

An overview of Australia's *Phytophthora* species assemblage in natural ecosystems recovered from a survey in Victoria

William A. Dunstan¹, Kay Howard¹, Giles E. StJ. Hardy¹, and Treena I. Burgess¹

¹Centre for *Phytophthora* Science & Management, School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, WA 6150, Australia; corresponding author e-mail: G.Hardy@murdoch.edu.au

Abstract: Although *Phytophthora* species cause serious diseases worldwide, until recently the main focus on disease in natural ecosystems in southern Australia has been on the distribution and impact of *P. cinnamomi*. However, new *Phytophthora* pathogens have emerged from natural ecosystems, and there is a need to better understand the diversity and distribution of these species in our natural forests, woodlands and heathlands. From a survey along a 70 km pipeline easement in Victoria, *Phytophthora* species were isolated from 249 rhizosphere samples and 25 bait bags deployed in 21 stream, river, or wetland locations. Of the 186 *Phytophthora* isolates recovered, 130 were identified to species based on ITS sequence data. Ninety-five isolates corresponded to 13 described *Phytophthora* species while additionally 35 isolates were identified as Clade 6 hybrids. *Phytophthora cinnamomi* was the most common species isolated (31 %), followed by *P. elongata* (6 %), both species were only recovered from soil. Samples from sites with the highest soil moisture at the time of sampling had the highest yield of isolates. Consistent with other studies throughout the world, Clade 6 species and their hybrids dominated water samples, although many of these species were also recovered less frequently from soil samples. Many of the species recovered in this study have not previously been reported from eastern Australia, reinforcing that *Phytophthora* species are widespread, abundant and diverse in natural ecosystems. We have probably been underestimating *Phytophthora* diversity in Australia.

Key words:

Eucalyptus
habitat disturbance
host preference
plant pathology
tree diseases

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INTRODUCTION

Although *Phytophthora* causes serious diseases worldwide in agriculture, horticulture and natural ecosystems, it is only recently that accurate identification of many species of these important plant pathogens has been made possible using molecular methods. The number of described species is rapidly increasing, with at least 124 species now described (Scott *et al.* 2013, Martin *et al.* 2014), allowing a more comprehensive understanding of the distribution of these destructive pathogens (Hansen *et al.* 2012). When developing a PCR-based molecular toolbox, Schena *et al.* (2008) focused on 15 *Phytophthora* species that cause a decline in forests and other natural ecosystems. However, Jung *et al.* (2005) suggest that all *Phytophthora* species have the potential to cause plant health decline in natural ecosystems, particularly when there are assemblages of different introduced species present, and conditions are conducive for disease development (Scott *et al.* 2013).

In natural communities in Australia, the attention has been largely on *P. cinnamomi*. Elsewhere, studies on *Phytophthora* species diversity have focussed on the few species most destructive in the horticultural and agricultural industries (Hansen *et al.* 2012). *Phytophthora cinnamomi* has the broadest host range of all *Phytophthora* species

and is considered an aggressive forest pathogen (Robin *et al.* 2012). *Phytophthora cinnamomi* occurs widely across Australia and is recognised by the Federal Government as 'a key threatening process' to Australia's biodiversity (Environment Protection and Biodiversity Conservation Act 1999, Environment Australia 2001). The impact of *P. cinnamomi* is greatest in the natural communities of the south-west and south-east of Australia, where it has caused the loss of animal species along with changes in species abundance and population structure (Cahill *et al.* 2008), also resulting in changes in floristic structure, diversity, and ecological processes (Shearer *et al.* 2004, McDougall *et al.* 2005). In south-west Western Australia (WA), which contains four of Australia's 15 biodiversity hotspots (Department of Environment 2015), it has been estimated that there are 2284 plant species that are susceptible to *P. cinnamomi* (Shearer *et al.* 2004). McDougall (2005) compiled a list of over 1460 species known to be susceptible, after *P. cinnamomi* had been isolated from infected plants or found in susceptibility testing. This represents only about 5 % of Australia's vascular plants and so the effect of *P. cinnamomi* on most Australian plants is still unknown (Cahill *et al.* 2008). Due to the focus on *P. cinnamomi*, particularly in natural ecosystems, there is a paucity of information on the nature and extent of other *Phytophthora* species present in natural ecosystems. We now

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know that there are several other pathogenic *Phytophthora* species in WA (Scott *et al.* 2009, Rea *et al.* 2010, 2011, Crous *et al.* 2011, 2012, 2014, Jung *et al.* 2011, Aghighi *et al.* 2012), but very little is known about their diversity and distribution across Australia.

Reports of *Phytophthora* in irrigation water date back to 1921 (Hong & Moorman 2005). Clade 6 *Phytophthora* species are dominant in water surveys (Reeser *et al.* 2011) where they may colonize leaves and other plant debris (Brasier *et al.* 2003, Jung *et al.* 2011, Reeser *et al.* 2011, Hansen *et al.* 2012). In northern Spain, High Throughput Sequencing (HTS) detected greater diversity of *Phytophthora* species in water than from soil samples indicating the importance of water environments as a source of inoculum (Català *et al.* 2015). In a small study in Victoria, isolation of *Phytophthora* species varied with season, bait type, and type of water filter used (Smith *et al.* 2009). Within one sampling season in WA, 12 *Phytophthora* species and two hybrids were isolated from baits in 48 waterways (Hüberli *et al.* 2013), emphasising the importance of surveying water bodies.

