Sequencing Historic Powdery Mildew Reference Collection Specimens to Resolve *Podosphaera* Species on Stone and Pome Fruit Crops Important to Australian Horticulture.

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Summary

The genus *Podosphaera* contains various morphologically similar powdery mildews on horticulturally important crops. Questions of taxonomy and identification in relation to *Podosphaera* in Australia were addressed by examining historic powdery mildew specimens in reference collections.

Firstly, methodologies appropriate for use on historic powdery mildew specimens were established. Thirteen different DNA extractions protocols, nine PCR barcodes and two NGS DNA sequencing library kits were tested on apple powdery mildew specimens. Fungal DNA concentration was more important than DNA quality. Given the fragmented nature of the DNA, PCR-based barcoding was unreliable and Illumina NGS with a bioinformatics approach was more suitable. By mining ITS and chloroplast genes from the NGS data, the powdery mildew fungi were confirmed to be *Podosphaera leucotricha* and the hosts to be *Malus* species.

Secondly, the presence or absence of cherry powdery mildew in Australia was addressed. *Podosphaera clandestina* was previously considered to infect both *Prunus* (cherry) and *Crataegus* (hawthorn) hosts, yet in Australia it had only been recorded on hawthorn and never observed on cherry. A phylogenetic study using DNA from powdery mildew herbarium specimens up to 130 years old demonstrated that Australian specimens are all *P. clandestina* in the strict sense on *Crataegus* and not *Podosphaera* species that infect cherry.

Thirdly, Australian specimens of *P. tridactyla* on stone fruit and ornamental *Prunus* were re-examined. *Podosphaera tridactyla* is a species complex containing at least 12 species. Phylogenetic analysis of 58 Australian specimens identified three species on Australian *Prunus*: *P. pannosa*, *P. ampla* and a new species I described as *P. cunningtonii*. Phylogenetic analysis was also used to identify the various hosts.

This research has enabled identification of both powdery mildew and host historic herbaria specimens using NGS and demonstrated its effectiveness to provide resolution of *Podosphaera* species of concern to the Australian cherry and stone fruit industries.

Statement of Authorship

This thesis consists primarily of work by the author that has been published or submitted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Reannon Louise Smith

Date: 6th January 2021

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Thesis Preface

This thesis consists of five chapters, with the original experimental research is presented in the form of two peer-reviewed journal articles and one manuscript that is presented in the journal article submission format. Chapter 1 provides a general overview of the literature in this area of research. The two published manuscripts are presented in Chapter 2 and Chapter 3 and with the final experimental manuscript presented in Chapter 4, which has been submitted for peer-review.

The research chapters are structured with its own introduction, methodology, results and discussion sections. Also, the research chapters are prefaced by a summary of the research completed, the manuscript publication details, contribution of the co-authors and a statement from the co-author confirming the authorship contribution of the PhD candidate. Chapter 5 provides a general discussion that integrates the major themes from the three manuscripts as well as providing suggestions for future research.

As each experimental chapter corresponds to a recently published or submitted manuscript, redundancy of content has arisen between the introduction and materials and methods sections of the respective journal articles. The three experimental chapters are presented with the respective referencing, citation and formatting styles of the corresponding journals. A single referencing and citation style which has been employed for chapters 1, 4, 5 and the reference list is provided at the end of this thesis.

Chapter 1

Introduction

1.1 Powdery mildews

Powdery mildew fungi (*Ascomycota*, *Helotiales*, *Erysiphaceae*) are some of the most frequently encountered and economically important plant pathogenic fungi worldwide (Braun 2011; Glawe 2008, Johnston et al. 2019). There are over 900 species of powdery mildew fungi that infect the leaves, stems, flowers and fruits of nearly 10,000 agricultural, horticultural and ornamental plant species (Braun and Cook 2012). Powdery mildew disease is characterised by white talcum powder-like colonies on the plant surface (Braun 1987). The disease causes significant crop losses by reducing the photosynthetic capability of the plant and, in severe cases, affects the fruit as well, causing additional losses (Grove and Boal 1991).

Figure 1: Apple powdery mildew *Podosphaera leucotricha* displaying the characteristic white powder-like colonies on a *Malus* species. Photo by R.L. Smith (2018).

1.1.1 The economic impact of powdery mildews

In Australia, studies on the economic impact of powdery mildew due to crop loss and control measures, have concentrated on three crops: wine grapes, wheat and barley. In Australian viticulture, powdery mildew caused by *Erysiphe necator* is a widespread, persistent disease that causes major crop losses annually in addition to decreased wine quality (Emmett and Edwards 2010). Australian wineries reject grapes from vineyard patches with more than 3% bunch disease severity. The economic impact was estimated to be $AUD76 million (M) in 2010 (Scholefield and Morison 2010). Barley powdery mildew, *Blumeria graminis* f. s. *hordei*, is considered one of the top three plant diseases affecting Australian barley crops, with annual losses of $AUD39 M with an additional cost of $AUD52 M costs in fungicide treatment (Murray and Brennan 2010). Wheat powdery mildew, B. *graminis* f. s. *tritici*, is not considered to be one of a major wheat pathogen, however, it was still estimated to cost the Australian wheat industry $AUD28 M in fungicide control and $AUD36 M in crop losses each year (Murray and Brennan 2009). There is little published data on the economic costs of other powdery mildew species on Australian agricultural and horticultural crops. In the 2019 Plant Health Australia National Plant Biosecurity Status Report, it was calculated that over $AUD1,108 M was spent on fungicides alone to control fungal pathogens in Australia between 2016 and 2019 (Plant Health Australia 2019). There was no analysis of the fungal pathogens that were targeted during this period, but powdery mildews are widespread across Australian agricultural and horticultural industries and their control costs would be included in the large volume of fungicide that was purchased and applied.

1.1.2 The importance of powdery mildews

The impact of powdery mildew fungi in food production is reflected in the significant research efforts in breeding powdery mildew resistance in both broadacre and horticultural crops. This has advanced considerably since the early 1990s after the development of molecular methods and recombinant DNA technologies that lead to the characterisation of plant disease resistance (*R*) genes (Kettles and Luna 2019). Over the last twenty years, research in Australia has concentrated on powdery mildew prevention through resistance breeding in the three crops mentioned in 1.1.1: grape, wheat and barley (Dreiseitl and Platz 2012; Dry et al. 2010; Feechan et al. 2010; Golzar et al. 2016; Hickey et al. 2012; Mcintosh et al. 1967). Additionally, research efforts have gone into investigating the rise of fungicide resistance observed in powdery mildews of many Australian crops such as apple, cucurbits, field pea, oilseed brassicas and strawberry (Davidson et al. 2004; Javid et al. 2015; O'Brien et al. 1988; O'Brien 1993; Uloth et al. 2016,2018; Washington et al. 1998). Despite this, the powdery mildews remain prominent as fungal pathogens with in our agricultural and horticultural systems (Glawe 2008).

1.2 The history of taxonomy of the *Erysiphales*

Powdery mildews have plagued farmers for centuries and powdery mildew fungi were among the first to be described in publications by Linnaeus (1753), Persoon (1796) and de Candolle (1805, 1815) (Braun and Cook 2012). Following the publication of Léveille's comprehensive species arrangement of the genus *Erysiphe* in 1851, which is considered to be the beginning of modern powdery mildew fungal taxonomy, there was increased interest in this fungal group over the following decades into the early 1900's (Braun 2011). Salmon's monograph of the *Erysiphales* (1900) described six genera, 49 species, and 11 varieties. It introduced a very wide species concept with classifications based solely on the morphology of the ascomata and did not consider biological specialisation or the taxonomic value of asexual characters (Braun and Cook 2012). Following Salmon, Neger (1901) described the germination of powdery mildew conidia, the anatomy of chasmothecia and carried out inoculation experiments. Neger's investigations contradicted Salmon's taxonomy of *Erysiphaceae* by demonstrating the morphological importance of conidia in species identification and the current scientific understanding supports Neger's ideas that powdery mildew asexual characters are integral taxonomic features (Braun and Cook 2012).

In 1927 Jaczewski summarised all members of *Erysiphaceae* known at the time and recognised nine genera and introduced a simple taxonomic system at the species level (Braun and Cook 2012). Jaczewski divided large, complex species into numerous species, assigning one powdery mildew species for each host genus. In Europe, comprehensive monographs were produced, starting with Blumer in 1933. Blumer formalised the narrow species concept established by Jaczewski by splitting some of Salmon's compound species complexes into new species. This narrowing of *Erysiphaceae* species influenced later taxonomy of this fungal group (Braun and Cook 2012). Various monographs were published over the next 50 years which were loosely based around Blumer (1933) and followed by Homma in 1937 who added subfamily *Leveilluloideae* and Viégas (1944) published on the ascomycetes from Brazil. Sawada (1951) studied the *Erysiphaceae* in the Tohoku district of Japan and Golovin (1956,1958) proposed changes to *Erysiphaceae* by generic concepts. Junell (1966, 1967) split Blumers remaining compound species which was followed by Blumer's second monograph (Blumer 1967) and Katumoto (1973) included the subfamily *Cystothecoideae* (Braun and Cook 2012).

The first worldwide comprehensive monograph of the *Erysiphales* was published by Braun (1987), in which the narrow species concept was utilised as established by Jaczewski (1927) and Blumer (1933, 1967). The monograph accepted two tribes (*Erysipheae* and *Cystotheceae*), five sub-tribes (*Erysiphinae, Microsphaerinae, Uncinulinae, Sawadaeinae* and *Typhulochaetinae*) and 18 genera (*Erysiphe, Setoerysiphe, Blumeria, Brasiliomyces, Microsphaera, Medusosphaera,*

Arthrocladiella, Uncinula, Uncinuliella, Sawadaea, Typhulochaeta, Cystotheca, Podosphaera, Sphaerotheca, Leveillula, Phyllactinia and *Pleochateta*). Following this, Cook et al. (1997) proposed two new tribes, *Golovinomycetinae* and *Neoerysiphinae*, and these additions were accepted by Braun and Cook (2012).

There have also been several publications providing a comprehensive understanding of powdery mildews. The first publication was *The Powdery Mildews*, complied by Spencer in 1978. This was the first book devoted entirely to the powdery mildews and provided thorough information on the history, epidemiology, chemical control, resistance and the genetics of powdery mildew. The first international powdery mildew conference held in 1999 highlighted the advancement in the study and understanding of powdery mildew fungi and diseases over the previous 20 years. From this, a new comprehensive treatise of the powdery mildews was proposed and published by Bélanger et al. (2002). This updated treatise covered a wide range of topics that included taxonomy, comparative genetics, epidemiology, disease resistance in crops, population genetics, breeding resistance and chemical control of powdery mildew disease.

1.2.1 Application of molecular data to the taxonomy of *Erysiphales*

The development of molecular technologies over the last 30 years such as DNA-DNA hybridization, restriction fragment length analysis (RFLP), PCR, DNA sequencing such as Sanger sequencing, advancing to whole genome Next Generation Sequencing (NGS) has enabled the revision of both the genera and species of *Erysiphales* through phylogenetic studies, leading to the resolution of taxonomic ambiguity (Bruns et al. 1990; Kettles and Luna 2020). Over the past 20 years, there have been several key revisions of the *Erysiphales* utilising molecular data. Using improved DNA extraction techniques, nested PCR and DNA purification by electrophoresis, Hirata and Takamatsu (1996) examined the rDNA sequences of the Internal Transcribed Spacer (ITS) ITS1-5.8S-ITS2 region and found it to be highly conserved within species and therefore useful for phylogenetic studies across the powdery mildew fungi. Braun (1999) and Braun and Takamatsu (2000) noted that *Golovinomyces, Neoerysiphe* and *Arthrocladiella* were phylogenetically similar and formed a separate lineage. This resulted in the introduction of a new tribe, *Golovinomyceteae*, and each genera contained within their own subtribes (Braun 1999; Braun and Takamatsu 2000). Traditionally, sections *Neoerysiphe* and *Golovinomyces* were included within the genus *Erysiphe*. Phylogenetic analysis found them to be genetically different, were split from *Erysiphe* and separated to form two independent genera (Braun and Takamatsu 2000). Several reductions of genera were completed with the small genera *Microsphaera* and *Uncinula* reduced to synonyms of *Erysiphe*; the genera *Podosphaera* and *Sphaerotheca* merged and, in addition, *Sawadaea* was placed in the tribe *Cystotheceae* (Braun and Takamatsu 2000). These new genera taxonomic revisions were made based on molecular phylogenetic analyses

of the ITS gene region and demonstrated that phylogenetic relationships in the *Erysiphales* are in line with the asexual characters rather than the sexual structures as was previously thought (Saenz and Taylor 1999a, b; Takamatsu et al. 1998, 2000; Takamatsu et al. 1999; Mori et al. 2000). At a taxonomic order level, Johnston et al. (2019) completed the most comprehensive phylogenetic analysis of the *Leotiomycetes* which includes *Erysiphaceae* and established that the *Erysiphales* should be included within the *Helotiales* order. Following this revision this thesis includes *Helotiales* in the taxonomic lineage of *Erysiphaceae*, however the inclusion of the *Erysiphales* within the order *Helotiales* has not yet been accepted by MycoBank or the taxonomy part of NCBI GenBank.

The usefulness of the rDNA ITS region in identifying powdery mildew fungi has not only made it possible for the taxonomic revisions previously mentioned, but also made it possible to identify specimens of powdery mildews which are lacking the sexual characters and therefore had not been assigned to species. This has been beneficial when reassessing reference collection specimens of powdery mildew fungi as demonstrated by Cunnington et al. (2003), who used the rDNA ITS region to genetically identify powdery mildews specimens held in the Victorian Plant Pathology Herbarium (VPRI) that lacked sexual structures. Cunnington et al. (2004a) completed a study on the asexual powdery mildews of the *Fabaceae* spp. in Australia and from 32 specimens identified four species. A study of asexual powdery mildews on the *Solanaceae* family in Australia revealed three broad taxa with affinities to *Golovinomyces* (Cunnington et al. 2005a). Several first report disease notes have been published on powdery mildew species in Australia such as *G*. *biocellatus* on *Mentha* (Liberato and Cunnington 2007), *Erysiphe heracleid* on carrot (Cunnington et al. 2008a) and *E. syringae* S-type on lilac (Cunnington and Brett 2009). To aid in the identification of powdery mildew fungi, Ellingham et al. (2019) investigated other potential DNA markers that could be used in conjunction with ITS as a secondary barcode for closely related taxa. They identified the *Mcm7* gene to be the most useful gene for powdery mildew as a secondary DNA barcode alongside the ITS and shows enormous potential for multiloci phylogenetic studies of the *Erysiphaceae*.

Unidentified asexual (or anamorphic) powdery mildews specimens referred to as *Oidium* sp., is referencing the Braun and Cook (2012) classification of anamorphic taxa. Braun and Cook (2012) put forward the generic name *Oidium* is *nomen conservandum* for anamorphs of powdery mildews. Under one fungus : one name, the genus *Oidium* (typified by *Oidium monilioides*) is a heterotypic synonym of *Blumeria* (typified by *Blumeria graminis*) (Braun 2013). *Blumeria* is a monotypic genus, containing the single species *B. graminis* that causes powdery mildew on cereals and grasses. However, material in reference collections is often still identified as "*Oidium* sp." as used formerly in a much broader sense for any asexual state of a powdery mildew.

Therefore, where collections borrowed from reference collections are referred to in this thesis as "*Oidium* sp.", it is in this broad sense.

Several studies of the phylogenetic relationships of powdery mildews were completed in the early 2000s using nucleotide sequences of the rDNA which included 18S, 5.8S and 28S, together with the ITS regions. Mori et al. (2000) phylogenetic analyses revealed *Uncinula* was the most basal taxa, with five major *Erysiphales* lineages characterised by the asexual structures and that the mycelioid appendage was actually a derived character which evolved independently by convergence several times throughout evolutionary history. Takamatsu (2004) calculated the time of the splitting of *Erysiphales* from *Myxotrichaceae* at 100 million years ago (MYA) and suggested that the first divergence of the *Erysiphales* occurred 76 MYA. Takamatsu (2004) also identified that the tree-parasitic powdery mildew taxa are the ancestral state and there have been multiple host expansion events from tree to herb over the evolutionary history of the powdery mildews (Takamatsu 2004).

1.2.2 Current molecular *Erysiphales* phylogeny

In 2012, Braun and Cook published the currently accepted phylogenetic tree of the *Erysiphales* tribes and genera, which was adapted from the earlier version published by Braun et al. (2006) (Figure 2). This phylogeny provided the most comprehensive account of *Erysiphales* at the time, with five tribes (*Erysipheae, Golovinomyceteae, Cystotheceae, Phyllactinieae* and *Blumerieae*), five subtribes (*Neoerysiphinae, Golovinomycetinae, Arthrocladiellinae, Cystothecinae* and *Sawadaeinae*), 17 genera (*Arthrocladiella, Blumeria, Brasiliomyces, Caespitothecea, Cystotheca, Erysiphe, Golovinomyces, Leveillula, Neoerysiphe, Parauncinula, Phyllactinia, Pleochaeta, Podosphaera, Queirozia, Sawadaea* and *Takamatsuella*). The genus *Microidium* contains species where no sexual states are known (Braun and Cook 2012) (Figure 1). Two recent studies have identified another two genera within the *Erysiphales*. *Bulbomicroidium* was described from Mexico (Marmolejo et al. 2018) and a study of powdery mildew fungal species in Australia by Kiss et al. (2020) resurrected the genus *Salmonomyces* increasing the number of genera to 19 (Figure 3).

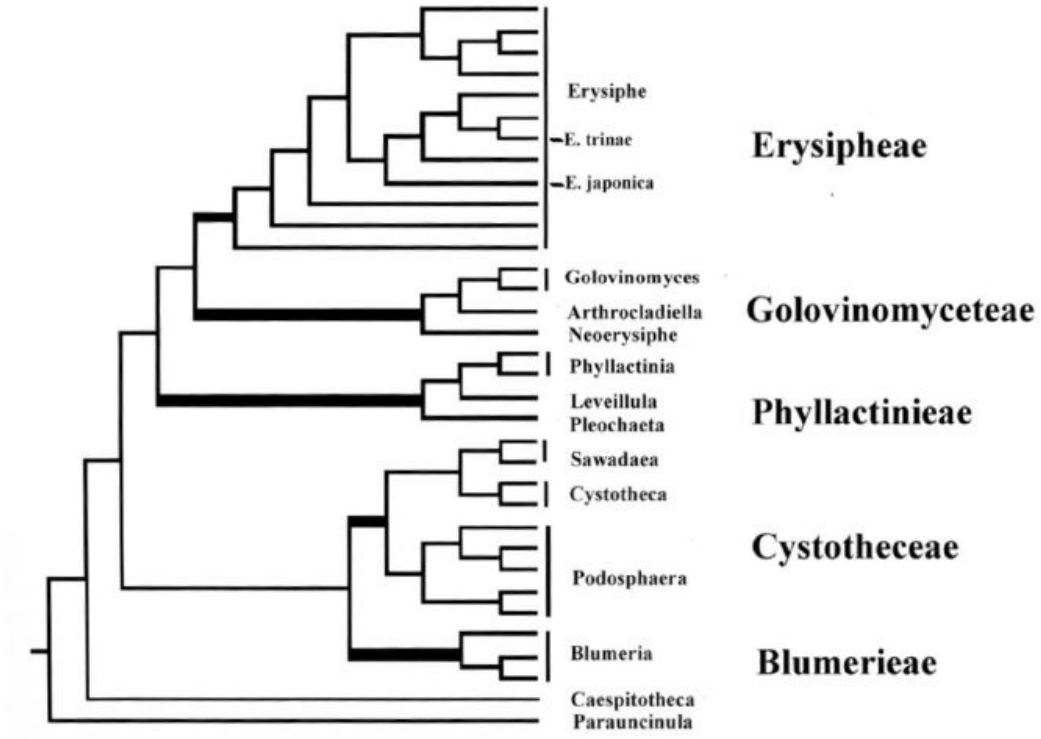

Figure 2: Phylogeny of *Erysiphales* based on 28S rDNA of 40 taxa representing the five tribes and genera. Reproduced from Braun and Cook (2012).

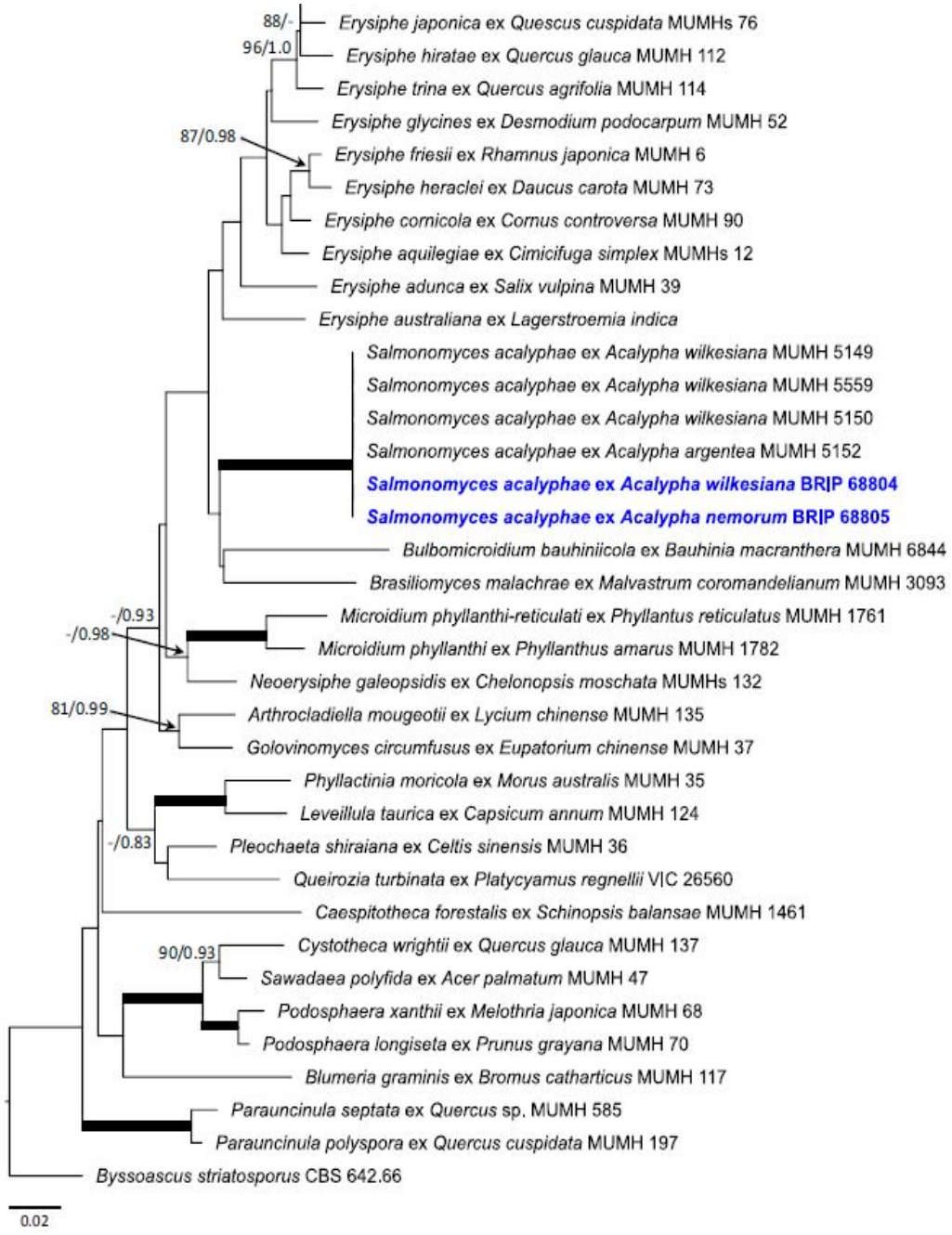

Figure 3: Maximum likelihood phylogeny of the 28S, 5.8S and 18S regions of representative genera the *Erysiphales*, with the exception of *Takamatsuella*. Reproduced from Kiss et al. (2020).

1.2.3 *Podosphaera*

The genus *Podosphaera* is characterised by non-mycelioid appendages which originate equatorially or in the upper half of the chasmothecium with dichotomously branched apices and conidia contain fibrosin bodies which are refractive particles but can only be observed in fresh specimens (Braun and Cook 2012). The genus contains an estimated 107 species which parasitise 250 host plant species covering 10 orders and 13 families, with 216 species of these host plant species (or 86.4%) belonging to the family *Rosaceae* (Braun and Cook 2012; Braun and Takamatsu 2000; Takamatsu et al. 2010). The genus *Podosphaera* is split into two sections. Section *Podosphaera* contains species such as *P. clandestina, P. leucotricha, P. pannosa* and the *P. tridactyla* complex, all of which infect woody plant hosts. *Podosphaera* section *Sphaerotheca* consists of species such as *P. aphanis, P. fugax, P. gunnerae* and *P. xanthii*, all of which infect herbaceous plant hosts (Braun and Cook 2012; Braun and Takamatsu 2010).

1.2.4 Taxonomic revision of *Podosphaera*

Powdery mildew fungi within *Podosphaera* infect many horticulturally important food crops such as stone fruit (including peaches, plum and apricots and cherries), pome fruit, cucurbits (squash, cucumber and watermelon), and strawberries (Gadoury et al. 2010; Meeboon et al. 2020; Moparthi et al. 2019; Pérez-Garcia et al. 2009; Urbanietz and Dunemann 2005). *Podosphaera* research has been limited thus far to evolutionary history, phylogenetic relationships to other powdery mildew fungi, and establishing powdery mildew resistant horticultural crops (Braun and Takamatsu 2000; Ito and Takamatsu 2009; Takamatsu et al. 2010; Shetty et al. 2012; Wolfenbarger et al. 2016; Kuzuya et al. 2006).

Recently, taxonomic revisions of two horticulturally important species of *Podosphaera* have been completed. *Podosphaera clandestina* was previously considered by Braun and Cook (2012) in a broad sense, including several morphologically different species that correlated with plant host genera. Moparthi et al. (2019) used phylogenetics to separate *P. clandestina* in the strict sense on *Crataegus monogyna* Jacq. (hawthorn) from the newly described *P. cerasi* on *Prunus avium* (L.) L. (cherry).

Cunnington et al. (2005b) studied specimens of *P. tridactyla* on *Prunus* hosts held in the Victorian Plant Pathology Herbarium (VPRI) using RFLP analysis and ITS sequencing. They identified previously unreported genetic variation within *P. tridactyla* and reported six RFLP groups. Their ITS neighbour-joining tree showed that the sequences representing these RFLP groups formed three clades, with members of each restricted to different subgenera of *Prunus*. Cunnington et al. (2005b) concluded that there were three taxa within *P. tridactyla*; this included *P. tridactyla* in the strict sense and two undescribed species.

Meeboon et al. (2020) published a comprehensive revision of the *Podosphaera tridactyla* species complex on *Prunus* spp. Their phylogenetic analyses identified 12 species of the *P. tridactyla* complex, which included three previously identified species, seven newly described species and two undescribed species from Australia; the latter based on the sequences obtained by Cunnington et al. (2005b). There are four powdery mildew species which infect horticulturally important *Prunus* species identified by Meeboon et al. (2020); these being *P. ampla, P. pruni-avium* and the two undescribed species from Australia.

1.3 Plant pathology in Australia

The introduction of non-native plants into Australia by European and Asian settlers throughout the 1800s also brought plant pathogens through accidental human-assisted introductions (Walker 1983). Due to regular rust epidemics which had been plaguing wheat crops every five to ten years, the Board of Agriculture was established in Victoria in 1851, and in 1864, a committee was appointed to inquire into the causes and prevention of rust (Fish 1970). The committee concluded the effect of agricultural practices were the cause of the rust epidemics and recommended early sowing and careful wheat variety selection as prevention (Fish 1970). This was the first comprehensive scientific inquiry into plant disease in Australia and was at the same time period that Anton de Bary was establishing plant pathology as a specific scientific discipline in Germany (Fish 1970). Successive Australian governments followed by instituting similar plant pathology committees and Departments of Agriculture in order to prevent and control plant pathogen outbreaks (Fish 1970).

The fundamental basic principle of plant pathology is the study of plant diseases and efforts to improve plant survival and growth when faced with the threat of pests and pathogens or adverse environmental conditions (Agrios 2005). The role of the plant pathologist in Australia is broad and varied with many facets, such as to identify plant pests and pathogens from specimens and collections; perform diagnostics to determine species; respond to pathogen incursions; specimen preservation; curate and maintain collections and data management of endemic pest species (Shivas and Beasley 2005). In the past, Australia plant pathologists worked together in an informal way to coordinate responses to biosecurity pest and pathogen outbreaks, which led to the creation of the National Plant Biosecurity Diagnostic Network (NPBDN) in 2011 as a part of the National Plant Biosecurity Diagnostic Strategy (Plant Health Australia 2012). Through the NPBDN, National Diagnostic Protocols (NDPs) have been established for endemic and exotic plant pests and pathogens which provide accurate and consistent identification for biosecurity surveillance and emergency responses (Plant Health Australia 2012). Having accurate knowledge of the plant pathogens within Australia's borders is

essential for the effectiveness of plant quarantine services to prevent or delay the arrival of unwanted pathogens (Hyde et al. 2010).

1.4 Victorian horticulture

In Australia, horticulture is one of the most important commodities for international and local trade with total Australian horticultural products generating $AUD14.37 Billion in 2018/19 (Horticulture Innovation Australia Limited 2019). Of that, fruit production was 2.79 M t. valued at $AUD5.53 B. Victoria is the largest horticultural producer in Australia and the top ten exported products are almonds, grapes, oranges, summer fruits (apricots, nectarines and peaches), cherries, asparagus, plums, pistachios, pears and mandarins. In the 2018/19 season, exports alone were valued at $AUD1.41 B (Horticulture Innovation Australia Limited 2019). Primary industries in Australia benefit from Australia's clean, green image through a world class biosecurity system which is a trade and economic asset (Craik et al. 2017).

1.5 The importance of biosecurity

Australia has a unique position as an island continent. This isolation has provided a physical barrier from other countries, preventing the introduction of a large diversity of plant biosecurity threats which could devastate Australia's agricultural and horticultural industries (Anderson et al. 2017). Currently, Australia is relatively free from many serious plant pathogens, keeping production costs proportionally low and providing Australian growers with secure access to international markets (McKirdy et al. 2012). The Australian biosecurity regulations not only protects the lucrative agricultural and horticultural export industries, which between 2018/20 was valued at $AUD53 Billion, but also serves to protect the unique Australian environment (Australian Agricultural Trade Report 2020; Horticulture Innovation Australia Limited 2019; McKirdy et al. 2012). From the early days of Australian plant scientific inquiry, plant pathology has evolved into an essential national biosecurity committee which provides effective surveillance and diagnostic services for exotic plant pests and pathogens (Craik et al. 2017).

In order to protect our Primary Industries, Australia maintains a plant biosecurity continuum coordinated into three management systems which are maintained through collaboration between national, state and local governments and non-government organisations and committees (Nelson et al. 2014). Australia's first system is pre-border exclusion to prevent the entry of exotic pests and pathogens into Australia. If that fails, the second system, at-the-border surveillance, minimises the likelihood of exotic pests and diseases entering and establishing in the country. The third system is post-border surveillance involving state governments and plant industries which monitor, and control established pests and pathogens in Australia (Nelson et al. 2014).

1.5.1 Biosecurity plant pathogen databases

The combined effort of Federal and State programmes performing border surveillance, in-country monitoring, and plant pathology for diagnostics and identifications has created an in-depth database of fungal plant pathogens which occur throughout Australia. As most pathogen identification is performed using morphological assessment, a physical sample, which can be a pure culture or a dried plant based collection, must be kept and held in reference collections such as VPRI (Victoria), BRIP (Queensland) and DAR (New South Wales) (Abd-Elsalam et al. 2010; Hyde et al. 2010). With the development of new technologies and inexpensive sequencing reagents, the shift into molecular identification has become widespread and providing genetic information for pathogen identification from reference specimens has become more readily utilised (Funk et al. 2017).

Reference collections are an essential part of the Australian biosecurity system enabling the trade of plant products; responding to emergency pathogen incursions and preventing exotic pests on imports from entering and establishing in Australia (Nelson et al. 2014). It is vital to maintain and revisit specimens of economically important plant pathogens, as over time, taxonomy and nomenclature is updated and changed making the old data redundant for biosecurity. Molecular revisions of reference collection specimens can be used to provide molecular taxonomic identifications to be used in conjunction with morphological descriptions to ensure plant pathogen species lists are correct and represent current taxonomic classifications (Brock et al. 2009; Hyde et al. 2010; Ristaino 2019).

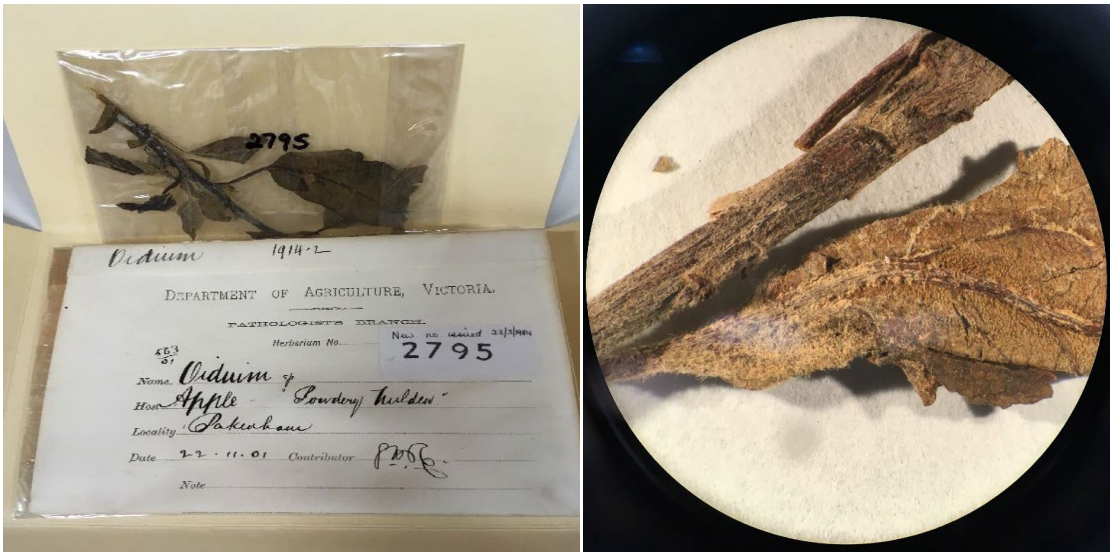

Figure 4: An example of a reference collection specimen of *Podosphaera leucotricha* in VPRI; A) in the packet and B) under the dissecting microscope prior to sampling. Photos by R.L. Smith (2018).

The Australian Plant Disease Database (APDD) is the collective database in which VPRI (Victoria), BRIP (QLD) and DAR (NSW) plant pathogen specimen record information is collated to provide accurate plant disease occurrence for Australian biosecurity (Shivas et al. 2006). The APPD is backed up by the specimen-based records of pathogens in these collections for future validation of pathogen incursions in disease outbreaks. The specimen records of APPD are used to assess plant pathogen intercepts from quarantine, to determine if they are endemic or exotic to Australia (Hyde et al. 2010).

1.6 The importance of reference collections

Millions of specimens are held within thousands of reference collections around the world. The primary aim of these collections is the permanent conservation of a comprehensive and diverse set of specimens of organisms such as plants, algae and fungi for documentation and comparative investigation, particularly taxonomy (Telle and Thines 2008). While the term "herbarium" has traditionally been applied to collections that include plant and fungi, increasingly collections of fungi are referred to as "fungaria" in recognition of the phylogenetic distinctiveness of Fungi from Plantae. In December 2020, there were 3324 active herbaria and fungaria across 178 countries around the world, containing over 390 million specimens which continuously increase each year (Thiers 2020). It was noted by Thiers (2016) that nearly one third of all institutional records have not been updated in more than ten years. The specimen numbers for these collections can then only be estimated and molecular revision of specimens held in these collections could potentially increase the worldwide specimen total dramatically. The importance of reference collections is highlighted by the functions that they can serve by resolving disputes over taxonomy, nomenclature, phylogenetics, function and evolution. For example, Ristaino (1998, 2019) was able to identify the strain of *Phytophthora infestans* through herbarium specimens which caused the great famine outbreaks during the 1800s and determined it to be an extinctic lineage. Ristaino (1998) also determined the life history of *P. infestans*, the centre of origin of the disease, changes in pathogen virulence and the genetic and evolutionary history of *P. infestans* through reference collection specimens.

1.6.1 Herbaria in Australia

The first Australian herbarium was opened in Melbourne in 1853 at the Royal Botanic Gardens and still holds the largest collection of specimens in Australia (Fish 1970; Thiers 2020). Australia's first government plant pathologist, Daniel McAlpine, was employed by the Victorian state government in 1890 as a vegetable pathologist (Parbery 2015). Throughout his career, McAlpine collected and retained specimens of diseased ornamental, native and agricultural plants which formed the basis of the Victorian Plant Pathogen Herbarium (VPRI). The three primary plant

pathogen collections in Australia which together hold most Australian plant pathogen specimens are VPRI, the Queensland Plant Pathogen Herbarium (BRIP); and the New South Wales Plant Pathology and Mycology Herbarium (DAR) (Shivas et al. 2006). These three reference collections form the National Collection of Fungi in Australia (Shivas et al. 2006).

1.6.2 The Victorian Plant Pathogen Herbarium (VPRI)

During the 1990s, the former VPRI curator Ian Pascoe instigated a project to catalogue the powdery mildew fungi of Australia, which was intended to lead to a treatise in the Australian Biological Resources Study (ABRS) *Fungi of Australia* series (I. Pascoe 2020, pers. comm. 24 September). This project coordinated the collection of powdery mildew fungi on agricultural, horticultural and ornamental plant hosts from across Australia and contributed over 2000 new specimens to VPRI (VPRI database accessed April 2018). Throughout the project, Pascoe and his collaborators A. Sivapalan and V. Beilharz completed detailed descriptions, documented the microscopic morphology through drawings and provided taxonomic classification to the majority of the newly acquired powdery mildew specimens and accessioned them into VPRI. Unfortunately, the *Fungi of Australia* treatment did not come to fruition and none of the morphological work completed by Pascoe and collaborators was ever published.

During the project, PhD student James Cunnington investigated the use of a molecular approach for taxonomic classification of the powdery mildew fungi. Through the early 2000s Cunnington published a number of articles using PCR and Sanger sequencing to provide taxonomic identification of Australian powdery mildew fungi which are lacking in sexual structures for traditional morphological identification (Cunnington and Brett 2009; Cunnington et al. 2004a,b; Cunnington et al. 2005a,b; Cunnington et al. 2008b; Cunnington et al. 2003; Cunnington et al. 2008a; Liberato and Cunnington 2006; Liberato and Cunnington 2007). However, since this extensive work was completed by Cunnington and his collaborators, very little further taxonomic research has been undertaken on powdery mildew fungi in Australia.

1.7 Ancient DNA

The concept of isolating ancient DNA (aDNA) from preserved specimens began with the DNA isolation from a museum specimen of the quagga, a zebra-like species which became extinct in 1883. Higuchi et al. (1984) proved that DNA can be recovered from preserved animal specimens and provide phylogenetic information on its evolutionary history by showing the quagga had a common ancestor 3-4 MYA with the extant mountain zebra. After this significate finding, aDNA research exploded after several key publications on aDNA was recovered from ancient human remains (Pääbo 1985a,b), extinct mammoths (Johnson et al. 1985) and insects preserved in amber (Cano et al. 1992).

Over the last 30 years, the use of molecular techniques for taxonomic classification has become critical. It is understood how important reference collections are for preserving and cataloguing plant and fungal species, and mycologists are recognising the value of reference collections as important resources for molecular genetic data (Bruns et al. 1990; O'Rourke et al. 1996; Yoshida et al. 2015). However, given the age and preservation quality of most collection specimens, can the DNA contained within herbaria be classified as aDNA? (Yoshida et al. 2015).

It is difficult to define aDNA and most researchers use a loose definition that excludes 'DNA samples from recently deceased individuals that may, nevertheless, be used in forensic and anthropological analysis' (O'Rourke et al. 1996). This definition is based on ancient human remains, but what of plant and fungal aDNA? The first published paper regarding DNA extraction and sequencing of dried fungi by Bruns et al. (1990) suggested that preserved fungal specimens contain aDNA, as the specimen is no longer alive and has been dried for long term preservation.

There is no exact defined minimum age that can be used to describe aDNA but there are several characteristic features which are synonymous with aDNA (Wieβ et al. 2016). aDNA obtained from historic specimens is characterised by low concentration DNA extractions which consists of short DNA fragment lengths, usually less than 500 bp. This fragmentation is caused by spontaneous depurination and hydrolysis of the DNA backbone, and a sign of depurination is excess adenine (A) and guanine (G) bases near DNA breakpoints in aDNA (Gutaker and Burbano 2017; Telle and Thines 2008; Wieβ et al. 2016). DNA degradation is also observed in aDNA with the spontaneous deamination of cytosine (C) to uracils (U) bases at the end of aDNA fragments, which are then read as thymine (T) during PCR and sequencing. Further damage can be caused by chemical and physical events during the preservation of the specimen which increase the DNA decay (McGaughran 2020; Telle and Thines 2008).

1.8 New sequencing technologies and their potential application to fungal aDNA

The aDNA characteristics result in poor quality DNA samples which have limitations in molecular applications such as PCR, where amplification length is restricted due to the short aDNA fragment lengths (Särkinen et al 2012). For aDNA PCR studies, it is recommended to use DNA extraction protocols to capture the shorter aDNA fragments and suitable DNA polymerases for aDNA amplification and to target gene regions which are less than 200 bp in length, or perform nested PCRs for greater amplification success (Bradshaw and Tobin 2020; Choi et al. 2015; Telle and Thines 2008). However, nested PCRs have the potential to increase the chances of amplifying DNA from other organisms present in the aDNA, highlighting the need for pure aDNA samples. Forin et al. (2018) published a study on the use of NGS for DNA barcodes on material in the Saccardo Mycological Herbarium, in which *Peziza* specimens collected during the 19th

century were sampled. A nested PCR approach was used to amplify the ITS2 gene region and the PCR products were sequenced on Illumina MiSeq. Forin et al. (2018) were able to obtain sequences for 23 of the 36 analysed specimens and through phylogenetic analysis, re-identify five specimens with species names and assign a further 18 specimens to genus or higher level. Delgat et al. (2019) also used NGS of the amplified ITS1 gene region to obtain a sequence from a specimen of *Lactifluus deceptivus* collected in 1885. While this approach is feasible when using a specimen of a macrofungus such as a *Peziza* or *Lactifluus*, it is not always practical for obligate biotrophic plant pathogens such as powdery mildew which are routinely preserved on plant material and will inevitably have a range of other microorganisms present on the leaf surface at the time of collection (Choi et al. 2015; Kistler et al. 2020).

