Characterising sub-species variation in New Zealand *Cylindrocarpon* species that cause black foot of grapevines

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Blessy Pathrose

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Blessy Pathrose

Black foot disease, caused by species of *Cylindrocarpon,* is a significant problem in New Zealand and throughout the world. From a 2005 study of symptomatic grapevine material contributed by 49 grape growers encompassing eight grape growing areas, 174 cultures of *Cylindrocarpon*-like isolates were recovered. The isolates were identified using a combination of morphological grouping, species specific PCR and sequencing of taxonomically informative genes. The collection contained five species of *Cylindrocarpon*, namely, *C. liriodendri* (57 isolates), *C. destructans* (53 isolates), *C. macrodidymum* (41 isolates),*C. pauciseptatum* (11 isolates) and *Cylindrocarpon* sp. (4 isolates).

All three of the main *Cylindrocarpon* species reported worldwide were found throughout New Zealand, however, they differed in their distribution. A high proportion of the isolates recovered in the South Island were *C. destructans* (39%, n=46/118) as compared to the North Island (17%, n=8/48). A high proportion of the isolates recovered in the North Island were *C. macrodidymum* (45%, n=22/48) as compared to South Island (17%, n=20/118). The proportion of *C. liriodendri* isolates recovered from the North Island (27%, n=13/48) and from South Island (37%, n=44/118) were similar. Similar numbers of *C. pauciseptatum* and *Cylindrocarpon* sp. were isolated from both islands. The distribution of the three main species correlated with optimal temperature for growth where *C. destructans* had the lowest optima of 17.7°C and *C. macrodidymum* had the highest optima of 19.3°C ($P<0.05$).

The data generated from UP-PCR analysis of the three main *Cylindrocarpon* species was used to create a neighbour joining tree which showed high inter- and intra-vineyard diversity, with few clonal isolates identified. The isolates in the neighbour joining trees formed four clades for *C. liriodendri* and *C. destructans* and six clades for *C. macrodidymum*. Nei's measure of genetic diversity for the *C. destructans*, *C. liriodendri* and *C. macrodidymum* populations were H=0.3346, 0.1816 and 0.2580, respectively. Vegetative compatibility tests with seven *C. destructans*, nine *C. liriodendri* and eight *C. macrodidymum* isolates selected from different branches of the neighbour joining tree produced three VCGs for *C.destructans* and two VCGs for both *C. liriodendri* and *C. macrodidymum*. Only for *C. destructans* did pairing of isolates give rise to an interaction characterised as partially incompatible. Microscopic analysis of compatible reactions for the three species revealed the presence of

hyphal anastomoses within and between the isolates and the formation of chlamydospores at the interaction zone of *C. destructans* isolates.

Virulence varied between genetically different isolates of *C. destructans*, *C. liriodendri* and *C.macrodidymum* on detached root assay and potted vine assay. In the detached root assay 17, 15 and 14 isolates of *C. liriodendri*, *C. destructans* and *C. macrodidymum* were analysed; with isolate Wpa1c of *C. liriodendri* producing the largest lesion and isolate Ack2d of *C. destructans* producing the smallest lesion. There were differences in the appearance of lesions with *C. macrodidymum* producing more mascerated root tissue than the other two species. In the potted vine assay, the furthest recovery at 3 and 6 cm was from vines infected by isolates of *C. macrodidymum*. At 8 cm isolates of both *C. liriodendri* and *C. macrodidymum* were recovered in similar proportions. In general, the virulence showed by isolates of *Cylindrocarpon* species on detached roots correlated with their abilities to move up the stem bases in the potted vine assay. No relationship was found between genetic groups and virulence. Attempts to produce a transformation system for *Cylindrocarpon* isolates by *Agrobacterium tumefaciens* or protoplast mediated transformation were unsuccessful.

Biochemical analysis using qualitative and quantitative assays showed that all three main species produced both laccase and acid protease *in vitro*. Among the three species, *C. macrodidymum* produced laccase activity that was up to 10 times greater than that of the other two species (P=0.000). For acid protease both *C. destructans* and *C. macrodidymum* produced greater activity than *C. liriodendri* (P=0.000). For both enzymes it was unclear whether greater activity was due to more enzyme or more active enzyme being produced. Using degenerate PCR the genes encoding lcc1 laccase and acid protease of the three *Cylindrocarpon* species were isolated. For the *lcc1* gene 519 bp (93%) of the predicted 558 bp gene was isolated and this was translated into a 173 residue polypeptide. The predicted amino acids showed variation between species and isolates with variable enzyme activity. Of the 31 polymorphic residues identified six non-conservative changes produced between species and eight produced non-conservative changes in isolates; and only one of these (Δ86D→R) affected a residue at a site conserved in laccase enzymes. For the acid protease gene 280 bp (87%) of the predicted 319 bp coding sequence was amplified and sequenced. The 85 residue predicted amino acid sequence showed no variation between isolates with differing acid protease activity.

Overall the study demonstrated substantial sub-species variation in genotype, virulence and enzyme production by species of *Cylindrocarpon* present in New Zealand vineyards.

Keywords: *Cylindrocarpon*, UP-PCR, genetic diversity, pathogenicity, transformation, enzymes.

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Chapter 1
Introduction

1.1 Viticulture and wine production in New Zealand

The grapevine is a fruit species of very ancient origins. From the Caucasian area, its cradle of origin, it spread first in the Mediterranean and later across the world. Grapes for wine making were first planted in New Zealand in the early nineteenth century by French settlers and religious missions (Jackson and Schuster, 1994). In New Zealand, grape production currently extends throughout the country and in 2011; the national vineyard area occupied 33,600 hectares (New Zealand Winegrowers Annual Statistics, 2011) and produced 328,000 tonnes of grapes. In the same year, about 235 million litres of wine was produced, of which nearly half was exported at a value of NZ$ 1,094 million (New Zealand Winegrowers Statistical Annual, 2011). Although the New Zealand wine industry has expanded rapidly, with the 2011 harvest almost four times that of ten years ago, New Zealand only plays a small part in the world wine market with about 1% of global production. The majority of wine is produced in ten major locations: Northland, Auckland, Bay of Plenty, Hawkes Bay, Gisborne, Wellington, Nelson, Marlborough, Canterbury and Central Otago. Gisborne, Hawkes Bay and Marlborough account for 90% of total grape production. These statistical data emphasise the importance of the viticulture and wine industry to New Zealand's economy.

The major varieties of grapevine (*Vitis vinifera*) in New Zealand are the white varieties Sauvignon Blanc, Chardonnay and Riesling and the red varieties, Pinot Noir, Cabernet Sauvignon and Merlot. The New Zealand viticulture industry's belief in the appropriateness of Pinot Noir to New Zealand's climate, grown not just in the smaller regions like Martinborough and Central Otago, but also on a bigger scale, has led in recent times to increased plantings in Marlborough. At present, the white wine grapes, Sauvignon blanc and Chardonnay dominate by occupying 51% and 11% of the total producing vineyard area, respectively. On the other hand, Pinot noir, is the dominant red variety (14%) and is followed by Merlot (4%) (New Zealand Winegrowers Statistical Annual report, 2011).The major New Zealand wine export varieties in 2011 were Sauvignon Blanc followed by Pinot Noir, Chardonnay, Pinot Gris and Merlot.

1.2 Grapevine rootstock varieties

In 1862, the importation of rooted vines from North America introduced phylloxera into Europe. By the end of the nineteenth century, the pest had caused complete destruction of the vineyards in southern France. Between 1885 and 1900, a significant effort to develop

resistant rootstock cultivars was made after the discovery by European investigators that native American *Vitis* species were resistant to the insect (Lider *et al.*, 1995). This led to the widespread practice of grafting scion wood to resistant rootstock. In 1895, when phylloxera was found in New Zealand soils, Romeo Bragato introduced grafted grapevines into New Zealand (Anonymous-Romeo Bragato Conference, 2010). Most of the grapevines in New Zealand (94%) were reported to be grafted onto *Phylloxera* resistant rootstock in 2011 (New Zealand Wine growers Statistical Annual, 2011).The primary rootstock varieties used in New Zealand are *V. riparia* (Riparia Gloire B), *V. riparia* x *V. rupestris* (3309 C, 101-14, Schwarzmann), *V. riparia* x *V. rupestris* x *V. berlandieri* (Gravesac), *V. berlandieri* x *V. riparia* (SO4, 5C, 420 A, 5 BB), *V. rupestris* x *V. berlandieri* (110 R, 1103 P) (Morton, 1995).

Research on rootstock varieties has mainly concentrated on their resistance to phylloxera and nematodes, their scion vigour, starch storage, nutrient uptake, suitability for soil types, adaptability to high or low soil pH, soil salinity and lime, as well as drought resistance. However, Scheck *et al*. (1998b) reported that vine decline, Petri disease and black foot were becoming more common on the rootstocks planted for *Phylloxera* control. More recently, research has investigated the susceptibility of grapevine rootstocks and varieties to several important trunk and root pathogens such as *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Eutypa lata* and *Armillaria mellea.* The results have shown that different levels of susceptibility exist amongst the rootstocks studied (Eskalen *et al.*, 2001; Feliciano *et al.*, 2004; Baumgartner and Rizzo, 2006; Sosnowski *et al.*, 2007). However, rootstock susceptibility to infection by *Cylindrocarpon* spp. (black foot) has not been well studied (Halleen *et al.*, 2006b). Rego *et al*. (2000) reported severe outbreaks of the disease, resulting in decline symptoms in grapevine and table grape cultivars grafted onto 101-14, 140 Ru, 161-49, 99 R, 1103 P and 5BB. Research by Gubler *et al*. (2004) found that rootstocks 101-14, 3309, 5 C and 110 R showed increased susceptibility to *Cylindrocarpon destructans*, compared with Freedom, 039-16 and *Vitis riparia,* which showed good resistance to this pathogen. In New Zealand, preliminary work conducted by Harvey and Jaspers (2006) assessed grapevine rootstocks commonly used in New Zealand for their susceptibility to *Cylindrocarpon* spp. under greenhouse conditions. Their results showed that all rootstock varieties included in their study were susceptible to *Cylindrocarpon* spp. to some degree, although Rupestris St George, RipariaGloire, R 140 and 3309 were least susceptible. Bonfiglioli (2005) reported that the rootstock 101-14, which was commonly used in New Zealand at that time, appeared to be more susceptible to black foot than some other rootstocks, which concurred with the results of Harvey and Jaspers, 2006.

1.3 Grapevine trunk diseases

Over the last 10-15 years, reduced survival rates of vines have been reported due to the effects of decline diseases in nurseries and in young vineyards (Oliveira *et al.*, 2004) worldwide. These diseases can affect the trunks or the roots of the grapevines and are most commonly caused by fungal pathogens. Diseased plants show symptoms, which include stunting, chlorosis, delayed budding and poor growth. Therefore, with a better awareness of grapevine diseases that may affect the long life of vineyards in New Zealand, it was considered suitable to present the summary of recent information of the trunk disease and their control methods. The diseases discussed here are Petri disease, esca, eutypa dieback and botryosphaeriaceae dieback. Petri disease, caused by *Phaeomoniella chlamydospora*, as well as several species of *Phaeoacremonium* is a trunk disease that has been implicated as a major contributor to the decline of young vines throughout the world (Halleen *et al.*, 2003; Fourie and Halleen, 2004). Esca is a disease complex that is also caused by Basidiomycota fungi, which often includes the pathogens of Petri disease; it causes structural and physiological changes in mature grapevines plants and ultimately their death (Mugnai *et al.*, 1999; Feliciano *et al.*, 2004). The pathogen, *Eutypa lata*, which causes Eutypa dieback, has also been studied extensively since it produces characteristic disease symptoms of dieback, cankers and wedge shaped staining in trunks or cordons (Pascoe, 1998). Foliar symptoms, caused by translocatable toxins produced by *E. lata* in the vascular tissue, include the dwarfing of internodes, tattering, chlorosis and necrosis of the leaves, and the eventual death of infected cordons (Lardner *et al.*, 2005). These symptoms have been associated with serious economic losses in many major grape producing countries (Mahoney *et al.*, 2003). For many years, these types of symptoms were thought to be solely due to *E. lata*, however, surveys in the Hunter Valley and subsequent laboratory studies conducted in 2002-2003 revealed that *E. lata* was not likely to be the main cause of these characteristic wood symptoms (Creaser *et al.*, 2003). Instead, *Botryosphaeriaceae* fungi were the most common species isolated from the trunks in almost all Australian vineyards surveyed (Creaser *et al.*, 2003). In New Zealand, the *Botryosphaeriaceae* species were isolated from most of the dark and discoloured grapevine wood found in a nation-wide survey with only rare isolations of *Eutypa* spp. (Baskarathevan *et al.*, 2012a).

Another trunk disease which rots the trunk base and roots of grapevines throughout the world is commonly known as ‘black foot’.

1.4 Black foot disease of grapevines

Decline symptoms in young grapevines, often followed by their death, were reported by growers throughout the world from the early 1990s, and this occurred mainly in sites converted from orchards or replanted from grapes (Oliveira *et al.*, 2004; Bonfiglioli, 2005). In

California and Brazil, decline was mainly observed on phylloxera resistant rootstocks (Scheck *et al.*, 1998b; Garrido *et al.*, 2004a, 2004b). The most commonly isolated fungi from symptomatic plants were *Phaeomoniella chlamydospora, Phaeoacremonium* species and *Cylindrocarpon* species, which have been identified as the causal agents of black foot disease (Oliveira *et al.*, 2004), with plants frequently being infected by more than one pathogen (Rego *et al.*, 2000; Armengol *et al.*, 2001; Halleen *et al.*, 2003; Oliveira *et al.*, 2004). Since then, black foot has been recognised as a serious threat to grapevines worldwide.

Black foot disease has been identified as a common cause of vine death in young vineyards, and in nursery sites where it contributes to the losses of grafted plants (Halleen *et al.*, 2004). However, it also occurs in mature vines and has been shown to cause the death of mother vines (Gubler *et al.*, 2004). This disease occurs in all major viticulture regions throughout the world. It was reported to be present in many of the young grapevines grown in California (Scheck *et al.*, 1998b), in France where losses of 50% or more were recorded in 2-8 year old vineyards (Larignon, 1999), Portugal (Rego *et al.*, 2000).

A number of *Cylindrocarpon* and related species have been reported to cause black foot. *Cylindrocarpon destructans* was recorded on grapevines in France in 1961 (Maluta and Larignon, 1991), Tasmania (Sweetingham, 1983), Italy (Grasso, 1984), Portugal (Rego *et al*., 2000) and New Zealand (Bleach *et al.*, 2007). In California, *C. destructans* and *C. obtusisporum* were reported as the causative agents of black foot disease of grapevines (Scheck *et al*., 1998a). In Sicily, *C. obtusisporum* was also identified as the causal agent of black foot disease (Grasso and Magnano Di San Lio, 1975). Various unidentified species of *Cylindrocarpon* have also been isolated from young grapevines and from declining vines with basal rot or root necrosis in Australia (Edwards and Pascoe, 2004) and South Africa (Fourie *et al.*, 2001).

In a recent New Zealand survey of declining vines, three different species were identified: *C. destructans*, *C. macrodidymum* and *C. liriodendri* (Bleach *et al.*, 2006). This New Zealand survey of declining vines, done in 2005, collected plant material from eight major grape growing areas (Bleach *et al.*, 2006) and isolated approximately 200 *Cylindrocarpon*-like isolates from 87% of the 121 symptomatic vines collected. These isolates were sent to Stellenbosch University (South Africa), where 60 isolates were selected for identification by their morphology and β-tubulin sequence. They were identified by Mostert *et al*. (2006b) as: *C. destructans* (35.6%), *C. macrodidymum* (30.5%) and *C. liriodendri* (27.1%) and at least one novel species (6.8%).

1.5 Taxonomy of *Cylindrocarpon* species

The generic name *Cylindrocarpon* was introduced by (Wollenweber, 1913). Teleomorphs with *Cylindrocarpon* anamorphs were traditionally classified in Nectria (Fr.) Fr.(Mantiri *et al.*, 2001) reported that the genus *Cylindrocarpon* contains approximately125 *Cylindrocarpon* spp. and varieties of this species have been described (Booth, 1966; Schroers *et al.*, 2008). Until recently, the *Cylindrocarpon* species identification and classification were based on their morphological characters in pure culture (Brayford, 1992). Booth (1966) grouped *Cylindrocarpon* species based on the presence or absence of chlamydospores and microconidia. The first group in which the chlamydospores are present and the microconida are absent, was represented by *Cylindrocarpon magnusianum* (Sacc.) Wollenw., which is the anamorph of the type species of *Neonectria* Wollenw. The second group was represented by *C. cylindroides* Wollenw. (lacks both chlamydospores and microconidia) which is the type species of the genus *Cylindrocarpon.* The members of *Cylindrocarpon* species connected with teleomorphs of *Nectria mammoidea* W. Phillips and Plowr. group (microconidia present and chlamydospores absent) represent the third group. These were the core members of the three anamorphic groups delineated by Booth (1966).

The reintroduction of *Neonectria* resulted from the realisation that *Nectria* was too broadly defined and that its segregation into numerous teleomorphic genera could be corroborated by anamorphic, phylogenetic and ecological characteristic patterns (Rehner and Samuels, 1995; Rossman *et al.*, 1999). *Cylindrocarpon destructans* was then considered to represent a fourth group, being the anamorph of *Neonectria radicicola*, which produces both chlamydospores and microconida. All the *Nectria* group representatives with anamorphs of *Cylindrocarpon* were transferred into *Neonectria* (Rossman *et al*., 1999; Mantiri *et al*., 2001; Brayford *et al.*, 2004).

Despite these attempts to resolve the taxonomy of the *Cylindrocarpon* species, (Halleen *et al.*, 2004) reported significant differences between isolates from the same species had been observed in New Zealand, South Africa, France and Australia. Their study included multiple isolates of four *Cylindrocarpon* and *Cylindrocarpon*-like taxa with which they examined morphological characters and completed phylogenetic analyses. They acknowledged three main groups. The first group of isolates was described as a new species, called *Cylindrocarpon macrodidymum* (*Neonectria macrodidyma* Halleen, Schroers and Crous). The genus *Campylocarpon* (Halleen, Schroers and Crous described from South Africa) was defined as a second group. The *C. destructans*/*Neonectria radicicola* species complex (Samuels and Brayford, 1990; Seifert *et al.*, 2003) was included in the third group. This third group had been found to be a collection of closely related phylogenetic species based on studies done from coniferous and angiosperm isolates (Seifert *et al*., 2003). Later, Halleen *et al*. (2006b) studied the isolates which were morphologically similar to the

C. destructans/*Neonectria radicicola* complex and so had been identified as such, and concluded that many of these isolates from grapevines should be attributed to a fourth species, *Neonectria liriodendri*, which is the teleomorph of *C. liriodendri*, a species reported previously to be associated with root rot of tulip poplar in the USA (Mac Donald and Butler, 1981). *Cylindrocarpon liriodendri* was reported in grapevines from France, Portugal, South Africa (Halleen *et al.*, 2006b), Australia (Whitelaw-Weckert *et al.*, 2007), Spain (Alaniz *et al.*, 2007) and Iran (Mohammadi *et al.*, 2009). In recent times, another species associated with grapevines, named, *Cylindrocarpon pauciseptatum* was reported in Slovenia, (Schroers *et al*., 2008). This species has also been isolated from roots of *Vitis* spp. in New Zealand (Schroers *et al*., 2008). However, its pathogenicity to grapevines has not been confirmed.

In a recent taxonomic study using the internally transcribed spacer (ITS) of the ribosomal RNA (rRNA) and β-tubulin gene sequences of *Cylindrocarpon* species associated with black foot disease of grapevines, three species were identified as the causative organisms, namely *C. destructans*, *C. macrodidymum* and *C. liriodendri* (Halleen *et al.*, 2004;Halleen *et al.*, 2006a; 2006b; Mostert *et al.*, 2006b). *Cylindrocarpon macrodidymum* has been recently reported in New Zealand, Australia and South Africa (Petit and Gubler, 2005). Furthermore, the South African study by Halleen *et al*. (2006b) found that many of the species previously identified by morphological characteristics as *Cylindrocarpon destructans* were more correctly reidentified by molecular techniques as *Cylindrocarpon liriodendri,* including the Portuguese isolates used by Rego *et al*. (2000). Subsequently, *Cylindrocarpon liriodendri* was reported by Alaniz *et al.*(2009b) in Spain and by Whitelaw-Weckert *et al*. (2007) in Australia. Halleen *et al*. (2004) reported a 'sister' group of *Cylindrocarpon* called *Campylocarpon*; *C. fasciculare* and *C. pesudofasciculare.* These two species were found in Australian, South African and Uruguayan vineyards (Abreo *et al.*, 2010).

Following the end of the experimental phase and during the writing phase of this PhD programme, three papers were published that reclassified *C. destructans* and *C. macrodidymum* on the basis of new DNA sequence data from multiple genes. As the research was completed and the budget exhausted there was no opportunity to address this experimentally. The two more important papers are reviewed below and details of how this new information has been incorporated into the thesis are explained.

A recent phylogenetic study by Chaverri *et al*. (2011) divided the *Neonectria* into four groups using morphology of anamorph and teleomorph as well as multilocus analyses. The four groups are 1) *Neonectria*/*Cylindrocarpon sensu stricto* 2) *Rugonectria* 3) *Thelonectria* and 4) *Ilyonectria*.

Cabral *et al*. (2012a) introduced 12 new taxa within the *Ilyonectria radicicola* (*C. destructans*) species complex. These taxa were delineated by the combined data from four genes, the

histone H3, β-tubulin, nuclear ribosomal RNA internal transcribed spacer (ITS) and translation elongation factor 1α gene. These taxa were reported to occur on a large variety of hosts and characteristics of their morphology like microconidia, macroconidia andconidiophore dimensions were described. The novel species were shown to be linked to previous names or taxa considered as synonyms of *C. destructans*. The taxa discriminated within the *Ilyonectria radicicola* complex were *Ilyonectria anthuriicola* (isolated from anthurium), *I. cyclaminicola* (isolated from a *Cyclamen* sp.), *I. europaea* (named for its broad geographic distribution), *I. gamsii* (named after Dr Walter Gams), *I. liliigena* (isolated from *Liliumregale*), *I. lusitanica* (named after the Latin name of Portugal), *I. panacis* (isolated from *Panax quinquefolium*), *I. pseudodestructans* (named due to its morphological similarity to *C. destructans*), *I. robusta* (from *Ramularia robusta*), *I. rufa* (name derived from *Coleomyces rufus*), *I. venezuelensis* (isolates collected from Venezuela) and *I. vitis* (isolated from *Vitis vinifera*). Their work demonstrated that accurate identification required information from all four taxonomically informative genes as the translation elongation factor 1α sequences could not discriminate the isolates designated as 'species 6' from *I. rufa*, *I. robusta*, *I. lusitanica*, *I. europaea* and *N. ditissima,* and the β-tubulin sequences were not able to discriminate 'species 4 and 6' and *I. robusta*.

A second publication by Cabral *et al*. (2012b) reported that there are six monophyletic species within the *Ilyonectria macrodidyma* complex (including isolates from 'species 4 and 6' above), and four of these were identified by DNA sequence analysis of multiple genes (β-tubulin, ITS, translation elongation factor 1α and histone H3) together with description of morphological characteristics. The four described species within the complex are *Ilyonectria alcacerensis*, *I. estremocensis*, *I. novozelandica* and *I. torresensis.* However, another two species, *Cylindrocarpon sp.*1 and *Cylindrocarpon sp.*2, were not identified by this study. Apart from *I. novozelandica,* these described species, are named after the city (in Portuguese) from where the holotype was collected. *Ilyonectria novozelandica* is named after the country (New Zealand) from which the initial isolate was collected.

Although methods have been developed for the identification of the different species, a comprehensive genotypic and phenotypic diversity study on the main pathogenic *Cylindrocarpon* species in New Zealand has not been done. Such studies could improve understanding of the biological characteristics of these species, including inoculum sources, dispersal, longevity and mode of pathogenesis by different species and isolates. By characterising different pathogen genotypes and assessing their specific interactions with cultivar and environmental variables, the spectrum of traits encompassed by the pathogen population in New Zealand may be better understood.

1.6 Descriptions of *Cylindrocarpon* species that commonly infect grapevines*

Cylindrocarpon species have a worldwide distribution, occurring from tropical rainforest litter to arctic tundra soils (Brayford, 1992). Host range varies between species within a genus, but is usually wide. The *Cylindrocarpon* species commonly found to infect in New Zealand grapevines are *C. destructans*, *C. macrodidymum* and *C. liriodendri*.

*The recent publications (Cabral *et al.*, 2012a; 2012b) that reported new taxonomical classifications of *Cylindrocarpon destructans* as *Ilyonectria radicicola* complex species, *C. macrodidymum* as *Ilyonectria macrodidymum* complex species are described on page 7.

1.6.1 *Cylindrocarpon destructans*

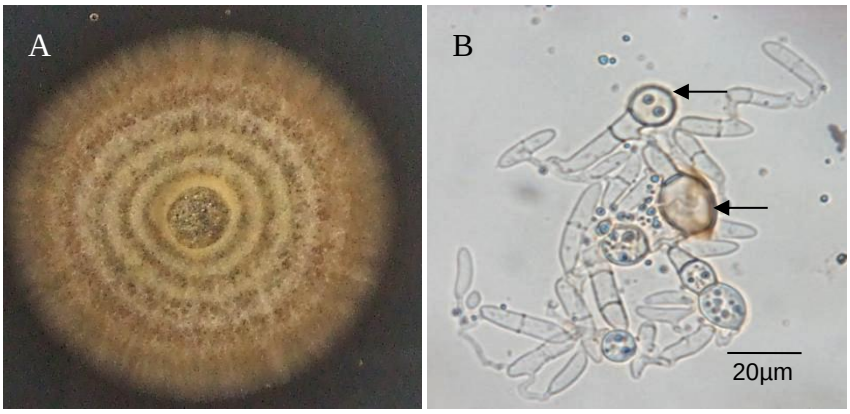


Figure 1.1 *Cylindrocarpon destructans* (A) adaxial surface on potato dextrose agar (B) macroconidia and chlamydospores (indicated by arrow, photographs by Carolyn Bleach).

Cylindrocarpon destructans forms microconidia, macroconidia and chlamydospores in cultures grown on potato dextrose agar (PDA). Colony texture is cottony/felty with aerial mycelium in the centre or over the entire colony. Its colour varies from white to cinnamon (Rego *et al.*, 2001). From the reverse side, the culture is reddish-brown in the central portion and beige at the edge. On 2% malt extract agar (MEA), *C. destructans* shows a buff reverse (Cedeno *et al.*, 2004). When the Petri dish is opened, it produces a distinctive odour of musty earth. Booth (1966) reported that the microconidia are hyaline, cylindrical to oval and measure 7.2 (5.6-8.8 µm) x 3.9 (3.3-4.5 µm). The macroconidia are hyaline, cylindrical or slightly wider at the distal end, straight or curved with rounded ends, and are 1-3 septate. The 1 septate macroconidia measure 23.1-29.2 µm x 5.2-6.2 µm and the 2-3 septate conidia measure 34.5-40.3 µm x 6.8-8.0 µm (Booth, 1966). The chlamydospores appear on and inside the substrate being terminal or intercalary and sometimes forming in chains. They are spherical to elliptical in shape, hyaline initially and then develop thick, golden brown walls. They measure 12.9 (11.3-14.5 µm) x 11.4 (10.1-12.7 µm) and are smooth, although deposits of wall substances make them appear rough on the surface (Booth, 1966).

According to Booth (1966), *C. destructans* is wide-spread and has been found in South Africa, Asia, Australia, New Zealand, Europe, Canada, Venezuela and the USA (Seifert and Axelrood, 1998). Cedeno *et al*. (2004) reported strains of *Cylindrocarpon destructans* var. *destructans* causing black foot rot on blackberry in Venezuela. *Cylindrocarpon destructans* is an important pathogen on forestry trees (especially silver firs), pears, apples, walnut, and stone fruits, as well as a number of agriculturally significant plants, such as strawberries, peas and bulbs (Bonfiglioli, 2005). It also attacks cyclamen, narcissus, ginseng, conifer, fruit trees and vines (Booth, 1966). *Cylindrocarpon destructans* was observed on and isolated from mouldy apple, apricot, and pear nursery stock and young fruit tree rots in cold storage (Traquair and White, 1992). Traquair and White (1992) reported the same fungus was identified on cold stored apple (*Maluspumila* L.) trees at the Horticultural Research Centre in Brooks, Canada.

1.6.2 *Cylindrocarpon macrodidymum*

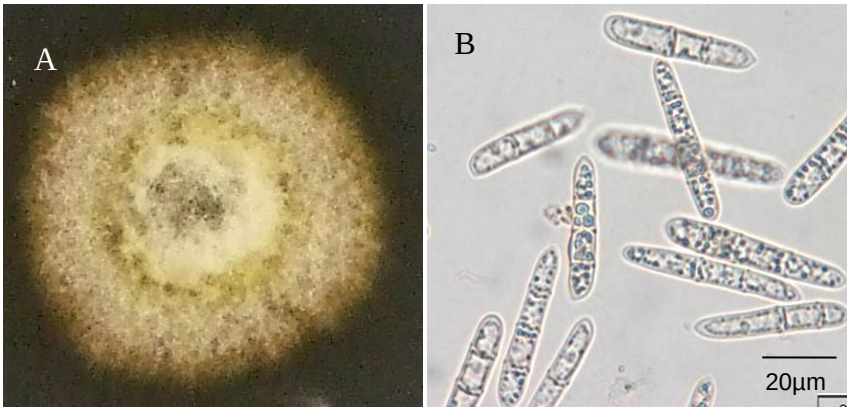


Figure 1.2 *Cylindrocarpon macrodidymum* (A) adaxial surface on potato dextrose agar (B) macroconidia (Photographs by Carolyn Bleach).

Cylindrocarpon macrodidymum forms microconidia and macroconidia on PDA but rarely chlamydospores. The colony surface is yellowish and felty with aerial mycelium abundantly formed, covering the whole colony or sectors of it. The microconidia measure 8-16 x 3.2-5.0 µm. The macroconidia are 1-3 septate. The mean of 1-septate macroconidia measure 14-27 x 4-6 µm. The 2-septate conidia measure 26-35 x 5-7.5 µm and the 3-septate conidia measure 31-41 x 6-8 µm (Halleen *et al*., 2004). On 2% MEA, Petit and Gubler (2005) reported that the colonies were orange-dark brown colour in reverse, a characteristic which helped to differentiate *C. macrodidymum* from *C. destructans* isolates, which had a buff reverse, in isolates from grapevines in California. They also observed that macroconidia of the *C. macrodidymum* were significantly larger than those of *C. destructans* and that *C. macrodidymum* grew more slowly than *C. destructans*.

Cylindrocarpon macrodidymum has been reported from grapevines in South Africa, Canada, Australia, USA, and New Zealand (Halleen *et al*., 2004), Chile (Auger *et al.*, 2007), Spain (Alaniz *et al*., 2009b) and Turkey (Özben *et al.*, 2012). Recently, this species was reported in

Italy to cause root rot in avocado (Vitale *et al.*, 2012) and olive trees in California (Urbez-Torres *et al.*, 2012).

1.6.3 *Cylindrocarpon liriodendri*

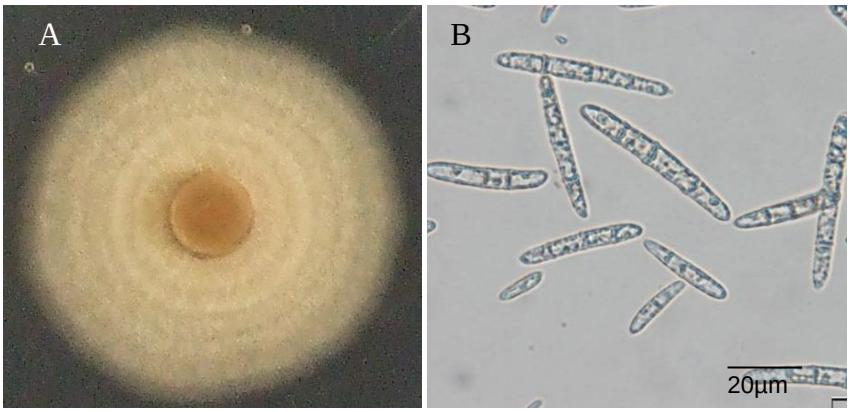


Figure 1.3 *Cylindrocarpon liriodendri* (A) adaxial surface on potato dextrose agar (B) macroconidia (photographs by Carolyn Bleach).

Cylindrocarpon liriodendri forms macroconidia and chlamydospores but has not been shown to form microconidia. Colonies on PDA are cinnamon to sepia with sparse aerial mycelium. On oatmeal agar (OA), the colonies are dark brick to fawn (surface and reverse). Yellow pigmentation was not observed. Macroconidia are three septate, straight or sometimes slightly curved. The mean size of macroconidia is 35-40 x 5.5-6 µm. Chlamydospores are medium brown, ovoid to ellipsoid, mostly short with intercalary chains and their mean size is 10-20 x 10-17 µm (Halleen *et al*., 2006b).

According to MacDonald and Butler (1981), *Cylindrocarpon liriodendri* has been reported on *Cyclamen* spp. in the Netherlands and on *Liriodendron tulipifera* in California. Halleen *et al* (2006b) reported *C. liriodendri* was isolated from *Vitis vinifera* in France, Portugal, South Africa and New Zealand. It has also been, reported in Australia by Whitelaw-Weckert (2007), Iran by Mohammedi *et al*. (2009) and in kiwifruit in Turkey (Erper *et al.*, 2010).

1.6.4 *Cylindrocarpon obtusisporum*

Cylindrocarpon obtusisporum produces macroconidia which are curved, cylindrical, and colourless. The 1-3 septate conidia vary in size with septation, overall mean sizes being 28-50 × 4-7.5 µm (Booth, 1966). Microconidia are ovoid or ellipsoid and colourless, 7-8 × 4-5 µm. Chlamydospores are formed sometimes singly or in chains. They are smooth, globose, hyaline to brown and 10-15 µm in diameter. Colony growth on PDA is slow, reaching 10 mm diameter after 7 days. The mycelium is white initially, floccose to felted, but darkening to light brown or sometimes brown with age.

Booth (1966) reported that *C. obtusisporum* has been found on *Acacia* spp. in the USA, *Apium* spp. in Cyprus, *Beilschmiedia* and *Coprosma* spp. in New Zealand, as well as

Glyceria and *Tilia* spp. in Britain. The association of this species with grapevines was reported by Maluta and Larignon (1991) in Uruguay, by Grasso and Magnana di San Lio (1975) in Italy and by Scheck *et al*. (1998a) in the USA. Petit and Gubler (2005) and Halleen *et al*. (2006a) concluded that *C. macrodidymum* isolates from grapevines may have been misidentified as *C. obtusisporum*.

1.7 Black foot symptoms on grapevines

Grapevines that typically show symptoms of this disease are often less than 5 years old and in some instances symptoms appeared during the first year of planting (Halleen *et al.*, 2005a). Diseased plants show a range of decline symptoms. The first visible symptoms in vineyards are usually delayed budding or absence of budding (Figure 1.4B; Halleen *et al.*, 2009). Other symptoms include reduced vigour, slower growth, sparse foliage and small leaves with interveinal chlorosis, shortened internodes, (Figure 1.4) and necrosis. In addition, some affected vines show retarded or absent sprouting, wilting and dieback, often leading to the death of the young vines (Grasso, 1984; Oliveira *et al*., 2004; Halleen *et al*., 2006a).

The below-ground symptoms were reported by Halleen *et al*. (2004) as abnormal development of roots with growth parallel to the soil surface, necrotic root crowns and development of secondary root crowns (Figure 1.5A). Affected grapevines may also show sunken necrotic root lesions with a reduction in root biomass. Removal of the rootstock bark may reveal pinkish/purple to black discoloration due to necrosis of the wood tissues (Figure 1.6B). Internal trunk symptoms often develop from the base of the rootstock which indicates the pathway of this disease (Alaniz *et al*., 2009a). In longitudinal section, brown to black streaks may be evident with broad blackened sectors near the base of the rootstock (Figure 1.5B). A cross section through the lesion reveals the development of necrosis from the bark to the pith (Figure 1.6A) (Larignon, 1999; Fourie and Halleen, 2001). These kinds of trunk symptom are usually described as typical of *Cylindrocarpon* species infection (Oliveira *et al*., 2004) and give the disease the name ‘black foot’. Sweetingham (1983) also described black foot discoloration of the phloem from which *C. destructans* was isolated. Microscopic examination showed that xylem vessels became occluded with fungal tissue, gums and tyloses. Similar symptoms have been observed on inoculated rooted vine cuttings infected with *Cylindrocarpon macrodidymum, C. destructans* and *C. liriodendri* (Rego *et al*., 2001; Petit and Gubler, 2005; Halleen *et al*., 2006a) three to six months after inoculation.

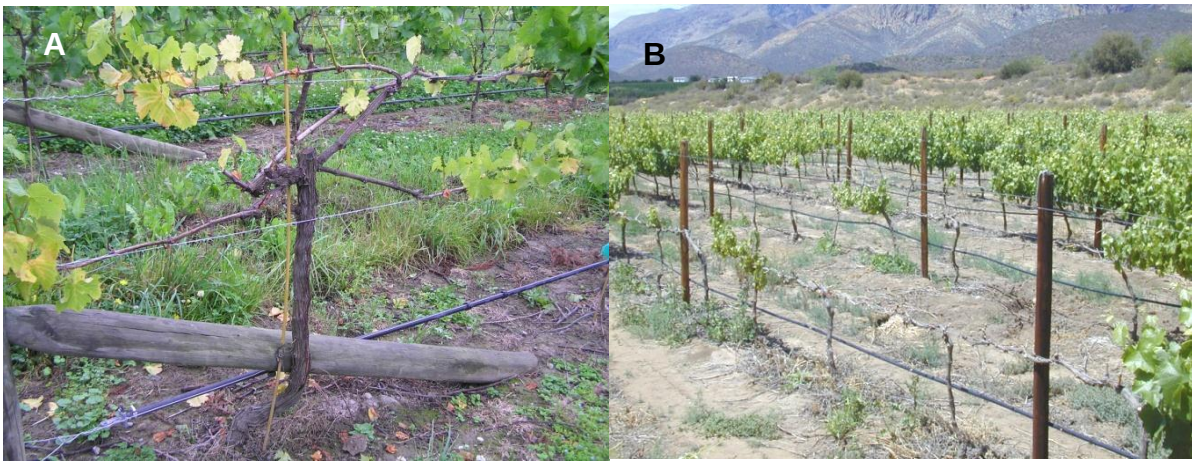


Figure 1.4 Above ground black foot symptoms (A) grapevines with sparse and chlorotic foliage (B) delayed bud break and stunded growth (Photographs by Marlene Jaspers).

Figure 1.5 Below ground black foot symptoms on grapevines: (A) reduced root biomass, few feeder roots, secondary root crown (B) longitudinal section: brown to black vascular streaks, from bark to pith (Photographs by Carolyn Bleach and Marlene Jaspers, respectively).

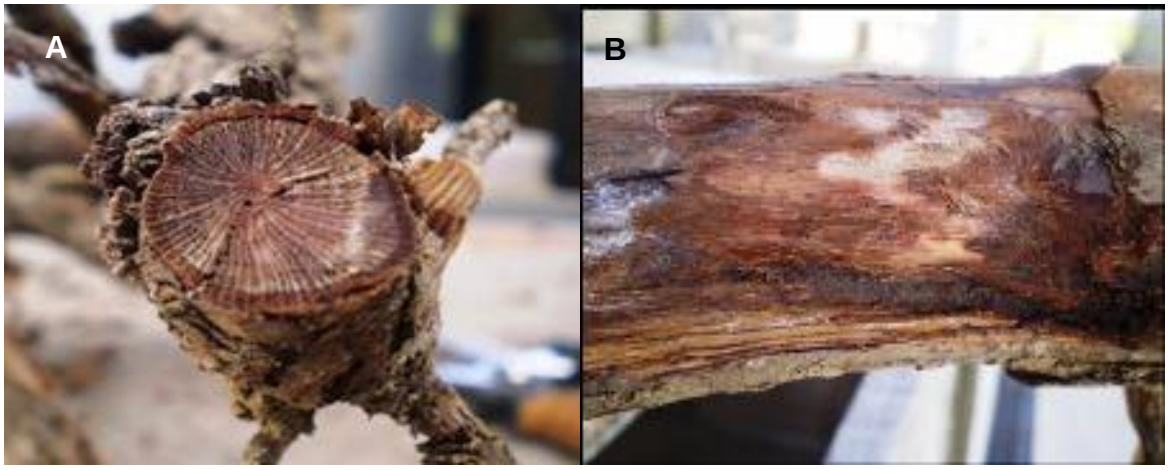


Figure 1.6 (A) Internal symptoms showing brown/black staining of xylem tissue and necrosis from bark to pith, (B) brown and pink discolouration of xylem on lower trunk. Courtesy of Dr Richard Pentreath, Corbans Viticulture Ltd, 2009.

1.8 Disease cycle

Several studies have been undertaken in different countries to find the initial sources of inoculum. In Portugal, Rego *et al.* (2000) reported that severe outbreaks occurred in young vineyards of certain grape cultivars, which were linked to the same regional source or the same nursery. Oliveira *et al*. (2004) isolated *Cylindrocarpon* species from 87.5% of vines with necrotic rootstock wood tissue that came from young vineyards. In Spain, *Cylindrocarpon* species were found only in rootstock sections of grafted vines (23.8%), mostly after being planted for rooting in the nursery field. In addition, a high number of young plants supplied by nurseries were diseased, with 37% of the plants being infected with *Cylindrocarpon* species (Armengol *et al.*, 2001). In California, USA Gubler *et al*. (2004) also reported that up to 5% of vines were infected from some nurseries, but they believed that the primary inoculum source was due to the presence of *Cylindrocarpon* species in vineyard soils. Therefore, it seems likely that *Cylindrocarpon* species inoculum can also build up in vineyard sites replanted from grapes or other fruit crops. Bonfiglioli (2005) suggested that the high rate of black foot infection in New Zealand vineyards may be due to the number of vineyard sites previously planted with apple trees. In New Zealand, the *Cylindrocarpon* species isolated from roots of declining apple trees were identified as *C. destructans* and these isolates were found to be similar to the isolates from infected grapevines (Mostert *et al*., 2006b).

An investigation of diseased vines in Tasmania, Australia showed that wood discoloration did not originate from the visible trunk base, but from the bottom of the buried portion of the trunk (Sweetingham, 1983). Extremely low levels of *Cylindrocarpon* species were found in canes of rootstock mother vines in South Africa indicating that the source was not aerial (Fourie and Halleen, 2002). An investigation of fungi occurring in asymptomatic nursery plants supported these findings since *Cylindrocarpon* species were hardly ever isolated from callused grafted cuttings prior to planting in nurseries (Halleen *et al*., 2003). However, once planted in the nurseries, *Cylindrocarpon* species were isolated from the roots, rootstocks and graft unions (Halleen *et al*., 2006a). This indicated that *Cylindrocarpon* species were already present in the nursery and vineyard soils and were likely to be living as saprobes on dead plant material, as soil inhabitants, root colonizers or pathogens (Brayford, 1992).

The *Cylindrocarpon* species may be present in soil as mycelia, conidia or chlamydospores. The production of chlamydospores could allow *Cylindrocarpon* species to survive for extended periods in soils (Booth, 1996; Halleen *et al*., 2004). However, very little information is currently available regarding the survival of these pathogens, and the role of chlamydospores during subsequent infections (Halleen *et al*., 2006a). Recent research at Lincoln University demonstrated that conidia and mycelium quickly converted to chlamydospores in the soil environment (Probst, 2011). Despite the importance of this disease, a comprehensive understanding of the ecology of *Cylindrocarpon* species infecting

grapevine is still lacking (Rego *et al.*, 2006). Recent studies at Lincoln University have shown great variability between isolates and species in pathogenicity, colony morphology (Probst, 2011) and susceptibility to different fungicides (Bleach, 2012).

1.9 Pathogenesis

Black foot is primarily a soil-borne disease. However, host stress and environmental factors may play an important role in disease development. Stress conditions that favour development of black foot disease include poor drainage, malnutrition, heavy crop loads on young plants, soil compaction, planting of vines in poorly prepared soil and improper plant holes (Larignon, 1999; Fourie and Halleen, 2001; Halleen *et al.*, 2004). The disease thrives in poorly drained and heavy soils, especially on sites where there may be standing water or the ground is sodden for significant periods of time (Gubler *et al*., 2004; Bonfiglioli, 2005). Poor root development is also caused by poor soil preparation and soil compaction (Fourie *et al*., 2000; Halleen *et al*., 2004). High temperatures during summer may also play an important part in expression of symptoms, because the diseased plants' vascular systems and compromised roots would not be able to supply enough water to compensate for the high transpiration rate during periods of high temperatures (Larignon, 1999).

Horsfall and Dimond (1960) reported that wounds for infection can be produced by the development of secondary roots, abrasion with soil particles or due to the activities of insects or nematodes. Brayford (1992) stated that *Cylindrocarpon* species are frequently part of disease complexes with other nematodes or fungi. Root infection progresses towards the trunk base as the pathogen moves into the vine. In addition, the butt end of the rootstock section, which is usually not completely covered in callus at the time of planting, may also provide a point of entry for the pathogen (Halleen *et al.*, 2007). When the pathogen moves further into the butt of the vines, the tissues become black and shows a dry cortical rot, and this leads to vine dieback and death. The xylem usually becomes occluded with fungal tissue, gums and tyloses which cause the vascular tissue to turn black (Whitelaw-Weckert *et al*., 2007).

The pathogen produces slimy spores which can spread in water (Brayford, 1992) and resistant chlamydospores, which allow the pathogen to survive in the soil. Halleen *et al*. (2003) reported that the pathogen was introduced to vineyards via contaminated nursery stock, but Gubler *et al*. (2004) concluded that the presence of the pathogen in vineyards could be from another source of inoculum, such as a previous crop. Maluta and Larignon (1991) reported that in vineyards, the infected grapevines were often located in lines or in patches, which indicated that infection spreads from one vine to another, possibly through rainwater, machinery or by human activities.

1.10 Production of cell wall degrading enzymes during pathogenesis

As with other plant pathogens *Cylindrocarpon* species are likely to produce an array of cell wall degrading enzymes that allow them to access the tissues of their plant hosts and derive nutrients from them. The cell wall degrading enzymes observed in other pathogens consist of cellulases, laccases, proteases, pectinases, xylanases, and a series of oligosaccharide degrading enzymes (Walton, 1994; Oliver and Osbourn, 1995; Annis and Goodwin, 1997).

Cellulases are a group of enzymes produced by a wide range of organisms, including bacteria (Doi *et al.*, 1998), insects, plants (Inoue *et al.*, 1997; Ohmiya *et al.*, 2000) and fungi (Teeri *et al.*, 1987). Among these organisms, fungal cellulases have been studied widely. The most extensively studied cellulolytic fungi are species of *Trichoderma* (Kubicek *et al.*, 1988; Penttila *et al.*, 1991; Teeri *et al.*, 1992). There are three main types of cellulases: (1) endo-β-D-1, 4-glucancases/endoglucanases which have a high affinity for soluble cellulose and attack by cleavage within the cellulose chain (endo), (2) β-glucosidases which hydrolyse the oligosaccharides and disaccharide cellobiose into glucose and so provide the fungus with carbon sources and (3) cellobiohydrolases which have affinity towards cellulose derivatives and attack these celluloses by endoaction (Henrissat *et al.*, 1985; Ilmen *et al.*, 1997).

The expression of the genes encoding fungal cellulases is tightly controlled at the transcription level (Ilmen *et al.*, 1997; Gielkens *et al.*, 1999; Tian *et al.*, 2009). Several studies with *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*) cellulase genes identified small metabolites like lactose, sophorose, cellobiose and xylose as inducers of cellulase genes (Mandels and Reese, 1960; Mandels *et al.*, 1962; Gielkens *et al.*, 1999; Kubicek *et al.*, 2009; Stricker *et al.*, 2008). In addition, Coradetti *et al*. (2012) reported that conserved and essential transcription factors like *clr-1* and *clr-2* (uncharacterised zinc binuclear cluster transcription factors) induced all major cellulase and hemicellulase genes in *Neurospora crassa*. A novel component, glucose-ribitol dehydrogenase 1 (GRD1) was also shown to induce the extracellular activity of the endo 1, 4-β-D glucanase activity, transcription and expression of the cellulase gene in *Trichoderma reesei* (Schuster *et al.*, 2011). Further, Schuster *et al*. (2012) found that protein kinase A and adenylate cyclase play a major role in light modulated cellulase regulation in *Trichoderma reesei*.

In addition to cellulases, laccase enzymes have been shown to play a role in breaking down plant tissue. Laccase, first described by Yoshida in 1883 (Thurston, 1994), belongs to the multicopper family of oxidases (Messerschmidt, 1997) also known as blue copper proteins or blue copper oxidases (Thurston, 1994). Laccases are found in plants, fungi and insects (Mayer and Staples, 2002). The fungal laccases play an important role in plant pathogen interactions (Zhu and Williamson, 2004) by delignifying the highly lignified xylem tissues of

plants (Gavnholt and Larsen, 2002). Moreover the laccases of plant pathogenic fungi are involved in degradation of lignocellulosic materials (Bourbonnais and Paice, 1990) and production of pigment (Aramayo and Timberlake, 1990). The six laccase genes, *lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5* and *lcc9* have been reported from the wilt fungus *Fusarium oxysporum* (Cañero and Roncero, 2008). These authors observed that *lcc1*, *lcc3* and *lcc9* were expressed in roots and stems of tomato plants during the infection process, with *lcc3* considered a potential virulence factor. According to Broda *et al*. (1995), different laccase genes are regulated by factors such as substrates, pH and fungal metabolism. Cañero and Roncero (2008) reported that in *F. oxysporum* the *lcc3* and *lcc5* genes contain three *PacC* binding consensus sites in the promoter region that allow modulation by ambient pH. In the plant pathogenic fungus, *Rhizoctonia solani*, four laccase genes, *lcc1*, *lcc2*, *lcc3* and *lcc4* have been identified (Wahleithner *et al.*, 1996). Another study of the *Botryosphaeriacaea* species showed that veratryl alcohol induced the secretion of intracellular laccases into the extracellular medium (Vasconcelos *et al.*, 2000).

In addition to those enzymes directly responsible for breaking down cellulose and lignin in the plant cell wall pathogens are also known to produces proteases during infection. Increased protease activity has been detected in a number of plant tissues infected by pathogenic fungi. Fungal proteases can enable localized penetration by the pathogen by breaking down the structural fibrous glycoproteins of the plant cell wall (Rauscher *et al.*, 1995) and membranes, thus making nutrients available for the fungi (Goldman *et al.*, 1994).

The proteases are classified into acidic, neutral and alkaline proteases according to the optimum pH of enzyme activity (Keay, 1971a; Keay, 1971b; Fogarty *et al.*, 1974). Robertsen (1984) observed that alkaline extracellular protease was produced by *Cladosporium cucumerinum,* a fungal pathogen which causes scab disease of cucumber seedlings. Kuc (1962) reported that *Cl. cucumerinum* secreted protease when grown in a liquid medium composed of soluble and insoluble components of cucumber seedlings. Poussereau *et al*. (2001) reported that *Sclerotinia sclerotiorum* produces acid proteases; which contain aspartyl protease and non aspartyl protease. These acid proteases are able to degrade the plant cell wall components and macerate the plant tissue. Their study also showed that acid protease (acp1 protein) expression was regulated at the transcription level by carbon and nitrogen availability and also by the ambient pH. Cotton *et al*. (2003) reported the secretion of acid proteases by *Scl*. *sclerotiorum*, when the environment has been acidified using oxalic acid and this enzyme is considered an important virulence factor (Rollins, 2003). Bueno *et al*. (2012) reported the secretion of acid proteases by *Scl. sclerotiorum* during *Phaseolus vulgaris* infection. In their study, *acp1* expression was low at the initial stage of infection and later increased quickly as the phase of necrosis spread. Another study on *Fusarium* species showed the secretion of acid proteases during the infection of maize seedlings and

suggested that these acid proteases may have a specific role in the pathogenic process (Urbanek and Yirdaw, 1978). Rolland *et al*. (2009) reported the secretion of acid protease (BcACP1), which was a new member of family G1 proteases, by *Botrytis cinerea* during infection; the production of BcACP1 was associated with the acidification of plant tissue.

Pectinases have also been identified as enzymes that are involved in plant infection. They are a group of enzymes that break down pectin, however, different pectinases use different mechanisms and it is their combined activity that completely breaks down pectin. They are classified into polygalacturonase (PG), pectin methylestrase and pectin pectatelyase (Fogarty and Kelly, 1982). Idnurm and Howlett (2001) reported that these enzymes are the first enzymes to be secreted by the fungus *Magnaporthe grisea* when attacking the plant cell wall. Zhang *et al*. (1999) demonstrated that polygalacturonase (PG) played a major role in the pathogenicity of *Didymella bryona* which causes black rot in cantaloupe fruit and that total fungal PG activity in decayed tissues and lesion size were related.

Rahman and Punja (2005) reported from their *in vitro* study that *C. destructans* isolates recovered from ginseng roots had marked differences in pectolytic enzyme production. Their pectinase and polyphenoloxidase enzymatic activity was measured by adding the *C. destructans* culture extract to a well in an agar medium containing pectin and phenol as the substrates. The diameter of the zone of clearance around the well where the *C. destructans* culture extract was placed showed higher pectinase activity for highly virulent isolates than with weakly virulent isolates. Another study on pectin lyase production by *C. destructans* isolated from ginseng was determined spectrophotometrically and showed significant difference in the quantities of pectin lyase and polygalacturonase between isolates, and the production levels were found to be reliant on culture conditions like incubation time, pH and temperature (Sathiyaraj *et al.*, 2011). In addition, pectinases have been shown to be involved in plant signalling that regulates pathogen developmental and plant defensive processes (Nighojkar *et al.*, 2006; Osman *et al.*, 2008).

Xylanases are enzymes that hydrolyse xylan, which is the second most common polysaccharide found in the plant cell wall. For the complete hydrolysis of xylan, two different types of xylanases are necessary: (1) endo 1, 4-β-xylanases which hydrolyse the back bone of xylan and (2) 1, 4-β-D-xylosidases which break off the small oligosaccharides (Subramaniyan and Prema, 2002). Wu *et al*. (2006) reported that xylanases are essential for the virulence of *Magnaporthe grisea*, the causative agent of rice blast disease, during pathogenesis and saprophytic growth.

1.11 Identification of *Cylindrocarpon* species based on morphology

One of the problems in further investigations of black foot epidemiology is the correct identification of the *Cylindrocarpon* species that are causing the disease. In the past, *Cylindrocarpon* species identification has relied on morphological features like colony pigmentation, growth rate, and production of chlamydospores, as well as microconidial/macroconidial shape and size (Booth, 1966). However, Brayford and Samuels (1993) argued that formation of abundant microconidia and or mycelial chlamydospores are species characteristics and do not delineate natural groups. They then used a holomorph approach for identifying *Cylindrocarpon*, but unfortunately, that approach has not been very successful. This was partly due to the great variation in morphology of isolates within the *Cylindrocarpon* species associated with black foot disease, as reported in California by Petit and Gubler (2005).

Although morphology is used extensively in fungal identification, it is often insufficient to differentiate species. As species may differ in minor morphological features, identification can be difficult for those not familiar with these fungi. Alaniz *et al*. (2009a) reported that the grapevine isolates identified as *Cylindrocarpon destructans* and *Cylindrocarpon liriodendri* had identical morphological characters. Halleen *et al*. (2006b) conducted morphological and phylogenetic studies from which they identified the *Cylindrocarpon* species which infect grapevines. Molecular tools are therefore needed for accurate identification of the New Zealand isolates of *Cylindrocarpon* species and to investigate the factors affecting their distribution and pathogenicity.

1.12 Molecular based *Cylindrocarpon* species identification

The advent of molecular biology has contributed to the diagnosis of plant pathogenic fungi by offering new methods for quicker and more accurate detection, identification and quantification. It can be used to detect pathogens within plant tissues, avoiding the need for pathogen isolation. In most cases, older methods of identification have relied on pathogen recovery on selective media. This was not always successful if the target organism was out-grown by faster competitors. In addition, morphological characters could be common for several species. Therefore, the application of molecular techniques for identifying fungi was an inevitable improvement. These methods are now routinely used in fungal identification.

The DNA encoding of ribosomal subunits and internally transcribed spacers (ITS) is usually targeted for the development of molecular diagnostic methods as it is often conserved within a species and the high copy number in the genome improves detection sensitivity of assays (Fox and Narra, 2006). However, for closely related species, variation in the ITS sequences is occasionally low or non-existent and the genes encoding β-tubulin, which is a component of microtubules or the translation elongation factor 1α, can also be used as alternatives

(Schmitt *et al.*, 2009). Thon and Rosye (1999) used the β-tubulin sequence to investigate the phylogeny between members of the Basidiomycotina. Groenewald *et al*. (2001) used ITS and β-tubulin sequences to separate the genus *Phaeomoniella* from *Phaeoacremonium*. Halleen *et al*. (2006b) recently reported that European and South African grapevine black foot isolates which were thought to be *C. destructans*, were morphologically and genetically identical and had identical morphology and 5.8 S rDNA/ITS and β-tubulin 2 gene sequences to *C. liriodendri*, and so were re-identified from *C. destructans* to *C. liriodendri*.

The development of species specific primers has provided a powerful tool for the detection of plant pathogens. The identification of fungal pathogens based on polymerase chain reaction (PCR) using species specific primers is now widely used, especially for economically important plant pathogens such as quarantine-listed fungi, those that are difficult to isolate or those that cause symptomless infections (Alaniz *et al*., 2009b). Several PCR-RFLP systems have also been developed to discriminate species of phytopathogenic fungi. For example, Alves *et al*. (2005) developed an amplified rDNA restriction analysis (ARDRA) procedure for identifying *Botryosphaeriaceae* species. Species specific primers are being developed for a wide range of plant pathogens and the development of quantitative PCR has proven to be an important tool to identify and quantify pathogens from seeds, plant tissues, water, air and soil samples (Schena *et al.*, 2004), providing information on the interaction between the host and the pathogen, the relationship between yield and loss due to a pathogen and the approach to manage the disease (McCartney *et al.*, 2003; Ward *et al.*, 2004).

Hamelin *et al*. (1996) designed species specific primers (Dest1 and Dest4) to detect *C. destructans* from conifer seedlings. Nascimento *et al*. (2001) obtained a DNA fragment of 400 bp using these primers. These authors also developed a nested PCR which used the universal primer ITS4 and the fungus specific primer ITS1F in a first stage fungus specific amplification, followed by a second stage amplification with the primers Dest1 and Dest4 using the PCR product from stage one. It was shown to be a reliable method for the detection of *Cylindrocarpon* species directly from infected grapevines (Nascimento *et al.*, 2001). However, they were unable to distinguish between *C. destructans* and *C. obtusisporum* when using these specific Dest1 and Dest4 primers, because an amplimer of the same size was obtained for isolates of both species. Furthermore, these primers were not able to detect *C. destructans* from artificially inoculated potted grapevines.

Alaniz *et al*. (2009a) developed species specific primers for the detection and identification of the three *Cylindrocarpon* species associated with grapevine, *C. liriodendri*, *C.macrodidymum* and *C. pauciseptatum*, from pure culture or infected grapevines and found that they clearly distinguished the three *Cylindrocarpon* species in a multiplex PCR assay. Moreover, using purified template DNA, all three pairs of primers have proven to be sufficiently sensitive to detect very low quantities of DNA. Probst (2011) optimised the PCR conditions for the New

Zealand *Cylindrocarpon* species isolates using the primers supplied by Dr Lizel Mostert (Stellenbosch University) for *C. liriodendri* and *C. macrodidymum,* and for *C*. *destructans* with primers supplied by Dr Hayley Ridgway (Lincoln University, New Zealand). These primers for the three *Cylindrocarpon* species were proven to be sensitive enough to detect as low a concentration as10 pg of template DNA.

1.13 Analysis of genetic diversity

An increased understanding of pathogen biology can be achieved by better understanding the genetic diversity of the fungal pathogens. Vegetative compatibility groupings (VCGs) have been extensively used to study the genetic relationships in anamorphic populations, in population structure and in diversity of many fungi, including several plant pathogenic fungi (Leslie, 1993; Cortesi and Milgroom, 1998; Milgroom and Cortesi, 1999). Valuable information about populations within the same species or individuals within a population can be provided by means of intraspecific units of vegetative compatibility groups (VCGs).

The formation of heterokaryons between different strains is an important and common component of the life cycle of many filamentous fungi. Lineages that are capable of fusing (anastomoses) and forming stable and functional heterokaryons are known as vegetatively compatible, and frequently described as members of the same group of vegetative compatibility or vegetative compatibility group (VCG) (Leslie, 1993). The development of vegetative and heterokaryon compatibility testing and the mechanisms behind these phenomena, have been thoroughly reviewed (Glass and Kuldau, 1992; Leslie, 1993; Glass *et al.*, 2000; Saupe, 2000). The genetic traits of vegetative compatibility have been investigated in many fungal species and found to be controlled by *vic* or *het* loci. Vegetative or heterokaryon incompatibility is due to a genetic mechanism that restricts the heterokaryosis between individuals who differ in one or more *het* or *vic* loci (Glass *et al.*, 2000; Xiang and Glass, 2004). These *vic* loci which are unlinked may be found dispersed throughout the fungal genome and play a crucial role in maintenance of a heterokaryon.

There are a number of molecular techniques that are used to investigate the intra-species genetic variability. One of the most common methods for investigating the genetic diversity of a fungus is the use of primers to amplify random DNA sequences from the genome. These techniques include the well-known random amplification of polymorphic DNA (RAPD) and universally primed PCR (UP-PCR) fingerprinting methods (Bulat *et al.*, 1998). RAPDs involve the amplification of random areas of DNA by short (10 bp) primers at low annealing temperatures. The use of low annealing temperatures causes a high degree of variability in RAPD amplification between laboratories. In contrast, UP-PCR primers are longer (15-20 bp), designed to amplify intergenic and therefore more variable areas of the genome and to anneal at higher temperatures (52-56°C). By annealing at high temperatures UP-PCR is

more reproducible between laboratories and experiments, which make the analysis and interpretation much easier and more consistent (Bulat *et al.*, 1998; Lubeck *et al.*, 1999). Lubeck *et al*. (1999) reported that the UP-PCR finger printing method was a highly robust method for studies on the genetic diversity of *Trichoderma harzianum*.

Another molecular method, the inter simple sequence repeat (ISSR) or random amplified microsatellites (RAMs) technique which consist of amplification of DNA fragments present at an amplifiable distance between two similar microsatellite repeat regions oriented in opposite direction (Reddy *et al.*, 2002). The primers are longer than UP-PCR primers (16-25 bp), and designed to target multiple genomic loci to amplify the ISSR sequences of different sizes. This method was used to study the genetic diversity of *Diplodia srobiculata* and showed a strong geographic separation between *D. scrobiculata* populations from diverse regions in North America (Burgess *et al.*, 2004). It has also been used to study the genetic diversity of *Cylindrocarpon* species from Spain and showed the correlation between genotype and pathogenicity in *C. macrodidymum* and *C. liriodendri* isolates. This study also showed a reasonable diversity in *C. macrodidymum* compared to *C. liriodendri* (Alaniz *et al*., 2009b).

Amplified fragment length polymorphism (AFLP) is another PCR based DNA analysis technique that can detect variation on a genome wide basis and is a very powerful DNA finger printing technique for DNA of any origin or complexity (Vos *et al.*, 1995). The AFLP method is based on the selective amplification of restriction fragments from the digested DNA. To produce the DNA fingerprints, the genomic DNA has to be digested using restriction enzymes and then adapters are ligated to restriction sites which enable selective amplification. Mostert *et al*. (2006a) studied the genetic diversity of *Ph. chlamydospora* isolates using the AFLP method. Both AFLP and UP-PCR techniques have been extensively used to study the genetic diversity of many fungal pathogens. In New Zealand, the UP-PCR technique was used for genetic diversity studies on *Neofusicoccum parvum* populations from blueberry farms (Che Omar, 2009), and vineyards (Baskarathevan *et al.*, 2012b) and on *N. luteum* populations isolated from grapevine nurseries (Billones, 2011), as well as *Spilocaea oleagina* populations infecting olives (Obanor *et al.*, 2010). The genetic diversity of *Ph. chlamydospora* populations in New Zealand was determined by combining the RAPD, RAMs, AFLP and UP-PCR (Pottinger *et al.*, 2002).

1.14 Fluorescent DNA tags in DNA of pathogenic fungi

Molecular technologies can also be used to transform fungi with fluorescent reporter genes that convey a detectable phenotype and are very useful for studying fungi in soil and plant material. Two reporter genes that have been shown to function in fungi are the green fluorescent protein (*gfp*) gene derived from the jellyfish *Aequoria victoria* (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Haseloff *et al.*, 1997) and the red fluorescent protein (DsRed-

Express and RsRed2) gene derived from the reef coral *Discosoma* sp. (Matz *et al.*, 1999). These reporter genes are particularly suited for dual colour labelling experiments of different fungi, since they have unique and different spectral properties that allow dual labelling and visualisation studies of fungi (McLean *et al.*, 2009). Expression of *gfp* in filamentous fungi requires a *gfp* variant (*sgfp*) that is efficiently translated in fungi together with a transformation system and a fungal promoter that satisfies the requirements of a given experimental objective. Fungi that have been transformed with *gfp* and are expressing the green fluorescent protein can be clearly visualised by fluorescence imaging (Lorang *et al.*, 2001). Most functional genomics methods depend upon the production of stable transformants. At present, there are successful transformation systems which are capable of transforming a wide range of filamentous fungi. Successful transformation of fungi has been achieved by a number of methods, including treatment of protoplast with $CaCl_2$/polyethylene glycol (PEG), electroporation, particle bombardment and *Agrobacterium tumefaciens*-mediated transformation (Weld *et al.*, 2006b; Utermark and Karlovsky, 2008).

A method frequently used for transforming filamentous fungi is PEG-mediated transformation (Riach and Kinghorn, 1996; Fitzgerald *et al.*, 2003). Transformation of protoplasts using $CaCl_2$/PEG typically occurs after 15-30 min incubation at room temperature in the presence of 10-50 mM $CaCl_2$ and high concentrations of PEG. Electroporation is also usually used to transform protoplasts; though sometimes germinating conidia are used. During electroporation, protoplasts or conidia are exposed briefly to a high amplitude electric field, which helps the uptake of foreign DNA by making the cell membrane permeable (Weld *et al.*, 2006). Although used widely, a major limitation of these methods is that they usually required the production of protoplasts. For particle bombardment (biolistics), the transforming DNA is coated on the gold or tungsten beads. These beads are then used to bombard the fungal tissue (Davidson *et al.*, 2000). The flexibility of target tissues used for biolistics has advantages in strains found to be unsuitable for protoplast production (Forbes *et al.*, 1998).

Another method is based on *Agrobacterium*-mediated transformation. At present, *Agrobacterium*-mediated transformation (AMT) is considered as the system of choice for many fungi, since *A. tumefaciens* can easily transfer the T-DNA to a broad range of fungi. Both targeted and random gene disruption can be achieved by this method (Michielse *et al.*, 2005a; 2005b). AMT of filamentous fungi was developed by de Groot *et al*. (1998), and was recently adapted for use in *Beauveria bassiana* by Fang *et al*. (2004) using the herbicide resistance gene as a selective marker. The advantage of AMT over biolistics is the production of more stable transformants with less complex insertional mutants than with biolistics.

1.15 Aims of this research

The general aim of this research is to investigate sub-species variation in New Zealand populations of *Cylindrocarpon* species infecting grapevines. This work will involve the identification of the species prevalent in New Zealand and analysis of the genetic variability of these populations. The variability in pathogenicity by different genotypes will be assessed and correlated to their ability to produce cell wall degrading enzymes *in vitro*. To achieve this aim, four objectives have been formulated as below.

Objective 1: To identify the *Cylindrocarpon* species and investigate their prevalence in New Zealand vineyard regions.

Objective 2: To determine the genetic variability of *Cylindrocarpon* species in New Zealand vineyards.

Objective 3: To correlate the genetic variability of *Cylindrocarpon* species with pathogenicity and to develop *gfp* transformed isolate(s) of *Cylindrocarpon* species to investigate the infection process.

Objective 4: To characterise the key cell wall degrading enzymes produced by *Cylindrocarpon* species and to assess any intraspecies variability in gene sequences or production levels.

Chapter 2
Identification of *Cylindrocarpon* species and their distribution in vineyards

2.1 Introduction

The 174 *Cylindrocarpon*-like isolates collected by Carolyn Bleach from diseased New Zealand grapevines were not all identified; the 60 isolates identified by molecular methods were predominantly *C. macrodidymum*, *C. destructans* and *C. liriodendri*. The isolate collection was obtained from 121 symptomatic grapevines contributed by 49 grape growers from the eight grape growing areas (18 isolates from Central Otago, 20 from Hawkes Bay, 10 from Waipara, 93 from Marlborough, 3 from Nelson, 13 from Gisborne, 14 from Auckland and 3 from Martinborough) in 2005. The samples were collected from grapevines showing some of the following symptoms: general decline, low vigour, stunted rootstock, short shoots, early senescence, dead scion, yellowing of the leaves, no bud burst, distorted leaves, no shoot growth and collapse during the growing season. Isolation was done by Ms Bleach from both roots and rootstock, trunks of the symptomatic grapevines, which represented most of the commonly grown rootstock varieties. The majority of the rootstock samples collected from the vineyards were 101-14 (68.1%), SO4 (11%), Gravesac (5.5%) and 15.4% for which the rootstock variety was unknown. However, identification of all the isolates collected during that survey is needed to provide information on species identification, regional variation and genetic diversity of this group of pathogens. This information could assist with the development of systems to control these diseases.

The genus *Cylindrocarpon* (teleomorph: *Neonectria* Wollenw.) contains more than 125 described species (Booth 1966; Schroers *et al*., 2008).A review of the taxonomy of the casual agents of black foot disease by Halleen *et al*. (2004), which included morphological characters and phylogenetic analysis of four *Cylindrocarpon* and *Cylindrocarpon*-like taxa isolated from symptomatic and asymptomatic grapevines from different parts of the world, identified three groups. The first group of isolates was defined as *Cylindrocarpon macrodidymum* Schroers, Halleen and Crous (*Neonectria macrodidyma* Halleen, Schroers and Crous) and included isolates from Australia, Canada, New Zealand and South Africa. This species was also reported in the USA (Petit and Gubler 2005), Chile (Auger *et al.*, 2007) and Spain (Alaniz *et al.*, 2007). The second group of isolates was described as a new genus called *Campylocarpon*. The third group of isolates was initially described as *Cylindrocarpon destructans*/*Neonectria radicicola*, and included isolates from France, New Zealand and South Africa. Later Halleen *et al*. (2006b) studied isolates morphologically assignable to the

C. destructans/*Neonectria radiciola* complex and concluded that many of the isolates from grapevines should be ascribed to *Neonectria liriodendri* Halleen, Rego and Crous, the teleomorph of *Cylindrocarpon liriodendri* J.D. MacDonald and E.E. Butler, a species previously associated with root rot of tulip poplar (*Liriodendron tulipifera*) in the USA (MacDonald and Butler 1981). *Cylindrocarpon lirodendri* was recorded in grapevines from France, Portugal, South Africa (Halleen *et al*. 2006b), Australia (Whitelaw-Weckert *et al*., 2007), the USA (Dubrovsky and Fabritius, 2007; Petit and Gubler, 2007), Spain (Alaniz *et al*., 2007) and Iran (Mohammadi *et al*., 2009). More recently, another species called *Cylindrocarpon pauciseptatum* was reported to be associated with grapevines (Schroers *et al*., 2008). This species was isolated from roots of *Vitis* spp. in New Zealand and Slovenia.

In the past, identification of *Cylindrocarpon* species has relied on morphological characteristics including conidial size and shape. However, overlapping morphological features, especially between the species, has meant that sole use of anamorph features for identification is inaccurate. Within a given *Cylindrocarpon* species, isolates have variable phenotypes and the range of these phenotypic characteristics overlap between species (Alaniz *et al*., 2009a). Consequently, it has not been possible to establish a reliable correspondence among these variables and the description of species (Halleen *et al.*, 2009). The development of species specific primers has provided a powerful tool for the detection of these pathogens (Mostert *et al*., 2006b; Alaniz *et al*., 2009a; Probst 2011). Therefore, these identification tools can be applied to provide accurate identification of *Cylindrocarpon* species from New Zealand vineyards.

The main goal of this objective was to use current molecular techniques to identify the *Cylindrocarpon* species present in a collection of 174 *Cylindrocarpon*-like isolates recovered from symptomatic plants in vineyards throughout the country.

2.2 Materials and methods

2.2.1 Species Identification

2.2.1.1 Sample Collection, Cultivation and storage of isolates

The 174 *Cylindrocarpon*-like isolates collected by Ms Carolyn Bleach that had been maintained in the Plant Pathology Research Group as an existing culture collection were used for this study. The nomenclature system for the collection (Appendix A.1) identifies the place where the sample was isolated, Ack (Auckland), CO (Central Otago), Mar (Marlborough), Mtb (Martinborough), Nel (Nelson), Gis (Gisborne), Wpa (Waipara), HB (Hawkes Bay). The number (1, 2, 3 etc.) denotes the vineyard and the letters (a, b, c etc.) denotes the isolations taken from the vine samples. For example, with isolate Ack1b and Ack1c, the Ack denotes Auckland, 1 denotes Nursery, “b” and “c” denote different isolations from vines.

2.2.2 Identification of *Cylindrocarpon* species based on colony morphology

In this study, the initial identification of *Cylindrocarpon* species was carried out based on their colony morphology on potato dextrose agar (PDA; Figure 2.1; Appendix A.3.1), using characteristics reported in the literature (Rego *et al*., 2001a; Halleen *et al*., 2004; Halleen *et al*., 2006b). For all *Cylindrocarpon*-like isolates, duplicate PDA plates were inoculated with 7 day old mycelial discs and incubated in 12 h dark:light conditions at 20°C for 7-14 days. The plates were observed by eye under standard fluorescent lights at 3day intervals for their morphology, and grouped on the basis of following characteristics: -

Group A (putative *C. destructans*) – Cottony or felty aerial mycelium initially white to cinnamon.In older colonies (9-12 days), production of reddish brown in the central portion and beige in the borders.

Group B (putative *C. liriodendri*) – Colonies were initially cream, becoming cinnamon to sepia with feathery aerial mycelium.

Group C (putative *C. macrodidymum*) – Colonies initially white becoming tinged with beige.In older colonies (9-12 days), production of yellow pigmentation with a floccose to feltedaerial mycelium covering the whole colony or in sectors.

Group D (Unknown) – Colonies that showed undulate, feathery edges which were orange in the middle and brown in the borders or over the whole colony.

2.2.3 Storage of *Cylindrocarpon* cultures

The cultures had been stored by Ms Bleach as single spore isolates on Spezieller Nährstoffarmer slopes at 4°C. For this experimental work, all isolates were subcultured by transferring a portion from the edges of the slopes to Spezieller Nährstoffarmer agar (SNA; Appendix A.3.3) plates and grown them at 20°C for approximately 7 days. As a culture resource for this work mycelia plugs (5-10) were taken from the growing edges of these colonies and transferred into sterile vials containing 20% glycerol for long term storage at −80°C (Thermo Scientific, Forma 900 series). When required, cultures were recovered from storage by plating a plug of the agar containing mycelium onto potato dextrose agar (PDA, Difco Laboratories, Detroit, USA) and growing at 20°C in 12:12h light:dark for 7 days.

2.2.4 Isolation of genomic DNA

For genomic DNA extraction, all isolates were subcultured by placing 2-3 agar plugs taken from the growing edge of a 14 day old culture that had been grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, USA; Appendix A.3.1) into a deep Petri dish containing potato dextrose broth (PDB; Difco Laboratories, Detroit, USA; Appendix A.3.2). These

cultures were grown at 20°C in 12:12h light:dark for 7 days or until the hyphae reached the edge of the Petri dish. Mycelial mats were then lifted from the broth using a sterile pipette tip and squeezed between sterile Miracloth™ (Calbiochem) and paper towels to remove excess liquid. The agar plug was removed and the mycelial mat was wrapped in aluminium foil and snap frozen in liquid nitrogen. Frozen mycelium was stored at −80°C until needed.

Genomic DNA was extracted from frozen mycelium using a PUREGENE genomic DNA isolation kit (PUREGENE®, Gentra Systems, Minneapolis, USA) according to the manufacturer's instructions, but adapted for use on fungi as follows. Frozen mycelium was ground to a fine powder in liquid nitrogen using a mortar and pestle and 100 mg transferred to a 1.7 mL tube. Five hundred µL of Cell Lysis solution was added to the tube, mixed with the ground mycelium by pipetting and incubated at 65°C for 1 h. The tube was inverted twice during the incubation. Once the tube had cooled to room temperature 1.5 µL RNase A solution (Invitrogen, Purelink™, RNase A, Carlsbad, CA, USA) was added to the cell lysate, mixed by inversion and incubated at 37°C for 15 min. The sample was cooled to room temperature and 167 µL Protein Precipitation solution was added, mixed by vortexing for 20 s and centrifuged at 16,000 × *g* for 3 min. The supernatant containing the DNA was transferred to a clean 1.7 mL tube, 500 µL of ice-cold isopropanol was added and the sample was mixed by inversion 20times. The precipitated DNA was pelleted by centrifugation at 16,000 ×*g* for 1 min, washed in 300 µL of 70% ethanol and then inverted to air dry for 10-15 min. The DNA was resuspended in 50 µL of DNA Hydration solution and stored at 4°C. The concentration of the extracted DNA was determined by spectrophotometry using a Nanodrop 3.0.0 spectrophotometer (Nanodrop technologies Inc. Delaware, USA) and diluted to 20 ng/µL for PCR.

