Range expansion of *Phytophthora,* particularly *Phytophthora cinnamomi* into colder environments: adaptation, a changing environment or both?

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A thesis submitted to Murdoch University
to fulfil the requirements for the degree of
Doctor of Philosophy

January 2019

Dedication

To my late parents, who unfortunately did not stay in this world long enough to see this dissertation completed

Declaration

I declare that this thesis is my own account of my research and contains as its main content work, which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been acknowledged.

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January 2019

Abstract

Alpine and sub-alpine regions were long considered free of *Phytophthora* species, especially *Phytophthora cinnamomi* due to restrictions on their growth from low temperatures. However, *P. cinnamomi* was isolated from a sub-alpine area 'Barrington Tops National Park' in the 1990s. Subsequent Australia wide surveys detected 68 *Phytophthora* species in Australia. Of these, 33 *Phytophthora* species, including *P. cinnamomi,* were detected in the alpine and sub-alpine areas on Kosciuszko National Park (KNP) alone. This suggested that *Phytophthora* species had adapted to cold environments. This project investigated the ability of *Phytophthora* species to produce infective propagules (zoospores) and cause disease at increasingly lower temperatures. *Phytophthora cinnamomi* was selected as a 'test' species due to its national and international significance. Initially, preliminary surveys were conducted in the sub-alpine and alpine areas of KNP and Tasmania to obtain living *Phytophthora* isolates. The lower temperature limit for growth and sporulation of Mediterranean (one isolate was from a sub-alpine area) *P. cinnamomi* isolates was determined and phenotypic plasticity experiments were established in an attempt to 'train' them to produce infective propagules and cause disease at increasingly lower temperatures. Finally, the distribution patterns of *Phytophthora* and vascular plants species in relation to disturbance and elevation were determined across elevation gradients in KNP. Preliminary surveys resulted in the isolation of eight *Phytophthora* species, including two new species that were formally described. *Phytophthora cinnamomi* was shown to produce infective propagules at temperatures lower (7.5 °C) than originally established (10 °C), and in a shorter time compared to original isolates when 'trained' under cold conditions. This suggests that *P. cinnamomi* responds rapidly to selection pressure and adapts to new environments. Although *P. cinnamomi* produced infective propagules at 7.5 °C, the pathogen could not be isolated from plants grown at 7.5 °C after three months. Therefore, more work is required to establish disease development at 7.5 °C and below. Results of surveys along elevation gradients showed *Phytophthora* and vascular plant species exhibited a linearly monotonic decline with increasing elevation on roads, but not in native vegetation. However, the elevation range of *Phytophthora* species was higher than vascular plants on both roads and in native vegetation. *Phytophthora* species did not show any habitat preference and exhibited similar composition and frequency on roads and in native vegetation; vascular plants showed the opposite trend with greater frequency in native vegetation. This suggests that *Phytophthora* richness at the plot level mimics that of vascular plants. A changing climate may permit invasion by other *Phytophthora* species not yet present.

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List of publications

Journal article

Chapter 2 published as: Khaliq I, Hardy GESJ, White D, Burgess TI (2018) eDNA from roots: a robust tool for determining *Phytophthora* communities in natural ecosystems. *FEMS Microbiology Ecology* 94: fiy048.

Chapter 3 published as: Khaliq I, Hardy GES, McDougall KL, Burgess TI (2018) *Phytophthora* species isolated from alpine and sub-alpine regions of Australia, including the description of two new species; *Phytophthora cacuminis* sp. nov and *Phytophthora oreophila* sp. nov recovered from these areas. *Fungal Biology* https://doi.org/10.1016/j.funbio.2018.10.006

Chapter 4 submitted as: Khaliq I, Hardy GES, Burgess TI (2018-in review) Phenotypic plasticity favours range expansion of *Phytophthora cinnamomi* into colder environments. *Plant Pathology*

Conference presentations

Khaliq I, Hardy GESJ, White D, Burgess TI. Comparison of traditional isolation methods for *Phytophthora* to sources of eDNA used for metabarcoding. The 8th Congress of IUFRO GOUP 7.02.09 “Phytophthora in forests and natural ecosystem ecosystems”. 18-25 March 2016, Sapa, Vietnam. Oral presentation.

Khaliq I, Hardy GESJ, Burgess TI. Can *Phytophthora cinnamomi* adapt to colder conditions? The 8th Congress of IUFRO GOUP 7.02.09 “Phytophthora in forests and natural ecosystem ecosystems”. 18-25 March 2016, Sapa, Vietnam. Oral presentation.

Acknowledgements

I would like to express my special appreciation and thanks to my supervisors Associate Professor Treena Burgess, Professor Giles Hardy and Dr. Keith McDougall, you have been tremendous mentors for me. I appreciate all your contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating.

I wouldn't have been able to survive without Diane White, as she was always available to show me molecular work and to double-check my protocols. I also thank Briony Williams for showing me traditional techniques used in chapter 2. I wouldn't have been able to complete the bioinformatics analysis of my last chapter without the help of Andrea Paparini, Alex Gofton and Rachael Lappan. I would also like to thank the Mountain Invasion Research Network (MIREN) for providing assistance during surveys.

My time in Perth was made enjoyable in large part due to amazing students of different cultural backgrounds at the Centre for Phytophthora Science & Management. Thank you for being a part of my life.

Statement of contributions

This thesis was co-supervised by Associate Professor Treena Burgess, Professor Giles Hardy and Dr. Keith McDougall. Burgess and Hardy contributed in the form of ideas and experimental design. McDougall collaborated on Chapter 3 where he assisted with samples collection, and Chapter 5 where he assisted with statistical analysis and provided useful insights on ecology related matters. Chris Shaw provided statistical assistance in Chapter 3.

Chapter 1: General Introduction and literature review

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What is a mountain?

A mountain is defined by a common ruggedness threshold (>200 m difference in elevation within a 2.5′ cell, 0.5′ resolution), arriving at 16.5 million km^2 or 12.3% of all terrestrial land area outside Antarctica being mountains (Körner *et al.* 2011). Ruggedness here refers to the maximum elevational difference among nearby grid points. Calculations are based on the digital elevation model (DEM) used by WorldClim (Hijmans *et al.* 2005). Australian mountains are unusual as compared to the rest of the world's mountains (Venn *et al.* 2017). The high mountains of Australia is comprised of approximately 11 700 km^2, and are comparatively low compared to the rest of the world with Mt Kosciuszko at 2228 m the highest point on mainland Australia (Venn *et al.* 2017). There is no 'nival zone' (areas with permanent snow cover) in Australia except for sub-Antarctic Heard Island that supports glaciers (Venn *et al.* 2017). The part above the treeline (treeless zone) is called the 'alpine zone'. Globally, treeline is determined by seasonal mean ground temperature of approximately 6.7 °C (Körner & Paulsen 2004). In Tasmania, Australia, a mean air temperature of the warmest month of 10 °C determines the treeline (Venn *et al.* 2017). In Australia, 'sub-alpine zone' is defined by woodlands dominated by trees related to *Eucalyptus pauciflora* and treeless plains of valleys; and 'montane zone' as the part below that (Venn *et al.* 2017). Alpine and sub-alpine vegetation have steep environmental gradients and highly localised and specialised communities. In Australia, the Snowy Mountains of New South Wales (NSW) contain the largest area of alpine vegetation extended over 100 km^2 (Venn *et al.* 2017).

Species in mainland Australia and alpine and sub-alpine areas respond markedly differently to competing land uses and climate change (Venn *et al.* 2017), therefore higher elevations are ideal to study the impact of climate change on species distribution. Mountains also allow 'natural experiments' due to sharp environmental gradients over short distances that test ecological theories and questions on adaptive evolution (Körner *et al.* 2011).

The uniqueness of mountain ecosystems

Globally, mountains provide critical ecosystem services such as fresh water, timber, medicinal plants, and recreational opportunities for surrounding lowland populations. Although mountains occupy only 12% of the land surface, their complex montage of microenvironments and ecoclines contribute to almost one quarter of global biodiversity (Körner *et al*., 2011). One fifth of the global area is mountains, which are inhabited by one fifth of the global population. Approximately one quarter of the global mountain areas are mountain forests (Körner 2004). It is therefore safe to conclude that water availability for half of the global population (Messerli *et al.* 2004), and safety and wellbeing of one fifth of the

population depend on the correct functioning of mountain ecosystems either directly or indirectly (Körner 2004, van den Bergh *et al.* 2018). Water from mountain catchments is used for drinking, irrigation and electricity production.

Half of the world's biodiversity hotspots and one-third of all protected areas (88 World Heritage Natural Sites, and 40% of all UNESCO MAB (Man and the Biosphere Reserves) are located in mountains (Chape *et al*., 2008). For example, in Australia, the Kosciuszko National Park (KNP) alone contains about 1100 vascular native plant species, which represents one quarter of the flora in New South Wales in only 10% of its land area (Doherty *et al.* 2015). About 40% of these species are endemic to the Australian Alps bioregion. Globally, in true alpine zones (above the treeline) flora covers only 3% of the vegetated area, but contain 4% of all known plant species. Vegetation above the treeline is very diverse due to smaller size of species and often half of the vegetation of a large area can be in found in a few square meters of dense vegetation (Körner 2004). Therefore, alpine zones are relatively species rich, especially when measured at large scales (Körner 1995).

Decline in species richness with altitude

Biotic interactions and diversity within mountains decrease as the elevation increases because the life conditions become adverse with increasing elevation (Körner 2004, Alexander *et al.* 2017). This natural loss in diversity is even more evident and fairly regular above the treeline with an average loss of ca. 40 species of flowering plants per 100 meters of elevation (Korner 2002). In most cases animal species' diversity also decreases with altitude, although the pattern of decline may vary from species to species. For example, beetles, moths and butterflies decline in number and diversity with altitude, but diptera may show higher diversity at higher elevations (Meyer & Thaler 1995). The possible causes of decline in species richness with altitude are mainly related to climatic and space and time limitations of evolution i.e. immigrant species need space to live and time to evolve or adapt (Körner 2003). Below is a breakdown of the major causes of decrease in species richness with altitude.

Adaptation limits: Species found at higher altitudes are well adapted to colder conditions and have already reached their adaptation limit. In addition, no physiological or reproductive limits have been identified in alpine species compared to their lowland relatives that can force them to evolve, which limits the selection for successful evolution (Körner 2004).

Space limitation: The decline in species number, especially flowering plants is directly proportional to the decline in land area at higher elevation. It is therefore possible that the

decrease in biodiversity across higher elevations has an evolutionary relationship with biogeography rather than climatic constraints (Körner 2004, Pauchard *et al.* 2009).

Limited functional space: Limited functional space means isolation, which is caused by missing or restricted migration leading to reduced functional space and the species pool which mountain biota can tap (Körner 2003, Pauchard *et al.* 2009).

Seasonal time constraints: Reduced season length for any season e.g. through influences like drought or extreme temperatures will reduce the time during which evolutionary processes can take place. Small organisms (e.g. soil microbes) may have several reproductive cycles at lower elevation, but only few at higher elevation leading to reduced chances of diversification (Körner 2003, Alexander *et al.* 2017).

Geological time constraints: Chances of diversification at higher elevations are also reduced by momentary or sporadically interrupted evolutionary history, for example habitat destruction due to glacial cycles or fire (Hawkins 2004, Camac *et al.* 2017).

Environmental filtering: Decline in species richness with increasing elevation is also due to progressive filtering out of species due to harsh climatic conditions along the elevation gradients. Anthropogenic activities can introduce organisms to cold environments but only those species survive and establish that are able to adapt to these extreme environments (Gerhardt & Collinge 2007, Alexander *et al.* 2011, Redondo *et al.* 2018). Although species richness can be reduced by processes like dispersal limitation or competitive exclusion, environmental filtering is more important in determining the pattern of species richness along an elevation gradients (Pauchard *et al.* 2009). The effect of environmental filtering will be evident if a species arrives at a certain location (i.e. there is no dispersal limitation), and its richness pattern is subsequently affected due to environmental conditions of an area in the absence of competitors; the latter can be validated by laboratory based measures of physiological tolerance (Kraft *et al.* 2015).

Decreased anthropogenic disturbance

Decreased introduction pathways result in reduced human related disturbance leading to low propagule pressure. This is especially true for non-native species because the impact of human related propagule pressure is stronger on non-native species than on native species (Marini *et al.* 2009). Decline in richness for non-native plant species along elevation gradients is especially pronounced along road corridors, as roads are the major pathways for dispersal of non-native species to higher elevation. This also explains the higher species richness of

non-native species on roads than in native vegetation. As area and temperature also decreases along elevation gradient, overall species richness (native and non-native) also decreases (Pauchard *et al.* 2009).

The genus *Phytophthora*

The name *Phytophthora* is derived from two Greek words, '*Phyto'* means plant and '*phthora'* means destroyer. *Phytophthora* spp. belong to the class Oomycetes that were traditionally placed in the kingdom Fungi but have now been placed in the kingdom Stramenopila together with diatoms and brown algae due to their structural, biochemical and genetic differences from true fungi (Irwin *et al.* 1995). They are commonly referred to as 'water moulds' as they require free water for sporulation and zoospores release (Erwin & Ribeiro 1996). The genus *Phytophthora* has been divided into 12 phylogenetic clades, and comprises of more than 140 described species that can be pathogens or saprophytes (Jung *et al.* 2018).

Distribution and origin

The genus *Phytophthora* is distributed worldwide and can be found in terrestrial, aquatic and aerial environments (Brasier & Hansen 1992, Erwin & Ribeiro 1996). *Phytophthora* species occur in a variety of climatic zones from temperate to tropical, some have a very broad host range, and some can survive as biotroph, hemibiotrophs and nectrotrophs (Erwin & Ribeiro 1996). Although their fossil record has remained inconsistent, the reports of fossils in the form of structurally preserved oogonium-antheridium complexes from Devonian and Carboniferous rocks demonstrate that they lived in plants as endophytes approximately 400 million years ago (Krings *et al.* 2011).

Anthropogenic related activities have resulted in the global movement of many *Phytophthora* species. The classical example is *P. infestans,* which has caused losses on potato and other solanaceous crops worldwide since the mid 19th century (Erwin & Ribeiro 1996). Other examples include, *P. cinnamomi,* which cause severe dieback in forest and heathlands in Western Australia (Shearer *et al.* 2004); *P. ramorum*, the cause of sudden oak and larch death in the USA and UK/Europe (Rizzo & Garbelotto 2003, Brasier & Webber 2010); *P. palmivora* causes disease in several hundred hosts, especially cocoa globally (Guest 2007); and species of the *P. alni* complex cause severe damage in the population of *Alnus* spp. across riverbank ecosystems in Europe (Jung & Blaschke 2004, Aguayo *et al.* 2014).

In Australia, emphasis is mainly placed on *P. cinnamomi* and *P. multivora* due to their devastating impact on natural and managed ecosystems (Burgess *et al.* 2018, Hardham &

Blackman 2018). There are several other *Phytophthora* species in Australia's natural ecosystems but there is limited information on their distribution and diversity (Dunstan *et al.* 2016). Irwin *et al.* (1995) reported 22 *Phytophthora* species in Australia; most were associated with agricultural crops and nursery plants with the exception of *P. cinnamomi.* Since 1982, the Vegetation Health Service of the Department of Parks and Wildlife in Western Australia has been isolating *Phytophthora* species during routine sampling for *P. cinnamomi* management. About 10% of the recovered isolates were not *P. cinnamomi* and their subsequent molecular re-evaluation identified many new *Phytophthora* species (Burgess *et al.* 2009), and many are awaiting description (Burgess *et al.* 2017b). Many of the newly described species were only found in Australia expect *P. multivora,* which has global distribution (Burgess *et al.* 2017a). Twenty seven taxa have been identified in combination with subsequent surveys of waterways in native ecosystems (Hüberli *et al.* 2013). Eight *Phytophthora* species were identified in soil samples collected from south-east QLD and central NSW (Scarlett *et al.* 2015), and five *Phytophthora* species were recovered in soil and water samples collected from Victoria (Dunstan *et al.* 2016).

Recently, Australia wide surveys were conducted to determine the diversity and distribution of *Phytophthora* species across Australia (Burgess *et al.* 2017b). A total of 68 species were detected in the study (Table 1.1). Of these, 33 *Phytophthora* species were detected in alpine and sub-alpine areas thought to be *Phytophthora* free (Podger *et al.* 1990, Brasier & Scott 1994). Detailed information on their respective clades, first record in Australian databases, habitat, putative centre of origin, and environmental predictors across Australian range has been briefly described (Burgess *et al.* 2017a, Burgess *et al.* 2018). Therefore, they will not be discussed here.

Table 1.1 Records of 91 *Phytophthora* species and taxa and their distribution within Australian states; Queensland (QLD), New South Wales (NSW), Victoria (VIC), Tasmania (TAS), Western Australia (WA) investigated by Bugess *et al*. (2017). The Australian Plant Pest Database (http://www.planthealthaustralia.com.au) was the primary database used. Additional records from other databases are acknowledged in footnotes. + indicates presence as recorded in databases, (+) indicates detection by metabarcoding. Species not detected by metabarcoding are shaded, while phylotypes detected only by metabarcoding are in bold.

	***Phytophthora* species**[1]	**Clade**[2]	**First record**[3]	**Habitat**[4]	**Status**[5]	**QLD**	**NSW**[6]	**VIC**[7]	**TAS**	**WA**[8]	**SA**[9]
1	*P. cactorum*[7]	1	1961	AH	I	+	+(+)	+(+)	+(+)	+	+
2	*P. clandestina*	1	1983	A	I		+	+		+	+
3	*P. infestans*	1	1909	A	I	+	+	+	+		+
4	*P. nicotianae*	1	1945	NAH	I	+(+)	+(+)	+(+)	+(+)	+(+)	+
5	**P. AUS1A**	**1**		**N**	**N**				**(+)**	**(+)**	
6	**P. AUS1B**	**1**		**N**	**N**		**(+)**		**(+)**		
7	**P. AUS1C**	**2**		**N**	**N**					**(+)**	
8	***P. capensis* complex**	**2**		**NH**	**I**		**(+)**		**(+)**	**(+)**	
9	*P. capsici*	2	1988	A	I	+					
10	*P. citricola* complex	2	1971	AH	I		+(+)	+(+)	(+)	+(+)	+
11	*P. citrophthora*	2	1919	AH	I	+	+	+		+	+
12	*P. elongata*	2	1989	N	N		(+)	+(+)	(+)	+(+)	
13	*P. frigida*	2	2015	NH	N		+(+)			(+)	
14	*P. meadii*	2	?	A	I	+					
15	*P. multivesiculata*	2	?	A	I		+				
16	*P. multivora*	2	1979	NH	I		+(+)	+(+)	(+)	+(+)	
17	*P. plurivora* complex	2	2009	NH	I		+(+)	(+)	(+)	(+)	
18	**P. AUS2A**	**2**		**N**	**N**	**(+)**	**(+)**	**(+)**	**(+)**	**(+)**	
21	*P. arenaria*	4	1986	N	N		(+)	(+)	(+)	+(+)	
22	*P. bisheria*	2	2008	A	I			+			
23	*P. boodjera*	4	2006	N	N		+(+)	(+)	(+)	+(+)	
24	***P. lichii***	**4**		**A**	**I**	**(+)**					
25	*P. palmivora*	4	1950	H	I	+	+(+)		(+)	+(+)	
26	*P. castaneae*	5	2000	NH	I	+(+)	(+)				
27	*P. heveae*	5	1975	H	I	(+)	+				
28	*P. amnicola*	6	2009	N	N		(+)	+(+)	(+)	+(+)	
29	*P. asparagi*	6	2007	A	I			+	(+)	+(+)	
47	*P. balyanboodja*	6	2014	N	N		(+)			+(+)	
30	*P. bilorbang*	6	2012	N	I		(+)	(+)	(+)	+(+)	
31	*P. chlamydospora*	6	2009	N	I	(+)	(+)	+	+	+(+)	
	P. condilina	6	2008	N	N					+	

	***Phytophthora* species**[1]	**Clade**[2]	**First record**[3]	**Habitat**[4]	**Status**[5]	**QLD**	**NSW**[6]	**VIC**[7]	**TAS**	**WA**[8]	**SA**[9]
	P. cooljarloo	6	1996	N	N					+	
32	*P. crassamura*	6	1992	N	I					+(+)	
33	*P. fluvialis*	6	1994	N	N			(+)	(+)	+(+)	
34	*P. gibbosa*	6	2009	N	N					+	
35	*P. gonapodyides*	6	2000	N	N	+	+(+)		(+)		
36	*P. gregata*	6	1965	NH	N		+(+)	+	(+)	+(+)	
37	*P. inundata*	6	1984	N	I	(+)	(+)	+(+)	(+)	+(+)	
	P. kwonganina	6	1993	N	N					+	
38	*P. litoralis*	6	2007	N	N	(+)	(+)	(+)	(+)	+(+)	
39	*P. megasperma*	6	1953	NH	I	+	+	+	+	+	+
40	*P. moyootj*	6	2006	N	N		(+)	(+)	(+)	+(+)	
	P. pseudorosacearum	6	1998	N	N					+	
41	***P. riparia***	**6**		**N**	**I**		**(+)**			**(+)**	
42	*P. rosacearum*	6	1993	NH	N?		(+)		(+)	+(+)	
43	*P. thermophila*	6	1980	N	N	(+)	(+)	+(+)	(+)	+(+)	
44	**P. AUS6A**	**6**		**N**	**N**		**(+)**			**(+)**	
45	**P. AUS6B**	**6**		**N**	**N**					**(+)**	
46	**P. AUS6C**	**6**		**N**	**N**				**(+)**	**(+)**	
48	**P. sp. forestsoil**	**6**		**N**	**I**			**(+)**		**(+)**	
49	**P. sp. hungarica**	**6**		**N**	**I**				**(+)**		
50	P. sp. personii	6	2005	NH	I		+	+	(+)	+	
51	P. sp. paludosa	6	2011	N	N			+			
52	P. sp. walnut	6	2015	NH	I		+(+)				
53	*P. cambivora*	7	1977	NH	I	+	+(+)	+(+)	(+)	+(+)	+
54	*P. cinnamomi*	7	1947	NH	I	+(+)	+(+)	+(+)	+(+)	+(+)	+
55	***P. europaea* complex**	**7**		**N**	**I**		**(+)**		**(+)**		
56	*P. fragariae*	7	1982	A	I		+	+			+
57	***P.* aff. melonis**	**7**		**A**	**I**					**(+)**	
58	*P. niederhauserii*	7	2002	NH	I		(+)	+(+)	(+)	+(+)	
59	*P. parvispora*	7	2000	NH	I	(+)	(+)	(+)		+(+)	
60	*P. rubi*	7	?	A	I		+	+			+
61	*P. sojae*	7	1980	A	I	+	+	+			
62	*P. vignae*	7	1960	A	I	+	+	+			
64	***P. brassicaceae***	**8**		**A**	**I**			**(+)**	**(+)**		
65	*P. cryptogea*	8	1942	NAH	N?	+	+(+)	+(+)	+(+)	+(+)	+
66	*P. drechsleri*	8	1967	NAH	I	+	+	+	+	+(+)	
67	*P. erythroseptica*	8	1968	A	I	+	+	+	+	+	+

	***Phytophthora* species**[1]	**Clade**[2]	**First record**[3]	**Habitat**[4]	**Status**[5]	**QLD**	**NSW**[6]	**VIC**[7]	**TAS**	**WA**[8]	**SA**[9]
68	*P. hibernalis*	8	1929	A	I		+			+	
69	***P. himalayensis***	**8**		**N**	**I**				**(+)**		
70	*P. medicaginis*	8	1971	A	I	+	+	+			
71	*P. porri*	8	1942	A	I		+	+		+	+
72	***P. primulae***	**8**		**A**	**I**				**(+)**		
73	*P. pseudocryptogea*	8	1981	NH	I		+(+)	+(+)	(+)	+(+)	
74	*P. syringae*	8	1979	N	I		+(+)	+(+)	(+)	+(+)	
75	***P. trifolii***	**8**		**A**	**I**			**(+)**			
76	P. sp kelmania	8	2010	A	I		+(+)			+(+)	
77	**P. AUS8A**	**8**		**N**	**N**				**(+)**	**(+)**	
	P. aquimorbida	9	2018							+	
78	*P. captiosa*	9	2015	N	N?					+(+)	
79	*P. constricta*	9	1981	N	N					+(+)	
80	*P. fallax*	9	2008	N	N		(+)	+(+)	+(+)	(+)	
81	*P. insolita*	9	2004	A	I					+	
82	*P. macrochlamydospora*	9	1984	NAH	N	+(+)	+(+)				
83	*P. richardiae*	9	1960	A	I		+				
84	P. sp. hennops	9	2012	N	N?			+			
85	**P. AUS9A**	**9**		**N**	**N**		**(+)**	**(+)**	**(+)**		
86	**P. AUS9B**	**9**		**N**	**N**			**(+)**	**(+)**		
87	**P. AUS9C**	**9**		**N**	**N**		**(+)**			**(+)**	
88	*P.* AUS9E	9	2012	N	N				(+)	+(+)	
89	*P. boehmeriae*	10	1962	AH	I	+	+	+		+	
90	P. *gondwanense*	10	2015	N	N		+				
91	**P. AUS10A**	**10**		**N**	**N**	**(+)**					
19	*P. versiformis*	11	2014	N	N		(+)	(+)	(+)	+(+)	
20	**P. AUS11A**	**11**		**N**	**N**			**(+)**		**(+)**	
	Total no. species[10]					29	60	52	49	61	14
	No. databases[11]					20	33	33	9	42	14
	No. HTS[12]					13	43	32	44	50	na
	No. Identical[13]					4	17	15	4	36	na

[1] *Phytophthora* species including several taxa with designated names that are not yet described. Potential new species as recognised by high throughput sequencing (HTS), are referred to as P. sp. nov followed by a code representing the clade number and a letter to distinguish new species in the same clade

[2] Phylogenetic Clade based on phylogeny of all known *Phytophthora* species (see Fig. 2)

[3] Date of the first record within Australian databases. ? denotes species reported within a database without a date attached to the record. Taxa without dates provided are those detected in the current HTS study.

[4] Known associations; A = annual crops H = perennial crops (including forestry), N = native ecosystems

[5] Known status of species I = introduced, N = native, N? = putatively native

[6] Additional species records for NSW were obtained from the CPSM, Royal Botanical Gardens and Scarlett *et al.* (2015). These identities have been confirmed by sequencing.

[7] Additional species for VIC recognised by Dunstan *et al.* (2016). These identities have been confirmed by sequencing.

[8] Additional species for WA come from VHS and CPSM collections. These identities have been confirmed by sequencing.

[9] No samples from South Australia were included in the metabarcoding study

[10] Total number of species/phylotypes from both databases and HTS

[11] Number of species known from Australian databases

[12] Number of phylotypes detected by HTS

[13] Number of species/phylotypes known both from databases and detection by HTS

Phytophthora cinnamomi

Phytophthora cinnamomi was first isolated from cinnamon (*Cinnamomum burmannii*) trees in Sumatra in 1922 (Rands 1922). *Phytophthora cinnamomi* has a worldwide distribution and has a host range approaching 5000 species (Cahill *et al.* 2008a, Jung *et al.* 2013). It has been detected in the 15 global biodiversity hotspots where 'exceptional concentration of endemic species are undergoing exceptional loss of habitat' (Myers *et al.* 2000). It is the only oomycete that is included in the top 100 of the world's worst invasive alien species as identified by the Invasive Species Specialist Group (Lowe *et al.* 2000). *Phytophthora cinnamomi* can outcompete other pathogens due to its short lifecycle, as it may take only eight hours to complete its asexual life cycle under optimal conditions, which is considered as one of the fastest developmental processes among any biological system (Walker & van West 2007, Crone 2012). *Phytophthora cinnamomi* infections can cause decline in species richness and abundance leading to faunal habitat degradation and increased incidences of soil erosion (Newhook & Podger 1972, Garkaklis *et al.* 2004).

The centre of origin of *P. cinnamomi* is still not clear, but theories on its centre of origin are based on two criteria: Firstly, genetic variability should be higher in the centre of origin than in areas where it has been introduced accidently (Vavilov's rule), and secondly it should co-evolve with the ecosystem without permanently altering it, implying that there should be tolerant and resistant vegetation in that ecosystem (Zentmyer 1980). However, there are some problems associated with this approach: it is difficult to obtain enough isolates to

determine genetic variability, resistant vegetation does not necessarily imply that a pathogen is indigenous to that area, and the flora might be susceptible but other interacting factors, such as suppressive soil might affect the actual disease potential of the pathogen (Zentmyer 1980). Different scientists have proposed different centres of origin for species. These have included Taiwan and islands of South-East Asia (Crandall & Gravatt 1967, Zentmyer 1988, Chang *et al.* 1996), and New Guinea/Celebes (Shepherd 1975). Arentz and Simpson (1986) and Arentz (2012) supported Shepherd's hypothesis but also stressed that this was only true for the A1 mating type and not the A2 mating type, which they concluded was introduced post-European colonization in the 19th century. A recent study hypothesized that the A1 mating type is Gondwanan in origin and it might have been present in New Guinea for about 10 million years. It is speculated that the A1 mating type has recently changed to the A2 due to genetic transformation (Arentz 2017).

Phytophthora cinnamomi in Australia

When Rands (1922) first described *P. cinnamomi* from Sumatra, this pathogen was already widespread in Australia, although it had not yet been isolated (Newhook & Podger 1972). Ashby first recorded *P. cinnamomi* causing disease in pineapple in Queensland in 1930 (Newhook & Podger 1972). First isolations from other parts of Australia were from New South Wales in 1948 (Fraser 1956); Western Australia in 1964 (Podger *et al.* 1965), Victoria in 1964 (Newhook & Podger 1972); Tasmania in 1965 (Newhook & Podger 1972); and South-Australia in 1969 (Davison 1970).

Phytophthora cinnamomi is widely distributed in Australia and is present in all states and territories causing disease in a wide range of hosts (Irwin *et al.* 1995). Extensive damage is caused in horticultural plants (Zentmyer & Thorn 1967), in exotic conifers (Oxenham & Winks 1963, Bertus 1968), in forests and heathlands of Western Australia (Hüberli *et al.* 2002a, Shearer *et al.* 2007), Victoria, Tasmania and New South Wales (Pratt *et al.* 1973). The pathogen causes serious losses in many species of Proteaceae and Leguminosae as well as *Rhododendron, Boronia, Eriostemon, Jacaranda, Eucalyptus, Thryptomene, Juglans, Olea, Passiflora, Prunus*, *Camellia, Erica, Calluna,* and many conifer species (Bertus 1968, Jenkins 1968, Dingley 1969, Newhook & Podger 1972). Of the 5710 described plant species in south-west Western Australia, 2284 are considered susceptible and 800 are highly susceptible to this pathogen (Shearer *et al.* 2004).

There are only three clonal lineages of *P. cinnamomi* in Australia, which implies infrequent sexual reproduction in *P. cinnamomi* (Dobrowolski *et al.* 2003). However, variation does occur in a single clonal lineage through mitotic recombination in the asexual phase (Hüberli *et al.* 2001, Dobrowolski *et al.* 2003). Therefore, *P. cinnamomi* has the capability to adapt to

new environmental conditions and cause disease in new hosts during its asexual phase (Hardham 2005). Detailed information about *P. cinnamomi* distribution, interactions with hosts, impacts on plant biodiversity in all Australian states, and horticultural impacts are adequately reviewed in (Newhook & Podger 1972, Cahill *et al.* 1993, Irwin *et al.* 1995, Hardham 2005, Cahill *et al.* 2008b, Hardham & Blackman 2018). Therefore, they will not be discussed here.

The biology and life cycle of Phytophthora cinnamomi

Just like other pathogens, disease development by *P. cinnamomi* requires the presence of the pathogen itself, a susceptible host, favourable environmental conditions and a sufficient period of time to allow disease development (Cahill *et al.* 2008b). Once the host is infected, *P. cinnamomi* produces mycelial strands that can grow within, or on the surface of host tissues (Fig. 1.1). These vegetative strands produce sporangia, which in turn release motile zoospores under warm, moist and aerobic conditions in stimulatory soil. Favourable soil temperature and pH for the production of sporangia is 25-30 °C and 5-6, respectively (Erwin & Ribeiro 1996). Even if conditions are conducive, certain stimulatory effects are required by *P. cinnamomi* to produce sporangia and release zoospores. These stimulatory effects are less understood but they are apparently influenced by soil type, soil micro-flora, and understory vegetation (Shearer & Tippett 1989). Sporangia release motile zoospores that can swim in free water towards hosts and cause infection.

Phytophthora cinnamomi also produces spherical, oblong or irregular shaped asexual spores called chlamydospores with a diameter ranging from 8 to 15 µm, which develop terminally or intercalary (Erwin & Ribeiro 1996). These are the hyphal protuberances filled with cytoplasm that are delimited from the hypha by septa and secondary thickening of the wall. They can be borne singly, in chains interspersed with undifferentiated hyphal swellings, or in grape-like clusters (Hemmes & Wong 1975). *Phytophthora cinnamomi* tends to produce chlamydospores when conditions are less favourable. They can survive in soil or plant material and germinate to produce mycelia, sporangia and then zoospores on the onset of favourable conditions (McCarren *et al.* 2005). More recently, *P. cinnamomi* has been shown to produce stromata, thick walled chlamydospores and selfed oospores within roots of tolerant and susceptible plant species (Crone *et al.* 2013b, Jung *et al.* 2013). *Phytophthora cinnamomi* is also believed to survive in lignitubers for extended periods (Jung *et al.* 2013). *Phytophthora cinnamomi* can be homothallic, heterothallic or neuter (Erwin & Ribeiro 1996). Homothallic species can produce oospores in a single culture without needing the opposite mating type, whereas heterothallic species require two compatible mating type A1 and A2 to produce oospores. In Australia, the A2 mating type is more frequently isolated than the A1

(Weste & Marks 1987). There are no direct reports of sexual reproduction in the field about this pathogen in Australia, the lack of presence of two mating types was assumed to be the reason for this (Cahill 1998). But now we know that that sexual reproduction does not occur even when two mating types are brought together in the same soil (Dobrowolski *et al.* 2002, Dobrowolski *et al.* 2003). It has also been found that *P. cinnamomi* is able to produce selfed oospores in the absence of opposite mating types (Jayasekera *et al.* 2007, Crone *et al.* 2013b, Jung *et al.* 2013). It has been also suggested that there is a recent change in mating type from A1 to A2 due to genetic transformation of A1 mating type (Arentz 2017).

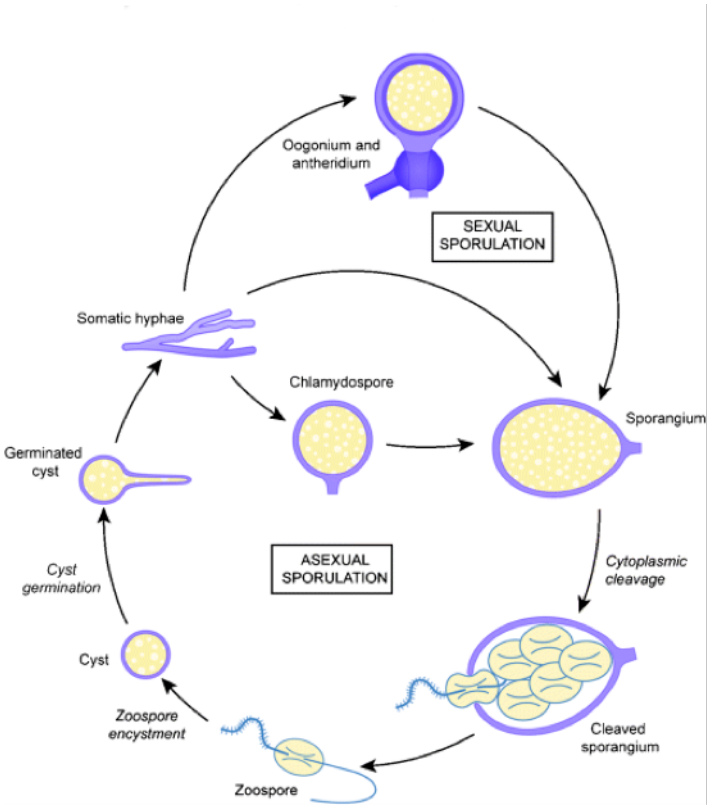

Figure 1.1 Diagram depicting life cycle of *Phytophthora cinnamomi* (Hardham 2005)

The biflagellate, motile and short-lived zoospores are the main infective propagules of the pathogen lifecycle that can move towards hosts in response to chemotactic stimuli for a short distance (Cahill *et al.* 1996). Zoospores encyst on or near the host roots and form a germtube that penetrates the outer epidermal or cortical tissues and rapidly colonise root and lower stem resulting in the necrosis and subsequent rotting of root and lower stem (Cahill *et al.* 2008b). Root to root contact may also facilitate the spread of disease between adjacent plants (Cahill *et al.* 2008b), whereas the movement of infested soil or plant materials are responsible for the spread of the disease across countries (McDougall *et al.* 2003). Oospores, mycelia, stromata, lignitubers, and hyphal aggregations formed within host tissues

that are in various stages of decomposition can also be likely sources of inoculum (Crone *et al.* 2013b, Jung *et al.* 2013).

Climate change and the distribution of pathogens

Climate affects the dynamics of host-pathogen interactions and thus climate change will have a strong effect on both spread (Pearson & Dawson 2003), and behaviour of plant species and pathogens (Sturrock *et al.* 2011). The classic disease triangle shows the interaction of plant host, pathogen and environment in causing disease (Agrios 2005). Over time, climate change can shift an environment from disease suppressive to disease conducive or vice versa (Fuhrer 2003, Perkins *et al.* 2011). Therefore, diseases are good indicators of climate change (Garrett *et al.* 2009). Atmospheric temperature has risen due to the release of latent heat from condensation of moisture, a result of evaporations from warm oceans surfaces (Graham 1995). Mountain temperatures have also risen resulting in the rapid melting of glaciers on high tropical mountains (Thompson *et al.* 1995) and upward shift in the freezing height (Diaz & Graham 1996).

Climate can affect pathogens either directly or indirectly. In the first case, pathogens can only cause diseases if their environmental requirements are met. For example, pathogens causing needle diseases are restricted by dry conditions. Therefore, their rates of reproduction, distribution and infection are greater in wet and moist conditions (Harvell *et al.* 2002). In the second case, pathogens indirectly affected by climate have the tendency to infect hosts that are already stressed by environmental factors. In this scenario, factors that stress their hosts are often critical to the successful invasion by pathogens of their hosts. For example, an extended summer drought will increase the probability of infection by pathogens whose ability to infect increases with host stress, such as root pathogens, wound colonizers and latent colonizers of sapwood (Brasier & Scott 1994, Lonsdale & Gibbs 1996, Desprez-Loustau *et al.* 2006). Similarly climate change can affect inoculum dispersal directly through changes to wind and turbulence, and indirectly through impacts on crop growth, phenology and agronomic practices (Margosian *et al.* 2009).

To date, many models have been developed to predict future distribution of perennial trees under changing climatic variables (Hamann & Wang 2006, Rehfeldt *et al.* 2006), but relatively a few have been developed to predict such distribution patterns for pathogens (Sturrock *et al.* 2011, Burgess *et al.* 2017a). The complex nature of interactions and feedback between climates, pathogens, and forest ecosystems make it nearly impossible to predict responses of specific pathogens to future climatic conditions at specific locations and times (Parker *et al.* 2000, Volney & Hirsch 2005). Similarly, it is also very difficult to illustrate the long term and progressive impacts of climate change on the dynamics of disease

epidemics (Jeger & Pautasso 2008). In order to accomplish this goal, long-term data must be available for both pathogens and the diseases they cause, which does not occur very often (Shaw *et al.* 2008).

