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ORIGINAL ARTICLE

Botrytis eucalypti, a novel species isolated from diseased *Eucalyptus* seedlings in South China

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Abstract *Eucalyptus* has become a preferred species for the production of industrial products and also for the protection of natural forests in South China. Many diseases affect these trees, both on plantations and in nurseries. One such disease in *Eucalyptus* nurseries is grey mould caused by a species of *Botrytis*. Symptoms of grey mould were recently observed on stems and leaves of *Eucalyptus urophylla* × *Eucalyptus grandis* seedlings in nurseries in ZhanJiang, GuangDong Province, South China. Diseased stems and leaves were covered with mycelium, conidiophores, and conidia of the causal pathogen. The fungus was identified on the basis of DNA sequence comparisons and morphological features, and its pathogenicity was tested on three *Eucalyptus* clones. Based on sequence comparisons of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA and partial DNA sequences of five nuclear gene regions, glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat-shock protein 60 (*HSP60*), DNA-dependent RNA polymerase subunit II (*RPB2*), necrosis and ethylene-inducing proteins (*NEP1* and *NEP2*), combined with morphological characteristics and culture growth rate, the fungus represents a previously undescribed species of *Botrytis*. This undescribed species is phylogenetically and morphologically closely to *Botrytis cinerea* and *B. pelargonii*, but can be distinguished from them by DNA sequences of *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene regions, conidial characteristics, and culture growth rate. Here, a description of the fungus, designated as *Botrytis eucalypti* sp. nov., is provided. The results of in vitro leaf inoculation tests demonstrate that *B. eucalypti* is virulent on all three tested *E. urophylla* × *E. grandis* clones.

Keywords Fungal pathogen · Grey mould · Nursery disease · Pathogenicity · Phylogeny

Introduction

Grey mould caused by *Botrytis* species is one of the most prevalent and devastating plant diseases globally (Elad et al. 2016a). *Botrytis cinerea*, the lectotype of *Botrytis*, can infect more than 200 plant species worldwide (Hahn et al. 2014). Under suitable weather conditions (humid and cool), grey mould can infect at any growth stage in susceptible plants (Davidson et al. 2004; Elad et al. 2004; Zhang 2006). The disease emerges as small water-soaked spots on leaves and stems that merge into large necrotic lesions covered with grey conidia and conidiophores, causing stem, leaf, and fruit rot (Zhang 2006; Williamson et al. 2007; Carisse 2016).

To meet increasing demands for pulp and paper, plantation forestry based on fast-growing trees, especially species of *Eucalyptus*, has expanded rapidly in South China during the past three decades (Zhou and Wingfield 2011). Currently, approximately 4.5 million ha of *Eucalyptus* plantations have been established in South China (Chen and Chen 2013). During the last two decades, however, several disease problems have emerged, either in plantations or nurseries. Diseases on *Eucalyptus* plantations include stem canker/wilt caused by species of *Botryosphaeria*, *Lasiodiplodia*, and *Neofusicoccum* in Botryosphaeriaceae (Chen et al. 2011a), *Ceratocystis* (Chen et al. 2013; Liu et al. 2015), *Chrysoporthe* (Chen et al. 2010),

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and *Teratosphaeria* (Chen et al. 2011b); leaf blight/spot caused by species of *Calonectria* (Lombard et al. 2010; Chen et al. 2011c), *Mycosphaerella* (Burgess et al. 2007), *Quambalaria* (Zhou et al. 2007), and *Teratosphaeria* (Burgess et al. 2006); and bacterial wilt associated with *Ralstonia solanacearum* (Old et al. 2003). In *Eucalyptus* nurseries, isolates of the genera *Botrytis* and *Calonectria* have been found (Lombard et al. 2010, 2015; Chen et al. 2011c; Li 2012). Diseases associated with *Calonectria* species include cutting rot and leaf blight (Lombard et al. 2010; Chen et al. 2011c). Several species of *Calonectria* have been isolated from diseased *Eucalyptus* seedlings in South China (Lombard et al. 2010; Chen et al. 2011c), and pathogenicity testing has revealed that *Calonectria* species are aggressive pathogens of two important *E. urophylla* × *E. grandis* clones extensively cultivated in nurseries in South China (Chen et al. 2011c). In *Eucalyptus*, *Botrytis* species cause stem wilt and leaf rot (Zaldúa and Sanfuentes 2010; Liao et al. 2013; Muñoz and Campos 2013). Only one species of *Botrytis* has been reported from *Eucalyptus* in China, symptoms of grey mould were observed on leaves of *Eucalyptus* trees, the pathogen's identification was based solely on morphological characteristics (Li 2012; Liao et al. 2013).

Recently, grey mould was observed on *E. urophylla* × *E. grandis* seedlings in one nursery in GuangDong Province in South China. A typical manifestation of *Botrytis* infection was observed on the leaves and stems of seedlings. The objectives of this study were to identify those fungal isolates based on sequence comparisons and morphological characteristics and to test its pathogenicity on three *E. urophylla* × *E. grandis* clones.

Materials and methods

Sample collection and isolation

In March 2015, leaves and stems exhibiting symptoms of grey mould were collected from *Eucalyptus urophylla* × *Eucalyptus grandis* seedlings at the South China Experiment Nursery of the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry, located in ZhanJiang, GuangDong Province, China. The diseased seedlings were collected from multiple sites in the nursery and placed in individual plastic bags and transported to the laboratory for isolation.

Conidial masses were transferred under a stereomicroscope AxioCam Stemi 2000C (Carl Zeiss, Germany) directly from the symptomatic tissues to 2 % malt extract agar (20 g malt extract powder and 20 g agar powder per liter water: malt extract powder from Beijing Shuangxuan microbial culture medium products factory, Beijing, China; agar powder from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) (MEA). After incubation at 20 °C for 24–48 h, single germinating conidia from the conidial masses were transferred onto fresh MEA plates and incubated at 20 °C in darkness for 12 days to obtain pure cultures. The cultures were deposited in the CERC culture collection. Representative isolates were deposited at the China Forestry Culture Collection Center (CFCC), Beijing, China. Representative isolates were stored in the China General Microbiological Culture Collection Center (CGMCC), Beijing, China. The specimens (fungal pure cultures) were deposited in the Collection of Central South Forestry Fungi of China (CSFF), GuangDong Province, China.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Fungal isolates grew on MEA for 7 days at 20 °C, after which mycelia were scraped from the culture surface with a sterilized scalpel and transferred to 2.0 mL Eppendorf tubes. Genomic DNA was extracted using the "Extraction method 5: grinding and CTAB," described by Van Burik et al. (1998). The extracted DNA was dissolved in 30 μL TE buffer (1 M Tris-HCl and 0.5 M EDTA, pH 8.0) and quantified using a Nano-Drop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

A 1 μL aliquot of genomic DNA was added as the template to each PCR reaction [TopTaq™ Master Mix 25 μL (Qiagen Inc., Hilden, Germany); forward primer: 1 μL, 10 μM (Invitrogen, Shanghai, China); reverse primer: 1 μL, 10 μM (Invitrogen, Shanghai, China); RNase-Free H_2O 22 μL (Qiagen Inc., Hilden, Germany)] to amplify the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA and the partial DNA sequences of five nuclear genes, glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat-shock protein 60 (*HSP60*), DNA-dependent RNA polymerase subunit II (*RPB2*), and necrosis and ethylene-inducing proteins (*NEP1* and *NEP2*). The ITS region was amplified with primers ITS1 and ITS4 (White et al. 1990). Primer pairs G3PDHfor/G3PDHrev, HSP60for/HSP60rev, RPB2for/RPB2rev, NEP1for/NEP1revB, NEP2forD/NEP2revD were used to amplify fragments of the *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* genes, respectively (Staats et al. 2005, 2007; Grant-Downton et al. 2014; Lorenzini and Zapparoli 2014; Ferrada et al. 2016). The amplifications were performed in 50 μL reaction volumes on an MJ Mini Cycler (BIO-RAD, Hercules, CA, USA) under the conditions described by Staats et al. (2005, 2007). The PCR amplification products were separated by 1 % agarose gel electrophoresis and visualized with SYBR Safe DNA gel stain (Thermo Fisher Scientific).

The PCR products were sequenced in both directions using the same primers as described above by the Beijing Genomics Institute, Guangzhou, China. After assembling with MEGA v. 6.0 (Tamura et al. 2007), sequences of the isolates obtained in this study were deposited in GenBank (Table 1).

Table 1 Isolates used for phylogenetic studies and pathogenicity tests in this study

Species	Isolate No. [a]	Host	Location	Year	GenBank accession No. [b]						Reference
					ITS	*G3PDH*	*HSP60*	*RPB2*	*NEP1*	*NEP2*	
Botrytis aclada	MUCL8415	*Allium* sp.	Germany	1965	N/A [c]	AJ704992	AJ716050	AJ745664	AM087059	AM087087	Staats et al. 2005, 2007
	PRI006	*Allium* sp.	N/A	N/A	AJ716295	AJ704993	AJ716051	AJ745665	N/A	N/A	Staats et al. 2005
B. allii	MUCL403	*Allium* sp.	The Netherlands	1957	N/A	AJ704996	AJ716055	AJ745666	N/A	N/A	Staats et al. 2005
B. byssoidea	MUCL94 [d]	*Allium* sp.	USA	1923	N/A	AJ704998	AJ716059	AJ745670	AM087045	AM087079	Staats et al. 2005, 2007
	OnionBC-76	*Allium* sp.	YunXi, HuBei, China	2008	FJ169671	FJ169652	FJ169661	FJ169681	N/A	N/A	Zhang et al. 2010b
B. californica	X655 [d]	*Vitis vinifera*	Reedley, California	2012	KJ937039	KJ937069	KJ937059	KJ937049	N/A	N/A	Saito et al. 2016
	X503	*V. vinifera*	Earlimart, California	2012	KJ937038	KJ937068	KJ937058	KJ937048	N/A	N/A	Saito et al. 2016
B. calthae	CBS 175.63	*Caltha palustris*	USA	1961	AJ716302	AJ704999	AJ716060	AJ745671	N/A	N/A	Staats et al. 2005
	MUCL1089	*C. palustris*	Belgium	1960	N/A	AJ705000	AJ716061	AJ745672	N/A	N/A	Staats et al. 2005
	MUCL2830	*C. palustris*	USA	1961	N/A	AJ705001	AJ716062	AJ745673	AM087031	AM087088	Staats et al. 2005, 2007
B. caroliniana	CB15 [d]	*Rubus fruticosus*	USA	2011	JF777531	JF811584	JF811587	JF811590	JF811593	N/A	Li et al. 2012
	CA3	*R. fruticosus*	USA	2011	JF777532	JF811585	JF811588	JF811591	JF811594	N/A	Li et al. 2012
B. cinerea	MUCL87 [d]	N/A	The Netherlands	1928	N/A	AJ705004	AJ716065	AJ745676	N/A	N/A	Staats et al. 2005
	B05.10	N/A	Germany	1994	AM235297	AJ705002	AJ716063	AJ745674	DQ211824	DQ211825	Staats et al. 2005, 2007
	SAS405	N/A	Italy	N/A	AM235293	AJ705005	AJ716066	AJ745678	AM087028	N/A	Staats et al. 2005, 2007
	SAS56	N/A	Italy	N/A	AJ716294	AJ705006	AJ716067	AJ745677	N/A	N/A	Staats et al. 2005
	Bc7	N/A	The Netherlands	1970	AM235296	AJ705003	AJ716064	AJ745675	AM087027	N/A	Staats et al. 2005, 2007
	BcB	*V. vinifera*	Italy	2011	N/A	N/A	N/A	N/A	AM087026	AM087090	Lorenzini et al. 2014
	B01G1	*Eucalyptus benthamii*	Brazil	2009	KJ476441	N/A	N/A	N/A	N/A	N/A	Sbravatti Júnior et al. 2013
	B6	*E. benthamii*	Brazil	2009	KJ476442	N/A	N/A	N/A	N/A	N/A	Sbravatti Júnior et al. 2013
	G1	*E. grandis*	Mexico	2002	AY550976	N/A	N/A	N/A	N/A	N/A	Pérez-Vera et al. 2005
	4879	*E. globulus*	Spain	2008	FR667990	N/A	N/A	N/A	N/A	N/A	Sánchez Márquez et al. 2011
	CB17	*R. fruticosus*	USA	2010	JN672673	JN672671	JN672675	JN672677	JN672679	N/A	Li et al. 2012
	D06_1-30	*Vitis* sp.	Germany	N/A	N/A	N/A	JX266714	N/A	N/A	JX266748	Leroch et al. 2013
	N11_K_W08b	*Vitis* sp.	Norway	N/A	N/A	N/A	N/A	N/A	N/A	JX266749	Leroch et al. 2013
	N11_K_W11	*Vitis* sp.	Norway	N/A	N/A	N/A	N/A	N/A	N/A	JX266756	Leroch et al. 2013
	N11_S_E08	*Fragaria ananassa*	Norway	N/A	N/A	N/A	JX266720	N/A	N/A	JX266757	Leroch et al. 2013
B. convoluta	9801	*Iris* sp.	The Netherlands	1998	AJ716304	AJ705007	AJ716068	AJ745679	AM087034	AM087061	Staats et al. 2005, 2007
	MUCL11595	*Iris* sp.	USA	1968	N/A	AJ705008	AJ716069	AJ745680	AM087035	AM087062	Staats et al. 2005, 2007
B. croci	MUCL436	*Crocus* sp.	The Netherlands	1968	N/A	AJ705009	AJ716070	AJ745681	AM087047	AM087065	Staats et al. 2005, 2007
B. deweyae	CBS 134649 [d]	*Hemerocallis* sp.	UK	2009	HG799533	HG799521	HG799519	HG799518	HG799527	HG799520	Grant-Downton et al. 2014
	CBS 134650	*Hemerocallis* sp.	UK	2009	HG799535	HG799523	N/A	N/A	HG799529	N/A	Grant-Downton et al. 2014
B. elliptica	BE0022	*Lilium* sp.	The Netherlands	2001	AM235307	AJ705010	AJ716071	AJ745682	AM087050	AM087081	Staats et al. 2005, 2007
	BE9610	*Lilium* sp.	The Netherlands	2005	AM235302	AJ705011	AJ716072	AJ745683	DQ211827	AM087082	Staats et al. 2005, 2007
	BE9714	*Lilium* sp.	The Netherlands	2005	AJ716300	AJ705012	AJ716073	AJ745684	AM087049	AM087080	Staats et al. 2005, 2007
B. eucalypti	CERC7170 [d,e] = CGMCC3.18028	*E. urophylla* × *E. grandis*	ZhanJiang, GuangDong, China	2016	**KX301016**	**KX301020**	**KX301024**	**KX301028**	**KX301032**	**KX301036**	This study
	CERC7160 [e] = CFCC51322	*E. urophylla* × *E. grandis*	ZhanJiang, GuangDong, China	2016	**KX301014**	**KX301018**	**KX301022**	**KX301026**	**KX301030**	**KX301034**	This study
	CERC7163 [e] = CFCC51323	*E. urophylla* × *E. grandis*	ZhanJiang, GuangDong, China	2016	**KX301015**	**KX301019**	**KX301023**	**KX301027**	**KX301031**	**KX301035**	This study
	CERC7208 [e] = CGMCC3.18029	*E. urophylla* × *E. grandis*	ZhanJiang, GuangDong, China	2016	**KX301017**	**KX301021**	**KX301025**	**KX301029**	**KX301033**	**KX301037**	This study
B. fabae	MUCL98 [d]	*Vicia faba*	Spain	1929	N/A	AJ705014	AJ716075	AJ745686	DQ211829	DQ211831	Staats et al. 2005, 2007

