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Genetic diversity and virulence variability in *Diplodia mutila* isolates from symptomatic grapevines in New Zealand

Virulence and genetic diversity of *Diplodia mutila*

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Summary

Genetic diversity and virulence variability of *Diplodia mutila* isolates recovered from grapevines in New Zealand were investigated. The universally primed PCR (UP-PCR) and vegetative compatibility group (VCG) methods were used to investigate the genetic diversity. Pathogenicity tests with 'Sauvignon Blanc' detached shoots and potted vines were used to determine the virulence diversity. UP-PCR analysis determined eight genetic groups of *D. mutila* with 70% of the population within one group. Phylogenetic analysis also determined that New Zealand isolates were more closely related to Australian isolates than Californian isolates. Vegetative compatibility grouping analysis placed the isolates into three VCG groups with 57% of isolates belonging to all three VCGs. Vegetative compatibility reactions were observed among isolates, but this was not correlated with the genetic clustering. Virulence assays proved that all isolates tested were pathogenic on grapevine stems. Differences in necrotic lesions lengths caused by *D. mutila* isolates were identified, indicating different virulence levels among isolates, however, no relationship was found between the genetic groups and the virulence. The results of the study indicated movement of *D. mutila* isolates between nurseries, vineyards, and other sources in New Zealand. This information will inform control strategies to limit the further spread of this pathogen into vineyards in the same region or new regions.

Keywords

anastomosis, *Botryosphaeria stevensii*, *Botryosphaeriaceae*, *Diplodia mutila*, pathogenicity, vegetative compatibility group, *Vitis vinifera*

Introduction

Diplodia mutila belongs to the Botryosphaeriaceae and, internationally, are well recognised as causal agents of dieback and decline of grapevines (Úrbez-Torres, 2011, Morales *et al.*, 2012, Carlucci *et al.*, 2015). However, for many of the Botryosphaeriaceae species, reports of disease differ between countries, with respect to the species responsible, the symptoms they cause on grapevines and the hosts. For example, *Botryosphaeria dothidea*, *D. seriata* and *D. mutila* were found to be the cause of "black dead arm" in France (Larignon and Dubos, 2001) but not in Portugal (Phillips, 2002). In addition to being recognised as grapevine pathogens, there have been an increasing number of recent reports of *D. mutila* causing disease on other hosts, such as, gummy canker in *Araucaria araucana* (Besoain *et al.*, 2017), canker disease on stone and pome fruit trees (Sessa *et al.*, 2016), dieback on apple trees (Úrbez-Torres *et al.*, 2016, Lódolo *et al.*, 2022), branch and trunk cankers of bristlecone fir (*Abies bracteata*; Sims *et al.*, 2016) and dieback of European ash (Fraxinus excelsior) trees (Kowalski *et al.*, 2016). In contrast to other Botryosphaeriaceae, such as *N. parvum* and *D. seriata* which are recognised to have a global distribution, *D. mutila* is reported to only be found in temperate and Mediterranean ecosystems (Batista *et al.*, 2021).

Differences between the relative pathogenicity of various Botryosphaeriaceae species on grapevine has also been found between countries. For example, *D. seriata* has been reported to be pathogenic on grapevine in Chile (Auger *et al.*, 2004; Morales *et al.*, 2012), the New South Wales region in Australia (Castillo–Pando *et al.*, 2001) and South Africa (van Niekerk *et al.*, 2004), but only considered weakly pathogenic in Portugal (Phillips, 2002) and in New Zealand (Amponsah *et al.*, 2011). Similarly, *D. mutila* was reported as a weak pathogen on grapevine in California (Úrbez-Torres and Gubler, 2009), however, it was reported as pathogenic and able to cause

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necrotic lesions on grapevine in New Zealand (Amponsah *et al.*, 2011), Australia (Taylor *et al.*, 2005), South Africa (van Niekerk *et al.*, 2004) and Chile (Morales *et al.*, 2012). Further, although the majority of studies have reported virulence variation between the species, some pathogenicity studies have shown that isolates of a single species of Botryosphaeriaceae can differ substantially in virulence (Baskarathevan *et al.*, 2012b; Baskarathevan *et al.*, 2017; van Niekerk *et al.*, 2004; Úrbez-Torres and Gubler, 2009). A preliminary study of eight *D. mutila* isolates recovered from nursery grapevine propagation material were shown to vary considerably in their virulence, with lesion lengths in green shoots varying 2.5 – 85.3 mm after 7 days, and in rooted canes 25.0 –137.5 mm after 28 days (Billones-Baaijens, 2011). However, the relative virulence of a larger number of New Zealand vineyard isolates of *D. mutila* has not been investigated.

Information about the genetic diversity within and between fungal populations may assist in understanding the possible sources of pathogen inoculum and spread that is vital to inform the development of effective control strategies. For instance, if the primary inoculum is mainly sources external to the vineyard, such as infected propagation material or windblown spores, low population differentiation between vineyards and regions is expected. If the primary inoculum originates from within the vineyard, such as from neighbouring vines or surrounding plants including shelterbelts, then high population differentiation among vineyards and regions is predicted (Li and Brewer, 2016). A study by Qiu *et al.* (2015) of the genetic diversity of four species of Botryosphaeriaceae from south-eastern Australian vineyards reported that *D. seriata* were genetically differentiated by region and in some cases vineyard. In contrast, *N. parvum* population were relatively genetically homogenous between vineyards and regions, with *B. dothidea* being homogenous between regions. A genetic study of variation in *D. seriata* isolates occurring on grapevines in Spain has reported no relationship between either geographic or host origin of isolates and the genetic clusters that were observed (Elena *et al.*, 2015). A study by Kraj *et al.* (2013) showed high intra population genetic diversity in *D. mutila* isolates recovered from declining *Fraxinus excelsior* from different regions in Poland. These studies indicate that the relative importance of different inoculum sources for different species of Botryosphaeriaceae, as well as those associated with different plants, may vary. To date, characterization of the genetic diversity of *D. mutila* isolates recovered from grapevine has not been studied in detail.

