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Phytophthora aff. *cactorum* from *Pinus radiata* in New Zealand plantation forests is a new species

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EXECUTIVE SUMMARY

Report Title: *Phytophthora* aff. *cactorum* from *Pinus radiata* in New Zealand plantation forests is a new species

Authors: Rebecca McDougal, Sarah Addison, Beccy Ganley, Nari Williams

The problem

The objective of this study was to genetically characterise isolates of *Phytophthora* aff. *cactorum* from *Pinus radiata* forests and forest nurseries in New Zealand. Sequence data and pathology observations indicated that these isolates were dissimilar to *Phytophthora cactorum* isolated from horticultural crops such as apple orchards. As the Healthy Trees, Healthy Future programme will test *P. cactorum* against the three host species (pine, apple and kauri), it is important to understand the genetic diversity of isolates for any resistance screening.

This project

Isolates of *P. cactorum sensu lato* were genetically compared using three gene regions; ITS, beta-tubulin and *coxI*.

Key Results

It was observed that all apple isolates had the same DNA sequence for the three gene regions analysed and all pine isolates also had the same DNA sequence but differed from the apple isolate sequences. However, the differences between the two groups of isolates was variable. The beta-tubulin regions were 99.7% similar, the ITS regions were 99.4% similar and the least similarity was observed in the *coxI* region with 97.9% similarity. Phylogenetic analysis showed clear clustering of these two groups of isolates, indicating that the pine isolates mostly likely represent a new species that clusters in *Phytophthora* clade 1.

Implications of Results for Client

For the Healthy Trees, Healthy Future research programme it would be recommended that the apple and pine isolates are considered two separate species *P. cactorum* and *Phytophthora* aff. *cactorum* respectively. It is also recommended that both species are tested in the programme.

Further Work

Further genetic analysis of the *ypt1* gene region would be recommended as this region is currently used for diagnostic analysis. In addition, the morphology of *Phytophthora* aff. *cactorum* needs to be characterised and Koch's postulates need to be demonstrated on *Pinus radiata*.

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Table of Contents

Introduction

For a number of years several *Pinus radiata* stands ranging in age (up to six years old) in Nelson have reported some trees with dieback symptoms, including lesions and stem resin bleeding. In 2010 material was sent to Scion's Forest Health Reference Laboratory included soil, root and wood samples taken from around the stem bleeding areas, for analysis. Wood samples from these areas were unusual in terms of where the lesions appeared (head height instead of the known root collar area) and symptom expression – the wood was moist, of 'soggy' consistency and had a pink discolouration. Isolations from soil and root material did not result in recovery of any likely pathogens. Isolations from the wood material yielded *Phytophthora cactorum* which is widespread in New Zealand and is known from the Nelson area, mostly from apple trees (cause of 'sour sap'). *P. cactorum* has been known to occur on *P. radiata* and was associated with the dieback of shelterbelts in the Auckland region in the late 1950s (Newhook, 1959). Furthermore it is known to be a pathogen of *P. radiata* seedlings in forest nurseries (Reglinski, et al., 2009). However, the height of the lesions and symptoms of *P. cactorum* from the Nelson sites was unusual and was considered to be a new behaviour.

Preliminary molecular analysis of the ITS region of the *P. cactorum* isolates showed they differed slightly from the *P. cactorum* sequences on GenBank. Broader comparison with other New Zealand isolates collected in association with *P. radiata* concurred with this sequence analysis. Although this brief analysis showed the ITS region of Nelson isolates and other *P. cactorum* from *P. radiata* were the same, the results did not clarify whether these isolates should be unequivocally identified as *P. cactorum*, nor did it indicate the level of genetic diversity within these gene regions.

In addition to the preliminary genetic analysis, phenotypic differences have been observed in the laboratory during zoospore production experiments (N. Williams, unpublished data) with *P. cactorum* isolates from *Pinus* for producing inoculum to trial these isolates for pine needle infection using the detached needle assay (Dick, et al., 2014). *P. cactorum* isolates from *P. radiata* and from *Malus domestica* (apple) were both trialled for zoospore production, however the pine isolates did not produce any zoospores using the optimised method whereas those from those apple produced an abundance of sporangia and zoospores (isolates obtained from Dr Ian Horner at Plant and Food Havelock North). In addition to this, Dr Ian Horner has recently tested one pine isolate in an apple pathogenicity assay alongside various apple isolates. The pine isolate did not produce any lesions or discolouration of the tissues, unlike the apple isolates which caused varying degrees of infection (I. Horner, pers. comm.).

A genus-wide phylogenetic structure of the *Phytophthora* genus was first established on the DNA sequences of the ribosomal ITS region of 50 morphological taxa in 2000 (Cooke, et al., 2000). The ITS region has been termed a biological clock in that its moderate evolutionary stability strongly supports species differentiation with sequence differences being a strong indication of species divergence. However, reliance on a single gene region does not always differentiate closely related species such as *P. rubi* and *P. fragariae* which are not differentiated by the ITS region (Blair, et al., 2012). Consequently, species descriptions and differentiation across the *Phytophthora* genus is better informed with a range of nuclear/genomic and mitochondrial genes (Kroon, et al., 2004). Such multi-locus phylogenies are best informed by both mitochondrial and genomic DNA sequences with DNA sequence divergence to detail population flow associated with sexual traits (homothallic and heterothallic species) and hybridisation events between parent species (Kroon, et al., 2004). Nuclear sequences (ITS and beta-tubulin) are inherited through both maternal and parental lines while mitochondrial genes (*coxI*) are maternally inherited (Whittaker, et al., 1994). Of these, the ITS, *coxI* and beta-tubulin were selected for this study as *P. cactorum* has been noted for its ability to form stable hybrids (Bonants, et al., 2000).

