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New *Ceratocystis* species associated with rapid death of *Metrosideros polymorpha* in Hawai`i

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Key words

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Abstract The native `ōhi`a lehua (*Metrosideros polymorpha*) has cultural, biological and ecological significance to Hawai`i, but it is seriously threatened by a disease commonly referred to as rapid `ōhi`a death (ROD). Preliminary investigations showed that a *Ceratocystis* species similar to *C. fimbriata* s.lat. was the cause of the disease. In this study, we used a combination of the phylogenetic, morphological and biological species concepts, as well as pathogenicity tests and microsatellite analyses, to characterise isolates collected from diseased `ōhi`a trees across Hawai`i Island. Two distinct lineages, representing new species of *Ceratocystis*, were evident based on multigene phylogenetic analyses. These are described here as *C. lukuohia* and *C. huliohia*. *Ceratocystis lukuohia* forms part of the Latin American clade (LAC) and was most closely associated with isolates from *Syngonium* and *Xanthosoma* from the Caribbean and elsewhere, including Hawai`i, and *C. platani*, which is native to eastern USA. *Ceratocystis huliohia* resides in the Asian-Australian clade (AAC) and is most closely related to *C. uchidae*, *C. changhui* and *C. cercfabiensis*, which are thought to be native to Asia. Morphology and interfertility tests support the delineation of these two new species and pathogenicity tests show that both species are aggressive pathogens on seedlings of *M. polymorpha*. Characterisation of isolates using microsatellite markers suggest that both species are clonal and likely represent recently-introduced strains. Intensive research is underway to develop rapid screening protocols for early detection of the pathogens and management strategies in an attempt to prevent the spread of the pathogens to the other islands of Hawai`i, which are currently disease free.

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INTRODUCTION

The Ascomycete genus *Ceratocystis* (*Sordariomycetes*, *Hypocreomycetidae*, *Microascales*) is one of 11 clearly defined genera in the *Ceratocystidaceae* (De Beer et al. 2014, 2017, Mayers et al. 2015, Nel et al. 2017). *Ceratocystis*, as currently recognized, includes 36 species (Marin-Felix et al. 2017, Liu et al. 2018) that are characterized by their mostly black, globose ascomatal bases with long, elongated necks terminating in an ostiole, through which sticky hat-shaped ascospores exude (Upadhyay 1981, Seifert et al. 1993). Based on phylogenetic inference, the species reside in four broadly defined geographic clades. These include the Latin American clade (LAC) (Harrington 2000, Engelbrecht & Harrington 2005), the North American clade (NAC) (Johnson et al. 2005), the African clade (AFC) (Heath et al. 2009, Mbenoun et al. 2014) and the Asian-Australian clade (AAC) (Johnson et al. 2005, Thorpe et al. 2005, Li et al. 2017).

Diseases caused by *Ceratocystis* spp. include vascular wilts, cankers, as well as rot of various root crops (Kile 1993, Roux & Wingfield 2009). Of particular concern in recent years has been the emerging epidemics caused by aggressive *Ceratocystis* spp., particularly those residing in the LAC. For example, *C. manginecans*, that was introduced into Oman and Pakistan (Al Adawi et al. 2014) has caused devastating losses to the mango industry (Al Adawi et al. 2006, Van Wyk et al. 2007) and resulted in apparent host jumps to native *Prosopis cineraria* and *Dalbergia sissoo*, causing branch and stem cankers (Al Adawi et al. 2013). A pathogen of the same name has emerged in South East Asia, causing a severe canker and wilt disease of plantation grown *Acacia mangium* (Tarigan et al. 2011, Thu et al. 2014, Brawner et al. 2015). *Ceratocystis platani*, also residing in the LAC, is the causal agent of a devastating canker and wilt disease of *Platanus orientalis* in Europe, where it was introduced from the USA in the early 1940s (Engelbrecht et al. 2004, Tsopelas et al. 2017). A strain of *C. fimbriata*, the type species of the genus, is well known as the causal agent of post-harvest black rot of sweet potato (*Ipomoea batatas*) (Li et al. 2016, Scruggs et al. 2017).

A new disease that is rapidly assuming crisis status in Hawai`i has colloquially been termed rapid `ōhi`a death (ROD). Hundreds of thousands of native *M. polymorpha* have recently died on the Big Island of Hawai`i (Keith et al. 2015, Mortenson et al. 2016). Dying trees were first noticed in the Puna district of Hawai`i Island as early as 2010, and the disease has now spread across most of the Big Island. This iconic tree species, more commonly known as `ōhi`a lehua, is the most common and widespread native tree species of Hawai`i, occurring from sea level to 2 500 m elevation in both dry and wet forests and on substrates ranging from 50 to 4 million years in age (Friday & Herbert 2006, Loope 2016). It is the most ecologically important native tree in Hawai`i, defining native forest succession and ecosystem function over broad areas, providing critical habitat for rare and endangered native bird and insect species, and

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exemplifying the strong links between native Hawaiian culture and the islands' environment (Dawson & Stemmermann 1990). Extensive mortality of `ōhi`a associated with abiotic factors, known as `ōhi`a dieback, has been noted in the past (Hodges et al. 1986), but the recent mortality appears to be caused by a new, biotic factor. Trees of all ages, across all environmental conditions, in native forests and residential areas, are currently affected, with mortality ranging from 10–90 % in certain areas (F. Hughes, pers. comm.).

A characteristic feature of much of the current mortality is the rapid development of symptoms from the onset of the first expression of the disease. Initially, leaves on individual branches in the crown appear to turn yellowish and become necrotic within days to weeks. The brown leaves typically remain attached to dead or dying branches as the disease symptoms rapidly develop throughout the canopy. Dark-brown to black radial discolouration or staining is observed in the outer woody xylem of affected trees that is typical of wilt symptoms caused by *Ceratocystis* species (Harrington 2013, Roux & Wingfield 2013).

The pathogen responsible for the wilt symptoms was tentatively identified as *C. fimbriata* s.lat., and the disease was referred to as Ceratocystis wilt (Keith et al. 2015). This identification was based on a combination of disease symptoms typical of *Ceratocystis* infections, morphological structures similar to those of *C. fimbriata* s.lat. and a 98 % similarity blast hit of the ITS (internal transcribed spacer) sequence to an isolate identified as *C. fimbriata* in GenBank (KC493164) (Keith et al. 2015). This isolate, CBS 115167, was obtained from basal rot symptoms on *Syngonium podophyllum* (arrowhead plant) in Florida, and its ITS sequence was identical to those of other *Syngonium* isolates in Florida, as well as *Syngonium* isolates from Brazil, Australia and Hawai`i (Uchida & Aragaki 1979, Thorpe et al. 2005). Other strains of *Ceratocystis* from Hawai`i include *C. fimbriata* from sweet potato, which has been known on this crop plant in Hawai`i since 1925 (Chung 1923, Brown & Matsuura 1941, Li et al. 2016), and strains from *Colocasia esculenta* (taro), which have been known in Hawai`i since the 1970s (Uchida & Aragaki 1979). These isolates from taro, also previously referred to as *C. fimbriata* (Thorpe et al. 2005), were recently described as a new species, *C. uchidae*, which resides in the AAC (Li et al. 2017).

The increasing importance of ROD in Hawai`i demands that the isolates of the *Ceratocystis* sp. associated with the disease on *M. polymorpha* be accurately identified. The objective of this study was to identify and describe a collection of isolates associated with ROD based on morphology, the biological species concept and by using a multigene phylogenetic approach. The study included closely related *Ceratocystis* species and isolates treated as *C. fimbriata* s.lat. previously reported from Hawai`i. The pathogenicity of the isolates from *M. polymorpha* were confirmed in inoculation studies and the ability of isolates to cross-infect other hosts was determined. In addition, isolates collected from different regions on the Island of Hawai`i were characterized using microsatellite markers to determine their genetic diversity.

MATERIALS AND METHODS

Sampling and isolations

Surveys were conducted in the Puna, Hilo and Kona districts of Hawai`i Island during 2014 and 2015 for *M. polymorpha* trees showing typical symptoms of ROD (Fig. 1). Wood samples were collected from *M. polymorpha* showing brown to black streaking in the woody xylem. Carrot baiting was used to isolate *Ceratocystis* from discoloured wood (Moller & Devay 1968). Primary isolations were made by transferring ascospore drops from the tips of the ascomata formed on the wood or carrot discs onto 10 % V8 agar. Pure cultures were stored at -80 °C in 15 % glycerol. Single ascospore cultures were generated by transferring single ascospores to 2 % malt extract agar (MEA, Biolab, Midrand, South Africa), supplemented with 100 µg/L thymine (Sigma-Aldrich, Germany). Cultures were incubated at 25 °C.

Cultures obtained in this study are stored at the USDA Agricultural Research Service, Hilo, HI; the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; or at Iowa State University (Table 1; Appendix). Representative cultures have been deposited at the Westerdijk Fungal Biodiversity Institute (CBS-KNAW), Utrecht, The Netherlands, Mycothèque de l'Université Catholique de Louvain, Belgium (MUCL) and dried specimens have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa (Table 1).

DNA extraction, PCR and sequencing

Cultures were grown for 2 wk on 2 % MEA. Mycelium was scraped from the surface of the agar plates, lyophilized and crushed using the Retsch® GmbH MM301 homogenizer (Haan, Germany). DNA was extracted using the phenol/chloroform method described by Goodwin et al. (1992). DNA quantity was measured using a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA) and diluted to 30 ng/µL working stock solutions.

For the phylogenetic analyses of *Ceratocystis* species, partial regions of five loci were amplified (Marin-Felix et al. 2017). These included the Internal Transcribed Spacer (ITS) rDNA regions and the 5.8S rRNA gene using the primers ITS1 and ITS4 (White et al. 1990), Beta-tubulin 1 (*bt1*) gene using primers Bt1a and Bt1b (Glass & Donaldson 1995), Transcription Elongation Factor-1 alpha (*tef1*) gene with primers TEF1F and TEF2R (Jacobs et al. 2004), the guanine nucleotide-binding protein subunit beta-like protein (*ms204*) using primers MS204F. cerato (AAG GGC ACC CTC GAG GGC CAC) and MS204R. cerato (GAT GGT RAC GGT GTT GAT GTA) (Fourie et al. 2015) and second largest subunits of RNA polymerase II (*rpb2*) using degenerate primers RPB2-5Fb (GAY GAY CGT GAT CAC TTY GG) and RPB2-7Rb (CCC ATR GCY TGY TTR CCC AT) (Fourie et al. 2015).

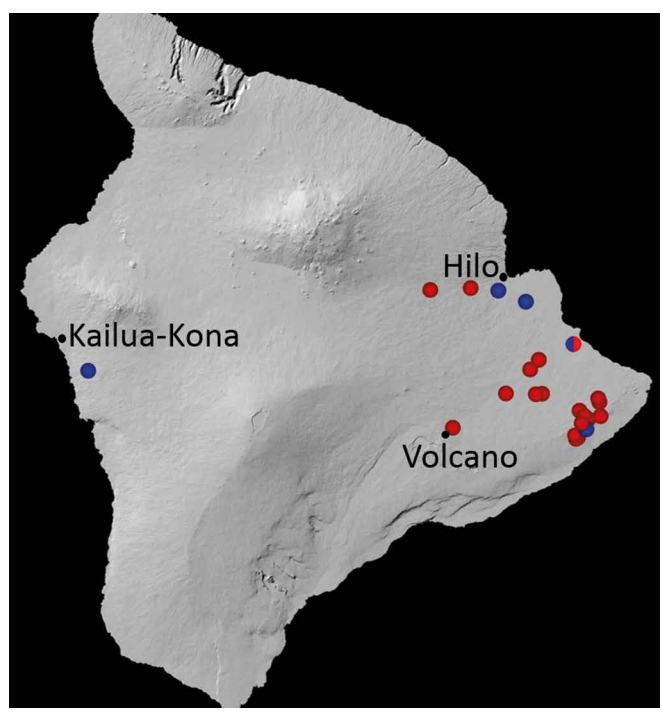

Fig. 1 Distribution of samples yielding isolates of *Ceratocystis lukuohia* (red circles) and *C. huliohia* (blue circles) on Hawai`i Island during 2014–2015.

Table 1 Details of the *Ceratocystis* isolates from the Latin American clade (LAC) and the Asian-Australian clade (AAC) used in the phylogenetic analyses, mating tests and pathogenicity tests in this study.

Species[1]	Isolate no.[2,3]	Substrate	Country	Locality	Year collected	GenBank accession numbers				
						ITS-rDNA	*bt1*	*tef1*	*ms204*	*rpb2*
C. adelpha[T]	CBS 115169; CMW 14809; PREM 61152; *C1833*	*Theobroma cacao*	Ecuador	Pichilingue	2001	DQ520637	KJ601509	KJ601516	KJ601563	KJ601599
C. adelpha	CBS 152.62; CMW 15051; C940	*Theobroma cacao*	Costa Rica	Atlantic side	1962	AY157951	KJ601510	KJ601517	KJ601564	KJ601600
C. albifundus	CBS 128992; CMW 4068	*Acacia mearnsii*	South Africa	KwaZulu-Natal, Bloemendal	1997	DQ520638	EF070429	EF070400	KY643987	KY644041
C. atrox[T]	CBS 120518; CMW 19385; PREM 59012	*Eucalyptus grandis*	Australia	Queensland	2005	NR_136981; EF070415	EF070431	EF070403	KY643976	KY644030
C. atrox	CBS 120517; CMW 19383	*Eucalyptus grandis*	Australia	Queensland	2006	EF070414	EF070430	EF070402	KY643975	KY644029
C. cacaofunesta[T]	CBS 115172; CMW 26375; C1983; BPI 843740	*Theobromae cacao*	Brazil	Rondonia	2002	AY157953	KJ601512	KJ601519	KJ601566	KJ601602
C. cacaofunesta	CBS 114722; CMW 14798; C1548; BPI 843730	*Theobromae cacao*	Costa Rica	La Lola Experiment Station	1999	AY157952	KJ601511	KJ601518	KJ601565	KJ601601
C. cercfabiensis[T]	CBS 139654; CMW 43029; CERC 2170; PREM 61229	*Eucalyptus* sp.	China	HaiNan Province, LinGao County	2013	KP727592 (Group 2, Clone F); KP727593 (Group 2, Clone G); KP727594 (Group 2, Clone K)	KP727618	KP727643	KY643963	KY644016
C. cercfabiensis	CBS 139656; CMW 42795; CERC 2687	*Eucalyptus* sp.	China	GuangDong	2013	same as KP727583 (Group 2, Clone A)	KP727619	KP727644	KY643968	KY644022
C. cercfabiensis	CMW 42736	*Eucalyptus* sp.	China	GuangDong	2013	KP727583 (Group 2, Clone A); KP727584 (Group 1, Clone B)	–	–	–	–
C. cercfabiensis	CMW 42741	*Eucalyptus* sp.	China	GuangDong	2013	KP727586 (Group 2, Clone A); KP727588 (Group 1, Clone E)	–	–	–	–
C. cercfabiensis	*C3301*; CERC 2548	*Eucalyptus* sp.	China	GuangDong	2013	KY306679	–	–	–	–
C. cercfabiensis	*C3302*; CERC 2549	*Eucalyptus* sp.	China	GuangDong	2013	KY306680	–	KP727623	–	–
C. cercfabiensis	CMW 42577; *C3305*; CERC 2552	*Eucalyptus* sp.	China	GuangDong	2013	KY306681	–	KP727624	–	–
C. cercfabiensis	C3356, CERC 2166	*Eucalyptus* sp.	China	Hainan	2013	–	–	–	–	–
C. cercfabiensis	CMW 42489; *C3358*; CERC 2168	*Eucalyptus* sp.	China	Hainan	2013	–	–	KP727630	–	–
C. changhui[T]	CBS 139797; CMW 43281; CERC 3615; PREM 61241	*Colocasia esculenta*	China	YunNan Province, KunMing	2014	KY643884	KY643919	KY643941	KY643962	KY644015
C. changhui	CBS 139798; CMW 43272; CERC 3605; PREM 61242	*Colocasia esculenta*	China	YunNan Province, KunMing	2015	KY643886	KY643915	KY643937	KY643958	KY644011
C. collisensis[T]	CBS 139679; CMW 42552; CERC 2459; PREM 61232	*Cunninghamia lanceolata*	China	FuJian Province, ZhangZhou Region	2013	KP727578	KP727614	KP727639	KY643970	KY644024
C. collisensis	CBS 139647; CMW 42554; CERC 2466	*Cunninghamia lanceolata*	China	FuJian Province, ZhangZhou Region	2013	KP727580	KP727615	KP727640	KY643972	KY644026
C. colombiana[T]	CBS 121792; CMW 5751; PREM 59434	*Coffea arabica*	Colombia	Valle del Cauca	2000	NR_119483; AY177233	AY177225	EU241493	KJ601567	KJ601603
C. colombiana	CBS 121791; CMW 5761; PREM 59435	*Coffea arabica*	Colombia	–	2000	AY177234	AY177224	EU241492	KJ601568	KJ601604
C. colombiana	*C1945*	*Coffea arabica*	Colombia	–	2002					
C. corymbiicola[T]	CBS 127215; CMW 29120; PREM 60431	*Corymbia variegata*	Australia	Wedding Bells State Forest, New South Wales	2008	NR_119830; HM071902	HM071914	HQ236453	KY643983	KY644037
C. corymbiicola	CBS 127216; CMW 29349; PREM 60433	*Eucalyptus pilularis*	Australia	Ingalba State Forest, New South Wales	2008	HM071919	HQ236455	HM071905	KY643984	KY644038
C. curvata[T]	CBS 122603; CMW 22442; PREM 60151	*Eucalyptus deglupta*	Ecuador	Salinas	2004	NR_137018; FJ151436	FJ151448	FJ151470	KJ601570	KJ601606
C. curvata	CBS 122604; CMW 22435	*Eucalyptus deglupta*	Ecuador	Salinas	2004	FJ151437	FJ151449	FJ151471	KJ601569	KJ601605
C. diversiconidia[T]	CBS 123013; CMW 22445; PREM 60160	*Terminalia ivorensis*	Ecuador	Salinas	2004	FJ151440	FJ151452	FJ151474	KJ601571	KJ601607
C. diversiconidia	CBS 122605; CMW 22448; PREM 60162	*Terminalia ivorensis*	Ecuador	Salinas	2004	FJ151441	FJ151453	FJ151475	KJ601572	KJ601608
C. ecuadoriana[T]	CBS 124020; CMW 22092; PREM 60155	*Eucalyptus deglupta*	Ecuador	Salinas	2004	FJ151432	FJ151444	FJ151466	KJ601573	KJ601609
C. ecuadoriana	CBS 124022; CMW 22097	*Eucalyptus deglupta*	Ecuador	Salinas	2004	FJ151434	FJ151446	FJ151468	KJ601574	KJ601610
C. eucalypticola[T]	CBS 124016; CMW 11536; PREM 60168	*Eucalyptus* sp.	South Africa	KwaZulu-Natal, KwaMbonambi	2002	FJ236723	FJ236783	FJ236753	KJ601576	KJ601612
C. eucalypticola	CBS 124019; CMW 10000	*Eucalyptus* sp.	South Africa	KwaZulu-Natal, KwaMbonambi	2002	FJ236722	FJ236782	FJ236752	KJ601575	KJ601611

Table 1 (cont.)