Table 1 (continued)

Species	Isolate No. [a]	Host	Location	Year	GenBank accession No. [b]						Reference
					ITS	*G3PDH*	*HSP60*	*RPB2*	*NEP1*	*NEP2*	
	CBS 109.57	*V. faba*	The Netherlands	1957	AJ716303	AJ705013	AJ716074	AJ745685	AM087025	N/A	Staats et al. 2005, 2007
	MUCL1104	*V. faba*	Italy	2008	N/A	N/A	N/A	N/A	N/A	JX266758	Leroch et al. 2013
	MUCL7923	*V. faba*	N/A	N/A	N/A	N/A	N/A	N/A	DQ211830	DQ211832	Staats et al. 2007
B. fabiopsis	BC-2 [d]	*V. faba*	WuHan, HuBei, China	2006	EU519204	EU519211	EU514482	EU514473	N/A	N/A	Zhang et al. 2010a
	BC-13	*V. faba*	XianNing, HuBei, China	2007	EU563122	EU563109	EU563100	EU563115	N/A	N/A	Zhang et al. 2010a
B. ficariarum	CBS 176.63 [d]	*Ficaria verna*	Belgium	1960	AJ716296	AJ705015	AJ716076	AJ745687	AM087055	AM087085	Staats et al. 2005, 2007
	MUCL376	*F. verna*	Belgium	1957	N/A	AJ705016	AJ716077	AJ745688	AM087056	N/A	Staats et al. 2005, 2007
B. galanthina	MUCL435	*Galanthus* sp.	The Netherlands	1958	N/A	AJ705018	AJ716079	AJ745689	AM087057	N/A	Staats et al. 2005, 2007
	MUCL3204	*Galanthus* sp.	The Netherlands	1963	N/A	AJ705017	AJ716078	AJ745690	AM087058	AM087067	Staats et al. 2005, 2007
B. gladiolorum	9701	*Gladiolus* sp.	N/A	1997	N/A	AJ705019	AJ716080	AJ745691	AM087040	AM087072	Staats et al. 2005, 2007
	MUCL3865	*Gladiolus* sp.	The Netherlands	1963	N/A	AJ705020	AJ716081	AJ745692	AM087041	N/A	Staats et al. 2005, 2007
B. globosa	MUCL444	*A. ursinum*	Belgium	1958	N/A	AJ705022	AJ716083	AJ745693	N/A	AM087071	Staats et al. 2005, 2007
	MUCL21514	*A. ursinum*	UK	1963	N/A	AJ705021	AJ716082	AJ745694	AM087044	AM087070	Staats et al. 2005, 2007
Botrytis Group S	D08_H_8-04	*F. ananassa*	Germany	N/A	N/A	N/A	JX266715	N/A	N/A	JX266750	Leroch et al. 2013
	D09_K_4-01	*F. ananassa*	Germany	N/A	N/A	N/A	JX266716	N/A	N/A	JX266751	Leroch et al. 2013
	D10_B_F1-06	*F. ananassa*	Germany	N/A	N/A	N/A	JX266717	N/A	N/A	JX266752	Leroch et al. 2013
	D10_B_F3-05	*F. ananassa*	Germany	N/A	N/A	N/A	JX266718	N/A	N/A	JX266753	Leroch et al. 2013
	G09_S33	*F. ananassa*	Greece	N/A	N/A	N/A	JX266719	N/A	N/A	JX266754	Leroch et al. 2013
	S10_C1	*F. ananassa*	Spain	N/A	N/A	N/A	N/A	N/A	N/A	JX266755	Leroch et al. 2013
B. hyacinthi	0001	*Hyacinthus* sp.	The Netherlands	1999	AJ716297	AJ705023	AJ716084	AJ745695	AM087048	AM087066	Staats et al. 2005, 2007
	MUCL442	*Hyacinthus* sp.	The Netherlands	1958	N/A	AJ705024	AJ716085	AJ745696	N/A	N/A	Staats et al. 2005
B. mali	BPI412756 [d]	*Malus*	USA	1932	N/A	EF367129	N/A	N/A	N/A	N/A	O'Gorman et al. 2008
	BPI411770	*Malus*	USA	1932	N/A	EF367121	N/A	N/A	N/A	N/A	O'Gorman et al. 2008
B. narcissicola	MUCL2120	*Narcissus* sp.	Canada	1961	N/A	AJ705026	AJ716087	AJ745697	AM087046	AM087078	Staats et al. 2005, 2007
	MUCL18857	*Narcissus* sp.	UK	1972	N/A	AJ705025	AJ716086	AJ745698	N/A	N/A	Staats et al. 2005
	F105830	*Rhododendron*	Argentine	N/A	KJ941071	N/A	N/A	N/A	N/A	N/A	Galán et al. 2015
B. paeoniae	MUCL16084	*Paeonia* sp.	Belgium	1970	N/A	AJ705028	AJ716089	AJ745700	AM087033	N/A	Staats et al. 2005, 2007
	0003	*Paeonia* sp.	The Netherlands	2002	AJ716298	AJ705027	AJ716088	AJ745699	AM087032	AM087064	Staats et al. 2005, 2007
B. pelargonii	CBS 497.50 [d]	*Pelargonium* sp.	Norway	1949	AJ716290	AJ704990	AJ716046	AJ745662	AM087030	N/A	Staats et al. 2005, 2007
	MUCL1152	*Pelargonium* sp.	Norway	1960	N/A	AJ705029	AJ716090	AJ745701	DQ211833	DQ211834	Staats et al. 2005, 2007
B. polyblastis	CBS 287.38 [d]	*Narcissus* sp.	UK	1938	AJ716291	AJ705030	AJ716091	AJ745702	AM087039	AM087074	Staats et al. 2005, 2007
	MUCL21492	*Narcissus* sp.	UK	1963	N/A	AJ705031	AJ716092	AJ745703	N/A	AM087073	Staats et al. 2005, 2007
B. porri	MUCL3234 [d]	*Allium* sp.	N/A	1926	AJ716292	AJ705032	AJ716093	AJ745704	AM087060	AM087063	Staats et al. 2005, 2007
	GarlicBC-16	*Allium sativum*	ZhuShan, HuBei, China	2007	EU519206	EU519219	EU514490	EU514481	N/A	N/A	Zhang et al. 2010b
B. prunorum	Bpru-21 [d]	*Prunus salicina*	Chile	2012	KP234036	KP339980	KP339994	KP339987	KR732658	KP400596	Ferrada et al. 2016
	Bpru-8	*P. salicina*	Chile	2012	KP234035	KP339979	KP339993	KP339986	KR732657	KP400595	Ferrada et al. 2016
B. pseudocinerea	VD110 [d]	*V. vinifera*	France	2007	N/A	KR030052	N/A	N/A	N/A	N/A	Walker et al. 2011
	10091	*V. vinifera*	France	2011	JN692379	JN692414	JN692400	JN692428	N/A	N/A	Zhou et al. 2014
	X1303	*Vaccinium bracteatum*	California	2012	KJ796646	KJ796654	KJ796658	KJ796650	N/A	N/A	Saito et al. 2014
	D08_H_8-15	*F. ananassa*	Germany	N/A	N/A	N/A	JX266721	N/A	N/A	N/A	Leroch et al. 2013

Table 1 (continued)

Species	Isolate No. [a]	Host	Location	Year	GenBank accession No. [b]						Reference
					ITS	*G3PDH*	*HSP60*	*RPB2*	*NEP1*	*NEP2*	
	N11_S_E06	*F. ananassa*	Norway	2012	N/A	N/A	JX266723	N/A	N/A	JX266759	Leroch et al. 2013
	VD256	*V. vinifera*	France	N/A	N/A	N/A	JX266722	N/A	N/A	N/A	Leroch et al. 2013
B. ranunculi	CBS 178.63 [d]	*Ranunculus* sp.	USA	1963	N/A	AJ705034	AJ716095	AJ745706	AM087054	AM087086	Staats et al. 2005, 2007
B. sinoallii	OnionBC-23 [d]	*A. cepa*	XianNing, HuBei, China	2006	EU519203	EU519217	EU514488	EU514479	N/A	N/A	Zhang et al. 2010b
	OninBC-59	*A. cepa*	JianShi, HuBei, China	2007	FJ169664	FJ169646	FJ169658	FJ169678	N/A	N/A	Zhang et al. 2010b
B. sinoviticola	GBC-3-2b [d]	*V. vinifera*	WuHan, HuBei, China	2009	JN692383	JN692411	JN692397	JN692425	N/A	N/A	Zhou et al. 2014
	GBC-9	*V. vinifera*	XinJiang, China	2010	JN692378	JN692408	JN692394	JN692422	N/A	N/A	Zhou et al. 2014
Botrytis sp.	B83	*V. vinifera*	Italy	2011	KC191680	KC191677	KC191678	KC191679	KC762944	KC762945	Lorenzini et al. 2014
B. sphaerosperma	MUCL21481	*A. triquetrum*	UK	1963	AJ716293	AJ705035	AJ716096	AJ745708	AM087042	AM087068	Staats et al. 2005, 2007
	MUCL21482	*A. triquetrum*	UK	1963	N/A	AJ705036	AJ716097	AJ745709	AM087043	AM087069	Staats et al. 2005, 2007
B. squamosa	MUCL1107 [d]	*A. cepa*	USA	1923	N/A	AJ705037	AJ716098	AJ745710	AM087052	AM087084	Staats et al. 2005, 2007
	MUCL9112	*A. cepa*	The Netherlands	1966	N/A	AJ705038	AJ716099	AJ745711	AM087053	N/A	Staats et al. 2005, 2007
	PRI026	*A. cepa*	N/A	N/A	AJ716299	AJ705039	AJ716100	AJ745707	AM087051	AM087083	Staats et al. 2005, 2007
B. tulipae	BT9001	*Tulipa* sp.	The Netherlands	2000	AM235308	AJ705040	AJ716101	AJ745712	AM087036	AM087075	Staats et al. 2005, 2007
	BT9830	*Tulipa* sp.	The Netherlands	2000	AJ716301	AJ705041	AJ716102	AJ745713	AM087037	AM087077	Staats et al. 2005, 2007
	BT9901	*Tulipa* sp.	The Netherlands	2000	AM235313	AJ705042	AJ716103	AJ745714	AM087038	AM087076	Staats et al. 2005, 2007
Sclerotinia sclerotiorum	484	N/A	N/A	N/A	N/A	AJ705044	AJ716048	AJ745716	N/A	N/A	Staats et al. 2005
	F113458	*Capsicum annuum*	Spain	N/A	KJ941074	N/A	N/A	N/A	N/A	N/A	Galán et al. 2015

[a] BPI, U.S. National Fungus Collection; CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CERC, Culture Collection of China Eucalypt Research Centre, Chinese Academy of Forestry, ZhanJiang, GuangDong, China; CFCC, China Forestry Culture Collection Center, Beijing, China; CGMCC, China General Microbiological Culture Collection Center, Beijing, China; MUCL, BCCM Belgium Coordinated Collection of Microorganisms.

