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Distribution and diversity of *Phytophthora* across Australia

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Abstract

The introduction and subsequent impact of *Phytophthora cinnamomi* within native vegetation is one of the major conservation issues for biodiversity in Australia. Recently, many new *Phytophthora* species have been described from Australia's native ecosystems; however, their distribution, origin, and potential impact remain unknown. Historical bias in *Phytophthora* detection has been towards sites showing symptoms of disease, and traditional isolation methods show variable effectiveness of detecting different *Phytophthora* species. However, we now have at our disposal new techniques based on the sampling of environmental DNA and metabarcoding through the use of high-throughput sequencing. Here, we report on the diversity and distribution of *Phytophthora* in Australia using metabarcoding of 640 soil samples and we compare the diversity detected using this technique with that available in curated databases. *Phytophthora* was detected in 65% of sites, and phylogenetic analysis revealed 68 distinct *Phytophthora* phylotypes. Of these, 21 were identified as potentially unique taxa and 25 were new detections in natural areas and/or new introductions to Australia. There are 66 *Phytophthora* taxa listed in Australian databases, 43 of which were also detected in this metabarcoding study. This study revealed high *Phytophthora* richness within native vegetation and the additional records provide a valuable baseline resource for future studies. Many of the *Phytophthora* species now uncovered in Australia's native ecosystems are newly described and

until more is known we need to be cautious with regard to the spread and conservation management of these new species in Australia's unique ecosystems.

Additional Keywords: amplicon pyrosequencing, eDNA, high-throughput sequencing, invasive species.

Introduction

Species within the genus *Phytophthora* (Stramenopiles: Oomycetes: Peronosporales) are known predominantly as serious pathogens of plants in native ecosystems, agriculture and horticulture. In 1995 there were ~50 described species and almost all were associated with diseases of food crops (Erwin and Ribeiro 1996). The origin of most of these species was unknown, but many had been distributed globally with their hosts. A few species were also known for their massive impact on native ecosystems, including one of the world's most devastating invasive species, *P. cinnamomi* (Burgess *et al.* 2016), a pathogen with a very broad host range whose biggest impact has been in native ecosystems in Australia (Garkaklis *et al.* 2004; Shearer *et al.* 2007), the Cape Province of South Africa (Von Broembsen and Kruger 1985) and the Iberian Peninsula in Europe (Brasier 1996). In all these regions, extensive conservation efforts are in place to minimise impact. The introduction and subsequent impact of *P. cinnamomi* on native vegetation is seen as one of the major conservation policy issues for biodiversity in Oceania (Kingsford *et al.* 2009), including, for example, the rare endemic mossy cloud forest of Lord Howe Island (Harris *et al.* 2005).

The emergence of *Phytophthora ramorum* as the causal agent of sudden oak death in California ignited interest in *Phytophthora* diseases of native ecosystems on all continents (Hansen *et al.* 2012). *P. ramorum* is a particular threat to tanoak (*Lithocarpus densiflorus*) and more than a million trees in California and Oregon have been killed (Parke and Rizzo 2011). More recently, plantations of Japanese larch (*Larix kaempferi*) in the United Kingdom have been decimated by *P. ramorum*infections (Brasier and Webber 2010). Suitable climatic regions for the persistence of *P. ramorum* and susceptible native hosts are widespread in Australia and New Zealand (Ireland *et*

al. 2013). *Phytophthora pluvialis*, a common and apparently benign species in western Oregon forests (Reeser *et al.* 2013), has become a major pathogen of *Pinus radiata* in New Zealand (Dick *et al.* 2014), but it has not been found on *P. radiata* within its native range in California. Also in New Zealand is the canker dieback of Kauri (*Agathis austalis*) caused by the newly described *Phytophthora agathidicida*, a pathogen of unknown origin (Weir *et al.* 2015). Outbreaks of *Phytophthora lateralis*, the causal agent of root disease in Port Orford Cedar (*Chamaecyparis lawsoniana*) in the Pacific North-west (Hansen *et al.* 2000), has been reported from dying *C. lawsoniana* in Europe (Hansen *et al.* 1999). In South America two very different *Phytophthora* diseases have emerged: *Phytophthora austrocedrae* causes root disease and extensive mortality through the root infection of *Austrocedrus chilensis* (Mal del Ciprés) on the eastern slopes of the Andes in Patagonia (Greslebin *et al.* 2007), and *Phytophthora pinifolia* causes a shoot blight of exotic *P. radiata* plantations (Daño Foliar del Pino) on the moist western slopes of the Chilean Andes (Durán *et al.* 2008). Elsewhere, *P. austrocedrae*has been reported as a serious pathogen of juniper (*Juniperus communis*) in the United Kingdom (Green *et al.* 2012). *Phytophthora* × *alni,* a natural hybrid thought to have originated in tree nurseries, has spread throughout Europe where it has caused massive mortality in natural populations and plantations of *Alnus* species (Brasier *et al.* 2004; Hansen 2012).

With increasing awareness of invasive *Phytophthora* species in native ecosystems on all continents, there has been a considerable impetus to understand the biology, epidemiology, origin and movement of these pathogens. These recent studies of impacts have led to the discovery of many new *Phytophthora* species. There are ~150 described species and over 50 have been described since 2010 (Scott *et al.* 2013). Most of these have been recovered from native ecosystems and waterways and currently little is known of their impact in native ecosystems, distribution, potential pathogenicity or host range. In fact, many of these newly described species have so far been recovered only from limited geographic areas where they may be endemic.

All known species of *Phytophthora* fall into 10 phylogenetic clades (Martin *et al.*2014). Most species are known pathogens; however, within Clades 6 and 9 there are many predominantly aquatic species

that are only opportunistically pathogenic (Brasier *et al.* 2003; Jung *et al.* 2011; Hansen *et al.* 2012; Hong *et al.* 2012). Many pathogens of agricultural crops cluster in Clades 1, 2, 7 and 8. However, in all phylogenetic clades there are pathogens of both annuals and perennials, some with broad host ranges, and some with a single known host.