Species[1]	Isolate no.[2,3]	Substrate	Country	Locality	Year collected	GenBank accession numbers				
						ITS-rDNA	*bt1*	*tef1*	*ms204*	*rpb2*
C. ficicola[T]	CMW 38543; NIAES 20600; C1355; BPI 843724; MAFF 625119	*Ficus carica*	Japan	Fukuoka Prefecture	1990	NR_119410	KY685077	KY316544	KY685080	KY685082
C. ficicola	CMW 38544	*Ficus carica*	Japan	Fukuoka Prefecture	1991	KY685076	KY685078	KY685079	KY685081	KY685083
C. fimbriata	CBS 114723; CMW 14799; C1421	*Ipomoea batatas*	USA	North Carolina	1998	KC493160	KF302689	KJ631109	KJ601578	KJ601614
C. fimbriata	CBS 123010; CMW 1547	*Ipomoea batatas*	Papua New Guinea	–	1984	AF264904	EF070443	EF070395	KJ601577	KJ601613
C. fimbriata	CMW 46734; P15-31	*Ipomoea batatas*	USA	Hawai`i Island, Hawai`i	2015	KY809154	KY809103	KY809116	KY809128	KY809141
C. fimbriata	*C4135*	*Ipomoea batatas*	USA	Hawai`i Island, Hawai`i	2016	same as KC493160	–	–	–	–
C. fimbriatomima[T]	CBS 121786; CMW 24174; PREM 59439	*Eucalyptus grandis* × *E. urophylla*	Venezuela	Portuguesa state, Acarigua	2006	EF190963	EF190951	EF190957	KJ601579	KJ601615
C. fimbriatomima	CMW 24377	*Eucalyptus grandis* × *E. urophylla*	Venezuela	Portuguesa state, Acarigua	2006	EF190966	EF190954	KJ601520	KJ601581	KJ601617
C. huliohia[T]	CBS 142794; CMW 47149; P15-58; PREM 61769; MUCL 56340	*Metrosideros polymorpha*	USA	Hōlualoa, North Kona District, Hawai`i	2015	KY809156	KY809105	KY809118	KY809130	KY809143
C. huliohia	CBS 142795; CMW 47135; P14-10; PREM 61768; MUCL 56341; *C4211*	*Metrosideros polymorpha*	USA	`Opihikao, Puna District, Hawai`i	2014	KY809155	KY809104	KY809117	KY809129	KY809142
C. huliohia	CMW 44104; P14-9-1	*Metrosideros polymorpha*	USA	`Opihikao, Puna District, Hawai`i	2014	same as KY809156	same as KY809105	same as KY809118	same as KY809130	same as KY809143
C. huliohia	CMW 46716; P14-14	*Metrosideros polymorpha*	USA	Hawaiian Paradise Park, Puna District, Hawai`i	2014	same as KY809156	same as KY809105	same as KY809118	same as KY809130	same as KY809143
C. huliohia	CMW 46721; P15-3	*Metrosideros polymorpha*	USA	Hilo, South Hilo District, Hawai`i	2014	same as KY809156	same as KY809105	same as KY809118	same as KY809130	same as KY809143
C. huliohia	CMW 46738; P15-59; *C4215*	*Metrosideros polymorpha*	USA	Hōlualoa, North Kona District, Hawai`i	2015	same as KY809156	same as KY809105	same as KY809118	same as KY809130	same as KY809143
C. huliohia	CMW 47143; P15-33	*Metrosideros polymorpha*	USA	Pana`ewa, South Hilo District, Hawai`i	2015	same as KY809156	same as KY809105	same as KY809118	same as KY809130	same as KY809143
C. huliohia	*C4190*	*Metrosideros polymorpha*	USA	Hawai`i Island, Hawai`i	2016	same as KY809156	–	–	–	–
C. huliohia	*C4191*	*Metrosideros polymorpha*	USA	Hawai`i Island, Hawai`i	2016	same as KY809156	–	–	–	–
C. huliohia	*C4192*	*Metrosideros polymorpha*	USA	Hawai`i Island, Hawai`i	2016	same as KY809156	–	–	–	–
C. larium[T]	CBS 122512; CMW 25434; PREM 60193	*Styrax benzoin*	Indonesia	North Sumatra	2007	NR_137016; EU881906	EU881894	EU881900	KY643981	KY644035
C. larium	CBS 122606; CMW 25435	*Styrax benzoin*	Indonesia	North Sumatra	2007	EU881907	EU881895	EU881901	KY643982	KY644036
C. lukuohia[T]	CBS 142792; CMW 44102; P14-1-1; PREM 61770; MUCL 56342; *C4212*	*Metrosideros polymorpha*	USA	Leilani Estates, Puna District, Hawai`i	2014	KP203957/ KY809157	KY809106	KY809119	KY809131	KY809144
C. lukuohia	CMW 44103; P14-6	*Metrosideros polymorpha*	USA	Orchidlands Estates, Puna District, Hawai`i	2014	same as KY809157	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CMW 44105; P14-12	*Metrosideros polymorpha*	USA	Hawaiian Acres, Puna District, Hawai`i	2014	same as KY809157	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CMW 44106; P14-13	*Metrosideros polymorpha*	USA	Hawaiian Paradise Park, Puna District, Hawai`i	2014	same as KY809157	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CMW 44107; P14-15	*Metrosideros polymorpha*	USA	Hawaiian Acres, Puna District, Hawai`i	2014	same as KY809157	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CBS 142793; CMW 46741; P15-64; PREM 61771; MUCL 56343; *C4210*	*Metrosideros polymorpha*	USA	Nānāwale Estates, Puna District, Hawai`i	2015	KY809158	KY809107	KY809120	KY809132	KY809145
C. lukuohia	CMW 47857; P15-11	*Metrosideros polymorpha*	USA	Keau`ohana Forest Reserve, Puna District, Hawai`i	2015	same as KY809158	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CBS 142591; CMW 47152; P15-65; PREM 61772; MUCL 56344; *C4213*	*Metrosideros polymorpha*	USA	Waiākea Forest Reserve, South Hilo District, Hawai`i	2015	haplotype A same as KY809157; haplotype C same as KY809158	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CMW 46743; P15-27	*Metrosideros polymorpha*	USA	Kopua Farm Lots, Puna District, Hawai`i	2015	haplotype A same as KY809157; haplotype C same as KY809158	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	CMW 47139; P15-17	*Metrosideros polymorpha*	USA	Mālama Kī Forest Reserve, Puna District, Hawai`i	2015	haplotype A same as KY809157; haplotype C same as KY809158	same as KY809106	same as KY809119	same as KY809131	same as KY809144
C. lukuohia	*C4133*	*Metrosideros polymorpha*	USA	Hawai`i Island, Hawai`i	2016	haplotype A same as KY809157	–	–	–	–
C. lukuohia	*C4185*	*Metrosideros polymorpha*	USA	Hawai`i Island, Hawai`i	2016	haplotype A same as KY809157	–	–	–	–

Table 1 (cont.)

Species[1]	Isolate no.[2,3]	Substrate	Country	Locality	Year collected	GenBank accession numbers				
						ITS-rDNA	*bt1*	*tef1*	*ms204*	*rpb2*
C. lukuohia	*C4186*	*Metrosideros polymorpha*	USA	Hawai`i Island, Hawai`i	2016	haplotype A same as KY809157	–	–	–	–
C. mangicola[T]	CBS 114721; CMW 14797; C1688; PREM 60182	*Mangifera indica*	Brazil	São Paulo State	2000	AY953382	EF433307	EF433316	KJ601582	KJ601618
C. mangicola	CMW 28907; PREM 60184	*Mangifera indica*	Brazil	São Paulo State	2008	FJ200257	FJ200270	FJ200283	KJ601583	KJ601619
C. manginecans[T]	CBS 121659; CMW 13851; PREM 59612	*Mangifera indica*	Oman	–	2002	NR_119532; AY953383	EF433308	EF433317	KJ601584	KJ601620
C. manginecans	CBS 121660; CMW 13852; PREM 59613	*Mangifera indica*	Oman	–	2002	AY953384	EF433309	EF433318	KJ601585	KJ601621
C. mangivora[T]	CBS 128340; CMW 27305; PREM 60570	*Mangifera indica*	Brazil	São Paulo State, Campinas	2001	FJ200262	FJ200275	FJ200288	KJ601587	KJ601623
C. mangivora	CBS 600.70; CMW 15052; PREM 60188	*Mangifera indica*	Brazil	São Paulo State	1970	EF433298	EF433306	EF433315	KJ601586	KJ601622
C. neglecta[T]	CBS 121789; CMW 17808; PREM 59616	*Eucalyptus grandis*	Colombia	–	2004	NR_137552; EF127990	EU881898	EU881904	KJ601588	KJ601624
C. neglecta	CMW 18194; PREM 59617	*Eucalyptus grandis*	Colombia	–	2004	EF127991	EU881899	EU881905	KJ601589	KJ601625
C. obpyriformis[T]	CBS 122511; CMW 23808; PREM 59796	*Acacia mearnsii*	South Africa	Mpumalanga, Piet Retief	2006	EU245003	EU244975	EU244935	KY643978	KY644032
C. obpyriformis	CBS 122608; CMW 23807; PREM 59797	*Acacia mearnsii*	South Africa	Mpumalanga, Piet Retief	2007	EU245004	EU244976	EU244936	KY643977	KY644031
C. papillata[T]	CBS 121793; CMW 8856; PREM 59438	*Citrus* × *Tangelo* hybrid	Colombia	Caldas	2001	NR_119486; AY233867	AY233874	EU241484	KJ601590	KJ601626
C. papillata	CMW 10844; PREM 60172	*Coffea arabica*	Colombia	Antioquia	1998	AY177238	AY177229	EU241481	KJ601591	KJ601627
C. pirilliformis[T]	CBS 118128; CMW 6579; PREM 57323	*Eucalyptus nitens*	Australia	Australian Capital Territory, Uriarra	2000	NR_119452; AF427105	DQ371653	AY528983	KJ601594	KJ601630
C. pirilliformis	CMW 6569; PREM 57322	*Eucalyptus nitens*	Australia	Australian Capital Territory, Uriarra	2000	AF427104	DQ371652	AY528982	KY643985	KY644039
C. platani[PT]	CBS 115162; CMW 14802; C1317	*Platanus occidentalis*	USA	Janet Day Plantation, North Carolina	1998	DQ520630	EF070425	EF070396	KJ601592	KJ601628
C. platani	CMW 23450	*Platanus orientalis*	Greece	Peloponese	2006	KJ631107	KJ601513	KJ601521	KJ601593	KJ601629
C. platani	CMW 1896	*Platanus* sp.	Switzerland	–	–	AF395681	KY809113	KY809126	KY809138	KY809151
C. platani	CMW 2219	*Platanus* sp.	France	Saint-Maurice	1991	AF395679	KY809114	AY528975	KY809139	KY809152
C. platani	CMW 9499	*Platanus* sp.	Italy	–	–	KY809159	KY809115	KY809127	KY809140	KY809153
C. platani	*C1329*	*Platanus occidentalis*	USA	Bertie, North Carolina	1998	–	–	–	–	–
C. platani	*C1339*	*Platanus occidentalis*	USA	Sussex, Virginia	1998	same as AY157958	–	–	–	–
C. platani	*C1342*	*Platanus occidentalis*	USA	Bertie, North Carolina	1998	–	–	–	–	–
C. polychroma[T]	CBS 115778; CMW 11424; PREM 57818	*Syzygium aromaticum*	Indonesia	Sulawesi, Touliang Oki	2002	AY528970	AY528966	AY528978	KY643973	KY644027
C. polychroma	CBS 115777; CMW 11436; PREM 57819	*Syzygium aromaticum*	Indonesia	Sulawesi, Touliang Oki	2003	AY528971	AY528967	AY528979	KY643974	KY644028
C. polychroma	CBS 115775; CMW 11449; *C2240*; PREM 57821	*Syzygium aromaticum*	Indonesia	Sulawesi, Touliang Oki	2003	AY528972	AY528968	KY316543	Pending	Pending
C. polyconidia[T]	CBS 122289; CMW 23809; PREM 59788	*Acacia mearnsii*	South Africa	Mpumalanga, Piet Retief	2006	EU245006	EU244978	EU244938	KY643979	KY644033
C. polyconidia	CBS 122290; CMW 23818; PREM 59789	*Acacia mearnsii*	South Africa	Mpumalanga, Piet Retief	2007	EU245007	EU244979	EU244939	KY643980	KY644034
Ceratocystis sp.	CBS 115167; CMW 48508; C1809	*Syngonium podophyllum*	USA	Florida	2001	AY526295	KY809108	KY809121	KY809133	KY809146
Ceratocystis sp.	CBS 114719; CMW 48500; *C1717*	*Syngonium* sp.	USA	Hawai`i	1987	AY526294	KY809110	KY809123	KY809135	KY809148
Ceratocystis sp.	CMW 50456; P16-1	*Syngonium podophyllum*	USA	Hawai`i Island, Hawai`i	2016	same as AY526294	–	–	–	–
Ceratocystis sp.	CMW 50457; P16-2	*Syngonium podophyllum*	USA	Hawai`i Island, Hawai`i	2016	same as AY526294	–	–	–	–
Ceratocystis sp.	*C4118*	*Syngonium podophyllum*	USA	Hawai`i Island, Hawai`i	2016	same as AY526294	–	–	–	–
Ceratocystis sp.	*C1774*	*Syngonium podophyllum*	USA	Florida	2000	AY526295	–	–	–	–
Ceratocystis sp.	CBS 115165; CMW 14805/48506; C1780	*Xanthosoma sagittifolium*	Costa Rica	Cartago	2001	AY526297	KY809111	KY809124	KY809136	KY809149
Ceratocystis sp.	CBS 114718; CMW 14794/48499; C1817	*Xanthosoma* sp. or *C. esculenta*	Cuba	Havana	2001	AY526298	KY809112	KY809125	KY809137	KY809150
C. uchidae[T]	CBS 115164; CMW 14804/48505; *C1714*; BPI 843732	*Colocasia esculenta*	USA	O`ahu, Hawai`i	1991	AY526306	KY643899	KY643921	KY643943	KY643996
C. uchidae	CBS 114720; CMW 14796/48501; *C1715*; BPI 843733	*Colocasia esculenta*	USA	Kaua`i, Hawai`i	1988	AY526307	KY643898	KY643920	KY643942	KY643995
C. uchidae	*C1931*; DAR 58902	*Xanthosoma sagittifolium*	Fiji	–	1987	AY526308	–	HM569618	–	–

[1] T = Extype material of *Ceratocystis* species used in the phylogeneic studies.

[2] BPI = US National Fungus Collections, Beltsville, Maryland, USA; C = Personal collection of Tom Harrington, Iowa State University; CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CERC = China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong Province, China; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MUCL = Mycothèque de l'Université Catholique de Louvain, Belgium; P = USDA Agricultural Research Service, Hilo, HI; PREM = National Collection of Fungi, Agricultural Research Council, Pretoria, South Africa.

[3] C numbers in italics were isolates used in the infertility tests (see Table 4, 5).

PCR amplification reactions and conditions, including annealing temperatures and $MgCl_2$ concentrations, for all gene regions were the same as those described by Fourie et al. (2015). When *rbp2* was difficult to amplify, a second round of PCR was carried out using the cleaned product of the first PCR reaction as a template. Successful PCR amplicons were determined by gel electrophoresis on 1 % agarose gels and visualized with GelRed™ (Biotium, Hayward, CA, USA) under UV illumination (Gel Doc EZ Imager, BioRad, Johannesburg, South Africa). PCR amplicons were purified through 6 % Sephadex G-50 (Sigma-Aldrich, Steinheim, Germany) using Centri-Sep spin columns (Princeton Separations, Inc., Adelphia, NJ, USA).

PCR amplicons were sequenced in both directions using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Thermo Fisher, Foster City, CA, USA) cleaned by filtration through 6 % Sephadex G-50 and run on an ABI 3100 Genetic Analyzer (Applied BioSystems, Thermo Fisher). In cases where the ITS region was difficult to sequence due to multiple ITS types (Marin-Felix et al. 2017), cloning and sequencing of the PCR product was carried out using the pGEM®-T Easy Vector System, according to manufacturer's instructions (Promega, Madison, WI, USA). Forward and reverse sequences were assembled into consensus sequences using CLC MainWorkbench v. 6.6.2 (CLC Bio, www.clcbio.com). All unique sequences generated in this study were submitted to GenBank.

Multi-gene phylogenetic analyses

Initial BLAST analyses of the sequences obtained against the NCBI GenBank database (NCBI; http://www.ncbi.nlm.nih.gov) were performed to identify the species of *Ceratocystis* most closely related to the isolates from Hawai`i and the biogeographic clade in which they reside. Representative sequences showing the highest similarity matches were downloaded and included in the datasets. Separate ITS datasets were generated using ex-type and ex-paratype sequences representing all described species of *Ceratocystis* (Li et al. 2017, Marin-Felix et al. 2017, Liu et al. 2018) for LAC and AAC. These datasets also included the different ITS types that have previously been described (Al Adawi et al. 2013, Naidoo et al. 2013, Harrington et al. 2014, Fourie et al. 2015, Liu et al. 2015). A third dataset included the combined sequences of the *bt1*, *ef1*, *ms204* and *rpb2* loci for both the LAC and AAC.

For phylogenetic tree construction, the consensus sequence reads were aligned using the online software MAFFT v. 7 (Katoh & Standley 2013) (http://mafft.cbrc.jp/alignment/server/) and the best alignment strategy for each locus was automatically selected by the software. Alignments were edited and trimmed in MEGA v. 7 (Kumar et al. 2016). Sequence alignments for all three datasets were deposited in TreeBASE (No. S22005) (http://purl.org/phylo/treebase/phylows/study/TB2:S22005).

Aligned sequences were used as input for phylogenetic tree construction using a maximum parsimony (MP) and maximum likelihood (ML) approach. *Ceratocystis albifundus*, residing in AFC, was used as the outgroup in all tree constructions (Table 1). Maximum parsimony analysis was performed with PAUP v. 4.0b10 (Swofford 2003), and most parsimonious trees were obtained using a heuristic search and TBR branch swopping strategy, based on 1 000 repeats. Gaps were treated as missing except for the LAC ITS dataset, where gaps were treated as a fifth character state. For the ITS datasets, sites 227–234 in the LAC dataset and sites 222–226 in the AAC dataset were excluded from the analyses because they contained repeating thymine nucleotides. Sites 867–880 in the combined dataset, forming part of the *ms204* region, were excluded due to poly-C nucleotides and ambiguous sites. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were all calculated. Branch confidence was determined using 1 000 bootstrap replicates.