Objectives

The objectives of this research were:

1. To determine the level of genetic variation within the ITS region, mitochondrial *coxI* region, and the beta-tubulin region.
2. Confirm the identification of all isolates and assist isolate selection for the Healthy Trees, Healthy Future (Phytophthora MBIE) programme.

Materials and Methods

Strains and culture conditions

All isolates used in this study are listed in Appendix Table A1. *Phytophthora* cultures were grown on carrot agar (Erwin, et al., 1996) with cellophane at 20°C for 3-10 days.

DNA extraction

DNA was extracted using a FastDNA kit (MP Biomedicals, OH, USA) according the manufacturers' instructions. Mycelium was scraped into lysing matrix A tubes and cell lysis solution CLS-Y was used. DNA was stored at -20°C. DNA was diluted 1:10 for PCR.

PCR

Three gene regions were amplified by PCR using the following primers (Table 1): ITS4 and ITS6 primers (Cooke, et al., 2000), Fm84 and Fm77 (or FM83) (Martin, et al., 2003) and BtubF1A and BtubR1A (Blair, et al., 2008).

PCRs with ITS and beta-tubulin primers were performed using the KAPA2G Robust HotStart Readymix (2X) (KAPA Biosystems), according to the manufacturer's instructions. Each 25 µl PCR reaction contained 7.75 µl of PCR grade water, 12.5 µl 2X KAPA2G Robust HotStart ReadyMix, 1.25 µl of each primer (10 µM), 1.25 µl DMSO, 1 µL DNA (1:10 dilution of gDNA). The cycling conditions consisted of an initial denaturation step of 95ºC for 1 min, then 35 cycles of 95ºC (15 sec), annealing at primer-specific temperatures (see Table 1) for (5 sec), and 72ºC (15 sec), and a final extension step of 72ºC (10 min). Gel electrophoresis was performed with 1.5% (wt/vol) agarose in TAE (120 V, 40 min), and stained with SYBR Safe® (Life Technologies) for UV transillumination. PCR primers used in this study are listed in Table 1.

PCR with the FM84 and Fm83 primers was the HOT FIREPol® Blend Master MIX PCR kit (Solis BioDyne, Tartu, Estonia). Each 20 µl PCR reaction typically contained 5× HOT FIREPol® Blend Master Mix, 1 µl of DNA (typically 1:10 dilution of gDNA), 0.6 µl of each primer (10 µM) and PCR-grade water (up to 20 µl total volume). The cycling conditions consisted of an initial denaturation step of 94°C for 10 min, then 35 cycles of 94°C (30 s), 55°C (40 s) and 72°C (1.5 min), and a final extension step of 72°C (10 min). PCR products were run on 1.5% (wt/vol) agarose in TAE (120 V, 40 min) and stained with SYBR Safe® (Life Technologies) for UV transillumination.

DNA sequencing and sequence analysis

Prior to DNA sequencing, PCR products were treated with Exonuclease I (Exo) and Fast Alkaline Phosphatase (FastAP) (Fermentas, Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturers' instructions. DNA sequencing was performed in both directions with forward and reverse target primers. DNA sequencing was performed on a 3500 Genetic Analyzer machine (Applied Biosystems), using BigDye® Terminator

v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions.

DNA sequences were analysed in Geneious 7.1.3. (Biomatters) and aligned using MAFTT v7.017 (Katoh, et al., 2002). The best nucleotide substitution model was determined using jModelTest v. 2.1.4 (Darriba, et al., 2012). Phylogenetic analyses was performed with the MrBayes v. 3.2.2. (Huelsenbeck, et al., 2001) using the Geneious plugin (Marc Suchard & Geneious team, Biomatters) applying the appropriate parameters as determined by jModelTest.

Table 1: Primers used in this study

Primer	Primer sequence	Gene target	Anneal (°C)	Ref.
ITS4	TCCTCCGCTTATTGATATGC	Ribosomal internal transcribed spacer (ITS)	56	(Cooke, et al., 2000)
ITS6	GAAGGTGAAGTC GTAACAAGG			
BtubF1A	GCCAAGTTCTGGGARGTSAT	Beta-tubulin (btub	58	(Blair, et al., 2008)
BtubR1A	CCTGGTACTGCTGGTAYTCMGA			
Fm83	CTCCAATAAAAAATAACCAAAAAT	Cytochrome oxidase I (*coxI*)	55	(Martin, et al., 2004)
Fm84	TTTAATTTTTAGTGCTTTTGC			

Results and Discussion

Comparison of DNA sequences

The ITS, *coxI* and beta-tubulin gene regions were used to compare the genetic diversity of *P*. aff *cactorum* isolates from *Pinus radiata* (n=11) to *P. cactorum* isolates from *Malus domestica* (apple, n=23), *Fragaria ananassa* (strawberry, n=2), *Diospyros kaki (*persimmon*,* n=1*), Meryta sinclairii* (puka or pukanui, n=1), as well as soil isolates from apple orchards (n=3) (Appendix Table A1). The isolates obtained from *P. radiata* were genetically uniform across each of these three regions. Similarly, New Zealand isolates of *P. cactorum* from apple were found to be genetically uniform across the three gene regions analysed. However, both groups were distinct both to each other and in comparison to the publically available sequences of the *P. cactorum* type-strain (see Appendix B) and other clade 1 *Phytophthora* species available on GenBank.

