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Research Papers

Fungal pathogens associated with black foot of grapevine in China

Qingtong YE[1,2], Wei ZHANG[1], Jingyi JIA[1], Xinghong LI[1], Yueyan ZHOU[1,2], Changping HAN[1,2], Xuehong WU[2], Jiye YAN[1,*]

[1] *Beijing Key Laboratory of Environment Friendly Management on Fruit Diseases and Pests in North China, Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China*
[2] *Department of Plant Pathology, China Agricultural University, Beijing 100193, China*
*Corresponding author. E-mail: jiyeyan@vip.163.com

Summary. Grapevine trunk diseases (GTDs) are the most destructive diseases in grape-growing regions worldwide. Black foot is one of the important GTDs affecting young vineyards and nurseries. This disease has not been reported in China. During 2017 and 2019, field surveys were carried out in the Guangxi, Hebei, Ningxia, Shanxi, and Xinjiang provinces of China. Incidence of plants with black foot symptoms was 0.1% to 1% in the surveyed vineyards. Plant samples with poorly developed shoots and canes, chlorotic leaves, and necrotic trunks or roots were collected from the five provinces. In total, 50 fungal isolates were obtained from symptomatic tissues. Based on morphological and multi-gene phylogenetic analyses, five species were identified as *Cylindrocladiella lageniformis*, *Dactylonectria torresensis*, *D. macrodidyma*, *D. alcacerensis* and *Neonectria* sp.1. Pathogenicity was assessed using young, healthy detached green shoots of grapevine 'Summer Black' and potted 3-month-old 'Summer Black' cuttings. Inoculated detached shoots developed necroses after 7 d, and inoculated cuttings after 80 d. Fungi were re-isolated from necrotic lesions. Among the five species, *D. macrodidyma* was the most aggressive. This is the first report of *C. lageniformis, D. torresensis*, *D. macrodidyma*, *D. alcacerensis,* and *Neonectria* sp. 1 associated with black foot in China. This study has enhanced knowledge of the fungi associated with black foot in China, and will assist development of control measures for this disease.

Keywords. Nectriaceae, morphological characteristics, phylogenetic analyses, *Vitis vinifera.*

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an economically important fruit crop, with global cultivation area of 7,449,000 hectares in 2018, and China is ranked the second in the world grapevine cultivation area (2019 OIV). More than 70 diseases have been reported in grapevines, most of which are caused by fungi or oomycetes (Wilcox *et al*., 2006), and among these, at least 27 diseases have been reported in China. Esca complex, Botryosphaeria dieback, black foot (BF), Eutypa dieback, and Phomopsis dieback are major fungal

grapevine trunk diseases (GTDs) worldwide. These diseases have been reported in almost all the main grape-growing countries (Gramaje *et al*., 2018). GTDs are complexes that affect grape yields, wine quality and lifespan of plants in many grape-growing regions. The global financial losses attributed to GTDs are estimated to be more than $US 1.5 billion per year (Hofstetter *et al*., 2012).

Black foot (BF) is one of the most significant GTDs, especially in nurseries and young plantations (Halleen *et al*., 2006). This disease has occurred in many viticulture regions during the last decade, including Australia, Brazil, California, Canada, (British Columbia, Quebec), France, Iran, Italy, New Zealand, Portugal, South Africa (Western Cape), Spain, Switzerland, Turkey, United States of America, and Uruguay (Agustí-Brisach and Armengol, 2013; Lombard *et al*., 2014; Carlucci *et al*., 2017; Aigoun-Mouhous *et al*., 2019; Lawrence *et al*., 2019; Berlanas *et al*., 2020). In France, 50% of losses caused by BF fungal pathogens were recorded in young vineyards of 2 to 8 years old (Larignon *et al*., 1999). In the Czech Republic, about 30% of plants showed root necroses, reduced root biomass and wood necroses in the basal ends of grapevine rootstocks, which are typical symptoms of BF that appear after one year of cultivation in 2015 (Pecenka *et al*., 2018). The most common symptoms of BF on grapevine plants include delayed or absent budding, stunted growth, shortened internodes with small trunks, chlorotic leaves with necrotic margins, brown to black necroses on rootstock bases and sunken necrotic root lesions, in nurseries and young plantations (Rego *et al*., 2000; Halleen *et al*., 2006; Alaniz *et al*., 2007; Abreo *et al*., 2010; Agustí-Brisach and Armengol, 2013).

Ilyonectria destructans (=*Cylindrocarpon destructans*) was first reported in France (Grasso and Magnano Di San Lio, 1975), and to date, 33 fungal species have been reported to be associated with BF. The most common fungal genera associated with BF are *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon* and *Thelonectria*. Fungal species that have been associated with BF include: *Campylocarpon fasciculare*, *Ca. pseudofasciculare*, *"Cylindrocarpon"* sp. 2, *Cylindrocarpon didymium*, *Cylindrocladiella parva*, *C. lageniformis*, *C. viticola*, and *C. peruviana*, *Dactylonectria alcacerensis*, *D. estremocensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. pinicola*, *D. torresensis*, *D. riojana*, *D. vitis, D. hordeicola*, *Ilyonectria destructans*, *I. europaea*, *I. liriodendri*, *I. lusitanica*, *I. pseudodestructans*, *I. robusta, I. vivaria*, *Neonectria obtusispora*, *N. quercicola, Neonectria* sp. 1, fungi in the *N. mammoidea* group, *Pleiocarpon algeriense*. *Thelonectria blackeriella*, *T. olida*, and *T. aurea*. (Agustí-Brisach and Armengol, 2013; Lombard *et al*., 2014; Carlucci *et al*., 2017; Aigoun-Mouhous *et al*., 2019; Lawrence *et al*., 2019; Berlanas *et al*., 2020). Among these species, *I. liriodendri* and *D. macrodidyma* are the most widely distributed ones (Agustí-Brisach and Armengol, 2013). These fungi are frequently isolated from BF symptoms in nursery and older grapevine plants (Petit *et al*., 2011; Carlucci *et al*., 2017), and from asymptomatic inner tissues from plants (Berlanas *et al*., 2020). Some BF fungi have also been detected from the soils of grapevine nurseries and vineyards, in Spain and South Africa. (Agustí-Brisach *et al*., 2013, 2014; Langenhoven *et al*., 2018).

Eutypa dieback was first reported in China in 2007, Botryosphaeria dieback in 2010, Diaporthe dieback in 2015 and Esca in 2020 (Li *et al*., 2007; Li *et al*., 2010; Dissanayake *et al*., 2015; Ye *et al*., 2020), while BF has not been reported in China previously.

MATERIALS AND METHODS

Vineyard surveys

Surveys were carried out in ten vineyards, located in Ningxia, Hebei, Shanxi, Guangxi and Xinjiang provinces of China, during 2017 and 2019 (Figure 1a). These provinces belong to the top ten grape cultivated grapevine areas in China, and Xinjiang province ranked the first, followed by Hebei province. The training systems used in the surveyed vineyards was mini "J". The vineyards were of similar age, from 5 to 6 years old. Typical symptoms associated with diseased vines were shortened shoot internodes, chlorotic leaves, and trunk and root necroses (Figure 1, b–h). Initial disease symptoms included root necroses (especially small roots). As the disease progressed, the above-ground plant parts developed shoot shortened internodes and chlorotic leaves in severe cases. Some grapevines were grafted (rootstock Fercal), and some others were self-rooted (Personal communication, some of the grape growers).