The development of reliable aDNA extraction methods which can retain short DNA fragments, together with an improved understanding of aDNA biochemical structure, has contributed to the increased success of NGS within the aDNA field (Gutaker and Burbano 2017; Linderholm 2016). Additionally, the development of robust NGS library preparation protocols specifically designed to handle poor quality and fragmented aDNA, using the fragmented nature of the aDNA as an advantage, has resulted in higher data yields compared to the use of DNA library protocols created for fresh DNA (Linderholm 2016; Nedoluzhko et al. 2020). There are capture or enhancement procedures which improve the aDNA libraries and make them more specific, especially in cases where the aDNA sample contains low amounts of endogenous DNA (Linderholm 2016). Incorporating uracil-removing enzymes into the library preparation can help repair aDNA damage prior to sequencing and contributes to more accurate sequencing (McGaughran 2020).

Using an NGS approach for aDNA enables the sequencing of whole genomic DNA and through bioinformatics, locating longer gene regions, genomes of informative genes, or whole organism genomes which is not possible in a PCR approach (Gutaker and Burbano 2017). The aDNA NGS data has been mapped to reference sequences or assembled *de novo* for use in evolutionary and phylogenetic studies investigating population genetics of ancient animal and plants (Linderholm 2016; Willerslev and Cooper 2005).

In the last decade, the use of NGS for aDNA samples from reference collections has been shown to be effective on the fragmented DNA and recent improvements in library protocols and more accurate sequencing platforms have improved the quality of the NGS data generated (Kistler 2020; Linderholm 2016; McGaughran 2020). There have been several key publications providing laboratory and bioinformatic protocols for genetic studies from historical collections of organism such as horseshoe bats (Bailey et al. 2016); insects (Timmermans et al. 2016; pigeons (Besnard et al. 2016); angiosperms (Bakker et al. 2016); palms (Heyduk et al. 2016), the olive

family (Zedane et al. 2016) and fungi (Dentinger et al. 2016). Dentinger et al. (2016) investigated the application of NGS on fungarium specimens from 30 families within the *Agaricales* and were able to generate whole genome sequence data from specimens collected from 1996 to 2014 from fungarium, culture and frozen specimens.

Following the Dentinger et al. (2016) study, there have been no further publications regarding whole genome NGS of fungal specimens held in reference collections despite the technological capability. Buerki and Baker (2016) discussed the need to incorporate NGS methods into biological reference collections and the incredible potential to reinvent collections-based research in the future. As NGS has proven ability to access the genetic material stored in collections, Buerki and Baker (2016) suggested that researchers and curators embrace the new genomic era and encourage cross-disciplinary research projects to protect and secure the role of reference collections in the future.

1.9 Research aims and objectives

The aim of this PhD study is to utilise novel molecular technologies to re-examine specimens held in Australian plant pathogen reference collections in order to update the taxonomy of the powdery mildew *Podosphaera* spp., especially in relation to species of importance to Victorian perennial horticultural crops.

This aim will be delivered through three key objectives:

1. Identify molecular diagnostic methodologies suitable for use on specimens of *Podosphaera* spp. up to 130 years old held in reference collections,
2. Apply the protocols established in objective 1 to confirm the absence in Australia of cherry powdery mildew (*Podosphaera cerasi*) on cherry (*Prunus avium*(L.) L.).
3. Apply the protocols established in objective 1 to determine the species of the *Podosphaera tridactyla* species complex that are present in Australia on stone fruit such as *Prunus armeniaca* L.*, Prunus cerasifera* Ehrh.*, Prunus domestica* L.*, Prunus persica* (L.) Batsch and other closely related *Prunus* species.

Chapter 2

Rediscovering an old foe: Optimised molecular methods for DNA extraction and sequencing application for fungarium specimens of powdery mildew (*Erysiphales*)

2.1 Chapter Preface

There is a need to molecularly re-evaluate powdery mildew fungi held in reference collections to resolve asexual *Oidium* sp. taxonomic classifications, address recent species revisions and confirm the powdery mildew species present in Australia. Currently, there are few diagnostic molecular methods suitable for use on preserved powdery mildew fungi. This chapter represents the first published manuscript of this thesis and presents the development of a next generation sequencing (NGS) protocol to re-examine powdery mildew fungi held in reference collections. Initial attempts at using cellophane strips and scalpel scrapings of powdery mildew on the surface of the dried leaves for DNA extraction were unsuccessful. This led to utilising a standardised leaf punch to collect material for testing with 13 DNA extraction methods. The most successful was a kit designed for use in forensics, the E.Z.N.A Forensic DNA kit.

This study found that due to the fragmented nature of the powdery mildew fungal DNA, Sanger sequencing of PCR barcoding regions provided inconsistent results. Therefore, short-read whole genome sequencing was explored as an alternative. Two NGS library kits were tested to find the most suitable for Illumina sequencing. Once again, a kit designed for forensic material proved the most suitable for purpose. Subsequent NGS produced consistent sequence data unhindered by the fragmented nature of the DNA. It also became evident that DNA quantity was more important than DNA quality for extracting the small amount of target sequence data from the much larger quantity of host and microflora sequence data.

These methods have been applied in a practical context in the subsequent two chapters which focus on resolving two taxonomic questions regarding *Podosphaera* species affecting Australian horticulture.

This chapter is presented in published format.

2.2 Publication details

Title: Rediscovering an old foe: Optimised molecular methods for DNA extraction and sequencing application for fungarium specimens of powdery mildew (*Erysiphales*)

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Authors: Reannon L. Smith, Tim I. Sawbridge, Ross C. Mann, Jatinder Kaur, Tom W. May and Jacqueline Edwards.

2.3 Statement of contribution of joint authorship

JE, TIS and RLS conceived the idea for the study and all the authors (RLS, TIS, RCM, JK, TWM and JE) contributed to the design. RLS researched and sourced the various extraction and library preparation kits available. RLS performed the experimental DNA extraction and sequencing, with assistance and advice from JK. RCM and TIS assisted RLS with the bioinformatics. Data analyses and interpretation were conducted by RLS with assistance and advice from all authors. RLS wrote the manuscript, which was reviewed by TKM and JE to shape the final version. All authors approved the final version of the manuscript.

2.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Smith, R.L., Sawbridge, T., Mann, R., Kaur, J., May, T.W. and Edwards, J., 2020. Rediscovering an old foe: Optimised molecular methods for DNA extraction and sequencing application for fungarium specimens of powdery mildew (*Erysiphales*). PLOS ONE 15(5), e0232535. https://doi.org/10.1371/journal.pone.0232535' I can confirm that Reannon L. Smith made the following contributions:

- Literature review
- Development of the experimental design
- Brought the concept of ancient DNA and use of forensic kits to our attention
- Preparation of powdery mildew specimens
- DNA extractions
- Library preparations
- PCR
- Illumina sequencing
- Sequence data extraction and analysis
- Generated all figures
- Writing the manuscript, critical appraisal of content and response to reviewers"

Dr. Jacqueline Edwards

Date: 13/12/2020

PLOS ONE

RESEARCH ARTICLE

Rediscovering an old foe: Optimised molecular methods for DNA extraction and sequencing applications for fungarium specimens of powdery mildew (Erysiphales)

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Abstract

The purpose of this study was to identify a reliable DNA extraction protocol to use on 25-year-old powdery mildew specimens from the reference collection VPRI in order to produce high quality sequences suitable to address taxonomic phylogenetic questions. We tested 13 extraction protocols and two library preparation kits and found the combination of the E.Z.N.A.® Forensic DNA kit for DNA extraction and the NuGen Ovation® Ultralow System library preparation kit was the most suitable for this purpose.

Introduction

Since analysis of the first DNA extractions from museum specimens was made possible through the development of PCR during the mid-1980's, the use of reference collection specimens for molecular phylogenetic studies has increased and several comparative studies have been published on ancient DNA (aDNA) and PCR amplification methods for plant and fungal specimens [1]. However, there is a knowledge gap regarding obligate biotrophic fungal plant pathogens such as powdery mildew–are we able to extract useable aDNA from powdery mildew on host leaf material for PCR and whole genome Next Generation Sequencing (NGS) applications?

aDNA from preserved specimens is subject to numerous natural processes such as degradation, fragmentation and the deamination of nucleotides, thus reducing the DNA quality and quantity that can be obtained, which reduces the efficacy of PCR [2]. There are many factors that can affect DNA in fungarium specimens such as the age and quality of the sample when collected, the preservation method used, exposure to chemicals and other mutagens, and temperature and relative humidity at which the specimens are stored [3, 4]. Accumulation of these effects results in small DNA fragments (40–400 bp) and low DNA concentration [5]. For

aDNA molecular studies the use of whole genome NGS applications has been shown to be more reliable than PCR- amplified gene regions, as the DNA strands are already fragmented, which is preferable for most sequencing platforms, whereas aDNA PCR is limited to shorter targeted gene regions which can reduce the phylogenetic capabilities of these regions [6, 7].

Accessing aDNA from specimens that were preserved primarily with the aim of conserving morphology rather than DNA presents complications when developing methods for the isolation, sequencing and analysis of aDNA [8]. Molecular methodologies have been developed for specific areas of aDNA research such as palaeontology, archaeology, forensics and reference collections of animals, plants and macro-fungi [9]. To date there has been limited research into fungal plant pathogens in reference collections, in particular obligate biotrophs such as powdery mildews (Erysiphales), although studies such as Ristaino [10] and Yoshida et al. [11] investigated the oomycete *Phytophthora infestans* from reference collections, using molecular tools to trace pathogen lineages to understand pathogen evolution.

Currently there are nearly four million algal, fungal and plant specimens held within just over three thousand herbaria and fungaria around the world [12]. The original purpose of these institutions was to provide permanent conservation of plant, algae and fungi collections for morphological analysis enabling research on taxonomy, nomenclature, phylogenetics and the evolution of species [10]. Accurate species identification supports our understanding of worldwide biodiversity; however, there is major discrepancy between the number of species that have been taxonomically classified in collections compared to the estimated species numbers which are still waiting for classification [13]. With the advancement of PCR and affordable sequencing technologies, aDNA molecular phylogenetic studies have seen a surge in the utilisation of herbaria, which have been relatively untouched for molecular analysis to date [14, 15].

The Victorian Plant Pathology Herbarium (VPRI) maintained by Agriculture Victoria at Bundoora, Victoria, Australia, is an example of a reference collection rich in historical collections of fungi. The collection was established in 1890 by Daniel Mc Alpine, the first Consulting Vegetable Pathologist to the Department of Agriculture of Victoria [16]. Specimen-based records of plant pathogens have been collected across Australia and preserved as a reference collection [17]. Currently, VPRI holds ca. 43,000 dried specimens and cultures. Online portals such as the Australian Plant Pest Database [18] utilise specimen-based collections such as VPRI to provide up to date information on current plant pathogen and pest status across Australia [17]. It is therefore vital that reference collections such as VPRI are accurate and up to date with current taxonomic classification.

Powdery mildews are the most commonly occurring plant pathogens worldwide, with ca. 900 species within 16 genera infecting thousands of plant species including ornamental, economically important agricultural and horticultural plants [19, 20,21]. Taxonomic classification of powdery mildews is complex due to the asexual and sexual lifecycles of the fungi. Traditionally, powdery mildew identification was based on morphology and host plant associations with morphological classification relying on specific descriptions of sexual characters to identify to species; however, when the sexual state was absent, identification was largely based on host association [21]. Currently, the use of phylogenetic analysis of nuclear ribosomal DNA has enabled researchers to identify five major lineages of powdery mildew, resolve genera and species delineation, understand powdery mildew evolutionary history, and the evolution of phenotypic characters used for identification purposes [22, 23, 24, 25, 26].

The subject of this study is the apple powdery mildew fungus *Podosphaera leucotricha*, which causes significant yield losses of cultivated apple (*Malus* x *domestica*) around the world [26]. The aim of this study was to test 13 DNA extraction protocols, which include 4 different DNA isolation methods including modifications, for use on preserved powdery mildew

specimens from the Victorian Plant Pathology Herbarium (VPRI), in order to obtain DNA suitable for use in species identification PCR and whole genome Next Generation Sequencing applications to provide molecular resolution of preserved powdery mildew specimens.

Results

Apple (*Malus* spp.) leaves infected with *Podosphaera leucotricha* collected between 1992–1994 were selected from VPRI (Table 1). A 6 mm leaf punch was used to sub-sample from VPRI *P. leucotricha* specimens as it was a standardised measure that could be used to compare DNA extraction protocols effectiveness. Infected leaf material was sub-sampled from VPRI *P. leucotricha* specimens using a leaf punch to cut leaf sections, which were then used to test 13 DNA extraction protocols. The 13 protocols tested were Chelex®100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+). These protocols were compared on the basis of DNA concentration and quality. PCR, ITS phylogeny and whole genome NGS library preparations were also performed. The DNA samples were expected to comprise *P. leucotricha* DNA, host DNA from apple as well as DNA from microorganisms present on the leaf tissue prior to its collection.

DNA concentration

The 13 different DNA extraction protocols generated variable concentrations of DNA from the five VPRI *P. leucotricha* specimens (Table 2). DNA was quantified using two methods, Qubit™ fluorometer (Life Technologies, Singapore) and Agilent 2200 TapeStation® (electrophoresis) (Agilent Technologies, Waldbronn, Germany) to eliminate instrument bias analysing poorer quality DNA samples. The two methods gave different estimates of DNA concentration. Qubit™ fluorometer consistently estimated lower concentrations than Agilent 2200 TapeStation®, except in two instances: EznS and EnzP. Based on Qubit™ fluorometer quantification the DNA extraction protocol which produced the highest DNA concentration was EznS (13.7 ng/μL), followed by EznP (10.9 ng/μL) and EznF (3.34 ng/μL) (Fig 1). WizG yielded 2.76 ng/μL; the remaining nine extraction protocols produced DNA concentrations < 1 ng/μL, with SDS producing the least DNA (0.107 ng/μL). Concentrations assessed with the Agilent 2200 TapeStation® followed a similar pattern to the Qubit™ fluorometer results with EznS, EznP, WizG and EznF showing the highest concentrations of 10.6 ng/μL, 8.89 ng/μL, 3.71 ng/μL and 3.64 ng/μL, respectively. However, the Agilent 2200 TapeStation® readings for the remaining 9 extraction methods were slightly higher with concentrations ranging between 2.22–2.93 ng/μL (Fig 2).

Table 1. Victorian plant pathology herbarium (VPRI) *P. leucotricha* specimens investigated.

VPRI NUMBER	LOCATION	COLLECTION YEAR	HOST SPECIES
18381	Queensland, Aust.	1992	*Malus pumila* L.
18536	Tasmania, Aust.	1992	*Malus domestica* Borkh.
18575	Tasmania, Aust.	1992	*Malus domestica* Borkh.
19785	South Australia, Aust.	1994	*Malus sylvestris* Mill.
19947	Tasmania, Aust.	1994	*Malus* sp.

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Table 2. Median and range of total DNA concentration (ng/μL) and DNA quality (A260/280) produced by 13 extraction protocols tested on five victorian plant pathology herbarium (VPRI) apple powdery mildew specimens.

Extraction Method	Median (Range) Invitrogen Qubit™ DNA Concentration (ng/μL)	Median (Range) Total DNA concentration Invitrogen Qubit™ (ng)	Median (Range) Agilent 2200 TapeStation® DNA concentration (ng/μL)	Median (Range) Total DNA concentration Agilent 2200 TapeStation® (ng)	Median (Range) NanoDrop 2000™ DNA Quality (A260/280)
CheX	0.52 (0–1.25)	104.4 (0–117.60)	2.22 (2.11–2.42)	444 (422–484)	1.36
InuP	0.30 (0–0.43)	60.4 (0–85.40)	3.67 (2.97–4.18)	734 (594–836)	1.29
SDS	0.11 (0–3.79)	5.4 (2.75–189.50)	2.45 (2.07–3.28)	122.5 (103.5–164)	2.68
EznS	13.7 (3.18–26.9)	1370 (318–2690)	10.6 (5.2–12.40)	1060 (520–1240)	2.17
DnaZ	0.99 (0.5–1.13)	49.9 (27.25–56.50)	2.62 (2.27–3.02)	131 (113.5–151)	2.06
EznF	3.34 (1.33–39.3)	334 (133–3930)	3.64 (2.91–9.41)	364 (291–941)	1.97
DneP	0.46 (0.38–3.66)	46.6 (43.9–366)	2.49 (1.14–2.66)	249 (114–266)	2.05
IspC	0.94 (0.25–1.93)	94.3 (24.6–193)	2.39 (0–3.19)	239 (0–319)	1.92
IspS	0.97 (0.21–2.12)	96.8 (21–138)	2.93 (2.31–4.91)	293 (231–491)	1.83
WizG	2.76 (0.74–8.89)	276 (74–889)	3.8 (2.82–9.71)	380 (282–971)	1.64
EznP	10.9 (2.82–12.7)	1090 (282–1270)	8.89 (3.51–16.60)	889 (351–1140)	1.87
CTAB	0.36 (0–0.55)	27.2 (0–41.25)	2.89 (2.19–3.17)	216.8 (164.3–237.75)	1.88
DneP+	0.36 (0.08–1.84)	36.3 (8.30–184)	2.42 (2.04–4.44)	242 (204–444)	1.33

Extraction method abbreviations: Chelex®100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

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DNA quality

The visual appearance of the extracted DNA varied between methods from colourless to brownish. In all cases the *P. leucotricha* DNA was highly fragmented, as indicated by the Agilent 2200 TapeStation® electrophoresis images with fragment sizes between 50 bp– 400 bp. DNA quality was measured using NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) absorbency measurement 260 nm /280 nm ratio; the optimum range indicating high quality DNA is 1.8–1.9 [27]. In general, the silica binding column methods (IspS, IspC, EznP and EznF) produced more consistent DNA quality than the precipitation-based methods (SDS, WizG® and DnaZ). The only method that consistently produced DNA quality within the 1.8–1.9 range was the IspS (Table 2 and Fig 3). The mean DNA quality produced by the EznF, EznP, IspC and CTAB protocols were within the ideal range, but the raw data included outliers either side of the required absorbency ratio. The absorbency ratio of the remaining DNA extraction protocols InuP, SDS, EznS, DnaZ, DneP and DneP+ were outside the required range. Precipitation-based methods produced less consistent DNA quality than the silica binding column methods (Fig 3).

DNA PCR amplification

Nine published PCR primer sets used in powdery mildew phylogenetic studies were tested for their suitability to amplify powdery mildew DNA extracted by the 13 protocols for species identification (Table 3). The nested PMITS1/PMITS2 and PMITS1/ITS4 [27] was the only set which amplified DNA extracted from all the methods tested (Table 4). Sanger sequencing of the nested PMITS1/PMITS2 and PMITS1/ITS4 PCR VPRI amplicons demonstrated that 64.6% matched GenBank Accession no. KY661076.1, the target *P. leucotricha* ITS region, at 98% identity or higher. The remaining amplicons matched other powdery mildew species (12.3%), undetermined fungi (4.6%), the host *Malus* (6.15%) or had failed amplification

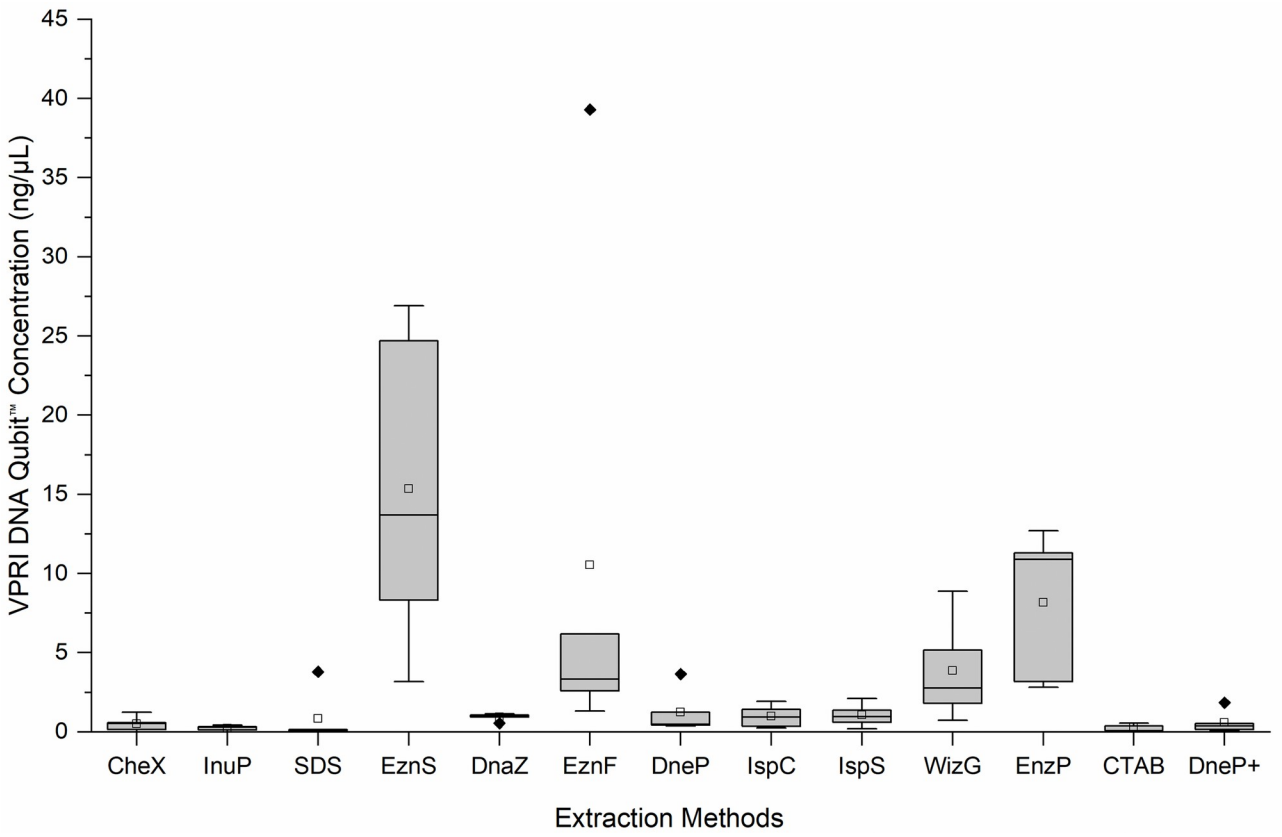

Fig 1. Boxplots of the DNA concentrations (ng/μL) of five victorian plant pathology herbarium (VPRI) apple powdery mildew specimens produced by 13 extraction protocols as measured by Invitrogen Qubit™ fluorometer. Median line —; Mean □; Outlier ◆Extraction method abbreviations: Chelex® 100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

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sequences (12.3%) (Table 4). DNA extracted using protocols CheX, WizG and DnaZ did not amplify well during PCR (Table 4). CheX and WizG resulted in only a single *P. leucotricha* ITS amplicon produced and DnaZ resulted in two *P. leucotricha* ITS amplicons. Only five amplicons derived from VPRI specimen 19947 were *P. leucotricha* ITS. There is reportedly no correlation between herbarium DNA concentration and PCR amplification success [28], yet the presence of other fungi and plant host DNA resulted in preferential amplification over *P. leucotricha* DNA in 21.5% of the PCR reactions during this study.

Phylogeny

Thirteen sequences were derived from *P. leucotricha* VPRI 18536 by nested PCR and Sanger sequencing, one from each extraction method. Two sequences were excluded from the analysis: the sequence generated from the extraction method DnaZ identified a contaminant (*Golovinomyces*) and the sequence generated from the extraction method CTAB was ambiguous and could not be aligned with the others. The maximum likelihood anaylsis (PhyML) including the other 11 VPRI 18536 sequences confirmed that VPRI 18536 was *P. leucotricha* (Fig 4). Sequences from other *Podosphaera* species sequences downloaded from GenBank were included and clustered in two separate clades. The first clade (bootstrap support 90.9%)

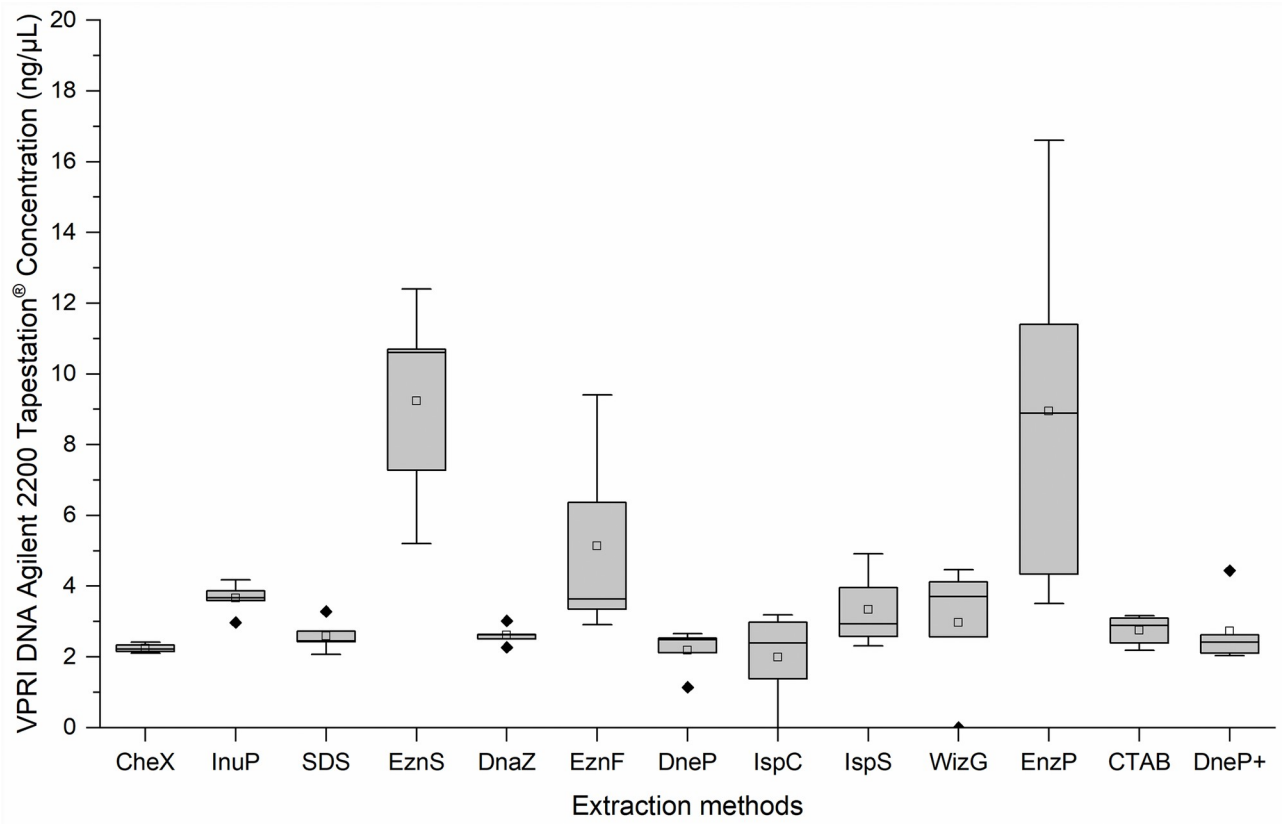

Fig 2. Box plots of the DNA concentrations (ng/μL) of five victorian plant pathology herbarium (VPRI) apple powdery mildew specimens produced by 13 extraction protocols, as measured by agilent 2200 Tapestation® DNA. Median line —; Mean □; Outlier Extraction method abbreviations: Chelex® 100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

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consisted of *P. clandestina*, *P. amelanchieris*, *P. leucotricha*, *P. ferruginea*, *P. pannosa*, *P. spiraeae*, *P. pannosa*, *P. aphanis*, *P. epilobii*, *P. erodii*, *P. caricicola* and an un-named *Podosphaera* collection from Japan. The second clade (bootstrap support 92.4%) consisted of sequences from *P. tridactyla* var. *tridactyla*, *P. longiseta*, *P. fuliginea* var. *sibirica*, *P. astericola*, *P. macrospora*, *P. balsaminae*, *P. cayratiae* and *P. fusca*. Within the first clade, sequences from *P. leucotricha* formed a tight clade (bootstrap support 99.8%) at the base of the clade. The sequences from VPRI 18536 and GenBank sequences of *P. leucotricha* from Australia, China, Korea, Japan, Hungary, UK and USA clustered together. Among *P. leucotricha* sequences, there were several base pair differences between some of the collections. Among the sequences of VPRI 18536 derived from different extraction methods, there was also a small amount of variation. In the alignment, sequences derived from extraction methods IspC, IspS and SDS had one missing base (T) at position 520 compared to sequences from the other nine extraction methods. CheX had one different base (G) at position 469 and IspC had one different base (T) at position 551.

Next generation sequencing VPRI 18536

Two library preparation kits, Illumina Nextera XT® (San Diego, California, USA) and NuGen Ovation® ultralow System V2 (San Carlos, California, USA), were compared using DNA

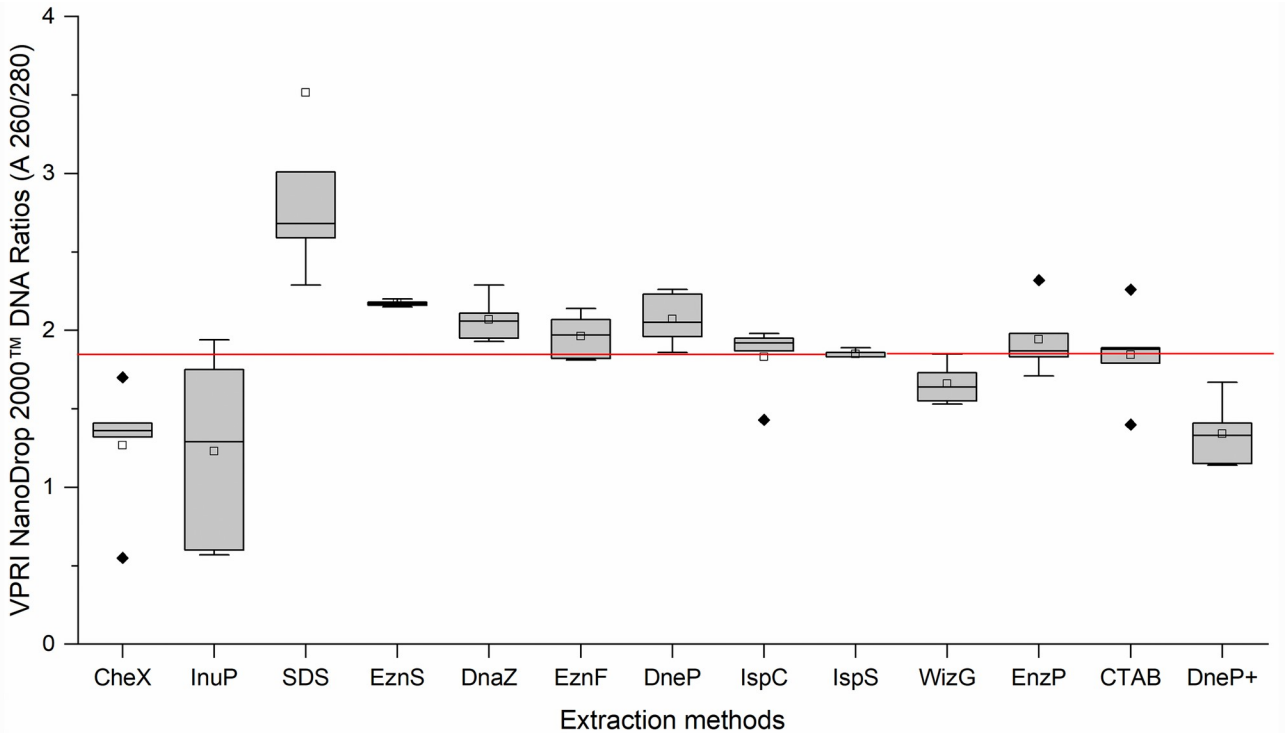

Fig 3. Boxplots of the DNA quality measured by thermo scientific nanodrop 2000™ absorbency measurement 260 nm / 280 nm ratios of five victorian plant pathology herbarium (VPRI) apple powdery mildew specimens produced by 13 DNA extraction methods. The red line indicates the desired target absorbency ratio 1.8–1.9. Median line —; Mean □; Outlier Extraction method abbreviations: Chelex® 100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

https://doi.org/10.1371/journal.pone.0232535.g003

extracts from the 13 DNA extraction protocols applied to VPRI specimen 18536. The genomic libraries were quantified by Promega Quantus™ fluorometer and Agilent 2200 TapeStation® and submitted for Illumina® HiSeq 3000 sequencing, except for DneP+ Illumina Nextera XT® and NuGen Ovation® ultralow System V2 libraries which were sequenced using

Table 3. Published primer sets tested on all DNA extracted from five VPRI *P. leucotricha* specimens from 1992–1994 (total = 65 reactions per extraction method), and the percentage of *P. leucotricha* amplicons generated per primer set.

PRIMER	TARGET GENE REGION	EXPECTED AMPLICON SIZE (BP)	REFERENCE	SUCCESSFUL AMPLICON %
ITS1F/ITS2	ITS 1	230	White et al. 1990 [29]	1.5%
PMITS1F/PMITS2	ITS 1, 5.8S, ITS 2	700	Cunnington et al. 2003 [30]	6.2%
PM5F/PM6	ITS 1, 5.8S, ITS 2	400	Takamatsu and Kano 2001 [31]	10.8%
PMITS1F/ITS4	ITS 1, 5.8S, ITS 2	600	White et al. 1990 [29]	56.9%
NESTED PMITS1/2 PMITS1/ITS4	ITS 1, 5.8S, ITS 2	550	Cunnington, Lawrie and Pascoe 2004 [32]	69.2%
PMBT1AF/BTMYCR	β-tubulin	400	Feau et al. 2011 [33]	55.4%
CHS79F/CHS354	Chitin Synthase	300	Carbone and Kohn 1999 [34]	60.0%
MCM7AF/MCM7A	Mini chromosome Maintenance Complex Component 7	550	Ellingham, David and Culham 2019 [35]	0.0%
MCM7SEQF/MCM7SEQ	Mini chromosome Maintenance Complex Component 7	550	Ellingham, David and Culham 2019 [35]	0.0%

https://doi.org/10.1371/journal.pone.0232535.t003

Table 4. Nested PMITS1/PMITS2 and PMITS1/ITS4 PCR results for VPRI apple powdery mildew *P. leucotricha* specimens.

VPRI #	18381	18536	18575	19785	19947
CheX	*Golovinomyces*	*P. leucotricha*		*Golovinomyces*	Undetermined fungi
InuP	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*Erysiphe*	Undetermined fungi
SDS	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*		
EznS	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*Malus* ITS
DnaZ	*Podosphaera*	*Golovinomyces*	*Podosphaera*	*P. leucotricha*	Undetermined fungi
EznF	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	Malus ITS
DneP	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	
IspC	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*Malus* ITS
IspS	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	
WizG	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*Malus* ITS
EznP	*P. leucotricha*	*P. leucotricha*			*P. leucotricha*
CTAB	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*Podosphaera*	
DneP+	*P. leucotricha*	*P. leucotricha*	*P. leucotricha*	*Podosphaera*	*P. leucotricha*

Light grey: *P. leucotricha* (matched to GenBank Accession no. KY661076.1), Dark Grey: other powdery mildew, White: *Malus*, shaded: undetermined fungi and Black: failed amplification. Extraction method abbreviations: Chelex® 100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

https://doi.org/10.1371/journal.pone.0232535.t004

Illumina® MiSeq V3 due to the Illumina® HiSeq 3000 being unavailable. Gydle programs were used for sequence read processing (https://www.gydle.com/). *P. leucotricha* VPRI sequences were filtered for quality using nuclear filter with a minimum score of 20, minimum length was set at 50 bp, and total length of 100 bp. Mapping to reference sequences was performed by nuclear search with sequence length set at 100, sensitivity set at 25, kmer 13 and mismatches set at 0. Six reference scaffolds were used for sequence read mapping: *P. leucotricha* ITS (GenBank accession number KX842350.1), *P. leucotricha* mitochondria and rRNA (S3 File) and host DNA *Malus* chloroplast (GenBank accession number KU851961) and *Malus* mitochondria (GenBank accession number FR714868.1). The mapped reads were used for creating viewable gym files using Gydle Gym-build. These files were visualised in Vision 2.6.24 (Gydle, Canada). Raw and QC read numbers were taken from the nuclear results before and after trimming. The mapped read numbers were obtained from the gym files displayed in the Vision program. Total read number of mapped sequences reads and mapped read percentages to total QC reads are presented in Tables 5 and 6.

There was a difference in the numbers of raw and quality-controlled (QC) sequence reads generated by each library kit. The Illumina Nextera XT® libraries highest QC reads were from the extraction protocol InuP (5,266,236) followed by EznP (5,063,754) and EznF (4,799,780) (Table 5), whereas the NuGen Ovation® Ultralow System V2 libraries from the extraction protocol EznF generated the highest number of QC reads (34,654,454) followed by WizG (6,936,414) and InuP (5,828,307) (Table 6). The percentage of reads aligned to *P. leucotricha* gene regions were $< 1\%$ in most cases for both Illumina Nextera XT® and NuGen Ovation® Ultralow System V2. These percentages taken from the total QC sequence reads are comparable with the number of total QC reads relative to the percentage of aligned ITS sequences and show that NuGen Ovation® Ultralow System V2 libraries provided a higher percentage of *P. leucotricha* sequence reads.

The mapping results of the two library kits highlighted that NuGen Ovation® Ultralow System V2 libraries performed better than Illumina Nextera XT® libraries with higher numbers

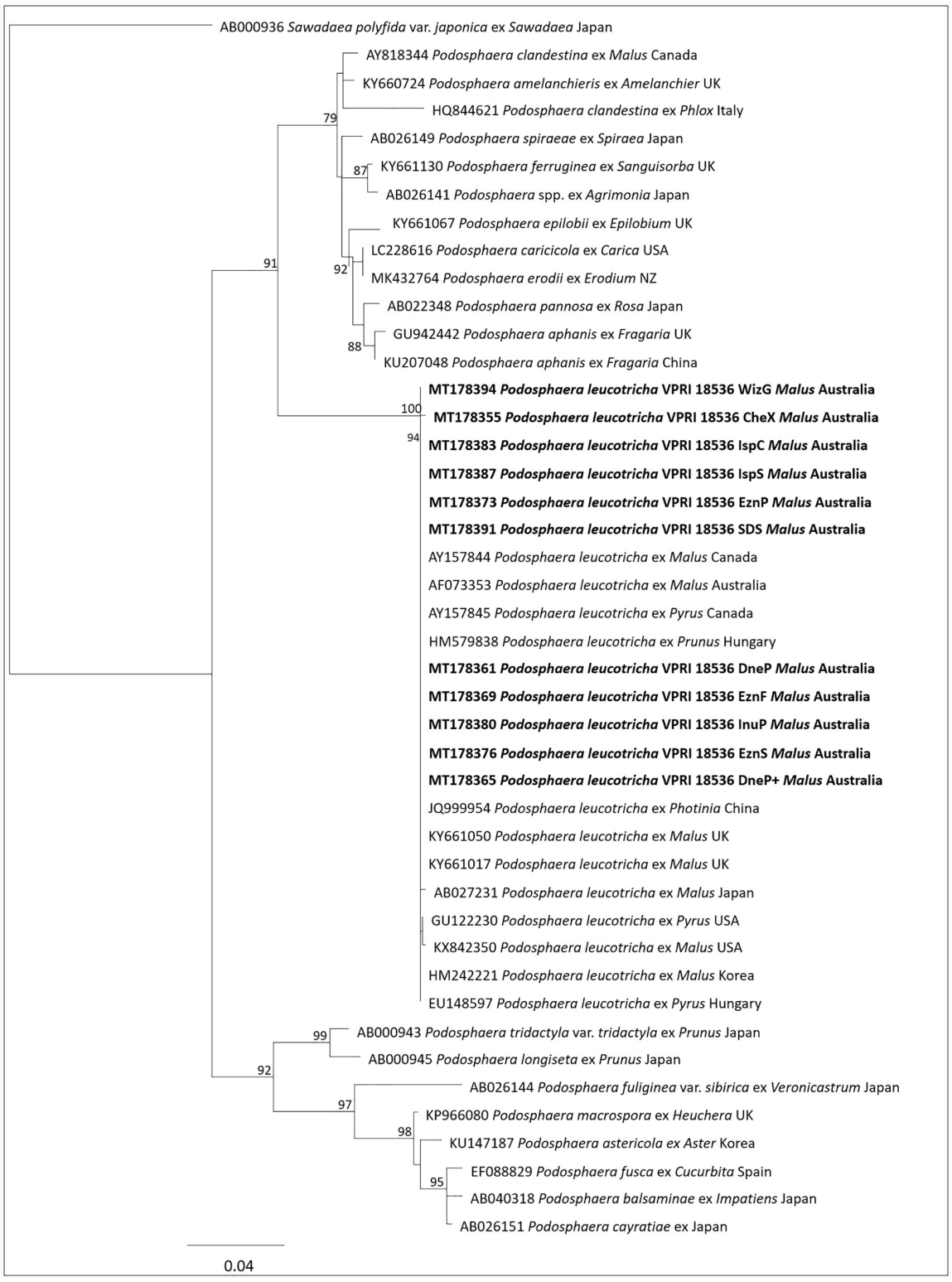

Fig 4. A Maximum Likelihood (ML) phylogenetic tree of the *podosphaera leucotricha* combined dataset of VPRI 18536 *podosphaera leucotricha* nested ITS PCR amplicons (bold) and other *podosphaera* species ITS sequence data taken from GenBank. This tree was generated using rDNA ITS1-5.8S-ITS2 sequences in PhyML with the GTR substitution model showing the relationship between VPRI *P. leucotricha* and GenBank *P. leucotricha* accession sequences. Bootstrap (BS) values of >70% were taken from 1000 replications and are shown on the respective branches and the scale bar equals 5 changes per 100 bases.

https://doi.org/10.1371/journal.pone.0232535.g004

Table 5. Illumina Nextera XT® VPRI *P. leucotricha* sequencing alignment results taken from vision alignment.