Since European colonisation, *Phytophthora* species have been translocated widely within Australia by anthropogenic activities. Likely modes of spread include the movement of contaminated mud between sites on vehicles and heavy machinery, including in road building and mining, recreational motorcyclists and off-road drivers, replanting using infected seedlings (a problem exacerbated by poor nursery hygiene), as well as by bushwalkers and apiarists (Cahill *et al.* 2008; Callaghan and Guest 2015). As a consequence of historical bias towards sampling sites showing symptoms of disease, and the variable effectiveness of traditional isolation methods for detecting different *Phytophthora* species (Hüberli *et al.* 2000), diversity in this genus is likely to have been underestimated. We now have at our disposal new techniques based on the sampling of environmental DNA (eDNA) and metabarcoding through the use of high-throughput sequencing (HTS). In general, the primers used target a wide range of organisms; however, an approach using *Phytophthora*-specific primers (Scibetta *et al.* 2012) was refined by Català *et al.*(2015). Application of these genus-specific primers, construction of a high-quality detailed phylogeny, and internally curated databases (together with considerable accumulated knowledge of the biology and ecology of the group) has greatly facilitated analysis and interpretation of eDNA sequences for *Phytophthora.*

Here, we report on *Phytophthora* diversity and distribution using metabarcoding of 640 soil samples collected from five (of six) Australian states (Queensland, New South Wales, Victoria, Western Australia and Tasmania). We compare the diversity detected using this technique with that available in curated databases. At a near-continental scale, the current study is among the largest assessments of microbial biogeography to date, and is by far the largest with respect to the economically and ecologically important genus *Phytophthora*.

Materials and methods

Sample collection and preparation

Sites ($n = 640$) were sampled during the summer and autumn of 2013, 2014 and 2015. Sites were situated within native asymptomatic vegetation in Tasmania (Tas.; 208 sites), Victoria (Vic.; 84 sites), New South Wales (including the Australian Capital Territory) (NSW; 117 sites), Queensland (Qld; 20 sites) and Western Australia (WA; 211 sites) (Fig. 1). These sites were selected to confirm or fill gaps in the known distribution of *P. cinnomomi* (Burgess *et al.* 2016). At each site, surface litter was removed and 8–12 scoops of topsoil (each ~100 g) from the first 10–15 cm were taken at random within a 5-m radius. Samples were bulked (totalling ~1 kg), air-dried, sieved (2 mm mesh size) and homogenised, and a portion (60–80 g) was crushed to a fine powder by using the TissueLyser LT (Qiagen). Between samples, the grinding tubes were cleaned using detergent (Pyroneg^{TM}), soaked in an acid bath for 5 min (HCl 0.4 M), rinsed with water and finally sprayed with 95% ethanol and allowed to air dry. All samples were stored frozen after disruption. Autoclaved sand was used for sieving and grinding controls.

eDNA extraction, 454-pyrosequencing

DNA was extracted in duplicate using the Mo Bio PowerSoil DNA isolation kit (Carlsbad, CA) according to the manufacturer's protocol, except for the first step, in which we replaced the buffer from the kit with 1 mL of saturated phosphate buffer (Na_2PO_4; 0.12 M; pH 8) to the soil sample (500 mg) in order to maximise extracellular DNA isolation (Taberlet *et al.* 2012). Final elutions were done in 60 µL of TE buffer. All DNA was stored at –20°C before amplicon generation. Amplicon libraries were created using the *Phytophthora*-specific primers (Scibetta *et al.* 2012) using a nested PCR approach as described by Català *et al.* (2015) with some variations. The primers amplify the ITS1 gene region. The PCR reaction mixture contained 2 µL of the genomic DNA (first round) or 1 µL of the PCR product (second round), 0.5 µL of each primer (10 pmol), 0.125 µL of Promega GoTaq Host Start Polymerase, 2.5 µL of Promega MgCl (25 nmole), 1.5 µL of dNTPs (10 nmole), 1 µL of Fisher bovine serum albumin (10 mg mL^{-1}) and 5 µL of 5X Promega Colourless GoTaq Flexi

buffer made up to 25 µL with PCR-grade water. The PCR conditions were: 95°C for 2 min, 30 cycles (first round) or 25 cycles (second round) of 95°C for 20 s, 60°C for 25 s, 72°C for 1 min, and a final extension of 72°C for 7 min. Negative PCR controls were included for every unique barcode in the first-round PCR and carried forward to the second round in the same manner as for the samples. If a band was visualised for the negative PCR controls after the second-round PCR the sample was discarded. First-round PCR was conducted in triplicate and, after visualisation on 2% agarose gels, the products were combined based on intensity of bands.

PCR products were cleaned twice with AMPure XP Beads (Beckman Coulter Genomics) following the Short Fragment removal protocol according to the manufacturer's instructions. After purification, the PCR products were visualised on agarose gels and then pooled (based on the band intensity) in an attempt 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.

A serial dilution was made from the cleaned 1/5000 dilution and quantified following the methodology proposed for ancient DNA (Bunce *et al.* 2012) with the following modifications: each qPCR reaction mixture contained 2 µL of diluted DNA, 10 µL of Sybr Green PCR master mix (Life technologies), 1 µL each of Lib-L Y adaptor forward and reverse primers and 6 µL of PCR-grade water. A dilution was selected such that 4.4 million copies could be added to the emulsion PCR reaction, which was carried out according to the Roche GS Junior emPCR Amplification Method Manual Lib-L (March 2012) except that a quarter of the recommended primer (5 µL) was used. The libraries were sequenced according to the Roche GS Junior Sequencing Method Manual (March 2012) using GS Junior Titanium chemistry and GS Junior Pico Titre Plates (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA).

