Copyright Statement

The digital copy of this dissertation is protected by the Copyright Act 1994 (New Zealand).

This dissertation may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- you will use the copy only for the purposes of research or private study
- you will recognise the author's right to be identified as the author of the dissertation and due acknowledgement will be made to the author where appropriate
- you will obtain the author's permission before publishing any material from the dissertation.
Identification of *Ilyonectria* species associated with and
determining their role in Avocado decline

A Dissertation
submitted in partial fulfilment
of the requirements for the Degree of
Bachelor of Agricultural Science with Honours

at
Lincoln University
by
Melissa C Herder

Lincoln University
2014
Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Agricultural Science with Honours.

Identification of *Ilyonectria* species associated with and determining their role in Avocado decline

by

Melissa C Herder

Black root rot is a significant problem for grapevine growers worldwide. In recent years, black root rot pathogens *Ilyonectria* and *Calonectria* species have been isolated from avocado roots of trees showing signs of decline. In 2013, avocado roots from an orchard in Northland showed signs of decline and Dr Eirian Jones isolated thirteen *Ilyonectria* and *Calonectria* type isolates from these roots. The aim of this study was to identify these *Ilyonectria* and *Calonectria* type isolates using molecular and morphological tools and to determine the pathogenicity of these species on avocado roots.

MultiNgene sequence analysis using the histone H3 gene and βNtubulin gene was able to identify all thirteen isolates down to species level. These thirteen isolates were identified into six species; *I. macrodidyma*, *I. iiriodendri*, *I. liligena*, *I. robusta*, *I. europaea* and *C. pacifica*. Of these species *I. macrodidyma* belongs to the *I. macrodidyma* complex and *I. liligena*, *I. robusta* and *I. europaea* belong to the *I. radicicola* complex. In this study the most informative gene was histone H3 that had high bootstrap values for the phylogenetic tree. The least informative gene was βNtubulin, which was unable to resolve between *I. robusta* and *I. europaea*.

Morphological observations identified different colony colours, textures and macroconidia size between the different isolates. The colony diameter measured over 14 days allowed for analysis to differentiate between the growth patterns of the different species x identified through sequencing. The results of the analysis found significant results (P<0.01) for the majority of assessment days. Colony diameter data also helped to confirm the differences within the *I. liligena* isolates seen from the sequence data, where isolate LUPP2503 was clustered in a different group to the other four New Zealand *I. liligena* isolates and showed significantly smaller colony diameter. Colony characteristics were however different to that of published descriptions for many of the species.
The pathogenicity of one isolate representing each of the four *Ilyonectria* species and one *Calonectria pacifica* isolate could not be determined in this study using a detached root assay as lesions also developed on uninoculated control roots. Isolates characteristic of *C. pacifica* was only recovered from lesions which developed on *C. pacifica* inoculated Bounty® rootstocks indicating that it maybe pathogenic to avocado roots. Unfortunately cross-contamination by *Ilyonectria* likely occurred in the glasshouse resulting in *Ilyonectria* type colonies being isolated from the majority of replicates for not only the *Ilyonectria* inoculated roots, but also the uninoculated control for all three avocado rootstocks used. To determine the pathogenicity of *Ilyonectria* and *Calonectria* species on avocado roots, another detached root assay should be done using rootstocks that were not clean of *Ilyonectria* and *Calonectria* contamination.

**Keywords:** *Ilyonectria macrodidyma, I. robusta, I. europaea, I. liligena, I. liriodendri, Calonectria pacifica, multi-gene sequence analysis, morphological characteristics, pathogenicity.*
Acknowledgements

Firstly, I would like to express my sincere gratitude to my main supervisor Dr Eirian Jones for her guidance, patience and support during my research and study this year. She has always been available to give me assistance to develop my skills and confidence in carrying out research. I would also like to thank my co-supervisor Dr Hayley Ridgway for her expertise and guidance in a field that I know very little about. I am very honoured to have been given the opportunity to work with such talented, kind and caring supervisors for my Honours year.

I would like to say a huge thank you to Candice Barclay and Megan Outram for their support and helping me in my laboratory experiments. Without Megan, this project would have been a lot more challenging but her guidance and her continual support was very much appreciated.

A huge thank you to Lincoln University for the research funds to undertake my research this year.

I would also like to particularly thank my wonderful fiancé for his continual support through all the breakdowns when everything went wrong. Without him pushing me and helping me clear my mind I would not have been able to get this project completed. I would like to thank him for all his assistance and sacrifices he had to make so that I could focus on my study.

Finally, a very special thank you to my mum and dad for their continual support over the course of this year. Without the hours of phone calls home for support and help I would not have been able to get through this year. I also want to thank my parents for sacrificing things in their life to help fund my studies. Without this support I would not have been able to get through university.
4.1.1 Species identified from avocado samples in New Zealand ........................................... 43
4.1.2 MultiNgene sequence analysis ..................................................................................... 44
4.2 Morphological identification of Ilyonectria and Calonectria isolates ............................. 45
  4.2.1 Colony colour and macro conidia size ........................................................................ 45
  4.2.2 Colony diameter .......................................................................................................... 48
4.3 Pathogenicity of Ilyonectria and Calonectria isolates on avocado roots using a detached root assay .................................................................................................. 50

Appendix A .......................................................................................................................... 53
  A.1 List of isolates used in this study ................................................................................. 53
  A.2 0.9% Agarose gel picture with labelled band sizes of the molecular ladder used in this study 53

Appendix B .......................................................................................................................... 54
  B.1 Media Recipes .............................................................................................................. 54
  B.2 Solutions ...................................................................................................................... 54

Appendix C .......................................................................................................................... 55
  C.1 List of isolates used for the detached root assay .......................................................... 55

References .......................................................................................................................... 56
List of Tables

Table 2.1 Nucleotide sequences of the primer pairs used to amplify βZtubulin and histone H3 on both sides of the 5.8S nuclear ribosomal RNA gene (Cabral, Rego, et al., 2012) .......................................................... 14

Table 2.2 Representative DNA sequences of the βZtubulin and histone H3 genes used for phylogenetic analysis (Cabral, Groenewald, et al., 2012; Lombard et al., 2010a) .......................................................... 15

Table 3.1 Mean lesion length (and standard deviation) that developed on roots of three avocado rootstocks (Dusa®, Bounty® and Duke 7) inoculated with four Ilyonectria spp. and one Calonectria sp. six days after inoculation .................................................................................................................................. 39

Table 3.2 Isolation of Ilyonectria or Calonectria type colonies from root pieces taken from within and beyond the lesions on inoculated Dusa® avocado rootstock detached roots inoculated with Ilyonectria spp., Calonectria sp. isolates or untreated control. Data presented represents the ratio out of the 5 replicate roots assessed per treatment positive for Ilyonectria or Calonectria type colonies .............................................. 40

Table 3.3 Isolation of Ilyonectria or Calonectria type colonies from root pieces taken from within and beyond the lesions which develop on inoculated Bounty® avocado rootstock detached roots inoculated with Ilyonectria spp., Calonectria sp. isolates or untreated control. Data presented represents the ratio out of the 5 replicate roots assessed per treatment positive for Ilyonectria or Calonectria type colonies. Where roots were too short, ratios are out of the number of roots assessed per positive treatment for Ilyonectria or Calonectria type colonies for that measurement. ........................................................................... 41

Table 3.4 Isolation of Ilyonectria or Calonectria type colonies from root pieces taken from within and beyond the lesions which develop on inoculated Duke 7 avocado rootstock detached roots inoculated with Ilyonectria spp., Calonectria sp. isolates or untreated control. Data presented represents the ratio out of the 5 replicate roots assessed per treatment positive for Ilyonectria or Calonectria type colonies. Where roots were too short, ratios are out of the number of roots assessed per positive treatment for Ilyonectria or Calonectria type colonies for that measurement. ........................................................................... 42
List of Figures

Figure 2.1. Each of the thirteen isolates with four replicates arranged in a complete randomised design. ................................................................. 17

Figure 2.2. Image (a) one of the Dusa® rootstock varieties avocado tree. Image (b) the selected roots for the detached root assay for the Dusa® rootstock. ....................................................................................... 19

Figure 2.3 Image (a) Bounty® roots placed inside 1.7 mL tubes with a Parafilm™ cap. Image (b) 9 mL of sterile water being pipetted into a deep petri dish containing 30 grams of silica sand. Image (c) Avocado root placed in petri dish with a control mycelial plug at the apical end of the root. Image (d) detached root dishes arranged per rootstock in the incubator ........................................................................ 20

Figure 2.4. Image (a) surface sterilisation process; right tray contains 0.35% sodium hypochlorite; middle and left tray contain sterile water. Image (b) the arrangement of roots around Petri dish containing PDA for Koch Postulates ....................................................................................... 21

Figure 3.1 PCR products of the histone H3 genes of thirteen Ilyonectria and Calonectria spp. isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; C1, LUPP2480; C3, LUPP2482; C4, LUPP2483; C5, LUPP2484; C6, LUPP2485; C7, LUPP2486; C11, LUPP2490; C14, LUPP2493; C18, LUPP2497; C19, LUPP2498; C22, LUPP2501; C24, LUPP2503; C26, LUPP2505; L, 1 kB PLUS DNA marker ........................................................................................... 22

Figure 3.2 PCR products of the B2tubulin genes of thirteen Ilyonectria or Calonectria spp isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; C1, LUPP2480; C3, LUPP2482; C4, LUPP2483; C5, LUPP2484; C6, LUPP2485; C7, LUPP2486; C11, LUPP2490; C14, LUPP2493; C18, LUPP2497; C19, LUPP2498; C22, LUPP2501; C24, LUPP2503; C26, LUPP2505; L, 1 kB PLUS DNA marker ........................................................................................... 23

Figure 3.3 PCR products of the histone H3 and B2tubulin genes of two Ilyonectria or Calonectria spp isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; HC26, histone H3 LUPP2505; HC19, histone H3 LUPP2498 (positive control); B, Negative control; BC26, B2tubulin LUPP2505; BC19, B2 tubulin LUPP2498 (positive control) ....................................................................................... 23

Figure 3.4 The neighbouring joining tree with bootstrap values using 1000 replicates generated in MEGA 5.1 using the Histone H3 gene sequences of thirteen isolates characteristic of Ilyonectria and Calonectria species isolated from New Zealand avocado trees and from five Ilyonectria spp. and one Calonectria sp. sourced from GenBank with accession number ....................................................................................... 25

Figure 3.5 The neighbouring joining tree with bootstrap values using 1000 replicates generated in MEGA 5.1 using the βZtubulin gene sequences of thirteen isolates characteristic of Ilyonectria and Calonectria species isolated from New Zealand avocado trees and from five Ilyonectria spp. and one Calonectria sp. sourced from GenBank with accession number ....................................................................................... 25

Figure 3.6 Colony morphology of isolate LUPP2480 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 26

Figure 3.7 Colony morphology of isolate LUPP2482 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 26

Figure 3.8 Colony morphology of isolate LUPP2483 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 27

Figure 3.9 Colony morphology of isolate LUPP2484 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 27

Figure 3.10 Colony morphology of isolate LUPP2485 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 28

Figure 3.11 Colony morphology of isolate LUPP2486 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 28

Figure 3.12 Colony morphology of isolate LUPP2490 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 28

Figure 3.13 Colony morphology of isolate LUPP2493 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm ........................................................................................... 29
Figure 3.14 Colony morphology of isolate LUPP2497 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Figure 3.15 Colony morphology of isolate LUPP2498 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Figure 3.16 Colony morphology of isolate LUPP2501 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Figure 3.17 Colony morphology of isolate LUPP2503 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Figure 3.18 Colony morphology of isolate LUPP2505 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Figure 3.19 Average colony diameter size (mm) for each of the thirteen isolates, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at P<0.05 at each assessment time.

Figure 3.20 Average colony diameter size (mm) for each of the six species, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at P<0.05 at each assessment time.

Figure 3.21 Average colony diameter size (mm) for each of the five isolates of the Ilyonectria liligena species, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at P<0.05 at each assessment time.

Figure 3.22 Average colony diameter size (mm) for each of the two isolates of the Ilyonectria europaea species, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at P<0.05 at each assessment time.

Figure 3.23 Average colony diameter size (mm) for each of the three isolates of the Ilyonectria liriodendri species, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at P<0.05 at each assessment time.
Chapter 1
Introduction and Literature Review

1.1 History and the uses of avocado (*Persea americana*)

The *Lauraceae* family is made up of 50 genera, one of which is *Persea* (Sampson, 2014). Within the *Persea* genus there are 150 species the most well-known one being *americana* (avocado) (Compendium, 2014). *Persea americana* is the only member of the *Lauraceae* family that produces edible fruit, with the family also including *Cinnamomum spp.* (cinnamon) and *Laurus nobilis* (bay trees) (Newcomb, 2000; Sampson, 2014). Although the distribution of avocado is through the tropical and temperate regions of Asia, Africa, North America, Central America and Caribbean, South America, Europe and Oceania, it originates from the eastern and central highlands of Mexico through Guatemala to the Pacific coast of Central America (Compendium, 2014; Inc., 2014). The avocado has been used in Mexico for 10,000 years and was first reported in 1519 by Martin Fernandez De Encisco (Inc., 2014; Whiley, Schaffer, & Wolstenholme, 2002).

Avocado was used by European sailors in the 1700s as a spread for biscuits, giving it the name ‘midshipman’s butter’ (Inc., 2014). One of the first cultivars was Fuerte which was the basis to the Californian avocado industry (Whiley et al., 2002). Fuerte was followed up later by the Hass variety in the late 1920’s discovered by Mr Rudolph Hass (Inc., 2014; Whiley et al., 2002). These two cultivars have become widely used in commercial avocado growing with Hass being the most popular choice for avocado producers (Inc., 2014; Whiley et al., 2002). The most common use of the avocado fruit is being eaten fresh by itself, in cold salads or ice cream for example (Avocado, 2014b; Whiley et al., 2002). As the avocado fruit has a good nutritional value being high in potassium and healthy monounsaturated fats it is recommended to be included as part of a healthy diet (Avocado, 2014b; Whiley et al., 2002). In New Zealand avocado oil is also made from the downgraded fruit not suitable for export. The flesh of the avocado fruit can also be used as an eye treatment, moisturiser, face mask and a hair conditioner (Avocado, 2014b). The Amazonians also used avocado fruit as a treatment for gout (Whiley et al., 2002).

