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BS1602 Development of a detection assay for the Brazilian isolate of *Ceratocystis fimbriata*

Templeton M, Andersen M

March 2018















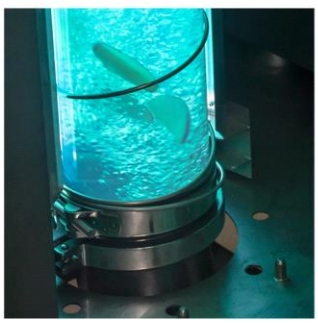


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EXECUTIVE SUMMARY

BS1602 Development of a detection assay for the Brazilian isolate of *Ceratocystis fimbriata*

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Plant & Food Research Auckland

March 2018

Kiwifruit wilt caused by several distinct isolates of *Ceratocystis fimbriata,* presents a significant biosecurity risk to New Zealand kiwifruit growers. A rapid DNA-based assay is essential if we are to be able to detect a potential incursion of these pathogens. Due to the presence of a number of isolates of *C. fimbriata* pathogenic on kiwifruit, there are additional complications to the development of a simple assay. Comparative genomics has ruled out the possibility a unique region common to all the kiwifruit pathogens could be used to design qPCR primers. The approach chosen was to design a set of primers to the Internal Transcribed Spacer I (ITS I) region, common to all members of the Latin American Clade of *C. fimbriata*. A second set of primers could be then used to identify the specific isolate involved, and distinguish the isolate of *C. fimbriata* (a pathogen of kumara) that is present in New Zealand. The primers were tested against soil and orchard samples. No false positive results were observed. The primers were also tested by Hill Laboratories and are found to be suitable to be outsourced to a commercial organisation for high-throughput sample analysis.

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1 INTRODUCTION

Ceratocystis fimbriata is a large complex of pathogens that collectively cause a wide range of diseases, predominantly on woody plants. The genus is found throughout the world but the continent with most genetic and pathogenic variation is Latin America and isolates from this region form the Latin American Clade (LAC). Recently, a major outbreak of a new disease on kiwifruit was found in Brazil. Disease symptoms were very serious, causing wilting and subsequent death of vines. This disease presents a significant biosecurity risk to New Zealand kiwifruit growers. A PCR-based detection system for this pathogen is essential for boarder screening or identifying for potential incursions. The project is further complicated by a report that the disease in Brazil is caused by several closely related but distinct isolates of *C. fimbriata* (Piveta et al. 2016; Figure 1). This means that it is unlikely that a single set of PCR primers will be sufficient to distinguish kiwifruit pathogens from all other isolates of Ceratocystis. In New Zealand, the only report of the presence of Ceratocystis is *C. fimbriata sensu stricto,* a pathogen of kumara. While historically this isolate has caused disease losses in kumara crops, it is usually well controlled by good grower management. It is not known whether any other introduced or native isolates of Ceratocystis are present in New Zealand. Any detection system developed, will need to ensure false positives are not generated by the isolate from kumara already present in New Zealand or from as yet unidentified isolates that might be present in or around New Zealand kiwifruit orchards.

2 COLLABORATIONS

International collaborations were a key part of the success of this project. An important requirement for this project was obtaining DNA from a variety of different isolates of Ceratocystis including those found in Brazil. To do this collaborations were developed with Professor Acelino Alfenas from the Departamento de Fitopatologia Universidade Federal de Viçosain in Brazil. Prof. Alfenas was the first person to identify the pathogens from kiwifruit and was able to provide us with two samples of DNA. One sample was from the Ceratocystis KiwiA pathogen, which is the predominant isolate found on infected kiwifruit vines. The second sample was from a related pathogen of Eucalyptus. The second collaborator contacted was Professor Tom Harrington at Iowa State University. Prof Harrington is the world's expert on Ceratocystis pathology, biology and taxonomy and was also able to provide us with several DNA samples including three kiwifruit pathogens. Combined, we had enough samples for the comparative genomics and to test qPCR primer sets we designed.

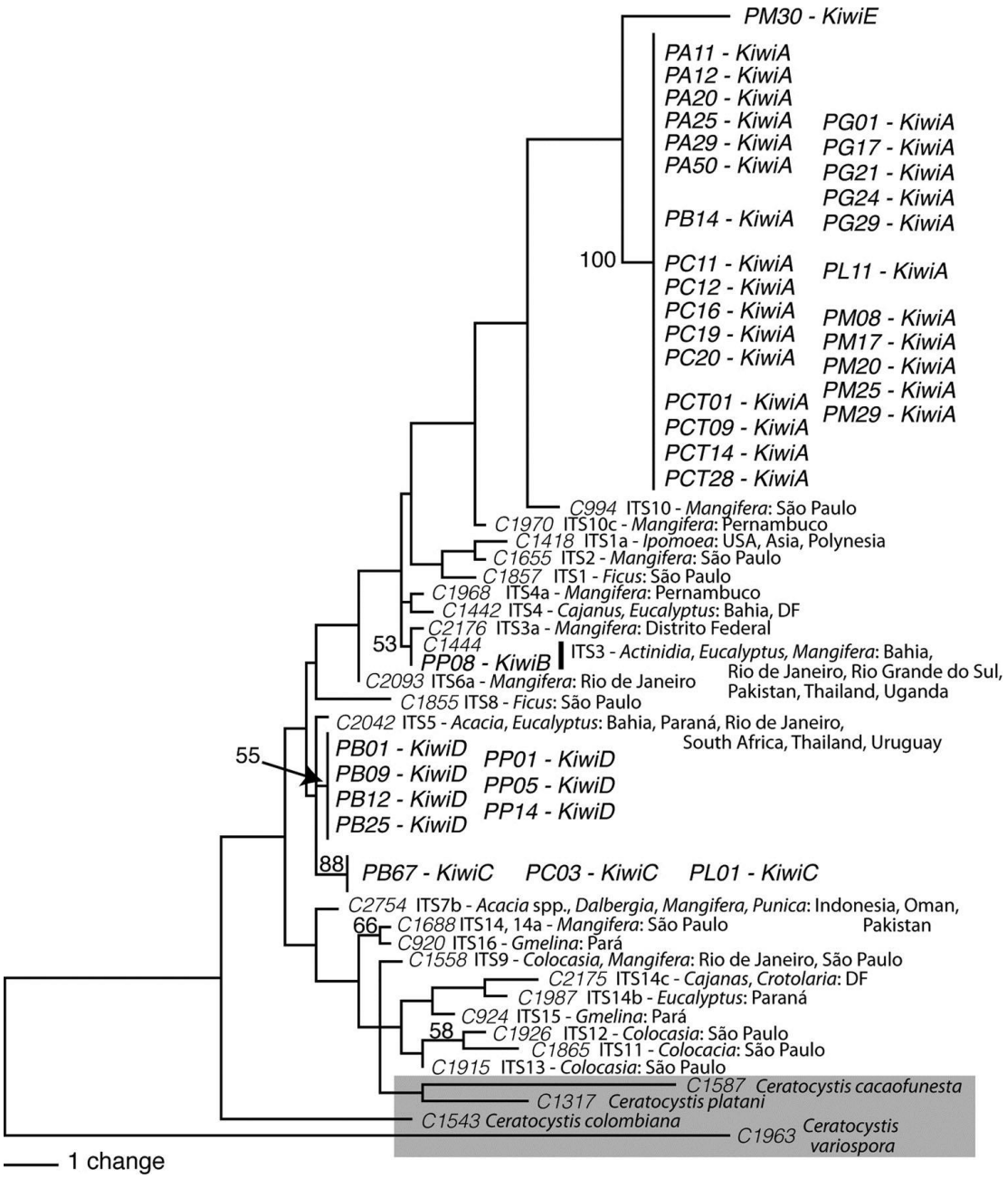


Figure 1. Phylogenetic tree generated from the ITS I sequences from members of the Latin American clade (LAC) of *C. fimbriata.* *T*he isolates pathogenic on kiwifruit are labelled KiwiA-E. From *Piveta et al. 2016.*

3 CO-FUNDING

This contract was supported in part by funding from Plant and Food Research SSIF for components of research required to follow the below rationale. The SSIF funding focused on the comparative genomics (assembling the sequences, gene annotation and phylogenetic analysis) and included a number of isolates that were not kiwifruit pathogens, for example the isolates from O'hia in Hawai'i. This provided a vital foundation for the practical goals of assay development within this project.