Genomic DNA quality and yield was confirmed by electrophoresis in 1% agarose gels. To prepare the gel, 1.5 g of agarose (Agarose Molecular Grade, Bioline, London) was dissolved in 150 mL 1 × TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5) by boiling. The molten agarose was cooled to 60°C before it was poured into a gel casting tray. A well-forming comb was inserted and the gel allowed to set for 30 min after which it was placed in an electrophoresis tank containing 1×TAE buffer. To prepare samples for gel loading, 5 µL aliquots of the DNA samples were each mixed with 3 µL of loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 40% w/v sucrose). Samples were separated by electrophoresis at a constant 10 V/cm for 45 min. Each agarose gel contained 5 µL of 1 Kb Plus DNA Ladder™ (0.1 ng/µL) (Invitrogen, California) for molecular weight comparisons. Gels were stained by immersion in 0.5 µg/ml ethidium bromide for 15 min and destained by soaking in tap water for 10 min. The DNA was visualised on a UV transilluminator and photographed under UV light using the VersaDoc™ Imaging System (Model 3000, Bio-Rad Laboratories Inc, CA, USA).

2.2.5 Species specific PCR

Species identification was performed as described in Probst (2011) using primers specific for *C. destructans*, designed by Dr Hayley Ridgway (Lincoln University, New Zealand), and for *C. macrodidymum* and *C. liriodendri*, which were designed by Dr Lizel Mostert (University of Stellenbosch). The DNA sequences of primers used for species specific PCR of the three *Cylindrocarpon* species are shown in Table 2.1. Each PCR was conducted in a 25 µL reaction volume that contained 1× PCR buffer (Roche Molecular Biochemicals, Germany), 200 µM of each dNTP, 10 pmol of each primer, 1.25 U Faststart Taq DNA polymerase (Roche Molecular Biochemicals, Germany) and 20 ng of DNA. Species specific amplification of *C. destructans* was conducted in an iCycler (Bio-Rad) using the following thermal cycle: 3 min at 94°C, then forty cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. For *C. macrodidymum* and *C. liriodendri* the thermal cycle consisted of 3 min at 94°C, followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s and extension at 72°C for 30 s, followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 62°C and an extension at 72°C for 30 s, followed by 23 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, followed by final extension at 72°C for 7 min. Negative control tubes in which the DNA template was substituted with sterile water were included in each set of reactions to ensure the reagents were free of contamination. PCR products (5 µL) were mixed with 3 µL loading dye (see Section 2.2.4) and separated by electrophoresis in a 1% agarose gel, using the same methodology described in Section 2.2.4.

Table 2.1 The names and DNA sequences of primers used for species specific PCR of three *Cylindrocarpon* species.

Species	Primer name	Sequence (5' to 3')	Expected amplicon (bp)
Cylindrocarpon macrodidymum	Cyma F1	CTG GGA CAT GAT GGC TAA TAT GAC TTC TTG	300 bp
	Cyma R1	GGT GGT GTG AGT TTC GTG C	
Cylindrocarpon liriodendri	Cyli F1	CTC CTC TTC AAC GAT CCG ACG TGC C	200 bp
	Cyli R1	GGG GCA GAG CAG ATT TCG	
Cylindrocarpon destructans	Cyde small	TGC RGG SAT TCG CTA ACG	200 bp
	Cyde R2	CYT GGA TAK GGG CAG ATG	

2.2.6 Confirmation by sequencing

From each group of identified *Cylindrocarpon* species isolates, the identity of 10% of the isolates was confirmed by DNA sequencing. As the ribosomal RNA region contains insufficient polymorphism to adequately distinguish the three species, the sequences of the translation elongation factor 1α, internal transcribed spacer region (ITS), and β-tubulin region were used. These regions were amplified by general primers specific for the regions (1) the translation elongation factor 1α using primers EF1–728F (5´ CATCGAGAAGTTCGAGAAGG 3') and EF1–968R (5´ TACTTGAAGGAACCCTTACC 3´) (Carbone and Kohn, 1999), (2) the internal transcribed spacer region, using primers ITS1 (5´ TCCGTAGGTGAACCTGCGG 3´) and ITS4 (5´ TCCTCCGCTTATTGATATGC 3´) (White *et al.*, 1990) and (3) the β-tubulin gene, using primers Bt2a (5´ GGTAACCAAATCGGTGCTGCTTTC 3´) and Bt2b (5´ ACCCTCAGTGTAGTGACC CCTTGGC 3´) (Slippers *et al.*, 2004). Each 25 µL reaction contained 1× PCR buffer, 200 µM of each dNTP, 10 pmol of each primer, 1.25 U Faststart Taq DNA polymerase (Roche Molecular Biochemicals) and 30 ng of DNA. Amplification was conducted in an iCycler (Bio-Rad) using the following thermal cycle: 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 90 s, followed by final extension at 72°C for 10 min. Negative controls in which the DNA template was substituted with sterile water were included in each set of reactions to ensure that the reagents were free of contamination. Products (5 µL) were mixed with 3 µL loading dye and routinely separated by 1% agarose gel electrophoresis as described in Section 2.2.4. The sequencing PCR was separated using the ABI PRISM® 310 Genetic analyser (Applied Biosystem, Foster city, California) automated sequencer in the Lincoln University Sequencing Facility. For each DNA template, forward and reverse sequences were obtained and aligned using the computer program DNAMAN version 4.0a (Lynnon Biosoft®) to obtain a complete consensus sequence. The sequences were then submitted to a Blastn search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank database.

2.2.7 Sequencing of the unidentified isolates

Isolates that were not amplified by the species specific primers were amplified using the general β-tubulin primers as described in Section 2.2.6. Then the PCR products were separated by 1% agarose gel electrophoresis as described in section 2.2.4. The PCR products were sequenced as described in Section 2.2.6.

2.2.8 Phylogenetic analysis of *Cylindrocarpon* species

A phylogenetic tree was produced using the translation elongation factor 1α, ITS and β-tubulin sequences of the New Zealand *Cylindrocarpon* isolates. The sequences were edited to remove the ambiguities at either end of the sequences using DNAMAN (Lynnon Biosoft version 4.0) and the representative *Cylindrocarpon* species retrieved from GenBank were compiled into a single fasta format file and aligned by CLUSTALW using Mega version 5.05(Tamura *et al.*, 2011). The aligned sequences were analysed using maximum likelihood based on the neighbour-joining method (Saitou and Nei, 1987) and 1000 bootstrap replications. The resultant trees were analysed by Mega version 5.05 to find the best DNA tree (Tamura *et al.*, 2011). *Campylocarpon fasciculare* was used as an out-group.

2.2.9 Determining the prevalence and distribution of *Cylindrocarpon* species in New Zealand vineyards

The number of successful isolations from symptomatic vines, their region of origin and their identification by morphology and molecular methods was used to investigate factors affecting the distribution of *Cylindrocarpon* species in symptomatic vine materials. To determine the predominant *Cylindrocarpon* species from each region, the proportion (percent) of isolates was calculated for each of the five *Cylindrocarpon* species (*C. destructans*, *C. liriodendri*, *C. macrodidymum*, *C. paucispetatum* and novel *Cylindrocarpon* species), one *Cylindrocladiella* sp. isolate, two *Neonectria hematococca* and five unidentified isolates. The data were analysed using Pearson's chi-square test (Minitab 16, Minitab Inc. United States) of independence at P≤0.05. This method determined whether the paired observations of two variables (i.e. *Cylindrocarpon* species incidence and their distribution in both North and South Islands) were independent or associated with each other.

2.2.10 Incidence of *Cylindrocarpon* species in infected rootstock varieties from New Zealand vineyards

For this study, the incidence of *Cylindrocarpon* species isolates and other identified and unidentified isolates from New Zealand vineyards was examined with respect to the rootstock varieties using one way ANOVA, Minitab. The rootstocks used to examine the incidence relationships were 101-14 (n=62), SO4 (n=10) and Gravesac (n=5). The data for other rootstocks were insufficient for inclusion in this study.

2.2.11 Effect of temperature on growth rate (*in vitro*) of *Cylindrocarpon* species

The effect of temperature on *invitro* growth rate of the three main *Cylindrocarpon* species isolated from grapevines was studied to determine the optimum temperature for growth. The growth rate was investigated at 5, 10, 15, 20, 25 and 30°C. Three isolates from each of the three species and from different wine growing regions were selected and grown on PDA at

20°C for 14 days to produce inoculum. Plates containing 15 mL of PDA (90 × 15 mm) were each inoculated centrally with a mycelial plug obtained from the edge of a 14 day old culture and sealed with cling film. The five replicates made for each temperature and each isolate were arranged in a completely randomised design in the incubator set to each of the respective temperatures. The plates were observed at two day intervals for their growth. On the 7th and 13th day after inoculation, colony growth was measured using a digital calliper in two perpendicular directions. The mean increase in diameter that occurred between these times was used to determine the growth rate (mm/24 h). To determine whether there were significant differences in optimum temperatures of the species, the growth rates were analysed with ANOVA, using SPSSv13.

2.3 Results

2.3.1 *Cylindrocarpon* species colony morphology

Of the *Cylindrocarpon*-like isolates grown on PDA, 90 were placed into group A as putative cultures of *C. destructans* (Table 2.2). These colonies were reddish brown in the central portion and beige in the borders after 7 days incubation (Figure 2.1 A). A further 40 isolates were placed into group B which consisted of those colonies with cinnamon to sepia centres radiating outwards to the edges. These colonies showed light brown colour on the reverse side after 7 days of incubation and were grouped as putative *C. liriodendri* (Figure 2.1B). Another 35 isolates that were yellow in colour with aerial mycelium that covered the whole colony or sectors of it were grouped as putative *C. macrodidymum* (Figure 2.1C). These 35 isolates had slower growth compared to other *Cylindrocarpon* species. The yellow pigment production was strong for the first week of incubation and reduced to lighter shade during the second week. Nine isolates which were different to the groups above were placed into an unknown group (Figure 2.1D). These isolates had undulate, feathery edges which were orange in middle and beige in borders or sometimes over the whole plate after 14 days of incubation at 20°C in 12:12 light:dark.

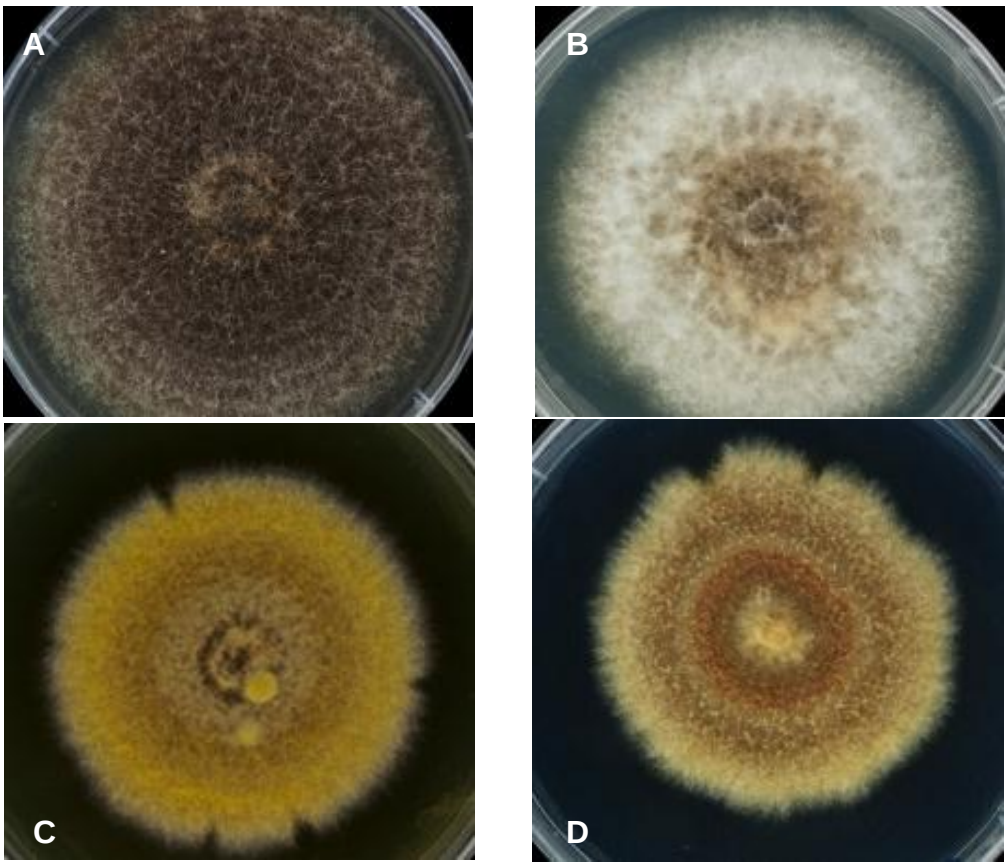


Figure 2.1 Grouping of *Cylindrocarpon* species isolates obtained from New Zealand grapevines based on their colony characteristics on PDA. A) Group A – putative *C.destructans* B) Group B – putative *C. liriodendri* C) Group C – putative *C. macrodidymum* D) Group D – Unknown group.

2.3.2 Molecular identification using species specific PCR

Of the 174 isolates amplified with primers Cyde small and Cyde R2 a total of 53 produced the expected 200 bp amplimer and were thus identified as *C. destructans* (Figure 2.2 A). Of these isolates 35 came from Group A and 18 isolates from Group B (Table 2.2). The remaining 121 isolates were amplified with CyliF1 and CyliR1, of which 57 isolates produced the expected 200 bp amplimer (Figure 2.2 B). These isolates were identified as *C. liriodendri,* of which 38 originated from Group A with 19 from Group B (Table 2.2). Of the remaining 64 isolates amplified using CymaF1 and CymaR1, 41 produced the expected 300 bp amplimer which identified them as *C. macrodidymum* (Figure 2.2 C). The majority (n= 33) of these isolates were from Group C with eight from Group A (Table 2.2). The remaining 23 isolates that were not identified by species specific PCR included the nine isolates from Group D (unknown), nine isolates from Group A, three isolates from Group B and two isolates from Group C (Table 2.2).

Table 2.2 The number of isolates of each of the five *Cylindrocarpon* species confirmed by molecular techniques that were placed into one of four morphological groupings.

Morphological group (isolates)	*C. destructans*	*C. liriodendri*	*C. macrodidymum*	*C. pauciseptatum*	*Cylindrocarpon sp.*	Other species
A (90)	35	38	8	0	2	7
B (40)	18	19	0	0	2	1
C (35)	0	0	33	2	0	0
D (9)	0	0	0	9	0	0
Total=174	53	57	41	11	4	8

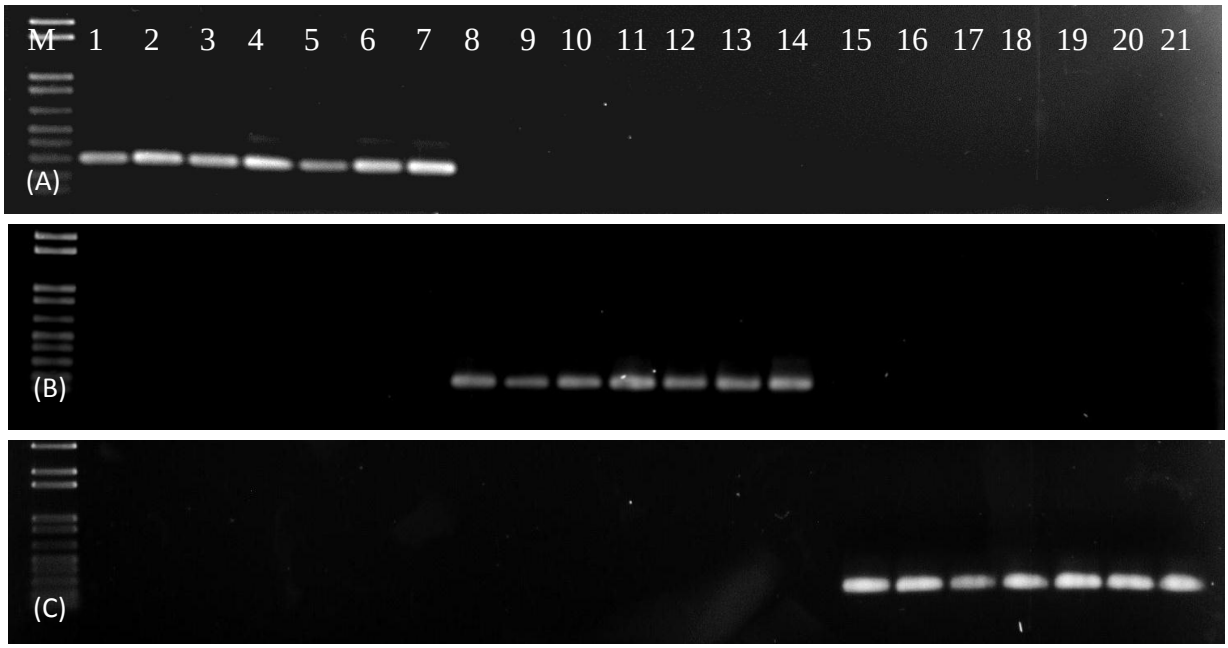


Figure 2.2 Species specific PCR products from 21 *Cylindrocarpon* isolates on 1% agarose gels. (A) *C. macrodidymum* (1-7) amplified with primers Cyma F1 and Cyma R1, (B) *C. liriodendri* (8-14) amplified with primers Cyli F1 and Cyli R1 and (C) *C.destructans* (15-21) amplified with primers Cyde small and Cyde R2. All gels show a 1Kb plus DNA Ladder (Invitrogen) on the left hand side.

2.3.3 Species confirmation by DNA sequencing

When the translation elongation factor 1α, ITS and β-tubulin genes of five isolates from each of three *Cylindrocarpon* species from different regions which were identified by species specific PCR were sequenced, all were confirmed as the species indicated by species specific PCR. Appendix A.2 contains a list of isolates and their sequence results; Figures 2.3, 2.4 and 2.5 show the phylogenetic trees. Isolates that were not identified by species specific PCR were identified by sequencing of the β-tubulin gene. They were confirmed as 11 isolates of *C. pauciseptatum*, four isolates of a novel *Cylindrocarpon* species (Figures 2.3,

2.4 and 2.5), one isolate of *Cylindrocladiella sp.* (Appendix A.2.6) and two isolates of *Neonectria haematococca* (Appendix A.2.7).

2.3.4 Phylogeny of *Cylindrocarpon* species

In the neighbour joining trees(Figures 2.3, 2.4 and 2.5), all New Zealand isolates grouped with the published sequences of the respective species, which confirmed the accuracy of the molecular species identification.

The ITS sequence tree placed the New Zealand *Cylindrocarpon* species in two major clades (Figure 2.3). Clade I was further divided into two sub-clades (A and B), of which Subclade A contained the *C. macrodidymum* and *Cylindrocarpon* sp. isolates clustered with representative international isolates, except for isolate Gis5a which was located on a separate branch. Subclade B contained New Zealand *C. pauciseptatum* isolates, which were identical to the international isolates. Clade II contained three subclades (Subclades C, D and E). Subclade C included four isolates of *C. destructans* clustered with *C. destructans* var *crassum* and *N. radicicola*. Subclade D contained only isolate Mar7a of *C. destructans,* which was similar to *N. radicicola,* and Subclade E contained *C. liriodendri* isolates.

The β-tubulin tree formed two main clades for the New Zealand *Cylindrocarpon* species. Clade I contained four subclades (Figure 2.4). Subclade A contained *C. macrodidymum* isolates grouping with representative international isolates. Subclade B contained *C. pauciseptatum* isolates and representative international isolates. Subclades C and D contained *Cylindrocarpon* sp. isolates. Clade II branched into three subclades. Subclade E and F contained *C. destructans* isolates and subclade G contained *C. liriodendri* isolates. Isolates Mar5d, Mtb1d, Mar6b and Mar9a were similar to *C. destructans* var *crassum* and isolate Mar7a was similar to *N. radicicola*.

The tree developed from translation elongation factor 1α sequences showed that the New Zealand *Cylindrocarpon* species grouped into three major clades (shown using brackets) (Figure 2.5). Clade I was divided into three sub-clades, namely, *C. macrodidymum* species (subclade A), and two subclades of different *Cylindrocarpon* sp. (subclades B and C). Of the isolates identified as *C. macrodidymum,* isolate Gis5a was located in a different branch to the other three isolates (Hb6a, Mar1a and Mar1d). Clade II was composed of *C. pauciseptatum* isolates and these were clearly distinct from other species. Clade III was also divided into subclades. All the *C. destructans* isolates were located in subclade D with isolates of *C. liriodendri* in subclade E. Isolate Mar7a clustered with *C. destructans* var *crassum* (*Ilyonectria crassa*) and the other four isolates with *C. destructans* (*Neonectria radicicola*).

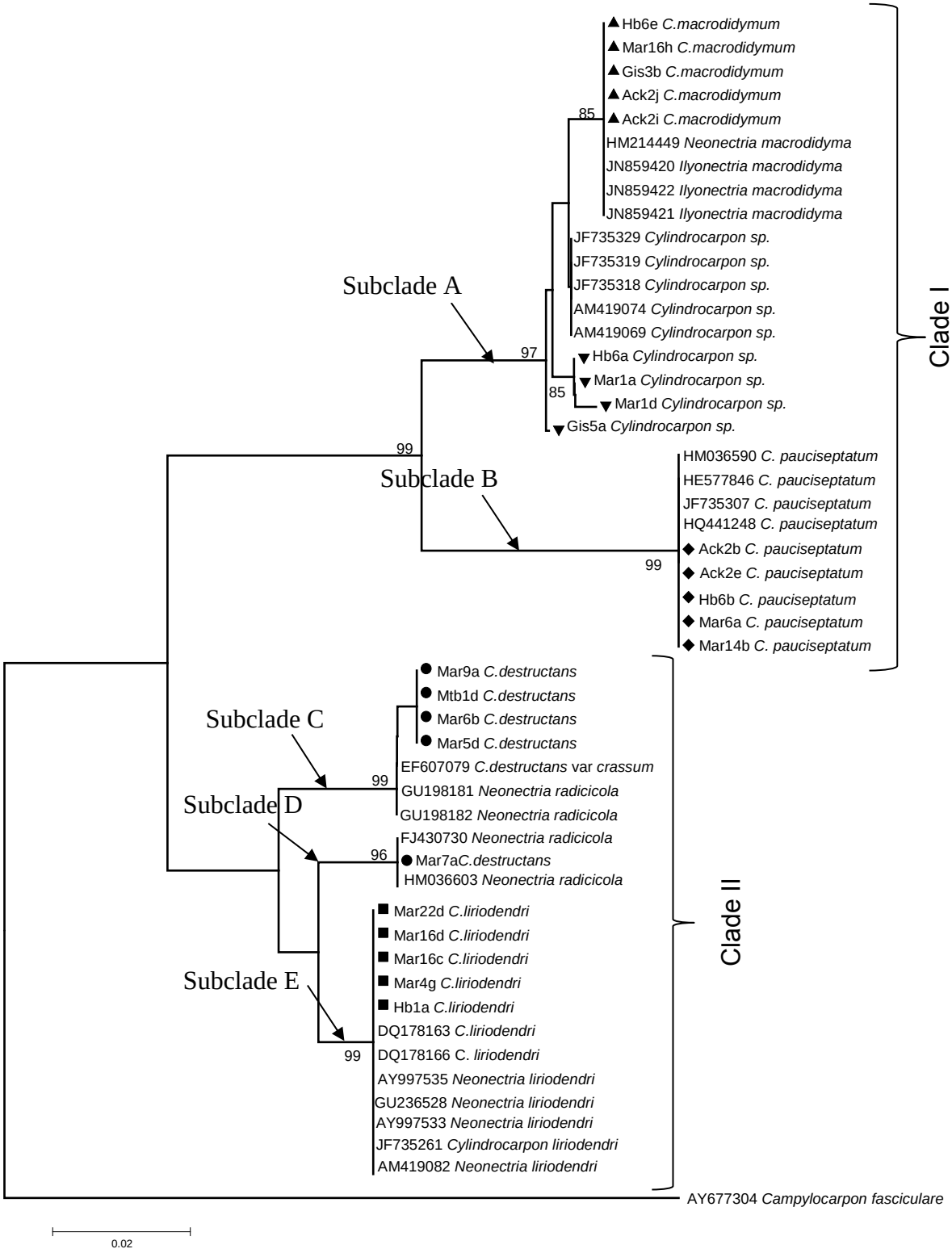


Figure 2.3 The neighbour joining tree with bootstrap values using 1000 replicates generated in MEGA 5.05 using the internal transcribed spacer (ITS) sequences of *Cylindrocarpon* species isolated from New Zealand vineyards (inserted with symbol) and from GenBank with accession number. *Campylocarpon fasciculare* was used as outgroup for the analysis.

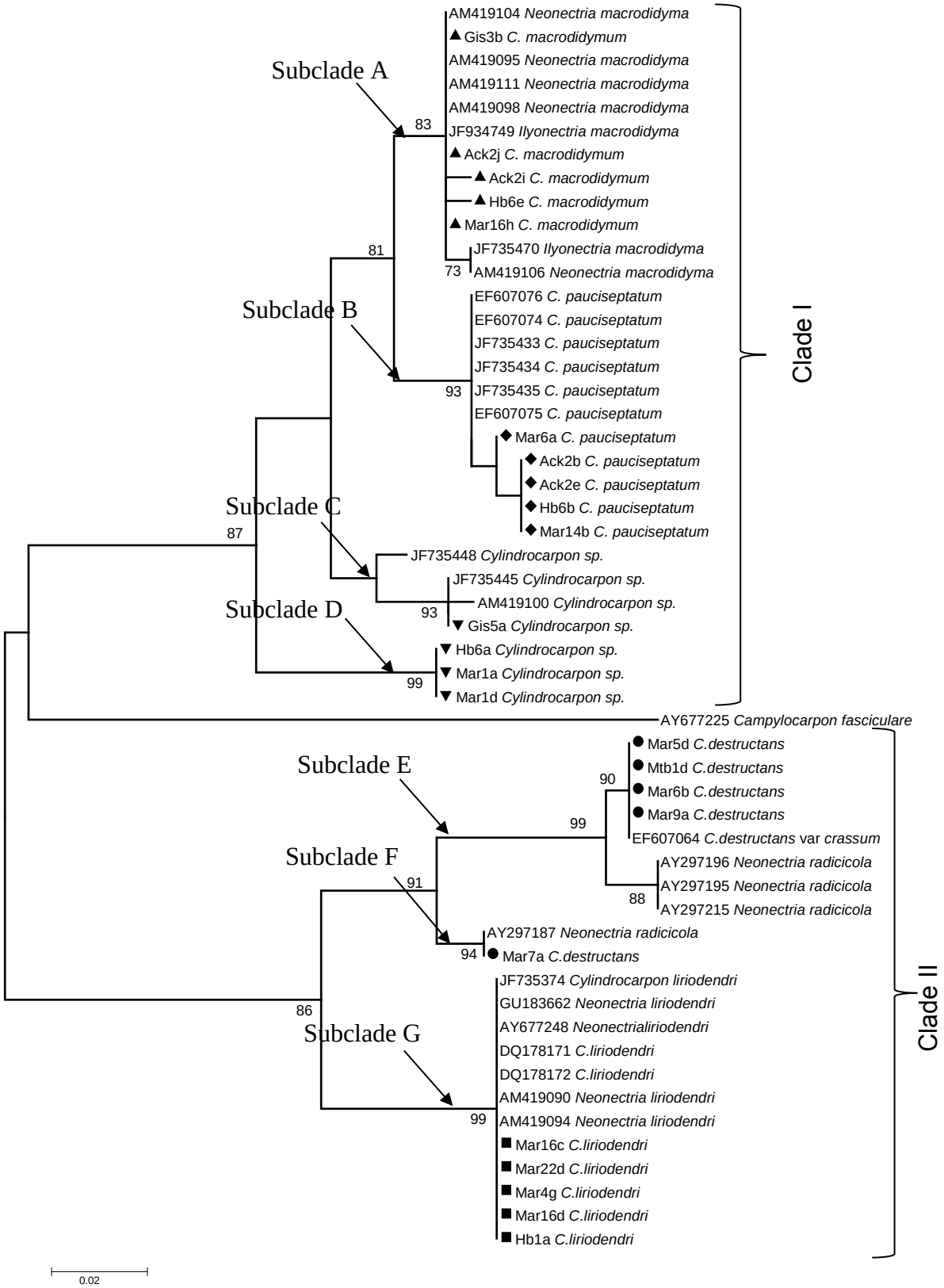


Figure 2.4 The neighbour joining tree with bootstrap values using 1000 replicates generated in MEGA 5.05 using β-tubulin gene sequences of *Cylindrocarpon* species isolated from New Zealand vineyards (inserted with symbol) and from GenBank with accession number. *Campylocarpon fasciculare* was used as out-group for the analysis.

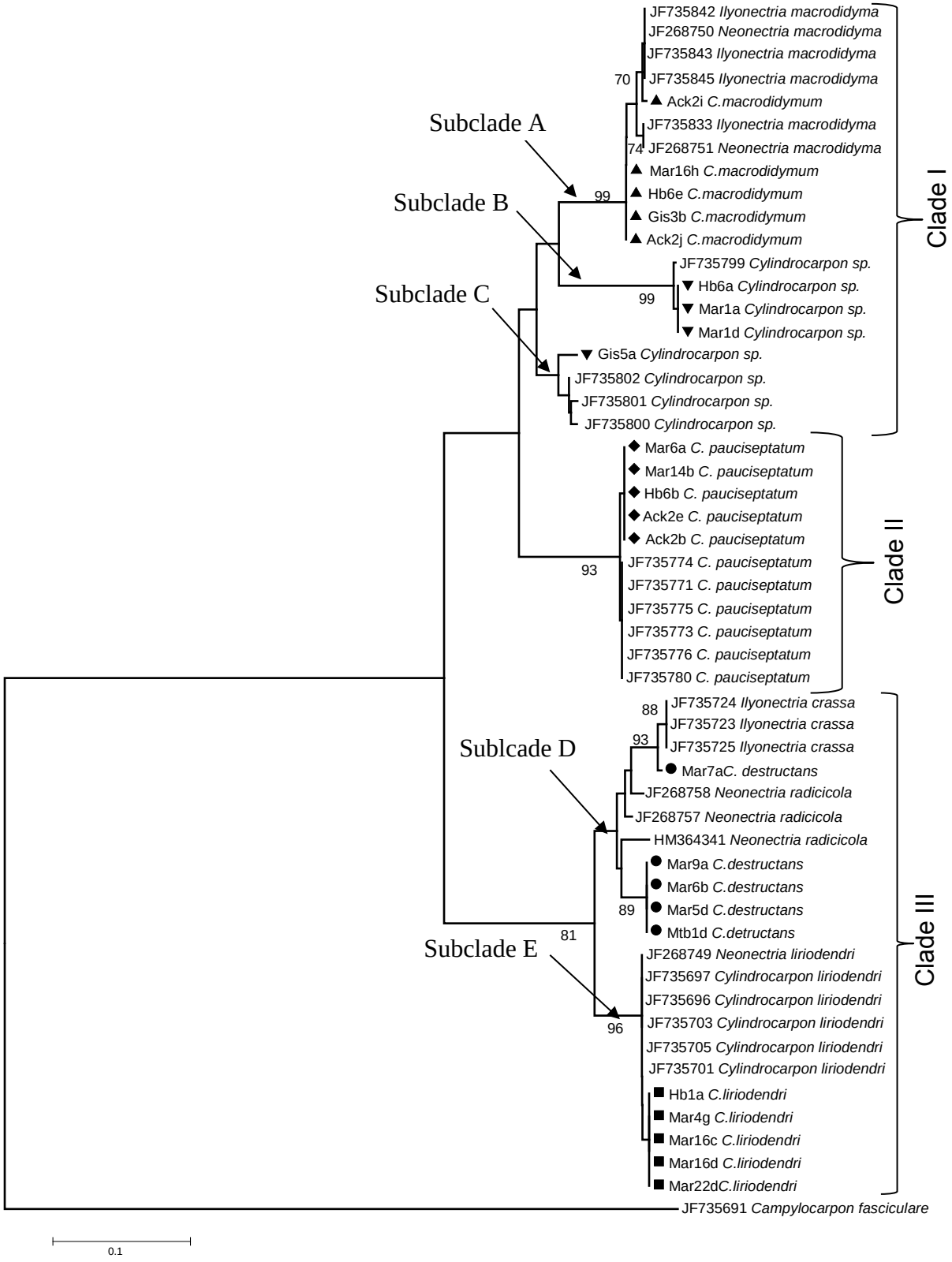


Figure 2.5 The neighbour joining tree with bootstrap values using 1000 replicates generated in MEGA 5.05 using TEF1-α gene sequences of *Cylindrocarpon* species isolated from New Zealand vineyards (inserted with symbols) and from GenBank with accession numbers. *Campylocarpon fasciculare* was used as outgroup for the analysis.

2.3.5 Accuracy of colony morphology for identification of *Cylindrocarpon* species

The molecular identification showed that only 53 isolates (59%) confirmed as *C. destructans* from the 90 isolates were originally placed into Group A (putative *C. destructans*). The remaining 37 isolates from group A were identified as *C. liriodendri* (20) and *C. macrodidymum* (17). A total of 57 isolates were identified using molecular techniques as *C. liriodendri* (putative group B), only 19 of these were from group B and 38 from group A. A total of 41 isolates were confirmed as *C. macrodidymum* and this included 33 isolates from Group C (putative *C. macrodidymum*) with an additional 8 isolates from Group A (Table2.2). Thus, the accuracy of morphological identification was: group A (59%), group B (50%) and group C (94%).

The nine unknown isolates (Group D) were identified as *C. pauciseptatum.* An additional two isolates from Group C were also identified as *C. pauciseptatum* (Table 2.2). The four novel *Cylindrocarpon* sp. isolates were equally divided between groups A and B. The *Cylindrocladiella* sp. isolate and two *Nectria hematococca* isolates were placed into groups A and B.

The correct identification achieved by the molecular methods using species specific PCR and sequencing of the taxonomically informative genes demonstrated a range of colony morphologies for each species. The range for *C. destructans* shown in Figure 2.6A (1-4), includes isolates numbered 2 and 3 from Figure A, which were misidentified as Group B (putative *C. liriodendri).* The range of colony morphologies for *C. liriodendri* is shown in Figure 2.6B, in which isolate 3 was misidentified as Group A (putative *C. destructans*).The range of colony morphologies for *C. macrodidymum* is shown in Figure 2.6C, of which isolate 1 was misidentified as Group B (putative *C. liriodendri*). The range of colony morphologies for *Cylindrocarpon* sp.is shown in Figure 2.6D, in which two isolates misidentified from Group A and the other two from Group B. The range of *C. pauciseptatum* shown in Figure 2.6E, in which two isolates misidentified from Group C. The *Cylindrocladiella* sp. shown in Figure2.6F and *Nectria hematococca* (Figure 2.6G) was misidentified from groups A and B.

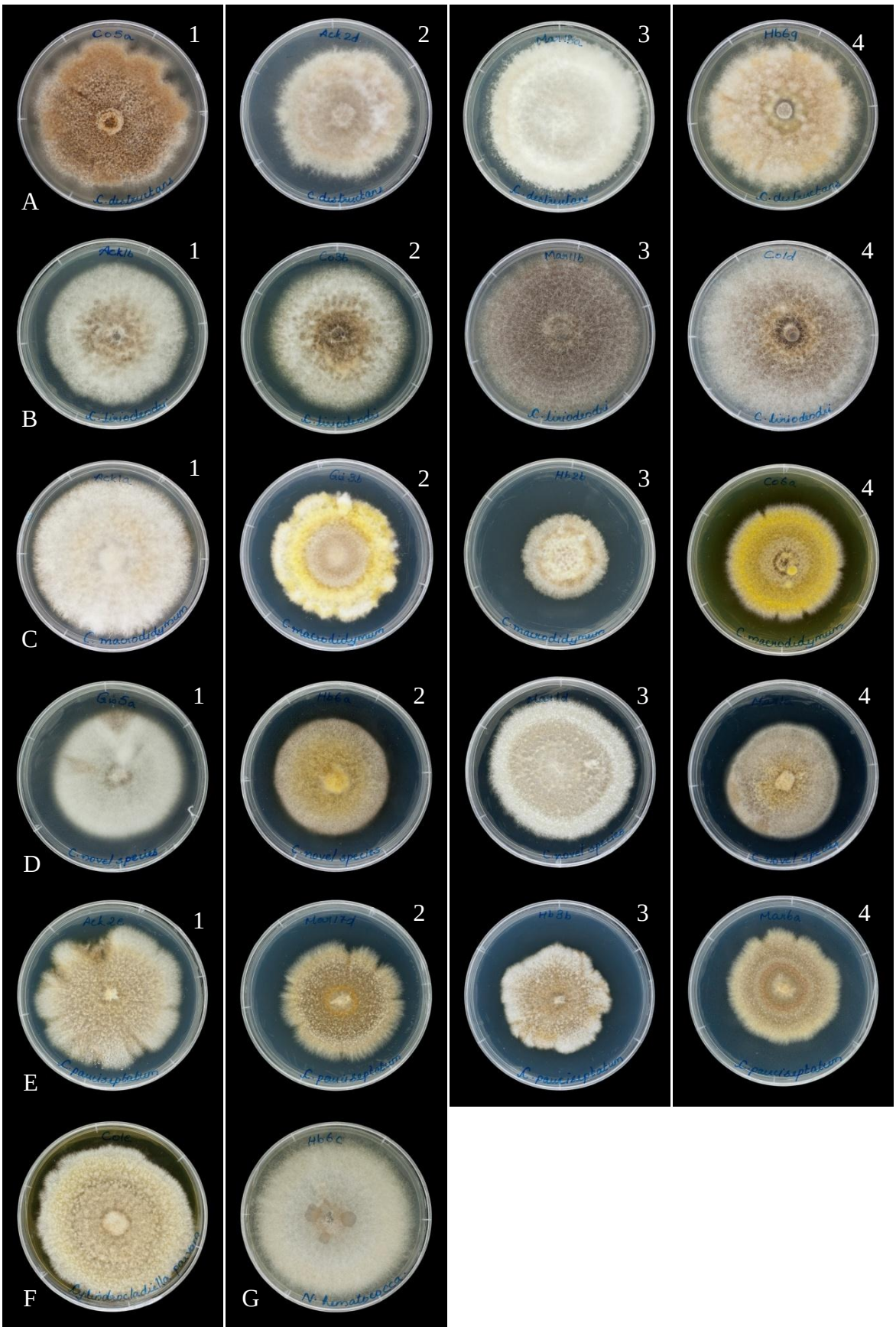


Figure 2.6 Varied colony morphologies of *Cylindrocarpon* species isolated from New Zealand grapevines. A) *C. destructans* isolates 1-4; B) *C. liriodendri* isolates 1-4; C) *C. macrodidymum* isolates 1-4; D) *Cylindrocarpon* species isolates 1-4; E) *C.pauciseptatum* isolates 1-4; F) *Cylindrocladiella sp.*; G) *Nectria hematococca*. All isolate identities were confirmed by molecular methods because of the overlapping colony morpholoy.

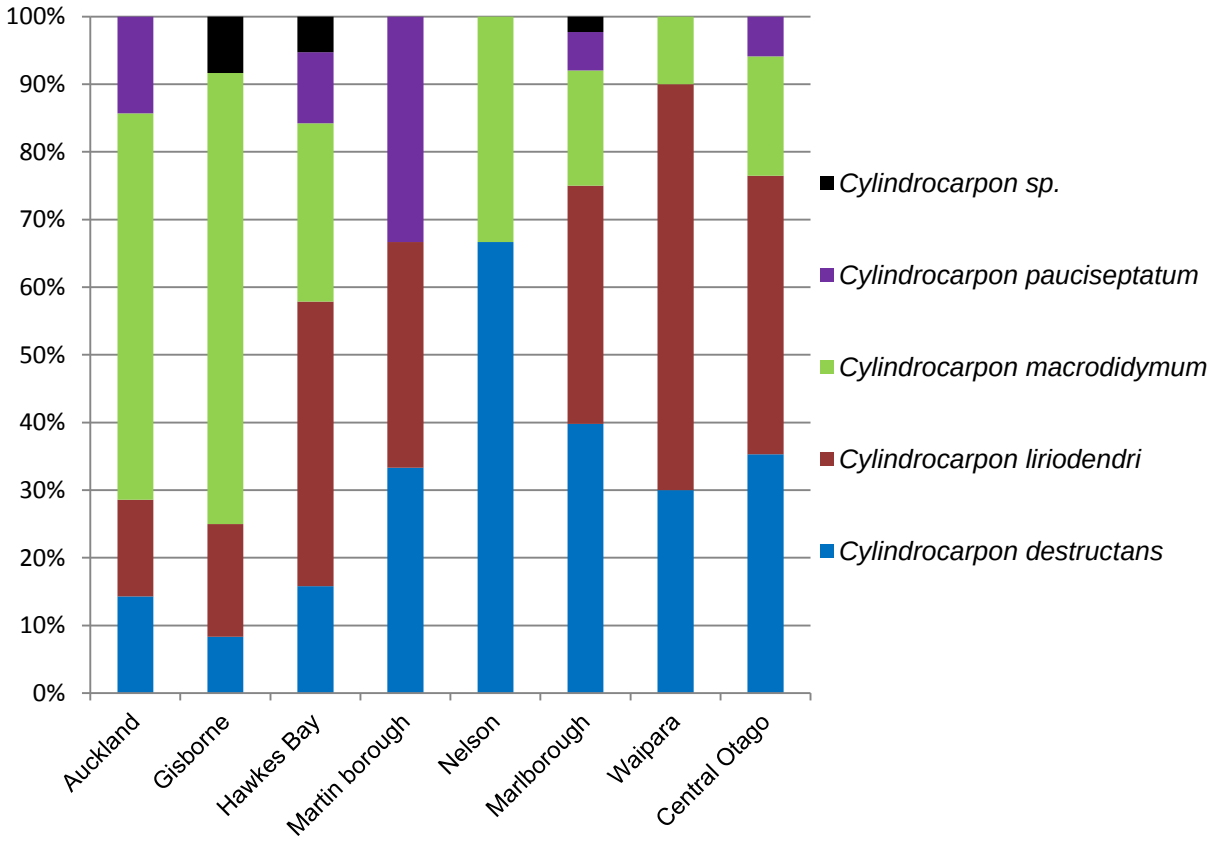
2.3.6 Regional distribution of *Cylindrocarpon* species

From 174 *Cylindrocarpon*-like isolates, 166 were identified as either *C. destructans*, *C. liriodendri*, *C. macrodidymum*, *C. pauciseptatum* and *Cylindrocarpon* sp. (Table 2.3) and the remaining eight isolates were *Nectria hematococca* (2), *Cylindrocladiella* sp. (1) and five were unidentified. Among these species, *C. liriodendri* was predominant (34%; n= 57), followed by *C. destructans* (32%; n=53); *C. macrodidymum* (25%; n=41), *C. pauciseptatum* (7%; n=11) and the novel *Cylindrocarpon* species (2%; n=4; Table 2.3). The three main *Cylindrocarpon* species, which comprised 90% of *Cylindrocarpon*-like isolates, were distributed throughout New Zealand (Figure 2.7), but with differences in the regional distribution (P=0.000; Pearson chi-square test) (Appendix A.5.1). A higher proportion of the isolates recovered in the South Island were *C. destructans* (39%, n=46/118) as compared to the North Island (17%, n=8/48). Almost half (46%, n=22/48) of the isolates recovered in the North Island were *C. macrodidymum* which was, proportionally, 2.5 times the number found in the South Island (17%, n=20/118; Figure 2.7). *Cylindrocarpon macrodidymum* was more frequently isolated from Auckland and Gisborne vines compared to other North Island regions. Fewer *C. liriodendri* isolates were recovered from the North Island (27%, n=13/48) than the South Island (37%, n=44/118). A higher proportion of *C. pauciseptatum* isolates were recovered from the North Island (10%, n=5/48) than from the South Island (5%, n=6/118). The novel *Cylindrocarpon* species was recovered from both the North Island (4%; n=2/48) and South Island (2%; n=2/118) in similar numbers.

Table 2.3 Incidence of *Cylindrocarpon* species isolated from diseased grapevines from eight major wine growing locations in New Zealand.

	Number of samples yielding *Cylindrocarpon* species				
Location	*C. destructans*	*C. liriodendri*	*C. macrodidymum*	*C. pauciseptatum*	*C. sp.*
Auckland*****	2	2	8	2	0
Gisborne*****	1	2	8	0	1
Hawkes Bay*****	3	8	5	2	1
Martinborough*****	1	1	0	1	0
Nelson[#]	2	0	1	0	0
Marlborough[#]	35	31	15	5	2
Waipara[#]	3	6	1	0	0
Central Otago[#]	6	7	3	1	0
Total	53	57	41	11	4

*North Island and [#]- South Island



Auckland (n=14), Gisborne (n=12), Hawkes Bay (n=19), Martinborough (n=3)
Nelson (n=3), Marlborough (n=88), Waipara (n=10), Central Otago (n=17)

Figure 2.7 Proportion of *Cylindrocarpon* species in major grape growing regions of New Zealand. The graph shows the percentage of each species identified in the different regions. The total number of isolates analysed from each region is shown at the base of the graph.

2.3.7 *Cylindrocarpon* species incidence from rootstocks

The survey data collected by Ms Bleach included isolates were recovered from 121 rootstock samples, however, *Cylindrocarpon* species were only isolated from 101 rootstock samples. These comprised 101-14 (n=62), SO4 (n=10), Gravesac (n=5), Riparia Gloire (n=3), 3309 (n=3), own rooted (n=2), 125AA (n=1), Schwarzmann (n=1) and unknown (n=14). Due to the limited numbers, only 101-14, SO4 and Gravesac were used to correlate infection with rootstock variety. For *C. destructans*, *C. liriodendri, Cylindrocladiella sp.*, *N.hematococca*, unidentified isolates and *Cylindrocarpon* sp., there was no significant difference in the proportion of isolates recovered from each of the three rootstocks ($P>0.05$) (Table 2.4; Appendix A.5.2). For *C. macrodidymum* and *C. pauciseptatum* more isolates were recovered from Gravesac than 101-14 and SO4 ($P<0.05$) (Table 2.4; Appendix A.5.2).

Table 2.4 Incidence of *Cylindrocarpon* species isolated from different rootstock varieties.

Species	% of rootstock infected		
	101-14 (n=62)	SO4 (n=10)	Gravesac (n=5)
Cylindrocarpon destructans	44%	60%	40%
*Cylindrocarpon macrodidymum**	21%a	40%b	80%b
Cylindrocarpon liriodendri	45%	40%	0%
*Cylindrocarpon pauciseptatum**	8%b	0%c	40%a
Cylindrocladiella sp.	2%	0%	0%
Nectria hematococca	2%	10%	0%
Unidentified isolates	5%	10%	0%
Cylindrocarpon spp.	5%	0%	0%

The symbol*and letters (a, b, c) indicate significant species differences (P<0.05) across the row of rootstock varieties (One way ANOVA test, Minitab). The LSD for *C. macrodidymum* is 0.435 and 0.287 for *C. pauciseptatum*.

2.3.8 Effect of temperature on growth rate (*in vitro*) of *Cylindrocarpon* species

The growth rates (mm/24 h) of the three main *Cylindrocarpon* species; *C. macrodidymum*, *C. liriodendri* and *C. destructans* over a range of incubation temperatures (5-30°C) are listed in Appendix A.6.3. The estimated optimum temperatures at which the maximum growth rates occurred varied significantly between species (P<0.05). Pairwise comparisons showed that all species differed from each other (P<0.001; Appendix A.6.1). The mean optimum temperatures were 17.7°C for *C. destructans*, 18.6°C for *C. liriodendri* and 19.3°C for *C. macrodidymum* (Table 2.5). The three isolates from each three *Cylindrocarpon* species showed significant differences (P<0.05) in their optimum temperatures for growth (Table 2.5; Appendix A.6.2). Amongst the nine isolates tested, *C. macrodidymum* isolate Ack1a had the highest optimum temperature for growth at 20.46°C. The lowest optimum temperature was for *C. destructans* isolate Mar7a at 16.95°C (Table 2.5).

Table 2.5 The optimum growth temperature for each of three isolates and the means for each species.

Species	Isolates	Optimum temperature (°C)	Mean optimum temperature(°C)
Cylindrocarpon destructans	Co1c	18.15 b	
	Mar7a	16.95 a	17.7 a
	Gis1b	17.85 b	
Cylindrocarpon liriodendri	Co3b	19.39 b	
	Mar11b	18.26 a	18.6 b
	Mar8i	18.06 a	
Cylindrocarpon macrodidymum	Ack1a	20.46 b	
	Gis2d	18.67 a	19.3 c
	Mar16i	18.80 a	
		P=0.000	P=0.000
LSD		0.441	0.537

Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

2.4 Discussion

This study used isolates collected during a nationwide sampling to investigate the prevalence and distribution of *Cylindrocarpon* species infecting grapevines in New Zealand. This is the first such study of a large representative collection of *Cylindrocarpon* species isolates that used molecular tools to identify the species present in symptomatic New Zealand grapevines. The work used a combination of species specific PCR and DNA sequencing to identify all isolates in a collection of 174 *Cylindrocarpon*-like isolates. The optimal growth temperatures of these species were investigated and correlations made with their common regional sources.

The results identified at least five species; *Cylindrocarpon destructans*, *C. liriodendri*, *C. macrodidymum*, *C. pauciseptatum* and *Cylindrocarpon* sp. These were isolated from both trunk and root sections of declining vines (101 samples) that were collected from 49 grape growers from eight major grapevine growing locations across New Zealand. Infection by *Cylindrocarpon* species occurred in 87% of the symptomatic material and was present in all eight locations. In the United States, a similar 'survey' of *Cylindrocarpon* species associated with black foot disease conducted in three states (Vermont, New York and Michigan) and one Canadian province, (Quebec) 90 symptomatic wood samples were collected from roots and trunks, of which 13% were infected with the *Cylindrocarpon* species (Petit *et al.*, 2011).

Clearly, the current New Zealand ‘sampling’ to collect *Cylindrocarpon* species causing black foot disease has provided greater proportion than the ‘survey’ by Petit *et al*. (2011) which also collected only symptomatic vines. A similar low proportion was reported from a random survey conducted in Spain by Aroca *et al*. (2006). Their survey randomly selected grapevines from nurseries, distributors and new vineyards to investigate incidence of trunk disease pathogens. They collected a total of 208 grafted and non-grafted grapevines in 2002 and 2004 and found *Cylindrocarpon* species in 16% and 15.3% of vines in 2002 and 2004, respectively. These two reports represent all the available literature on surveys for grapevine black foot disease and appeared to show that the incidence of black foot was much less in other countries than in New Zealand.

The proportion of black foot disease in New Zealand vineyards reported here was not a true reflection of the disease incidence since it was biased by the collection of only symptomatic vine samples. It also reflected the situation in a particular region, where a greater perceived problem by grape growers caused them to contribute more symptomatic vines. Most vine samples were collected from Marlborough (55%) and least from Martinborough (2.4%), which reflected the age of the vines in those areas. In Marlborough, there had been rapid expansion in vineyard area during the 2-3 years prior to the survey, whereas there had been little expansion in the Martinborough area during that time (New Zealand Winegrowers Statistical Annual report, 2011). Since young vines are more susceptible to damage by black foot pathogens than mature vines and the disease is more common in heavy soils than light ones (Gubler *et al.*, 2004), such factors can influence survey results.

In this study, the higher proportion of *Cylindrocarpon* species observed in the symptomatic vines than in the United States survey (Petit *et al*., 2011) indicated that it is a major pathogen that contributes to grapevine disease and decline symptoms in New Zealand vineyards. The highest incidence in the study conducted by Petit *et al*. (2011) was in Quebec vineyards, in which 48% were infected with *Cylindrocarpon* species from 23 diseased wood samples. The greater proportion in New Zealand vineyards may be associated with age of vineyards, soil types or even use of land on which the previous crops had suffered from *Cylindrocarpon* spp. infection. More international surveys with a focus on isolation from roots and lower trunks of symptomatic vines may determine whether New Zealand’s incidence level is remarkably high or within the range observed worldwide. Alternatively, a New Zealand survey, which might be used to indicate the disease status of the national vineyard, should aim to randomly collect similar numbers of samples from all regions. Inclusion of apparently healthy plants in the survey could also determine whether there was latent infection within roots. However, growers are naturally reluctant to contribute healthy vines so such a plan might not be feasible.

Of the identified species, *C. liriodendri*, *C. destructans* and *C. macrodidymum* were the most predominant (95%) species found in New Zealand vineyards. In New Zealand these three *Cylindrocarpon* species have been demonstrated to be pathogenic (Bleach *et al.*, 2007; Pathrose *et al.*, 2010; Probst *et al.*, 2012). All of the species found in New Zealand have been reported as pathogens in other grape growing regions worldwide (Sweetingham, 1983; Grasso, 1984; Maluta and Larignon, 1991; Scheck *et al.*, 1998a; Rego *et al.*, 2000; Nascimento *et al.*, 2001; Garrido *et al.*, 2004b; Petit and Gubler, 2005; Whitelaw-Weckert *et al.*, 2007). In particular, the literature indicates that *Cylindrocarpon liriodendri* and *C. macrodidymum* are the major threat to grapevines worldwide (Halleen *et al*., 2004; Halleen *et al*., 2006a; 2006b; Auger *et al*., 2007; Petit and Gubler, 2007; Alaniz *et al*., 2009b).

In some countries, different combinations of pathogens were reported to be present. In Chile only *C. macrodidymum* has been reported (Auger *et al*., 2007), while in South Africa Halleen *et al*. (2004; 2006b) reported five species causing black foot disease in vineyards and nurseries. They were *C. destructans*, *C. liriodendri*, *C. macrodidymum*, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare*. In Canada, three *Cylindrocarpon* species (*C. destructans*, *C. liriodendri* and *C. macrodidymum*) and also *C. didymium* and *N. mammoidea*-like species have been reported (Petit *et al*., 2011). Alaniz *et al*. (2009a) reported three *Cylindrocarpon* species (*C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum*) in Spain but not *C. destructans* which has also not been reported in Uruguay (Abreo *et al.*, 2010). In Iran only *C. liriodendri* and *C. destructans* have been reported (Mohammedi *et al*., 2009) but not *C. macrodidymum*. In New Zealand, five *Cylindrocarpon* species, *C. destructans*, *C. liriodendri*, *C. macrodidymum*, *C.pauciseptatum* and *Cylindrocarpon* sp., were found in grapevines.

In California four *Cylindrocarpon* species, *C. destructans*, *C. liriodendri*, *C. macrodidymum* (Petit and Gubler, 2007) and *C. obtusisporum* (Scheck *et al*., 1998a) have been reported. However, the identity of *C. obtusisporum* isolates from grapevines has been questioned. Booth (1966) reported that *C. obtusisporum* had been found on *Beilschmiedia* and *Coprosma* spp. The international collection of microorganisms from plants (ICMP) collection, New Zealand also lists two isolates of *C. obtusisporum* from collapsed stem of *Panax quinquefoilum* (ICMP 14372) which had been contributed by Braithwaite, M (2001) and from roots of *Vitis riparia* (ICMP 17329 and contributed by Ho, W. (2007). However, Halleen *et al*. (2006a) reported that *C. macrodidymum* had been misidentified in *Vitis vinifera* as *C. obtusisporum* since they found that the "*C. obtusisporum"* isolates formed perithecia when mated with *C. macrodidymum*. In 2005, Petit and Gubler reported *C. macrodidymum* in California using the ITS and β-tubulin sequences and as a result, *C. obtusisporum* is generally believed to not cause black foot on grapevines.

In New Zealand the incidence of *C. destructans* was similar to that of *C. macrodidymum* and *C. liriodendri.* In New Zealand, the presence of *C. destructans* inoculum in the soil is thought to be high because vineyards have often replaced orchards of apple and stone fruit which are susceptible to this pathogen (Bonfiglioli, 2005). Infection by these *Cylindrocarpon* species might also have originated from infected mother plants which supply the scion and rootstock cuttings for propagation, or by contamination or infection of grafted plants in the course of propagation process prior to planting (Halleen *et al.*, 2005b). It would be beneficial to test the genetic similarity of the *Cylindrocarpon* grapevine isolates by comparing with the *Cylindrocarpon* apple isolates maintained in the ICMP culture collection.

The sequencing results for four *C. destructans* isolates showed that both *N. radicicola* and *C. destructans* var *crassum* were present. Seifert *et al*. (2003) hypothesised the existence of different phylogenetic species among isolates grouped as *C. destructans* (teleomorph – *N. radicicola*). *Cylindrocarpon destructans* var *destructans* and *C.destructans* var *crassum* are included in the *N. radicicola* complex. However, in this research programme the species specific PCR methods were unable to discriminate between the *C. destructans* var *crassum* and *N. radicicola* or other members of the *C. destructans* complex. Further development of new primers to alternative taxonomic genes, such as the translation elongation factor 1α may improve resolution. Other researchers have reported the use of translation elongation factor 1α in species identification (Amatulli *et al.*, 2010; Hasegawa *et al.*, 2010; Wright and Harmon, 2010).

Cylindrocarpon pauciseptatum was recovered from symptomatic vines either alone or in association with the other main *Cylindrocarpon* species. However, no other studies showed that the association of *C. pauciseptatum* with other main *Cylindrocarpon* species. This indicates that *C. pauciseptatum* might also be a grapevine pathogen in New Zealand. Schroers *et al*. (2008) isolated *C. pauciseptatum* from the roots of *Vitis* species in Slovenia, while Alaniz *et al*. (2009a) isolated *C. pauciseptatum* from the grapevines roots with decline symptoms and demonstrated that this species was able to produce necrotic lesions on 110R rootstock. Martin *et al*. (2011) reported *C. pauciseptatum* as a causal agent of black foot disease of grapevines in Spain with potentially significant impact on grapevine nurseries. They identified this species based on morphology, gene analysis (ITS) and pathogenicity tests. In addition, in Southern Italy Yaseen *et al*. (2012) reported *C. pauciseptatum* to be associated with root rot and decline in peach. In their study, peach plants inoculated with *C. pauciseptatum* isolates showed black foot symptoms within 3 months. Another study done by Agustí-Brisach *et al*. (2011) reported damping off of *Pinus radiata* caused by *C. pauciseptatum* in Spain. However, there are no reports of *C. pauciseptatum* on other species in New Zealand. However, only Alaniz *et al*. (2009a) have demonstrated its pathogenicity on the grapevines. The low proportion (6%) in grapevines reported in this study may suggest the

recent introduction of this pathogen to grapevines or that it is not highly pathogenic on this host.

The identification of *C. destructans*, *C. liriodendri* and C*. macrodidymum* in this study was by species specific PCR using primers designed to amplify the β-tubulin gene. Their specificity for the respective *Cylindrocarpon* species was demonstrated in the current study and previous research (Probst, 2011). In addition to the primers used here, other species specific primers were designed by Alaniz *et al*. (2009a) to bind to the ITS region of the rDNA for *C. macrodidymum*, *C.liriodendri* and *C. pauciseptatum.* These primers could detect as little as 100 pg of *C. liriodendri* and *C. pauciseptatum* genomic DNA and 100 fg of *C. macrodidymum* genomic DNA extracted from pure cultures and also from 0.2 g of necrotic roots. They used nested PCR in a multiplex system to increase the sensitivity in plant material which had been artificially inoculated with the pathogen. These primers were unsuitable for use in the current research due to cross reactivity with *C. destructans* (Probst. 2011), although Alaniz *et al*. (2009a) used them to discriminate between *C. macrodidymum* and *C. liriodendri*. Other techniques that may also be considered to discriminate between *Cylindrocarpon* species include the development of a PCR-RFLP or PCR-SSCP method, such as those described for members of the *Botryosphariaceae* (Alves *et al.*, 2005; Ridgway *et al.*, 2011). At least two different novel *Cylindrocarpon* species, designated as *Cylindrocarpon* sp., were not identified by species specific PCR and were identified by DNA sequencing of three taxonomic genes (rDNA, β-tubulin and TEF-1α). The pathogenicity of these isolates has yet to be confirmed on grapevines.

DNA sequencing identified two further species, *Cylindrocladiella sp., Neonectria hematococca* that had been isolated from the symptomatic vines. Infection by *Cylindrocladiella* sp*.* was always found to be in association with one of the three main *Cylindrocarpon* species. This pathogen was also isolated from a declining grapevine in a newly established vine block in South Africa (Coller *et al.*, 2005). Another study reported that *Cylindrocladiella* sp*.* was present in different hosts in South Africa including *Vitis vinifera* (Crous and Wingfield, 1993; Victor *et al.*, 1998;Crous, 2002). The isolates recovered in the current study were used in further pathogenicity assays, which included rootstocks 101-14 and Schwarzmann. A first report of *Cylindrocladiella parvum* presence in New Zealand also demonstrated that it was pathogenic towards grapevines (Jones *et al.*, 2011). During that time, the isolate was identified as *Cylindrocladiella parvum*. However, the identified *Cylindrocladiella parvum* is later re-identified as *Cylindrocladiella sp*. according to the recent taxonomic classification (Lombard *et al.*, 2012). Given its close association with the other three main *Cylindrocarpon* species in symptomatic vines, it is possible that this pathogen may form part of a pathogen complex responsible for black foot disease of grapevines in New Zealand.

Nectria hematococca (anamorph=*Fusarium solani*) was isolated from infected grapevines. The two isolates identified (1% of total incidence) were both associated with infection by one of the three main *Cylindrocarpon* species. This fungus has been reported to cause disease in many crops, such as, sudden death syndrome of soybean (Aoki *et al.*, 2003), root and fruit rot in *Cucurbita* species (Mehl and Epstein, 2007), crown root in grafted cucumber (Li *et al.*, 2010), dry rot of potato, root rot of bean and pea (Eskandari *et al.*, 2010). There are a few reports of this species causing disease in grapevines. *Fusarium solani* has been associated with or shown to cause root rots (Lele *et al.*, 1978; Andrade, 1993; Gugino *et al.*, 2001). van Coller *et al*. (2005) tested the pathogenicity of *F. solani*, *F. proliferatum* and *F. oxysporum* on grapevines grafted onto 101-14 Mgt and 99 Richter and showed that the three *Fusarium* species were pathogenic to the rootstocks tested. Thus, it is possible that the *Nectria hematococca* isolates recovered in the current study are pathogenic to grapevines but further experiments would be needed to confirm this. The low proportion suggests that, if it is pathogenic, it is either not prevalent in this host, is acting as a secondary coloniser or that it is an emerging grapevine pathogen in New Zealand.

The initial procedure for grouping isolates in this study was morphotyping based on colony characteristics. The production of pigmentation on PDA has been widely used to distinguish *Cylindrocarpon* species, including *C. destructans*, *C. macrodidymum* and *C .liriodendri* (Rego *et al*., 2001; Halleen *et al*., 2004; Halleen *et al*., 2006b). However, previous research has shown that morphological characterisation of *Cylindrocarpon* species and other fungal pathogens such as *Fusarium* spp. and *Phaeoacremonium* spp., is difficult and can lead to inaccurate identification of recovered isolates (Groenewald *et al*., 2001; Alaniz *et al*., 2009b). Comparison of the results from the molecular identification with the morphology based groupings showed that morphology was most successful for *C. macrodidymum* (94%) and least successful for *C. destructans* and *C. liriodendri*. This demonstrates the limitations of this as a method of species identification. This problem has also been demonstrated by other groups. For example, isolates from *C. macrodidymum* have previously been misidentified as *C. obtusisporum* (Halleen *et al*., 2004; Petit and Gubler, 2005) and the *C. liriodendri* isolates have been misidentified as *C. destructans* (Halleen *et al*., 2006b; Petit and Gubler, 2007; Alaniz *et al*., 2009b). The PCR based detection methods that used species specific primers overcame these identification problems for the three main species.

Molecular methods have been commonly used for identifying plant pathogenic fungi for which morphological identification is inaccurate (Wang *et al.*, 2007; Miyazaki *et al.*, 2009). However, for *Cylindrocarpon* species the ongoing reclassification of these species has caused associated problems in the design of species specific primers. In 1996 a set of primers, Dest 1 and Dest 4, for *Cylindrocarpon destructans* were shown to also amplify a 400 bp band for *C. obtusisporum* isolates (Rego *et al*., 2001). However, there was doubt over the

accuracy of the identification of the *C. obtusisporum* isolates used by Rego *et al*. (2001) and this was later confirmed as *C. macrodidymum* after DNA sequencing of taxonomically informative genes (Halleen *et al*., 2004; Petit and Gubler, 2005). Nascimento *et al*. (2001) evaluated (Dest1/Dest4) primers to detect *C. destructans* from grapevine plants with DNA from pure cultures, but amplified both *C. destructans* and *C. obtusisporum*. As previously described, the primers designed by Alaniz *et al*. (2009b) based on the rDNA are unable to distinguish *C. macrodidymum* from *C. destructans*.

The distribution of the five *Cylindrocarpon* species from the survey differed between the North Island and South Island. Similar to this, Petit *et al*. (2011) reported differences in the distribution of *Cylindrocarpon* species associated with black foot disease of grapevines between the northeastern United States and southeastern Canada. They showed that *C.liriodendri* and *C. macrodidymum* were found in Canada, but not in the northeastern United States. Also they reported that *C. liriodendri* and *C. macrodidymum* had a broader geographic distribution than the other two new species, not previously identified in grapevines (*C. didymium* and *N. mammcodea*-like species), which had a narrow geographic range. They suggested that the diversity of *Cylindrocarpon* species was related to latent infection in the nursery plants which had been grown from a wide range of imported *Vitis* genotypes and differed between vineyard regions. In contrast, they suggested that the *C. didymium* and *N. mammoidea*-like species might be endemic to the regions. A study on botryosphaeriaceous grapevine trunk pathogens in New Zealand showed differences in species distribution between the North Island and South Island which also suggested that these species might be influenced by climatic conditions (Baskarathevan *et al.*, 2012a).

In New Zealand *C. destructans* is a major pathogen causing black foot and occurs and is isolated more frequently from symptomatic material than has been observed in other countries. The majority of *C. destructans* isolates were recovered from Marlborough, where the disease was wide spread. The reason(s) for this are not clear, although the higher proportion of the disease in Marlborough could be related to the region's earlier history of high density apple orchards. Over time many orchards have been replanted as vineyards and, thus, the soil inoculum that remained from their root diseases may have caused black foot disease to develop in subsequent grapevine crops (Mostert *et al*., 2006b). The cooler and wetter climatic conditions in New Zealand, especially in South Island may be influencing the survival and activity of *C. destructans* in soils and grapevines. Traquair and White (1992) reported occurrence of *C. destructans* as a mould on fruit trees in cold storage and that it had significant saprophytic ability at 10°C (Taylor, 1964). Therefore, it suggests that *C. destructans* might have adapted to cooler regions and this could explain why this species was more frequently isolated from South Island than the North Island.

It is possible that nursery soils and plants with infections may have been responsible for introducing black foot disease into the New Zealand vineyards. Olivera *et al*. (2004) reported that black foot disease is considered to be a major problem in young vineyards, which suggests the possibility that the plants may have been infected in the nursery fields. Gubler *et al*. (2004) reported that 5% of grapevines from some nurseries were infected with this pathogen but they believed that the presence of *Cylindrocarpon* species in vineyard soil was a primary source of inoculum. However, Halleen *et al.* (2003) showed that the pathogen was present in the nursery soils from which it infected the young plants; less than 1% of the grafted plants were infected with *Cylindrocarpon* spp. prior to planting in the nurseries, but at 7 months after planting out in nursery soils more than 50% of the sampled plants were infected.

In this study, the differences in optimum temperatures might explain some of the variation in regional distribution of species. The higher optimum growth temperature for *C. macrodidymum* (19.3ºC) was matched by greater distribution in the warmer regions of the North Island (Auckland and Gisborne). Petit and Gubler (2005) also reported a similar result and their study showed that the optimal growth for *C. macrodidymum* occurred at 25°C, which was much higher than that recorded in this study. In contrast, the lower optimum temperature of *C. destructans* (17.7ºC) was matched by greater distribution in the South Island. *Cylindrocarpon liriodendri* whose optimum was an intermediate 18.6ºC was distributed equally throughout both islands. These result suggest that for *Cylindrocarpon* species distribution there is a pattern, which may be due to their varied temperature tolerances; since *C. macrodidymum* had the highest optimum temperature for growth and *C. destructans* had the lowest. However, *C. liriodendri* is spread throughout New Zealand because of their wide temperature tolerance as demonstrated by their ability to grow across temperature between 5 and 30°C. In addition, the differences between studies may reflect isolate variation. These studies suggest that *C. macrodidymum* might have adapted to grow at more temperate climatic conditions rather than cooler regions. There was no apparent correlation of growth temperature with the distribution of *C. liriodendri* and no reports were found stating the geographic distribution of *C. liriodendri*.

In addition to the optimum temperature for growth, the growth rate of New Zealand *Cylindrocarpon* species differed to each other. At 5°C only two of the three main species, *C. destructans* and *C. liriodendri,* were able to grow. This result was in contrast to Petit and Gubler (2005), who showed that 18 *C. macrodidymum* isolates were able to grow at all temperatures tested including 5°C. Similarly Alaniz *et al*. (2007) reported that *C. liriodendri* and *C. macrodidymum* grew at 5°C. Compared to *C. macrodidymum, C. destructans* and *C. liriodendri* isolates grew faster at the different temperatures tested except at 30°C. Similarly Petit and Gubler (2005) reported that 17 *C. destructans* isolates grew significantly

faster than 18 *C. macrodidymum* isolates at the optimum temperature of 25°C. Another study done by Alaniz *et al*. (2007) showed that a total of 82 isolates of *C. liriodendri* and *C. macrodidymum* grew on potato dextrose agar (PDA) at 5°C-30°C, but not at 35°C.

The New Zealand isolates generally grew on PDA at faster rates than those reported for the same species in the literature. The New Zealand *C. macrodidymum* isolates grew 2.6 times faster (1.3 mm/day) at 10°C than the Spanish isolates (0.50 mm/day) (Alaniz *et al*., 2007). The New Zealand *C. liriodendri* isolates grew 2.5-4 times faster at 5 and 10°C than Spanish (Alaniz *et al*., 2007) and eight Iranian (Mohammadi *et al*., 2009) isolates which were also grown on potato dextrose agar (PDA). The maximum growth rate for *C. destructans* and *C. macrodidymum* at 25°C was 6 mm and 4.4 mm/day, respectively. Petit and Gubler (2005) showed the maximum growth rate per day for Californian *C. destructans* and *C. macrodidymum* on potato sucrose agar (PSA) were 3.4 mm/day and 3.0 mm/day respectively. The variations in the optimum temperature for maximum growth rate of a species is likely to reflect differences in laboratory conditions, isolate storage, agar constituents and the range of isolates tested.

The survey data also allowed the correlation between infection incidence and some rootstock cultivars to be tested. The incidence of *C. macrodidymum* and *C. pauciseptatum* isolates was higher in Gravesac than 101-14 rootstock. This data may reflect a true correlation of susceptibility or it may be the result of other factors such as number of samples, nursery of origin or vineyard location. Among the collected symptomatic vines, 101-14 was the most commonly collected rootstock. The reason for the high representation of 101-14 is unlikely to be its susceptibility to infection by *Cylindrocarpon* species but rather that it was the most widely planted rootstock variety during the development of the Marlborough area, which provided most of the samples. Another study in Uruguay showed that *C. macrodidymum* was commonly isolated from SO4 rootstocks, which was suggested to be because this was the most widely planted rootstock variety in Uruguay (Abreo *et al*., 2010). In contrast Rego *et al*. (2000) reported that randomly sampled vines from nurseries around Portugal showed that 101-14 (47%) had a higher disease incidence than SO4 (33.4%) from the nine different rootstock varieties. Harvey and Jaspers (2006) tested 14 rootstock varieties commonly grown in New Zealand and found that all varieties were susceptible to some degree. However, 101-14 was more susceptible to black foot than 5C, which has a similar parentage to SO4 (*Vitis berlandieri* x *Vitis rupestris)* (Jackson and Schuster, 1994). The different susceptibility of grapevine rootstocks to *C. liriodendri* and *C. macrodidymum* in Spain has been reported (Alaniz *et al.*, 2010); however, the varieties grown in Spain were generally different to those grown in New Zealand. The current study did not employ a random sampling technique to collect equal numbers of samples from different rootstock varieties. Thus, the association between rootstock varieties infection and species incidence found

probably reflected the sampling method rather than the real situation. To understand the true susceptibility of different rootstocks to different *Cylindrocarpon* species experiments using multiple rootstocks in equal numbers and representative isolates should be undertaken.

In conclusion, this study has showed that, as in other countries, *Cylindrocarpon* species are the predominant cause of black foot disease in grapevines. The species assemblage in New Zealand was similar to that in other countries except for the high proportion of *C. destructans* and the absence of *C. obtusisporum*. The five *Cylindrocarpon* species found were distributed throughout the main grape growing regions in New Zealand; however, the distribution of *C. destructans* and *C. macrodidymum* appeared to reflect the correlation between optimum temperatures for growth and the average ambient temperatures. Other factors such as soil type, moisture, stress conditions and rootstock varieties along with vineyard practices may also influence the distribution of *Cylindrocarpon* species in New Zealand and these require further study.

2.5 Implications of recent taxonomical classification

In this section the sequence data from isolates of *C. destructans (*n=*5)*, *C. macrodidymum (*n=*5)* and *Cylindrocarpon* sp. (n=4) were further analysed according to the recent reclassification (Cabral *et al*., 2012a; 2012b). Two aspects were investigated: (1) whether the species specific primers used in chapter 2 could distinguish any of the newly classified *Ilyonectria* species, and (2) whether the data from the sequenced New Zealand isolates were sufficient to assign a species name within the new classification. The following information details the methodology, results and discussion for that process.

2.5.1 Material and Methods

2.5.1.1 Analysis of binding sites for the species specific primers for the Ilyonectria radicicola and I. macrodidyma species complexes

The β-tubulin sequences of the five New Zealand *C. destructans* isolates and representative isolates of species within the *Ilyonectria radicicola* complex retrieved from GenBank were aligned using DNAMAN (Lynnon Biosoft version 4.0). The binding sites for primers Bt2a and Bt2b were identified and assessed for specificity to species within the *Ilyonectria radicicola* complex. This process was repeated for five of the New Zealand *C. macrodidymum* isolates, published (GenBank) representatives of the *I. macrodidyma* complex species and the primers CymaF1 and CymaR1.

2.5.1.2 Phylogenetic analysis of Cylindrocarpon species

Phylogenetic trees were produced using the translation elongation factor 1α and β-tubulin sequences of the New Zealand *Cylindrocarpon* isolates and also representative isolates of

the newly classified *Ilyonectria* species (Cabral *et al*., 2012a; 2012b). The sequences were retrieved from GenBank and aligned by CLUSTALW using Mega version 5.05 as described in Section 2.2.8.

2.5.2 Results

2.5.2.1 Results for the primer binding site

The binding sites for the *C. destructans* and *C. macrodidymum* species specific primers were conserved in all members of the *Ilyonectria radicicola* and *I. macrodidyma* complexes, respectively (Appendix A.7). The binding sites for the *C. macrodidymum* species specific primers were not conserved in *Cylindrocarpon* sp.1 and *Cylindrocarpon* sp. 2 (Appendix A.8).

2.5.2.2 Phylogenetic comparison of New Zealand Cylindrocarpon species with Ilyonectria radicicola complex and I. macrodidyma complex species

The β-tubulin and TEF-1α neighbour joining trees of New Zealand isolates of *C. macrodidymum*, *C. destructans, C. liriodendri* and four *Cylindrocarpon* sp. isolates analysed together with the published sequence data retrieved from GenBank (Cabral *et al*., 2012a; 2012b) were able to resolve some of the species within the complexes.

For *C. destructans* the β-tubulin and TEF-1α sequences identified four isolates (Mar5d, Mtb1d, Mar6b and Mar9a) as *Ilyonectria europaea* (Appendix A.6.4, Figure A.7) and one isolate (Mar7a) as "*Cylindrocarpon"* sp. This indicates that approximately 80% of the *C. destructans* isolates are likely to be *I. europaea*.

For *C. macrodidymum* the β-tubulin and TEF-1α sequences identified one isolate (Ack2i) as *I. macrodidyma* and four isolates (Gis3b, Ack2j, Mar16h and Hb6e) were placed within the *I. macrodidyma* complex but the individual species remained unresolved mainly because further alignment using the sequence of histone3 are required.

The β-tubulin and TEF-1α sequences of the novel *Cylindrocarpon* sp. isolates placed three (Mar1a, Mar1d and Hb6a) within *Cylindrocarpon* sp. 1 and one (Gis5a) within *Cylindrocarpon* sp. 2.

2.6 Discussion

The aim of this later study was to use the sequence data obtained in the current research to try and confirm the identities of the New Zealand isolates according to the new taxonomical classification by Cabral *et al*., 2012a; 2012b.

Using two taxonomically informative genes, the majority (80%) of *C. destructans* isolates probably belonged to the new species *I. europaea* with a few (20%) identified as "*Cylindrocarpon"* sp. *Ilyonectria europaea* has previously been isolated from the basal end of 2 year old *Vitis vinifera*, the stem of *Phragmites australis* (common reed), wood of *Aesculus hippocastanum* (horse chestnut) and from an internal stem lesion of *Actinidia chinesis* (kiwifruit) (Cabral *et al*., 2012a). The "*Cylindrocarpon"* sp. is not yet classified. The species specific primers could not discriminate between these species.

For the 53 isolates identified as *C. destructans* there was a differential distribution with the majority of isolates found in the South Island. It would be interesting to investigate the individual species that make up the *Ilyonectria radicicola* complex in each of the islands to determine if this correlates with regional differences. The optimum temperature for growth of *C. destructans* was shown to be the lowest of all three species. Although multiple isolates were used in this analysis, it is possible that the optimal growth temperatures for each of the two species are different. This was reinforced by the temperature experiment where "*Cylindrocarpon*" sp. isolate Mar7a (genetic group III) had lower optimum temperature, whereas the other two isolates (genetic group II) had similar optimum temperature, likely to be *I. europaea*. Similarly, for *C. macrodidymum*, the isolate Ack1a (genetic group I) had a higher optimum temperature, whereas the other two isolates (genetic group IV) had lower optimum temperature.