In each run there were three types of process controls using autoclaved sand: (a) sieving controls (i.e. sand was passed through sieve and then processed as a sample), (b) grinding controls (after each day of grinding samples, sterile sand was ground and then processed as a sample), and (c) DNA extraction controls (with each set of DNA extractions autoclaved sand was treated as a sample). Sieving,

grinding and DNA extraction controls were assigned MIDs and taken through to the pyrosequencing step even though no product could be visualised on agarose gels.

Trimming and clustering of molecular operational taxonomic units (MOTUs)

The sequences that passed on the basis of quality scores were imported into Geneious ver. R9 (http://www.geneious.com/) and sorted into separate files based on their unique barcode. During this process the end adaptors, primers and barcode were automatically trimmed. For each barcode (sample) a *de novo*assembly was performed in Geneious under the following conditions: reads were reverse complemented, 53 bp were trimmed from 5′ and 20 bp were trimmed from 3′ end of the reads (to obtain sequence of the complete ITS1 gene region), samples were filtered for a minimum overlap identity of 99% and homopolymer variants were merged. Consensus sequences were then aligned using MAFFT alignment within Geneious using default parameters, and putative chimeras were discarded. Identities were first assigned to MOTUs by conducting an internal blast search against a customised reference database. The reference database consists of ITS1 sequence of 171 *Phytophthora* species and undescribed (but designated) taxa. Second, all MOTUs were then separated into clades and phylogenetic analyses were conducted using Geneious tree builder using verified sequences of all known *Phytophthora* species (Fig. 2). For the purpose of phylogenetic identification, the database was divided into (a) Clades 1 and 2, (b) Clades 3–5, (c) Clade 6, (d) Clade 7, (e) Clade 8 and (f) Clades 9 and 10 (Fig. 2). 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.

Phylotypes for each site were assigned an abundance rating: 1, <1% of reads; 2, 1–10% of reads; 3, >10% of reads. Species were identified as rare (<1%), occasional (1–10%), common (10–50%) or very common (>50%) based on the proportion of sites from which they were obtained. These data were used to generate the heatmap (Fig. 3).

Extrapolated species richness was calculated by the Chao method from a species accumulation model using the function specaccum in the vegan package of R (Oksanen *et al.* 2016). The random method was used with 100 permutations.

Historical Phytophthora records and their classification

Historical *Phytophthora* records are based on the isolation of living cultures and are documented by state administrative boundaries. Data are available for Tas., Vic., NSW, Qld, South Australia (SA) and WA. The data for SA are not matched with new data from the metabarcoding study as no samples were taken from this state. This did not influence the total number of species known from databases as there are no unique taxa in SA. All historical records were obtained from the Australian Plant Pest Database (APPD) (http://www.planthealthaustralia.com.au) and complemented with records from the collections of (a) the Vegetation Health Service (VHS) Department of Parks and Wildlife, WA, (b) the Centre for Phytophthora Science and Management (CPSM), WA, collection and (c) the Royal Botanical Gardens, Sydney, NSW. Many records from the APPD predate the current practice of molecular identification and will have been classified on the basis of the host they infected and morphological features.

The data of the first record in Australia were also recorded for each species where available. Each species was also classified based on its known association with agriculture, horticulture or native ecosystems (species can have multiple known associations). Data on association were obtained from (Erwin and Ribeiro 1996), the Phytophthora Database (http://www.phytophthoradb.org/), the APPD and the CPSM. Using current distribution and historical records of occurrence, we designated each species detected in this study as either introduced, if they are known globally (from agriculture and horticulture), or native, if they are currently known only from Australia (Table 1). In addition, Phytophthora fallax and Phytophthora captiosa, two species first described from Eucalyptus leaves in New Zealand (Dick et al. 2006), were classified as native to Australia.

Results

Sequencing throughput and quality control

Across all runs, an average of 132 694 ± 7851.33 reads was obtained and, of these, an average of 67% of wells passed filter to produce good-quality reads. The average read length was 259 ± 2.76 bp. On average, 99.5% of the reads corresponded to the genus *Phytophthora*. Other reads were attributed to other oomycetes, including *Halophytophthora*, *Peronospora* or *Phytopythium*, and were excluded from further analysis. The number of reads producing chimeras ranged from 0.4 to 0.7%. There were no reads obtained for the three process controls. Some closely related species relevant to this study cannot be separated solely on the basis of ITS1 (Fig. 2): (1) *P. citricola* and *P. pachypleura*, (2) *P. plurivora*, *P. acerina*and *P. pini*, (3) *P. capensis* and *P.* sp. *emanzi*, (4) *P. europea* and *P. uliginosa*, and (5) *P. gregata*, *P. gibbosa* and *P.* sp. raspberry. For simplicity, only the first species name followed by the word 'complex' is used herein.

Phytophthora species detected from soil eDNA

Phytophthora was detected in 415 sites (65%). MOTU clustering followed by phylogenetic analysis revealed 68 distinct *Phytophthora* phylotypes that largely correspond to species (or clusters of related species). Of these, 14 were identified as potentially unique taxa (GenBank Accession numbers KY110340–KY110359) and eight correspond to known taxa that have been assigned a provisional name by various researchers but have not been formally described: *P.* sp. cyperaceae, *P.* sp. forestsoil, *P.* sp. hungarica, *P.* sp. personii, *P.* sp. walnut, *P.* cf. cryptogea, *P.* aff. melonis, *P.* sp. kelmania (Table 1, Fig. 1). Of these 68 phylotypes, 25 can be considered new detections in native areas and/or new introductions to Australia (Table 1). Thirteen of these are potentially new species in Clades 1, 2, 3, 8, 9 and 10 (*P.* sp. nov. 9E had been isolated in a previous study by the CPSM); 10 correspond to known species or designated taxa (*P. litchii*, *P. riparia*, *P.* sp. forestsoil, *P.* sp. hungarica, *P.* cf. cryptogea, *P. brassicaceae*, *P. himalayensis*, *P. primulae*, *P.* aff. melonis and *P. trifolii)* and two phylotypes correspond to species complexes that cannot be separated on ITS sequence alone (*P. capensis* complex and *P. europea*complex) (Table 1).