In Victoria, most of the published research on *Phytophthora* in natural ecosystems has focussed on *P. cinnamomi* across the south of the state in the Grampians, the Brisbane Ranges, Wilson's Promontory and East Gippsland (Cahill *et al.* 2008). Gibson *et al.* (2002) compiled a map of 495 sites where *P. cinnamomi* had been isolated between 1976 and 2001, with isolations occurring across most of the state. A formal assessment of the presence of *P. cinnamomi* within a 70 km survey corridor was undertaken prior to the commencement of construction activities of the Sugarloaf Pipeline, north east of Melbourne, in southern Victoria. Opportunistically isolating all *Phytophthora* species recovered from water baits and soil samples provided an insight into the assemblage of *Phytophthora* species in the region.

MATERIALS AND METHODS

Site description

As part of the Melbourne metropolitan water supply, the Sugarloaf Pipeline carries water from the Goulburn River at Killingworth (165 m AMSL) north of Yea (ca 100 km northeast of central Melbourne) to the Sugarloaf Reservoir in the Yarra Ranges (ca 5 km west of Yarra Glen) (Fig. 1). The parent material of the soils were principally siltstone and minor sandstone of the Humevale Formation, Quaternary alluvium on drainage line, and a minor occurrence of siltstone and shale of the Dargile Formation within the Sugarloaf reserve. The soil pH was mostly <5.5, with a couple of instances of pH 5.5 to 6.5. Along the corridor, the median annual rainfall ranged from 700 mm (Yea) to 1300 mm on the Great Dividing Range (GDR) to <600 mm around Sugarloaf Reservoir (Fig. 1). The study area encompassed two catchments separated by the GDR. Locations 1–3 are north of the GDR (Fig. 1).

Along the corridor there were approximately 12 of Victoria's 300 Ecological Vegetation Communities (EVCs) (DSE 2003) including nine classes of forest and complexes: lowland, riparian, heathy dry, shrubby dry, grassy dry, herb-rich, damp, wet and swampy forests; and three classes of woodlands: grassy, gully, and riparian (Table 1).

Experimental design

Stream baiting was used to isolate *Phytophthora* species from streams and minor water bodies at 21 locations (Table 2) along the entire length of the pipeline easement – 25 bait bags were deployed. In addition, 249 soil samples were collected for baiting from native forest and woodland sites, where the risk of damage to vegetation from *Phytophthora* was greatest.

Stream baiting

Floating mesh bait bags (30 x 30 cm) were left *in situ* for 9 to 10 d in the water bodies (Table 2) before all baits were retrieved in one operation. All streams, whether perennial or intermittent, were flowing at the time they were baited, although rainfall records for five weather stations on, or adjacent to, the survey corridor showed a median reduction in mean monthly rainfall of 64 % for the month prior to baiting (September). All static water bodies were at, or near, full capacity.

Baits included in each bag were 2-d-old lupin seedlings (*Lupinus angustifolius*; Erwin & Ribiero 1996), young, near fully expanded leaves of oak (*Quercus robur*; Jung *et al.* 1996, Balci *et al.* 2007), and immature leaves of sweet pittosporum (*Pittosporum undulatum*; Smith *et al.* 2009). Segments of leaves with signs of infection (water soaked to brown or black lesions) were plated onto a selective agar medium (NARPH; Hüberli *et al.* 2000), from which primary isolates were again transferred to NARPH medium and then to half strength potato dextrose agar (PDA, or ½ PDA; Becton Dickinson, Sparks, MD, USA).

Soil baiting

Soil samples were collected from 10 EVCs and two complexes (Table 1), often from multiple sites. These samples were collected from the pipeline easement from Glenburn to south of Kinglake (14 km) incorporating Toolangi State Forest, Paul's Range State Forest and Sugarloaf Reservoir Reserve (1.75 km).

Each soil sample (250–400 g) was taken, where possible, from within the 30 m pipeline easement. The elevation range for sample collection was between 185 and 390 m AMSL. Individual sampling points were generally selected using two criteria: the presence of dead or unhealthy individuals of known *P. cinnamomi* host species; or if potential hosts were absent, then suitable topography or presence of disturbance that were likely to be favourable for the introduction and survival of the pathogen (i.e. earthworks for roads, forest tracks, low relief and/or water gaining damp microsites).

Within 16 d of collection, the presence of *Phytophthora* species in soils were assessed using 2-d-old lupin seedlings (*Lupinus angustifolius*) and immature near fully expanded leaves of holm oak (*Quercus ilex*) as baits following the methods of Marks & Kassaby (1974). All lupin roots, and sections of oak leaves showing signs of infection, were plated onto NARPH medium. A second round of baiting was conducted 9 d after the first baiting using *Eucalyptus sieberi* cotyledons. All *Phytophthora* species were further purified on NARPH medium and maintained on ½ PDA for longer-term storage.