[b] ITS, internal transcribed spacer; *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSP60*, heat-shock protein 60; *RPB2*, DNA-dependent RNA polymerase subunit II; *NEP1* and *NEP2*, necrosis and ethylene-inducing proteins 1 and 2. GenBank accession No. in boldface are sequenced in this study.

[c] N/A = Not Available.

[d] Isolates are ex-type.

[e] Isolates used for pathogenicity tests

Phylogenetic analysis

Sequences of the ex-type strains of *Botrytis* species and other published strains closely related to the *Botrytis* isolates sequenced in this study were downloaded from NCBI (http://www.ncbi.nlm.nih.gov) and used for phylogenetic analysis (Table 1). Sequences of the *Botrytis* isolates collected in this study and those from NCBI were aligned using the online version of MAFFT (http://mafft.cbrc.jp/alignment/server) with the interactive refinement method (FFT-NS-i) setting. The alignments were further edited manually in MEGA v. 6.0 and deposited in TreeBASE (http://treebase.org).

Phylogenetic analyses were conducted on both individual ITS, *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* sequence datasets, but also with combined datasets for two to five gene regions, depending on the *Botrytis* species/strains availability. Two methods were used for phylogenetic analyses. Maximum parsimony (MP) analyses were performed using PAUP v. 4.0 b10 (Swofford 2003) and maximum likelihood (ML) analyses were conducted with PhyML v. 3.0 (Guindon and Gascuel 2003).

For MP analyses, gaps were treated as a fifth character, and characters were unordered and of equal weight with 1000 random addition replicates. A partition homogeneity test (PHT) using PAUP v. 4.0 b10 (Swofford 2003) was conducted to determine whether data for the two to five genes could be combined. The most parsimonious trees were obtained using the heuristic search option with stepwise addition, tree bisection, and reconstruction branch swapping. MAXTREES was set to 5,000 and zero-length branches were collapsed. A bootstrap analysis (50 % majority rule, 1,000 replicates) was carried out to determine statistical support for internal nodes in trees. Tree length (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) were used to assess phylogenetic trees (Hillis and Huelsenbeck 1992).

For ML analyses, the best nucleotide substitution model was established with jModeltest v. 2.1.5 (Posada 2008). In PhyML, the maximum number of retained trees was set to 1,000, and nodal support was determined by non-parametric bootstrapping with 1,000 replicates. For both MP and ML analyses, the phylogenetic trees were viewed using MEGA v. 6.0.

Morphology

Representative isolates identified by DNA sequence comparisons and from different *Eucalyptus* seedlings were selected to determine morphological characteristics. Mycelial agar plugs (6 mm diameter) of selected isolates were transferred using a cork borer onto 90 mm Petri dishes containing MEA and potato dextrose agar (200 g diced potato, 20 g dextrose and 20 g agar powder per liter water: dextrose from Shantou Xilong Chemical Co., Ltd., Guangdong, China; agar powder from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) (PDA) and incubated at 20 °C in the dark to induce sclerotia formation for five replicates of each isolate. Sclerotia were examined microscopically (AxioCam Stemi 2000C stereomicroscope; AxioCam ERc 5S digital camera, Carl Zeiss Ltd., Munchen, Germany). Mycelial agar plugs (6 mm diam) were transferred onto water agar (20 g agar power per liter water, agar powder from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) (WA) with sterilized *Eucalyptus* leaves placed on the agar surface and incubated at 20 °C under continuous near-fluorescent light to induce sporulation. Conidiophores and conidia that emerged on the surface of the *Eucalyptus* leaves were mounted in one drop of 85 % lactic acid on glass slides and examined under an Axio Imager A1 microscope (Carl Zeiss Ltd., Munchen, Germany) and an AxioCam ERc 5S digital camera with Zeiss Axio Vision Rel. 4.8 software (Carl Zeiss Ltd., Munchen, Germany).

The size dimensions of 25–50 sclerotia, conidiophores and conidia of each isolate were measured. Minimum, maximum, and average (mean) values were determined and presented in the taxonomic description as follows: [minimum – (average – standard deviation)] – [(average + standard deviation) – maximum]. Ratios of average length to average width were also calculated.

Culture characteristics of the *Botrytis* isolates were determined using cultures grown in 90 mm Petri dishes containing MEA for 3–12 days. To determine the effect of temperature on mycelial growth rate of the representative isolates, mycelial agar plugs (6 mm diam) were transferred from these cultures to new MEA and PDA Petri dishes and incubated in darkness under different temperatures ranging from 5 °C to 40 °C at 5 °C intervals. Five replicates of each isolate (CERC7160, CERC7163, CERC7170, and CERC7208) were incubated at each temperature. Colony diameters were measured at the 24 h intervals up to 48 h (for 2 days), and the data were used to calculate growth rates. The average growth rate was calculated for each of the eight temperatures and the experiment was repeated once.

Pathogenicity tests

To identify whether the *Botrytis* strains isolated from *E. urophylla* × *E. grandis* seedlings were pathogenic to different *Eucalyptus* clones, isolates (CERC7160, CERC7163, CERC7170, and CERC7208) were used in pathogenicity tests against three different genotypes of *E. urophylla* × *E. grandis* clones (CEPT-11, CEPT-12, and CEPT-13). The CEPT-11 is the clone from which *Botrytis* was originally isolated, this clone was used for pathogenicity tests to fulfil Koch's postulates. The three *Eucalyptus* clones were planted in the South China Experiment Nursery in ZhanJiang.

Young healthy leaves were collected, surface-sterilized in 5 % bleach for 2 min, and then rinsed three times with sterilized water. Mycelial agar plugs (6 mm diam) were transferred from 2-day-old MEA cultures and placed face down on the detached leaflets. Ten detached leaves of each *Eucalyptus* clone were inoculated with each of the representative isolates. Additional ten detached leaves from each *Eucalyptus* clone were inoculated with sterile MEA plugs as negative controls. All inoculated leaves were placed in sealed plastic boxes, the bases of which were lined with moist pads to maintain humidity. These boxes were kept in a moist chamber at 25 °C. After 1 week, lesions on each leaf were measured, and the experiment was repeated once. Re-isolations were made by cutting small pieces (0.04 mm^2) of leaf tissue from the diseased/wounded edges and transferring them to MEA at 25 °C. Re-isolations were made from all leaves inoculated as controls and from four randomly selected leaves per isolate. The lesion lengths were analyzed with a one-way analysis of variance (ANOVA) in SPSS Statistics v. 20 (SPSS 2011). Duncan's multiple range test was applied, and *F*-values with $P < 0.05$ were considered significant.

Results

Isolation

Fungal strains were isolated from four *E. urophylla* × *E. grandis* seedlings exhibiting grey mould symptoms, the sclerotia and conidia of these strains consistent with the typical morphological characteristics of *Botrytis* species. Four isolates (CERC7160, CERC7163, CERC7170, and CERC7208) collected from four seedlings in one nursery were selected for further study.

Phylogenetic analysis

To obtain preliminary identification, the ITS, *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* sequences of the four Chinese *Botrytis*

Table 2 Statistics resulting from phylogenetic analyses

Dataset	No. of taxa	No. of bp [a]	Maximum parsimony					
			PIC [b]	No. of trees	Tree length	CI [c]	RI [d]	HI [e]
ITS	52	450	12	5000	20	0.650	0.837	0.350
G3PDH	76	887	138	449	289	0.609	0.904	0.391
HSP60	82	980	181	172	369	0.637	0.930	0.363
RPB2	72	1095	200	12	367	0.670	0.940	0.330
NEP1	52	742	256	45	564	0.610	0.891	0.390
NEP2	53	802	251	5000	523	0.608	0.902	0.392
G3PDH/HSP60/RPB2	72	2960	516	60	1060	0.617	0.915	0.383
NEP1/NEP2	39	1579	472	16	1058	0.578	0.850	0.422
G3PDH/HSP60/RPB2/NEP1/NEP2	37	4519	807	2	1735	0.588	0.851	0.412

Dataset	Maximum likelihood							
	Subst. model [f]	NST [g]	Rate matrix					Rates
ITS	HKY + I	2						equal
G3PDH	TrNef + I + G	6	1.0000	1.9179	1.0000	1.0000	9.8933	gamma
HSP60	SYM + I + G	6	1.0661	3.5088	1.2085	0.4296	8.8406	gamma
RPB2	GTR + I + G	6	1.6458	4.4648	0.8771	0.4427	10.8437	gamma
NEP1	SYM + G	6	2.5059	6.6551	2.6626	1.9389	10.6922	gamma
NEP2	TIM1 + G	6	1.0000	3.0042	0.7049	0.7049	4.0569	gamma
G3PDH/HSP60/RPB2	GTR + I + G	6	1.2815	3.7009	1.0442	0.6173	10.1654	gamma
NEP1/NEP2	TIM2 + G	6	1.3205	4.0609	1.3205	1.0000	5.4934	gamma
G3PDH/HSP60/RPB2/NEP1/NEP2	TIM2 + I + G	6	1.3409	4.0324	1.3409	1.0000	7.6628	gamma

[a] bp, base pairs.

[b] PIC, number of parsimony informative characters.

[c] CI, consistency index.

[d] RI, retention index.

[e] HI, homoplasy index.

[f] Subst. model, best fit substitution model.

[g] NST, number of substitution rate categories

isolates were subjected to BLAST searches using the NCBI nucleotide database. Among the four Chinese isolates, differences were not observed among the *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* sequences, although a single nucleotide polymorphism was observed in the ITS sequences (genotype 1: CERC7160 and CERC7163; genotype 2: CERC7170 and CERC7208). The PHT comparing the *G3PDH*/*HSP60*/*RPB2*, *NEP1*/*NEP2*, *G3PDH*/*HSP60*/*RPB2*/*NEP1*/*NEP2* gene datasets generated the *P* values as 0.250, 0.333, 0.250, respectively, which indicated that no significant conflict existed between these datasets. These datasets were consequently combined and subjected to phylogenetic analyses. The sequence alignments for ITS (52 taxa, 450 characters), *G3PDH* (76 taxa, 887 characters), *HSP60* (82 taxa, 980 characters), *RPB2* (72 taxa, 1095 characters), *NEP1* (52 taxa, 742 characters), *NEP2* (53 taxa, 802 characters), and the combined *G3PDH*/*HSP60*/*RPB2* (72 taxa, 2960 characters), *NEP1*/*NEP2* (39 taxa, 1579 characters), *G3PDH*/*HSP60*/*RPB2*/*NEP1*/*NEP2* (37 taxa, 4519 characters) were deposited in TreeBASE (TreeBASE No. 18759).