Climate change and range expansion

Climate change not only results in the introduction of novel pathogens into an area (Stenlid *et al.* 2011), but also helps in further establishment and spread of existing pathogens (Hannukkala 2011, Burgess *et al.* 2017a). Climate can restrict the growth and development of pathogens at higher altitudes. For example, susceptible plants in the alpine areas of Australian Alps can be asymptomatic hosts to *Phytophthora* species but disease outbreaks can only occur when the temperature is warm enough for disease expression (Burgess *et al.* 2017b), which can have a huge impact on plant communities (Cahill *et al.* 2008a). Similarly, the range expansion and occurrence of plant communities at higher altitudes might also be restricted by plant pathogens (Brown & Vellend 2014). For example, *Phytophthora cinnamomi,* already present in the sub-alpine areas of Australia, might not only affect the existing vegetation but could also restrict the upward movement of exotic plants and other *Phytophthora* species from lower elevations (Burgess *et al.* 2017b).

Low temperatures have been reported to affect the survival of some *Phytophthora* species such as, *P*. x *alni, P. ramorum* and *P. cinnamomi* (Podger *et al.* 1990, Ireland *et al.* 2013, Aguayo *et al.* 2014). However, in the last 60 years, changes in climatic conditions, such as higher mean winter temperatures, seasonal precipitation shifts from summer into winter, and an inclination towards heavy rainfall are favouring disease development by several *Phytophthora* species, such as *P. cinnamomi* and *P. ramorum* (Sturrock *et al.* 2011). CLIMEX models predict more widespread *Phytophthora* root rot disease in the UK and much of the Coastal Europe and a marked increase of the disease in temperate zones of the northern and southern hemispheres globally (Brasier & Scott 1994, Brasier 1996, Burgess *et al.* 2017a). Models projecting the impacts of temperature on the geographic range of *P. cinnamomi* suggest the range expansion of this pathogen in warming climates (Brasier & Scott 1994, Bergot *et al.* 2004, Burgess *et al.* 2017a).

Many species living in the tropics have very narrow elevational ranges, and experience very little local environmental fluctuations (Janzen 1967, Ghalambor *et al.* 2006, Huey *et al.* 2009). As regional temperature changes, these species may not able to survive unless they move towards higher elevations (Forero- Medina *et al.* 2011). Species may also move to elevations that are higher than those occupied by other species in order to fill gaps in their current distribution (Forero- Medina *et al.* 2011). Climate change has resulted in the change in timings of life cycles during the year (Root *et al.* 2005, Menzel *et al.* 2006, Rosenzweig *et*

al. 2008, Thackeray *et al.* 2010), and range shifts towards higher latitudes and elevations of many species (Hickling *et al.* 2006, Thomas 2010). Using meta-analysis Chen *et al.* (2011) estimated that species have recently shifted to higher elevations at a median rate of 11.0 meters per decade, and to higher latitudes at a median rate of 16.9 kilometres per decade. It has also been revealed that elevational and latitudinal shifts were significantly greater in studies dealing with high levels of warming (Chen *et al.* 2011). In the case of Swiss needle cast disease caused by *Phaeocryptopus gaeumanii,* a 0.2-0.4 °C increase in winter temperature and 0.7 - 1.5 cm increase in spring precipitation per decade since 1970 have led to increased severity and distribution of the disease in the Oregon Coast Range; and this is likely to increase even more due to a projected 0.4 °C increase in temperature per decade through to 2050 (Stone *et al.* 2008).

A lag phase may occur in range shifts towards higher elevations if new suitable conditions may not be accessed easily. For example, suitable conditions maybe at some other mountain peaks, or may not be accessed easily due to topographic and microclimatic complexity of mountainous terrain, such as cooler locations on poleward facing slopes (Suggitt *et al.* 2011)]; or some other geological, climatic and ecological constraints (Pounds *et al.* 2006, Forero- Medina *et al.* 2011). It should be noted that individual species show variations in physiological responses to different climatic stresses. Therefore, these responses will combine with climatic extremes at different times of their life cycles for an unusual change to take place (Easterling *et al.* 2000).

Phenotypic plasticity and range expansion

Phenotypic plasticity is the capacity of organisms to express different phenotypes in response to changes in biotic or abiotic environments (Agrawal 2001). Evolutionary biologists have been working on the genetic basis of phenotypes, and earlier work was specifically focused on traits presumed to be unaffected by the environment because environmentally affected phenotypes were assumed to have no genetic basis (Agrawal 2001). But now it has been shown that phenotypic plasticity can be adaptive i.e. heritable (Dudley & Schmitt 1996, Kasuga *et al.* 2016). This is supported by Lamarck's First Law, which states that organisms go through an adaptive change in response to environment, and such changes can be inherited (Lamarck 1809). Phenotypic plasticity occurs frequently in nature in response to environmental heterogeneity, and is often adaptive (Holeski *et al.* 2012). It has been shown that phenotypes induced by such environments have higher relative fitness than the alternative phenotypes (Schmitt *et al.* 1995), and the alternative ("wrong") phenotype is not expressed in the changed/new environment (Dudley & Schmitt 1996).

Plasticity may lead to ecological success in novel habitats, and its ecological/evolutionary consequence depends on whether it evolves in response to certain environmental changes or as an overall strategy of organisms to change i.e. associative learning (Agrawal 2001). For most traits, the response is usually intermediate (Agrawal 2001). In any case, plasticity enhances ecological niche breadth because it allows organisms to express useful phenotypes in diverse environments (Bradshaw 1965). When a certain trait is canalised (a lack of plasticity), it is well adapted to a certain environment, but when a trait is plastic, it is well adapted to many environments (Pigliucci 2001). According to the theoretical models for the evolution of adaptive phenotypic plasticity, selection will favour adaptive plasticity for given variations when (i) populations are exposed to different environments, (ii) environments produce reliable cues, (iii) selection favours varying phenotypes in each environment, and (iv) no individual phenotype shows superior fitness across all environments. Plasticity is necessary for organisms to persist in novel environments, and once established, heritable differences accumulate by natural selection and the initially achieved phenotypes become genetic (Baldwin 1896).

The rate of adaptation to new environments is different, and it depends on the relative closeness of plastic phenotype to the desired trait in the novel environment (Price *et al.* 2003). Populations stop adaptive genetic differentiation when phenotypic plasticity matches with the optimum phenotype in the new environment (Price *et al.* 2003). Phenotypic plasticity is a characteristic of certain traits in certain environments; therefore, it is incorrect to think of an organism or genotype as a whole to be more or less plastic than others. A certain genotype maybe plastic for specific traits in a particular environment and non-plastic for other specific traits in the same environment (Bradshaw 1965, Pigliucci 2001).

An example of plasticity in *Phytophthora* is the production of autoinducer-2 by *P. nicotianae.* Autoinducer-2 facilitates interspecific communication among bacterial populations and its production had never been reported for *Phytophthora* species before. The frequent co-isolation of bacteria with *Phytophthora* species suggest possible inter-communication between these organisms (Kong *et al.* 2010). Another example of phenotypic plasticity is the variation in virulence among U.S isolates of *P. ramorum* on different hosts (Rizzo *et al.* 2005). Hüberli *et al.* (2001) examined variation among 73 *P. cinnamomi* isolates collected from *E. marginata* and *Corymbia calophylla* trees in the southwest of Western Australia and found a broad range of phenotypic and pathogenic differences, such as differential growth rate and colony morphology on potato dextrose agar at different temperatures, varied sporangial and gametangial morphology, and their ability to form lesions and cause death. Another example of plasticity in *P. cinnamomi* is the decreased sensitivity to the fungicide phosphite after its extended use in horticultural environments (Dobrowolski *et al.* 2008).

Range expansion of *Phytophthora,* in particular *Phytophthora cinnamomi* into colder environments: a changing environment, adaptation or both?

Temperature directly affects the growth rate of *P. cinnamomi* in host tissues, the optimal temperature for growth is 28 °C (Zentmyer 1980, Grant & Byrt 1984). It has been shown that *P. cinnamomi* becomes inactive when temperature drops below 10 °C in soil (Weste & Marks 1987), and no growth is shown on artificial media below 5 °C (McConnell & Balci 2015). Although many factors other than winter temperatures limit the canker development by *P. cinnamomi,* (for example, summer temperature, precipitations, and host susceptibility), they most likely act as secondarily modulating factors (Robin *et al.* 1992, Marçais *et al.* 1993). Delatour (1986) proposed the hypothesis that the range of ink disease in oaks in Europe caused by *P. cinnamomi* is seriously limited by lethal frosts, as the pathogen still had a restricted host range even after a century of its supposed introduction in France in 1860.

Sub-alpine regions were considered free of disease caused by *P. cinnamomi* due to presumed restrictions on its growth from lower temperatures (Podger *et al.* 1990). However, *P. cinnamomi* was isolated from soil beneath dying *Oxylobium arborescens* (Mills 1999, McDougall *et al.* 2003) at the elevation of 1560 m at Barrington Tops, NSW Australia where mean maximum and minimum temperatures are likely to be 16 °C and 3 °C, respectively (Zoete 2000). As a result of this finding, Burgess *et al.* (2017b) sampled two Mountain Invasion Research Network (MIREN) transects in Kosciuszko National Park (KNP), six peaks in Tasmania and Mt Toolbranup in WA (110 individual samples), as well as over 500 additional sites from across much of Australia. Many sites in NSW, VIC and Tasmania were above the tree line. Rather than using traditional baiting to isolate *Phytophthora*, DNA was extracted from these soils and subsequent amplicon pyrosequencing revealed 33 *Phytophthora* species (including *P. cinnamomi*) in Kosciuszko National Park (KNP) as high as 2100 m asl (i.e. almost at the highest point of mainland Australia) in asymptomatic vegetation. The recovery of *P. cambivora* from *Nematolepis ovatifolia* a shrub endemic to sub-alpine and alpine areas of Snowy Mountains, Australia in 2014/015 further strengthened these observations. The detection of such a diverse *Phytophthora* community, including *P. cinnamomi*, at high elevations above the tree line was unexpected and suggests that either climatic condition have changed or the pathogen has adapted to colder environments. A caveat to the finding that certain *Phytophthora* species were widespread across elevation and disturbance is HTS techniques can detect DNA of species even if they have no longer persisted (Carini *et al.* 2017).

Published distributions of *P. cinnamomi* have traditionally mapped observations of disease symptoms on susceptible plants. Also, vegetation surveys from the 1940s were conducted

under the assumption that the Snowy mountains were too cold for *P. cinnamomi* (Green 2016). However, the findings of Crone *et al.* (2013a) have shown that the pathogen infects and survives on some native plant species without observable disease symptoms. The pathogen has also been shown to produce selfed oospores along with other survival structures, such as thick walled chlamydospores, stromata and lignitubers (Crone *et al.* 2013b, Jung *et al.* 2013). Additionally, anthropogenic activities are known to have distributed this exotic pathogen widely since European colonization, for example through contaminated mud on vehicles and heavy machinery, road building and mining (Cahill *et al.* 2008a, Callaghan & Guest 2015). The extent of the true distribution of *P. cinnamomi* may be seriously underestimated, as distribution is not always associated with disease. *Phytophthora* spp. are elusive pathogens; traditional plant pathology techniques such as plating infected material onto agar to isolation are generally ineffective (Hüberli *et al.* 2000).

Range expansion of *P. cinnamomi* into cold environments is of great importance as this puts the pathogen into contact with new environments and hosts. Cold environment communities have very restricted climatic envelopes for their distribution, so that any stress, biotic or abiotic, can have devastating and irreversible consequences. If host susceptibility or pathogen virulence or aggressiveness is increased by climatic conditions, a disease outbreak or epidemic is likely to occur (Agrios 2005). Keeping in view the plastic nature of *Phytophthora* species and the possible climate change, it can be predicted that the pathogen is far more widely distributed than has been mapped based on symptoms, and is very likely to be present in the majority of sub-alpine areas.

We propose three overarching hypotheses to explain the occurrence of a *Phytophthora* community in colder environments (Fig. 2.1).

1. The environmental conditions in alpine and sub-alpine areas at higher elevations are no longer unfavourable for survival of *Phytophthora* species,
2. The *Phytophthora* community has adapted to alpine and sub-alpine areas at higher elevations and can now survive under colder conditions, and/or
3. a combination of both.

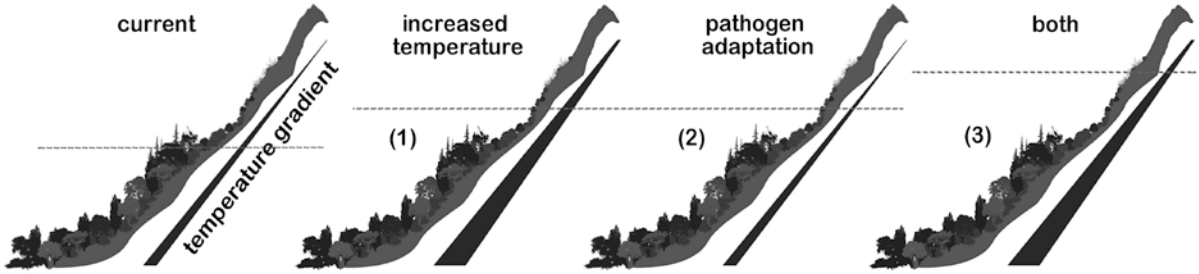

Figure 1.2 Three overarching hypotheses to explain *Phytophthora* occurrence into colder ecosystems.

Thesis aims

The aims of this thesis were to:

1. Determine the best substrate (soil, soil roots, filters or baited roots) and isolation technique most suitable for determining *Phytophthora* community, which could be later on used for *Phytophthora* surveys in mountains,
2. Survey alpine and sub-alpine areas to isolate/determine baseline *Phytophthora* species, and to confirm that species detected through high throughput sequencing previously were viable.
3. Examine the adaptive capability of *P. cinnamomi* and its ability to cause disease in alpine and sub-alpine regions, and
4. Update current understanding of *Phytophthora* diversity and distribution in mountain ecosystems, and to determine patterns of *Phytophthora* species richness in relation to elevation and disturbance and assess whether they are the same as for vascular plants.

Chapter 2: eDNA from roots: a robust tool for determining *Phytophthora* communities in natural ecosystems

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Khaliq I, Hardy GESJ, White D, Burgess TI (2018) eDNA from roots: a robust tool for determining *Phytophthora* communities in natural ecosystems. *FEMS Microbiology Ecology* 94: fiy048.

Khaliq conducted the survey, performed the lab work and was responsible for the writing and correspondence with the journal. White provided technical assistance, and Burgess and Hardy were supervisors.

Abstract

Proper isolation and identification of *Phytophthora* species is critical due to their broad distribution and huge impact on natural ecosystems throughout the world. In this study, five different sites were sampled and seven methods were compared to determine the *Phytophthora* community. Three traditional isolation methods were conducted (1) soil baiting, (2) filtering of the bait water, and (3) isolation from field roots using Granny Smith apples. These were compared to four sources of eDNA used for metabarcoding using *Phytophthora* specific primers on (1) sieved field soil, (2) roots from field, (3) filtered baiting water, and (4) roots from bait plants grown in the glasshouse in soil collected from these sites. Six *Phytophthora* species each were recovered by soil baiting using bait leaves and from the filtered bait water. No *Phytophthora* species were recovered from Granny Smith apples. eDNA extracted from field roots detected the highest number of *Phytophthora* species (25). These were followed by direct DNA isolation from filters (24), isolation from roots from bait plants grown in the glasshouse (19), and DNA extraction from field soil (13). Therefore, roots were determined to be the best substrate for detecting *Phytophthora* communities using eDNA.

Introduction

Phytophthora diseases cause significant losses to plants in agriculture, horticulture and natural ecosystems throughout the world. Many species, including *Phytophthora cinnamomi* one of the world's most devastating invasive species, are also known for their huge impact on natural ecosystems. *Phytophthora cinnamomi* has a massive impact on the natural ecosystems in Australia, the Cape Province of South Africa, and the Iberian Peninsula in Europe (Burgess *et al.* 2017a) . Interest in *Phytophthora* diseases of natural ecosystems has increased since the emergence of *P. ramorum* as the causal agent of sudden oak death in California (Hansen *et al.* 2012). About 50 new species have been described since 2010; most of these species have been isolated from natural ecosystems, and very little is known about their distribution and impact on natural ecosystems (Burgess *et al.* 2017b). Proper detection and identification of *Phytophthora* species is of great importance for biosecurity and quarantine. The number of *Phytophthora* species has risen to over 150 and this number will likely increase due to extensive surveys of previously unexplored forest and river ecosystems.

Non-selective media used for isolating true fungi are not suitable for *Phytophthora* species due to antagonism and rapid growth of secondary microorganisms, and slow revival of dormant *Phytophthora* survival structures, such as oospores and chlamydospores (Tsao 1990a). The genus *Phytophthora* is difficult to isolate from infected plants and soil, but the efficiency of isolation has greatly increased by the use of baiting techniques and selective isolation media (Tsao 1990a). Eckert and Tsao (1962b) reported the first medium 3P (pimaricin-penicillin-polymyxin) for selective isolation of *Phytophthora.* Different media have been used for selective isolation of *Phytophthora* since then (see supplementary Table 2.S1), most recently used include NARH (Simamora *et al.* 2017), *Phytophthora* selective medium (PSM) of Burgess *et al.* (2008), and CMA-PARPBH (Pérez-Sierra *et al.* 2010).

Phytophthora species are disseminated through soil, water and aerially (Erwin & Ribeiro 1996), and can be isolated directly from plant tissues, such as leaves, roots, stem and twigs without any surface sterilization when infection is active by plating sections of an advancing lesion margin onto a selective medium (Streito *et al.* 2002). However, the presence of *Phytophthora* is not always associated with visible symptoms and it can be recovered from symptomless plant tissues (Hüberli *et al.* 2000). The rate of success of isolation also depends on the pathogen's activity, which varies between seasons. Autumn was found to be the best season for isolation of alder *Phytophthora* from plant tissues (Streito *et al.* 2002). Similarly, saprophytic microorganisms and antagonistic bacteria affect the efficiency of isolation (Hüberli *et al.* 2000, Streito *et al.* 2002, Jung & Blaschke 2004). Therefore, surface

sterilization with 70% ethanol followed by drying on paper towel prior to plating can increase chances of isolation (Martin *et al.* 2012). Finally, washing plant tissues to leach out phenolic compounds from plants like *Eucalyptus* and *Alnus* species prior to plating can increase isolation of *Phytophthora* species (Hüberli *et al.* 2000, Streito *et al.* 2002).

Various traditional methods have been reported for the isolation of *Phytophthora* species from soils. Direct plating of soil onto selective media is not suitable for recovery of *Phytophthora* species, as there are few viable propagules per gram of soil and lots of contaminants (Hendrix & Kuhlman 1965). Soil baiting is more effective for isolation of *Phytophthora* species for a number of reasons. First, a large amount of soil can be tested, which increases chances of isolation when species are present at a low population density (Martin *et al.* 2012). Secondly, it is more effective for isolation of homothallic species, which often survive as dormant oospores (Jeffers & Aldwinckle 1987). Species are more frequently isolated when soil is kept between 15 and 20 ^{o}C and the bait is not wounded, which discourages *Pythium* and bacterial colonization (Hwang *et al.* 2008, Ghimire *et al.* 2009). Leaf tissues are more commonly used as baits than fruits, but all leaf tissues are not equally attractive to multiple *Phytophthora* species. Young and succulent leaves of *Camellia*, *Rhododendron*, and *Quercus* spp., and *Pimelea ferruginea* and *Eucalyptus sieberi* cotyledons have been successfully used in recent years (Jung *et al.* 2000, McDougall *et al.* 2002, Fichtner *et al.* 2007, Hwang *et al.* 2008).

Currently, baits and filter-based approaches are mainly used to isolate *Phytophthora* species from water. A variety of plant baits (Klotz *et al.* 1959, McIntosh 1966, Erwin & Ribeiro 1996, Oudemans 1999, Hüberli *et al.* 2013, Dunstan *et al.* 2016) as well as filter membranes and filtering methods (MacDonald *et al.* 1994, Von Broembsen & Wilson 1998, Hong *et al.* 2002) have been used. However, not all membranes are equally efficient for recovery of diverse *Phytophthora* species. Hong *et al.* (2002) compared nine hydrophilic membranes for isolation of Pythiaceous species in water, and found Durapore5 and Millipore5 to be more efficient than other membranes. Filtration has been found to be more efficient for isolation of *Phytophthora* in water than baiting (Hwang *et al.* 2008).

Fruit baits are also used for isolation of *Phytophthora.* These have included apple (Campbell 1949, Newhook 1959), lemon (Klotz & DeWolfe 1958), avocado (Zentmyer *et al.* 1960), tomato (Reis *et al.* 2003), and pear and cucurbits (Gevens *et al.* 2007). Apples are usually not very effective for isolation of *Phytophthora* species from soil because saprophytic fungi, such as *Mucor*, *Rhizopus* and *Penicillium* species produce rapid soft rots, which inhibit the growth of *Phytophthora* (Chee & Newhook 1965b). Also, *Trichoderma viride* is commonly present in soil and causes a hard rot of apple. Although both may cause distinct isolatable rots in a single apple, it is more likely that *T. viride* will suppress growth of *Phytophthora*

(Chee & Newhook 1965b). Furthermore, Jeffers and Aldwinckle (1987) compared different baits, such as apple, pear, apple seedlings, cotyledons, and seedling leaf pieces, and found that apple and pear were not suitable for isolation of *P. cactorum* from naturally infested soil. However, pears have been reported as useful baits for isolation of *P. cinnamomi* from naturally infested soil (Greenhalgh 1978).

Although these conventional methods are useful for the isolation of many *Phytophthora* species (Erwin & Ribeiro 1996, Drenth *et al.* 2006), it can be a laborious and difficult task. Additionally, it can be difficult to identify species based on morphology, especially now that so many new species have been described in the past 10 years. Some species, such as *P. mirabilils*, *P*. *infestans*, and *P. ipomoeae* have similar sporangia (semi-papillate and caducous) and oospore characteristics, and therefore, cannot be distinguished by morphology alone (Erwin & Ribeiro 1996, Flier *et al.* 2002). Moreover, morphology of *Phytophthora* is plastic (Braiser 1992), and not all *Phytophthora* species can be cultured on agar media (Mircetich 1970). DNA based identification is a fast and reliable method for the identification of *Phytophthora* species (Martin *et al.* 2014) and has been used in numerous studies (Ristaino *et al.* 1998, Burgess *et al.* 2009, Oh *et al.* 2013, Català *et al.* 2015).

The term environmental DNA (eDNA) refers to DNA isolated from environmental samples (e.g. air, soil and water) without first isolating any desired organism (Taberlet *et al.* 2012). This term was first used by Ogram *et al.* (1987) while isolating microbial DNA from a range of sediment types. It is composed of intracellular DNA from living cells and extracellular DNA from naturally lysed cells. High Throughput Sequencing (HTS) has made it possible to characterize microbial and fungal communities in eDNA without time consuming and expensive cloning (Sogin *et al.* 2006, Coince *et al.* 2013). Environmental substrates are usually easy to sample and can be collected by non-specialists (Lear *et al.* 2018). HTS is an effective tool for epidemiological studies when description of new or rare taxa is required (Vannini *et al.* 2013). Previous studies have mainly concentrated on detection of all organisms that can be present in environmental samples by targeting barcoding genes, such as ITS and 18S (Nakayama *et al.* 2013, Weber *et al.* 2013), but only a few have focused on targeting specific organisms (Bergmark *et al.* 2012, Li *et al.* 2013).

Although the metabarcoding approach has greatly improved the detection of *Phytophthora* species in environmental samples (Vannini *et al.* 2013, Català *et al.* 2015, Prigigallo *et al.* 2015, Català *et al.* 2016), none of the studies have been specifically targeted to determine the best substrate for isolation and detection of *Phytophthora* species. The current study compared traditional isolation methods to metabarcoding using *Phytophthora* specific primers on samples taken in natural environments.

Materials and Methods

Sampling sites and sampling procedure

Rhizosphere soil including roots was collected from five different urban parks in Perth; Bold Park, Kings Park, Attadale Foreshore, Manning Park, and Bibra Lake in the late (June) autumn (Table 2.1). At each site, a bulked soil sample was collected (3 kg comprised of 10 x 300g sampled from ten different locations to a depth of 10-20 cm). Emphasis was placed on the collection of rhizosphere soil containing fine roots. The soil samples were placed into plastic bags and kept in an insulated box to protect samples from high temperature and direct sunlight and carried to the laboratory.

In the laboratory, the samples were mixed thoroughly. Some fine roots were removed from the soil, rinsed with tap water to remove soil particles and chopped into 1-2 mm segments. Chopped roots (approximately 1000mg) were placed into three Eppendorf tubes and frozen at -20 ^{o}C until used for DNA extraction while others were used in apple baits. A sub-sample of soil (200g) was air dried for DNA extraction from soil, and three sub-samples of soil (each approx. 400g) were taken for traditional baiting. Finally, four subsamples of soil (approx. 1500g) were used for growing *Eucalyptus sieberi* and *Banksia attenuata* seedlings in the glasshouse as susceptible living ‘baits’ for *Phytophthora.*

Table 2.1 Site location and host plants from which *Phytophthora* isolates were obtained

Location	Site key	GPS location	Vegetation type
Bold Park	1	31°56'47.68"S 115°46'41.96"E	Mixed *Eucalyptus gomphocephala* and *Banksia* woodlands, with mixed *Acacia rostellifera* and *Melaleuca acerosa* understory
Kings Park	2	31°57'50.08"S 115°49'19.60"E	Mixed *Eucalyptus, Banksia* and *Xanthorrhoea preissii* woodlands
Attadale foreshore	3	32° 1'2.05"S 115°47'52.85"E	Mixed *Eucalyptus marginata* and *Corymbia calophylla* woodlands, with *Banksia, Melaleuca*, and *Agonis flexuosa* midstory, *and Sporobolus virginicus* open grassland
Manning Park	4	32° 5'31.76"S 115°45'58.49"E	Mixed *Eucalyptus gomphocephala* and *E. decipiens* woodland, with *Acacia*, *Melaleuca hugelii* and *M. acerosa* understory
Bibra Lake	5	32° 5'41.96"S 115°49'14.33"E	Mixed *Eucalyptus* woodland with *Banksia attenuata, B. menziesii* midstory and *Melaleuca teretifolia* and/or *Astartea* aff. *fascicularis* understory

Traditional isolation from soil using bait leaves

Soils from each sample were placed in 1.5 L rectangular polypropylene containers (167 mm × 108 mm). Each soil sample was replicated three times. Combined, the roots and soil occupied one-third of the container. The soil/root mix was then pre-moistened with distilled water overnight to stimulate pathogen activity. The next morning, the samples were flooded with distilled water in a 1:3 soil/water ratio and young leaves of *Quercus ilex*, *Q. suber*, *Pimelea ferruginea*, *Poplar* sp., *Scholtzia involucrata*, and *Hedera helix* were floated on the surface [Fig. 2.1a, c]. The containers were incubated at 20°C (±5°C) under ambient conditions.

The baited leaves were observed for the appearance of lesions every 1-2 days for seven days. Leaves with brownish lesions were blotted dry on paper toweling, the lesions were cut into 2 × 2 mm pieces, and plated onto NARH (Simamora *et al.* 2017). The plates were incubated at 20°C (±5°C) in the dark and examined under 10X magnification for the presence of hyphae typical for *Phytophthora*. After 1-2 days, any *Phytophthora*-like cultures

were transferred onto fresh plates of NARH twice and finally transferred onto individual vegetable juice agar (V8A) plates [100 ml/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery products Ltd., Norfolk, UK), 900 ml/L distilled water, 0.1 g/L $CaCO_{3,}$ pH adjusted to 7 and 17 g Grade A Agar (Becton, Dickenson and Company, Sparks, MD, USA]. After seven days, the soil was allowed to air dry and then baiting was repeated (double baiting) to increase isolation (Jeffers & Aldwinckle 1987, Davison & Tay 2005).

Isolation from filtered bait water

Filtration was performed with a filtering funnel (Nihon Millipore K.K, Tokyo-Japan) and a glass flask connected to a vacuum pump [Fig. 2.1d]. Between samples, plastic containers (funnel, porous plate, rubber bung) and a glass flask were placed in a detergent Pyroneg (L88Z, Diversey) and then washed in a separate container containing water, sterilised with 4% sodium hypochlorite for a minute, and then finally placed in a separate container containing water for approximately five minutes before handling another bait sample.

Approximately, 250 mL of bait water was filtered from each 'bait tray' each time after finishing baiting (on day 7) through a 47 mm circular filter paper with 5 µm pore size UltraSep™ Polyetherasulfone (GVS Life Sciences, Sanford-USA). Three filters were collected for each sample. Finally, tap water was passed through a fresh filter as a filter control. Each filter was cut into two halves. Half of each filter was placed topside down onto the surface of NARH medium. After 12 hours, the filter was removed and colonies were transferred to fresh NARH plates (5-6 sub-cultures per plate). *Phytophthora* like cultures were transferred onto V8A plates after 2-3 days. Soil used in each 'bait tray' was allowed to air dry and the same procedure was repeated for filtered bait water from the second round of baiting (double baiting).

Isolation using Granny Smith apple baits

Granny Smith apples were used as baits for roots from the field sites. Briefly, two holes (about 10 mm width) were made with a sterile scalpel on opposite sides of each apple; the column was taken out and chopped fine roots were placed into each hole and blocked with the removed apple column and sealed with glad-wrap (plastic film) [Fig. 2.1e]. Each apple was kept for 5-7 days in a separate zip-lock bag (Sandvik, Australia) at 20 ± 5 °C and brown discoloured lesions around holes were plated onto NARH.

Bait plants in glasshouse

Seed of *B. attenuata* was grown in sterilized sand for two weeks and transplanted to free-draining pots (18 x 6.5 cm L x W) containing soil collected from each site and placed into an evaporatively cooled glasshouse. *E. sieberi* seed was then directly germinated in the same pots and watered daily (Fig. 2.1f). There were four replicate pots for each site. Seedlings showing disease symptoms while growing were cut into 1-2 cm segments and plated onto NARH. After 2-3 days, any *Phytophthora*-like cultures were transferred onto individual V8A plates. After nine weeks, the *E. sieberi* and *B. attenuata* seedlings were harvested by severing shoots from roots. Roots were carefully washed over a 1-mm sieve immediately after harvesting to remove soil particles and stored in collection tubes in triplicates at -20 ^{o}C for DNA extraction.

Morphological and molecular identification of isolates

Living isolates were maintained on V8A. Isolates were divided into morphotypes based on their gross colony morphology and hyphal characteristics examined at 10x magnification under a light microscope. Finally, two to three isolates from each morphotype were selected for sequence-based identification using the ITS gene region. ITS sequence data were obtained for all isolates, and their identity was confirmed by conducting BLAST search in GenBank (www.ncbi.nlm.nih.gov/genbank/).

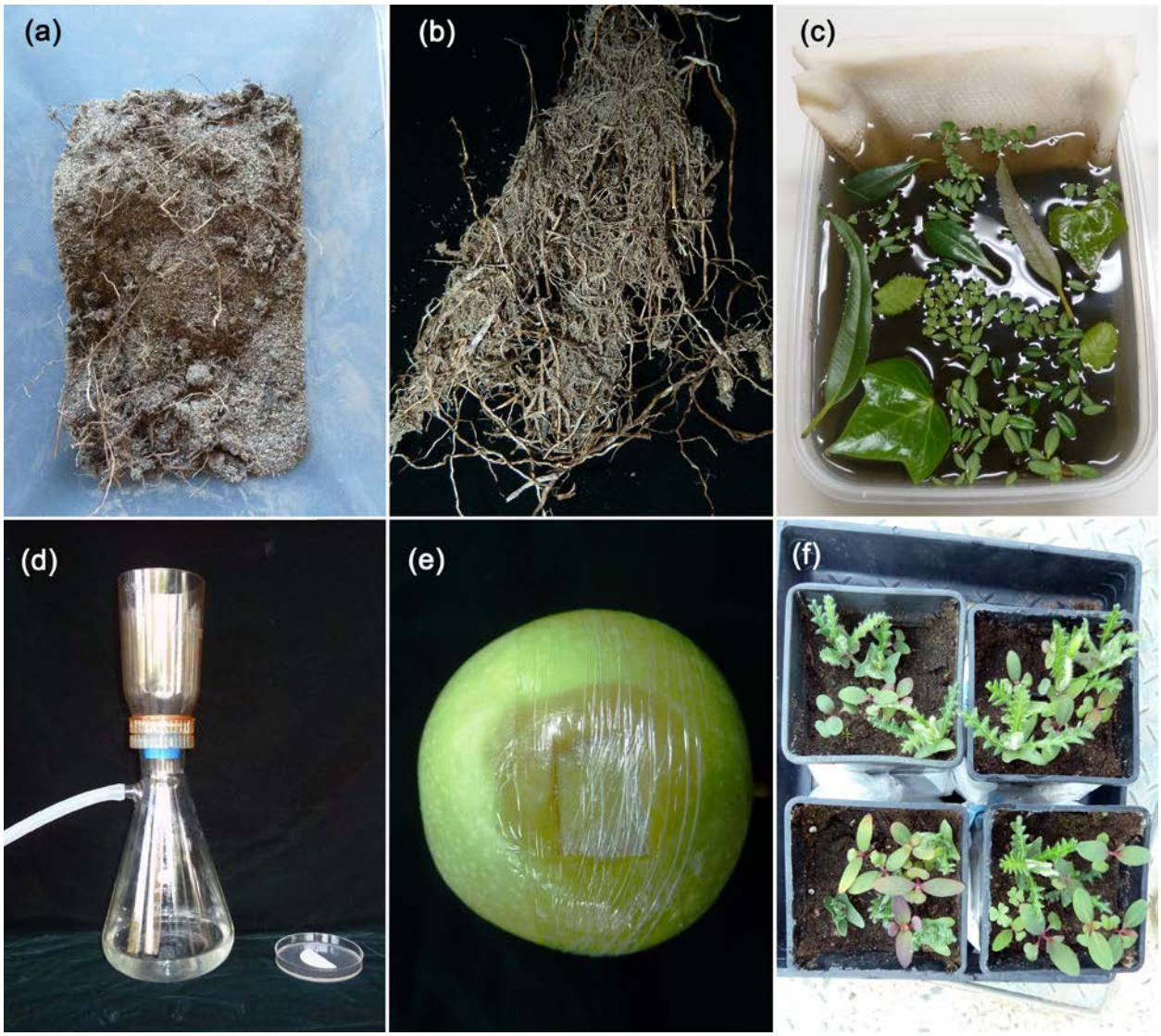

Figure. 2.1 Techniques for isolating *Phytophthora* from soil and root samples; (a) a typical soil sample in a baiting tray; (b) fine roots collected from soil for eDNA extraction and placement into Granny Smith apples; (c) traditional baiting assay from a soil and root samples; (d) filtration apparatus for filtering bait water; (e) isolation from field roots using Granny Smith apples; and (f) isolation from field soil using bait plants (*Banksia attenuata* and *Eucalyptus sieberi)* grown in the glasshouse.

DNA extraction

DNA was extracted in triplicate from (i) fine roots collected from field soil samples using the PowerPlant® DNA isolation kit following the manufacturer's instructions; (ii) the air-dried soil sample was sieved and 100g of this soil was crushed to a fine powder using the TissueLyser LT (Qiagen, Haan, Germany) and DNA was extracted using the Mo Bio PowerSoil® DNA isolation kit following the manufacturer's instructions except for the first step the buffer from the kit was replaced with 1 ml of saturated phosphate buffer (Na2PO 4; 0.12 M; pH 8) to the samples (500 mg) to maximize extracellular DNA isolation (Taberlet *et al.* 2012); (iii) the remaining two halves of filters from the first and second round of filtered bait water using

PowerSoil® DNA isolation kit (filter halves obtained from the first and second round were bulked to reduce the cost), and (iv) fine roots recovered from glasshouse bait plants using the PowerPlant® DNA isolation kit. Extreme care was taken to avoid any possible contamination during extraction and extraction controls were also included.

Amplicon pyrosequencing and clustering

Amplicon libraries for ITS gene region were created using the *Phytophthora*-specific primers (Scibetta *et al.* 2012) and Promega GoTaq Host Start Polymerase using a nested PCR approach as optimised by (Burgess *et al.* 2017b). Negative controls were included each time a PCR reaction was setup, and carried forward to the second round in the same manner as for the samples. PCR products were cleaned twice with AMPure XP Beads (Beckman Coulter Genomics) following the Short Fragment removal protocol according to manufacturer's instructions. After purification, the PCR products were visualized on agarose gels and then pooled (based on the band intensity) to standardise each sample's DNA contribution to pooled samples. The final pooling was diluted to 1/5000 of the original concentration, and 50 µl of the dilution was again cleaned with AMPure XP Beads. DNA was quantified as described previously (Burgess *et al.* 2017b). The emulsion PCR reactions were carried out according to the Roche GS Junior emPCR Amplification Method Manual Lib-L (March 2012). The libraries were sequenced using Junior Genome Sequencer plates (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA). Bioinformatics was conducted in GENEIOUS version R9 (http://www.geneious.com/). Reads were then clustered into molecular operational taxonomic units (MOTU) based on 99% sequence similarity, which allows identification of closely related species. Identities were assigned to MOTUs after phylogenetic analysis against a dataset containing verified sequences of all known *Phytophthora* species. These identities are considered phylotypes acknowledging that this is based on sequence data rather than a living isolate. Chimeras were discarded after making alignments of consensus MOTUs for each barcode. Identities were assigned to phylotypes as described by (Burgess *et al.* 2017b).

Data processing and statistical analysis

One-way Analysis of Variance (ANOVA) test was used to determine the differences in the number of unique *Phytophthora* species isolated and detected by the different techniques. Additionally, a Welch's T-test (Welch 1951) was used to compare the number of unique *Phytophthora* species recovered by traditional isolations and metabarcoding. Assumptions of normality were assessed using the Shapiro-Wilk test and observation frequency histograms. Both the Levene's and Bartlett's tests of homogeneous variance were undertaken before the

analysis. Three of the six detection methods were not normally distributed. An ANOVA was performed as distribution were not similar and the test was robust enough to handle violations of normality assumption (Schmider *et al.* 2010). After performing the ANOVA, a Tukey HSD *post hoc* test was conducted when the predictive variable was significant. All analyses were performed in R (R Core Team 2015)(Anonymous) using the "stats", "graphics", and "car" (John & Sanford 2011) packages. Diversity indices were calculated for metabarcoding techniques using the R package "vegan" (Oksanen *et al.* 2017).

It should be noted that the Granny Smith apple technique (in which no *Phytophthora* was recovered), and failed PCR runs from two sites (2 and 3) in case of field roots eDNA were excluded from the analysis because no amplifications were achieved for these sites.

Results

Traditional isolation from soil using bait leaves

All baits developed brownish necrotic lesions within 2-4 days. However, *Phytophthora* species could only be recovered from sites 3, 4 and 5. These included *P. thermophila*, *P. rosacearum*, *P.* 'oreophila'*, P*. *amnicola*, *P*. *multivora* and *P. inundata* (Table 2.1, supplementary Table S2.2). *Pythium* species were also readily isolated from all sites.

Isolation from filtered bait water

No *Phytophthora* species were recovered from sites 1, 2 and 4. Species recovered from the other two sites included *P. amnicola, P. thermophila*, *P. multivora, P. rosacearum, P.* 'oreophila', and *P. gregata* (Table 2.1, supplementary Table S2.2).

Isolation using Granny Smith apple baits

No *Phytophthora* species were recovered by using this technique. However, a few unidentified *Pythium* (data not shown) isolates were recovered.

Phytophthora species detected from eDNA

All the soil, filters and glasshouse root samples, and nine of 15 extractions from field roots from five sites yielded PCR products. Across all runs, a total of 81 324 quality reads were produced from samples that yielded PCR products. Across all sites, 30 phylotypes corresponding to 25 known species, three designated but undescribed species and two potentially new species were obtained. Some closely related species relevant to this study cannot be separated based solely on ITS1 (Fig. 2.S1): (i) *P. citrophthora* and *P. terminalis*,

(ii) *P. capsici* and *P. glovera,* (iii) *P. arenaria, P. boodjera* and *P. alticola,* and (iv) *P. versiformis, P. quercina and P. castenatorum*. With the exception of *P. arenaria* and *P. boodjera* which are both found in Australia, for the other groups only the first named species in known in Australia.