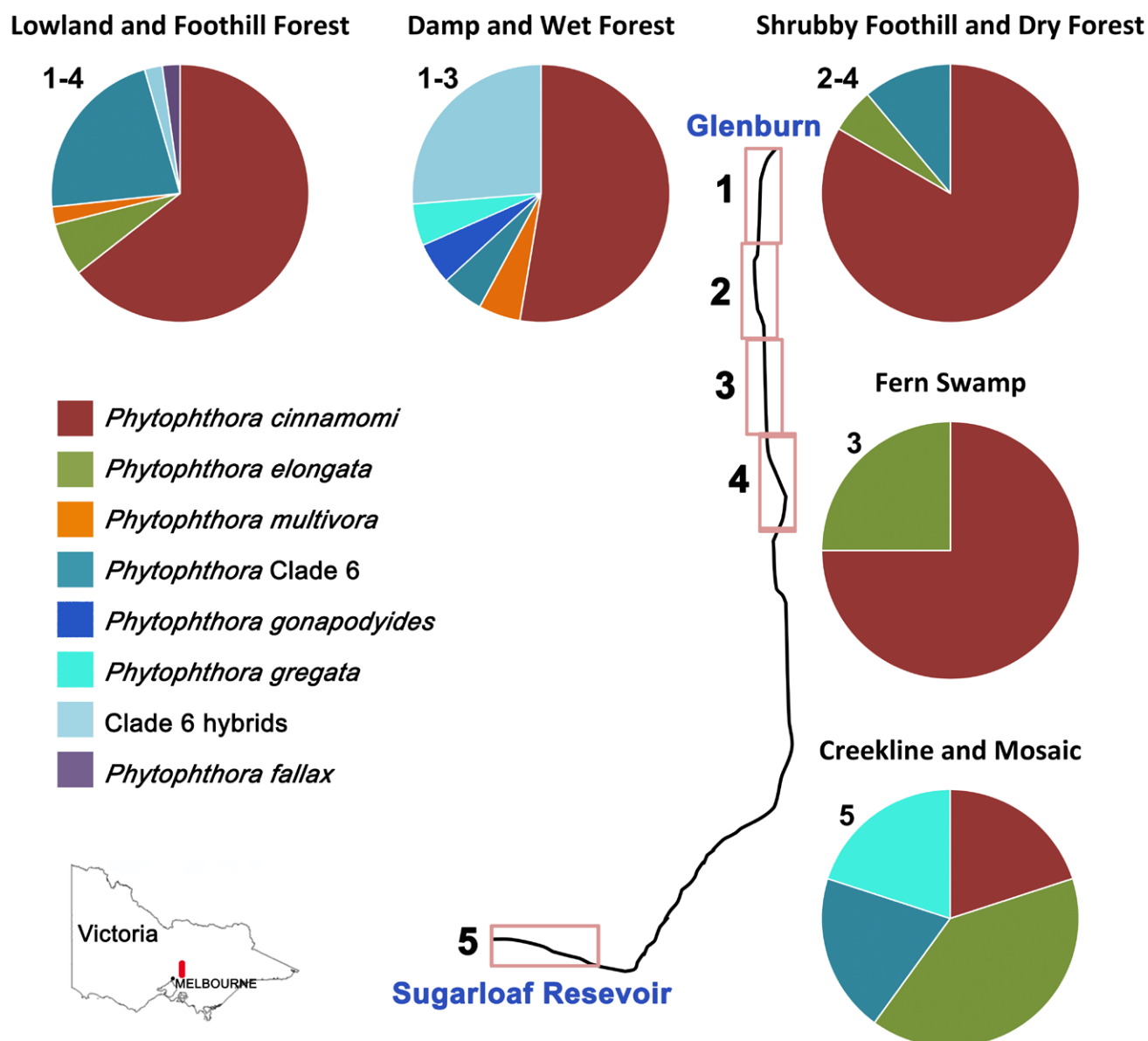


Fig. 1 Location of survey northeast of Melbourne, Victoria (bottom left). Proportion of each *Phytophthora* species to total number of isolates from soil in Victoria's Ecological Vegetation Classes (EVC). Lowland and Foothill Forest is a combination of EVC16 and 23, Damp and Wet Forest is a combination of EVC29 and 30. Shrubby Foothill and Dry Forest is a combination of EVC20 and 45, while Creekline and Mosaic is a combination of EVC164 and Mosaic. Fern Swamp is EVC721. The location (1–5) along Sugarloaf Pipeline Alliance easement, from Glenburn to Sugarloaf Reservoir, is noted next to the relevant pie chart. Locations 1–3 are north of the Great Dividing Range (GDR). Data not included if fewer than 4 isolates were recovered from EVC.

Isolate identification

After subculture on NARPH medium, putative *Phytophthora* isolates were grown on ½ PDA, sorted based on colony morphology, and representative isolates were selected for molecular identification (130 of 186 isolates).

DNA extraction from mycelium was carried out using the method of Andjic *et al.* (2007). For each isolate, the region spanning the internal transcribed spacers (ITS1-5.8S-ITS2) region of the ribosomal DNA was amplified using the primers ITS-6 (Cooke *et al.* 2000) and ITS-4 (White *et al.* 1990), with the PCR reaction reagents and conditions as described by Andjic *et al.* (2007). For identification purposes, ITS templates were sequenced with an ITS-4 primer. The cleanup of PCR products and sequencing reactions were as

described in Sakalidis *et al.* (2011). Sequence data for the ITS region were initially cleaned and subsequent manual adjustments made in Geneious v. 7.0.4 (Drummond *et al.* 2011). Ambiguous bases were coded according to the IUPAC nucleotide code. Identification was initially based on BLAST searches in GenBank (<http://blast.ncbi.nlm.nih.gov>), but in all cases, sequence data from closely related species were obtained from GenBank or from our own unpublished sequence data, and alignments and analysis were carried out in Geneious.

Almost 20 % of isolates had ITS sequences that were highly polymorphic, and these were identified as Clade 6 hybrids by comparing these sequences to those obtained in previous studies (Nagel *et al.* 2013a, Burgess 2015).

Table 1. Ecological Vegetation Classes (EVC) sampled for *Phytophthora* species in southern Victoria. Map locations are shown in Figure 1.