The ITS sequence data consisted of 786 nucleotides. Sequences obtained for the ITS region from the apple or apple-orchard-soil *P. cactorum* isolates differed from the sequence of the type strain by two nucleotides and the pine-isolates by five nucleotides (Table 2). The pine isolates also differed by three nucleotides compared to the *P. cactorum* type strain (ATCC10091). *P. hedraiandra* was described in 2004, and distinguished from *P. cactorum* and *P. pseudotsugae* based on the DNA sequences of the ITS (nuclear) and *cox1* (mitochondrial gene regions). *P. hedraiandra* differed from *P. cactorum* in 4-9 positions (over approximately 800bp) in the ITS region (de Cock, et al., 2004). Similar levels of genetic diversity have been observed in the ITS region in this study.

Table 2: ITS gene similarities between the *Phytophthora cactorum* type strain (ATCC10091), and isolates from apple and pine

ITS - pairwise % ID (identical sites)*	*P. cactorum* Type strain ATCC10091	Apple isolates (n = 23)	Pine isolates (n = 11)
P. cactorum ATCC10091		99.7 (784/786)	99.6 (783/786)
Apple isolates (n = 23)			99.4 (781/786)
Pine isolates (n = 11)			

* % = identical sites/ungapped length of sequences in alignment × 100

DNA sequence of approximately 1195 bp was obtained for the *coxI* region of most isolates. From this sequence, there were 20 variable nucleotide positions for the pine isolates whereas the apple isolates only varied at one location in comparison to the type strain of *P. cactorum* (see Appendix Table C2). Comparison across the 610 bp region in which all sequences could be directly aligned reduced the number of variable nucleotide locations for the pine isolates to 13, which is still relatively high for this size fragment, and also for the *coxI* gene region of *Phytophthora* (Martin, et al., 2003). The pine isolates were therefore less similar at this location compared to the other two genetic loci analysed (Table 3).

Table 3: *coxI* gene similarities between the *Phytophthora cactorum* type strain (ATCC10091), and isolates from apple and pine

coxI - pairwise % ID (identical sites)*	*P. cactorum* Type strain ATCC10091	Apple isolates (n = 23)	Pine isolates (n = 11)
P. cactorum ATCC10091		99.8 (609/610)	97.7 (596/610)
Apple isolates (n = 23)			97.9 (596/610)
Pine isolates (n = 11)			

* % = identical sites/ungapped length of sequences in alignment × 100

The beta-tubulin sequence data was 1108 nucleotides in length and was the least variable of the three loci analysed (Table 4). The pine isolates only had one variable nucleotide location and the apple isolates had three variable nucleotides.

Table 4: Beta-tubulin gene similarities between the *Phytophthora cactorum* type strain (ATCC10091), and isolates from apple and pine

Btub - pairwise % ID (identical sites)*	*P. cactorum* Type strain ATCC10091	Apple isolates (n = 23)	Pine isolates (n = 11)
P. cactorum ATCC10091		99.7 (1105/1108)	99.5 (1102/1108)
Apple isolates (n = 23)			99.7(1104/1108)
Pine isolates (n = 11)			

* % = identical sites/ungapped length of sequences in alignment × 100

Of the isolates not recovered from apple or pine, isolates NZFS 119.03 (*Meryta sinclairii*) and H388 (ICMP 20004, *Diospyros kaki*) had variable nucleotides in each of the three gene regions analysed. In the ITS and beta-tubulin region these isolates contained double-peaks in the DNA sequencing chromatograms which are characteristic of hybrid species (Bertier, et al., 2013; Bonants, et al., 2000). *P. cactorum* is well-known for its ability to hybridize with other species, however cloning and re-sequencing of PCR amplicons are required to identify the parental lines (Bonants, et al., 2000). No further analysis of these strains was performed as it was out-of-scope for this study.

Phylogenetic analysis *Phytophthora cactorum sensu lato*

DNA sequences were aligned using MAFTT and Bayesian analysis was performed in Geneious. The analyses were performed on the different gene regions separately and also as concatenated sequences. Phylogenetic trees for individual genes are presented in (Appendix D). Concatenated sequences were run using two different nucleotide substitution models (Appendix E outlines parameters used for Bayesian analyses); the first being GTR (+G rate variation parameter) as determined by jModelTest and the second being JC69 (with equal rate variation parameter) to determine if any difference would be observed. The JC69 model would possibly make less assumptions about the evolutionary rates which could be different for each partition in the concatenated data (M. Buys, pers. comm). Very little difference was observed between tree topologies obtained using the two different models (data not shown), and the phylogeny obtained with the JC69 model is presented (Fig 1).

The tree topology indicates that the pine isolates form a clade of their own, quite separate to the apple isolates and the type strain of *P. cactorum*. These isolates do not closely cluster with any of the Clade 1 *Phytophthora* type-strain species. The topology of the phylogenetic trees was consistent for the single gene analyses (Appendix D) and the concatenated dataset (Fig 1).