There were 25 phylotypes from field roots, 24 from filters, 19 from glasshouse bait roots, and 12 from soil (Table 2.2, supplementary Table S2.4). The three most abundant phylotypes were *P. multivora* (66.73 %), followed by *P. pseudocryptogea* (12.69 %) and *P. amnicola* (3.38 %) (see supplementary Table S2.3). Diversity indices were calculated to determine species richness and diversity. According to all the alpha diversity indices (α, $\alpha_{SI,}$ α_S) (Simpson 1949), higher diversity in detections was displayed in the field roots eDNA having had the most diverse *Phytophthora* community than other substrates (Table 2.3). According to multiplicative beta diversity ($\beta_{\gamma/\alpha}$), higher novelty in detection was displayed in filters' eDNA than any other substrate. Finally, Bray-Curtis dissimilarity index (Bray & Curtis 1957) showed that *Phytophthora* species detected in field roots eDNA in different sites were more dissimilar than other substrates (Table 2.3).

Soil: Thirteen phylotypes were detected by metabarcoding; *Phytophthora* AUS 1D, *P. multivora, P. amnicola, P. inundata, P. versiformis, P. constricta, P. rosacearum, P.* 'oreophila', *P. kwongonina*, *P. thermophila, P. cinnamomi, P. melonis,* and *P. pseudocryptogea* (Table 2.2). Species distribution varied across sites with *P. multivora* and *P. pseudocryptogea* were detected in all sites, *P*. AUS 1D was detected in four sites, and *P. thermophil*a were detected in three sites, *P. versiformis*, *P. amnicola, P.* 'oreophila', *P. rosacearum,* and *P. inundata* were detected in two sites, and *P. kwongonina, P. cinnamomi*, *P. melonis* and *P. constricta* were detected at only one site (Table S2.4).

Field roots: A total of 25 phylotypes were detected in field roots by metabarcoding: *P. nicotianae*, *P*. AUS 1D, *P. capensis, P. elongata, P. multivora*, *P. pachypleura, P*. 'acacia', *P. citrophthora*, *P*. AUS 2C, *P. amnicola, P. fluvialis, P. gregata, P. inundata, P. litoralis, P. moyootj, P. rosacearum, P.* 'oreophila'*, P. kwongonina, P. thermophila, P. pseudocryptogea, P. versiformis, P. arenaria, P. cinnamomi, P. '*kelmania' and *P. constricta* (Table 2.2). Species distribution was uneven across sites; *P. nicotianeae, P*. AUS 1D, *P. multivora, P. amnicola, P.* 'oreophila'*, P. thermophila, P. cinnamomi, P. pseudocryptogea,* and *P. constricta* were detected in three sites, *P. citrophthora, P. inundata, P. rosacearum,* and *P. kwongonina* were detected in two sites, and *P. capensis, P. elongata, P. pachypleura, P.* 'acacia'*, P*. AUS 2C*, P. arenaria, P. fluvialis, P. gregata, P. litoralis, P. moyootj, P.* 'kelmania'*,* and *P. versiformis* were detected at only one site (Table S2.4).

Filtered bait water: A total of 24 phylotypes were detected by metabarcoding: *P. nicotianae, P.* AUS 1D, *P. capensis, P. capsici, P. elongata, P. frigida, P. multivora, P. pachypleura, P. citrophthora, P. arenaria, P. palmivora, P. amnicola, P. gregata, P. inundata, P. moyootj, P.* 'oreophila'*, P. rosacearum, P. kwongonina, P. thermophila, P. cambivora, P. cinnamomi, P. pseudocryptogea, P. constricta* and *P. versiformis* (Table 2.2). The occurrence of phylotypes also varied across sites; *P. multivora, P. amnicola, P.* 'oreophila'*, P. kwongonina, P. thermophila* and *P. pseudocryptogea* were detected in five sites; *P. inundata* was detected in four sites; *P. nicotianae* was detected in three sites; *P.* AUS 1D, *P. cinnamomi,* and *P. constricta* was detected in two sites, and *P. capensis, P. capsici, P. elongata, P. frigida, P. pachypleura, P. citrophthora, P. arenaria, P. palmivora, P. gregata, P. moyootj, P. rosacearum, P. cambivora* and *P. versiformis* were detected in only one site (Table S2.4).

***Glasshouse bait roots*:** Nineteen phylotypes were detected by metabarcoding; *P. nicotianae*, *P*. AUS 1D, *P. capensis, P. multivora*, *P. capsici*, *P.* 'acacia'*, P. citrophthora, P. versiformis, P. arenaria, P. amnicola, P. inundata, P. rosacearum, P. kwongonina*, *P. thermophila, P.* 'oreophila', *P. cinnamomi, P*. *pseudocryptogea*, *P. frigida* and *P. constricta* (Table 2.2). Considerable differences were observed in species distribution across sites. *P. nicotianae, P. multivora, P. amnicola, P. '*oreophila'*, P. thermophila, P. cinnamomi* and *P. pseudocryptogea* were detected in all five sites; *P. inundata* was detected in four sites; *P. capensis, P. citrophthora, P. arenaria, P. kwongonina,* and *P. constricta* were detected in three sites; *P*. AUS 1D and *P. rosacearum* were detected in two sites, and *P. capsici, P. frigida, P.* 'acacia' and *P. versiformis* were detected in only one site (Table S2.4).

Comparison of traditional isolations to metabarcoding

Significant differences were observed between traditional isolations and metabarcoding techniques tested for the isolation and detection of *Phytophthora* t(23.0.16) = 6. 827, p = 0.000). Significantly more *Phytophthora* were detected by molecular techniques (10. 88 average) compared with traditional (1.4 average). Differences among the analysed substrates used for metabarcoding studies were also significant [$F(5,22) = 10.34$, $p = 0.000$). Of the 30 *Phytophthora* phylotypes detected in this study, all were recovered in eDNA from a variety of sources and only seven of these were recovered by traditional methods (traditional isolation using bait leaves and filtered bait water). The highest number of *Phytophthora* species was detected in field soil roots eDNA (25); the lowest numbers were detected with traditional isolation using bait leaves and filtered bait water (6 *Phytophthora* species each), while no *Phytophthora* species were detected in Granny Smith apple baits (Table 2.2).

Certain *Phytophthora* species were detected by all techniques tested in this study except Granny Smith apples. For example, *P. multivora, P. amnicola, P. inundata, P.* 'oreophila'*, P. rosacearum,* and *P. thermophila.* However, *P.* AUS 1D, *P. kwongonina, P. cinnamomi*, *P. pseudocryptogea, P. constricta,* and *P. versiformis* were detected from all sources of eDNA (soil, filters, roots from field, and bait plants in the glasshouse), but these were not recovered by traditional isolations (baiting, and filtered bait water and Granny Smith apple baits). Certain *Phytophthora* species were only detected with one technique (Table 2.2). For example, *P. melonis* was detected from field soil eDNA; *P. palmivora* and *P. cambivora* were detected from filters' eDNA, and *P.* AUS 2C, *P. fluvialis, P. litoralis,* and *P.* 'kelmania' were detected from field soil roots eDNA only (Table 2.2).

Table 2.2 Number of sites (from total of 5) from which each *Phytophthora* species was (A) isolated using traditional techniques, or (B) detected by metabarcoding

Species	Clade	(A) Isolations			(B) Metabarcoding			
		Baiting	Filters	Apple	Soil	Filters	Field[1] roots	Glasshouse bait roots
P. nicotianeae	1					3	3	5
P. AUS 1D	1				4	2	3	2
P. capensis	2					1	1	3
P. capsici	2					1		1
P. elongata	2					1	1	
P. frigida	2					1		1
P. multivora	2	2	1		5	5	3	5
P. pachypleura	2					1	1	
P. 'acacia'	2						1	1
P. citrophthora	2					1	2	3
P. AUS 2C	2						1	
P. arenaria	4					1	1	3
P. palmivora	4					1		
P. amnicola	6	1	1		2	5	3	5
P. fluvialis	6						1	
P. gregata	6		1			1	1	
P. inundata	6	1			2	4	2	4
P. litoralis	6						1	
P. moyootj	6					1	1	
P. 'oreophila'	6	1	1		2	5	3	5
P. rosacearum	6	2	2		2	1	2	2
P. kwongonina	6				1	5	2	3
P. thermophila	6	1	1		3	5	3	5
P. cambivora	7					1		
P. cinnamomi	7				1	2	3	5
P. melonis	7				1			
P. psuedocryptogea	8				5	5	3	5
P. 'kelmania'	8						1	
P. constricta	9				1	2	3	3
P. versiformis	11				2	1	1	1
Total no. of species		6	6	0	13	24	25	19

[1] amplification was only achieved for 3 of the 5 sites

Table 2.3 The table below displays gamma, alpha and beta diversity indices. Indices that account for species abundance (α_{SW}, α_S, β_{BC},) were calculated with the number of reads. Symbols: γ represent gamma diversity; symbol α represent species richness; α_{SI} means Shannon Index (Shannon, 1948); α_S, mean Simpson diversity (Simpson, 1949); $\beta_{\gamma/\alpha}$, multiplicative beta diversity; β_{BC} means Bray-Curtis dissimilarity (Bray and Curtis, 1957).

Metabarcoding substrate	**Number of sites of sites in which *Phytophthora* species were detected**	**Gamma**	**Alpha**			**Beta**	
		γ	α	α_{SI}	α_S	$\beta_{\gamma/\alpha}$	β_{BC}
Soil	5	13	6.20	0.50	0.24	2.10	0.50
Filters	5	24	11.2	0.81	0.38	2.14	0.53
Roots	3	25	15.67	1.71	0.72	1.59	0.75
Glasshouse roots	5	19	12.4	1.44	0.61	1.53	0.49

DISCUSSION

This study provides a comparison of traditional isolation methods to metabarcoding, and for the first time, an evaluation of different substrates used for isolation and detection of *Phytophthora* species was conducted. This research will have a great impact on *Phytophthora* diagnostics and its isolation and detection in natural ecosystems and is particularly relevant to studies of other root infecting organisms. Different techniques and substrates used for the isolation and detection of *Phytophthora* species showed variable results. Of the 30 *Phytophthora* phylotypes detected in this study, all were identified in eDNA from a variety of sources and only seven of these were recovered by traditional methods. We were also able to recognize two potentially new phylotypes, both of which had been detected in Australia previously (Burgess *et al.* 2017b).

Traditional techniques tested for isolation of *Phytophthora* species showed variable results. One interesting finding is that one additional species, *P. gregata,* was recovered from filtered bait water compared to traditional isolation from soil using bait leaves. This result further confirms the association between *Phytophthora* species and the type of bait leaves used (Erwin & Ribeiro 1996). It also supports the idea of using multiple bait leaves to avoid host preference and competition for food among *Phytophthora* species (Scibetta *et al.* 2012). *Scholtzia involucrata* (spiked scholtzia) and *Pimelea ferruginea* (rice flower) were found to be more successful in isolation of *Phytophthora* species in the current study; *P. ferruginea* has been successfully used in previous research (McDougall *et al.* 2002). Further work is suggested to establish the relationship between *Phytophthora* species and different baits. Moreover, inoculum level varies between seasons; therefore baiting assays need to be conducted at different times of the year to get a good picture of *Phytophthora* species

present (Balci & Halmschlager 2003). Also, the successful recovery of *Phytophthora* species in about one third of the resampled sites by Balci *et al.* (2007) emphasizes the need for sampling throughout the year to avoid false negative results on sites. No *Phytophthora* species were isolated from field soil roots using apple baits. Although there had been some previous reports on successful isolation of *Phytophthora cinnamomi* from soil using fruit baits (Greenhalgh 1978), no *Phytophthora* species were recovered from field soil roots using apple as a bait, this was also the case for Aghighi (2013).

Although one additional *Phytophthora* species were recovered in filtered bait water (*P. gregata* that was not recovered with traditional baiting), no *Phytophthora* species were recovered from site 4 using this technique. This was surprising because *Phytophthora* species were recovered from this site by traditional baiting and one would expect zoospores to be picked up on the filters as water was filtered from the same 'bait trays'. This result may be explained by the fact that colonies are difficult to purify in case of filtering method because colonies are concentrated in the area where membrane is placed (Hong *et al.* 2002). A single filter was used to filter bait water from each 'bait tray' in the current study. It has been shown that the density of *Phytophthora* propagules (cfu/L) from a single filter decreases with the increase in the amount of water filtered because multiple fast-growing *Phytophthora* and *Pythium* species interfere with the identification and growth of slow growing colonies (Reeser *et al.* 2011). Therefore, the amount of water to be filtered should be divided onto several filters to accurately measure *Phytophthora* diversity (Reeser *et al.* 2011).

The metabarcoding results were also variable. Among the different sources of eDNA tested for metabarcoding, the lowest number (13) of *Phytophthora* phylotypes was detected from field soil eDNA. It is believed that the lower detection of *Phytophthora* from soil eDNA can be due to the presence of humic acid inhibitors and high DNA degradation (Català *et al.* 2015). The humic acid inhibitors' interpretation is not valid for the current study as we used the Mo Bio PowerSoil® DNA isolation kit, which efficiently removes humic acid and other inhibitors. (Anonymous , Lear *et al.* 2018) reviewed practices for the extraction, storage and amplification of environmental samples for a wide range of taxa from 2010 to 2015 and found that Mo Bio PowerSoil and PowerMax Soil DNA isolation kits (now rebranded as DNeasy PowerSoil and DNeasy PowerMax by Qiagen, Carlsbad, USA) were used in almost all of the studies dealing with the detections from soil or sediment material; and has been recommended by a number of international standards consortia following comparisons with many other methods (Gilbert *et al.* 2014). Therefore, a more likely explanation for the low detections from soil is that because *Phytophthora* species mainly live as parasites in plants, and survive as resting structures (mainly oospores and chlamydospores) and mycelium in soil. There is a higher chance of degradation in soil as these survival structures are more

exposed to environmental extremes compared to roots where they are protected by thick layers of host tissues. *Phytophthora* species are known to respond differently to environmental extremes even in different parts of a single plant. For example, *P. cinnamomi* is more vulnerable to temperature extremes in bark tissues rather than in root tissues deep under soil (Marçais *et al.* 1996).

Determining fungal diversity in soil eDNA has always been a challenge and several techniques have been used to improve the amount of DNA extracted with sufficient purity. For example, liquid nitrogen and phenol/chloroform in combination with powdered skim milk was used to reduce PCR inhibitors in case of *Fusarium oxysporum* f. sp *ciceris (García-Pedrajas et al. 1999).* Okubara *et al.* (2007) used pressure cycling technology (samples are subjected to alternate cycles of high and ambient pressure), rather than mechanical disruption for detecting *Rhizoctonia* and *Pythium* species from soil. Probably the most common approach used to reduce the amount of inhibitors is the dilution of DNA extract. Although this often results in the amplification, it is not very useful to detect low levels of pathogen inoculum (Bilodeau *et al.* 2012). Alternatively, aluminum sulphate (Dong *et al.* 2006) and Sephadex column (Tsai & Olson 1992) can be used to reduce humic substances. Humic acid substances can also be reduced by supplementing PCR mixtures with adjuvants, such as BSA (Lear *et al.* 2018). It is advisable to get an accurate estimation of consistency of results between replicates to get accurate results because inoculum is not always uniformly distributed in soil. Three to four replications for each extraction with sample tubes holding a total volume of 0.5 g of soil each has been reported to provide sufficient consistency in replicate results without increasing cost (Bilodeau *et al.* 2012). Further work is suggested to evaluate different soil DNA extraction techniques and optimization of sensitivity of detections from soil.

In comparison to field soil eDNA, 24 *Phytophthora* phylotypes were detected in eDNA from filters (extracted onto filters by filtered bait water). This result maybe explained by the fact that a large amount of soil (approx. 400g) was used in filtered bait water, whereas only 50 mg soil was used for pyrosequencing analysis of soil eDNA.

The highest number (25) of *Phytophthora* phylotypes was detected in the eDNA of fine roots collected from field soil. These results are contrary to the hypothesis proposed by Coince *et al.* (2013), that high-throughput sequencing can rule out the niche differentiation between fine roots and soil, and can detect most oomycete and fungal MOTUs present in fine roots in soil as well. Prigigallo *et al.* (2015) detected three additional *Phytophthora* species out of nine species belonging to definite taxonomic groups in soil rather than roots. Landeweert *et al.* (2005) determined the diversity of an active ectomycorrhizal fungal community in root tips and total soil DNA, and did not detect a single fungal species in root tips that was not present

in soil. However, Dickie *et al.* (2002) found that mycorrhizal fungi obtained from fine roots can be different from those obtained from soil. Our research matches these latter findings as 15 additional *Phytophthora* species not found in the soil were detected in fine roots, which raise the possibility of using fine roots as an effective substitute for other substrates for metabarcoding studies. An issue with the detection directly from soil could be that the DNA extracted could be from dead organisms (Nocker *et al.* 2006). Roots act as a biological filter; if an organism is present in the roots it must have been alive to get there. However, we did only detect *Phytophthora* in three of the five root samples, which could be somehow linked to the type of roots collected and the relatively small sample size (as inoculum is not uniformly dispersed). More work needs to be done to optimize this methodology.

Nineteen *Phytophthora* phylotypes were detected in the roots of bait plants grown in the glasshouse. These results are consistent with those of McDougall *et al.* (2002), who detected *P. cinnamomi* in twice as many areas and five times as many samples with *in situ* baiting with *Banksia grandis* than *ex situ* soil and root baiting. A possible explanation for this might be that bait leaves used in traditional isolation from soil were not as attractive to *Phytophthora* as the roots of bait plants grown in the glasshouse. Another possible explanation for this could be that *Phytophthora* survival structures (oospores and chlamydospores) were dormant and the traditional baiting technique was unable to break their dormancy, even after double baiting (Balci *et al.* 2007). Similarly, antagonistic microorganisms could have suppressed the growth and germination of these viable propagules during traditional baiting (Balci & Halmschlager 2003), while living roots in soil stimulated their growth and germination. Furthermore, air-drying followed by remoistening and incubation up to three days might be required for recovery of some *Phytophthora* species (e.g. *P. cactorum*). In a study by Jeffers and Aldwinckle (1987) recovery occurred in 100% of replicate subsamples when subsamples were remoistened and incubated for three days before flooding, but only in 17 and 72% of sub-samples when remoistened and incubated for 1 and 2 days, respectively before flooding.

Baiting is a complicated process that shows variable results in soils with different physiochemical and biological characteristics (Williams *et al.* 2009). Chemical composition of the soil can affect zoospore release and hence subsequent bait infection. It has been shown that the use of soil with high levels of N, P, K and organic matter resulted in the increased levels of zoospore production (Broadbent & Baker 1974, Shearer 2003). Messenger *et al.* (2000) showed that high calcium levels were necessary for zoospore production. Duncan (1976) reported that one germinating oospore (producing a sporangium, which then produces 8-14 zoospores) of *P. fragariae* was able to cause detectable infection in a strawberry bait plant under optimum conditions, especially when zoospores were produced

close to the roots and not at the soil surface. The quality of water used can also affect baiting as zoospores show sensitivity to toxic ions present in the un-purified water (Tsao 1983). Gerrettson-Cornell, quoted in Tsao (1983), found that the frequency of isolation of *P. cinnamomi* was 94, 32 and 0%, respectively when glass de-ionized water, deionized water, and de-ionized water from a metal still was used. Lastly, the rate of positive detection of *Phytophthora* by baiting is usually very low (0.4 to 10%) in Western Australian soil (Podger 1978, Blowes 1980) compared to New South Wales and Queensland (27 to 58%) (Blowes 1980, Pryce *et al.* 2002). It is not clear whether the reason for this difference in recovery is due to difference in soil composition or different climates (O'Brien *et al.* 2009).

In conclusion, all the techniques tested for the isolation and detection of *Phytophthora* species showed variable results. Although traditional baiting assays are important for obtaining living isolates, they do not represent the actual *Phytophthora* community present in a location. High-throughput amplicon pyrosequencing of eDNA detected the highest number of *Phytophthora*, therefore it is a very useful tool for assessing *Phytophthora* diversity in environmental samples. The ITS region can fail to discriminate some species complexes (Català *et al.* 2015, Burgess *et al.* 2017b). However, clustering at 99 % of similarity or above may help in differentiating closely related species (clustering was done at 99% sequence similarity level in the present study). Català *et al.* (2015) obtained 20 % more differentiation of closely related species by including a control species mixture and clustering at 99% threshold. Despite these shortcomings, ITS is still very useful to differentiate known species and identify new ones. ITS is the main locus for molecular identification due its easy amplification for most species (Ristaino *et al.* 1998), availability of large sequence data deposited in GenBank, its importance in phylogenetic analysis (Grünwald *et al.* 2011), and more commonly the targeted region for fungal analysis than other gene regions (Lear *et al.* 2018). As the highest number of *Phytophthora* species were detected in field roots eDNA, it could be substituted for other substrates to assess *Phytophthora* diversity in environmental samples. Finally, certain *Phytophthora* could be only detected by one method; therefore a combination of these techniques may be necessary to accurately assess the presence or absence of *Phytophthora* species.

Chapter 3: *Phytophthora* species isolated from alpine and sub-alpine regions of Australia, including the description of two new species; *Phytophthora cacuminis* sp. nov and *Phytophthora oreophila* sp. nov

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Khaliq I, Hardy GES, McDougall KL, Burgess TI (2018) *Phytophthora* species isolated from alpine and sub-alpine regions of Australia, including the description of two new species; *Phytophthora cacuminis* sp. nov and *Phytophthora oreophila* sp. nov recovered from these areas. *Fungal Biology https://doi.org/10.1016/j.funbio.2018.10.006*.

Khaliq performed the lab work and was responsible for the writing and correspondence with the journal. McDougall collected samples from New South Wales, and Burgess and Hardy were supervisors

Abstract

Plant deaths had been observed in the sub-alpine and alpine areas of Australia. Although no detailed aetiology was established, patches of dying vegetation and progressive thinning of canopy suggested the involvement of root pathogens. Therefore, surveys were conducted in alpine and sub-alpine regions of New South Wales and Tasmania to determine if *Phytophthora* species were involved. Baiting of roots and associated rhizosphere soil resulted in the isolation of eight *Phytophthora* species; *Phytophthora cactorum*, *Phytophthora cryptogea, Phytophthora fallax, Phytophthora gonapodyides, Phytophthora gregata, Phytophthora pseudocryptogea,* and two new species, *Phytophthora cacuminis* sp. nov and *Phytophthora oreophila* sp. nov, described here. The new species *P*. *cacuminis* sp. nov is closely related to *P. fallax*, and was isolated from asymptomatic *Eucalyptus coccifera* and species from the family *Proteaceae* in Mount Field NP in Tasmania. The other new species, *P. oreophila* sp. nov, was isolated from a disturbed alpine herbfield in Kosciuzsko National Park. New species low cardinal temperature for growth suggests that they have well adapted to survive under these conditions, and should be regarded as potential threats to the diverse flora of sub-alpine/alpine ecosystems. *Phytophthora gregata* and *P. cryptogea* have already been implicated in poor plant health. Of the eight species recovered, the native or introduced status of the two new species and *P. gregata* is not clear, *P. fallax* is considered to be native while the remainder are thought to have been introduced. Tests on a range of alpine / subalpine plant species are now needed to determine their pathogenicity, host range and invasive potential.

Introduction

Mountains have been recognised as one of the world's richest biodiversity hotspots. Although mountains occupy about 12 % of the land surface, their complex mosaic of microenvironments and ecoclines support almost one quarter of its biodiversity (Körner *et al.* 2011). The sub-alpine zone alone occupies only 3 % of the global area, yet supports around 10,000 vascular plant species, most of which are endemic to mountains (Körner 2004). In Australia, Kosciuszko National Park (KNP) alone contains about 1100 vascular native plant species, which represents one quarter of the New South Wales (NSW) flora in only 10 % of its land area (Doherty *et al.* 2015). Due to the steep environmental gradients over small spatial scales, mountainous regions are useful model systems for understanding ecological and evolutionary processes associated with biological invasions (Pauchard *et al.* 2016, Petitpierre *et al.* 2016). Any stress, biotic or abiotic, can have devastating and irreversible consequences on the distribution of species due to its very restricted climatic envelope.

Plants deaths had been observed in sub-alpine areas of Australia leading to concerns among land managers. Although no comprehensive aetiology had been established due to the assumption that lower temperatures in sub-alpine areas restricts the growth of *Phytophthora* species, such as *Phytophthora cinnamomi* (Podger *et al.* 1990), the progressive thinning of canopy and patches of dying vegetation in Barrington Tops National Park of KNP in the 1990's suggested the involvement of root pathogens. This assumption was confirmed when *P. cinnamomi* was isolated from dying *Oxylobium arborescens* and associated rhizosphere soil at Barrrington Tops National Park at an elevation of 1560 m above sea level (asl) (Mills 1999, McDougall *et al.* 2003). Barrington Tops is a sub-alpine area with annual mean temperature 9.5 °C (extracted from downscaled 30 arc second resolution Worldclim layers) (Hijmans *et al.* 2005), with mean maximum and minimum temperatures of 16 °C and 3 °C, respectively at the highest altitude (Zoete 2000).

In 2013, surveys were conducted in sub-alpine areas of Tasmania (TAS), KNP and Mt Toolbranup in Western Australia (WA) (Burgess *et al.* 2017b). Using high throughput sequencing (HTS), 33 *Phytophthora* species were detected in KNP, including *P. cinnamomi* that was detected at an elevation of 2100 m asl (almost at the highest point of mainland Australia) in asymptomatic vegetation, and in lower elevation ecosystems thought to be non-conducive, such as tall forests with deep loam soil. The detection of such a diverse *Phytophthora* community at such higher elevations was unexpected, as *Phytophthora* diseases are usually not associated with higher altitudes and cold climate vegetation (McDougall *et al.* 2003). The annual mean temperature in the sub-alpine area (Charlottes Pass; elevation 1757 m asl) of KNP is approximately 3.4°C (Barrows *et al.* 2001, Edmonds *et*

al. 2006). At Kiandra in KNP (1395 m asl) it is recorded as 6.8 °C, and for Kosciuszko Hotel (1530 m asl) it is 6.1 °C (Costin 1954). Temperature at higher elevations would be even lower. These temperatures (except for Barrington Tops) are much lower than the mean annual temperature limit of 7.5 °C predicted for disease expression by *P. cinnamomi* (Podger *et al.* 1990).

A recent CLIMEX model predicted increased climate suitability for the growth of *P. cinnamomi* in most sub-alpine areas under present environmental conditions and increased suitability for the growth and survival of the pathogen under changing climatic variables (higher mean winter temperatures, seasonal precipitation shifts from summer into winter, and global warming) (Burgess *et al.* 2017a). This model supersedes the previous models that had only mapped the presence of the pathogen based on disease symptoms on susceptible plants, not on its survival, growth and lifecycle. The presence of a pathogen does not automatically lead to infection and disease rather the following conditions must be satisfied (i) a virulent pathogen, (ii) susceptible host(s), (iii) favourable environmental conditions, and (iv) favourable conditions for long enough for a pathogen to cause disease, and host(s) to express symptoms.

Although many *Phytophthora* species were detected in alpine and sub-alpine areas through HTS in 2013, they were not proof of living organisms, as HTS can detect DNA from dead organisms. The current study was conducted to systematically survey sub-alpine and alpine areas to isolate living *Phytophthora* species to determine baseline *Phytophthora* species in these areas.

Materials and methods

Samples collection and isolation

Rhizosphere soil and associated roots were collected from asymptomatic vegetation within 5 m of roads and track edges in the sub-alpine and alpine areas of NSW (KNP) in spring 2015/16, and asymptomatic vegetation in sub-alpine areas in TAS adjacent to walking tracks in May 2016. Special emphasis was placed on collecting rhizosphere soil including roots. The soil samples were placed into zip-lock plastic bags and kept in an insulated box to protect samples from high temperature and direct sunlight. In the laboratory, about 300 g of each soil sample was baited with juvenile leaves of *Quercus ilex, Q. suber*, *Pimelea ferruginea*, *Poplar* sp., *Scholtzia involucrata*, *Hedera helix* (Ivy), and *Hibbertia scandens.* The baited leaves were observed daily for a week. Leaves with brownish lesions were blotted dry on paper towelling, cut into 3 × 3 mm pieces, and plated onto modified NARH (Simamora *et al.* 2017). Plates were observed microscopically and any *Phytophthora*-like cultures were

transferred to vegetable juice agar V8A plates [100 ml/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery products Ltd., Norfolk, UK), 900 ml/L distilled water, 0.1 g/L $CaCo_{3,}$ pH adjusted to 7, and 17 g Grade A Agar (Becton, Dickenson and Company, Sparks, MD, USA]. After a week, the soil was allowed to air dry and re-baited (double baiting) to increase isolation (Jeffers & Aldwinckle 1987).

DNA isolation, amplification and sequencing

All the isolates were grown on half strength potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, USA, 19.5 g PDA, 7.5 g of agar and 1 L distilled water) for 7 days. Mycelia was harvested by scraping the agar surface with a sterile blade and placed it in a 1.5 ml sterile eppendorf® tubes. The mycelium was ground to a fine powder and genomic DNA was extracted using ZR Fungal/Bacterial DNA Miniprep™ (Zymo Research, Irvine, California). The region spanning the internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the primers DC6 (Cooke *et al.* 2000) and ITS-4 (White *et al.* 1990). The mitochondrial gene cox1 (COX) was amplified with primers FM84 and FM83 (Martin & Tooley 2003). Heat shock protein 90 (HSP) was amplified with primers HSP 90-Fint and HSP-90 R1 (Blair *et al.* 2008). ß- tubulin (TUB) was amplified with primers BTF1A and BTR1 (Kroon *et al.* 2004). NADH dehydrogenase subunit 1 was amplified with NADH-F1 and NADH-R1 primers according to Kroon *et al.* (2004).

Templates were sequenced in both directions with primers used in amplification for all gene regions. The clean up of PCR products and sequencing were performed as described by Sakalidis *et al.* (2011). All sequences derived in this study were deposited in GenBank and their accession numbers are given in Table 3.1. Cultures were maintained under long-term storage in water at CPSM (Centre for *Phytophthora* Science & Management), Murdoch University following identity confirmation through sequencing.

Phylogenetic analysis

The data set comprised of sequences of the new species *P. '*cacuminis', *P. '*oreophila', and those of closely related species in (Fig. 3.2), which were manually edited and compiled in Geneious v. R10 (http://www.geneious.com/). Parsimony analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) (Swofford 2003), and Bayesian analysis with MrBayes (Ronquist *et al.* 2012) as plugins within Geneious software. Bayesian analyses were performed with applying a general time reversible (GTR) substitution model with inverse gamma (I). Alignment files and trees can be viewed on TreeBase (https://treebase.org/).

Cultural characteristics

Isolates were grown on V8A for seven days in the dark at 20 ˚C. Circular inoculum plugs were cut with a sterile cork borer (5 mm in diameter) from the colony edges and placed centrally in 90 mm Petri dishes of the test media. Colony growth patterns were described from 7-day-old cultures grown at 20 ˚C in the dark on V8A, 2 % malt extract agar (MEA; 20 g malt extract, 17 g agar and 1 L distilled water), carrot agar (CA; 0.1 L filtered carrot juice, 17 g agar and 0.9 L distilled water), and half-strength PDA for all species except *P. fallax* and *P. '*cacuminis*'*. Colony growth patterns were described after 18 days for *P.* 'cacuminis' and *P. fallax* due to their very slow growth. Colony growth patterns were described according to Erwin and Ribeiro (1996). For temperature-growth relationship, 5 mm diameter agar plugs of all isolates were placed centrally onto V8A and incubated at 20 ˚C for 20 hours to stimulate growth. The margins were marked and the isolates were then moved to incubators set at temperatures of 4, 10, 15, 20, 25, 30, 32.5, 35, and 37.5 ˚C. Plates were observed daily to make sure colonies did not reach the edges; radial growth rate was measured after 7 days for *P. '*oreophila' and after 24 days for *P. '*cacuminis' and *P. fallax.* Plates showing no growth at higher temperatures were returned to 20 ˚C to determine their viability.

Morphology of sexual and asexual structures

Isolates were grown on V8A for seven days and 3-4 agar plugs (5 mm diameter) were taken from the edges and placed in sterile empty Petri dishes. Each Petri dish was flooded with 10% clarified V8 broth (Erwin & Ribeiro 1996) until the broth was just above the surface of the agar plugs, and kept in an incubator set at 20 ˚C to stimulate mycelial growth overnight. The following day, plates were flooded with deionized water. This water was decanted and replaced twice (after 4 and 6 h). In the final change, 7–10 drops of non-sterile pine (*Pinus radiata*) bark extract were added to the water in each plate. The pine bark extract was made by suspending 100 g of pine bark potting mixture in 1 L distilled water, and incubated overnight. After 18-22 h, dimensions and characteristic features of 50 mature sporangia, selected at random, were measured at 40x in a BX51 Olympus microscope for each isolate.

*Phytophthora '*oreophila' was homothallic. *Phytophthora '*cacuminis' was crossed with A1 and A2 mating types of two different species (*P. nicotianae* and *P. cryptogea*), but no oospore formation was observed. After four weeks, dimensions and characteristics of 50 randomly selected mature oogonia and oospores were measured at 40x for *P. '*oreophila'. The oospore wall index was calculated as described by Dick (1990).

Results

Phytophthora species isolated from sub-alpine and alpine areas

Eight *Phytophthora* species were recovered from 11 (46 %) of the 24 soil samples tested. Thirty-two isolates corresponding to three species (*Phytophthora* 'cacuminis', *Phytophthora fallax* and *Phytophthora gregata*) were recovered from baiting the six samples collected from asymptomatic vegetation adjacent to walking tracks in TAS, and 57 isolates corresponding to six species (*Phytophthora cactorum, Phytophthora gonapodyides, Phytophthora pseudocryptogea, Phytophthora cryptogea, Phytophthora gregata* and *Phytophthora* 'oreophila') were recovered from baiting 18 samples collected from asymptomatic vegetation within 5 m of roads and track edges in NSW (Table 3.2). Isolates of all *Phytophthora* species with their closest relatives, considered in this study, and locations of isolation and altitude are listed in (Table 3.1, Supplementary Table 3.1). Of the six *Phytophthora* species recovered from KNP, four species; *P. cactorum, P. gonapodyides*, *P. pseudocryptogea,* and *P.* 'oreophila' were isolated from this region for the first time through baiting. The most frequently isolated species in KNP from all sites were *P. cactorum* and *P. gregata. Phytophthora cryptogea* was isolated from the alpine area at the summit of Mt Kosciuszko (2228 m asl). This is also the first record on the recovery of living isolates of *P. fallax* and *P.* 'cacuminis' in TAS. The most frequently isolated species in TAS was *P. gregata,* and it was the only species isolated in both states. It has also been implicated in *Pimelea bracteatea* dieback in Rocky Plains in KNP (Fig. 3.1)

Figure. 3.1 Dieback disease symptoms on *Pimelea bracteatea* caused by *Phytophthora gregata* in Kosciuszko National Park. (A) healthy plants; (B) severe dieback and thinning leading to loss of aerial canopy giving the plants 'sticks' like appearance; and (C) root collar showing necrotic lesions resulting in the death of aerial stem.

Table 3.1 Identity, date and location of isolation, host information and GenBank accession numbers for isolates of *Phytophthora* species considered in this study. Shaded rows represent isolates that were recovered in this study. Additional information for isolates can be found in Table S1.

Isolate	Organism	Location	Vegetation	Date	GenBank Accession number				
					ITS	TUB	HSP	Cox	NADH
QLD13E	*Phytophthora* sp.	Australia, QLD[a], Koombooloomba	Tropical rain forest	2013	MG542958	MG543047	MG543034	MG543012	MG543024
U40	*P. cacuminis*	Australia, TAS[a], Mt Field NP[b]	*Eucalyptus coccifera*	2016	MG542997	MG543045	MG543032	MG543010	MG543019
U41	*P. cacuminis*	Australia, TAS, Mt Field NP	*Eucalyptus coccifera*	2016	MG542998	MG543046	MG543033	MG543011	MG543020
U11	*P. oreophila*	Australia, NSW[a], Merritts Creek	Disturbed alpine herbfield	2016	MG542976	MG543037	MG543025	MG543002	MG543013
VHS26182	*Phytophthora* sp.	Australia, WA, Fitzgerald River NP	Kwongan heathland	2006	MG543000				
TAS34	*P. cactorum*	Australia, TAS, Pine Lake	*Athrotaxis selaginoides*	2013	MG542959				
U1	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	MG542966				
U2	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	MG542967				
U3	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	MG542968				
U4	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	MG542969				
U5	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	MG542970				
U6	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	MG542971				
U7	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	MG542972				
U8	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	MG542973				
W1846	*P. cambivora*	Australia, NSW, Charlottes Pass	*Nematolepis ovatifolia*	2014	MG543001				
TAS188	*P. cinnamomi*	Australia, TAS, Condominium Creek	Riparian rain forest	2013	MG542963				
VHS16127	*P. constricta*	Australia, WA, Fitzgerald River NP	Kwongan heathland	2006	HQ013224				
VHS16130	*P. constricta*	Australia, WA, Fitzgerald River NP	Kwongan heathland	2006	HQ01327				
U21	*P. cryptogea*	Australia, NSW, Mt Kosciuszko	Walking track edge in alpine heath	2016	MG542983				
U22	*P. cryptogea*	Australia, NSW, Mt Kosciuszko	Walking track edge in alpine heath	2016	MG542984				
TAS126	*P. elongata*	Australia, TAS, Mt Field NP	Riparian rain forest	2013	MG542960				
U34	*P. fallax*	Australia, TAS, Hartz Mountain NP	Melaleuca	2016	MG542991	MG543043	MG543030	MG543008	MG543017
U35	*P. fallax*	Australia, TAS, Hartz Mountain NP	Alpine heath	2016	MG542992	MG543044	MG543031	MG543009	MG543018
U36	*P. fallax*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	MG542993				
U37	*P. fallax*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	MG542994				
U14	*P. gonapodyides*	Australia, NSW, Smiggins Hole	Road edge in subalpine heath	2016	MG542979	MG543038	MG543026	MG543003	MG543014
U15	*P. gonapodyides*	Australia, NSW, Kosciuszko Road	Disturbed alpine herbfield	2016	MG542980	MG543039	MG543027	MG543004	MG543015
TAS206	*P. gregata*	Australia, TAS, Pine Lake	Moorland	2013	MG542964				
TAS207	*P. gregata*	Australia, TAS, Pine Lake	Moorland	2013	MG542965				
U9	*P. gregata*	Australia, NSW, Pipers Gap	Road edge in subalpine heath	2016	MG542974				
U10	*P. gregata*	Australia, NSW, Pipers Gap	Road edge in subalpine heath	2016	MG542975				
U12	*P. gregata*	Australia, NSW, Perisher	Disturbed subalpine wetland	2016	MG542977				
U13	*P. gregata*	Australia, NSW, Perisher	Disturbed subalpine wetland	2016	MG542978				

Isolate	Organism	Location	Vegetation	Date	GenBank Accession number				
					ITS	TUB	HSP	Cox	NADH
U18	*P. gregata*	Australia, NSW, Pipers Gap	Road edge in subalpine heath	2016	MG542981				
U32	*P. gregata*	Australia, NSW, Perisher	Disturbed subalpine wetland	2016	MG542989				
U38	*P. gregata*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	MG542995				
U39	*P. gregata*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	MG542996				
U42	*P. gregata*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	MG542999				
CBS139749	*P. pseudocryptogea*	Australia, WA, Fitzgerald River NP	*Isopogon buxifolius*	2006	KP288376	KP288392	KP288426	KP288342	KP288360
VHS5380	*P. pseudocryptogea*	Australia, WA, Fitzgerald River NP	*Xanthorrhoea preissii*	1992	KP288374	KP288390	KP288424	KP288340	KP288358
TAS143	*P. pseudocryptogea*	Australia, TAS, Steppes	Woodland	2013	MG542962				
U20	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	MG542982				
U23	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	MG542985				
U24	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	MG542986				
U30	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	MG542987				
U31	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	MG542988				
U33	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	MG542990				
CBS119107	*P. captiosa*	New Zealand, Rotoehu Forest	*Eucalyptus saligna*	1995	DQ297402				
NZFS310.35	*P. captiosa*	New Zealand, Rotoehu Forest	*Eucalyptus saligna*	1998	DQ297405				
MUCC761	*P. gonapodyides*	Australia, VIC[a], Toolangi North	*Eucalyptus oblique* forest	2008	HQ012937	JN547598	HQ012896	HQ012850	JN547686
CBS127954	*P. thermophila*	Australia, WA, Dwellingup	*Eucalyptus marginata*	2004	EU301155	JN547613	HQ012916	HQ012872	JN547700
TP13.29	*P. versiformis*	Australia, WA, Naturaliste	*Corymbia calophylla*	2013	KX011277	KX011318	KX011254	KX011220	KX011299
CBS 142005	*P. versiformis*	Australia, WA, Williams	*Corymbia calophylla*	2013	KX011279	KX011321	KX011256	KX011222	KX011302
HAS2313	*P. cooljarloo*	Australia, WA, Cooljarloo	Swamp native vegetation	1996	HQ012961	MF326817	HQ012929	HQ012885	MF326911
VHS24266	*P. pseudorosacearum*	Australia, WA, Albany	*Xanthorrhoea platyphylla*	2010	JN547637	MF326826	MF326877	MF326857	MF326909
OSU55	*P. rosacearum*	USA, Maryland	*Prunus armeniaca*		KJ372271	MF326833	MF326882	MF326854	MF326902
VHS29592	*P. pseudorosacearum*	Australia, WA, Jarrahdale	*Persoonia longifolia*	2013	KJ372267	MF326827	MF326878	MF326858	MF326907
VHS23298	*P. kwongonina*	Australia, WA, Bunbury	*Banksia grandis*	2010	JN547636	MF326824	MF326876	MF326847	MF326914
TAS35	*P. gonapodyides*	Australia, Tas, Houn River	Native vegetation	2009	JN547620	JN547642	MG543031	JN547581	JN547669
IMI389735	*P.* taxon walnut	USA, California, Merced County	*Juglans hindsii*	1988	AF541910				
CLJ0100	*P.* cooljarloo	Australia, WA, Cooljarloo	Hibbertia sp	2008	HQ012957	MF326816	HQ012925	HQ012881	MF326910
CBS124696	*P. rosacearum*	USA, California			EU925376				
P10725	*P. fallax*	New Zealand	*Eucalyptus fastigata*	2004	HQ261557				
CBS125801	*P. constricta*	Australia, WA, Fitzgerald River NP	Kwongon heathland	2006	HQ013225				
NZFS310.25	*P. captiosa*	New Zealand, Rotoehu Forest	*Eucalyptus saligna*	1998					

Phylogenetic analysis

The alignments for TUB, HSP, ITS, COX and NADH consisted of 1178, 936, 846,1236 and 837 characters, respectively. Trees for the individual datasets produced similar topology (TreeBASE 22955) and the nuclear and mitochondrial gene regions were combined separately for the analyses presented here.