EVC number and name	Map	Occurs	Contains
16	1	A small area of the Melba Highway road reserve, it has a species poor ground-layer. Within the Toolangi State Forest it occurs on flats and lower foothill slopes, on well drained soils of moderate fertility where it has a relatively species-rich ground layer.	Overstory is dominated by <i>Eucalyptus obliqua</i> and <i>E. radiata</i> (to 25 m) with an understorey of <i>Hakea decurrens</i> , <i>Kunzea ericoides</i> and <i>Acacia</i> spp., <i>Grevillea alpina</i> , <i>Banksia marginata</i> , <i>B. spinulosa</i> , <i>Spyridium parvifolium</i> , <i>Pultenaea muelleri</i> and <i>Olearia lirata</i> .
20	3	With shallow rocky soils with low fertility or water holding capacity, in the southern section of the Toolangi SF in areas of moderate to high rainfall areas.	The overstorey is dominated by <i>Eucalyptus macrorhyncha</i> , <i>E. radiata</i> , <i>E. goniacalyx</i> and <i>E. polyanthemos</i> (15 - 20 m). The understorey consists of a low shrub layer including <i>Acrotiche prostrata</i> , <i>A. serrulata</i> , <i>Epacris impressa</i> and <i>Acacia verniciflua</i> .
Heathy Dry Forest	4	Within Sugarloaf Reservoir. It occurs in lower rainfall areas on sedimentary soils.	A medium, open forest to woodland (to 20 m) dominated by <i>Eucalyptus goniacalyx</i> , <i>E. macrorhyncha</i> and <i>E. polyanthemos</i> over a sparse shrub layer and a relatively species-rich ground layer dominated by drought-tolerant grasses, herbs, forbs and hardy fern species.
22	5	At the southern end of Toolangi SF.	Relatively moist forest type which has a mixed eucalypt overstorey, scattered tall, medium and low shrubs, over a species-rich ground layer.
Grassy Dry Forest	3		
16/23	4	Restricted to the extreme southern end of the Toolangi SF found growing on fertile, well-drained clay loam soils on a range of parent rock types in areas with moderate to high rainfall.	Medium height forest is dominated by a mixture of <i>Eucalyptus obliqua</i> , <i>E. radiata</i> and <i>E. cypellocarpa</i> (20 - 30m), over a diverse ground layer with scattered shrubs including <i>Coprosma quadrifida</i> , <i>Cassinia aculeata</i> , <i>Spyridium parvifolium</i> and <i>Leptospermum scoparium</i> .
Lowland Forest and Herb-rich Foothill Forest complex	3	Restricted to a single gully in Toolangi SF on a diverse range of fertile, deep, well-structured soils. Much of the understorey has been removed in areas.	Tall forest (35 - 40m tall) dominated by <i>Eucalyptus cypellocarpa</i> , <i>E. obliqua</i> and <i>E. radiata</i> over a diverse mid-story including <i>Coprosma quadrifida</i> , <i>Acacia dealbata</i> and <i>Bedfordia arborescens</i> .
23	2	Found on well-drained loamy soils of relatively high fertility, restricted to a single gully within the Toolangi SF. This EVC has close affinities with Damp Forest.	Overstorey of <i>Eucalyptus obliqua</i> (70 m) co-dominated with <i>Coprosma quadrifida</i> , <i>Cyathea australis</i> , <i>Hedyccarya angustifolia</i> , <i>Pomaderris aspera</i> , <i>Acacia dealbata</i> and a wide diversity of ferns.
Herb-rich Foothill Forest	4	Fertile, well drained, loam and clay soils commonly found on ridges and exposed slopes often in connection with Damp or Wet Forest in the Toolangi SF.	Tall forest (25 - 40 m) dominated by <i>Eucalyptus obliqua</i> , <i>E. cypellocarpa</i> and <i>E. radiata</i> with a dense mid-story of <i>Coprosma quadrifida</i> , <i>Acacia melanoxylon</i> , <i>Pultenaea gunni</i> and <i>P. muelleri</i> . Relatively species poor ground layer dominated by ferns.
29	5	Fertile, well drained, loamy soils with annual rainfall of 700 – 800 mm on gently undulating lower slopes and valley floors within Sugarloaf Reservoir.	Open forest (20 - 30m tall) dominated by <i>Eucalyptus obliqua</i> , <i>E. ovata</i> and/or <i>E. radiata</i> . The ground layer comprises an array of herbs, lilies, grasses and sedges in spring, less diverse in winter.
Damp Forest			
30	2		
Wet Forest			
45			
Shrubby Foothill Forest			
47			
Valley Grassy Forest			

Table 1. (Continued).

EVC number and name	Map	Occurs	Contains
164 Creekline Herb-rich Woodland	5	Restricted sampling sites within Sugarloaf Reservoir Reserve, on alluvial sand, silt and clay soils in low-gradient swampy gullies.	Overstory of <i>Eucalyptus ovata</i> and <i>E. yarraensis</i> . The mid-story contained scattered shrubs with a ground layer of grassy/seedg species with a highly modified understory dominated by introduced grasses.
721 Fern Swamp	3	Restricted to a high rainfall area near the headwaters of the Yea River, this perched wetland is surrounded by Lowland Forest.	Vegetation is treeless except for fringing <i>Acacia melanoxylon</i> and <i>Eucalyptus ovata</i> . The rest is dominated by dense thicket of <i>Gleichenia microphylla</i> .
Mosaic	5	Sections of this mosaic have affinities with both Grassy Dry and Valley Grassy Forest.	A mosaic of non-native vegetation/ <i>Microlaena stipoides</i> , native grassland and <i>Pteridium esculentum</i> ; a result of clearing.