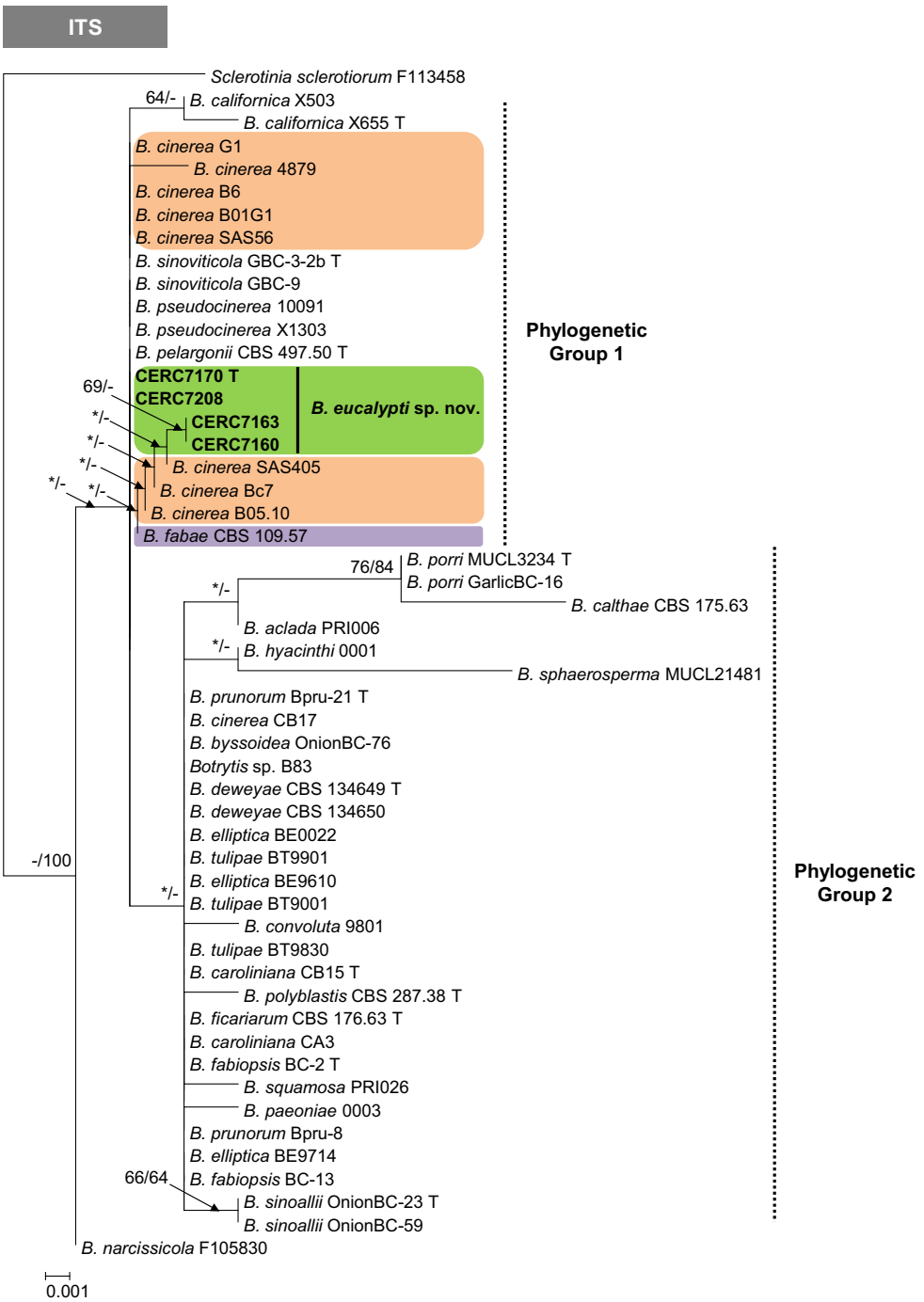

Fig. 1 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of DNA sequence data of the ITS region. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. *Sclerotinia sclerotiorum* (isolate No. F113458) was the outgroup

Phylogenetic analyses of individual ITS, *G3PDH*, *HSP60*, *RPB2*, *NEP1*, *NEP2*, and combined sequence datasets were conducted using both MP and ML methods. The number of parsimony informative

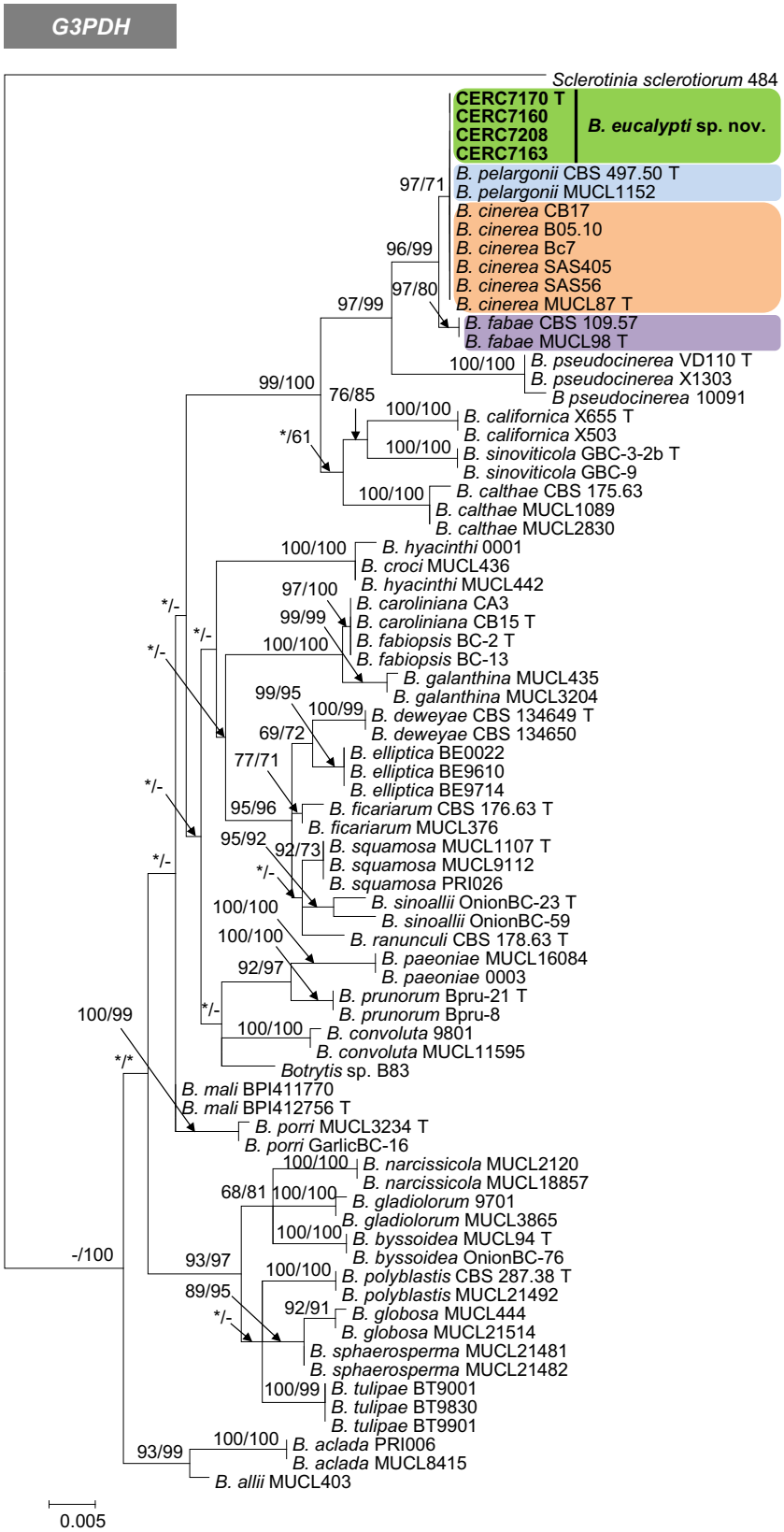

Fig. 2 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of *G3PDH* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. *Sclerotinia sclerotiorum* (isolate No. 484) was the outgroup

characters, the statistical values for phylogenetic trees of MP analyses, and the parameters for best-fit substitution models of ML analyses are shown in Table 2. Although the relative positions of individual *Botrytis* species differed slightly between MP and ML trees, the overall topologies were similar.

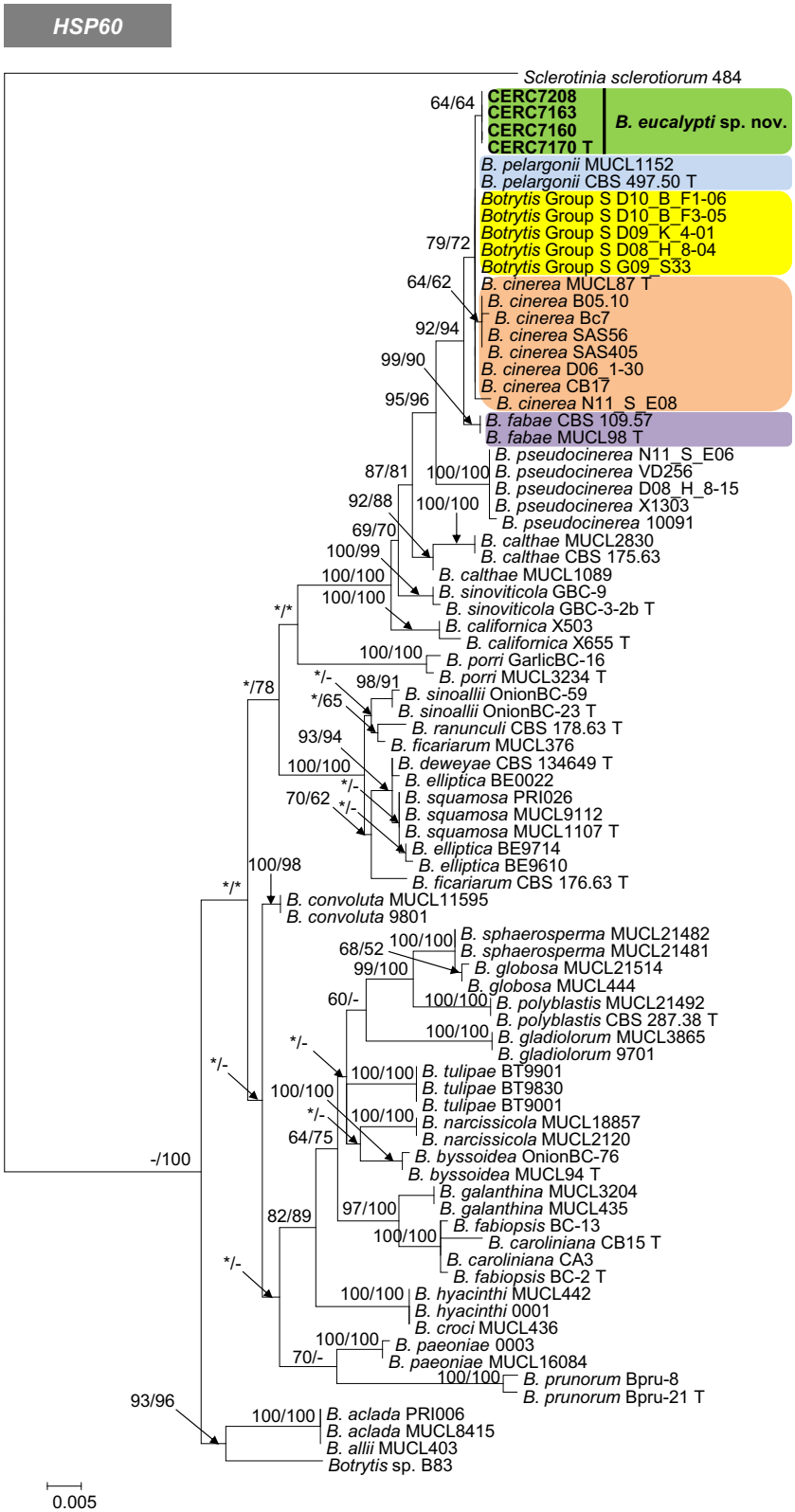

Fig. 3 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of *HSP60* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. *Sclerotinia sclerotiorum* (isolate No. 484) was the outgroup

In the tree based on ITS sequences (Fig. 1), the *Botrytis* species were separated into two main phylogenetic groups (Fig. 1), strains of some *Botrytis* species were grouped in the same phylogenetic clade in each of the two phylogenetic groups. For example, in Phylogenetic Group 1, *B. sinoviticola* (ex-type strain GBC-3-2b) and *B. pelargonii* (ex-type strain CBS 497.50) were grouped together, in Phylogenetic Group 2, *B. fabiopsis* (ex-type strain BC-2), *B. ficariarum* (ex-type