Support for terminal clades and their clustering was equivalent in both analyses and the Bayesian analysis is presented here (Fig. 3.2). All species reside in highly supported terminal clusters. *Phytophthora* 'oreophila' resides in clade 6a and is related to *P. rosacearum* and *P. pseudorosacearum*, but differs from them across the gene regions sequenced here by 39 and 64 polymorphisms, respectively. *Phytophthora* 'cacuminis' resides in clade 9 and is closely related to the known eucalypt pathogens *P. fallax and P. captiosa,* although it differs from them by 75 and 116 fixed polymosphisms, respectively

Fig. 3.2. Bayesian trees of (A) concatenated nuclear regions and (B) concatenated mitochondrial regions showing the phylogenetic position of *P. oreophila* (orange) and *P. cacuminis* (blue) in relation to related species. Bayesian posterior probabilities are listed above the branches.

Phytophthora oreophila I. Khaliq and T.I Burgess, **sp. nov**.

Mycobank MB825232

(Figs. 3.3 and 3.5)

Etymology: '*oreos'* refers to a Greek word for a mountain and '*phila*' means loving. The name is given to the species due to its mountainous origin, and its ability to grow at extremely low temperatures (less than 4 °C).

Type: **Australia**: New South Wales, Merritts Creek; by baiting rhizosphere soil and associated roots collected from a disturbed alpine herbfield, January 2016. Collected by *Keith McDougall* (Office of Environment and Heritage, PO Box 733, Queanbeyan NSW 2620). Holotype MURU 483 (dried culture on V8A, Herbarium of Murdoch University, Western Australia), cultures ex-type U11. ITS, cox1, NADH, HSP90 and β tubulin sequence GenBank numbers are MG542976, MG543002, MG543013, MG543025, and MG543037, respectively.

Original Description: Sporangia were exclusively non-papillate, persistent and frequently produced in non-sterile pine bark extract. They were borne terminally on simple sporangiophores rarely with globose swellings (2%) produced near the base of sporangia (Fig. 3.3G). Although predominantly ovoid (80%, Fig. 3.3A, B, F, H, I), a few ellipsoid (12%, Fig. 3.3C-E, G), and distorted shaped (8%, Fig. 3.3J) sporangia were also observed. Sporangia averaged 40.9 x 26.7 µm, ranged 19.9 X 59.9 – 13.4 x 38.5 µm, exit pores 12.8 µm in diameter, and length: breadth ratio was 1.5 (Table 3.1). Sporangia proliferated internally in both an extended (Fig. 3.3H) and a nested way (Fig. 3.3I, K). Intercalary hyphal swellings with radiating hyphae formed occasionally in non-sterile pine bark extract (Fig. 3.3 L, M). Zoospore cysts were spherical with average diameter 9.7 µm (Table 3.1).

Phytophthora oreophila is homothallic, readily produces oogonia, oospores and antheridia in single culture on CA, MEA and V8A. Time to oospore maturity was between 25 to 30 days. Oogonia averaged 36.8 µm in diameter ranging from 29.3 to 48.1 µm (Table 3.1). Plerotic oospores containing ooplasts when semi-mature to mature (Fig. 3.3 O-R). Oospores averaged 33.8 µm in diameter ranging from 26.8 to 42 µm. Oospore walls were relatively thick (2.2 µm) (Fig. 3.3 N-V), and oospore wall index was 0.34 µm (Table 3.1). Paragynous antheridia (Fig. 3.3 N-R, U, V) averaged 10.9 x 10.6 µm in diameter often (16 %) with multiple antheridium (Fig. 3.3 O, P, Q, V). Most of the oospores (90%) observed aborted after wall formation (Fig. 3.3 S-V).

Cultures: Phytophthora oreophila produced a slightly petaloid growth pattern on CA, petaloid growth pattern on V8A and MEA, and rosaceous growth pattern on PDA (Fig. 3.5). The colony morphology of *P. oreophila* was clearly distinguishable from *P. rosacearum* as the latter produced uniform colonies on V8A and MEA compared to the petaloid growth pattern of *P. oreophila.* Optimum temperature for the growth on V8A was 20 °C, and the average growth rate was 4.92 mm day^{-1} at this temperature. The maximum temperature for growth was 32.5 °C, and the lethal temperature for growth was recorded as 35°C (Table 3.1).

Diagnosis: *Phytophthora oreophila* is closely related to *P. rosacearum* and *P. pseudorosacearum* but there are several differences; (1) *P*. *oreophila* has lower minimum, optimal and maximum temperatures for growth (Table 3.1, Fig. 3.6); (2) *P*. *oreophila* grows faster than related species at temperatures less than 20 °C (Fig. 3.6); (3) colony morphologies also differ on V8A and MEA, as *P. oreophila* produced a petaloid growth pattern compared to uniform colony growth by *P. rosacearum,* and *P. pseudorosacearum* (Fig. 3.5)*;* and (4) *P*. *oreophila* has smaller sporangia and slightly larger oogonia and oospores (Table 3.1)

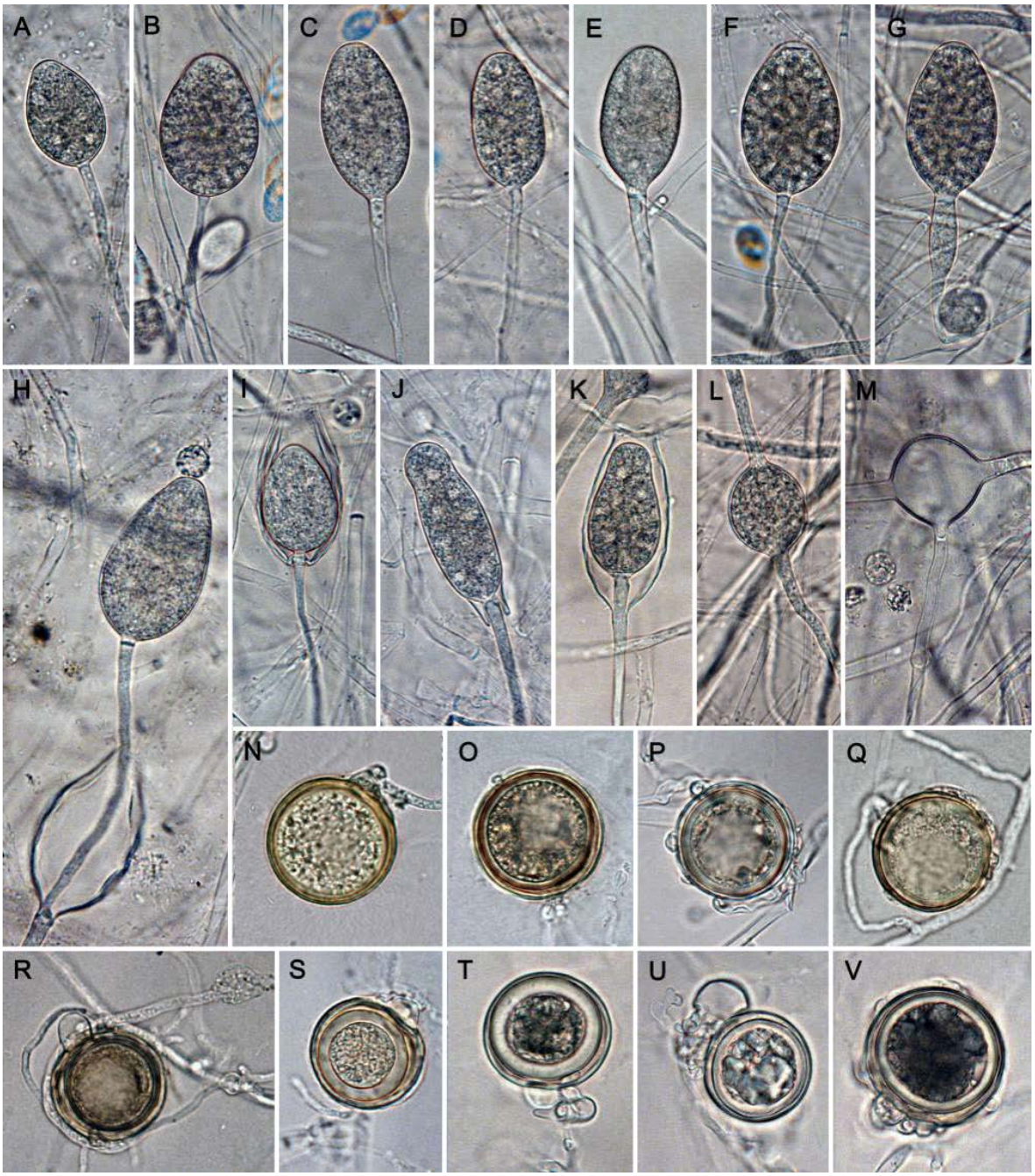

Figure 3.3. Persistent non-papillate sporangia of *P. oreophila* formed on V8A flooded with pine bark extract; ovoid (A, B, F, H, I), ellipsoid (C-E, G), and distorted (J). Internal proliferations in a nested (I, K) and an extended way (H) were observed. Intercalary hyphal swellings (L,M). Swollen sporangiophore (G) rarely observed. Sporangiophores were occasionally twisted (F, I). Oogonia formed on solid media; globose oogonia with smooth margins that turned pale brown (N-R) on maturity, with plerotic oospores (N-R) and paragynous antheridia (N-R, U, V). Oospore with more than one antheridium was occasionally observed (O, P, Q, V). Oospores often abort after wall formation (S-V). Oospores rarely surrounded by hyphal coil (R). Scale bar = 25 µm

Table 3.3. Comparison of morphological characters and dimensions, and temperature-growth relations of *P. rosacearum, P. pseudorosacearum, P. oreophila, P. fallax* and *P. cacuminis.* All measurements are in µm

Species	*P. rosacearum*	*P. pseudorosacearum*	*P. oreophila*	*P. fallax*	*P. cacuminis*
no of isolates	6	3	1	9	2
sporangia					
LxB mean ± SD	46.4 ± 8.7 x 28.7 ± 4.8	52.7 ± 10.0 x 34.1 ± 5.6	40.9 ± 10.3 x ± 26.7 ± 6.08	55.5 ± 5 x 32 ± 3	27.4 ± 5.9 x 22.4 ± 4.42
Total Range	22.5 – 73.4 x 16.7 – 40.1	32.7 - 59.3 x 19.4 - 38.3	19.98 - 59.99 x 13.4 – 38.5	50.5 – 61.5 x 28 - 34	14.5 – 40.2 x 10.8 – 37.3
Range of isolates means	43.7 - 47.9 x 23.7 - 31.9	49.4 - 56.0 x 30.7 - 37.8	na[a]	nd[b]	27.4 – 22.4 x 27.4 – 22.4
L/B ratio (range)	1.63 ± 0.25 (1.05 - 2.36)	1.57 ± 0.31 (1.02 - 2.48)	1.54 ± 0.2 (0.79 - 2.35)	1.7	1.22 ± 0.13 (0.76 – 1.57)
Features	Terminal, persistent, non papillate	Terminal, persistent, non papillate	Terminal, persistent, non papillate	Terminal, persistent, non papillate	Terminal, persistent, non papillate
Sporangiophores	Simple	Simple	Simple	Simple	Simple, but frequently slightly twisted and narrowed
Shapes	ovoid 56% ellipsoid ovoid 29% broad ovoid 2% ellipsoid 10% limonoform 2%	ovoid 55%, elongated ovoid 30%, lemoniform 5% ellipsoid 5% broad ovoid 5%	ovoid 80%, ellipsoid 12% distorted 8%	Obpyriform to distorted, often with a distinctive elongated neck and conspicuous basal plugs, hyphal projections at apex	ovoid 90%, globose 6% lemoniform 4%
Proliferation	Internal, both nested and extended	Internal, both nested and extended	Internal, both nested and extended	Internal, both nested and extended	Internal, both nested and extended
Exit pores					
Width (range)	12.6 ± 2.8 (5. 7- 18.6)	14.9 ± 2.7 (8.8 - 20.2)	12.84 ± 4.13 (5.88 – 19.83)	nd	9.88 ± 1.2 (7.4 – 11.6)
Zoospore cysts	11.9 ± 1.2 (9.6 – 16.0)	11.6 ± 1.8 (8.0 - 19.9)	9.70 ± 2.87 (6.11 – 13.13)	nd	10.68 ± 2.69 (8 - 15.69)
Chlamydospores	Absent	present	present	present	present
Diameter (range)		28.4 ± 5.3 (20.1 - 42.7)		12 – 26	30.2 ± 2.6
Hyphal swellings	present	present	present	absent	absent
Features	predominantly spherical and intercalary with radiating hyphae	predominantly spherical and intercalary with radiating hyphae	predominantly spherical and intercalary with radiating hyphae	nd	absent
Mean diam	17.6 ± 5.7 (9.0 - 27.8)	17.8 ± 6.0 (6.1 - 30.9)	20.7 ± 5.6 (10.9 – 31.2)		18.13
Breeding system	Homothallic	Homothallic	Homothallic	Homothallic	Sterile in culture
Oogonia					
Features	slightly wavy walls	wavy walls, sometimes with a slightly tapering base	spherical, pale brown on maturity	spherical, pale brown on maturity	
Mean diam	36.1± 3.9 (23.8 - 47.3)	35.8 ± 4.9 (23.8 - 49.0)	36.8 ± 4.8 (29.3 – 48.1)	33.5 ± 3	
Range of isolates means	32.6 - 38.8	33.1 - 37.4	na	30 – 39	

Species	*P. rosacearum*	*P. pseudorosacearum*	*P. oreophila*	*P. fallax*	*P. cacuminis*
Oospores					
Features	Slightly aplerotic, pale on maturity	aplerotic, slightly golden on maturity and often slightly eccentric	plerotic, pale on maturity	Initially plerotic, become aplerotic with age	
Abortion	59%	20%	90%	nd	
Mean diam	31.2 ± 3.4 (20.3 - 41.0)	30.8 ± 3.3 (22.3 - 38.1)	33.8 ± 4.49 (26.8 - 42)	31.5 ± 2.5	
Range of isolates means	28.4 - 35.4	29.5 - 31.8	na	29 – 35	
Wall diameter	2.05 ± 0.47	2.46 ± 0.47	2.23 ± 0.86	2 ± 0.5	
Oospore wall index	0.34 ± 0.06	0.41 ± 0.06	0.34 ± 0.11	nd	
Antheridia					
Features	Paragynous round-club shaped, predominantly adjacent to oogonial stalk, very few amphigynous in some isolates	Paragynous round-club shaped, predominantly adjacent to oogonial stalk	Paragynous round-club shaped, often with multiple antheridium	Paragynous (globose), amphigynous (cylindrical and single celled), attached near stalk	
LxB mean	12.9 ± 2.5 x 9.4 ± 2.1	13.8 ± 3.9 x 11.4 ± 3.2	10.9 ± 2.8 x 10.6 ± 14.7	18.5 ± 4 x 14 ± 1	
LxB range	7.5 – 19.2 x 4.7 - 13.9	6.1 - 26.6 x5.5 - 22.1	5.65 – 16.9 x 3.7 – 11.9	nd	
Growth Characteristics					
Max temp (ºC)	37.5	37.5	32.5	30	25
Opt temp (ºC)	25-30	30	20	20	20
Min temp (ºC)	4	4	<4	2	4
Lethal temp (ºC)	>37.5	>37.5	35	>30<32.5	>25<30
Growth rate on V8A at optimum (mm day^{-1})	5.94 ± 0.1	5.2 ± 0.40	4.9 ± 0.06	0.83	1.24 ± 0.02

Table 3.2. Number of isolates of each *Phytophthora* species recovered through baiting in Kosciuszko National Park and Tasmania.

***Phytophthora* species**	**Clade**	**Number of isolates recovered**	
		NSW (KNP)	**TAS**
P. cactorum	1	24	
P. gregata	6	15	16
P. pseudocryptogea	8	8	
P. cryptogea	8	5	
P. fallax	9		14
P. gonapodyides	6	4	
P. cacuminis	9		2
P. oreophila	6a	1	
Total		57	32

Phytophthora cacuminis I. Khaliq and T.I Burgess, **sp. nov**

MycoBank MB825231

(Figs. 3.4-5)

Etymology: The species name *cacuminis* is derived from a Latin word '*cacumen*' for a 'peak'. The name is given to the species based on its isolation from a peak in Tasmania

Type: **Australia:** south Australia: Tasmania, Mount Field NP*,* from asymptomatic vegetation (*Eucalyptus coccifera* and species in *Proteaceae*), May 2016, collected by *Treena Burgess*, holotype MURU 482 (dried culture on V8A, Herbarium of Murdoch University, Western Australia), cultures ex-type U40. ITS, cox1, NADH, HSP90 and β tubulin sequence GenBank numbers are MG542998, MG543011, MG543020, MG543033, and MG543046, respectively.

*Original Description***:** Exclusively non-papillate, terminal, persistent and predominately ovoid sporangia (90%, Fig. 3.4A-D, F, G, I, J), but a few globose sporangia (6%, Fig. 3.4H) and lemoniform (4%, Fig. 3.4E) sporangium were also observed. Sporangia averaged 27.4 x 22.4 µm in diameter ranging from 14.5 x 40.2 to 10.8 x 37.3 µm (Table 3.3). Internal proliferations both in a nested (Fig. 3.4F, H, I, J) and extended (Fig. 3.4G) way were observed. Exit pores diameter averaged 9.9 µm in diameter. Sporangiophores were mostly slightly twisted and narrowed (Fig. 3.4 A-C, E-I). Zoospore cysts were spherical with average diameter 10.7 µm. Chlamydospores were present (Fig. 3.4 K,L), with average diameter 30.2 µm (Table 3.3). No hyphal swellings were observed.

Phytophthora cacuminis isolates were sterile in culture; no oogonia or oospores were formed when isolates were crossed with A1 and A2 mating type isolates of two different species (*P. nicotianae* and *P. cryptogea)*.

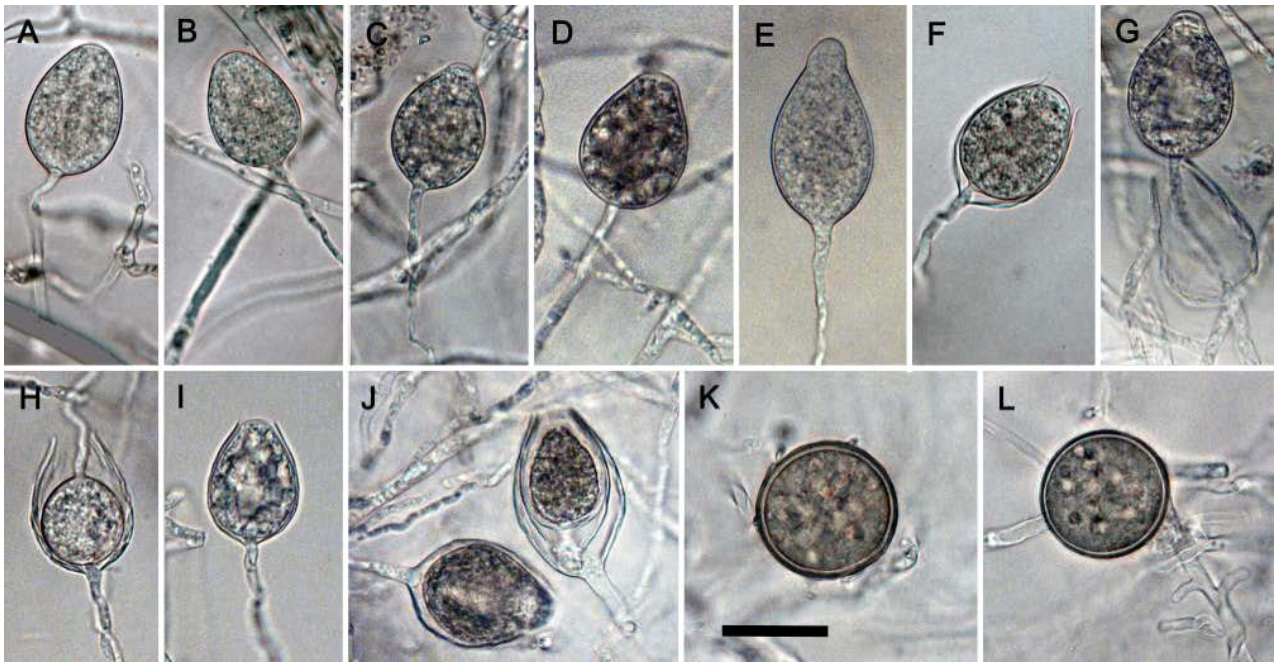

Figure 3.4. Persistent non-papillate sporangia of *P. cacuminis* formed on V8A flooded with pine bark extract; predominantly ovoid (A-D, F, G, I, J) with internal proliferations in a nested (F, H, I, J) or an extended way (G). Lemoniform (E) and globose (H) sporangia were also observed. Chlamydospores were frequently observed (K, L). Sporangiophores frequently slightly twisted and/or narrowed (A-C, E-I). Scale bar = 25 µm

Cultures: All isolates produced colonies with distinctive growth patterns on different media (Fig. 3.5). Colonies had a halo of submerged hyphae on CA, plumose growth pattern on V8A, uniform growth on MEA and dense growth on half PDA. The colony morphology of *P. cacuminis* was clearly distinguishable from *P. fallax* on V8A as the former produced plumose growth pattern on V8A compared to uniform growth pattern of *P. fallax*. The optimum temperature for growth on V8A was 20 °C with a growth rate of 1.2 mm/day. The maximum temperature for growth was recorded as 25 °C (Table 3.3). No growth occurred at 30 °C, and this temperature was found to be lethal as isolates did not resume growth when subsequently incubated at 20 °C. *Phytophthora fallax* was markedly slower growing than *P. cacuminis* (Table 3.3, Fig. 3.6).

*Diagnosis***:** *Phytophthora cacuminis* is closely related to *P. fallax* but it distinguishable from *P. fallax* in many ways; (1) *P. cacuminis* is sterile in culture compared to the homothallic nature of *P. fallax*; (2) *Phytophthora cacuminis* produces on average smaller sporangia; (3) sporangiophores are frequently slightly twisted and narrowed, similar to *P. constricta,* a closely related species in the same clade. It had been suggested by (Jung *et al.* 2011) that *P. constricta* was in the process of becoming caducous because of this feature; (4) colony morphologies also differ, as *P. cacuminis* produces a plumose growth pattern on V8A, while *P. fallax* has a uniform growth pattern on the same medium; and (5) maximum temperature for growth for *P. cacuminis* is 25 °C, and for *P. fallax* is 30 °C. The later has also markedly slower growth rate than *P. cacuminis* (Fig. 3.6): 30 °C was found to be lethal for *P. cacuminis,* but not for *P. fallax.*

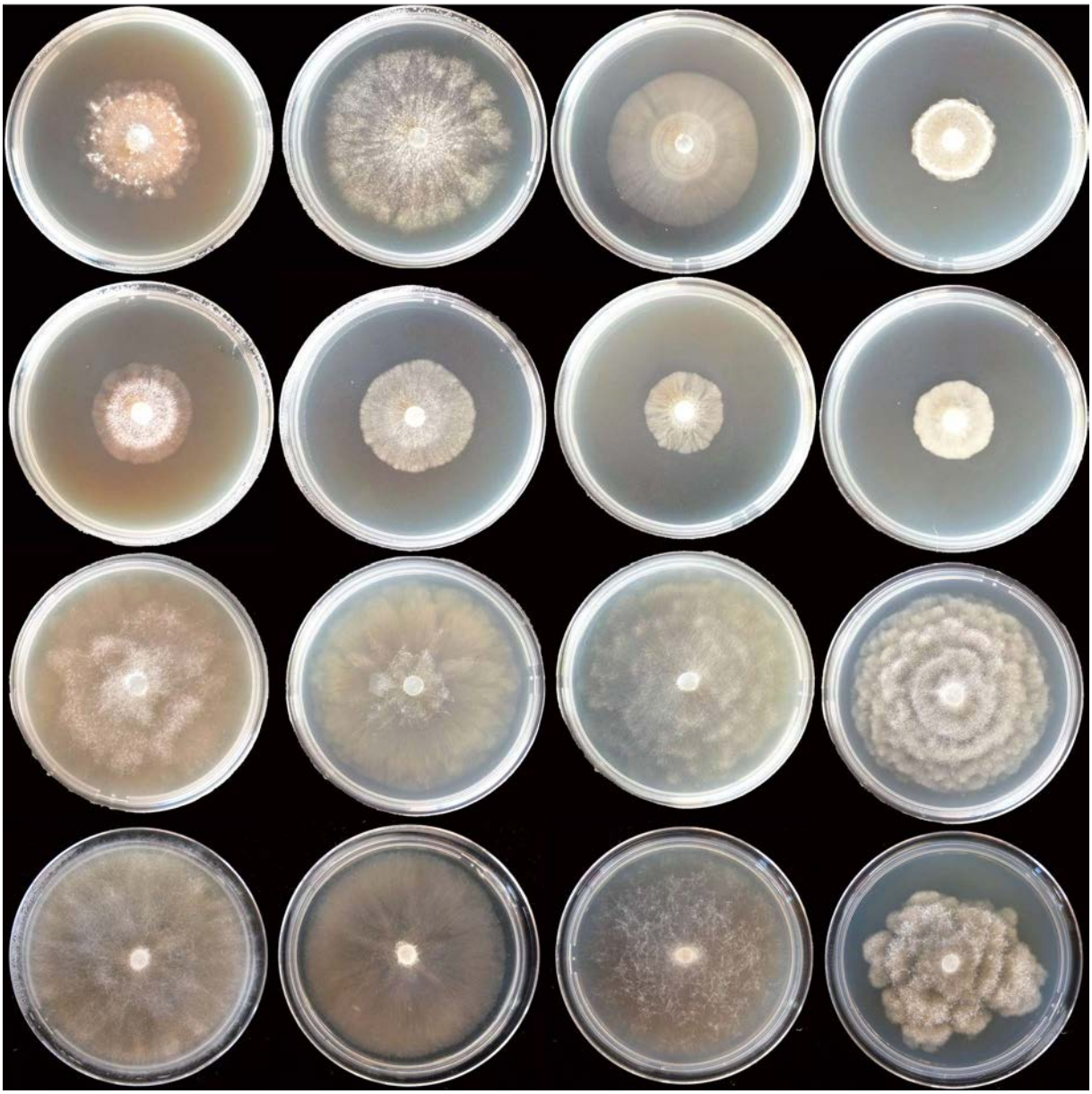

Figure 3.5 Colony morphology (top to bottom) of isolates *P. cacuminis, P. fallax, P. oreophila,* and *P. rosacearum* on CA, V8A, MEA and half strength PDA (left to right)

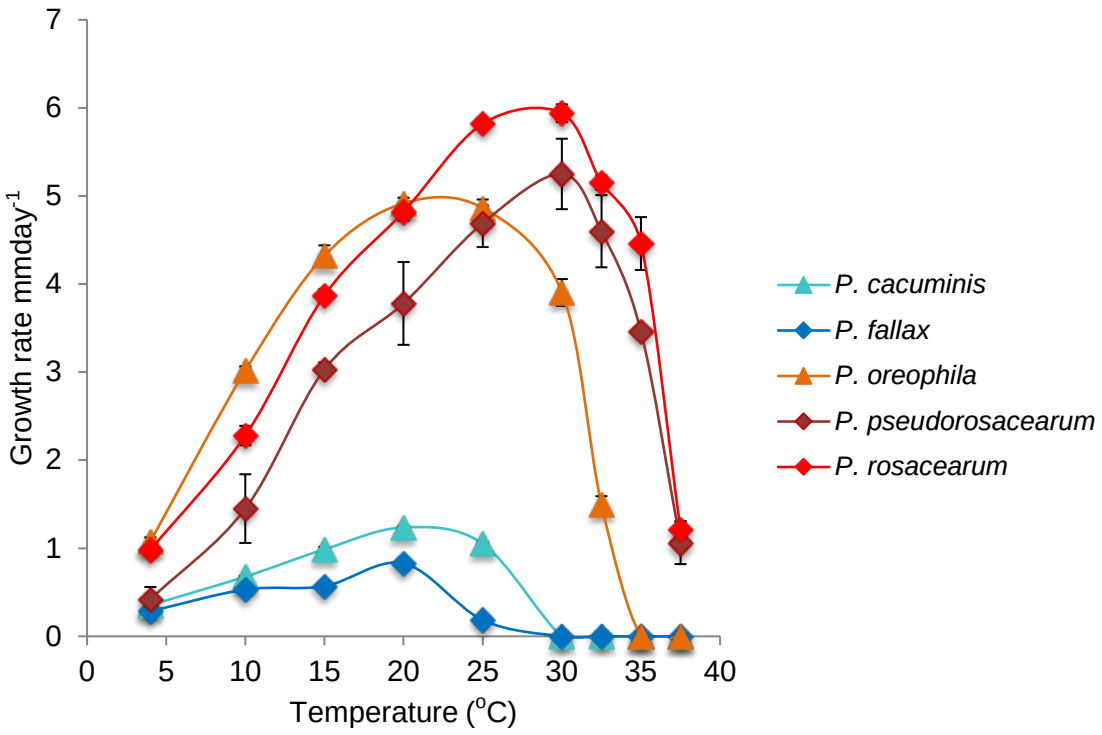

Figure 3.6 Average growth rate (mm day^{-1}) of *P. cacuminis, P. fallax, P. oreophila, P. pseudorosacearum* and *P. rosacearum* on V8A across the temperature range from 4 - 37.5 °C.

Discussion

Eight *Phytophthora* species from phylogenetic clades 1, 6, 8 and 9 were recovered from alpine and sub-alpine areas of NSW and TAS by baiting rhizosphere soil and associated roots; two of these were new species. This is the first record on the recovery *Phytophthora cactorum, Phytophthora gonapodyides*, *Phytophthora pseudocryptogea,* and *Phytophthora oreophila* in KNP, and for *Phytophthora fallax,* and *Phytophthora cacuminis* in TAS. The other species have been recorded before, but this is the first time that any species, in this case *Phytophthora cryptogea,* has been recovered from the summit of Mt Kosciuszko- the highest point on mainland Australia (2228 m asl).

Phytophthora gregata was one of the most frequently isolated species. *Phytophthora gregata* belongs to phylogenetic clade 6 and has been previously isolated from natural vegetation in WA, and formally described by Jung *et al.* (2011). It had been referred to as *P.* taxon raspberry previously (Brasier *et al.* 2003, Jung *et al.* 2011). This species was also recovered from soil and water samples (Dunstan *et al.* 2016), and raspberry roots (Brasier *et al.* 2003) in Victoria, Pine Lake in TAS (Brasier *et al.* 2003, Jung *et al.* 2011), China (Huai *et al.* 2013), the US (Aram 2017), and Sweden and France (Brasier *et al.* 2003, Redondo *et al.* 2018). It was also detected in KNP and TAS in 2013 using HTS (Burgess *et al.* 2017b). *Phytophthora gregata,* along with *P. cryptogea,* is implicated in the widespread death of the endemic shrub

Pimelea bracteata in wetlands and riparian vegetation in northern KNP and surrounding areas. Plants at all growth stages are affected and there is very little or no regeneration (McDougall et al. 2018- unpublished). *Phytophthora gregata* has been shown to significantly reduce shoot/and or root growth of *Eucalpytus marginata, Corymbia calophylla, Banksia occidentalis, B. litoralis,* and *Lambertia infermis* in recent pathogenicity trials, although it did not kill them (Belhaj *et al.*). It is not known if this species is native or introduced to Australia, however its widespread occurrence in Europe in cooler climates suggests it is probably introduced.

Phytophthora cactorum was the other most frequently isolated species; it is a species in phylogenetic clade 1, has a broad host range affecting 150 plant species (Nienhaus 1960), and causes diseases from topical to temperate climates (Rytkönen *et al.* 2008, Liu *et al.* 2018). It has been isolated in agricultural systems in much of temperate eastern Australia, and was also detected in KNP and TAS using HTS (Burgess *et al.* 2017b). Although it has not been associated with a disease in the sub-alpine areas in Australia so far, it should be regarded as a serious threat due to its capability to cause disease at relatively low temperatures, and in many hosts. Liu *et al.* (2018) studied the effect of temperature on infection and development of fruit rot caused by *P. cactorum* and observed that young apple fruits inoculated with zoospores of *P. cactorum* developed visible lesions from 10-30 °C, with optimum temperature being 23.5 °C. Similarly, incidence and severity of *P. cactorum* increased with increased wetness duration (1-12 hours) over a temperature range of 10-30 °C on pears, and 7 – 10 °C on apples under controlled environmental conditions (Grove & Boal 1991). *Phytophthora cactorum* has been reported to readily form chlamydospores in V8A juice broth and mycelial mats buried in pasturized soil at 4 °C after 20 days of incubation (Darmono & Parke 1990). Chlamydospores had high (60-80%) germination rates even after incubating at -23 °C for 24 hours (Darmono & Parke 1990). It is considered to have been introduced to lowlands in Australia and then spread to mountain ecosystems by human activities (Burgess *et al.* 2017b).

Phytophthora fallax belongs to phylogenetic clade 9, and was first described on *Eucalyptus,* causing leaf spots, petiole, twigs and small branches infection in New Zealand (Dick *et al.* 2006). It has previously been recovered in *Eucalyptus regnans* forests in Victoria (Cunnington *et al.* 2010, Dunstan *et al.* 2016), and in sub-alpine areas using HTS (Burgess *et al.* 2017b). Dick *et al.* (2006) evaluated its temperature-growth relationships when describing this species and suggested that it had adapted to colder temperatures due to its low cardinal temperatures. To date, no disease has been associated with this species in Australia. The reason could be the nature of the lesions they cause, which usually have no distinctive patterns (Dick *et al.* 2006). Another reason could be its mode of infection. It has

been observed to cause disease only in the inaccessible crown of trees, 6-20 m high, with an unknown mode of dispersal (Dick *et al.* 2006). Therefore, it is difficult to observe symptoms from the ground. Finally, due to its affinity to lower temperatures, it is also possible that *P. fallax* may have been living in the alpine and sub-alpine regions for a long time, and may have co-evolved in these ecosystems attaining an equilibrium and is, therefore, not causing any observable symptoms. This species appears to be native to Australia's mountain ecosystems due to its adaptability to these colder ecosystems and its growth and survival at relatively lower temperatures.

Phytophthora gonapodyides belongs to phylogenetic clade 6 and was first described from submerged fruits and twigs in Denmark by Petersen (1909). It has a worldwide distribution in all climates except the tropics (Zeng *et al.* 2009), and is ubiquitous in aquatic ecosystems in northwestern USA and Europe (Jung *et al.* 1996, Brasier *et al.* 2003, Reeser *et al.* 2011). It has been found in Denmark, New Zealand, Chile, Australia, UK, USA, France, and Czechoslovakia infecting minor roots and small seedlings of a limited number of hosts, such as *Tsuga, Pseudotsuga, Rhododendron* and *Hebe* (Brasier *et al.* 1993, Erwin & Ribeiro 1996, Brasier *et al.* 2003). In Australia, it has been isolated from native vegetation in Pine Lake in TAS (Brasier *et al.* 2003), and from soil baiting in Victoria (Dunstan *et al.* 2016). It has also been detected in KNP through HTS (Burgess *et al.* 2017b). *Phytophthora gonapodyides* has higher maximum (up to 38 °C) and lower minimum temperatures (3 °C) for growth (Brasier *et al.* 2003, Nechwatal *et al.* 2013). Its isolation from colder environments or even arctic alpine environments, and its relatively higher optimum (up to 33 °C) and maximum temperature (up to 38 °C) for growth is rather contrasting/surprising. A possible explanation for still remaining as a 'high temperature taxon' could be its physiological adaptation to certain aspects of its ecology, such as litter breakdown, rather than climatic adaption (Brasier *et al.* 2003). Further research is recommended to investigate its survival strategies at these higher altitudes considering it does not produce chlamydospores. The origin of this species is uncertain due to its wide distribution due to anthropogenic activities (Jung *et al.* 2011). It has perhaps been introduced to Australia's lowland ecosystems, and from these to mountain ecosystems.

Phytophthora cryptogea belongs to phylogenetic clade 8, and was first described from foot root of tomato in 1919 (Pethybridge & Lafferty 1919). This species has been associated with *Rubus anglocandicans* decline in WA (Aghighi *et al.* 2016), and had also been isolated from aquatic ecosystems in WA (Hüberli *et al.* 2013). *Phytophthora cryptogea* has been reported to cause a collar rot in *Pimelea bracteata* in south-eastern NSW (Bago State Forest; McDougall et al. 2018- unpublished). *Phytophthora pseudocryptogea* also belongs to phylogenetic clade 8 and was formally described in 2015; it was given the name

pseudocryptogea based on its physiological and morphological resemblance to *P. cryptogea* (Safaiefarahani *et al.* 2015). Recent pathogenicity trials have shown that this species is not pathogenic on *Corymbia calophylla* (Croeser *et al.* 2018), and it has not been associated with a disease in KNP. This is the first record on the recovery of living isolates of *P. pseudocryptogea* in KNP, although it had been detected in TAS through HTS (Burgess *et al.* 2017b).

