Increasing accuracy of Powdery Mildew (Ascomycota, Erysiphales) identification using previously untapped DNA regions

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

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Plant and Fungal Systematics Research

April, 2017

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Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

The powdery mildews (Ascomycota, Erysiphales) are a group of obligate biotrophic fungi found on nearly 10,000 angiosperm plant hosts globally including many that are important horticultural and agricultural plants. Infection can greatly reduce the appearance and vigour of the host therefore reducing attractiveness and yields significantly. A reliable and efficient method is required for unambiguous identification of these often cryptic species such that spread to new areas and/or new hosts can be detected rapidly and controlled early. This research aims to combine currently accepted techniques – host identification, fungal morphological analysis, DNA sequencing of the fungal rDNA ITS region – with sequencing of additional nuclear DNA regions in order to increase the reliability of the identification process via BLAST, DNA Barcoding, and phylogenetic reconstruction. Samples were collected through the Powdery Mildew Survey (a citizen science scheme), begun in 2014 and concluded in 2016. Generic fungal DNA primers were found to amplify non-powdery mildew species, some of which were mycoparasites, as well as powdery mildews, and were therefore not a useful technique for accurate identification of powdery mildews. Consequently specific primers were developed for the amplification of the Actin, β-tubulin, Chitin synthase, *Mcm7*, Translation elongation factor 1-α, and *Tsr1* regions. Results indicate that several of these regions could be used alongside ITS to increase identification power (reliability and accuracy), with regions *Mcm7* and β-tubulin performing particularly well. These rapid diagnostic techniques could provide a valuable tool for plant quarantine, and plant breeding, particularly for greater security in the movement of plants and plant products in trade.

Acknowledgements

It is my great pleasure to reach this stage and be able to thank everyone involved in the powdery mildew PhD. I offer sincere thanks to the funders who made the research possible; the Biotechnology and Biological Sciences Research Council and the Royal Horticultural Society. As well as my initial supervisor, Béatrice Henricot, and principal supervisor Alastair Culham for the inception of the project and seeing a fungal systematist in me. I am also indebted to John David who stepped in for the lion's share as my secondary supervisor. Thanks also must go to my lab mates, particularly Maria Christodoulou for her time and Masters student Waheed Arshad for his help in 2014.

I am grateful to Roger Cook, whose early masterclasses set me on my powdery way and to Levente Kiss for the inception and organisation of the Powdery mildew summer school.

Special thanks must go to the powdery mildews themselves whose lifestyle and beautiful forms are always varied and rarely boring. Thanks for providing me with such a fascinating study organism and introducing me to the wonders and utility of fantastic Fungi.

I wish to show my gratitude to the science outreach staff of the British Mycological Society, Botanical Society of Britain & Ireland, Plant Heritage (NCCPG), and the British Society of Plant Pathology for help promoting the Powdery Mildew Survey. I am also grateful to the outreach staff of the Royal Horticultural Society as well as their pathology and media teams who hosted me on numerous occasions.

I would like to use this opportunity to thank my friends and family and the welcoming staff at C.U.P. for offering me refuge from academia and fellow researchers who I am honoured to call friends: Lionel Smith, Matthew McFrederick, Gedimimas Baltulionis, and Irene Teixidor.

Finally, I am truly indebted and thankful to Halala Hewazy for her unwavering supply of great food, friendship, smiles, and understanding.

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List of Abbreviations

Throughout this work the following abbreviations apply:

PM	Powdery mildew
ITS	Internal transcribed spacer
Mcm7	Mini-chromosome maintenance protein 7
Chs	Chitin synthase
EF1-α	Translation elongation factor 1-α
BI	Bayesian inference
PP	Posterior probability
K2P	Kimura 2-parameter

Chapter 1: Introduction

1.1: Powdery mildews

Powdery mildews (PMs) (Ascomycota, Erysiphales) are some of the most diverse and frequently encountered plant pathogenic fungi in the world (Braun, 1987). The 872 different species form a characteristic white talcum-powder like coating on the leaves, shoots, buds and fruits of approximately 10,000 different host plants (Braun, 1987) including many economically important crops such as grains, fruit trees, grapes and ornamentals (Figure 1.1). These obligate biotrophic fungi have historically posed problems for mycological taxonomists as many species are morphologically almost identical and require expert knowledge or molecular techniques to discriminate between them.

Figure 1.1: Characteristic white talcum-powder-like colonies forming on the leaf surface of *Geranium phaeum* L. Photo by O. Ellingham.

1.2: The importance of fungal diseases

Fungal infections of plants are hugely diverse in their morphology, biology, ecology, damage caused, and host plants infected. Fungi are known to infect plants in the majority of terrestrial environments, and can cause large-scale damage to natural ecosystems (Fisher *et al.*, 2012) and farmed plants used in agriculture (Oerke, 2006), arboriculture (Pawsey, 1973), and horticulture (Verma & Sharma, 1999). Fungal diseases are key players in the continuous evolutionary arms-races of, and between, animals, plants, fungi and their competitors; infecting various hosts in order to transfer the flow of nutrients into their own cells. Damage can be quantified in terms of loss of ecosystem services and loss of crop production. The Food and Agriculture Organization (FAO) estimates that pests and diseases are responsible for about 25% of crop loss (Martinelli *et al.*, 2015). This thesis will focus on the diagnostic techniques for identifying the fungal plant disease predicted to be one of the greatest threats to global, future food security (Bebber & Gurr, 2015): powdery mildew (Figure 1.2).

Figure 1.2: PM is forecast to be the fungal plant pathogen with greatest future threat to food security. Here *Blumeria graminis* (DC.) Speer infects *Triticum aestivum* L. Photo by O. Ellingham.

1.3: Importance of powdery mildew

The major economic impact of PMs is the result of just a handful of the overall diversity of species infecting our most important crops. As a result, PMs known to cause the most extensive crop damage are now well studied to the genome level (Spanu *et al.*, 2010, Wicker *et al.*, 2013, Jones *et al.*, 2014). However, the majority of PM species are poorly known and there is little DNA sequence data available for them. PMs infect numerous plants important to horticulture and agriculture. The disease causes early defoliation, stunted growth, and discolouration or malformation of leaves, all resulting in decline of growth in infected plants, reduced aesthetic value, negative effects on yield quality (Zhang *et al.*, 2005) and quantity (Conner *et al.*, 2003) and reduction in product quality (Limkaisang *et al.*, 2006, Mmbaga *et al.*, 2016).

Host plants are distributed globally, though the majority studied thus far are found in temperate regions. Studies have shown PM to adversely affect numerous abundant and economically important genera of plants in the Northern Hemisphere including, but not limited to *Pisum* (Munjal *et al.*, 1963, Gritton & Ebert, 1975, Warkentin *et al.*, 1996, Tiwari *et al.*, 1997), *Quercus* (Manos *et al.*, 1999), *Fragaria* (Xiao *et al.*, 2001), *Malus* (Pessina *et al*., 2014), *Prunus* (Lalancette *et al.*, 2014), *Vitis* (Fuller *et al.*, 2014), and *Cornus* (Mmbaga *et al*., 2016), as well as many within the families Cucurbitaceae (Sitterly, 1978, McGrath & Thomas, 1996), Solanaceae (Kiss *et al.*, 2001, Lebeda *et al.*, 2014), and Poaceae (Inuma *et al.*, 2007, Jankovics *et al.*, 2015). Barley and wheat PMs are major problems in the crop producing regions of Asia, North and East Africa, North and South America and northern Europe, causing a loss of yield up to 20% (Curtis *et al.*, 2002). Similarly, PMs cause 10-15% yield loss in peppers grown in the USA each year (Sabaratnam, 2012).

PMs in tropical or subtropical regions are less well studied than those of the temperate regions and, like all PMs, often lack the teleomorphic life stage; therefore their ecology and classification often remain uncertain (Limkaisang *et al.*, 2006). The cultivated plants of tropical and subtropical regions that PMs infect are however economically important. These include, but are not

limited to, *Hevea brasiliensis* (Shaw, 1967), *Bixa orellana* (Peregrine & Siddiqi, 1972), *Mangifera indica* (Boesewinkel, 1980), *Citrus* spp. (Boesewinkel, 1981), *Anacardium occidentale* (Sijaona *et al.*, 2001), and *Acacia* spp. (Takamatsu *et al.*, 2007). As a result billions are spent annually on resistant cultivars and fungicides intended to aid the control of PMs that infect economically important plants (Savary *et al.*, 2012).

Losses can be limited via a suite of varying prevention techniques including use of resistant plant varieties, application of fertilisers and fungicides at the right time and dose, and limiting the spread of potential detrimental fungi by monitoring the trade and transport of plants (Schrader & Unger, 2003). Each of these is aided by knowledge of fungal biology (life-cycle); in order to apply fungicides at the most efficient time and even detect the fungi in the first instance. Diagnostic techniques can therefore be of huge importance for detection and identification of potentially harmful plant diseases in order to develop the best coping strategies.

The importation of plants to the UK for use in horticulture has inevitably brought associated pathogens. Horticulture makes up 1.7% of all retail sales (£9 billion to the economy each year as an industry), and employs 300,000 people (The Royal Horticultural Society, 2013). The annual value of UK grower production of herbaceous perennials is estimated at £97m (Denny, 2014). Gardening, a pastime enjoyed throughout the UK, is an aspect of horticulture which the masses can understand and therefore connect with. PMs with detrimental effects upon such a pursuit are therefore likely to produce emotive responses from hobbyists and this connection can be the starting point for knowledge dissemination from members of industry and research to wider audiences. Any threat to such a thriving industry and popular pastime should be considered seriously and give studies of PM great importance.

PMs have been shown to regularly increase their host ranges and adapt to form new species (Schnathorst, 1959a, Ale-Agha *et al.*, 2000, Kiss, 2005, Seko *et al.*, 2008, Menardo *et al.*, 2016). The approximately 80 different PM species found in the UK (Braun *et al.*, 2014) seems to be an

underestimate, particularly when trade between countries is so common. Importation of exotic plants may bring with it invasive PMs but can also expose such exotics to PMs already present in the UK (Pettitt *et al.*, 2010). Fast and effective identification of PM species is therefore important. Screening of exotic plants as they enter the UK for invasive PMs and knowing their potential host ranges could help to alleviate one of the many stresses on the horticultural industry.

It is clear that amongst the wide-ranging detrimental effects resulting from PM infection, its significant economic impact makes it a disease worthy of further study. This has driven basic and applied research efforts in phytopathology for many years, and will continue to do so. The ability to diagnose and identify the species involved in infection is one aspect of the research into reducing PM economic impact. Such identification is critical to other aspects of disease treatment as it can enable targeted treatment and even initial prevention through breeding strategies and good growing practice. Investment in research into PM diagnostics and identification is therefore key to mitigating these economic impacts. This thesis will therefore focus on diagnostics of the PM; the first step towards controlling it.

1.4: PM biology

PM will rarely cause plant death as the biotrophic relationship between fungus and plant relies upon the survival of the plant in order for the fungus to continue to thrive and proliferate. The species most frequently reproduce asexually (this form is called the anamorph), and have been shown to disperse spores on the wind (Willocquet *et al.*, 1998, Willocquet & Clerjeau, 1998, Willocquet *et al.*, 2008). However, PM species are able to produce both the anamorph and the sexual structures (known as the teleomorph) at any time. The teleomorphic structures aid with perennation through adverse conditions such as winter (Liyanage & Royle, 1976, Grove, 2004). Anamorphs and teleomorphs are often found separately meaning that they were previously classified as separate species. It is only since the 1981 Sydney Congress (Voss *et al.*, 1983) that anamorph and teleomorph names have been united (names typified by an asexual morph were not permitted to be included in

a genus with a sexual type, and any such names were ruled as illegitimate); resulting in complete holomorphic descriptions (Hawksworth *et al.*, 2011) and, thanks to molecular methods, to link anamorph and teleomorph, the eventual end of dual nomenclature (Crous *et al.*, 2015).

1.5: Established Species Identification

Species identification and classification provide the nomenclatural backbone of biology. Classification has traditionally been the specialist domain of taxonomists; each with knowledge of niche clades and a grounding in common names, naming systems and scientific binomials that are regularly changed and updated. Fungi were traditionally included in the study of botany and remain under the ICN (previously ICBN) – International Code of Nomenclature for algae, fungi, and plants. However, with the continuous progression of molecular biology comes the possibility of developing working systems for species identification in tandem with computer-based techniques; removing the necessity for such specialist knowledge and aiding the identification process. Numerous issues, crucial to biology (and the world), including but not limited to: biodiversity, biosecurity, food security, and pandemics rely on consistent, accurate identification of species in order to ensure that the most applicable and efficient methods are employed to combat global challenges that arise.

Many of the 872 named PM species have features requiring specialist knowledge to separate them — particularly within clades of relatively fast-evolving, closely-related, phylogenetically-young species such as the tribe Erysiphaeae (Meeboon & Takamatsu, 2015a, Meeboon & Takamatsu, 2015b, Meeboon & Takamatsu, 2015c, Takamatsu *et al.*, 2015a, Takamatsu *et al.*, 2015b) and the genus *Golovinomyces* (Takamatsu *et al.*, 2013). Clear species boundaries are often lacking, making the discovery of life's true diversity — differentiation of individual organisms as members of the same entity or not — problematic (Dayrat, 2005).

Although PMs are easily recognised due to their characteristic talcum-powder-like appearance on plant surfaces, species level identifications are difficult when teleomorphs are available and can be impossible when anamorphs occur alone (Braun, 1987). Like other

microorganisms, PM therefore require a look beyond the limits of ordinary observation (Kimmerer, 2003), using microscopes to view micro-features and molecular techniques in order to examine the building-blocks of life — DNA. While a correct species identification is not vital for initial prevention or immediate control, it becomes critical when determining the fate of imported plants in quarantine (Schrader & Unger, 2003), for modelling forecasting systems (Caffi *et al.*, 2013), and resistance breeding (Debener & Byrne, 2014).

