Plant Disease • 2022 • 106:661-668 • https://doi.org/10.1094/PDIS-08-21-1639-RE

e-Xtra*

A New Real-Time PCR Assay for Detecting Fungi in Genus *Ceratocystis*

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Abstract

The genus *Ceratocystis* contains several significant plant pathogens, causing wilt and canker disease on a wide range of plant species. There are >40 known species of *Ceratocystis*, some of which are becoming increasingly important in agricultural or natural ecosystems. The diagnostic procedures for most *Ceratocystis* species rely on time-consuming and labor-intensive culturing approaches. To provide more time-efficient and sensitive molecular diagnostic tools for *Ceratocystis*, a generic TaqMan real-time PCR assay was developed using the ITS gene. This novel two-probe TaqMan assay amplified DNA from all tested *Ceratocystis* species. Some nonspecific amplification of a few species from closely related genera was observed under certain conditions; however, these false-positive detections could be ruled out using the additional PCR primers developed for further sequence-based identification of the detected species. The assay was found to be highly sensitive, as it detected 0.2 pg/µl of *Ceratocystis* DNA in water as well as in host DNA matrix. Further validation with artificially inoculated fig stem tissue demonstrated that the assay was also able to effectively detect the pathogen in infected asymptomatic stem tissue. This newly developed real-time PCR assay has practical applications in biosecurity, conservation, and agriculture; it will enable the detection of *Ceratocystis* species directly from plant material to facilitate more sensitive screening of imported plant germplasm, and allow rapid tracking of pathogens in the case of disease outbreaks.

Keywords: biosecurity, Ceratocystidaceae, Ceratocystis wilt, pathogen detection, plant disease diagnostics

Ceratocystis species are significant plant pathogens, infecting a wide range of herbaceous and woody plant species, both in agricultural production and in natural ecosystems (Heath 2009; Roux and Wingfield 2009). The symptoms vary depending on the pathogen species and host, but most *Ceratocystis* species cause infections on roots, stems, leaves, and fruit, with wilt and canker diseases being the most common (Al Adawi et al. 2014; Barnes et al. 2018; Heath et al. 2009). While some of the species are weak pathogens, others can be highly aggressive (Cho et al. 2020; Harrington 2015).

The genus *Ceratocystis* currently contains 42 species placed in four geographical clades (Barnes et al. 2018; Cho et al. 2020; Liu et al. 2018; Marincowitz et al. 2020; Marin-Felix et al. 2017). The largest of these, often referred to as the most aggressive group of *Ceratocystis* pathogens, is the Latin American clade. It contains several well-known pathogens, such as the plantain wilt pathogen *C. platani*; the sweet potato black rot pathogen *C. fimbriata* (note: the *C. fimbriata* strains also cause kiwifruit wilt, which are different from the sweet potato strains); and the ʻōhiʻa wilt pathogen *C. lukuohia* (Barnes et al. 2018; Engelbrecht and Harrington 2005; Piveta et al. 2016). The North American clade contains tree pathogens (e.g., the economically significant almond canker pathogen *C. destructans*) that are mostly associated with wounded trunks (Holland et al. 2019; Johnson et al. 2005). The Asian–Australian clade contains less aggressive pathogens, such as the ʻōhiʻa canker pathogen *C. huliohia* and the taro root pathogens *C. uchidae* and *C. changhui* (Barnes et al. 2018; Li et al. 2017). The African clade contains pathogens that mostly affect tree species native to Africa, such as the wattle wilt pathogen *C. albifundus*, which has a very broad host range (Heath et al. 2009; Mbenoun et al. 2014; Roux et al. 2007).

Because of the limited variation in the morphological characters and the presence of several different spore stages, inconsistencies in *Ceratocystis* taxonomy prevailed for decades. Then, de Beer et al. (2014) used DNA sequences of three gene regions (LSU, 60S, and MCM7) from 79 species classified as *Ceratocystis*, and showed them belonging to seven distinct genera in the family Ceratocystidaceae: *Ceratocystis* (*sensu stricto*), *Charalopsis*, *Endoconidiophora*, *Davidsoniella*, *Thielaviopsis*, *Huntiella*, and *Ambrosiella*. While the internal transcribed spacer (ITS) region is the most commonly used region for fungal identification (Schoch et al. 2012), the species recognition within *Ceratocystis* has been based on multiple molecular markers such as ITS, BT, and TEF-1 (Marin-Felix et al. 2017). This is because high intragenomic variation has been detected in the ITS region of some *Ceratocystis* species (Harrington et al. 2014; Roux et al. 2020). Kanzi et al. (2020) have further demonstrated that a phylogenomic approach would be more appropriate for species delimitation within *Ceratocystis*, as there is also evidence indicating hybridization between different species.

Specific PCR assays have been developed for the detection of significant pathogens like *C. platani* (Luchi et al. 2013; Lumia et al. 2018; Pilotti et al. 2012), *C. lukuohia*, and *C. huliohia* (Heller and Keith 2018). A generic PCR-based restriction fragment length polymorphism assay was developed for *Ceratocystis* in the late 1990s (Witthuhn et al. 1999), but because this method is time-consuming, and the taxonomy of the genus has been substantially revised, it is no longer fit-for-purpose in diagnostics. Therefore, the detection of most *Ceratocystis* species from suspected diseased plant material as of this writing relies on symptom examination and isolation-based methods, followed by morphological and/or DNA sequence-based identification. This diagnostic approach is labor-intensive and time-consuming, and could also give false-negative results, especially in the case of early or nonsymptomatic infections.

Given their broad geographical distribution and pathogenicity toward a wide range of host plants, *Ceratocystis* species pose a threat

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Funding: This work was supported by the Ministry for Primary Industries, Operational Research Programmes under Project 405749.

*The *e*-Xtra logo stands for "electronic extra" and indicates there are two supplementary figures and one supplementary table published online.

The author(s) declare no conflict of interest.

Accepted for publication 28 September 2021.

to horticultural and forest industries as well as natural ecosystems worldwide. Over the last decade, a large number of new *Ceratocystis* species have been described (e.g., Barnes et al. 2018; Cho et al. 2020; Li et al. 2017; Liu et al. 2018), accounting for more than half of the recognized species in the genus. Given the possibility of undiscovered cryptic species and their unknown potential host range, it is critical to focus on the whole *Ceratocystis* genus rather than selected species when conducting diagnostics. Therefore, a rapid and sensitive assay for early detection of any *Ceratocystis* species is highly desirable, for containing the spread of unwanted pathogens.