RESULTS

The soil and water sampling yielded 186 *Phytophthora* isolates; based on molecular identification 130 of these isolates corresponded to species and hybrids from Clades 2, and 6–9 (Tables 2–3). The remaining 56 isolates had the same cultural morphology as species from Clade 6 but were not sequenced and, thus, cannot be confidently assigned to a species (Table 3). *Phytophthora cinnamomi* was the most commonly found species, comprising 31 % of all isolations, and almost half of the soil isolations. Of the species isolated, only *P. fallax* and Clade 6 hybrids were recovered from both soil and water.

Stream baiting

Sixty-four *Phytophthora* isolates were obtained from the water baits (Table 3), with one to three *Phytophthora* isolates recovered from each baiting location (Table 2). All but *P. cryptogea* and *P. fallax* were from Clade 6 (Table 3). *Phytophthora thermophila* was the most widespread species, with nine isolates found at eight different locations, both at permanent and intermittent water bodies (Table 3). Half of the isolates were unidentified Clade 6 *Phytophthora* species and were widespread as they were collected from 18 of the 21 locations sampled. While there were six species of *Phytophthora* isolated from both types of water bodies, there were more isolates from permanent (40) than intermittent water bodies (24).

Soil samples

From the 249 soil samples, we obtained 122 isolations of *Phytophthora*, which included six identified species, while 24 isolates were not identified to species level. *Phytophthora* species recovered were from Clades 2, 6, 7, and 9 (Table 3). The most commonly isolated species, *P. cinnamomi* was found in all but four of the EVCs and these were all EVCs from which low numbers of samples were collected; Grassy Dry Forest (8 samples), Herb-rich Foothill Forest (7), Wet Forest (4), and Mosaic (8).

The EVCs with the highest yield of *Phytophthora* isolates were the vegetation types with the greatest soil moisture (EVC 16, 47, 164 and 721). The drier forest classes (EVC 20, 22, 23 and 45) yielded *Phytophthora* from 8.8 to 37 % of the samples. The Damp Forest, found between Glenburn and Kinglake (Fig. 1, map location 1), yielded the greatest number of *Phytophthora* species. While over half of the isolates were either *P. cinnamomi* or unidentified clade 6 *Phytophthora* species, there were an additional nine isolates representing four species; *P. elongata*, *P. gonapodyides*, *P. gregata*, and *P. multivora* (Fig. 1). Most recoveries of *P. cinnamomi* were from the Toolangi State Forest, north of the Kinglake-Healesville Road (Fig. 1, map locations 2–4).

The Clade 6 hybrids were found in map locations 1–3 (Fig. 1), and only occurred in the Lowland Forest, Lowland Forest and herb-rich Foothill Forest complex, Damp Forest and the Shrubby Foothill Forest.

The lowest diversity of *Phytophthora* species was in the area sampled close to Sugarloaf Reservoir (map sector 5), with the Valley Grassy Forest, Creekline Herb-rich Woodland and the Mosaic EVCs only yielding nine *Phytophthora* isolates; three *P. cinnamomi*, one *P. gregata*, one *P. elongata*, and four unidentified *Phytophthora* isolates (Fig. 1, Table 4). This is an area with vegetation and soil conditions that are generally unfavourable for the recovery of *Phytophthora*, mostly occurring on relatively shallow soils with northern and western aspects.

Table 2. Site descriptions of stream baiting locations and the recovery of *Phytophthora* species along the Sugarloaf Pipeline, in southern Victoria. The confirmation of presence (+) for each *Phytophthora* species is indicated for each wetland.

Location	Description	Latitude	Longitude	<i>Phytophthora</i> species							
Goulburn River (Killingworth)	Riverine, permanent, fringing vegetation	37°09'49.4"	145°25'33.1"								
Yea Wetlands	Billabong, permanent, fringing vegetation	37°12'26.4"	145°25'48.0"								
Ewing Creek	Intermittent creek, fringing vegetation	37°14'59.9"	145°27'22.7"								
Triangle Creek	Intermittent creek	37°16'01.6"	145°27'27.5"								
Ti-Tree Creek	Intermittent creek	37°17'57.5"	145°27'45.9"								
Rellimeiggam Creek	Intermittent creek	37°18'29.0"	145°27'32.8"								
Yea River (Devlin's Bridge)	Riverine, permanent, fringing vegetation	37°22'57.7"	145°28'21.8"								
Kalatha Creek	Permanent, fringing vegetation and pasture	37°24'11.3"	145°27'26.7"								
Katy's Creek	Permanent, fringing vegetation, pasture	37°24'43.1"	145°26'47.4"								
Eagle Nest Creek	Intermittent creek	37°25'02.3"	145°25'55.2"								
Wee Creek	Permanent, fringing vegetation, pasture	37°26'20.5"	145°25'08.3"								
Yea River	Permanent stream, fringing vegetation	37°30'50.7"	145°25'29.5"								
Dixon's Creek (Upper)	Intermittent stream	37°33'03.0"	145°24'59.7"								
Dixon's Creek (Lower)	Permanent, fringing vegetation, pasture	37°37'18.4"	145°24'35.4"								
Steel's Creek (Gulf Road)	Permanent, fringing vegetation, pasture	37°37'54.4"	145°23'30.5"								
Steel's Creek (Willowbank)	Intermittent, fringing vegetation, pasture	37°36'30.7"	145°22'34.9"								
Maroondah Aqueduct	Intermittent, fringing vegetation, pasture	37°38'45.9"	145°21'51.0"								
Sugarloaf (Dam 1)	Permanent, fringing vegetation, pasture	37°39'38.0"	145°19'17.8"								
Sugarloaf (Dam 2)	Permanent, fringing vegetation, pasture	37°39'38.9"	145°19'30.9"								
Sugarloaf (Dam 3)	Intermittent, EVC 22/pasture	37°39'59.0"	145°19'58.6"								
Sugarloaf (Dam 4)	Intermittent, degraded	37°39'46.7"	145°19'01.8"								

DISCUSSION

From an area north-east of Melbourne, Victoria (approximately 140 km²), 13 *Phytophthora* species were isolated by soil and water baiting. Most of these species have host ranges that include native and/or horticultural important plant taxa (Hüberli *et al.* 2013). *Phytophthora cinnamomi* was the most commonly isolated species from the rhizosphere, even from beneath asymptomatic vegetation. Overall, more *Phytophthora* species were recovered from the lower lying areas of wetter vegetation than from the dry vegetation classes. Every water bait yielded *Phytophthora*. This is one of the first studies in Victoria to survey soils in natural systems for *Phytophthora* species other than *P. cinnamomi*.

