Botryosphaeriaceous infection in New Zealand vineyards: Identification, population structure and genetic diversity

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by

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A survey of 43 vineyards from six wine growing regions in New Zealand collected 238 grapevine wood samples displaying characteristic symptoms, including cankers, trunk necrosis, dieback and decline. Isolation from the symptomatic material showed that botryosphaeriaceous species infection was present in 88% of the vineyards and 68% of the 238 samples, which yielded 336 isolates of botryosphaeriaceous species. The incidence of infection varied between regions (P<0.001), being highest in Gisborne (96%) and lowest in Otago (23%), and between age groups (P<0.001), being highest in grapevines 6 –10 years old. Infection incidence differed between scion varieties (P<0.001), being highest in Sauvignon blanc and higher in grafted than non-grafted grapevines (P<0.001).

Analysis of all isolates using morphological characteristics and the molecular tool ARDRA identified nine species as *Neofusicoccum parvum, N. luteum, N. australe, N. ribis, Diplodia mutila, D. seriata, Dothiorella sarmentorum, Botryosphaeria dothidea* and also *Do. iberica,* which was a first record in New Zealand. The relative frequencies of these species showed that *N. parvum* was predominant (34%) followed by *D. mutila* (18%), *D. seriata* (16%), *N. luteum* (14%), *N. australe* (11%), *N. ribis* (3%), *Do. iberica* (2%), *Do. sarmentorum* (1%) and *B. dothidea* (1%). *Neofusicoccum parvum* and *N. luteum* were isolated most frequently from the North Island, and *N. australe* most frequently from the South Island. The *Diplodia* species were mostly found in South Island vineyards. The species, *N. parvum, N. luteum, N. australe, D. mutila* and *D. seriata,* varied significantly (P<0.05) in their optimum temperatures for maximum growth rate.

High genetic diversity intra- and inter-vineyard and between regions was demonstrated for populations of *N. parvum, N. luteum, N. australe* and *D. mutila* using UP-PCR and neighbour joining analysis. Nei's genetic diversity using data from 8 UP-PCR primers for *N. parvum* was H=0.2581 and with 5 UP-PCR primers for *N. luteum, N. australe* and *D. mutila* populations was 0.1791, 0.2417 and 0.2347, respectively. Vegetative compatibility tests with 11 *N. parvum* isolates selected from different branches of the neighbour joining tree showed incompatible, partially compatible and compatible interactions, with overlap between three of the four VCGs identified. For *D. mutila* the 14 isolates within three VCGs largely overlapped between groups. Microscopic analysis of different compatibility

reactions for *N. parvum* and *D. mutila* revealed many anastomoses within and between the isolates' colonies.

Pathogenicity varied between isolates of *N. parvum*, *N. luteum*, *N. australe* and *D. mutila* in assays with excised grapevine green shoots and 1 year old potted grapevines. However, the isolates were ranked in a similar order in both assays with respect to their mean lesion lengths. On potted grapevines, the greatest ranges in lesion lengths were caused by isolates of *N. parvum* (0–56 mm) and *N. australe* (47–139 mm). On potted vines, *N. luteum* isolates caused the largest lesions (100–178 mm) with the greatest endophytic movement (153–268 mm) beyond lesions. Isolates from the same genetic groups in *N. parvum* and *N. luteum* neighbour joining tree generally showed similar pathogenicity levels. Co-inoculation with *N. parvum* and *N. luteum* isolates on potted grapevine showed that the infections were not synergistic or competitive, although co-inoculation caused greater downward movement from the inoculation point than single inoculations. Preliminary assays showed that *N. parvum* isolates could produce laccase *in vitro* but the variable levels from different isolates showed no relationship with their pathogenicity.

From the UP-PCR fingerprints, endogenous markers were identified in *N. parvum* isolate B2141 and *N. luteum* isolate G51a2, so PCR assays were developed to identify them in environmental samples. For *N. parvum* B2141, a nested PCR-RFLP based on sequence polymorphism allowed the *Taq*I restriction endonuclease to differentiate it from other *N. parvum* isolates and to detect as little as 0.5 pg genomic DNA. For *N. luteum* G51a2, a standard PCR assay at an annealing temperature of 63ºC amplified a unique 510 bp product for isolate G51a2 but not for other *N. luteum* isolates, and had a sensitivity of 5 pg of genomic DNA. Neither assay amplified bands in the other botryosphaeriaceous species tested. In a field experiment with conidia of these endogenous markers of *N. parvum* and *N. luteum*, the method could detect rainwater splashed propagules up to 2 m from the conidium source.

Keywords: Grapevine, botryosphaeriaceous species, *Neofusicoccum*, *Diplodia*, UP-PCR, genetic diversity, pathogenicity, endogenous marker, rainwater dispersal

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Chapter 1

Introduction

1.1 Viticulture industry in New Zealand

Viticulture is one of the fastest growing sectors in New Zealand. Grape production extends throughout the country and according to the New Zealand Winegrowers Statistical Report (2010), in the last 7 years, the New Zealand vineyard area has doubled (from 15,800 ha to 33,428 ha; Figure 1.1) and wine export values increased by 370%. Over 57% of the national grapevine area is in the Marlborough region, 15% in Hawke's Bay and 6% in the Gisborne region (Winegrowers' Annual Report, 2010). Other grape production areas include Auckland, Wairarapa, Nelson, Canterbury and Otago, which together represent 17% of the vineyard area. In 2010, 142 million litre of wine was exported at a value of NZ$ 1041 million (Winegrowers' Annual Report, 2010). These statistical data emphasise the importance of the viticulture and wine industry to New Zealand's economy.

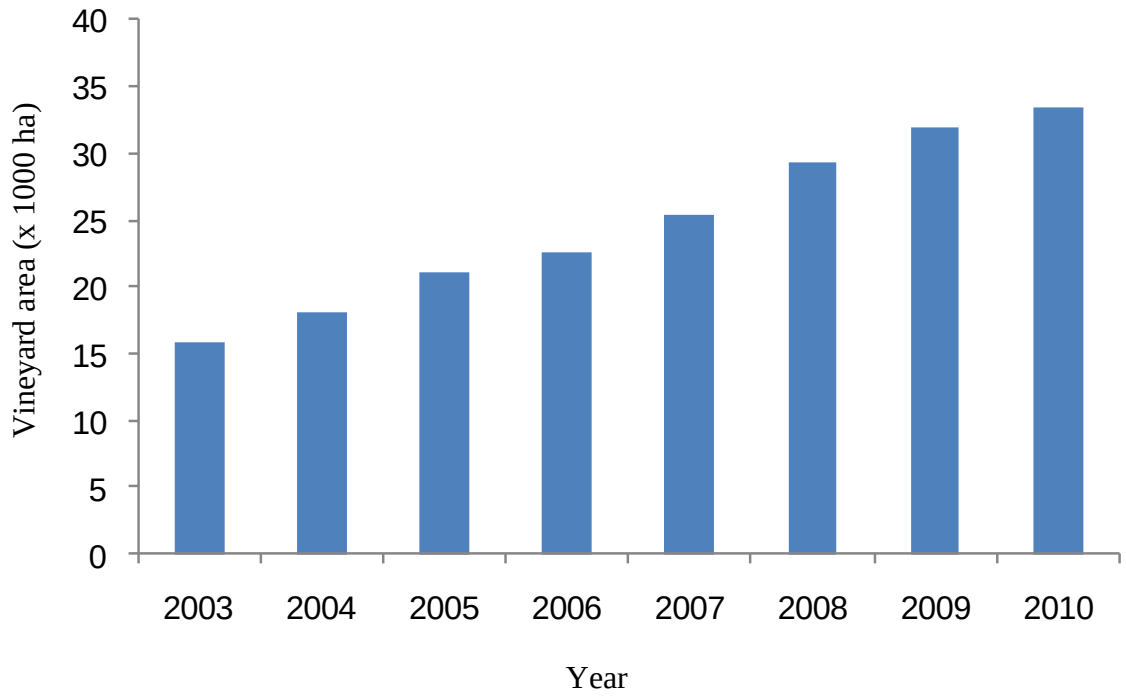

Figure 1.1: Extent of land area planted with grapevines in New Zealand from 2003 to 2010 (data obtained from Winegrowers' Annual Report, 2010).

1.2 Grapevine trunk diseases

As with grapevines all over the world, pests and diseases are major problems which reduce the yield and quality of the grapes in New Zealand. Among grapevine diseases, the grapevine trunk diseases are responsible for reduction in both quantity and quality of the grapes and causing significant economic

losses to the wine industry worldwide (Gubler *et al*., 2010). In California, the losses caused by a grapevine trunk pathogen *Eutypa lata* has reported the yield reduction ranges from 30% to 60% in the vineyards depending on the severity of the disease (Munkvold *et al*., 1994).

Recently several studies have shown that many fungal pathogens are associated with decline and dieback of grapevines which threaten the long-term productivity of grapevines (Larignon & Dubos, 1997; Mugnai *et al*., 1999; Halleen *et al*., 2003). These pathogens infect grapevines mainly through pruning wounds (van Niekerk *et al*., 2005) and lead to the development of wide ranges of symptoms including stunted growth (Munkvold *et al*., 1994), dieback, cankers and different types of wood necrosis (van Niekerk *et al*., 2006) and in some cases, deformed leaves and shoots caused by fungal toxins (Amborabe *et al*., 2001).

The major fungal pathogens associated with trunk diseases are *Eutypa lata* (Eutypa dieback; Munkvold *et al*., 1994), *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. (Petri disease; Mostert *et al*., 2006), *Phomopsis* spp. (Phomopsis cane and leaf spot; van Niekerk *et al*., 2005) and several botryosphaeriaceous species (dieback and decline; van Niekerk *et al*., 2004). All of these fungal pathogens have been reported to produce overlapping symptoms and it has also been shown that the symptom expression varies from year to year and among grape varieties, partly because of variability in susceptibility, making it difficult to identify the causal agents of grapevine trunk diseases (Rolshausen *et al*., 2010).

1.2.1 Eutypa dieback

The pathogen, *Eutypa lata* has been identified as the causal agent of Eutypa dieback also known as dead arm disease (Carter, 1991; Péros & Larignon, 1998). Since then *E. lata* has been recognised as an aggressive pathogen on grapevines, causing decline and dieback which results in economic losses due to lower grape yields (Siebert, 2001; van Niekerk *et al*., 2003). This disease has been extensively reported in most of the grapevine growing regions in the world including New Zealand (Mundy & Manning, 2006).

The first symptom of Eutypa dieback becomes visible at least 2-3 years after the infection took place (Munkvold & Marois, 1995), and severe dieback is often observed on infected grapevines that are more than 10 years old (Munkvold *et al*., 1994). The foliar symptoms caused by *E. lata* comprise stunted shoots with chlorotic leaves that are often cupped and with necrotic margins. The cankers are normally formed on trunks or cordons where the initial infection occurred and they penetrate deep into the xylem of grapevines, causing dieback or death of grapevines (Figure 1.2A; Munkvold & Marois, 1995). Internally, necrosis of the xylem tissue that develops as a result of infection can be observed as a wedge-shaped discoloured sector in the cross-sections of infected trunk and cordons (Figure 1.2C; Carter, 1988; Chapuis *et al*., 1998). In some cases foliar symptoms appear on the vine without

showing any internal wood necrosis and this is believed to be associated with the effect of phytotoxins produced by this fungus in infected grapevines (Figure 1.2E; Tey-Rulh *et al*., 1991).

1.2.2 Petri disease

Petri disease, which is also known as "black goo", "slow dieback" and "slow decline, is caused by *Phaeomoniella chlamydospora* and or several *Phaeoacremonium* species (Mugnai *et al*., 1999; Mostert *et al*., 2006). This disease is a problem in most grapevine growing areas in the world and was reported as one of the most destructive disease in Europe and California (Larignon & Dubos, 1997). The causal fungi occur asymptomatically in planting materials (Halleen *et al*., 2003) and in infected vines and only express symptoms under stress conditions (Borie *et al*., 2002). Vineyards of 1 - 5 years are most susceptible to this disease (Fourie & Halleen, 2004) and may suffer severe losses (Pascoe and Cottral, 2000). This disease has reported from many countries including South Africa (Ferreira *et al*., 1994), United States (Morton, 1997), Italy (Mugnai *et al*., 1999), Australia (Pascoe, 1999) and New Zealand (Whiteman *et al*., 2000).

The grapevines affected by this disease show stunted growth and signs of declining and dieback symptoms. The shoots appeared to have shortened internodes and leaves displayed chlorosis that sometimes developed into necrosis which led to leaf drop (Fourie & Halleen, 2004). Black to brown streaking could be observed in the longitudinal sections of the diseased grapevine wood, with black to brown spots in the cross sections of xylem tissue (Laukart *et al*., 2001). These symptoms may be observed in the rootstocks, graft unions, scion trunks, scion arms or cordons, and to small extent in roots (Morton, 2000).

1.2.3 Esca disease

Esca disease is regarded as a complex disease with several fungal pathogens involved (Surico, 2000). The white rot observed in the older vines affected by esca distinguishes it from Petri disease, which often occurs in the same vines (Graniti *et al*., 2000). Several basidiomycetes are believed to cause Esca disease, since they have been shown to be associated with the internal wood decay symptoms in affected grapevines (Larignon and Dubos, 1997). The esca disease has not been reported in New Zealand, although the Petri disease fungi have been isolated from New Zealand grapevines (Clearwater *et al*., 2000; Whiteman *et al*., 2002).

Esca disease is characterised by variety of foliar, fruit and internal symptoms. The foliar symptoms include light green to chlorotic spots which later turn brown or dark red (Figure 1.2F; Mugnai *et al*., 1999). In some regions, the ripe fruit show dark brown to black speckles and smudges, called black measles (Fig 1.2B). A wide range of internal symptoms are observed in the vines showing these foliar

symptoms. The most characteristic internal symptom is a soft white rot of the trunk wood, which is separated from the healthy tissue by a dark brown margin (Mugnai *et al*., 1999). The Petri disease symptoms of black spots can be observed in the cross sections of the adjacent non-rotted wood of trunk and cordons of the diseased vines (Figure 1.2D; Surico *et al*., 2000) (Mugnai *et al*., 1999).

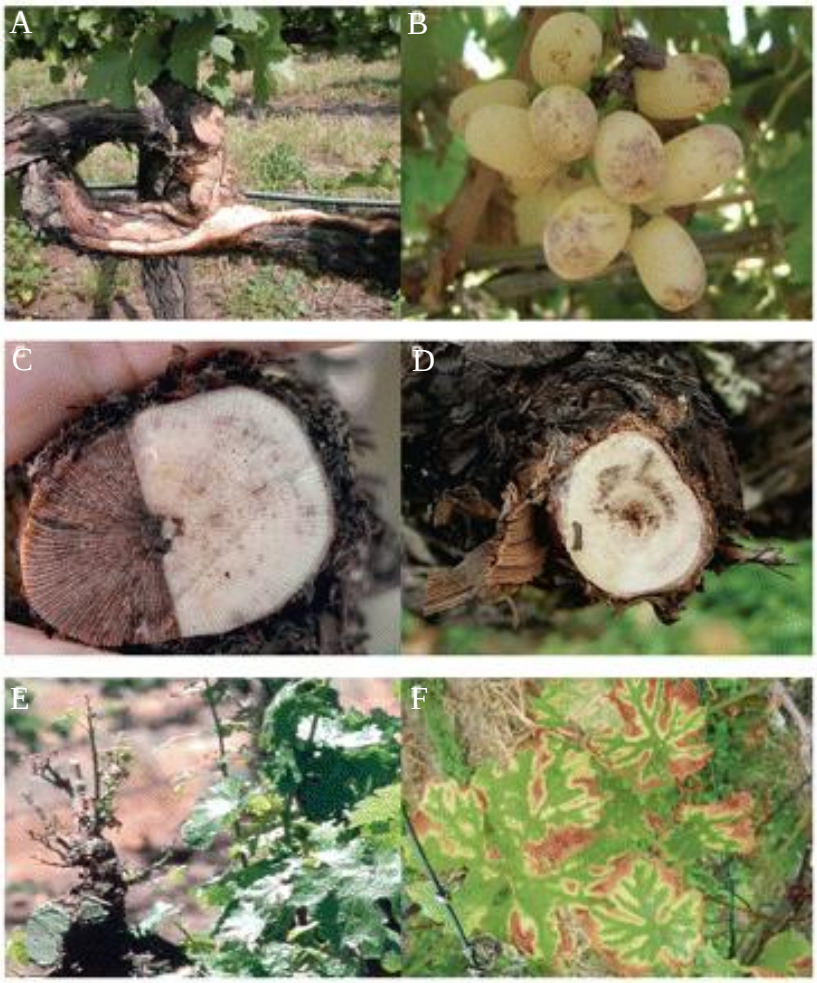

Figure 1.2: Symptoms of eutypa and esca diseases on grapevine. A) Dieback on cordon, B) spotting on grape berries characteristics of esca disease, C) cross section of grapevine cordon showing the necrotic lesion characteristic dieback, D) cross section of grapevine cordon showing dark vascular streaking characteristic of young esca disease, E) stunted shoots and leaves characteristic eutypa dieback foliar symptoms and F) foliar symptom characteristic to esca disease (Adopted from Rolshausen *et al*., 2010).

1.2.4 Phomopsis cane and leaf spot

The phomopsis cane and leaf spot of grapevine is caused by more than 15 *Phomopsis* species, with *Phomopsis viticola* being the most virulent grapevine pathogen (van Niekerk *et al*., 2005). The disease caused by *Phomopsis* spp. has a significant impact on grapevine production mostly in older

grapevines, in which 30% - 50% losses are reported to be caused by *Pm. viticola* (Schilder *et al*., 2005).

The phomopsis cane and leaf spot symptoms are visible as dark round lesions with surrounding yellow haloes on leaves and also on the internodes of young shoots that later turn brown to black. These lesions develop from near the bases of shoots and lead to weakening of shoots, which could break off in strong wind (Scheper *et al*., 1995; Rawnsley *et al*., 2004). Fruits rots also have been observed with diseased berries turning brown and shrivelled (Hewitt and Pearson, 1988). Apart from cane and leaf spot symptoms, *Pm. viticola* has also been associated with internal stem necrosis which is very similar to the wedge-shape necrosis characteristic of *E. lata* infection, leading to incorrect identification of the associated causal organism (Castillo-Pando *et al*., 2001).

1.3 Botryosphaeriaceous species disease on grapevine

For many years, dieback and decline symptoms were thought to be due only to *E. lata*, however, surveys in the Hunter Valley (Australia) and subsequent laboratory studies conducted in 2002-2003 revealed that *E. lata* was not likely to be the main cause of these characteristic symptoms. Instead, botryosphaeriaceous species were the most common species isolated in almost all the vineyards surveyed (Creaser *et al*., 2003). The losses attributed to *Eutypa* in the past, which have been estimated to be worth tens of millions in dollars in the USA and Australia may well have been in part due to diseases caused by botryosphaeriaceous species. In the past, botryosphaeriaceous species were isolated very commonly from dark and discoloured vine wood, but their role in vine disease was uncertain, because they were frequently isolated from wood that also contained *Eutypa, Phaeomoniella,* and *Phaeoacremonium* spp. as well as other wood-inhabiting fungi (Pascoe *et al*., 2000). Recently however, botryosphaeriaceous species have gained more recognition due to the fact that they have increasingly been the only die-back pathogen isolated from grapevines which exhibit dieback of shoots, spurs and arms, as well as severe internal wood necrosis symptoms.

To date, 13 botryosphaeriaceous species have been reported to cause disease symptoms in grapevines in different production regions of the world (Úrbez-Torres, *et al*., 2006a). Different botryosphaeriaceous species have been reported as the major cause of grapevine dieback and decline disease from different countries. *Diplodia seriata* has been associated with the decline of mature vines in Canada (Chamberlain *et al*., 1964), Italy (Rovesti & Montermini 1987), France (Larignon & Dubos, 2001) and Hungary (Horváth & Schweighardt, 1991). Similarly, *Diplodia mutila* has also been reported to cause the decline of mature grapevines in Canada (Shoemaker, 1964) and Hungary (Lehoczky, 1974). In California, Arizona and North Mexico, *L. theobromae* (*B. rhodina*) was found associated with the dieback of grapevines (Leavitt, 2003). In Australia, Castillo-Pando *et al*., (2001) demonstrated that *D. seriata*, could be isolated from declining mature grapevines in the Hunter Valley wine region of South Wales and was able to infect grapevines via injury to aerial parts of the vine,

causing internal symptoms such as dark streaking in the bark. Wood and Wood (2005) have reported cane dieback of grapevines in Western Australia caused by infection of pruning wounds by *L. theobromae.* Also some botryosphaeriaceous species have been isolated from 1-year-old rootstock cane and certified rootstock mother vines in South Africa (Fourie & Hallen 2004). The botryosphaeriaceous species *Botryosphaeria dothidea, D. seriata* and *Neofusicoccum parvum* were isolated from young vines in Portugal showing these species were responsible the graft failure (Phillips, 2002). Species occurring on grapevines in different countries have been shown to differ in pathogenicity; this has led to confusion and conflicting reports about which botryosphaeriaceous species are important pathogens of grapevines (Phillips, 2002).

1.3.1 Symptoms of botryosphaeriaceous species infection on grapevine

The botryosphaeriaceous species are reported to cause canker, dieback, fruit rots and other symptoms on a wide range of host plants (Maas & Uecker, 1984). In grapevines the symptoms caused by botryosphaeriaceous species has been shown to differ from region to region and with different cultivars (van Niekerk *et al*., 2004). Also the symptoms vary with the pathogen species, age of the grapevine and the environmental factors. As a result, different disease names such as Diplodia cane dieback, Macrophoma rot, excoriose, bot canker and black dead arm have been given to the different types of symptoms (Urbez-Torres & Gubler, 2009). The grapevine symptoms associated with these species worldwide are summarised in Table 1.1. The major symptoms include bud mortality, dieback, decline (Figure 1.3A), internal wood necrosis (Figure 1.3B), vascular streaking and various other symptoms attributed to the infection of the vascular tissue (Luque *et al*., 2005; Taylor *et al*., 2005; van Niekerk *et al*., 2006). These symptoms develop slowly and lead to a gradual decline in vigour and yield (Phillips, 1998). This is probably the reason why the most severe losses occur in mature (>8-years-old) grapevines (Larignon & Dubos, 2001; van Niekerk *et al*., 2004). These species can endophytically inhabit vines without causing any symptoms and severe symptoms are usually only observed when the vine has been subjected to stress conditions (Stanosz *et al*., 2001; Burgess *et al*., 2005).

Wound infections, especially of pruning wounds, lead to arm and trunk dieback, with a dark brown discoloration of the wood starting in the pruning wounds and spreading down the trunk. Within the infected trunks and cordons, wide varieties of internal necroses have often been observed and these include wedge- and arc-shaped necrotic lesions and brown vascular streaking (Castillo-Pando, 2001; Gubler *et al*., 2005; van Niekerk *et al*., 2006). The botryosphaeriaceous species, *D. mutila* and *D. seriata* have frequently been isolated from dark, wedge-shaped lesions in the trunks of the affected grapevines (Whitelaw-Weckert *et al*, 2006).

Table 1.1: Different symptoms associated with major botryosphaeriaceous species infection on grapevines (Adopted from van Niekerk *et al*., 2006 and modified).

Symptoms	[a]*B.d*	*N.p*	*D.s*	*D.m*	*N.l*	*N.r*	*L.t*	*N.a*
Bud mortality	X	X	X					
Shoot dieback	X					X	X	
Cankers	X					X	X	
Bleached canes	X	X	X	X	X		X	
Trunk dieback	X	X	X	X	X		X	
Wedge-shape necrotic lesion	X	X	X	X	X	X	X	X
Arch-shaped necrotic lesion								X
Dark brown wood discolouration	X	X	X	X	X		X	
Brown streaking, black spots	X	X	X	X	X		X	
Infected pruning wounds		X	X					
Leaf chlorosis		X	X	X		X		
Fruit rot	X					X	X	
Graft union failure	X	X	X	X	X		X	
Asymptomatic								X

[a]*B.d-Botryosphaeria dothidea, N.p- Neofusicoccum parvum, D.s- Diplodia seriata, D.m- D. mutila, N.l- N. luteum, N.r- N. ribis, L.t- Lasiodiplodia theobromae* and *N.a- N. australe*

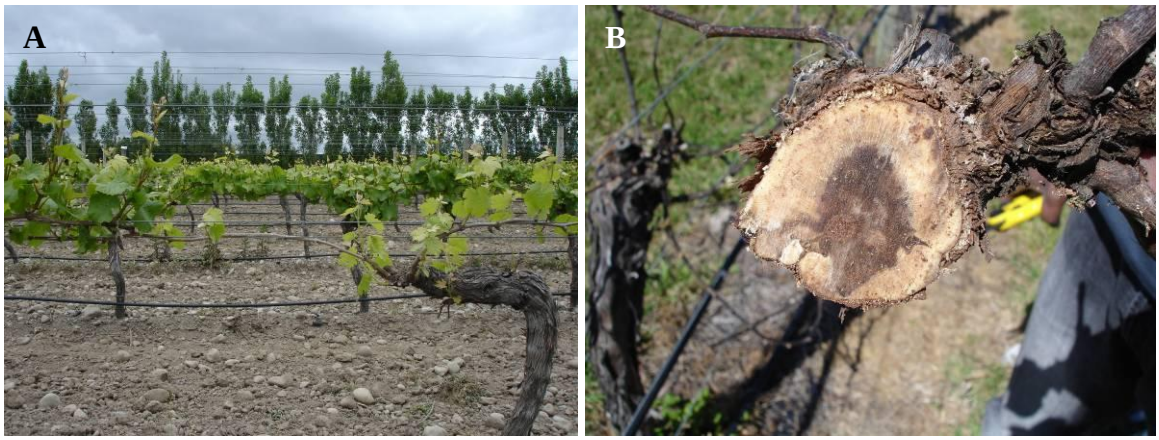

Figure 1.3: Symptoms of botryosphaeria disease on grapevines. A) Vine declining symptoms indicated by arrow and B) internal necrotic lesion in the cross section of grapevine trunk.

Other symptoms have been associated with botryosphaeriaceous species, including bud mortality, which leads directly to a reduction in yield, and a mild leaf chlorosis, but the aetiology of the disease interaction has not been elucidated. Fruit rot symptoms associated with botryosphaeriaceous species have also been reported from France (Larignon & Dubos, 2001) and the USA (Leavitt, 2003). Infections by botryosphaeriaceous species can also be a problem in young vines. These species have been isolated from 1-year-old rootstock cane and certified rootstock mother vines in South Africa (Fourie & Hallen 2004). Also the graft failure of young vines in Portugal was associated with *B. dothidea, D. seriata* and *N. parvum* (Phillips, 2002).

1.3.2 Epidemiology of botryosphaeria disease

Very little is currently known about the epidemiology of botryosphaeria diseases on grapevine. The large and increasing number of botryosphaeriaceous species is also complicating epidemiological studies of this pathogen. Botryosphaeriaceous species infection takes place either through wounds, or directly through the stomata or other openings (Kim *et al*., 2001). Successful infection and susceptibility of the infected trees is closely linked to environmental conditions, where high temperatures, water logging and other forms of stress favour colonization (Ahimera *et al*., 2003). In Australia, Castillo-Pando *et al*., (2001) demonstrated that *D. seriata*, isolated from declining mature grapevines in the Hunter Valley wine region of South Wales, was able to infect grapevine via injury to aerial parts of the vine, causing internal symptoms such as dark streaking in the bark. Wood and Wood (2005) reported cane dieback of grapevines in Western Australia caused by infection of pruning wounds with *L. theobromae.* Lehoczky (1988) reported that *D. mutila*, the causal agent of black dead arm in Hungary, over-wintered in diseased wood of grapevines, and pycnidial development and spore release coincided with rainy weather. Furthermore, the longer the period of wetness and high humidity, the more spores were produced and released (Michailides & Morgan, 1992). Hewitt (1988) stated that *L. theobromae*, the causal organism of diplodia cane dieback over-winters inside diseased wood and on pruning debris on vineyard floors where spores were released during wet periods. Van Niekerk *et al*. (2004) isolated several botryosphaeriaceous species from pruning debris, further confirming the importance of debris as a source of inoculum.

Apart from infection of grapevines in the field, infection can take place during the propagation stages. Part of the life-cycle of these fungi is spent as an endophyte within healthy plant tissue (Smith *et al*., 1996). Halleen *et al*., (2003) isolated botryosphaeriaceous species from a number of young, apparently healthy nursery plants. For this reason, their introduction into new environments on germplasm could go unnoticed. In a new environment, botryosphaeriaceous species have the potential to infect different hosts or to contributing to the genetic diversity and fitness of an existing population (Wingfield *et al*., 2001). Damm *et al*. (2005) highlighted the possibility of stone fruit trees as potential inoculum sources for grapevine trunk diseases, since they found that botryosphaeriaceous species from peach and plum could be transferred to grapevines growing in close proximity. Identification and knowledge of the botryosphaeriaceous species that occur on plants moved across the world for commercial purpose is, therefore, crucially important (Wingfield *et al*., 2001).

1.3.3 Pathogenicity of botryosphaeriaceous species

For many years the botryosphaeriaceous species have been considered as saprophytic or secondary pathogens of many hosts including grapevines. Recent pathogenicity studies conducted using botryosphaeriaceous species on grapevines showed that these species are virulent on grapevines (van Niekerk *et al*., 2005; Urbez-Torres & Gubler, 2009). However, different botryosphaeriaceous species have been reported as major pathogenic species on grapevines in different countries. In South Africa the pathogenicity studies conducted by van Niekerk *et al*. (2005) revealed that *N. australe, N. parvum,*

N. ribis and *D. mutila* were the most pathogenic species in vineyards. *Lasiodiplodia theobromae*, *N. luteum*, *N. parvum* and *N. australe* were categorized as highly virulent species on grapevine in California, whereas *D. mutila*, *D. seriata*, *Do. iberica* and *D. viticola* were reported to be weakly pathogenic on grapevines (Urbez-Torres & Gubler, 2009).

There are contrasting reports about the pathogenicity of the botryosphaeriaceous species between the countries. For example, the *L. theobromae* was reported as highly virulent on grapevine in California (Urbez-Torres & Gubler, 2009), Australia (Taylor *et al.*, 2005), Mexico (Urbez-Torres *et al.*, 2008) and Spain(Aroca *et al.*, 2008) but in South Africa the same species was the least pathogenic (van Niekerk *et al.*, 2004). Similar to this *D. seriata* was considered as a virulent species on grapevine in Chile (Auger *et al.*, 2004), South Africa (van Niekerk *et al.*, 2004) and Eastern Australia (Savocchia *et al.*, 2007), but weakly pathogenic in California (Urbez-Torres & Gubler, 2009), Portugal (Phillips, 2002) and New Zealand (Amponsah *et al*., 2008). The botryosphaeriaceous species, *N. parvum* and *N. luteum* were reported to be the most pathogenic species in several countries including eastern Australia (Savocchia *et al.*, 2007), Spain (Luque *et al*., 2007), South Africa (van Niekerk *et al.*, 2004) and California (Urbez-Torres & Gubler, 2009). Similarly, *D. mutila* was highly virulent in South Africa (van Niekerk *et al.*, 2004) but it was considered weakly pathogenic in California (Urbez-Torres & Gubler, 2009), Portugal (Phillips, 2002), Australia (Taylor *et al.*, 2005) and New Zealand (Amponsah *et al*., 2008). These differences could be due to variability in isolate virulence, or as a result of the experimental assay used, such as type of inoculum used, type of inoculated tissue, incubation period, age of the host, differences in cultivar susceptibility and other possible factors (Urbez-Torres & Gubler, 2009). For example, *D. seriata* was more virulent than *N. parvum* and *N. luteum* when inoculated into green shoots but not in cane inoculation (Urbez-Torres & Gubler, 2009). This indicates that the type of tissue used for the pathogenicity assay could lead to false conclusions about the virulence of the botryosphaeriaceous species. These findings emphasise that, although some similarities are observed in the virulence of botryosphaeriaceous species between the countries, pathogenicity studies of botryosphaeriaceous species on grapevines need to be investigated with the isolates obtained locally in order to determine the pathogenicity of species in a particular locality.

1.3.4 Enzymes and toxins produced by botryosphaeriaceous species

The pathogenicity of botryosphaeriaceous species is likely to be related to their ability to produce phytotoxic metabolites. Several phytotoxic metabolites are known to be produced by different fungal species that cause grapevine dieback and decline. For examples, two phytotoxic metabolites were isolated from the culture filtrate of *Pm. aleophilum* (Evidente *et al*., 2000) and Tabacchi *et al*. (2000) identified different phytotoxic metabolites in the liquid culture filtrate of four esca-related fungal species. The phytotoxin eutypine is known to be produced by *E. lata*, the causal agent of Eutypa dieback of grapevines (Lardner *et al*., 2006). However, only a few reports are available on the phytotoxic metabolites produced by botryosphaeriaceous species associated with grapevine diseases.

Several isolates of botryosphaeriaceous species obtained from non-grapevine hosts are known to producing bio-active toxic metabolites, which are probably involved in the disease development (Venkatasubbaiah & Chiltorn, 1990; Venkatasubbaiah *et al*., 1991; Barbosa *et al*., 2003). Venkatasubbaiah & Chiltorn (1990) reported two phytotoxic, hydroxylated mellein derivatives from culture filtrates of isolates of *D. seriata* which caused frogeye leaf spot and black rot on apple. The mullein was the most abundant toxin isolated from the culture fluid of *D. seriata* which was used in a leaf bioassay to determine the phyto-toxicity against 17 apple cultivars and showed that most of the cultivars were highly sensitive to the toxins (Venkatasubbaiah *et al*., 1991). They also concluded that there was no correlation between the pathogenicity of the isolates and the amount of the toxin production in culture but the presence of mullein could be used as a molecular marker for the detection of *D. seriata* in the diseased plants (Venkatasubbaiah *et al*., 1991).

Similar to the study by Venkatasubbaiah *et al*. (1991), four phytotoxic compounds (melleins) were isolated from a *D. seriata* isolate obtained from grapevine which showed leaf necrosis in a grapevine leaf bioassay (Djoukeng *et al*., 2009). This study also showed that *D. seriata* was able to produce these toxins on natural medium using grapevine wood powder but their production in the field infected grapevine was not investigated (Djoukeng *et al*., 2009). In another study the culture filtrate of five botryosphaeriaceous species isolated from grapevine (*B. dothidea, D. seriata, Do. viticola, N. luteum* and *N. parvum*) showed phytotoxic activity on tobacco leaves, with varied incubation periods of 14 to 21 days being required to reach the maximum phytotoxicity (Martos *et al*., 2008). The culture filtrate of *N. parvum* also caused symptoms ranging from discoloured or necrotic spots to total withering of grapevine leaves (Martos *et al*., 2008). This study suggested that most of the botryosphaeriaceous species were able to produce phytotoxic metabolites and these could be involved in virulence of these species *in planta* but no reports were available on their production in the infected grapevines in the field.

In addition to the phytotoxic metabolites, some of the botryosphaeriaceous species were reported to produce laccases. The laccases are multi-copper oxidases, which are widely distributed among fungi and reported to be involved in pathogenesis (Gianfreda *et al*., 1999). A botryosphaeriaceous species isolate (*Botrysophaeria* spp. MAMB-5, not identified to species level) obtained from a stem canker on a eucalyptus tree was shown to be lignolytic and actively produce laccase (Barbosa *et al*., 1996), with enzyme production being strongly induced by 3,4-dimethoxybenzyl (veratryl) alcohol (Vasconcelos *et al*., 2000; Dekker *et al*., 2001).. The laccase production level was shown to vary between three botryosphaeriaceous species including *Botrysophaeria* isolate MAMB-5, *N. ribis* and *L. theobromae* (Vasconcelos *et al*., 2001). In addition to laccase, the production of pectinase was observed when the fungus was cultured on pectin (Dekker *et al*., 2001), confirming that some botryosphaeriaceous species may be pectinolytic (Saldanha *et al*., 2007). The pectinolytic nature of the botryosphaeriaceous species was first reported in *N. ribis* (McClendon *et al*., 1960). The enzyme lipase is also produced by isolates of botryospaeriaceous species, with the highest production levels shown by *N. ribis* and lowest levels by *L. theobromae* (Messias *et al*., 2009). Although many of these enzymes are reported to be

produced by several botryosphaeriaceous species, their role in disease development on grapevines or any other hosts needs to be investigated.

1.4 Reports of botryosphaeriaceous species from New Zealand

Previously, several botryosphaeriaceous species have been isolated in New Zealand, mostly from non-grapevine hosts, but few published reports are available. Data obtained from the Landcare research culture collection (http://nzfungi.landcareresearch.co.nz) showed these species were mainly isolated from fruit trees with a variety of symptoms (Table 1.2). The few reports of botryosphaeriaceous species are being isolated from symptomatic grapevine material specified cane dieback, failed grafts, necrotic buds and excoriose symptoms (Bonfiglioli & McGregor, 2006; Mundy & Manning, 2010). A study conducted by Amponsah, (2011) reported five botryosphaeriaceous species isolated from grapevines in New Zealand including *N. parvum, N. luteum, N. australe, D. mutila* and *D. seriata*. Among them *N. australe* was a first record for New Zealand (Amponsah *et al*., 2009). In addition to this, a survey conducted in grapevine nurseries in New Zealand has identified seven botryosphaeriaceous species including a new record of *N. macroclavatum* in New Zealand (Billones *et al*., 2010). These studies indicated that the botryosphaeriaceous species were present in grapevine material and may be important pathogens of grapevines in New Zealand.

1.5 Taxonomy of botryosphaeriaceous species

Members of the fungal family botryosphaeriaceae were first described in the 1820's and currently more than 2000 names are linked to this family (Slippers & Wingfield, 2007). This family consist of many species with a diverse cosmopolitan distribution (Denman *et al*., 2000). Due to the large number of species accommodated in this family, the taxonomy of the botryosphaeriaceae family has been confused for a long period (Slippers & Wingfield, 2007). Although the teleomorph stage of some these fungi belonging to botryosphaeriaceae family have been identified in nature, they have rarely been produced in cultures (Denman *et al*., 2000). Even for those species with a teleomorph stage, identification based on the teleomorph characteristics was not sufficient as they varied on different hosts (Slippers *et al*., 2004a). Denman *et al*. (2000) concluded that to simplify the grouping of botryosphaeriaceae, two genera such as *Fusicoccum* and *Diplodia* could be considered. The *Fusicoccum* taxa was characterised with hyaline and narrower conidia (<10 µm) with thinner walls and the *Diplodia* taxa with wider (>10 µm) conidia with thicker walls and were often pigmented with age (Denman *et al*., 2000; Alves *et al*., 2004). Subsequently another anamorphic group, *Dothiorella,* with pigmented conidia was included into the botryosphaeriaceae (Phillips *et al*., 2005). Based on the anamorph genera, the botryosphaeriaceae were assigned to 18 coelomycetes genera (Phillips *et al*., 2005). However, there are numerous conidial anamorphs having morphological characteristics intermediate between *Fusicoccum* and *Diplodia,* and there are several species with anamorphs typical to botryosphaeriaceae classified outside the botryosphaeriaceae (Crous *et al*., 2006).

Table 1.2: Botryosphaeriaceous species isolated from different hosts in New Zealand (Data obtained from Landcare research web page).

Species	ICMP no	Host	Plant tissue	Collector	Year
N. parvum	8002	Black poplar	Dead twig	G.J. Samuels	1981
	7818	Apple	Rot fruit	S.R. Pennycook	1982
	7812	Kiwifruit	Rot fruit	S.R. Pennycook	1982
	8848	Avocado	Fruit lesion	M.A. Manning	1985
	9476	Apricot	Stem dieback	P.G. Brodhurst	1988
	15809	Grapevine	Cuttings	A. Reed	2005
	16594	Karaka	Stem	J. Pay	2006
	16595	Black matipo	Dying tree	J. Pay	2006
B. dothidea	2096	Gooseberry	Not specified	G.F. Laundon	1967
	10083	Black current	Basal rot	G.F. Laundon	1976
	7007	Blueberry	Tip dieback	P. R. Johnston	1976
	7006	Blueberry	Tip dieback	P. R. Johnston	1979
	7697	Kiwifruit	Ripe fruit rot	S.R. Pennycook	1982
	9702	Avocado	Stem and fruit rot	W.F.T. Hartill	1986
	12043	Broom	N/A	P. R. Johnston	1992
	15108	Apple	Dieback	S. Ganev	2003
N. luteum	8835	Avocado	Peduncle	M.A. Manning	1988
	15894	Walnut	Nut	M. Brathwaite	2005
	16678	Rhododendron	Leaf spots	P. R. Johnston	2006
	17508	Kiwifruit	Fruit rot	P. Rheinlander	2007
	17697	Grapevine	Fruit rot	P.K. Buchanan	2008
D. mutila	7811	Kiwifruit	Fruit rot	S.R. Pennycook	1982
	17889	Encephalartos	Leaf	C.F. Hill	2009
	18232	Kawakawa	Stem	R. Thangavel	2009
	6830	Apple	Rotted fruit	S.R. Pennycook	-
	13909	Avocado	Stem dieback	C.F. Hill	-
B. rhodina	17526	Begonia	Stem	C.F. Hill	2008
D. seriata	10080	Apricot	Canker	G.F. Laundon	1976
	10082	Apple	Not specified	G.F. Laundon	1976

Recent advances in DNA-based molecular techniques have provided efficient tools to further define species within the botryosphaeriaceae. The current revision of the botryosphaeriaceae using the morphological characteristic and DNA sequence data has identified at least 10 different lineages including *Diplodia/ Lasiodiplodia* (several teleomorph genera), *Botryosphaeria* (anamorph *Fusicoccum*), *Macrophomina* (teleomorph unknown), *Neoscytalidium dimidiatum* (teleomorph unknown), *Dothidotthia* (anamorph *Dothiorella*), *Neofusicoccum* (teleomorph *Botryosphaeria*- like), *Pseudofusicoccum* (teleomorph unknown), *Botryosphaeria quercuum* (anamorph *Diplodia*-like), *Saccharata* (*Fusicoccum*- and *Diplodia*-like) and *Guignardia* (anamorph *Phyllosticta*) (Crous *et al*., 2006). The authors of this paper also mentioned that the taxonomy of some of these groups remains unresolved due to the absence of ex-type cultures for molecular confirmation. The name '*Botryosphaeria'* does not refer to the whole group any longer, and is now restricted to only *B. dothidea* and closely related species (Slippers & Wingfield, 2007). Therefore, the name '*Botryosphaeria'* used for other botryosphaeriaceous species in previous publications will be cited

with their anamorphs names in this study. The botryosphaeriaceous species reported from grapevines worldwide can be placed into the anamorph genera *Diplodia/ Lasidiplodia*, *Botryosphaeria*, *Neofusicoccum* and *Dothidotthia* (Crous *et al*., 2006; Urbez-Torres *et al*., 2006a).

1.6 Identification of botryosphaeriaceous species based on morphology

In the past, colony morphology, anamorph morphology and temperature effects on mycelial growth rate have been used to identify the botryosphaeriaceous species (Shoemaker, 1964; Laundon, 1973; Pennycook & Samuels, 1985). Pure cultures of the botryosphaeriaceous species are relatively easily distinguished from other grapevine fungal pathogen species by their grey to black aerial mycelium and the grey to black pigment that is visible from the reverse side of the Petri dishes (Figure 1.4A) (Slippers & Wingfield, 2007). Pennycook & Samuels (1985) reported that *N. luteum* produced a characteristic yellow pigment into the growing medium after 3-4 days of incubation and that this could be used to identify this species from other botryosphaeriaceous species (Figure 1.4B). However, production of this yellow pigment was also observed in other *Neofusicoccum* species, including *N. australe* (Slippers *et al*., 2004b). Therefore, although these characters are often useful for initial grouping of related isolates in large scale isolation, they are insufficient for accurate species identification. Some researchers have used the optimum temperatures at which maximum growth rate occurred to assist with identification of the botryosphaeriaceous species (Jacobs & Rehner, 1998; Pennycook & Samuels, 1985; Urbez-Torres *et al.*, 2006a). However, reports of divergent optimun temperatures for the same botryosphaeriaceous species between countries makes this method invalid for accurately identification of species.

Mainly, identification of the botryosphaeriaceous species has relied on morphological features of conidia (Denman *et al*., 2000). Unlike the teleomorphs, these anamorphs are frequently observed in nature, many sporulate readily in culture, and have great variability in spore morphology, including size, shape, colour, septation, wall thickness and texture (Figure 1.4C-I) (Barber *et al*., 2005). However, some morphological characters exhibit extensive plasticity (Laundon, 1973). Thus, size ranges of conidia of different species overlap, while age and state of maturity affects conidial pigmentation and septation. The morphological characters can also be influenced by the substrate on which the fungus is growing. Moreover, some species are able to colonize a wide range of hosts, and multiple species may be found in the same host (Denman *et al*., 2000). As species may differ in minor morphological features, identification can be difficult for those not familiar with these fungi (Shoemaker, 1964). Thus, the species identification based on anamorph morphology alone has become very difficult due to the fact that closely related species are often morphologically indistinguishable (Slippers *et al.*, 2004a; 2004b). Recent studies have shown that combining both morphological characteristic and molecular data can clearly define species within the genera (Phillips *et al*., 2002,

Slippers *et al*., 2004a). This highlights the importance of molecular tools for the accurate and reproducible identification of botryosphaeriaceous species.

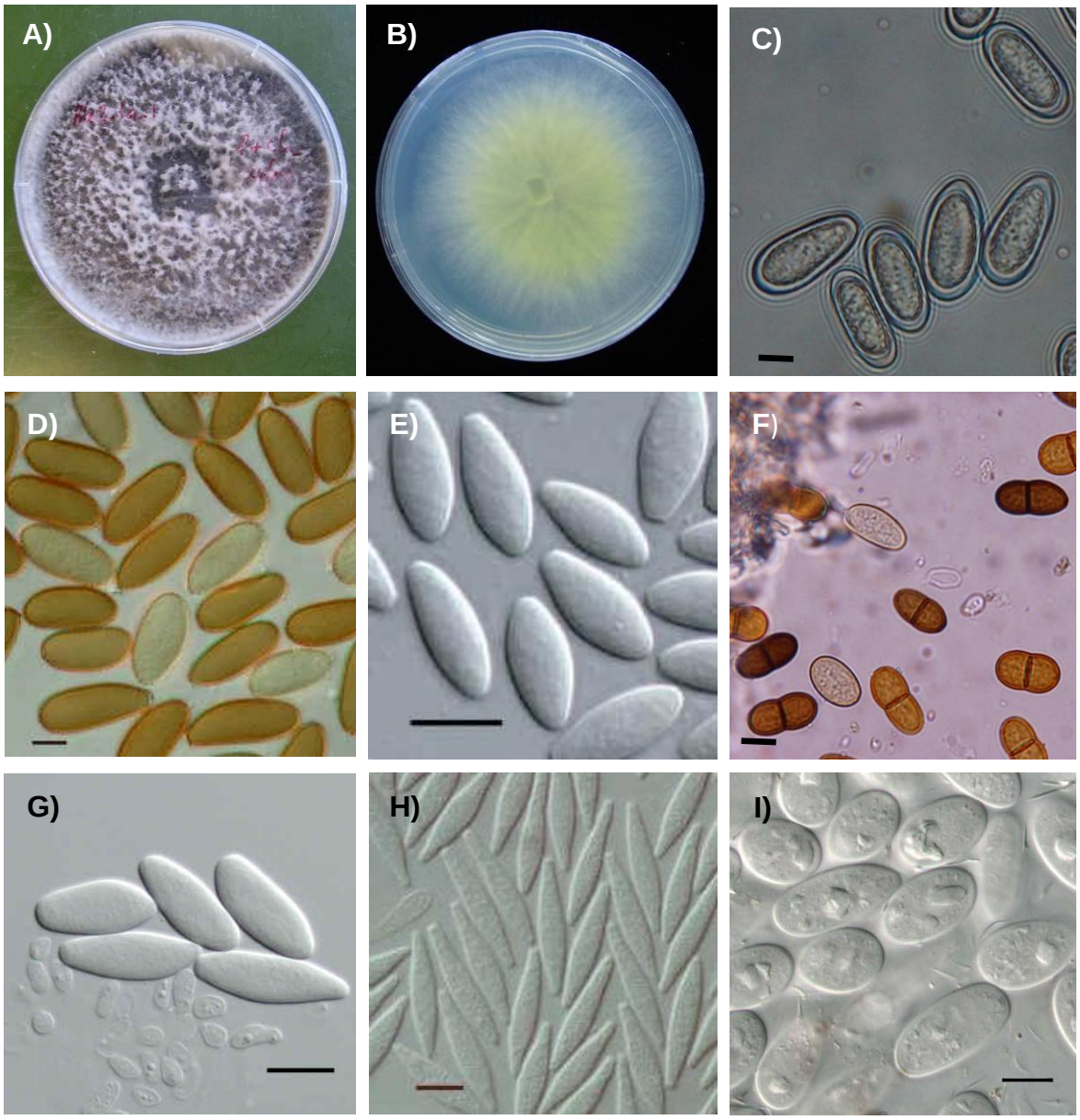

Figure 1.4: Culture and conidial morphology of botryosphaeriaceous species most commonly found in grapevines. A) A culture of botryosphaeriaceous species showing grey to black aerial mycelium, B) reverse side of the *N. luteum* culture showing yellow pigment, C) conidia of *D. mutila*, D) young and mature conidia of *D. seriata*, E) conidia of *N. parvum*, F) young and mature conidia of *Do. iberica*, G) micro and macro conidia of *N. luteum*, H) conidia of *B. dothidea* and I) immature conidia of *L. theobromae*. Scale bar= 10 µm. (Pictures D, E, G, H and I are obtained from source http://www.crem.fct.unl.pt/botryosphaeria_site/descriptions).

1.7 Identification of botryosphaeriaceous species using molecular methods

In recent years, molecular techniques have increasingly been used for the identification of micro-organisms. These methods are faster, more accurate and have greater reproducibility than traditional morphological methods. Molecular techniques, especially sequence analysis of variable regions of the internally transcribed spacer (ITS) of the ribosomal RNA gene region (rRNA), have been used to clarify the taxonomy of the botryosphaeriaceous fungi and have become powerful tools for differentiating the species. The rRNA gene regions have been most widely used to distinguish botryosphaeriaceous species, often in combination with morphological characters (Jacobs & Rehner, 1998, Phillips *et al*., 2002). For example, some cryptic species like *Neofusicoccum ribis* and *N. parvum,* or *N. luteum* and *N. australe,* are very similar morphologically, and it is difficult to accomplish reliable identification without the use of molecular methods (Slippers *et al*., 2004b). To support discrimination of fungal species based on ITS sequences, other housekeeping genes have also been used, including β-tubulin (Hirsch *et al*., 2000), actin (Weiland & Sundsbak, 2000), elongation factor 1- alpha (EF1-α; Jimenez-Gasco *et al.*, 2002) and mating type genes (Foster *et al*., 2002). In addition to sequence data, different DNA fingerprinting methods have been used to identify the botryosphaeriaceous species, including microsatellite-primed polymerase chain reaction (MSP-PCR; also called ISSR) and repetitive-sequence-based polymerase chain reaction (rep-PCR) and amplified ribosomal DNA restriction analysis (ARDRA; Alves *et al.*, 2005, 2007).

1.7.1 Identification using taxonomically informative gene regions

In the fungal genome, the rDNA occurs as repeated, structured units consisting of three, relatively conserved, ribosomal RNA (rRNA) subunit genes which are separated by internally transcribed spacers (ITS). This ITS region is an area of particular importance in fungal identification since it contains areas of relatively high variability, which allows classification over a wide range of taxonomic levels (White *et al*., 1990), sometimes even below species level (Atkins *et al*., 2003). However, ribosomal sequences do not always reflect sufficient sequence variation to discriminate between particular species (Tooley *et al*., 1996).

The rDNA sequence data has been widely used to identify the botryosphaeriaceous species by comparing the sequence data of the target isolate with the sequence data available in GenBank (Jacobs & Rehner, 1998; Smith & Stanosz, 2001; Zhou & Stanosz, 2001). In recent studies using the use of molecular data combined with morphological characters has clearly defined some botryosphaeriaceous species, which were incorrectly identified in the past based on morphological characters alone. For example, isolates previously identified as *B. dothidea* have been shown to include the three species, *B. dothidea*, *N. parvum* and *N. ribis* (Jacobs & Rehner, 1998; Smith & Stanosz, 2001). Similarly, isolates previously identified using morphology as *D. seriata*, *D. mutila* and *B. quercuum* were not always

confirmed in these species groups when sequence data was used (Alves *et al.*, 2004; Zhou & Stanosz, 2001). A study that investigated the minimal DNA sequence of the ITS region required for the identification of botryosphaeriaceous species had reported the 200 bp sequence from the start of the ITS1 and end of the ITS2 was sufficient to identify 11 of the 13 botryosphaeriaceous species studied (Vilas-Boas *et al*., 2007).

Although most taxonomic studies of botryosphaeriaceous species used DNA sequence differences in the ITS region of the rRNA gene to construct phylogenies this single gene region alone could not clearly discriminate between some closely related botryosphaeriaceous species such as *N. parvum* and *N. ribis*. In these cases, a combination of different gene regions, together with morphological characters, was necessary to delimit and describe the species (Slippers *et al*., 2004a). Alves *et al*. (2004) also stated that the sequence analysis of the ITS region in combination with the introns of protein-encoding genes, such as β-tubulin and EF1-α, was essential to distinguish closely related botryosphaeriaceous species. For example, the multi-allelic DNA sequence data from the rRNA gene (ITS1, 5.8S, and ITS2), β-tubulin and EF1-α genes were used to characterize *B. dothidea,* and showed that this species was distinct from *N. ribis* (Slippers *et al*., 2004a). Furthermore, this study reported that *N. ribis* could be distinguished from *N. parvum* based on β-tubulin and EF1-α sequence data. A similar approach was used to distinguish other closely related botryosphaeriaceous species such as *N. luteum*/ *N. australe* (Slippers *et al.*, 2004b) and *Dothiorella sarmentorum*/ *Do. iberica* (Phillips *et al.*, 2005).

1.7.2 Identification using DNA fingerprinting methods

Alves *et al.* (2005) developed an ARDRA (amplified ribosomal DNA restriction analysis) procedure for the identification of botryosphaeriaceous species. The method relies on PCR amplification of a rRNA gene fragment followed by restriction analysis of the amplicons with restriction endonucleases. For ARDRA, amplification using primers ITS1 (White *et al*., 1990) and NL4 (O'Donnell, 1993) produced a ~1200 bp product that included ITS1, 5.8S, ITS2 and the first 614 bp of the large ribosomal subunit (28S rDNA) which contained the D1/D2 domains. Restriction digestion of this large amplimer allowed differentiation between even closely related botryosphaeriaceous species. Using a combination of different restriction enzymes in ARDRA, it was possible to identify 10 botryosphaeriaceous species. ARDRA fingerprinting is an inexpensive and simple alternative to sequencing and is especially useful when dealing with a large number of isolates.

In a similar process to that of Alves *et al*. (2005) PCR amplification of the rRNA gene using the ITS1 and ITS4 primers followed by restriction digestion using *Cfo*I produced species specific fingerprints for botryosphaeriaceous species isolated from Eucalyptus in Venezuela (Mohali *et al*., 2007). However, this method failed to distinguish *N. parvum* and *N. ribis*. In other work, PCR amplification using microsatellite primers BOT15 and BOT16 (Slippers *et al*., 2004a) followed by restriction

digestion using *Cfo*I produced different fingerprints which could separate these two species (Mohali *et al*., 2007).

Other PCR-based fingerprinting methods using random primers provide a good alternative to methods that rely on primers targeted to genes. These techniques, which analyze the whole genome, have been shown to be relatively robust and discriminatory (Olive & Bean, 1999). The microsatellite-primed polymerase chain reaction (MSP-PCR) uses single primers to generate DNA fingerprints that are useful for discriminating between fungal species and strains (Meyer *et al*., 1993). Previous studies with other fungi have shown that MSP-PCR fingerprinting was well suited for detecting intraspecific variability (Czembor & Arseniuk, 1999). Similarly, rep-PCR, which is a genomic fingerprinting technique, has been used to study the inter- and intra-specific variability of many fungal genera. Both of these methods were reported to be useful for identifying botryosphaeriaceous species and could clearly differentiate cryptic species such as *N. ribis* and *N. parvum* or *N. luteum* and *N. australe* (Alves *et al*., 2007). Initially, MSP-PCR was used to characterize *B. dothidea* populations (Ma & Michailides, 2002) and later this technique proved to be a useful tool for differentiating botryosphaeriaceous species. Zhou *et al*. (2001) showed that MSP-PCR (or Inter Simple or Short Sequence Repeats; ISSR) was capable of differentiating 10 botryosphaeriaceous species and associated anamorphic fungi. Simple sequence repeat (SSR) or microsatellite markers have also been developed for the identification of some botryosphaeriaceous species with *Diplodia* anamorphs, such as *Diplodia pinea* and *L. theobromae* (Burgess *et al*., 2003). Additionally, species specific molecular markers based on the mitrochondrial small subunit ribosome gene (mt SSU rDNA) have been reported to successfully detect *D. pinea* and *D. scrobiculata* (Smith & Stanosz, 2006).

1.8 Detection of grapevine trunk disease pathogens from field samples

Identification of the causal agent of a grapevine trunk disease based on the symptoms is often difficult due to the overlapping symptoms caused by different species and certain symptoms that closely resemble those of other diseases such as Phomopsis dead arm and Eutypa dieback (Castillo-Pando *et al*., 2001). The wedge-shape internal necrosis in the infected grapevine trunk has been previously used as characteristic symptom to identify Eutypa disease but it can also be produced by *Pm. viticola* and botryosphaeriaceous species. Therefore, the identification of the pathogens causing grapevine trunk diseases has relied on isolation of the pathogen from the symptomatic diseased samples onto artificial media and subsequent identification based on colony characteristic or anamorph morphology. This is a time-consuming process and requires destructive sampling of vines. Also these colony and morphological characters are insufficient to identify some of the grapevine trunk pathogen species. For example, the hyphae of *E. lata* have no unique diagnostic features which make the identification of this species dependent on conidial characteristic. However, production of conidia from *E. lata* colonies may take up to 2 months and some isolates do not produce conidia in cultures (Carter, 1991), making it difficult to distinguish *E. lata* from other ascomycetes, in particular diatrypaceous fungi (Rolshausen

et al., 2004). Similarly, the detection of another grapevine trunk pathogen, *Pa. chlamydospora,* from field samples also required destructive sampling for the isolation of fungi and identification. However, slow growth of the fungus in culture could delay the identification for several weeks (Pascoe & Cottral, 2000). Therefore, there is a requirement for a rapid, reliable and non-destructive sampling technique which would enable growers to identify the grapevine trunk pathogens even before the appearance of symptoms.

Recent advancement in molecular techniques can provide rapid, accurate and reliable detection of fungal pathogens. Since the introduction of PCR (Mullis & Faloona, 1987), nucleic acid base methods have been increasingly developed for the detection and identification of plant pathogens. The PCR is a procedure which allows amplification of a specific DNA sequence of the target species and is considered a powerful technique for detecting species because of its specificity and sensitivity (McCartney *et al*., 2003). The PCR-based detection methods are very sensitive and can be used to detect very small quantities of pathogen DNA (Lee and Taylor, 1990). Therefore, the PCR based methods are useful for the identification of early stage or latent infections of plant pathogens. Development of species specific PCR assays for the detection of a species based on the unique DNA sequence of the target species is important. Different studies have shown that species specific PCR assays developed for *Pa. chlamydospora* could detect this species in grapevines (Ridgway *et al*., 2002; Retief *et al*., 2006). Similarly, species specific primers designed based on the ITS sequences for *E. lata* was used to detect this species from infected grapevines (Lecomte *et al*., 2000). However, in some cases combination of two different molecular techniques was required to identify the species. For example, Rolshausen *et al*. (2004) developed a PCR-RFLP based protocol capable of identifying *E. lata* following amplification of DNA using the universal ITS1 and ITS4 PCR primers and digestion of the PCR product with the restriction enzyme *Alu*I. However, in some assays the sensitivity of the conventional PCR assay was sometimes inadequate to detect the target species especially from environmental samples. The nested-PCR method, which includes an extra step of amplification within the target sequence, has been used to improve the sensitivity of detection (Whiteman *et al*., 2002). Similar to other grapevine trunk pathogens, there are number of PCR based detection methods published for the detection of different botryosphaeriaceous species from different hosts.

1.8.1 Detection of botryosphaeriaceous species from field samples

There is a need for a rapid and effective tool to identify the botryosphaeriaceous species, especially in a host infected by multiple species (Mohali *et al*., 2007). In prior research, different molecular markers have been developed for PCR- based identification of some botryosphaeriaceous species (Zhou *et al*., 2001; Burgess *et al*., 2003; Slipper *et al*., 2004a). Most of these molecular markers have been used for botryosphaeriaceous species identification in laboratory conditions, using pure cultures. A quantitative PCR assay using species specific primers designed based on the sequence of the small subunit of rRNA along with a TaqMan^{TM} probe has been used for the detection and quantification of *Sphaeropsis sapinea* (*D. pinea*) from pine trees (Luchi *et al*., 2005). This quantitative PCR has been reported to detect *S. sapinea* even at low amounts of inoculum (0.1 pg) (Luchi *et al*., 2005). Smith & Stanosz

(2006) developed species specific PCR assays based on the mitochondrial small subunit ribosome gene (mt SSU rDNA) for the detection of *D. pinea* and *D. scrobiculata* in dead red and jack pines in which the pathogens caused collar rot. They designed species specific forward primers DpF and DsF for *D. pinea* and *D. scrobiculata,* respectively, and used the nonspecific reverse primer BotR to produce species specific detection. The resultant assay was useful to detect these species from the stem or root of the diseased plant. Further to this, Luchi *et al.* (2011) recently developed a PCR method using high resolution melting analysis (HRMA) for the detection of *D. pinea* in pine. This method used the sequence of mitochondrial SSU rRNA to detect *D. pinea* and could distinguish *D. pinea* from the closely related species *D. scrobiculata* without the need for a post-PCR procedure (Luchi *et al*., 2011).

There are few reports available about the application of molecular markers for the detection of botryosphaeriaceous species infecting grapevines. Recently, a nested PCR assay was developed for the detection of botryosphaeriaceous species with particular emphasis on the *N. parvum*/ *N.ribis* complex (Spagnolo *et al*., 2010). This method used the ITS5 and ITS4 (White *et al*., 1990) along with the specific internal primers NprcA and NprcB designed for the *N. parvum*/ *N. ribis* complex and BoitsA and BoitsB for botryosphaeriaceous species. This method was reported as specific and sensitive, being able to detect these species in symptomatic and non-symptomatic vines (Spagnolo *et al*., 2010). In addition to this, a PCR-SSCP assay was developed using ITS based multi genus primer pairs BOT100F and BOT472 which were able to differentiate four botryosphaeriaceous species found on grapevine including *N. australe*, *N. luteum*, *D. mutila* and *D. seriata* (Ridgway *et al*., 2011). The sensitivity of this assay was 0.1 pg in nested PCR assay and it was able to detect multiple botryosphaeriaceous species in rainwater samples collected from the vineyards (Ridgway *et al*., 2011). These methods are useful to screen the grapevine planting material for botryosphaeriaceous species infection.

1.9 Genetic variability of botryosphaeriaceous species

The genetic variability of different botryosphaeriaceous species populations from different hosts and localities have been studied in using a morphological method such as vegetative compatibility grouping (Ragazzi *et al*., 1997; Burgess *et al*., 2001) and using different fingerprinting methods, including simple sequence repeat marker (SSR; Burgess *et al*., 2004), MSP-PCR (Ma *et al*., 2001; 2003), Random Amplified Polymorphic DNA (RAPD; Smith & Stanosz, 1995; Stanosz *et al*., 1996; Saldanha *et al*., 2007) and Amplified Fragment Length Polymorphism (AFLP; Piškur *et al*., 2010).

1.9.1 Genetic diversity analysis using vegetative compatibility groupings

Vegetative compatibility groupings (VCGs) have been extensively used to describe fungal population structure and diversity (Leslie, 1993). In many filamentous fungi, physiologically distinct individuals of the same species can fuse asexually to form a heterokaryon. When the heterokaryon is stable, then the participants are said to be vegetatively compatible and to belong to the same vegetative compatibility group (VCG). The isolates belonging to the same VCG are presumed to be more genetically similar to each other. The mycelia of different isolates of a same species that did not fuse or produced unstable heterokaryons are called vegetatively incompatible. The genetics of vegetative compatibility has been shown in many fungal species. The vegetative incompatibility (*vic*) loci which are unlinked loci found dispersed throughout the fungal genome play a crucial role in the maintenance of the heterokaryon. The number of *vic* loci responsible for the VCG phenotypes varies in different fungal species (Cortesi & Milgroom, 1998). The different VCGs may differ from one another at one, some, or all of the *vic* loci that are responsible for the VCG phenotype (Leslie, 1996).

Although VCGs have been reported in many fungal species, this phenomenon has been reported for relatively few botryosphaeriaceous species namely *B. dothidea* from pistachio, *S. sapinea* from pine and *Neofusicoccum* species from *Eucalyptus* spp. (Ma *et al*., 2001; Smith *et al*., 2000; Slippers & Wingfield, 2007). For these species, different numbers of VCGs were identified in the different botryosphaeriaceous species. For example, 20 VCGs were identified from 378 *B. dothidea* isolates obtained from pistachio in California (Ma *et al*., 2004) and 62 VCGs from 107 isolates of *S. sapinea* from pine in South Africa (Smith *et al*., 2000). In another study, Burgess *et al*. (2001) identified 67 VCGs in 252 *S. sapinea* isolates obtained from pine. This study showed that different levels of genetic diversities measured in different *S. sapinea* populations using VCGs ranged from extremely low in Australia (ranges 1.6% - 7.4%) to very high in South Africa (52.7% -86.2%) with New Zealand having an intermediate genotypic diversity (28.2%; Burgess *et al*., 2001). Clearly, some of the botryosphaeriaceous species populations had greater genetic diversity than others.

1.9.2 Genetic diversity analysis using different fingerprinting methods

Many studies have shown that molecular fingerprinting can provide more detailed information on the genetic diversity and population structure of fungal pathogens than VCG analysis (Parker *et al*., 1998). There are a number of PCR-based techniques available for the analysis of genetic diversity of fungal populations. The most commonly used methods include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and universally primed PCR (UP-PCR).

RAPD can be used for genotyping and provide markers that vary between the populations and species (Williams *et al*., 1990). The RAPD analysis involves random amplification of DNA without previous knowledge of DNA sequences using a 10 base pair (bp) primer which anneals to the complementary

sites in the genome and produces several different sized bands. Many of these bands could be polymorphic among the individuals and populations. RAPD has been used to produce fingerprints for the genetic diversity analysis in many fungal species including the grapevine trunk pathogen *E. lata* (Peros & Larignon, 1998). RAPD is one of the most common PCR-techniques for population studies as it is simple and inexpensive compared to other PCR-based methods. However, RAPD has several disadvantages such as the need for specific reaction conditions, reagents and equipment can affect the reproducibility of the results between the studies and laboratories (Tommerup *et al*., 1995). Also, the genetic basis of the RAPD bands is usually unknown and to confirm the homology of the same size bands sequencing is required (Brown, 1996).

The UP-PCR fingerprinting method is similar to the RAPD technique but the primers are longer than RAPD primers. UP-PCR primers are not random primers like those used in RAPD and are designed to target intragenic, more variable areas of the genomes (Bulat *et al*., 1998). There are several advantages of using UP-PCR compared with RAPD including greater reproducibility and generation of much more complex banding pattern. Due to the robustness of the UP-PCR method, it has been used to study the genetic relatedness of different fungal populations including *Pa. chlamydospora* population in grapevines (Pottinger *et al*., 2002).

AFLP fingerprinting technique is a robust and reliable technique based on selective amplification of restriction fragments from the digest of genomic DNA. To generate AFLP fingerprints, the genomic DNA is cut by two restriction enzymes then adapters are ligated to the restriction sites which facilitate selective amplification and reduce the number of bands generated. This technique is expensive and more laborious than RAPD and UP-PCR methods. However, AFLP can produce highly complex fingerprint profiles and is useful to distinquish fungal isolates of a species (Vos *et al*., 1995). AFLPs have been widely used to study the genetic variability of a range of fungal species (Gonzalez *et al*., 1998; Schnieder *et al*., 1998).

A binomial matrix data can be generated from the above methods, based on the presence and the absence of the specific sized bands in the fingerprints produced. The matrix data can be used to produce phylogenetic trees to show the genetic relatedness of the individuals of the populations using different phylogenetic analysis software. Also, the matrix data is useful to enable and the genetic diversities of different populations to be calculated using programs such as Popgene or other relevant software.

Only a very few studies have reported analysis of the genetic diversity of botryosphaeriaceous species isolated from different hosts using molecular methods (Ma *et al*., 2001; Burgess *et al*., 2004). A study conducted on *B. dothidea* populations obtained from pistachio and other hosts showed different levels of genetic variability in botryosphaeriaceous species population in different hosts and localities (Ma *et*

al., 2001). However, there are no reports of the genetic variation for populations of any of the botryosphaeriaceous species obtained from grapevines.

1.10 Aim and objectives of this research

To date, no detailed studies have been done to identify and characterise populations of botryosphaeriaceous species present in New Zealand vineyards. The overall aim of this PhD research is to study the population composition and genetic variability of botryosphaeriaceous species in New Zealand vineyards and to use this information to develop molecular tools for field level detection. To achieve this aim four objectives have been formulated as below:

Objective 1: To investigate the population composition of botryosphaeriaceous species in New Zealand vineyards. A survey will be carried out to provide information on species identification, prevalence, regional variation and species diversity of this group of pathogens.

Objective 2: To determine the genetic diversity of populations of up to four major botryosphaeriaceous species found in New Zealand vineyards. Single spore isolates will be produced and their genetic diversity will be assessed using the UP-PCR method.

Objective 3: To investigate the intra-species virulence variability of the four major botryosphaeriaceous species populations found in New Zealand vineyards. The intra-species virulence variability will be assessed using genetically distinct isolates of these species in both *in vitro* and glass house experiments.

Objective 4: To develop and optimise isolate specific molecular markers identified in the genetic variation analysis. The developed markers will be validated in the vineyard where they will be used to track the marked isolate.

Chapter 2
Population composition and distribution of botryosphaeriaceous species infecting grapevines

2.1 Introduction

For many years botryosphariaceous species have mainly been considered to be saprophytes or secondary colonizers of grapevines (Phillips, 2002), and so their pathogenicity has been underestimated and their epidemiology not fully investigated. In New Zealand and worldwide, *E. lata*, the causal agent of Eutypa dieback, was thought to be the most important die-back pathogen of grapevine. However, botryosphaeriaceous species have gained recognition as black rot canker and fruit rot pathogens on fruit trees, mainly being reported from apple (Laundon, 1973) and kiwifruit (Pennycook & Samuels, 1985) in New Zealand. Die-back and decline disease problems have been increasingly observed in New Zealand and in overseas vineyards 13 botryosphariaceous species have been reported as the causes of die-back disease (Úrbez-Torres, *et al*., 2006a). Until now, a major problem facing the grapevine industry was the correct identification of the botryosphariaceous species causing disease on vines from different cultivars, localities and countries. In addition, species occurring on grapevines in different countries have been shown to differ in pathogenicity and this has led to confusion and conflicting reports about which species of botryosphariaceous are important pathogens of grapevines (Phillips, 2002).

Research on botryosphaeriaceous species infecting grapevine has demonstrated that different species composition occurs in different countries. In New Zealand very little is known about which botryosphaeriaceous species are responsible for dieback disease of grapevines since no detailed surveys have been carried out in vineyards or on other woody hosts. A comprehensive survey of New Zealand vineyards is needed to provide information on species identification, regional variation and genetic diversity of this group of pathogens. A study of the botryosphaeriaceous species population composition in association with symptomatic grapevines would provide the foundation for further detailed research to develop systems to control these diseases.

In the past, identification of botryosphaeriaceous species has relied on anamorphic characteristics including conidial size, shape and texture (Jacobs & Rehner, 1998). However, overlapping morphological features, especially between the several new species recently introduced into this group, has meant that sole use of anamorph features for identification is inaccurate (Alves *et al*., 2005). Introduction of molecular techniques including polymerase chain reaction (PCR) and sequencing of taxonomically important gene regions has significantly improved identification of botryosphaeriaceous species. Using a combination of morphological characteristics and molecular

data, species within this genus have been clearly defined (Denman *et al*., 2000; Phillips *et al*., 2002; Slippers *et al*., 2004a). Therefore, these identification tools can be applied to provide accurate and unambiguous identification of botryosphaeriaceous species in symptomatic material collected from New Zealand vineyards.

The aim of this study was to investigate the population composition of botryosphaeriaceous species in New Zealand vineyards to provide information on the species present and their prevalence. This study will also identify any differences in the geographical distribution of botryosphaeriaceous species, for example between North and South Island vineyards. To achieve this aim, samples of diseased grapevine materials were collected from throughout the country for the isolation of botryosphaeriaceous species. Species identification of botryosphaeriaceous species was carried out using a combination of culture morphology and conidial characteristics followed by molecular confirmation using a published ARDRA method (Alves *et al*., 2005) and sequencing of ITS, β-tubulin and elongation factor (EF1-α) gene regions.

2.2 Materials and methods

2.2.1 Vineyard sampling

The purpose of sampling diseased vine material showing decline and die-back disease symptoms from vineyards throughout New Zealand was to isolate botryosphaeriaceous fungi. In June–December 2007, symptomatic grapevine samples were requested from at least 25 growers in each of the major grapevine growing areas in New Zealand including Otago, Canterbury, Marlborough (Blenheim), Nelson, Eastland (Gisborne) and Auckland (Figure 2.1). Disease information leaflets (Appendix A.1) and survey request forms (Appendix A.2) were prepared and sent to the vineyard owners or vineyard managers by post asking them to supply at least 10 suspected decline and dieback vine specimens. Information about the age of the vine, vine cultivar, growing conditions and any special management practices (eg: top grafting) of the sampled vine were requested on the survey form and recorded for later analysis. Regional visits were conducted to six vineyards in Gisborne, six vineyards in Otago, and four vineyards in Canterbury to ensure that sufficient specimens were obtained from each region. All the samples from the Auckland region were collected by a consultant (Allan D. Clarke, viticulture specialist) who was familiar with viticulturists in the region.

The collected vines were systematically numbered, packed and kept in a cool room at 4ºC until the beginning of the isolation process, which was within 2-3 weeks of sample receipt. Isolation and identification was carried out according to the procedure outlined in Figure 2.2.

