Genetic diversity in *Dactylonectria pauciseptata* associated with black foot disease in New Zealand

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Abstract The fungal genera *Ilyonectria* and *Dactylonectria* are causal agents of black foot disease affecting grapevines. A New Zealand survey of grapevines symptomatic for this disease was conducted in 2005, and 11 *D. pauciseptata* isolates were recovered. The incidence of this disease has increased over the last decade so a multi-gene approach was used to accurately identify these isolates and to determine whether they could infect grapevine roots *in vitro*. Eight of the original 11 *D. pauciseptata* isolates remained viable and were recovered from storage. DNA sequencing, universally primed polymerase chain reaction (UP-PCR) and a detached root assay were used to characterise these eight isolates. Phylogenetic analyses of ITS, β-tubulin, EF1-α and histone gene regions showed a monophyletic clade using the rRNA gene, EF1-α and histone gene, but paraphyletic based on β-tubulin. UP-PCR showed genetic diversity, with five major groups ($P<0.01$), with major groups concordant with the β-tubulin phylogeny. Pathogenicity tests with two common rootstocks showed that isolates produced lesions. Two distinct groups of *D. pauciseptata* were identified. Both groups are capable of infecting grapevine roots.

Keywords Black foot, *Dactylonectria pauciseptata*, grapevine, pathogenicity

INTRODUCTION

The fungal genera *Ilyonectria* and *Dactylonectria* (Lombard et al. 2014) contain species that are known causal agents of black foot disease, which affects grapevines (*Vitis* spp.) both in nurseries and vineyards throughout the world. Over the last decade, the incidence of this disease has increased significantly in major viticulture regions, including South Africa, New Zealand, Italy, Spain, Portugal, Australia, North America and Brazil (Halleen et al. 2004; Petit & Gubler 2005; Alaniz et al. 2009; Cabral et al. 2012; Úrbez-Torres et al. 2014; dos Santos et al. 2014). Infected vines typically show a range of decline symptoms, including delayed or absent budding, sparse foliage and small leaves with interveinal chlorosis, slower growth, shortened internodes and necrosis. *Dactylonectria pauciseptata* (syn. *Cylindrocarpon paucisepatum*) was first recovered from necrotic regions on roots, wood and trunk bases of infected *Vitis* spp. rootstocks and dying plants with similar symptoms to black foot disease by Schroers et al. (2008). To date, it has been characterised by morphology, DNA sequencing and pathogenicity assays as a pathogen of grapevine and other hosts in many countries, including Spain, Turkey, Canada, Brazil, and Italy (Martin et al. 2011; Erper et al. 2013; Úrbez-Torres et al. 2014; dos Santos et al. 2014; Aiello et al. 2014). However, it has not been associated with black foot disease in New Zealand and there are no studies describing the

genetic diversity of this species.

Dactylonectria spp. are difficult to identify and cannot be accurately differentiated by morphological methods alone. The recent work of Cabral et al. (2012) highlighted the need to use the sequence of four gene regions to accurately identify *Dactylonectria/Ilyonectria* species. In that work, the authors used multi-gene analysis (rRNA, β-tubulin, translation elongation factor 1- α, histone H3) to distinguish New Zealand *D. pauciseptata* strains from either Slovenian or Portuguese strains. In recent work by Úrbez-Torres et al. (2014) in Canada, a multi-gene analysis was applied to identify five different black foot fungi associated with declining young vines, namely *D. pauciseptata, I. liriodendri, D. macrodidyma, I. robusta* and *D. torresensis*. However, there has been no analysis of New Zealand isolates using a four-gene phylogeny to identify this species.

In other countries, researchers have studied the pathogenicity of *D. pauciseptata* isolates recovered from woody plants of grapevines and other hosts. In South Africa, four species (*"C." destructans, I. liriodendri, D. macrodidyma, D. pauciseptata*) recovered from apple roots were able to induce lesion development on seedling roots with variable pathogenicity, except for one isolate of *D. pauciseptata* (STE-U6630), which did not cause a significant amount of root rot or reduction of weight and height (Tewoldemedhin et al. 2011). In Turkey, Erper et al. (2013) tested the pathogenicity of selected isolates from a group identified as *Dactylonectria* and *Ilyonectria* species, including a *D. pauciseptata* isolate from kiwifruit. Results showed that 10 out of 11 isolates, including the isolate of *D. pauciseptata*, were able to induce typical root-rot disease symptoms, affecting plant development and leading to the death of some kiwifruit plants.