The earliest identification methods for PM species involved the identification of host plants. However, varying host specificity from highly-specialised (host-specific) to generalist PMs means that identification is rarely possible based purely upon host identification. Salmon (1900) considered the PMs to be polyphagous fungi and used a very wide species concept: *Erysiphe alphitoides* has been shown to infect plants from a number of different families (Figure 1.3) (Desprez-Loustau *et al.*, 2010). However, such generalist PMs can be considered outliers as the majority are specialised pathogens, with host ranges limited to a single family, genus, or species (Braun, 1995). For example, each one of five major groups within *Golovinomyces* is restricted to a tribe in the host family Asteraceae. Coevolution of PMs in association with their hosts seems plausible (Matsuda & Takamatsu, 2003) (Figure 1.4). PMs in the tribe Blumerieae are the most extreme examples of host specialisations as they adapt within the species (intraspecifically) to infect individual cereal crops (Troch *et al.*, 2014). These have been split into *formae speciales* which have been shown to develop specificity such that most can no longer infect other grasses (Table 1.1).

Table 1.1: General adaptation of *Blumeria graminis formae speciales* (ff.spp.) to cultivated cereal hosts*.

Source hosts (origin of isolates)	Inoculated host†				
	Oat	Barley	Wheat	Rye	Triticale
B. graminis f. sp. *avenae*	+++	-	-	-	-
B. graminis f. sp. *hordei*	-	+++	-	-	-
B. graminis f. sp. *tritici*	±	±	+++	-	+
B. graminis f. sp. *secalis*	-	-	±	+++	+
B. graminis f. sp. 'triticale'	-	-	++	±	+++

*Based on Eshed and Wahl (1970), Wyand and Brown (2003), Walker *et al.* (2011), Troch (2012), Troch *et al.* (2014).

†+, infection; –, no infection; more + corresponds to a higher aggressiveness.

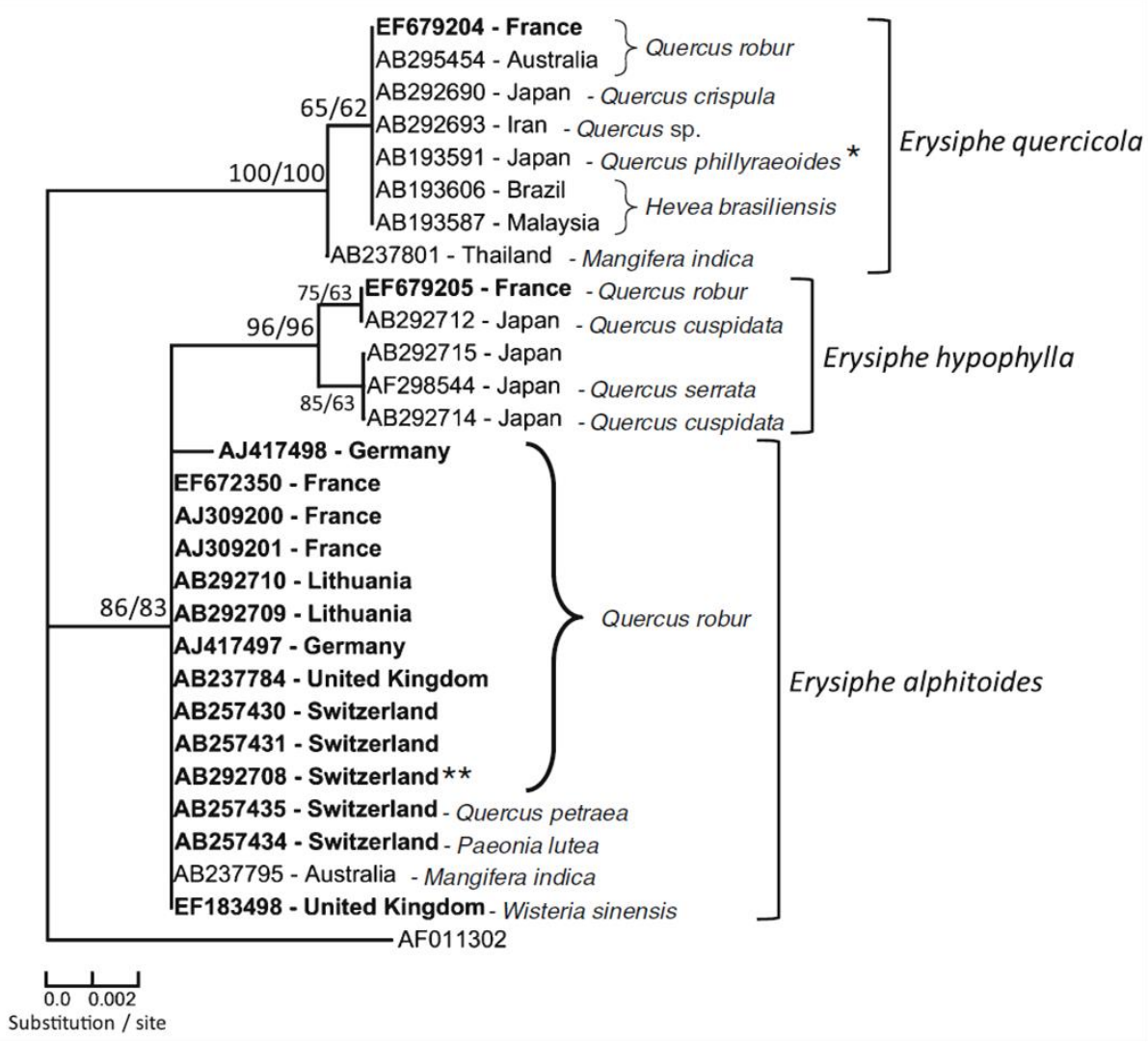

Figure 1.3: Phylogenetic relationships among species of the oak PM complex on *Quercus* spp. and non-oak hosts. An indication of the generalist nature of *Erysiphe alphitodies*. From Desprez-Loustau et al. (2010).

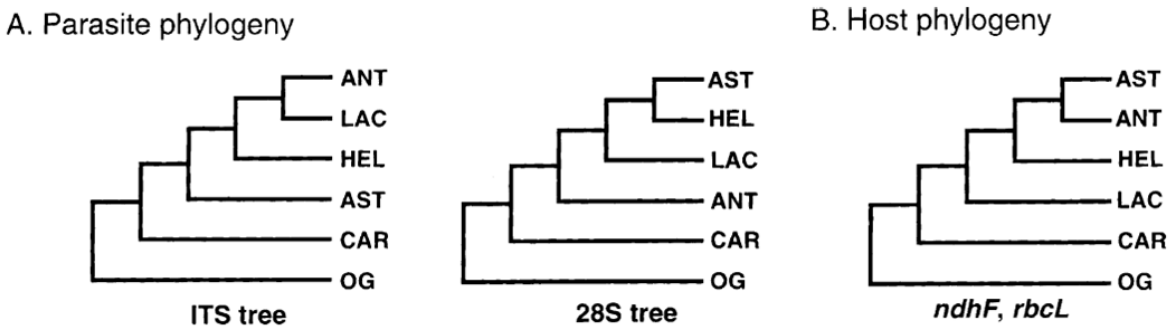

Figure 1.4: Branching order of the five major groups of Golovinomyces (A) indicated using the ITS and 28S regions and their corresponding host tribes of the Asteraceae (Anthemideae, Astereae, Carduoideae, Helenieae, Lactorideae) (B). The branching order of host and parasite is similar enough to hypothesise coevolution between the two. OG = outgroup. From Matsuda and Takamatsu (2003).

Studies have shown that more than one species of PM can grow on a single host plant, on a single leaf at any given time (Cook *et al.*, 2006, Takamatsu *et al.*, 2009). Individual identifications based on a single host leaf can therefore be ambiguous as a second or third species may be overlooked in morphological and/or molecular analyses. Furthermore, PMs rarely exist alone (Topalidou & Shaw, 2015). Instead they are accompanied by a whole community of associated fungi and bacteria (Topalidou, 2014); an example of the 'microbiome' (Berendsen *et al.*, 2012, Turner *et al.*, 2013). Alongside PMs, an interesting fungal genus of this particular microbiome, *Ampelomyces*, have been shown to interact with the PMs as generalist mycoparasites (Szentivanyi *et al.*, 2005, Pintye *et al.*, 2015) and have even been trialled as a biocontrol agents against the action of PMs (Pintye *et al.*, 2012). Early researchers thought of the mycoparasitic *Ampelomyces* as an extension of PMs, including their structures in their observations (Figure 1.5 (shown as spores exiting the Chasmothecium in number 3)) (Tulasne & Tulasne, 1863).

These remarkably detailed early drawings (Figure 1.5) (Tulasne & Tulasne, 1863) show PM microscopic detail. Analysis of key features of both teleomorphic (Gadoury & Pearson, 1988) and anamorphic stages (Cook *et al.*, 1997) have since been key to differentiation of the hundreds of PM species. Initially phylogenies and species characteristics were based solely on morphology of the teleomorph. However it was later found that anamorphic features, such as conidiogenesis type, conidia shape, and presence/absence of fibrosin bodies within conidia, were consistent with

molecular analyses and as such were accepted as more phylogenetically informative characteristics for evolutionary groupings (summarised in Braun (1987)).

In 1997 Cook *et al.* used scanning electron microscopy (SEM) to analyse conidial structures in greater detail. They showed that patterns on conidial surfaces were consistent with accepted genera, thereby allowing identifications to be made in this way (Figure 1.6). This work is reproducible, though requires advanced and expensive equipment, time to carry out sample preparation, and samples of good quality in good condition in order to discern the different conidial surface patterns (To-anun *et al.*, 2005).

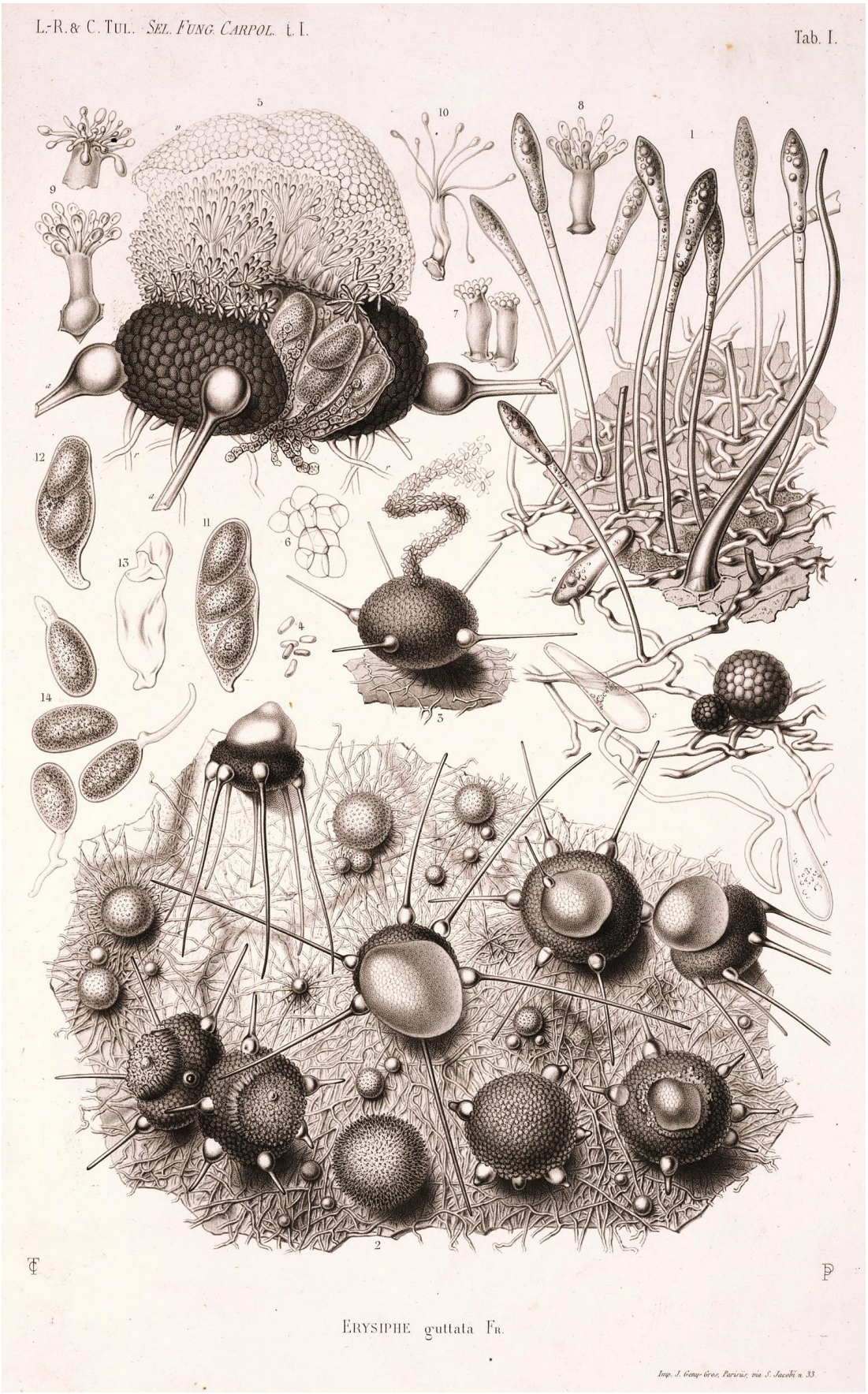

Figure 1.5: Illustrations of Tulasne and Tulasne (1863) showing the beautiful anamorphic (image 1) and teleomorphic (images 2-13) forms of PMs.