4 PROJECT RATIONALE

The overall aim of the project was to design qPCR primers that were specific to the Ceratocystis pathogens of kiwifruit found in Brazil. There were three key objectives:

1. Development of a strategy for designing qPCR primers for detection of the kiwifruit pathogens
2. Design and test qPCR primers against DNA from the Ceratocystis isolates available
3. Validate qPCR primers:
    a. Against a wide range of soil samples collected from around New Zealand.
    b. Against a set of samples from kiwifruit orchards
    c. Using a high-throughput assaying regime tested at Hill Laboratories

5 DEVELOPMENT OF A STRATEGY FOR DESIGNING QPCR PRIMERS FOR DETECTION OF THE KIWIFRUIT PATHOGENS

A number of strategies are available for designing qPCR primers to detect specific fungal pathogens. These include: a traditional ITS-based method; use of mitochondrial sequences or the identification of a virulence factor unique to the kiwifruit pathogens (Table 1).

Table 1. The evaluation of different DNA detection strategies. The table highlights the advantages and disadvantages of some of the various strategies used to design a DNA detection method.

Target DNA	Sensitivity	Specificity
ITS	High (copy number >100/cell)	Challenging if differentiating closely related isolates
Mitochondria	High (copy number >100/cell)	Best for single target isolate
Unique pathogenicity factor	Low (single copy)	Ideal if target is unique to all pathogen variants

Ideally, the primers should be highly specific, sensitive and informative. Additionally, strategies may vary depending on the circumstances dictating the nature and prevalence of the pathogen. In the case of the Ceratocystis pathogens of kiwifruit in Brazil, an additional complication was that the disease appeared to be caused by a range of distinct isolates identified as KiwiA-E. It could not be assumed that these were the only isolates capable of causing disease on kiwifruit. Only *Ceratocystis fimbriata senso stricto* is reported to be present in New Zealand, this isolate appears to be specific for kumara and sweet potato. However, it is not known whether there are other native Ceratocystis isolates in New Zealand. This pathogen is known to consist of a large variable species complex with the potential for cryptic host-specific species or sub-species. It was essential that any assay distinguish the kumara isolate from the kiwifruit pathogens known to be present. Equally important was to ensure as much as possible that any other Ceratocystis isolates present in New Zealand but not known to cause disease, particularly in kiwifruit orchards, did not interfere with the test and produce significant numbers of false positives.

5.1 Identification of DNA unique to the Ceratocystis pathogens of kiwifruit

A possible explanation for the polyphyletic nature of the Ceratocystis pathogens of kiwifruit is that they all have in common the genes responsible for kiwifruit pathogenicity. There is a precedence for this. Isolates of *Fusarium oxysporum* f.sp *lycopersici,* a soil-borne pathogen of tomato, are also polyphyletic. This is due to the fact that the genes required for pathogenicity on tomato reside on a small dispensable chromosome that can be transferred horizontally between *F. oxysporum* isolates. To determine whether this might be the case for the Ceratocystis pathogens of kiwifruit, we developed a bioinformatic pipeline to identify DNA sequences common to the isolates pathogenic on kiwifruit, but absent in all other Ceratocystis isolates. Technical details of the pipeline are shown in Appendix IA. The pipeline was validated using genomes of *F. oxysporum* as a proof of concept. It was shown that the pipeline was able to identify the genes unique to the *F. oxysporum* f.sp *lycopersici* isolates. When we applied the pipeline to the Ceratocystis genomes we have, we were not able to identify any DNA unique to the kiwifruit pathogens (Appendix IB).

We also rejected the idea of using mitochondrial DNA as a template. The reason for this was twofold. Firstly, the majority of the assembly programmes resulted in fragmentation of the mitochondria genome. Secondly, the polyphyletic nature of the kiwifruit pathogens adds an extra layer of complexity.

5.2 Use of ITS I sequences as a template for designing specific primers for Ceratocystis detection and identification

There are many examples of where ITS I sequences have been used for pathogen detection and identification. Advantages of using this region for detection are that there are around 100 copies of this region per genome, thus potentially increasing the sensitivity of a qPCR assay by ~5 C_t. Disadvantages are that, although the ITS I region is highly variable, it does not have that many variable characters, and hence is certainly limited for discriminating taxonomy (particularly below the species level). It was also obvious that the five kiwifruit pathogens identified to date did not fall into a neat clade (phylogenetic grouping) and thus could not be specifically amplified using a single set of primers. Our strategy was therefore to design a set of generic primers that would amplify all members of the LAC. We would also design a secondary sets of primers to identify individual kiwifruit pathogens from any positive results from the first qPCR reaction. Complicating the design is the presence of *C. fimbriata senso stricto* in New Zealand. However, this pathogen is part of a clonal population and most likely restricted to the kumara-growing regions of New Zealand. In addition, because its ITS I sequence is known, it could be distinguished from other isolates from either melting temperature analysis or directly sequencing the qPCR product.

6 DESIGN AND TEST QPCR PRIMERS AGAINST DNA FROM THE CERATOCYSTIS ISOLATES AVAILABLE

We found all the ITS I sequences for Ceratocystis isolates that were available to us or present in GenBank. These were lined up using ClustalW (Figure 2). Three sets of primers were designed in the first instance (Table 2). One set were universal primers that would amplify all fungi. This set was to act as a positive control for soil and plant samples. The second set was designed to amplify all members of the LAC of Ceratocystis, and the third set were specific for the predominant kiwifruit pathogen designated KiwiA. These three primer sets were used for all testing. Following the successful testing of this strategy, we designed primers specific to KiwiA-E.

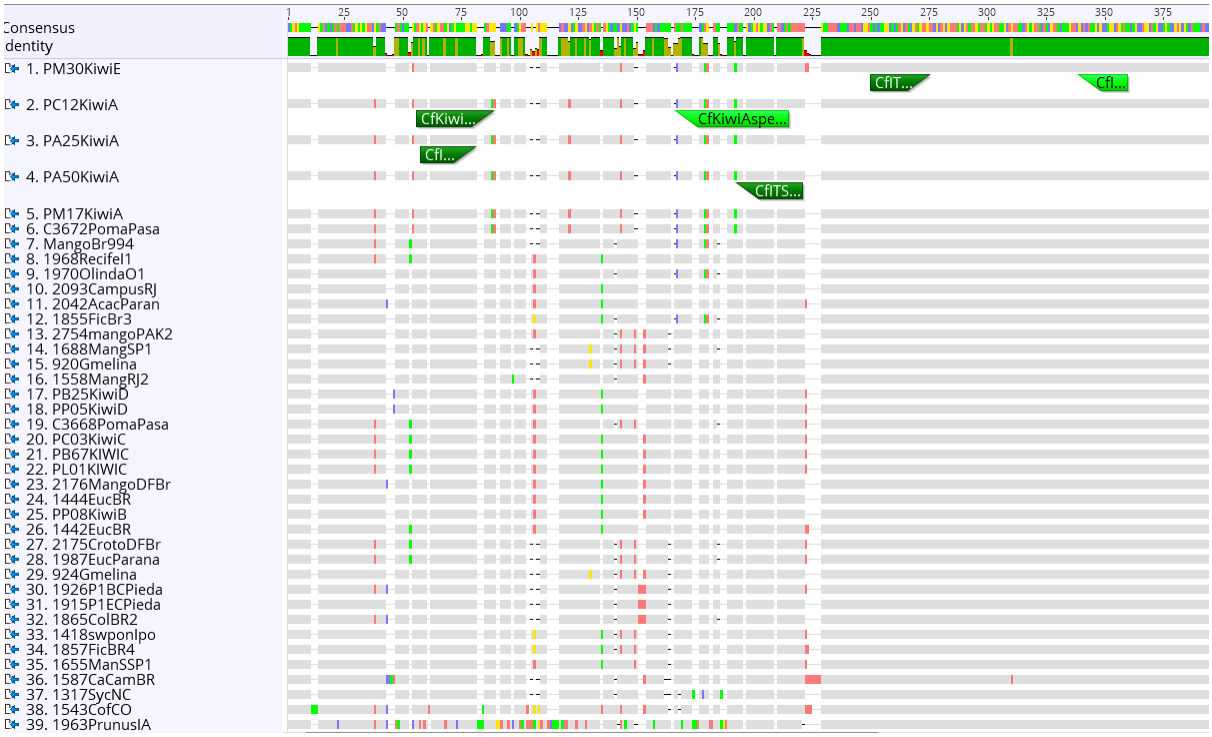


Figure 2. Line-up of Ceratocystis ITS I sequences. The primers shown are the generic ITS I primers at positions including bases 250 and 350, the LAC specific primers including bases 60 and 220 and the KiwiA specific primers around bases 85 and 175 ….