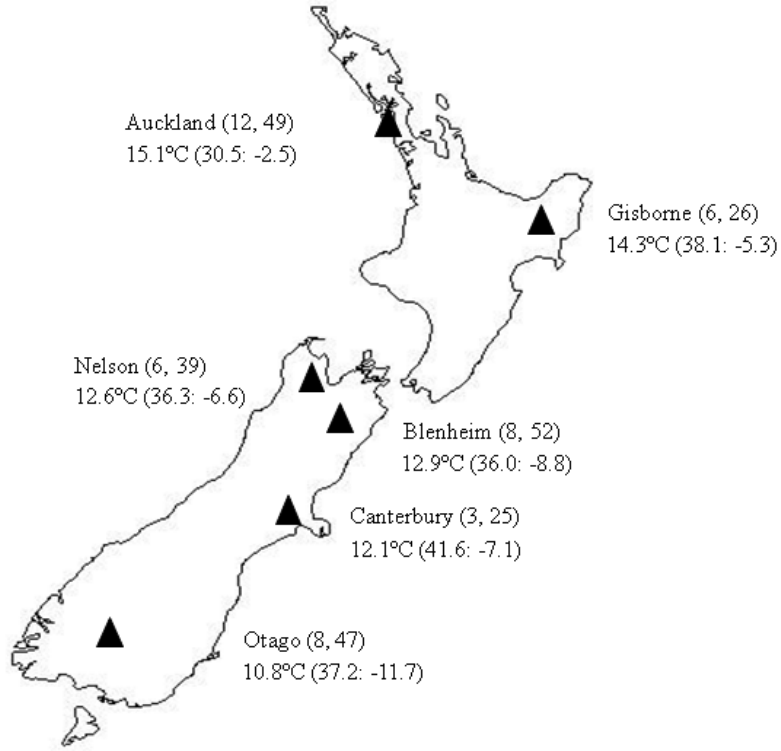

Figure 2.1: New Zealand map indicating the wine growing regions surveyed in this study. (Adopted from http://www.maps-gps-info.com/mp-nz.html and modified). The number of vineyards sampled per region followed by the number of diseased samples collected is provided in brackets beside the name of the region. Immediately below the region is the avearge (mean annual values for 1971-2000 period) temperature for each region in brackets (Source: http://www.niwa.co.nz/our-science/climate).

2.2.2 Data collection and maintenance

Maintaining an accurate and detailed database was important to keep all the information collected by the survey for later analysis. An Excel® (Microsoft Office version 2003) spreadsheet was used to store the information including grower's details, vineyard information and management practices. Vineyard details consisted of the history of the vine such as rootstock and/or scion cultivar, nursery of origin, date of planting and external symptoms of the collected vine samples. Management practices included information about soil nutrient status, irrigation type, crop history of the site, method of pruning and disposal methods for pruning debris. To protect the privacy of growers, all collected information was kept confidential and the isolate names did not reflect the identity of the vineyard. Vineyards were allocated numbers and codes referring to region of origin (Appendix A.3) and these were used throughout the analysis.

2.2.3 Isolation of botryosphaeriaceous species from grapevine lesions

The process of isolation and identification of botryosphaeriaceous species collected by the survey is outlined schematically in Figure 2.2. Vine trunk materials were visually examined, and shape and size of the internal lesions were recorded and digitally photographed. The lesion area and adjacent non-symptomatic wood was sliced into 1 cm thick sections using a hand saw. The vine slices were surface sterilized with 70% ethanol for 30 s, 1% sodium hypochlorite (NaOCl) for 1 min and finally with 70% ethanol for 30 s. They were then rinsed with sterile water and air dried in a laminar flow hood (van Niekerk *et al*., 2004a). Surface sterilized vine slices were cut repeatedly into small segments (2–3 mm) using sterile secateurs and placed onto half strength potato-dextrose agar plates (½ PDA; Difco™ Becton, Dickinson and Company, Maryland, USA) supplemented with chloramphenical; ½ PDA-Chl; Whiting *et al*., 2000) and incubated at room temperature (approximately 20ºC). After four days the individual colonies growing from the vine segments that were characteristic of botryosphaeriaceous species were sub-cultured onto fresh PDA plates. These pure cultures were assigned with a specific code and maintained as independent isolates. For rapid and cost effective preservation of these isolates different storage methods were evaluated using four botryosphaeriaceous species. The results showed among the 11 methods evaluated, the mycelial plugs stored in sterile water at 4ºC was the best method for maintaining the viability and preserving the growth rates of botryosphaeriaceous species (Baskarathevan *et al*., 2009). This method was used to maintain the isolates collected from this study and those obtained from culture collection of other research groups.

To determine whether multiple species and/or isolates were present within a lesion, 10 representative diseased grapevine specimens with internal lesions of a large size (>50 mm) obtained from different areas were selected for isolation. The single lesion region was divided lengthwise into sequential 1 cm zones and isolation was done from each zone individually as previously described.

2.2.4 Induction of sporulation on culture medium

Most botryosphaeriaceous species do not readily produce spores on culture media, thus several different media were assessed for their ability to induce spore production. In this study a sporulation induction experiment was carried out with 37 botryosphaeriaceous species isolates (Appendix A.4) that had already been identified to species level based on the morphology of conidia that were produced on plant tissue (provided by Nicholas Amponsah, Lincoln University). The first two media comprised potato dextrose agar (PDA) and tap water agar (TWA; Appendix A.5) overlaid with 3 cm double-autoclaved pine needles (*Pinus radiata*) as a substrate (Burgess *et al*., 2005) and the third medium was Prune extract agar medium (PEA; Appendix A.5) which was known to induce sporulation (pers. com. Bob Fullerton, Plant & Food Research, Auckland). All the culture plates were maintained under fluorescent lights (providing near UV light) at room temperature to induce the sporulation (Figure 2.3). After 3 weeks growth the plates were examined under a stereo microscope

(×60) for pycnidium formation. The pycnidia were separated from the pine needles and prune agar and 5–10 pycnidia collected from each of the isolates into a 1.5 mL tube. To release the conidia from the pycnidia, 100 µL sterile water was added to each tube, and the pycnidia were crushed with a sterile micro pestle. A sample of each conidial suspension was observed under a light microscope (×100). The medium that produced the most conidia was subsequently used to produce conidia from all the isolates obtained in this study.

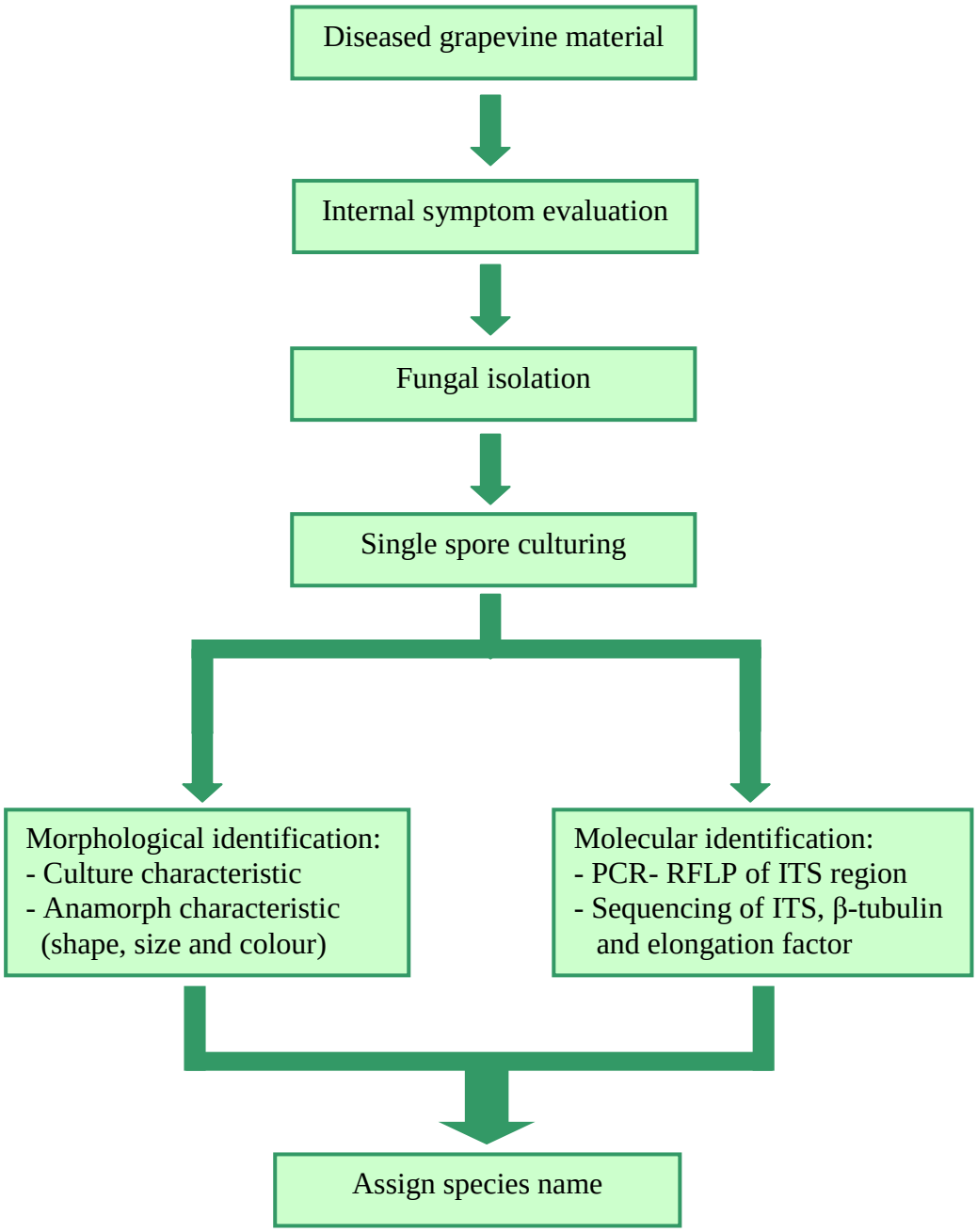

Figure 2.2: Schematic representation of the isolation and identification process of the botryosphaeriaceous species from a diseased grapevine sample.

Figure 2.3: Layout of fluorescent light beams used to produce near UV light to induce the *in vitro* sporulation of botryosphaeriaceous species. Lights are 75 cm from the surface of the plates. The light overhead beam consisted of four 40 W cool white fluorescent bulbs (Philips TLD 36W/840) arranged by two on each sides with a single 36 W blue bulb (SYLVANIA FL36W/ Blacklight blue) in the centre.

2.2.5 Identification of botryosphaeriaceous species based on colony and conidium morphology

Identification of botryosphaeriaceous species largely depends on the taxonomy of the anamorphs (Smith & Stanosz, 2001). The morphological characteristics of the anamorphs that are considered useful for identification include conidial size, shape, colour and septation (Pennycook & Samuels, 1985). Denman *et al.* (2000) placed *Botryosphaeria* anamorphs into two groups based on conidium colour. Accordingly, species with hyaline conidia were placed in the *Fusicoccum* group while those with dark conidia were placed in the *Diplodia* group.

In this study, the initial identification of botryosphaeriaceous species was carried out based on the colony morphology on PDA and anamorph characteristics reported in the literature. Production of characteristic yellow pigment on PDA after 3 d incubation was used to group *Neofusicoccum luteum*, *N. australe* and *N. parvum* species. The conidial morphology was studied using a microscope (at ×100 and ×400) after crushing a pycnidium on a microscopic slide with a water drop to release the conidia. Conidial characteristics were observed under a microscope and digitally imaged using an Olympus BX51 microscope with a built-in Olympus DP12 camera (Olympus optical, Japan). The length and width of the conidia were measured using the AnalySIS® software, and the shape and colour recorded. The conidial morphology of the botryosphaeriaceous isolates from New Zealand vineyards were compared to published descriptions of the botryosphaeriaceous species to assign species names.

2.2.6 Single spore culture production

Establishing a large number of single-spore isolates is essential for studies of genotype diversity in fungi (Ho & Ko, 1997). Fungal colonies which grow from single spores are derived from a single nucleus and therefore represent genetic individuals from the population.

Single spore cultures were produced by spreading 20 µL of each conidium suspension onto TWA plates. After incubation at 23.5ºC for 24 h each plate was examined under a stereomicroscope (×100) and the colonies that originated from a single conidium were marked and transferred to separate PDA plates (Ko *et al.*, 1986). Cultures were incubated at 23.5ºC for 7 d and kept in the cool room (4ºC) for short term storage.

For isolates that failed to produce pycnidia on pine needles embedded in TWA, hyphal tip culturing was used (van Niekerk *et al*., 2004, Úrbez-Torres, *et al*., 2006a). In this process, the isolates were grown on TWA at 23.5ºC for 24 h and then examined under a stereomicroscope (×100). Individual hyphal tips containing a single cell were marked, excised using a sterile scalpel and transferred onto PDA plates.

2.2.7 Molecular based identification using ARDRA and sequencing

Species identification based on anamorph morphology alone can be difficult due to some closely related species being morphologically indistinguishable (Slippers *et al*., 2004a). The molecular confirmation of species that had been identified based on their anamorph morphology and identification of non-sporulating isolates was done using a published ARDRA method (Alves *et al*., 2005). A slight modification was made from the published protocol in that the gel electrophoresis of digested PCR products was done on 1.5% Agarose (molecular grade; Bioline) gels using 1×TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0) under a constant voltage of 10 V/cm for 1 h instead of 4% Agarose 3:1 HRBe (Amresco) gels under a constant voltage of 80 V for 2.5 h (Alves *et al*., 2005).

2.2.7.1 DNA extraction from fungal mycelium

For species confirmation of the entire collection of botryosphaeriaceous isolates using molecular methodology, either of two standard rapid DNA extraction methods was used. These methods were faster than the standard genomic DNA extraction method and provided DNA suitable for PCR. The first method (Chelex method) was done using 5% Chelex solution made up by suspending 0.5 g of Chelex®100 resin (BioRAD) in 10 mL of sterile water. A small amount of aerial mycelium was taken from a young (2–3 days old) culture using a sterile 10 µL pipette tip and added directly into 50 µL of suspended Chelex resin. The sample was heated at 99ºC for 20 min using a heating block then frozen

by placing at –20ºC for 20 min. Subsequently, the sample was allowed to thaw completely at room temperature, gently mixed by a brief pulse on a vortex then centrifuged at 15,700 $\times g$ for 2 min. The supernatant (15 µL) was transferred to a new 1.5 mL tube and used as a template for PCR.

Due to inconsistent results from the method described, a second rapid DNA extraction method, REDExtract-N-Amp™ PCR kit (Sigma), was used. The same amount of mycelium was added into 50 µL extraction solution and incubated at 95ºC for 10 min using a heating block. Then 50 µL of dilution solution was added and vortex briefly. This solution was stored at –20°C until used for PCR, which was carried out with REDExtract-N-Amp™ PCR reagents according to the manufacturer's instructions.

For genotyping studies high quality genomic DNA was extracted from cultures grown on potato dextrose broth (PDB) at 23.5°C in 12/12 h light/dark conditions for 4–6 days. Mycelium was harvested onto sterile Miracloth™ (Calbiochem), squeezed between paper towels to remove excess moisture, immediately wrapped with aluminium foil and transferred into liquid nitrogen to snap freeze. Harvested mycelium was stored at –80°C until use for DNA extraction. Genomic DNA was extracted from the mycelium using the plant tissue DNA isolation protocol of the PUREGENE® genomic DNA isolation kit (Gentra systems, USA). About 200–500 mg of frozen mycelium was ground to a fine powder in a cooled mortar and pestle then used for DNA extraction according to the manufacturer's instructions. In the final step of extraction, the DNA pellet was rehydrated in 50 µL DNA hydration solution. To check final DNA quality a 2 µL aliquot of purified DNA was separated by electrophoresis in a 1% agarose gel in 1 × TAE buffer at 10 V/cm for 50 min. Gels were stained with ethidium bromide (0.05 µg/ml) or Sybersafe™ (Invitrogen; 5 µL/100 ml 1% agarose gel), visualized on a UV transilluminator (Versadoc imaging system 3000™, USA) and photographed. The concentration of the DNA solution was measured by spectrophotometry (NanoDrop –NanoDrop technologies, USA). All genomic DNA samples were diluted to 20–30 ng/µL for use in PCR.

2.2.7.2 PCR amplification with ITS primers

The rDNA gene region of the botryosphaeriaceous species including ITS1, 5.8S, ITS2 and the first 614 bp of the 28S was amplified using the primer sets ITS1 (TCCGTAGGTGAACCTGCGG; White *et al*., 1990) and NL4 (GGTCCGTGTTTCAAGACGG; O'Donnell, 1993). Initial PCR was done with the DNA extracted using Chelex solution. For these PCR the reaction mixture contained 1 × PCR buffer (with 1.5 mM $MgCl_2$; Roche), 200 µM each of dATP, dTTP, dGTP, dCTP (Fermentas), 5 pmole of each primer (Invitrogen), 1 U of FastStart Taq polymerase (Roche) and 2 µL of template DNA extracted using Chelex solution. Each reaction volume was made up to 25 µL with sterile water. Negative controls with sterile water instead of the template DNA were used in every PCR.

For DNA extracted using the REDExtract-N-Amp™ kit a 20 µL PCR mixture was prepared with 10 µL of REDExtract-N-Amp™ solution, 5 pmole of each primer (Invitrogen), and 4 µL of extracted DNA. The reactions were done in a BioRad iCycler Thermal cycler (Germany). The amplimers from the two rapid methods were used for RFLP according to the method of Alves *et al.* (2005).

To obtain high quality PCR products for direct sequencing, 20 ng of genomic DNA extracted using the PUREGENE® kit was used as a template for PCR. Other reagents were as described for the Chelex methodology.

For all reactions the amplification conditions were as follows: initial denaturation of 5 min at 95ºC, followed by 30 cycles of 30 s at 94ºC, 30 s at 50ºC, and 1 min at 72ºC, and a final extension period of 10 min at 72ºC. After amplification, 2 µL of each PCR product was separated by electrophoresis in 1% agarose gels in 1× TAE buffer at 10 V/cm for 50 min. Gels were stained and photographed as described in Section 2.2.7.1.

2.2.7.3 Restriction digestion of ITS region

The amplified ribosomal DNA restriction analysis (ARDRA) technique was used in this study as described by Alves *et al*. (2005). An iterative process of restriction digestion was done to confirm the identity of *Neofusicoccum* group species (Figure 2.4).

The PCR product obtained from the amplification of the ITS region of *Neofusicoccum* group isolates using ITS1/NL4 primer set was digested with the *Hae*III restriction endonuclease to distinguish the *N. parvum*/*N.ribis* group from the rest of the *Neofusicoccum* species. Typically, 10 µL of PCR product was digested with 2 U of *Hae*III (BioLabs) enzyme for 12 h at 37 ºC and the resulting fragments were separated by electrophoresis on 1.5 % agarose gel for 50 min at 10 V/cm in 1 × TAE. The gels were visualized as described in Section 2.2.7.1. For the remaining *Neofusicoccum* isolates, a second 10 µL aliquot of the PCR product was digested with 2 U Taq$^{\alpha}$1(*Taq*I; BioLab) restriction endonuclease at 65ºC for 2 h followed by heat inactivation at 80ºC for 20 min to confirm the *N. luteum/N. australe* group. The identity of the *Diplodia mutila* and *D. seriata* isolates was confirmed by restriction digestion using *Sau*96I (*Asu*I; BioLabs) and *Nco*I (BioLabs) enzymes at 37ºC for 12 h, respectively.

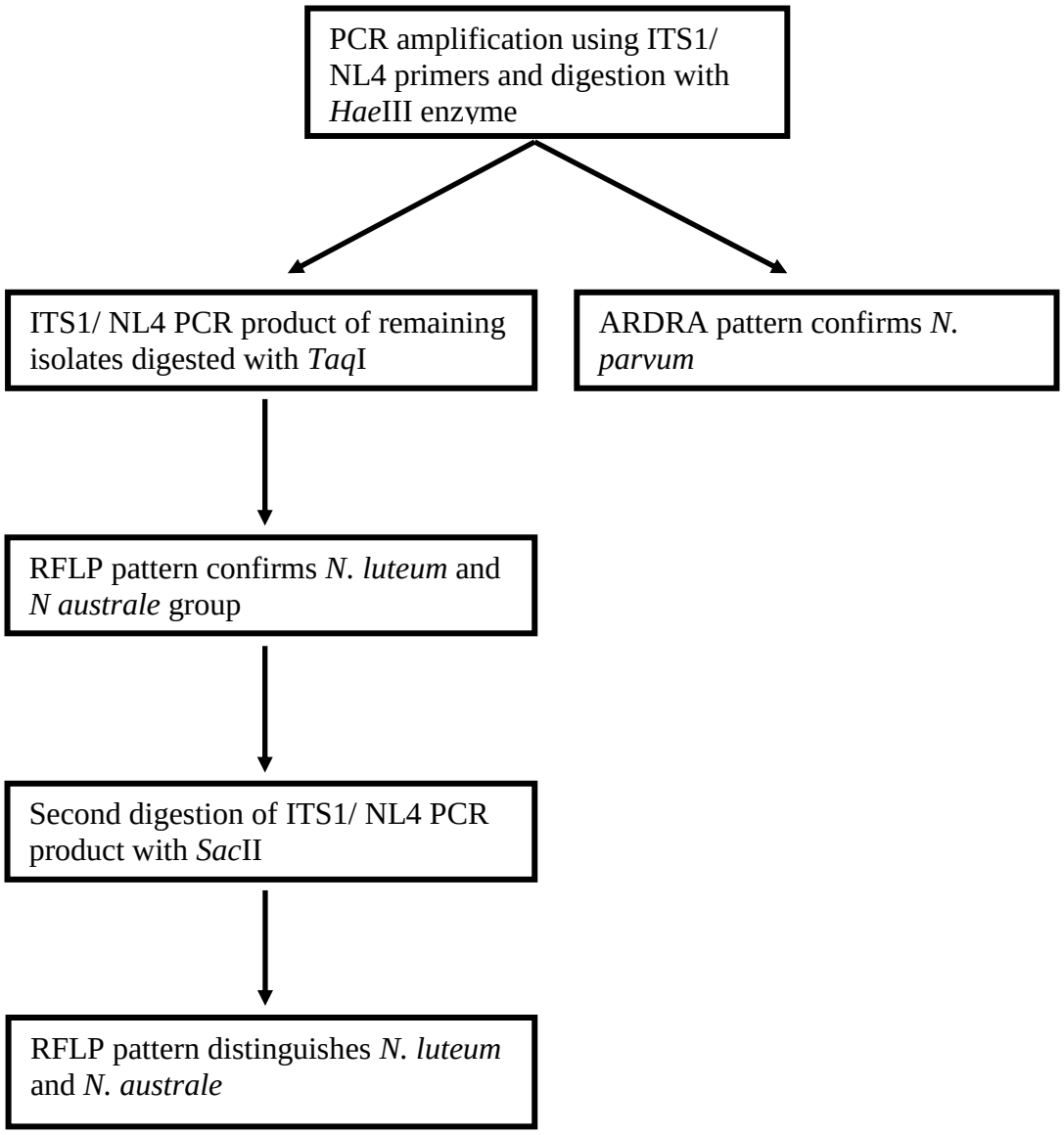

Figure 2.4: Diagram of the iterative restriction digestion process of rDNA genes with different restriction endonucleases to confirm the identity of *Neofusicoccum* species isolated from grapevine.

2.2.7.4 Increased species resolution by restriction analysis of ITS and β-tubulin gene regions

Since the published ARDRA technique of Alves *et al.* (2005) could not distinguish between *N. luteum* and *N. australe* a further method needed to be developed. *In silico* analysis of published sequences of *N. luteum* and *N. australe* using DNAMAN software (Version: 4.0a; Lynnon BioSoft) showed that the number of recognition sites for the restriction endonuclease *Sac*II differed between these two species and thus this enzyme could be used to distinguish them (Figure 2.5). Ten µL of PCR product was

digested with 2 U of *Cfr*42I (*Sac*II; Fermentas) at 37ºC for 12 h according to manufacturer's instructions to differentiate the *N. luteum* and *N. australe* isolates.

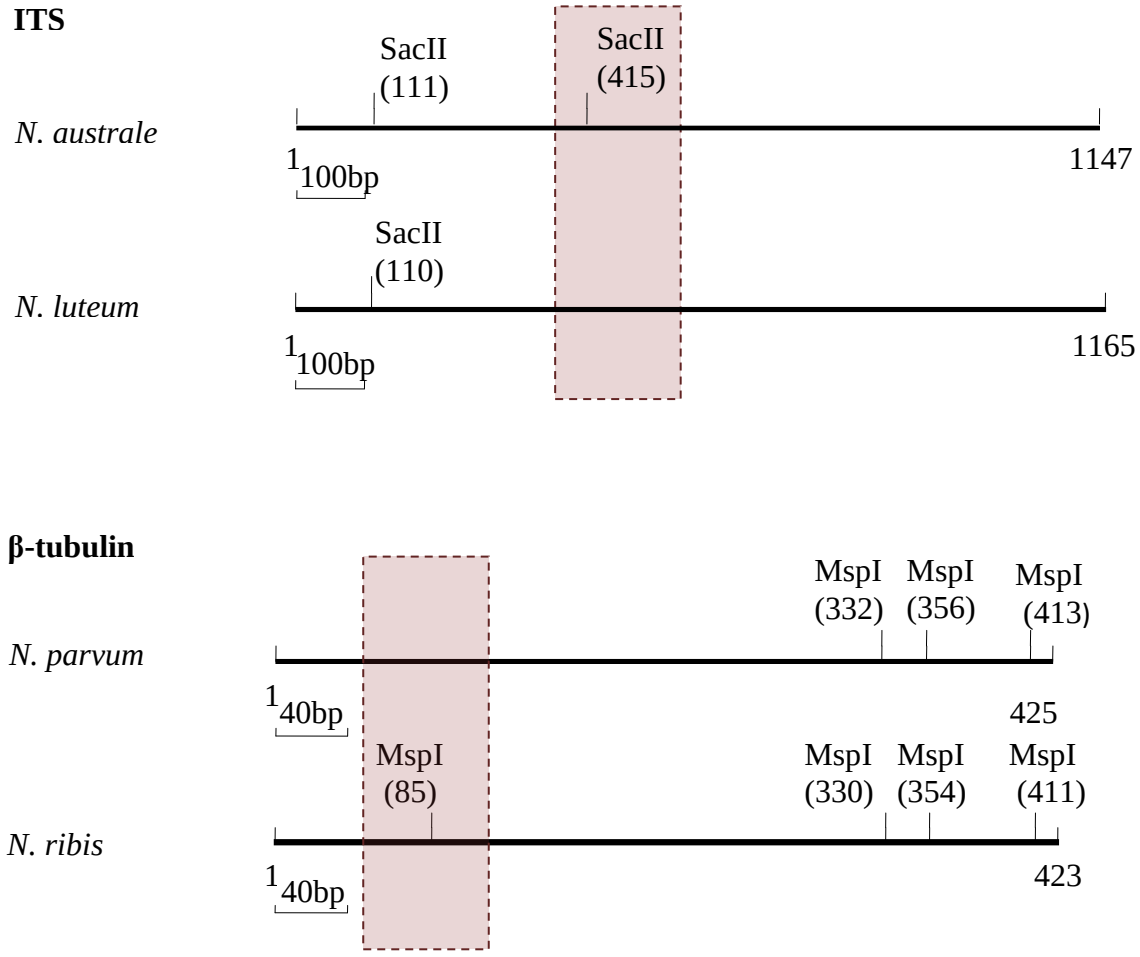

Figure 2.5: Diagram of restriction endonuclease sites in rRNA and β-tubulin gene regions that were used to differentiate closely related botryosphaeriaceous species. Red box highlights the region that contains the discriminating restriction endonuclease site.

Although the published technique (Alves *et al.,* 2005) has reported that the *Hae*III enzyme could distinguish *N. parvum* from *N. ribis,* it did not produce a distinct restriction pattern on the agarose gels used in this study. Therefore, *in silico* analysis of published ITS and β-tubulin sequences for both species was done using DNAMAN (Version: 4.0a; Lynnon BioSoft). The sequence analysis of the β-tubulin region showed that the recognition site for the restriction endonuclease *Msp*I differed between these two species. The 420 bp β-tubulin region was amplified using primer set Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (AACCTCAGTGTAGTGACCCTTGGC; Glass & Donaldson, 1995). The reaction mix was as described in Section 2.2.7.2. The amplification conditions were as follows: initial denaturation of 5 min at 95ºC, followed by 30 cycles of 30 s at

94ºC, 30 s at 58ºC, and 1 min at 72ºC, and a final extension period of 10 min at 72ºC. Ten µL of PCR product was digested using the *Msp*I enzyme at 37ºC for 12 h according to the manufacturer's instructions.

Similarly, to the published method of Alves *et al*. (2005) the restriction endonuclease *Asu*I could not distinguish the species *Do. sarmentorum* from *Do. iberica*. To identify these species the sequence data of ITS, β-tubulin and elongation factor (EF1-α) regions were used.

2.2.7.5 Sequencing of ITS, β-tubulin and elongation factor regions

Five representative isolates from each species that had been identified by ARDRA were selected for sequencing of the rRNA (specifically the ITS regions) gene region to confirm their identity. The ITS region was amplified using the ITS1 and ITS4 primer set as described in Section 2.2.7.2. The PCR product was sequenced directly in both directions in a 3130xl Genetic Analyzer (Applied Biosystems) at the Lincoln University Sequencing Facility. The sequence data was analysed using sequence scanner software (Version:1.0; Applied Biosystems) and edited to remove the usually ambiguous area close to the sequencing primer using DNAMAN (Version: 4.0a; Lynnon BioSoft). The sequence data was submitted to the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the basic local alignment search tool (BLAST) function to confirm matching species. For *Dothiorella* isolates and the remaining cryptic isolates that failed to produce characteristic species specific DNA ARDRA patterns, the ITS, β-tubulin and elongation factor (EF1-α) regions were sequenced. The elongation factor region was amplified using the primer set EF1-728F (CATCGAGAAGTTCGAGAAGG) and EF1-986R (TACTTGAGGGAACCCTTACC; Carbone & Kohn 1999). The amplification conditions were as follows: initial denaturation of 5 min at 95ºC, followed by 30 cycles of 30 s at 94ºC, 30 s at 58ºC, 1 min at 72ºC, and a final extension period of 10 min at 72ºC.

A phylogenetic tree was produced using the sequences of the New Zealand isolates and the ITS sequences of representative botryosphaeriaceous species retrieved from GenBank (Appendix A.6) based on the neighbour-joining method (Saitou and Nei, 1987) using MEGA version 4.0.2 (Tamura *et al*., 2007).

2.2.8 Determining the prevalence and distribution of botryosphaeriaceous 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 determine the incidence of botryosphaeriaceous species in symptomatic vine materials obtained from vineyards. The overall relative proportion (percent) of each species was used to determine the predominant botryosphaeriaceous species. The relative proportion (percent) of individual species per region was used to assess the population composition within each region. Correlations between the

botryosphaeriaceous species found in a vineyard and the vine characteristics such as age, cultivar and special management practices were analysed using the data collected through the survey by a statistical consultant (Dr Chris Frampton, Statistecol Consultants Ltd, Christchurch). The analysis included correlations between geographical distribution and population composition of botryosphaeriaceous species with respect to North and South Island vineyards. All the data was statistically analysed with Pearson Chi-Square tests using SPSS V12.0.

2.2.9 Effect of temperature on growth rate of botryosphaeriaceous species

The effect of temperature on growth rate of the botryosphaeriaceous species isolated from the vineyards was studied to find the optimum temperature for the maximum growth rate. The growth rate of five of the most abundant botryosphaeriaceous species was investigated at 10, 15, 20, 25, 30 and 35ºC. Three isolates from each species and from different wine growing regions were selected and grown on PDA at 23.5ºC for 3 d to produce inoculum. PDA plates each containing 15 mL of PDA in a Petri dish (90 × 15 mm) were each inoculated centrally with a mycelial plug obtained from the edge of a 3 d old culture and sealed with cling film. Three replicates were made for each temperature for all isolates and arranged in a completely randomised design. After 24 h and 48 h of incubation the radial growth was marked using a fine permanent marker. The radial growth between 24 h and 48 h (growth rate per day) was measured using a digital calliper in two perpendicular directions and the mean value was used for statistical analysis. The growth rate data were analysed using a modified Ratkowski model (Ratkowsky *et al*., 1983) to fit regression curves in SPSSv13. Using this model the optimum temperature was defined as the temperature at which the maximum growth rate occurred. ANOVA was used to determine whether there was a significant difference in optimum temperature between species using SPSSv13.

2.3 Results

2.3.1 Field symptoms and internal wood symptoms

Symptoms associated with infection by botryosphaeriaceous species, such as internal necrosis, decline, stunted growth and dead vines were observed in the vineyards from which samples were collected (Figure 2.6). From field observation it appeared that there was much greater internal trunk necrosis and dieback in older grapevines (15–20 years) than in young grapevines (3−6 years). Larger external trunk lesions (cankers) were observed in older vines. In most of the vineyards, infected vines displayed stunted foliar growth, which was especially common in the young vines. Stunted foliar growth was observed in vines from Otago region where most of the vineyards were recently established and had young vines (5 – 10 years old). Sporadic patches of stunted and dead vines were observed in a few vineyards. Occasionally cane bleaching symptoms were observed in some of the vines (Figure 2.6F).

Figure 2.6: Various symptoms associated with botryosphaeria trunk disease observed in the vineyards. A) Necrotic trunk canker on a very old vine, B) poorly developed shoots, C) necrotic trunk lesion in young vine, D) a young vine infected by botryosphaeriaceous fungi showing poor shoot growth, E) dead vines in a section of the vineyard, F) a vine with bleached canes which produced yellow leaves and G) dead arm from a diseased trunk.

Internal vine symptoms were examined by cutting across the trunks and cordons of diseased vines. A wide range of different shapes and sizes of internal, brown coloured necrotic lesions were observed including the characteristic wedge– or V– shaped lesions and various irregular shapes (Figure 2.7). From the pattern of the lesions it appeared that most of them had developed from the surfaces of the vines towards internal tissues. Internal necrosis was also observed in the centre of some cross-sectioned vines. In some cases vines were still alive despite 90% of the cross-section area being necrotic (Figure 2.7F).

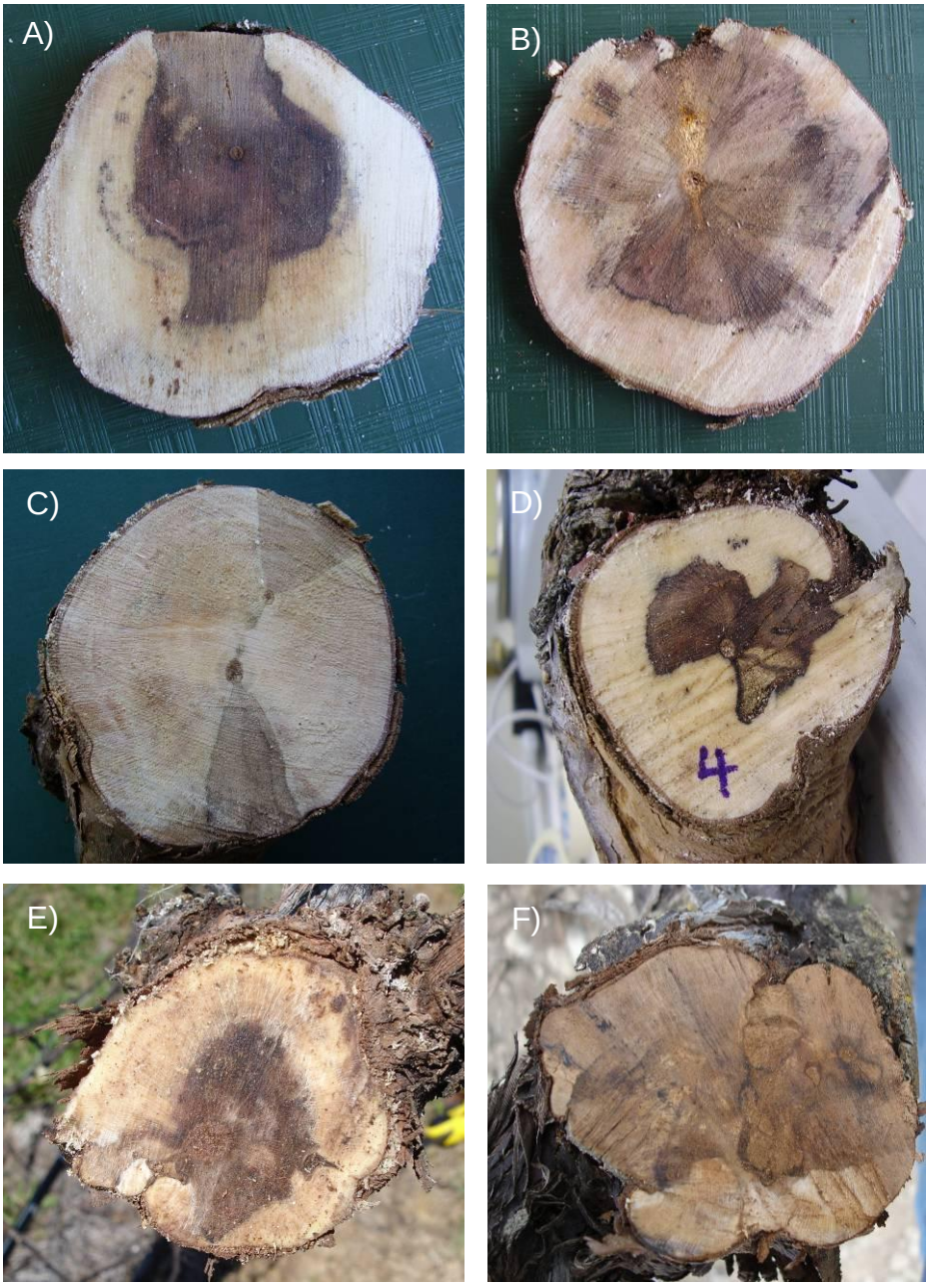

Figure 2.7: Range of internal wood necrosis observed in cross-sectioned diseased grapevines. A, B, D & E) Necrosis originating from the surface of the trunk, C) characteristic V-shaped lesion caused by botryosphaeriaceous species and F) a live vine in which more than 90% of the cross-sectioned trunk area was necrotic.

2.3.2 Incidence of botryosphaeriaceous species isolated in symptomatic vines

The 336 isolates of botryosphaeriaceous species and 35 isolates of other fungal species, including *E. lata, Phaeomoniella chylamydospora* and *Fusarium* spp., were isolated from 238 diseased grapevine samples collected from 43 vineyards across six wine growing areas. Botryosphaeriaceous species were isolated from most (38 vineyards; 88%) of the vineyards surveyed from all areas, but only from six out of eight vineyards in Blenheim and from five out of eight in Otago. The overall incidence of botryosphaeriaceous species in the diseased grapevine samples was 68% (162 samples that were positive out of 238). The incidence of botryosphaeriaceous species infection in diseased grapevine samples was significantly different between the locations investigated (P<0.001; Appendix A.7.1). The highest incidence of botryosphaeriaceous species was recorded from Gisborne (n = 26; 96%) and the lowest from Otago (n = 47; 23%). In symptomatic vines the incidence of botryosphaeriaceous species was 76%, 90%, 77% and 56%, in Auckland (n =49), Nelson (n =39), Blenheim (n =52) and Canterbury (n =25), respectively (Table 2.1).

Table 2.1: Incidence of botryosphaeriaceous species isolated from diseased grapevines which showing dieback, decline and poor foliar growths from six major wine growing regions in New Zealand.

Region	No. of Vineyards [a]	Vineyards positive for botryosphaeriaceous species (%) [b]	Total number of samples [c]	Samples positive for botryosphaeriaceous species (%)[d,*]
Auckland	12	12 (100)	49	37 (76) cd
Gisborne	6	6 (100)	26	25 (96) d
Blenheim	8	6 (75)	52	40 (77) bc
Nelson	6	6 (100)	39	35 (90) cd
Canterbury	3	3 (100)	25	14 (56) b
Otago	8	5 (63)	47	11 (23) a
Totals	43	38 (88)	238	162 (68)

[a] Number of vineyards sampled, [b] number of vineyards (and percentage of total number of vineyards sampled per region) yielding botryosphaeriaceous species, [c] total number of diseased vine samples collected, [d] number of samples (and percentage of total number of samples per region) yielding botryosphaeriaceous species. *The values with the same letters are not significantly different from each other by Fisher's protected LSD test ($P \leq 0.05$).

2.3.3 Sporulation of botryosphaeriaceous species

After 3 weeks, 57% (21 out of 37) of the isolates were able to produce pycnidia and conidia on tap water agar embedded with autoclaved pine needles and grown under fluorescent lights at room temperature (approximately 20 ºC). All the pycnidia were produced on the pine needles (Figures 2.8A & 2.8B) and not in the agar. Among the sporulating isolates, 95% were *Diplodia* species, including *D. mutila* and *D. seriata*. From the *Neofusicoccum* species, including *N. parvum* and *N. luteum*, only one isolate produced pycnidia containing conidia and the other isolates produced sterile pycnidia (no conidia). All of the isolates belonging to the *Diplodia* species produced conidia when grown on all three media tested (Table 2.2). Among the *Neofusicoccum* species 95% of *N. luteum* isolates sporulated only on prune agar extract (PEA). A very low number of of *N. parvum* isolates sporulated on PDA (2%), water agar embedded with pine needles (5%) and prune extract agar (5%). From the results of this experiment, PEA was selected for further use to induce *in vitro* sporulation of collected isolates through the survey for the purpose of species identification and production of single spore cultures (Figure 2.8D).

Table 2.2: Sporulation of botryosphaeriaceous species on different substrates

Species	Substrates		
	Pine needle* (3 weeks)[#]	Prune extract agar (3 weeks)	PDA (6 weeks)
N. parvum	5%	5%	2%
N. luteum	0%	95%	0%
D. seriata	100%	100%	100%
D. mutila	100%	100%	85%

* Water agar embedded with pine needle. #Incubation time.

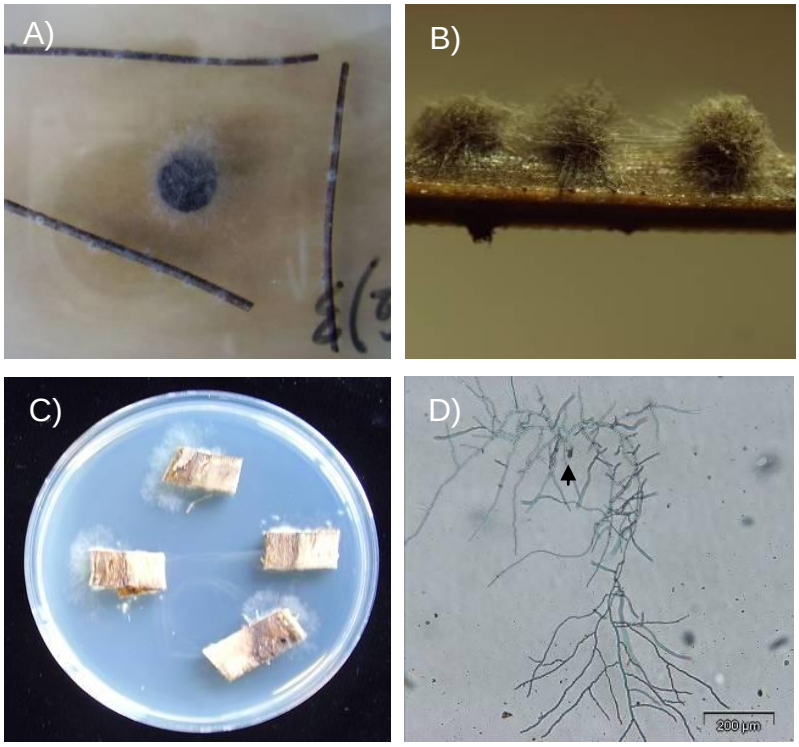

Figure 2.8: Pycnidial formation on pine needles. A) Botryosphaeriaceous fungi growing on pine needle embedded on water agar, B) pycnidial colonized pine needle, C) botryosphaeriaceous fungi growing from grapevine wood chips and D) germination of a single spore (indicated by arrow) of *D. seriata* on water agar.

2.3.4 Botryosphaeriaceous species colony morphology

For identification based on colony morphology, all the isolates growing from grapevine wood samples (Figure 2.8C) were subcultured onto PDA and grown at 23.5 ºC in 12/12 light conditions for 3 d (pers. comm. Nicholas Amponsah, Lincoln University). The initial identification of isolates was carried out by grouping them according to their colony characteristics such as mycelial growth and production of yellow pigment on PDA. Two main groups were formed, group A with a production of yellow pigment and/or aerial mycelium and group B with no or very little aerial mycelium and no yellow pigment production. Within group A two sub−morphological groups were identified. Sub-group 1 had fast growth, producing much aerial mycelium, deep yellow pigment (Figure 2.9A) on PDA, by 6–7 d it became violaceous(Figure 2.9B) visible from the reverse side of the Petri dish and the central area became clear with little aerial mycelium which decreased with age (Figure 2.9C). Sub-group 2 had slightly slower growth and was characterised by light yellow pigment (Figure 2.9D), and/or less aerial mycelium (Figures 2.9E &F). Three morphological sub–groups were identified in group B according to the colour development of the colony on the upper side of the PDA plate. In sub-group 1, colonies

grown on PDA started to become olivaceous green from the centre with age (Figure 2.9G); sub-group 2 had colonies that were grey or black on PDA and/or wavy growth margins (Figure 2.9H) and sub-group 3 had dark olivaceous brown colonies (Figure 2.9I). Conidial characteristics such as colour, shape and size were used to identify the botryosphaeriaceous species further to species level.

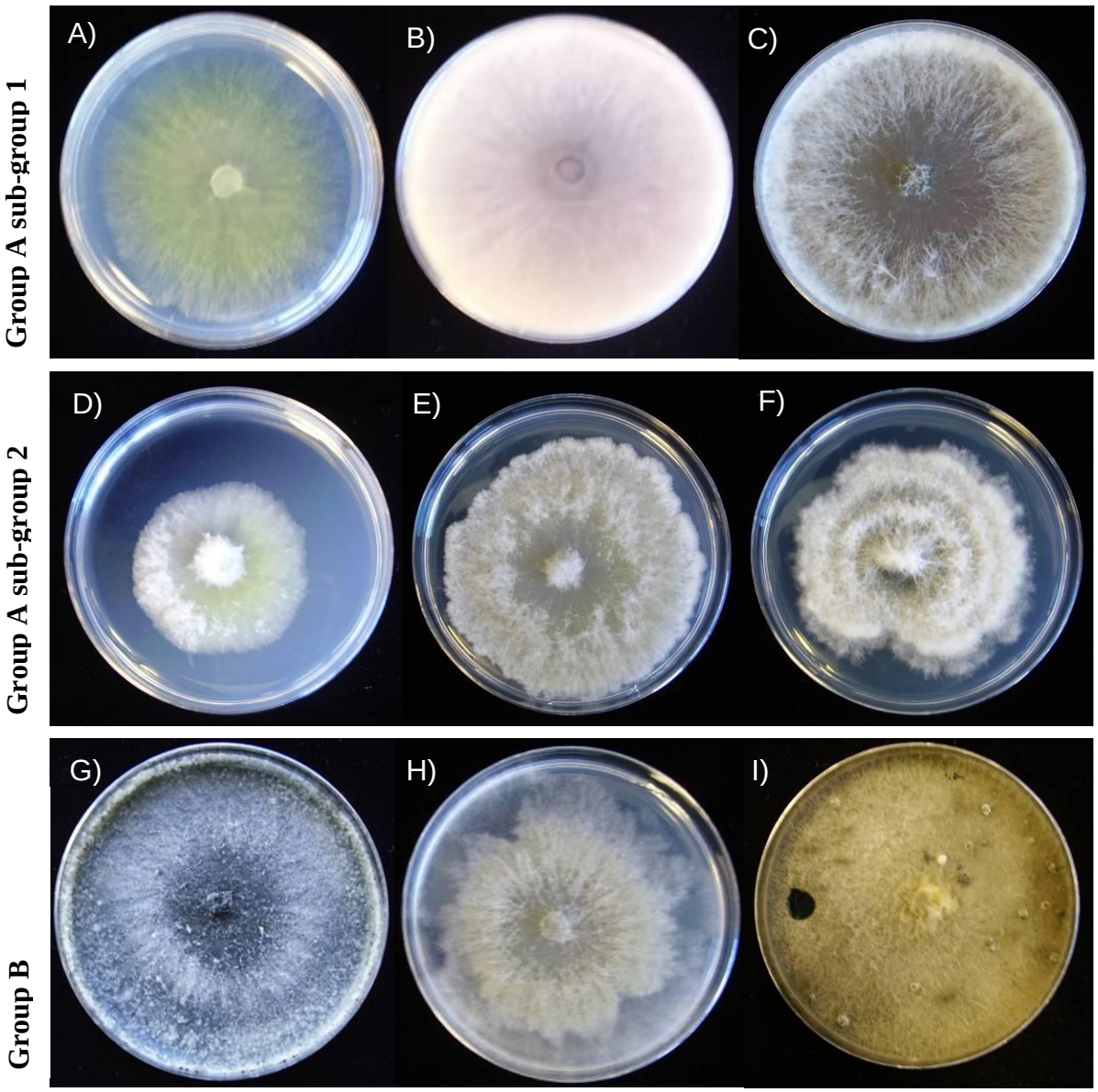

Figure 2.9: Grouping of botryosphaeriaceous species isolates obtained from New Zealand grapevines based on their colony characteristic on PDA. Group A is divided into two sub- groups. Letters A - I are showing the different morphologies observed within these groups.

2.3.5 Presumptive identification of the botryosphaeriaceous species based on conidial characteristics

From group A (yellow pigment producers), most of the isolates from sub–group 1 were able to produce conidia on PEA. The conidia of this sub–group were characterised by being hyaline, thin-walled, aseptate and fusiform in shape (Figure 2.10A). By using both the conidial morphology and culture characteristics this group was identified as *N. luteum* . Only 35% of the isolates from sub–group 2 in group A sporulated on PEA. Two different conidial morphologies were identified in this group type (a) and (b). Most of the conidia produced by sporulating isolates from sub–group 2 were type (a) being hyaline, thin-walled, aseptate and ellipsoidal in shape (Figure 2.10B). The conidial characteristic of this group overlapped with sub–group 1 and these isolates were later confirmed *N. australe* using molecular methods. The conidia of the few remaining sporulating isolates of sub–group 2 were type (b) being hyaline, thin walled and fusiform to ellipsoidal and some became septate when mature (Figure 2.10C). These isolates were identified as *N. parvum* species. The remaining non-sporulating isolates from sub–group 2 were subsequently identified using molecular methods.

Most of the isolates belonging to group B produced conidia on PEA. Different conidial characteristics were associated with each of the sub–groups belonging to group B. Isolates of sub–group 1 produced conidia that were ovoid, broadly rounded at both ends, hyaline, unicellular and smooth walled (Figure 2.10D). Isolates belonging to this sub–group were identified as *D. mutila* species. The sub–group 2 isolates were identified as *D. seriata* based on their unique conidial characteristics being ovoid, brown, aseptate and with rough walled surface (Figure 2.10E). Conidia of sub–group 3 were characterised by being ovoid, dark–brown and 1-septate, which made them clearly distinct from the other groups (Figure 2.10F). Based on these conidial characteristics, this group of isolates were identified as *Dothiorella* species. Although conidial morphology of this group differentiated them from other botryosphaeriaceous species the exact *Dothiorella* species could not be determined by morphology. Isolates belonging to this group were later identified to species level using molecular methods.

2.3.6 Molecular confirmation of botryosphaeriaceous species

A single PCR product of approximately 1200 bp was amplified from the DNA of all botryosphaeriaceous species in all three DNA extraction methods, using the ITS1 and NL4 primers (Figure 2.11). However, the amplification obtained using the template DNA from the Chelex extraction was inconsistent, whereas the REDExtract™ process produced a consistent amplification for a large number of isolates.

The PCR product obtained using the ITS1 and NL4 primers for all the isolates belonging to Group A (yellow-pigment producers) was first digested with the *Hae*III restriction endonuclease. The ARDRA

pattern clearly distinguished *N. parvum* from all other botryosphaeriaceous species with four visible bands (253-254-258, 203, 157, 98-83 and 88 bp; Figure 2.11: pattern B).

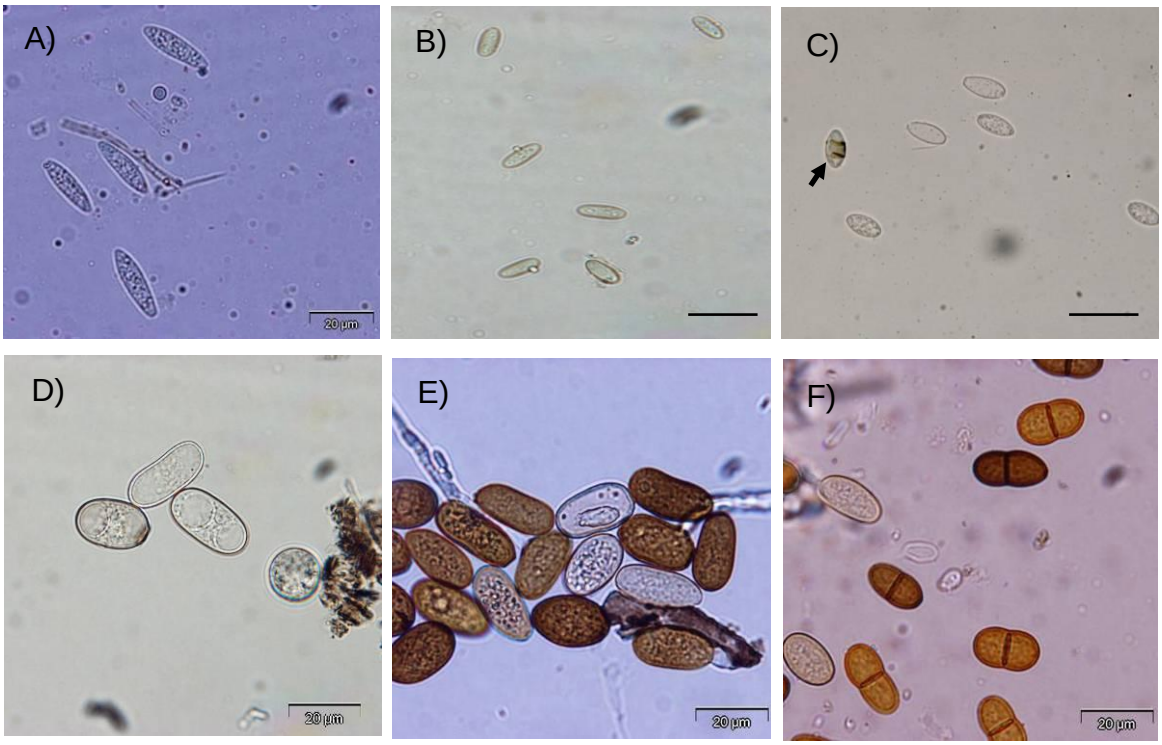

Figure 2.10: Anamorph morphology of botryosphaeriaceous species isolated from New Zealand grapevines. A) Conidia of *N. luteum*, B) conidia of *N. australe* (later confirmed by molecular methods), C) *N. parvum* conidia (mature septate conidia indicated by arrow), D) conidia of *D. mutila*, E) conidia of *D. seriata* and F) conidia of *Dothiorella* group species. Scale bar = 20 µM

The PCR product of the remaining isolates (Figure 2.11: pattern A) belonging to Group A digested with *Taq*I produced five visible bands (364, 292, 189, 92 and 63-41 bp) clearly confirming them as *N. luteum* and/or *N. australe* (Figure 2.11: pattern C). The ARDRA pattern produced with *Taq*I digestion was similar for both of these species. Further restriction analysis of the PCR products with the *Sac*II enzyme produced two bands (1057 and 110 bp) for *N. luteum* (Figure 2.11; pattern E) and three bands (732, 304 and 111 bp) for *N. australe* (Figure 2.11; pattern F).

The second banding pattern produced by *Taq*I digestion of sub–group 2 of group A isolates contained five visible bands (428, 291, 189, 92 and 41-59 bp; Figure 2.11; pattern D) indicating that they were either *N. ribis* or *D. corticola*, as the first restriction digestion using *Hae*III enzyme had confirmed that these isolates were not *N. parvum*. The morphological characteristics had confirmed these isolates were not *D. corticola*, thus these isolates were confirmed as *N. ribis.* As *N. parvum* and *N. ribis* are very closely related species based on morphology and the ITS sequence, their identity was further resolved by *Msp*I restriction digestion of the PCR product obtained using Bt2a and Bt2b primer pairs

which produced three bands (245, 85 and 24-57 bp) for *N. ribis* (Figure 2.11; pattern G) and two bands (332 and 24-57 bp) for *N. parvum* (Figure 2.11; pattern H).

Most of the isolates from Group B (non-yellow pigment producers), except the *Dothiorella* group, were clearly identified to species level using anamorph characteristics. The identified species from this group were confirmed using the ARDRA method with a specific restriction enzyme for each species. *D. mutila* identity was confirmed using *Asu*I restriction enzyme with two visible bands (495-499 and 35-63 bp; Figure 2.11; pattern I). The identity of *D. seriata* was confirmed by ARDRA using *Nci*I, which produced 2 bands (747 and 334 bp; Figure 2.11; pattern J) and it clearly differenciated from *D. mutila* (1 band 1079 bp; Figure 2.11; pattern k).

A few isolates belonging to Group B did not produce spores and the ARDRA banding profile obtained was different from the above identified species. Those isolates were identified as *Botryosphaeria dothidea* based on sequencing results. The *Dothiorella* isolates were also identified to species level as *Do. sarmentorum* and *Do. iberica* using the sequence data of ITS, β-tubulin and EF1-α regions. The ITS and β-tubulin sequence data did not clearly confirm the identity of the *Dothiorella* species. However, the sequence alignment of the EF-1α sequences of the *Dothiorella* isolates obtained in this study along with the sequences of *Do. iberica* and *Do. sarmentorum* obtained from GenBank confirmed the identity of *Do. iberica* (100% identity, accession numbers EU768884.1, EU768883.1, EU768882.1 and EU768881.1) and *Do. sarmentorum* (97% identity, accession numbers HM236496.1, GU251303.1, GU251302.1 and GU251301.1) .

A neighbour-joining tree was generated with the sequence data of representative isolates from each species identified along with the sequence data obtained from GenBank. The New Zealand isolates were grouped with the published sequences of the respective species and confirmed the accuracy of the morphological and molecular (ARDRA) species identification (Figure 2.12). The botryosphaeriaceous species present in New Zealand fell into four major clades of the neighbour-joining tree. Clade one comprised species with *Diplodia* anamorphs, including *D. mutila* and *D. seriata*. The second clade included species with *Fusicoccum* anamorphs, which accommodated *B. dothidea* only. The *Dothiorella* anamorph species, *Do. sarmentorum* and *Do. iberica* were grouped into clade three. Clade four comprised four species with *Neofusicoccum* anamorphs, including *N. parvum*, *N. luteum*, *N. australe* and *N. ribis*, which were all in separate sub groups with respective international isolates.

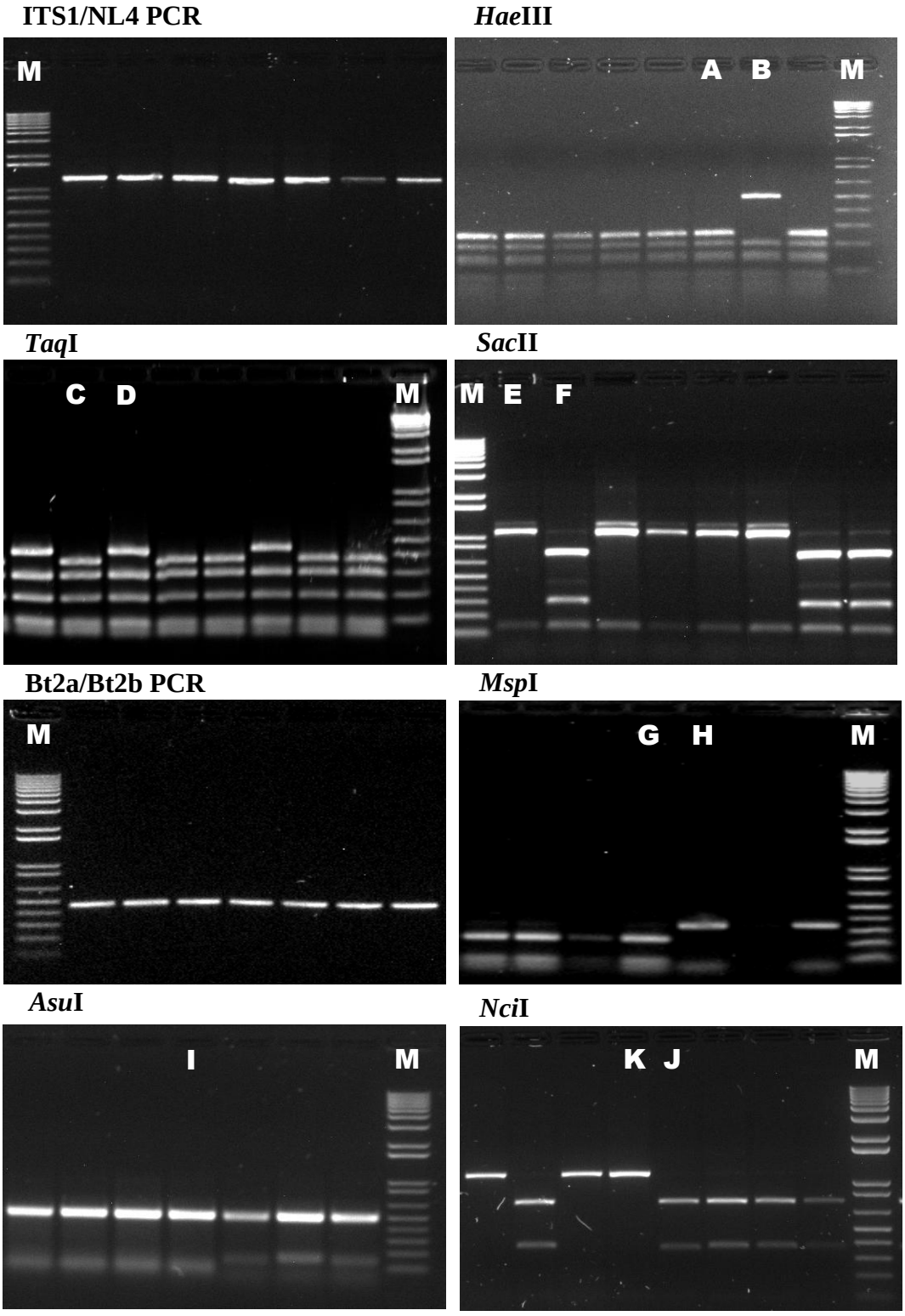

Figure 2.11: ARDRA pattern of PCR products of the rRNA and β-tubulin gene regions digested with different restriction endonucleases to identify the botryosphaeriaceous species isolated from New Zealand vineyards. Letters A-J on the pictures indicates the different RFLP patterns used to confirm the species and M is 1kb^{+} plus DNA Ladder (Invitrogen). A) *N. parvum*, B) all non- *N. parvum* isolates, C) *N. luteum*/ *N. australe* group, D) all non- *N. luteum*/ *N. australe* isolates, E) *N. luteum*, F) *N. australe*, G) *N. ribis*, H) *N. parvum*, I) & K) *D. mutila* and *D. seriata.*

2.3.7 Correlation of botryosphaeriaceous species occurrence with vine age, scion variety and grafted/non-grafted vines

The age of the grapevines significantly influenced the incidence of botryosphaeriaceous species (*P*=0.001; Appendix A.7.2). In this study, six different age groups were analysed with variable number of samples from each group, and the percentage of incidence of botryosphaeriaceous species ranged from 30% to 93% (Table 2.3). Most of the species found in New Zealand were isolated from vines of all age groups, except for *D. seriata* which was not isolated from the youngest age group (0–5 years). The occurrence of two or more botryosphariaceous species within a single vine, such as *N. australe* and *D. seriata*, was significantly higher in older vines (over 21 years; age groups 5 & 6) than in younger vines.

Scion varieties differed significantly with respect to botryosphaeriaceous species (*P*=0.001; Appendix A.7.3). Higher infection was found in scion varieties Sauvignon Blanc and Pinot Noir with both scion varieties comprising 58% of the total samples collected. The percentage infection of the other seven scion varieties (number of samples ranged between 3 and 25) ranged from 50 to 100%. Incidence of botryosphaeriaceous species was significantly higher in grafted grapevines than in non-grafted grapevines (*P*=0.015; Appendix A.7.4).

Table 2.3: Incidence of botryosphaeriaceous (Bot) species (%) in different age group of grapevines

Age group	Total number of samples collected	Samples positive for Bot species	Incidence of Bot species (%)
1 (0- 5 yr)	27	8	29.6
2 (6- 10 yr)	70	43	61.4
3 (11- 15 yr)	41	34	82.9
4 (16- 20 yr)	25	14	56
5 (21- 25 yr)	20	16	80
6 (> 26 yr)	14	13	92.9

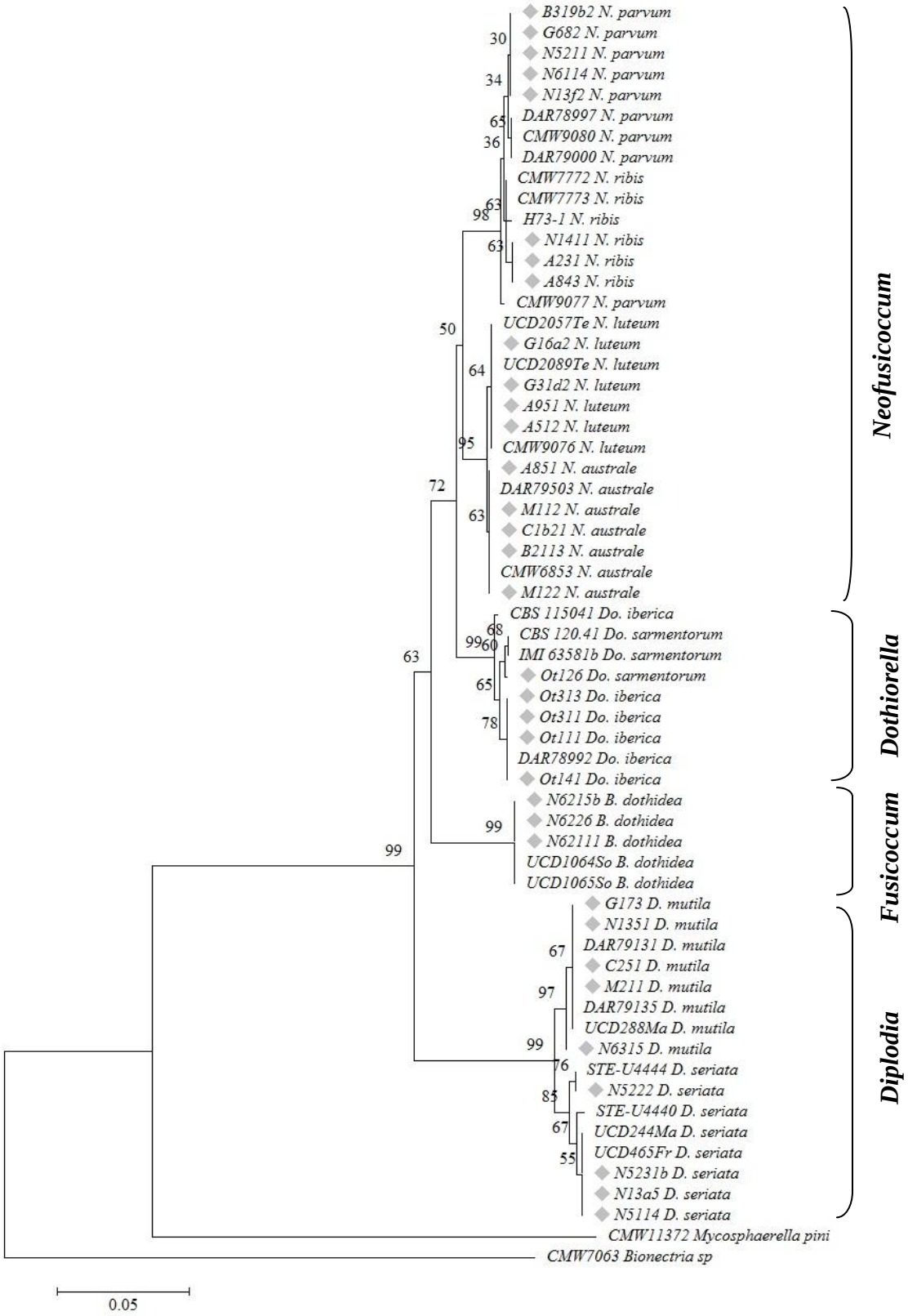

Figure 2.12: The neighbour-joining tree with bootstrap values using 1000 replicates generated in MEGA 4.0.2 using the internal transcribed spacer (ITS) sequences of Botryosphaeriaceous species isolated from New Zealand vineyards (♦) and from GenBank. *Mycosphaerella* and *Bionectria* spp. were used as outgroups for the analysis.

2.3.8 Botryosphaeriaceous species composition and distribution in New Zealand vineyards

Species identification using combined morphological characteristics and PCR-RFLP confirmation along with neighbour joining analysis of the ITS sequences of representative isolates confirmed that at least nine botryosphaeriaceous species were present in symptomatic New Zealand grapevines. These were *N. parvum, N. luteum, N. australe, N. ribis, D. mutila, D. seriata, B. dothidea, Do. sarmentorum* and *Do. iberica.* This is the first report of *Do. iberica* in New Zealand.

Among these species *N. parvum* was found in 31 out of 43 vineyards (72%) surveyed from six wine growing locations in New Zealand. It was isolated from all 18 vineyards surveyed in the North Island and from 13 of 25 South Island vineyards. *Neofusicoccum luteum* was isolated from 18 vineyards, and mainly in the North Island vineyards (78%). The majority of *N. australe* (80%) and *D. mutila* (85%) isolates were found in South Island vineyards. The botryosphaeriaceous species *D. seriata*, *Do. sarmentorum, Do. iberica* and *B. dothidea* were only isolated from South Island vineyards (Table 2.4).

Among the botryosphaeriaceous species, *N. parvum* was predominant (n= 72; 35% of the total occurrence) in New Zealand grapevines (Figure 2.13). The occurrences of other *Neofusicoccum* species were *N. luteum* 14%, *N. australe* 11% and *N. ribis* 3%, comprising 63% *Neofusicoccum* species of the total botryosphariaceous species recovered from symptomatic material. Among these species, 87% of *N. luteum* were found in the North Island and, in contrast, 92% of the *N. australe* were isolated from the South Island in the Blenheim and Canterbury regions. *N. ribis* was isolated from Auckland and Nelson vineyards only.

Table 2.4: Incidence of botryosphaeriaceous species isolated from diseased grapevines from six major wine growing regions in New Zealand.

Region	Number of samples yielding botryosphaeriaceous species								
	Neofusicoccum				*Diplodia*		*Dothiorella*		*Botryosphaeria*
	luteum	*parvum*	*australe*	*ribis*	*mutila*	*seriata*	*sarmentorum*	*iberica*	*dothidea*
Auckland*	15	24	1	4					
Gisborne*	11	18	1		3				
Blenheim #		9	16		20	11			
Nelson #	2	14		2	10	21			3
Canterbury #		4	6		4	2	1		
Otago #	2	3					1	4	
Totals	30	72	24	6	37	34	2	4	3

*-North Island and #- South Island

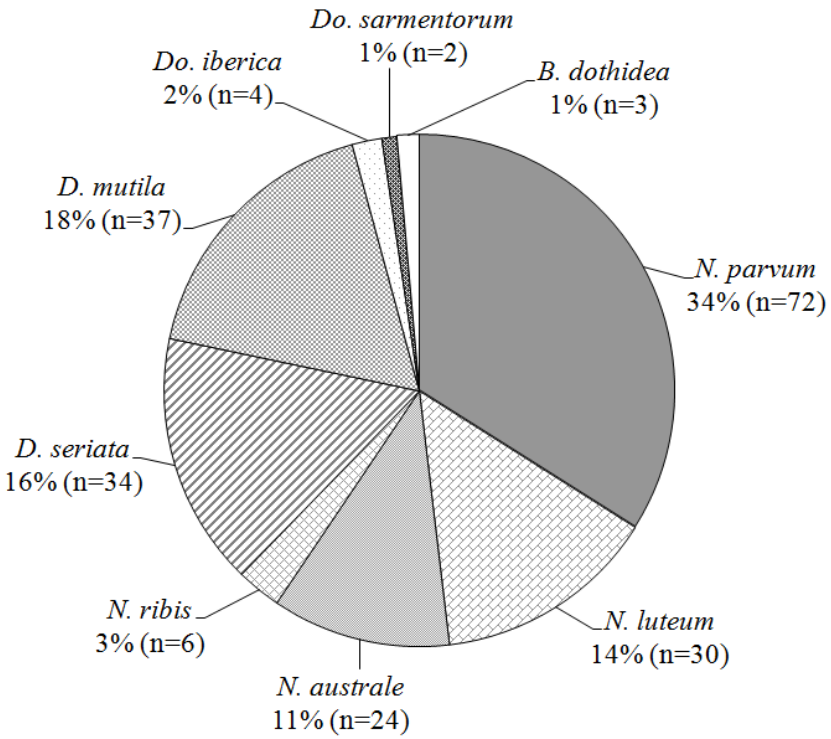

Figure 2.13: Proportions of botryosphaeriaceous species isolated from New Zealand grapevines.

Among the *Diplodia* species, most (92%) of the *D. mutila* isolates were recovered from the South Island, mainly from Blenheim and Nelson, and the remainder (n=3) came from Gisborne in the North Island. *Diplodia seriata* was only isolated from South Island vineyards and 61% of the isolates were recovered from Nelson. *Botryosphaeria dothidea* was only isolated from Nelson and comprised only 1% of the total botryosphaeriaceous isolates recovered. *Dothiorella* species were only isolated from Canterbury and Otago vineyards. *Dothiorella sarmentorum* was found in both regions while *Do. iberica* was found only in Otago. These results showed a difference in species composition of botryosphaeriaceous species between the wine growing areas (Figure 2.14).

Differences in distribution of the botryosphaeriaceous species was observed between the North and South Islands. The *Neofusicoccum* group species *N. parvum* and *N. luteum* were more frequently isolated from North Island vineyards compared to South Island vineyards. *Neofusicoccum australe* was more frequently isolated from South Island vineyards. The *Diplodia* group species *D. mutila* and *D. seriata* also dominated in samples from South Island vineyards (Figure 2.15).