In vitro, both *P. cryptogea* and *P. pseudocrytogea* have high optimum (25 °C) and maximum temperatures (33 °C for *P. cryptogea* and 35 °C for *P. pseudocryptogea*) for growth (Safaiefarahani *et al.* 2015). On the other hand, their minimum temperature for growth is also very low (3 °C) (Safaiefarahani *et al.* 2015). This shows that these pathogens have the ability to grow at both low and high temperatures. Therefore, they have the capability to cause disease both in tropical and temperate climates. Their centre of origin is unknown, but it is believed that anthropogenic activities associated with nurseries, horticulture, and agriculture dispersed these pathogens globally (Brasier 2008, Stukenbrock & McDonald 2008). These invasive species were probably introduced to Australia's lowland ecosystems and then spread to mountains. It is interesting that *P. cryptogea* was isolated at these higher altitudes, and not *P. pseudocryptogea,* because the most frequently isolated species in the 'cryptogea' complex in lowlands in Australia is *P. pseudocryptogea* (CPSM unpublished data).

The two new *Phytophthora* species, *P. cacuminis* and *P. oreophila* described here have not been isolated anywhere in the world before. Therefore, it is not clear whether they are native to Australia or introduced. Nonetheless, their temperature growth-relationships suggests that these species are well adapted to these colder conditions. *Phytophthora oreophila* has a higher growth rate at temperatures less than 20 °C, and has a much lower optimal temperature than related species, *P. rosacearum, pseudorosacearum, P. kwongonina,* and *P. cooljarloo* and is the only species to have been isolated from cold environments. Additionally, *P. oreophila* exhibit prolific growth at temperatures less than 4 °C. *Phytophthora cacuminis* is closely related to *P. fallax* and *P. constricta,* and all of them have low optimum and minimum temperature for growth. *P. fallax* and *P. cacuminis* have been isolated from mountains, but *P. constricta* has only been isolated from lowlands.

Although *P. cacuminis* failed to produce oospores, its low cardinal temperatures and the ability to produce chlamydospores suggest that this species is well adapted to colder environments. The establishment of *Phytophthora* species in colder environments is mainly determined by low cardinal temperatures for growth and asexual structures rather than the ability to produce sexual structures (Redondo *et al.* 2018). Further research is recommended

to understand their distribution, ecology, host-pathogen interactions, and to determine centres of origin of these new species.

Whilst the incidence of *Phytophthora* diseases is well documented in horticultural environments, ornamental plants grown in nurseries, and other lowland ecosystems (Henricot *et al.* 2014, Català *et al.* 2015, Jung *et al.* 2016), sub-alpine regions have received little attention. In our literature searches, there appear to be a very few records on the occurrence of *Phytophthora* species in the sub-alpine ecosystems (Newby 2014, Scarlett *et al.* 2015, Green 2016). This is due to the presumed assumption that lower temperatures restrict the growth and sporulation of *Phytophthora* and are not conducive to disease expression (Chee & Newhook 1965a, Shepherd & Pratt 1974, Phillips & Weste 1985, Shearer *et al.* 1987, Podger *et al.* 1990, Shelley *et al.* 2017, Rafiei *et al.* 2018). As such, the recovery of such a diverse *Phytophthora* community in sub-alpine and alpine areas previously thought to be pathogen-free leads to many concerns, and raises questions about their introduction, survival and subsequent dispersal, as they could potentially have a devastating effect on the rare and threatened species in these ecosystems.

Anthropogenic activities are known to have distributed *Phytophthora* species widely since European colonization, for example through contaminated mud on vehicles, road building and mining, replanting using infected seedlings because of poor hygiene, bushwalkers, feral horses and apiarists (Brasier 2008, Cahill *et al.* 2008a, Callaghan & Guest 2015). Once introduced, *Phytophthora* species are dispersed by root to root contact between adjacent plants (Shearer *et al.* 2010), and movement of infested soil attached to visitor's boots, bicycles, management vehicles tires and feral horses (McDougall *et al.* 2003). Self sexual reproduction and parasexuality (Desprez-Loustau *et al.* 2007), phenotypic plasticity (Mariette *et al.* 2016), evolutionary potential (McDonald & Linde 2002), survival in host tissues in deep soil layers (Marçais *et al.* 1996), and the ability to form asexual survival structures and low cardinal temperature for growth can assist *Phytophthora* species to survive/invade extreme environments (Crone *et al.* 2013b, Redondo *et al.* 2018)

Thee recovery of apparently introduced (invasive) species *P. cactorum, P. gonapodyides, P. cryptogea* and *P. pseudocryptogea* surviving in these sub-alpine environments is of great concern. The involvement of *P. cryptogea* and the *P. gregata* in the decline of *Pimelea bracteata* in Bago State Forest at an elevation of 1160 m asl, and Kellys Plains in KNP at an elevation of 1270 m asl, respectively shows that *Phytophthora* species present in the sub-alpine ecosystems have the potential to cause disease. While the other species have not been associated with a disease, it does not necessarily mean they are not causing disease because no studies or surveys have been conducted on these pathogens in relation to diseases/hosts in these areas. Also, *Phytophthora* species have been known to live in plants

as biotrophs without causing observable disease symptoms (Crone *et al.* 2013a, Crone *et al.* 2013b). Therefore, asymptomatic areas where *Phytophthora* species have been detected/isolated should be explored more for soil suppression, host resistance and asymptomatic presence of *Phytophthora* species. Further work is required to determine the susceptibility of sub-alpine flora to a range of *Phytophthora* species. Glasshouse trials have recently tested the susceptibility of nine KNP sub-alpine shrub species to *P. cinnamomi* and *P. cambivora,* and found that one species *Phebalium squamulosum* was especially susceptible to both pathogens (Rigg *et al.* 2018). This is particularly important when temperature is rising globally, which will shift the climatic range of *Phytophthora* species and other pathogens, and render some host species more susceptible to disease.

In conclusion, the occurrence of such a diverse *Phytophthora* community in alpine and sub-alpine ecosystems, previously considered not suitable for *Phytophthora* indicate that alpine and sub-alpine areas of KNP are at risk. It is now important to restrict its further spread to protect the diverse and unique flora in alpine/sub-alpine ecosystem. Road closure is the best management strategy to reduce the spread of invasive pathogens. In areas where road closure is not possible, roads should be engineered in a way to stop the flow of water from roads to adjacent areas and/or reduce the chances of infested soil uptake by vehicle from wet areas (Colquhoun & Hardy 2000, Hansen *et al.* 2000). Besides landscape features, the inclusion of non-host and resistant vegetation can also reduce the dispersal of the pathogens (Holdenrieder *et al.* 2004). Visitors and staff need to be educated on hygiene and the potential spread of these pathogens via vehicles, cycling and walking. Overall, strict measures need to be taken to decrease introduction pathways, human land use, and habitat disturbance to reduce the spread of pathogens into colder environments.

Chapter 4: Phenotypic plasticity favours range expansion of *Phytophthora cinnamomi* into colder environments

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Khaliq I, Hardy GES, Burgess TI (2018) Phenotypic plasticity favours range expansion of *Phytophthora cinnamomi* into colder environments. Submitted to Plant Pathology

Khaliq performed the lab work and was responsible for the writing and correspondence with the journal. Burgess and Hardy were supervisors

Abstract

Phytophthora cinnamomi has recently been found in highly diverse and fragile alpine/sub-alpine environments previously considered pathogen and disease free due to low temperatures. We investigated the ability of *P. cinnamomi* to adapt to cold and cause disease in the laboratory under conditions comparable to alpine/sub-alpine environments. Initially, the ability of *P. cinnamomi* isolates to grow and sporulate at 10°C was demonstrated (2 °C lower than previously thought possible). The phenotypic plasticity of isolates was then explored *in planta* in three successive experiments comparing cold (8, 9, 7.5°C) and ambient conditions. Isolates grown under cold conditions produced sporangia and released zoospores (infective propagules) at 7.5°C, even lower than originally considered possible. No changes were observed for isolates grown under glasshouse conditions. Although *P. cinnamomi* isolates could produce infective propagules at 7.5 °C *in vitro*, they could not be recovered from plants grown at this temperature after three months.

Introduction

Phytophthora cinnamomi is one of the world's most devastating plant pathogens. It is the only oomycete, and one of the only three plant pathogens listed as one of the 100 worst invasive alien plant pathogens in The Global Invasive Species Database (Lowe *et al.* 2000, Burgess *et al.* 2017a). Sub-alpine regions were long considered free of *P. cinnamomi* because harsh environmental conditions were thought to limit its survival. Podger *et al.* (1990) modeled damage caused by *P. cinnamomi* in Tasmania and hypothesized that it is unlikely to cause disease in areas where annual mean temperature is less than 7.5 ˚C. *Phytophthora cinnamomi* becomes inactive when temperature drops below 10 °C (Weste & Marks 1987) in soil, and no growth is shown on artificial media below 5 °C (McConnell & Balci 2015). However, *P. cinnamomi* was isolated at an elevation of 1560 masl (metres above sea level) at Barrington Tops National Park from dying *Oxylobium arborescens* and associated rhizosphere soil (Mills 1999, McDougall *et al.* 2003), where average annual maximum and minimum temperatures are 16 °C and 3 °C, respectively (Zoete 2000).

In 2013, 640 soil samples were obtained from across Australia, including 110 samples from above the tree line in sub-alpine regions of Tasmania, (TAS) Victoria (VIC) and New South Wales (NSW). Rather than traditional baiting, DNA was extracted from these soils, and subsequent high through-put sequencing (HTS) detected 68 *Phytophthora* species (Burgess *et al.* 2017b). *Phytophthora cinnamomi* was detected up to 2100 (i.e. almost at the highest point of mainland Australia), 580 and 1433 masl in NSW, VIC and TAS, respectively. The previous distribution model using 7.5 °C as the lowest temperature limit for *P. cinnamomi* predicted these areas to be pathogen free (Podger *et al.* 1990); however, detailed data for the distribution of *P. cinnamomi* across a latitudinal gradient in North America (Thompson *et al.* 2014) was used to recalibrate the cold stress and a new model was generated (Burgess *et al.* 2017a). This new model predicted the climate to be suitable for the growth of *P. cinnamomi* in most sub-alpine regions of the world under the present environmental conditions and increased suitability/range expansion under expected climatic change (higher mean winter temperatures, seasonal precipitation shifts from summer into winter, global warming). Although the model provides an excellent projection of the pathogen distribution, it does not explain disease development in the presence of susceptible hosts because the presence of a pathogen does not automatically lead to disease development. Disease occurs due to complex interactions between a virulent pathogen, a susceptible host(s) and an environment favorable for long enough for the pathogen to cause a disease. Although *P. cinnamomi* was detected in the sub-alpine/alpine areas, it is not proof of a living organism as HTS can detect (e)DNA even if an organism is dead.

Published distributions of *P. cinnamomi* have been traditionally mapped based on observations of disease symptoms on susceptible plants. However, the pathogen infects and survives on some native plant species without causing observable disease symptoms (Crone *et al.* (2013a). The pathogen can also produce selfed oospores along with other survival structures, such as thick walled chlamydospores, stromata and lignitubers (Crone *et al.* 2013b, Jung *et al.* 2013), thus able to survive in harsh climatic conditions. Additionally, anthropogenic activities are known to have distributed this exotic pathogen across most of the high rainfall areas of Australia widely since European colonization, for example through contaminated mud on vehicles and heavy machinery, road building and mining (Cahill *et al.* 2008a, Callaghan & Guest 2015). Therefore, the extent of the true distribution of *P. cinnamomi* may be seriously underestimated, as distribution is not always associated with disease. Vegetation surveys since the 1940s were conducted under the assumption that the Snowy Mountains were too cold for *P. cinnamomi* (Green 2016). This background assumption does not consider its phenotypic plasticity.

Phenotypic plasticity is the ability of organisms to express different phenotypes in response to changes in living or non-living environments. Such changes occur frequently in nature in response to heterogenic environments and can be inherited (Holeski *et al.* 2012, Kasuga *et al.* 2012). Plasticity is essential for organisms to survive in novel habitats, and once established, all the heritable differences are accumulated by natural selection and the phenotypes achieved become genetic (adaptive) (Baldwin 1896). However, the rate of adaption is different depending on the relative closeness of a phenotype to the desired trait in a novel environment (Price *et al.* 2003, Garbelotto *et al.* 2015). When the phenotype matches with the optimum phenotype in a novel environment, adaptive genetic differentiation stops (Price *et al.* 2003). The recent detection of *P. cinnamomi* at alpine/sub-alpine regions shows that the pathogen could be exhibiting adaptive phenotypic plasticity.

In Australia, there are hosts susceptible to *P. cinnamomi* above the tree line in alpine and sub-alpine regions (Rigg *et al.* 2018). Thus, in order to get disease, a warming climate must be present, which is predicted by the model of Burgess *et al.* (2017a), and/or the pathogen has adapted to colder environments as indicated by its detection from sub-alpine and alpine areas in Australia. Therefore, the current study asked the following questions: can individual *P. cinnamomi* isolates be 'trained' under cold conditions to (1) grow, produce sporangia and release infective propagules (zoospores) at temperatures lower than they were originally capable of, and (2) infect and cause disease at temperatures lower than they were originally capable of?

Materials and methods

Isolates and media

A total of 30 *Phytophthora cinnamomi* isolates were used. Of these, 22 isolates were from the culture collection of Hüberli *et al.* (2000), seven isolates were originally isolated from Queensland, and the remaining isolate was isolated from a sub-alpine area in Tasmania. Details of location and substrates of isolation of all isolates used in the study are given in Table 4.1.

Table 4.1. Host and location detail of *Phytophthora cinnamomi* isolates studied

Isolate code	Substrate	Location
MP62	*Eucalyptus marginata*	Jarrahdale, WA
MP80	*Eucalyptus calophylla* tap root	Jarrahdale, WA
MP84	*Eucalyptus calophylla* lignotuber	Jarrahdale, WA
MP88	*Eucalyptus calophylla* lignotuber	Jarrahdale, WA
MP89	*Eucalyptus calophylla* root	Jarrahdale, WA
MP114	*Eucalyptus calophylla* lignotuber	Willowdale, WA
MP119	*Eucalyptus calophylla* lignotuber	Willowdale, WA
MP126	*Eucalyptus marginata*	Huntly, WA
MP129	*Eucalyptus marginata*	Jarrahdale, WA
MP133	Avocado	Gatton, QLD
MP94-05	*Eucalyptus marginata* collar	Willowdale, WA
MP94-09	*Eucalyptus marginata* collar & root	Willowdale, WA
MP94-10	*Eucalyptus marginata* collar & root	Willowdale, WA
MP94-11	*Eucalyptus marginata* root	Willowdale, WA
MP94-12	*Eucalyptus marginata* collar & root	Willowdale, WA
MP94-15	*Eucalyptus marginata* lateral root	Willowdale, WA
MP94-18	*Eucalyptus calophylla*	Willowdale, WA
MP94-20	*Eucalyptus marginata*	Willowdale, WA
MP94-27	*Eucalyptus marginata* collar & stem	Willowdale, WA
MP94-33	*Eucalyptus calophylla* lignotuber	Willowdale, WA
MP94-39	*Eucalyptus marginata* collar	Willowdale, WA
MP94-48	*Eucalyptus marginata*	Willowdale, WA
MP94-49	*Eucalyptus marginata*	Willowdale, WA
MUCC813	Native Forest	Mount Lewis Road, QLD
MUCC814	Native Forest	Mount Lewis Road, QLD
MUCC815	Native Forest	Mount Lewis Road, QLD
MUCC816	Native Forest	Shoteel Creek, QLD
MUCC817	Native Forest	Tully Falls, QLD
MUCC818	Rainforest	Tully Falls, QLD
TAS188	Riparian rain forest	Condominium Creek, TAS

All isolates were passaged through Granny Smith apples to ensure they were at the same physiological stage. Briefly, two holes were made with a sterile scalpel of about 15 x 15 mm in each apple. The cores were removed and a 10 mm^2 colonized agar plug was placed into each hole; the cores were replaced and sealed with Glad® wrap (a plastic film). Each apple was kept for 2-3 days in a separate zip-lock bag (Sandvik, Australia) at 24 °C (±1°C) and tissues from the edge of brown discoloured lesions were plated onto modified *Phytophthora* selective medium 'NARH' (Simamora *et al.* 2017). The plates were incubated at 24 °C (±1°C) and examined under 10x magnification for the presence of characteristics typical for *P. cinnamomi*, specifically coralloid hyphae, pronounced hyphal swellings, and clusters of chlamydospores (Erwin & Ribeiro 1996). When confirmed, cultures were transferred onto individual vegetable juice agar (V8A) plates [100 ml/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery products Ltd., Norfolk, UK), 900 ml/L distilled water, 0.1 g/L CaCo3, pH adjusted to 7, and 17 g Grade A Agar (Becton, Dickenson and Company, Sparks, MD, USA) after sub-culturing onto NARH twice. The isolates were maintained at 24 °C (±1°C) on V8A for the entire experiment with regular sub-culturing.

Effect of temperature on radial growth

For growth rate studies, all 30 isolates were grown on V8A for four days at 24 °C (±1°C). Inoculum disks of 5 mm diameter were then cut from the margins of actively growing 4-day-old cultures in triplicates and inoculated centrally onto fresh V8A plates with mycelium placed face down. Plates were incubated at 24 °C (±1°C) for 24 hours to stimulate growth, colony margins were marked, and three replicate plates for each isolate were transferred to individual incubators set at 4, 7.5, 10, 15, 20, 25, 30, 35 and 37.5 °C in the dark. Incubators could fluctuate in temperature by ±1°C. Colony diameters were checked daily until the mycelial colony was about to reach the plate margins. Colony diameter at all temperatures was measured at two perpendicular points on each Petri dish. Radial growth was determined by averaging the two measurements per Petri dish and then the diameter of the agar disk used at the start of the trial was subtracted. Growth rates were calculated by dividing the radial growth rates on day 4, since the colonies reached the plate margins at day 4 at the optimum temperature. Plates showing no growth at higher and lower temperature were returned to 20 ˚C to check if the temperatures were lethal.

Method of sporangia production and subsequent count

The isolates were grown on V8A agar for 4 days at 24 °C (±1 °C). About 3-4 agar plugs (5 mm diameter) were then cut from the growing edge of the colony in triplicates from each isolate and transferred to sterile empty Petri dishes. Each Petri dish was flooded with 10%

clarified V8 broth (Erwin & Ribeiro 1996) until the broth was just above the surface of the agar plugs and all the plates were then directly transferred to incubators set at 4, 7.5, 10, 12.5, 15, 20, 25, 30, 32.5, 35 and 37.5 °C. The plates were incubated for 2-3 days at the higher temperatures (20, 25, 30, 32.5, 35 and 37.5 °C), a week at 15 °C and up to two weeks at lower temperatures (4, 7.5, 10 and 12.5 °C), to allow sufficient mycelium to be produced. The cultures were then rinsed thrice with deionised water, soaking for one hour for each rinse. Finally, all the cultures were flooded with 10% non-sterile pine (*Pinus radiata*) bark extract (100 g of pine bark potting mix in 1 L water, and left overnight) and incubated under light to encourage sporangia production. The number of intact and empty sporangia were counted in three fields of view at 20 and 25 °C and in six fields of view at all other temperatures at 10x magnification after 18-22 hours at higher temperatures (32.5, 35 and 37.5 °C), 24-48 hours at medium temperatures (20, 25 and 30 °C), 10 days at 15 °C, 15-20 days at 12.5 °C, and 20-24 days at 7.5 and 10 °C. Isolates kept at 4 °C were observed every week for eight weeks for sporangia production and zoospore release.

Effect of temperature on sporulation and zoospore release

From 30 isolates used in the radial growth trial, nine isolates (Fig. 4.1) were selected based on their growth rates at different cardinal temperatures to establish their temperature profile for sporulation and zoospore release. Their ability to produce sporangia and release zoospores was determined at 4, 7.5, 10, 12.5, 15, 20, 25, 30, 32.5, 35 and 37.5 °C. The number of intact and empty sporangia were counted as described above. The experiment was repeated to confirm the observations. Isolates showing differential sporulation and zoospore release were selected from this sporulation experiment for three successive experiments (below) each conducted over a six-month period in an attempt to 'train' isolates to grow faster, and sporulate and release zoospores at temperatures lower than originally established.

Phenotypic plasticity experiment 1

The inoculum was prepared by using vermiculite (1 L), millet (*Panicum miliaceaum*) seeds (10 g), and V8 broth (600 mL) as described by Simamora *et al.* (2016). The amount of inoculum used in all trials was 1% of the weight of the sand in pots.

An experiment was established with four isolates (MP94-48-0, TAS188-0, MP119-0 and MP114-0, where '0' designates original isolates) to establish the lower temperature limit (*in planta*) for these isolates inoculated on *Oxylobium ellipticum* seedlings and placed in a growth chamber set at 8 °C. Additionally, *O. ellipticum* seedlings were inoculated with MP94-

48-0 as an inoculum control and placed in an evaporatively cooled glasshouse at 25 (±5 °C). Each isolate was replicated three times. Briefly, the sand was first steam sterilised in hessian bags in an aluminium container for two hours at 98 °C. Seed trays (Punnets, Garden City Plastics, 90 mL) were also steam sterilised. *Oxylobium ellipticum* seedlings were germinated in sterilised river sand under glasshouse conditions. Plants were watered with deionised water and fertilised with soluble fertiliser Thrive® (Yates Company) when required at the recommended rates. Three months after sowing, the individual seedlings were pricked out into three 10 cell (to be placed in a growth chamber set at 8 °C) and one 10 cell punnet (to be placed in the glasshouse) (Punnets, Garden City Plastics, 90 mL) also contained sterilised sand. Three seedlings were placed in a 10 cell punnet and 12 seedlings were placed at random in the three 10 cell punnets leaving one empty cell between seedlings. When placing the seedlings into the punnets, a sterile 10 mL diameter plastic tube was also inserted into the sand in each cell for introducing inoculum later, without causing damage to the roots. Ten days after transplanting the seedlings to the punnets, seedlings in the three 10 cell punnet (to be transferred to the growth chamber) were inoculated with the four *P. cinnamomi* isolates by removing the plastic tubes from each cell and placing 1 g (1% of the weight of the sand) inoculum into each hole. The other punnet (to be kept in the glasshouse) was inoculated with MP94-48-0 in the same manner. The holes were then filled with sand. The punnets were then flooded with deionised water overnight to stimulate sporangia production and subsequent zoospore release and host infection by zoospores.

Disease progression was recorded over time and the time to seedling death was also recorded. Re-isolations were made from dying plants during growth on modified NARH to confirm Koch's postulates. Three months after inoculation, the remaining seedlings were gently removed from the punnets and sand was gently rinsed with tap water. Roots and shoots were cut into 2 x 2 cm segments, blotted dry on paper towels and plated onto modified NARH. *Phytophthora cinnamomi* was recovered only from plants inoculated with TAS188-0 at 8 °C. This isolate is henceforth known as TAS188-1. Isolate MP94-48-0 was also recovered from the seedlings grown in the glasshouse at ambient temperature (henceforth known as MP94-48-1). TAS188-1 was stored on V8 agar at 12.5 °C to make sure it stays at low temperature at all times and MP94-48-1 was stored in a controlled temperature room (24 °C ±1 °C). Radial growth rates on agar, and number of intact and empty sporangia for these two and original isolates were determined across the same temperature range as described above. These characteristics were compared to the original isolates to see if there was any change in the number of days radial growth took to reach the plate edge, or the number of days it took to sporulate and release zoospores, and shift in temperature range in the case of sporulation.

Phenotypic plasticity experiment 2

Five isolates were used in this experiment. Of these, two isolates (TAS188-1 and MP94-48-1) were recovered in phenotypic plasticity experiment 1; and three isolates (TAS188-0, MP80-0 and MP89-0) were selected from the original sporulation experiment based on their different growth characteristics at different temperatures in an attempt to 'train' them to grow faster, and to sporulate and release zoospores at temperatures lower than originally established.

Zoospores and mycelial plugs were used as an inoculum source instead of vermiculite, as only one of the four isolates was recovered by using vermiculite inoculum. For the zoospore inoculum, isolates were grown on V8A agar for 4 days at 24 °C (±1 °C) and sporangia were produced as described above. The cultures were then cold shocked at 4 °C for 30 minutes to encourage zoospore release. The cultures were left at room temperature for one hour following cold shock to ensure maximum zoospore release. The concentration of zoospores in the suspension was calculated by placing 10 x 2 µL drops of encysted zoospore suspension on a slide and counting zoospores present at 10x magnification. The suspension was diluted with deionized water to a zoospore concentration of approximately 1 x 10^5/mL. For the mycelial inoculum, all isolates were grown on V8A for 4 days and small agar plugs (5 mm diameter) were taken from the growing edge of the colonies to inoculate plants.

Oxylobium ellipticum seedlings were germinated in sterilised river sand under glasshouse conditions. The sand and punnets were steam sterilised as described above. Three months after sowing, the individual seedlings were pricked out into six punnets each consisted of six cells containing sterilised sand. When planting the seedlings, two sterile 10 mL diameter plastic tubes were also inserted on either side of the seedling into the sand in each cell, one for placing the *P. cinnamomi* colonised agar plug and the other for introducing the zoospores.

After 10 days of acclimatisation, the seedlings were watered thoroughly and immediately inoculated with the five *P. cinnamomi* isolates by removing the plastic tubes from each cell and placing a 5 mm diameter colonised agar inoculum into one hole and pouring 1 x 10^5/mL zoospores suspension into the other hole. One seedling in each punnet was left as a non-inoculated control. The holes were then filled with sand. The punnets were immediately flooded overnight with deionised water to stimulate sporangia production and provide a moist environment for the zoospores to infect the host. After flooding overnight, one set of plants (6 punnets) was transferred to a growth chamber set at 9 °C. The other set of plants (6 punnets) was left in an evaporatively cooled glasshouse at ambient temperature 25 (±5 °C) for comparison to ensure changes in growth characteristics or sporulation are due to exposure to cold temperatures, not due to a host effect. All punnets were randomised weekly

and flooded every fortnight until harvested. Plants were watered with deionised water and fertilised with soluble fertiliser Thrive® (Yates Company) when required at recommended rates.

Disease progression was recorded over time and the time to seedling death was also recorded. Re-isolations were made from dying plants and plated on NARH to confirm Koch's postulates. After identity confirmation, the re-isolated *P. cinnamomi* isolates grown at 9 °C *in planta* were stored at 12.5 °C on V8A, and the others grown at ambient temperature were stored in a controlled temperature room (24 °C ±1) on V8A. Three months after inoculation, surviving seedlings were harvested, and re-isolations were made from 2 x 2 mm surface sterilised root and collar tissues on NARH. Roots were cleared and stained using the method described by Brundrett *et al.* (1996). Briefly, for clearing, small roots were flooded in 10% KOH overnight and rinsed with de-ionised water. For staining, roots were immersed in lactoglycerol trypan blue (88% lactic acid, glycerol and distilled water (1:1:1 + 0.05% w/v trypan blue). The roots were de-stained for 12 h using lactoglycerol (1:1 of 88% lactic acid, glycerol). The same solution was used for root storage. Sections of fine roots were place on slides, covered with lactoglycerol and gently squeezed with a cover slip. Slides were examined for morphological structures typical of *P. cinnamomi* at 40x under a BX51 Olympus microscope. Growth rate, numbers of intact and empty sporangia were determined across the same temperature range as described above. The radial growth rate and sporulation experiments were repeated for lower temperatures (7.5, 10 and 12.5 °C) to confirm the observations.

Phenotypic plasticity experiment 3

Phenotypic plasticity experiment 3 was identical to phenotypic plasticity experiment 2 except temperature in the growth chamber was dropped from 9 ˚C to 7.5 ˚C.

Data analysis

For radial growth rate experiment, one-way ANOVA was performed in Microsoft Excel to analyse the effect of different temperatures on radial growth rates of different *P. cinnamomi* isolates. The means of different treatments were compared using Tukey's test.

Results

Effect of temperature on radial growth

All 30 isolates showed different growth rates at different temperatures. The isolate MP89 grew faster (mean 3.64 mm day^{-1}) than all other isolates except MP84 (mean 3.37 mm day^{-1}). The isolates MP89 had significantly ($p < 0.05$) higher growth rate than all other isolates, while MUCC815 was the only isolate to show significantly slower growth rate (1.46 mm day^{-1}) at all temperatures than all other isolates (Table 4.S1). The optimum temperature for growth for all isolates, except MP114, was observed over a temperature range of 25-30 ˚C. The optimum growth temperature for 19 of 30 isolates on V8A was found to be 30 ˚C, while 10 of the 30 isolates exhibited an optimum growth at 25 ˚C. Whilst, MP114 exhibited an optimal growth at 20 ˚C. All isolates exhibited significantly ($p < 0.05$) slower growth rates at 4, 7. 5 and 35 ˚C, and a rapid increase in growth almost in a linear fashion was observed from 10-30 ˚C. A rapid decline in growth rate for all isolates was observed at temperatures greater than 30 ˚C. The radial growth rate of all 30 isolates is shown in Table 4.S1, Fig. 4.S1.

Effect of temperature on sporulation and zoospore release

The temperature range for sporulation and zoospore release was narrower than the range over which it grew on V8A. Sporangia production and zoospore release were observed between 10-30 ˚C in the non-sterile pine bark extract (Fig. 4.1). Sporulation was prolific between 25 and 30 ˚C; however, one isolate (MUCC815) only produced sporangia at 20 and 25 ˚C. The highest number of sporangia mm^{-2} was produced at 25 ˚C for all nine isolates tested. Similarly, the optimum temperature for zoospore release mm^{-2} was 25 ˚C for seven of the nine isolates. The optimum temperature for zoospore release for the remaining two isolates (MP89 and MP84) was 30 ˚C. Similar to radial growth, the number of sporangia produced increased rapidly in a linear fashion from 10-30 ˚C. A few aborted sporangia with shrunken cytoplasm were observed at 32.5 ˚C. Sporangia production was infrequent at 10 and 12.5 ˚C and zoospore release was limited at temperatures less than 15 ˚C. Additionally, the rate of sporulation and zoospore release was much slower at 10 and 12.5 ˚C, as they were observed after 20-24 days compared to 24-48 hours at the optimum temperatures. Sporulation and zoospore release/sporangium at lower temperature increased with increasing incubation period in both V8A and pine bark extract. Mean radial growth rates, number of sporangia produced and number of empty sporangia (indicating zoospore release) for the nine *P. cinnamomi* isolates at different temperatures are shown in Fig. 4.1.

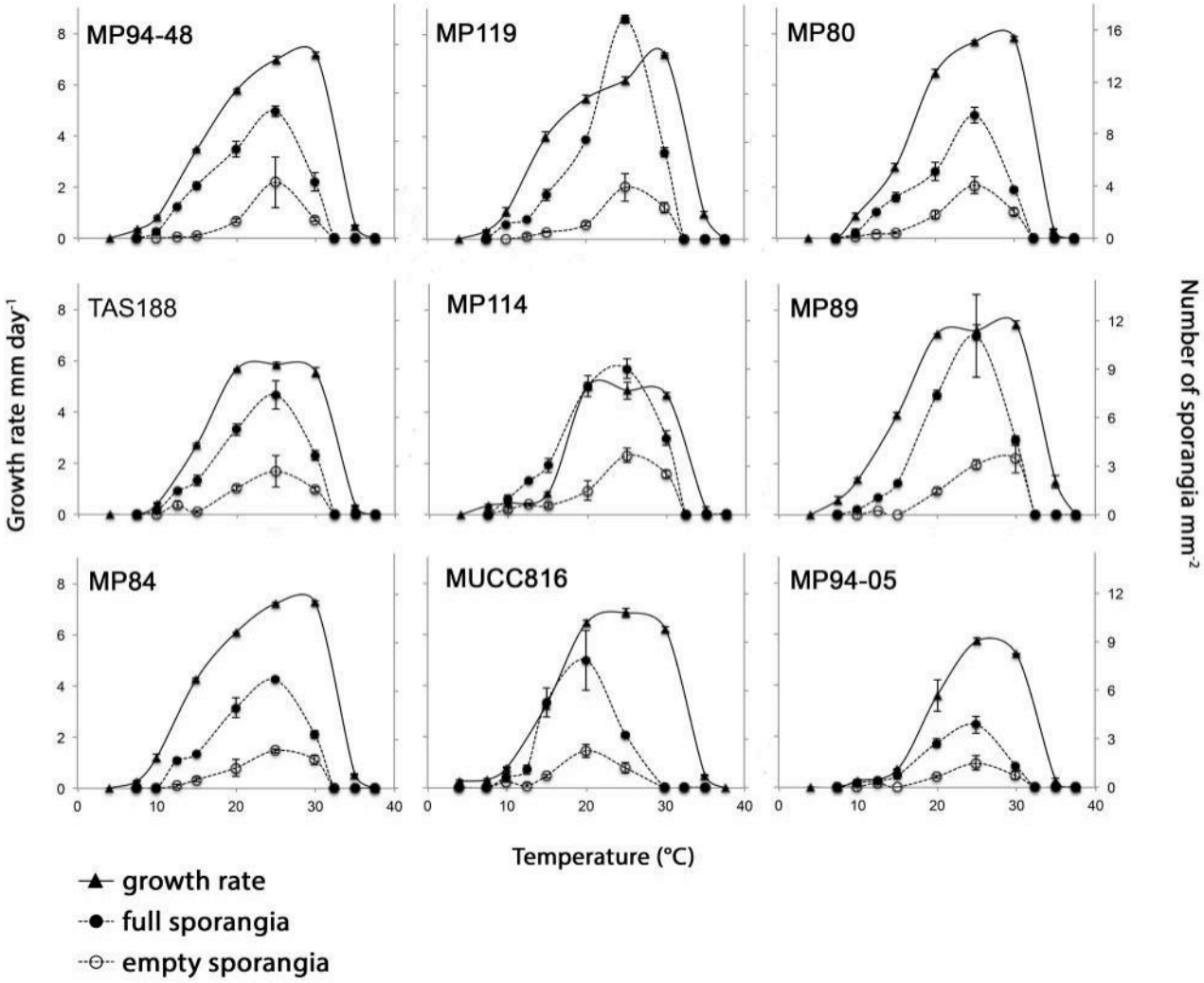

Figure 4.1. Mean radial growth rate (mm day^{-1}), number of intact and empty (indicative of zoospore release) sporangia (mm^{-2}) for nine *P. cinnamomi* isolates at different temperatures.

Phenotypic plasticity experiment 1

In the phenotypic plasticity experiment 1, disease symptoms started to appear on seedlings grown in the glasshouse two weeks after inoculation. Some seedlings died during the trial and isolate MP94-48, inoculated onto seedlings as an inoculum control, was recovered (henceforth referred to as MP94-48-1). In contrast to seedlings grown in the glasshouse, no visual increase in height was observed for seedlings grown in the growth chamber. Lower leaves developed chlorotic lesions 32 days after inoculation, which later became necrotic and some seedlings died possibly due to cold stress. Also, seedling roots had less roots mass and were shorter. Of the four isolates inoculated on seedlings grown in the growth chamber

at 8 ˚C, only one isolate TAS188-0 (henceforth referred to as TAS188-1) was recovered at harvest.

The recovered isolates (from glasshouse and growth chamber) grew faster and took three days to reach plate edges as compared to four days by taken by original isolates at optimum temperature (25 ˚C), which was probably due to host effect. Temperature range for sporulation (10-30 ˚C) was similar to the original isolates. In summary, no phenotypic plasticity features were observed in phenotypic plasticity experiment 1 (Fig 4.2).

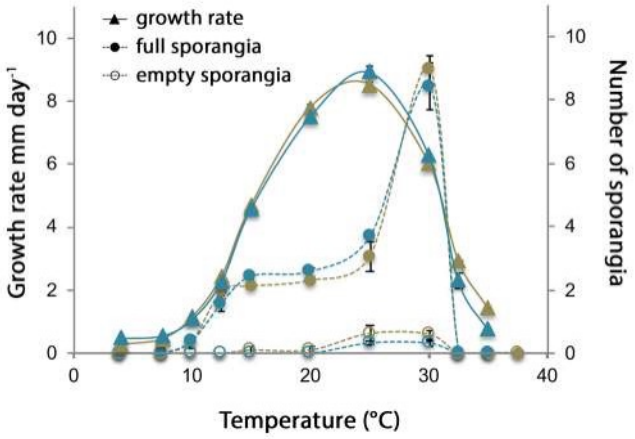

Figure 4.2. Comparison of mean radial growth rates (mm day^{-1}) and numbers of intact and empty (indicative of zoospore release) sporangia (mm^{-2}) for the isolate TAS-188, where orange lines indicate original isolates (TAS188-0) and blue lines indicate the isolate (TAS188-1) recovered from plants grown in the growth chamber at 8 ˚C for three months. Bars = SE

Phenotypic plasticity experiment 2

In the phenotypic plasticity experiment 2, all five isolates were recovered from seedlings grown in the growth chamber at 9 ˚C along with all the five isolates inoculated on seedlings grown in the glasshouse at ambient temperature 25 ˚C (±5 °C). Seedlings grown in the glasshouse became chlorotic two weeks after inoculation and later died. Some seedlings died due to collar rot. Seedlings grown at 9 ˚C exhibited stunted growth as compared to the seedlings grown in the glasshouse. Lower leaves developed chlorotic lesions 25 days after inoculation, which later became necrotic and a few seedlings died due to collar rot. Root systems of seedlings grown in the glasshouse were healthier and larger than of seedlings grown in the growth chamber at 9 ˚C. Isolates were recovered from dead and surviving seedling on NARH. No considerable variation in growth rate was observed between isolates recovered from the growth chamber and glasshouse.

A range shift in sporulation was observed for three isolates (TAS188-1, TAS188-2 and MP80) recovered from seedlings grown in the growth chamber at 9 ˚C, as they were able to

sporulate and release zoospores at 7. 5 ˚C (Fig. 4.3); compared to the original isolates where the lowest temperature they were able to sporulate and release zoospores at was 10 ˚C (Fig. 4.1). Additionally, these isolates took only 15 days to produce sporangia at 7.5 ˚C compared to 20-24 days at 10 ˚C for the original isolates (Table 4.2). However, two of the five isolates recovered from the growth chamber did not produce at 7.5 ˚C (Fig. 4.S2). No viable sporangia or zoospore release were observed at 7.5 ˚C for isolates recovered from seedlings grown in the glasshouse (Fig. 4.3).

Figure 4.3. Comparison of mean number of intact and empty (indicative of zoospore release) sporangia (mm^{-2}) between isolates recovered from plants grown in the glasshouse (orange) at ambient temperature and those recovered from plants grown in the growth chamber at 9 ˚C (blue) for three months. Bars = SE

Phenotypic plasticity experiment 3

In the phenotypic plasticity experiment 3, no isolates of *P. cinnamomi* were recovered from seedlings grown in the growth chamber set at 7.5 ˚C. Seedlings growing in the glasshouse showed severe disease symptoms and had been killed by *P. cinnamomi.*

Table 4.2. Incubation period in non-sterile potting mix extract required by *P. cinnamomi* to produce sporangia at different temperatures in each phenotypic plasticity experiment. No isolates were recovered from seedlings grown in the chamber at 7.5 ˚C in phenotypic plasticity experiment 3.

Temperature (°C)	Number of days to produce sporangia		
	Original experiment	Phenotypic plasticity experiment 1	Phenotypic plasticity experiment 2
7.5			15
10	20-24	20-24	18-20
12.5	15-20	15-20	15-18
15	10-15	10-15	10
20	1-2	1-2	1-2
25	1-2	1-2	1-2
30	1-2	1-2	1-2

Discussion

In response to cold condition comparable to that in alpine/sub-alpine environments, three of the five *Phytophthora cinnamomi* isolates evaluated exhibited phenotypic plasticity and were able to produce sporangia and release zoospores at lower temperature than they were originally capable of. Although *P. cinnamomi* isolates could produce infective propagules at 7.5 °C *in vitro*, they could not be recovered from plants grown at this temperature after three months.