Table 2. Sequence of primers used for detection each Ceratocystis isolate.

Primer name	Primer sequence	Description
5.8rDNAF	TCTCTTGGCTCTAGCATCGATGAAGA	forward ITS primer binds to all fungal sequences
5.8rDNAR	AATACTGCCAGGCGCAATGTGC	reverse ITS primer that binds to all fungal sequences
LACF	TGAGATGAATGCTGTTTTGGTGG	Forward primer for LAC
LACR	TTTTTTATAGTTATGCCACTCAGCAAT	Reverse primer for LAC
CfKiwiAspecificF	AGTGAGATGAATGCTGTTTTGGTGGTAGTA	forward ITS primer specific for KiwiA
CfKiwiAspecificR	CCACTCAGCAATAAAAAATTAAGAAAATAG	reverse ITS primer specific for KiwiA and E
CfKiwiAEspecificF	CCATGTGTGAACATACCTATCTTA	forward ITS primer specific for KiwiA and KiwiE
CfKiwiB-DspecificF	GGTGGTAGGGCCCTTCTGAAGAGA	forward ITS primer specific for KiwiC and KiwiD
CfKiwiB-DR	CCACTCAGCAATAAAAAATTAAGAAAATAA	reverse ITS primer specific for KiwiB-D

7 TESTING OF PRIMERS ON PURIFIED DNA FROM VARIOUS CERATOCYSTIS ISOLATES.

We had available to us a small number of DNA samples provided by Profs Alfenes and Harrington. hese enabled us to test whether the primers we designed were specific and if our strategy was likely to be successful. As we can see from Table 3, the generic fungal primers designed to the ITS 1 (5.8 region) of rDNA amplify all isolates strongly (last column). The primers designed to amplify all member of LAC also amplified all Ceratocystis DNA samples. This was to be expected since most of the isolates were members of the LAC. Importantly the KiwiA specific primers only amplified DNA from the two KiwiA samples (Table 3).

Table 3. qPCR results using the KiwiA and LAC primers. Cq numbers below 35 are considered to be a positive result and are highlighted in yellow.

			KiwiA		LAC		5.8rDNA	
Isolate designation	Country/state of origin	host	Cq	MCA	Cq	MCA	Cq	MCA
SBS1-1	Brazil	eucalyptus	N/A	79.3	33	82.3	16	80.2
PG01-1 (KiwiA)	Brazil	kiwifruit	26	79	28	79.3	12	80.2
KUMARA3	New Zealand	kumara	N/A	N/A	24	81.7	13	80.2
C1714	Hawai'i	taro	N/A	N/A	32.4	80.2	15	80.2
C3649 (KiwiD)	Brazil	kiwifruit	N/A	N/A	17.0	81.4	15	80.2
C3668 (KiwiD)	Brazil	kiwifruit	N/A	N/A	16.4	81.1	15	79.9
C3672 (KiwiA)	Brazil	kiwifruit	28	79.3	18.9	79	16	80.2
C4118	Hawai'i	syngonium	44	80.2	18.1	80.5	15	80.2
C4184	Hawai'i	Ohi'a	N/A	N/A	17.0	80.2	14	79.9
C4186	Hawai'i	Ohi'a	N/A	N/A	16.1	81.1	14	79.9
C4191	Hawai'i	Ohi'a	N/A	N/A	32.2	80.5	15	80.2
NTC			N/A	N/A	N/A	N/A	N/A	80.2
NTC			N/A	N/A	N/A	N/A	N/A	N/A

Additionally, the purified DNA from Ceratocystis that the Ministry for Primary Industries (MPI) held, also had a collection of DNA samples from closely related genera such as Ophiostoma. These were tested with the generic fungal and the LAC primers. The only sample that tested positive was the isolate from Ohi'a with the LAC specific primers. This confirms that the primers designed are specific for the isolates they are designed to amplify and fit for purpose.

Table 4. Testing of qPCR primers against related fungal genera. PCR positive amplifications with a Cq below 35 cycles are highlighted in yellow. Most of the samples also amplified with the generic fungal primers indicating these form a good PCR competent DNA isolation control.

			KiwiA		LAC		5.8S RNA	
Sample	Host	Fungal species	Cq	MCA	Cq	MCA	Cq	MCA
1	Musa sp.	*Ceratocystis musarum*	N/D	N/D	N/D	N/D	24.75	79.5
2		*Ophiostoma quercus*	N/D	N/D	N/D	N/D	21.68	80.5
3		*Ophiostoma quercus*	N/D	N/D	N/D	N/D	22.37	80.5
4	Hibiscus sp.	*Ophiostoma sp.*	N/D	N/D	N/D	N/D	19.49	79
5	*Pinus radiata*	*Ophiostoma setosum*	N/D	N/D	N/D	N/D	N/D	N/D
6	*Pinus radiata*	*Ophiostoma setosum*	N/D	N/D	N/D	N/D	N/D	N/D
7	*Pinus radiata*	*Ophiostoma setosum*	N/D	N/D	N/D	N/D	20.75	80.5
8	*Ulmus glabra*	*Ophiostoma novo-ulmi*	N/D	N/D	N/D	N/D	32.03	78.5
9	*Scolytus multistriatus*	*Ophiostoma novo-ulmi*	N/D	N/D	N/D	N/D	30.37	78.5
10	Ulmus sp.	*Ophiostoma novo-ulmi*	N/D	N/D	N/D	N/D	20.47	80.5
11	*Pinus radiata*	*Ophiostoma piceae*	N/D	N/D	N/D	N/D	30.45	80.5
12	*Pinus radiata*	*Ophiostoma piceae*	N/D	N/D	N/D	N/D	23.64	80.5
13	*Pinus radiata*	*Ophiostoma piceae*	N/D	N/D	N/D	N/D	26.2	80.5
14		*Chalara fraxinea*	N/D	N/D	N/D	N/D	25.16	78.5
15		*Chalara fraxinea*	N/D	N/D	N/D	N/D	24.76	78.5
16		*Hymenoscyphus pseudoalbidus*	N/D	N/D	N/D	N/D	25.55	78.5
17		*Opiostoma penicillatum*	N/D	N/D	N/D	N/D	N/D	78
18	Banana	*Ceratocytis paradoxa*	N/D	N/D	N/D	N/D	22.6	79.5
19	Banana	*Ceratocytis paradoxa*	N/D	N/D	N/D	N/D	21.65	79.5
20	*Pisum sativum*	*Ophiostoma ips*	N/D	N/D	N/D	N/D	25.5	79
21	Golden Elm	*Ophiostoma nova ulmi*	N/D	N/D	N/D	N/D	20.91	80.5
22		*Thielaviopsis basicola*	N/D	N/D	N/D	N/D	15	78.5
23	*Metrosideros polymorpha*	*Ceratocystis fimbriata*	43.58	72.5+78.5	21.16	79.5	16.79	78.5
24	N/A	NTC	N/D	N/D	N/D	N/D	N/D	N/D

The next objective was to test these against samples from New Zealand to determine whether there was DNA from environmental samples that might test positive for these primers.