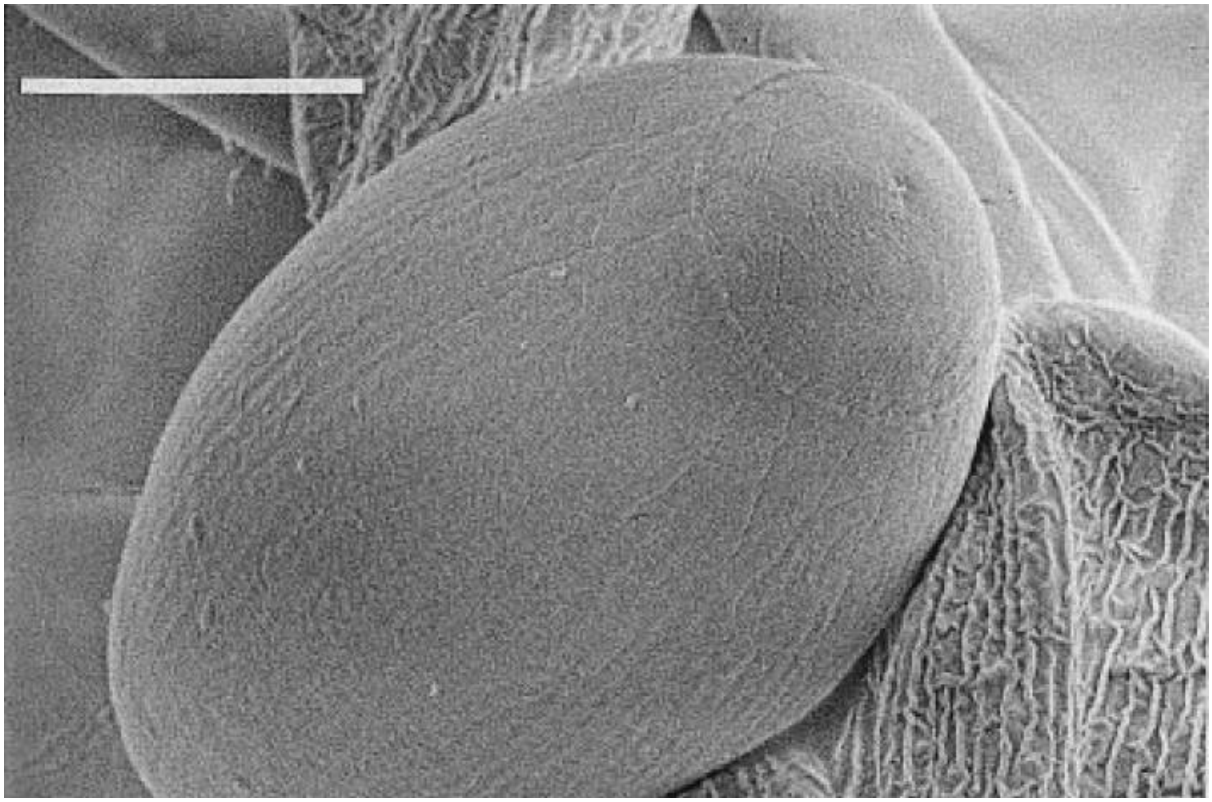

Figure 1.6: Scanning electron micrograph of turgid conidia with ‘striate' outer wall and ‘fibrillar' septa typical of the *Oidium* subgen. *Striatoidium* anamorph of *Erysiphe*. Here *Neoerysiphe galeopsidis* ex *Stachys sylvatica* showing a finely ribbed outer wall. Bar = 10 µm. From Cook *et al.* (1997).

Morphological analyses have allowed substantial progress in species identification, relatedness, biology, and evolutionary history. Initial species delimitation of the current study would therefore be completed by examining host plants and fungal morphology. However, reapplication of these methods often can be problematic, requiring specific knowledge, and without measurement of multiple characters can leave ambiguities. The use of molecular technologies is helping to reduce such ambiguities but requires links to morphologically examined type material in order to enable interpretation. Increasingly specialised equipment is necessary for molecular studies, but costs per-nucleotide of DNA sequence data have dropped and accuracy and ease of techniques are improving. It is therefore necessary to supplement morphological analyses with DNA data to understand patterns and processes behind PM biodiversity and thereby extrapolate highly resolved species delimitations and estimations of species histories.

Five PM genomes have been sequenced to date (Spanu *et al.*, 2010, Wicker *et al.*, 2013, Jones *et al.*, 2014) and it is the intention of the global PM community to sequence a further 13 in the next five years as part of the Joint Genome Institute, Community Science Programme (JGI CSP). Genome sequencing however remains costly and still requires further research to develop a reproducible protocol for the diverse array of PMs difficult to culture (Kenyon *et al.*, 1995, Nicot *et al.*, 2002). Sequencing of short DNA fragments is considerably more achievable and has been used alongside phylogenetic and barcoding analyses for improved identification of numerous fungal groups including *Cylindrocladium* (Crous *et al.*, 2000), *Stemphylium* (Câmara *et al.*, 2002) *Alternaria* (Kang *et al*., 2002), *Armillaria* (Keča *et al.*, 2006, Maphosa *et al.*, 2006), *Phytophthora* (Schena *et al.*, 2008), *Fusarium* (Amatulli *et al.*, 2010), *Cladosporium* (Bensch *et al.*, 2012), *Cercospora* (Groenewald *et al.*, 2013), *Fomitopsis* (Haight *et al*., 2016), Clavariaceae (Birkebak *et al.*, 2016), and many more (Table 1.2). Currently the internal transcribed spacer (ITS) region has received most attention for successful identification of the broadest range of fungi, with the most clearly defined barcode gap between inter- and intra-specific variation (Schoch *et al.*, 2014). However, in each of the fungal examples the ITS has been used as an 'anchor'; complemented by additional 'identifier' regions.

Table 1.2: Additional loci used in identification within fungal groups

Fungal group	**Regions used for ID**
Cylindrocladium	ITS, ß-tubulin
Stemphylium	ITS, Glyceraldehyde-3-phosphatedehydrogenase
Alternaria	ITS, Histone H3
Armillaria	ITS, IGS, Translation elongation factor 1-α gene (*TEF1*)
Phytophthora	ITS, Ypt1
Fusarium	ITS, *TEF1*
Cladosporium	ITS, Actin, *TEF1*
Cercospora	ITS, Actin, Calmodulin, Histone H3 and *TEF1*
Clavariaceae	ITS, RNA polymerase II (*RPB2*), 28S
Fomitopsis	ITS, *TEF1*, *RPB2*

Intensive sampling and analyses of PM morphology and genetics will further elucidate PM speciation and biodiversity. The result will be a more complete picture of PM species; integrating morphological, ecological, biogeographical, and DNA sequence data (Amalfi *et al.*, 2012) to achieve the "integrative taxonomic approach" (Puillandre *et al.*, 2012) or "consolidated species concept" (Quaedvlieg *et al.*, 2014) recently highlighted within Ascomycota. The use of morphology and rDNA ITS to identify PM species will be explored in Chapter 3 while additional 'identifiers' will be explored in Chapters 4-7.

1.5.1: Established relationships, phylogeny, and taxonomy

Knowledge of the evolutionary development of current species helps to infer relationships between them. As stated previously in section 1.5 early reconstructions were dependent upon morphological characteristics: initially those of the teleomorph and later of the anamorph due to its congruence with molecular findings (summarised in Braun (1987)). As a result the most complete phylogenies are based on ITS sequence data and help to clarify the evolution of morphological features (reviewed in Takamatsu (2013a)). These group the PM species into one family, five distinct tribes, and 16 holomorphic genera (and one asexual genus, *Oidium*) (Figure 1.7).

Each of these can then be traced back to early diverging ancestral genera: (*Para*)*uncinula* and *Caespitotheca* emerged from basal lineages restricted to the Pacific ring (Takamatsu, 2013b). These genera are therefore commonly used to root PM phylogenetic trees. From these narrow areas of Asia and South America Takamatsu (2013b) shows that sequential invasions and radiations of PM species to new locations have meant that large clades can be clearly defined by their geographical distributions and host plants as well as using molecular data. For example 65, rather over-replicated samples of ITS sequences of the genus *Neoerysiphe* were shown to follow this trend under phylogenetic analysis (Heluta *et al.*, 2010). The three clades formed according to their hosts (*Lamiaceae*, *Asteraceae*, and *Rubiaceae* and *Geraniaceae*) and geographical origin (Global, New World and East Asia, and Eurasia respectively) (Figure 1.8).

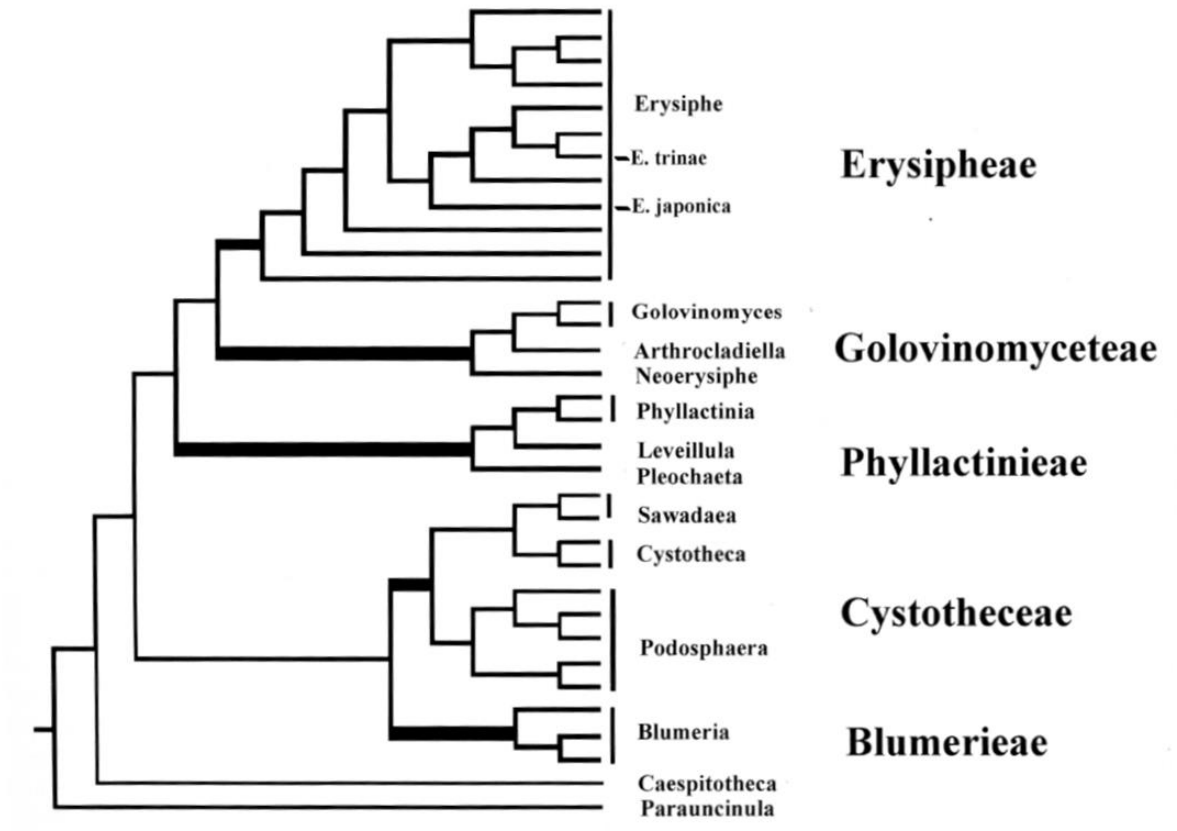

Figure 1.7: Schematic of current PM phylogeny of tribes and genera. From Braun and Cook (2012).

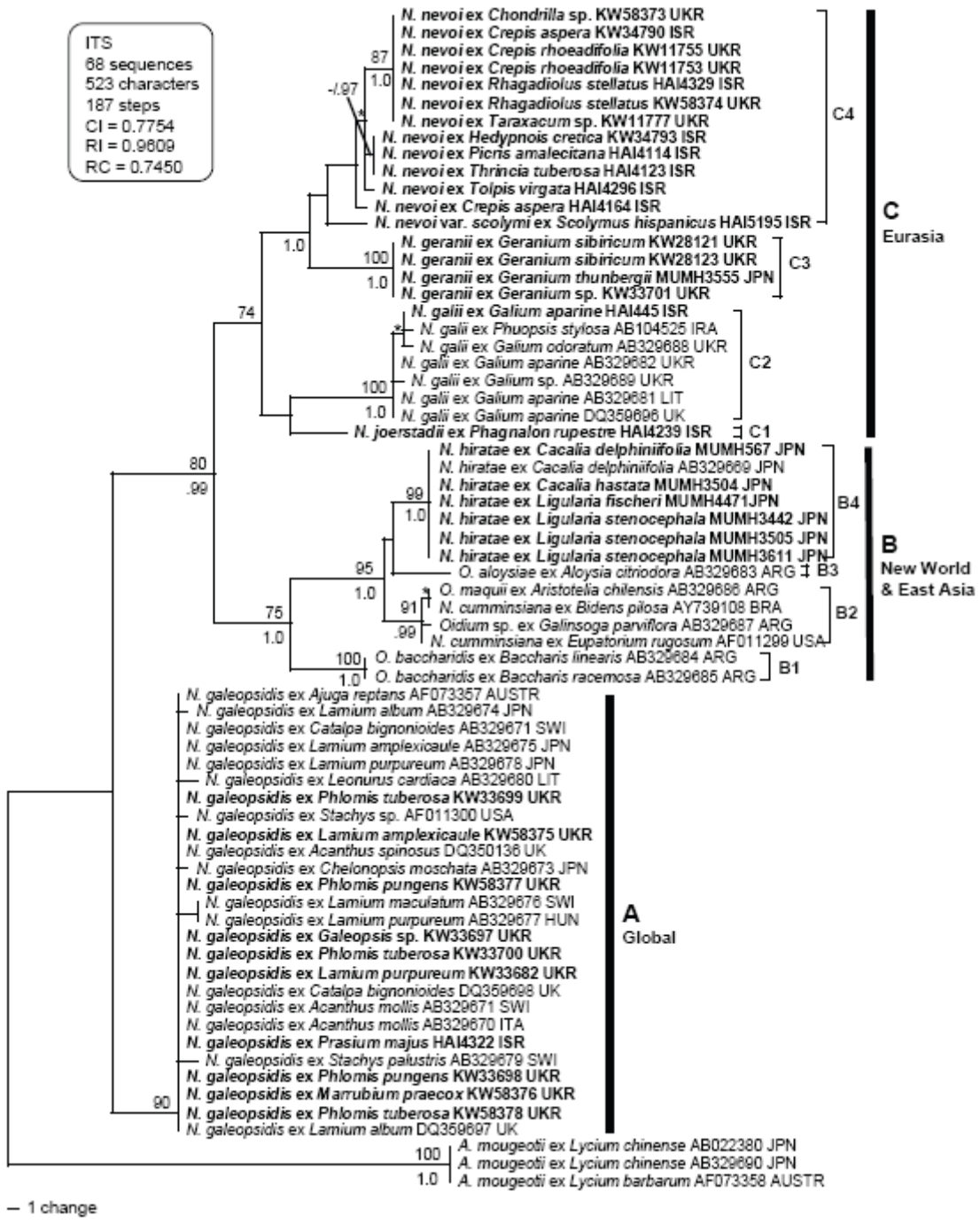

Figure 1.8: Phylogenetic reconstruction of PMs based on ITS. From Heluta *et al.* (2010).