Sample collection, fungus isolation and morphology of the pathogens

Samples were collected from *V. rotundifolia* Michx., and *V. vinifera* cvs Marselan, Cabernet Franc or Cabernet Sauvignon. Typical symptoms were recorded by taking appropriate photographs. The samples were kept at 4°C for further study, and the presence of spores or structures on the surfaces of trunks or roots were detected using a microscope. Isolations were made from

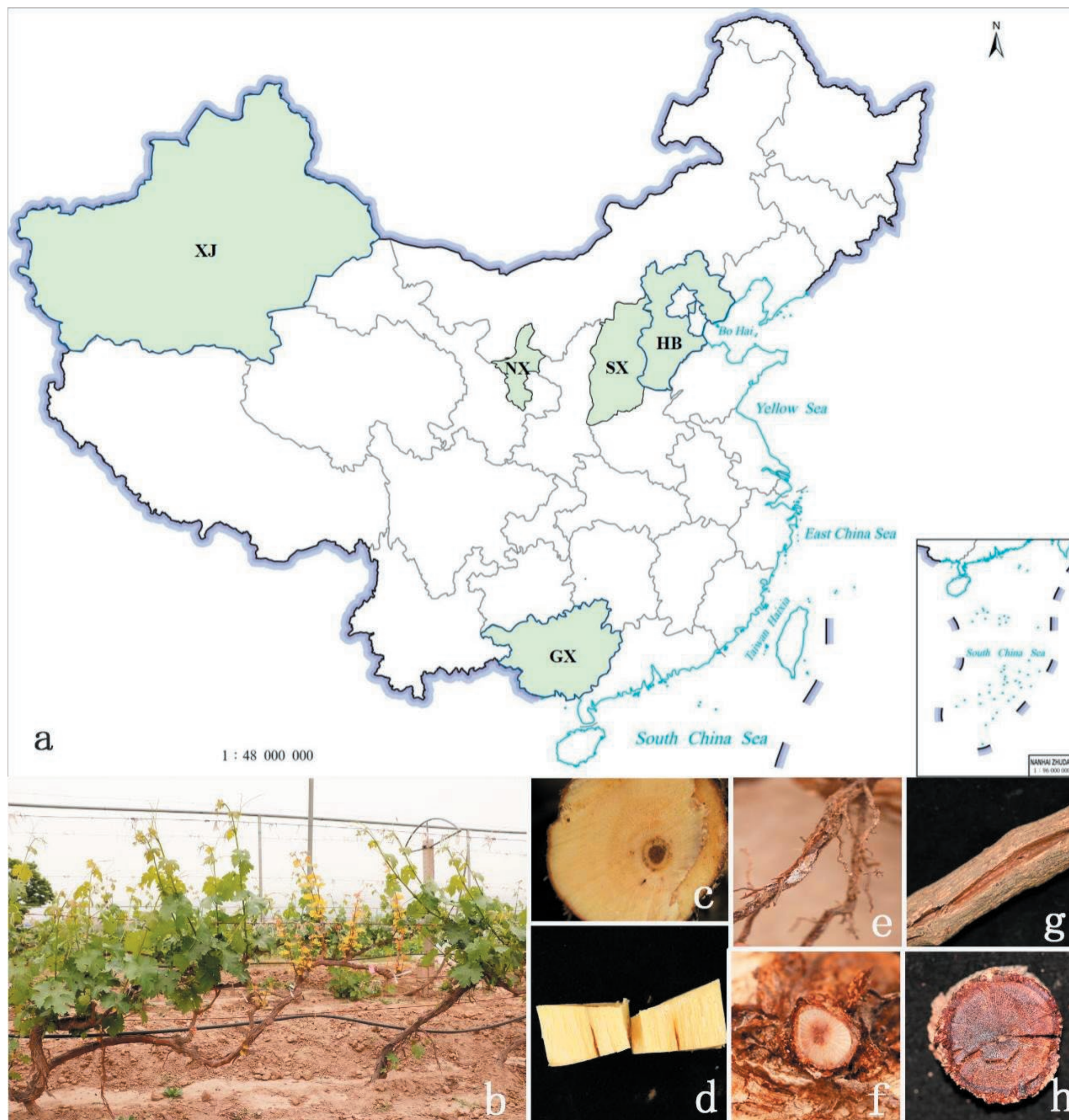

Figure 1. Sample collection sites and disease symptoms. Field surveys were carried out in five provinces of China. GX: Guangxi; HB: Hebei; NX: Ningxia;SX: Shanxi; XJ: Xinjiang (**a**). Diseased plant (Chardonnay, 5-year-old) showing shortened internodes of shoots (**b**).Necrotic grapevine roots and trunks (**c**–**h**).

symptomatic trunks and roots. Necrotic root and trunk samples were debarked and cut into small pieces (4–5 mm^2). These small pieces were then surface-sterilized in 75% ethanol for 30 s, rinsed three times with sterilized water, dried, and cultured on potato dextrose agar (PDA; 20% potatoes, 2% dextrose, 1.5 to 2% agar) in Petri plates. The plates were incubated at 25°C. Fungi growing from tissue pieces were transferred onto new PDA plates after 7 d, and pure cultures were obtained by isolating single spores. Pure cultures were grown on PDA and malt extract agar (MEA) and incubated at 25°C in the dark for 7 d. Conidia and colonies on the MEA plates

were observed and photographed using the Axio Imager Z2 photographic microscope (Carl Zeiss Microscopy).

DNA amplification and phylogenetic analyses

Single-spore purification were done for all the isolates before DNA extractions. Total genomic DNA was extracted from 50–100 mg of mycelium after 14 d of incubation on PDA (Guo *et al*., 2000). For initial genus identification, the internal transcribed spacer and intervening 5.8S gene regions (ITS) were amplified and sequenced for all the isolates, and the resulting sequences were searched using BLASTN within GenBank/NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), as described by Manawasinghe *et al.* (2019). All the isolates in the present study belonged to *Cylindrocladiella*, *Neonectria* or *Dactylonectria*.

For species confirmation, phylogenetic analyses were conducted using multigene phylogenies. For *Cylindrocladiella*, histone H3 (*his3*), β-tubulin (*tub2*), and partial translation elongation factor 1-alpha (*tef1*) were sequenced (Marin-Felix *et al*., 2019). ITS, *tub2*, *his3*, and *tef1* gene regions were sequenced for *Dactylonectria* and *Neonectria* species (Berlanas *et al*., 2020). The primer pairs and amplification protocols used in the present study are summarized in Table 1. Each PCR mixture comprised 1.0 μL of genomic DNA, 0.6 μL of TaKaRa ExTaq DNA polymerase, 5.0 μL of 10 × ExTaq DNA polymerase buffer, 4.0 μL of dNTPs, and 1.0 μL of each primer, and was adjusted with sterilized double-distilled water to a final volume of 50.0 μL. The PCR reactions were carried out in a thermal cycler (Bio-Rad, model C1000). Amplification products were visualized on 1% agarose electrophoresis gels under UV light using a Gel DocTM XR+ Molecular Imager (Bio-Rad). All positive bands obtained by PCR amplification were sequenced by Tsingke Company, Beijing, China, and the sequence data obtained were deposited in GenBank (Table 2).

Reference sequences of related taxa were obtained from GenBank (Marin-Felix *et al*., 2019; Berlanas *et al*., 2020). The sequence data generated in the present study were included, and individual gene regions were aligned using the MAFFT v. 7 webserver (Kuraku *et al*., 2013; Katoh *et al*., 2019) (https://mafft.cbrc.jp/alignment/server/). The alignments were checked and edited manually, where necessary using BioEdit v7.0.9 (Hall, 1999). Phylogenetic trees were generated using Maximum Likelihood (ML) in RAxML (Silvestro and Michalak, 2016) and Maximum Parsimony (MP) in PAUP (v4.0) (Swofford, 2002). The ML and MP trees were constructed using the methods described by Manawasinghe *et al.* (2019). For MP, heuristic searches were conducted with 1000 bootstrap replicates by random addition. All characters were unordered and equally weighted. Gaps were treated as missing data, and the steepest descent option not in effect, whereas the MulTrees option was used. The Tree Length (TL), Consistency Index (CI), Retention Index (RI), Relative Consistency Index (RC), and Homoplasy Index (HI) were calculated in PAUP. All the resulting trees were saved and checked using Kishino-Hasegawa tests (Kishino and Hasegawa, 1989). The ML analyses of single genes and combined multiple genes were accomplished using the RAxML-HPC2 on XSEDE (8.2.8) in the CIPRES Science Gateway (https://www.phylo.org/portal2/createTask!create.action). Phylogenetic trees were visualized using FigTree v1.4.4 (Rambaut, 2018) and were annotated in Microsoft PowerPoint 2016.

Pathogenicity tests

Pathogenicity tests of potential BF pathogens were conducted on detached green shoots or potted 3-month-

Table 1. The primer pairs and their amplified protocols used in present study.