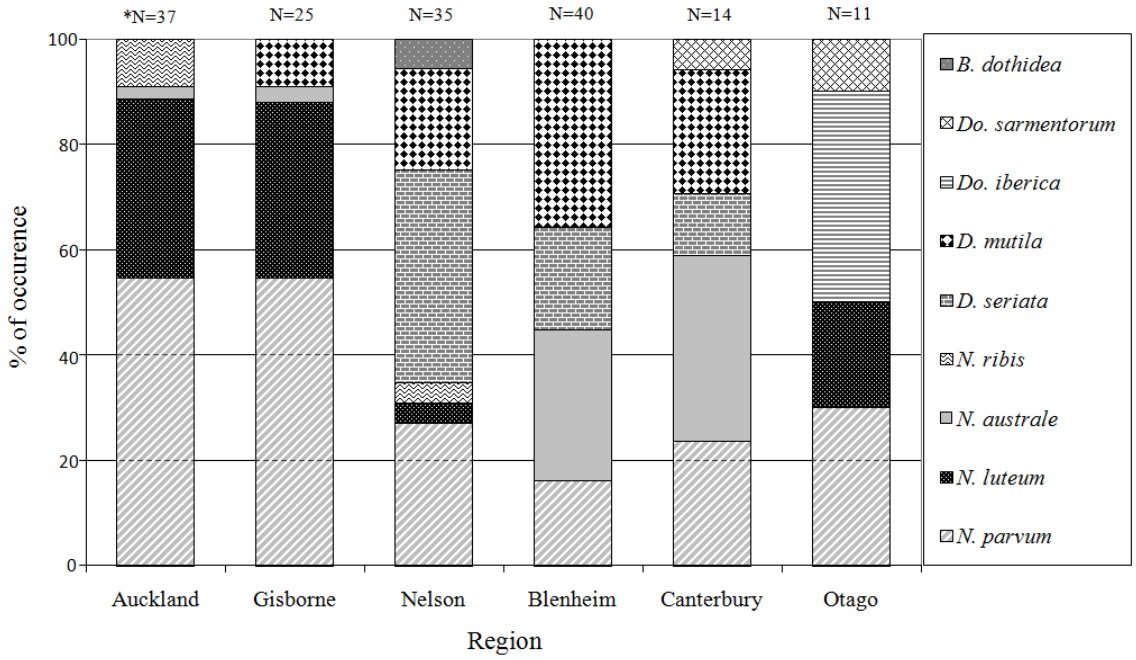

Figure 2.14: Distribution of botryosphaeriaceous species in different vine growing regions in New Zealand. *N value indicates the number of grapevine samples positive for infection by botryosphaeriaceous species.

2.3.9 Multiple botryosphaeriaceous species in a vineyard and a lesion

In most of the vineyards surveyed multiple botryosphaeriaceous species were isolated (Appendix A.8). A maximum of five botryosphaeriaceous species was found in one vineyard from Nelson and this was obtained from a collection of 12 grapevine samples. Among the botryosphaeriaceous species, *N. parvum* and *N. luteum* were most frequently found together in a vineyard.

From the 18 lesions that were systematically sampled to determine if there were multiple species present, 12 were found to contain more than one species and two lesions contained three botryosphaeriaceous species. *Neofusicoccum* group species *N. parvum* and *N. luteum* were found more frequently together (20%) than *Diplodia* group species *D. mutila* and *D. seriata* (16%). In multiple species occurrence, *D. seriata* was found most frequently (40% of the multiple species occurrence) in combination with other botryosphaeriaceous species (Table 2.5) despite *N. parvum* being the most frequently isolated species.

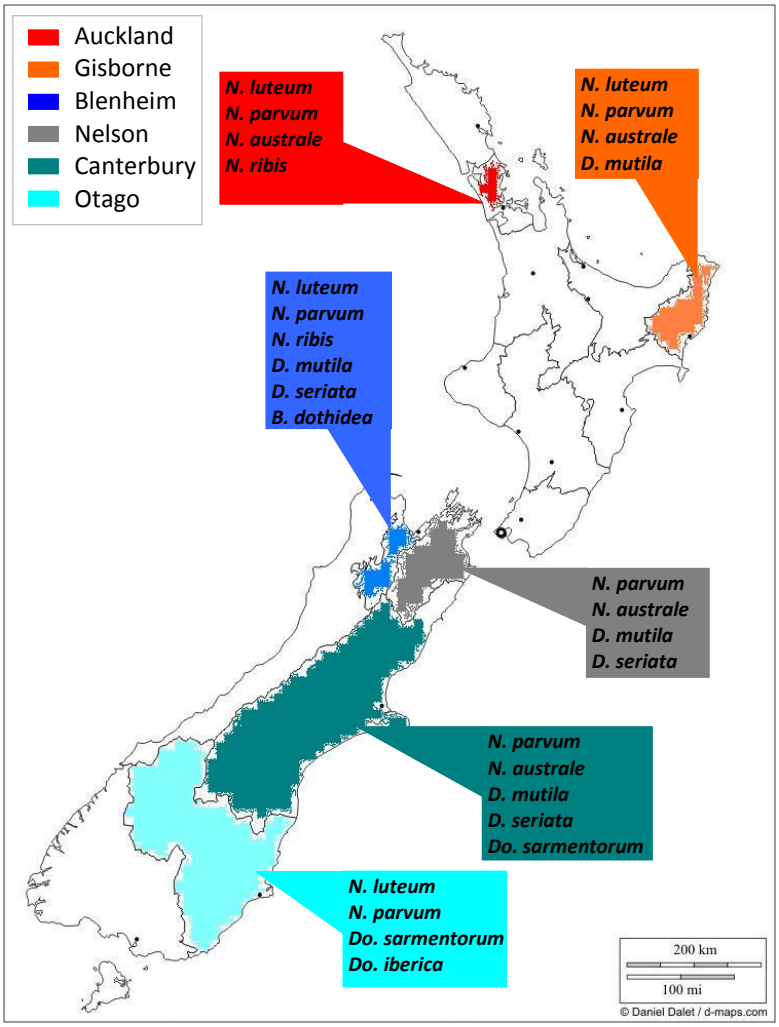

Figure 2.15: Geographical distribution of botryosphaeriaceous species isolated from symptomatic New Zealand grapevines.

Table 2.5: Frequency of occurrence of cohabiting botryosphaeriaceous species in diseased grapevine sample

Cohabiting species	Frequency of occurrence (%)
N. parvum– N. luteum	20
N. parvum– D. seriata	12
N. parvum – D. mutila	10
N. australe– D. mutila	16
N. australe– D. seriata	4
N. luteum – D. seriata	4
N. luteum–D. mutila	2
N. ribis –D. seriata	2
D. seriata– D. mutila	16
B. dothidea–D. seriata	2

2.3.10 Effect of temperature on growth rate of botryosphaeriaceous species

The growth rates (mm/24 h) of three *Neofusicoccum* species; *N. parvum*, *N. luteum* and *N. australe* and two *Diplodia* species; *D. mutila* and *D. seriata* over a range of incubation temperatures (10–40ºC) are shown in Figure 2.16. The estimated optimum temperatures at which the maximum growth rate occurred varied significantly between species (P=0.05; Appendix A.9.1). These temperatures were 26.1ºC for *N. luteum*, 26.3ºC for *D. seriata*, and 25.9ºC for *N. parvum*, which were significantly greater than 24.6 ºC for *D. mutila* and 23.4 ºC for *N. australe*. The growth rates of these species at the optimum temperature were also significantly different between the species (P<0.05; Appendix A.9.2). The highest growth rate was 38.7 mm/day for *N. parvum* and the lowest 28.1 mm/day for *D. mutila* (Table 2.6).

Table 2.6: Estimate of optimum temperatures and maximum growth rates of botryosphaeriaceous species

Species	Opt. T (ºC)[a]	Growth rate (mm/day)[b]
N. australe	23.4a	33.0ab
N. luteum	26.1b	28.7a
D. seriata	26.3b	35.4b
N. parvum	25.9b	38.7b
D. mutila	24.6a	28.1a

a- Estimated optimum temperature (Opt T) at which maximum growth rate occur (P=0.05) and b- growth rate of each species at optimum temperature (P<0.05). Values with same letters are statistically not different from each other.

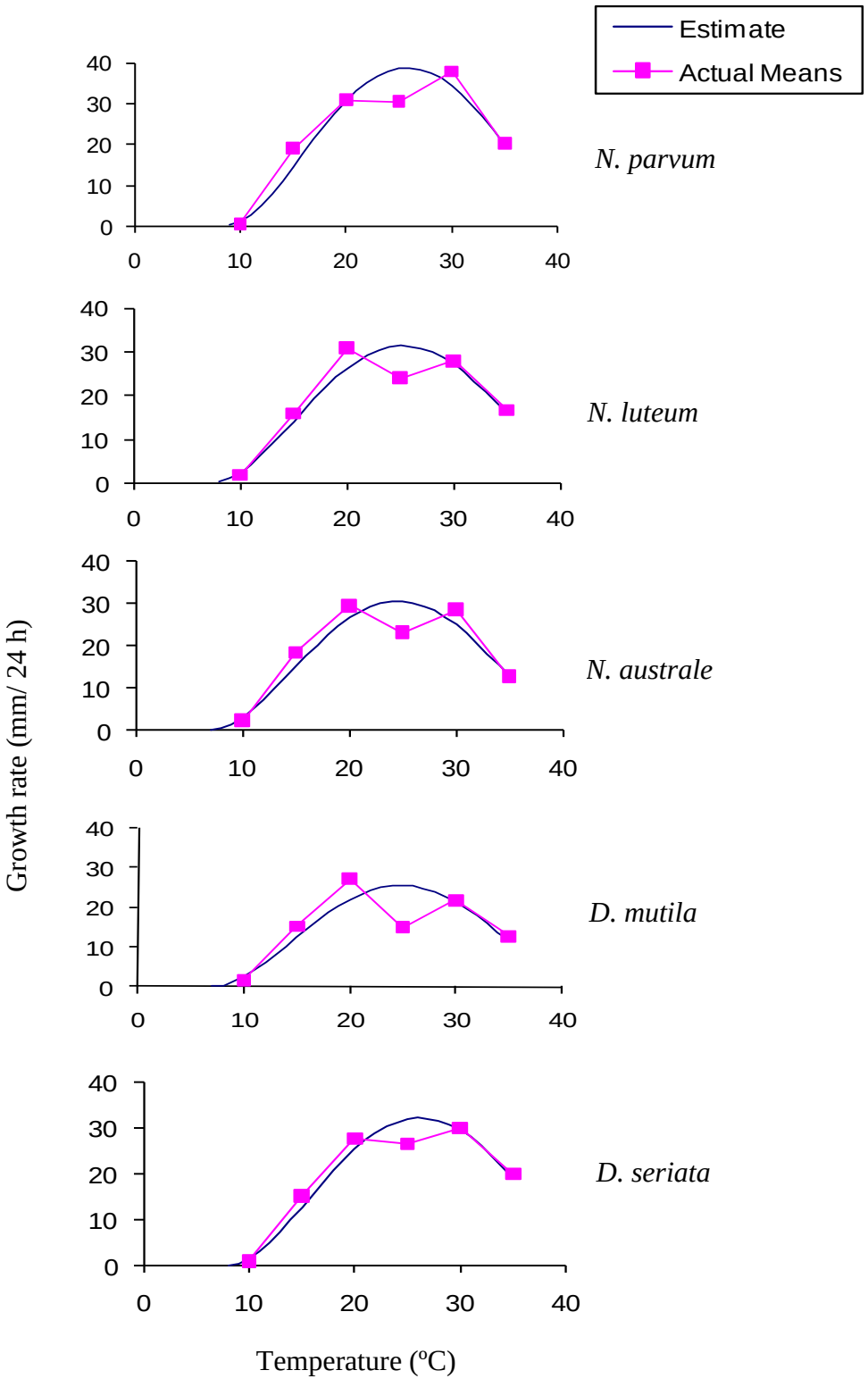

Figure 2.16: Temperature effect on growth rate of five botryosphaeriaceous species. Measured temperatures are shown in blocks (■) and line and fitted regression curves are shown by line (—). Each growth rate data points were the mean value of eight replicate measurements.

2.4 Discussion

The objective of this study was to determine the incidence of botryosphaeriaceous pathogens present in symptomatic grapevines in vineyards nationwide. This investigation was timely due to the recent recognition of botryosphaeriaceous species as serious pathogens that produced trunk decline and dieback in mature vineyards and also due to the increasing age profile of vineyards in New Zealand. Although some botryosphaeriaceous species have been isolated previously from grapevines in New Zealand (Bonfiglioli & McGregor, 2006; Amponsah *et al*., 2007) this was the first extensive study of these grapevine trunk pathogens in New Zealand. This survey recovered 336 isolates from 43 vineyards in six major vine growing areas in New Zealand which comprised at least nine botryosphaeriaceous species isolated from a range of symptomatic grapevine materials. In addition this study provided details about the symptoms related to this disease and their distribution throughout the country.

The symptoms associated with infection by botryosphaeriaceous species included necrotic cankers on mature grapevines associated with V-shaped necrosis, internal trunk necrosis, decline and stunted growth in young grapevines, cane bleaching and vine deaths. Similar symptoms in grapevines have been reported in Australia (Castillo-Pando *et al*., 2001; Taylor *et al*., 2005; Wood & Wood 2005; Pitt *et al*., 2010), South Africa (van Niekerk *et al*., 2004), California (Úrbez-Torres *et al*., 2006a, 2007), Northern Mexico (Úrbez-Torres *et al*., 2008), Spain (Úrbez-Torres *et al*., 2006b) and Portugal (Phillips, 2002). However, it was also possible that symptomless infection were present in New Zealand vineyards.

In this study, larger external lesions (cankers) and wood/ trunk internal necrosis were observed in older vines, whereas most of the young vines showed stunted foliar growth. Some of the older vines were still alive even when most (99%) of the trunk cross–section area was necrotic internally. Many researchers reported that botryosphaeriaceous species produce a characteristic wedge- or V- shaped internal necrosis in cross-sectioned cordons and trunks of declining vines (Pitt *et al*., 2010; Úrbez-Torres *et al*., 2006a). However, this survey demonstrated that the shapes of the internal necroses caused by botryosphaeriaceous species varied in the cross-sections of the grapevine trunks. Of the young vines sampled from the Otago region which displayed stunted growth (5 – 10 years old), only 23% proved to be infected with botryosphaeriaceous species. This was less than the overall average of 68% demonstrating the difficulty of using external symptoms as an indicator of infection by botryosphaeriaceous species as it may also be caused by other factors (nutritional deficiency or stress). The overall distribution of infected vines was patchy in most vineyards. In Gisborne, where some of the oldest vines were sampled, the patches included dead and symptomatic vines from which botryosphaeriaceous species were isolated. As the patches of infected vines included plants from adjacent rows, it suggested that the infection had occurred in the field rather than by infection from the nursery.

The wide-spread sampling from six major vine growing areas provided good representative samples and provided a detailed view of the botryosphaeriaceous species incidence in New Zealand vineyards. A total of 238 samples were collected from 43 vineyards across the country planted with 25,355 ha of grapevines in 2008 (1 sample per 106 ha grapevines; Winegrowers Report, 2008). In a survey of botryosphaeriaceous disease conducted in California, USA, 1735 diseased grapevine samples were collected from 166 vineyards out of 345,200 ha total vineyard area (1 sample per 200 ha; Úrbez-Torres *et al*., 2006a). Among the major vine growing regions in New Zealand, no samples were collected from Hawkes Bay region (18% of total vineyard area) due to time and financial constraints.

The overall incidence of botryosphaeriaceous species was 68% of the symptomatic vine samples. This indicated that the majority of trunk disease and decline in New Zealand vineyards can be attributed to botryosphaeriaceous pathogens. In a similar study conducted in California, 89% of the vineyards were positive for botryosphaeriaceous infection with an overall incidence of 47% (Úrbez-Torres *et al*., 2006a). Two separate studies conducted in Australia reported different incidences of botryosphaeriaceous infection. A survey conducted in New South Wales and South Australia by Pitt *et al*. (2010) showed that 98% of vineyards contained botryosphaeriaceous infections with a mean incidence of 46% in the 2239 samples collected from 91 vineyards. Another study conducted in the Hunter Valley and Mudgee grape growing regions of New South Wales, Australia demonstrated 36% of botryosphaeriaceous infection in 450 wood samples collected from 11 vineyards (Qiu *et al*., 2011). This suggested that the number and types of grapevine samples, such as from symptomatic, non-symptomatic, trunks and cordons used for the study, may influence the results of botryosphaeriaceous species incidence in different regions/ countries.

Thus, the vineyards in New Zealand appear to be more affected by this disease than California and Australia. Further surveys in other countries would demonstrate whether New Zealand's infection levels are unusually high or within the range observed globally. In this study, only symptomatic vine trunk or cordon samples were collected. However, botryosphaeriaceous species have also been isolated from other grapevine parts including buds and leaves (Bonfiglioli & McGregor, 2006; Amponsah *et al*., 2007) and therefore the true incidence in New Zealand vineyards may have been even greater. In this study, the grapevine samples with internal necrotic lesions, which were mostly obtained from mature vines, were 100% positive for botryosphaeriaceous species infection. In contrast, the samples collected from the stunted grapevines without internal necrotic lesions showed a low percentage of botryosphaeriaceous species infection. The botryosphaeriaceous species can be endophytes (Slippers & Wingfield, 2007) and have been shown to cause disease mostly following the onset of stress (Schoeneweiss, 1981). However, the botryosphaeriaceous species have also been isolated from apparently healthy planting materials from New Zealand nurseries (Billones *et al*., 2009). However, these isolates were demonstrated to be pathogenic on grapevines in later experiments

(Chapter 4). This study suggests that apparently healthy vines may be infected with this disease and present a potential future problem for vineyard owners. A fully randomised survey would be required to establish the exact incidence of infection by botryosphaeriaceous pathogens in the national vineyard.

This study found significant effects of age group on the presence of botryosphaeriaceous species infection in grapevines, with highest infection found in age group 2 (6-10 yrs old). Larignon & Dubos (2001) reported that botryosphaeriaceous species infection caused the most severe losses in mature (>8 years old) grapevines while Úrbez-Torres *et al*. (2006a) reported that a Californian survey showed no significant correlation between the vine age groups (three groups; 10-20, 21-30 and >30 yrs) and infection incidence. However, New Zealand vineyards are generally younger than this as 53% of the vineyards area is planted with less than seven years old. The difference observed in California may be due to the much older age groupings of the vines (> 8 years).

Most of the scion varieties sample collected in this study (58% of the total sample) were Sauvignon Blanc (46 samples) and Pinot Noir (50 samples). Although relatively equal numbers of these scion varieties were collected, Sauvignon Blanc had a higher percentage of infection (83%) than Pinot Noir (42%). The occurrence of particular botryosphaeriaceous species did not correlate with scion varieties. In a Californian study, they did not investigate the correlation of wine grape scion variety and the infection by botryosphaeriaceous species. Instead they compared the combined incidence in all wine grape varieties to that of table-raisin grapes (Úrbez-Torres *et al*., 2006a). The results of the current study suggested that some scion varieties may be more susceptible to botryosphaeriaceous species infection than others. However, a previous study conducted in New Zealand to test the susceptibility of the grapevine varieties, Chardonnay, Pinot noir, Riesling, Cabernet sauvignon and Sauvignon blanc to three botryosphaeriaceous species (*N. luteum*, *N. parvum* and *D. mutila*) using pathogenicity assays with *in vitro* green shoots reported no significant differences in susceptibility between the grapevine varieties (Amponsah *et al*., 2008). This suggested that susceptibility of grapevine varieties to botryosphaeriaceous species infection may differ under field conditions or with vine maturity. Therefore, further studies are desirable to investigate the susceptibility of different grapevine scion varieties to botryosphaeriaceous species infection under field conditions.

The colony characteristic used in initial differentiation of the isolates was the production of yellow pigment on PDA after 3 days incubation. The production of yellow pigment on PDA after 3 days of incubation has been widely used to distinguish *Neofusicoccum* species, including *N. luteum* (Pennycook & Samuels 1985; Phillips *et al*., 2002) and *N. australe* (Slippers *et al*., 2004b) from other botryosphaeriaceous species. In addition to these two species, the production of yellow pigment was also observed in other *Neofussicoccum* species, including *N. parvum* and *N. ribis* that were later identified to species level using molecular methods. However, only the *N. luteum* could be identified

to species level using colony colour, as it produced a stronger yellow pigment than other *Neofussicoccum* species, and turned violaceous by 7 days. The yellow pigment producing isolates belonging to sub-group 2 were identified using molecular methods and identified as *N. parvum*, *N. ribis* and *N. australe* which all produced a light yellow pigment on PDA. Although different culture morphologies were observed in the non-yellow producing isolate group, the identification of these isolates to species level was carried out using conidial morphology and molecular methods that confirm the species *D. mutila*, *D. sariata*, *B. dothidea*, *Do. sarmentorum* and *Do. iberica* belonging to this group. This shows that the colony morphology of botryosphaeriaceous species was only useful to group the isolates, and that conidial morphology and molecular methods were necessary to identify them to species level.

Attempts to induce *in vitro* sporulation were successful for all four botryosphaeriaceous species tested, except for *N. parvum*. This may be because most of the *N. parvum* isolates found in New Zealand were sterile, a similar result reported by Ploetz *et al*. (2009). They reported that *N. parvum* isolates from *Syzygium paniculatum* did not produce fertile pycnidia and were referred to as sterile strains. However, *N. luteum* and *N. parvum* isolates were not able to sporulate on pine needle which is not in agreement with other research reports (Slippers *et al*., 2004b; van Niekerk *et al*., 2004). Unlike these research results, the current study showed that only *Diplodia* group species produced fertile pycnidia on pine needles. Since other substrates, such as acidified potato dextrose agar (Sánchez-Hernández *et al*., 2002), malt yeast extract agar (Slippers *et al*., 2004) and carnation leaf agar (Denman *et al*., 2003) have also been used to induce sporulation of botryosphaeriaceous species and this indicates that other researchers have found that different substrates may influence the production of fertile pycnidia of particular botryosphaeriaceous species.

The conidial characteristics of the New Zealand isolates were useful to identify *D. mutila*, *D. seriata* and *Dothiorella* group species in this study. However, the conidial characteristic of *N. luteum* and *N. australe* did not allow them to be separated as the shape, colour and size of the conidia (data not shown) of these species overlapped. Slippers *et al*. (2004b) also reported that these two species are morphologically very similar and molecular methods are required to distinguish them. In this study, the isolates belonging to *Dothiorella* group were identified to species level using molecular methods as identification based on conidial morphology is very difficult because they are very similar in shape, size and colour (Phillips *et al*., 2005).

Many researchers have stated that closely related botryosphaeriaceous species only show minor morphological differences and therefore, molecular methods are needed to provide accurate identification of these species (Denman *et al*., 2000; Zhou & Stanosz, 2001). In this study the botryosphaeriaceous species isolates unidentified by culture morphology and conidial characteristics were identified to species level using molecular methods PCR-RFLP and sequencing of the ITS gene

regions. The molecular confirmation of species identity was possible for the majority of isolates using a published PCR-RFLP method (ARDRA; Alves *et al*., 2005) and sequencing of taxonomically useful genes could identify representative or cryptic species. However, this method was unable to distinguish *N. luteum* and *N. australe*. The distinction between *N. luteum* and *N. australe* was not recognised in previous studies based only on ITS sequence data (Smith & Stanosz, 2001, Denman *et al*., 2003). These studies only analysed the sequence of a small portion (~600 bp) of the ITS region to differentiate these two species and the alignment results showed very low variation. In this study therefore a new restriction digestion method was developed based on a longer size ITS sequence (~1200 bp) of the same amplimer used in the Alves *et al.* (2005) study which was digested with *Sac*II enzyme. This method was able to clearly distinguish these two species by producing different RFLP patterns. The methods described by Alves *et al.* (2005) were not able to distinguish *N. parvum* from *N. ribis* in this study, possibly because the resolving capability of the standard agarose was used not enough to separate the restriction digestion of the ITS1/NL4 amplimer with *Asu*I. The ITS sequence alignment of these two species showed 99% identity and so another restriction digestion method was developed which successfully amplified the β-tubulin gene region with primers Bt2a and Bt2b followed by restriction digestion using *Msp*I enzyme, using this method, it was possible to distinguish *N. parvum* from *N. ribis*.

The species confirmation of *Dothiorella* species using the published ARDRA method with *Asu*I did not distinguish the individual species. A further attempts to confirm identity using ITS, β-tubulin sequences found conflicting data on GenBank. However, the 9 base pair deletion in the EF-1α sequence of *Do. iberica* was used to distinguish the *Do. iberica* from *Do. sarmentourm*, and has been reported as useful character to differentiate these two species in a previous study (Pitt *et al*., 2010).

This was the first study to isolate nine botryosphaeriaceous species from New Zealand grapevines being *N. parvum*, *N. luteum*, *N. australe*, *N. ribis*, *B. dothidea*, *D. mutila*, *D. seriata*, *Do. sarmentorum* and *Do. iberica*. Previous studies in New Zealand have reported botryosphaeriaceous species from grapevines (Bonfiglioli & McGregor, 2006; Mundy & Manning 2006), although their distribution and occurrence was not fully investigated. In a preliminary survey conducted by Amponsah *et al.* (2007) five botryosphaeriaceous species (*N. parvum*, *N. luteum*, *N. australe*, *D. mutila* and *D. seriata*) were isolated from 20 vineyards in New Zealand. Thus, this study has extended that list and provides a more comprehensive list of species that are infecting vines. Similar to this, studies conducted in California reported nine botryosphaeriaceous species (Úrbez-Torres *et al*. 2006a, 2007) while in Australia eight species have been reported (Pitt *et al*., 2010). All of the species found in New Zealand have been reported in other grape growing regions worldwide (van Niekerk *et al*., 2004; Taylor *et al*., 2005; Úrbez-Torres *et al*., 2006a and b; Urbez-Torres *et al*., 2008; Úrbez-Torres and Gubler 2009; Úrbez-Torres *et al*., 2009; Pitt *et al*., 2010; Qiu *et al*., 2010). A notable difference between New Zealand and other countries is the absence of *L. theobromae* (=*B. rhodina*) in the current study. This

species has been reported as present in New Zealand, being isolated from a few infected sweet potato tubers in Avondale, Auckland in 1963 and as only a single culture in the ICMP culture collection, which was isolated from the stem of *Begonia* × *hiemalis* in Ramarama, Auckland in 2008 (http://nzfungi.landcareresearch.co.nz). There are no other reports available of it being in any other hosts including grapevines. The regions overseas where *L. theobromae* has established are characterised by a semi-arid climate with mild winters and hot summers (Burruano *et al*., 2008), with a broad distribution over tropical and subtropical climate regions (Punithalingam, 1980). In particular, *L. theobromae* is found in Mexico (Úrbez-Torres *et al*., 2008), Texas (Úrbez-Torres *et al*., 2009), the southern desert region of California (Leavitt, 1990; Úrbez-Torres *et al*., 2006a), Upper Hunter Valley of New South Wales (NSW; Qiu *et al*., 2010), Northern Rivers region of NSW (Pitt *et al*., 2010), Western Australia (Taylor *et al*., 2005), Italy (Burruano *et al*., 2008) and South Africa (van Niekerk *et al*., 2004). New Zealand is characterised by a temperate climate and therefore, it is likely that *L. theobromae* is absent from New Zealand grapevines because of the relatively cooler and wetter climatic conditions that are not favourable for their survival.

The representative isolates of five botryosphaeriaceous species grew over a range of temperatures (10ºC –35ºC), but the optimal temperature for maximum growth rate varied depending on the species. In this study the actual growth rate measured at 25ºC was lower than the growth rate measured at 20ºC and 30ºC for all five species. This was possibly due to the fact that the incubator used at 25ºC was previously used for growth of *Trichoderma* spp. and persistence of any volatile residues produced by *Trichoderma* spp. may have suppressed the growth of the botryosphaeriaceous species. However, the estimated regression model curves fitted well with the data and were used to determine the optimum temperatures at which the maximum radial growth rate occurred. The optimum temperatures obtained for *N. parvum*, *N. luteum* and *N. australe* were slightly lower (2.3– 4.4ºC) than reported in other studies (Pennycook & Samuels, 1985; Úrbez-Torres *et al*., 2006a). However, the optimum temperatures obtained for *D. mutila* and *D. seriata* in this study were similar to those reported from California (Úrbez-Torres *et al*., 2006a) and Spain (Sanchez *et al*., 2003). In addition, all five botryosphaeriaceous species grew at 35 º C, which conflicted with studies by Pennycook & Samuels (1985) and Jacobs & Rehener (1998) but agreed with the study of Úrbez-Torres *et al*. (2006a).

The maximum growth rate at the optimum temperatures also varied between the species, with *N. parvum* having the highest radial mycelial growth rate and *D. mutila* having the lowest. This result is different from that observed by Úrbez-Torres *et al*. (2006a), as they found the highest radial mycelial growth rate for *L. theobromae* and lowest for *N. australe* species. Variations in the optimum temperature for maximum growth rate of a species between countries are likely to depend on the spectrum of isolates used for the study. This makes it difficult to compare the results of the effect of temperature on growth rates between different countries and studies, although it may be useful to compare the temperature ranges over which growth occurs.

Among the botryosphaeriaceous species, *N. parvum* was the predominant species found in New Zealand vineyards, followed in order by *D. mutila, D. seriata, N. luteum, N. australe, N. ribis, Do. iberica, Do. sarmentorum* and *B. dothidea*. The predominance of *N. parvum* in New Zealand vineyards was in contrast to results of surveys that found *D. seriata* to be the predominant species in California (Úrbez-Torres *et al*. 2006a), Western Australia (Taylor *et al*., 2005), New South Wales (Pitt *et al*., 2010; Qiu *et al*., 2010), South Africa (van Niekerk *et al*., 2004) and Spain (Martin & Cobos, 2007). In Californian vineyards *N. parvum* was the fourth most abundantly isolated species, preceded by *D. mutila, B. dothidea* and *L. theobromae* (Úrbez-Torres *et al*., 2006a). However, the incidence of *D. seriata* (16%) in New Zealand is similar to those found in Australian studies (15 – 19 %; Taylor *et al*., 2005; Pitt *et al*., 2010). It is unclear why *N. parvum* is predominant in New Zealand while *D. seriata* is predominant in the vineyards of some other countries. It is possible that New Zealand cool temperature may influence the establishment of these species, or it may be simply due to isolate differences. More research is needed involving a greater number of species and isolates to determine the effect of temperature and other environmental factors on New Zealand botryosphaeriaceous species.

In the current study, similar incidence levels were found for *D. mutila* (18%), *D. seriata* (16%), *N. luteum* (14%), and *N. australe* (11%). Although similar results were reported in California (Úrbez-Torres *et al*., 2006a) and Australia (Taylor *et al*., 2005; Pitt *et al*., 2010), variable incidence levels were reported in South Africa (van Niekerk *et al*., 2004) and Mexico (Úrbez-Torres *et al*., 2008). *Neofusicoccum ribis* (3%), *Do. iberica* (2%), *B. dothidea* (1%) and *Do. sarmentorum* (1%) had the lowest incidence in the current study. Interestingly, higher incidences of *B. dothidea* have been reported in California (Urbez-Torres *et al*., 2006a) and Australia (Qiu *et al*., 2010) than that found in New Zealand and the reason for these differences is unclear. The overall incidence was low for *N. ribis, Do. iberica, B. dothidea* and *Do. sarmentorum* in New Zealand grapevines. It is possible that these species are emerging as pathogens in response to changes in climate, management practices or selection pressure. All of these are important aspects that need to be researched in order to ascertain the importance of these species in the disease complex.

The survey results showed differences in botryosphaeriaceous species distribution between the North and South Islands in New Zealand wine growing regions. Results suggested that the botryosphaeriaceous species distribution in New Zealand could be influenced by climatic conditions, a phenomenon which has been reported in California (Úrbez-Torres *et al*., 2006a) and Australia (Pitt *et al*., 2010). Among the nine botryosphaeriaceous species identified in New Zealand grapevines, *N. parvum* was found in most of the vineyards in all six wine growing areas surveyed in both the North and South Island. In contrast, the distribution of *N. parvum* species in California showed that it was restricted to cooler regions of Northern California (6.8–23.7ºC; Urbez-Torres *et al*., 2006a) and the

Northern Slopes (11.3-25ºC) and Northern Rivers (13-25ºC) regions in NSW (Pitt *et al*., 2010). *Neofusicoccum lutem, D. mutila, N. ribis* and *N. australe* were isolated from both the North and South Island vineyards, while *D. seriata, Do. sarmentorum, B. dothidia* and *Do. iberica* were only isolated from South Island vineyards. Although *N. australe* and *N. luteum* isolates were distributed in both the North and South Islands, the majority of *N. australe* isolates were found in the South Island (Blenheim and Canterbury) while the majority of *N. luteum* isolates were found in the North Island (Auckland and Gisborne). These results suggest that there is a pattern to their distribution, which may be due to their different temperature tolerances, since *N. australe* had the lowest optimum temperature (23.4°C) for maximum growth and *N. luteum* had the highest optimum temperature (26.3°C) for maximum growth. However, *N. parvum, N. lutem, D. mutila* and *N. australe* are spread throughout New Zealand because they have a wide temperature tolerance as evidenced by their ability to grow across temperatures between 10 and 35°C. However, *D. seriata* also grew between these temperature ranges but was only found in the South Island. Although *Do. sarmentorum* and *Do. iberica* were not included in the temperature study, overseas reports found that the highest incidence of *Do. sarmentorum* was associated with cooler climatic conditions while optimum temperature growth data was 21.8°C for *Do. iberica*. It is possible that *Do. sarmentorum* and *Do. iberica* are more adapted to cooler regions and may help explain why they were only isolated from Canterbury and Otago regions, which have the lowest mean temperatures in the sampled regions (NIWA). This suggests that temperature may influence the distribution patterns of botryosphaeriaceous species in New Zealand vineyards.

In summary, this study has demonstrated that, as in other countries, botryosphaeriaceous species are the predominant cause of dieback and decline in grapevines. The nine botryosphaeriaceous species are distributed throughout the six main grape growing regions of New Zealand. Their incidence and distribution is likely to be influenced by climatic conditions and different sources of inoculum in the regions sampled. Other factors such as wind, rainfall and humidity, along with viticulture practices may influence the distribution of botryosphaeriaceous species. The results provided in this research clearly identify the target species for development of management strategies in New Zealand.

Chapter 3

Genetic diversity of *Neofusicoccum parvum*, *N. luteum*, *N. australe* and *Diplodia mutila*

3.1 Introduction

The genetic diversity of a fungal population is an important feature as it encompasses dispersal and distribution of particular genotypes in different localities. Detailed knowledge of genetic diversity within a pathogen population can also provide a basis for the selection of potential control strategies (Reeleder, 2003). The relative contributions of asexual and sexual reproductive activities to the disease cycle can influence the genetic diversity of a fungal species population. A fungal species with high levels of sexual reproduction is thought to build up a genetically more diverse population than a fungal species that reproduces mainly asexually (Cortesi & Milgroom, 2001). In nature, Ascomycete fungi mainly reproduce asexually (Zeigler, 1998), and for botryosphaeriaceous species many researchers believe asexual spores are the main type of inoculum. A genetic diversity study of *Botryosphaeria dothidea* in California, using 120 isolates, showed that among the *B. dothidea* population obtained from pistachio in different locations there was a high level of genetic similarity (genetic identity >98%), however, the non- Californian *B. dothidea* population were genetically diverse (Ma *et al*., 2004).

The use of vegetative compatibility groups (VCG) or mycelial compatibility groups (MCG) can provide information on the genetic diversity of many fungi. Vegetative compatibility studies can be used to assess genetic compatibility at multiple loci (Milgroom & Cortesi, 1999), and has been used extensively to describe fungal population structures and their genetic diversity. In the botryosphaeriaceous species, VCG have been reported for *B. dothidea* (Ma *et al*., 2004). However, the effectiveness of this method in estimating genetic relatedness of a population has been questioned for many fungal species. Consequently, molecular methods that produce information from DNA-based polymorphisms are now widely accepted as the best estimate of genetic diversity (Waugh, 1997). Many types of molecular markers have been used to characterize genetic diversity in fungi (Milgroom, 1996). These include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) methods. In addition universally primed PCR (UP-PCR) can be used to amplify DNA from any organism without previous knowledge of DNA sequences and generates profiles containing multiple bands when the products are separated by gel electrophoresis, called 'fingerprints'(Bulat *et al*., 1998). The UP-PCR fingerprinting technique is a useful tool for the characterization and grouping of fungal strains in order to explain their genetic relatedness (Lübeck *et al*., 1999). This technique has been successfully used in genetic variation

analysis of fungal pathogens (Yli-mattila *et al*., 1997; Bulat *et al*., 1998; Lubeck *et al*., 1999) and plants (Bulat *et al*., 1994). However, the genetic diversity of the botryosphaeriaceous species populations obtained from vineyards has not been studied extensively in New Zealand or overseas.

The aim of this study was to determine the genetic diversity within populations of four botryosphaeriaceous species isolated from New Zealand vineyards using the UP-PCR method. The regional, inter- and intra-vineyard genetic variability were analysed and compared to the genetic variability of international isolates. The vegetative compatibility groups of *N. parvum* and *D. mutila* were also investigated using a subset of isolates from the genetic variation analysis.

3.2 Materials and methods

3.2.1 Selection of botryosphaeriaceous species and isolates for the genetic diversity study

The genetic diversity of the four most commonly isolated botryosphaeriaceous species; *N. parvum*, *N. luteum*, *N. australe* and *Diplodia mutila* was studied. Multiple isolates were selected from a single vineyard to study the intra-vineyard genetic diversity and from different vineyards and locations to analyse inter-vineyard and inter-regional genetic diversity. International isolates from Australia, South Africa and USA were also included in this study. The number of isolates of each species obtained from different locations in New Zealand and from overseas is summarized in Table 3.1. A full list of the isolates of the four species and their origin is in Appendix B.1.

3.2.2 Genomic DNA extraction

All the selected isolates from the four species were grown on potato dextrose broth and the genomic DNA was extracted using PUREGENE® genomic DNA isolation kit as detailed in Section 2.2.7.1**.** All the genomic DNA samples were diluted to 20-30 ng/µL for use in UP-PCR reactions.

3.2.3 Genetic variation analysis using UP-PCR method

3.2.3.1 Primer testing

In order to choose the most informative primers, each of the eleven UP-PCR primers (Table 3.2) was tested against three representative isolates from each of the four botryosphaeriaceous species. The three representative isolates were chosen from different locations and/or countries in order to test isolates that were most likely to be diverse from each other. The criteria used to select primers were number of polymorphic bands, total number of bands and visual assessment of band distribution on the agarose gel to facilitate scoring them visually.

Table 3.1: Isolates of four botryosphaeriaceous species from different locations in New Zealand and other countries that were used for the genetic variation study.

Species	Region/ Country	No of isolates
N. parvum	Auckland	17
	Gisborne	20
	Blenheim	4
	Nelson	4
	Hawkes Bay	3
	Bay of Plenty	1
	Australia	4
	South Africa	5
	USA	3
N. luteum	Auckland	18
	Gisborne	8
	Hawkes Bay	6
	Bay of Plenty	2
	Nelson	1
	USA	5
N. australe	Auckland	7
	Gisborne	1
	Blenheim	11
	Nelson	2
	Canterbury	9
	Hawkes Bay	2
D. mutila	Auckland	2
	Gisborne	4
	Blenheim	25
	Nelson	5
	Canterbury	4
	Australia	5
	USA	3

Table 3.2: Sequence and annealing temperature of UP-PCR primers used in this study

Name	Sequence	Annealing	Reference
AA2M2	$^{5'}$CTGCGACCCAGAGCGG$^{3'}$	50	Lübeek *et al*., 1998
AS4	$^{5'}$TGTGGGCGCTCGACAC$^{3'}$	50	Lübeek *et al*., 1998
AS15	$^{5'}$GGCTAAGCGGTCGTTAC$^{3'}$	52	Bulat *et al*., 1994
AS15inv	$^{5'}$CATTGCTGGCGAATCGG$^{3'}$	52	Cumagun *et al*., 2000
L15	$^{5'}$GAGGGTGGCGGTTCT$^{3'}$	52	Tyson *et al*., 2002
L15/AS19	$^{5'}$GAGGGTGGCGGCTAG$^{3'}$	52	Lübeek *et al*., 1998
3-2	$^{5'}$TAAGGGCGGTGCCAGT$^{3'}$	52	Bulat *et al*., 1994
L21	$^{5'}$GGATCCGAGGGTGGCGGTT$^{3'}$	55	Lübeek *et al*., 1998
L45	$^{5'}$GTAAAACGACGGCCAGT$^{3'}$	51	Bulat *et al*., 1994
Fok1	$^{5'}$GGATGACCCACCTCCTAC$^{3'}$	52	Lübeek *et al*., 1998
0.3-1	$^{5'}$CGAGAACGACGGTTCT$^{3'}$	50	Bulat *et al*., 1994

3.2.3.2 UP-PCR procedure

Each UP-PCR was done in a 25 µl reaction volume consisting of 10 mM Tris pH 8.0, 50 mM KCl, 200 µM of each dNTPs, 2.5 mM $MgCl_2$, 20 pmoles of primer (Table 3.2), 1.25 U of FastStart® *Taq* DNA polymerase (Roche Diagnostics) and 20-30 ng of genomic DNA from each of the isolates. The amplification was performed as follows: denaturation at 94ºC for 5 min, 5 cycles of 94ºC for 50 s, annealing at respective primer temperature (Table 3.2) for 2 min and extension at 72 ºC for 1 min, followed by 34 cycles of 94ºC for 50 s, annealing at respective primer temperature (Table 3.2) for 90 s, and extension at 72ºC for 1 min, with a final extension of 72ºC for 10 min.

3.2.3.3 Electrophoresis and scoring of DNA fingerprints

UP-PCR amplification products were separated by 1% agarose gel electrophoresis at 5 V/cm for 3 h in 1× TAE (Appendix B.2). Agarose gels were stained with Sybersafe (5 µl/100 ml 1 × TAE) and visualized on a UV transilluminator (Versadoc™). Only strongly fluorescent bands were considered for scoring. If a band was poorly amplified and faintly fluorescent it was not included. Scoring was done as follows: for each position where a band was present it scored as "1" and if absent it was scored as "0". For each species, the binomial data were prepared as a matrix in Microsoft Excel® spreadsheet and saved as a 'text tab delimited file' to enable them use for genetic variation analysis in another program.

3.2.3.4 Genetic diversity analysis

Genetic diversity among isolates of the same species was analysed individually for each species. Intra-vineyard, inter-vineyard and inter regional genetic diversity was analysed for each species. Genetic diversity of the New Zealand isolates was compared to the international isolates obtained from Australia, California (USA) and South Africa. For each species, the binomial matrix data file generated from the banding profile was analysed using the distance based method of neighbour-joining (NJ) as a tree building algorithm using the PAUP software version 4.0b10 (Swofford, 1999). The NJ tree was generated based on the total character differences and the tree was rooted to the midpoint. Genetic groups were identified in NJ trees of each species by drawing arbitrary lines at a set distance from the roots to produce genetic groups with similar percentage 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 $H=\Sigma\ (1-\Sigma\ \chi_k^{\ 2}\ )/h$, χ is 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 a normalized Shannon's diversity index (I) (Sheldon, 1969): $I= -\ \Sigma_{pi}\ \mathrm{In}P_i/\mathrm{In}N$, where 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.

3.2.4 Examination of vegetative compatibility

From the four predominant species, a single species from the *Neofusiccocum* (*N. parvum*) and *Diplodia* (*D. mutila*) groups was chosen for vegetative compatibility analysis. This extension of genetic diversity analysis was conducted to determine the relationship between VCG and the genetic diversity, for *N. parvum* and *D. mutila*. The *N. parvum* and *D. mutila* isolates that were selected from the different genetic clusters of the neighbour joining trees included 11 isolates of *N. parvum* and 14 isolates of *D. mutila*.

Two different strengths of PDA medium, ½ PDA (19.5 g PDA /L) and full- strength (39 g PDA /L) as well as prune extract agar (PA) were used to test the vegetative compatibility of *N. parvum* isolates. Only ½ PDA was used to test *D. mutila* isolates. For each species the selected isolates were grown on PDA at 23.5ºC under 12/12 h light/dark conditions for 3 days prior to setting up the experiment. For setting up the two isolates in a single Petri dish (pairing), a 3 mm diameter mycelial plug was taken from the edges of 3 day old colonies of the selected isolates, which were placed 2.5 cm apart in the middle of the Petri dish (Ø9 cm) containing the appropriate agar medium. All isolates belonging to the same species were tested in pairs against each other (self versus non-self) and themselves (self versus self). Each combination of isolates was prepared in triplicate. Observations were made after 7, 10 and 21 days of incubation at 23.5 ºC under 12/12 h light/ dark conditions. The plates were visually examined in both upper and reverse side and scored a) vegetatively compatible, when mycelia of two isolates merged together uniformly, b) vegetatively incompatible, when mycelia of two isolates grew to a meeting point on the agar but remained separated by a "barrage-like" reaction formed along the line of contact between paired isolates and c) partially incompatible which is any other intermediate reactions different from self versus self reaction. Any type of merging reaction at the interaction zone which was different from self versus self pairing was considered as either incompatible (presence of barrage line) or partially incompatible (no merge but did not display a barrage line), depending on the presence or absence of a barrage line and imaged using a digital camera.

3.2.5 Microscopic examination of vegetative compatibility

Microscopic analysis of the vegetatively incompatible reactions was carried out for *N. parvum* and *D. mutila*. Based on vegetative compatibility reactions observed in the previous experiment, two isolates from each compatibility reaction were selected and paired against each other and themselves on a microscopic slide. The method previously described (Aimi *et al*., 2002) used two 5 mm cubes of 1/8

strength PDA media which contained 1% agar, placed 1 cm apart on a sterile glass microscopic slide. Each agar cube was inoculated on its top with a 2 mm cube of mycelium colonized agar obtained from 3-4 day old cultures on PDA, with each isolate paired against itself and against other test isolates. 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 five times. These slides were incubated at 23.5ºC in 12 light /12 dark conditions until the hyphae from each isolates 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). The leading hyphae from each isolate colony were followed under the microscope to identify the anastomoses between the isolates and digitally imaged using Olympus BX51 microscope with a built-in Olympus DP12 camera (Olympus optical, Japan).

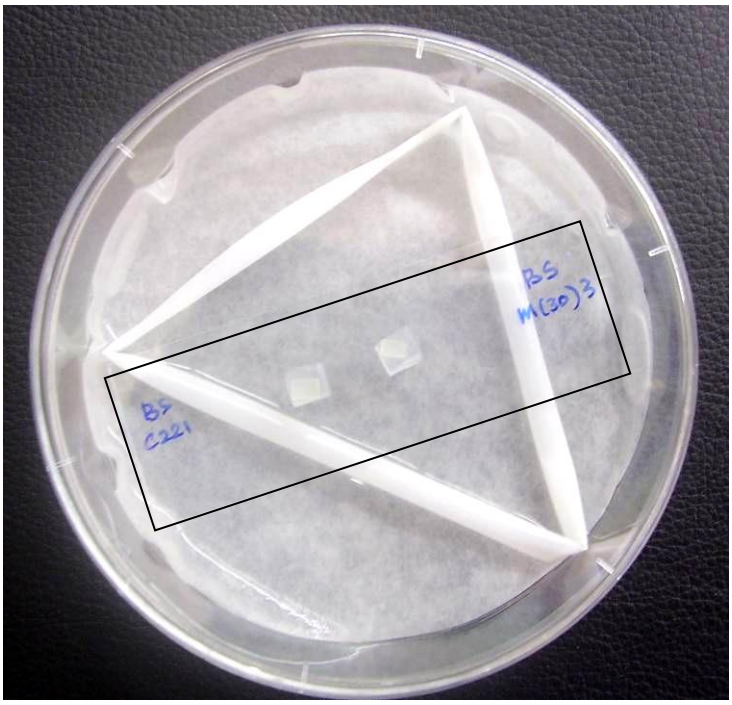

Figure 3.1: Growing *D. mutila* isolate pairs on a glass microscopic slide for microscopic examination of vegetative compatibility reactions.

3.3 Results

3.3.1 UP-PCR banding pattern and primer selection

Different DNA banding patterns were generated by each of the 11 UP-PCR primers when they were tested with representative isolates of the four botryosphaeriaceous species. The total number of bands amplified for each primer and species combination varied only slightly, however, the number of these

bands that were polymorphic varied considerably (eg: 0–58% for primer Fok1; Table 3.3). Examples of UP-PCR fingerprint profiles generated with different botryosphaeriaceous species and different UP-PCR primers are shown in Figure 3.2.

Table 3.3: Details of the UP-PCR primers tested for genetic variation study of four botryosphaeriaceous species with number of total bands generated (AB), number of polymorphic bands (PB) and the percentage of polymorphism (P%). The primers selected for each species is indicated in bold in the P% column.

Primer	*N. parvum*			*N. luteum*			*N. australe*			*D. mutila*		
	AB	PB	P%	AB	PB	P%	AB	PB	P%	AB	PB	P%
AA2M2	12	3	**25**	13	4	**31**	10	2	**20**	9	4	**44**
AS4	14	2	**14**	10	2	20	11	1	9	9	1	11
AS15	8	2	25	9	4	**44**	10	3	**30**	6	1	17
AS15inv	10	2	**20**	9	3	**33**	11	2	**18**	10	0	0
Fok1	14	7	**50**	0	0	0	0	0	0	12	7	**58**
L15	13	4	**31**	12	1	8	12	2	**17**	10	2	20
L15/AS19	11	3	27	14	6	**43**	12	3	**25**	9	2	22
L21	9	3	33	7	0	0	7	0	0	8	2	**25**
L45	15	3	**20**	7	1	14	7	1	14	14	3	**21**
0.3-1	12	5	**42**	0	0	0	4	1	25	10	4	**40**
3-2	17	5	**29**	9	3	**33**	8	2	25	8	1	13

The primers selected for the genetic variation analysis of each species (bold in Table 3.3) were those that gave the greatest number of polymorphic bands, and differed among species. For *N. parvum*, amplifications were obtained with all the 11 UP-PCR primers tested. Among the tested primers the highest percentage of polymorphic bands (50%) were generated with primer Fok1 (Figure 3.3) and the least (14%) with primer AS4. For *N. luteum*, primers Fok1 and 0.3-1 did not produce any amplification and no polymorphic bands were produced by primer L21, thus, these were not used for analysis of this population. No amplification and no polymorphic bands were obtained in *N. australe* species with primers Fok1 and L21, respectively, and these were not used to analyse *N. australe* populations. Amplifications were obtained with all 11 UP-PCR primers in *D. mutila* but no polymorphism was seen for the primer AS15inv. Eight primers were selected for *N. parvum* but only five primers were selected for the genetic diversity analysis of *N. luteum*, *N. australe* and *D. mutila* due to the available time and in consideration of cost.

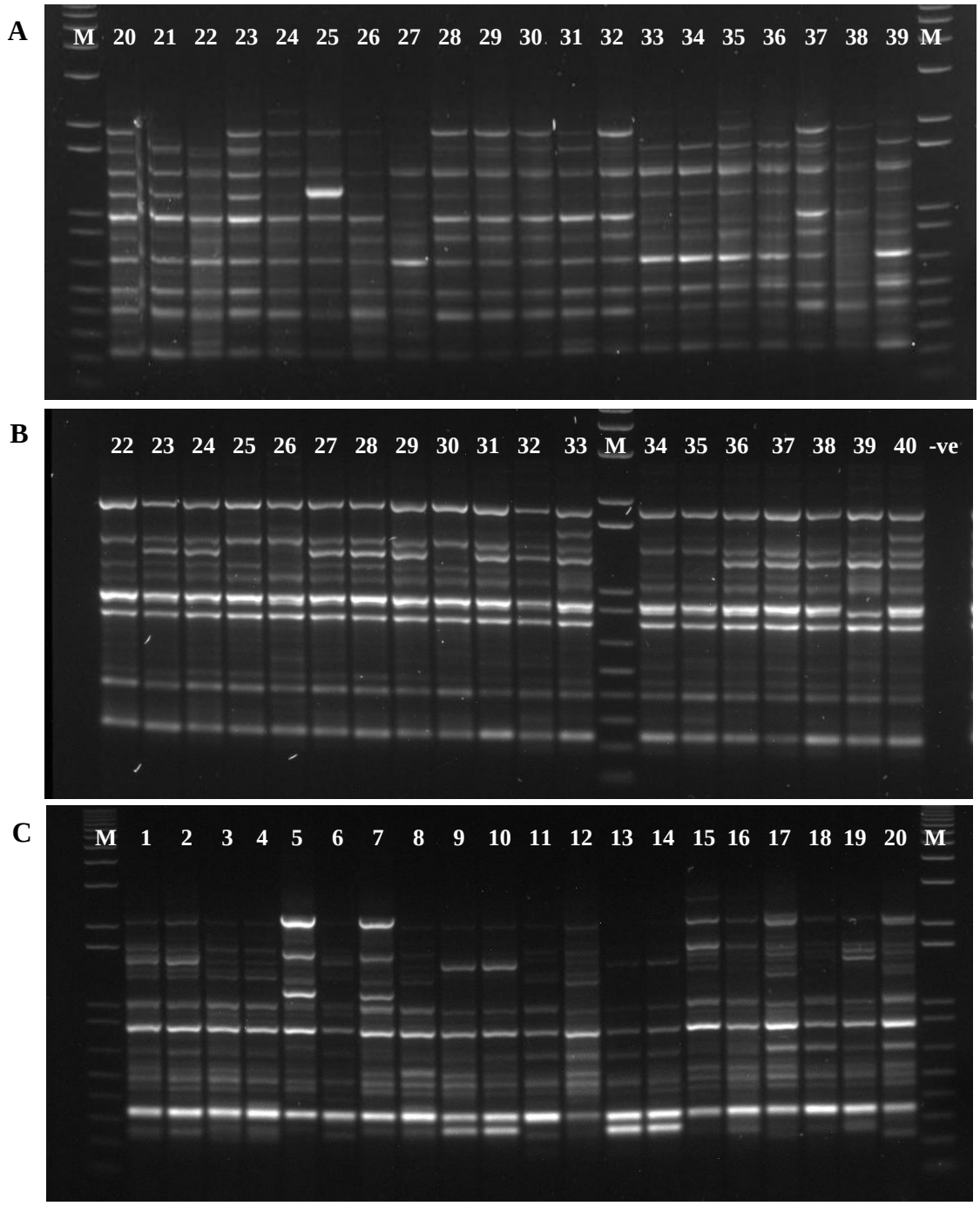

Figure 3.2: UP-PCR fingerprints generated by different UP-PCR primers in different botryosphaeriaceous species. A) Fingerprints of AA2M2 primer with *N. parvum* isolates, B) fingerprints of AA2M2 primer with *N. luteum* isolates and C) fingerprints of L21 primer with *D. mutila* isolates. Lane numbers indicate the isolates number (Appendix B.1) and M- 1Kb^{+} marker (Invitrogen).

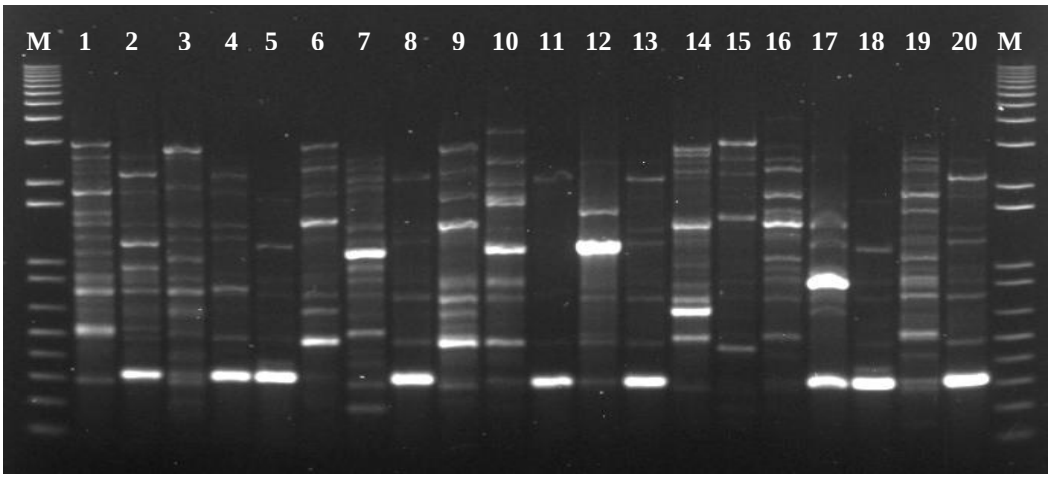

Figure 3.3: UP-PCR fingerprints generated by Fok1 UP-PCR primers with *N. parvum* isolates. Lanes 1-20 indicates the isolate numbers (Appendix B.1) and M, the 1Kb^{+} marker (Invitrogen).

3.3.2 Genetic diversity analysis of *Neofusicoccum parvum*

The eight UP-PCR primers generated a total of 82 bands (loci) from 61 *N. parvum* isolates studied, of which 73 (89.02%) were polymorphic bands. A neighbour joining tree created in PAUP, using combined binomial data generated by the eight UP-PCR primers, showed the *N. parvum* population found in New Zealand vineyards was genetically diverse (Figure 3.4). Only four *N. parvum* isolate pairs were identified as clonal populations. Seven main clusters were identified by drawing an arbitrary line at 2.5 character changes (2.5/82 x100= 3% change) from the root of the neighbour joining tree. Each genetic cluster contained a range of isolates from different regions and vineyards.

The *N. parvum* isolates from different regions dispersed in different clusters showing regional genetic variability. For example the Gxxx isolates from Gisborne were located in genetic groups 1, 2, 4 and 5. Genetic variability was also observed at both inter- and intra-vineyards levels. The isolates G2xxx and G6xxx obtained from different vineyards in the same region (Gisborne) were placed in genetic groups 1, 2, 4 and 5 which indicated genetic diversity of *N. parvum* between the vineyards. The isolates G2xxx were obtained from the same vineyard in Gisborne and were located in genetic groups 1, 2 and 5 which showed the intra-vineyard genetic diversity. The results showed that inter-vineyard genetic diversity was higher than the intra-vineyard diversity. Although the three isolates obtained from a single lesion (isolates A102b4, A102b6 and A102b9) were placed in genetic group 2, they were not genetically identical (clonal) as each of them occupied separate branches of genetic group 1. This showed the existence of genetic diversity of the *N. parvum* isolates within a lesion.

The Australian (DAR) and Californian (UCD) *N. parvum* isolates clustered with the New Zealand isolates in genetic groups 1, 2 and 5. Among the four Australian isolates (DAR), three of them were in separate sub-branches in genetic group 2 and one of them in genetic group 1 with New Zealand isolates. All of the South African *N. parvum* isolates (STE-U) were grouped in a separate cluster. This analysis showed that the Australian and Californian isolates are genetically closer to the New Zealand population than the South African isolates.

Genetic diversity analysis of seven *N. parvum* sub-populations including four New Zealand sub-populations obtained from different regions and three international isolates showed differences in percentage of polymorphic loci produced. The percentage of polymorphic loci produced varied between the populations with the highest (67.07%) in the Auckland population and lowest (17.07%) in the Californian isolates. The overall mean genetic diversity of the total population calculated by Nei's genetic diversities (*H*; Nei, 1973) was 0.2581, showing that 26% of all the isolates in *N. parvum* populations were genetically diverse. Among the *N. parvum* populations obtained from different regions the most genetically diverse population was found in Auckland (0.2488) and least in Nelson (0.1082), while that of Californian isolates was 0.0759. The genotypic diversity calculated by Shannon's diversity index ranged from 0.1087 in the Californian isolates to 0.3674 in the Auckland population (Table 3.4).

Table 3.4: The percentage of polymorphic loci, mean genetic diversity (*H*) and Shannon diversity index (*I*) of *N. parvum* populations

Population	Sample size	% polymorphic loci	H^a	I^b
Auckland	17	67.07	0.2488	0.3674
Gisborne	17	60.98	0.2019	0.3050
Nelson	4	28.05	0.1082	0.1609
Blenheim	4	47.56	0.1905	0.2802
Californian isolates	3	17.07	0.0759	0.1087
Australia isolates	4	20.73	0.0854	0.1246
South Africa isolates	5	50.00	0.1815	0.2734
Total population	54	89.02	0.2581	0.3946

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

The Nei's (Nei, 1973) measure of genetic identity and genetic distance demonstrated the genetic distance of the different populations (Table 3.5). The dendogram generated from Nei's measure of genetic distance showed that the Auckland and Gisborne populations were genetically the most similar to each other. The Australian isolates were more similar to the New Zealand population than either the Californian or South African isolates (Figure 3.5). This was a similar result to that shown by the neighbour joining analysis.

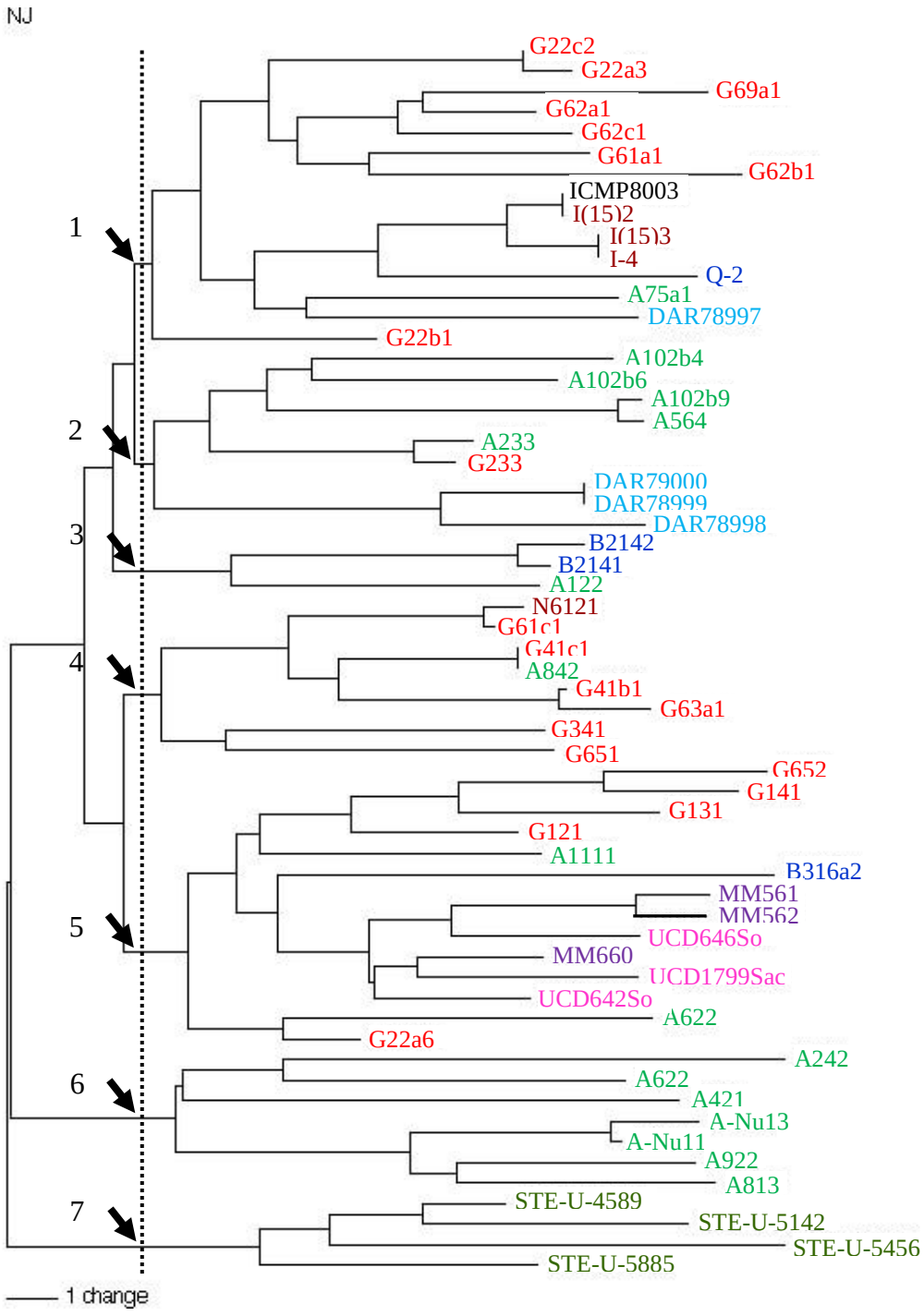

Figure 3.4: Neighbour joining tree based on DNA fingerprints generated with eight UP-PCR primers of the *N. parvum* grapevine isolates collected from different regions in New Zealand. International isolates from Australia (DAR), USA (UCD) and South Africa (STE-U) also included in this analysis. The arbitrary line (····) drawn at 2.5 changes from the root of the tree is used to differentiate genetic groups (1-7).

Table 3.5: Nei's measures of genetic identity and genetic distance of different *N. parvum* populations.

Population	Auck	Gis	Nel	Ble	Cal	Aus	SA
Auckland (Auck)	[a]****	0.9556	0.8999	0.9363	0.8785	0.8814	0.8519
Gisborne (Gis)	0.0454	****	0.9228	0.9315	0.9077	0.8753	0.8274
Nelson (Nel)	0.1055	0.0803	****	0.9115	0.7870	0.8659	0.7686
Blenheim (Ble)	0.0658	0.0709	0.0927	****	0.8660	0.8610	0.8248
California (Cal)	0.1296	0.0969	0.2395	0.1439	****	0.8335	0.7600
Australia (Aus)	0.1262	0.1332	0.1440	0.1496	0.1822	****	0.7991
South Africa (SA)	0.1603	0.1895	0.2631	0.1927	0.2745	0.2242	****

[a]Nei's genetic identity based on 82 loci is above the diagonal, and Nei's genetic distance coefficients are below the diagonal.

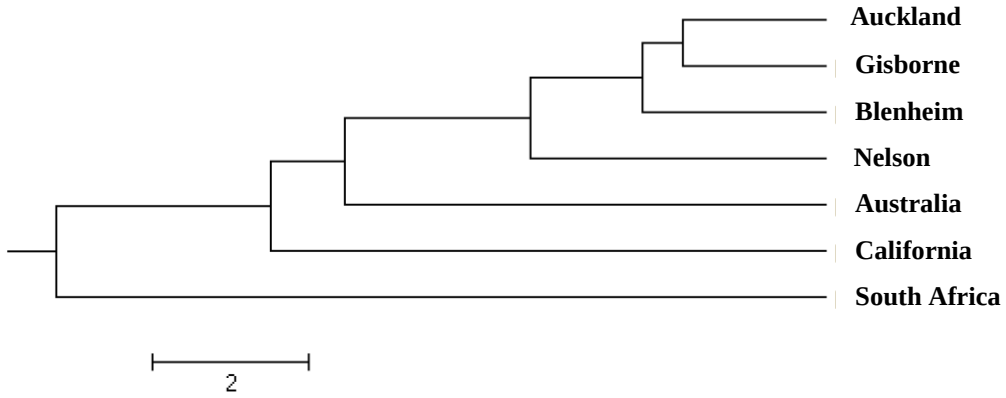

Figure 3.5: UPGMA dendogram showing the genetic relatedness of the *N. parvum* populations studied based on Nei's (1973) genetic distance.

3.3.3 Genetic diversity analysis of *Neofusicoccum luteum*

From the 40 *N. luteum* isolates, 51 scorable DNA bands were produced with five UP-PCR primers, of which 68.63% were polymorphic. A genetically diverse *N. luteum* population was found in the New Zealand, at both the inter- and intra-vineyard level (Figure 3.6) which was similar to the results observed in neighbour joining analysis of *N. parvum*. Seven genetic clusters were identified in the *N. luteum* neighbour joining tree by drawing an arbitrary line of 1 character change (1/51 x100=2% change) from the root of the neighbour joining tree. The number of isolates grouped in each genetic group varied from 1 in genetic group 6 to 12 in genetic group 3. Only one pair of isolates (A512 and A526) was clonal, and they were isolated from same vineyard in Auckland.

The *N. luteum* isolates obtained from different locations were dispersed in the different genetic clusters identified, which was a similar pattern to that seen for the *N. parvum* population. For example the Gxxx isolates obtained from the Gisborne region were placed in genetic groups 1, 3 and 7. Non-grapevine *N. luteum* isolates (CC445 and CC440) included in this study were also distributed in different genetic clusters showing that grapevine isolates were not genetically distinct from non-

grapevine isolates. Most of the Californian isolates (UCD) grouped in genetic group 1 and only a single isolate grouped with a New Zealand isolates in genetic group 4.

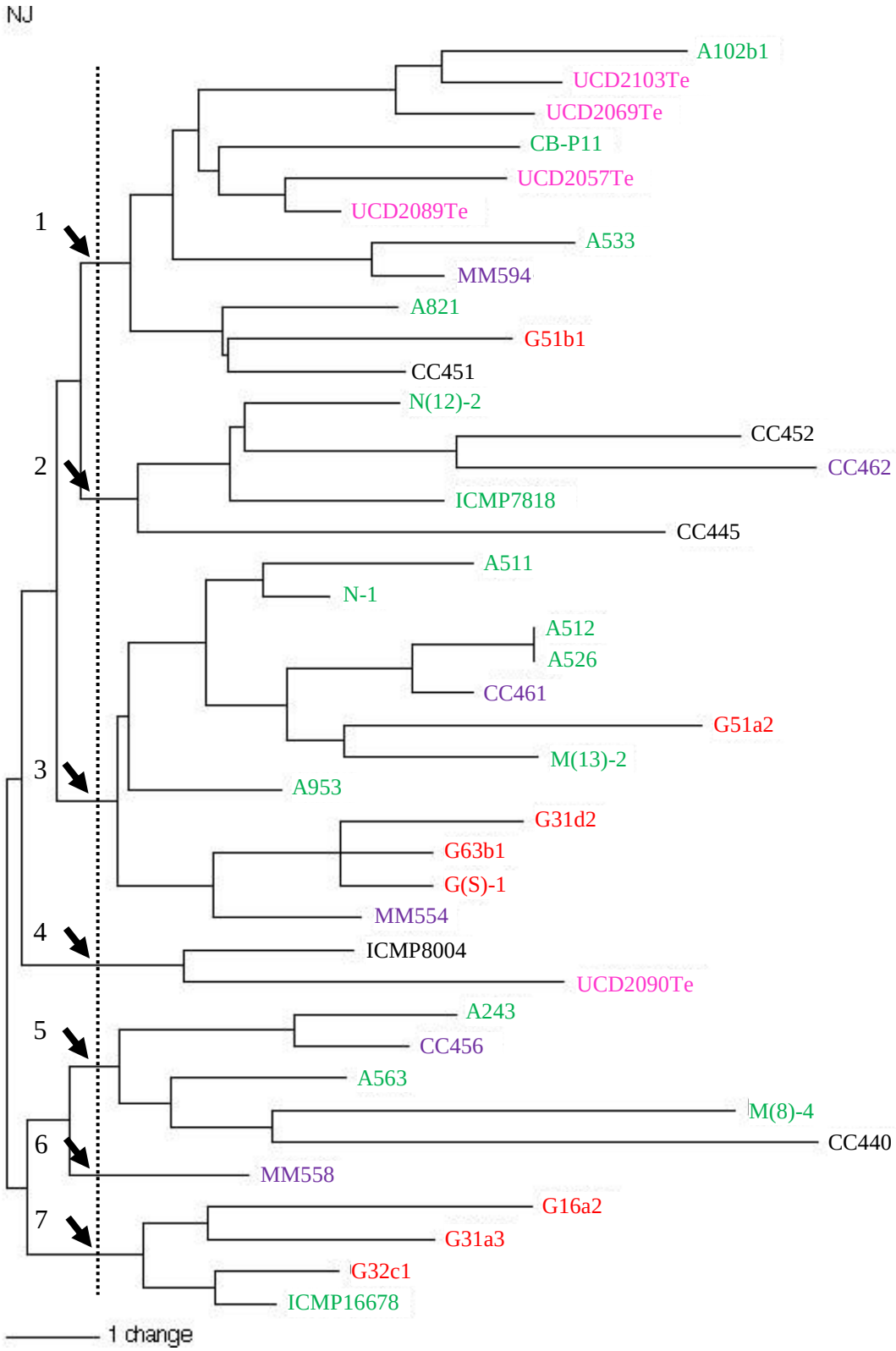

Figure 3.6: Neighbour joining tree based on DNA fingerprints generated with five UP-PCR primers of the *N. luteum* isolates collected from different regions in New Zealand. California isolates (UCD) also were included in this analysis. The arbitrary line (····) drawn at 1.0 changes from the root of the tree is used to define genetic groups (1-7).

The percentages of polymorphic loci produced varied between populations, with 29.41% in the Californian isolates and 52.94% in the Auckland population (Table 3.6). The overall mean genetic diversity of the *N. luteum* population was 0.1791 which was lower than the mean genetic diversity of the *N. parvum* population (0.2581). The range of genetic diversities varied between the populations from 0.1129 (Californian isolates) to 0.1673 (Hawkes Bay- Plant & Food Research collection). The genotypic diversity calculated by Shannon's diversity index ranged from 0.1675 in the Californian isolates to 0.2517 in the Hawkes Bay population. The Nei's measures of genetic identity and genetic distance of *N. luteum* populations showed that the Auckland and Hawkes Bay populations (Plant & Food Research collection) were genetically the most similar and the Californian isolates was genetically dissimilar to the other populations studied (Table 3.7 & Figure 3.7).

Table 3.6: The percentage of polymorphic loci, mean genetic diversity (*H*) and Shannon diversity index (*I*) of *N. luteum* populations

Population	Sample size	% polymorphic loci	*H*[a]	*I*[b]
Auckland	14	52.94	0.1573	0.2442
Gisborne	8	39.22	0.1409	0.2112
Hawkes bay	8	47.06	0.1673	0.2517
Californian isolates	5	29.41	0.1129	0.1675
Non-grapevine	5	41.18	0.1537	0.2297
Total population	40	68.63	0.1791	0.2827

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

Table 3.7: Nei's measures of genetic identity and genetic distance of different *N. luteum* populations

Population	Auck	Gis	Hwb	Cal	NGr
Auckland (Auck)	[a]****	0.9531	0.9822	0.9447	0.9519
Gisborne (Gis)	0.0481	****	0.9650	0.9254	0.9360
Hawkes bay (Hwb)	0.0179	0.0356	****	0.9496	0.9723
California (Cal)	0.0569	0.0775	0.0517	****	0.9279
Non-grapevine (NGr)	0.0492	0.0662	0.0281	0.0749	****

[a]Nei's genetic identity based on 51 loci is above the diagonal, and Nei's genetic distance coefficients are below the diagonal.

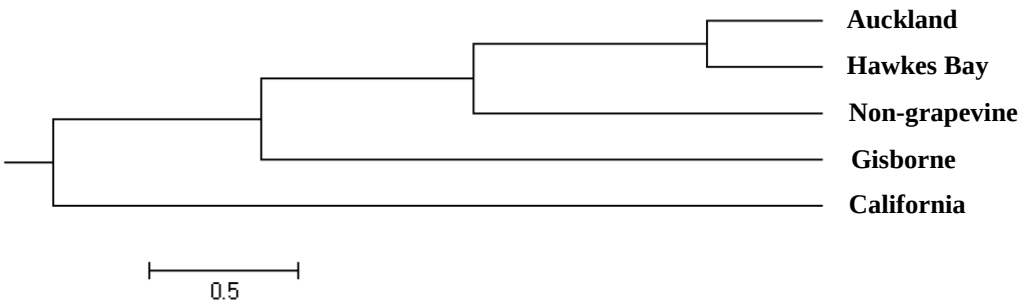

Figure 3.7: UPGMA dendogram showing the genetic relatedness of the *N. luteum* populations studies based on Nei's (1973) genetic distance.