1.6: DNA sequence analyses

1.6.1: NCBI GenBank Nucleotide BLAST

With DNA data comes also an investigation into the most efficient and accurate analysis method. Existing online databases in the public domain, such as NCBI's GenBank contain extensive datasets. For the PMs this has been used as a tool for putative identification when using a single region (ITS) of the PM genome. However this resource is far from complete and rarely 100% reliable (Kovács *et al.*, 2011). This is due to the quality of previously submitted sequences: numerous samples are incorrectly identified (Kovács *et al.*, 2011). Other, potentially more informative regions of the PM genome remain understudied and as such have few representative samples available on this, or any similar database (Table 1.3). Current species identification via the use of the NCBI Basic Local Alignment Search Tool (BLAST) alone should not be relied on (Schoch *et al.*, 2014).

Table 1.3: Number of PM sequences available of regions examined in the current study from NCBI GenBank.

Gene region	**Sequences available on GenBank**
ITS	2836
Actin	164
β-tubulin	419
Calmodulin	58
Chitin Synthase	82
Elongation factor 1-α	135
Mcm7	0
Tsr1	0

1.6.2: Phylogenetics

Phylogenetic analysis of DNA sequence data can offer insight into species delimitations and the evolutionary relationships between species, identification of unknown isolates, and mapping of various character profiles against isolates (Guadet *et al.*, 1989, O' Donnell *et al.*, 1998a). Datasets should be well tailored and include predominantly well-characterised isolates; previously identified and preferably accompanied by isolate specific features such as host, geographic origin and pathogenicity. Discordance in classification schemes has been caused by misidentification of isolates

(Thrane, 2001) and this may increase difficulties associated with consistent species delimitation and obscure true evolutionary relationships between species (Kristensen *et al.*, 2005). Usefully, sequences from different gene markers from the same organisms can be concatenated to produce a phylogenetic tree from more than one dataset. Phylogenies can then be constructed using the main techniques of distance, parsimony, likelihood, and Bayesian analyses (Harrison & Langdale, 2006).

1.6.3: DNA barcoding

DNA barcoding is a technique used to identify unknown isolates (Hebert *et al.*, 2003a). A DNA barcode represents a unique DNA sequence pattern 400–800 nucleotides in length that can be quickly processed from thousands of specimens or cultures and unambiguously analysed by computer programmes to identify species. It uses DNA sequence data from specific regions and relies upon a barcode reference library. A barcode region should therefore be variable enough to resolve closely related species and short enough for easy experimental manipulation and low cost. The flanking regions should be well-conserved in order to facilitate the design of primers with high PCR and sequencing success (Dong *et al.*, 2015).

Barcoding for animal life identified the cytochrome *c* oxidase subunit 1 (COI) region of mitochondrial DNA which has enabled discrimination of closely allied species in all animal phyla (Hebert *et al.*, 2003b). The maturase K (matK) region of plastid DNA has shown success in plant species discrimination, though often requires additional identifying regions to enable definite identification (CBOL Plant Working Group *et al.*, 2009). From the fungal markers evaluated, the ITS appeared to be the main candidate because of its broad utility as a species marker in taxonomic and ecological studies and the ease of amplification across the kingdom. The ITS has since been proposed as a standard barcode for fungi (Schoch *et al.*, 2012); consistently identifying many fungal genera (Schoch *et al.*, 2014), but rarely enabling species identification. Identifying, secondary barcodes is therefore needed in such cases. These have frequently incorporated protein-coding genes, but have

varied depending on the genus investigated. Regions of PM DNA have, as yet, not been tested for their DNA barcoding efficacy.

1.7: UK PM baseline review

Many PMs come from the putative PM origin and centre of diversity, South East Asia (Takamatsu, 2013b). This project however, focused purely on PMs of the UK. The diversity of PMs in the UK is less in number than that of the rest of the world (Braun & Cook, 2012), however to what degree has not previously been clarified. As cited in section 1.3: the Erysiphales database website of Braun *et al.* (2014) lists 82 PM species to have been found in the UK. This is a strong starting point, however supplementing this with additional trusted records (Royal Botanic Gardens Kew, 2016, British Mycological Society, 2017) makes for a greater existing number of species within the UK: 196 (Table 1.4).

Table 1.4: PM species previously recorded in the UK

Genus	**Kew Fungarium**	**Additional sp. from Erysiphales Database**	**Additional sp. from BMS Fungal Records**	**Combined**
Erysiphe	76	9	22	107
Golovinomyces	5	8	9	22
Arthrocladiella	1	0	0	1
Neoerysiphe	4	0	0	4
Phyllactinia	5	2	3	10
Leveillula	0	1	1	2
Sawadaea	2	0	0	2
Podosphaera	14	8	11	33
Blumeria	1	0	0	1
Oidium	14	0	0	14
Total	122	28	46	**196**

PMs have been shown to have a UK-wide distribution (British Mycological Society, 2017, National Biodiversity Network, 2017). Collection of a wide array of samples for the current study in order to give a true representation of UK PMs would therefore require extensive sampling.

1.8: Aims

Improving diagnostic techniques of PM, one of the greatest threat to global, future food security (Bebber & Gurr, 2015), will enable more efficient future control and accurate future monitoring. The study aimed to achieve this by diverse sample collection with the aid of a citizen science scheme. Diagnostically informative regions of the PM genome would subsequently be identified and reproducible protocols for sequencing them would be developed. New sequences would be linked to identified type accessions with established morphological and molecular techniques to ensure accuracy, before sequences would be deposited in a universally accessible database to enable samples to be compared to these new standards in future.

Hypotheses were as follows:

1. The launch and maintenance of a citizen science scheme will provide the project with sufficient number, quality, and diversity of PM samples to test the accuracy of established and newly developed identification techniques.
2. The combination of established morphological analysis and sequencing of the ITS region will provide sufficient accuracy to identify PM species.
3. Currently available molecular data will be sufficient for identification of diagnostically informative PM regions.
4. Previously designed primers will enable amplification and sequencing of PM species from environmental samples.
5. Currently available molecular data will be sufficient for design of primers specific to PMs such that amplification and sequencing will be reproducible.
6. Candidate PM identifying regions will enable greater accuracy in identification of PM species.

Chapter 2: Sampling – The Powdery Mildew Citizen Science Scheme

2.1: Introduction

2.1.1: Citizen science

2.1.1.1 Value, usage, and caution

The involvement of volunteers in science is an increasingly popular approach to undertaking monitoring over much larger spatial and temporal extents and much finer resolutions than would otherwise be possible (Pocock *et al.*, 2014). One way to obtain data is through citizen science, a research technique that enlists the public in gathering scientific information (Bhattacharjee & Boyce, 2005). This has the potential to engage members of the public, industry, and government (funders and those who research may affect) directly in the science.

Citizen science offers a great opportunity for connection between science and the public as volunteering participants feel they can then make a contribution to science and learn at the same time (Rotman *et al.*, 2012). Crowd-sourcing is one element of citizen science. It is generally completed exclusively online; participants completing small, cognitive tasks of problem solving or pattern recognition (Pocock *et al.*, 2014). Collection or recording of physical samples is a separate aspect of citizen science with its main benefit being the potential for collection of diverse datasets. Drawbacks include the potential for poor quality data and time taken for training participants, continued engagement, and continued feedback. Therefore the most successful citizen science schemes tend to be simple (Pocock *et al.*, 2014).

Numerous studies have used the principle of citizen science; some of these have run for more than a hundred years. The Christmas Bird Count (Butcher & Niven, 2007) has been run since 1900, the Botanical Society of Britain & Ireland's Distribution Database (Botanical Society of Britain & Ireland, 2016) since pre-1930, and the British Mycological Society's Fungal Records Database of Britain and Ireland since pre-1900. These latter two have devolved regional recorders compiling data

from local participants and contributing towards enhancing knowledge of UK plant and fungus species and their distributions. More recent projects such as the Conker Tree Science Project (Pocock & Evans, 2014) and the Starling Survey (University of Gloucestershire, 2016) have sourced non-expert data from across the UK, while the Living Ash Project has developed a suite of ways to contribute to aiding the survival of European ash trees (MacLean, 2014, Saunders *et al.*, 2014, Sollars *et al.*, 2017). Cape Citizen Science, a project run from South Africa, seems to be most similar to the current study as it sources *Phytophthora* samples from around the country in order to better understand the plant disease and educate participants at the same time (Hulbert, 2016). Additionally organisations such as Open Air Laboratories (OPAL) offer large-scale funding and programmes to increase public engagement with, and understanding of, the environment. Citizen scientists have participated in projects on climate change, invasive species, conservation biology, ecological restoration, water quality monitoring, population ecology (Silvertown, 2009), astronomy, and now fungal disease identification.

2.1.1.2 Use in the project

The aim of sampling in this study was to collect PMs spanning the order Erysiphales, from a broad range of hosts with a spread from across the UK. This enabled current identification methods to be tested and novel molecular methods for identification to be developed. If successful, this would fuel the development of fast, accurate identification which could take place when traded plants, valuable to UK horticulture, were suspected of spreading potentially harmful disease. A truly accurate method would be effective on all of the 872 PM species. Sampling therefore attempted to include multiple samples from each of the five Erysiphaceae tribes and more than one sample from each of the 16 PM genera (Braun & Cook, 2012) and enabled developed molecular markers to be tested. The 196 PM species present in the UK (section 1.7) are found in each of the five tribes, but just nine of these 16 genera. Discovery of PM species not currently listed in the UK was possible, but it was unlikely that these would be from the six additional genera. Therefore collection of repeat samples

from nine PM genera spanning the five tribes was deemed sufficient. This was achieved with a UK-wide citizen science scheme.

Consultation of the decision framework of Pocock *et al.* (2014) (Figure 2.1) confirmed that a citizen science approach was appropriate for this project. The need for large scale sampling and lack of need for specialised equipment and specialist, public knowledge made it a good option. Thus a citizen science scheme requesting PM infected plant material from the public was the study's main sampling technique. This offered a diverse array of samples from all over the UK over the space of the three years of collection. Of equal importance to sampling was augmenting the awareness and interest of the UK public. This was achieved through the offer of an identification service for UK PMs, concurrent with an explanatory, interactive blog, active social media accounts, and face-to-face interaction at horticultural shows, conferences, and specialist society meetings.

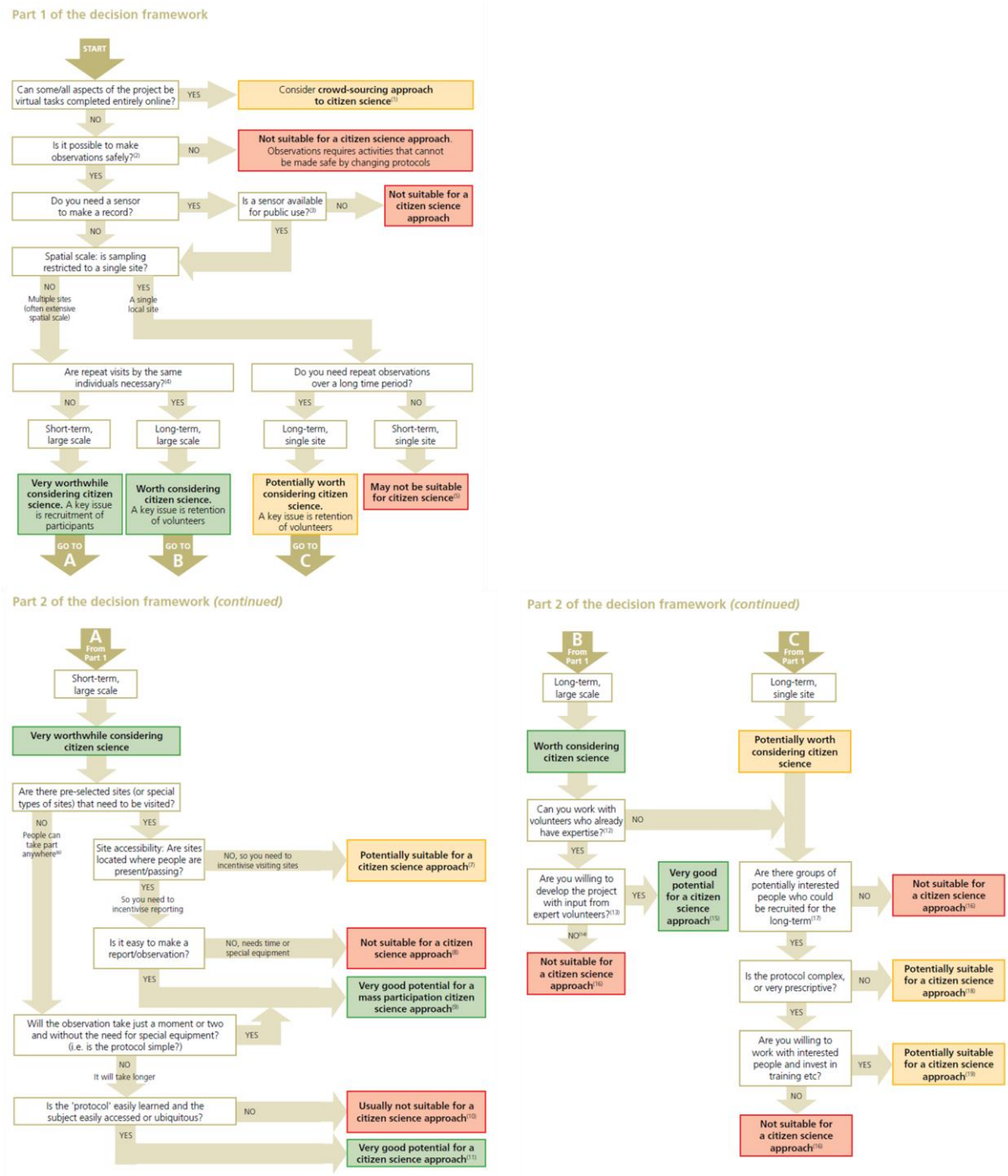

Figure 2.1: The decision framework for choosing and using citizen science. From Pocock *et al.*, 2014.