The genetic diversity of a fungal species is influenced by the relative contributions of asexual and sexual reproductive activities of the disease cycle within a population. A fungal species with high levels of sexual reproduction will be genetically more diverse than a fungal species that reproduces mainly asexually, thus information from such studies can be used to infer the frequency of sexual recombination (Cortesi and Milgroom, 2001). Molecular markers that produce information from DNA-based polymorphisms are widely used to characterise genetic diversity among fungal species (Waugh 1997; Milgroom, 1996). Older methods include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) methods. In addition, universally primed PCR (UP-PCR), which was developed specifically for fungi, can be used to generate 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 method has been successfully used in genetic variation analysis of grapevine pathogens such as *N. parvum* (Baskarathevan *et al.*, 2012b), *N. luteum* and *N. australe* (Baskarathevan *et al.*, 2017) and *Togninia minima* (Gramaje *et al.*, 2013). Vegetative compatibility group (VCG) studies can also be used to assess the genetic compatibility of fungal species at multiple loci (Milgroom and Cortesi, 1999). This method has been widely used for indirectly assessing the genetic variability among isolates of fungal plant pathogens (Leslie, 1993). Characterization of fungal populations into different VCGs is relevant to provide more information on the potential for genetic exchange (parasexual recombination) in species where sexual reproduction is not prevalent. This method has been used to study the genetic diversity of different Botryosphaeriaceae species isolated from grapevines (Baskarathevan *et al.*, 2012b; Elena *et al.*, 2015). These two studies reported similar results for *N. parvum* and *D. seriata*, with the lack of clear VCGs indicating weak barriers to anastomosis in these species resulting in genetic exchange between isolates. However, this has not been investigated for *D. mutila.*

The aim of this study was to determine the intraspecific genetic diversity and virulence variability of *Diplodia mutila* populations isolated from grapevines in New Zealand vineyards. Forty-eight isolates of *D. mutila* recovered from a nationwide survey and international isolates were assessed for genetic relatedness using UP-PCR primers, and pathogenicity on detached grapevine shoots and whole plants. The outcome of this study will provide information on the distribution of *D. mutila* genotypes both within and between vineyards and regions and therefore dispersal dynamics and potential inoculum sources. This will inform the development of management strategies to mitigate spread of this pathogen into vineyards.

Material and Methods

Fungal isolates used in the study

A total of 48 isolates of *D. mutila* were used in this study. This group comprised 36 isolates recovered from symptomatic tissue of grapevines with trunk decline from five different wine growing regions of New Zealand (Baskarathevan *et al.*, 2012a). A few *D. mutila* isolates obtained from non-grapevine hosts were also included to expand the comparative scope of the study. Four *D. mutila* isolates were recovered from lesions on non-grapevine hosts located adjacent to vineyards and these were willow (*Salix* sp.), plum (*Prunus domestica*), apple (*Malus domestica*) and oak (*Quercus* sp.) (Table 1). Five *D. mutila* isolates were obtained from Australia (DAR) and three from California, USA (UCD). For this study, all isolates were identified using morphological characteristics, sequence of the ITS region and using amplified ribosomal DNA restriction

Table 1: *Diplodia mutila* isolates used in the study with details of vine number, vineyard number and geographical region from which the isolates were collected

Geographic origin	Host	Vineyard number[a]	Vine number[b]	Isolate ID
Marlborough, NZ	Grape	2	1	M211, M213
		2	2	M221, M226
		2	3	M232
		2	4	M246, M248
Blenheim, NZ	Grape	1	3	B1326
		3	1	B3111, B31012, B31111, B31213
		3	2	B32011, B32012, B32112
		3	4	B3413
		3	6	B3612
		3	8	B3811
		-		F(20)-1, F(12)-2
		-		D(6)-1, Q
	Willow	-		F-1
	Plum	-		A-3
	Apple	-		A-2
Gisborne, NZ	Grape	1	6	G16a1 G16a3
		1	7	G173
		2	1	G211
Canterbury, NZ	Grape	2	2	C221
		2	5	C251
		2	6	C261
		-		Rd3
Nelson, NZ	Grape	1	3	N13c3, N1351, N13b1
		2	-	M(30)-3, M(7)-6
		6	3	N6315
	Oak	-		J-4
NSW, Australia	Grape	-		DAR79131, DAR79132, DAR79135 DAR79136, DAR79137
Madera, CA, USA	Grape	-		UCD288Ma
Santa Barbara, CA, USA	Grape	-		UCD1953SB, UCD1965SB

[a] This is a unique identifying number given to a vineyard within a region during a nationwide survey to protect grower anonymity
[b] This is a unique identifier of a vine within a vineyard that was sampled during a nationwide survey

analysis (ARDRA; Alves *et al.*, 2005) as described in Baskarathevan *et al.* (2012a). All isolates were maintained in 10% glycerol at -80°C.

DNA extraction and UP-PCR amplification

Isolates were grown on potato dextrose broth inoculated with a single mycelial plug for 3 days at 23°C ± 0.5°C with a 12:12 h photoperiod and the genomic DNA was extracted from the mycelial mat using the PUREGENE® genomic DNA isolation kit (Gentra Systems) as per manufacturer's instructions. The genomic DNA samples were diluted to 20-30 ng μL^{-1} for use in UP-PCR. UP-PCR with primers AA2M2 ($^{5'}$CTGCGACCCAGAGCGG$^{3'}$), L21 ($^{5'}$GGATCCGAGGGTGGCGGTT$^{3'}$), 0.3-1 ($^{5'}$CGAGAACGACGGTTCT$^{3'}$), Fok1 ($^{5'}$GGATGACCCACCTCCTAC$^{3'}$) and L45 ($^{5'}$GTAAAACGACGGCCAGT$^{3'}$) was done as described by Baskarathevan *et al.* (2012b). UP-PCR amplification products were separated by 1% agarose gel electrophoresis at 5 V cm^{-1} for 3 h in 1× TAE. Agarose gels were stained with Sybersafe (5 µL per 100 mL 1× TAE) and visualized on a UV transilluminator (Versadoc™). Only strongly fluorescent and reproducible bands were considered for scoring.

