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Taxonomic revision and multi-locus phylogeny of the North American clade of *Ceratocystis*

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Abstract: The North American clade (NAC) of *Ceratocystis* includes pathogenic species that infect a wide range of woody hosts. Previous phylogenetic analyses have suggested that this clade includes cryptic species and a paraphyletic *C. variospora*. In this study, we used morphological data and phylogenetic analyses to characterize NAC taxa, including *Ceratocystis* isolates causing a serious disease of almond trees in California. Phylogenetic analyses based on six gene regions supported two new species of *Ceratocystis*. *Ceratocystis destructans* is introduced as the species causing severe damage to almond trees in California, and it has also been isolated from wounds on *Populus* and *Quercus* in Iowa. It is morphologically similar to *C. tiliae*, a pathogen on *Tilia* and the most recently characterized species in the NAC. *Ceratocystis betulina* collected from *Betula platyphylla* in Japan is also newly described and is the sister taxon to *C. variospora*. Our six-locus phylogenetic analyses and morphological characterization resolved several cryptic species in the NAC.

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INTRODUCTION

The genus *Ceratocystis* (*Sordariomycetes*, *Microascales*, *Ceratocystidaceae*) was proposed in 1890 based on *C. fimbriata*, which was first described as the causal agent of black rot of sweet potato (*Ipomoea batatas*) in the USA (Halsted 1890). The genus now comprises 39 species (Marin-Felix *et al*. 2017, Barnes *et al*. 2018, Liu *et al*. 2018) and consists of a complex of many cryptic and some host-specialized species (Harrington 2000, Oliveira *et al*. 2015a) that cause various wilt and canker diseases on a wide range of economically important crops around the world (Kile 1993, Harrington 2013). Hosts impacted by *Ceratocystis* species include *Coffea arabica* (coffee), *Eucalyptus* spp., *Ficus carica* (fig), *Hevea brasiliensis* (rubber tree), *Mangifera indica* (mango), *Platanus* spp. (sycamore or plane trees), *Populus* spp. (aspen and other poplars), *Prunus* spp. (almond and other stone fruits), *Quercus* spp. (oak) and *Theobroma cacao* (cacao) (Harrington 2000, 2013, de Beer *et al*. 2014). Recently, *Ceratocystis s. lat*. was split into 11 genera (*Ambrosiella*, *Berkeleyomyces*, *Bretziella*, *Ceratocystis*, *Chalaropsis*, *Davidsoniella*, *Endoconidiophora*, *Huntiella*, *Meredithiella*, *Phialophoropsis*, and *Thielaviopsis*) based on morphological observations, namely perithecial and ascospore characters, and to a greater extent based on phylogenetic placement (de Beer *et al*. 2014, 2017, Mayers *et al*. 2015, 2018, Nel *et al*. 2018).

Ceratocystis is morphologically defined as species that produce hat-shaped ascospores from brown to black, globose, unornamented perithecial bases with elongated perithecial necks that terminate as aseptate, divergent, and blunt-tipped ostiolar hyphae (de Beer *et al*. 2014). Long-necked perithecia release sticky masses of ascospores at their terminus (Upadhyay 1981, Seifert *et al*. 1993, Harrington 2013, de Beer *et al*. 2014). The asexual thielaviopsis-like morph, is characterized by phialidic conidial ontogeny producing chains of hyaline, single-celled, cylindrical-shaped conidia, called endoconidia (de Beer *et al*. 2014). Barrel-shaped conidia (doliiform conidia) may also be produced from similar endoconidiophores, and most species produce dark, thick-walled aleurioconidia that facilitate survival in wood or in soil (Harrington 2013, de Beer *et al*. 2014).

Ceratocystis species are mainly wound colonizers and include weak to highly virulent pathogens causing disease on diverse woody plant hosts. However, *Ceratocystis* disease cycles are not well understood due to the diversity of spore types, inoculum sources, and dispersal mechanisms, such as insect vectors, wind, infected planting material, root grafting or mechanical transmission during pruning and harvesting (Harrington 2013). Many *Ceratocystis* species are adapted for insect dispersal by producing sweet-smelling or fruity volatiles that attract insect vectors (Harrington 1993, Kile 1993, Wingfield *et al*. 1993). The sticky ascospore masses adhere to insect bodies where they can be easily vectored from one host to another (Malloch & Blackwell 1993). *Ceratocystis fimbriata* has nonspecific associations with insects such as sap-feeding beetles (*Coleoptera*; *Nitidulidae*), flies (*Diptera*; *Drosophilidae*) and ambrosia beetles (*Coleoptera*; *Curculionidae*) (Kile 1993). In addition to insect dispersal, *Ceratocystis* species that produce aleurioconidia are typically soilborne and can be transported in water (Kile 1993, Harrington 2013).

Currently, phylogenetic hypotheses have placed *Ceratocystis* in four broad geographical clades, 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).

The LAC is represented by *C. fimbriata* which is the pathogen that causes black rot of sweet potato. This pathogen is native to South and Central America and the Caribbean (Harrington *et al*. 2011) and causes wilt or cankers on coffee, *Eucalyptus* spp., rubber trees, and mango (Harrington 2013). Species in the LAC are considered to be aggressive pathogens responsible for emerging epidemics when introduced to new hosts and locations, such as the recent outbreak of Ceratocystis wilt on mango in Oman and Pakistan (Al Adawi *et al*. 2014). Other economically important LAC species include *C. platani*, the causal agent of canker stain on *Platanus* spp. and *C. cacaofunesta*, the causal agent of Ceratocystis wilt of *T. cacao* (Engelbrecht & Harrington 2005). In California, *C. platani* from the LAC has caused mortality of California sycamores (*Platanus racemosa* var. *racemosa*) and plane trees in Modesto, California (Perry & McCain 1988), and the pathogen was apparently introduced from the eastern USA (Engelbrecht *et al*. 2005). Most recently, a new *Ceratocystis* species belonging to the LAC, *C. lukuohia*, was identified in Hawai'i and associated with rapid death of `ōhi`a lehua (*Metrosideros polymorpha*), a devastating disease on an ecologically important native tree species (Barnes *et al*. 2018).

The AFC includes *C. albofundus*, a pathogen of black wattle (*Acacia mearnsii*) in Africa (Wingfield *et al*. 1996). This species is thought to be native to Africa with two genetically isolated populations, in Uganda and South Africa (Barnes *et al*. 2005). The AAC is represented by *C. pirilliformis* (Johnson *et al*. 2005, Thorpe *et al*. 2005), a pathogen discovered on *Eucalyptus nitens* in Australia (Barnes *et al*. 2003). Other species residing in the AAC include *C. changhui* from *Colocasia esculenta* in China (Liu *et al*. 2018) and *C. uchidae* from *C. esculenta* in Hawai'i (Li *et al*. 2017). However, species boundaries within the AAC are unclear and require phylogenetic and taxonomic re-examination (Li *et al*. 2017). Recently, *C. huliohia*, a newly identified species in the AAC, was described together with *C. lukuohia* (LAC), as a second causal agent of rapid death of `ōhi`a lehua in Hawai'i (Barnes *et al*. 2018).

Morphological features have been used to distinguish isolates from the NAC and LAC, most notably, slightly smaller ascospores in the NAC and the presence of a collar at the base of the neck of the perithecium; this diagnostic feature is absent in members of the LAC (Johnson *et al*. 2005). Within the NAC, *Ceratocystis* species are distinguished from one another based on the presence or absence of conidial stages, host range, isozyme alleles, and DNA-based phylogenetic analyses (Johnson *et al*. 2005, de Beer *et al*. 2014, Oliveira *et al*. 2015a). Yet, the taxonomy and systematics in the NAC needs more rigorous investigation (Johnson *et al*. 2005, Oliveira *et al*. 2015a).

The NAC currently includes five *Ceratocystis* species that have been isolated from various tree hosts, including *Betula*, *Carya*, *Celtis*, *Ostrya*, *Populus*, *Prunus*, *Quercus*, *Tilia*, and *Ulmus* in Europe, Asia, and North America (Johnson *et al*. 2005). Currently, the NAC of *Ceratocystis* is comprised of four strongly supported species including *C. caryae* (*Carya* spp. and other hosts), *C. harringtonii* (synonym *C. populicola*; *Populus* spp.), *C. smalleyi* (*Carya* spp. and an associated bark beetle, *Scolytus quadrispinosus*), *C. tiliae* (*Tilia americana*), and the paraphyletic taxon *C. variospora*. Traditionally, the name *C. variospora* has been used to describe the species infecting oaks (*Quercus* spp.) in the midwestern USA, but it has been isolated from other hardwood species, and *C. variospora* currently includes the pathogen on *Prunus* spp. in California (Johnson *et al*. 2005). Although isolates of *C. variospora* from oak and *Prunus* differ in their ITS sequences, they could not be distinguished based on morphology nor host association (Johnson *et al*. 2005). A new species within *C. variospora* was recently described as *C. tiliae*, a wound-associated pathogen of basswood (*Tilia americana*) in Nebraska and Iowa (Oliveira *et al*. 2015a). Individual phylogenetic analysis across three loci (LSU, *TEF1*, and Cerato-platanin) strongly suggests that *C. variospora* is a paraphyletic taxon as currently defined (Oliveira *et al*. 2015a). Interfertility tests have shown that isolates from the *Quercus* lineage (*C. variospora s. str*.) are only interfertile with each other and not with isolates collected from *Betula*, *Prunus*, or *Tilia* (*C. variospora s. lat*.) (Johnson *et al*. 2005), thus supporting a biological species concept in conjunction with host specialization (Oliveira *et al*. 2015a).

Johnson *et al*. (2005) proposed that the name *C. variospora* should be applied to the *Prunus* pathogen in California. The fungus causes Ceratocystis canker of almond (*Prunus dulcis*) (DeVay *et al*. 1960) and infects other stone fruits, including apricot (*P. armeniaca*) and prune (*P. domestica*) (DeVay *et al*. 1962). The fungus is thought to colonize wounds made on the bark of trees during mechanical harvest. This disease is ubiquitous in older almond orchards and has recently become a growing concern for young orchards. Almonds are California's most economically important agricultural crop and over 80 % of the global supply is grown in California. Disease symptoms appear as brown to dark brown, shallow (not extending far beyond the cambium), and sunken, cankers. Canker expansion is rapid during the growing season, eventually girdling infected limbs, causing leaves to wilt and branches to dieback. The use of mechanical shakers has led to bark injuries on the trunks of trees and a high incidence of Ceratocystis canker.

The aim of this study was to revisit the taxonomy and phylogeny of *Ceratocystis* isolates recovered from symptomatic almond trees in California. DNA from cultures linked to ex-type and representative specimens for each species in the NAC were obtained and included in a six-gene phylogeny, utilizing portions of 28S (LSU) rDNA, β-tubulin (*TUB2*), translation elongation factor 1-alpha (*TEF1*), mini-chromosome maintenance complex component 7 (*MCM7*), 60S ribosomal protein RPL10 (60S), and Cerato-platanin (CP) gene fragments to further resolve cryptic species within the NAC of *Ceratocystis*.

MATERIALS AND METHODS

Collection of isolates

Isolates were collected from symptomatic almond trees throughout the major almond producing regions in the Central Valley of California (Table 1). Frequently, isolates were collected from trees that were damaged by mechanical harvesting at the trunk or near large pruning wounds made on the scaffolding branches. Gummosis delineated the margins of the cankers in most cases. Trees exhibiting gummosis and sunken lesions in the bark were sampled using a hatchet. Fungi were isolated from pieces of inner bark (50 × 50 × 5 mm) from the margins of active cankers; the pieces were surface disinfested in 0.6 % sodium hypochlorite for 2 min, rinsed twice with sterile water and patted dry with a paper towel. The inner bark pieces were incubated

Table 1. *Ceratocystis* species used for phylogenetic analyses in this study. Newly generated sequences appear in **bold**.