Gene region	Primers	Sequence 5'-3'	Protocols for PCR	References
ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	94°C: 3 min, (94°C: 30 s, 52°C: 30 s, 72°C: 1 min) × 34 cycles 72°C: 7 min	White *et al.* (1990)
HIS	CYLH3F CYLH3R	AGGTCC ACTGGTGGCAAG AGCTGGATGTCCTTGGACTG	94°C: 3 min, (94°C: 30 s, 58°C: 30 s, 72°C: 1 min) × 34 cycles 72°C: 7 min	Crous *et al.* (2004)
β-tubulin	T1 Bt2b	AACATGCGTGAGATTGTAAGT ACCCTCAGTGTAGTGACCCTTGGC	94°C: 3 min, (94°C: 30 s, 58°C (62°C): 30 s, 72°C: 1 min) × 34 cycles 72°C: 7 min	O'Donnell and Cigelnik (1997) Glass and Donaldson (1995)
EF1-α	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	94°C: 3 min, (94°C: 30 s, 54°C: 30 s, 72°C: 1 min) × 34 cycles 72°C: 7 min	Carbone and Kohn (1999) Udayanga *et al.* (2012a ; b)

Table 2. Reference sequence data obtained from GenBank and isolate sequence data from the present study which were used for phylogenetic tests.

Species	Isolates	GenBank accession No.			
		ITS	tub2	his3	tef1
Neonectria coccinea	CBS 119158	JF268759	KC660727	N/A	DQ789749.1
N. confusa	CBS 127484	KM515889	KM515886	N/A	N/A
N. confusa	CBS 127485	FJ560437	FJ860054	N/A	JF268736.1
N. ditissima	**CBS 226.31**	JF735309	DQ789869	JF735594	JF735783
N. ditissima	CBS 835.97	JF735310	DQ789880	JF735595	JF735784
N. faginata	**CBS 217.67**	HQ840385	JF268730	N/A	JF268746.1
N. faginata	CBS 119160	HQ840384	DQ789883	N/A	N/A
N. fuckeliana	CBS 119200	HQ840387	JF268731	N/A	JF268747.1
N. fuckeliana	CBS 239.29	HQ840386	DQ789871	N/A	JF268748.1
N. hederae	CBS 714.97	N/A	DQ789878	N/A	KC660461
N. hederae	**IMI 058770**	N/A	DQ789895	N/A	DQ789752
N. lugdunensis	CBS 125475	KM231762	KM232019	KM231482.1	KM231887.1
N. lugdunensis	CBS 125485	KM231762	KM232019	KM231482	KM231887
N. major	**CBS 240.29**	JF735308	DQ789872	JF735593	JF735782
N. neomacrospora	**CBS 198.62**	AJ009255	HM352865	KM231481	HM364351
N. neomacrospora	CBS 324.61	JF735312	DQ789875	JF735599	JF735788
N. neomacrospora	CBS 503.67	AY677261	JF735436	JF735600	JF735789
N. obtusispora	CBS 183.36	AM419061	AM419085	JF735607	JF735796
N. obtusispora	CPC 13544	AY295306	JF735443	JF735608	JF735797
N. punicea	CBS 242.29	KC660522	DQ789873	N/A	DQ789730
N. punicea	CBS 119724	KC660496	DQ789824	N/A	DQ789681
N. quercicola	**CBS 143704**	KY676880	KY676874	KY676862	KY676868
N. quercicola	CPC 13530	AY295302	JF735441	JF735605	JF735794
N. ramulariae	MAFF411012	JX034565.1	JX034567.1	N/A	N/A
N. ramulariae	CBS 151.29	AY677291	JF735438	JF735602	DQ789720
N. ramulariae	CBS 182.36	HM054157	JF735439	JF735603	JF735792
Neonectria sp. 1	CPC 13545	N/A	JF735437	JF735601	JF735790
Neonectria sp. 1	JZB3210004	MN988722	MN958534	MN958545	MN956387
N. tsugae	**CBS 788.69**	KM231763	KM232020	N/A	DQ789720
Dactylonectria alcacerensis	**CBS 129087**	JF735333	N/A	JF735630	JF735819
D. alcacerensis	Cy134	JF735332	N/A	JF735629	JF735818
D. alcacerensis	JZB3310007	MN988716	MN958528	MN958539	MN956381
D. amazonica	**MUCL 55430**	MF683706	MF683643	MF683686	MF683664
D. anthuriicola	CBS 564.95	JF735302	JF735430	JF735579	JF735768.1
D. ecuadoriensis	**MUCL 55424**	MF683704	MF683641	MF683684	MF683662
D. ecuadoriensis	MUCL55425	MF683705	MF683642	MF683684	MF683663
D. estremocencis	CPC 13539	JF735330	JF735458	JF735627	JF735816
D. estremocencis	**CBS 129085**	JF735320	JF735448	JF735617	JF735806
D. hispanica	**CBS 142827**	KY676882	KY676876	KY676864	KY676870
D. hispanica	Cy228	JF735301	JF735429	JF735578	JF735767
D. hordeicola	**CBS 162.89**	AM419060	AM419084	JF735610	JF735799
D. macrodidyma	CBS 112601	MH862898	AY677229	JF735644	JF735833
D. macrodidyma	**CBS 112615**	AY677290	AY677233	JF735647	JF268750
D. macrodidyma	Cy258	JF735348	JF735477	JF735656	JF735845
D. macrodidyma	CBS 112604	AY677284	AY677229	JF735644	JF735833
D. macrodidyma	JZB3310008	MN988717	MN958529	MN958540	MN956382
D. macrodidyma	JZB3310009	MN988718	MN958530	MN958541	MN956383

(Continued)

Table 2. (Continued).

Species	Isolates	GenBank accession No.			
		ITS	tub2	his3	tef1
D. macrodidyma	JZB33100010	MN988719	MN958531	MN958542	MN956384
D. novozelandica	CBS 112608	AY677288	AY677235	JF735632	JF735821
D. palmicola	**MUCL55426**	MF683708.1	MF683645.1	MF683687.1	MF683666.1
D. pauciseptata	**CBS 120171**	EF607089	EF607066	JF735587	JF735776
D. pinicola	CBS 159.43	JF735318	JF735446	JF735613	JF735802
D. pinicola	**CBS 173.37**	JF735319	JF735447	JF735614	JF735803
D. polyphaga	**MUCL55209**	MF683689	MF683626	MF683668	MF683647
D. torresensis	CBS 119.41	JF735349	JF735478	JF735657	JF735846
D. torresensis	Cyl102	KP823905	KP823885	KP823894	KP823874
D. torresensis	Cyl106	KP823907	KP823887	KP823895	KP823876
D. torresensis	Cyl110	KP823908	KP823888	KP823896	KP823877
D. torresensis	Cyl124	KP823912	KP823891	KP823900	KP823881
D. torresensis	**CBS 129086**	JF735362	JF735492	JF735681	JF735870.1
D. torresensis	JZB33100011	MN988720	MN958532	MN958543	MN956385
D. torresensis	JZB33100012	MN988721	MN958533	MN958544	MN956386
D. vitis	**CBS 129082**	JF735303	JF735431	JF735580	JF735769.1
D. valentina	**CBS 142826**	KY676881	KY676875	KY676863	KY676869
Cylindrocladiella addiensis	**CBS 143794**	MH111383	MH111388	N/A	MH111393
C. addiensis	CBS 143793	MH111385	MH111390	N/A	MH111395
C. addiensis	CBS 143795	MH111384	MH111389	N/A	MH111394
C. arbusta	**CMW47295/ CBS 143546**	MH017015	MH016958	MH016996	MH016977
C. arbusta	CMW 47296; CBS 143547	MH017016	MH016959	MH016997	MH016978
C. australiensis	**CBS 129567**	JN100624	JN098747	JN098932	JN099060
C. brevistipitata	**CBS 142786**	N/A	MF444926	N/A	MF444940
C. camelliae	IMI 346845	AF220952	AY793471	AY793509	JN099087
C. clavata	**CBS 129564**	JN099095	JN098752	JN098858	JN098974
C. cymbiformis	**CBS 129553**	JN099103	JN098753	JN098866	JN098988
C. elegans	**CBS 338.92**	AY793444	AY793474	AY793512	JN099039
C. ellipsoidea	**CBS 129573**	JN099094	JN098757	JN098857	JN098973
C. hahajimaensis	**MAFF238172**	JN687561	N/A	N/A	JX024570.1
C. hawaiiensis	**CBS 129569**	JN100621	JN098761	JN098929	JN099057
C. horticola	**CBS 142784**	MF444911	MF444924	N/A	MF444938
C. humicola	**CBS 142779**	MF444906	MF444919	N/A	MF444933
C. infestans	**CBS 111795**	AF220955	AF320190	AY793513	JN099037
C. kurandica	**CBS 129577**	JN100646	JN098765	JN098953	JN099083
C. lageniformis	CBS 111060	JN100611	JN098770	JN098918	JN099046
C. lageniformis	CBS 111061	JN100606	JN098771	JN098913	JN099040
C. lageniformis	CBS 112898	AY793445	AY725652	AY725699	JN098990
C. lageniformis	**CBS 340.92**	MH862360	AY793481	AY793520	JN099003
C. lageniformis	JZB3320001	MN988714	MN958526	MN958537	MN958535
C. lageniformis	JZB3320002	MN988715	MN958527	MN958538	MN958536
C. lanceolata	**CBS 129566**	JN099099	JN098789	JN098862	JN098978
C. lateralis	**CBS 142788**	MF444914	MF444928	N/A	MF444942
C. longiphialidica	**CBS 129557**	JN100585	JN098790	JN098851	JN098966
C. longistipitata	**CBS 116075**	AF220958	AY793506	AY793546	JN098993
C. malesiana	CBS 143549	MH017017	MH016960	MH016998	MH016979
C. microcylindrica	**CBS 111794**	AY793452	AY793483	AY793523	JN099041
C. natalensis	**CBS 114943**	JN100588	JN098794	JN098895	JN099016