1.2 Avocado industry in New Zealand

Avocados are thought to have been introduced into the Gisborne area of New Zealand around 1920 by Charles Grey (Avocado, 2014a). In 1926, Charles and his son Len together grew many avocado seedlings and first marketed good quality avocados from these seedlings in 1939 (Avocado, 2014a). In the 1940s Charles and Len imported avocado varieties from California and in 1965 started a
commercial orchard with around 600 trees (Avocado, 2014a) These avocado trees included many varieties including Hass, Fuerte, Nabal and Zutano (Avocado, 2014a). Since then production of avocados has spread through the warmer regions of New Zealand to the Bay of Plenty, Whangarei and the Far North (Authority, 2012).

In New Zealand there are 1600 growers that supply both local and export markets with top quality avocados (Aitken & Hewett, 2013; Authority, 2012). In 2012, the New Zealand avocado industry was worth 63 million dollars, producing 33,997 tonnes for export and local markets (Aitken & Hewett, 2013; Authority, 2012). Of the varieties available Hass is the most suitable for export in New Zealand as consumers globally identify it as the best quality avocado available (Barber et al., 2004). Other varieties that may be grown in New Zealand are Fuerte, Reed or Zutano, however their ability to commercially produce good quality fruit is low (Barber et al., 2004).

1.3 Avocado rootstock varieties

Grafting fruiting scion to seedling or colonial rootstocks is a necessary practice to have a fully producing avocado tree. This is because it difficult to get roots to develop on shoot cuttings (Witney, 2002). Grafting is carried out to give the best shoot and root combination for production that would take a very long time to achieve if it was done by conventional breeding (Witney, 2002). In New Zealand most fruiting scions are grafted onto seedling rootstocks (Zutano) (Barber et al., 2004; Nursery, 2014). Seedling rootstocks are used for grafting over clonal rootstocks (Duke 7, Bounty® or Dusa®) because the propagating technique is more difficult for clonal rootstocks (Barber et al., 2004; Nursery, 2014). Studies in California have shown that clonal rootstocks can have higher production than seedling rootstocks, however in New Zealand selection of rootstock should be based on the soil type of the orchard (Barber et al., 2004). Some clonal rootstocks for example Dusa® and Bounty® have a medium or high tolerance to the damaging Phytophthora root rot that often occurs in avocado production (Limited, 2014).

The scions that are used for grafting to produce fruiting trees are commonly Hass and Reed (Nursery, 2014). Pollinating scions are also grafted to rootstocks; these are Bacon, Zutano and Fuerte. Fuerte can also be used as a fruiting scion (Nursery, 2014). Care must be taken with young rootstocks as no rootstock is able to withstand being in waterlogged soils for extended periods of time (Limited, 2014).

1.4 Avocado root diseases

In New Zealand the biggest disease facing avocado growers is Phytophthora cinnamomi (avocado root rot). Phytophthora cinnamomi is a problem for most avocado producing countries e.g. Australia, Mexico, South Africa, USA (Whiley et al., 2002). There are over 1000 host plants for P. cinnamomi
including pineapple and kiwifruit (Whiley et al., 2002). Trees that are infected with *P. cinnamomi* show gradual decline in growth due to the death of the feeder roots which reduces water intake (Barber et al., 2004; Whiley et al., 2002). The disease is hard to identify at first as the feeder roots are affected by going black and brittle, but as the infection spreads the leaves reduce in size, become a pale green to yellow colour and eventually wilt and fall off (Barber et al., 2004; Whiley et al., 2002). Trees that are most susceptible are those younger than five years and older than fifteen (Barber et al., 2004). The most common source of *P. cinnamomi* is from nursery rootstocks, so ensuring clean planting material can reduce the incidence in the avocado orchard (Whiley et al., 2002).

### 1.5 Black root rot

Black root rot of avocado is a very recent disease that has been isolated from avocado roots in South Africa, Israel, Italy, Australia, Spain and Chile (Besoain & Piontelli, 1999; E. Dann et al., 2011; E. K. Dann et al., 2012; Vitale et al., 2012; Zilberstein et al., 2007). In New Zealand, black root rot has been reported on grapevines but to date has not been reported on avocado. Black root rot on avocado has been reported to occur in young trees less than 4 years old in the nursery (Besoain & Piontelli, 1999; E. K. Dann et al., 2012; Vitale et al., 2012; Zilberstein et al., 2007). In avocado, black root rot has been reported to be associated with the fungi *Calonectria ilicicola* (E. Dann et al., 2011), *Ilyonectria macrodidyma* (Vitale et al., 2012) and *Ilyonectria radicicola* (Zilberstein et al., 2007).

On grapevines black root rot is a serious disease in young vineyards and nurseries and some mature vines throughout the world (AgustiNBrisach & Armengol, 2013; Halleen, Schroers, Groenewald, & Crous, 2004; Pedro, Cabral, Nascimento, Oliveira, & Rego, 2013). This disease has spread throughout the major grape growing regions of the world after first being discovered in 1961 on grapevines in France (Halleen, Schroers, et al., 2006; Petit & Gubler, 2005). In recent years, incidence of black root rot has increased and in New Zealand grapevine plant losses are estimated to be between 20% and 40% (C. M. Probst, 2011). These plant losses from black root rot is worth approximately $25 million and loss yield from mother vines is thought to be worth approximately $3 million annually (C. M. Probst, 2011). The occurrence of black root rot in New Zealand grapevine regions is relatively low, however in Marlborough the incidence of black root rot is high (Outram, 2013). It is thought the infection of black root rot may have arisen from those grapevines planted previously on apple orchards infected with the disease (Outram, 2013).

#### 1.5.1 Taxonomy of “*Cylindrocarpon*” and “*Calonectria*” species

The genus “*Cylindrocarpon*” was introduced by Wollenweber in 1913 for anamorphs that belonged to the *Nectria* section *Willkommiotes* Wollenw (AgustiNBrisach & Armengol, 2013; Cabral, Groenewald, Rego, Oliveira, & Crous, 2012). In this section only species without chlamydospores were included,
but in 1917 Wollenweber expanded the section to include species that produced chlamydospores, such as *C. destructans* (AgustiNBrisach & Armengol, 2013). In 1966, the *Cylindrocarpon* genus was divided up into four groups based on the presence or absence of micro conidia or chlamydospores (Booth, 1966). Group one was “*Cylindrocarpon*” *magnusianum* (Sacc.) Wollenw., which was the anamorph of *Neonectria rumulariae* (no micro conidia, mycelial chlamydospores present) (AgustiNBrisach & Armengol, 2013; D Brayford, 1993; Cabral, Rego, et al., 2012). The second group includes *Cylindrocarpon cylindroides* that is the type species of the genus *Cylindrocarpon* (micro conidia present, mycelial chlamydospores not present (AgustiNBrisach & Armengol, 2013; D Brayford, 1993; Cabral, Rego, et al., 2012). Group three includes those species of “*Cylindrocarpon*” that are connected to telemorphs of the ‘*Nectria*’ *mammoidea* group (no micro conidia and mycelial chlamydospores) (AgustiNBrisach & Armengol, 2013; D Brayford, 1993; Cabral, Rego, et al., 2012).

Lastly group four includes *Cylindrocarpon destructans*, which was the anamorph of *Neonectria radicicola* (micro conidia and mycelial chlamydospores present) (AgustiNBrisach & Armengol, 2013; D Brayford, 1993; Cabral, Rego, et al., 2012).

Traditionally, species that represented groups of *Nectria* with “*Cylindrocarpon*” anamorphs were redefined as *Neonectria* (AgustiNBrisach & Armengol, 2013; D. Brayford, Honda, Mantiri, & Samuels, 2004; Halleen et al., 2004). Redefining *Neonectria* was due to the broad definition of *Nectria*, and that this genus could be separated into several teleomorphic genera verified by anamorphic, phylogenetic, and ecological character patterns (Halleen et al., 2004). For some of the species in the *Neonectria/“Cylindrocarpon”* Mantiri *et al.* (2001) and Brayford *et al.* (2004) analysed mitochondrial small subunit ribosomal DNA sequence data and found that these grouped species were monophyletic. Mantiri *et al.* (2001) and Brayford *et al.* (2004) however, also found that *Neonectria/“Cylindrocarpon”* included distant sub clades matching to three of the four groups defined by Booth (1966).

Regardless of the work by Mantiri *et al.* (2001) , Brayford *et al.* (2004) and Booth (1966), defining the taxonomy of “*Cylindrocarpon”/Neonectria” has been difficult as there is considerable variation in cultural and morphological characters from isolates within the same species collected from grapevines in nurseries and vineyards (AgustiNBrisach & Armengol, 2013; Halleen et al., 2004). Through morphological and phylogenetic identification it has been found that the main species that cause black root rot are “*C*. destructans, “*C*. liriiodendri , “*C*. macrodidymum , “*C*. obtusisporum and “*C*. pauciseptatium” (AgustiNBrisach & Armengol, 2013; Halleen et al., 2004; Halleen, Schroers, et al., 2006).

Recently a phylogenetic study by Chaverri *et al.* (2011) of *Neonectria, “Cylindrocarpon”* and related genera with “*Cylindrocarpon”Nlike anamorphs, were found to not form a monophyletic group. This
result proposes that *Neonectria*/*Cylindrocarpon* signify more than one genus (AgustiNBrisach & Armengol, 2013; Chaverri et al., 2011). Based on a combination of characters linked to perithecial anatomy and conidial septation *Neonectria* was split into four genera: *Neonectria/Cylindrocarpon sensu stricto* (Booth’s groups 1 and 4), *Rugonectria, Thelonectria* (group 2) and *Ilyonectria* (group 3) (Chaverri et al., 2011). According to this, only *Neonectria* has “*Cylindrocarpon*” anamorphs, whereas the remaining genera have “*Cylindrocarpon*”-like anamorphs (AgustiNBrisach & Armengol, 2013; Cabral, Rego, et al., 2012; Chaverri et al., 2011). This reclassification led to “C”. *liriodendri* (*I. liriodendri*) and “C”. *macrodidymum* (*I. macrodidymum*) being included into the *Ilyonectria* genus, with *I. radicicola* (previously “C”. *destructans*) as the type species (AgustiNBrisach & Armengol, 2013; Chaverri et al., 2011). Taxonomic studies by Cabral *et al.* (2012) using multigene analysis found twelve new taxa in the *I. radicicola* complex. These are *Ilyonectria* *anthuriicola*, *I. cyclaminicola*, *I. europaea*, *I. gamsii*, *I. liligena*, *I. lusitanica*, *I. panacis*, *I. pseudodestructans*, *I. robusta*, *I. rufa*, *I. venezuelensis* and *I. vitis* (Cabral, Groenewald, et al., 2012).

The genus *Calonectria* (Ca.) was first described in 1867 by De Notaris with *Ca. daldiniana* as the type (Lombard, Crous, Wingfield, & Wingfield, 2010a, 2010b). *Calonectria* genra is part of the *Nectriaceae* family restricted as having uniloculate ascomata that are orange to purple and not immersed in well developed stromata (Lombard et al., 2010b). In the *Nectriaceae* family there are twenty genra in which *Calonectria* is clearly characterised from the other genra by its *Cylindrocladium* anamorphs (Lombard et al., 2010b). Morphologically the anamorph *Cylindrocladium* offers the largest number of unique characters for *Calonectria* identification particularly vesicle shape, stipe extension length and conidial septation (Lombard et al., 2010a).

In 1950, the first monograph of *Cylindrocladium* was done by Boedijn and Reitsma (1950). They presented seven *Cylindrocladium* species with one *Calonectria* connection (Boedijn & Reitsma, 1950). In a monograph by Peerally (1991) documented ten *Calonectria* species with their *Cylindrocladium* anamorphs, as well as sixteen other *Cylindrocladium* anamorphs not associated with a telemorph. A monograph by Crous and Wingfield (1994) further established the importance of *Cylindrocladium* anamorph traits in the identification of *Calonectria* species. In this monograph they identified twenty two *Cylindrocladium* species that are associated with sixteen *Calonectria* species (Crous & Wingfield, 1994). In 2002, Crous further identified twenty eight *Calonectria* species all of which were related to *Cylindrocladium* anamorphs (Crous, 2002). On top of this an extra eighteen *Cylindrocladium* species were identified that had unknown telemorphs (Crous, 2002). As of 2010, there were thirty seven *Calonectria* and fifty two *Cylindrocladium* species that have been identified (Lombard et al., 2010a, 2010b). So that there is a single nomenclature for pleomorphic fungi, the telemorph name *Calonectria* takes superiority over the anamorph name *Cylindrocladium* when both types belong to
the same helomorph (Lombard et al., 2010a). Those *Cylindrocladium* species that do not have a *Calonectria* telemorph are also transferred to *Calonectria* (Lombard et al., 2010a).

### 1.5.1 Symptoms of black root rot

In avocado plants grown in Australia, Israel and Chile it has been reported that black root rot has caused tree decline and death in young trees (E. K. Dann et al., 2012). These avocado trees show wilting, leaf chlorosis, leaf drop and necrosis (E. K. Dann et al., 2012). In Italy, where it was first reported that *Ilyonectria macrodidyma* caused black root rot on avocado it was found to be on young nursery plants (Vitale et al., 2012). These young trees were 6 months old and 4 years old and had dark black discolouration on the roots (Vitale et al., 2012). With time the root rot caused the leaves to go yellow and develop necrosis, with the plants then wilting before dying (Vitale et al., 2012).

In grapevine, plants that have black root rot show signs of being stressed or have stunted growth (Jaspers, 2013). Typical external symptoms in grapevine are slow growth, reduced vigour, shortened internodes, chlorosis and necrosis of the leaves, sparse foliage, thin trunks and small leaf size (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, & Crous, 2006; C. M. Probst, 2011). Once infected with black root rot the grapevine will start to wilt and in young trees death will occur very quickly. The older the grapevine is the slower the infection will spread and vine death can take up to a year (AgustiNBrisach & Armengol, 2013). Underground symptoms are decline in root biomass and the root hairs will have sunken necrotic root lesions (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; C. M. Probst, 2011). The pith of the grapevine will show brown to black discolouration and will be compacted (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; C. M. Probst, 2011). When the roots are cut open the xylem vessels may have gum and fungal tissue blocking them (Halleen, Fourie, et al., 2006; Pathrose, 2012; C. M. Probst, 2011). It may also be noticed when the soil around the roots is removed that there is a secondary crown formed with roots growing parallel to the soil surface (AgustiNBrisach & Armengol, 2013; Pathrose, 2012; C. M. Probst, 2011). As very little research has been carried out on the symptoms of black root rot in the avocado orchard, it would be predicted that symptoms would be similar to grapevine.