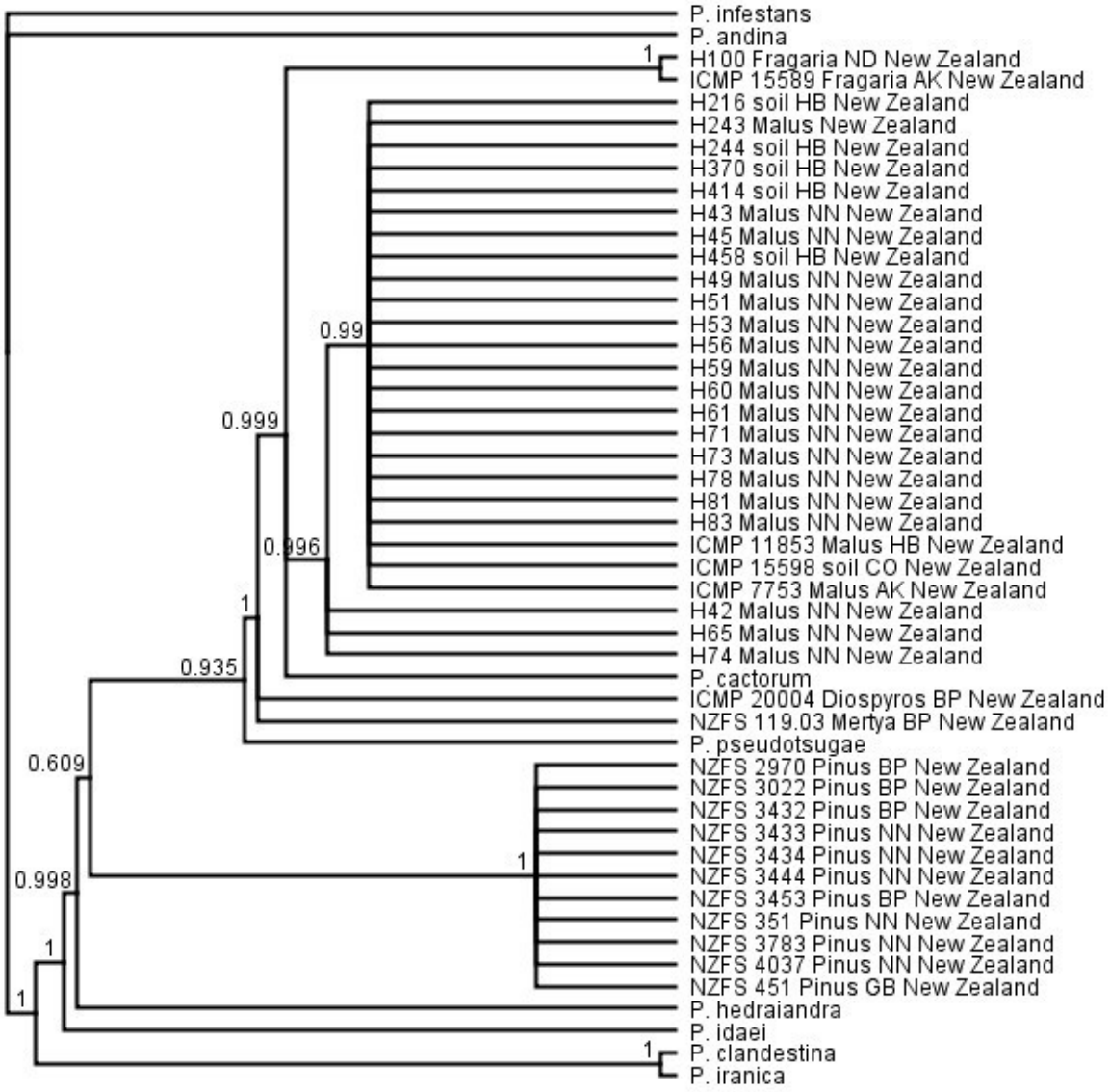

Fig 1. Bayesian inference tree using concatenated sequences. The source of type-strain sequences are listed in Appendix table B1. Numbers at the nodes indicate posterior probability based on bayesian analysis of the dataset. The *P. infestans* sequence was selected as the outgroup.

While the overall structure of the *Phytophthora* genus has been stably characterised for some time (Blair, et al., 2008; Cooke, et al., 2000; Kroon, et al., 2004; Martin, et al., 2014), there remain numerous ambiguities for closely related species that require taxonomic clarification. This has seen the recent revision of numerous species complexes including those in the clade 6 '*P. megasperma* complex' and the revision of the *P. citricola'* complex in clade 2 (Jung, et al., 2009). Such revisions have resulted in the recognition of numerous new species and clarifications of host range, specificity and biosecurity considerations. Previous studies have shown strong host-affiliations in strains of *P. cactorum* internationally (Bhat, et al., 2006; Eikemo, et al., 2004; Hantula, et al., 2000). In our studies, the differentiation of New Zealand *P.* aff cactorum isolates from apple and pine to those of the type strain would support these being described as separate species with support from both the multi-locus phylogenies presented here along with differential

pathogenicity and characteristics in culture. There is considerable evidence that both of the new strains have been present in New Zealand for an extended period dating back to 1998 and 1999 for the apple and pine strains, respectively (these are the collection dates for the oldest isolates tested in this study).

Conclusions

From analysis of three gene regions it can be concluded the *P*. aff. *cactorum* isolates from *Pinus radiata* are most likely a new species that falls in the Clade 1 *Phytophthora* group (Cooke, et al., 2000).

Future Work

Further genetic analysis of the *ypt1* gene could show further evidence for the diversity of the pine isolates. It would also be recommended that this region is analysed as it is used for *P. cactorum* diagnostics and current PCR primers for this do amplify DNA from both *P. cactorum* from apple and *P*. aff. *cactorum* from pine.