Genetic diversity analysis

The binomial matrix generated from the combined UP-PCR primers was analysed using Primer version 7.0.5 (Primer E Ltd). The presence or absence of a specific band was interpreted as a positive or null allele, respectively. A resemblance matrix was generated using Jaccard similarity analysis. Analysis of the resultant clusters was done using similarity profiling (SIMPROF) to determine which isolates were significantly

different (P<0.05) from each other (Clarke, 1993). All other measures of genetic diversity were calculated using POPGENE version 1.32 (Yeh *et al.*, 1999). 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. Genotypic diversity was determined by a normalized Shannon's diversity index (I) (Sheldon, 1969): $I= - \Sigma_{pi} \ln P_i/\ln N$, 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 with the same genotype) to 1 (individual with different genotype).

Characterisation of vegetative compatibility groups (VCGs)

Fourteen *D. mutila* isolates representative of the different vineyards and genetic groups were selected for VCG analysis. The *D. mutila* isolates were grown on PDA at 23°C ± 0.5°C under 12:12 h photoperiod for 3 days. A 3 mm diameter mycelial plug was taken from the growing margin of each 3-day-old colony of the selected isolates and placed in pairs 2.5 cm apart in the middle of a Petri dish (Ø9 cm) containing ½ strength PDA. All isolates were tested in pairs against each other (self *vs* non-self) and themselves (self *vs* self). Each combination of isolates was prepared in triplicate. Observations were made after 7, 10 and 21 days of incubation at 23°C ±0.5°C under 12 h/12 h light/dark conditions. The plates were visually examined in both upper and reverse side and scored a) vegetatively compatible (C), when mycelia of two isolates merged together uniformly, b) vegetatively incompatible (IC), 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.

To investigate the hyphal interactions in the compatible and incompatible reactions microscopic observations was carried out. Based on the vegetative compatibility reactions, two isolates from each of the compatibility reactions were selected and paired against each other and themselves on a microscopic slide as described by Aimi *et al.* (2002). Each combination of isolates was replicated five times. Microscopic observations of the interaction zone between the two isolate's hyphae were observed at x100, x400 and x600 magnification using an Olympus BX51 microscope with a built-in Olympus DP12 camera (Olympus Optical, Japan).

Pathogenicity tests

Virulence variability of *D. mutila* was studied using selected isolates from different genetic groups. Fourteen isolates were used in the green shoot assay which was reduced to six isolates representing low, medium and high levels of virulence for the potted vine assay.

Detached green shoot assay

Grapevine green shoots were obtained from 6-month-old 'Sauvignon Blanc' potted vines. Grapevine green shoots of approximately 25 – 30 cm in length and of a uniform thickness were excised using a sterile scalpel and the basal leaves removed to provide 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 sterile pumice granules and the universal bottle filled with sterile water. 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 10−25°C) 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.

Potted grapevine assay

Three-month-old 'Sauvignon Blanc' vines in 3 L pots containing potting mix (80% composted bark, 20% pumice with 0.005% Osmocote exact 15-3.9-9.1 (N-P-K), 0.001% lime) were used for this assay. 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 mycelium 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 by wrapping with cling film. The control vines were wounded and inoculated in a similar manner with non-colonized PDA plugs. Eight potted vines were inoculated for 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 15-25°C) until harvest.

Disease assessment

The external lesion measurements (mm) of *in vitro* inoculated green shoots were made 7 days after inoculation using digital calliper (Mitutoyo). The lesion measurements of potted vines were made 6 weeks after inoculation. For the potted vine assay, the internal lesion length was measured after debarking the main trunk using a sterile knife. The total lengths of the internal dark lesions on the grapevine trunks were measured. Pathogen re-isolation was carried out to confirm the lesions were caused by the inoculated *D. mutila* isolates. The lesion length data was statistically analysed by one way ANOVA using GenStat 9.0 and the means were separated using a post-hoc Tukey's HSD test (P≤0.05).

Results

Genetic diversity of Diplodia mutila isolates

The five UP-PCR primers produced a total of 48 informative bands (loci), of which 90% were polymorphic (Table 2). Analysis of similarity using the Jaccard similarity index produced eight different genetic groups (SIMPROF; P<0.05; Fig. 1) with a few groups only containing single isolates (Groups 1 to 4). Genetic groups 5, 6 and 8 contained 4, 2 and 4 isolates, respectively. Genetic group 7 was the largest which contained 34 isolates and included isolates from Australia, USA and the alternate hosts (plum and oak). Two of the Australian isolates from grapevines were grouped with the New Zealand isolates from apple and willow but not with other grapevine isolates. Genetic group 5 only contained isolates from a single vineyard in Blenheim although other isolates from this vineyard were also found in genetic groups 4 and 7. All USA isolates grouped together with isolates from New Zealand in Genetic group 7.