Maximum likelihood analysis was performed using RAxML (Stamatakis 2014) with standard parameters and the GTR-GAMMA setting to determine the optimal nucleotide substitution model. Branch confidence for ML was determined using 1 000 bootstrap replicates. Single gene trees for *bt1*, *ef1*, *ms204* and *rpb2* and a species tree based on the combined sequences of the four genes were generated using both MP and ML analysis. Phylogenetic trees were edited using MEGA v. 7 (Kumar et al. 2016) and Adobe Illustrator CS6.

Culture characteristics and morphology

Isolates used for morphological characterisation were grown on 2 % MEA and incubated for 3 wk at 22 °C with natural day/night light intervals. Colony colour was determined using the colour charts of Rayner (1970). Fungal material was mounted in 80 % lactic acid amended with bromothymol blue on glass slides. Observations of structures were made using the Nikon SMZ 18 stereo microscope and Nikon Eclipse N*i* compound microscope with differential interference contrast (DIC) illumination. Measurements were made, and electronic images captured, with a Nikon DS-Ri2 camera and NIS-Elements BR (Nikon Instruments Software-Elements Basic Research) software program.

Up to 50 measurements were taken for each characteristic morphological structure including ascomata, ascospores, phialides and the different conidial forms for isolates representing the holotypes and paratypes for all new species. Measurements are presented as (min–)mean-SD – mean+SD(–max), height × width. All relevant data pertaining to type specimens were deposited in MycoBank (www.MycoBank.org).

Growth in culture

In order to determine growth rate in culture, 4 mm agar plugs covered with mycelium were taken from the outer edges of 10-d-old cultures and placed face down in the centres of 90 mm Petri dishes containing 2 % MEA. Four replica plates were made for all isolates chosen to represent holotypes and paratypes and grown in the dark in incubators at temperatures ranging from 10–30 °C at 5 °C intervals. Measurements of colony diam were taken for each culture every 2nd d for 14 d and averages calculated.

Genotyping of Ceratocystis isolates

Twenty-seven microsatellite markers (Barnes et al. 2001, Steimel et al. 2004, Fourie et al. 2016) were used to determine the genetic diversity of the isolates obtained from Hawai`i. PCR reactions and conditions were the same as those described by Fourie et al. (2015). PCR amplicons were visualised on 2 % agarose gels. Products were pooled into three panels and run on an ABI 3500xl Genetic Analyzer (Thermo Fisher Scientific) and sized using GeneScan software and Liz500 (-250) size standard (Thermo Fisher Scientific). Alleles, based on fragment length, were determined using GeneMapper® v. 4.1 software (Applied Biosystems, Thermo Fisher Scientific). Microsatellite profiles of isolates that were phylogenetically most closely related to those from Hawai`i were also generated and included for comparative purposes.

Interfertility tests

Two series of mating tests were performed on strains of the identified new species and their close relatives as described by Li et al. (2017). Most field isolates of *Ceratocystis* are MAT2

and self-fertile, but self-sterile sectors of MAT2 isolates that had lost female characteristics (loss of protoperithecium production) are often found in laboratory cultures and can be used as male testers (Harrington & Mcnew 1997, 1998, Ferreira et al. 2010, Oliveira et al. 2015). Such self-sterile MAT2 tester strains were recovered as V-shaped sectors during the cultivation of field isolates on malt yeast extract agar (MYEA; 2 % malt extract, 0.2 % yeast extract, 2 % agar). Female, MAT1 tester strains that produced protoperithecia were obtained by recovering single-ascospore progeny from self-fertile field isolates, which yield both self-fertile (MAT2) and self-sterile (MAT1) strains.

Two putative species of *Ceratocystis*, referred to as species A and species B, were recovered from symptomatic `ōhi`a trees. Suitable male, MAT2 tester strains were derived from three isolates of *Ceratocystis* sp. A and two isolates of species closely related to it in the phylogenetic analyses. These were a North Carolina isolate of *C. platani* and a Florida isolate of a *Ceratocystis* sp. from *Syngonium podophyllum* (Thorpe et al. 2005). In two replicate experiments, the MAT2 testers were used to spermatize MAT1 testers from eight field isolates of *Ceratocystis* sp. A, *C. platani* and *Ceratocystis* sp. from *Syngonium*, plus MAT1 testers of *C. colombiana*, *C. cacaofunesta* and *C. fimbriata* of the LAC of *Ceratocystis*. In the second series of pairings, suitable male, MAT2 testers were derived from two isolates of *Ceratocystis* sp. B, two isolates of *C. uchidae* and an isolate of *C. cercfabiensis* (Li et al. 2017). These MAT2 testers were used to spermatize 11 MAT1 testers derived from field isolates of *Ceratocystis* sp. B, *C. uchidae*, *C. cercfabiensis* and *C. polychroma*, all of the AAC of *Ceratocystis* (Li et al. 2017).

The tester strains were grown on MYEA for 7 d at room temperature and lighting before spermatization. Sterile water was added to the male tester, and a suspension of conidia, conidiophores and mycelial fragments was scraped from the agar surface. An aliquot of 1–2 mL of the suspension was dispersed over the colony of the female tester. The plates were incubated at room temperature and lighting for 7 d and examined through a dissecting microscope for the presence of ascomata and ascospore masses that accumulated at the tip of the ascomatal necks. If ascospore masses were evident, the ascospores were examined and rated by making microscope slides of ascospore masses from three ascomata mounted in cotton blue and examining at 1125× with Normarski interference microscopy. Mounts of normal ascospore masses had greater than 90 % uniform ascospores, while abnormal ascospore masses had 10 % or more of the spores misshapen or without cytoplasm, and there was generally debris within the spore mass, assumed to be aborted asci (Li et al. 2017).

Germination of the ascospores from the crosses was evaluated by placing an ascospore mass from atop one perithecium into 25 µL of light oil (Isopar M) on a MYEA plate. Small amounts of the oil suspension were taken with a wire loop (bacterial loop) and thoroughly streaked across each of two additional plates of MYEA. The remaining oil suspension on the original plate was also thoroughly spread with the wire loop. Germination was evaluated on the three plates after 6–8 d incubation at room temperature (Li et al. 2017). If the colonies on the three plates were too numerous to count (TNTC), then the germination percentage was considered high. If fewer than 40 total colonies developed on the three plates, the germination was considered low. If there were 40 or more total colonies on the three plates, but sill countable, then the germination was considered medium. The phenotypes of the colonies were also evaluated after incubating for 1–2 wk to confirm that a cross had occurred, that is, that the progeny colonies were from a genetic recombination between the two parent strains and not from a selfing event. In the case of recombination, the streaks of the ascospore masses resulted in colonies of differing phenotype (the fluffy mycelial phenotype of the male parent and the flat phenotype of the female parent), and some of the colonies produced ascomata and ascospores (self-fertile, MAT2), while others produced no ascomata.

Pathogenicity tests

Metrosideros polymorpha plants were propagated from seeds or cuttings taken from 6–12-mo-old seedlings and grown in 10 cm pots. *Platanus* × *acerifolia* 'Bloodgood' was imported as 1.5 m bare rooted whips and planted into 38 L pots. *Colocasia esculenta* 'Lehua' and 'Bun-long' plants were propagated from corms grown in 15 cm pots, divided and individually transplanted into 15 cm pots. *Syngonium podophyllum* cv. 'White Butterfly' plants were propagated by rooting cuttings from locally purchased stock plants and grown in 10 cm pots. All host plants were grown in Sunshine Mix #4 (Sun Gro Horticulture, Agawam, MA, USA), fertilized every 3 months with Nutricote Total 13-11-11 Type 100 (Arysta LifeScience) and watered twice daily for 5 min with an automatic irrigation system. All plants were grown in a greenhouse in Hilo, HI, with shade ranging from 30 to 72 %, except for *C. esculenta*, which was grown outdoors in a partially shaded area.

Pathogenicity tests were conducted on 1–2-year-old, ~3–12 mm diam, ~13–96 cm tall *M. polymorpha* plants; 1-mo-old, ~19–38 mm diam, ~147–177 cm tall *Platanus* × *acerifolia*; ~30 cm tall *C. esculenta* plants with only one fully expanded leaf; and 11-mo-old transplanted *S. podophyllum* plants. Trial inoculations of *M. polymorpha* using multiple isolates of *Ceratocystis* spp. were previously conducted to assess if variation in pathogenicity and/or virulence existed in the population. Since no variation was observed (data not shown), one isolate of *Ceratocystis* sp. A (P14-1-1), one isolate of *Ceratocystis* sp. B (P15-59), and two isolates of *Ceratocystis* from *Syngonium* (P16-1, P16-2) were used. Three to six plants were inoculated for each isolate and tests were repeated at least once. Plants were inoculated either by a stem injection method or a stem-flap wound method (Uchida & Aragaki 1979, Harrington 2004, Thorpe et al. 2005, Keith et al. 2015). For both methods, an inoculum suspension was produced from 7-d-old cultures grown on 10 % V8 agar at 24 °C under 24 h continuous fluorescent lighting and adjusted to 1×10^6 spores/mL using a Bright-Line haemocytometer. *Meterosideros polymorpha*, *C. esculenta* and *S. podophyllum* were inoculated with a 31 gauge insulin syringe (BD brand) with ± 1 µL of inoculum injected into the stems, pseudopetioles and pseudostems, respectively. *Meterosideros polymorpha* and *Platanus* × *acerifolia* were also wound inoculated using a sterile scalpel to produce a longitudinal stem flap ~0.5 mm deep × 10–15 mm long and the approximate width of the stem. Inoculum for stem flaps included 5 mm diam filter paper discs soaked in the conidial suspension, plated onto fresh 10 % V8 agar, and grown for 2 d at 24 °C under 24 h continuous fluorescent lighting. Two filter paper discs were inserted into the stem flap of *Platanus* × *acerifolia* plants; a single strip of filter paper was trimmed from one of the discs and inserted into the stem of *M. polymorpha* plants. Control plants were injected with sterile distilled water or inoculated with filter paper discs soaked in sterile distilled water. All stem flaps were wrapped with parafilm. All plants, except for *Platanus* × *acerifolia*, were placed in a growth chamber set at 24 °C with 12-h light and ~70 % relative humidity. *Platanus* × *acerifolia* plants were placed in a greenhouse with 70 % shade and daily temperatures ranging from 22 °C to 32 °C. All inoculated plants were observed for wilting of leaves, stem discolouration, and plant death every 3–7 d for as little as 2 wk and up to 1 yr depending on the host. *Ceratocystis* spp. were reisolated using carrot baiting and identified by morphology and PCR amplification and sequencing of the ITS region of rDNA.

Fig. 2 Typical symptoms associated with *Ceratocystis lukuohia* (sp. A). a, b. Affected trees exhibiting rapid, synchronized death of leaves on a major trunk fork or the entire crown; c. dark brown to black vertical streaks of discolouration seen in the woody xylem of an affected tree; d–e. radiating pattern of sapwood discolouration in stem cross-sections of wilting trees; f–g. ascomata of *C. lukuohia* with long necks supporting sticky masses of ascospores.

RESULTS

Sampling and isolations

Trees exhibiting wilt symptoms of the foliage, including death of the entire canopy (Fig. 2, 3), were sampled by chopping into the trees with a hatchet or axe, while the stems of other trees were dissected with a chain saw. Internal signs of infection included brown to black discolouration of the woody xylem. The symptomatic trees lacked bark cankers, and mycelial mats and/or ascomata were not observed on the surface of intact bark of dead trees. Symptoms were not directly associated with obvious wounding. Tunnels and boring frass of ambrosia beetles were evident in dead trees at some locations, especially in the Puna district, where there was widespread mortality and boring dust/frass could be readily found.

The majority of the sampled symptomatic trees exhibited vertical streaks in the wood that were dark brown-black in colour (Fig. 2). When transverse sections of the trunk were made, the darkest discolouration appeared to follow radially along the ray parenchyma (Fig. 2). In some of the sampled trees, including some completely dead trees and also some trees with partial crown death, a second pattern of wood discolouration was ob-

Fig. 3 Typical symptoms associated with *Ceratocystis huliohia* (sp. B). a. Affected trees exhibiting rapid, synchronized death of leaves on individual branches and spreading to the entire crown; b. brown to grey-black vertical streaks of discolouration in the woody xylem of an affected tree; c–e. blotchy, diffuse black to grey staining of sapwood; and thin black lines of host reaction following the contours of the cambium (arrows); f–g. ascomata of *C. huliohia* with necks supporting sticky masses of ascospores.

served in which the vertical streaks were lighter, more diffuse and brown to grey-black in colour. Discolouration in transverse sections of the second pattern was typically diffuse and blotchy and in a few cases, black lines of discolouration appeared in the woody xylem that followed the contours of the cambium (Fig. 3, arrows).

Successful isolations of *Ceratocystis* spp. were made from both the radial and diffuse patterns of xylem discolouration using the carrot baiting technique. A total of 68 isolates were obtained from the discoloured xylem of wilted trees ranging in age from young saplings to mature trees at 17 different sites across Hawai`i Island (Table 1; Fig. 1; Appendix).

PCR and sequencing

The ITS region was amplified and sequenced for 64 isolates, with an amplicon size of ± 550 bp in length. The PCR products were directly sequenced with the PCR primers, but the electropherograms of the PCR products from the extracted DNA of 35 isolates showed overlapping base calls indicative of intragenomic variation among the repeats of the rDNA operon. These PCR products were cloned, and at least 4–6 cloned products were sequenced per isolate. Three ITS haplotypes were obtained from the cloned PCR products or from direct sequencing: A haplotype 'A' was obtained from 18 isolates, a haplotype 'B' was obtained from nine isolates, and a haplotype 'C' obtained from two isolates (Table 1; Appendix; GenBank accession numbers KY809157, KY809156 and KY809158). Both haplotype 'A' and 'C' sequences were obtained from the cloned fragments of each of the 35 samples that could not be directly sequenced. When the ITS sequences of haplotypes A (KY809157) and C (KY809158) were aligned with each other, they differed at six base positions: at site 103 in the ITS1 region (T in haplotype A vs C in haplotype C), at site 373 in the ITS2 region (an extra T present in haplotype C), and at sites 517–519 in the ITS2 region where three extra cytosine bases were present in haplotype A that were absent in haplotype C. No recombinants among the three polymorphisms were present in any of the direct sequences or cloned fragments of the A and C haplotypes. The haplotype A and C sequences were most similar to the ITS sequences of members of the LAC. The sequence of the haplotype B was most similar to the ITS sequences of members of the AAC.

Based on the results of the ITS sequencing, isolates representing the three haplotypes, and mixtures of haplotypes A and C from different locations on the island, were chosen for further PCR and sequencing of the *bt1*, *ef1*, *ms204* and *rpb2* loci. These amplified products were ~610 bp, ~780 bp, ~970 bp and ~1200 bp, respectively.

Multi-gene phylogenetic analyses

The total number of aligned characters used for the phylogenetic analysis of the ITS (LAC) dataset (56 isolates), the ITS (AAC) dataset (48 isolates) and the combined dataset of *bt1*, *ef1*, *ms204* and *rpb2* (82 isolates) were 549, 630 and 3264, respectively. The number of parsimony-informative characters were 157, 273 and 510, and the number of parsimony-uninformative characters were 66, 36 and 14, respectively. MP analyses for the ITS_LAC loci resulted in 205 most parsimonious trees with TL = 383, CI = 0.736, RI = 0.892 and RC = 0.656. The ITS_AAC loci resulted in 34 most parsimonious trees with TL = 571, CI = 0.758, RI = 0.917 and RC = 0.695. The combined dataset had 15 most parsimonious trees with TL = 763, CI = 0.765, RI = 0.952 and RC = 0.729. The log-likelihood of the most likely tree obtained from ML analyses were -1871.29 (ITS_LAC), -2203.75 (ITS_AAC) and -8552.56.55 (combined). Bootstrap confidence values > 60 % for both MP and ML analyses are presented on a single most parsimonious tree based on MP for each dataset (Fig. 4–6).

Table 2 Number of fixed nucleotide differences between *Ceratocystis lukuohia* (sp. A) and close relatives of the Latin American clade, and *C. huliohia* (sp. B) and close relatives from the Asian-Australian clade, observed at five loci used in the phylogenetic analyses.

	ITS	*bt1*	*ms204*	*rpb2*	*ef1*	Total
***C. lukuohia* compared with**						
C. cacaofunesta	33	3	19	6	1	61
C. colombiana	30	2	18	10	5	69
C. fimbriata (*Ipomoea batatas*)	29	5	18	6	1	55
Ceratocystis sp. from *Syngonium*	2	0	2	5	1	8
Ceratocystis sp. from *Xanthosoma*	2	0	2	4	0	6
C. platani	13	1	1	2	1	18
***C. huliohia* compared with**						
C. atrox	36	6	6	6	13	67
C. cercfabiensis	23	2	1	4	5	35
C. changhui	9	0	2	4	5	20
C. corymbiicola	32	4	20	4	7	67
C. polychroma	34	2	0	6	5	47
C. uchidae	13	0	1	5	6	25

For all loci tested, the isolates from *M. polymorpha* in Hawai`i separated into two biogeographical clades, viz. LAC and AAC in both the ML and MP analyses (Fig. 4–6). Although the ITS phylogenies differed in topology with that of the combined phylogeny, the isolates from *M. polymorpha* formed two monophyletic lineages supported with high bootstrap values and were distinct from all other described *Ceratocystis* species. These unique lineages represent two new species of *Ceratocystis* from Hawai`i, hereafter referred to as *Ceratocystis* sp. A and *Ceratocystis* sp. B.

In both the ITS_LAC and the combined phylogeny (Fig. 4, 5), one of the emerging new taxa (*Ceratocystis* sp. A) grouped most closely with isolates collected from *Xanthosoma* sp. from Cuba and Costa Rica and those from *Syngonium* sp. from Hawai`i and Florida. There was little or no sequence difference observed between *Ceratocystis* sp. A and these isolates based on the *bt1* and *ef1* loci. *Ceratocystis* sp. A differed, however, from these isolates by up to six and eight fixed polymorphisms, respectively, across all five loci (Table 2). In the combined phylogeny (Fig. 4), *Ceratocystis* sp. A also grouped closely to *C. platani* (differing at five fixed nucleotide sites), *C. cacaofunesta* (differing at 29 sites) and *C. colombiana* (differing at 35 sites) (Table 2). In the ITS_LAC phylogeny, the two ITS types of *Ceratocystis* sp. A (haplotypes A and C) were clustered together and were designated as Group 1 and Group 2 in Fig. 5.