Several hypotheses were presented for the high incidence of *C. destructans* in New Zealand compared to other countries. However, resolving the members of the species complex recovered from grapevines and also those from other hosts may help to clarify whether isolates from orchards represent a significant inoculum source. The apparently lower frequency of "*Cylindrocarpon"* sp. may be due to a recent introduction into grapevines or weaker pathogenicity on this host. Several studies have demonstrated isolate variability in pathogenicity (Rego *et al*., 2001; Rahman and Punja, 2005; Bleach *et al*., 2007; Probst *et al*., 2012) and it possible that this is related to different species within the *I. radicicola* complex. Further studies should be conducted to investigate the pathogenicity of individual species within the complex. Also previous studies have shown that susceptibility of rootstock varieties to black foot disease (Rego *et al*., 2000; Harvey and Jaspers. 2006; Alaniz *et al*., 2010; Probst *et al*., 2012), so it is possible that this may be related to the different species within the *I. radicicola* complex. Clearly, resolving the identities of the 53 isolates identified as

belonging to the *I. radicicola* complex will shed more light on the significance of these species in New Zealand.

Similarly, for the 41 isolates identified as *C. macrodidymum*, there was a differential distribution with the majority of isolates found in North Island. It would be beneficial to study the individual species that make up the *Ilyonectria macrodidyma* complex in each of the islands to correlate their identity with the regional differences. The optimum temperature for growth of *C. macrodidymum* was shown to be the highest of all the three species. Although multiple isolates were used in this analysis, it is possible that the optimal temperatures for each of the species in the complex are different. Agrios (2005) reported that optimal temperatures for *in vitro* growth are not necessarily the same as those for disease development. For example, the optimum growth temperature for *Thielaviopsis basicola* ranges from 22-28°C and for the optimal temperature for development of black root rot disease ranges from 17-23°C (Agrios, 2005). These may account for the comparative differences in optimal growth temperature for New Zealand *C. macrodidymum* isolates compared to those of other countries. Studies have shown different levels of virulence between *C. macrodidymum* isolates (Alaniz *et al*., 2009b) and Petit *et al*. (2011) reported that *C. macrodidymum* showed broader geographic distribution than found in New Zealand. This may possibly be related to different species within the *I. macrodidyma* complex. It would be advantageous to examine the pathogenicity of individual species within the *I. macrodidyma* complex. Similar to *C. destructans*, Alaniz *et al*. (2010) showed *C. macrodidymum* to have different levels of susceptibility to different rootstock varieties. Therefore, it is possible that this may be due to the presence of different species within the *I. macrodidyma* complex. Clearly, resolving the identities of the 41 isolates identified as belonging to the *I. macrodidyma* complex will shed more light on the significance of these species in New Zealand.

Using two taxonomically informative genes to determine the identity of novel *Cylindrocarpon sp.* isolates, the majority (75%) were found to be *Cylindrocarpon* sp. 1, with a single isolate (25%) identified as *Cylindrocarpon* sp. 2. The isolates were found in combination with the main *Cylindrocarpon* species and the pathogenicity of these isolates has yet to be confirmed on grapevines. However, Cabral *et al*. (2012a) reported six types of *Cylindrocarpon* sp. isolates, (*Cylindrocarpon* sp. 1-6). Among these six Cabral *et al*. (2012b) identified all except *Cylindrocarpon* sp. 1 and 2, as *I. macrodidyma* complex and reported that *Cylindrocarpon* sp. 1 and 2 needed further study. In their study, the *Cylindrocarpon* sp. 1 isolate was isolated from root of *Hordeum vulgare*, Netherlands and *Cylindrocarpon* sp. 2 isolate was isolated from *Vitis vinifera* in Portugal and from *Pinus* (UK). So it would be beneficial to study the role of this species, its pathogenicity to grapevines and susceptibility to different rootstocks.

The design of a species specific primer, PCR-SSCP or PCR-RFLP (ARDRA) method as has been developed for *Botryosphaeriaceae* species (Alves *et al*., 2005; Ridgway *et al*., 2011) would be useful to discriminate each species within the complex. This would enable the rapid, cost effective and high throughput analysis of populations recovered in surveys such as that described here.

In conclusion, this study showed that *I. europaea* species were predominant among the *C. destructans* isolates and for *C. macrodidymum* species, the isolates predominantly belonged to the *I. macrodidyma* complex, which could not be resolved as an individual species. However, further studies need to done before assigning name to isolates within the species complex.

Chapter 3
Genetic variability of *Cylindrocarpon* species in New Zealand vineyards

3.1 Introduction

In fungal pathogens, genetic diversity is mainly influenced by the relative contributions of asexual and sexual reproduction to the disease cycle (Anderson and Kohn, 1998). Although asexual strategies can produce genetically different progeny the degree of variability is generally lower compared to progeny produced by sexual reproduction (Agrios, 2005). In plant pathogens, genetic diversity in a species population can be responsible for a range of adaptations which suit different environmental factors and, therefore, improve their ability to survive and infect their hosts (Gilbert, 2002). Reeleder (2003) reported that detailed understanding of the genetic diversity in a pathogen population can provide information that assists in the selection of suitable control strategies.

Limited information is available on the genetic diversity of *Cylindrocarpon* species, which are known to reproduce in a predominantly asexual fashion (Cedeno *et al*., 2004). A recent study of Spanish populations showed that variation in the virulence of isolates of *C. liriodendri* and *C. macrodidymum* correlated to different genotypes when isolates were analysed with inter simple sequence repeat (ISSR) markers (Alaniz *et al*., 2009b). In that study isolates from two main clades were significantly more virulent than isolates placed in other clades. Similarly, when New Zealand isolates of *N. luteum* and *N. parvum* were analysed by UP-PCR Baskarathevan (2011) showed that the genetic groups correlated with pathogenicity; however, the relationships were comparatively weak.

Molecular methods that produce information based on polymorphism in the DNA are widely accepted as the best tools for assessment of genetic diversity (Waugh, 1997). Many molecular techniques have been used to study the genetic diversity in fungi and these include AFLP (Vos *et al*., 1995), ISSR (Reddy *et al*., 2002), RAPD and UP-PCR (Bulat *et al.*, 1998; Lynch and Milligan, 1994). UP-PCR is a method that is similar to the traditional RAPD technique (Williams *et al.*, 1990), but it employs longer primers (15-20 nucleotides) which anneal at higher temperatures (Bulat *et al*., 1998). As the UP-PCR primers selected for fungi primarily target intergenic, more variable areas of the genome, this method is especially suitable for detecting intraspecific variation (Bulat *et al*., 1998). The main advantage of UP-PCR compared with RAPD is that the higher annealing temperatures (52-56°C) results in a high degree of reproducibility and the generation of more complex banding patterns, which improves the likelihood of identifying isolate specific and pathotype-specific bands (Bulat *et*

al., 1998; Lubeck *et al*., 1999). The UP-PCR technique has been successfully used in studying the genetic diversity of other fungi, including *Trichoderma* spp. (Cumagun *et al.*, 2000), *Phaeomoniella chlamydospora* (Pottinger *et al.*, 2002), *Fusarium* species (Yli-Mattila *et al.*, 1997; 2004), *Spilocaea oleagina* (Obanor *et al.*, 2010) and *Neofusicoccum spp.* (Baskarathevan *et al.*, 2012b).

In addition to molecular tools, macroscopic methods such as vegetative compatibility grouping (VCG) can be used to assess genetic diversity (Leslie, 1993). This method is based on the formation of compatible and incompatible interactions on agar plates, in which compatible reactions allow anastomosis of hyphae between colonies and incompatible reactions lead to hyphal death and barrage reactions between colonies. Although a convenient method for classification this method is sometimes difficult to assess due to obscure interactions between colonies. This method has been used successfully for several fungi, such as, *Diaporthe ambigua* (Smit *et al.*, 1997), *Rhizina undulata* (Lygis *et al.*, 2005), *Sclerotinia sclerotiorum* (Schafer and Kohn, 2006; Irani *et al.*, 2011), *Fusarium oxysporum* and *Fusarium pseudocircinatum* (Shiraishi *et al.*, 2011).

In this chapter, the genetic diversity of *Cylindrocarpon* species in New Zealand vineyards was investigated using UP-PCR and VCG.

3.2 Materials and Methods

3.2.1 Selection of *Cylindrocarpon* isolates

The genetic diversity of 151 New Zealand isolates representing the three most commonly isolated *Cylindrocarpon* species, namely, *C. macrodidymum* (n=41; Appendix B.1.1), *C. liriodendri* (n=57; Appendix B.1.3) and *C. destructans* (n=53; Appendix B.1.2), was studied. These isolates had been derived from single spores (C. Bleach, pers. comm.) prior to storage on SNA slopes at 4°C. International isolates were obtained from Australia and South Africa. The number of isolates of each species obtained from the different locations in New Zealand and overseas is summarised in Table 3.1.

3.2.2 Genomic DNA extraction

Each *Cylindrocarpon* isolate was grown on PDB and the genomic DNA was extracted using the PUREGENE® genomic DNA isolation kit as detailed in Section 2.2.4**.** All genomic DNA was diluted to 20 ng/µL for use in UP-PCR reactions.

3.2.3 Selection of UP-PCR primers for genetic diversity analysis

Initially all 11 UP-PCR primers were tested to select the five best primers most likely to detect polymorphism. This was done by amplifying DNA extracted from three test isolates from each of the three species (randomly selected from different locations) with each of the 11 UP-PCR primers. The sequence of each UP-PCR primer and their respective annealing temperature is shown in (Table 3.2).

Table 3.1 Isolates of three *Cylindrocarpon* species from different locations in New Zealand and other countries that were used for the genetic variation study.

Species	**Region/Country**	**No. of isolates**
Cylindrocarpon macrodidymum	Auckland	8
	Central Otago	3
	Gisborne	8
	Hawkes Bay	5
	Marlborough	15
	Waipara	1
	Nelson	1
	Australia	3
	South Africa	3
Cylindrocarpon liriodendri	Auckland	2
	Central Otago	7
	Gisborne	2
	Hawkes Bay	8
	Marlborough	31
	Waipara	6
	Martinborough	1
	Australia	1
	South Africa	3
Cylindrocarpon destructans	Auckland	2
	Central Otago	6
	Gisborne	1
	Hawkes Bay	3
	Marlborough	35
	Waipara	3
	Martinborough	1
	Nelson	2
	South Africa	1

Table 3.2 The name and DNA sequence of the UP-PCR primers and their respective annealing temperatures.

Primer name	Sequence 5' - 3'	Annealing temperature (°C)
AA2M2	GAGCGACCCAGAGCGG	50
AS4	TGTGGGCGCTCGACAC	50
AS15	GGCTAAGCGGTCGTTAC	52
AS15inv	CATTGCTGGCGAATCGG	52
FOK1	GGATGACCCACCTCCTAC	52
L15	GAGGGTGGCGGTTCT	52
L15/ASI9	GAGGGTGGCGGCTAG	52
L21	GGATCCGAGGGTGGCGGTTCT	55
L45	GTAAAACGACGGCCAGT	51
0.3-1	CGAGAACGACGGTTCT	50
3-2	TAAGGGCGGTGCCAGT	50

3.2.4 Universally Primed PCR (UP-PCR)

Each 25 µl UP-PCR contained 1 × PCR buffer (FastStart™, Roche), 200 µM of each dNTP, 10 pmol of each primer, 2.0 mM $MgCl_2$,1.25 U FastStart™ *Taq* Polymerase and 20 ng of DNA. The thermal cycle was conducted in a Veriti 96 well Thermal cycler (Applied Biosystems, California, USA). It consisted of an initial denaturation of 3 min at 94°C, followed by 5 cycles of denaturation at 94°C for 30 s, annealing for 2 min at the optimal temperature for each primer (Table 3.2) and extension at 72°C for 1 min, followed by 25 cycles in which the annealing step was shortened to 1.5 min with a final extension of 10 min at 72°C. Negative controls in which the DNA template was substituted with sterile water were included in each set of reactions to ensure they were free of contamination.

The same conditions were repeated for the international isolates of *C. macrodidymum*, *C. liriodendri* and *C. destructans* to amplify the genomic DNA using the primers selected for each species.

3.2.5 Electrophoresis and scoring of DNA fingerprints

UP-PCR amplification products (5 µL) were combined with 2 µL of loading dye and separated by 1% agarose gel electrophoresis at 5 V/cm for 3 h 20 min in 1× TAE (Appendix B.2). The 1 kb plus™ DNA ladder (Invitrogen, Carlsbad, USA) was included as a molecular weight marker in each gel. Agarose gels were stained with ethidium bromide and visualized on a UV transilluminator (Versadoc™) as described in Section 2.2.4.

UP-PCR profiles were analysed from prints of the agarose gels. Only clear and reproducible bands were included in the analysis. Scoring was done as follows: If a band was present it was indicated by a "1" and absent by means of "0". Bands that were not distinct (not clearly separate and of average intensity) were not counted. In this process, given the high conservation in genomes within a species, it was assumed that bands of the same molecular weight were homologous and that isolates with similar banding patterns were genetically similar. The binomial table created using Microsoft Excel® was converted to a "nex" file using Mesquite 2.74 program (Mesquite Software Inc. Oregon state University and University of British Columbia, USA) and analysed with PAUP version 4.0b 10 (Phylogenetic Analysis Using Parsimony, United States) to construct phylograms from the collective binomial data of all five primers. A phylogram was generated for the three *Cylindrocarpon* species isolates using a neighbour joining (nj) tree.

3.2.6 Genetic diversity analysis

Intra-vineyard, inter-vineyard and inter regional genetic diversity amongst isolates of the same species was analysed individually for each of the three *Cylindrocarpon* species. For each species, the binomial matrix data file generated from the banding profile was analysed using the distance based method of neighbour joining as a tree building algorithm using the PAUP software version 4.0b10 (Swofford, 1999). The neighbour joining tree was generated based on the total character differences and the tree was rooted to the midpoint. Genetic groups were identified in neighbour joining trees of each species by drawing arbitrary lines at a set distance from the roots to produce genetic groups with similar percentages of intra-group similarity.

All genetic diversity parameters were calculated using the software POPGENE version 1.32 (Yeh *et al.*, 1999). The average gene diversity (H; Nei, 1973) was calculated for each population using binomial matrix data, where $H = \Sigma\ (1\text{-}\Sigma_{\chi k}{}^2)/h$, χ was the allele frequency of the k^{th} UP-PCR cluster, and h was the number of UP-PCR loci. In addition, genotypic diversity was quantified by normalized Shannon's diversity index (I; Sheldon, 1969) $I = - \Sigma_{pi} \ln P_i / \ln N$, in which P_i is the frequency of the i^{th} haplotype and N is the number of isolates in each population. The value of 'I' ranges from 0 (individuals in the sample having a same

genotype) to 1 (each individual in the sample having a different genotype). The presence or absence of a specific band was interpreted as a positive or null allele, respectively.

3.2.7 Genetic diversity of North, upper South and lower South Island populations of *Cylindrocarpon* species

The comparative genetic diversity of species populations from the North Island (Auckland, Gisborne, Hawkes bay and Martinborough), upper South Island (Nelson and Marlborough), and lower South Island (Waipara and Central Otago) were calculated using the software POPGENE version 1.32 as described in the previous section.

3.2.8 Optimising the agar medium for vegetative compatibility groupings

To determine the optimum medium for VCG, six isolates belonging to each of the three main *Cylindrocarpon* species selected from different branches of the neighbour joining tree were used for this experiment. For pairing of isolates of the same species, 3 mm diameter mycelial plugs were taken from the edges of 7 day old colonies grown on PDA. The respective isolate plugs were placed 2 cm apart onto PDA, ½PDA, czapek dox agar (CDA) or water agar. Each isolate was paired with itself and with each of the five other isolates within a species, and each combination of isolates was replicated three times for each agar. Observations were made after 7 days, 10 days and one month incubation at 20°C in 12:12 h light:dark.

The paired isolates were visually examined on both the upper and reverse side and scored for vegetative compatibility. The criteria used were as follows: when mycelia of two isolates grew together uniformly and merged, without distinction between colonies, they were classed as compatible (C). When mycelia of two isolates grew to a meeting point on the agar but were separated by a barrage reaction formed along the line of contact between paired isolates they were classed as incompatible (IC). When mycelia of two isolates grew to a meeting point on the agar, but were joined with a mild barrage line along the line of contact, they were classed as partially incompatible (PI).

3.2.9 Vegetative compatibility grouping using potato dextrose agar

Nine isolates of *C. liriodendri*, eight isolates of *C. macrodidymum* and seven isolates of *C. destructans,* including those isolates tested previously, were selected randomly from the different clades of the neighbour joining tree. At the time of this experiment, no international isolates available, so only New Zealand isolates were used. Pairing on PDA was done as described in Section 3.2.8.

3.2.10 Microscopic examination of vegetative compatibility

Microscopic evaluation was also carried out for one pair of isolates from compatible, incompatible and partially incompatible reaction of each species. The isolates were paired

against themselves and against each other on microscopic slides. The method, described previously (Aimi *et al.*, 2002), used two 5 mm cubes of 1/8 strength PDA medium which contained 1% agar, placed 1 cm apart on a sterile glass microscopic slide. Each agar cube was inoculated with a 2 mm cube of mycelium colonized agar obtained from a 3-4 day old PDA culture, with each isolate paired against. Each slide was placed on a sterile plastic straw in a Petri dish lined with moist filter paper, covered and sealed with cling film (Figure 3.1). Each combination of isolates was replicated three times. These slides were incubated at 20ºC in 12:12 h light:dark conditions until the hyphae from each isolate began to merge on the centre of the glass slide. The agar cubes were then gently pushed off the slides and a drop of sterile water was added onto the hyphae in the centre of the slide and covered with a cover slip for microscopic examination. The hyphal merging zone between the isolates was microscopically observed at different magnifications (×100, 400 and 600) and digitally imaged using a Lena Microscope with a built in Olympus DP12 camera.

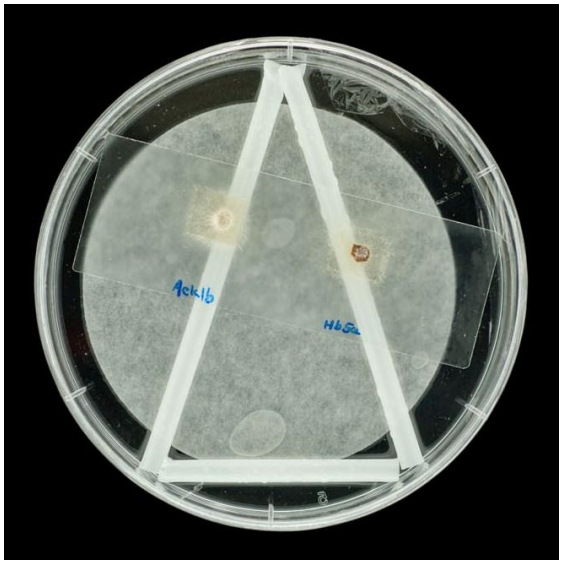


Figure 3.1 Experimental set-up for the microscopic examination of vegetative compatibility reactions of isolates of *Cylindrocarpon macrodidymum*, *C. liriodendri* and *C. destructans*

3.3 Results

3.3.1 Selected UP-PCR primers

For the three *Cylindrocarpon* species, bands were amplified by all 11 UP-PCR primers. The total number of bands amplified varied for each UP-PCR primer and species combination. The primers selected for the genetic variation analysis of each species were those that gave a high number of clear, well-distributed bands with relatively high rate of polymorphism and had different effects amongst the three species. Of the 11 primers tested, primer 3-2 produced the most and 0.3-1 the least bands. For each of the three species, the five different primers selected are shown in Table 3.3.

Table 3.3 The number of bands (NB), polymorphic bands (PB) and percentage polymorphic bands (P%) for the different UP-PCR primers tested for *C. destructans*, *C. liriodendri* and *C. macrodidymum*. Primers selected for each species are shown in red.

Primer name	***Cylindrocarpon destructans***			***Cylindrocarpon liriodendri***			***Cylindrocarpon macrodidymum***		
	NB	PB	P%	NB	PB	P%	NB	PB	P%
L15/As19	8	3	38	6	4	66	6	4	66
L15	**12**	**7**	**58**	9	4	44	**8**	**5**	**62**
Fok 1	6	3	50	**15**	**5**	**33**	5	1	20
3-2	**15**	**6**	**40**	**13**	**4**	**30**	**17**	**7**	**41**
AS15	**9**	**5**	**55**	**14**	**3**	**21**	**16**	**4**	**25**
AS15inv	**14**	**4**	**28**	4	1	25	14	4	29
AA2M2	3	1	33	**13**	**4**	**30**	**9**	**5**	**55**
0.3-1	2	1	50	6	3	50	6	2	33
L45	**14**	**6**	**43**	7	2	28	6	4	66
L21	5	3	60	6	3	50	**12**	**4**	**33**
AS4	6	3	50	**11**	**3**	**27**	10	3	30

NB = Total no. of bands, PB = Number of polymorphic bands, P% = Percentage of polymorphism. **Selected primers**.

For *C. destructans*, of the five selected primers, the highest percentage of polymorphic bands (58%) was generated with primer L15 and the least (28%) with primer AS15inv (Table 3.3). From the five selected primers for *C. liriodendri*, the highest percentage of polymorphic bands (33%) was generated with primer Fok1 and the least (21%) with primer AS15. From the five selected primers for *C. macrodidymum*, the highest percentage of polymorphic bands (62%) was generated with the primer L15 and the least (25%) with the primer AS15.

3.3.2 Scoring of DNA fingerprints

DNA fingerprints were generated for all 151 isolates. Figures 3.2 A-C show examples of the banding profiles generated for *C. liriodendri* (Figure 3.2A), *C. macrodidymum* (Figure 3.2B) and *C. destructans* (Figure3.2C) isolates by UP-PCR primers.

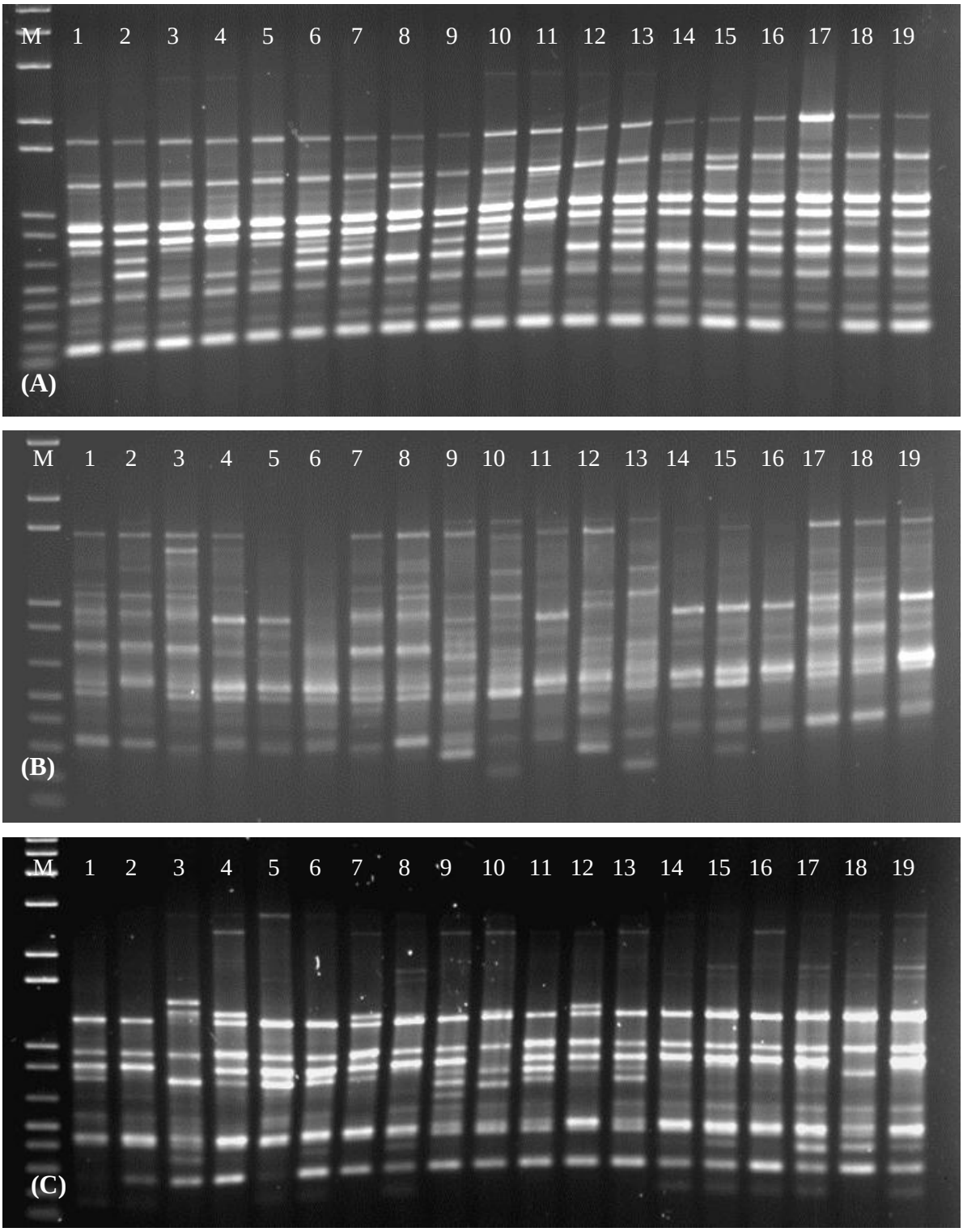


Figure 3.2 Representative images of UP-PCR finger printing of (A) *C. liriodendri* amplified with primer AA2M2, (B) *C. macrodidymum* amplified with 3-2 and (C) *C. destructans* amplified L15. Lanes 1 to 19 contain representative isolates. M - Marker (1Kb plus ladder, Invitrogen).

For *C. liriodendri* isolates the five primers generated a total of 66 scorable bands of which 59% were polymorphic. The bands ranged in size from 150 to approximately 3000 bp. For *C. macrodidymum* a total of 62 scorable bands were generated from five primers of which 74% were polymorphic. The bands ranged in size from 150 to 2500 bp. For *C. destuctans,* the five primers produced a total of 64 scorable bands of which 92% were polymorphic. The bands ranged in size from 200 to 1650 bp.

3.3.3 Genetic diversity of *Cylindrocarpon macrodidymum*

The neighbour joining (nj) tree showed that the *C. macrodidymum* isolates found in New Zealand vineyards were genetically diverse (Figure 3.3). An arbitrary line drawn at 3 changes from the root of the neighbour joining tree identified six main groups (Figure 3.3). The *C. macrodidymum* isolates from different geographic locations were dispersed in different groups. For example, the Auckland isolates (Ack) were located in genetic groups I, II, III, IV and V. Inter-vineyard and intra-vineyard genetic variability was observed, for example, isolates from single Auckland (Ack2k, Ack2j, Ack2h, Ack2i and Ack2c), Central Otago (Co6c and Co6a) and Gisborne (Gis3d and Gis3b) vineyards were located in different groups. Also isolates from a single grapevine, Ack2h/Ack2i and Gis3c/Gis3d, were located in different groups. Three pairs of clonal isolates, Mar5b/Mar9b, Mar8g/Mar16f and Mar11f/Mar16h, were observed. The clonal isolates were from the same region but from five different vineyards and were part of the large (n=95) collection obtained from Marlborough.

Only a single Australian (MW) and South African (SA) isolate were placed with the New Zealand isolates in group I and IV. The remaining two Australian and two South African isolates were in a separate group (VI).

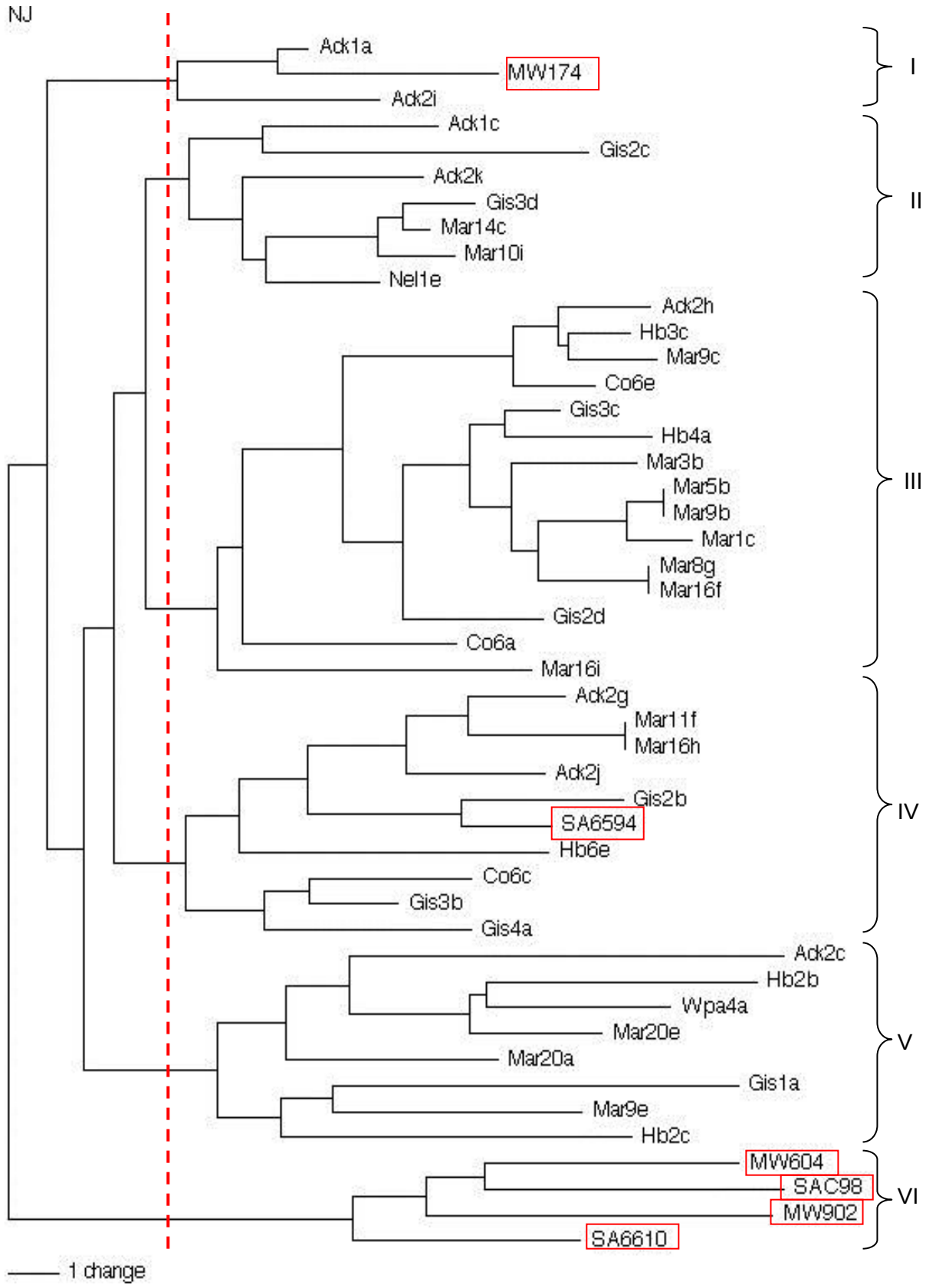


Figure 3.3 Neighbour joining tree generated in PAUP 4.0b10 using UP-PCR fingerprinting data shows the genetic diversity of the *Cylindrocarpon macrodidymum* population in New Zealand vineyards. International isolates are shown in boxes. Clades are designated on the right hand side.

The overall mean genetic diversity of the New Zealand *C. macrodidymum* population calculated by Nei's genetic diversity (H; Nei, 1973) was 0.2580. Among the *C. macrodidymum* isolate groups obtained from different geographic locations, the most genetically diverse group was found in Hawkes Bay (0.2555) and least in Central Otago (0.1577), while that of South Africa and Australia was 0.1720 and 0.1864, respectively (Table 3.4). The genotypic diversity of each group, calculated by Shannon's diversity index, ranged from 0.2259 in Central Otago to 0.3682 in Hawkes Bay. Two isolates of *C. macrodidymum* were not included in the regional analysis as there was only one isolate from each of the two locations. These isolates were included in the total New Zealand isolate collection.

Table 3.4 The mean genetic diversity (*H*) and Shannon diversity index (*I*) of *Cylindrocarpon macrodidymum* populations.

Population	Sample size	H^a	I^b
Auckland	8	0.2263	0.3410
Central Otago	3	0.1577	0.2259
Gisborne	8	0.2263	0.3390
Hawkes Bay	5	0.2555	0.3682
Marlborough	15	0.2271	0.3400
Australia	3	0.1864	0.2669
South Africa	3	0.1720	0.2464
Total NZ population	41	0.2580	0.3845
Total population	47	0.2829	0.4286

[a] Nei's (1973) genetic diversity.
[b] Shannon diversity index (Sheldon, 1967).

Nei's (Nei, 1973) measure of genetic identity and genetic distance for the different isolate groups is shown in Table 3.5. The dendrogram generated from Nei's genetic distance showed that the Central Otago and Gisborne isolate groups as well as the Hawkes Bay and Marlborough isolate groups were genetically the most similar to each other (Figure 3.4).The Australian and the South African isolates were located on a separate branch. This was a similar result to that shown by the neighbour joining analysis.

Table 3.5 Nei's measures of genetic identity and genetic distance of different *Cylindrocarpon macrodidymum* populations.

Population	Ack	CO	Gis	HB	Mar	Aus	SA
Auckland (Ack)	[a]****	0.9232	0.9505	0.9218	0.9318	0.8208	0.8237
Central Otago (CO)	0.0799	****	0.9515	0.9396	0.9435	0.7685	0.7962
Gisborne (Gis)	0.0508	0.0497	****	0.9388	0.9534	0.7852	0.8220
Hawkes Bay (HB)	0.0814	0.0623	0.0632	****	0.9537	0.7695	0.7902
Marlborough (Mar)	0.0706	0.0581	0.0478	0.0474	****	0.7444	0.7801
Australia (Aus)	0.1975	0.2633	0.2418	0.2620	0.2952	****	0.8778
South Africa (SA)	0.1939	0.2279	0.1960	0.2354	0.2484	0.1304	****

[a]Nei's genetic identity based on 62 loci is above the diagonal and the genetic distance coefficients are below the diagonal.

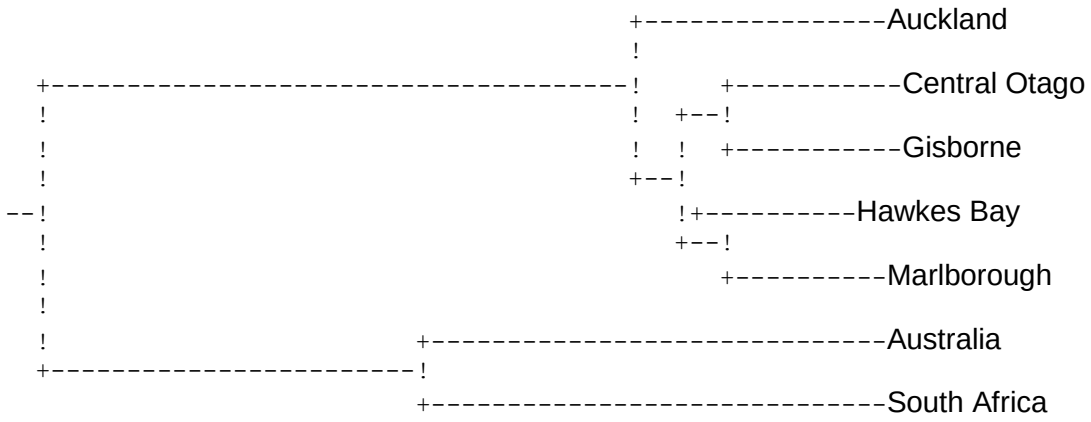


Figure 3.4 UPGMA dendrogram showing the genetic relatedness of the *Cylindrocarpon macrodidymum* populations studied based on Nei's (1973) genetic distance.

3.3.4 Genetic diversity of *Cylindrocarpon destructans*

An arbitrary line drawn at 1.5 changes from the root of the tree identified four main groups in the *C. destructans* neighbour joining tree (Figure 3.5). Genetically diverse *C. destructans* isolates were present at both the inter- and intra-vineyard level (Figure 3.5), which was similar to the results observed in the *C. macrodidymum* neighbour joining tree. The number of isolates placed in each genetic group was highly variable ranging from one isolate in genetic group lV to 32 in genetic group ll. Isolates obtained from different locations were dispersed in different genetic groups. For example, isolates from Central Otago and Marlborough locations were placed in genetic groups l, ll and lll. The majority of the isolates from North Island locations were placed within group l except one isolate from Hawkes Bay which was placed in genetic group lll (Hb6g), one isolate (Gis1b) from Gisborne and one isolate from Martinborough (Mtb1d) which were placed in genetic group ll. The single South African isolate was situated alone in the genetic group IV. Isolates from single vineyards in Marlborough (Mar7c and Mar7a) and Nelson (Nel1b and Nel1d) were located on different branches. Three groups of isolates, Mar6d/Nel1b, Wpa1d/Mar22a/Mar2a and Mtb1d/Mar1b, were clonal and these originated from different vineyards and geographic locations.

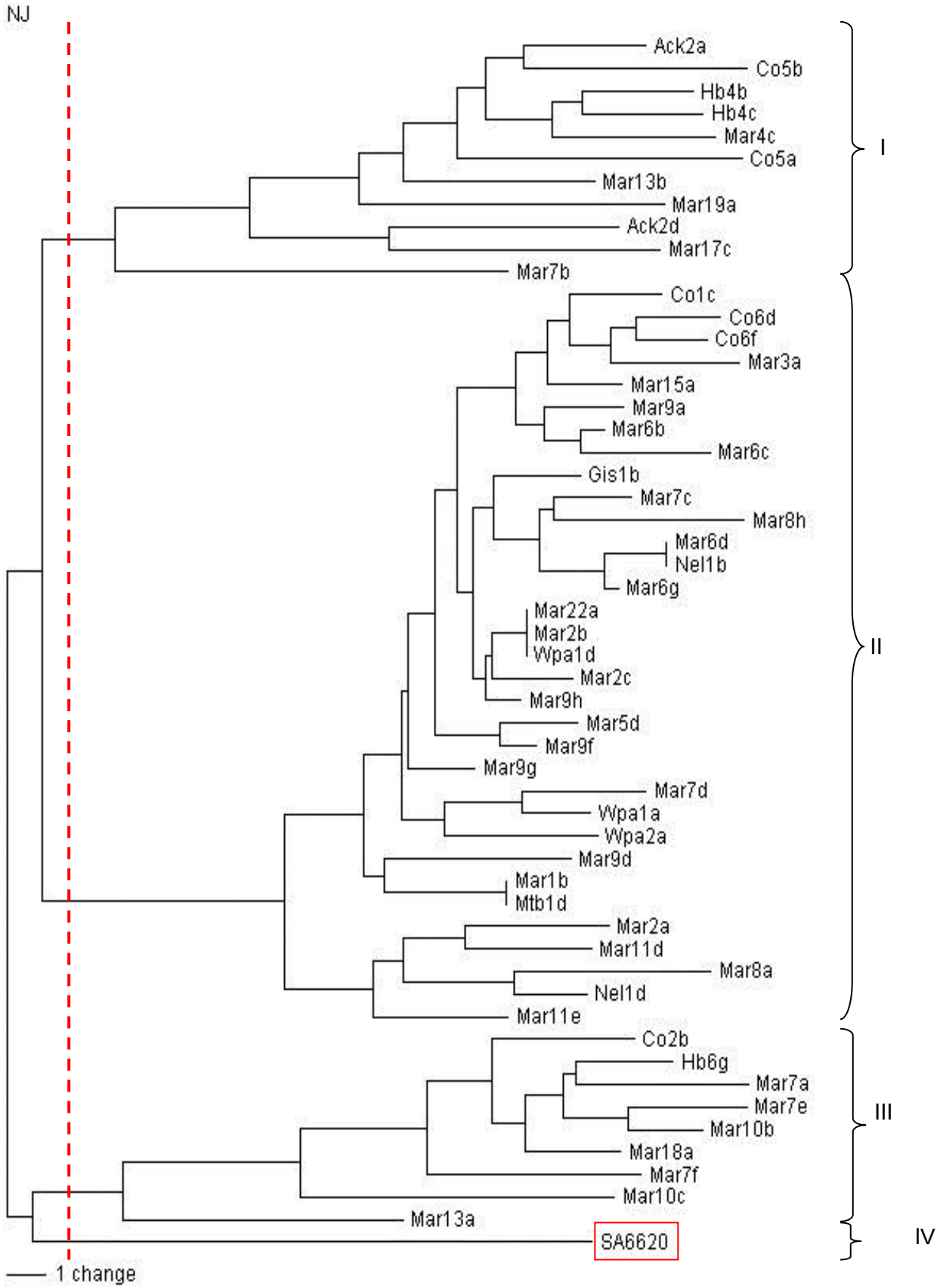


Figure 3.5 Neigbour joining tree generated in PAUP 4.0b10 using UP-PCR finger printing data shows the genetic diversity of the *Cylindrocarpon destructans* population in New Zealand vineyards. International isolates are shown in boxes. Clades are designated on the right hand side.

The overall mean genetic diversity of the *C. destructans* population calculated by Nei's genetic diversity (H; Nei, 1973) was 0.3346. Among the *C. destructans* populations obtained from the different geographic locations the most genetically diverse population was found in Central Otago (0.3325) and least in Waipara (0.0833). The genotypic diversity calculated by Shannon's diversity index ranged from 0.1193 in Waipara to 0.4829 in Marlborough. In this analysis four geographic locations were not included in the population because there were less than two isolates from each of the four places. These isolates were included in the total New Zealand population and the single South African isolate was included in the total population.

Table 3.6 The mean genetic diversity (*H*) and Shannon index (*I*) of *Cylindrocarpon destructans* populations.

Population	Sample size	H^a	I^b
Central Otago	6	0.3325	0.4816
Hawkes Bay	3	0.2292	0.3282
Marlborough	35	0.3192	0.4829
Waipara	3	0.0833	0.1193
Total NZ population	53	0.3346	0.5013
Total population	54	0.3389	0.5078

[a] Nei's (1973) genetic diversity.
[b] Shannon diversity index (Sheldon, 1967).

Table 3.7 Nei's measures of genetic identity and genetic distance of different *Cylindrocarpon destructans* populations.

Population	CO	HB	Mar	Wpa
Central Otago (CO)	[a]****	0.8289	0.9409	0.8489
Hawkes Bay (HB)	0.1876	****	0.7744	0.5886
Marlborough (Mar)	0.0609	0.2556	****	0.9311
Waipara (Wpa)	0.1638	0.5300	0.0713	****

[a]Nei's genetic identity based on 64 loci is above the diagonal and the genetic distance coefficients are below the diagonal.

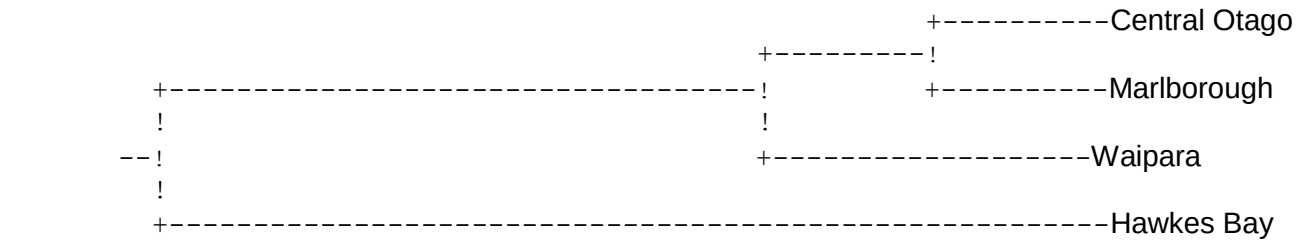


Figure 3.6 UPGMA dendrogram showing the genetic relatedness of the *Cylindrocarpon destructans* populations based on Nei's (1973) genetic distance.

3.3.5 Genetic diversity of *Cylindrocarpon liriodendri*

The neighbour joining tree analysis of *C. liriodendri* showed that the population found in New Zealand was genetically diverse (Figure 3.7).

For *Cylindrocarpon liriodendri*, an arbitrary line drawn at 3 changes from the root of the neighbour joining tree identified four main groups (Figure 3.7). Each genetic group contained a range of isolates from different geographic locations and vineyards. There was no clustering by region indicating that genetically dissimilar individuals were found at all sites. For example, the isolates from Hawkes Bay and Central Otago were located in genetic groups I and III and the isolates from Marlborough region were located in genetic group I, II and III. Genetic diversity was observed inter-vineyard and intra-vineyard. For example, isolates from single Auckland (Ack1b and Ack1d), Central Otago (Co4a and Co1a), Marlborough (Mar6e and Mar6f; Mar16b and Mar16d; Mar8b and Mar8f) and Hawkes Bay (Hb2d and Hb2a) vineyards, were found on different branches. Also isolates, such as Mar10a and Mar10f, isolated from a single grapevine were found in different branches. Clonal isolates were found in groups I and III. The Australian and South African isolates were placed in genetic group IV which did not contain any New Zealand isolates.

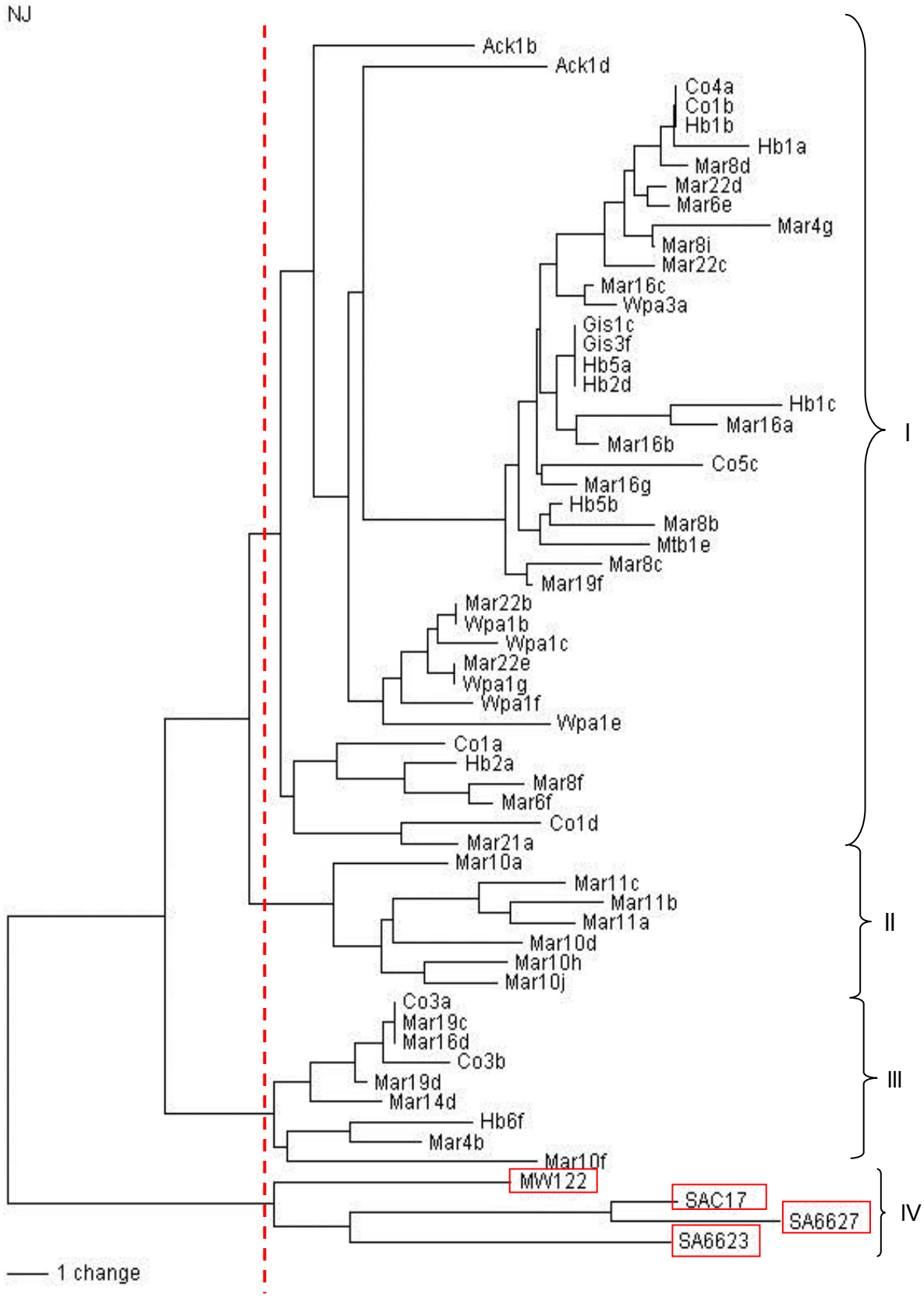


Figure 3.7 Neighbour joining tree generated in PAUP 4.0b10 using UP-PCR finger printing data shows the genetic diversity of the *Cylindrocarpon liriodendri* population in New Zealand vineyards. International isolates are shown in boxes. Clades are designated on the right hand side.

Nei's genetic diversity and the Shannon diversity index calculated for *C. liriodendri* isolate groups ranged from 0.0816 to 0.1928 and from 0.1250 to 0.2884, respectively (Table 3.8). The highest genetic diversity calculated was 0.1928 in Marlborough and the lowest was 0.0816 in Waipara. For the South African isolates, the genetic diversity was calculated as 0.1414. Nei's measure of genetic identity and genetic distance analysis showed that the *C. liriodendri* isolate groups obtained from the Marlborough and Central Otago locations were genetically more similar to each other than to other isolate groups (Table 3.9 and Figure 3.8). In this analysis four geographic locations were not included in the population because there were less than two isolates from each. These isolates were included in the total New Zealand population and the single Australian isolate was included in the total population (Table 3.8).

Table 3.8 Mean genetic diversity (*H*) and Shannon index (*I*) of *Cylindrocarpon liriodendri* populations.

Population	Sample size	H^a	I^b
Central Otago	7	0.1843	0.2731
Hawkes Bay	8	0.1392	0.2119
Marlborough	31	0.1928	0.2884
Waipara	6	0.0816	0.1250
South African	3	0.1414	0.2025
Total NZ population	57	0.1816	0.2770
Total population	61	0.2163	0.3435

[a]Nei's (1973) gene diversity.
[b] Shannon diversity index (Sheldon, 1967).

Table 3.9 Nei's measures of genetic identity and genetic distance of different *Cylindrocarpon liriodendri* populations.

Population	CO	HB	Mar	Wpa	SA
Central Otago (CO)	[a]****	0.9706	0.9901	0.9591	0.6164
Hawkes Bay (HB)	0.0298	****	0.9730	0.9415	0.5919
Marlborough (Mar)	0.0099	0.0273	****	0.9649	0.6223
Waipara (Wpa)	0.0418	0.0602	0.0357	****	0.5773
South Africa (SA)	0.4838	0.5244	0.4743	0.5494	****

[a]Nei's genetic identity based on 66 loci is above the diagonal and the genetic distance coefficients are below the diagonal.

Figure 3.8 UPGMA dendrogram showing the genetic relatedness of the *C. liriodendri* populations studied based on Nei's (1973) genetic distance.

3.3.6 Genetic diversity of North, upper South and lower South Island populations

Isolates obtained from North Island, upper South Island, lower South Island were grouped and analysed. For *C. macrodidymum*, the genetic diversity and Shannon diversity index was most similar between the North Island and upper South Island. The lowest genetic diversity was in the lower South Island which also had the smallest sample size (Table 3.10). The dendrogram based on Nei's measure of genetic distance (Table 3.11) showed that the North Island and the upper South Island populations were more similar to each other than to the lower South Island population as demonstrated by branch length (Figure 3.9).

Table 3.10 The mean genetic diversity (*H*) and Shannon diversity index (*I*) of *Cylindrocarpon macrodidymum* populations.

Population	Sample size	H^a	I^b
North Island	21	0.2636	0.3944
Upper South Island	16	0.2256	0.3376
Lower South Island	4	0.1935	0.2820

[a] Nei's (1973) gene diversity.
[b] Shannon diversity index (Sheldon, 1967).

Table 3.11 Nei's measures of genetic identity and genetic distance of different *Cylindrocarpon macrodidymum* populations.

Population	NI	USI	LSI
North Island	[a]****	0.9673	0.9578
Upper South Island	0.0332	****	0.9541
Lower South Island	0.0431	0.0470	****

[a]Nei's genetic identity based on 62 loci is above the diagonal and the genetic distance coefficients are below the diagonal.

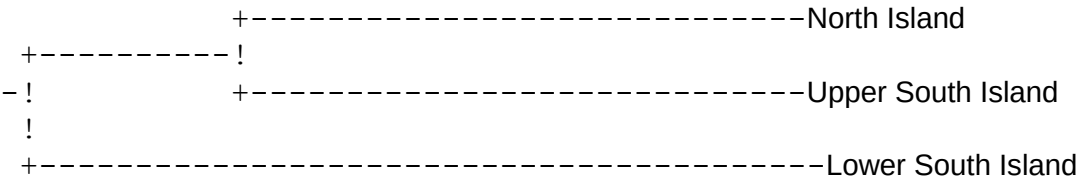


Figure 3.9 UPGMA dendrogram showing the genetic relatedness of the *Cylindrocarpon macrodidymum* populations studied based on Nei's (1973) genetic distance.

For *C. destructans*, the genetic diversity and Shannon diversity index were similar for all three populations despite the differences in sample sizes (Table 3.12). The dendrogram produced based on Nei's measure of genetic distance (Table 3.13) showed that the two South Island populations were more similar to each other than to the North Island population as demonstrated by branch length (Figure 3.10).

Table 3.12 Mean genetic diversity (*H*) and Shannon diversity index (*I*) of *Cylindrocarpon destructans* populations.

Population	Sample size	H^a	I^b
North Island	7	0.3112	0.4581
Upper South Island	37	0.3157	0.4791
Lower South Island	9	0.3063	0.4546

[a] Nei's (1973) gene diversity.
[b] Shannon diversity index (Sheldon, 1967).

Table 3.13 Nei's measures of genetic identity and genetic distance of different *Cylindrocarpon destructans* populations.

Population	NI	USI	LSI
North Island	[a]****	0.8980	0.8888
Upper South Island	0.1076	****	0.9712
Lower South Island	0.1179	0.0293	****

[a]Nei's genetic identity based on 64 loci is above the diagonal and the genetic distance coefficients are below the diagonal.

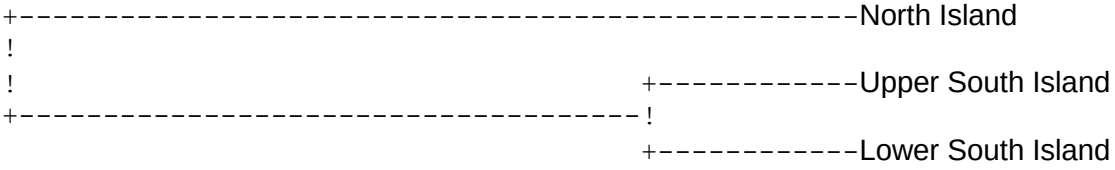


Figure 3.10 UPGMA dendrogram showing the genetic relatedness of the *Cylindrocarpon destructans* populations studied based on Nei's (1973) genetic distance.

For New Zealand isolates of *C. liriodendri*, the genetic diversity and Shannon index calculated ranged from 0.1348 to 0.1928 and from 0.2118 to 0.2884, respectively. The highest genetic diversity was in the upper South Island and the lowest in the North Island (Table 3.14). The dendrogram based on Nei's measure of genetic distance (Table 3.15) showed that the two South Island populations were more similar to each other than to the North Island population as demonstrated by branch length (Figure 3.11).

Table 3.14 Mean genetic diversity (*H*) and Shannon diversity index (*I*) of *Cylindrocarpon liriodendri* populations.

Population	Sample size	H^a	I^b
North Island	13	0.1348	0.2118
Upper South Island	31	0.1928	0.2884
Lower South Island	13	0.1553	0.2396

[a] Nei's (1973) gene diversity.
[b] Shannon diversity index (Sheldon, 1967).

Table 3.15 Nei's measures of genetic identity and genetic distance of different *Cylindrocarpon liriodendri* populations.

Population	NI	USI	LSI
North Island	[a]****	0.9753	0.9677
Upper South Island	0.0250	****	0.9883
Lower South Island	0.0329	0.0118	****

[a]Nei's genetic identity based on 66 loci is above the diagonal and the genetic distance coefficients are below the diagonal.

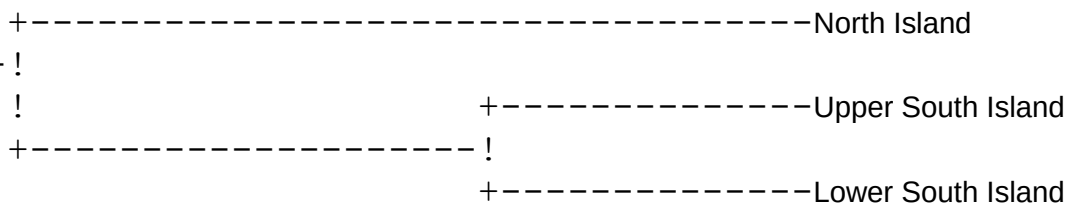


Figure 3.11 UPGMA dendrogram showing the genetic relatedness of the *Cylindrocarpon liriodendri* populations studied based on Nei's (1973) genetic distance.

3.3.7 Optimising the agar medium for vegetative compatibility groupings

Amongst the four media used for the vegetative compatibility groupings, PDA gave the clearest demarcation between two incompatible isolates (Figures 3.12A; 3:13A and 3.14A). The colonies grew rapidly and were lightly pigmented providing good contrast with the agar background. In ½PDA, the colonies were generally darker hued which made it more difficult to discern the reaction zone between the two isolates (Figures 3.12B, 3.13B and 3.14B). On CDA, the incompatible reaction was well defined for *C. macrodidymum* but less distinct for the other two species (Figures 3.12C; 3.13C and 3.14 C). In water agar, the incompatible reaction was not distinct for any of the three species (Figures 3.12D, Figure 3.13D and Figure 3.14D). Thus, PDA was chosen for further work on a greater number of isolates.

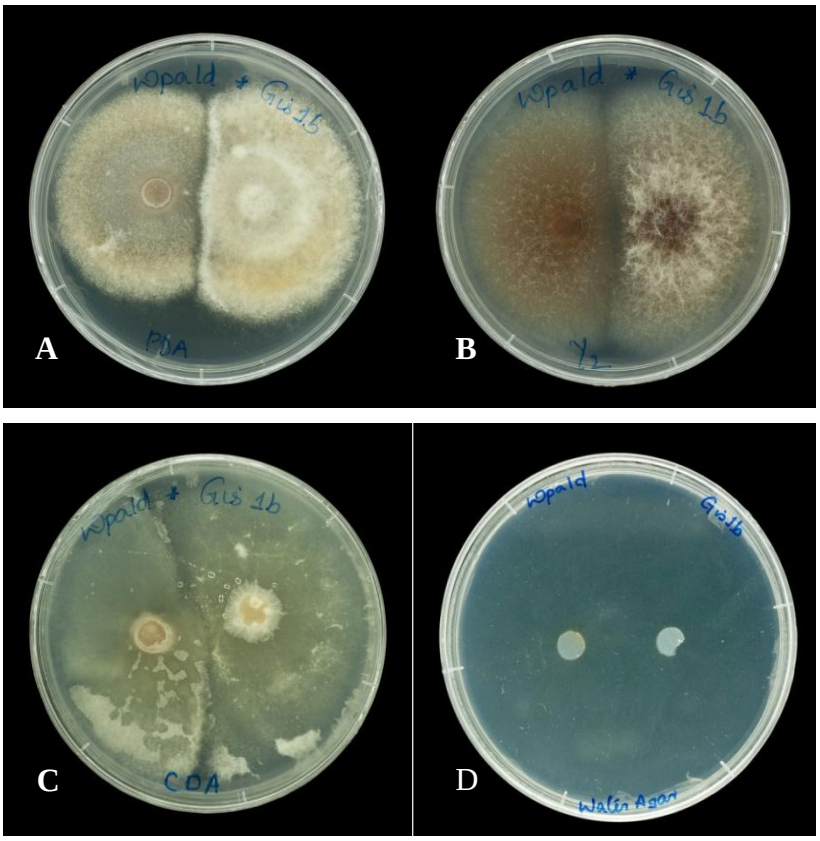


Figure 3.12 Vegetative incompatibililty reaction of *Cylindrocarpon destructans* on (A) PDA, (B) ½ PDA, (C) CDA and (D) Water agar.

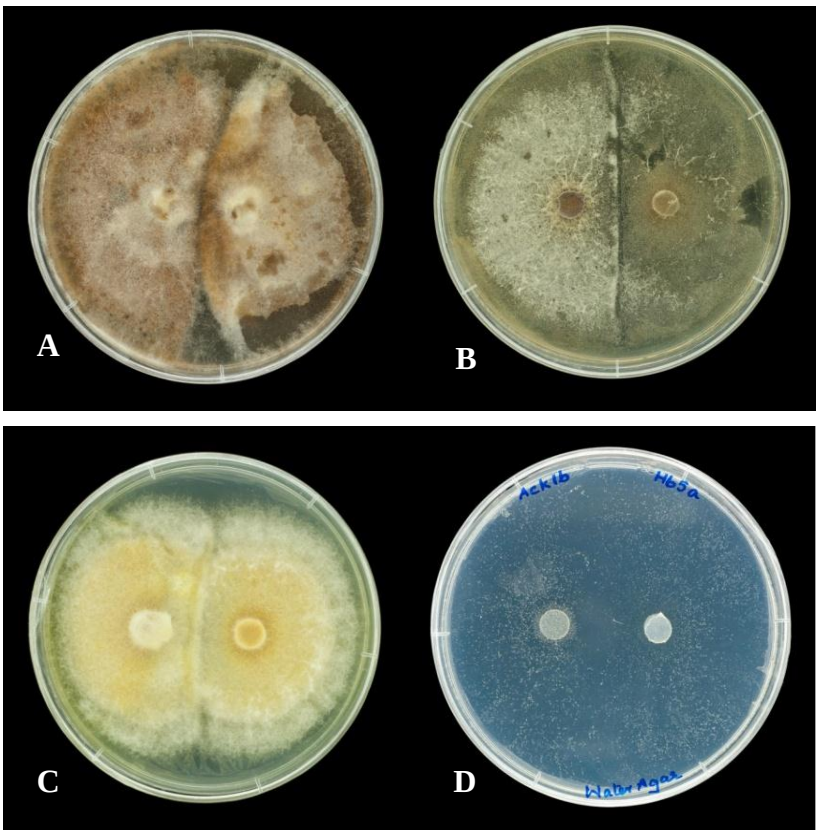


Figure 3.13 Vegetative incompatibililty reaction of *Cylindrocarpon liriodendri* on (A) PDA, (B) ½ PDA, (C) CDA and (D) Water agar.

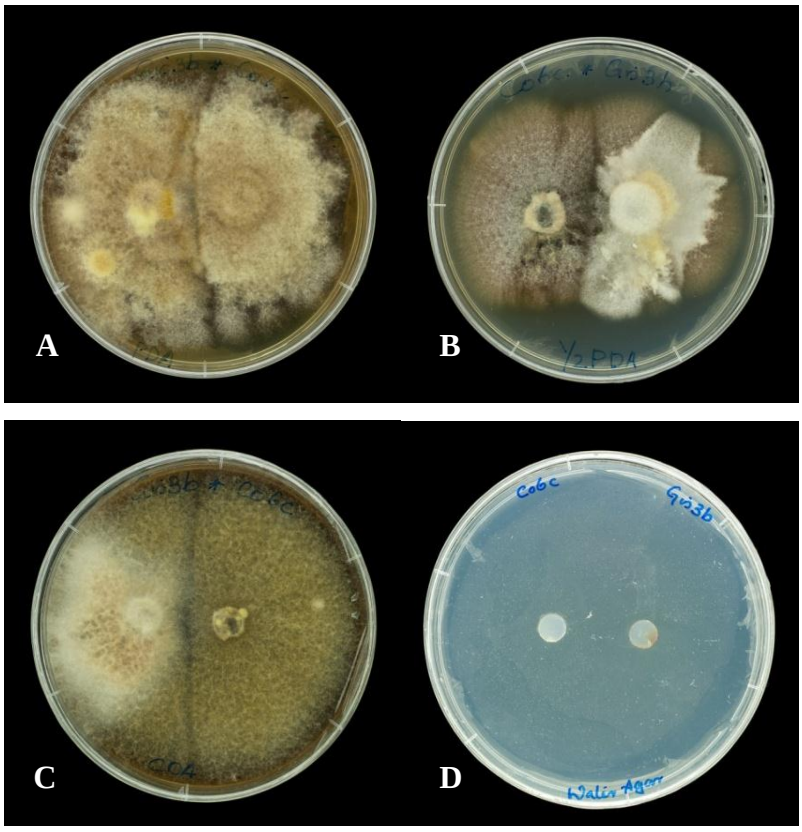


Figure 3.14 Vegetative incompatibililty reaction of *Cylindrocarpon macrodidymum* on (A) PDA, (B) ½ PDA, (C) CDA and (D) Water agar.

3.3.8 Vegetative compatibility groups

For *C. destructans* the majority (68%) of pairings produced compatible results (Table 3.16) with uniform merging of the hyphae along the contact zone. Pairings between three isolates (Hb6g:Gis1b, Hb6g:Wpa1d) were identified as partially incompatible as they produced a mild barrage line with a dark black line in the mycelial meeting point when observed from the reverse side and from the upper surface of the Petri dish. Seven pairs of isolates produced incompatible reactions (Wpa1d:Gis1b and the six isolates paired with Ack2d), which were characterised by the formation of clear zones between the growing edges of the paired isolates. The incompatible reactions mainly resulted from the pairing of isolates from different genetic groups (Figures 3.3; 3.7) but *C. destructans* isolates Wpa1d and Gis1b were from the same group (Figure 3.5). Even though the incompatible and partial incompatible groups caused clearly different interactions on agar, the placing of isolates into VCGs required them to be designated as either compatible or incompatible. Therefore, the incompatible and partially incompatible groups were merged for the purpose of designating VCGs (Figure 3:15). VCG1 contained the isolate Ack2d which showed an incompatible reaction with all other isolates. Three isolates from VCG2 overlapped with VCG3.

Table 3.16 Vegetative compatibilty testing of *Cylindrocarpon destructans.*

Isolates	Gis1b	Wpa1d	Nel1d	Co1c	Hb6g	Mar7a	Ack2d
Gis1b	C	IC	C	C	PI	C	IC
Wpa1d		C	C	C	PI	C	IC
Nel1d			C	C	C	C	IC
Co1c				C	C	C	IC
Hb6g					C	C	IC
Mar7a						C	IC
Ack2d							C

*C = Compatible, IC = Incompatible, PC = Partially Incompatible

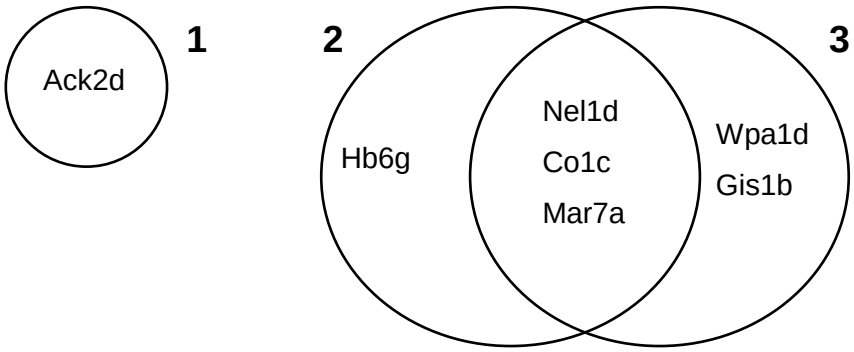


Figure 3.15 The isolates belonging to three vegetative compatibility groups of *Cylindrocarpon destructans* are shown in circles. The isolates in intersection are common to the respective VCGs.

For *C. liriodendri* the majority (98%) of pairings produced compatible results with uniform merging of the hyphae along the contact zone (Table 3.17). One pair of isolates (Hb5a:Ack1b) was identified as incompatible as they produced a barrage line. The *C. liriodendri* isolates did not produce any partially incompatible reactions among the test isolates. There were two vegetative compatibility groups for *C. liriodendri* isolates (Figure 3:16) represented by isolates Ack1b and Hb5a which are from the same genetic group on the neighbour joining tree.

Table 3.17 Vegetative compatibility testing of *Cylindrocarpon liriodendri.*

Isolates	Gis1c	Mar8i	Co1b	Hb5a	Ack1b	Wpa3a	Co3b	Co1d	Mar11b
Gis1c	C	C	C	C	C	C	C	C	C
Mar8i		C	C	C	C	C	C	C	C
Co1b			C	C	C	C	C	C	C
Hb5a				C	IC	C	C	C	C
Ack1b					C	C	C	C	C
Wpa3a						C	C	C	C
Co3b							C	C	C
Co1d								C	C
Mar11b									C

*C = Compatible, IC = Incompatible

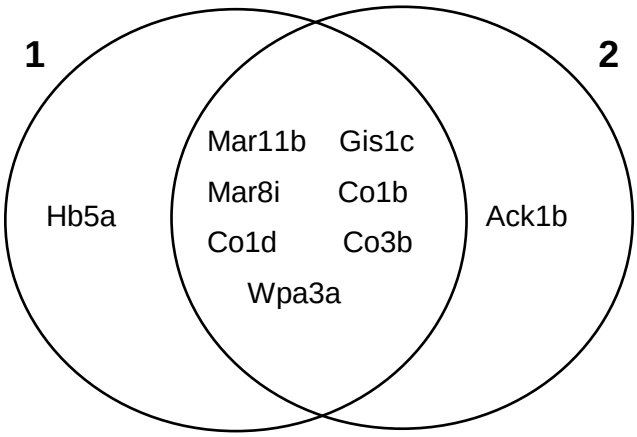


Figure 3.16 The isolates belonging to each vegetative compatibility group of *Cylindrocarpon liriodendri* are shown in circles. The isolates in intersection are common to both VCGs.

For the *C. macrodidymum* the majority (94%) of pairings produced compatible results with uniform merging of the hyphae along the contact zone (Table 3.18). Two pairs of isolates (Gis3b:Co6c and Gis3b:Hb2b) were identified as incompatible as they produced a barrage line. The isolates Gis3b and Co6c were from the same genetic group of the neighbour joining tree. There was no partially incompatible reaction among the *C. macrodidymum* isolates. The *C. macrodidymum* isolates produced two vegetative compatibility groups. The five isolates from VCG1 overlapped with VCG2 (Figure 3.17).

Table 3.18 Vegetative compatibility testing of *Cylindrocarpon macrodidymum*

Isolates	Co6c	Hb2b	Gis3b	Ack2c	Wpa4a	Ack2h	Ack1a	Mar16i
Co6c	C	C	IC	C	C	C	C	C
Hb2b		C	IC	C	C	C	C	C
Gis3b			C	C	C	C	C	C
Ack2c				C	C	C	C	C
Wpa4a					C	C	C	C
Ack2h						C	C	C
Ack1a							C	C
Mar16i								C

*C = Compatible, IC = Incompatible

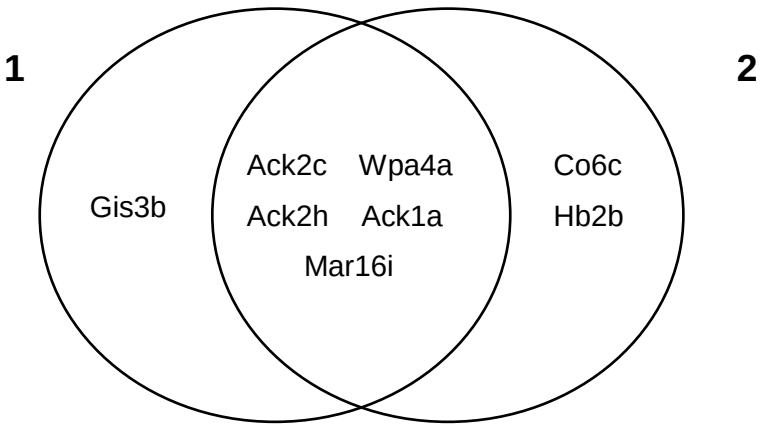


Figure 3.17 The isolates belonging to two vegetative compatibility groups of *Cylindrocarpon macrodidymum* shown in circles. The isolates in the intersection are common to the respective VCGs.

3.3.9 Microscopic examination of vegetative compatibility reactions

Anastomoses were observed in self-self and non-self pairing of isolates with compatible isolate pairs selected from all *Cylindrocarpon* species, but not from incompatible pairs.

In self-self pairing and compatible isolate pairings frequent hyphal fusion was observed (Figure 3.18C and D). Two types of anastomosis were observed 1) fusion of growing hyphal tips and 2) hyphal wall to wall fusion both in distal and proximal sections of the colony. In compatible non-self pairings, it was not possible to confirm the identity of the individual hyphae from each colony as they formed a hyphal network (Figure 3.18D). In some cases of compatible, partially incompatible and incompatible reactions, only a few hyphae grew from the PDA cubes into the space between colonies. However, in other compatible reactions the growing hyphae from both colonies thickly merged in the middle of the slide. The same was observed in partially incompatible reactions, which occurred only in *C. destructans* and which also produced chlamydospores in the interaction zone but not in other types of interactions (Figure 3.18E and F).

In incompatible reactions, no hyphal merging was observed. In a few cases, hyphal death and malformation of the leading hyphae was observed (Figure 3.18A and B) or the disintegration of the hyphal tip before hyphal fusion could take place (Figure 3.18B).

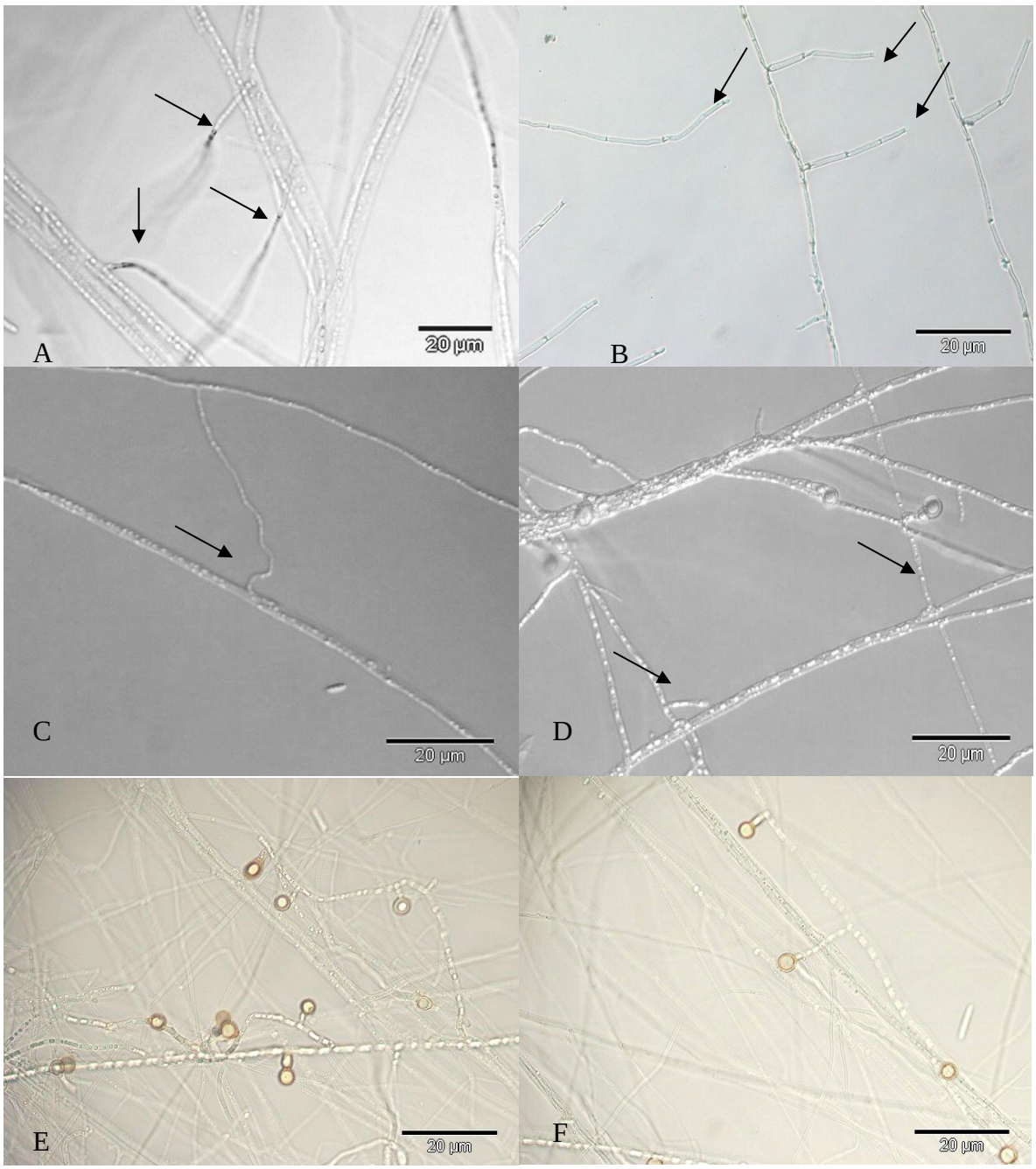


Figure 3.18 Types of compatible, incompatible and partially incompatible reaction (A) Incompatible: the hyphal tip appears to die before the two hyphae touch as shown by the arrows (B) Incompatible: the hyphae have stopped growing when near the incompatible partner (C) and (D) Compatible: the anastomosis in two near by hyphae (E) and (F) Partially incompatible: mycelial clustering and formation of chlamydospores.