Because several *Ceratocystis* species are of biosecurity concern in many countries (e.g., Australia, the United Kingdom, New Zealand, and the United States of America) they also need to be screened for in imported plant germplasm (ABARES 2021; EPPO 2021; MPI 2020; ONZPR 2021). Using a single assay that covers a broad range of species could therefore be beneficial for improved efficiency and assurance. This study presents the development of a two-probe–based, real-time PCR assay, for the genus *Ceratocystis*, for enabling the detection of these significant pathogens directly from plant material.

Materials and Methods

Fungal isolates. A test panel of 96 fungal isolates from a range of geographical origins and all phylogenetic clades was gathered for assay validation. These isolates were obtained from the Forestry and Agriculture Biotechnology Institute in South Africa (CMW), the Westerdijk Fungal Biodiversity Institute in the Netherlands (CBS), the International Collection of Microorganisms from Plants in New Zealand (ICMP), and the in-house collection of the Plant Health and Environment Laboratory. The panel contained 55 isolates of 35 different *Ceratocystis* species, 15 isolates of 12 species from six genera that are closely related to *Ceratocystis*, and 26 isolates of 21 other fungal species that could be associated with some of the host plants (Table 1). Fungal cultures were grown on potato dextrose agar (PDA) at room temperature and stored in glycerol at –80°C.

DNA extraction, amplification, and sequencing. Two-to-three agar plugs with mycelium (5 mm in diameter) were transferred into 2-ml tubes of MagNA Lyser Green Beads (Roche Life Sciences, Basel, Switzerland). Tissue was lysed using a bead-beater (BioSpec Products, Bartlesville, OH) at 7,000 rpm for 1 min before adding 600 µl of cetyltrimethylammonium bromide (CTAB) lysis buffer (2.0% CTAB, 1.0% polyvinylpyrrolidone, 1.4 M of NaCl, 20 mM of EDTA, and 100 mM of Tris-HCl at pH 8.0) and incubating it at 65°C for 30 min with occasional shaking for 20 s at 2,000 rpm with 2-min intervals in a Thermomixer (Eppendorf, Hamburg, Germany). The content was centrifuged at 14,000 *g* for 2 min and 420 µl of the supernatant was used for DNA extraction with the automated system KingFisher (Thermo Fisher Scientific, Waltham, MA) using the InviMag Plant DNA Mini Kit protocol (STRATEC Molecular, Berlin, Germany), following manufacturer's instructions. Before using the DNA for assay validation, the identity of the isolates was confirmed by amplifying the ITS region using primers ITS5 and ITS4 (White et al. 1990) and sequencing at EcoGene (Auckland, New Zealand). The sequences were analyzed using the program Geneious v.10.2.5 (Biomatters Ltd, Auckland, New Zealand).

Primer and probe design. To design *Ceratocystis* genus-specific primers and probes, a total of 1,051 ITS sequences, reported to originate from *Ceratocystis* and closely related genera, were obtained from the GenBank. Of these, 612 *Ceratocystis sensu stricto* ITS sequences were aligned using the alignment option 'MUSCLE' in the program Geneious v.10.2.5. The alignment was manually searched for potential regions for specific primer and probe design. These regions were then *in silico* tested for *Ceratocystis sensu stricto* specificity through BLASTn analysis on GenBank and for secondary structures in Geneious 10.2.5 (Biomatters Ltd). Two primers, Cer-All F1 and Cer-All R1, were designed for the real-time PCR assay and synthesized by Thermo Fisher Scientific. Two TaqMan hydrolysis probes, Cer-All P1 and Cer-All P2, were designed with 6-carboxyfluorescein and hexachlorofluorescein fluorophores, respectively, and synthesized by BioSearch Technologies (Hoddleston, U.K.). In addition, primers Cer-All F2 and Cer-All F3 were designed to amplify a longer PCR product in combination with Cer-All R1 in an end-point PCR assay (Table 2). The longer PCR product is for species-level identification via sequencing in the case of a positive real-time PCR signal.

Real-time PCR assay optimization. A gradient end-point PCR was performed to verify the performance of Cer-All F1 and Cer-All R1 primers on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA). The reaction mix contained 1 µl (250 nM) each of Cer-All F1 and Cer-All R1 primers, 10 µl of 2× Go *Taq* Green master mix (Promega, Madison, WI), 1 µl of Bovine Serum albumin (10 mg/ml), 2 µl of DNA template, and sterile water to bring the total volume to 20 µl. Initial denaturation was performed at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59, 60, 61, and 62°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The PCR products were examined in gel electrophoresis and sequenced. The optimization of the real-time PCR assay was performed on a CFX96 Real-Time System Thermocycler (Bio-Rad, Hercules, CA). The reaction mix contained 10 µl of master mix (2× PerfeCTa qPCR ToughMix [Quanta BioSciences, Gaithersburg, MD] or 2× Platinum Quantitative PCR SuperMix-UDG [Invitrogen, Waltham, MA], below referred to as "SuperMix-UDG"), 1.2 µl each of Cer-All F1 (300 nM) and Cer-All R1 (300 nM), 0.8 µl of Cer-All P1 (200 nM), and 0.4 µl of Cer-All P2 (100 nM), and 2 µl of DNA, and sterile water was used to bring the total volume to 20 µl. A gradient real-time PCR was used to optimize the annealing temperature of the primers and probes using the following PCR conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, then annealing and elongation at 60, 60.3, 60.8, 61.6, 62.6, 63.4, 63.8, and 64°C for 40 s. The products of these real-time PCR reactions were analyzed using gel electrophoresis and sequenced to confirm that the assay amplified the ITS region of interest. For pathogen–host matrix testing, healthy peach leaf, peach stem, and plum stem samples spiked with *Ceratocystis* DNA were used.