	Reads Raw	Reads Passed QC	PM ITS Raw	PM ITS	PM Mito1 Raw	PM Mito1	PM Mito2 Raw	PM Mito2	PM rRNA Raw	PM rRNA	*Malus* Mito Raw	*Malus* Mito	*Malus* Chloro Raw	*Malus* Chloro
CheX	3,301,690	2,125,386	142	0.01%	2,395	0.11%	1,603	0.08%	3,382	0.16%	1,915	0.09%	2,956	0.14%
InuP	7,172,861	5,266,236	837	0.02%	13,406	0.26%	10,011	0.19%	5,696	0.11%	4,223	0.08%	5,210	0.10%
SDS	6,913,188	3,950,749	477	0.01%	7,518	0.19%	5,551	0.14%	1,805	0.05%	5,479	0.14%	6,237	0.16%
EznS	919,376	746,040	80	0.01%	1,544	0.21%	1,120	0.15%	806	0.11%	59,470	7.97%	164,254	22.02%
DnaZ	6,236,108	2,948,446	21	0.00%	457	0.02%	304	0.01%	72	0.00%	3,060	0.10%	10,743	0.36%
EznF	5,911,120	4,799,780	408	0.01%	1,773	0.04%	1,353	0.03%	1,849	0.04%	20,072	0.42%	24,437	0.51%
DneP	5,999,284	3,837,027	88	0.00%	403	0.01%	350	0.01%	494	0.01%	7,692	0.20%	10,775	0.28%
IspC	241,089	195,337	99	0.05%	635	0.33%	412	0.21%	1,013	0.52%	23,867	12.22%	29,683	15.20%
IspS	140,095	110,579	16	0.01%	87	0.08%	71	0.06%	334	0.30%	5,711	5.17%	5,737	5.19%
WizG	4,517,829	3,631,938	72	0.00%	161	0.00%	120	0.00%	1,657	0.05%	14,064	0.39%	25,543	0.70%
EznP	6,256,598	5,063,754	142	0.00%	481	0.01%	360	0.01%	1,494	0.03%	35,642	0.70%	67,163	1.33%
CTAB	6,357,620	3,373,175	46	0.00%	692	0.02%	469	0.01%	1,230	0.04%	8,304	0.25%	5,959	0.18%
DneP +	640,221	572,874	9	0.00%	140	0.02%	75	0.01%	66	0.01%	1,337	0.23%	2,376	0.41%

Total raw reads, QC reads and mapped raw reads and percentage of aligned sequence reads to reference genes: *P. leucotricha* ITS (PM ITS), *P. leucotricha* mitochondria 1 and 2 scaffolds (PM Mito 1/2), *P. leucotricha* rRNA scaffold (PM rRNA)s, *Malus* mitochondria and *Malus* chloroplast genomes. Extraction method abbreviations: Chelex®100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

https://doi.org/10.1371/journal.pone.0232535.t005

Table 6. NuGen Ovation® Ultralow system V2 VPRI *P. leucotricha* sequencing alignment results taken from vision alignment.

	Reads Raw	Reads Passed QC	PM ITS Raw	PM ITS	PM Mito1 Raw	PM Mito1	PM Mito2 Raw	PM Mito2	PM rRNA Raw	PM rRNA	*Malus* Mito Raw	*Malus* Mito	*Malus* Chloro Raw	*Malus* Chloro
CheX	6,269,905	5,725,643	26	0.00%	2,038	0.04%	1,352	0.02%	856	0.02%	247	0.00%	487	0.01%
InuP	6,333,104	5,828,307	1471	0.03%	112,097	1.92%	79,256	1.36%	14,398	0.25%	10,338	0.18%	24,331	0.42%
SDS	2,011,540	1,817,078	741	0.04%	43,443	2.39%	30,016	1.65%	6,494	0.36%	7,275	0.40%	13,892	0.77%
EznS	4,782,426	4,283,523	80	0.00%	1,544	0.04%	1,120	0.03%	806	0.02%	59,470	1.39%	164,254	3.84%
DnaZ	2,587,961	2,327,882	95	0.00%	6,200	0.27%	4,067	0.18%	217	0.01%	7,911	0.34%	66,157	2.84%
EznF	38,206,866	34,654,454	3291	0.01%	90,080	0.26%	63,198	0.18%	15,598	0.05%	268,897	0.78%	543,306	1.57%
DneP	6,292,079	5,726,089	211	0.00%	5,821	0.10%	3,959	0.07%	1,086	0.02%	24,778	0.43%	62,759	1.10%
IspC	3,562,523	3,137,760	99	0.00%	635	0.02%	412	0.01%	1,013	0.03%	23,867	0.76%	29,683	0.75%
IspS	846,555	758,044	16	0.00%	87	0.01%	71	0.01%	334	0.04%	5,711	0.75%	5,737	0.76%
WizG	7,642,944	6,936,414	233	0.00%	2,523	0.04%	1,811	0.03%	3,818	0.06%	56,928	0.82%	142,744	2.06%
EznP	3,352,226	2,795,537	45	0.00%	552	0.02%	454	0.02%	185	0.01%	13,004	0.47%	38,916	1.39%
CTAB	2,446,075	2,211,347	101	0.01%	4,613	0.21%	3,254	0.15%	3,363	0.15%	14,643	0.66%	19,590	0.89%
DneP +	5,757,754	5,145,556	201	0.00%	4,499	0.09%	3,255	0.06%	1,054	0.02%	22,404	0.44%	46,878	0.91%

Total raw reads, QC reads and mapped raw and percentage of aligned sequence reads to reference genes: *P. leucotricha* ITS (PM ITS), *P. leucotricha* mitochondria 1 and 2 scaffolds (PM Mito 1/2), *P. leucotricha* rRNA scaffold (PM rRNA), *Malus* mitochondria and *Malus* chloroplast genomes. Extraction method abbreviations: Chelex®100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

https://doi.org/10.1371/journal.pone.0232535.t006

of *P. leucotricha* reads mapping to the references. For both libraries total mapped read numbers obtained from the six reference Gym-files highlighted the nominal amount of *P. leucotricha* DNA sequences which mapped to the references compared to the total QC reads, most notably the ITS gene region (Tables 5 and 6). However, the number of ITS reads which mapped to the *P. leucotricha* ITS reference was higher in those protocols that generated the higher number of QC reads as shown in Figs 5 and 6. The Illumina Nextera XT® libraries with the highest number of mapped ITS reads were InuP (837), SDS (477) and EznF (408) (Table 5). The Illumina Nextera XT® ITS Vision image shows the overall reduced number of aligned ITS reads and reduced sequencing coverage across all DNA extraction methods, indicated by gaps in the alignment (Fig 5). The Vision image shows that InuP, SDS and EznF sequentially have the most coverage of the *P. leucotricha* ITS regions. This differs from the percentage of ITS reads from the total QC reads which shows that IspC has the highest mapped ITS percentage compared to the remaining 12 protocols; this is due to IspC having the second lowest QC read total (Table 5).

The NuGen Ovation® Ultralow System V2 libraries which produced the highest mapped ITS reads were extraction protocols EnzF (3291), InuP (1471) and SDS (741) (Table 6). The aligned ITS Vision image for the 13 NuGen Ovation® Ultralow System V2 libraries shows the overall increase in aligned ITS sequence reads and increased ITS gene regions coverage, indicated by the minimal gaps across the alignment (Fig 6). The NuGen Ovation® Ultralow System V2 Vision image visually highlights the superior sequencing results of EznF extraction kit compared to the 12 other protocols tested. It shows that higher total QC read numbers resulted in better coverage of the ITS region (Fig 6). The alignment image shows that EznF, InuP and

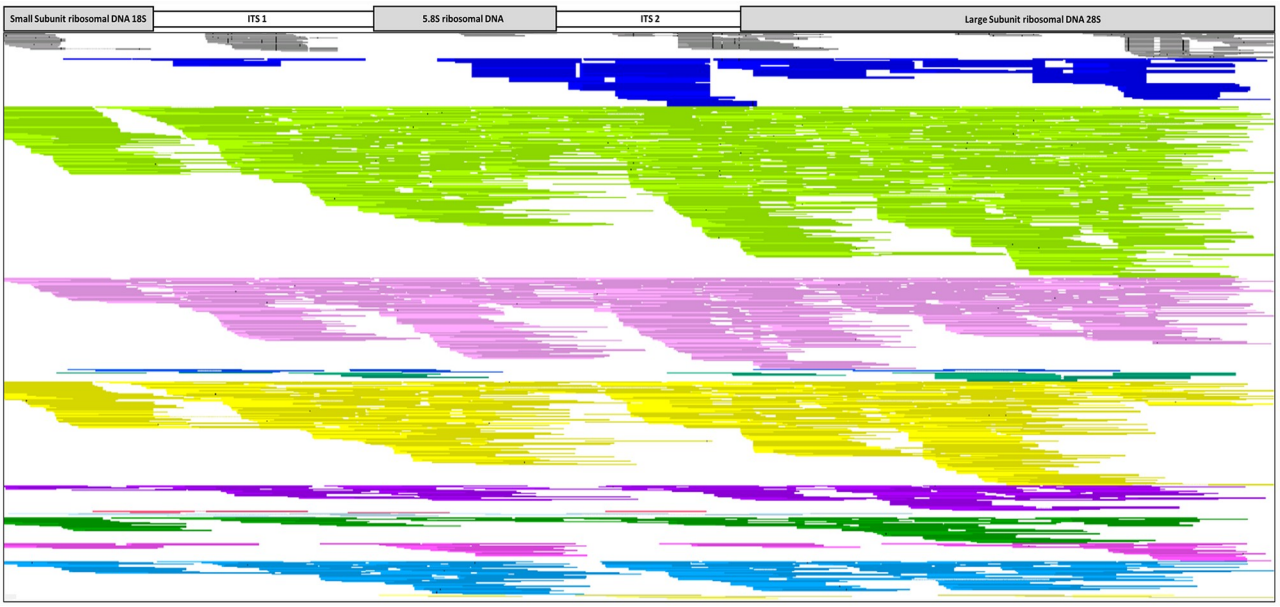

Fig 5. Vision 2.6.24 image of 13 illumina nextera XT® VPRI 18536 DNA extraction protocol libraries mapped to *P. leucotricha* ITS (GenBank accession no. KX842350.1) including *P. tridactyla* as an outgroup for comparison. Continuous unbroken lines represent sequence reads that completely align to the reference sequence. Gaps in the alignment indicates no mapping sequence reads, and SNPs between the mapped read and the reference are represented as black bars. Colour code: Grey- *P. tridactyla*, Dark Blue- CheX (Chelex®100), Light Green- InuP (innuPrep Plant DNA), Light Pink- SDS (sodium dodecyl sulphate), Blue- EznS (E.Z.N.A.® SP Plant), Green- DnaZ (DNAzol™), Yellow- EznF (E.Z.N.A.® Forensic DNA), Purple- DneP (Qiagen DNeasy® Plant), Red- IspC (Isolate II Plant DNA Lysis buffer PA1 C), Light Blue- IspS (Isolate II Plant DNA Lysis buffer PA2 S), Dark Green- WizG (Wizard® Genomic DNA Purification), Light Blue- EznP (E.Z.N.A.® Plant), Dark Pink- CTAB (Cetyl trimethyl ammonium bromide) and Light Yellow- DneP+ (Qiagen DNeasy® Plant plus PTB).

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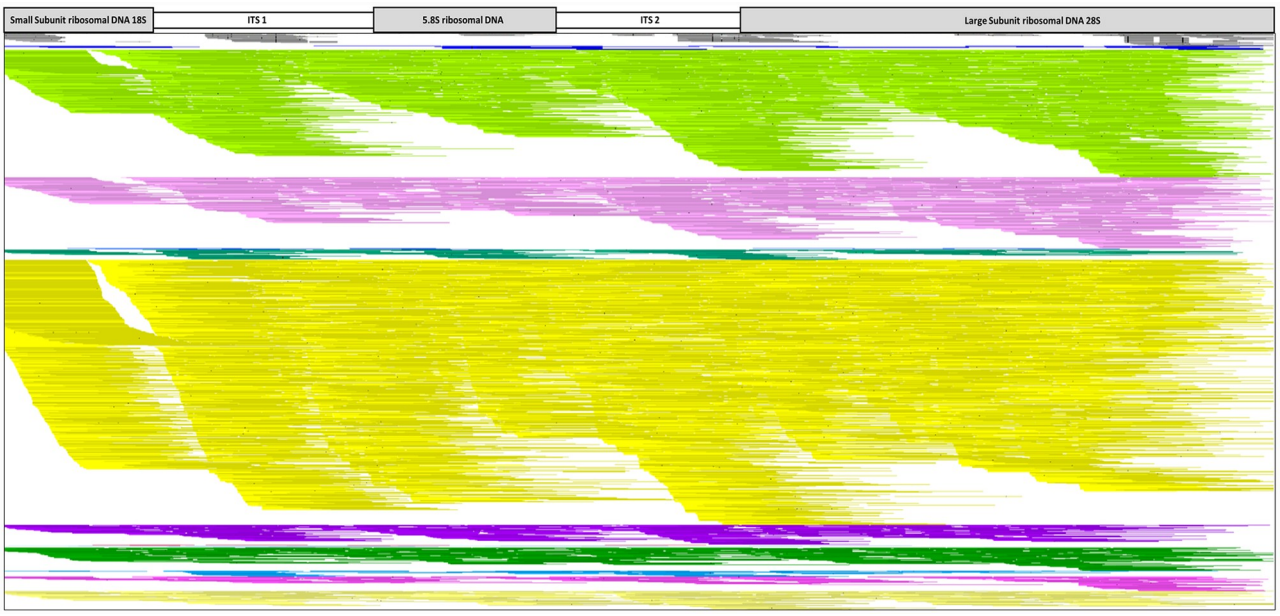

Fig 6. Vision 2.6.24 image of 13 NuGen Ovation® ultralow system V2 VPRI 18536 DNA extraction protocol libraries mapped to *P. leucotricha* ITS (GenBank accession no. KX842350.1) including *P. tridactyla* as an outgroup for comparison. Continuous unbroken lines represent sequence reads that completely align to the reference sequence. Gaps in the alignment indicates no mapping sequence reads, and SNPs between the mapped read and the reference are represented as black bars. Colour code: Grey- *P. tridactyla*, Dark Blue- CheX (Chelex® 100), Light Green- InuP (innuPrep Plant DNA), Light Pink- SDS (sodium dodecyl sulphate), Blue- EznS (E.Z.N.A.® SP Plant), Green- DnaZ (DNAzol™), Yellow- EznF (E.Z.N.A.® Forensic DNA), Purple- DneP (Qiagen DNeasy® Plant), Red- IspC (Isolate II Plant DNA Lysis buffer PA1 C), Light Blue- IspS (Isolate II Plant DNA Lysis buffer PA2 S), Dark Green- WizG (Wizard® Genomic DNA Purification), Light Blue- EznP (E.Z.N.A.® Plant), Dark Pink- CTAB (Cetyl trimethyl ammonium bromide) and Light Yellow- DneP+ (Qiagen DNeasy® Plant plus PTB).

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SDS gave the best coverage of the ITS; this differed from the percentage of aligned ITS reads, which showed SDS as the highest, followed by InuP and EznF (Table 6).

The *P. leucotricha* mitochondria and rRNA mapping for both library kits tested showed an increase in the numbers of reads which mapped to these references (Tables 5 and 6). For Illumina Nextera XT® libraries protocol InuP had the highest number of mapped reads for all three *P. leucotricha* references Mito1 (13,406), Mito2 (10,011) and rRNA (5696), closely followed by protocols SDS and EnzF (Table 5). Plant host mapping was completed using *Malus* mitochondria and chloroplast. The Illumina Nextera XT® libraries extraction protocol EznS had the highest number of mapped reads to these references with 59,470 reads aligning to the mitochondria reference and 164,254 reads aligned to the chloroplast reference (Table 5). The NuGen Ovation® Ultralow System V2 libraries mapping results showed InuP with the highest number of reads mapped to Mito1 (112,097) and Mito2 (79,256) but EznF had the greatest number of aligned reads for rRNA with 15,598 (Table 6). EznF also had the highest number of mapped reads for the plant host references *Malus* mitochondria and chloroplast with 268,897 and 543,306 respectively.

Discussion

This is the first study to systematically compare different DNA extraction methods and sequencing capabilities on powdery mildew reference collection specimens and highlights the difficulties to extract, isolate and sequence powdery mildew DNA from preserved leaf material. We found that DNA concentration was more important than DNA quality for molecular applications of DNA from powdery mildew plant pathogens as the increased DNA

concentration will provide greater chance of containing the target DNA. We also found that PCR barcoding and Sanger sequencing were not suitable for identifying preserved powdery mildew specimens due to the variability of correct fungal DNA amplification, and that NGS was more applicable for molecular analysis of preserved powdery mildew specimens. This result is not consistent with studies by Särkinen et al. [36] who compared DNA extraction methods on herbarium plant specimens and showed DNA purity was the most important factor for PCR amplification of barcode regions. The difference between the two studies is likely to be due to the different end points. Särkinen et al. [36] focused on PCR applications for herbarium plant DNA, in which plant DNA is by far the dominant DNA type extracted from the specimens. Therefore, DNA purity improves the success rate of subsequent PCR amplification. In our study, the target powdery mildew DNA was a tiny proportion of the total DNA extracted from the specimen and therefore higher total DNA concentrations increased the chance of sequencing powdery mildew DNA using NGS.

Although the methods tested in this study successfully obtained powdery mildew DNA from the VPRI specimens, the DNA samples were very low yielding, heavily fragmented and degraded, consistent with previous reports when using herbarium specimens [15, 28, 37, 38, 39]. More selective sampling from preserved powdery mildew specimens, by reducing the amount of leaf material sampled, could improve the DNA concentration of the target fungus. None of the 13 DNA extraction methods tested provided both high quality and high concentration. Regarding concentration, this study found that the better performing protocols were commercially available kits that used silica binding columns such as InuP, DneP, IspC and IspS rather than precipitation (WizG or CTAB) or chelating based methods (CheX). This result suggests an increased retention of fragmented DNA in column-based DNA extractions over precipitation methods. For the recovery of powdery mildew DNA from a mixed preserved DNA sample the EznF, EznP and EznS kits produced more DNA from VPRI specimens compared with the other silica binding kits. Previous studies on herbarium DNA extractions highlighted CTAB or commercially available kits such as Qiagen DNeasy® Plant to be reliable for herbarium DNA extractions [15]. However, this study has shown that for older preserved powdery mildew specimens, these methods did not produce comparative concentrations of fungal DNA compared to the EznF, EznP and EznS extraction kits.

DNA quality from preserved plant and fungal specimens is often compromised due to contaminants such as plant-based PCR inhibitors and microflora present on the specimen at time of collection [40], which can confound PCR amplicon sequencing [36, 15]. The IspS extraction method produced the best quality DNA, although it produced one of the lowest DNA concentrations. Several protocols produced less than optimal DNA quality (IspC, EznF, EznP, CTAB and EznS), and it was decided that further DNA cleaning steps during extraction to improve DNA quality would be detrimental, based on the low DNA sample concentrations that were obtained from the VPRI specimens. Further cleaning would potentially reduce the DNA concentration below that required for NGS library preparation, given most of the extraction protocols yielded < 1 ng/μL of DNA [28, 41].

Most herbarium phylogenetic studies to date have relied on the analysis of PCR products for species identification but a major factor that strongly influences preserved specimen PCR success is target amplicon size. The application of PCR-based approaches for phylogenetic studies using aDNA is problematic; aDNA can be highly fragmented and there are few small loci (less than 500 bp) which are phylogenetically informative that can be used [36, 42]. This study tested nine published primer sets for potential gene regions, that could be utilised as barcodes for powdery mildew molecular species identification [29, 30, 31, 32, 33, 34, 35]. These primer sets proved to be unsuitable in most cases as the target gene regions are too long in length (greater than 550 bp) for the amplification of the fragmented aDNA. The tested primers

also showed inconsistent amplification across the five VPRI samples. The poor PCR results in the present study highlight the difficult nature of working with preserved plant pathogen specimens.

For this study we found that the nested PCR primers PMITS1/PMITS2 and PMITS1/ITS4 provided the most consistent amplification results for VPRI powdery mildew DNA. Currently for the construction of powdery mildew phylogenies, ITS is the most commonly used gene region, although it does not always provide adequate resolution between closely related species [43]. However, for *P. leucotricha* ITS was sufficient to demonstrate that the VPRI 18536 specimens were correctly identified (Fig 4). Molecular sequence data produced for species identification must be specific and reliable for accurate identifications, but many of the common fungal ITS primers are hindered by multiple types of biases, such as length bias, taxonomic bias and primer mismatch bias [44]. Together with fungal primer bias low DNA concentrations and variable DNA quality from fungarium DNA reduces PCR capabilities for molecular identification.

An alternative method to overcome the limitations of PCR approaches with preserved fungal specimens is to use a sequencing platform that is designed for fragmented DNA [42]. Whole genome NGS requires DNA strand lengths less than 500 bp and it was hypothesised that NGS would be suitable for fungarium DNA, which is already naturally fragmented. However, library preparation kits developed for fresh DNA have a fragmentation step incorporated into the protocol to create uniform DNA fragments. In this study, we compared two different library preparation kits to investigate whether DNA from preserved specimens would generate better sequence data using a kit specifically designed for low quality and fragmented DNA (NuGen Ovation® Ultralow System V2) over a kit for fresh DNA (Illumina Nextera XT®).

Analysis of the sequencing data for VPRI *P. leucotricha* DNA showed the NuGen Ovation® Ultralow System V2 kit outperformed Illumina Nextera XT® in library concentration, read quality and generation of reads that aligned to *P. leucotricha* reference sequences. The results demonstrated the ability to generate sequence data from unrepaired aDNA of VPRI *P. leucotricha* that could be confidently aligned to *P. leucotricha* reference scaffolds. However, a greater depth of sequencing is required to generate whole genome phylogenetic data.

When comparing the library kits, the NuGen Ovation® Ultralow System V2 outperformed the Illumina Nextera XT® consistently in both raw and QC reads except for SDS, DnaZ, EznP and CTAB, which yielded higher Illumina Nextera XT® raw and QC reads (Tables 5 and 6). Illumina Nextera XT® requires excellent quality DNA for library preparation whereas NuGen Ovation® Ultralow System V2 has been tailored for degraded and poorer quality DNA, resulting in higher library efficiency [45]. Illumina Nextera XT® has a tagmentation step to fragment the DNA and attach adapters to the DNA fragments, and aDNA which is already fragmented could pose issues during adapter and index reactions when dealing with DNA of varying lengths [46]. In comparison NuGen Ovation® Ultralow System V2 library preparation uses targeted sonication to fragment the DNA sample prior to processing resulting in a higher percentage of equally fragmented DNA strands. Nascimento et al. [45] systematically compared four library preparations including Illumina Nextera XT® and NuGen Ovation® Ultralow System V2 and found the latter outperformed in terms of library sample concentration, library fragment length (ca. 300–500 bp), good quality sequences and produced the best assemblies from the sequence data.

From this study, we conclude that the EznF DNA extraction method (based on DNA concentration, quality, PCR and sequencing performance), together with the NuGen Ovation® Ultralow System V2 library kit gave the best results for use on preserved specimens of powdery mildew, as shown by the Vision alignment image (Fig 6). DNA concentration and selection of the appropriate library preparation kit were the major contributors to successful aDNA

sequencing. Higher starting amounts of aDNA requires less amplification during library preparation and results in improved DNA library complexity, as amplification can preferentially select and amplify a portion of DNA present therefore losing genetic diversity within the library [47]. This is especially important when working with an epiphytic, biotrophic fungus such as powdery mildew which constitutes only a small proportion of the extracted DNA.

In summary, our key findings when working with plant pathogenic fungi from reference collections include: (1) selective sampling from the specimens to maximise the target fungus and minimise the contribution of other phylloplane microphylla and host DNA; (2) PCR amplification success was limited due to the fragmentation of fungarium DNA and whole genome NGS overcame this limitation; (3) DNA concentration was more important than DNA quality for whole genome NGS purposes; (4) a library preparation kit designed for degraded and fragmented DNA outperformed a standard use kit to generate fungarium sequence data.

Methods

Sampling

Five 25-year-old *Podosphaera leucotricha* reference collection specimens were sampled from the Victorian Plant Pathology Herbarium, Agriculture Victoria (Bundoora, Victoria, Australia). Specimens sampled were VPRI 18536 (collected 1992), VPRI 19785 (1994), VPRI 18575 (1992), VPRI 19947 (1994) and VPRI 18381 (1992). For standardisation of starting material, a 6 mm leaf punch was selected to cut sections of infected leaf material to be used in the DNA extraction protocol study. Powdery mildew conidia and mycelia were collected from leaves and stems by using a 6 mm leaf punch; specimen VPRI 19785 included chasmothecia.

DNA extraction

Thirteen DNA extraction protocols were selected to cover the main DNA extraction methods such as chelating, silica binding and precipitation outlined in Table 7. Commercial DNA extraction kits manufacturer's instructions and DNA extractions protocols from published sources were followed as per instructed, full methods outlined in S1 File.

DNA was processed from VPRI powdery mildew infected plant material placed in 2 mL Eppendorf tubes containing a metal bead and was homogenized on Tissuelyser II (Qiagen) for

Table 7. DNA extraction protocols tested on five VPRI apple powdery mildew *P. leucotricha* specimens in this study.

Method or kit name	Protocol Code	Reference or supplier (catalogue no.)	Extraction Method
Chelex® 100	CheX	Hirata & Takamatsu 1996 [48]	Chelating
innuPREP Plant DNA	InuP	Telle and Thines 2008 [15] (Analytik-jena 845-KS-10600)	Silica binding
SDS	SDS	Edwards, Johnstone and Thompson 1991 [49], Pintye et al., 2012 [50]	Precipitation
E.Z.N.A.® SP Plant	EznS	Omega Bio-tek (D5511-00)	Silica binding
DNAzol™ with MinElute® PCR Purification kit	DnaZ	Richards et al. 2019 [51]	Precipitation + Silica Binding
E.Z.N.A.® Forensic DNA	EznF	Telle and Thines 2008 (D3591-00) [15]	Silica binding
Qiagen DNeasy® Plant	DneP	Telle and Thines 2008 (69104) [15]	Silica binding
Isolate II Plant DNA Lysis buffer PA1 C	IspC	Bioline (BIO-52070)	Silica binding
Isolate II Plant DNA Lysis Buffer PA2 S	IspS	Bioline (BIO-52070)	Silica binding
Wizard® Genomic DNA Purification	WizG	Promega (A1120)	Precipitation
E.Z.N.A.® Plant	EznP	Telle and Thines 2008 [15] (Omega Bio-tek D3485-00)	Silica binding
CTAB	CTAB	Särkinen et al., 2012 [36]	Precipitation
Qiagen DNeasy® Plant plus PTB	DneP+	Lister et al., 2008 [52]	Silica binding

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two rounds of 30 seconds at 30 Hz or until all plant material was broken down. For all protocols, a Ribonuclease A (RNase A) treatment was included to remove RNA during processing. DNA was eluted in sterile water or the elution buffer provided by the commercial kits. NanoDrop 2000™ was used to assess DNA quality using the 260/280 nm absorbency ratio (1.8–1.9). DNA concentrations were quantified using two methods: Invitrogen Qubit™ fluorometer and Agilent Tapestation® electrophoresis.

PCR amplification and sanger sequencing

PCR amplification and Sanger sequencing were used to confirm the presence of *P. leucotricha* in DNA samples from the thirteen different extraction methods. A powdery mildew specific nested PCR was used spanning the ITS1, 5.8S and ITS2 (Fig 7) [27]. Primers used were PMITS1 (`5'-TCG GAC TGG CCY AGG GAG A-3'`)/ PMITS2 (`5'-TCA CTC GCC GTT ACT GAG GT-3'`). The initial PMITS1 and PMITS2 PCR was performed in 20 μL reactions using the Dreamtaq 2x master mix, 500 nM forward and reverse primers, DSMO 5%, 5 μL dH_2O and 2 μL DNA template. Thermal cycling conditions included an initial denaturing at 94˚C for 10 minutes, followed by 35 cycles of denaturation at 94˚C for one minute, annealing at 65˚C for one minute and extension at 72˚C for one minute; final extension at 72˚C for 10 minutes. PCR products were confirmed on 2% agarose gel. DNA extracted from fresh *Podosphaera tridactyla* (GenBank accession MT309052) and *Podosphaera* xanthii (Genbank Accession MT309053) using the SDS method were used as positive controls for each PCR round as no fresh *Podosphaera leucotricha* was available at the time.

The nested PCR PMITS1 and ITS4 (`5'-TCC TCC GCT TAT TGA TAT GC-3'`) reaction mix was set up as previously mentioned for ITS 1 and ITS 2 except the primer concentration was increased to 1000 nM and included 1 μL of the first round PCR product as the DNA template. Thermal cycling conditions for the nested PCR were the same as the first round except the annealing temperature was lowered to 60˚C. PCR products were confirmed on 2% agarose gel.

Successful nested PCR products were sent to Macrogen (Seoul, Korea) for Sanger sequencing. All VPRI *Podosphaera leucotricha* ITS sequences generated in this study were accessioned to GenBank (Table 8).

Phylogenetic analysis

ITS sequences from *P. leucotricha* VPRI 18536 from the 13 different extraction methods were aligned with sequences of *P. leucotricha* and *Podosphaera* species obtained from GenBank on the basis of the phylogeny published by Takamatsu, Hirata and Sato [53]. Extra *Podosphaera* species sequences were obtained using BLASTn. Initial alignment used the MUSCLE 3.8.425 package [54]. The alignment was visually refined and trimmed using Geneious 11.1.4 [55]. A

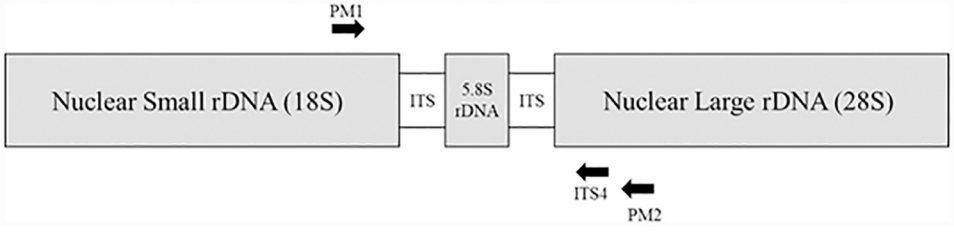

Fig 7. Primer map indicating nested PMITS1/PMITS2 and PMITS1/ITS4 amplified regions used in this study.

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Table 8. Successful nested ITS PCR *P. leucotricha* amplicons GenBank accession numbers generated in this study.

Protocol	18381	18536	18575	19785	19947
CheX	-	MT178355	-	-	-
InuP	MT178379	MT178380	MT178381	-	-
SDS	MT178390	MT178391	MT178392	-	-
EznS	MT178375	MT178376	MT178377	MT178378	-
DnaZ	-	-	-	MT178359	-
EznF	MT178368	MT178369	MT178370	MT178371	-
DneP	MT178360	MT178361	MT178362	MT178363	-
IspC	MT178382	MT178383	MT178384	MT178385	-
IspS	MT178386	MT178387	MT178388	MT178389	-
WizG	MT178393	MT178394	MT178395	MT178396	-
EznP	MT178372	MT178373	-	-	MT178374
CTAB	MT178356	MT178357	MT178358	-	-
DneP+	MT178364	MT178365	MT178366	-	MT178367

Extraction protocol abbreviations: Chelex® 100 (CheX), innuPrep Plant DNA (InuP), sodium dodecyl sulphate (SDS), E.Z.N.A.® SP Plant (EznS), DNAzol™ (DnaZ), E.Z.N.A.® Forensic DNA (EznF), Qiagen DNeasy® Plant (DneP), Isolate II Plant DNA Lysis buffer PA1 C (IspC), Isolate II Plant DNA Lysis buffer PA2 S (IspS), Wizard® Genomic DNA Purification (WizG), E.Z.N.A.® Plant (EznP), Cetyl trimethyl ammonium bromide (CTAB) and Qiagen DNeasy® Plant plus PTB (DneP+).

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maximum likelihood tree was generated from the aligned sequences using PhyML 3.3.20180621 [56] using the Hasegawa, Kishino and Yano 1985 evolutionary model with fixed proportion of invariable sites 0, number of substation rate 4 and estimated Gamma distribution parameter. Branch support was calculated with 1000 bootstrap replicates. *Sawadaea polyfida* var. *japonica* was chosen as the outgroup following the phylogeny published by Takamatsu, Hirata and Sato [49].

Powdery mildew fungarium specimens next generation sequencing

VPRI specimen 18536 was used as a DNA representative from each of the 13 DNA extraction protocols, in a comparison study of two library preparation kits, Illumina Nextera XT® (New England Biolabs) and NuGen Ovation® Ultralow System V2 (NuGen).

Illumina Nextera XT® double indexed and NuGen Ovation® single indexed sequencing library preparations were completed for 13 VPRI 18536 DNA samples as per manufacturer's instructions (S1 File). No DNA repair was performed on the fungarium DNA samples. The NuGen Ovation® Ultralow System V2 libraries DNA samples were fragmented to 350 bp by sonication using Covaris S-Series Focused ultrasonicator. Fragmentation sonication settings are shown in S2 File. DNA library concentrations were quantified using Promega Quantus™ fluorometer and Agilent 2200 TapeStation®. The finalised Illumina Nextera XT® and NuGen Ovation® Ultralow System V2 libraries were paired-end sequenced on the Illumina® HiSeq 3000 platform. Except for DneP+ Illumina Nextera XT® and NuGen Ovation® Ultralow System V2 libraries which were sequenced on Illumina® MiSeq using the reagent V3 600 cycles kit due to a changeover in sequencing platforms in our facility and Illumina® HiSeq 3000 is no longer available.

Read processing and mapping

Reads were assigned to each sample based on their indices. Gydle programs were used for sequence read processing (https://www.gydle.com/). *P. leucotricha* VPRI sequences were filtered for quality using nuclear filter with a minimum score of 20, minimum length was set at

50 bp, and length total of 100. Mapping to reference sequence was performed by nuclear search with sequence length set at 100, sensitivity set at 25, kmer 13 and mismatches set at 0. Gym-build created files of mapped VPRI sequences reads to be visualised in Vision 2.6.24 (Gydle, Canada). References used for read mapping were a *P. leucotricha* series of reference scaffolds, which included *P. leucotricha* ITS (GenBank accession no. KX842350.1), *P. leucotricha* mitochondria and rRNA (generated using fresh *P. leucotricha* DNA, S3 File) and host DNA *Malus* chloroplast (GenBank Accession no. KU851961) and *Malus* mitochondria (GenBank Accession no. FR714868.1). Raw and QC read numbers were taken from total sequence reads before and after trimming. The mapped read numbers were displayed from the gym files by the Vision program (Figs 5 and 6). The total number of mapped sequence reads were converted to a percentage of the total QC read numbers.

Supporting information

S1 File. DNA extraction protocols and library preparation.
(DOCX)

S2 File. DNA extractions raw data.
(DOCX)

S3 File. Apple powdery mildew *podosphaera leucotricha* mitochondria genome.
(DOCX)

S4 File. Apple powdery mildew *podosphaera leucotricha* rRNA genome.
(DOCX)

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Supervision: Tom W. May, Jacqueline Edwards.

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References

1. Thomas M, Gilbert P, Bandelt H-J, Hofreiter M, Barnes I. Assessing ancient DNA studies. TRENDS in Ecology and Evolution. 2005; 20(10):541–4. https://doi.org/10.1016/j.tree.2005.07.005 PMID: 16701432
2. Pääbo S, Poinar H, Serre D, Jaenicke-Després V, Hebler J, Rohland N, et al. Genetic anaylsis from ancient DNA. Annual Review of Genetics. 2004; 38:645–79. https://doi.org/10.1146/annurev.genet.37.110801.143214 PMID: 15568989

3. Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993; 362:709–15. https://doi.org/10.1038/362709a0 PMID: 8469282
4. Staats M, Cuenca A, Richardson JE, Vrielink-van Ginkel R, Petersen G, Seberg O, et al. DNA damage in plant herbarium tissue. Plos One. 2011; 6(12):1–9.
5. Wieß CL, Schuenemann VJ, Devos J, Shirsekar G, Reiter E, Gould BA, et al. Temporal patterns of damage and decay kinetics of DNA retrieved from plant herbarium specimens. Royal Society Open Science. 2016; 3 (160239).
6. Hart ML, Forrest LL, Nicholls JA, Kidner CA. Retrieval of hundreds of nuclear loci from herbarium specimens. Taxon. 2016; 65(5):1081–92.
7. Zeng C-X, Hollingsworth PM, Yang J, He Z-S, Zhang Z-R, Li D-Z, et al. Genome skimming herbarium specimens for DNA barcoding and phylogenetics. Plant Methods. 2018; 14(43).
8. O'Rourke DH, Hayes MG, Carlyle SW. Ancient DNA studies in physical anthropology. Annual Review of Anthropology. 2000; 29:217–42.
9. Taylor JW, Swann EC. DNA from herbarium specimens. In: Herrmann B, Hummel S, editors. Ancient DNA: Recovery and analysis of genetic material from paleontological, archaeological, museum, medical, and forensic specimens. 1st ed. New York: Springer-Verlag New York Inc; 1994. p. 165–81.
10. Ristaino JB. The importance of archival and herbarium materials in understanding the role of oospores in Late Blight epidemics of the past. Phytopathology. 1998; 88:1120–30. https://doi.org/10.1094/PHYTO.1998.88.11.1120 PMID: 18944844
11. Yoshida K, Schuenemann VJ, Cano LM, Pais M, Mishra B, Sharma R, et al. The rise and fall of the *Phytophthora infestans* lineage that triggered the Irish potato famine. Plos eLIFE. 2013; 2(e00731).
12. Thiers BM. Index Herbarium: A global directory of public herbaria and associated staff. New York New York Botanical Garden's Virtual Herbarium; 2016.
13. Bebber DP, Carine MA, Wood JRI, Wortley AH, Harris DJ, Prance GT, et al. Herbaria are a major frontier for species discovery. PNAS. 2010; 107(51):22169–71. https://doi.org/10.1073/pnas.1011841108 PMID: 21135225
14. Sproul JS, Maddison DR. Sequencing historical specimens: successful preparation of small specimens with low amounts of degraded DNA. Molecular Ecology Resources. 2017; 17:1183–201. https://doi.org/10.1111/1755-0998.12660 PMID: 28199781
15. Telle S, Thines M. Amplification of *cox2* (~620 bp) from 2mg of Up to 129 years old herbarium specimens, comparing 19 extraction methods and 15 polymerases. Plos One 2008; 3(10):1–8.
16. Fish S. The history of plant pathology in Australia. Annual Review of Phytopathology. 1970; 8:13–36.
17. Shivas RG, Beasley DR, Pascoe IG, Cunnington JH, Pitkethley RN, Priest MJ. Specimen-based databases of Australian plant pathogens: Past, present and future. Australiasian Plant Pathology. 2006; 35:195–8.
18. Plant Health Australia 2001 [Online Database]. https://www.planthealthaustralia.com.au/.
19. Glawe DA. The Powdery Mildews: A review of the world's most familiar (yet poorly known) plant pathogens. Annual Review of Phytopathology. 2008; 46:27–51. https://doi.org/10.1146/annurev.phyto.46.081407.104740 PMID: 18680422
20. Ridout CJ. The Mycota. In: Deising HB, editor. A comprehensive treatise on fungi as experimental systems for basic and applied research. 2. Berlin Heidelberg: Springer-Verlag; 2009. p. 51–68.
21. Braun U, Cook RTA. Taxonomic manual of the Erysiphales (Powdery Mildews). Samson RA, editor. The Netherlands: CBS-KNAW Fungal Biodiversity Center; 2012.
22. Takamatsu S. Phylogeny and evolution of the powdery mildew fungi (Erysiphales, Ascomycota) inferred from nuclear ribosomal DNA sequences. Mycoscience. 2004; 45(2):147–57.
23. Takamatsu S, Inagaki M, Niinomi S, Khodaparast SA, Shin H-D, Grigaliunaite B, et al. Comprehensive molecular phylogenetic analysis and evolution of the genus *Phyllactinia* (Ascomycota: Erysiphales) and its allied genera. Mycological Research. 2008; 112(3):299–315.
24. Takamatsu S, Niinomi S, Harada M, Havrylenko M. Molecular phylogenetic analyses reveal a close evolutionary relationship between *Podosphaera* (Erysiphales: Erysiphaceae) and its rosaceous hosts. Persoonia: Molecular Phylogeny and Evolution of Fungi. 2010; 24:38.
25. Takamatsu S. Molecular phylogeny reveals phenotypic evolution of powdery mildews (Erysiphales, Ascomycota). Journal of general plant pathology. 2013; 79(4):218–26.
26. Urbanietz A, Dunemann F. Isolation, identification and molecular characterization of physiological races of apple powdery mildew (*Podosphaera leucotricha*). Plant Pathology. 2005; 54:125–33.
27. Matlock B. Assessment of nucleic acid purity. Technical Note 52646: Thermo Fisher Scientific Inc.; 2015.

28. Drábková L, Kirschner J, Vlček Č. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of *Junaceae*. Plant Molecular Biology Reporter. 2002; 20:161–75.

29. White TJ, Bruns TD, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. California, USA: Academic Press Inc.; 1990.

