Development of a PCR-RFLP method to distinguish species within the *Ilyonectria macrodidyma* complex

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Abstract Species within the *Ilyonectria macrodidyma* complex are known plant pathogens and several are implicated as the causal agents of black foot disease of grapevines. The seven species within the complex can be identified by DNA sequencing of the histone H3 gene. In this study, a PCR-RFLP method to identify the species was developed. *In silico* digestion of the 500 bp histone H3 amplimer using *Mnl*I showed that it could identify *Ilyonectria* sp. 1, *Ilyonectria* sp. 2, *I. alcacerensis* and *I. macrodidyma*. Subsequent *in silico* digestion with *Hinf1* identified *I. estremocensis, I. novozelandica* and *I. torresensis*. The PCR-RFLP was validated using a collection of 40 *I. macrodidyma* complex isolates that had been recovered from symptomatic grapevines. *Ilyonectria macrodidyma, I. novozelandica* and *I. torresensis* were detected in that collection. Intraspecific polymorphism was only detected in *I. torresensis*. This method provides a rapid procedure for identifying individual species of the *I. macrodidyma* species complex.

Keywords *Cylindrocapon, Ilyonectria macrodidyma, I. torresensis, I. novozelandica, Ilyonectria* sp. 1, *Ilyonectria* sp. 2, *I. alcacerensis*, PCR-RFLP.

INTRODUCTION

Black foot disease of grapevine is a major disease associated with decline and death of vines, particularly in young vineyards and nurseries, in all major viticulture regions worldwide (Agusti-Brisach & Armengol 2013). This disease has been reported to have an increased incidence and severity over the past few years causing substantial economic losses (Oliveira et al. 2004; Halleen et al. 2006a).

Several *Cylindrocarpon*-like species residing in the genera *Campylocarpon* and *Ilyonectria* have been identified as the causal agents of this disease. The predominant pathogens are *Ilyonectria liriodendri* and the *I. macrodidyma* complex, and two *Campylocarpon* species, *C. fasciculare* and *C. pseudofasciculare* (Cabral et al. 2012b). Although the relative importance, frequency and geographic distribution of these pathogens are still poorly understood, the *I. liriodendri* and *I. macrodidyma* complex are most commonly isolated from affected grapevines (Petit & Gubler 2005; Halleen et al. 2006a; Alaniz et al. 2007). In a New Zealand survey, Pathrose (2012) also found the *I. radicicola* complex was predominantly isolated from symptomatic grapevine material. Recent reclassification of *Ilyonectria* spp. has shown, however, that many of the earlier records actually represent some

newly described species (Cabral et al. 2012a, b). These include *I. alcacerensis* A. Cabral, Oliveira & Crous, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. novozelandica* A. Cabral & Crous and *I. torresensis* A. Cabral, Rego & Crous, which were described from within the *I. macrodidyma* species complex (Cabral et al. 2012b).

At present, sequence analysis is the only reliable method for the detection of the individual species of this complex. Sequence analysis is, however, expensive to carry out, especially when many isolates need to be analysed on a routine basis. Other methods include the use of morphological and cultural characteristics. Reis et al. (2013) found that conidial measurements allowed for clustering of isolates into seven distinct groups in accordance to the species groups as described by Cabral et al. (2012b), but cultural characteristics including mycelium coloration and growing margin revealed nine different groups of isolates. RAPD and ISSR markers have been successfully used to differentiate between the individual species, although low reproducibility of results means that their use is limited (Reis et al. 2013). This research aimed to develop a PCR-RFLP method to provide a rapid and sensitive method that could be routinely used to distinguish the individual species within the *I. macrodidyma* species complex recovered from grapevines.