Effect of temperature on radial growth

The optimum temperature for growth rate for 29 of 30 *P. cinnamomi* isolates on V8A was observed over a range of 25-30 ˚C. Only one isolate exhibited optimum growth at 20 ˚C and no growth was observed outside 5-35 ˚C. These results agree with those obtained by Shepherd *et al.* (1974) for 50 Australian *P. cinnamomi* isolates using the same medium. Similar results were obtained by Phillips and Weste (1985) using PDA. In contrast to original isolates, the recovered isolates from the growth chamber and glasshouse exhibited faster growth rate in the present study. The radial growth rates are not directly comparable because the recovered isolates took three days to reach plate margins as compared to four days taken by original isolates. This can probably be attributed to host effect, as the original

isolates were stored on agar for a long time; host infection enhanced their pathogenicity and growth rate. There is discrepancy in the literature regarding differentiating *P. cinnamomi* based on growth rates. Temperature characteristics show little variation after subculturing for a long period, and isolates of the same species collected from different hosts and origins do not exhibit much variation in response to different temperatures (Leonian 1925, Tucker 1931, Chee & Newhook 1965a, Zentmyer *et al.* 1976). In contrast, considerable variation was observed among isolates of the same species from different (White 1937, cited in Shepherd and Pratt 1974) and both the same and different geographic origins (Shepherd & Pratt 1974).

Effect of temperature on sporulation and zoospore release

Sporangia production was observed over a range of temperatures from 10-30 ˚C in the initial sporulation experiment. Unlike the radial growth on agar, no sporangia production was observed over 30 ˚C. This result supports the findings of Chee and Newhook (1965a) who showed that temperature range for sporangia production was narrower than the range at which mycelium grew. No relationship was found between the optimum temperature for sporulation/zoospore release, and geographical origin of the isolates, which is in line with the findings of previous studies (Chee & Newhook 1965b, Halsall & Williams 1984). The optimum temperature for sporangia production and zoospore release was observed at 25 ˚C, suggesting that pathogen will probably be more virulent at this temperature. No viable sporangia were observed over 30 ˚C. The pathogen produced a few sporangia at 32.5 ˚C, but these were aborted due to the higher temperature.

The lower temperature limit for sporangia production by *P. cinnamomi* was found to be 12 ˚C by reviewing the literature (Chee & Newhook 1965a, Nesbitt *et al.* 1979, Shearer 2014). However, seven of nine isolates produced sporangia at 10 ˚C, prior to the phenotypic plasticity experiments, in the current study. This difference between this study and the previous ones could be due to increased incubation period (two weeks) in V8A broth to allow sufficient mycelium development, and in pine bark extract (20-24 days) to allow sufficient time for sporangia induction by pine bark and associated microflora at lower temperatures 7.5, 10 and 12.5 ˚C. Whereas, Chee and Newhook (1965a) incubated mats of aerial mycelium for just 48 hours, while Nesbitt *et al.* (1979) did not specify any incubation period. Incubation period has been reported to markedly affect the rate of sporangia production. The number of sporangia significantly increased after a 12 day incubation period compared to a 2 day incubation period (Shearer 2014).

Phenotypic plasticity in relation to sporulation, zoospore release and disease

A range shift in sporulation temperature from 10-30 ˚C to 7.5 to 30 ˚C was observed in the phenotypic plasticity experiment 2 for three (TAS188-1, TAS188-2 and MP80) of the five *P. cinnamomi* isolates grown in the growth chamber for three months at 9 ˚C. Also, a shorter incubation period was required (15 days) to produce sporangia at this temperature compared to the 20-24 days required for sporulation to occur at 10 ˚C in the initial experiment. This implies that *P. cinnamomi* is exhibiting phenotypic plasticity and producing sporangia and releasing zoospores at temperatures lower than it was originally capable of, and in a shorter period when 'trained' under cold conditions. This also suggests that *P. cinnamomi* has a plastic genotype and is able to persist in a broad range of environmental conditions. Phenotypic plasticity is a very common and efficient mechanism organisms use to adapt to changing climatic variables, such as a change in temperature (Via & Lande 1985, Laine 2008). A number of studies have demonstrated phenotypic plasticity in *Phytophthora* species. An example is the decreased sensitivity to a fungicide phosphite after its prolonged use in an horticultural environment (Dobrowolski *et al.* 2008). Hüberli *et al.* (2001) examined variation among 73 *P. cinnamomi* isolates collected from *E. marginata* and *Corymbia calophylla* trees in the southwest of Western Australia and found a broad range of phenotypic and pathogenic differences between them. Similarly, a significant variation in virulence was found among *P. ramorum* isolates when inoculated on different hosts, which indicates high levels of phenotypic plasticity (Rizzo *et al.* 2005, Kasuga *et al.* 2016). Mariette *et al.* (2016) tested the performance of *P. infestans* isolates (within a single clonal lineage) collected from different geographic origins across a range of temperatures and observed that isolates collected from colder climates performed better (produced more sporangia) at lower temperatures, than those collected from Mediterranean environments.

In addition to *Phytophthora,* the role of phenotypic plasticity in thermal adaptation has been examined in several true fungi (Zhan & Mcdonald 2011, Stefansson *et al.* 2013). Phenotypic plasticity can also enable pathogens to infect different hosts closely related to their ancestral hosts, although the infection could be mild at first (De Vienne *et al.* 2009). Phenotypic plasticity contributes to the success of invasiveness of species (Davidson *et al.* 2011). The theory is based on the assumptions that (i) invasive species come to a new area with a few members (usually with relatively low genetic diversity) and experience a different environment in which they evolved. Phenotypic plasticity will help these species in coping and establishing in the new environment (Schlichting & Levin 1986), and (ii) phenotypic plasticity will assist in taking benefit of environmental fluctuations, thus helping in the establishment of the species in a novel environment (van Kleunen & Richardson 2007). An

example is the invasion of semi arid areas by pathogens, including *P. infestans* where they were hardly expected to survive (Bashi & Rotem 1974).

Until recently, the literature on the invasive potential of plant pathogens has mainly focused the qualitative traits related to their successful infection, such as the presence/absence of compatible resistance and virulence genes in hosts and pathogens (Garbelotto *et al.* 2015). This resulted in the excessive usage of "lack of co-evolution" hypothesis to interpret the invasive potential of plant pathogens, which further led to fewer studies on infection capability, latent period, sporulation rate and spore size (Lannou 2012). Traits related to enhanced transmission, such as enhanced spore production and small spore size have been reported to be favoured over traits associated with increased virulence and competitive ability for successful invasions, as increased virulence results in hosts' death and complete arrest of sporulation (Garbelotto *et al.* 2015). Analysis of gene expression on dead and living tissues has revealed a trade off between parasitism and saprotrophic wood decay. While living on host tissues, gene expression favour more toxin production, protection against plant defences and managing abiotic stresses at the expense of carbohydrate decomposition and membrane transport capacity, indicating that pathogens do not use their full capacity for energy acquisition during saprophytic growth and rather keep a full arsenal of wood degrading enzymes (Olson *et al.* 2012).

Phytophthora species richness decreases with increasing elevation due to harsh climatic conditions (Redondo *et al.* 2018). *Phytophthora* species can be introduced to cold environments due to human related activities but only those manage to establish and spread which have a plastic genotype and can adapt to cold conditions, the rest are progressively filtered out along the elevation gradients (Redondo *et al.* 2018). Due to the isolation and detection of *P. cinnamomi* in alpine and sub-alpine environments (Mills 1999, McDougall *et al.* 2003, Burgess *et al.* 2017b) once considered pathogen free due to cold temperature (Podger *et al.* 1990), we hypothesize that the pathogen has adapted to cold environments. Our findings in the laboratory further confirm this observation; *P. cinnamomi* has a plastic genotype that responds rapidly to selection pressure and adapts to new environments. Therefore, models for *P. cinnamomi* distribution solely based on cardinal temperatures for growth have limited value, and need to be modified owing to the plastic genotype of the pathogen. This also suggests the need of taking preventive measures to stop further spread and establishment of the pathogen into sub-alpine and alpine areas.

Although *P. cinnamomi* produced infective propagules (zoospores) at 7.5 ˚C in pine bark extract in phenotypic plasticity experiment 2, the pathogen was not recovered from inoculated plants grown at this temperature after three months in phenotypic plasticity

experiment 3. It is not clear whether the pathogen was unable to infect the host at this temperature, or it could not withstand cold conditions for three months. There are discrepancies in the literature about the minimum, optimum and maximum temperature required by *P. cinnamomi* for host infection and survival under harsh conditions. For example, Shearer *et al.* (1987) reported the minimum, optimum and maximum temperature for *P. cinnamomi,* in roots of *Banksia grandis* and *Eucalyptus marginata,* to be 5, 29 and 34 ˚C, respectively. However, these temperature limits cannot be generalized because they vary with different media and substrates (Shearer *et al.* 1987). Whilst, Halsall and Williams (1984) showed that zoospores were formed, attracted to hosts (*Eucalyptus pilularis* and *E. maculata*) chemotactically, and infected them at temperatures as low as 6 ˚C. Although infection was slow and no symptoms were observed at 6 ˚C, *P. cinnamomi* was recovered from washed roots 6-8 weeks after inoculation. A significant reduction in growth was observed at temperatures as low as 10 ˚C (Halsall & Williams 1984). The authors further concluded that although little infection can occur at 6 ˚C, the pathogen is not pathogenic below 10 ˚C.

Pryce *et al.* (2002) found that there was no significant difference in the rate of isolation of *P. cinnamomi* in diseased and asymptomatic sites in the north Queensland. *Phytophthora cinnamomi* isolation from asymptomatic sites suggest that the environment at these locations is suitable for the pathogen but not for disease expression. This further suggests that, *P. cinnamomi* could be infecting plants in the alpine/sub-alpine areas asymptomatically under the current environmental conditions, and symptom expression may occur when temperature become warmer. This is further supported by the findings of Kliejunas and Nagata (1979) who assessed soil from three ohia forest sites in Hawaii for population levels of *P. cinnamomi* over an 8-13 month period, and found that the pathogen was either undetectable or its population was at the lowest during winter when soil temperature was near 10 ˚C; the detection rate increased with increasing temperature. *Phytophthora cinnamomi* has also been observed to infect plants asymptomatically even in a highly susceptible Mediterranean climate (Crone *et al.* 2013a, Crone *et al.* 2013b). Such behaviour makes the detection and management of *P. cinnamomi* very difficult (Newby 2014).

There could be several other reasons for the lack of disease expression in alpine/sub-alpine areas in NSW, including greater host resistance of local endemic flora to *P. cinnamomi* as demonstrated by McCredie *et al.* (1985). *Phytophthora cinnamomi* is known to have patchy distribution in soil (McDougall *et al.* 2003), therefore susceptible hosts may escape infection. Antagonism and competition between *P. cinnamomi* and other soil microorganisms is also known to affect the distribution (Broadbent & Baker 1974, Weste & Vithanage 1978) as well as disease expression (Broadbent & Baker 1974, Marks & Smith 1981) by *P. cinnamomi.*

Perhaps the most likely explanation for the lack of disease expression is related to climate. Temperature and moisture are the major limiting factors for the growth and development of *P. cinnamomi,* so if there is sufficient moisture availability, temperature will be the major important restrictive factor (Thompson *et al.* 2012). There is sufficient moisture availability in the sub-alpine/alpine areas, but temperatures at higher elevations (especially above 1400 masl) are likely to be too cold for *P. cinnamomi* disease expression.

Although conditions could be too cold for disease expression in sub-alpine regions, the pathogen is still able to survive. *Phytophthora cinnamomi* mainly infects roots (hence protected by host tissues) deep under soil layers where temperature seldom reaches less than 10 ˚C (Van Steekelenburg 1973, Marçais *et al.* 1996). This idea can be supported by the fact that *P. cambivora,* the species associated with the decline of *Nematolepis ovatifolia* in KNP, has proven difficult to isolate from rhizosphere soil*,* suggesting the soil is not favourable for the pathogen's survival structures at the higher elevations and the pathogen may only survive inside host tissues and spread through root-to-root contact rather than through soil via zoospores (Ristaino & Gumpertz 2000, Green 2016). *Phytophthora cinnamomi* has also been known to produce survival structures capable of withstanding unfavourable conditions (dry, hot and cold) for several years due to the chemical composition and thickness of their walls (Jung *et al.* 1999, Crone *et al.* 2013b, Jung *et al.* 2013).

Our finding that *P. cinnamomi* can exhibit plasticity and produce sporangia and release infective propagules at temperatures lower than originally thought possible (and in a shorter period) when 'trained' under cold conditions, and its isolation and detection at higher elevations previously considered pathogen free has significant ecological implications. Sub/alpine areas of KNP are at risk due to the presence of the pathogen and susceptible hosts. It is now important to restrict its further spread to protect the diverse and unique flora in alpine/sub-alpine ecosystems. As described above, the reasons for the lack of disease expression appear complex and need further investigation. The areas where *P. cinnamomi* has been detected should be explored more for soil suppression, host resistance and asymptomatic presence of *P. cinnamomi.* In vitro trials need to be conducted to investigate the interplay between different hosts, soil type, and low temperatures on disease expression. Pathogenicity trials should be set up with cold climate vegetation in the sub-alpine and alpine areas during different seasons without manipulating environmental conditions to get a real insight into host-pathogen interactions. In addition, *in vitro* trials need to be established at different temperatures to predict the pathogen behaviour under the changing climate. Only one isolate was from a sub-alpine area in the current study. Future studies should include multiple isolates from alpine/sub-alpine and Mediterranean environments i.e. demographic plasticity (Garbelotto *et al.* 2015), and compare growth rates x temperature, sporulation x

temperatures, spore size and pathogenicity of these isolates to get real insights on the pathogen adaption to colder environments.

Chapter 5: Comparison of distribution of *Phytophthora* and vascular plant species along a steep elevation gradient

Abstract

This study was conducted to compare how *Phytophthora* and vascular plant species' richness, composition and distribution vary along a steep elevation gradient. *Phytophthora* and vascular plant species were recorded over a wide range of elevation gradient (410-2125 m) in two 2 x 50 m^2 plots at each sampling point, one plot parallel to the road edge, and the other perpendicular to the road plot. *Phytophthora* and vascular plant species exhibited a linearly monotonic decline with increasing elevation on roads, but not in native vegetation. However, the elevation range of *Phytophthora* species was greater than vascular plants on both roads and in native vegetation. *Phytophthora* species exhibited similar composition and frequency on roads and in native vegetation, whilst vascular plants had greater frequency in native vegetation. Many *Phytophthora* species, including five regarded as non-native, are widespread in our study area (Kosciuszko National Park) but their richness at the plot level mimics that of vascular plants. The occurrence of a diverse *Phytophthora* community, which includes non-native species, is likely to affect resident vascular plant species, many of which are endemic to mountain environments; a changing climate may permit invasions by other *Phytophthora* species not yet present.

Introduction

Non-native plant species' richness within mountains usually decreases as elevation increases, and as such higher elevations are considered resistant to invasion. This decline along elevation gradients is typically continuous (Becker *et al.* 2005) in temperate areas or hump shaped (Arévalo *et al.* 2005) in tropical areas. The reasons for this decline are mainly related to harsh climatic conditions, decreased anthropogenic disturbance resulting in low propagule pressure, and lack of pre-adaptation and plasticity in non-native species (Pauchard *et al.* 2009, Alexander *et al.* 2011, Seipel *et al.* 2012). As roads are the primary reason for the introduction of non-native plant species, this pattern is especially pronounced along road corridors (Seipel *et al.* 2012). Conversely, changes in native plant species' richness vary with increasing elevation, and are scale-dependent (Nogués-Bravo *et al.* 2008); but typicaly hump-shaped patterns are observed in tropical areas (Kessler *et al.* 2011), and both continuous and hump-shaped patterns are observed in temperate areas (Grytnes & Vetaas 2002, Grytnes *et al.* 2006, Kluge *et al.* 2006, Nogués-Bravo *et al.* 2008).

While there is plant species movement from low elevations to high elevations along roads in mountains, there does not appear to be much exchange of species between roads and adjoining native vegetation (Seipel *et al.* 2012). The reasons could be different introduction history, intentional sowing of certain vegetation along roads, and increased competition from native vegetation (Seipel *et al.* 2012). For example, species richness 75 m away from roads was lower and independent of species richness along roads in the Australian Alps (Seipel *et al.* 2012). Additionally, species turnover is typically greater in native vegetation (Sandoya *et al.* 2017), and non-native plant species tend to be restricted to roads due to human related propagules pressure and exploitation of resources not fully utilized by native vegetation (Pauchard *et al.* 2009). Non-native plant species capable of establishing away from roads in native vegetation tend to prefer moist and shady habitat as opposed to the non-native species established on roads, which prefer open and well-drained habitat (Forman & Alexander 1998, McDougall *et al.* 2018).

Pathogens belonging to the genus *Phytophthora* cause significant losses to plants in agriculture, horticulture and natural ecosystems throughout the world. Globally, more than 66% of all fine root diseases and more than 90% of all collar rots of woody plants are caused by *Phytophthora* species (Tsao 1990b). Despite this, studies on ecological filtering for *Phytophthora* species are rare and with the exception of a few *Phytophthora* species that cause emerging diseases in natural (e.g., *Phytophthora cinnamomi* and *P. ramorum*) or agricultural (e.g., *P. infestans*) ecosystems, their ecological impacts have not yet been established adequately (Redondo *et al.* 2015, Redondo *et al.* 2018). Unlike vascular plants, *Phytophthora*

species are difficult to detect unless special cultural or molecular techniques are used (Jung *et al.* 2018, Khaliq *et al.* 2018b), and their native range often remains unknown even if they are novel and are causing major ecological impacts (Rizzo 2005).

Phytophthora species can be highly invasive and can destabilize whole ecosystems once introduced into host-pathogen non-coevolved ecosystems (Jung *et al.* 2016). Anthropogenic activities can introduce *Phytophthora* species into colder environments, but only ecologically compatible species will be able to survive and establish due to climate filtering (Redondo *et al.* 2015). Only a few small-scale studies have been conducted on *Phytophthora* in temperate mountain ecosystems (McDougall *et al.* 2003, Green 2016), but they do not represent the complex *Phytophthora* diversity, or their variation in relation to elevation and disturbance gradients. No studies have been conducted globally to compare the distribution patterns of *Phytophthora* and vascular plant species along elevation and disturbance gradients in a temperate mountain. This is particularly important when temperature is rising globally and expanding the range of *Phytophthora* species, which may render some host species more susceptible to disease (Sturrock *et al.* 2011).

The aim of the current study was to combine high throughput sequencing (HTS) with the traditional *Phytophthora* isolation method 'baiting' to determine whether patterns of *Phytophthora* species richness, composition and distribution in relation to elevation and disturbance are the same as for vascular plants. The patterns of species richness, composition and disturbance were determined for all (i.e. native and non-native) vascular plants and *Phytophthora* species due to lack of knowledge on the native or non-native origin of *Phytophthora* species.

Methods

Study area description

The study was conducted in the Kosciuszko National Park (KNP), New South Wales (NSW). The area of KNP comprises of approximately 790,000 hectares and most is subjected to severe winter weather from mid-May to mid-August (Jenkins & Morris 2003). The average rainfall ranges from 558 to 2343 mm and the mean daily temperatures in summer ranges from 2.4 to 29.7 ˚C and in winter from -5.6 to 11.1 ˚C (Bureau of Meteorology 1975). The alpine area around Mt Kosciuszko consists of 100 km^2 of true alpine vegetation starting from the treeline at approximately 1830 m to the top of Mt Kosciuszko at 2125 m (Costin *et al.* 2000).

Survey design and sampling

For vascular plants, sampling followed the protocol developed by the Mountain Invasion Research Network (Seipel *et al.* 2012). For the purposes of the current study, only selected elements of the protocol were used as follows. Three roads were selected for sampling in the KNP (Seipel *et al.* 2012). The roads extended over a wide range of elevation gradients (410-2125 m above sea level). The bottom of each road was the National Park boundary and the top of the roads was the highest point of the roads. Each road was divided into 19 equally spaced parts using Geographic Information System (GIS), giving 20 sampling points per road (Appendix S1). Sampling locations were pre-determined and located using a global positioning system (GPS) and photos of the sites. Sample locations were allotted numbers from lowest (1) to highest (20) based on elevation. Each transect consisted of two 2 x 50 m^2 plots, one plot parallel to the road edge (the road plot), and one plot perpendicular to the road plot (50-100 m from the road), together forming a "T". In all cases, the road plot was highly disturbed and modified by road construction and maintenance, while the perpendicular plot was dominated by native vegetation with little evidence of disturbance, apart from native animal digging and fire in a few plots. All vascular plant species[1] were recorded in each plot.

For *Phytophthora* species, rhizosphere soil samples including fine roots were collected in each sample plot for baiting and HTS. Soil was collected from the native vegetation plots first to avoid the accidental introduction of *Phytophthora* species from road plot to native vegetation plots. Briefly, a bulked rhizosphere soil sample was collected from five different places (approximately 10-15 m apart) and mixed thoroughly in a tray. Emphasis was placed on the collection of fine roots. This sample was then processed in two ways: firstly, approximately 20 g of fine roots were removed from the soil and placed in a small zip lock bag for environmental DNA (eDNA) extraction; secondly, about 300 g of soil was placed in a large zip lock bag for traditional baiting. The excess dirt from the tray was removed with a brush and then thoroughly cleaned using methylated spirit and paper towels. The same sampling procedure was repeated for sampling from the disturbed/roads plot. The soil samples were kept in an insulated icebox to protect samples from high temperatures and direct sunlight and carried to the laboratory.

In the laboratory, root samples were rinsed with tap water to remove soil particles and chopped into 1-2 mm segments. Chopped roots (approximately 5 g) were placed into three Eppendorf tubes and frozen at -20 ˚C until used for DNA extraction while soil samples were used for traditional baiting. Roots were chosen as a substrate for the HTS based on the findings of Khaliq *et al.* (2018b).

[1] Data for vascular plants was collected by Mountain Invasion Research Network (MIREN)

Baiting

Rhizosphere soil was baited with young leaves of *Rhododendron* spp., *Quercus ilex*, *Q. suber*, *Pimelea ferruginea*, *Poplar* sp., *Scholtzia involucrata*, and *Hedera helix* as described by Khaliq *et al.* (2018b). Baits developing necrotic lesions were plated directly onto a *Phytophthora* selective medium NARH (Simamora *et al.* 2017), and any *Phytophthora* like colonies (Erwin & Ribeiro 1996) were transferred onto individual vegetable juice agar (V8A) plates [100 ml/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery products Ltd., Norfolk, UK), 900 ml/L distilled water, 0.1 g/L $CaCo_3$, pH adjusted to 7 and 17 g Grade A Agar (Becton, Dickenson and Company, Sparks, MD, USA] after subculturing on NARH twice. Isolates were divided into morphotypes based on their gross colony morphology and hyphal characteristics examined at 10x magnification under a light microscope. Finally, two to three isolates from each morphotype were selected and genomic DNA was extracted using ZR Fungal/Bacterial DNA Miniprep™ (Zymo Research, Irvine, California). The region spanning the internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the primers DC6 (Cooke *et al.* 2000) and ITS-4 (White *et al.* 1990), and sequences were compared with those of *Phytophthora* by conducting BLAST search in GenBank (www.ncbi.nlm.nih.gov/genbank/).

DNA extraction and amplicon sequencing

DNA was extracted from fine roots using the Mo Bio PowerPlant® DNA isolation kit (Carlsbad, CA) following the manufacturer's instructions except for the first step where the buffer from the kit was replaced with 1 ml of saturated phosphate buffer (Na_2PO_4; 0.12 M; pH 8) to the roots (500 mg) to maximize extracellular DNA extraction (Taberlet *et al.* 2012). Final elutions were done in 60 µL of TE buffer. Extractions and PCRs were carried out in a separate laboratory using equipment that had never been used for *Phytophthora,* and extreme care was taken to avoid cross contamination between samples. All genomic DNA was frozen at - 80 ˚C until sequencing preparation.

ITS 18s rRNA gene sequences (~250 bp) were amplified by a nested PCR approach using primary *Phytophthora*-specific primers 18h2f and 5.8RBis in the first round, and nested primers ITS6 and 5.8S-IR (Català *et al.* 2015) in the second round with Illumina MiSeq adapter sequences attached to the 5' end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocols: Metagenomic Sequencing Library Preparation). The PCRs were performed in 25 µl volume tubes containing 12.5 µl of PCR buffer KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 8 µl of PCR grade water, 1 µM of each primer and 2.5 µl of genomic DNA (first round) or 1 µl of the PCR product (second round). No-template negative

PCR controls were included each time a PCR reaction was set up and carried forward to the second round in the same manner as for the samples. PCR cycling conditions were 94 °C for 2 min, 30 cycles of 95 °C for 20 s, 60 °C for 25 s and 72 °C for 1 min before a final 72 °C for 7 min and holding at 4 °C. If a band was visualised in the negative PCR controls, the products were discarded. First round PCR was conducted in duplicate and second round RCR products were combined based on intensity of bands on 2% agarose gels.

All PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter) as described in the Illumina protocol. All the samples were then uniquely barcoded and prepared for sequencing according to Illumina recommended protocol (Illumina Demonstrated Protocols: Metagenomic Sequencing Library Preparation), and sequenced on an Illumina Miseq using 500-cycle V2 chemistry (250 bp paired-end reads), following the manufacturer's recommendation.

Bioinformatics analysis

Paired end reads were received in FASTQ format from the sequencing centre. Reads were imported to Geneious ver. R10 (https://www.geneious.com/); reads that did not have perfect primer sequences (no mismatches allowed) were removed from the dataset, and primer sequences and distal bases were trimmed from the 5' and 3' ends of the reads. Paired end reads were then merged using USEARCH v10.0.240 (Edgar 2010). The reads were then quality filtered (sequences with >1% expected errors were excluded from the dataset), de-replicated, and singletons were discarded using USEARCH v10.0.240. After chimera filtering was carried out, the reads were clustered into zero radius operational taxonomic units (OTUs) i.e. ZOTUs with the UNOISE (de-noising) algorithm (Edgar & Flyvbjerg 2015). The original raw paired end reads were aligned to the high quality ZOTUs representative set with USEARCH v10.0.240 to assign a ZOTU to each sequence and a ZOTU table was created. From this point, the sequences were processed within Geneious in order to use phylogeny to assign species names. Consensus sequences were aligned using MAFFT alignment within Geneious with default parameters. Identities were first assigned to ZOTUs by conducting an internal blast search against a customised reference database. The reference database consists of ITS1 sequence of 300 *Phytophthora* species, undescribed (but designated) taxa and *Phytophthora* phylotypes recognised through metabarcoding from global studies. After that, all ZOTUs were then separated into clades and phylogenetic analyses were conducted using Geneious tree builder using verified sequences of all known *Phytophthora* species (Fig. S5.1). These final identities are considered to be phylotypes, acknowledging that their identity is based on sequence data rather than a living isolate. A phylotype was considered to represent a putative new species if it did not match any known species in the phylogenetic analysis. These

phylotypes were named with a number representing the phylogenetic clade and a letter to distinguish between putative new phylotypes in the same clade. New phylotypes belonging to unknown clades were allocated in clade(s) 'X'. For phylotypes that did not match any species in the curated database, BLASTn (GenBank database) searches were conducted to assign identities if possible.

Statistical analysis

1) Is there an effect of disturbance and elevation on species richness?

The influence of disturbance (as a factor: either roads or native vegetation) and elevation (m) and their interaction on species richness i.e. number of species / sample plot of a) *Phytophthora* species, and b) vascular plant species was investigated by fitting generalised linear models (Poisson family with a log link function). First and second order polynomial terms of elevation were included to determine whether patterns were monotonic or hump-shaped. Analyses were performed using the *stats* package in R (R Development Core Team 2014).

The relationship between *Phytophthora* and vascular plant species richness (at the plot scale) was investigated by fitting a generalised linear model (Poisson family with a log link function).

2) Is there an effect of disturbance and elevation on species composition?

The influence of disturbance and elevation on species composition was investigated using distance based linear models in the Permanova+ add-in to Primer v6 (Anderson et al. 2008; Clarke and Gorley 2006). Plots with one or fewer species were removed from the *Phytophthora* species matrix (this was not the case for vascular plants, as they were more in number). Resemblance matrices were then created using the Bray Curtis metric for *Phytophthora* and vascular plant species separately. Distance based linear models were fitted for two predictor variables: elevation (m) and disturbance (roads = 1, native vegetation = 0) and an adjusted R^2 was calculated. The matrices were plotted using principal coordinates in Permanova+; vectors for elevation and disturbance were overlayed on the plots.

3) Is there an effect of disturbance and elevation on species distribution?

The distributions of *Phytophthora* and vascular plant species in relation to disturbance were investigated by calculating the Pearson's product-moment correlation between the frequency of occurrences on roads and in native vegetation. A strong correlation would suggest there is little effect of disturbance on species distribution. The distributions of *Phytophthora* and vascular plant species in relation to elevation were investigated by determining the elevation range of all

species with 5 or more records in either native vegetation or on roads. The significance of the difference in mean range between *Phytophthora* species and vascular plant species was determined by analysis of variance. Tukey's HSD test was used to perform multiple pairwise-comparisons between the means of groups (*Phytophthora* and vascular species x roads and native vegetation plots).

Results

Bioinformatics analysis

Eighty-two of 118 extractions from 59 sites (two extractions per site; one from road and the other from native vegetation) yielded PCR products. Across all runs, a total of 5 092 729 reads were obtained from samples that yielded PCR products. On average, 48.2% of the reads corresponded to the genus *Phytophthora* and the remaining were attributed to other oomycetes, including *Halophytophthora, Peronospora, Pythium or Phytopythium;* unidentified oomycetes and *Lagena* species that were excluded from further analysis (Table S5.1).

Total species richness

Across all sites, 25 *Phytophthora* species corresponding to 14 known species and 11 potentially new phylotypes were detected (Table S5.2). Of the 25 *Phytophthora* species/ phylotypes detected in this study, 18 phylotypes were detected by HTS and 9 species were recovered by traditional baiting, while two species (*P. elongata* and *P. pseudocryptogea*) were detected by both techniques. The *Phytophthora* species resided in clades 1, 2, 4, 6, 7, 8, 9, 10, 11 and unknown clade(s) (X). *Phytophthora* distribution was uneven across clades. The highest number of *Phytophthora* species were detected in clade 1 (7 species), followed by unknown clade(s) (4 species), clades 2 and 6 (3 species), clades 8 and 9 (2 species), and one *Phytophthora* species was detected in each of clades 4, 7, 10 and 11.

In road plots, 24 species of *Phytophthora* and 341 species of vascular plants were recorded. In the adjoining native vegetation plots, 22 species of *Phytophthora* and 458 species of vascular plants were recorded. The mean number of *Phytophthora* species in native vegetation plots was 4.2 ± 0.4, and was not significantly different from that in road plots (4.6 ± 0.4) as determined by a paired t-test ($t = -0.66$, $P > 0.05$). The mean number of vascular plant species in native vegetation plots was 35.9 ± 1.3, was significantly greater than that in roads plots (32.4 ± 1.4) as determined by a paired t-test ($t = -2.09$, $P < 0.05$).

1) Is there an effect of disturbance and elevation on species richness?

There was an effect of disturbance on species richness for both *Phytophthora* species ($P < 0.01$) and vascular plant species ($P < 0.001$) but no effect of elevation. There was a significant interaction between disturbance and elevation for both *Phytophthora* species ($P < 0.01$) and vascular plant species ($P < 0.001$; Table S5.3). In post hoc generalised linear models of species richness and elevation fitted for each disturbance type, there was a significant decline in species richness with increasing elevation for *Phytophthora* species ($z = -3.33$, $P < 0.001$) and vascular plant species ($z = -7.71$, $P < 0.001$) on roads but no significant ($P > 0.05$) effect in native vegetation (Fig. 5.1). The second order polynomial model of elevation was not supported for vascular plant species. For *Phytophthora* species richness, the second order polynomial model was significant ($P < 0.05$) but not as well supported as the first order polynomial model ($P < 0.001$). That is, there is little support for a hump shaped relationship between elevation and species richness for either *Phytophthora* or vascular plant species.

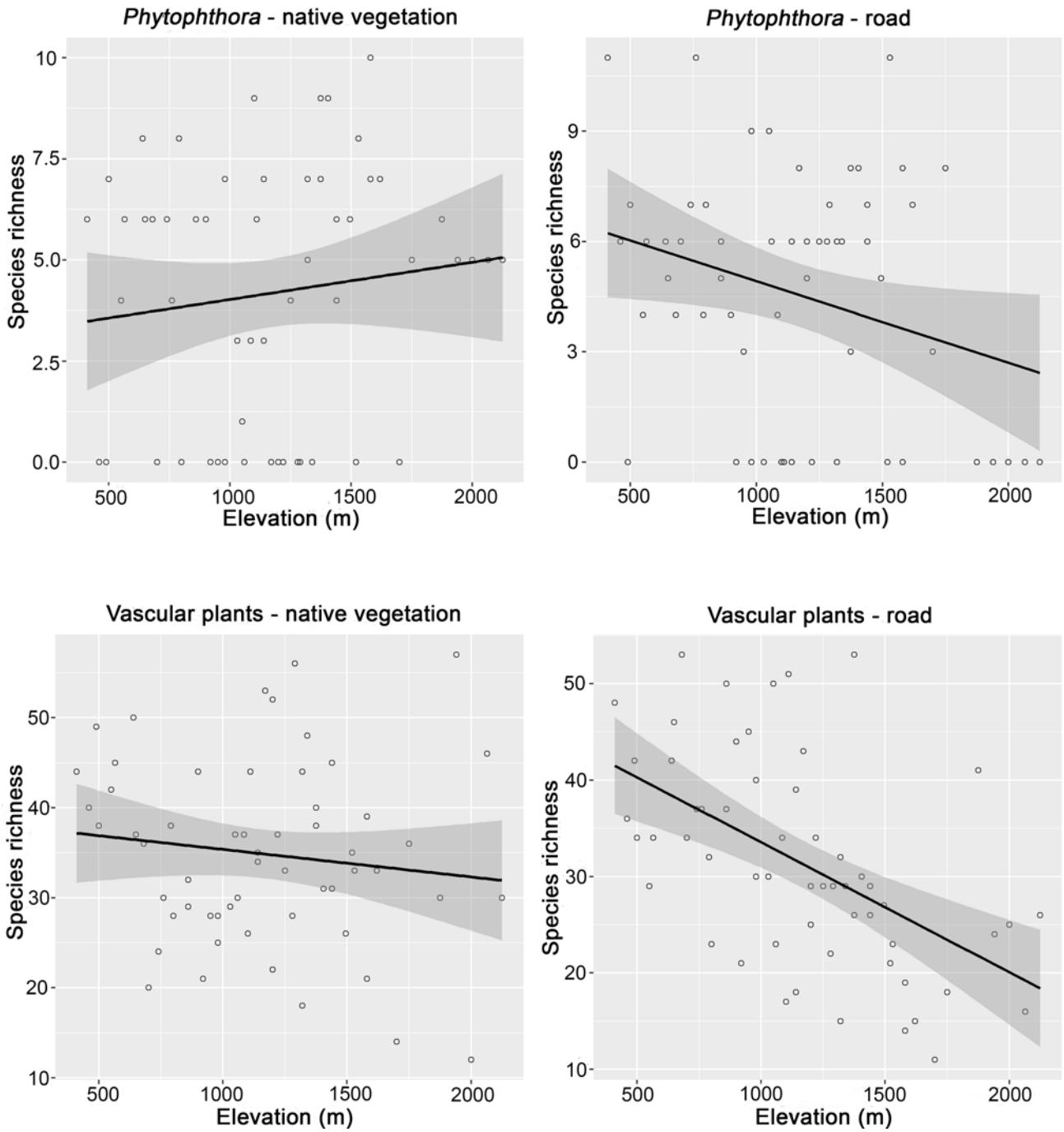

Figure 5.1 Comparison between *Phytophthora* and vascular plant species richness and elevation on roads and in native vegetation (significant for roads only, $P < 0.001$). The least squares regression lines and 95% confidence intervals (grey ribbons) are show

The generalised linear model fitted for the relationship between *Phytophthora* and vascular plant species richness was weakly significant (z = 2.16, P < 0.05; Fig. 5.2).

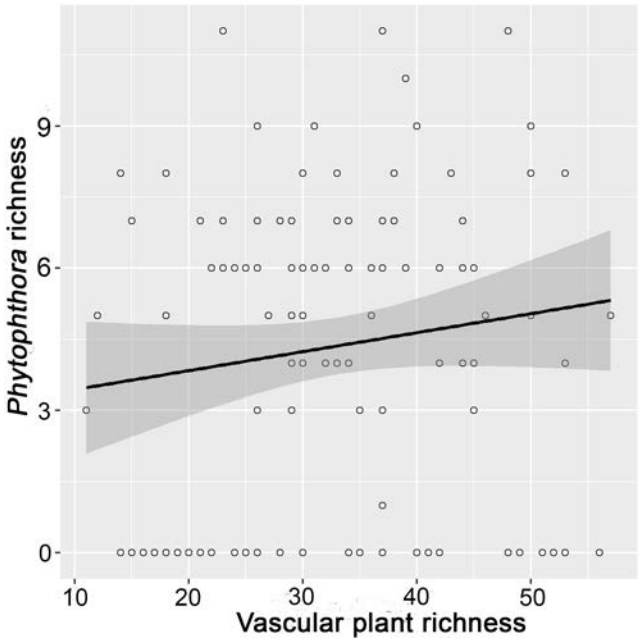

Fig. 5.2 The relationship between *Phytophthora* and vascular plant species richness as fitted with a generalised linear model (z = 2.16, P < 0.05). The least squares regression line and 95% confidence interval (grey ribbons) is shown

2) Is there an effect of disturbance and elevation on species composition?

Species composition changed significantly (P < 0.01) with elevation for both *Phytophthora* and vascular plant species. There was, however, only a significant (P < 0.01) difference in composition between roads and native vegetation for vascular plant species (Fig. 5.3). The model for *Phytophthora* explained 11% of total variation in species composition while the model for vascular plants explained 21% of variation.

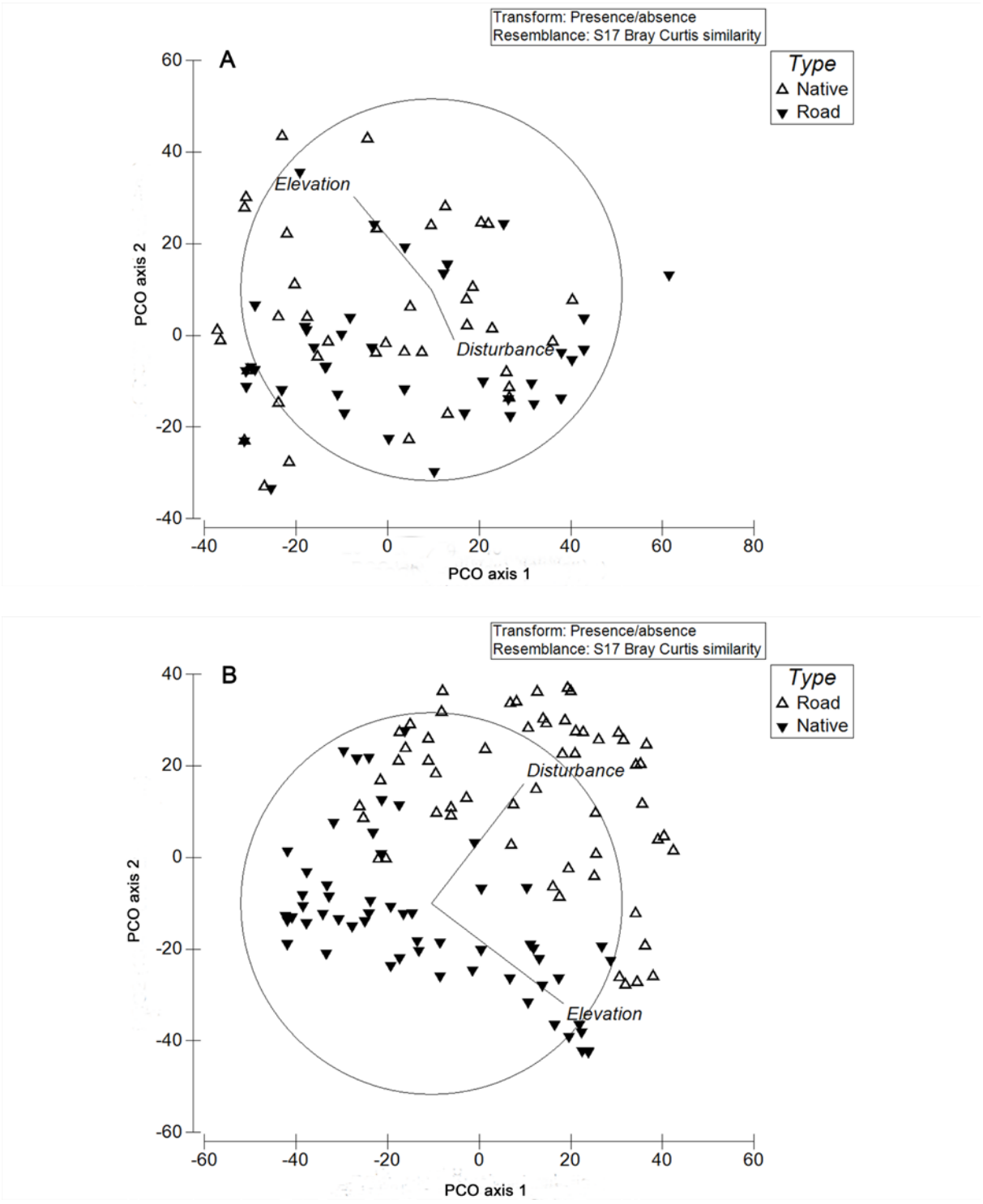

Figure 5.3 Principal co-ordinates plot of a) *Phytophthora* and b) vascular plant species composition with the vectors of environmental variables (elevation and disturbance) shown. Elevation explained a significant amount of variation in both *Phytophthora* and vascular plant species composition, whilst disturbance explained a significant amount of variation only for vascular plants.