8 VALIDATION OF QPCR PRIMERS AGAINST ENVIRONMENTAL SAMPLES FROM NEW ZEALAND SOILS AND KIWIFRUIT ORCHARDS

Ceratocystis is largely a soil-borne pathogen. While it is known that the kumara pathogen *C. fimbriata senso stricto* is present in New Zealand, it is not known whether there are related Ceratocystis genera, either native or exotic, present in New Zealand. If so, it is possible that DNA from these putative isolates might amplify with our primers and give a false positive result. To test this possibility we collaborated with a group that was part of the Natural Heritage National Science project, who had collected a diverse set of environmental DNA (eDNA) samples from around the country. This collection was designed to include a broad range of environments and soil types from around the country, and as such was an ideal collection to screen the primers against. None of the eDNA samples was positive for the Ceratocystis primers whereas all samples were positive for the universal fungal control primers (Appendix 2). These results suggest that there is not a widespread presence of isolates of the kumara, native or exotic Ceratocystis complex in New Zealand soils.

The primers were further tested on 12 DNA extracts from kiwifruit plants growing in New Zealand orchards and glass houses. The results show that all DNA samples apart from the roots amplifies with the generic fungal primers (Fungal ITS and 5.8S RNA) but that all samples are negative with the LAC and KiwiA primers. While not a comprehensive survey, it indicates that fungal DNA can be amplified from kiwifruit samples and that Ceratocystis DNA is not present in those samples, suggesting the presence of Ceratocystis strains that can confound the qPCR test are at least not likely to be widely present in New Zealand.

Table 5. Testing of orchard samples

			KiwiA		LAC specific		Fungal ITS		5.8s RNA	
Tissue type	Orchard	Region	Cq	MCA	Cq	MCA	Cq	MCA	Cq	Mca
Leaf	Orchard A	Te Puke	ND	64	N/A	N/A	35.3	73	26.9	80.5
Flower sepal	Orchard A	Te Puke	ND	ND	N/A	N/A	27.1	79.9	28.2	80.5
Cane	Orchard A	Te Puke	ND	ND	42.1	73.6	34.3	81.1	36	81.4
Flower sepal	Orchard B	Aongatete	ND	ND	N/A	N/A	26.9	80.2	24.4	80.8
Leaf	Orchard B	Aongatete	ND	69.7	N/A	N/A	34.8	81.1	25.1	81.1
Leaf	Orchard B	Aongatete	ND	ND	N/A	N/A	34.2	81.4	25.4	81.1
Stem	PFR glasshouse	Lincoln	ND	ND	N/A	N/A	34.3	79.9	27.1	81.1
Stem	PFR glasshouse	Lincoln	ND	ND	N/A	N/A	34	80.2	26.4	81.4
Leaf	PFR glasshouse	Lincoln	ND	ND	N/A	N/A	35.5	80.5	27.1	81.4
Leaf	PFR glasshouse	Lincoln	ND	ND	N/A	N/A	35.1	81.4	26.2	81.1
Fruit	Orchard D	Riwaka	ND	ND	N/A	N/A	32.2	79.6	N/D	81.1
Roots	Orchard C	Te Puke	ND	ND	N/A	72.4	ND	ND	ND	ND
Positive control			17.3	79	19.0	79	12.8	79.9	24.3	81.4
Positive control			17.1	79	16.3	81.1	12.1	79.9	24.1	81.4
NTC			N/A	N/A	N/A	N/A	42	78.7	N/A	N/A
NTC			N/A	N/A	N/A	N/A	43.4	87.1	N/A	N/A

A more comprehensive survey of kiwifruit orchard samples was made in conjunction with Hill Laboratories. The aim of this was twofold. Firstly a requirement for any high-throughput DNA assay is that it can be used by an external provider. We chose Hill Laboratories as our provider as they have previous experience testing kiwifruit samples for Psa using qPCR. The second aim was to test more kiwifruit samples, and Hill's was able to do this by screening legacy kiwifruit orchard samples. The consistent result showed that all samples were positive using the generic fungal primers but none amplified product with either the LAC or KiwiA primers (Appendix 3).

9 FINAL CONCLUSIONS AND FUTURE DIRECTIONS

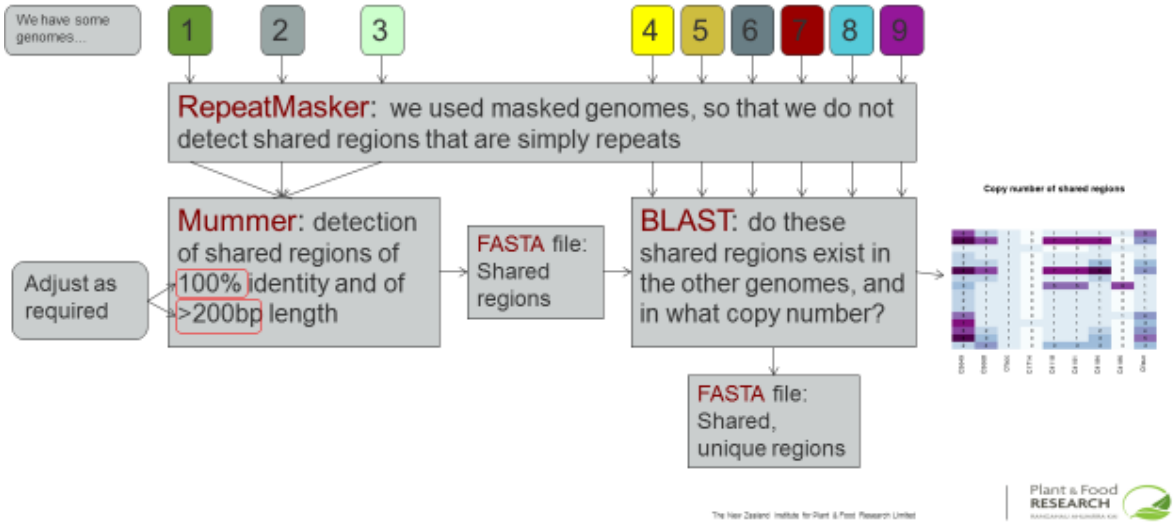
We have designed a robust qPCR-based assay that can detect all isolates of *C. fimbriata* that are pathogenic on kiwifruit. The assay has been tested extensively against DNA from isolates of Ceratocystis and related genera. The primers have not generated false positives against a wide range of soil and kiwifruit vine samples. The only non-target samples that might be amplified by these primers are those that contain DNA from the kumara pathogen of Ceratocystis. This potential false positive result will be eliminated by the second set of primers designed to specifically identify any positive results from the first set of primers. While we are confident these primers will work as a rapid response to a potential incursion, we have not been able to test them against kiwifruit material infected with these fungi. This work could only be done in collaboration with our partners in Brazil, or by import of DNA from infected plants. We recommend that this last set of experiments be carried out to fully verify the assay.