3.4 Discussion

The aim of this study was to analyse the genetic diversity within populations of *C. destructans*, *C. macrodidymum* and *C. liriodendri* found in New Zealand vineyards. This is the first study to assess genetic diversity of three *Cylindrocarpon* species using the UP-PCR method and the results showed that the isolates were genetically diverse. Genetically different individuals were observed within vineyards and between vineyards. The majority of New Zealand isolates were genetically different from Australian and South African isolates.

The UP-PCR method has been used extensively to study the genetic variability of fungi including other grapevine pathogens, such as, *Phaeomoniella chlamydospora* (Pottinger *et al*., 2002) and *Botryosphaeria* species (Baskarathevan, 2011). All 11 UP-PCR primers produced different numbers of polymorphic bands with a range of 4-7 for *C.destructans* isolates, 3-5 for *C. liriodendri* isolates and 4-7 for *C. macrodidymum* isolates. This is similar to results from other New Zealand studies on the *Botryosphaeriaceae*, a predominantly asexual group of pathogen of grapevines. Baskarathevan, (2011) used UP-PCR primers to generate different numbers of bands for different botryosphaeriaceous species. However, Pottinger *et al*. (2002) found that five out of nine UP-PCR primers they used did not produce polymorphic bands for *P.chlamydospora* isolates. This suggests that the number of polymorphic bands generated by each UP-PCR primer is dependent on fungal species and may depend on the reproductive mechanism such as the relative frequencies of asexual and sexual reproduction. The production of polymorphic bands by UP-PCR by this method provided useful data to study the genetic diversity of *Cylindrocarpon* species.

In the current study, the polymorphic bands produced by the UP-PCR method were easily scored and the consistency of this method was checked with repeating atleast 50% of the isolates and it was a rapid and cost effective technique. This study used a single method to assess genetic diversity; however, it may be beneficial to apply another method, which may be able to produce additional information or amplify different areas of the genome. Alaniz *et al*. (2009b) assessed the genetic diversity of the Spanish population of *C. liriodendri* and *C. macrodidymum* using the ISSR method. Applying the same method (ISSR) to the New Zealand population, would allow the two populations to be compared. Other studies have used methods such as AFLP or RAPDs and some have combined the data from different methods. Some studies showed that use of different molecular techniques for genetic variation analysis could show different levels of genetic diversity within the same population (Steele *et al.*, 2001; Tooley *et al.*, 2000). For example, the genetic variation study of *P.chlamydospora* in New Zealand using the AFLP method produced a greater number of genotypes than the UP-PCR method (Pottinger *et al*., 2002). Another study, with combined data from UP-PCR and RAPDs, showed six genotypes in 51 New Zealand isolates of *Sclerotium cepivorum*, whereas, the data from each method showed very little genetic

diversity (Tyson *et al.*, 2002). Similarly, a study with combined data from RAPD and AFLP showed little genetic variability in Algerian *Fusarium oxysporum* forma specialis *lentis* isolates (Belabid *et al.*, 2004).

The UP-PCR technique was able to distinguish a large number of genotypes for each of the *Cylindrocarpon* species, 50 genotypes from 54 *C. destructans* isolates, 44 genotypes from 47 *C. macrodidymum* isolates and 52 genotypes from 61 *C. liriodendri* isolates. Similarly, another study by van Toor *et al*. (2005) using UP-PCR on *Ciborinia camelliae* produced 33 genotypes from 36 isolates tested. In contrast, using ISSR, Alaniz *et al*. (2009b) reported 18 genotypes from 57 *C. macrodidymum* isolates and 3 genotypes from 30 *C. liriodendri* isolates, forming a total of 21 different genotypes from a total of 87 isolates, which is substantially lower than the number of genotypes identified by UP-PCR in this study. Although both studies showed the least genotypes for *C. liriodendri*. However, the ISSR method allowed Fan *et al*. (2010) to show high genetic diversity in 164 isolates of *Monilinia fructicola* which usually reproduces asexually and only rarely by sexual mechanisms, and did not identify any clonal isolates.

It seems unlikely that the Spanish *Cylindrocarpon* spp. populations reproduce differently from the New Zealand populations, which appears to indicate that the results are affected by the methods used. In addition, it is possible that the relative frequencies of sexual and asexual reproduction differ. However, another study using AFLP on the genetic diversity of *Leptosphaeria maculans*, the fungus that cause blackleg of canola in Australia, showed low genetic diversity (Barrins *et al.*, 2004) within the population. In contrast to this, for the same pathogen, Gout *et al*. (2006) used minisatellite markers, reported high genetic diversity in France. This demonstrates that populations of the same pathogen may have different diversity in different countries and this could be explained by differences in the method used. These methods target different areas of the genome. Microsatellites are generally not functionally constrained and known to accumulate mutation rapidly due to slippage during replication. In contrast, AFLP amplifies random sections of the genome which may or may not encode functional traits. It is likely that whether the area sampled by the genetic diversity method is under functional constraint, i.e; encodes a protein, will influence how diverse it is within a population. However, it could also be explained by different founder populations, differences in the frequency of introductions or in the frequency of genetic recombination. As the two methods used to study *Cylindrocarpon* species were different it is also possible that the technique of UP-PCR is able to identify greater diversity than ISSR in *Cylindrocarpon* spp. However, as Fan *et al*. (2010) reported ISSR markers can identify high genetic diversity in pathogens, which have both a sexual and asexual cycle. Whether this is true could be established by applying the same molecular method to both populations. Regardless, the large number of genotypes in the New Zealand *Cylindrocarpon* species populations suggests that there is an active mode of recombination.

Using the UP-PCR method, the *C. destructans* population found in New Zealand vineyards showed a higher level of genetic diversity (H=0.3346) compared to the genetic variability of *C. liriodendri* (H=0.1816) and *C. macrodidymum* (H=0.2580). This is the first study to show the genetic diversity of *C. destructans* in grapevines. However, Korean *C. destructans* isolates recovered from ginseng were shown as a single genetic group when investigated using RAPD analysis (Seo *et al.*, 2011). The genetic diversity of the *C. destructans* population was also higher than New Zealand populations of some other predominantly asexual pathogens analysed using UP-PCR. These included the grapevine trunk pathogen *N. parvum* (H=0.2581) (Baskarathevan *et al.*, 2012b) and the foliar pathogen of olive, *S. oleagina* (H=0.1322) (Obanor *et al*., 2010). However, the genetic diversity of *C. destructans* is similar to the genetic diversity (H=0.36) of the Ludhiana population of *Botryodiplodia theobromae* (syn*. Lasidiplodia theobromae*), which was isolated from pear trees showing die-back and bark canker in Punjab (Shah *et al.*, 2011). These results indicate that the genetic diversity of fungal species with mainly asexual reproduction can be variable. The greater diversity of *C. destructans* when compared to the other two *Cylindrocarpon* species could be explained in several ways 1) a greater frequency of sexual recombination, 2) a more active parasexual cycle, 3) the introduction and recombination with genetically distinct isolates of *C. destructans* species from neighbouring non-grapevine hosts. The frequent introduction of new isolates could also be associated with non-grapevine plants. Researchers have been reported the isolation of *C. destructans* from non-grapevine like strawberry (Booth, 1967), apple trees (Braun, 1995; Mostert *et al.*, 2006b) and blackberry (Cedeno *et al.*, 2004) and 4) more frequent introduction of new isolates 5) the presence of multiple species within *C. destructans* described in Section 3.4.

For all three *Cylindrocarpon* species the genotype data showed the presence of more than one genotype within a vineyard. Similar to this result, inter and intra-vineyard genetic diversity was found in *Botryosphaeria* species populations (Baskarathevan, 2011) and *P. chlamydospora* populations (Pottinger *et al*., 2002) in New Zealand. Both of these are predominantly asexually reproducing grapevine pathogens. Furthermore, the genetically distinct isolates from different vineyards were randomly distributed among the different clades for the three *Cylindrocarpon* species suggesting either frequent introduction of new genotypes or recombination of existing genotypes. The distribution of *C. destructans*, *C. macrodidymum* and *C. liriodendri* genotypes throughout different geographic locations may demonstrate the free movement of these fungal species between the geographic locations in New Zealand.

A likely explanation for the inter- and intra-vineyard genetic diversity in all three species is that there were multiple introductions of these pathogens into the vineyards. Another study conducted by Pottinger *et al*. (2002) showed a similar result for *P. chlamydospora* population in New Zealand and they also hypothesised multiple introductions into vineyards. Later work

by Whiteman *et al*. (2007) demonstrated that *P. chlamydospora* created latent infections in grafted cuttings in the nursery system, which were then sold throughout the country, providing for frequent introduction of new genotypes. Similarly, Baskarathevan *et al*. (2009) also found high inter-vineyard and intra-vineyard variability among New Zealand *N. parvum* isolates. Later work by Billones (2011) demonstrated that mothervines were infected with these pathogens as were cuttings provided for sale. In the USA Gubler *et al*. (2004) stated the black foot disease is linked to nursery production, as they found up to 5% of vines from nurseries were infected with *Cylindrocarpon* species. In South Africa Halleen *et al*. (2007) reported black foot infection in grapevine cuttings obtained from nurseries and showed that *Cylindrocarpon* species can be introduced into vineyards via contaminated rootstocks (Halleen *et al*., 2003). Thus, it is likely that the use of propagation material from different nurseries is the main source of the genetic diversity, since grape growers frequently purchase new vines to replace dead ones or may replant whole blocks within existing vineyards when different varieties are required by wine makers.

The frequent introduction of new isolates could also be associated with non-grapevine plants. The likelihood of non-grapevine hosts acting as inoculum sources is supported by *Cylindrocarpon* species being isolated from other hosts like strawberry (Booth, 1967), apple trees (Braun, 1995; Mostert *et al.*, 2006b) and blackberry (Cedeno *et al.*, 2004). In the absence of an airborne dispersal mechanism, it is probable that mobile water tables and other forms of water moving through the soil may also assist in the spread of the disease into and through a vineyard (Bonfiglioli, 2005). However, such movement between alternate hosts is not supported by the results from the neighbour joining tree, in which the majority of the international *C. macrodidymum* isolates (Australian and South African) were found in a separate clade. In addition, all of the international *C. liriodendri* isolates were in a separate clade, which were likely to have been related to their host source. The majority of the *C*. *liriodendri* isolates from South Africa were isolated from apple and only one isolate, obtained from Australia, was isolated from newly planted Traminer on Ramsey, which is not a common rootstock and cultivar in New Zealand. In contrast, the single South African *C. destructans* isolate recovered from apple grouped with New Zealand grapevine isolates. This suggests that isolates on other hosts may be genetically similar to those on grapevines. It would be beneficial to test the genetic similarity of the *Cylindrocarpon* grapevine isolates by comparing them with the *Cylindrocarpon* apple isolates maintained in the ICMP culture collection.

In addition, Bonfiglioli (2005) also reported that previous land use is also thought to be important, and old orchard ground is considered to be a prime risk, as *C. destructans* is known to be a major pathogen of apple trees. *Cylindrocarpon* isolates from roots of declining apple trees in New Zealand were identified as *C. destructans* and found to be genetically

similar to isolates from New Zealand grapevines (Mostert *et al*., 2006b). This suggested that cross infection of genetically distinct *Cylindrocarpon* species isolates from infected roots or from infected orchards may occur to vineyards that are located in close proximity or were planted in the same sites. Planting vines in sites previously used to grow pears or stone fruit, or used as a forestry nursery also carries a risk, since all these sites are likely to contain residual infection (Bonfiglioli. 2005).Further isolation and comparison of *Cylindrocarpon* isolates from nearby alternate hosts would be necessary to clarify this in New Zealand.

Only a small number of isolates, six of *C. macrodidymum*, seven of *C. destructans* and 14 of *C. liriodendri*, were identified as clonal amongst the three *Cylindrocarpon* species. This contrasts with the study by Alaniz *et al*. (2009b) who used the ISSR method on *C. liriodendri* and *C. macrodidymum* which showed that the majority of the isolates in each species were clonal. The higher number of clonal isolates found in their study may be the result of the choice of molecular technique as previously discussed. Similar to that study analysis of 39 isolates of *P. chlamydospora* isolates by Pottinger *et al*. (2002) produced nine branches on the neighbour joining tree and six of them consisted of a large number of clonal isolates that varied from 3 to 10 (Pottinger *et al*., 2002). For all *Cylindrocarpon* species the clonal isolates were present in different vineyards and were often from different geographic locations. As previously discussed the dispersal of clonal isolates around the country may be due to latent infection of nursery rootstock sourced from the same nurseries. This was substantiated in some cases by the nursery of origin details provided by the vineyard owners. For example, clonal isolates Wpa1d and Mar22a were collected from Waipara and Marlborough, respectively; however, the planting material came from the same nursery.

Further analysis with combined isolate groups from the North Island, upper South Island and lower South Island demonstrated that there was geographical distinction in the relationships between populations of the different *Cylindrocarpon* species. For *C. destructans*, although the genetic diversity was very similar, the genetic distance between the North Island and South Island groups was high. This may possibly be likely due to the presence of *C. destructans* var *crassum* and *N. radicicola.* In contrast, no distinction was apparent for *C. liriodendri* and *C.macrodidymum*. Similar to this result, Bayraktar (2010) reported that genetic diversity of *F. oxysporum* f. sp. *cepae* isolates, a causal agent of basal plate rot on onion and are closely related to *Cylindrocarpon species*. Using RAPD markers they showed that *F. oxysporum* f. sp. *cepae* isolates from Turkey were different from the Colorado isolates with the Bursa population being the most diverse and stated that the Nei's genetic distance supported the populations grouping according to geographical locations.

This research also demonstrated that genetically distinct isolates of a single *Cylindrocarpon* species can be present in a single lesion and that these isolates can be located in different branches of the neighbour joining tree. These isolates may have either originated from

multiple infections of the wound by genetically distinct isolates or have been generated by recombination mechanisms occurring within the lesion. A likely mechanism is through the introduction of a new isolate within a replant which is then planted into infested soil.

According to Carlile *et al*. (2001), although 20% of all known fungi reproduce asexually, some genetic recombination has been observed in most species. Unlike other organisms, fungi have novel mechanisms of genetic exchange and recombination that are not limited to sexual reproduction, which include heterokaryosis and parasexuality (Anderson and Kohn, 1998). However, parasexual crosses are believed to occur less frequently than sexual ones and their occurrence in natural population has not been proven conclusively (Anderson and Kohn, 1998). Clearly, the reproductive mechanisms of *Cylindrocarpon* species have produced higher genetic diversity when compared to other predominantly asexual grapevine pathogens such as *P. chlamydospora* (Pottinger *et al*., 2002). This may suggest more frequent use of parasexual recombination than in other species. Since species with high genetic diversity are more likely to survive the challenges of a changing environment (Carlile *et al*., 2001). The high genetic diversity among *C. destructans*, *C. macrodidymum* and *C. liriodendri* isolates indicates that they are likely to be more flexible in environmental changes and would probably be more difficult to control than the pathogens which have low genetic variability. Therefore, this genetic diversity should be taken into consideration in developing control strategies.

Parasexual recombination only occurs between members of the same VCG (Leslie, 1993) and therefore, isolates from each of the three *Cylindrocarpon* species were investigated for VCGs. This is the first study of VCG for the three *Cylindrocarpon* species and three different types of reactions, compatible, partially incompatible and incompatible, were evident on PDA. The partially incompatible reaction was observed only for *C. destructans* and not for *C. liriodendri* and *C. macrodidymum*. For *C. destructans* the formation of a partially incompatible pairing is unusual but has been observed before for *Botryosphaeria* species (Baskarathevan, 2011). This type of reaction was also reported by Zeigler *et al*. (1997) who showed evidence of parasexual recombination between isolates of *Magnaporthe grisea.* Tyson *et al*. (2002) reported the different appearance of incompatible reactions observed between *S. cepivorum* isolates, with faint to dark reaction lines. They hypothesised that isolate pairs with greater genetic difference, as shown by UP-PCR, produced stronger incompatible reactions than genetically more similar isolate pairs. This could explain the different types of incompatible reactions produced by pairing *C. destructans* isolates selected from the different genetic groups based on the neighbour joining tree analysis.

In the current study, the *C. destructans* isolates showed partially incompatible reactions and produced many chlamydospores, which were not observed for the other two species. It is possible that the formation of chlamydospores may incorporate mechanisms for genetic

exchange or recombination in this species. The meeting of two hyphae with dissimilar nuclei may initiate the formation of chlamydospores and the merging of genetic information. In her thesis, Probst (2011) showed that two chlamydospores were often formed by the conversion of four-celled conidia, when inoculated in soil. The chlamydospores were also able to be formed by the merging of cytoplasm from one to multiple conidia and germinated hyphae. Similarly, Matturi and Stenton (1964) reported chlamydospores formed from germ tubes or hyphal cells and in conidia where one or two cells formed resistant walls. They also reported the transfer of contents of one conidium to a second conidium connected by germ tubes and the formation of two to three cell chlamydospore clusters of *C. destructans*. The presence of recombinant nuclei in the chlamydospores could be established by collecting the chlamydospores formed at the hyphal interaction zone of two isolates, generating colonies from single chlamydospores and analysing their genotypes using UP-PCR to see if they differed from the parent isolates.

The isolates chosen for VCG pairings were selected from different genetic groups of the neighbour joining tree. For *C. destructans* isolates forming incompatible (group I) and partially incompatible (groups II and III) reactions were located in different genetic groups of the neighbour joining tree. The incompatible reaction were produced by the single group I isolate tested and it would be interesting to see if other members of this group were also incompatible with isolates from groups II and III. Similarly, further analysis of group III isolates would indicate the prevalence of partially incompatible reactions. Barcelos *et al*. (2011) showed that there was an association between the RAPD groups and VCG for *Colletotrichum lindemuthianum* isolates from Brazil. Vegetative incompatibility is due to a genetic mechanism that restricts the heterokaryosis between individuals that differ in one or more *het* or *vic* loci (Glass *et al*., 2000; Xiang and Glass, 2004). In contrast to *C. destructans* isolates, isolates of *C. macrodidymum* and *C. liriodendri* that were incompatible were from the same groups of the respective neighbour joining trees and, thus, were genetically similar. In fact, for *C. macrodidymum,* incompatible isolates Gis3b and Co6c occupied the same branch within group IV. The placement of *C. liriodendri* isolates Hb5a and Ack1b was less convincing; although they were placed in the same group, Ack1b was located on a branch by itself. Thus, in these two species VCG was not correlated to genetic group.

This is the first study to show the VCG groupings in *Cylindrocarpon* species. However, other studies have shown variable number of VCGs. In a study onVCG of *Fusarium oxysporum* f. sp. c*epae* isolates isolated from onion in Colorado, five VCGs were identified from a total of 19 isolates (Swift *et al*., 2002). Similarly, Smith *et al*. (2000) identified 62 VCGs from 107 *Sphaeropsis sapinea* isolates isolated from three plantations in South Africa and four VCGs from 83 isolates from Northern Sumatra. In addition, another study on *Neofusicoccum* species isolated from individual lesions on a Eucalyptus leaf identified 14 VCGs (Smith,

2001; Slippers and Wingfield, 2007). These results indicate that the number of VCGs found in a population varies considerably and may depend on species, host and location. Unlike other studies, some isolates of the three *Cylindrocarpon* species belonging to different VCGs overlapped. Similar to this result Baskarathevan (2011) identified the overlapping of *N. parvum* isolates belonging to different VCGs. This suggests that the overlapping may result in enhanced formation of anastomosis between genetically different isolates which leads to hyphal fusion and subsequent gene flow between isolates (Leslie, 1993). However, no studies have been done to investigate the *vic* loci found in *Cylindrocarpon* species, so a detailed study of VCGs in *Cylindrocarpon* species isolates with molecular characterisation of the *vic* loci may deliver information that may possibly help the partial incompatible reaction observed in this study.

The relationship between the VCGs in New Zealand populations and international isolates needs to be investigated to determine whether they are compatible or incompatible, thereby providing a barrier to recombination between NZ isolates and introductions from overseas. Alaniz *et al*. (2009b) reported the low genetic diversity in Spanish of *C. macrodidymum* and *C. liriodendri* isolates but did not identify the VCGs to which these isolates belonged. The low diversity may mean that there are fewer VCG in Spain, however, a lack of correlation between the genotypic diversity of these species and VCG was demonstrated for New Zealand isolates. It would be interesting to conduct VCG analysis on the Spanish population and to test the compatibility of representative isolates with their New Zealand counterparts. A study on *Me. grisea* isolates from rice also showed that both compatible and incompatible reactions can occur between isolates of the same genetic groups generated from RAPD fingerprints (Rathour *et al.*, 2004). In addition, a VCG study conducted with six *P. chlamydospora* isolates (highly clonal population) and 11 isolates of *N. parvum* (highly diverse population) showed that isolates from different genetic groups of the neighbour joining tree were all compatible (Pottinger *et al*., 2002, Baskarathevan, 2011). Fungal vegetative compatibility groups are regulated by multiple unlinked loci (*vic*) (Glass *et al*., 2000) where individuals having the same vegetative incompatibility loci *(vic)* can fuse to form a heterokaryon and are considered to belong to the same vegetative compatibility group.

Microscopic examination of the VCG reactions of the three *Cylindrocarpon* species isolates grown on microscope slides showed that there was anastomosis occurring within the actively growing hyphae of isolates in compatible reactions. Similarly, a study on VCG reactions of *Botryosphaeriacea* species (Baskarathevan, 2011) also showed partially compatible reaction. However, in this study it was not possible to confirm whether anastomoses were formed between the hyphae of two isolates or of the isolate with itself. Zeigler *et al*. (1997) made similar observations on the formation of anastomoses in pairing of *Me. grisea* isolates but

could not distinguish whether they were within or between isolates. However, incompatible reactions were easy to identify because the isolates did not merge and in some cases malformation and hyphal death was noticed at the interaction zone. In partially compatible reaction, the isolates produced chlamydospores at the place of barrage line formation between two isolates of *C. destructans* and this was not observed in incompatible or compatible reactions. The formation of chlamydospores was not observed in pairings of *C. liriodendri* and *C. macrodidymum*. The frequent formation of anastomoses within a colony may facilitate asexual recombination of this fungus and possibly plays a major role in increasing the genetic diversity of the *Cylindrocarpon* species population in New Zealand vineyards. It would be beneficial to test the genetic similarity of the *Cylindrocarpon* grapevine isolates by comparing with the *Cylindrocarpon* apple isolates from international collection of microorganisms from plant (ICMP) culture collection.

In summary, the genetic diversities of populations of the three *Cylindrocarpon* species namely, *C. destructans*, *C. macrodidymum* and *C. liriodendri,* in New Zealand were comparatively higher than other pathogens with similar asexual reproduction strategies such as *P. chlamydospora,* but similar to that observed for *Botryosphaeria* spp. Mechanisms suggested for the relatively high diversity included multiple introductions of these species into vineyards, cross infection from alternate hosts and/ or a strong mechanism of asexual recombination. The mechanisms appeared to have caused some differences between species as shown by the correlation of VCG with genetic group and the formation of chlamydospores in the interaction zone of *C. destructans*.

3.5 Implications of the recent taxonomical classification

The section discusses the implications of recent reclassification of *C. destructans* and *C. macrodidymum* species by Cabral *et al*. (2012a; 2012b) on the analysis of genetic diversity.

Using the UP-PCR method, the *C. destructans* population found in New Zealand vineyards showed a high level of genetic diversity and was able to distinguish 50 genotypes from 54 isolates. The neighbour joining tree identified four main groups and it is possible that each group may correspond to different species within the *Ilyonectria radicicola* complex. This is supported by results from Chapter 2, which showed at least two species of *Ilyonectria radicicola* complex were likely to be present in New Zealand. The isolates identified as *I. europaea* were located in genetic group II, which contained the majority of *C. destructans* isolates, whereas the isolate identified as "*Cylindrocarpon"* sp. was located in genetic group III. This could be accurately established by sequencing the histone gene of some of the isolates from each of the main branches of the neighbour joining tree. The small amount of sequencing done in this chapter 2 suggested that approximately 80% of the isolates are likely to be *I. europaea* and 20% *"Cylindrocarpon"* sp. This placement was reinforced by the

temperature experiment where "*Cylindrocarpon*" sp. isolate Mar7a had lower optimum temperature and was found in clade III, whereas the other two isolates, likely to be *I.europaea,* were found in clade II. It is likely that the three clades in the neighbour joining tree attributed to *C. destructans* are actually the different species within the *I. radicicola* complex. Similarly, for *C. macrodidymum*, the isolate Ack1a (clade I) had higher optimum temperature for growth. Thus, the other two isolates (clade IV), are likely to be a different species within the *I. macrodidyma* complex. The presence of several clades on the dendrogram suggests that the population structure of species within the *I. macrodidyma* complex in New Zealand is likely to be complex.

For *C. destructans*, although the genetic diversity of the North Island and South Island populations was very similar, the genetic distance between them was high. This may indicate a regional distribution of different species within the *I. radicicola* complex (Cabral *et al*., 2012a). Also in Chapter 2 it was shown that approximately 80% of the *C. destructans* were *I. europaea* and 20% *Cylindrocarpon* sp. and that there were fewer isolates from the North Island (n=7) than the South Island (n=46). As Chapter 2 also showed that *Cylindrocarpon sp*. (Mar7a; Chapter 2) from genetic group III had a lower optimum temperature for growth than *I. europaea*. It would be interesting to determine whether these temperature optima are related to distribution of these two species from the *Ilyonectria radicicola* complex.

In the vegetative compatibility grouping for *C. destructans*, isolate Ack2d was the only isolate from genetic group I tested and it was incompatible with all other isolates tested. This may indicate that group I isolates are a distinct species within the *I. radicicola complex*. Similarly, this may be true for isolate Hb6g from genetic group III which was partially incompatible with two isolates from group II. Baayen *et al*. (2000) reported that different species in the *Fusarium oxysporum* complex were vegetatively incompatible; however, they also reported that two strains of *F. oxysporum* f. sp. *dianthi* isolated from the same carnation plant were vegetatively incompatible even though they were phylogenetically similar. In addition, Appel and Gordon (1996) reported that *Fusarium oxysporum* isolates from their culture collection were vegetatively compatible with *F. oxysporum* f. sp. *melonis* and stated that in some cases vegetative compatibility may be coincidental, possibly arising by convergence rather than common descent. Sequencing of the histone region will allow the relationship between VCG and species within the *I. radicicola* complex to be determined.

Several hypotheses were presented for the comparatively high genetic diversity of the New Zealand *C. destructans* population. Resolving the members of the species complex recovered from grapevines and their relationship to genetic group will produce a more accurate estimation of the genetic diversity of each species within the complex. The apparent lower frequency of the isolates in genetic group I and III may be due to more recent introduction of these species and/ or a less active parasexual recombination process in these

species. Of particular interest will be identifying those isolates that were partially compatible and the species identity of the chlamydospores formed at the interaction zone.

Similarly, there was high genetic diversity in the *C. macrodidymum* population with 44 genotypes identified from 47 *C. macrodidymum* isolates. The neighbour joining tree identified six main groups and it is possible that each group may contain different species within the *Ilyonectria macrodidyma* complex (Cabral *et al*., 2012b). As for the *I. radicicola* complex sequencing the histone gene from representatives of each genetic group may clarify this. In the vegetative compatibility grouping of *C. macrodidymum*, two isolates (Co6c and Hb2b) were incompatible with isolate Gis3b but all remaining isolates were compatible with all three of these isolates. In this case, two isolates (Gis3b and Co6c) were from the same branch in group IV. This does not support each genetic group being a different species within the *I. macrodidyma* complex. There are conflicting reports in the literature of the reaction that occurs between isolates of different but closely related species. A study on *Me. grisea* by Rathour *et al*. (2004) identified both compatible and incompatible isolates within the same genetic groups generated from RAPD fingerprints. In addition, Pottinger *et al*. (2002) reported that six isolates of *P. chlamydospora* from different genetic group were all compatible to each other. Similarly, Correl (1991) reported that isolates of *F. oxysporum* f.sp. *apii* race 2, a pathogen of celery and of *F. oxysporum* f.sp.*vasinfectum* race 3, a pathogen of cotton, formed a single VCG. Once the identity of the isolates have been confirmed it would be useful to study the interactions microscopically.

Several hypotheses were presented for the comparative difference in genetic diversity of *C. macrodidymum* in New Zealand when compared to Spain. The Spanish study showed different levels of virulence between *C. macrodidymum* isolates (Alaniz *et al*., 2009b). It is possible that this may be due to the presence of different species of the *I. macrodidyma* complex. Once the identity of the New Zealand isolates has been confirmed it will allow studies on the virulence of isolates within the complex.

In conclusion, this study showed that the high genetic diversity among the *C. destructans* isolates and for *C. macrodidymum* species is likely to be due, in part, to the presence of multiple species within the *I. radicicola* and *I. macrodidyma* complexes. The relationship between VCG and species remains to be elucidated as does the potential for genetic recombination between species.

Chapter 4
Variabilty in virulence of *Cylindrocarpon* species

4.1 Introduction

Previous studies have shown that pathogenicity differs between isolates for some fungal pathogens. Thus, isolates of the same *Cylindrocarpon* species may differ in their virulence, as shown by Rego *et al*. (2001) for *C. destructans.* Seifert *et al*. (2003) reported significant differences in virulence between isolates of *C. destructans* in ginseng and conifers. Alaniz *et al*. (2009b) reported isolate differences and observed a correlation between genotype and virulence among isolates of *C. liriodendri* and *C. macrodidymum,* but *C. destructans* was not included in that study. However, when Petit and Gubler (2005) tested Californian isolates of *C. destructans* and *C. macrodidymum* for their pathogenicity to grapevine rootstock variety 5C, they found that all isolates caused significant root rot on grapevine, with no significant variation between isolates or species. The same observation was made by Alaniz *et al*. (2009b) with Spanish *C. liriodendri* isolates.

In New Zealand, *C. liriodendri*, *C. macrodidymum* and *C. destructans* were found to be equally common, although there was some differences in distribution though the North and South Islands (Chapter 2, Bleach *et al*., 2006), however, the relative pathogenicity of the species and isolates was not tested. Probst (2011) showed that, although all three *Cylindrocarpon* species were capable of infecting grapevines through wounded roots or callused basal ends, the three isolates within each species that she tested varied in their pathogenicity. The study by Alaniz *et al*. (2009b) showed that it was possible to link specific isolate genotypes to virulence but the link between genotypic diversity and virulence has not been studied for New Zealand isolates.

In experiments with *Cylindrocarpon* isolates on grapevines, pathogenicity tests have generally used vines grown in autoclaved soil or potting mixture (Rego *et al*., 2001; Halleen *et al*., 2003) and they were inoculated with conidium suspensions to prove Koch's postulates. Studies of *Cylindrocarpon* species pathogenicity on grapevines have generally used conidium suspensions of 10^6 to 10^8 conidia/mL, in which grapevine cuttings were soaked for 30 min, and then grown for 2 to 5 months before being assessed for infection incidence. In Portugal, Rego *et al*. (2000) inoculated rooted cuttings of rootstock variety 99R by soaking washed roots of the plants for 30 min in conidium suspensions (10^8/mL) made from different isolates of *C. destructans*. Three months after inoculation, the pathogen was re-isolated from vines, of which 16 to 66% developed root symptoms and 33 to 67% died, with differences in symptoms being associated with different isolates.

The purpose of this study is to determine the difference in virulence of genetically different isolates of *Cylindrocarpon liriodendri*, *C. macrodidymum* and *C. destructans* under both *in vitro* and *in vivo* conditions. To achieve this aim a detached root assay will be developed to provide a rapid *in vitro* method to screen a large number of isolates for their relative virulence on grapevine roots. If variable virulence is observed then the results will be confirmed on whole plants. In addition, in order to investigate the infection process, an attempt will be made to introduce a *gfp* gene into a wild type *Cylindrocarpon* species by using either *Agrobacterium*-mediated transformation (AMT) or protoplasts mediated transformation to obtain a traceable isolate that is morphologically stable and pathologically similar to the wild type.

4.2 Materials and methods

4.2.1 Development of a grapevine detached root assay

To enable the virulence of isolates of *Cylindrocarpon* species to be rapidly assessed, a grapevine detached root assay was developed.

4.2.1.1 Fungal cultures

Three isolates were selected from *C. macrodidymum* (C06a, Hb4a, and Nel1b) and *C. liriodendri* (Co5c, Hb5a and Hb2d)and two of *C. destructans* (Mar11e and Mar15a), grown on potato dextrose agar (PDA; Difco) for 7 days at 20°C with 12:12 h dark:light. These isolates had been stored to maintain viability and virulence as described in Section 2.2.1.3.

4.2.1.2 Selection of roots

As the physical characteristics of grapevine roots vary significantly, care was taken to collect roots of similar diameter and pigmentation. The younger outer feeder roots (Figure 4.1A and B) with a diameter range of 1.5 to 2.0 mm were removed from potted one year old 101-14 rootstock plants, washed in tap water to remove the soil and trimmed to remove the lateral roots. The roots were used immediately for the assay. The roots of rootstock 101-14 were used as this variety had previously been shown to be susceptible to *Cylindrocarpon* species infection (Probst, 2011).

4.2.1.3 Detached root assay pilot study

The three replicate roots (7-8 cm long) per *Cylindrocarpon* isolate were placed individually into Petri dishes (90 x 15 mm) containing 30 g of silica sand (Fulton Hogan, New Zealand) wetted with 9 mL of sterile water. 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 during the experiment (Figure 4.1C). The apical end of each root was cut across and a mycelial plug taken from the growing edge of a 7 day old fungal colony placed against the cut end (Figure 4.1D). The experiment was duplicated using mycelial plugs grown on either half (½PDA;

Difco™, Becton, Dickinson & company, USA) or full strength PDA. Control roots had plugs of half or full strength PDA placed against the cut ends. The Petri dishes were sealed with clingfilm, incubated at room temperature arranged in randomised complete block design and after one month, the lesion lengths were measured using a digital calliper (Mitutoyo, U.K Ltd). The lesion length data were analysed by ANOVA using GenStat version 12, with significant differences between treatment means determined using Fisher's Protected LSD at P=0.05.

Figure 4.1 Experimental set-up for the detached root assay, A) harvested grapevine roots B) root section used for selection for the assay, C) trimmed root inserted into 1.7 mL tube filled with sterile water, D) root placed onto wetted silica sand and mycelial plug placed against the cut end.

4.2.1.4 Reisolation

To confirm Koch's postulates the pathogen was reisolated from the inoculated root tissue. The roots were surface sterilized for 3 min in 0.35% sodium hypochlorite and washed in sterile water twice for 2 min. Each root was sequentially cut (1 cm lengths) and the pieces from both the lesions and the apparently healthy root sections were placed onto PDA amended with chloramphenicol (250 mg/L). The plates were incubated at 20°C in 12:12 h light:dark for 7 days. Colony and conidium morphology was used to identify the *Cylindrocarpon* species.

4.2.2 Virulence of genotypically different isolates of *C. macrodidymum*, *C. liriodendri* and *C. destructans* using the detached root assay

The virulence of *C. macrodidymum*, *C. liriodendri* and *C. destructans* isolates from different genetic groups of the neighbour joining tree produced in Chapter 3 were assessed using the *in vitro* detached root assay.

4.2.2.1 Fungal cultures

From a total of 41 isolates of *C. macrodidymum*, 57 isolates of *C. liriodendri* and 53 isolates of *C. destructans* 30% of the isolates were selected for each species (Table 4.2). The isolates selected were from different genetic groups of the neighbour joining tree (Section 3.3.6 to 3.3.8) and from different geographical locations. For *C. macrodidymum* 14 isolates were selected, for *C. liriodendri* 17 isolates and for *C. destructans* 15 isolates (Appendix C.2). The isolates were grown on½PDAfor 2 weeks at 20°C with 12:12 h dark:light. From the previous pilot study, the ½PDA treatment gave the broader range of virulence compared to PDA treatment, so this study was carried out using ½PDA.

4.2.2.2 Detached root assay

For each *Cylindrocarpon* isolate, five replicate detached roots were harvested as described in Section 4.2.1.2. The experimental set up and the reisolation of the pathogen was the same as described in Section 4.2.1.3 and 4.2.1.4 except that sterile river sand and not silica sand was used as it made a more stable base. The experiment was arranged as a completely randomised design. The detached root assay lesion length data at 1 and 2 months after inoculation were subjected to analysis of variance (ANOVA) to evaluate the isolate and species effects on lesion length over time and least significant difference (LSD) was calculated.

4.2.3 Potted Vine Assay 1 – Virulence of *C. macrodidymum*, *C. liriodendri* and *C. destructans* isolates on four rootstock varieties

4.2.3.1 Rootstocks

Unrooted canes of four rootstock varieties, 101-14, 3309, Schwarzmann, SO4 were obtained from Corbans Viticulture, Auckland. The basal10 cm of the canes were placed into a tray filled with wetted pumice (Atiamuri Sand and Pumice Co., New Zealand) and kept on a heat pad at 25°C for callusing for at least 6 weeks to allow root development.

4.2.3.2 Fungal Isolates

Three isolates of *C. macrodidymum* and five isolates each of *C. liriodendri* and *C. destructans* were chosen from different geographic locations (Table 4.5) and they were grown on PDA at 20°C for 4 weeks with 12:12 h dark:light. A conidial suspension was made by placing 10 mL of sterile distilled water (SDW) onto the plate surface, scraping the culture surface with a glass slide and sieving the solution through a sterile 150 µm pore size sieve to

remove the mycelia fragments. The concentration of the resulting conidial suspension was determined with a haemocytometer. For all isolates of both *C. liriodendri* and *C. macrodidymum* the conidial suspensions were diluted to 1×10^5 conidia/mL using SDW to a final volume of 1 L. However, due to insufficient conidia for *C. destructans* isolates, the conidial suspensions were diluted to 2×10^3 conidia/mL using SDW to a final volume of 1 L. The spore suspensions were prepared and used on the same day within 3 h of preparation.

4.2.3.3 Inoculation

Rooted cuttings were removed from the pumice and the roots trimmed. The plants were inoculated in batches of 10 by soaking plant bases (~15 cm) for 30 min in a conidial suspension of the appropriate isolate prior to planting. The plants were placed into a 1.5 L pot with potting mix (80% composted bark, 20% pumice, 2kg/m3 Osmocote® Extract Standard 3 – 4 months gradual release fertiliser (16:3.5:10; N:P:K, respectively plus trace elements), $1kg/m^3$ agricultural lime and $500g/m^3$ Hydraflo® [granular wetting agent, Scott Product New Zealand Ltd]). The control rooted vines were treated in the same manner except the bases were soaked in tap water prior to potting. The treatments were arranged in a randomised complete block design (RCBD) with 10 replicates for each isolate and the four rootstock varieties and allowed to grow in the glasshouse for 3 months (Figure 4.2) between December 2009 and February 2010. The plants were watered daily being careful to avoid splashing between pots.

Figure 4.2 Experimental set-up for the virulence studies with 1 year old grapevines maintained in the Lincoln University glass house.

4.2.3.4 Assessment

The plants were assessed after 3 months growth. Plants were removed from their pots and their roots were shaken to remove loose potting mix. The roots of the plants were removed from the stem base. The main stems (trunks) were thoroughly washed with running tap water, air dried and the top sections removed to enable the remaining stems (approx. 20 cm) to be fully immersed in the sterilisation tanks. The method developed by Halleen *et al*. (2003) was used to reisolate the pathogen from the infected plants as described next.

4.2.3.5 Surface sterilisation

The plants were sterilised in batches of the same treatment. The plants were washed as described in Section 4.2.3.4. The sterilisation process involved the plants being dipped for 30 s in 70% ethanol, then 5 min in 0.35% sodium hypochlorite and finally in 70% ethanol for 30s. The plants were then placed into a sterile bag ready for immediate tissue isolation.

4.2.3.6 Tissue isolation

Tissue isolation was carried out under sterile conditions (Class I laminar flow, AIRPURE* Laminar flow cabinet, Westinghouse Pty Ltd, Incorporated in NSW). The root crown comprising the lowest 1-2 cm of the trunk base was removed using ethanol sterilised secateurs so that isolations could be taken from the basal tissue. A 1-2 mm transverse section of tissue was sliced from the basal end of the stem, cut into four equal pieces of approximately 3 mm width and placed equidistance from each other near the edge of a plate containing PDA with chloramphenicol (250 mg/L) to reduce bacterial growth. Further isolations were made using sterile secateurs to cut tissue sections at 1, 3, 6 and 8 cm from above the base of the stem and these were placed in Petri dishes containing PDA with chloramphenicol (250 mg/mL). All isolation plates were incubated for 1 week at 20°C in 12:12 h light:dark.

4.2.3.7 Identification and analysis

Fungal isolates growing from the wood pieces were identified as *C. destructans*, *C. macrodidymum* and *C. liriodendri* by comparison with the colony morphology and conidium morphology of the isolates used for inoculation. The number of root pieces infected was noted as well as the isolation distance from the trunk base. The pot experiment data were subjected to analysis of variance (ANOVA) using SPSS to evaluate the effect to rootstock, isolate or species, and any interactions.

4.2.4 Potted Vine Assay 2 – Virulence of different *C. macrodidymum*, *C. liriodendri* and *C. destructans* isolates on rootstock 101-14

The investigation into virulence of *C. macrodidymum*, *C. liriodendri* and *C. destructans* isolates was repeated (Chapter 3) using a potted grapevine assay on rootstock 101-14. In this second assay all species were used at the same inoculum concentration.

4.2.4.1 Rootstock

Non-rooted canes of rootstock variety 101-14 were obtained from Corbans Viticulture, Auckland. To allow root development, the basal 10 cm of the canes were placed into trays filled with wetted pumice and kept on a heat pad at 25°C for at least 6 weeks.

4.2.4.2 Fungal isolates

In this study, four isolates of *C. macrodidymum* of which two isolates were from potted vine assay 1 and two isolates from the different clades of the neighbour joining tree were selected. Five isolates of *C. liriodendri* were selected of which one isolate was from the potted vine assay 1 and four isolates from different clades of the neighbour joining tree (Table 4.7). For *C. destructans* three isolates were selected from the potted vine assay 1 and two isolates were selected from the different clades of the neighbour joining tree. A conidial suspension was prepared for each isolate as described in Section 4.2.3.2 and the conidial concentration adjusted to 1×10^4 conidia/mL using a haemocytometer. The inoculation, assessment and isolation were conducted as described in Sections 4.2.3.3 to 4.2.3.7.

4.3 Transformation of isolates of *Cylindrocarpon* species

The purpose of this experiment was to determine if *Cylindrocarpon* species could be transformed by *A. tumefaciens* or by protoplast mediated transformation so that an isolate containing a fluorescent marker could be used to investigate the infection process.

4.3.1 Growth of fungal isolates

Two isolates from *C. liriodendri* (Ack1b, Hb5a), *C. destructans* (Hb6g, Wpa1d) and *C. macrodidymum* (Co6c, Gis3b) were selected and they were grown on PDA at 20°C for 4 weeks with 12:12 h dark:light. These isolates were tested for susceptibility to hygromycin to determine whether they had natural hygromycin resistance by plating three replicates of each onto PDA plates amended with 100 µg/mL, 200 µg/mL or 300 µg/mL of hygromycin (Sigma-Aldrich, Co., USA) and incubated at 20°C for 1 week with 12:12 h dark:light. For collection of conidia these isolates were plated onto a PDA plate and incubated at 20°C for 1 week with 12:12 h dark:light. Conidia were harvested as described in Section 4.2.3.3.

4.3.2 Strains and plasmids

Three binary vectors were used for transformation of *Cylindrocarpon* species. These were two variants of pYT6 (Appendix C.6) containing GFP (pYT6GFP1 and pYT6GFP2) and also p0300GFP, previously used for the transformation of *Beauveria bassiana* (Wu *et al*., 2008). These vectors had been inserted into *Agrobacterium tumefaciens* strain EHA105 and were kindly provided by Dr Richard Weld. The bacterium was cultured in Luria Bertani (LB) agar (Appendix C.5.2) amended with rifampicin (50 µg/mL, Sigma-Aldrich, Co., USA) and kanamycin (25 µg/mL, Sigma-Aldrich, Co., USA). The plasmid, mCherry, kindly provided by Dr Artemio Mendoza was used for protoplast mediated transformation (Appendix C.7).

4.3.3 *Agrobacterium* mediated transformation

Agrobacterium mediated transformation was conducted as described by Weld *et al.* (2006a). *Agrobacterium tumefaciens* EHA105 containing binary vector pYT6GFP1, pYT6GFP2 or p0300GFP was streaked onto LB agar amended with antibiotics (50 µg/mL of rifampicin and 25 µg/mL of kanamycin) and incubated at room temperature for 2 days. After 2 days a single colony was transferred to 5 mL LB amended with antibiotics (50 µg/mL of rifampicin and 25 µg/mL of kanamycin) and incubated overnight at 28°C, with shaking at 200 rpm in a shaking incubator (Labnet 211DS, Labnet International, Inc.). A 0.5 mL aliquot of the *A. tumefaciens* EHA105 culture was placed into 50 mL minimal media (MM; 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM $(NH_4)SO_4$, 10 mM glucose) amended with rifampicin (50 µg/mL) and kanamycin (25 µg/mL) and incubated overnight at 28°C with shaking at 200 rpm. The overnight culture was diluted to an optical density at 660 nm OD_{660} of 0.15 using the induction medium (IM) (MM plus 40 mM 2-N-Morpholino ethane sulfonic acid [MES] buffer; Sigma-Aldrich, Co., USA, 5 g/L of glycerol, 200 µM acetosyringone;Sigma-Aldrich, Co., USA) without antibiotics and cultured at 24°C for 4-6 h with shaking at 140 rpm in a shaking incubator (Labnet 211DS).

Transformation was done by co-cultivation of conidia with the *A. tumefaciens* solution. Conidia from 4 week old cultures of *Cylindrocarpon* isolates on PDA were suspended in sterile water to a concentration of 1×10^6 spores/mL. Each conidial suspension, 50-100 mL, was poured into the Stericup®, which consisted of a sterile membrane filter that separated the conidia from the solution by vacuum filtration (Stericup® and Steritop®, Millipore Corporation, Billerica, U.S.A) and the conidia collected onto the surface. Two mL of *A. tumefaciens* solution was added to the membrane, the Steritop® used to cap the stericup, and the suspension was mixed by hand, by swirling for 3-4 s. The liquid was removed from the suspension by applying a vacuum to the Stericup. The filter surface of the Stericup containing the conidia and *A. tumefaciens* mixture was excised using a sterile scalpel, cut into at least 10 wedges and placed onto water agar amended with MES buffer, glycerol and acetosyringone without antibiotics or selection and incubated at room temperature for 2-3 days. Filters were then transferred onto PDA amended with 300 µg/mL hygromycin and 200 µg/mL timentin (known to kill the *Agrobacterium*) and incubated at 20°C in light for 2 weeks.

4.3.4 Protoplast mediated transformation

4.3.4.1 Fungal isolates chosen for protoplast

In this study the same isolates of *Cylindrocarpon* species were used as described in Section 4.3.1.

4.3.4.2 Digestion of plasmid for transformation

The plasmid used to transform the two isolates from each of the three *Cylindrocarpon* species was mCherry. The mCherry plasmid was digested with 10 U *Xmn*I in a total volume of 60 µL for 3 h at 37 ºC and then heat inactivated at 65ºC for 20 min. Complete digestion of the plasmid was confirmed by 1% agarose gel electrophoresis. The plasmid suspension was precipitated by adding an equal volume of phenol and chloroform (Sigma-Aldrich, Co., USA; Fischer Scientific) (450 µL:450 µL), mixed by vortexing vigorously for one min at room temperature until an emulsion formed. The aqueous phase was transferred to a new tube and stored at −20°C until used.

4.3.4.3 Preparation of protoplasts

One hundred mL of PDB was inoculated with a conidial suspension of 1×10^8 conidia prepared as described in Section 4.2.3.2 and incubated at 20°C for 15-16 h in a shaking incubator at 140 rpm (Labnet 211DS, Labnet International, Inc.).The germinated conidia were collected on sterile miracloth and rinsed twice with 50 mL of sterile water, then twice with 25 mL of osmotic medium (OM) buffer (Appendix C.5.5). Glucanex was dissolved in OM buffer (350 mg/mL) and sterilized by filtration through a sterile miracloth then 500 mg of the washed germinated conidia were resuspended in 35 mL of OM/Glucanex and incubated at 20°C for 2 h in a shaking incubator at 140 rpm. Protoplasts were observed after 2 h by viewing 20 µL aliquots with a microscope (Lena Microscope with a built in Olympus DP12 camera, Magnification 400 times).

The protoplasts were separated from the cell debris by filtering through two layers of sterile miracloth and collected into a sterile 50 mL tube. To this, 30 mL of sorbitol tris (ST) buffer (Appendix C.5.6) was added slowly to allow the formation of two layers and centrifuged at 1369 × *g* (Heraeus Multifuge X1R, Thermoscientific) for 7 min to form a sandwich layer containing the protoplasts. The sandwich layer was recovered using a 10 mL pipette and placed into a new 50 mL sterile tube. To this 20 mL of cold sorbitol tris calcium chloride (STC) buffer (Appendix C.5.7) was added and centrifuged at 1369 × *g* for 7 min and the supernatant was discarded. This step was repeated thrice. The protoplasts were finally resuspended in 2.0 mL ice cold STC and kept on ice until used.

Protoplasts were tested for susceptibility to hygromycin by plating each protoplast suspension onto three replicate PDA plates amended with 100 µg/mL, 200 µg/mL or 300 µg/mL of hygromycin and incubated at 20°C for 2 days with 12:12 h dark:light.

4.3.4.4 Transformation of protoplasts using mCherry

For the transformation reaction, 20 µL of plasmid (5-20 µg in STC), 2 µL of sterile heparin (10 mg/mL) (Sigma-Aldrich, Co., USA) and 2 µL of spermidine (10 mg/mL) (Sigma-Aldrich, Co., USA) was added to 240 µL of protoplast suspension, and incubated by placing the tube in ice for 20 min. After incubation 260 µL of 40% (v/v) PEG solution was added and mixed slowly by inversion 6-8 times and incubated at room temperature for 30 min. After incubation the protoplasts were mixed with 8 mL of regeneration overlay medium (Appendix C.5.9) mixed gently by inversion 6-8 times and poured over plates of regeneration medium (Appendix C.5.10) containing 300 µg/mL of hygromycin. The plates were incubated at 20°C for 3-5 days in dark:light 12:12 h.

4.4 Results

4.4.1 Development of detached root assay

Control roots (Fig. 4.3 A) had only minor discolourations at the apical cut ends after one month incubation. All inoculated roots (Fig.4.3 B) had dark brown lesions that were clearly distinguishable from the remaining healthy root.

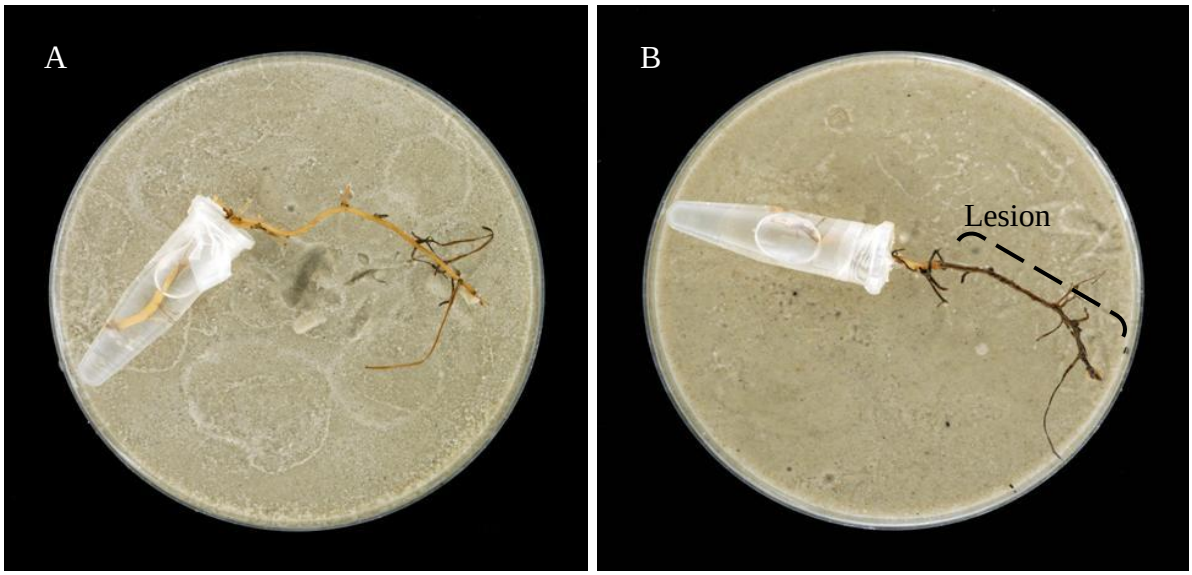


Figure 4.3 Experimental set-up for the detached root assay one month after inoculation. A) Control root showing healthy root material and B) root inoculated with *C. liriodendri* isolate Co5c showing development of the black lesion from the apical end.

There was a significant isolate effect on mean lesion lengths ($P<0.05$) (Table 4.1).The largest lesions were produced on ½PDA by *C. liriodendri* isolate C05c (40.4 mm) and on PDA by *C. liriodendri* isolate Hb5a and *C. destructans* isolate Mar11e (39.3 and 38.9 mm, respectively). The smallest lesions were produced by *C. destructans* isolates Mar11e on ½PDA and Mar15a on PDA (28.7 and 30.0 mm, respectively; Appendix C.1).The three

species were also isolated from the detached root which was inserted inside the sterile water and this portion had no lesion.

There was a significant interaction between agar type and isolate (P<0.001) (Table 4.1; Appendix C.1.1; C.1.2). Several isolates (Co5c, Nel1b and Mar15a) that produced large lesions when inoculated as colonised ½PDA plugs produced smaller lesions when inoculated as colonised PDA plugs. For isolates Hb4a and Mar11e the reverse was true with larger lesions produced when the inoculum was in PDA rather than ½PDA. Notably, *C. destructans* isolate Mar11e produced the smallest lesion when inoculated in ½PDA and the largest lesion when inoculated in PDA. The additional observation found in this study was the infection caused by *C. destructans* was initially slower than for the other species. Also the degradation of the detached roots appeared different. For *C. macrodidymum*, infection appeared highly necrotrophic with the tissue containing the lesion soft and highly degraded, whereas, *C. liriodendri* and *C. destructans,* although creating a black lesion, did not cause the root tissue to soften and degrade substantially.

Table 4.1 Mean lengths (mm) of lesions produced on detached roots of rootstock 101-14 one month after inoculation with a PDA or half strength (½) PDA plug colonised with isolates of either *C. liriodendri*, *C. macrodydimum* or *C. destructans*.

Species	Isolate	Lesion Length (mm)	
		½ PDA	PDA
Cylindrocarpon liriodendri			
	Co5c*	[1]40.4 a	34.6 b
	Hb5a *	32.4 cd	39.3 a
	Hb2d	34.9 bc	34.8 b
Cylindrocarpon macrodidymum			
	Co6a	35.0 c	34.3 b
	Hb4a *	29.8 de	37.1 ab
	Nel1b	37.4 ab	35.0 b
Cylindrocarpon destructans			
	Mar11e*	28.7 e	38.9 a
	Mar15a*	37.5 ab	30.0 c
Control		12.2 f	10.0 d
LSD		3.44	3.38

[1]Means followed by the same letters within each column are not significantly different according to Fishers LSD P<0.05 test. The symbol * denotes isolates whose lesion lengths differed significantly (P<0.05) between agar types.

4.4.2 Virulence of different *C. destructans*, *C.liriodendri* and *C. macrodidymum* isolates using the detached root assay

The results showed that all detached roots inoculated with isolates of the three *Cylindrocarpon* species produced lesions (Table 4.2). There was a significant species effect (i.e; for *C. destructans*, *C. liriodendri* and *C. macrodidymum)* on mean lesion lengths at 1 month (P=0.000; Appendix C.2.1) and 2 month (P=0.000; Appendix C.2.2) assessments. There was also an isolate effect on lesion lengths for all three *Cylindrocarpon* species (P<0.05; Table 4.2; Appendix C.2.3).

There was a significant isolate effect for *C. destructans* at both assessment times (P<0.001), with all isolates, apart from Co2b and Co1c after 1 month, producing longer mean lesions than the untreated control (Table 4.2). The isolates differed with respect to lesion lengths (P=0.000). After 1 month incubation, the longest mean lesion was caused by Mar8a isolate (8.6 mm), followed by Hb6g (7.9 mm) and the shortest mean lesion caused by Co2b (2.0 mm) and Co1c (2.5 mm). After 2 months, the longest mean lesions were produced by Mar13a, Mar7b and Co1c (47.3, 45.6 and 43.5 mm, respectively). The shortest mean lesions were produced by Ack2d and Nel1b (14.3 and 14.4 mm, respectively). Isolate Mar8a, which produced the longest mean lesion (8.6 mm) at the 1 month assessment, produced one of the shortest lesion lengths (23.2 mm) compared to the other isolates after 2 months. In contrast, isolate Co1c which produced the shortest mean lesion after one month produced the third longest mean lesion after 2 months.

For *C. liriodendri* at both the 1 and 2 month assessment times all isolates produced longer lesions (P=0.000) compared with the untreated control (Table 4.2). After one month, the longest mean lesion was caused by Wpa1c (22.9 mm) and the shortest mean lesion was caused by Mar19f (4.0 mm). At the 2 months assessment, the longest mean lesions were caused by Wpa1c, Mar8b and Co4a (51.5, 48.2 and 47.9 mm, respectively) and the shortest mean lesion was produced by Mar10a (18.5 mm).

For *C. macrodidymum* at both assessment times all isolates, apart from Mar9e and Mar20a after 1 month, produced longer lesions compared with the untreated control (Table 4.2). Again the lesion lengths differed between isolates (P=0.000). The longest mean lesion after 1 month was caused by isolate Gis2d (24.5 mm) and the shortest mean lesions were caused by Mar9e and Mar20a (3.5 and 4.1 mm, respectively). After 2 months, the longest mean lesion was caused by Mar11f (49.9 mm) and the shortest lesions were produced by Mar5b, Hb2b and Mar16i (30.7, 30.9 and 33.3 mm respectively).

Table 4.2 Mean lesion lengths on detached root assay produced by isoates of the three *Cylindrocarpon* species selected from different genetic groups.

Species	Genetic group	Isolate	One Month	Two Month
Cylindrocarpon destructans	2	Mar8a	8.6 e	23.2 c
	3	Hb6g	7.9 de	24.8 c
	2	Wpa1a	7.1 de	31.9 ef
	2	Mar11e	7.0 de	29.5 de
	2	Mar5d	6.9 de	25.9 cd
	1	Mar7b	6.6 de	45.6 h
	3	Mar13a	6.5 cde	47.3 h
	2	Mar22a	6.2 cde	35.6 fg
	2	Mar6c	5.8 cde	33.4 ef
	2	Nel1b	5.5 cd	14.4 b
	1	Co5a	5.1 cd	26.4 cd
	1	Ack2d	5.1 cd	14.3 b
	1	Ack2a	3.7 bc	39.2 g
	2	Co1c	2.5 ab	43.5 h
	3	Co2b	2.0 ab	34.8 f
		Control	0.7 a	3.5 a
		LSD	2.841	4.031
Cylindrocarpon liriodendri	1	Wpa1c	22.9 i	51.5 i
	1	Hb5a	12.1 h	43.4 h
	1	Hb2a	11.8 h	33.9 fg
	1	Wpa1e	11.6 h	43.8 h
	1	Co4a	8.9 g	47.9 i
	1	Ack1b	8.0 fg	32.2 efg
	1	Mar8b	7.6 fg	48.2 i
	2	Mar10d	6.9 ef	31.9 efg
	1	Mar22c	6.6 def	28.6 cde
	3	Mar4b	6.5 def	32.3 efg
	1	Co1d	6.3 cdef	31.9 efg
	1	Co5c	5.4 bcde	34.7 g
	1	Mar10j	5.4 bcde	29.9 def
	1	Mar6e	5.4 bcde	29.7 def
	3	Co3a	4.9 bcd	26.3 cd
	2	Mar10a	4.4 bc	18.5 b
	1	Mar19f	4.0 b	24.2 c
		Control	0.7 a	3.5 a
		LSD	1.987	4.412
Cylindrocarpon macrodidymum	3	Gis2d	24.5 g	42.5 e
	3	Mar5b	19.7 f	30.7 b
	3	Mar1c	18.2 f	36.9 cd
	4	Mar11f	11.8 e	49.9 f
	1	Ack1a	11.3 de	41.5 e
	3	Co6a	10.9 de	42.9 e
	5	Hb2b	9.1 cde	30.9 b
	3	Mar16i	8.3 cde	33.3 bc
	3	Hb4a	7.2 bcd	42.7 e
	4	Gis3b	6.1 bcd	39.8 d
	3	Ack2h	5.9 bcd	43.3 e
	2	Gis2c	5.7 bcd	34.6 bc
	5	Mar20a	4.1 abc	36.1 cd
	5	Mar9e	3.5 ab	36.4 cd
		Control	0.7 a	3.5 a
		LSD	4.59	4.577

Values within a column with the same letters are not significantly different [Fishers LSD test (P<0.05)].

4.4.3 Potted Vine Assay 1 – Virulence of different isolates of *C. destructans*, *C. liriodendri* and *C. macrodidymum* on four rootstock varieties

The result showed that the trunks of all plants inoculated with the three *Cylindrocarpon* species were infected. In all cases, the *Cylindrocarpon* colonies recovered from inoculated plant tissues had similar morphologies to the isolates used to inoculate the respective tissues. The analysis showed that there was a significant effect for the inoculation treatments (control and three *Cylindrocarpon* species), a significant interaction between rootstock and species and also significant differences between the isolates within each species.

4.4.3.1 Cylindrocarpon *species effect*

The results of inoculation by the three *Cylindrocarpon* species across all rootstocks are shown in Table 4.3 (Appendix C.3.1). There was a significant species effect on the numbers of live plants and on infection incidence at the crown and at 1, 3 and 6 cm from the trunk base. Infection incidence at the stem base of the plants inoculated with all three species was higher than for the control (P=0.05) but did not differ between species (P=0.05). Recovery of *C. destructans* from the *C. destructans* inoculated plants at 1, 3 and 6 cm was lower (P=0.05) than for the other two species. Recovery of all species at 8 cm was low, with no difference between species.

Table 4.3 Proportion of live plants and incidence of recovery of *Cylindrocarpon* species from tissue at the stem bases and 1, 3, 6 and 8 cm above the stem base, in young grapevine plants inoculated with *Cylindrocarpon destructans*, *C. liriodendri* and *C. macrodidymum.*

Species	**Proportion of live plants**	**Proportion of *Cylindrocarpon* species recovered from the trunk tissue**				
		Stem base	**1 cm**	**3 cm**	**6 cm**	**8 cm**
Control	0.68a	0.05b	0.00c	0.00c	0.00c	0.00
C. destructans	0.62a	0.99a	0.69b	0.36b	0.14b	0.00
C. liriodendri	0.41b	0.99a	0.90a	0.64a	0.38a	0.05
C. macrodidymum	0.19c	1.00a	0.91a	0.64a	0.31a	0.03
P-value	0.000	0.000	0.000	0.000	0.000	0.059
LSD	0.135	0.052	0.079	0.0975	0.091	NSD

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

4.4.3.2 Rootstock effects

The rootstock variety effect was evident for proportions of live plants (P=0.006), being lower for varieties 3309 and Schwarzmann than for 101-14 and SO4. The effect of rootstock varieties on incidence of *Cylindrocarpon* species was not significant for stem base or 8 cm isolations but it was significant for isolations from 1, 3 and 6 cm above the crown. At 1 cm, incidence was lower for rootstock 101-14 than for the other three cultivars (Table 4.4). At

both 3 and 6 cm, incidence of *Cylindrocarpon* species recovery was higher from 3309 rootstocks compared with the other cultivars. There was no significant interaction effect between the four rootstock varieties and *Cylindrocarpon* species used for inoculation.

Table 4.4 Proportion of live plants and recovery of *Cylindrocarpon* species from tissues at the stem bases and 1, 3, 6 and 8 cm above the stem bases of young grapevine plants for four rootstocks inoculated with *Cylindrocarpon destructans*,*C. liriodendri* and *C. macrodidymum*.

Rootstock	**Proportion of live plants**	**Proportion of *Cylindrocarpon* species recovered from the tissue**				
		Stem base	**1 cm**	**3 cm**	**6 cm**	**8 cm**
101-14	0.53ab	0.77	0.65b	0.39c	0.18b	0.03
3309	0.36c	0.74	0.94a	0.81a	0.51a	0.01
Schw	0.43bc	0.77	0.88a	0.47bc	0.17b	0.03
SO4	0.59a	0.75	0.86a	0.56b	0.24b	0.04
P-value	0.006	0.508	0.000	0.000	0.000	0.363
LSD	0.135	NSD	0.089	0.113	0.105	NSD

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

4.4.3.3 Isolate effects

Table 4.5 shows the relative infection incidence caused by inoculation with each of the different isolates for each species. For *C. destructans* there was an isolate effect on proportions of live plants, being higher for isolate Co1c than with all other isolates. There was no isolate effect on incidence of pathogen recovery for the tissues taken from the stem base or any distances from them (Appendix C.3.2).

For *C. liriodendri* there was no isolate effect on the proportion of live plants. Similarly, there was no isolate effect on incidence of this species in tissues taken at the stem base but there were significant effects at 1, 3, 6 and 8 cm. At both 1 and 3 cm, the pathogen was recovered at higher incidences from rootstocks treated with isolates Hb5a and Ack1b than with isolate Co1b (1 and 3 cm) and isolate Gis1c (3 cm). At 6 and 8 cm, isolation of the pathogen was higher from rootstocks treated with isolate Hb5a compared with all other isolates (Appendix C.3.3).

For *C. macrodidymum*, there was no isolate effect on the proportion of live plants. There were also no isolate effects on the incidence of the pathogen in tissues taken from the trunk bases or at 1, 3 and 8 cm, however there was a significant difference between isolates at 6 cm, with greater incidence for isolates Gis3b and Hb2b compared with Co6c (Appendix C.3.4).

Table 4.5 Proportion of live plants and incidence of *Cylindrocarpon* species recovered from tissues at the stem bases and 1, 3, 6 and 8 cm above the trunk bases of young grapevine plants inoculated with isolates of *Cylindrocarpon destructans*, *C. liriodendri* and *C. macrodidymum*.

Species	**Isolate**	**Proportion of live plants**	**Proportion of *Cylindrocarpon* species isolates recovered from the tissue**				
			Stem base	**1cm**	**3 cm**	**6 cm**	**8 cm**
Cylindrocarpon destructans	Co1c	0.83a	1.00	0.53	0.33	0.05	0.00
	Gis1b	0.43c	1.00	0.75	0.43	0.18	0.00
	Hb6g	0.63b	1.00	0.73	0.38	0.13	0.00
	Nel1d	0.55bc	0.98	0.73	0.45	0.18	0.00
	Wpa1d	0.66ab	0.97	0.75	0.41	0.13	0.02
	P-value	0.003	0.546	0.104	0.772	0.577	0.466
	LSD	0.197	NSD	NSD	NSD	NSD	NSD
Cylindrocarpon liriodendri	Ack1b	0.29	1.00	0.95a	0.74ab	0.43b	0.03b
	Co1b	0.43	0.98	0.80b	0.45d	0.30bc	0.03b
	Gis1c	0.52	0.95	0.90ab	0.52cd	0.21c	0.00b
	Hb5a	0.49	1.00	0.99a	0.87a	0.69a	0.18a
	Mar8i	0.31	1.00	0.89ab	0.67bc	0.28bc	0.00b
	P-value	0.082	0.288	0.045	0.000	0.000	0.000
	LSD	NSD	NSD	0.123	0.192	0.190	0.091
Cylindrocarpon macrodidymum	Co6c	0.24	1.00	0.90	0.61	0.19b	0.08
	Gis3b	0.15	1.00	0.83	0.65	0.28a	0.00
	Hb2b	0.24	1.00	0.97	0.49	0.32a	0.01
	P-value	0.584		0.085	0.512	0.027	0.143
	LSD	NSD	NSD	NSD	NSD	0.205	NSD

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

4.4.4 Potted Vine Assay 2 – Virulence of *C. destructans*, *C. liriodendri* and *C. macrodidymum* isolates on rootstock 101-14

The results showed that all plants inoculated with the three *Cylindrocarpon* species were infected. The analysis showed that there were significant species effects for isolation only at 3 and 6 cm (Table 4.6), but there were significant incidence effects of isolates within the species at most isolation distances for *C. destructans* and *C. liriodendri* but not all distances

for *C. macrodidymum.* For numbers of live plants the isolate effects were significant for all species (Tables 4.7 – 4.9; Appendix C.4).

4.4.4.1 Cylindrocarpon *species effects*

The results of inoculation by the three *Cylindrocarpon* species are shown in Table 4.6. There was no difference (P>0.05) in the proportion of the live plants between the three *Cylindrocarpon* species. Infection incidences at the stem bases of plants inoculated with all three species were not different from each other (P>0.05). At 3 and 6 cm *Cylindrocarpon* species recovery was higher from the *C. macrodidymum* inoculated rootstocks compared with those inoculated with *C. destructans*, and significantly higher than *C. liriodendri* at 6 cm (P<0.05). At 1 and 8 cm there was no difference in *Cylindrocarpon* species recovery between the three species (P>0.05).

Table 4.6 Proportion of live plants and recovery incidence of *Cylindrocarpon* species from tissues at the stem bases and 1, 3, 6 and 8 cm above the trunk bases of young grapevine plants inoculated with *Cylindrocarpon destructans*, *C. liriodendri* and *C. macrodidymum*.

Species	Proportion of live plants	Stem base	1 cm	3 cm	6 cm	8 cm
Cylindrocarpon destructans	0.72	1.00	0.88	0.47b	0.13b	0.00
Cylindrocarpon liriodendri	0.55	1.00	0.90	0.68ab	0.30ab	0.15
Cylindrocarpon macrodidymum	0.56	1.00	0.97	0.88a	0.47a	0.09
P-value	0.31		0.41	0.003	0.01	0.84
LSD	NSD	NSD	NSD	0.214	0.212	NSD

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

4.4.4.2 Effects of different genetic isolates of the three Cylindrocarpon species

Tables 4.7, 4.8 and 4.9 show the relative incidence of infection by each of the different isolates for each species. For *C. destructans*, the proportion of live plants was significantly lower for isolate Gis1b than for all other isolates. There were no differences in the recovery incidences of *C. destructans* isolates from tissue taken from the stem base. However, at 1 and 3 cm, the pathogen was recovered at higher incidences from rootstocks treated with Hb6g, Gis1b and Ack2d than isolate Nel1b which was not recovered beyond 1 cm. At 6 cm, isolation incidence was highest from rootstocks treated with Hb6g.

For *C. liriodendri* isolates, the proportion of live plants was higher for isolates Mar10a and Co1b than with all other isolates. There was no isolate effect on their recovery incidences

from the stem bases. However, at 1 cm, incidence was lowest for isolate Hb6f and at 3 cm incidence was lower for isolates Hb6f and Co1b compared to isolates Co5c and Wpa1e. Mar1a and HB6f were not recovered beyond 3 cm. Only isolates Co1b and Wpa1e were recovered at 8 cm, incidence being highest from rootstocks treated with Wpa1e.

For *C. macrodidymum*, the proportion of live plants was higher for isolates Mar10i and Hb2b than for other isolates. There were no significant isolate effects in the recovery incidence of the pathogen from the stem base or the closer distances until 6 cm when isolate Ack2h had the highest incidence, followed by isolates Co6c and Hb2b. Isolate Mar10i was not recovered beyond 3 cm and isolate Ack2h was the only one recovered at 8 cm.

Table 4.7 Proportion of live plants and recovery of *Cylindrocarpon* species from tissues at the stem bases and 1, 3, 6 and 8 cm above the trunk bases of young grapevine plants inoculated with isolates of *Cylindrocarpon destructans*.

Isolates	Proportion of live plants	Stem base	1 cm	3 cm	6 cm	8 cm
Gis1b	0.25 b	1.00	1.00 a	0.63 ab	0.00 b	0.00
Ack2d	0.75 a	1.00	1.00 a	0.38 bc	0.13 b	0.00
Nel1b	1.00 a	1.00	0.50 b	0.00 c	0.00 b	0.00
Hb6g	0.88 a	1.00	1.00 a	0.88 a	0.38 a	0.00
P-value	0.004		0.000	0.000	0.000	>0.05
LSD	0.453	NSD	0.252	0.380	0.244	NSD

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

Table 4.8 Proportion of live plants and recovery of *Cylindrocarpon* species from tissue at the stem bases and 1, 3, 6 and 8 cm above the trunk bases of young grapevine plants inoculated with isolates of *Cylindrocarpon liriodendri.*

Isolates	Proportion of live plants	Stem base	1 cm	3 cm	6 cm	8 cm
Mar10a	0.75 ab	1.00	1.00 a	0.63 ab	0.00 c	0.00 c
Hb6f	0.50 bc	1.00	0.63 b	0.25 b	0.00 c	0.00 c
Co1b	0.75 ab	1.00	0.88 a	0.50 b	0.25 b	0.25 b
Co5c	0.50 bc	1.00	1.00 a	1.00 a	0.38 b	0.00 c
Wpa1e	0.25 c	1.00	1.00 a	1.00 a	0.88 a	0.50 a
P-value	0.004		0.000	0.000	0.000	0.000
LSD	0.453	NSD	0.252	0.380	0.244	0.242

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

Table 4.9 Proportion of live plants and recovery of *Cylindrocarpon* species from tissue at the stem bases and 1, 3, 6 and 8 cm above the trunk bases of young grapevine plants inoculated with isolates of *Cylindrocarpon macrodidymum*.

Isolates	Proportion of live plants	Stem base	1 cm	3 cm	6 cm	8 cm
Co6c	0.38 ab	1.00	1.00	1.00	0.50 b	0.00 b
Mar10i	0.88 a	1.00	1.00	0.75	0.00 c	0.00 b
Hb2b	0.75 a	1.00	0.88	0.75	0.38 b	0.00 b
Ack2h	0.25 b	1.00	1.00	1.00	1.00 a	0.38 a
P-value	0.004		>0.05	>0.05	0.000	0.000
LSD	0.453	NSD	NSD	NSD	0.244	0.242

NSD = No significant difference. Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

4.4.5 Screening of *Cylindrocarpon* isolates for transformation

When six isolates from each of the three *Cylindrocarpon* species were subcultured onto PDA medium containing various concentrations of hygromycin B, mycelial growth and protoplast regeneration of all isolates were completely inhibited by 300 µg/mL hygromycinB but not at lower concentrations.

4.4.6 *Agrobacterium* mediated transformation

Although 1×10^6 conidia were used in each of three separate rounds of attempted Agrobacterium mediated transformation, for each of the six isolates of the three *Cylindrocarpon* species, no transformants were evident for any of them, indicating that no isolates were transformed with the three vectors.

4.4.7 Protoplast collection

The protoplasts were successfully generated and were diluted using ice cold STC buffer to a concentration of 1×10^6 (Figures 4.5A and B).

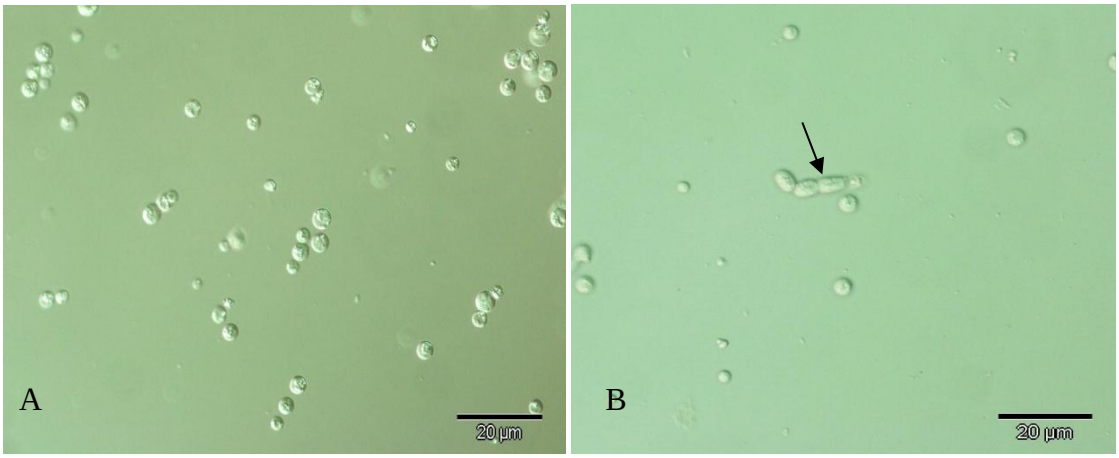


Figure 4.4 (A) Microscopic image of protoplasts after recovering them from the sandwich layer after centrifugation (B) the arrow shows separate protoplasts for multicellular conidia.

4.4.8 Transformation by protoplasts

No growth of protoplasts was observed from a total of 1×10^6 protoplasts for each of the six isolates on the regeneration overlay medium amended with hygromycin.

4.5 Discussion

The study demonstrated that genetically different isolates of *C. destructans*, *C. liriodendri* and *C. macrodidymum* differed in their virulence on detached roots and potted vines. Isolates of all three species showed variation in lesion development.

When 30% of the identified isolates were initially assessed for virulence using a detached root assay, the novel method was able to show different levels of virulence between the isolates of all three species. This result was supported by earlier studies using potted rootstock plants when three isolates from each species progressed into trunk bases at different rates (Probst *et al.*, 2007). The inoculated roots in this study showed typical infection symptoms of black discolouration within a month, which was faster than disease development in the trunk bases of inoculated vines, which usually takes 3 to 6 months (Petit and Gubler, 2005; Halleen *et al*., 2006a). This may have been due to the young soft roots selected for the bioassay, the absence of plant defence responses, as the roots were detached from plants (Elżbieta *et al*., 2013), or the absence of competing microbial flora which would naturally interact with *Cylindrocarpon* species in the soil. In addition to rapid symptom development, this assay can also be conducted at any time during the year, unlike potted vine trials which are more seasonally restricted. The potential for greater replication and tighter control over light, temperature and humidity also means that this type of assay is subject to fewer variable factors than in potted vine experiments or field trials. In such whole-vine experiments, the variability of the growing season greatly affects results, for example, high temperatures during summer play an important role in symptom expression (Halleen *et*

al., 2006a). This assay could also be used to test the virulence of *Cylindrocarpon* species on other grapevine rootstock varieties.