						GenBank Accession Nos.					
Species	**Isolate[a]**	**Host**	**Geographic origin**	**Year**	**Collector**	**LSU**	***TUB2***	***TEF1***	***MCM7***	**60S**	**CP[b]**
Ceratocystis betulina	**C1709/CBS 144246**	*Betula platphylla*	Japan, Morioke, Iwate	2000	H. Masuya	**MG980939**	**MG980839**	**MG980743**	**MG980985**	**MG980789**	**MG980889**
	C1770	*Betula platphylla*	Japan, Morioke, Iwate	2000	H. Masuya	**MG980940**	**MG980840**	**MG980744**	**MG980986**	**MG980790**	**MG980890**
C. caryae	**C1829/CBS 114716**	*Carya cordiformis*	Linn Co., Iowa	2001	J.A. Johnson	**MG980929**	**MG980829**	**MG980733**	**MG980978**	**MG980781**	**MG980879**
	C1827/CBS 115168	*Carya ovata*	Boone Co., Iowa	2001	J.A. Johnson	**MG980928**	**MG980828**	**MG980732**	KM495414	KM495502	**MG980878**
C. destructans	KARE219	*Prunus dulcis*	Fresno Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980949**	**MG980849**	**MG980753**	**MG980995**	**MG980799**	**MG980899**
	KARE230	*Prunus dulcis*	Fresno Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980951**	**MG980851**	**MG980755**	**MG980997**	**MG980801**	**MG980901**
	KARE300	*Prunus dulcis*	Fresno Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980952**	**MG980852**	**MG980756**	**MG980998**	**MG980802**	**MG980902**
	KARE490	*Prunus dulcis*	Kern Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980953**	**MG980853**	**MG980757**	**MG980999**	**MG980803**	**MG980903**
	KARE494	*Prunus dulcis*	Kern Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980954**	**MG980854**	**MG980758**	**MG981000**	**MG980804**	**MG980904**
	KARE495	*Prunus dulcis*	Kern Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980955**	**MG980855**	**MG980759**	**MG981001**	**MG980805**	**MG980905**
	KARE223	*Prunus dulcis*	Merced Co., California	2015	F.P. Trouillas & M.T. Nouri	**MG980950**	**MG980850**	**MG980754**	**MG980996**	**MG980800**	**MG980900**
	KARE978	*Prunus dulcis*	Colusa Co., California	2016	L.A. Holland	**MG980956**	**MG980856**	**MG980760**	**MG981002**	**MG980806**	**MG980906**
	KARE979	*Prunus dulcis*	Colusa Co., California	2016	L.A. Holland	**MG980957**	**MG980857**	**MG980761**	**MG981003**	**MG980807**	**MG980907**
	KARE994	*Prunus dulcis*	Colusa Co., California	2016	L.A. Holland	**MG980958**	**MG980858**	**MG980762**	**MG981004**	**MG980808**	**MG980908**
	KARE1427	*Prunus dulcis*	Madera Co., California	2016	L.A. Holland & F.P. Trouillas	**MG980959**	**MG980859**	**MG980763**	**MG981005**	**MG980809**	**MG980909**
	KARE1428/CBS 144247	*Prunus dulcis*	Madera Co., California	2016	L.A. Holland & F.P. Trouillas	**MG980960**	**MG980860**	**MG980764**	**MG981006**	**MG980810**	**MG980910**
	KARE1447	*Prunus dulcis*	Fresno Co., California	2016	L.A. Holland & F.P. Trouillas	**MG980961**	**MG980861**	**MG980765**	**MG981007**	**MG980811**	**MG980911**
	KARE1448	*Prunus dulcis*	Fresno Co., California	2016	L.A. Holland & F.P. Trouillas	**MG980962**	**MG980862**	**MG980766**	**MG981008**	**MG980812**	**MG980912**
	KARE1609	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980963**	**MG980863**	**MG980767**	**MG981009**	**MG980813**	**MG980913**
	KARE1610	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980964**	**MG980864**	**MG980768**	**MG981010**	**MG980814**	**MG980914**

Table 1. (Continued).

Species	Isolate[a]	Host	Geographic origin	Year	Collector	GenBank Accession Nos.					
						LSU	*TUB2*	*TEF1*	*MCM7*	60S	CP[b]
	KARE1611	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980965**	**MG980865**	**MG980769**	**MG981011**	**MG980815**	**MG980915**
	KARE1612	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980966**	**MG980866**	**MG980770**	**MG981012**	**MG980816**	**MG980916**
	KARE1613	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980967**	**MG980867**	**MG980771**	**MG981013**	**MG980817**	**MG980917**
	KARE1614	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980968**	**MG980868**	**MG980772**	**MG981014**	**MG980818**	**MG980918**
	KARE1622	*Prunus dulcis*	Merced Co., California	2016	F.P. Trouillas	**MG980969**	**MG980869**	**MG980773**	**MG981015**	**MG980819**	**MG980919**
	KARE1624	*Prunus dulcis*	Glenn Co., California	2016	F.P. Trouillas	**MG980970**	**MG980870**	**MG980774**	**MG981016**	**MG980820**	**MG980920**
	KARE1627	*Prunus dulcis*	Glenn Co., California	2016	F.P. Trouillas	**MG980971**	**MG980871**	**MG980775**	**MG981017**	**MG980821**	**MG980921**
	KARE1628	*Prunus dulcis*	Glenn Co., California	2016	F.P. Trouillas	**MG980972**	**MG980872**	**MG980776**	**MG981018**	**MG980822**	**MG980922**
	C578	*Prunus dulcis*	California	1989	R. Bostock	**MG980945**	**MG980845**	**MG980749**	**MG980991**	**MG980795**	**MG980895**
	C821	*Prunus dulcis*	Colusa Co., California	1996	D. Rizzo	**MG980948**	**MG980848**	**MG980752**	**MG980994**	**MG980798**	**MG980898**
	C856	*Prunus dulcis*	Merced Co., California	1996	T.C. Harrington	**MG980946**	**MG980846**	**MG980750**	**MG980992**	**MG980796**	**MG980896**
	C1822	*Prunus dulcis*	Stanislaus Co., California	2001	T.C. Harrington	**MG980947**	**MG980847**	**MG980751**	**MG980993**	**MG980797**	**MG980897**
	C1953	*Populus* sp.	Boone Co., Iowa	2002	J.A. Johnson	**MG980942**	**MG980842**	**MG980746**	**MG980988**	**MG980792**	**MG980892**
	C1956	*Quercus macrocarpa*	Lucas Co., Iowa	2002	J.A. Johnson	**MG980941**	**MG980841**	**MG980745**	**MG980987**	**MG980791**	**MG980891**
	C1957	*Celtis* sp.	Lucas Co., Iowa	2002	J.A. Johnson	**MG980943**	**MG980843**	**MG980747**	**MG980989**	**MG980793**	**MG980893**
	C1963	*Prunus serotina*	Boone Co., Iowa	2002	J.A. Johnson	**MG980944**	**MG980844**	**MG980748**	**MG980990**	**MG980794**	**MG980894**
C. fimbriata	C1476/C1099	*Ipomoea batatas*	Papua New Guinea	1984	E.H.C. McKenzie & F.M. Quin	**MG980927**	**MG980827**	**MG980731**	**MG980977**	**MG980781**	**MG980877**
C. harringtonii	**C685/CBS 115161**	*Populus tremuloides*	Québec, Canada	1993	E. Smalley	**MG980932**	**MG980832**	**MG980736**	**MG980980**	**MG980784**	**MG980882**
	C1485/ATCC 24096	*Populus tremuloides*	Colorado	1999	T.E. Hinds	**MG980933**	**MG980833**	**MG980737**	**MG980981**	**MG980785**	**MG980883**
	C995/CBS 119.78	*Populus* sp.	Poland	1978	J. Gremmen	**MG980934**	**MG980834**	**MG980738**	KM495435	KM495523	**MG980884**
C. smalleyi	**C684/CBS 114724**	*Carya cordiformis*	Wisconsin	1993	E. Smalley	**MG980930**	**MG980830**	**MG980734**	KM495463	KM495553	**MG980880**
	C682	*Carya cordiformis*	La Crosse Co., Wisconsin	1986	E. Smalley	**MG980931**	**MG980831**	**MG980735**	**MG980979**	**MG980783**	**MG980881**

Table 1. (Continued).

Species	Isolate[a]	Host	Geographic origin	Year	Collector	GenBank Accession Nos.					
						LSU	*TUB2*	*TEF1*	*MCM7*	60S	CP[b]
C. tiliae	C2131/CBS 137355	*Tilia americana*	Story Co., Iowa	2004	T.C. Harrington	**MG980975**	**MG980875**	**MG980779**	**MG981021**	**MG980825**	**MG980925**
	C2622/CBS 137356	*Tilia americana*	Douglas Co., Nebraska	2001	T.C. Harrington	**MG980973**	**MG980873**	**MG980777**	**MG981019**	**MG980823**	**MG980923**
	C1954/CBS 137354	*Tilia americana*	Boone Co., Iowa	2002	J.A. Johnson	**MG980974**	**MG980874**	**MG980778**	**MG981020**	**MG980824**	**MG980924**
	C1959	*Tilia americana*	Iowa	2002	J.A. Johnson	**MG980976**	**MG980876**	**MG980780**	**MG981022**	**MG980826**	**MG980926**
C. variospora	**C1009/CBS 773.73**	*Quercus ellipsoidalis*	Carlton Co., Minnesota	1955/1956	R.N. Campbell	**MG980935**	**MG980835**	**MG980739**	**MG980982**	**MG980786**	**MG980885**
	C1483/ATCC 12866	*Quercus ellipsoidalis*	Minnesota	1955/1956	R.N. Campbell	**MG980936**	**MG980836**	**MG980740**	**MG980983**	**MG980787**	**MG980886**
	C1843/CBS 114715	*Quercus alba*	Allamakee Co., Iowa	2001	J.A. Johnson	**MG980937**	**MG980837**	**MG980741**	KM495471	KM495561	**MG980887**
	C1846/CBS 114714	*Quercus robur*	Marshall Co., Iowa	2001	J.A. Johnson	**MG980938**	**MG980838**	**MG980742**	**MG980984**	**MG980788**	**MG980888**

[a]Isolates in bold represent type specimens. Isolates with the prefix "C" are from the culture collection from T. C. Harrington at Iowa State University, isolates with the prefix "KARE" are from the culture collection at University of California Kearney Agricultural Research and Extension Centre.

[b] Cerato-platanin.

bark-side down (cambium-side up) in a moist chamber (metal mesh rack placed over moistened paper towels in clear plastic boxes) at room temperature for one wk in the laboratory under natural photoperiod to promote perithecia formation. Mats of mycelium typical of *Ceratocystis*, namely a white wiry mycelium with black, long-necked perithecia extending from the surface of diseased tissue was observed after 5–6 d. Masses of ascospores exuding from the tips of the perithecia were transferred with a sterilized needle to fresh acidified potato dextrose agar (APDA; 2.6 mL of 25 % [vol/vol] lactic acid per liter of medium) plates followed by hyphal-tip purification to fresh PDA (Potato Dextrose Agar, Difco) filled Petri dishes for additional morphological and phylogenetic analyses. Twenty-six isolates including five cultures linked to ex-type specimens of *Ceratocystis* were obtained from the culture collection of Dr. Thomas C. Harrington, Department of Plant Pathology and Microbiology, Iowa State University, corresponding to isolates lodged at the Westerdijk Fungal Biodiversity Institute (former CBS) and are presented in Table 1.

Phylogenetic analyses

Total genomic DNA was isolated from 24 Californian isolates and an additional 26 NAC isolates from mycelium scraped with a sterile scalpel from the surface of 14-d-old PDA cultures using the DNeasy Plant Kit (Qiagen, Valencia, California), following the manufacturer's instructions. Amplification of translation elongation factor 1-α (*TEF1*) fragments utilized the primer set EFCF1 and EFCF6 (Harrington 2009), β-tubulin (*TUB2*) utilized primers Bt1a and Bt1b (Glass & Donaldson 1995), the 28S (LSU) rDNA region utilized primers LROR and LR7 (Vilgalys & Hester 1990), Cerato-platanin (CP) utilized primers CP-2F and CP-1R (Pazzagli *et al*. 1999, Chen *et al*. 2013), 60S ribosomal protein (60S) utilized primers 60S-506F and 60S-908R (Stielow *et al*. 2015), and the mini-chromosome maintenance complex component 7 (*MCM7*) utilized primers Cer-MCM7F and Cer-MCM7R (de Beer *et al*. 2014). PCR amplification conditions for the *TUB2* and *TEF1* regions were the same as those described by Oliveira *et al*. (2015b); amplification conditions for the cerato-platanin region were the same as those described by Oliveira *et al*. (2015a), and amplification conditions for the LSU region were the same as those described by Vilgalys & Hester (1990). A slightly modified PCR program from de Beer *et al*. (2014) was used for *MCM7* and 60S [initial denaturation (96 °C, 5 min) followed by 35 cycles of denaturation (95 °C, 45 s), annealing (58 °C for *MCM7* and 56 °C for 60S, 45 s), extension (72 °C, 60 s), and a final extension (72 °C, 10 min)]. PCR products were visualized on a 1.5 % agarose gel (120 V for 25 min) to validate presence and size of amplicons, purified *via* Exonuclease I and recombinant Shrimp Alkaline Phosphatase (Affymetrix, Santa Clara, California), and sequenced in both directions via BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fischer Scientific, Waltham, Massachusetts) on an ABI 3730 Capillary Electrophoresis Genetic Analyzer (College of Biological Sciences Sequencing Facility, University of California, Davis).