3) Is there an effect of disturbance and elevation on species distribution?

The frequency of *Phytophthora* species on roads was highly significantly ($r^2 = 0.75$, $P < 0.001$) correlated with the frequency of *Phytophthora* species in native vegetation, while the frequency of vascular plant species was weakly significantly ($r^2 = 0.03$, $P < 0.05$) correlated with the frequency of vascular plant species in native vegetation. As with the relationship between disturbance and *Phytophthora* species composition (i.e. the assemblages of species), disturbance has no apparent effect on overall *Phytophthora* species distribution. Conversely, many vascular plant species were found mostly on either roads or in native vegetation (Fig. 5.4).

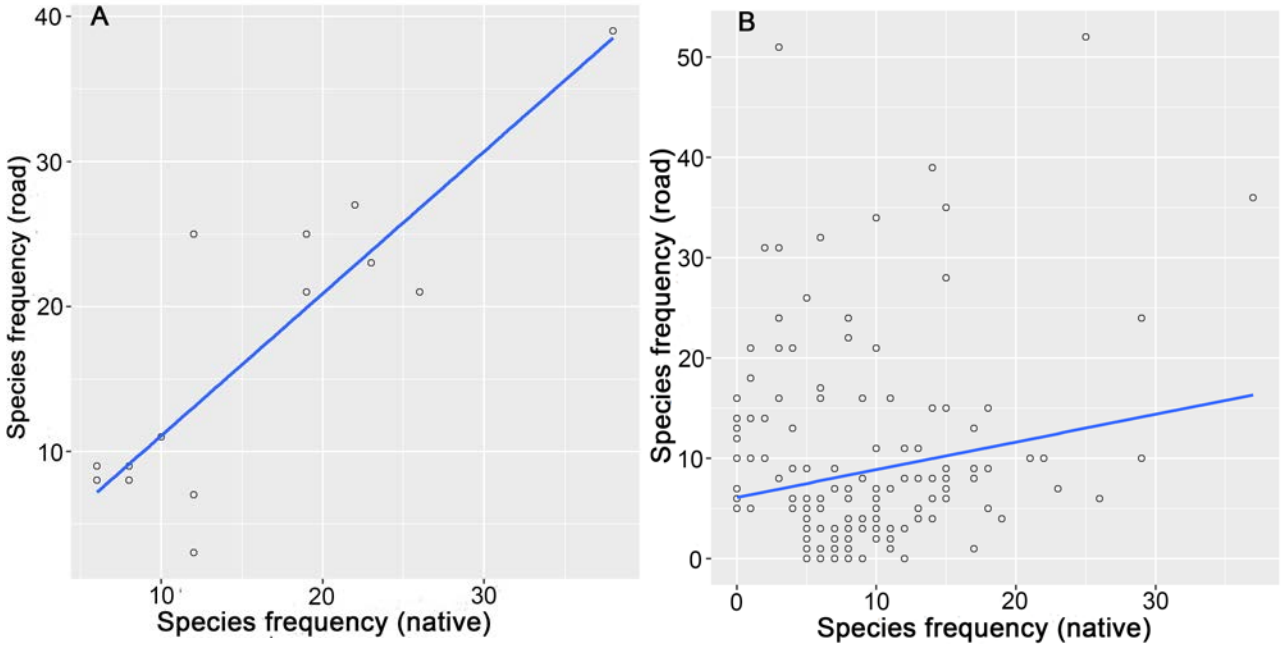

Figure 5.4 Scatterplot of species frequencies on roads and native vegetation for a) *Phytophthora* and b) vascular plants. The least squares regression lines have been added. The graphs show that a particular *Phytophthora* species is found as often on roads as in native vegetation but most native species are likely to be found mostly in native vegetation.

Elevation range differed significantly ($F = 19.9$, $P < 0.001$) between categories of species x disturbance type (i.e. *Phytophthora* on roads, *Phytophthora* in native vegetation, vascular species on roads and vascular species in native vegetation). Using Tukey's HSD test of multiple comparisons of means, there was a significant ($P < 0.001$) difference between species categories (i.e. the mean elevation range of *Phytophthora* species was greater than the mean elevation range of vascular plants), but not between disturbance types ($P > 0.05$; Fig. 5.5). Most *Phytophthora* species occurred throughout much of the elevation range of the sample plots (minimum = 410 m, maximum = 2125 m, range = 1715 m). Despite that, some species were more frequently found at particular elevations (e.g. *P. arenaria* at low elevation, *P*. AUS XD at mid elevations and *P*. AUS 8E and *P. europea* at high elevations; Fig. 5.6).

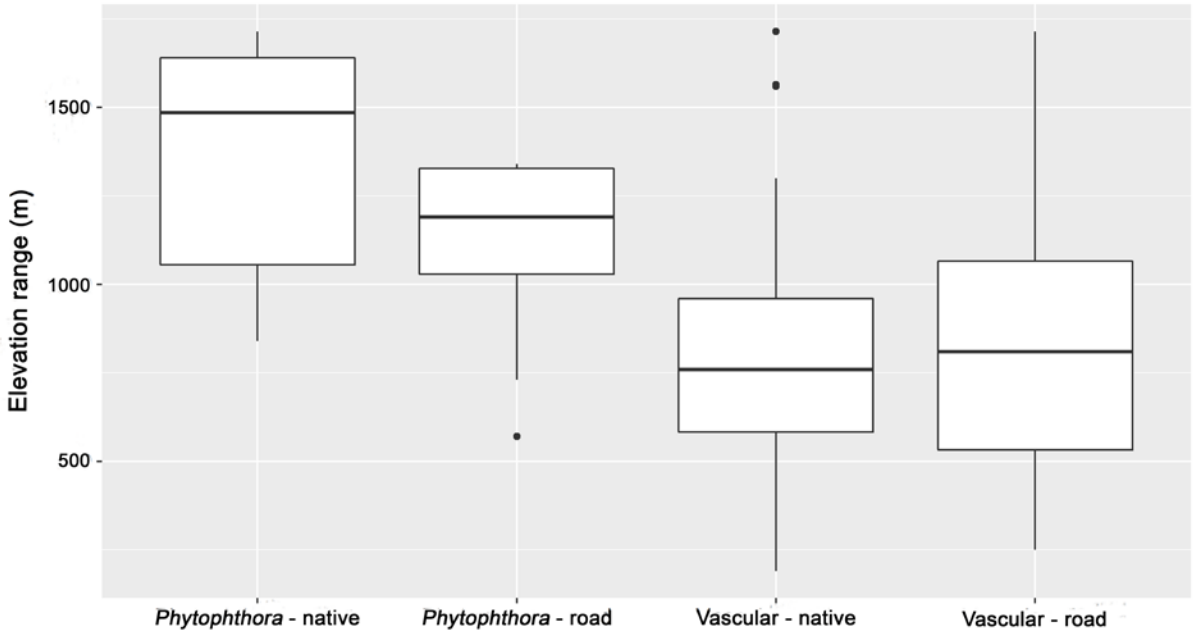

Figure 5.5 Boxplots of elevation range (m) for *Phytophthora* and vascular plant species on roads and in native vegetation. *Phytophthora* species had a significantly ($P < 0.001$) greater elevation range than vascular plants on both roads and in native vegetation. There was no significant ($P > 0.05$) difference in elevation range between native vegetation and roads for either species group. The line in the middle of each boxplot is the median, the box limits are the quartiles (25 and 75%) and the dots are outlying points

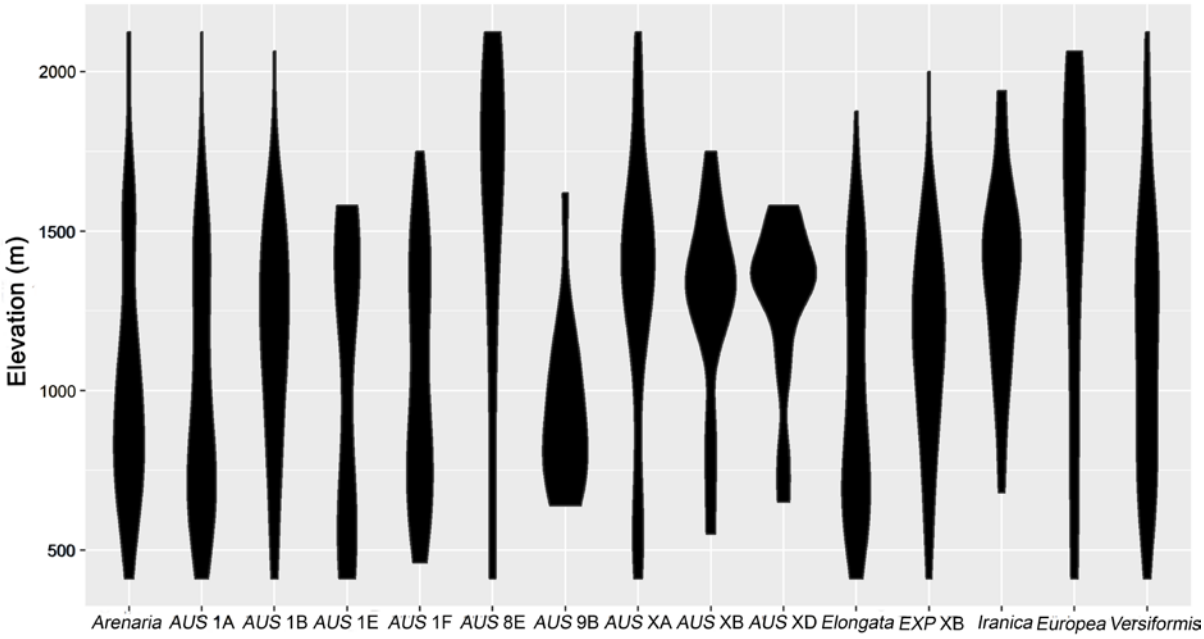

Fig. 5.6 Violin plots of *Phytophthora* species with 14 or more occurrences in total (roads and native vegetation plots) across different elevations. The width of the column is related to frequency of occurrence at that elevation

Discussion

Is there an effect of disturbance and elevation on species richness?

There was a significant decline in species richness of both vascular plants and *Phytophthora* species with increasing elevation on roads, but not in native vegetation. For vascular plants, this is not surprising as roads are generally invaded by non-native species for which species richness at higher elevations is strongly hampered by climatic harshness and decreasing human related propagule pressure (Grytnes & Vetaas 2002, Alexander *et al.* 2011, Kessler *et al.* 2011). Harsh climatic conditions and decreased human related propagule pressure with increasing elevation also act as a filter for *Phytophthora* species richness (Redondo *et al.* 2018), although their native and non-native origin often remain uncertain (Rizzo 2005).

Non-native plant species with broader climatic amplitude can establish at higher elevations, the rest are progressively filtered out with increasing elevation (Alexander *et al.* 2011). In the Australian Alps, non-native plant species introduced to sub-alpine regions, especially due to anthropogenic disturbance, were able to germinate and grow, but were unable to reproduce due to climatic harshness (Mallen-Cooper 1990). Similar to vascular plants, only newly introduced *Phytophthora* species with the ability to overcome harsh climatic conditions manage to survive and spread in colder ecosystems; the rest are progressively filtered out with increasing elevation. Their ability to overcome harsh climatic conditions is mainly associated with their ability to form asexual survival structures and lower cardinal temperatures for growth (Redondo *et al.* 2018).

No decline in species richness of vascular plants and *Phytophthora* species was observed with increasing elevation in native vegetation plots. In the case of vascular plants, the difference in richness patterns is probably attributable to the greater proportion of non-native species occurring on roads. When road habitat is created, it is largely bare and establishment by non-native plants relies largely on dispersal of propagules from lowlands (Alexander et al. 2011). Time (the time taken for dispersal from lowland to mountain tops) and climate tend to favour higher species richness at low elevation and a decline in species richness with increasing elevation. This slower colonisation by plants at high elevations on roads may in turn have resulted in lower *Phytophthora* species richness. Recolonisation of roads by *Phytophthora* species directly relies on the colonisation of roads by plant species as *Phytophthora* species are poor saprophytes (Erwin & Ribeiro 1996), and are unlikely to survive in soil without a host(s) at higher elevations in temperate climates (Van Steekelenburg 1973, Marçais *et al.* 1996, Green 2016). This suggests that the richness of *Phytophthora* species mimics the richness of their hosts.

Is there an effect of disturbance and elevation on species composition?

Vascular plants and *Phytophthora* species composition changed significantly with increasing elevation. This can be mainly related to climate harshness and decreasing anthropogenic disturbance with increasing elevation, which has widely been studied before (Alexander *et al.* 2011, Seipel *et al.* 2012, Redondo *et al.* 2015, Redondo *et al.* 2018). In the case of vascular plants, a significant difference in species composition was found on roads and within native vegetation. This is principally related to the low richness of non-native plants in native vegetation, which can be attributed to different environmental conditions (microclimate) on roads, landscape barriers, seed morphological characteristics, different introduction history, decreased human related land use in native vegetation, dense vegetation resulting in an increased habitat resistance in native vegetation, and lack of plasticity in non-native species (Parendes & Jones 2000, Petryna *et al.* 2002, Hansen & Clevenger 2005, Delgado *et al.* 2007, Alexander *et al.* 2009, Seipel *et al.* 2012).

In contrast to vascular plants, no significant difference in *Phytophthora* species composition was found between the road and native vegetation plots suggesting an efficient dispersal of *Phytophthora* propagules. This efficient dispersal is mainly related to the relatively short lifecycle of *Phytophthora* species. Also, each propagule of *Phytophthora* species have the ability to infect and rapidly propagate compared to vascular plants that are mainly dispersed through seeds. The seeds are non-motile and above ground, so they need to be blown or carried by vectors. After dispersal, they typically need bare ground to germinate and have to compete with other plants for light, water and nutrients. In contrast, we believe *Phytophthora* species detected in the current study are soil borne. Therefore, they could be easily dispersed by root to root contact and water and animal mediated soil movement (Hansen *et al.* 2000, Jules *et al.* 2002). *Phytophthora* propagules could also be easily washed down from road into native vegetation plots, as many native vegetation plots were located below the roads.

Is there an effect of disturbance and elevation on species distribution?

Phytophthora species had broader ecological niches along the elevation gradient than vascular plants and many species were recorded from the bottom to the top of the mountain. Self-sexual reproduction by homothallic species and parasexuality has enabled *Phytophthora* species to establish and persist in novel environments in spite of limited genetic variation (Desprez-Loustau *et al.* 2007). Evolutionary potential (McDonald & Linde 2002), survival under deep soil layers in plant tissues (Marçais *et al.* 1996), the ability to produce resistant

structures (Crone *et al.* 2013b, Jung *et al.* 2013) and phenotypic plasticity (Chapter 04 in this thesis) can further assist in their persistence and establishment in novel environments.

Although most *Phytophthora* species occurred throughout much of the elevation range of the sample plots, some species were more frequently found at particular elevations, which could be related to a preference for specific hosts. A caveat to the finding that certain *Phytophthora* species were widespread across elevation and disturbance is HTS techniques can detect DNA of species even if they have no longer persisted (Carini *et al.* 2017). No living *Phytophthora* species were recovered using traditional baiting above 1750 m. The elevation range for survival and reproduction may therefore be narrower for the species detected through HTS but, at times, they may have occupied a broad elevation range. However, there is a strong probability that species detected through HTS were alive at the time of detection, as they were detected in roots, not in soil. Roots act as a biological filter; if an organism is detected in the roots, it must have been alive to get there (Khaliq *et al.* 2018b). Vascular plants had a narrower elevational range than *Phytophthora* species. For native plant species, this probably reflects millennia of selection and specialisation, with many species now restricted to particular biomes. The alpine area of KNP for instance, has a large number of local endemics (Costin *et al.* 2000). For non-native species, climate filtering has been shown to limit elevational distribution of species that are mainly climate generalists. With time, many non-native plants may have much broader distributions like most of the *Phytophthora* species.

Disturbance had no apparent effect on overall *Phytophthora* species distribution suggesting wider ecological niches of these species. Factors responsible for similar *Phytophthora* species composition on roads and within native vegetation seem to be responsible for the similar frequency of *Phytophthora* species on roads and within native vegetation. Conversely, disturbance had a significant effect on vascular plants distribution i.e. many vascular plant species were found mostly on either roads or in native vegetation. The reasons for this lack of colonisation of plants between roads and native vegetation have been described above (species composition section).

Implications for nature conservation

Phytophthora cinnamomi, a non-native pathogen known to cause widespread damage to native vegetation in lowland Australia (Cahill *et al.* 2008a), was not detected in the current study and has not been isolated in KNP using traditional baiting techniques. Disease caused by *P. cinnamomi* has been estimated to occur only in areas with a mean annual temperature > 7.5°C (Podger *et al.* 1990). *Phytophthora cinnamomi* dieback is currently below about 1300 m elevation but could rise to as high as 1820 m by 2070 under climate projections (Rigg *et*

al. 2018), encompassing almost the entire park. This is particularly important when seven of nine alpine and subalpine species recently tested from KNP were susceptible to infection by *P. cinnamomi,* and one of those (*Phebalium squamulosum*) was highly susceptible (Rigg *et al.* 2018). At least five of the species detected in this study are believed to be non-native (Burgess *et al.* 2017b, Burgess *et al.* 2018). The occurrence of non-native *Phytophthora* species in both road and native vegetation plots suggests that non-native species capable of surviving in mountain conditions spread rapidly once introduced. It is highly likely that some native plant species in KNP will be affected by non-native *Phytophthora* species. For example, *Phytophthora gregata* and *P. cryptogea* have recently been found to cause severe damage in populations of a native shrub '*Pimelea bracteata*' in KNP (Khaliq *et al.* 2018a). Although seven of the 25 *Phytophthora* species detected in the current study were regarded as native to Australia by Burgess *et al.* (2017b), they may not be native to KNP, or other stresses on native vegetation may increase their effect on native hosts. Eighteen of the 25 species detected in the current study have unknown origin, but it is likely that some of them could be non-native.

In conclusion, vigilance for the appearance of disease and monitoring of *Phytophthora* distribution will be essential for the long-term protection of the KNP native flora. Vehicle hygiene is impractical in catchments traversed by high volume public roads but is paramount in remote areas accessible only to management vehicles. Therefore, boot cleaning for bushwalkers and tyre cleaning for mountain bike riders may be worthwhile in those areas, but dispersal by native and non-native herbivores may undermine efforts to protect native vegetation. Ultimately, protection of highly susceptible species (by phosphite application or ex situ conservation) may be required. Globally, understanding the interaction between *Phytophthora* and vascular plant species in other temperate areas of the world will play a major role in conserving the rare and vulnerable alpine and sub-alpine flora under the increasing threat of *Phytophthora* invasion due to projected climate change and human related activities.

Chapter 6: General Discussion

Overview of the study

This study is the first to investigate the ability of *Phytophthora cinnamomi* to exhibit plasticity to colder environments. No research has been conducted in Australia or worldwide around this theme on *P. cinnamomi*. I was able to demonstrate that isolates originally from Mediterranean, and temperate environments can exhibit phenotypic plasticity and produce sporangia and release zoospores at temperatures lower than originally established, and in a shorter period when 'trained' under cold conditions. My research also looked into the extent of invasion of the alpine, and sub-alpine ecosystems by other *Phytophthora* species and created baseline knowledge on *Phytophthora* diversity in these ecosystems. This study is also the first to compare the distribution pattern of *Phytophthora* species and vascular plants in relation to elevation and disturbance gradients in Australia.

Phytophthora cinnamomi has a worldwide distribution and causes diseases in agricultural, horticultural and natural ecosystems. However, it has never been regarded as a pathogen of colder environments due to the assumption that lower temperatures limit the growth and survival of the pathogen (Shearer *et al.* 1987, Podger *et al.* 1990). My finding that the pathogen can exhibit phenotypic plasticity and produce sporangia and release zoospores in cold environments has major ecological and management implications in terms of restricting its introduction and subsequent spread in mountain ecosystems to protect the diverse and threatened flora, and in turn fauna. My findings can be used to update the current species distribution models. For example, Burgess *et al*. 2017 used 9 ˚C as the lower temperature limit for sporangia production by *P. cinnamomi,* but based on my data the pathogen can produce sporangia at 7.5 ˚C. This would change the cold limits in the model and thus extend areas of suitability for growth of *P. cinnamomi* into colder areas in the northern hemisphere and up higher elevations in mountainous regions. The data for the biology and physiology of *P. cinnamomi* used by previous models is based on the earlier studies of Zentmyer (1980), which need to be updated owing to the plastic nature of *P. cinnamomi.* My findings can also be used by land managers and policy makers to protect areas at risk, and prevent spread into areas that might be kept pathogen-free, which is discussed below under 'Future risks'. An overview of the thesis is now provided.

A preliminary experiment comparing metabarcoding with traditional isolation techniques revealed metabarcoding to be more effective in determining *Phytophthora* diversity in natural ecosystems; and eDNA extracted from field roots was found to be the best substrate for metabarcoding studies (Chapter 2). However, amplifications were achieved for only three of the five sites investigated, which could be linked to the type of roots collected and the relatively small sample size (as inoculum is not uniformly dispersed). More work is required

to optimize this methodology. Also, certain *Phytophthora* species were detected by only one technique; therefore, a combination of techniques should be used to get an accurate image of *Phytophthora* diversity in an area. Preliminary surveys conducted in the sub-alpine and alpine areas of areas of New South Wales (NSW) and Tasmania (TAS) to determine baseline *Phytophthora* species, and to confirm that *Phytophthora* species detected by a previous study resulted in the isolation of eight *Phytophthora* species, including two new species; *P. oreophila* sp. nov and *P. cacuminis* sp. nov that were formally described (Chapter 3).

The occurrence of a diverse *Phytophthora* community in sub-alpine and alpine ecosystems previously thought to be 'pathogen-free' (Podger *et al.* 1990) was surprising and suggested that *Phytophthora* species had adapted to cold conditions. Alternatively, they have always been present, or present for a long time but no one had looked in detail for *Phytophthora* species at these altitudes. Extended experiments were established (Chapter 4) to test the first theory. *Phytophthora cinnamomi* was selected as a 'test' species because of its national and international significance. *Phytophthora cinnamomi* produced sporangia and released zoospores at 7.5 °C lower than originally (10 °C) established in a shorter period when 'trained' under cold conditions. *Phytophthora cinnamomi* was 'trained' under cold conditions in an inoculated host i.e. *Oxylobium ellipticum* rather than in the form of mycelium to enhance the pathogen's physiological response to changing temperatures in the presence of a plant. These findings show that *P. cinnamomi* responds rapidly to selection pressure and can adapt to new environments.

Although it was established that *P. cinnamomi* has the ability to adapt to colder conditions, and it had been isolated/detected in the sub-alpine and alpine areas previously (McDougall *et al.* 2003, Burgess *et al.* 2017b), the pathogen was not detected in the sub-alpine and alpine areas in the current study. This could be explained by the fact that *P. cinnamomi* typically has patchy distribution in the soil (Weste & Taylor 1971, Weste & Kennedy 1997, Brown *et al.* 2002, Pryce *et al.* 2002, McDougall *et al.* 2003); therefore, it could have been missed easily by sampling (also potential hosts may have escaped infection). The reason for the patchy distribution of *P. cinnamomi* is not clear, but it is likely related to hosts, availability of soil water, soil texture, organic matter and soil microbes (Newby 2014). Antagonism and competition from soil microbes are known to effect the distribution and disease expression by *P. cinnamomi* (Broadbent & Baker 1974). Alternatively, *P. cinnamomi* detected in the sub-alpine and alpine areas by Burgess *et al.* (2017b) could be dead, as in the current study the emphasis was on collecting roots and not rhizosphere soil as done by Burgess *et al.* (2017b). It has been shown that *P. cinnamomi* is unlikely to survive in temperate areas in soil due to harsh climatic conditions, and it mainly survives in host tissues (Van Steekelenburg 1973).

Phytophthora cinnamomi isolates were 'trained' over time to make them produce sporangia and release zoospores at temperatures lower than originally thought possible. This suggests *P. cinnamomi* isolates introduced into higher elevation may not be able to sporulate immediately, but rather adapt to these conditions before it is able to sporulate.

Detailed surveys were conducted to determine the distribution pattern of *Phytophthora* species and vascular plants in relation to disturbance and elevation along elevation gradients in KNP (Chapter 5). Species richness of both *Phytophthora* and vascular plants decreased with increasing elevation on roads but not in native vegetation (100 m away from roads); and the elevation range of *Phytophthora* species was higher than vascular plants both on roads and within native vegetation. Species composition changed significantly for both *Phytophthora* and vascular plants with elevation, however the effect of disturbance on species composition was only significant for vascular plants. In contrast to vascular plants, *Phytophthora* species exhibited similar composition on roads and native vegetation. This suggests that the distribution of *Phytophthora* and vascular plant species is more related to climate filtering than to human activities in a temperate mountain.

Apart from the involvement of *P. gregata* and *P. cryptogea* in *Pimelea bracteata* dieback in KNP, the eight *Phytophthora* species had not been associated with a disease. In fact, the *Phytophthora* species were isolated underneath healthy vegetation. There are several reasons for the lack of disease expression. *Phytophthora cinnamomi* can live in plants as a biotroph without killing them (Crone *et al.* 2013a, Crone *et al.* 2013b). Host resistance can also suppress disease, which is recognised in some NSW *Banksia* species (McCredie *et al.* 1985). Since the composition of *Phytophthora* species was the same on roads and within native vegetation, it is possible that these species have been established for decades and have attained equilibrium with the ecosystem. It is also possible that *Phytophthora* species are surviving in plants asymptomatically. It has been shown that *Phytophthora* species can infect plants without causing observable symptoms (Hüberli *et al.* 2000, Crone *et al.* 2013a). In a random survey in north Queensland, there was no significant difference in the rate of isolation of *P. cinnamomi* from symptomatic and asymptomatic vegetation (Pryce *et al.* 2002). Such behaviour in infected hosts accompanied by cold conditions will make the isolation and management of *P. cinnamomi* very difficult (Newby 2014). This also suggests that environment is favourable for the pathogen, but not for disease expression (Pryce *et al.* 2002). In such a case, a disease outbreak is likely to occur under the expected climate change (Burgess *et al.* 2017a). The wide distribution across elevation gradients, higher elevation range and similar composition on roads and native vegetation also suggest that the survival and continued spread of existing *Phytophthora* species is more dependent on

climatic and topographic factors than anthropogenic disturbance. Anthropogenic introduction of new species may however pose a significant risk.

Anthropogenic introductions and subsequent invasion can be divided into a series of stages (Blackburn *et al.* 2011). Following Blackburn's definition of invasive species, only those non-native *Phytophthora* species can be considered invasive that are able to establish and spread across a range of habitats regardless of their impact. At each stage there are different barriers that needed to be overcome in order to establish and spread into new environments and become invasive (Fig. 6.1). These barriers, for example barrier for survival and introduction can affect the life cycle of the invader and can be used to study the characteristics of a location, such as climatic factors and hosts, which in turn can be used to investigate the impact of environmental filtering on *Phytophthora* species once they are introduced into a new area. The main barriers for *Phytophthora* species could be the absence of susceptible hosts or harsh climatic conditions (Ireland *et al.* 2013, Burgess *et al.* 2017a).

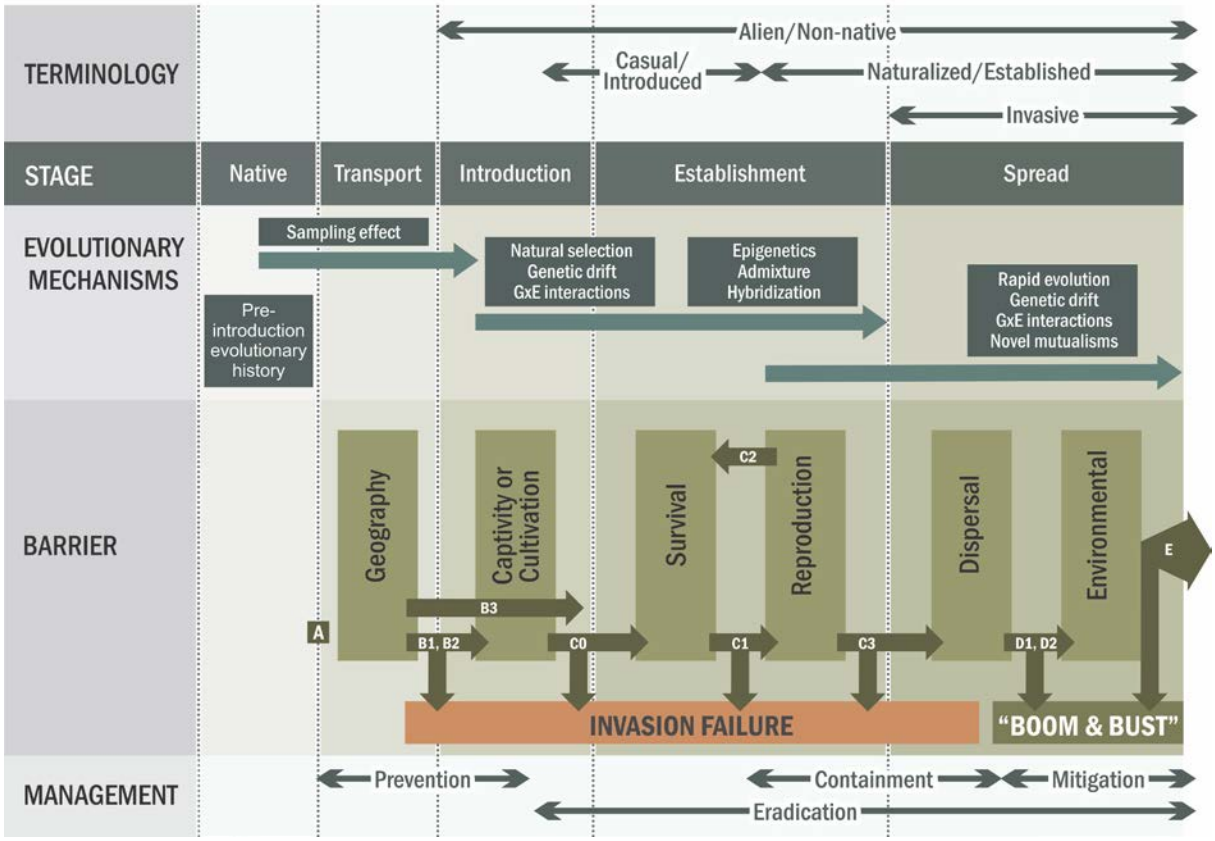

Figure. 6.1 The proposed unified framework for biological invasions by Blackburn *et al.* (2011). Species are classified based on where in the invasion process they have reached along with different management practices that can be adapted at different stages of invasion. Species are not dispersed beyond their native ranges (A), species are dispersed beyond their native range and are either restricted in captivity or quarantine (B1), or in cultivation i.e. species are cultivated but explicit measures are taken to prevent dispersal (B2), species are transported beyond their native ranges and

are directly released into new environments (B3), species are released into the wild i.e. outside of captivity or cultivation and are either incapable of surviving for extended periods (C0), or capable of surviving but unable to reproduce (C1), reproduction is occurring but population is not self-sustaining (C2), reproduction is occurring but population self-sustaining (C3), self-sustained population is surviving at a significant distance from the initial point of introduction (D1), self-sustained population is surviving and reproducing at a significant distance from the initial point of introduction (D2), species become fully invasive and disperse, survive and reproduce across a different range of habitats (E).

Future directions/research

Best methodology for determining Phytophthora diversity

eDNA extracted from field roots was found to be the best substrate for metabarcoding studies (Chapter 2). However, amplifications were achieved for only three of the five sites investigated, which could be linked to the type of roots collected and the relatively small sample size (as inoculum is not uniformly dispersed). More work is required to optimize this methodology. Also, certain *Phytophthora* species were detected by only one technique, therefore a combination of techniques maybe used to accurately assess *Phytophthora* diversity in a certain area.

Determination of lower temperatures for infection

Although *P. cinnamomi* produced sporangia and released infective propagules (zoospores) at 7.5 ˚C in non-sterile pine bark extract, the pathogen could not be isolated from plants grown at 7.5 ˚C three months after inoculation. It is not clear whether the pathogen failed to cause infection at this temperature or it could not withstand lower temperature for three months. More work is needed to establish the lower temperature limit for infection and disease development by *P. cinnamomi*. For this purpose, growth chambers need to be set at temperatures from 1 to 8 ˚C with 14 h day length (5 am-7 pm). Temperatures need to be incrementally changed (for plants grown at each temperature) with the minimum temperature being maintained for four hours during the night (2 am-6 am) and the maximum for four hours during the day (1 pm-5 pm) to mimic sub-alpine conditions. Plants should be inoculated with *P. cinnamomi* and healthy plants should be placed alongside inoculated ones to see if the pathogen can infect/transfer to healthy plants (through root to root contact or zoospore infection). Punnets should be flooded every fortnight and re-isolations should be made from inoculated and non-inoculated plants 90 days (to allow sufficient time for training) after inoculation to confirm Koch's postulates. Flood water should also be baited at 1-8 ˚C to check if zoospores are produced and baits are infected. The ability of recovered isolates to

produce sporangia and release zoospores should be determined at temperatures 7.5 ˚C and below. Other *Phytophthora* species and hybrids should also be included in the trial.

A relatively faster way to establish if *P. cinnamomi* can infect at lower temperatures is to do traditional baiting at lower temperatures (8 ˚C and below), with non-inoculation controls (water with floated bait leaves) to make sure that baits are not already infected by *P. cinnamomi.* Similarly, bait trays need to be incubated at room temperature as positive controls to test the viability of inoculum. *Phytophthora cinnamomi* isolates could be grown in sterilised parboiled rice as an inoculum source. Other more natural source of inoculum could be *P. cinnamomi* infected host tissues. The recovered isolates could be re-baited at increasingly lower temperatures to observe whether baits are infected at these temperatures. The ability of recovered isolates to produce sporangia and release zoospores should be determined at temperatures 7.5 ˚C and below. Other *Phytophthora* species and hybrids should also be included in the trial.

The reasons for the lack of disease expression in areas from where *Phytophthora* species have been isolated should be explored in detail. The role of soil suppression, cold climate vegetation resistance against *Phytophthora* species, biotrophic behaviour of *Phytophthora* species, and the interaction between different moisture levels and temperatures on disease expression of *Phytophthora* species should be investigated in detail both *in vivo* and *in vitro.*

Phenotypic plasticity experiments- a step further

Initially, the lower temperature limit (10 ˚C) for the production of sporangia and zoospore release by *P. cinnamomi* was determined and then phenotypic plasticity experiments were carried out at increasingly lower temperatures (9, 8, 7.5 ˚C) to explain the range expansion of *P. cinnamomi* to sub-alpine and alpine regions. The same experiments can be carried out at higher temperatures to demonstrate that *P. cinnamomi* has a plastic genotype. The highest at which *P. cinnamomi* produced sporangia and released zoospores was 30 ˚C; therefore, phenotypic plasticity trials need to be established (*in planta)* at 32.5 ˚C and higher to determine whether the pathogen could be 'trained' to produce sporangia at temperatures greater than 30 ˚C. Similarly, phenotypic plasticity experiments can be conducted with non-hosts, resistant hosts or hosts closely related to susceptible hosts to determine if the pathogen could exhibit plasticity and infect non-hosts/resistant hosts. This will further support the hypothesis that *P. cinnamomi* has a plastic genotype that responds rapidly to selection pressure and adapts to new environments and hosts. Other *Phytophthora* species and hybrids should also be included in the trial.

Future risks

Climate change/changing environment

Climate change is a major threat to the diverse flora in mountain ecosystems. Warming temperatures not only facilitate the invasion of colder environments, but also affect plants' resistance to pathogens. Hüberli *et al.* (2002b) studied the effect of temperature changes on *E. marginata* clonal lines inoculated with *P. cinnamomi* and found that temperatures of 25-30 ˚C dramatically decreased resistance of all clonal lines. Increased species invasions of higher elevations due to shift in global temperature is well known (Sturrock *et al.* 2011). The finding that increased temperature can affect host resistance to pathogens is yet another issue making climate change an important threat to the unique biodiversity of colder environments. Theoretically, global warming led to increased oak decline caused by *P. cinnamomi* in Europe (Brasier & Scott 1994). A CLIMEX model extended this theory to the rest of the world and predicted an increase in activity of *P. cinnamomi* in the colder regions of the world (Burgess *et al.* 2017a).

It is impossible to exactly predict the weather conditions over the next 50 or 100 years, but certain approaches can be used to push the limits of host plants to pathogen invasion. These include analysis of resistance of cold climate vegetation to *Phytophthora* species in controlled environments against a range of temperatures. For this purpose, one set of plants could be grown at temperatures ranging 7.5 to 15 ˚C and another set of plants could be grown at temperatures ranging 25-30 ˚C to establish temperature by host-pathogen interactions. Field trials should be set up in the sub-alpine and alpine areas and monitored over time across different seasons without manipulating environmental conditions to get a real insight into host-pathogen interactions. Transparent plastic structures need to be installed in asymptomatic areas where the *Phytophthora* species have been detected to see if disease expression occurs. These structures should be built in such a way that they ensure water incursion under them is limited and that they heat up the area covered. This will in turn provide favourable conditions for disease expression by *Phytophthora* species i.e. create warmer and drought stressed conditions. Increased understanding of plant pathogen interactions will not only answer questions on cold climate vegetation resistance to *Phytophthora* species, but will also improve distribution models which consider only environmental covariates, such as weather for mapping spatial distribution of *P. cinnamomi,* for example (Bishop *et al.* 2016). Models predicting the impact of climate change on *Phytophthora* dieback need to consider the varying distribution of hosts and non-uniform changes in the local climate for predicted for NSW (https://www.environment.nsw.gov.au/research-and-publications/publications-search/greater-

blue-mountains-world-heritage-area-strategic-plan). Global warming not only affects host resistance against pathogens, but also negatively affects the efficacy of control treatments, such as fungicides (Coakley *et al.* 1999). For example, elevated atmospheric carbon dioxide level could result in thickened epicuticular wax layer, which could reduce uptake of systemic fungicides, such as phosphite (Hüberli 2001). Little is known about the effect of temperature (both higher and lower) on phosphite and its ability to control *Phytophthora in planta*. Further research in this regard is suggested to get an understanding of the effect of temperature on phosphite and host-pathogen interactions. For this purpose, one set of plants could be grown at temperatures ranging from 7.5 to 15 ˚C and other set of plants could be grown at temperatures ranging 25-30 ˚C to determine phosphite by host-pathogen interactions.