### 1.5.1 Disease cycle

The disease cycle of black root rot in avocado has not yet been studied. However, in a study by Vitale *et al* (2012) it was found that *Ilyonectria macrodidyma* produced macro conidia, chlamydospores and mentioned that it could be a saprobe. Black root rot generally occurs in the early stage of the crop cycle when the plants are young. This is likely due to the main source of contamination being from nursery plants. Black root rot tends to quickly kill young trees as it blocks the xylem tissue (Outram, 2013). In some cases older avocado trees may be affected if the conditions change and dormant
Chlamydospores start to germinate in infected soils. In grapevines the disease cycle of black root rot causing pathogens *Cylindrocarpon* and *Ilyonectria* species is not clearly understood as of present (Petit, Barriault, Baumgartner, Wilcox, & Rolshausen, 2011).

*Cylindrocarpon* and *Ilyonectria* spp. are thought to survive in soil as chlamydospores for long periods of time after previously infected crops have been removed (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Jaspers, 2013; Outram, 2013; Pathrose, 2012; Petit et al., 2011; C. M. Probst, 2011). Mycelium and macroconidia are also present in infected soils but under acid and alkaline conditions cannot survive and production of chlamydospores is started (Outram, 2013; C. M. Probst, 2011). Chlamydospores in soil remain dormant until germination is initiated by plant exudates or are broken down by other microorganisms (C. M. Probst, 2011). It is thought that *Cylindrocarpon* and *Ilyonectria* spp. are saprobes and feed on dead plant material when hosts are not present (AgustiNBrisach & Armengol, 2013; Halleen et al., 2004; Outram, 2013; C. M. Probst, 2011). It has been found in New Zealand that *Cylindrocarpon* species that were found in apple orchards are similar to species found in vineyards (C. M. Probst, 2011). It is thought that grapevines that have been planted in past infected apple orchards have been infected by the chlamydospores that have remained dormant in the soil over extended periods of time (AgustiNBrisach & Armengol, 2012; Bleach, Jones, Ridgway, & Jaspers, 2013; Jaspers, 2013; Outram, 2013; Pathrose, 2012; C. Probst, Jones, Ridgway, & Jaspers, 2012; C. M. Probst, 2011). *Cylindrocarpon* and *Ilyonectria* spp. are also very prevalent in nursery soils (AgustiNBrisach & Armengol, 2013; AgustiNBrisach, Gramaje, GarciaN Jimenez, & Armengol, 2013; Bleach et al., 2013; Outram, 2013). In South Africa it was reported that more than 50% of grafted nursery vines showed signs of infection after 7 months (Bleach et al., 2013). It has also been said that rootstocks kept in propagation material before planting rarely had infection from *Cylindrocarpon* and *Ilyonectria* spp., but once planted in open rooted nursery soil signs of infection occurred (AgustiNBrisach & Armengol, 2013).

The main form of black root rot dispersal is via water as it is soilborne. Splash dispersal of conidia by rainfall or irrigation can lead to infection of the lower parts of the trunk (Jaspers, 2013; C. M. Probst, 2011). Spores can also be dispersed in free water that spreads conidia around the vineyard as the water drains away causing plant to plant infection (Jaspers, 2013; Outram, 2013; Petit et al., 2011; C. M. Probst, 2011). One other method of dispersal is via hydration tanks. If the water sourced for watering the vines is contaminated with black root rot then the conidia will be spread through the water to healthy vines (AgustiNBrisach et al., 2013). *Cylindrocarpon* and *Ilyonectria* spp. can also be dispersed through human activities such as using scissors on an infected vine and not disinfecting them before using them on a healthy vine (AgustiNBrisach et al., 2013; Outram, 2013; C. M. Probst, 2011). Using machinery in the grafting process or in the removal of infected vines and then not cleaning it before using again can spread the pathogen to healthy vines (AgustiNBrisach et al., 2013;
Outram, 2013; C. M. Probst, 2011). Moving contaminated soil and soil water to uninfected areas will also disperse black root rot pathogens (Jaspers, 2013).

Infection of healthy vines can occur by planting in soil that is contaminated with *Cylindrocarpon* and *Ilyonectria* spp. (Bleach et al., 2013; Jaspers, 2013). If the vines have any natural openings or wound in the root then the pathogen present in the soil will infect the plant (AgustiNBrisach & Armengol, 2013; AgustiNBrisach et al., 2013; Bleach et al., 2013; Halleen, Fourie, et al., 2006; Jaspers, 2013; C. M. Probst, 2011). Wounds can be caused by disbudding, young callus roots breaking, an opening at the nonNcallused part of the trunk/stem base, in the canes or by insects or birds (AgustiNBrisach & Armengol, 2013; AgustiNBrisach et al., 2013; Bleach et al., 2013; Halleen, Fourie, et al., 2006; Jaspers, 2013; C. M. Probst, 2011). A major form of black root rot infection is from nursery soil in newly grafted vines (AgustiNBrisach & Armengol, 2013; AgustiNBrisach et al., 2013; Bleach et al., 2013; Halleen, Fourie, et al., 2006; Outram, 2013). The basal ends of the cuttings can come into contact with contaminated soil during nursery practices (AgustiNBrisach & Armengol, 2013; AgustiNBrisach et al., 2013; Bleach et al., 2013; Halleen, Fourie, et al., 2006; Outram, 2013). As it is hard to remove the pathogen in the soil in nurseries the pathogen numbers build up in soil increasing the likelihood of infection (Halleen, Fourie, & Crous, 2007; Outram, 2013; C. Probst et al., 2012). A final way of infection by black root rot is through root exudates interacting with the chlamydospores allowing them to germinate and produce hyphae and grows towards the roots of healthy plants (C. M. Probst, 2011).

### 1.5.2 Factors that influence black root rot infection

The development of black root rot is reliant on an initial inoculum source. In avocado, studies have shown that severe outbreaks that occur in young plants connect back to infection in the nursery from a build up of pathogens in the soil (E. K. Dann et al., 2012; Vitale et al., 2012; Zilberstein et al., 2007). Infection can also occur from planting in previously infected apple orchards as mentioned in the disease cycle (Bleach et al., 2013). When root exudates of a host of black root rot are produced then the chlamydospores in soil will germinate and produce hyphae that enter into wounds and natural openings in the feeder roots of avocado trees and grapevines breaking down the cortex cells (Halleen, Fourie, et al., 2006; C. M. Probst, 2011).

Any stress that the plant is under can increase the pathogenesis of *Cylindrocarpon* and *Ilyonectria* species increasing their development and prevalence in the soil. Host stress can make the plant vulnerable to infection by *Cylindrocarpon* and *Ilyonectria* spp. (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007). If the soil is lacking in essential plant elements then the plant could suffer from malnutrition and show signs of deficiency making the plant susceptible to infection by black root rot pathogens (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006;
If the site of the orchard or vineyard has bad soil drainage causing waterlogged soils, it reduces the oxygen present in the soils and puts the trees and vines under stress (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). Soil compaction also reduces the amount of oxygen in the soil and reduces water flow increasing plant stress (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). Young plants also cannot have too much fruit on them, as they will become stressed (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). Plants need to be planted in well prepared soil with large plant holes to ensure that the soil is easy for the roots to penetrate and access water and nutrients so they do not become stressed (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011).

Environmental factors can influence the pathogenesis of *Cylindrocarpon* and *Ilyonectria* spp. (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). Soil texture and structure have a huge influence on how easily compacted the soil can get, reducing the ability of roots to penetrate soil and develop a healthy root system (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). High temperatures during summer result in increased transpiration rate from the plant (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). If the plant is infected with black root rot then the damage to the vascular system will prevent the plant from taking up water from the soil to make up for that lost from the leaves (AgustiNBrisach & Armengol, 2013; Halleen, Fourie, et al., 2006; Halleen et al., 2007; Outram, 2013; Pathrose, 2012; C. Probst et al., 2012; C. M. Probst, 2011). The ideal temperature for maximum growth is 20 to 25°C (AgustiNBrisach & Armengol, 2012). Growth of mycelium and conidia is optimum at pH 7.0 but can grow in a pH range from 4.0 to 8.0 (AgustiNBrisach & Armengol, 2012). Pests such as insects, birds or rodents are able to make wounds in the bark or root tissue that gives the black root rot pathogen a place to access the vascular system and increase the infection area (C. M. Probst, 2011).

### 1.5.3 Identification based on morphology

The identification of the causal agents of black root rot has traditionally been carried out based on morphological characteristics until the development of molecular identification in the 1990’s (Lombard et al., 2010b). These characteristics include colony texture, colour, density and zonation.
when grown on potato dextrose agar (PDA) as well as the size, shape and number of septate of conidia produced (Cabral, Groenewald, et al., 2012). The colour of the surface and reverse sides of colonies are in most cases determined using a mycological colour chart (Rayner, 1970). Using morphological identification can be unreliable due to variation in colony characteristics from different exposure to light, oxygen and nutrients (Petit & Gubler, 2005). Since the development of molecular tools for identification in the 1990’s, morphological identification is no longer relied on for identification of a species. In most cases morphological characteristics are a minor part of the experimental design but is used for the description of taxonomy.

Morphological characteristics of black root rot causing *Ilyonectria radicicola* complex include a cottony/felty colony texture with aerial mycelium in the centre or all over the entire colony when grown on PDA (Pathrose, 2012). The colour varies from white to cinnamon on the surface and reddish-brown to beige from the centre outward (Pathrose, 2012). Macroconidia are 1 to 3 septate, and are a cylindrical shape with curved ends. The 1 septate macroconidia measure 23.1N29.2 μm x 5.2N6.2 μm and the 2 to 3 septate macroconidium measure 34.5N40.03 μm x 6.8N8.0 μm (Booth, 1966). There are microconidia present (Booth, 1966). For *I. macrodidyma* the colony can be identified by a yellowish and felty surface and with plentiful aerial mycelium covering the whole colony (Pathrose, 2012). The macroconidia are 1 to 3 septate, the 1 septate macroconidia measuring 14N27 x 4N6 μm (Halleen et al., 2004). Those macroconidia that are 2 septate measure 26N35 x 5.7.5 μm and the 3 septate measure 31N41 x 6N8 μm (Halleen et al., 2004). There are microconidia present (Pathrose, 2012). Black root rot causing species *I. liriodendri* has a cinnamon to sepia coloured colony with sparse aerial mycelium (Pathrose, 2012). The macroconidia are straight and slightly curved at the ends with three septate (Pathrose, 2012). Macroconidia have an average size of 35N40 x 5.5N6 μm and no microconidia are produced (Pathrose, 2012). Black root rot caused by *Calonectria pacifica* when grown on PDA is luteous to sienna in colour on the surface of the colony (Kang, Crous, & Schoch, 2001). The macroconidia measure (40N) 55N68 (N75) x 4N5 μm, one septate and have no microconidia (Kang et al., 2001).

1.5.4 Identification using molecular tools

Molecular biology provides a key tool for the identification (Papломатас, 2006), detection, and quantification of plant pathogens (Papломатас, 2006). This is because only small amounts of DNA are required to carry out the techniques and this can be gathered from any life stage of the pathogen. Through the process of polymerase chain reaction (PCR) a specific region of DNA can be quickly amplified through repeated cycles of denaturation, annealing and extension that simulate the natural process of DNA synthesis (McCartney, Foster, Fraaije, & Ward, 2003; Outram, 2013).
At species level fungi can be identified from PCR products generated by primers that have been
designed to recognise conserved sequences such as the nuclear and mitochondrial rRNA cluster that
contain numerous copies per genome (Papomatas, 2006). In particular the internal transcribed
spacers (ITS) have been used frequently to identify fungi to species level (Papomatas, 2006). Protein
encoding genes can also be used as another option, for example, RNA polymerases (RPB1 and RPB2),
βNtubulin, γNactin, ATP synthase (ATP6), and elongation factor EFN1α (TEF1α) (Schmitt et al., 2009).

Molecular techniques have been important for identifying black root rot pathogens (AgustiNBrisach et
al., 2013). Halleen et al., (2004) compared the Neonectria/Cylindrocarpon taxa using large subunit
rDNA, ITS and partial βNtubulin gene to identify the different groups within the taxa. In 2007,
Dubrovsky and Fabritius developed PCR primers that were specific to “Cylindrocarpon” species and
were able to identify “C”.liriodendri and “C”.macrodidymum (Dubrovsky & Fabritius, 2007). In the
same year it was published by Alaniz et al., (2007) that a conserved 52 base pair insertion from the βN
tubulin gene was able to distinguish “C”. macrodidymum and “C”. liriodendri.

Recently, Cabral, Groenewald, et al., (2012) used a multiNgene approach with the partial βNtubulin,
histone H3, translation elongation factor 1Nα and nuclear ribosomal RNAinternal transcribed spacer
(nrRNANITS) to identify Ilyonectria radicicola and “CylindrocarponNlike anamorph species complex. It
was found that nrRNANITS sequences were the least informative, where as the histone H3 sequences
were the most informative at identifying taxa (Cabral, Groenewald, et al., 2012). This multiNgene
analysis resulted in the identification of twelve new taxa within the I. radicicola complex (Cabral,
Ilyonectria species were distinguished using the same multiNgene approach . As with the I. radicicola
complex it was found that the histone H3 gene sequences was the most informative at identifying
taxa (Cabral, Rego, et al., 2012). This multiNgene analysis of novel Ilyonectria species led to the
discovery of six monophyletic species within the I. macrodidyma complex (Cabral, Rego, et al., 2012).