Phylotype recognition was uneven across the *Phytophthora* clades, with almost one-third (22 phylotypes) grouping in Clade 6, while only a single (undescribed) phylotype was detected from Clade 10 (Fig. 3). The breadth of phylotype occurrence across sites also varied widely as most phylotypes were rare, with the majority (~68%) occurring in fewer than 2.5% of sites. Two phylotypes (corresponding to *P. multivora* and *P. cinnamomi*) were detected in over half the sites (286 and 219, respectively) and 11 phylotypes were present in more than 10% of sites: *P. nicotianae*, *P. elongata*, *P. plurivora* complex, P. sp. *versiformis*, *P. arenaria*, *P. boodjera*, *P. amnicola*, *P. inundata*, *P. thermophila*, *P.* cf. cryptogea and *P. pseudocryptogea* (Fig. 3).

Six phylotypes corresponding to *P. nicotianae*, *P.* sp. nov 2A, *P. inundata*, *P. litoralis*, *P. thermophila* and *P. cinnamomi* were detected in all states sampled. Seventeen additional phylotypes were detected in all states except Qld: *P. citricola*, *P. elongata*, *P. multivora*, *P. plurivora* complex, P. sp. *versiformis*, *P. arenaria*, *P. boodjera*, *P. amnicola*, *P. bilorbang*, *P. moyootj*, *P. cambivora*, *P. niederhauserii*, *P.* cf. cryptogea, *P. cryptogea*, *P. pseudocryptogea*, *P. syringae* and *P. fallax*. There were fewer sites for Qld than other states and yet several phylotypes were detected exclusively (*P. litchii*, *P. heveae*, *P.* sp. nov 10A) or in a high proportion of sites (*P. macrochlamydospora*, *P. castaneae*) in Qld (Fig. 3).

The species richness estimate (with 95% confidence limits) across all sites was 74 (71, 76), slightly higher than the observed species count of 68. Mean species richness per site where *Phytophthora* was detected was 5.0 ± 3.5 (range 1 to 17). Of the 68 *Phytophthora* phylotypes detected, 35 were considered to be introduced (~51%) (Table 1).

Comparison of metabarcoding results with historical records

Records of historical *Phytophthora* detection differ greatly between states. Most notably, of the 66 species reported in Australian databases, only nine had been reported for Tasmania (Table 1). Of the 66 *Phytophthora* species recorded in databases, 18 introduced species known exclusively from agriculture and five taxa reported from native ecosystems were not detected in the current metabarcoding study (Table 1).

Altogether, 91 taxa have been reported in databases or detected by metabarcoding in Australia. In total, 60% of the taxa are considered to be introduced to Australia and all these taxa have a known association with cultivated crops (both agriculture and horticulture). Western Australia has the most reported species, the most detected by metabarcoding and the highest number of similar species.

Discussion

This metabarcoding study revealed high *Phytophthora* richness within native vegetation across five states in Australia. These additional records provide a valuable baseline resource for future studies. Such diversity has historically been underestimated in databases and the literature. The spatially extensive sampling likewise allowed for improved knowledge of the geographic range of *Phytophthora*in Australia; most species previously known only from Western Australia were detected in eastern Australia. However, the two most widely distributed species were known invasive species: *P. cinnamomi* and *P. multivora*. We recognised 14 potentially new species, none of which were common. Of the described species, most have documented associations with native ecosystems but seven rarely encountered species are considered to be exclusive pathogens of non-woody agricultural crops. The species richness estimate based on metabarcoding was lower than the actual number of species known from Australia when the results of this metabarcoding study were combined with species reported in databases. This was most likely because 19 of the 23 *Phytophthora* species reported exclusively from databases were species known to be confined to agricultural settings and our samples were from asymptomatic vegetation in native ecosystems.

Nevertheless, we also detected some known agricultural crop pathogens, though they tended to be rare. For example, *P. brassiceae* was recovered from a road verge in Victoria and from a tourist site at Pine Lake in Tasmania, *P. primulae* was recovered from the same tourist site in Tasmania, and *P. trifolii* was recovered from vegetation adjacent to a paddock in Victoria. This suggests moderate spillover into native ecosystems, though in all cases there was proximity to human activity. Of interest is the fact that although many agricultural pathogens have global distributions, these three species had

never before been reported in Australia. Also of interest is that numerous agricultural *Phytophthora* species previously reported in Australia were not detected in this study; for example, *P. clandestina* (clover), *P. citrophthora* (citrus), *P. erythroseptica* (potato) and *P. hibernalis* (citrus) (Shivas 1989). This is not to say, of course, that these species are absent from natural ecosystems, though it appears that they are not abundant and/or widespread.

The sites where crop or pasture pathogens were detected were not the only sites subjected to current or historical anthropogenic disturbance either during road-building activities, walking-track establishment or during forestry operations (and subsequent movement of vehicles, hikers and livestock). As such, if all *Phytophthora* species were equally invasive we would expect to see all the same species as detected in agriculture. However, while some species with horticultural associations such as *P. niederhauserii*, *P. cambivora* and *P. parvispora* were common in native ecosystems, most species known as pathogens of annual crops or non-woody plants were detected in low frequency or not at all. It appears that only some agricultural species can invade and persist in native ecosystems.