End-point PCR assay optimization. The optimization of the end-point PCR was completed on a Veriti 96-Well Thermocycler (Applied Biosystems). The PCR reaction contained 10 µl of master mix (2× GoTaq Green Master Mix; Promega), 1 µl (250 nM) each of forward (Cer-All F2 or Cer-All F3) and reverse primers (Cer-All R1), 1 µl of bovine serum albumin (10 mg/ml), 2 µl of DNA, and sterile water up to a total volume of 20 µl. The two primer pairs were individually tested in a gradient PCR using the following thermocycler conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 54, 55, 56, and 57°C for 50 s, elongation at 72°C for 50 s, and a final elongation at 72°C for 6 min. The products of these end-point PCR reactions were analyzed using gel electrophoresis and sequenced to confirm that the assay amplified the ITS region of interest.

Analytical specificity and sensitivity testing. The fungal isolates listed in Table 1 were used for specificity testing. To verify the sensitivity of the assay, genomic DNA from *C. destructans* isolate CBS 114717 (20 ng/µl) was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) in sterile water, peach leaf, peach stem, and plum stem DNAs. The following PCR conditions were used for the specificity and sensitivity assays: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing and elongation at 61°C for 40 s.

***In planta* detection assay.** Three young fig plants (*Ficus carica* 'Mrs Williams') were infected with *C. ficicola* CMW 83544 to obtain infected plant material. A small section of the bark on the main stem was removed with a sterile scalpel and an agar plug from a 2-week-old *C. ficicola* culture on PDA was placed over the cut with the sporulating side against the stem. The plug was secured into place with parafilm and plants were incubated with an 8-h photoperiod at 25°C for 4 weeks. One fig plant was used as a control, using the same process as described above, but with an agar plug from a noninoculated media plate. After the incubation period, stem material was sampled for DNA extraction from the following areas: the inoculation site, the internal discoloration periphery above and below the

Table 1. The specificity results of the generic *Ceratocystis* real-time PCR assay using Cer-All F1, Cer-All R1 primers and probes Cer-All P1 (P1) and Cer-All P2 (P2), and the SuperMix-UDG (Invitrogen)[a]

Taxon name	Isolate	Host/substrate	Country of origin	PCR result	
				P1	P2
Ambrosiella roeperi	T19_01731A	*Quercus* sp.	New Zealand	–[b]	–
A. xylebori	CBS 110.61	*Coffea arabica*	Côte d'Ivoire	–	–
Armillaria limonea	ICMP 13891	*Beilschmiedia tawa*	New Zealand	–	–
A. novae-zelandiae	ICMP 13880	*Pinus nigra*	New Zealand	–	–
Berkeleyomyces basicola	CBS 414.52	*Primula*	Netherlands	+	+
	ICMP 15838	*Actinidia deliciosa*	New Zealand	+	+
	T13_03309A	*Musa* sp.	Ecuador	+	+
B. ethacetica	ICMP 13062	*Triticum aestivum*	New Zealand	–	–
B. musarum	ICMP 15221	*Musa* sp.	New Zealand	–	–
Bretziella fagacearum	CBS 130770	*Quercus rubra*	USA	–[c]	–
Ceratocystis adelpha	CMW 14809	*Theobroma cacao*	Ecuador	+	+
	CBS 115163	*T. cacao*	Ecuador	+	+
C. adiposa	CBS 136.34	*Sacchrum officiarum*	Japan	–[c,d]	–
C. albifundus	CBS 128992	*Acacia mearnsii*	South Africa	+	+
C. atrox	CBS 120517	*Eucalyptus grandis*	Australia	+	+
C. cacaofunesta	CBS 114722	*T. cacao*	Costa Rica	+	+
	CMW 14803	*T. cacao*	Ecuador	+	+
C. caryae	CBS 114716	*Carya cordiformis*	USA	+	+
	CBS 115168	*Carya ovata*	USA	+	+
C. colombiana	CBS 121792	*C. arabica*	Colombia	+	+
C. corymbiicola	CBS 127217	*Brachypeplus planus*	Australia	+	+
C. curvata	CBS 122603	*Eucalyptus deglupta*	Ecuador	+	+
C. destructans	CBS 114717	*Prunus dulcis*	USA	+	+
C. diversiconidia	CBS 122605	*Terminalia ivorensis*	Ecuador	+	+
C. ecuadoriana	CBS 124020	*E. deglupta*	USA	+	+
C. eucalypticola	CBS 124016	*Eucalyptus* sp.	South Africa	+	+
C. ficicola	CMW 38544	*Ficus carica*	Japan	+	+
C. fimbriata	CBS 114718	*Colocasia esculenta*	Cuba	+	+
	CBS 114723	*Ipomoea batatas*	USA	+	+
	CBS 115173	*Gmelina arborea*	Brazil	+	+
	CBS 141.37	*I. batatas*	Unknown	+	+
	CBS 103.40	*C. arabica*	Guatemala	+	+
	CMW 4901	*E. grandis*	Brazil	+	+
	A35_1[e]	*Actinidia* sp.	Brazil	+	+
	CF249_1[e]	*Actinidia* sp.	Brazil	+	+
	CF286_1[e]	*Actinidia* sp.	Brazil	+	+
	CF261_1[e]	*Actinidia* sp.	Brazil	+	+
	JO03_1[e]	*Actinidia* sp.	Brazil	+	+
	P1A1_1[e]	*Actinidia* sp.	Brazil	+	+
C. fimbriatomima	CBS 121786	*E. grandis*	Venezuela	+	+
C. huliohia	C4191	*Metrosideros polymorpha*	USA	+	+
	P15-59	*M. polymorpha*	USA	+	+
C. larium	CBS 122512	*Styrax benzoin*	Indonesia	+	+
C. lukuohia	C4186	*M. polymorpha*	USA	+	+
C. manginecans	CMW 22562	*Acacia mangium*	Indonesia	+	+
	CMW 22581	*A. mangium*	Indonesia	+	+
C. neglecta	CBS 121789	*E. grandis*	Colombia	+	+
C. obpyriformis	CBS 122511	*Acacia mearnsii*	South Africa	+	+
C. pirilliformis	CBS 118128	*Eucalyptus nitens*	Australia	+	+
C. platani	CMW 1896	*Platanus* sp.	Switzerland	+	+
	CBS 115162	*Platanus occidentalis*	USA	+	+
C. polychroma	CBS 115778	*Syzygium aromaticum*	Indonesia	+	+
C. polyconidia	CBS 122289	*A. mearnsii*	South Africa	+	+
C. populicola	CBS 119.78	*Populus* sp.	Netherlands	+	+
	CBS 114725	*Populus tremuloides*	USA	+	+
C. smalleyi	CBS 114724	*C. cordiformis*	USA	+	+
Ceratocystis sp.	CMW 50456	*Syngonium podophyllum*	USA	+	+
C. tanganyicensis	CBS 122295	*A. mearnsii*	Tanzania	+	+
C. thulamelensis	CMW 35972	*Colophospermum mopane*	South Africa	+	+
C. tsitsikammensis	CBS 121018	*Rapanea melanophloeos*	South Africa	+	+
C. uchidae	CBS 114720	*Colocasia esculenta*	USA	+	+
C. variospora	CBS 773.73	*Quercus ellipsoidalis*	USA	+	+