In New Zealand, a national survey in 2005 recovered 174 isolates from symptomatic vines of eight major grape-growing regions. Most of these isolates (95%) belonged to the *D. macrodidyma* complex, *I. radicicola* complex and *I. liriodendri* (Bleach et al. 2006) and were shown to be causal agents of black foot disease in New Zealand (Pathrose, 2012). A low frequency (6.3%; n=11) of isolates identified by morphology and β–tubulin sequences as *D. pauciseptata* were also isolated during the survey but their genetic diversity and pathogenicity were not assessed. The overall aim of this study was to use a multi-gene approach to accurately identify these 11 New Zealand isolates and to determine whether they could infect grapevine roots *in vitro*.

MATERIALS AND METHODS

Fungal cultures

Dactylonectria pauciseptata isolates collected from New Zealand in 2005 were obtained from the Lincoln University culture collection where they had been stored as mycelia plugs in 20% glycerol at -80°C. All isolates were subcultured onto a potato dextrose agar (PDA; Difco) and grown at 20°C 12 h light:dark conditions for 7 days. Of the original 11 *D. pauciseptata* isolates that had been stored, only eight (Co6g, Mar5a, Hb3b, Mtb1a, Mar14b, Ack2b, Mar6a and Ack2e) remained viable and could be recovered (Table 1). One isolate of *I. liriodendri* (CO1b) was selected as a positive control from isolates previously demonstrated to be pathogenic on grapevines (Pathrose 2012).

Genomic DNA extraction

DNA had been extracted each of the 11 isolates using the PureGene DNA extraction kit (Gentra systems, USA) prior to storage. The DNA samples had been stored at -20°C. The DNA samples were checked by gel electrophoresis and spectrophotometry and found to still be intact; thus, genetic material for all 11 isolates was available for the current study. The DNA sample of an isolate (AvoC2) from a root lesion on a symptomatic avocado plant was also included in the molecular identification (Table 1). This sample was included because it had been identified by DNA sequencing of the β-tubulin gene as *D. pauciseptata.*

Molecular identification

The identity of all 11 *D. pauciseptata* isolates from grapevine was confirmed by DNA

Table 1 List of New Zealand *Dactylonectria* isolates for phylogenetic analysis and pathogenicity studies.

Species	Isolate Code	Host	NZ Origin
D. pauciseptata	Ack2b	*Vitis vinifera*	Auckland
D. pauciseptata	Ack2e	*Vitis vinifera*	Auckland
D. pauciseptata	CO6g	*Vitis vinifera*	Central Otago
D. pauciseptata	Hb3b	*Vitis vinifera*	Hawkes Bay
D. pauciseptata	Hb6b	*Vitis vinifera*	Hawkes Bay
D. pauciseptata	Mar5a	*Vitis vinifera*	Marlborough
D. pauciseptata	Mar6a	*Vitis vinifera*	Marlborough
D. pauciseptata	Mar14b	*Vitis vinifera*	Marlborough
D. pauciseptata	Mar17d	*Vitis vinifera*	Marlborough
D. pauciseptata	Mar17e	*Vitis vinifera*	Marlborough
D. pauciseptata	Mtb1a	*Vitis vinifera*	Martinborough
D. pauciseptata	AvoC2	*Persea americana* (avocado)	Northland
I. liriodendri	CO1b	*Vitis vinifera*	Central Otago

sequencing of four taxonomically informative genes (rRNA, β-tubulin, translation elongation factor 1- α, histone H3) as described by Cabral et al. (2012). The ribosomal DNA was amplified using the universal primers ITS4 and ITS1F (White et al. 1990), the elongation factor 1-α gene using primers EF1 and EF2 (Carbone et al. 1999), the β-tubulin gene using primers T1 and Bt2b (Cabral et al. 2012) and the histone gene using primers CYLH3F and CYLH3A (Crous et al. 2004). The PCR products were sequenced directly at the Lincoln University Sequencing Facility. The sequences were submitted to a blastn search (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm matching species in the GenBank database. The sequences were aligned with closely related species using the CLUSTALW function of Mega software (Version 5.05; Tamura et al. 2011). A phylogenetic tree for each gene was produced using maximum likelihood based on the neighbour-joining method (Saitou & Nei 1987) and 1000 bootstrap replications.