3.3.4 Genetic diversity analysis of *Neofusicoccum australe*

A total of 57 scorable bands were generated from 33 isolates of *N. australe* of which 85.96% were polymorphic loci. The neighbour joining analysis of *N. australe* species also showed that the population found in New Zealand was genetically diverse (Figure 3.8), which was similar to the results of *N. parvum* and *N. luteum*. No clonal isolates of *N. australe* were found among the 33 isolates studied. An arbitrary line of 4 character changes (4/57 x100= 7% change) from the root of the neighbour joining tree identified six genetic groups. The number of isolates in each of the genetic groups varied with the highest of 16 isolates in genetic group 2 whereas the genetic group 3 and 6 had only a single isolate each. Isolates obtained from different locations, including Auckland, Gisborne, Blenheim and Canterbury, were sub-grouped into genetic group 2 showing that they were genetically closer but not identical. The majority of the isolates obtained from nurseries (R0xxx) were also found in genetic group 2 with the isolates from vineyards, showing that nursery isolates did not belong to a distinct genetic group.

The genetic diversity and Shannon diversity index calculated for *N. australe* populations ranged from 0.1856 to 0.2549 and from 0.2885 to 0.3789, respectively (Table 3.8). The highest genetic variability calculated was 0.2549 in Auckland and the lowest was 0.1856 in Blenheim. The Nei's measure of genetic identity and genetic distance analysis showed that the *N. australe* populations obtained from the Blenheim and Canterbury regions were genetically more similar to each other than to the Auckland population (isolates obtained from nurseries) (Table 3.9 & Figure 3.9).

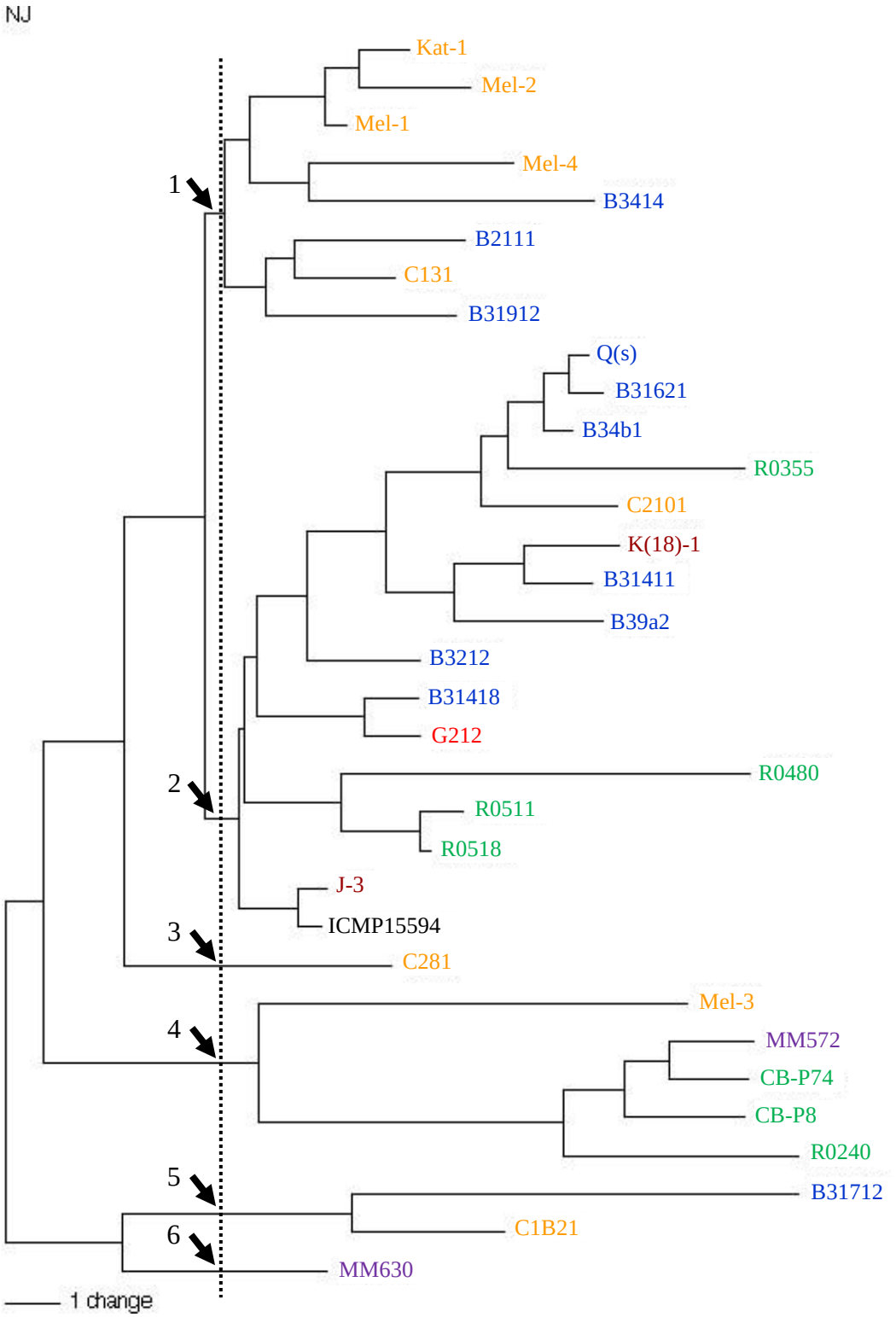

Figure 3.8: Neighbour joining tree based on DNA fingerprints generated with five UP-PCR primers of the *N. australe* isolates collected from different regions in New Zealand. The arbitrary line (···) drawn at 4 changes from the root of the tree is used to define genetic groups (1-6).

Table 3.8: The percentage of polymorphic loci, mean genetic diversity (*H*) and Shannon diversity index (*I*) of *N. australe* populations

Population	Sample size	% polymorphic loci	H^a	I^b
Auckland	7	64.91	0.2549	0.3740
Blenheim	11	61.40	0.1856	0.2885
Canterbury	9	57.89	0.1906	0.2920
Total population	27	85.96	0.2417	0.3789

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

Table 3.9: Nei's original measures of genetic identity and genetic distance of different *N. australe* populations

Population	Auck	Ble	Can
Auckland (Auck)	[a]****	0.9173	0.9065
Blenheim (Ble)	0.0864	****	0.9545
Canterbury (Can)	0.0981	0.0466	****

[a]Nei's genetic identity based on 57 loci is above the diagonal, and Nei's genetic distance coefficients are below the diagonal.

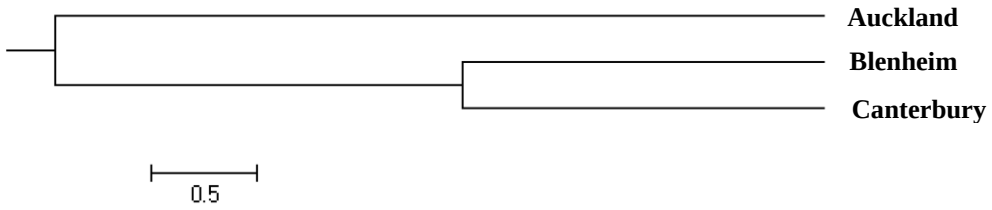

Figure 3.9: UPGMA dendogram showing the genetic relatedness of the *N. australe* populations studies based on Nei's (1973) genetic distance.

3.3.5 Genetic diversity analysis of *Diplodia mutila*

A total of 48 scorable bands were produced with five UP-PCR primers. Like the *Neofusicoccum* species studied, the neighbour joining analysis of *D. mutila* population also showed genetic diversity at inter- and intra-vineyards levels. Eight genetic clusters were identified by an arbitrary line of 1 character change (1/48 x 100= 2% change) from the root of the neighbour joining tree (Figure 3.10). Genetic group 2 had the highest number of isolates (25) with only a single isolate in genetic group 6. The isolates obtained from non-grapevine hosts were also spread throughout the clusters and were not genetically distinct from grapevine isolates. The international isolates from Australia and California also grouped with New Zealand isolates. The results showed these populations were genetically similar.

The mean genetic diversity of the *D. mutila* population was calculated as 0.2347. The genetic variability was highest (0.2165) in the Blenheim population and lowest (0.1204) in the Californian isolates (Table 3.10). The Nei's measures of genetic identity and genetic distance analysis showed that the Gisborne and Blenheim populations were genetically more similar to each other than to the other populations studied. Also the Australian and non-grapevine isolates (New Zealand) were genetically more related than to the others (Table 3.11 & Figure 3.11).

Table 3.10: The percentage of polymorphic loci, mean genetic diversity (*H*) and Shannon diversity index (*I*) of *D. mutila* populations

Population	Sample size	% polymorphic loci	H^a	I^b
Gisborne	6	47.92	0.1863	0.2745
Nelson	5	43.75	0.1633	0.2441
Blenheim	22	72.92	0.2165	0.3341
Canterbury	3	29.17	0.1296	0.1856
Australian isolates	5	54.17	0.2133	0.3142
Californian isolates	3	27.08	0.1204	0.1724
Non-grapevine	4	39.58	0.1589	0.2335
Total population	48	89.58	0.2347	0.3722

[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 *D. mutila* populations

Population	Gis	Nel	Ble	Can	Aus	Cal	NGr
Gisborne (Gis)	[a]****	0.9334	0.9650	0.9036	0.9470	0.9207	0.9129
Nelson (Nel)	0.0689	****	0.9275	0.8903	0.9132	0.8759	0.9002
Blenheim (Ble)	0.0356	0.0753	****	0.9071	0.9507	0.9403	0.9122
Canterbury (Can)	0.1014	0.1162	0.0975	****	0.8896	0.8730	0.8299
Australia (Aus)	0.0545	0.0908	0.0505	0.1170	****	0.9216	0.9438
California (Cal)	0.0826	0.1325	0.0615	0.1358	0.0816	****	0.8416
Non-grapevine (NGr)	0.0912	0.1051	0.0919	0.1865	0.0579	0.1724	****

[a]Nei's genetic identity based on 48 loci is above the diagonal, and Nei's genetic distance coefficients are below the diagonal.

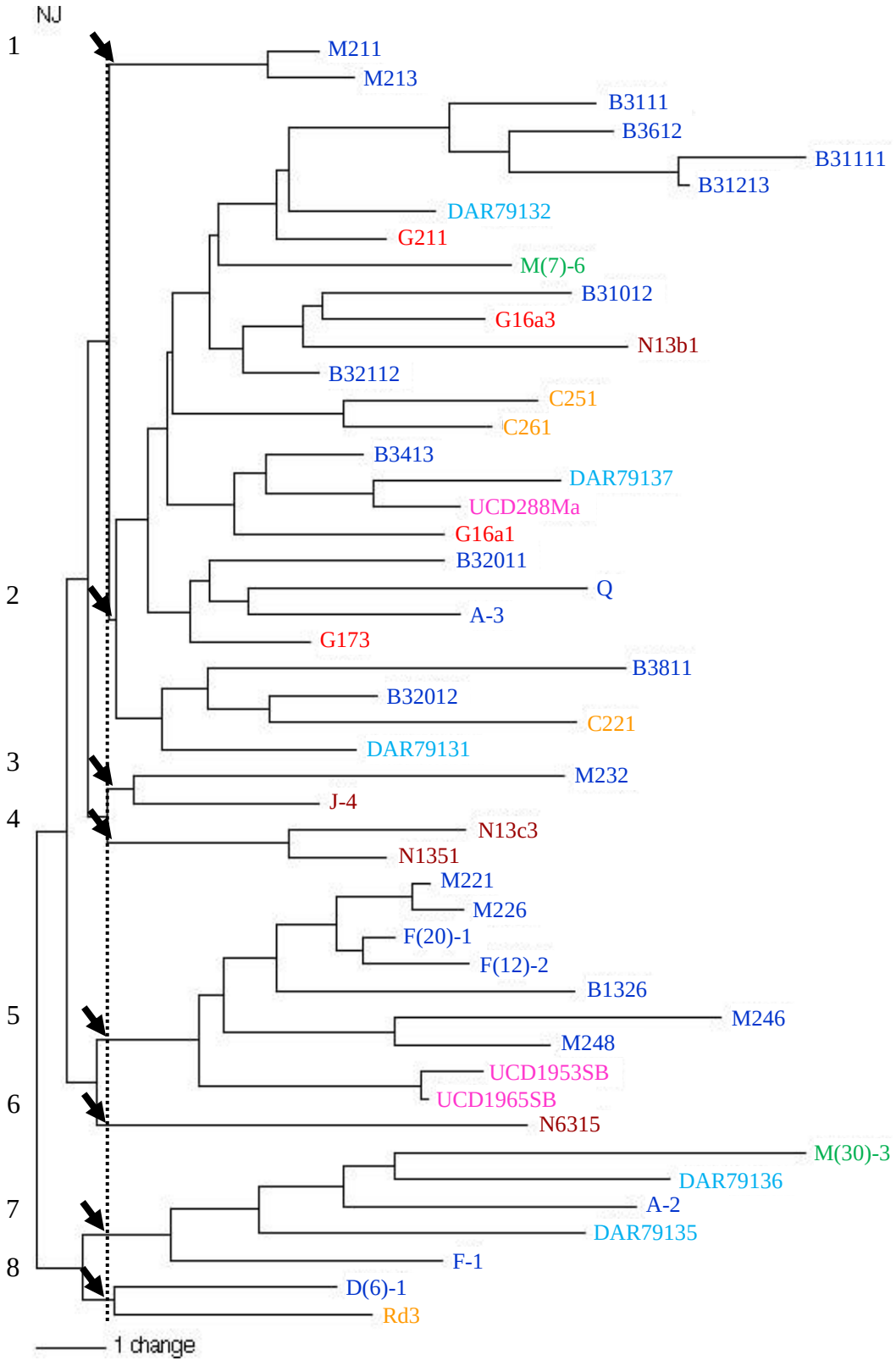

Figure 3.10: Neighbour joining tree based on DNA fingerprints generated with five UP-PCR primers of the *D. mutila* isolates collected from different regions in New Zealand. International isolates from Australia (DAR) and USA (UCD) also included in this analysis. The arbitrary line (···) drawn at 1 changes from the root of the tree is used to define genetic groups (1-8).

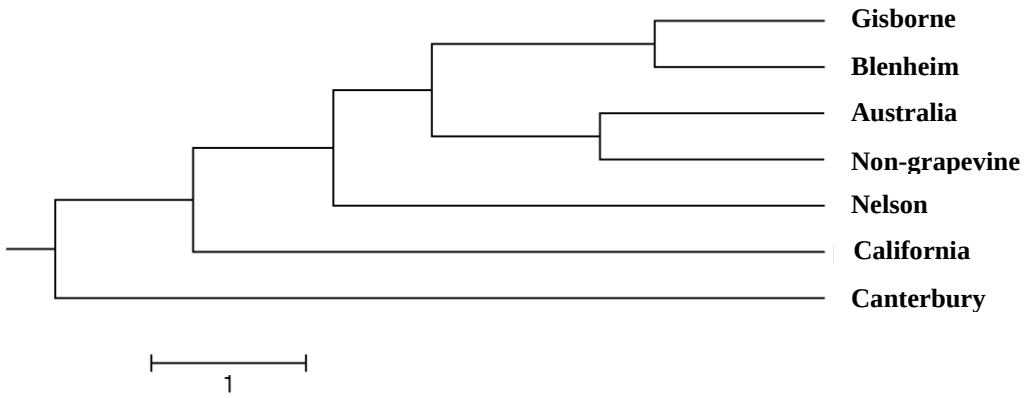

Figure 3.11: UPGMA dendogram showing the genetic relatedness of the *D. mutila* populations studies based on Nei's (1973) genetic distance.

3.3.6 Vegetative compatibility test

The 11 isolates of *N. parvum* paired on PDA plates produced a large amount of mycelial growth, and no clear interaction zones were observed due to the density of mycelium at the interaction zone. In contrast, a clear reaction between the *N. parvum* isolates pairs was produced on ½PDA and on PA. Formation of pycnidia was observed on both sides of the interaction zone in 22% (12/55) of the paired isolates plated on PA. Microscopic examination of the pycnidia showed that only asexual conidia and no sexual reproductive structures or spores were present. Based on this result, ½PDA medium was selected to analyse the vegetative compatibility groups of *N. parvum* and *D. mutila* isolates.

3.3.6.1 Vegetative compatibility groups of Neofusicoccum parvum *isolates*

Amongst the 11 *N. parvum* isolates paired on ½PDA, none showed a clear "barrage-like" reaction at the interaction zone. Compare to the self versus self pairing of isolates (Figure 3.12A), the self versus non-self pairings produced three different types of reactions at the interaction zone (Table 3.12). The Type A reaction had no black line observed at the interaction zone when observed from the reverse side of the Petri dish and no mycelial separation observed on the upper surface (Figure 3.12B). The Type A reaction was very similar to the self-self pairing reaction and differences appeared to be due to pigmentation patterns, therefore this group was considered as a vegetatively compatible group. The Type B reaction had a faint and often interrupted black line, observed at the interaction zone from the reverse side of the Petri dish, and dense aerial mycelium or a white mycelial line on the upper surface at the interface between the isolates (Figure 3.12C). This interrupted barrage line was distinct and reproducible on repeated paring of isolates. The Type B group isolates were considered as partially

incompatible. The third group, Type C, had a dark black line in the interaction zone when observed from the reverse side of the Petri dish, which was also visible within the aerial mycelium on the upper surface (Figure 3.12D). Isolates that had a Type C reaction were considered to be vegetatively incompatible.

Table 3.12: Vegetative compatibility reactions of *Neofusicoccum parvum* isolate pairs. The compatible (C), partially incompatible (PI) and incompatible (IC) reaction of all the possible combination of isolate pairs is shown.

		N. parvum isolates										
Genetic Group	*N. parvum* isolates	G22a3	G69a1	A75a1	A122	B2141	A842	MM562	G652	A421	A931	A813
1	G22a3		PI	PI	PI	PI	IC	C	PI	PI	PI	PI
1	G69a1			PI	PI	IC	PI	C	PI	PI	PI	IC
1	A75a1				PI	PI	IC	C	PI	PI	PI	IC
3	A122					C	PI	C	C	C	PI	IC
3	B2141						C	C	PI	C	C	IC
4	A842							C	C	IC	C	IC
5	MM562								C	C	C	C
5	G652									PI	PI	PI
6	A421										PI	IC
6	A931											C
6	A813											

Most of the isolates paired from the same genetic group showed either a partially incompatible or compatible reaction. For example the three isolates from genetic group 1 showed partially incompatible reactions between them. The incompatible reactions mostly resulted from pairing of isolates of different genetic groups, except with isolates A421 and A813 which both belonged to genetic group 6 but showed an incompatible reaction. Although the partial incompatibility and incompatibility groups had several apparent differences the allocation of isolates into VCGs required them to be designated as ‘compatible’ or ‘incompatible’. Therefore, these incompatibility types were merged into one type, and by doing so reanalysis of the results produced four vegetative compatibility groups (VCGs). The isolate MM562 showed a compatible reaction with all other isolates. Two isolates from VCG 1 overlapped with VCG 2 and a single isolate overlapped with VCG 3 (Figure 3.13). All the isolates from VCG 2 either overlapped with VCG 1 or with VCG 3. A single isolate from VCG 1 and three isolates from VCG 3 did not overlap with any other VCG. The VCG 4 had only 2 isolates, of which the isolate G22a3 did not overlap with any other VCG (Figure 3.13). Although some of the isolates selected for this experiment were from the same genetic group, they produced different types of incompatibility reactions. This result indicates that the vegetative compatibility groups of *N. parvum* did not correlate with the genetic groups identified in the neighbour joining analysis.

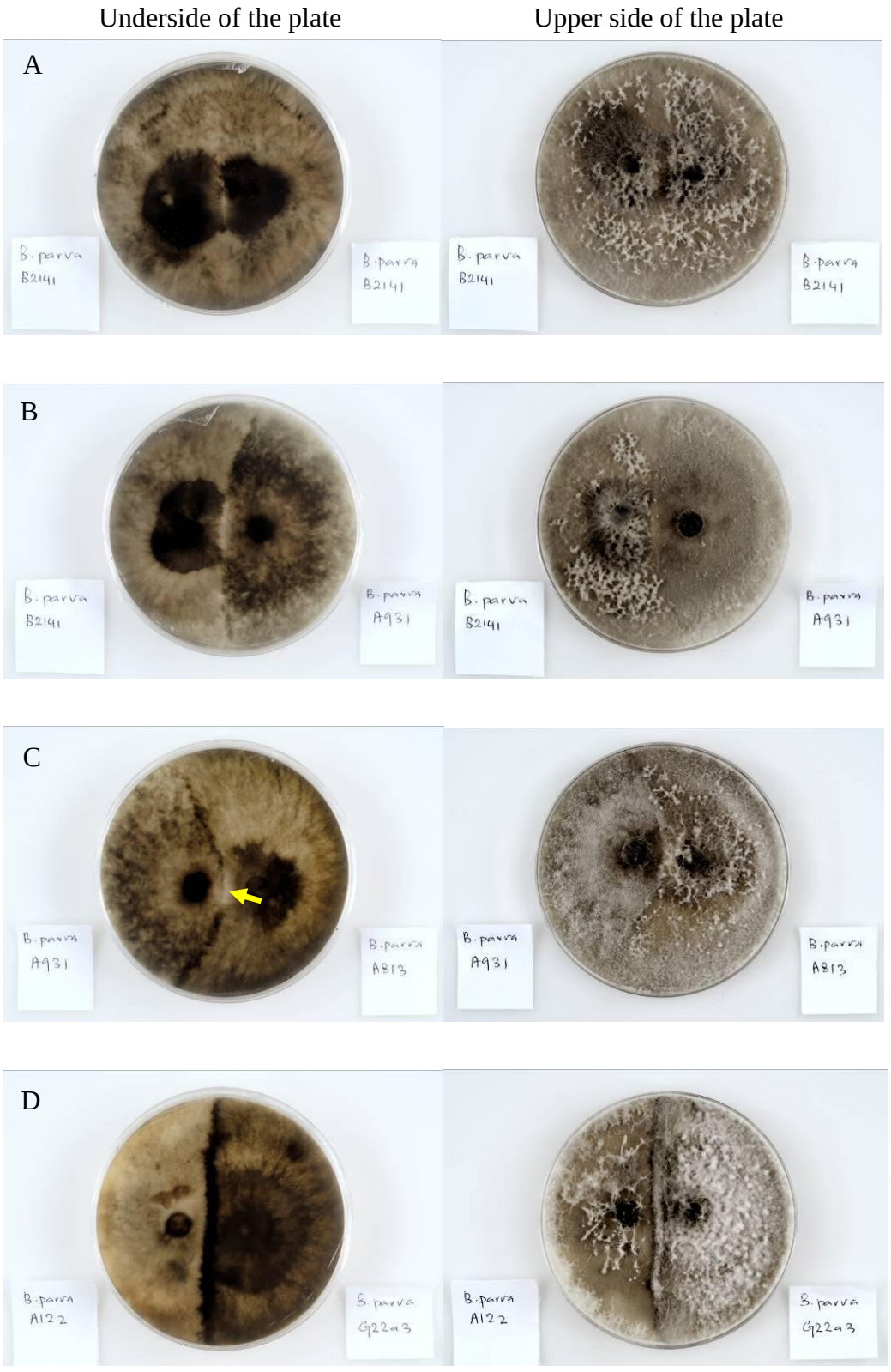

Figure 3.12: Types of vegetative compatibility reactions produced by pairing *N. parvum* isolates. A) Self- self pairing of isolate B2141, B) compatible reaction (Type A) between isolates B2141 and A931, C) partially incompatible reaction (Type B) between isolates A931 and A813 with imperfect barrage reaction line shown by arrow and D) incompatible reaction (Type C) between isolates A122 and G22a3.

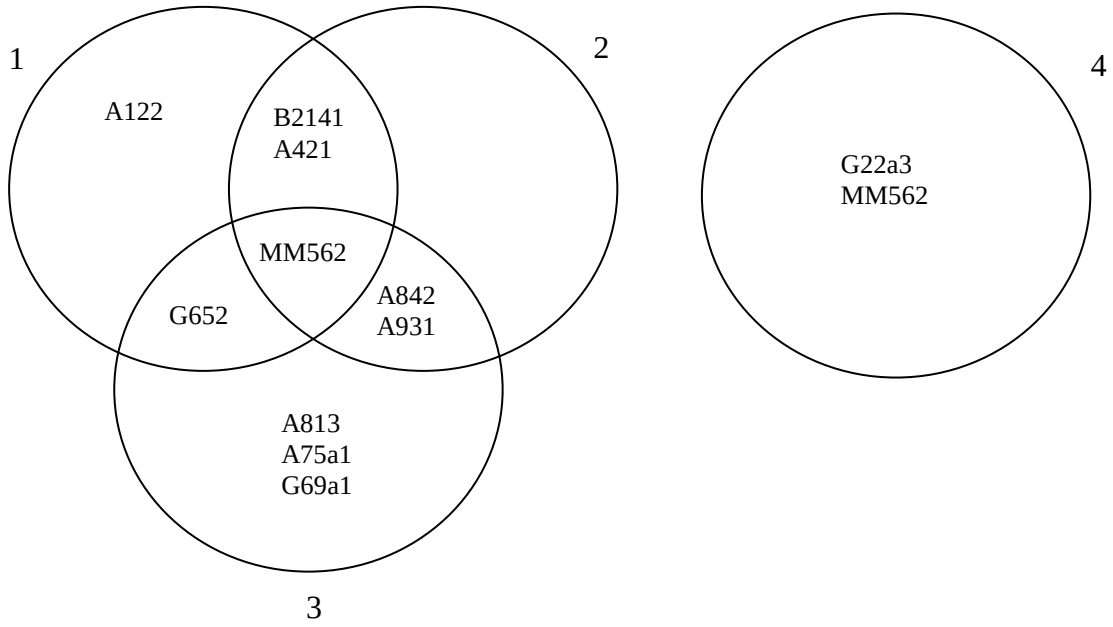

Figure 3.13: The isolates belonging to four vegetative compatibility groups of *N. parvum* indicated in circles. The isolates in the intersection of the circles are common to the respective vegetative compatibility groups.

3.3.6.2 Vegetative compatibility groups of Diplodia mutila *isolates*

Among the fourteen *D. mutila* isolates paired, clear vegetatively compatible and incompatible reactions were observed. Vegetatively compatible reactions were obtained for most of the isolates paired and only 28% pairs (26 out of 91) produced incompatible reactions (Table 3.13). Incompatible reactions were characterized by the formation of clear zones between the growing edges of paired isolates (Figure 3.14A) whereas in compatible reactions the growing edges of paired isolates merged completely and no reaction zone was observed (Figure 3.14B).

Among the fourteen *D. mutila* isolates, three VCGs were identified. Out of the 14 isolates, 8 belonged to all three VCG, creating a large amount of overlap (Figure 3.15). Like the *N. parvum* population, the compatible and incompatible reactions occurred between the isolates of the same genetic group. The results showed that the genetic groups from neighbour joining analysis did not correlate to the vegetative compatibility groups.

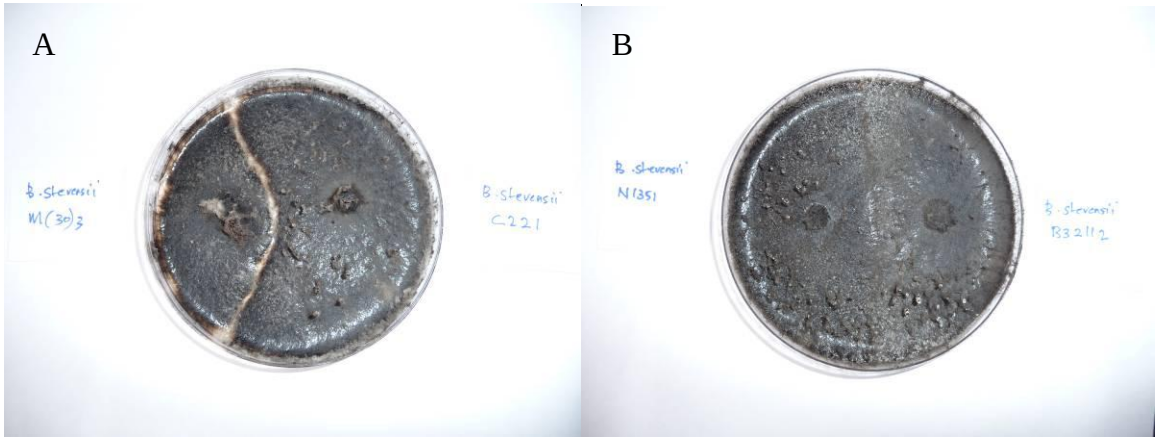

Figure 3.14: Vegetatively incompatible (A) and compatible (B) reactions formed in *Dioplodia mutila* isolate interactions

Table 3.13: Vegetative compatibility reactions of *Diplodia mutila* isolate pairs. The vegetatively compatible reaction (C) and vegetatively incompatible (IC) are presented for all possible combination of isolates

		D. mutila isolates													
Genetic group	*D. mutila* isolates	G173	Q	B32112	C221	B3612	G211	B32012	M232	J-4	N1351	F(12)2	N6315	M(30)3	D(6)1
2	G173		C	C	IC	C	C	C	IC	C	C	IC	C	IC	C
2	Q			C	C	C	C	C	C	C	C	C	C	C	IC
2	B32112				C	IC	IC	C	C	C	C	C	C	IC	IC
2	C221					IC	C	C	IC	C	C	IC	C	IC	IC
2	B3612						IC	C	IC	C	C	C	C	IC	C
2	G211							C	C	C	C	C	C	IC	IC
2	B32012								IC	C	C	C	C	C	C
3	M232									C	C	C	IC	IC	C
3	J-4										C	C	C	C	C
4	N1351											C	C	C	IC
5	F(12)2												C	IC	C
6	N6315													IC	C
7	M(30)3														IC
8	D(6)1														

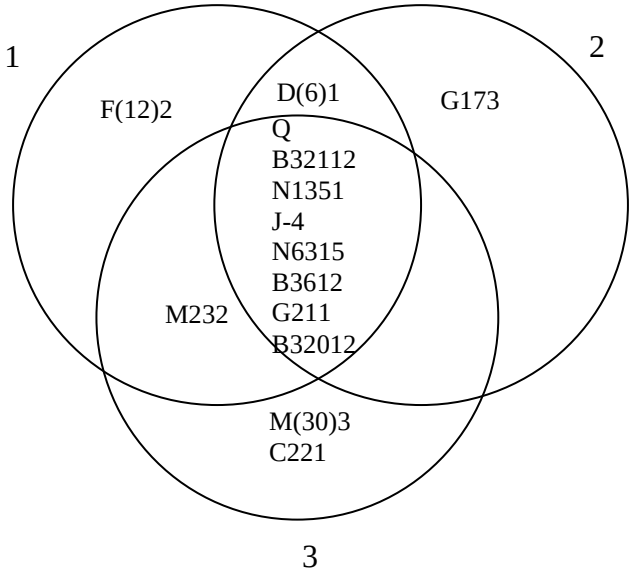

Figure 3.15: The isolates belonging to three vegetative compatibility groups of *D. mutila* indicated in circles. The isolates in the intersection of the circles are common to the respective vegetative compatibility groups.

3.3.6.3 Microscopic examination of vegetative compatibility reactions of N. parvum *and* D. mutila *isolates*

Hyphal fusions (anastomoses) were observed in self-self and non-self pairing of *N. parvum* isolates. In self-self pairing, hyphae growing from both PDA cubes on each side of the slides merged. Anastomoses were mostly observed within the interior of the colony close to the PDA cubes. Different types of anastomoses were observed, including the fusion of growing hyphal tips and formation of hyphal pegs (Figure 3.16A). Most of the anastomoses were formed by hyphal peg fusion (Figure 3.16B). Different lengths of the hyphal pegs were observed depending on the distance between the two growing hyphae. Hyphal wall to wall fusion was observed when the two hyphae grew very close (Figure 3.16C). The number of anastomoses events observed was high in older hyphae, but with only a single anastomosis observed per hyphal compartment. Only on a few occasions were the growing hyphae from both colonies thickly merged in the middle of the slide.

In self versus self pairing of isolates, many anastomoses were observed, with almost all hyphae in close contact or crossing over each other. It was not possible to confirm the identity of the individual hyphae from each colony as they formed a hyphal network. Only in a few vegetatively compatible reactions, hyphae growing from both isolates were merged and many anastomoses were observed behind the merge line (Figure 3.16D). Only two partially compatible isolate pairs were observed to have merging hyphae, and the anastomoses were only observed within each colony rather than at the interaction zone. For only a few of the non-self paired isolates, were the growing hyphae merged. No hyphal merging was observed in incompatible reactions. In a few cases, hyphal death and

malformation of leading hyphae was observed in both partially compatible and incompatible reactions (Figure 3.16E). Occasionally, hyphal compartment death was observed after the formation of anastomoses, in which only the anastomosed compartment was alive and the other compartments had died. This phenomenon was mostly observed with partially compatible reactions (Figure 3.16F).

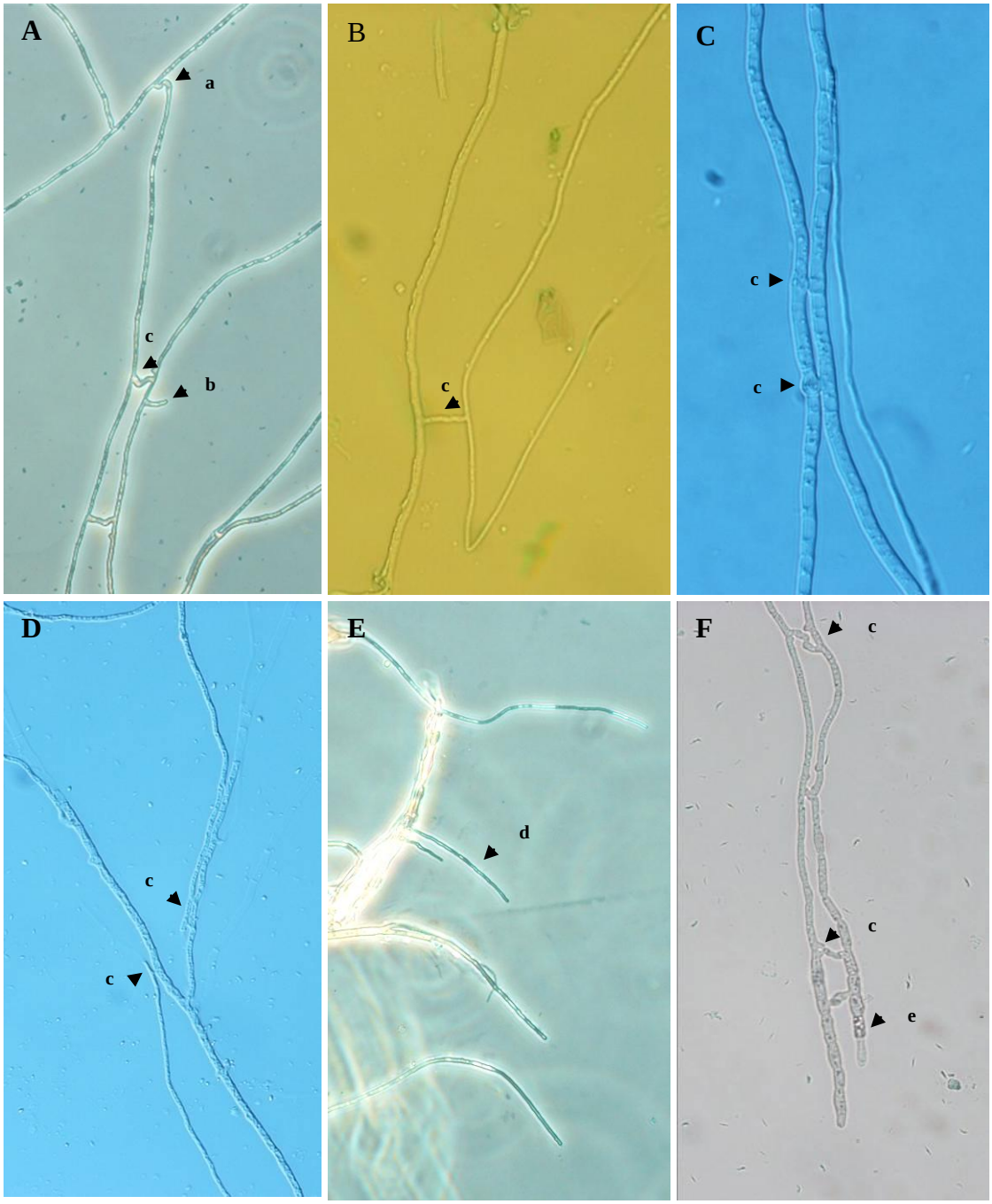

Figure 3.16: Microscopic examination of vegetative compatibility reactions of *N. parvum*. A) Anastomosis of *N. parvum* isolate A75a1 showing the hyphal tip fusion (a), formation of hyphal peg (b) and hyphal peg fusion (c), B) anastomosis of hyphae in *N. parvum* isolate G22a3 paired with A122, C) anastomosis in very close hyphae of G22a3 paired with G69a1, D) multiple hyphal fusion in A122 paired with B2141, E) dead hyphal tips (d) in A75a1 paired with G69a1 and F) malformed growing hyphal tips in G69a1 paired with B2141.

The microscopic examination of self versus self and non-self isolate pairs of *D. mutila* showed formation of anastomoses with compatible and incompatible reactions which was similar to *N. parvum* isolates. However, formation of hyphal pegs were more frequent in *D. mutila* isolates (Figure 3.17A). On a few occasions two hyphal pegs from one hyphae was fused with a single hyphal peg from another hyphae (Figure 3.17B). The length of the hyphal pegs was dependent on the distance between the hyphal pegs or mycelium. Like *N. parvum* isolates, anastomosis was formed by fusion of hyphal pegs or fusion of a hyphal peg to the compartment wall of the other hyphae. Multiple point anastomosis was observed in many cases (Figure 3.17C). Single anastomosis was observed per compartment, at the ends or the middles of the compartment (Figure 3.17D).

In compatible interactions, *D. mutila* isolates merged in the centres of the slides. However, the anastomoses were mostly observed within each colony. Due to the excessive mycelial growth, it was difficult to confirm the anastomoses between the isolates. In incompatible interactions no hyphal merging was observed but formation of malformed hyphal structures were observed. No dead leading hyphae were observed and in few cases hyphal coiling was also observed. In incompatible interactions there were higher numbers of self-anastomosis within each isolates.

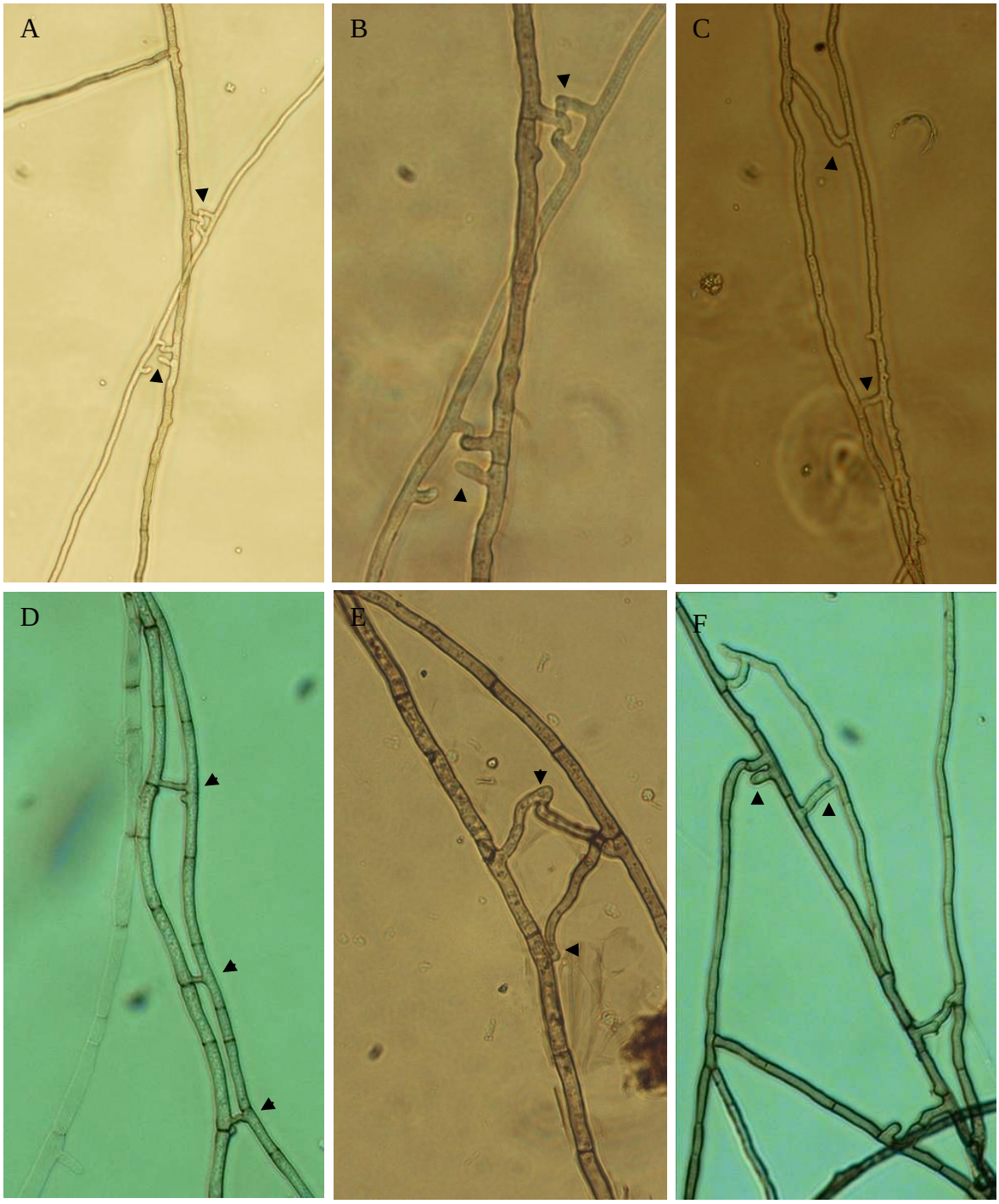

Figure 3.17: Microscopic examination of vegetative compatibility reactions of *D. mutila*. A) Hyphal peg formation and fusion in isolate M(30)3 paired with C221, B) higher magnification of picture A showing the peg fusion (anastomosis), C) formation of multiple anastomoses in isolate M232 paired with G173, D) formation of single anastomosis per cell in isolate M(30)3 paired with N6315, E) anastomoses of hyphal peg fusion and peg fusion to hyphal wall in isolate M(30)3 paired with C221 and F) multiple anastomoses in isolate M(30)3 paired with C221. Hyphal pegs and the anastomosis are indicated by arrows.

3.4 Discussion

The aim of this study was to analyse genetic diversity within populations of *N. parvum, N. luteum, N. australe* and *D. mutila* found in New Zealand vineyards. This is the first study to assess genetic diversity of botryosphaeriaceous species using the UP-PCR method. The results showed there were different genetic groups in the New Zealand populations. Genetically different individuals were observed within a lesion, within a vineyard and between vineyards. The genetic variation analysis also showed the New Zealand botryosphaeriaceous species population had some genetic similarity to Australian, South African and Californian isolates. In the following sections the results for the *N. parvum* population will be discussed in detail and then compared to the genetic variability observed for *N. luteum, N. australe* and *D. mutila*.

This was the first study reporting the genetic diversity of a *N. parvum* population. The UP-PCR method has been used extensively to study the genetic variability of many fungi including another grapevine trunk pathogen, *Phaeomoniella chlamydospora,* in New Zealand (Pottinger *et al*., 2002). All 11 UP-PCR primers produced different number of bands with the range of 8 – 17 in *N. parvum* isolates and the percentage of polymorphic bands produced with all primers ranged from 14 – 50%. This is different from the results for *P. chlamydospora* obtained by Pottinger *et al*. (2002) as five out of nine UP-PCR primers they used did not produce polymorphic bands in *P. chlamydospora*. In another New Zealand study of the olive leaf spot pathogen *Spilocaea oleagina*, using five UP-PCR primers, all produced polymorphic bands (Obanor *et al*., 2010). The production of polymorphic bands by UP-PCR indicates that it is an appropriate method to study the genetic diversity of botryosphaeriaceous species.

The UP-PCR method produced polymorphic bands that were easily scored. It was also rapid and cost effective; however, another method may have produced more information. It has been demonstrated that use of different molecular techniques for genetic variation analysis can show different levels of genetic diversity within the same population (Steele *et al*., 2001; Tooley *at al*., 2000). For example, a greater number of genotypes were identified using the AFLP method, which produced greater number of polymorphic bands than the UP-PCR method in a genetic variation analysis of *P. chlamydospora* in New Zealand (Pottinger *et al*., 2002). In another study six genotypes were identified in 51 New Zealand isolates of *Sclerotium cepivorum* using the combined data from UP-PCR and RAPD, whereas, the data from each method individually showed very little genetic diversity (Tyson *et al*., 2002).

Using the UP-PCR method, the *N. parvum* population found in New Zealand vineyards showed a high level of genetic diversity (Nei's genetic diversity H=0.2581) compared to the genetic variability reported for other botryosphaeriaceous species (Ma *et al*., 2004; Burgess *et al*., 2004). This result was unexpected since sexual reproduction has been reported very rarely (Denman *et al*., 2000) and predominant asexual reproduction was expected to result in a high number of clonal populations and

limited genetic diversity. The large number of different genotypes (93% of individuals were unique) in the *N. parvum* population is in contrast to the results reported for the genetic diversity analysis of *B. dothidea* (378 isolates), isolated from pistachio in California (Ma *et al*., 2004). This population was analysed using six microsatellite primers and showed a low level of genetic diversity (>98% identity) with large numbers of clonal isolates. In another genetic diversity study which included New Zealand isolates of *Diplodia pinea* that used the simple sequence repeat (SSR) method, low allelic diversity was observed both regionally and also between countries (Burgess *et al*., 2004). However, similar to *N. parvum,* high levels of genetic diversity were found in a small population of *L. theobromae* (8 isolates) obtained from *Eucalyptus* and from different fruits and vegetables studied with eight RAPD primers in Brazil (Saldanha *et al*., 2007). The genetic diversity of the New Zealand *N. parvum* population (H=0.2581) was higher than the genetic diversity of the New Zealand *S. oleagina* population (H=0.1322) using UP-PCR method, which is causal agent of olive leaf spot, (Obanor *et al*., 2010) and the genetic diversity analysis using ISSR method of the Spanish population of the grapevine black foot causing fungi *Cylindrocarpon liriodendri* (H=0.06) and *C. macrodidymum* (H=0.16) (Alaniz *et al*., 2009). However, this result is similar to the genetic diversity (H=0.337) of the Chinese population of the brown rot causing fungus *Monilinia fructicola,* which is also reported to have uncommon sexual reproduction (Fan *et al*., 2010). The author suggested the reason for high genetic variability of *Ma. fructicola* was that this species could have been introduced to China from different countries and had existed in China for a long time period. These results from a diverse range of fungi show that the genetic diversity of fungal species with predominantly asexual reproduction can be variable.

The eight UP-PCR primers distinguished 56 genotypes (branches) from 60 *N. parvum* isolates studied which, for ease of analysis, were grouped into seven genetic groups with 97% (2.5 changes out of 82 loci) similarity between the groups. However, four of these genotypes were represented by only two isolates in each. The low number of clonal isolates was also unexpected in the *N. parvum* population as other asexually reproducing fungi frequently have populations with many clonal isolates (Pottinger *et al*., 2002). For example analysis of 39 isolates of *P. chlamydospora* produced nine branches on the neighbour joining tree and six of them consisted of a large number of clonal isolates that varied in number from 3 to 10 (Pottinger *et al*., 2002). In contrast, analysis of *Ciborinia camelliae,* which reproduces only by sexual means, used six UP-PCR primers and produced a result more similar to the current study of 33 genotypes from the 36 isolates tested (van Toor *et al*., 2005). However, analysis using ISSR markers showed that genetic diversity was high in 164 isolates of *Ma. fructicola* with no clonal isolates (Fan *et al*., 2010), which is similar to the results obtained in *N. parvum* population. The large number of genotypes in the *N. parvum* population suggests that there is an active method of recombination in this species.

Genetic diversity analysis of the *N. parvum* population in New Zealand indicated the presence of more than one genotype within a vineyard. Also the results showed variable levels of genetic diversity in *N. parvum* sub-populations from different regions in New Zealand. Similar to this result, inter- and intra-vineyard genetic diversity was found in *P. chlamydospora* populations in New Zealand (Pottenger *et al*., 2002). The genetic variation analysis of *S. oleagina* populations in olive also showed different levels of genetic diversity within and between regions in New Zealand (Obanor *et al*., 2010). Among the sub-population in New Zealand, greater genetic diversity was found in Auckland (H=0.2488) and Gisborne (H=0.2019) than Nelson (H=0.1082) and Blenheim (H=0.1905). This result was likely to be influenced by the population size as 62% of the *N. parvum* isolates were obtained from Auckland and Gisborne regions and only 15% of the *N. parvum* isolates from the Nelson and Blenheim regions. Also the higher genetic diversity in Auckland and Gisborne could be due to the occurrence of multiple introduction of *N. parvum* over a long period of time into these regions in New Zealand. A similar explanation was made for the higher genetic diversity of *S. sapinea* on pine in South Africa (Smith *et al*., 2000) and *Ma. fructicola* on stone fruit in China (Fan *et al*., 2010).

The inter- and intra-vineyard genetic diversity in the *N. parvum* population indicates the possibility that there were multiple introductions of this pathogen into New Zealand vineyards. Similar to this result the *P. chlamydospora* population showed inter-vineyard and intra-vine genetic variability in New Zealand. This diversity could be due to multiple introductions associated with infected grapevine or non-grapevine planting materials. This is supported by a study conducted by Pennycook & Samuels (1985) in which botryosphaeriaceous species were isolated from introduced hosts such as *Malus*, *Populus* and *Actinidia* species. The *N. parvum* isolates from Australia (n=4) and California (USA; n=3) dispersed in the neighbour joining tree and grouped with New Zealand isolates suggesting these are genetically similar. This was reinforced by Nei's genetic distance analysis, in which they were most similar populations to those in New Zealand. However, the four South African *N. parvum* isolates grouped in a separate branch and were less similar to the New Zealand population. This suggests the *N. parvum* population may have introduced into New Zealand from Australia and/or California (USA). Similar to the results of the current study, similar genotypes were found in the *P. chlamydospora* populations in New Zealand and Italy which suggests the movement of this fungal species between these two countries. However, the only international isolates included in this study were from Italy (Pottinger *et al*., 2002). Burgess *et al*. (2004) stated that *Pinus radiata* planting materials (seeds and cuttings) had been exchanged between Australia, South Africa and New Zealand and that it was likely that *D. pinea* had been distributed with these planting materials between these countries. Similar to this example other botryosphaeriaceous species could be introduced to New Zealand, however, a larger number of isolates from Australia, California and other grape-growing countries should be tested to identify the most likely source of New Zealand isolates of botryosphaeriaceous species.

The dispersal of *N. parvum* genotypes throughout different regions demonstrates the free movement of this fungal species between the regions in New Zealand. The possibility of nurseries being an infection source was supported by a nursery survey that showed the presence of botryosphaeriaceous species in non-symptomatic grapevine propagation materials (Billones *et al*., 2009). Thus, multiple introductions into a vineyard could occur through the grapevine propagation materials originating from different nurseries. In addition, it is possible that there is introduction of genetically distinct isolates of *N. parvum* species into a vineyard from neighbouring non-grapevine hosts. This is supported by the isolation of botryosphaeriaceous species from other fruit trees and vines in New Zealand (Laundon, 1973; Pennycook & Samuels, 1985). In a recent survey botryosphaeriaceous species were also isolated from non-grapevine hosts including apple, plum, cherry, lemon, pine, oak, broom and willow in New Zealand (Amponsah *et al*., 2008). Ma *et al.* (2001) studied *B. dothidea* populations from pistachio and reported that other host species may serve as sources of inoculum and increase genetic diversity. Isolates from grapevine have also been shown to be pathogenic on other hosts such as blueberry and eucalyptus (Che Omar pers comm.2009; Billones *et al*., 2010). This suggests that cross infection of genetically distinct *N. parvum* isolates from infected orchard trees or other woody plants may occur to vineyards that are located in close proximity.

This research demonstrated that genetically distinct *N. parvum* isolates can be present in a grapevine lesion and these isolates were located in separate branches of the neighbour joining (NJ) tree. These isolates may have either originated from multiple infections of the wound by genetically distinct isolates of *N. parvum* species or been generated by recombination mechanisms occurring within the lesion. There are different mechanisms proposed for the dispersal of botryosphaeriaceous species in the field. The conidia of *B. dothidea* had been reported to be dispersed mainly by rainwater splash, whereas, the ascospores were dispersed by both air and water splash (Sutton, 1981). Other researchers have suggested that, for botryosphaeriaceous species in vineyards, rain splash plays an important role in liberating conidia from the gelatinous exudation from pycnidia into aerosols, which are subsequently wind dispersed (van Niekerk *et al*., 2010). Although there was no previous report available on how far the botryosphaeriaceous species spores can be dispersed by this mechanism the work in this thesis measured the distance of rainwater splash dispersal of *N. parvum* and *N. luteum* up to 2 m from a single inoculation point (Section 5.3.5.1). Long distance dispersal of botryosphaeriaceous species spores is unlikely to occur by this mechanism. However, other factors such as wind assistance of the splashed droplets may increase dispersal distance. The reproductive mechanisms of *N. parvum* that produce the higher genetic diversity observed when compared to other predominantly asexual grapevine pathogens, such as *P. chlamydospora* and *Cylindrocarpon* spp. is unknown (Pottinger *et al*., 2002; Alaniz *et al*., 2009). However, parasexual recombination could play a role in development of genetic diversity of *N. parvum* isolates within a lesion. Both of these potential mechanisms could explain multiple isolates within a lesion but further work is required to clarify which is most likely.

The potential of sexual reproduction contributing to the genetic diversity of *N. parvum* isolates is supported by a study of *D. scrobiculata* populations, which showed that even though no sexual stage has been found, evidence of sexual recombination was revealed in multi locus DNA analysis (Burgess *et al*., 2004). Similar analysis of *N. parvum* populations from *Eucalyptus* confirmed that, despite widespread asexual reproduction, sexual out-crossing does occurs at low frequencies (Slippers & Wingfield, 2007). In this study no sexual reproductive structures were identified for any of the botryosphaeriaceous species including *N. parvum*. The failure to detect sexual reproductive structures in the vineyards could be because sexual reproduction occurs either in a particular season of the year or in a non-grapevine host. For *Ma. fructicola*, genetic variation analysis showed that high genetic diversity was found in the blossom blight phase and low diversity during the fruit rot phase which was explained by the population establishment in spring from ascospores and subsequent spread by conidia in summer (Scherm & Emery, 2003). The possibility that sexual reproduction of botryosphaeriaceous species occurs on other hosts is supported by a genetic diversity analysis conducted on a *B. dothidea* population from pistachio, which showed the isolates obtained from other hosts including blackberry, eucalyptus, walnut and willow, in which a low frequency of the sexual stage had been observed, had higher genetic diversity when compared to isolates obtained from Pistachio (Ma *et al*., 2001). Milgroom (1996) stated that relatively low frequencies of sexual reproduction should be sufficient to cause appreciable genotypic variability in a fungal population. To confirm the possibility of sexual reproduction in New Zealand more careful observations need to be made in year round surveys of non-grapevine and grapevine hosts.

Alternatively, other reproductive mechanisms, such as frequent parasexual recombination, may be occurring in the *N. parvum* population to produce this genetically diverse population. Parasexual recombination has been proven to play a major role in the genetic diversity of a fungal species which mainly reproduce asexually (Zeigler, 1998). For example, parasexual recombination was reported to have a major impact on the genetic structure of *Magnaporthe grisea* which is also reported to have a predominantly asexual mode of reproduction (Zeigler *et al*., 1997). However, no information is available on parasexual recombination in botryosphaeriaceous species. This could be investigated by analysing the genetic variability of the isolates obtained from the interaction zone of paired *N. parvum* isolates by producing hyphal tip cultures as demonstrated for *Me. grisea* (Zeigler *et al*., 1997). Parasexual recombination only occurs between members of the same VCG and therefore determination of VCGs in a fungal population is necessary prior to investigating the potential for parasexuality (Leslie, 1993). Development of heterokaryons may also lead to greater genetic diversity, therefore, in this research a subset of the *N. parvum* population was investigated for VCGs.

The VCG study of a subset of *N. parvum* isolates showed three different types of reactions, compatible, partially incompatible and incompatible. The vegetative compatibility reaction allows the

transfer of genetic material through exchange of nuclei and therefore important for generating genetic diversity. This result was different from VCG tests conducted on other botryosphaeriaceous species, including *B. dothidea* (Ma *et al*., 2003), *Sphaeropsis sapinea* (Smith *et al*., 2000) and *Neofussicoccum* species (Sánchez-Hernández *et al*., 2002), as these studies all reported either a compatible reaction with growing hyphae merging together or an incompatible reaction with a dark reaction line at the colony intersection. However, a similar type of partial incompatibility reaction to that observed in *N. parvum* was shown in isolates of *Me. grisea,* which produced weak barrage-like reactions to some extent but no clear incompatibility reactions. The authors concluded that the vegetative incompatibility system of this species was weak (Zeigler *et al*., 1997) and they showed evidence of parasexual recombination between the isolates of *Me. grisea* with weak barrage-like reactions. Thus it is possible that the partial incompatibility reaction in *N. parvum* isolates may facilitate parasexual recombination. Tyson *et al.* (2002) also reported the different appearance of incompatible reactions observed between *S. cepivorum* isolates, with faint to dark reaction lines, and explained that the isolate pairs with greater genetic differences produced stronger incompatible reactions than genetically more similar isolate pairs. This could explain the results obtained in the current study with different types of incompatible reactions produced by pairing *N. parvum* isolates selected from unique genotypes with different genetic distances based on the NJ analysis.

To facilitate the identification of the VCGs among the 11 *N. parvum* isolates tested, the partially incompatible and incompatible reactions were grouped together, resulting in four VCG's. Variable numbers of VCGs have been reported from other studies in different botryosphaeriaceous species. In a previous study, 14 VCGs of *Neofusicoccum* species were identified on a single leaf of a *Eucalyptus* plant where all the isolates were obtained from individual lesions (Slippers &Wingfield, 2007). Among 390 *B. dothidea* isolates from pistachio, 20 VCGs were identified and 94% of the isolates assigned into one of two VCGs (Ma *et al*., 2003). In another study on *S. sapinea* 62 VCGs were identified in 107 isolates from three plantations in South Africa and four VCGs among 83 isolates from three plantations in Northern Sumatra (Smith *et al*., 2000). This shows that the number of VCGs found in a population varies considerably, and may depend on species, host and location. Unlike other botryosphaeriaceous species studies, some of the *N. parvum* isolates belonging to different VCGs overlapped, which may result in enhanced formation of anastomosis between genetically different isolates, leading to hyphal fusion and subsequent gene flow between isolates (Leslie, 1993).

Neofusicoccum parvum isolates belonging to the same genetic group (isolates with ≥97% similarity to each other) in the NJ tree had different vegetative compatibility reactions. For example, in group 6 the *N. parvum* isolate pairs A421-A931 were partially incompatible, A421-A813 incompatible and A931-A813 compatible. However, *N. parvum* isolate A842 belonging to genetic group 4 was compatible with isolates G652 and A931, from genetic groups 5 and 6, respectively. These results show that for *N. parvum* isolates, their position in genetic groups did not indicate position in VCGs. Another study

reported similar results as both compatible and incompatible isolates of *Me. grisea* were identified within the same genetic groups generated from RAPD fingerprints (Rathour *et al*., 2004). In addition, a VCG study conducted with only six *P. chlamydospora* isolates from different genetic groups of a NJ tree were all compatible (Pottinger *et al*., 2002). The lack of such relationship is explained by the genetic basis of fungal vegetative compatibility groups which are regulated by multiple unlinked loci (*vic*) that range in number from six in *Cryphonectria parasitica* (Cortesi & Milgroom, 1998) to 11 in *Neurospora crassa* (Glass *et al*., 2000). Individuals of a fungal species having the same vegetative incompatibility (*vic*) loci can fuse to form a heterokaryon (Glass *et al*., 2000) and are considered to belong to the same VCG. In contrast, isolates that are different at one or more of the *vic* loci will not anastomose and those isolates represent different VCGs. No studies have been done to investigate the number of *vic* loci found in botryosphaeriaceous species. A more extensive study of VCGs in *N. parvum* isolates with molecular characterisation of the *vic* loci may provide information that could help explain the partially incompatible phenotypes observed.

Microscopic examination of VCG reactions of *N. parvum* isolates grown on microscope slides found anastomoses of several different types within the actively growing hyphae of isolates. The main type of anastomosis produced was the fusion of hyphal peg tips, which is typical of filamentous ascomycetes (Glass *et al*., 2000). Unlike the interaction on Petri plates, the leading hyphae from paired isolates grown on the microscopic slides did not usually merge with each other, even in self-self pairings. In only in a few instances where the growing hyphal fronts of the self-self and compatible isolates pairs observed to merge with each other and anastomoses were identified between the isolates by following a single growing hyphae from a colony under the microscope. However, in many cases it was difficult to confirm whether the 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 anastomosis in pairings of *Me. grisea* isolates but could not distinguish whether they were within or between isolates. Anastomoses between isolates of *P. chlamydospora* were studied using a method similar to that used here and the authors reported being able to track the mycelium of the individual colonies by following the growing hyphae (Smetham, 2008). The fast growing nature of *N. parvum* made it difficult to track the individual growing hyphae from a colony. The leading hyphae of incompatible isolates did not merge and in some cases malformation and hyphal death was noticed. 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 *N. parvum* population in New Zealand vineyards.

This was the first study of genetic variation analysis of *N. luteum* and *N. australe*. Similar to *N. parvum*, the 11 UP-PCR primers each produced different numbers of bands, 7- 14 in *N. luteum* and 4-12 in *N. australe*. Unlike the results obtained in *N. parvum*, the UP-PCR primer Fok1 did not produce any bands and the primer L21 did not produce any polymorphic bands in both species. Using five UP-

PCR primers the number of loci produced for *N. luteum* and *N. australe* species was 51 and 57, respectively, but the percentage of polymorphic bands differed considerably being 68.63% and 85.96% in *N. luteum* and in *N. australe,* respectively. The genetic diversity measured in the *N. luteum* population was H=0.1791 with two clonal isolates and in the *N. australe* population it was H=0.2417 with no clonal isolates. This showed that the genetic diversity of the *N. australe* population was greater than for the *N. luteum* population. The genetic diversity measured in these species was within the range of genetic diversity measured in some other species with predominantly asexual reproduction (Alaniz *et al*., 2009; Fan *et al*., 2010). The genetic diversity of the *N. australe* population was similar to *N. parvum* population (H=0.2581). This was unexpected as both of these species are more closely related to each other than to other botryosphaeriaceous species (Slippers *et al*., 2004).

Different numbers of genetic groups were defined in NJ trees; for *N. luteum* seven genetic groups were identified at 98% similarity between the groups whereas for *N. australe* six groups were identified in at 93% similarity. Unlike the *N. luteum* genetic groups which each contained similar numbers of isolates, genetic group 2 of the *N. australe* population accounted for 60% of the total population. This indicated that a large proportion of the *N. australe* population was genetically similar and that introduction of genetically distinct *N. australe* isolates may occur into an existing population.

Inter- and intra-vineyard variability was observed in both species and there appeared to be no geographical affect on the variation of these species within New Zealand vineyards. In a recent survey of New Zealand nurseries *N. luteum* was predominantly isolated (Billones *et al*., 2009), supporting the hypothesis that nurseries are a potential source of this fungus. The non-grapevine *N. luteum* isolates obtained from kiwifruit and apple were located in different genetic groups that also contained grapevine isolates, indicating that they are genetically similar and that cross infection between these hosts is possible. *Neofusicoccum luteum* was first isolated from imported non-grapevine hosts in New Zealand (Pennycook & Samuels, 1985). Thus, multiple introduction of this species could have occurred into New Zealand through introduction of these hosts from different countries. Californian *N. luteum* isolates were the only international population included in this study and since four of these were located in genetic group 1 and one in genetic group 4 it is possible that some New Zealand isolates had originated from California. However, much larger samples of Californian and *N. luteum* isolates from other countries need to be included to investigate whether Californian *N. luteum* isolates are likely to have been introduced into the New Zealand population.

Although *N. australe* has been isolated from grapevines in Australia and California (Úrbez-Torres, 2006a; Pitt *et al*., 2010), this species has only recently been isolated from grapevines in New Zealand (Amponsah *et al*., 2009). The high level of genetic diversity (comparable to *N. parvum*) found in the population suggests that either this species has been resident in New Zealand for some time without being identified or a diverse population of this species has been recently transferred to grapevines

from other hosts. *N. australe* is known to infect many different hosts (Burgess *et al*., 2006), however, isolations of the pathogen from these alternate hosts has not been made in New Zealand according to the Landcare culture collection.

The same explanation for the genetic diversity of *N. parvum* is applicable to these two species, namely, multiple introductions of these species may have occurred and/or that the reproductive mechanism plays a major role. Thus, a similar type of asexual recombination process may be involved in these species to generate the genetically diverse populations identified in New Zealand grapevines.

For *D. mutila* the 11 UP-PCR primers also produced variable numbers of bands ranging from 6–14 with the highest percentage of polymorphic bands obtained with the Fok1 primer. UP-PCR primer AS15inv did not produce any polymorphic bands, which is different from the *Neofusicoccum* group species where 18–33 % of bands generated by this primer were polymorphic. Genetic diversity analysis with five UP-PCR primers produced 48 loci and 89.58 % of the loci were polymorphic with genetic diversity measured as H=0.2347, which is similar to the genetic diversity of *N. parvum* and *N. australe*. A genetic diversity analysis of global populations of *D. pinea*, a pathogen of *Pinus radiata*, using ISSR markers showed different levels of genetic diversity in north-east America (H=0.223), Michigan (H=0.192), Europe (H=0.027), New Zealand (H=0.206), Western Australia (H=0.152) and South Africa (H=0.218) (Burgess *et al*., 2004). The reason for this variability in genetic diversity is unknown but it was suggested to be influenced by multiple introductions of this species in some of these populations. The *D. mutila* diversity was equivalent to that observed for *D. pinea* in America and South Africa. In this study, eight genetic groups of *D. mutila* were identified with 98% similarity between the groups in the NJ tree, however, 52% of the total population was located in genetic group 2 which was similar to the pattern observed in *N. australe*.

Inter- and intra-vineyard genetic variation was observed in the *D. mutila* population which can be explained by the free movement of this species between the nurseries, vineyards and other sources in New Zealand. The non-grapevine *D. mutila* isolates grouped with isolates from grapevine indicating that they were genetically similar. Similarly, the international isolates from Australia and California also grouped with New Zealand isolates which is different from *N. parvum* and *N. luteum* and shows that some might have common source to those in New Zealand. Since the Australian *D. mutila* population was the most similar to the New Zealand population this may indicate the introduction of this species from Australia to New Zealand. However, investigation of a larger number of isolates from Australia is necessary to confirm this.

The vegetative compatibility study of a subset of *D. mutila* isolates selected from different genetic groups in the neighbour joining tree identified three VCGs in 14 isolates. As was shown for *N. parvum*, *D. mutila* isolates belonging to the same genetic group had both compatible and incompatible

reactions. Microscopic analysis of interactions between *D. mutila* isolates also showed that there were frequent anastomoses. Given this observation the same hypotheses could be suggested for the observed high genetic diversity, namely multiple introduction of this species into New Zealand and/or active recombination in this species.

In summary, the genetic diversities of populations of four botryosphaeriaceous species, namely, *N. parvum, N. luteum, N. australe* and *D. mutila* in New Zealand vineyards were generally higher than for other grapevine pathogens such as *Cylindrocarpon* spp. and *P. chlamydospora* which appeared to have similarly asexual reproduction strategies. The genetic diversities of these botryosphaeriaceous species were more similar to asexually reproducing pathogens such as *Ma. fruticola*. Mechanisms suggested for the relatively higher diversity were proposed including multiple introductions of these species into New Zealand, cross infection from alternate hosts (possibly on which the pathogen undergoes a sexual stage) and/or a strong mechanism of asexual recombination. Isolates from nurseries and vineyards were genetically similar and often located within the same genetic group suggesting that some infection may be originating from nurseries. The placement of isolates from non-grapevine hosts within genetic groups containing grapevines isolates suggested that there is cross infection between grapevine and other non-grapevine hosts for some botryosphaeriaceous species.

Chapter 4

Intra-species variability in virulence of *Neofusicoccum parvum*, *N. luteum*, *N. australe* and *Diplodia mutila*

4.1 Introduction

Reports of disease caused by the botryosphaeriaceous fungi differ between countries, with respect to the species responsible, the symptoms they cause on grapevines and the cultivars infected. For example, *B. dothidea*, *D. seriata* and *D. mutila* were found to be the cause of "black dead arm" in France (Larignon *et al*., 2001). In contrast, no symptoms of "black dead arm" were found associated with the same species on grapevines in Portugal (Phillips, 2002). The relative virulence of the various botryosphaeriaceous species on grapevine has also been found to vary between countries and the grapevine cultivars. The best example of this discrepancy concerns virulence of *D. seriata*. Although this species has been reported as virulent on grapevine in Chile (Auger *et al*., 2004), the New South Wales region in Australia (Castillo-Pando *et al*., 2001) and South Africa (van Niekerk *et al*., 2004), it has been considered to be weakly virulent in Portugal (Phillips, 2002) and in New Zealand (Amponsah *et al*., 2008). The majority of these pathogenicity studies conducted in New Zealand and elsewhere, have solely reported differences in virulence levels between the species. However, some other studies have shown isolates of a botryosphaeriaceous species can also differ substantially in virulence (van Niekerk *et al*., 2004; Urbez-Torres & Gubler, 2009). A recent study conducted on *Cylindrocarpon* species, which are grapevine root pathogens, demonstrated that genetically different isolates varied significantly in their virulence on grapevine (Alaniz *et al*., 2009).

An added layer of complexity was demonstrated in a survey carried out in New Zealand vineyards (Chapter 2) which identified multiple botryosphaeriaceous species present in a single lesion. No information is available on the relative virulence of the co-habiting species in these multiple infection situations. Among the co-habiting botryosphaeriaceous species *N. parvum* and *N. luteum* were most frequently found together in a grapevine lesion (Section 2.3.9).

Isolates of botryosphaeriaceous species isolated from non-grapevine hosts have been reported to produce bio-active metabolites, which are probably involved in their disease development (Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah *et al*., 1991; Barbosa *et al*., 2003). Martos *et al*., (2008) characterized different phytotoxic metabolites produced by five botryosphaeriaceous species which were isolated from grapevine. They suggested that these phytotoxic metabolites could be involved in the pathogenicity of botryosphaeriaceous species *in planta*. In addition, to these metabolites, intra- and extra-cellular laccases have been reported to be produce by some

botryosphaeriaceous species, including *L. theobromae* and *Neofusicoccum ribis* (Vasconcelos *et al*., 2001). Laccases, which are classified as multi-copper oxidases, are widely distributed among fungi associated with wood decay, basidiomycetes and ascomycetes alike, and are involved in plant pathogenesis (Barbosa *et al*., 2007). Two activity types of extracellular laccases (PPO-I and PPO-II) were produced by these botryosphaeriaceous species and their production could be induced in the presence of veratryl alcohol on Vogel's minimum salt medium (Vasconcelos *et al*., 2000). A further study showed that different genetic groups of *L. theobromae* isolates produced different levels of laccase (Barbosa *et al*., 2007).

The purpose of this study was to estimate the relative virulence of genetically distinct isolates of *N. parvum*, *N. luteum*, *N. australe* and *D. mutila* individually under both *in vitro* and *in vivo* conditions. Further, this study was extended to evaluate the virulence level of co-inoculated *N. parvum* and *N. luteum* isolates which were frequently found to co-habit in lesions. The laccase production levels of *N. parvum* isolates selected from different genetic groups were also assessed.

4.2 Materials and methods

4.2.1 Virulence of different genotypic isolates of *N. parvum*, *N. luteum*, *N. australe* and *D. mutila*

Virulence of four botryosphaeriaceous species, *N. parvum*, *N. luteum*, *N. australe* and *Diplodia mutila* was studied using isolates from different genetic groups of the neighbour joining (NJ) tree produced in the genetic diversity analysis (Appendix C.1). The number of isolates selected from the culture collection for each species varied for *in vitro* green shoot and the glasshouse potted vine virulence assays (Table 4.1). The isolates used in the glasshouse potted vine assay were a subset of the ones in the green shoot assay and were selected based on the results obtained in the *in vitro* green shoots assays, with representatives that had low, medium and high levels of virulence.

Table 4.1: Number of isolates selected from each of the four botryosphaeriaceous species for *in vitro* green shoots and *in vivo* potted vine assays

Species	Green shoot assay	Potted vine assay
N. parvum	15	6
N. luteum	18	5
N. australe	15	6
D. mutila	14	6

4.2.1.1 Inoculum production

The virulence studies were conducted using mycelial colonized agar plugs as the inoculum source. All the selected isolates were grown on PDA at 23.5ºC under 12/12 h light/dark conditions for 3 days. Two different sized mycelial agar plugs were used from 3 day old cultures. Three mm diameter agar

plugs were used for *in vitro* inoculation of green shoots and 5 mm diameter agar plugs were used for *in vivo* potted vine assays. Plugs were taken from the agar plate immediately prior to inoculation.

4.2.1.2 In vitro green shoots assays

Grapevine green shoots were obtained from 6 months old Sauvignon Blanc potted vines as this cultivar was reported as more susceptible to botryosphaeriaceous species infection (Amponsah, 2011). Grapevine green shoots of about 25 – 30 cm in length and of a uniform thickness were excised using a sterile scalpel and the basal leaves removed to provide approximately 5-7 cm of bare stem. Each of the green shoots were separately placed into a 25 mL sterile universal bottle half filled with 1–4 mm Pumice granules (Atiamuri sand and pumice company, New Zealand) and the universal bottle filled with sterile water. The universal bottles were sealed around the stems of the shoots with Parafilm®. A wound was made on the shoot about 10 cm from the basal cut end using a sterile 3 mm diameter cork borer and scalpel. A single mycelium colonized PDA plug obtained from the growing margin of a 3 day old culture was placed onto the wound with the mycelium facing the plant and then covered with cling film to fix it in place. The control shoots were wounded and inoculated with non-colonized PDA plugs in a similar manner. Each isolate was replicated five times on separate green shoots. The inoculated green shoots were randomly arranged in a transparent plastic chamber at room temperature (range of 10−25ºC) under natural daylight conditions. Initially (first 1−2 days) high humidity was maintained by frequently misting the chamber with water using a hand sprayer. Subsequently, the shoots were sprayed with water twice a day until harvest.