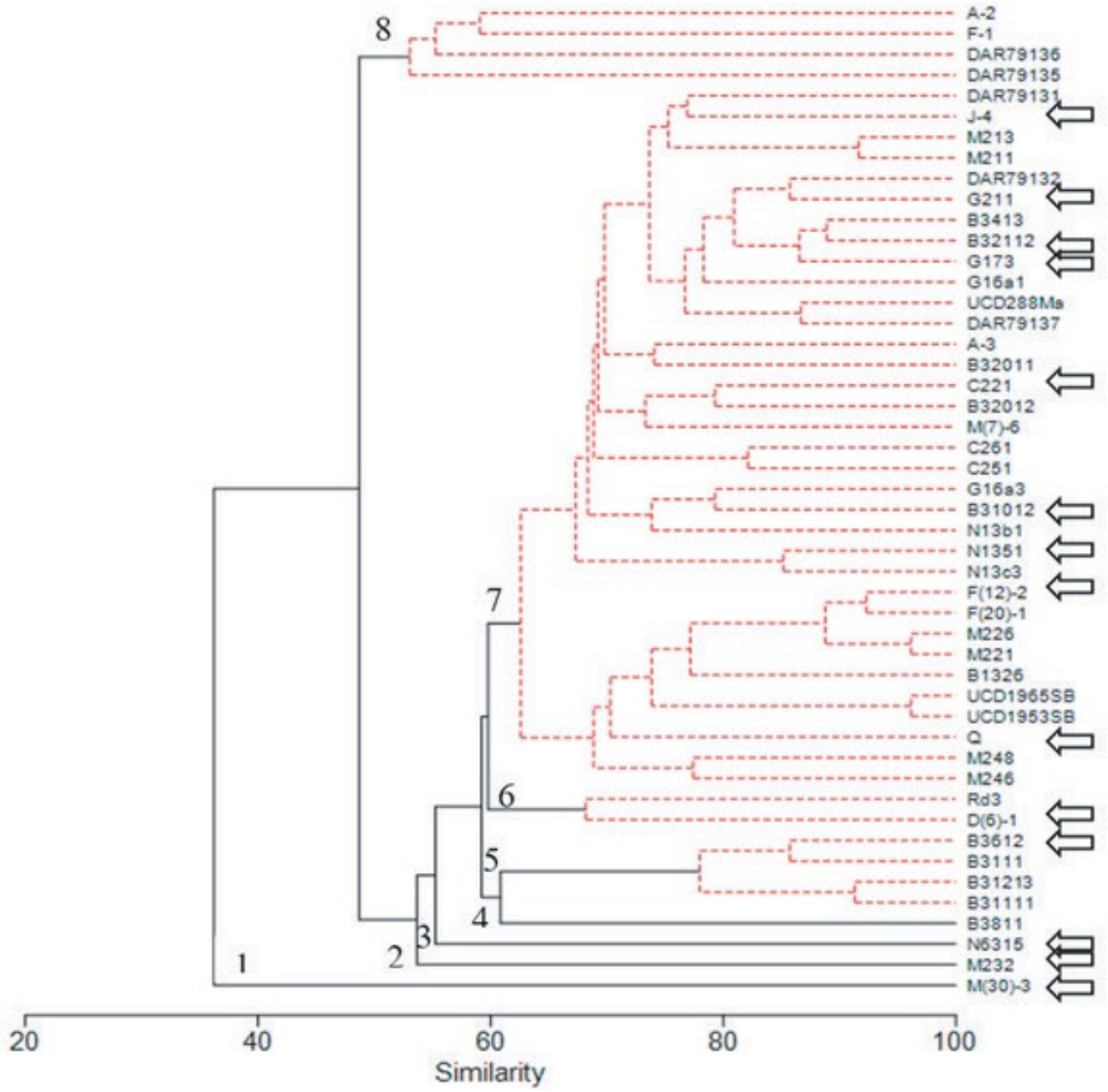

Fig. 1: Neighbourhood joining tree based on DNA fingerprints generated with 5 UP-PCR primers of 48 *Diplodia mutila* isolates from New Zealand, Australia and California, USA. Isolates separated by solid lines are genetically different from each other based on a Jaccard similarity index (P<0.05). Genetic groups numbered 1-8 are labelled on the solid lines. Arrows indicate those isolates selected for pathogenicity assays.

Table 2: 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
Australia	5	54.17	0.2133	0.3142
California	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 (Nei, 1973) gene diversity.
[b] Shannon diversity index (Sheldon, 1969).

The mean genetic diversity of the *D. mutila* isolates was 0.2347. The genetic variability was highest (0.2165) in the Blenheim subset and lowest (0.1204) in the Californian subset (Table 2). Nei's measures of genetic identity and genetic distance analysis showed that the Gisborne and Blenheim subsets were genetically more similar to each other than to the other subsets studied. Also, the Australian and non-grapevine isolates (New Zealand) were more related to each other than other subsets (Table 3).

Vegetative compatibility groups of Diplodia mutila isolates

Among the 14 *D. mutila* isolates paired, clear vegetatively compatible and incompatible reactions were observed. Vegetatively compatible reactions in which paired isolates merged completely were obtained for most of the isolates (Fig. 2A). Hyphal peg formation and multiple anastomoses were seen in microscopic observations (Fig. 3). Only 28% of the paired isolates (26 out of 91) produced incompatible reactions with the formation of clear zones between the growing edges of paired isolates (Fig. 2B) were observed. Out of the 14 isolates, 8 (57%) belonged to all three VCG, creating a large amount of overlap (Fig. 2C). The compatible and incompatible reactions occurred between the isolates of the same genetic group.

Virulence of Diplodia mutila isolates

Detached green shoot assay

After 7 days no lesions developed on the control (PDA) inoculated grapevine shoots. The lesion lengths produced by the *D. mutila* isolates on grapevine green shoots varied significantly between the isolates (ANOVA; $P<0.001$) with means ranging from 8.3 to 52.0 mm for isolates B32112 and N1351, respectively (Fig. 4). Inoculation with isolate N1351 resulted in significantly longer lesions compared with all other isolates, apart from isolate B3612. 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, produced a mean lesion of 12 mm on the grapevine green shoots which did not differ significantly from those produce by all grapevine isolates apart from B3612 and N1351 (Fig. 4). Isolates G211, Q, G173, F(12)2, B3612 and N1351 representing low, medium and high virulence were selected for the potting vine assay.