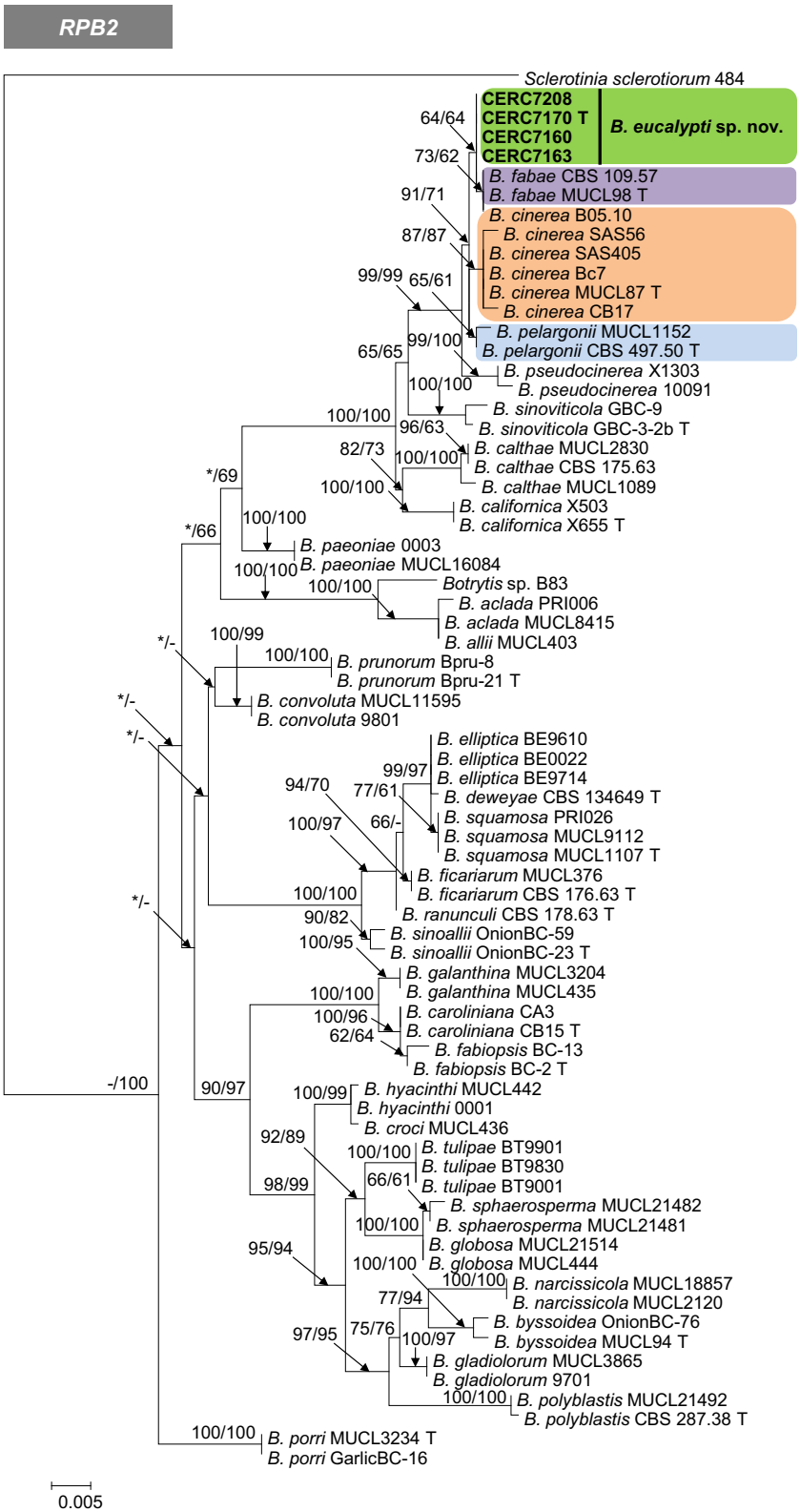

Fig. 4 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of *RPB2* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. *Sclerotinia sclerotiorum* (isolate No. 484) was the outgroup

strain CBS 176.63), *B. caroliniana* (ex-type strain CB15), and *B. deweyae* (ex-type strain CBS 134649) were in the same clade (Fig. 1). The four Chinese *Botrytis* isolates collected in this study from *Eucalyptus* seedlings reside in Phylogenetic Group 1, and were subdivided into two phylogenetic clades (clade 1: CERC7170, CERC7208; clade 2: CERC7160, CERC7163) (Fig. 1). Similar to other *Botrytis* isolates collected from *Eucalyptus* species in other countries (Brazil: isolates B01G1 and B6; Mexico: isolate G1; Spain: isolate 4879), which were also grouped in Phylogenetic Group 1, the ITS sequences alone did not provide sufficient resolution to distinguish different *Botrytis* species (Fig. 1).

In the trees based on *G3PDH* sequences (Fig. 2), *B. cinerea*, *B. pelargonii* and the four Chinese isolates (CERC7160, CERC7163, CERC7170, and CERC7208) were indistinguishable. In the *HSP60* tree, the Chinese isolates were grouped in one separate phylogenetic clade, closely related to *B. pelargonii*, *B. cinerea* and "Botrytis Group S", a novel clade in Leroch et al. (2013) (Fig. 3). In the *RPB2* tree, the Chinese isolates were closest to *B. fabae*, but formed a single

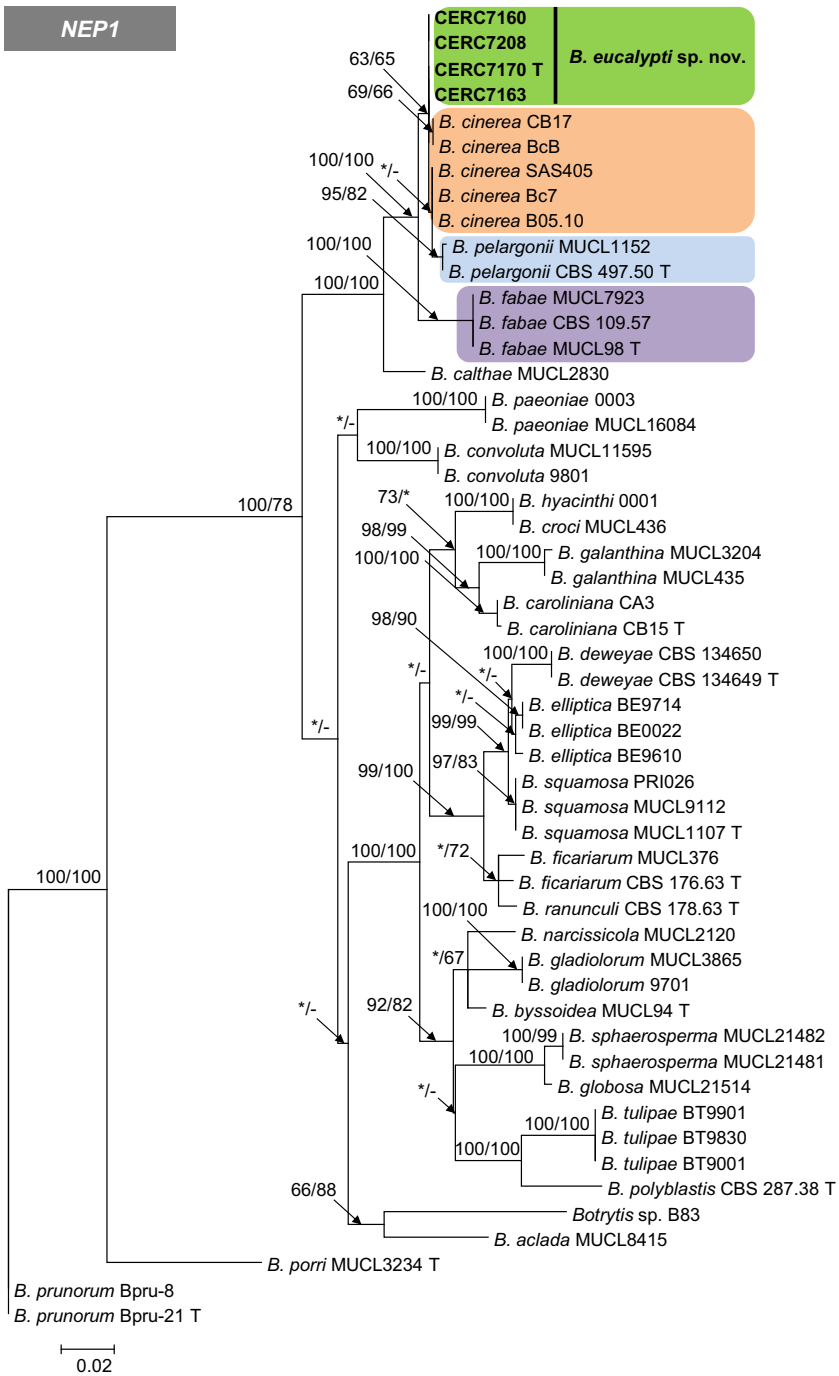

Fig. 5 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of *NEP1* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. The tree was un-rooted

separate phylogenetic clade (Fig. 4). In the *NEP1* tree, the Chinese isolates were separated from other phylogenetically closely related species, including *B. cinerea* and *B. pelargonii* (Fig. 5). In the *NEP2* tree, the four Chinese isolates and six isolates of "Botrytis Group S" (Leroch et al. 2013) formed one group which is phylogenetically different from *B. cinerea* and *B. pelargonii*, this is supported by bootstrap values of 85 %/83 % (ML/MP), and the four Chinese isolates formed a clade separated from the six isolates of "Botrytis Group S" (Fig. 6).

Phylogenetic analyses of each of the combined datasets of *G3PDH*/*HSP60*/*RPB2*, *NEP1*/*NEP2*, and *G3PDH*/*HSP60*/*RPB2*/*NEP1*/*NEP2* indicated that the Chinese isolates were more closely related to *B. cinerea* and *B. pelargonii* than *B. fabae* (Figs. 7, 8, and 9), and formed an independent clade distinct from *B. cinerea*, *B. fabae* and *B. pelargonii* (ML/MP bootstrap values: *G3PDH*/*HSP60*/*RPB2*, 82 %/62 %; *NEP1*/*NEP2*, 100 %/98 %; *G3PDH*/*HSP60*/*RPB2*/*NEP1*/*NEP2*, 100 %/99 %).

In the individual *HSP60*, *RPB2*, *NEP1*, and *NEP2* trees and in the trees based on combined sequences of *G3PDH*/*HSP60*/*RPB2*, *NEP1*/*NEP2*, and *G3PDH*/*HSP60*/*RPB2*/*NEP1*/*NEP2*, the four Chinese isolates clustered together and formed an independent clade distinct from other previously described *Botrytis* species. The results of these phylogenetic analyses indicate that the Chinese isolates represent a distinct, undescribed species.

The phylogenetic analyses indicated that the Chinese isolates were most closely related to *B. cinerea* and *B. pelargonii*, while they could be distinguished from *B. cinerea* and *B. pelargonii* using single nucleotide polymorphism (SNP) analyses for each of *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene regions sequenced (Tables 3 and 4). The Chinese group could be separated from *B. cinerea* and *B. pelargonii* by nine and 12 unique SNPs for the four gene regions (Tables 3 and 4) .

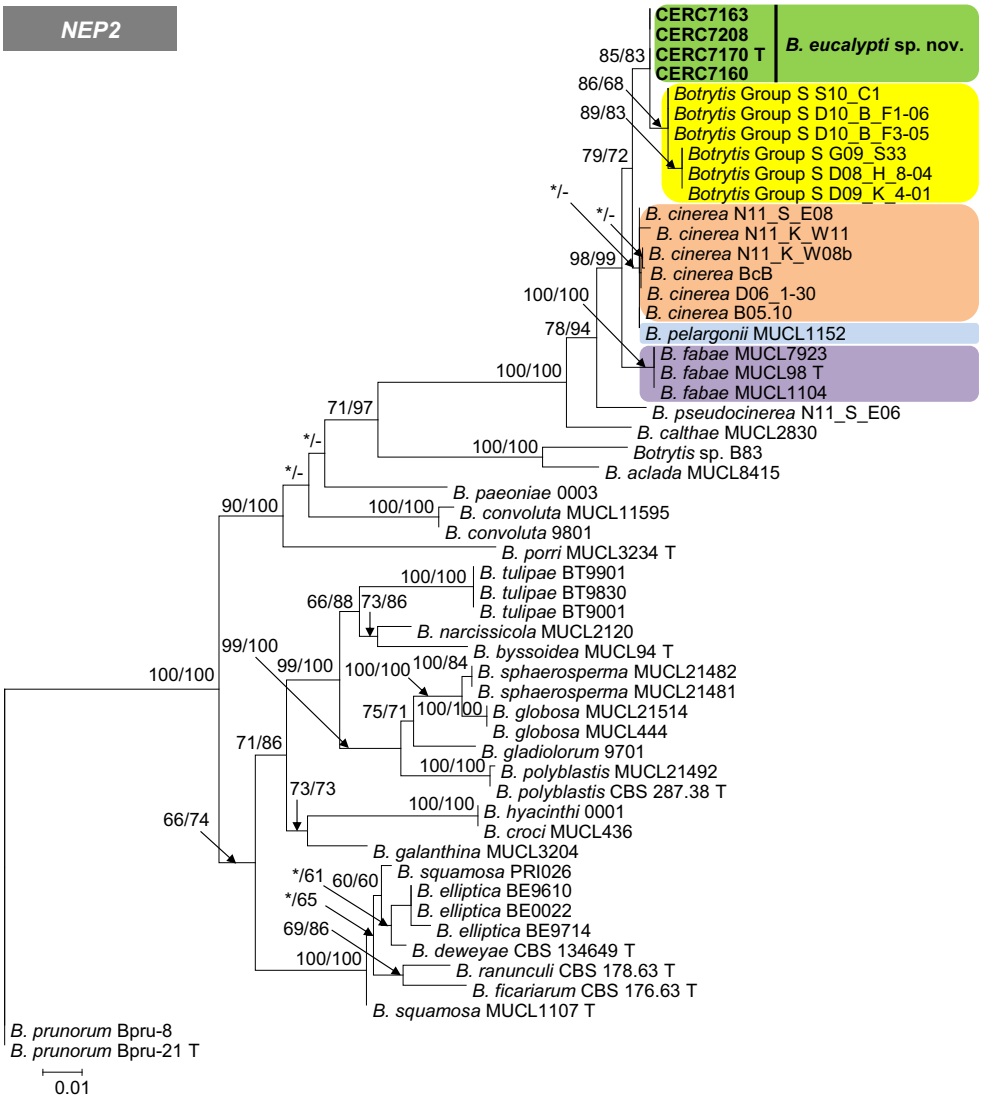

Fig. 6 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of *NEP2* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. The tree was un-rooted