Additional work looking at the pathogenicity would also be interesting as Koch's postulates have not yet been satisfied for the pine isolates. This is also important for comparison to work done by Dr Ian Horner looking at pathogenicity of the isolates on apple.

Finally a species description, including detailed morphological analyses, of the *P*. aff. *cactorum* from pine would further contribute to international knowledge of *Phytophthora* species.

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Appendix A: Strains used in this study

ICMP	NZFS	Alternate isolate number	Host	Substrate	Date Collected	Crosby Region	Location
ICMP 15589	2543		*Fragaria ananassa*	Necrotic crown with black vascular tissue	1999	AK	Massey
	3578	H243	soil	by baiting apple orchard soil, using apple cotyledon leaves			
	3579, 3857 (received twice)	H244	soil	by baiting apple orchard soil, using apple cotyledon leaves	2010	HB	Hawkes Bay
ICMP 11853	2509		*Malus domestica*	Root lesions	1993	HB	Pakowai
ICMP 7753	2660		*Malus domestica*			AK	Auckland
	119.03		*Meryta sinclairii*		1998	BP	Tauranga, Park.
	451		*Pinus radiata*		2000	GB	Nuhaka Forest, Cpt 0027
ICMP 16150	351		*Pinus radiata*		1999	NN	Nelson, Spring Grove Nursery, Boundary Blk
	2970		*Pinus radiata*	Root rot in cutting stool plants. Lesions along base of stem and in roots	2007	BP	Rotorua, Te Ngae Nursery
	3022		*Pinus radiata*	Plants with root rot - discolouration extending into stems	2008	BP	Rotorua, Te Ngae Nursery
	3432[2,3,4]		*Pinus radiata*	Stem lesion	2010	BP	TeTeko, Taumata Estate, Kelly Road, Arborgen
	3433[2]		*Pinus radiata*	Stem lesion	2010	NN	33_ITS
	3434[2]		*Pinus radiata*	Isolated from stem lesion	2010	NN	Nelson Forests Ltd, Golden Downs Forest, Stand 179/2
	3444[2]		*Pinus radiata*	Collar and stem infection	2010	NN	Golden Downs Forest, Cpt 100/15
	3453		*Pinus radiata*	isolated from root collar of 2-year-old plants	2011	BP	Kinleith Forest
	3783		*Pinus radiata*	Root Collar	2014	NN	Nelson Forests Ltd
	4037		*Pinus radiata*	Branch	2014	NN	Nelson Forests Ltd

ICMP	NZFS	Alternate isolate number	Host	Substrate	Date Collected	Crosby Region	Location
ICMP 15598	2664, 3831 (received twice)	H220	Soil	Soil with specific apple replant disease from apple orchard	2004	CO	Clyde
	3846	H45	*Malus domestica*	wood	1998		Nelson
	3826	H49	*Malus domestica*	wood	1998		Nelson
	3827	H59	*Malus domestica*	wood	1998		Nelson
	3828	H60	*Malus domestica*	wood	1998		Nelson
	3829	H78	*Malus domestica*	wood	1998		Nelson
	3830	H83	*Malus domestica*	wood	1999		Nelson
	3858	H370	*Malus domestica*	soil	2011		Hawkes Bay
	3860	H414	*Malus domestica*	soil	2012		Hawkes Bay
	3861	H458	*Malus domestica*	soil	2014		Hawkes Bay
	3844	H042	*Malus domestica*	wood	1998		Nelson
	3845	H043	*Malus domestica*	wood	1998		Nelson
	3847	H051	*Malus domestica*	wood	1998		Nelson
	3852	H073	*Malus domestica*	wood	1998		Nelson
	3855	H100	*Fragaria ananassa*	stem	1999		Coatesville
	3856	H216	*Malus domestica*	soil	2003		Hawkes Bay
	3849	H061	*Malus domestica*	wood	1998		Nelson
	3848	H053	*Malus domestica*	wood	1998		Nelson
	3853	H074	*Malus domestica*	wood	1998		Motueka
	3851	H071	*Malus domestica*	wood	1998		Nelson
	3850	H065	*Malus domestica*	wood	1998		Nelson
	3854	H081	*Malus domestica*	fruit	1999		Nelson
20004	3859	H388	*Diospyros kaki*	fruit	2011		Tauranga
	3862	H056	*Malus domestica*	fruit	1998		Nelson

Appendix B: DNA sequences obtained for this study

***Phytophthora* species**	**Accession number[1]**	**Alternative number[2]**
P. andina (T)[3]	HQ261598.1	P13365
P. andina[4]	PD_01016	P13660
P. cactorum[5]	FJ801257.1	P0714, ATCC10091, CBS231.30
P. clandestina	HQ261538.1	P3942, ATCC58715, CBS349.86
P. hedraiandra[4]	PD_02050	P11678
P. hedraiandra	FJ802065.2	P11056
P. idaei (T)	HQ261579.1	P6767,CBS971.95, IMI313728
P. infestans	HQ261589.1	P10560
P. iranica (T)	HQ261598.1	P3882, ATCC60237, CBS374.72, IMI158964
P. pseudotsugae (T)	HQ261654.1	P10339, IMI331662

[1] Sequences obtained from NCBI or the Phytophthora database
[2] Martin et al. 2014
[3] (T) denotes the type strains, used where available
[4] Sequences used for the coxI locus were used from a different strain as sequences were not available for the type strains of *P. andina* or *P. hedraiandra*
[5] It is unclear from the literature whether this is the type strain or neotype, however it is the strain used in Martin et al. 2014 (the most recent *Phytophthora* phylogeny).