MATERIALS AND METHODS
Fungal isolates
This study included 40 isolates of "*C.*" *macrodidyma* fungi, previously identified using morphological and molecular methods (Pathrose 2012). These isolates were obtained in a 2005 survey of symptomatic grapevine material of vineyards across seven regions (Auckland, Marlborough, Nelson, Gisborne, Central Otago, Waipara and Hawke's Bay) in New Zealand and were maintained as agar slopes at 4°C (Bleach 2013). In the current study, these isolates were sequenced for part of the histone H3 gene (previously shown to be a very informative locus; Cabral et al. (2012b)) to identify the individual species within the *I. macrodidyma* complex. Sequencing reactions were performed by the Bio-Protection Research Centre, Lincoln University, Canterbury, New Zealand. The sequences obtained were then edited to remove any ambiguities using DNAMAN™ (LynnonBiosoft version 5.0) and compared to sequences present in the GenBank database (www.ncbi.nlm.nih.gov/Genbank/) using the nucleotide basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.giv/BLAST/).

DNA extraction and amplification
For each isolate, genomic DNA was extracted using the PUREGENE genomic DNA isolation kit (PUREGENE®, GentraSystems, Minneapolis, USA) according to the manufacturer's instructions. PCR amplification of the histone H3 gene was performed using the protocol described by Cabral et al. (2012b) using the CYLH3F and CYLH3R primers (Crous et al. 2004). PCR products were separated on a 1% agarose gel and stained by immersion in ethidium bromide (0.5 μg/ml) for 15 min and visualised under UV light. Molecular masses of products were estimated by comparison to a 1 kB Plus DNA ladder™ (Invitrogen).

Design of PCR-RFLP analysis
To rapidly identify the seven species belonging to the *I. macrodidyma* complex, a PCR-RFLP method was designed based on the nucleotide sequence of histone H3 gene of *I. macrodidyma* complex representatives (Table 1). These sequences were aligned by multiple species alignment using DNAMAN™ (LynnonBiosoft version 5.0). Areas of variability between the species were identified and at least 20 bp fragments were examined using the database of 180 frequently used enzymes available in DNAMAN™. Restriction digestion *in silico* was carried out and predicted restriction profiles were determined for each of the species. A list of readily available, inexpensive enzymes able to distinguish between the different species was compiled. Enzyme systems were then designed, beginning with an enzyme that was able to distinguish between three or more species in the initial digest. Several systems were developed *in silico* and the system that used the fewest number of enzymes was chosen.

Table 1 Representative DNA sequences of the histone H3 gene used for *in silico* PCR-RFLP analysis.

Species	GenBank accession numbers for histone H3 gene
Ilyonectria sp. 1	JF35610
Ilyonectria sp. 2	JF735611
I. alcacerensis	JF735630
I. novozelandica	JF735633
I. macrodidyma	JF735647, JF735648
I. torresensis	JF735681, AM419087
I. estremocensis	JF735617

Restriction endonuclease digestion

Restriction digestion *in silico* was carried out on representative sequences (Table 1) using a *Mnl*I/*Hinf*I system using DNAMAN™ (LynnonBiosoft version 5.0). Experimentally, amplimers of the histone H3 gene generated from the 40 "*C.*" *macrodidyma* isolates were digested with *Mnl*I and *Hinf*I (New England Biolabs). The reaction composition was as follows: (1) for *Mnl*I, 5 U of enzyme was added to 14 µl of PCR product in 1× CutSmart™ Buffer (New England Biolabs, Inc.) in a total volume of 50 µl and (2) for *Hinf*I, 10 U of enzyme was added to 14 µl of PCR product in 1× SuRE/Cut Buffer H (Roche, New Zealand) in a total volume of 50 µl. Digestions were carried out at 37ºC for 16 h and stopped by heating to 65ºC for 20 min as recommended by the manufacturer.