This study also showed that the virulence of a particular isolate was significantly affected by the medium on which it had been grown. Isolates that were highly virulent when applied as mycelium in ½PDA were less pathogenic in PDA and *vice versa*. This was surprising, however, it may reflect differences in the saprophytic ability of the individual isolates; those that were highly virulent when applied in ½PDA may have been better able to colonise and access the plant nutrients than other more saprophytic isolates and, thus, were less reliant on the agar medium. In full strength PDA isolates with greater saprophytic ability could produce a larger biomass and, thus, produced larger lesions by virtue of inoculum density. Overall the ½PDA treatment gave the broader range of virulence (28.7-40.4 mm lesions) compared to the PDA treatment (30.0-39.3 mm lesions). To better reflect the natural processes of infection in soil, the assay could be modified to use chlamydospores, which may be produced in liquid culture (Yoo *et al.*, 1996).However, Matturi and Stenton (1964) observed that chlamydospores needed a period to become mature and newly formed chlamydospores of *C. destructans* were damaged by pea root substances. Also Probst (2011) stated that *Cylindrocarpon* species chlamydospores needed a longer time to overcome the dormancy and infect plants. Similarly, Probst (2011) stated that in soil environment, single conidia formed chlamydospores within their cells or combined the protoplasms of multiple conidia to form multiple chlamydospores. So for this assay, it is likely that mycelia inoculum rather than chlamydospores should still be used.

The use of detached roots has been reported for testing the virulence of plant pathogenic microorganisms. van der Merwe *et al*. (1992) used detached roots to evaluate the resistance of avocado rootstocks using *Phytophthora cinnamomi* zoospores and mycelium as inoculum source. Their result showed that mycelium gave significant difference in lesion length but not zoospores. Similarly, Zilberstein and Pinkas (1987) reported that detached root technique used as a screening method to differentiate the resistance of avocado rootstocks and susceptibility to *Phytophthora* root rot disease. In addition to detached root assays, the use of detached leaf assays has been frequently reported for investigations into fungicide efficacy, induced resistance assays and isolate variation in virulence. Pettitt *et al*. (2011) reported the development of a detached leaf assay to determine the virulence of *Pythium* isolates to cut-flower chrysanthemum roots. The necrosis was rapid on the detached leaf and within a few days the whole leaf was covered with necrosis. In addition, they stated that the detached leaf assay was advantageous by allowing a large number of isolates to be screened in a survey of *Pythium* isolates from samples collected from chrysanthemum nurseries. Thus, the detached root assay adds to the *in vitro* leaf assays described in the literature. As with the detached leaf assays, this root assay is rapid and allows the screening of large number of

isolates. *In vitro* assays are generally acknowledged to provide a quick and economic method that correlates well with similar assays on whole plants.

Despite the artificial nature of the detached root assay, when used with ½PDA as the inoculation medium the lesion length in the detached root assay and the isolate recovery from the potted vine virulence assays showed good correlation for all three species. For *C. macrodidymum*, isolate Ack2h produced the second longest lesion among 14 isolates tested and was recovered at 8 cm in the whole plant assay which was significantly greater than for the other isolates (Co6c, Mar10i and Hb2b). Recovery of isolate Hb2b was at a significantly lower distance than Ack2h and this isolate gave the shortest lesion amongst the isolates tested in detached root assay. A similar outcome was evident for *C. destructans* isolate Hb6g and *C. liriodendri* isolates Hb5a and Wpa1e, which produced large lesions in the detached root assay and had moved longer distances into the stem base in the potted vine assays. These results suggested that the mechanism of lesion development by *Cylindrocarpon* species is similar in both detached root and potted vine assay. However, the inoculum used in the detached root assay was mycelia, whereas in the potted vine assay the inoculum was conidia, and both were able to infect root tissue that had been wounded.

The detached root assay allowed some differences in the infection process to be observed. The infection caused by *C. destructans* was initially slower than for the other species. Also the degradation of the detached roots appeared different. For *C. macrodidymum* infection appeared highly necrotrophic with the tissue containing the lesion soft and highly degraded ("mushy"), whereas, the *C. liriodendri* and *C. destructans,* although creating a black lesion, did not cause the root tissue to soften and degrade substantially (roots were still firm) suggesting that they moved inside the roots endophytically without causing as much damage to the plant tissue. For *C. macrodidymum* infection, the soft and highly degraded ("mushy") lesions may have been caused by different physiological processes than with *C. liriodendri* and *C. destructans* lesions which were black and firm, suggesting less damage to the plant tissue. The differences in symptoms may have been due to different infection mechanisms with differing amounts of cell wall degrading enzymes being produced. Lyr and Kluge (1968) studied nine isolates of *N. radicicola* as pathogens of *Pinus sylvestris* and grouped these isolates into three classes according to their virulence. They demonstrated that the difference in virulence was associated with toxin production and also the virulent isolates had slightly higher pectinase and cellulase activity. Evans and White (1966) isolated a phytotoxin from *N. radicicola*, which they initially identified as brefeldin A or nectrolide that stunted *Eucalyptus pilularis* growth. In the current study, *C. macrodidymum* may also have been reacting to differences in the plant status, such as the absence of plant resistance mechanism, which allow it to produce necrotic enzymes unchecked (Unestam *et al.*, 1989; Brayford, 1991). An additional observation was that all species were able to reach the end of the detached root which was inserted inside the sterile water and during isolation the *Cylindrocarpon* species

were recovered from this portion even in the absence of a lesion. This result showed that these pathogens can grow in different extra-radical environments such as water immersion and also indicates endophytic movement of this pathogen beyond the lesion. Unestam *et al*. (1989) reported that infection by *Cylindrocarpon* species may be facilitated by the stress caused by oxygen deficiency due to excess of water in the soil, which weakens the root system; however there was no evidence of such a phenomenon in this study.

There appeared to be no relationship between the genetic group that the isolates belonged to in the neighbour joining tree and their virulence level. For example, isolates within *C. liriodendri* genetic group 1 isolates gave the longest and shortest lesions in the detached root assay. This was in contrast to the study of Alaniz *et al*. (2009b) who investigated the genetic diversity of the two *Cylindrocarpon* species; i.e; *C. liriodendri* and *C. macrodidymum,* using ISSR analysis and separated the different genotypes into seven groups. They showed significance differences in virulence on grapevine seedlings between the ISSR groups. In a similar outcome to the current study, Petit and Gubler (2005) reported that there was no variation in virulence between genetically different isolates of *C. destructans* and *C. macrodidymum* assessed 4 months after inoculation on 6 month old vines. They had selected the isolates from multigene analysis and assessed the presence of the pathogen in grapevines by recovery onto PDA plates. In another study, genetically diverse isolates of *E. lata* selected from different populations produced by RAPD markers showed a range of severity in the disease development on grapevines (Peros and Berger, 2003). This study also showed no correlation between geographic region and virulence, since isolates from the same region produced significantly different lesions for all the three *Cylindrocarpon* species.

The UP-PCR method used in this research was effective in showing the genetic diversity of different isolates of *Cylindrocarpon* species; however this method was not able to provide any relationship between genotypic variability and virulence diversity. The lack of correlation observed might be due to the limitations of UP-PCR primers, which are designed to target intergenic and non-coding areas of the genome (Bulat *et al*., 1998). Since, the virulence of pathogens may be regulated by single or multiple genes (Agrios, 2005) these specific areas of the genome might not be targeted by the UP-PCR primers. Therefore, it would be advantageous to use methods like RAPD, AFLP and ISSR (Hu *et al.*, 1995; McDonald, 1997; Alaniz *et al.*, 2009b) which are more random and may sample coding gene regions in addition to non- coding areas. This may explain the correlations found by Alaniz *et al*. (2009b) but not in this study. It would be interesting to apply ISSR methodology to the NZ population for which virulence has already been characterised. In addition, it may be useful to do targeted analysis of pathogenesis genes such as the protease, cellulase and laccase genes which are reported to be involved in the pathogenesis (Poussereau *et al*., 2001; Lev and Horwitz, 2003; Cañero and Roncero, 2008).

In this study, rootstock variety 101-14 was selected for the detached root assay for two reasons 1) a previous study done by Probst (2011) found no significant difference in susceptibilities of the two rootstock variety 101-14 and 5C and 2) it is one of the rootstocks commonly used in New Zealand. In a previous New Zealand study, Harvey and Jaspers (2006) reported that the susceptibility to black foot was found to be high for 101-14; however the results were not statistically analysed. An experiment by Dore (2009) found that susceptibility to *C. destructans* was not statistically different between the 101-14 and 5C rootstock varieties.

The current study used a greater number of isolates than the study by Harvey and Jaspers (2006). It investigated virulence of the three main *Cylindrocarpon* species with many isolates, being *C. destructans* (n=15), *C. liriodendri* (n=17), and *C. macrodidymum* (n=14). It demonstrated different virulence levels among the isolates from a single species, which occurred in the three main species of *Cylindrocarpon*. Similarly, Halleen *et al*. (2006a) reported that 13 *C. destructans* isolates had different levels of virulence, which also correlated with the age of cultures, but that *C. macrodidymum* and *C. liriodendri* isolates did not produce such a wide range of virulence. Alaniz *et al*. (2009b) reported variable virulence between 14 *C. macrodidymum* isolates inoculated on three to four leaf grapevine seedlings, but not between the five *C. liriodendri* isolates. This indicates that susceptibility testing is likely to be influenced by the number of isolates chosen and their virulence. Given the good correlation between the detached root assay developed in this study and whole plant assays it may provide a useful *in vitro* method for screening a large number of isolates against rootstocks to determine their relative susceptibility. The process could then be validated on whole plants with fewer isolates.

The pot trial assays showed that all 21 isolates from the three *Cylindrocarpon* species were able to infect the roots of wounded plants and that the four different varieties of rootstocks were all susceptible to infection. Under greenhouse conditions *C. destructans*, *C. macrodidymum* and *C. liriodendri* showed external symptoms of black foot disease within 3 months, however, the response to inoculation varied among the grapevine rootstocks. The rootstocks 101-14 and SO4 had fewer dead plants than Schwarzmann and 3309, with rootstock 3309 having the greatest number of dead plants. Of the four rootstock varieties, 101-14, 3309, Schwarzmann and SO4, used in this study there was a higher incidence of *Cylindrocarpon* species recovery from rootstock 3309 at 6 cm than for the other varieties. In contrast, the number of live plants was higher in 101-14 and SO4 irrespective of the *Cylindrocarpon* species causing the infection. In addition, the disease severity was lower in these two varieties compared to 3309 and Schwarzmann.

Among the three species tested, the disease severity was highest for *C. liriodendri* and *C.macrodidymum* and lowest for *C. destructans*. In similar work Probst (2011) also showed

that *C. liriodendri* produced consistently high disease severity independent of inoculum type used. The proportion of live plants was highest with the plants inoculated with *C. destructans*, which did not differ significantly from the control. In the first trial, this might have been due to the low concentration of the conidial suspension of *C. destructans* used as the trend was not shown in the second trial for rootstock 101-14. However, other overseas researchers have reported that *C. macrodidymum* and *C. liriodendri* are the major threats to grapevines (Halleen *et al*., 2004; Halleen *et al*., 2006b; Petit and Gubler, 2007; Alaniz *et al*., 2009b). It would be beneficial to compare the virulence of international isolates to the New Zealand *Cylindrocarpon* isolates to determine whether our populations are of similar virulence. However, this was not possible in standard laboratory conditions due to the requirement for a PC2 glass house in which to test international isolates *in planta*.

The virulence and progression of the pathogen up the stem of the plants showed differences between isolates and species. Other studies have not shown these differences (Rego *et al*., 2000, Petit and Gubler, 2005; Alaniz *et al*., 2009b) however, most researchers have only isolated at 1 cm, although Probst (2011) also isolated from 5 cm. In this study, the isolation was done at four intervals up to 8 cm providing more data about the relative movement of isolates and species. In the first assay, *C. destructans* was only recovered from the base, and in the second assay (with higher inoculum concentrations), it was recovered at up to 6 cm, although incidence was much lower at 3 and 6 cm than for the other species. This may have been due to the different inoculum concentrations, however, Probst (2011) showed that increasing spore concentration led to higher incidence at the trunk base but not at 5 cm from the trunk base. For *C. macrodidymum,* recovery distances and incidences were similar to those of *C. liriodendri* in the first assay, but were higher than for *C. liriodendri* in the second assay. However, both species had similar incidence at 8 cm. This indicates that, both *C. liriodendri* and *C. macrodidymum* isolates are more invasive than *C. destructans* which may account for their reputation as being the most important black foot pathogens worldwide (Halleen *et al*., 2004; Halleen *et al*., 2006b; Petit and Gubler, 2007; Alaniz *et al*., 2009b). However, in New Zealand Bleach *et al*. (2006) reported that *C. destructans* was present in similar proportions of vines as *C. liriodendri* and *C. macrodidymum* which may be due to the fact that many vineyards replaced orchards of apples which are susceptible to *C. destructans* (Bonfiglioli, 2005).

In this study, the potted vines displayed obvious signs of decline within 3 months. This is a relatively rapid onset of decline symptoms which normally take up to 6 months to become visible under field conditions. This may be due to a number of factors. Firstly, the rapid onset of symptoms may be due to the inoculum concentration used which led to higher disease severity than would normally be present in the field. Researchers have reported that the application of increased inoculum concentrations to plants generally resulted in increased

disease severity under artificial conditions (Pegg and Dixon, 1969; Shaw *et al.*, 1997; Sippell and Hall, 1982). Probst (2011) showed that increasing spore concentration, applied into the soil, led to higher incidence and severity at the trunk base in potted plants treated with the same three *Cylindrocarpon* species as tested in this study. In both her study and this study, the plants were grown in potting mix which does not reflect the natural environment of the *Cylindrocarpon* species in vineyards, where it interacts with other microorganisms present in the soil. Douglas (1970) demonstrated the role of other soil microorganisms by adding different concentration of *Fusarium oxysporum* f. sp. *melonis* to autoclaved and non-autoclaved soil planted with muskmelons. His study showed that the plants survived better in natural soil compared to autoclaved soil at the two highest inoculum concentrations, indicating that inoculum efficiency was reduced in non-sterile soil. Secondly, the plants may have been stressed due to the high temperatures during summer in the glass house where the temperature often reached 25°C and the pots had limited temperature buffering due to surface area, unlike field soil. This may also account for the high mortality in the control plants where approximately one-third died. Larignon (1999) reported that high temperatures during summer also played an important role in symptom expression, since the compromised root and vascular system of diseased plants would not be able to supply enough water to compensate for the higher transpiration rates.

In an effort to provide a greater understanding of the infection mechanisms for the three species several attempts were made to created transgenic strains containing selectable markers. There are no prior reports regarding the transformation of *Cylindrocarpon* species and this was the first attempt to transform *Cylindrocarpon* species. The vectors that were used had all been successful in transformation of other ascomycetes. The vector p0300GFP was used to transform the entomopathogen *Beauveria bassiana* (Wu *et al.*, 2008); the vectors pYT6GFP1 and pYT6GFP2 were previously used for the plant pathogen *Sclerotinia sclerotiorum* (Weld *et al*., 2006a) and the vector mCherry for *Trichoderma* spp. (A. Mendoza pers comm. (2012). To increase the potential success rate of transformation, two different methods of transformation were attempted (*Agrobacterium* mediated transformation and protoplast mediated). This transformation study used conidia, which were also used by Wu *et al*. (2008) who transformed the *Beauveria bassiana.* Conidia are also the preferred substrate for *Trichoderma* species (A. Mendoza pers comm.); however Weld *et al*. (2006a) transformed *S. sclerotiorum* using ascospores.

Several reasons can be postulated for the lack of AMT success. Unsuitable plasmids may not have been able to integrate into the genomic DNA or may be degraded by cellular enzymes (Fitzgerald *et al*., 2003). Weld *et al*. (2006b) also suggested that mitotic instability of the DNA, instability of the mRNA or protein or lack of function of the promoters could interfere with the process. However, in this case it was considered unlikely that the vectors

were unsuitable as they had been successfully used in other systems and shown to express the selectable markers. Lack of function of a promoter was also considered unlikely because the promoter linked to the gene for hygromycin resistance has been shown to work in a wide range of fungi (Jones *et al.*, 1999; Rho *et al.*, 2001; Atkins *et al.*, 2004; Weld *et al.*, 2006a; Wu *et al.*, 2008). A more likely reason is that the material was recalcitrant to transformation. The method used here was based on conidia as the starting material; however, these are multicellular and so multinucleate, the nuclei possibly being different. Other research has shown that the existence of two or more genetically different nuclei in the same cell can hamper transformation. Maor *et al*. (1998) reported that some *Trichoderma* isolates form heterokaryons spontaneously, which makes it very difficult to isolate single spore hygromycin resistant lines that will fluoresce. It is also possible that a particular genomic control system might be present in *Cylindrocarpon* species that could interfere with the integration or the expression of foreign genes (Rolland *et al.*, 2003). Another reason may be that the conditions themselves need optimisation. Although the system used here was one that had been optimised for the recalcitrant fungus *S. sclerotiorum* (Weld *et al*., 2006a) further optimisation may be required. Yang *et al*. (2007) reported that, infection of *Trichoderma harzianum* by *A. tumefaciens* was time dependent. Thus, an iterative time course study may be needed to optimise co-cultivation conditions. However, in this study, incubation of spores on co-cultivation plates for up to 48 hours did not improve transformation. Similar difficulties were observed with *Colletotrichum graminicola* and the co-cultivation period was extended to 5 days (Flowers and Vaillancourt, 2005). Therefore, the transformation process could be repeated using hyphae and longer co-cultivation times.

The PEG-mediated transformation was also not successful, despite a high protoplast yield which should have improved transformation frequency. In this study the protoplast concentration used was 1×10^6 protoplast/mL. Fitzgerald *et al*. (2003) used the protoplast concentration of $1\text{-}9 \times 10^6$ of *Venturia inaequalis* and stated that the higher number of protoplasts led to an increased number of transformants. Atkins *et al*. (2004) used a concentration of 10^7 protoplasts/mL for the transformation of *Pochonia chlamydospora*, which indicated that a higher protoplast concentration might help identify stable transformants. These results indicate that a higher concentration of protoplasts might assist the recovery of transformants.

In summary, this study can report that the three main *Cylindrocarpon* species were able to cause disease in grapevines. There was a substantial isolate variation both in the results from the detached root assay and the potted vine assay, although both were related. The detached root assay represented a useful method for screening a large number of isolates for virulence and is likely to facilitate the identification of resistant rootstocks. The results reinforced the need to use multiple isolates when undertaking virulence assays. However,

there was no relationship between the genetic group developed by UP-PCR and virulence of the isolates of the three *Cylindrocarpon* species.

4.6 Implications of recent taxonomical classification

The section discusses the implications of recent reclassification of *C. destructans* and *C. macrodidymum* species by Cabral *et al*. (2012a; 2012b) on the isolate variation shown by the pathogenicity tests.

In this study, the results for *C. destructans* showed that virulence differed among isolates. Several hypotheses were presented for the isolate variation of *C. destructans* in New Zealand. However, it may also be due to the presence of multiple species of the *I. radicicola* complex species. Therefore, resolving the members of the species complex recovered from grapevines will help clarify whether some species have greater or lesser virulence on grapevines. Several studies have demonstrated variability in virulence among isolates of the same *Cylindrocapon* species (Rego *et al*., 2001; Rahman and Punja, 2005; Bleach *et al*., 2007; Pathrose *et al*., 2010; Probst *et al*., 2012) which may be due to different species within the *I. radicicola* complex. Further studies should be conducted to investigate the pathogenicity of individual species within the complex. Data presented in Chapter 2 suggested that the majority of the *C. destructans* isolates were likely to be *I. europaea* with only 20% of these isolates identified as "*Cylindrocapon"* sp. The apparently lower frequency of this last group may be due to a recent introduction into grapevines or weaker virulence on this host. However, the results in this chapter showed that high or low virulence was not correlated with a particular group in the neighbour joining tree. Instead, isolates with different levels of pathogenicity were found in each group. This suggests that even if different species are present in the neighbour joining tree that variability in isolate virulence is still likely. Also, the inconsistent variability in susceptibility of different rootstock varieties to black foot disease shown by previous studies (Rego *et al*., 2000; Harvey and Jaspers. 2006; Alaniz *et al*., 2010 and Probst *et al*., 2012) indicate that this variability may reflect different assemblages of species from the *I. radicicola* complex were used in the pathogenicity experiments.

The *C. macrodidymum* isolates also varied in virulence in this study as well as in Spanish populations, where genetic group correlated with virulence diversity (Alaniz *et al*., 2009b). It is likely that more than one species of the *I. macrodidyma* complex were present in both studies and, if the ISSR markers used in the Spanish study were able to differentiate species within the complex, then it may indicate that some species are more pathogenic on grapevines than others. In contrast, there was no correlation found in the New Zealand study suggesting that either 1) only one species is present and the isolates have variable virulence or 2) all the species present had variable virulence within the isolates. Accurate identification of the species of the *I. macrodidyma* complex present in New Zealand would be useful to

determine if there are significant differences between the New Zealand and Spanish populations. The variable effects from previous studies into susceptibility of different rootstocks (Harvey and Jaspers, 2006; Bleach *et al*., 2007; Pathrose *et al*., 2010; Alaniz *et al*., 2010) may also be due to the presence of different species within the *I. macrodidyma* complex. Once the identity of the *C. macrodidymum* isolates has been confirmed it will allow studies on the virulence of isolates within the complex.

In conclusion, this study showed difference in virulence among the *C. destructans* isolates and the *C. macrodidymum* isolates, which may be due, in part, to the presence of multiple species within the *I. radicicola* and *I. macrodidyma* complexes. The varied virulence of these isolates within the species remains to be elucidated.

Chapter 5
Characterisation of key cell wall degrading enzymes produced by *Cylindrocarpon* species

5.1 Introduction

The plant cell wall is made of cellulose, hemicelluloses, pectic substances and proteinaceous compounds (Albersheim *et al.*, 1969). Plant pathogens secrete enzymes capable of degrading the substances in the plant cell wall (An *et al.*, 2005; Moreira *et al.*, 2005; Bolton *et al.*, 2006) to obtain important nutrients and also to enable cell penetration and progress of the pathogen inside the plant (Moreira *et al.*, 2005). To achieve this plant pathogenic fungi are known to produce a wide variety of enzymes; which include cellulase (Zaldivar *et al.*, 2001; Moreira *et al.*, 2005), laccase (Wahleithner *et al.*, 1996; Valderrama *et al.*, 2003), protease (Billon-Grand *et al.*, 2002; Ten Have *et al.*, 2004), pectinase (Reignault *et al.*, 2008) and xylanase (Brutus *et al.*, 2005; Brito *et al.*, 2006). Among these enzymes cellulase, pectinase and xylanase are reported to degrade plant polysaccharides (Cotton *et al.*, 2003; Olivieri *et al.*, 2004; Moreira *et al.*, 2005; Brito *et al.*, 2006).

Cellulases are among the most well studied of these enzymes, and many plant pathogens are reported to secrete cellulase including *Pythium oligandrum* (Picard *et al.*, 2000), *Trichoderma reesei* (Stricker *et al.*, 2008; Kubicek *et al.*, 2009; Schuster *et al.*, 2011; Schuster *et al.*, 2012), *Trichoderma viride* (Montenecourt and Eveleigh, 1977; Khare and Upadhyay, 2011) and *Fusarium oxysporum* (Ramanathan *et al.*, 2010).

In addition to enzymes capable of degrading polysaccharides plant pathogens secrete an array of other enzymes. These include proteases, which facilitate host penetration and colonization of plant cell walls by degrading structural proteins and enzymes which may be embedded in the plant cell wall (Rolland *et al.*, 2009). The proteases have been studied in several fungi including *Fusarium* species (Urbanek and Yirdaw, 1978), *Metarhizium anisopliae* (Freimoser *et al.*, 2003), *Magnaporthe grisea*, *Fusarium graminearum* (Hu and St. Leger, 2004), *Sarocladiumoryzae* (Garcia *et al.*, 2003), *Botrytis cinerea* (Rolland *et al.*, 2009) and *Sclerotinia sclerotiorum* (Poussereau *et al.*, 2001; Bueno *et al.*, 2012).

Laccases are a family of fungal enzymes that have been implicated in pathogenesis and the degradation of lignin (Vasconcelos *et al.*, 2001). They have been reported to be produced by the grapevine pathogens *Botryosphaeria* sp. isolate MAMB-5, *Neofusicoccum ribis, N. parvum* and *Lasiodiplodia theobromae* which have been shown to be lignolytic (Barbosa *et al.*, 1996; Vasconcelos *et al.*, 2001; Baskarathevan, 2011). These enzymes were also reported to be secreted by other plant pathogenic fungi including *Rhizoctonia solani*

(Wahleithner *et al.*, 1996), *Gaeumannomyces graminis* var. *tritici* (Litvintseva and Henson, 2002) and *Fusarium oxysporum* (Cañero and Roncero, 2008).

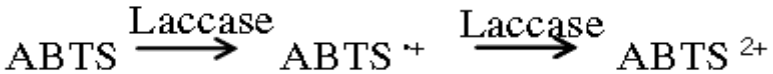
Tonukari (2003) stated that the cell wall degrading enzymes are involved in tissue maceration, penetration and virulence. In the previous chapter, the three *Cylindrocarpon* species inoculated onto grapevine detached roots and whole plants showed tissue maceration and caused plant death. Therefore, it is likely that the three species produced a range of cell wall degrading enzymes and that differences between enzyme activity may explain variation in virulence. This hypothesis is supported by a study on ginseng, which showed that *C. destructans* isolates, which were pathogenic to ginseng, produced varied levels of pectinolytic enzymes (Rahman and Punja, 2005).

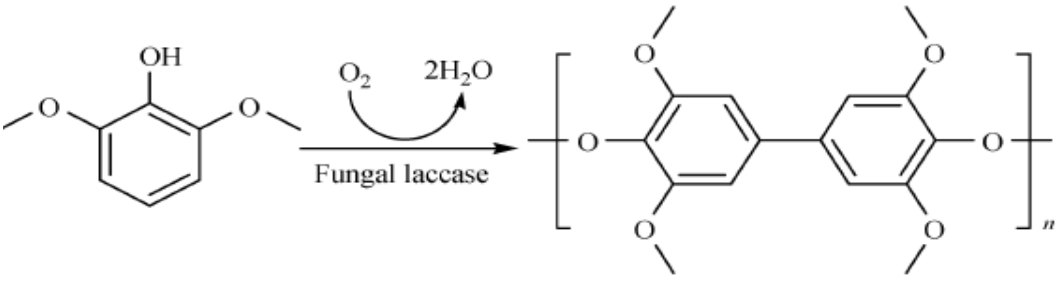
Therefore, the aim of this study was to confirm that *Cylindrocarpon* species secrete some of the plant cell wall degrading enzymes like laccase, protease and cellulase to identify genes encoding the enzymes and to investigate isolate variation in the activity of these enzymes.

5.2 Methods

5.2.1 Laccase production by *Cylindrocarpon* species

Laccase activity was measured using two substrates, ABTS (2,2[1]-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid; Sigma) and DMP (2,6-dimethoxy-phenol; Global) according to the method described by Vasconcelos *et al*. (2000). Oxidation of ABTS by laccase results in a green-blue coloured radical cation (ABTS•+) measurable at 420 nm (Figure 5.1), whereas for the oxidation of DMP by laccase results in 2,2',6,6'-tetramethoxydibenzo-1-1'-diquinone and was determined by increasing absorbance at 468nm.

A 

B 

DMP 2,2’,6,6’-tetramethoxydibenzo-1-1'-diquinone

Figure 5.1 Oxidation of A) ABTS by laccase results in a green–blue coloured radical cation (ABTS•+) and B) 2, 6-dimethoxy-phenol by fungal laccases results in forming 2,2',6,6'-tetramethoxydibenzo-1-1'-diquinone.

5.2.1.1 Culture production

A total of 17 isolates, comprising six isolates of *C. liriodendri* (Ack1b, Co1d, Co3b, Hb2a, Mar11b and Mar8i) and five isolates each of *C. destructans* (Ack2d, Co1c, Mar13a, Nel1d and Wpa1d) and *C. macrodidymum* (Ack1a, Co6a, Gis3b, Hb2b and Mar16i) were selected to represent a range of genetic groups (from the neighbour-joining tree, Figures 3.3, 3.5 and 3.7) and pathogenicity levels (Chapter 4). Isolate G121of *N. parvum* was also included as a positive control. For each isolate three 5 mm diameter mycelial colonized agar plugs from the growing edge of a 14 day old PDA plates was used to inoculate 25 mL of minimal medium (Appendix C.5.3) in 100 mL flasks and sealed by cotton wool plug replicated three times. Extra-cellular laccase production was induced by the addition of 119.7 µL veratryl alcohol (3, 4-dimethyozybenzyl alcohol, 96%; Sigma) to the minimal medium to achieve a final concentration of 30.4 mM, immediately prior to inoculating with the fungi (Vasconcelos *et al.*, 2000). All the flasks were incubated at 20°C on a rotary shaker (Chiltern Scientific) at 140 rpm under 12:12 h light:dark condition for 7 days.

5.2.1.2 Laccase enzymatic assay

After 7 days incubation, each liquid culture was transferred to a 50 mL tube and centrifuged at 12,500 × *g* for 30 min. The mycelium-free extracellular fluid was decanted and used as the extracellular enzyme extract for the enzyme assay. The activity towards ABTS, which is specific for PPO-1 (polyphenol oxidase-1) laccase, was determined at an acidic pH of 3.0 and a temperature of 50°C (Barbosa *et al*., 1996), while the activity against DMP, which is specific for PPO-Ⅱ laccase, was measured at pH of 6.5 and a temperature of 45°C (Vasconcelos *et al*., 2000).

For PPO-1 analysis McIlvaine's citrate-phosphate buffer was prepared based on McIlvaine's buffer system which is volumetrically set for pH values in a range from 2.2 to 8.0 (Appendix D.1.5). Twenty mL of buffer was prepared by mixing 4.11 mL of 0.2 M Na_2HPO_4 and 15.89 mL of 0.1 M citric acid to produce a solution with a pH of 3.0. The reaction mixture for PPO-1 assay was prepared with aliquots of 0.15 mL ABTS (40 mM in water), 0.15 mL McIlvaine's citrate-phosphate (60 mM, pH 3.0) and 0.7 mL of the extracellular fluid recovered from the inoculated flask cultures in a final volume of 1 mL and incubated at 50°C for 5 min. The absorbance measurement was made at 420 nm using a spectrophotometer (Model 6305; Jenway Ltd, England) at 420 nm.

For PPO-Ⅱ analysis McIlvaine's citrate-phosphate buffer was prepared by mixing 14.2 mL of 0.2 M Na_2HPO_4 and 5.8 mL of 0.1 M citric acid to produce a solution with a pH of 6.5. The reaction mixture of PPO-Ⅱ assay was prepared with aliquots of 0.15 mL aliquot of DMP (10 mM in water), 0.15 mL McIlvaine's citrate-phosphate buffer (170 mM, pH 6.5) and 0.7 mL of extracellular fluid recovered from the inoculated flask cultures were combined. After 5 min at 45°C, the absorbance measurement was made at 468 nm using a spectrophotometer.

One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of substrate per min. Laccase activity was quantified in units as µmol oxidised product formed min^{-1} mL^{-1} of enzyme solution for each substrate. Both types of laccase produced by the three *Cylindrocarpon* species were quantified and compared between the isolates. The laccase activity of the three *Cylindrocarpon* species were statistically analysed using ANOVA.

5.2.2 Design of degenerate primers for the laccase gene

The DNA sequences of the *lcc1* laccase gene from *Fusarium oxysporum* (GenBank accession EF990894), *Trichoderma* sp. (EU526310), *Magnaporthe oryzae* (XM001522482), *Myceliophthora thermophilia* (XM003659795), and *Nectria haematococca* (XM003050908) were retrieved from GenBank and aligned using DNAMAN (Version: 4.0a; Lynnon BioSoft). A forward and a reverse primer containing degenerate bases were designed to bind to conserved regions of the *lcc1* gene (Appendix D.2.1).

5.2.3 Degenerate PCR of the *Cylindrocarpon* laccase gene

Genomic DNA of six isolates, comprising two isolates each of *C. liriodendri* (Hb2a and Wpa1e), *C. destructans* (Mtb1d and Mar13a) and *C. macrodidymum* (Gis3b and Ack2g) were extracted as descirbed in Section 2.2.4. Each PCR reaction was conducted in a 25 µL reaction volume that contained 1× PCR buffer, 200 µM of each dNTP, 20 pmol of each primer,1.25 U Faststart *Taq* DNA polymerase (Roche Molecular Biochemicals, Germany) and 20 ng of DNA. Negative control tubes in which the DNA template was substituted with sterile water were included in each set of reactions to ensure they were free of contamination. Amplification was done in a Veriti 96 well Thermal cycler (Applied Biosystems, California, USA) using the following thermal cycle: 3 min at 94°C, then forty cycles of denaturation at 94°C for 30 s, annealing at either 58, 59 or 60°C for 45 s and extension at 72°C for 30 s, followed by final extension at 72°C for 7 min. PCR products (5 µL) were mixed with 3 µL loading dye (Section 2.2.4) and separated by electrophoresis in a 1% agarose gel as described in Section 2.2.4.

5.2.4 Sequencing of the amplified bands for the laccase

The amplified laccase bands were sequenced using the reverse laccase degenerate primer in the Lincoln University Sequencing Facility. The reverse sequences were submitted to a blastx search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank database to confirm their identity. The sequences from all the three species were aligned using the computer program DNAMAN version 4.0a (Lynnon Biosoft®) and specific primers (F1 and R579) designed (Appendix D.2.2).

5.2.5 Amplification and sequencing of the *Cylindrocarpon* laccase genes

The genomic DNA from a total of nine isolates used in the quantitative assay, comprising three each of *C. liriodendri* (Hb2a, Co1d and Mar11b), *C. destructans* (Mar13a, Co1c and Nel1d) and *C. macrodidymum* (Ack1a, Hb2b and Mar16i), which were amplified using the *Cylindrocarpon* laccase specific primers (F1 and R579). Each PCR reaction was conducted as described in Section 5.2.3, except that 10 pmol of each primer was used, with an annealing temperature of 58°C for 30 s. The PCR products were separated as described in Section 2.2.4.

The amplified laccase bands genes from all nine isolates were sequenced using the forward primer at the Lincoln University Sequencing Facility. The resultant sequences were aligned using DNAMAN version 4.0a. This programme was used to translate the DNA sequences and align the predicted amino acid sequences. The aligned sequences were annotated for characteristic copper binding regions Cbr1 and CbrII, putative glycosylation sites (Appendix D.2.3) and also for domain structures in the protein using blastp (http://www.ncbi.nlm.nih.gov/BLAST/) and alignment with *Melanocarpus albomyces* laccase for which the crystal structure is available (Hakulinen *et al.*, 2002).

5.2.5.1 Phylogenetic analysis of the laccase enzyme

A phylogenetic tree was produced in MEGA version 5.05 (Tamura *et al.*, 2011) using the predicted amino acid sequences of laccase from the three *Cylindrocarpon* species together with laccase from other plant pathogens, industrially important ascomycetes and basidiomycetes. Representatives of different members of the laccase family (*lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5*, *lcc9*) were included. The protein sequence of laccase from the bacterium *Bacillus subtilis* was used as an out-group. The aligned sequences were analysed to find the best model, using maximum likelihood with 500 bootstrap replications.

5.2.6 Pilot study of agars for protease plate assay

Two agars were compared for the ease of measurement. 1) Potato dextrose agar (PDA) supplemented with 5% skimmed milk (Dore, 2009) and 2) Czapek Dox agar (CDA) supplemented with 5% skimmed milk powder (Damare *et al.*, 2006). Three isolates each of *C. destructans* (Nel1d, Ack2d and Mar13a), *C. macrodidymum* (Ack1a, Gis3b and Hb2b) and *C. liriodendri* (Ack1b, Hb2a and Co3b) were randomly chosen from the different clades of neighbour joining tree. A 3 mm diameter mycelial plug was taken from the edge of 14 day old colony and placed on the middle of the agar plate. The isolates were replicated thrice and incubated at 20°C for 12:12 h light:dark condition for 7 days. Clearance zones produced around the fungal colonies indicated a positive reaction for protease production.

5.2.6.1 Quantitative analysis of protease production by Cylindrocarpon isolates

Protease activity was measured using azocasein as a substrate as described by Damare *et al*. (2006). For this study the same isolates used for laccase enzyme activity were selected which includes five isolates of *C. destructans* and *C. macrodidymum* and six isolates of *C. liriodendri* (Section 5.2.1.1). The 25 mL aliquots of Czapek Dox Broth (CDB, Sigma) containing 0.3% skimmed milk powder (Anchor) in 100 mL flasks were each inoculated with a 5 mm mycelial colonized agar plugs obtained from the growing edge of a 14 day old PDA culture. The flasks which were sealed with cotton wool plugs and incubated at 20°C on a rotary shaker (Chiltern Scientific) at 180 rpm under 12:12 h light:dark condition for 7 days.

After 7 days incubation, each liquid culture was transferred to 50 mL tube and centrifuged at 10,000 × *g* for 20 min. The supernatant containing mycelium-free extracellular fluid was decanted and used for the enzyme assay. Protease activity was measured by adding 150 µL of extracellular enzyme extract to 250 µL of the substrate azocasein at 2% (w/v) concentration prepared in 100 mM sodium acetate buffer, pH 5 (Sigma-Aldrich) and incubating at 20°C for 30 min. The reaction was stopped by addition of 1.2 mL of 10% trichloroacetic acid and the contents centrifuged at 12,500 × *g* for 10 min. To the supernatant, 1.4 mL of 1N NaOH was added and the absorbance read immediately at 440 nm using a spectrophotometer. One ACU (Azocasein Digestion Unit) was defined as the increase in absorbance by 0.001 min^{-1} under the assay condition (Hamamato *et al.*, 1995). The protease activity of the three *Cylindrocarpon* species were statistically analysed using ANOVA.

5.2.7 Design of degenerate primers for the acid protease gene

The DNA sequences of acid protease genes from six species of fungi, namely *Aspergillus fumigatus* (GenBank accession XM743526), *Neosartorya fischeri* (XM001259015), *Aspergilus nidulans* (XM675692), *Pencillium marneffei* (XM002147174), *Gibberella zeae* (XM388372) and *Sclerotinia sclerotiorum* acid protease (AF221843) were retrieved from GenBank and aligned using DNAMAN Version: 4.0a. Forward and reverse primer containing degenerate bases were designed to bind to conserved regions of the acid protease genes (Appendix D.4.1).

5.2.7.1 Degenerate PCR of the acid protease gene from Cylindrocarpon species

Genomic DNA from four isolates encompassing the three randomly selected *Cylindrocarpon* species (Ack1a, Mar13a, Mar11f and Nel1d) was used. Each PCR reaction contain reagents as described in Section 5.2.3 except that annealing was done at five different temperatures (61°C, 62°C, 63°C, 64°C and 65°C).PCR products (5 µL) were mixed with 3 µL loading dye

(Section 2.2.4) and separated by electrophoresis in a 1% agarose gel as described in Section 2.2.4. Since 64°C was most effective, the amplification was then repeated at 64°C using the degenerate primers F265 (forward) and R632 (reverse) for the five isolates of *C. destructans* and *C. macrodidymum* and also for the six isolates of *C. liriodendri* used in the quantitative assay.

The amplified bands were sequenced using the forward degenerate primer in the Lincoln University Sequencing Facility. The sequences were submitted to a blastx search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank database to confirm their identity. The sequences from all the three *Cylindrocarpon* species were aligned using the computer program DNAMAN version 4.0a and specific primers designed (Appendix D.4.2).

5.2.7.2 Amplification using specific primers and sequencing of acid protease genes from Cylindrocarpon species

For this study, four selected isolates from each of the three *Cylindrocarpon* species were those that produced high, medium and low protease activity in the quantitative assay (Section 5.2.7). Each PCR reaction was conducted as described in Section 5.2.3 except that the annealing temperature for the specific primers was at 66ºC.

The amplified bands from all 12 isolates were sequenced using the reverse primer at the Lincoln University Sequencing Facility. The resultant sequences were aligned using DNAMAN version 4.0a. This programme was used to translate the DNA sequences and align the predicted amino acid sequences. The aligned predicted amino acid sequences were analysed for polymorphism between isolates. The aligned sequences were annotated for the regions of domains present in the protein.

5.2.7.3 Phylogenetic analysis of the acid protease enzyme

A phylogenetic tree was produced in MEGA version 5.05 (Tamura *et al*., 2011) using the predicted amino acid sequences of proteases from the three *Cylindrocarpon* species together with acid proteases from other plant pathogens and basidiomycetes. Representatives of acid protease 1 and acid protease B were included. The protein sequence of alkaline protease from a *Fusarium* species (GenBank accession# AAC60571) was used as an out-group. The aligned sequences were analysed to find the best model, using maximum likelihood with 500 bootstrap replications.

5.2.8 Cellulase production by *Cylindrocarpon* species

For this study two media were chosen 1) Carboxy methyl cellulose agar medium (Pointing, 1999) and 2) Carboxy methyl cellulose congo red agar (Jo *et al.*, 2009). The carboxy methyl cellulose agar medium contained 4% w/v cellulose (Sigma) and 1.6% w/v agar. Incorporation of cellulose into solid agar media resulted in an opaque substrate due to the insolubility of the cellulose. The carboxy methyl cellulose (CMC) congo red agar medium was the same

medium except that 0.02% congo red was added. Four isolates each of *C. destructans* (Nel1d, Ack2d, Mar2c and Mar13a), *C. macrodidymum* (Ack1a, Gis3b, Co6e and Hb2b) and *C. liriodendri* (Ack1b, Hb2a, Mar11b and Co3b) were chosen from the different clades of neighbour joining tree. A 3 mm diameter mycelial plug was taken from the edges of 14 day old colony of the selected isolates, which were placed on the middle of the Petridish containing appropriate medium. The isolates were replicated thrice and incubated at 20°C for 12:12 h light:dark condition for 7 days. A clearance zone around the colony indicates production of a cellulase enzyme. An isolate of *Pythium* sp. (LUPP1177) and *Trichoderma* sp. (LUPP555) were included as positive controls.

5.2.8.1 Design of degenerate primers for the endo β-D-1, 4-glucanasegene

The DNA sequences of endo β-D-1, 4-glucanase gene from *Aspergillus nidulans* (AF420021), *Bispora sp.* (FJ695140)*, Pencillium sp.* (JF827297), *Neurospora crassa* (XM951338), *Neosartorya fischeri* (XM 001262789), *Penicillium marneffi* (XM 002152933), *Pyrenophora sp.* (XM 003300681) and *Thielavia terrestris* (XM 003653428) were retrieved from GenBank and aligned using DNAMAN version 4.0a. Forward and reverse degenerate primers were designed to conserved regions of the endo β-D-1, 4-glucanase (Appendix D.4.3).

5.2.8.2 Degenerate PCR for the endo β-D-1, 4-glucanasegene

For this experiment the genomic DNA of one isolate from each of the three *Cylindrocarpon* species (Mar14c, Mar5d and Mar10f) for provision of the genomic DNA extracted as described in Section 2.2.4.The PCR reaction was conducted as described in Section 5.2.3 except the annealing was done at 55°C and 56°C for 30 s. PCR products (5 µL) were mixed with 3 µL loading dye (Section 2.2.4) and separated by electrophoresis in a 1% agarose gel, using the same methodology described in Section 2.2.4.

5.2.8.3 Isolation of cellulase bands and reamplification by touch up PCR

Single bands were excised from 1% agarose gels of PCR products generated from Mar14c (*C. macrodidymum*), Mar5d (*C. destructans*) and Mar10f (*C. liriodendri*) by the degenerate primers using a sterile scalpel blade. The band was placed in a 1.7 mL sterile tube containing 10 µL of sterile water and the tube was placed on a block heater (Stuart®) to melt the agarose at 60°C for 5 min. The resuspended agarose containg the band/ sterile water mixture was used as a template and reamplified with the primers F605 and R1222 using touch up PCR. Each PCR reaction contained reagents as described in Section 5.2.3. Amplification was conducted in a Veriti 96 well Thermal cycler using the following thermal cycle: 3 min at 94°C, then forty cycles of denaturation at 94°C for 30 s, annealing at 54°C, 57°C and 58°C for 30 s and extension at 72°C for 30 s, followed by final extension at 72°C for 10 min. PCR products (5 µL) were mixed with 3 µL loading dye (Section 2.2.4) and

separated by electrophoresis in a 1% agarose gel, using the same methodology described in Section 2.2.4.

5.2.8.4 Sequencing

Single bands were sequenced using the F605 cellulase degenerate primer. The sequences were aligned using the computer program DNAMAN version 4.0a.

5.3 Results

5.3.1 Laccase activity by *Cylindrocarpon* species

All three *Cylindrocarpon* species produced measureable levels of both PPO-1 and PPO-II laccase. There was a significant species effect on the average amount of PPO-1 type laccase activity ($P<0.05$; Appendix D.1.1; Table 5.1) between the three *Cylindrocarpon* species, but the PPO-Ⅱ type laccase activity was not affected by species ($P>0.05$; Appendix D.1.2; Table 5.1). There was also an isolate effect ($P<0.001$) in the amounts of both the PPO-1 and PPO-II laccase activity ($P<0.001$ for both; Appendix D.1.3; D.1.4; Table 5.2).

For PPO-1, among the three *Cylindrocarpon* species, *C. macrodidymum* produced the highest activity with a mean of 0.835 U mL^{-1} of PPO-1 (Table 5.1) and three isolates produced the maximum measurable level of ≥1.000 U mL^{-1} (Table 5.2). The mean activity was five times greater for *C. macrodidymum* than for the other two *Cylindrocarpon* species. There was no difference in activity between *C. liriodendri*, *C. destructans* or the *N. parvum* (positive control). *Cylindrocarpon destructans* isolate Nel1d yielded the least laccase activity compared to the other isolates tested ($P<0.05$).

For PPO-Ⅱ type laccase there was no difference in mean activity between the three *Cylindrocarpon* species and the positive control, *N. parvum* ($P>0.05$). There was isolate variability in PPO-II laccase production ($P<0.05$) (Table 5.2). All species showed isolate variation although the greatest PPO-II laccase activity was for *C. liriodendri* isolate Hb2a (Table 5.2).

Table 5.1 Mean PPO-1 and PPO-Ⅱ type laccase activity (U mL^{-1}) by multiple isolates of three *Cylindrocarpon* species and one isolate of *Neofusicoccum parvum.*

Species	**PPO-1(U mL^{-1})**	**PPO-Ⅱ(U mL^{-1})**
C. destructans	0.147 b	0.025 a
C. liriodendri	0.156 b	0.027 a
C. macrodidymum	0.835 a	0.030 a
N. parvum	0.126 b	0.017 a
	P=0.000	P=0.928
LSD	0.1351	0.0324

Numbers in a column followed by different letters are significantly different from each other.

Table 5.2 Mean PPO-1 and PPO-Ⅱ type laccase activity (U mL^{-1}) produced by isolates of three *Cylindrocarpon* species and one isolate of *Neofusicoccum parvum.*

Species	Isolate	PPO-1(U mL^{-1})	PPO-Ⅱ(U mL^{-1})
C. destructans	Ack2d	0.173 b	0.051 b
	Co1c	0.134 c	0.000 d
	Mar13a	0.177 a	0.066 a
	Nel1d	0.072 d	0.002 c
	Wpa1d	0.178 a	0.003 c
C. liriodendri	Ack1b	0.114 e	0.010 c
	Co1d	0.149 d	0.000 d
	Co3b	0.197 b	0.028 b
	Hb2a	0.205 a	0.119 a
	Mar11b	0.108 f	0.000 d
	Mar8i	0.162 c	0.001 d
C. macrodidymum	Ack1a	1.000 a	0.038 b
	Co6a	1.000 a	0.054 a
	Gis3b	1.000 a	0.000 d
	Hb2b	0.766 b	0.028 c
	Mar16i	0.407 c	0.027 c
N. parvum	G121	0.125	0.017
		P =0.000	*P=0.000*
LSD		0.0010	0.0010

Numbers in a column for each species followed by different letters are significantly different from each other.

5.3.2 Amplification and sequencing of the laccase (*lcc1*) gene

The laccase degenerate primers F140 (5' GAR GCV AAC TGG GGH GAC A 3') and R880 (5' AKG KTG SWV CGC ATC CAG 3') (Appendix D.2.1) produced the expected 740 bp for two randomly selected isolates for each of the three *Cylindrocarpon* species at annealing temperatures of 58°C, 59°C and 60°C. For all isolates, additional fainter bands were produced of approximately 350 bp and 250 bp at 58°C and 60°C. At 59°C, only two bands were present, a strong 740 bp and a 400 bp band for *C. liriodendri* isolates and a single 740 bp band for *C. macrodidymum* and *C. destructans* (Figure 5.2).

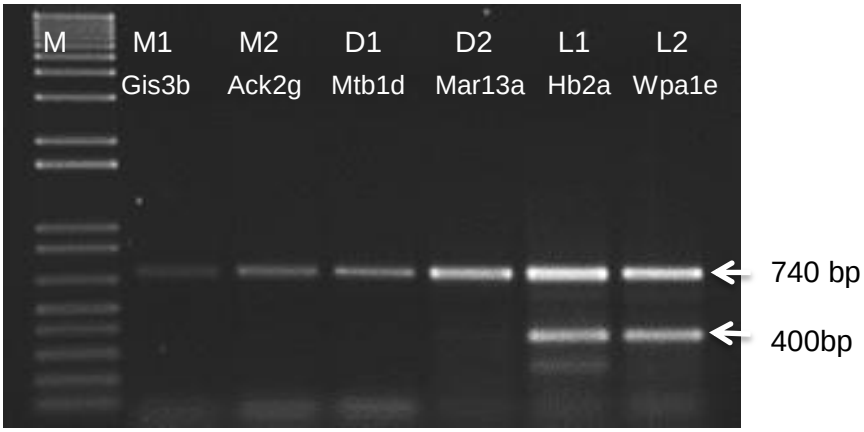


Figure 5.2 Agarose gel (1%) of the laccase gene amplified from representative *Cylindrocarpon macrodidymum* isolates (M1, M2), *C. destructans* (D1, D2) and *C. liriodendri* (L1, L2), and at 59°C using the laccase degenerate primers F140 and R880. M indicates 1Kb plus ladder. The arrows indicate 740 bp and 400 bp bands.

Sequencing of the band produced by the degenerate primers for each of the three *Cylindrocarpon* species resulted in 580 bp of usable sequence (Appendix D.2.2). Comparison of the 580 bp of sequence from *C. destructans*, *C. liriodendri* and *C. macrodidymum* isolates with the known sequences in the GenBank database revealed that they had 75% similarity to the *lcc1* gene of *Fusarium oxysporum* (GenBank accession# EF990894), 76% similarity to the *Fusarium proliferatum lac3* laccase gene (AF312321), 75% similarity to *Myceliophthora thermophila* ATCC 42464 laccase copper binding domain (XM003659795) and showed 80% similarity to *Trichoderma* species T01 laccase gene (EU526310).

5.3.2.1 Laccase specific primers for Cylindrocarpon species

The sequence generated by the degenerate primers led to design the laccase specific primers F1 (5' GAC ACC ATY CAR GTS ACC GT 3') and R579 (5' GGA CGA AGT CRT TGG CGA T 3') for *Cylindrocarpon* species (Appendix D.2.2). These primers produced a strong single band of 558 bp for all the nine isolates tested of each species (Figure 5.3).

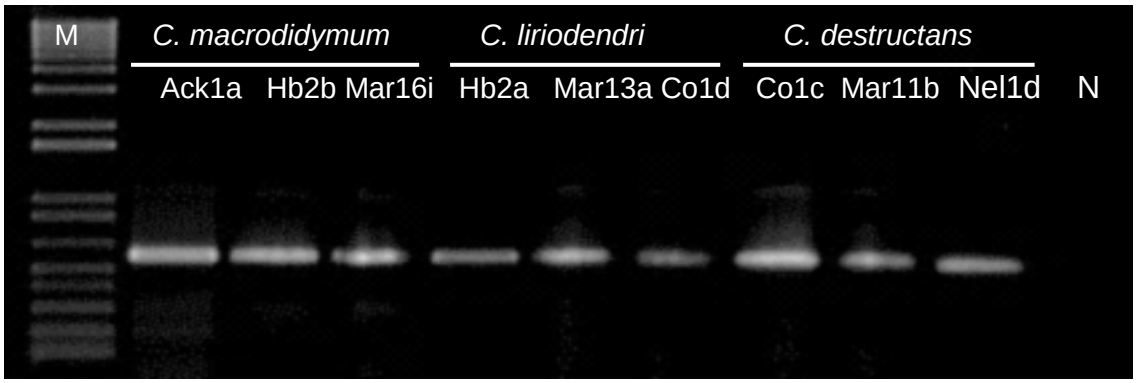


Figure 5.3 Agarose gel (1%) showing bands amplified from isolates of three *Cylindrocarpon* speciesusing the laccase specific primers F1 and R579 at 59°C. Each lane is denoted with the species and isolate names; M indicates1Kb plus ladder and N denotes negative control.

Sequencing of the 558 bp bands produced by using the laccase specific primer F1 for each of the three *Cylindrocarpon* species resulted in 519 bp of usable sequence. Comparison of these sequences from *C. destructans*, *C. liriodendri* and *C. macrodidymum* isolates with the known sequences in the GenBank database showed that they had a 75% similarity to *Fusarium oxysporum lcc1* gene (GenBank accession# ABS19938), 71% similarity to *Metarhizium acridum lcc1* gene (EFY87101) and 74% similarity to *Gibberella intermedia* laccase (AAK72901). The 519 bp was translated into a predicted 173 amino acid sequence. The alignment of the predicted amino acid sequence showed that there were polymorphic residues amongst the three *Cylindrocarpon* species and also between the high, medium and

low laccase activity isolates within the species and across species. The aligned amino acid sequences of *Cylindrocarpon macrodidymum*, *C. liriodendri* and *C. destructans* isolates showed 94.12% similarity. The aligned amino acid sequences showed conservation of the two copper binding regions located at positions aa1-7 and 41-50. A putative glycosylation site at position115-117 (NCS) was conserved (Appendix D.2.3). The aligned predicted amino acid sequence on standard protein blast showed that the protein was composed of multiple domains. They are 1) a Cu-oxidase 3 domain (pfam07732) (amino acids 1-68) and 2) a non-specific Cu-oxidase domain (amino acids 75-173). The aligned protein sequences of *Cylindrocarpon* species with that of *Melanocarpus albomyces* laccase (GenBank accession# IGWO_A) showed 88.32% similarity. Comparison with the known domains of *Melanocarpus albomyces* confirmed that the protein was composed of two cupredoxin-like domains in domain A (1-77) and domain B (78-173) which matched well to those indicated by blastp. The conserved amino acids are marked with asterisks; one helix in domain A and another one in domain B are shown in red and six β-strands highlighted with blue.

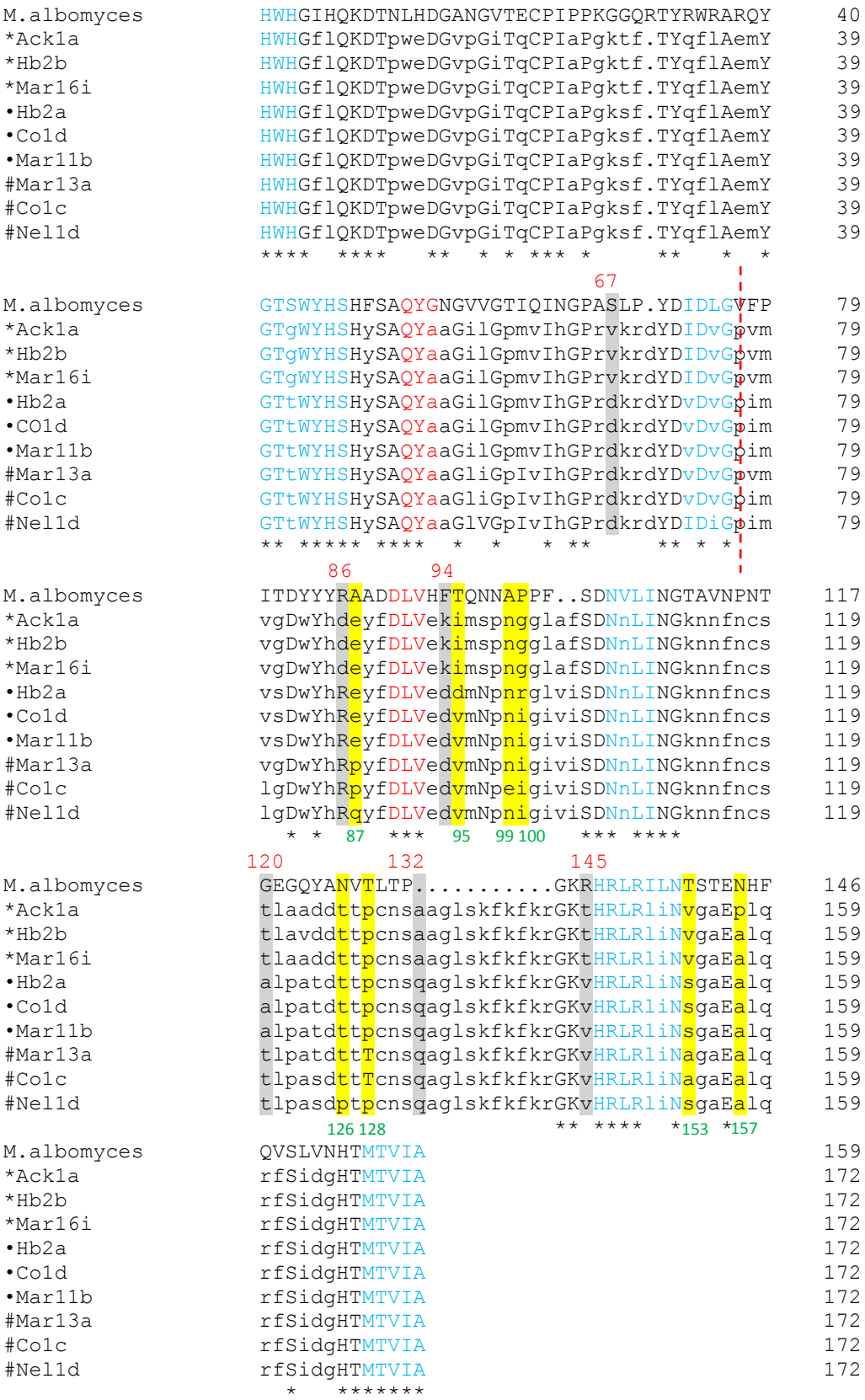


Figure 5.4 Alignments of multiple amino acid sequences of laccase from isolates of *Cylindrocarpon* species and *Melanocarpus albomyces* retrieved from Genbank. Amino acids (1-77) are the domain A and the remainder are in domain B. Conserved amino acids are marked with asterisks. Helices in red and β-strands in blue. The domain A containing red residue participate in copper binding at the trinuclear site and domain B contains blue residues participate in copper binding at the mononuclear, trinuclear and in substrate binding. Non-conservative changes in species were highlighted (grey) with numbers on top in red and non-conservative changes in isolates were highlighted (yellow) with numbers below in green. The symbol * indicates *C. macrodidymum* isolates, •*C.liriodendri* isolates and # *C. destructans* isolates.

Between isolates of the three species, there were a total of 31 amino acid differences in the translated sequences (Table 5.3). Of these, 15 were solely differences between the species and not between isolates of each species. The translated sequence of *C. liriodendri* isolates Co1d and Mar11b were identical to each other. In 11 out of those 15 substitutions (positions 29, 42, 67, 86, 94, 97, 103, 104, 122, 132 and 145) *C. destructans* and *C. liriodendri* were identical and *C. macrodidymum* differed. Of the 15 changes nine were conservative substitutions (positions 29, 42, 56, 60, 81, 97, 103, 104 and 122) in which the amino acids substitutions were to those with similar properties, for example the Δ29S→T where both serine (S) and threonine (T) have polar side chains. In six substitutions there were non-conservative changes (positions 67, 86, 94, 120, 132 and 145, Table 5.4) in which the amino acid substitutions were to those with dissimilar properties, for example, Δ67V→D where valine (V) has a hydrophobic side chain and aspartic acid (D) has an acidic side chain. Of the six non-conservative changes five (positions 67, 86, 94, 132 and 145) were between *C .macrodidymum* and the other two species.

Of the 16 substitutions in which there were differences between isolates two were in *C. macrodidymum* isolates (position 123 and 157), three in *C. liriodendri* isolates (positions 95, 100 and 102) and 11 in *C. destructans* isolates (positions 57, 73, 75, 78, 80, 87, 99, 124, 126, 128 and 153). Most (68%) of the *C. destructans* polymorphism was in isolate Nel1d. Of the 16 substitutions eight were conservative (positions 57, 73, 75, 78, 80, 102, 123 and 124) and eight were non-conservative (positions 87, 95, 99, 100, 126, 128, 153 and 157). Of the non-conservative substitutions, one was in *C. macrodidymum,* two in *C. liriodendri* and five in *C. destructans.*

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Table 5.3 Amino acid polymorphism in the translated sequences from isolates of *Cylindrocarpon macrodidymum*, *C. liriodendri* and *C. destructans* with high, medium and low laccase activity.

Amino acid polymorphism positions in *Cylindrocarpon* species																														
Isolate	**29**	**42**	**56**	**57**	**60**	**67**	**73**	**75**	**78**	**80**	**81**	**86**	**87**	**94**	**95**	**97**	**99**	**100**	**102**	**103**	**104**	**120**	**122**	**123**	**124**	**126**	**128**	**132**	**145**	**153**	**157**
Ack1a	**T**	**G**	I	**L**	M	**V**	**I**	V	**V**	V	G	**D**	E	**K**	**I**	**S**	N	**G**	**L**	**A**	**F**	T	**A**	A	**D**	T	P	**A**	**T**	**V**	**P**
Hb2b	**T**	**G**	I	**L**	M	**V**	**I**	V	**V**	V	G	**D**	E	**K**	**I**	**S**	N	**G**	**L**	**A**	**F**	T	**A**	**V**	**D**	T	P	**A**	**T**	**V**	A
Mar16i	**T**	**G**	I	**L**	M	**V**	**I**	V	**V**	V	G	**D**	E	**K**	**I**	**S**	N	**G**	**L**	**A**	**F**	T	**A**	A	**D**	T	P	**A**	**T**	**V**	A
Hb2a	S	T	I	**L**	M	D	V	V	I	V	**S**	R	E	D	**D**	N	N	**R**	**L**	V	I	**A**	P	A	T	T	P	Q	V	S	A
Co1d	S	T	I	**L**	M	D	V	V	I	V	**S**	R	E	D	V	N	N	I	I	V	I	**A**	P	A	T	T	P	Q	V	S	A
Mar11b	S	T	I	**L**	M	D	V	V	I	V	**S**	R	E	D	V	N	N	I	I	V	I	**A**	P	A	T	T	P	Q	V	S	A
Mar13a	S	T	**L**	**I**	**I**	D	V	V	**V**	V	G	R	**P**	D	V	N	N	I	I	V	I	T	P	A	T	T	**T**	Q	V	**A**	A
Co1c	S	T	**L**	**I**	**I**	D	V	V	I	**L**	G	R	**P**	D	V	N	**E**	I	I	V	I	T	P	A	**S**	T	**T**	Q	V	**A**	A
Nel1d	S	T	**L**	**V**	**I**	D	**I**	**I**	I	**L**	G	R	**Q**	D	V	N	N	I	I	V	I	T	P	A	**S**	**P**	P	Q	V	S	A

***Cylindrocarpon macrodidymum* isolates**; ***C. liriodendri* isolates**; ***C. destructans* isolates**.The laccase activity of the isolates is in descending order with the isolate with the highest activity at the top. Amino acid differences soley between species are shaded, residues that show isolate variation are not shaded. Non-conservative substitutions are shown in red.The letters in the column 29-157 denotes the amino acids. Amino acid with polar residues (T – Threonine, S – Serine, G – Glycine,Q – Glutamine, N – Asparagine). Non-polar residues (I – Isoleucine, L – Leucine, V – Valine, M – Methionine, A – Alanine, F – Phenylalanine, P – Proline (α-imino acid).Acidic (D – Aspartic acid, E – Glutamic acid). Basic (R – Arginine, K – Lysine).

5.3.2.2 Phylogenetic analysis of the laccase enzyme

A phylogenetic tree was produced for the laccase enzyme. The best model was analysed using the MEGA version 5.05 and found to be WAG with gamma distributed and invariant sites substitution model (WAG+G+I model). The neighbour joining tree produced 5 major clades (Figure 5.5).

The *Cylindrocarpon* species grouped together in one branch and each species formed a separate sub branch (Figure 5.5). The *Cylindrocarpon* species clustered with the *lcc1* enzyme of other ascomycetes. For several of the laccase sequences from saprophytes and pathogens the specific laccase type was not designated in GenBank. The grapevine pathogen, *Lasiodiplodia theobromae* shared the clade with *Cylindrocarpon* species. The basidiomycetes were placed in a separate group. The *lcc2* genes shared the same clade as the *lcc4* genes. The *lcc5* genes formed their own group. The *lcc3* and *lcc9* genes shared a clade.

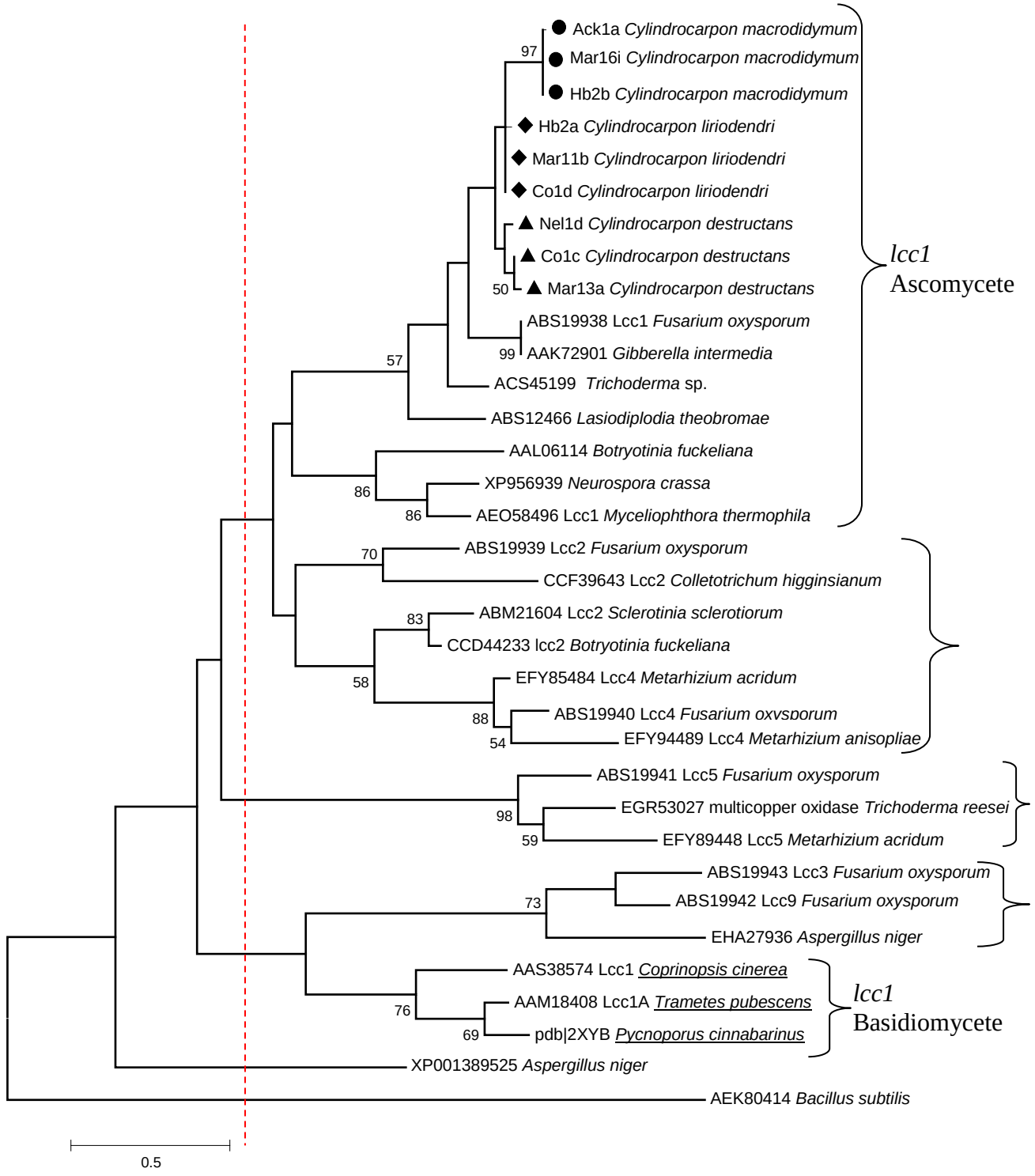

Figure 5.5 Neighbour joining tree produced for the laccase enzyme of *Cylindrocarpon* species and other ascomycetes and basidiomycetes, with the out-group *Bacillus subtilis*, using the best protein model with MEGA version 5.05. The distance bar represents the Kimura distance. Bootstrap values greater than 50% are indicated at branch nodes.

5.3.3 Qualitative analysis of protease production

A zone of clearance was produced on both media for the same isolates of *C. destructans* and *C. liriodendri*. Isolates of *C. macrodidymum* did not produce any zone of clearance. The zone of clearance was more distinct using CDA and easier to measure due to the greater clarity (Figure 5.6). For some isolates the radius of clearance zone was small, but the clarity of the clearance zone was very high. For example, *C. liriodendri* isolate Hb2a (Figure 5.6C) produced a small zone of clearance in CDA, but the transparency was much higher than on PDA (Figure 5.6B). CDA supplemented with 5% skim milk powder was chosen as the best agar for the protease assay.

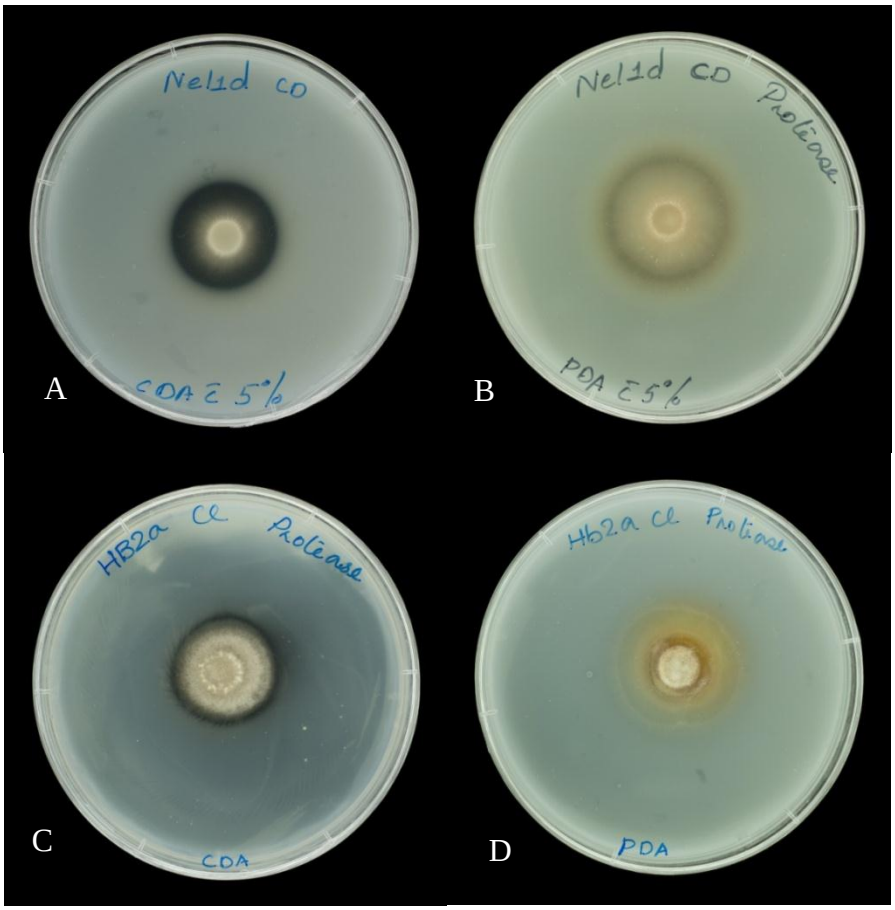

Figure 5.6 The zone of clearance on two media (A) *C. destructans* isolate Nel1d on CDA with 5% skim milk (B) *C. destructans* isolate Nel1d on PDA with 5% skim milk. (C) *C. liriodendri* isolates Hb2a on CDA with 5% skim milk (D) *C. liriodendri* isolates Hb2a on PDA with 5% skim milk.

5.3.4 Quantification of protease production by *Cylindrocarpon* species

There was a species effect (P=0.001) on the protease activity, with *C. liriodendri* having lower activity than *C. destructans* and *C. macrodidymum* (P<0.05; Appendix D.3.1), which were similar (P>0.05; Table 5.4).There was also an isolates effect (P<0.05; Table 5.5; Appendix D.3.2) on protease activity, which were apparent between isolates of all species.

Table 5.4 Mean protease activity of the three *Cylindrocarpon* species towards the substrate azocasein measured in ACU min^{-1} (Azocaesin Digestion Units).

Species	Protease activity (ACU min^{-1})
C. liriodendri	0.117 b
C. destructans	0.146 a
C. macrodidymum	0.145 a
	P=0.000
LSD	0.0245

Numbers in a column followed by different letters are significantly different from each other.

Table 5.5 Mean protease activity of isolates of each of three *Cylindrocarpon* species towards substrate azocasein measured in ACU min^{-1} (Azocaesin Digestion Units).

Species	Isolates	Protease activity (ACU min^{-1})
C. liriodendri	Ack1b	0.101 c
	Co1d	0.147 a
	Co3b	0.092 d
	Hb2a	0.139 b
	Mar11b	0.147 a
	Mar8i	0.074 e
C. destructans	Ack2d	0.112 d
	Co1c	0.133 c
	Mar13a	0.154 b
	Nel1d	0.166 a
	Wpa1d	0.165 a
C. macrodidymum	Ack1a	0.163 a
	Co6a	0.111 c
	Gis3b	0.145 b
	Hb2b	0.164 a
	Mar16i	0.143 b
		P=0.000
LSD		0.0017

For each species, numbers in a column for each species followed by different letters are significantly different from each other.

5.3.5 Isolation of the gene encoding acid protease using degenerate PCR

Two forward degenerate primers (F172 and F265) and two reverse degenerate primers (R557 and R632) were designed to bind to conserved areas of the genes encoding acid protease (Table 5.6). The expected band sizes generated by these primers ranged from 292 bp to 460 bp.

Table 5.6 The sequences and Tm of the degenerate primers designed to bind the conserved areas of gene encoding acid protease.

Primer name	Sequence 5' - 3'	Tm
F172	5' AAC TGG KCY GGW GCH GTS C 3'	63°C
F265	5' GCY TGG GTY GGY MTY GAY GG 3'	66°C
R557	5' TC RGC RTT RKW CTC GCA VAG 3'	62°C
R632	5' CY SST RAA KGT VAC RGT GCC 3'	61°C

Only degenerate primers F265 and R632 amplified a band in all four isolates tested at all six annealing temperatures tested. The optimal annealing temperature was 64°C and at this temperature, primers F265 and R632 produced the expected 367 bp product (Figure 5.7), although isolates Mar13a and Nel1d also had fainter bands at lower molecular weights apparent on the agarose gel. Primer combination F172 and R557 produced multiple bands in two isolates which were larger than the expected 385 bp product. Primer combination F172 and R632 produced the expected 460 bp product only for isolate Mar13a, and primer combination, F265 and R557 produced multiple bands in two isolates.

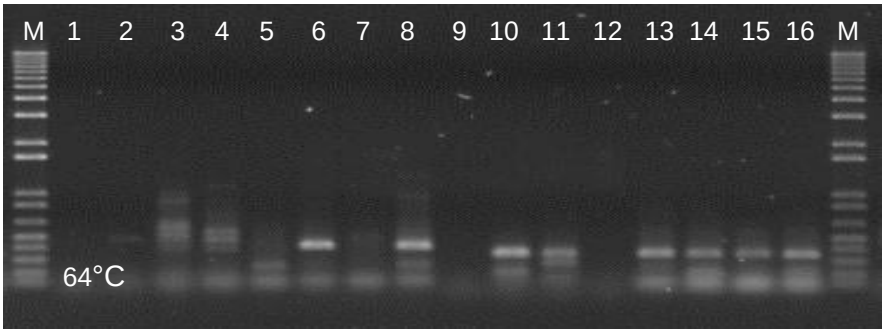

Figure 5.7 Agarose gel (1%) showing the bands produced by *Cylindrocarpon macrodidymum* isolate (Ack1a), *C. destructans* (Mar13a and Nel1d) and *C. liriodendri* (Mar11b) at an annealing temperature of 64°C using four different pairs of degenerate primers designed to amplify the acid protease gene. Lanes 1-4 amplified with primers F172 and R557, 5-8 is amlified with primer F172 and R632, 9-12 is with the primer F265 and R557, 13-16 is amplified using the primer F265 and R632. The letter M denotes the 1Kb plus ladder.

All isolates of *Cylindrocarpon* species used in the quantitative analysis of protease activity produced amplimers using primers F265 and R632 at an annealing temperature of 64°C (Figure 5.8) except *C. macrodidymum* isolate Gis3b (Figure 5.8B 4) and *C. destructans* isolate Mar13a which was not amplified due to low DNA concentration (Figure 5.8C 4), but was previously amplified (Figure 5.7 lane -14) producing a band of the expected size (367 bp).

