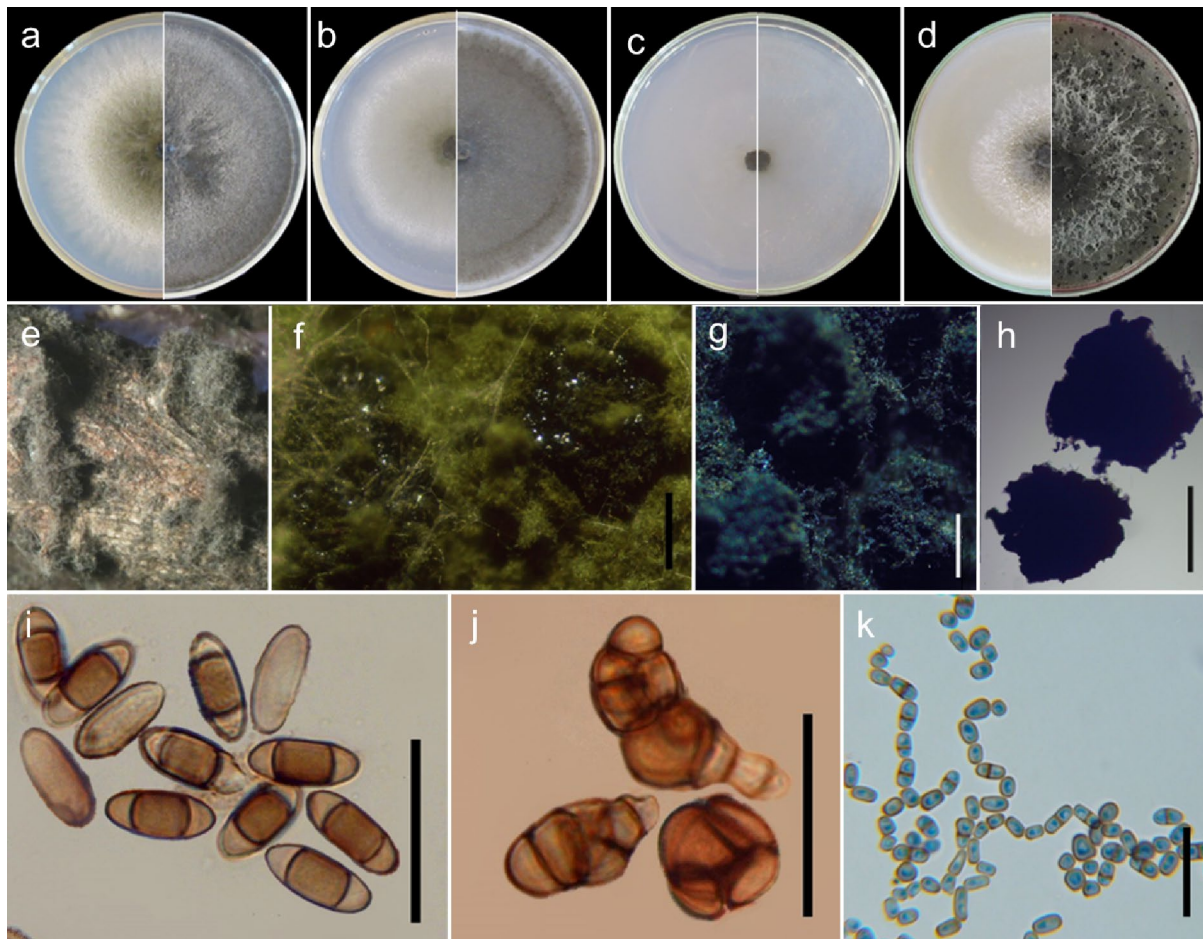


Fig. 4 *Neoscytalidium novaehollandiae* (IRAN 4172C). (a–c) Colony after 4 d (right) and 14 d (left) at 28 °C on (a) PDA, (b) CMA, (c) WA, and (d) OA. (e) Pycnidia developing on pine needle in culture.