For each species, 20 µl of the restriction digest product was mixed with 5 µl of loading dye (40% [w/v] sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol). The first and last wells on the gel were loaded with 7 µl of 1 kB Plus DNA Ladder™ (0.1 ng/µl) (Invitrogen, New Zealand) and 3 µl of loading dye. DNA fragments were separated by gel electrophoresis in a 10% polyacrylamide gel (acrylamide to bis ratio 19:1; Biorad) in 1× TBE buffer under a constant voltage of 270 V for 2 h 30 min. Gels were stained by immersion in ethidium bromide (0.5 µg/ml) for 15 min and visualised under UV light. Resultant DNA banding patterns were visually analysed to determine restriction profiles.

RESULTS

Restriction analysis of representative sequences *in silico*

The *in silico* restriction patterns generated by *Mnl*I are shown in Figure 1 and fragment sizes in Table 2. Isolates belonging to the species *Ilyonectria* sp. 1 produced banding pattern A (10 bands: range 9-192 bp), *Ilyonectria* sp. 2 produced banding pattern B (8 bands: range 9-201 bp), *I. alcacerensis* produced banding pattern C (9 bands: range 9-99 bp) and *I. macrodidyma* produced banding pattern E (8 bands: range 9-136 bp). The banding patterns produced from *I. estremocensis* (D) (8 bands: range 9-133), *I. novozelandica* (F) (7 bands: range 23-137) and *I. torresensis* (G) (8/9 bands: range 9-137) were unable to be differentiated from one another as

Table 2 Restriction patterns of representative DNA sequences using restriction enzymes *Mnl*I and *Hinf*I applied to the histone H3 500 bp amplimer *in silico*.

| Species	*Mnl*I RFLP pattern	*Mnl*I Fragment size (bp)	*Hinf*I RFLP pattern	*Hinf*I Fragment size (bp)
Ilyonectria sp. 1	A	192, 62, 54, 46, 37, 33, 24, 23, 20, 9		No digestion
Ilyonectria sp. 2	B	201, 70, 61, 40, 36, 36, 24, 23, 9		No digestion
I. alcacerensis	C	99, 79, 68, 61, 56, 40, 31, 23, 9		No digestion
I. estremocensis	D	133, 127, 75, 62, 40, 31, 23, 9	J	187, 94, 85, 70, 55, 9
I. macrodidyma	E	136, 130, 68, 61, 33, 23, 23, 16, 9		No digestion
I. novozelandica	F	137, 130, 70, 68, 39, 32, 23	H	351, 84, 50, 9
I. torresensis	G	137, 99, 68, 61, 40, 32, 31, 23, 9 137, 130, 68, 61, 40, 32, 23, 9	I	161, 127, 84, 63, 56, 9

the restriction fragments were ≤4 bp different in size or were reliant on the presence/absence of a 9 bp fragment. Digestion of *I. estremocensis, I. novozelandica* or *I. torresensis* using the restriction endonuclease *Hinf*I produced distinctive banding patterns for these three species. *Ilyonectria estremocensis* produced banding pattern J (six bands: range 9-187 bp), *I. novozelandica* produced banding pattern H (four bands: range 9-351 bp) and *I. torresensis* produced banding pattern I (six bands: range 9-161 bp) (Figure 2 & Table 2).

PCR using primers CYLH3F and CYLH3R yielded a single amplimer of approximately 500 bp for each isolate. The PCR-RFLP rapid identification system using restriction enzymes *Mnl*I and *Hinf*I was applied to the collection of 40 New Zealand isolates. It was found that the collection comprised three species *I. macrodidyma* (58%; n=23), *I. novozelandica* (22%; n=9) and *I. torresensis* (20%; n=8). Isolates of *I. macrodidyma* produced banding pattern E when digested with *Mnl*I, while *I. novozelandica* and *I. torresensis* produced banding patterns F and G, respectively (Figure 3). Isolates belonging to *I. torresensis* produced the expected two variants in the banding pattern with *Mnl*I digestion. *Ilyonectria novozelandica* and *I. torresensis* isolates were then digested with *Hinf*I and produced banding patterns H and I (Figure 4).