Species diversity in soil and water samples

There was low diversity in the *Phytophthora* species isolated from individual soil samples. After *P. cinnamomi* (47 % of soil recoveries), the second most common species was *P. elongata*, comprising almost 10 % of soil recoveries. *Phytophthora elongata* was recovered from five of the moist EVCs containing sandy loams and clay soils, and known hosts from *Myrtaceae*, *Proteaceae*, *Epacridaceae*, *Xanthorrhoeaceae*, and *Iridaceae* (Rea *et al.* 2010) were all abundant in these EVCs. *Phytophthora elongata* was found to be as aggressive as *P. cinnamomi* in soil infestation tests (Rea *et al.* 2010). Clade 6 species and hybrids comprised almost 20 % of soil isolations, challenging the assumption that they are generally associated with waterways. Further surveys, combined with pathogenicity testing of isolates, are required to determine what role Clade 6 species and hybrids play in natural ecosystems.

Isolates identified from water samples belonged predominantly to Clade 6, with only one isolate of *P. cryptogea* from Clade 8 and an isolate of *P. fallax* from Clade 9. There were more recoveries from the permanent water bodies than the intermittent sites, but there were an equal number of species from the two types of water bodies, however only Clade 6 species were recovered from intermittent water bodies. *Phytophthora thermophila*, the most widespread species, is closely related to three species known only from Western Australia, *P. litoralis*, *P. fluvialis*, and *P. moyootj* (Crous *et al.* 2014). The high optimum and maximum temperatures for growth of *P. thermophila* (Jung *et al.* 2011) might contribute to its recovery from intermittent sources as well as permanent water bodies. Based on our stream recoveries, it could be speculated that many of the unidentified isolates recovered from intermittent sites would belong to *Phytophthora* Clade 6.

Distribution of *Phytophthora cinnamomi*

The high recovery of *P. cinnamomi* within the study area was anticipated because there are records of the presence of this pathogen from locations along the GDR, both east and west of the study area, at Toolangi (Marks *et al.* 1972) and Kinglake National Park in 1973 (Weste & Marks 1974, Duncan & Keane 1996, Gibson *et al.* 2002). The survey followed a route that has been used for passage across the GDR from Melbourne to the Murray-Goulburn pastoral regions and goldfields since European settlement in 1835.

Given the history of the travel route, the proximity to areas that were converted to agriculture, and a long history of timber harvesting in the area, it is conceivable that exotic species such as *P. cinnamomi* have been introduced to the area.

Phytophthora cinnamomi was recovered most frequently on the northern side of the GDR, frequently from sampling points where there were no signs of disease in plants that could have been attributed to *Phytophthora*. In contrast, *P. cinnamomi* was recovered from only one of 45 samples (in EVC20) immediately south of the crest of the GDR. The difference in recoveries between north and south of the GDR probably cannot be attributed to the relative abundance of hosts within EVCs. The total number of plant species present known to be susceptible to *P. cinnamomi* (Podger & Brown 1989, Gibson *et al.* 2002, Weste *et al.* 2002) was 29–30 species north of the GDR, and 19–20 species south of the GDR (Sugarloaf Pipeline Alliance 2008). The mean number of dominant susceptible hosts (ground covers, over- and understory) within each EVC ranged between one and six species, with means of 2.2 and 3.25, north and south of the GDR, respectively. Given the abundance of possible hosts, environmental factors, such as topography and soils, might explain the difference in frequency of recovery of *P. cinnamomi*. Four common overstory species within the survey area (*Eucalyptus obliqua*, *E. macrorhyncha*, *E. radiata*, and *E. baxteri*) have been shown to be highly susceptible to *P. cinnamomi* at some sites within Victoria. This occurs on some soils that can be alternatively very wet, which favours rapid proliferation of the pathogen, then dry, leading to death of infected plants through drought stress, with mortality rates between 40 and 100 % (Weste 1975, Weste *et al.* 1973, 1976, 2002). Nearly all sampling points north of the GDR were located 10s to 100s of metres downslope of high points in the landscape, and therefore water gaining, in contrast to south of the range where sampling points were at, or near, a ridgeline and therefore relatively dry and less likely to be conducive to disease expression.

Recent drought and fire concealed signs of disease caused by *P. cinnamomi* and other *Phytophthora* species. The survey was conducted toward the end of the Millennium Drought (2001–2009; van Dijk *et al.* 2013), and drought effects on plants were most likely to have been confused with signs of disease. Part of the survey area had been burnt 2–3 yr prior to the survey which would have removed diseased and dead host plants and therefore reduced the opportunity to sample symptomatic plants and recover *Phytophthora* species, potentially leading to an underestimation of presence and diversity.