Forward and reverse nucleotide sequences were assembled, proofread, and edited in Sequencher v. 5 (Gene Codes Corporation, Ann Arbor, Michigan) and deposited in GenBank (Table 1). Sequences from type and non-type *Ceratocystis* isolates (n = 5 and 21, respectively) in the NAC were included for phylogenetic reference (de Beer *et al*. 2014,

Oliveira *et al*. 2015a) (Table 1). Multiple sequence alignments were performed in MEGA v. 6 (Tamura *et al*. 2013) and manually adjusted where necessary in Mesquite v. 3.10 (Maddison & Maddison 2016). Alignments were submitted to TreeBASE under accession number S22454. Concordance among datasets ($P \geq 0.010$) was evaluated with the partition homogeneity test (PHT) conducted in PAUP v. 4.0b162 (Swofford 2002). Datasets were analyzed using two different optimality search criteria, maximum parsimony (MP) and maximum likelihood (ML), in MEGA v. 6 (Tamura *et al*. 2013). For MP analyses, heuristic searches with 1 000 random sequence additions were implemented with the Tree-Bisection-Reconnection algorithm, gaps were treated as missing data. Bootstrap analysis with 1 000 pseudoreplicates was used to estimate branch support. For ML analyses, MEGA was used to infer a model of nucleotide substitution for each dataset, using the Akaike Information Criterion (AIC). ML analysis utilized the Nearest-Neighbor-Interchange heuristic method and branch stability was determined by 1 000 bootstrap pseudoreplicates. Sequences of *Ceratocystis fimbriata s. str*. isolate C1476 from the LAC served as the outgroup taxon in all analyses.

Morphological characterization

Novel fungal species identified during this study were characterized for morphology. Representative isolates (KARE1428, KARE1610, C578, C821, C1709, and C1770) selected based on phylogenetic results were cultured on MEA (2 % Malt Extract Agar; Difco) and PDA. Subculturing was performed by transferring triplicate 5-mm diam mycelial plugs from the colony periphery of a pure culture to the center of fresh Petri dishes filled with MEA and PDA. Cultures were incubated for up to 14 d at room temperature (24 +/- 1 °C) with natural ambient day light and darkness at night (Sep. 2017). Radial growth was measured after 7 d of incubation by taking two measurements at right angles to each other. This experiment was repeated once. Descriptions of colony color (Rayner 1970) and morphology was conducted on day 14. Morphological characterization included measuring the diameter of perithecia and length of ostiolar neck (n = 30), length of ostiolar hyphae (n = 30), ascospore dimensions (n = 30), conidiophores (n = 10), dimensions of cylindrical and doliiform conidia (n = 30), and aleurioconidia (n = 30) at 1000× magnification from 14-d-old cultures by mounting and/or squashing perithecia and other structures in a sterile 50 % glycerol solution on glass slides followed by covering with a glass coverslip and observing structures with a Leica DM500B compound microscope (Leica microsystems CMS GmbH, Wetzlar, Germany). No stain was applied in order to preserve the natural pigmentation of the fungal specimens. Morphological measurements are represented by the mean and a range depicting the standard deviation in the center with minima and maxima in parentheses, respectively.

Optimal growth temperature for the representative isolates (KARE1428, KARE1610, C578, C821, C1709, and C1770) was assessed by culturing isolates as described above on MEA and PDA in the dark and incubating them at temperatures ranging from 5 °C to 40 °C in five degree increments for up to 14 d. Radial colony growth was measured as described above every two days and average colony growth rate and average colony diameter were calculated. Three individual colony replicates per isolate were measured for each temperature. This experiment was repeated once.

RESULTS

Collection of isolates

Surveys of almond orchards in California revealed that Ceratocystis canker was widespread throughout the Central Valley region where almond trees are grown. Infections produced gummosis at the margin of active cankers (Fig. 1A, B). Internal symptoms in infected trunks or scaffolds included death of cambium and bark tissues as well as diffuse, dark brown discoloration that extended into the primary and secondary xylem (Fig. 1C). Cankers were generally associated with wounds created by mechanical harvesting and pruning equipment on the tree trunks (Fig. 1A, D–F) or main scaffold branches (Fig. 1B). Wounds caused by mechanical harvesters typically ruptured the bark, thus exposing the susceptible cambial tissues. Cankers expanded along the main axis of the tree, sometimes extending into one of the main scaffolds (Fig. 1A). Isolations from 4–15-yr-old trees symptomatic of Ceratocystis canker yielded 87 *Ceratocystis* isolates from 20 almond orchards in six California counties.

Phylogenetic analyses

Tests for concordance between datasets using PHT revealed that these data were not significantly inconcordant (P = 0.10) and were combined and analyzed as above. For ML analyses, the best-fit model of nucleotide evolution was selected based on the AIC (K2 for 60S and LSU; K2+G for *TEF1*, *TUB2*, *MCM7*, CP, and the combined analysis).

Alignment of the combined sequences (*TEF1*+*TUB2*+CP +60S+*MCM7*+LSU) resulted in a 4905-character dataset, in which 4 432 characters were constant, 198 characters were parsimony-uninformative, and 275 characters were parsimony-informative (6 %). MP analysis generated 8 equally most parsimonious trees of 553 steps and consistency index (CI), retention index (RI), and rescaled consistency index (RC) of 0.8951, 0.9580, and 0.8523, respectively. MP and ML analyses of the combined six-gene dataset revealed seven strongly supported lineages (≥ 91 % / ≥ 99 % MP and ML bootstrap values, respectively) within the NAC (Fig. 2). Of these seven lineages, two represent the newly described species hereinafter identified as *Ceratocystis betulina* sp. nov. and *Ceratocystis destructans* sp. nov. *Ceratocystis betulina* was revealed to be the sister taxon to *C. variospora s. str*., while *C. destructans* includes the almond pathogen and a group of *Ceratocystis* isolates collected from *Populus*, *Celtis* sp., black cherry (*Prunus serotina*), and *Quercus macrocarpa* in Iowa. The branch that included only the California isolates (including a single isolate from *Populus* in Iowa) was strongly supported (91 % / 99 %), but there was only weak support (75 % / < 70 %) for the broader *C. destructans* lineage that included all the Iowa isolates. The *C. destructans* lineage is sister to the recently described species *C. tiliae*. The order of divergence within the NAC was almost fully resolved, thus providing the first strongly supported hypotheses concerning speciation order within the NAC as depicted in Fig. 2. Thus, the six-gene analysis provides strong support not only for species delineation but also for early and late bifurcations of independently evolving lineages within the NAC.

PCR amplification of the *TEF1* locus produced 1 433–1 473 bp fragments and resulted in a 1 473-character dataset, in which 1 351 characters were constant, 39 were parsimony

Fig. 1. Symptoms of Ceratocystis canker of almond in California. **A.** Gummosis and canker associated with a large pruning wound on trunk. **B.** Scaffold canker. **C.** Transverse cut of a tree trunk infected with Ceratocystis canker and showing dead cambium and bark tissues as revealed by the brown discoloration extending into the primary and secondary xylem. **D–E.** Damaged bark and active Ceratocystis cankers developing on the trunk of young almond trees. **F.** Damaged bark and active Ceratocystis cankers developing on the trunk of a mature almond tree.

uninformative, and 83 were parsimony informative (6 %). The MP analysis produced eight equally most parsimonious trees of 146 steps and a CI, RI, and RC of 0.8290, 0.9529, and 0.8223, respectively (Fig. 3A). PCR amplification of the *TUB2* locus produced 535–547 bp fragments and resulted in a 547-character dataset, in which 480 characters were constant, 33 were parsimony uninformative, and 34 were parsimony informative (6 %). The MP analysis produced eight equally most parsimonious trees of 76 steps and a CI, RI, and RC of 0.8604, 0.9625, and 0.8870, respectively (Fig. 3B). PCR amplification of the *MCM7* locus produced 628 bp fragments and resulted in a 628-character dataset, in which 570 characters were constant, 20 were parsimony uninformative, and 38 were parsimony informative (6 %). The MP analysis produced 10 equally most parsimonious trees of 68 steps and a CI, RI, and RC of 0.9166, 0.9823, and 0.9254, respectively (Fig. 3C). PCR amplification of the 60S locus produced 415–429 bp fragments and resulted in a 429-character dataset, in which 386 characters were constant, 18 were parsimony uninformative, and 25 were parsimony informative (6 %). The MP analysis produced 10 equally most parsimonious trees of 47 steps and a CI, RI, and RC of 0.9574, 0.9800, and 0.9385, respectively (Fig. 3D). PCR amplification of

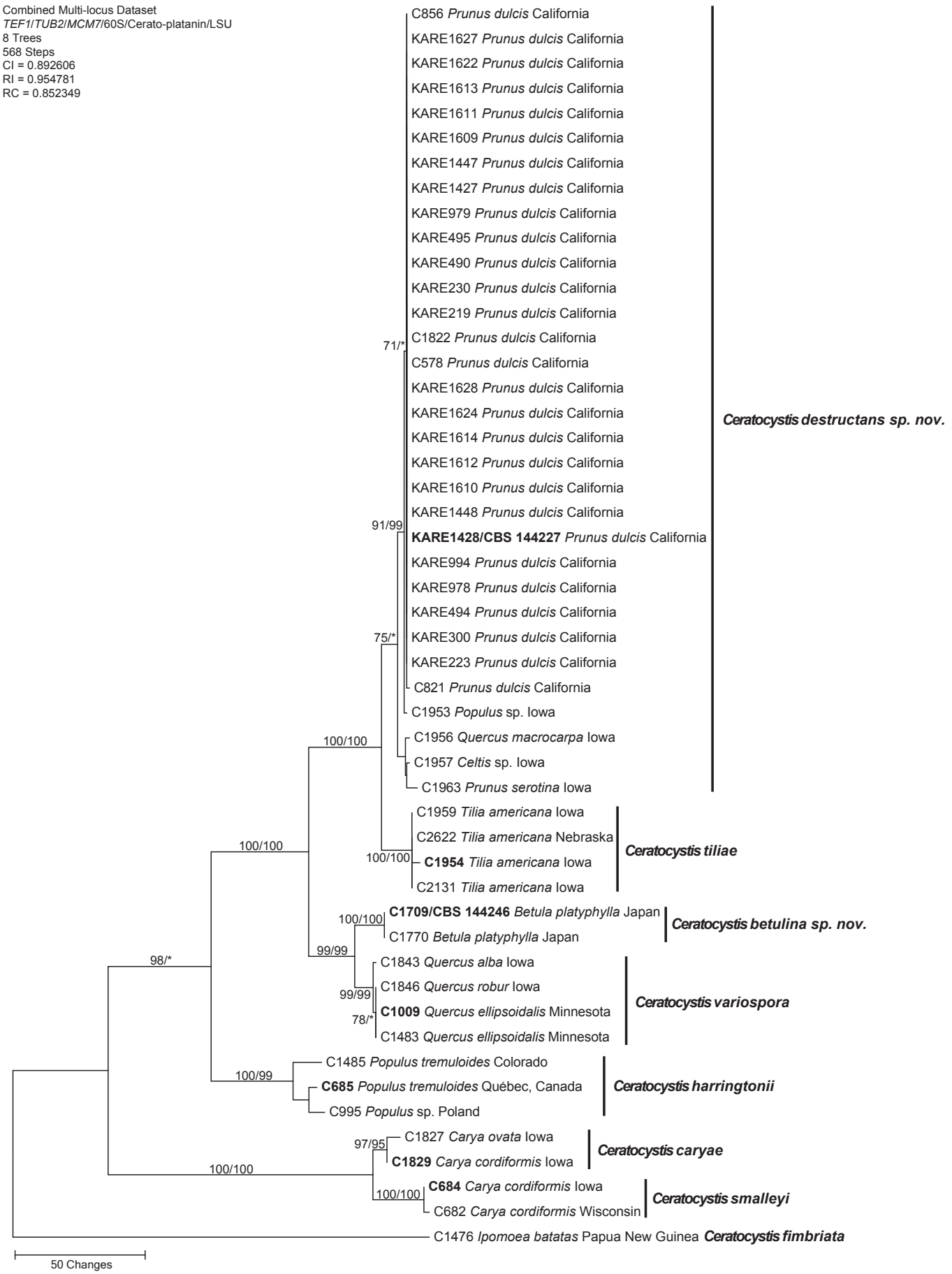

Fig. 2. One of eight equally most parsimonious trees generated from maximum parsimony analysis of the six-gene (*TEF1*+*TUB2*+CP+60S+*MCM7*+LSU) combined dataset. Numbers in front and after the slash represent parsimony and likelihood bootstrap values from 1 000 pseudoreplicates, respectively. Values represented by an asterisk were less than 70 % for the bootstrap analyses. Bar indicates the number of nucleotide changes. Ex-type isolates are in **bold**.