Molecular techniques have also been important for the identification of Calonectria species and its
Cylindrocladium anamorphs (Lombard et al., 2010b). In the first study carried out using 5.8S
ribosomal RNA gene and internally transcribed spacers, the sequences produced were unable to
distinguish between Cy. scoparium and Cy. floridanum (Jeng, Dumas, Liu, Wang, & Hubbes, 1997). It
was found later that this gene region contains few informative characters (Lombard et al., 2010b;
Schoch, Crous, Wingfield, & Wingfield, 2001). To identify the differences between species Schoch et
Schoch et al., (2001) completed the first DNA sequence based phylogenetic study on Cylindrocladium
using the partial βNtubulin gene sequences. The ITS, βNtubulin and histone H3 gene region DNA
sequence data has been used in the taxonomy of Calonectria and Cylindrocladium. Research using
the translation elongation 1Nα (TEF1Nα) and calmodulin gene regions for identification of *Calonectria* and *Cylindrocladium* has also been carried out, however, there is not enough data available in these gene regions to make them valuable for comparative analysis (Crous, Groenewald, Risede, Simoneau, & Hywel-N-Jones, 2004; Lombard et al., 2010b).

1.6 Aims of this research

In New Zealand, black root rot on avocado trees caused by *Ilyonectria* or *Calonectria* species has not yet been reported. Internationally in Australia, South Africa, Italy, and Israel it has been reported that *Ilyonectria* and *Calonectria* species can cause black root rot in avocado. *Ilyonectria* species have been reported to cause black root rot on grapevine in New Zealand and in wine growing regions throughout the world.

Firstly, the aim of this research was to determine the morphology and molecular identification of thirteen isolates of suspected *Ilyonectria* or *Calonectria* species that had been previously isolated by Dr Eirian Jones at Lincoln University from avocado roots. Secondly, the aim of this research was to determine if a representative of suspected *Ilyonectria* or *Calonectria* species are pathogenic on different colonial rootstocks of avocado with varying tolerance to *Phytophthora cinnamomi* root rot.

**Objective 1:**
To identify the *Ilyonectria* and *Calonectria* species through observation of culture morphology, conidial morphology and size. Further identification of *Ilyonectria* and *Calonectria* species involves the sequencing of taxonomic informative genes using the βNtubulin and histone gene regions.

**Objective 2:**
To determine the pathogenicity of the *Ilyonectria* isolates in a detached root assay using avocado roots of three rootstocks.
Chapter 2
Methods and Materials

2.1 Experiment 1: Molecular identification of *Ilyonectria* and *Calonectria* isolates

2.1.1 General background and source of fungal cultures

In 2013 roots with distinct lesions from relatively newly planted avocado plants showing decline from an orchard in Northland were sent to Lincoln University. Isolations were carried out from the edge of the lesions by Eirian Jones resulting in the recovery of 27 fungal isolates characteristic of *Ilyonectria*, *Cylindrocarpon* or *Calonectria* spp. These isolates were purified by culturing from single spores and stored in 20% glycerol at N80°C in the Plant Microbiology Group culture collection. Of these, 12 isolates morphologically characteristic of *Ilyonectria/Cylindrocarpon* species LUPP2480, LUPP2482, LUPP2483, LUPP2484, LUPP2485, LUPP2486, LUPP2490, LUPP2493, LUPP2497, LUPP2498, LUPP2501, LUPP2503 and one isolate morphologically characteristic of *Calonectria* sp. (LUPP2505) were selected to represent the range of isolate morphologies and the different zones and blocks in which they were sourced. These thirteen isolates were grown on potato dextrose agar (PDA; Oxoid) for 6 days in an incubator set at 12 hours light and 12 hours dark at 20°C.

2.1.1 Isolation of genomic DNA

Genomic DNA was isolated from thirteen isolates (Section 2.1.1) using a rapid DNA extraction method. A small amount of mycelium was scraped off the colony growing on a PDA plate using a sterile pipette tip and added to 100 μL of Extraction solution (SIGMANALDRICH) and heated for 10 minutes at 95°C. After heating, 100 μL of dilution solution (SIGMANALDRICH) was added to the mixture and vortexed to mix. After mixing the DNA samples were placed in the fridge.

2.1.2 MultiZgene phylogeny

Identification of isolates morphologically identified as *Ilyonectria/Cylindrocarpon* species was carried out using the current multiNgene approach as described by Cabral et al. (2012). This approach included the amplification and sequencing of the βNtubulin and histone H3 gene that provide greatest resolution of *Ilyonectria/Cylindrocarpon* species.

2.1.3 MultiZgene PCR

The βNtubulin and histone H3 genes were amplified using polymerase chain reaction (PCR). Each PCR was carried out in a 20 μL reaction volume that contained 4 μL of sterile water, 10 μL ExtractNNN
Amp™ PCR ReadyMix, 1 μL of each of the gene specific forward and reverse primers (5 μM) (Table 2.1) and 4 μL of DNA extract. For each reaction a negative control was carried out where the DNA extract was substituted with sterile water, to ensure reagents were free of contamination.

Amplification of genes was conducted on a Veriti (Life Technologies) using the following thermal cycle conditions: an initial denaturation period of 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 80 seconds, followed by a final extension of 72°C for 7 minutes.

To confirm amplification, 7 μL of PCR product was combined with 3 μL of loading dye (40% [w/v] sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) and separated by electrophoresis using 1% agarose gels (BIOLINE Agarose Molecular Grade, London) in 1 x TAE buffer (Appendix). DNA samples were separated at a constant 100 V for 45 minutes. For each gel 7 μL of 1 kB Plus DNA Ladder™ (0.1ng/μL) (Invitrogen, New Zealand) was included for comparisons of molecular weight.

The agarose gels were immersed in ethidium bromide (0.5 μg/mL) for 15 minutes to stain the gel. Soaking them in distilled water for a further 10 minutes then destained the gels. Under UV light the DNA was visualised using a FireReader™ imaging system (UviTec, Cambridge, UK) and photographs were taken.

Table 2.1 Nucleotide sequences of the primer pairs used to amplify βZtubulin and histone H3 on both sides of the 5.8S nuclear ribosomal RNA gene (Cabral, Rego, et al., 2012).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pair</th>
<th>Sequence (5’N3’)</th>
<th>Expected Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β6tubulin</td>
<td>T1</td>
<td>AAC ATG CGT GAG ATT GTA AGT</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td>Bi2h</td>
<td>ACC CTC AGT GTA GTG ACC CTT GGC</td>
<td></td>
</tr>
<tr>
<td>Histone H3</td>
<td>CYLH3F</td>
<td>AGG TCC ACT GGT GGC AAG</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>CYLH3R</td>
<td>AGC TGG ATG TCC TTG GAC TG</td>
<td></td>
</tr>
</tbody>
</table>

2.1.4 Sequencing of amplification products

The βNtubulin and histone H3 products were sequenced using the T1 and CYLH2R primers respectively. This was carried out using the dideoxynucleotide chain termination method using BigDye chemistry and subsequent separation and detection on an ABI Prism 3130xl Genetic Analyser (Applied Biosystems, Foster City, California) by the BioNProtection Research Centre, Lincoln University, Canterbury, NZ.

The DNA sequences were obtained and then edited to remove any uncertainties at either end of the sequence using DNAMAN™ (Lynnon Biosoft version 5.0) and compared to sequences present in the GenBank database (www.ncbi.nlm.nih.gov/Genbank/) using the nucleotide basic local alignment.
A species was considered a match if it had 98 to 100% identity and coverage to *Ilyonectria* or *Calonectria* species present in GenBank records.

### 2.1.5 Phylogenetic analysis

A phylogenetic tree was produced for each of the βNtubulin and histone H3 genes. This included the alignment of edited DNA sequences with representative sequences of *Ilyonectria macrodidyma, I. liriodendri, I. liligena, I. robusta, I. europaea, Calonectria pacifica* that were obtained from the GenBank database (Table 2.2). These sequences were chosen as they represented the known nucleotide differences found within these species (Cabral, Groenewald, et al., 2012; Lombard et al., 2010a).

The DNA sequences were then aligned using the CLUSTALW algorithm in MEGA (Molecular Evolutionary Genetics Analysis) version 5.1 (Outram, 2013). After manual adjustment by eye where needed, a phylogenetic analysis was performed on the alignment. This analysis was done using the maximum likelihood based on the neighbourNjoining method, and 1000 bootstrap replications. The histone H3 analysis included sequences from thirteen New Zealand isolates and six international representative sequences. For the βNtubulin analysis not all of the isolates were successfully amplified so only seven New Zealand isolates and six international representative sequences were included in the analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession numbers</th>
<th>βNtubulin</th>
<th>Histone H3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ilyonectria robusta</em></td>
<td>JF735390</td>
<td>JF735531</td>
<td></td>
</tr>
<tr>
<td><em>I. europaea</em></td>
<td>JF735421</td>
<td>JF735567</td>
<td></td>
</tr>
<tr>
<td><em>I. liligena</em></td>
<td>JF735426</td>
<td>JF735574</td>
<td></td>
</tr>
<tr>
<td><em>I. macrodidyma</em></td>
<td>AY677233</td>
<td>JF735647</td>
<td></td>
</tr>
<tr>
<td><em>I. liriodendri</em></td>
<td>AY677241</td>
<td>JF735512</td>
<td></td>
</tr>
<tr>
<td><em>Calonectria pacifica</em></td>
<td>GQ267213</td>
<td>GQ267255</td>
<td></td>
</tr>
</tbody>
</table>
2.2 Experiment 2: Morphological identification of *Ilyonectria* and *Calonectria* isolates

Morphological identification of the isolates based on the colony colour, growth rate and conidial morphology and size were determined by comparisons with published descriptions of these fungal genera and species (Cabral, Groenewald, et al., 2012; Cabral, Rego, et al., 2012; Lombard et al., 2010a, 2010b). The same thirteen isolates used in Experiment 1 were subcultured onto Petri dishes (90 x 15mm) containing PDA and incubated for 7 days at 20°C in 12 hours light and 12 hours dark.

Seven day old isolate cultures were subcultured onto Petri dishes (90 x 15 mm) containing PDA and Spezieller Nährstoffarmer agar (SNA; Appendix B) and sealed with Clingfilm. For each of the thirteen isolates there were four replicates subcultured onto PDA to determine the colony colour and diameter. Two more replicates were subcultured onto SNA to determine conidia size. The four replicates on PDA were arranged in a randomised complete block design [Figure 2.1] and placed in an incubator for 14 days at 20°C in 12 hours light and 12 hours dark. The two replicates on SNA were arranged in a randomised complete design and placed in an incubator for 14 days at 20°C in 12 hours light and 12 hours dark.
2.2.1 Mycelial growth colour identification and measurement

During the 14 days four measurements of the mycelial growth were taken on day 3, 7, 10 and 14. The colony diameter was measured in two perpendicular directions across the Petri dish using a digital calliper (Mitutoyo, U.K. Ltd). The average colony diameter for each plate was then determined. When the colony mycelium had reached the edge of the plate the diameter measurement was 85 mm. On day 14 using a mycological colour chart (Rayner, 1970) the colour of each colony was determined.

2.2.2 Conidia measurement

Conidia were harvested from 14 day old cultures growing on SNA at 14 days at 20°C in 12 hours light and 12 hours dark. A solution made up of 1 litre of sterile water mixed with one drop of Tween 80 was used to make up a spore suspension. Of the solution 2 mLs was pipetted onto the colony surface and a glass slide was used to scrape the colony surface to release the conidia into the solution. The spore suspension was filtered through Miracloth™ to remove any mycelium. One drop of filtered suspension was placed on a clean glass slide and a cover slip was placed on top. Under a compound microscope with a camera at 40x/0.65 magnification the width and length of 15 macro conidia were measured and the relative number of micro conidia (classified as very few, few, many) was noted.
2.2.3 Statistical analysis

Statistical analysis was performed using Genstat oneNway ANOVA in randomised blocks for comparisons from the mycelial growth data between isolates and within species that had more than one isolate. A Genstat unbalanced ANOVA was performed comparing the differences between species from the mycelial growth data. Fishers LSD test was carried out to determine where the significant differences occurred for each analysis. For each of the thirteen isolates the average value and standard deviation for the length and width of fifteen macro conidia was calculated.

2.3 Experiment 3: Pathogenicity of *Ilyonectria* and *Calonectria* isolates on avocado roots using a detached root assay

2.3.1 Fungal cultures

One isolate was randomly selected from each of *I. liligena* (LUPP2493), *I. liriodendri* (LUPP2486), *I. europaea* (LUPP2498), *I. robusta* (LUPP2484) and *C. pacifica* (LUPP2505). These isolates were subcultured onto Petri dishes (90 x 15 mm) containing PDA and incubated for 7 days at 20°C in 12 hours light and 12 hours dark.