Within the AAC, *Ceratocystis* sp. B formed a distinct lineage in the *C. polychroma* cluster and separated from six closely related species (*C. uchidae C. changhui*, *C. polychroma*, *C. atrox*, *C. cerfabiensis* and *C. corymbiicola*) with a minimum of 20 fixed nucleotide differences across the five loci (Table 2; Fig. 4, 6). In the ITS_AAC phylogeny (Fig. 6), *Ceratocystis* sp. B was sister to *C. changhui*, known only from China, and *C. uchidae*, known from Hawai`i and Fiji. In the combined analyses, *Ceratocystis* sp. B was closest to *C. polychroma*, known from *S. aromaticum* from Indonesia. In the AAC, the *ef1* and the ITS loci showed the greatest differences among species. *Ceratocystis* sp. B could not be distinguished from *C. changhui* and *C. uchidae* using *bt1* or from *C. polychroma* using *ms204*.

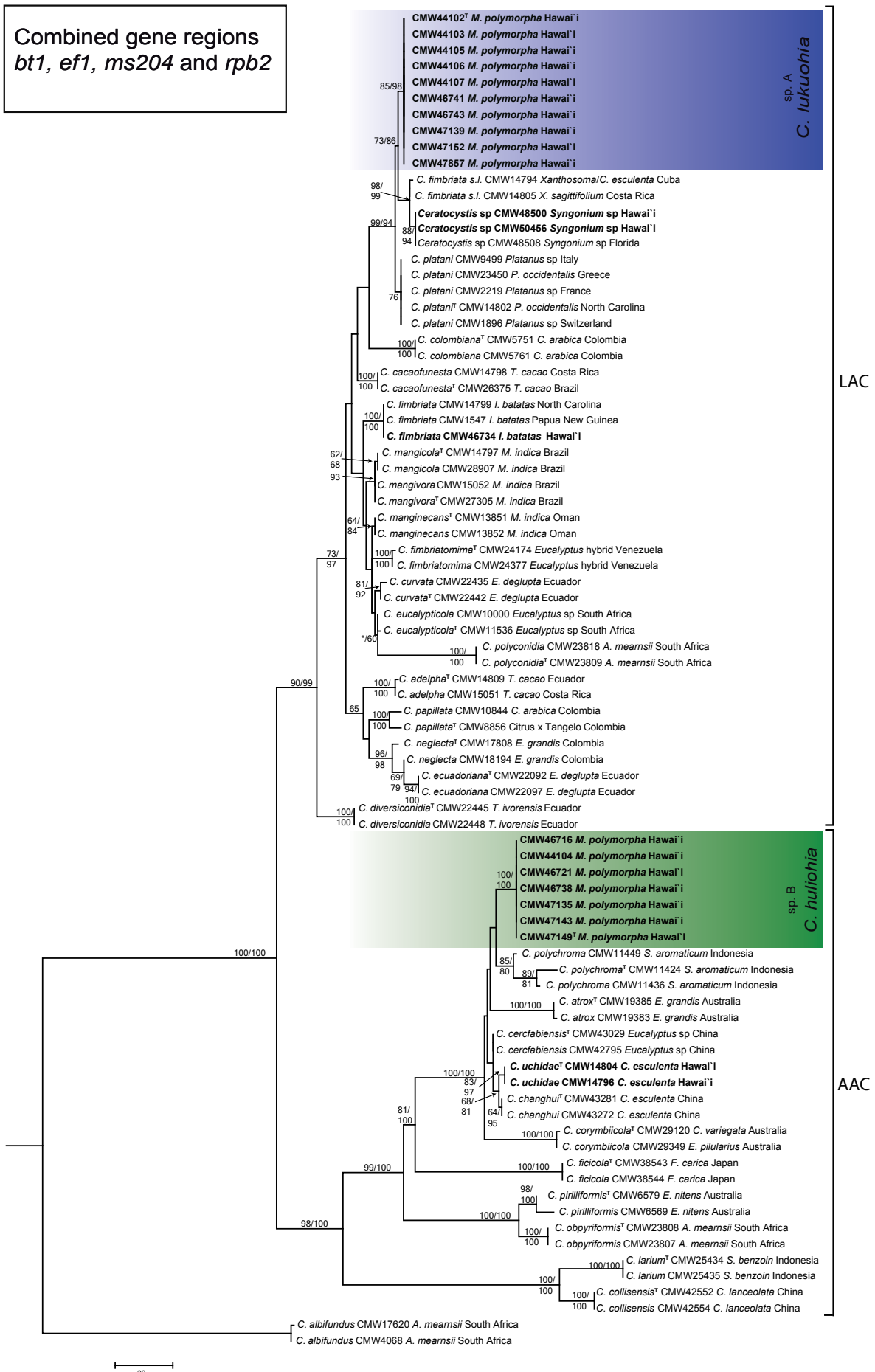

Fig. 4 Maximum parsimony tree based on the combined dataset of four gene regions (*bt1*, *tef1*, *ms204* and *rpb2*) for species in the Latin American clade (LAC) and the Asian-Australian clade (AAC) of *Ceratocystis*. Isolates sequenced in this study from *M. polymorpha* in Hawai`i are highlighted in blue in the LAC, as *C. lukuohia* (sp. A), and in green in the AAC, as *C. huliohia* (sp. B). Bootstrap values > 60 % for MP/ML are presented at the branches. Bootstrap values lower than 70 % are indicated with *. *Ceratocystis albifundus* (CMW 4068) from the African clade was used as the outgroup.

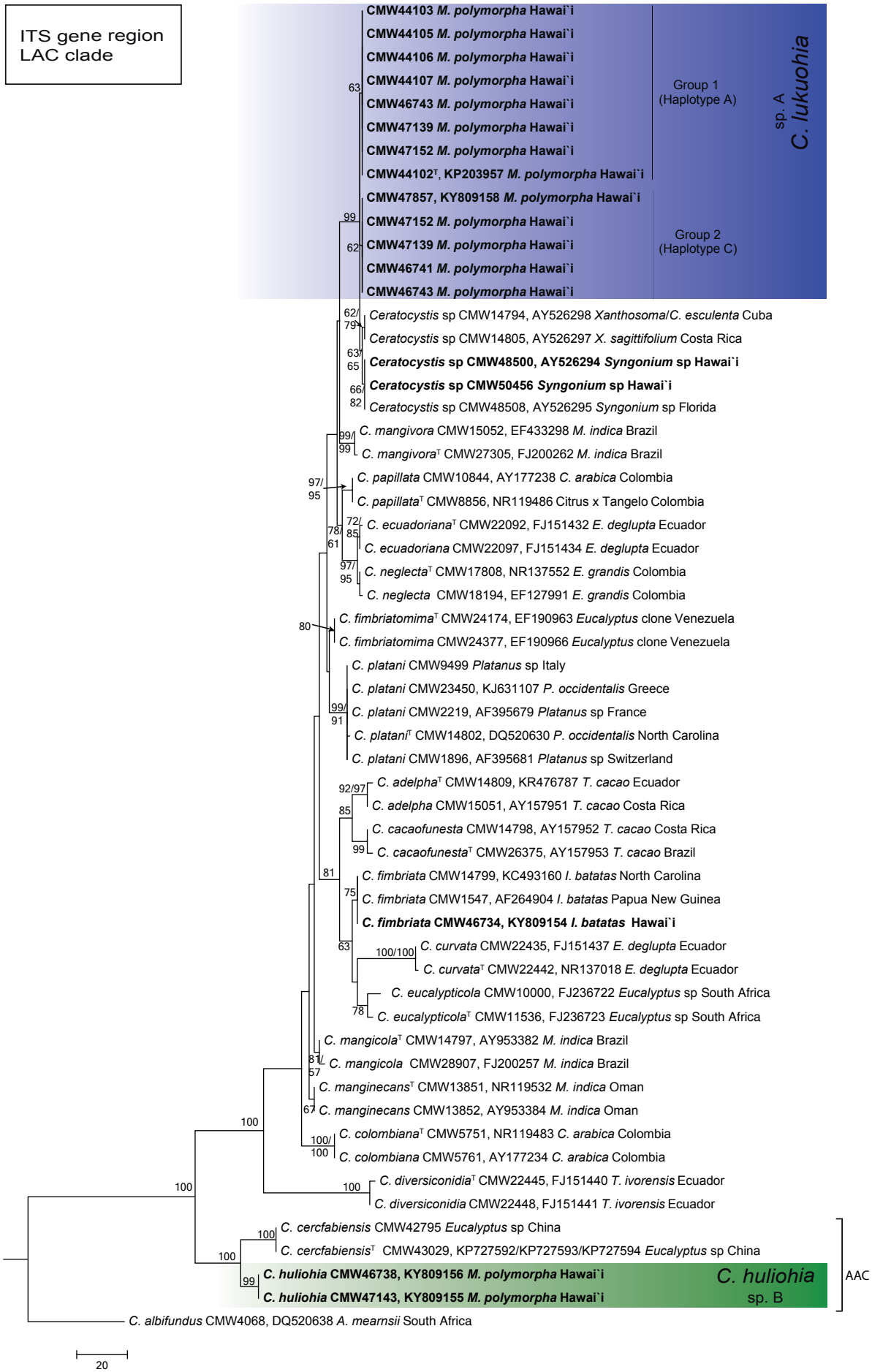

Fig. 5 Phylogenetic tree based on MP of the ITS-rDNA of *Ceratocystis* species in the Latin American clade (LAC). Two ITS haplotypes (Group 1 and Group 2) were evident in the isolates of *C. lukuohia* (sp. A) from *M. polymorpha* in Hawai`i. These grouped closest to isolates from *Xanthosoma* sp. from Cuba and Costa Rica and *Syngonium* sp. from Hawai`i and Florida. Bootstrap values > 70 % for MP/ML are presented at the branches. *Ceratocystis albifundus* (CMW 4068) from the African clade was used as the outgroup.

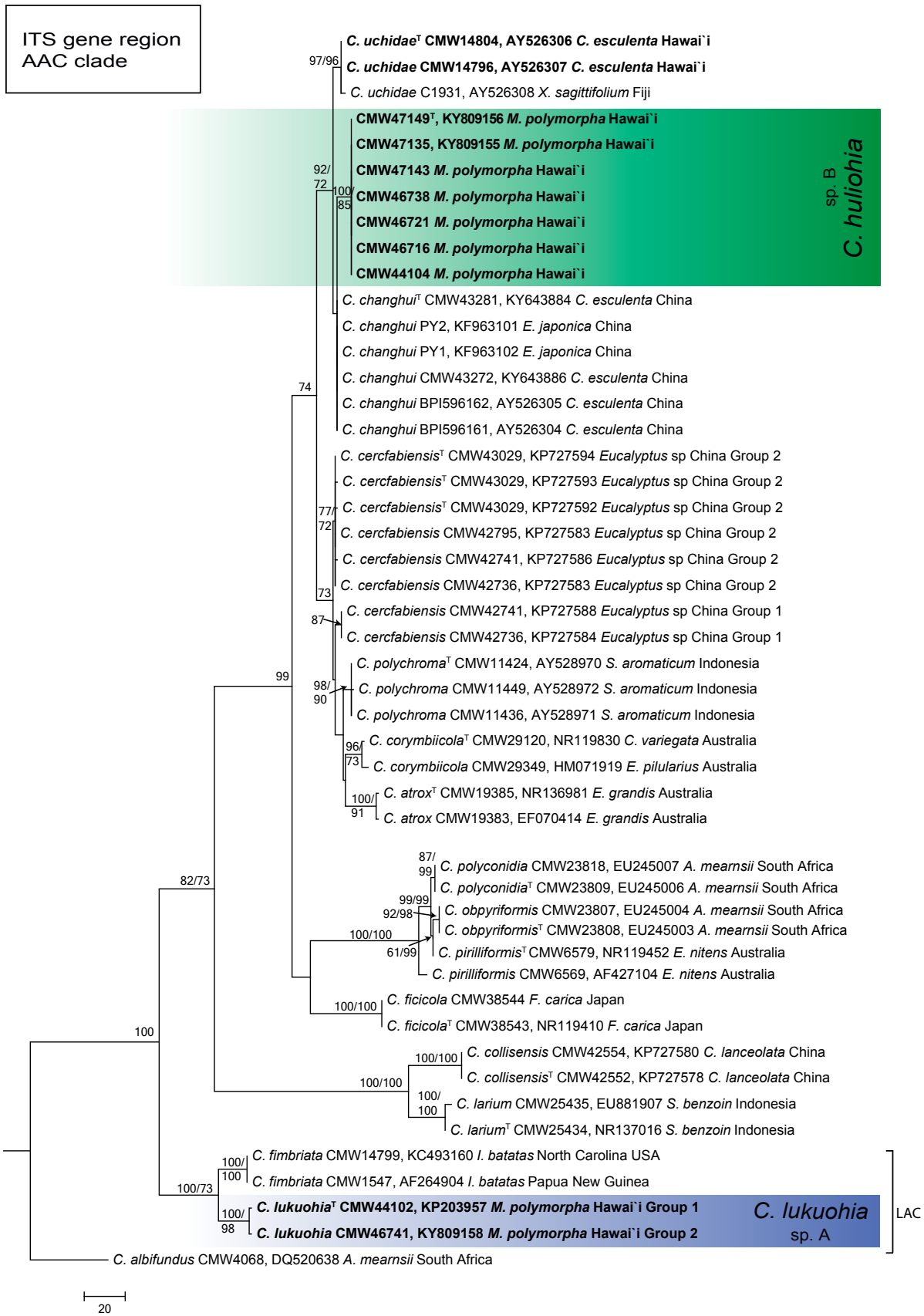

Fig. 6 Phylogenetic tree based on MP of the ITS-rDNA of *Ceratocystis* species in the Asian-Australian clade (AAC). *Ceratocystis huliohia* (sp. B) forms a monophyletic clade and is closest to species of *C. uchidae*, also described from Hawai`i and *C. changhui*. Bootstrap values > 70 % for MP/ML are presented at the branches. *Ceratocystis albifundus* (CMW 4068) from the African clade was used as the outgroup.

Table 3 Morphological measurements (in µm) of isolates of *Ceratocystis lukuohia* (sp. A) and *C. huliohia* (sp. B) on 2 % MEA taken after 2 wk of growth.

	C. lukuohia CMW 44102/P14-1-1 (ITS_A)	*C. lukuohia* CMW 47152/P15-65 (ITS_AC)	*C. lukuohia* CMW 46741/P15-64 (ITS_C)	*C. huliohia* CMW 47149/P15-58	*C. huliohia* CMW 47135/P14-10
Ascomatal base	(151–)161.5–239(–308) × (146.5–)153.5–241.5(–311)	(157.5–)173.5–242(–271) × (142–)169–229(–250.5)	(134–)163–244.5(–306.5) × (121–)161.5–244.5(–272.5)	(128–)175.5–258.5(–310.5) × (116–)156–234(–287.5)	(169.5–)196.5–279(–331.5) × (161–)182–257(–321.5)
Av. ascomatal base	200 × 198	208 × 199	204 × 203	217 × 195	238 × 220
Neck length	(324.5–)534.5–833.5(–971.5)	(560–)727–1022.5(–1244)	(503.5–)690.5–1002.5(–1144.5)	(287.5–)480–824.5(–928.5)	(504–)573.5–781(–871.5)
Av. neck length	684	875	847	652	677
Neck width at base	(12–)22–37(–42.5)	(16–)23.5–31.5(–33.5)	(25–)28.5–35.2(–42.5)	(26–)31–40(–43)	(24–)29.5–39(–41.5)
Av. neck width at base	30	28	32	35.5	34
Neck width at tip	(9–)15–26(–31.5)	(13–)15–21(–28)	(15–)16–20(–22)	(13.5–)16.5–24(–34)	(14–)16.5–22(–28)
Av. neck width at tip	20	18	18	20	19
Ostiolar hyphae length	(40–)56.5–89.5(–111.5)	(38.5–)49–82.5(–98)	(48–)61.5–86.5(–102)	(30–)37.5–56(–70)	(26.5–)29–49.5(–65)
Av. ostiolar hyphae length	73	66	74	47	39
Conidiogenous cell length	(28–)42.5–65.5(–81)	(24.5–)38–53.5(–57.5)	(22.5–)29.5–86.5(–178.5)	(48.5–)50–64.5(–72)	(20.5–)26.5–51(–61.5)
Av. conidiogenous cell length	54	46	58	57	39
Cylindrical conidia	(10–)13–19(–24.5) × (3.5–)4–5	(9.5–)12–19.5(–24) × (2–)3–4(–6)	(11–)14–26(–34.5) × 3–4.4(–6)	(12–)16.5–23.5(–28.5) × (2.5–)3–4(–4.5)	(10–)13–23(–39.5) × 3–4(–5)
Av. cylindrical conidia length	16 × 4	16 × 4	20 × 4	20 × 3.5	18 × 4
Aleurioconidia	(11–)13–15(–16.5) × (8.5–)9.5–10.5(–11.5)	(11–)12–14(–14.5) × (7.5–)8.5–10.5(–12)	(12–)12.5–15.5(–18) × (8–)9.5–11(–11.5)	(10–)12–14.5(–17) × (8–)9.5–12(–14)	(10–)11.5–13.5(–14.5) × (9.5–)10–12(–13.5)
Av. aleurioconidia	14 × 10	13 × 10	14 × 10	13 × 11	13 × 11
Ascospores top view	(4.5–)5–5.5(–6) × (3.5–)4–4.5(–5)	(4–)4.5–5.5 × (3–)3.5–4.5(–5)	(5–)5.5(–6) × 4–4.5	(4–)4.5–5.5(–6) × 3.5–4(–4.5)	4.5–5.5(–6) × (3–)3.5–4(–4.5)
Av. ascospores top view	5 × 4	5 × 4	5 × 4	5 × 4	5 × 4
Ascospores side view	(4.5–)5–6 × 2.5–3(–3.5)	(4–)4.5–5.5 × (2–)2.5–3(–3.5)	5–6 × (2–)2.5–3(–3.5)	(3.5–)4.5–5(–5.5) × (1.5–)2–2.5(–3)	(3–)4–5(–6) × 2–3(–3.5)
Av. ascospores side view	5 × 3	5 × 3	5.5 × 3	5 × 2	4 × 2

Culture characteristics and morphology

Isolates representing the different ITS types of *Ceratocystis* sp. A (CMW 44102/P14-1-1 = haplotype A, CMW 46741/P15-64 = haplotype C and CMW 47152/P15-65 = mixture of haplotypes A and C) and the haplotype B of *Ceratocystis* sp. B (CMW 47149/P15-58 and CMW 47135/P14-10) were used to characterise the culture and morphological characteristics of these fungi (Table 3; Fig. 7–10).