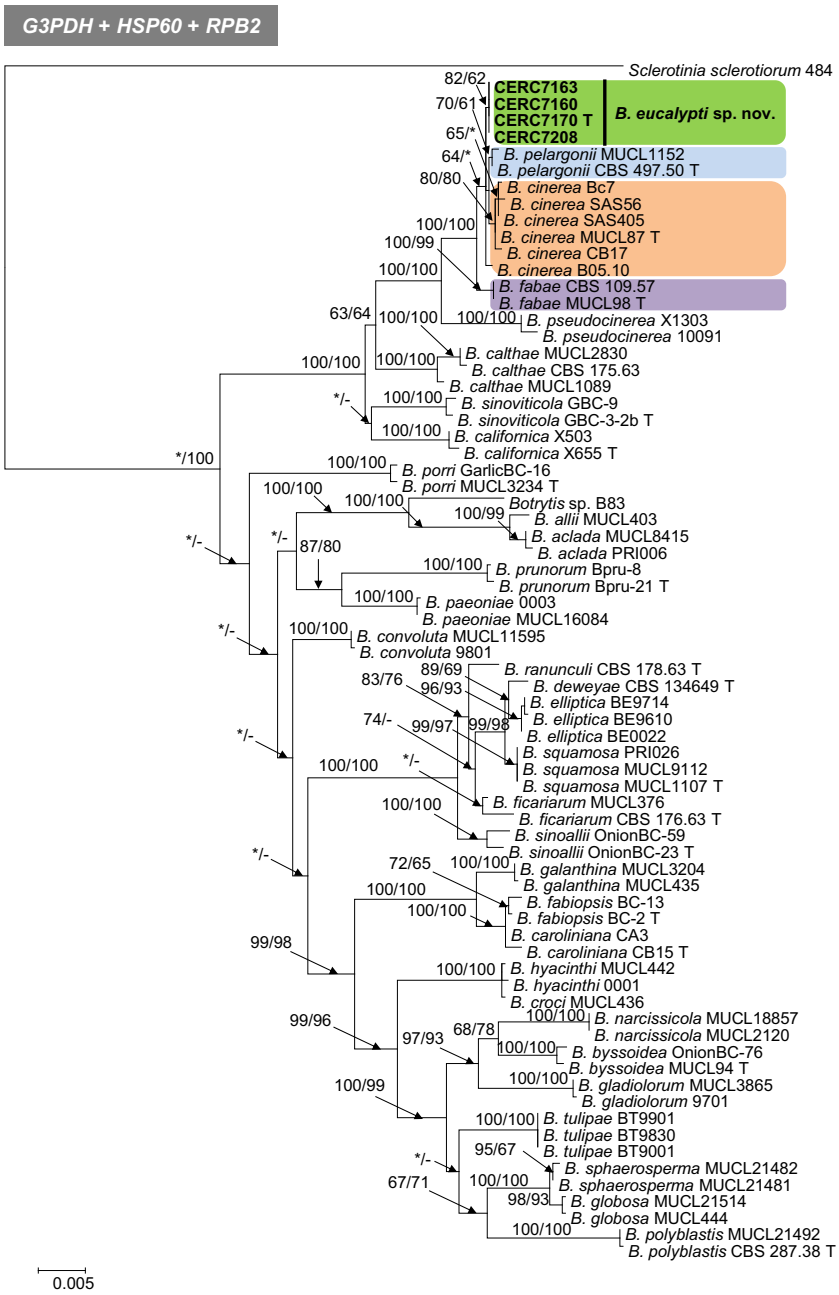

Fig. 7 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of combination of *G3PDH*, *HSP60*, and *RPB2* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. *Sclerotinia sclerotiorum* (isolate No. 484) was the outgroup

Taxonomy

The four Chinese *Botrytis* isolates collected from *E. urophylla* × *E. grandis* seedlings produced sclerotia on MEA, but not on PDA and formed conidiophores and conidia on MEA and WA medium within 2 weeks. Sexual structures were not observed.

The size of sclerotia, conidiophores and conidia were used to distinguish different species of *Botrytis*. The morphological characteristics of sclerotia, conidiophores and conidia were very similar between Chinese *Botrytis* species and other phylogenetically closely related species that have been characterized previously, including *B. cinerea* and *B. pelargonii*, while the conidia of Chinese *Botrytis* species (up to 8.5 μm) are narrower than that of *B. cinerea* (up to 12.0 μm) and wider than *B. pelargonii* (up to 6.5 μm) (Table 5).

Based on the phylogenetic analyses and morphological characterization, the fungus isolated from diseased *Eucalyptus* seedlings was identified as an undescribed species of *Botrytis*. The new species is described as follows:

Botrytis eucalypti Q.L. Liu & S.F. Chen, sp. nov. (Fig. 10)
MycoBank MB817103

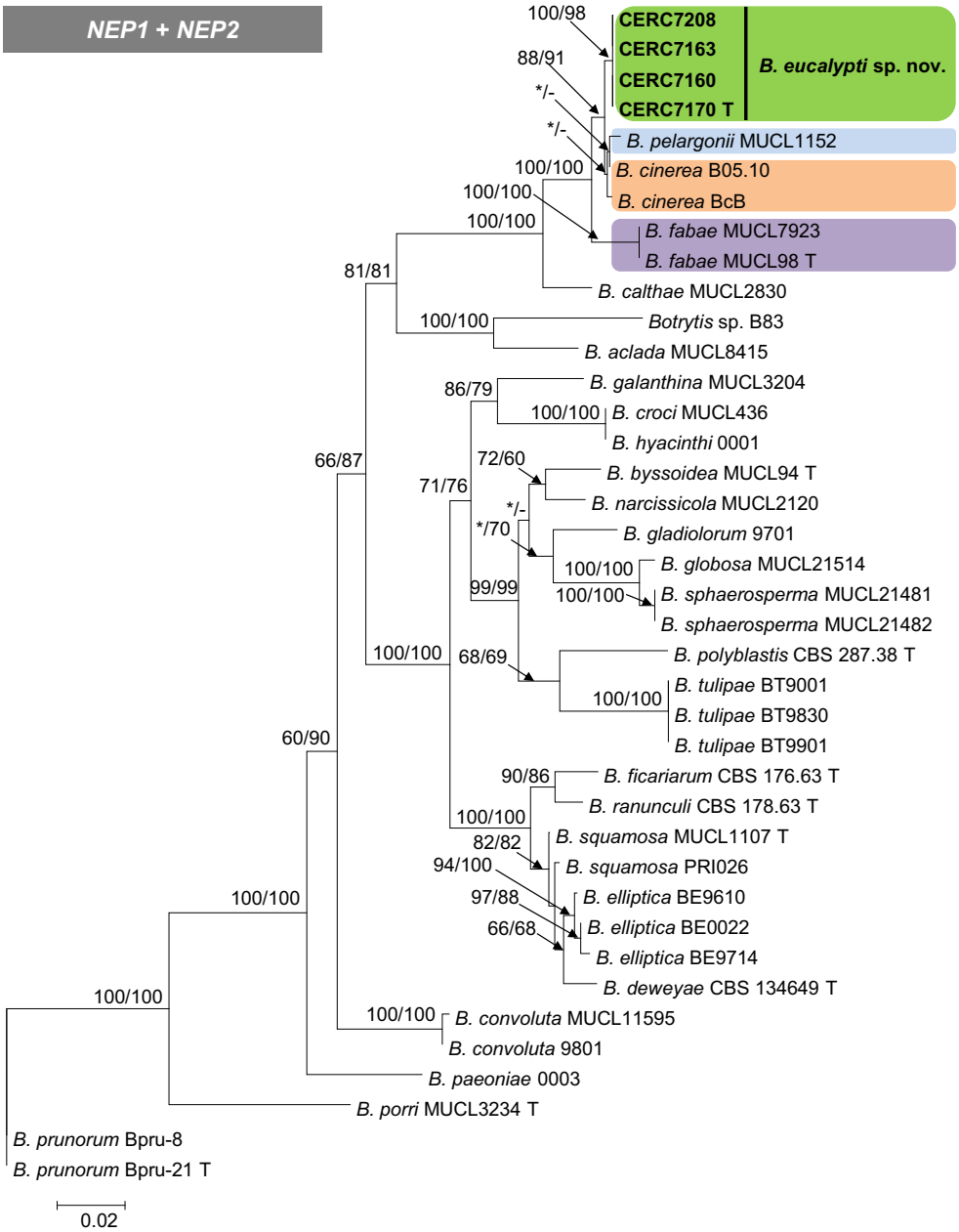

Fig. 8 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of combination of *NEP1* and *NEP2* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. The tree was un-rooted

Etymology: "*eucalypti*" refers to the plant genus *Eucalyptus*, the host from which this fungus was first isolated.

Description: On MEA and PDA at 10, 15, 20, 25, and 30 °C, the mycelial growth rate and colony morphology is similar, no growth at 5 or 35 °C. On MEA at 20 °C in the dark, after 3 days colony surface white, mycelium fluffy (Fig. 10a), after 12 days mycelium became buff brown (Fig. 10b). On PDA, no sclerotia were produced, on MEA at 20 °C, **sclerotia** formed on the surface of the medium after 12 days, small, grey to black, irregular to spherical, scattered or aggregated (Fig. 10c and d), (0.9–)1.0–1.5(–1.9) × (0.8–)0.9–1.4(–1.8) mm. Sclerotia could also formed on the surface of detached *Eucalyptus* leaf tissues on WA (inoculated with *B. eucalypti*). Conidiophores, conidiogenous cells and conidia were produced on MEA and *Eucalyptus* leaves on WA within 2 weeks (Fig. 10e and f), but not on PDA media. Conidiophores, conidiogenous cells and conidia formed on MEA and WA were similar. **Conidiophores** erect, septate, branched at the top, brown, (558–)894–1574(–1887) × (11–)13–15(–17) µm. **Conidiogenous cells** inflated and swollen at the apex, (8–)9–11(–13) × (8–)10–14(–16) µm (average 10.3 × 11.7 µm). **Conidia** three dimensional, botryoidal, obovate to elliptical, unicellular, hyaline to pale brown, (6.5–)8.0–11.0(–12.5) × (4.0–)5.5–7.0(–8.5) µm (average 9.6 × 6.3 µm; length/width = 1.5) (Fig. 10i, Table 5).

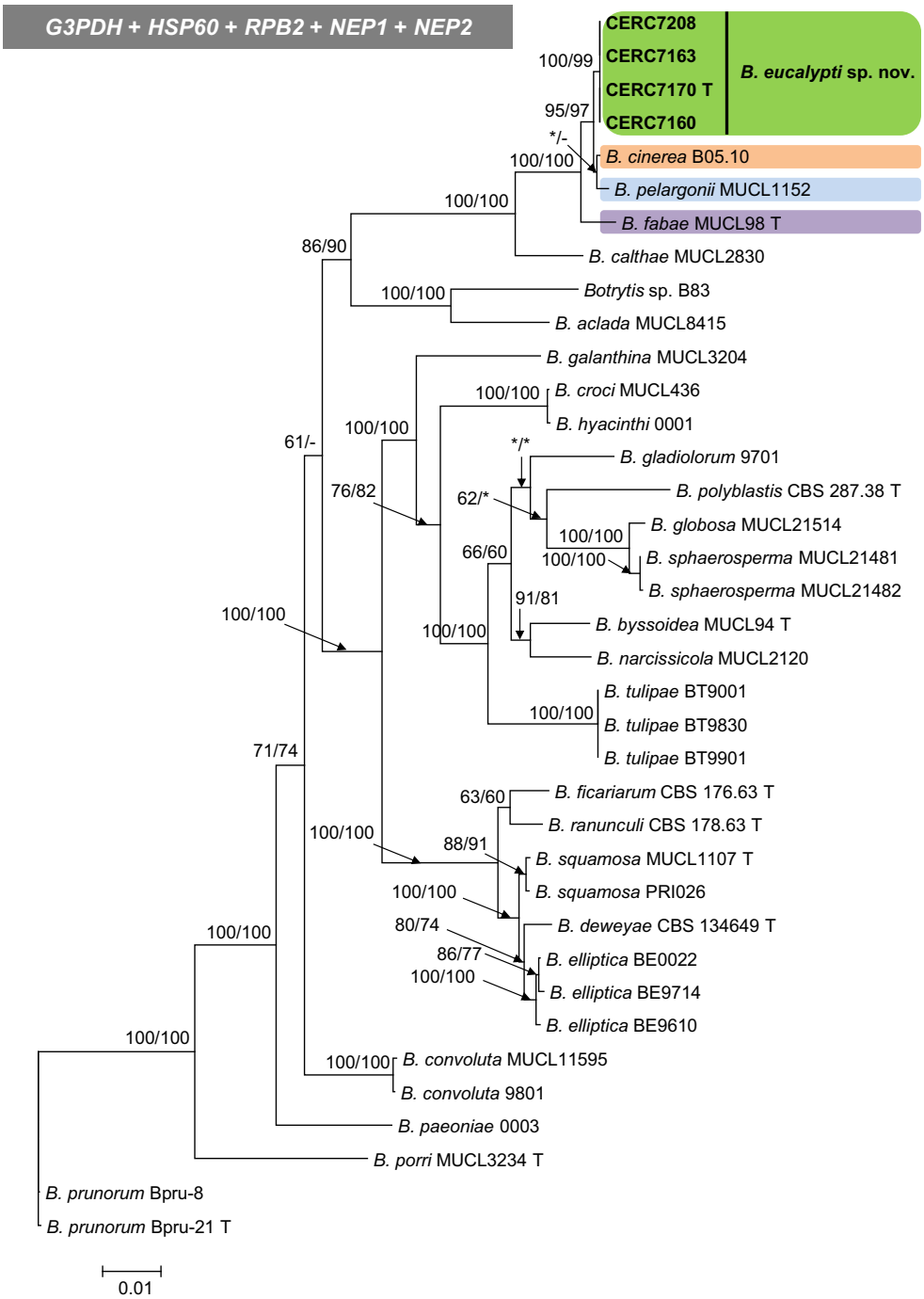

Fig. 9 Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of combination of *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene sequences. Isolates highlighted in bold were sequenced in this study. ML and maximum parsimony (MP) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. T refers to ex-type isolates. The tree was un-rooted

Growth characteristics: Colonies on MEA after 48 h at 10, 15, 20, 25, and 30 °C reaching 25, 26, 56, 49, and 12 mm, respectively. Optimal growth at 20 °C. No growth at 5, 35, or 40 °C.