2.3.2 Selection of roots

Avocado roots were harvested from 15 potted *Persea americana* rootstock plants obtained from commercial nurseries [Figure 2.2]. Three rootstock cultivars used were Duke 7, Dusa® (Lynwood Avocado Nursery Ltd) and Bounty® (Riversun Nursery Ltd) having five plants for each. These rootstocks were selected for their tolerance to *Phytopthora* root rot; low N medium (Duke 7), medium – high (Bounty®) and high Dusa® (Riversun). Due to overwatering the roots became waterlogged and although the watering was reduced the selection of roots was greatly reduced. The roots selected were of similar pigmentation, width (2 mm to 3 mm) and length (6 – 7 cm). For the Duke 7 and Bounty rootstocks the length of roots was 4 N 5 cm due to the roots taking longer to recover from the effects of waterlogging. The roots were washed down with tap water to remove any soil and used immediately in the detached root assay.
2.3.3 Detached root assay

This was carried out as described for grapevine detached root assay by Pathrose et al. (2010). For each rootstock, 60 roots were cut to size as mentioned in Section 2.4.2. The basal cut end of each root was inserted through a Parafilm™ cap into a 1.7 mL tube filled with sterile water to keep the root hydrated through the experiment [Figure 2.3]. Each root in the tube was individually placed into a deep Petri dish (90 x 25 mm) containing 30 g of sterile silica sand (Fulton Hogan, New Zealand) wetted with 9 ml of sterile water. The apical end of each root was cut across and a mycelial plug (size???) taken using a cork borer from the growing edge of the fungal colony. The mycelial plug placed underneath the apical end of the root so the root was resting on it. The negative control contained a plug of sterile PDA that was placed under the apical end of the each root. The negative controls were set up before the fungal treatments to reduce the chance of cross-contamination. The Petri dishes were sealed with Clingfilm. For each of the five *Ilyonectria* spp. and *Calonectria* sp. isolates and the negative control ten replicates were set up for each of the three avocado rootstocks, with each rootstock being set up on a separate day. Replicates of each rootstock was arranged in a completely randomised design on an individual shelf in an incubator set at 20°C with 24 hours dark and left for 6 days. Following incubation the lesion length on each root was measured using a digital calliper (Mitutoya, U.K. Ltd). Visual observations were made on the colour of any lesions that developed on the inoculated roots.
2.3.4 Koch’s Postulates

To determine Koch’s postulates it is necessary to confirm the identity of the pathogen species by re-isolation from the lesions that develop on the inoculated roots. Infected roots were surface sterilised by placing in 0.35% sodium hypochlorite (bleach) for 3 minutes and washed with sterile water twice for 2 minutes [Figure 2.4]. Where the lesion ended the root was cut and a 5 mm piece of root was cut into the lesion. To determine whether the isolates colonised beyond the lesion, into the apparently healthy root 5 mm pieces were cut at 5 mm, 10 mm, 15 mm, 20 mm and 25 mm from the lesion [Figure 2.4]. In some cases the healthy section of the root was too short and only two to four pieces of root from the healthy tissue could be cut. When only two pieces of healthy tissue were available another piece of root was cut at 10 mm into the lesion. The pieces of root were placed in a Petri dish (90 x 15 mm) containing PDA and arranged evenly in order starting at the lesion point around the dish. The dish was sealed with Clingfilm and incubated at 20°C in 12 hours light and 12 hours dark for 7 days. For each isolate and the negative control, 5 of the 10 replicates where chosen at random for Koch’s postulates. Koch’s postulates were done for each of the three avocado rootstocks.
Figure 2.4. Image (a) surface sterilisation process; right tray contains 0.35% sodium hypochlorite; middle and left tray contain sterile water. Image (b) the arrangement of roots around Petri dish containing PDA for Koch Postulates.
3.1 Molecular identification of *Ilyonectria* and *Calonectria* isolates

3.1.1 MultiZgene PCR and sequencing

Amplification products of approximately 500 base pairs (bp) for the histone H3 gene were acquired for twelve of the thirteen fungal isolates characteristic of *Ilyonectria* and *Calonectria* spp (Figure 3.1 and Figure 3.3). For isolate LUPP2505 no amplification product was produced, however a DNA sequence from a non single spore LUPP2505 isolate was used for sequence analysis. For the βN tubulin gene amplification products of 630 bp were acquired for the thirteen fungal isolates characteristic of *Ilyonectria* or *Calonectria* spp (Figure 3.2 and Figure 3.3). Sequencing of the amplification products of the histone H3 and βN tubulin gene showed that isolates belonged to one of *I. macrodidyma*, *I. liliodendri*, *I. illigena*, *I. robusta*, *I. europaea* or *C. pacifica*. For six isolates (LUPP2486, LUPP2486, LUPP2490, LUPP2497, LUPP2498 and LUPP2505) the sequencing reaction failed and unclear sequences were produced that were unable to be used.

![PCR products of the histone H3 genes of thirteen *Ilyonectria* and *Calonectria* spp. isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; C1, LUPP2480; C3, LUPP2482; C4, LUPP2483; C5, LUPP2484; C6, LUPP2485; C7, LUPP2486; C11, LUPP2490; C14, LUPP2493; C18, LUPP2497; C19, LUPP2498; C22, LUPP2501; C24, LUPP2503; C26, LUPP2505; L, 1 kB PLUS DNA marker.](image)

Figure 3.1 PCR products of the histone H3 genes of thirteen *Ilyonectria* and *Calonectria* spp. isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; C1, LUPP2480; C3, LUPP2482; C4, LUPP2483; C5, LUPP2484; C6, LUPP2485; C7, LUPP2486; C11, LUPP2490; C14, LUPP2493; C18, LUPP2497; C19, LUPP2498; C22, LUPP2501; C24, LUPP2503; C26, LUPP2505; L, 1 kB PLUS DNA marker.
Figure 3.2  PCR products of the βZtubulin genes of thirteen *Ilyonectria* or *Calonectria* spp isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; C1, LUPP2480; C3, LUPP2482; C4, LUPP2483; C5, LUPP2484; C6, LUPP2485; C7, LUPP2486; C11, LUPP2490; C14, LUPP2493; C18, LUPP2497; C19, LUPP2498; C22, LUPP2501; C24, LUPP2503; C26, LUPP2505; L, 1 kB PLUS DNA marker.

Figure 3.3  PCR products of the histone H3 and βZtubulin genes of two *Ilyonectria* or *Calonectria* spp isolates on 1% agarose gels. L, 1 kB PLUS DNA marker; B, Negative control; HC26, histone H3 LUPP2505; HC19, histone H3 LUPP2498 (positive control); B, Negative control; BC26, βZtubulin LUPP2505; BC19, βZtubulin LUPP2498 (positive control).
3.1.2 MultiZgene phylogenetic analysis

The neighbouring joining trees are based on the histone H3 and \( \beta \)Ntubulin gene analysis (Figure 3.4, Figure 3.5) of the *Ilyonectria* and *Calonectria* spp. isolated from New Zealand avocados grouped with international representative isolates of the species summarised as described by (Cabral, Groenewald, et al., 2012; Lombard et al., 2010a).

The histone H3 phylogenetic tree, with a branch length of 0.4945 (Figure 3.4), lead to the clustering of the thirteen *Ilyonectria* and *Calonectria* New Zealand isolates with the six international representative sequences, *I. liligena*, *I. robusta*, *I. europaea*, *I. macrodidyma*, *I. liriodendri* and *C. pacifica*. The bootstrap values were high for the *I. robusta*, *I. liriodendri*, *I. europaea*, *I. macrodidyma* and *C. pacifica* clusters (range 80N100). The bootstrap value for the *I. liligena* cluster was low at 48. For four of the New Zealand *I. liligena* species the bootstrap value was 100, and one was 55.

The \( \beta \)Ntubulin phylogenetic tree with a branch length of 0.3943 (Figure 3.5) lead to the clustering of the seven of the thirteen *Ilyonectria* and *Calonectria* New Zealand isolates with the four international representative sequences, *I. liligena*, *I. robusta*, *I. macrodidyma* and *I. liriodendri*. The bootstrap value for the *I. liligena* cluster was high at 90, and the *I. macrodidyma* and *I. liriodendri* clusters also had high the bootstrap values (100). For the *I. robusta* cluster the bootstrap value was average at 78, however, this cluster also included the *I. europaea* representative strain. Therefore the \( \beta \)Ntubulin gene can resolve between *I. liligena*, *I. liriodendri* and *I. macrodidyma* but is unable to differentiate between *I. robusta* and *I. europaea*. 
Figure 3.4  The neighbouring joining tree with bootstrap values using 1000 replicates generated in MEGA 5.1 using the Histone H3 gene sequences of thirteen isolates characteristic of *Ilyonectria* and *Calonectria* species isolated from New Zealand avocado trees and from five *Ilyonectria* spp. and one *Calonectria* sp. sourced from GenBank with accession number.

Figure 3.5  The neighbouring joining tree with bootstrap values using 1000 replicates generated in MEGA 5.1 using the βZtubulin gene sequences of thirteen isolates characteristic of *Ilyonectria* and *Calonectria* species isolated from New Zealand avocado trees and from five *Ilyonectria* spp. and one *Calonectria* sp. sourced from GenBank with accession number.
3.2 Morphological identification of *Ilyonectria* and *Calonectria* isolates

3.2.1 Colony colour and macro conidia size

Colony morphology for isolate LUPP2480 at Day 14. The reverse side of the colony from the centre outward had chestnut and umber zonation that changed to umber and luteous zonation with the outside zonation being straw (Figure 3.6 A). The surface side of the colony from the centre was amber to straw to buff at the edge of the Petri dish (Figure 3.6 B). Macroconidia average size 19.15(+/N 6.53) x 5.00 (+/N 1.25) μm with two septate and very few microconidia (Figure 3.6 C).

![Figure 3.6 Colony morphology of isolate LUPP2480 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.](image)

Colony morphology for isolate LUPP2482 at Day 14. The reverse side of the colony from the centre outward was sepia then umber to honey (Figure 3.7 A). The surface side of the colony from the centre was umber to ochreous buff to white at the edge of the Petri dish (Figure 3.7 B). Macroconidia average size 15.61(+/N 2.56) x 6.05 (+/N 1.70) μm with one septate and many micro conidia (Figure 3.7 C).

![Figure 3.7 Colony morphology of isolate LUPP2482 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.](image)
Colony morphology for isolate LUPP2483 at Day 14. The reverse side of the colony from the centre outward was chestnut to umber then sienna to honey then straw (Figure 3.8 A). The surface side of the colony from the centre was hazel to white then cinnamon to buff to white at the edge of the Petri dish (Figure 3.8 B). Macro conidia average size 23.35 (+/N 2.74) x 6.03 (+/N 0.74) μm with one septate and very few micro conidia (Figure 3.8 C).

Figure 3.8  Colony morphology of isolate LUPP2483 growing on PDA A) reverse side of the colony , B) surface side of the colony , C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Colony morphology for isolate LUPP2484 at Day 14. The reverse side of the colony from the centre outward was chestnut then sepia to sienna then pale luteous to straw (Figure 3.9 A). The surface side of the colony from the centre was cinnamon to buff then straw at the edge of the Petri dish (Figure 3.9 B). Macro conidia average size 31.39 (+/N 7.61) x 7.05 (+/N 1.38) μm with two septate and few micro conidia (Figure 3.9 C).

Figure 3.9  Colony morphology of isolate LUPP2484 growing on PDA A) reverse side of the colony , B) surface side of the colony , C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.
Colony morphology for isolate LUPP2485 at Day 14. The reverse side of the colony from the centre outward was umber then chestnut to dark brick then cinnamon to pale luteous then buff then amber to straw then white in zonations (Figure 3.10 A). The surface side of the colony from the centre was white to cinnamon then straw to white at the edge of the Petri dish (Figure 3.10 B). Macroconidia average size 14.48 (+/N 3.01) x 5.44 (+/N 0.85) μm with one septate and many micro conidia (Figure 3.10 C).

Figure 3.10 Colony morphology of isolate LUPP2485 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Colony morphology for isolate LUPP2486 at Day 14. The reverse side of the colony from the centre outward was umber and honey zonations followed by honey and amber zonations to white (Figure 3.11 A). The surface side of the colony from the centre was hazel then honey to buff then white at the edge of the Petri dish (Figure 3.11 B). Macro conidia average size 14.63 (+/N 7.29) x 3.93 (+/N 1.09) μm with one septate and many micro conidia (Figure 3.11 C).

Figure 3.11 Colony morphology of isolate LUPP2486 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.
Colony morphology for isolate LUPP2490 at Day 14. The reverse side of the colony from the centre outward was a ochreous and chestnut the ochreous and umber to hazel/ocherous then pale luteous to buff (Figure 3.12 A). The surface side of the colony from the centre was hazel to buff to white at the edge of the Petri dish (Figure 3.12 B). Macro conidia average size 18.89 (+/N 4.04) x 5.60 (+/N 1.19) μm with one septate and few micro conidia (Figure 3.12 C).

Figure 3.12 Colony morphology of isolate LUPP2490 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Colony morphology for isolate LUPP2493 at Day 14. The reverse side of the colony from the centre outward was sepia then dark brick to fulvous then ochreous to buff to white (Figure 3.13 A). The surface side of the colony from the centre was fawn to sepia then bay to umber then buff at the edge of the Petri dish (Figure 3.13 B). Macro conidia average size 17.67 (+/N 3.90) x 5.69 (+/N 1.12) μm with one septate and many micro conidia (Figure 3.13 C).

Figure 3.13 Colony morphology of isolate LUPP2493 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.
Colony morphology for isolate LUPP2497 at Day 14. The reverse side of the colony from the centre outward was isabelline then honey and amber rings to straw then white (Figure 3.14 A). The surface side of the colony from the centre was buff to amber then honey to white at the edge of the Petri dish (Figure 3.14 B). Macro conidia average size 23.41 (+/N 5.78) x 6.25 (+/N 0.94) μm with two septate and few micro conidia (Figure 3.14 C).

Figure 3.14 Colony morphology of isolate LUPP2497 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Colony morphology for isolate LUPP2498 at Day 14. The reverse side of the colony from the centre outward was chestnut to pale luteous then umber to cinnamon followed by honey to amber then straw (Figure 3.15 A). The surface side of the colony from the centre was umber to hazel then honey to buff followed by white at the edge of the Petri dish (Figure 3.15 B). Macro conidia average size 15.15 (+/N 3.81) x 4.97 (+/N 1.30) μm with one septate and few micro conidia (Figure 3.15 C).

Figure 3.15 Colony morphology of isolate LUPP2498 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.
Colony morphology for isolate LUPP2501 at Day 14. The reverse side of the colony from the centre outward was umber and isabelline rings followed by honey and hazel rings then straw to buff (Figure 3.16 A). The surface side of the colony from the centre was hazel to honey followed by buff to white at the edge of the Petri dish (Figure 3.16 B). Macro conidia average size 22.32 (+/N 10.27) x 6.21 (+/N 2.57) μm with two septate and few micro conidia (Figure 3.16 C).

Figure 3.16  Colony morphology of isolate LUPP2501 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.

Colony morphology for isolate LUPP2503 at Day 14. The reverse side of the colony from the centre outward was sienna and chestnut rings to dark brick followed by ochreous to isabelline to honey then pale luteous to buff (Figure 3.17 A). The surface side of the colony from the centre was umber to buff then cinnamon and rosy buff rings then hazel to honey buff at the edge of the Petri dish (Figure 3.17 B). Macro conidia average size 28.27 (+/N 4.49) x 8.00 (+/N 1.59) μm with two septate and very few micro conidia (Figure 3.17 C).

Figure 3.17  Colony morphology of isolate LUPP2503 growing on PDA A) reverse side of the colony, B) surface side of the colony, C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.
Colony morphology for isolate LUPP2505 at Day 14. The reverse side of the colony from the centre outward was chestnut to sepia then dark brick to fawn (Figure 3.18 A). The surface side of the colony from the centre was sepia to dark brick then cinnamon to buff at the edge of the Petri dish (Figure 3.18 B). The aerial mycelium on top was a vinaceous buff (Figure 3.18 B) Macro conidia average size 26.88 (+/N 4.09) x 2.86 (+/N 0.59) μm with no septate and very few micro conidia (Figure 3.18 C).