(f–h) Pycnidia on OA. (i–j). Conidia of coelomycetous state. (k) Arthric chains of conidia

Field disease incidence

In recent years, widespread presence and high incidence of dieback and sooty canker on trunks and branches associated with tree decline were observed in some areas of Khuzestan province. Field disease symptoms of the different hosts are

Table 4 Pathogenicity of representative isolates inoculated onto stem fragments 10 days after inoculation

Treatments	disease severity (mean PWD* \pm standard deviation)
<i>Bauhinia purpurea</i>	11.25 \pm 2.58
<i>Conocarpus erectus</i>	67.5 \pm 5.59
<i>Eucalyptus camaldulensis</i>	95.75 \pm 1.92
<i>Ficus religiosa</i>	34.5 \pm 3.84
<i>Morus nigra</i>	93.25 \pm 2.04
control	0

* Percentage of wood discoloration

shown in Fig. 1. In detail, all investigated hosts showed various external disease symptoms including shoot and twig dieback, sooty canker, and cracking of the bark. Internal observation of symptomatic tissues revealed a brown wood discoloration. Isolations were made from the discolored edge of cankers on branches and stems, dried shoots and branches, and also from the inner bark and woody tissues of the hosts. In our study, 29 isolates of *Neoscytalidium novaehollandiae* were recovered from various ornamental and fruit trees including, *Citrus sinensis*, *Cassia fistula*, *C. floribunda*, *Conocarpus erectus*, *Cordia myxa*, *Cupressus sempervirens*, *Eucalyptus camaldulensis*, *Ficus religiosa*, *F. benghalensis*, *Juglans regia*, *Hibiscus rosa-sinensis*, *Morus nigra*, *Prunus persica*, *Punica granatum*, *Nerium oleander*, *Syzygium cumini* and *Ziziphus spina-christi*. Visual inspection of the trees showed that in some cases, young shoots were affected initially, and as the disease progressed, branches and stems were affected as well. In most cases, necrotic girdling of stem tissues resulted in death of shoots and branch. This survey included about 5850 trees in all studied regions.

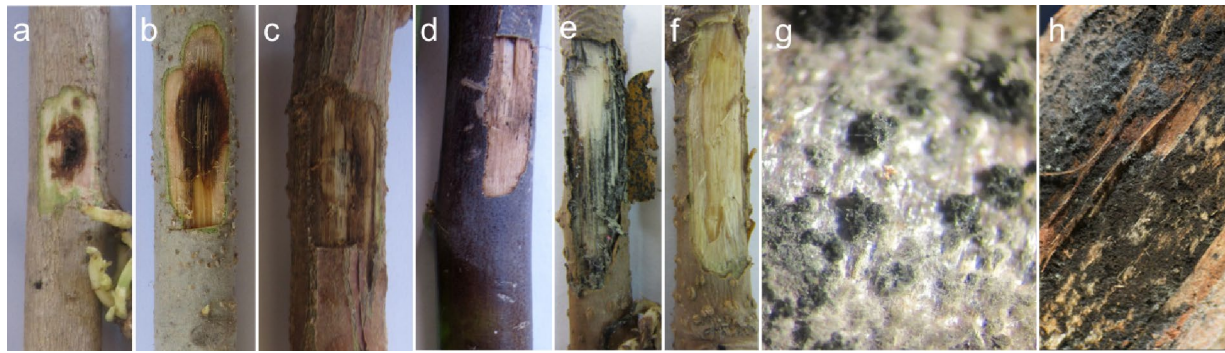


Fig. 5 Pathogenicity tests. (a–e) Necrosis on a stem fragment of *Bauhinia purpurea*, *Ficus religiosa*, *Conocarpus erectus*, *Eucalyptus camaldulensis*, and *Morus nigra* respectively. (f) compared to a

control fragment. (g–h) Pycnidia and masses of black fungal spores on *Eucalyptus camaldulensis* and *Conocarpus erectus* respectively

According to the disease estimation (Table 5), disease incidence and severity varies between sampling areas (ranging from 5 to 30 percent) and trees (ranging from 11.1 to 28.2 percent). Results show that Ahvaz, Masjed-soleyman and Molasani had the most disease incidence and severity (Table 5).

Discussion

The present study is the first to investigate the incidence and etiology of dieback and sooty canker of various ornamental and fruit trees in the Khuzestan province of Iran. All fungal isolates obtained from symptomatic materials were

Table 5 Incidence and severity of sooty canker and dieback on green space trees of Khuzestan province