Phytophthora species detected within Australia

Irwin *et al.* (1995) reported 22 *Phytophthora* species in Australia, all of which, with the exception of *P. cinnamomi*, were considered pathogens of agricultural crops and nursery plants. At that time, very little was known either in Australia or elsewhere about other *Phytophthora* species associated with native vegetation. However, since 1982 the Vegetation Health Service of the Department of Parks and Wildlife in WA has been curating *Phytophthora* isolates recovered during routine sampling for *P. cinnamomi* management as legislated by the WA state government. About 10% of isolates recovered were not *P. cinnamomi.* A subsequent molecular re-evaluation of these isolates identified numerous new *Phytophthora* species (Burgess *et al.* 2009). In combination with subsequent sampling of waterways within native ecosystems (Hüberli *et al.* 2013), 27 taxa have now been identified, including seven known species, 14 species described as a result of these studies (Scott *et al.* 2009; Rea *et al.* 2010, 2011; Crous *et al.* 2011, 2012, 2014; Jung *et al.*2011; Aghighi *et al.* 2012; Simamora *et al.* 2015; Safaiefarahani *et al.* 2015) and six taxa awaiting description. Most of these newly described species were known only from WA, with only *P. multivora* known to have a

global distribution. Detailed soil sampling in south-east Qld and central NSW identified eight *Phytophthora* species (Scarlett *et al.* 2015), but did not recover any of the new species from WA, except *P. multivora*. However, five species (*P. thermophila*, *P. amnicola*, *P. elongata*, *P. gregata*and *P. multivora*) were recovered during sampling in Victoria from soil and water (Dunstan *et al.* 2016). There had been speculation that the species originally described from WA were endemic to the region, but in the light of their detection in the current study across much of Australia, this assumption was premature.

Most *Phytophthora* taxa in the current study were rare, but 13 were recovered from more than 10% of the sites in all states except Queensland. Other than the well known global pathogens *P. cinnamomi*, *P. nicotianae*, and *P. inundata*, eight of the remaining species have been described recently from native ecosystems in Western Australia: *P. multivor*a, *P. elongata*, *P.* sp. *versiformis*, *P. arenaria*, *P. boodjera*, *P. amnicola*, *P. thermophila* and *P. pseudocryptogea*. One phylotype corresponds to *P.* cf. *cryptogea*, a long recognised, but as yet undescribed species in the *P. cryptogea* complex (Martin *et al.* 2014; Safaiefarahani *et al.* 2015). The remaining phylotype belongs to a complex of species in Clade 2 that cannot be separated from other species in the clade (namely *P. acerina*, *P. plurivora* and *P. pini*) on the basis of the ITS1 gene region. *P. plurivora* is common in Europe (Jung and Burgess 2009; Schoebel *et al.* 2014), *P. pini* is common in North America (Hong *et al.* 2011) and *P. acerina* to date has only been recovered from Italy (Ginetti *et al.*2014). To date, the only species in this complex isolated in Australia is *P. plurivora* (from NSW).

Using traditional isolation techniques, *Phytophthora* assemblages have been characterised in Europe and North America (Hansen *et al.* 2012), Argentina (Greslebin *et al.* 2005) and South Africa (Oh *et al.* 2013) with many of the same taxa isolated from trees, soil or water samples. The few metabarcoding studies of *Phytophthora* communities reveal an ever-increasing diversity. Prigigallo *et al.*(2016) examined soil and roots from eight nurseries, detecting 25 *Phytophthora*phylotypes. For each nursery at least twice as many species were detected using metabarcoding compared with using a cloning/Sanger sequencing approach. Italian chestnut groves produced a diverse assemblage of *Phytophthora* (up to 15 species), well beyond the two species normally isolated and implicated in

the chestnut disease (Vannini *et al.* 2013). Català *et al.* (2015) detected over 40 *Phytophthora* phylotypes from forests in northern Spain, many of which were new species or new records for the region. More recently, in a study confined only to oak (*Quercus* spp.) from two sites in Spain, Català *et al.* (2016) found seven known species and several new species. It will take some time to unravel the biological relevance of these new findings; perhaps many species have a broader host range than previously though or can infect non-hosts without causing disease. The differences between species recovered using standard isolation techniques and metabarcoding of eDNA samples indicates that many species may be unculturable. All species of *Peronospora*, a genus closely related to *Phytophthora* (Runge *et al.*2011), cannot be isolated and grown in the laboratory in pure culture, and it is possible that there are *Phytophthora* species with the same life strategy. Català *et al.* (2016) detected an unknown phylotype tentatively named '*P.* taxon ballota', and designed species-specific primers for this phylotype. Thereafter, it could readily be detected from samples and yet this species has never been isolated (Català *et al.*2016). The potential for detecting unculturable species using HTS technologies has opened up the field of fungal biodiversity.

In forests from northern Spain, almost three times as many *Phytophthora*phylotypes were detected from water (35 phylotypes) than from soil samples (13 phylotypes) (Català *et al.* 2015). Sampling in the current study focussed only on soil and, as such, we may have missed a significant component of the *Phytophthora*diversity. However, in the current study we sampled many more sites over a larger geographical area than any previous studies, and detected phylotypes of all the Clade 6 species (*P. inundata*, *P. amnicola*, *P. thermophila*, *P. litoralis*, *P. fluvialis* and *P. moyootj*) previously isolated from water bodies in Australia (Jung *et al.* 2011; Hüberli *et al.* 2013; Nagel *et al.* 2013; Burgess 2015; Dunstan *et al.* 2016). In particular, two species, *P. thermophila* and *P. litoralis*, were common in all states and although they were first reported from water they are also now commonly isolated from soil (CPSM database).

Native versus introduced Phytophthora species

Many *Phytophthora* species have an asymptomatic phase and can even survive and reproduce on a wide range of plants not typically thought of as hosts without causing any signs of disease (Crone *et*

al. 2013). Additionally, many species have several adaptations that facilitate survival in highly variable or unfavourable conditions, in particular the production of thick-walled oospores that enable them to survive in the absence of hosts in, for example, mud adhering to shoes or car tyres. Together these strategies allow long-distance, human-aided dispersal (Scott *et al.* 2013). For these reasons, it is extremely difficult to control the spread of *Phytophthora* species, several of which have established globally, moving around the world either before the introduction of quarantine and biosecurity regulations during the transport of soil and plants, or more recently as cryptic hitchhikers via travel and trade (Brasier 2008; Cahill *et al.* 2008; Migliorini *et al.* 2015; Callaghan and Guest 2015).