(Continued)

Table 2. (Continued).

Species	Isolates	GenBank accession No.			
		ITS	tub2	his3	tef1
C. nederlandica	**CBS 152.91**	JN100603	JN098800	JN098910	JN099033
C. novazelandica	**CBS 486.77**	AF220963	AY793485	AY793525	JN099050
C. nauliensis	**CBS 143792**	MH111387	MH111392	N/A	MH111397
C. nauliensis	CBS 143791	MH111386	MH111391	N/A	MH111396
C. obpyriformis	**CMW47194/ CBS 143552**	MH017022	MH016965	MH017003	MH016984
C. parvispora	**CMW 47197/ CBS 143554**	MH017025	MH016968	MH017006	MH016987
C. parva	CBS 114524	AF220964	AY793486	AY793526	JN099009
C. peruviana	**IMUR 1843**	AF220966	AY793500	AY793540	JN098968
C. pseudocamelliae	**CBS 129555**	JN100577	JN098814	JN098843	JN098958
C. pseudohawaiiensis	**CBS 210.94**	JN099128	JN098819	JN098890	JN099012
C. pseudoinfestans	**CBS 114531**	AF220957	AY793508	AY793548	JN099004
C. pseudoparva	**CBS129560**	JN100620	JN098824	JN098927	JN099056
C. queenslandica	**CBS 129574**	JN099098	JN098826	JN098861	JN098977
C. reginae	**CBS 142782**	MF444909	MF444922	N/A	MF444936
C. solicola	**CMW47198/** CBS 143551	MH017021	MH016964	MH017002	MH016983
C. stellenboschensis	**CBS 110668**	JN100615	JN098829	JN098922	JN099051
C. terrestris	**CBS 142789**	MF444915	MF444929	N/A	MF444943
C. thailandica	**CBS 129571**	JN100582	JN098834	JN098848	JN098963
C. variabilis	**CBS 129561**	JN100643	JN098719	JN098950	JN099080
C. viticola	**CBS 112897**	AY793468	AY793504	AY793544	JN099064
C. vitis	**CBS 142517**	KY979751	KY979918	N/A	KY979891
Campylocarpon fasciculare	CBS 112613	AY677301.1	AY677221.1	JF735502.1	JF735691.1
C. pseudofasciculare	CBS 112679	AY677306.1	KJ022328.1	JF735503.1	JF735692.1
Gliocladiopsis sagariensis	**CBS 199.55**	NR147628	JQ666141	JQ666031	JQ666107

[a] CBS**:** Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CPC**:** Culture collection of Pedro Crous, housed at CBS. MUCL: Mycothèque de l'Université catholique de Louvain; JZB: Beijing Academy of Agriculture and Forestry Sciences Culture Collection, China; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K; IMUR: Institute of Mycology, University of Recife, Recife, Brazil; MAFF: Genetic Resources Centre, National Agriculture and Food Research Organization (NARO), NARO GenBank, Ibaraki, Japan; **Cy:** Cylindrocarpon collection housed at Laboratório de Patologia Vegetal "Veríssimo de Almeida" - ISA, Lisbon, Portugal;
[b] Ex-type were in bold.
N/A: The sequence is not available or not applicable to the present study.

old healthy plants of grapevine cv. 'Summer Black'. Five isolates (JZB3320001, JZB3310007, JZB3310008, JZB33100011 and JZB3210004) were selected randomly for pathogenicity tests. Mycelium discs (4 mm diam.) were obtained from the edges of PDA colonies which were grown for 10 d at 25°C.

Detached shoots were surface-disinfected in 75% ethanol and then dried, and each shoot was then wounded (4 mm) using a sterilized scalpel. The mycelium discs were placed onto the wound sites and covered with parafilm (Bemis). Non-colonized sterile PDA plugs were used as negative controls. The shoots were then inserted into moist soil and kept at 25°C. Each experiment included ten shoots for each fungus isolate, with a total of three parallel experiments conducted. The lengths of the lesions were measured after 7 d, and meanwhile photos were taken.

Pathogenicity tests of BF fungal agents were further conducted on the 3-month old grapevine cuttings which were inoculated in a manner similar to the detached green shoots. The experiment was performed in six cuttings for each tested isolate and the negative controls. The plants were kept in a greenhouse maintained at 25°C, and the trial was conducted twice. Shoots were collected, and lesion lengths were measured upward and downward from the points of inoculation after 80 d.

Fungi were re-isolated from necroses on the test plants in all pathogenicity tests, and fungus identifications were based on cultural and morphological characters. The lesion dimension data were statistically ana-

lyzed with IBM SPSS Statistics 21.0 (IBM Corp.) using a one-way analysis of variance (at $P = 0.05$) to determine differences in shoot lesion dimensions resulting from different fungus isolate inoculations.

RESULTS

Fungus isolation and initial species identifications

Incidence of BF-like symptoms in the investigated vineyards was 0.1% to 1%.

Colony morphology of all the isolates distinguished after 14 d of growth on PDA. In total, 50 isolates were obtained from the symptomatic grapevine tissues. For genus confirmation of the isolates, the ITS regions were amplified for all the isolates. The products of the ITS regions were approx. 0.5 kb. All sequences obtained were compared to those deposited in GenBank, and the isolates possessed 95%-99% similarity with sequences from the genera *Cylindrocladiella*, *Dactylonectria* or *Neonectria*. One or two isolates were selected from each of these three genera for pathogenicity tests.

The ML MP trees had similar topologies, so only the ML tree is presented in this study, with ML and MP bootstrap support values.

Identification of Cylindrocladiella *species* The optimization likelihood value of the final ML tree was -9044.171598. The matrix had 563 distinct alignment patterns, with 12.52% of undetermined characters or gaps. The parameters for the GAMMA+P-Invar model were as follows: estimated base frequencies A = 0.217646, C = 0.325293, G = 0.206497, T = 0.250564; substitution rates include TL = 1.662695, AC = 1.021632, AG = 3.427269, AT = 1.399071, CG = 0.562601, CT = 4.088874, GT = 1.000000; proportion of invariable sites (I) = 0.346767, and gamma distribution shape parameter (α) = 0.401996. In the MP tree, the heuristic search produced 1000 trees (length = 1415, CI = 0.529, RI = 0.840, RC = 0.445 and HI = 0.471), and the dataset consisted of 1527 total characters. Of these characters, 1000 were constant, 158 variable characters were parsimony-uninformative and 369 were parsimony-informative. In the ML tree (Figure 2), generated using the combined data, two isolates (JZB3320001 and JZB3320002) clustered with *C. lageniformis* (CBS 340.92) with bootstrap value 100 and 99 obtained from the ML and MP tree, respectively. Based on morphological characteristics and phylogenetic results, isolates JZB3320001 and JZB3320002 were identified as *Cylindrocladiella lageniformis* Crous, M.J. Wingf. & Alfenas.