Tree species	Disease incidence (DI) and severity (DS)															
	Ahvaz		Andimeshk		Baghmalek		Behbahan		Dezful		Izeh		Masjed-soleyman		Molasani	
	DI%	DS%	DI%	DS%	DI%	DS%	DI%	DS%	DI%	DS%	DI%	DS%	DI%	DS%	DI%	DS%
<i>Albizia lebbek</i>	25	13.9	17	13.6	12	11.1	15	12.5	16	11.1	–	–	13	15.9	21	13.3
<i>Bauhinia purpurea</i>	9	13.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
<i>Callistemon viminalis</i>	20	14.4	12	11.1	–	–	20	12.2	11	13.3	–	–	–	–	20	15.5
<i>Cassia fistula</i>	17	14.8	12	11.1	–	–	–	–	11	11.1	–	–	–	–	–	–
<i>Cassia floribunda</i>	10	11.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
<i>Citrus aurantifolia</i>	26	18.8	14	15.9	13	12.9	12	14.8	13	15.9	7	11.1	–	–	22	20.3
<i>Citrus limetta</i>	27	17.1	17	11.1	10	11.1	14	14.3	13	14.8	11	11.1	–	–	25	17.6
<i>Citrus sinensis</i>	17	18.5	15	14.3	12	12.9	14	15.9	14	12.7	–	–	–	–	17	19.7
<i>Conocarpus erectus</i> var <i>erectus</i>	27	20.6	24	16.6	17	14.8	14	12.7	21	17.2	11	16.7	24	20.3	25	19.6
<i>Cordia myxa</i>	8	19.4	7	14.8	–	–	8	16.6	5	11.1	–	–	–	–	8	16.6
<i>Cupressus sempervirens</i>	13	16.6	7	11.1	7	16.6	9	15.5	6	14.8	8	11.1	–	–	–	–
<i>Eucalyptus camaldulensis</i>	28	20.6	26	17.1	21	15.2	23	18.5	24	16.6	19	12.3	26	19.6	27	19
<i>Ficus benghalensis</i>	26	28.2	17	16	21	14.1	23	17.6	16	16.6	11	13.3	–	–	24	19.4
<i>Ficus religiosa</i>	30	17	26	14.5	23	13	23	14.8	25	13.7	21	13.1	–	–	27	15.9
<i>Hibiscus rosa-sinensis</i>	30	18.5	27	15.9	25	13.7	28	15.1	24	14.8	20	11.1	25	17.1	27	17.5
<i>Juglans regia</i>	–	–	–	–	–	–	–	–	–	–	28	23	–	–	–	–
<i>Morus nigra</i>	31	24.7	30	17.7	30	17	28	18.3	30	17.7	28	18.3	28	19.8	29	19.3
<i>Nerium oleander</i>	12	13	7	11.1	6	11.1	9	15.5	5	11.1	6	11.1	13	14.8	11	15.5
<i>Prunus persica</i>	–	–	–	–	–	–	–	–	–	–	17	18.5	–	–	–	–
<i>Punica granatum</i>	–	–	–	–	–	–	–	–	–	–	21	16.2	–	–	–	–
<i>Syzygium cumini</i>	21	14.1	11	13	–	–	–	–	10	11.1	–	–	–	–	19	13.3
<i>Ziziphus spina-christi</i>	14	15.9	11	13.3	7	11.1	14	14.3	10	13.3	10	11.1	15	16.6	17	19.7

identified to represent the same botryosphaeriaceous species, *Neoscytalidium novaehollandiae*, based on the production of typical muriform, Dichomera-like conidia (Pavlic et al. 2008), as well as their phylogenetic placement in a monophyletic clade, alongside the type specimens of the species. However, the newly collected isolates, which appear to represent the same strain based on available sequence data, formed a discrete subclade resulting from two point-mutations detected in the *tef-1a* gene sequence. The UPGMA dendrogram based on *tef1-a* of a global population of *N. novaehollandiae* reveals that there are at least three genetic groups associated with this fungus. All three groups occur in Turkey and two have been detected in Iran. But more generally the dendrogram shows that a greater diversity of *N. novaehollandiae* exists in Asia where all three groups are present, as compared to Australia, for instance, where only one group has been detected so far. Therefore, one can arguably assume that the diversification center of this fungus would be located in Asia, even-though a more representative sampling throughout the world will be needed to reach robust conclusions.

Artificially inoculating the fungus back onto various original host materials resulted in a pathogenic interaction in all cases, but it also highlighted a variation in susceptibility among hosts. For instance, wood discolorations and black powdery appearance in *Conocarpus erectus*, *Eucalyptus camaldulensis* and *Morus nigra* stems were significantly greater than those in *Ficus religiosa* and *Bauhinia purpurea*. This conformed with the results of disease assessment in the field that showed a variation in disease incidence and severity among investigated tree species.