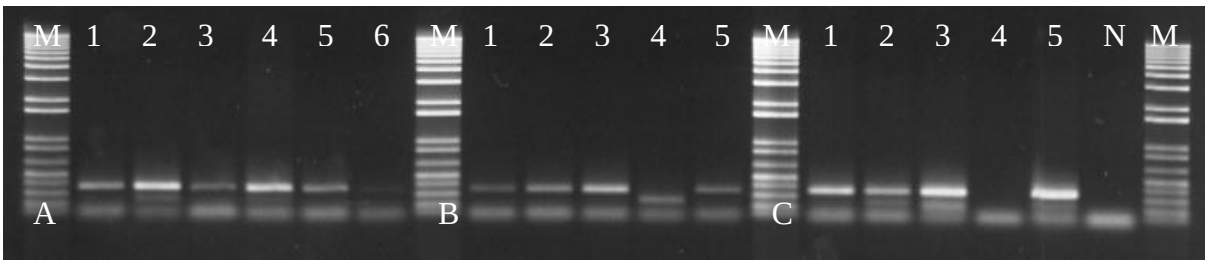

Figure 5.8 Agarose gel (1%) showing the bands produced by (A) *C. liriodendri* (Mar8i, Ack1b, Mar11b, Co3b, Hb2a and Co1d) (B) *C. macrodidymum* (Hb2b, Co6a, Mar16i, Gis3b and Ack1a) and (C) *C.destructans* (Wpa1d, Ack2d, Nel1d, Mar13a and Co1c) amplified with the primers F265 and R632. The letter M denotes the 1Kb plus ladder and N denotes the negative.

Direct sequencing of the amplimers produced by primers F265 and R632 produced 258 bp of unambiguous sequence. Comparison of the 258 bp translated nucleotide fragment from isolates of *C. liriodendri*, *C. macrodidymum* and *C. destructans* with the known sequences in the GenBank database revealed an identity (88%) to hypothetical protein FOXB 13593 of *F. oxysporum* Fo5176 (Genbank accession# EGU75890), (73%) identity to peptidase A4 family protein of *Glomerella graminicola* M1.001 (Genbank accession# EFQ27406) and 52% identity of *Scl. sclerotiorum*, acid protease partial (Genbank accession# AEY84333).

Using the sequence information a protease specific reverse primer (proteaseR1; 5' AAT GTG ACG GTG CCA AAG TCG 3') was designed for the three *Cylindrocarpon* species to improve the amplification consistency and sequencing. This primer combination with F265 produced a 319 bp band in all 16 isolates used in the quantitative analysis (Figure 5.9).

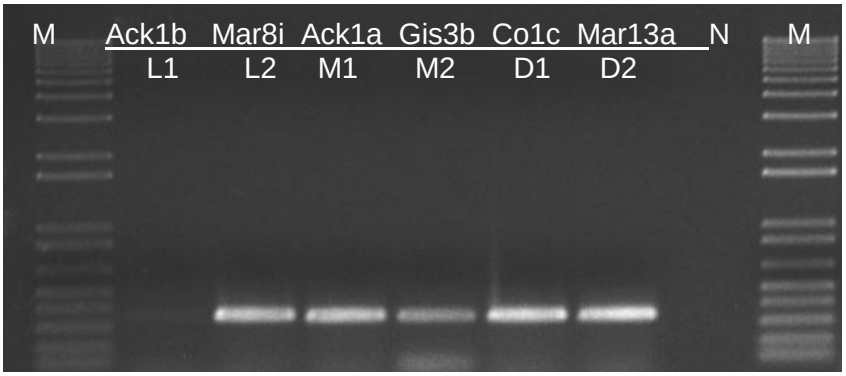

Figure 5.9 Agarose gel (1%) showing the 319 bp bands amplified by the representative isolates from the quantitative analysis by *C. liriodendri* (Ack1b, Mar8i), *C. macrodidymum* (Ack1a, Gis3b) and *C. destructans* (Co1c, Mar13a) using primers F265 and proteaseR1. The letter M denotes the 1Kb plus ladder and N denotes the negative. The absence of band in the Ack1b is due to the less concentration of DNA.

5.3.5.1 Protein sequence analysis of acid protease gene from isolates of each Cylindrocarpon species producing high and low protease activity

Sequence analysis of 319 bp bands produced from each of the isolates produced 280 bp of unambiguous sequence. Each DNA sequence was translated into a putative protein sequence of 85 amino acids. The amino acid sequence was polymorphic (97.65% similarity) among the three *Cylindrocarpon* species, but was not polymorphic within each species (Table 5.7).

Table 5.7 Amino acid polymorphism in the protein sequence of isolates of the three *Cylindrocarpon* species producing high and low protease activity

		Amino acid position					
Species	**Isolates**	**27**	**28**	**58**	**61**	**66**	**72**
C. macrodidymum	Hb2b[3]	A	H	E	T	I	S
C. macrodidymum	Ack1a[4]	A	H	E	T	I	S
C. macrodidymum	Mar16i[7]	A	H	E	T	I	S
C. macrodidymum	Co6a[10]	A	H	E	T	I	S
C. liriodendri	Mar11b[5]	A	Y	Q	S	V	N
C. liriodendri	Co1d[6]	A	Y	Q	S	V	N
C. liriodendri	Co3b[11]	A	Y	Q	S	V	N
C. liriodendri	Mar8i[12]	A	Y	Q	S	V	N
C. destructans	Nel1d[1]	S	Y	Q	S	V	N
C. destructans	Wpa1d[2]	S	Y	Q	S	V	N
C. destructans	Co1c[8]	S	Y	Q	S	V	N
C. destructans	Ack2d[9]	S	Y	Q	S	V	N

The numbers[1-12] indicates isolates producing high to low protease activity. The letters in the columns 27 – 72 denotes the amino acids. The amino acid with polar residues (S – Serine, Y – Tyrosine, Q – Glutamine, T – Threonine, N – Asparagine. Non-polar residues are (A – Alanine, I – Isoleucine, V – Valine. H – Histidine (basic), E – Glutamic acid (acidic).

5.3.5.2 Phylogenetic analysis of the acid protease enzyme

A phylogenetic tree was produced for the acid protease enzyme. The best model was found to be the WAG model. The neighbour joining tree produced two major clades (Figure 5.10). The *Cylindrocarpon* species grouped together in one branch, with each species i.e. *Cylindrocarpon macrodidymum*, *C. liriodendri* and *C.destructans* forming a separate sub clade (Figure 5.10). The acid protease enzyme analysis caused the *Cylindrocarpon* species to cluster with the acid protease enzyme of other ascomycetes. The basidiomycetes were placed in a separate clade.

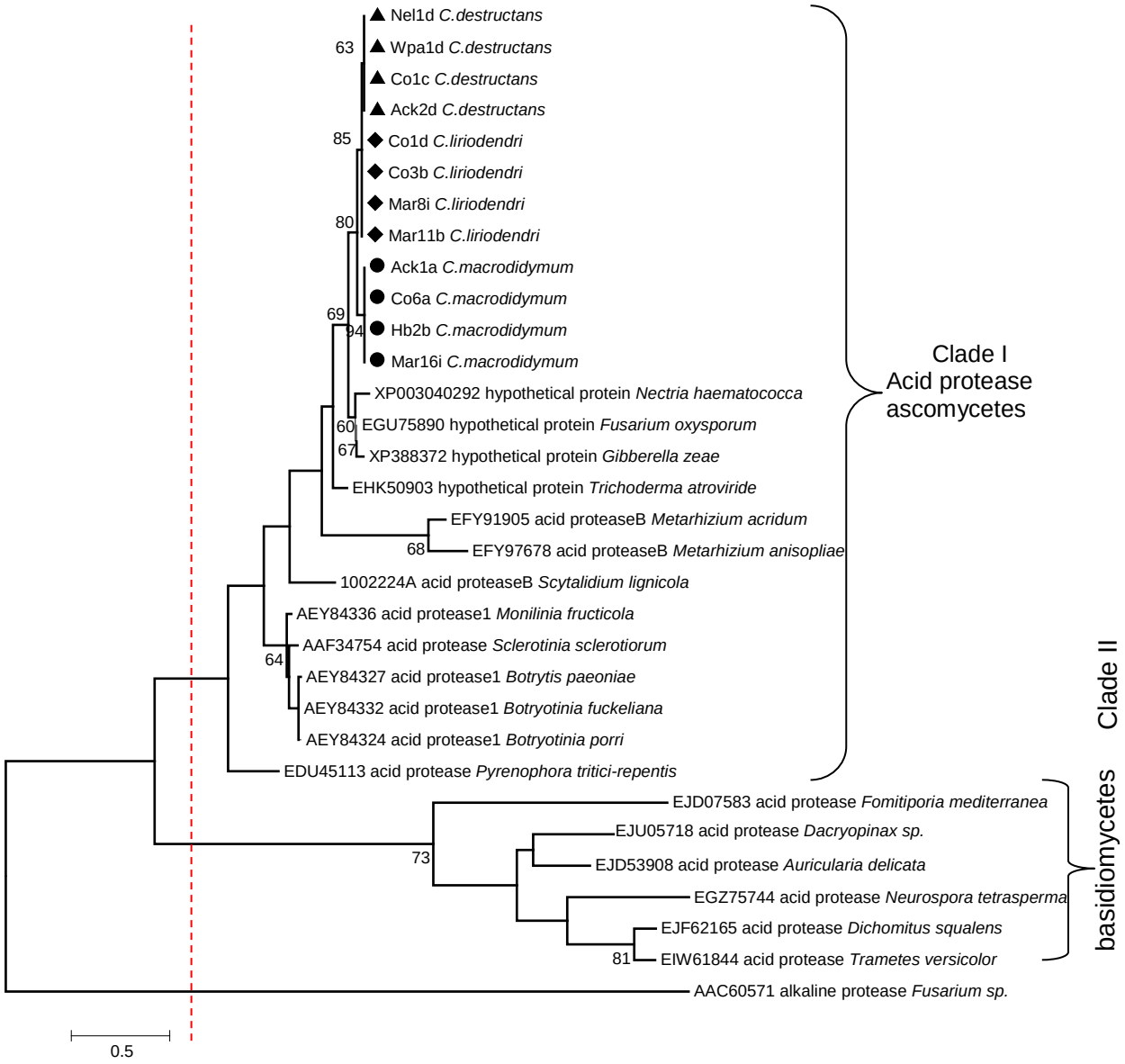

Figure 5.10 Neighbour joining tree produced for the acid protease enzyme of *Cylindrocarpon* species and other ascomycetes and basidiomycetes, with the alkaline protease enzyme of *Fusarium* species as an out-group, using the best protein model with MEGA version 5.05. The distance bar represents the Kimura distance. Bootstrap values greater than 50% are indicated at branch nodes.

5.3.6 Qualitative analysis of cellulase production

Cellulase was not detected for the three *Cylindrocarpon* species or the two positive controls, *Trichoderma* sp. and *Pythium* sp. (Figure 5.11A, B, C and D).

* This experiment repeated twice. However, the continued earthqaukes disrupted this experiment meaning that no useful data was collected.

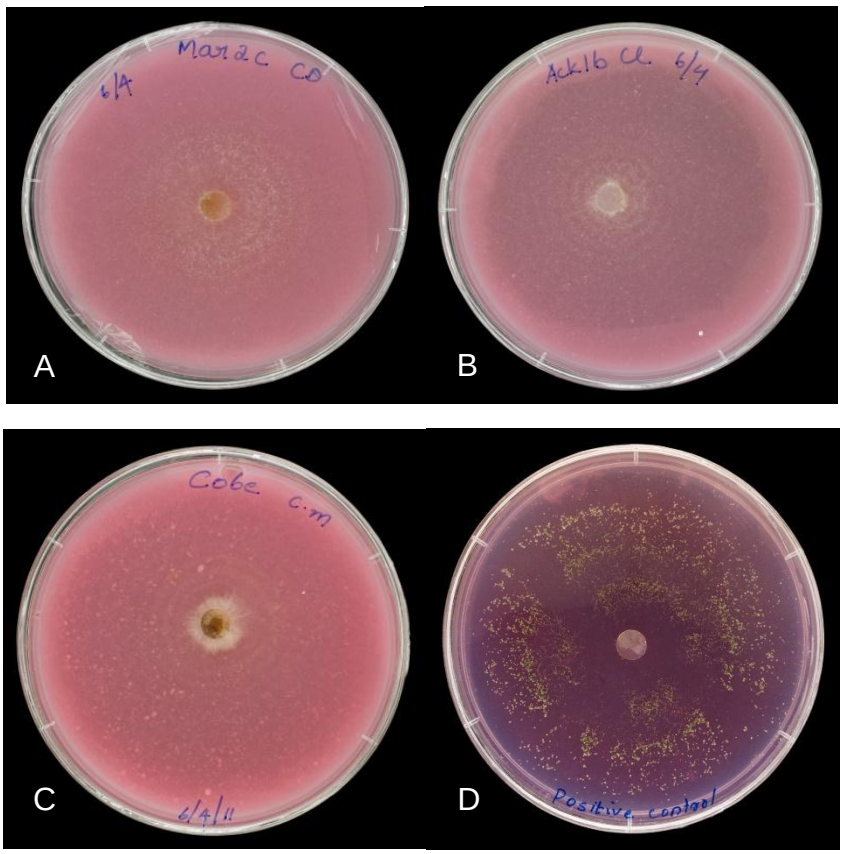

Figure 5.11 Carboxy methyl cellulase media that incoprated congo red dye with no zone of clearance around the inoculated colonies (A) *C.destructans,* (B) *C. liriodendri,* (C) *C. macrodidymum* or (D) the positive control *Trichoderma* sp.

5.3.7 Isolation of the gene encoding endo β-D-1, 4-glucanase

Two forward and two reverse degenerate primers that were designed to anneal to conserved areas of the endo β-D-1, 4-glucanase genes are shown in Table 5.8 and the entire sequence with primer regions in Appendix D.4.3. The expected band size with the primer combination F518 and R1222 was 704 bp, for F605 and R1222 it was 617 bp and for primers F605 and R1378 it was 773 bp.

Table 5.8 The sequence of the cellulase degenerate primers designed to bind the conserved areas of gene encoding endo β-D-1, 4-glucanase.

Primer name	Sequence 5' - 3'	Tm
F518	5' IRW RAA CGG IGC BYT ITA 3'	54°C
F605	5' BTA YTG YGA YGC BCA RTG 3'	54°C
Rev1222	5' TIA WIR YIR GIA CCA TIC C 3'	57°C
Rev1378	5' TDS WDC CVA TMT CIC CCC A 3'	59°C

5.3.8 Specificity of cellulase degenerate primers

The cellulase degenerate primers amplified multiple bands in the three isolates from each of the *Cylindrocarpon* species tested (Figure 5.12).

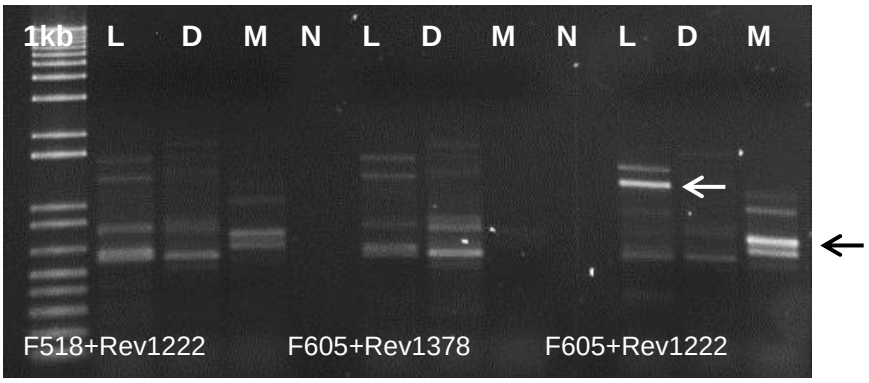

Figure 5.12 Agarose gel (1%) showing bands from amplification of *Cylindrocarpon* isolates using different cellulase degenerate primer combination. L, D, M represents *C. liriodendri* isolate (L), *C. destructans* (D), *C. macrodidymum* isolate (M) and Negative (N). 1kb denotes 1Kb plus ladder.

5.3.8.1 Amplification of the excised bands using touch up PCR

Reamplification of the bands excised from amplimers generated by primers F605 and R1222 (annotated using arrows in Figure 5.12) using touch up PCR produced a single band for *C. macrodidymum* isolate Mar14c of approximately 700 bp (Figure 5.13-1). Single bands were not able to be reamplified for isolates Mar10f and Mar5d.

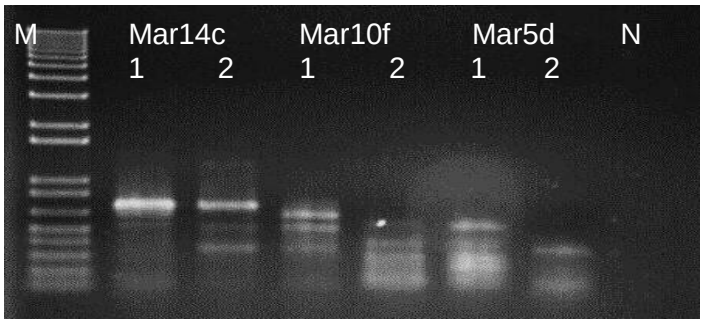

Figure 5.13 Agarose gel (1%) showing the amplification of excised bands from *C. macrodidymum* isolate (Mar14c), *C. liriodendri* (Mar10f) and *C. destructans* (Mar5d). The letter M denotes the 1Kb plus ladder and N denotes the negative control.

5.3.8.2 Sequencing analysis

Sequencing of the band from *C. macrodidymum* isolate Mar14c resulted in 200 bp of usable sequence. Comparison of this sequence with the known sequences in the GenBank database revealed that they had 53% similarity to the cellulose synthase catalytic subunit of *Verticillium albo-atrum VaMs.102* (GenBank accession# XP002999868), 40% similarity to the *Aspergillus niger* cellulose synthase gene (XP001390453), 35% identity to the *Pyrenophora*

tritici-repentis cellulose synthase catalytic subunit (XP001940444) and showed 73% similarity to the glycosyl transferase gene of *Nectria haematococca* (XP0030435830).

5.3.9 Relationship between virulence and enzyme activity by *Cylindrocarpon* species

This research showed that there was a relationship between laccase enzyme activity and virulence by the three *Cylindrocarpon* species. However, no relationship was found for protease enzyme activity (Table 5.9). For *C. macrodidymum*, the isolates Ack1a, Co6a and Gis3b isolates produced high levels of laccase activity and these isolates were relatively high virulent in detached root assay (Chapter 4). The isolate Hb2b produced medium level of laccase activity and this isolate was relatively medium virulent in potted vine assay (Chapter 4; Table 4.9). For *C. destructans*, the isolate Mar13a produced high level of laccase activity and this isolate was relatively high virulent in detached root assay. The isolate Nel1d produced low laccase activity and this isolate was relatively low virulent in potted vine assay. For *C. liriodendri*, the isolate Hb2a produced high level laccase activity and this isolate was relatively high virulent in detached root assay.

Table 5.9 Relationship between virulence and enzyme activity in three *Cylindrocarpon* species isolates.

Isolate	Genetic group	Virulence		Laccase (PPO-1)	Protease
		Detached root	Potted vine assay		
*Ack1a	I	high virulent	-	1.00a	0.16a
*Co6a	III	high virulent	-	1.00a	0.11c
*Gis3b	IV	high virulent	high virulent	1.00a	0.15b
*Hb2b	V	-	medium virulent	0.77b	0.16a
*Mar16i	III	low virulent	-	0.41c	0.14b
#Wpa1d	II	-	-	0.18a	0.17a
#Mar13a	III	high virulent	-	0.18a	0.15b
#Ack2d	I	medium virulent	medium virulent	0.17b	0.11d
#Co1c	II	low virulent	low virulent	0.13c	0.13c
#Nel1d	II	-	low virulent	0.07d	0.17a
•Hb2a	I	high virulent	-	0.21a	0.14b
•Co3b	III	-	-	0.19b	0.09d
•Mar8i	I	-	medium virulent	0.16c	0.07e
•Co1d	I	medium virulent	-	0.15d	0.15a
•Ack1b	I	medium virulent	medium virulent	0.11e	0.10c
•Mar11b	II	-	-	0.10f	0.15a

**C.macrodidymum* isolates, #*C. destructans*isolates, •*C. liriodendri* isolates. – indicates isolates not used in those experiments. The letters after the decimal indicates high to low enzyme activity. The isolates were ranked from high to low virulent.

5.4 Discussion

The aim of this chapter was to measure activity of some plant cell wall degrading enzymes that may have a role in virulence of *Cylindrocarpon* species (Chapters 3 and 4) to determine whether there were differences in enzyme activity between the species and/or isolates. The biochemical analyses were accompanied by gene discovery of the genes encoding these enzymes and to determine whether any predicted amino acid sequence variation correlated with measured differences in enzyme activity. The results showed that all three *Cylindrocarpon* species analysed produced laccase and acid protease although there was substantial variation in the activity measured amongst isolates and species. Subsequent isolation of the genes encoding these enzymes showed predicted variation in amino acid sequence between species but only the laccase was variable between isolates. This is the first report on laccase production by *Cylindrocarpon* species.

The three *Cylindrocarpon* species were shown to produce both type of laccase activity (PPO-1 and PPO-II) and that the amount of activity produced varied between species and isolates, there was relationship between pathogenicity and PPO-1 laccase activity (Table 5.9). Polyphenol oxidase-1 and 2 (PPO-1 and PPO-II) are two extracellular laccase enzymes. Another grapevine pathogen *Neofusicoccum parvum* (a botryosphaeriaceous species) was also shown to produce both PPO-1 and PPO-II laccase and the amount varied between isolates, but there was no correlation between pathogenicity (Baskarathevan, 2011). In that study the activity of PPO-1 and PPO-II were measured using the same assay described here. In another study botryosphaeriaceous species isolated from eucalyptus stem canker also produced laccase *in vitro* (Barbosa *et al*., 1996). The laccase produced by botryosphaeriaceous species is likely to be involved in pathogenesis by this species, as it is known to degrade plant cells.

Multiple laccase genes have been identified in other soil-borne pathogens. For example *Rhizoctonia solani,* was shown to have four laccase genes (Wahleithner *et al*., 1996). Similarly, *Gaeumannomyces graminis* var. *tritici* was shown to have three laccase genes. A study on the vascular wilt fungus, *Fusarium oxysporum* identified six laccase genes, *lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5* and *lcc9* and among these genes *lcc1*, *lcc3* and *lcc9* were expressed in roots and stems during the infection process (Cañero and Roncero, 2008). The laccase assay used for this study is similar to those used in other studies of botryosphaeriaceous species (Barbosa *et al.,* 1996; Vasconcelos *et al*., 2001; Baskarathevan, 2011). However, there were some modifications made for the analysis of *Cylindrocarpon* species. For example, the incubation temperature used for growth of *N. parvum* was 28°C (Basakarathevan, 2011), whereas, 20°C was used for *Cylindrocarpon* species as Chapter 2 showed that the optimum temperature for growth of *Cylindrocarpon* species isolates ranged between 18 and 20°C, and no growth was observed at 30°C. The medium was also modified

slightly from Vogel's minimum salt medium and glucose (1% w/v) (Vasconcelos *et al*., 2001; Basakarathevan, 2011) to the minimal medium used for growth in the transformation studies (Chapter 4).

In this study, the activity of the extracellular laccases produced by the three *Cylindrocarpon* species differed. The difference in activity may be due to different amounts of the enzyme being secreted extracellularly or by the production of enzymes with different efficiencies. Interestingly, *C. macrodidymum* produced a much higher level (in some cases greater than 10 times) of activity for extracellular laccase (PPO-1) when compared to the other species. Variation in activity between species of the same genus with similar modes of infection has been reported previously. Vasconcelos *et al*. (2001) showed variation in the level of laccase activity between three botryosphaeriaceous species isolated from eucalyptus; with *Botryosphaeria* sp. isolate MAMB-5 having the highest level of PPO-1 laccase activity compared to *N. ribis* and *L. theobromae*. In addition, their study showed that PPO-1 activity was always higher than PPO-II and they showed that PPO-II was likely to be produced intracellularly by these species. Another study by Baskarathevan (2011) on *N. parvum* isolated from New Zealand grapevines also showed that the PPO-1 activity was higher than PPO-II type laccase.

If the different levels of laccase activity between the three species was due to different levels of production and secretion this may not necessarily be because *C. macrodidymum* produces more PPO-1 laccase during infection. It could also be explained by differences in the substances required to induce the expression of the genes encoding these enzymes for each fungal species. Vasconcelos *et al.* (2001) reported that the laccase activity differed depending of presence or absence of veratryl alcohol. They showed that veratryl alcohol in the media stimulated the production of PPO-1 and PPO-II type laccase in botryosphaeriaceous species. They also stated that veratryl alcohol can cause some changes in cell membrane structure and properties which leads to the secretion of laccase into the medium. Similarly, in *Cylindrocarpon* species, the addition of veratryl alcohol stimulated the secretion of PPO-1 and PPO-II type laccase into the media, but the degree of response may differ between isolates and species. Other studies have shown that the production of laccase can be stimulated by the presence of other compounds, for example, extracellular laccase was induced by the activity of copper ions in *Botryosphaeria rhodina* (Dekker *et al.*, 2007). Similarly, Vázquez-Garcidueñas *et al*. (2012) reported that the production of laccase was significantly higher in *Botryosphaeria* strains in the presence of copper sulfate and ethanol. Crowe and Olsson (2001) reported that *Rhizoctonia solani* showed increased activity when exposed to isopropanol and ethanol, which destabilise the cell membrane and induces the release of extracellular laccase into an aqueous medium. Further, Fortina *et al*. (1996) reported that the production of laccase enzyme was enhanced

in *Botrytis cinerea* using gallic acid. In basidiomycetes, ferulic acid, 2,5-xylidine, syringaldazine, guaiacol and ethanol have all been shown to enhance laccase production (Rescigno *et al.*, 1993; Eggert *et al.*, 1996; Skorobogatko *et al.*, 1996; Lee *et al.*, 1999; Farnet *et al.*, 2004). In addition to a possible requirement for different specific compounds to induce laccase in the three *Cylindrocarpon* species, variable induction may be the result of differences in response to nutrient composition of the media and/or environmental factors. Kunamneni *et al*. (2007) reported that factors like carbon source, nitrogen content, pH and temperature influenced the ability of basidiomycetes to produce laccase activity, and that different strains reacted differently to these factors.

It is unclear whether the differences in laccase activity between the three species were due to differences in the amount of laccase secreted or in the efficacy of the enzymes. The relative amounts of enzyme in the extracellular medium, could be determined by methods to estimate protein concentration such as the Bradford, Biuret or Lowry assays (Hartree, 1972; Beyer, 1983; Compton and Jones, 1985). However, since these protein and enzyme assays do not distinguish between the types of extracellular proteins secreted into the medium during the laccase assay, further identification would also be needed, such as by running the filtrate through a protein separation system such as column chromatography and testing the individual fractions for laccase activity. If standard concentrations were made of the laccase in the protein extract then studies similar to those used here could indicate the activities of the laccases from the three *Cylindrocarpon* species. Such a series of experiments could determine whether more laccase enzyme was produced by *C. macrodidymum* or whether the enzyme was more effective.

For all the different isolates of the three species, there was variation in the amount of laccase activity produced. Isolate variation in laccase activity has been reported previously for other pathogens. For example, Saldanha *et al*. (2007) reported that the laccase activity in eight isolates of *L. theobromae* (also a grapevine pathogen) varied significantly and found that there was a correlation between laccase activity and genetic groups. For *C. macrodidymum* isolates, a similar pattern of isolate variation was noted; with isolate Mar16i producing approximately half the activity of PPO-1 compared to other *C. macrodidymum* isolates. However, it should be noted that for some isolates of the measured activities exceeded the maximum level of 1.00. Another isolate of this species (Gis3b) produced no PPO-II laccase and there was no correlation between activities of PPO-1 and PPO-II produced. A similar lack of correlation was seen for the other two species although there was lower overall laccase activity. Similarly, Vasconcelos *et al*. (2001) found difference between the activity of PPO-1 and PPO-II type laccase, with *B. ribis* producing a lower activity level of PPO-II compared to PPO-1. However, the biological implications of the different levels are not known and it may just reflect the intracellular rentension of PPO-II as reported by

Vasconcelos *et al*. (2001). Therefore, it would be advantageous to study the role of PPO-1 and PPO-II in the virulence of *Cylindrocarpon* species using gene knock-outs.

The results also showed that there was a relationship between laccase production and virulence (Section 5.3.9; Table 5.9). This higher laccase activity by *C. macrodidymum* could explain the greater degree of degradation of the grapevine roots observed in the detached root assay (Chapter 4) and may have contributed to the virulence of this species in the potted vine assay where *C. macrodidymum* killed the most plants. *Cylindrocarpon liriodendri* and *C. destructans* had lower laccase activity compared to *C. macrodidymum* and were observed to move inside the root endophytically without causing substantial deterioration of the root tissue (detached root assay, Chapter 4). A relationship between laccase activity and virulence was also observed for some isolates. The *C. destructans* isolate Mar13a, *C.liriodendri* isolate Hb2a and *C. macrodidymum* isolates Co6a and Ack1a produced high levels of PPO-1 laccase activity and these isolates were relatively more virulent than others in detached root assays (Chapter 4). Similarly, the isolates with low laccase activity showed lower virulence in detached root assays. For example, the *C. macrodidymum* isolate Mar16i produced low level of PPO-1 laccase activity and this isolate was relatively less virulent than others in detached root assays. For a few isolates this trend was also observed in potted vines. The *C. macrodidymum* isolate Gis3b produced high levels of PPO-1 and this isolate was relatively more virulent than others in potted vine assay. The *C. destructans* isolates, Co1c and Nel1d produced low levels of PPO-1 and these isolates were relatively low virulent than others in potted vine assay. In a similar study done on *N. parvum* Baskarathevan (2011) showed that the amount of laccase produced varied between isolates, however, no relationship was found between laccase activity and virulence. However, since the relationship did not appear to occur for all isolates of a species in this study, and Baskarathevan used only three isolates a greater sample may be shown a different effect. Although Saldanha *et al*. (2007) showed that laccase activity differed between the eight isolates of *L. theobromae* and correlated this with genetic group they did not show a relationship with virulence. Given the clearly higher activity in *C. macrodidymum* it would be interesting to investigate the role of this enzyme in pathogenesis. To undertake functional genomics research both a transformation system must be developed and the gene encoding laccase must be isolated. The isolation of the laccase gene was attempted using the process of degenerate PCR.

Research has shown that multiple laccase genes are found in many fungi including ascomycetes and basidiomycetes, with up to 17 genes in *Coprinus cinereus* (Kilaru *et al.*, 2006). When they compared *Botryosphaeria rhodina* with other ascomycetes and basidiomycetes, Castilho *et al*. (2009) found that there was sequence diversity and species variation in laccase genes. Thus, it seems reasonable to suggest that sequence variation in

the laccase genes of *Cylindrocarpon* species may be responsible for the differences in enzyme activity between isolates. To investigate this hypothesis, degenerate primers were made from the conserved region of *lcc1* gene of ascomycetes. This gene was initially targeted as it is the most well studied in ascomycetes and basdidiomycetes and therefore substantial sequence data could be retrieved from the GenBank. Furthermore, research has indicated that this gene is involved in pathogenesis by other species. Cañero and Roncero (2008) reported that *lcc1* gene was expressed in roots and stems during the infection process of *Fusarium oxysporum* in tomato plants. Catalano *et al*. (2011) reported the involvement of *lcc1* in the degradation of sclerotia by *Trichoderma virens* and Jiang *et al*. (2009) reported that *lac1* is a copper regulated laccase, which acted as a virulence factor in *Cryptococcus neoformans*.

The degenerate primers made from conserved regions of the *lcc1* gene successfully amplified a 580 bp band for the three *Cylindrocarpon* species and this band had 75% similarity to the *lcc1* gene of other ascomycetes. Phylogenetic analysis of the predicted laccase amino acid sequence showed high similarity to the *lcc1* enzymes produced by other fungi and grouped in a clade containing the *lcc1* amino acid sequences of other ascomycetes. The other laccase enzymes (*lcc2*, *lcc3*, *lcc4*, *lcc5* and *lcc9*) were placed in separate clades, as were the laccase enzymes of basidiomycetes. This indicates that the *Cylindrocarpon* laccase gene amplified here is likely to be the *lcc1* gene that was targeted by the degenerate PCR. These degenerate primers may also be useful to isolate the *lcc1* gene from other ascomycetes.

Alignment of the predicted 173 amino acid sequence obtained from the three *Cylindrocarpon* species with the *lcc1* encoded laccase enzymes from other ascomycetes and basidiomycete showed two copper binding domains that are conserved motifs in laccase enzymes and one possible N-glycosylation site. Similar to this study Castilho *et al*. (2009) reported amplification of a 900 bp fragment encoding the copper oxidase domain of the laccase gene from *Botryosphaeria rhodina;* however, that study did not distinguished different laccase genes. Litvintseva and Henson (2002) identified four putative copper binding regions in *lac1*, *lac2* and *lac3* amino acid sequences of *Gaeumannomyces graminis* var. *tritici*. The conservation of the copper binding regions of laccase genes have been reported in the thermophilic ascomycete *Melanocarpus albomyces* (Kiiskinen and Saloheimo, 2004) and the basidiomycetes *Pycnoporus cinnabarinus* (Eggert *et al*., 1998), *Coprinus cinerus* (Yaver *et al.*, 1999), and *Trametes* sp. (Fan *et al.*, 2011).

Analysis of the translated DNA sequence of the three *Cylindrocarpon* species showed that the predicted amino acid sequences differed between species and isolates. There were some non-conservative substitutions in the amino acid residues between high and low laccase producing isolates and these may be responsible for a greater activity of the *C.*

macrodidymum laccase. The *C. macrodidymum* amino acid sequence had only 87% similarity with C*. destructans* and *C. liriodendri,* which had substantially lower laccase activity. Of the 15 predicted amino acid differences between the species, the majority (10) were instances where *C. destructans* and *C. liriodendri* were the same but differed from *C. macrodidymum*. Thus, the differences appeared to correlate with taxonomic relationships and laccase activity. Of the 10 differences, six were non-conservative changes that may be responsible for the potentially greater enzyme activity of *C. macrodidymum*. The remaining 16 predicted amino acid substitutions included those in which there were differences between the isolates, eight of which were non-conservative changes that may be responsible for the difference in the level of enzyme activity between isolates. However, the amino acid sequence of *C. macrodidymum* isolates were identical to each other except for a single substitution of a proline for an alanine residue at position 157 in Ack1a (Δ157A→P), the isolate producing one of the highest laccase activities. It would be interesting to sequence isolates Co6a and Gis3b, which had similar laccase activity to Ack1a, to see if this substitution was conserved in those isolates.

There are two-crystal structures for different species for the lcc1 laccase of ascomycetes (Hakulinen *et al.*, 2002; Kallio *et al.*, 2011). The crystal structure of the laccase from the ascomycete *Melanocarpus albomyces* showed that this protein is composed of two cupredoxin-like domains (Domain A and Domain B). Analysis of the amino acid sequences of the *Cylindrocarpon* species showed that the laccase was composed of two domains, a specific Cu-oxidase 3 domain and a non-specific Cu-oxidase domain which are both cupredoxin-like domains. Thus, the *M. albomyces* and *Cylindrocarpon* species laccase proteins were aligned to determine which part of the protein the six non-conservative substitutions between species and the eight non-conservative substitutions between isolates, affected. *Cylindrocarpon macrodidymum* clearly had higher laccase activity than the other two species and there were five non-conservative changes between this species and the other two species. Of these only Δ86R→D altered the amino acid at a conserved site. Of the eight non-conservative substitutions between isolates none affected conserved sites. Even though they did not modify a conserved amino acid residue, it is possible that the changes could alter the three dimensional structure of the protein and thus alter activity. For example, proline is known to allow sharper bends to be formed in proteins because of its small size as was provided by Δ157A→P. As only 93% of the predicted laccase gene was isolated and sequenced it is also possible that important amino acid differences that occured in the remainder of the gene were not determined. It would be beneficial to sequence the remainder of the gene and study the crystal structure of *Cylindrocarpon* laccase enzyme.

In addition to changes in the activity of the enzyme, the increased activity of laccase in *C. macrodidymum* may be the result of more laccase being secreted either due to inducers in

the media or differences in gene promoters. Analysis of the promoter controlling the *lcc1* gene may improve understanding of how this gene is regulated in the different species. If the promoter region of *C. macrodidymum*, *C. liriodendri* and *C. destructans* contain the same regulatory motifs, then they are likely to be regulated in a similar manner and it is unlikely that the greater activity of *C. macrodidymum* laccase is simply through increased mRNA expression. Transcription factors that bind to the regulatory motifs of the laccase gene could also be induced by factors like carbon availability, nitrogen or pH. According to Broda *et al*. (1995), different laccase genes are regulated by different factors such as substrate conditions, pH and fungal metabolism, and these may also differ between the *Cylindrocarpon* species.

The transcription factors themselves may also differ between the *Cylindrocarpon* species and it may be via upstream differences in signal recognition that differ between the species. Cañero and Roncero (2008) reported that in *F. oxysporum* the *lcc3* and *lcc5* genes promoter regions contained three *PacC* binding consensus sites (recognition motif for ambient pH) and also showed that the expression of *lcc3* occurred in acidic conditions (pH 5.5 to 6.0). Whether greater upregulation of the laccase gene is occurring in *C. macrodidymum* than in *C. liriodendri* and *C. destructans* can be determined by quantitative PCR, or by northern blotting. Using these techniques the response of the laccase gene in the different species to known inducers could also be investigated and the results may shed light on whether the laccase genes are under similar regulation in all three species. Further extension of the laccase gene fragment to encompass the promoter region could be done using thermal asymmetric interlaced (TAIL) PCR (Liu *et al.*, 1995) which uses a specific primer within the known gene sequence together with a series of randomly binding nested primers to amplify the flanking region. Alignment and analysis of the promoter regions from the three *Cylindrocarpon* species could identify regulatory motifs and their level of conservation between the species.

This chapter also showed that the three *Cylindrocarpon* species secreted acid protease. Plant pathogens are known to degrade various proteins in the plant cell wall (Agrios, 2005) using protease enzymes, allowing penetration by the pathogen (Rauscher *et al.*, 1995). Proteases are classified into acidic, neutral and alkaline according to the optimum pH of enzyme activity (Keay, 1971a; Fogarty *et al*., 1974). The main role of acid proteases is to degrade the plant cell wall components and macerate the plant tissue, and so they have a specific role in the pathogenic process (Poussereau *et al*., 2001; Garcia *et al*., 2003; Hu and St. Leger, 2004; Rolland *et al*., 2009). There are no prior reports on the role of the protease enzyme in this genus but it has been studied in many other plant pathogenic fungi. The secretion of acid proteases by *Sclerotinia* species in infected plant tissues has been reported (Khare and Bompeix, 1976; Poussereau *et al.*, 2001). Urbanek and Yirdaw (1978) reported

the secretion of acid proteases by *Fusarium* species during infection of maize seedlings and suggested that the acid proteases have a specific role in pathogenic process. Another study on *Cladosporium cucumerium* reported that it produced an alkaline extracellular protease, which does not play an important role in pathogenesis (Robertsen, 1984).

The results from this study showed that the protease activity varied between the three *Cylindrocarpon* species. At the species level *C. destructans* and *C. macrodidymum* produced greater protease activity than *C. liriodendri.* Similarly, another study by Oyeleke *et al*. (2010) using isolates from a local rice husk dumpsite in Nigeria found *Aspergillus flavus* produce higher protease activity (0.9 µg/mL/min) than *Aspergillus fumigatus* (0.8 µg/mL/min). Another study on acid protease activity by *Candida* species isolated from clinical specimens showed that among the three *Candida* species; *C. parapsilosis*, C. *glabrata* and *C. albicans* the higher activity was shown by *C. parapsilosis* (69%) and the lowest by *C. albicans* (48%) (D'Eça Júnior *et al.*, 2011).

There was no correlation between protease activity and observed pathogenicity of the three *Cylindrocarpon* species, nor any reports of such a relationship between the protease activity with virulence for any plant pathogenic fungi. In contrast to the qualitative plate assay result, the quantitative assay showed that isolates of the three *Cylindrocarpon* varied in their protease activity. Similarly, Djamel *et al*. (2009) reported the acid protease production by *Penicillium* species and showed that 250 *Penicillium* isolates from decomposing debris of the clam *Ruditapes decussatus* from El-Mellah Lake in northeast Algeria had different levels of acid protease activity. Another study on protease activity by (Dhar and Kaur, 2010) on 14 isolates of *Metarhizium anisopliae* showed different level of protease activity and suggested that this variation may be due to geographical origin of the isolate. Another study by Dore (2009) reported that bacterial isolates recovered from grapevine rhizosphere showed variation in protease activity.

The activity of extracellular acid protease produced by *Cylindrocarpon* was quantified using a spectrophotometry method that allowed quantification of protease activity as described by Damare *et al*. (2006). In that study used eight buffers with different pH (from acidic, neutral to basic) which can determine the production of acid or alkaline proteases by deep sea fungi. There are similar methods also reported except the buffer used to quantify the extracellular acid protease was different. Garcia *et al*. (2003) reported that in their study on the protease activity of *Sarocladium oryzae* that cause rice sheath rot disease, the reaction buffer used was 0.1 M citric acid-NaOH with pH 3.0-6.0. Siala *et al*. (2009) used the similar quantitative assay as used in this study except that the buffer used was 100 mM glycine-HCL with pH 3.0 for the quantification of the extracellular acid protease from *Aspergillus niger*.

In the qualitative assay, *C. macrodidymum* isolates did not produce any clearance zones, but isolates of this species were able to produce high levels of protease activity in broth medium. This may have been due to the different concentration of nutrients in the broth as compared to the agar medium. Srinubabu *et al*. (2007) screened the protease enzyme production by *Aspergillus oryzea* in a basal medium containing different nutrients like glucose, malt extract, yeast extract, peptone, K2HPO4, MgSO4 and FeSO4. They found that glucose was the best carbon source for the enzyme production, and poor enzyme production and enzyme activity was found in yeast extract. However, they reported that addition of 1% cottonseed followed by 2% soya bean meal induced protease enzyme production and the enzyme activity was influenced by culture time, pH and interaction between these two. Similarly, Bueno *et al*. (2012) showed differences in the protease activity of *S. sclerotiorum* in medium containing different carbon sources like pectin, glucose and cell wall materials of *Phaseolus vulgaris.* In their study, pectin was the carbon source that produced the highest level of protease activity. Therefore, it would be advantageous to screen the protease enzyme activity using different carbon source in the medium.

The difference in activity may also be due to differences in the pH optima of the enzymes from each of the three species. The pH of agar media used in this study was around 6-7 and the quantification of protease activity was done at pH 5. Pappinen and Von Weissenberg, (1997) showed that extracellular protease from fungal pathogens (*Gremmeniella abietina*, *Endocronartium pini* and *Heterobasidon annosum*) showed greatest proteolytic activity at acidic pH 3.3-4.4. Urbanek and Yirdaw (1978) reported that the activity of a protease produced by *Fusarium* species was high at acidic pH and reduced when the pH of the medium was increased to 6.0. Thus, media with a range of pH should be tested to determine how pH affects the activity of the protease produced by the three *Cylindrocarpon* species. In addition, adding plant cell walls or inducers like glucose or collagen into the media, and changing the environmental conditions like (e.g, pH and temperature) may alter the amount and activity of proteolytic enzymes secreted into the extracellular media. Pursuing this may shed light on differences in pathogenesis by the three *Cylindrocarpon* species.

The amount of protease activity produced by *Cylindrocarpon* species by the quantitative analysis varied both within and between species. To determine whether the variation observed was the result of differences in the encoding gene further effort was made to isolate the gene responsible for the enzymatic activity. To isolate the acid protease gene, degenerate primers were made using sequences for acid proteases that were present in GenBank. Comparison of these DNA sequences showed the presence of conserved regions to which degenerate primers for acid protease could be made.

The degenerate primers made from the conserved region of the acid protease gene successfully amplified a 367 bp that had 80% similarity to reported protease genes. It was

interpreted as being able to produce an amino acid sequence that was 97.7% among the three *Cylindrocarpon* species. Similar to this study, Poussereau *et al*. (2001) designed degenerate primers for the isolation of the acp1 gene for non-aspartyl acid proteases in *Scl. sclerotiorum* and produced a 204 bp amplimer. However, their primers were not used in this study as they produced a smaller 204 bp amplimer, whereas the primer designed in this study produced a much larger 367 bp (56% bigger) fragment of the acid protease gene. The degenerate primers described here may be useful to isolate the acid protease gene from other ascomycetes.

Although, *C. destructans* and *C. macrodidymum* showed higher protease activity than *C. liriodendri* there were no non-conservative amino acid substitutions that seemed to explain this difference in activity. Of the six predicted amino acid differences between the species, the majority (five) were instances where *C. destructans* and *C. liriodendri* were the same but these differed from *C. macrodidymum*. Thus, the differences appeared to reflect taxonomic relationships rather than enzyme activity. Of the single remaining difference at position 27, *C. destructans* had a polar serine and the other two species a non-polar alanine residue. The amino acid sequence of the acid protease showed that this protein is a part of peptidase A4 domain. However, given that both *C. destructans* and *C. macrodidymum* have the same level for protease activity it is unlikely that this substitution affects the activity of the enzyme. As only 87% of the predicted acid protease gene was isolated it is possible that the important amino acid differences occur in the remainder of the gene.

As analysis of the predicted protein sequence of acid protease between the three *Cylindrocarpon* spp. showed that they were highly conserved, it is unlikely that there is a difference in protein activity or, therefore, environmental optima for its activity. In contrast, it is more likely that *C. destructans* and *C. macrodidymum* secrete more acid protease than *C. liriodendri*. Using the protein concentration estimation methods previously suggested for laccase, the amount of protease present in the medium could be measured. As described for laccase, efforts could be made to expand the genes sequence to investigate regulatory motifs in the promoter region and also to undertake quantitative PCR analysis of gene expression. As there was variability in the activity of protease between isolates, it is likely that some of this may be reflected in the expression profile and/ or promoter region sequence of different isolates.

In addition to laccase and proteases there are several other enzymes likely to be involved in pathogenesis. One of these is cellulase and the measurement of this enzyme and the isolation of this gene was also attempted in this chapter. The cellulolytic capability of the plant pathogenic fungi plays a significant role in their ability to attack hosts (Ilmen *et al.*, 1997). In fungal cellulases, the most extensively studied cellulolytic fungi are *Trichoderma* species (Kubicek *et al.*, 1988; Penttila *et al.*, 1991; Teeri *et al.*, 1992). In this study, two

methods of plate clearing assay, which had been previously used to show the cellulolytic activity in fungi, were used (Pointing, 1999; Kasana *et al*., 2008). Neither plate assay showed cellulolytic activity by *Cylindrocarpon* species or by the positive controls *Trichoderma* sp. and *Pythium* sp. Similar studies on celluloytic activity in *Ganoderma lucidum* inoculated onto chromogenic media incorporating either Congo red, Phenol red, Remazol Brilliant blue and Trypan blue with a positive control, *Trichoderma sp.,* produced clear zone around the colony (Jo *et al*., 2009). Kasana *et al*. (2008) reported that cellulase producing microorganisms includes bacteria such as *Pseudomonas* sp., *Bacillus* sp. and fungi such as *Penicillium chrysogenum* and *Aspergillus nige r*which produced a zone of clearance on CMC Congo red plates. However, they also reported difficulties in differentiating the enzyme lysis zones as the intensity of clearance was usually low.

In parallel to this, degenerate primers were made to target the endo β-D-1, 4-glucanase gene, which can cleave the β1, 4-glycosidic bonds present in cellulose. Sequencing of a band isolated and reamplified from a *Cylindrocarpon liriodendri* isolate produced a small amount of unambiguous useful sequence that had no similarity to endo β-D-1, 4-glucanase gene. Further cloning and sequencing of the multiple products generated by the degenerate primers may yield cellulases including an endo β-D-1, 4-glucanase gene. Given the likely role of these enzymes in the pathogenicity of *Cylindrocarpon* species, it would be beneficial to do further detailed study on cellulase gene in *Cylindrocarpon* species.

There are several reports that cellulases are expressed during pathogenesis. Lev and Horwitz (2003) reported the mitogen activated protein kinase pathway modulates the expression of the two cellulase genes, cellobiohydrolase, *CBH7*, and an endoglucanase, *EG6* in *Cochilobolus heterostrophus* during maize plant infection. Another study by Ramanathan *et al*. (2009) reported that *Fusarium oxysporum* isolated from infected tomato plants were able to produce maximum cellulase at an optimum pH of 6 and a temperature of 50°C incubated for 12 days. Akintobi *et al*. (2012) reported that the extracts collected from *Penicillium* infected cocoa beans showed cellulase activities*.* In addition to a role in pathogenesis DNA sequences for this enzyme are well represented on GenBank. Despite being developed to conserved sites the degenerate primers amplified multiple bands. This may be because the region the primer bound to is conserved across different cellulases or possibly the sequences of cellulase gene families may present in multiple copies within the *Cylindrocarpon* species.

In summary, this is the first study reporting the production of laccase and protease by *Cylindrocarpon* species. This study showed that the activity of these enzymes varied amongst the three species, *C. destructans*, *C. liriodendri* and *C. macrodidymum* and between isolates of these species under standard conditions*.* For laccase, the variation in activity was correlated to variation in pathogenicity but this was not apparent for protease.

Analysis of genes encoding *lcc1* and acid protease showed that there was variation in the predicted amino acid sequence between species. There was no evidence found for the presence of cellulase although it is likely that these species produce these enzymes. In order to prove the link between enzyme activity, gene and pathogenicity suggested here it would be necessary to undertake expression studies and transformation to produce a knock-out strain.

5.5 Implications of recent taxonomical classification

This section discusses the implications of the recent reclassification of *C. destructans* and *C. macrodidymum* species by Cabral *et al.* (2012a; 2012b) on the variation within and between species shown by the laccase and protease enzyme tests.

In this study, *C. macrodidymum* isolates showed the highest level of laccase activity and this was up to greater than ten times higher than other species. There was a single amino acid substitution in isolate Ack1a, which was one of three isolates (including Co6a and Gis3b) that produced the greatest amount of laccase. It is possible that this single amino acid substitution indicates that isolate Ack1a is from a different species within the *Ilyonectria macrodidyma* complex, since its location (clade I) in the neighbour joining tree (Chapter 3) is different to isolates Co6a and Gis3b, which had similar high activity, and were also located in different clades (clades III and IV). Furthermore, isolate Mar16i (clade III) which produced the least laccase activity shared the clade with Co6a. Isolate Gis3b was identified as being in the *I. macrodidyma* complex by sequencing in Chapter 2. In addition, other studies have demonstrated differences in laccase activity among the isolates of *N. parvum* (Baskarathevan, 2011) and for *L. theobromae* (Saldanha *et al*., 2007). Thus, it is likely that laccase activity does not reflect species variation but is part of isolate variation as seen in other species. Resolving the members of the species complex will confirm whether laccase activity varies between isolate of a single species.

For *C. destructans* the laccase activity of isolates was low compared to that of *C. macrodidymum* isolates. Some of this variation in enzyme activity could be attributed to variation in amino acid residues. In contrast to *C. macrodidymum* there were 10 sites at which amino acid residues varied between isolates of *C. destructans* and, of these, six differentiated isolate Nel1d from the other two isolates tested. The information presented in Chapter 2 suggested that the majority of the *C. destructans* isolates were likely to be *I. europaea* with only 20% of these isolates identified as "*Cylindrocapon"*sp. Unfortunately, none of the isolates used in these laccase assay were identified by sequencing in Chapter 2. Thus, the relatively high number of polymorphic residues in *C. destructans* isolates may be due to the presence of multiple species of the *I. radicicola* species complex and accurate identification of the members of the species complex recovered from grapevines will help to

clarify whether the isolates within a species have high or low laccase activity and whether laccase activity varies between isolates of the same species.

In addition, the results in this chapter showed that level of laccase activity was often associated with the level of virulence. Given the clearly high laccase activity in *C. macrodidymum* further investigations could be conducted in the role of this enzyme in pathogenesis to provide a better understanding of infection and disease progression

Similarly, the *C. destructans* and *C. macrodidymum* showed more protease activity compared to the other *Cylindrocarpon* species and isolates of these two species showed differences in the level of acid protease activity. This may be due to the presence of multiple species of the *I. macrodidyma* and *I. radicicola* complex. Therefore, resolving the members of the species complex recovered from grapevines will also help to clarify whether some have high protease activity.

In summary, differences in enzyme activity among the *C. destructans* and *C. macrodidymum* isolates may be due, in part, to the presence of multiple species. These differences may account for varied levels of enzyme activity in isolates and so re-identification of isolates after sequencing of histone gene as shown by Cabral *et al*. (2012b) could clarify whether different levels of enzyme production were due to species identity or were a component of isolate variation.

Chapter 6
Concluding discussion

Black foot disease was reported in major viticulture areas worldwide in the 1990s; including Australia, California, Portugal, South Africa (Scheck *et al*., 1998a; Rego *et al*., 2001), and was also shown to be a major concern for New Zealand vineyards in 2005 (Bleach *et al*., 2007). Research has demonstrated that the main causative agents differ between countries (Halleen *et al*., 2004; 2006b; Bleach *et al*., 2007; Alaniz *et al*., 2009b; Mohammedi *et al*., 2009; Abreo *et al*., 2010; Petit *et al*., 2011). In New Zealand, a subset of 60 isolates from the 174 isolates recovered from symptomatic vines in a survey conducted by Carolyn Bleach in 2005 were identified by South African collaborators and contained three described *Cylindrocarpon* species: *C. destructans*, *C. liriodendri* and *C. macrodidymum*. The overall aim of this research was to use that collection to understand the population structure and genetic diversity of *Cylindrocarpon* species in infected vines, to study sub-species variation in virulence and correlate that with the production of cell wall degrading enzymes

Identification of the collection of *Cylindrocarpon*-like isolates recovered by Ms Bleach using species specific PCR and DNA sequencing has shown that *Cylindrocarpon* species were obtained from all eight grape growing areas from which samples were obtained (Central Otago, Hawkes Bay, Waipara, Marlborough, Nelson, Gisborne, Auckland and Martinborough) and were representative of the national vineyard. The originally identified three species, *C. macrodidymum*, *C. destructans* and *C. liriodendri* within the subset of 60 were predominant, comprising 87% (151) of the isolates. The species distribution between the regions varied, with *C. destuctans* being more prevalent in the South Island and *C. macrodidymum* in North Island. *Cylindrocarpon liriodendri* was evenly distributed throughout both islands. There were a small number of *C. pauciseptatum* isolates recovered, which although a significant grapevine and non-grapevine pathogen in other countries (Alaniz *et al*., 2009a; Martin *et al*., 2011; Agustí-Brisach *et al*. 2011; Yaseen *et al*., 2012), appeared to be less important in New Zealand due to its low observed prevalence. The exact regional distribution of species was difficult to estimate due to the substantial variation in number and type of samples received from each area. The highest proportion of *Cylindrocarpon* species infection was in Marlborough (53%) and the lowest in Martinborough and Nelson (1.8% each). It was not possible to determine the exact regional proportion of species because the ‘survey’ comprised samples contributed by growers in each area. Ideally, a study to establish exact incidence per region would sample equal numbers of vineyards in each region and randomly select the same number of samples from each of a variety of rootstock varieties. Inclusion of samples from apparently healthy material would allow the presence of latent

infections to be determined and provide a more accurate estimate of the incidence of this disease; however, vineyard owners are likely to be unwilling to sacrifice healthy vines.

This study identified isolates of five *Cylindrocarpon* species, namely, *C. destructans*, *C. liriodendri*, *C. macrodidymum*, *C. pauciseptatum* and a yet unnamed *Cylindrocarpon* sp. The relative incidences showed that *C. liriodendri* (33%; n=57) was the most predominant species followed by *C. destructans* (30%; n=53) and then *C. macrodidymum* (24%; n=41). This relative frequency of isolates was similar to studies in South Africa (Halleen *et al*., 2006a) but contrasted with a study in Portugal (Rego *et al*., 2000) where the predominant species isolated was *C. destructans* and one in Spain where *C. liriodendri* and *C. macrodidymum* were significant pathogens but there was no report of *C. destructans* (Alaniz *et al*., 2007). The reasons for the relative differences in predominance between countries could be explained by several factors including the climate, rate of introduction and proximity of different hosts. In addition, the early Portuguese and possibly South African reports had relied on an erroneous method of identification which misidentified *C. liriodendri* as *C. destructans*, which was also reported to be a pathogen of apples (Bonfiglioli, 2005). In New Zealand, it is highly likely that the replanting of vineyards on old apples orchard sites may have contributed to the higher incidence of *C. destructans*. Further work investigating apple isolates could be done by obtaining isolates from ICMP and from infected apple trees and then analysing these by UP-PCR. The fingerprints could then be compared to those produced by isolates in this study to see if the isolates were similar or formed a clade distinct from grapevine isolates. In addition, more accurate identification of species could be provided with the new classification of Cabral *et al*. (2012a, 2012b).

At the outset of this research species specific PCR represented a rapid and highly accurate method to identify the isolates which overcame the issues with morphological identification, as demonstrated in chapter 2. However, recent reclassifications of the *C. destructans* and *C. macrodidymum* species to *Ilyonectria radicicola* and *I. macrodidyma* species complexes, respectively, were presented in taxonomic papers published by Cabral *et al*., (2012a, 2012b). In the individual chapters, the implications of this reclassification have been discussed.

Reclassification of *C. destructans* revealed 12 new taxa within *I.radicicola* complex and therefore, it would be beneficial to resolve the identities of the 53 isolates designated as *C. destructans* by species specific PCR with respect to the 12 new taxa described using sequencing of taxonomically informative genes which includes β-tubulin, TEF-1α, ITS and histone H3. The small amount of sequencing done in chapter 2 suggested that approximately 80% of the isolates are likely to be *I. europaea* and 20% *"Cylindrocarpon"* sp. This placement was reinforced by the temperature experiment in which "*Cylindrocarpon*" sp. isolate Mar7a had a lower optimum temperature than the other two isolates. In addition, it was found in

clade III, whereas the other two isolates, likely to be *I. europaea,* were found in clade II. It is likely that the three clades in the neighbour joining tree attributed to *C. destructans* are actually different species within the *I. radicicola* complex. This has been shown previously as Varga *et al*. (2011) reported that UP-PCR analysis distinguished *Aspergillus awamoria*, a cryptic species within the *A. niger* species complex and they stated that UP-PCR analysis was useful for species delineation. The preliminary indications from the data presented here, including DNA sequencing, temperature experiments and dendrogram, suggest that there are three species from the *I. radicicola* complex present in New Zealand vineyards. These are likely to comprise *I. europaea* (clade II; n=33) which was predominant, "*Cylindrocarpon"* sp. (clade III; n=9) which includes a South African isolate from apple and an as yet unidentified species (clade I; n=11). Further work may show whether each species has a different optimum temperature for growth and the identity of isolates in clade I.

Similarly, the reclassification of *C. macrodidymum* revealed six new species within the *I. macrodidyma* complex. The limited amount of sequencing done in this research project was unable to resolve any of the species present in the collection according to the new classification and, therefore, this could be done by sequencing the histone3 genes of the 41 isolates. However, if the different clades within the dendrogram can be considered as representing species groups then it suggests that up to five species are present in New Zealand vineyards. The largest group of isolates are those in clade III (n=15), followed by clade IV (n=9), clade V (n=8), clade II (n=7) and clade I (n=2). Interestingly, only clades I and IV contained single international isolates and the majority of international isolates (n=4) were placed in a separate clade (VI) despite originating from two different countries (South Africa and Australia). As for the *C. destructans* isolates, the isolates from clade I had higher optimum temperature and the other two isolates, which were similar and both from clade IV. The presence of several clades on the dendrogram suggests that the population structure of species within the *I. macrodidyma* complex in New Zealand is likely to be composed of several species that may differ in some respects to the species found in Australia and South Africa.

To facilitate the differentiation of the species within *I. radicicola* and *I. macrodidyma* complexes it would be beneficial to design a rapid molecular test similar to the species-specific primers used here. It is unlikely that sufficient sequence differences are present to allow the production of a number of species specific primers. In addition, the iterative use of primers specific for one of the 12 *I. radicicola* and six *I. macrodidyma* species would be cumbersome and time consuming. A more useful system could use the existing species specific primers to produce an amplimer and then distinguish the different species by either RFLP (such as the ARDRA system of Alves *et al*., 2005), SSCP (such as the system for the *Botryosphaeriaceae* of Ridgway *et al*., 2011) or high resolution melting (such as the system

of Ganopoulos *et al*. (2012) for the *Fusarium oxysporum* formae speciales complex. This would utilise existing technology (primers) and provide cost effective and rapid resolution of individual species within the complex.

This study also identified *C. pauciseptatum* isolates taken from the symptomatic vines, which has been identified previously (Schroers *et al*., 2008) from *Vitis* species, but their pathogenicity on grapevines has not yet been elucidated. Given the low number of isolates collected in this survey, it is unclear whether *C. pauciseptatum* is a weak pathogen or is an emerging pathogen of grapevines. Pathogenicity tests with the 11 isolates recovered on potted vines of a range of cultivars and comparison with the pathogenicity of representative isolates of the three predominant species could show the relative pathogenicity of *C. pauciseptatum*. If it proves to be highly pathogenic then that would suggest that it has only recently become a pathogen of grapevine – potentially through a recent introduction. If it is pathogenic then it is also possible that symptoms used here as criteria to collect infected material are not indicative of infection by this pathogen. Further sampling studies with a greater number of vines may be needed to assess prevalence and distribution.

UP-PCR was a useful technique to measure genetic diversity as a good number of polymorphic loci were produced. However, it would be useful to study genetic diversity with other genotyping methods such as ISSR, AFLP and microsatellites. Analysis using the same ISSR primers used by Alaniz *et al*. (2009b) would allow direct comparison of the New Zealand and Spanish populations to confirm whether the relatively high genetic diversity observed for New Zealand populations was due to the choice of method. In addition, Pottinger *et al*. (2002) showed that AFLP gave more information than UP-PCR when it was used to investigate genetic diversity in *P. chlamydospora*. As AFLP provides more data and samples the genome more randomly than UP-PCR, it is possible that the genetic groups produced may correlate with pathogenicity of isolates. The development of microsatellites is time consuming but, if developed for *Cylindrocarpon* species, would be an interesting tool to investigate gene flow between regions and countries. However, since microsatellites are often species specific the recent reclassification of species may indicate that this could be difficult. By using microsatellites to understand the relationship between isolates within New Zealand and their international counterparts, it might possibly to prove a connection between the nursery and vineyard, and to determine how the pathogens distributes themselves around the country and their route of entry into New Zealand. In this study, the one isolate of *C. destructans* from South Africa, isolated from apple, grouped with New Zealand isolates from grapevines and therefore it would also be interesting to determine whether there is exchange of isolates between hosts.

Even if the clades within the dendrograms represented different species, there were still few clonal isolates within the New Zealand *Cylindrocarpon* populations, which was surprising

when compared to the situation for other predominantly asexually reproducing grapevine pathogens such as *P. chlamydospora*. The pattern observed for *Cylindrocarpon* species is more similar to that recently described by Baskarathevan *et al*. (2012b) for the grapevine pathogen *Neofusicoccum parvum*. The exact mechanism for the higher than expected genetic diversity is unknown. It could be explained by frequent asexual recombination and also the introduction of genetically diverse *Cylindrocarpon* species from other countries. Therefore, it would be beneficial to develop a nuclear *gfp* and *rfp* tag for two compatible *Cylindrocarpon* species to test the recombination process. A preliminary study of vegetative compatibility showed that hyphal fusion occurred between *Cylindrocarpon* isolates within each species. In *C. destructans* there was also formation of chlamydospores by the merging of cytoplasm from multiple cells. The merging of cellular contents may promote the parasexual cycle in these species and allow the introduction of genetic diversity due to parasexual recombination. However, a detailed study is necessary to investigate the role of hyphal fusion and formation of chlamydospores in genetic recombination. This could be done by comparing the DNA fingerprint of the parent isolates with those of the progeny (conidia and chlamydospores) produced at the interaction zone *in planta* and *in vitro* on plates using a technique such as UP-PCR, AFLP or ISSR. For *C. destructans* there was a partially incompatibility reaction. This suggests that there may be weak barriers/weak incompatibility reactions to anastomosis in this species. The genetic basis of the vegetative compatibility groups in *Cylindrocarpon* species is unknown. In ascomycetes, the formation of heterokaryotic cells by hyphae is controlled by *het* or *vic* genes (Be´gueret *et al.*, 1994; Smith *et al.*, 2006) and these genes vary from one species to another (Saupe *et al*., 2000), therefore use of a strategy such as degenerate PCR is unlikely to prove useful in attempts to identify these genes in *Cylindrocarpon* species. Some isolates of the three *Cylindrocarpon* species belonging to different VCGs overlapped. This may result in enhanced formation of anastomosis between genetically different isolates, leading to hyphal fusion and subsequent gene flow between isolates (Leslie, 1993). Therefore, although it would be beneficial to study the genetic basis of VCGs in *Cylindrocarpon* species by molecular characterisation of the *vic* loci but this may be difficult to achieve.

Also, in the vegetative compatibility grouping, the isolates of *C. destructans* and *C.macrodidymum* were from different clades. Despite the suggestion that each clade in the neighbour joining tree represents each species within the complex, the fact that isolates of *C. destructans* and *C. macrodidymum* from different clades showed what appeared to be a compatible reactions seems to argue against this. For example, the *C. destructans* isolate Mar7a (clade III) later identified as "*Cylindrocarpon*" sp. was compatible with the isolates from clade II. Similarly, isolates of *C. macrodidymum* from different clades appeared to be compatible. Clearly, it is unlikely that pairs of different species will undergo a truly compatible reaction resulting in hyphal anastomosis and the formation of a stable heterokaryon. It is

more likely that there is simply a lack of recognition between the two opposing colonies and they are just growing together or across each other. Appel and Gordon (1996) reported that *Fusarium oxysporum* isolates representing different *formae specialis* from their culture collection were vegetatively compatible and stated that in some cases vegetative compatibility may be coincidental, possibly arising by convergence rather than common descent i.e; the isolates had compatible loci that were produced by converging evolution rather than because they originated from a similar ancestral species.

Isolates of all three species had variable pathogenicity on whole plants and detached roots. Generally, *C. destructans* produced the shortest lesions out of the three main species suggesting that it is the weakest of the three pathogens. However, the individual pathogenicity of the isolates overlapped with that of isolates from other species. A similar level of symptom variation between isolates of *Botryosphaeria* species was reported by Amponsah *et al*. (2011), also indication that pathogenicity is unlikely to be a useful way to discriminate between species.

The intra species variation in pathogenicity may perhaps also be the result of differences in host specificity. As in other countries, *Cylindrocarpon* species have been isolated from many hosts in New Zealand and it is possible that the isolates with weak pathogenicity on grapevines may be more pathogenic on other hosts. Determining the host range of *Cylindrocarpon* species may provide information about the potential risk posed by neighbouring crops. It may also be possible that a broad host range may also underpin the drive for genetic recombination and the diversity observed in the *Cylindrocarpon* species population. Experiments to determine the host range will also rely on accurate resolution of the species within the *I. macrodidyma* and *I. radicicola* complexes as some of those species may be reported as more common on different hosts. In addition, although the work presented her focuses on horticulture it may be useful to determine the risk these pathogens pose to native plants, especially as viticulture expands to new sites.

This work is the first attempt to transform *Cylindrocarpon* species. Transformation was unsuccessful despite attempts with two starting substrates and transformation systems (AMT and protoplast mediated). The reasons for the lack of success are not clear and require further research, especially as the production of a *gfp* marked strain would be a valuable tool to improve understanding of the pathogenesis processes used by these species. For example, it would be interesting to infect grapevine roots with the *gfp* marked strain at specific sites (root tips, wounds, branch sites, callused stem base) and track the movement of the pathogen through the plant stem. Sprague *et al*. (2007) reported the use of *gfp* transformed *L. maculans* to study the colonisation pathway in *Brassica napus* roots and found that *L. maculans* can directly infect intact roots. Transformation using hyphae of *Cylindrocarpon* species could also be attempted as other studies have shown that hyphae

are very robust and they have been successfully used in transformation of *Fusarium oxysporum* f. sp. *cubense* (Visser *et al.*, 2004; Li *et al.*, 2011).

The preliminary study on *in vitro* production of laccase by three *Cylindrocarpon* species showed varied levels of laccase activity. Among the three *Cylindrocarpon* species; *C. macrodidymum* had highest level of laccase activity. However, it was unresolved whether the *C. macrodidymum* produced more enzyme or a more active enzyme. The observed differences in the amino acid sequence between the three species suggested that amino acid substitutions could underlay differences in enzyme activity. To resolve this question, lcc1 laccase extract obtained from isolates of all three species could be purified and diluted to the same concentration prior to being tested in similar assays. Addition of the purified enzyme preparations to plant roots, followed by an assessment of the level of maceration produced could be used to investigate the role of this enzyme in pathogenicity. One way to investigate the role of laccase in the pathogenicity of *C. macrodidymum* is to use transgenic methods to create a gene knock out and to then observe any alteration in pathogenicity. However, this relies on the production of a working transformation system. This work demonstrated that the laccase gene amongst the three species was polymorphic and the production of a crystal structure of the *Cylindrocarpon* laccase enzyme using X-ray diffraction/neutron diffraction could allow understanding of where in the protein the polymorphic amino acid residues are placed and, therefore, whether they might alter the activity of the enzyme.

Similarly, the preliminary study on *in vitro* production of protease by the three *Cylindrocarpon* species showed varied levels of protease activity. Among the three *Cylindrocarpon* species, *C. liriodendri* had lowest protease activity. It may be possible that acid protease was not efficiently induced using the media for quantitative assay for some of the *Cylindrocarpon* species. Therefore, iterative testing of a range of inducers may show whether they can alter the protease secretion. The outcome of the analysis of the protease gene suggested that the differences in activity were not the result of differences in amino acid residues, although not all of the gene was sequenced. By investigating the regulatory motifs in the promoter region and analysing gene expression, differences in the regulation of this gene amongst different *Cylindrocarpon* species could be determined. The addition of purified acid protease into the plant roots could investigate any role in pathogenicity. In addition, as described for laccase, it would be interesting to study the role of protease in the pathogenicity in *Cylindrocarpon* species by creating a gene knockout mutant. Overall the sequencing of the two cell wall degrading enzymes showed greater variation in the laccase gene between species and suggested that this may be more important in pathogenesis by *C. macrodidymum* than the other species. Once again the resolution of species within the *I. macrodidyma* and *I.*

radicicola complexes may determine whether the observed polymorphism was indicative of different species.

It is very likely that *Cylindrocarpon* species produce cellulases; however, the assay to measure cellulase did not work. Other methods for measuring cellulase activity have been reported such as using a chromogenic medium that incorporated either congo red, phenol red, remazol brilliant blue or trypan blue (Jo *et al*., 2009). Other methods of inducing cellulase production involve optimising the pH, temperature, carbon and nitrogen sources in the media with the addition of inducers like lactose, cellobiose, sophorose, L-sorbose, L-arabitol and D-galactose (Kubicek *et al*., 1988; Bisaria and Mishra, 1989; Margolles-Clark*et al*., 1997; Karaffa *et al*., 2006; Ramanathan *et al*., 2010). Testing some of these methods with representative isolates may determine whether they are more useful for *Cylindrocarpon* species. Also by modifying the media used in this research by adding plant material may help to stimulate the secretion of cellulase by *Cylindrocarpon* species. In attempted gene discovery the degenerate primers amplified multiple bands whose identity was not resolved. Further cloning and sequencing of these multiple products may indicate presence of cellulases including the targeted endo β-D-1, 4-glucanase gene. As for laccase and protease, investigating the coding sequence and the putative translation may help to improve understanding of the roles of these enzymes in pathogenicity.

Future studies that measure the laccase, protease and cellulase activity together with other plant cell wall degrading enzymes, such as pectinase and xylanase, of a large number of *Cylindrocarpon* isolates with known pathogenicity levels would improve a study of how these enzymes act in pathogenicity. The ever increasing use of pyrosequencing to generate genome and transcriptome data may be used in the future to identify the cell wall degrading enzymes and then to follow their activation during pathogenesis. The genetically characterised and representative *Cylindrocarpon* species collection described in this research would be a useful resource for this work.

In summary, this research has produced new information on distribution, incidence, sub-species variation and enzyme production of *Cylindrocarpon* species associated with black foot disease of grapevines in New Zealand. The use of molecular tools for identification of the isolate collection has characterised at least five *Cylindrocarpon* species; *C.liriodendri*, *C. pauciseptatum, Cylindrocarpon* sp. and members of the *I. radicicola* and *I. macrodidyma* complexes.

Publications and presentation from thesis

Journal Publications:

- **Pathrose, B**., Jones, E. E., Jaspers, M. V. and Ridgway, H. J. (2013). High genotypic and virulence diversity in *Ilyonectria liriodendri* isolates associated with black foot disease in New Zealand vineyards. *Plant Pathology*. (Accepted for publication).
- Jones, E. E., Brown, D. S., Bleach, C. M., **Pathrose, B**., Barclay, C., Jaspers, M. V. and Ridgway, H. J. (2011). First report of *Cylindrocladiella parva* as a grapevine pathogen in New Zealand. *Plant Disease, 96*(1), 144-144.
- **Pathrose, B**., Jones, E. E., Jaspers, M. V. and Ridgway, H. J. (2010). Development of a grapevine detached root assay for assessing pathogenicity of *Cylindrocarpon* spp. *New Zealand plant protection, 63*, 24-27.

Conference presentation:

- **Pathrose, B**., Jones, E.E., Jaspers, M. V. and Ridgway, H. J. (2011). *Species composition and genetic diversity of Cylindrocarpon species found commonly infecting New Zealand grapevines.* Paper presented at the 4th Asian Conference on Plant Pathology concurrent with the 18th Biennial Australasian Plant Pathology Society Conference, Darwin, Australia.