Although there were abundant susceptible hosts, particularly among the *Eucalyptus* overstory, there was only one only area with consistent signs of disease typical of *P. cinnamomi*; a relatively high proportion of death of *Xanthorrhoea minor* within Lowland Forest (EVC16; map location 1) where *P. cinnamomi* was recovered from most samples collected. In WA, disease fronts are often apparent, but in the eastern states of Australia the presentation of symptoms in the natural ecosystem can be described as a mosaic (O'Gara *et al.* 2005), making the identification of infested areas very difficult. The current survey shows how cryptic this pathogen can be as there were few signs of

Table 3. Number of isolates of *Phytophthora* species from soil and water baiting (permanent or intermittent water bodies) from the Sugarloaf Pipeline easement between Yea and Sugarloaf Reservoir, Victoria.

Identification	Clade	Soil baiting	Water baiting		Total isolates
			permanent	intermittent	
<i>P. elongata</i>	2	11			11
<i>P. multivora</i>	2	3			3
<i>P. amnicola</i>	6		1	2	3
<i>P. chlamydospora</i>	6		2		2
<i>P. gonapodyides</i>	6	1			1
<i>P. gregata</i>	6	2			2
<i>P. thermophila</i>	6		5	4	9
<i>P. "personii"</i>	6			1	1
<i>P. "hennops"</i>	6		1		1
<i>P. "paludosa"</i>	6		1		1
Clade 6 hybrids	6	22	10	3	35
Clade 6 (unidentified)		24	20	12	56
<i>P. cinnamomi</i>	7	58			58
<i>P. cryptogea</i>	8			1	1
<i>P. fallax</i>	9	1		1	2
Total		122	64		186

disease in spite of the abundance of susceptible hosts and a high frequency of recovery of the pathogen. The survey area contains an old infested site (map location 2–4), where a decline in recovery over time has been recorded (Weste 2003).

Comparison to other regions in Australia and worldwide

Soil sampling in southeast Queensland and central New South Wales revealed eight *Phytophthora* species (Scarlett et al. 2015). *P. cinnamomi* was also the most frequently isolated species, and like the current study, *P. multivora* and *P. cryptogea* were also isolated. While there were only six species identified from all the soil samples in the current study, the use of amplicon HTS may have resulted in many more species being identified. For instance, in Italian chestnut (*Castanea sativa*) groves, Vannini et al. (2013) found nine and 15 species using baiting and HTS, respectively. HTS in northern Spain detected 35 *Phytophthora* species, with species from all clades except Clades 5 and 9 (Català et al. 2015). Thus, the Australian assemblages may be more complex than what are detected by baiting alone.

The dominant *Phytophthora* species found in soil samples varies from region to region throughout the world, and would be largely dependent on host species, environmental conditions and human and animal vectored spread. Australian studies, including the current study, have identified *P. cinnamomi* (Clade 7) as the dominant soil species (Smith et al. 2009, Hüberli et al. 2013), while in South Africa *P. multivora* (Clade 2) comprised 40 % of isolates (Oh et al. 2013), *P. syringae* (Clade 8) was the dominant species in Argentina (Greslebin et al. 2005), *P. plurivora* (Clade 2) from Spanish oak forests (Perez-Sierra et al. 2013), chestnut forests (Vannini et al. 2013) and *P. chlamydospora* (Clade 6) in Chinese oak forests (Huai et al. 2013). Despite having different dominant species,

there were more similarities between the *Phytophthora* assemblages found in Australia and South Africa where there were five species in common, than with species reported in the northern hemisphere (0–2; Perez-Sierra et al. 2013, Vannini et al. 2013, Huai et al. 2013) and Argentina (2; Greslebin et al. 2005). However, until systematic surveys are undertaken across continents with similar sampling intensity, these comparisons are purely speculative.

Within Australia, *Phytophthora* species also appear to be relatively abundant in natural streams as found in the US (Hansen & Delatour 1999, Hwang et al. 2007, Sutton et al. 2009, Sims et al. 2015), South America (Greslebin et al. 2005), Asia (Ho et al. 2002) and South Africa (Nagel et al. 2013b, Oh et al. 2013). In an intrastate comparison, stream baiting for *Phytophthora* species yielded nine species isolated from eight sites east of Melbourne (Smith et al. 2009), but had no definitive overlap with species identified from water samples in the current study although there is only 50 km between the two survey sites. These differences can be attributed to the season of the survey and the bait types used. For instance, Smith et al. (2009) showed that different bait types yielded different *Phytophthora* species from the same source, which also varied according to season. While Smith et al. (2009) isolated *P. cinnamomi*, *P. "citricola"* (potentially *P. multivora* or *P. elongata*) and *P. gonapodyides* from water samples, *P. cinnamomi* and *P. gonapodyides* were present only in soil samples in the current study. None of these species were found in WA waterways where *P. inundata* and *P. thermophila* were common and where most species were recovered from *B. attenuata* and *P. undulatum* leaves (Hüberli et al. 2013). The current survey found six species that were not detected previously in Victoria *P. amnicola*, *P. thermophila*, *P. "personii"*, *P. "hennops"*, *P. "paludosa"* and *P. fallax*. Most notably, Smith et al. (2009) recovered *P. cinnamomi* from water using mature eucalypt leaves as bait, and only in their

Table 4. Yield of *Phytophthora* isolates from soil samples taken in relation to Ecological Vegetation Class (EVC details in Table 1).