10 REFERENCES

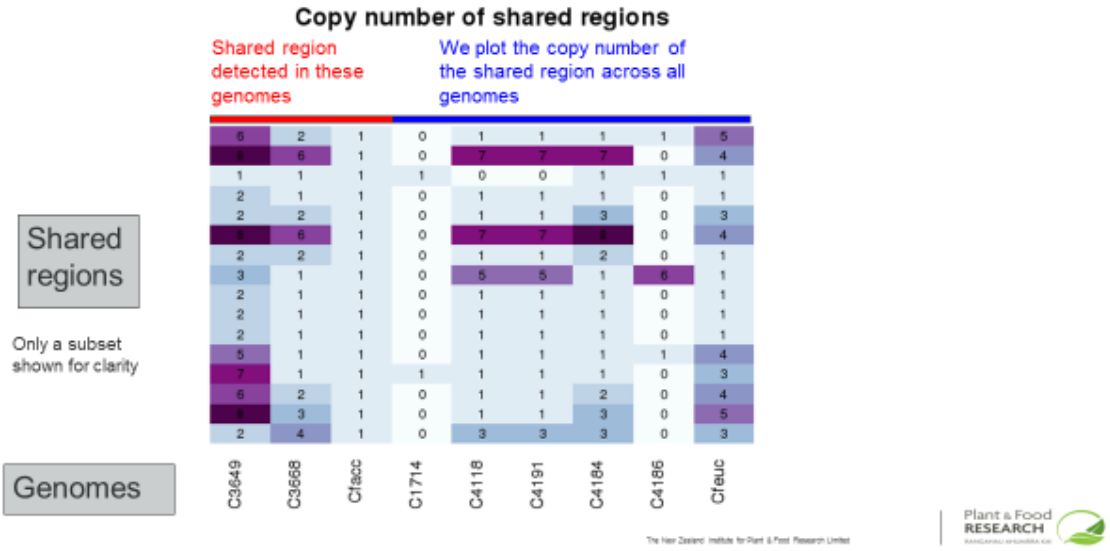
G Piveta, MA Ferreira, M FB Muniz, D Valdetaro, R Valdebenito-Sanhueza, T Harrington & AC Alfenas (2016) *Ceratocystis fimbriata* on kiwifruit (*Actinidia* spp.) in Brazil, New Zealand Journal of Crop and Horticultural Science, 44:1, 13-24, DOI: 10.1080/01140671.2016.1143020

APPENDIX I. BIOINFORMATIC PIPELINE FOR IDENTIFYING DNA UNIQUE TO CERATOCYSTIS PATHOGENS

The pipeline



Applying the pipeline to Ceratocystis



APPENDIX 2. SUMMARY OF TESTING OF ENVIRONMENTAL SOIL DNA SAMPLES USING CERATOCYSTIS PRIMERS

The primer sets were:

Forward Primer	Reverse Primer	Target
ITS-1F	ITS-4R	All fungal DNA
CfITSallF	CfITSallR	General Ceratocystis primers
CfKiwiA F	CfKiwiA R	Specific to Cf KiwiA virulent on kiwifruit
CfITSHRM F	CfITSHRM R	Designed to amplify the LAC and distinguish by melt curve analysis

PCR conditions were:

Polymerase Activation	95°C	03:00	1x
PCR Cycling	95°C	00:05	45x
	60°C	00:07	
	72°C	00:10	
Melt Curve	95°C	00:15	
	55°C	00:15	
	95°C	00:15	

125 samples were diluted 1/10 and tested using three sets of primers.

C3672 (Cf isolate KiwiA) 1/10 dilution was used as a positive control for all primer sets, and a Non-template Control (NTC) where water was added to the mix instead of DNA was used as a negative control.

Summary of Key Results

- All positive controls were positive. All Non Template Ccontrols (NTC) samples were negative.
- All samples tested negative for CfKiwiA F/R except for positive controls.
- All samples tested negative for CfITSHRM F/R except for positive controls.
- Details of results are shown in the Table below.

Results Table

Sample No.	Landcare Plate and Position	Landcare Name	ITS 1/4	Cf All 1F/1R	Cf Kiwi A F/R	Cf HRM F/R
1	P1-A1	AM1	24	27	N/A	N/A
2	P1-A2	AN2F	23	27	N/A	41.1
3	P1-A3	AS1H	25	27	N/A	N/A
4	P1-A4	AS5F	23	25	N/A	N/A
5	P1-A5	BM4F	22	24	N/A	N/A
6	P1-A6	BN5	22	25	N/A	N/A
7	P1-A7	BS4	26	28	N/A	N/A
8	P1-A8	CM5	22	27	N/A	N/A
9	P1-A9	CN4	21	26	N/A	39.9
10	P1-A10	CS5H	25	27	N/A	N/A
11	P1-B1	AM2	25	27	N/A	N/A
12	P1-B2	AN3	23	26	N/A	44.6
13	P1-B3	AS1N	34	28	N/A	44.0
14	P1-B4	BM1	23	26	N/A	40.7
15	P1-B5	BM5	23	25	N/A	44.9
16	P1-B6	BS1H	25	26	N/A	N/A
17	P1-B7	BS5	23	25	N/A	40.3
18	P1-B8	CN1	36	38	N/A	N/A
19	P1-B9	CN5	22	27	N/A	39.8
20	P1-B10	CS5N	22	27	N/A	43.1
21	P1-C1	AM3	24	29	N/A	42.5
22	P1-C2	AN4H	24	28	N/A	42.5
23	P1-C3	AS1F	24	27	N/A	N/A
24	P1-C4	BM2H	24	27	N/A	N/A
25	P1-C5	BN1	24	28	N/A	42.4
26	P1-C6	BS1N	26	27	N/A	40.3
27	P1-C7	CM1	26	27	N/A	41.5
28	P1-C8	CN2H	22	28	N/A	38.1
29	P1-C9	CS1	22	28	N/A	42.2
30	P1-C10	CS5F	25	26	N/A	43.8
31	P1-D1	AM4	27	29	N/A	43.9
32	P1-D2	AN4N	24	28	N/A	41.4
33	P1-D3	AS2	22	24	N/A	40.6
34	P1-D4	BM2N	23	26	N/A	38.6
35	P1-D5	BN2H	23	25	N/A	40.6
36	P1-D6	BS1F	24	25	N/A	N/A
37	P1-D7	CM2	23	26	N/A	40.6
38	P1-D8	CN2N	24	25	N/A	38.2
39	P1-D9	CS2	21	23	N/A	40.8
40	P1-E1	AM5	23	26	N/A	43.9
41	P1-E2	AN4F	27	29	N/A	41.6
42	P1-E3	AS3	24	28	N/A	44.2

Sample No.	Landcare Plate and Position	Landcare Name	ITS 1/4	Cf All 1F/1R	Cf Kiwi A F/R	Cf HRM F/R
43	P1-E4	BM2F	24	26	N/A	44.4
44	P1-E5	BN2N	22	27	N/A	39.2
45	P1-E6	BS2H	25	26	N/A	N/A
46	P1-E7	CM3H	24	26	N/A	38.7
47	P1-E8	CN2F	23	26	N/A	41.0
48	P1-E9	CS3	24	26	N/A	39.6
49	P1-F1	AN1	23	26	N/A	43.3
50	P1-F2	AN5H	25	27	N/A	43.1
51	P1-F3	AS4	24	27	N/A	39.6
52	P1-F4	BM3	22	25	N/A	42.3
53	P1-F5	BN2F	23	28	N/A	43.1
54	P1-F6	BS2N	24	26	N/A	40.0
55	P1-F7	CM3N	24	28	N/A	39.7
56	P1-F8	CN3H	24	28	N/A	41.4
57	P1-F9	CS4H	24	26	N/A	42.0
58	P1-G1	AN2H	27	28	N/A	41.4
59	P1-G2	AN5N	24	27	N/A	44.9
60	P1-G3	AS5H	26	25	N/A	42.3
61	P1-G4	BM4H	24	25	N/A	41.0
62	P1-G5	BN3	24	26	N/A	40.0
63	P1-G6	BS2F	25	27	N/A	44.5
64	P1-G7	CM3F	25	27	N/A	38.8
65	P1-G8	CN3N	22	25	N/A	41.0
66	P1-G9	CS4N	26	25	N/A	40.9
67	P1-H1	AN2N	26	28	N/A	44.9
68	P1-H2	AN5F	26	29	N/A	41.4
69	P1-H3	AS5N	23	24	N/A	41.1
70	P1-H4	BM4N	23	24	N/A	43.9
71	P1-H5	BN4	25	28	N/A	N/A
72	P1-H6	BS3	23	28	N/A	N/A
73	P1-H7	CM4	22	26	N/A	43.6
74	P1-H8	CN3F	23	27	N/A	45.0
75	P1-H9	CS4F	26	26	N/A	44.9
76	P2-A1	DM1	25	28	N/A	43.3
77	P2-A2	DM5F	23	27	N/A	N/A
78	P2-A3	DN4	22	28	N/A	41.4
79	P2-A4	DS3	24	26	N/A	41.4
80	P2-A5	EN1H	22	25	N/A	39.7
81	P2-A6	EN5	24	25	N/A	42.3
82	P2-A7	ES5N	20	26	N/A	40.1
83	P2-B1	DM2H	25	28	N/A	44.8
84	P2-B2	DM6	25	27	N/A	40.1
85	P2-B3	DN5H	25	N/A	N/A	45.0