Genetic diversity

Genetic diversity was assessed using five UP-PCR primers (Table 2). Each 25 μL PCR contained 1× PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 μM each of dGTP, dCTP, dATP and dTTP), 20 pmol of UP-PCR primer, 2.5 mM MgCl2, 1.25 U FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) and 20–25 ng template DNA. The PCR program consisted of an initial step of 3 min at 94°C, followed by 5 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature (Table 2) for 30 s, and elongation at 72°C for 1 min. The next step was 34 cycles of 94°C for 30 s, annealing at the appropriate temperature (Table 2) for 90 s and 1 min primer extension at 72°C. A final extension was performed at 72°C for 10 min. All UP-PCR amplification products were separated by 1% agarose gel electrophoresis. A binomial matrix was generated based on the presence and absence of bands and these data were analysed using Primer software (Version 7.0.5; Primer E Ltd). 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 ($P<0.01$) from each other (Clarke & Ainsworth 1993).

Table 2 Nucleotide sequence and respective annealing temperatures of the UP-PCR primers.

Primer Name	Primer Sequence	Annealing Temperature (°C)	Reference
AA2M2	5'CTGCGACCCAGAGCGG3'	50	Lübeck et al. 1998
AS15	5'GGCTAAGCGGTCGTTAC3'	55	Bulat et al. 1998
AS15 Inv	5'CATTGCTGGCGAATCGG3'	52	Cumagun et al. 2000
L15/AS19	5'GAGGGTGGCGGCTAG3'	52	Lübeck et al. 1998
3-2	5'TAAGGGCGGTGCCAGT3'	52	Bulat et al. 1998

Detached root assays

Pathogenicity assays were carried out as described by Pathrose et al. (2010) on detached roots of either *Vitis vinifera* var. 'Schwarzmann' or *Vitis vinifera* var. 'Riparia Gloire'. A mycelial plug from each of the eight *D. pauciseptata* (Co6g, Mar5a, Hb3b, Mtb1a, Mar14b, Ack2b, Mar6a and Ack2e) isolates and one isolate of *I. liriodendri* (CO1b) as a positive control were taken from the edge of a colony grown on ½ PDA for 7 d at 20°C under 12:12 h dark:light conditions. Young feeder roots of similar age, diameter (1.5–2.0 mm) and pigmentation were randomly selected from one-year-old rootstock plants from both cultivars and washed with tap water to remove soil. They were cut into 6-cm lengths. A mycelial plug infested with a fungal colony was placed on the cut end of detached root with the other end placed in a 1.75-mL tube filled with sterile water. Negative controls were inoculated with a disc of uninoculated ½ PDA. Eight replicates for each isolate were incubated on moist sterile sand at room temperature arranged in a completely randomised design without light for 4 weeks on rootstock 'Schwarzmann' and both 4 and 8 weeks on rootstock 'Riparia Gloire'. Measurement of lesion length using a digital calliper (Mitutoyo, U.K Ltd) was done after each incubation period. Visual observations were made on the type/colour of any lesions which developed on the inoculated roots by eye and under the stereo microscope. The lesion length data were analysed by analysis of variance (ANOVA) using Genstat software (Version 16), with significant differences between treatment means determined using Fisher's protected least significant difference (LSD) (Baskarathevan et al. 2012). To complete Koch's postulates (Agrios 2005), infected roots were surface sterilised and 0.3-cm lengths of roots at 0, 0.5 and 1.5 cm from the lesion margin were taken and placed separately onto PDA plates amended with chloramphenicol. The plates were incubated at 20°C under 12:12 h dark:light growing conditions for 1 week before any colonies growing from the roots were identified based on colony and conidial morphology.