4.2.1.3 Potted vine assays in the shade house

The potted vine assay of *N. parvum* species was conducted during the summer in November 2008 and for other species in November 2009. One year old dormant Sauvignon Blanc cuttings were collected in winter from the Lincoln University vineyard. The cuttings were rooted in pumice granules for 3–4 weeks prior to potting them. Cuttings with well-developed roots were selected and potted into 3 litre pots using a 1 year potting mix (Appendix C.2). The potted vines were grown in a glasshouse for 3 months prior to being inoculated. Healthy potted grapevines without any external disease symptoms were selected for inoculation. A wound was made on the main trunk, 15 cm above the soil line, using a sterile 5 mm diameter cork borer and scalpel. A mycelial colonized PDA plug, obtained from the growing margins of a 3 day old culture, was placed onto the wound with the mycelium facing the plant and held in place (Figure 4.1A) by wrapping with Parafilm®. The control vines were wounded and inoculated in a similar manner with non-colonized PDA plugs. Eight potted vines were inoculated with each isolate and control. After the inoculation, all the inoculated vines were sprayed with water and individually covered with transparent plastic bags for 24 h and then the vines were arranged in a completely randomised design in a shade house under ambient temperature (range of 15-25ºC) until harvest (Figure 4.1B).

4.2.1.4 Lesion length measurements

The external lesion length measurements of *in vitro* inoculated green shoots were made as describe by Amponsah *et al*. (2011) for *N. parvum*, *N. australe* and *D. mutila* 7 days after inoculation and, due to the faster lesion development of *N. luteum*, these lesion lengths were measured five days after inoculation. The total length of the dark lesion on the green shoots was measured using digital callipers (Mitutoyo). The lesion measurements of potted vines were made 6 weeks after inoculation for all four species. For the potted vine assay the internal lesion length was measured after debarked the main trunk using a sterile knife (Figure 4.1C). The total lengths of the dark lesions on the grapevine trunks were measured using a standard ruler. The lesion length data was statistically analysed by one way ANOVA using GenStat 9.0 and the means were separated by Fisher's protected LSD test ($P \leq 0.05$).

4.2.1.5 Endophytic pathogen movement within inoculated vines

Total endophytic pathogen movement in the inoculated potted vines was measured by re-isolation of the pathogen onto PDA plates after the lesion measurements were made. Due to time constrain the endophytic pathogen movements were measured only for *N. parvum, N. luteum* and *N. australe.* The vine section was surface sterilized by dipping into 70% ethanol for 3–5 s and briefly flaming. The lesion area and up to 5 cm beyond the lesion, in both the upwards and downwards directions from the inoculation point, was aseptically cut into 1 cm sections (Figure 4.1D). The 1 cm vine pieces were placed onto half strength potato dextrose agar plates (Figure 4.1E) supplemented with chloramphenical (½ PDA-Chl; Whiting *et al*., 2000) and kept at room temperature (approximately 20ºC). After 2–3 days, the plates were visually examined and the presence or absence of the botryosphaeriaceous pathogens determined by colony morphology (Figure 4.1F). The data obtained from this plate assessment was used to measure the extent of endophytic movement of the pathogens into the vine tissue beyond the visible lesion. The data was statistically analysed by one way ANOVA using GenStat 9.0 and the means were separated by Fisher's protected LSD test ($P \leq 0.05$).

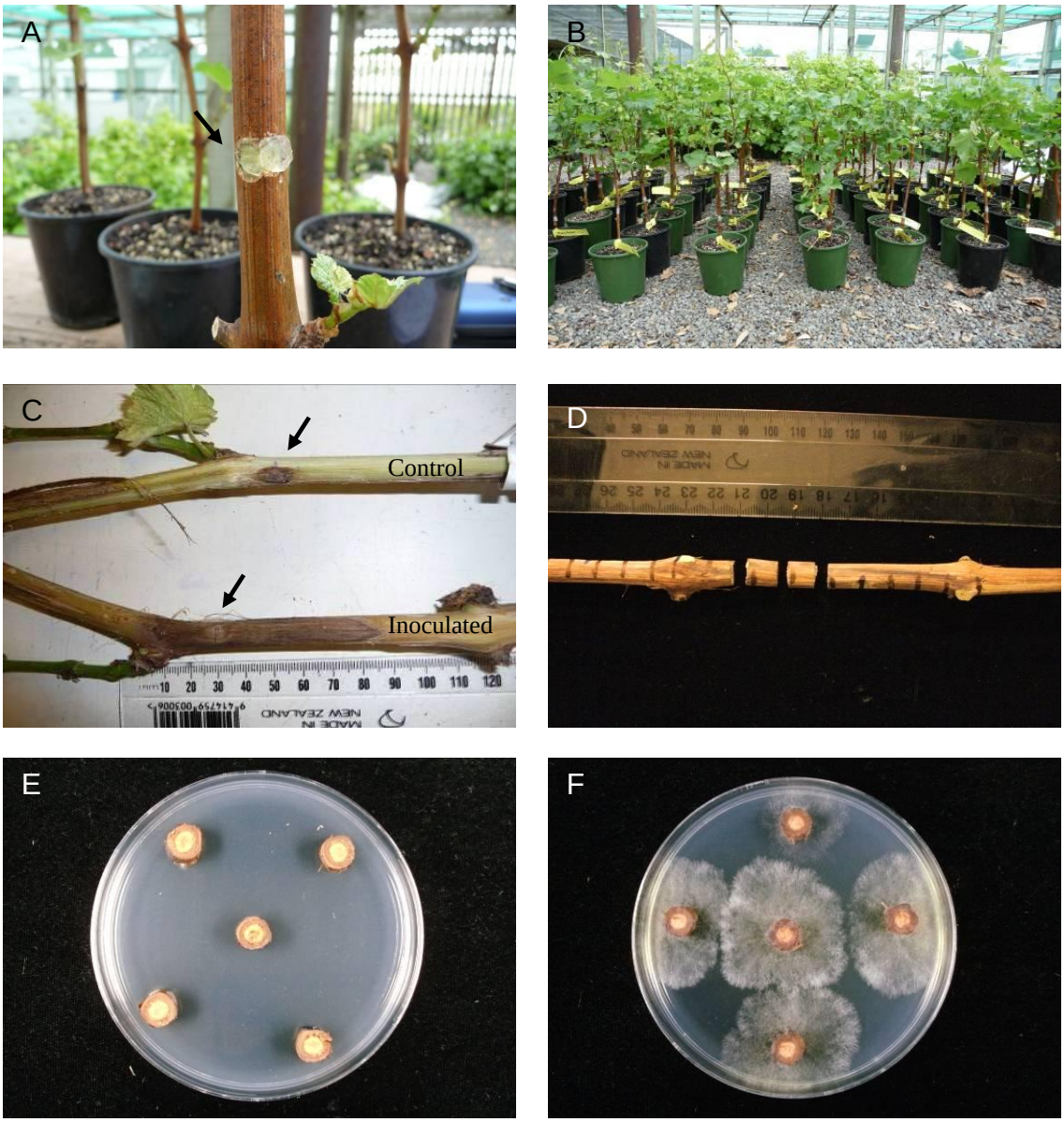

Figure 4.1: Stages in an experiment assessing inoculation of potted grapevines by botryosphaeriaceous species. A) Artificial wound created in vine between the internodes using a mycelium colonised agar plug for inoculation, B) inoculated vines arranged randomly in the shade house, C) an internal lesion that developed on an inoculated vine and the absence of a lesion on a control vine, D) the inoculated vine lesion region that was sectioned into 1 cm pieces for pathogen re-isolation, E) vine pieces obtained from different sections placed onto ½ PDA-Chl plates and F) botryosphaeriaceous fungi growing from the vine pieces after 3 days.

4.2.2 Intra-genetic group virulence of *N. parvum* and *N. luteum* isolates

A group of isolates of *N. parvum* and *N. luteum* from within a genetic group (and therefore with similar genotypes) were selected from the neighbour joining trees. Within these groups, the relative virulence of individual isolates was assessed by lesion lengths to determine whether genotype and thus presence on a particular branch of the neighbour joining tree correlated to their virulence. The isolates from the two most common species, *N. parvum* and *N. luteum* showed high variability in virulence on both green shoots and potted vines. Therefore, the variability in virulence of isolates within genetic groups selected from both species was studied on potted vines in the shade house as this was assumed to be more representative of the field situation. Isolates that comprised the high virulent genetic group (HVG) and low virulent genetic group (LVG) were selected based on the results obtained in the previous virulence assays (Sections 4.2.1.2). Among the six *N. parvum* isolates, three were selected from the sub-branches of genetic group 1 (HVG) that contained isolate G22a3 which had produced the largest lesion and three from genetic group 6 (LVG) (two of which were from the same sub-branch) that included isolate A421 which produced the smallest lesion in the previous studies (Section 4.3.1.1). Four *N. luteum* isolates were selected from genetic group 3 that contained the isolate (G51a2) which had produced the largest lesion in the previous studies. Two of the isolates came from the same sub-branch (N-1 and A511) and the other two came from separate sub–branches. Four *N. luteum* isolates were selected from genetic group 5 that contained the isolate (A563) which had produced the smallest lesion in the previous studies (Section 4.3.1.2).

Separate potted vine virulence assays were carried out using mycelial colonized agar plugs for each isolate from both species as described in Section 4.2.1.3. After six weeks the lesion lengths were measured on debarked stems. The data were statistically analysed by one way ANOVA using GenStat 9.0, to determine the significance of variation in virulence for the HVG and LVG isolates of each species, and the means were separated by Fisher's protected LSD test ($P \leq 0.05$).

4.2.3 Virulence of cohabitant species

As *N. luteum* and *N. parvum* were the species most frequently isolated together from lesions, the virulence level was assessed to determine when these pathogens were co-inoculated whether these species were supportive, synergistic or antagonist towards each other. The relative virulence was studied using potted vines grown in a shade house under ambient temperature (range of 15–25ºC). Two isolates, representative of high and low virulent, were selected for each of these two species based on their virulence level obtained in the previous assays. The *N. parvum* isolates G22a3 (highly virulent) and A421 (weakly virulent) and the *N. luteum* isolates G51a2 (highly virulent) and A563 (weakly virulent) were co-inoculated onto potted vines in all possible combinations. The potted vines were inoculated as described in Section 4.2.1.3 with the following modification to the inoculation process. The co-inoculation was performed by creating two immediately adjacent wounds on the vines

using a 5 mm diameter sterile cork borer and inoculating with two mycelial plugs obtained from an isolate of each species. As a control to determine the relative pathogenicity of the species combinations, each of the isolates was also inoculated individually using two mycelial plugs obtained from the same isolate. The control plants were wounded and inoculated in a similar manner with two PDA plugs. After six weeks the internal lesion lengths were measured from the inoculation point in both the upward and downward directions. The upward, downward and the total lesion length data were analysed individually as described previously in Section 4.2.1.4.

The endophytic pathogen movement beyond the lesion was assessed by the pathogen re-isolation as described in Section 4.2.1.5. The isolation plates were visually assessed and the botryosphaeriaceous colonies identified morphologically to determine the length of movement of each species in co-inoculated grapevines. Although both of these species produced yellow pigment on ½ PDA from the 3rd day onwards the intensity of the yellow pigment varied between them, with the stronger yellow pigment being produced by *N. luteum*. Initially both species were identified by visual examination of the re-isolation plates and later their identity was confirmed by molecular analysis using PCR-RFLP (Section 4.2.3.1). The distances of endophytic pathogen movement beyond the lesions were also statistically analysed in both upward and downward directions from the inoculation points and the total lengths of movement of each species individually assessed.

4.2.3.1 DNA extraction and PCR amplification of co-inoculated species

Genomic DNA was extracted from the mycelium growing from all the vine pieces on ½ PDA-Chl plates using a REDExtract-N-Amp™ PCR kit (Sigma) as described in Section 2.2.7.1. The ITS region of the nuclear ribosomal RNA gene region (rRNA) was amplified using the multi genus specific primers BOT100F ($^{5'}$AAACTCCAGTCAGTRAAC$^{3'}$) and BOT454R ($^{5'}$TTTCTCAAGGMTGACC TCGGA$^{3'}$; Ridgway *et al.*, 2011). The PCR was carried out using the reagents supplied by the REDExtract-N-Amp™ kit according to the manufacturer's instructions. Each 20 µL PCR reaction contained 10 µL of REDExtract-N-Amp™ solution, 5 pmol of each primer (Invitrogen), and 4 µL of extracted DNA. The reactions were performed in a BioRad iCycler Thermal cycler (Germany). The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 58ºC, and 1 min at 72 ºC, and a final extension period of 10 min at 72 ºC. After the amplification, electrophoresis was conducted with 5 µL of each PCR product which had been loaded into a 1% agarose gel, placed in a 1 × TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0) and run at 10 V/cm for 50 min. Gels were stained with ethidium bromide (0.05 µg/ml) for 15 min, destained for 10 min in water and visualized on a UV transilluminator (Versadoc™, BioRad Laboratories).

4.2.3.2 Restriction endonuclease digestion of the BOT100F-BOT454R PCR product

In silico analysis of restriction endonuclease sites within the 374 bp amplimer of the rRNA gene region produced from isolates of *N. parvum* and *N. luteum* with primers BOT100F and BOT454R was done using DNAman™ (Lynnon Biosoft). The software identified that the restriction endonuclease *Mbo*II, which recognised the sequence GATC, could be used to distinguish these two species. Eight µl of the PCR product obtained with primers BOT100F and BOT454R was digested with 3 U of *Mbo*II (Biolab) at 37ºC for 2 h and followed by heat inactivation at 65ºC for 30 min. The digested product was separated by electrophoresis in a 1.3% agarose gel at 10 V/cm for 75 min. The gel was stained with ethidium bromide and visualized on a UV transilluminator as described in Section 2.2.7.1.

4.2.4 Preliminary study of laccase production level of *N. parvum* isolates

As previous studies have identified that variation in laccase production can correlate with relative pathogenicity, the laccase production by *N. parvum* isolates was measured using enzymatic assays (Vasconcelos *et al*., 2000). Laccase activity is commonly determined spectrophotometrically and is based on the ability of this enzyme to oxidize–colorize specific aromatic compounds such as guaiacol, syringaldazine, or 2,6-dimethoxyphenol (2,6-DMP) and the subsequent monitoring of the coloured oxidation products using a spectrophotometer. 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). Oxidation of ABTS by laccase will result in a green–blue coloured radical cation (ABTS+•) measurable at 436 nm (Figure 4.2).

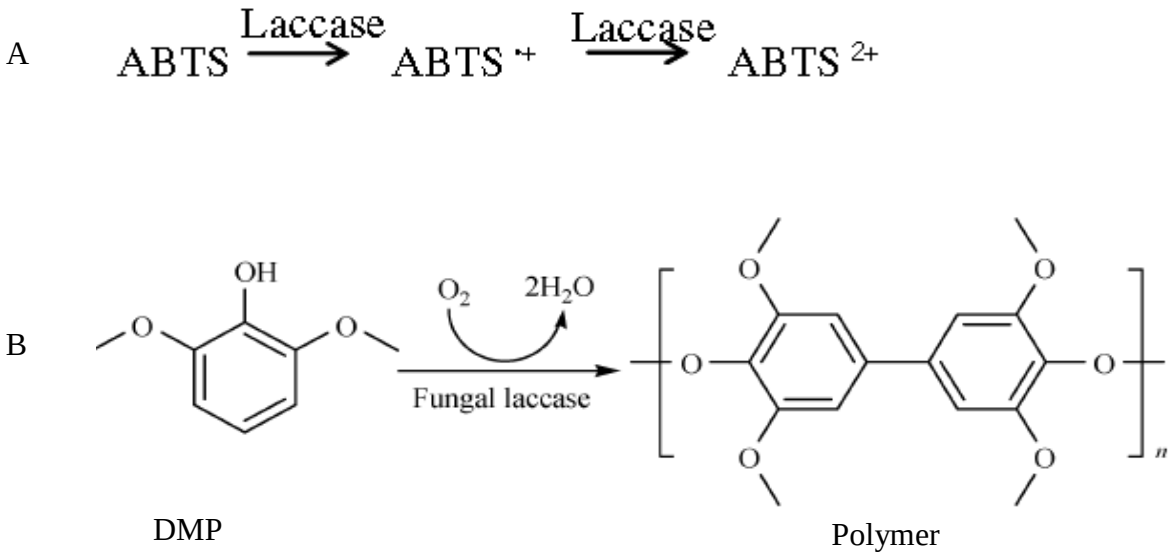

Figure 4.2: Oxidation of A) ABTS by laccase results in a green–blue coloured radical cation (ABTS+•) and B) 2, 6-dimethoxyphenol by fungal laccases.

4.2.4.1 Culture production

Three *N. parvum* isolates maintained on PDA in cool storage (4ºC) were selected from different genetic groups (neighbour-joining tree; Figure 4.2) and variable pathogenicity levels as determined by the green shoot and potted vine assays. The *N. parvum* isolates used in this study were G22a3 (highly virulent), MM562 (average pathogenicity) and G121 (weakly pathogenic). Inoculum was prepared by growing each of the selected *N. parvum* isolates individually on agar plates containing minimal salt medium (Vogel, 1956; Appendix C.3), agar (2% w/v) and glucose (1% w/v) at 23.5ºC for 4 days. To recover the laccase enzyme, three mycelial colonized agar plugs obtained from the growing edge of the culture plates using a sterile 5 mm diameter cork borer was used to inoculate 25 mL of basal medium (Vogel minimal salt medium and glucose (1% w/v) as the carbon source, pH adjusted to 6.0) in 100 mL flasks which were sealed by cotton wool plugs. Extra-cellular laccase production was induced by the addition of 119.7 µL veratryl alcohol (3, 4-dimethyozybenzyl alcohol, 96%; Sigma) to the basal medium to a final concentration of 30.4 mM, before inoculating the fungi (Vasconcelos *et al*., 2000). All the flasks were incubated at 28ºC on a rotary shaker (Chiltern Scientific) at 180 rpm under 12/12 light/dark condition for 5 days.

4.2.4.2 Preparation of culture extract and enzymatic assay

After 4–5 days incubation, each liquid culture was transferred to a 50 mL tube and centrifuged at 12500 × *g* for 30 min. The mycelium–free extracellular fluid was decanted and used as the extracellular enzyme extract for the enzymatic assay. The activity towards ABTS, which is specific for PPO-I (Polyphenol oxidase-I) laccase, was determined at an acidic pH 3.0 and temperature of 50ºC (Barbosa *et al*., 1996), while the activity against DMP, which is specific for PPO-II laccase, was measured at an almost neutral pH of 6.5 and temperature of 45ºC (Vasconcelos *et al*., 2000).

The 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 C.4). Twenty mL of buffer for PPO-I analysis 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 3.0. Twenty mL of buffer for PPO-II analysis 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 6.5. The reaction mixture for the PPO-I (ABTS) assay was prepared with 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. The absorbance measurement was made after inoculation at 50ºC for 5 min using a spectrophotometer (Model 6305; Jenway Ltd, England) at 420 nm.

The reaction mixture for the PPO-II (DMP) assay was prepared with 0.15 mL 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 in a final volume of 1 mL. After 5 min at 45ºC, 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 *N. parvum* isolates were quantified and compared between the isolates.

4.3 Results

4.3.1 Virulence of different genotypic isolates of four botryosphaeriaceous species

4.3.1.1 Virulence of N. parvum *isolates*

All isolates selected from the genetic groups in the NJ tree caused visible, brown necrotic lesions on green shoots 2 days after inoculation, which progressed both upwards and downwards from the inoculation point. No lesion was produced on control shoots inoculated with non-colonized PDA plugs (Figure 4.3). After 7 days the mean lesion lengths ranged between the 11 and 82.5 mm produced by *N. parvum* isolates A421 and G22a3, respectively. Lesion lengths varied significantly ($P<0.001$; Appendix C.5.1) between the isolates (Figure 4.4). However, the isolates selected from the same genetic group in the NJ tree produced significantly different mean lesion lengths indicating that virulence of these isolates differed within this genetic group. Three isolates out of the four selected from genetic group 1 were highly virulent and two isolates from genetic group 6 were weakly virulent.

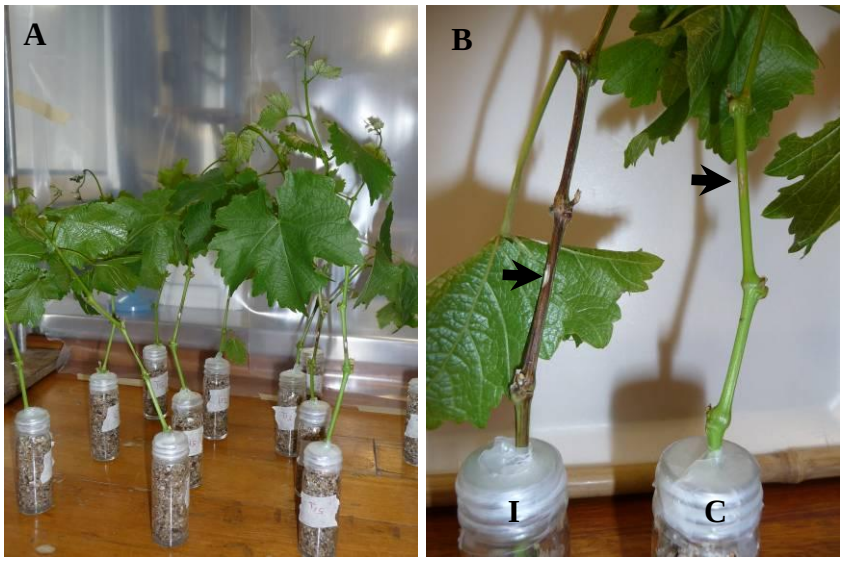

Figure 4.3: *In vitro* green shoot pathogenicity assay of *N. parvum*. A) Experimental set-up of grapevine green shoots arranged in a transparent plastic chamber after inoculation, B) brown necrotic lesion development in an inoculated green shoot (I) and absence of lesions in control green shoots (C). Arrows indicate the inoculation point.

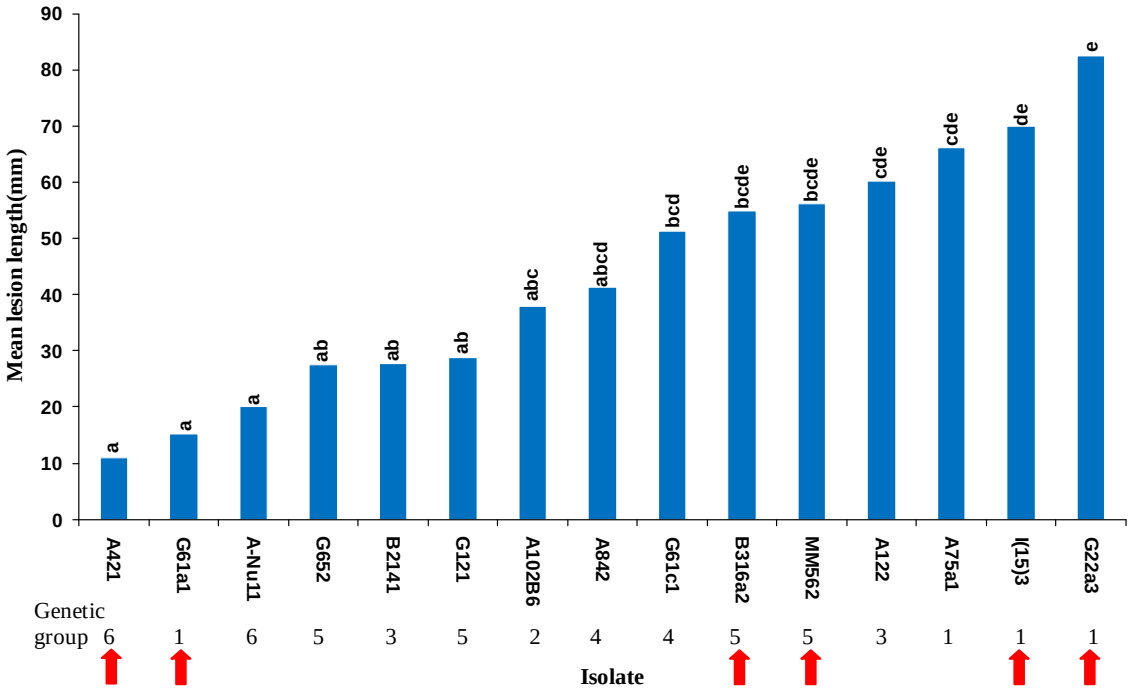

Figure 4.4: Mean lesion lengths on grapevine green shoots seven days after inoculation with *N. parvum* isolates belonging to different genetic groups. Bars with different letters are significantly different ($P \leq 0.05$) by Fisher's protected LSD test. Arrows indicate those isolates that were selected for potted vine experiments.

No external symptoms were observed in the potted vines 6 weeks after inoculation with the selected isolates. Examination for internal lesions after debarking the vines revealed brown necrotic lesions that had developed in both an upwards and downwards direction from the inoculation point and differed significantly ($P<0.001$; Appendix C.5.2) between isolates (Figure 4.5). Only three isolates (MM562, I(15)3 and G22a3) produced lesions, while isolates A421 and G61a1 which produced the smallest mean lesions on the green shoot assay did not produced any lesions on potted grapevines. Among the isolates that had produced lesions of average length on the green shoots, isolate B316a2 did not produce a lesion on the potted grapevine, whereas isolate MM562 did. The isolates which had produced the largest mean lesions when inoculated onto green shoots, I(15)3 and G22a3, also produced the largest lesions on potted grapevines with the means being 47 mm and 56 mm, respectively (Figure 4.5), which were significantly larger ($P \leq 0.05$) than the lesions caused by other isolates.

The endophytic pathogen movement beyond the visible lesion or inoculation point, for those that had not produced a lesion, were observed in all isolates on potted grapevines. For those isolates that had produced lesions the lengths of endophytic pathogen movement was greater than the total visible lesion length produced and was significantly different between the isolates ($P<0.001$; Appendix C.5.3). The greatest and the least mean endophytic pathogen movements were measured for isolates

A421 (7 mm) and G22a3 (69 mm), respectively (Figure 4.5). The results of the green shoot and potted vine assays showed that the pathogenicity levels of the *N. parvum* isolates from each genetic group varied within the group but for each isolate it was consistent in both tissue types.

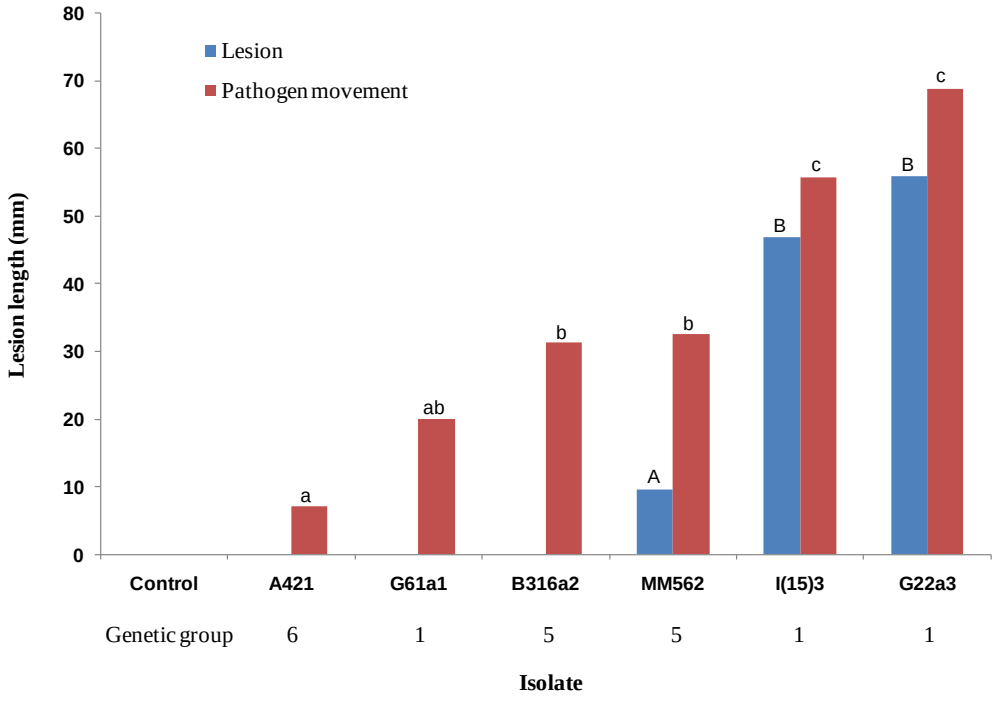

Figure 4.5: Mean lesion lengths and distances of endophytic movement of *N. parvum* isolates from different genetic groups in the trunk of potted grapevines six weeks after inoculation. Bars with different letters are significantly different ($P \leq 0.05$) from each other according to a Fisher's protected LSD test.

4.3.1.2 Virulence of N. luteum *isolates*

The development of the external brown lesion on grapevine green shoots by *N. luteum* isolates selected from different genetic groups was faster than observed for the *N. parvum* isolates. Accordingly, the lesion length measurements were made earlier than for the *N. parvum* green shoot assays, at 5 days after inoculation. Significant variation was observed in the lengths of lesions produced by *N. luteum* isolates ($P<0.001$; Appendix C.5.4). Mean lesion lengths ranged from 10 to 89 mm (Figure 4.6) with little relationship with their genetic group. Two of the non-grapevine *N. luteum* isolates, ICMP16678 isolated from *Rhododendron vireya* and CC445 isolated from *Actinidia arguta*, produced lesion lengths of 58 mm and 76 mm on grapevine shoots, respectively.

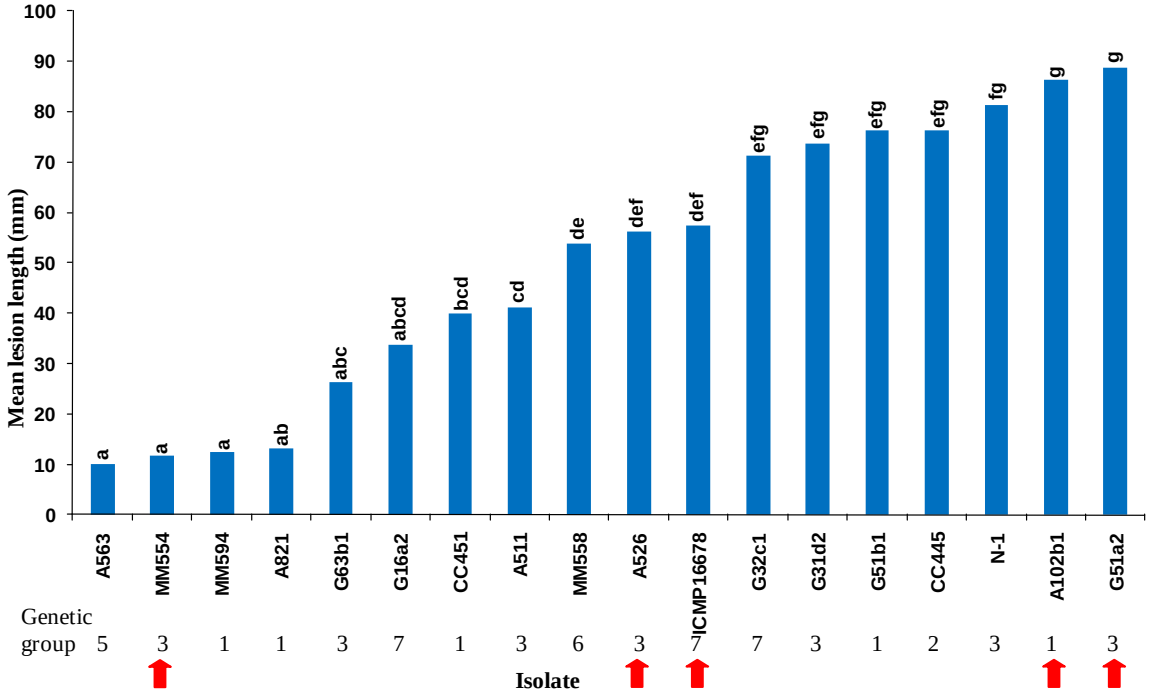

Figure 4.6: Mean lesion lengths measured on grapevine green shoots 5 days after inoculation with *N. luteum* isolates belonging to different genetic groups. Bars with different letters are significantly different ($P \leq 0.05$) by Fisher's protected LSD test. Arrows indicate those isolates selected for potted vine assays.

The five isolates selected for inoculation onto potted grapevines, caused visible symptoms after 6 weeks growth. The lateral shoots arising from the internodes just above the inoculation point were dead in all inoculated grapevines (Figure 4.7), which was unlike the *N. parvum* symptoms. All isolates produced lesions and the mean lesion lengths produced by the different *N. luteum* isolates were significantly different ($P<0.001$; Appendix C.5.5) from each other. The mean lesion lengths ranged from 100 mm produced by isolate A526 to 178 mm by isolate A102b6 (Figure 4.8) and were larger than those noted for *N. parvum* isolates under similar conditions. For each isolate its relative pathogenicity obtained in the potted vine assay correlated well with the results obtained in the green shoot pathogenicity assay. When the pathogen was re-isolated from the lesions and the apparently healthy tissue above and below the lesions the results showed the *N. luteum* isolates had moved endophytically beyond the lesion region in both directions. The distances of endophytic movement by different isolates of *N. luteum* were statistically different ($P=0.03$; Appendix C.5.6) and ranged from 153 mm to 268 mm. The furthest endophytic pathogen movement by isolate G51a2 was significantly greater than for the other isolates which were similar for the other four *N. luteum* isolates although they produced different lesion lengths (Figure 4.8).

Figure 4.7: External symptoms observed in *N. luteum* inoculated potted grapevines. A) Dieback symptom of total vine, B) dieback of lateral shoot above the inoculation point and C) control inoculated debarked vine. The inoculation point is indicated by arrows.

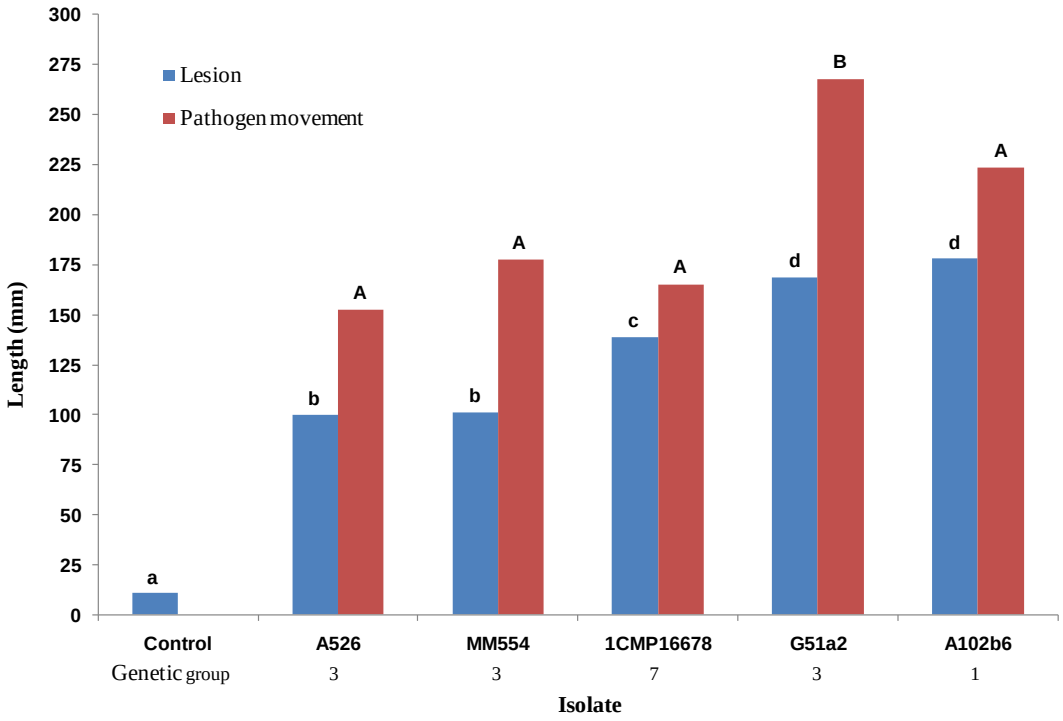

Figure 4.8: Mean lesion lengths and endophytic movement of genetically distinct *N. luteum* isolates on trunks of potted 1 year old grapevines six weeks after inoculation. Bars with different letters are significantly different ($P \leq 0.05$) by Fisher's protected LSD test.

4.3.1.3 *Virulence of* N. australe *isolates*

The external brown lesions that developed 7 days after inoculation on grapevine green shoots were similar in appearance to the lesions produced by *N. parvum* and *N. luteum*. The mean lesion lengths produced on green shoots differed significantly ($P<0.001$; Appendix C.5.7) for the 15 *N. australe* isolates, ranging from 30 to 83 mm for isolates C1B21 and G212, respectively (Figure 4.9). The non-grapevine *N. australe* isolate J-3 isolated from broom (*Cytisus* spp.) produced a mean lesion of 45 mm on grapevine green shoots. Isolates from within the same genetic group produced significantly different mean lesion lengths ($P\leq0.05$) indicating that their pathogenicity level was variable within genetically similar isolates.

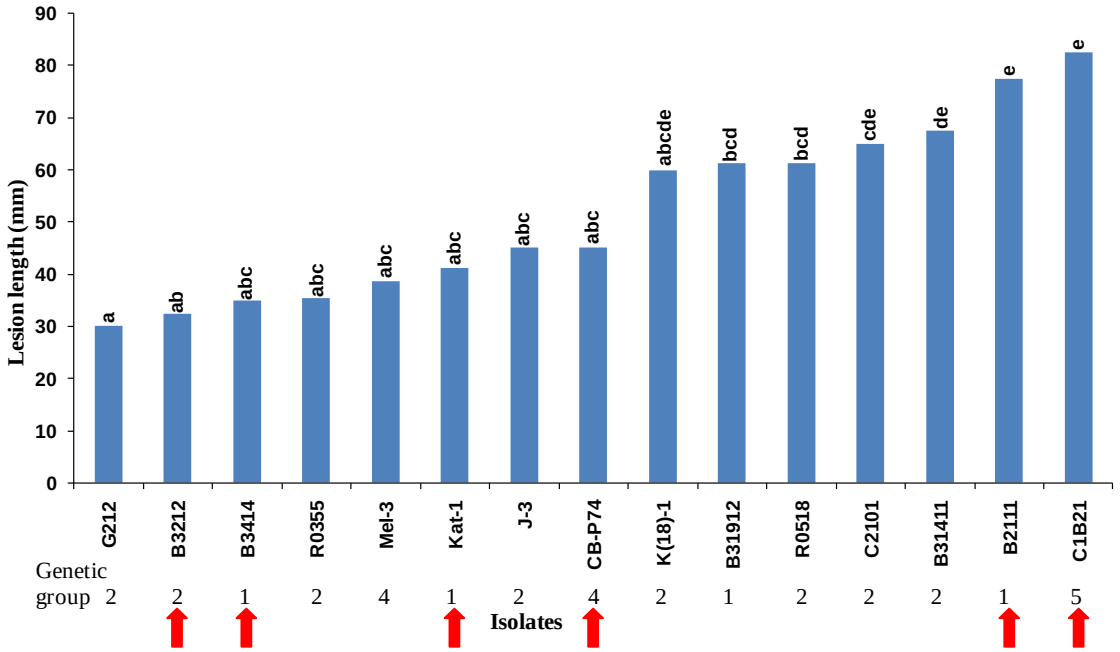

Figure 4.9: Mean lesion lengths measured on grapevine green shoots seven days after inoculation with *N. australe* isolates belonging to different genetic groups. Bars with different letters are significantly different ($P\leq0.05$) by Fisher's protected LSD test. Arrows indicate those isolates selected for potted vine assays.

On potted vines no external disease symptoms were observed at six weeks after *N. australe* inoculation. The 6 isolates selected from the 15 used in the green shoot assays, differed significantly with respect to the lengths of the internal lesions that developed in the potted vines ($P<0.001$; Appendix C.5.8) with means ranging from 47 mm to 139 mm for isolates C1B21 and B3212, respectively (Figure 4.10). The relative virulence levels of isolates in the potted grapevine assay correlated well with their virulence on green shoots.

The results of the endophytic movement of *N. australe* isolates in grapevine were similar to *N. parvum* and *N. luteum* isolates in that they moved into non-symptomatic vine tissues beyond the visible lesions. The endophytic movement of the six *N. australe* isolates used in the potted vine assay

followed the same trend as observed in lesion development and means ranged from 93 mm to 164 mm. The endophytic movement was significantly different between the *N. australe* isolates ($P<0.001$; Appendix C.5.9). The greatest mean endophytic movement of 164 mm in 6 weeks was observed for isolate B2111, which was not significantly different from the mean endophytic movement of 162 mm by isolate C1B21 (Figure 4.10). The endophytic movement observed for the other four *N. australe* isolates was not significantly different from each other.

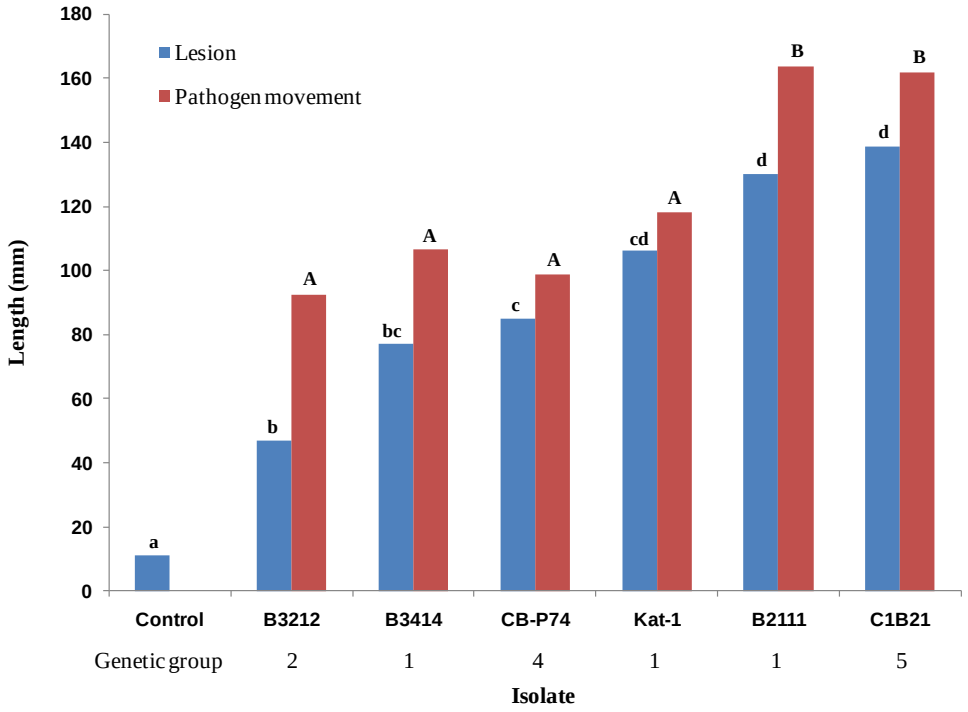

Figure 4.10: Mean lesion lengths measured on trunks of potted 1 year old grapevines six weeks after inoculation with *N. australe* isolates from different genetic groups. Bars with different letters are significantly different ($P \leq 0.05$) from each other by Fisher's protected LSD test.

4.3.1.4 Virulence of D. mutila *isolates*

After 7 days the lesion lengths produced by the *D. mutila* isolates on grapevine green shoots varied significantly between the isolates ($P<0.001$; Appendix C.5.10), means ranging from 8.3 to 52 mm for isolates B32112 and N1351, respectively. The lesion lengths produced by the majority (79%) of the isolates ranged between 8 and 20 mm after 7 days. The non-grapevine *D. mutila* isolate J4, which was isolated from oak (*Quercus* sp.), produced a mean lesion of 12 mm on the grapevine green shoots (Figure 4.11). Similar to other botryosphaeriaceous species included in this study, the *D. mutila* isolates within the same genetic group produced different mean lesion lengths indicating that their virulence differed.

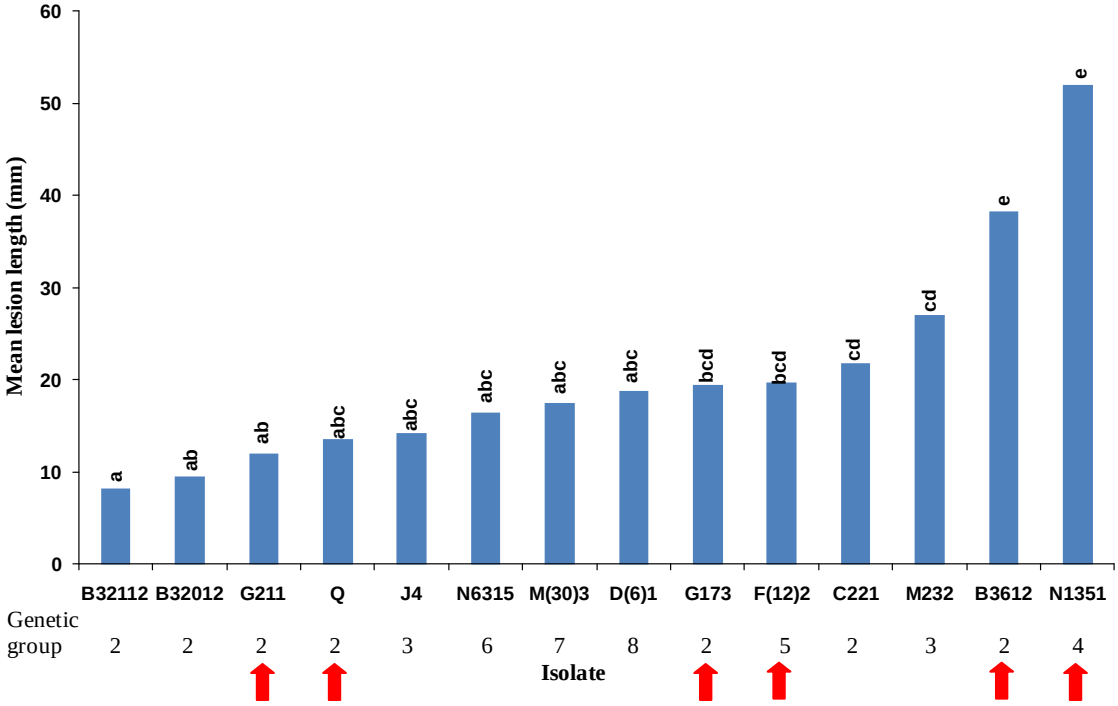

Figure 4.11: Mean lesion lengths measured on grapevine green shoots seven days after inoculation with *D. mutila* isolates belonging to different genetic groups. Bars with different letters were significantly different ($P \leq 0.05$) by Fisher's protected LSD test. Arrows indicate those isolates selected for potted vine assays.

No external disease symptoms were observed on grapevines inoculated with *D. mutila* isolates after 6 weeks. For the six selected isolates the internal necrotic lesions produced on potted vines were similar to those caused by the *Neofusicoccum* species on potted grapevines. Lesion lengths of isolates differed significantly ($P<0.001$; Appendix C.5.11), means ranging from 39 mm to 84 mm for isolates G173 and N1351, respectively (Figure 4.12). The virulence of isolates on potted vines after 6 weeks did not correlate well with the virulence of the same isolates on the green shoots. Isolate G173 produced a medium size lesion (mean of 20 mm) on green shoots but produced the smallest mean lesions (39 mm) on potted vines, whereas the isolates G211 and Q which produced smaller mean lesions on green shoots than isolates G173, produced larger mean lesions of 42 mm and 63 mm, respectively, on potted vines.

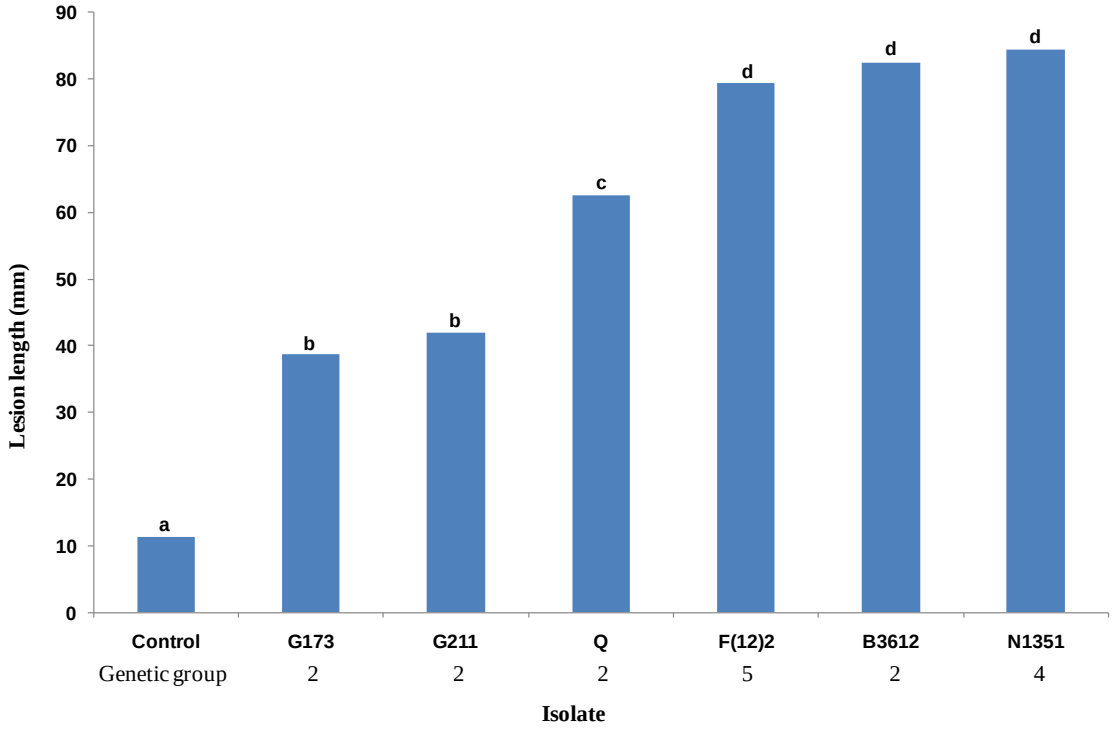

Figure 4.12: Mean lesion length measured on trunks of 1 year old potted grapevines after six weeks inoculation with *D. mutila* isolates belonging to different genetic groups. Bars with different letters are significantly different ($P \leq 0.05$) by Fisher's protected LSD test.

The results of the virulence assays of all four species on both green shoots and potted vines are summarised in Table 4.2. On green shoots, all four species produced necrotic lesions, although lesion development was fastest with *N. luteum* inoculation than with the other species and slowest with *D. mutila*. On potted grapevines external disease symptoms were only observed in *N. luteum* inoculations. The lengths of the internal necrotic lesions on trunks of potted grapevines six weeks after inoculation produced by these four species were variable. The longest mean lesion length on trunks of potted vines was produced by *N. luteum* (138.7 mm) and the smallest (18.7 mm) by *N. parvum*, because three isolates of *N. parvum* did not produce any lesions on potted vines. The result of the reisolation of *Neofusicoccum* species from inoculated potted vines showed that the pathogens endophytically moved beyond the lesion in upwards and downwards directions. Of the four species, the greatest endophytic movement was obtained for *N. luteum* inoculations, being in the range of 153 - 268 mm and the least for *N. parvum* in the range of 7-69 mm.

Table 4.2: The lesion lengths caused by four botryosphaeriaceous species and endophytic pathogen movement (by re-isolation) in grapevine green shoots and trunks of potted vines

Species	Green Shoot Assay		Potted Vine Assay		
	Lesion size (range in mm)	Mean lesion (mm)	Lesion size (range in mm)	Mean lesion (mm)	Endophytic movement (mm)
N. parvum	11–83	43.4	0–56	18.7	7–69
N. luteum	10-89	50.6	100-178	138.7	153-268
N. australe	30-83	51.8	47-139	97.4	93-164
D. mutila	8-52	20.6	39-84	64.9	Not assessed

4.3.2 Intra-genetic group variability in virulence of *N. parvum isolates*

The six *N. parvum* isolates which were selected from genetic group 1 (HVG) and genetic group 6 (LPG) produced significantly different lesion sizes ($P<0.001$; Appendix C.5.12) on trunks of potted grapevines after 6 weeks, with mean lesion lengths ranging from 22 mm for A931 to 178 mm for G69a1(Figure 4.13). Among the LVG isolates of genetic group 6, the mean lengths of necrotic lesion caused by isolates A931 and A421, which were from the same sub-branch of genetic group 6 but from two different vineyards, were not significantly different from the control treatment. The mean length of lesions produced by the LVG isolate A813 was 160 mm and it was significantly longer ($P\leq0.05$) than for lesions caused by two of the HVG isolates (G22a3 and G22c2). All of the isolates belonging to HVG of genetic group 1 produced large lesions with means ranging from 110 mm to 178 mm. Among the HVG isolates the mean lengths of lesions produced by the isolates G22a3 and G22c2 were not significantly different. Although HVG isolate G69al belonged to the same genetic group the mean lengths of lesions produced was significantly different from the other two isolates. The three LVG isolates also differed with one producing large lesions and two producing small lesions. There was a relationship between mean lesion lengths and the genetic similarities of the isolates as shown by the NJ tree (Figure 3.4) but there were exceptions in each group.

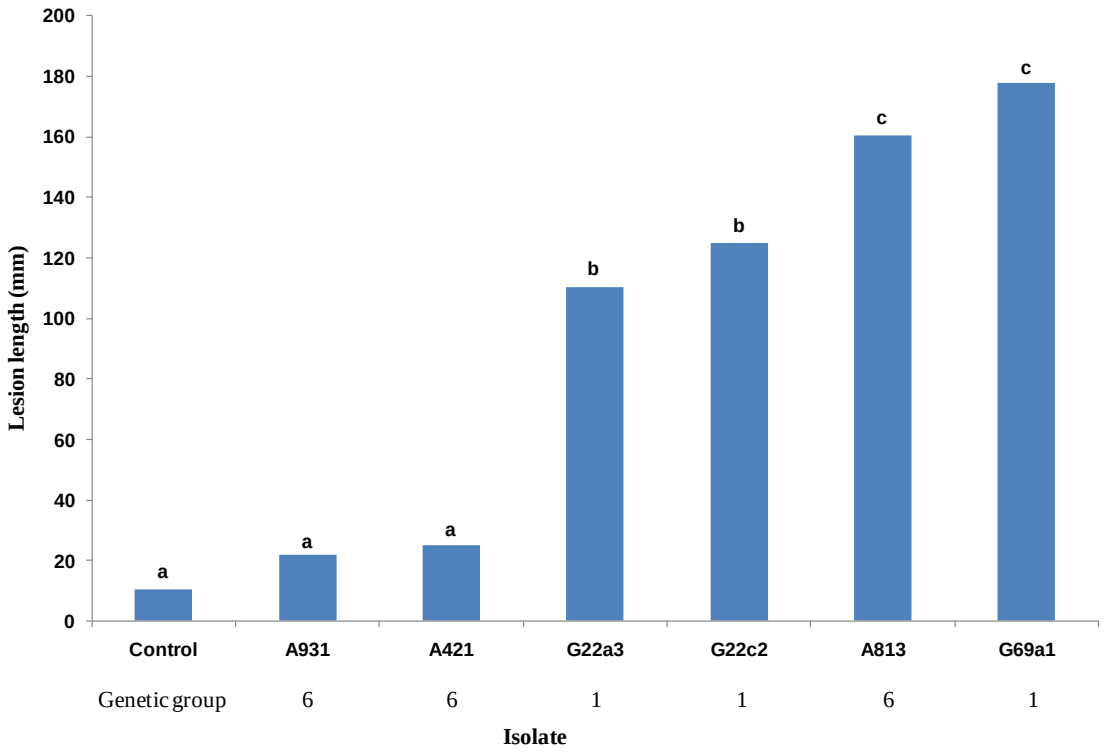

Figure 4.13: Lesion length of two different genetic groups (group 1 and 6) of *N. parvum* isolates in 1 year old potted grapevines after 6 weeks of inoculation. Bars with the same letters are not differ significantly ($P \leq 0.05$) by Fisher's protected LSD test.

4.3.3 Intra-genetic group variability in pathogenicity of *N. luteum* isolates

The lesion size produced on potted grapevines by the eight *N. luteum* isolates belonging to genetic groups 3 (HVG) and 5 (LVG) were significantly different ($P<0.001$; Appendix C.5.13). Of the four isolates in HVG (genetic group 3) the mean sizes of the lesions produced by three of the isolates (A511, N-1 and G51a2) were large and not significantly different from each other. The lesion produced by the other group 3 isolate A526 was small (mean of 100 mm) and not significantly different from two of the LVG isolates (A563 and A243). The smallest lesion was produced by the LVG isolate M(8)4, which was not significantly different from the control vines, however the fourth LVG isolate CC456 produced the largest mean lesion of 288 mm (Figure 4.14). These results showed that most of the isolates within each genetic group had similar pathogenicity but that there were exceptions in each groups.

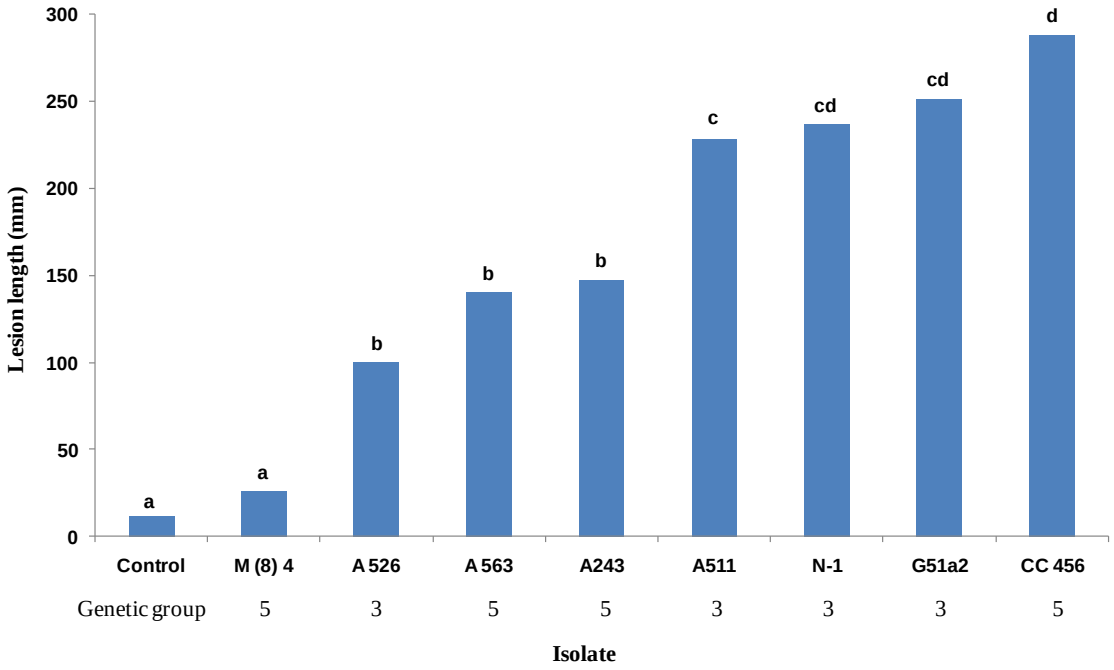

Figure 4.14: Lesion length of isolates from within two different genetic groups (3 and 5) of *N. luteum* on trunks of 1 year old potted grapevines 6 weeks after inoculation. Bars with different letters are significantly different ($P \leq 0.05$) by Fisher's protected LSD test.

4.3.4 Virulence levels of cohabitant species *N. parvum* and *N. luteum*

Mean lesion lengths differed between the co-inoculations of isolates of *N. parvum* and *N. luteum* of trunks of potted vines ($P<0.001$; Appendix C.5.14). The largest mean lesion of 201 mm was produced by the co-inoculation of *N. parvum* isolate NP G22a3 and *N. luteum* isolate NL G51a2 (Figure 4.15), two isolates that were highly virulent in the previous experiments (Sections 4.3.1.1 and 4.3.1.2). The total mean lesion length produced by the co-inoculation of these isolates was not significantly different from the lesion length produced by isolate NL G51a2 alone, although it was significantly larger from the mean lesion lengths produced by isolate NP G22a3 alone. In the downward direction mean lesion lengths produced by the co-inoculation of these highly virulent isolates was significantly larger than that produced by either alone ($P \leq 0.05$).

The co-inoculation of a highly virulent *N. parvum* isolate (NP G22a3) and a weakly virulent *N. luteum* isolate (NL A563) resulted in a significant reduction in the total lesion length relative to that produced by isolate NL A563 alone ($P \leq 0.05$). The combination produced a greater downward lesion development than either produced alone ($P \leq 0.05$).

Co-inoculation of two low virulent isolates (NP A421 and NL G51a2) resulted in the production of a total mean lesion length which was not significantly different in length from that produced by either

isolate alone. However, the mean upward lesion length was significantly larger in NL G51a2 inoculation (Figure 4.15) than in the combined inoculations with *N. parvum* isolate NP A421.

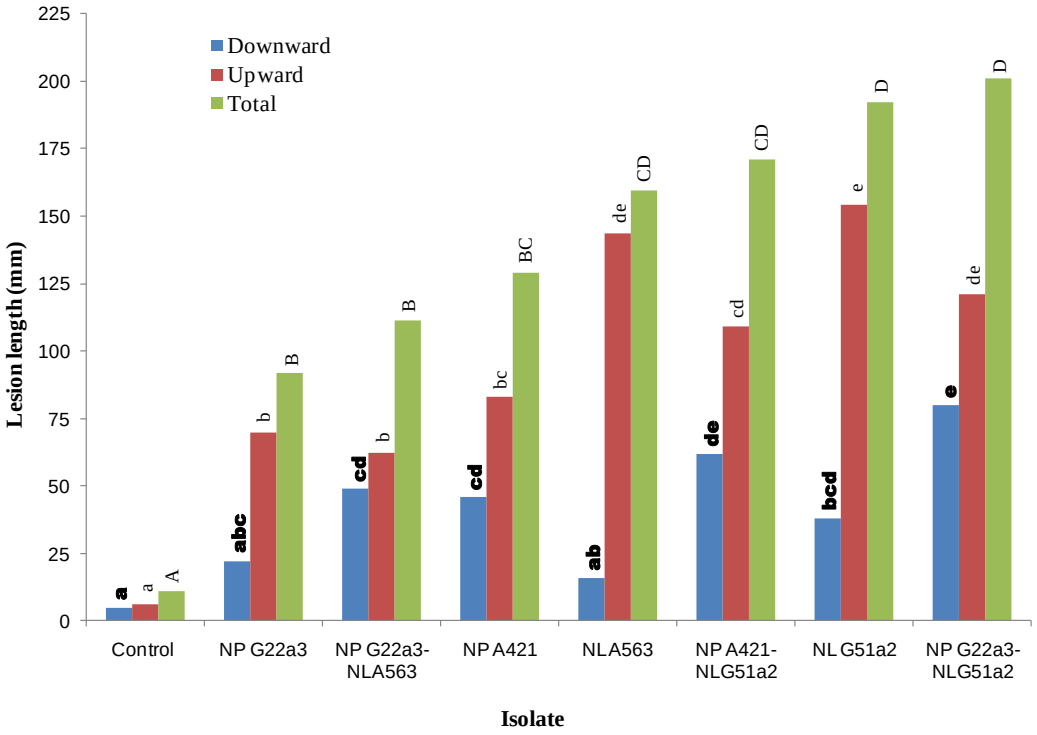

Figure 4.15: Mean lesion lengths of the co-inoculated botryosphariaceous species *N. parvum* (NP) and *N. luteum* (NL) on 1 year old potted grapevines. The graph shows upward and downward lesion length from the inoculation point and the total length of the lesion. Bars with the same letters are not significantly different ($P \leq 0.05$) by Fisher's protected LSD test.

The pathogen re-isolation from co-inoculated grapevines followed by morphological identification measured the endophytic movements of *N. parvum* (NP) and *N. luteum* (NL) species in co-inoculated grapevines. For morphological identification the development of yellow pigment after 3 days incubation was used as the distinguishing criterion for the two species (Figure 4.16). Subsequently, a 25% sub-sample of the isolated colonies was confirmed to species level using the molecular method of PCR-RFLP. During confirmation of the species identification by the molecular method, a single PCR product of approximately 374 bp was obtained with the botryosphariaceae multi-species primers BOT100F and BOT454R (Figure 4.17). The identification of each of the species was confirmed by a specific banding profile produced in a restriction endonuclease digestion with *Mbo*II. A single *Mbo*II restriction site in the sequence of *N. parvum* produced two bands of 100 bp and 274 bp size and two restriction sites found in *N. luteum* produced two visible bands (100 bp+101 bp and 172 bp in size) (Figure 4.18). The molecular confirmation of species identity using this method confirmed a 100% accuracy of the morphological assessment of the re-isolation plates.

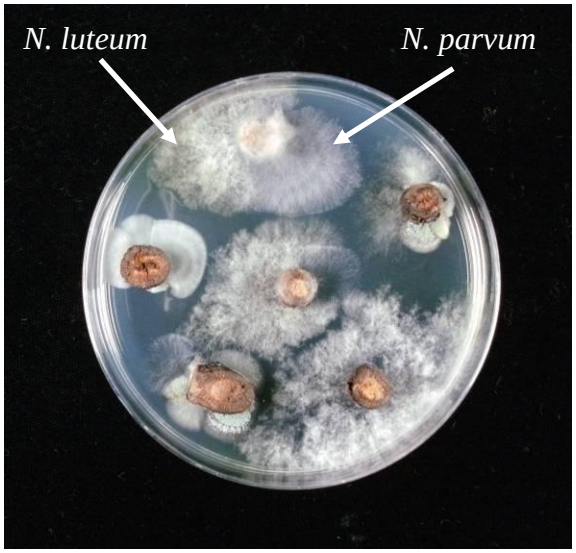

Figure 4.16: Agar plate of wood discs from grapevines co-inoculated with *N. luteum* and *N. parvum*. Arrows show both *N. luteum* (yellow pigment) and *N. parvum* species growing from a co-inoculated piece of grapevine on ½ PDA plates after 4 days of incubation.

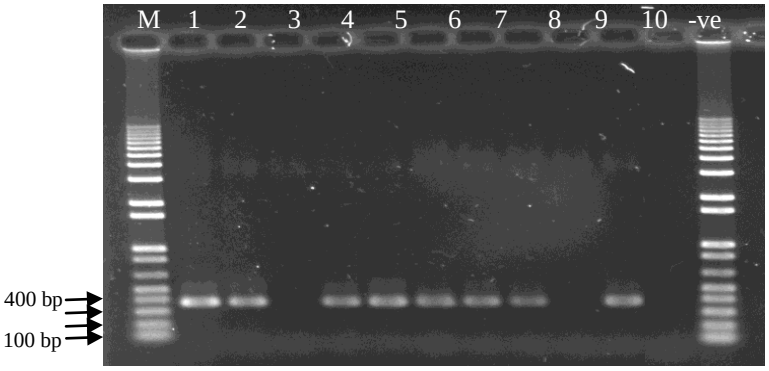

Figure 4.17: PCR product obtained from co-inoculated grapevines using botryosphariaceous species multi-genus primers BOT100F and BOT454R. Lane 1 – 10 indicates the DNA extracted from colonies obtained at 1 cm intervals from the inoculation point, -ve – negative control and M– 1Kb+ DNA ladder (Invitrogen).

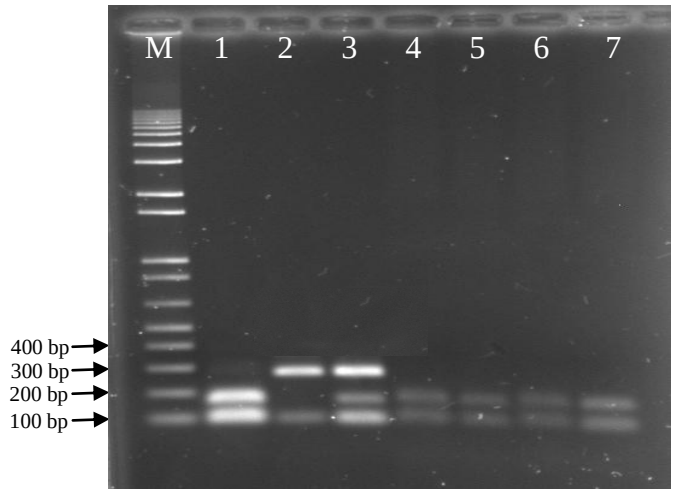

Figure 4.18: PCR-RFLP banding patterns of *Mbo*II digested PCR products. Samples 1 (*N. luteum* type specimen), 2 (*N. parvum* type specimen) and 3 (*N. luteum* and *N. parvum* co-digestion) were the RFLP pattern of PCR product obtained from genomic DNA of representative isolates. Samples 4- 7 were the RFLP pattern of PCR product obtained from the DNA extracted from co-inoculated samples. M- 1Kb+ DNA ladder (Invitrogen).

The data of endophytic movement of both inoculated species, including the area beyond the lesion, in both the upward and downward directions from the inoculation point were highly variable within replicates of each treatment. However, the total endophytic movement significantly differed between the co-inoculation treatments (P=0.012; Appendix C.5.15). The greatest endophytic movement of 258 mm was recorded for the co-inoculation with NP G22a3 and NL G51a2 and the least of 130 mm with NP G22a3 and NL A563.

The data of the endophytic pathogen movement for each species analysed separately in both the upward and downward direction showed the endophytic movement of *N. luteum* isolates in co-inoculated grapevines varied significantly from each other in both upward (P=0.012) and downward (P=0.021) directions from the inoculation points (Figure 4.19). In all the inoculations the upward endophytic movement of *N. luteum* was larger than the downward movements. Among the *N. luteum* isolates the furthest upward endophytic movement of 172.4 mm was obtained with isolate A563 when inoculated alone. This isolate (A563) produced the least downward endophytic movement when inoculated alone. A decrease in *N. luteum* endophytic movement was observed in all co-inoculations with *N. parvum* isolates when compared to their movement when inoculated alone.

Although the isolates were selected on the basis of low and high relative pathogenicity, the relative endophytic movement of the *N. parvum* isolates were not significantly different from each other in either the upward (P=0.092) or downward (P=0.090) directions from the inoculation points. However,

in single and co-inoculation treatments the endophytic movement of *N. parvum* was higher in an upward rather than downward direction from the inoculation point. In two co-inoculation treatments, NP A421+NL G51a2 and NP G22a3+NL G51a2 the upward endophytic movement of *N. parvum* was higher than when either of the *N. parvum* isolates were inoculated alone. In the remaining co-inoculation treatment, NP G22a3 and NL A563 the upward endophytic movement was reduced in both species relative to when they were inoculated alone (Figure 4.19).

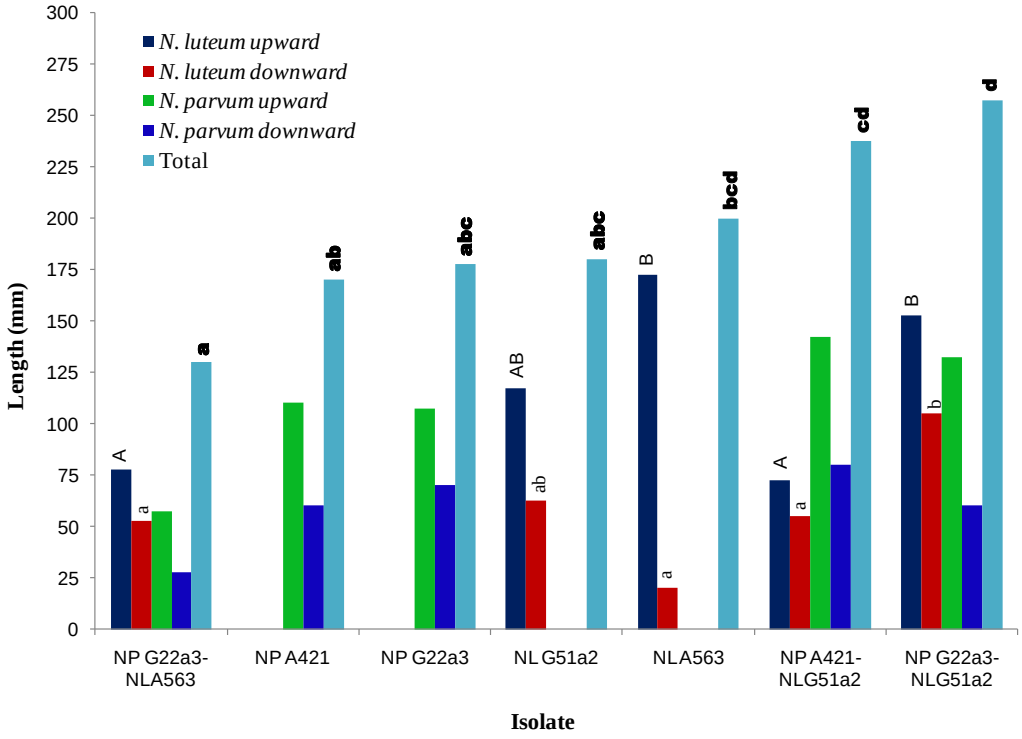

Figure 4.19: Mean endophytic movement of the isolates of *N. parvum* (NP) and *N. luteum* (NL) either alone or co-inoculated on the trunks of potted grapevines. The data presented consists of the mean lengths of upward and downward endophytic movement of each of these species from the inoculation point and the total mean lengths of movement. The bars with the same letters are not significantly different ($P \leq 0.05$) from each other.

4.3.5 Production of laccase by *Neofusicoccum parvum* isolates

The activity of both types of laccases, PPO-I and PPO-II, were measured in three *N. parvum* isolates, chosen for low (G121), medium (MM562) and high (G22a3) virulent towards grapevine. The data obtained in reactions with the DMP substrate, which is used to measure the PPO-II activity, was inconsistent and it was therefore eliminated from the analysis.

The activity level of PPO-I type laccase was significantly different between the three *N. parvum* isolates ($P<0.001$; Appendix C.5.16). Isolate G121 produced the highest level of 0.134 U ml^{-1} of PPO-I and this was five times that produced by isolate MM562, which was 0.027 U ml^{-1}. Although isolates G121 and MM562 grouped in the same genetic cluster they produced different lesion lengths on green shoots with 29 mm and 56 mm, respectively (Figure 4.4). An intermediate level of 0.086 U ml^{-1} of PPO-I was produced by isolate G22a3 (Figure 4.20) which had produced some of the largest lesions in both the green shoot and potted grapevine virulence assays.