Sexual morph: Not observed.

Habitat: Leaves and stems of *Eucalyptus* seedlings in nurseries.

Host and distribution: *Eucalyptus urophylla* × *E. grandis*, GuangDong Province, China.

Typification: CHINA. GuangDong Province, ZhanJiang Region, SuiXi County, LingBei Town, South China Experiment Nursery, 21°16′0″N, 110°05′14″E, on leaves of a *E. urophylla* × *E. grandis* seedling, 2 March 2015, S.F. Chen & Q.L. Liu, a *Eucalyptus* leaf sample on the surface of WA medium inoculated with isolate CERC7170 was dried and deposited as Herb. CSFF2013 (HOLOTYPE), culture EX-TYPE CERC7170 = CFCC51324 = CGMCC3.18028.

Table 3 Summary of polymorphic nucleotides found within the *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene regions generated for the phylogenetic groups of *B. eucalypti*, *B. cinerea*, and *B. pelargonii*

Species	Isolate number	*HSP60* [a]			*RPB2*									
		54 [b]	184	429	5	84	249	282	468	741	855	981	1086	1087
B. eucalypti	CERC7170 [c]	G	*T* [d]	C	T	T	T	A	C	G	T	C	*C*	T
	CERC7160	G	*T*	C	T	T	T	A	C	G	T	C	*C*	T
	CERC7163	G	*T*	C	T	T	T	A	C	G	T	C	*C*	T
	CERC7208	G	*T*	C	T	T	T	A	C	G	T	C	*C*	T
B. cinerea	MUCL87 [c]	G	C	C	T	T	T	*G*	C	G	T	*T*	A	T
	SAS405	A	C	C	T	T	T	*G*	C	G	T	*T*	A	T
	SAS56	A	C	C	T	T	C	*G*	C	A	T	*T*	A	T
	Bc7	A	C	T	T	T	T	*G*	C	G	T	*T*	A	T
	CB17	G	C	C	T	T	T	*G*	T	G	G	*T*	A	T
B. pelargonii	CBS 497.50 [c]	G	C	C	T	*C*	T	A	C	G	T	C	A	T
	MUCL1152	G	C	C	C	*C*	T	A	C	G	T	C	A	G

Speices	Isolate number	*NEP1*							*NEP2*					
		173	189	190	228	432	459	459	108	235	305	316	317	377
B. eucalypti	CERC7170 [c]	A	G	T	T	C	C	C	C	*C*	*C*	*A*	*A*	*T*
	CERC7160	A	G	T	T	C	C	C	C	*C*	*C*	*A*	*A*	*T*
	CERC7163	A	G	T	T	C	C	C	C	*C*	*C*	*A*	*A*	*T*
	CERC7208	A	G	T	T	C	C	C	C	*C*	*C*	*A*	*A*	*T*
B. cinerea	MUCL87 [c]	N/A [e]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	B05.10	A	G	T	T	T	C	C	C	T	T	G	T	C
	SAS405	A	G	T	T	T	C	C	N/A	N/A	N/A	N/A	N/A	N/A
	Bc7	A	G	T	T	T	C	C	N/A	N/A	N/A	N/A	N/A	N/A
	BcB	A	G	T	T	C	T	T	T	T	T	G	T	C
	CB17	A	G	T	T	C	T	T	N/A	N/A	N/A	N/A	N/A	N/A
B. pelargonii	CBS 497.50 [c]	A	*A*	*C*	*C*	T	C	C	N/A	N/A	N/A	N/A	N/A	N/A
	MUCL1152	G	*A*	*C*	*C*	T	C	C	C	T	T	G	T	C

[a] Only polymorphic nucleotides occurring in all of the isolates are shown.

[b] Numerical positions of the nucleotides in the DNA sequence alignments are indicated.

[c] Isolates are ex-type.

[d] Fixed polymorphisms for each group are highlighted in italics.

[e] N/A = Not Available

Additional specimens: CHINA. GuangDong Province, ZhanJiang Region, SuiXi County, LingBei Town, South China Experiment Nursery, 21°16′0″N, 110°05′14″E, on leaves of a *E. urophylla* × *E. grandis* seedling, 27 February 2015, S.F. Chen & Q.L. Liu, a *Eucalyptus* leaf sample on the surface of WA medium inoculated with isolate CERC7160 was dried and deposited as Herb. CSFF2014, culture CERC7160 = CFCC51322, culture CERC7163 = CFCC51323; CHINA. GuangDong Province, ZhanJiang Region, SuiXi County, LingBei Town, South China Experiment Nursery, 21°16′03″N, 110°05′14″E, on leaves of a *E. urophylla* × *E. grandis* seedling, 6 March 2015, S.F. Chen & Q.L. Liu, a *Eucalyptus* leaf sample on the surface of WA medium inoculated with isolate CERC7208 was dried and deposited as Herb. CSFF2015, culture CERC7208 = CFCC51325 = CGMCC3.18029.

Table 4 Number of unique alleles in *B. eucalypti*, *B. cinerea*, and *B. pelargonii*

G3PDH/HSP60/RPB2/NEP1/NEP2 [a]	*B. cinerea*	*B. pelargonii*
B. eucalypti	9 (0/1/3/0/5)	12 (0/1/2/4/5)
B. cinerea		6 (0/0/3/3/0)

[a] The order of the five genes: total numbers (*G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2*)

Notes: *Botrytis eucalypti* is phylogenetically closely related to *B. cinerea* and *B. pelargonii*. However, morphological differences exist among these three species, namely the conidia of *B. eucalypti* (width up to 8.5 μm)

Table 5 Morphological characteristics of *B. eucalypti*, *B. cinerea*, and *B. pelargonii*

Species [a]	Sclerotia [b]				Conidiophores [c]	Conidia [c]				Reference
	Color	Shape	(l × w) Size (mm) [d]	No. per dish	(l × w) Size (μm)	Shape	(l × w) Size (μm)	Mean (μm)	l/w [e]	
B. eucalypti	Black	Irregular, Oblong/Elliptical, Spherical	(0.9–)1.0–1.5(–1.9) × (0.8–)0.9–1.4(–1.8)	98–121	(558–)894–1574(–1887) × (11–)13–15(–17)	Elliptical, Ovoid	(6.5–)8.0–11.0(–12.5) × (4.0–)5.5–7.0(–8.5)	9.6 × 6.3	1.53	This study
B. cinerea	Black	Irregular, Oblong/Elliptical, Spherical	0.7–27.5 × 0.5–12	34–51	712–1745 × 10–17	Elliptical, Ovoid	7.7–12.9 × 6.6–10.5	N/A [f]	N/A	Zhang et al. 2010a
B. cinerea	N/A	Irregular, Spherical	1.0–12.9 × 0.7–6.1	57–111	N/A	N/A	7–14 × 6–13	N/A	1.28	Zhang et al. 2010b
B. cinerea	Black	N/A	N/A	N/A	741–3925 (length)	N/A	N/A	N/A	N/A	Li et al. 2012
B. cinerea	Black	Irregular, Oblong/Elliptical, Spherical	1.0–4.5 × 1.0–4.0	45–54	1033–2178 × 8–17	Elliptical, Ovoid	7.0–15.7 × 6.6–11.9	N/A	1.36	Zhou et al. 2014
B. cinerea	N/A	N/A	3.9–6.9 × 3.3–5.4	N/A	464–2555 × 7.3–17.7	N/A	7.4–12.8 × 5.2–10.0	9.6 × 7.3	1.27–1.33	Ferrada et al. 2016
B. cinerea	N/A	N/A	0.9–6.1 × 0.9–4.0	67–184	397–2943 × 8–17	N/A	5.6–15.8 × 4.5–9.8	9 × 6.4	1.32–1.48	Saito et al. 2016
B. cinerea	N/A	Irregular, Oblong/Elliptical, Spherical	0.8–9.5 × 0.8–7.0	N/A	N/A	Elliptical, Ovoid	6.3–13.0 × 5.0–10.5	N/A	N/A	Zhang et al. 2016
B. pelargonii	N/A	Hemispherical	2.5–4.8 × 1.9–2.4	N/A	N/A	Elliptical, Ovoid	9.9–11.0 × 6.0–6.4	N/A	N/A	Zhang et al. 2016

[a] Morphological data of *B. cinerea* and *B. pelargonii* from recently published articles.

[b] Culture incubated at 20 °C for 12 days to record the color, shape, size and number per dish of sclerotia produced on PDA or MEA.

[c] Culture grown at 20 °C after 12 days to calculate the size of conidiophores and conidia produced on PDA, MEA, or WA.

[d] l × w, length × width, [minimum – (average – standard deviation)] – [(average + standard deviation) – maximum] or minimum – maximum.

[e] l/w, average length/average width.

[f] N/A = Not Available

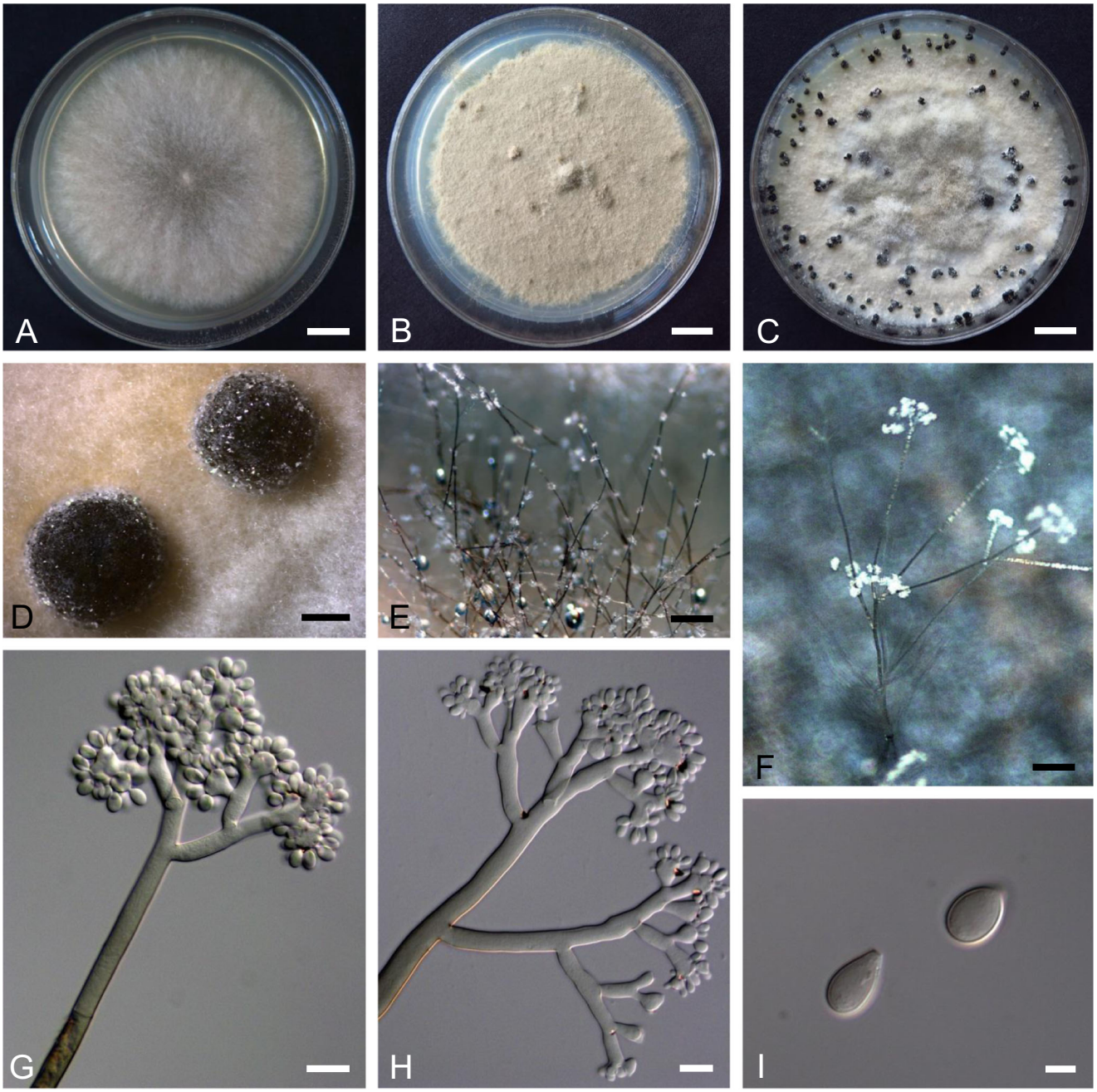

Fig. 10 Morphological characteristics of *Botrytis eucalypti*. A. Colony of *B. eucalypti* on MEA after 3 days at 20 °C; B, C. Colony of *B. eucalypti* on MEA after 12 days at 25 °C and 20 °C, respectively; D. Sclerotia produced on the medium; E, F. Sporulation of *B. eucalypti* on the medium and a *Eucalyptus* leaf at 20 °C, respectively; G, H. Conidiophores; I. Conidia. Bars: A, B, C = 1 cm; D, E = 500 μm; F = 200 μm; G, H = 20 μm; I = 5 μm