RESULTS

Molecular identification using four taxonomically informative genes

Comparison of ITS, β-tubulin, EF1-α and histone genes among all 11 *D. pauciseptata* isolates showed that they were 99–100% identical to each other. These sequences were also compared with data present in the GenBank database from previously sequenced isolates (GenBank accession numbers: JF735307 for isolate Cy238 from Portugal, EF607067, JF735582 and JF735771 for isolate CBS100819 from New Zealand, JF735775 for isolate CBS120171 from Slovenia, and JF735434 for isolate Cy217 from Portugal). All of the GenBank isolates used for comparison had been cited in the taxonomy work of Cabral et al. (2012) as *Cylindrocarpon paucisepatum*. The ITS, translation elongation factor 1-α and histone H3 neighbour-joining (NJ) trees each contained a single clade of *D. pauciseptata* isolates. The NJ tree generated from β-tubulin sequences placed the 11 isolates into two clades (Fig. 1). The first clade contained seven isolates and was moderately supported (64%). The isolate recovered from a symptomatic avocado plant was also placed into

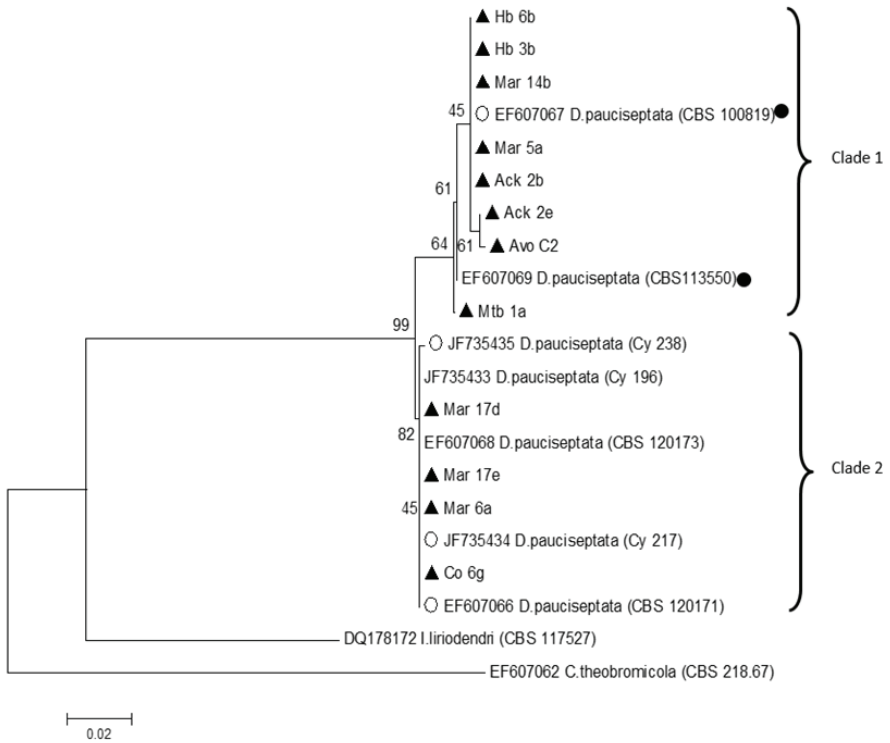

Figure 1 The neighbour joining tree with 1000 replicates of bootstrap values generated by Kimura 2-parameter model of MEGA 5.05 using β–tubulin sequences of *Dactylonectria pauciseptata* isolates from New Zealand vineyards and from GenBank with accession numbers. Isolates with a white circle at left side are from voucher specimens identified using the β–tubulin gene. Isolates: JF735435 – Portugal, JF735433 – Portugal, EF607068 – Slovenia, JF735434 – Portugal, EF607066 – Slovenia. Isolates highlighted with a black triangle represent New Zealand *Dactylonectria pauciseptata* isolates from the present study. Isolates followed by a black circle are previously published New Zealand strains.

clade 1 and clustered with Ack2e in a sub-group (61% bootstrap value). This sub-group contained 1 bp substitution from the other five isolates in clade 1. Isolate Mtb1a was distinct from the other seven isolates in clade 1. DNA from two *D. pauciseptata* voucher isolates (CBS100819 from *Erica melanthera* and CBS113550 from *Vitis* sp.) previously collected from New Zealand (Schroers et al. 2008) were also placed in this clade. The second clade (Mar17d, Mar17e, Mar6a, Co6g) was highly supported (82%) and clustered with five *D. pauciseptata* sequences obtained from GenBank, including the voucher isolates Cy238 and Cy217 from Portugal, and CBS120171 from Slovenia. The isolates in clade 1 had nine single base-pair substitutions compared with isolates in clade 2. *Colleototrichum theobromicola* and *I. liriodendri* were used as outgroups.