Appendix C1: Nucleotide variance tables

Position	Overall consensus	H388, 119.03	2664, H73, H43, H71, H81, H61, H45, H458, H414, 3578, H216, H370, H244, H59, H220, H53, H49, H60, H51, 3579, 2509, 2660, H56, H78, H83	H65, H42, H74	3022, 3433, 3444, 2970, 3434, 3783, 3029, 3432, 3453, 351, 451, 4037	H100, 2543	Number of Variants
Coverage							
132	C		T				1
140	:				T		1
141	C				:		1
159	:				C		1
160	A	M			:		2
195	G	R					1
235	C		T	T			2
381	G				A		1
399	A	R					1
745	A	:					1
746	:	R					1
747	C	Y					1
773	C	Y					1
Total Differences		**7**	**2**	**1**	**5**	**0**	

Appendix C2: *coxI* nucleotide variance table

Position		Overall consensus	3783, 3432, 3433, 3434, 2970, 3022, 3444, 3029, 3453, 451, 351, 4037	H100, 2543	H388, 119.03	3579, H244, H56, H370, H81, H65, H74, H51, 2509, H53, H61, H73, 2664, H59, H71, H49, H83, H220, 3578, H42, H414, H78, H458, H244, H216, 2660, H45, H43, H60	Number of Variants
91		G	A				1
145		T	A				1
211		T	A				1
283		R	A	G	G	A	4
301		A	C				1
424		A	G				1
496		T	G				1
534		C	T				1
544		C	T				1
550		A		T			1
553		T	G				1
604		G	T				1
646		A	T				1
712		G		A			1
715		T	A				1
853		T	A				1
919		T	A				1
970		A	G				1
1,021		C	T				1
1,078		G	A				1
1,093		C	T				1
1,108		C	T				1
Total Differences			**20**	**3**	**1**	**1**	

Nucleotide variants indicated are relative to the consensus sequence obtained from the DNA sequence alignment.

Appendix C3: Beta-tubulin nucleotide variance table

Position	Overall Consensus	119.03, H388	3783, 3433, 3444, 3434, 3022, 2970, 351, 451, 3453, 4037, 3432	H60, H458, H370, H73, H53, H43, 3579, H65, H49, 2660, H220, H51, H216, H71, H61, 2664, H42, H83, H414, H100, 2509, H81, H244, H59, H45, H78, H74, H56, 3578, 2543.	Number of Variants
155	C	Y		T	2
545	R	G	A	G	3
569	T			G	1
665	G	R			1
Total Differences		**3**	**1**	**3**	

Nucleotide variants indicated are relative to the consensus sequence obtained from the DNA sequence alignment.