2.2: Materials and methods

2.2.1: Citizen science launch & engagement

The Powdery Mildew Citizen Science Scheme was launched via online blog post on May 21st 2014. It was relaunched annually at the start of the UK spring season (March 3rd, 2015 and March 21st, 2016) (Appendix 1 for launch blog posts). The scheme was promoted through an array of printed and social media. This included society publications from the Royal Horticultural Society (RHS), British Mycological Society (BMS), Botanical Society of Britain & Ireland (BSBI), Plant Heritage (NCCPG), and British Society of Plant Pathology (BSPP), through social media platforms Twitter (@PowderyM) and Facebook and in person at shows and conferences. In 2016 the scheme became an optional practical activity in GCSE classrooms through support from Science & Plants for Schools (Appendix 2).

Feedback was delivered via email on receipt of samples stating that samples had been received and whether the samples were of sufficient quality to be identified. If so, this was followed up with a further email of detailed feedback of the information from the PM identification process. Completed individual records for each contributor were sent via email when results were ready in order to provide feedback and encourage repeat contributions. Annual collection results were published in a blog (Ellingham, 2017) towards the end of the season to inform contributors of the overall state of the project and the relevance of their own samples.

2.2.2: Sample collection

Collection of fresh samples was favoured to sourcing them from herbaria as it would ensure the PM study and methods developed from it were relevant to the current threats seen in UK horticulture. Herbaria collections were therefore not used.

Before the launch of the scheme in 2014 samples were collected in 2013 from the University of Reading Harris Garden and RHS Garden Wisley. These were sites likely to contain plants common to other UK gardens, along with the associated microorganisms, and made for good model sites for

initial sampling. Many of these plants were imported from other UK or European nurseries and may be exported in future. This made them possible sinks and sources of disease.

Instructions to citizen science participants were clearly stated on a linked, supplementary blog post (Ellingham, 2017). In 2016 a printed handout was put together by Phil Smith of the BSPP for dissemination at events (Appendix 3). Instructions required participants to locate PM infected plant material in their locale, detach whole leaves from the infected plant, and send via mail in a slightly inflated, sealed bag. A grid reference/GPS/postcode of the sample's collection site and email address of participant were also required and supplementary data such as host plant identity, images, and information of growing conditions were welcomed but not essential. Instructions purposefully excluded specific requirements for collection of particular host plants. It was felt that such direct instruction would reduce the likelihood of citizens participating. It was decided that PM from all hosts and all sites in the United Kingdom would be accepted in order to ensure maximum participation. Quality control (removing samples mistaken for PM and excluding samples arriving in poor condition or of a host already abundantly present in the dataset) would then occur on receipt of samples. Certain samples contributed by RHS members for identification by the RHS Advisory Service were forwarded to the scheme.

Following receipt of PM on *Heuchera* sp. from RHS Garden Wisley in 2015, five samples of PM infected *Tellima grandiflora* were requested from Kew Fungarium. There was no previous record of PM infecting *Heuchera* sp. in the UK. Reliable, conspecific samples of the PM were therefore required for comparison.

2.2.3: Sample handling

Samples were processed on receipt. If host plants were native or naturalised to the UK, they were identified using the Vegetative Key to the British Flora (Poland & Clement, 2009). Samples were analysed morphologically (see section 3.2.2). Samples were then stored and labelled in 12 x 6 cm resealable, polyethene bags with 2 - 5 mm non-toxic silica gel (Figure 2.2). If a large amount of the

sample was present then it was also pressed in a standard herbarium press with newspaper and blotting paper (Figure 2.3). Collector, collector's email, accession number, extraction code, collection site (GPS (latitude, longitude)) and generic site name (e.g. Kingston Upon Thames), host plant identity (host family, genus, and species), and potential PM species based on host identity were recorded in a Microsoft Excel Spreadsheet. Samples were transferred to fungarium packets made from A4 paper (Figure 2.4) and stored in fungarium drawers (Figure 2.5) in the University of Reading Herbarium (RNG) after initial molecular analyses (see section 3.2.3 and 3.2.4).

Figure 2.2: Citizen science samples processed for storage in resealable bags with silica gel, ready for further analyses. Photo O. Ellingham.

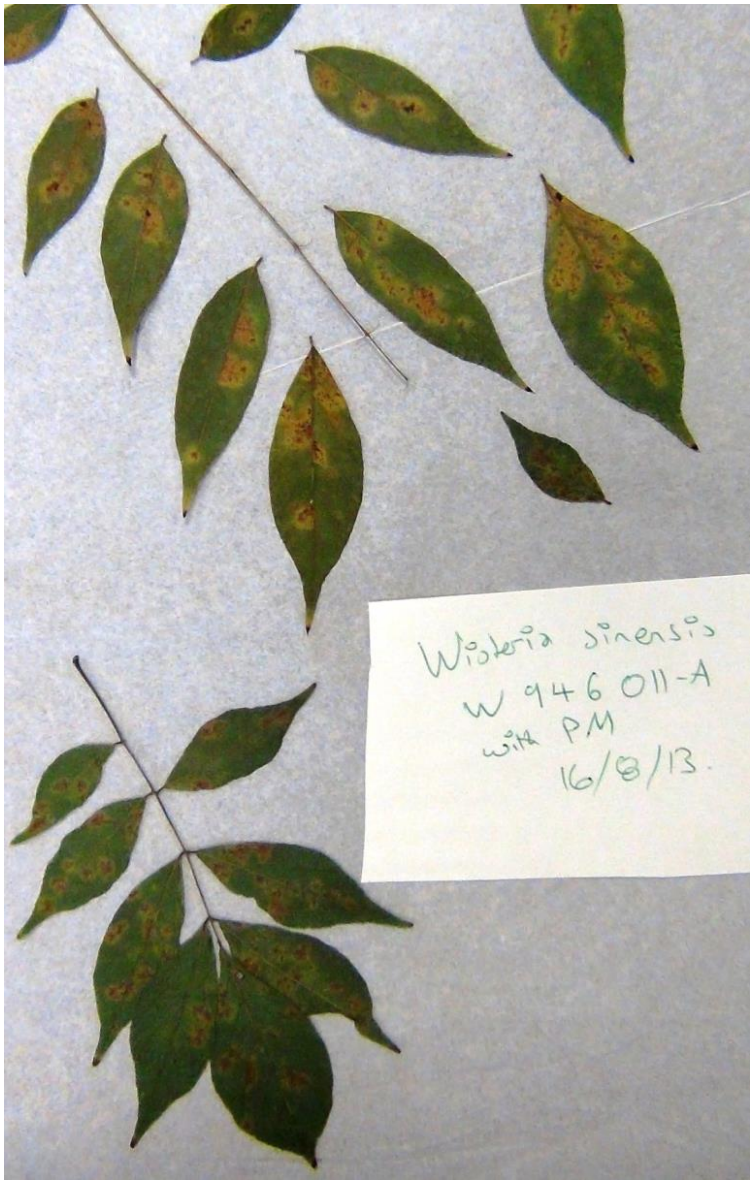

Figure 2.3: Citizen Science sample processed for storage in herbarium press. Photo O. Ellingham.

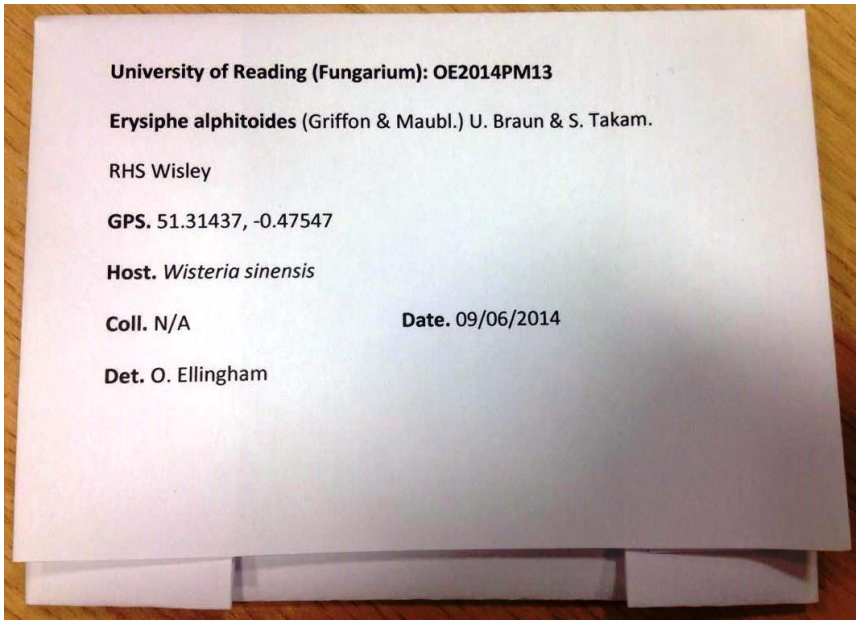

Figure 2.4: Fungarium packet for long term storage of PM samples. Photo O. Ellingham.

Figure 2.5: Fungarium packets for storage in RNG. Photo O. Ellingham.

2.2.4: Culturing of PM samples

PMs were cultured in the autumn of 2013 in order to achieve growth of clonal, single culture PM colonies. Successful growth would enable a lasting stock of PMs to be regularly sourced from colonies of few potential contaminants. Forty-eight seeds of *Triticum aestivum* 'Cerco', *Hordeum vulgare* 'Golden Promise', *Cucumis sativus* 'Marketmore', and *Pisum sativum* 'Hurst Greenshaft' were planted and grown in 5 x 5 cm plug trays in John Innes Compost No 1 and covered with vermiculite. Leaves emerged in 2-4 weeks and 25% of all whole leaves were harvested.

Harvested leaves were added to collected leaves of *Rhodonendron* 'Karen Tripitta' and surface sterilised using 70% ethanol. Leaves were laid on water agar (5 g Agar Agar (Fisher Scientific), 900 ml RO water, and 100 ml Benzimidazol (1 g per litre)) in glass petri dishes of diameter 10 cm in a laminar flow hood. PM from infected detached leaf material of each individual host was then transferred onto detached leaves on water agar with an artist's paint brush and lids were applied before storing in controlled environment of 20°C with day length of 14 hours. Signs of PM infection were recultured onto fresh leaf material after 21 days.

2.3: Results

2.3.1: Samples received and social engagement

A total of 596 PM infected samples were received (160 in 2014, 353 in 2015, and 83 in 2016) and added to the 43 samples collected in 2013. This made for a total of 639 samples (see Appendix 4). 90% of these were collected between June and October of each year (Figure 2.6).

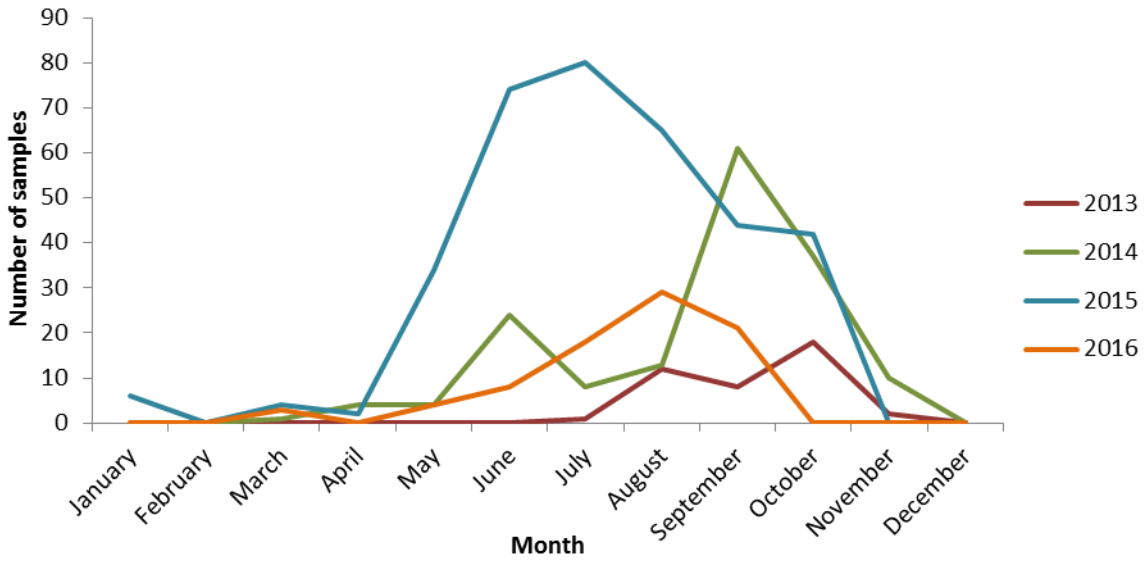

Figure 2.6: Number of PM samples collected each month during each year of study. 2013 corresponds to samples collected by the researcher, 2014-2016 are those of citizen science collection.

Seventy-nine people contributed samples to the Powdery Mildew Citizen Science Scheme. Samples were contributed on more than one occasion by 42 people. Nine of these people contributed samples in two different years and two contributed in each of the three years the scheme ran. However, many more individuals than this have been engaged with the scheme. This has happened: at 12 conferences and society meetings, four horticultural shows, and two UK Fungus Days; in printed media via publications in The Rock Garden (Ellingham, 2016), The Plantsman (The Royal Horticultural Society, 2015b), and The Garden (The Royal Horticultural Society, 2015a, The Royal Horticultural Society, 2016), and on the BSBI (Marsh, 2014, Marsh, 2015, Marsh, 2016), BSPP (Ellingham, 2015b), and BMS (Ellingham, 2015a) websites; and on social media via the RHS Facebook

and Twitter accounts and the @PowderyM Twitter account, which has amassed 355 followers and tweeted 547 times (January, 2017).

2.3.2: Sample distribution

Samples from 2013 were collected exclusively from the University of Reading and RHS Garden Wisley (Figure 2.7). Samples from 2014, 2015, and 2016 were collected from across the UK (Figure 2.8). The combination of all four years collection offered a UK-wide distribution (Figure 2.8) of samples for further analyses. Citizen science samples were received from the SE of England each year. This sampling bias was shifted due to contributions from the SW, East Anglia, Scotland and particularly the NW: in 2015 212 of the 353 samples came from a single contributor in the area around Merseyside (NW England).

2.3.3: Host profiling

Of the 639 PM samples, 638 host plants were identified at least to genus. The identity of the ten most frequently identifed plant families and genera from individual years and all years combined are shown in Figure 2.9.