Genetic diversity

A total of 65 scorable bands were generated by five primers, of which 46% were polymorphic. Analysis of similarity using the Jaccard similarity index (Baskarathevan et al. 2012) produced a dendrogram with five significantly different groups ($P<0.01$) (Fig. 2). Isolates Ack2b, Ack2e, Mar14b, Mar5a, Hb3b and Hb6b formed a single group of six isolates. Mar17d and Mar17e formed

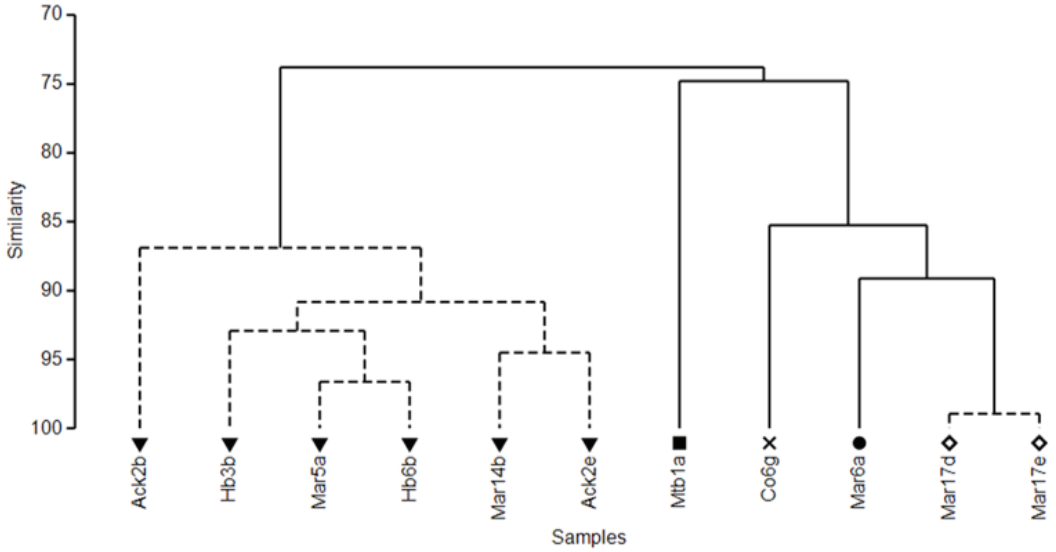

Figure 2 Dendrogram of five significantly different groups (P<0.01) labelled with different shapes above isolates name produced by the analysis of similarity using the Jaccard similarity index. Groups connected by horizontal unbroken lines are significantly different at P<0.01, with those connected by horizontal broken lines not significantly different.

a group of two isolates and the remaining three isolates were each placed on separate branches. These clusters showed similar trends in isolate groupings as the distinction of isolates shown by the β–tubulin NJ tree.

Detached root assay

For both *Vitis vinifera* 'Schwarzmann' and 'Riparia Gloire' rootstocks, the roots inoculated with *D. pauciseptata* showed black discolouration as typical infection symptoms at 4- and 8-week assessment times.

For rootstock Schwarzmann, *D. pauciseptata* isolates CO6g and Mar14b produced lesions (14.7 mm and 13.9 mm, respectively) that were significantly different from the uninoculated control (P<0.001). The remaining six isolates of *D. pauciseptata* were not significantly different from the control (Table 3). The virulence of the D. pauciseptata *isolates within and between* each genetic group generated by UP-PCR was variable. The positive control CO1b (*I. liriodendri*) produced the largest mean lesion overall (36.4 mm). *Dactylonectria* species were not re-isolated from control roots as observed by colony morphology of any fungi emerging from the root pieces.

For 'Riparia Gloire' (Table 3), the positive control *I. liriodendri* CO1b and *D. pauciseptata* isolates Ack2e and Mtb1a were pathogenic. Lesions of 12.5 mm, 9.1 mm and 14.5 mm, respectively (P=0.012) were produced at the 4-week assessment time. At 8 weeks after inoculation, the positive control *I. liriodendri* CO1b (23.0 mm) and *D. pauciseptata* isolates Hb3b (18.6 mm) and Mar14b (24.0 mm) produced the longest lesions but these were not significantly different from isolates Mtb1a, Ack2e and Mar5a.

