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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.

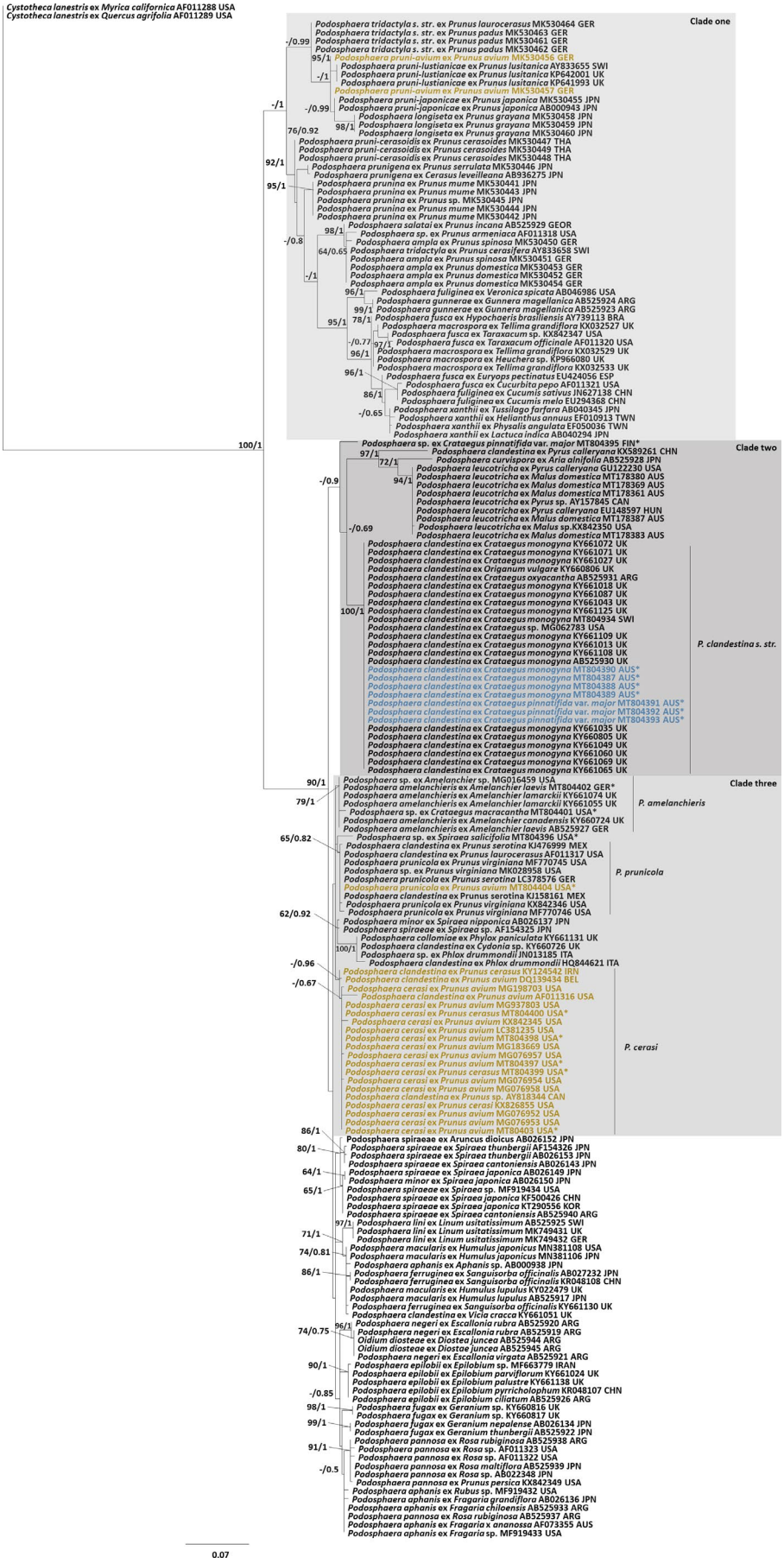
[a]Listed host species of spore trap sample with no plant material present.

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
P. clandestina s. str.
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
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
P. amelanchieris
Podosphaera sp. ex Crataegus macracantha MT804401 USA*
Podosphaera amelanchieris ex Amelanchier canadensis KY660724 UK
Podosphaera amelanchieris ex Amelanchier laevis AB525927 GER
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
P. prunicola
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
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
P. cerasi
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
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 diosteae ex Diostea juncea AB525944 ARG
Oidium diosteae 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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