30. Cunnington JH, Takamatsu S, Lawrie AC, Pascoe IG. Molecular identification of anamorphic powdery mildew (Erysiphales). Australiasian Plant Pathology 2003; 32:421–8.

31. Takamatsu S, Kano Y. PCR primers useful for nucleotide sequencing of rDNA of the powdery mildew fungi. Mycoscience 2001; 42:135–9.

32. Cunnington JH, Lawrie AC, Pascoe IG. Molecular determination of anamorphic powdery mildew fungi on the *Fabaceae* in Australia. Australasian Plant Pathology. 2004; 33:281–4.

33. Feau N, Decourcelle T, Husson C, Desprez-Loustau M-L, Dutech C. Finding single copy genes out of sequences genomes for multilocus phylogenetics in non-model fungi. Plos One. 2011; 6(4):e18803. https://doi.org/10.1371/journal.pone.0018803 PMID: 21533204

34. Carbone I, Kohn LM. A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia. 1999; 91(3):553–6.

35. Ellingham O, David J, Culham A. Enhancing identification accuracy for powdery mildews using previously underexploited DNA loci. Mycologia. 2019;In Press

36. Särkinen T, Staats M, Richardson JE, Cowan RS, Bakker FT. How to open the treasure chest? Optimising DNA extraction from herbarium specimens. Plos One. 2012; 7(8).

37. Hyde KD, Chomnunti P, Crous PW, Groenewald JZ, Damm U, Ko Ko TW, et al. A case for re-inventory of Australia's plant pathogens. Persoonia. 2010; 25:50–60. https://doi.org/10.3767/003158510X548668 PMID: 21339966

38. Bruns TD, Fogel R, Taylor JW. Amplification and sequencing of DNA from fungal herbarium specimens. Mycologia. 1990; 82(2):175–84.

39. Li W, Brlansky RF, Hartung JS. Amplification of DNA of *Xanthomonas axonopodis* pv. citri from historic citrus canker herbarium specimens. Journal of Microbiological Methods. 2006; 65:237–46. https://doi.org/10.1016/j.mimet.2005.07.014 PMID: 16099061

40. Choi J, Lee H, Shipunov A. All that is gold does not glitter? Age, taxonomy, and ancient plant DNA quality. PeerJ Life & Environment. 2015; 3(e1087).

41. Kemp BM, Monroe C, Smith DG. Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 2006; 33:1680–9.

42. Burrell AS, Disotell TR, Bergey CM. The use of museum specimens with high-throughput DNA sequences. Journal of Human Evolution. 2015; 79:35–44. https://doi.org/10.1016/j.jhevol.2014.10.015 PMID: 25532801

43. Schmitt I, Crespo A, Divakar PK, Fankhauser JD, Herman-Sackett E, Kalb K, et al. New primers for promising single-copy genes in fungal phylogenetics and systematics. Persoonia. 2009; 23:35–40. https://doi.org/10.3767/003158509X470602 PMID: 20198159

44. Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. ITS as an environmental DNA barcode for fungi: an in-silico approach reveals potential PCR biases. BMC Microbiology. 2010; 10 (189).

45. Nascimento FS, Wei-Pridgeon Y, Arrowood MJ, Moss D, da Silva AJ, Talundzic E, et al. Evaluation of library preparation methods for Illumina next generation sequencing of small amounts of DNA from foodborne parasites. Journal of Microbiological Methods. 2016; 130:23–6. https://doi.org/10.1016/j.mimet.2016.08.020 PMID: 27553132

46. Gutaker RM, Burbano HA. Reinforcing plant evolutionary genomics using ancient DNA. Current Opinion in Plant Biology. 2017; 36:38–45. https://doi.org/10.1016/j.pbi.2017.01.002 PMID: 28160617

47. Head SR, Kormori HK, LaMere SA, Whisenant T, Van Nieuwerburgh F, Salomon DR, et al. Library construction for next-generation sequencing: Overviews and challenges. BioTechniques. 2014; 56:61–77. https://doi.org/10.2144/000114133 PMID: 24502796

48. Hirata T, Takamatsu S. Nucleotide sequence diversity of rDNA internal transcribed spacer extracted from conidia and cleistothecia of several powdery mildew fungi. Mycoscience. 1996; 37:283–8.

49. Edwards K, Johnstone C, Thompson C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research. 1991; 19(6):1349. https://doi.org/10.1093/nar/19.6.1349 PMID: 2030957

50. Pintye A, Bereczky Z, Kovács GM, Nagy LG, Xu X, Legler SE, et al. No indication of strict host associations in a widespread mycoparasite: Grapevine powdery mildew (*Erysiphe necator*) is attacked by

phylogenetically distant Ampelomyces strains in the field. Phytopathology. 2012 102(7):707–16. https://doi.org/10.1094/PHYTO-10-11-0270 PMID: 22512466

51. Richards SM, Hovhannisyan N, Gilliham M, Ingram J, Skadhauge B, Heiniger H, et al. Low-cost cross-taxon enrichment of mitochondrial DNA using in-house synthesised RNA probes. Plos One. 2019; 14(2: e0209499). https://doi.org/10.1371/journal.pone.0209499 PMID: 30716066

52. Lister DL, Bower MA, Howe CJ, Jones MK. Extraction and amplification of nuclear DNA from herbarium specimens of emmer wheat: A method for assessing DNA preservation by maximum amplicon length recovery. Taxon. 2008; 57(1):254–8.

53. Takamatsu S, Hirata T, Sato Y. A parasitic transition from trees to herbs occurred at least twice in tribe *Cystotheceae* (*Erysiphaceae*): evidence from nuclear ribosomal DNA. Mycological Research. 2000; 104(11):1304–11.

54. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research. 2004; 32(5).

55. Geneious. 2019 [Online] www.geneious.com.

56. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate Maximum-Likelihood phylogenies: Assessing the performance of PhyML 3.0. Systematic Biology. 2010; 59(3):307–21. https://doi.org/10.1093/sysbio/syq010 PMID: 20525638

Chapter 3

Molecular data from up to 130-years-old herbarium specimens do not support the presence of cherry powdery mildew in Australia

3.1 Chapter Preface

Cherry powdery mildew has not been reported in Australia and the cherry-infecting strain of *Podosphaera clandestina* is listed as a high priority exotic pathogen for the Australian cherry industry. Previously, *P. clandestina* was believed to have a wide host range, including both *Crataegus monogyna* Jacq. (hawthorn) and *Prunus avium* (L.) L. (cherry). Recent taxonomic revisions have determined that *P. clandestina* is a species complex and that the cherry-infecting form is a genetically different species from *P. clandestina* in the strict sense, described as *P. cerasi*. Australian plant pathogen reference collections such as the Victorian Plant Pathology Herbarium (VPRI) hold specimens of *Podosphaera clandestina* on *Crataegus* spp. from Australia and on *Prunus* from Europe and USA. Using the protocol developed in chapter 2, *P. clandestina* specimens held in Australian plant pathogen reference collections were re-examined to confirm the absence of *P. cerasi* collected from within Australia. There were several outcomes from this study: the successful Illumina sequencing of reference collection specimens up to 130 years old; computational analysis enabling extraction of *Podosphaera* sequence data from host and microflora sequence data and phylogenetic analysis demonstrating that all Australian specimens were *P. clandestina* in the strict sense on *Crataegus* hosts. Additionally, three species of *Podosphaera* (*P. cerasi*, *P. prunicola* and *P. pruni-avium*) were identified on cherry specimens from Europe and USA. It is now evident that three *Podosphaera* species are capable of infecting cherry, which is important for Australian biosecurity authorities to be aware of. This study provided validation of the protocol developed for in chapter 2 for its usefulness on old powdery mildew specimens held in reference collections.

This chapter is presented in published format.

3.2 Publication details

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3.3 Statement of contribution of joint authorship

RLS, JE, TIS and TWM conceived the idea for the study and all the authors (RLS, TIS, RCM, JK, TWM and JE) contributed to the design. RLS performed the experimental DNA extraction and sequencing, with assistance and advice from JK. RCM and TIS assisted RLS with the bioinformatics. TWM assisted and advised RLS with the phylogenetic analyses. Data analyses and interpretation were conducted by RLS with assistance and advice from all authors. RLS wrote the manuscript, which was reviewed by TKM and JE to shape the final version. All authors approved the final version of the manuscript.

3.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Smith, R.L., May, T.W., Kaur, J., Sawbridge, T., Mann, R., and Edwards, J., 2020. Molecular data from up to 130-year-old herbarium specimens do not support the presence of cherry powdery mildew in Australia.

Plant Pathology 00:1-10, DOI: 10.1111/ppa.13316' I can confirm that Reannon L. Smith made the following contributions:

- Literature review
- Development of the experimental design
- Preparation of powdery mildew specimens
- DNA extractions
- Library preparations
- Illumina sequencing
- Sequence data extraction and analysis
- Phylogenetic analysis
- Generated all figures
- Writing the manuscript, critical appraisal of content and response to reviewers"

Dr. Jacqueline Edwards

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Molecular data from up to 130-year-old herbarium specimens do not support the presence of cherry powdery mildew in Australia

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Abstract

A strain of *Podosphaera clandestina* has been highlighted as a priority pest threat to the Australian cherry industry. Australia currently has no records of powdery mildew on cherry (*Prunus avium*). *P. clandestina* is reported to cause disease on a range of Rosaceae genera including *Crataegus* and *Prunus*; in Australia, *P. clandestina* has only been recorded on *Crataegus*. A recent species revision identified *Podosphaera cerasi* on *P. avium* as a separate species from *P. clandestina*. Therefore, a revision of which powdery mildew species is present in Australia on *Crataegus* is required to inform Australian plant biosecurity. Reference collection specimens from the Victorian Plant Pathology Herbarium (VPRI) recorded as *Podosphaera* spp. collected between 1889 to 2008 on cherry and three other host plant genera from Australia and overseas were sampled for DNA extraction and next-generation sequencing (NGS). Sequence data from preserved specimens were successfully mapped to internal transcribed spacer (ITS) sequences of *P. clandestina* in the strict sense, *P. cerasi*, and *Podosphaera prunicola*, and chloroplast *matK* sequences were used to identify plant hosts. Australian specimens on *Crataegus* hosts were *P. clandestina* in the strict sense and specimens on *Prunus* from the USA were identified as *P. cerasi* and *P. prunicola*. The outcome of this study confirmed the powdery mildew on Australian *Crataegus* specimens to be *P. clandestina* and none of the cherry powdery mildews (*Podosphaera pruni-avium*, *P. cerasi*, or *P. prunicola*) are present on Australian specimens in the VPRI collection, which suggests they are not present in Australia.

KEYWORDS

cherry, *Crataegus*, *Podosphaera clandestina*, reference collections

1 | INTRODUCTION

Powdery mildews are some of the most commonly occurring obligate plant pathogens worldwide, with approximately 900 species and 19 genera infecting over 10,000 plant species including ornamental, agricultural, and horticultural plants (Braun & Cook, 2012; Kiss et al., 2020; Marmolejo et al., 2018). Horticulture in Australia was established by the immigration of European and Asian settlers who introduced horticultural plant species from Europe, Asia, and the Americas (Kiss et al., 2020; Virtue et al., 2004). The introduction of exotic plant species to a new environment also brought exotic plant pathogens, including powdery mildews (Brewer & Milgroom, 2010;

Kiss et al., 2020; Walker, 1983). Powdery mildews are relatively understudied in Australia, with fewer than 60 species recorded. There is doubt on the accuracy of some identifications, which were often originally based on host plant–pathogen associations alone (Kiss et al., 2020).

Australia is a primary producer of agricultural and horticultural products for international trade and there is a large diversity of plant biosecurity threats that could impact Australia's production and international exports (Anderson et al., 2017). The Australian plant biosecurity continuum is coordinated into three management systems: preborder exclusion; at the border surveillance (minimizes the likelihood of pest and disease entering the country); and postborder surveillance; all involving federal and state governments, plant industries, and growers. The plant biosecurity system relies on accurate records of plant pathogen status, which is largely based on specimens stored in reference collections such as the Victorian Plant Pathology Herbarium (VPRI). However, in order to provide current status of presence or absence of plant pathogens in Australia, these records need to be up to date with current taxonomy and nomenclature and have identifications based on reliable characters such as DNA sequences (Hyde et al., 2010).

In Australian horticulture, cherry (*Prunus avium*) is a high-value crop for domestic and international markets. In 2018, 15,560 t of cherries were produced with a wholesale value of A$135.1 million, of which 70% were consumed domestically as fresh cherries and cherry preserves. The Australian cherry industry exported 4,114 t of fruit valued at A$62.2 million to Asia and North America (Horticulture Innovation Australia Limited, 2019). A serious plant biosecurity threat to the Australian cherry industry is cherry powdery mildew, which to date has not been recorded on cherry in Australia (Plant Health Australia, 2017). Cherry powdery mildew affects tree foliage by reducing photosynthesis, resulting in decreased fruit production. Severe powdery mildew infection can lead to infection of the fruit, resulting in further crop losses. Cherry powdery mildew can overwinter as chasmothecia in tree bark or on the orchard floor, providing inoculum for the next season (Grove & Boal, 1991).

There are three powdery mildew species in the genus *Podosphaera* that infect cherry: *P. cerasi*, *P. prunicola*, and *P. pruniavium* (a newly named species within the *P. tridactyla* complex) (Meeboon et al., 2020; Moparthi et al., 2019). In addition, *P. clandestina* was formerly applied in a much wider sense that included powdery mildews with cherry as a host, such as by Braun (1987). Braun and Cook (2012) considered that *P. clandestina* in the broad sense represented several species based on morphological differences that were correlated with plant host genera. Consequently, they recognized *P. amelanchieris* on *Amelanchier* and *P. minor* and *P. spiraeicola* on *Spiraea*. Furthermore, they narrowed the circumscription of *P. clandestina* in the strict sense to a fungus occurring on *Crataegus* and a few other hosts in the Rosaceae distinct from a similar *Podosphaera* on *Prunus*, indicated as a novel species by the preliminary molecular analysis of Takamatsu et al. (2010). In addition to the type variety, *P. clandestina* var. *clandestina*, Braun and Cook (2012) recognized three other varieties of *P. clandestina*: var. *cydoniae* on *Cydonia* (Rosaceae) and two varieties on *Crataegus* (var. *luxurians* and var. *perlonga*) that exhibited morphological differences; but they treated most reports from *Crataegus* under *P. clandestina* var. *clandestina*. In addition, Braun and Cook (2012) described *P. prunicola* for a powdery mildew from *Prunus melanocarpa*, that also parasitizes *Prunus virginiana*, *Prunus serotina*, and *P. avium* (Pandey et al., 2018). Based on morphological and phylogenetic analysis, Moparthi et al. (2019) recently described *P. cerasi* on *P. avium* as a separate species, distinguished from *P. clandestina* in the strict sense on *Crataegus* and *P. prunicola*. According to Braun and Cook (2012), original material of *Erysiphe oxyacanthae* was used to lectotypify *Alphitomorpha clandestina*, which means that *Podosphaera oxyacanthae* is an obligate synonym of *P. clandestina*.

Plant Health Australia have highlighted *P. clandestina* var. *clandestina* causing powdery mildew of cherry as a high priority pest threat to the Australian cherry industry (Plant Health Australia, 2017). However, to date, powdery mildews recorded as *P. clandestina* from Australia have not been verified through sequencing. Furthermore, listing of *P. clandestina* as the cause of powdery mildew of cherry, even as "var. *clandestina*" could well be based on the older taxonomy, when *P. clandestina* was used in a much broader sense. A recent study by Smith et al. (2020) established the effectiveness of DNA extraction, next-generation sequencing (NGS), and bioinformatic analysis to successfully generate sequence data from preserved powdery mildew specimens from plant pathogen reference collections.

Therefore, the purpose of this study was to use NGS to reexamine powdery mildew specimens from Australia and from overseas held in Australian plant pathogen reference collections that have been identified as *Podosphaera* spp. on cherry (*P. avium* or *Prunus cerasus*), *Amelanchier*, *Crataegus*, and *Spiraea*, all of which are genera of Rosaceae, in order to resolve the species present in Australia.

2 | MATERIALS AND METHODS

A request for powdery mildew specimens identified as *P. clandestina* in the broad sense on plant hosts identified as cherry (*P. avium* and *P. cerasus*) or on the genera *Amelanchier*, *Crataegus*, and *Spiraea* was sent to the three major Australian plant pathogen herbaria (Queensland Plant Pathogen Herbarium [BRIP], New South Wales Plant Pathology Biosecurity Collections [DAR], and Victorian Plant Pathogen Herbarium [VPRI]). VPRI was the only collection with any, and held 32 specimens, collected from Armenia, Australia, Finland, Germany, Italy, Japan, Korea, Switzerland, the UK, and the USA.

Each VPRI specimen was inspected for DNA extraction suitability based on number of leaves in the specimen packet, level of powdery mildew infection present on the leaves, and if the specimens were glued to mounting paper. Using these criteria, 19 were suitable for DNA extraction (Table 1). Among these, VPRI 32542 was a piece of spore trap filter paper collected from a *P. avium* orchard in Washington, USA.

TABLE 1 Collection details for the 19 specimens of *Podosphaera* used in this study with fungus and plant host species as listed on the specimen, and GenBank accession numbers for the internal transcribed sequence (ITS) data generated in this study

VPRI accession	Fungus name as listed	Collection date	Country	Plant host as listed	ITS GenBank accession
6225	*P. oxyacanthae*	1889	USA	*Prunus cerasus*	MT804403
6221	*P. oxyacanthae*	1890	USA	*P. cerasus*	MT804400
6222	*P. oxyacanthae*	1890	USA	*Crataegus spathulata*	—
40176	*P. oxyacanthae*	1904	USA	*P. cerasus*	MT804399
40177	*P. oxyacanthae*	1904	USA	*Prunus avium*	MT804404
6224	*P. oxyacanthae*	1904	USA	*Crataegus* sp.	MT804401
6223	*P. oxyacanthae*	1908	USA	*Spiraea salicifolia*	MT804396
20388	*P. clandestina*	1976	Finland	*C. sanguinea*	MT804395
19079	*P. clandestina*	1993	Australia	*Crataegus* sp.	MT804388
19634	*P. clandestina*	1993	Australia	*Crataegus* sp.	MT804390
19170	*P. curvispora*	1993	Australia	*Crataegus oxyacantha*	MT804389
18863	*P. clandestina*	1993	Australia	*C. oxyacantha*	MT804387
19938	*P. clandestina*	1994	Australia	*Crataegus* sp.	MT804391
19984	*P. clandestina*	1994	Australia	*Crataegus* sp.	MT804392
19985	*P. clandestina*	1994	Australia	*Crataegus* sp.	MT804393
32210	*P. clandestina*	1995	Switzerland	*C. oxyacantha*	MT804394
21116	*P. clandestina*	1996	USA	*Prunus* sp.	MT804398
32542	*P. clandestina*	2000	USA	*P. avium*	MT804397
41640	*P. clandestina*	2008	Germany	*Amelanchier laevis*	MT804402

2.1 | Sampling

Sampling was completed under clean room conditions to minimize contamination with modern DNA. Powdery mildew conidia, mycelia, and chasmothecia, where present, were collected from the specimens by using a 6 mm leaf punch or scraping with a blade.

2.2 | DNA extraction and quantification

DNA was extracted following the E.Z.N.A. Forensic DNA protocol (Smith et al., 2020). The initial DNA extraction incubation step was increased to 1 hr and included a repeated final elution step (50 µl elution buffer was added to the filter column with a 5 min incubation for a final volume of 100 µl). NanoDrop 2000 (Thermo Fisher Scientific) was used to assess DNA quality using the 260/280 nm absorbance ratio (1.8–1.9). DNA concentrations were quantified using two methods: Quantus fluorometer (Promega) and Agilent Tapestation electrophoresis (Agilent Technologies).

2.3 | Library preparation and sequencing

Whole-genome library preparation was as described by Smith et al. (2020). Libraries were paired-end sequenced on the Illumina HiSeq 3000 platform.

2.4 | Sequence analysis

Sequence reads were assigned to each sample based on their indices. Raw DNA-Seq sequence files were trimmed using the filter option of the program Nuclear v. 3.3.6 (Gydle Inc. Bioinformatics Service; http://www.gydle.com) A minimum read length of 100 bp was used and 20 mismatches. The library preparations were expected to contain *Podosphaera* DNA and host DNA, as well as DNA from microbes present on the leaf surface at the time of collection. Therefore, the filtered, high-quality (HQ) reads were mapped as paired-end reads to *Podosphaera* and plant host reference sequences. For *Podosphaera*, reference sequences were the internal transcribed spacer region (ITS, comprising ITS1-5.8S-ITS2) of *P. clandestina* (GenBank accession AB525930 and KY661125), *P. cerasi* (KX826855), and *P. prunicola* (LC378576). For hosts, reference sequences were the maturase K (*matK*) chloroplast gene from *P. avium* (GenBank accession FJ899109), *P. cerasus* (FJ899111), *Crataegus monogyna* (FJ899108), *Spiraea salicifolia* (JQ041795), and *Amelanchier laevis* (JQ390945). The sequence mapping was performed by Nuclear, generating reference-initiated sequence alignments to be viewed in Vision v. 3.3.6 software (Gydle Inc.). In Vision, ITS and *matK* sequences were edited to incorporate single nucleotide polymorphisms (SNPs) and indels that related to the sequence data of each individual VPRI specimen. A mapping threshold was determined by a minimum 5 × coverage of the complete reference sequence and sequence files that did not meet these criteria were excluded. Mapping success was determined from

the Vision images of each sequence file, by calculating the total number of aligned DNA sequence reads and converting it into a percentage from the total number of HQ reads. The mapped ITS and *matK* sequences generated for this study were exported from Vision for BLASTn and phylogenetic analysis.

2.5 | Phylogenetic analysis

Eighteen ITS sequences of *Podosphaera* species generated for this study were lodged in GenBank; VPRI 6222 was omitted due to low mapped sequence numbers (Table 1). Phylogenetic analysis included these sequences along with 165 sequences within the *P. clandestina* complex (section *Podosphaera* subsection *Podosphaera*) obtained from GenBank that were selected from published studies (Meeboon et al., 2020; Moparthi et al., 2019; Smith et al., 2020) and NCBI searches of *Podosphaera* ITS sequences. The phylogenetic analysis of 183 sequences included *Cystotheca lanestris* as the outgroup taxon, as per Moparthi et al. (2019). Alignments were generated in Geneious Prime using Muscle v. 3.8.425 (Biomatters Ltd, 2020; Edgar, 2004) alignment tool with suggested settings. Alignments were refined manually in Geneious Prime. Phylogenetic trees were obtained from the aligned sequence data by maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed in PhyML (3.3.20180621) (Guindon et al., 2010) with the general time reversible (GTR) substitution model, optimization for topology/length/rate with proportion of invariable sites set at 0 and number of substitution rate categories 4. The bootstrap analysis was set at 1,000 replications with the stepwise addition option set as simple. BI analysis was completed in MrBayes v. 3.2.6 (Huelsenbeck & Ronquist, 2001) using two runs with four chains each under the GTR model and run assuming a gamma-distribution variation. Four heated chains and a single cold chain were used in all Markov chain Monte Carlo (MCMC) analyses, which were run for 1,100,000 generations and sampled one tree every 200 generations. Burn-in length was set at 100,000.

3 | RESULTS

The 19 VPRI collections of *P. clandestina* in the broad sense that were suitable for DNA extraction were from Australia and elsewhere on four host genera: *Prunus*, *Crataegus*, *Spiraea*, and *Amelanchier*, dating from 1889 to 2008 (Table 1). The collections from Australia were all on *Crataegus*.

3.1 | Sequence analysis

The number of mapped sequence reads to the powdery mildew ITS was low compared to the total number of HQ reads from each sample library. The sequence lengths mapped to ranged between 612 and 1,509 bp, including partial sequences of 18S rRNA, the complete ITS region and partial 28S rRNA regions. The specimens with the highest percentage of mapped ITS sequence reads were VPRI 40176 (0.046%), VPRI 40177 (0.019%), and VPRI 6224 (0.008%) (Table 2). The age of the specimen and the amount of sequence data generated did not affect sequencing success, with US specimens collected from 1890 to 1904 (VPRI 40176, VPRI 40177, VPRI 6221, and VPRI 6224) having higher percentages of aligned sequences to reference scaffolds compared to younger specimens that generated more sequence data. The ITS mapping of specimen VPRI 6222 only had 28 sequence reads that partially mapped to the reference sequence; this was considered below the mapping threshold and was excluded from further analysis (Table 2).

All specimens that were successfully mapped to reference sequences matched to a GenBank accessioned *Podosphaera* ITS sequence with a BLASTn percentage identity of at least 98% and as high as 100%, and E values were 0.0 (Table 2). All but one of the specimens matched to named *Podosphaera* species, specifically *P. clandestina*, *P. cerasi*, *P. prunicola*, and *P. amelanchieris*. The ITS sequence for specimen VPRI 6224 was the only sequence that failed to match a fully described *Podosphaera* species at 99.7% (Table 2).

The host plant *matK* sequence mapping produced similar mapped read percentages as the *Podosphaera* ITS sequencing results with VPRI 6221 (0.0215%), VPRI 19634 (0.0169%), and VPRI 40177 (0.0088%) having the highest percentages (Table 3). Plant hosts identified to species with BLASTn identities between 99% and 100% and E values of 0.0, except for VPRI 18863 (E value of 2e−170) and VPRI 32210 (2e−170) due to shorter sequences exported from the Vision alignment (Table 3). For seven of the 19 sequences from VPRI specimens, plant host species indicated by BLASTn matched the listed plant species recorded on the VPRI specimen. For a further seven collections, sequencing of the host provided a species-level identification where the existing identification was only to genus, as for VPRI 6224 (*Crataegus macracantha*), VPRI 21116 (*P. avium*) and two collections identified specifically as *C. monogyna* and three as *Crataegus pinnatifida* var. *major* (Table 3). For four collections, the host was reidentified: the host of VPRI 6222 was previously listed as *Crataegus spathulata* but has been reidentified as *Crataegus punctata*, and the hosts of VPRI 19170, 18863, and 32210 were originally recorded as *Crataegus oxyacantha* but have been updated to *C. monogyna*. The sequence for VPRI 32452 was generated from DNA isolated from filter paper from a spore trap; the sample after mapping to host *matK* references returned a BLASTn identity of *P. avium* at 99.9%.

3.2 | Phylogeny

The phylogenetic analysis based on 183 sequences for the 501 bp of the ITS region formed a tree with three main clades: Clade 1 consisted of sequences related to the *P. tridactyla* species complex including *P. pruni-avium*, Clade 2 contained sequences related to *P. clandestina* in the strict sense, and Clade 3 consisted of *P. cerasi* and sister taxa. *Podosphaera* spp. on cherry hosts were found in two clades across the phylogenetic tree, *P. pruni-avium* in Clade 1, *P.*

TABLE 2 Next-generation sequencing data for VPRI *Podosphaera clandestina* sensu lato specimens

VPRI accession	Raw reads	HQ reads	Mapped ITS reads	Mapped ITS (%)	GenBank BLAST accession	GenBank BLAST *Podosphaera* species result	GenBank ID (%)
6225	9,233,801	8,083,669	216	0.0027	MG183669	*P. cerasi*	100
6221	13,469,787	12,286,161	393	0.0032	MG183669	*P. cerasi*	100
6222	15,430,574	13,984,003	28	0.0002	—	—	—
40176	16,132,212	14,522,628	6,641	0.0457	KX826855	*P. cerasi*	100
40177	13,601,902	12,174,908	2,331	0.0191	LC378576	*P. prunicola*	99.6
6224	17,161,316	15,467,497	1,208	0.0078	MG016459	*Podosphaera* sp.	99.7
6223	20,073,310	17,298,244	1,160	0.0067	LC378576	*P. prunicola*	99.3
20388	12,638,054	10,987,636	110	0.0010	LC378576	*P. prunicola*	98.4
19079	22,399,573	20,254,690	260	0.0013	AB525930	*P. clandestina*	100
19634	21,614,487	19,640,082	2,095	0.0107	KY660805	*P. clandestina*	100
19170	18,166,170	16,395,803	82	0.0005	KY661125	*P. clandestina*	100
18863	29,315,042	26,641,664	261	0.0010	KY661125	*P. clandestina*	100
19938	18,406,522	16,487,326	1,139	0.0069	AB525930	*P. clandestina*	100
19984	26,182,198	23,584,588	397	0.0017	KY661125	*P. clandestina*	100
19985	21,989,378	19,760,034	140	0.0007	KY661125	*P. clandestina*	100
32210	18,351,898	16,469,900	1,081	0.0066	AB525930	*P. clandestina*	100
21116	21,462,558	19,521,704	3,051	0.0156	KX826855	*P. cerasi*	100
32542	17,479,940	15,891,629	1,163	0.0073	KX826855	*P. cerasi*	100
41640	20,830,005	18,592,795	1,674	0.0090	AB525927	*P. amelanchieris*	99.9

Note: Raw sequence read numbers and high quality (HQ) read numbers are compared with the number of mapped ITS sequence reads and the percentage ITS sequences that map to *Podosphaera* references. ITS1-5.8S-ITS2 region (GenBank accessions *P. clandestina* AB525930 and KY661125, *P. cerasi* KX826855, and *P. prunicola* LC378576). GenBank BLAST results for each VPRI collection show accession number, species name, and percentage ID.

cerasi and *P. prunicola* in Clade 3 (Figure 1). The VPRI specimens of *P. cerasi* on cherry were all collected in the USA.

All the Australian specimens were on *Crataegus* and fell into Clade 2 in a well-supported clade comprising 28 sequences with high posterior probability (BI) and bootstrap (BS) support, 100 and 1, respectively (Figure 1). This clade also included sequences of *P. clandestina* on *Crataegus* from Argentina, the UK, and the USA. A sequence from VPRI 20388 *Crataegus* from Finland (MT804395) was placed in Clade 2 but was not close to any other sequences and fell outside of the *P. clandestina* clade.

In Clade 3, VPRI specimens from the USA, including 6225 (MT804403), 21116 (MT804398), and 32542 (MT804397) on *P. avium* and VPRI 6221 (MT804400) and 40176 (MT804399) on *P. cerasus*, fell in a clade comprising 19 sequences including 11 sequences identified as *P. cerasi* by Moparthi et al. (2019), with BI support 0.67 but BS support below the 60% threshold (Figure 1). Two sequences misidentified as *P. clandestina* (DQ139434 occurring on *P. avium* in Belgium and KY124542 on *P. cerasus* from Iran) were immediately adjacent to the clade composed of *P. cerasi*, and these two sequences had BI support of 0.96 but very low BS support. A sequence labelled *P. clandestina* on *P. avium* from the USA (AF011316) fell inside of the *P. cerasi* clade but differed at four base positions from other *P. cerasi* sequences.

Also, in Clade 3, four sequences on *P. serotina* and *P. virginiana* identified as *P. prunicola* by Moparthi et al. (2019) formed a clade with five other sequences including the sequence from VPRI 40177 (MT804404) on *P. avium* from the USA (Figure 1). In addition, this clade included three sequences originally labelled as *P. clandestina* (on *Prunus laurocerasus* and *P. serotina*) and one sequence not previously identified to species (on *P. virginiana*). The BS support for this clade was 65, with BI providing stronger support at 0.82.

The sequence for VPRI 6223 (collected in 1908) on *S. salicifolia* (MT804396) was sister to the *P. prunicola* clade, but the node basal to it had low branch support (Figure 1). The sequence from VPRI 41640 (MT804402) on *A. laevis* from Germany fell within a strongly supported clade (BS 79, BI 1) with sequences that had been identified as *P. amelanchieris*. VPRI 6224 (collected in 1904) on *C. macracantha* (MT804401) was also located in the *P. amelanchieris* clade; it differed at three base positions compared to other sequences of *P. amelanchieris*.

4 | DISCUSSION

The focus of this study was to reexamine specimens of *Podosphaera* on *P. avium* and closely related hosts held in Australian reference collections using NGS to clarify which species of the *P. clandestina* complex are present in Australia, in light of the newly described *P. cerasi* on *P. avium* and *P. cerasus* (Moparthi et al., 2019). Illumina

TABLE 3 Next-generation sequencing data for plant hosts

VPRI accession	Mapped *mat*K reads	Mapped *mat*K reads (%)	Listed plant host	GenBank BLAST accession	GenBank BLAST *Podosphaera* species result	GenBank ID (%)
6225	327	0.0040	*Prunus cerasus*	FJ899111	*P. cerasus*	99.3
6221	2,638	0.0215	*P. cerasus*	FJ899111	*P. cerasus*	99.7
6222	120	0.0009	*Crataegus spathulata*	KC173404	***Crataegus punctata***	100
40176	143	0.0010	*P. cerasus*	FJ899111	*P. cerasus*	99.4
40177	1,077	0.0088	*Prunus avium*	NC_044701	*P. avium*	99.5
6224	641	0.0041	*Crataegus* sp.	HQ593252	***Crataegus macracantha***	100
6223	110	0.0006	*Spiraea salicifolia*	JQ041795	*S. salicifolia*	100
20388	213	0.0019	*Crataegus sanguinea*	KY419945	***Crataegus pinnatifida* var. *major***	99.9
19079	807	0.0040	*Crataegus* sp.	KC206945	***Crataegus monogyna***	100
19634	3,318	0.0169	*Crataegus* sp.	JQ391067	***C. monogyna***	99.6
19170	432	0.0026	*Crataegus oxyacantha*	KC206945	***C. monogyna***	100
18863	494	0.0019	*C. oxyacantha*	FN687519	***C. monogyna***	100
19938	183	0.0011	*Crataegus* sp.	KY419945	***C. pinnatifida* var. *major***	100
19984	1,267	0.0054	*Crataegus* sp.	KY419945	***C. pinnatifida* var. *major***	100
19985	700	0.0035	*Crataegus* sp.	KY419945	***C. pinnatifida* var. *major***	100
32210	23	0.0001	*C. oxyacantha*	FN687519	***C. monogyna***	100
21116	605	0.0031	*Prunus* sp.	NC_044701	***P. avium***	100
32542	151	0.0010	*P. avium*[a]	NC_044701	*P. avium*[a]	99.9
41640	354	0.0019	*Amelanchier laevis*	JQ390945	*A. laevis*	99.9

Note: Mapped *mat*K sequence reads and the percentage *mat*K sequences that map to GenBank *mat*K references for *P. avium* accession FJ899109, *P. cerasus* accession FJ899111, *C. monogyna* accession FJ899108, *S. salicifolia* accession JQ041795, and *A. laevis* accession JQ390945. GenBank BLAST results for each VPRI collection show accession number, species name, and percentage ID. Reidentifications or more specific identifications are in bold.

[a]Listed host species of spore trap sample with no plant material present.

HiSeq 3000 sequence data were successfully generated for 19 specimens, and ITS sequences were able to be retrieved from 18 of the 19 specimens and mapped to known *Podosphaera* ITS sequence data. Phylogenetic analysis showed that all the specimens collected within Australia are *P. clandestina* in the strict sense on *Crataegus* hosts. The cherry powdery mildews, *P. cerasi* and *P. prunicola*, were only identified from herbarium material collected from outside of Australia.

Plant hosts for powdery mildew specimens were confirmed by mapping the sequence data generated to the *matK* chloroplast gene and we were able to resolve several previously unlisted host plant species names to update specimen records in the VPRI database. This molecular approach could be used to identify closely related and morphologically similar plant species from herbaria.

The phylogenetic analysis showed that Australian *P. clandestina* VPRI specimen sequences form a well-supported clade with other sequences of *P. clandestina* in the strict sense on *Crataegus*. This confirms that the powdery mildew species present on *Crataegus* in Australia is not the newly recognized *P. cerasi*. However, the phylogenetic analysis highlights three powdery mildew species, *P. cerasi*, *P. prunicola*, and *P. pruni-avium* (the latter within the *P. tridactyla* species complex), that infect cherry and therefore pose significant threats to the Australian cherry industry. VPRI specimens collected from the USA between 1890 and 1904 (VPRI 6221, 6225, 21116, 32542, and 40176) generated sequences that were all able to be aligned with modern sequences of *P. cerasi* or of *P. prunicola* (VPRI 40177) and *P. amelanchieris* (VPRI 6224 and VPRI 41640). However, sequences from specimens VPRI 6223 (from the USA collected in 1908) and VPRI 20388 (from Finland collected in 1976) failed to cluster with any other sequences. A possible explanation is nucleotide misincorporations (C → T/G → A) due to the age of the specimens (Staats et al., 2013), but other

FIGURE 1 Maximum-likelihood (ML) phylogenetic analysis of internal transcribed spacer (ITS) sequences of VPRI *Podosphaeraclandestina* and other *Podosphaera* spp. Bootstrap values and posterior probability (≥65%) obtained for ML and Bayesian inference (BI) analyses are shown for well-supported branches. Sequences on cherry are in yellow, sequences from Australian collections are in blue. Sequences generated for the study are indicated by *. Clade 1 (highlighted) *P. tridactyla* in the strict sense and relatives; Clade 2 (highlighted) *P. clandestina* in the strict sense and relatives; Clade 3 (highlighted) *P. cerasi* and relatives