Ceratocystis sp. A and *Ceratocystis* sp. B could be distinguished from each other based on culture characteristics and some morphological features. In culture, the colony growth of *Ceratocystis* sp. A was circular to irregular in form with undulate to lobate colony margins, whereas the colonies of *Ceratocystis* sp. B were circular with smooth to undulate colony margins (Fig. 10). The cultures of *Ceratocystis* sp. A were olivaceous green to brown in colour due to the production of excessive quantities of aleurioconidia in long chains in the medium, with grey aerial mycelium. Cultures of *Ceratocystis* sp. B varied in colour from white to grey to olivaceous green. Sectoring occurred in both taxa. Some isolates of *Ceratocystis* sp. B produced cultures with concentric rings of black ascomata (Fig. 10), and occasionally, multiple necks were observed on single ascomatal bases, particularly in culture CMW 47135/P14-10. In *Ceratocystis* sp. A, the light- to dark-brown to black ascomata were generally scattered throughout the colony. The ascomatal bases of *Ceratocystis* sp. A were generally more globose (av. = 204 × 200 µm) than the more pyriform bases (av. = 227.5 × 207.5 µm) of *Ceratocystis* sp. B, but the differences were minor.

The most obvious morphological characters distinguishing *Ceratocystis* sp. A and *Ceratocystis* sp. B were the average lengths of the necks and ostiolar hyphae. *Ceratocystis* sp. A had longer necks (av. ranging from 684–875 µm among the three isolates) and ostiolar hyphae (av. ranging from 66–74 µm among the three isolates) compared to the shorter necks (av. of the two isolates = 652 and 677 µm) and ostiolar hyphae (av. of the two isolates = 39 and 47 µm) of *Ceratocystis* sp. B. Both cylindrical conidia and the thick-walled aleurioconidia were more abundant in cultures of *Ceratocystis* sp. A, and the aleurioconidia of *Ceratocystis* sp. A were produced in long chains, whereas they were only produced in short chains in *Ceratocystis* sp. B.

Growth in culture

For three isolates of *Ceratocystis* sp. A (CMW 46741, P15-64; CMW 47152, P15-65 and CMW 44102, P14-1-1) and for four isolates of *Ceratocystis* sp. B (CMW 47149, P15-58; CMW 46716, P14-14; CMW 46738, P15-59 and CMW 47135, P14-10), the optimal temperature for growth was 25 °C (Fig. 9). Overall, *Ceratocystis* sp. B grew faster than *Ceratocystis* sp. A at 15, 20, 25 and 30 °C and grew an average of 4.3 mm/d at 25 °C, while *Ceratocystis* sp. A grew an average of 3.6 mm/d. For both taxa, there was a significant reduction in growth at 10 °C. Although there was a sharp decline in growth at 30 °C as compared to 25 °C for both taxa, *Ceratocystis* sp. B (43 mm diam) had almost double the growth of *Ceratocystis* sp. A (25 mm diam) at this temperature after 14 d. For both taxa, ascomatal production was most abundant when grown at 20 and 25 °C, and no ascomata were produced at 10 and 30 °C.

TAXONOMY

Phylogenetic inference based on five gene regions showed conclusively that there are two distinct taxa associated with the death of *M. polymorpha* trees on the Island of Hawai`i. *Ceratocystis* sp. A and *Ceratocystis* sp. B reside in the LAC and AAC biogeographical clades of *Ceratocystis* s.lat., respectively, and they are phylogenetically distinct from the other described

species in those clades. These two putative new taxa could be distinguished from each other based on morphology. These results provide robust evidence to justify describing the two taxa as new species as follows (where *Ceratocystis* sp. A = *Ceratocystis lukuohia* and *Ceratocystis* sp. B = *Ceratocystis huliohia*):

Ceratocystis lukuohia I. Barnes, T.C. Harr. & L.M. Keith, *sp. nov.* — MycoBank MB824050; Fig. 2, 7

Etymology. The name lukuohia is derived from the Hawaiian words luku (destruction) and `ōhi`a, and reflects the fact that this fungus destroys `ōhi`a trees.

Type. USA, Hawai`i, Puna District, Leilani Estates, isolated from diseased *Metrosideros polymorpha*, 20 Feb. 2014, *J.B. Friday* (holotype PREM 61770, culture ex-type CMW 44102 = P14-1-1 = CBS 142792 = MUCL 56342). ITS sequence GenBank KY809157.

Ascomata perithecial, developing within 8 d and mature within 12 d, mostly scattered, sometimes gregarious, partially embedded or superficial in agar; *bases* subglobose to globose (151–)161.5–239(–308) × (146.5–)153.5–241.5(–311) µm, dark brown to black, covered with undifferentiated hyphae and conidiophores with aleurioconidia. *Necks* straight, occasionally curved, (324.5–)534.5–833.5(–971.5) µm long, smooth, brown to black at base (12–)22–37(–42.5) µm wide

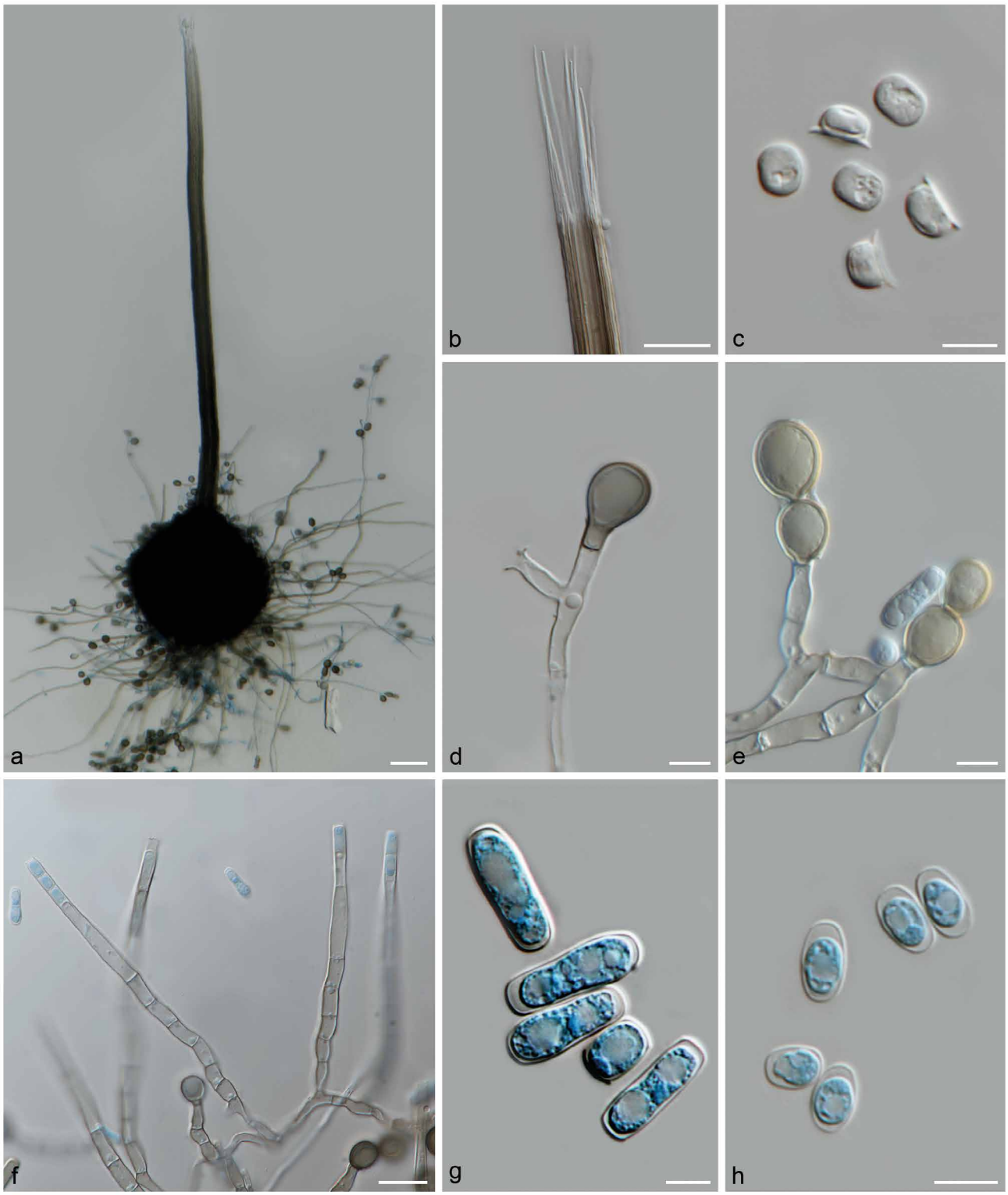

Fig. 7 Morphological characteristics of *Ceratocystis lukuohia* (sp. A). a. Globose ascomata base with long neck; b. straight ostiolar hyphae; c. hat-shaped ascospores from top and side view; d. terminal thick-walled aleurioconidium; e. aleurioconidia in chains; f. flask-shaped conidiophores; g. cylindrical conidia; h. short, barrel-shaped conidia. — Scale bars: a = 50 µm; b, f = 20 µm; c, g–h = 5 µm; d–e = 10 µm.

and becoming paler towards tapering apex (9–)15–26(–31.5) µm wide. *Ostiolar hyphae* mostly straight, occasionally divergent, non-septate, (40–)56.5–89.5(–111.5) µm long, hyaline. *Asci* evanescent, not seen. *Ascospores* accumulate in white to creamy masses at tips of necks, single-celled, hyaline, ellipsoidal in top view (4.5–)5–5.5(–6) × (3.5–)4–4.5(–5) µm with gelatinous sheath giving hat-shaped appearance in side view (4.5–)5–6 × 2.5–3(–3.5) µm. *Conidiophores* branched or unbranched, straight or flexuous, hyaline to pale brown, multiseptate, smooth-walled. *Conidiogenous cells* endophialidic with enteroblastic conidium ontogeny, flask-shaped (lageniform) or tubular (28–)42.5–65.5(–81) µm long. *Conidia* mostly cylindrical with apices rounded, smooth, non-septate, hyaline, (10–)13–19(–24.5) × (3.5–)4–5 µm, borne in long chains; other conidia barrel-shaped with truncate bases, smooth-walled, non-septate, hyaline, 4–5.5(–6.5) × 3–4.5(–5) µm. *Aleurioconidia* abundant, pale brown to brown, pyriform to ovoid (11–)13–15(–16.5) × (8.5–)9.5–10.5(–11.5) µm, thick-walled, formed singly or in long chains.

Cultural characteristics — Colonies on 2 % MEA circular to irregular with undulate to lobate colony margins, mycelium submerged to aerial, producing fruity aroma; submerged mycelium greenish olivaceous, mainly due to aleurioconidia; aerial mycelium light grey, frequently sectoring. Optimal temperature for growth 25 °C, with colony diam reaching 66.5 mm after 14 d. Optimal temperature for ascomatal production 20–25 °C.

Substratum — Woody xylem of trunk, stems and branches of *M. polymorpha*.

Distribution — Hawai`i Island, HI, USA.

Additional specimens. USA, Hawai`i, Puna District, Nānāwale, isolated from diseased *M. polymorpha*, 3 June 2015, *T. Sowards* (paratype PREM 61771, culture ex-paratype CMW 46741 = P15-64 = CBS 142793 = MUCL 56343) (ITS haplotype C); South Hilo District, Waiākea Forest Reserve, isolated from diseased *M. polymorpha*, 27 May 2015, *T. Sowards* (paratype PREM 61772, culture ex-paratype CMW 47152 = P15-65 = CBS 142591 = MUCL 56344) (ITS haplotype A/C).

Notes — Typical symptoms associated with *C. lukuohia* include rapid, synchronized death of the entire crown and extensively stained sapwood.

Ceratocystis lukuohia has two different ITS haplotypes (GenBank KY809157 and KY809158), and most isolates appear to have a mixture of the two haplotypes among the tandem repeats of the rDNA operons. It is phylogenetically most closely related to *C. platani* (Engelbrecht & Harrington 2005) and an undescribed species of *Ceratocystis* isolated from *Xanthosoma* sp. in Cuba and Costa Rica and from *Syngonium* in Hawai`i and Florida (Thorpe et al. 2005). *Ceratocystis lukuohia* can be distinguished from these *Ceratocystis* spp. based on ITS, *ms204* and *rpb2* and from *C. platani* with ITS, *ms204*, *rpb2*, *ef1* and *bt1* sequence data. Morphologically, *C. lukuohia* is very similar to these other *Ceratocystis* spp. and they cannot reliably be distinguished from each other, although isolates from *Syngonium* and *Xanthosoma* generally have less aerial mycelium.

Ceratocystis huliohia I. Barnes, T.C. Harr. & L.M. Keith, *sp. nov.* — MycoBank MB824051; Fig. 3, 8

Etymology. The name huliohia is derived from the Hawaiian words huli (to turn) and `ōhi`a and refers to the fact that this fungus changes the natural state of `ōhi`a trees.

Type. USA, Hawai`i, North Kona District, Hōlualoa, isolated from diseased *M. polymorpha*, 10 June 2015, *J.B. Friday* (holotype PREM 61769, culture ex-type CMW 47149 = P15-58 = CBS 142794 = MUCL 56340). ITS sequence GenBank KY809156.

Ascomata perithecial, developing within 8 d and mature within 12 d, aggregated, partially embedded in agar; *bases* subglobose to pyriform (128–)175.5–258.5(–310.5) × (116–)156–234(–287.5) µm, dark brown to black, covered with undifferentiated hyphae. Spines and ornamentations absent. *Necks* straight or occasionally slightly curved and (287.5–)480–824.5(–928.5) µm long, wider at base (26–)31–40(–43) µm and tapering towards apex (13.5–)16.5–24(–34) µm, unbranched, light brown to dark brown or black, smooth to crenulate. *Ostiolar hyphae* straight to mostly divergent, non-septate, hyaline to light brown, (30–)37.5–56(–70) µm long. *Asci* evanescent, not seen. *Ascospores* accumulate in white to creamy masses at tips of necks, single-celled, hyaline, ellipsoidal in top view (4–)4.5–5.5(–6) × 3.5–4(–4.5) µm with gelatinous sheath giving hat-shaped appearance in side view (3.5–)4.5–5(–5.5) × (1.5–)2–2.5(–3) µm. *Conidiophores* branched or unbranched, straight or flexuous, hyaline to pale brown, multiseptate, smooth-walled. *Conidiogenous cells* endophialidic, flask-shaped (lageniform) (48.5–)50–64.5(–72) µm in length producing primary conidia in chains by means of enteroblastic conidium ontogeny. *Conidia* hyaline, non-septate, smooth-walled, cylindrical or bacilliform with rounded apices, mostly (12–)16.5–23.5(–28.5) µm long × (2.5–)3–4(–4.5) µm wide. *Aleurioconidia* ovoid, thick-walled, hyaline when young, becoming brown when mature, smooth, formed mostly singly (terminal) or in short chains, (10–)12–14.5(–17) × (8–)9.5–12(–14) µm in size.

Culture characteristics — Colonies on 2 % MEA showing circular growth with entire (smooth) to undulate colony margins. Mycelium submerged to aerial, producing fruity aroma; submerged mycelium greenish olivaceous, aerial mycelium light grey, turning dark olivaceous green to brown with age. Ascomata/perithecia either produced in clumps or in a concentric 'ring-like' fashion. Optimal temperature for growth 25 °C, with colony diam reaching 58.4 mm after 14 d. Optimal temperature for ascomatal production 20–25 °C.

Substratum — Woody xylem of trunk, stems and branches of *M. polymorpha*.

Distribution — Hawai`i Island, HI, USA.

Additional specimens. USA, Hawai`i, Puna District, `Opihikao, isolated from diseased *M. polymorpha*, 8 July 2014, *T. Sowards* (paratype PREM 61768, culture ex-paratype CMW 47135 = P14-10 = CBS 142795 = MUCL 56341).

Notes — *Ceratocystis huliohia* has been isolated from stained sapwood of trees showing rapid, synchronized death of major branches or death of the entire crown. However, it has also been isolated from trees that have appeared to be dying gradually over a period of years. Phylogenetically, *C. huliohia* groups in the *C. polychroma* cluster of the AAC and is closest to *C. uchidae*, *C. changhui*, *C. cercfabiensis* and *C. polychroma*, which are generally less aggressive plant pathogens than members of the LAC. Morphological features within individuals of the same species can show great variation in the dimensions of measured morphological characteristics and in some cases, these overlap with other species (Li et al. 2017, Liu et al. 2018). However, based on average measurements, *C. huliohia* can be distinguished from its closest relatives based on ascomatal size, the length of the neck and ostiolar hyphae (Li et al. 2017, Liu et al. 2018). The ascomatal bases of *C. huliohia* (av. of two isolates = 227.5 × 207.5 µm, Table 3) are somewhat larger than those of *C. uchidae* (range 95–190 diam), *C. changhui* (av. = 182 × 180.5 µm) and *C. cercfabiensis* (av. = 178 × 184 µm). The ascomatal necks of *C. huliohia* (av. of two isolates = 664.5 µm) are longer than *C. uchidae* (av. = 422.5 µm) but shorter than *C. changhui* (av. = 702 µm), *C. cercfabiensis* (av. = 1 114.5 µm) and *C. polychroma* (av. = 960 µm). The length of ostiolar hyphae of *C. huliohia* (av. of two isolates = 43 µm) is shorter than that of *C. uchidae* (av. = 57.5 µm), *C. changhui* (av. = 82 µm) and *C. cercfabiensis* (av. = 59 µm), but slightly longer than *C. polychroma* (av. = 38 µm).