Potted grapevine assay

No external disease symptoms were observed on potted grapevines inoculated with the *D. mutila* isolates 6 weeks after inoculation. For the non-inoculated control, examination of the internal lesions after debarking the vines showed no lesions developed apart from some discolouration at the

Table 3: Nei's measures of genetic identity and genetic distance of different *Diplodia 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.

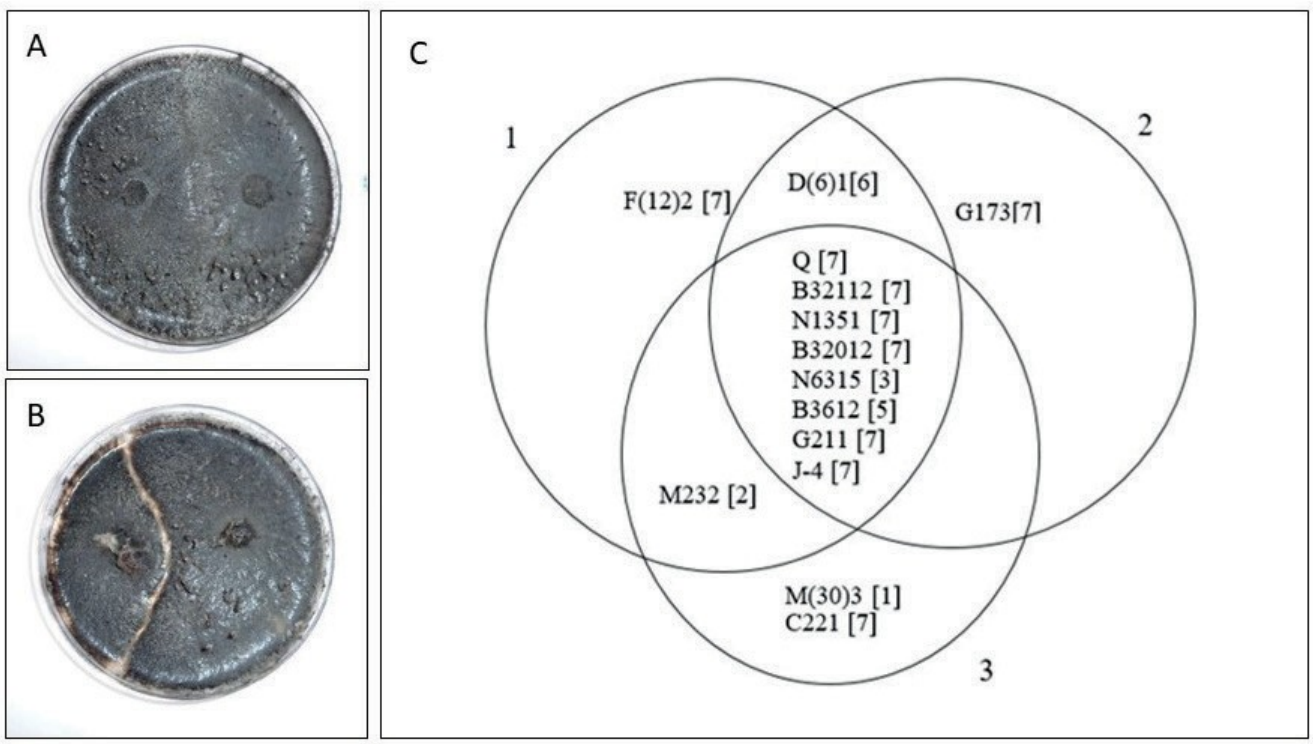

Fig. 2: Vegetatively (A) compatible (isolates N1351 and B32012) and (B) incompatible (isolates M232 and C221) reactions formed in *Diplodia mutila* isolate interactions. (C) The isolates belonging to three vegetative compatibility groups of *D. mutila* indicated in circles. Figure in square brackets indicates the UP-PCR genetic group for that isolate that was identified by Jaccard similarity index. The isolates in the intersection of the circles are common to the respective vegetative compatibility group