Identification of the species of Dactylonectria *and* Neonectria *species* The ML optimization likelihood value was -12848.031218. The matrix had 916 distinct alignment patterns, with 16.70% of undetermined characters or gaps. Parameters for the GAMMA+P-Invar model were: estimated base frequencies A = 0.216108, C = 0.328348, G = 0.225791, T = 0.229753; substitution rates include TL = 1.593283, AC = 1.370476, AG = 3.210224, AT = 1.626079, CG = 0.702088, CT = 5.644898, GT = 1.000000; proportion of invariable sites (I) = 0.388010, and gamma distribution shape parameter (α) = 0.971160. In the MP tree, the heuristic search produced 1000 trees (length = 1981, CI = 0.610, RI = 0.911, RC = 0.556 and HI = 0.390), and the dataset consisted of 2041 total characters. Of these, 1225 were constant, 88 variable characters were parsimony-uninformative and 728 were parsimony-informative. In the ML tree (Figure 3), generated using the combined data, three isolates (JZB3310008, JZB3310009 and JZB33100010) collected from Ningxia province clustered with *D. macrodidyma* (CBS 112615) and one isolate (JZB3210004) clustered with *Neonectria* sp. 1(CPC 13545); In addition, the isolate (JZB3310007) clustered with *D. alcacerensis* (CBS 129087) and two isolates (JZB33100011 and JZB33100012) clustered with *D. torresensis*(CBS 119.41).

Morphological characteristics

Morphological observations for the five identified species are outlined below.

Cylindrocladiella lageniformis Crous, M.J. Wingf. & Alfenas

Pathogenic on trunks and rootstocks of *Vitis vinifera*. **Asexual morph**: Conidiophores were hyaline and penicillate. Conidia were hyaline, cylindrical, one septate or aseptate, with dimensions of 5.3–9.5 × 1.5–2.8 μm, mean ± SD = 7.8 ± 1.1 × 2.1 ± 0.3 μm. The terminal vesicles were lageniform to ovoid. (Figure 4 c-d). **Sexual morph**: undetermined.

Culture characteristics: Colonies on PDA reached 74.9 ± 0.8 mm diam. after 6 d incubation at 25°C in the dark, and were yellow to tan, with flourish aerial mycelium (Figure 4 a-b).

Material examined: CHINA, Guangxi province, on trunk and rootstock of *Vitis vinifera*, 8 April 2018, Xinghong Li, living cultures, JZB3320001, JZB3320002.

Dactylonectria macrodidyma (Halleen, Schroers & Crous) L. Lombard & Crous

Pathogenic on trunks and roots of *Vitis vinifera*. **Asexual morph**: the isolates rarely formed chlamydospores and microconidia, producing abundant macroconidia on MEA. Macroconidia hyaline, cylindrical, straight to slightly curved, one to four septate, with dimensions

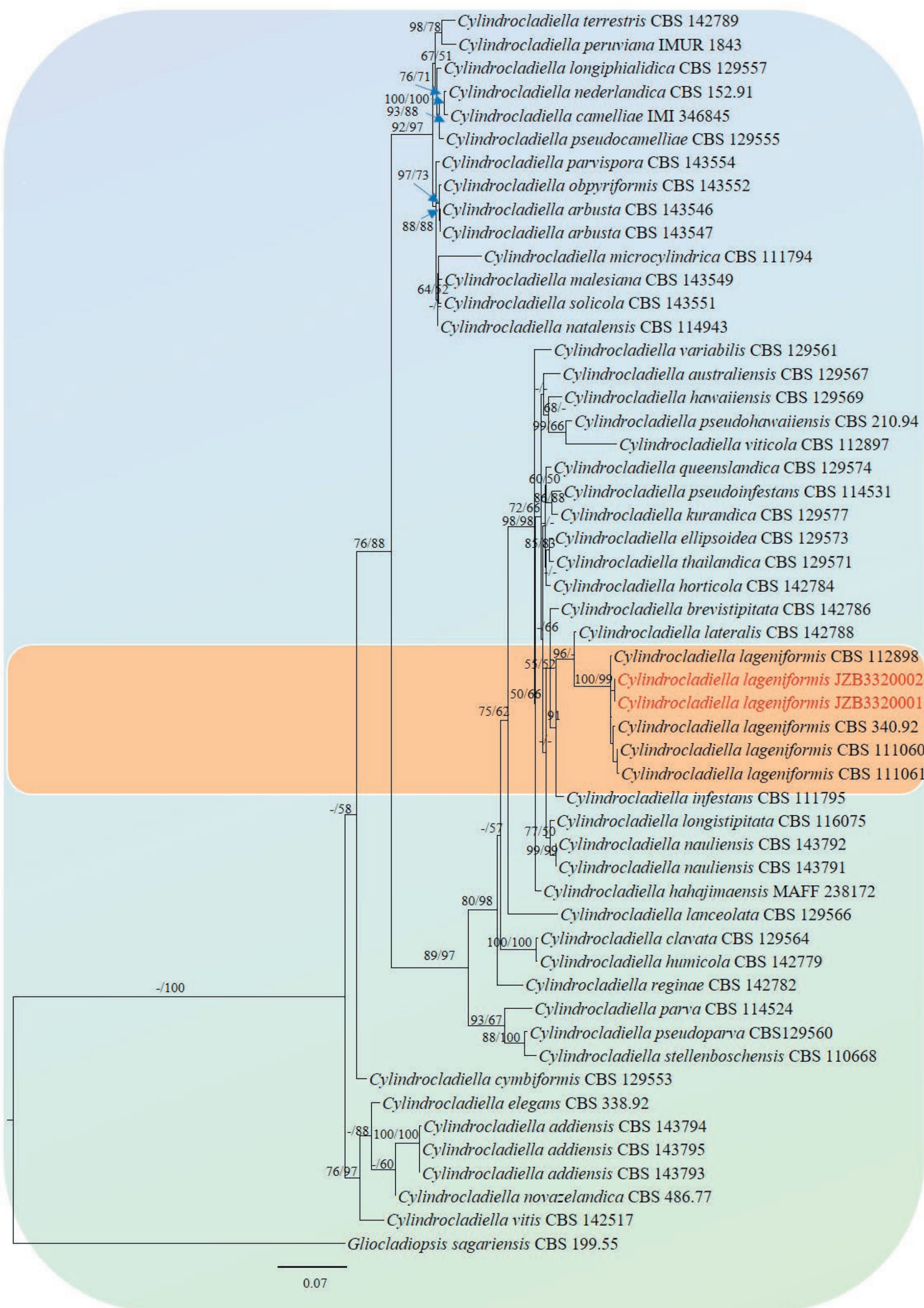

Figure 2. Maximum likelihood tree obtained from the phylogenetic analysis based on *tef1*, *his3* and *tub2* sequence alignments. The scale bar represents 0.07 changes. The tree is rooted in *Gliocladiopsis sagariensis* (CBS 199.55).