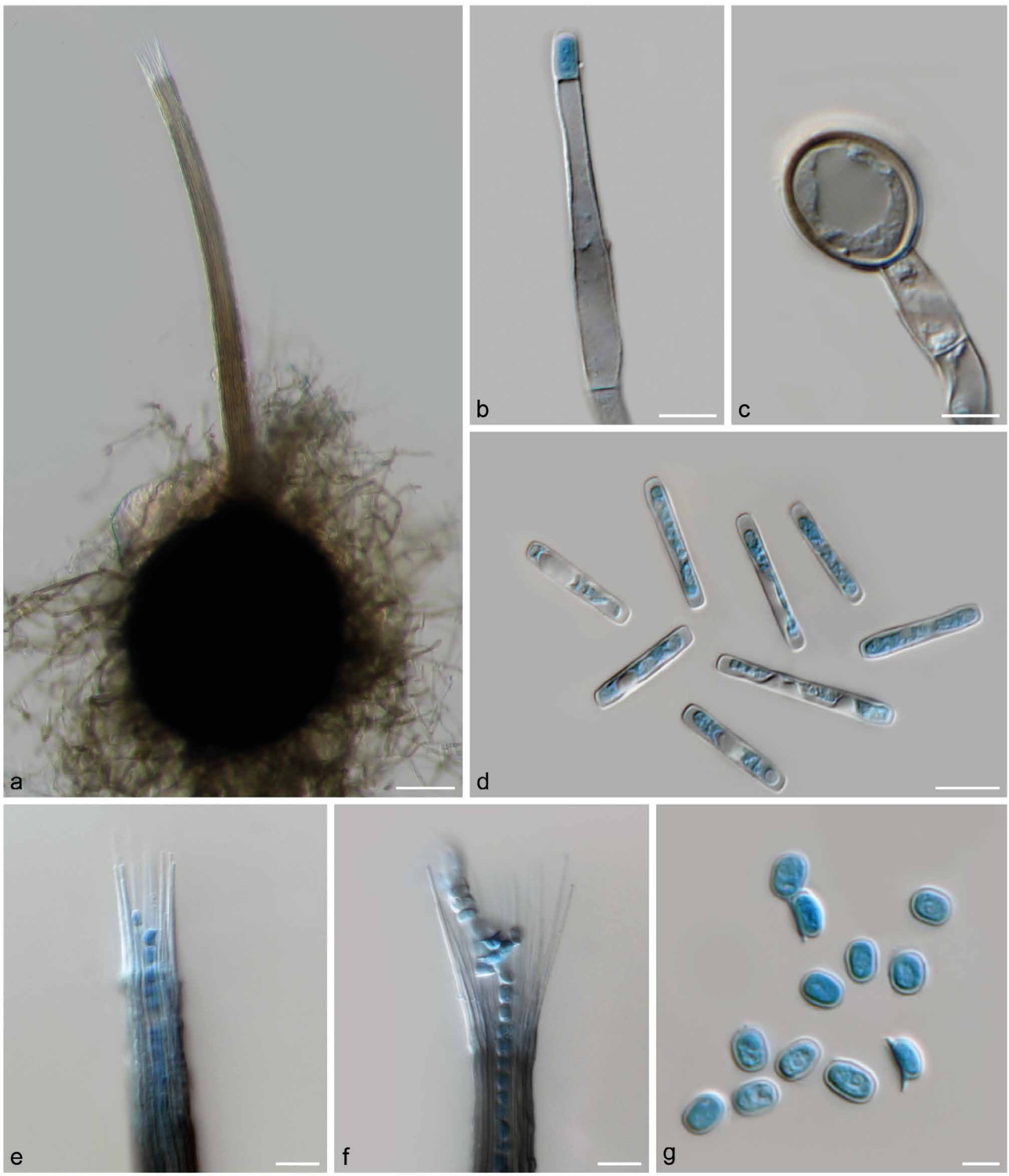

Fig. 8 Morphological characteristics of *Ceratocystis huliohia* (sp. B). a. Ascomata with globose to obpyriform base and short neck; b. conidiophore with emerging, short, barrel-shaped conidium; c. terminal aleurioconidium; d. cylindrical conidia; e. short, straight ostiolar hyphae; f. divergent ostiolar hyphae; g. hat-shaped ascospores in side and top view. — Scale bars: a = 50 µm; b, d–f = 10 µm; c, g = 5 µm.

Ceratocystis lukuohia and *C. huliohia* can clearly be differentiated from each other based on culture characteristics and morphology. On MEA, *C. lukuohia* grows more slowly than *C. huliohia* (Fig. 9) and has smaller, more rounded ascomata that are distributed evenly in olivaceous green to brown cultures. Sectoring is more common in cultures of *C. lukuohia* than in cultures of *C. huliohia*, but both species frequently change mycelial phenotype. The culture colour in *C. huliohia* varies considerably and can be white, to grey, to olivaceous green (Fig. 10). The larger ascomata of *C. huliohia* are often clumped together (Fig. 3) and in some cases, multiple necks are seen from the same ascomatal base. In both species, optimal growth is at 25 °C (Fig. 9) with ascomata production occurring between 20–25 °C. The most distinguishing morphological features of these two species are the longer necks and ostiolar hyphae in *C. lukuohia*. *Ceratocystis lukuohia* also produces prolific amounts of conidia and aleurioconidia in long chains.

Genotyping of Ceratocystis isolates

The 10 *C. lukuohia* isolates used in the phylogenetic studies for the LAC were clonal at 27 microsatellite loci. The remaining 49 isolates that were screened with a reduced number of markers (10–20) were also all clonal (Appendix). Isolates having the A

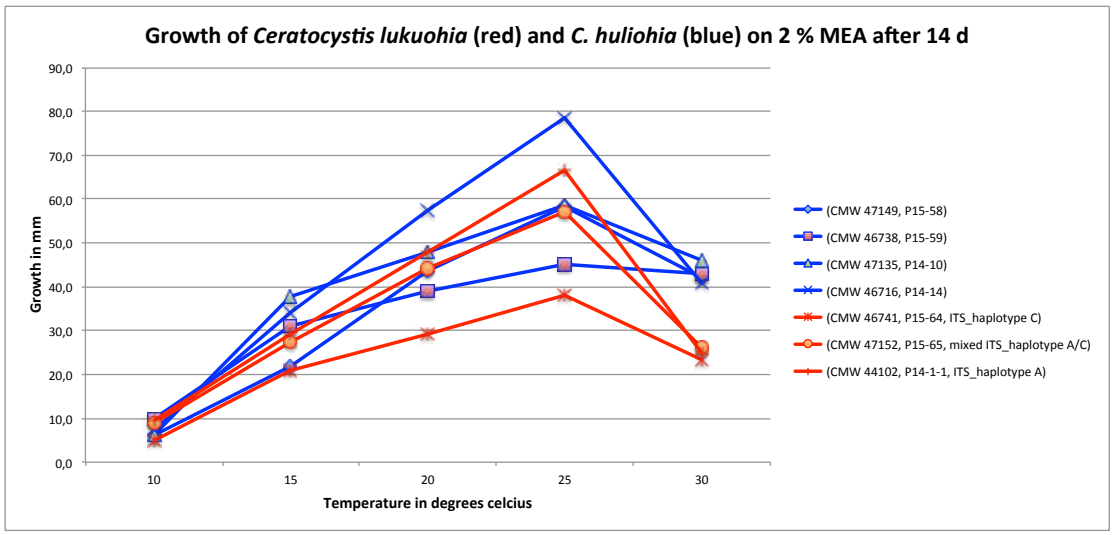

Fig. 9 Diameter of colonies in mm of *Ceratocystis lukuohia* (sp. A) and *C. huliohia* (sp. B) isolates on 2 % malt extract agar at five different temperatures taken after 14 d growth. *Ceratocystis huliohia* grows slightly faster than *C. lukuohia*. Both species have optimal growth at 25 °C.

ITS haplotype (18 isolates), the C ITS haplotype (2 isolates) or mixtures of A and C haplotype (35 isolates) did not differ in their multilocus microsatellite profile. The six *Ceratocystis* isolates from *Xanthosoma* and *Syngonium*, which were phylogenetically closest to *C. lukuohia*, had ± 20 % alleles in common with this species. Similarly, the clonal lineages of *C. platani* isolates from continental USA, Greece, Switzerland, France and Italy were identical to *C. lukuohia* at only 33.3 % of the scorable loci (Appendix). The multilocus genotype (MLG) of the *C. fimbriata* isolate from sweet potato in Hawai`i was very different to that of *C. lukuohia* and only shared 7.4 % of the total alleles.

The nine *C. huliohia* isolates from Hawai`i all shared the same MLG and were, therefore, clonal at all 27 microsatellite loci. Isolates of *C. huliohia* shared 47.80 % similar alleles with *C. polychrome*, 52.17 % with *C. uchidae*, 60 % with *C. changhui* and 70.83 % with *C. cercfabiensis*. The two *Ceratocystis* species causing disease on *M. polymorpha* only shared a single allele, out of a total of 26. Primer set AF6 did not amplify in either species.

Interfertility tests

In the parings among closely-related species in the LAC or in pairings among closely-related species in the AAC, almost all of the crosses between male, MAT2 and female, MAT1 testers resulted in ascomata with ascospore masses at the tips of their necks. Ascospores germinated when they were streaked onto the surface of MYEA, but the germination percentages were relatively low in crosses between different species (Table 4, 5). When ascospores were produced in either interspecific or intraspecific pairings, progeny arising from the ascospores plated

Table 4 Mating reactions (production of normal or abnormal ascospores/with high, medium, low or no germination) in two replicated pairing experiments between MAT1, female tester strains of *Ceratocystis* spp. in the Latin American clade and MAT2, male testers of *Ceratocystis lukuohia*, *C. platani* and *Ceratocystis* sp. from *Syngonium*.

		MAT2 males				
		C. lukuohia			*C. platani*	*Ceratocystis* sp. from *Syngonium*
	MAT1 females	From C4133	From C4185	From C4186	From C1342	From C1774
C. lukuohia	From C4185	Normal/high, Normal/high[1]	Normal/high, Normal/high	Normal/high, Normal/high	Abnormal/NT, Abnormal/medium	Abnormal/medium, Normal/medium
	From C4210 (P15-64)	Abnormal/high, Normal/high	Normal/high, Normal/high	Normal/high, Normal/high	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium
	From C4212 (P14-1-1)	Abnormal/high, Abnormal/high	Normal/high, Abnormal/high	Normal/high, Normal/high	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium
	From C4213 (P15-65)	Abnormal/high, Normal/high	Normal/high, Normal/high	Normal/high, Normal/high	No ascomata, Abnormal/medium	Abnormal/medium, Abnormal/medium
C. platani	From C1339	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Normal/high, Normal/high	Abnormal/low, Abnormal/medium
	From C1329	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Normal/high, Normal/high	Abnormal/medium, Abnormal/medium
Ceratocystis sp. from *Syngonium*	From C1717	Abnormal/high, Abnormal/high	Abnormal/medium, Abnormal/low	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Normal/medium, Normal/high
	From C4118	Abnormal/medium, Abnormal/medium	Abnormal/high, Normal/high	Abnormal/high, Abnormal/medium	Abnormal/medium, Abnormal/medium	Normal/high, Normal/high
C. colombiana	From C1945	Abnormal/low, Abnormal/medium	Abnormal/low, Abnormal/medium	Abnormal/low, Abnormal/low	Abnormal/medium, Abnormal/medium	Abnormal/low Abnormal/low
C. cacaofunesta	From C1833	Abnormal/low, Abnormal/none	Abnormal/none, Abnormal/none	Abnormal/low, Abnormal/none	Abnormal/none, Abnormal/low	Abnormal/low, Abnormal/medium
C. fimbriata	From C4135	No ascomata, No ascomata	No ascomata, No ascomata	No ascomata, No ascomata	No ascomata, No ascomata	No ascomata, No ascomata

[1] Microscopic examination of abnormal ascospore masses showed more than 10 % of the ascospores misshapen or empty and typically with ascus or ascospore debri. Streaked ascospore mass on malt yeast extract agar: high germination = colonies too numerous to count on three plates; medium germination = 40 or more colonies on three plates; and low germination = fewer than 40 colonies on the three plates. Pairings or ascospore germinations not tested are indicated by NT.

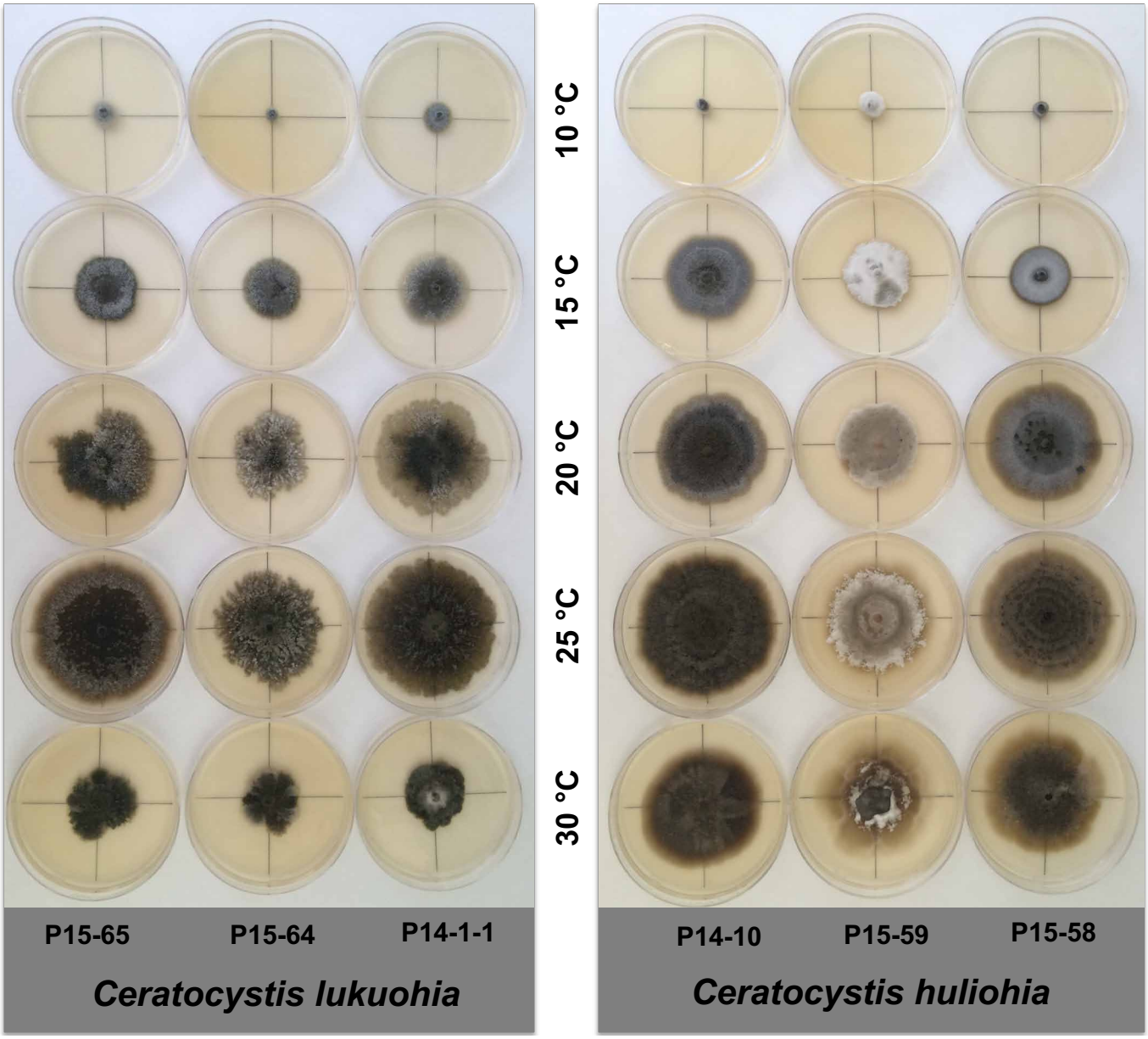

Fig. 10 Variation in culture morphology of isolates of *Ceratocystis lukuohia* (sp. A) and *C. huliohia* (sp. B) after 14 d growth on 2 % MEA at temperatures 10, 15, 20, 25 and 30 °C.

onto MYEA showed some variation in the mycelial phenotype (colony texture and in presence/absence of ascomata), which suggested that sexual recombination had occurred. However, microscopic examination of the ascospores showed substantial variation among interspecific crosses in the abundance and proportion of misshapen ascospores. In most cases of pairings between two strains of the same species, less than 10 % of the ascospores were misshapen or empty, but in most interspecific pairings, greater than 10 % of the ascospores were misshapen (Table 4, 5). In most of the cases, where a pairing of isolates was conducted twice, the identical results were obtained.

With few exceptions, the female, MAT1 testers were only fully interfertile (production of normal appearing ascospores and high germination percentage) with male testers of the same species. For *C. lukuohia*, three of the four female testers produced normal ascospores with high germination percentages with each of the three male testers of *C. lukuohia* in at least one of the two tests (Table 4). The fourth female tester (from C4212) produced abnormal ascospores in both crosses with the male tester from isolate C4133 of *C. lukuohia*, but the percentage of germinating ascospores was high in both cases. The four female testers of *C. lukuohia* produced abnormal ascospores and only medium germination percentages with the male testers of *C. platani* and the strain isolated from *Syngonium*. The female tester strains of *C. platani* produced normal ascospores with high germination percentage only with the male tester of *C. platani*. The female testers of the *Syngonium* strain produced normal ascospores with high germination percentages with the male *Syngonium* tester, but high germination percentages also were obtained for some of the ascospore masses taken from the *Syngonium* MAT1 testers after spermatization with male testers of *C. lukuohia* (Table 4). The female tester strains of *C. colombiana* and *C. cacaofunesta* produced abnormal ascospores with low or medium germination percentages when crossed with the male testers, and in some cases ascospores taken from female *C. cacaofunesta* ascomata spermatized with *C. lukuohia* male testers failed to germinate (Table 4). The female tester of the sweet potato strain of *C. fimbriata* produced no ascomata with any of the male tester strains.

Similar results were found in pairings among representative tester strains of the AAC (Table 5). The four female testers of *C. huliohia* produced normal ascospores and high germination percentages only in pairings with the two male testers of *C. huliohia*; the two female testers of *C. uchidae* produced

Table 5 Mating reactions (production of normal or abnormal ascospores/with high, medium, low or no germination) in two replicated pairing experiments between MAT1, female tester strains of *Ceratocystis* spp. from the Asian-Australian clade and MAT2, male testers of *Ceratocystis huliohia*, *C. uchidae*, *C. cercfabiensis* and *C. polychroma*.

		MAT2 males				
		C. huliohia		*C. uchidae*		*C. cercfabiensis*
	MAT1 females	From C4215 (P15-59)	From C4192	From C1714	From C1931	From C3358
C. huliohia	From C4191	Normal/high, Normal/high[1]	Normal/high, Normal/high	Abnormal/low, Abnormal/low	Abnormal/medium, Abnormal/medium	Abnormal/medium, NT
	From C4192	Normal/high, Normal/high	Normal/high, Normal/high	Abnormal/medium, Abnormal/low	Abnormal/medium, NT	Abnormal/low, Abnormal/medium
	From C4211	Normal/high, Normal/high	Normal/high, Normal/high	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium
	From C4190	Normal/high, Normal/high	Normal/NT, Normal/high	Abnormal/low, Abnormal/low	Abnormal/medium, Abnormal/medium	Abnormal/high, Abnormal/medium
C. uchidae	From C1714	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Abnormal/high, Normal/high	Normal/high, Normal/high	Abnormal/medium, Abnormal/medium
	From C1715	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Normal/high, Normal/high	Normal/high, Normal/high	Abnormal/medium, Abnormal/medium
C. cercfabiensis	From C3301	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/low	Abnormal/low, Abnormal/medium	Normal/medium, Normal/medium	Normal/high, Normal/high
	From C3356	Abnormal/medium, Abnormal/low	Abnormal/NT, Abnormal/medium	Abnormal/medium, Abnormal/medium	Abnormal/high, Abnormal/medium	Normal/high, Normal/high
	From C3302	Normal/medium, NT	Normal/medium, NT	Abnormal/medium, NT	Abnormal/medium, NT	Normal/high, NT
	From C3305	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Abnormal/medium, Abnormal/medium	Normal/medium, Normal/medium	Normal/high, Normal/medium
C. polychroma	From C2240	Sterile ascomata	Sterile ascomata	Abnormal/low, Abnormal/low	Abnormal/low, Abnormal/medium	Abnormal/medium, Abnormal/medium

[1] Microscopic examination of abnormal ascospore masses showed more than 10 % of the ascospores misshapen or empty and typically with ascus or ascospore debri. Streaked ascospore mass on malt yeast extract agar: high germination = colonies too numerous to count on three plates; medium germination = 40 or more colonies on three plates; and low germination = fewer than 40 colonies on the three plates. Pairings or ascospore germinations not tested are indicated by NT.

normal ascospores with high germination when paired with the two male testers of *C. uchidae*; and the four female testers of *C. cercfabiensis* only produced normal ascospores with high germination with the male testers of *C. cercfabiensis*. Normal ascospores with low to medium germination were found in some crosses between three of the *C. cercfabiensis* female testers and males of either *C. huliohia* or *C. uchidae* (Table 5). The female tester of *C. polychroma* produced only sterile ascomata (no ascospore masses) when paired with *C. huliohia* and only abnormal ascospores with medium to low germination percentages with *C. uchidae* and *C. cercfabiensis* male tester strains (Table 5).