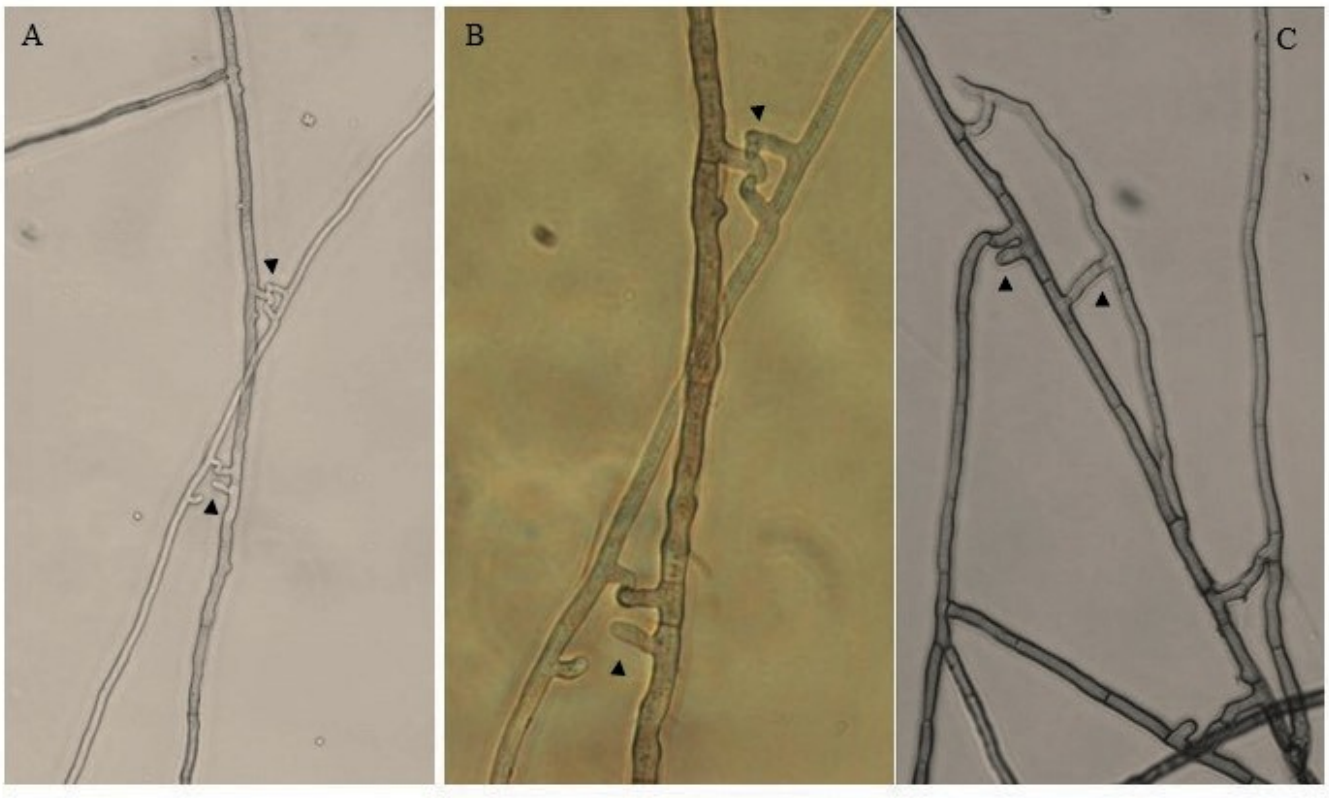

Fig. 3: Microscopic examination of vegetative compatibility reactions of *Diplodia mutila* isolate M(30)3 paired with isolate C221. (A) Hyphal peg formation and fusion, (B) higher magnification of picture A showing the peg fusion (anastomosis), and (C) multiple anastomose. Hyphal pegs and anastomosis are indicated by arrows.

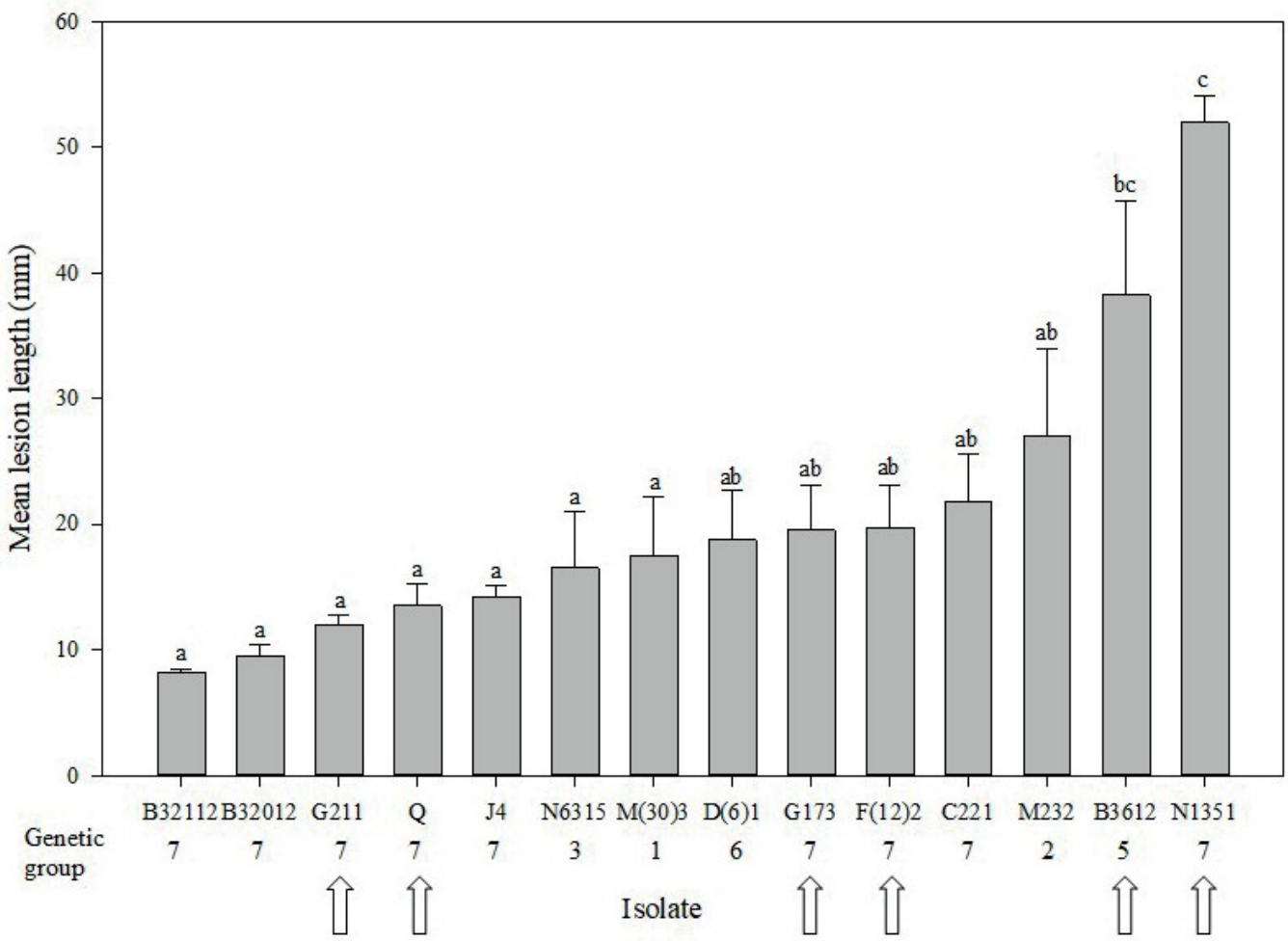

Fig. 4: Mean lesion lengths measured on grapevine green shoots seven days after inoculation with *Diplodia mutila* isolates belonging to different genetic groups. Bars with different letters were significantly different ($P \leq 0.05$) based on Tukey's HSD test. Error bars indicate the standard deviation. Arrows indicate those isolates selected for potted vine assays