Figure 3.18  Colony morphology of isolate LUPP2505 growing on PDA A) reverse side of the colony , B) surface side of the colony , C) macroconidia. All images were taken on Day 14 of the experiment. Bar represents 8 μm.
3.2.2 Colony size

3.2.2.1 Analysis of colony size for all isolates

The average colony diameter of each of the thirteen *Ilyonectria* and *Calonectria* sp. was significantly different overall isolates at each assessment time point (*P*<0.01) (Figure 3.19). The largest colony diameter at day 3 was seen for isolate LUPP2505, and at day 7 isolate LUPP2498, and 10 for isolate LUPP2498. By day 14 isolates LUPP2482, LUPP2483, LUPP2485, LUPP2493, LUPP2498 and LUPP2505 had all reached the edge of the Petri dish.

At day 3 the isolate with the least colony growth was LUPP2503, at day 7 and 10 was isolate LUPP2497, and at day 14 isolate LUPP2503. At day 3 isolates LUPP2505 and LUPP2498 had a significantly larger colony diameter compared with all the other isolates, and in particular LUPP2503 by 55 to 60%, respectively. On day 7 there are two clear groups with one outlier. The group with the smaller colony diameters (LUPP2480, LUPP2486, LUPP2497, LUPP2501 and LUPP2503) are on average 36% smaller than the group with larger colony diameters (LUPP2482, LUPP2483, LUPP2484, LUPP2485, LUPP2490, LUPP2493 and LUPP2505). Isolate LUPP2498 is separate from these two groups being 58% and 16% larger than the smallest and middle isolates respectively. On day 10 similar differences are seen as described for day 7. The isolates with the smallest colonies (at day 7) were on average 33% smaller than the isolates with the largest colonies (at day 7). Isolates LUPP2498 and LUPP2505 were separated from these two groups being 49% and 12% larger than the smaller and larger colonies, respectively. On day 14 the isolates split into roughly two groups. The isolates with the largest colonies were those that had reached the edge of the plate as mentioned previously or one millimetre from the edge (LUPP2484 and LUPP2490). The isolates with a smaller colony diameter (LUPP2480, LUPP2486, LUPP2497, LUPP2501, LUPP2503) were on average 18% smaller than the colonies that had reached the edge of the plate.
Figure 3.19 Average colony diameter size (mm) for each of the thirteen isolates, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at $P<0.05$ at each assessment time.
3.2.2.2 Analysis of between species colony size

The average colony diameter of each of the *Ilyonectria* and *Calonectria* sp. was significantly different at each assessment time point (*P*<0.01)(Figure 3.20). At all assessment times *Ilyonectria lirioidendri* and *I. macrodidyma* had the smallest colony diameter whilst *I. europaea* and *Calonectria pacifica* had the largest colony diameter, with *I. robusta* and *I. liligena* being intermediate in colony diameter. These differences were most significant on days 7 and 10 when *I. lirioidendri* and *I. macrodidyma* were 39% and 40% smaller than *I. europaea* and *C. pacifica* respectively.

![Graph showing average colony diameter size (mm) for each of the six species, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at *P*<0.05 at each assessment time.](image)

*Figure 3.20* Average colony diameter size (mm) for each of the six species, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at *P*<0.05 at each assessment time.
3.2.2.3 Analysis of within species colony size

The average colony diameter of the five isolates of the *Ilyonectria liligena* species at each of the four assessment days is summarised in Figure 3.21. There was a significant difference between the five isolates (*P*<0.01). This significant difference was due to LUPP2503 having a significantly smaller average colony diameter in comparison to the other four isolates at each assessment time. On assessment days 3, 7, 10 and 14 the colony diameter of isolate LUPP2503 was 39%, 43%, 42% and 27% smaller, respectively than the average colony diameter of the four other tested *I. liligena* isolates.

![Graph showing average colony diameter size for five isolates of Ilyonectria liligena](image)

**Figure 3. 21** Average colony diameter size (mm) for the five isolates of *Ilyonectria liligena*, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at *P*<0.05 at each assessment time.
Figure 3.22 shows the average colony diameter of the two *Ilyonectria europaea* isolates at the four assessment times. There was a significant difference between the average colony diameter for days 3, 7 and 10 ($P<0.01$) with LUPP2498 having larger colonies at each assessment time. At day 3, 7 and 10 colonies of LUPP2498 were 14%, 19% and 16% larger colony diameter respectively than LUPP2485. On day 14 there was no significant difference between the isolates as the colonies of both isolates reached the edge of the Petri dish.

**Figure 3.22** Average colony diameter size (mm) for the two isolates of *Ilyonectria europaea*, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at $P<0.05$ at each assessment time.
Figure 3.23 below illustrates the average colony diameter for the three *Ilyonectria liriodendri* isolates on the four assessment days. There was no significant difference in colony diameter between the three isolates of *I. liriodendri* for all time periods except day 7. At day 7 there was a significant difference between the isolates (P<0.01) where isolate LUPP2486 had significantly larger diameter compared with LUPP2497 and LUPP2501. Colonies of isolate LUPP2486 were 12% larger in colony diameter than LUPP2497 and 8% larger than LUPP2501 on assessment day 7.

![Graph showing average colony diameter](image)

**Figure 3.23** Average colony diameter size (mm) for the three isolates of *Ilyonectria liriodendri*, measured at day 3, day 7, day 10 and day 14. The red bar represents the LSD at P<0.05 at each assessment time.
3.3 Pathogenicity of *Ilyonectria* and *Calonectria* isolates on avocado roots using a detached root assay

3.3.1 Lesion lengths from the detached roots

Table 3.1 Mean lesion length (and standard deviation) that developed on roots of three avocado rootstocks (Dusa®, Bounty® and Duke 7) inoculated with four *Ilyonectria* spp. and one *Calonectria* sp. six days after inoculation.

<table>
<thead>
<tr>
<th>Species/Isolate</th>
<th>Dusa®</th>
<th>Bounty®</th>
<th>Duke 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average Lesion Length (mm)</strong></td>
<td><strong>Standard Deviation (mm)</strong></td>
<td><strong>Average Lesion Length (mm)</strong></td>
<td><strong>Standard Deviation (mm)</strong></td>
</tr>
<tr>
<td>Control</td>
<td>17.9</td>
<td>10.1</td>
<td>15.9</td>
</tr>
<tr>
<td><em>I. robusta</em> LUPP2484</td>
<td>20.6</td>
<td>5.8</td>
<td>17.5</td>
</tr>
<tr>
<td><em>I. liriodendri</em> LUPP2486</td>
<td>22.3</td>
<td>6.0</td>
<td>20.0</td>
</tr>
<tr>
<td><em>I. liligena</em> LUPP2493</td>
<td>20.3</td>
<td>6.6</td>
<td>21.7</td>
</tr>
<tr>
<td><em>I. europaea</em> LUPP2498</td>
<td>22.7</td>
<td>6.5</td>
<td>18.9</td>
</tr>
<tr>
<td><em>C. pacifica</em> LUPP2505</td>
<td>31.5</td>
<td>6.6</td>
<td>22.2</td>
</tr>
</tbody>
</table>

All isolates produced a visible lesion on the three avocado rootstocks (Dusa®, Bounty® and Duke 7) six days after inoculation. Table 3.1 shows the average lesion length that was caused by the four *Ilyonectria* spp. and one *Calonectria* sp. were similar within a rootstock, however, differed between rootstocks. There was a difference between the lesion lengths which developed on the different avocado rootstocks mainly due to the roots of Bounty® and Duke 7 that were used for the assay were shorter due to a slower recovery from waterlogging affects. The isolate that produced the greatest lesion on all three rootstocks was *C. pacifica* (LUPP2505) and the smallest lesions produced on all three rootstocks were by *I. robusta* (LUPP2484). However, as lesions developed on uninoculated control no statistical analysis was carried out, as there was no way to differentiate between lesions that developed on uninoculated controls and those inoculated with *Ilyonectria* spp. or *Calonectria* sp. (Alison Lister, Pers. Comm.).
3.3.2 Isolation from lesions on detached roots to confirm pathogenicity

To confirm pathogenicity *Ilyonectria* or *Calonectria* type colonies were isolated within the lesion and from five distances beyond the edge of the lesions on the inoculated and uninoculated control Dusa\textsuperscript{*} roots (Table 3.2), Bounty\textsuperscript{*} roots (Table 3.3) and Duke 7 roots (Table 3.4).

Table 3.2 Isolation of *Ilyonectria* or *Calonectria* type colonies from root pieces taken from within and beyond the lesions on inoculated Dusa\textsuperscript{*} avocado rootstock detached roots inoculated with *Ilyonectria* spp., *Calonectria* sp. isolates or untreated control. Data presented represents the ratio out of the 5 replicate roots assessed per treatment positive for *Ilyonectria* or *Calonectria* type colonies.

<table>
<thead>
<tr>
<th>Species/Isolate</th>
<th>At Lesion</th>
<th>5 mm</th>
<th>10 mm</th>
<th>15 mm</th>
<th>20 mm</th>
<th>25 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td><em>I. robusta</em> LUPP2484</td>
<td>4/5</td>
<td>4/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td><em>I. liliodendri</em> LUPP2486</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
<td>5/5</td>
</tr>
<tr>
<td><em>I. liligena</em> LUPP2493</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
<td>4/5</td>
</tr>
<tr>
<td><em>I. europaea</em> LUPP2498</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
</tr>
<tr>
<td><em>C. pacifica</em> LUPP2505</td>
<td>5/5</td>
<td>4/5</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
<td>4/5</td>
</tr>
</tbody>
</table>

*Ilyonectria* type colonies were isolated from within the lesion and at all distances tested beyond the lesion for all isolates on inoculated Dusa\textsuperscript{*} avocado rootstock detached roots (Table 3.2). No isolates characteristic of *Calonectria* sp. were recovered from any of the roots including those inoculated with *C. pacifica*. Within the five replicated roots, no greater than 2/5 replications had no *Ilyonectria* type colonies isolated from them. Where this occurred, other fungal colonies were isolated which were green, with white fluffy growth and were morphologically identified as *Trichoderma* spp.
Table 3.3  Isolation of *Ilyonectria* or *Calonectria* type colonies from root pieces taken from within and beyond the lesions which develop on inoculated Bounty® avocado rootstock detached roots inoculated with *Ilyonectria* spp., *Calonectria* sp. isolates or untreated control. Data presented represents the ratio out of the 5 replicate roots assessed per treatment positive for *Ilyonectria* or *Calonectria* type colonies. Where roots were too short, ratios are out of the number of roots assessed per positive treatment for *Ilyonectria* or *Calonectria* type colonies for that measurement.

<table>
<thead>
<tr>
<th>Species/Isolate</th>
<th>Within Lesion</th>
<th>At Lesion</th>
<th>5 mm</th>
<th>10 mm</th>
<th>15 mm</th>
<th>20 mm</th>
<th>25 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/1</td>
<td>2/5</td>
<td>2/5</td>
<td>2/5</td>
<td>2/4</td>
<td>2/4</td>
<td>1/2</td>
</tr>
<tr>
<td><em>I. robusta</em> LUPP2484</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
<td>3/5</td>
<td>3/4</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td><em>I. liriodendri</em> LUPP2486</td>
<td>4/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>2/5</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td><em>I. liligena</em> LUPP2493</td>
<td>3/4</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
<td>3/4</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td><em>I. europaea</em> LUPP2498</td>
<td>1/2</td>
<td>3/5</td>
<td>4/5</td>
<td>3/5</td>
<td>3/5</td>
<td>1/3</td>
<td>0/1</td>
</tr>
<tr>
<td><em>C. pacifica</em> LUPP2505</td>
<td>1/1</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
<td>2/4</td>
<td>0/2</td>
</tr>
</tbody>
</table>

NB: There are no results for isolate LUPP24864 and LUPP 2486 as the length of the root was long enough to get four or five isolations beyond the lesion.

*Ilyonectria* or *Calonectria* type colonies were isolated from within the lesion and at all distances tested beyond the lesion for all isolates on inoculated Bounty® roots (Table 3.3). In those roots that were inoculated with *C. pacifica*, 3/5 five replicate root colonies produced a colony that from the reverse side of the plate had similar colours and textures to the isolate identified in Section 3.2.1..

Within the replicated roots, no greater than 1/4 replications had no *Ilyonectria* and *Calonectria* type colonies isolated from them aside from the negative control. Where this happened other fungal colonies were isolated. These other fungi produced a green white fluffy colony (morphologically identified as *Trichoderma* spp.), a fluffy pink colour colony (morphologically identified as *Fusarium* spp.) or a flat green grey colony (morphologically identified as *Penicillium* spp.).
Table 3.4  Isolation of *Ilyonectria* or *Calonectria* type colonies from root pieces taken from within and beyond the lesions which develop on inoculated Duke 7 avocado rootstock detached roots inoculated with *Ilyonectria* spp., *Calonectria* sp. isolates or untreated control. Data presented represents the ratio out of the 5 replicate roots assessed per treatment positive for *Ilyonectria* or *Calonectria* type colonies. Where roots were too short, ratios are out of the number of roots assessed per positive treatment for *Ilyonectria* or *Calonectria* type colonies for that measurement.

<table>
<thead>
<tr>
<th>Species/Isolate</th>
<th>Within Lesion</th>
<th>At Lesion</th>
<th>5 mm</th>
<th>10 mm</th>
<th>15 mm</th>
<th>20 mm</th>
<th>25 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2/5</td>
<td>2/5</td>
<td>2/5</td>
<td>2/5</td>
<td>2/4</td>
<td>2/3</td>
</tr>
<tr>
<td><em>I. robusta</em> LUPP2484</td>
<td>1/2</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
<td>2/2</td>
<td>1/1</td>
</tr>
<tr>
<td><em>I. liriodendri</em> LUPP2486</td>
<td>1/1</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/4</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td><em>I. liligena</em> LUPP2493</td>
<td>1/1</td>
<td>3/5</td>
<td>4/5</td>
<td>4/5</td>
<td>3/4</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td><em>I. europaea</em> LUPP2498</td>
<td></td>
<td>2/5</td>
<td>3/5</td>
<td>2/5</td>
<td>3/5</td>
<td>3/3</td>
<td>0/1</td>
</tr>
<tr>
<td><em>C. pacifica</em> LUPP2505</td>
<td>1/1</td>
<td>5/5</td>
<td>2/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/4</td>
<td>1/3</td>
</tr>
</tbody>
</table>

NB: There are no results for isolate LUPP2498 and the control as the length of the root was long enough to get four or five isolations beyond the lesion for all replicates. For isolate LUPP2493 the replicate roots were too short to get isolations 25 mm beyond the lesion.