Sample No.	Landcare Plate and Position	Landcare Name	ITS 1/4	Cf All 1F/1R	Cf Kiwi A F/R	Cf HRM F/R
86	P2-B4	EM1H	N/A	27	N/A	41.1
87	P2-B5	EN1N	N/A	25	N/A	37.0
88	P2-B6	ES1	N/A	25	N/A	N/A
89	P2-B7	ES5F	19	26	N/A	41.0
90	P2-C1	DM2N	24	27	N/A	39.3
91	P2-C2	DM7	25	28	N/A	39.3
92	P2-C3	DN5N	26	28	N/A	44.9
93	P2-C4	EM1N	22	24	N/A	41.2
94	P2-C5	EN1F	20	26	N/A	39.8
95	P2-C6	ES2	22	26	N/A	41.8
96	P2-D1	DM2F	25	27	N/A	38.3
97	P2-D2	DM8	26	28	N/A	38.5
98	P2-D3	DN5F	26	28	N/A	39.6
99	P2-D4	EM1F	22	24	N/A	40.0
100	P2-D5	EN2	23	25	N/A	40.1
101	P2-D6	ES3	21	25	N/A	42.6
102	P2-E1	DM3	25	27	N/A	41.7
103	P2-E2	DN2	34	35	N/A	N/A
104	P2-E3	DS1H	23	27	N/A	38.3
105	P2-E4	EM2	21	27	N/A	37.7
106	P2-E5	EN3H	N/A	44	N/A	44.7
107	P2-E6	ES4H	20	24	N/A	40.1
108	P2-F1	DM4	23	27	N/A	39.2
109	P2-F2	DN3H	24	28	N/A	39.7
110	P2-F3	DS1N	21	26	N/A	39.3
111	P2-F4	EM3	21	27	N/A	38.7
112	P2-F5	EN3N	22	24	N/A	43.7
113	P2-F6	ES4N	22	26	N/A	40.1
114	P2-G1	DM5H	25	26	N/A	41.6
115	P2-G2	DN3N	29	28	N/A	37.8
116	P2-G3	DS1F	23	27	N/A	40.6
117	P2-G4	EM4	22	25	N/A	39.0
118	P2-G5	EN3F	22	25	N/A	39.0
119	P2-G6	ES4F	25	27	N/A	38.5
120	P2-H1	DM5N	23	27	N/A	39.5
121	P2-H2	DN3F	23	28	N/A	38.4
122	P2-H3	DS2	24	28	N/A	42.0
123	P2-H4	EM5	21	25	N/A	41.9
124	P2-H5	EN4	22	25	N/A	43.4
125	P2-H6	ES5H	21	26	N/A	39.1

APPENDIX 3. HILL LABORATORIES QUOTATION

Hill Laboratories
TRIED, TESTED **AND TRUSTED**

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Private Bag 3205
Hamilton 3240 New Zealand

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E mail@hill-labs.co.nz
W www.hill-laboratories.com

ANALYSIS REPORT

Client:	Plant & Food Research	**Lab No:**	1845357	SPv2
Contact:	Mark Andersen	**Date Received:**	19-Sep-2017	
	C/- Plant & Food Research	**Date Reported:**	16-Oct-2017	**(Amended)**
	Private Bag 92169	**Quote No:**	87304	
	Auckland Mail Centre	**Order No:**	PFR 2018329	
	Auckland 1142	**Client Reference:**		
		Submitted By:	Mark Andersen	

Analyst's Comments

Amended Report: This report replaces an earlier report issued on 05 Oct 2017 at 9:53 am
Reason for amendment: The title of Figure 1 has been corrected.

Appendix No.1 - Ceratocytis PCR Report

These samples were collected by yourselves (or your agent) and analysed as received at the laboratory.

Samples are held at the laboratory after reporting for a length of time depending on the preservation used and the stability of the analytes being tested. Once the storage period is completed the samples are discarded unless otherwise advised by the client.

Eilidh Mowat PhD
Senior Technologist - Plant Pathology

1. Aim

To evaluate the specificity of three sets of Ceratocytis oligonucleotide primers supplied by the client using a panel of DNA extracted from kiwifruit cane material.

2. Method

A panel of 40 retained DNA samples were selected for analysis. All DNA samples were extracted from kiwifruit cane material and had been stored at -20ºC for a maximum of 6 months. The quantity and quality of each DNA sample was verified using plant specific (COX) real time PCR assay. The presence or absence of Ceratocytis DNA in this panel of DNA samples had not been investigated prior to this study being undertaken.

Three sets of Ceratocysis primers (HRM, Kiwi and ITS) were received from the client. Each 10µL PCR reaction contained 0.5µM forward and reverse primer, 1× Kapa Sybr Fast (Kapa Biosystems) and 2µL template DNA. All PCR assays were completed using the Eco (Illumina) qPCR instrument using the following conditions: 95ºC for 3min, followed by 45 cycles of 95ºC for 3s and 60ºC for 7s. A melt temperature step was completed at the end of each PCR assay and conditions were as follows: 95ºC 15s, 55ºC for 15s, increasing to 95ºC for 15s. No further optimisation of the PCR conditions was undertaken. Data analysis was completed using the Eco Illumina software (v4.0). Presumptive positive results were considered when a DNA sample produced a Cq <45 along with an associated melt temperature within ±1ºC of the Ceratocytis positive control DNA. Any amplification with a melt temperature outside of ±1ºC of the Ceratocytis positive control DNA melt temperature was considered to be non-specific.

3. Results

3.1. Verification of Plant DNA

All 40 selected DNA samples extracted from kiwifruit cane material were tested using plant specific (COX) primers to confirm a sufficient quantity of DNA was present in the retained samples. Plant DNA was successfully detected in all 40 samples (Table 1, Figure 1). COX Cq's ranged between 23.06 – 31.72 with associated melt temperatures of 78.4 – 78.7ºC. No plant DNA was detected in the Ceratocytis DNA control sample supplied by the client.

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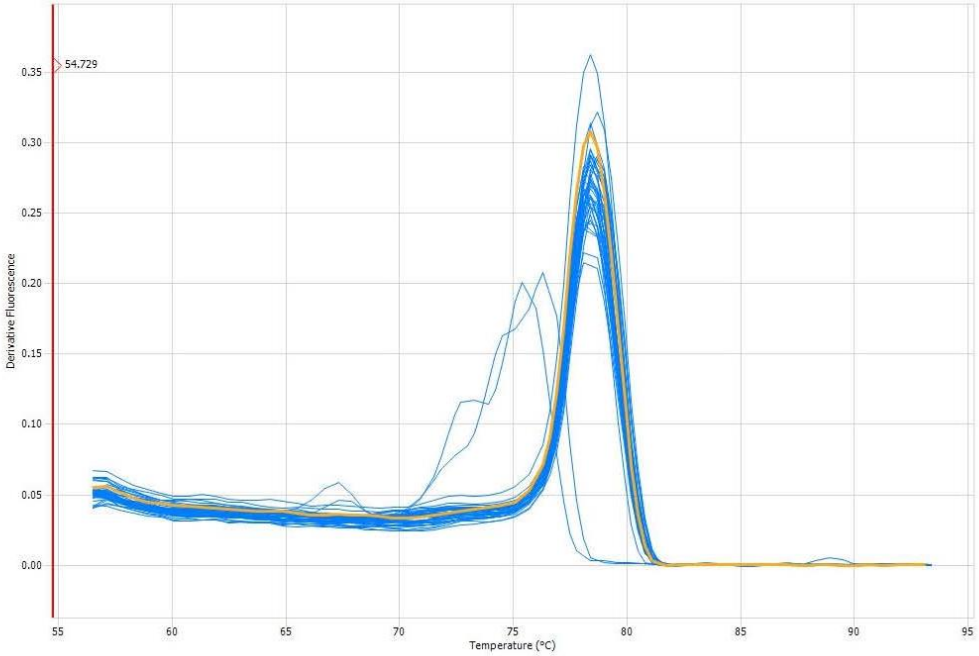
Figure 1. COX primer results.