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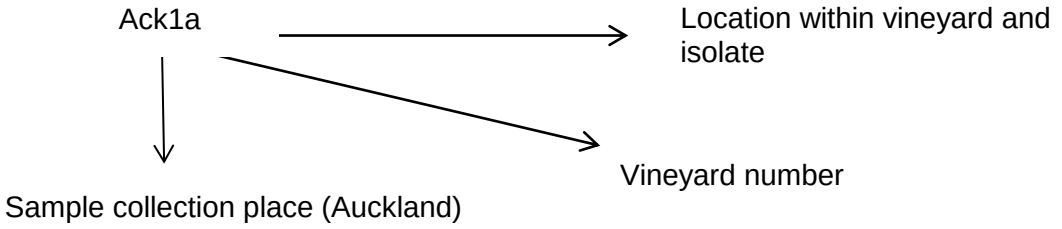
Appendix A

A.1 Codes and numbers used to name the samples and isolates obtained in the survey

Place of sample	Code used
Auckland	Ack
Gisborne	Gis
Hawkes Bay	Hb
Martinborough	Mtb
Nelson	Nel
Marlborough	Mar
Waipara	Wpa
Central Otago	CO

For example; culture code Ack1a indicates;



A.2 List of *Cylindrocarpon* species isolates used for sequencing with β-tubulin, translation elongation factor 1α and ITS gene sequences

A.2.1 Beta tubulin sequences of *Cylindrocarpon macrodidymum* isolates

Ack2i

```
1       CTACTTCAAC GAGGTACGTG ATCAAACCCT GCTGCCTGCT CTGCCTCTGG
51      AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
101     CGCCGTCCTT GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG
151     GCCCCTTCGG CCAGCTCTTC CGCCCCGACA ACTTCGTTTT CGGTCAGTCC
201     GGTGCTGGAA ACAACTGGGC CAAGGGTCAC T
```

Mar16h

```
1       CTACTTCAAC GAGGTACGTG ATCAAACCCT GCTGCCTGCT CAGCCTCTGG
51      AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
101     CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG
151     GCCCCTTCGG CCAGCTCTTC CGCCCCGACA ACTTCGTTTT CGGTCAGTCC
201     GGTGCTGGAA ACAACTGGGC CAAGGGTCAC T
```

Hb6e

```
1       CTACTTCAAC GAGGTACGTG ATCAAACCCT GCTGCCTGCT CAGCCTCTGG
51      AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
101     CGCCGTCCTC GTCGATCTTG AGCCCGGTAC CATGGACGCC GTCCGTGCCG
151     GCCCCTTCGG CCAGCTCTTC CGCCCCGACA ACTTCGTTTT CGGTCAGTCC
201     GGTGCTGGAA ACAACTGGGC CAAGGGTCAC T
```

Gis3b

```
1       CTACTTCAAC GAGGTACGTG ATCAAACCCT GCTGCCTGCT CAGCCTCTGG
51      AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
101     CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG
151     GCCCCTTCGG CCAGCTCTTC CGCCCCGACA ACTTCGTTTT CGGTCAGTCC
201     GGTGCTGGAA ACAACTGGGC CAAGGGTCAC T
```

Ack2j

```
1       CTACTTCAAC GAGGTACGTG ATCAAACCCT GCTGCCTGCT CAGCCTCTGG
51      AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
101     CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG
151     GCCCCTTCGG CCAGCTCTTC CGCCCCGACA ACTTCGTTTT CGGTCAGTCC
201     GGTGCTGGAA ACAACTGGGC CAAGGGTCAC T
```

A.2.2 Beta tubulin sequences of *Cylindrocarpon pauciseptatum* isolates

Mar6a

```
1       TACTTCAACG AGGTACGTGA ATAAACTCTG CTGCCTGCTC GGCCGCTGGA
51      ACCACGAAAC TCACACCACC CAGGCCTCTG GCAACAAGTA TGTCCCTCGC
101     GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCCG TCCGTGCCGG
151     CCCCTTCGGC CAGCTCTTCC GTCCCGACAA CTTTGTTTTC GGTCAGTCCG
201     GTGCTGGAAA CAACTGGGCC AAGGGTCAC
```

Mar14b

```
1       TACTTCAACG AGGTACGTGA ATAAACTCTG CTGCCTGCTC GGCCGCTGGA
51      ACCACGAAAC TCACACCACC CAGGCTTCTG GCAACAAGTA TGTCCCTCGC
101     GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCCG TCCGTGCCGG
151     CCCCTTCGGC CAGCTCTTCC GTCCCGACAA CTTTGTTTTC GGTCAGTCCG
201     GTGCTGGAAA CAACTGGGCC AAGGGTCAC
```

Hb6b

```
1       TACTTCAACG AGGTACGTGA ATAAACTCTG CTGCCTGCTC GGCCGCTGGA
51      ACCACGAAAC TCACACCACC CAGGCTTCTG GCAACAAGTA TGTCCCTCGC
101     GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCCG TCCGTGCCGG
151     CCCCTTCGGC CAGCTCTTCC GTCCCGACAA CTTTGTTTTC GGTCAGTCCG
201     GTGCTGGAAA CAACTGGGCC AAGGGTCAC
```

Ack2e

```
1       TACTTCAACG AGGTACGTGA ATAAACTCTG CTGCCTGCTC GGCCGCTGGA
51      ACCACGAAAC TCACACCACC CAGGCTTCTG GCAACAAGTA TGTCCCTCGC
101     GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCCG TCCGTGCCGG
151     CCCCTTCGGC CAGCTCTTCC GTCCCGACAA CTTTGTTTTC GGTCAGTCCG
201     GTGCTGGAAA CAACTGGGCC AAGGGTCAC
```

Ack2b

```
1       TACTTCAACG AGGTACGTGA ATAAACTCTG CTGCCTGCTC GGCCGCTGGA
51      ACCACGAAAC TCACACCACC CAGGCTTCTG GCAACAAGTA TGTCCCTCGC
101     GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCCG TCCGTGCCGG
151     CCCCTTCGGC CAGCTCTTCC GTCCCGACAA CTTTGTTTTC GGTCAGTCCG
201     GTGCTGGAAA CAACTGGGCC AAGGGTCAC
```

A.2.3 Beta tubulin sequences of *Cylindrocarpon destructans* isolates

Mar7a

```
1       ACTTCAACGA GGTACGTGAA ATCTACTCAT CTGCCCCTAT CCAGGAGGTG
51      TCGAACTCAC ACCACGCAGG CTTCTGGAAA CAAGTATGTC CCTCGCGCCG
101     TTCTCGTCGA TCTCGAGCCC GGTACCATGG ACGCTGTCCG TGCCGGTCCT
151     TTCGGCCAGC TCTTCCGCCC CGACAACTTC GTCTTCGGTC AGTCCGGTGC
201     CGGCAACAAC TGGGCCAAGG GTCACT
```

Mar9a

```
1       ACTTCAACGA GGTACGTGGA ATCTACTCAT CTGCCCCTAT CCAAGAGCTG
51      TTGAACTCAC ACTAGGCAGG CCTCTGGAAA CAAGTATGTC CCTCGCGCCG
101     TCCTTGTCGA TCTCGAGCCC GGTACCATGG ACGCTGTCCG TGCTGGTCCT
151     TTCGGCCAGC TCTTCCGCCC CGACAACTTC GTCTTCGGTC AGTCCGGTGC
201     TGGCAACAAC TGGGCCAAGG GTCACT
```

Mar6b

```
1       ACTTCAACGA GGTACGTGGA ATCTACTCAT CTGCCCCTAT CCAAGAGCTG
51      TTGAACTCAC ACTAGGCAGG CCTCTGGAAA CAAGTATGTC CCTCGCGCCG
101     TCCTTGTCGA TCTCGAGCCC GGTACCATGG ACGCTGTCCG TGCTGGTCCT
151     TTCGGCCAGC TCTTCCGCCC CGACAACTTC GTCTTCGGTC AGTCCGGTGC
201     TGGCAACAAC TGGGCCAAGG GTCACT
```

Mar5d

```
1       ACTTCAACGA GGTACGTGGA ATCTACTCAT CTGCCCCTAT CCAAGAGCTG
51      TTGAACTCAC ACTAGGCAGG CCTCTGGAAA CAAGTATGTC CCTCGCGCCG
101     TCCTTGTCGA TCTCGAGCCC GGTACCATGG ACGCTGTCCG TGCTGGTCCT
151     TTCGGCCAGC TCTTCCGCCC CGACAACTTC GTCTTCGGTC AGTCCGGTGC
201     TGGCAACAAC TGGGCCAAGG GTCACT
```

Mtb1d

```
1       ACTTCAACGA GGTACGTGGA ATCTACTCAT CTGCCCCTAT CCAAGAGCTG
51      TTGAACTCAC ACTAGGCAGG CCTCTGGAAA CAAGTATGTC CCTCGCGCCG
101     TCCTTGTCGA TCTCGAGCCC GGTACCATGG ACGCTGTCCG TGCTGGTCCT
151     TTCGGCCAGC TCTTCCGCCC CGACAACTTC GTCTTCGGTC AGTCCGGTGC
201     TGGCAACAAC TGGGCCAAGG GTCACT
```

A.2.4 Beta tubulin sequences of *Cylindrocarpon liriodendri* isolates

Hb1a

```
1       CTACTTCAAC GAGGTACGCG AAATCTGCTC TGCCCCTATA CATGAAGTGT
51      CAAACTCACA CCACGTAGGC CTCTGGAAAC AAGTATGTCC CTCGCGCCGT
101     CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCTGTCCGT GCCGGTCCTT
151     TCGGCCAGCT CTTCCGCCCC GACAACTTCG TCTTCGGTCA GTCCGGTGCT
201     GGCAACAACT GGGCCAAGGG TCACTAC
```

Mar4g

```
1       CTACTTCAAC GAGGTACGCG AAATCTGCTC TGCCCCTATA CATGAAGTGT
51      CAAACTCACA CCACGTAGGC CTCTGGAAAC AAGTATGTCC CTCGCGCCGT
101     CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCTGTCCGT GCCGGTCCTT
151     TCGGCCAGCT CTTCCGCCCC GACAACTTCG TCTTCGGTCA GTCCGGTGCT
201     GGCAACAACT GGGCCAAGGG TCACTAC
```

Mar16c

```
1       CTACTTCAAC GAGGTACGCG AAATCTGCTC TGCCCCTATA CATGAAGTGT
51      CAAACTCACA CCACGTAGGC CTCTGGAAAC AAGTATGTCC CTCGCGCCGT
101     CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCTGTCCGT GCCGGTCCTT
151     TCGGCCAGCT CTTCCGCCCC GACAACTTCG TCTTCGGTCA GTCCGGTGCT
201     GGCAACAACT GGGCCAAGGG TCACTAC
```

Mar16d

```
1       CTACTTCAAC GAGGTACGCG AAATCTGCTC TGCCCCTATA CATGAAGTGT
51      CAAACTCACA CCACGTAGGC CTCTGGAAAC AAGTATGTCC CTCGCGCCGT
101     CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCTGTCCGT GCCGGTCCTT
151     TCGGCCAGCT CTTCCGCCCC GACAACTTCG TCTTCGGTCA GTCCGGTGCT
201     GGCAACAACT GGGCCAAGGG TCACTAC
```

Mar22d

```
1       CTACTTCAAC GAGGTACGCG AAATCTGCTC TGCCCCTATA CATGAAGTGT
51      CAAACTCACA CCACGTAGGC CTCTGGAAAC AAGTATGTCC CTCGCGCCGT
101     CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCTGTCCGT GCCGGTCCTT
151     TCGGCCAGCT CTTCCGCCCC GACAACTTCG TCTTCGGTCA GTCCGGTGCT
201     GGCAACAACT GGGCCAAGGG TCACTAC
```

A.2.5 Beta tubulin sequences of *Cylindrocarpon sp.* isolates

Hb6a

```
1       TCTCGACAGC AATGGTGTCT ACAACGGCAC CTCCGAGCTC CAGCTCGAGC
51      GTATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
101     CGCCCTTGGT AGCACGAAAC TCACACTATT TAGGCCGCTG GCAACAAGTA
151     TGTCCCTCGC GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCTG
201     TCCGTGCCGG TCCCTTCGGC CAGCTCTTCC GCCCTGACAA CTTCGTTTTC
251     GGTCAGTCCG GTGCTGGAAA CAACTGGGCC AAGGGTCACT
```

Mar1a

```
1       TCTCGACAGC AATGGTGTCT ACAACGGCAC CTCCGAGCTC CAGCTCGAGC
51      GTATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
101     CGCCCTTGGT AGCACGAAAC TCACACTATT TAGGCCGCTG GCAACAAGTA
151     TGTCCCTCGC GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCTG
201     TCCGTGCCGG TCCCTTCGGC CAGCTCTTCC GCCCTGACAA CTTCGTTTTC
251     GGTCAGTCCG GTGCTGGAAA CAACTGGGCC AAGGGTCACT
```

Mar1d

```
1       TCTCGACAGC AATGGTGTCT ACAACGGCAC CTCCGAGCTC CAGCTCGAGC
51      GTATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
101     CGCCCTTGGT AGCACGAAAC TCACACTATT TAGGCCGCTG GCAACAAGTA
151     TGTCCCTCGC GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCTG
201     TCCGTGCCGG TCCCTTCGGC CAGCTCTTCC GCCCTGACAA CTTCGTTTTC
251     GGTCAGTCCG GTGCTGGAAA CAACTGGGCC AAGGGTCACT
```

Gis5a

```
1       TCTCGACAGC AATGGTGTCT ACAACGGCAC CTCCGAGCTC CAGCTCGAGC
51      GCATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT ACTGCCTGCG
101     TTTTGGAAGC ACGAAACTCA CACACCACCT AGGCTTCTGG CAACAAGTAT
151     GTCCCTCGCG CCGTCCTCGT CGATCTCGAG CCCGGTACCA TGGACGCTGT
201     CCGTGCCGGC CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTCGTTTTCG
251     GTCAGTCCGG TGCTGGAAAC AACTGGGCCA AGGGTCACT
```

A.2.6 Beta tubulin sequences of *Cylindrocladiella sp.* isolate

Co1e

```
1       TACTTCAACG AGGTACGTGA TTATAACACT CATTTTATCA CATTGAAGAT
51      TCTCAATGTA CTCACACATT CTAGGCTTCC GGCAACAAGT ATGTCCCTCG
101     CGCCGTCCTC GTCGATCTTG AGCCCGGTAC CATGGATGCC GTCCGTGCCG
151     GTCCTTTCGG TCAGCTCTTC CGCCCCGACA ACTTCGTCTT CGGTCAGTCC
201     GGTGCCGGAA ACAACTGGGC CAAGGGTCAC TACACTGAG
```

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN098743.1	Cylindrocladiella sp. CBS 122595 beta-tubulin gene, partial cds	442	442	100%	5e-121	100%
JN098730.1	Cylindrocladiella sp. CBS 199.62 beta-tubulin gene, partial cds	442	442	100%	5e-121	100%
JN098823.1	Cylindrocladiella sp. LL-2011l strain CBS 122594 beta-tubulin gene, partial cds	442	442	100%	5e-121	100%
JN098724.1	Cylindrocladiella sp. CBS 139.26 beta-tubulin gene, partial cds	436	436	100%	3e-119	99%

A.2.7 Beta tubulin sequences of *Nectria hematococca* isolate

Hb6c

```
1       TACTTCAACG AGGTATGTTG TCCGGCAGCT GTGCAAATCT AGCTGACATT
51      TGTAGGCCTC TGGAACCAAG TACGTCCCTC GCGCCGTCCT CGTCGATCTT
```

```
101     GAGCCCGGTA CCATGGACGC CGTTCGTGCT GGTCCCTTCG GTCAGCTCTT
151     CCGTCCCGAC AACTTCGTCT TCGGTCAGTC CGGTGCTGGC AACAACTGGG
201     CCAAGGATCA CTAC
```

Mar10e

```
1       TTACAACGGC ACCTCTGAGC TCCAGCTCGA GCGCATGAGC GTCTACTTCA
51      ACGAGGTATG TTGTCCGGCA GCTGTGCCAA ATCTAGCTGA CATTTGTAGG
101     CCTCTGGCAA CAAGTACGTC CCTCGCGCCG TCCTCGTCGA TCTTGAGCCC
151     GGTACCATGG ACGCCGTTCG TGCTGGTCCC TTCGGTCAGC TCTTCCGTCC
201     CGACAACTTC GTCTTCGGTC AGTCCGGTGC TGGCAACAAC TGGGCCAAGG
251     GTCACTAC
```

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JQ265767.1	Nectria haematococca strain 156-30-6 beta-tubulin gene, partial cds >gb|JQ265768.1| Nectria haematococca strain 241-1-1 beta-tubulin gene, partial cds	477	477	100%	2e-131	100%
JQ265764.1	Nectria haematococca strain 62-22 beta-tubulin gene, partial cds	477	477	100%	2e-131	100%
JQ265766.1	Nectria haematococca strain 55-5-1 beta-tubulin gene, partial cds	368	368	100%	8e-99	98%
JQ265765.1	Nectria haematococca strain 196-10-7 beta-tubulin gene, partial cds	368	368	100%	8e-99	98%
JQ265763.1	Nectria haematococca strain 96-17 beta-tubulin gene, partial cds	368	368	100%	8e-99	98%

A.2.8 Translation elongation factor 1α gene sequences of *Cylindrocarpon macrodidymum* isolates

Ack2i

```
1       GATCGTCGGT CGATTCCCAC GTCGCCGGTC CTGCACCCGA CGCACATTCT
51      CACCCCTCGA TCAAAATTTC CACACTGCAA TTTTTTTGGT GGGGCGAATT
101     TTACCCCTCC ACACATTTGT GGTTGAAATT TGCCCCGCCC CACCCTAGCA
151     TCTCGACCAC AAACTCGAGC CTCGTCACAT ACTATGCACA GAATACTGAC
201     AACGTCGCCT TACAGGAAGC CGCTGAGCTC GGTAAGGGTT CCTTCAAGT
```

Mar16h

```
1       GATCGTCGGT CGATTCCCAC GTCGCCGGTC CTGCACCCGA CGCACATTCT
51      CACCCCTCGA TCAAAATTTC CACACTGCAA TTTTTTTGGT GGGGCGAATT
101     TTACCCCTCC ACACATTTGT GGTTGAAATT TGCCCCGCCC CACCCTAGCA
151     TCTCAACCAC CAACTCGAGC CTCGTCACAT ACTATGCACA GAATACTGAC
201     AACGTCGCCT TACAGGAAGC CGCTGAGCTC GGTAAGGGTT CCTTCAAGT
```

Hb6e

```
1       GATCGTCGGT CGATTCCCAC GTCGCCGGTC CTGCACCCGA CGCACATTCT
51      CACCCCTCGA TCAAAATTTC CACACTGCAA TTTTTTTGGT GGGGCGAATT
101     TTACCCCTCC ACACATTTGT GGTTGAAATT TGCCCCGCCC CACCCTAGCA
151     TCTCAACCAC CAACTCGAGC CTCGTCACAT ACTATGCACA GAATACTGAC
201     AACGTCGCCT TACAGGAAGC CGCTGAGCTC GGTAAGGGTT CCTTCAAGT
```

Gis3b

```
1       GATCGTCGGT CGATTCCCAC GTCGCCGGTC CTGCACCCGA CGCACATTCT
51      CACCCCTCGA TCAAAATTTC CACACTGCAA TTTTTTTGGT GGGGCGAATT
101     TTACCCCTCC ACACATTTGT GGTTGAAATT TGCCCCGCCC CACCCTAGCA
151     TCTCAACCAC CAACTCGAGC CTCGTCACAT ACTATGCACA GAATACTGAC
201     AACGTCGCCT TACAGGAAGC CGCTGAGCTC GGTAAGGGTT CCCTTCAAGT
```

Ack2j

```
1       GATCGTCGGT CGATTCCCAC GTCGCCGGTC CTGCACCCGA CGCACATTCT
51      CACCCCTCGA TCAAAATTTC CACACTGCAA TTTTTTTGGT GGGGCGAATT
101     TTACCCCTCC ACACATTTGT GGTTGAAATT TGCCCCGCCC CACCCTAGCA
151     TCTCAACCAC CAACTCGAGC CTCGTCACAT ACTATGCACA GAATACTGAC
201     AACGTCGCCT TACAGGAAGC CGCTGAGCTC GGTAAGGGTT CCTTCAAGT
```

A.2.9 Translation elongation factor 1α gene sequences of *Cylindrocarpon pauciseptatum* isolates

Mar6a

```
1       TCGGTCGATT CCCACGTTGC GCTGCTTATG CGCTCGACGC ATATACTCAT
51      CCCTCGATCA ATTTTTTTCC CCACACACTG CATTGTTTTT TTGGTGGGGC
101     GAATTTTACC CCTCCACACA ATCGTGGTCG AAATTTGCCC CACCCCACCC
151     CAGCAACACT GAACCACCAA CCCGAGCGTC GTCACATGCT ATGCACAGAA
201     TACTGACAGA GACGTTTGCA GGAAGCCGCC GAGCTCGGTA AGGGTTCCTT
251     CAAGTAA
```

Mar14b

```
1       TCGGTCGATT CCCACGTTGC GCTGCTTATG CGCTCGACGC ATATACTCAT
51      CCCTCGATCA ATTTTTTTTT CCCACACACT GCATTGTTTT TTTGGTGGGG
101     CGAATTTTAC CCCTCCACAC AATCGTGGTC GAAATTTGCC CCACCCCACC
151     CCAGCAACAC TGAACCACCA ACCCGAGCGT CGTCACATGC TATGCACAGA
201     ATACTGACAG AGACGTTTGC AGGAAGCCGC CGAGCTCGGT AAGGGTTCCT
251     TCAAGTAA
```

Hb6b

```
1       TCGGTCGATT CCCACGTTGC GCTGCTTATG CGCTCGACGC ATATACTCAT
51      CCCTCGATCA ATTTTTTTTT CCCACACACT GCATTGTTTT TTTGGTGGGG
101     CGAATTTTAC CCCTCCACAC AATCGTGGTC GAAATTTGCC CCACCCCACC
151     CCAGCAACAC TGAACCACCA ACCCGAGCGT CGTCACATGC TATGCACAGA
201     ATACTGACAG AGACGTTTGC AGGAAGCCGC CGAGCTCGGT AAGGGTTCCT
251     TCAAGTAA
```

Ack2e

```
1       TCGGTCGATT CCCACGTTGC GCTGCTTATG CGCTCGACGC ATATACTCAT
51      CCCTCGATCA ATTTTTTTTT CCCACACACT GCATTGTTTT TTTGGTGGGG
101     CGAATTTTAC CCCTCCACAC AATCGTGGTC GAAATTTGCC CCACCCCACC
151     CCAGCAACAC TGAACCACCA ACCCGAGCGT CGTCACATGC TATGCACAGA
201     ATACTGACAG AGACGTTTGC AGGAAGCCGC CGAGCTCGGT AAGGGTTCCT
251     TCAAGTAA
```

Ack2b

```
1       TCGGTCGATT CCCACGTTGC GCTGCTTATG CGCTCGACGC ATATACTCAT
51      CCCTCGATCA ATTTTTTTTT CCCACACACT GCATTGTTTT TTTGGTGGGG
101     CGAATTTTAC CCCTCCACAC AATCGTGGTC GAAATTTGCC CCACCCCACC
151     CCAGCAACAC TGAACCACCA ACCCGAGCGT CGTCACATGC TATGCACAGA
201     ATACTGACAG AGACGTTTGC AGGAAGCCGC CGAGCTCGGT AAGGGTTCCT
251     TCAAGTAA
```

A.2.10 Translation elongation factor 1α gene sequences of *Cylindrocarpon destructans* isolates

Mar7a

```
1       GATTTCAACG TCGCTGCGTC TGCCCACGAA ACACAACCCC TCACCCTTCG
51      ATCAAAATTT TTCACCCACC CTCTATTGTT TTTTTTGGTG GGGCGAATTT
101     TACCCCGCCG CACACTGGTG GTTGGAATTT GCCCCGCCCC ACCATAGCAT
151     CATTAAATCA TCATCGCGGG ACTCTTCACA CGCTATGCAC AGAATACTGA
201     CAGTGCCCTC TTACAGGAAG CTGCCGAGCT CGGTAAGGGT T
```

Mar9a

```
1       GATTTCAACG TCGCTGCGTC TGCCCACGAA ACACAACCCC TCACCCTTCG
```

```
51      ATCAAAAATT TTCACCCACC CTCCATTGTT TTTTGGTGGG GGCGAATTTT
101     ACCCCGCCGC ACACTGGTGG TTGAAATTTA CCCCGCCCCA CCACAGCATC
151     ATTAAATCAT CATCGCGGGA CTCTTCAAAC GCTAGGCACA GAATACTGAC
201     AGTGCCCTCT TACAGGAAGC TGCCGAGCTC GGTAAGGGTT
```

Mar6b

```
1       GATTTCAACG TCGCTGCGTC TGCCCACGAA ACACAACCCC TCACCCTTCG
51      ATCAAAAATT TTCACCCACC CTCCATTGTT TTTTGGTGGG GGCGAATTTT
101     ACCCCGCCGC ACACTGGTGG TTGAAATTTA CCCCGCCCCA CCACAGCATC
151     ATTAAATCAT CATCGCGGGA CTCTTCAAAC GCTAGGCACA GAATACTGAC
201     AGTGCCCTCT TACAGGAAGC TGCCGAGCTC GGTAAGGGTT
```

Mar5d

```
1       GATTTCAACG TCGCTGCGTC TGCCCACGAA ACACAACCCC TCACCCTTCG
51      ATCAAAAATT TTCACCCACC CTCCATTGTT TTTTGGTGGG GGCGAATTTT
101     ACCCCGCCGC ACACTGGTGG TTGAAATTTA CCCCGCCCCA CCACAGCATC
151     ATTAAATCAT CATCGCGGGA CTCTTCAAAC GCTAGGCACA GAATACTGAC
201     AGTGCCCTCT TACAGGAAGC TGCCGAGCTC GGTAAGGGTT
```

Mtb1d

```
1       GATTTCAACG TCGCTGCGTC TGCCCACGAA ACACAACCCC TCACCCTTCG
51      ATCAAAAATT TTCACCCACC CTCCATTGTT TTTTGGTGGG GGCGAATTTT
101     ACCCCGCCGC ACACTGGTGG TTGAAATTTA CCCCGCCCCA CCACAGCATC
151     ATTAAATCAT CATCGCGGGA CTCTTCAAAC GCTAGGCACA GAATACTGAC
201     AGTGCCCTCT TACAGGAAGC TGCCGAGCTC GGTAAGGGTT
```

A.2.11 Translation elongation factor 1α gene sequences of *Cylindrocarpon liriodendri* isolates

Hb1a

```
1       GTCGATTTCC ACGTCGCTGC GTGTGCACAC GAAGCACAAC CCCTCACCCT
51      CCGATCAAAA ATTTTCATCC ACCCACCATT ATTTTTTGGT GGGGGCGAAT
101     TTTACCCCGC CGCACACTGG TGGTTGGAAT TTGCCCCGCC CCACCACAGC
151     ATCATCAAAT CATCATCGTG GGCCTCTTCA CATGCTATGC ACAGAATACT
201     GACAGTGCCC CCTTACAGGA AGCTGCCGAG CTCGGTAAGG GTTCCTTCAA
251     GTAA
```

Mar4g

```
1       GTCGATTTCC ACGTCGCTGC GTGTGCACAC GAAGCACAAC CCCTCACCCT
51      CCGATCAAAA ATTTTCATCC ACCCACCATT ATTTTTTGGT GGGGGCGAAT
101     TTTACCCCGC CGCACACTGG TGGTTGGAAT TTGCCCCGCC CCACCACAGC
151     ATCATCAAAT CATCATCGTG GGCCTCTTCA CATGCTATGC ACAGAATACT
201     GACAGTGCCC CCTTACAGGA AGCTGCCGAG CTCGGTAAGG GTTCCTTCAA
251     GTAA
```

Mar16c

```
1       GTCGATTTCC ACGTCGCTGC GTGTGCACAC GAAGCACAAC CCCTCACCCT
51      CCGATCAAAA ATTTTCATCC ACCCACCATT ATTTTTTGGT GGGGGCGAAT
101     TTTACCCCGC CGCACACTGG TGGTTGGAAT TTGCCCCGCC CCACCACAGC
151     ATCATCAAAT CATCATCGTG GGCCTCTTCA CATGCTATGC ACAGAATACT
201     GACAGTGCCC CCTTACAGGA AGCTGCCGAG CTCGGTAAGG GTTCCTTCAA
251     GTAA
```

Mar16d

```
1       GTCGATTTCC ACGTCGCTGC GTGTGCACAC GAAGCACAAC CCCTCACCCT
51      CCGATCAAAA ATTTTCATCC ACCCACCATT ATTTTTTGGT GGGGGCGAAT
101     TTTACCCCGC CGCACACTGG TGGTTGGAAT TTGCCCCGCC CCACCACAGC
151     ATCATCAAAT CATCATCGTG GGCCTCTTCA CATGCTATGC ACAGAATACT
201     GACAGTGCCC CCTTACAGGA AGCTGCCGAG CTCGGTAAGG GTTCCTTCAA
251     GTAA
```

Mar22d

```
1       GTCGATTTCC ACGTCGCTGC GTGTGCACAC GAAGCACAAC CCCTCACCCT
51      CCGATCAAAA ATTTTCATCC ACCCACCATT ATTTTTTGGT GGGGGCGAAT
101     TTTACCCCGC CGCACACTGG TGGTTGGAAT TTGCCCCGCC CCACCACAGC
151     ATCATCAAAT CATCATCGTG GGCCTCTTCA CATGCTATGC ACAGAATACT
201     GACAGTGCCC CCTTACAGGA AGCTGCCGAG CTCGGTAAGG GTTCCTTCAA
251     GTAA
```

A.2.12 Translation elongation factor 1α gene sequences of *Cylindrocarpon sp.* isolates

Hb6a

```
1       TCGATTCCTA CGTCGCCGCT CCTGCACACG ACGCACACTT TTACCCCTCG
51      ATCAAATTTT CCACACTGCA ATTTTTTCTG GTGGGGCGAA TTTTACCCCT
101     CCACAGAATT GTGGTCGAAA TTTGCCCCAC CCCACCCCAG CATCTGAACC
151     ACCAACCCGA GCCTCGTTAT ATGCCATGCA CAGAATACTG ACAGCGTCAT
201     CTTATAGGAA GCCGCCGAGC TCGGTAAGGG TTCCTTCAAG TAA
```

Mar1a

```
1       TCGATTCCTA CGTCGCCGCT CCTGCACACG ACGCACACTT TTACCCCTCG
51      ATCAAATTTT CCACACTGCA ATTTTTTCTG GTGGGGCGAA TTTTACCCCT
101     CCACAGAATT GTGGTCGAAA TTTGCCCCAC CCCACCCCAG CATCTGAACC
151     ACCAACCCGA GCCTCGTTAT ATGCCATGCA CAGAATACTG ACAGCGTCAT
201     CTTATAGGAA GCCGCCGAGC TCGGTAAGGG TTCCTTCAAG TAA
```

Mar1d

```
1       TCGATTCCTA CGTCGCCGCT CCTGCACACG ACGCACACTT TTACCCCTCG
51      ATCAAATTTT CCACACTGCA ATTTTTTCTG GTGGGGCGAA TTTTACCCCT
101     CCACAGAATT GTGGTCGAAA TTTGCCCCAC CCCACCCCAG CATCTGAACC
151     ACCAACCCGA GCCTCGTTAT ATGCCATGCA CAGAATACTG ACAGCGTCAT
201     CTTATAGGAA GCCGCCGAGC TCGGTAAGGG TTCCTTCAAG TAA
```

Gis5a

```
1       TCGATTCCCA CGTCGCCGCT TCTGCATCCG ACGCACACAC TCACCCCTCG
51      ATCAAAATTT CCACACTGCA ATTTTTTTTG GTGGGGCGAA TTTTACCCCT
101     CCACACAATT GTGGTCGAAA TTTGCCCCGC CCCACCCCAG CATTTGAACC
151     ACCATTCCGA GCCTCGTCAC ATGCTATGCA CAGGATACTG ACAGCCTCGT
201     CTTACAGGAA GCCGCCGAGC TCGGTAAGGG TTCCTTCAAG TAA
```

A.2.13 Internal transcribed spacer (ITS) region sequences of *Cylindrocarpon macrodidymum* isolates

Ack2i

```
1       CCTGCGGAGG GATCATTACC GAGTTTACAA CTCCCAAACC CCTGTGAACA
51      TACCTATTTG TTGCCTCGGC GGTGCCTGTT CCGACAGCCC GCCAGAGGAC
101     CCCAAACCCT GATTACATTT AAGAAGTCTT CTGAGTAAAC CGATTAAATA
151     AATCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
201     GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
251     AATCTTTGAA CGCACATTGC GCCCGCTAGT ATTCTGGCGG GCATGCCTGT
301     CCGAGCGTCA TTTCAACCCT CAAGCCCCCG GGCTTGGTGT TGGGGATCGG
351     CGAGCCTCCG CGCCCGCCGT CCCCTAAATC TAGTGGCGGT CTCGCTGTAG
401     CTTCCTCTGC GTAGTAGCAC ACCTCGCACT GGGAAACAGC GCGGCCACGC
451     CGTTAAACCC CCAA
```

Mar16h

```
1       CCTGCGGAGG GATCATTACC GAGTTTACAA CTCCCAAACC CCTGTGAACA
51      TACCTATTTG TTGCCTCGGC GGTGCCTGTT CCGACAGCCC GCCAGAGGAC
101     CCCAAACCCT GATTACATTT AAGAAGTCTT CTGAGTAAAC CGATTAAATA
151     AATCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
201     GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
251     AATCTTTGAA CGCACATTGC GCCCGCTAGT ATTCTGGCGG GCATGCCTGT
301     CCGAGCGTCA TTTCAACCCT CAAGCCCCCG GGCTTGGTGT TGGGGATCGG
```

```
351    CGAGCCTCCG CGCCCGCCGT CCCCTAAATC TAGTGGCGGT CTCGCTGTAG
401    CTTCCTCTGC GTAGTAGCAC ACCTCGCACT GGGAAACAGC GCGGCCACGC
451    CGTTAAACCC CCAA
```

Hb6e

```
1      CCTGCGGAGG GATCATTACC GAGTTTACAA CTCCCAAACC CCTGTGAACA
51     TACCTATTTG TTGCCTCGGC GGTGCCTGTT CCGACAGCCC GCCAGAGGAC
101    CCCAAACCCT GATTACATTT AAGAAGTCTT CTGAGTAAAC CGATTAAATA
151    AATCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
201    GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
251    AATCTTTGAA CGCACATTGC GCCCGCTAGT ATTCTGGCGG GCATGCCTGT
301    CCGAGCGTCA TTTCAACCCT CAAGCCCCCG GGCTTGGTGT TGGGGATCGG
351    CGAGCCTCCG CGCCCGCCGT CCCCTAAATC TAGTGGCGGT CTCGCTGTAG
401    CTTCCTCTGC GTAGTAGCAC ACCTCGCACT GGGAAACAGC GCGGCCACGC
451    CGTTAAACCC CCAA
```

Gis3b

```
1      CCTGCGGAGG GATCATTACC GAGTTTACAA CTCCCAAACC CCTGTGAACA
51     TACCTATTTG TTGCCTCGGC GGTGCCTGTT CCGACAGCCC GCCAGAGGAC
101    CCCAAACCCT GATTACATTT AAGAAGTCTT CTGAGTAAAC CGATTAAATA
151    AATCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
201    GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
251    AATCTTTGAA CGCACATTGC GCCCGCTAGT ATTCTGGCGG GCATGCCTGT
301    CCGAGCGTCA TTTCAACCCT CAAGCCCCCG GGCTTGGTGT TGGGGATCGG
351    CGAGCCTCCG CGCCCGCCGT CCCCTAAATC TAGTGGCGGT CTCGCTGTAG
401    CTTCCTCTGC GTAGTAGCAC ACCTCGCACT GGGAAACAGC GCGGCCACGC
451    CGTTAAACCC CCAA
```

Ack2j

```
1      CCTGCGGAGG GATCATTACC GAGTTTACAA CTCCCAAACC CCTGTGAACA
51     TACCTATTTG TTGCCTCGGC GGTGCCTGTT CCGACAGCCC GCCAGAGGAC
101    CCCAAACCCT GATTACATTT AAGAAGTCTT CTGAGTAAAC CGATTAAATA
151    AATCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
201    GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
251    AATCTTTGAA CGCACATTGC GCCCGCTAGT ATTCTGGCGG GCATGCCTGT
301    CCGAGCGTCA TTTCAACCCT CAAGCCCCCG GGCTTGGTGT TGGGGATCGG
351    CGAGCCTCCG CGCCCGCCGT CCCCTAAATC TAGTGGCGGT CTCGCTGTAG
401    CTTCCTCTGC GTAGTAGCAC ACCTCGCACT GGGAAACAGC GCGGCCACGC
451    CGTTAAACCC CCAA
```

A.2.14 Internal transcribed spacer (ITS) region sequences of *Cylindrocarpon pauciseptatum* isolates

Mar6a

```
1      AACCTGCGGA GGGATCATTA CCGAGTTTTC AACTCCCAAA CCCCTGTGAA
51     CATACCATTT TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG
101    ACCCCAAACC CAAACTTCCT TGAGTGAGTC TTCTGAGTAA CCGATTAAAT
151    CAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
201    CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC
251    GAATCTTTGA ACGCACATTG CGCCCGCCAG TATTCTGGCG GGCATGCCTG
301    TTCGAGCGTC ATTACATCCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG
351    GCGAGCCTCA GCGCCCGCCG TCCCCTAAAT TTAGTGGCGG TCACGCTGTA
401    ACTTCCTCTG CGTAGTAGCA CACTTAGCAC TGGGAAACAG CGCGGCCACG
451    CCGTAAAACC CCCCAA
```

Mar14b

```
1      AACCTGCGGA GGGATCATTA CCGAGTTTTC AACTCCCAAA CCCCTGTGAA
51     CATACCATTT TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG
101    ACCCCAAACC CAAACTTCCT TGAGTGAGTC TTCTGAGTAA CCGATTAAAT
151    CAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
201    CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC
251    GAATCTTTGA ACGCACATTG CGCCCGCCAG TATTCTGGCG GGCATGCCTG
```

```
301    TTCGAGCGTC ATTACATCCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG
351    GCGAGCCTCA GCGCCCGCCG TCCCCTAAAT TTAGTGGCGG TCACGCTGTA
401    ACTTCCTCTG CGTAGTAGCA CACTTAGCAC TGGGAAACAG CGCGGCCACG
451    CCGTAAAACC CCCCAA
```

Hb6b

```
1      AACCTGCGGA GGGATCATTA CCGAGTTTTC AACTCCCAAA CCCCTGTGAA
51     CATACCATTT TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG
101    ACCCCAAACC CAAACTTCCT TGAGTGAGTC TTCTGAGTAA CCGATTAAAT
151    CAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
201    CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC
251    GAATCTTTGA ACGCACATTG CGCCCGCCAG TATTCTGGCG GGCATGCCTG
301    TTCGAGCGTC ATTACATCCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG
351    GCGAGCCTCA GCGCCCGCCG TCCCCTAAAT TTAGTGGCGG TCACGCTGTA
401    ACTTCCTCTG CGTAGTAGCA CACTTAGCAC TGGGAAACAG CGCGGCCACG
451    CCGTAAAACC CCCCAA
```

Ack2e

```
1      AACCTGCGGA GGGATCATTA CCGAGTTTTC AACTCCCAAA CCCCTGTGAA
51     CATACCATTT TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG
101    ACCCCAAACC CAAACTTCCT TGAGTGAGTC TTCTGAGTAA CCGATTAAAT
151    CAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
201    CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC
251    GAATCTTTGA ACGCACATTG CGCCCGCCAG TATTCTGGCG GGCATGCCTG
301    TTCGAGCGTC ATTACATCCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG
351    GCGAGCCTCA GCGCCCGCCG TCCCCTAAAT TTAGTGGCGG TCACGCTGTA
401    ACTTCCTCTG CGTAGTAGCA CACTTAGCAC TGGGAAACAG CGCGGCCACG
451    CCGTAAAACC CCCCAA
```

Ack2b

```
1      AACCTGCGGA GGGATCATTA CCGAGTTTTC AACTCCCAAA CCCCTGTGAA
51     CATACCATTT TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG
101    ACCCCAAACC CAAACTTCCT TGAGTGAGTC TTCTGAGTAA CCGATTAAAT
151    CAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
201    CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC
251    GAATCTTTGA ACGCACATTG CGCCCGCCAG TATTCTGGCG GGCATGCCTG
301    TTCGAGCGTC ATTACATCCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG
351    GCGAGCCTCA GCGCCCGCCG TCCCCTAAAT TTAGTGGCGG TCACGCTGTA
401    ACTTCCTCTG CGTAGTAGCA CACTTAGCAC TGGGAAACAG CGCGGCCACG
451    CCGTAAAACC CCCCAA
```

A.2.15 Internal transcribed spacer (ITS) region sequences of *Cylindrocarpon destructans* isolates

Mar7a

```
1      ACTCCCAAAC CCCTGTGAAC ATACCATTTG TTGCCTCGGC GGTGCCTGCT
51     TCGGCAGCCC GCCAGAGGAC CCAAACCCTT GATTTTATAC AGTATCTTCT
101    GAGTAAATGA TTAAATAAAT CAAAACTTTC AACAACGGAT CTCTTGGTTC
151    TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG
201    AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC CGCCAGTATT
251    CTGGCGGGCA TGCCTGTTCG AGCGTCATTT CAACCCTCAA GCCCCCGGGC
301    TTGGTGTTGG AGATCGGCGT GCCCCCCGGG GCGCGCCGGC TCCCAAATAT
351    AGTGGCGGTC TCGCTGTAGC TTCCTCTGCG TAGTAGCACA CCTCGCACTG
401    GAAAACAGCG TGGCCACGCC GTTAAACCCC CC
```

Mar9a

```
1      ACTCCCAAAC CCCTGTGAAC ATACCATATT GTTGCCTCGG CGGTGCCTGT
51     TTCGGCAGCC CGCCAGAGGA CCCAAACCCT AGATTACATT AAAGTATCTT
101    GTGAGTCAAT GATTAAATCA ATCAAAACTT TCAACAACGG ATCTCTTGGT
151    TCTGGCATCG ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
201    AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGTA
251    TTCTGGCGGG CATGCCTGTC CGAGCGTCAT TTCAACCCTC AAGCCCCCGG
301    GCTTGGTGTT GGAGATCGGC GAGCCCTCCG GGGCGCGCCG TCTCCCAAAT
```

```
351    ATAGTGGCGG TCCCGCTGTA GCTTCCTCTG CGTAGTAGCA CACCTCGCAC
401    TGGGAAACAG CGTGGCCACG CCGTGAAACC CCCC
```

Mar6b

```
1      ACTCCCAAAC CCCTGTGAAC ATACCATATT GTTGCCTCGG CGGTGCCTGT
51     TTCGGCAGCC CGCCAGAGGA CCCAAACCCT AGATTACATT AAAGTATCTT
101    GTGAGTCAAT GATTAAATCA ATCAAAACTT TCAACAACGG ATCTCTTGGT
151    TCTGGCATCG ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
201    AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGTA
251    TTCTGGCGGG CATGCCTGTC CGAGCGTCAT TTCAACCCTC AAGCCCCCGG
301    GCTTGGTGTT GGAGATCGGC GAGCCCTCCG GGGCGCGCCG TCTCCCAAAT
351    ATAGTGGCGG TCCCGCTGTA GCTTCCTCTG CGTAGTAGCA CACCTCGCAC
401    TGGGAAACAG CGTGGCCACG CCGTGAAACC CCCC
```

Mar5d

```
1      ACTCCCAAAC CCCTGTGAAC ATACCATATT GTTGCCTCGG CGGTGCCTGT
51     TTCGGCAGCC CGCCAGAGGA CCCAAACCCT AGATTACATT AAAGTATCTT
101    GTGAGTCAAT GATTAAATCA ATCAAAACTT TCAACAACGG ATCTCTTGGT
151    TCTGGCATCG ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
201    AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGTA
251    TTCTGGCGGG CATGCCTGTC CGAGCGTCAT TTCAACCCTC AAGCCCCCGG
301    GCTTGGTGTT GGAGATCGGC GAGCCCTCCG GGGCGCGCCG TCTCCCAAAT
351    ATAGTGGCGG TCCCGCTGTA GCTTCCTCTG CGTAGTAGCA CACCTCGCAC
401    TGGGAAACAG CGTGGCCACG CCGTGAAACC CCCC
```

Mtb1d

```
1      ACTCCCAAAC CCCTGTGAAC ATACCATATT GTTGCCTCGG CGGTGCCTGT
51     TTCGGCAGCC CGCCAGAGGA CCCAAACCCT AGATTACATT AAAGTATCTT
101    GTGAGTCAAT GATTAAATCA ATCAAAACTT TCAACAACGG ATCTCTTGGT
151    TCTGGCATCG ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
201    AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGTA
251    TTCTGGCGGG CATGCCTGTC CGAGCGTCAT TTCAACCCTC AAGCCCCCGG
301    GCTTGGTGTT GGAGATCGGC GAGCCCTCCG GGGCGCGCCG TCTCCCAAAT
351    ATAGTGGCGG TCCCGCTGTA GCTTCCTCTG CGTAGTAGCA CACCTCGCAC
401    TGGGAAACAG CGTGGCCACG CCGTGAAACC CCCC
```

A.2.16 Internal transcribed spacer (ITS) region sequences of *Cylindrocarpon liriodendri* isolates

Hb1a

```
1      CATACCATTA TCGTTGCCTC GGCGGTGCCC GCTTCGGCGG CCCGCCAGAG
51     GACCCAAACC CTTGATTTTT ATAACAGTAT CTTCTGAGTA AATGATTAAA
101    TCAATCAAAA CTTTCAACAA CGGATCTCTT GGCTCTGGCA TCGATGAAGA
151    ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT
201    CGAATCTTTG AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT
251    GTTCGAGCGT CATTTCAACC CTCAAGCCCC CGGGCTTGGT GTTGGAGATC
301    GGCGAGCCCT CCGGGGCGCG CCGGCTCCCA AATATAGTGG CGGTCCCGCT
351    GTAGCTTCCT CTGCGTAGTA GCACACCTCG CACTGGAAAA CAGCGCGGCC
401    A
```

Mar4g

```
1      CATACCATTA TCGTTGCCTC GGCGGTGCCC GCTTCGGCGG CCCGCCAGAG
51     GACCCAAACC CTTGATTTTT ATAACAGTAT CTTCTGAGTA AATGATTAAA
101    TCAATCAAAA CTTTCAACAA CGGATCTCTT GGCTCTGGCA TCGATGAAGA
151    ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT
201    CGAATCTTTG AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT
251    GTTCGAGCGT CATTTCAACC CTCAAGCCCC CGGGCTTGGT GTTGGAGATC
301    GGCGAGCCCT CCGGGGCGCG CCGGCTCCCA AATATAGTGG CGGTCCCGCT
351    GTAGCTTCCT CTGCGTAGTA GCACACCTCG CACTGGAAAA CAGCGCGGCC
401    A
```

Mar16c

```
1       CATACCATTA TCGTTGCCTC GGCGGTGCCC GCTTCGGCGG CCCGCCAGAG
51      GACCCAAACC CTTGATTTTT ATAACAGTAT CTTCTGAGTA AATGATTAAA
101     TCAATCAAAA CTTTCAACAA CGGATCTCTT GGCTCTGGCA TCGATGAAGA
151     ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT
201     CGAATCTTTG AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT
251     GTTCGAGCGT CATTTCAACC CTCAAGCCCC CGGGCTTGGT GTTGGAGATC
301     GGCGAGCCCT CCGGGGCGCG CCGGCTCCCA AATATAGTGG CGGTCCCGCT
351     GTAGCTTCCT CTGCGTAGTA GCACACCTCG CACTGGAAAA CAGCGCGGCC
401     A
```

Mar16d

```
1       CATACCATTA TCGTTGCCTC GGCGGTGCCC GCTTCGGCGG CCCGCCAGAG
51      GACCCAAACC CTTGATTTTT ATAACAGTAT CTTCTGAGTA AATGATTAAA
101     TCAATCAAAA CTTTCAACAA CGGATCTCTT GGCTCTGGCA TCGATGAAGA
151     ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT
201     CGAATCTTTG AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT
251     GTTCGAGCGT CATTTCAACC CTCAAGCCCC CGGGCTTGGT GTTGGAGATC
301     GGCGAGCCCT CCGGGGCGCG CCGGCTCCCA AATATAGTGG CGGTCCCGCT
351     GTAGCTTCCT CTGCGTAGTA GCACACCTCG CACTGGAAAA CAGCGCGGCC
401     A
```

Mar22d

```
1       CATACCATTA TCGTTGCCTC GGCGGTGCCC GCTTCGGCGG CCCGCCAGAG
51      GACCCAAACC CTTGATTTTT ATAACAGTAT CTTCTGAGTA AATGATTAAA
101     TCAATCAAAA CTTTCAACAA CGGATCTCTT GGCTCTGGCA TCGATGAAGA
151     ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT
201     CGAATCTTTG AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT
251     GTTCGAGCGT CATTTCAACC CTCAAGCCCC CGGGCTTGGT GTTGGAGATC
301     GGCGAGCCCT CCGGGGCGCG CCGGCTCCCA AATATAGTGG CGGTCCCGCT
351     GTAGCTTCCT CTGCGTAGTA GCACACCTCG CACTGGAAAA CAGCGCGGCC
401     A
```

A.2.17 Internal transcribed spacer (ITS) region sequences of *Cylindrocarpon sp.* isolates

Hb6a

```
1       AGGGATCATT ACCGAGTTTA CAACTCCCAA ACCCCTGTGA ACATACCTAT
51      TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG ACCCCAAACC
101     CTGATTACAT TAAAGAATTC TTCTGAGTAA CCGATTAAAT AAATCAAAAC
151     TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAA
201     TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
251     ACGCACATTG CGCCCGCTAG TATTCTGGCG GGCATGCCTG TTCGAGCGTC
301     ATTTCAACCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG GCGAGCCTCC
351     GCGCCCGCCG TCCCCTAAAT CTAGTGGCGG TCTCGCTGTA GCTTCCTCTG
401     CGTAGTAGCA CACCTCGC
```

Mar1a

```
1       AGGGATCATT ACCGAGTTTA CAACTCCCAA ACCCCTGTGA ACATACCTAT
51      TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG ACCCCAAACC
101     CTGATTACAT TAAAGAATTC TTCTGAGTAA CCGATTAAAT AAATCAAAAC
151     TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAA
201     TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
251     ACGCACATTG CGCCCGCTAG TATTCTGGCG GGCATGCCTG TTCGAGCGTC
301     ATTTCAACCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG GCGAGCCTCC
351     GCGCCCGCCG TCCCCTAAAT CTAGTGGCGG TCTCGCTGTA GCTTCCTCTG
401     CGTAGTAGCA CACCTCGC
```

Mar1d

```
1       AGGGATCATT ACCGAGTTTA CAACTCCCAA ACCCCTGTGA ACATACCTAT
51      TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG ACCCCAAACC
101     CTGATTACAT TAAAGAATTC TTCTGAGTAA CCGATTAAAT AAATCAAAAC
```

```
151    TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAA
201    TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
251    ACGCACATTG CGCCCGCTAG TATTCTGGCG GGCATGCCTG TTCGAGCGTC
301    ATTTCAACCC TCAAGCCCCC GGGCTTGGTG TCGGGGATCG GCGAGCCTCC
351    GCGCCCGCCG TCCCCTAAAT CTAGTGGCGG TCTCGCTGTA GCTTCCTCTG
401    CGTAGTAGCA CACCTCGC
```

Gis5a

```
1      AGGGATCATT ACCGAGTTTA CAACTCCCAA ACCCCTGTGA ACATACCTAT
51     TGTTGCCTCG GCGGTGCCTG TTCCGACAGC CCGCCAGAGG ACCCCAAACC
101    CTGATTACAT TAAAGAAGTC TTCTGAGTAA CCGATTAAAT AAATCAAAAC
151    TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAA
201    TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
251    ACGCACATTG CGCCCGCTAG TATTCTGGCG GGCATGCCTG TTCGAGCGTC
301    ATTTCAACCC TCAAGCCCCC GGGCTTGGTG TTGGGGATCG GCGAGCCTCC
351    GCGCCCGCCG TCCCCCAAAT CTAGTGGCGG TCTCGCTGTA GCTTCCTCTG
401    CGTAGTAGCA CACCTCGC
```

A.3 Media Recipes

A.3.1 Potato Dextrose Agar (PDA)

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

A.3.2 Potato Dextrose Broth (PDB)

24 g of potato dextrose broth powder (Difco Laboratories, USA) was added to 1 L of distilled water and sterilised by autoclaving.

A.3.3 Spezieller Nährstoffarmer Agar (SNA)

Sucrose	0.2 g
Glucose	0.2 g
Potassium nitrate (KNO_3)	1.0 g
Potassium phosphate monobasic (KH_2PO_4)	1.0 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5 g
Sodium chloride (NaCl)	0.5 g
Agar	12 g

The above ingredients was added to 1 L of distilled water and sterilized by autoclaving. The agar was cooled to 50°C and then poured into sterile petri dishes and allow to solidify.

A.4 Table shows the distribution of *Cylindrocarpon* species among the main grape growing areas in New Zealand and also includes the analysis of 174 isolates collected from the infected grapevines.

		Species Identification								
		Cylindrocarpon destructans	*Cylindrocarpon liriodendri*	*Cylindrocarpon macrodidymum*	*Cylindrocarpon pauciseptatum*	*Cylindrocarpon sp.*	*Cylindrocladiella parva*	*Neonectria hematococca*	Unidentified	Total
Auckland	Count	2	2	8	2	0	0	0	0	14
	% within place	14.3%	14.3%	57.1%	14.3%	.0%	.0%	.0%	.0%	100.0%
	% within spp. identification	3.8%	3.5%	19.5%	18.2%	.0%	.0%	.0%	.0%	8.0%
Central Otago	Count	6	7	3	1	0	1	0	0	18
	% within place	33.3%	38.9%	16.7%	5.5%	.0%	5.6%	.0%	.0%	100.0%
	% within spp. identification	11.3%	12.3%	7.3%	9.1%	.0%	100.0%	.0%	.0%	10.3%
Gisborne	Count	1	2	8	0	1	0	0	1	13
	% within place	7.7%	15.4%	61.5%	.0%	7.7%	.0%	.0%	7.7%	100.0%
	% within spp. identification	1.8%	3.5%	19.5%	.0%	25.0%	.0%	.0%	20.0%	7.5%
Hawkes Bay	Count	3	8	5	2	1	0	1	1	21
	% within place	14.2%	38.1%	23.8%	9.5%	4.8%	.0%	4.8%	4.8%	100.0%
	% within spp. identification	5.7%	14.0%	12.2%	18.2%	25.0%	.0%	50.0%	20.0%	12.1%
Marlborough	Count	35	31	15	5	2	0	1	3	92
	% within place	38.0%	33.7%	16.3%	5.4%	2.2%	.0%	1.1%	3.%	100.0%
	% within spp. identification	66.0%	54.4%	36.7%	45.5%	50.0%	.0%	50.0%	60.0%	53.0%
Martin borough	Count	1	1	0	1	0	0	0	0	3
	% within place	33.3%	33.3%	.0%	33.3%	.0%	.0%	.0%	.0%	100.0%
	% within spp. identification	1.9%	1.8%	.0%	9.1%	.0%	.0%	.0%	.0%	1.7%
Nelson	Count	2	0	1	0	0	0	0	0	3
	% within place	66.7%	.0%	33.3%	.0%	.0%	.0%	.0%	.0%	100.0%
	% within spp. identification	3.8%	.0%	2.4%	.0%	.0%	.0%	.0%	.0%	1.7%
Waipara	Count	3	6	1	0	0	0	0	0	10
	% within place	30.0%	60.0%	10.0%	.0%	.0%	.0%	.0%	.0%	100.0%
	% within spp. identification	5.7%	10.5%	2.4%	.0%	.0%	.0%	.0%	.0%	5.7%
Total	Count	53	57	41	11	4	1	2	5	174
	% within place	30.5%	32.6%	23.6%	6.3%	2.3%	0.6%	1.1%	2.8%	100.0%
	% within spp. identification	100.0%	100.0%	100.0%	100.0%	100.0%	100%	100.0%	100.0%	100.0%

A.5 Result of statistical analysis

A.5.1 Pearson Chi-Square test for the regional distribution of *Cylindrocarpon* species in New Zealand vineyards

Species	North Island	South Island	Total
Cylindrocarpon destructans	7	46	53
Cylindrocarpon liriodendri	13	44	57
Cylindrocarpon macrodidymum	21	20	41
Cylindrocarpon pauciseptatum	5	6	11
Cylindrocarpon sp.	2	2	4
Total	48	118	166

	Value	df	P
Pearson Chi - Square	19.649	4	0.001

3 cells with expected count less than 5.

A.5.2 Incidence of *Cylindrocarpon* species infection on five different rootstock varieties using one way ANOVA

For *Cylindrocarpon destructans*

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	0.877	0.219	0.88	0.482	0.500
Error	78	19.509	0.250			
Total	82	20.386				

For *Cylindrocarpon macrodidymum*

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	2.662	0.666	3.51	0.011	0.436
Error	78	14.808	0.190			
Total	82	17.470				

For *Cylindrocarpon liriodendri*

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	0.984	0.246	1.01	0.410	0.495
Error	78	19.088	0.245			
Total	82	20.072				

For *Cylindrocarpon pauciseptatum*

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	1.560	0.390	4.71	0.002	0.288
Error	78	6.463	0.083			
Total	82	8.024				

For *Cylindrocladiella parva*

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	0.004	0.001	0.08	0.988	0.112
Error	78	0.9839	0.013			
Total	82	0.9880				

For *Nectria hematococca*

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	0.068	0.017	0.70	0.592	0.155
Error	78	1.884	0.024			
Total	82	1.952				

For unidentified isolates

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	0.277	0.0693	1.22	0.308	0.238
Error	78	4.421	0.057			
Total	82	4.699				

For *Cylindrocarpon sp.* isolates

	df	Sum of squares	Mean square	f	P	LSD
Variety	4	0.286	0.071	1.58	0.187	0.213
Error	78	3.521	0.045			
Total	82	3.807				

A.6 Univariate analysis of variance results for the temperature for fastest growth rate of each *Cylindrocarpon* species

A.6.1 Estimated Marginal means

Species

Estimates

Dependent variable: Optimum temperature

Species			95% confidence Interval	
	Mean	Std. Error	Lower Bound	Upper Bound
C. destructans	17.655[a]	.092	17.468	17.842
C. liriodendri	18.574[a]	.092	18.387	18.761
C. macrodidymum	19.315[a]	.092	19.128	19.502

a. Based on modified population marginal mean.

Pairwise Comparisons

Dependent Variable: Optimum temperature

		c			95% Confidence Interval for Difference	
Species (I)	Species (J)	Mean difference (I – J)	Std. Error	Sig. [c]	Lower Bound	Upper Bound
C. destructans	*C. liriodendri*	-.919[*,a,b]	.130	.000	-1.183	-.655
	C. macrodidymum	-1.660[*,a,b]	.130	.000	-1.924	-1.395
C. liriodendri	*C. destructans*	.919[*,a,b]	.130	.000	.655	1.183
	C. macrodidymum	-.741[*,a,b]	.130	.000	-1.005	-.476
C. macrodidymum	*C. destructans*	1.660[*,a,b]	.130	.000	1.395	1.924
	C. liriodendri	.741[*,a,b]	.130	.000	.476	1.005

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. An estimate of the modified population marginal mean (I)

b. An estimate of the modified population marginal mean (J).

I. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

A.6.2 ANOVA results of the optimum temperature for the growth of isolates of *Cylindrocarpon* species

Isolates

Dependent Variable: Optimum temperature

Source	Sum of squares	df	Mean square	F	P value
Hypothesis	19.035	6	3.173	24.91	0.001
Error	4.585	36	0.127		

Dependent Variable: Optimum temperature

			95% Confidence Interval		
Isolate	Species	Mean	Std. Error	Lower Bound	Upper Bound
Ack1a	*C. macrodidymum*	20.469	0.16	20.145	20.793
Co1c	*C. destructans*	18.152	0.16	17.828	18.476
Co3b	*C. liriodendri*	19.392	0.16	19.068	19.715
Gis1b	*C. destructans*	17.859	0.16	17.535	18.182
Gis2d	*C. macrodidymum*	18.671	0.16	18.347	18.995
Mar11b	*C. liriodendri*	18.264	0.16	17.94	18.588
Mar16i	*C. macrodidymum*	18.805	0.16	18.481	19.128
Mar7a	*C. destructans*	16.955	0.16	16.632	17.279
Mar8i	*C. liriodendri*	18.067	0.16	17.743	18.391

A.6.3 Growth rates of *Cylindrocarpon* species at different temperatures

Species	**Temperatures and Growth rate / 24 hr. (mm)**					
	5°C	**10°C**	**15°C**	**20°C**	**25°C**	**30°C**
Cylindrocarpon destructans	1.99	3.51	5.22	6.87	6.02	0.48
Cylindrocarpon liriodendri	1.69	2.09	3.89	6.45	5.63	0.73
Cylindrocarpon macrodidymum	0	1.33	2.76	5.22	4.41	0.76

A.6.4 Phylogenetic tree of *Cylindrocarpon* species

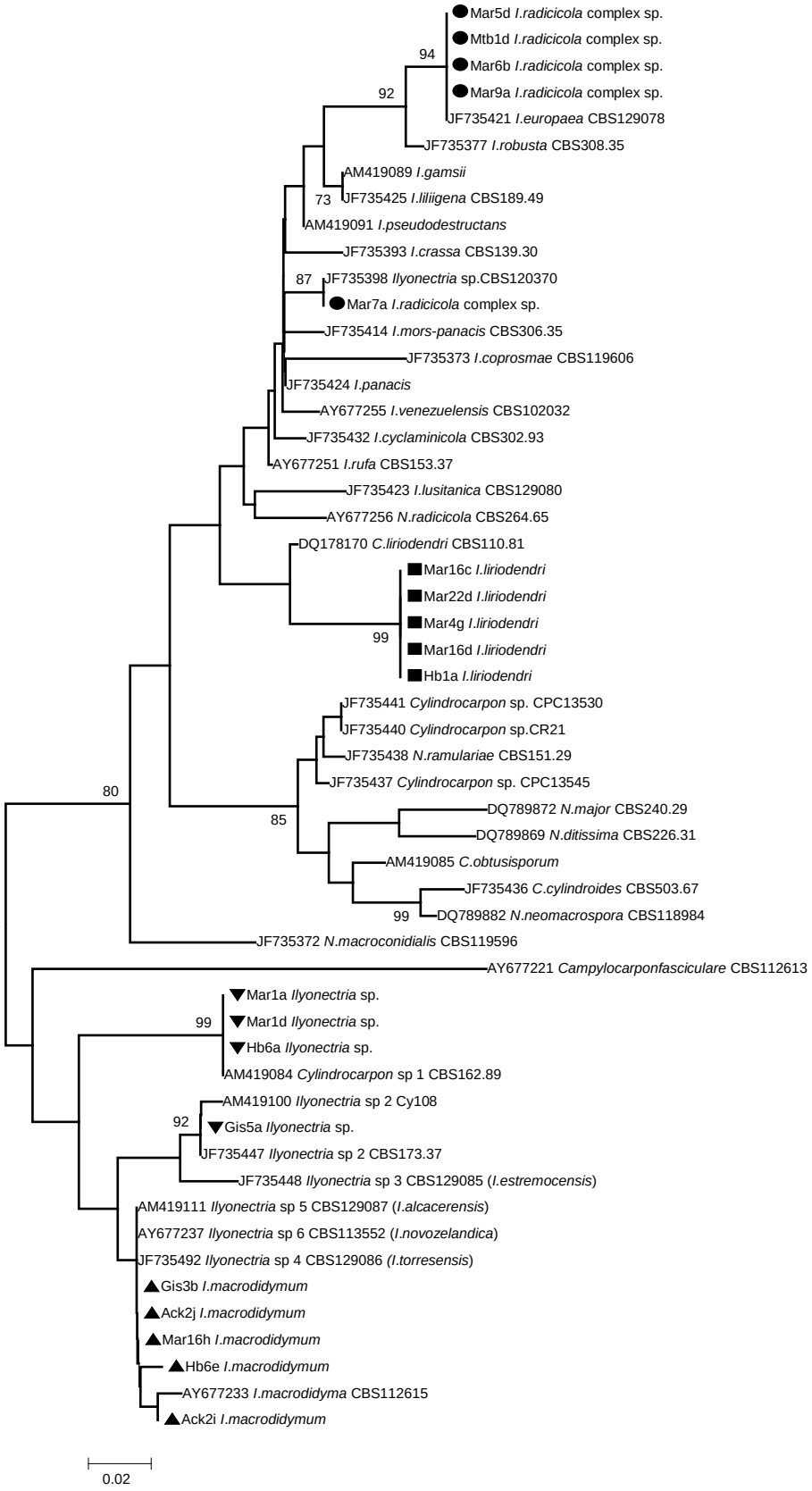


Figure A.7 The neighbour joining tree with bootstrap values using 1000 replicates generated in MEGA 5.05 using β-tubulin gene sequences of*Ilyonectria* species isolated from New Zealand vineyards (inserted with symbols) and isolates representative of the recent classification from GenBank with accession numbers. *Campylocarpon fasciculare* was used as outgroup for the analysis.

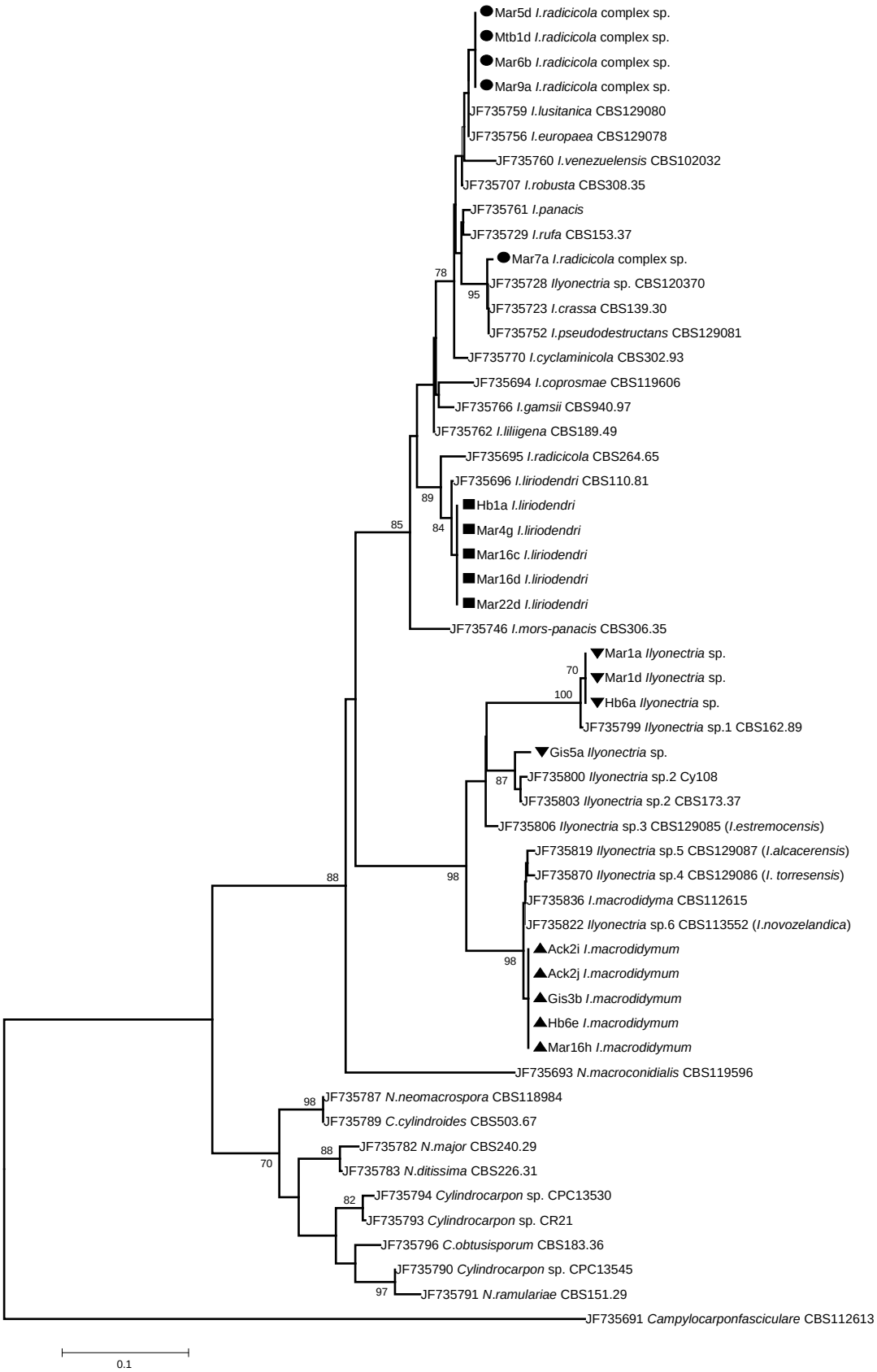


Figure A.8 The neighbour joining tree with bootstrap values using 1000 replicates generated in MEGA 5.05 using translation elongation factor 1α gene sequences of *Ilyonectria* species isolated from New Zealand vineyards (inserted with symbols) and isolates representative of the recent classificationfrom Genbank with accession numbers. *Campylocarpon fasciculare* was used as outgroup for the analysis.

A.7 Beta tubulin primer binding sites for New Zealand *C. destructans* isolates and representatives of the *Ilyonectria radicicola* complex