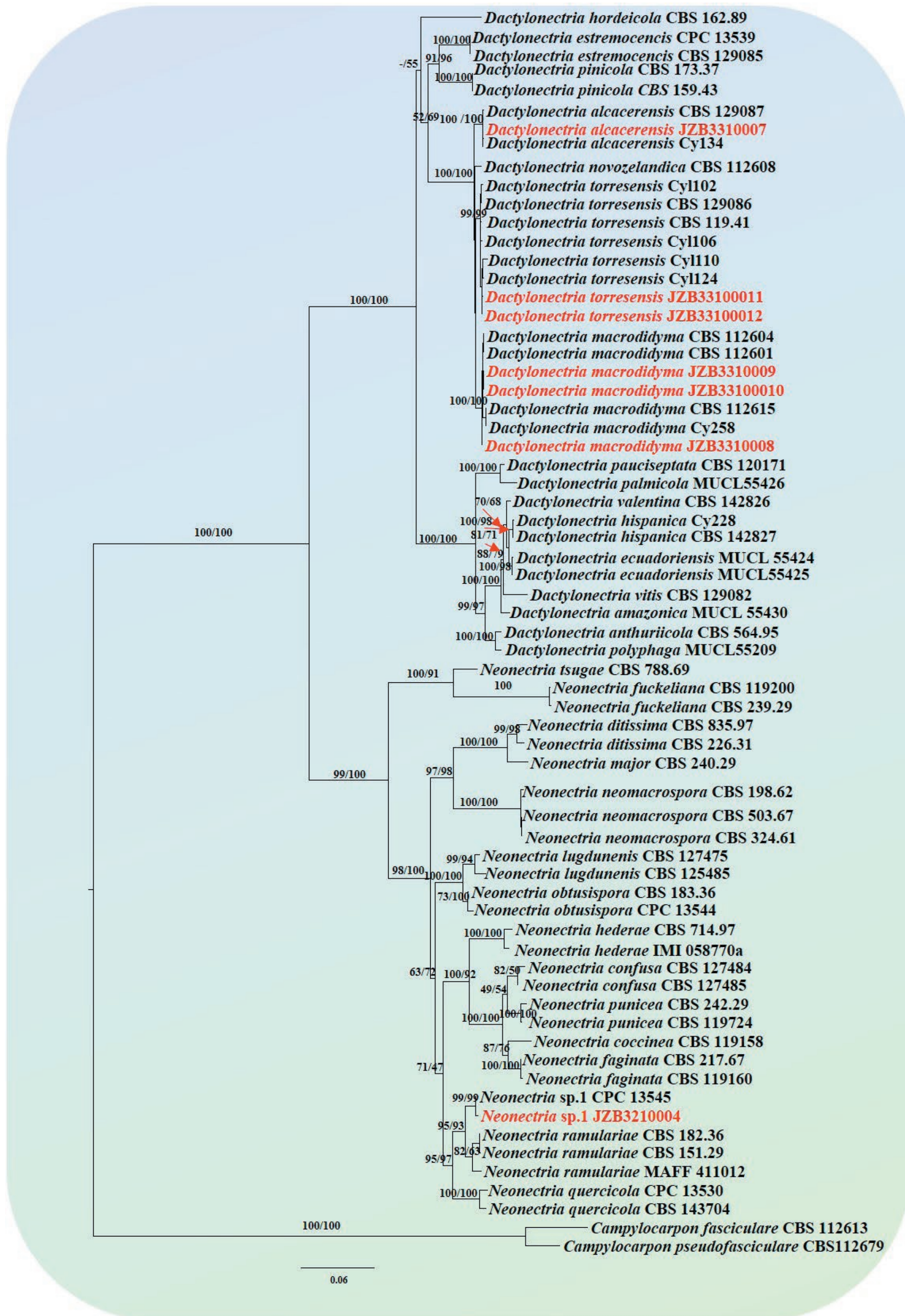

Figure 3. Maximum likelihood tree obtained from the phylogenetic analysis based on combined ITS, *tub2*, *his3*, and *tef1* sequence alignments. The scale bar represents 0.05 changes. The ex-type strains are in bold font. The outgroups of the tree are *Campylocarpon fasciculare* (CBS 112613) and *C. pseudofasciculare* (CBS 112679).

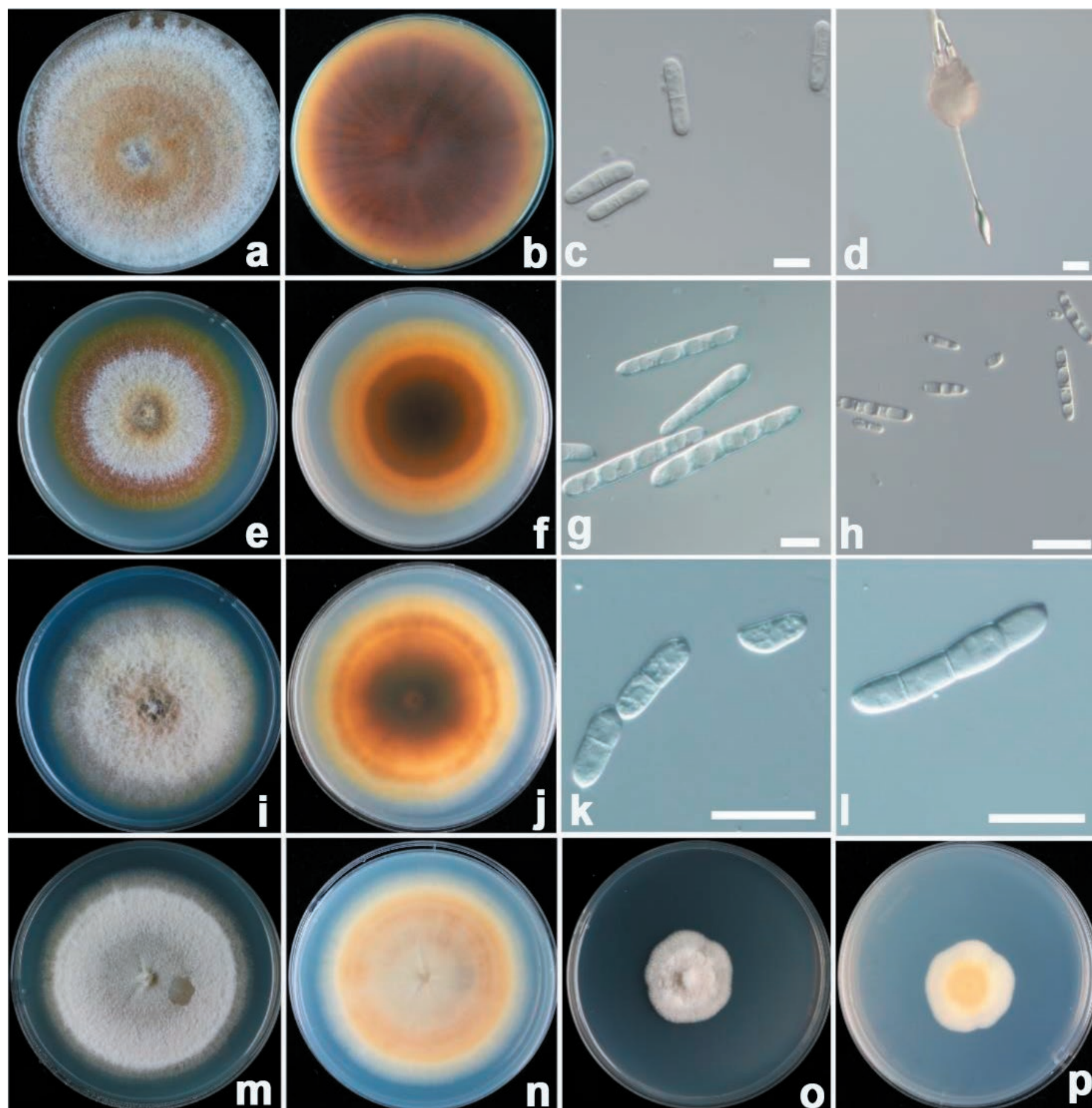

Figure 4. Photographs of isolated fungi and their morphological characterization. Colonies (top and bottom views of cultures) of *Cylindrocladiella lageniformis* (**a and b**), *Dactinonectria macrodidyma* (**e and f**), *D. torresensis* (**i and j**), *D. alcaceresis* (**m and n**), or *Neonectria* sp.1 (**o and p**). Conidia and terminal vesicles of *C. lageniformis* (**c and d**). Conidia of *D. macrodidyma* (**g and h**), and *D. torresensis* (**k and l**). All the fungi were grown on PDA for 14 d. Bars = 10 μm (**c and d**) or 20 μm (**g, h, k and l**).

of 14.4–44.2 × 4.0–8.2 μm, mean ± SD = 31.1 ± 7.8×6.2 ± 0.9 μm (Figure 4, g and h). Microconidia ellipsoid to ovoid, hyaline, straight, aseptate to one septate. **Sexual morph**: undetermined.