DNA Sample	COX Cq	COX Tm1(°C)	Result
1	24.40	78.4	Positive
2	25.35	78.4	Positive
3	26.23	78.4	Positive
4	25.43	78.4	Positive
5	31.72	78.4	Positive
6	25.34	78.4	Positive
7	24.69	78.4	Positive
8	25.25	78.4	Positive
9	24.20	78.4	Positive
10	25.40	78.4	Positive
11	23.87	78.1	Positive
12	23.95	78.4	Positive
13	23.52	78.4	Positive
14	23.06	78.4	Positive
15	25.06	78.4	Positive
16	25.66	78.4	Positive
17	26.38	78.4	Positive
18	25.64	78.4	Positive
19	28.86	78.4	Positive
20	25.90	78.4	Positive
21	24.76	78.4	Positive
22	25.34	78.4	Positive
23	27.09	78.4	Positive
24	25.79	78.4	Positive
25	26.76	78.4	Positive
26	26.04	78.4	Positive
27	25.52	78.4	Positive
28	24.49	78.7	Positive
29	25.42	78.7	Positive
30	26.32	78.7	Positive
31	28.90	78.4	Positive
32	25.44	78.4	Positive
33	24.99	78.7	Positive
34	24.96	78.7	Positive
35	27.11	78.7	Positive
36	25.98	78.7	Positive
37	28.63	78.7	Positive
38	25.40	78.7	Positive
39	27.28	78.7	Positive
40	27.30	78.4	Positive
COX DNA Control	26.07	78.7	Positive
Ceratocytis DNA	37.11	75.4	Not Detected
NTC	39.84	73	Not Detected

Figure 1. Melt temperature profiles of COX PCR products. The melt curve of the COX positive control DNA is highlighted in gold.



3.2. HRM Primer Evaluation

The Ceratocytis positive control DNA was successfully amplified by the HRM primers producing a Cq of 17.90 and a melt temperature of 79.3ºC. Non-specific amplification was noted in all 40 DNA samples from kiwifruit cane material with Cq's ranging between 32.29 – 34.26 and associated melt temperatures of 75.1 – 82.6ºC (Table 2, Figure 2).

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Table 2. HRM Ceratocytis primer results.

DNA Sample	HRM Cq	HRM Tm1 (ºC)	HRM Result
1	32.29	75.7	Not Detected
2	33.50	75.4	Not Detected
3	34.09	75.7	Not Detected
4	33.71	75.4	Not Detected
5	33.88	75.1	Not Detected
6	33.65	75.7	Not Detected
7	33.76	75.4	Not Detected
8	33.98	75.7	Not Detected
9	33.17	82.6	Not Detected
10	32.52	75.7	Not Detected
11	34.19	75.7	Not Detected
12	33.49	75.4	Not Detected
13	33.19	75.4	Not Detected
14	32.70	75.1	Not Detected
15	34.26	75.4	Not Detected
16	33.66	75.7	Not Detected
17	33.55	75.4	Not Detected
18	33.01	75.7	Not Detected
19	33.84	75.1	Not Detected
20	33.16	75.7	Not Detected
21	33.45	75.7	Not Detected
22	32.82	75.7	Not Detected
23	33.91	75.1	Not Detected
24	33.30	75.7	Not Detected
25	33.90	74.5	Not Detected
26	33.24	75.7	Not Detected
27	32.84	75.1	Not Detected
28	33.17	75.4	Not Detected
29	33.37	75.4	Not Detected
30	33.18	75.7	Not Detected
31	33.17	75.1	Not Detected
32	33.75	75.7	Not Detected
33	33.84	75.4	Not Detected
34	33.85	75.4	Not Detected
35	33.49	75.4	Not Detected
36	33.10	75.7	Not Detected
37	32.99	75.1	Not Detected
38	34.11	74.5	Not Detected
39	32.97	75.4	Not Detected
40	32.63	75.7	Not Detected
Ceratocytis DNA	17.90	79.3	Positive
NTC	33.01	75.1	Not Detected

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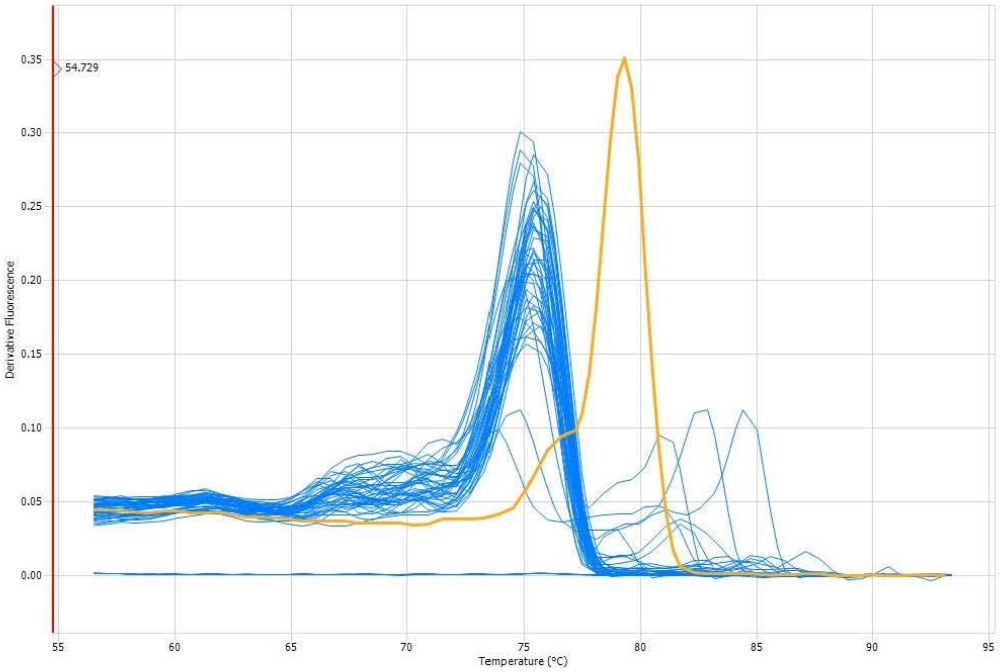
Figure 2. Melt temperature profiles of HRM PCR products. The melt curve of the Ceratocytis positive control DNA is highlighted in gold.



3.3. Kiwi primer Evaluation

The Ceratocytis positive control DNA was successfully detected using the Kiwi primer set producing a Cq of 17.37 and a melt temperature of 79.3ºC. There was no detectable amplification in 14 of the DNA samples. Non-specific amplification was noted in 24 DNA samples from kiwifruit cane material with Cq's ranging between 36.04 to 44.23 and associated melt temperatures of 64.6 – 76ºC (Table 3, Figure 3). The two exceptions were DNA samples 9 and 22 that produced melt temperatures of 79.6 and 79ºC respectively. Repeat PCR results of these two samples were not detected with melt temperatures of 75.4 ºC.