(*Continued on next page*)

[a] CMW, cultures from the Forestry and Agriculture Biotechnology Institute, South Africa; CBS, cultures from the Westerdijk Fungal Biodiversity Institute, Netherlands; ICMP, cultures from the International Collection of Microorganisms from Plants, New Zealand; T, cultures from the in-house collection of the Plant Health and Environment Laboratory, New Zealand.

[b] These isolates produced a flat curve with a very low relative fluorescence unit value (40 to 60). These results were not considered as positive (see Supplementary Fig. S1).

[c] These species are only detected with P1 when using ToughMix (Quanta BioSciences). No detection was found with SuperMix-UDG (Invitrogen).

[d] While this species is named *Ceratocystis*, phylogenetic analyses have demonstrated that while related to *Ceratocystis*, this species does not fall into genus *Ceratocystis* or any of the closely related groups (de Beer et al. 2014).

[e] Only DNA available that was obtained from overseas by the New Zealand Institute for Plant and Food Research Limited.

Table 1. (*Continued from previous page*)

Taxon name	Isolate	Host/substrate	Country of origin	PCR result	
				P1	P2
	CBS 114714	*Quercus robur*	USA	+	+
	CBS 114715	*Unknown*	USA	+	+
C. zambeziensis	CMW 35958	*Combretum imberbe*	South Africa	+	+
C. zombamontana	CBS 122296	*Eucalyptus* sp.	South Africa	+	+
Cercospora beticola	T15_06249C	*Beta vulgaris*	New Zealand	–	–
Cercospora sp.	T17_03175B	*Musa* sp.	Fiji	–	–
	T19_06818D	*Carica papaya*	Fiji	–	–
Chalaropsis thielavioides	CMW 37309	*Pseudolachnostylis* sp.	South Africa	+	+
	ICMP 2116	*Rosa* sp.	Australia	+	+
Chondrostereum purpureum	ICMP 2881	*Prunus persica*	New Zealand	–	–
Cryptosporiopsis keinholzii	T14_00854A	*Malus sylvestris*	New Zealand	–	–
Cylindrocladiella parva	ICMP 19020	*Prunus armeniaca*	New Zealand	–	–
Cylindrocladiella sp.	T18_01697D	*Metrosideros excels*	New Zealand	–	–
C. stellenboschensis	T18_04209C	*Castanea sativa*	New Zealand	–	–
Davidsoniella australis	CMW 2333	*Nothofagus cunninghamii*	Australia	+	+
D. eucalypti	CMW 3254	*Eucalyptus sieberi*	Australia	+	+
Diaporthe citri	ICMP 10355	*Citrus* × *reticulata*	New Zealand	–	–
Ganoderma australe	T19_00255	*Pyrus communis*	New Zealand	–	–
Huntiella chinaeucensis	ICMP 21168	*Tectona grandis*	Thailand	–	–
H. sumatrana	CBS 124011	*A. mangium*	Indonesia	–	–
Lophiostoma sp.	T18_02462L	*Lophomyrtus bullata*	New Zealand	–	–
Nectria hematococca	T18_00456D	*Albizia julibrissin*	New Zealand	–	–
Neofusicoccum australe	T18_00387 2A	*Metrosideros umbellata*	New Zealand	–	–
N. luteum	T17_03036 B	*Actinidia* sp.	New Zealand	–	–
N. parvum	T17_03601J	*Phoenix* sp.	New Zealand	–	–
Neonectria microconidia	T18_04083 2A	*Actinidia* sp.	New Zealand	–	–
Ophiostoma novo-ulmi subsp. *americana*	CBS 116564	*Ulmus minor*	Austria	–	–
Phaeobotryosphaeria citrigena	ICMP 16629	*Citrus* sp.	New Zealand	–	–
Pseudocercospora atromarginalis	T20_03055A	*Solanum aviculare*	New Zealand	–	–
P. coprosmae	T20_03080A	*Coprosma* sp.	New Zealand	–	–
P. eucalyptorum	T20_03057A	*Eucalyptus nitens*	New Zealand	–	–
Pseudocercospora sp.	T21_01064	*Rhododendron ponticum*	New Zealand	–	–
Stigmina carpophila	ICMP 11202	*P. persica*	New Zealand	–	–
Verticillium dahlia	ICMP 8139	*Actinidia deliciosa*	New Zealand	–	–

infection site, and asymptomatic tissue 3 cm above and below the internal discoloration periphery. Only the infection site was sampled from the control plant. After homogenizing 500 mg of plant material (healthy and infected) in 5 ml of CTAB lysis buffer using an automated tissue homogenizer (Bioreba, Reinach, Switzerland), DNA extraction was carried out as described above. To determine the ability of the assay to detect the pathogen in bulked plant samples, DNA extractions were also performed from samples containing 500 mg of plant tissue in a ratio of 1 part of infected stem tissue with 4 or 9 parts of healthy stem tissue. This DNA was tested using the real-time PCR assay, as well as the end-point PCR assays, as described above. Two PCR products from each end-point PCR assay were sequenced for confirmation.

To confirm the presence of the pathogen in the infected plant material, stem sections from the above-described sampling sites of all four plants were plated on PDA and incubated at room temperature. The plates were regularly screened for the presence of *Ceratocystis* colonies. The identity of the fungi with *Ceratocystis*-like morphological characters was determined by amplifying and sequencing the ITS region as described above.