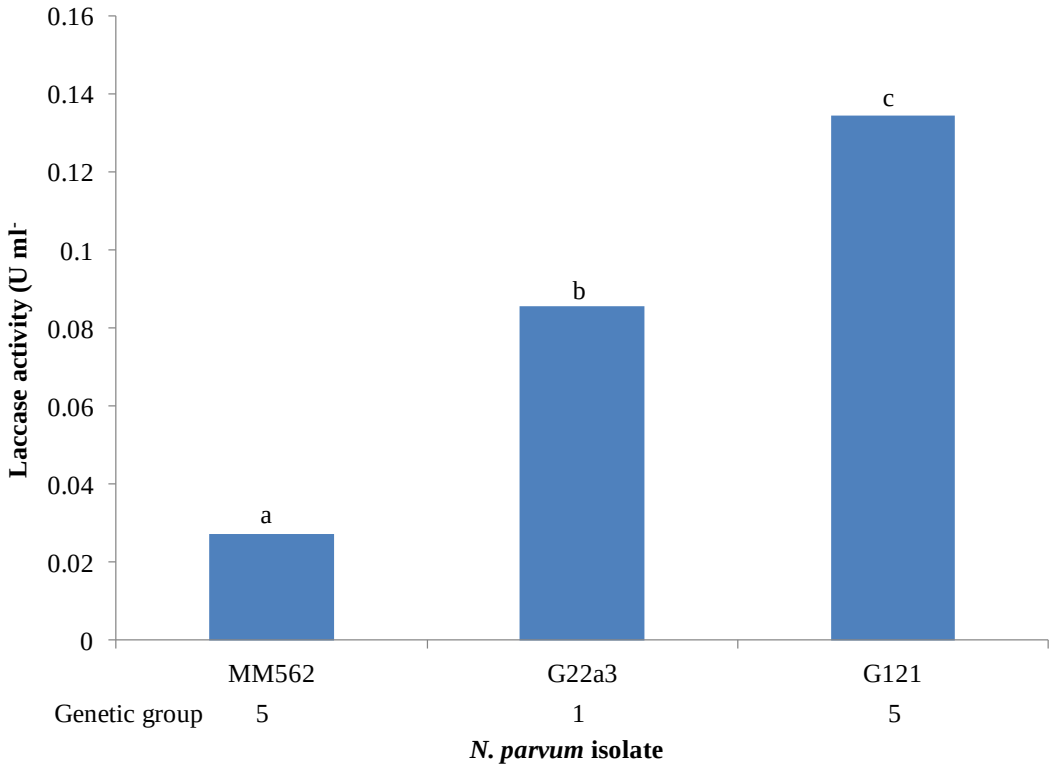

Figure 4.20: Extracellular laccase production (PPO-I) by three *Neofusicoccum parvum* isolates on Vogel basal salt medium in the present of veratryl alcohol. Bars with the same letters are statistically not significantly different ($P \leq 0.05$) by Fisher's protected LSD test.

4.4 Discussion

The aim of this study was to measure the intra-species variability in virulence of the botryosphaeriaceous species for which genetic diversity had been studied previously (Chapter 3), including *N. parvum*, *N. luteum*, *N. australe* and *D. mutila*, using grapevine green shoot and potted grapevine inoculations. For all four species the results showed large intra-species variations in virulence in both lesion development and endophytic pathogen movement in the grapevine tissue. For three of the species (*N. parvum*, *N. luteum* and *N. australe*), there was good relationship between the

lesions in green shoot and potted vine virulence assays, however, for the other species (*D. mutila*) there were differences in the relative ranking between the two assays. The relationship between the genetic groups the isolates belonged to in the neighbour joining tree and their virulence level was not strong but for some species (*N. parvum* and *N. luteum*) there appeared to be some relationship. The studies on pathogenicity of dual inoculations by *N. parvum* and *N. luteum* did not show any major effects on their levels of virulence on potted grapevines, indicating that there was no synergy or antagonism between these two species. However, co-inoculation by some isolates of these species did produce larger lesions downward from the inoculation point than those produced by either alone. A preliminary study indicated that both types of laccases, PPO-I and PPO-II were produced by *N. parvum*. The results from the four species are discussed in the following text in the same chronological order as the results section.

The *N. parvum* isolates produced a broad range of lesion lengths (11 mm to 83 mm) on grapevine green shoots 7 days after inoculation and 0 to 56 mm on potted vines 6 weeks after inoculation. *Neofusicoccum parvum* isolate variability in lesion lengths was also shown in a previous study of an additional three New Zealand isolates (Amponsah *et al*., 2008). Differences in the lesion sizes produced by *N. parvum* isolates is not restricted to grapevines but has also been reported on other hosts, including *Syzygium paniculatum* (Ploetz *et al*., 2009), *Eucalyptus* spp. (Gezahgne *et al*., 2003; Mohali *et al*., 2009) and *Terminalia* spp. (Begoude *et al*., 2010). This indicates that, irrespective of the host plant species, *N. parvum* isolates produce different levels of disease severity. This phenomenon has also been observed in other botryosphaeriaceous species. For example, van Niekerk *et al*. (2004) reported that some isolates of *D. seriata* and *N. australe* produced grapevine lesions that were twice the size of those caused by the remaining isolates. Similarly, Larignon *et al*. (2001) reported that isolates of *D. seriata* could be divided into four virulent groups based on the size of the lesions they produced on grapevines. Savocchia *et al*. (2007) also found significant differences in lesion lengths caused by *D. seriata* isolates both in green shoots and potted grapevine assays. This indicates that isolates within *N. parvum* can also be divided into different virulent groups. The range of lesion lengths produced by these *N. parvum* isolates reflects the relatively high degree of genetic diversity observed in the *N. parvum* population in New Zealand (Chapter 3). The genetic diversity of isolates within a population may be related to variability in physiological traits such as temperature tolerance, growth rate, virulence or host specialisation.

The *N. parvum* isolates used in this study were chosen to represent different genetic groups in the neighbour joining tree. This study extends the observations made in previous work which only studied the differences in virulence of *N. parvum* isolates without the knowledge of their genetic background (Amponsah *et al*., 2008; Plotz *et al* 2009; Gezahgne *et al*., 2003; Mohali *et al*., 2009; Begoude *et al*., 2010; Savocchia *et al*., 2007). The aim of this work was to determine whether any relationship could be made between the pathogenicity of *N. parvum* isolates and their position in the neighbour joining

tree. The majority (75%; or 3 out of 4) of the highly virulent *N. parvum* isolates that were tested were located in genetic group 1 in the neighbour joining tree and the weakly virulent isolates in genetic group 6 with one exception. This was the first study to investigate a relationship between genetic groups and virulence of botryosphaeriaceous species in grapevines. However, there has been a report of relationship between genetic groupings and virulence of some other grapevine fungal pathogens, the *Cylindrocarpon* spp. (Alaniz *et al*., 2009). Alaniz *et al*. (2009) reported that the *C. macrodidymum* isolates, which were selected from different genetic groups based on the ISSR fingerprints, showed significant differences in pathogenicity on grapevine seedlings between the ISSR groups.

In this study the lack of relationship between the genetic variability and their virulence suggests that the UP-PCR fingerprinting has little value for monitoring the development of new virulent genotypes of this fungal species. It is possible that the absence of strong relationship between the UP-PCR data and the virulence of isolates is due to the UP-PCR primers not amplifying the genes related to virulence such as those involved in the regulation and production of cell wall degrading enzymes. The UP-PCR primers were designed to primarily target intergenic, more variable regions of the genome (Bulat *et al*., 1998) perhaps other random molecular markers like AFLP or RAPDs may have produced better relationship between genetic diversity and pathogenicity of *N. parvum*. McDonald *et al*. (1995) suggested that it would be unlikely to find an association in a randomly mating population between DNA markers and pathogenicity or fungicide resistance genes. They believed that the best chance to find associations between DNA markers and other loci which are subjected to selection pressure (such as pathogenicity or fungicide resistance), is in a population which reproduces exclusively by asexual means. Therefore, further analysis with different molecular tools such as RAPD and RFLP and a greater number of loci or isolates may prove successful.

Plant pathogenic botryosphaeriaceous species primarily direct their attack onto the plant cell wall by producing key enzymes associated with its hydrolysis (Dekker *et al*., 2001). Thus, there are several virulent factors in these pathogens which may be important for their disease causing capacity, e.g. the cell wall degrading enzymes laccase and pectinase production. In previous work a botryosphaeriaceous species isolated from a stem canker on a eucalyptus was shown to be lignolytic and produce laccase constitutively *in vitro* (Barbosa *et al*., 1996). The results from this study demonstrated that *N. parvum* does produce both types of laccase (PPO-I and PPO-II) and that the amounts produced vary between isolates. However, in this preliminary study there was no relationship between the virulence of the isolate and the production of laccase. There are no prior reports of laccase production by *N. parvum* and only a single report available on the relationship between enzyme production (laccase and pectinase) and the genetic diversity of *L. theobromae* isolates (Saldanha *et al*., 2007). That research work measured the production of laccase and pectinase from eight *L. theobromae* which belonged to three RAPD genetic groups, and showed laccase production varied significantly between the eight isolates of *L. theobromae* with a strong relationship found between the genetic

groups and the production of laccase. However, there was no relationship found in pectinase production between the *L. theobromae* isolates from the different genetic groups (Saldanha *et al*., 2007). Also included in that study a single *N. ribis* isolate which was reported to produce the highest level of pectinase relative to the eight *L. theobromae* isolates. These results suggested that different botryosphaeriaceous species may produce different types of cell wall degrading enzymes that allow them to become successful pathogens in particular hosts. However, no data was provided in that study to help understand the relationship between the level of laccase production and the virulence of *L. theobromae* isolates. It is likely that multiple enzymes are involved and therefore correlation with a single enzyme may be unlikely. Further study is needed to measure the laccase production levels of a large number of *N. parvum* isolates which are from different virulent groups.

As variable levels of laccase did not explain the higher pathogenicity of some *N. parvum* isolates it is possible that other enzymes and or molecules involved in infection may be responsible. In addition to cell wall degrading enzymes, production of different phytotoxic metabolites have been identified in some botryosphaeriaceous species and may be involved in the pathogenicity of *N. parvum* and *N. luteum* in grapevine (Martos *et al*., 2008). Other studies have reported the production of bio-active toxic metabolites by isolates of botryosphaeriaceous species obtained from grapevine and non-grapevine hosts (Venkatasubbaiah & Chilton, 1990; Venkatasubbaiah *et al*., 1991; Barbosa *et al*., 2003; Djoukeng *et al*., 2009). To identify the mechanisms by which some *N. parvum* isolates are more pathogenic than others a more extensive study is needed to measure the production of different enzymes and/or phytotoxic metabolites using a larger number of isolates.

In addition to differences in enzyme production the variation in virulence observed between the isolates may be the result of an isolate being less adapted to that host and, therefore, having less efficient processes for recognition and initiation of infection. Many other studies have reported that *N. parvum* was isolated from other non-grapevine hosts in New Zealand including apple, black poplar, *Tibouchina* spp. and blueberry (Pennycook & Samuels, 1985; Sammonds *et al*., 2009). The presence of alternate hosts, many of which are grown in close proximity to viticulture areas, provides the potential for cross infection by the same *N. parvum* isolate. Unpublished work has demonstrated that isolates of *N. parvum* obtained from New Zealand grapevines were also pathogenic on blueberry (Che Omar pers. comm.). In addition, isolates of *D. mutila* and *N. australe* obtained from non-grapevine hosts, such as native ngaio, oak, plum, willow, apple and broom produced lesions on grapevines that were equivalent to those produced by grapevine isolates (Amponsah *et al*., 2008). Other studies have reported that cross-infection and movement of isolates of botryosphaeriaceous species between different hosts is most likely in affected areas (Ma *et al*., 2001; Pavlic *et al*., 2007). Recently Billones *et al*. (2010) showed that botryosphaeriaceous species can increase their known host range as *N. macroclavatum*, previously reported as a pathogen *Eucalyptus globulus* in Australia was recently reported on a grapevine sample in New Zealand. Therefore, *N. parvum* as a species is known to have a

broad host range, however, specific isolates may have different levels of adaptation to a particular host and this may explain their observed differences in virulence levels.

For the most virulent *N. parvum* isolate a lesion of 83 mm was produced on green shoots in 7 days and this demonstrated that this species was highly virulent to grapevines. In a previous study, Amponsah *et al.* (2008) reported that the lesion lengths produced by *N. parvum* isolates on green shoots of grapevine (Pinot noir) after 10 days was 59 mm which was smaller than the lesion lengths produced in this current study. The reason for these differences could be attributed to several factors such as grapevine cultivar, maturity of the green shoots, environmental conditions and isolate variations. This can be supported by the results obtained in a pathogenicity study conducted with nine botryosphaeriaceous species in California (Úrbez-Torres & Gubler, 2009). The results showed the most pathogenic species varied depending on the types of assays (green shoots and potted vine), inoculation types (spur and cordon inoculations) and grapevine cultivars. Overall in that study *N. parvum* was ranked as the third most virulent botryosphaeriaceous species on grapevine next to *L. theobromae* and *N. luteum*. However, different botryosphaeriaceous species have been reported as major pathogenic species on grapevines in different countries. This suggests that there are number of factors which determine the major virulence botryosphaeriaceous species on grapevine in different countries.

The relationship between the lesion lengths produced on green shoots and those on potted vines suggests that similar mechanisms of infection are operating in both systems. However, the mean lesion length produced by the most virulent isolate (G22a3) was 33% less (83 mm versus 56 mm) 6 weeks following inoculation on potted vines compared to that observed after 7 days in green shoots. In addition, the isolate with the smallest lesion on green shoots did not produce a lesion on the potted vines. Thus, although isolates of *N. parvum* can infect wounded grapevine tissues at different stages of phenological development (green shoots and mature cane) the rate of disease progression is decreased in older more woody material. This is probably due to the thicker and highly lignified cell wall in mature plant tissues which slows down fungal colonization. Furthermore, the potted vines may also produce physical and chemical defence mechanisms that are absent in detached shoots. The colonisation of the vascular system by some botryosphaeriaceous species on other hosts have been reported to cause tissue discolouration and clogging of vessels with tyloses (Maas & Uecker, 1984; Ramoes *et al*., 1991). Despite these differences, the consistent ranking of isolates between the assays suggests that the *in vitro* green shoot assay is useful as a rapid method to test the virulence of a large number of isolates (Savocchia *et al*., 2007; Amponsah *et al*., 2008; Úrbez-Torres and Gubler, 2009; Úrbez-Torres *et al*., 2009).

The pathogen re-isolation results from the inoculated grapevines showed that *N. parvum* could move endophytically within the potted vines. For most isolates the endophytic growth showed a similar

trend to the lesion data, with the pathogen progressing an extra ~25% further than the lesion in the two most pathogenic isolates. However, for an isolate with medium virulent (MM562) the endophytic movement was almost three times that of the visible lesion. For two isolates there were no visible lesions on the vines despite the pathogen progressing 8 to 30 mm. The browning (necrosis) of the vine tissue could be caused by either the phytotoxic metabolites produced by the pathogen (Martos *et al*., 2008) and/or the induction of plant defences (Maas & Uecker, 1984; Ramoes *et al*., 1991). The inability to produce lesions by some of the *N. parvum* isolates may be due to absent or low production of phytotoxic metabolites. In contrast, those isolates with high levels of phytotoxic metabolites or cell wall hydrolysis enzymes may colonize the grapevine tissue faster and produce larger lesions. An *in vitro* study of the grapevine trunk pathogen *E. lata* showed that there was a good relationship between the virulence and the toxicity of culture filtrate (Mauro *et al*., 1988). Although the infection and colonisation process of botryosphaeriaceous fungi is not well studied at cytological levels a few reports have suggested that after penetration the fungi can spread through the vascular system. A detailed study of the role of phytotoxic metabolites or cell wall hydrolysis enzymes in the process of tissue colonisation by botryosphaeriaceous fungi would be necessary as a first step in understanding why some isolates of *N. parvum* produce larger lesions than others on grapevine.

The mean lesion lengths caused by *N. luteum* inoculation on green shoots ranged from 10 to 89 mm in 5 days which showed that it was able to progress faster than *N. parvum*. In a previous study, Amponsah *et al.* (2008) reported that the lesion lengths produced by *N. parvum* isolates (59 mm) on green shoots of grapevine (Pinot noir) after 10 days were smaller than for *N. luteum* (76 mm). In that report *N. luteum* was reported as more virulent on grapevine green shoots than *N. australe*, *N. parvum*, *D. mutila* and *D. seriata* (Amponsah *et al*., 2008). In a similar observation from a pathogenicity study conducted with nine botryosphaeriaceous species in California (Úrbez-Torres & Gubler, 2009) *N. parvum* was ranked as the third most virulent species in Californian grapevine (Úrbez-Torres & Gubler, 2009) after *L. theobromae* and *N. luteum*. Their results are in agreement with the results of the current study that *N. luteum* is the most virulent species in New Zealand grapevines, despite *N. parvum* being the most predominant. However, different countries report different botryosphaeriaceous species as major virulent species on grapevine. This suggests that there are number of factors which influence the major virulent botryosphaeriaceous species on grapevine in different countries.

Unlike *N. parvum*, all five selected *N. luteum* isolates produced a lesion on potted grapevines. The mean lesion lengths measured on potted vines inoculated with *N. luteum* isolates ranged from 100 to 178 mm 6 weeks after inoculation, which was longer than the maximum length of 56 mm produced by *N. parvum* isolates. In a study conducted in Australia using excised one year old canes, *N. luteum* isolates produced different lesion lengths ranging from 49 mm to 52 mm on Chardonnay and from 22 mm to 23 mm on Shiraz grapevines 21 days after inoculation and one isolate of *N. luteum* produced a

mean lesion length of 27.2 mm on glasshouse potted grapevine variety Chardonnay 41 weeks after inoculation (Savocchia *et al*., 2007). Similar observation was made in a pathogenicity study conducted in California which also reported mean lesion lengths of 71.8 mm produced by a *N. luteum* isolate on 2 years old Thompson seedless grapevine (Úrbez-Torres & Gubler, 2009). This indicates that the lesion lengths produced by *N. luteum* isolates vary considerably and depend on the grapevine cultivar and types of grapevine tissue used for inoculation. Thus, absolute lesion length may not be the best measure of virulence. The larger mean lesion lengths of *N. luteum* indicates this species causes faster lesion development than *N. parvum* on potted vines and therefore may be producing different types or quantities of cell wall degrading enzymes and/or phytotoxic metabolites. Although a single report provides evidence of the production of phytotoxic compounds by *N. luteum* (Martos *et al*., 2008), there are no other reports regarding the production of cell wall degradation enzymes. The endophytic pathogen movement in potted vines showed that *N. luteum* moved beyond the necrotic lesion area which was similar to *N. parvum*. However, the endophytic movement of *N. luteum* beyond the lesion area was greater (98 mm) than for *N. parvum* (31 mm). This indicates that *N. luteum* isolates moved the furthest endophytically in grapevine tissue without causing a lesion. The reason for this difference could be related to their enzyme profile during the infection process as explained previously for their virulence.

Although isolates of *N. luteum* were the most virulent among the tested species in both assays the relationship between the green shoot and potted vine assays was not strong between the weakly virulent isolates (A526 and MM554). This result combined with the overall greater virulent of the *N. luteum* isolates may indicate a slightly different mechanism of infection for green shoots and potted vines. In an experiment carried out using detached grapevine green shoots inoculated with *D. seriata* and *N. luteum* it was shown that the lesion produced by *N. luteum* was not significantly different from the un-inoculated control, whereas, the same isolates produced substantial lesions (49 to 52.3 mm) on one-year old canes of grapevine cultivar Chardonnay (Savocchia, *et al*., 2007). In another example, results showed that *L. theobromae* and *D. porosum* were the most virulent in an *in vivo* mature cane assay but least virulent in an *in vitro* mature cane (Nickerk *et al*., 2004). This author attributed the variability to the interaction of the fungi with the host tissue at different stages of phenological development, the physiological characters of the host tissue, cultivar susceptibility, environmental conditions and length of incubation.

Similar to the results observed in *N. parvum*, there was no relationship between the virulence of the *N. luteum* isolates and their genetic groups on the neighbour joining tree. The lesion lengths produced by the isolates from the same genetic group varied significantly. For example, *N. luteum* isolates A526 and G51a2, which belong to genetic group 3, produced lesion lengths of 100 mm and 169 mm, respectively 6 weeks after inoculation on potted vine. A similar trend was also observed in green shoots assays, since isolates belonging to the same genetic group produced significantly different sized

lesions. This indicates that isolates from the same genetic groups varied in their virulence. The same explanation for *N. parvum* is valid for the lack of relationship between the virulence and the genetic groups of *N. luteum* isolates.

The lesion length produced on green shoots by the *N. australe* isolates ranged from 30 mm to 83 mm after 7 days which was a similar range to the lesions caused by *N. parvum* although the range was narrower. The range of lesion lengths produced on potted vine was 47 mm to 139 mm 6 weeks after inoculation which showed that *N. australe* was the second most virulent species on grapevines. Although *N. luteum* and *N. australe* are very closely related species (Slippers *et al*., 2004b), this result indicates that this species is less virulent on grapevine than *N. luteum*. In a previous study *N. australe* produced lesion of 31 –36 mm on green shoots and 77 mm on cordons of grapevine variety Red Globe and was ranked the second most virulent species on grapevine next to *L. theobromae* (Úrbez-Torres & Gubler, 2009). However, in the same study *N. australe* produced mean lesion lengths of 40 mm on 2 years old Thomson seedless grapevine variety and was ranked as the fourth most virulent species after *L. theobromae*, *N. luteum* and *N. parvum* (Úrbez-Torres & Gubler, 2009). This indicates that the virulence of *N. australe* is determined by several factors including grapevine cultivar, types of virulence assays and the isolate variation.

Similar to *N. parvum*, a good relationship was observed between the green shoots and potted vine assays. This indicates the mechanism of infection process of *N. australe* is similar in different grapevine tissues. Endophytic movement of *N. australe* was also observed beyond the lesion in grapevine tissue, but, unlike *N. parvum* and *N. luteum*, the endophytic movement of the low virulent isolates (B3212 and B3414) which produced small mean lesion lengths were equal to the medium virulent isolates (CB-P74 and Kat-1). This indicates that there is greater endophytic movement of the low virulent isolates in grapevine tissue compared with the external symptoms on the grapevine trunks. The reason for these differences is unknown and it is possibly related to their enzyme profile during the infection process as explained previously for *N. parvum* and *N. luteum*. There was no relationship between the genetic groups and the virulence of *N. australe* isolates in both assays and this is similar to that seen for *N. parvum* and *N. luteum*. The reason for the lack of relationship could be as described for *N. parvum*.

The mean lesion lengths of *D. mutila* ranged from 8 to 52 mm on green shoots 7 days after inoculation and 39 to 84 mm on potted vines 6 weeks after inoculation. However, the majority of isolates (86% or 12/15) produced lesions ≤ 25 mm, with only two isolates exceeding that in the green shoots. However, in the potted vine assay a greater range in lesion sizes were observed in the five isolates tested. The reason for there being less variability in the mean lesion lengths produced by *D. mutila* isolates on green shoots compared to potted vines is unknown. Similar to the *Neofusicoccum* species, the disease development on potted vines was slower than on green shoots. This suggests that as for *N. parvum* and

N. australe, D. mutila has a similar infection mechanism for different grapevine tissues. The results of the green shoots showed *D. mutila* was less virulent on grapevine compared to the *Neofusicoccum* species tested in this study. This result is in agreement with the previous virulence study conducted in New Zealand using grapevine green shoots, where *D. mutila* was ranked as the fourth most virulent after *N. luteum, N. australe* and *N. parvum* (Amponsah *et al*., 2008). However the mean lesion lengths caused by *D. mutila* isolates on potted vines were greater than for *N. parvum* and smaller than for *N. luteum* and *N. australe*. There are conflicting reports on the virulence of *D. mutila* in other countries. This species was first reported as a causal agent of black dead arm disease of grapevine in Hungary (Lehoczky, 1988). It was considered as a weak pathogen in Portugal (Phillips, 2002), Australia (Taylor *et al*., 2005) and California (Úrbez Torres & Gubler, 2009). In contrast, *D. mutila* was considered as highly virulent in South Africa (van Niekerk *et al*., 2004). This suggests that different factors might be involved in the selection or expression of virulence by this species in different countries. Factors may include variation in the genetic background of isolates, grapevine cultivars or the environmental conditions.

The relationship between position in the neighbour joining tree and relative virulence for *N. parvum* and *N. luteum* was further investigated by selecting three isolates each from branches containing the isolates displaying the weakest and strongest virulent. Similar to this experiment, in another study by Alaniz *et al*. (2009) selected isolates from different ISSR genetic groups of *C. macrodidymum* and showed variation in virulence between the genetic groups. In another study, the isolates selected from different populations of *E. lata* with variable genetic diversity showed a range of aggressiveness in disease development on grapevine (Péros and Berger, 2003). In contrast, the isolates of this study were selected by different criteria, being from the genetic groups that included either high or low virulent isolates based on the results obtained from the previous experiments. The results showed that within each genetic group variable virulence levels for *N. parvum* and *N. luteum* isolates were observed on potted grapevine after 6 weeks of inoculation.

The *N. parvum* isolates from two genetic groups, groups 6 (low virulent) and 1 (high virulent) produced different lesion lengths. All three *N. parvum* isolates belonging to a putative highly virulent group produced lesions ranging 110 mm to 178 mm consistent with a relationship between position on the neighbour joining tree and high virulence. In contrast, although the lesions produced by two isolates from the low virulent group did not significantly differ from the control, one isolate (A813) produced a mean lesion of 161 mm which showed that it was highly virulent. Thus, five out of the six isolates selected in this work showed good relationship between position in the neighbour joining tree and virulence. Similar to this result, Alaniz *et al*. (2009) showed that although seven genetic groups were identified in the *C. macrodidymum* population, the isolates belonging to two ISSR genetic groups were highly virulent on grapevines compared with isolates from other genetic groups. They also reported that a few isolates within the highly virulent genetic groups varied significantly in their

virulence. This indicated that although the isolates belonged to the same genetic group their virulence could vary considerably. In contrast to the results for *N. parvum*, there was less association between position in the neighbour joining tree and virulence of *N. luteum* isolates. Out of four *N. luteum* isolates from the low virulent group (genetic group 5), three isolates produced mean lesion lengths ranging from 21 mm to 147 mm and the fourth isolate CC456, produced the greatest mean lesion lengths among all the tested *N. luteum* isolates. Similarly the *N. luteum* isolate A526 belonging to high virulent group (genetic group 3) produced a lesion length which was not significantly different from the mean lesion lengths of the low virulent group. Although most of the isolates belonging to the high virulent group produced greater mean lesion lengths and the low virulent group produced lower mean lesion lengths in both species, there was also variability in the virulence of isolates within a genetic group. The lack of relationship indicates that, the UP-PCR genetic group does not reflect virulence levels as explained previously. Further work that increases the number of loci used to construct the neighbour joining tree and assesses a larger number of isolates from each group may strengthen the association.

Although several authors have shown that grapevines are often infected by multiple botryosphaeriaceous species in a single vineyard (Úrbez-Torres *et al*., 2006a; Pitt *et al*., 2010; Amponsah *et al*., 2007) this is the first study of how multiple infections within a lesion area affect virulence of botryosphaeriaceous species. In this study multiple botryosphaeriaceous species were isolated from a single grapevine lesion (Chapter 2). Among them *N. luteum* and *N. parvum* were identified as the most frequently isolated co-habiting species (20%). Although different botryosphaeriaceous species were found together, the reason is unknown for why these two species are most frequently found together. There are no detailed reports available on occurrence of multiple botryosphaeriaceous species in a lesion on grapevine or any other host. The mean lesion lengths caused by co-inoculation of *N. luteum* and *N. parvum* isolates showed variation compared to the respective isolates inoculated alone on potted grapevine.

As the work in this chapter had already identified strong and weak virulent for *N. parvum* and *N. luteum* this information was incorporated into the experimental process by combining isolates of different virulence. There were some differences observed in virulence ranking of *N. parvum* and *N. luteum* isolates in this assay when inoculated alone, compared to the results of the previous assays. However, the overall results showed that there was no synergistic or antagonistic effect in co-inoculation, as measured by total lesion lengths after 6 weeks of inoculation onto whole plants. In contrast, when distance from the inoculation point was analysed separately for each direction there were some differences noted. There was a significant increase in downward mean lesion lengths from the inoculation point when these species were co-inoculated at all combinations. The greater downward lesion lengths may be explained by either or both species trying to colonize unoccupied tissue to avoid competition for nutrients and/or the combined action of the enzymes from both species permitting greater downward movement of these species.

The movement of *N. parvum* isolates did not show any significant variation both in the upward and downward directions. However, the *N. luteum* isolates showed significant variation in movement and the trend for greater downward movement when co-inoculated with *N. parvum* compared to individual isolate inoculation. This reinforces the earlier statement that this is due to either avoiding competition for nutrients or combined enzyme activity.

In summary this study reports the virulence of four commonly isolated botryosphaeriaceous species, *N. parvum*, *N. luteum*, *N. australe* and *D. mutila.* Although all four botryosphaeriaceous species were able to cause disease there was substantial variation between the disease-causing ability of individual isolates on the grapevine cultivar Sauvignon Blanc. As expected the disease progression of these four species was slower in potted vines than in green shoots. There was good relationship between green shoot and potted vine assays for *N. parvum*, *N. australe* and *D. mutila* but less for *N. luteum*. The most virulent species based on total mean lesion length on potted vines was *N. luteum* followed by *N. australe*, *D. mutila* and *N. parvum*. The non-grapevine botryosphaeriaceous isolates were equally virulent on grapevines indicating that there is no impediment to movement of botryosphaeriaceous species between these hosts. There was some relationship between genetic group and the virulence of the isolates for *N. parvum* but this was not demonstrated for *N. luteum*. The co-inoculation of *N. luteum* and *N. parvum* species did not show any synergy or antagonism overall but there was some evidence that co-infection could increase downward movement from the inoculation point. The results showed that *N. parvum* produces laccase but the quantity of this enzyme produced by isolates does not appear to be the main driver of their virulence.

Chapter 5
Development of isolate-specific markers for *Neofusicoccum parvum* and *N. luteum* and their use to study splash dispersal of conidia

5.1 Introduction

Generally, molecular identification of plant pathogenic fungi is based on the detection of polymorphisms in ubiquitously conserved genes, such as ribosomal RNA, β-tubulin or translation elongation factor (EF)-1α (McCartney *et al*., 2003; Lievens *et al*., 2005a). Although this strategy is successful for species identification, even when different species can only be discriminated by a single nucleotide polymorphism (Lievens *et al*., 2006), these genes do not generally allow discrimination below species level (Lievens *et al*., 2003). Therefore, additional strategies have been use to identify DNA sequences that could be used to distinguish individuals within a population. Isolate-specific markers have been developed using approaches such as random-amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) or amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995). Markers identified with these approaches can be used to design specific sequence-characterized amplified region (SCAR) primers (Goldstein *et al.*, 2000) that specifically amplify the selected markers in individual isolates resulting in a robust identification assay (McDermott *et al*., 1994; Larsen *et al*., 2002).

SCAR markers are widely used in all areas of fungal biology studies with many examples of use in field detection of isolates (Hermosa *et al*., 2000; Nunes *et al*., 2008). For example, an isolate-specific marker was developed to detect *Colletotrichum coccoides*, a prospective bioherbicide for velvetleaf (*Abutilon theophrasti*), in field samples (Dauch *et al*., 2003). In another study, isolate-specific primers were developed to distinguish an isolate of the sclerotial mycoparasite *Coniothyrium minitans* (Goldstein *et al.*, 2000). The PCR assay developed was used to study the ecology of this potential biological control agent in soil (Ridgway & Stewart, 2000). In a genetic variation study of *Phaeomoniella chlamydospora* using UP-PCR, an isolate-specific marker was identified and developed for tracking *P. chlamydospora* infection in grapevine (Ridgway *et al*., 2005). These studies illustrate that isolate-specific markers are powerful molecular tools for studying the ecology of a target fungal species in its ecological niche.

In recent years, various molecular techniques have been used to develop species-specific markers for botryosphaeriaceous species. These techniques include dominant and codominant molecular markers such as RAPDs, ISSRs and microsatellites (Burgess *et al*., 2001; Smith and Stanosz 2001; Zhou *et al*., 2001) and sequence data of ITS, β-tubulin and EF1-α DNA regions (Jacobs and Rehner 1998; Denman

et al., 2000; Zhou and Stanosz 2001a; Phillips *et al.*, 2002; Slippers *et al*., 2004a). Simple sequence repeat (SSR) or microsatellite markers have been developed for some botryosphaeriaceous fungi such as *Diplodia pinea* and *Lasiodiplodia theobromae* (Burgess *et al*., 2003). In another study SSR markers were developed for the identification of *N. parvum* (Slippers *et al*., 2004b). Although several species-specific markers have been developed, no one has developed isolates-specific markers for any of the botryosphaeriaceous species. Appropriate storage of isolates is important to preserve the integrity of the assays as many plant fungal pathogens can undergo recombination in nature. Epidemiological studies using marker strains should always utilize the stored isolates and not that recovered from the environment.

The aim of this study was to develop molecular assays for the isolate-specific bands that had been identified in genetic diversity analysis of *N. parvum* and *N. luteum* using UP-PCR. Initially the specificity and sensitivity of the developed markers were optimised and then the molecular tool applied to determine the distance of rainwater splash dispersal of *N. parvum* and *N. luteum* in the field.

5.2 Materials and methods

5.2.1 Isolation of marker bands

During genetic variation analysis using UP-PCR primer AA2M2, two isolate-specific bands (later termed as isolate-specific markers) were identified in *N. parvum* isolates B2141 and UCD646So and an isolate-specific band was identified in *N. luteum* isolate G51a2 (later termed as marker isolate). For the *N. parvum* markers, the isolate-specific marker was only developed for *N. parvum* isolate B2141 (later termed as the marker isolate) which was isolated from grapevines in New Zealand. The other isolate-specific marker identified in the international isolate UCD646So was not developed due to the restriction of using imported organisms in field experiments (ERMA NZ regulations). The identified markers were converted into specific sequence-characterized amplified regions (SCAR) and primers were designed for use in PCR assays.

The isolate-specific bands were excised from agarose gels using a 200 µL sterile pipette tip and used as template for subsequent re-amplification with the respective UP-PCR primer as described in Section 3.2.3.2. To confirm the correct size of the excised and reamplified band it was run on a 1% agarose gel alongside the UP-PCR fingerprint from which it was derived at 10 V/cm for 50 min as described in Section 3.2.3.3. PCR products were used directly for cloning if they were single bright bands. If multiple or weak bands were produced following excision and re-amplification, the original isolate-specific band was excised from the gel using a sterile scalpel and the PCR product was extracted using HiYield Gel/ PCR DNA extraction kit (Real Genomics) according to manufacturer's

instructions. Gel extracted PCR products were checked for purity and quantity by agarose gel electrophoresis and then used for cloning without re-amplification.

5.2.2 Cloning of isolates specific band into the pGEM-T vector

Purified isolate-specific bands were ligated individually into the pGEM-T®Easy vector using TA cloning according to manufacturer's instructions (Promega Corporation). Each 10 µL ligation mixture consisted 5 µL of 2 x buffer, 1 µL of pGEM-T®Easy vector, 1 µL of PCR product, 1 µL T4 DNA ligase (3 U/µL) and 2 µL of sterile water. A positive control was prepared using the DNA supplied with the kit. The ligations were incubated overnight at 4ºC.

5.2.3 Transformation of cloned pGEM-T vectors into *Escherichia coli*

Each ligated isolate-specific band was transformed into *Escherichia coli* strain DH5α competent cells (Promega). A 40 µL aliquot of DH5α *E. coli* competent cells was allowed to thaw on ice and 2 µL of ligated vector was added and mixed by stirring gently with a sterile pipette tip. The resultant mixture was incubated on ice for 30 min, heat shocked for 40 s at 42ºC and placed on ice for 2 min. A 250 µL aliquot of SOC medium (Appendix D.1) was added and the samples were incubated for 1 h at 37ºC to allow expression of ampicillin resistance. Agar plates (Luria Bertani [LB]; Appendix D.2) supplemented with ampicillin (100 µg/ mL) were each spread with 20 µL of X-Gal (bromo-chloro-indolyl-galactopyranoside; 50 mg/mL) and pre-heated to 37ºC. A 100 µL aliquot of each transformant mixture was spread onto a separate LB plate using a sterile metal loop and the plates were incubated at 37ºC overnight. Three replicate plates were made for each transformation.

5.2.4 Colony PCR to confirm *E. coli* containing the isolate-specific band

The incubated plates were kept in the fridge (4ºC) for 1 h to develop a blue colour and differentiate the non-transformed *E. coli* from the transformed *E. coli* cells. Colony PCR was done in a 25 µL reaction volume that consisted of 10 mM Tris pH 8.0, 50 mM KCl, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 10 pmole of primer M13f (5'-GTTTTCCCAGTCACGACGTTGTA-3'), 10 pmole of primer M13r (5'-CAGGAAACAGCTATACC-3') and 1.25 U *Taq* DNA polymerase (Roche Diagnostics). Up to ten white colonies from each of the LB plates were picked up using a sterile 10 µL pipette tip and directly added to the PCR mixture as template. The PCR amplification was performed as follows: denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 30 s, annealing temperature of 60 ºC for 20 s and extension at 72 ºC for 1 min, with the final extension of 72ºC for 10 min. At the same time the same colonies were used to inoculate 10 mL of LB broth supplemented with ampicillin (50 µg/ mL) in a 25 mL universal bottle. The LB broth cultures were incubated at 37ºC overnight under continuous shaking at 180 rpm (Ratek Orbital mixer incubator, Australia). The amplified PCR products and the UP-PCR product of

the respective isolates were separated by 1% agarose gel electrophoresis at 10 V/cm for 3 h in 1 × TAE. Gels were stained and visualized as described in Section 3.2.3.3.

5.2.5 Plasmid isolation and *EcoR*I restriction digestion

Colonies containing a PCR product of the correct size were selected for plasmid isolation. Plasmid isolation was done from broth culture using the Zyppy™ plasmid isolation miniprep kit (Zymo Research) according to the manufacturer's instructions. To confirm the size of the insert, 10 µL of the isolated plasmids were digested with *EcoR*I restriction endonuclease (BioLabs) according to the manufacturer's instructions at 37ºC for 1 h, followed by heat inactivation at 65ºC for 20 min. The digested products were separated by 1% agarose gel electrophoresis at 10 V/cm for 3 h as described in Section 3.2.3.3.

5.2.6 Sequencing and primer design

After confirming the plasmid contained an insert of the correct size, each insert was sequenced in both directions using the M13f and M13r primers at the Lincoln University Sequencing Facility. The returned sequence data of each isolate-specific marker was analysed using DNAMAN (Version: 4.0a; Lynnon BioSoft) to remove the portions of the vector sequence. The isolate-specific marker sequences were submitted to GenBank using the BLAST function to identify any similarity to known fungal DNA sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The translated isolate-specific marker was also submitted to GenBank using the tblastx search tool. Forward and reverse primers were designed to the DNA sequence of these isolate-specific SCAR markers. The criteria were used to select primer as described by McPherson and Moller (2006).

5.2.7 Specificity of the isolate specific PCR

Each isolate-specific PCR was tested for specificity with the DNA of all the isolates for that species that had been used in the genetic diversity study (Chapter 3; approximately 50 isolates). Each PCR was done in a 25 µL reaction volume consisting of 10 mM Tris pH 8.0, 50 mM KCl, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 10 pmole of forward primer, 10 pmole of reverse primer and 1.25 U *Taq* DNA polymerase (Roche Diagnostics). PCR amplification of the *N. parvum* isolate-specific marker (BP-1-42) was done as follows: denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 30 s, annealing temperature of 60ºC for 30 s and extension at 72ºC for 90 s, with a final extension at 72ºC for 10 min. The same amplification conditions were used for *N. luteum* isolate-specific marker (BL-1-14) except the extension was at 72 ºC for 1 min. The amplified PCR products were separated by 1% agarose gel electrophoresis at 10 V/cm for 50 min and visualised as described in Section 2.2.7.1. The specificity of *N. parvum* and *N. luteum* isolate-specific markers was tested against two representative isolates of

each of the seven other botryosphaeriaceous species found in New Zealand grapevines namely, *N. parvum, N. luteum, N. australe, N. ribis, D. mutila, D. seriata, Do. iberica* and *Do. sarmentorum*.

5.2.8 Development of PCR-RFLP for *N. parvum* marker isolate

In silico restriction analysis was carried out to determine if the polymorphisms present in the sequence of the *N. parvum* isolate-specific marker (BP-1-42) could be used to differentiate marker isolate B2141 from other *N. parvum* isolates. The forward sequences of *N. parvum* marker isolate B2141 and the forward sequence of *N. parvum* non- marker isolates G233 was used for the restriction analysis using DNAMAN (Version: 4.0a; Lynnon BioSoft).

The *Taq*I restriction enzyme was used to differentiate the *N. parvum* marker isolate B2141 from non-marker isolates. The PCR amplification of 60 *N. parvum* isolates including the marker isolate B2141 was done using *N. parvum* isolate-specific marker (BP-1-42) primers as described in Section 5.2.8. A 10 µL aliquot of the PCR product was digested with 2 U Taq$^{\alpha}$1 (*Taq*I; BioLab) restriction endonuclease at 65ºC for 2 h followed by heat inactivation at 80ºC for 20 min and the resulting fragments were separated by electrophoresis on 1.5 % agarose gel for 50 min at 10 V/cm in 1 × TAE.

5.2.9 Sensitivity of the isolate specific PCR

The sensitivity of both isolate-specific PCR assays were tested with different concentrations of genomic DNA from the isolate that produced the marker band (termed marked isolate) in 10 fold dilutions from 10 ng/µL to 50 fg/µL. To increase the sensitivity of the PCR the number of cycles was increased to 40.

For the *N. parvum* isolate-specific marker BP-1-42 the sensitivity of PCR was increased by nested PCR. To increase the sensitivity in the nested PCR assay, internal (nested) forward and reverse primers were designed. The internal forward primer BP-1-42intF (5'-CCATGTGAAGTGGACCCAGA-3') with a melting temperature (Tm) of 62.0°C was 71 bp from 5'-end of the marker band and the reverse primer BP-1-42intR (5'-CTTCCTGAAATGACACCCGA-3') with a Tm of 60.0°C was 1099 bp from 3'- end of the marker band. Together they would amplify a 403 bp internal segment of the marker band. For nested PCR a primary PCR was done using primers BP-1-42F and BP-1-42R for 40 cycles. This product was diluted 50× and a 1 µL aliquot of the dilution used as the template for the secondary PCR using the internal primer pair BP-1-42intF and BP-1-42intR with the same amplification conditions.

5.2.10 Dispersal distance of *N. parvum* and *N. luteum* conidia during rainfall

A field experiment using the marked isolates was done to determine the distance of conidial dispersal by *N. parvum* and *N. luteum* during rainfall. This experiment was done in the Lincoln University vineyard during early spring 2009 using *in-vitro* inoculated grapevine shoots as a source of conidia.

5.2.10.1 In vitro inoculum production

Grapevine shoots with oozing pycnidia were produced *in vitro* by inoculating grapevine green shoots with the marker strains of *N. parvum* or *N. luteum* as described by Amponsah *et al*. (2008). Green shoots were obtained from 1 year old shade house grown potted Sauvignon Blanc grapevines and 15 shoots used for *in vitro* inoculation as described in Section 4.2.1.2 with the isolates of each species from which the SCAR markers were derived. Once the lesion had developed for 5 days post inoculation, the lesion areas of the green shoots were excised and allowed to air dry for 2 days then stored at 4ºC in a sealed container until used for field inoculations. Immediately prior to placing them in the field the grapevine shoot lesions were removed from storage at 4°C and incubated in a deep Petri dish lined with moist filter paper, covered and sealed to maintain high moisture condition for 24 h to induce the oozing of conidia.

5.2.10.2 Experimental set up in the vineyard

The experiment was set up in three independent locations in the vineyard (total area of 1 ha) prior to budburst in early spring 2009 during the start of a forecast rain event. A grapevine with a lateral cordon was selected and two oozing grapevine shoot lesions for each marker isolate were fixed to the top of the vine as shown in Figure 5.1. Ten cm diameter plastic water collection cups (500 mL) were placed on the ground at 50 cm intervals for up to 3 m in four directions (North, East, West, South) from the site of the inoculated canes (Figure 5.2). To confirm there was no natural infection of *N. parvum* and *N. luteum* in the experimental vineyard, the water sample (-ve sample) also collected 10 rows away (25 m) from the inoculation point.

5.2.10.3 Water sample collection and microscopic observation

After overnight rainfall, the rainwater in the collection cups was analysed for the detection of both marker isolates. Water samples from the three sites were stored frozen at -20ºC until used for DNA extraction. The rainfall and wind speed data at the experimental area (Lincoln) was obtained from the NIWA website using the database query form for the sample collection data (http://cliflo.niwa.co.nz).

The thawed water samples were centrifuged at 10ºC for 15 min at 10,000 × *g* to collect the conidia and the resulting pellet was re-suspended in 500 µL sterile water. Duplicate water samples from each collection point were examined using a light microscope (×100 and ×400; Olympus Optical, Japan) to determine whether conidia of *N. parvum* and *N. luteum* were present.

Figure 5.1: The oozing pycnidial colonised grapevine shoots were fixed to the top of the grapevine in the vineyard. The yellow arrow indicates the pycnidia that were oozing conidia at the time of placement.

Figure 5.2: The plastic water collection cups were placed on the ground around the inoculum that had been fixed to a grapevine, and positioned in four directions at 50 cm intervals for up to 3 m.

5.2.10.4 DNA extraction and PCR amplification

DNA was extracted from a 300 µL aliquot (60%) of the water sample using the PowerSoil™ DNA isolation kit (MO BIO laboratories, USA) according to manufacturer's instructions. The isolate-specific PCR reactions were carried out using the extracted DNA from the water samples as templates with the *N. parvum* or *N. luteum* isolate-specific marker. The same PCR reaction conditions were used for both markers as described in Section 5.2.8 except that the number of PCR cycles were increased from 35 to 40. For the detection of the *N. parvum* marked isolate, a nested PCR amplification was performed with external and internal primers of the *N. parvum* isolate-specific PCR-RFLP as described in Section 5.2.8.

5.3 Results

5.3.1 Identification of marker bands

The UP-PCR fingerprints (Section 3.3.2) revealed two isolate-specific bands when *N. parvum* isolates were amplified with primer AA2M2. An isolate-specific band of ~1500 bp was identified in *N. parvum* isolate B2141 and a ~400 bp isolate specific band was identified in *N. parvum* isolate UCD646So which had been imported from Californian, USA (Figure 5.3).

Among the *N. luteum* isolates used in genetic diversity analysis, isolate G51a2 which was isolated from the grapevine samples from Gisborne, produced an isolate-specific band of ~850 bp size in the UP-PCR fingerprints generated with primer AA2M2 (Figure 5.4).

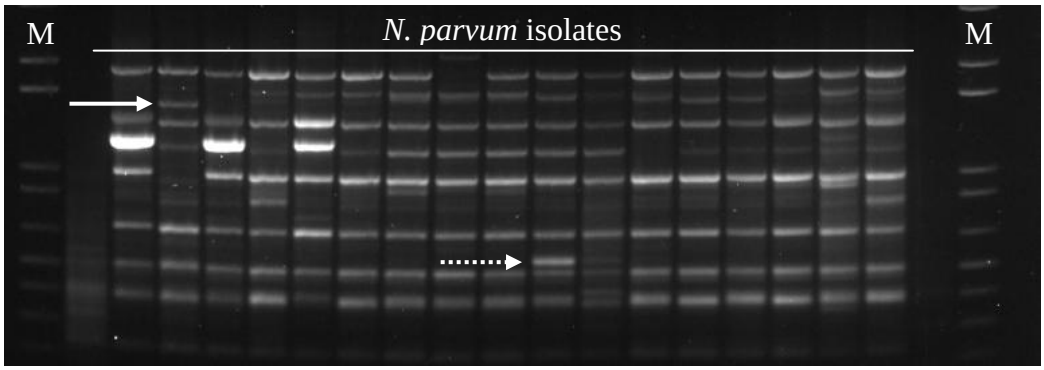

Figure 5.3: UP-PCR (AA2M2) fingerprint of *N. parvum* isolates showing two potential isolate-specific bands identified, in a New Zealand isolate (solid arrow, B2141) and in a Californian isolate (dotted arrow, UCD646So). M = 1 kb plus DNA ladder (Invitrogen)

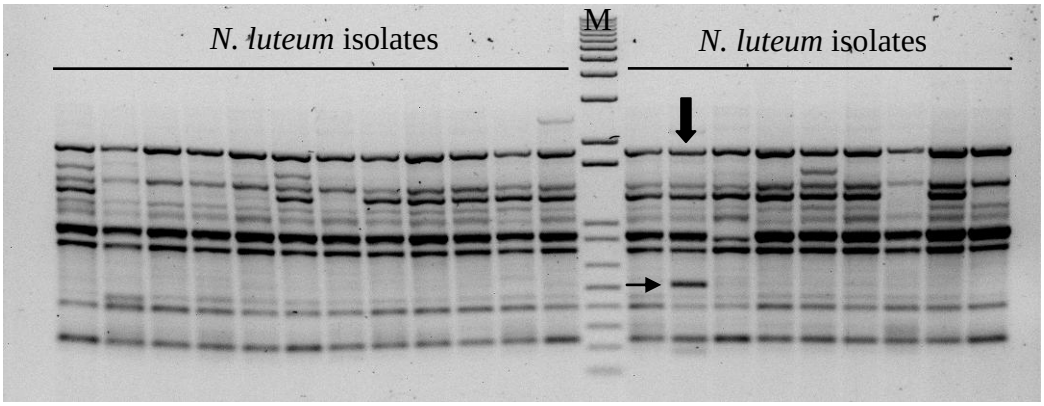

Figure 5.4: UP-PCR fingerprints of *N. luteum* isolates generated with the primer AA2M2 showing potential isolate-specific band (~850 bp) in a New Zealand isolate G51a2. M = 1 kb plus DNA ladder (Invitrogen).

5.3.2 Sequence characterisation of the molecular markers

The identified isolate-specific bands of *N. parvum* and *N. luteum* were converted into isolate-specific SCAR markers by cloning and sequencing. For the *N. luteum* isolate G51a2, the specific band obtained in the PCR amplification was excised and directly used for cloning as it produced a strongly amplified isolate-specific band (Figure 5.6). However, similar PCR amplification of the *N. parvum* isolate-specific band produced a large number of non-specific bands and therefore was unsuitable for direct cloning (Figure 5.5). Extraction of the isolate-specific band using a gel PCR extraction kit yielded a single band that was used for cloning (results not shown).

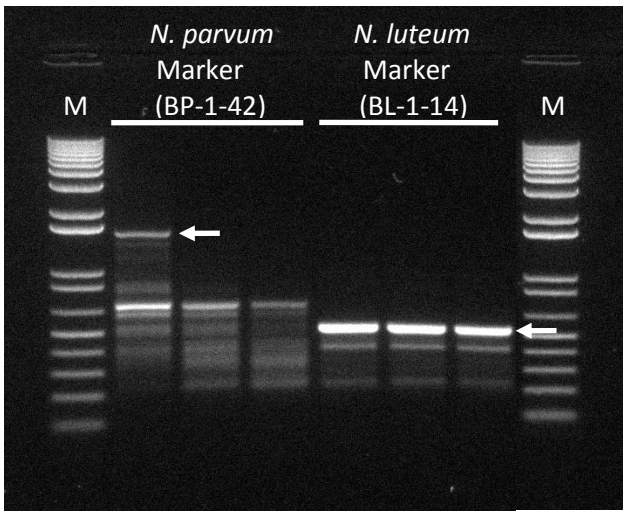

Figure 5.5: PCR amplification of isolate-specific bands using the templates excised from previous UP-PCR gels. The isolate-specific bands are indicated by arrows. M = 1 kb plus DNA ladder (Invitrogen)

5.3.3 Development of *N. parvum* isolate specific marker

5.3.3.1 Selection of E. coli transformants with N. parvum *isolate-specific band (BP-1-42)*

After cloning and transformation of *N. parvum* isolate-specific band BP-1-42 into *E. coli*, a colony with the expected PCR product size of ~1750 bp (colony 11) was selected (Figure 5.6A). The expected ~1750 bp amplicon consisted of the isolate-specific band of ~1500 bp and the 257 bp vector flanking region. The correct size of the insert was confirmed using *EcoR*I restriction digestion to remove the vector flanks from the ~1750 bp PCR product and demonstrating that the band was of equivalent size to the original band in the UP-PCR fingerprint with primer AA2M2 (Figure 5.6B).

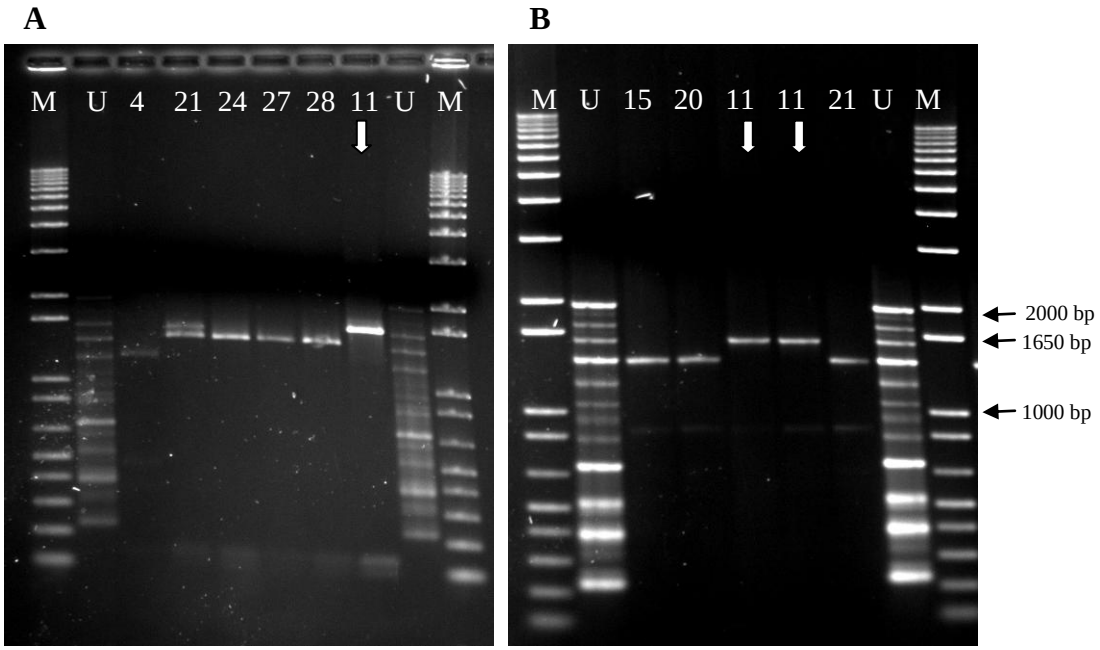

Figure 5.6: Confirmation of the size of the *N. parvum* BP-1-42 isolate-specific band using *EcoR*I restriction digestion of the colony PCR product indicated by arrow (A) and UP-PCR amplification with AA2M2 primer (B) of the plasmid extracted from *E. coli* colonies. Letter U is the UP-PCR product of *N. parvum* isolate B2141, numbers indicate the colony numbers and M- 1 kb plus DNA ladder (Invitrogen).

5.3.3.2 Marker sequence analysis and primer designing

DNA sequencing of the insert from colony 11 showed that the isolate-specific band was 1573 bp in size. A forward primer BP-1-42F (5-AGAGCGGATACAACGTCAGT-3') with a Tm of 60.0°C was designed 9 bp from 5' end and a reverse primer BP-1-42R (5'-GGAGAGGAGAAAGTAGTGTG-3') with a Tm of 63.0°C was designed 14 bp from 3' end to amplify a product of 1550 bp (Appendix D.3).

5.3.3.3 Development of the isolate-specific PCR-RFLP assay

A single PCR amplicon (1550 bp) was obtained in *N. parvum* isolates amplified with BP-1-42F and BP-1-42R primers (Figure 5.7). All the *N. parvum* isolates produced the same size band. The alignment of the sequence of the 1573 bp band of isolate B2141 with the 1474 bp nucleotide sequence from five other *N. parvum* isolates obtained using primers BP-1-42F and BP-1-42R showed that the sequence of isolate B2141 contained five single nucleotide polymorphisms (SNPs) at positions 168, 526, 775, 1220 and 1278 (0.34% polymorphism) which were different from non-marker isolates (Appendix D.4). The alignment of the 1474 bp sequences from isolates other than B2141 was 99.4% identical to each other (0.6% polymorphism).

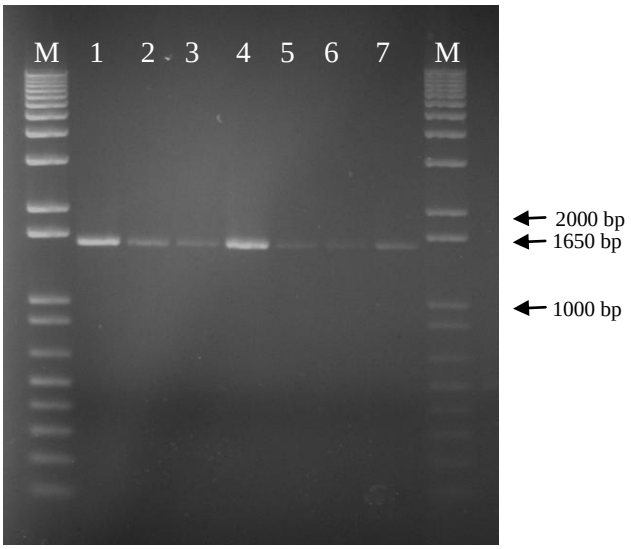

Figure 5.7: PCR products produced by amplification of *N. parvum* isolates using primers BP-1-42F and BP-1-42R. Number 1 is *N. parvum* strain B2141 containing the SCAR marker and 2- G61a1, 3- G233, 4- B316a2, 5- MM561, 6- DAR78999 and 7- UCD646So are *N. parvum* non-marker isolates. M– 1 kb plus DNA ladder (Invitrogen).

In silico restriction analysis carried out to determine whether these polymorphisms could be used to differentiate isolate B2141 from other *N. parvum* showed that there were five *Taq*I restriction sites in the sequence from isolate B2141 (termed the marker isolate) and four sites in the other isolates (Figure 5.8). Digestion with the restriction endonuclease *Taq*I differentiated the marker isolate by cleaving the new recognition site created by the polymorphism at position 168 (T/C**G**A) in isolate B2141. The *Taq*I restriction digestion of the amplimer produced by primers BP-1-42F and BP-1-42R produced four visible bands (51-64, 97-157, 405 and 776 bp) on 1% agarose gel and it was clearly different from other *N. parvum* isolates which also produced 4 bands (51-97, 221, 405 and 776 bp) (Figure 5.9).

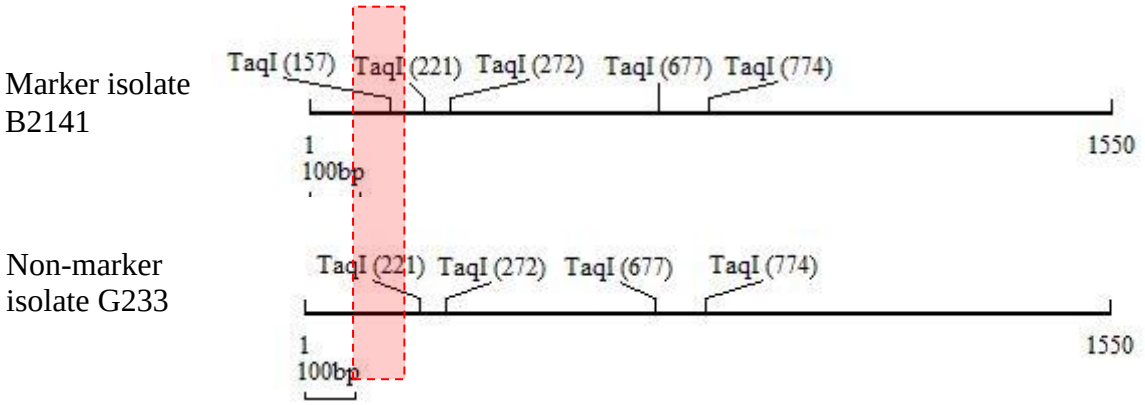

Figure 5.8: *Taq*I restriction map of 1550 bp sequence of *N. parvum* marker isolate B2141 (five restriction sites) and non-marker isolate G233 (four restriction sites) amplified with BP-1-42F and BP-1-42R primers.

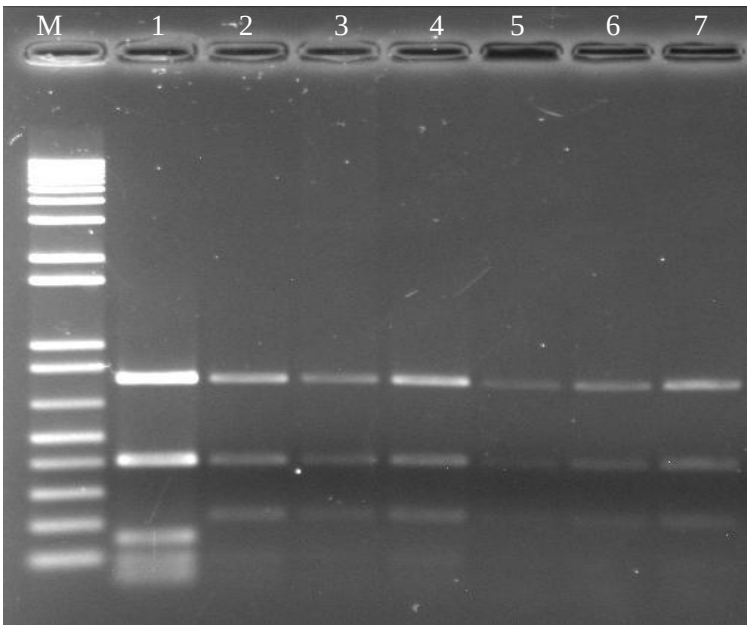

Figure 5.9: RFLP pattern of the ~1550 bp PCR product from *N. parvum* marker isolate B2141 and 6 other *N. parvum* isolates generated by amplification with primers BP-1-42F and BP-1-42R and digestion with *Taq*I restriction endonuclease. Lane 1 is the marker strain B2141 and lanes 2–7 are other *N. parvum* isolates. M- 1 kb plus DNA Ladder (Invitrogen).

5.3.3.4 Characterisation of the N. parvum *marker gene (BP-1-42)*

The BLAST search of GenBank provided no DNA sequences homologous to the BP-1-42 sequence from isolate B2141. However, similarity to hypothetical proteins containing zinc finger MYND (myeloid, Nervy, and DEAF-1) domains produced by other organisms were found using tblastx

(Appendix D.5). The closest match to the translated -3 open reading frame (-3 ORF) of the *N. parvum* marker sequence showed that the protein sequence encoded by nucleotides 186–299 had 50% (19/38) identity to part of the zinc finger MYND (myeloid, Nervy, and DEAF-1) domain-containing protein of *Culex quinquefasciatus* (Accession number XM_001849894.1). The same protein match also found in some other organisms including *Toxoplasma gondii* (Accession number XM_002366403.1), *Laccaria bicolour* (Accession number XM_001877867.1) and *Coccidioides posadasii* (Accession number XM_003066974.1) with a query coverage range of 5-7%. The hypothetical protein match to the *N. parvum* marker gene encompassed only 20% (320 bp/1573 bp) of the marker sequence. Further comparison of the translation of the -3 ORF of the *N. parvum* marker sequence showed that the protein sequence encoded by nucleotides 84–155 and 174–311 had 46% (21/46) and 50% (12/24) identity, respectively, to two parts of a hypothetical protein (Accession number AM_269981.1) produced by *Aspergillus niger* (Figure 5.10). The nucleotides sequence 180-287 (+3 ORF) and 85-156 (+1 ORF) of the *N. parvum* marker sequence had 50% (18/36) and 50% (12/24) identity, respectively, to two parts of a hypothetical protein produced by *Phaeosphaeria nodorum* (XM_001793389.1).

Only one of the polymorphisms was found within the sequence that had similarity to proteins from other fungal species (Appendix D.4). This polymorphism at nucleotide 168 was present in the area encoding the four amino acids beyond the protein match to *P. nodorum* (+1 ORF) but before a stop codon. In the +1 ORF the polymorphism is TCA to TCG and both sequences encode the amino acid serine, therefore, the mutation is silent in this frame. The second, third, fourth and fifth polymorphisms at nucleotides 526, 775, 1220 and 1278 bp were within a sequence area that had no match to known or hypothetical proteins.

A)

```
61    TAGCCACATTTCCATGTGAAGTGGACCCAGATAAGCTGGGGCTTTGCGAGGTCCGGTGAG
+1      *  P  H  F  H  V  K  W  T  Q  I  S  W  G  F  A  R  S  G  E
        Phaeosphaeria nodorum →  T  Q  t  i  r  G  F  s  s  S  s  a
+2       S  H  I  S  M  *  S  G  P  R  *  A  G  A  L  R  G  P  V  R
+3        A  T  F  P  C  E  V  D  P  D  K  L  G  L  C  E  V  R  *  E

121   AAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGTGGTGAAGTCGAACTGTGAGTAG
+1      K  R  M  P  R  K  Y  S  E  L  G  R  V  V  K  S  N  C  E  *
        K  k  M  P  R  *  c  t  g  L  G  R
+2       R  G  C  R  G  N  T  R  S  W  D  G  W  *  S  R  T  V  S  R
+3        E  D  A  E  E  I  L  G  A  G  T  G  G  E  V  E  L  *  V  G
                                                P. nodorum →        G

181   GCGCGACAGAGCAGCTTATGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGTAC
+1      A  R  Q  S  S  L  *  V  G  Q  S  A  V  W  H  S  F  E  Q  Y
+2       R  D  R  A  A  Y  E  W  A  S  R  R  S  G  I  P  S  S  S  T
+3        A  T  E  Q  L  M  S  G  P  V  G  G  L  A  F  L  R  A  V  P
          A  a  E  Q  L  M  d  G  P  V  G  l  L  A  h  v  *  A  V  t

241   CAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGGCTTCGAGCATGCAGCACACAGG
+1      Q  M  Q  *  Q  R  P  Q  A  C  *  C  G  F  E  H  A  A  H  R
+2       R  C  S  D  S  D  R  K  P  A  D  A  A  S  S  M  Q  H  T  G
+3        D  A  V  T  A  T  A  S  L  L  M  R  L  R  A  C  S  T  Q  A
          D  g  a  a  t  r  A  v  v  h  a  q  i  R  A

301   CTTTTCGGGTCGGCCATCGTGCAGTGAATAACACAGGAGAGGGACCGGAGGAATTTTCAG
+1      L  F  G  S  A  I  V  Q  *  I  T  Q  E  R  D  R  R  N  F  Q
+2       F  S  G  R  P  S  C  S  E  *  H  R  R  G  T  G  G  I  F  R
+3        F  R  V  G  H  R  A  V  N  N  T  G  E  G  P  E  E  F  S  E
```

B)

```
Score = 29.9 bits (59), Expect = 1e-11
Identities = 12/24 (50%), Positives = 15/24 (63%), Gaps = 0/24 (0%)
Frame = -3/+2

N. parvum(B2141) 155    RPSSEYFLGILFSPDLAKPQLIWV  84
                        RPS E+   I F  D A+P LIW+
A. niger         66359  RPSPEHRRAIFFPVDKAEPILIWI  66430
```

```
Score = 68.4 bits (143), Expect = 1e-11
Identities = 21/46 (46%), Positives = 32/46 (70%), Gaps = 0/46 (0%)
Frame = -3/+2

N. parvum(B2141)311   DPKSLCAACSKPHQQACGRCHCIWYCSKECQTADWPTHKLLCRAYS  174
                      +P+  C  C+K   + CG C  I+YCSK CQ +D+P+HKLLC+ ++
A. niger        66209 EPEDCCVMCNKTPARRCGCCRSIYYCSKNCQESDFPSHKLLCKKFA  66346
```

Figure 5.10: Translation of 61- 360 bp of SCAR sequence for *N. parvum* showing similarity to hypothetical proteins from *A. niger* and *P. nodorum*. A) The nucleotide sequence is shown in bold and the translations of the forward ORF's (+1, +2, +3) are in italics. The area matching the sequence of the *P. nodorum* genes is shown in red italics. B) The amino acid sequence of the translated reverse ORF's that are similar to *A. niger* hypothetical proteins is shown in the red and blue boxes. The corresponding nucleotides of the marker sequence that encode these amino acids are also contained within red and blue boxes (A). The sequence in green is the polymorphism identified in marker sequence.

5.3.3.5 Specificity and sensitivity of the N. parvum *isolate specific marker*

The specificity test using the marker primers BP-1-42F and BP-1-42R against 60 *N. parvum* isolates used for genetic variation analysis showed PCR amplification was obtained only in 40% (24/60) of the isolates. Isolate-specific PCR-RFLP using the *Taq*I restriction enzyme on these 24 isolates showed that a unique pattern was produced in *N. parvum* marker isolate B2141. The specificity test of marker primers BP-1-42F and BP-1-42R against 7 other botryosphaeriaceous species did not produce amplimers from any of the tested species (Figure 5.11).

Serial dilution demonstrated that these primers could produce an amplimer from as little as 50 pg of purified *N. parvum* genomic DNA. A single band was obtained by nested PCR with internal primers in marker isolate B2141 and in non-marker isolates. The sensitivity of the PCR assay was increased 100× to 0.5 pg (500 fg) using the nested PCR assay (Figure 5.12).

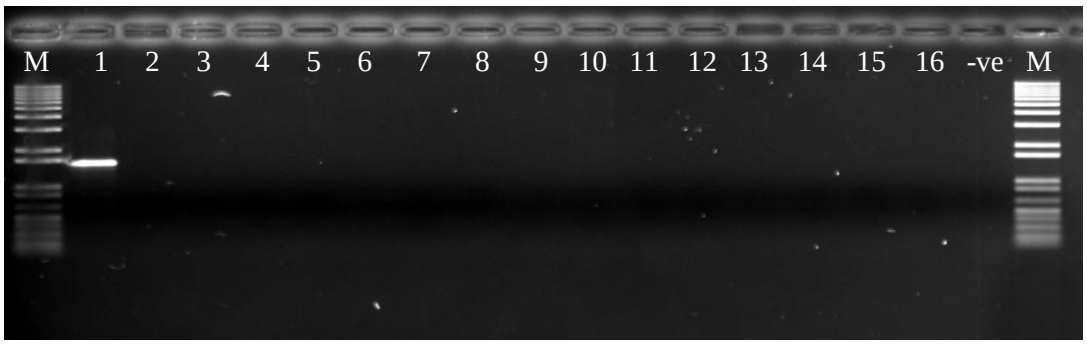

Figure 5.11: Specificity testing of *N. parvum* SCAR primers BP-1-42F and BP-1-42R with other botryosphaeriaceous species. Lane 1- *N. parvum* (marker isolate B2141), 2-4: *N. luteum*, 5-6: *N. australe*, 7-9: *D. mutila*, 10-11: *D. seriata*, 12-13: *N. ribis*, 14-15: *Do. iberica* and 16 : *Do. sarmentorum*. M- 1kb^{+} is a molecular ladder (Invitrogen).

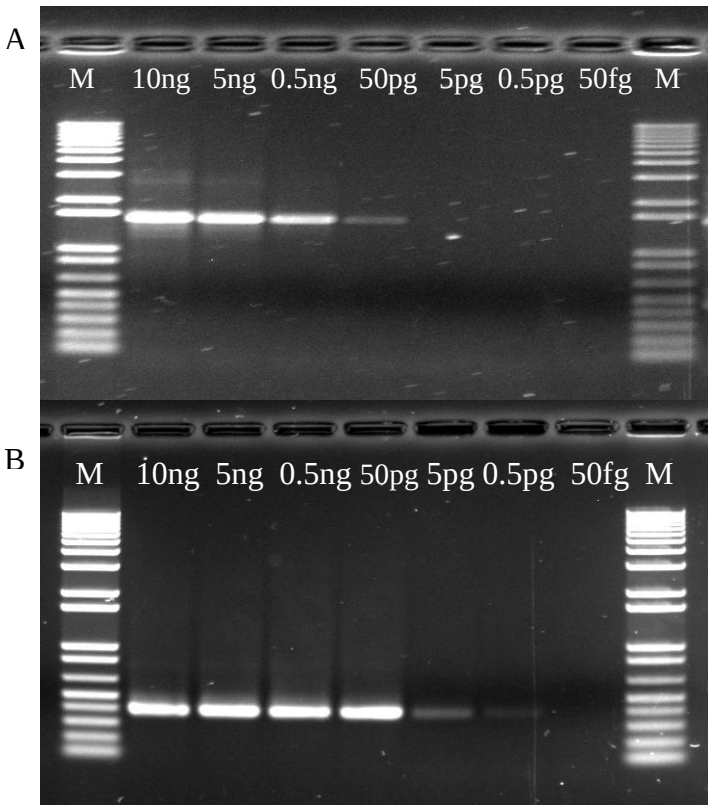

Figure 5.12: Sensitivity of the *N. parvum* isolate-specific PCR assay for differing amounts of genomic DNA template. A) Sensitivity of the standard PCR assay and B) sensitivity of the nested PCR assay. The concentration of the template DNA used in the PCR amplification is denoted above each lane. M- 1 kb plus DNA ladder (Invitrogen).

5.3.4 Development of the *N. luteum* isolate-specific marker

5.3.4.1 Selection of E. coli *transformants containing the* N. luteum *isolate-specific band*

An *E. coli* colony containing an insert of the expected size, approximately 750 bp (~500 bp marker band + 257 bp flanks), was selected by colony PCR with M13f and M13r primers. This produced many *E. coli* colonies with the correct sized insert and *E. coli* colony number 1 was selected for plasmid extraction and sequencing (Figure 5.13A). The ~500 bp insert was confirmed by *EcoR*I restriction digestion of the plasmid extracted from the selected *E. coli* colony number 1 (Figure 5.13B).