Of the identified samples 48 of the 191 families of flowering plants found in the UK (David, 2010) and 134 of the 14,559 genera of the worlds flowering plants (The Plant List, 2010) were included. Ninety-six samples were contributed from the Rosaceae, largely from genera *Rosa* (22 samples), *Crataegus* (17), *Filipendula* (11), *Geum* (11), and *Prunus* (11). Similarly, 77 Asteraceae samples largely of *Taraxacum* (20), *Centaurea* (9), *Senecio* (9), and *Sonchus* (9) were included. Of the 47 Fabaceae samples *Trifolium* (14) was the most prominent genus, and *Lamium* (16) made up almost half of the 36 Lamiacae samples. The majority of the 46 Sapindaceae samples were *Acer* (41), and similarly the 34 Ranunculaceae samples were made up largely of *Aquilegia* (20). Selected host infections are shown in Figure 2.11.

Fourteen samples of the Saxifragaceae were included. Amongst these were four samples of *Heuchera* sp.; a new record within the UK (Ellingham *et al.*, 2016).

Figure 2.7: Distribution map of samples collected in 2013. Arrows show enlarged maps of local sites.

Figure 2.8: Distribution maps of citizen science samples from individual years (2014-16) and years 2013-16 combined.

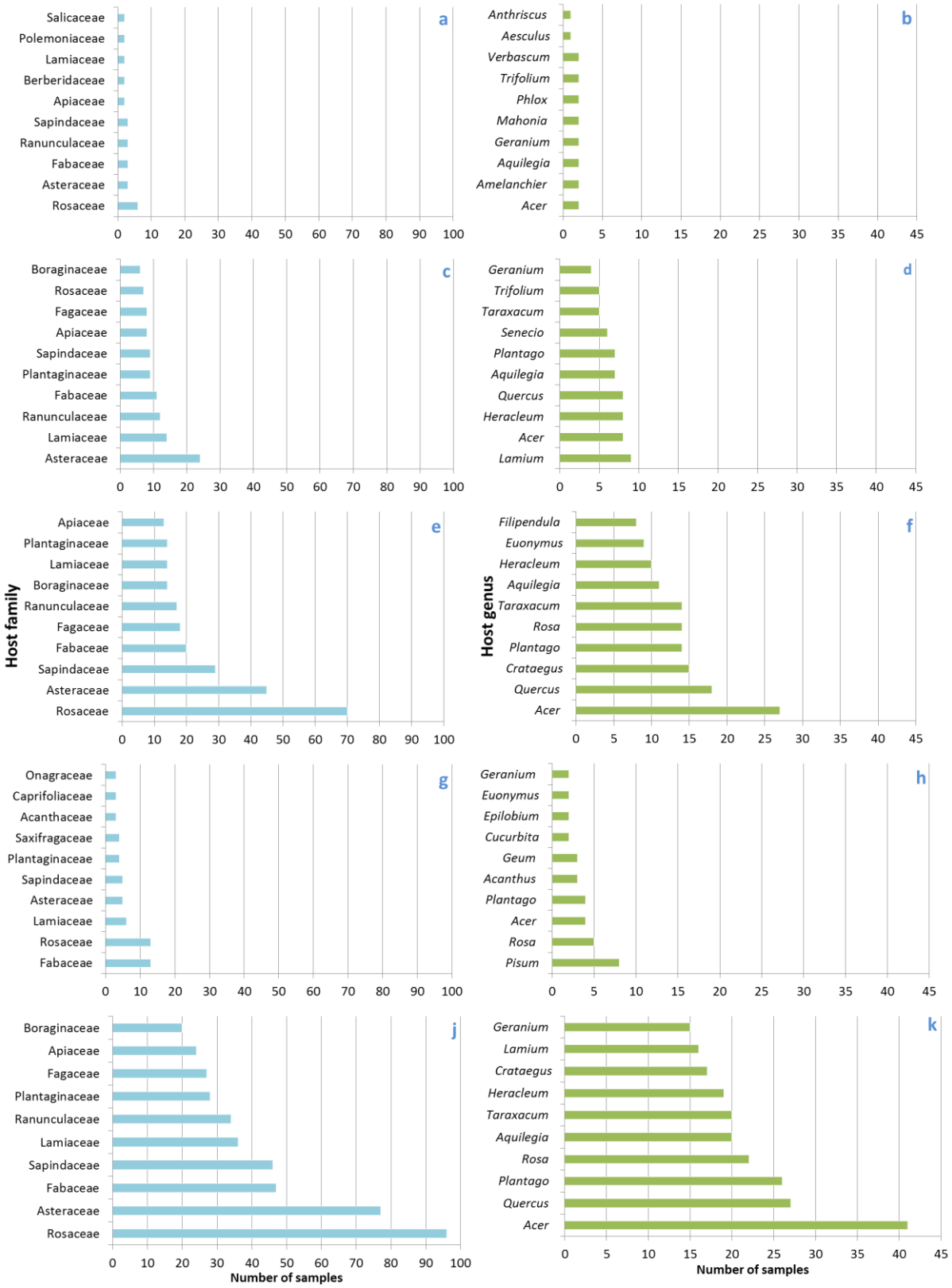

Figure 2.9: Bar charts showing the top 10 PM host plant families (blue) and genera (green) of 2013 (a, b), 2014 (c, d), 2015 (e, f), 2016 (g, h), and all years combined (j, h).

2.3.4: Culturing of PM samples

Detached leaves regularly became infected with non-PM fungal contaminants (Figure 2.10). The method was inaccurate and inefficient, producing profuse growths of fungi such as *Botrytis cinerea*.

Figure 2.10: Cultures of *Rhodonendron* 'Karen Tripitta' show evidence of damage to plant cells and profuse growth of *Botrytis cinerea*.

Figure 2.11: A selection of PM infected hosts: (a) unidentified PM on *Monarda didyma*, (b) *Neoerysiphe galeopsidis* on *Acanthus spinosus*, (c) *E. pisi* on *Pisum sativum*, (d) *Podosphaera leucotricha* on *Malus domestica*, (e) *E. trifoliorum* on *Trifolium arvense*, (f) *E. aquilegiae* on *Aquilegia vulgaris*, (g) *E. berberidis* on *Berberis thunbergii*, and (h) *E. catalpae* on *Catalpa bignonioides*.

2.4: Discussion

2.4.1: Samples received and social engagement

Citizen science schemes can receive hundreds to millions of new recordings (Silvertown, 2009). There is such scope due to variable inputs of time and money and different sorts of data being required for different schemes. The uptake of the Powdery Mildew Citizen Science Scheme (measured in number of contributors, number of samples, and number of engagements) falls towards the lower end of these. A citizen science project's funding is likely to be an important factor in its success (Whitelaw *et al.*, 2003) as this generally correlates to input of time, promotion, and continued maintenance (Conrad & Hilchey, 2011). With funding coming from a single PhD studentship and input largely from one person the number of samples and relatively small reach of this project are unsurprising. The aid of various British societies for promotion was invaluable as it enabled access to already established, potentially interested audiences. The launch and maintenance of this scheme has engaged hundreds of people with the important and problematic PM fungus and has resulted in the contribution of hundreds of PM samples to RNG; providing a unique snapshot into PM diversity in the UK. These provided the necessary samples on which to develop and test increasingly efficient identification techniques and as such, the scheme can be seen as a success.

Initial aims of the scheme were to collect PMs from across all five tribes such that developed molecular methods could be tested on samples spanning the entire Erysiphales order (success is reviewed in Chapter 3). This was different from the majority of published PM research, which tends to focus on specific host plants such as cereal crops (Wyand & Brown, 2003, Troch *et al.*, 2014), peas (Fondevilla *et al.*, 2006, Fondevilla & Rubiales, 2012), crucifers (Adam *et al.*, 1999), cucurbits (Sitterly, 1978, Vela-Corcía *et al.*, 2014), or grapes (Brewer & Milgroom, 2010, Brewer *et al.*, 2011), to name a few. For these it is important to maintain a constant supply of infected research material. Therefore cultures are kept in field or greenhouse environments or on artificial growing media. In the case of

novel PM species identifications a subsample of closely related Erysiphales species are necessary in order to show clear species separation (Cho *et al.*, 2014, Kabaktepe *et al.*, 2017, Tang *et al.*, 2017). This will regularly come as ITS sequence data, which is sourced from online databases and compared with the newly generated species sequence data in question.

In terms of a national scale of sampling, the series of papers regarding PM phylogenetics (Meeboon *et al.*, 2015, Meeboon & Takamatsu, 2015a, Meeboon & Takamatsu, 2015b, Meeboon & Takamatsu, 2015c) is most similar to this one. Meebon and Takamatsu relied on neglected, environmental collections dating back to 1993, supplemented by constant present-day collection, enabling a study of the 264 species recorded in Japan. Similarly, the present study required plenty of samples and plenty of DNA from them to develop and test new methods. This was achieved with collection of fresh samples for testing the accuracy of current identification techniques and was followed up with the development of novel methods to increase accuracy and efficiency of the process. Given the specificity of PM samples to their host plants, the 134 genera of plants collected from 48 families provide samples likely to host PM species ranging across all five tribes and multiple genera.

The number of samples received increased from 2014 to 2015 due to increased publicity of the scheme. This level of outreach via article publication and presence at conferences and flower shows was maintained during 2016, however sample number declined. This is likely due to the weight of samples already present from previous years collections which resulted in the discard of numerous new samples. These were those of the top 10 most abundant genera; a saturation point had been reached for these, most common, PM host plants of the UK. It is not yet known whether abundance or diversity of PM samples received were sufficient testing the accuracy of established and newly developed identification techniques. This will be explored fully in Chapter 3.

Sampling could have been more targeted, particularly during 2016, by communicating the need for particular host plants. Such directives should occur in future years of the scheme: samples

of the most common hosts have reached a saturation point, with little to no new information coming from their repeated collection. Collection of species already present in the dataset but lacking repeats and species known to be present in the UK but missing from the dataset should therefore be aimed for by requesting host plants likely to harbour particular PMs.

It is unknown if the peaks of received samples shown in the results were due to the greater prominence of PMs during these months or greater publicity for the scheme itself. It is clear that PMs are seasonal and that their main growing season is known to be when their angiosperm hosts are in leaf. The data of this project concurs with such seasonality, although it was not specifically designed to do so.

2.4.2: Sample distribution

The sampling sites of 2013 (University of Reading Harris Garden and RHS Garden Wisley) are sites of horticultural excellence containing plants common to UK gardens and wilder areas, along with their associated microorganisms. This is exemplified by seven of the top ten families over the whole study time being collected in 2013 (Figure 2.9a, j). RHS Garden Wisley, in particular, is a site at which import and export of plants via trade is common. Trade is one of the major sources of inoculum for plant diseases (Fisher *et al.*, 2012); particularly diseases establishing themselves across physical borders which cannot be crossed easily, such as the English Channel. These sites can therefore be good indicators of new PM species, potentially threatening to UK horticulture.

The use of a citizen science scheme for the collection of samples in 2014, 2015 and 2016 allowed for the contribution of a greater diversity of plants and PMs. The scheme was promoted largely by the RHS who have a preponderance of members in SE England (The Royal Horticultural Society, 2013). We can therefore expect a bias of samples from this same area of the UK. The distribution maps show this to be the case, but the contributions of others from Scotland, SW England, East Anglia, and particularly Merseyside result in well balanced UK sampling.

2.4.3: Host profiling

The use of citizen science was always likely to produce convenience sampling; it was not possible to control who contributed to the scheme as collectors contributed on a purely voluntary basis. Influencing which infected host plants caught their attention was also not possible. The convenience sampling resulted in a good representation of the British flora, with many of the most common UK species (The Plant List, 2010) amongst the samples.

2.4.4: Culturing of PM samples

This method was halted due to its inaccuracies and inefficiencies. This was in line with numerous culturing trials (Morrison, 1960, Kenyon *et al.*, 1995, Álvarez & Torés, 1997, Nicot *et al.*, 2002) which have rarely resulted in reproducible results. Efforts were instead concentrated on developing molecular markers from environmental PM samples collected from the citizen science scheme.

2.5: Conclusions

The Powdery Mildew Citizen Science Survey proved to be a useful and fruitful method of PM sampling. Samples were collected from a broad array of flora within the UK, with certain well-known genera making up large amounts of the overall sampling. However, a saturation point for the most common host genera was quickly reached and thus a more targeted approach became necessary. Receipt of 134 host genera provided the basis for evaluation of current identification techniques. This collection has resulted in a much needed update of PM host records and their distribution within the UK.

Promotion of the scheme via physical and online publications, and at horticultural shows and conferences drove increased popularity and knowledge of the scheme. However, a more diverse array of promotion methods could be used in future to reach a greater audience. Participation in trade shows and special interest groups, as well as more sustained online presence would have enabled this.

Chapter 3: Species Identification using established techniques

3.1: Introduction

Accurate identification of PM species can be a difficult task as many share morphological and molecular features. Methods to date have concentrated on analyses via host plant identification, fungal morphology, and more recently sequencing of genomic rDNA regions (see section 1.5 for greater detail). Prior to molecular works, monographs of the PMs were published based largely on the teleomorphic stage of the PM lifecycle and their available morphological characters (Salmon, 1900, Braun, 1987). Further morphological characters have since been discovered including differences in the outline of conidial chains (Shin & La, 1993), differences in the conidial surface when viewed by Scanning Electron Microscopy (SEM) (Cook *et al.*, 1997), the position of conidiophores on the mother cell (Shin & Zheng, 1998), shape and size of the penicillate cells in the upper half of chasmothecia of *Phyllactinia* (Shin & Lee, 2002), and details of the patterns of conidial germination (Cook & Braun, 2009). Descriptions of the anamorphic stage of the PM life cycle were also added to this; uniting sexual teleomorphs with their asexual anamorphic forms and resulting in complete holomorphic descriptions (Hawksworth *et al.*, 2011). This, allied with the development of DNA based identification, has since coincided with the end of dual nomenclature for fungi (Crous *et al.*, 2015) (see http://www.fungaltaxonomy.org).