Cystotheca lanestris ex Myrica californica AF011288 USA
Cystotheca lanestris ex Quercus agrifolia AF011289 USA
Clade one
Podosphaera tridactyla s. str. ex Prunus laurocerasus MK530464 GER
Podosphaera tridactyla s. str. ex Prunus padus MK530463 GER
Podosphaera tridactyla s. str. ex Prunus padus MK530461 GER
Podosphaera tridactyla s. str. ex Prunus padus MK530462 GER
Podosphaera pruni-avium ex Prunus avium MK530456 GER
Podosphaera pruni-lustanicae ex Prunus lusitanica AY833655 SWI
Podosphaera pruni-lustanicae ex Prunus lusitanica KP642001 UK
Podosphaera pruni-lustanicae ex Prunus lusitanica KP641993 UK
Podosphaera pruni-avium ex Prunus avium MK530457 GER
Podosphaera pruni-japonicae ex Prunus japonica MK530455 JPN
Podosphaera pruni-japonicae ex Prunus japonica AB000943 JPN
Podosphaera longiseta ex Prunus grayana MK530458 JPN
Podosphaera longiseta ex Prunus grayana MK530459 JPN
Podosphaera longiseta ex Prunus grayana MK530460 JPN
Podosphaera pruni-cerasoidis ex Prunus cerasoides MK530447 THA
Podosphaera pruni-cerasoidis ex Prunus cerasoides MK530449 THA
Podosphaera pruni-cerasoidis ex Prunus cerasoides MK530448 THA
Podosphaera prunigena ex Prunus serrulata MK530446 JPN
Podosphaera prunigena ex Cerasus leveilleana AB936275 JPN
Podosphaera prunina ex Prunus mume MK530441 JPN
Podosphaera prunina ex Prunus mume MK530443 JPN
Podosphaera prunina ex Prunus sp. MK530445 JPN
Podosphaera prunina ex Prunus mume MK530444 JPN
Podosphaera prunina ex Prunus mume MK530442 JPN
Podosphaera salatai ex Prunus incana AB525929 GEOR
Podosphaera sp. ex Prunus armeniaca AF011318 USA
Podosphaera ampla ex Prunus spinosa MK530450 GER
Podosphaera tridactyla ex Prunus cerasifera AY833658 SWI
Podosphaera ampla ex Prunus spinosa MK530451 GER
Podosphaera ampla ex Prunus domestica MK530453 GER
Podosphaera ampla ex Prunus domestica MK530452 GER
Podosphaera ampla ex Prunus domestica MK530454 GER
Podosphaera fuliginea ex Veronica spicata AB046986 USA
Podosphaera gunneracae ex Gunnera magellanica AB525924 ARG
Podosphaera gunneracae ex Gunnera magellanica AB525923 ARG
Podosphaera fusca ex Hypochaeris brasiliensis AY739113 BRA
Podosphaera macrospora ex Tellima grandiflora KX032527 UK
Podosphaera fusca ex Taraxacum sp. KX842347 USA
Podosphaera fusca ex Taraxacum officinale AF011320 USA
Podosphaera macrospora ex Tellima grandiflora KX032529 UK
Podosphaera macrospora ex Heuchera sp. KP966080 UK
Podosphaera macrospora ex Tellima grandiflora KX032533 UK
Podosphaera fusca ex Euryops pectinatus EU424056 ESP
Podosphaera fusca ex Cucurbita pepo AF011321 USA
Podosphaera fuliginea ex Cucumis sativus JN627138 CHN
Podosphaera fuliginea ex Cucumis melo EU294368 CHN
Podosphaera xanthii ex Tussilago farfara AB040345 JPN
Podosphaera xanthii ex Helianthus annuus EF010913 TWN
Podosphaera xanthii ex Physalis angulata EF050036 TWN
Podosphaera xanthii ex Lactuca indica AB040294 JPN
Clade two
Podosphaera sp. ex Crataegus pinnatifida var. major MT804395 FIN*
Podosphaera clandestina ex Pyrus calleryana KX589261 CHN
Podosphaera curvispora ex Aria alnifolia AB525928 JPN
Podosphaera leucotricha ex Pyrus calleryana GU122230 USA
Podosphaera leucotricha ex Malus domestica MT178380 AUS
Podosphaera leucotricha ex Malus domestica MT178369 AUS
Podosphaera leucotricha ex Malus domestica MT178361 AUS
Podosphaera leucotricha ex Pyrus sp. AY157845 CAN
Podosphaera leucotricha ex Pyrus calleryana EU148597 HUN
Podosphaera leucotricha ex Malus domestica MT178387 AUS
Podosphaera leucotricha ex Malus sp.KX842350 USA
Podosphaera leucotricha ex Malus domestica MT178383 AUS
Podosphaera clandestina ex Crataegus monogyna KY661072 UK
Podosphaera clandestina ex Crataegus monogyna KY661071 UK
Podosphaera clandestina ex Crataegus monogyna KY661027 UK
Podosphaera clandestina ex Origanum vulgare KY660806 UK
Podosphaera clandestina ex Crataegus oxyacantha AB525931 ARG
Podosphaera clandestina ex Crataegus monogyna KY661018 UK
Podosphaera clandestina ex Crataegus monogyna KY661087 UK
Podosphaera clandestina ex Crataegus monogyna KY661043 UK
Podosphaera clandestina ex Crataegus monogyna KY661125 UK
Podosphaera clandestina ex Crataegus monogyna MT804934 SWI
Podosphaera clandestina ex Crataegus sp. MG062783 USA
Podosphaera clandestina ex Crataegus monogyna KY661109 UK
Podosphaera clandestina ex Crataegus monogyna KY661013 UK
Podosphaera clandestina ex Crataegus monogyna KY661108 UK
Podosphaera clandestina ex Crataegus monogyna AB525930 UK
Podosphaera clandestina ex Crataegus monogyna MT804390 AUS*
Podosphaera clandestina ex Crataegus monogyna MT804387 AUS*
Podosphaera clandestina ex Crataegus monogyna MT804388 AUS*
Podosphaera clandestina ex Crataegus monogyna MT804389 AUS*
Podosphaera clandestina ex Crataegus pinnatifida var. major MT804391 AUS*
Podosphaera clandestina ex Crataegus pinnatifida var. major MT804392 AUS*
Podosphaera clandestina ex Crataegus pinnatifida var. major MT804393 AUS*
Podosphaera clandestina ex Crataegus monogyna KY661035 UK
Podosphaera clandestina ex Crataegus monogyna KY660805 UK
Podosphaera clandestina ex Crataegus monogyna KY661049 UK
Podosphaera clandestina ex Crataegus monogyna KY661060 UK
Podosphaera clandestina ex Crataegus monogyna KY661069 UK
Podosphaera clandestina ex Crataegus monogyna KY661065 UK
P. clandestina s. str.
Clade three
Podosphaera sp. ex Amelanchier sp. MG016459 USA
Podosphaera amelanchieris ex Amelanchier laevis MT804402 GER*
Podosphaera amelanchieris ex Amelanchier lamarckii KY661074 UK
Podosphaera amelanchieris ex Amelanchier lamarckii KY661055 UK
Podosphaera sp. ex Crataegus macracantha MT804401 USA*
Podosphaera amelanchieris ex Amelanchier canadensis KY660724 UK
Podosphaera amelanchieris ex Amelanchier laevis AB525927 GER
P. amelanchieris
Podosphaera sp. ex Spiraea salicifolia MT804396 USA*
Podosphaera clandestina ex Prunus serotina KJ476999 MEX
Podosphaera clandestina ex Prunus laurocerasus AF011317 USA
Podosphaera prunicola ex Prunus virginiana MF770745 USA
Podosphaera sp. ex Prunus virginiana MK028958 USA
Podosphaera prunicola ex Prunus serotina LC378576 GER
Podosphaera prunicola ex Prunus avium MT804404 USA*
Podosphaera clandestina ex Prunus serotina KU158161 MEX
Podosphaera prunicola ex Prunus virginiana KX842346 USA
Podosphaera prunicola ex Prunus virginiana MF770746 USA
P. prunicola
Podosphaera minor ex Spiraea nipponica AB026137 JPN
Podosphaera spiraeae ex Spiraea sp. AF154325 JPN
Podosphaera collomiae ex Phylox paniculata KY661131 UK
Podosphaera clandestina ex Cydonia sp. KY660726 UK
Podosphaera sp. ex Phlox drummondii JN013185 ITA
Podosphaera clandestina ex Phlox drummondii HQ844621 ITA
Podosphaera clandestina ex Prunus cerasus KY124542 IRN
Podosphaera clandestina ex Prunus avium DQ139434 BEL
Podosphaera cerasi ex Prunus avium MG198703 USA
Podosphaera clandestina ex Prunus avium AF011316 USA
Podosphaera cerasi ex Prunus avium MG937803 USA
Podosphaera cerasi ex Prunus cerasus MT804400 USA*
Podosphaera cerasi ex Prunus avium KX842345 USA
Podosphaera cerasi ex Prunus avium LC381235 USA
Podosphaera cerasi ex Prunus avium MT804398 USA*
Podosphaera cerasi ex Prunus avium MG183669 USA
Podosphaera cerasi ex Prunus avium MG076957 USA
Podosphaera cerasi ex Prunus avium MT804397 USA*
Podosphaera cerasi ex Prunus cerasus MT804399 USA*
Podosphaera cerasi ex Prunus avium MG076954 USA
Podosphaera cerasi ex Prunus avium MG076958 USA
Podosphaera clandestina ex Prunus sp. AY818344 CAN
Podosphaera cerasi ex Prunus cerasi KX826855 USA
Podosphaera cerasi ex Prunus avium MG076952 USA
Podosphaera cerasi ex Prunus avium MG076953 USA
Podosphaera cerasi ex Prunus avium MT80403 USA
P. cerasi
Podosphaera spiraeae ex Aruncus dioicus AB026152 JPN
Podosphaera spiraeae ex Spiraea thunbergii AF154326 JPN
Podosphaera spiraeae ex Spiraea thunbergii AB026153 JPN
Podosphaera spiraeae ex Spiraea cantoniensis AB026143 JPN
Podosphaera spiraeae ex Spiraea japonica AB026149 JPN
Podosphaera minor ex Spiraea japonica AB026150 JPN
Podosphaera spiraeae ex Spiraea sp. MF919434 USA
Podosphaera spiraeae ex Spiraea japonica KF500426 CHN
Podosphaera spiraeae ex Spiraea japonica KT290556 KOR
Podosphaera spiraeae ex Spiraea cantoniensis AB525940 ARG
Podosphaera lini ex Linum usitatissimum AB525925 SWI
Podosphaera lini ex Linum usitatissimum MK749431 UK
Podosphaera lini ex Linum usitatissimum MK749432 GER
Podosphaera macularis ex Humulus japonicus MN381108 USA
Podosphaera macularis ex Humulus japonicus MN381106 JPN
Podosphaera aphanis ex Aphanis sp. AB000938 JPN
Podosphaera ferruginea ex Sanguisorba officinalis AB027232 JPN
Podosphaera ferruginea ex Sanguisorba officinalis KR048108 CHN
Podosphaera macularis ex Humulus lupulus KY022479 UK
Podosphaera macularis ex Humulus lupulus AB525917 JPN
Podosphaera ferruginea ex Sanguisorba officinalis KY661130 UK
Podosphaera clandestina ex Vicia cracca KY661051 UK
Podosphaera negeri ex Escallonia rubra AB525920 ARG
Podosphaera negeri ex Escallonia rubra AB525919 ARG
Oidium diostede ex Diostea juncea AB525944 ARG
Oidium diostede ex Diostae juncea AB525945 ARG
Podosphaera negeri ex Escallonia virgata AB525921 ARG
Podosphaera epilobii ex Epilobium sp. MF663779 IRAN
Podosphaera epilobii ex Epilobium parviflorum KY661024 UK
Podosphaera epilobii ex Epilobium palustre KY661138 UK
Podosphaera epilobii ex Epilobium pyrricholophum KR048107 CHN
Podosphaera epilobii ex Epilobium ciliatum AB525926 ARG
Podosphaera fugax ex Geranium sp. KY660816 UK
Podosphaera fugax ex Geranium sp. KY660817 UK
Podosphaera fugax ex Geranium nepalense AB026134 JPN
Podosphaera fugax ex Geranium thunbergii AB525922 JPN
Podosphaera pannosa ex Rosa rubiginosa AB525938 ARG
Podosphaera pannosa ex Rosa sp. AF011323 USA
Podosphaera pannosa ex Rosa sp. AF011322 USA
Podosphaera pannosa ex Rosa maltiflora AB525939 JPN
Podosphaera pannosa ex Rosa sp. AB022348 JPN
Podosphaera pannosa ex Prunus persica KX842349 USA
Podosphaera aphanis ex Rubus sp. MF919432 USA
Podosphaera aphanis ex Fragaria grandiflora AB026136 JPN
Podosphaera aphanis ex Fragaria chiloensis AB525933 ARG
Podosphaera pannosa ex Rosa rubiginosa AB525937 ARG
Podosphaera aphanis ex Fragaria x ananassa AF073355 AUS
Podosphaera aphanis ex Fragaria sp. MF919433 USA
0.07

VPRI specimens that were collected during the same time period showed no DNA degradation and formed clades with modern sequences with exactly the same characteristic bases present as in more recent collections, and therefore this explanation is unlikely. Further collections with sequences that match these two sequences are required before their taxonomic placement can be resolved. In relation to host range, a collection on *P. avium* (VPRI 40177) was identified as *P. prunicola*. This species was described from *P. melanocarpa* and *P. virginiana*, but the extension of the host range to *P. avium* presents a previously undocumented potential threat for cherry.

Specimens available for this study highlight the lack of recently collected powdery mildew specimens from Australia in plant pathogen reference collections (Kiss et al., 2020). To rectify this lack of collections, mycologists and plant pathologists should instigate new collecting initiatives across Australia to sample and recollect powdery mildew specimens, similar to that undertaken in the mid-1990s by VPRI (Pascoe & Sivapalan, 1995). With fresh powdery mildew sampling, collections can be made with the aim of preserving both the DNA and morphological characters used for species identification. Plant pathogen specimens, either live cultures or preserved host and fungal material, will continue to provide the basis for taxonomy and species identification. However, as researchers are now routinely using collections to prepare DNA sequences without necessarily examining morphological characters, it is therefore desirable to make DNA preparations at the time of collection of new specimens and to preserve sufficient pathogen material for future reference to allow for potential destructive sampling (Funk et al., 2017).

Obtaining DNA from powdery mildew specimens up to 130 years old has highlighted the importance of plant pathogen reference collections such as VPRI. These collections hold invaluable resources that can be used to follow plant pathogen introduction and determine species distributions. This information informs plant biosecurity in relation to which pathogens are known to be present in a country and which are not, to assist in preventing entry of new and potentially economic threatening pathogens (Hyde et al., 2010; Sikes et al., 2018).

The applications of the NGS techniques described in this study are not limited to simple species identification but can be applied to specimens that represent cryptic or species complexes, which are difficult to accurately identify through morphology alone. This can resolve complicated species questions for plant biosecurity purposes and clear potential trade issues by confirming the presence or absence of plant pathogen species present in Australia (McTaggart et al., 2016). Molecular techniques such as the ones described can be used to enhance plant pathogen reference collections by not only providing plant pathogen species distributions, but also by generating sequences that increase accessibility to the collections with molecular data in global online databases (Heberling et al., 2019). The ability to generate molecular data from plant pathogen collections such as VPRI could also be used to trace obligate plant pathogen lineages through history. Ristaino (1998) highlighted the importance of herbaria and museum collections by tracking the role of oospores in the biology of *Phytophthora infestans* from historical documentation and herbarium specimens together with molecular analysis to determine the earliest records of *P. infestans* in the USA. Following this, Ristaino (2002) used herbarium specimens to determine the haplotypes of *P. infestans* that lead to the late blight epidemic and subsequent epidemics worldwide over the following 150 years.

This study used post-sequencing bioinformatic analysis, in which we were able to isolate powdery mildew ITS and host chloroplast *matK* gene sequences from a mixed DNA extraction by mapping the sequences to reference sequences. This bioinformatic approach has been used previously for other purposes such as to map chloroplast genomes of Australian eucalypts, dehydrin genes in Pinaceae for drought response expression, and agronomically important regions within the wheat genome (Bayly et al., 2013; Keeble-Gagnère et al., 2018; Stival Sena et al., 2018). Until this study, this bioinformatic approach had not been used for mapping plant pathogen DNA for molecular identification from NGS data.

Traditionally, powdery mildew identification was based on morphology and host plant associations, with morphological classification relying on specific descriptions of sexual characters to identify to species; however, when the sexual state was absent, identification was largely based on host association (Takamatsu, 2004). More recently, PCR-based molecular identification is routinely used together with morphology for powdery mildew species identification; this is valid for fresh powdery mildew samples, but when powdery mildew specimens are preserved some important taxonomic characters are lost and DNA can become degraded and fragmented, thus reducing PCR success (Särkinen et al., 2012). Smith et al. (2020) demonstrated that PCR amplification of preserved powdery mildew specimens can be difficult and too inconsistent to be used for accurate molecular identification. One factor affecting PCR success is the degraded nature of the DNA caused by ageing processes that fragments the DNA into short (<500 bp) lengths (Staats et al., 2011; Wieß et al., 2016).

A recent study by Bradshaw and Tobin (2020) developed a new sequencing protocol for use on herbarium powdery mildew specimens up to 130 years old. This sequencing protocol amplifies the ITS and the large ribosomal subunit (LSU) regions through a nested PCR amplification approach. Bradshaw and Tobin (2020) recommend performing separate ITS and LSU PCRs due to the fragmented nature of herbarium DNA, and further recommend amplifying smaller sections within ITS and LSU for very difficult specimens. In contrast, the NGS approach taken in this study does not require multiple amplification steps and assembly. Even though the DNA from herbarium powdery mildew is fragmented, it can be handled by NGS and mapping to reference sequences as an effective way of assembling the shorter sequences.

We have confirmed that *P. cerasi* and *P. prunicola* on *Prunus* are phylogenetically different species from *P. clandestina* in the strict sense on *Crataegus*. The nine sequences identified as *P. clandestina* that failed to form a clade with other sequences of *P. clandestina* require further investigation for taxonomic revision. Through NGS applications we were able to identify to species powdery mildews and their host plants from specimens up to 130 years old. The

results from this study will be used to update plant pathogen reference collections and Plant Health Australia with correct species name for cherry powdery mildew (replacing *P. clandestina* var. *clandestina*). Presence or absence of *P. cerasi*, *P. prunicola*, and *P. pruni-avium* on cherry in Australia is an issue of high significance for the Australian cherry industry. Powdery mildew has never been reported on cherry in Australia and this study provides confirmation that the powdery mildew on Australian *Crataegus* specimens are *P. clandestina* and that none of the cherry powdery mildews, *P. cerasi*, *P. pruni-avium*, and *P. prunicola*, are present on Australian specimens in the VPRI collection.

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

DATA AVAILABILITY STATEMENT

The data sets generated and analysed during the current study are available from the corresponding author on reasonable request.

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REFERENCES

Anderson, C., Low-Choy, S., Whittle, P., Taylor, S., Gambley, C., Smith, L. et al. (2017) Australian plant biosecurity surveillance systems. *Crop Protection*, 100, 8–20.

Bayly, M.J., Rigault, P., Spokevicius, A., Ladiges, P.Y., Ades, P.K., Anderson, C. et al. (2013) Chloroplast genome analysis of Australian eucalypts–*Eucalyptus*, *Corymbia*, *Angophora*, *Allosyncarpia* and *Stockwellia* (Myrtaceae). *Molecular Phylogenetics and Evolution*, 69, 704–716.

Biomatters Ltd (2020) *Geneious Prime 2020.0.5*. Available at: https://www.geneious.com/about/. [Accessed 26 November 2020].

Bradshaw, M. & Tobin, P.C. (2020) Sequencing herbarium specimens of a common detrimental plant disease (powdery mildew). *Phytopathology*, 110, 1248–1254.

Braun, U. (1987) A monograph of the Erysiphales (powdery mildews). *Beihefte zur Nova Hedwigia*, 89, 1–700.

Braun, U. & Cook, R.T.A. (2012) *Taxonomic manual of the Erysiphales (powdery mildews)*. Netherlands: CBS-KNAW Fungal Biodiversity Centre.

Brewer, M.T. & Milgroom, M.G. (2010) Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse *Vitis* species. *BMC Evolutionary Biology*, 10, 268.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792–1797.

Funk, V.A., Gostel, M., Devine, A., Kelloff, C.L., Wurdack, K., Tuccinardi, C. et al. (2017) Guidelines for collecting vouchers and tissues intended for genomic work (Smithsonian Institution): Botany Best Practices. *Biodiversity Data Journal*, 5, e11625.

Grove, G.G. & Boal, R.J. (1991) Factors affecting germination of conidia of *Podosphaera clandestina* on leaves and fruit of sweet cherry. *Ecology and Epidemiology*, 81, 1513–1518.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, 59, 307–321.

Heberling, J.M., Prather, L.A. & Tonsor, S.J. (2019) The changing uses of herbarium data in an era of global change: an overview using automated content analysis. *BioScience*, 69, 812–822.

Horticulture Innovation Australia Limited (2019) *Australian Horticulture Statistics Handbook Fruit 2017/2018*. Sydney, Australia: Freshlogic, pp. 91–95.

Huelsenbeck, J.P. & Ronquist, F. (2001) MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17, 754–755.

Hyde, K.D., Chomnunti, P., Crous, P.W., Groenewald, J.Z., Damm, U., Ko Ko, T.W. et al. (2010) A case for re-inventory of Australia's plant pathogens. *Persoonia*, 25, 50–60.

Keeble-Gagnère, G., Rigault, P., Tibbits, J., Pasam, R., Hayden, M., Forrest, K. et al. (2018) Optical and physical mapping with local finishing enables megabase-scale resolution of agronomically important regions in the wheat genome. *Genome Biology*, 19. 112.

Kiss, L., Vaghefi, N., Bransgrove, K., Dearnaley, J.D.W., Takamatsu, S., Pei Tan, Y. et al. (2020) Australia: a continent without native powdery mildews? The first comprehensive catalogue indicates recent introductions and multiple host range expansion events, and leads to the re-discovery of *Salmonomyces* as a new lineage of the Erysiphales. *Frontiers in Microbiology*, 11, 1571.

Marmolejo, J., Siahaan, S.A., Takamatsu, S. & Braun, U. (2018) Three new records of powdery mildews found in Mexico with one genus and one new species proposed. *Mycoscience*, 59, 1–7.

McTaggart, A.R., Shivas, R.G., Doungsa-ard, C., Weese, T.L., Beasley, D.R., Hall, B.H. et al. (2016) Identification of rust fungi (Pucciniales) on species of *Allium* in Australia. *Australasian Plant Pathology*, 45, 581–592.

Meeboon, J., Takamatsu, S. & Braun, U. (2020) Morphophylogenetic analysis revealed that *Podosphaera tridactyla* constitutes a species complex. *Mycologia*, 112, 244–266.

Moparthi, S., Grove, G.G., Pandey, B. & Bradshaw, M. (2019) Phylogeny and taxonomy of *Podosphaera cerasi*, sp. nov., and *Podosphaera prunicola* sensu lato. *Mycologia*, 111, 647–659.

Pandey, B., Naidu, R.A. & Grove, G.G. (2018) Next generation sequencing analysis of double-stranded RNAs from sweet cherry powdery mildew fungus *Podosphaera prunicola*. *Journal of Plant Pathology*, 100, 435–446.

Pascoe, I.G. & Sivapalan, A. (1995) Revision of Erysiphales (powdery mildew fungi) in Australia, and reassessment of the classification of anamorphic mildews. In: *Proceedings of the 10th Australasian Plant Pathology Society Conference, Lincoln University, Christchurch, 28–30 August 1995*; Abstract 40. Lincoln: APPS.

Plant Health Australia (2017) *National Plant Biosecurity Status Report*. Canberra, ACT: Plant Health Australia.

Ristaino, J.B. (1998) The importance of archival and herbarium materials in understanding the role of oospores in late blight epidemics of the past. *Phytopathology*, 88, 1120–1130.

Ristaino, J.B. (2002) Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. *Microbes and Infection*, 4, 1369–1377.

Särkinen, T., Staats, M., Richardson, J.E., Cowan, R.S. & Bakker, F.T. (2012) How to open the treasure chest? Optimising DNA extraction from herbarium specimens. *PLoS One*, 7, e43808.

Sikes, B.A., Bufford, J.L., Hulme, P.E., Cooper, J.A., Johnston, P.R. & Duncan, R.P. (2018) Import volumes and biosecurity interventions shape the arrival rate of fungal pathogens. *PLoS Biology*, 16, e2006025.

Smith, R.L., Sawbridge, T., Mann, R., Kaur, J., May, T.W. & Edwards, J. (2020) Rediscovering an old foe: optimised molecular methods for

DNA extraction and sequencing applications for fungarium specimens of powdery mildew (Erysiphales). *PLoS One*, 15, e0232535.

Staats, M., Cuenca, A., Richardson, J.E., Vrielink-van Ginkel, R., Petersen, G., Seberg, O. et al. (2011) DNA damage in plant herbarium tissue. *PLoS One*, 6, e28448.

Staats, M., Erkens, R.H., Van De Vossenberg, B., Wieringa, J.J., Kraaijeveld, K., Stielow, B. et al. (2013) Genomic treasure troves: complete genome sequencing of herbarium and insect museum specimens. *PLoS One*, 8, e69189.

Stival Sena, J., Giguère, I., Rigault, P., Bousquet, J. & Mackay, J. (2018) Expansion of the dehydrin gene family in the Pinaceae is associated with considerable structural diversity and drought-responsive expression. *Tree Physiology*, 38, 442–456.

Takamatsu, S. (2004) Phylogeny and evolution of the powdery mildew fungi (Erysiphales, Ascomycota) inferred from nuclear ribosomal DNA sequences. *Mycoscience*, 45, 147–157.

Takamatsu, S., Niinomi, S., Harada, M. & Havrylenko, M. (2010) Molecular phylogenetic analyses reveal a close evolutionary relationship between *Podosphaera* (Erysiphales: Erysiphaceae) and its rosaceous hosts. *Persoonia*, 24, 38–48.

Virtue, J.G., Bennett, S.J. & Randall, R.P. (2004) Plant introductions in Australia: how can we resolve 'weedy 'conflicts of interest. In: Sindel, B.M. and Johnson, S.B. (Eds.) *Proceedings of the 14th Australian Weeds Conference*. Wagga Wagga: Weed Society of New South Wales, pp. 42–48.

Walker, J. (1983) Pacific mycogeography: deficiencies and irregularities in the distribution of plant parasitic fungi. *Australian Journal of Botany Supplementary Series*, 13, 89–136.

Wieß, C.L., Schuenemann, V.J., Devos, J., Shirsekar, G., Reiter, E., Gould, B.A. et al. (2016) Temporal patterns of damage and decay kinetics of DNA retrieved from plant herbarium specimens. *Royal Society Open Science*, 3, 160239.

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Chapter 4

Re-evaluation of the *Podosphaera tridactyla* species complex in Australia

4.1 Chapter Preface

Podosphaera tridactyla causes powdery mildew of many species of *Prunus,* including horticultural important stone fruit. It is morphologically variable, and a recent study revealed that *P*. *tridactyla* is a species complex consisting of 12 genetic species. Using the newly described species of the *P*. *tridactyla* complex as references, 58 *P*. *tridactyla* specimens held in Australian plant pathology reference collections were re-analysed using the protocol described in chapter 2. Phylogenetic analysis revealed that most Australian *P*. *tridactyla* specimens on stone fruit and closely related *Prunus* species belonged to *P*. *ampla,* with six belonging to an unidentified species. Additionally, two were identified as *P. pannosa*, the rose powdery mildew fungus, which is not part of the *P*. *tridactyla* species complex but is known to jump hosts when conditions are favourable. The undescribed powdery mildew species was restricted to Australian peach specimens from quarantine glasshouses and we describe it here as *P. cunningtonii*. Its origin is unknown. The protocol developed in Chapter 2 was demonstrated to have additional benefits over PCR-based sequence barcode methods. The chloroplast gene, *matK*, was extracted from the sequence data and used to confirm the specimen hosts. Four had been wrongly identified as *Prunus* and were, in fact, *Malus prunifolia* (Willd.) Borkh.*,* commonly known as plum-leaf crab apple. Other hosts were only identified as *Prunus* sp. and many of these were able to be updated.

This chapter is presented in published format.

4.2 Publication details

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4.3 Statement of contribution of joint authorship

RLS, JE, TIS and TWM conceived the idea for the study and all the authors (RLS, TIS, RCM, JK, TWM, IGP and JE) contributed to the design. RLS performed the experimental DNA extraction and sequencing, with assistance and advice from JK. TIS assisted RLS with the bioinformatics. TWM and JE assisted and advised RLS with the phylogenetic analyses. IGP provided the morphological data obtained from the fresh specimens in 1995 and the taxonomic drawings for the new species description. Data analyses and interpretation were conducted by RLS with assistance and advice from all authors. RLS wrote the manuscript, which was reviewed by TWM and JE to shape the final version. All authors approved the final version of the manuscript.

4.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Smith, R.L., May, T.W., Kaur, J., Sawbridge, T., Mann, R., Pascoe, I.G. and Edwards, J., 2021. Re-evaluation of the *Podosphaera tridactyla* species complex in Australia. J. Fungi 2021, 7, 171 DOI:10.3390/jof7030171' I can confirm that Reannon L. Smith made the following contributions:

- Literature review
- Development of the experimental design
- Preparation of powdery mildew specimens
- DNA extractions
- Library preparations
- Illumina sequencing
- Sequence data extraction and analysis
- Phylogenetic analyses of fungal and host data
- Generated all figures except the taxonomic drawing of the new species description
- Writing the manuscript and critical appraisal of content"

Dr. Jacqueline Edwards

Date: 13/12/2020

Journal of *Fungi*

MDPI

Article

Re-Evaluation of the *Podosphaera tridactyla* Species Complex in Australia

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Abstract: The *Podosphaera tridactyla* species complex is highly variable morphologically and causes powdery mildew on a wide range of *Prunus* species, including stone fruit. A taxonomic revision of the *Po. tridactyla* species complex in 2020 identified 12 species, seven of which were newly characterised. In order to clarify which species of this complex are present in Australia, next generation sequencing was used to isolate the fungal ITS+28S and host *matK* chloroplast gene regions from 56 powdery mildew specimens of stone fruit and ornamental *Prunus* species accessioned as *Po. tridactyla* or *Oidium* sp. in Australian reference collections. The specimens were collected in Australia, Switzerland, Italy and Korea and were collected from 1953 to 2018. Host species were confirmed using *matK* phylogenetic analysis, which identified that four had been misidentified as *Prunus* but were actually *Malus prunifolia*. *Podosphaera* species were identified using ITS+28S phylogenetic analysis, recognising three *Podosphaera* species on stone fruit and related ornamental *Prunus* hosts in Australia. These were *Po. pannosa*, the rose powdery mildew, and two species in the *Po. tridactyla* species complex: *Po. ampla*, which was the predominant species, and a previously unidentified species from peach, which we describe here as *Po. cunningtonii*.

Keywords: stone fruit; powdery mildew fungi; *Prunus*; *Podosphaera ampla*; *Podosphaera cunningtonii*

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1. Introduction

Stone fruit such as peaches (*Prunus persica*), cherries (*Pr. avium*), apricots (*Pr. armeniaca*), plums (*Pr. domestica*, *Pr. salicina* and *Pr. cerasifera*) and almonds (*Pr. dulcis*) belong to the genus *Prunus* (Rosaceae), which contains approximately 250 species distributed across temperate regions worldwide [1]. In Australia, there are only two native *Prunus* species, *Pr. brachystachya* and *Pr. turneriana*; both are in the subgenus *Cerasus* and found in tropical rainforests of north east Australia. A further 15 species of *Prunus* have been introduced to Australia as horticultural crops and for use in gardens [2]. On the basis of phylogenetic analysis of multiple genes, the genus is subdivided into three subgenera: *Cerasus* (cherries), *Padus* (bird cherries, including species formerly placed in *Laurocerasus*) and *Prunus* (plums) and the latter subgenus is further subdivided into seven sections: *Amygdalus*, *Armeniaca*, *Emplectocladus*, *Microcerasus*, *Persicae*, *Prunocerasus* and *Prunus* [3]. The three subgenera are also distinguished on morphology according to inflorescence structure, where subgenus *Cerasus* has corymbose inflorescences, *Padus* has racemose inflorescences and *Prunus* has solitary inflorescences [4].

The Australian stone fruit industry was established in the late 1800s by European and Chinese settlers who introduced apricot, peach, nectarine and plums that were in

cultivation across Asia, Europe and the USA. [5]. By 2017-18, the Australian stone fruit industry (comprising apricots, nectarines, peaches and plums) produced 153,148 tons (t) of fruit with a wholesale value of AUD 391.7 M. [6]. The 2017-18 net stone fruit supply was divided into export (17,769 t), processing (31,790 t) and domestic fresh supply (106,684 t). The export market is predominantly China, Indonesia, Singapore, Saudi Arabia and United Arab Emirates. Stone fruit is grown in temperate regions of all states of Australia, with Victoria producing the majority (108,197 t). Peaches and nectarines are the dominant crop (88,787 t), followed by plums (15,099 t) and apricots (4311 t) [6].

Stone fruit are affected by several powdery mildew fungi, including *Podosphaera tridactyla*, which is known to be highly variable morphologically, with a wide host range among *Prunus* species [7]. Powdery mildew infects leaves and stems, reducing the plant's photosynthetic capabilities and fruit production [8]. Severe infection causes cupping and malformation of the leaves and infected fruit, resulting in further crop losses [9].

In 2005, Cunnington and co-workers investigated genetic variation within *Po. Tridactyla*, studying specimens from Australia, South Korea and Switzerland, using restriction fragment length polymorphism (RFLP) and rDNA ITS (internal transcribed spacer) sequence analyses [10]. RFLP analysis divided the specimens into six groups, with four of the groups (1–4) differing by a single restriction enzyme pattern. Based on the ITS sequence analysis, there were three well-supported clades. Clade 1 contained RFLP Group 5 from hosts including an unidentified *Prunus* sp., *Pr. cerasifera* and *Pr. armeniaca* (all belonging to *Prunus* subgenus *Prunus*). Clade 2 contained RFLP Groups 1–4 from *Pr. persica*, *Pr. japonica*, *Pr. padus*, *Pr. laurocerasus* and *Pr. lusitanica* (*Prunus* subgenera *Cerasus*, *Padus* and *Prunus*). Clade 3 contained RFLP Group 6 from an unknown *Prunus* sp. and *Pr. apetala* (*Prunus* subgenus *Cerasus*). The clade on hosts from *Prunus* subgenera *Cerasus*, *Padus* and *Prunus* represented *Po. tridactyla* in the strict sense, originally described from *Pr. padus*, while the other two taxa were undescribed species morphologically indistinguishable from *Po. tridactyla* in the strict sense.

In their taxonomic revision of Erysiphales, Braun and Cook [7] treated *Po. tridactyla* as a species complex, with no clear morphological delimitation between "typical" collections of *Po. tridactyla* and deviating forms. They did recognise *Podosphaera longiseta* as a distinct species within the *Po. tridactyla* species complex, but concluded that further molecular, biological and morphotaxonomic studies were required to fully recognise species diversity within the species complex.

Meeboon et al. [11] published a wide ranging morphological and molecular taxonomic revision of powdery mildew fungi on *Prunus*, examining 30 specimens from 16 hosts and five countries across Asia and Europe. They characterised the *Po. tridactyla* species complex as comprising 12 species, of which seven were newly described: *Po. ampla*, *Po. pruni-avium*, *Po. pruni-cerasoidis*, *Po. prunigena*, *Po. pruni-japonicae*, *Po. pruni-lusitanicae* and *Po. prunina*. Additionally belonging to the complex were *Po. tridactyla* in the strict sense, *Po. longiseta* and *Po. salatai* and the two undescribed *Podosphaera* species from Australia. These two undescribed species formed distinct lineages but could not be described as the authors did not have access to the physical specimens, only DNA sequence data from Cunnington et al. [10]. Additionally, Meeboon et al. [11] identified host specificity at the subgenus level in *Prunus*, suggesting a degree of coevolution between species of the *Po. tridactyla* complex and their hosts. The radiation of *Prunus* subgenera identified by Chin et al. [4] conforms with the divergence of *Po. tridactyla* complex species across Eurasia, for example, *Po. tridactyla* in the strict sense is a European species found on hosts within *Prunus* subgenus *Padus* and *Po. ampla* is of Asian origin, infecting hosts within the *Prunus* subgenus *Prunus* [11].

The objective of the current study was to clarify which species of the *Po. tridactyla* complex are present in Australia, utilising the next generation sequencing (NGS) methods developed by Smith et al. [12], based on a re-examination of powdery mildew collections from horticultural *Prunus* species and closely related ornamental *Prunus* species in Australia held in Australian plant pathogen reference collections.

2. Methods

All powdery mildew collections identified as *Podosphaera tridactyla* or *Oidium* sp. on *Prunus* hosts were obtained from the three major Australian plant pathogen reference collections (Queensland Plant Pathogen Herbarium (BRIP—two collections), New South Wales Plant Pathology Biosecurity Collections (DAR—32 collections) and Victorian Plant Pathogen Herbarium (VPRI—125 collections)). Collections in BRIP and DAR were all from Australia, while those in VPRI were from Australia, Switzerland, Italy and Korea. All collections were inspected for DNA extraction suitability as described by Smith et al. [12], based on number of leaves in the packet, level of powdery mildew infection present on the leaves and if the specimens were glued to mounting paper. Using these criteria, 58 collections contained specimens that were suitable for DNA extraction (Table 1).

Table 1. Collection details for the 58 powdery mildew collections on *Prunus* used in this study with fungus and plant host species as listed with collection and GenBank accession numbers. For hosts, abbreviations in brackets refer to divisions within *Prunus*: AM: subgenus *Prunus* section *Amygdalus*, CR: subgenus *Cerasus*, LR: subgenus *Padus* section *Laurocerasus*, PD: subgenus *Padus* section *Padus* and PR: subgenus *Prunus* section *Prunus*. *Po.* = *Podosphaera*, *Pr.* = *Prunus*.

Collection Number	Date	Country	Fungus	GenBank ITS+28S	Host	GenBank *matK*
BRIP 8323	1958	Australia	*Po. tridactyla*	MW364487	*Pr. persica* (AM)	MW369028
BRIP 15118	1986	Australia	*Oidium* sp.	MW364537	*Pr. persica* (AM)	MW369020
DAR 12478	1962	Australia	*Oidium* sp.	MW364486	*Pr. armeniaca* (PR)	MW369006
DAR 28962	1977	Australia	*Oidium* sp.	MW364533	*Pr. persica* (AM)	MW369032
DAR 28963	1977	Australia	*Oidium* sp.	MW364528	*Pr. mahaleb* (CR)	MW369056
DAR 35281	1980	Australia	*Oidium* sp.	-	*Pr. laurocerasus* (LR)	-
DAR 64667	1989	Australia	*Po. tridactyla*	-	*Pr. persica* (AM)	-
DAR 71638	1996	Australia	*Oidium* sp.	MW364532	*Pr. laurocerasus* (LR)	MW369033
VPRI 12495	1984	Australia	*Po. tridactyla*	MW364488	*Pr. persica* (AM)	MW369060
VPRI 18452	1992	Australia	*Po. tridactyla*	MW364489	*Prunus* sp.	MW369058
VPRI 18514	1992	Australia	*Po. tridactyla*	MW364490	*Pr. domestica* (PR)	MW369061
VPRI 18600	1992	Australia	*Po. tridactyla*	MW364521	*Prunus* sp.	MW369043
VPRI 18885	1993	Australia	*Po. tridactyla*	MW364522	*Prunus* sp.	MW369044
VPRI 19000	1993	Australia	*Po. tridactyla*	MW364523	*Prunus* sp.	MW369045
VPRI 19006	1993	Australia	*Po. tridactyla*	MW364529	*Prunus* sp.	MW369050
VPRI 19008	1993	Australia	*Po. tridactyla*	MW364524	*Pr. armeniaca* (PR)	MW369007
VPRI 19015	1993	Australia	*Po. tridactyla*	MW364538	*Pr. domestica* (PR)	MW369021
VPRI 19017	1993	Australia	*Po. tridactyla*	MW364525	*Pr. domestica* (PR)	MW369022
VPRI 19022	1993	Australia	*Po. tridactyla*	MW364520	*Prunus* sp.	MW369046
VPRI 19164	1993	Australia	*Po. tridactyla*	MW364519	*Pr. armeniaca* (PR)	MW369008
VPRI 19238	1993	Australia	*Po. tridactyla*	MW364526	*Pr. cerasifera* (PR)	MW369015
VPRI 19248	1993	Australia	*Po. tridactyla*	MW364518	*Prunus* sp.	MW369047
VPRI 19319	1993	Australia	*Po. tridactyla*	MW364517	*Pr. domestica* (PR)	MW369023
VPRI 19591	1993	Australia	*Po. tridactyla*	MW364516	*Pr. persica* (AM)	MW369035
VPRI 19788	1994	Australia	*Po. tridactyla*	MW364515	*Prunus* sp.	MW369048
VPRI 19837	1994	Australia	*Po. tridactyla*	MW364527	*Pr. cerasifera* (PR)	MW369016

Table 1. *Cont.*

Collection Number	Date	Country	Fungus	GenBank ITS+28S	Host	GenBank *matK*
VPRI 19864	1994	Australia	*Po. tridactyla*	MW364514	*Pr. armeniaca* (PR)	MW369009
VPRI 19865	1994	Australia	*Po. tridactyla*	MW364513	*Pr. armeniaca* (PR)	MW369010
VPRI 19868	1994	Australia	*Po. tridactyla*	MW364512	*Pr. persica* (AM)	MW369036
VPRI 19871	1994	Australia	*Po. tridactyla*	MW364511	*Pr. domestica* (PR)	MW369024
VPRI 19872	1994	Australia	*Po. tridactyla*	MW364510	*Pr. domestica* (PR)	MW369025
VPRI 19873	1994	Australia	*Po. tridactyla*	MW364509	*Pr. domestica* (PR)	MW369026
VPRI 20027	1994	Australia	*Po. tridactyla*	MW364508	*Prunus* sp.	MW369049
VPRI 20040	1994	Australia	*Po. tridactyla*	MW364507	*Prunus* sp.	MW369051
VPRI 20041	1994	Australia	*Po. tridactyla*	MW364506	*Prunus* sp.	MW369052
VPRI 20045	1994	Australia	*Po. tridactyla*	MW364505	*Pr. salicina* (PR)	MW369039
VPRI 20097	1994	Australia	*Po. tridactyla*	MW364504	*Pr. armeniaca* (PR)	MW369011
VPRI 20231	1990	South Korea	*Po. tridactyla*	MW364503	*Pr. yedoensis* (CR)	MW369055
VPRI 20233	1993	South Korea	*Po. tridactyla*	MW364534	*Pr. padus* (PD)	MW369030
VPRI 20490	1993	South Korea	*Po. tridactyla*	MW364536	*Pr. padus* (PD)	MW369031
VPRI 20491	1993	South Korea	*Po. tridactyla*	MW364502	*Pr. sargentii* (CR)	MW369042
VPRI 20514	1995	Australia	*Po. tridactyla*	MW364496	*Pr. cerasifera* (PR)	MW369017
VPRI 20687	1995	Australia	*Po. tridactyla*	MW364491	*Prunus* sp.	MW369059
VPRI 20705	1995	Australia	*Po. tridactyla*	MW364497	*Pr. persica* (AM)	MW369037
VPRI 20706	1995	Australia	*Po. tridactyla*	MW364540	*Pr. persica* (AM)	MW369038
VPRI 20797	1996	Australia	*Po. tridactyla*	MW364498	*Pr. armeniaca* (PR)	MW369057
VPRI 20811	1996	Australia	*Po. tridactyla*	MW364499	*Pr. armeniaca* (PR)	MW369012
VPRI 21717	1998	Australia	*Po. tridactyla*	MW364531	*Prunus* sp.	MW369053
VPRI 22156	1995	Switzerland	*Po. tridactyla*	MW364539	*Pr. laurocerasus* (LR)	MW369029
VPRI 22159	1994	Switzerland	*Po. tridactyla*	MW364535	*Pr. padus* (PD)	MW369034
VPRI 22167	1995	Switzerland	*Po. tridactyla*	MW364500	*Pr. domestica* (PR)	MW369027
VPRI 22232	2000	Australia	*Po. tridactyla*	MW364541	*Pr. salicina x persica* (PR/AM)	MW369040
VPRI 22233	2000	Australia	*Po. tridactyla*	MW364501	*Pr. salicina x persica* (PR/AM)	MW369041
VPRI 41641	2006	South Korea	*Po. tridactyla*	MW364530	*Pr. armeniaca* (PR)	MW369013
VPRI 43878	2018	Australia	*Podosphaera* sp.	MW364492	*Prunus* sp.	MW369054
VPRI 43879	2018	Australia	*Podosphaera* sp.	MW364493	*Pr. cerasifera "elvins"* (PR)	MW369018
VPRI 43880	2018	Australia	*Podosphaera* sp.	MW364495	*Pr. cerasifera "elvins"* (PR)	MW369019
VPRI 43881	2018	Australia	*Podosphaera* sp.	MW364494	*Pr. armeniaca* (PR)	MW369014

2.1. Fungal Sampling and Morphological Characterisation

Powdery mildew conidia, mycelia and chasmothecia were collected from the specimens by using a 6 mm leaf punch or scraping with a blade. Sampling was completed under clean room conditions to minimise contamination with modern DNA.

The VPRI specimens collected between 1993 and 1995 were morphologically examined while fresh at the time of collection. The hyphae, conidiophores and conidia were collected off the leaf surface with clear cellotape, which was then mounted on a microscope slide with lactofuchsin mountant and observed using light microscopy. Lactofuchsin preparation was as follows: lactic acid 20 g, glycerol 40 g, water (H_2O) 20 mL and acid fuchsin 0.1 g; this was adapted from the Johnson and Booth [13] lactophenol mountant recipe minus the phenol. Germination patterns were studied on host tissue by pressing a sporulating colony onto a fresh, healthy leaf and incubating the leaf in a petri dish with moist filter paper, then examined by cellotape strip after 24 and 48 h. Reference collection specimens collected between 1977 and 1992 were rehydrated before examination, as described by Shin [14] and Shin and La [15], by placing a small section of infected leaf upside down in a drop of lactic acid on a microscope slide, then placing a flame underneath to boil. Once boiled, the rehydrated fungal material was scraped off the leaf surface and mounted in lactic acid for examination by light microscopy [14,15].

Morphological characters were recorded for both fresh and reference collection specimens as follows: mycelium growth pattern and hyphal structure, mycelial appressoria shape, conidiophore characteristics (length, shape, position of basal septum), conidial chain edge line, conidial size and shape, presence or absence of fibrosin bodies and germination characters.