Pathogenicity tests

Inoculations of *M. polymorpha* with isolates of *C. lukuohia* and *C. huliohia* resulted in wilt symptoms in 2–4 wk. As symptoms progressed, leaves withered, died and remained attached to the plants. Complete plant mortality occurred between 4 wk to 1 yr for 91.7 % of the seedlings inoculated with *C. lukuohia* and 70 % of the seedlings inoculated with *C. huliohia* (Table 6). For both species, xylem discolouration was observed in the main stem and the pathogen was re-isolated from all inoculated plants. The reisolated cultures were the same *Ceratocystis* species inoculated into the plant based on morphology and sequencing the ITS region. All control plants remained healthy. *Metrosideros polymorpha* inoculated with the *Ceratocystis* isolates from *Syngonium* did not produce symptoms (Table 6). Similarly, no wilt symptoms were observed on seedlings of *Platanus* × *acerifolia* regardless of whether they had been inoculated with either of the new *Ceratocystis* spp. or with the isolates from *Syngonium* (Table 6).

Syngonium podophyllum inoculated with *Ceratocystis* isolates from *Syngonium* resulted in tissue collapse and discolouration of the petiole. Inoculated pseudostems of *S. podophyllum* died within two weeks. No symptoms were observed on *Syngonium* inoculated with either of the two new species of *Ceratocystis*

Table 6 Percent mortality of four host species with isolates of *Ceratocystis lukuohia*, *C. huliohia* and *Ceratocystis* sp. from *Syngonium*.

Inoculated host	Source of inoculum[1]	Total no. of inoculated plants	Mortality (%)
Metrosideros polymorpha	*C. lukuohia* from *M. polymorpha* (CMW 44102, P14-1-1)	12	91.7
	C. huliohia from *M. polymorpha* (CMW 46738, P15-59)	10	70.0
	Ceratocystis sp. from *Syngonium* (CMW 50456, P16-1)	8	0.0
Platanus × *acerifolia*	*C. lukuohia* from *M. polymorpha* (CMW 44102, P14-1-1)	8	0.0
	C. huliohia from *M. polymorpha* (CMW 46738, P15-59)	8	0.0
	Ceratocystis sp. from *Syngonium* (CMW 50456, P16-1)	8	0.0
Syngonium podophyllum	*C. lukuohia* from *M. polymorpha* (CMW 44102, P14-1-1)	8	0.0
	C. huliohia from *M. polymorpha* (CMW 46738, P15-59)	8	0.0
	Ceratocystis sp. from *Syngonium* (CMW 50456, P16-1 and CMW 50457, P16-2)	16	75.0
Colocasia esculenta	*C. lukuohia* from *M. polymorpha* (CMW 44102, P14-1-1)	6	16.7[2]
	C. huliohia from *M. polymorpha* (CMW 46738, P15-59)	6	16.7[2]

[1] CMW = Culture collection of the Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; P = culture collection of the USDA Agricultural Research Service, Hilo, HI.

[2] One pseudopetiole of one plant.

from *M. polymorpha* (Table 6). One pseudopetiole of *Colocasia esculenta* inoculated with *C. lukuohia* and one inoculated with *C. huliohia* died (Table 6). Mortality was most likely due to natural senescence and not the pathogen since observed discolouration was no more than in the controls.

DISCUSSION

Results of this study have shown that two new *Ceratocystis* species, *C. lukuohia* and *C. huliohia*, are associated with the devastating mortality seen in native *M. polymorpha* forests, known as rapid `ōhi`a death (ROD). Recognition of these species as novel taxa in *Ceratocystis* was based on a combination of phenotypic, biological, morphological and phylogenetic data. Phylogenetically, *C. lukuohia* resides in the Latin American clade and *C. huliohia* in the Asia-Australian clade of *Ceratocystis*. Neither species was fully interfertile with their closest relatives in those respective clades, and neither appeared to be pathogenic to *Syngonium podophylum*, *Colocasia esculenta* or *Platanus* × *acerifolia*, the hosts of their nearest known relatives. Microsatellite analyses have shown that they represent two clonal lineages, suggesting that they represent recent introductions into Hawai`i.

Widespread mortality of *M. polymorpha* has been noted in past decades (Hodges et al. 1986), but in the current epidemic, the trees die more rapidly (Keith et al. 2015). In most ROD cases, a major branch or the whole crown suddenly shows wilt symptoms, and discolouration that follows along the ray parenchyma of the woody xylem is strongly evident. These symptoms are characteristic of wilt or canker-stain diseases caused by those *Ceratocystis* spp. that reside in the Latin American clade (Harrington 2013, Roux & Wingfield 2013). The initial *Ceratocysis* sp. associated with *M. polymorpha* mortality was originally identified as *C. fimbriata* based on morphology and ITS sequences, which were similar to those of other members of the LAC, including the canker-stain pathogen *C. platani* (Baker et al. 2003) and a *Syngonium* strain that had been previously reported in Hawai`i (Thorpe et al. 2005, Keith et al. 2015). Koch's postulates were completed with small seedlings of *M. polymorpha*, and the disease was reported as Ceratocystis wilt (Keith et al. 2015). The present study confirms the association of this *Ceratocystis* sp. with the Ceratocystis wilt epidemic and describes the pathogen as a new species, *C. lukuohia*. This study also associates a second *Ceratocystis* sp., *C. huliohia*, with the *M. polymorpha* mortality, and seedling inoculations confirm its capacity to cause wilt-like symptoms in `ōhi`a.

Minor differences in symptoms and rate of symptom development are associated with the two *Ceratocystis* species. For example, mortality caused by *C. lukuohia* typically occurs rapidly and is associated with an extensive staining of the wood that is brown-black in colour, with discolouration developing in a radial pattern. In contrast, *C. huliohia* is commonly isolated from dying trees that show partial crown death, and the staining of the sapwood is often more diffuse and brown to grey-black in colour. In transverse sections of wood, the staining associated with *C. huliohia* appears more blotchy and can be seen following the contours of the cambium, perhaps due to host defence responses in sapwood tissues near the cambium. Inoculation of seedlings indicates that *C. huliohia* is aggressive on *M. polymorpha*, but field observations on the progression of symptoms in naturally infected trees suggest that it is less aggressive than *C. lukuohia*. This is consistent with the generalization that species of *Ceratocystis* in the Asian-Australian clade are less aggressive pathogens than species in the Latin American clade (Harrington 2013, Li et al. 2016). Further work is needed to characterize the disease on *M. polymorpha* caused by *C. huliohia* and its role in the currently widespread mortality known as rapid `ōhi`a death. In the present study, *C. lukuohia* was the more commonly isolated pathogen from suddenly wilting trees, and it has been consistently associated with the dramatic mortality found in new outbreak areas on Hawai`i Island.

Thus far, the distribution of the two *Ceratocystis* pathogens appears to be limited to the Island of Hawai`i. The spread of the pathogens, from the initial discovery in the Puna district on the southeast side of the island in 2010, has been hypothesised to be due to a combination of anthropogenic movement of infected material used for firewood or contaminated tools and aerial dispersal of infected insect boring dust. Some *Ceratocystis* species are known for their loose association with bark beetles (*Scolytinae*), ambrosia beetles (*Scolytinae* and *Platypodinae*), nitidulid beetles (*Coleoptera*: *Nitidulidae*) and flies (*Diptera*) that act as pathogen vectors (Wingfield et al. 2017). However, members of the LAC have been more commonly associated with wind dispersal of aleurioconidia in the frass of wood-boring ambrosia beetles (Harrington 2013). Non-native ambrosia beetles, such as *Xyleborinus saxesenii* and various Asian *Xyleborus* spp., bore into the colonised sapwood of killed *M. polymorpha* trees (Samuelson 1981), and both species of *Ceratocystis* have been detected in the frass expelled to the outside of the trees during beetle tunnelling. The frass, contaminated with abundant aleurioconidia, could then be airborne and dispersed in winds. Infections of healthy trees would then take place via natural or induced wounds. However, the modes of transmission and the associated insects involved, require further investigation.

Ceratocystis species are capable of selfing through unidirectional mating type switching, but they can also outcross (Harrington & Mcnew 1997, Witthuhn et al. 2000, Wilken et al. 2014, Wilson et al. 2015). Thus, introduced strains are capable of reproducing clonally through sexual as well as asexual reproduction, and introduced populations typically show only limited genetic variation (Engelbrecht et al. 2004, 2007, Ocasio-Morales et al. 2007, Al Adawi et al. 2014, Oliveira et al. 2015, Li et al. 2016, Scruggs et al. 2017). Isolates of both *C. lukuohia* and *C. huliohia*, collected across three different districts of Hawai`i Island, showed no variation in alleles at 23 microsatellite loci. The genetic homogeneity of both pathogens indicates that they are each reproducing clonally and more importantly, this signifies recent introductions of single genotypes of each of the pathogens into Hawai`i. Distinct microsatellite alleles and phylogenetic concordance among the DNA sequences of each of the nuclear genes show that the two genotypes are members of the respective Asian-Australian and Latin American geographic clades, which are only distantly related. Thus, it is unlikely that the two species are two genotypes of a single hybridization event between an Asian species and a Latin American species. However, further genetic studies are needed to confirm intersterility and reproductive isolation between the two species.

The possible origins of *C. lukuohia* and *C. huliohia* and their pathways into Hawai`i are unknown, but species of *Ceratocystis* have been frequently dispersed to new regions in vegetatively propagated plant material (Thorpe et al. 2005, Engelbrecht et al. 2007, Ferreira et al. 2011, Harrington 2013, Oliveira et al. 2015, Li et al. 2016). Phylogenetically, *C. lukuohia* and *C. huliohia* are distinct from each other, and they reside in distinctly different geographical clades of *Ceratocystis*. *Ceratocystis lukuohia* resides in the LAC, and its closest relatives are *C. platani* and strains of an undescribed *Ceratocystis* species on *Xanthosoma* and *Syngonium* (Thorpe et al. 2005, Li et al. 2017). *Ceratocystis platani* is an aggressive canker and wilt pathogen of *Platanus* spp. that is specific to this host (Panconesi 1999, Engelbrecht et al. 2004). It is considered to be indigenous to the south-eastern

USA, but it has been spread to California and several countries in southern Europe (Tsopelas et al. 2017). As demonstrated in the present study, *C. lukuohia* and *C. platani* do not infect each others' hosts, they are only partially interfertile and they differ by more than 66 % in their microsatellite profiles. Phylogenetically, *C. lukuohia* is very similar to isolates from *Xanthosoma* and *Syngonium*, and these strains are believed to be native to the Caribbean region (Thorpe et al. 2005). A strain on the American ornamental *Syngonium podophyllum* has apparently been moved in infected cuttings to other continents by the commercial nursery trade, and this strain was reported from an ornamental nursery in Hilo, Hawai`i (Uchida & Aragaki 1979, Thorpe et al. 2005), close to the area where Ceratocystis wilt of *M. polymorpha* was first recognized. However, *C. lukuohia* is morphologically distinct from the *Syngonium* strain, it has mostly distinct microsatellite alleles, and the two species are only partially interfertile. In addition, *Syngonium* isolates did not cause disease in `ōhi`a seedlings. In general, isolates from *Araceae* are not aggressive on dicotyledonous hosts and vice versa (Baker et al. 2003, Thorpe et al. 2005), and in the present study, *Ceratocystis* isolates from *M. polymorpha* did not cause disease in the aroids *S. podyphyllum* or *Colocasia esculenta*. Nonetheless, it is possible that *C. lukuohia*, or something closely related, was introduced to Hawai`i in plant propagative material, and it may have hybridized with the *Syngonium* strain.

Black rot of sweet potato caused by *C. fimbriata* has been known in Hawai`i since 1925, but it is host specialized and infects only sweet potato (Brown & Matsuura 1941, Li et al. 2016). *Ceratocystis lukuohia* is clearly distinct from the sweet potato pathogen. A sweet potato isolate from Hawai`i Island was found to have the same combination of microsatellite alleles as other sweet potato isolates from around the world (Li et al. 2016). Although the sweet potato strain resides in the LAC, the present study confirmed that it is not interfertile with *C. platani* (Engelbrecht & Harrington 2005), and it is not interfertile with *C. lukuohia*.

Ceratocystis huliohia falls in the *C. polychroma* cluster in the AAC, where the taxonomic placement and differences among species have recently been well debated and characterised (Li et al. 2017, Liu et al. 2018). The species most closely related to *C. huliohia* based on ITS sequence are the recently described *C. uchidae* and *C. changhui* (Li et al. 2017, Liu et al. 2018). Both of these species are pathogens of root crops in the *Araceae*. *Ceratocystis uchidae* was described from Hawai`i and Fiji on *C. esculenta* and *X. sagittifolium*, while *C. changhui* is currently only known from China on *Eucalyptus* sp. and *Colocasia esculenta*. Thorpe et al. (2005) hypothesized that *C. uchdiae* was introduced from Asia to Hawai`i many years ago on corms of *Colocasia esculenta*. Interestingly, *C. huliohia* was not pathogenic on *Colocasia esculenta*, and it is distinct from the other species in the ACC (including *C. polychroma* and *C. cercfabiensis*) based on phylogenetic inference, partial interfertility and host range. The origin and introductory pathway of *C. huliohia* will need further study.

The ITS region is considered the barcoding region of choice for species identification of fungi (Schoch et al. 2012, 2014, Stielow et al. 2015), and it has been used extensively for delimiting species in *Ceratocystis* (Fourie et al. 2015). However, Harrington et al. (2014) recognised that this region is very variable and heterogeneous and needs to be used with caution for species delimitation in *Ceratocystis*. Even in the current study, the topology of the species in the ITS phylogeny differed somewhat to the other gene regions used. In addition, the discovery of multiple ITS types within a single spore can complicate species delineation (Al Adawi et al. 2013, Naidoo et al. 2013, Harrington et al. 2014, Li et al. 2017, Liu et al. 2018), and at least two ITS types were found in most isolates of *C. lukuohia*. However, both of these sequences clustered closely together in the ITS phylogeny and were separated from the *Araceae* isolates with moderate bootstrap support. Although species concepts in *Ceratocystis* remain open to debate, it is clear that the two species associated with ROD are phylogenetically distinct from all other described species of *Ceratocystis* based on multiple gene regions.

Ceratocystis lukuohia and *C. huliohia* now represent the fourth and fifth species of *Ceratocystis* to have been introduced into Hawai`i. It is evident that these two species did not arise through sympatric speciation on the same host because they reside in distinct phylogeographic lineages of *Ceratocystis*. The ROD epidemic provides a sobering example of where introduced tree pathogens can threaten natural ecosystems. The rapid spread of *C. lukuohia* and *C. huliohia* in Hawai`i presents a great threat to the diversity, structure and function of *M. polymorpha* forests and the services they provide. If the disease is not contained, much of the landscape is likely to be replaced by non-native species (Mortenson et al. 2016). These pathogens not only threaten the natural biodiversity of Hawai`i, but also the culture of a people that is intricately tied to this native tree species. The implementation of strict quarantine and biosecurity measures are, therefore, crucial to prevent the spread of the pathogens to other continents and islands, such as New Zealand, that have extensive *Meterosideros* forests.

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Appendix Multilocus microsatellite profiles of isolates used in this study using primers developed for *Ceratocystis* sp. (Part 1)