are wider than *B. pelargonii* (width up to 6.5 μm), but narrower than *B. cinerea* (width up to 12 μm) (Table 5). *Botrytis eucalypti* differed from *B. cinerea* in culture mycelial growth rate (the growth information of *B. pelargonii* is not available), on PDA at 5 °C, *B. cinerea* grew more than 3 mm/d, but *B. eucalypti* did not grow; on PDA at 10, 15, and 25 °C, *B. eucalypti* grew slower than *B. cinerea* (Fig. 11).

Pathogenicity tests

All four tested *B. eucalypti* isolates (CERC7160, CERC7163, CERC7170, and CERC7208) produced lesions on detached leaves collected from three *Eucalyptus* clones after 1 week, while no disease symptoms and only wounds were observed on leaves treated with the negative controls (Fig. 12). Mean comparison tests showed that the lesions produced by the

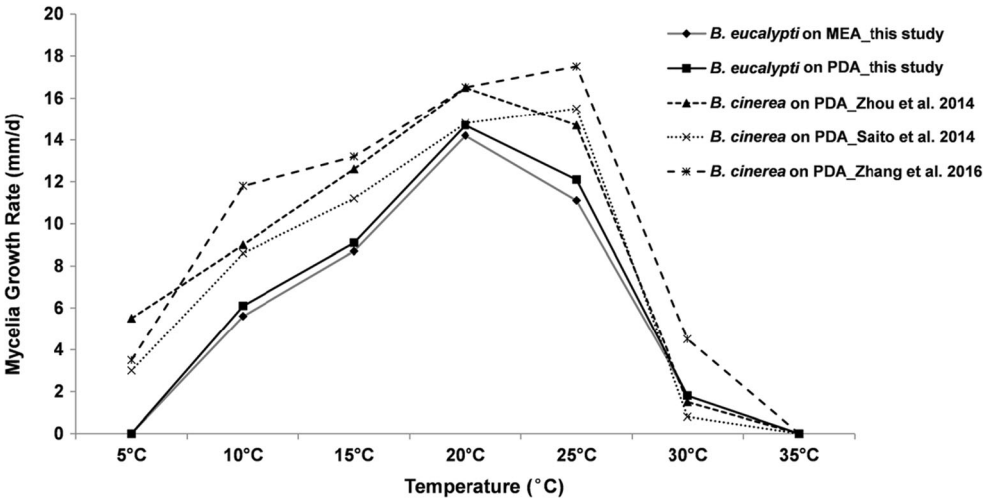

Fig. 11 Effect of temperature on mycelial radial growth of two species, *Botrytis eucalypti* (on MEA and PDA) and *B. cinerea* (on PDA) in the dark. Each value represents the average of 5 replicates

tested isolates were all significantly longer ($P < 0.05$) than the wounds caused by the negative controls (Fig. 13). An ANOVA revealed significant differences among the three *Eucalyptus* clones in susceptibility to the tested isolates ($P < 0.05$). Not all the *B. eucalypti* isolates reacted in the same manner to the tested *Eucalyptus* clones. For example, lesions produced by isolate CERC7160 on *Eucalyptus* clones CEPT-11 and CEPT-12 were significantly longer than those on CEPT-13, whereas lesions produced by isolate CERC7163 on CEPT-11 and CEPT-12 were significantly shorter than those on CEPT-13 (Fig. 13). Lesions produced by isolates CERC7170 and CERC7208 on *Eucalyptus* clones CEPT-11 were significantly longer than those on CEPT-13 and CEPT-12, and lesions produced by CERC7208 on three *Eucalyptus* clones are significantly longer than CERC7170. The inoculated isolates were re-isolated from the lesions, while no *Botrytis* species was isolated from the negative controls.

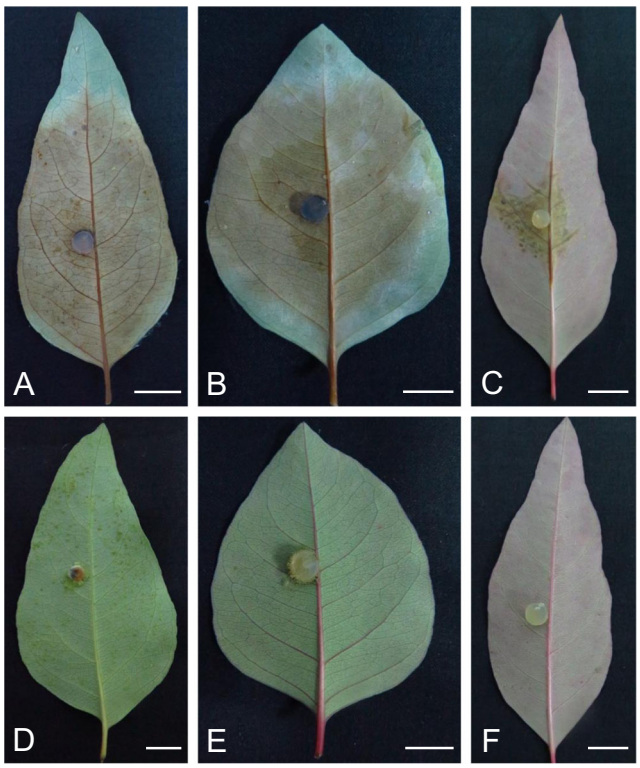

Fig. 12 Symptoms of *Botrytis eucalypti* (CERC7160) inoculated on detached leaves of three *Eucalyptus* clones compared with responses of negative controls. A–C. Lesions produced by isolate CERC7160 on leaves of *Eucalyptus* clones CEPT-11, CEPT-12, and CEPT-13, respectively. D–F. Respective negative controls of *Eucalyptus* clones CEPT-11, CEPT-12, and CEPT-13 were marked by the absence of lesions. Bars: A–F = 10 mm

Discussion

In this study, a previously undescribed species of *Botrytis* was identified and described. This species was isolated from

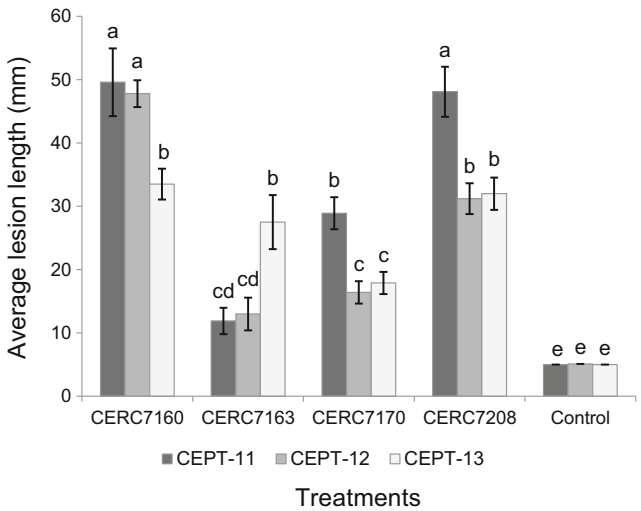

Fig. 13 Column chart showing average lesion lengths (mm) resulting from inoculation trials on detached leaves of three *Eucalyptus* clones (CEPT-11, CEPT-12, and CEPT-13). Four isolates of *B. eucalypti* were used. Vertical bars represent the standard error of means. Different letters above the bars indicate treatments that were statistically significantly different ($P = 0.05$)

diseased leaves of *Eucalyptus* seedlings in South China and named *B. eucalypti*. Identification of this fungus as a novel species is supported by phylogenetic analyses, morphological characteristics, and culture growth rates. Pathogenicity tests demonstrated that *B. eucalypti* is pathogenic to all three tested *Eucalyptus grandis* clones.

DNA sequence comparison of *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene regions has recently become an effective and increasingly important method for species identification of *Botrytis* (Zhang et al. 2010a; Li et al. 2012; Khan et al. 2013; Leroch et al. 2013; Grant-Downton et al, 2014; Lorenzini and Zapparoli 2014; Zhou et al. 2014; Ferrada et al. 2016; Saito et al. 2016). In this study, the Chinese *Botrytis* isolates were identified as a new species, the conclusions of which are supported by phylogenetic analyses of each of *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene sequences, as well as by the combined sequences of *G3PDH*/*HSP60*/*RPB2*, *NEP1*/*NEP2*, and *G3PDH*/*HSP60*/*RPB2*/*NEP1*/*NEP2*. Consistent with previous findings, ITS sequences could not provide sufficient resolution for the identification and differentiation of *Botrytis* species (Zhang et al. 2010a; Li et al. 2012; Ferrada et al. 2016; Saito et al. 2016). Morphological differences of conidial size were also observed among *B. eucalypti* and its phylogenetically closest relatives, including *B. cinerea* and *B. pelargonii* (Zhang et al. 2010a, b, 2016; Li et al. 2012; Zhou et al. 2014; Ferrada et al. 2016; Saito et al. 2016).

Except for *B. cinerea*, which infects over 200 eudicot hosts in various families (MacFarlane 1968; Williamson et al. 2007), and *B. fabae*, which can infect some species in genera *Lens*, *Phaseolus*, *Pisum*, and *Vicia* of the Fabaceae (Jarvis 1977; Zhang 2006), most species of *Botrytis* are considered specialists with a narrow host range, infecting only one or a few closely related species within the same plant genus (Mansfield 1980; Staats et al. 2005; Elad et al. 2016b). In regard to the three species phylogenetically closest to *B. eucalypti*, isolates of *B. cinerea* and *B. fabae* have been obtained from hosts in multiple genera, whereas *B. pelargonii* has only been isolated from *Pelargonium* species (Zhang 2006; Saito et al. 2016).

Prior to this study, the only *Botrytis* species reported to infect *Eucalyptus* has been *B. cinerea* (Old et al. 2003; Pérez-Vera et al. 2005; Muñoz and Campos 2013; Sánchez Márquez et al. 2011; Sbravatti Júnior et al. 2013; Elad et al. 2016b). Grey mould caused by *B. cinerea* is one of the most severe diseases in *Eucalyptus* nurseries worldwide (Zaldúa and Sanfuentes 2010; Muñoz and Campos 2013; Caires et al. 2015). Before this study, only one species of *Botrytis*, *B. cinerea* was identified from *Eucalyptus* leaves solely based on morphological characteristics (Liao et al. 2013). In this study, *B. eucalypti* was discovered on naturally infecting *Eucalyptus* seedlings. Morphological differences exists between the Chinese *B. cinerea* and *B. eucalypti* isolated from *Eucalyptus*, for example, the conidia of *B. cinerea* (9.0–16.0 × 6.0–10.0 μm) are larger than that of *B. eucalypti* (6.6–12.7 × 4.2–8.4 μm) (Liao et al. 2013).

The pathogenicity tests conducted in this study revealed that *B. eucalypti* is pathogenic to all three tested *Eucalyptus* clones and it is capable of producing necrotic lesions on *Eucalyptus* leaves within a short time. This fungus has the potential to cause serious disease in *Eucalyptus* seedlings in nurseries and may be distributed to other nurseries in South China. Careful monitoring is needed to detect the spread of this fungus to other regions. Extensive epidemiological studies should be conducted to help control grey mould caused by *B. eucalypti* on *Eucalyptus* seedlings.

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