Ecological Vegetation Class (EVC #)	Total samples collected	Number of samples yielding <i>Phytophthora</i>	Yield for vegetation class (%)
Fern Swamp (721)	4	4	100.0
Valley Grassy Forest (47)	7	6	85.7
Lowland Forest (16)	52	37	71.2
Creepline Herb-rich Woodland (164)	3	2	66.7
Damp forest/Shrubby Foothill Forest complex (29/45)	22	15	68.2
Lowland and Herb-rich Foothill Forest complex (16/23)	32	18	56.3
Wet forest (30)	4	2	50.0
Damp Forest (29)	50	24	48.0
Grassy Dry Forest (22)	8	3	37.5
Herb-rich Foothill Forest (23)	7	2	28.6
Heathy Dry Forest (20)	18	5	27.8
Mosaic	8	1	12.5
Shrubby Foothill Forest (45)	34	3	8.8
Total	249	122	

summer survey. Neither the current study, nor Huberli *et al.* (2013) used mature eucalypt leaves or sampled during the height of summer. The multi seasonal surveys and comparison of baits undertaken by Smith *et al.* (2009) has demonstrated a more extensive study, of preferred baits comparing local species, mature and immature leaves, and seedlings, needs to be undertaken to determine the optimal bait, along with consideration of seasonal variations including water temperature and flow rates.

The species recovered by stream baiting in the current study that were in common with the WA study (Hüberli *et al.* 2013) were *P. amnicola*, *P. cryptogea* and *P. thermophila*. From WA, there were 12 *Phytophthora* species and two hybrids recovered (Hüberli *et al.* 2013). The total number of isolates and hybrids found were similar, and *P. thermophila* was found to be widespread in waterways, while *P. cryptogea* and *P. multivora* were rarely encountered (Hüberli *et al.* 2013). *Phytophthora gonapodyides* was only recovered once from soil from the Damp Forest EVC in the current study, and once from water by Smith *et al.* (2009), while *P. chlamydospora* was isolated infrequently in soil and water samples in Victoria, but never in WA (Hüberli *et al.* 2013). The Clade 6 hybrids were found in both soil and water samples in the current study, with nearly 40 % of the soil isolates identified as hybrids being recovered from Damp or Wet forest EVCs (29 and 30), and all from wet microsites regardless of EVC. Clade 6 hybrids (*P. amnicola* x *P. chlamydospora* and *P. thermophila* x *P. amnicola*) were also detected in water samples in South Africa (Nagel *et al.* 2013a). This suggests that Clade 6 hybrids are predominantly aquatic or riparian.

While the key species isolated from water are Clade 6 and deemed to be largely saprophytic (Brasier *et al.* 2003, Hansen *et al.* 2012), morphologically similar species such as *P. gonapodyides* and *P. chlamydospora* (Oh *et al.* 2013) can be opportunistic and sometimes aggressive tree pathogens (Brown & Brasier 2007, Jung *et al.* 2011). *Phytophthora gonapodyides* and *P. chlamydospora* are widespread species

commonly isolated from waters and riverbank soils around the world (Brasier *et al.* 2003, Greslebin *et al.* 2005, Reeser *et al.* 2011, Scibetta *et al.* 2012, Nagel *et al.* 2013a). While these species are common in the Northern Hemisphere and the one study undertaken in South America (Greslebin *et al.* 2005), however, it is not the case for South Africa (Oh *et al.* 2013) or Australia. *Phytophthora gonapodyides* and *P. chlamydospora* are rarely recovered in Australia, instead other Clade 6 *Phytophthora* species and hybrids are more common (Hüberli *et al.* 2013, Burgess 2015). Regardless of the continent, Clade 6 species dominate the waterways.

There are several potential explanations for the community differences in the intrastate comparison; variation in seasonal and geographical differences have been reported (Smith *et al.* 2009, Reeser *et al.* 2011, Hansen *et al.* 2012) and sampling bias has been demonstrated depending on different baits used (Smith *et al.* 2009, Sutton *et al.* 2009, Hüberli *et al.* 2013). Hüberli *et al.* (2013) demonstrated that no single plant bait isolated all species. However, Reeser *et al.* (2011) attests that the diversity of *Phytophthora* species recovered from forest streams in Oregon and Alaska was not affected by the type of bait used but it did affect the number of isolations. Filtration, rather than baiting, provided a greater diversity and has the potential to capture the slow growing species and those with particular growth requirements (Reeser *et al.* 2011, Català *et al.* 2015). This emphasizes the need for sampling to be undertaken over different seasons with multiple methods, using a range of baits, and from multiple locations in order to thoroughly survey an area.

CONCLUSIONS

This study has demonstrated that, like many other regions in the world, there is a diverse assemblage of *Phytophthora* species in natural ecosystems in Victoria. Using the term “assemblage” to describe the groups of *Phytophthora* species

rather than “community” shows our limited knowledge of the taxonomy, diversity and dynamics of this pathogenic genus (Reeser *et al.* 2011, Scibetta *et al.* 2012). *Phytophthora cinnamomi* remains the most prominent species in the soil; and Clade 6 species were frequently recovered from water bodies. While the new metagenomic approach of Scibetta *et al.* (2012) shows promise in being able to discriminate multiple species within samples and reduce the incidence of false negatives, allowing an estimate of diversity, it will not provide isolates for identification or further studies. Català *et al.* (2015) presents HTS as a fast, cheap method to determine the species diversity of *Phytophthora* in environmental samples. However, future studies should consider baiting along with HTS to more accurately identify *Phytophthora* species present at a site. Australian natural ecosystems are managed to reduce the risk of spreading *P. cinnamomi*. Accurate mapping involving both baiting and HTS will facilitate identification in regions where presence cannot be determined by the death of susceptible hosts. There have been very few studies of *Phytophthora* diversity and distribution in natural ecosystems in Australia, and there is limited knowledge of which species are endemic or invasive. Studies such as this provide the baseline of data upon which new invasive species can be identified and managed.

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