2.2. DNA Extraction and Quantification

DNA was extracted following the E.Z.N.A.® Forensic DNA protocol [12]. The DNA extraction initial incubation step was increased to one hour and included a repeated final elution step (50 µL of elution buffer were added to the filter column with a 5 min incubation for a final volume of 100 µL). A NanoDrop 2000™ (Thermo Fisher Scientific, Waltham, Massachusetts) was used to assess DNA quality using the 260/280 nm absorbency ratio (1.8–1.9). DNA concentrations were quantified using two methods: Quantus™ fluorometer (Promega) and Agilent Tapestation® electrophoresis (Agilent Technologies, Santa Clara, CA, USA).

2.3. Library Preparation and Sequencing

Library preparation was as described in Smith et al. [12]. Libraries comprising VPRI DNA were paired-end sequenced on the Illumina HiSeq 3000 platform (San Diego, CA, USA). Libraries comprising BRIP and DAR DNA were paired-end sequenced on the Illumina MiSeq platform, due to sequencing platform accessibility constraints at the time.

2.4. Sequence Analysis

Sequence reads were assigned to each sample based on their indices. Raw DNA-Seq files were filtered using the Nuclear program to trim adapters and retain only the sequences above the cut-off value of 100 bp read length and 20 mismatches. The library preparations were expected to contain *Podosphaera* DNA and plant host DNA, as well as DNA from microbes present on the leaf surface at the time of collection. Therefore, the filtered, high-quality (HQ) reads were mapped as paired-end reads to *Podosphaera* and plant host reference sequences. For *Podosphaera*, the reference sequences used were the internal transcribed spacer ITS (ITS1-5.8S-ITS2) and 28S rDNA regions of *Po. ampla* (GenBank accession MK530453), *Po. cerasi* (KX826855), *Po. clandestina* (KY660805), *Po. leucotricha* (KX842350), *Po. longiseta* (MK530459), *Po. pannosa* (KX842349), *Po. prunigena* (AB936275), *Po. prunina* (MK530442), *Po. pruni-avium* (MK530457, KP641982), *Po. pruni-cerasoides* (MK530448), *Po. pruni-japonicae* (MK530455), *Po. pruni-lusitanica* (KP641993), *Po. salatai* (AB525929), *Po.* sp (AY833653) and *Po. tridactyla s. str.* (MK530462). The plant host

was identified by mapping to *matK* chloroplast plastid regions. Reference sequences were those identified by Chin et al. [4] or taken directly from GenBank. The *matK* accessions from Chin et al. [4] were: *Pr. armeniaca* (HQ235100), *Pr. cerasifera* (HQ619834), *Prunus domestica* (HQ235146), *Pr. laurocerasus* (HQ235181), *Pr. mahaleb* (HQ235184), *Pr. padus* (HQ235216), *Pr. persica* (HQ235409), *Pr. salicina* (HQ235252), and the others from GenBank were *Pr. yedoensis* (GQ248191) and *Malus prunifolia* (JQ391019). The sequence mapping was performed by the program Nuclear, generating reference-initiated sequence alignments to be viewed in Vision software (version 3.3.6 Gydle Inc. Bioinformatics Service, Québec City, Québec; http://www/gydle.com, accessed on 3 February 2021). In Vision, ITS+28S and *mat*K sequences were edited to incorporate single nucleotide polymorphisms (SNPs) and indels which related to each individual specimen's sequence data. A mapping threshold was determined by a minimum of 5x coverage of the complete reference sequence and sequence files which did not meet this criterion were excluded. Mapping success was determined from the Vision images of each sequence file by calculating the total number of aligned DNA sequence reads and converting it into a percentage from the total number of HQ reads. The mapped ITS+28S and *mat*K sequences generated for this study were exported from Vision for identification to species by BLASTn and phylogenetic analysis.

2.5. Phylogenetic Analysis

Based on preliminary phylogenetic analysis, two data sets were generated for the sequences originally identified as *Po. tridactyla*. The first data set (Figure 1) was constructed for ITS (rDNA ITS1-5.8S-ITS2) sequences from VPRI *Po. tridactyla* specimens reidentified as *Po. leucotricha* and *Po. pannosa*, which included sequences from closely related *Podosphaera*. The second data set contained ITS+28S (rDNA ITS1-5.8S-ITS2 + rDNA 28S large subunit) sequences from the *Po. tridactyla* complex (Figure 2). Both data sets included sequences generated from this study and sequences of *Podosphaera* obtained from GenBank that were selected from published studies [11,16,17] and NCBI searches of *Podosphaera* spp. ITS and ITS+28S sequences. Phylogenetic analyses of these two data sets used *Cystotheca lanestris* (GenBank accessions AF011289 and AF011288) as the outgroup taxon, in line with Meeboon et al. [11]. The phylogenetic analysis of host *matK* sequences included sequences from *Prunus* species subgenera *Cerasus*, *Padus* and *Prunus* obtained from Chin et al. [4] and the outgroup was *Oemleria cerasiformis* (*matK* AF288110). Alignments were generated in Geneious Prime using the Muscle 3.8.425 [18,19] alignment tool with suggested settings followed by manual refinement. Phylogenetic trees were obtained from the aligned sequence data by maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed in PhyML (3.3.20180621) [20] with the general time-reversible (GTR) substitution model with optimization for topology/length/rate with the proportion of invariable sites set at 0 and number of substitution rate categories at 4. The bootstrap analysis was set at 1000 replications with the stepwise addition option set as simple. BI analysis was completed in MrBayes (3.2.6) [21] using two runs with four chains each under the GTR model and run assuming a gamma distribution variation. Four heated chains and a single cold chain were used in all Markov chain Monte Carlo (MCMC) analyses, which were run for 1,100,000 generations and sampled one tree every 200 generations. Burn-in length was set at 100,000.

To identify characteristic bases for sets of closely related species, summaries of variable base positions were generated for (1) *Po. ampla* and sister taxa and (2) species closely related to *Po. pruni-avium*. For each set of sequences, outgroup sequences were removed, and the sequences of interest were realigned. Masking of the amended alignments by removing sites containing identical bases, leaving only sites with variable bases (including gaps), was performed by the Mask Alignment tool in Geneious Prime. In addition, positions where only one sequence within a species varied from the consensus were removed manually in Geneious Prime. Base position numbers of the variable sites were manually edited in Microsoft PowerPoint to reflect base positions in the original alignment.

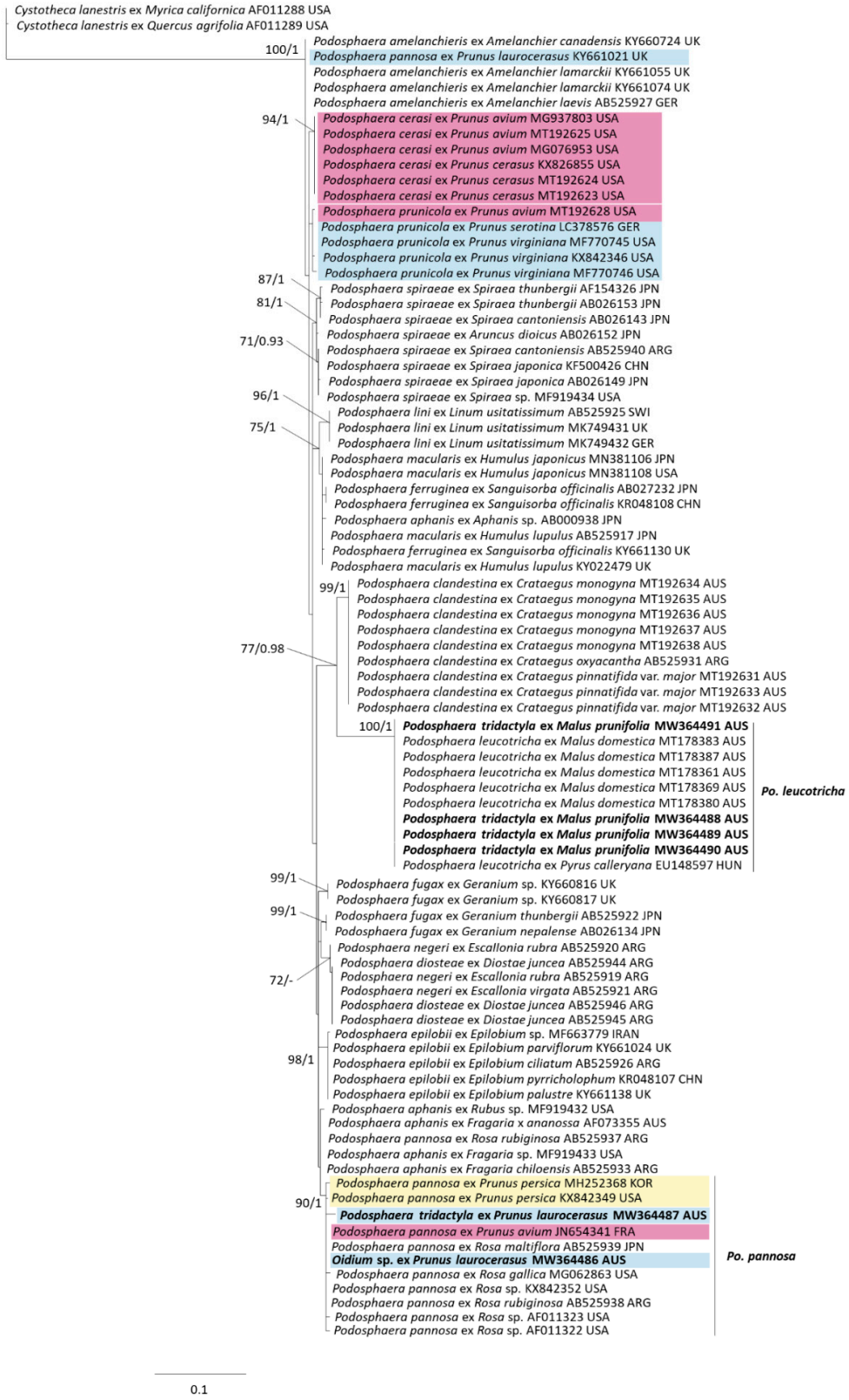

Figure 1. Maximum likelihood phylogenetic analysis of nuclear rDNA ITS (ITS1-5.8-ITS2) sequences for *Podosphaera pannosa* and *Po. leucotricha* and closely related species. Branch support values for maximum likelihood and Bayesian inference analyses are shown when >70% and 0.85, respectively. Sequences generated in this study are shown in bold with names as originally listed in reference collections. Label colours represent *Prunus* subgenera; *Cerasus* is pink, *Padus* is blue and *Prunus* is yellow.

Figure 2. Maximum likelihood phylogenetic analysis of nuclear rDNA ITS1-5.8S-ITS2 and 28S sequences for the *Po. tridactyla* species complex and closely related species. Branch support values for maximum likelihood and Bayesian inference analyses are shown when >70% and 0.85, respectively. *Type indicates sequences obtained from isotype or holotype specimen for that species. Sequences generated in this study are shown in bold with names as originally listed in reference collections. Colours represent *Prunus* subgenera; *Cerasus* is pink, *Padus* is blue and *Prunus* is yellow.

3. Results

3.1. Sequencing

DNA from 56 of the 58 collections were successfully mapped to reference sequences of the powdery mildew (*Podosphaera*) ITS+28S region and host plant chloroplast gene *matK*. The exceptions were DAR 35281 and DAR 64667, which failed to meet the mapping threshold. The ITS+28S and *matK* sequences generated from this study were confirmed as belonging to *Podosphaera* and *Prunus*, respectively, using BLASTn. The BLASTn analysis of the powdery mildews detected six collections which did not match with species of the *Po. tridactyla* complex and matched to either *Po. pannosa* or *Po. leucotricha*. These six sequences were placed in a data set with other sequences identified at *Po. leucotricha* and *Po. pannosa* as well as closely related *Podosphaera* species, as they are not part of the *Po. tridactyla* species complex. The remaining ITS+28S sequences returned BLASTn percentage identities of at least 98% and up to 100% (E values were 0.0) with members of the *Po. tridactyla* species complex.

3.2. Phylogeny

Phylogenetic analysis of the first data set compared ITS sequences of the six collections reidentified as *Po. pannosa* and *Po. leucotricha* with closely matched *Podosphaera* from GenBank and published sources [16,17] to total 88 sequences of 508 base pairs (Figure 1). A well-supported clade (bootstrap (BS) support 90 and posterior probability (BI) 1) consisted of nine sequences identified as *Po. pannosa* along with sequences from BRIP 8232 (originally identified as *Po. tridactyla*) and DAR 12478 (originally identified as *Oidium* sp.) Another well-supported clade (BS 100, BI 1) consisted of seven sequences identified as *Po. leucotricha* along with sequences from VPRI 12495, 18514, 18452 and 20687 (all originally identified as *Po. tridactyla*) (Figure 1).

Phylogenetic analysis of the second data set, comprising 75 ITS+28S sequences of 1251 base pairs, showed that collections from Australia represented two species of the *Po. tridactyla* species complex: *Po. ampla* on *Prunus* subgenus *Prunus* hosts and a previously undescribed *Podosphaera* species on *Prunus persica* and *Pr. mahaleb* (from *Prunus* subgenus *Prunus* and *Cerasus* (Figure 2)). A well-supported clade (BS 100, BI 1) consisted of five sequences from collections identified as *Po. ampla*, all from Germany, along with 37 sequences from Australian collections previously identified as *Po. tridactyla* and *Oidium* sp. (BRIP 15118). Within this clade, there was a moderately supported BI 0.85 subclade containing the *Po. ampla* reference sequences from Germany and nine sequences from Australian collections (Figure 2). Within this subclade, two of the collections from Germany (MK530450 and MK530451) fell into a further subclade (BS 87, BI 1). Additional VPRI collections from outside Australia fell within four of the established species: *Po. prunigena* (VPRI 20231 and 20491, South Korea), *Po. prunina* (VPRI 41641, South Korea), *Po. pruni-japonicae* (VPRI 20233 and 20490 South Korea) and *Po. pruni-avium* (VPRI 22156, Switzerland and VPRI 22159, Switzerland) (Figure 2) (Table 2).

A summary of variable bases for the *Po. ampla* sequences shows that the subclade containing the *Po. ampla* sequences from Germany, when compared to the remaining members of the clade, exhibits a one base pair difference at position 445 where a T is present instead of a C (Figure S1). Sequences MK530450 and MK530451 from Germany have two additional base changes at positions 813 (G instead of T) and 816 (A instead of G) (Figure S1). Sequence AF154321 displayed identical base pairs to other *Po. ampla* sequences, although it was significantly shorter at 479 bp in length. Sequence AY833656 was also shorter in length (503bp) but included three variants at positions 545 (A instead of G), 546 (T instead of A) and 550 (A instead of G).

Table 2. Powdery mildew fungal specimens with updated fungus and host species names. These revised identifications are based on ITS+28S and chloroplast gene *matK* phylogenies, respectively. For hosts, abbreviations in brackets refer to divisions within *Prunus*: AM: subgenus *Prunus* section *Amygdalus*, CR: subgenus *Cerasus*, LR: subgenus *Padus* section *Laurocerasus*, PD: subgenus *Padus* section *Padus* and PR: subgenus *Prunus* section *Prunus*. The *Prunus domestica* group includes *Pr. domestica*, *Pr. cerasifera* and *Pr. salicina*, which are not readily distinguishable in the host *matK* phylogeny. * Indicates a host that was originally misidentified. *Po.* = *Podosphaera*, *Pr.* = *Prunus*.

Collection Number	Original Fungus	Reidentified Fungus	Original Host	Reidentified Host
BRIP 8323	*Po. tridactyla*	*Po. pannosa*	*Pr. persica* (AM) *	*Pr. laurocerasus* (LR)
BRIP 15118	*Oidium* sp.	*Po. ampla*	*Pr. persica* (AM) *	*Pr. domestica* group (PR)
DAR 12478	*Oidium* sp.	*Po. pannosa*	*Pr. armeniaca* (PR)	*Pr. armeniaca* (PR)
DAR 28962	*Oidium* sp.	*Po. cunningtonii*	*Pr. persica* (AM)	*Pr. persica* (AM)
DAR 28963	*Oidium* sp.	*Po. cunningtonii*	*Pr. mahaleb* (CR)	*Pr. mahaleb* (CR)
DAR 71638	*Oidium* sp.	*Po. cunningtonii*	*Pr. laurocerasus* (LR) *	*Pr. persica* (AM)
VPRI 12495	*Po. tridactyla*	*Po. leucotricha*	*Pr. persica* (AM) *	*Malus prunifolia*
VPRI 18452	*Oidium* sp.	*Po. leucotricha*	*Pr. laurocerasus* (LR) *	*M prunifolia*
VPRI 18514	*Po. tridactyla*	*Po. leucotricha*	*Pr. persica* (AM) *	*M. prunifolia*
VPRI 18600	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 18885	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)
VPRI 19000	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 19006	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 19008	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. armeniaca* (PR)
VPRI 19015	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 19017	*Po. tridactyla*	*Po. ampla*	*Pr. armeniaca* (PR)	*Pr. domestica* group (PR)
VPRI 19022	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)
VPRI 19164	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. armeniaca* (PR)
VPRI 19238	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 19248	*Po. tridactyla*	*Po. ampla*	*Pr. armeniaca* (PR)	*Pr. domestica* group (PR)
VPRI 19319	*Po. tridactyla*	*Po. ampla*	*Pr. cerasifera* (PR)	*Pr. domestica* group (PR)
VPRI 19591	*Po. tridactyla*	*Po. cunningtonii*	*Prunus* sp.	*Pr. persica* (AM)
VPRI 19788	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)
VPRI 19837	*Po. tridactyla*	*Po. ampla*	*Pr. persica* (AM)	*Pr. domestica* group (PR)
VPRI 19864	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. armeniaca* (PR)
VPRI 19865	*Po. tridactyla*	*Po. ampla*	*Pr. cerasifera* (PR)	*Pr. armeniaca* (PR)
VPRI 19868	*Po. tridactyla*	*Po. ampla*	*Pr. armeniaca* (PR) *	*Pr. domestica* group (PR)
VPRI 19871	*Po. tridactyla*	*Po. ampla*	*Pr. armeniaca* (PR)	*Pr. domestica* group (PR)
VPRI 19872	*Po. tridactyla*	*Po. ampla*	*Pr. persica* (AM) *	*Pr. domestica* group (PR)*
VPRI 19873	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)
VPRI 20027	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)
VPRI 20040	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)
VPRI 20041	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 20045	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 20097	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 20231	*Po. tridactyla*	*Po. prunigena*	*Pr. salicina* (PR)	*Pr. yedoensis* (CR)
VPRI 20233	*Po. tridactyla*	*Po. pruni-japonicae*	*Pr. armeniaca* (PR)	*Pr. padus* (PD)
VPRI 20490	*Po. tridactyla*	*Po. pruni-japonicae*	*Pr. yedoensis* (CR)	*Pr. padus* (PD)
VPRI 20491	*Po. tridactyla*	*Po. prunigena*	*Pr. padus* (PD)	*Pr. sargentii* (CR)
VPRI 20514	*Po. tridactyla*	*Po. ampla*	*Pr. padus* (PD)	*Pr. domestica* group (PR)
VPRI 20687	*Po. tridactyla*	*Po. leucotricha*	*Pr. sargentii* (CR) *	*M. prunifolia*
VPRI 20705	*Po. tridactyla*	*Po. cunningtonii*	*Pr. cerasifera* (PR)	*Pr. persica* (AM)
VPRI 20706	*Po. tridactyla*	*Po. cunningtonii*	*Prunus* sp.	*Pr. persica* (AM)
VPRI 20797	*Po. tridactyla*	*Po. ampla*	*Pr. persica* (AM)	*Pr. armeniaca* (PR)
VPRI 20811	*Po. tridactyla*	*Po. ampla*	*Pr. armeniaca* (PR)	*Pr. armeniaca* (PR)
VPRI 21717	*Po. tridactyla*	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 22156	*Po. tridactyla*	*Po. pruni-avium*	*Pr. laurocerasus* (LR)	*Pr. laurocerasus* (LR)
VPRI 22159	*Po. tridactyla*	*Po. pruni-avium*	*Pr. padus* (PD)	*Pr. padus* (PD)
VPRI 22167	*Po. tridactyla*	*Po. ampla*	*Pr. domestica* (PR)	*Pr. domestica* group (PR)

Table 2. *Cont.*

Collection Number	Original Fungus	Reidentified Fungus	Original Host	Reidentified Host
VPRI 22232	*Po. tridactyla*	*Po. ampla*	*Pr. salicina x persica* (PR/AM)	*Pr. persica* (AM)
VPRI 22233	*Po. tridactyla*	*Po. ampla*	*Pr. salicina x persica* (PR/AM)	*Pr. persica* (AM)
VPRI 41641	*Po. tridactyla*	*Po. prunina*	*Pr. armeniaca* (PR)	*Pr. armeniaca* (PR)
VPRI 43878	*Podosphaera* sp.	*Po. ampla*	*Prunus* sp.	*Pr. domestica* group (PR)
VPRI 43879	*Podosphaera* sp.	*Po. ampla*	*Pr. cerasifera "elvins"* (PR)	*Pr. domestica* group (PR)
VPRI 43880	*Podosphaera* sp.	*Po. ampla*	*Pr. cerasifera "elvins"* (PR)	*Pr. domestica* group (PR)
VPRI 43881	*Podosphaera* sp.	*Po. ampla*	*Pr. armeniaca* (PR)	*Pr. armeniaca* (PR)

Separate analyses of alignments of each of ITS (ITS1-5.8S-ITS2), including 134 sequences covering 486 base pairs, and of 28S (large subunit) including 73 sequences of 808 base pairs, formed the same overall structure regarding all major clades. All Australian sequences generated from this study fell into two well-supported clades (*Po. ampla* and the unknown species of *Podosphaera*). Support values for the *Po. ampla* clade, including the Australian sequences, were as follows: ITS (BS 80, BI 1) and 28S (BS 100, BI 1) (Figures S2 and S3). In the ITS tree, two Australian sequences (AY833656 and AF154321) published by Cunnington et al. [10] and labelled as *Podosphaera* sp. by Meeboon et al. [11] fell into the *Po. ampla* clade.

Eight Australian *Podosphaera* sequences on *Pr. persica* and *Pr. mahaleb* hosts, including two Australian sequences (AY833651 and AY833653) published by Cunnington et al. [10] and also labelled as *Podosphaera* sp. by Meeboon et al. [11], formed an independent lineage from other species of the *Po. tridactyla* complex. In the ITS+28S phylogeny, support for this new *Podosphaera* species is high (BS 84 and BI 1) (Figure 2). The individual ITS and 28S phylogenies also have strong support for this clade: ITS (BS 87, BI 1) and 28S (BS -, BI 0.98) (Figures S2 and S3). A summary of variable bases for the novel *Podosphaera* species shows five base-pair positions in 1234 characters at which there are differences from the two sister taxa, with differences at three positions compared to *Po. pruni-avium* and two positions compared to *Po. pruni-japonicae* (Figure S4). The new sequence generated for VPRI 19591 by next generation sequencing in the current study was taken from the same specimen that was used to generate sequence AY833653 by Sanger sequencing in 2005; the two sequences are identical.

3.3. Fungus–Host Relationships

The ITS+28S phylogeny of the fungi showed several sequences from hosts belonging to different *Prunus* subgenera compared to the hosts detected by Meeboon et al. [11] (Figure 2). Three species, *Po. pruni-avium*, *Po. pruni-japonicae* and *Po. prunigena*, were all originally described from *Prunus* hosts within the subgenus *Cerasus*, but in the ITS+28S phylogeny, the clades for these species included VPRI 22159, VPRI 20490, VPRI 20233 and VPRI 20491 on *Pr. padus*, which is in subgenus *Padus*. The *Podosphaera pruni-japonicae* clade in the ITS tree also included sequences from fungi collections on *Prunus davidiana* (subgenus *Prunus*) (Figure S2). The ITS tree includes additional fungi sequences from GenBank, in which *Po. pruni-avium* forms two separate lineages; the first lineage consists of collections on both *Pr.* subgenus *Cerasus* and *Padus* and the second lineage has collections only on *Pr.* subgenus *Cerasus* (Figure S2). The clade consisting of collections of the undescribed powdery mildew on peach, *Prunus persica* (subgenus *Prunus*), also included a sequence from a collection on *Pr. mahaleb* (subgenus *Cerasus*). Both *Po. prunina* and *Po. ampla* clades contained powdery mildew sequences generated from *Prunus* hosts within subgenus *Prunus* in all three phylogenetic analyses.

3.4. Plant Host Phylogeny

There were nine different *Prunus* species originally listed as the plant hosts for Australian *Po. tridactyla* complex collections (*Pr. armeniaca*, *Pr. cerasifera*, *Pr. domestica*, *Pr. laurocerasus*, *Pr. mahaleb*, *Pr. padus*, *Pr. persica*, *Pr. salicina* and *Pr. yedoensis*). The plant host species were analysed in a *matK* phylogenetic analysis containing 133 sequences of 1288 base pairs, including representatives of the three subgenera of *Prunus* (Figure 3).

The phylogeny forms three main clades which represented the *Prunus* subgenera *Cerasus* (clade 1), *Padus* (clade 2) and *Prunus* (clade 3). The *matK* sequences for DAR 28963, VPRI 20491 and VPRI 20231 fell into clades with sequences for *Pr. mahaleb* and *Pr. yedoensis* belonging to subgenus *Cerasus* within the *Padus* clade, VPRI 22156 and BRIP 8323 formed a well-supported clade (BS 90, BI 1) with sequences identified as *Pr. laurocerasus*. This is a host reidentification for BRIP 8323 which had *Pr. persica* listed on the specimen. Three VPRI sequences, 20490, 20233 and 22159, formed a clade with no BS support and BI support below the 0.85 threshold with *Pr. padus* sequences and close relatives such as *Pr. grayana* and *Pr. virginiana*. Within the subgenus *Prunus*, section *Amygdalus* formed a clade that was only supported by BI (0.91) containing sequences identified as *Pr. persica* along with sequences VPRI 19591, 20705, 20706, 22232 and 22233 (all originally identified as *Pr. persica* and *Pr. salicina* x *persica*) along with DAR 28962 and DAR 71638 (originally misidentified as *Pr. laurocerasus*). In section *Prunus*, the sequences identified as *Pr. armeniaca* formed a clade including eleven VPRI and DAR specimens identified as *Pr. armeniaca* with BI support of 0.95. The remaining VPRI sequences (originally identified as *Prunus* sp., *Pr. cerasifera*, *Pr. domestica*, *Pr. salicina* and *Pr. persica*) fell within a clade comprising sequences identified as Eurasian plums of sect. *Prunus* with high BI support (0.92) and no BS support. The Eurasian plums included *Pr. domestica*, *Pr. cerasifera* and *Pr. salicina*. There are minimal differences between these species within the *matK* gene region and therefore, for the purpose of identification, this group is referred to as the *Pr. domestica* group which includes the previously mentioned *Prunus* species as well as *Pr. brigantina*, *Pr. consociiflora*, *Pr. simonii* and *Pr. spinosa*. Additionally included in the clade of Eurasian plums were sequences from BRIP 15118 and VPRI 19868, which were both previously misidentified as *Pr. persica* (Figure 3). There were 14 specimens with plant hosts originally listed only as *Prunus* sp., in the *matK* phylogeny, these sequences fell within the clade of Eurasian plums in section *Prunus* (Table 2).

There were four VPRI specimens, VPRI 12495, 18452, 18514 and 20687, which are labelled with plant hosts listed as *Prunus persica*, *Pr.* sp., *Pr. domestica* and *Pr.* sp., respectively. However, the *matK* sequences identified the hosts as *Malus prunifolia* (plum-leaf crab apple) and the associated powdery mildews were all re-identified as *Po. leucotricha*.

3.5. Morphological Characterisation

The phylogenetic analysis showed a well-supported independent lineage of an undescribed species which is sister to *Po. pruni-avium* and *Po. pruni-japonicae*. No sexual morphs were found among material of this species. The morphologies of the sister taxa barely differ from *Po. tridactyla* in the strict sense and the asexual characters of this undescribed species on *Prunus persica* and *Pr. mahaleb* are the shorter conidiophore foot cells, crenate conidial chains and smaller conidia when compared to characters of the asexual morph of *Po. tridactyla*, which has long foot cells and ellipsoid(-doliform) conidia catenescent with crenate edge line (Figure 4).

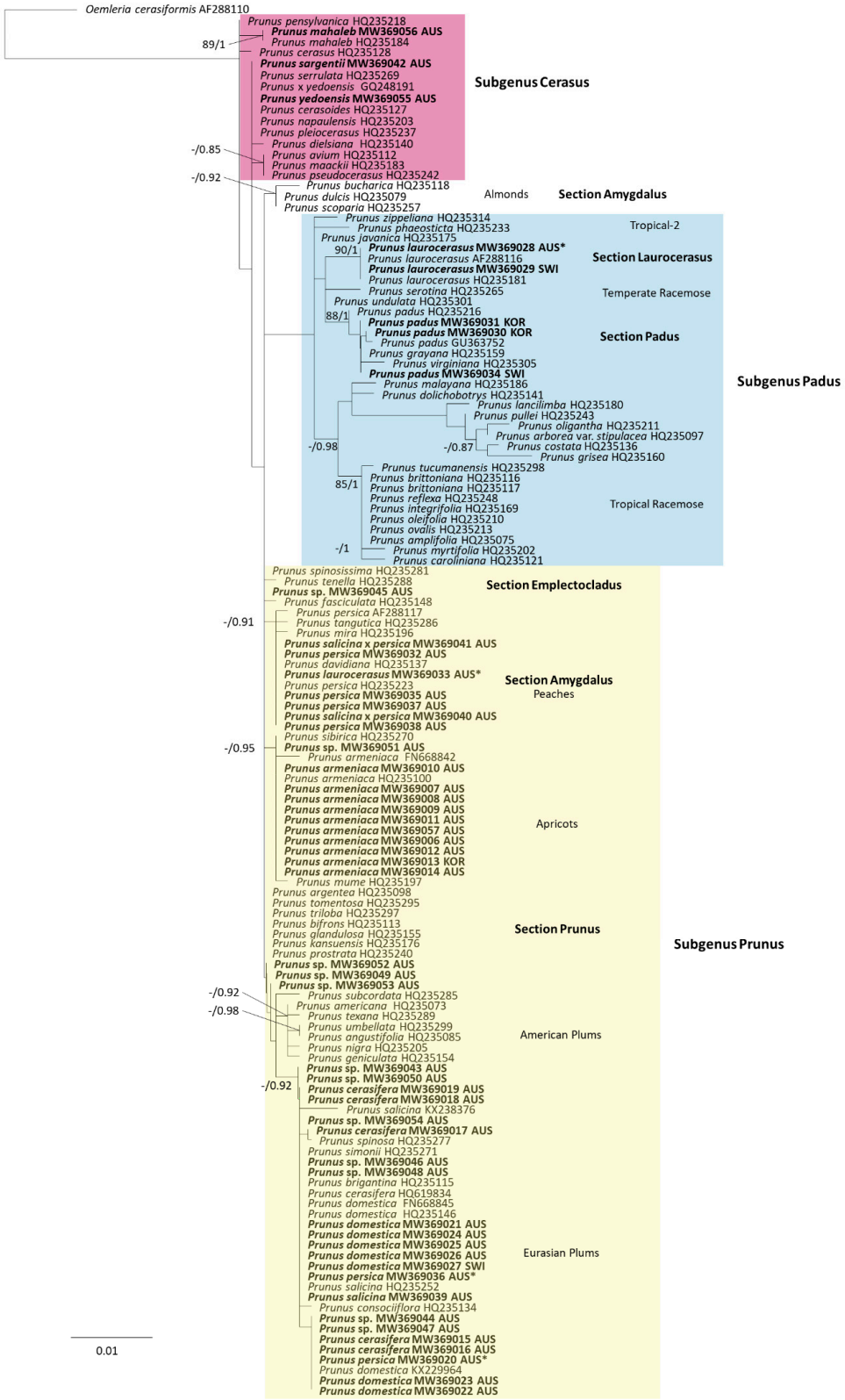

Figure 3. Maximum likelihood phylogenetic analysis of *matK* chloroplast gene sequences for host *Prunus* species. Branch support values for maximum likelihood (bootstrap (BS)) and Bayesian inference (BI) analyses are shown when >70% and 0.85, respectively. Colours represent *Prunus* subgenera; *Cerasus* is red, *Padus* is purple and *Prunus* is green. Sequences generated from this study are in bold and sequence names as deposited in reference collections. * Indicates a host that was originally misidentified (under original name).

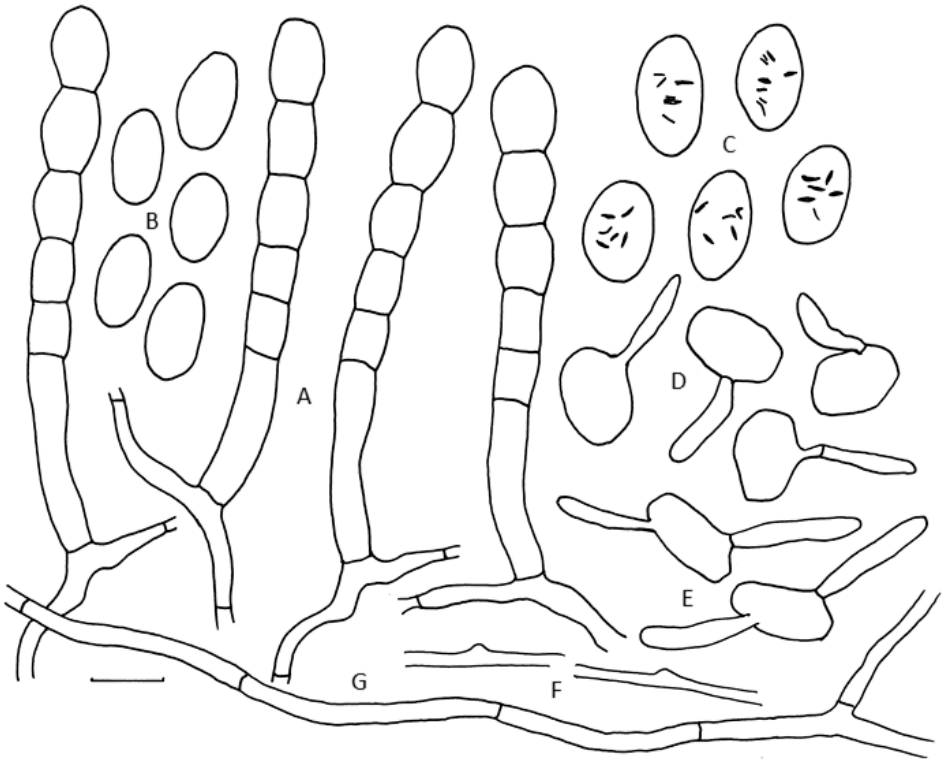

Figure 4. *Podosphaera cunningtonii* on *Prunus persica* morphology. (**A**) Conidiophores on Sellotape strip in lactic acid. (**B**) Conidia (mounted in lactofuchsin). (**C**) Conidia (mounted in water), showing fibrosin bodies. (**D**) Conidia germinated on host leaf for 24 h (mounted in lactofuchsin). (**E**) Conidia germinated on host leaf for 48 h (mounted in lactofuchsin). (**F**) Inconspicuous, nipple-shaped appressoria (mounted in lactofuchsin). (**G**) Hyphae on Sellotape strip (mounted in lactofuchsin). Scale bar = 20 μm. (All drawings from holotype VPRI 19591, except D & E from VPRI 20705).

Based on the morphological and sequence differences, we propose the following new species.

Podosphaera cunningtonii R.L. Smith, I. Pascoe, T.W. May and J. Edwards. Mycobank Number: MB838823.

Typification. AUSTRALIA, VICTORIA: Burnley, isolated from *Prunus persica,* 28 October 1993, I. Pascoe (holotype VPRI 19591, dried culture; ITS+28S: MW364516).

Etymology: the epithet commemorates Dr. James Cunnington, the first person to conduct molecular examination of this species.

Mycelium hyphae branched, thin walled, sinuous. Hyphal appressoria nipple shaped. Conidiophores straight, basal septum slightly displaced at junction, foot cells (30–) 43–60 × 8–11 μm, succeeded by 2–3 following cells, forming conidia in chains with crenate edge lines. Conidia ovoid(-doliform), 23–25 × 12–15 μm, fibrosin bodies present in water mounts, lacking in lactic acid mounts. Germination on host tissue at 24 h, producing single oblong-clavate, simple germ tubes 20–30 × 5–6 μm; at 48 h, producing 1–2 simple germ tubes 25–35 μm. No sexual morph observed.

Natural distribution: currently unknown as isolates were collected in quarantine glasshouses in Australia.

Additional collections examined: AUSTRALIA, VICTORIA: Knoxfield, isolated from *Pr. persica,* 7 March 1994, A. Sivapalan VPRI 19,868; isolated from *Pr. persica*, 27 September 1995, V. Beilharz VPRI 20,705; isolated from *Pr. persica*, 27 September 1995, V. Beilharz VPRI 20706; NEW SOUTH WALES: Rydalmere, isolated from *Pr. persica,* 3 June 1977, L. Penrose and J. Walker DAR 28,962; isolated from *Pr. mahaleb*, 3 June 1977, L. Penrose and J. Walker DAR 28,963; isolated from *Pr. persica*, 22 January 1996, G. Stovold DAR 71638 (sequence only, not examined morphologically).

4. Discussion

This study re-evaluated reference collection specimens of *Po. tridactyla* and *Oidium* sp. on *Prunus* hosts and, through phylogenetic analysis, found seven different powdery mildew species and corrected misidentified plant hosts of eight specimens. The main species present on *Prunus* in Australia was *Po. ampla* and we also found that Australian collections of *Po. pannosa* and *Po. leucotricha* had been misidentified as *Po. tridactyla* and *Oidium* sp. in two reference collections. Finally, we characterised a new species in the *Po. tridactyla* complex which we name *Po. cunningtonii* from *Pr. persica* and *Pr. mahaleb* plant hosts.

The phylogenetic analysis based on sequences derived from NGS shows that 37 of the 56 specimens fell within a clade alongside sequences identified as *Po. ampla* by Meeboon et al. [11]. Most Australian sequences assigned to *Po. ampla* had a one base pair difference compared to the *Po. ampla* sequences analysed in previous studies. The revision of the *Po. tridactyla* species complex by Meeboon et al. [11] included a number of sequences containing several bases varying from the *Po. ampla* holotype MK530453 but were still included under *Po. ampla*. Therefore, most of the Australian powdery mildew fungi on stone fruit (*Prunus armeniaca*, *Pr. cerasifera*, *Pr. domestica* and *Pr. salicina*) formerly identified as *Po. tridactyla* are in fact *Po. ampla*.

The ITS phylogeny and 28S phylogenies also confirmed that most of the Australian sequences were *Po. ampla* and fell within a well-supported clade with *Po. ampla* sequences analysed by Meeboon et al. [11] (Figures S2 and S3). *Podosphaera tridactyla* sequences AF154321 and AY883656 generated by Cunnington et al. [10] were included in the ITS phylogeny and summary of variable sites and they both fell in with the other *Po. ampla* sequences, the only differences being that the sequence lengths were significantly shorter than the ITS+28S sequences which were generated by this study and Meeboon et al. [11] and AY883656 contained three variable base pairs towards the end of the sequence. We are confident in reidentifying these two sequences as *Po. ampla*.

The range of hosts for *Po. ampla* observed in this study included *Pr. cerasifera* and *Pr. domestica* as well as other species within subgenus *Prunus* section *Prunus* (Figure 3). In Meeboon et al. [11], *Po. ampla* was identified from *Pr. domestica* and *Pr. spinosa*, as well as from *Pr. armeniaca* and *Pr. cerasifera*. These *Prunus* hosts for *Po. ampla* were observed in this study as well as *Pr. salicina*, which is also within subgenus *Prunus* sect. *Prunus*. It would be expected in ideal conditions such as in a glasshouse for *Po. ampla* to infect a close relative of its listed hosts.

The results of the three phylogenetic analyses from this study contradict host specificity at the subgenus level in *Prunus* for *Po. prunigena*, *Po. pruni-japonicae* and *Po. pruni-avium* species, as suggested by Meeboon et al. [11] because each fungus species clade contained *Prunus* hosts from subgenera *Cerasus* and *Padus*. Chin et al. [4] suggested that species within the subgenus *Cerasus* have a close alliance with some temperate racemose species found within subgenus *Padus*, such as *Pr. padus* and *Pr. laurocerasus*. This might offer an explanation as to the inclusion of supposedly different *Prunus* host subgenera in these host genera-specific powdery mildew fungi, as the remaining species of the *Po. tridactyla* complex are specific at the host subgenus level.

The remaining species which were identified from the Australian reference collections were: *Po. pannosa*, *Po. leucotricha*, *Po. pruni-japonicae*, *Po. pruni-avium*, *Po. prunina* and *Po. prunigena*. *Podosphaera pannosa* and *Po. leucotricha* were identified from Australian collections which had misidentified fungus and plant hosts recorded. The phylogeny showed four VPRI specimens were actually *Po. leucotricha* or the apple powdery mildew on plum-leaf crab apple (*M. prunifolia*) and two specimens from BRIP and DAR were *Po. pannosa*, which is more commonly seen on roses. The remaining *Po. tridactyla* complex species were identified from collections made in Switzerland and South Korea but held at VPRI.

While most Australian specimens were *Po. ampla*, six of the sequences newly generated by NGS were of an unknown species which did not match any identified sequences on

GenBank. Cunnington et al. [10] and Meeboon et al. [11] both stated that two of the six sequences represented in their studies were an independent genetic lineage within the *Po. tridactyla* complex. In the Cunnington et al. [10] study, the two sequences AY833651 and AY833653 formed a clade which was sister to a clade comprising *Podosphaera* on *Pr. japonica*, which was renamed *Po. pruni-japonicae* by Meeboon et al. [11], within clade 2, which contained sequences of *Po. tridactyla* not on *Prunus* subgenus *Prunus*. In the Meeboon et al. [11] study, the phylogeny showed sequences AY833651 and AY833653 forming a separate clade sister to a clade comprising *Po. pruni-japonicae* and *Po. pruni-avium*. The results of the present phylogenetic study align with both Cunnington et al. [10] and Meeboon et al. [11], where the ITS+28S phylogeny shows that sequences of *Po. cunningtonii* including additional sequences generated in this study form a well-supported clade which is sister to a clade comprising *Po. pruni-japonica* and *Po. pruni-avium*.