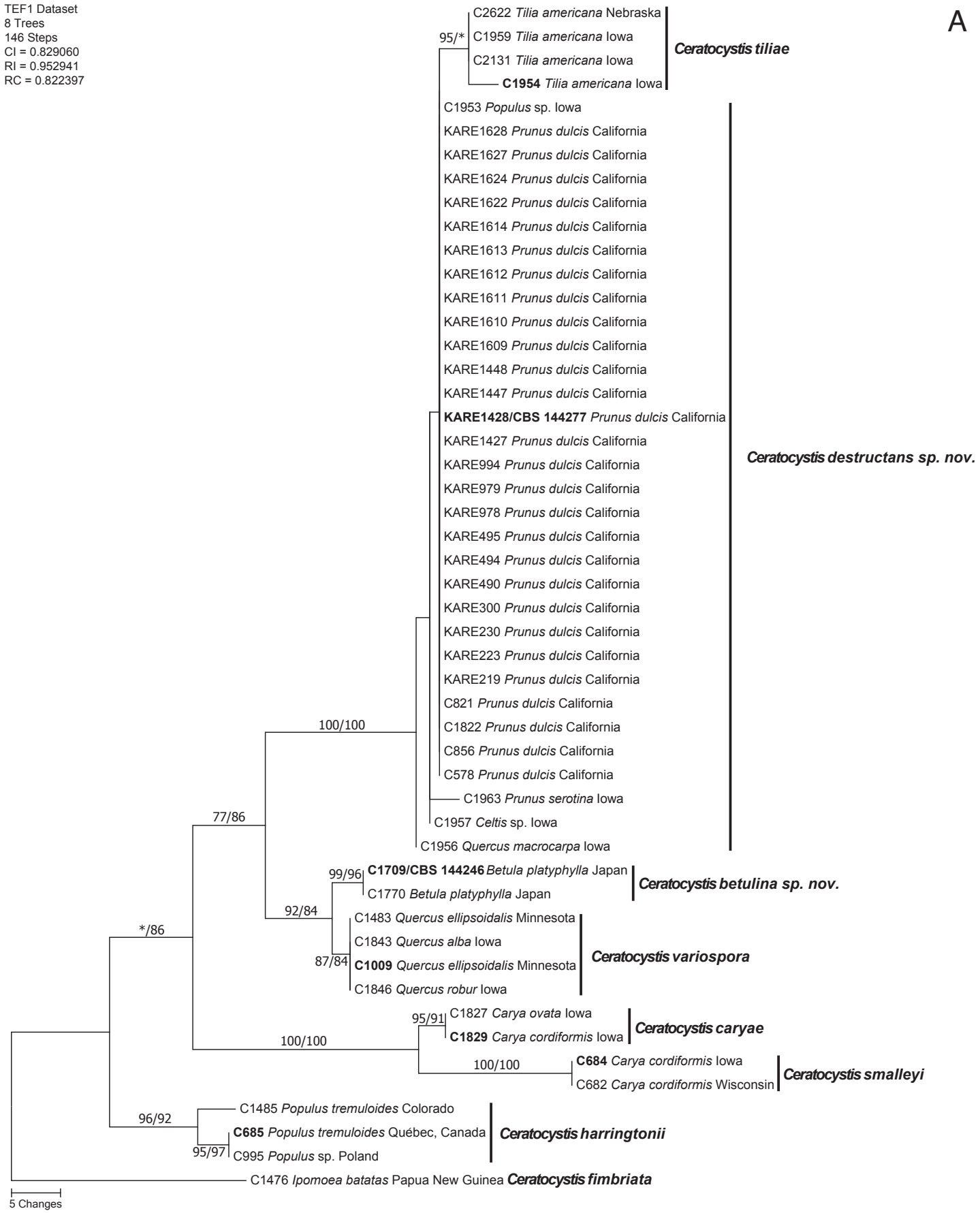

Fig. 3. Equally most parsimonious trees from single-locus analyses. Numbers in front and after the slash represent parsimony and likelihood bootstrap values from 1 000 pseudoreplicates, respectively. Values represented by an asterisk were less than 70 % for the bootstrap analyses. The scale bar indicates the number of nucleotide changes. Ex-type isolates are in **bold**. **A.** One of eight equally most parsimonious trees for the *TEF1* analyses. **B.** One of eight equally most parsimonious trees for the *TUB2* analyses. **C.** One of 10 equally most parsimonious trees for the *MCM7* analyses. **D.** One of 10 equally most parsimonious trees for the 60S analyses. **E.** One of 10 equally most parsimonious trees for the Cerato-platanin analyses. **F.** One of 10 equally most parsimonious trees for the LSU analyses.

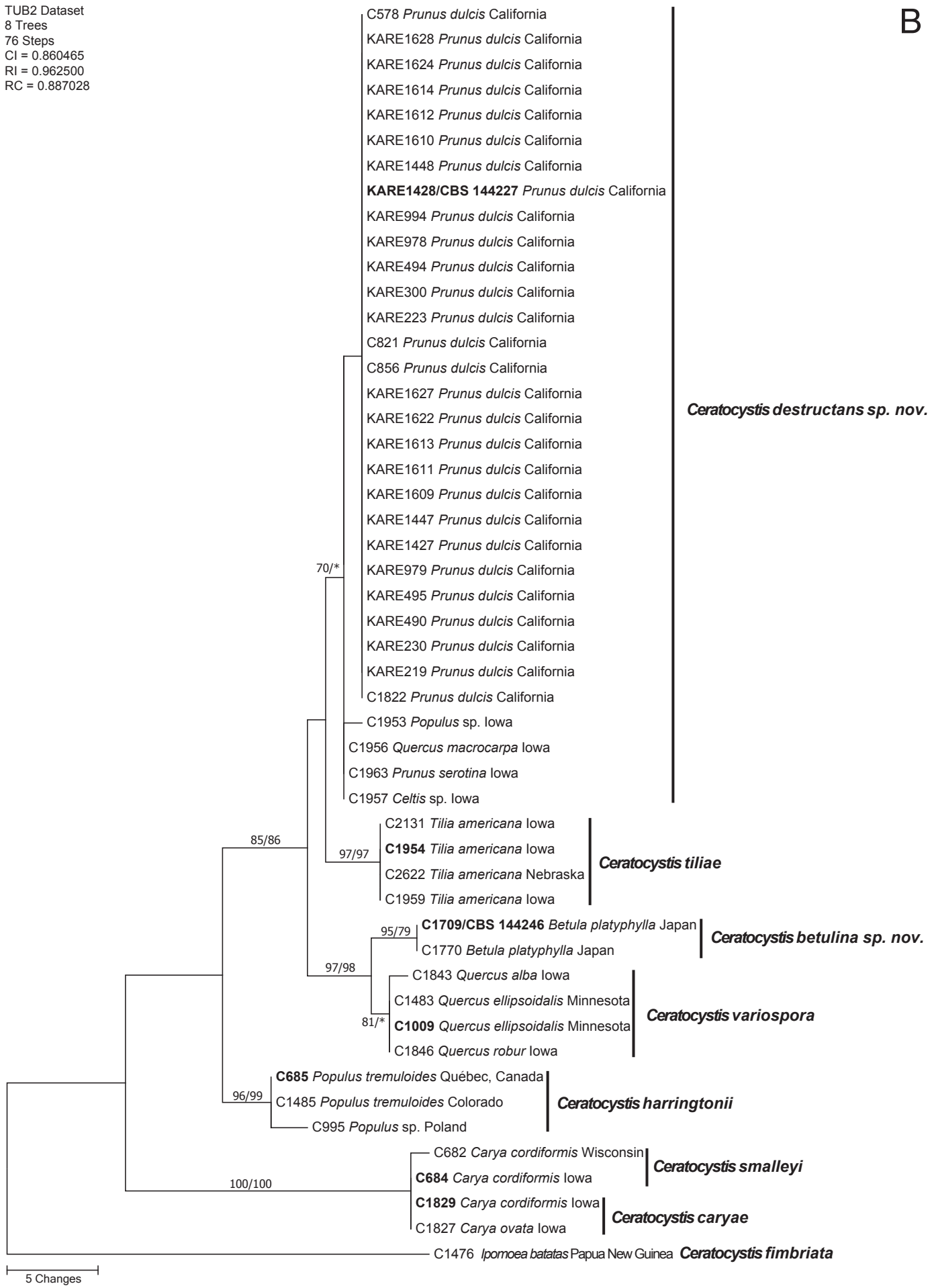

Fig. 3. (Continued).

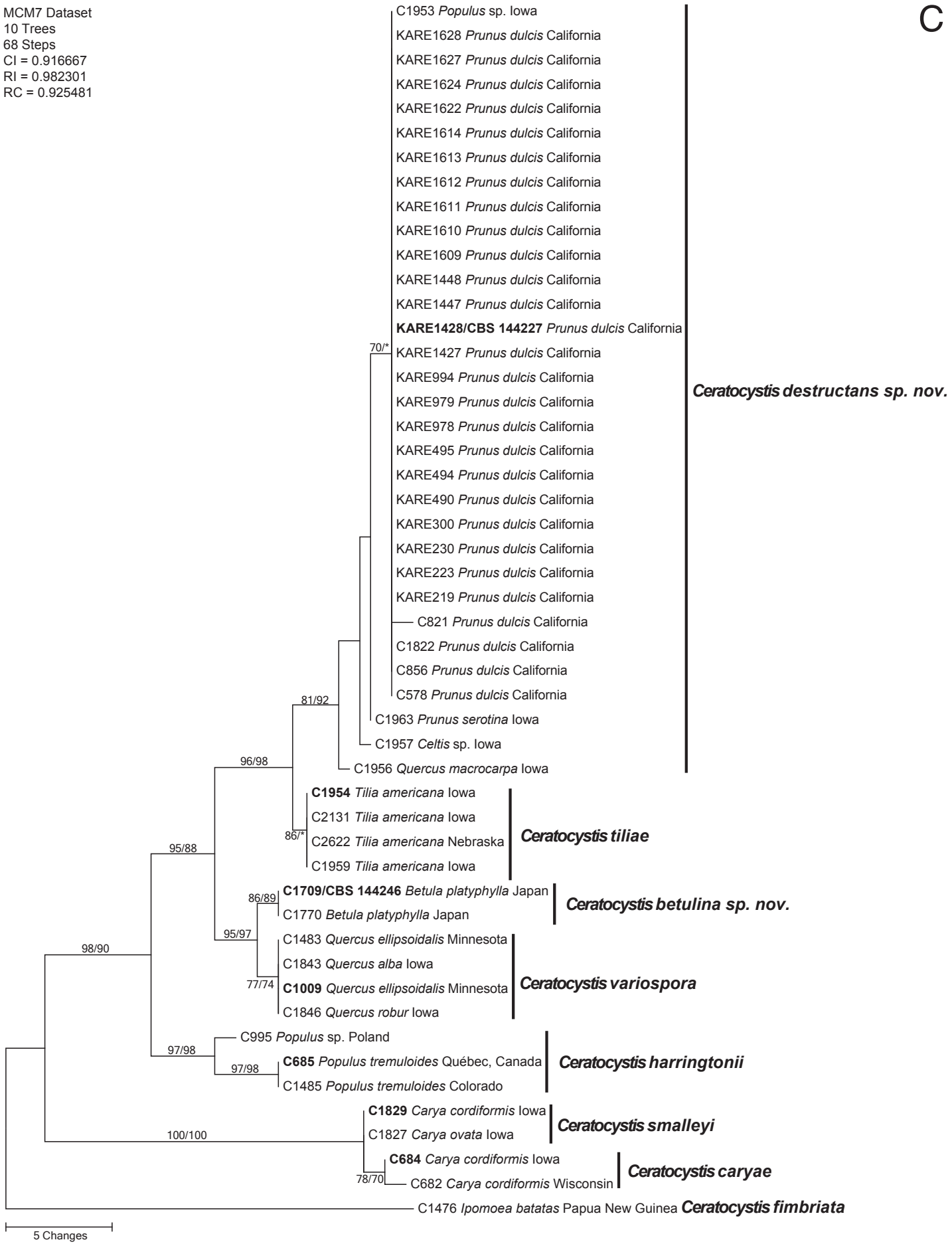

Fig. 3. (Continued).