DISCUSSION

The histone H3 gene has been shown to be useful in the distinction of the seven monophyletic species within the *I. macrodidyma* complex as described by Cabral et al. (2012b). In the present study a PCR-RFLP technique was developed using the 500 bp DNA fragment amplified from part of the histone H3 gene. PCR-RFLP analysis has previously been shown to be useful as a rapid method for taxonomic resolution of fungal plant pathogens, including closely
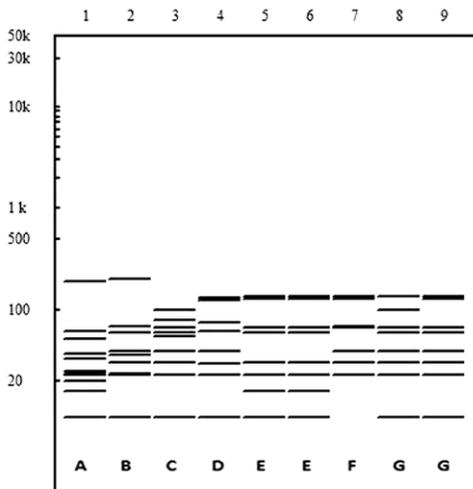

Figure 1 *In silico* analysis of international representative sequences using the restriction enzyme *Mnl*I. Lane 1: *Ilyonectria* sp. 1 (JF35610); Lane 2: *Ilyonectria* sp. 2 (JF735611); Lane 3: *I. alcacerensis* (JF735630); Lane 4: *I. estremocensis* (JF735617); Lanes 5-6: *I. macrodidyma* (JF735647, JF735648); Lane 7: *I. novozelandica* (JF735633); and Lanes 8-9: *I. torresensis* (JF735681, AM419087). The groups to which these species were allocated (A–G) are indicated at the bottom.
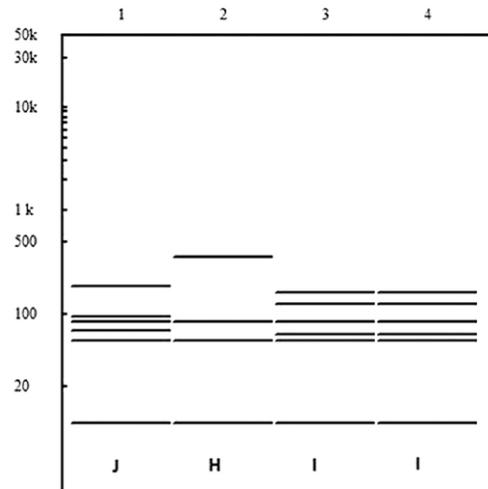
Figure 2 *In silico* analysis of international representative sequences using the restriction enzyme *Hinf*I. Lane 1: *I. estremocensis* (JF735617)*;* Lane 2: *I. novozelandica* (JF735633); and Lanes 3-4: *I. torresensis* (JF735681, AM419087). The groups to which these species were allocated (H–J) are indicated at the bottom.
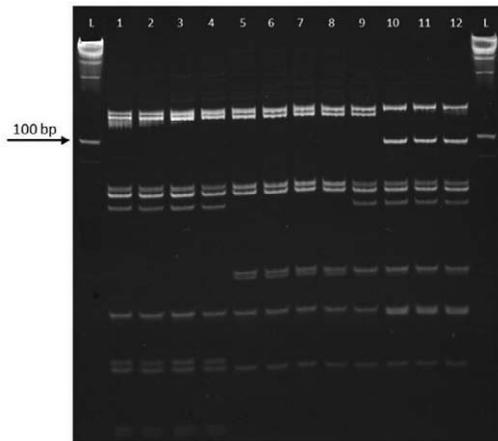
Figure 3 Histone H3 PCR products digested with *Mnl*I restriction enzyme visualised on a 10% polyacrylamide gel. Lanes L: 1 kB plus DNA ladder; Lanes 1-4: *I. macrodidyma* – Ack1c, Gis3d, Ack1a, Mar9c; Lanes 5-8: *I. novozelandica* – Ack2g, Co6c, Mar11f, Gis4a; and Lanes 9-12: *I. torresensis* – Ack2c, Hb2b, Wpa4a, Hb2c.
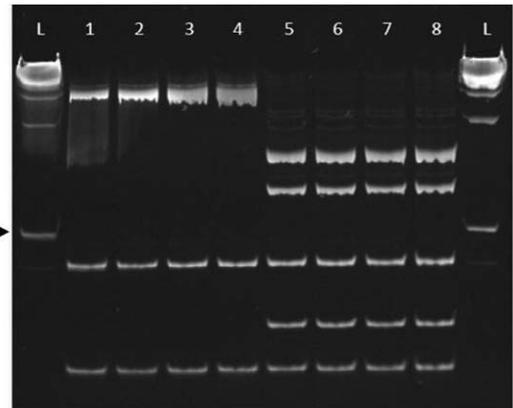
Figure 4 Histone H3 PCR products digested with *Hinf*I restriction enzyme visualised on a 10% polyacrylamide gel. Lanes L: 1 kB plus DNA ladder; Lanes 1-4: *I. novozelandica* – Ack2g, Co6c, Mar11f, Gis4a; and Lanes 5-8: *I. torresensis* – Ack2c, Hb2b, Wpa4, Hb2c.