A GenBank search for sequences of *Podosphaera* species on *Prunus persica* returned 26 sequences of two powdery mildew species, *Po. pannosa* and *Po. leucotricha* (peach rusty spot), which are both found on peach [22–24]. However, the sequences of this new species do not match either of these powdery mildew species, further confirming a previously undescribed powdery mildew. Braun and Cook [7] list three powdery mildew species with *Pr. persica* included in the host ranges, these are *Po. tridactyla* in the strict sense, *Po. prunicola* and *Po. pannosa*, which are quite separate to *Po. cunningtonii* in the phylogeny.

At the time of the first collections of *Po. cunningtonii* in Victoria (mid-1990s), it was assumed that the quarantine glasshouse-grown *Pr. persica* had been infected by powdery mildew blowing into the glasshouse (Cunnington 2020 pers. comm.). However, in this study, we identified the same powdery mildew fungi on three specimens from New South Wales, two of which (DAR 28962 and DAR 28963) were also collected in quarantine glasshouses. The two specimens from quarantine glasshouses in N.S.W. are on two different hosts—*Pr. persica* (subgenus *Prunus*) and *Pr. mahaleb* (subgenus *Cerasus*). Notes in the specimen packet reported that the *Pr. mahaleb* seedlings were growing next to heavily powdery mildew-infected *Pr. persica* seedlings in the same glasshouse. The determiner, Dr. John Walker, noted at the time of collection (1977), "mildew development very light and feel that this is an adventitious development of mildew on this host from the heavily mildewed peach seedlings growing nearby". We suggest that the primary host of this new powdery mildew is *Pr. persica* but under glasshouse conditions it can infect other *Prunus* species from different subgenera as accidental hosts.

A third specimen of *Po. cunningtonii*, from N.S.W. (DAR 71638), was collected from *Pr. persica* on a private property in 1996, in a locality with several orchards that grew peach and other stone fruit in the near vicinity. This is the only report of *Po. cunningtonii* from outside of quarantine glasshouses and therefore we cannot confirm if it is established in Australia. Two specimens of powdery mildew on *Pr. persica* collected in 1991 from a quarantine facility in Alice Springs, Northern Territory, are lodged in the Northern Territory Plant Pathogen Collection (DNAP). Unfortunately, we were unaware of them at the time of commencement of the present study, so they were not included. At the time of collection, they were identified morphologically as *Po. pannosa* by determiner Dr. Jose Liberato. The two native *Prunus* species, *Pr. brachystachya* and *Pr. turneriana*, are only found in tropical rainforests in the far north of Queensland of Australia. Horticultural *Prunus* species, including stone fruit, are grown in the temperate regions of Australia further south [2,6]. Geographical distance and climatic differences would prevent the spread of a "native" *Prunus* powdery mildew fungus onto commercial stone fruit crops and to date there are no records of powdery mildew fungi infecting these native hosts. It is highly unlikely that *Po. cunningtonii* has a native area of distribution in the natural distribution of *Pr. persica* which originates from Northwest China. New peach varieties are imported into Australia as budwood and grown under quarantine prior to release. Potential explanations as to why *Po. cunningtonii* has not been observed more widely in Australia could be that infected plants would have not been released from quarantine. Another reason could be that the local climate in peach-growing regions in Australia is not climatically suitable for

Po. cunningtonii development, whereas the quarantine glasshouse conditions provided an artificial environment for *Po. cunningtonii* growth [25,26].

Australia is believed to be a continent without native powdery mildew fungi, with accidental human-assisted powdery mildew introductions occurring post-European settlement on non-native agricultural, horticultural, ornamental and pasture plant species [27]. Further evidence for the lack of native powdery mildew fungi in Australia is the evolution of native plant species in long isolation from continents that now make up the northern hemisphere, which is where powdery mildew fungi appear to have originated and subsequently co-evolved with plant hosts [27]. Walker [28] investigated the distribution of plant-parasitic fungi across Australia and observed that most accounts of powdery mildew infection were on introduced monocot and dicot plant species and only a few on native plant species, with these infections occurring in artificial settings such as glasshouses or nurseries. After analysis of records of powdery mildew fungi from Australia, Kiss et al. [27] concluded that through European settlement, almost all of the agricultural and horticultural crops grown in Australia were recently introduced and with them powdery mildew. They also found that freshly collected powdery mildew specimens from Australia were phylogenetically similar to species known overseas and were collected mostly on imported plant hosts but were also identified from 13 native plant species [27].

Over the last 50 years, there have been changes in the way that specimens are collected, preserved, studied and recorded in reference collections [29]. These changes include increased use of molecular analysis for identification, publication of sequence data in online resources such as GenBank, digitisation of reference collection specimens and exposure via associated online catalogues. We detected numerous misidentified *Po. tridactyla* specimens and we also identified and described a previously unrecorded powdery mildew species, *Po. cunningtonii*. Outcomes such as these highlight the need to re-evaluate important plant pathogen groups held in reference collections, in particular unculturable pathogens and complexes of cryptic species [30]. Australia implements a high level of biosecurity and quarantine measures to prevent entry of unwanted pests and pathogens which can affect the Australian horticultural industry and trade (Hyde et al. 2010). In order to provide this pest and pathogen information, the specimen records must be up to date with current taxonomic classification and names in order to provide accurate species lists of pathogens currently present in Australia [31].

Through NGS applications, re-examination of selected powdery mildew specimens from Australian plant pathogen reference collections has demonstrated that one species, *Po. ampla*, is the dominant powdery mildew infecting stone fruit in Australia. In addition, the phylogenetic study confirmed the presence of *Po. pannosa* in Australia and allowed delimitation of a new powdery mildew species within the *Po. tridactyla* species complex, *Po. cunningtonii* on *Pr. persica*, for which we provide a morphological and molecular description. This study resolved powdery mildew species but also identified *Prunus* plant hosts and reclassified previously misidentified or incompletely identified hosts of specimens held in Australian plant pathogen reference collections. The information generated in this study will be used to update BRIP, DAR and VPRI powdery mildew specimens to provide accurate data for Australian biosecurity agencies.

Supplementary Materials: The following are available online at https://www.mdpi.com/2309-608X/7/3/171/s1, Figure S1: Summary of variable sites for *Podosphaera ampla* and sister taxa *Po. prunina* and *Po. prunigena*, Figure S2: Maximum likelihood phylogenetic analysis of nuclear rDNA ITS1-5.8S-ITS2 sequences for the *Po. tridactyla* species complex and closely related species, Figure S3: Maximum likelihood phylogenetic analysis of nuclear rDNA 28S sequences for the *Po. tridactyla* species complex and closely related species, Figure S4: Summary of variable sites for *Po. cunningtonii* sequences compared to sister taxa *Podosphaera pruni-avium* and *Po. pruni-japonicae*.

Author Contributions: R.L.S., J.E., T.I.S. and T.W.M. conceived the idea for the study and all the authors (R.L.S., T.I.S., R.C.M., J.K., T.W.M., I.G.P. and J.E.) contributed to the design. R.L.S. performed the experimental DNA extraction and sequencing, with assistance and advice from J.K. T.I.S. and R.C.M. assisted R.L.S. with the bioinformatics. T.W.M. and J.E. assisted and advised R.L.S. with the

phylogenetic analyses. I.G.P. provided the morphological data and taxonomic drawings for the new species description. Data analyses and interpretation were conducted by R.L.S. with assistance and advice from all authors. R.L.S. wrote the manuscript, which was reviewed by T.W.M. and J.E. to shape the final version. All authors have read and agreed to the published version of the manuscript.

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References

1. Jun, W.; Berggren, S.T.; Chung-Hee, L.; Ickert-Bond, S.; Ting-Shuang, Y.; Ki-Oug, Y.; Lei, X.; Joey, S.; Potter, D. Phylogenetic inferences in *Prunus* (Rosaceae) using chloroplast ndhF and nuclear ribosomal ITS sequences. *J. Syst. Evol.* **2008**, *46*, 322–332. [CrossRef]
2. Jeanes, J.A.; Jobson, P.C. Rosaceae. In *Flora of Victoria, Dicotyledons Winteraceae to Myrtaceae*; Walsh, N.G., Entwisle, T.J., Eds.; Inkata Press: Melbourne, Australia, 1996; Volume 3.
3. Shi, S.; Li, J.; Sun, J.; Yu, J.; Zhou, S. Phylogeny and classification of *Prunus* sensu lato (Rosaceae). *J. Integr. Plant Biol.* **2013**, *55*, 1069–1079. [CrossRef]
4. Chin, S.-W.; Shaw, J.; Haberle, R.; Wen, J.; Potter, D. Diversification of almonds, peaches, plums and cherries–molecular systematics and biogeographic history of *Prunus* (Rosaceae). *Mol. Phylogenetics Evol.* **2014**, *76*, 34–48. [CrossRef] [PubMed]
5. George, A. Deciduous fruit production in Australia. *RAP Publ. (FAO) Eng No. 1999/10*. Available online: http://www.fao.org/3/ab985e/ab985e04.htm (accessed on 21 September 2020).
6. Horticulture Innovation Australia Limited. *Australian Horticulture Statistics Handbook Fruit 2017/2018*; Freshlogic: Sydney, Australia, 2019; pp. 91–95.
7. Braun, U.; Cook, R.T.A. *Taxonomic Manual of the Erysiphales (Powdery Mildews)*; CBS-KNAW Fungal Biodiversity Center: Utrecht, The Netherlands, 2012; Volume 1, p. 2.
8. Jeon, C.; Kang, S.; Choi, W.; Shin, H.-D. First report of powdery mildew caused by *Podosphaera tridactyla* on *Prunus davidiana* in Korea. *Plant Dis.* **2019**, *103*, 158. [CrossRef]
9. García-Ruiz, M.; Galicia-Buendia, N.; Leyva-Mir, S.; Correia, K.; Nieto-López, E.; Camacho-Tapia, M.; Tovar-Pedraza, J. First report of powdery mildew of European plum caused by *Podosphaera tridactyla* in Mexico. *Plant Dis.* **2019**, *103*, 587. [CrossRef]
10. Cunnington, J.H.; Lawrie, A.C.; Pascoe, I.G. Genetic variation within *Podosphaera tridactyla* reveals a paraphyletic species complex with biological specialization towards specific *Prunus* subgenera. *Mycol. Res.* **2005**, *109*, 357–362. [CrossRef] [PubMed]
11. Meeboon, J.; Takamatsu, S.; Braun, U. Morphophylogenetic analysis revealed that *Podosphaera tridactyla* constitutes a species complex. *Mycologia* **2020**, *112*, 244–266. [CrossRef] [PubMed]
12. Smith, R.L.; Sawbridge, T.; Mann, R.; Kaur, J.; May, T.W.; Edwards, J. Rediscovering an old foe: Optimised molecular methods for DNA extraction and sequencing applications for fungarium specimens of powdery mildew (Erysiphales). *PLoS ONE* **2020**, *15*, e0232535. [CrossRef] [PubMed]
13. Johnston, A.; Booth, C. *Plant Pathologist's Pocketbook*; C.M.I.: Kew, UK, 1983.
14. Shin, H.-D. Erysiphaceae of Korea. Ph.D. Thesis, Seoul National University, Seoul, Korea, 1988.
15. Shin, H.-D.; LA, Y.-J. Morphology of edge lines of chained immature conidia on conidiophores in powdery mildew fungi and their taxonomic significance. *Mycotaxon* **1993**, *46*, 445–451.
16. Moparthi, S.; Grove, G.G.; Pandey, B.; Bradshaw, M.; Latham, S.R.; Braun, U.; Meeboon, J.; Romberg, M. Phylogeny and taxonomy of *Podosphaera cerasi*, sp. nov., and *Podosphaera prunicola* sensu lato. *Mycologia* **2019**, *111*, 647–659. [CrossRef]
17. Smith, R.L.; May, T.W.; Kaur, J.; Sawbridge, T.; Mann, R.; Edwards, J. Molecular data from up to 130-year-old herbarium specimens do not support the presence of cherry powdery mildew in Australia. *Plant Pathol.* **2020**. [CrossRef]
18. Biomatters Ltd. Geneious Prime 2020.0.5. Available online: https://www.geneious.com/ (accessed on 3 August 2020).
19. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef]
20. Guindon, S.; Dufayard, J.-F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst. Biol.* **2010**, *59*, 307–321. [CrossRef] [PubMed]

21. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **2001**, *17*, 754–755. Available online: http://146.6.100.192/users/CH391L/Handouts/MRBAYES.pdf (accessed on 15 January 2020). [CrossRef]
22. Leus, L.; Dewitte, A.; Van Huylenbroeck, J.; Vanhoutte, N.; Van Bockstaele, E.; Höfte, M. *Podosphaera pannosa* (syn. *Sphaerotheca pannosa*) on *Rosa* and *Prunus* spp.: Characterization of pathotypes by differential plant reactions and ITS sequences. *J. Phytopathol.* **2006**, *154*, 23–28. [CrossRef]
23. Jankovics, T.; Dolovac, N.; Bulajić, A.; Krstić, B.; Pascal, T.; Bardin, M.; Nicot, P.C.; Kiss, L. Peach rusty spot is caused by the apple powdery mildew fungus, *Podosphaera leucotricha*. *Plant Dis.* **2011**, *95*, 719–724. [CrossRef] [PubMed]
24. Marimon, N.; Eduardo, I.; Martínez-Minaya, J.; Vicent, A.; Luque, J. Disease progression of peach powdery mildew in Catalonia, Spain–Towards a decision support system based on degree-days to initiate fungicide spray programs. 2020. Available online: http://hdl.handle.net/20.500.11824/1148 (accessed on 14 November 2020).
25. Lu, W.; Newlands, N.K.; Carisse, O.; Atkinson, D.E.; Cannon, A.J. Disease risk forecasting with Bayesian learning networks: Application to grape powdery mildew (*Erysiphe necator*) in Vineyards. *Agronomy* **2020**, *10*, 622. [CrossRef]
26. Asalf, B.; Onofre, R.B.; Gadoury, D.M.; Peres, N.A.; Stensvand, A. Pulsed water mists for suppression of strawberry powdery mildew. *Plant Dis.* **2020**. [CrossRef] [PubMed]
27. Kiss, L.; Vaghefi, N.; Bransgrove, K.; Dearnaley, J.D.W.; Takamatsu, S.; Tan, Y.P.; Marston, C.; Liu, S.-Y.; Jin, D.-N.; Adorada, D.L.; et al. Australia: A continent without native powdery mildews? The first comprehensive catalog indicates recent introductions and multiple host range expansion events, and leads to the re-discovery of *Salmonomyces* as a new lineage of the Erysiphales. *Front. Microbiol.* **2020**, *11*. [CrossRef] [PubMed]
28. Walker, J. Pacific mycogeography: Deficiencies and irregularities in the distribution of plant parasitic fungi. *Aust. J. Bot. Suppl. Ser.* **1983**, *13*, 89–136. [CrossRef]
29. Schindel, D.E.; Cook, J.A. The next generation of natural history collections. *PLoS Biol.* **2018**, *16*, e2006125. [CrossRef] [PubMed]
30. Hyde, K.D.; Chomnunti, P.; Crous, P.W.; Groenewald, J.Z.; Damm, U.; Ko, T.K.; Shivas, R.G.; Summerell, B.A.; Tan, Y.P. A case for re-inventory of Australia's plant pathogens. *Persoonia* **2010**, *25*, 50–60. [CrossRef] [PubMed]
31. Shivas, R.; Beasley, D.; Pascoe, I.; Cunnington, J.; Pitkethley, R.; Priest, M. Specimen-based databases of Australian plant pathogens: Past, present and future. *Australas. Plant Pathol.* **2006**, *35*, 195–198. [CrossRef]

Chapter 5

Discussion

5.1 Context of research

This thesis investigated species of powdery mildew fungi within the genus *Podosphaera* which infect important horticultural crops of Victoria utilising preserved plant specimens held in Australian plant pathogen reference collections. The results of the research undertaken in this thesis have provided new molecular taxonomic methods which can be applied to powdery mildew herbarium specimens and used to provide molecular taxonomic classification when morphological characters are absent for traditional identification or when the taxon is known to be a species complex.

Powdery mildews in Australia are relatively understudied. Walker (1983) performed a survey of foliar plant pathogens in Australia and concluded that powdery mildew species present in Australia are almost entirely exotic introductions. This was based on the absence of powdery mildews on native hosts in natural settings. The powdery mildews infections that were observed on native hosts were identified as known introduced powdery mildew species and appeared to be the result of host jumps in situations such as glasshouses and nurseries.

An Australian Biological Resources Study (ABRS) project was conducted on powdery mildews for over a decade between the early 1990s to the early 2000s, during which over 1000 specimens were collected from across Australia representing agricultural, horticultural and ornamental plant host species (Cunnington et al. 2003). These were accessed into the Victorian Plant Pathology Herbarium (VPRI) for morphological analysis and storage. A book on the powdery mildew fungi of Australia was planned for publication as a part of the ABRS series *Fungi of Australia*. This was to have a complete list of *Erysiphales* species present in Australia at the time, but unfortunately was not completed (I. Pascoe 2020, pers. comm. 24 September).

It was estimated during the ABRS project there were up to 100 different *Erysiphales* species in Australia, but only 20 species were observed to produce both the asexual and sexual structures required for morphological identification to species (Cunnington et al. 2003). Currently within VPRI there are over 1000 specimens that have been lodged under the name *Oidium* sp., which is the genus formerly used to place asexual forms, prior to the abandonment of dual taxonomy for pleiomorphic fungi (those with asexual and sexual stages). The common presence of asexual forms in Australia is most likely due to only one mating type having been introduced resulting in the absence of sexual reproduction (Braun and Cook 2012; Cunnington et al. 2003).

The presence of such a high number of asexual specimens in the VPRI was one of the drivers for investigating appropriate molecular methods for identification. Cunnington continued research on Australian powdery mildews in the early 2000's by using molecular analyses such as RFLP and rDNA ITS sequencing to resolve asexual species and provide disease reports of newly identified species in Australia (Cunnington and Brett 2009; Cunnington et al. 2004a,b; Cunnington et al. 2003; Liberato and Cunnington 2006; Liberato and Cunnington 2007). An important study from this period was the identification of genetic variation within *Po*. *tridactyla* on VPRI *Prunus* specimens, indicating that there were at least three taxa within the complex (Cunnington et al. 2005b).

The monograph of the *Erysiphales* by Braun and Cook (2012) has only ~50 powdery mildew species with Australia listed in the geographic range. Even this number is doubtful as some of these records were based on plant host specificity which has been shown not to be accurate in determining species. Also, the high numbers of asexual morphs lacking species identification and morphologically cryptic species highlight the lack of knowledge regarding the number of species in Australia (Kiss et al. 2020). A recent publication by Kiss et al (2020) investigated the hypothesis of a continent with no native powdery mildews by cataloguing the *Erysiphales* species present in Australia. This was achieved by curating a list of (1) taxa that have been previously identified based on published DNA barcode sequences, coupled with (2) morphological and molecular identification of 117 fresh specimens and 30 herbarium specimens. This study confirmed 42 *Erysiphales* species representing 10 genera, including two genera and 13 species previously unrecorded in Australia. Additionally, the study resurrected the *Salmonomyces* genus and identified that powdery mildew on 13 native plant species was caused by known *Erysiphales* species from overseas. Their conclusion was similar to that of Walker (1983), i.e. that Australia does not have any native powdery mildews and all the endemic *Erysiphales* species have been introduced through European colonisation and settlers from around the world (Kiss et al. 2020).

Horticulture in Australia was established through European and Asian settlement during the 1800s with the introduction of horticultural plant species brought with the settlers from their home countries (George 1999). This inadvertently introduced plant pathogens on the imported plants into an environment which has evolved in the absence of many of these pathogens (Kiss et al. 2020). Currently, the Australian horticultural industry produces high-value commodities for Australia, producing 6.73 M t. of produce valued at $AUD14.37 B for domestic and export fresh markets in 2019 (Horticulture Innovation Australia Limited 2019). Australian fruit production in which the state of Victoria was the major producer of almonds, summer fruit (apricots, nectarines and peaches), cherries, plums, grapes, pome, citrus and pistachios totalling 347,745 t. at a value of $AUD1.41 B (Horticulture Innovation Australia Limited 2019).

With high demand for Australian produce, protecting these crops from pest and pathogen threats is of the utmost importance (Anderson et al. 2017). One of the most common diseases of horticultural crops is powdery mildew (Kiss et al. 2001; McTaggart et al. 2012; Palmer 2007; Tucker et al. 2013; Uloth et al. 2008). Powdery mildew can cause significant crop damage and losses through infection of the leaves, reducing the plants' photosynthetic capabilities and fruit production (Jeon et al. 2019). In severe cases, powdery mildew can cause cupping and malformation of the leaves, restrict plant growth and infect fruit resulting in further crop losses (García-Ruiz et al. 2019).

In order to protect local industries as well as our unique flora, Australia has a high level of plant biosecurity measures in place to prevent the introduction of unwanted plant pests and pathogens. To maintain this surveillance, specimen-based records of pest and pathogen species already present in Australia are curated to provide accurate species databases, which are cross referenced when a quarantine intercept or an incursion occurs (Hyde et al. 2010). It is important that these pathogen records are accurately updated with recent taxonomic classification and nomenclature, as well having molecular and morphological identifications (Bieker and Martin 2018; Hyde et al. 2010).

Plant pathogen reference collections in Australia have been operating for over 100 years and over that time have evolved from specimens accessioned purely on morphological identification to the use of genomic information for identifying cryptic species or pathogens which do not have all the morphological characters to provide a full species identification (Shivas et al. 2006). The majority of the powdery mildew specimens lodged prior to the early 2000s were identified solely on the basis of morphology; therefore, revision of horticulturally important specimens is required. In order to undertake this task, molecular methods suitable for use on preserved powdery mildew specimens needed to be identified and a protocol developed. This thesis presented the molecular protocol that was developed and subsequently applied to resolve two taxonomic questions regarding *Podosphaera* species affecting *Prunus* species of high horticultural significance to Victoria.

5.2 Working with plant pathogen reference collections

Working with plant pathogen reference collections presents several challenges. Firstly, the specimen needs to be retained within the reference collection and cannot be used in its entirety, in order to maintain the specimen-based records which underpin biosecurity in Australia (Nelson et al. 2014). Utilisation of destructive sampling to extract DNA places limitations on sub-sampling from specimens. In some cases, the specimens are not suitable due to the tiny quantity of infected material collected and accessioned. Indeed, some specimen packets contain a single infected leaf which displays low level infection. In order to extract pathogen DNA in that situation the whole specimen would need to be used. Also, some older or duplicate specimens are mounted on card with glue preventing sampling for DNA extraction.

Sampling methods established for fresh powdery mildew specimens were initially tested and found to be unsuitable for the reference collection specimens. For sampling of powdery mildew fungi, it is very common to use cellotape or a scalpel blade to collect mycelia and conidia from the leaf surface (Brewer and Milgroom 2010). While these methods are effective on fresh powdery mildew samples, specimens from reference collections are dried and depending on the plant host, the leaf material becomes very brittle and sticks to the cellotape or the blade shatters the leaf during mycelial scrapings, so it was decided not to continue with these methods. Instead, a decision was made to standardise the amount of material sampled using a leaf punch on a portion of specimen that contained the most visible infection, accepting that inclusion of host material was unavoidable (Chapter 2).

Museum and reference collections hold valuable genetic resources which document the evolutionary and ecological history of organisms. DNA extracted from these specimens is considered to be ancient DNA (aDNA) and the characteristics of aDNA are well-documented – highly fragmented, degraded and usually obtained in low concentrations (Bieker et al. 2020; Wieβ et al. 2016). Over the last decade, the technical issues faced when working with aDNA have been addressed through the development of new molecular technologies designed for aDNA and collection-based research is being encouraged to adopt and understand the benefits of these techniques for molecular characterisation of biological collections (Buerki and Baker 2016). Using molecular methods to explore plant pathogen reference collections can enable the identification of plant pathogens and their hosts, and also the microphylla (the microorganisms present on the specimen at the time of collection) (Bieker et al. 2020). These methods have been used to characterise the metagenomic microbe assemblage throughout time and from different environments, as well as track the movement of plant, animal and pathogen distributions during epidemics and as a result of increased human and trade movement around the world (Bebber et al. 2014; Meineke et al. 2018; Ristaino 2019). Reference collections can also provide information

regarding how plants and their pathogens respond to change throughout history, which may assist ecological and agricultural research into the impacts of climate change (Buerki and Baker 2016; Meineke et al. 2018; Ristaino 2019; Schindel and Cook 2018). These areas of study can provide new research avenues for use of reference collections and potentially increase interest to fund, maintain and contribute to reference collections worldwide (Buerki and Baker 2016).

5.2.1 Establishing a protocol for molecular taxonomic revision of powdery mildew specimens in reference collections

Establishing molecular methods for revising powdery mildew specimens in plant pathology reference collections proved to be more challenging than expected. Since the early 2000s there have been significant advances in molecular technologies which have enabled improvements in DNA extraction, creation of DNA libraries designed for poor quality and fragmented DNA and the evolution of more accurate sequencing platforms (Linderholm 2016; McGaughran 2020). The first outcome of this research was the development of a next generation sequencing (NGS) pipeline to access the DNA of powdery mildew specimens held in VPRI. There was a significant knowledge gap regarding DNA extraction of obligate biotrophs such as powdery mildew fungi from reference collections for use in NGS applications. The lack of previous studies on biotrophic fungi necessitated the testing of extraction protocols in relation to DNA quality, DNA concentration and the performance in PCR amplification to determine what factors are important for use on preserved powdery mildew specimens. Using publications on DNA extraction from herbaria as a basis, DNA quality was expected to be more important than DNA quantity (Choi et al. 2015; Särkinen et al. 2012). However, for the powdery mildew specimens, DNA concentration rather than quality was critical to ensure enough target fungal DNA was present in the sample and not overwhelmed by DNA from the plant host and microphylla. (Choi et al. 2015; Särkinen et al. 2012). PCR-based single- and multi-locus sequencing has been the main element of phylogenetic studies over the last 20 years, but for aDNA this can be problematic due to the fragmented nature and poor quality of the DNA (Brotherton et al. 2007). With the development of cheaper reagents, new sequencing platforms and easier sequencing protocols, the use of NGS for aDNA has started to occur (Linderholm 2016; McGaughran 2020).

In chapter 2, methods were tested on apple powdery mildew specimens that were 25 to 30 years old. PCR primers developed specifically for *Erysiphales* taxonomy were unreliable in their ability to amplify the aDNA, presumed to be due to the short aDNA fragments. Illumina short-read sequencing which is designed for fragment lengths of 120-140 bp proved to be highly successful, allowing extraction of the target DNA reads by mapping against a reference sequence for subsequent assembly and use in phylogenetic studies.

The development of this DNA extraction and NGS pipeline for obtaining target DNA from old reference specimens containing obligate biotrophic plant pathogens contributes to the unlocking of a treasure trove of plant pathogen reference specimens for future phylogenetic and phylogenomic studies.

5.2.2 Cherry powdery mildew in Australia

The NGS pipeline developed in chapter 2 was applied to a biosecurity question regarding the presence or absence of cherry powdery mildew in Australia in light of a recent molecular taxonomic revision of *Podosphaera clandestina* (Moparthi et al. 2019). Of the 19 *Po*. *clandestina* and *Po*. *oxyacanthae* specimens revisited in this study nine were collected from U.S.A., seven were Australian collections, and Finland, Germany and Switzerland had one specimen each. I found that the newly described cherry powdery mildew fungus, *Po*. *cerasi*, was not present on Australian specimens held in plant pathogen reference collections from Australia but was confirmed on cherry specimens from U.S.A. This supports the lack of any reports of cherry powdery mildew in Australia.

In this study, the capabilities of the DNA extraction methods and NGS pipeline demonstrated on powdery mildew specimens up to 130 years old. Reliable sequence data was generated which could be mapped to the ITS gene regions of the target fungi, as well as to chloroplast genes which allowed confirmation of the plant host identity. The chloroplast gene *matK* was used to generate sequences and the identity of these sequences was checked using BLASTn. The hosts of *Po. clandestina* in the strict sense and of *Po*. *cerasi* are from two different genera, *Crataegus* and *Prunus* respectively. Therefore, confirmation of the host identity only required simple BLASTn searches. For closely related hosts within a single genus, confident identification would require a more in-depth approach such as phylogenetic analysis.

All Australian specimens were *Po*. *clandestina* in the strict sense on *Crataegus* spp. hosts and the specimens from Switzerland was also confirmed to be *Po*. *clandestina*. The Finland specimen failed to form a clade with other *Po*. *clandestina* sequences but was closely related. Eight specimens from the U.S.A. were found to be *Po*. *cerasi* and one specimen was *Po*. *prunicola* on *Prunus avium* (L.) L., demonstrating that there are in fact three species, *Po*. *cerasi*, *Po*. *prunicola* and *Po*. *pruni-avium*, are capable of infecting cherry.

The results of chapter 3 have been published and brought to the attention of Australian plant biosecurity personnel in DAWE and Plant Health Australia in order to update the cherry industry biosecurity plan and support the Australian cherry industry in maintaining international trade and market access.

5.2.3 *Podosphaera tridactyla* on stone fruit in Australia

The second taxonomic question that was addressed in this thesis was to resolve the identity of members of the *Po*. *tridactyla* species complex occurring on stone fruit and closely related *Prunus* plant hosts in Australia.

NGS data were generated for 56 specimens, enabling fungal ITS+28S and plant chloroplast *matK* gene regions to be mapped and extracted for phylogenetic analysis. Using ITS and 28S, the majority of the specimens previously referred to as *Oidium* sp. or *Po*. *tridactyla* were confirmed to be *Po*. *ampla*, which was recently described by Meeboon et al. (2020). Six specimens were determined to be an undescribed species. *Podosphaera ampla* infects Eurasian plum species within subgenus *Prunus* section *Prunus*. In addition, the new species was characterised, described and proposed as a separate taxon, *Po*. *cunningtonii,* within the *Po*. *tridactyla* species complex. This lineage was first identified as a distinct clade by Cunnington et al. (2005b), who generated the first ITS sequences, and again by Meeboon et al. (2020), who incorporated the original sequences in their phylogeny and recommended morphological description for this distinct phylogenetic lineage. The current study generated a further six sequences of *Po*. *cunningtonii* from five Australian peach (*Pr*. *persica* (L.) Batsch) and one *Pr*. *mahaleb* L. specimen which were collected from quarantine glasshouses in Victoria and New South Wales and one private property in New South Wales between 1977-1996. Given that there have been no new reports of powdery mildew on peach since 1996, it is unlikely that *Po*. *cunningtonii* has become established in Australia.

The European settlement of Australia during the 1800s started the introduction of agricultural and horticultural plant species to establish food crops to support a growing population (George 1999). The European origin of many horticultural plant species now present in Australia is consistent with the presumed European origin of *P*. *ampla* (Meeboon et al. 2020). The origin of *Po*. *cunningtonii* is unknown, but given the centre of origin for peach is in the area between west Asia to eastern Asia (China), it may have Asian origins. The phylogenetic analysis showed the sister taxa of *Po*. *cunningtonii* are *Po*. *pruni-japonicae*, from Japan and *Po*. *pruni-avium* from Europe. The distributions of the sister taxa do not shed light on the potential origin of *Po*. *cunningtonii*.

Another outcome from chapter 4 was the identification of six misidentified powdery mildew specimens held in three separate reference collections. Four specimens listed as *Po*. *tridactyla* on *Pr. persica* (L.) Batsch , *Pr*. *domestica* L. and *Prunus sp*. from VPRI were re-identified in the phylogenetic analysis as *Po. leucotricha* and the hosts were re-identified as *Malus prunifolia* (Willd.) Borkh or the plum-leaf crab apple. The other two specimens, one from the Queensland Plant Pathogen Herbarium, BRIP 8232 (originally identified as *Po*. *tridactyla* on *Pr*. *persica* (L.) Batsch) and one from the New South Wales Plant Pathology and Mycology Herbarium, DAR 12478 (listed as *Oidium* sp. on *Pr*. *laurocerasus* L.), were identified as *Po*. *pannosa,* and the host for BRIP 8232 was re-identified as *Pr*. *laurocerasus* L. (Chapter 4). The use of the protocol developed in chapter 2 to re-examine specimens originally identified based on morphology has highlighted the benefits of the NGS pipeline to not only revise pathogen identity but also confirm host identity, which is important for Australian biosecurity.

5.3 New research since this thesis commenced

While this thesis was in preparation, several molecular phylogenetic studies have been published which have provided resolution of the taxonomy of several *Podosphaera* species, directly influencing the research completed. Moparthi et al. (2019) revised *Po*. *clandestina* and described the cherry powdery mildew *Po*. *cerasi* as a genetically separate species. Meeboon et al. (2020) published a worldwide revision of *Po*. *tridactyla* species complex, which included 12 species consisting of seven newly described species, three known taxa and two undescribed, provided the reference sequences used to identify *Po*. *ampla* specimens identified in chapter 4.

Other studies of the genus *Podosphaera* published since the start of this research include the release of the *Po*. *xanthii* mitochondrial genome (Kim et al. 2019) and the first genome assembly for *Po*. *leucotricha* (Gañán et al. 2020), as well as several first disease reports such as *Po*. *erigerontis-canadensis* on dandelion (*Taraxacum officinale*) in the U.S.A. (Yang et al. 2018) and *Po*. *pannosa* on *Eucalyptus* in Brazil (Fonseca et al. 2017), and investigation into the host plants of *Po*. *xanthii* in Taiwan (Yeh et al. 2020). None of these have any direct bearing on the work herein.

5.3.1 Powdery mildew molecular updates

Most recently, Bradshaw and Tobin 2020 published new powdery mildew specific PCR primers that are effective for powdery mildew aDNA from reference collections. They generated six new powdery mildew specific primers for a nested PCR approach which target the ITS and LSU (28S) regions. Using these primers and nested PCR Bradshaw and Tobin (2020) were able to sequence powdery mildew specimens up to 130 years old. The new powdery mildew specific primers that

they published will provide access to powdery mildew DNA stored in reference collections worldwide as PCR amplification is still currently the main molecular tool used. However, Bradshaw and Tobin (2020) did not address the issues of working with aDNA and their PCR protocol requires a nested PCR approach in order to amplify the short DNA regions. This may increase the likelihood of amplifying non-target DNA present in the aDNA sample, which was observed by Smith et al. (2020a) when other fungi and *Malus* ITS gene regions were amplified.

Ellingham et al. (2019) explored potential secondary DNA barcodes to complement the ITS for improved identification in closely related taxa of *Erysiphales*. They tested seven genes (*actin*, β-*tubulin*, *calmodulin*, *Chs*, elongation factor 1-α [*EF1*-α], *Mcm7* and *Tsr1*) and determined *Mcm7* was the most effective in differentiating between closely related species. For their study, Ellingham et al. (2019) utilised fresh powdery mildew samples collected during a U.K. citizen science initiative to test newly designed primers for the seven gene regions. Based on the recommendations of the authors, *Mcm7* was tested on VPRI aDNA and unfortunately failed to amplify during PCR. It was concluded the fragmented nature of the aDNA inhibited the primers from binding and amplifying the large gene of *Mcm7.* Mapping VPRI NGS data to the *Mcm7* gene was also tested during the *Po*. *tridactyla* bioinformatics analysis but this also failed with no sequence reads aligning to the reference *Mcm7*. Due to time constraints this was not investigated further.

5.4 Future research

5.4.1 Use of molecular methods to classify *Oidium* sp. in VPRI

As an extension of this project, it would be useful to complete the re-evaluation of all powdery mildew specimens held in the VPRI using the new molecular diagnostic tools established in this thesis. There are still hundreds of specimens identified only as *Oidium* sp. which need to be identified to species level in order update the pathogen names in the VPRI database, which feeds directly into the Australian Plant Disease Database (APDD). Also, there are many VPRI powdery mildew specimens which are only identified to genus, including six additional genera (*Erysiphe*, *Golovinomyces*, *Leveillula*, *Microsphaera*, *Podosphaera* and *Sawadaea)* not examined in this thesis. Questions such as distinguishing the mating types of *Leveillula* species on *Solanaceae* (biosecurity risk), resolving the *Golovinomyces biocellatus* species complex on *Laminaceae*, more investigation into powdery mildews collected on Australian native flora and identification of powdery mildews of berries and nuts in Australia can all be addressed using the molecular methods developed in this thesis. If funding became available, this would allow revision and completion of the species identifications undertaken as part of the initial ABRS-funded project and potential publication of the *Erysiphales* of Australia.

5.4.2 Powdery mildew DNA in herbaria over time

During the literature search for powdery mildew DNA extraction methods, it was highlighted that there was no published literature regarding the effect of long-term storage on powdery mildew fungal DNA in reference collections. Using NGS data generated from VPRI and DAR apple powdery mildew specimens collected over the last century, there is the opportunity to study the effects of DNA preservation over time. This study would focus on how the powdery mildew fungal DNA changes over time, looking at fragment lengths, deamination and calculating DNA decay rates. This will provide the framework for future powdery mildew reference collection studies, enabling researchers to have background information in what to expect from powdery mildew specimens that are 20, 40, 60, 80 or 100 years old. Also, this NGS data generated from all specimens could be interrogated further for the suggested secondary barcode regions (Ellingham et al. 2019) and determine why previous attempts to map sequences reads to these gene references failed.

5.4.3 Australian powdery mildew sampling initiative

Throughout the research undertaken in this thesis, it became evident how few powdery mildew specimens have been collected since the end of the ABRS funding in the early 2000s. I propose that a new powdery mildew collection initiative be instigated, similar to the ABRS project in the 1990s which contributed over 2000 new powdery mildew collections to VPRI alone and similar to the UK citizen science collection scheme organised by Ellingham et al. (2019). This will complement the work recently published by Kiss et al. (2020) by adding to the powdery mildew species list they compiled and providing further clarity on what powdery mildews occur in Australia and on what hosts. It would be beneficial to resurvey agricultural and horticultural powdery mildews as well as encourage specimen collecting from private gardens to increase the representation of powdery mildews on ornamental plant species in Australian reference collections.

5.5 Conclusions

This project demonstrated that advances in molecular technologies have enabled access to the DNA held in plant pathogen reference collections. Generation of sequences of this DNA allow reanalysis of the classification of an important group of obligate biotrophic fungal pathogens using a phylogenetic approach. The revisions of *Podosphaera* taxonomy completed in this project have provided clarity for two keystone Victorian horticultural industries and resulted in the description of a new species infecting peach, observed so far only in Australia.

The molecular methods that were developed in this thesis provide the beginning of a new era in collection-based research from plant pathogen reference collections. Previous studies have used a mixed approach of DNA barcoding using PCR-based amplification followed by NGS to obtain sequence data (Delgat et al. 2019; Forin et al. 2018). The novel approach used in this thesis utilised whole genome NGS on aDNA containing host and target DNA, and then used reference-initiated bioinformatics to separate the target fungal DNA sequence reads from the other sequence reads. To my knowledge, this is the first time this approach has been used on plant pathogen reference specimens in order to confirm the identities of both the pathogen and the host. This NGS pipeline can be applied to a wide range of obligate biotrophic pathogens for re-identification and can be extended to investigate environmental microorganism assemblages on specimens, study the geographic distributions of plant pathogens in changing climates and evolutionary histories of important plant pathogens. The genetic data collected from these historical collections will be added to the worldwide genetic repository and contribute to the under-explored plant pathogen aDNA.

Two taxonomic questions were resolved by the research undertaken in this project. However, there are still thousands of powdery mildew specimens in Australian reference collections that need to be reassessed. Kiss et al. (2020) documented powdery mildew species from fresh collections, reference collections and previously published research articles to summarise the powdery mildew species present in Australia. Their study concentrated on species found in the northern half of Australia due to a prerequisite of the funding source (L. Kiss 2018, pers. comm.). The identification of an undescribed species among collections in VPRI indicates the potential for species discovery from temperate regions of Australia. By completing the molecular revision of unidentified or incompletely identified specimens of powdery mildews in reference collections together with a new Australia-wide powdery mildew survey, an accurate species list of powdery mildews in Australia can be curated for Australian biosecurity.

References

Abd-Elsalam, K. A., Yassin, M. A., Moslem, M. A., Bahkali, A. H., de Wit, P. J., McKenzie, E. H., Stephenson, S. L., Cai, L., and Hyde, K. D. 2010. Culture collections, the new herbaria for fungal pathogens. Fungal Diversity 45:21-32.

Agrios, G. N. 2005. chapter one - INTRODUCTION. Pages 3-75 in: Plant Pathology (Fifth Edition). G. N. Agrios, ed. Academic Press, San Diego.

Anderson, C., Low-Choy, S., Whittle, P., Taylor, S., Gambley, C., Smith, L., Gillespie, P., Löcker, H., Davis, R., and Dominiak, B. 2017. Australian plant biosecurity surveillance systems. Crop Protection 100:8-20.

Asalf, B., Onofre, R. B., Gadoury, D. M., Peres, N. A., and Stensvand, A. 2020. Pulsed water mists for suppression of strawberry powdery mildew. Plant Disease 104. PDIS-04-20-0735-RE

Bailey, S. E., Mao, X., Struebig, M., Tsagkogeorga, G., Csorba, G., Heaney, L. R., Sedlock, J., Stanley, W., Rouillard, J.-M., and Rossiter, S. J. 2016. The use of museum samples for large-scale sequence capture: a study of congeneric horseshoe bats (family Rhinolophidae). Biological Journal of the Linnean Society 117(1):58-70.

Bakker, F. T., Lei, D., Yu, J., Mohammadin, S., Wei, Z., van de Kerke, S., Gravendeel, B., Nieuwenhuis, M., Staats, M., and Alquezar-Planas, D. E. 2016. Herbarium genomics: plastome sequence assembly from a range of herbarium specimens using an Iterative Organelle Genome Assembly pipeline. Biological Journal of the Linnean Society 117:33-43.