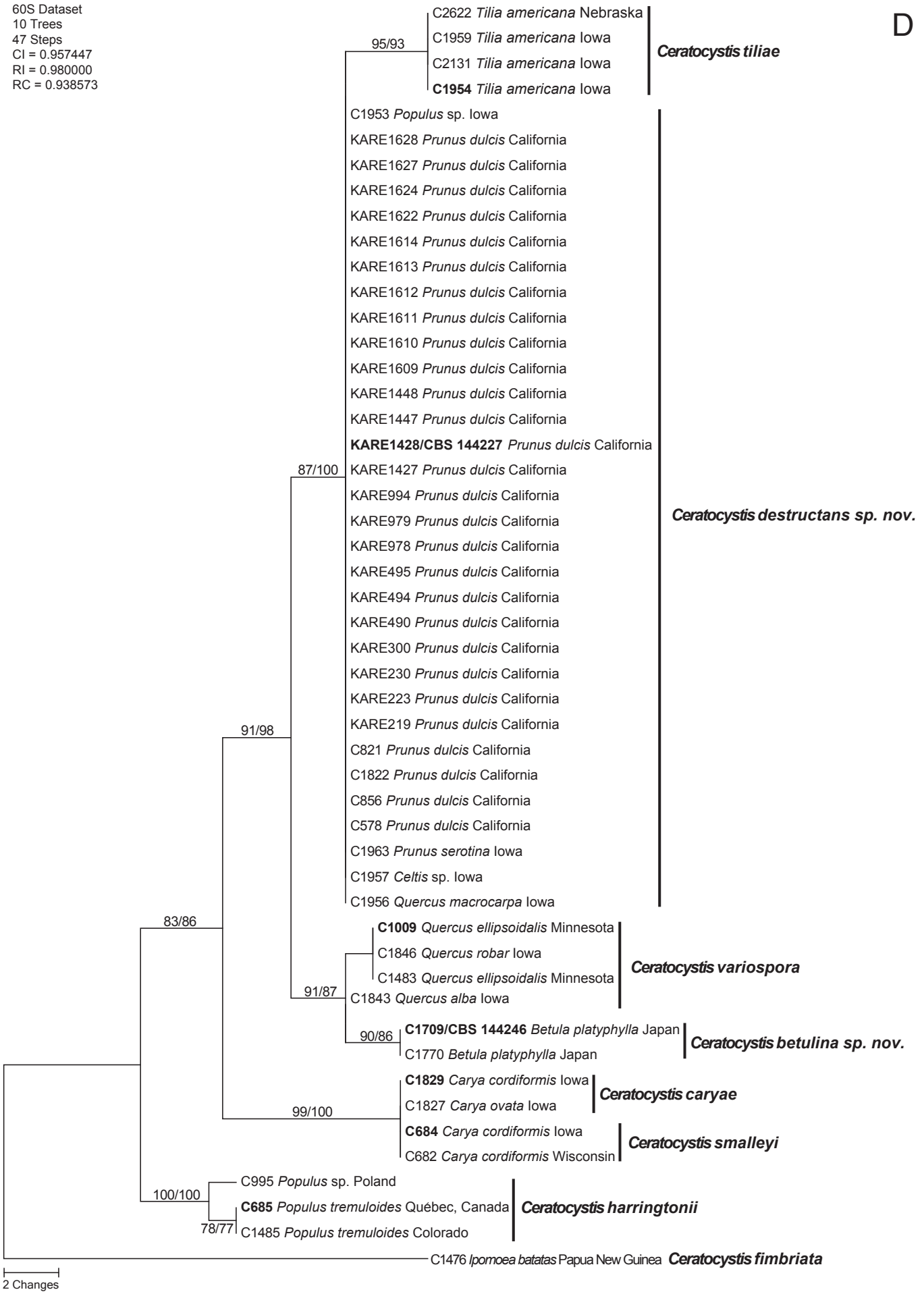

Fig. 3. (Continued).

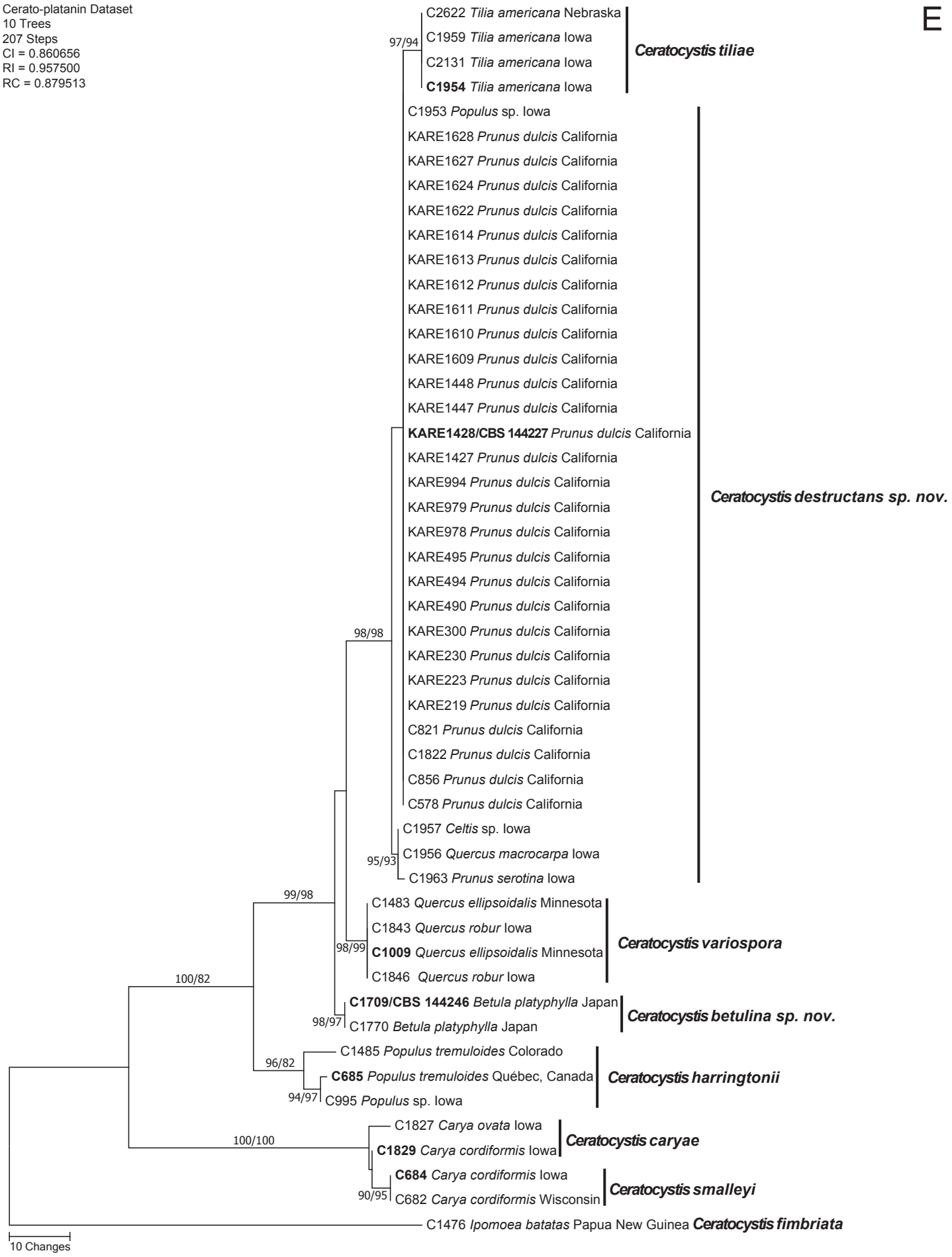

Fig. 3. (Continued).

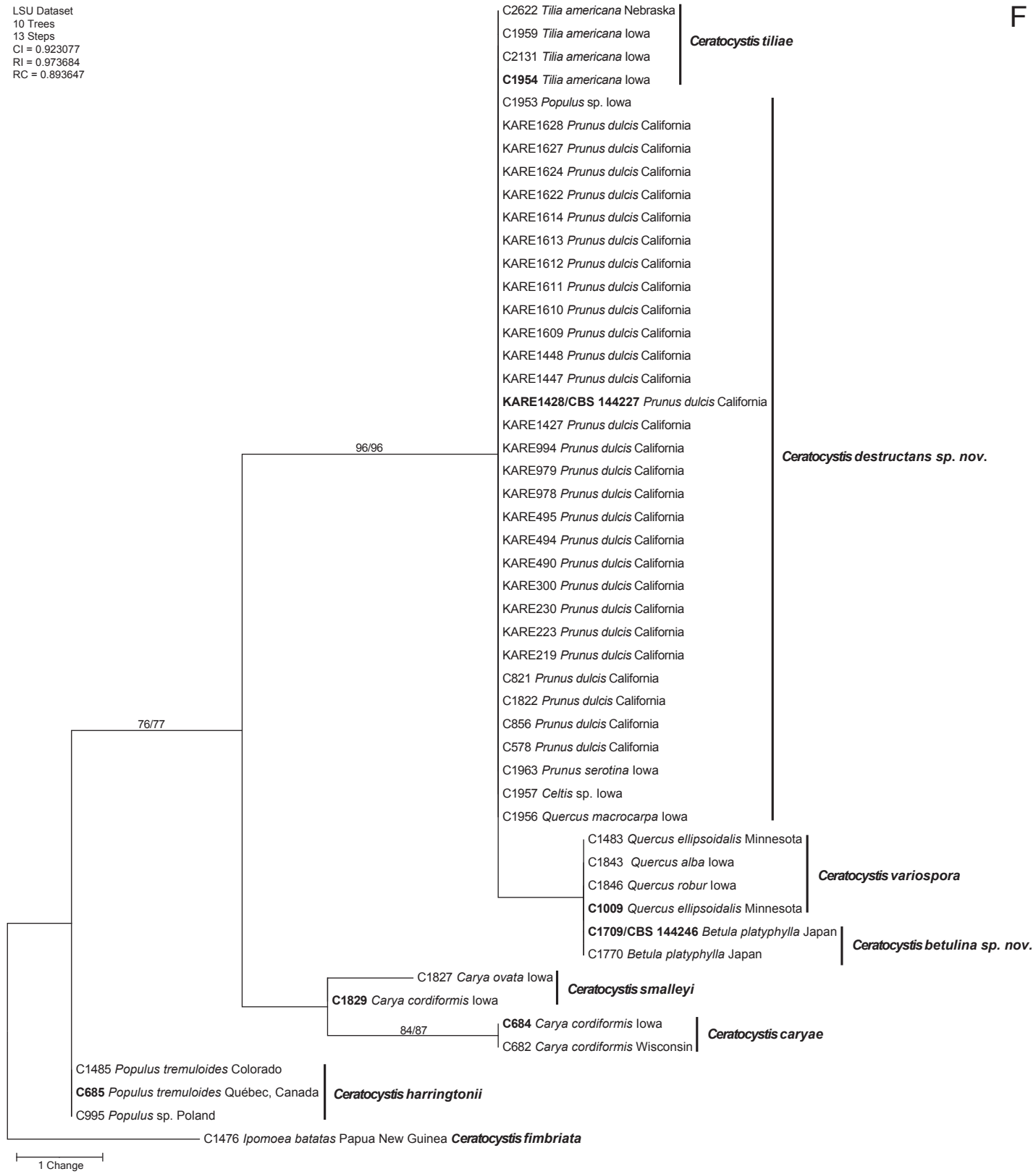

Fig. 3. (Continued).

the Cerato-platanin locus produced 487–498 bp fragments and resulted in a 498-character dataset, in which 327 characters were constant, 85 were parsimony uninformative, and 86 were parsimony informative (17.2 %). The MP analysis produced 10 equally most parsimonious trees of 207 steps and a CI, RI, and RC of 0.8606, 0.9575, and 0.8795 respectively (Fig. 3E). PCR amplification of LSU produced 1 330 bp fragments and resulted in a 1 330-character dataset, in which 1 318 characters were constant, three were parsimony uninformative, and nine were parsimony informative (1 %). The MP analysis produced 10 equally most parsimonious trees of 13 steps and a CI, RI, and RC of 0.9230, 0.9736, and 0.8936, respectively (Fig. 3F). No single gene fragment was able to confidently recognize all seven lineages within the NAC. Many single gene analyses produced tree topologies that separated most if not all species; however, a lack of support for some phylogenetic positions was realized.

Results strongly suggest that multiple gene regions are required to accurately separate the more recently diverged species *C. tiliae* and *C. destructans*, while earlier diverging members of the NAC (i.e. *C. harringtonii* and *C. smalleyi*) were typically discernible based on fewer loci or even a single locus (i.e. *TEF1*) (Fig. 3A–F). Only the *TEF1* gene region was able to discern the recently diverged *C. caryae* and *C. smalleyi* as separate phylogenetic species, further supporting the use of multiple loci for accurate identification of *Ceratocystis* species in the NAC.