In the current study we have considered species only found in Australia to be native and those with global distribution to be introduced. This is surely not completely accurate, but represents a first attempt at trying to uncover patterns of global phylogeography in a highly interconnected world. Roughly equal numbers of species assigned to each category (native versus introduced). Clades 1, 7 and 8 were dominated by globally distributed species common in agriculture and horticulture (with the exception of phylotypes identified as potential new species, which by virtue of only being detected in Australia were designated as putatively native). Conversely, Clades 4, 6 and 9 predominately comprise native species under our paradigm, as they were first described from Australia. There were common species in both categories. It is important to note that the factors influencing the movement of introduced species will also affect local native species, i.e. any pathway that can spread an introduced species such as *P. cinnamomi* could also spread a native species such as *P. arenaria.* This could explain why many species first described from Western Australia appear to be common across Australia.

Conclusion

Until the late 20th century, *Phytophthora* research was focussed predominantly on damaging pathogens of agricultural crops. As interest built on *Phytophthora* diseases in native ecosystems, increased surveying intensity also led to recognition of many new species with limited regional rather

than global distributions. In the current study there were common globally distributed species, species known only from Australia and rare species with limited distribution. Metabarcoding of eDNA using genus-specific primers has proven to be invaluable in recognising *Phytophthora* diversity and distribution in native vegetation across Australia. The number of species detected in Western Australia, a region where there has been a lot of research conducted in native ecosystems, corresponded closely to the species known from databases. Elsewhere, however, there was a large discrepancy and the knowledge gathered here will help to focus future research efforts. The biology, ecology, pathology, and impact management of *P. cinnamomi* in Australia has been studied for over 50 years, and while some issues still remain unanswered there is universal acceptance of the need to implement hygiene and management to prevent its spread into pristine ecosystems.

Many *Phytophthora* species now uncovered in Australia's native ecosystems are newly described (or undescribed) and there is limited information on their distribution, host range and potential impact. Until we know more, and based on the experience of the numerous new *Phytophthora* diseases in native ecosystems across the globe, we need to be cautious with regard to the spread and conservation management of these new species in Australia's unique ecosystems.

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Fig. 1. Map of Australia showing the locations where soil samples were collected for this study.

Fig. 2. Phylogenetic comparisons of all known Phytophthora species based on ITS1 gene region. For visualisation purposes and for the identification of MOTUs the phylogeny was divided into six separate analyses: (a) Clades 1 and 2, (b) Clade 6, (c) Clade 7, (d) Clades 3–5, (e) Clades 9–10, and (f) Clade 8. Sequences of representative MOTUs recovered in the current study are interspersed throughout all clades. MOTUs were assigned to new phylotypes if they did not match any known Phytophthora species. The ITS1 gene region ranges in size from 150 to 250 bp and is highly variable. However, some species cannot be separated on the basis of ITS1 alone. Such species relevant to this study (i to v) are contained within the same coloured block and referred to as a complex throughout the paper.

Fig. 3. Heat map of Phytophthora phylotype proportion and abundance as detected by metabarcoding of eDNA extracted from soil collected across Australian states: Queensland (Qld), New South Wales (NSW), Victoria (Vic.), Tasmania (Tas.), Western Australia (WA). The phylogenetic tree on the left is based on ITS1 gene region and includes all phylotypes detected in this study. The number following the name indicates to which ITS clade they belong. The column on the far right gives the average abundance rating for each phylotype.

Table 1. Records of 91 *Phytophthora* species and taxa and their distribution within Australian states

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 indicated by shading, while phylotypes detected only by metabarcoding are shown in bold. Qld, Queensland; NSW, New South Wales; Vic.,Victoria; Tas.,Tasmania; WA, Western Australia

Fig. 1.

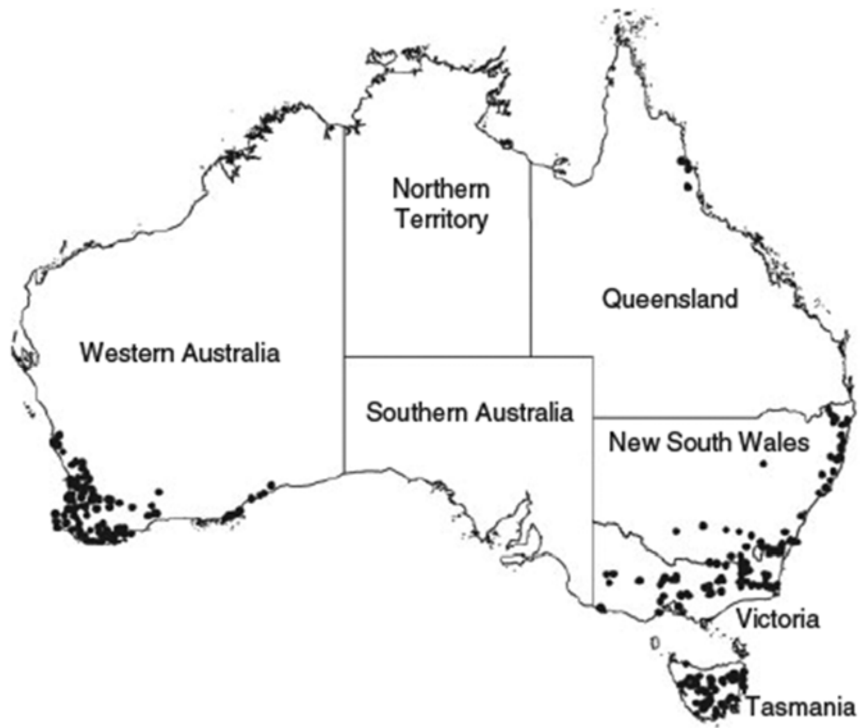

Fig. 2.