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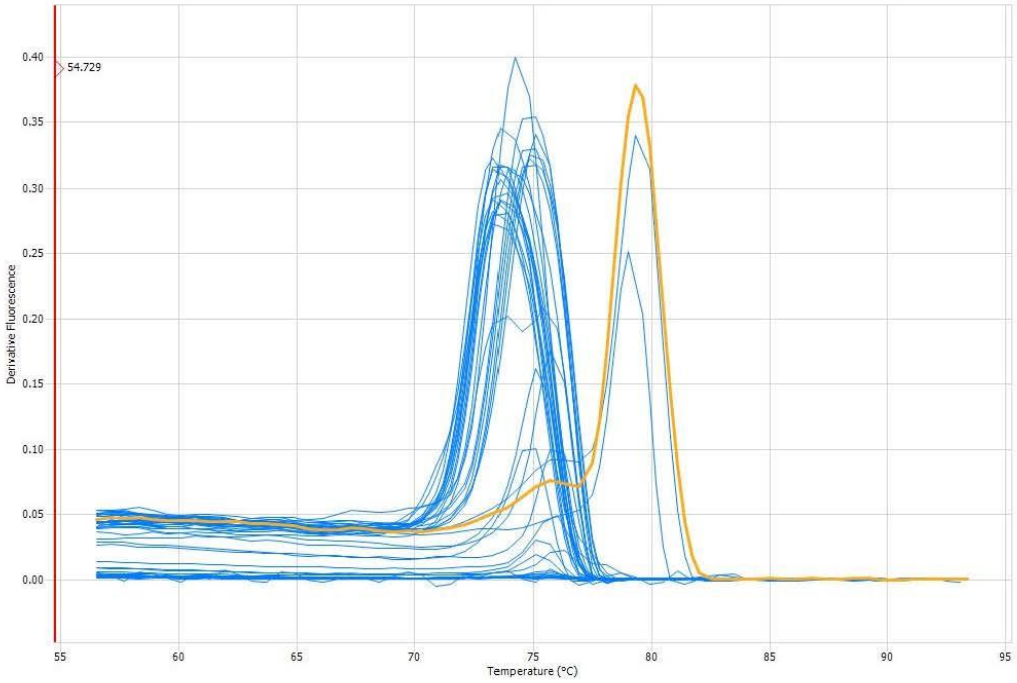
Table 3. Kiwi primer results.

DNA Sample	Kiwi Cq	Kiwi Tm1 (ºC)	Kiwi Result
1	44.23	76	Not Detected
2	N/A	82.9	Not Detected
3	37.41	73.6	Not Detected
4	37.48	73.6	Not Detected
5	36.68	74.8	Not Detected
6	37.84	75.1	Not Detected
7	N/A	N/A	Not Detected
8	37.22	73.9	Not Detected
9	37.56	73.6	Not Detected
10	N/A	N/A	Not Detected
11	N/A	N/A	Not Detected
12	37.84	73.9	Not Detected
13	N/A	75.7	Not Detected
14	43.12	75.7	Not Detected
15	37.33	73.9	Not Detected
16	N/A	75.4	Not Detected
17	38.74	73.6	Not Detected
18	N/A	75.7	Not Detected
19	36.04	79.6	Repeat to confirm
19 – repeat	35.56	75.4	Not Detected
20	36.67	74.2	Not Detected
21	N/A	76	Not Detected
22	42.11	79	Repeat to confirm
22 - repeat	35.13	75.4	Not Detected
23	40.04	75.1	Not Detected
24	N/A	75.7	Not Detected
25	37.28	73.6	Not Detected
26	N/A	N/A	Not Detected
27	36.81	74.8	Not Detected
28	37.21	74.8	Not Detected
29	37.27	74.8	Not Detected
30	N/A	N/A	Not Detected
31	38.37	73.6	Not Detected
32	43.26	75.1	Not Detected
33	N/A	64.6	Not Detected
34	N/A	N/A	Not Detected
35	39.73	73.6	Not Detected
36	N/A	N/A	Not Detected
37	36.49	73.6	Not Detected
38	40.32	75.7	Not Detected
39	44.22	74.8	Not Detected
40	36.47	73.3	Not Detected
Ceratocytis DNA	17.37	79.3	Positive
NTC	N/A	75.4	Not Detected

Figure 3. Melt temperature profiles of Kiwi PCR products. The melt curve of the Ceratocytis positive control DNA is highlighted in gold.



3.4. ITS primer Evaluation

The Ceratocytis positive control DNA was successfully detected by the ITS primers producing a Cq of 14.24 and melt temperature of 80.2ºC. Ceratocytis DNA was not detected in DNA samples 5 and 31. Sample 17 produced an inconclusive initial PCR result, but the presence of Ceratocytis DNA was classified as not detected on repeat PCR analysis (Table 4, Figure 4). The results for the remaining 37 DNA samples were undetermined as they all produced Cq values <45 and melt temperatures within ±1ºC of the Ceratocytis positive control DNA sample on repeat PCR testing.

Table 4. ITS Primer results.

	Initial PCR Screen			Repeat PCR		
DNA Sample	ITS Cq	ITS Tm1 (ºC)	ITS Result	ITS Cq	ITS Tm1 (ºC)	ITS Result
1	23.07	80.5	Repeat to confirm	22.93	80.5	UN
2	21.38	80.8	Repeat to confirm	21.36	80.8	UN
3	26.08	80.8	Repeat to confirm	24.25	80.5	UN
4	25.60	80.8	Repeat to confirm	26.15	80.8	UN
5	37.52	79	Not Detected			
6	20.90	80.8	Repeat to confirm	19.32	80.8	UN
7	21.29	80.8	Repeat to confirm	20.69	80.5	UN
8	23.31	80.8	Repeat to confirm	22.45	80.5	UN
9	21.42	80.8	Repeat to confirm	20.61	80.5	UN
10	24.94	80.5	Repeat to confirm	25.07	80.5	UN
11	26.46	80.8	Repeat to confirm	21.20	80.8	UN
12	30.46	80.8	Repeat to confirm	25.82	80.8	UN
13	27.46	80.8	Repeat to confirm	25.01	80.5	UN
14	24.85	80.8	Repeat to confirm	22.40	80.8	UN
15	30.46	80.5	Repeat to confirm	22.10	80.5	UN
16	27.03	80.5	Repeat to confirm	22.42	80.5	UN
17	27.59	80.5	Repeat to confirm	28.01	82	Not Detected
18	26.31	80.8	Repeat to confirm	27.05	80.5	UN
19	26.31	80.8	Repeat to confirm	26.99	80.5	UN
20	28.35	80.5	Repeat to confirm	28.99	80.2	UN
21	25.58	80.5	Repeat to confirm	26.67	80.5	UN
22	24.50	80.5	Repeat to confirm	26.67	80.5	UN
23	27.53	80.5	Repeat to confirm	29.18	80.5	UN
24	30.86	79.9	Repeat to confirm	27.09	80.2	UN
25	33.36	80.5	Repeat to confirm	28.19	80.2	UN
26	30.93	80.5	Repeat to confirm	27.24	80.2	UN
27	N/A	79.3	Repeat to confirm	27.79	80.2	UN
28	41.02	79.3	Repeat to confirm	27.05	80.2	UN
29	33.60	80.5	Repeat to confirm	26.32	80.5	UN
30	34.29	80.5	Repeat to confirm	27.56	80.5	UN
31	37.19	78.7	Not Detected			
32	N/A	80.2	Repeat to confirm	28.08	80.2	UN
33	28.28	80.2	Repeat to confirm	28.06	80.2	UN
34	29.35	80.5	Repeat to confirm	27.26	80.2	UN
35	29.67	80.5	Repeat to confirm	29.90	80.5	UN
36	26.37	80.8	Repeat to confirm	26.52	80.5	UN
37	27.90	80.8	Repeat to confirm	28.30	80.5	UN
38	25.50	80.5	Repeat to confirm	25.14	80.5	UN
39	23.27	80.5	Repeat to confirm	25.11	80.5	UN
40	30.86	79.9	Repeat to confirm	25.70	80.8	UN
Ceratocytis DNA	14.24	80.2	Positive	14.05	80.2	Positive
NTC	36.56	78.6	Not Detected	34.95	80.5	UN

4. Summary

Overall the results indicate the HRM and Kiwi primers are suitable for the specific detection of Ceratocytis DNA. The Cq values of the non-specific amplification detected with the Kiwi primers were >36 and further optimisation of the PCR conditions, could resolve its frequency. Further work is recommended to confirm the specificity of the ITS primers, as the panel of DNA samples testing in this study produced a number of undetermined results.

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