Other invasive Phytophthora species

Initially, much emphasis was placed on *P. cinnamomi* as the main *Phytophthora* pathogen in the current study because of its catastrophic effects on vegetation in Mediterranean climates. Therefore, its range expansion to mountain ecosystems was a real concern. For this reason, *P. cinnamomi* was used as the model organism in the phenotypic plasticity experiments. However, as outlined in Chapters 2 and 4, the recovery of other apparently introduced (invasive) *Phytophthora* species (*P. cactorum, P. gonapodyides, P. cryptogea* and *P. pseudocryptogea*) in sub-alpine and alpine environments is of great concern. Therefore, it is important to look for other *Phytophthora* species that may cause diseases in mountain ecosystems either alone or in combination with *P. cinnamomi*. Although *Phytophthora* species had previously been overlooked and were not considered as a potential threat to the Australian sub-alpine and alpine ecosystems, some have since been shown to cause disease in the sub-alpine flora. For example, *P. cryptogea* and *P. gregata* are a serious problem in *Pimelea bracteata* populations in the Northern KNP and wetlands in surrounding areas. Plants at all growth stages are affected and there is very little or no regeneration (McDougall et al. unpublished). *Phytophthora cryptogea* and *P. gregata* have a wide host range, therefore other KNP vegetation is at risk.

The involvement of *P. gregata* and *P. cryptogea* in the decline of *P. bracteata* shows that *Phytophthora* species already present in the sub-alpine ecosystems have the potential to cause disease. Therefore, work is required to determine the susceptibility of sub-alpine flora to a range of *Phytophthora* species, not just *P. cinnamomi*. Little is known about the response of sub-alpine flora to invasive *Phytophthora* species. This is particularly important when temperature is rising globally, which may render some host species more susceptible to diseases caused by *Phytophthora* and other pathogens. Detailed surveys need to be conducted to investigate *Phytophthora* dieback in these areas.

Recreation and tourism

Nature-based tourism and recreational activities in protected areas are increasing throughout the world, including Australia, leading to catastrophic impacts on soil properties and diverse and threatened flora (Newsome *et al.* 2012). A study comparing the vegetation type and soil properties on roads and 10 m into the forest in the sub-alpine areas of KNP revealed that soil alongside roads had significantly lower levels of humus, higher levels of gravel and sand, fewer nutrients and lower pH and electrical conductivity than soil 10 m into the forest. Roadsides had 28% weed and bare cover compared to 2% bare ground and 6% weed cover 10 m into the forest (Johnston & Johnston 2004). Tourism leads to the introduction and spread of weeds and pathogens, vegetation trampling, fire scars, littering, soil and water nutrients manipulation, and illegal collection of rare and threatened plants (Willard & Marr 1970, McDougall *et al.* 2003). Management practices to reduce losses from tourism should include hardening of walkways with pathogen-free gravel, elevated wood or metal walkways to reduce contact of visitors with the flora and limit trampling, and pavers along with controlled drainage systems (Good & Grenier 1994). These management practices are expensive and more than Aus$420,000 were spent on the rehabilitation of the summit of Mt Kosciuszko and surrounding area (NPWS 1997). Although KNP flora is of national and international significance, very limited research has been carried out to determine the direct and indirect impacts of tourism. Therefore, future research should be directed to investigate the impacts of tourism in this area (Pickering & Hill 2007).

Management strategies

An integrative research and management approach needs to be undertaken to stop the introduction of new species and reduce the spread and impact of the existing *Phytophthora* community in KNP. These include strictly monitored quarantine procedures, the use of conventional hygiene measures (for example, foot scrubbing stations at track heads, vehicle wash down, signage for visitors, and training of staff members), determination of susceptibility of sub-alpine flora to *Phytophthora* species, the inclusion of non-host and resistant vegetation to reduce the spread, re-routing walk tracks to low risk areas, or closing walk trails during periods of high rainfall to prevent further spread via vehicles, cycling and foot walking. Remote sensing tools (e.g. multi and hyperspectral imagery through satellite, fixed wing aircraft and unmanned aerial vehicles) should be regularly used to detect early changes in vegetation health, especially susceptible species.

In conclusion, this study has shown alpine and sub-alpine ecosystems to be at risk from a range of *Phytophthora* species, including *P. cinnamomi*. As described above, reasons for the

lack of disease expression in areas where *Phytophthora* species have been isolated/detected appear complex and need further investigation. Kosciuszko National Park heathlands contain species from many families that are very susceptible to *Phytophthora* infection in other areas in Australia; these include Proteaceae, Xanth orrhoeaceae and Fabaceae (Newhook & Podger 1972, Podger 1972). *Pimelea bracteata* already infected with *Phytophthora* species should possibly be eradicated to stop further spread. If eradication is not practical, they should not be used in other areas for re-vegetation to and should be treated with systemic fungicides, such as phosphite. Further work is suggested to establish the efficacy of phosphite against *Phytophthora* species including *P. cinnamomi*, especially those isolated from these environments. Also, further work is required to determine the persistence, concentration (amount) and appropriate time (season) of application of phosphite in these areas. Also, the efficacy of *Phytophthora* should be tested against a range of *Phytophthora* species to determine its efficacy, as this information is not known. Since no attempts have been made to determine *Phytophthora* dieback in these ecosystems, detailed surveys should be conducted to investigate *Phytophthora* infestation in these areas.

Supplementary Material

Supplementary material for Chapter 2

Table S2.1 Selective media used for isolation of *Phytophthora* species. Ingredients are given to make the final volume one liter

Isolation Medium	Ingredients (µg/mL = mg/L = ppm)	Reference
3-P Medium	Difco cornmeal agar 17 g Pimaracin 100 ppm[1] Penicillin 50 ppm Polymyxin 50 ppm	(Eckert & Tsao 1960, Eckert & Tsao 1962a)
P_{10}VP	Difco cornmeal agar 17 g Pimaracin 10 ppm Vancomycin 200 ppm Pentachloronitrobenzene 100 ppm	(Tsao & Ocana 1969)
Media utilizing Hymexazol	Potato-dextrose agar (1 % agar) Benomyl 10 ppm Pentachloronitrobenzene 25 ppm Nystatin 25 ppm Ampicillin 500 ppm Rifampicin 10 ppm Hymexazol 25-50 ppm	(Masago *et al.* 1977)
P_{10}ARP	Difco cornmeal agar 17 g Pimaracin 10 ppm Ampicillin 250 ppm Rifampicin 10 ppm Pentachloronitrobenzene 100 ppm	(Kannwischer & Mitchell 1978)
P_5ARP	Difco cornmeal agar 17 g Pimaracin 5 ppm Ampicillin 250 ppm Rifampicin 10 ppm Pentachloronitrobenzene 100 ppm	(Jeffers & Martin 1986)
Media utilizing Benomyl	Agar 20 g Benomyl 25 ppm Pentachloronitrobenzene 30 ppm Penicillin 60 ppm Chloramphenicol 30 ppm Polymyxin 50 ppm Hymexazol 25 ppm	(Sneh & Katz 1988)
Media utilizing Benomyl	Benomyl 10 ppm Pimaracin 10 ppm Rifampicin 10 ppm Hymexazol 50 ppm	(George & Milholland 1986)
Media utilizing Benomyl	Benomyl 25 ppm Pimaracin 5 ppm Vancomycin 200 ppm Penicillin 100 ppm Pentachloronitrobenzene 100 ppm Hymexazol 20 ppm	(Papavizas *et al.* 1981)
Media utilizing Benomyl	Benomyl 15 ppm Penicillin G 250 ppm Polymyxin B 250 ppm Pentachloronitrobenzene 100 ppm	(Ponchet *et al.* 1972, Ricci 1972)
Media utilizing Benomyl	Benlate 20 ppm Hymexazol 20 ppm Mycostatin 10 ppm	(Bisht & Nene 1988)

	Pentachloronitrobenzene 20 ppm Pimaracin 5 ppm Vancomycin 200 ppm Rifampicin 10 ppm	
Media utilizing Cholaramphenicol (PBNC)	A weak V8 juice 40 mL	(Schmitthenner 1973)
	$CaCO_3$ 0.6 g Yeast extract 0.2 g Sucrose 1 g Cholestrol N, N-dimethyl formadide (0.01 g in2 mL) Agar 20 g Benlate (50 % benomyl) 20 ppm Pentachloronitrobenzene 27 ppm Neomycin sulfate 100 ppm Chloramphenicol 10 ppm	
PCH	KH_2PO_4 1.0 g $MgSO_4.7H_20$ 0.5 g $CaCl_2.2H_20$ 0.01 g $FeSO_4.7H_20$ 0.02 g Thiamine HCl 0.001 g KCl 0.5 g Yeast extract 0.3 g $NaNO_3$ 1.0 g Agar 20 g Dextrose 15 ppm Pentachloronitrobenzene 35 ppm Chloramphenicol (10 ppm in 2 mL of ethanol) Hymexazol 50 ppm Pimaracin 5 ppm	(Shew & Benson 1982)
NARPH	Cornmeal agar 17 g Nystatin 1 mL Ampicillin 100 ppm Rifampicin 10 ppm Pentachloronitrobenzene 100 ppm Hymexazol 50 ppm	(Hüberli *et al.* 2000)
Phytophthora Selective Medium (PSM)	Agar 8 g	(Burgess *et al.* 2008)
	Carrot puree 20 mL Potato puree 80 mL Hymexazol 3.7 mL of stock solution in water Pimaracin 400 µl Penicillin 200 ppm	
CMA-PARBH	Difco cornmeal agar 17 g Pimaracin 5 ppm Ampicillin 250 ppm Rifampicin 10 ppm Pentachloronitrobenzene 100 ppm Benomyl 0.02 g Hymexazol 0.069 g	(Pérez-Sierra *et al.* 2010)
NARH	Cornmeal agar 17 g Nystatin 1 mL Ampicillin 100 ppm Rifampicin 10 ppm Hymexazol 50 ppm	Simamora et al. (2017)

Table S2.2 Number of isolates of each *Phytophthora* species recovered from each site using soil baiting and filtering. No isolates were obtained using the apple baiting method

Species	Clade	Soil baiting					Filters				
		1	2	3	4	5	1	2	3	4	5
P. multivora	2			1	40				6		
P. amnicola	6					8					15
P. gregata	6										1
P. inundata	6					30					
P. 'oreophila'	6					13					6
P. rosacearum	6			1		24			1		5
P. thermophila	6					16					39
Total				2	40	91			7		66

Table S2.3 Proportion of total reads of each *Phytophthora* species detected

Species	Clade	% reads
P. multivora	2	66.73
P. psuedocryptogea	8	12.69
P. amnicola	6	3.38
P. thermophila	6	2.95
P. AUS 1D	1	2.57
P. rosacearum	6	2.35
P. nicotianeae	1	2.03
*P. '*oreophila'	6	1.43
P. cinnamomi	7	1.37
P. kwongonina	6	0.68
P. 'kelmania'	8	0.66
P. capensis	2	0.66
P. arenaria complex	4	0.45
P. constricta	9	0.44
P. versiformis	11	0.44
P. inundata	6	0.44
P. melonis	7	0.21
P. citrophthora	2	0.17
P. gregata	6	0.11
P. moyootj	6	0.08
P. 'acacia'	2	0.05
P. elongata	2	0.04
P. frigida	2	0.03
P. pachypleura	2	0.03
P. palmivora	4	0.02
P. AUS 2C	2	0.02
P. capsici	2	0.02
P. fluvialis	6	0.02
P. litoralis	6	0.01
P. cambivora	7	0.004

Table S2.4 *Phytophthora* species detected from each site from each substrate by metabarcoding. Rows that are shaded indicate species that were isolated using standard isolation techniques.

Species	Clade	Soil					Filters					Field roots			Glasshouse roots				
		1	2	3	4	5	1	2	3	4	5	1	4	5	1	2	3	4	5
P. nicotianeae	1							+	+		+	+	+	+	+	+	+	+	+
P. AUS1D	1	+	+	+		+	+	+				+	+	+				+	+
P. capensis	2								+				+		+	+	+		
P. capsici	2								+										+
P. elongata	2								+				+						
P. frigida	2								+										+
P. multivora	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. pachypleura	2								+				+						
P. 'acacia'	2												+			+			
P. citrophthora	2								+				+	+		+	+		+
P. AUS2C	2												+						
P. arenaria complex	4								+				+		+	+	+		
P. palmivora	4								+										
P. amnicola	6	+			+		+	+	+	+	+	+	+	+	+	+	+	+	+
P. fluvialis	6												+						
P. gregata	6										+		+						
P. inundata	6		+			+		+	+	+	+	+	+			+	+	+	+
P. litoralis	6												+						
P. moyootj	6										+		+						
P.' oreophila'	6	+		+			+	+	+	+	+	+	+	+	+	+	+	+	+
P. rosacearum	6		+		+				+			+	+			+	+		
P. kwongonina	6	+					+	+	+	+	+		+	+		+	+	+	
P. thermophila	6	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. cambivora	7								+										
P. cinnamomi	7			+				+	+			+	+	+	+	+	+	+	+
P. melonis	7			+															
P. psuedocryptogea	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. 'kelmania'	8												+						
P. constricta	9				+				+	+		+	+	+		+	+		+
P. versiformis	11	+		+					+				+			+			
Total		8	6	7	5	5	7	10	21	8	10	11	25	11	9	16	14	10	13

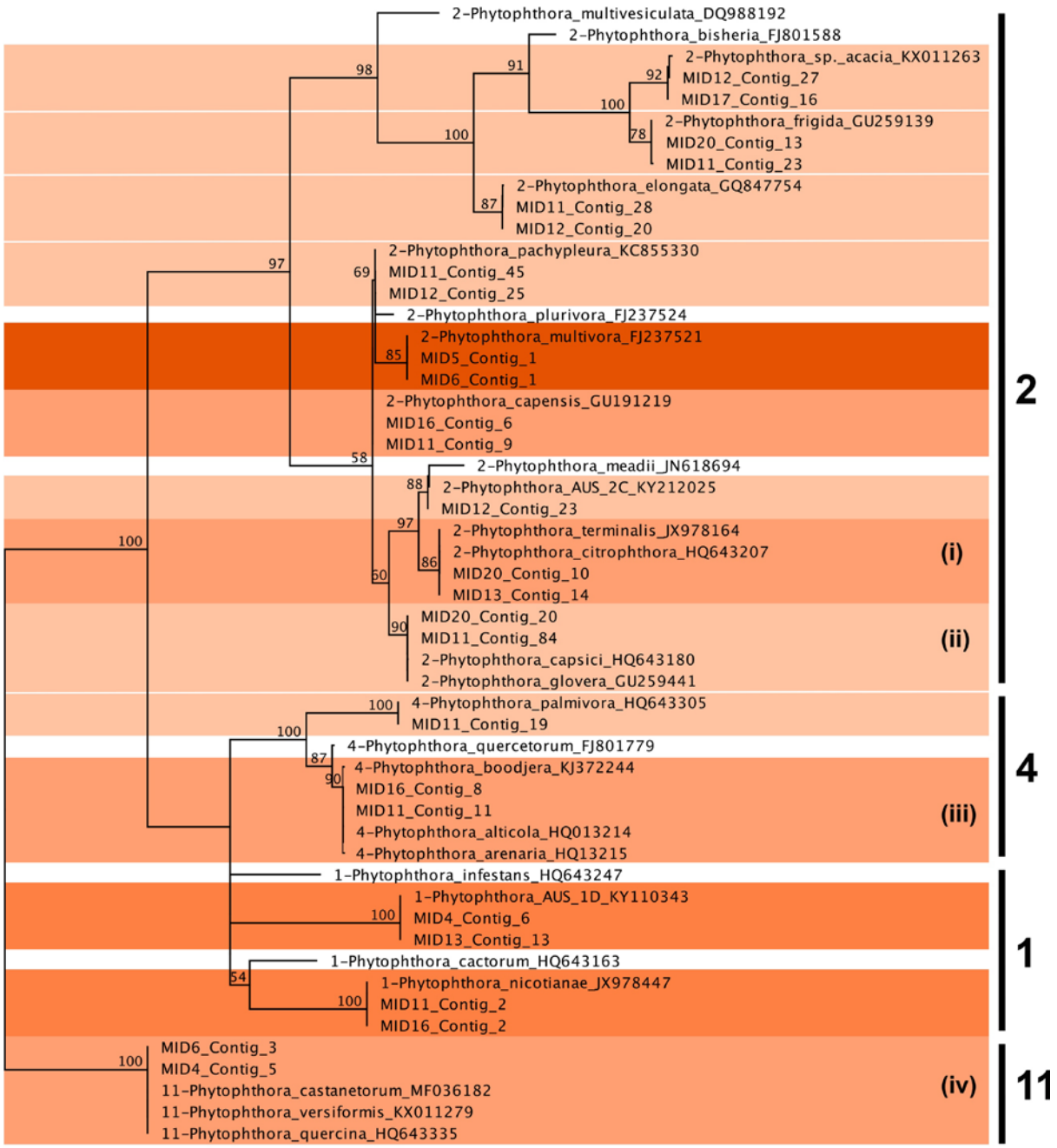

Figure S2.1 Phylogenetic comparisons of MOTUs detected in the current study within Clades 1, 2, 4 and 11 and all closely related known *Phytophthora* species and taxa species based on ITS1 gene region. The darker the background colour, the more common the species. MOTUs were assigned to new phylotypes if they did not match any known *Phytophthora* species. The ITS1 gene region ranges in size from 150-250 bp and is highly variable. However, some species cannot be separated based on ITS1 alone. Such species relevant to this study are designated i to iv.

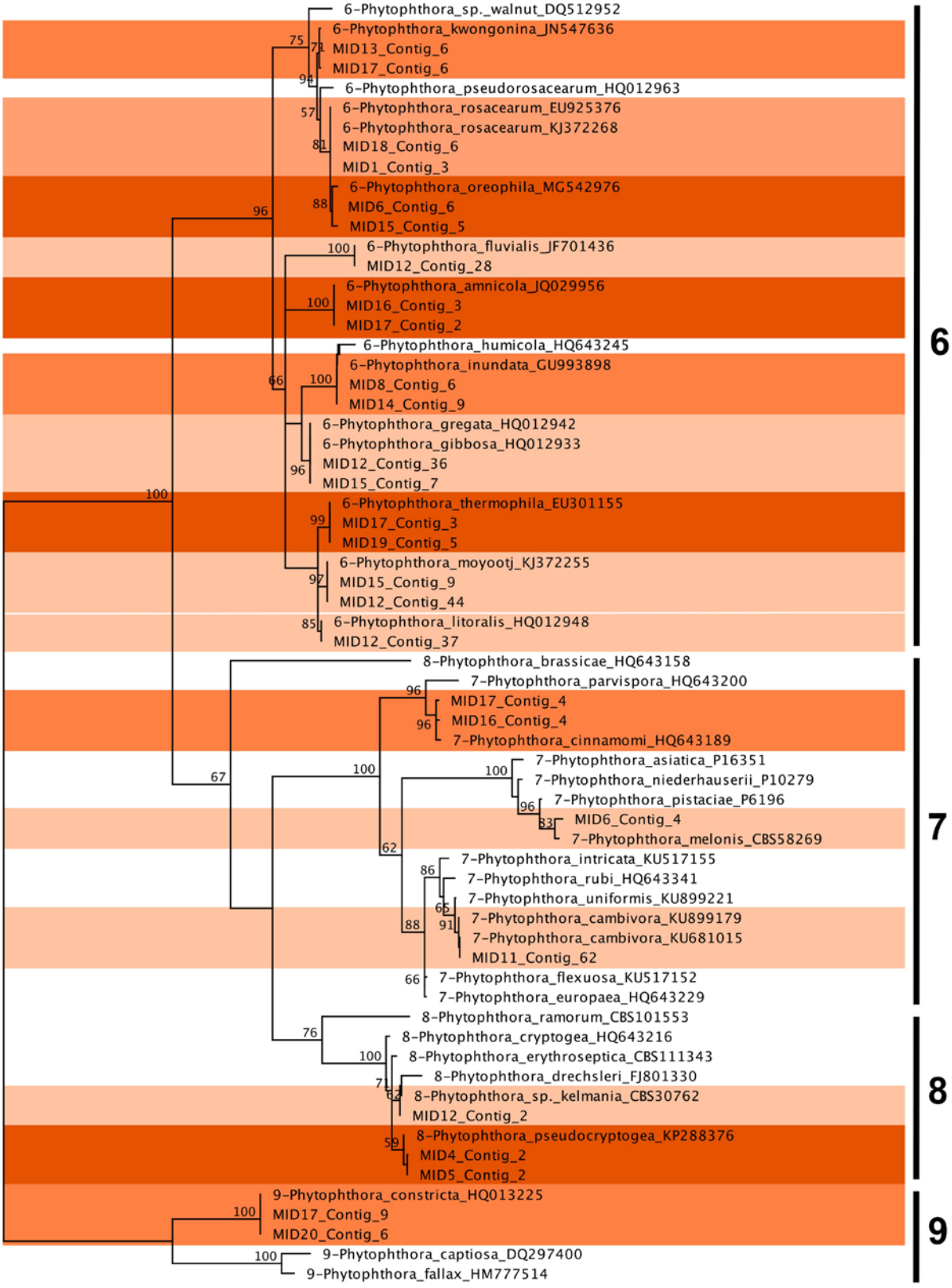

Figure S2.2 Phylogenetic comparisons of MOTUs from Clades 6-9 detected in the current study within Clades 1-5 and all closely related known *Phytophthora* species and taxa species based on ITS1 gene region. The darker the background colour, the more common the species

Supplementary material for Chapter 3

Table S3.1. Location, including altitude, and host association of isolates considered in this study. Shaded rows represent isolates that were recovered in this study. The remaining isolates were included in the phylogenetic analysis.

Isolate	Organism	Location	Vegetation	Date	Latitude	Longitude	Altitude (m)
QLD13E	*Phytophthora* sp.	Australia, QLD[a], Koombooloomba	Tropical rain forest	2013	17°49'52.00"S	145°35'43.01"E	735
U40	*P. cacuminis*	Australia, TAS[a], Mt Field NP[b]	*Eucalyptus coccifera*	2016	42°40'49.00"S	146°35'26.09"E	735
U41	*P. cacuminis*	Australia, TAS, Mt Field NP	*Eucalyptus coccifera*	2016	42°40'50.28"S	146°35'20.63"E	1434
U11	*P. oreophila*	Australia, NSW[a], Merritts Creek	Disturbed alpine herbfield	2016	36°27'28.80"S	148°18'10.80"E	1830
VHS26182	*Phytophthora* sp.	Australia, WA, Fitzgerald River NP	Kwongan heathland	2006	33°53'11.09"N	119°53'06.88"E	44
TAS34	*P. cactorum*	Australia, TAS, Pine Lake	*Athrotaxis selaginoides*	2013	41°44'34.60"S	146°42'32.34"E	1207
U1	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	36°27'28.80"S	148°18'10.80"E	1830
U2	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	36°27'28.80"S	148°18'10.80"E	1830
U3	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	36°27'28.80"S	148°18'10.80"E	1830
U4	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	36°26'02.90"S	148°19'46.83"E	1830
U5	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	36°26'02.90"S	148°19'46.83"E	1830
U6	*P. cactorum*	Australia, NSW, Merritts Creek	Disturbed alpine herbfield	2016	36°27'28.80"S	148°18'10.80"E	1830
U7	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	36°26'02.90"S	148°19'46.83"E	1830
U8	*P. cactorum*	Australia, NSW, Charlottes Pass	*Eucalyptus niphophila*	2016	36°26'02.90"S	148°19'46.83"E	1830
W1846	*P. cambivora*	Australia, NSW, Charlottes Pass	*Nematolepis ovatifolia*	2014	36°26'02.90"S	148°19'46.83"E	1830
TAS188	*P. cinnamomi*	Australia, TAS, Condominium Creek	Riparian rain forest	2013	42°57'35.26"S	146°21'41.21"E	350
VHS16127	*P. constricta*	Australia, WA, Fitzgerald River NP	Kwongan heathland	2006	33°53'20.54"N	119°53'01.75"E	44
VHS16130	*P. constricta*	Australia, WA, Fitzgerald River NP	Kwongan heathland	2006	33°53'20.54"N	119°53'01.75"E	44
U21	*P. cryptogea*	Australia, NSW, Mt Kosciuszko	Walking track edge in alpine heath	2016	36°27'21.6"S	148°15'50.40"E	2228
U22	*P. cryptogea*	Australia, NSW, Mt Kosciuszko	Walking track edge in alpine heath	2016	36°27'21.6"S	148°15'50.40"E	2228
TAS126	*P. elongata*	Australia, TAS, Mt Field NP	Riparian rain forest	2013	42°40'54.12"S	146°38'49.2E	1029
U34	*P. fallax*	Australia, TAS, Hartz Mountain NP	Melaleuca	2016	43°13'13.70"S	146°46'18.98"E	1254
U35	*P. fallax*	Australia, TAS, Hartz Mountain NP	Alpine heath	2016	43°13'26.39"S	146°46'12.08"E	1254
U36	*P. fallax*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	43°13'13.70"S	146°46'18.98"E	1254
U37	*P. fallax*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	43°13'13.70"S	146°46'18.98"E	1254
U14	*P. gonapodyides*	Australia, NSW, Smiggins Hole	Road edge in subalpine heath	2016	36°23'38.40"S	148°25'40.8"E	1660
U15	*P. gonapodyides*	Australia, NSW, Kosciuszko Road	Disturbed alpine herbfield	2016	36°27'14.40"S	148°18'10.80"E	1870

Isolate	Organism	Location	Vegetation	Date	Latitude	Longitude	Altitude (m)
TAS206	*P. gregata*	Australia, TAS, Pine Lake	Moorland	2013	41°44'36.24"S	146°42'42.84"E	1200
TAS207	*P. gregata*	Australia, TAS, Pine Lake	Moorland	2013	41°44'36.24"S	146°42'42.84"E	1200
U9	*P. gregata*	Australia, NSW, Pipers Gap	Road edge in subalpine heath	2016	36°24'00.00"S	148°25'08.40"E	1740
U10	*P. gregata*	Australia, NSW, Pipers Gap	Road edge in subalpine heath	2016	36°24'00.00"S	148°25'08.40"E	1740
U12	*P. gregata*	Australia, NSW, Perisher	Disturbed subalpine wetland	2016	36°24'10.8"S	148°25'01.2"E	1730
U13	*P. gregata*	Australia, NSW, Perisher	Disturbed subalpine wetland	2016	36°24'10.8"S	148°25'01.2"E	1730
U18	*P. gregata*	Australia, NSW, Pipers Gap	Road edge in subalpine heath	2016	36°24'00.00"S	148°25'08.40"E	1740
U32	*P. gregata*	Australia, NSW, Perisher	Disturbed subalpine wetland	2016	36°24'10.8"S	148°25'01.2"E	1730
U38	*P. gregata*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	43°13'26.39"S	146°46'12.08"E	1254
U39	*P. gregata*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	43°13'26.39"S	146°46'12.08"E	1254
U42	*P. gregata*	Australia, TAS, Hartz Mountain NP	*Melaleuca* sp.	2016	43°13'13.70"S	146°46'18.98"E	1254
TAS143	*P. pseudocryptogea*	Australia, TAS, Steppes	Woodland	2013	42°07'43.32"S	146°58'17.76"E	797
U20	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	36°19'22.80"S	148°28'40.80"E	1275
U23	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	36°19'22.80"S	148°28'40.80"E	1275
U24	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	36°19'22.80"S	148°28'40.80"E	1275
U30	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	36°19'22.80"S	148°28'40.80"E	1275
U31	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	36°19'22.80"S	148°28'40.80"E	1275
U33	*P. pseudocryptogea*	Australia, NSW, Island Bend	Highly modified montane forest	2016	36°19'22.80"S	148°28'40.80"E	1275
CBS119107	*P. captiosa*	New Zealand, Rotoehu Forest	*Eucalyptus saligna*	1995	38°01'12.45"S	176°31'56.97"E	292
NZFS310.35	*P. captiosa*	New Zealand, Rotoehu Forest	*Eucalyptus saligna*	1998	38°01'12.45"S	176°31'56.97"E	292
MUCC761	*P. gonapodyides*	Australia, VIC[a], Toolangi North	*Eucalyptus oblique* forest	2008	37°32'19.30"S	145°31'12.88"E	741
CBS127954	*P. thermophila*	Australia, WA, Dwellingup	*Eucalyptus marginata*	2004	32°44'09.61"S	116°03'09.34"E	237
TP13.29	*P. versiformis*	Australia, WA, Naturaliste	*Corymbia calophylla*	2013	33°37'51.96"S	115°03'27.79"E	94
CBS 142005	*P. versiformis*	Australia, WA, Williams	*Corymbia calophylla*	2013	33°02'00"S	116°53'00.00"E	289
HAS2313	*P. cooljarloo*	Australia, WA, Cooljarloo	Swamp native vegetation	1996	30°42'27.28''S	115°23'12.30"E	72
VHS24266	*P. pseudorosacearum*	Australia, WA, Albany	*Xanthorrhoea platyphylla*	2010	35°01'44"S	117°54'49"E	103
OSU55	*P. rosacearum*	USA, Maryland	*Prunus armeniaca*				
VHS29592	*P. pseudorosacearum*	Australia, WA, Jarrahdale	*Persoonia longifolia*	2013	32°19'21.37"S	116°01'30.48"E	142
VHS23298	*P. kwongonina*	Australia, WA, Bunbury	*Banksia grandis*	2010	33°19'32.29"S	115°38'22.74"E	10
TAS35	*P. gonapodyides*	Australia, TAS, Houn River	Native vegetation	2009	43°08'22.88"S	146°42'53.12E	134
IMI389735	*P.* taxon walnut	USA, California, Merced County	*Juglans hindsii*	1988	37°12'03.52"N	120°42'43.21"S	30
CBS124696	*P. rosacearum*	USA, California					
P10725	*P. fallax*	New Zealand	*Eucalyptus fastigata*				

Isolate	Organism	Location	Vegetation	Date	Latitude	Longitude	Altitude (m)
CBS139749	*P. pseudocryptogea*	Australia, WA, Fitzgerald River NP	*Isopogon buxifolius*	2006	33°53'20.54"N	119°53'01.75"E	44
VHS5380	*P. pseudocryptogea*	Australia, WA, Fitzgerald River NP	*Xanthorrhoea preissii*	1992	33°53'20.54"N	119°53'01.75"E	44
CBS125801	*P. constricta*	Australia, WA, Fitzgerald River NP	Kwongon heathland	2006	33°53'20.54"N	119°53'01.75"E	44
CLJ0100	*P. cooljarloo*	Australia, WA, Cooljarloo	Hibbertia sp.	2008	30°42'27.28''S	115°23'12.30"E	72
NZFS310.25	*P. captiosa*	New Zealand, Rotoehu Forest	*Eucalyptus saligna*	1998	38°01'12.45"S	176°31'56.97"E	292

[a] QLD = Queensland, TAS = Tasmania, VIC = Victoria, NSW = New South Wales
[b] NP = National Park

Supplementary material for Chapter 4

Table S4.1 Comparison of mean radial growth rates (mm day^{-1}) of all 30 isolates from slowest to fastest. Isolates with different letters indicate significantly ($p < 0.05$) different growth

Isolate	Mean	Rank
MUCC815	1.46a	1
MP94-05	1.99b	2
MP126	2.01b	3
MP94-18	2.01b	4
MP114	2.06bc	5
MP94-15	2.09bc	6
MP133	2.09bc	7
MP62	2.09bcd	8
MP94-09	2.14bcd	9
MP94-27	2.15bcd	10
MP129	2.22bcde	11
MUCC817	2.23bcde	12
MP94-33	2.23bcde	13
MUCC813	2.25bcde	14
MUCC814	2.30cde	15
MP94-10	2.35def	16
MP94-49	2.39def	17
MP94-39	2.47ef	18
TAS188	2.60fg	19
MP94-12	2.64fg	20
MP94-20	2.77gh	21
MP88	2.79gh	22
MUCC818	2.82ghi	23
MP94-11	2.94hij	24
MUCC816	3.07ijk	25
MP94-48	3.16jkl	26
MP119	3.18jkl	27
MP80	3.30kl	28
MP84	3.37lm	29
MP89	3.64m	30

Figure S4.1 Average growth rate (mm day^{-1}) of 30 *P. cinnamomi* isolates on V8A across the temperature range from 4 - 37.5 °C. The isolates were divided into five profiles A, B, C, D and E (top to bottom) based on their differential growth rates. Profile A (MUCC813- MP80), profile B (MUCC816- MP89), profile C (MP114), profile D (MP94-05, MP94-27), and profile E (MUCC815)

6

Figure S4.2 Comparison of mean number of intact and empty (indicative of zoospore release) sporangia (mm^{-2}) between isolates recovered from plants grown in the glasshouse (orange) at ambient temperature and those recovered from plants grown in the growth chamber at 9 ˚C (blue) for three months. Bars = SE

Supplementary material for Chapter 5

Table S5.1 Proportion of total reads of each *Phytophthora* species and other organisms detected

Organisms	Clade	% reads
Phytophthora versiformis	11	19.90
Phytophthora AUS XA	X	5.55
Phytophthora AUS 1B	1	3.96
Phytophthora EXP XB	X	3.58
Phytophthora AUS 1A	1	2.89
Phytophthora AUS 1F	1	2.09
Phytophthora elongata	2	1.92
Pythium rostratum		1.33
Phytophthora AUS XB	X	1.32
Phytophthora AUS 8E	8	1.30
Phytophthora iranica	1	1.22
Phytophthora europea	7	1.22
Unidentified oomycete 2		1.20
Phytophthora AUS 9B	9	1.19
Pythium attrantheridium		1.16
Phytophthora AUS XD	X	0.93
Unidentified oomycete 1		0.83
Phytophthora arenaria	4	0.46
Phytophthora AUS 1E	1	0.44
Phytopythium citrinum		0.23
Pythium rostratifingens		0.08
Lagena sp.		0.06
Phytophthora pachypleura	2	0.04
Phytophthora pseudocryptogea	8	0.02
Pythium segnitium		0.01
Phytophthora AUS 1G	1	0.01
Non-oomycete		**48.28**

Table S5.2 *Phytophthora* species detected along an elevation gradient in Kosciuszko National Park. Shaded rows represent species isolated obtained by traditional (non-molecular) isolation methods. Only two *Phytophthora* species (*P. elongata* and *P. pseudocryptogea*) were detected with both high-throughput sequencing and traditional baiting.

Species name	Clade
Phytophthora AUS 1A	1
Phytophthora AUS 1B	1
Phytophthora AUS 1F	1
Phytophthora AUS 1G	1
Phytophthora AUS 1E	1
Phytophthora iranica	1
Phytophthora cactorum	1
Phytophthora elongata	2
Phytophthora pachypleura	2
Phytophthora multivora	2
Phytophthora arenaria	4
Phytophthora gregata	6
Phytophthora thermophila	6
Phytophthora chlamydospora	6
Phytophthora europea	7
Phytophthora AUS 8E	8
Phytophthora pseudocryptogea	8
Phytophthora AUS 9B	9
Phytophthora fallax	9
Phytophthora gondwanense	10
Phytophthora versiformis	11
Phytophthora AUS XA	X
Phytophthora AUS XB	X
Phytophthora AUS XD	X
Phytophthora EXP XB	X
Total	**25**

Table S5.3 Estimates, standard error, z value and probability (** P < 0.01; *** P < 0.001) for the models fitted for *Phytophthora* and vascular plant species richness in relation to disturbance (factor: roads or native vegetation) and elevation (m).

	Estimate	**Std. Error**	**z value**	**P(>|z|)**
***Phytophthora* species**				
(Intercept)	0.99	0.35	2.79	**
Elevation (m)	0.01	0.01	1.27	n.s.
Disturbance (roads / native)	1.54	0.48	3.25	**
Interaction (elevation*disturbance)	-0.04	0.01	-3.12	**
Vascular plant species				
(Intercept)	3.77	0.12	31.74	***
Elevation (m)	-0.01	0	-1.86	n.s.
Disturbance (roads / native)	0.61	0.17	3.59	***
Interaction (elevation*disturbance)	-0.02	0.01	-4.29	***

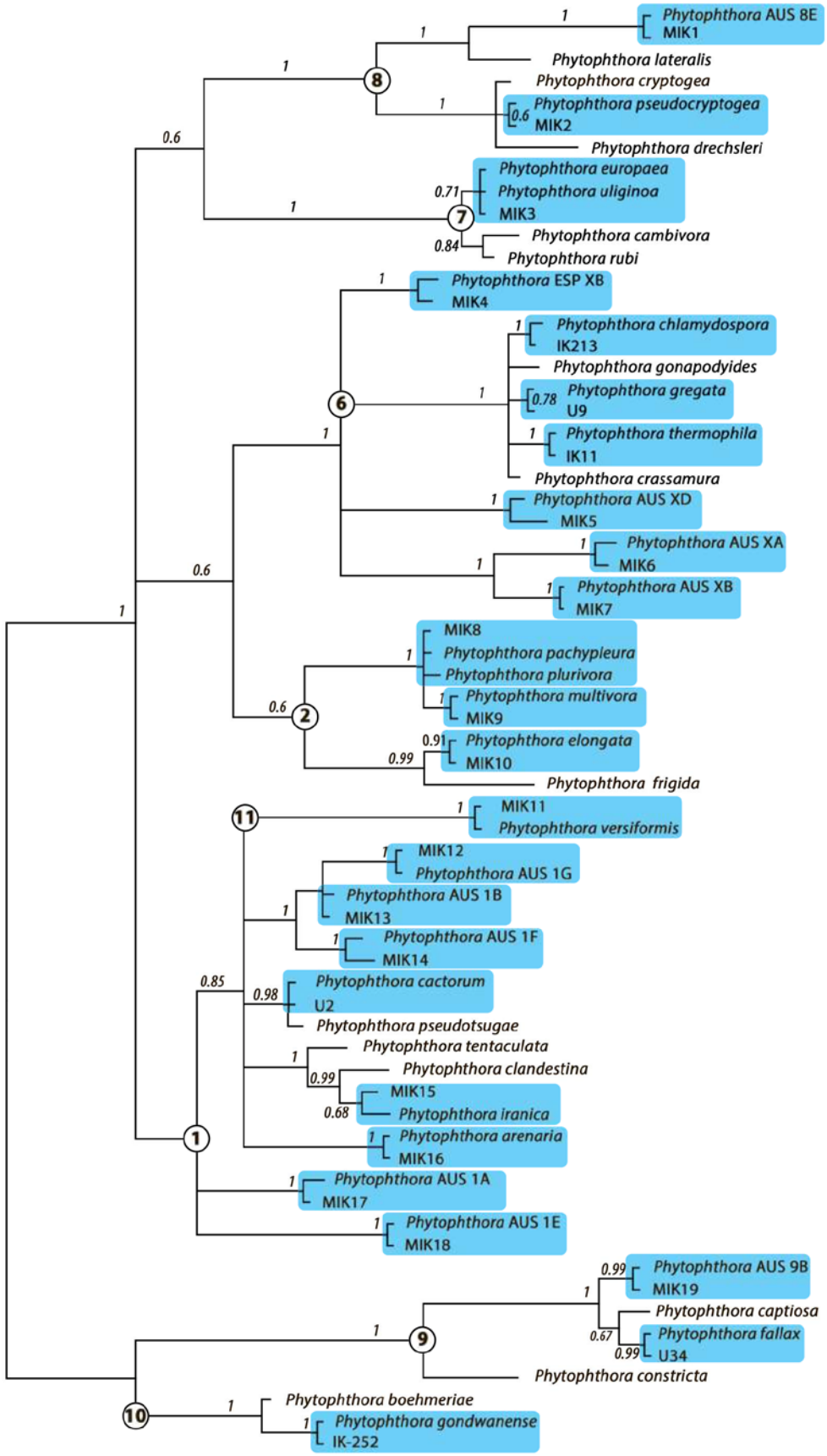

Figure S5.1 Phylogenetic comparisons of *Phytophthora* species detected in the current study (blue) with all closely related known *Phytophthora* species and taxa species based on ITS1 gene region. Code 'MIK' represents Phylotypes and codes 'U' and 'IK' represent isolations. Bayesian posterior probabilities are listed above the branches.

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