*Ilyonectria* type colonies were isolated from within the lesion and at all distances tested beyond the lesion for all isolates on inoculated Duke 7 roots (Table 3.4). No isolates characteristic of *Calonectria* sp. were recovered from any of the roots including those inoculated with *C. pacifica*. Within the replicated roots, no greater than 1/5 replications had no *Ilyonectria* type colonies isolated from them aside from *I. europaea* 25 mm beyond the lesion. Where this occurred other fungal colonies were isolated. These other fungi produced a green white fluffy colony (morphologically identified as *Trichoderma* spp.), a fluffy pink colour colony (morphologically identified as *Fusarium* spp.) or a flat green grey colony (morphologically identified as *Penicillium* spp.).
Chapter 4
Discussion

4.1 Molecular identification of Ilyonectria and Calonectria isolates

4.1.1 Species identified from avocado samples in New Zealand

This research confirmed the species identity of thirteen morphologically identified Ilyonectria and Calonectria type isolates that were isolated from avocado roots by Dr Eirian Jones. This was the first study in New Zealand to identify Ilyonectria and Calonectria species isolated from the symptomatic roots of avocado plants. In this study the current taxonomic process for identifying Ilyonectria spp. in grapevine, as described by Cabral et al. (2012), was used. This process involved PCR and multiGene sequence analysis using part of the histone H3 and the BNTubulin gene, as the study by Cabral et al. (2012) showed these were the most informative genes.

The results of this research identified that the thirteen isolates belonged to one of either I. liligena, I. robusta, I. liriodendri, I. macrodidyma, I. europaea and C. pacifica species. The most common species identified, with five isolates (LUPP2482, LUPP2483, LUPP2490, LUPP2493 and LUPP2503), was I. liligena. Other species identified as belonging in the I. radicicolaM complex were one isolate (LUPP2484) identified as I. robusta and two isolates (LUPP2485 and LUPP2498) which were identified as I. europaea. The other species within the I. radicicolaM complex were not identified in this collection. This study also resulted in the identification of three isolates (LUPP2486, LUPP2497 and LUPP2501) as I. liriodendri, one isolate (LUPP2480) was identified as belonging to the I. macrodidyma species complex, I. macrodidyma, and one isolate was identified as Calonectria pacifica.

To date there have been no comparable studies carried out in New Zealand, and limited studies internationally. However, of the studies carried out three of the six species identified in this study have been previously reported from avocado roots. A paper by Dannet al. (2012) isolated I. liriodendri from avocado trees in Australia that were showing signs of decline. In Israel, Zilberstein et al. (2007) also found that wilting disease on young avocados was caused by the Ilyonectria radicicola / Cylindrocarpon destructans complex. Black root rot in avocado has also been reported to be caused by Ilyonectria macrodidyma in Italy (Vitale et al., 2012). Dann et al. (E. K. Dann et al., 2012) also found that Calonectria ilicicola was a severe root pathogen on avocados. The species C. ilicicola is a sister group to C. pacifica that was isolated from avocado roots in this study (Lombard et al., 2010a). As C. pacifica is closely related to C. ilicicola it is highly likely that it has similar pathogenicity on avocado roots. This could be very damaging to the avocado industry in New Zealand, as C. pacifica could have
the potential to severely damage avocado plant roots if found to be pathogenic. This is however the first report of *C. pacifica* in New Zealand.

Isolates from species within both *I. macrodidyma* and *I. radicicola* complex and *I. liriodendri* have previously been isolated from symptomatic grapevine roots in New Zealand (Pathrose, (2012) Outram, (2013). To date the exact identify of the *I. radicicola* complex species isolated from grapevine in New Zealand is not known, but three species within the *I. macrodidyma* complex; *I. macrodidyma*, *I. novozelandica* and *I. torresensis* have been identified (Outram, 2013). Therefore it is likely there could be more *Ilyonectria* or *Calonectria* species present in soil surrounding avocado roots, however, because of the limited sample size in the present study, this has not been represented. In the future, isolations from more samples of avocado roots showing symptoms of black root rot would provide a better representation of the *Ilyonectria* and *Calonectria* species affecting avocados in New Zealand.

### 4.1.2 MultiZgene sequence analysis

In studies by Cabral *et al.* (2012) and Lombard *et al.* (2010a) the use of multiZgene sequence analysis has been shown to identify the differences in DNA sequences to a sufficient level to distinguish between the individual species within the *Ilyonectria radicicola* complex, the *I. macrodidyma* complex and between *Calonectria* species and their *Cylindrocladium* anamorphs. In this study, the use of the histone H3 and βNtubulin genes in this multiZgene sequence analysis was able to show sufficient differences between the DNA sequences to distinguish between the six *Ilyonectria* and *Calonectria* species and classify them as per their relatedness to each other.

Of the two genes used in this study the histone H3 gene had the highest resolving capacity. All of the New Zealand isolates could be differentiated between one another (Figure 3.4). However, for one isolate, LUPP2505, the DNA sequence used was not from a single spored isolate so should be repeated in the future to ensure the validity of the results. A study by Cabral *et al.* (2012) also found that the histone sequence data alone was sufficient to identify species belonging to the *I. radicicola* and *I. macrodidyma* complexes.

For all *Ilyonectria* and *Calonectria* species the bootstrap values were high (80 N 100) except for *I. liligena* cluster. However, the bootstrap values for *I. liligena* were low. From comparing the DNA sequence for all of the *I. liligena* isolates including the international representative there was some variation in the sequences. In several areas of the DNA sequence one or two bases of LUPP2503 are different to the other four New Zealand *I. liligena* isolates. Cabral *et al.* (2012) reported that there was a small split between the *I. liligena* isolates giving a bootstrap value of 85 to the cluster of which the international representative isolate used in this study was chosen. Some of the differences in the
nucleotide base sequence for isolate LUPP2503 were not the same as that in the international representative isolate, which could explain its position in the phylogenetic tree. This international representative I. liligena isolate used in the present study was different from the other representative isolates as it was isolated from a Lilium sp. bulb not from Vitis vinifera as for the other isolates investigated by Cabral et al. (2012). The location of the isolate was also from the Netherlands; whereas the other representative isolates came from Portugal and South Africa (Cabral, Rego, et al., 2012).

The βNtubulin gene had the lowest resolving capacity and is not suitable to be used alone for identification purposes. This gene was unable to resolve between the closely related I. robusta and I. europaea species. Using the histone H3 sequence, isolate LUPP2484 was identified as I. robusta being in the same cluster as the international representative. However, when the βNtubulin gene sequence was analysed, the isolate was a sister group to the I. robusta and I. europaea international representatives. Similar results were reported by Cabral et al. (2012) where I. robusta and I. liligena could not be separated by the βNtubulin gene and had low bootstrap values.

In this study, six of the isolates LUPP2486, LUPP2486, LUPP2490, LUPP2497, LUPP2498 and LUPP2505 had failed sequencing reactions and subsequently unclear sequences were produced for the βNtubulin gene leading to an absence of data. However, as the histone H3 gene had sufficient resolving capacity to enable species identification of the isolates no further sequencing reactions were carried out. In the future, given more time, these reactions should be repeated to ensure accurate identification of species.

4.2 Morphological identification of Ilyonectria and Calonectria isolates

4.2.1 Colony colour and macro conidia size

Morphological identification of the thirteen isolates of Ilyonectria and Calonectria species has shown some similarities within isolates molecularly identified as the same species however, there is still variability between isolates of a species.

Isolate LUPP2480 was molecularly identified as I. macrodidyma, as there was only one isolate identified the morphological comparison must be made with published data. The colony colour and texture of LUPP2480 was similar to what was has been previously published, having a yellowish surface and an orange dark brown reverse (Halleen et al., 2004). The conidia size of LUPP2480 was slightly smaller than what has been published with two septate macroconidia measuring 26 to 35 μm in length, the width was similar (Pathrose, 2012). Only isolate LUPP2484 was molecularly identified as I. robusta from the thirteen unknown isolates. When compared with published data LUPP2484 had very similar colony descriptions with a cinnamon to buff surface and a sepia to sienna reverse.
(Cabral, Groenewald, et al., 2012). The macroconidia size of LUPP2484 was very similar to the size of the two and three septate macroconidia found by Cabral et al. (2012).

From the thirteen unknown isolates LUPP2482, LUPP2483, LUPP2490, LUPP2493 and LUPP2503 were molecularly identified as *I. liligena*. Of these five isolates, isolates LUPP2483 and LUPP2493 have similar surface colony colours and textures and isolates LUPP2482 and LUPP2490 have similar surface colony colours and textures. All of these four isolates have similar colours and rough pattern on the reverse colony. However, isolate LUPP2503 is very different from the other four isolates with a lighter colour and clear zonation on the surface and reverse side of the colony. When compared with published descriptions there are some similarities in the colour of the colony with the all being buff and cinnamon in colour (Cabral, Groenewald, et al., 2012). The morphological difference of LUPP2503 and the other *I. liligena* isolates is mirrored with the molecular identification. The histone H3 phylogenetic tree probably suggests that LUPP2503 is a new species as although the nearest species identity is *I. liligena*, it is on a separate branch to the other *I. liligena* species. The size of the macroconidia are also slightly smaller in length for LUPP2482, LUPP2490 and LUPP2493 compared with the descriptions of Cabral et al. (2012), but are slightly bigger in width.

Three of the unknown isolates LUPP2486, LUPP2497 and LUPP2501 were molecularly identified as *I. liriodendri*. All three of the isolate colonies in this study have the same colony colour and pattern, however they do not match up with what has been published. Published results have found that *I. liriodendri* are cinnamon and sepia in colour and the isolates in this study were honey and buff (Pathrose, 2012). The macroconidia in this study were also one or two septate and 15 to 25 μm in length in comparison to three septate and 35 to 40 μm in length (Pathrose, 2012). Two isolates (LUPP2485 and LUPP2498) were molecularly identified as *I. europaea*. In the study the two isolates look very similar to each other, but the morphology is different to what has been published (Cabral, Groenewald, et al., 2012). The colony colour as reported by Cabral et al. (2012) was sienna to saffron, whereas in this study *I. europaea* was umber, cinnamon and hazel. The length of the macroconidia in this study were one septate and on average 15 μm in length, where as the published description of *I. europaea* states that they are one to three septate and are 21 to 24 μm in length for one septate macroconidia (Cabral, Groenewald, et al., 2012).

Lastly, isolate LUPP2505 was molecularly identified as *Calonectria pacifica*. In the study the morphological colony description is different to what has been published (Kang et al., 2001). The colony colour reported by Kang et al., (2001) was luteous to sienna however, in this experiment the colour was sepia to cinnamon. The macroconidia size reported is also larger ranging from 55 – 66 μm in length and 4N5 μm in width (Kang et al., 2001). This is compared to the results of this study where the macroconidia size was 26.88 μm in length and 2.86 μm in width.
Even though all the isolates within the same species look morphologically similar to each other, using morphological identification to determine an unknown species identity is not very accurate. In this study as previously mentioned the colony colours and conidia size that results from one study may vary to that of another study. These differences in morphology identification; particularly the colour of the colony can vary between the people identifying the colony as the colour charts are used for identification could be different or what one person classes as a particular colour another person may class as another colour. To try and minimise this variation between studies, the same colour chart (Rayner, Mycological colour charts) as used by Cabral et al. (2012) was used in the present study. However, differences were still seen between the colony colours for the isolates of the same species between the two studies. A possible reason for the differences in colony morphological identification could be the conditions in which the isolates were grown. In this study the isolates were incubated in 12 hours light and 12 hours dark at 20°C for 14 days. In the study by Cabral et al. (2012) the isolates were incubated at 20°C for 14 days in 24 hours dark. As the isolates in this study were exposed to light this could have been a factor responsible for the differences observed in fungi colour between the species in this study and published species. The amount of PDA nutrient agar available to the fungal colonies may have had an influence on the colour of the colonies, as there may have been more or less PDA agar poured into the Petri dishes in this study in comparison to published studies. These isolates were also stacked on top of each other in the incubator. This stacking of the plates could have reduced the amount of oxygen available to the isolates and caused variation in the colour of the colony. The isolates were grown at the same temperatures and for the same length of time, so these factors are probably not responsible for the changes in colour morphology.

For many of the species identified in this study the macroconidia were smaller in length and had fewer septate than what has been previously published. In this study the same nutrient agar (SNA) was used to determine the characteristics of macroconidia and the presence or absence of microconidia. In this study however, the isolates growing on SNA were only incubated for two weeks whereas in the published studies the isolates were growing for up to five weeks (Cabral, Groenewald, et al., 2012). This longer growth period would have allowed for the macroconidia to mature and develop more in size and for more macroconidia to be present. The method used in this study to observe the conidia was through production of a spore suspension with aliquots of this then placed onto the glass slide, from which measurements of the macroconidia was taken using a microscope with a camera attached. In the study by Cabral et al. (2012) a 1Ncm² agar plug was removed from the colony margin and placed on a microscope slide with a drop of water on top and measurements taken of the macroconidia. This method used by Cabral et al. (2012) would have resulted in a higher number of macroconidia present to take measurements from and given a better representation of
the macroconidia and microconidia present. With the spore suspension method used in this study there were few macroconidia present and for some isolates it was difficult to find fifteen macroconidia to take measurements from. This could have been as a result of the isolates not having enough time to develop macroconidia in the incubator or that the volume of liquid used to prepare the conidial suspension was too high.

A limitation to this study was the number of replicates for each of the isolates. If this study was repeated again, at least 10 replicates for each isolate should be investigated to enable a true representative of the colony morphology to be represented. Only one nutrient agar was used to grow the isolates on to determine the colour and texture morphology of the isolates in this study. In all other morphological studies two nutrient medias have been used to give a more accurate representation of the colour morphology of the isolates (Cabral, Groenewald, et al., 2012; Pathrose, 2012). If this study was repeated the use of two growing media should be used to gain a greater understanding of the colony morphology. The way that the replicates were arranged in the incubator is thought to have had an effect on the colony colour and texture in this study. To try eliminate the effect of stacking on the colony morphology, using a bigger incubator or spreading the replicates out in the incubator so that there is less stacking. This would allow more even distribution of light and oxygen to all of the isolate replicates.