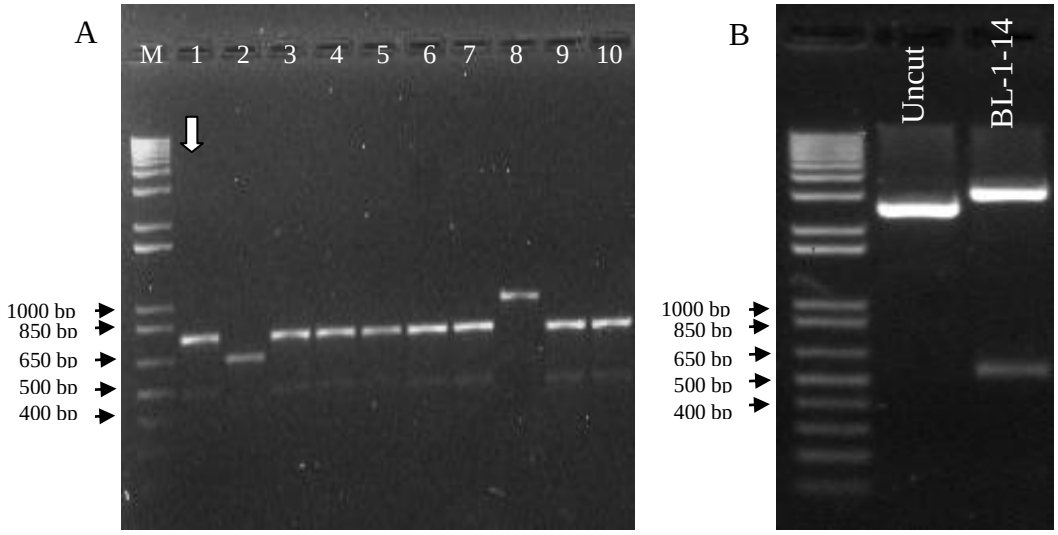

Figure 5.13: Confirmation of the size of the *N. luteum* BL-1-14 marker band using colony PCR amplification (A) and *EcoR*I restriction digestion of the plasmid (B). Lanes numbers 1-10indicate the *E. coli* transformants colony numbers. M- 1 kb plus DNA ladder (Invitrogen).

5.3.4.2 Sequence analysis and primer design

DNA sequencing of the insert showed that the isolate-specific band was 524 bp in size. A forward primer BL-1-14F (5'- GACCCAGAGCGGCATAAGTG -3') with a Tm of 63.0°C was designed at 5 bp from 5' end and a reverse primer BL-1-14R (5'- GAGCGGTGAATGAATGGGAC -3') with a Tm of 63.0°C was designed at 11 bp from 3' end to amplify a product of 510 bp (Appendix D.6).

5.3.4.3 PCR amplifications using N. luteum *isolate-specific primers*

Amplification at an annealing temperature of 63.0°C generated a 510 bp amplimer from isolate G51a2 from which the marker was derived, and not from other isolates (Figure 5.14A). However, at a lower annealing temperature of 50ºC the primers produced multiple bands in a range of *N. luteum* isolates with a strong 510 bp band produced only in isolate G51a2 (Figure 5.14B). This result demonstrated that the isolate-specific primers were able to bind and amplify sequences from other *N. luteum* isolates under less stringent conditions. As the *N. luteum* primers BL-1-14F and BL-1-14R only amplified the isolate-specific band at an annealing temperature of 63ºC in isolate G51a2 this PCR was used as the isolate-specific PCR assay.

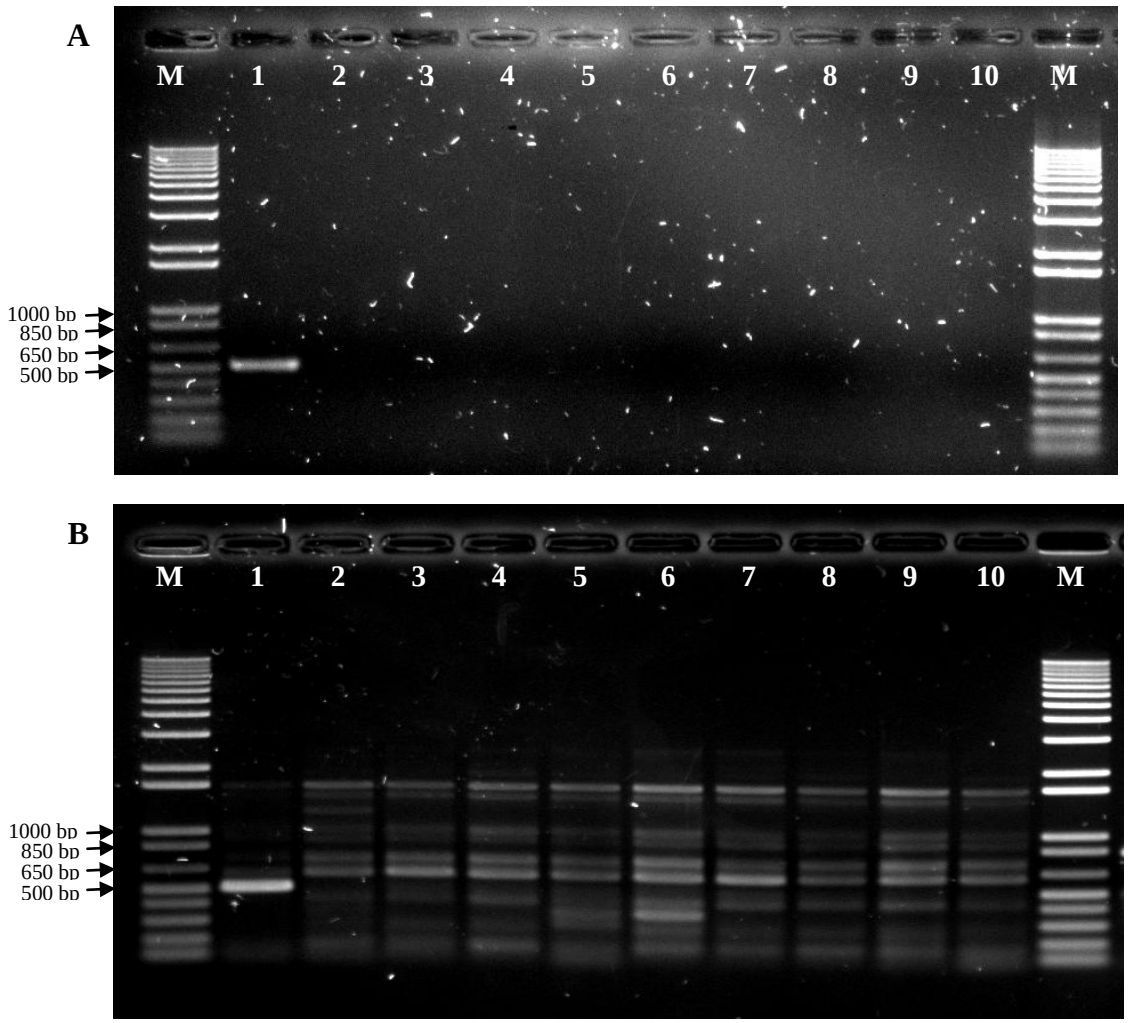

Figure 5.14: PCR amplification of ten *N. luteum* isolates using the isolate-specific primers BL-1-14F and BL-1-14R at annealing temperatures of 63ºC (A) and 50ºC (B). Lane 1 is the *N. luteum* marker isolate, 2 - A526, 3 - A821, 4 - A102b1, 5 - G16a2, 6 - G63b1, 7 - MM558, 8 - ICMP16678, 9 - CC445 and 10 - CC451, 2-10 being non-marker *N. luteum* isolates. M- 1 kb plus DNA ladder (Invitrogen).

5.3.4.4 Characterisation of N. luteum *isolate-specific DNA sequence*

The nucleotide sequence of the 524 bp band (BL-1-14) did not match any DNA sequences in GenBank. However, when translated and submitted to GenBank using the tblastx function the results showed that in ORF +2 the translated sequence of nucleotides at positions 62-163 had 50% (17/ 34) and 52% (17/ 33) identity, respectively, to mRNA contigs 4697 and 2920 of the fungal pathogen *Claviceps purpurea* (Accession numbers FM991520.1 and FM989743.1; Appendix D.7). The putative function of contig-4697 was as a chitin synthase involved in cell wall synthesis (Oeser *et al*., 2009). In addition, similarity to a hypothetical protein produced by other fungal species, including *Nectria haematococca* and *Gibberella zeae*, was also found. In ORF +2 the translated sequence of nucleotides at position 65-115 had 89% (15/17) and 83% (14/17) similarity to hypothetical proteins of *N.*

haematococca and *G. zeae*, respectively (Accession numbers XM 003049666.1 and XM 382557.1). The functions of these proteins are unknown. A further match to microsatellite DNA of *Platichthys flesus* was identified in ORF +3 (Accession number AJ538312.1). Some of these protein matches overlapped and no matches were found in the reverse frames. (Figure 5.15)

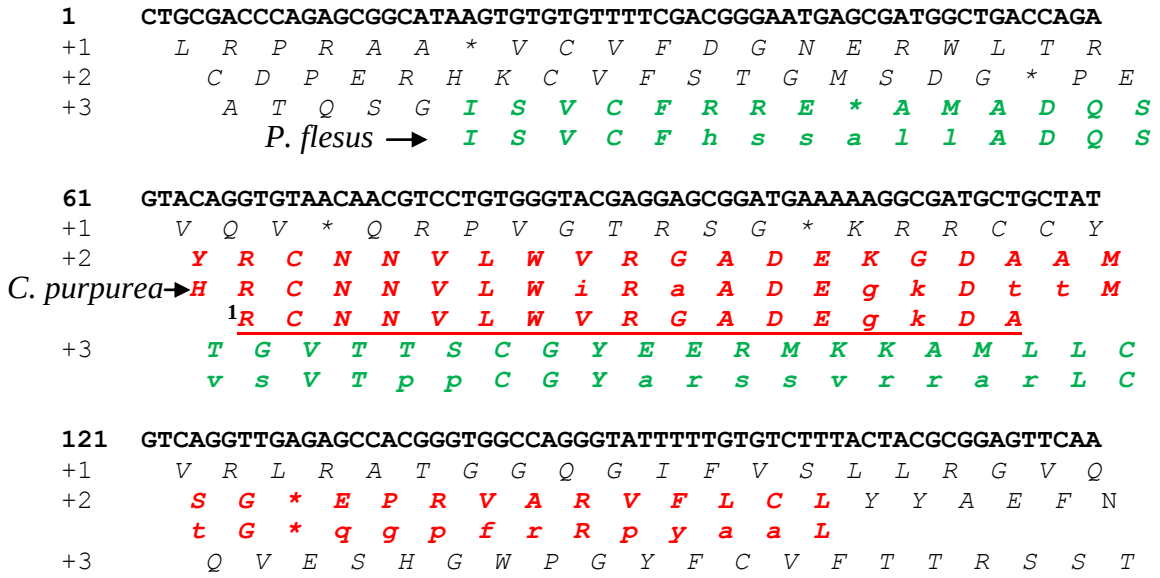

Figure 5.15: The translation of 180 bp of the *N. luteum* SCAR (isolate G51a2) and the corresponding protein match found in *C. purpurea*, *P. flesus*, *G. zeae* and *N. haematococca*. The open reading frame (+1, +2, +3) is shown on the left. The letters in red, bold and italics is the protein sequence produced by translation of +2 ORF with the matching protein sequences of *C. purpurea* and *G. zeae* aligned underneath. The letters in green, bold and italics is the translated protein sequencein the +3 ORF with the matching protein sequence of *P. flesus* aligned underneath.

5.3.4.5 Specificity and sensitivity of the N. luteum *marker*

The specificity test of the *N. luteum* isolate-specific PCR assay using DNA from other botryosphaeriaceous species showed no amplification even at low annealing temperatures (results not shown). Serial dilution of genomic DNA from the marker isolate G51a2 demonstrated that it could produce an amplimer at a template concentration as low as 5 pg (Figure 5.16).

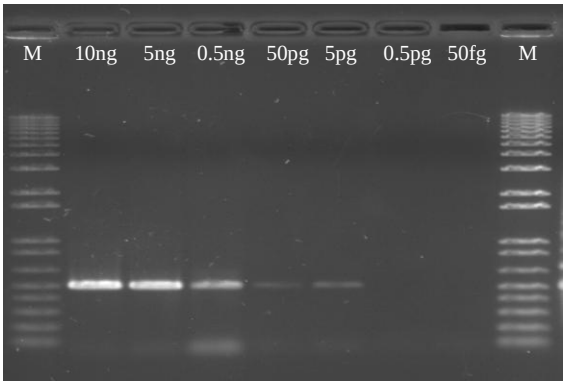

Figure 5.16: PCR amplification of *N. luteum* isolate G51a2 using serial dilution of genomic DNA. The concentration of template DNA added to the PCR is denoted above the gel. M – 1 kb plus DNA ladder (Invitrogen).

5.3.5 Distance of rainwater dispersal of *N. parvum* and *N. luteum* conidia

Microscopic examination confirmed the presence of *Neofusicoccum* species conidia in most of the water samples from one site (13 samples out of 17 in a site) with variable numbers (1-6/ 5µL) in the water samples collected at different distances from the inoculation point. Identification of *N. parvum* and *N. luteum* isolates in rainwater traps was by the isolate-specific PCR-RFLP and PCR assays generated for isolates B2141 (*N. parvum*) and G51a2 (*N. luteum*), respectively.

The isolate-specific PCR using the BL-1-14 marker primers for the *N. luteum* marker isolate G51a2 detected the rainwater splash dispersal of this isolate up to 2 m from the inoculation point (Figure 5.17). Stronger PCR amplification of the *N. luteum* marker was obtained in the water samples collected from downwind directions (South and West) than upwind. No amplification of the *N. luteum* marker was obtained in water samples collected 10 rows away (25 m) from the inoculation point in the same vineyard (-ve sample) demonstrating the specificity of the marker.

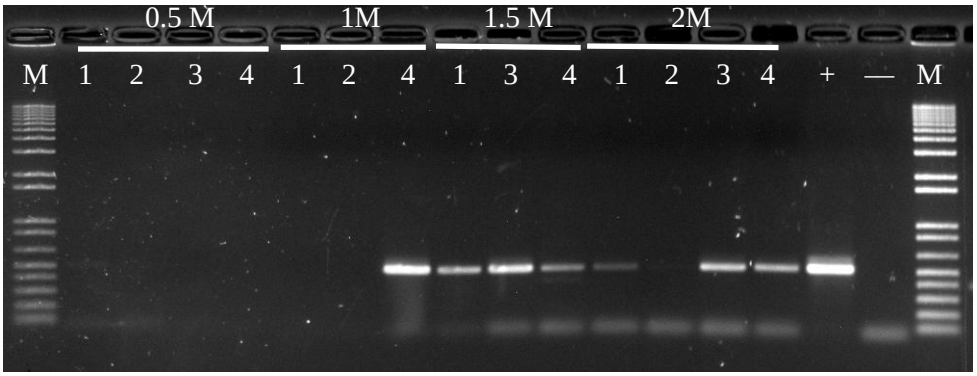

Figure 5.17: PCR amplification of DNA extracted from water samples collected at different distances (a few water traps were blown over by wind and no water collected) from the inoculation point using *N. luteum* isolate-specific PCR assay. Numbers above the lanes indicate the four directions of sampling points, 1- North, 2- East, 3-South, and 4-West. Genomic DNA from isolate G51a2 (marked isolate) was used as a positive (+) control and (—) is the water sample collected 10 rows from the inoculation point in the vineyard. M – 1 kb plus DNA ladder (Invitrogen).

The primary PCR amplification of the *N. parvum* marker was obtained from 0.5 m to 2 m distance from the inoculation point with stronger amplification in the water samples collected from downwind directions (Figure 5.18A). Subsequent nested PCR using the internal (nested) primers for the *N. parvum* isolate-specific PCR-RFLP assay increased the number and intensity of amplimers obtained, presumably due to the increased sensitivity of the nested PCR. (Figure 5.18B). The *Taq*I restriction digestion of the nested PCR product produced two visible bands (95 bp and 159-210 bp) in all amplimers obtained from the water collections. This confirmed, via the isolate-specific PCR-RFLP assay, the recovery of isolate G51a2 from rainwater traps up to 2 m from the inoculation point. (Figure 5.18C).

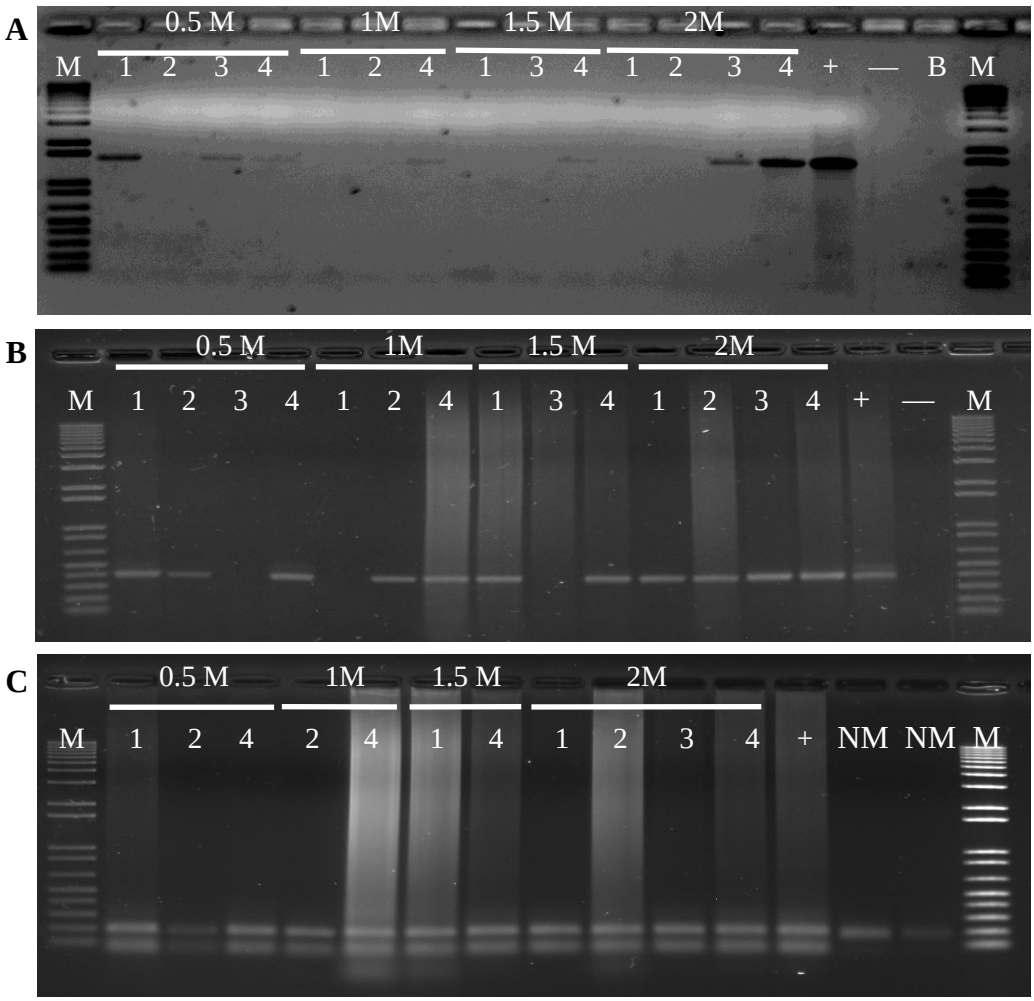

Figure 5.18: Analysis of water samples collected at different distances from the inoculation point using the nested PCR-RFLP isolate-specific assay for *N. parvum* isolate B2141. A) Initial PCR amplification using primary primers BP-1-42F and BP-1-42R, B) secondary PCR amplification using internal primers BP-1-42intF and BP-1-42intR and C) RFLP of the amplimer from nested PCR using *Taq*I . Numbers above the lanes indicate the four directions from which samples were collected; 1-North, 2- East, 3-South, and 4-West. Genomic DNA from the marker strain was used as positive (+) control, (—) is the negative water sample collected 10 rows from the inoculation point in the vineyard, B is the blank (minus template) control reaction for the PCR and NM is non-marker *N. parvum* isolates. M – 1 kb plus DNA ladder (Invitrogen).

5.4 Discussion

The aim of this study was to develop isolate-specific PCR assays for isolates of *N. parvum* and *N. luteum* for which unique bands had been identified using UP-PCR primer AA2M2.

Isolate-specific bands were identified in the fingerprints of two *N. parvum* isolates, B2141 and UCD646So (imported from California, USA). Due to the restrictions in using imported organisms in the field experiments in New Zealand (ERMANZ regulations), the isolate-specific band identified in *N. parvum* isolate UCD646So was not developed further. Primers designed to amplify the 1573 bp isolate-specific band from *N. parvum* isolate B2141 produced a band of the similar size (1550 bp) in 40% of the tested *N. parvum* isolates. However, polymorphism in the band from B2141 allowed an isolate-specific PCR-RFLP assay to be developed to specifically distinguish isolate B2141. The sensitivity of the PCR-RFLP system for *N. parvum* was 0.5 pg of DNA using the nested PCR and was specific for isolate B2141. A 524 bp isolate-specific band was identified in *N. luteum* isolate G51a2. Primers designed to amplify this band were specific for isolate G51a2 at an annealing temperature of 63ºC and the sensitivity of the *N. luteum* isolate-specific PCR was 5 pg of DNA. Both markers were tested in a field experiment to study the rainwater splash dispersal of these species in the vineyard.

Neofusicoccum parvum isolate B2141 belonged to genetic group 3 in the neighbour joining tree (Section 3.3.2) and showed medium pathogenic on detached grapevine green shoots (Section 4.3.1). In contrast, *N. luteum* isolate G51a2 belonged to genetic group 3 in the neighbour joining tree (Section 3.3.3) and showed highly pathogenic on grapevines in both *in vitro* green shoots and potted vine assays (Section 4.3.2). As both isolates had been shown to be virulent and had been isolated from field grown plants in New Zealand the isolate-specific bands identified in UP-PCR fingerprints had the potential to be developed as PCR assays to detect and monitor the respective isolates in field experiments (Dauch *et al*., 2003; Ridgway *et al*., 2005).

This research adds to the few reports available on the development of UP-PCR derived isolate-specific bands as SCAR markers. Bulat and colleagues (2000) identified an isolate-specific band in *Clonostachys rosea* and another UP-PCR derived isolate-specific band was identified in the grapevine pathogen *P. chlamydospora* (Ridgway *et al*., 2005). There are more reports available on the use of other fingerprinting techniques such as RAPD and microsatellite PCR (MP-PCR) for generation of SCAR markers for other fungal species. For example, an isolate-specific marker was identified in genetic variation analysis of mycorrhizal fungi using RAPDs (Abbas *et al*., 1996) and species-specific markers were developed for *Monilinia fructicola,* which causes fruit rot in stone fruit, and *Botryosphaeria dothidea,* which causes panicle and shoot blight in pistachio, using microsatellite PCR (MP-PCR) fingerprints (Ma *et al*., 2003). The demonstrated success of the UP-PCR method indicates that it also has good potential for identifying isolate-specific markers in fungal species and this may

lead to greater adoption of the technique for this purpose. As UP-PCR primers are designed to amplify intergenic, more variable regions of the genome (Bulat *et al*., 1994) they may have a greater likelihood of identifying polymorphism as these intergenic regions of the genome are typically more polymorphic than gene regions.

The primers for *N. parvum* isolate-specific PCR-RFLP assay were designed inside the UP-PCR primer binding sites and produced a band of the same size (1550 bp) in all the tested *N. parvum* isolates. This indicated that the polymorphism contributing to the different size of the unique band observed in UP-PCR fingerprints was not within the sequence region amplified by this primer pair as it was not possible to differentiate the marker isolate B2141 from non-marker isolates based on the size of PCR product. The polymorphism creating the unique band in B2141 was therefore likely to be present at the UP-PCR primer-binding site, creating a new primer binding site in the marker isolate. Similar to this, a single nucleotide polymorphism created a novel binding site for UP-PCR primer 3-2 producing an isolate-specific band in *P. chlamydospora* isolate A21 (Ridgway *et al*., 2005). To investigate this in the isolate-specific band of *N. parvum* isolate B2141, molecular methods such as inverse PCR (Ochman *et al*., 1990) or thermal asymmetric interlaced PCR (TAIL-PCR; Wang *et al*., 2007) could be used, which permits the amplification of upstream and downstream regions flanking a specific gene region.

In *N. luteum* isolate G51a2, unlike *N. parvum*, primers designed based on the sequence of the unique 524 bp band amplified a 510 bp amplimer only in isolate G51a2 at an annealing temperature of 63ºC. However, lowering the annealing temperature to 50ºC produced multiple bands in other *N. luteum* isolates indicating these primers are able to bind and amplify the sequences from the *N. luteum* non-marker isolates. Therefore, the PCR with these marker primers was directly used as isolate-specific assay to identify *N. luteum* isolate G51a2 at an annealing temperature of 63ºC. In a system similar to this, an isolate-specific band was identified in RAPD fingerprints for isolates of *C. minitans* and *C. coccoides* and they were developed as isolate-specific markers (Goldstein *et al*., 2000; Dauch *et al*., 2003).

To differentiate the *N. parvum* marker isolate B2141 from the *N. parvum* non-marker isolates, a PCR-RFLP assay was developed using the nucleotide polymorphisms found in the DNA sequence from isolate B2141. Similarly, an isolate-specific PCR-RFLP system was also used to identify the marker isolate of *P. chlamydospora* from non-marker isolates (Ridgway *et al*., 2005). The primers were based on the sequence of the unique band identified in *P. chlamydospora* isolate A21 and to enable the marker isolate to be distinguished, a PCR-RFLP system was developed using the *Bsr*I restriction enzyme (Ridgway *et al*., 2005). As for the *P. chlamydospora* system, the PCR-RFLP for *N. parvum* isolate B2141 required two molecular procedures (PCR and subsequent restriction digestion) to confirm the identity of the marker isolate and this is not ideal for rapid confirmation of isolate identity. Also PCR-RFLP systems are not suitable as quantitative PCR assays (qPCR). To improve this,

identification of the likely polymorphism present in the UP-PCR primer binding site of the marker sequence for *N. parvum* isolate B2141 could help to develop an isolate-specific PCR assay to detect the isolates in single molecular procedure. It may then be possible to develop an isolate-specific TaqMan^{TM} probe based on the nucleotide polymorphism found in the sequence of marker isolates for qPCR (Luchi *et al*., 2005).

In order to demonstrate specificity, the isolate-specific assays were tested with multiple isolates from each of the respective species. The results showed that PCR with primers designed for the *N. parvum* isolate specific PCR-RFLP assay produced amplimers in 40% of the *N. parvum* population, however, the RFLP was specific for isolate B2141. Similarly when the *N. luteum* marker was tested against other *N. luteum* isolates multiple bands were produced in all of the isolates at the lower annealing temperature of (50ºC), but at a more stringent temperature the 510 bp isolate-specific band was only obtained in isolate G51a2. The ability to produce amplimers in other isolates for both markers suggests that the gene region is not unique in the two target isolates but that the unique bands generated by UP-PCR were the result of polymorphism that altered primer binding. This is important as for the marked isolates to be useful for field studies they should be representative of the species and not genetically unusual. Indeed, it can be hypothesised that, if average mutation rates apply, then 1 in every 1000 bp is polymorphic (Lewin, 2004) and this would mean up to 37,000 polymorphic sites in the average fungal genome of 37 Mb (Gregory *et al*., 2007). The specificity of the *N. parvum* and *N. luteum* markers was further confirmed by testing against two representative isolates from seven botryosphaeriaceous species which were isolated from grapevines in New Zealand. There was no amplification of any bands by either marker in any other botryosphaeriaceous species tested using the genomic DNA suggesting these markers were specific to their respective species. However, testing these primers against other grapevine pathogens would be an advantage to further prove their specificity.

Sensitivity of the isolate-specific PCR assays is another important issue especially if the ultimate goal is to detect an isolate in the field. The sensitivity of the *N. parvum* isolate-specific marker using the standard PCR assay was 50 pg and it was increased 100 times to 0.5 pg (500 fg) by nested PCR. This was similar to the sensitivity of the isolate-specific marker derived for *P. chlamydospora* which was increased to 50 fg in a nested PCR assay (Ridgway *et al*., 2005). The *N. luteum* isolate-specific marker was ten fold more sensitive (5 pg) than the *N. parvum* marker (50 pg) using a standard PCR assay. Therefore, a nested PCR assay was not developed for this marker as the detection sensitivity was deemed sufficient to allow its use in field experiments. The sensitivity of the *N. parvum* and *N. luteum* markers could be calculated in terms of number of conidia as follows; if the haploid genome of a botryosphaeriaceous species is considered to have approximately the same C-value as *Fusarium oxysporum* with 0.0377 pg (Pasquali *et al*., 2006) then the DNA in a conidium of *N. parvum* would weigh 0.0377 pg. Therefore, the sensitivity of the *N. parvum* marker in nested PCR assay was equivalent to approximately 13 conidia (0.0377 pg/ conidium x 13 conidia = 0.5 pg). The sensitivity of

the *N. luteum* marker in the standard PCR assay was approximately 132 conidia (0.0377 pg/ conidium x 132 conidia = 5 pg). However, the total volume in which the DNA solution was eluted and the volume of sample from which that DNA originated would have to be included in these calculations to produce the exact detection sensitivity of the assay. The detection sensitivity of the assays was considered biologically relevant for the detection of these species in field experiments.

The detection sensitivity of PCR assays designed for multiple copy genes are typically more sensitive than those of the *N. parvum* and *N. luteum* markers described here. For example, the sensitivity of the multi-species primer pairs, designed based on ITS sequences to identify six botryosphaeriaceous species including *N. australe, N. luteum, N. parvum, N. ribis*, *D. mutila* and *D. seriata,* was 1 pg in standard PCR and 0.1 pg in a nested PCR assay which was equivalent to 2-5 conidia (Ridgway *et al*., 2011). In another study a species-specific marker designed for *Sphaeropsis sapinea* detected as little as 0.1 pg of DNA in a quantitative PCR-assay (Luchi *et al*., 2005) and the MP-PCR-derived primers for *B. dothidea* detected as little as 1 fg (0.001 pg) in a nested PCR assay (Ma *et al*., 2003). The reason for the high sensitivity of the ITS and MP-PCR derived PCR assays is likely to be the multiple copies of these regions found in eukaryotic genomes (Balloux and Lugon-Moulin, 2002; Lecomte *et al*., 2000). However, the isolate-specific marker developed for *P. chlamydospora* was reported as a single copy and had 10 times greater sensitivity in a nested PCR assay than the *N. parvum* marker described here (Ridgway *et al*., 2005). Although the number of copies of the *N. parvum* marker gene was not determined in this study, Southern blot hybridization analysis could be used to determine the copy number of this gene in a similar manner to that described for *C. rosea* (Bulat *et al*., 2000).

The 1573 bp sequence of the *N. parvum* marker band showed no homology to nucleotide sequences available on the GenBank database. However, searching using the translated sequence showed that the first 320 bp (20%) of the sequence had similarity to a protein match to part of the zinc finger MYND of *Culex quinquefasciatus* (Accession number XM_001849894.1) and some other organisms including *Toxoplasma gondii* (Accession number XM_002366403.1), *Laccaria bicolour* (Accession number XM_001877867.1) and *Coccidioides posadasii* (Accession number XM_003066974.1). The match to these hypothetical proteins indicated that the SCAR contained a zinger finger MYND (named after myeloid translocation protein 8, Nervy, and DEAF-1) domain protein. The zinc finger (Znf) domains are relatively small protein motifs, which contain multiple finger-like protrusions that make tandem contacts with their target molecule. Some of these domains bind zinc, but many do not and instead bind to other metals such as iron, or no metal at all. The zinc fingers (ZnFs) are extremely abundant in eukaryotes and were considered to function exclusively as sequence-specific DNA-binding motifs, but are now known to have additional activities such as the recognition of RNA and other proteins (Gamsjaeger *et al*., 2006). The MYND domain is a conserved zinc binding domain. It is defined by seven conserved cysteine residues and a single histidine residue that are arranged in a C4–C2HC consensus. MYND domains exist in a large number of proteins that play important roles in

development or are associated with protein–protein interactions, mainly in the context of transcriptional regulation (Spadaccini *et al*., 2006). The homology found suggests that the marker gene characterised in *N. parvum* could be a part of zinger finger MYND protein.

The nucleotide BLAST search of GenBank using the 524 bp sequence of the *N. luteum* marker gene sequence showed no sequence homology to any sequence in the database. However, when the translated sequence was used protein matches were identified in the first 163 bp sequence of the *N. luteum* marker (31%) and all of them represented hypothetical proteins with unknown functions. Only one match for an mRNA contig (contig 4697; FM991520.1) of *Claviceps purpurea* had a putative function which was as a chitin synthase involved in fungal cell wall synthesis and modification (Oester *et al*., 2009). This result suggested that the *N. luteum* marker gene could be a part of chitin synthase gene.

PCR products were obtained using both *N. parvum* and *N. luteum* isolate-specific markers with DNA extracted from the water samples collected during rainfall events in vineyards at different distances from the inoculation point. This indicated that both of these markers were able to detect the conidia of their respective isolates within the environmental samples. PCR amplification was obtained using the *N. parvum* markers in both standard and nested PCR assays, but the detection capacity of the nested PCR assay was greater. Similarly, a species-specific molecular marker was used to detect the presence of *P. chlamydospora* in the water samples obtained from the nursery propagation process (Whiteman, 2004). Other studies have also shown that the isolate-specific marker derived from UP-PCR fingerprints for *C. rosea* was able to detect the marked isolate in inoculated soil samples (Bulat *et al*., 2000). This shows the potential use of species- and isolate-specific marker in epidemiological studies. The *N. parvum* and *N. luteum* isolates-specific markers developed in this study could be used to identify the possible infection pathways of these species in nurseries by using them to test solutions, media, equipment and cuttings used in the grafting process.

Different studies have shown that the conidia of the botryosphaeriaceous species can be trapped in rainwater samples collected from vineyards and these studies have indicated that there are some species difference in the timing and amount of spore release (Úrbez-Torres *et al*., 2010b; van Nierkerk *et al*., 2010; Amponsah *et al*., 2009). However, this is the first report that indicates how far the botryosphaeriaceous species conidia can be disseminated by rainwater splash from a single inoculation point during a single rainfall event. In a single rainfall event, both the *N. parvum* and *N. luteum* isolates were detected up to 2 m from the inoculation point using the isolate-specific markers. It is probable that some conidia were dispersed further than this but the detection sensitivity of the PCR assays would have been insufficient to detect these due to the dilution effect on condial concentrations as the distance from the inoculation point increased. As the *N. parvum* and *N. luteum*

isolate-specific markers do not detect any other isolates of botryosphaeriaceous species the data produced by this study is a highly accurate and reliable estimation of dispersal by these two species.

The sensitivities of the *N. parvum* and *N. luteum* markers were equivalent to 13 and 132 conidia respectively. A study conducted by Amponsah (2011) showed that using 20 µL of a 10^2 conidia / mL (~2 conidia/ inoculation) suspension of *N. luteum* was able to produced lesions on grapevine shoots. Thus, the detection of spores at 2 m from the inoculation point would have been in numbers sufficient to cause infection if they landed on a suitable site within the grapevine, such as a fresh pruning wound. Although the sensitivity of these markers was biologically useful it is clear that botryosphaeriaceous species can infect grapevines at conidial concentrations lower than the detection threshold of the PCR assays developed here. The sensitivity of the *N. luteum* marker could be increased by developing a nested PCR assay. Similarly, a study of PCR detection of *Sclerotinia sclerotium* in oil seed rape reported that that between 50 and 100 ascospores per petal was needed to cause infection (McCartney *et al*., 2001). The *S. sclerotiorum*-specific PCR assay was able to detect this species when petals contained 50 ascospores or more (Freeman *et al*., 2002) and therefore was able to help growers make timely decisions on fungicide application.

Development of qPCR system for *N. parvum* and *N. luteum* markers would be beneficial for use in epidemiological studies of these species. As discussed previously, the qPCR system could be developed for this marker by designing isolates-specific primers based on the likely polymorphism present in the UP-PCR primer binding site or using Taqman™ probe technique. A Taqman™ probe was used to detect the *S. sapinea* from pine using a species-specific marker and was reported to be useful to detect this pathogen in plant tissues even in very low quantities (Luchi *et al*., 2005). As the sensitivity of the *N. luteum* marker is lower than *N. parvum* marker, nested PCR assay could be developed for *N. luteum* marker to increase the sensitivity of the marker.

The isolate-specific markers developed here should be further optimised for detection in grapevine wood to improve their usefulness as tools to study epidemiology of these pathogens. Ridgway *et al*. (2002) demonstrated that a species-specific marker developed for *P. chlamydospora* was useful to detect the pathogen in grapevine wood and to identify infected mothervines. A similar process using the *N. luteum* and *N. parvum* isolate-specifc markers could address the potential for transmission of the pathogen from infected mothervines into new season's canes and this would provide important information to the industry about potential infection pathways. Pathogenicity studies conducted using these species showed that both species can moved endophytically beyond the lesions in grapevine tissues (Chapter 4). By using these isolate-specific markers as qPCR systems to study the endophytic movement of these pathogens in grapevine wood, information on the relative amounts of biomass present beyond a lesion could be gathered, and this may be of practical relevance to growers when treating infection by pruning to remove symptomatic wood. As botryosphaeriaceous species have also

been reported to survive in pruning debris (Amponsah, 2011) the markers could be used to study the longevity of propagules in these substrates. Thus, the isolates-specific PCR assays developed for *N. parvum* and *N. luteum* in this study have potential for use in future research, either in their present form, or as improved qPCR assays.

In summary, the UP-PCR derived isolate-specific marker of *N. parvum* and *N. luteum* are specific to their target isolates. The specificity and sensitivity of these markers was demonstrated to be sufficient to detect conidial dispersal in the field. The conidia of *N. parvum* and *N. luteum* were shown to disperse up to 2 m by rain splash and were influenced by wind direction. These isolate-specific markers will be useful for future epidemiological studies of these fungi in the vineyards and potentially in other crops.

Chapter 6
Concluding discussion

The overall aim of this research was to investigate the species composition, prevalence, distribution and genetic diversity of botryosphaeriaceous species associated with grapevine dieback and decline disease in New Zealand. In this chapter, the key findings of this research are discussed with a view to making suggestions for further improvements in future research.

This study has shown that the botryosphaeriaceous species are the major fungal pathogens associated with grapevine dieback and decline disease in New Zealand, which is consistent with similar reports from California (Úrbez-Torres *et al*., 2006a), Australia (Pitt *et al*., 2010) and South Africa (Fourie and Halleen, 2004a). The sampling process (June–December 2007) recovered symptomatic grapevine samples from 43 vineyards in six major grapevine growing areas (Otago, Canterbury, Marlborough (Blenheim), Nelson, Eastland (Gisborne) and Auckland) and 88% of the vineyards were positive to botryosphaeriaceous species infection. Of the 238 symptomatic samples obtained the overall incidence of infection by botryosphaeriaceous species was 68%. Although other grapevine trunk pathogens, including *Eutypa* spp. and *Phaeomoniella* spp. were also isolated, they represented in only 5% of the total symptomatic vine samples collected. The incidence of the botryosphaeriaceous species infection varied between the areas studied with the highest in Gisborne (96%) and lowest in Otago (23%). When incidence was correlated with vine age, the results suggested that vine age was the main reason for the variable distribution as the least infection (30%) was observed in age group 1 (1 - 5 yrs old) compared to other age groups. The lowest incidence of botryosphaeriaceous species infection was found in Otago where sampled vines were 2 to 9 yrs old and showed no prominent external lesions. This finding correlates well with a previous study which showed that the botryosphaeriaceous species infection was largely observed in older vines (>8 yrs old) (Larignon and Dubos, 2001).

The initial aim of the survey was to collect at least 10 symptomatic grapevine samples from 25 growers in each wine growing area in New Zealand using a postal survey system. However, due to a poor response from the growers, the number of samples was supplemented by collection during personal visits to vineyards in Gisborne, Canterbury and Otago with a consultant hired to collect samples in Auckland. This resulted in variation in the number of samples collected from each vineyard, the total number of vineyards sampled in each region and the total number of symptomatic samples obtained from each region. Also, due to time and money constraints, this sample collection did not cover some major wine growing areas such as Hawkes Bay and Bay of Plenty. Although not ideal, this strategy did collect symptomatic material in an amount per ha that was equivalent to that of international surveys such as grapevine survey in California (Úrbez-Torres *et al*., 2006a) and Australia (Pitt *et al*., 2010). These two surveys used a similar sampling strategy to the one in this study, in that

only the symptomatic samples were collected for the investigation of botryosphaeriaceous species. Ideally, a study to establish exact incidence per region would sample equivalent numbers of vineyards in each region and would attempt to get equivalent material from different vine age groups and scion varieties, with random selection wherever possible. Several botryosphaeriaceous species have been reported to have an endophytic phase without showing any symptoms in hosts (Slippers and Wingfield, 2007). A study conducted by Billiones *et al*. (2010) showed the endophytic infection of botryosphaeriaceous species in healthy looking grapevine planting materials obtained from the nurseries. The selection of symptomatic tissues in this study may have influenced the relatively high number of *N. parvum* isolates recovered, since this pathogen has been demonstrated to cause visible cankers, unlike some other botryosphaeriaceous species which often cause die-back after periods of dormancy (Amponsah *et al.,* 2011). Thus, a completely randomized sampling of grapevine materials from a uniform number of vineyards in all major wine growing areas in New Zealand would provide a better estimate of the botryosphaeriaceous species infection (symptomatic and endophytic). A survey which assessed infection in asymptomatic and symptomatic material may provide different incidence of the major species. However, the aim of this research was to identify the primary fungal pathogen causing grapevine dieback and decline thus symptomatic material was targeted.

From the 238 symptomatic grapevines samples, 336 botryosphaeriaceous isolates were obtained and identified to species level using combined morphological and molecular methods which confirmed at least nine botryosphaeriaceous species to be present in New Zealand grapevine including *N. parvum, N. luteum, N. australe, N. ribis, D. mutila, D. seriata, B. dothidea, Do. sarmentorum* and *Do. iberica.* No novel species were identified in this research. Among these, this is the first report of *Do. iberica* isolated from grapevine in New Zealand. This study has identified a greater number of botryosphaeriaceous species compared to the smaller previous study conducted by Amponsah, (2011) which had isolated five botryosphaeriaceous species in New Zealand grapevines. The number and composition of botryosphaeriaceous species isolated from grapevines is similar to the results obtained in other studies; the Californian grapevine survey which had identified seven botryosphaeriaceous species (Úrbez-Torres *et al*., 2006a) and Australian survey had reported eight botryosphaeriaceous species (Pitt *et al*., 2010). A notable difference observed in this study was the absence of *L. theobromae* which was present in other studies. There is a single report of this species in the Landcare culture collection and it was isolated from a Begonia plant in Auckland in 2008. The absence of this species in any other hosts including grapevine, suggests that this species could have been introduced recently to New Zealand through importation of planting materials, which is believed to be a common route of introduction for botryosphaeriaceous species (Pennycook and Samuels, 1985). The other reason for not detecting *L. theobromae* in grapevines could be that the temperature is not favourable for the establishment of *L. theobromae* in New Zealand; this species was shown to be established in other countries which are characterised by a semiarid climate with mild winters and hot summers (Burruano *et al*., 2008; Punithalingam 1980). It is important to prevent the entry of *L. theobromae* into

New Zealand through planting materials by following strict border security procedures because this species have been reported as vigorous pathogen in grapevine (Úrbez-Torres *et al*., 2008; Burruano *et al*., 2008) and many other hosts in different countries.

The relative frequencies of the nine botryosphaeriaceous species indentified in grapevines showed that the *N. parvum* was the most predominant species isolated from the symptomatic vine samples in most of the vineyards in New Zealand. This result was in contrast to the report from the studies in California (Úrbez-Torres *et al*., 2006a) and Australia (Pitt *et al*., 2010) which both identified *D. seriata* to be the most predominant species in the vineyards. The reason why *D. seriata* was the predominant species in other countries and *N. parvum* in New Zealand vineyard could be explained by several reasons including the climate, frequency of introduction and proximity of alternate hosts. Previous reports from California and Australia showed that *N. parvum* was mostly found in cooler regions (Úrbez-Torres *et al*., 2006a; Pitt *et al*., 2010). In contrast to the results from the vineyard survey a study conducted in New Zealand nurseries showed that *N. luteum* was the most frequently isolated species (Billiones, 2011). The difference between vineyard and nursery prevalence could be explained by the sole collection of symptomatic samples from vineyards whereas in the nursery study most samples were asymptomatic material. These contrasting results suggest that *N. parvum* produces more obvious visible symptoms, so the infected plants or cuttings are likely to be discarded when nursery material is inspected for use or sale. In contrast, *N. luteum* is more likely to cause a latent endophytic infection. Pathogenicity assays conducted in this study supported this hypothesis as lesions were less severe (often intermittent) and endophytic movement was greater in *N. luteum* infections.

This research supported observations that were made in the Californian and Australian studies (Úrbez-Torres *et al*., 2006a; Pitt *et al*., 2010) that the distribution of botryosphaeriaceous species was influenced by climatic conditions. The distribution of species was different between the North and South Island vineyards and the optimum temperatures for growth of the pathogen species correlated well with their distribution. In this study the optimum temperatures for the maximum growth rate of five botryosphaeriaceous species showed significant variation and supported the argument that their distribution in New Zealand could be related to climatic conditions. This observation could be further strengthened by a random sample collection of grapevine and other non-grapevine hosts to isolate botryosphaeriaceous species.

The genetic diversity analysis of four major botryosphaeriaceous species (*N. parvum*, *N. luteum*, *N. australe* and *D. mutila*) found that populations of these species were genetically diverse in New Zealand. Although, the number of UP-PCR loci used for the analysis varied between the species it was sufficient to measure the genetic diversities of these populations. However, it would be advantageous to study the genetic diversity of this population with another genotyping method such as RAPD or AFLP to confirm the results obtained in this study; another study showed that AFLP was more

informative than UP-PCR in genetic diversity analysis of *Pa. chlamydospora* (Pottinger *et al*., 2002). It would be interesting to compare the genetic diversities of the New Zealand populations with a large number of isolates from different countries and it may provide more details about the relationship between these populations and possibly identify the likely path of entry of botryosphaeriaceous species into New Zealand.

The high genetic diversities obtained for the botryosphaeriaceous species in this study was unexpected as these species are reported to reproduce mainly asexually, with the sexual stage of these fungi being very rarely observed in nature. The genetic diversities of these species were greater than for other grapevine fungal pathogens including *Pa. chlamydospora*, *C. liriodendri* and *C. macrodidymum* which are also reported to have predominantly asexual reproduction (Pottinger *et al*., 2002; Alaniz *et al*., 2009). However, they were similar to the results obtained in other pathogen populations with predominantly asexual reproduction, such as a Chinese population of *Ma. fruiticola,* which was reported to have very rare sexual reproduction (Fan *et al*., 2010). Although the exact mechanism underlying the relatively high genetic diversity of botryosphaeriaceous species is unknown it could be explained by several reasons including the presence of an active asexual recombination strategy, that these species have been present in New Zealand for a long time or that multiple introductions of these species from genetically diverse populations from other countries have occurred.

Although, the mechanism which is generating the relatively high genetic diversity in botryosphaeriaceous populations is unknown, a preliminary investigation of VCGs showed a large number of fungal anastomoses and hyphal fusions between the isolates of each species and this could provide the opportunity for parasexual recombination. However, further study is required to investigate the role of these hyphal fusions in increasing genetic diversity. This could be done by comparing the DNA fingerprint profiles of the parent isolates with the DNA fingerprints of the isolates from the interaction zone using the DNA fingerprinting methods such as UP-PCR, RAPD and AFLP. In a similar study conducted in *Me. grisea,* isolates obtained from the interaction zone produced different fingerprints to the parent isolates confirming vegetative recombination between those two isolates (Zeigler *et al*., 1997). The suggestion that there is an active vegetative recombination is further strengthened by the research in this study which showed that, in the field, isolates obtained from a single grapevine lesion were genetically diverse, suggesting that hyphal fusion and vegetative recombination may be occurring within the host tissues. A future research focus in this area could provide understanding of the role of the host in fungal hyphal fusion and recombination on the generation of diverse populations of botryosphaeriaceous species.

Although other studies have reported that sexual reproduction does occur in low frequencies, the sexual stage of the botryosphaeriaceous species was never observed on the diseased grapevine samples collected in this study. However, botryosphaeriaceous species are reported to infect a wide range of

host plants in New Zealand and it could be possible that the sexual reproduction of these fungi may occur in another host and subsequently recombinants spread to grapevines or that the sexual reproduction may occur in a particular season of the year. Support for this argument is provided by a genetic diversity analysis of a *B. dothidea* population from pistachio in California which identified the sexual stage of this fungus from a number of non-pistachio hosts in the surrounding area and reported that the genetic diversity of the non-pistachio population was greater than that of the pistachio population (Ma *et al*., 2001). In future work extensive investigation of non-grapevine hosts close to the vineyards to detect sexual reproductive structures of botryosphaeriaceous species would be useful. This may clearly identify a role for sexual reproduction in the creation of genetically diverse populations.

Virulence assays conducted using genetically distinct isolates of the four main species (*N. parvum, N. luteum, N. australe* and *D. mutila*) showed that there was a large amount of intra-species variation in virulence. On grapevine green shoots the genetically distinct isolates of *N. parvum* and *N. luteum* produced lesion lengths of 11 – 82.5 mm and 10 – 89 mm, respectively, with *N. australe* and *D. mutila* isolates producing smaller ranges of 30 –83 mm and 39 –84 mm, respectively. However, on 1 year old plants lesion lengths produced by the *N. parvum* and *N. australe* isolates were much greater than for the other two species. This suggests that some of these botryosphaeriaceous species may be specialized to different grapevine tissues. Reports from other pathogenicity studies of botryosphaeriaceous species have also found intra-species variation in virulence although these studies were done without knowledge of the genetic similarity of the isolates (Savocchia *et al*., 2007; Begoude *et al*., 2010) and infection was variable in different pathogenicity assays (Úrbez- Torres and Gubler, 2009). To determine whether some isolates of the botryosphaeriaceous species show tissue specificity, virulence studies should be conducted on different tissues such as trunks, cordons, green shoots, flower buds and fruits. In addition, as all the virulence studies conducted in this research used grapevine variety Sauvignon Blanc it would be advantageous to test the same isolates on different grapevine varieties, especially as other studies have reported some of the grapevine scion and rootstock varieties are more susceptible to botryosphaeriaceous species infection than others (Úrbez-Torres and Gubler, 2009; Billoons *et al*., 2011). The preliminary study on *in vitro* production of laccase by three *N. parvum* isolates showed variation in laccase production levels although this did not appear to correlate with virulence. Future research to measure laccase and other plant degrading enzymes, such as cellulase and protease levels, in a larger number of isolates of *N. parvum* and other botryosphaeriaceous species with known virulence data would help to investigate the role of these enzymes in the virulence of the isolates. The genetically characterised and extensive botryosphaeriaceous collection produced by this research would be an ideal resource for this work. The intra-species variation in pathogenicity of botryosphaeriaceous species could also be explained by differences in host specificity. As in other countries botryosphaeriaceous species have been isolated from several hosts in New Zealand and it is possible that the isolates with weak virulent could be

recently introduced to grapevine from other hosts on which they were more pathogenic. However virulence studies conducted on grapevine using the isolates obtained from non-grapevine hosts, such as broom and native ngaio, showed strong virulent (Amponsah *et al*., 2008). Similarly, isolates obtained from grapevine were equally virulent on blueberry (Che Omar, 2010). Therefore, conducting virulence studies with a larger number of isolates of each botryosphaeriaceous species on wide range of hosts could provide more understanding about the host specificity of these species. This may include quantifying the relative influence of enzyme production and perhaps correlating the results with genetic grouping.

As multiple botryosphaeriaceous species were isolated from a single grapevine lesion it was unknown whether these species were acting synergistically in disease development on grapevines or each other competing for nutrients. In a novel virulence study conducted by co-inoculation of *N. parvum* and *N. luteum* isolates on potted grapevines it was demonstrated that there was no synergy or competition effect on either lesion development or the endophytic movement of each species in grapevine tissue 6 weeks after inoculation. However, this experiment was of short duration and this may have decreased the likelihood of observing any significant differences. In future this experiment could be repeated for a longer period with many more isolates. In addition it is well known that stress can cause symptom expression in vines with latent infections of botryosphaeriaceous species. Thus, repeating the co-inoculations and creating artificial stress on the grapevines, such as by wounding the trunk and roots or water stress, could provide more information on how two species act together in multiple infection conditions on grapevines. Under these conditions, plant death or production losses may become more evident.

Endogenous isolate-specific markers were developed for an *N. parvum* and *N. luteum* isolate based on unique bands identified in the UP-PCR fingerprints. These endogenous markers were obtained from two New Zealand isolates of the most prevalent botryosphaeriaceous species making them ideal tools to track and improve understanding of how these species behave in the field. The sensitivity and specificity of the resultant PCR assays were evaluated and proved enough to detect the endogenous marker of *N. parvum* and *N. luteum* when conidia of the marker isolates were dispersed in rainwater splash for up to 2 m from the source of conidia. However, these markers should also be used to test different materials from the vineyards such as grapevine wood, pruning debris and soil, to provide information on sources of infection and survival of these species in pruning debris and soil. As shown in other studies isolate specific endogenous markers have great potential for use in field experiments (Goldstein *et al*., 2000; Ridgway *et al*., 2005).

The nested PCR-RFLP assay developed for the *N. parvum* marker isolate allowed for field detection of this isolate. However, this molecular assay was not ideal as it required two separate molecular procedures (PCR and subsequent restriction digestion) and it was not possible to adapt the final assay

to quantitative PCR (qPCR). To refine this PCR assay further work, potentially including the identification of the likely polymorphism present in the UP-PCR primer binding site, may identify sufficient polymorphism from which an isolate specific PCR could be developed. This could be achieved by using molecular methods such as inverse PCR or TAIL-PCR (Ochman *et al*., 1990; Wang *et al*., 2007). Alternatively, designing a TaqMan™ probe based on the nucleotide polymorphism found in the sequence of marker isolates may allow the production of a qPCR system as this technique is powerful enough that a single nucleotide polymorphism can distinguish two individuals (Luchi *et al*., 2005). Production of a qPCR technique would allow epidemiological questions to be answered in greater depth, such as determining the amount of inoculum in water splash or the amount of fungal material present as the pathogen progresses through plant tissue. The *N. luteum* marker could also be improved by adaptation to a qPCR, and this would provide information on the factors affecting its endophytic colonisation within vines.

Given the high incidence of nine botryosphaeriaceous species in vineyards, and their high genetic diversity, it is important to plan multiple control strategies to control such a variable pathogen population due to the large genetic reservoir for responding to a control measures. The presence of botryosphaeriaceous species in so many New Zealand vineyards is a serious concern for the New Zealand winegrowers. Since 53% of the planted grapevine area is currently <7 years old, the proportion of infected vines can be expected to increase. Multiple control strategies should aim to prevent introduction and spread of this fungal pathogen in New Zealand vineyards. One source of these pathogens was identified in a study conducted in New Zealand nurseries, which isolated botryosphaeriaceous species from 23% of the planting materials sampled, most of which appeared healthy (Billiones *et al*., 2010). To prevent these pathogens entering new vineyards in this way, it is important to test the planting materials for botryosphaeriaceous species infection. An integrated approach could also help to prevent the spread of this pathogen within and between the vineyards. Since infection of botryosphaeriaceous species mainly occurs through pruning wounds, the hard pruning wounds should be protected from botryosphaeriaceous species infection using fungicides (Bester *et al*., 2007) or using a commercial formulation of biological control agents like *Trichoderma harzianum* which was able to prevent infection by *E. lata* (John *et al*., 2005). A study conducted by Amponsah *et al*. (2011) isolated botryosphaeriaceous species from the pruning debris in the vineyards, so it is also important to maintain good sanitation practices in the vineyards to prevent the spread of botryosphaeriaceous species infection within and between vineyards.

This study has provided new information on species composition, distribution and incidence of botryosphaeriaceous species associated with grapevine dieback and decline in New Zealand. The use of molecular tools for identification means that the isolate collection has been characterised with a high degree of accuracy to define nine species as present in New Zealand. The large collection of identified isolates allowed new questions to be addressed, such as the genetic diversity of the regional

populations and the relationship between genetic group and pathogenicity, enzyme production and correlation with VCGs. The genetic diversity analysis also underpinned the development of molecular assays for the analysis of pathogen processes in the field situation. Overall, the study has provided New Zealand viticulturalists with a broad knowledge on population structure that will facilitate the development of control strategies to manage this disease.

List of presentations and publications from thesis

Conference presentations

Ridgway H.J., Probst C., **Baskarathevan J.**, Jones E.E. and Jaspers M.V. (2007). Developing molecular markers for grapevine trunk diseases. Proceedings of the 16th Biannual Australian Plant Pathology Society Conference, Adelaide, Australia, 24-27 September 2007.

Baskarathevan J., Jaspers M.V., Jones E.E. and Ridgway H.J. (2008). Distribution of *Botryosphaeria* species causing grapevine dieback and decline in New Zealand vineyards. Proceedings of the Romeo Bragato 14th Annual Conference, Christchurch, New Zealand, 21-23 August 2008.

Baskarathevan. **J.,** Jaspers M.V., Jones E.E. and Ridgway H.J. (2008). Distribution of *Botryosphaeria* species causing grapevine dieback and decline in New Zealand vineyards. 61st New Zealand Plant Protection Society Annual conference, Paihia, New Zealand 12-14 August 2008.

Baskarathevan J., Jaspers M.V., Jones E.E. and Ridgway H.J. (2009). Genetic diversity of *Botryosphaeria parva* (*Neofusicoccum parvum*) in New Zealand vineyards. Paper presented at the 17th Biannual Australian Plant Pathology Society Conference, Newcastle, NSW, Australia.

Baskarathevan J., Jaspers M.V., Jones E.E. and Ridgway H.J. (2009). Occurrence and distribution of *Botryosphaeria* species causing grapevine dieback and decline in New Zealand. Paper presented at the postgraduate conference, Lincoln University, Christchurch 31st August- 1st September 2009.

Baskarathevan. **J,** Jaspers M.V., Jones E.E. and Ridgway H.J. (2010). Use of endogenous molecular markers to measure rain water splash dispersal of *Neofusicoccum* species in New Zealand vineyards. Paper presented at the 7th International Workshop on Grapevine Trunk Diseases (IWGTD) Santa Cruz, Chile 17-21 January 2010.

Billones. R.G., **Baskarathevan J.**, Jones E.E., Ridgway H.J. and Jaspers M.V. (2010). Botryosphaeria infection in grapevine- where is it coming from*?* Paper presented at the 16th Romeo Bragato Annual Conference. Marlborough 26-28 August 2010.

Journal publications

Baskarathevan. **J,** M.V. Jaspers, E.E. Jones and H.J. Ridgway (2009). Evaluation of different storage methods for rapid and cost-effective preservation of *Botryosphaeria* species. *New Zealand Plant Protection 62:234-237*.

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Appendix A

A.1 Disease information leaflet

Botryosphaeria grapevine dieback

The *Botryosphaeria* fungi cause dieback and decline in grapevines, usually causing severe losses only in vineyards that are over 8 years old. The disease generally builds up progressively in a vineyard over a number of years, leading to a gradual decline in vigour and yield of vineyard, contributing to "grapevine decline". Recent studies have shown that *Botryosphaeria* species are present in New Zealand vineyards showing dieback of shoots, spurs, buds and cordons. The dieback symptoms often start at large pruning wounds and spread inwards or downwards.

The primary symptoms include:

- dieback of shoots, spurs and arms
- cane bleaching
- bud necrosis
- trunk cankers
- wood necrosis (discoloured dead tissue in trunk sections)
- failure of graft unions
- decline or death of young vines

* Pictures illustrating typical symptoms are shown overleaf.


Symptoms of *Botryosphaeria* Grapevine Dieback
Die back from pruning wounds
Dead arm
Death of young vine
Cane bleaching
Bud necrosis
Poorly developed buds
Trunk symptom
Wedge-shape wood necrosis
Trunk canker

A.2 Survey form used for the data collection along with disease information leaflet

Botryosphaeria Dieback Disease Survey Form

(Please place this form in plastic bags with vine sample)

Contact details

Growers name	
Vineyard name	
Address	
Contact Phone No	
E-mail address	

Vine details (Please fill in as many details as possible)

Total Vineyard area	
Vines	Grafted / not grafted
	If grafted; Root stock variety:
	Scion variety :
Nursery of Origin	
Type of plant	dormant rooted plant yes / no greenhouse-grown green plant yes / no callused grafted cutting yes / no
Date of planting (years is sufficient)	
Date of first harvest	
Pruning method	
Method of disposing of pruning debris	
Vine's external symptoms & symptom history	
Other trunk disease diagnosed?	
Any special vineyard practices? eg: Vine reworking or top grafting	

Soil & management

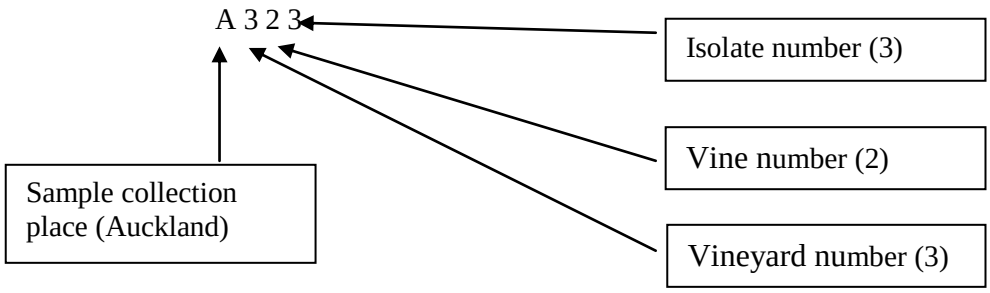
Previous crop in site	
Soil nutrient status & fertiliser	
Irrigation type	

Other comments:

A.3 Codes and numbers used to name the samples and isolates obtained in the survey

Place of sample	Code used
Auckland	A
Gisborne	G
Mulbrough	M
Blenheim	B
Nelson	N
Canterbury	C
Otago	O

For Example; Culture code A323 indicates;



A.4 Isolates of botryosphaeriaceous species used for *in vitro* sporulation experiment

No	Isolate No	Identity	Host	Location	Region/ Area
1	Q	*Diplodia mutila*	Grapevine	Ashmora	Auckland
2	Q-2	*Neofusicoccum. parvum*	Grapevine	Ashmora	Auckland
3	Q(s)	*Neofusicoccum. luteum*	Grapevine	Ashmora	Auckland
4	Q-1	*Neofusicoccum. parvum*	Grapevine	Ashmora	Auckland
5	F(20)-1	*Diplodia mutila*	Grapevine	Brancott Estate	Blenheim
6	F(12)-2	*Diplodia mutila*	Grapevine	Brancott Estate	Blenheim
7	R(s)	*Diplodia seriata*	Grapevine	Framingham	Blenheim
8	L(17)-4	*Diplodia seriata*	Grapevine	Greenhough	Nelson
9	L(17)-3	*Neofusicoccum. parvum*	Grapevine	Greenhough	Nelson
10	L-1	*Diplodia seriata*	Grapevine	Greenhough	Nelson
11	L-5	*Neofusicoccum. luteum*	Grapevine	Greenhough	Nelson
12	L(16)-2	*Diplodia seriata*	Grapevine	Greenhough	Nelson
13	H(1)-2	*Diplodia seriata*	Grapevine	Herman Siefried	Nelson
14	H(2)-3	*Neofusicoccum. parvum*	Grapevine	Herman Siefried	Nelson
15	H(1)-1	*Neofusicoccum. parvum*	Grapevine	Herman Siefried	Nelson
16	H(2)-5	*Diplodia mutila*	Grapevine	Herman Siefried	Nelson
17	I(15)-3	*Neofusicoccum. parvum*	Grapevine	Kahurangi	Nelson
18	I-4	*Neofusicoccum. parvum*	Grapevine	Kahurangi	Nelson
19	I(15)-2	*Neofusicoccum. parvum*	Grapevine	Kahurangi	Nelson
20	G(26)-2	*Neofusicoccum. luteum*	Grapevine	Kowhai	Auckland
21	M(13)-2	*Neofusicoccum. luteum*	Grapevine	Kumea river	Auckland
22	M(8)-4	*Neofusicoccum. luteum*	Grapevine	Kumea river	Auckland
23	M(30)-3	*Diplodia mutila*	Grapevine	Kumea river	Auckland
24	M(7)-6	*Diplodia mutila*	Grapevine	Kumea river	Auckland
25	RGSC-1	*Neofusicoccum. parvum*	Grapevine	Linnaeus	Gisborne
26	RGSC-3	*Neofusicoccum. parvum*	Grapevine	Linnaeus	Gisborne
27	G(s)-1	*Neofusicoccum. luteum*	Grapevine	Linnaeus	Gisborne
28	Sch	*Neofusicoccum. luteum*	Grapevine	Linnaeus	Gisborne
29	O-3-1	*Neofusicoccum. parvum*	Grapevine	Matua Valley	Auckland
30	J(22)-3	*Neofusicoccum. parvum*	Grapevine	Neudorf	Nelson
31	J(12)-1	*Neofusicoccum. parvum*	Grapevine	Neudorf	Nelson
32	K(18)-1	*Neofusicoccum. luteum*	Grapevine	Neudorf 2	Nelson
33	D(6)-1	*Diplodia mutila*	Grapevine	Roger Rose	Blenheim
34	Rd3	*Diplodia mutila*	Grapevine	Rossendales	Canterbury
35	N(12)-2	*Neofusicoccum. luteum*	Grapevine	Rothesay	Auckland
36	N-1	*Neofusicoccum. luteum*	Grapevine	Rothesay	Auckland
37	F-1	*Diplodia mutila*	Willow	Brancott	Blenheim

A.5 Recipes of media types used to induce *in vitro* sporulation of botryosphaeriaceous species

Tap water agar medium (TWA)

20 g agar powder (Danisco® Bacteriological agar)
Tap water to make 1 L
Autoclave

Prune Extract Agar (PEA)

Use 25 g prunes per 500 mL flask.
De-stone and chop up prunes. Fill flask with distilled water to just under neck; cap flask. Place in pressure cooker (half filled with water) and boil in free steam for 30 minutes.

Filter product through filters – Whatman filter paper no. 3 (or two tissues) then Whatman filter paper no. 1. Add 400 mL aliquots to 500ml flasks.

Autoclave then refrigerate

To make 1L agar:

Sucrose	5 g
Yeast extract	1 g
Agar(Danisco® Bacteriological agar)	30 g
Prune extract	100 mL
Distilled water	900 mL

Boil to dissolve before autoclaving.

A.6 The ITS sequences of botryosphaeriaceous and associated species retrieved from the GenBank used in this study

Isolate	Species	Host	Origin	GenBank accession
DAR78997	*N. parvum*	*Vitis vinifera*	Australia	EU919694
DAR79000	*N. parvum*	*V. vinifera*	Australia	EU919701
CMW9080	*N. parvum*	*Populus nigra*	New Zealand	AY236942
CMW9077	*N. parvum*	*Actinidia deliciosa*	New Zealand	AY236939
CMW7773	*N. ribis*	*Ribes* sp.	USA	AY236936
CMW7772	*N. ribis*	*Ribes* sp.	USA	AY236935
H73-1	*N. ribis*	*V. vinifera*	Australia	HQ392733
UCD2057Te	*N. luteum*	*V. vinifera*	USA	DQ233604
UCD2089Te	*N. luteum*	*V. vinifera*	USA	DQ233605
CMW9076	*N. luteum*	*Malus × domestica*	New Zealand	AY236946
DAR79503	*N. australe*	*V. vinifera*	Australia	FJ422898
CMW6853	*N. australe*	*Sequiadendron giganteum*	Australia	AY339263
CBS115041	*Do. iberica*	*Quercus ilex*	Spain	AY573202
DAR78992	*Do. iberica*	*V. vinifera*	Australia	EU768874
DAR78992	*Do. iberica*	*V. vinifera*	Australia	EU768874
CBS 120.41	*Do. sarmentorum*	*Pyrus communis*	Norway	AY573207
IMI 63581b	*Do. sarmentorum*	*Ulmus* sp.	United kingdom	AY573212
UCD1064So	*B. dothidea*	*V. vinifera*	USA	DQ233600
UCD1065So	*B. dothidea*	*V. vinifera*	USA	DQ233601
DAR79135	*D. mutila*	*V. vinifera*	Australia	EU919691
DAR79131	*D. mutila*	*V. vinifera*	Australia	EU919687
UCD288Ma	*D. mutila*	*V. vinifera*	USA	DQ008313
STE-U4444	*D. seriata*	*V. vinifera*	South Africa	AY343424
STE-U4440	*D. seriata*	*V. vinifera*	South Africa	AY343420
UCD244Ma	*D. seriata*	*V. vinifera*	USA	DQ008314
UCD465Fr	*D. seriata*	*V. vinifera*	USA	DQ008316
CMW11372	*Mycosphaerella pini*	*Pinus radiata*	South Africa	AY808277
CMW7063	*Bionectria* sp.	*Not known*	South Africa	AY236956

A.7 Results of Pearson Chi-Square Test

A.7.1 Incidence of botryosphaeriaceous species infection on grapevines in different winegrowing area

	Value	df	P
Pearson Chi-Square	65.801[a]	5	0.000
Likelihood Ratio	67.701	5	0.000
Number of valid cases	238		

a- 0 cells (0%) have expected count less than5. The minimum expected count is 7.98.

A.7.2 Incidence of botryosphaeriaceous species infection on different age group of grapevines

	Value	df	P
Pearson Chi-Square	28.666[a]	5	0.000
Likelihood Ratio	29.994	5	0.000
Number of valid cases	197		

a- 1 cell (8.3%) has expected count less than 5. The minimum expected count is 4.90.

A.7.3 Incidence of botryosphaeriaceous species infection on different scion variety of grapevines

	Value	df	P
Pearson Chi-Square	28.039[a]	9	0.001
Likelihood Ratio	31.056	9	0.000
Number of valid cases	165		

a- 11 cells (55.0%) have expected count less than 5. The minimum expected count is 1.

A.7.4 Incidence of botryosphaeriaceous species infection on grafted and non-grafted grapevines

	Value	df	P
Pearson Chi-Square	5.873[a]	1	0.015
Likelihood Ratio	5.640	1	0.018
Number of valid cases	168		

a- 0 cells (0%) have expected count less than5. The minimum expected count is 8.71.