wound site. For the six selected isolates the internal necrotic lesions lengths differed significantly (ANOVA; $P<0.001$) with treatment means ranging from 39 (G173) to 84 mm (N1351) (Fig. 5). Isolate Q which produced one of the smallest lesion length (14 mm) on green shoots was produced a medium size lesion (62 mm lesion length) on potted grapevine. Whereas, isolate G173 which produced a medium size lesion (20 mm length) on green shoots produced a smaller lesion (38 mm length) on potted grapevine. Isolate N1351 produced longer lesions compared with all other isolates apart from isolates B3612 and F(12)2.

Fungal colonies morphologically identified as *D. mutila* were recovered from the lesion edges of the inoculated shoots, and no colonies were recovered from the control shoots.

Discussion

This is the first study to assess genetic diversity of the *D. mutila* population in New Zealand, and using the UP-PCR method, with the results showing limited genetic diversity in the *D. mutila* population. Genetically different individuals were observed within a vineyard and between vineyards. Eight genetic groups of *D. mutila* were identified with 98% similarity between the groups in the NJ tree, however, 70% of the total population was located in genetic group 7 which was similar to the pattern observed in *N. australe* (Baskarathevan *et al.*, 2017). In contrast, high intra-population genetic variation was seen for *D. mutila* isolates recovered from declining *Fraxinus excelsior* from different regions in Poland investigated by Kraj *et al.* (2013) using Random Amplified Microsatellites (RAMS). The authors suggested this was likely related to the variation in climatic conditions in the areas where the *D. mutila* isolates were collected for the study. This is in alignment with the report that *D. mutila* can adapt to a range of climatic conditions and can be an active or latent pathogen in a wide range of woody hosts (Crous *et al.*, 2006; Slippers and Wingfield, 2007). In the current study, the genetic diversity of the *D. mutila* population could be the result of multiple introductions of the fungus or 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 similar to that observed for *N. luteum* (Baskarathevan *et al.*, 2017) and *N. parvum* (Baskarathevan *et al.*, 2012b). Since the Australian *D. mutila* isolates were the most similar to the New