Culture characteristics: Colonies on PDA reached 57.3 ± 5.4 mm diameter after 9 d at 25°C in the dark, and were yellowish, with abundant aerial mycelium (Figure 4e). Colony reverse sides were burnt umber to raw sienna or brownish yellow on PDA (Figure 4f).

Material examined: CHINA, Ningxia province, on trunk and rootstock of *Vitis vinifera*, 8 April 2018, Qingtong Ye and Xinghong Li, living cultures, JZB3310008, JZB3310009, JZB3310010.

Dactylonectria torresensis (A. Cabral, Rego & Crous) L.

Lombard & Crous

Pathogenic on trunks and rootstocks of *Vitis vinifera*. **Asexual morph**: The isolates rarely formed chlamydospores and microconidia, producing some macroconidia on MEA. Macroconidia straight or minutely curved, cylindrical, one to four septate. Microconidia zero to one septate, ellipsoid to ovoid. **Sexual morph**: undetermined.

Culture characteristics: Colonies on PDA reached 55.5 ± 3.6 mm diam. after 9 d at 25°C in the dark, and were pale buff to chestnut (Figure 4, i and j). Colony reverse sides were buff to umber to chestnut on PDA.

Material examined: CHINA, Shanxi and Hebei province, on trunk and rootstock of *Vitis vinifera*, 8 April 2018, Qingtong Ye and Xinghong Li, living cultures, JZB3310011, JZB3310012.

Dactylonectria alcacerensis (A. Cabral, H. Oliveira & Crous) L. Lombard & Crous

Pathogenic on roots of *Vitis vinifera*. **Asexual morph**: isolates did not produce macroconidia, microconidia, or chlamydospores on MEA. **Sexual morph**: undetermined.

Culture characteristics: Colonies on PDA reached 49.3 ± 2.2 mm diam. eafter 9 d at 25°C in the dark, and were felty to slightly cottony (Figure 4 m-n).

Material examined: CHINA, Shanxi province, on trunk and rootstock of *Vitis vinifera*, 8 May 2018, Qingtong Ye and Xinghong Li, living culture, JZB3310007.

Neonectria sp. 1

Pathogenic on the bark of trunk of *Vitis vinifera*. **Asexual morph**: In the present study, the isolates did not produce macroconidia, microconidia, or chlamydospores on PDA. **Sexual morph**: undetermined.

Culture characteristics: Colonies on PDA reached 61.1±1.1 mm diameter after 15 d of incubation at 20°C in dark (Figure 4 o-p).

Material examined: CHINA, Xinjiang province, on trunk and rootstock of *Vitis vinifera*, 16 April 2018, Qingtong Ye and Xinghong Li, living culture,

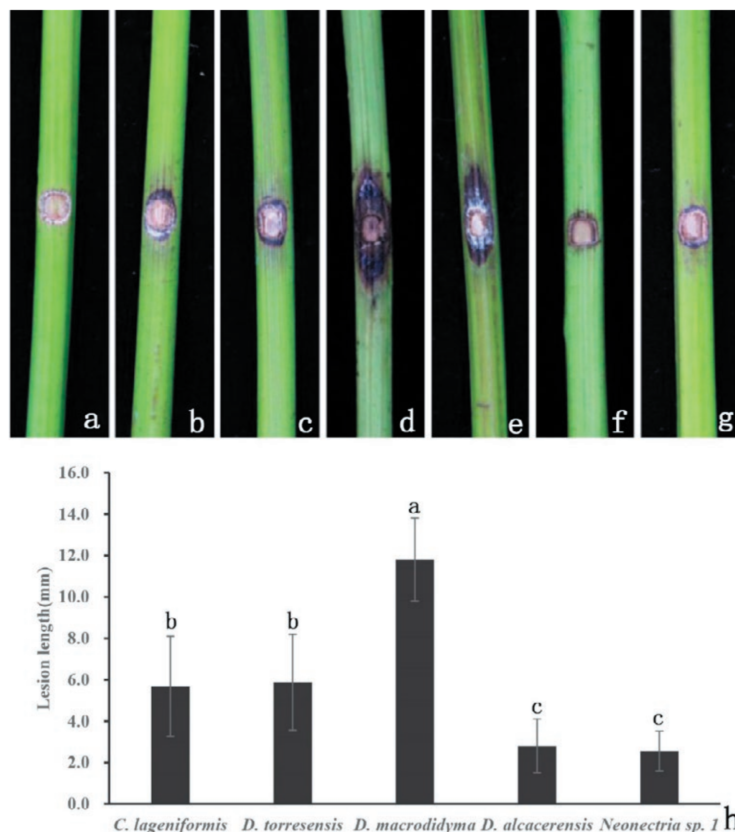

Figure 5. Pathogenicity tests results (after 7 d) of BF fungal agents inoculated onto detached green shoots of grapevine 'Summer Black'. Control (**a**), *Cylindrocladiella lageniformis*(**b**), *Dactinonectria torresensis* (**c**), *D. macrodidyma* (**d and e**), *D. alcacerensis*(**f**), or *Neonectria* sp. 1(**g**). Histogram (**h**) of mean lesion lengths on wood shoots after inoculations with the different fungi. Means accompanied by different letters are significantly different ($P = 0.05$).

JZB3210004.

Pathogenicity tests

In the pathogenicity tests conducted with detached green shoots, the non-inoculated shoots did not develop any symptoms (Figure 5a). In contrast, shoots inoculated with mycelium discs resulted in necroses. The lesions were brown to black, and the mean lesion lengths differed among the different inoculated fungi ($P < 0.05$) (Figure 5, b to h). *Dactylonectria macrodidyma* was the most aggressive pathogen (mean lesion length = 1.18 cm) among the five species (Figure 5). The re-isolation rates of isolates *C. lageniformis*, *D. torresensis*, *D. macrodidyma*, *D. alcacerensis,* and *Neonectria* sp. 1. were between 70% with 100% from the lesions.

Pathogenicity tests on 3-month-old grapevine cuttings showed different results for the different inoculated pathogens, as well. The non-inoculated controls showed no symptoms on the shoots (Figure 6a). *Dactylonectria macrodidyma* caused brown to black necrotic lesions on the shoots (mean lesion length = 1.95 cm) (Figure 6, d and e). Less necrosis was observed in the cuttings inoculated in *C. lageniformis*, *D. torresensis*, *Neonectria* sp. 1., or *D. alcacerensis* (Figure 6, b to c', f to g'). The re-isolation rates of the different inoculated fungi from the respective lesions were between 70% with 100%.

This is the first report of *C. lageniformis*, *D. torresensis*, *D. macrodidyma*, *D. alcacerensis* and *Neonectria* sp. 1 associated with BF of grapevines in China.

DISCUSSION

Grapevines can be affected by several diseases throughout each year, especially during fruit production. In the present study, 50 isolates obtained from diseased grapevine samples in five provinces of China were identified as *C. lageniformis*, *D. torresensis*, *D. macrodidyma*, *D. alcacerensis,* or *Neonectria* sp. 1. To date, *D. torresen-*