Morphological characterization

Isolates representing *C. betulina* (C1709 and C1770) and *C. destructans* (C578, C821, KARE1428, and KARE1610) were used for morphological characterization (Table 2 and Supplementary Table 1). For *C. betulina* isolates C1709 and C1770, the average colony diameter after 7 d on PDA and MEA at room temperature (24 +/- 1 °C) was 27 (PDA)/24.2 (MEA) and 23/22 mm, respectively. For *C. destructans* isolates C578, C821, KARE1428 and KARE1610 the average colony diameter after 7 d on PDA and MEA at room temperature (24 +/- 1 °C) was 36/30, 35/32, 40/20.3 and 35/17.3 mm, respectively. In culture, both *C. betulina* and *C. destructans* were slow-growing with even to uneven margins. The colonies of *C. betulina* varied in color from white to grey to olivaceous green. Colonies of *C. destructans* were grey to olivaceous green. *Ceratocystis betulina* isolate C1770 produced few perithecia in culture, and the perithecia often lacked necks or had short necks. Ascospores were not observed for this isolate. Isolates of *C. destructans* produced black ascomata scattered throughout the colony with many perithecia near the colony centre. The ascomatal bases of *C. destructans* isolates ((118–)197(–358) µm) were larger than those produced by isolates of *C. betulina* ((103–)162(–220) µm). The morphological characters that distinguished *C. betulina* from *C. destructans* were the average diameter of the ascomata and lengths of the necks. *Ceratocystis destructans* had larger ascomatal diameters (av. ranging from 163–220 µm among the four isolates) and longer necks (av. ranging from 379–623 µm among the four isolates) compared to smaller ascomata (av. of the two isolates = 149 µm and 175 µm, respectively) and shorter necks (av. of the two isolates = 142 µm and 298 µm, respectively) of *C. betulina*. Cylindrical conidia and thick-walled aleurioconidia were abundant in both species and of similar dimensions. Aleurioconidia of *C. betulina* were often found in short chains compared to *C. destructans*, whose aleurioconidia were found singly or in short chains. Doliiform conidia were abundant in *C. betulina* isolates and sparse or absent in *C. destructans* isolates C578 and C821.

For two isolates of *C. betulina* (C1709 and C1770) and four isolates of *C. destructans* (C578, C821, KARE1428 and KARE1610) the optimal temperature for growth was 25 °C. In general, *Ceratocystis destructans* grew faster than *C. betulina* at 5, 10, 15, 20 and 35 °C on PDA. On the other hand, *C. betulina* isolates grew faster on PDA at the optimal temperature of 25 °C at an average of 3.5 mm/d, while *C. destructans* isolates grew at an average of 3.1 mm/d on PDA at 25 °C. All isolates grew slower on MEA, with an average growth rate of 0.8 mm/d and 1.3 mm/d at 25 °C for *C. destructans and C. betulina*, respectively. No growth was observed at 40 °C for any isolates on either growth medium. For both taxa, growth at 5 and 10 °C was reduced, and an abrupt decline was observed at 35 °C, however *C. destructans* (25 mm diam) had almost double the growth of *C. betulina* (15 mm diam) at this temperature after 14 d on PDA. For both taxa ascomatal production was most abundant when grown at 20 and 25 °C, and no ascomata were produced at 10 and 35 °C.

TAXONOMY

Morphological comparisons coupled with multi-locus phylogenetic analyses (MP and ML) of the combined six-gene dataset identified two distinct and strongly supported lineages for which no apparent species names exists. Thus, we propose the following new species names to properly circumscribe these unique taxa and to further resolve paraphyletic and cryptic taxa in the NAC.

Ceratocystis betulina D.P. Lawr., L.A. Holland & Trouillas, ***sp. nov.*** MycoBank MB824502. Figs 2, 4.

Etymology: The name refers to the host, *Betula platyphylla*, from which this fungus was isolated.

Typus: **Japan**, Morioke, Iwate, isolated from sporulating fungal mat on a log of *Betula platyphylla*, 22 Sep. 2000, *H. Masuya* No. C1709 (**holotype** BPI 910648, dried culture; ex-type culture CBS 144246).

Colonies 24.2 mm after 7 d at 25 °C on MEA, slow-growing with uneven margins and copious aerial hyphae. *Hyphae* initially hyaline, smooth, straight, branched, septate, becoming dark with age. *Mycelium* submerged, olivaceous green, aerial mycelium white, producing ascomata in clumps, odor sweet, with banana-like scent. *Ascomata* perithecial, with bases superficially to partially immersed in the substrate, mostly black, globose, (102.5–)124–174(–193) µm diam, unornamented or with undifferentiated hyphae, collar (32–)43–54(–59.5) µm wide at the base of the perithecial neck. *Perithecial necks* black, slender, (144.5–)227.5–368(–438) µm long, (14.5–)16.5–22(–24.5) µm wide at the base, (11.5–)13.5–19.5(–20) µm wide at the apex. *Ostiolar hyphae* hyaline, aseptate, straight to flexuous, 22–55 µm long. *Asci* not seen. *Ascospores* (4.5–)4.5–5(–5.5) × (2.5–)3–3.5(–5) µm with outer sheath forming a hat-shaped brim. *Conidiophores* of three types: endoconidiophores lageniform, hyaline to pale brown, septate, 29–67 µm in length, 3–6.5 µm wide at the base and 3–5 µm wide at the mouth, producing hyaline, concatenated, cylindrical conidia (10–)11–15.5(–19) × (2–)2.5–3(–3) µm; other endoconidiophores shorter, 14–33 µm in length, 3–6 µm wide at the base and 3.5–5.5 µm wide at the mouth, producing hyaline, concatenated, smooth-walled, doliiform conidia (5.5–)6–8(–9.5) × (4.5–)5.5–6(–6.5) µm; and less abundant, simple conidiophores (21.5–)23–30(–30) × (2.5–)3–5(–6) µm, producing smooth- and thick-walled, dark brown, ellipsoid to clavate, aleurioconidia (9–)9.5–10.5(–11) × (7.5–)8–8.5(–8.5) µm either singly or in short chains of 2–3.

Distribution: Morioke, Iwate (Japan).

Additional material examined: **Japan**, Morioke, Iwate, isolated from *Carpophilus sibiricus* from a log of *Betula platyphylla*, 22 Sep. 2000, *H. Masuya* (C1770).

Notes: *Ceratocystis betulina* was isolated from a log of *Betula platyphylla* located near *Prunus* and *Quercus* trees. Prior to our analyses, isolates C1709 and C1770 were considered to be *C. variospora*. Phylogenetically, *C. betulina* is strongly supported

Table 2. Comparative morphological characteristics of *Ceratocystis fimbriata* and *Ceratocystis platani* (Latin American clade) and *Ceratocystis* species in the North American clade of *Ceratocystis*.

Species	Hosts/Insects	Cultures examined in referenced studies[a]	Perithecia diameter (µm)	Width of collar (µm) at base of perithecial neck	Length of perithecial neck (µm)	Ascospores (µm)	Length of ostiolar hyphae (µm)	Flask-shaped conidiophores (µm)	Cylindrical conidia (µm)	Wide-mouthed conidiophores (µm)	Doliform conidia (µm)	Aleurioconidia (µm)	Reference
Ceratocystis fimbriata	*Ipomoea batatas*	C1418, C1354, C1476	110–250	Absent	440–770	5.5–7 × 3.5–5	53–136	55–120 × Mouth 3–6 Base 3–8	9–33 × 3–5	Absent	Absent	11–16 × 6.5–12	Engelbrecht & Harrington 2005
Ceratocystis platani	*Platanus* spp.	C1351, C1317, C1339	175–290	Absent	535–835	4–6.5 × 3–4.5	20–90	55–165 × Mouth 2.5–7.5 Base 3.5–7.5	11–22 × 3–5	35–50 × Mouth 5.5–6.5 Base 4.5–5	6–10 × 3.5–5	10–20 × 6–12	Engelbrecht & Harrington 2005
Ceratocystis destructans[*]	*Prunus dulcis*	C578, C821, **KARE1428**, KARE1610	122–208	44–64	381–626	4–5 × 3–3.5	38–56	33–113 × Mouth 2–3 Base 3–5	12–26 × 2–3	26–69 × Mouth 3–4 Base 5–6	7–11 × 4–6	8–13 × 7–12	This study
Ceratocystis tiliae	*Tilia americana*	**C1954**, C2131, C2622	175–350	50–100	425–915	5–6 × 4–4.5	40–90	80–160 (330) × Mouth 3.5–4.5 Base 4–5.5	15–40 × 3–5.5	50–90 × Mouth 4–6 Base 4.5–5.5	6.5–9 × 4.5–6	7.5–12.5 × 8–11.5	Oliveira *et al*. 2015
Ceratocystis betulina[*]	*Betula platyphylla*/ *Carpophilus sibiricus*	**C1709**, C1770	103–192	32–60	145–438	4.5–6 × 3–4	22–55	29–67 × Mouth 3–5 Base 2–6.5	10–19 × 2–3	14–33 × Mouth 3–5 Base 3–6	6–9 × 5–7	9–11 × 8–9	This study
Ceratocystis variospora	*Quercus alba*, *Q. ellipsoidalis*, *Q. robur*	**C1009**, C1483, C1843, C1846	130–350 (425)	51–80	≤ 830	3.5–6 × 3–5	22–50	52–98 × Mouth 2.5–4.5 Base 4.5–7	6–30 × 2.5–5	32–90 × Mouth 4.5–7.5 Base 4–5.5	5.5–10 × 5–8	6.5–9 × 7.5–14	Johnson *et al*. 2005
Ceratocystis harringtonii (formerly *C. populicola*)	*Populus tremuloides*, *Populus* hybrid	C89, **C685**, C947, C995, C1485	110–275	Present	≤ 665	4.5–6.5 × 3–5	42–75	45–200 × Mouth 3.5–4.5 Base 3.5–7	10–33 × 2–5 (5.5)	17–95 (125) × Mouth 3.5–8.5 Base 3.5–6	6.5–12 × 3.5–5	9–18.5 × 8–17.5	Johnson *et al*. 2005
Ceratocystis caryae	*Carya cordiformis*, *C. ovata*, *Ostrya virginiana*	C1827, **C1829**, C1412, C1413, C1845, C1971	135–340	48–103	≤ 950	4–6 × 3.5–4.5	32–80	42–510 × Mouth 3.2–4.8 Base 3.8–7.5	8.5–27 (43) × 2.5–6	40–100 × Mouth 5.5–8 Base 5–6.5 (7)	6–13.5 (16) × 5.5–9.5	9–21.5 × 8.5–16.5	Johnson *et al*. 2005
Ceratocystis smalleyi	*Carya cordiformis*, *C. ovata*/*Scolytus quadrispinosus*	C682, C683, **C684**, C1410, C1411, C1828, C1839, C1840, C1842, C1844, C1952	100–300 (350)	42–73 (85)	≤ 570	4–6 × 3.5–5	54–101	Absent	Absent	35–105 × Mouth 4–7.5 Base 4–6	7.5–31.5 × 4–7.5	Absent	Johnson *et al*. 2005

[a]Type specimen in bold.

[*]Measurements based on the type specimen.

Fig. 4. Morphological characteristics of *Ceratocystis betulina*. **A.** 14-d-old PDA culture. **B.** Close-up of perithecia from 7-d-old culture. **C.** Globose unornamented ascomata base with elongated neck. **D.** Straight to flexuous ostiolar hyphae. **E.** Hat-shaped ascospores from top and side view. **F.** Cylindrical conidia. **G.** Short, barrel-shaped conidia. **H.** Thick-walled aleurioconidia. Scale bars: C = 100 µm; D = 20 µm; E = 5 µm; F–G = 20 µm; H = 10 µm.

as the sister taxon to *C. variospora*. Morphologically, *C. betulina* is similar to other members in the NAC and cannot be easily distinguished, though it has somewhat smaller perithecia (102.5–)149(–192) µm diam, shorter, flask-shaped conidiophores (29–67 µm), shorter, wide-mouth conidiophores (14–33 µm), and smaller aleurioconidia, (9–)10(–11) × (7.5–)8(–8.5) µm. *Ceratocystis betulina* can be distinguished from *C. variospora* based on slightly smaller cylindrical conidia (10–19 × 2–3.5 µm for *C. betulina* and 6–30 × 2.5–5 µm for *C. variospora*) (Table 2; Supplementary Table 1).

Ceratocystis destructans L.A. Holland, D.P. Lawr., & Trouillas, ***sp. nov.*** MycoBank MB824558. Figs 2, 5.

Etymology: The name refers to this fungus causing destructive cankers in almond.

Typus: **USA**, California, Madera County, 36°52'50.3"N 119°51'25.4"W, isolated from wood canker of *Prunus dulcis*, 19 Jul. 2016, *L.A. Holland* No. KARE1428 (**holotype** BPI 910649, dried culture; ex-type culture CBS 144247).