The beta tubulin forward and reverse primer binding sites were indicated in red.

```
1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         ....................................      0
AM419091 Cylindroca aacatgcgtgagattgtaagttgcctgcctcttgtggt..     38
JF735393 I.crassa   aacatgcgtgagattgtaagttgcctgcctcttgtggt..     38
JF735421 I.europaea .........gagattgtaagttgcttgcctcctgtggt..     29
AM419089 C.destruct aacatgcgtgagattgtaagttgcctgcctcttgtggt..     38
JF735425 I.liliigen aacatgcgtgagattgtaagttgcctgcctcttgtggt..     38
JF735424 I.panacis  aacatgcgtgagattgtaagttgcctacctcttgtggt..     38
JF735377 I.robusta  aacatgcgtgagattgtaagttgcctgcatcttgtggt..     38
RC primer Bt2b      ....................................      0

1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         ....................................      0
AM419091 Cylindroca ..tgcccgttcccaacgacgcgttctgttacctgc.cggg     75
JF735393 I.crassa   ..tgcccgctcccaacgacgcgttctgttacctgctcggg     76
JF735421 I.europaea ..tgcccgctcccaacgacgcgttctgttacctgctcggg     67
AM419089 C.destruct ..tgctcgctcccaacgacgcgttctgtcacctgct..gg     74
JF735425 I.liliigen ..tgctcgctcccaacgacgcgttctgttacctgct..gg     74
JF735424 I.panacis  ..tgcccgctcccaacgacgcgttctgttacctgctcggg     76
JF735377 I.robusta  ..tgcccgctcccaacgacgcgttctgttacctgctcggg     76
RC primer Bt2b      ....................................      0

1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         ....................................      0
AM419091 Cylindroca cga...tgctgcccctgattctaccccgccgcagcatttc    112
JF735393 I.crassa   cga...tgctgcccctgattctaccccgccgcagcatttc    113
JF735421 I.europaea cgatgctgctgcccctgattctaccccgccgcagcagttc    107
AM419089 C.destruct cga...tgctgcccctgattctaccccgccgcagcatttc    111
JF735425 I.liliigen cga...tgctgcccctgattctaccccgccgcagcatttc    111
JF735424 I.panacis  cga...tgctgcccctgattctaccccgccgcagcatttc    113
JF735377 I.robusta  cgatgctgctgcccctgattctaccccgccgcagcatttc    116
RC primer Bt2b      ....................................      0


1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD MtB1D-Bt2a       ....................................      0
PRIMER Bt2a         ....................................      0
AM419091 Cylindroca caccgcctttaggacaacaaagctcgggacttcaaccacg    152
JF735393 I.crassa   caccgcctttaggacaacaaagctcgggacttcaaccacg    153
JF735421 I.europaea caccgcctttaggacaacaaagctcgggacttcaaccacg    147
AM419089 C.destruct caccgccttcaggacaacaaagctcgggacttcaaccacg    151
JF735425 I.liliigen caccgccttcaggacaacaaagctcgggacttccaccacg    151
JF735424 I.panacis  caccgcctttaggacaacaaagctcgggacttcaaccacg    153
JF735377 I.robusta  taccgccttcaggacaacaaagctcgggacttcaaccacg    156
RC primer Bt2b      ....................................      0

1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         ....................................      0
AM419091 Cylindroca acgtgattttgggacaagatggctgaccatgactttcttc    192
JF735393 I.crassa   acgtgattttgggacaagatggctgaccatgactttcttc    193
JF735421 I.europaea acgtgattttgggacaagatggctgaccatgactttcttc    187
AM419089 C.destruct acgtgattttgggacaagatggctgaccatgact...ttc    188
JF735425 I.liliigen acgtgattttgggacaagatggctgaccatgactttcttc    191
JF735424 I.panacis  acgtgattttgggacaagatggctgaccatgactttcttc    193
JF735377 I.robusta  acgtgattttgggacaagatggctgaccatgactttcttc    196
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```
RC primer Bt2b      ....................................      0


1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         ....................................      0
AM419091 Cylindroca tgcaatataggtccacctccagaccggccagtgcgtaagt    232
JF735393 I.crassa   tgcaatataggtccacctccagaccggccagtgcgtaagt    233
JF735421 I.europaea tgcaatataggtccacctccagaccggccagtgcgtaagt    227
AM419089 C.destruct tgcaatataggtccacctccagaccggccagtgcgtaagt    228
JF735425 I.liliigen tgcaatataggtccacctccagaccggccagtgcgtaagt    231
JF735424 I.panacis  tgcaatataggtccacctccagaccggccagtgcgtaagt    233
JF735377 I.robusta  tgcaatataggtccacctccagaccggccagtgcgtaagt    236
RC primer Bt2b      ....................................      0


1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         ............................tgcrggsattc     11
AM419091 Cylindroca gcttcatcct...cttcaacgatccgacgtgcggggattc    269
JF735393 I.crassa   gcttcatcct...cttcaacgatccgacgtgcggggattc    270
JF735421 I.europaea gcttcatcct...cttcaacgatccgacgtgcgggcattc    264
AM419089 C.destruct gcttcatcct...cttcaacgatccgacgtgcggggattc    265
JF735425 I.liliigen gcttcatcct...cttcaacgatccgacgtgcggggattc    268
JF735424 I.panacis  gcttcatcct...cttcaacgatccgacgtgcggggattc    270
JF735377 I.robusta  gcttcatcct...cttcaacgatccgacgtgcaggcattc    273
RC primer Bt2b      ....................................      0

1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
PRIMER Bt2a         gctaacg                                    18
AM419091 Cylindroca gctaacgatgcgtggatagggtaaccaaattggtgctgct    309
JF735393 I.crassa   gctaacgatgcctcgatagggtaaccaaattggtgctgct    310
JF735421 I.europaea gctaacgatgcgtggatagggtaaccaaatcggtgctgct    304
AM419089 C.destruct gctaacgatgcgtggatagggtaaccaaattggtgctgct    305
JF735425 I.liliigen gctaacgatgcgtggatagggtaaccaaattggtgctgct    308
JF735424 I.panacis  gctaacgatgcgtggatagggtaaccaaattggtgctgct    310
JF735377 I.robusta  gctaacgatgcgtggatagggtaaccaaatcggtgctgct    313
RC primer Bt2b      ....................................      0
Consensus

1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
AM419091 Cylindroca ttctggcagaccatctctggcgagcacggtctcgacagca    349
JF735393 I.crassa   ttctggcagaccatctctggcgagcacggtctcgacagca    350
JF735421 I.europaea ttctggcagaccatctctagcgagcacggtctcgacagca    344
AM419089 C.destruct ttctggcagaccatctccggcgagcacggtctcgacagca    345
JF735425 I.liliigen ttctggcagaccatctctggcgagcacggtctcgacagca    348
JF735424 I.pancis   ttctggcagaccatctctggcgagcacggtctcgacagca    350
JF735377 I.robusta  ttctggcagaccatctctagcgagcacggtctcgacagca    353
RC primer Bt2b      ....................................      0


1D Mar9a-Bt2a       ....................................      0
2D Mar6b-Bt2a       ....................................      0
CD Mar7a-Bt2a       ....................................      0
AM419091 Cylindroca atggtgtctacaacggtacctccgagctgcagctcgagcg    389
JF735393 I.crassa   atggtgtctacaacggtacctccgagctgcagctcgagcg    390
JF735421 I.europaea atggtgtctacaacggtacctccgagctgcagctcgagcg    384
AM419089 C.destruct atggtgtctacaacggtacctccgagctgcagctcgagcg    385
JF735425 I.liliigen atggtgtctacaacggtacctccgagctgcagctcgagcg    388
JF735424 I.panacis  atggtgtctacaacggtacctccgagctgcagctcgagcg    390
JF735377 I.robusta  atggtgtctacaacggtacctccgagctgcagctcgagcg    393
RC primer Bt2b      ....................................      0
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1D Mar9a-Bt2a          ..........ACTTCAACGAGGTACGTGGAATCTACTCA     29
2D Mar6b-Bt2a          ..........ACTTCAACGAGGTACGTGGAATCTACTCA     29
CD Mar7a-Bt2a          ..........ACTTCAACGAGGTACGTGGAATCTACTCA     29
AM419091 Cylindroca catgagcgtctACTTCAACGAGGTACGTGaAATCTACTCA     429
JF735393 I.crassa   catgagcgtctACTTCAACGAGGTAtGTGGAATCTACTCA     430
JF735421 I.europaea catgagcgtctACTTCAACGAGGTACGTGGAATCTACTCA     424
AM419089 C.destruct catgagcgtctACTTCAACGAGGTACGTGaAATCcACTCA     425
JF735425 I.liliigen catgagcgtctACTTCAACGAGGTACGTGaAATCcACTCA     428
JF735424 I.panacis  catgagcgtctACTTCAACGAGGTACGTGaAATCTACTCA     430
JF735377 I.robusta  catgagcgtctACTTCAACGAGGTACGTGaAATCTACcCA     433
RC primer Bt2b         ....................................CA       2


1D Mar9a-Bt2a          TCTGCCCCTATCCAAGAGCTGTTGAACT..CACACTAGGC      67
2D Mar6b-Bt2a          TCTGCCCCTATCCAAGAGCTGTTGAACT..CACACTAGGC      67
CD Mar7a-Bt2a          TCTGCCCCTATCCAAGAGCTGTTGAACT..CACACTAGGC      67
AM419091 Cylindroca TCTGCCCCTATCtAgGAGgTGTTGAACT..CACACcAcGC     467
JF735393 I.crassa   TCTGCCCCTATCCAgGAGgTGTcGAACT..CACACcAcGC     468
JF735421 I.europaea TCTGCCCCTATCCAAGAGCTGTTGAACT..CACACTAGGC     462
AM419089 C.destruct TCTGCCCaTATCCAgGAGgTGTTGAACT..CACACcAcGC     463
JF735425 I.liliigen TCTGCCCaTATCCAgGAGgTGTTGAACT..CACACcAcGC     466
JF735424 I.panacis  TCTtCCCCTATCCAgGAGgTGTcGAACT..CACACcAcGC     468
JF735377 I.robusta  TCTGCCCCTATCCAAGAGCTGTTGAACT..CACACTAGGC     471
RC primer Bt2b         TCTGCCCmTATCCArG                           18


1D Mar9a-Bt2a          AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     107
2D Mar6b-Bt2a          AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     107
CD Mar7a-Bt2a          AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     107
AM419091 Cylindroca AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTcGT     507
JF735393 I.crassa   AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTcGT     508
JF735421 I.europaea AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     502
AM419089 C.destruct AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     503
JF735425 I.liliigen AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     506
JF735424 I.panacis  AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTcGT     508
JF735377 I.robusta  AGGCCTCTGGAAACAAGTATGTCCCTCGCGCCGTCCTTGT     511
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A.8 Beta tubulin primer binding site for New Zealand *C. macrodidymum* isolates and representative of the *I. macrodidyma* complex

The beta tubulin reverse and forward primer binding sites were indicated in red.

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AM419084_Cylindroca CCCCTGATTCTACCCCGCTGCAGTATTTCCACCGCCTCGA     40
CM Ack2i-Bt2a          ........................................      0
CM Hb6e-Bt2a           ........................................      0
M1 Gis3b-Bt2a          ........................................      0
M3 Ack2j-Bt2a          ........................................      0
M5 Mar16h-Bt2a         ........................................      0
RevCom.c.macro revp    ........................................      0
C. macro forward pr    ........................................      0
JF735448 Ilyonectri CCCCTGATTCTACCCCGCTGCAGcATTTCCACCGCCTCGA     40
AM419111 Ilyonectri CCCCTGATTCTACCCCGCTGCAGcATTTCCACCGCCTCGA     40
AY677237 Ilyonectri CCCCTGATTCTACCCCGCTGCAGcATTTCCACCGCCTCGA     40
JF735492 Ilyonectri CCCCTGATTCTACCCCGCTGCAGcATTTCCACCGCCTCGA     40
AY677233 I.macrodid CCCCTGATTCTACCCCGCTGCAGcATTTCCACCGCCTCGA     40
AM419100 Ilyonectri CCCCTGATTCTACCCCGCTGCAGcATTTCCACCGCCTCGA     40


AM419084_Cylindroca GCAAAACAATGCCGCGGCTTCAACCACGACGTGATTATGG     80
CM Ack2i-Bt2a          ........................................      0
CM Hb6e-Bt2a           ........................................      0
M1 Gis3b-Bt2a          ........................................      0
M3 Ack2j-Bt2a          ........................................      0
M5 Mar16h-Bt2a         ........................................      0
RC C.macro primer      ........................................      0
C. macro forward pr    ....................................cTGG      4
JF735448_Ilyonectri GCAAAACAAaGCCGCGGCTTCAACCACGgCGTGATTcTGG     80
AM419111_Ilyonectri GCAAAACAAaGCCaCGGCTTCAACCACGACGTGATTcTGG     80
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AY677237_Ilyonectri GCAAAACAAaGCCaCGGCTTCAACCACGACGTGATTcTGG     80
JF735492_Ilyonectri GCAAAACAAaGCCaCGGCTTCAACCACGACGTGATTcTGG     80
AY677233_I.macrodid GCAAAACAAaGCCaCGGCTTCAACCACGACGTGATTcTGG     80
AM419100_Ilyonectri GCAAAACAAaGCCGCGGtTTCAACCACGACGTGATTcTGG     80

AM419084_Cylindroca GACGTGATGGCTAATATGACTTCTTCTGCAATATAGGTCC    120
CM Ack2i-Bt2a       .........................................      0
CM Hb6e-Bt2a        .........................................      0
M1 Gis3b-Bt2a       .........................................      0
M3 Ack2j-Bt2a       .........................................      0
M5 Mar16h-Bt2a      .........................................      0
RevCom.c.macro revp .........................................      0
C. macro forward pr GACaTGATGGCTAATATGACTTCTTg                     30
JF735448_Ilyonectri GACaTGATGGCTAATATGtCTTCTTCTGCAATATAGGTCC    120
AM419111_Ilyonectri GACaTGATGGCTAATATGACTTCTTgTGCAATATAGGTCC    120
AY677237_Ilyonectri GACaTGATGGCTAATATGACTTCTTgTGCAATATAGGTCC    120
JF735492_Ilyonectri GACaTGATGGCTAATATGACTTCTTgTGCAATATAGGTCC    120
AY677233_I.macrodid GACaTGATGGCTAATATGACTTCTTgTGCAATATAGGTCC    120
AM419100_Ilyonectri GACaTGATGGCTAATATGtCTTCTTCTGCAATATAGGTCC    120


AM419084_Cylindroca ACCTCCAGACCGGCCAGTGCGTAAGTGCTTCCTCCTCCTC    160
CM Ack2i-Bt2a       .........................................      0
CM Hb6e-Bt2a        .........................................      0
M1 Gis3b-Bt2a       .........................................      0
M3 Ack2j-Bt2a       .........................................      0
M5 Mar16h-Bt2a      .........................................      0
RevCom.c.macro revp .........................................      0
JF735448_Ilyonectri ACCTCCAGACCGGCCAGTGCGTAAGTGCTTCCTCCTCCTt    160
AM419111_Ilyonectri ACCTCCAGACCGGCCAGTGCGTAAGTGCcTCCTatTCCTC    160
AY677237_Ilyonectri ACCTCCAGACCGGCCAGTGCGTAAGTGCcTCCTCtTCCTC    160
JF735492_Ilyonectri ACCTCCAGACCGGCCAGTGCGTAAGTGCcTCCTCtTCCTC    160
AY677233_I.macrodid ACCTCCAGACCGGCCAGTGCGTAAGTGCcTCCTCtTCCTC    160
AM419100_Ilyonectri ACCTCCAGACCGGCCAGTGCGTAAGTGCTTCCTCCcCCcC    160

AM419084_Cylindroca GACAACACGACGGAA.GAC.TCTAACAATACGTCGATAGG    198
CM Ack2i-Bt2a       .........................................      0
CM Hb6e-Bt2a        .........................................      0
M1 Gis3b-Bt2a       .........................................      0
M3 Ack2j-Bt2a       .........................................      0
M5 Mar16h-Bt2a      .........................................      0
RevCom.c.macro revp .........................................      0
JF735448_Ilyonectri ctCgACgacACGGcAgGAC.TCTAACAAcgCGTgGATAGG    199
AM419111_Ilyonectri GttgACAacACGGcggagatTCTAACAAcgCGTgGATAGG    200
AY677237_Ilyonectri GttgACAacACGGcggagatTCTAACAAcgCGTgGATAGG    200
JF735492_Ilyonectri GttgACAacACGGcggagatTCTAACAAcgCGTgGATAGG    200
AY677233_I.macrodid GttgACAacACGGcggagatTCTAACAAcgCGTgGATAGG    200
AM419100_Ilyonectri ttCAACgacACGacAgGAC.TCTgACAAcgCGcgGATAGG    199


AM419084_Cylindroca GCAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCCGG    238
CM Ack2i-Bt2a       .........................................      0
CM Hb6e-Bt2a        .........................................      0
M1 Gis3b-Bt2a       .........................................      0
M3 Ack2j-Bt2a       .........................................      0
M5 Mar16h-Bt2a      .........................................      0
RevCom.c.macro revp .........................................      0
JF735448_Ilyonectri GtAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCCaG    239
AM419111_Ilyonectri GtAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCtGG    240
AY677237_Ilyonectri GtAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCtGG    240
JF735492_Ilyonectri GtAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCtGG    240
AY677233_I.macrodid GtAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCtGG    240
AM419100_Ilyonectri GtAACCAAATTGGTGCTGCTTTCTGGCAGACCATCTCCGG    239


AM419084_Cylindroca CGAGCATGGTCTCGACAGCAATGGTGTCTACAACGGCACC    278
CM Ack2i-Bt2a       .........................................      0
CM Hb6e-Bt2a        .........................................      0
M1 Gis3b-Bt2a       .........................................      0
M3 Ack2j-Bt2a       .........................................      0
M5 Mar16h-Bt2a      .........................................      0
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RevCom.c.macro revp ....................................      0
JF735448_Ilyonectri CGAGCATGGTCTtGACAGCAATGGTGTCTACAACGGCACC    279
AM419111_Ilyonectri CGAGCATGGTCTtGACAGCAATGGTGTCTACAACGGCACC    280
AY677237_Ilyonectri CGAGCATGGTCTtGACAGCAATGGTGTCTACAACGGCACC    280
JF735492_Ilyonectri CGAGCATGGTCTtGACAGCAATGGTGTCTACAACGGCACC    280
AY677233_I.macrodid CGAGCATGGTCTtGACAGCAATGGTGTCTACAACGGCACC    280
AM419100_Ilyonectri CGAGCATGGTCTCGACAGCAATGGTGTCTACAACGGCACC    279


AM419084_Cylindroca TCCGAGCTCCAGCTCGAGCGTATGAGCGTCTACTTCAACG    318
CM Ack2i-Bt2a       ............................CTACTTCAACG     11
CM Hb6e-Bt2a        ............................CTACTTCAACG     11
M1 Gis3b-Bt2a       ............................CTACTTCAACG     11
M3 Ack2j-Bt2a       ............................CTACTTCAACG     11
M5 Mar16h-Bt2a      ............................CTACTTCAACG     11
RevCom.c.macro revp ....................................      0
JF735448_Ilyonectri TCCGAGCTCCAGCTCGAGCGcATGAGCGTCTACTTCAACG    319
AM419111_Ilyonectri TCCGAGCTCCAGCTCGAGCGcATGAGCGTCTACTTCAACG    320
AY677237_Ilyonectri TCCGAGCTCCAGCTCGAGCGcATGAGCGTCTACTTCAACG    320
JF735492_Ilyonectri TCCGAGCTCCAGCTCGAGCGcATGAGCGTCTACTTCAACG    320
AY677233_I.macrodid TCCGAGCTCCAGCTCGAGCGcATGAGCGTCTACTTCAACG    320
AM419100_Ilyonectri TCCGAGCTCCAGCTCGAGCGcATGAGCGTCTACTTCAACG    319


AM419084_Cylindroca AGGTACGTGAATAAACTCTGCTGCCTGCTCGCCCT....T    354
CM Ack2i-Bt2a       AGGTACGTGAtcAAACcCTGCTGCCTGCTCt...gcctcT     48
CM Hb6e-Bt2a        AGGTACGTGAtcAAACcCTGCTGCCTGCTCa...gcctcT     48
M1 Gis3b-Bt2a       AGGTACGTGAtcAAACcCTGCTGCCTGCTCa...gcctcT     48
M3 Ack2j-Bt2a       AGGTACGTGAtcAAACcCTGCTGCCTGCTCa...gcctcT     48
M5 Mar16h-Bt2a      AGGTACGTGAtcAAACcCTGCTGCCTGCTCa...gcctcT     48
RevCom.c.macro revp ....................................      0
JF735448_Ilyonectri AGGTACGTaAATAAACTCTGCTGCCTGCTtt...T....T    352
AM419111_Ilyonectri AGGTACGTGAtcAAACcCTGCTGCCTGCTCt...gcctcT    357
AY677237_Ilyonectri AGGTACGTGAtcAAACcCTGCTGCCTGCTCa...gcctcT    357
JF735492_Ilyonectri AGGTACGTGAtcAAACcCTGCTGCCTGCTCt...gcctcT    357
AY677233_I.macrodid AGGTACGTaAtcAAACcCTGCTGCCTGCTCt...gcctcT    357
AM419100_Ilyonectri AGGTACGTGAATAAACTCTaCTGCaTGCgtt...T....T    352


AM419084_Cylindroca GGTAGCACGAAACT..CACACTATTTAGGCCGCTGGCAAC    392
CM Ack2i-Bt2a       GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC     86
CM Hb6e-Bt2a        GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC     86
M1 Gis3b-Bt2a       GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC     86
M3 Ack2j-Bt2a       GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC     86
M5 Mar16h-Bt2a      GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC     86
RevCom.c.macro revp ....GCACGAAACT..CACACcAcc19
JF735448_Ilyonectri GGaAGCACGAAACT..CACACcAccTAGGCttCTGGCAAC    390
AM419111_Ilyonectri GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC    395
AY677237_Ilyonectri GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC    395
JF735492_Ilyonectri GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC    395
AY677233_I.macrodid GGaAGCACGAAACT..CACACcAcccAGGCCtCTGGCAAC    395
AM419100_Ilyonectri GGaAGCACGAAACTcaCACACcAccTAGGCttCTGGCAAC    392

AM419084_Cylindroca AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    432
CM Ack2i-Bt2a       AAGTATGTCCCTCGCGCCGTCCTtGTCGATCTCGAGCCCG    126
CM Hb6e-Bt2a        AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTtGAGCCCG    126
M1 Gis3b-Bt2a       AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    126
M3 Ack2j-Bt2a       AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    126
M5 Mar16h-Bt2a      AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    126
JF735448_Ilyonectri AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    430
AM419111_Ilyonectri AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    435
AY677237_Ilyonectri AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    435
JF735492_Ilyonectri AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    435
AY677233_I.macrodid AAGTATGTCCCTCGCGCCGTCCTtGTCGATCTCGAGCCCG    435
AM419100_Ilyonectri AAGTATGTCCCTCGCGCCGTCCTCGTCGATCTCGAGCCCG    432


AM419084_Cylindroca GTACCATGGACGCTGTCCGTGCCGGTCCCTTCGGCCAGCT    472
CM Ack2i-Bt2a       GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    166
CM Hb6e-Bt2a        GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    166
M1 Gis3b-Bt2a       GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    166
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M3 Ack2j-Bt2a        GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    166
M5 Mar16h-Bt2a       GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    166
JF735448_Ilyonectri GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    470
AM419111_Ilyonectri GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    475
AY677237_Ilyonectri GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    475
JF735492_Ilyonectri GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    475
AY677233_I.macrodid GTACCATGGACGCcGTCCGTGCCGGcCCCTTCGGCCAGCT    475
AM419100_Ilyonectri GTACCATGGACGCTGTCCGTGCCGGcCCCTTCGGCCAGCT    472


AM419084_Cylindroca CTTCCGCCCTGACAACTTCGTTTTCGGTCAGTCCGGTGCT    512
CM Ack2i-Bt2a        CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    206
CM Hb6e-Bt2a         CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    206
M1 Gis3b-Bt2a        CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    206
M3 Ack2j-Bt2a        CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    206
M5 Mar16h-Bt2a       CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    206
JF735448_Ilyonectri CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    510
AM419111_Ilyonectri CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    515
AY677237_Ilyonectri CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCG        510
JF735492_Ilyonectri CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    515
AY677233_I.macrodid CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGC     514
AM419100_Ilyonectri CTTCCGCCCcGACAACTTCGTTTTCGGTCAGTCCGGTGCT    512


AM419084_Cylindroca GGAAACAACTGGGCCAAGGGTCAC                    536
CM Ack2i-Bt2a        GGAAACAACTGGGCCAAGGGTCAC                    230
CM Hb6e-Bt2a         GGAAACAACTGGGCCAAGGGTCAC                    230
M1 Gis3b-Bt2a        GGAAACAACTGGGCCAAGGGTCAC                    230
M3 Ack2j-Bt2a        GGAAACAACTGGGCCAAGGGTCAC                    230
M5 Mar16h-Bt2a       GGAAACAACTGGGCCAAGGGTCAC                    230
JF735448_Ilyonectri GGAAACAACTGGGCCAAGGGTCAC                    534
AM419111_Ilyonectri GGAAACAACTGGGCCAAGGGTCAC                    539
JF735492_Ilyonectri GGAAACAACTGGGCCAAGGGTCAC                    539
AM419100_Ilyonectri GGAAACAACTGGGCCAAGGGTCAC                    536
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Appendix B

B.1 List of isolates each of *Cylindrocarpon* species used for the genetic diversity study

B.1.1 *Cylindrocarpon macrodidymum* isolates

No	Isolate ID	Origin
1	Ack1a	Auckland, New Zealand
2	Ack2i	Auckland, New Zealand
3	Ack1c	Auckland, New Zealand
4	Ack2k	Auckland, New Zealand
5	Ack2h	Auckland, New Zealand
6	Ack2g	Auckland, New Zealand
7	Ack2j	Auckland, New Zealand
8	Ack2c	Auckland, New Zealand
9	Gis2c	Gisborne, New Zealand
10	Gis3d	Gisborne, New Zealand
11	Gis3c	Gisborne, New Zealand
12	Gis2d	Gisborne, New Zealand
13	Gis2b	Gisborne, New Zealand
14	Gis3b	Gisborne, New Zealand
15	Gis4a	Gisborne, New Zealand
16	Gis1a	Gisborne, New Zealand
17	Mar14c	Marlborough, New Zealand
18	Mar10i	Marlborough, New Zealand
19	Mar9c	Marlborough, New Zealand
20	Mar3b	Marlborough, New Zealand
21	Mar5b	Marlborough, New Zealand
22	Mar9b	Marlborough, New Zealand
23	Mar1c	Marlborough, New Zealand
24	Mar8g	Marlborough, New Zealand
25	Mar16f	Marlborough, New Zealand
26	Mar16i	Marlborough, New Zealand
27	Mar11f	Marlborough, New Zealand
28	Mar16h	Marlborough, New Zealand
29	Mar20e	Marlborough, New Zealand
30	Mar20a	Marlborough, New Zealand
31	Mar9e	Marlborough, New Zealand
32	Nel1e	Nelson, New Zealand
33	Hb3c	Hawkes Bay, New Zealand
34	Hb4a	Hawkes Bay, New Zealand
35	Hb6e	Hawkes Bay, New Zealand
36	Hb2b	Hawkes Bay, New Zealand
37	Hb2c	Hawkes Bay, New Zealand
38	Co6a	Central Otago, New Zealand
39	Co6c	Central Otago, New Zealand
40	Co6e	Central Otago, New Zealand
41	Wpa4a	Waipara, New Zealand
42	MW174	NSW, Australia
43	MW604	NSW, Australia
44	MW174	NSW, Australia
45	SAC98	Stellenbosch University, South Africa
46	SA6610	Stellenbosch University, South Africa
47	SA6594	Stellenbosch University, South Africa

B.1.2 *Cylindrocarpon destructans* isolates

No	Isolate ID	Origin
1	Ack2a	Auckland, New Zealand
2	Ack2d	Auckland, New Zealand
3	Co5a	Central Otago, New Zealand
4	Co5b	Central Otago, New Zealand
5	Co1c	Central Otago, New Zealand
6	Co2b	Central Otago, New Zealand
7	Co6d	Central Otago, New Zealand
8	Co6f	Central Otago, New Zealand
9	Hb4b	Hawkes Bay, New Zealand
10	Hb4c	Hawkes Bay, New Zealand
11	Hb6g	Hawkes Bay, New Zealand
12	Gis1b	Gisborne, New Zealand
13	Nel1b	Nelson, New Zealand
14	Nel1d	Nelson, New Zealand
15	Wpa1d	Waipara, New Zealand
16	Wpa1a	Waipara, New Zealand
17	Wpa2a	Waipara, New Zealand
18	Mtb1d	Martinborough, New Zealand
19	Mar4c	Marlborough, New Zealand
20	Mar13b	Marlborough, New Zealand
21	Mar19a	Marlborough, New Zealand
22	Mar17c	Marlborough, New Zealand
23	Mar7b	Marlborough, New Zealand
24	Mar3a	Marlborough, New Zealand
25	Mar15a	Marlborough, New Zealand
26	Mar9a	Marlborough, New Zealand
27	Mar6b	Marlborough, New Zealand
28	Mar6c	Marlborough, New Zealand
29	Mar7c	Marlborough, New Zealand
30	Mar8h	Marlborough, New Zealand
31	Mar6d	Marlborough, New Zealand
32	Mar6g	Marlborough, New Zealand
33	Mar22a	Marlborough, New Zealand
34	Mar2b	Marlborough, New Zealand
35	Mar2c	Marlborough, New Zealand
36	Mar9h	Marlborough, New Zealand
37	Mar10b	Marlborough, New Zealand
38	Mar7f	Marlborough, New Zealand
39	Mar5d	Marlborough, New Zealand
40	Mar9f	Marlborough, New Zealand
41	Mar9g	Marlborough, New Zealand
42	Mar7d	Marlborough, New Zealand
43	Mar9d	Marlborough, New Zealand
44	Mar1b	Marlborough, New Zealand
45	Mar2a	Marlborough, New Zealand
46	Mar11d	Marlborough, New Zealand
47	Mar8a	Marlborough, New Zealand
48	Mar11e	Marlborough, New Zealand
49	Mar7a	Marlborough, New Zealand
50	Mar7e	Marlborough, New Zealand
51	Mar18a	Marlborough, New Zealand
52	Mar10c	Marlborough, New Zealand
53	Mar13a	Marlborough, New Zealand
54	SA6620	Stellenbosch University, South Africa

B.1.3 *Cylindrocarpon liriodendri* isolates

No	Isolate ID	Origin
1	Ack1ba	Auckland, New Zealand
2	Ack1d	Auckland, New Zealand
3	Co4a	Central Otago, New Zealand
4	Co1b	Central Otago, New Zealand
5	Co5c	Central Otago, New Zealand
6	Co1a	Central Otago, New Zealand
7	Co1d	Central Otago, New Zealand
8	Co3a	Central Otago, New Zealand
9	Co3b	Hawkes Bay, New Zealand
10	Hb1b	Hawkes Bay, New Zealand
11	Hb1a	Hawkes Bay, New Zealand
12	Hb5a	Hawkes Bay, New Zealand
13	Hb2d	Hawkes Bay, New Zealand
14	Hb1c	Hawkes Bay, New Zealand
15	Hb5b	Hawkes Bay, New Zealand
16	Hb2a	Hawkes Bay, New Zealand
17	Hb6f	Hawkes Bay, New Zealand
18	Mar8d	Marlborough, New Zealand
19	Mar22d	Marlborough, New Zealand
20	Mar6e	Marlborough, New Zealand
21	Mar4g	Marlborough, New Zealand
22	Mar8i	Marlborough, New Zealand
23	Mar22c	Marlborough, New Zealand
24	Mar16c	Marlborough, New Zealand
25	Mar16a	Marlborough, New Zealand
26	Mar16b	Marlborough, New Zealand
27	Mar16g	Marlborough, New Zealand
28	Mar8b	Marlborough, New Zealand
29	Mar8c	Marlborough, New Zealand
30	Mar19f	Marlborough, New Zealand
31	Mar22b	Marlborough, New Zealand
32	Mar22e	Marlborough, New Zealand
33	Mar8f	Marlborough, New Zealand
34	Mar6f	Marlborough, New Zealand
35	Mar21a	Marlborough, New Zealand
36	Mar10a	Marlborough, New Zealand
37	Mar11c	Marlborough, New Zealand
38	Mar11b	Marlborough, New Zealand
39	Mar11a	Marlborough, New Zealand
40	Mar10d	Marlborough, New Zealand
41	Mar10h	Marlborough, New Zealand
42	Mar10j	Marlborough, New Zealand
43	Mar19c	Marlborough, New Zealand
44	Mar19d	Marlborough, New Zealand
45	Mar14d	Marlborough, New Zealand
46	Mar4b	Marlborough, New Zealand
47	Mar10f	Marlborough, New Zealand
48	Mar16d	Marlborough, New Zealand
49	Wpa1b	Waipara, New Zealand
50	Wpa1c	Waipara, New Zealand
51	Wpa1e	Waipara, New Zealand
52	Wpa1f	Waipara, New Zealand
53	Wpa1g	Waipara, New Zealand
54	Wpa3a	Waipara, New Zealand
55	Gis1c	Gisborne, New Zealand
56	Gis3f	Gisborne, New Zealand
57	Mtb1e	Martinborough, New Zealand
58	MW122	NSW, Australia
59	SAC17	Stellenbosch University, South Africa
60	SA6623	Stellenbosch University, South Africa
61	SA6623	Stellenbosch University, South Africa

B.2 Recipes

B.2.1 TAE used for gel electrophoresis

To prepare 50X TAE

Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH: 8)	100 mL

Make upto 1 L using sterile water

B.2.2 Gel loading dye

40% (w/v) of sucrose

0.25% (w/v) of bromophenol blue

0.25% (w/v) of xylene cyanol

B.2.3 Czapek Dox Broth (CDB)

35 grams of CDB (Sigma Chemicals, USA) was added to 1 L of distilled water and sterilised by autoclaving.

Appendix C

C.1 ANOVA for the pilot detached root assay

C.1.1 ANOVA for the isolates each of the three *Cylindrocarpon* species on PDA

Source	Type III Sum of Squares	df	Mean square	F pr.
Isolates	1669.006	8	208.626	<0.001
Residual	82.875	18	4.604	
Total	1751.880	26		

C.1.2 ANOVA for the isolates each of the three *Cylindrocarpon* species on ½PDA

Source	TypeIII Sum of Squares	df	Mean square	F pr.
Isolates	1918.137	8	239.767	<0.001
Residual	76.422	18	4.246	
Total	1994.559	26		

C.2 List of isolates each of the three *Cylindrocarpon* species used for the detached root assay

Species	**Genetic group**	**Isolate**	**One Month**	**Two Month**
Cylindrocarpon destructans	2	Mar8a	8.6 e	23.2 c
	3	Hb6g	7.9 de	24.8 c
	2	Wpa1a	7.1 de	31.9 ef
	2	Mar11e	7.0 de	29.5 de
	2	Mar5d	6.9 de	25.9 cd
	1	Mar7b	6.6 de	45.6 h
	3	Mar13a	6.5 cde	47.3 h
	2	Mar22a	6.2 cde	35.6 fg
	2	Mar6c	5.8 cde	33.4 ef
	2	Nel1b	5.5 cd	14.4 b
	1	Co5a	5.1 cd	26.4 cd
	1	Ack2d	5.1 cd	14.3 b
	1	Ack2a	3.7 bc	39.2 g
	2	Co1c	2.5 ab	43.5 h
	3	Co2b	2.0 ab	34.8 f
		Control	0.7 a	3.5 a
		LSD	2.841	4.031
Cylindrocarpon liriodendri	1	Wpa1c	22.9 i	51.5 i
	1	Hb5a	12.1 h	43.4 h
	1	Hb2a	11.8 h	33.9 fg
	1	Wpa1e	11.6 h	43.8 h
	1	Co4a	8.9 g	47.9 i
	1	Ack1b	8.0 fg	32.2 efg
	1	Mar8b	7.6 fg	48.2 i
	2	Mar10d	6.9 ef	31.9 efg
	1	Mar22c	6.6 def	28.6 cde
	3	Mar4b	6.5 def	32.3 efg
	1	Co1d	6.3 cdef	31.9 efg
	1	Co5c	5.4 bcde	34.7 g

	1	Mar10j	5.4 bcde	29.9 def
	1	Mar6e	5.4 bcde	29.7 def
	3	Co3a	4.9 bcd	26.3 cd
	2	Mar10a	4.4 bc	18.5 b
	1	Mar19f	4.0 b	24.2 c
		Control	0.7 a	3.5 a
		LSD	1.987	4.412
Cylindrocarpon macrodidymum	3	Gis2d	24.5 g	42.5 e
	3	Mar5b	19.7 f	30.7 b
	3	Mar1c	18.2 f	36.9 cd
	4	Mar11f	11.8 e	49.9 f
	1	Ack1a	11.3 de	41.5 e
	3	Co6a	10.9 de	42.9 e
	5	Hb2b	9.1 cde	30.9 b
	3	Mar16i	8.3 cde	33.3 bc
	3	Hb4a	7.2 bcd	42.7 e
	4	Gis3b	6.1 bcd	39.8 d
	3	Ack2h	5.9 bcd	43.3 e
	2	Gis2c	5.7 bcd	34.6 bc
	5	Mar20a	4.1 abc	36.1 cd
	5	Mar9e	3.5 ab	36.4 cd
		Control	0.7 a	3.5 a
		LSD	4.59	4.577

C.2.1 ANOVA for lesion length by three *Cylindrocarpon* species in a detached root assay after one month

Multiple comparisons

Species (I)	Species (J)	Mean Difference (I – J)	Std. Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
Cylindrocarpon liriodendri	*Cylindrocarpon destructans*	2.2878*	.39474	.000	1.5094	3.0663
	Cylindrocarpon macrodidymum	-2.0732*	.40164	.000	-2.8653	-1.2811
Cylindrocarpon destructans	*Cylindrocarpon liriodendri*	-2.2878*	.39474	.000	-3.0663	-1.5094
	Cylindrocarpon macrodidymum	-4.3610*	.41290	.000	-5.1753	-3.5467
Cylindrocarpon macrodidymum	*Cylindrocarpon liriodendri*	2.0732*	.40164	.000	1.2811	2.8653
	Cylindrocarpon destructans	4.3610*	.41290	.000	3.5467	5.1753

The error term is Mean square (Error) = 6.599.
*. The mean difference is significant at the .05 level.

C.2.2 ANOVA for lesion length by three *Cylindrocarpon* species in a detached root assay after two months

Multiple comparisons

Species (I)	Species (J)	Mean Difference (I – J)	Std. Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
Cylindrocarpon liriodendri	*Cylindrocarpon destructans*	3.3219*	.52883	.000	2.2789	4.3648
	Cylindrocarpon macrodidymum	-3.4200*	.53809	.000	-4.4812	-2.3588
Cylindrocarpon destructans	*Cylindrocarpon liriodendri*	-3.3219*	.52883	.000	-4.3648	-2.2789
	Cylindrocarpon macrodidymum	-6.7419*	.55316	.000	-7.8328	-5.6510
Cylindrocarpon macrodidymum	*Cylindrocarpon liriodendri*	3.4200*	.53809	.000	2.3588	4.4812
	Cylindrocarpon destructans	6.7419*	.55316	.000	5.6510	7.8328

The error term is Mean square (Error) = 11.845.
*. The mean difference is significant at the .05 level.

C.2.3 ANOVA for lesion length by isolates each of the three *Cylindrocarpon* species in detached root assay after one month and two months

Dependent Variable: Lesion length of isolates of *Cylindrocarpon macrodidymum* after one month

Source	TypeIII Sum of Squares	df	Mean square	F	Sig.
Isolates	3020.843	14	215.775	16.376	.000
Error	790.577	60	13.176		
Total	11047.386	75			

Dependent Variable: Lesion length of isolates of *Cylindrocarpon macrodidymum* after two month

Source	TypeIII Sum of Squares	df	Mean square	F	Sig.
Isolates	7756.253	14	554.018	42.296	.000
Error	785.921	60	13.099		
Total	107522.170	75			

Dependent Variable: Lesion length of isolates of *Cylindrocarpon destructans* after one month

Source	TypeIII Sum of Squares	df	Mean square	F	Sig.
Isolates	370.086	15	24.672	4.883	.000
Error	323.394	64	5.053		
Total	3079.609	80			

Dependent Variable: Lesion length of isolates of *Cylindrocarpon destructans* after two months

Source	TypeIII Sum of Squares	df	Mean square	F	Sig.
Isolates	10889.170	15	725.945	71.405	.000
Error	650.660	64	10.167		
Total	81567.526	80			

Dependent Variable: Lesion length of isolates of *Cylindrocarpon liriodendri* after one month

Source	TypeIII Sum of Squares	df	Mean square	F	Sig.
Isolates	1950.877	17	114.757	46.030	.000
Error	179.502	72	2.493		
Total	7534.919	90			

Dependent Variable: Lesion length of isolates of *Cylindrocarpon liriodendri* after two months

Source	TypeIII Sum of Squares	df	Mean square	F	Sig.
Isolates	11377.607	17	669.271	54.451	.000
Error	884.974	72	12.291		
Total	109727.521	90			

C.3 ANOVA for the first pot trial assay

C.3.1 Effects of *Cylindrocarpon* species and rootstocks

Dependent Variable: Live/Dead

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	5.739	3	1.913	20.633	.000
Rootstock	Hypothesis	1.217	3	.406	4.376	.006
Rootstock*Species	Hypothesis	1.014	9	.113	1.215	.291
	Error	12.239	132	.093 [b]		

[b]MS (Error)

Dependent Variable: Crown

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	25.219	3	8.406	602.505	.000
Rootstock	Hypothesis	.033	3	.011	.779	.508
Rootstock*Species	Hypothesis	.095	9	.011	.755	.658
	Error	1.842	132	.014[b]		

[b]MS (Error)

Dependent Variable: 1 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	1.200	2	.600	19.444	.000
Rootstock	Hypothesis	1.351	3	.450	14.590	.000
Rootstock*Species	Hypothesis	.147	6	.025	.794	.577
	Error	3.025	98	.031[b]		

[b]MS (Error)

Dependent Variable: 3 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	1.597	2	.799	16.731	.000
Rootstock	Hypothesis	2.959	3	.986	20.663	.000
Rootstock*Species	Hypothesis	.463	6	.077	1.615	.151
	Error	4.677	98	.048[b]		

[b]MS (Error)

Dependent Variable: 6 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	1.265	2	.632	15.125	.000
Rootstock	Hypothesis	2.226	3	.742	17.748	.000
Rootstock*Species	Hypothesis	.434	6	.072	1.731	.122
	Error	4.097	98	.042[b]		

[b]MS (Error)

Dependent Variable: 8 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	.038	2	.019	2.921	.059
Rootstock	Hypothesis	.021	3	.007	1.077	.363
Rootstock*Species	Hypothesis	.022	6	.004	.570	.753
	Error	.635	98	.006[b]		

[b]MS (Error)

C.3.2 Effects of *Cylindrocarpon destructans* isolates and rootstocks

Dependent Variable: Live/dead

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	3.933	3	1.311	6.493	.000
Isolates	Hypothesis	3.461	5	.692	3.428	.005
Rootstock*Isolates	Hypothesis	5.410	15	.361	1.786	.038
	Error	41.598	206	.202[b]		

[b]MS (Error)

Dependent Variable: Crown

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	.046	3	.015	1.212	.306
Isolates	Hypothesis	.038	5	.008	.602	.698
Rootstock*Isolates	Hypothesis	.176	15	.012	.928	.534
	Error	2.612	206	.013[b]		

[b]MS (Error)

Dependent Variable: 1 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	4.119	3	1.373	7.463	.000
Isolates	Hypothesis	1.476	5	.295	1.605	.160
Rootstock*Isolates	Hypothesis	4.532	15	.302	1.642	.065
	Error	37.899	206	.184[b]		

[b]MS (Error)

Dependent Variable: 3 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	7.438	3	2.479	12.166	.000
Isolates	Hypothesis	.391	5	.078	.383	.860
Rootstock*Isolates	Hypothesis	3.319	15	.221	1.086	.371
	Error	41.979	206	.204[b]		

[b]MS (Error)

Dependent Variable: 6 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	1.992	3	.664	5.883	.001
Isolates	Hypothesis	.431	5	.086	.764	.577
Rootstock*Isolates	Hypothesis	.712	15	.047	.421	.972
	Error	23.257	206	.113[b]		

[b]MS (Error)

Dependent Variable: 8 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	.013	3	.004	.997	.395
Isolates	Hypothesis	.020	5	.004	.962	.442
Rootstock*Isolates	Hypothesis	.062	15	.004	.991	.466
	Error	.862	206	.004[b]		

[b]MS (Error)

C.3.3 Effects of *Cylindrocarpon liriodendri* isolates and rootstocks

Dependent Variable: Live/dead

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	5.362	3	1.787	8.663	.000
Isolates	Hypothesis	1.738	4	.434	2.105	.082
Rootstock*Isolates	Hypothesis	2.863	12	.239	1.156	.319
	Error	34.459	167	.206[b]		

[b]MS (Error)

Dependent Variable: Crown

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	.052	3	.017	1.173	.322
Isolates	Hypothesis	.075	4	.019	1.260	.288
Rootstock*Isolates	Hypothesis	.123	12	.010	.686	.764
	Error	2.486	167	.015[b]		

[b]MS (Error)

Dependent Variable: 1 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	.985	3	.328	4.244	.006
Isolates	Hypothesis	.772	4	.193	2.494	.045
Rootstock*Isolates	Hypothesis	.726	12	.061	.782	.669
	Error	12.925	167	.077[b]		

[b]MS (Error)

Dependent Variable: 3 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	4.314	3	1.438	7.639	.000
Isolates	Hypothesis	4.465	4	1.116	5.930	.000
Rootstock*Isolates	Hypothesis	2.799	12	.233	1.239	.260
	Error	31.439	167	.188[b]		

[b]MS (Error)

Dependent Variable: 6 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	6.860	3	2.287	12.431	.000
Isolates	Hypothesis	5.621	4	1.405	7.640	.000
Rootstock*Isolates	Hypothesis	2.107	12	.176	.955	.494
	Error	30.719	167	.184[b]		

[b]MS (Error)

Dependent Variable: 8 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	.096	3	.032	.773	.511
Isolates	Hypothesis	.883	4	.221	5.327	.000
Rootstock*Isolates	Hypothesis	.295	12	.025	.594	.845
	Error	6.920	167	.184[b]		

[b]MS (Error

C.3.4 Effects of *Cylindrocarpon macrodidymum* isolates and rootstocks

Dependent Variable: Live/dead

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	1.991	3	.664	4.536	.005
Isolates	Hypothesis	.286	3	.095	.652	.584
Rootstock*Isolates	Hypothesis	.820	8	.102	.700	.691
	Error	17.556	120	.146[b]		

[b]MS (Error)

Dependent Variable: Crown

Source		TypeIII Sum of Squares	df	Mean square
Rootstock	Hypothesis	.000	3	.000
Isolates	Hypothesis	.000	3	.000
Rootstock*Isolates	Hypothesis	.000	8	.000
	Error	.000	120	.000[b]

[b]MS (Error)

Dependent Variable: 1 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	2.394	3	.798	10.343	.000
Isolates	Hypothesis	.523	3	.174	2.258	.085
Rootstock*Isolates	Hypothesis	.516	8	.065	.836	.572
	Error	9.258	120	.077[b]		

[b]MS (Error)

Dependent Variable: 3 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	4.194	3	1.398	6.124	.001
Isolates	Hypothesis	.529	3	.176	.772	.512
Rootstock*Isolates	Hypothesis	1.019	8	.127	.558	.810
	Error	27.399	120	.228[b]		

[b]MS (Error)

Dependent Variable: 6 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	3.435	3	1.145	6.437	.000
Isolates	Hypothesis	1.689	3	.563	3.165	.027
Rootstock*Isolates	Hypothesis	2.115	8	.264	1.487	.169
	Error	21.344	120	.178[b]		

[b]MS (Error)

Dependent Variable: 8 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Rootstock	Hypothesis	.054	3	.018	.640	.591
Isolates	Hypothesis	.156	3	.052	1.840	.143
Rootstock*Isolates	Hypothesis	.108	8	.014	.477	.871
	Error	3.399	120	.028[b]		

[b]MS (Error)

C.4 ANOVA for the second pot trial

Dependent Variable: Live/dead

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	.593	2	.296	1.183	.311
	Error	23.561	94	.251[b]		
Isolates	Hypothesis	6.712	12	.559	2.693	.004
	Error	17.442	84	.208[b]		

[b]MS (Error)

Dependent Variable: 1 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	.152	2	.076	.913	.405
	Error	7.848	94	.083[b]		
Isolates	Hypothesis	2.596	12	.216	3.363	.000
	Error	5.404	84	.064[b]		

[b]MS (Error)

Dependent Variable: 3 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	2.641	2	1.320	6.361	.003
	Error	19.513	94	.208[b]		
Isolates	Hypothesis	9.885	12	.824	5.639	.000
	Error	12.269	84	.146[b]		

[b]MS (Error)

Dependent Variable: 6 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	1.891	2	.945	4.632	.012
	Error	19.186	94	.204[b]		
Isolates	Hypothesis	10.885	12	.907	7.475	.000
	Error	10.192	84	.121[b]		

[b]MS (Error)

Dependent Variable: 8 CM

Source		TypeIII Sum of Squares	df	Mean square	F	Sig.
Species	Hypothesis	0.402	2	.201	2.541	.084
	Error	7.444	94	.079[b]		
Isolates	Hypothesis	2.846	12	.237	3.985	.000
	Error	5.000	84	.060[b]		

[b]MS (Error)

C.5 Media and solution recipies

C.5.1 Luria Bertani (LB) broth

Bacto-Tryptone - 10 g

Bacto-Yeast extracts - 5 g

NaCl - 10 g

- Dissolve in 900 mL water
- Adjust pH to 7.0 with 10M NaOH
- Adjust the volume to 1 L, sterilise by autoclaving and store at room temperature

C.5.2 Luria Bertani (LB) agar

Additionally add 10 g agar to the LB broth ingredient

C.5.3 Minimal medium

For 50 mL

Solution 1 - 200 g/l K_2HPO_4, 145 g/l KH_2PO_4 - 500 µL

Solution 2 - 30 g/l $MgSO_4.7H_2O$, 15 g/l NaCl - 1 mL

Solution 3 - 1% w/v $CaCl_2.2H_2O$ - 50 µL

Solution 4 - 20% glucose - 500 µL

Solution 5 - 0.01% w/v FeSO4 - 500 µL

Solution 6 - 20% w/v $(NH_4)2SO_4$ - 125 µL

C.5.4 Preparation of induction medium supplemented with acetogyringone (IMAS)

2 mL	40 mM MES buffer (9.8 g MES in 40 mL of water, adjust to pH 5.3 with Sodium hydroxide and finally adjust the volume to 50 mL and sterilise by autoclaving and store at room temperature).
500 µL	50% (v/v) glycerol and sterilize by autoclaving
100 µL	acetosyringone (In 2.4 mL ethanol stock add 48 mg of acetosyringone).

Add these quantities to minimal medium to produce IMAS.

C.5.5 Preparation of osmotic medium buffer (OM Buffer)

For 1 L

1.2 M $MgSO_4.7H_2O$	296 g
10 mM Na_2HPO_4	100 mL (from stock)

Stock 100mM Na_2HPO_4: 1.42 g/100 mL.

Add 100 mL sterile water to dissolve then add 200 µLof 100 mM $NaH_2PO_4.2H_2O$ (1.56 g/100 mL) or until pH reaches 5.8 and then top up to 1 L. Sterilse the solution by passing through the filter (0.45 µm).

C.5.6 Preparation of sorbitol tris buffer (ST Buffer)

For 100 mL

0.6 M Sorbitol	10.93 g
100 mM Tris HCl pH 8.0	10 mL (from 1 M stock)

Adjust the quantities to 100 mL with sterile water and filter sterilise at 0.45 µm.

C.5.7 Preparation of sorbitol tris calcium chloride buffer (STC Buffer)

For 500 mL

1.2 M Sorbitol	109.25 g
10 mM Tris-HCl pH 7.5	5 mL (from 1M stock)
10 mM $CaCl_2.2H_2O$	0.55 g

Add 500 mL of sterile water and filter sterilise.

C.5.8 Preparation of 40% polyethylene glycol (PEG) solution

For 100 mL

40% PEG 3350	40 g
50 mM Tris-HCl pH 7.5	5 mL (from 1M stock)
50 mM $CaCl_2$	0.56 g

Adjust the volume to 100 mL using sterile water and sterilise the solution by autoclaving.

C.5.9 Preparation of Regeneration Overlay Media (ROM)

Yeast extract	0.5 g
0.8 M sucrose	273.8 g
0.8% agar	8.0 g

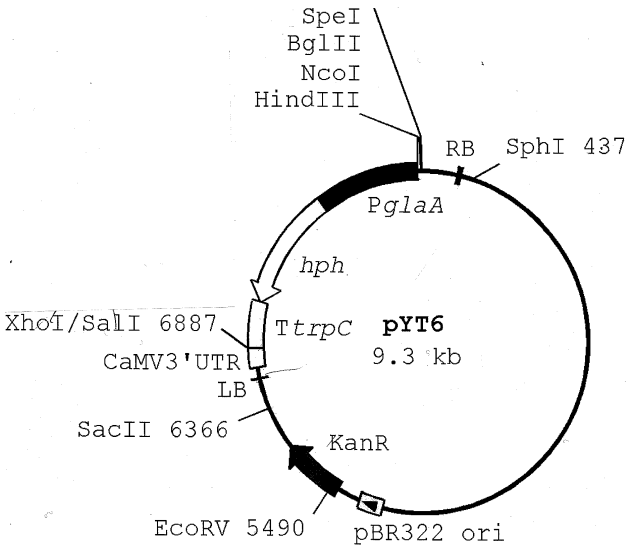
Add the ingredients into 1 L of nanopure water and sterilise the media by autoclaving.

C.5.10 Preparation of Regeneration Media (RM)

Yeast extract	0.5 g
0.8 M sucrose	273.8 g
1.5% agar	15.0 g

Add the ingredients into 1 L of nanopure water and sterilise the media by autoclaving.

C.6 Map of pYT6 binary vector



Abbreviations:

RB – right border

LB – left border

CaMV3'UTR – cauliflower mosaic virus 3' untranslated region

Ori – origin of replication

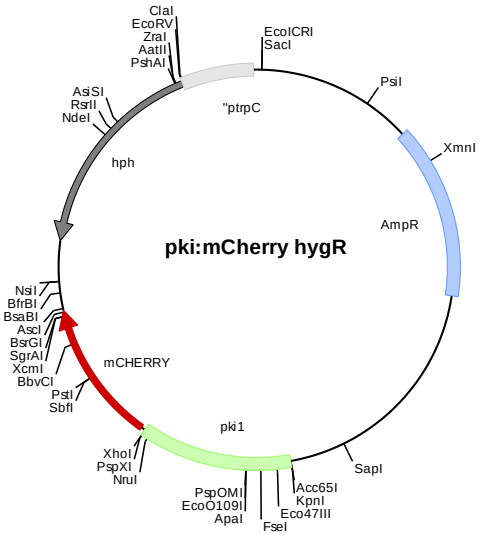
TtrpC – terminator from trpC gene

PglaA – promoter from glaA gene

Hph – hygromycin resistance gene (coding region)

KanR – kanamycin resistance gene

C.7 Map of mCherry vector



Abbreviations:

Hph – hygromycin phosphor transferase

AmpR – Ampicillin resistance gene

ptrpC – Tryptophan promoter gene

pki 1 – Pyruvate kinase 1 gene

Appendix D

D.1 Anova results for the quantification of laccase produced by *Cylindrocarpon* species

D.1.1 ANOVA for the quantification of laccase enzyme (PPO-1) produced by *Cylindrocarpon* species

Source	df	Sum of squares	Mean square	F	P
Species	3	1.6568	0.5523	25.12	0.000
Error	13	0.2858	0.0220		
Total	16	1.9426			

D.1.2 ANOVA for the quantification of laccase enzyme (PPO-II) produced by *Cylindrocarpon* species

Source	df	Sum of squares	Mean square	F	P
Species	3	0.00016	0.00005	0.04	0.928
Error	13	0.01649	0.00127		
Total	16	0.01665			

D.1.3 ANOVA for the PPO-1 laccase enzyme by isolates of *Cylindrocarpon* species

Source	df	Sum of squares	Mean square	F	P
Isolate	16	5.82773	0.36423	395232.32	0.000
Error	34	0.00003	0.00000		
Total	50	5.82777			

D.1.4 ANOVA for the PPO-II laccase enzyme by isolates of *Cylindrocapon* species

Source	df	Sum of squares	Mean square	F	P
Isolate	16	0.049955	0.0031	3249.67	0.000
Error	34	0.000032	0.0000		
Total	50	0.499884			

D.1.5 McIlvaine's Buffer System

A citrate/phosphate buffer system that can be volumetrically set for pH in a wide range (2.2 to 8) and is therefore useful for enzyme kinetic studies etc.

To prepare 20 mL of the buffer, mix 0.2 M disodium hydrogen phosphate and 0.1 M citric acid as shown below in the Table.

pH required.	0.2 M Na_2HPO_4.	0.1 M citric acid.
	cc.	cc.
2.2	0.40	19.60
2.4	1.24	18.76
2.6	2.18	17.82
2.8	3.17	16.83
3.0	4.11	15.89
3.2	4.94	15.06
3.4	5.70	14.30
3.6	6.44	13.56
3.8	7.10	12.90
4.0	7.71	12.29
4.2	8.28	11.72
4.4	8.82	11.18
4.6	9.35	10.65
4.8	9.86	10.14
5.0	10.30	9.70
5.2	10.72	9.28
5.4	11.15	8.85
5.6	11.60	8.40
5.8	12.09	7.91
6.0	12.63	7.37
6.2	13.22	6.78
6.4	13.85	6.15
6.6	14.55	5.45
6.8	15.45	4.55
7.0	16.47	3.53
7.2	17.39	2.61
7.4	18.17	1.83
7.6	18.73	1.27
7.8	19.15	0.85
8.0	19.45	0.55

D.2 Degenerate and specific primer sequences for laccase

D.2.1 Alignment of laccase (*lcc1*) genes. Sites from which degenerate primers were designed are shown by arrows

```
                                                  ---------->
XM001522482  TACCCGGGACCCATGATCGAGGCCAACTGGGGAGACACCG    160
EF990894     TttCCtGcgCCtcTGATtGAaGCaAACTGGGGtGACACCa    160
XM003659795  TtCCCcGGgCCgcTGgTCGAGGCCAACTGGGGcGACACga    160
XM003050908  TttCCcGGgCCCAccATCGAGGCCAACTGGGGcGACACga    160
EU526310     TtCCCcGGcCCatTGATCGAGGCgAACTGGGGtGACAtta    160

XM001522482  TCCAGGTCACTGTTCACAACAACATCACCGGCCCGGAAGA    200
EF990894     TCCAaGTCACTGTTCAtAACgACAT....GGaC..GAtGA    194
XM003659795  TCgtGGTggaTGTcCAtAACgACATtACCGGgCCGGAgGA    200
XM003050908  TtCAGGTgACaGTgagCAACgACAT....tGaa..GAcGA    194
EU526310     TtCAaGTCACTaTgCACAACAACATCACCGGaCCaGAcGA    200
.............................................................

                                          <----------
XM001522482  ..CGACCAGGCCCCTGGCTCCGGTTTCTGGATGCGCAGCA    859
EF990894     ..tGAtggc...aaattggaCtcaTaCTGGATGCGCAGCA    844
```

```
XM003659795  gtCGgCgAG...CCcacCTCgGccTTCTGGATGCGgtcCA    853
XM003050908  ..CGACggc...gaTctagaCGccTTCTGGATGCGCtcCA    844
EU526310     ..aGACggt...aaacttgaCGcTTaCTGGATGCGtAGCA    844

XM001522482  CCCTGGCCGC...CTGCTCTGAGGCTCGCCAGCCCAACGC    896
EF990894     aCaTttCtGCcatCTGtagTctGGgTCGtgccCCagc...    881
XM003659795  aCCTGtCgtC...gTGCctgccGGCgCGgCAaCCCtAtGC    890
XM003050908  aCaTctCtaCaaagTGCagcctGtCaCaCgccCCggA...    881
EU526310     aCaTtagCa....CTaaatgctccaTCaCCAGCCagcCca    880
```

D.2.2 Alignment of the laccase *(lcc1)* gene from *Cylindrocarpon* species. Primer sites are shown by the arrows

```
RC GIS3B-LR880      GACACCATCCAGGTGACCGTCCACAACGAGCTTGAGGATG     40
RC MAR3A-LR880      GACACCATtCAaGTcACCGTtCACAAtGAGgTTGAGGATG     40
RC MAR6D-LR880      GACACCATtCAaGTcACCGTtCACAAtGAGgTTGAGGATG     40
RC MTB1D-LR880      GACACCATtCAaGTcACCGTtCACAAtGAGgTTGAGGATG     40
RC Hb2A-LR880       GACACCATtCAGGTcACCGTCCACAAtGAGgTTGAGGATG     40
RC Ack2g-LR880      GACACCATCCAGGTGACCGTCCACAACGAGCTTGAGGATG     40

RC GIS3B-LR880      AAGGTATGGCCCTTCACTGGCATGGCTTCCTTCAGAAAGA     80
RC MAR3A-LR880      AgGGTgTcGCCgTTCACTGGCAcGGgTTCCTcCAGAAgGA     80
RC MAR6D-LR880      AgGGTgTcGCCgTTCACTGGCAcGGgTTCCTcCAGAAgGA     80
RC MTB1D-LR880      AgGGTgTcGCCgTTCACTGGCAcGGgTTCCTcCAGAAgGA     80
RC Hb2A-LR880       AgGGTgTcGCCCTgCACTGGCAcGGgTTCCTcCAGAAgGA     80
RC Ack2g-LR880      AAGGTATGGCCCTTCACTGGCATGGCTTCCTTCAGAAAGA     80

RC GIS3B-LR880      CACCCCCTGGGAGGATGGTGTCCCTGGTATCACACAATGC    120
RC MAR3A-LR880      CACCCCCTGGGAGGATGGTGTCCCTGGTATtACcCAATGt    120
RC MAR6D-LR880      CACCCCCTGGGAGGATGGTGTCCCTGGTATtACcCAATGt    120
RC MTB1D-LR880      CACCCCCTGGGAGGATGGTGTCCCTGGTATtACcCAATGt    120
RC Hb2A-LR880       CACCCCCTGGGAGGATGGTGTCCCTGGcATCACcCAATGC    120
RC Ack2g-LR880      CACCCCCTGGGAGGATGGTGTCCCTGGTATCACACAATGC    120
.....................................................................

RC GIS3B-LR880      CGTCATCGCCAACGACTTCGTCCA                    584
RC MAR3A-LR880      gGTCATCGCCAAtGACTTCGTCCA                    584
RC MAR6D-LR880      gGTCATCGCCAAtGACTTCGTCCA                    584
RC MTB1D-LR880      gGTCATCGCCAAtGACTTCGTCCA                    584
RC Hb2A-LR880       gGTCATCGCCAAtGACTTCGTCCA                    584
RC Ack2g-LR880      CGTCATCGCCAACGACTTCGTCCA                    584
```

D.2.3 Copper binding regions (Cbr1 and CbrII) and one N-glycosylation site found in the laccase enzyme of *Cylindrocarpon* species

Cbr1

```
ACK1A (CM)           HWHGFLQKDTPWEDGVPGITQCPIAPGKTFTYQF.LAEMY     39
HB2b (CM)            HWHGFLQKDTPWEDGVPGITQCPIAPGKTFTYQF.LAEMY     39
MAR16I (CM)          HWHGFLQKDTPWEDGVPGITQCPIAPGKTFTYQF.LAEMY     39
Hb2a  (CL)           HWHGFLQKDTPWEDGVPGITQCPIAPGKsFTYQF.LAEMY     39
MAR13A (CD)          HWHGFLQKDTPWEDGVPGITQCPIAPGKsFTYQF.LAEMY     39
CO1D (CL)            HWHGFLQKDTPWEDGVPGITQCPIAPGKsFTYQF.LAEMY     39
Co1c (CD)            HWHGFLQKDTPWEDGVPGITQCPIAPGKsFTYQF.LAEMY     39
MAR11B (CL)          HWHGFLQKDTPWEDGVPGITQCPIAPGKsFTYQF.LAEMY     39
Nel1d (CD)           HWHGFLQKDTPWEDGVPGITQCPIAPGKsFTYQF.LAEMY     39
P.anserina P78722    HWHGlhQKgTnmhDGanGvTeCPIpPkggsriyrfrAqqY     40
F.oxysporumABS19939 HWHGlrQleTfemDGVnGvTQCPIAPGdsyTYtF.rAvqY     39
N.crassa M18334      HWHGmhQrnsniqDGVnGvTeCPIpPrggskvyrwrAtqY     40
N.crassa P06811      HWHGmhQrnsniqDGVnGvTeCPIpPrggskvyrwrAtqY     40
Trametes sp.48424    HWHGFfQKgTnWaDGpafInQCPIssGhsFlYdFqvpdqa     40
```

Cbr II

```
ACK1A (CM)           GTGWYHSHYSAQYAAGILGPMVIHG...PRVKRDYDIDVG     76
HB2b  (CM)           GTGWYHSHYSAQYAAGILGPMVIHG...PRVKRDYDIDVG     76
MAR16I (CM)          GTGWYHSHYSAQYAAGILGPMVIHG...PRVKRDYDIDVG     76
```

```
Hb2a (CL)             GTtWYHSHYSAQYAAGILGPMVIHG...PRdKRDYDvDVG     76
MAR13A (CD)           GTtWYHSHYSAQYAAGliGPiVIHG...PRdKRDYDvDVG     76
CO1D (CL)             GTtWYHSHYSAQYAAGILGPMVIHG...PRdKRDYDvDVG     76
Co1c (CD)             GTtWYHSHYSAQYAAGliGPiVIHG...PRdKRDYDvDVG     76
MAR11B (CL)           GTtWYHSHYSAQYAAGILGPMVIHG...PRdKRDYDvDVG     76
Nel1d (CD)            GTtWYHSHYSAQYAAGlvGPiVIHG...PRdKRDYDIDiG     76
P.anserina P78722     GTsWYHSHfSAQYgnGvvGtiVvnG...Pasvpydidlgv     77
F.oxysporumABS19939   GTsWYHSHYSlQYAdGlaGPitIyG...Pssapfdegrn.     75
N.crassa M18334       GTsWYHSHfSAQYgnGIvGPiVInG...Pasanydvdlgp     77
N.crassa P06811       GTsWYHSHfSAQYgnGIvGPiVInG...Pasanydvdlgp     77
Trametes sp.48424     GTfWYHSHlStQYcdGlrGPfVvydpndPaadlydvdnd.     79

ACK1A (CM)            PVMVGDWYHDEYFDLVEKIMSPNGGLAFSDNNLI...NGK    113
HB2b (CM)             PVMVGDWYHDEYFDLVEKIMSPNGGLAFSDNNLI...NGK    113
MAR16I (CM)           PVMVGDWYHDEYFDLVEKIMSPNGGLAFSDNNLI...NGK    113
Hb2a (CL)             PiMVsDWYHrEYFDLVEddlnPNrGLviSDNNLI...NGK    113
MAR13A (CD)           PVMVGDWYHrpYFDLVEdvMnPNiGiviSDNNLI...NGK    113
CO1D (CL)             PiMVsDWYHrEYFDLVEdvMnPNiGiviSDNNLI...NGK    113
Co1c (CD)             PiMlGDWYHrpYFDLVEdvMnPeiGiviSDNNLI...NGK    113
MAR11B (CL)           PiMVsDWYHrEYFDLVEdvMnPNiGiviSDNNLI...NGK    113
Nel1d (CD)            PiMlGDWYHrqYFDLVEdvMnPNiGiviSDNNLI...NGK    113
P.anserina P78722     fpitdyyhkpadvlveEtmnggpppsdt...vLf...kGh    111
F.oxysporumABS19939   PilitDWsHrsaFqswqrelvPNptrpmmngvLI...NGv    112
N.crassa M18334       fpltdyyYdtadrlvlltqhagpppsnn...vLf...NGf    111
N.crassa P06811       fpltdyyYdtadrlvlltqhagpppsnn...vLf...NGf    111
Trametes sp.48424     ........dtvitlvdwyhvaaklGpAFplgadatliNGK    111

ACK1A (CM)            NNFNCSTLAADDTTPCNSAAGLSKFKFKRGKTHRLRLINV    153
HB2b (CM)             NNFNCSTLAvDDTTPCNSAAGLSKFKFKRGKTHRLRLINV    153
MAR16I (CM)           NNFNCSTLAADDTTPCNSAAGLSKFKFKRGKTHRLRLINV    153
Hb2a (CL)             NNFNCSaLpAtDTTPCNSqAGLSKFKFKRGKvHRLRLINs    153
MAR13A (CD)           NNFNCSTLpAtDTTtCNSqAGLSKFKFKRGKvHRLRLINa    153
CO1D (CL)             NNFNCSaLpAtDTTPCNSqAGLSKFKFKRGKvHRLRLINs    153
Co1c (CD)             NNFNCSTLpAsDTTtCNSqAGLSKFKFKRGKvHRLRLINa    153
MAR11B (CL)           NNFNCSaLpAtDTTPCNSqAGLSKFKFKRGKvHRLRLINs    153
Nel1d (CD)            NNFNCSTLpAsDpTPCNSqAGLSKFKFKRGKvHRLRLINs    153
P.anserina P78722     gknpqtgagkfanvtltpgkrhrlriiintsthdhfqLklq    151
F.oxysporumABS19939   gNFagSfprerfnmtvtkgrkyv...........LRvINt    141
N.crassa M18334       akhpttgagqyaTvsltkgkkhrlrlintsvenhfqLslV    151
N.crassa P06811       akhpttgagqyaTvsltkgkkhrlrlintsvenhfqLslV    151
Trametes sp.48424     grspstTtAdlsvisvtpgkryrfrlvslscdpnytfsid    151


ACK1A (CM)            GAEPLQRFSIDGHTMTVIA                         172
HB2b (CM)             GAEaLQRFSIDGHTMTVIA                         172
MAR16I (CM)           GAEaLQRFSIDGHTMTVIA                         172
Hb2a (CL)             GAEaLQRFSIDGHTMTVIA                         172
MAR13A (CD)           GAEaLQRFSIDGHTMTVIA                         172
CO1D (CL)             GAEaLQRFSIDGHTMTVIA                         172
Co1c (CD)             GAEaLQRFSIDGHTMTVIA                         172
MAR11B (CL)           GAEaLQRFSIDGHTMTVIA                         172
Nel1d (CD)            GAEaLQRFSIDGHTMTVIA                         172
P.anserina P78722     nhtmtiiaadmvpvqaqtv                         170
F.oxysporumABS19939   svdttwiFSIDnHnfTVms                         160
N.crassa M18334       nhsmtiisadlvpvqpykv                         170
N.crassa P06811       nhsmtiisadlvpvqpykv                         170
Trametes sp.48424     GhnmtiietdsinTaplvv                         170
```

Figure D.1 Alignment of multiple amino acid sequences of laccase proteins of *Cylindrocarpon* species and other fungal species retrieved from GenBank. Accession numbers are cited for species other than *Cylindrocarpon*. Two conserved copper binding regions (Cbr1 and Cbr II) are shown as shaded regions. Possible N-glycosylation sites are underlined bold for (___) *Cylindrocarpon* species and (___) for the other fungal species.

D.3 ANOVA results for the quantification of protease produced by *Cylindrocarpon* species

D.3.1 ANOVA for the quantification of protease activity of *Cylindrocarpon* species

Source	df	Sum of squares	Mean square	F	P
Species	2	0.009424	0.004712	7.83	0.001
Error	45	0.027067	0.000601		
Total	47	0.036492			

D.3.2 ANOVA for the quantification of protease activity by isolates of *Cylindrocarpon* species

Source	df	Sum of squares	Mean square	F	P
Isolates	15	0.0363937	0.0024262	792.24	0.000
Error	32	0.0000980	0.0000031		
Total	47	0.0364917			

D.4 Degenerate and specific primer sequences for protease and cellulase

D.4.1 Alignment of protease genes. Arrows show areas to which degenerate primers were designed

```
                                                                  →
Aspergillus fumiga  GCTC..AGCAACACCACCCAGGTCGAGTACAGCTCCAACT    199
Neosartorya fischer GCTC..AGCAACACCACCCAGGTCGAGTACAGCTCCAACT    199
Aspergillus nidulan cCTC..tcCAAtgagACCCAGGTaGAGTACAGCTCCAACT    202
Penicllium marneffe caTC..AcCggtACCAagCAcGTCGAGTACAGCTCtAACT    274
Gibberella zeae DNA tgTCagcGatgCcaacgttc.....AGTACtctggaAACT    190
Sclerotinia hypoth  GgaCcaAcCAACAagACCgAtGTttccTACtcCTCCAACT    175

                    ──────────→
Aspergillus fumiga  GGGCTGGTGCCGTCCTCATCGGCACAGGCTACACGGCTGT    239
Neosartorya fischer GGGCTGGTGCCGTCCTCATCGGCtCAGGCTACACtGCcGT    239
Aspergillus nidulan GGGCcGGTGCCGTgCTCATtGGCACAGGCTACACctCcGT    242
Penicllium marneffe GGtCTGGTGCtGTCCTtATCGGaACtGGCTACAgtGCTGT    314
Gibberella zeae DNA GGGCcGGaGCaGTCCagATtGGCAgtGGCTAtAacaagGT    230
Sclerotinia hypoth  GGGCTGGTGCCGTCCTCgTCGGCACtGGtTACACttCcGT    215

Aspergillus fumiga  GACTGGCGAGTTCGTCGTCCCTACCCCCAGCGTCCCAAGC    279
Neosartorya fischer GACcGGCGAGTTCGTCGTCCCTACCCCCcaCGcCCCgAGC    279
Aspergillus nidulan aACcGGCGAaTTtacCGTgCCcACtCCCtcCcTgCCAAcg    282
Penicllium marneffe cACTGcCGAaTTCactGTCCCcACCCCCcataTtCCcAGt    354
Gibberella zeae DNA GcagGGCactaTCacCGTCCCTgaagtgAGCGgttCcAat    270
Sclerotinia hypoth  aACcGGtaccTTCactGcCCCatCCCCaAGC.........    246

                                                        →
Aspergillus fumiga  GGTGGCTCTTCCAGCAAGCAGTACTGCGCCTCCGCTTGGG    319
Neosartorya fischer GGTGGCTCTTCCAaCgAGCAGTACTGCGCCTCCGCcTGGG    319
Aspergillus nidulan GGTGcaagccgCAaCAAGCAGTACTGCGCCTCtGCcTGGG    322
Penicllium marneffe GGTGGCagcagCgGaAcaCAGTACTGCGCCTCtGCcTGGG    394
Gibberella zeae DNA GGTGctgCT..................GCCTCtGCcTGGG    292
Sclerotinia hypoth  ...............AcagccggaTctGgaTCCGCcTGGG    271

                    ──────────→
Aspergillus fumiga  TCGGTATCGACGGTGACACCTGCAGCTCTGCCATCCTGCA    359
Neosartorya fischer TCGGTATCGACGGTGACACCTGCAGCTCTGCCATCCTGCA    359
Aspergillus nidulan TtGGcATtGAtGGcGAtACCTGCAGCaCcGCgATtCTGCA    362
Penicllium marneffe TtGGTcTtGACGGgGACACCTGCgGCaCctCCATtCTcCA    434
Gibberella zeae DNA TCGGcATtGACGGaGAtACtTGCcaaaCTGCtATaCTGCA    332
Sclerotinia hypoth  TtGGTATtGACGGTGACACCTGtgGtaCcGCCATtCTcCA    311

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                                                  ←──────────
Aspergillus fumiga  CCTTCACCGGCGGCGTGGACGGCAATCTGTGCGAGTACAA    599
Neosartorya fischer CCTTCACtGGtGGCGTGGACGGCAATCTGTGCGAGTACAA    599
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```
Aspergillus nidulan CCTTtACCGGtGGCGTGGAtGGagATCTcTGCGAGTAtAA    602
Penicllium marneffe aCTTCtC...tGGtGaatcCGaCAAgCTcTGCGAGTACAA    671
Gibberella zeae DNA gCTTCACCaaCacCccatctac...TCTtTGCGAGacCAA    560
Sclerotinia hypoth  CCTTCAgCGGtGGtGTtGAtGGagAcCTcTGCGAGTACAA    551

Aspergillus fumiga  TGCCGAG..............TGGATCGTTGAAGACTT    623
Neosartorya fischer TGCCGAG..............TGGATCGTTGAgGACTT    623
Aspergillus nidulan TGCCGAG..............TGGATCGTcGAgGACTT    626
Penicllium marneffe TGCtGAG..............TGGATCGTgGAgGACTT    695
Gibberella zeae DNA cGCtGAtataactacaacttcacTGGAcaaTgtActgagg    600
Sclerotinia hypoth  cGCCGAG..............TGGATCGTTGAgGAtTT    575

Aspergillus fumiga  TGAGTCCAACGGGTCTCTGGTGCCGTTTGCTAACTTTGGC    663
Neosartorya fischer TGAGTCCAACGGGTCTCTaGTGCCGTTTGCTAACTTTGGC    663
Aspergillus nidulan cGAaagCAAtGGacagCTGGTcCCcTTTGCggAtTTTGGC    666
Penicllium marneffe TGAagaaggCGGcTCctTGGTtCCcTTcGCcAACTTcGGC    735
Gibberella zeae DNA gagGTCatgaGaGTggaTGagGCtGTTaataAggagataa    640
Sclerotinia hypoth  cGAGgaaggtaGcTCcCTcGTcCaGTTcGCcAACTTcGGC    615

Aspergillus fumiga  ACTGTCACCTTCACCGGGGCTCAGGCTACCGATGGCGGTT    703
Neosartorya fischer ACTGTCACCTTCACCGGtGCcCAGGCTACCGATGGCGGTT    703
Aspergillus nidulan ACcGTgACaTTtACgaGtGCTgAGGtgACCaATGatGGga    706
Penicllium marneffe ACcGTtACCTTCAgCGGtGCTaAGGCTACCaAgaGCGGcT    775
Gibberella zeae DNA ....................................    640
Sclerotinia hypoth  ACTGTCACCTTCACCGGtGCctcaGCcACCcAaaaCGGag    655
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D.4.2 Alignment of *Cylindrocarpon* species acid protease genes. Sites where specific primers were designed are shown by arrows

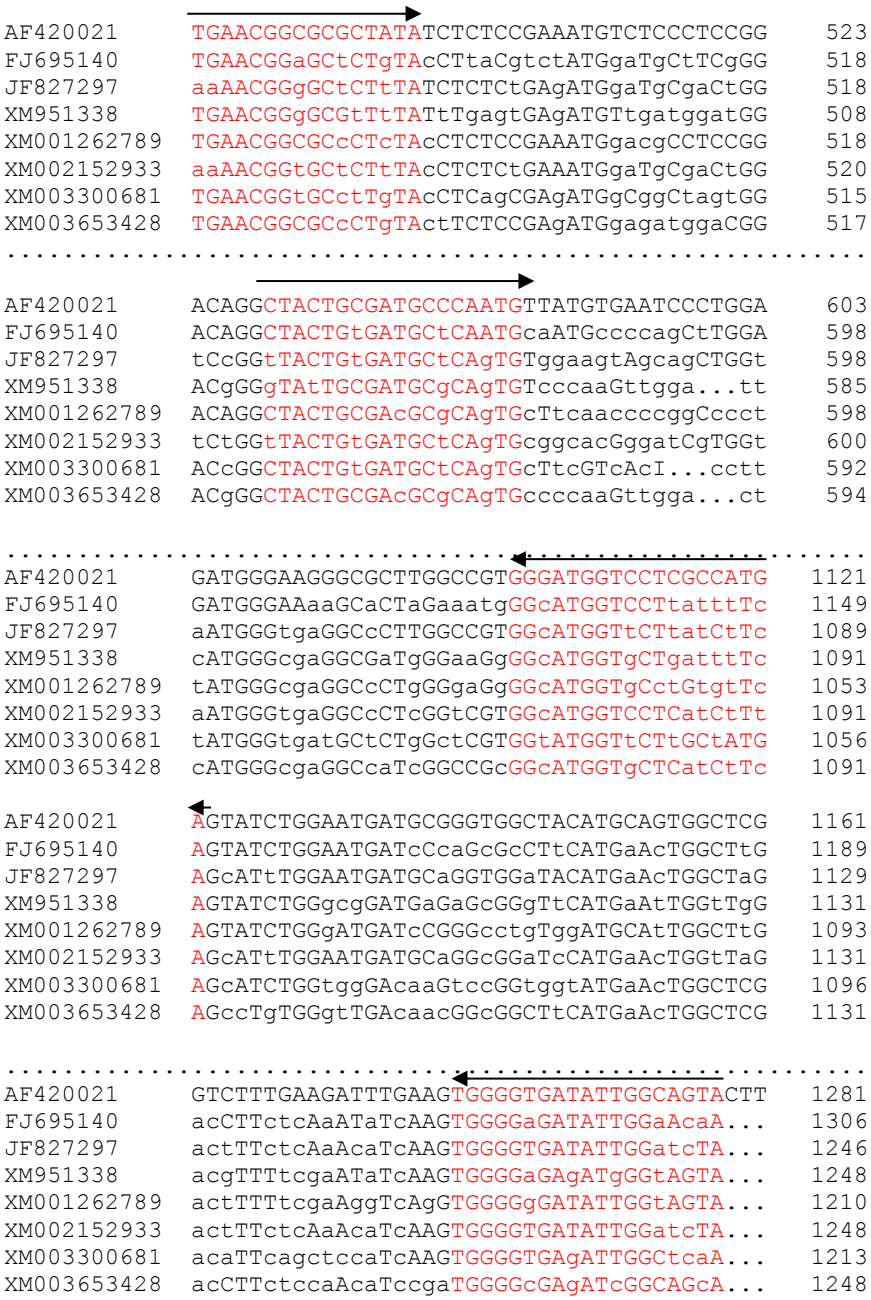
```
11-F265 protease ACATTTACCAACACGCCCTCTACCCTCTGCGAGACCAATG     40
25-F265 protease ACcTTTACCAACACtCCCTCgACCCTCTGCGAGACCAATG     40
27-F265 protease ACATTTACCAACACGCCCTCTACCCTCTGCGAGACCAATG     40
31-F265 protease ACcTTTACCAACACtCCCTCgACCCTCTGCGAGACCAATG     40
36-F265 protease ACcTTTACCAACACtCCCTCgACCCTCTGCGAGACCAATG     40
38-F265 protease tCgTTcAgCAgCACcCCtTCcACgCTCTGtGAGACCAATG     40
65-F265 protease ACcTTTACtAgCACtCCtTCgACCCTCTGCGAaACCAATG     40
67-F265 protease ACcTTTACtAgCACtCCCTCaACCCTCTGCGAaACCAATG     40

11-F265 protease CCGAGTGGATCGTTGAGGCGTTCCAGGAGAATGGCAGCCA     80
25-F265 protease CCGAGTGGATtGTTGAGGCcTTCCAGGAGAATGGCAGCCA     80
27-F265 protease CCGAGTGGATCGTTGAGGCGTTCCAGGAGAATGGCAGCCA     80
31-F265 protease CCGAGTGGATtGTTGAGGCcTTCCAGGAGAATGGCAGCCA     80
36-F265 protease CCGAGTGGATtGTTGAGGCcTTCCAGGAGAATGGCAGCCA     80
38-F265 protease CCGAGTGGATtGTcGAGGCcTTCCAGGAGAATGGCAGCCA     80
65-F265 protease CCGAGTGGATCGTTGAGGCcTTCCAGGAGAATGGCAGCCA     80
67-F265 protease CCGAGTGGATCGTTGAGGCcTTCCAGGAGAATGGCAGCCA     80

11-F265 protease AGTCACCCTCGTCGACTTTGGCACCGTCACATTCAGGA      118
25-F265 protease AGTCACCCTCGTCGACTTTGGCACCGTCACATTCAGGA      118
27-F265 protease AGTCACCCTtGTCGACTTTGGCACCGTCACATTCAGGA      118
31-F265 protease AGTCACCCTCGTCGACTTTGGCACCGTCACATTCAGGA      118
36-F265 protease AGTCACCCTCGTCGACTTTGGCACCGTCACATTCAGGA      118
38-F265 protease gGTCACtCTCGcCGACTTTGGCACCGTCACATTCAGGA      118
65-F265 protease AGTCACCCTCGTCGACTTTGGCACCaTgACATTC          114
67-F265 protease AGTCACCCTCGTCGACTTTGGCACCGTgACATTCA         115
```

D.4.3 Alignment of cellulase genes. Arrows show areas to which degenerate primers were designed

```
AF420021     TTTACCTTCACTGTCTACGTCTCCGCGCTCCCATGCGGGA    483
FJ695140     aTTAgtTTtACaGTCgAtGTtTCtaacCTgCCtTGtGGaA    478
JF827297     aTTAgCTTtgaTGTagAtGcCTCCaactTaCCATGtGGtg    478
XM951338     aTctCgTTCgaTGTggAtGTtagtaattTgCCtTGtGGcA    468
XM001262789  TTcACCTTCgacGTCgACGcCTCCaaGCTCCCcTGCGGcA    478
XM002152933  aTTAgCTTtgacGTagAtGTCTCtaactTaCCATGCGGcg    480
XM003300681  TTTACCTTtgaTGTCgACaTgTCaagGCTCCCATGCGGtA    475
XM003653428  cTcAgCTTCgacGTCgACGTgTCCcaGCTCgtcTGCGGcA    477
```


AF420021 TGAACGGCGCGCTATATCTCTCCGAAATGTCTCCCTCCGG 523
FJ695140 TGAACGGaGCtCTgTAcCTtaCgtctATGgaTgCtTCgGG 518
JF827297 aaAACGGgGCtCTtTATCTCTCtGAgATGgaTgCgaCtGG 518
XM951338 TGAACGGgGCGtTtTATtTgagtGAgATGTtgatggatGG 508
XM001262789 TGAACGGCGCcCTcTAcCTCTCCGAAATGgacgCCTCCGG 518
XM002152933 aaAACGGtGCtCTtTAcCTCTCtGAAATGgaTgCgaCtGG 520
XM003300681 TGAACGGtGCctTgTAcCTCagCGAgATGgCggCtagtGG 515
XM003653428 TGAACGGCGCcCTgTActTCTCCGAgATGgagatggaCGG 517
AF420021 ACAGGCTACTGCGATGCCCAATGTTATGTGAATCCCTGGA 603
FJ695140 ACAGGCTACTGtGATGCtCAATGcaATGccccagCtTGGA 598
JF827297 tCcGGtTACTGtGATGCtCAgTGTggaagtAgcagCTGGt 598
XM951338 ACgGGgTAtTGCGATGCgCAgTGTcccaaGttgga...tt 585
XM001262789 ACAGGCTACTGCGAcGCgCAgTGcTtcaaccccggCccct 598
XM002152933 tCtGGtTACTGtGATGCtCAgTGcggcacGggatCgTGGt 600
XM003300681 ACcGGCTACTGtGATGCtCAgTGcTtcGTcAcI...cctt 592
XM003653428 ACgGGCTACTGCGAcGCgCAgTGccccaaGttgga...ct 594
AF420021 GATGGGAAGGGCGCTTGGCCGTGGGATGGTCCTCGCCATG 1121
FJ695140 GATGGGAAaaGCaCTaGaaatgGGcATGGTCCTtatttTc 1149
JF827297 aATGGGtgaGGCcCTTGGCCGTGGcATGGTtCTtatCtTc 1089
XM951338 cATGGGcgaGGCGaTgGGaaGgGGcATGGTgCTgatttTc 1091
XM001262789 tATGGGcgaGGCcCTgGGgaGgGGcATGGTgCctGtgtTc 1053
XM002152933 aATGGGtgaGGCcCTcGGtCGTGGcATGGTCCTCatCtTt 1091
XM003300681 tATGGGtgatGCtCTgGctCGTGGtATGGTtCTtGCtATG 1056
XM003653428 cATGGGcgaGGCcaTcGGCCGcGGcATGGTgCTCatCtTc 1091
AF420021 AGTATCTGGAATGATGCGGGTGGCTACATGCAGTGGCTCG 1161
FJ695140 AGTATCTGGAATGATcCcaGcGcCTtCATGaAcTGGCTtG 1189
JF827297 AGcATtTGGAATGATGCaGGTGGaTACATGaAcTGGCTaG 1129
XM951338 AGTATCTGGgcgGATGaGaGcGGgTtCATGaAtTGGtTgG 1131
XM001262789 AGTATCTGGgATGATcCGGGcctgTggATGCAtTGGCTtG 1093
XM002152933 AGcATtTGGAATGATGCaGGcGGaTcCATGaAcTGGtTaG 1131
XM003300681 AGcATCTGGtggGAcaaGtccGGtggtATGaAcTGGCTCG 1096
XM003653428 AGccTgTGGgtTGAcaacGGcGGCTtCATGaAcTGGCTCG 1131
AF420021 GTCTTTGAAGATTTGAAGTGGGGTGATATTGGCAGTACTT 1281
FJ695140 acCTTctcAaATaTcAAGTGGGGaGATATTGGaAcaA... 1306
JF827297 actTTctcAaAcaTcAAGTGGGGTGATATTGGatcTA... 1246
XM951338 acgTTTtcgaATaTcAAGTGGGGaGAgATgGGtAGTA... 1248
XM001262789 actTTTtcgaAggTcAgGTGGGGgGATATTGGtAGTA... 1210
XM002152933 actTTctcAaAcaTcAAGTGGGGTGATATTGGatcTA... 1248
XM003300681 acaTTcagctccaTcAAGTGGGGTGAgATTGGCtcaA... 1213
XM003653428 acCTTctccaAcaTccgaTGGGGcGAgATcGGCAGcA... 1248