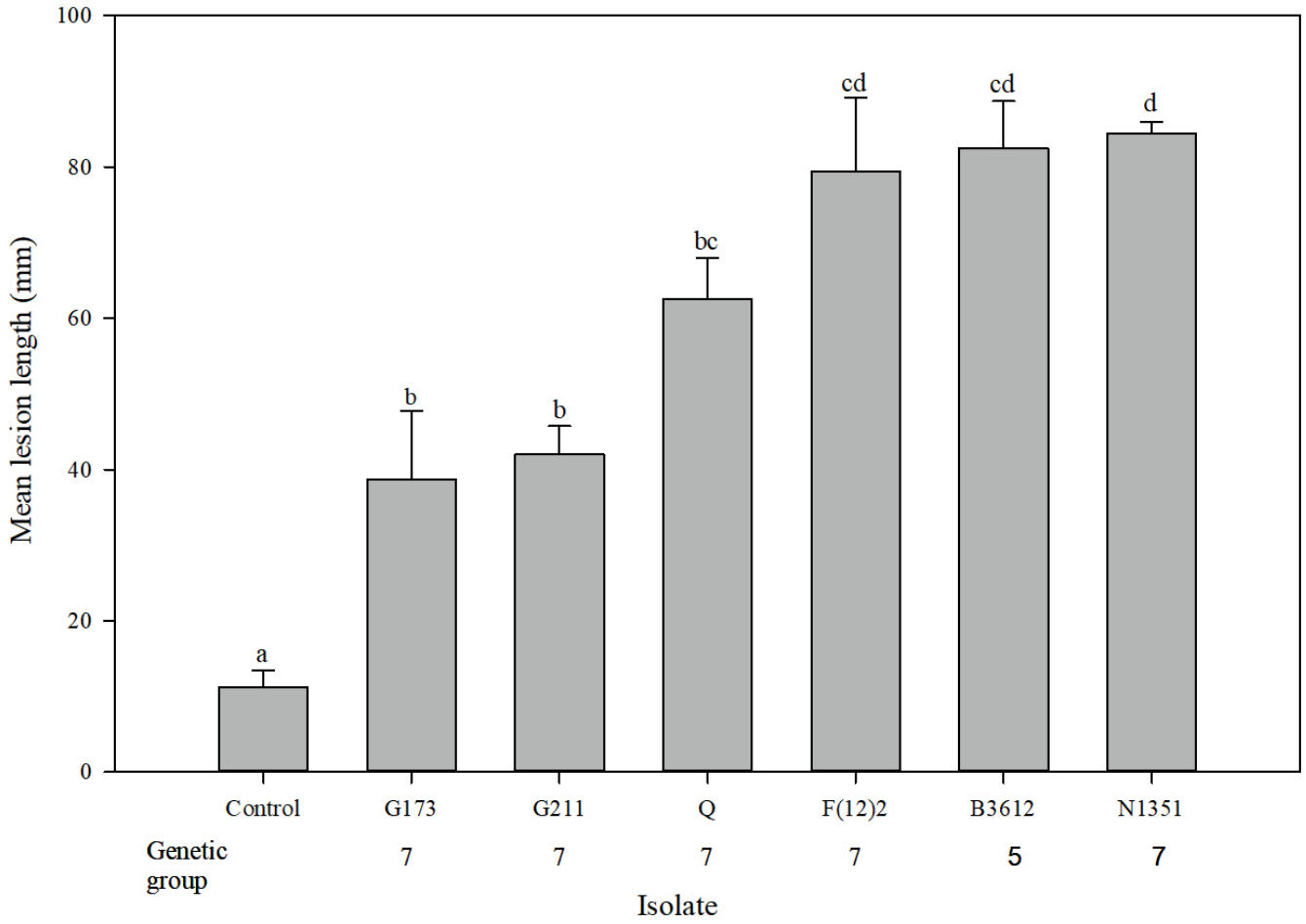

Fig. 5: Mean internal lesion length measured on trunks of 1 year old potted grapevines after six weeks inoculation with *Diplodia mutila* isolates belonging to different genetic groups. Bars with different letters are significantly different ($P \leq 0.05$) based on Tukey's HSD test. Error bars indicate the standard deviation

Zealand population this may indicate a common source of introduction to these two countries and/or exchange between the two countries.

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 with only 28% producing incompatible reactions. 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. This is comparable to the four overlapping VCGs identified in 11 isolates of *N. parvum* (Baskarathevan *et al.*, 2012b) and the lack of clear VCG groups reported by Elena *et al.* (2015) for *D. seriata* and collectively suggests that members of the Botryosphaeriaceae have weak barriers to anastomoses. These results support the hypothesis of multiple introductions of this species into New Zealand and/or that active recombination in this species has occurred.

Although there were some slight variations in the relative ranking of some isolates on the two tissue types, overall, the results on the green shoots and potted vine assays were similar, with the isolates (B3612 and N1351) most virulent on green shoots also the most virulent on potted vines. 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, on the green shoots the majority of isolates (79% or 11/14) produced lesions ≤ 25 mm, with only three isolates exceeding this. In the potted vine assay, the lesions were overall longer with a greater range in lesion sizes observed in the six isolates tested. Billones-Baaijens *et al.* (2013b) also reported *D. mutila* and *D. seriata* to cause longer lesions on rooted canes compared with detached green shoots suggesting these two species were tissue specific. In contrast, for *Neofusicoccum* species (*N. australe*, *N. luteum* and *N. parvum*) lesion development was slower in potted vines compared with detached shoots (Baskarathevan *et al.*, 2012b, 2017; Billones-Baaijens *et al.*, 2013b). Variability in the virulence of *D. mutila* isolates between countries has been previously reported. This species was first reported as a causal agent of black dead arm disease of grapevine in Hungary (Lehoczky, 1988) and highly pathogenic in South Africa (van Niekerk *et al.*, 2004) although it was considered a weak pathogen in Portugal (Phillips, 2002), Australia (Taylor *et al.*, 2005) and California (Úrbez-Torres and Gubler, 2009). This suggests that different factors might be involved in the selection or expression of pathogenicity by this species in different countries. These factors may include

variation in the genetic background of isolates, grapevine cultivars or the environmental conditions. In the current study an isolates recovered from oak was equally as pathogenic on grapevine detached shoots as isolates originating from grapevine. Similarly, isolate of species of Botryosphaeriaceae obtained from apple, pear and peach hosts were reported to cause disease symptoms on the other hosts, suggesting that cross-infection are possible between the susceptible hosts (Sessa *et al.*, 2016).

There was no relationship between the virulence of the isolates and their genetic groups on the neighbour joining tree. This is similar to that reported in other studies using UP-PCR where there was no obvious genotype-pathotype relationship for *N. australe*, *N. luteum* or *N. parvum* (Baskarathevan *et al.*, 2012b; Baskarathevan *et al.*, 2017; Billones-Baaijens *et al.*, 2013b). It is likely that the absence of a strong relationship between the UP-PCR data and the virulence of isolates in this and other studies is due to the UP-PCR primers not targeting genes related to pathogenicity 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). Kraj *et al.* (2013) used RAMS to determine the intra- and inter-genetic diversity of *D. mutila* recovered from ash but the relative pathogenicity of these isolates was not investigated. However, Elena *et al.* (2015) showed there was no relationship between genetic diversity in *D. seriata*, determined using inter-simple sequence repeat (ISSR), and virulence. McDonald *et al.* (1995) suggested that it would be unlikely to find an association in a randomly mating population between DNA markers and virulence 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 provide additional understanding.

The study showed that although genetic diversity exists within New Zealand's *D. mutila* population, that the majority of isolates were genetically similar not only for isolates recovered from grapevines but also other woody hosts and international isolates. An isolate from oak was similarly pathogenic on detached grapevine to isolates sourced from grapevines. This, and the fact that *D. mutila* is known to have a wide host range, indicates that cross-infection is possible between grapevines and neighbouring woody hosts including fruit tree crops and shelter belt species. This should be considered when developing appropriate measures for the prevention and management of Botryosphaeria dieback in vineyards planted in close proximity to orchards and other susceptible hosts. In addition, since *D. mutila* has been recovered from asymptomatic grapevine nursery material (Billones-Baaijens *et al.*, 2013a) infected grapevine planting material may pass through the nursery grading process undetected and contribute to the spread of genotypes into vineyards and regions as indicated in this study. Therefore, ensuring the health status of nursery mother vines and propagation material entering the nursery and during the propagation process is essential to reducing the spread and introduction of these pathogens into vineyards.

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Conflicts of interest

The authors declare that they do not have any conflicts of interest.

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