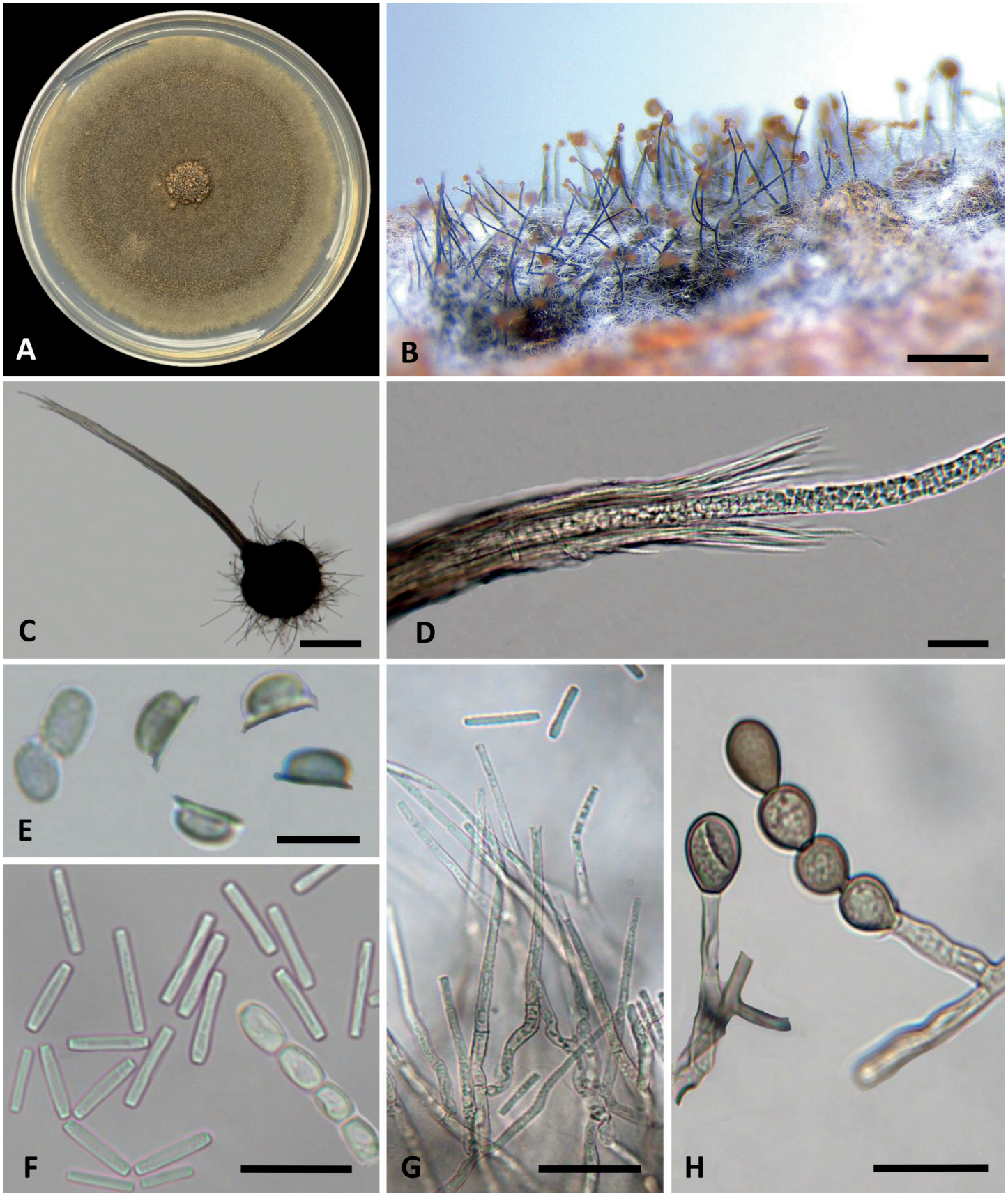

Fig. 5. Morphological characteristics of *Ceratocystis destructans*. **A.** 14-d-old PDA culture. **B.** Close-up of perithecia from 7-d-old culture. **C.** Globose unornamented ascomata base with elongated neck. **D.** Mostly straight ostiolar hyphae. **E.** Hat-shaped ascospores from top and side view. **F.** Cylindrical and short, barrel-shaped conidia. **G.** Flask-shaped conidiophores. **H.** Thick-walled aleurioconidia. Scale bars: B = 500 µm; C = 100 µm; D = 25 µm; E = 5 µm; F = 25 µm; G = 20 µm; H = 25 µm.

Colonies 20.3 mm after 7 d at 25 °C on MEA, slow-growing with uneven margins and copious aerial hyphae, olivaceous green to brown, aerial hyphae white, odor sweet, with banana-like scent, ascomata produced in clumps or in concentric rings.

Hyphae initially hyaline, smooth, straight, branched, septate, becoming dark with age. *Ascomata* perithecial, with bases superficially to partially submerged, mostly black, globose, (121.5–)142–184(–208) µm diam, unornamented or with undifferentiated hyphae, collar (44–)47.5–57(–64) µm wide at the base of the perithecial neck. *Perithecial necks* black, slender, (382–)434–573(–626) µm long, (13–)18–23.5(–24.5) µm wide at the base, (11–)12–17(–22) µm wide at the apex. *Ostiolar hyphae* hyaline, aseptate, mostly straight, 38–56 µm long. *Asci* not seen. *Ascospores* (5–)4.5–5(–5) × (2.5–)3–3.5(–3.5) µm with outer sheath forming a hat-shaped brim. *Conidiophores* of three types: endoconidiophores lageniform, hyaline to pale brown, septate, 34–113 µm in length, 3–5 µm wide at the base and 2–3 µm at the mouth; producing concatenated, hyaline, cylindrical conidia (12–)14.5–20.5(–26) × (1.5–)2–2.5(–3) µm; other endoconidiophores less prevalent and shorter, 26–69 µm in length, 3.5–4 µm wide at the base and 4.5–5.5 µm wide at the mouth, producing hyaline, concatenated, smooth-walled, doliiform conidia (7–)8–10(–10.5) × (4–)4.5–6(–6.5) µm; and simple conidiophores not as prevalent, (18–)24–41(–45.5) × (2.5–)3–3.5(–4), producing smooth- and thick-walled, dark brown, clavate, aleurioconidia (7.5–)9–12(–13) × (7–)8.5–11(–13) µm either singly or in short chains of up to four.

Distribution: California (USA), *Prunus dulcis*; Iowa (USA), *Prunus serotina*, *Populus*, *Celtis* and *Quercus.*

Additional materials examined: **USA**, California, Colusa County, isolated from bark canker of *P. dulcis*, 1989, *R. Bostock* (C578); California, Colusa County, isolated from stem canker of *P. dulcis*, 24 Feb. 1996, *D. Rizzo* (C821); California, Merced County, isolated from bark canker of *P. dulcis*, 8 Sep. 2016, *F. Trouillas* (KARE1610).

Notes: *Ceratocystis destructans* has been isolated from almond trees throughout the Central Valley Region of California from necrotic inner bark and wood tissues of trees showing sunken cankers and gummosis. *Ceratocystis destructans* is morphologically similar to the sister species *C. tiliae*. However, these species can be distinguished based on average ascospore dimensions, with *C. destructans* having slightly smaller ascospores (4–5 × 2.5–3.5 µm) than *C. tiliae* (5–6 × 4–4.5 µm) and smaller ascomata (Table 2; Supplementary Table 1).

DISCUSSION

Morphological and phylogenetic analyses revealed two novel *Ceratocystis* species, *C. betulina* and *C. destructans*, that reside in the North American clade of *Ceratocystis.* The NAC of *Ceratocystis* was established, and the first species delineated by Johnson *et al*. (2005) based on ITS-rDNA phylogeny, electrophoretic phenotypes, interfertility tests, and cross-inoculations experiments. The host-associated lineages included the aspen lineage with *C. harringtonii* (synonym *C. populicola*), the hickory lineage with two species, *C. caryae* and the closely related species *C. smalleyi*, and a third lineage represented by the earlier described *C. variospora* (Davidson 1944), with two well-supported subclades: the "oak lineage" associated with oak (*Quercus*) and birch (*Betula*) and the 'cherry lineage,' mainly associated with cherry and almond (*Prunus* spp.), *Populus*, and basswood (*Tilia americana*). Johnson *et al*. (2005) hypothesized that host-associated isolates of *C. variospora* from *Prunus*, *Quercus*, and *Tilia* could represent three separate species, respectively, but these intersterile lineages could not be clearly distinguished by phenotypic traits, i.e., morphology or host specialization to *Quercus* vs. *Prunus* spp. Oliveira *et al*. (2015a), using three individual gene analyses (LSU, *TEF1*, and CP), showed that *Ceratocystis* isolates recovered from *Tilia* clustered as a well-supported monophyletic group sister to the cherry lineage of *C. variospora* in the *TEF1* and CP analyses. Not surprising, limited sequence variation failed to identify sublineages within *C. variospora* in the analysis of LSU, the least informative marker in that study and in our study. Inoculation of *Quercus macrocarpa* and *Tilia americana* seedlings demonstrated that only *Tilia*-derived isolates were aggressive on *T. americana*, cultures of the *Tilia* pathogen were distinguished morphologically, and *C. tiliae* was described as new (Oliveira *et al* 2015a). Like Johnson *et al*. (2005), Oliveira *et al*. (2015a) maintained the name *C. variospora* to accommodate isolates recovered from *Betula*, *Celtis*, *Populus*, *Prunus* and *Quercus*, thus leaving *C. variospora* as a paraphyletic species.

Our individual gene analyses produced similar topologies and support values for species assignments as in de Beer *et al*. (2014) and Oliveira *et al*. (2015a), highlighting the need for combined multi-locus analyses to discriminate closely related species and to estimate species relationships in the NAC. For example, of the six loci tested, only *TEF1* was able to confidently delineate the sister species *C. caryae* and *C. smalleyi*, and all loci except LSU and 60S were able to discern *C. betulina* and *C. variospora* as well-supported sister groups. All loci supported the close relationship of *C. betulina*, *C. variospora*, *C. tiliae*, and *C. destructans*, while *C. harringtonii* and *C. caryae*/*smalleyi* lineages were more distantly related, in agreement with previous studies (Johnson *et al*. 2005, Oliveira *et al*. 2015a).

The de Beer *et al*. (2014) multi-locus analysis (LSU+*MCM7* +60S) involving NAC members distinguished the ex-type cultures of *C. caryae* C1829, *C. harringtonii* C685, *C. smalleyi* C684, and *C. variospora* C1009, but they did not examine the intraspecies diversity of the NAC. Analyses with more isolates of the NAC with phylogenetically informative loci such as *TEF1* and CP have revealed greater diversity within the oak and cherry lineages of *C. variospora* (Oliveira *et al*. 2015a) as compared to ITS analyses (Johnson *et al*. 2005). Oliveira *et al*. (2015a) did not perform a multi-locus analysis because they reported a low *P* value (*P* = 0.01) for their three-gene PHT. The topology and support values for our *TEF1* and CP phylograms are very similar to those reported by Oliveira *et al*. (2015a). The results of our PHT (*P* = 0.10) and examination of tree topology and support for phylogenetic species recognition utilizing six loci revealed no significant incongruence amongst loci, and the combined analyses resulted in a more robustly supported inference about species recognition (Taylor *et al*. 2000) and species relationships within the NAC.

The use of multiple phylogenetically informative gene regions has allowed for further taxonomic refinement of species assignments within both the oak and cherry lineages of *C. variospora*. The oak lineage now consists of two robust lineages, which are defined by the species *C. betulina* and *C. variospora*, which was hypothesized by Johnson *et al*. (2005) and is now strongly supported by multi-locus analyses. The former cherry lineage now consists of two strongly supported phylogenetic lineages, *C. tiliae* and *C. destructans*, as predicted by interfertility tests and distinct mycelial phenotypes, namely the former with slower growth and less pigmentation as compared to the latter

(Johnson *et al*. 2005, Oliveira *et al*. 2015a). The phylogenetic position of three isolates (C1956 from *Quercus*, C1957 from *Celtis*, and C1963 from *Prunus*) from Iowa is very close to *C. destructans*, and can be considered this species, but further intersterility testing and phylogenetic analyses would be needed to confidently resolve this small group.