Bayly, M. J., Rigault, P., Spokevicius, A., Ladiges, P. Y., Ades, P. K., Anderson, C., Bossinger, G., Merchant, A., Udovicic, F., and Woodrow, I. E. 2013. Chloroplast genome analysis of Australian eucalypts–*Eucalyptus*, *Corymbia*, *Angophora*, *Allosyncarpia* and *Stockwellia* (*Myrtaceae*). Molecular Phylogenetics and Evolution 69:704-716.

Bebber, D. P., Carine, M. A., Wood, J. R. I., Wortley, A. H., Harris, D. J., Prance, G. T., Davidse, G., Paige, J., Pennington, T. D., Robson, N. K. B., and Scotland, R. W. 2010. Herbaria are a major frontier for species discovery. PNAS 107(51):22169-22171.

Bebber, D. P., Holmes, T., Smith, D., and Gurr, S. J. 2014. Economic and physical determinants of the global distributions of crop pests and pathogens. New Phytologist 202:901-910.

Bélanger, R. R., Bushnell, W. R., Dik, A. J., and Carver, T. L. 2002. The powdery mildews: a comprehensive treatise. American Phytopathological Society (APS Press), U.S.A.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., and Kauserud, H. 2010. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiology 10: 189.

Besnard, G., Bertrand, J. A., Delahaie, B., Bourgeois, Y. X., Lhuillier, E., and Thébaud, C. 2016. Valuing museum specimens: high-throughput DNA sequencing on historical collections of New Guinea crowned pigeons (*Goura*). Biological Journal of the Linnean Society 117:71-82.

Bieker, V. C., and Martin, M. D. 2018. Implications and future prospects for evolutionary analyses of DNA in historical herbarium collections. Botany Letters 165:409-418.

Bieker, V. C., Sánchez Barreiro, F., Rasmussen, J. A., Brunier, M., Wales, N., and Martin, M. D. 2020. Metagenomic analysis of historical herbarium specimens reveals a post-mortem microbial community. Molecular Ecology Resources 20:1206-1219.

Biomatters Ltd, 2020. Geneious Prime 2020.0.5.

Blumer, S. 1933. Die Erysiphaceen Mitteleuropas unter besonderer Berucksichtigung der Schweiz. Beitr. Krypt.-F1. Schweiz 7:1-483. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Blumer, S. 1967. Echte Mehltaupilze (*Erysiphaceae*) G. Fischer Verlag, Jena. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Bradshaw, M., and Tobin, P. C. 2020. Sequencing Herbarium Specimens of a Common Detrimental Plant Disease (Powdery Mildew). Phytopathology: PHYTO-04-20-0139-PER.

Braun, U. 1987. A monograph of the *Erysiphales* (powdery mildews). Beihefte zur Nova Hedwigia. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Braun, U. 1999. Some critical notes on the classification and the generic concept of the Erysiphaceae. Schlechtendalia 3:48-54.

Braun, U. 2011. The current systematics and taxonomy of the powdery mildews (*Erysiphales*): an overview. Mycoscience 52:210-212.

Braun, U. 2013. (2210–2232) Proposals to conserve the teleomorph–typified name Blumeria against the anamorph–typified name Oidium and twenty–two teleomorph–typified

powdery mildew species names against competing anamorph–typified names (Ascomycota: Erysiphaceae). TAXON 62:1328-1331.

Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Braun, U., and Takamatsu, S. 2000. Phylogeny of *Erysiphe*, *Microsphaera*, *Uncinula* (*Erysipheae*) and *Cystotheca*, *Podosphaera*, *Sphaerotheca* (*Cystotheceae*) inferred from rDNA ITS sequences. Schlechtendalia 4:1-33.

Braun, U., Takamatsu, S., Heluta, V., Limkaisang, S., Divarangkoon, R., Cook, R., and Boyle, H. 2006. Phylogeny and taxonomy of powdery mildew fungi of *Erysiphe* sect. *Uncinula* on *Carpinus* species. Mycological Progress 5:139-153.

Brewer, M. T., and Milgroom, M. G. 2010. Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse *Vitis* species. BMC Evolutionary Biology 10:268.

Brock, P. M., Döring, H., and Bidartondo, M. I. 2009. How to know unknown fungi: the role of an herbarium. New Phytologist 181:719-724.

Brotherton, P., Endicott, P., Sanchez, J. J., Beaumont, M., Barnett, R., Austin, J., and Cooper, A. 2007. Novel high-resolution characterization of ancient DNA reveals C> U-type base modification events as the sole cause of post-mortem miscoding lesions. Nucleic acids research 35:5717-5728.

Bruns, T. D., Fogel, R., and Taylor, J. W. 1990. Amplification and sequencing of DNA from fungal herbarium specimens. Mycologia 82:175-184.

Buerki, S., and Baker, W. J. 2016. Collections-based research in the genomic era. Biological Journal of the Linnean Society 117:5-10.

Burrell, A. S., Disotell, T. R., and Bergey, C. M. 2015. The use of museum specimens with high-throughput DNA sequences. Journal of Human Evolution 79:35-44.

Cano, R., Poinar, H., Roubik, D., and Poinar, G. 1992. Enzymatic amplification and nucleotide sequencing of portions of the 18s RNA gene of the bee *Proplebeia dominicana* (*Apidae*: *Hymenoptera*) isolated from 25-40 million year old Dominican amber. Medical Science Research 20:619-622.

Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553-556.

Chin, S.-W., Shaw, J., Haberle, R., Wen, J., and Potter, D. 2014. Diversification of almonds, peaches, plums and cherries–molecular systematics and biogeographic history of *Prunus* (*Rosaceae*). Molecular Phylogenetics and Evolution 76:34-48.

Choi, J., Lee, H., and Shipunov, A. 2015. All that is gold does not glitter? Age, taxonomy, and ancient plant DNA quality. PeerJ 3: e1087.

Cook, R., Inman, A., and Billings, C. 1997. Identification and classification of powdery mildew anamorphs using light and scanning electron microscopy and host range data. Mycological Research 101:975-1002.

Craik, W., Palmer, D., and Sheldrake, R. 2017. Priorities for Australia's Biosecurity System – An Independent Review of the Capacity of the National Biosecurity System and its Underpinning Intergovernmental Agreement.'. Commonwealth of Australia: Canberra, ACT.

Cunnington, J. H., and Brett, R. W. 2009. S-type powdery mildew on lilac in Australia. Australasian Plant Disease Notes 4:21-22.

Cunnington, J. H., Jones, R. H., and de Alwis, S. K. 2008b. First record of powdery mildew on the *Cephalotaceae*. Australasian Plant Disease Notes 3:51-52.

Cunnington, J. H., Lawrie, A. C., and Pascoe, I. G. 2004. Molecular determination of anamorphic powdery mildew fungi on the *Fabaceae* in Australia. Australasian Plant Pathology 33:281-284.

Cunnington, J. H., Lawrie, A. C., and Pascoe, I. G. 2004b. Unexpected ribosomal DNA internal transcribed spacer sequence variation within *Erysiphe aquilegiae* sensu lato. Fungal Diversity 16:1-10.

Cunnington, J. H., Lawrie, A. C., and Pascoe, I. G. 2005a. Molecular identification of *Golovinomyces* (*Ascomycota*: *Erysiphales*) anamorphs on the *Solanaceae* in Australia. Australasian Plant Pathology 34:51-55.

Cunnington, J. H., Lawrie, A. C., and Pascoe, I. G. 2005b. Genetic variation within *Podosphaera tridactyla* reveals a paraphyletic species complex with biological specialization towards specific *Prunus* subgenera. Mycological Research 109:357-362.

Cunnington, J. H., Takamatsu, S., Lawrie, A. C., and Pascoe, I. G. 2003. Molecular identification of anamorphic powdery mildew (*Erysiphales*). Australiasian Plant Pathology 32:421-428.

Cunnington, J. H., Watson, A., Liberato, J. R., and Jones, R. H. 2008. First record of powdery mildew on carrots in Australia. Australasian Plant Disease Notes 3:38-41.

Davidson, J. A., Krysinska-Kaczmarek, M., Kimber, R. B. E., and Ramsey, M. D. 2004. Screening field pea germplasm for resistance to downy mildew (*Peronospora viciae*) and powdery mildew (*Erysiphe pisi*). Australasian Plant Pathology 33:413-417.

Delgat, L., Dierickx, G., De Wilde, S., Angelini, C., De Crop, E., De Lange, R., Halling, R., Manz, C., Nuytinck, J., and Verbeken, A. 2019. Looks can be deceiving: the deceptive milkcaps (*Lactifluus*, *Russulaceae*) exhibit low morphological variance but harbour high genetic diversity. IMA Fungus 10:14.

Dentinger, B. T., Gaya, E., O'Brien, H., Suz, L. M., Lachlan, R., Díaz-Valderrama, J. R., Koch, R. A., and Aime, M. C. 2016. Tales from the crypt: genome mining from fungarium specimens improves resolution of the mushroom tree of life. Biological Journal of the Linnean Society 117:11-32.

Drábková, L., Kirschner, J., and Vlček, Č. 2002. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of Junaceae. Plant Molecular Biology Reporter 20:161-175.

Dreiseitl, A., and Platz, G. 2012. Powdery mildew resistance genes in barley varieties grown in Australia. Crop and Pasture Science 63:997-1006.

Dry, I. B., Feechan, A., Anderson, C., Jermakow, A. M., Bouquet, A., Adam-Blondon, A.-F., and Thomas, M. R. 2010. Molecular strategies to enhance the genetic resistance of grapevines to powdery mildew. Australian Journal of Grape and Wine Research 16:94-105.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-1797.

Edwards, K., Johnstone, C., and Thompson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research 19:1349.

Ellingham, O., David, J., and Culham, A. 2019. Enhancing identification accuracy for powdery mildews using previously underexploited DNA loci. Mycologia 111:798-812.

Emmett, R. W., and Edwards, J. 2010. Economic evaluation of grapevine powdery mildew management R&D: An evaluation of integrated pest and disease management case

study. Department of Primary Industries, ed. The State Government of Victoria, Melbourne.

Feau, N., Decourcelle, T., Husson, C., Desprez-Loustau, M.-L., and Dutech, C. 2011. Finding single copy genes out of sequences genomes for multilocus phylogenetics in non-model fungi. PLOS ONE 6(4):e18803.

Feechan, A., Kabbara, S., and Dry, I. B. 2011. Mechanisms of powdery mildew resistance in the *Vitaceae* family. Molecular Plant Pathology 12:263-274.

Fish, S. 1970. The history of plant pathology in Australia. Annual Review of Phytopathology 8:13-36.

Fonseca, N. R., Guimarães, L. M., Pires, R. P., Klopfenstein, N. B., and Alfenas, A. C. 2017. Eucalypt powdery mildew caused by *Podosphaera pannosa* in Brazil. Tropical Plant Pathology 42:261-272.

Forin, N., Nigris, S., Voyron, S., Girlanda, M., Vizzini, A., Casadoro, G., and Baldan, B. 2018. Next generation sequencing of ancient fungal specimens: the case of the Saccardo Mycological Herbarium. Frontiers in Ecology and Evolution 6:129.

Funk, V. A., Gostel, M., Devine, A., Kelloff, C. L., Wurdack, K., Tuccinardi, C., Radosavljevic, A., Peters, M., and Coddington, J. 2017. Guidelines for collecting vouchers and tissues intended for genomic work (Smithsonian Institution): Botany Best Practices. Biodiversity data Journal 5: e11625.

Gadoury, D. M., Asalf, B., Heidenreich, M. C., Herrero, M., Welser, M., Seem, R. C., Tronsmo, A. M., and Stensvand, A. 2010. Initiation, development, and survival of cleistothecia of *Podosphaera aphanis* and their role in the epidemiology of strawberry powdery mildew. Phytopathology 100:246-251.

Gañán, L., White, R. A., Friesen, M. L., Peever, T. L., and Amiri, A. 2020. A Genome Resource for the Apple Powdery Mildew Pathogen *Podosphaera leucotricha*. Phytopathology 110:1756-1758.

García-Ruiz, M., Galicia-Buendia, N., Leyva-Mir, S., Correia, K., Nieto-López, E., Camacho-Tapia, M., and Tovar-Pedraza, J. 2019. First report of powdery mildew of European plum caused by *Podosphaera tridactyla* in Mexico. Plant Disease 103:587.

George, A. 1999. Deciduous fruit production in Australia. RAP Publication (FAO) eng no. 1999/10.

Glawe, D. A. 2008. The Powdery Mildews: A review of the world's most familiar (yet poorly known) plant pathogens. Annual Review of Phytopathology 46:27-51.

Golovin, P. 1956. Materialy k monografii mučnisto-rosjanyh gribov (semejstvo *Erysiphaceae*) v SSSR (rody *Arthrocladiella*, *Podosphaera*, *Microsphaera*). Tr. Bot. Inst. Im. VL Komarova 2:309-366. Cited in Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Golovin, R. 1958. Review of the genera of the *Erysiphaceae*. Sbornik rabot Instituta prikladnoi zoologii i fitopalologif 5:101-139. Cited in Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Golzar, H., Shankar, M., and D'Antuono, M. 2016. Responses of commercial wheat varieties and differential lines to western Australian powdery mildew (*Blumeria graminis* f. Sp. *tritici*) populations. Australasian Plant Pathology 45:347-355.

Grove, G. G., and Boal, R. J. 1991. Factors affecting germination of conidia of *Podosphaera clandestina* on leaves and fruit of Sweet Cherry. Ecology and Epidemiology 81:1513-1518.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59:307-321.

Gutaker, R. M., and Burbano, H. A. 2017. Reinforcing plant evolutionary genomics using ancient DNA. Current Opinion in Plant Biology 36:38-45.

Hart, M. L., Forrest, L. L., Nicholls, J. A., and Kidner, C. A. 2016. Retrieval of hundreds of nuclear loci from herbarium specimens. Taxon 65:1081-1092.

Head, S. R., Kormori, H. K., LaMere, S. A., Whisenant, T., Nieuwerburgh, F. V., Salomon, D. R., and Ordoukhanian, P. 2014. Library construction for next-generation sequencing: Overviews and challenges. BioTechniques 56:61-77.

Heberling, J. M., Prather, L. A., and Tonsor, S. J. 2019. The changing uses of herbarium data in an era of global change: an overview using automated content analysis. BioScience 69:812-822.

Heyduk, K., Trapnell, D. W., Barrett, C. F., and Leebens-Mack, J. 2016. Phylogenomic analyses of species relationships in the genus *Sabal* (*Arecaceae*) using targeted sequence capture. Biological Journal of the Linnean Society 117:106-120.

Hickey, L. T., Lawson, W., Platz, G. J., Fowler, R. A., Arief, V., Dieters, M., Germán, S., Fletcher, S., Park, R. F., and Singh, D. 2012. Mapping quantitative trait loci for partial resistance to powdery mildew in an Australian barley population. Crop Science 52:1021-1032.

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O. A., and Wilson, A. C. 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature 312:282.

Hirata, T., and Takamatsu, S. 1996. Nucleotide sequence diversity of rDNA internal transcribed spacers extracted from conidia and cleistothecia of several powdery mildew fungi. Mycoscience 37:283-288.

Homma, Y. 1937. *Erysiphaceae* of Japan. Journal of the Faculty of Agriculture, Hokkaido Imperial University 38:183-461. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Horticulture Innovation Australia Limited, 2019. Australian Horticulture Statistics Handbook Fruit 2017/2018. HIA Ltd, ed. Sydney, Australia: Freshlogic, 91-5.

Howden, M., Nelson, R., and Cameron, A. 2020. Agricultural Commodities. Australian Bureau of Agricultural and Resource Economics (ABARE), Australia.

Huelsenbeck, J. P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755. doi:146.6.100.192

Hyde, K. D., Chomnunti, P., Crous, P. W., Groenewald, J. Z., Damm, U., Ko Ko, T. W., Shivas, R. G., Summerell, B. A., and Tan, Y. P. 2010. A case for re-inventory of Australia's plant pathogens. Persoonia 25:50-60.

Ito, M., and Takamatsu, S. 2010. Molecular phylogeny and evolution of subsection *Magnicellulatae* (*Erysiphaceae*: *Podosphaera*) with special reference to host plants. Mycoscience 51:34-43.

Jaczewski, A. 1927. Karmannyj opredelitel'gribov, II. Mucnisto-rosjanye griby, Leningrad:65-75. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Jankovics, T., Dolovac, N., Bulajić, A., Krstić, B., Pascal, T., Bardin, M., Nicot, P. C., and Kiss, L. 2011. Peach rusty spot is caused by the apple powdery mildew fungus, *Podosphaera leucotricha*. Plant Disease 95:719-724.

Javid, M., Rosewarne, G. M., Sudheesh, S., Kant, P., Leonforte, A., Lombardi, M., Kennedy, P. R., Cogan, N. O. I., Slater, A. T., and Kaur, S. 2015. Validation of molecular markers associated with boron tolerance, powdery mildew resistance and salinity tolerance in field peas. Frontiers in Plant Science 6:917.

Jeanes, J. A., and Jobson, P. C. 1996. *Rosaceae*. in: Flora of Victoria, *Dicotyledons Winteraceae* to *Myrtaceae* vol. 3. N. G. Walsh and T. J. Entwisle, eds. Inkata Press, Melbourne.

Jeon, C., Kang, S., Choi, W., and Shin, H.-D. 2019. First report of powdery mildew caused by *Podosphaera tridactyla* on *Prunus davidiana* in Korea. Plant Disease 103:158-158.

Johnston, A., and Booth, C. 1983. Plant Pathologist's Pocketbook. C.M.I, Kew.

Johnston, P. R., Quijada, L., Smith, C. A., Baral, H.-O., Hosoya, T., Baschien, C., Pärtel, K., Zhuang, W.-Y., Haelewaters, D., and Park, D. 2019. A multigene phylogeny toward a new phylogenetic classification of Leotiomycetes. IMA fungus 10:1-22.

Johnson, P. H., Olson, C. B., and Goodman, M. 1985. Isolation and characterization of deoxyribonucleic acid from tissue of the woolly mammoth, *Mammuthus primigenius*. Comparative biochemistry and physiology. Comparative Biochemistry Physiology B. 81:1045-1051.

Junell, L. 1966. A revision of *Sphaerotheca fuliginea* ([Schlecht.] Fr.) Poll. s. lat. Svensk Botanisk Tidskrift 60:365-392. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Junell, L. 1967. *Erysiphaceae* of Sweden. Symbolae Botanicae Upsaliensis 14:1-117. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Keeble-Gagnère, G., Rigault, P., Tibbits, J., Pasam, R., Hayden, M., Forrest, K., Frenkel, Z., Korol, A., Huang, B., Cavanagh, C., Taylor, J., Abrouk, M., Sharpe, A., Konkin, D., Sourdille, P., and Darrier, B. 2018. Optical and physical mapping with local finishing enables megabase-scale resolution of agronomically important regions in the wheat genome. Genome Biology 19:112.

Kemp, B. M., Monroe, C., and Smith, D. G. 2006. Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 33:1680-1689.

Kettles, G. J., and Luna, E. 2019. Food security in 2044: How do we control the fungal threat? Fungal Biology 123:558-564.

Kim, S., Jung, M., Oh, E. A., Ho Kim, T., and Kim, J.-G. 2019. Mitochondrial genome of the *Podosphaera xanthii*: a plant pathogen causes powdery mildew in cucurbits. Mitochondrial DNA Part B 4:4172-4173.

Kiss, L., Vaghefi, N., Bransgrove, K., Dearnaley, J. D. W., Takamatsu, S., Tan, Y. P., Marston, C., Liu, S.-Y., Jin, D.-N., Adorada, D. L., Bailey, J., Cabrera de Álvarez, M. G., Daly, A., Dirchwolf, P. M., Jones, L., Nguyen, T. D., Edwards, J., Ho, W., Kelly, L., Mintoff, S. J. L., Morrison, J., Németh, M. Z., Perkins, S., Shivas, R. G., Smith, R., Stuart, K., Southwell, R., Turaganivalu, U., Váczy, K. Z., Blommestein, A. V., Wright, D., Young, A., and Braun, U. 2020. Australia: A continent without native powdery mildews? The first comprehensive catalog indicates recent introductions and multiple host range expansion events and leads to the re-discovery of Salmonomyces as a new lineage of the *Erysiphales*. Frontiers in Microbiology 11:1571.

Kistler, L., Bieker, V. C., Martin, M. D., Pedersen, M. W., Ramos Madrigal, J., and Wales, N. 2020. Ancient Plant Genomics in Archaeology, Herbaria, and the Environment. Annual Review of Plant Biology 71:605-629.

Kuzuya, M., Yashiro, K., Tomita, K., and Ezura, H. 2006. Powdery mildew (*Podosphaera xanthii*) resistance in melon is categorized into two types based on inhibition of the infection processes. Journal of Experimental Botany 57:2093-2100.

Leus, L., Dewitte, A., Van Huylenbroeck, J., Vanhoutte, N., Van Bockstaele, E., and Höfte, M. 2006. *Podosphaera pannosa* (syn. *Sphaerotheca pannosa*) on *Rosa* and *Prunus* spp.: characterization of pathotypes by differential plant reactions and ITS sequences. Journal of Phytopathology 154:23-28.

Li, W., Brlansky, R. F., and Hartung, J. S. 2006. Amplification of DNA of *Xanthomonas axonopodis* pv. citri from historic citrus canker herbarium specimens. Journal of Microbiological Methods 65:237-246.

Liberato, J. R., and Cunnington, J. H. 2006. First record of *Erysiphe aquilegiae* on a host outside the *Ranunculaceae*. Australasian Plant Pathology 35:291-292.

Liberato, J. R., and Cunnington, J. H. 2007. Powdery mildew on Mentha in Australia. Australasian Plant Disease Notes 2:83-86.

Lindahl, T. 1993. Instability and decay of the primary structure of DNA. Nature 362:709-715.

Linderholm, A. 2016. Ancient DNA: the next generation–chapter and verse. Biological journal of the Linnean society 117:150-160.

Lister, D. L., Bower, M. A., Howe, C. J., and Jones, M. K. 2008. Extraction and amplification of nuclear DNA from herbarium specimens of emmer wheat: A method for assessing DNA preservation by maximum amplicon length recovery. Taxon 57:254-258.

Lu, W., Newlands, N. K., Carisse, O., Atkinson, D. E., and Cannon, A. J. 2020. Disease risk forecasting with Bayesian learning networks: Application to grape powdery mildew (*Erysiphe necator*) in vineyards. Agronomy 10:622.

Marimon, N., Eduardo, I., Martínez-Minaya, J., Vicent, A., and Luque, J. 2020. Disease progression of peach powdery mildew in Catalonia, Spain–Towards a decision support system based on degree-days to initiate fungicide spray programs. Plant Disease 104:9, 2418-2425.

Marmolejo, J., Siahaan, S. A., Takamatsu, S., and Braun, U. 2018. Three new records of powdery mildews found in Mexico with one genus and one new species proposed. Mycoscience 59:1-7.

Matlock, B. 2015. Assessment of nucleic acid purity. in: Technical Note 52646 Thermo Fisher Scientific Inc.

McGaughran, A. 2020. Effects of sample age on data quality from targeted sequencing of museum specimens: what are we capturing in time? BMC Genomics 21:1-10.

Mcintosh, R., Luig, N., and Baker, E. 1967. Genetic and Cytogenetic Studies of Stem Rust, Leaf Rust, and Powdery Mildew Resistances in Hope and Related Wheat Cultivars. Australian Journal of Biological Sciences 20:1181-1192.

McKirdy, S., Rodoni, B., Moran, J., and Sharma, S. 2012. Microbial threat–a growing challenge for plant biosecurity. Microbiology Australia 33:12-14.

McTaggart, A. R., Nest, M. A. v. d., Steenkamp, E. T., Roux, J., Slippers, B., Shuey, L. S., Wingfield, M. J., and Drenth, A. 2016. Fungal genomics challenges the dogma of name-based biosecurity. PLOS Pathogens 12(5): e1005475.

Meeboon, J., Takamatsu, S., and Braun, U. 2020. Morphophylogenetic analysis revealed that *Podosphaera tridactyla* constitutes a species complex. Mycologia 112:244-266.

Meineke, E. K., Davis, C. C., and Davies, T. J. 2018. The unrealized potential of herbaria for global change biology. Ecological Monographs 88:505-525.

Moparthi, S., Grove, G. G., Pandey, B., Bradshaw, M., Latham, S. R., Braun, U., Meeboon, J., and Romberg, M. 2019. Phylogeny and taxonomy of *Podosphaera cerasi,* sp. nov., and *Podosphaera prunicola* sensu lato. Mycologia 111:647-659.

Mori, Y., Sato, Y., and Takamatsu, S. 2000. Evolutionary analysis of the powdery mildew fungi using nucleotide sequences of the nuclear ribosomal DNA. Mycological Society of America 92:74-93.

Murray, G. M., and Brennan, J. P. 2009. Estimating disease losses to the Australian wheat industry. Australasian Plant Pathology 38:558-570.

Murray, G. M., and Brennan, J. P. 2010. Estimating disease losses to the Australian barley industry. Australasian Plant Pathology 39:85-96.

Nascinmento, F. S., Wei-Pridgeon, Y., Arrowood, M. J., Moss, D., Silva, A. J. d., Talundzic, E., and Qvarnstrom, Y. 2016. Evaluation of library preparation methods for Illumina next generation sequencing of small amounts of DNA from foodborne parasites. Journal of Microbiological Methods 130:23-26.

Nedoluzhko, A. V., Sharko, F. S., Tsygankova, S. V., Boulygina, E. S., Barmintseva, A. E., Krasivskaya, A. A., Ibragimova, A. S., Gruzdeva, N. M., Rastorguev, S. M., and Mugue, N. S. 2020. Molecular phylogeny of one extinct and two critically endangered Central Asian sturgeon species (genus *Pseudoscaphirhynchus*) based on their mitochondrial genomes. Scientific Reports 10:722

Neger, F. W. 1901. Beitrage zur Biologie der Erysipheen. Flora 88:333-370. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Nelson, M., Roffey, P., McNevin, D., Lennard, C., and Gahan, M. E. 2014. An overview of biosecurity in Australia. Australian Journal of Forensic Sciences 46:383-396.

O'Brien, R., Vawdrey, L., and Glass, R. 1988. Fungicide resistance in cucurbit powdery mildew (*Sphaerotheca fuliginea*) and its effect on field control. Australian Journal of Experimental Agriculture 28:417-423.

O'Brien, R. G. 1994. Fungicide resistance in populations of cucurbit powdery mildew (*Sphaerotheca fuliginea*). New Zealand Journal of Crop and Horticultural Science 22:145-149.

O'Rourke, D. H., Carlyle, S. W., and Parr, R. L. 1996. Ancient DNA: Methods, progress and perspectives. American Journal of Human Biology: The Official Journal of the Human Biology Association 8:557-571.

O'Rourke, D. H., Hayes, M. G., and Carlyle, S. W. 2000. Ancient DNA studies in physical anthropology. Annual Review of Anthropology 29:217-242.

Pääbo, S. 1985a. Molecular cloning of ancient Egyptian mummy DNA. Nature 314:644-645.

Pääbo, S. 1985b. Preservation of DNA in ancient Egyptian mummies. Journal of Archaeological Science 12:411-417.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., and Hofreiter, M. 2004. Genetic analysis from ancient DNA. Annual Review of Genetics 38:645-679.

Palmer, S. A. 2007. Strawberry powdery mildew: epidemiology and the effect of host nutrition on disease. University of Adelaide.

Pandey, B., Naidu, R. A., and Grove, G. G. 2018. Next generation sequencing analysis of double-stranded RNAs from sweet cherry powdery mildew fungus *Podosphaera prunicola*. Journal of Plant Pathology 100:435-446.

Parbery, D. G. 2015. Daniel McAlpine and The Bitter Pit. Springer International Publishing, Switzerland.

Pascoe, I. G., and Sivapalan, A. 1995. Revision of *Erysiphales* (powdery mildew fungi) in Australia, and reassessment of the classification of anamorphic mildews. in: 10th Australasian Plant Pathology Society Conference, 28–30 August APPS, ed. Proceedings of the 10th Australasian Plant Pathology Society Conference, Lincoln University, Christchurch.

Pérez-García, A., Romero, D., Fernández-Ortuño, D., López-Ruiz, F., De Vicente, A., and Tores, J. A. 2009. The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*), a constant threat to cucurbits. Molecular Plant Pathology 10:153-160.

Pintye, A., Bereczky, Z., Kovács, G. M., Nagy, L. G., Xu, X., Legler, S. E., Váczy, Z., Váczy, K. Z., Caffi, T., Rossi, V., and Kiss, L. 2012 No indication of strict host associations in a widespread

mycoparasite: Grapevine powdery mildew (*Erysiphe necator*) is attacked by phylogenetically distant *Ampelomyces* strains in the field. Phytopathology 102:707-716.

Plant Health Australia 2001 [Online Database]. https://www.planthealthaustralia.com.au.

Plant Health Australia. 2017. The National Plant Biosecurity Diagnostic Strategy. Canberra, ACT, Australia.

Plant Health Australia. 2017. National Plant Biosecurity Status Report. Canberra, ACT, Australia.

Plant Health Australia. 2019. National Plant Biosecurity Status Report. Canberra, ACT, Australia.

Richards, S. M., Hovhannisyan, N., Gilliham, M., Ingram, J., Skadhauge, B., Heiniger, H., Llamas, B., Mitchell, K. J., Meachen, J., Fincher, G. B., Austin, J. J., and Cooper, A. 2019. Low-cost cross-taxon enrichment of mitochondrial DNA using in-house synthesised RNA probes. PLOS ONE 14(2): e0209499.

Ridout, C. J. 2009. The Mycota. Pages 51-68 in: A comprehensive treatise on fungi as experimental systems for basic and applied research, vol. 2. H. B. Deising, ed. Springer-Verlag, Berlin Heidelberg.

Ristaino, J. B. 1998. The importance of archival and herbarium materials in understanding the role of Oospores in Late Blight epidemics of the past. Phytopathology 88:1120-1130.

Ristaino, J. B. 2002. Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. Microbes and infection 4:1369-1377.

Ristaino, J. B. 2019. The Importance of Mycological and Plant Herbaria in Tracking Plant Killers. Frontiers in Ecology and Evolution 7:521.

Saenz, G. S., and Taylor, J. W. 1999a. Phylogeny of the *Erysiphales* (powdery mildews) inferred from internal transcribed spacer ribosomal DNA sequences. Canadian Journal of Botany 77:150-168.

Saenz, G. S., and Taylor, J. W. 1999b. Phylogenetic relationships of *Meliola* and *Meliolina* inferred from nuclear small subunit rRNA sequences. Mycological Research 103:1049-1056.

Särkinen, T., Staats, M., Richardson, J. E., Cowan, R. S., and Bakker, F. T. 2012. How to open the treasure chest? Optimising DNA extraction from herbarium specimens. PLOS ONE 7(8): e43808.

Sawada, K. 1951. Researches on fungi in the Tôhoku district of Japan (I). *Erysiphaceae*. Bulletin of the Government Forest Experiment Station. 50:97-140. Cited by Braun, U.,

and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Schindel, D. E., and Cook, J. A. 2018. The next generation of natural history collections. PLOS Biology 16(7):e2006125.

Schmitt, I., Crespo, A., Divakar, P. K., Fankhauser, J. D., Herman-Sackett, E., Kalb, K., Nelsen, M. P., Nelson, N. A., Rivas-Plata, E., Shimp, A. D., Widhelm, T., and Lumbsch, H. T. 2009. New primers for promising single-copy genes in fungal phylogenetics and systematics. Persoonia 23:35-40.

Scholefield, P., and Morison, J. 2010. Assessment of economic cost of endemic pests and diseases on the Australian grape and wine industry. Grape and Wine Research and Development Corporation (GWRDC) project. Project number: GWR 8.

Shetty, R., Jensen, B., Shetty, N. P., Hansen, M., Hansen, C. W., Starkey, K. R., and Jørgensen, H. J. L. 2012. Silicon induced resistance against powdery mildew of roses caused by *Podosphaera pannosa*. Plant pathology 61:120-131.

Shi, S., Li, J., Sun, J., Yu, J., and Zhou, S. 2013. Phylogeny and classification of *Prunus sensu lato* (*Rosaceae*). Journal of Integrative Plant Biology 55:1069-1079.

Shin, H. 1988. *Erysiphaceae* of Korea [PhD thesis]. Seoul National University, South Korea.

Shin, H.-D., and La, Y.-J. 1993. Morphology of edge lines of chained immature conidia on conidiophores in powdery mildew fungi and their taxonomic significance. Mycotaxon 46:445-451.

Shivas, R., and Beasley, D. 2005. Management of plant pathogen collections. Office of the Chief Plant Protection Officer, Department of Agriculture, Fisheries and Forestry, Australian Government.

Shivas, R., Beasley, D., Pascoe, I., Cunnington, J., Pitkethley, R., and Priest, M. 2006. Specimen-based databases of Australian plant pathogens: past, present and future. Australasian Plant Pathology 35:195-198.

Sikes, B. A., Bufford, J. L., Hulme, P. E., Cooper, J. A., Johnston, P. R., and Duncan, R. P. 2018. Import volumes and biosecurity interventions shape the arrival rate of fungal pathogens. PLOS Biology 16(5): e2006025.

Smith, R. L., Sawbridge, T., Mann, R., Kaur, J., May, T. W., and Edwards, J. 2020a. Rediscovering an old foe: Optimised molecular methods for DNA extraction and

sequencing applications for fungarium specimens of powdery mildew (*Erysiphales*). PLOS ONE 15(5): e0232535.

Smith, R. L., May, T. W., Kaur, J., Sawbridge, T., Mann, R., and Edwards, J. 2020b. Molecular data from up to 130-year-old herbarium specimens do not support the presence of cherry powdery mildew in Australia. Plant Pathology. DOI: 10.1111/ppa.13316.

Spencer, D. M. 1978. The Powdery Mildews. London (UK) Academic Press.

Sproul, J. S., and Maddison, D. R. 2017. Sequencing historical specimens: successful preparation of small specimens with low amounts of degraded DNA. Molecular Ecology Resources 17:1183-1201.

Staats, M., Cuenca, A., Richardson, J. E., Vrielink-van Ginkel, R., Petersen, G., Seberg, O., and Bakker, F. T. 2011. DNA damage in plant herbarium tissue. PLOS ONE 6(12): e28448.

Staats, M., Erkens, R. H., van de Vossenberg, B., Wieringa, J. J., Kraaijeveld, K., Stielow, B., Geml, J., Richardson, J. E., and Bakker, F. T. 2013. Genomic treasure troves: complete genome sequencing of herbarium and insect museum specimens. PLOS One 8(7): e69189.

Takamatsu, S. 2004. Phylogeny and evolution of the powdery mildew fungi (*Erysiphales*, *Ascomycota*) inferred from nuclear ribosomal DNA sequences. Mycoscience 45:147-157.

Takamatsu, S. 2013. Molecular phylogeny reveals phenotypic evolution of powdery mildews (*Erysiphales*, *Ascomycota*). Journal of general plant pathology 79:218-226.

Takamatsu, S., Hirata, T., and Sato, Y. 1998. Phylogenetic analysis and predicted secondary structures of the rDNA internal transcribed spacers of the powdery mildew fungi (*Erysiphaceae*). Mycoscience 39:441-453.

Takamatsu, S., Hirata, T., and Sato, Y. 2000. A parasitic transition from trees to herbs occurred at least twice in tribe *Cystotheceae* (*Erysiphaceae*): evidence from nuclear ribosomal DNA. Mycological Research 104:1304-1311.

Takamatsu, S., Hirata, T., Sato, Y., and Nomura, Y. 1999. Phylogenetic relationships of *Microsphaera* and *Erysiphe* section *Erysiphe* (powdery mildews) inferred from the rDNA ITS sequences. Mycoscience 40:259-268.

Takamatsu, S., Inagaki, M., Niinomi, S., Khodaparast, S. A., Shin, H.-D., Grigaliunaite, B., and Havrylenko, M. 2008. Comprehensive molecular phylogenetic analysis and evolution of

the genus *Phyllactinia* (*Ascomycota*: *Erysiphales*) and its allied genera. Mycological Research 112:299-315.

Takamatsu, S., and Kano, Y. 2001. PCR primers useful for nucleotide sequencing of rDNA of the powdery mildew fungi. Mycoscience 42:135-139.

Takamatsu, S., Niinomi, S., Harada, M., and Havrylenko, M. 2010. Molecular phylogenetic analyses reveal a close evolutionary relationship between *Podosphaera* (*Erysiphales*: *Erysiphaceae*) and its rosaceous hosts. Persoonia: Molecular Phylogeny and Evolution of Fungi 24:38.

Taylor, J. W., and Swann, E. C. 1994. DNA from herbarium specimens. Pages 166-181 in: Ancient DNA. Springer.

Telle, S., and Thines, M. 2008. Amplification of *cox2* (~620 bp) from 2mg of Up to 129 years old herbarium specimens, comparing 19 extraction methods and 15 polymerases. PLoS ONE 3:1-8.

Thiers, B. M. 2016. Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium, New York.

Thomas, M., Gilbert, P., Bandelt, H.-J., Hofreiter, M., and Barnes, I. 2005. Assessing ancient DNA studies. TRENDS in Ecology and Evolution 20:541-544.

Timmermans, M. J., Viberg, C., Martin, G., Hopkins, K., and Vogler, A. P. 2016. Rapid assembly of taxonomically validated mitochondrial genomes from historical insect collections. Biological Journal of the Linnean Society 117:83-95.

Uloth, M. B., You, M. P., and Barbetti, M. J. 2016. Cultivar resistance offers the first opportunity for effective management of the emerging powdery mildew (*Erysiphe cruciferarum*) threat to oilseed brassicas in Australia. Crop and Pasture Science 67:1179-1187.

Uloth, M. B., You, M. P., and Barbetti, M. J. 2018. Plant age and ambient temperature: significant drivers for powdery mildew (*Erysiphe cruciferarum*) epidemics on oilseed rape (*Brassica napus*). Plant Pathology 67:445-456.

Urbanietz, A., and Dunemann, F. 2005. Isolation, identification and molecular characterization of physiological races of apple powdery mildew (*Podosphaera leucotricha*). Plant Pathology 54:125-133.

Viégas, A. P. 1944. Alguns fungos do Brasil: II. ascomicetos. Bragantia 4:5-392. Cited by Braun, U., and Cook, R. T. A. 2012. Taxonomic manual of the *Erysiphales* (Powdery Mildews). CBS-KNAW Fungal Biodiversity Center, The Netherlands.

Virtue, J. G., Bennett, S. J., and Randall, R. P. 2004. Plant introductions in Australia: how can we resolve 'weedy' conflicts of interest. Pages 42-48 in: Proceedings of the 14th Australian Weeds Conference, eds BM Sindel and SB Johnson.

Walker, J. 1983. Pacific mycogeography: deficiencies and irregularities in the distribution of plant parasitic fungi. Australian Journal of Botany Supplementary Series 13:89-136.

Washington, W. S., Villalta, O. N., Ingram, J., and Bardon, D. 1998. Susceptibility of apple cultivars to apple scab and powdery mildew in Victoria, Australia. Australian Journal of Experimental Agriculture 38:625-629.

Weiß, C. L., Schuenemann, V. J., Devos, J., Shirsekar, G., Reiter, E., Gould, B. A., Stinchcombe, J. R., Krause, J., and Burbano, H. A. 2016. Temporal patterns of damage and decay kinetics of DNA retrieved from plant herbarium specimens. Royal Society open science 3:160239.

Wen, J., Berggren, S. T., Lee, C-H., Ickert-Bond, S., Yi, T-S., Yoo, K-O., Xie, L., Shaw, J., and Potter, D. 2008. Phylogenetic inferences in *Prunus* (*Rosaceae*) using chloroplast ndhF and nuclear ribosomal ITS sequences. Journal of Systematics and Evolution 46:322-332.

White, T. J., Bruns, T. D., Lee, S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press Inc., California, USA.

Willerslev, E., and Cooper, A. 2005. Ancient DNA. Proceedings of the Royal Society B: Biological Sciences 272:3-16.

Wolfenbarger, S. N., Massie, S. T., Ocamb, C., Eck, E. B., Grove, G. G., Nelson, M. E., Probst, C., Twomey, M. C., and Gent, D. H. 2016. Distribution and characterization of *Podosphaera macularis* virulent on hop cultivars possessing R6-based resistance to powdery mildew. Plant disease 100:1212-1221.

Yang, W., Shi, A., Ma, J., Correll, J., Evans, M., Motes, D., Xiong, H., Weng, Y., and Qin, J. 2018. Identification of the pathogen *Podosphaera erigerontis-canadensis* causing powdery mildew disease on dandelion (*Taraxacum officinale*) in US Arkansas state. Australasian Plant Disease Notes 13:12.

Yeh, Y.-W., Wu, T.-Y., Wen, H.-L., Jair, H.-W., Lee, M.-Z., and Kirschner, R. 2020. Host plants of the powdery mildew fungus *Podosphaera xanthii* in Taiwan. Tropical Plant Pathology:1-18.

Yoshida, K., Sasaki, E., and Kamoun, S. 2015. Computational analyses of ancient pathogen DNA from herbarium specimens: Challenges and prospects. Frontiers in Plant Science 6.

Yoshida, K., Schuenemann, V. J., Cano, L. M., Pais, M., Mishra, B., Sharma, R., Lanz, C., Martin, F. N., Kamoun, S., Krause, J., Thines, M., Weigel, D., and Burbano, H. A. 2013. The rise and fall of the *Phytophthora infestans* lineage that triggered the Irish potato famine. eLIFE 2: e00731.

Zedane, L., Hong-Wa, C., Murienne, J., Jeziorski, C., Baldwin, B. G., and Besnard, G. 2016. Museomics illuminate the history of an extinct, paleoendemic plant lineage (*Hesperelaea*, *Oleaceae*) known from an 1875 collection from Guadalupe Island, Mexico. Biological Journal of the Linnean Society 117:44-57.

Zeng, C.-X., Hollingsworth, P. M., Yang, J., He, Z.-S., Zhang, Z.-R., Li, D.-Z., and Yang, J.-B. 2018. Genome skimming herbarium specimens for DNA barcoding and phylogenetics. Plant Methods 14.