Appendix D: Phylogenetic trees for individual genes

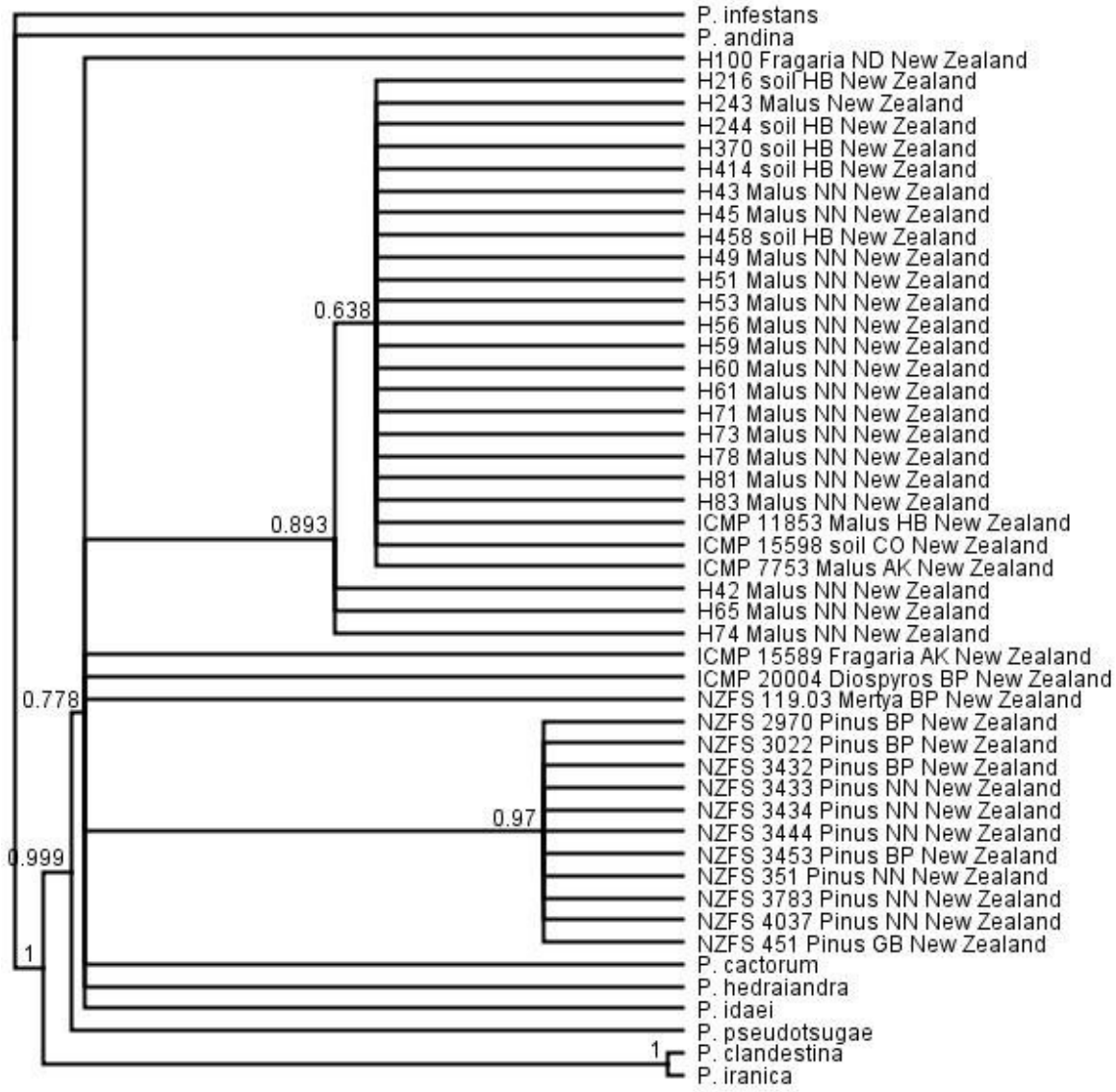

Figure D1: Bayesian inference tree using ITS DNA sequences. The source of type-strain sequences are listed in Appendix table B1. Numbers at the nodes indicate Posterior probability based on Bayesian analysis of the dataset. The *P. infestans* sequence was selected as the outgroup.

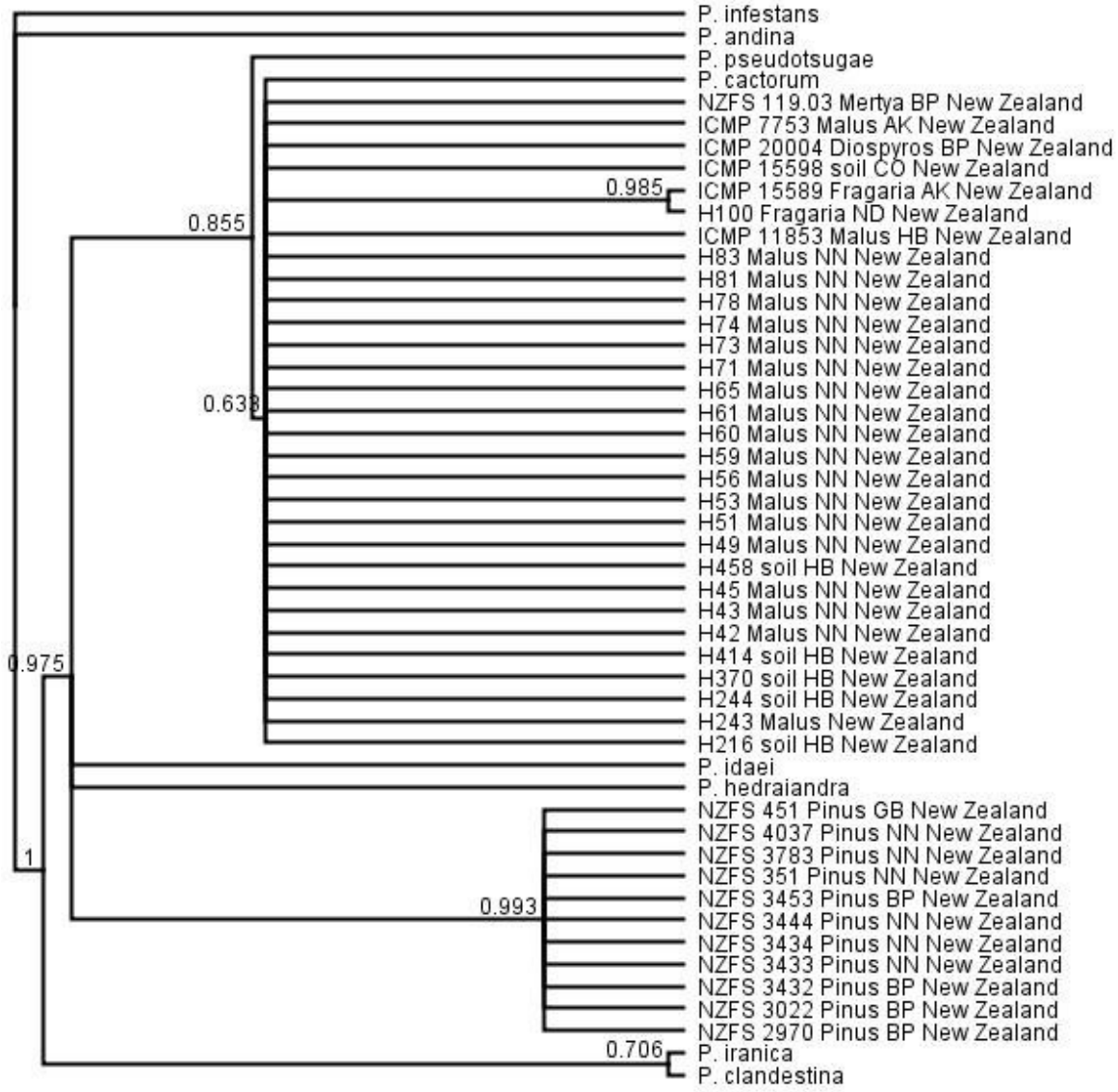

Figure D2: Fig 1. Bayesian inference tree using *coxI* DNA sequences. The source of type-strain sequences are listed in Appendix table B1. Numbers at the nodes indicate Posterior probability based on Bayesian analysis of the dataset. The *P. infestans* sequence was selected as the outgroup.