Duplex assay development. To develop a duplex real-time PCR assay for simultaneous detection of the pathogen and host plant DNA, primers Cer-All F1 and Cer-All R1, and probes Cer-All P1 and Cer-All P2, were combined with plant *cox* gene-specific primers COX-F, COX-R, and probe COX-P (Weller et al. 2000; Table 2). For validation, DNA from *C. destructans* isolate CBS 114717 was serially diluted in healthy peach leaf, peach stem, and plum stem DNA, and prepared as a test panel. Initially, simplex assays for *Ceratocystis* and the *cox* gene were carried out independently. Later, the duplex assay was performed in a reaction containing 10 µl of ToughMix (Quanta BioSciences), 1.2 µl (300 nM) each of Cer-All F1 and Cer-All R1, 0.8 µl (200 nM) of Cer-All P1, 0.4 µl (100 nM) of Cer-All P2, 1 µl (250 nM) of COX-F and COX-R, 0.6 µl (150 nM) of TaqMan COX-P, 2 µl of DNA template, and sterile water to a total volume of 20 µl. The thermocycler program was initiated with denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 10 s and annealing and elongation at 61°C for 40 s.

Assay reproducibility testing. Reproducibility of the assay was evaluated using different real-time PCR machines at different times and by different users. Interlaboratory testing was carried out at the Department of Agriculture, Water and Environment laboratory in Mickleham, Australia using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The submitted DNA panel was tested using 20-µl reactions with the above-detailed primer and probe concentrations and cycling conditions. A different master mix, GoTaq Probe Real-Time PCR Master Mix (Promega), was used according to the manufacturer's instructions, and assay result threshold values were applied automatically with the software CFX Manager v.3.1 (Bio-Rad).

Results

Primers and probes for genus *Ceratocystis*. Two areas of the ITS1 region were identified as highly similar among *Ceratocystis* species, and the primers Cer-All F1 and Cer-All R1 were designed to amplify a 163-bp product (Table 2). *In silico* analysis of the Cer-All F1 primer showed specificity to *Ceratocystis* species and species from closely related genera, as well as some other Ascomycetes fungi. The Cer-All R1 primer was specific mostly to species of *Ceratocystis* and genera that were previously included in *Ceratocystis*. The primers designed for end-point PCR (Cer-All F2 and Cer-All F3) were shown to amplify all *Ceratocystis* species and species from closely related genera.

During probe design within the selected 163-bp region, two regions with a high sequence similarity were identified in all *Ceratocystis* species. Cer-All P1 region showed varying degrees of matchless nucleotide stretches because of single-nucleotide polymorphisms, insertions, and deletions in some sequenced isolates. To cover the

sequence variation in some of these *Ceratocystis* strains and maximize the assay specificity, an additional probe, Cer-All P2, was designed for a region that was found to be uniform in the sequences with mismatches.

PCR assay optimization. A gradient real-time PCR detected no difference in the amplification of the target DNA when testing the annealing and elongation temperatures between 60 and 64°C. The *Ct* values for both Cer-All probes were low, ranging between 16 and 18. The amplification of the target region was confirmed using gel electrophoresis that showed the expected amplicon size of 163 bp, as well as sequencing of the real-time PCR product. The real-time PCR annealing and elongation at 61°C for 40 s was selected for further assay validation.

Similarly, to optimize Cer-All F2, Cer-All F3, and Cer-All R1 primers, a gradient end-point PCR at the annealing temperatures between 54 and 57°C was performed. The Cer-All F2 and Cer-All R1 primers amplified the expected 297-bp product, and the Cer-All F3 and Cer-All R1 primers amplified the expected 340-bp product at all annealing temperatures used, without showing any decrease in PCR product concentration.

Assay specificity. The assay detected DNA of all 55 tested *Ceratocystis* isolates with both master mixes. The assay also detected a few species in closely related genera, namely *Berkeleyomyces basicola*, *Davidsoniella australis*, *D. eucalypti*, and *Chalaropsis thielavioides* with both probes in both master mixes. When using ToughMix, *C. adiposa*, *Bretziella fagacearum*, *Ambrosiella xylebori*, and *A. roeperi* were also detected but only with the Cer-All P1 probe. When using the more stringent SuperMix-UDG, the detection of *C. adiposa* and *B. fagacearum* was eliminated, but *A. xylebori* and *A. roeperi* were still detected with the Cer-All P1 probe. However, the amplification curve was flat throughout the assay and low CFU values were observed for the *Ambrosiella* species (Supplementary Fig. S1). The assay did not amplify any other fungal species tested.

Cer-All F3 and Cer-All R1 primers amplified a single PCR product in a size range of 340 to 400 bp from all *Ceratocystis* species and closely related genera tested. The Cer-All F2 and Cer-All R1 primer pair amplified a single PCR product in a size range of 295 to 370 bp in all of the *Ceratocystis* species and most of the closely related genera tested, with the exception of *C. thielavioides*, *B. fagacearum*, and *A. roeperi*, for which the amplified product was ~1 kb in size. The Cer-All F3 and Cer-All R1 primer combination was shown to be less sensitive than the combination of Cer-All F2 and Cer-All R1 primers. No cross amplification occurred when testing healthy host plant DNA.

Sensitivity of the real-time PCR assay. The assay sensitivity was initially assessed using *Ceratocystis* DNA diluted in water, and detection was observed in samples containing 0.2 pg/µl *Ceratocystis* DNA. The assay sensitivity in the stem and leaf host DNA matrix was slightly affected by the host matrix, resulting in amplification from material containing 0.2 to 2 pg/µl of *Ceratocystis* DNA (Fig. 1). As Cer-All P1 appeared to have lower sensitivity than the Cer-All P2 probe to detect samples at higher dilutions, the assay limit of detection was set at 2 pg/µl. PCR efficiency for Cer-All P1 and Cer-All P2 probes was 91.14 and 92.31%, respectively.

Duplex assays for codetection of *Ceratocystis* and the host (COX) were tested using serially diluted *Ceratocystis* DNA in peach leaf, peach stem, and plum stem DNAs. Codetection of *Ceratocystis* and COX occurred without compromising each other's amplification (Fig. 2; Supplementary Table S1) and no differences were found between the hosts. The detection of COX from peach and plum DNA was observed at low *Ct* values (20 to 22) in all samples.