Species	CMW Culture number	Personal Collection number	Host	ITS haplotype	Country/Locality	Date collected	Fourie et al. 2016								
							AF2	AF3	AF4	AF5	AF6	AF7	AF8	AF9	AF11
Latin American Clade (LAC)															
C. fimbriata	46734	P15-31	*Ipomoea batatas*	-	USA, Hawai`i Island	2015	198	215	246	242	285	331	349	415	460
Ceratocystis sp.	48499	C1817	*Xanthosoma* sp.	-	Cuba	2001	206	203	254	262	300	325	334	415	432
Ceratocystis sp.	48506	C1780	*Xanthosoma sagittifolium*	-	Costa Rica	2001	209	203	257	262	300	325	334	415	432
Ceratocystis sp.	48500	C1717	*Syngonium* sp.	-	USA, Hawai`i	-	192	206	252	258	294	325	334	415	432
Ceratocystis sp.	48508	C1809	*Syngonium podophyllum*	-	USA, Florida	2001	192	206	252	258	294	325	334	415	432
Ceratocystis sp.	50456	P16-1	*Syngonium podophyllum*	-	USA, Hawai`i, Hilo, South Hilo District	2016	192	206	252	258	294	325	334	415	432
Ceratocystis sp.	50457	P16-2	*Syngonium podophyllum*	-	USA, Hawai`i, Hilo, South Hilo District	2016	192	206	252	258	294	325	334	415	432
C. lukuohia	44102	P14-1-1	*Metrosideros polymorpha*	A	USA, Hawai`i, Leilani Estates, Puna District	2014 Feb 20	195	215	249	262	NR	316	334	412	432
C. lukuohia	44105	P14-12	*Metrosideros polymorpha*	A	USA, Hawai`i, Hawaiian Acres, Puna District	2014 Jul 31	195	215	249	262	NR	316	334	412	432
C. lukuohia	44106	P14-13	*Metrosideros polymorpha*	A	USA, Hawai`i, Hawaiian Paradise Park, Puna District	2014 Jul 8	195	215	249	262	NR	316	334	412	432
C. lukuohia	44107	P14-15	*Metrosideros polymorpha*	A	USA, Hawai`i, Hawaiian Acres, Puna District	2014 Jul 31	195	215	249	262	NR	316	334	412	432
C. lukuohia	44103	P14-6	*Metrosideros polymorpha*	A	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	NR	316	334	412	432
C. lukuohia	47857	P15-11	*Metrosideros polymorpha*	C	USA, Hawai`i, Keau`ohana Forest Reserve, Puna District	2015 Jan 6	195	215	249	262	NR	316	334	412	432
C. lukuohia	47139	P15-17	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Mālama Kī Forest Reserve, Puna District	2015 Feb 2	195	215	249	262	NR	316	334	412	432
C. lukuohia	46743	P15-27	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Kopua Farm Lots, Puna District	2015 Mar 12	195	215	249	262	NR	316	334	412	432
C. lukuohia	46741	P15-64	*Metrosideros polymorpha*	C	USA, Hawai`i, Nānāwale Estates, Puna District	2015 Jun 3	195	215	249	262	NR	316	334	412	432
C. lukuohia	47152	P15-65	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Waiākea Forest Reserve, South Hilo District	2015 May 27	195	215	249	262	NR	316	334	412	432
C. lukuohia	47153	P15-67	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Keau`ohana Forest Reserve, Puna District	2015 Jun 9	195	215	249	262	–	316	334	412	432
C. lukuohia	47154	P15-68	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Volcano, Puna District	2015 Jul 15	195	215	249	262	–	316	334	412	432
C. lukuohia	46702	P14-1-3	*Metrosideros polymorpha*	A	USA, Hawai`i, Leilani Estates, Puna District	2014 Feb 20	195	215	249	262	–	316	334	412	–
C. lukuohia	46706	P14-4-3	*Metrosideros polymorpha*		USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	–
C. lukuohia	46707	P14-4-4	*Metrosideros polymorpha*		USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	–
C. lukuohia	46711	P14-7	*Metrosideros polymorpha*	A	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	–
C. lukuohia	47856	P15-10	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Keau`ohana Forest Reserve, Puna District	2015 Jan 26	195	215	249	262	–	316	334	412	–
C. lukuohia	46725	P15-15	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Nānāwale Estates, Puna District	2015 Jan 29	195	215	249	262	–	316	334	412	–
C. lukuohia	47140	P15-18	*Metrosideros polymorpha*	A	USA, Hawai`i, Mālama Kī Forest Reserve, Puna District	2015 Feb 2	195	215	249	262	–	316	334	412	–
C. lukuohia	47858	P15-20	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Mālama Kī Forest Reserve, Puna District	2015 Feb 2	195	215	249	262	–	316	334	412	–
C. lukuohia	47854	P15-6	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Leilani Estates, Puna District	2015 Jan 13	195	215	249	262	–	316	334	412	–
C. lukuohia	46722	P15-7	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Leilani Estates, Puna District	2015 Jan 15	195	215	249	262	–	316	334	412	–
C. lukuohia	47855	P15-8	*Metrosideros polymorpha*	A	USA, Hawai`i, Leilani Estates, Puna District	2015 Jan 15	195	215	249	262	–	316	334	412	–
C. lukuohia	46723	P15-9	*Metrosideros polymorpha*		USA, Hawai`i, Leilani Estates, Puna District	2015 Jan 15	195	215	249	262	–	316	334	412	–
C. lukuohia	46710	P14-6-1	*Metrosideros polymorpha*	A	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	46714	P14-11	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hawaiian Acres, Puna District	2014 Jul 31	195	215	249	262	–	316	334	412	432
C. lukuohia	46718	P14-16	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hawaiian Acres, Puna District	2014 Jul 31	195	215	249	262	–	316	334	412	432
C. lukuohia	46703	P14-2	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	46704	P14-2-1	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	46705	P14-3	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	46712	P14-7-3	*Metrosideros polymorpha*		USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	46713	P14-8	*Metrosideros polymorpha*	A	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	46719	P15-1	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Waiākea Forest Reserve, South Hilo District	2014 Nov 20	195	215	249	262	–	316	334	412	432
C. lukuohia	47138	P15-13	*Metrosideros polymorpha*	A	USA, Hawai`i, Nānāwale Estates, Puna District	2015 Jan 29	195	215	249	262	–	316	334	412	432
C. lukuohia	46724	P15-14	*Metrosideros polymorpha*	A	USA, Hawai`i, Nānāwale Estates, Puna District	2015 Jan 29	195	215	249	262	–	316	334	412	432
C. lukuohia	46726	P15-16	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Nānāwale Estates, Puna District	2015 Jan 29	195	215	249	262	–	316	334	412	432
C. lukuohia	46727	P15-19	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Mālama Kī Forest Reserve, Puna District	2015 Feb 2	195	215	249	262	–	316	334	412	432
C. lukuohia	46720	P15-2	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Waiākea Forest Reserve, South Hilo District	2014 Nov 20	195	215	249	262	–	316	334	412	432
C. lukuohia	46728	P15-21	*Metrosideros polymorpha*	A	USA, Hawai`i, Leilani Estates, Puna District	2014 Jun 17	195	215	249	262	–	316	334	412	432
C. lukuohia	46729	P15-22	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Leilani Estates, Puna District	2015 Jan 27	195	215	249	262	–	316	334	412	432
C. lukuohia	46730	P15-24	*Metrosideros polymorpha*	A	USA, Hawai`i, `Opihikao, Puna District	2015 Mar 3	195	215	249	262	–	316	334	412	432
C. lukuohia	46731	P15-25	*Metrosideros polymorpha*	A	USA, Hawai`i, `Opihikao, Puna District	2015 Mar 3	195	215	249	262	–	316	334	412	432
C. lukuohia	46732	P15-28	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hawaiian Acres, Puna District	2015 Mar 12	195	215	249	262	–	316	334	412	432

Appendix (cont.) (cont. Part 1)

Species	CMW Culture number	Personal Collection number	Host	ITS haplotype	Country/Locality	Date collected	Fourie et al. 2016								
							AF2	AF3	AF4	AF5	AF6	AF7	AF8	AF9	AF11
C. lukuohia	46733	P15-29	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Pu'u Kali'u, Puna District	2015 Mar 6	195	215	249	262	–	316	334	412	432
C. lukuohia	47142	P15-32	*Metrosideros polymorpha*	A	USA, Hawai`i, Pu'u Kali'u, Puna District	2015 Mar 6	195	215	249	262	–	316	334	412	432
C. lukuohia	46735	P15-34	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hawaiian Paradise Park, Puna District	2015 Mar 12	195	215	249	262	–	316	334	412	432
C. lukuohia	47144	P15-35	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hawaiian Paradise Park, Puna District	2015 Mar 12	195	215	249	262	–	316	334	412	432
C. lukuohia	47145	P15-36	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Volcano, Puna District	2015 Mar 3	195	215	249	262	–	316	334	412	432
C. lukuohia	47146	P15-38	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Lava Tree, Puna District	2015 Mar 3	195	215	249	262	–	316	334	412	432
C. lukuohia	47147	P15-39	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Lava Tree, Puna District	2015 Mar 3	195	215	249	262	–	316	334	412	432
C. lukuohia	47136	P15-4	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Dec 12	195	215	249	262	–	316	334	412	432
C. lukuohia	46708	P14-5	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Jun 11	195	215	249	262	–	316	334	412	432
C. lukuohia	47137	P15-5	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2014 Dec 12	195	215	249	262	–	316	334	412	432
C. lukuohia	46736	P15-53	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Orchidlands Estates, Puna District	2015 Mar 31	195	215	249	262	–	316	334	412	432
C. lukuohia	47148	P15-54	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Leilani Estates, Puna District	2015 May 27	195	215	249	262	–	316	334	412	432
C. lukuohia	46737	P15-55	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hilo Watershed Forest Reserve, South Hilo District	2015 Jun 8	195	215	249	262	–	316	334	412	432
C. lukuohia	47150	P15-61	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, Hilo Watershed Forest Reserve, South Hilo District	2015 Jul 1	195	215	249	262	–	316	334	412	432
C. lukuohia	46740	P15-63	*Metrosideros polymorpha*	Mix A/C	USA, Hawai`i, `Opihikao, Puna District	2015 May 27	195	215	249	262	–	316	334	412	432
C. lukuohia	46742	P15-66	*Metrosideros polymorpha*	A	USA, Hawai`i, Keau`ohana Forest Reserve, Puna District	2015 Jun 9	195	215	249	262	–	316	334	412	432
C. platani	9499	CF20	*Platanus sp.*	-	Italy	-	203	239	243	262	NR	322	334	418	432
C. platani	1896	BRENO10	*Platanus sp.*	-	Switzerland	-	203	239	243	262	NR	322	334	418	432
C. platani	2219	1012/D0	*Platanus sp.*	-	France, Saint-Maurice	1991	203	239	243	262	NR	322	334	418	432
C. platani	23450	c-1	*Platanus orientalis*	-	Greece	2006	203	239	243	262	NR	322	334	418	432
C. platani	14802	C1317	*Platanus occidentalis*	-	USA, North Carolina	1998 Jul	201	215	243	262	–	322	334	426	–
C. platani	9496	CF34	*Platanus sp.*	-	Italy	-	203	239	243	262	–	322	334	418	–
C. platani	9000	CF8	*Platanus sp.*	-	Italy	2002 Feb	203	239	243	262	–	322	334	418	–
C. platani	2218	1012/D4	*Platanus sp.*	-	France, Saint-Maurice	1991	203	239	243	262	–	322	334	418	–
C. platani	23918		*Platanus sp.*	-	Greece	2006 Jul	203	239	243	262	–	322	334	418	–
Asian_Australian Clade (AAC)															
C. huliohia	47135	P14-10	*Metrosideros polymorpha*	B	USA, Hawai`i, `Opihikao, Puna District	2014 Jul 8	212	194	234	226	NR	328	328	401	444
C. huliohia	46716	P14-14	*Metrosideros polymorpha*	B	USA, Hawai`i, Hawaiian Paradise Park, Puna District	2014 Jul 8	212	194	234	226	NR	328	328	401	444
C. huliohia	44104	P14-9-1	*Metrosideros polymorpha*	B	USA, Hawai`i, Hawaiian Paradise Park, Puna District	2015 Feb 04	212	194	234	226	NR	328	328	401	444
C. huliohia	46721	P15-3	*Metrosideros polymorpha*	B	USA, Hawai`i, Hilo, South Hilo District	2014 Nov 26	212	194	234	226	NR	328	328	401	444
C. huliohia	47143	P15-33	*Metrosideros polymorpha*	B	USA, Hawai`i, Pana`ewa, South Hilo District	2015 Mar 3	212	194	234	226	NR	328	328	401	444
C. huliohia	47149	P15-58	*Metrosideros polymorpha*	B	USA, Hawai`i, Hōlualoa, North Kona District	2015 Jun 10	212	194	234	226	NR	328	328	401	444
C. huliohia	46738	P15-59	*Metrosideros polymorpha*	B	USA, Hawai`i, Hōlualoa, North Kona District	2015 Jun 10	212	194	234	226	NR	328	328	401	444
C. huliohia	46739	P15-60	*Metrosideros polymorpha*	B	USA, Hawai`i, Hōlualoa, North Kona District	2015 Jun 10	212	194	234	226	–	328	328	401	–
C. huliohia	47151	P15-62	*Metrosideros polymorpha*	B	USA, Hawai`i, Hōlualoa, North Kona District	2015 Jun 10	212	194	234	226	–	328	328	401	–
C. uchidae	14796	CBS 114720	*Colocasia esculenta*	-	USA, Hawai`i, O`ahu	1991	219,68	194	249	226	270	328	–	401	444
C. uchidae	14804	CBS 115164	*Colocasia esculenta*	-	USA, Hawai`i, Kaua`i	1988	219,75	194	249	226	270	328	–	401	444
C. cercfabiensis	43029	CERC 2170	*Eucalyptus* sp.	-	China, HaiNan Province	2013	203	194	237	226	294	328	–	401	444
C. cercfabiensis	42795	CERC 2687	*Eucalyptus* sp.	-	China, GuangDong	2013	206	194	237	226	291	334	328	401	447
C. changhui	43272	CERC 3605	*Colocasia esculenta*	-	China, YunNan Province	2015	220	194	237	226	270	328	–	401	444
C. changhui	43281	CERC 3615	*Colocasia esculenta*	-	China, YunNan Province	2014	220	194	237	226	270	328	–	401	444
C. changhui	49317	C3371	*Eucalyptus* sp.	-	China, YunNan Province	2014	232	194	243	226	270	328	328	401	444
C. changhui	49318	C3372	*Eucalyptus* sp.	-	China, YunNan Province	2014	232	194	243	226	270	328	328	401	444
C. changhui	49319	C3373	*Eucalyptus* sp.	-	China, YunNan Province	2014	232	194	243	226	270	328	328	401	444
C. changhui	49320	C3374	*Eucalyptus* sp.	-	China, YunNan Province	2014	232	194	243	226	270	328	328	401	444
C. changhui	49321	C3375	*Eucalyptus* sp.	-	China, YunNan Province	2014	232	194	243	226	270	328	328	401	444
C. changhui	49322	C3376	*Eucalyptus* sp.	-	China, YunNan Province	2014	232	194	243	226	270	328	328	401	444
C. polychroma	11424		*Syzygium aromaticum*	-	Indonesia	2002	209	194	234	226	294	328/334	–	401	444
C. polychroma	11436		*Syzygium aromaticum*		Indonesia	2002	209	194	234	226	294	334	–	401	444

MB = multiple bands observed during PCR, did not do fragment analyses; - = no data available; – = PCR was not done; FP = finger print pattern that was difficult to score; NR = no PCR amplification; MA = multiple alleles observed.

Appendix (cont.) (Part 2)

Species	CMW Culture number		Stiemel et al. 2004												Barnes et al. 2001				
			AAG8	AAG9	CAA9	CAA10	CAA15	CAA38	CAA80	CAT1	CAT3K	CAT9X	CAT1200	CAG5	GACA650	AG1/ AG2	AG7/ AG8	CF13/ CF14	CF15/ CF16	CF23/ CF24
Latin American Clade (LAC)																				
C. fimbriata	46734		185	397	206	138	320	150	301	255	322	280	376	319	219	269	284	406	465	155
Ceratocystis sp.	48499	171.37 close to bin171	392	199.66	131	314	184	295	263	MA	281	374	328	212	270	302	401	490	153	
Ceratocystis sp.	48506	171.49 close to bin171	392	199.66	131	314	184	295	263	MA	281	374	328	212	270	304	401	490	174	
Ceratocystis sp.	48500		165	392	254	131	314	250	263	269	MA	281, MA	374	328	212	270	302	401	466	172
Ceratocystis sp.	48508		165	392	254	131	314	250	263	269	MA	281, MA	374	328	212	270	302	401	466	172
Ceratocystis sp.	50456		165	392	254	131	314	250	263	269	MA	281, 287	374	328	212	270	302	401	466	172
Ceratocystis sp.	50457		165	392	254	131	314	250	263	269	MA	281, 287	374	328	212	270	302	401	466	172
C. lukuohia	44102		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	44105		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	44106		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	44107		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	44103		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	47857		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	47139		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	46743		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	46741		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	47152		188	392	266	129	314	232	313	257	MA	284, MA	MB	328	227	269	293	FP	471	161
C. lukuohia	47153		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	47154		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46702		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46706		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46707		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46711		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	47856		188	392	266	129	314	232	–	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46725		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	47140		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	47858		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	47854		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46722		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	47855		188	392	266	129	314	232	–	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46723		188	392	266	129	314	232	313	–	–	–	–	328	227	–	293	FP	471	161
C. lukuohia	46710		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46714		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46718		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46703		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46704		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46705		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46712		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46713		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46719		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47138		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46724		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46726		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46727		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46720		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46728		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46729		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46730		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46731		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46732		–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–

Appendix (cont.) (cont. Part 2)

Species	CMW Culture number	Stiemel et al. 2004												Barnes et al. 2001				
		AAG8	AAG9	CAA9	CAA10	CAA15	CAA38	CAA80	CAT1	CAT3K	CAT9X	CAT1200	CAG5	GACA650	AG1/ AG2	AG7/ AG8	CF13/ CF14	CF15/ CF16	CF23/ CF24
C. lukuohia	46733	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47142	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46735	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47144	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47145	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47146	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47147	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47136	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46708	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47137	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46736	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47148	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46737	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	47150	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46740	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. lukuohia	46742	–	–	266	–	–	–	313	–	–	–	–	–	–	–	–	–	–	–
C. platani	9499	174	406	294	129	314	157	295	257	MA	281, MA	393	328	287	271	300	FP	480	161
C. platani	1896	174	406	294	129	314	157	295	257	MA	281, MA	393	328	301	271	300	FP	480	161
C. platani	2219	174	406	294	129	314	157	295	257	MA	281, MA	393	328	286.9	271	300	FP	480	161
C. platani	23450	174	406	294	129	314	157	295	257	MA	281, MA	393	328	316	271	300	FP	480	161
C. platani	14802	174	406	367	129	285	133	295	–	–	–	–	328	-	–	300	406.47	482	161
C. platani	9496	174	406	294	129	314	157	295	–	–	–	–	328	287	–	300	FP	480	161
C. platani	9000	174	406	294	129	314	157	295	–	–	–	–	328	287	–	300	FP	480	161
C. platani	2218	174	406	294	129	314	157	295	–	–	–	–	328	287	–	300	FP	480	161
C. platani	23918	174	406	294	129	314	157	295	–	–	–	–	328	287	–	300	FP	480	161
Asian_Australian Clade (AAC)																			
C. huliohia	47135	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	46716	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	44104	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	46721	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	47143	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	47149	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	46738	174	395	166	126	383	136	304	232	306	270	355	> 500	182	266	278/291	417	471	157
C. huliohia	46739	174	395	166	126	383	136	304	–	–	–	–	–	182	–	278/291	417	471	157
C. huliohia	47151	174	395	166	126	383	136	304	–	–	–	–	–	182	–	278/291	417	471	157
C. uchidae	14796	171	392	147	126	–	139	284	232	306	267	355	–	182	266	278	446	–	157
C. uchidae	14804	171	392	147	126	–	139	284	232	306	267	355	–	182	266	278	446	–	157
C. cercfabiensis	43029	174	392	160	126	–	136	304	232	306	270	355	–	182	266	278	431	–	157
C. cercfabiensis	42795	174	392	160	126	–	136	304	232	306	270	355	–	182	266	278	431	–	157
C. changhui	43272	171	392	147	126	–	143	304	232	306	267	355	–	182	266	278	431	–	157
C. changhui	43281	171	392	147	126	–	143	304	232	306	267	355	–	182	266	278	431	–	157
C. changhui	49317	171	392	147	126	–	136	292	232	306	267	355	–	182	266	278/291	431	472,8	157
C. changhui	49318	171	392	147	126	–	136	292	232	306	267	355	–	182	266	278/291	431	472,8	157
C. changhui	49319	171	392	147	126	–	136	292	232	306	267	355	–	182	266	278/291	431	472,8	157
C. changhui	49320	171	392	147	126	–	136	292	232	306	267	355	–	182	266	278/291	431	472,8	157
C. changhui	49321	171	392	147	126	–	136	292	232	306	267	355	–	182	266	278/291	431	472,8	157
C. changhui	49322	171	392	147	126	–	136	292	232	306	267	355	–	182	266	278/291	431	472,8	157
C. polychroma	11424	188	389	153	126	–	139	307	229	–	264	355	–	182	266	278	431	–	157
C. polychroma	11436	188	389	153	126	–	139	307	229	306	264	355	–	182	266	278	431	–	157