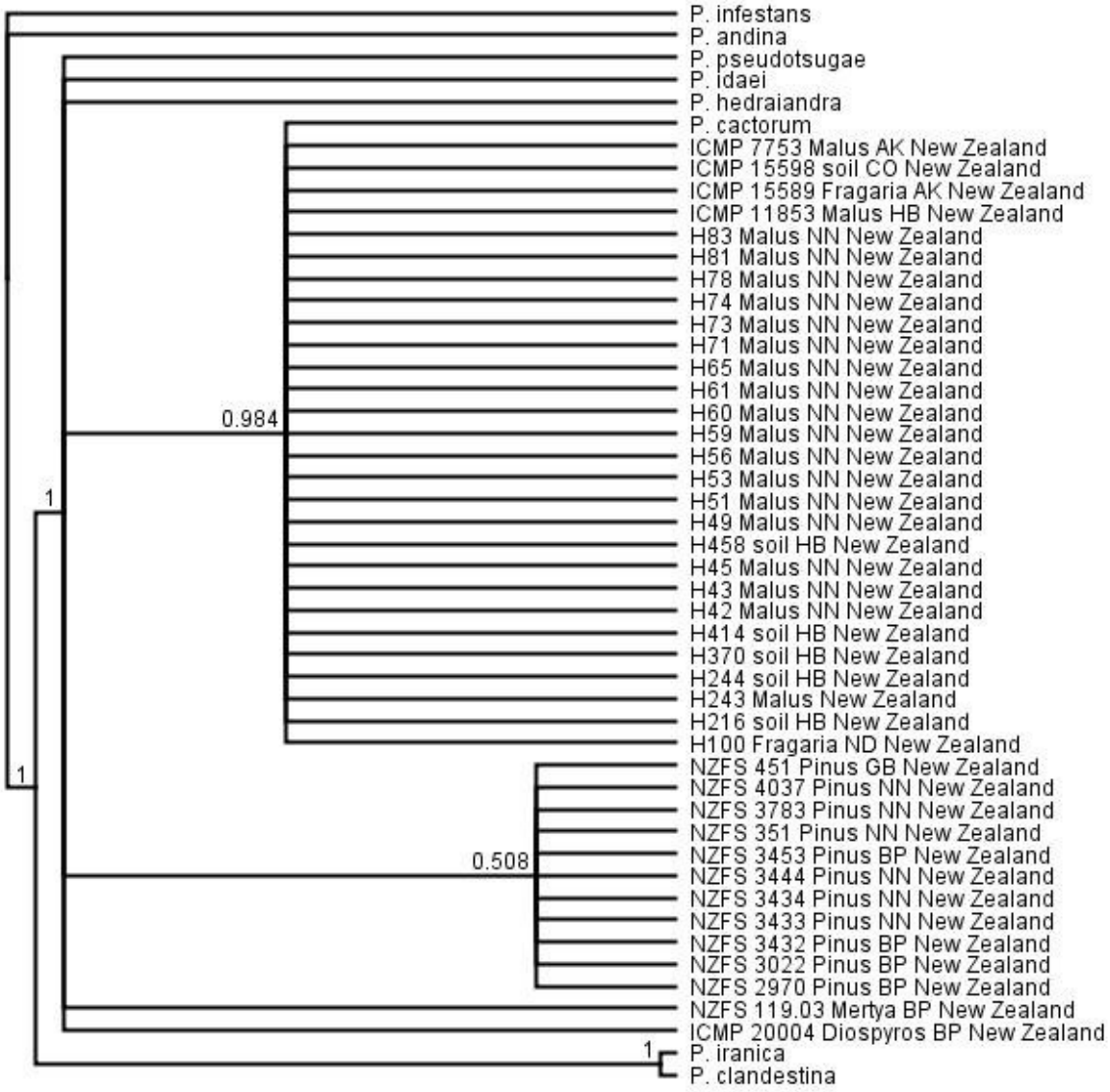

Figure D3: Fig 1. Bayesian inference tree using beta-tubulin DNA sequences. The source of type-strain sequences are listed in Appendix table B1. Numbers at the nodes indicate Posterior probability based on Bayesian analysis of the dataset. The *P. infestans* sequence was selected as the outgroup.

Appendix E: MrBayes parameters used in Geneious

Parameter setting in Geneious	ITS	*coxI*	beta-tubulin	concatenated sequences
Substitution model	HKY85	HKY85	GTR	JC69
Rate variation	gamma	propinv	propinv	equal
Outgroup	*P. infestans*	*P. infestans*	*P. infestans*	*P. infestans*
MCMC settings:				
Chain length	1,100,000	1,100,000	1,100,000	1,100,000
Subsampling frequency	200	200	200	200
Heated Chains	4	4	4	4
Burn-in length	100,000	100,000	100,000	100,000
Heated chain Temp	0.2	0.2	0.2	0.2
Random Seed	15, 021	28, 470	6,336	17,286
Priors:				
Unconstrained Branch lengths: Exponential	10	10	10	10