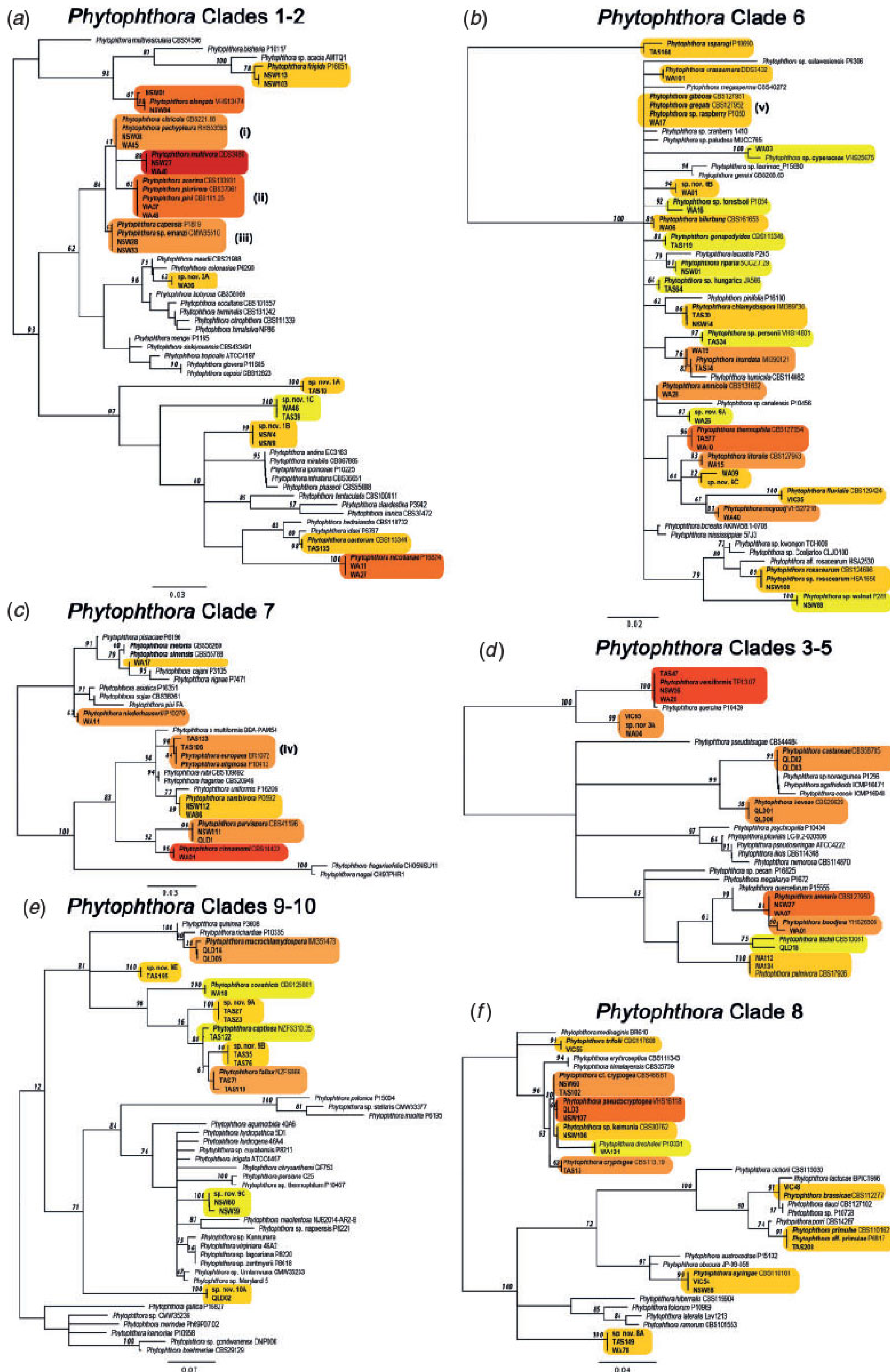

Fig. 3.