The introduction of molecular methods provided further insight into the species and often resulted in new interpretations of their boundaries and relationships (Saenz & Taylor, 1999, Hirata *et al.*, 2000, Mori *et al.*, 2000, Matsuda & Takamatsu, 2003, Ito & Takamatsu, 2010). The ITS region has been used extensively in early phylogenetic studies, along with the small (18S) and large (28S) flanking ribosomal subunits (Bruns *et al.*, 1991). The ribosomal repeat unit was a focus of the current study due largely to its large number of tandem copies and accompanying concerted evolution that allowed ease of amplification by PCR (Bruns *et al.*, 1991). As such, it has formed the backbone of molecular analyses in the Erysiphales (Kovács *et al.*, 2011, Wang *et al.*, 2013) and fungi as a whole

(Schoch *et al.*, 2009), and was proposed as the anchoring barcoding region for fungal identification (Schoch *et al.*, 2012).

Developments of PM knowledge have resulted in more detailed morphological descriptions of species and uncovered new taxa. Approximately 864 PM species are now recognised (Braun & Cook, 2012); an increase from 515 in 1987 (Braun). The efficiency and accuracy of identification of PM species has therefore been furthered, but scope for improvement still remains; discrepancies remain in the consistent separation via phylogenetic and barcoding analyses of closely-related, phylogenetically young species (Cunnington *et al.*, 2004, Pirondi *et al.*, 2015, Takamatsu *et al.*, 2015a). The trend in increasing numbers of species seen from 1987 to 2012 is therefore likely to continue as DNA based identification continues to develop.

The aims were:

- to assess the ability of currently accepted morphological techniques for identifying PM accessions to genus and species level;

- to assess the ability of currently accepted molecular techniques for identifying PM accessions to genus and species level;

- and to assess the ability of combining currently accepted techniques for identifying PM accessions to genus and species level.

3.2: Materials and methods

3.2.1: Sample collection

Samples were collected via the powdery mildew citizen science scheme (Chapter 2). All 596 of these samples were morphologically examined and 507 were successfully sequenced (Appendix 5).

3.2.2: Morphological analyses

Fungi were mounted on slides and imaged using the Leica DM2000 LED with associated Leica Application Suite. Putative species identity was based on Braun and Cook (2012). Conidiogenesis type, appressoria form, presence/absence of fibrosin bodies, and conidia size and shape were recorded in asexual forms (Figure 3.1). Chasmothecium size, shape, and colour, appendage length and form, and asci and associated ascospore number and size were recorded in sexual forms when present (Figure 3.2).

3.2.3: DNA extraction

DNA was extracted from 0.01-0.02g dry weight of infected leaf material. This was frozen using liquid nitrogen and ground with two tungsten carbide ball bearings and acid washed silica sand using the Qiagen TissueLyser II. The Qiagen DNeasy Plant Mini Kit protocol was then followed without modification.

3.2.4: Polymerase chain reaction (PCR) and sequencing protocol

PCR was performed using published PM specific primers (PMITS1 and PMITS2 (Cunnington *et al.*, 2003)). The conditions were 12.5 µl BioMix™ Red (Bioline), 0.5 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 ng μl^{-1}, 9.25 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}; in 25 µl final volume. Cycling parameters were an initial denaturation step of 95 °C for five minutes, followed by 35 cycles of denaturation at 95 °C for 15 seconds, annealing at 56 °C for 20 seconds, and elongation at 72 °C for one minute and a final elongation at 72 °C for five minutes.

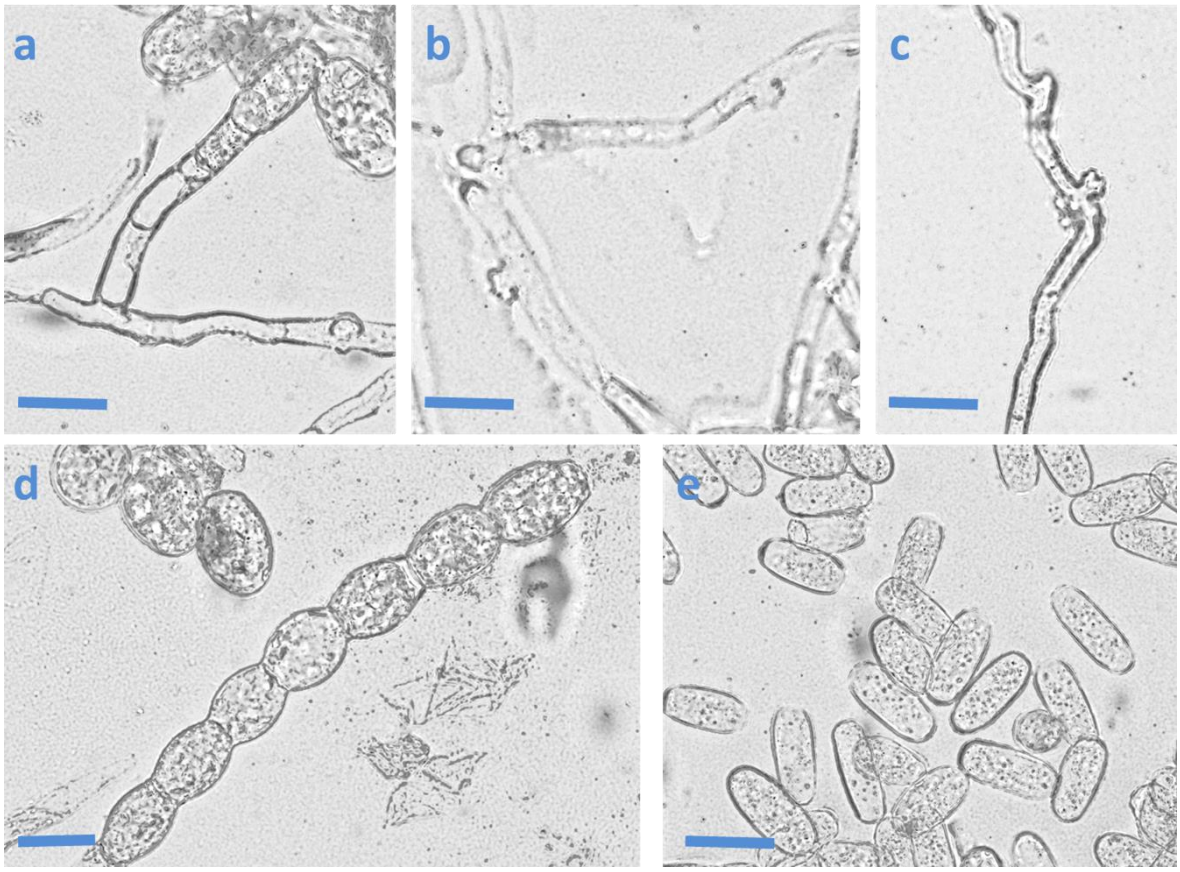

Figure 3.1: PM anamorphic structures: (a) pseudoidium conidiogenesis type; (b) nipple shaped appressoria; (c) nipple shaped appressorium; (d) euoidium conidiogenesis type; and (e) condidia with fibrosin bodies present. Bars = 20 um. Photos O. Ellingham.

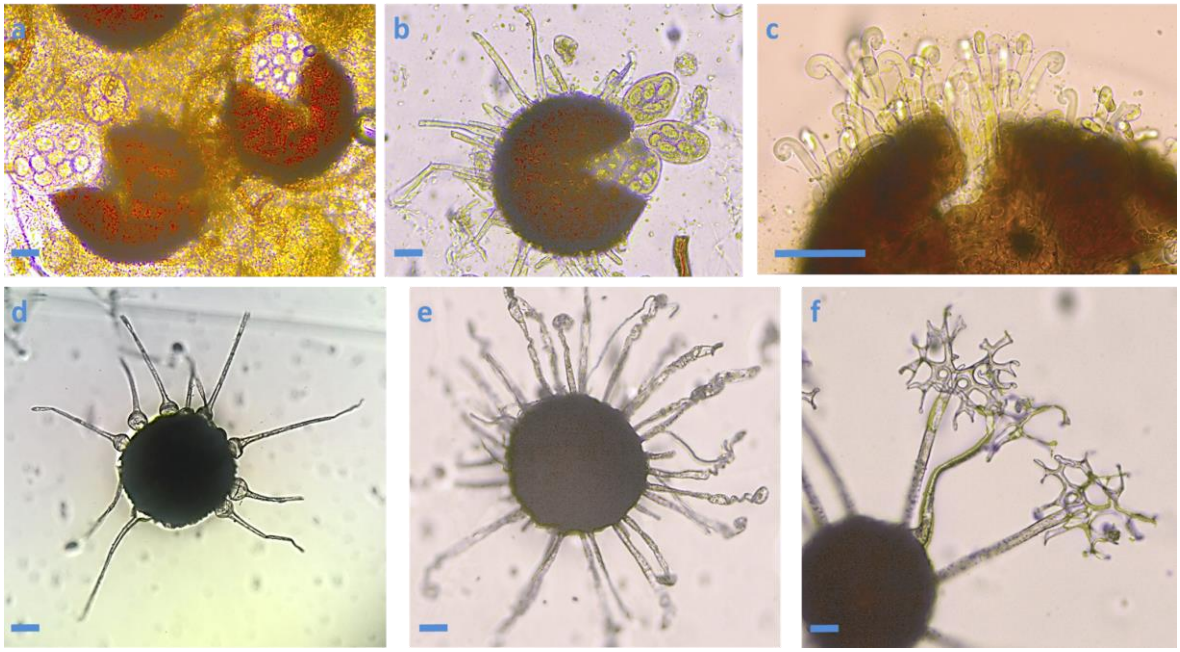

Figure 3.2: PM teleomorphic chasmothecial structures: (a) single ascus with 8 ascospores; (b) multiple asci with 5-6 ascospores and simple-mycelioid appendages; (c) uncinate-circinate, dichotomously branched appendages; (d) hyaline appendages with bulbous base; (e) helically twisted, uncinuloid appendages; and (f) club-shaped appendages. Bars = 20 um. Photos O. Ellingham.

The PCR products were separated by electrophoresis at 10V cm^{-1}, for one hour, in 1% agarose gels in Tris base (pH 8.3), acetic acid and 100mM EDTA buffer (pH 8.0) (TAE) at 1x concentrate (Sambrook *et al.*, 1989) and stained with ethidium bromide (0.5 µg ml^{-1}) in 2014 and 2015 and GelRed (Biotium) in 2016. HyperLadder™ 1kb (Bioline) was used to indicate product size. The gel was visualised on the T:Genius – Syngene UV transilluminator. Single amplicons of more than 10 ng $band^{-1}$ were then sent to Source BioScience in 2014 and 2015, and GATC in Germany in 2016 for sequencing.

Complementary forward and reverse sequences generated in this study were assembled and manually edited using SeqMan Pro software (DNASTAR, Madison, WI, USA). These were submitted to GenBank with accession numbers KY653161 – KY653211 and KY660722 – KY661161 (Appendix 5).

3.2.5: Species identification via the Basic Local Alignment Search Tool (BLAST)

DNA sequences were copied into the NCBI GenBank Nucleotide BLAST and highly similar sequences (megablast) were searched for. The query cover and identity of closest matches were recorded with a 99% identity threshold for a definite identification of a PM species (Tang *et al.*, 2017).

3.2.6: Sequence alignment

Sequence alignment of ITS data was performed using MUSCLE (Edgar, 2004) and manually edited to form the complete ITS dataset. This was treated in two ways. Firstly, the alignment was split into samples of the suspected species from three, well represented, PM tribes (Cystotheceae, Golovinomyceteae, and Erysipheae). Each alignment was saved separately and further edited to remove gaps. Secondly, samples from the complete dataset were compared by pairwise alignment and those with identical sequences were combined into single units in order to optimize computation time when analysing for the overall topology of the Erysiphales. Each alignment was deposited in TreeBASE (Accession S20958).

3.2.7: Phylogenetic analyses

The optimal nucleotide substitution model was selected for each alignment via the AIC criterion (Akaike, 1974) using PAUP (Swofford, 2003) and the MrModelblock command (from MrModeltest (Nylander, 2004)). Bayesian inference (BI) was performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Stationarity using a plot of –ln was tested for, a 25% burn-in was used and all trees were rooted with the outgroup *Blumeria graminis* ("5_86_Blumeria_graminis_ex_Poa_trivialis"). All other variables followed default settings of BI. Parameter states and trees were stored every 10,000 generations to avoid autocorrelation via a check using Excel. To ensure convergence was reached, the average standard deviation of split frequencies was monitored to ensure that it fell below 0.05, and trace files of all parameters were examined using Tracer v1.6 (Rambaut *et al.*, 2015) to ensure proper mixing. Consensus of resultant trees was built and visualised using BayesTrees V1.3 (http://www.evolution.reading.ac.uk/BayesTrees.html).

For BI of the ITS dataset with duplicate DNA sequences removed the GTR+I+G model was used and was run for 10,000,000 generations. For BI of ITS samples of Cystotheceae the SYM+I+G model was used and was run for 5,000,000 generations. For BI of ITS samples of Golovinomyceteae the GTR+G model was used and was run for 5,000,000 generations. For BI of ITS samples of Erysipheae the GTR+I+G model was used and was run for 5,000,000 generations, at a temperature of 0.2 in order to reach the reach the optimal solution most efficiently.

3.2.8: DNA barcoding analysis

Samples in the ITS sequence dataset were renamed, to species where possible, according to DNA sequence and morphological data from all previous analyses. The dataset was imported into Taxon DNA/SpeciesIdentifier 1.8 (Meier *et al.*, 2006). The Species Summary, Pairwise Summary, Pairwise Explorer, Distance Analysis, Extreme Pairwise, Best Match/Best Close Match, All Species Barcodes, Cluster, and Overlap Analysis were calculated with pairwise distances using Kimura 2-parameter corrected distances (K2P) (Kimura, 1980). Resultant data were stored in a Word document. Pairwise

Summary results were imported into Microsoft Excel 2007. Bar plots were then generated from these data to compare the differences between intra- and interspecific distances.

3.3: Results

3.3.1: PM identification

All 596 PM samples were morphologically examined. 507 of these were successfully sequenced. Morphological examinations of 596 samples enabled identification to PM genus 80% of the time and to PM species 65% of the time. ITS sequencing of 507 samples enabled 461 of these to be identified to PM genus (91% of samples successfully sequenced and 77% of all 596 samples), however identification to PM species via BLAST was often not possible due to the intrageneric sequence similarity of PMs and incorrect records available in NCBI GenBank. Combining inferences from morphological examination with ITS sequence data enabled identification to PM genus in 94% of all samples and to PM species in 80% of all samples. The most commonly identified PM species are shown in Figure 3.3.