Assay repeatability and reproducibility. Little or no difference was found in the assay results when using different PCR machines and operators. The assay was also validated at the Australian Post Entry Quarantine Plant Laboratory, Mickleham, Victoria, where similar results using a different PCR machine and master mix were obtained (Table 3).

Detection of *Ceratocystis* in infected plant material. The ability of the assay to detect *Ceratocystis* from infected plant material was assessed using fig tree stems artificially infected with *C. ficicola* isolate CMW 83544. Four weeks after inoculation, extensive wilting of the plant parts above the infection site and some necrosis symptoms around the infection site were observed. Longitudinal cuts of the infected stems revealed reddish-brown internal discoloration around the infection site, which had spread in both directions within the stem (Fig. 3). The intensity of the discoloration varied between the plants, as did the total span of symptomatic tissue ranging from 4.3 to 11 cm. No external or internal symptoms developed in the control plant. The fungus was successfully isolated only from the plant material that was taken from the infection site but not from the tissue of the lesion periphery or the asymptomatic material that was 3 cm from the lesion periphery. No *Ceratocystis* culture was obtained from the asymptomatic tissue.

The duplex assay using DNA from the sampled plant material detected *Ceratocystis* in all stem samples with varying intensities. The amplification of the target at the inoculation site had a *Ct* value of 17 to 18. At the lesion periphery, slightly higher *Ct* values were obtained (18 to 28) and a weaker amplification with *Ct* values of 29 to 33 was detected for the asymptomatic material at 3 cm from the lesion periphery. *Ceratocystis* was also successfully detected from all bulked samples. Testing symptomatic samples bulked in 1:4 and 1:9 ratios resulted in low *Ct* values (21 to 22 [average 21.67] and 22 to 25 [average 24.02], respectively), whereas testing asymptomatic tissue bulked in 1:4 and 1:9 ratios showed higher *Ct* values (35 to 36 [average 35.38] and 35 to 37 [average 36.69], respectively). Amplification of COX was observed at the *Ct* value range of 16 to 20 for all samples.

The end-point PCR assay successfully detected the infection in the samples that had *Ct* values <23, which represented most of the samples at the inoculation site and at the lesion periphery. The pathogen was not detected from the asymptomatic tissue. When testing bulked samples, infection was detected in samples bulked in 1:4

Table 2. Primers and probes targeting the *Ceratocystis* genus and the plant internal control gene, used in this study[a]

Primer/probe name	Sequences (5′ to 3′)	Target	Application	Source
Cer-All F1	CAACGGATCTCTTGGCTCTAGCA	ITS1	Real-time PCR	This study
Cer-All F2	AGATGAATGCTGTTTTGGTGGT	ITS1	End-point PCR	This study
Cer-All F3	TTGTACTCTATAAACCATGTGTGAAC	ITS1	End-point PCR	This study
Cer-All R1	TGAGTGGTGAAATGACGCTCGG	ITS1	Real-time and end-point PCR	This study
Cer-All P1	FAM[b]-GCACATTGCGCCTGGCAGT-BHQ-1	ITS1	Real-time PCR	This study
Cer-All P2	HEX[c]-TTCTGCCAGGCATGCCTGT-BHQ-1	ITS1	Real-time PCR	This study
COX–F	CGTCGCATTCCAGATTATCCA	Plant COI	Real-time PCR	Weller et al. (2000)
COX–R	CAACTACGGATATATAAGAGCCAAAACTG	Plant COI	Real-time PCR	Weller et al. (2000)
COX–P	CAL Fluo Red-610–TGCTTACGCTGGATGGAATGCCCT–BHQ2	Plant COI	Real-time PCR	Weller et al. (2000)

[a] ITS, internal transcribed spacer gene 1; COI, cytochrome oxidase subunit 1 gene.
[b] FAM, 6-Carboxyfluorescein.
[c] HEX, Hexachlorofluorescein.

ratio, although primer pair Cer-All F2/Cer-All R1 produced a weaker band than Cer-All F3/Cer-All R1. Weak detection was also seen in symptomatic samples bulked in 1:9, but only with primer pair Cer-All F3/Cer-All R1 (Fig. 4).

Discussion

As a primary fungal barcode (Schoch et al. 2012) and being the region with the most publicly available sequences for *Ceratocystis* (NCBI 2021; https://www.ncbi.nlm.nih.gov/), ITS was selected for assay development to ensure that maximal coverage of sequence diversity was included in the analysis. This increases the likelihood that the developed assay would be able to detect all *Ceratocystis* species. ITS is also represented in the genome in multiple copies (Schoch et al. 2012), which increases the chances of detecting the pathogen at low levels. While ITS should not be used as a single barcode for delimiting species of *Ceratocystis* (Marin-Felix et al. 2017), a region that is common between all the species was found in this study and can therefore be used for genus level detection. Using a two-probe approach further ensures the coverage of any nucleotide polymorphisms and increases the likelihood that the assay will also be suitable for detecting cryptic species, even if polymorphisms are present in one of the probe-binding regions.

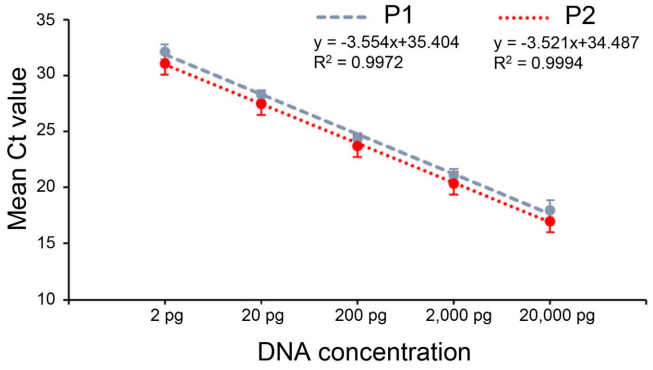

Fig. 1. Standard curve analysis of the real-time PCR using *Ceratocystis destructans* (CBS 114717) DNA serially diluted in healthy peach stem DNA. Linear response was observed for both probes (Cer-All P1 and Cer-All P2) when using 2–20,000 pg of target DNA. Error bars show standard deviation.