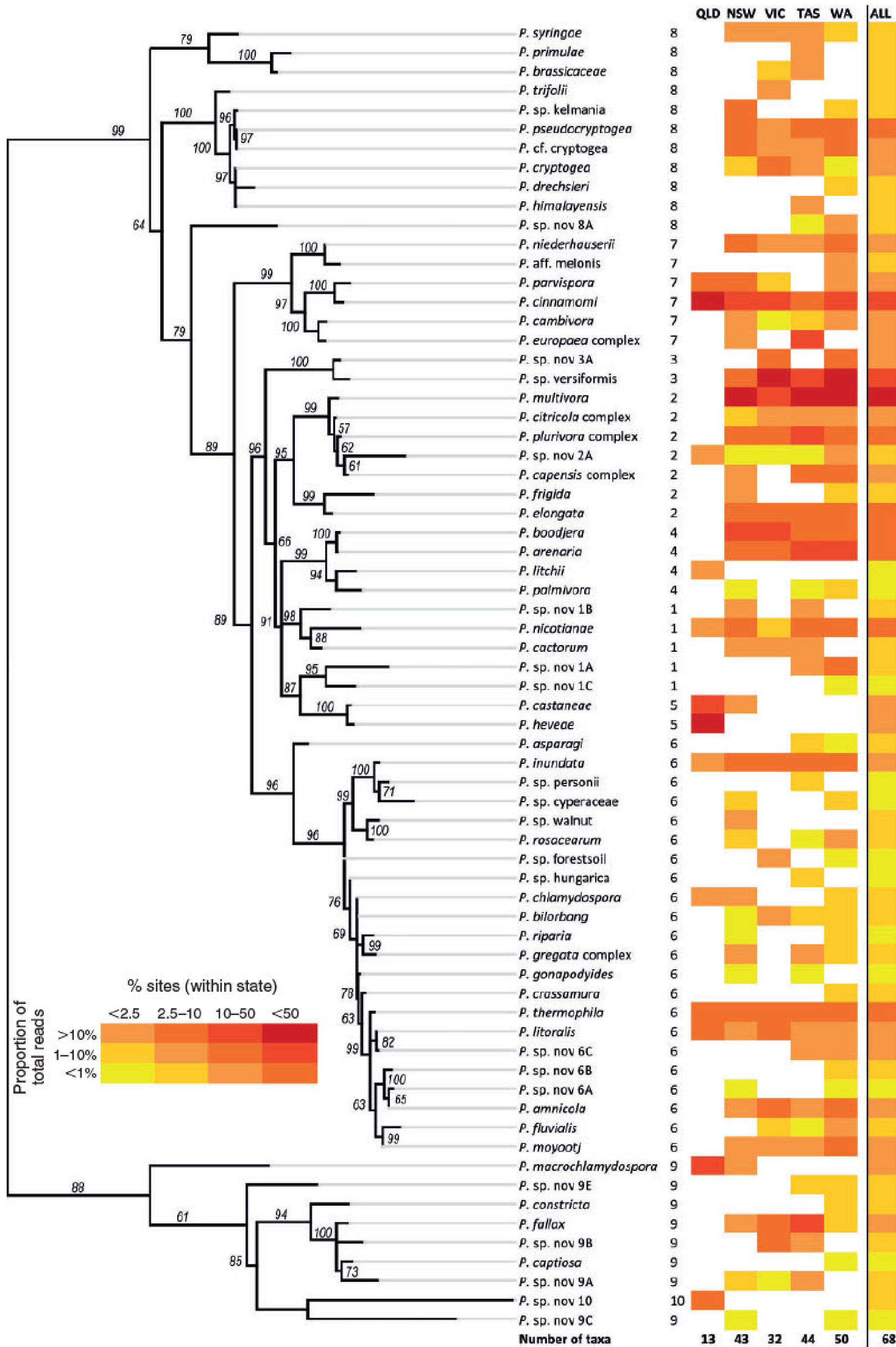

Table 1.

	Phytophthora species[A]	Clade[B]	First record[C]	Habitat[D]	Status[E]	Qld	NSW[F]	Vic.[G]	Tas.	WA[H]	SA[I]
1	*P. cactorum*	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. sp. nov 1A**	**1**		**N**	**N**				(+)	(+)	
6	**P. sp. nov 1B**	**1**		**N**	**N**		(+)		(+)		
7	**P. sp. nov 1C**	**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. sp. nov 2A**	**2**		**N**	**N**	(+)	(+)	(+)	(+)	(+)	
19	*P.* sp. versiformis	3	2014	N	N		(+)	(+)	(+)	+(+)	
20	**P. sp. nov 3A**	**3**		**N**	**N**			(+)		(+)	
21	*P. arenaria*	4	1986	N	N		(+)	(+)	(+)	+(+)	
22	*P. bisheria*	4	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			+	(+)	+(+)	
30	*P. bilorbang*	6	2012	N	I		(+)	(+)	(+)	+(+)	
31	*P. chlamydospora*	6	2009	N	I	(+)	(+)	+	+	+(+)	
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	(+)	(+)	+(+)	(+)	+(+)	
38	*P. litoralis*	6	2007	N	N	(+)	(+)	(+)	(+)	+(+)	
39	*P. megasperma*	6	1953	NH	I	+	+	+	+	+	+
40	*P. moyootj*	6	2006	N	N		(+)	(+)	(+)	+(+)	
41	***P. riparia***	**6**		**N**	**I**		(+)			(+)	
42	*P. rosacearum*	6	1993	NH	N?		(+)		(+)	+(+)	
43	*P. thermophila*	6	1980	N	N	(+)	(+)	+(+)	(+)	+(+)	
44	**P. sp. nov 6A**	**6**		**N**	**N**		(+)			(+)	
45	**P. sp. nov 6B**	**6**		**N**	**N**					(+)	
46	**P. sp. nov 6C**	**6**		**N**	**N**				(+)	(+)	
47	P. sp. cyperaceae	6	2014	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	+	+	+			
63	**P. cf. cryptogea**	**8**		**NAH**	**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	+	+	+	+	+	+
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. sp. nov 8A**	**8**		**N**	**N**				(+)	(+)	
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. sp. nov 9A**	**9**		**N**	**N**		(+)	(+)	(+)		
86	**P. sp. nov 9B**	**9**		**N**	**N**			(+)	(+)		
87	**P. sp. nov 9C**	**9**		**N**	**N**		(+)			(+)	
88	*P.* sp. nov 9E	9	2012	N	N				(+)	+(+)	
89	*P. boehmeriae*	10	1962	AH	I	+	+	+		+	
90	P. sp. gondwanense	10	2015	N	N		+				
91	**P. sp. nov 10**	**10**		**N**	**N**	(+)					
	Total no. of species[J]					29	60	52	49	61	14
	No. of databases[K]					20	33	33	9	42	14
	No. of HTS[L]					13	43	32	44	50	na
	No. identical[M]					4	17	15	4	36	na

[A]*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.
[B]Phylogenetic Clade based on phylogeny of all known *Phytophthora* species (see Fig. 2).
[C]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.
[D]Known associations: A, annual crops; H, perennial crops (including forestry); N, native ecosystems.
[E]Known status of species: I, introduced; N, native; N?, putatively native.
[F]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.
[G]Additional species for Vic. were recognised by Dunstan *et al.* (2016). These identities have been confirmed by sequencing.
[H]Additional species for WA come from VHS and CPSM collections. These identities have been confirmed by sequencing.
[I]No samples from South Australia were included in the metabarcoding study.
[J]Total number of species/phylotypes from both databases and HTS.
[K]Number of species known from Australian databases.
[L]Number of phylotypes detected by HTS.
[M]Number of species/phylotypes known both from databases and detection by HTS.