Most *Ceratocystis* species within the NAC are not only supported by multi-locus phylogenetic analyses but also by host specialization, a biological species concept, and in some cases morphological characters. Host specificity through pathogenicity tests has been demonstrated in the aspen (*C. harringtonii*) and hickory lineages (*C. caryae* and *C. smalleyi*) (Johnson *et al*. 2005, Oliveira *et al*. 2015a). Now, with additional locus sampling, the former oak lineage of *C. variospora* consists of two host-associated species, *C. betulina* and *C. variospora*, which have only been isolated from *Betula* and *Quercus*, respectively. A clear pattern of host specialization was not as evident in the oak and cherry lineage cross-inoculations, with one exception. *Ceratocystis tiliae* was more aggressive to *Tilia* than close relatives isolated from *Quercus* and *vice versa* (Oliveira *et al*. 2015a). *Ceratocystis betulina* is represented by isolates from a single *Betula* log, and it is not clear if the log was saprobically colonized or if the fungus was native to Japan. To our knowledge no pathogenicity trials have compared the host associated sister clades *C. betulina* and *C. variospora*, but we predict that some level of host specialization will be realized in this clade as suggested by phylogenetic results. Preliminary pathogenicity trials have shown that isolates of *C. destructans* are highly pathogenic to almond trunks and branches (Holland, unpublished data); however, no cross-inoculation experiments have been performed. *Ceratocystis destructans* appears to have a rather broad geographic and host range, including *Prunus* spp. in California as well as *Celtis*, *Populusk*, *Prunus* and *Quercus* in the Midwest of the USA.

Intersterility tests by Johnson *et al*. (2005) revealed that MAT-2 testers from the cherry, oak and *Tilia* lineages of *C. variospora* were only interfertile with MAT-1 testers from the same respective lineages, which are now recognized as *C. destructans*, *C. variospora* and *C. tiliae*. Two MAT-2 testers from almond trees in California (i.e. C578 and C856) were only interfertile with other isolates now defined as *C. destructans*, including other Californian isolates from almond and Iowa isolates from *Populus* and *Quercus.* Furthermore, C1709, the ex-type culture of *C. betulina*, was not interfertile with the MAT-2 testers of these species, nor were MAT-1 strains of *C. harringtonii*, *C. caryae* and *C. smalleyi* interfertile with the MAT-2 testers of *C. destructans*, *C. variospora* and *C. tiliae.* These examples of reproductive isolation support the recognition of biological species, which together with phylogenetic evidence, supports designation of these lineages as distinct taxa.

Morphology in the NAC was similar for all isolates with some unifying features, such as the ability to produce a second endoconidial stage of doliiform conidia from wide-mouth phialides and a distinct collar at the base of the perithecial neck (Johnson *et al*. 2005). Within the NAC, morphological features vary only slightly among the different species. For instance, *C. variospora*, which formerly encompassed what is now identified as *C. tiliae* (Oliveira *et al*. 2015a), *C. destructans* and *C. betulina*, differs morphologically from these species. For example, *C. variospora* possesses larger perithecia (130–350 µm), on average, than *C. destructans* (122–208 µm) and *C. betulina* (103–192 µm). *Ceratocystis variospora* also produces slightly larger cylindrical endoconidia than *C. betulina*. However, the overall lack of morphological distinction makes it difficult to recognize these species without molecular characterization. *Ceratocystis destructans* and *C. betulina* are similar to other species in the NAC, with a dark green to grey colony color, fruity odor, and both cylindrical and doliiform endoconidia, as well as aleurioconidia (Table 2; Supplementary Table 1). *Ceratocystis caryae* and *C. smalleyi* (hickory lineage) have very similar ITS sequences and allozyme phenotypes, and they appear to be sexually interfertile (Johnson *et al*. 2005), but they differ greatly in morphology and biology. *Ceratocystis smalleyi* lacks cylindrical conidia from flask-shaped phialides and aleurioconidia (Johnson *et al*. 2005).

Several species in the NAC, including *C. caryae*, *C. harringtonii*, *C. tiliae* and *C. variospora*, are most commonly associated with wounded trunks and branches of trees (Johnson *et al*. 2005, Oliveira *et al*. 2015a), suggesting that members of this clade may act primarily as wound colonizers. *Ceratocystis destructans* is proposed as the new name for the causal agent of Ceratocystis canker of almond in California. The disease is common in California almond orchards where the trees have suffered repeated bark injuries during mechanical harvest, and *C. destructans* has been routinely isolated from discolored inner bark of almond trees that have been damaged by mechanical harvesting equipment. *Ceratocystis destructans* can also infect almond trees at wounds caused by pruning, producing branch cankers that result in extensive branch dieback. Several insects have been identified in California almond orchards as potential vectors, including several species of sap-feeding beetles (*Coleoptera*; *Nitidulidae*) and fruit flies (*Diptera*: *Drosophilidae*) (Moller & DeVay 1968). These insects are attracted to the sweet-smelling volatile compounds produced by *Ceratocystis*, and thus the insects may transport infectious spore inoculum from one tree to another. The pathogenicity to almond of *C. destructans* isolates collected for this study was recently investigated. Results showed that this fungus can produce cankers and cause extensive gumming in trunks and branches of almond (Holland *et al*. 2017). The host range of *C. destructans* in California and the occurrence of putative natural inoculum sources in the native vegetation surrounding almond orchards are unknown. Yet, *C. destructans* has been isolated from *Populus* spp. and *Quercus* spp. in the eastern USA. The occurrence in California of *C. destructans* on similar or related plant species should be investigated to better understand the pathogens' biology and putative origin as Ceratocystis canker continues to threaten the almond industry in California.

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REFERENCES

Al Adawi AO, Barnes I, Khan IA, *et al*. (2014). Clonal structure of *Ceratocystis manginecans* populations from mango wilt disease in Oman and Pakistan. *Australasian Plant Pathology* **43**: 393–402.

Barnes I, Roux J, Wingfield BD, *et al*. (2003). *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Barnes I, Nakabonge G, Roux J, *et al*. (2005). Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. *Plant Pathology* **54**: 189–195.

Barnes I, Fourie A, Wingfield MJ, *et al*. (2018). New *Ceratocystis* species associated with rapid death of *Metrosideros polymorpha* in Hawai'i. *Persoonia* **40**: 154–181.

Chen H, Kovalchuck A, Keriö S, *et al*. (2013). Distribution and bioinformatic analysis of the cerato- platanin protein family in *Dikarya*. *Mycologia* **105**: 1479–1488.

Davidson RW (1944). Two American hardwood species of *Endoconidiophora* described as new. *Mycologia* **36**: 300–306.

de Beer ZW, Duong TA, Barnes I, *et al*. (2014). Redefining *Ceratocystis* and allied genera. *Studies in Mycology* **79**: 187–219.

de Beer ZW, Marincowitz S, Duong TA, *et al*. (2017). *Bretziella*, a new genus to accommodate the oak wilt fungus, *Ceratocystis fagacearum* (*Microascales*, *Ascomycota*). *MycoKeys* **27**: 1–19.

DeVay JE, English H, Lukezix FL, *et al*. (1960). Mallet wound canker of almond trees. *California Agriculture* **14**: 8–9.

DeVay JE, Lukezix FL, English H, *et al*. (1962). Ceratocystis canker. *California Agriculture* **16**: 1–3.

Engelbrecht CJB, Harrington TC (2005). Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **6**: 1323–1330.

Halsted BD (1890). Some fungous diseases of the sweet potato. *Bulletin of the New Jersey Agricultural Experiment Station* **76**: 3–32.

Harrington TC (1993). Biology and taxonomy of fungi associated with bark beetles. In: *Beetle-pathogen Interactions in Conifer Forests* (Schowalter TD, ed). Academic Press, USA: 37–58.

Harrington TC (2000). Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brasileira* **25** (Suppl): 262–263.

Harrington TC (2009). The genus *Ceratocystis*: Where does the oak wilt fungus fit? In: *Proceedings of the National Oak Wilt Symposium, 2nd* (Appel DN, Billings RF, eds). USA: 27–41.

Harrington TC (2013). *Ceratocystis* diseases. In: *Infectious Forest Diseases* (Gonthier P, Nicolotti G, eds). CAB International, England: 230–255.

Harrington TC, Thorpe DJ, Alfenas AC (2011). Genetic variation and variation in aggressiveness to native and exotic hosts among Brazilian populations of *Ceratocystis fimbriata*. *Phytopathology* **101**: 555–566.

Heath RN, Wingfield MJ, Wingfield BD, *et al*. (2009). *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* **34**: 41–68.

Holland LA, Nouri MT, Morris N, *et al*. (2017). Almond Trunk and Scaffold Canker Diseases in California: Diagnosis, Pathogenicity, and Management [Abstract]. American Phytopathological Society Annual Meeting, San Antonio, TX.

Johnson JA, Harrington TC, Engelbrecht CJB (2005). Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092

Kile GA (1993). Plant diseases caused by species of *Ceratocystis* sensu stricto and *Chalara*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds). APS Press, USA: 173–183.

Li Q, Harrington TC, McNew D, *et al*. (2017). *Ceratocystis uchidae*, a new species on *Araceae* in Hawai`i and Fiji. *MycoScience* **58**: 398–412.

Liu FF, Barnes I, Roux J, *et al*. (2018). Molecular phylogenetics and microsatellite analysis reveal a new pathogenic *Ceratocystis* species in the Asian-Australian clade. *Plant Pathology* **67**: 1097-1113. https://doi.org/10.1111/ppa.12820.

Maddison WP, Maddison DR (2016). Mesquite: a modular system for evolutionary analysis. Version 3.10 http://mesquiteproject.org.

Malloch D, Blackwell M (1993). Dispersal biology of the ophiostomatoid fungi. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds). APS Press, USA: 195–206.

Marin-Felix Y, Groenewald JZ, Cai L, *et al*. (2017). Genera of phytopathogenic fungi: GOPHY 1. *Studies in Mycology* **86**: 99–216.

Mayers CG, McNew DL, Harrington TC, *et al*. (2015). Three genera in the *Ceratocystidaceae* are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. *Fungal Biology* **119**: 1075–1092.

Mayers CG, Bateman CC, Harrington TC (2018). New *Meredithiella* species from mycangia of *Corthylus* ambrosia beetles suggest genus-level coadaptation but not species-level coevolution. *Mycologia* **110**: 63–78.

Mbenoun M, Wingfield MJ, Begoude Boyogueno AD, *et al*. (2014). Molecular phylogenetic analyses reveal three new *Ceratocystis* species and provide evidence for geographic differentiation of the genus in Africa. *Mycological Progress* **13**: 219–240.

Moller WJ, DeVay JE (1968). Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Nel WJ, Duong TA, Wingfield BD, *et al*. (2018). A new genus and species for the globally important, multihost root pathogen *Thielaviopsis basicola*. *Plant Pathology* **67**: 871–882.

Oliveira LSS, Harrington TC, Freitas RG, *et al*. (2015a). *Ceratocystis tiliae* sp. nov., a wound pathogen on *Tilia americana*. *Mycologia* **107**: 986–995.

Oliveira LSS, Harrington TC, Ferreira MA, *et al*. (2015b). Species or genotypes? Reassessment of four recently described species of the Ceratocystis wilt pathogen, *Ceratocystis fimbriata*, on *Mangifera indica*. *Phytopathoogy*. **105**: 1229–1244.

Pazzagli L, Cappugi G, Manao G, *et al*. (1999). Purification, characterization and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. *platani*. *Journal of Biological Chemistry* **274**: 24949–24964.

Perry E, McCain AH (1988). Incidence and management of canker stain in London plane trees in Modesto, California. *Journal of Arboriculture* **14**: 18–19.

Rayner RW (1970). *A mycological colour chart*. Commonwealth Mycological Institute and British Mycological Society.

Seifert KA, Wingfield MJ, Kendrick WB (1993). A nomenclator for described species of *Ceratocystis*, *Ophiostoma*, *Ceratocystiopsis*, *Ceratostomella* and *Sphaeronaemella*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds). APS Press, USA: 269–287.

Stielow JB, Lévesque CA, Seifert KA *et al*. (2015). One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia* **35**: 242–263.

Swofford DL (2002). PAUP* 4: phylogenetic analysis using parsimony (*and other methods). Sunderland, Massachusetts: Sinauer Associates.

Tamura K, Stetcher G, Peterson D, *et al*. (2013). MEGA 6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Taylor JW, Jacobson DJ, Kroken S, *et al*. (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.

Thorpe DJ, Harrington TC, Uchida JY (2005). Pathogenicity, internal transcribed spacer-rDNA variation, and human dispersal of

Ceratocystis fimbriata on the family *Araceae*. *Phytopathology* **95**: 316–323.

Upadhyay H (1981). *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press, USA.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **174**: 4238–4246.

Wingfield MJ, Seifert KA, Webber JF (1993). *Ophiostoma and Ceratocystis: Taxonomy, Ecology and Pathogenicity*. APS Press, USA.

Wingfield MJ, De Beer C, Visser C, *et al*. (1996). A New *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Applied Microbiology* **19**: 191–202.

Supplementary Material: http://fuse-journal.org/

Table. S1. Additional specimens of *C. betulina* and *C. destructans* examined.