DISCUSSION

A multi-gene sequence analysis was applied to accurately identify 6.3% of the isolates recovered during a nationwide survey of symptomatic vines that had been tentatively identified as *D. pauciseptata* using morphology and β-tubulin sequences. The 11 isolates formed a monophyletic unit in the rRNA gene, EF1-± and histone gene tree, while they were paraphyletic based on

Table 3 Mean lesion lengths (mm) produced on detached roots of *Vitis vinifera* rootstocks 'Schwarzmann' and 'Riparia Gloire' at various times after inoculation. *Ilyonectria liriodendri* (CO1b) is included as a positive control.

Species	Isolate	Mean lesion length (mm)		
		Schwarzmann	Riparia Gloire	
		4 weeks	4 weeks	8 weeks
I. liriodendri	CO1b	36.4c	12.5cd	23.0c
D. pauciseptata	Mar6a	0.2a	2.2ab	2.3ab
	Mtb1a	3.6a	14.5d	15.2abc
	CO6g	14.7b	2.0ab	3.2ab
	Ack2e	0.5a	9.1bcd	16.8abc
	Ack2b	1.6a	4.3ab	5.2ab
	Mar5a	2.0a	3.1ab	8.7abc
	Hb3b	3.8a	6.6abcd	18.6bc
	Mar14b	13.9b	6.2abc	24.0c
	Control	0.9a	0.7a	1.1a
	*LSD	9.74	8.11	16.38
	P value	<0.001	0.012	0.028

*Fisher's protected LSD test (P<0.05).

β-tubulin phylogenies. For the NJ tree generated from β-tubulin sequences, eight isolates, including an avocado isolate, were identical to the New Zealand isolates recovered previously from voucher specimens of *Erica melanthera* (CBS100819) and *Vitis* spp. (CBS113550), but did not group with voucher isolates from other countries. There were nine bp differences between these eight isolates and the other four isolates which had grouped with international voucher specimens in clade 2. Isolate Mtb1a was consistently located on a separate branch from other isolates on the NJ trees based on sequences of the β-tubulin and EF1-α gene. It would be interesting to determine if isolates with the distinct β-tubulin gene sequence are only been found in New Zealand, however, a greater number of isolates from more hosts would be required to do this.

The *Dactylonectria/Ilyonectria* genus is a complex group which has undergone several reclassifications over the last decade (Agustí-Brisach & Armengol 2013). The taxonomy of the genus *Ilyonectria* and other black foot pathogens has been modified very recently (Lombard et al. 2014). A new genus, *Dactylonectria*, with 10 new combinations, several of which were previously treated in *Ilyonectria*, was introduced from this work. Future studies of this genus using molecular tools may clarify any possible intra- or interspecific variation within *D. pauciseptata* strains. Analysis of these 11 isolates of *D. pauciseptata* showed that they were genetically diverse in New Zealand. When compared to the results for other grapevine pathogens, this result is more polymorphic than the results for *Phaeomoniella chlamydospora* obtained by Pottinger et al. (2002) but similar to those of Pathrose (2012) for other *Dactylonectria* species and Baskarathevan et al. (2012) for botryosphariaceous species. The total number of isolates collected in the survey was small and analysis of a greater number of isolates could help determine the relative frequency of members of each of the genetic groups.

Pathogenicity tests on detached roots in comparison with a representative isolate of *I. liriodendri* indicated that *D. pauciseptata* was

pathogenic towards grapevines with lesions apparent in detached root material. The lesion length of *D. pauciseptata* on detached roots was generally smaller in comparison with the positive control and there was no correlation between genetic groups and pathogenicity. Thus, *D. pauciseptata* may be less pathogenic than other *Dactlyonectria* species. Deliberate co-inoculation of *D. pauciseptata* isolates with other *Dactylonectria* spp. may also be valuable to try in the future work to determine if they are synergistic with other pathogens. Further work on whole plants is also warranted. *Dactylonectria pauciseptata* may also be more aggressive on hosts other than grapevines, such as avocado. A survey of more hosts with symptoms characteristic of black foot is required to clarify relative pathogenicity on different hosts.

In summary, this study has shown that *D. pauciseptata* is present as at least two genetic groups, one of which appeared unique to New Zealand and potentially deserving of reclassification. There was no apparent difference in pathogenicity between the two genetic groups although different isolates were variable in their pathogenicity on the two rootstocks tested.

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