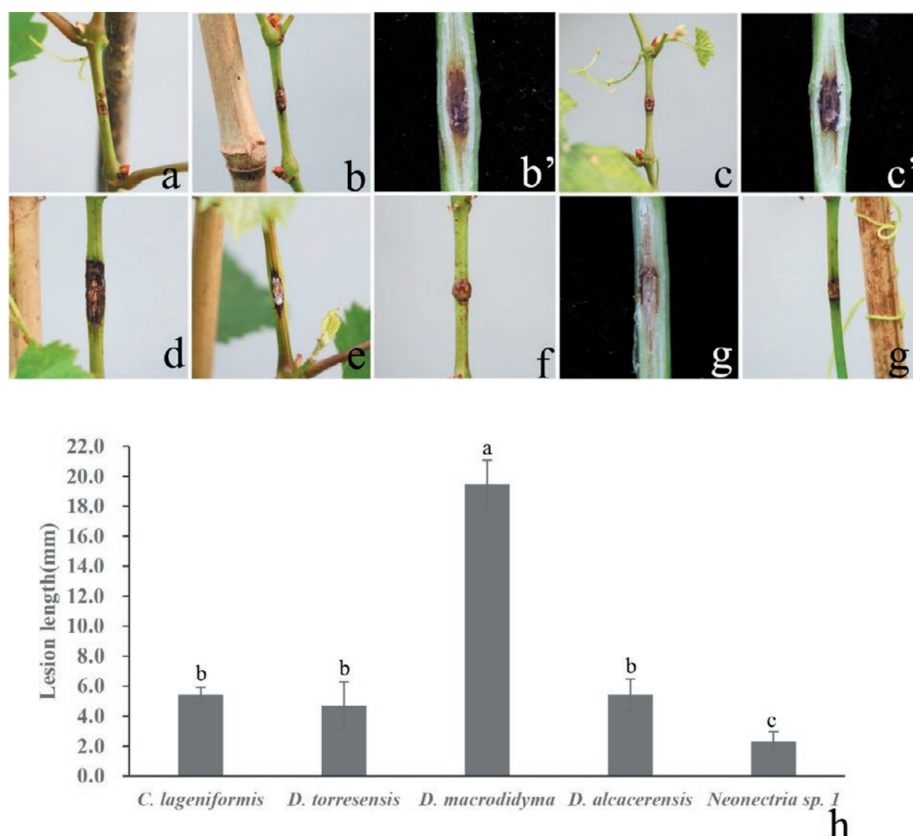

Figure 6. Pathogenicity tests results (after 80 d) of BF fungal agents inoculated onto 3-month-old 'Summer Black' grapevine plants in greenhouse. Control (**a**), *Cylindrocladiella lageniformis* (**b and b'**), *Dactinonectria torresensis*(**c and c'**), *D. macrodidyma*(**d and e**), *D. alcacerensis* (**f and g**), *Neonectria* sp. 1 (**g'**). Histogram (**h**) of mean lesion lengths caused by inoculations with the different fungi. Means accompanied by different letters are significantly different ($P = 0.05$).

sis has been reported as grapevine pathogen in Australia, Canada, Czech Republic France, Italy, New Zealand, Portugal, South Africa, Spain and USA (Agustí-Brisach *et al*., 2013; Carlucci *et al*., 2017; Pecenka *et al*., 2018; Pintos *et al*., 2019), and *Cylindrocladiella lageniformis* has been reported mainly from California and South Africa (Van Coller *et al*., 2005; Koike *et al*., 2016).

The BF pathogens are soil-borne organisms that affect roots and the basal ends of rootstock vines, and most of the fungi reported in the present study were also reported in California and Spain (Koike *et al*., 2016; Berlanas *et al*., 2020). However, whole grapevines in the north grape-growing regions of China are routinely buried under the soil in winter, to allow survival during low temperature winter conditions. It has been proposed that the soil-borne pathogens could infect plants through wounds (to roots, rootstocks, trunks, canes and shoots). Pathogenicity tests of BF pathogens were carried out with canes or roots in previous studies (Koike *et al*., 2016; Berlanas *et al*., 2020). Nevertheless, pathogenicity tests of *Cylindrocladiella lageniformis* have also been conducted with green shoots (Van Coller *et al.*, 2005; Koike *et al*., 2016). Therefore, in the present study, it was important to determine whether the soil-borne fungi could infect host canes or shoots. The fungi were inoculated by wounding between two nodes of each cutting. Among the tested fungi, *D. macrodidyma* produced the longest lesions in the pathogenicity tests. However, Berlanas *et al.* (2020) reported that virulence of *D. alcacerensis* was greater than that of *D. macrodidyma* and *Neonectria* sp. 1., while *D. macrodidyma* was found to be more virulent than *D. alcacerensis* in the present study. Differences in virulence of *D. alcacerensis* or the other species could be attributed to: (1) strain origins (Probst *et al*., 2019), (2) host genotype susceptibility to black foot fungus infections (Berlanas *et al*., 2020), (3) methods of inoculation (Alaniz *et al*., 2009b; Probst *et al.,* 2019; Berlanas *et al*., 2020), or (4) inoculum dose.

The distribution of BF fungal pathogens in the present study may have been influenced by climate. The climate system of China is diverse due to the varied topography and vast area, including climates of Tibetan plateau, temperate continental, subtropical monsoon, and tropical monsoon (Yan *et al*., 2013). The characteristics of BF pathogens are likely to vary due to the diverse temperature of China. Based on the present study (data not shown), colony diameter on PDA after 6 d of *C. lageniformis* from the south of China reached up to 60.4 ± 3.3 mm at 30°C while the other fungi (*D. torresensis*, *D. macrodidyma*, *D. alcacerensis* and *Neonectria* sp. 1.) from the north of China hardly grew at 30°C. Most of these fungi could grow below 5°C in PDA, except for *C. lageniformis*.

Although the incidence of diseased plants with BF symptoms was about 1% in the surveyed vineyards in China, which is much less than in France (losses of 50%: Larignon *et al*., 1999), BF pathogens can infect grapevine roots and trunks in young nurseries and plantations, and the pathogenic fungi can be transmitted to new vineyards by cuttings (De la Fuente *et al*., 2016). The fungi *C. lageniformis*, *D. torresensis*, *D. macrodidyma*, *D. alcacerensis,* and *Neonectria* sp. 1. are all soilborne, and can infect hosts through the soil (Halleen *et al*., 2003). In the north of China, grapevines need to be buried under the soil for survival during cold weather, resulting in small wounds that are likely to be susceptible to infection by soilborne fungi, so more attention should be paid to BF in China in future.

Grapevine BF is prevalent in nurseries and new plantations (De la Fuente *et al*., 2016), and the current strategies for controlling this disease include good hygiene or sanitation, which are the most important means of obtaining healthy vines (Gramaje and Armengol 2011), including treatments with hot water, (Gramaje *et al*., 2010; Halleen and Fourie 2016), fungicides (Halleen *et al*., 2007; Rego *et al*., 2009; Alaniz *et al*., 2011) and biological control agents (Berbegal *et al*., 2020; Martínez-Diz *et al*., 2021; van Jaarsveld *et al*., 2020, 2021). Chemical treatments during propagation processes in nurseries for control of BF pathogens have been evaluated, including treating cutting prior to cold storage, cutting prior to callusing, rooting pre- and post-grafting, and pre-planting fungicide treatments of rooted cuttings, to eliminate or reduce potential fungal agents before planting (Halleen *et al*., 2007; Rego *et al*., 2009, Alaniz *et al*., 2011, Gramaje *et al*., 2018). Based on previous research, benomyl was effective for elimination or reducing *Cylindrocarpon destructans* infections (Rego *et al.* 2006). Reductions of *D. torresensis* and *D. macrodidyma* incidence and disease severity on the bases of 2-year-old plants have been reported from applications of *Streptomyces* sp. E1+R4 before preplanting (Martínez-Diz *et al*., 2021).

Some practices, such as hot water treatments, are useful for sanitizing commercially produced plants. Generally, this practice entails treating the plants at 50°C for 30 min. However, this is stressful for the plants (Waite *et al*., 2013). Despite treated with these practices, diseases in symptomless plants can still be transmitted to non-infested areas (De la Fuente *et al*., 2016). The detection of BF fungi in soils or vines is essential for controlling the disease in nurseries and new plantations. Alaniz *et al.* (2009a) reported a multiplex PCR system for specific and early detection of *Ilyonectria liriodendri* (=*Cylindrocarpon liriodendra*), *Dactylonectria macro-*

didyma (=*Cylindrocarpon macrodidymum*), and *Dactylonectria pauciseptata* (=*Cylindrocarpon pauciseptatum*) from pure fungus cultures or diseased plants. Martínez-Diz *et al.* (2020) attempted to detect *I. liriodendri* in bulk soils, rhizosphere soils, and grapevine endorhizospheres using Droplet Digital PCR (ddPCR) and real-time PCR (qPCR) techniques. They showed that ddPCR was more sensitive than qPCR to lower target concentrations. Nevertheless, the ddPCR technique has not been used for detection of *C. lageniformis*, *D. torresensis*, *D. macrodidyma*, *D. alcacerensis* or *Neonectria* sp.1, and this technology could be useful for detection of BF in China. Further study should also be conducted to develop specific protocols for effective BF management.

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