A.8 Number of vineyards with multiple botryosphaeriaceous species infection from six wine growing regions

Region	No. of Vineyards [a]	Vineyards positive for botryosphaeriaceous species (%) [b]	No. of vineyards with multiple botryosphaeriaceous species infection
Auckland	12	12 (100)	10
Gisborne	6	6 (100)	5
Blenheim	8	6 (75)	6
Nelson	6	6 (100)	4
Canterbury	3	3 (100)	3
Otago	8	5 (63)	2
Totals	43	38 (88)	30

[a] Number of vineyards sampled, [b] number of vineyards (and percentage of total number of vineyards sampled per region) yielding botryosphaeriaceous species

A.9 Analysis of variance of effect of temperature on growth of botryosphaeriaceous species

A.9.1 ANOVA results of effect of temperature on mycelia growth rate of botryosphaeriaceous species

Source	Sum of Squares	df	Mean Square	F	p-value
Species	215.87	4	53.968	5.63	0.043
Region	42.391	5	8.478	0.884	0.552
Error	47.931	5	9.586		
Total	15640.61	15			

A.9.2 ANOVA results optimum temperature for maximum growth rate of botryosphaeriaceous species

Source	Sum of Squares	df	Mean Square	F	p-value
Species	8.446	4	2.111	5.017	0.053
Region	4.468	5	0.894	2.123	0.214
Error	2.104	5	0.421		
Total	9681.153	15			

Appendix B

B.1 Botryosphaeriaeceous isolates used for the genetic diversity study

B.1.1 *Neofusicoccum parvum* isolates

No	Isolate ID	Origin
1	A122	Auckland, New Zealand
2	A233	Auckland, New Zealand
3	A242	Auckland, New Zealand
4	A421	Auckland, New Zealand
5	A564	Auckland, New Zealand
6	A622	Auckland, New Zealand
7	A75a1	Auckland, New Zealand
8	A813	Auckland, New Zealand
9	A842	Auckland, New Zealand
10	A922	Auckland, New Zealand
11	A931	Auckland, New Zealand
12	A102b4	Auckland, New Zealand
13	A102b6	Auckland, New Zealand
14	A102b9	Auckland, New Zealand
15	A1111	Auckland, New Zealand
16	A-Nu11	Auckland, New Zealand
17	A-Nu13	Auckland, New Zealand
18	G121	Gisborne, New Zealand
19	G131	Gisborne, New Zealand
20	G141	Gisborne, New Zealand
21	G22a3	Gisborne, New Zealand
22	G22a6	Gisborne, New Zealand
23	G22b1	Gisborne, New Zealand
24	G22c2	Gisborne, New Zealand
25	G233	Gisborne, New Zealand
26	G341	Gisborne, New Zealand
27	G41b1	Gisborne, New Zealand
28	G41c1	Gisborne, New Zealand
29	G61a1	Gisborne, New Zealand
30	G61c1	Gisborne, New Zealand
31	G62a1	Gisborne, New Zealand
32	G62b1	Gisborne, New Zealand
33	G62c1	Gisborne, New Zealand
34	G63a1	Gisborne, New Zealand
35	G651	Gisborne, New Zealand
36	G652	Gisborne, New Zealand
37	G69a1	Gisborne, New Zealand
38	B2141	Blenheim, New Zealand
39	B2142	Blenheim, New Zealand
40	B316a2	Blenheim, New Zealand
41	Q-2	Blenheim, New Zealand
42	N6121	Nelson, New Zealand
43	I(15)2	Nelson, New Zealand
44	I(15)3	Nelson, New Zealand
45	I-4	Nelson, New Zealand
46	MM561	Hawkes bay, New Zealand
47	MM562	Hawkes bay, New Zealand
48	MM660	Hawkes bay, New Zealand
49	ICMP8003	Bay of plenty, New Zealand
50	DAR78997	NSW, Australia
51	DAR78998	NSW, Australia
52	DAR78999	NSW, Australia
53	DAR79000	NSW, Australia
54	STE-U-4589	South Africa
55	STE-U-5142	South Africa
56	STE-U-5456	South Africa
57	STE-U-5885	South Africa
58	UCD1799Sac	Stanislaus Co, CA, USA
59	UCD642So	Sonoma Co, CA, USA
60	UCD646So	Sonoma Co, CA, USA

B.1.2 *Neofusicoccum luteum* isolates

No	Isolate ID	Origin	Non-grapevine host
1	A243	Auckland, New Zealand	
2	A511	Auckland, New Zealand	
3	A512	Auckland, New Zealand	
4	A526	Auckland, New Zealand	
5	A533	Auckland, New Zealand	
6	A563	Auckland, New Zealand	
7	A821	Auckland, New Zealand	
8	A953	Auckland, New Zealand	
9	A102b1	Auckland, New Zealand	
10	CB-P11	Auckland, New Zealand	
11	G16a2	Gisborne, New Zealand	
12	G31a3	Gisborne, New Zealand	
13	G31d2	Gisborne, New Zealand	
14	G32c1	Gisborne, New Zealand	
15	G51a2	Gisborne, New Zealand	
16	G51b1	Gisborne, New Zealand	
17	G63b1	Gisborne, New Zealand	
18	M(13)-2	Auckland, New Zealand	
19	M(8)-4	Auckland, New Zealand	
20	G(S)-1	Gisborne, New Zealand	
21	N(12)-2	Auckland, New Zealand	
22	N-1	Auckland, New Zealand	
23	ICMP8004	Bay of Plenty, New Zealand	*Actinidia deliciosa*
24	ICMP16678	Auckland, New Zealand	*Rhododendron vireya*
25	ICMP7818	Auckland, New Zealand	*Malus X domestica*
26	MM558	Hawkes bay, New Zealand	
27	MM554	Hawkes bay, New Zealand	
28	MM594	Hawkes bay, New Zealand	
34	CC440	Takaka, New Zealand (nelson)	*Actinidia arguta*
31	CC445	Bay of Plenty, New Zealand	*Actinidia arguta*
29	CC451	Pukekohe, New Zealand	
32	CC452	Pukekohe, New Zealand	
35	CC456	Hawkes bay, New Zealand	
30	CC461	Hawkes bay, New Zealand	
33	CC462	Hawkes bay, New Zealand	
36	UCD2057Te	Temecula Valley, CA, USA	
37	UCD2089Te	Temecula Valley, CA, USA	
38	UCD2090Te	Temecula Valley, CA, USA	
39	UCD2103Te	Temecula Valley, CA, USA	
40	UCD2069Te	Temecula Valley, CA, USA	

B.1.3 *Neofusicoccum australe* isolates

No	Isolate ID	Origin	Non-grapevine host
1	Kat-1	Canterbury, New Zealand	
2	Mel-1	Canterbury, New Zealand	
3	Mel-2	Canterbury, New Zealand	
4	Mel-3	Canterbury, New Zealand	
5	Mel-4	Canterbury, New Zealand	
6	Q(s)	Blenheim, New Zealand	
7	K(18)-1	Nelson, New Zealand	
8	J-3	Nelson, New Zealand	Broom
9	ICMP15894	New Zealand	*Juglans sp*
10	B2111	Blenheim, New Zealand	
11	B31411	Blenheim, New Zealand	
12	B31418	Blenheim, New Zealand	
13	B31621	Blenheim, New Zealand	
14	B31712	Blenheim, New Zealand	
15	B31912	Blenheim, New Zealand	
16	B3212	Blenheim, New Zealand	
17	B3414	Blenheim, New Zealand	
18	B34b1	Blenheim, New Zealand	
19	B39a2	Blenheim, New Zealand	
20	C131	Canterbury, New Zealand	
21	C1B21	Canterbury, New Zealand	
22	C2101	Canterbury, New Zealand	
23	C281	Canterbury, New Zealand	
24	G212	Gisborne, New Zealand	
25	MM630	Hawkes bay, New Zealand	
26	MM572	Hawkes bay, New Zealand	
27	CB-P74	Auckland, New Zealand	
28	CB-P8	Auckland, New Zealand	
29	R0355	Auckland, New Zealand	
30	R0480	Auckland, New Zealand	
31	R0511	Auckland, New Zealand	
32	R0518	Auckland, New Zealand	
33	R0240	Auckland, New Zealand	

B.1.4 *Diplodia mutila* isolates

No	Isolate ID	Origin	Non-grapevine host
1	M211	Blenheim, New Zealand	
2	M213	Blenheim, New Zealand	
3	M221	Blenheim, New Zealand	
4	M226	Blenheim, New Zealand	
5	M246	Blenheim, New Zealand	
6	M232	Blenheim, New Zealand	
7	M248	Blenheim, New Zealand	
8	B1326	Blenheim, New Zealand	
9	B3111	Blenheim, New Zealand	
10	B3612	Blenheim, New Zealand	
11	B3811	Blenheim, New Zealand	
12	B31012	Blenheim, New Zealand	
13	B31111	Blenheim, New Zealand	
14	B31213	Blenheim, New Zealand	
15	B32011	Blenheim, New Zealand	
16	B32012	Blenheim, New Zealand	
17	B32112	Blenheim, New Zealand	
18	B3413	Blenheim, New Zealand	
19	G16a1	Gisborne, New Zealand	
20	G16a3	Gisborne, New Zealand	
21	G173	Gisborne, New Zealand	
22	G211	Gisborne, New Zealand	
23	C221	Canterbury, New Zealand	
24	C251	Canterbury, New Zealand	
25	C261	Canterbury, New Zealand	
26	N13c3	Nelson, New Zealand	
27	N1351	Nelson, New Zealand	
28	N13b1	Nelson, New Zealand	
29	N6315	Nelson, New Zealand	
30	Q	Blenheim, New Zealand	
31	F(20)-1	Blenheim, New Zealand	
32	F(12)-2	Blenheim, New Zealand	
33	M(30)-3	Auckland, New Zealand	
34	M(7)-6	Auckland, New Zealand	
35	D(6)-1	Blenheim, New Zealand	
36	Rd3	Canterbury, New Zealand	
37	F-1	Blenheim, New Zealand	Willow
38	A-3	Blenheim, New Zealand	Plum
39	A-2	Blenheim, New Zealand	Apple
40	J-4	Nelson, New Zealand	Oak
41	DAR79131	NSW, Australia	
42	DAR79132	NSW, Australia	
43	DAR79135	NSW, Australia	
44	DAR79136	NSW, Australia	
45	DAR79137	NSW, Australia	
46	UCD288Ma	Madera, CA, USA	
47	UCD1953SB	Santa Barbara, CA, USA	
48	UCD1965SB	Santa Barbara, CA, USA	

B.2 TAE used for gel electrophoresis

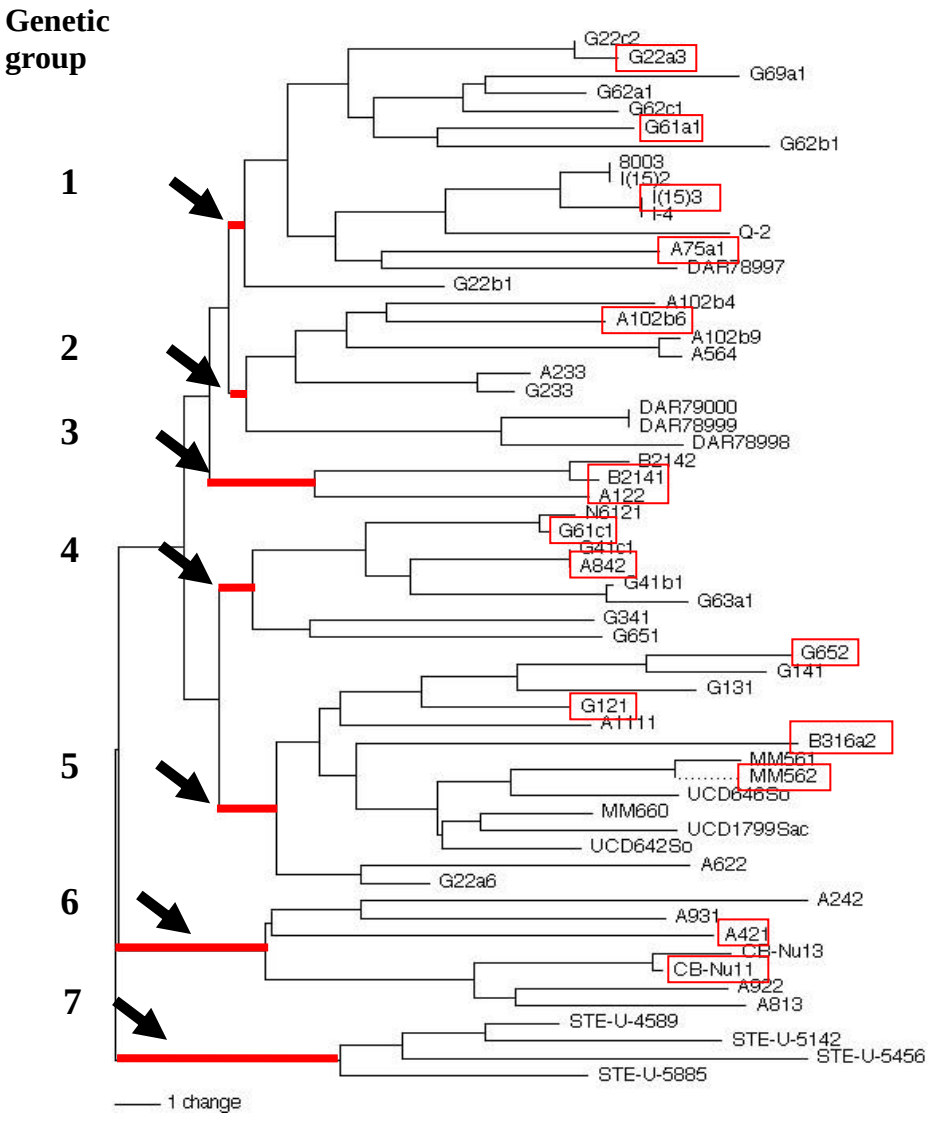
To prepare 50X TAE;

Tris- base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8)	100 mL
Sterile water	-make up to 1 L

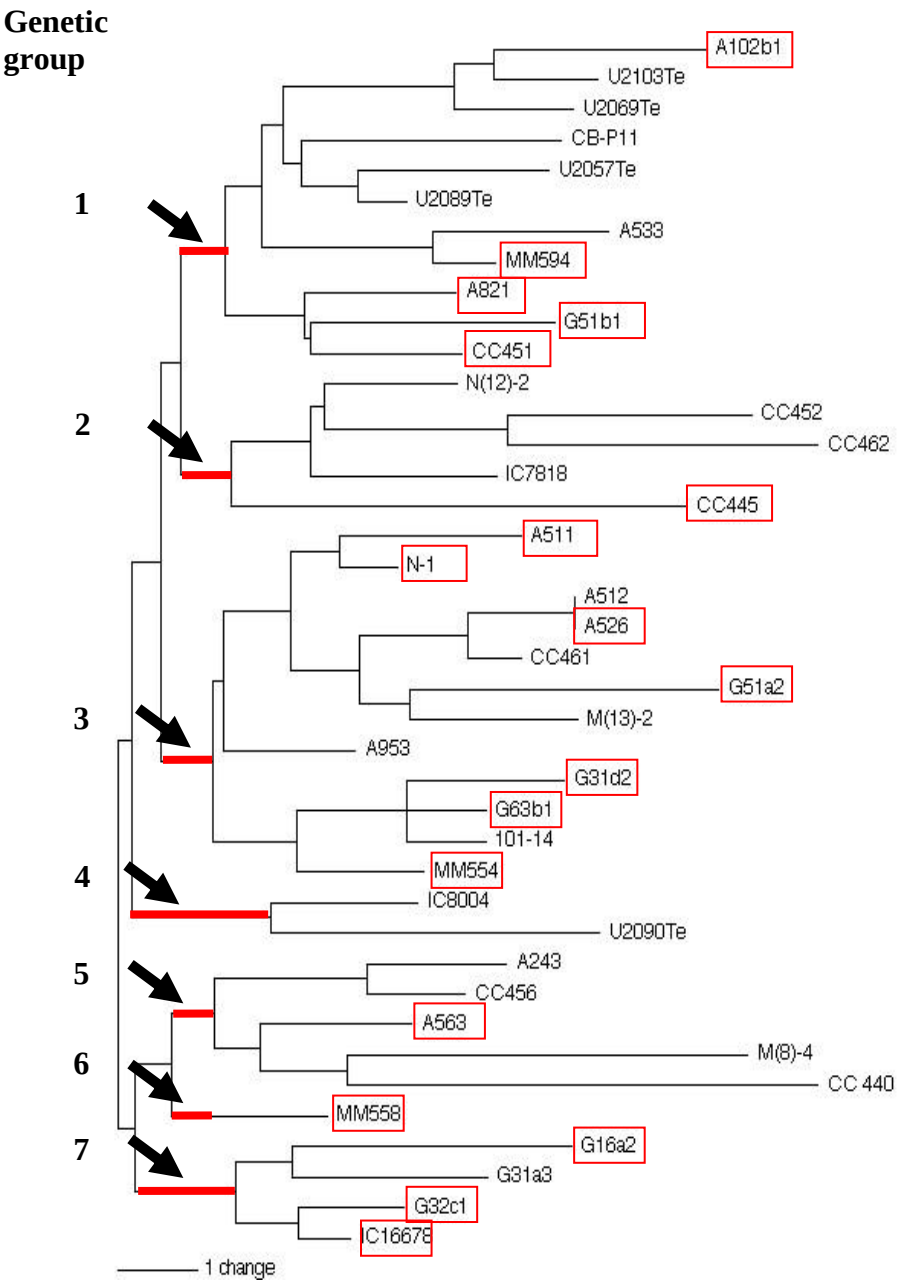
Appendix C

C.1 Botryosphaeriaeceous isolates used for the pathogenicity studies selected from different genetic groups of neighbour joining (NJ) tree

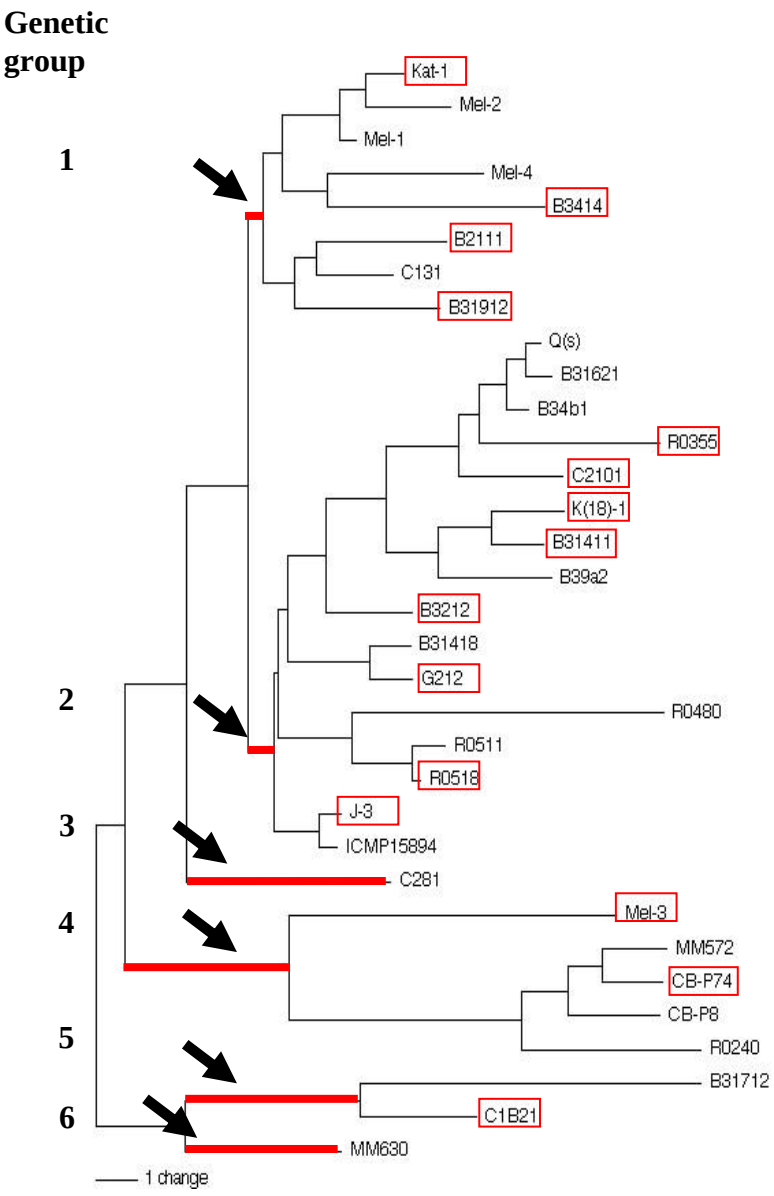
C.1.1 *Neofusicoccum parvum* isolates used for the pathogenicity studies (indicated in boxes)



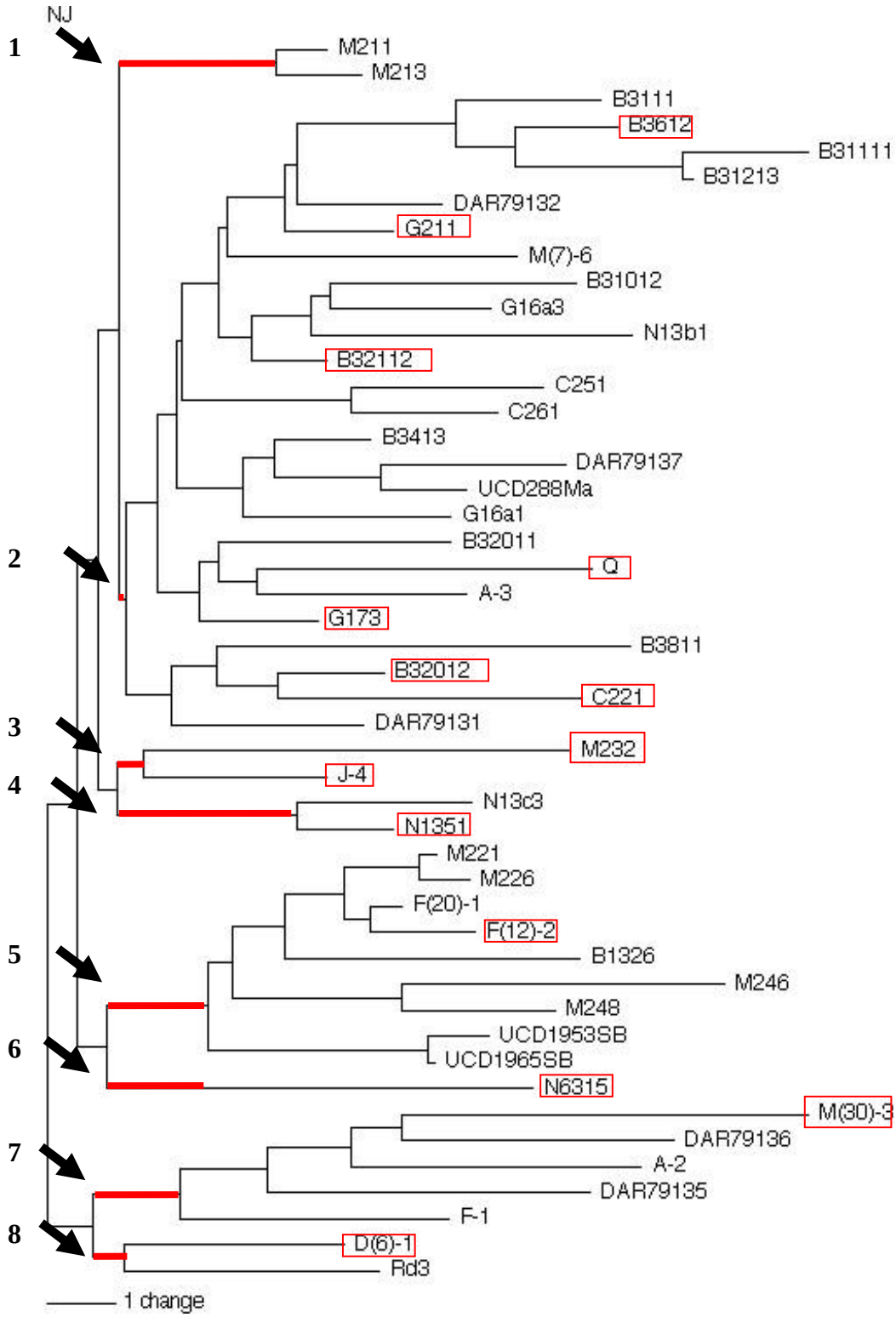
C.1.2 *Neofusicoccum luteum* isolates used for the pathogenicity studies (indicated in boxes)



C.1.3 *Neofusicoccum australe* isolates used for the pathogenicity studies (indicated in boxes)



C.1.4 *Diplodia mutila* isolates used for the pathogenicity studies (indicated in boxes)



C.2 Potting mix (1 year) used for grapevines

To prepare 1000 litres;

Media - 800 litres composted bark
200 litres pumice

Fertilisers - Osmocote exact 15-3.9-9.1 (N,P,K) 12 -14 months 5000g
- Agricultural Lime 1000g
- Hydraflo 1000g

C.3 Vogal's minimum salt medium

Distilled water	500 ml
Vogel's N conc salts (see below)	10 ml
Sucrose	10 g
KNO_3	0.505 g
Agar	7.5 g

Auotclave at 121 C for 15 min

A) Concentrated salts solution

In 775 ml distilled water the following salts were added in order and while stirring over heat on a magnetic stirrer. Each one was dissolved before the neaxt one was added. One gram of calcium chloride ($CaCl_2$) was added at a time.

Na_3 citrate $2H_2O$	125 g
KH_2PO_4	250 g
$MgSO_4$. $7H_2O$	10 g
$CaCl_2$. $2H_2O$	5 g

Add following to salts before first use:
Trace element solution (see B) 5.0 ml

B) Trace element solution

In 95 ml distilled water the following compounds were added:

Citric acid H_2O	5 g
$ZnSO_4$. $7H_2O$	5 g
$Fe(NH_4)2(SO_4)2$. 6 H_2O	1g
$CuSO_4$. $5H_2O$	0.25 g
$MnSO_4$. H_2O	50 mg
Boric acid (anhydrous)	50 mg
Na_2MoO_4. $2H_2O$	50 mg

The resulting solution was 50x concentrated; 2 ml chloroform were added as a preservative and the solution can then be stored at room temperature

C.4 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 in therefore useful for enzyme kinetic studies.

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 (http://stanxterm.aecom.yu.edu/wiki/index.php?page=McIlvaine_buffer).

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

C.5 Analysis of variance of pathogenicity studies

C.5.1 ANOVA results of lesion lengths produced by *N. parvum* isolates on green shoots after 7 days

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	14	26158.4	1868.5	4.08	<.001
Residual	45	20612.0	458.0		
Total	59	46770.4			

C.5.2 ANOVA results of lesion lengths produced by *N. parvum* isolates on pottedvines after 6 weeks

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	5	25052.7	5010.5	21.10	<.001
Residual	41	9734.0	237.4		
Total	46	34786.7			

C.5.3 ANOVA results of endophytic movements of *N. parvum* isolates in pottedvines after 6 weeks

Variate: endophytic movement

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	5	18915.3	3783.1	7.63	<.001
Residual	38	18839.3	495.8		
Total	43	37754.5			

C.5.4 ANOVA results of lesion lengths produced by *N. luteum* isolates on green shoots after 5 days

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	17	52457.3	3085.7	8.44	<.001
Residual	54	19752.5	365.8		
Total	71	72209.8			

C.5.5 ANOVA results of lesion lengths produced by *N. luteum* isolates on pottedvines after 6 weeks

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	5	148851.5	29770.3	35.69	<.001
Residual	33	27523.0	834.0		
Total	38	161573.6			

C.5.6 ANOVA results of endophytic movements of *N. luteum* isolates in pottedvines after 6 weeks

Variate: endophytic movement

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	4	65366	16342	3.57	0.033
Residual	14	64125	4580		
Total	18	96895			

C.5.7 ANOVA results of lesion lengths produced by *N. australe* isolates on green shoots after 7 days

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	14	16156.9	1154.1	2.46	0.011
Residual	45	21128.0	469.5		
Total	59	37284.9			

C.5.8 ANOVA results of lesion lengths produced by *N. australe* isolates on pottedvines after 6 weeks

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	6	98466	16411	13.66	<.001
Residual	44	52867	1202		
Total	50	46651			

C.5.9 ANOVA results of endophytic movements of *N. australe* isolates in pottedvines after 6 weeks

Variate: endophytic movement

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	5	39676	7935	6.29	<.001
Residual	24	30261	1261		
Total	29	57174			

C.5.10 ANOVA results of lesion lengths produced by *D. mutila* isolates on green shoots after 7 days

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	13	7247.86	557.53	9.10	<.001
Residual	42	2573.50	61.27		
Total	55	9821.36			

C.5.11 ANOVA results of lesion lengths produced by *D. mutila* isolates on pottedvines after 6 weeks

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	6	36641.3	6106.9	35.81	<.001
Residual	42	7162.0	170.5		
Total	58	41452.4			

C.5.12 ANOVA results lesion lengths produced by *N. parvum* isolates belonging in different genetic groups (HVG and LVG) pottedvine after 6 weeks

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	6	236458.4	39409.7	44.09	<.001
Residual	47	42009.2	893.8		
Total	53	275944.0			

C.5.13 ANOVA results of lesion lengths produced by *N. luteum* isolates belonging in different genetic groups (HVG and LVG) pottedvine after 6 weeks

Variate: Lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	8	635259	79407	23.10	<.001
Residual	53	182186	3437		
Total	61	782236			

C.5.14 ANOVA results lesion lengths produced by *N. parvum* and *N. luteum* isolates in co-inoculation on pottedvines after 6 weeks

Variate: Total lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	213762	30537	16.46	<.001
Residual	40	74215	1855		
Total	47	250960			

Variate: Upward lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	131472	18782	14.33	<.001
Residual	40	52421	1311		
Total	47	159776			

Variate: Upward lesion

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	34508.9	4929.8	6.46	<.001
Residual	40	30523.4	763.1		
Total	47	54744.0			

C.5.15 ANOVA results of endophytic movements of *N. parvum* and *N. luteum* isolates in co-inoculation on pottedvines after 6 weeks

Variate: endophytic movement

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	6	88527	14755	3.74	0.012
Residual	20	78825	3941		
Total	26	123163			

Variate: upward endophytic movement of *N. parvum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	4	34508	8627	2.45	0.092
Residual	15	52900	3527		
Total	19	70200			

Variate: downward endophytic movement of *N. parvum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	4	12407	3102	2.46	0.090
Residual	15	18875	1258		
Total	19	25095			

Variate: upward endophytic movement of *N. luteum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	4	62793	15698	4.62	0.012
Residual	15	50975	3398		
Total	19	82455			

Variate: downward endophytic movement of *N. luteum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	4	29581	7395	4.00	0.021
Residual	15	27750	1850		
Total	19	42580			

C.5.16 ANOVA results of laccase enzyme production of *N. parvum* isolates

Variate: downward endophytic movement of *N. luteum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	2	0.0344871	0.0172436	30.95	<.001
Residual	8	0.0044567	0.0005571		
Total	10	0.0188767			

Appendix D

D.1 SOC culture medium for growing *E. coli*

Bacto-Tryptone -20g

Bacto-Yeast extracts -5g

NaCl -0.5g

1 M KCl -2.5 mL

1 M glucose -20 mL

- Dissolve in 900 mL water

- Adjust pH to 7.0 with 10 M NaOH and add water up to 990 mL

- Sterilize by autoclaving and store at room temperature

- Add 10 mL sterile 1 M $MgCl_2$

D.2 Luria Bertani (LB) Broth / Agar

LB broth

Bacto-Tryptone -10g

Bacto-Yeast extracts -5g

NaCl -10g

- Dissolve in 900 mL water

- Adjust pH to 7.0 with 10 M NaOH

- Adjust the volume to 1 L and sterilize by autoclaving and store at room temperature

LB agar

Additionally add 10 g agar to the LB broth incredient

D.3 Marker band sequence of *N. parvum* marker isolate B2141 showing the primer binding sites

The strandard PCR primer (external primers) annealing sites were indicated in red and the nested-PCR primer (internal primers) annealing sites indicated in green.

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ORIGIN
1       CTGCGACCCA GAGCGGATAC AACGTCAGTG CCAAGGTGAG GTGACGAGTC
51      TGCGTACGGA TAGCCACATT TCCATGTGAA GTGGACCCAG ATAAGCTGGG
101     GCTTTGCGAG GTCCGGTGAG AAGAGGATGC CGAGGAAATA CTCGGAGCTG
151     GGACGGGTGG TGAAGTCGAA CTGTGAGTAG GCGCGACAGA GCAGCTTATG
201     AGTGGGCCAG TCGGCGGTCT GGCATTCCTT CGAGCAGTAC CAGATGCAGT
251     GACAGCGACC GCAAGCCTGC TGATGCGGCT TCGAGCATGC AGCACACAGG
301     CTTTTCGGGT CGGCCATCGT GCAGTGAATA ACACAGGAGA GGGACCGGAG
351     GAATTTTCAG AAGATGGTTG GAAGAAGAGG GTGAGTTGTG TGAGCGAAGG
401     CTATGTCATT GCGGACGCGC AACGCTAAAG CCAATGCAGA AGCGCAGTCA
451     GAGGTCGGGT GTCATTTCAG GAAGTCACGC TCACTTCCAT TCGTTCTACC
501     AGATTCTGTA GGGCTGCAGA GCTTCATGGG CCATCCGCTC TCCCAGCCTT
551     TATCTCTGAA ATTCAAGTCG TTTGTTCTTT CTTTACTACG CGCACTTCCG
601     TGGGCAAACA TGTTCAGGCA TATTTTCTGC GCATTCTCCA CTTGGTCACT
651     TCACTCCTGG CAAAGACATA TCTTCTCCAC ACGTATCGAT CTTCCACAGG
701     ATGATAGCCG TCTCGCCGAA ACTTCTCTTG GCTAAAATCA TCAATTTTCC
751     AGGCTCTTCA AGCCCTGTCT GGGGAAGTGC TATCGATCGC ATACTCCTGT
801     AGCTTTGGCC CTACCGGCAG CAGCAAGATC TCTCTTATTC TTTCCCGGAT
851     GCTTTTTCTA TGCCCTCTAG CCTCTTGATC ATCTCACTTG TGGAAGAAGG
901     CATCTCGCTA ATTGCTACAC CATCCATCTT GTGAAACCGT TTCCTCAAGA
951     GTTCAGGACA TCCAGATCCC AGAAGTCTGA TCCCACGCTT GTTCTTAATG
1001    ACAGTCATAT CTTTGGAAGG GCTGATCCTG ATCCCGATAG AAGCTGTCCC
1051    GGGTTTCAGA GAGGATGGAA GATCAGATAA TCTGGCGATC TTGCCGCTAC
1101    AGATGACACG TCTGAGAGTT TAATCACAGG TTTCACAAAG CCCATCAAAC
1151    CGAGAGGCCG TGGTATTCGG ACAAAACAAT TCTGCATGGC GTGAAGACGG
1201    CGTGATGTTG CGTCTCACAT ACGCCGTCAC CTCTGAAACA CTTCGTTGCG
1251    GCGTAGCAAC GAAGATCAGC AGACTTCTAA CCCCTGCGGA GAAAAATGCG
1301    GGGGTCTATG CGCCACGACA CGGCTTCTCG CGCTATCTAC GCCTAAGAAT
1351    ATTCACGGAA TAACATATTC GGGGTCGGAA TCGCGTTCGG TCTTAATAGT
1401    TGATTATTCA TTAAGTGTAG GTCCTTTTCG GACGGCCTTG GCAGTGACGA
1451    CAAACAACCA GATATCCAGG CGCTTTCAGA TACTGGCCAA AAAGATACAC
1501    GCTCCCGCAC TTGCAATACT CCTGCCCGTT CCTATCTTAC ACACTACTTT
1551    CTCCTCTCCG CTCTGGGTCG CAG //
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D.4 Sequence alignments of *N. parvum* marker isolate B2141 with *N. parvum* non-marker isolates

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ORIGIN
B2141                CTGCGACCCAGAGCGGATACAACGTCAGTGCCAAGGTGAG     40
DAR78999             ....................................cGtt      4
UCD646So             ...................................aata      4
B316a2               ..................................cGGTtAa      7
G233                 ...................................caGta      5
G61a1                ....................................cGtt      4
Consensus

B2141                GTGACGAGTC.TGCGTACGGATAGCCACATTTCCATGTGA     79
DAR78999             aaGgCagagt.TGCGTACGGATAGCCACATTTCCATGTGA     43
UCD646So             agttCagagtcTGCGTACGGATAG.CACATTTCCATGTGA     43
B316a2               GTtcaaAGTC.TGCGTACGGATAGCCACATTTCCATGTGA     46
G233                 aaagCagagt.TGCGTACGGATAGCCACATTTCCATGTGA     44
G61a1                agtgCagagt.TGCGTACGGATAGCCACATTTCCATGTGA     43
Consensus                     tgcgtacggatag cacatttccatgtga
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B2141              AGTGGACCCAGATAAGCTGGGGCTTTGC.GAGGTCCGGTG    118
DAR78999           AGTGGACCCAGATAAGCTGGGGCTTTcCgGAGGTCCGGTG     83
UCD646So           AGTGGACCCAGATAAGCTGGGGCTTTcCgGAGGTCCGGTG     83
B316a2             AGTGGACCCAGATAAGCTGGGGCTTTGC.GAGGTCCGGTG     85
G233               AGTGGACCCAGATAAGCTGGGGCTTTGC.GAGGTCCGGTG     83
G61a1              AGTGGACCCAGATAAGCTGGGGCTTTcCgGAGGTCCGGTG     83
Consensus          agtggacccagataagctggggcttt c gaggtccggtg

B2141              AGAAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGT    158
DAR78999           AGAAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGT    123
UCD646So           AGAAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGT    123
B316a2             AGAAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGT    125
G233               AGAAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGT    123
G61a1              AGAAGAGGATGCCGAGGAAATACTCGGAGCTGGGACGGGT    123
Consensus          agaagaggatgccgaggaaatactcggagctgggacgggt

B2141              GGTGAAGTCGAACTGTGAGTAGGCGCGACAGAGCAGCTTA    198
DAR78999           GGTGAAGTCaAACTGTGAGTAGGCGCGACAGAGCAGCTTA    163
UCD646So           GGTGAAGTCaAACTGTGAGTAGGCGCGACAGAGCAGCTTA    163
B316a2             GGTGAAGTCaAACTGTGAGTAGGCGCGACAGAGCAGCTTA    165
G233               GGTGAAGTCaAACTGTGAGTAGGCGCGACAGAGCAGCTTA    163
G61a1              GGTGAAGTCaAACTGTGAGTAGGCGCGACAGAGCAGCTTA    163
Consensus          ggtgaagtc aactgtgagtaggcgcgacagagcagctta

B2141              TGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGT    238
DAR78999           TGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGT    203
UCD646So           TGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGT    203
B316a2             TGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGT    205
G233               TGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGT    203
G61a1              TGAGTGGGCCAGTCGGCGGTCTGGCATTCCTTCGAGCAGT    203
Consensus          tgagtgggccagtcggcggtctggcattccttcgagcagt

B2141              ACCAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGG    278
DAR78999           ACCAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGG    243
UCD646So           ACCAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGG    243
B316a2             ACCAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGG    245
G233               ACCAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGG    243
G61a1              ACCAGATGCAGTGACAGCGACCGCAAGCCTGCTGATGCGG    243
Consensus          accagatgcagtgacagcgaccgcaagcctgctgatgcgg

B2141              CTTCGAGCATGCAGCACACAGGCTTTTCGGGTCGGCCATC    318
DAR78999           CTTCGAGCATGCAGCACACAGGCTTTTCGGGTCGGCCATC    283
UCD646So           CTTCGAGCATGCAGCACACAGGCTTTTCGGGTCGGCCATC    283
B316a2             CTTCGAGCATGCAGCACACAGGCTTTTCGGGTCGGCCATC    285
G233               CTTCGAGCATGCAGCACACAGGCTTTTCGGGTCGGCCATC    283
G61a1              CTTCGAGCATGCAGCACACAGGCTTTTCGGGTCGGCCATC    283
Consensus          cttcgagcatgcagcacacaggcttttcgggtcggccatc

B2141              GTGCAGTGAATAACACAGGAGAGGGACCGGAGGAATTTTC    358
DAR78999           GTGCAGTGAATAACACAGGAGAGGGACCGGAGGAATTTTC    323
UCD646So           GTGCAGTGAATAACACAGGAGAGGGACCGGAGGAATTTTC    323
B316a2             GTGCAGTGAATAACACAGGAGAGGGACCGGAGGAATTTTC    325
G233               GTGCAGTGAATAACACAGGAGAGGGACCGGAGGAtTTTTC    323
G61a1              GTGCAGTGAATAACACAGGAGAGGGACCGGAGGAATTTTC    323
Consensus          gtgcagtgaataacacaggagagggaccggagga ttttc

B2141              AGAAGATGGTTGGAAGAAGAGGGTGAGTTGTGTGAGCGAA    398
DAR78999           AGAAGATGGTTGGAAGAAGAGGGTGAGTTGTGTGAGCGAA    363
UCD646So           AGAAGATGGTTGGAAGAAGAGGGTGAGTTGTGTGAGCGAA    363
B316a2             AGAAGATGGTTGGAAGAAGAGGGTGAGTTGTGTGAGCGAA    365
G233               AGAAGATGGTTGGAAGAAGAGGGTGAGTTGTGTGAGCGAA    363
G61a1              AGAAGATGGTTGGAAGAAGAGGGTGAGTTGTGTGAGCGAA    363
Consensus          agaagatggttggaagaagagggtgagttgtgtgagcgaa
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B2141              GGCTATGTCATTGCGGACGCGCAACGCTAAAGCCAATGCA    438
DAR78999           GGCTATGTCATTGCGGACGCGCAACcCTAAAGCCAATGCA    403
UCD646So           GGCTATGTCATTGCGGACGCGCAACGCTAAAGCCAATGCA    403
B316a2             GGCTATGTCATTGCGGACGCGCAACGCTAAAGCCAATGCA    405
G233               GGCTATGTCATTGCGGACGCGCAACGCTAAAGCCAATGCA    403
G61a1              GGCTATGTCATTGCGGACGCGCAACGCTAAAGCCAATGCA    403
Consensus          ggctatgtcattgcggacgcgcaac ctaaagccaatgca

B2141              GAAGCGCAGTCAGAGGTCGGGTGTCATTTCAGGAAGTCAC    478
DAR78999           GAAGCGCAGTCAGAGGTCGGGTGTCATTTCAGGAAGTCAC    443
UCD646So           GAAGCGCAGTCAGAGGTCGGGTGTCATTTCAGGAAGTCAC    443
B316a2             GAAGCGCAGTCAGAGGTCGGGTGTCATTTCAGGAAGTCAC    445
G233               GAAGCGCAGTCAGAGGTCGGGTGTCATTTCAGGAAGTCAC    443
G61a1              GAAGCGCAGTCAGAGGTCGGGTGTCATTTCAGGAAGTCAC    443
Consensus          gaagcgcagtcagaggtcgggtgtcatttcaggaagtcac

B2141              GCTCACTTCCATTCGTTCTACCAGATTCTGTAGGGCTGCA    518
DAR78999           GCTCACTTCCATTCGTTCTACCAGATTCTGTAGGGCTGCA    483
UCD646So           GCTCACTTCCATTCGTTCTACCAGATTCTGTAGGGCTGCA    483
B316a2             GCTCACTTCCATTCGTTCTACCAGATTCTGTAGGGCTGCA    485
G233               GCTCACTTCCATTCGTTCTACCAGATTCTGTAGGGCTGCA    483
G61a1              GCTCACTTCCATTCGTTCTACCAGATTCTGTAGGGCTGCA    483
Consensus          gctcacttccattcgttctaccagattctgtagggctgca

B2141              GAGCTTCATGGGCCATCCGCTCTCCCAGCCTTTATCTCTG    558
DAR78999           GAGCTTCgTGGGCCATCCGCTCTCCCAGCCcTTATCTCTG    523
UCD646So           GAGCTTCgTGGGCCATCCGaTCTCCCAGCCTTTATCTCTG    523
B316a2             GAGCTTCgTGGGCCATCCGCcCTCCCAGCCTTTATCTCTG    525
G233               GAGCTTCgTGGGCCATCCGCTCTCCCAGCCcTTATCTCTG    523
G61a1              GAGCTTCgTGGGCCATCCGCTCTCCCAGCCcTTATCTCTG    523
Consensus          gagcttc tgggccatccg  ctcccagcc ttatctctg

B2141              AAATTCAAGTCGTTTGTTCTTTC.TTTACTACGC.GCACT    596
DAR78999           AAATTCAAGTCGTTTGTTCTTTC.TTTACTACGC.GCACT    561
UCD646So           AAATTCAAGTCGTTTGTTCTTTC.TTTACTACGC.GCACT    561
B316a2             AAATTCAAGTCGTTTGTTCTTTCtTTTACTACGCgGCACT    565
G233               AAATTCAAGTCGTTTGTTCTTTC.TTTACTACGC.GCACT    561
G61a1              AAATTCAAGTCGTTTGTTCTTTC.TTTACTACGC.GCACT    561
Consensus          aaattcaagtcgtttgttctttc tttactacgc gcact

B2141              TCCGTGGGCAAACATGTTCAGGCATA.TTTTCTGCGCATT    635
DAR78999           TCCGTGGGCAAACATGTTCAGGCATAtTTTTCTGCGCATT    601
UCD646So           TCCGTGGGCAAACATGTTCAGGCATA.TTTTCTGCGCATT    600
B316a2             TCCGTGGGCAAACATGTTCAGGCATA.TTTTCTGCGCATT    604
G233               TCCGTGGGCAAACATGTTCAGGCATA.TTTTCTGCGCATT    600
G61a1              TCCGTGGGCAAACATGTTCAGGCATA.TTTTCTGCGCATT    600
Consensus          tccgtgggcaaacatgttcaggcata ttttctgcgcatt

B2141              CTCCACTTGGTCACTTCACTCCTGGCAAAGACATATCTTC    675
DAR78999           CTCCACTTGGTCACTTCACTCCTGGCAAAGACATATCTTC    641
UCD646So           CTCCACTTGGTCACTTCACTCCTGGCAAAGACATATCTTC    640
B316a2             CTCCACTTGGTCACTTCACTCCTGGCAAAGACATATCTTC    644
G233               CTCCACTTGGTCACTTCACTCCTGGCAAAGACATATCTTC    640
G61a1              CTCCACTTGGTCACTTCACTCCTGGCAAAGACATATCTTC    640
Consensus          ctccacttggtcacttcactcctggcaaagacatatcttc

B2141              TCCACACGTATCGATCTTCCACAGGATGATAGCCGTCTCG    715
DAR78999           TCCACACGTATCGATCTTCCACAGGATGATAGCCGTCTCG    681
UCD646So           TCCACACGTATCGATCTTCCACAGGATGATAGCCGTCTCG    680
B316a2             TCCACACGTATCGATCTTCCACAGGATGATAGCCGTCTCG    684
G233               TCCACACGTATCGATCTTCCACAGGATGATAGCCGTCTCG    680
G61a1              TCCACACGTATCGATCTTCCACAGGATGATAGCCGTCTCG    680
Consensus          tccacacgtatcgatcttccacaggatgatagccgtctcg
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B2141              CCGAAACTTCTCTTGGCTAAAATCATCAATTTTCCAGGCT    755
DAR78999           CCGAAACTTCTCTTGGCTAAAATCATCAATTTTCCAGGCT    721
UCD646So           CCGAAACTTCTCTTGGCTAAAATCATCAATTTTCCAGGCT    720
B316a2             CCGAAACTTCTCTTGGCTAAAATCATCAATTTTCCAGGCT    724
G233               CCGAAACTTCTCTTGGCTAAAATCATCAATTTTCCAGGCT    720
G61a1              CCGAAACTTCTCTTGGCTAAAA..ATCAATTTTCCAGGCT    718
Consensus          ccgaaacttctcttggctaaaa  atcaattttccaggct

B2141              CTTCAAGCCCTGTCTGGGGAAGTGCTATCGATCGCATACT    795
DAR78999           CTTCAAGCCCTGTCTGGGGgAGTGCTATCGATCGCATACT    761
UCD646So           CTTCAAGCCCTGTCTGGGGgAGTGCTATCGATCGCATACT    760
B316a2             CTTCAAGCCCTGTCTGGGGgAGTGCTATCGATCGCATACT    764
G233               CTTCAAGCCCTGTCTGGGGgAGTGCTATCGATCGCATACT    760
G61a1              CTTCAAGCCCTGTCTGGGGgAGTGCTATCGATCGCATACT    758
Consensus          cttcaagccctgtctgggg agtgctatcgatcgcatact

B2141              CCTGTAGCTTTGGCCCTACCGGCAGCAGCAAGATCTCTCT    835
DAR78999           CCTGTAGCTTTGGCCCTACCGGCAGCAGCAAGATCTCTCT    801
UCD646So           CCTGTAGCTTTGGCCCTACCGGCAGCAGCAAGATCTCTCT    800
B316a2             CCTGTAGCTTTGGCCCTACCGGCAGCAGCAAGATCTCTCT    804
G233               CCTGTAGCTTTGGCCCTACCGGCAGCAGCAAGATCTCTCT    800
G61a1              CCTGTAGCTTTGGCCCTACCGGCAGCAGCAAGATCTCTCT    798
Consensus          cctgtagctttggccctaccggcagcagcaagatctctct

B2141              TATTCTTTCCCGGATGCTTTTTCTATGCCCTCTAGCCTCT    875
DAR78999           TATTCTTTCCCGGATGCTTTTTCTATGCCCTCTAGCCTCT    841
UCD646So           TATTCTTTCCCGGATGtTTTTTCTATGCCCTCTAGCCTCT    840
B316a2             TATTCTTTCCCGGATGCTTTTTCTATGCCCTCTAGCCTCT    844
G233               TATTCTTTCCCGGATGCTTTTTCTATGCCCTCTAGCCTCT    840
G61a1              TATTCTTTCCCGGATGCTTTTTCTATGCCCTCTAGCCTCT    838
Consensus          tattctttcccggatg tttttctatgccctctagcctct

B2141              TGATCATCTCACTTGTGGAAGAAGGCATCTCGCTAATTGC    915
DAR78999           TGATCATCTCACTTGTGGAAGAAGGCATCTCGCTAATTGC    881
UCD646So           TGATCATCTCACTTGTGGAAGAAGGCATCTCGCTAATTGC    880
B316a2             TGATCATCTCACTTGTGGAAGAAGGCATCTCGCTAATTGC    884
G233               TGATCATCTCACTTGTGGAAGAAGGCATCTCGCTAATTGC    880
G61a1              TGATCATCTCACTTGTGGAAGAAGGCATCTCGCTAATTGC    878
Consensus          tgatcatctcacttgtggaagaaggcatctcgctaattgc

B2141              TACACCATCCATCTTGTGAAACCGTTTCCTCAAGAGTTCA    955
DAR78999           TACACCATCCATCTTGTGAAACCGTTTCCTCAAGAGTTCA    921
UCD646So           TACACCATCCATCTTGTGAAACCGTTTCCTCAAGAGTTCA    920
B316a2             TACACCATCCATCTTGTGAAACCGTTTCCTCAAGAGTTCA    924
G233               TACACCATCCATCTTGTGAAACCGTTTCCTCAAGAGTTCA    920
G61a1              TACACCATCCATCTTGTGAAACCGTTTCCTCAAGAGTTCA    918
Consensus          tacaccatccatcttgtgaaaccgtttcctcaagagttca

B2141              GGACATCCAGATCCCAGAAGTCTGATCCCACGCTTGTTCT    995
DAR78999           GGACATCCAGATCCCAGAAGTCTGATCCCACGCTTGTTCT    961
UCD646So           GGACATCCAGATCCCAGAAGTCTGATCCCACGCTTGTTCT    960
B316a2             GGACATCCAGATCCCAGAAGTCTGATCCCACGCTTGTTCT    964
G233               GGACATCCAGATCCCAGAAGTCTGATCCCACGCTTGTTCT    960
G61a1              GGACATCCAGATCCCAGAAGTCTGATCCCACGCTTGTTCT    958
Consensus          ggacatccagatcccagaagtctgatcccacgcttgttct

B2141              TAATGACAGTCATATCTTTGGAAGGGCTGATCCTGATCCC   1035
DAR78999           TAATGACAGTCATATCTTTGGAAGGGCTGATCCTGATCCC   1001
UCD646So           TAATGACAGTCATATCTTTGGAAGGGCTGATCCTGATCCC   1000
B316a2             TAATGACAGTCATATCTTTGGAAGGGCTGATCCTGATCCC   1004
G233               TAATGACAGTCATATCTTTGGAAGGGCTGATCCTGATCCC   1000
G61a1              TAATGACAGTCATATCTTTGGAAGGGCTGATCCTGATCCC    998
Consensus          taatgacagtcatatctttggaagggctgatcctgatccc
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B2141              GATAGAAGCTGTCCCGGGTTTCAGAGAGGATGGAAGATCA   1075
DAR78999           GATAGAAGCTGTCCCGGGTTTCAGAGAGGATGGAAGATCA   1041
UCD646So           GATAGAAGCTGTCCCGGGTTTCAGAGAGGATGGAAGATCA   1040
B316a2             GATAGAAGCTGTCCCGGGTTTCAGAGAGGATGGAAGATCA   1044
G233               GATAGAAGCTGTCCCGGGTTTCAGAGAGGATGGAAGATCA   1040
G61a1              GATAGAAGCTGTCCCGGGTTTCAGAGAGGATGGAAGATCA   1038
Consensus          gatagaagctgtcccgggtttcagagaggatggaagatca

B2141              GATAATCTGGCGATCTTGCCGCTACAGATGACACGTCTGA   1115
DAR78999           GATAATCTGGCGATCTTGCCGCTACAGATGACACGTCTGA   1081
UCD646So           GATAATCTGGCGATCTTGCCGCTACAGATGACACGTCTGA   1080
B316a2             GATAATCTGGCGATCTTGCCGCTACAGATGACACGTCTGA   1084
G233               GATAATCTGGCGATCTTGCCGCTACAGATGACACGTCTGA   1080
G61a1              GATAATCTGGCGATCTTGCCGCTACAGATGACACGTCTGA   1078
Consensus          gataatctggcgatcttgccgctacagatgacacgtctga

B2141              GAGTTTAATCACAGGTTTCACAAAGCCCATCAAACCGAGA   1155
DAR78999           GAGTTTAATCACAGGTTTCACAAAGCCCATCAAACCGAGA   1121
UCD646So           GAGTTTAATCACAGGTTTCACAAAGCCCATCAAACCGAGA   1120
B316a2             GAGTTTAATCACAGGTTTCACAAAGCCCATCAAACCGgGA   1124
G233               GAGTTTAATCACAGGTTTCACAAAGCCCATCAAACCGAGA   1120
G61a1              GAGTTTAATCACAGGTTTCACAAAGCCCATCAAACCGAGA   1118
Consensus          gagtttaatcacaggtttcacaaagcccatcaaaccg ga

B2141              GGCCGTGGTATTCGGACAAAACAATTCTGCATGGCGTGAA   1195
DAR78999           GGCCGTGGTATTCGGACAAAACAATTCTGCATGGCGTGAA   1161
UCD646So           GGCCGTGGTATTCGGACAAAACAATTCTGCATGGCGTGAA   1160
B316a2             GGCCGTGGTATTCGGACAAAACAATTCTGCATGGCGTGAA   1164
G233               GGCCGTGGTATTCGGACAAAACAATTCTGCATGGCGTGAA   1160
G61a1              GGCCGTGGTATTCGGACAAAACAATTCTGCATGGCGTGAA   1158
Consensus          ggccgtggtattcggacaaaacaattctgcatggcgtgaa

B2141              GACGGCGTGATGTTGCGTCTCACATACGCCGTCACCTCTG   1235
DAR78999           GACGGCGTGATGTTGCGTCTCACAcACGCCGTCACCTCTG   1201
UCD646So           GACGGCGTGATGTTGCGTCTCACAcACGCCGTCACCTCTG   1200
B316a2             GACGGCGTGATGTTGCGTCTCACAcACGCCGTCACCTCTG   1204
G233               GACGGCGTGATGTTGCGTCTCACAcACGCCGTCACCTCTG   1200
G61a1              GACGGCGTGATGTTGCGTCTCACAcACGCCGTCACCTCTG   1198
Consensus          gacggcgtgatgttgcgtctcaca acgccgtcacctctg

B2141              AAACACTTCGTTGCGGCGTAGCAACGAAGATCAGCAGACT   1275
DAR78999           AAACACTTCGTTGCGGCGTAGCAACGAAGATCAGCAGACT   1241
UCD646So           AAACACTTCGTTGCGGCGTAGCAACGAAGATgAGCAGACT   1240
B316a2             AAACACTTCGTTGCGGCGTAGCAACGAAGATCAGCAGACT   1244
G233               AAACACTTCGTTGCGGCGTAGCAACGAAGATCAGCAGACT   1240
G61a1              AAACACTTCGTTGCGGCGTAGCAACGAAGATCAGCAGACT   1238
Consensus          aaacacttcgttgcggcgtagcaacgaagat agcagact

B2141              TCTAACCCCTGCGGAGAAAAATGCGGGGGTCTATGCGCCA   1315
DAR78999           TCcAACCCCTGCGGAGAAAAATGCGGGGGTCTATGCGCCA   1281
UCD646So           TCcAACCCCTGCGGAGAAAAATGCGGGGGTCTATGCGCCA   1280
B316a2             TCcAACCCCTGCGGAGAAAAATGCGGGGGTCTATGCGCCA   1284
G233               TCcAACCCCTGCGGAGAAAAATGCGGGGGTCTATGCGCCA   1280
G61a1              TCcAACCCCTGCGGAGAAAAATGCGGGGGTCTATGCGCCA   1278
Consensus          tc aacccctgcggagaaaaatgcgggggtctatgcgcca

B2141              CGACACGGCTTCTCGCGCTATCTACGCCTAAGAATATTCA   1355
DAR78999           CGACACGGCTTCTCGCGCTATCTACGaCTAAGAATATTCA   1321
UCD646So           CGACACGGCTTCTCGCGCTATCTACGaCTAAGAATATTCA   1320
B316a2             CGACACGGCTTCTCGCGCTATCTACGCCTAAGAATATTCA   1324
G233               CGACACGGCTTCTCGCGCTATCTACGaCTAAGAATATTCA   1320
G61a1              CGACACGGCTTCTCGCGCTATCTACGaCTAAGAATATTCA   1318
Consensus          cgacacggcttctcgcgctatctacg ctaagaatattca
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B2141              CGGAATAACATATTCGGGGTCGGAATCGCGTTCGGTCTTA   1395
DAR78999           CGGAATAACATATTCGGGGTCGGAATCGCGTTCGGTCTTA   1361
UCD646So           CGGAATAACATATTCGGGGTCGaAATCGCGTTCGGTCTTA   1360
B316a2             CGGAATAACATATTCGGGGTCGGAATCGCGTTCGGTCTTA   1364
G233               CGGAATAACATATTCGGGGTCGGAATCGCGTTCGGTCTTA   1360
G61a1              CGGAATAACATATTCGGGGTCGGAATCGCGTTCGGTCTTA   1358
Consensus          cggaataacatattcggggtcg aatcgcgttcggtctta

B2141              ATAGTTGATTATTCATTAAGTGTAGGTCCTTTTCGGACGG   1435
DAR78999           ATAGTTGATTATTCATTAAGTGTAGGTCCTTTTCGGACGG   1401
UCD646So           ATAGTTGATTATTCATTAAGTGTAGGTCCTTTTCGGACGG   1400
B316a2             ATAGTTGATTATTCATTAAGTGTAGGTCCTTTTCGGACGG   1404
G233               ATAGTTGATTATTCATTAAGTGTAGGTCCTTTTCGGACGG   1400
G61a1              ATAGTTGATTATTCATTAAGTGTAGGTCCTTTTCGGACGG   1398
Consensus          atagttgattattcattaagtgtaggtccttttcggacgg

B2141              CCTTGGCAGTGACGACAAACAACCAGATATCCAGGCGCTT   1475
DAR78999           CCTTGGCAGTGACGACAAACAACCAGATATCCAGGCGC.T   1440
UCD646So           CCTTGGCAGTGAtGACAAACAACCAGATATCCAGGCGC.T   1439
B316a2             CCTTGGCAGTGACGACAAACAACCAGATATCCAGGCGCTT   1444
G233               CCTTGGCAGTGACGACAAACAACCAGATATCCAGGCGC.T   1439
G61a1              CCTTGGCAGTGACGACAAACAACCAGATATCCAGGCGC.T   1437
Consensus          ccttggcagtga gacaaacaaccagatatccaggcgc t

B2141              TCAGATACTGGCCAAAAAGATACACG.CTCCCGCACTTGC   1514
DAR78999           TCAGATACTGGCCAAAAAGATACACG..TCCCGCACTTGC   1478
UCD646So           TCAGATACTGGCCAAAAAGATACACGc.TCCCGCACTTGC   1478
B316a2             TCAGATACTGGCCAAAAAGATACACG.CTCCCGCACTTGC   1483
G233               TCAGATACTGGCCAAAAAGATACACG..TCCCGCACTTGC   1477
G61a1              TCAGATACTGGCCAAAAAGATACACGc.TCCCGCACTTGC   1476
Consensus          tcagatactggccaaaaagatacacg  tcccgcacttgc

B2141              AATACTCCTGCCCGTTCCTATCTTACACACTACTTTCTCC   1554
DAR78999           AATACTCCTGCCCcggCC                        1496
UCD646So           AATACTCCTGCCtaTc                          1494
B316a2             AATACTCCTGCCCGccC                         1500
G233               AATACTCCTGCCtacc                          1493
G61a1              AATACTCCTGCCCGcc                          1492
Consensus          aatactcctgcc

B2141              TCTCCGCTCTGGGTCGCAG                       1573
Consensus
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D.5 Results of GenBank search of *N. parvum* marker gene sequence using tblastx

Accession	Description	Max score	Total score	Query coverage	E value
XM_002149417.1	*Penicillium marneffei* ATCC 18224 hypothetical protein, mRNA	68.4	101	13%	2e-12
AM269981.1	*Aspergillus niger* contig An01c0340, genomic contig	68.4	98.4	13%	1e-11
FQ790347.1	*Botryotinia fuckeliana* isolate T4 SupSuperContig_79_291_1 genomic supercontig, whole genome	53.3	97.9	10%	1e-11
XM_001793389.1	*Phaeosphaeria nodorum* SN15 hypothetical protein partial mRNA	55.1	155	12%	7e-10
XM_001793397.1	*Phaeosphaeria nodorum* SN15 hypothetical protein partial mRNA	70.7	70.7	9%	5e-09
XM_001794325.1	*Phaeosphaeria nodorum* SN15 hypothetical protein partial mRNA	69.3	69.3	8%	1e-08
XM_001215440.1	*Aspergillus terreus* NIH2624 predicted protein (ATEG_06262) partial mRNA	55.1	85.5	14%	7e-08
XM_001849894.1	*Culex quinquefasciatus* zinc finger MYND domain-containing protein 10, mRNA	66.6	66.6	7%	9e-08
XM_001934873.1	*Pyrenophora tritici*-repentis Pt-1C-BFP conserved hypothetical protein, mRNA	62.9	62.9	8%	1e-06

Accession	Description	Max score	Total score	Query coverage	E value
XM_001437395.1	*Paramecium tetraurelia* hypothetical protein (GSPATT00007475001) partial mRNA	62.9	62.9	7%	1e-06
XM_002366403.1	*Toxoplasma gondii* ME49 zinc finger MYND domain-containing protein, mRNA	62.5	62.5	7%	2e-06
XM_002960560.1	*Selaginella moellendorffii* hypothetical protein, mRNA	61.1	61.1	7%	4e-06
XM_001633763.1	*Nematostella vectensis* predicted protein (NEMVEDRAFT_v1g165958) partial mRNA	61.1	61.1	8%	4e-06
XM_967190.1	PREDICTED: *Tribolium castaneum* similar to CG11253 CG11253-PA (LOC661000), mRNA	61.1	61.1	7%	4e-06
NM_200397.1	Danio rerio zgc:63660 (zgc:63660), mRNA >gb|BC054130.1| Danio rerio zgc:63660, mRNA (cDNA clone MGC:63660 IMAGE:5612622), complete cds	61.1	61.1	8%	4e-06
XM_001877867.1	*Laccaria bicolor* S238N-H82 ectomycorrhiza-upregulated zf-MYND domain-containing protein partial mRNA	60.2	60.2	5%	8e-06
XM_001601884.1	PREDICTED: *Nasonia vitripennis* similar to conserved hypothetical protein (LOC100117786), mRNA	60.2	60.2	8%	8e-06
XM_001996571.1	*Drosophila grimshawi* GH23322 (Dgri\GH23322), mRNA	59.3	59.3	7%	1e-05
XM_001976525.1	*Drosophila erecta* GG22945 (Dere\GG22945), mRNA	59.3	59.3	8%	1e-05
XM_001793388.1	*Phaeosphaeria nodorum* SN15 hypothetical protein partial mRNA	41.8	77.3	11%	2e-05
FP929134.1	*Leptosphaeria maculans* v23.1.3 lm_SuperContig_13_v2 genomic supercontig, whole genome	58.3	101	8%	3e-05
XM_003081228.1	*Ostreococcus tauri* MYND domain protein, putative (ISS) (Ot09g02860) mRNA, complete cds	58.3	58.3	7%	3e-05
XM_002969203.1	*Selaginella moellendorffii* hypothetical protein, mRNA	58.3	58.3	7%	3e-05
XM_002084697.1	*Drosophila simulans* GD12679 (Dsim\GD12679), mRNA	58.3	58.3	8%	3e-05
XM_002048402.1	*Drosophila virilis* GJ13970 (Dvir\GJ13970), mRNA	58.3	58.3	7%	3e-05
XM_001654384.1	*Aedes aegypti* hypothetical protein partial mRNA	58.3	58.3	7%	3e-05
XM_003066974.1	*Coccidioides posadasii* C735 delta SOWgp MYND finger family protein, mRNA	57.9	57.9	7%	4e-05
XM_002786877.1	*Perkinsus marinus* ATCC 50983 hypothetical protein, mRNA	57.9	57.9	7%	4e-05
XM_002092744.1	*Drosophila yakuba* GE14382 (Dyak\GE14382), mRNA	57.9	57.9	8%	4e-05
XM_001783584.1	*Physcomitrella* patens subsp. patens predicted protein (PHYPADRAFT_98941) mRNA, complete cds	57.9	57.9	7%	4e-05
XM_001239399.1	*Coccidioides* immitis RS hypothetical protein (CIMG_09021) partial mRNA	57.9	57.9	7%	4e-05
FR823391.1	*Neospora caninum* Liverpool complete genome, chromosome X	57.4	57.4	6%	5e-05
FP929114.1	*Leptosphaeria maculans* v23.1.3 lm_SuperContig_31_v2 genomic supercontig, whole genome	57.4	57.4	9%	5e-05
NM_140368.2	*Drosophila melanogaster* CG11253 (CG11253), mRNA	57.4	57.4	8%	5e-05
BT044380.1	*Drosophila melanogaster* FI08044 full insert cDNA	57.4	57.4	8%	5e-05
XM_002030323.1	*Drosophila sechellia* GM24612 (Dsec\GM24612), mRNA	57.4	57.4	8%	5e-05
AY113309.1	*Drosophila melanogaster* AT27448 full insert cDNA	57.4	57.4	8%	5e-05
XM_003301001.1	*Pyrenophora teres* f. teres 0-1 hypothetical protein, mRNA	57.0	57.0	8%	7e-05
XM_002676841.1	*Naegleria gruberi* hypothetical protein, mRNA	57.0	57.0	7%	7e-05
XM_001604953.1	PREDICTED: *Nasonia vitripennis* similar to GA18420-PA (LOC100121386), mRNA	57.0	57.0	8%	7e-05
XM_001026696.1	*Tetrahymena thermophila* MYND finger family protein, mRNA	57.0	57.0	7%	7e-05
XM_002008648.1	*Drosophila mojavensis* GI13633 (Dmoj\GI13633), mRNA	56.5	56.5	7%	1e-04
XM_968449.2	PREDICTED: *Tribolium castaneum* similar to CG8503 CG8503-PA (LOC662348), mRNA	56.5	56.5	8%	1e-04
XM_001602955.1	PREDICTED: *Nasonia vitripennis* similar to Zmynd10 protein (LOC100119187), mRNA	56.5	56.5	7%	1e-04
XM_003141369.1	*Loa loa* hypothetical protein (LOAG_05832) mRNA, complete cds	56.1	56.1	7%	1e-04
XM_002561999.1	*Penicillium chrysogenum* Wisconsin 54-1255 hypothetical protein (Pc18g02010) mRNA, complete cds	56.1	56.1	8%	1e-04
XM_002390159.1	*Moniliophthora perniciosa* FA553 hypothetical protein (MPER_10561) mRNA, partial cds	56.1	56.1	6%	1e-04
AM920433.1	*Penicillium chrysogenum* Wisconsin 54-1255 complete genome, contig Pc00c18	56.1	56.1	8%	1e-04
XM_002082889.1	*Drosophila simulans* GD11837 (Dsim\GD11837), mRNA	56.1	56.1	8%	1e-04
XM_002037482.1	*Drosophila sechellia* GM18310 (Dsec\GM18310), mRNA	56.1	56.1	8%	1e-04
XM_001972580.1	*Drosophila erecta* GG13785 (Dere\GG13785), mRNA	56.1	56.1	7%	1e-04
AY113356.1	*Drosophila melanogaster* GM13546 full insert cDNA	56.1	56.1	8%	1e-04
XM_003304703.1	*Pyrenophora teres f. teres* 0-1 hypothetical protein, mRNA	55.6	55.6	9%	2e-04
XM_003296533.1	*Pyrenophora teres f. teres* 0-1 hypothetical protein, mRNA	55.6	55.6	9%	2e-04
XM_003033434.1	*Schizophyllum commune* H4-8 hypothetical protein, mRNA	55.6	55.6	7%	2e-04
XM_002606726.1	*Branchiostoma floridae* hypothetical protein, mRNA	55.6	55.6	7%	2e-04

Accession	Description	Max score	Total score	Query coverage	E value
BT044442.1	*Drosophila melanogaster* FI09725 full insert cDNA	55.6	55.6	8%	2e-04
XM_002062049.1	*Drosophila willistoni* GK17346 (Dwil\GK17346), mRNA	55.6	55.6	7%	2e-04
XM_001601707.1	PREDICTED: *Nasonia vitripennis* similar to conserved hypothetical protein (LOC100117557), mRNA	55.6	55.6	8%	2e-04
XM_001596401.1	*Sclerotinia sclerotiorum* 1980 predicted protein (SS1G_02671) partial mRNA	55.6	55.6	5%	2e-04
AE013599.4	*Drosophila melanogaster* chromosome 2R, complete sequence	55.6	154	8%	2e-04
AY814101.1	*Schistosoma japonicum* SJCHGC05428 protein mRNA, complete cds	55.6	55.6	7%	2e-04
NM_138046.1	*Drosophila melanogaster* zinc finger protein RP-8 (Zfrp8), mRNA	55.6	55.6	8%	2e-04
AC012167.5	*Drosophila melanogaster*, chromosome 2R, region 60B-60C, BAC clone BACR27M17, complete sequence	55.6	55.6	8%	2e-04
XM_003203858.1	PREDICTED: *Meleagris gallopavo* n-lysine methyltransferase SMYD2-like (LOC100538978), mRNA	55.1	55.1	7%	3e-04
XM_003036494.1	*Schizophyllum* commune H4-8 hypothetical protein, mRNA	55.1	55.1	5%	3e-04
XM_002094628.1	*Drosophila yakuba* GE20079 (Dyak\GE20079), mRNA	55.1	55.1	8%	3e-04
XM_001881748.1	*Laccaria bicolor* S238N-H82 hypothetical protein partial mRNA	55.1	55.1	7%	3e-04
XM_001510111.1	PREDICTED: *Ornithorhynchus anatinus* hypothetical protein LOC100079176 (LOC100079176), mRNA	55.1	55.1	7%	3e-04
XM_419420.2	PREDICTED: *Gallus gallus* SET and MYND domain containing 2 (SMYD2), mRNA	55.1	55.1	7%	3e-04
NM_001004284.1	*Rattus norvegicus* zinc finger, MYND-type containing 10 (Zmynd10), mRNA >gb|BC079255.1| Rattus norvegicus zinc finger, MYND-type containing 10, mRNA (cDNA clone MGC:94352 IMAGE:7133212), complete cds	55.1	55.1	9%	3e-04
BX950800.1	*Gallus gallus* finished cDNA, clone ChEST243a10	55.1	55.1	7%	3e-04
XM_003063627.1	*Micromonas pusilla* CCMP1545 hypothetical protein, mRNA	54.7	54.7	5%	4e-04
XM_003035727.1	*Schizophyllum commune* H4-8 hypothetical protein, mRNA	54.7	54.7	8%	4e-04
XM_003033245.1	*Schizophyllum commune* H4-8 hypothetical protein, mRNA	54.7	54.7	7%	4e-04
NM_206851.1	*Rattus norvegicus* SET and MYND domain containing 2 (Smyd2), mRNA	54.7	54.7	9%	4e-04
XM_001956329.1	*Drosophila ananassae* GF25173 (Dana\GF25173), mRNA	54.7	54.7	7%	4e-04
XM_001505238.1	PREDICTED: *Ornithorhynchus anatinus* similar to Zinc finger, MYND domain-containing 10 (LOC100073489), mRNA	54.7	54.7	8%	4e-04
XM_001374849.1	PREDICTED: *Monodelphis domestica* SET and MYND domain containing 2 (SMYD2), mRNA	54.7	54.7	7%	4e-04
BK001057.1	TPA: TPA_exp: *Rattus norvegicus* SET and MYND domain protein 2 (Smyd2) mRNA, complete cds	54.7	54.7	9%	4e-04
NM_001022435.1	*Schizosaccharomyces pombe* 972h- histone lysine methyltransferase Set6 (predicted) (set6), mRNA	54.7	54.7	6%	4e-04
NM_001096803.1	*Xenopus laevis* zinc finger, MYND-type containing 10 (zmynd10), mRNA >gb|BC089198.1| Xenopus laevis zinc finger, MYND domain-containing 10, mRNA (cDNA clone MGC:98297 IMAGE:5516052), complete cds	54.7	54.7	8%	4e-04
XM_002945874.1	*Volvox carteri f. nagariensis* hypothetical protein, mRNA	54.2	54.2	7%	5e-04
XM_002935633.1	PREDICTED: *Xenopus (Silurana)* tropicalis zinc finger MYND domain-containing protein 10-like (LOC100497840), mRNA	54.2	54.2	8%	5e-04
XM_002284350.1	PREDICTED: *Vitis vinifera* hypothetical protein LOC100267791 (LOC100267791), mRNA	54.2	54.2	6%	5e-04
XM_382355.1	*Gibberella zeae* PH-1 hypothetical protein partial mRNA	54.2	54.2	6%	5e-04
FQ790255.1	*Botryotinia fuckeliana* isolate T4 SuperContig_7_1 genomic supercontig, whole genome	53.8	53.8	8%	7e-04
XM_003190692.1	*Aspergillus oryzae* RIB40 MYND domain protein, mRNA	53.8	53.8	7%	7e-04
XM_002758385.1	PREDICTED: *Callithrix jacchus* zinc finger, MYND-type containing 10, transcript variant 2 (ZMYND10), mRNA	53.8	53.8	9%	7e-04
XM_002758384.1	PREDICTED: *Callithrix jacchus* zinc finger, MYND-type containing 10, transcript variant 1 (ZMYND10), mRNA	53.8	53.8	9%	7e-04
XM_002377415.1	Aspergillus flavus NRRL3357 MYND domain protein, putative, mRNA	53.8	53.8	7%	7e-04
XM_002191428.1	PREDICTED: *Taeniopygia guttata* SET and MYND domain containing 2 (LOC100230503), mRNA	53.8	53.8	7%	7e-04
XM_001839486.1	*Coprinopsis cinerea* okayama7#130 t## hypothetical protein, mRNA	53.8	53.8	8%	7e-04
CU689717.1	Synthetic construct Homo sapiens gateway clone IMAGE:100018295 3' read ZMYND10 mRNA	53.8	53.8	11%	7e-04
XM_001745475.1	*Monosiga brevicollis* MX1 predicted protein MONBRDRAFT_25193 mRNA, complete cds	53.8	53.8	7%	7e-04
DQ851823.1	Danio rerio SET and MYND domain containing 2a (smyd2a) mRNA, partial cds	53.8	53.8	9%	7e-04
XM_001182407.1	PREDICTED: *Strongylocentrotus purpuratus* hypothetical LOC576498 (LOC576498), mRNA	53.8	53.8	7%	7e-04

Accession	Description	Max score	Total score	Query coverage	E value
XM_776799.2	PREDICTED: *Strongylocentrotus purpuratus* hypothetical LOC576498 (LOC576498), mRNA	53.8	53.8	7%	7e-04
AP007171.1	*Aspergillus oryzae* RIB40 DNA, SC011	53.8	53.8	7%	7e-04
NM_001013550.1	*Danio rerio* SET and MYND domain containing 2a (smyd2a), mRNA >gb|BC091465.1| Danio rerio SET and MYND domain containing 2a, mRNA (cDNA clone MGC:110553 IMAGE:7157766), complete cds	53.8	53.8	9%	7e-04

D.6 Marker band sequence of *N. luteum* marker isolate G51a2 showing the primer binding sites (indicated in red)

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ORIGIN
1       CTGCGACCCA GAGCGGCATA AGTGTGTGTT TTCGACGGGA ATGAGCGATG
51      GCTGACCAGA GTACAGGTGT AACAACGTCC TGTGGGTACG AGGAGCGGAT
101     GAAAAAGGCG ATGCTGCTAT GTCAGGTTGA GAGCCACGGG TGGCCAGGGT
151     ATTTTTGTGT CTTTACTACG CGGAGTTCAA CTGGATAGAG GGATCTGCTT
201     CCACGCCGAC TGAGAGCATC AATGCGACAA CATTTGCGCC ATTCCGTTCC
251     GAGACCGATG ACTAACGATT GACGAATGTT TTGCGACGAT CTGAGCGCCT
301     TCTGTGATGA ATCTGGTGGT GGGCGCGGCG GTTCCAGTTC GTACGCTGGT
351     AAACTCGGTG GTTGAGTTGA CGAAGTTGGA GGCACGACGT CTCTGGTGGA
401     CTAGATCGCT ATGACATAGC ATCTCTGCAT TGGCTATCAG GTTTCATCAC
451     CTCCTGCACA CTCGGTCTAG TGAGCCCGCA TCATCGCCTC TGCCGTCCCA
501     TTCATTCACC GCTCTGGGTC GCAG//
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D.7 Results of GenBank search of *N. luteum* marker gene sequence using tblastx

Accession	Description	Max score	Total score	Query coverage	E value
XM_003049666.1	*Nectria haematococca* mpVI 77-13-4 predicted protein, mRNA	40.0	40.0	9%	2.3
XM_757006.1	*Ustilago maydis* 521 hypothetical protein (UM05952.1) partial mRNA	38.6	38.6	40%	6.1
CP002480.1	*Acidobacterium* sp. MP5ACTX9, complete genome	38.2	38.2	13%	8.3
XM_381557.1	*Gibberella zeae* PH-1 hypothetical protein partial mRNA	38.2	38.2	9%	8.3
AJ538312.1	*Platichthys flesus* microsatellite DNA, locus StPfl014	38.2	38.2	19%	8.3
BX294142.1	*Rhodopirellula baltica* SH 1 complete genome; segment 10/24	38.2	38.2	25%	8.3