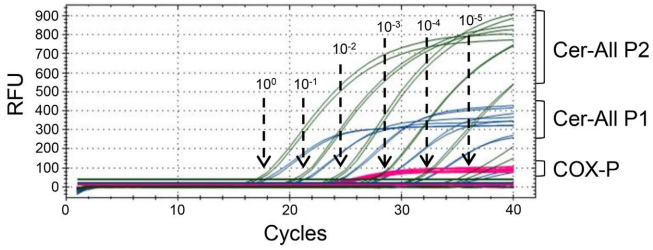

Fig. 2. The duplex assay results when using *Ceratocystis destructans* (CBS 114717) DNA serially diluted in peach stem DNA (original sample contained 20,000 pg of target DNA). Note that the relatively flat curve for plant internal control is characteristic for *Prunus* DNA and was shown not to be a result of the *Ceratocystis* assay inhibiting the amplification of the plant DNA. Significantly higher values were seen when testing the fig stem material.

For the assay development, sequence data from all currently accepted *Ceratocystis* species was analyzed, and where possible, DNA was used in specificity testing. Given the unavailability of the cultures for nine *Ceratocystis* species at the time of the assay development, *C. betulina*, *C. cercfabiensis*, *C. changhui*, *C. collisensis*, *C. mangicola*, *C. mangivora*, *C. papillata*, *C. quercicola*, and *C. tiliae* could not be included in laboratory testing. However, all these were shown to be detected during *in silico* analysis, except for *C. quercicola*, a species that was described after the completion of this assay development without published ITS sequence data (Cho et al. 2020).

A positive result from the generic real-time PCR assay should be followed up further to determine the identity of the detected fungus. For that purpose, an end-point PCR assay was developed to amplify a longer section of the ITS1 region with primers Cer-All F2 and Cer-All F3 in combination with Cer-All R1. These primer sets should produce PCR products of the expected size for *Ceratocystis* and will allow subsequent sequence analysis to differentiate between most *Ceratocystis* species. In cases where the sequenced DNA region is not found to be variable enough, it will help to narrow expected targets to just a handful of species and assist with further diagnostics, for example, isolation from the original plant material.

The aim of this project was to develop an assay that detects any *Ceratocystis* species present in a plant sample. Despite efforts to find regions that are highly specific to *Ceratocystis*, some species in genera closely related to *Ceratocystis* were also detected. These included species of *Berkleyomyces*, *Chalaropsis*, and *Davidsoniella*, even when using the more stringent SuperMix-UDG master mix. While a detection was also noted for *Ambrosiella*, especially when using SuperMix-UDG, the amplification curves in these cases were flat and with very low relative fluorescence unit values. Based on these additional observations, these detections can be ruled out as negative for *Ceratocystis*. Some of the species in the detected nontarget genera are also significant plant pathogens that can cause similar symptoms to *Ceratocystis* infections. Therefore, their detection could potentially add diagnostic value to the assay. However, the end-point PCR primers Cer-All F2 and Cer-All F3, in combination with Cer-All R1 as a secondary assay, will help to rule out these nontarget detections – either by examining the PCR product size on gel or by sequencing the amplicon.

Assay sensitivity is important in quarantine testing and for detecting pathogens in asymptomatic or recently infected plants. The limit of detection of the developed real-time PCR assay was shown to be as low as 0.2 pg/µl of pathogen DNA in some samples and at least 2 pg/µl of pathogen DNA in host matrix. Knowing that the haploid genome size of *Ceratocystis* species ranges between 0.025 and 0.03

Table 3. The interlaboratory testing results of a panel containing a range of *Ceratocystis* isolates; the results show very little intra- and interassay variation in the *Ct* values obtained with the Cer-All P1 (P1) and Cer-All P2 (P2) probes[a]

Ceratocystis isolate	In-house assay results		Interlaboratory assay results		Intra-assay CV%		Interassay CV%	
	Ct value ± STD P1	*Ct* value ± STD P2	*Ct* value ± STD P1	*Ct* value ± STD P2	P1	P2	P1	P2
CBS 115173	21.98 ± 0.134	23.20 ± 0.056	23.32 ± 0.532	22.98 ± 0.479	0.61	0.24	2.28	2.08
CBS 114717	20.39 ± 0.141	21.90 ± 0.098	21.72 ± 0.662	21.64 ± 0.340	0.69	0.45	3.04	1.57
CBS 114720	20.67 ± 0.106	22.12 ± 0.014	22.23 ± 0.298	21.99 ± 0.279	0.51	0.06	1.34	1.27
CBS 115162	20.60 ± 0.035	22.03 ± 0.028	22.23 ± 0.249	21.98 ± 0.244	0.17	0.12	1.12	1.11
CBS 114714	19.26 ± 0.028	20.79 ± 0.007	20.89 ± 0.450	20.67 ± 0.425	0.14	0.03	2.15	2.05
CBS 121792	20.12 ± 0.176	21.55 ± 0.042	21.62 ± 0.409	21.40 ± 0.359	0.87	0.19	1.89	1.67
CBS 122605	20.38 ± 0.098	21.77 ± 0.148	21.95 ± 0.455	21.69 ± 0.441	0.48	0.68	2.07	2.03
CBS 103.40	19.84 ± 0.480	21.04 ± 0.035	21.20 ± 0.400	20.85 ± 0.350	2.42	0.16	1.88	1.67
CBS 114717	25.28 ± 0.134	26.42 ± 0.035	26.14 ± 0.405	25.83 ± 0.402	0.53	0.13	1.55	1.55

[a] CV, coefficient of variance; STD, standard deviation; CBS, cultures from the Westerdijk Fungal Biodiversity Institute, Netherlands.

pg (van der Nest et al. 2014, 2019), and that the ITS region is present at high copy numbers (>100 per cell; Templeton and Andersen 2018), the assay is considered to be able to detect the pathogen even if just a few cells are present in the sample. The same sensitivity level was also obtained in the duplex assay, which allowed simultaneous amplification of *Ceratocystis* and plant DNA. An internal control is essential to verify that competent DNA was successfully extracted from the sample. Using the duplex assay will save time and resources by amplifying the DNA of the plant host and the potential pathogen in a single reaction.