Visual assessment of sooty canker and dieback of trees in areas under investigation showed that the disease index and tree mortality positively correlate with environmental stress. At least 5–30 percent of ornamental and fruit trees were affected by *Neoscytalidium novaehollandiae* in Khuzestan Province (Table 5). Since drought and extremely hot summers became more common in Khuzestan during the last decade, higher than usual incidence of die back and sooty canker diseases may be due to drought stresses and higher annual temperatures that made trees more susceptible to the disease. During this period, some members of the family Botryosphaeriaceae, including *Botryosphaeria*, *Lasiodiplodia*, *Neofusicoccum* and *Neoscytalidium* species, have emerged as destructive pathogens of many tree species, mainly causing canker and dieback (Manawasinghe et al. 2016; Alizadeh et al. 2022). Observational assessment showed that there was a clear increase in decline symptoms in the zones with high light intensity, low fertility soils, drought, water-logging, prolonged exposure to extremely high temperatures, summer sunscald, nutrient deficiency, soil compaction, changes in the soil grade and mechanical injuries. Previous studies showed that weakening by abiotic

stress factors, such as high temperatures and drought periods could play a role in increasing the virulence and expansion of the Botryosphaeriaceae and other decline pathogens (Smith et al. 1996; Kim et al. 2009; Arnold and Herre 2003; Desprez-Loustau et al. 2007; Slippers and Wingfield 2007; Botella et al. 2010; Dissanayake et al. 2015; Fan et al. 2016; Anonym 2017; Delgado-Cerrone et al. 2016). Results of this study show that the canker and dieback of fruit and ornamental trees caused by *N. novaehollandiae* is a serious epidemic, that affects a broad range of tree species in Iran. In recent years, there are many scattered reports of detection of this fungus associated with emerging diseases prevalence on various plants from many regions of Asia and Australia. In view of the fact that this fungus is typically widespread in tropical and subtropical regions (Crous et al. 2006), it can also be assumed that it is well adapted to the environmental conditions of southwestern and southern Iran (Goudarzi and Moslehi 2020).

Most members of the Botryosphaeriaceae have a cosmopolitan distribution and cause disease on a broad host range of woody plants (Slippers and Wingfield 2007). *N. novaehollandiae* has been reported from a wide range of plants from Australia, China and Turkey and a few plants from Iran. The present study reports a wide range of new hosts (22 tree species) for *N. novaehollandiae* in Iran, in association with dieback and sooty canker. Previously, this fungus has been reported as causal agent of various diseases on *Acacia synchronica*, *Adansonia gibbosa*, *Carpinus betulus*, *Crataegus pentagyna*, *Crotalaria medicaginea*, *Diospyros kaki*, *Fagus orientalis*, *Grevillia agrifolia*, *Mangifera indica*, *Morus alba*, *Pinus eldarica*, *Pistacia vera*, *Prunus dulcis*, *Pyrus communis*, *Quercus brantii*, *Salvia officinalis*, *Solanum lycopersicum*, *Ulmus densa*, and *Vitis vinifera* (Rahmani 2018; Sabernasab et al. 2019; Kazemzadeh Chakusary et al. 2019; Pavlic et al. 2008; Ray et al. 2010; Sakalidis et al. 2011; Zhu and Liu 2012; Kurt et al. 2019; Ören et al. 2020a, b; Derviş et al. 2021; Oksal and Özer 2021; Alizadeh et al. 2022). Furthermore, Alizadeh et al. (2022) presented the host range of this pathogen, and showed that these potential hosts are prone to this pathogen under high temperature and low humidity.

In conclusion, *Neoscytalidium* species cause canker and dieback diseases on a large number of shrubs and trees with agricultural, industrial, forestry and ornamental importance. This study establishes *N. novaehollandiae* as the causal agent of dieback and sooty canker on many fruit and ornamental trees in Khuzestan province, Iran. The great number of affected tree species, together with the high disease indices estimated suggest that we are dealing with a problem that is growing in importance, probably in connection with global environmental change. This also means that numerous woody plants are at risk of severe disease due to *N. novaehollandiae* in various regions of the world.

This study lays a foundation for further investigation into the economic impact of this pathogen, factors affecting disease development and dissemination, strategies to prevent and manage diseases and minimizing diseases losses in different climates.

Acknowledgements Authors would like to appreciate the administration of plant protection Department, Faculty of Agriculture, Shahid Chamran University of Ahvaz for their support in accomplishment of this study. The authors are thankful to anonymous reviewers for beneficial comments and suggestions on the manuscript.

Authors' contributions Seyedeh Akram Ahmadpour carried out sample preparation, fungal isolation and purification, morphometric and morphological determination, DNA isolation and amplification, DNA and phylogenetic analysis and the writing of the manuscript. Mehdi Mehrabi-Koushki carried out the design and implementation of the research and revising of the manuscript. Reza Farokhinejad contributed to the implementation of the research and revising of the manuscript. Zahra Mirsoleymani, provided some fungal isolates and revising of the manuscript.

Funding This work was financially supported by grant (SCU. AP1401.294) from Research Council of Shahid Chamran University of Ahvaz.

Data availability The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov>.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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