### 4.2.2 Colony diameter

In Figure 3.19 there was significant differences seen at each assessment day between the isolates. This significant difference was a result of there being six different species compared and not all the species having the same diameter growth between the assessment days. The results in Figure 3.20 are able to separate the differences in colony diameter between the species explaining the significant differences found when all the isolates were compared irrespective of species identity. The species that had the smallest diameter over all the assessment days are *I. macrodidyma* and *I. liriodendri*. These two species were found to be in different clusters than *I. europaea*, *I. liligena* and *I. robusta* in the phylogenetic tree based on the histone H3 gene sequences (Figure 3.4). This is because *I. europaea*, *I. liligena* and *I. robusta* are all part of the *I. radicicola* complex whereas, *I. macrodidyma* and *I. liriodendri* are not part of this species complex. In other studies *I. macrodidyma* and *I. liriodendri* after seven days were reported to have a colony diameter of 30 to 45 mm (Halleen et al., 2004; Halleen, Schroers, et al., 2006). This is smaller than for colonies of the three species in the *I. radicicola* complex that range in diameter from 35 to 52 mm after 7 days (Cabral, Groenewald, et al., 2012). Within this complex however, it was found that *I. robusta* had the largest diameter followed by *I. europaea* (Cabral, Groenewald, et al., 2012). However, in the presents study *I. europaea* had the larger diameter out of the two species. These species are very closely related and
because there was only one isolate identified as *I. robusta* from these unknown isolates there was not a true representation of the diversity of *I. robusta* isolates in this study. For *I. europaea* there were two isolates identified from the unknown isolates, of which one had slightly larger diameter growth from day 7 through to day 10. *Calonectria pacifica* had high colony diameters at each of the four assessment days. There is very little information on *C. pacifica*, however from information about other closely related species of *Calonectria* they all appear to be fast growing (Crous et al., 2004). This could explain why *C. pacifica* had one of the largest colony diameters of the six species studied. The rate of growth was the highest from day 3 to day 10, with the colony diameter at least doubling for *I. robusta*, *I. europaea*, *I. liligena* and *C. pacifica* over this time.

There were five unknown isolates identified as *I. liligena*. The colony growth results showed that of the five *I. liligena* isolates LUPP2503 has a significantly smaller colony diameter than the other four isolates. Based on the colony characteristics and the results of the histone H3 phylogenetic tree isolate LUPP2503 is different to the other four isolates. The colour and pattern of isolate LUPP2503 was very different to the other identified *I. liligena* species and when clustered in a phylogenetic tree it was separate from the other four isolates. Currently *I. liligena* has only been isolated from *Lilium* bulbs in the Netherlands (Cabral, Groenewald, et al., 2012). The four New Zealand isolates of *I. liligena* that are the same may be a new species closely related to *I. liligena* that has adapted to living on a new host such as avocado in this case. Isolate LUPP2503 that is different from the other four isolates could be a variation between the representative international *I. liligena* isolate and the other four New Zealand isolates. As the colony diameter of LUPP2503 is much smaller in comparison to the other isolates, this form of *I. liligena* species could be less pathogenic or easier to control than the other isolates as it has a low growth rate. However this would need to be confirmed on lower nutrient agar, which would more closely represent the lower nutrient availability common in soil. The results of the colony growth rate support the findings that LUPP2503 was a sister group to the other *I. liligena* isolates (LUPP2482, LUPP2483, LUPP2490 and LUPP2493) identified in this study. All these isolates were isolated from avocado roots obtained from either zone two or three and were each taken from a separate block within each zone within the orchard. Since the four other *I. liligena* isolates were all identified with high bootstrap values despite being isolated from different blocks, the location of LUPP2503 does not explain the difference in the identity of this isolate. It does however indicate that a high diversity of species were associated with the root lesions recovered in the orchard.

For the isolates (LUPP2485 and LUPP2498) identified by sequencing as *I. europaea* there was a very high similarity in the histone H3 phylogenetic tree. For this reason the results of the colony growth rate is unexpected. On assessment day 7 and day 10 colonies of LUPP2498 were approximately 10 mm larger in size than those of LUPP2485 but by assessment day 14 the colony diameters were the
same. One possible reason for this result could be how much light, nutrients and oxygen the replicate colonies of each species were exposed to. For assessment day 7 and day 10 isolate LUPP2498 may have consumed the nutrients available faster thus the colony grew quicker to get access to more nutrients to survive. However, other researchers have also reported high variability in the growth parameters of isolates within a species for *Ilyonectria* sp. (Pathrose, 2012) and these isolates might represent the natural variability between isolates of this species. Similarly, a difference although smaller was observed in the colony size for *I. liriodendri* isolates and again could be due to the positions of the isolates in the incubator and how much light they were exposure to, or due to natural variation between isolates of this species.

As discussed for colony colour and conidia size if this were repeated the use of more replicates would give a better representation of the colony diameter. There were also a very small number of isolates compared in this study. If more isolates were sourced from avocado roots it may lead to the identification of more *I. macrodidyma, I. robusta* and *C. pacifica* isolates, allowing better conclusions about the colony morphology.

### 4.3 Pathogenicity of *Ilyonectria* and *Calonectria* isolates on avocado roots using a detached root assay

In this study, the pathogenicity of *Ilyonectria* and *Calonectria* isolates on avocado roots was unable to be determined. When the detached root assay was performed it took six days for lesions to develop on the avocado roots. The quick development of lesions on the avocado roots was not expected. In the past when a detached root assay has been carried out using *Ilyonectria* spp. on grapevine it had taken one month for lesions to develop on the roots (Outram, 2013; Pathrose, 2012). This indicates that avocado roots could be more susceptible to *Ilyonectria* and *Calonectria*. The avocado roots were quite fleshy and easy to break in comparison to grapevine roots and therefore may be more susceptible to pathogen infection.

In this detached assay however, lesions developed on the uninoculated control avocado roots for all three avocado rootstocks although these were smaller than the lesions that developed on the inoculated roots (Table 3.1). The development of lesions on the uninoculated controls meant that there was no way to differentiate between the lesions which developed due to inoculation of the fungal isolates and those that developed due to other factors, including background infection, as seen on the uninoculated control. This meant that no statistical analysis was carried out on the results. In order to confirm pathogenicity of the isolates and complete Koch’s Postulates, reN isolations were carried out from the lesions that developed on the roots and into the apparently healthy tissue. These results showed that *Ilyonectria* type colonies were isolated from lesions on both the inoculated and the uninoculated control roots for all three of the avocado rootstocks (Table
From the Bounty® rootstock inoculated with *C. pacifica*, isolates morphologically similar to the inoculated isolate was recovered from three of the five replicates. This result suggests that Bounty® rootstocks could be susceptible to *C. pacifica*. For the replicates where *Ilyonectria* type colonies were not isolated from other fungi such as *Trichoderma* spp and *Penicillium* spp. were recovered and as previously mentioned these are typical inhabitants of potting mix soil (Table 3.2, Table 3.3 and Table 3.4) (Dr Eirian Jones, Pers. Comm.).

At some point the avocado rootstocks were cross-contaminated and infected with an *Ilyonectria* type fungus. The three avocado rootstocks were sourced from two different nurseries, therefore it is probably unlikely that the cross-contamination occurred at the nursery. If cross-contamination did occur at the nursery then it would be expected that not all of the three rootstocks would be contaminated by *Ilyonectria* type colonies. Since the assay for each of the rootstocks were set up on separate days with the roots from different rootstocks selected and processed on different days, the rootstocks could not have contaminated each other with the *Ilyonectria* type fungus. Further, when the inoculation of the roots using mycelial/agar plugs was carried out, the uninoculated control was done first and all tools used were sterilised using 96% ethanol before and after using them on the uninoculated PDA and each mycelial plug. As the whole method involved using sterile tools and was carried out using standard aseptic techniques, cross-contamination was unlikely to have occurred at any stage in the set up of the detached root assay.

Where cross-contamination was likely to have occurred was in the glasshouse where the avocado rootstocks were grown and left to develop abundant healthy roots. The glasshouse where the avocado rootstocks were grown is where past experiments have been carried out on grapevines to determine the pathogenicity and epidemiology of *Ilyonectria* species. As the grapevines were inoculated with different *Ilyonectria* species in the glasshouse, this is where the cross-contamination was likely to come from. There was a large amount of grapevine debris around where the avocado rootstocks were placed and there were grapevines growing parallel to the avocado rootstocks. In addition, it has been reported (Bleach, 2013; Probst, 2011) that rootstocks and grafted grapevines obtained from nurseries are often infected with *Ilyonectria* spp. and this may have been the case with the grapevine plants growing in close proximity to the avocado plants. The grapevines and avocado rootstocks were watered using a garden hose, which may have resulted in splash dispersal of *Ilyonectria* spores between pots, and there were no collection trays under any of the pots that both plant species were growing in. This is likely how the *Ilyonectria* species was able to colonise the avocado rootstocks as *Ilyonectria* species spores are known to be spread through movement of free water (Jaspers, 2013). As the avocado rootstock roots were unhealthy due to waterlogging, they may have been more susceptible to the *Ilyonectria* species pathogen as has been shown for grapevines under waterlogged conditions (Probst, 2011). When the roots were selected from rootstocks...
Bounty® and Duke 7 for the detached root assay, it was difficult to find roots that looked healthy, with many having blackish/brown small lesions on the root. After the detached root assay was set up the replicates were placed in the incubator the *Ilyonectria* species pathogen which would have been optimal conditions for growth and lesion development on the roots, especially since the roots were detached from the plant reducing their natural resistance to pathogenic infection. Further all the roots were wounded by removing the tip prior to inoculation and this again would be likely to promote the development of any fungi present on or in the roots.

To examine the pathogenicity of *Ilyonectria* and *Calonectria* species on avocado in the future, the rootstocks should be grown in a glasshouse that contains no other hosts of *Ilyonectria* and *Calonectria*, and where experiments using these species have not been conducted. As avocado roots are more fragile than grapevine roots, perhaps too much handling of the roots increased the susceptibility to *Ilyonectria* and *Calonectria* species. The determine the pathogenicity of *Ilyonectria* and *Calonectria* species instead of the detached root assay used in this study, the method used by Vitale *et al.* (2012) including whole *Persea americana* seedlings, may give a better indication of the pathogenicity of the *Ilyonectria* and *Calonectria* species isolated from symptomatic New Zealand avocado roots.
## Appendix A

Replace the content of this page with your own content. The appendix heading uses the ‘App1’ style.

### A.1 List of isolates used in this study

<table>
<thead>
<tr>
<th>Code</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUPP2480</td>
<td>Ilyonectria</td>
<td>macrodidyma</td>
</tr>
<tr>
<td>LUPP2482</td>
<td>Ilyonectria</td>
<td>liligena</td>
</tr>
<tr>
<td>LUPP2483</td>
<td>Ilyonectria</td>
<td>liligena</td>
</tr>
<tr>
<td>LUPP2484</td>
<td>Ilyonectria</td>
<td>robusta</td>
</tr>
<tr>
<td>LUPP2485</td>
<td>Ilyonectria</td>
<td>europaea</td>
</tr>
<tr>
<td>LUPP2486</td>
<td>Ilyonectria</td>
<td>liriodendri</td>
</tr>
<tr>
<td>LUPP2490</td>
<td>Ilyonectria</td>
<td>liligena</td>
</tr>
<tr>
<td>LUPP2493</td>
<td>Ilyonectria</td>
<td>liligena</td>
</tr>
<tr>
<td>LUPP2497</td>
<td>Ilyonectria</td>
<td>liriodendri</td>
</tr>
<tr>
<td>LUPP2498</td>
<td>Ilyonectria</td>
<td>europaea</td>
</tr>
<tr>
<td>LUPP2501</td>
<td>Ilyonectria</td>
<td>liriodendri</td>
</tr>
<tr>
<td>LUPP2503</td>
<td>Ilyonectria</td>
<td>liligena</td>
</tr>
<tr>
<td>LUPP2505</td>
<td>Calonectria</td>
<td>pacifica</td>
</tr>
</tbody>
</table>

### A.2 0.9% Agarose gel picture with labelled band sizes of the molecular ladder used in this study

Figure removed due to copyright.  
Appendix B

B.1 Media Recipes

B.1.1 Potato Dextrose Agar (PDA)

39 g potato dextrose agar (Difco Laboratories, USA) was added to 1 L of distilled water and sterilised by autoclaving at 121°C for 15 minutes. The agar was cooled at 50°C and then poured into sterile Petri dishes and allowed to solidify.

B.1.2. Spezieller Nährstoffarmer Agar (SNA)

All ingredients except agar were dissolved in 1 L of SDW and the pH adjusted to 6N6.5. The agar was added and dissolved before the solution was autoclaved (20 min at 121°C and 15 psi) and allowed to cool to 50°C (Nirenberg, 1976 in Brayford, 1993). 1.0 g KH2PO4, 1.0 g KNO3, 500 mg MgSO4.7H2O, 500 mg KCl, 200 mg Glucose, 200 mg Sucrose, 20 g Agar, 1 L Distilled water.

B.2 Solutions

B.2.1 1x TAE buffer

40 mM acetic acid, 20 mM acetic acid, and 1 mM EDTA
Appendix C

C.1 List of isolates used for the detached root assay

<table>
<thead>
<tr>
<th>Code</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUPP2484</td>
<td><em>Ilyonectria</em></td>
<td>robusta</td>
</tr>
<tr>
<td>LUPP2486</td>
<td><em>Ilyonectria</em></td>
<td><em>liriodendri</em></td>
</tr>
<tr>
<td>LUPP2493</td>
<td><em>Ilyonectria</em></td>
<td><em>liliigena</em></td>
</tr>
<tr>
<td>LUPP2498</td>
<td><em>Ilyonectria</em></td>
<td><em>europaea</em></td>
</tr>
<tr>
<td>LUPP2505</td>
<td><em>Calonectria</em></td>
<td><em>pacific</em>a</td>
</tr>
</tbody>
</table>
References


Limited, R. N. (2014). Avocado rootstock characteristics


Outram, M. A. (2013). *Identification of the individual species within the Ilyonectria macrodidiyma complex that cause black foot disease of grapevines in New Zealand*. (Bachelor of Science (Honours)), Lincoln University.


: Commonwealth Mycological Institute, British Mycological Society.