As *Ceratocystis* species can be vectored by ambrosia beetles (*Xyleborus* spp.; Harrington et al. 2010; Roy et al. 2018; Souza et al. 2013), the assay might also be useful for testing for the presence of *Ceratocystis* species in the insects' gut. During the assay development, *A. roeperi*, naturally carried by a beetle, was detected using the less stringent ToughMix and the Cer-All P1 probe (Supplementary Fig. S2). This suggests that the assay may be useful for *Ceratocystis* detection in insects and should be considered for further validation, including optimization of DNA extraction methods from insects to obtain high-quality material.

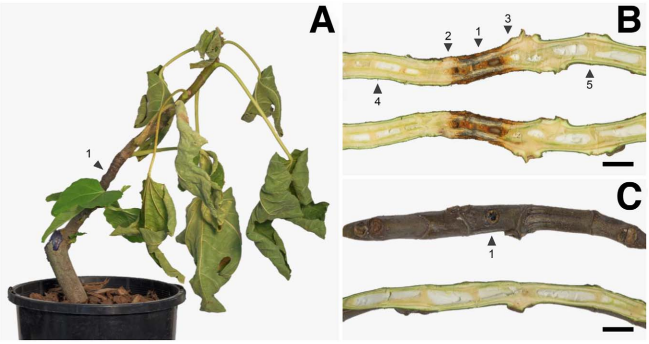

Fig. 3. *Ceratocystis* in planta infection assay. **A,** Fig plant showing wilting 4 weeks after inoculation with *Ceratocystis ficicola* (CMW 83544). Note the wilting above the infection site and green vigorous leaves at the base of the plant. **B,** Longitudinal cut of the *C. ficicola*-inoculated fig stem showing internal discoloration above and below the infection site. **C,** Longitudinal cut of the stem of the control plant. Arrows indicate the following sampling areas: **1,** infection site; **2,** periphery of the discoloration above the infection site; **3,** periphery of the discoloration below the infection site; **4,** asymptomatic area 3 cm above the infection site; and **5,** asymptomatic area 3 cm below the infection site. Scale bar = 1 cm.

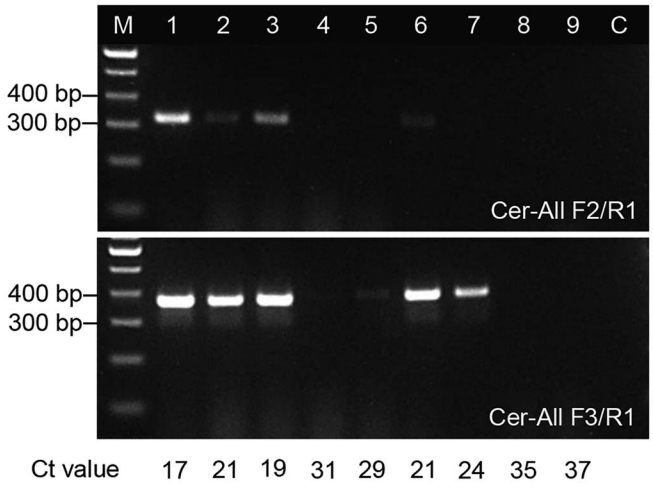

Fig. 4. Gel electrophoresis image showing the end-point PCR results when testing the plant material from *in planta* assay where fig stems were infected with *Ceratocystis ficicola*. Primers Cer-All F2/Cer-All R1 (top) and Cer-All F3/Cer-All R1 (bottom) amplified a 310- and 340-bp product, respectively. The samples are as follows: **M,** ladder marker; **1,** infection site; **2,** periphery of the discoloration above the infection site; **3,** periphery of the discoloration below the infection site; **4,** asymptomatic area 3 cm above the infection site; **5,** asymptomatic area 3 cm below the infection site; **6,** symptomatic bulked sample in ratio 1:4; **7,** symptomatic bulked sample in ratio 1:9; **8,** asymptomatic bulked sample in ratio 1:4; **9,** asymptomatic bulked sample in ratio 1:9; and **C,** no template control. The *Ct* values given below the gel picture were obtained for the respective samples in a duplex real-time PCR (probes Cer-All P1 and P2 gave similar values).

The assay has been used for routine diagnostics at the Plant Health and Environment Laboratory since late 2019, processing over 300 samples of *Prunus* spp. and *Citrus* spp. growing in post-entry quarantine. In these, only one nonspecific detection was found with the real-time assay. The end-point PCR produced an amplicon with an unexpected band size and thus confirmed that the detection did not represent *Ceratocystis*. Further sequencing found the detection to be a fungus belonging to Ascomycetes (potentially endophytic or lichen associated), with no close matches to any described species. The assay has also been successfully used at the Australian Post-Entry Quarantine Plant Laboratory, with different reagents and equipment, clearly demonstrating that the method is transferrable between laboratories. This proves that the assay can be effectively used for routine testing and the proposed additional testing methodology is effective in ruling out any nonspecific detections, should they occur.

Little is known about the biology of many fungi in the genus *Ceratocystis* and therefore it has been largely unknown if the fungus can be spread with asymptomatic plant material or not. In our *in planta* infection assay, *C. ficicola* was successfully detected in asymptomatic fig stem parts. Because the fungus was not successfully isolated from these areas via culturing, it is likely that the presence of *C. ficicola* in such tissue would have gone unnoticed if tested with the culture-based approach. *Ceratocystis ficicola* was also not detected from asymptomatic material when using the end-point PCR. Therefore, using the real-time PCR assay presented in this study provides additional assurance that even asymptomatic infections will be effectively detected. It also presents a new opportunity to research and better understand the spread of *Ceratocystis* within plants.

Acknowledgments

We thank the project team members Robert Taylor, Abigail Durrant, Sue Escott-Brown, Richard Lardner, Claire McDonald, and Zhidong Yu for their suggestions and advice throughout the work. Mike Wingfield kindly provided numerous isolates of *Ceratocystis* species that were invaluable for the assay development and validation. Mark Andersen and Matt Templeton provided DNA from *C. fimbriata* isolates obtained from kiwifruit and provided sequence data that was used for initial primer and probe design. Ruth Griffin, Leanne Woodward, and Anthony D'Agata assisted with blind panel testing. Katharina Hofer, Robert Taylor, and Michael Gemmell are acknowledged for their critical review of an earlier version of this manuscript and Matthew Leamy assisted with taking images of infected fig plants.

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