related grapevine pathogens species such as *Botryosphaeria* species (Alves et al. 2005). However, PCR-RFLP has never been applied to the *I. macrodidyma* species complex.

The design of the PCR-RFLP procedure presented here involved analysis of 150 available restriction enzymes for the detection of variable regions in the histone H3 gene between the species within the *I. macrodidyma* species complex. The restriction enzyme, *Mnl*I was chosen as it was able to distinguish the greatest number of species in a single digest (four species). It was necessary to also use the restriction enzyme *Hinf*I as the banding patterns of *I. estremocensis, I. novozelandica* and *I. torresensis* following digestion with *Mnl*I could not be differentiated from one another due to insufficient resolution of restriction fragments that were ≤4 bp different in molecular weight. These enzymes were also chosen as they are commonly available and inexpensive, meaning that this technique is cost effective, especially in comparison to DNA sequencing. This rapid and inexpensive tool will facilitate large scale studies on the population composition of these pathogen species in vineyards and other crop

systems. As this technique involves sequence analysis it is reproducible and definitive for species identification and amenable to computer database analysis. However, this technique is unable to detect sequence differences at sites other than the *Mnl*I and *Hinf*I recognition sites resulting in its inability to detect other sequence variations.

The PCR-RFLP procedure designed in this study was able to differentiate between these cryptic species using restriction analysis of the histone H3 gene. This gene was chosen as it has been shown by Cabral et al. (2012b) to have the highest resolving power (4% percent diversity or 20 bp difference in nucleotide sequence). Other genes including the β-tubulin (0.8% diversity) and the internal transcribed spacers on both sides of the 5.8S nuclear ribosomal RNA gene (ITS) (0% diversity) are ineffective at resolving these species.

The *Mnl*I/*Hinf*I PCR-RFLP procedure presented here identified all seven of the *I. macrodidyma* complex species *in silico* and experimentally between isolates belonging to *I. macrodidyma, I. torresensis* and *I. novozelandica.* Intraspecific variability was detected in the

representative sequences of the international *I. torresensis* isolates *in silico* and in the New Zealand *I. torresensis* isolates experimentally, following digestion with the restriction endonuclease, *Mnl*I. Two distinctive patterns were produced for this species due to expected single nucleotide polymorphisms in the DNA sequence. However, despite this variation further digestion with *Hinf*I produced a single distinctive pattern for this species allowing for definitive identification.

The PCR-RFLP procedure presented here confirmed for the first time that three species belonging to the *I. macrodidyma* species complex are found in New Zealand. The most predominant species found in this collection of isolates was *I. macrodidyma,* with *I. novozelandica* and *I. torresensis* being present in approximately equal numbers.

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