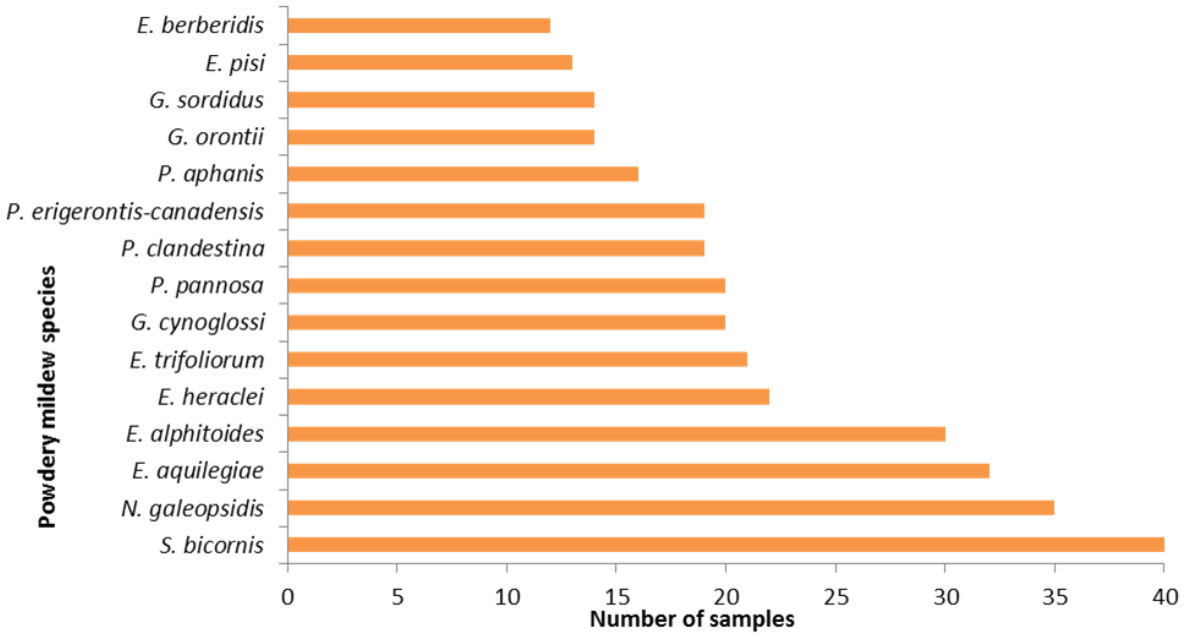

Figure 3.3: Fifteen most common PM species of the study.

Identification of host plants resulted in immediate delimitation of potential PM species. For certain hosts this can mean a reduction from 864 potential species to one. Even so, morphological and ITS analyses were performed in order to back an identification up with additional data. The asexual form of PMs was observed in 95% of all samples and morphological analysis of the

associated structures was a reliably useful tool. Asexual features such as the combination of conidiogenesis type and presence/absence of fibrosin bodies in conidia were a crude method of initial delimitation to potential genera (Figure 3.4). The combination of pseudoidium conidiogenesis with no fibrosin bodies was common to *Erysiphe* and *Phyllactinia*. Euoidium conidiogenesis with no fibrosin bodies was common to *Blumeria*, and genera of the Golovinomyceteae (*Arthrocladiella*, *Golovinomyces*, and *Neoerysiphe*). Euoidium conidiogenesis with fibrosin bodies present was common to genera of the Cystotheceae (*Podosphaera* and *Sawadaea*). These features were supplemented with observations on conidial and appressorial shape where possible. Sexual forms were less common; observed in 20% of all samples. When observed, features such as the number of asci per chasmothecium, ascospores per ascus, and particularly appendage morphology were useful as they enabled delimitation to genus level (Figure 3.2). Combining available features with host identification enabled identification of PM to a single species 65% of the time. For the remaining samples it was known that more than one PM species with identical morphological features had been recorded on the given host. Therefore further analyses were required to identify the species.

A species formerly unreported in the UK was characterised (Ellingham *et al.*, 2016). Detailed morphological analyses enabled the separation of potential PM taxa *Podosphaera alpina* and *P. macrospora* on *Heuchera* cultivars. Measurements of the mean dimensions of ascospores enabled final identification of *P. macrospora* when a lack of previous, accurate ITS sequence data meant that identification was not possible.

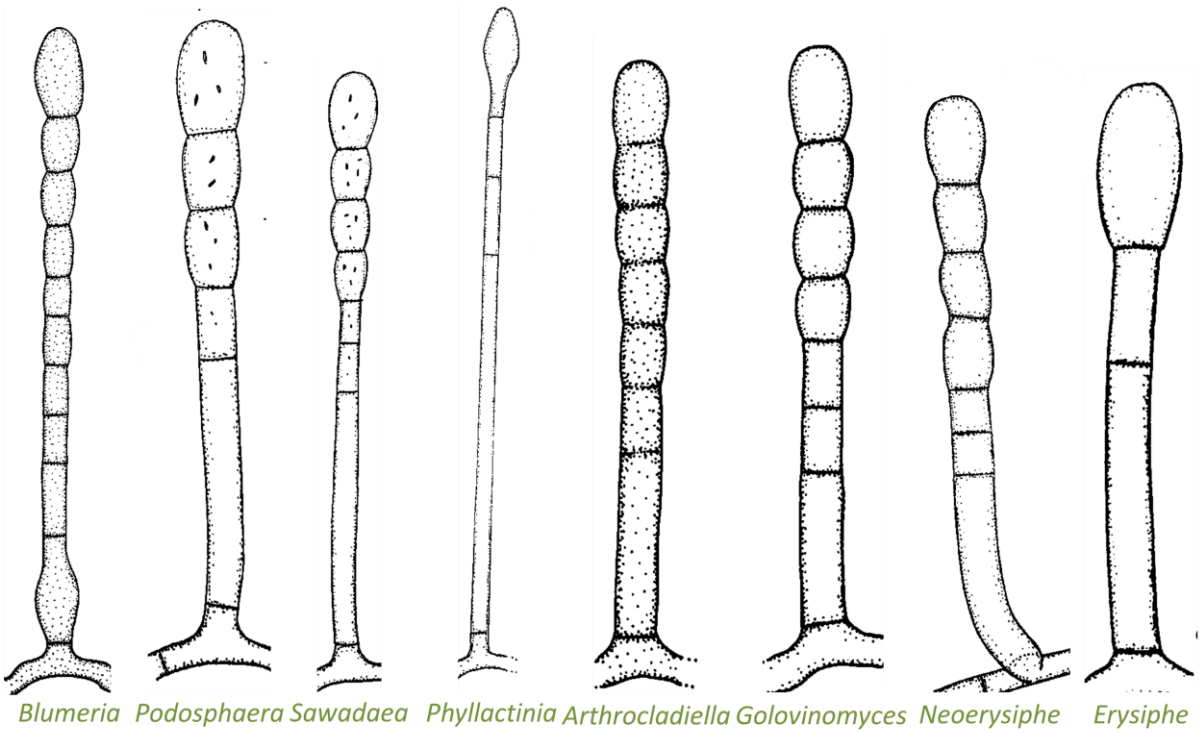

Figure 3.4: Diagrams of anamorphic forms of the PM genera sampled in the current study. Conidiogenesis type, conidial shape, and presence/absence of fibrosin bodies are evident. From Braun and Cook (2012).

3.3.2: Phylogenetic analyses

Removal of accessions with the same DNA sequence of the 507 sequenced resulted in a dataset of 173 accessions covering each of the five PM tribes. The final alignment was 881 bp in length with 370 variable sites (42%). BI of this overall PM phylogeny resulted in clear discrimination of each PM tribe represented by three or more accessions. Posterior probabilities (PPs) of the overall topology were high. Tribes Cystotheceae, Golovinomyceteae, and Erysipheae were monophyletic from the *Blumeria* root, with PP of 83%, 96%, and 99% respectively (Figure 3.5). However accessions of tribe Phyllactineae were polyphyletic; split between a monophyletic clade and that of the Cystotheceae. Apart from these *Pyllactinia* spp., each genus included in the sampling proved to be monophyletic as *Podosphaera* had 86% PP, *Neoerysiphe* had 100% PP, *Arthrocladiella* had 96% PP, *Golovinomyces* had 88% PP, and *Erysiphe* had 99% PP.

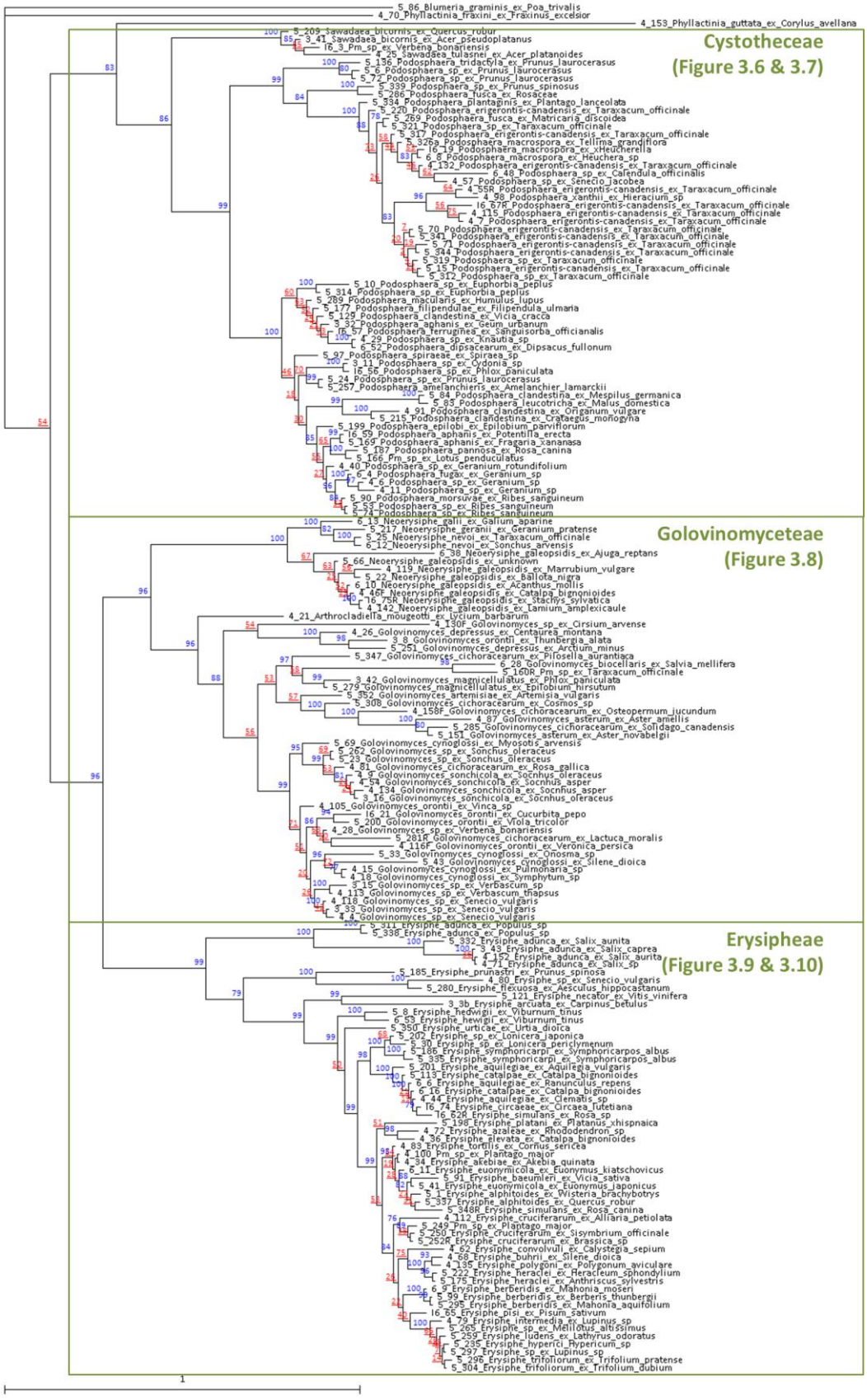

Figure 3.5: BI using 173 sequences of the ITS region. Green line separations show the three main sampled tribes and reference BI figures of the individual tribes. Accession names include accession code, PM name, and host identity. Posterior probabilities (PPs) above 75% are shown in blue and below in red.

3.3.2.1 Cystotheceae

The division of the dataset into the Cystotheceae reduced it from 507 to 193 taxonomic units. BI of the resultant 738 bp region resulted in a split into two monophyletic groups from the rooted *Blumeria graminis* accession (Figure 3.6 & Figure 3.7). The clade comprising 37 accessions of *Sawadaea* had PP of 97% (Figure 3.6) and the clade comprising 155 accessions of *Podosphaera* had PP of 45% (Figure 3.6 & Figure 3.7).

Within the *Sawadaea*, *S. tulasnei* (two accessions with 99% PP) and *S. bicornis* (35 accessions with 94% PP) were shown to be monophyletic. *S. bicornis* split further according to host species: the clade comprising ten accessions with 98% PP on *Acer campestre* and the clade comprising 25 accessions with 81% PP on *Acer pseudoplatanus*.

Within the *Podospharea*, *P. tridactyla* (five accessions with 98% PP), *P. leucotricha* (nine accessions with 100% PP), *P. clandestina* (18 accessions with 100% PP), *P. amelanchieris* (six accessions with 13% PP), and *P. euphorbiae-helioscopiae* (two accessions with 99% PP) were shown to be monophyletic. *P. macrospora* accessions (four accessions with 48% PP and 12 accessions with 33% PP) were grouped together but paraphyletically amongst species of *P. plantaginis* and *P. fusca*. *P. pannosa* accessions were grouped into two separate, clades (four accessions with 43% PP and ten accessions with 20% PP). *P. aphanis* accessions formed a single main group (eight accessions with 80% PP). However five other accessions of *P. aphanis* were placed in other groupings of the *Podosphaera*.

The remaining *Podosphaera* species were scattered into four mixed groups. These were made up of: (1) *P. fusca, P. plantaginis, P. macrospora, P. erigerontis-canadensis,* and *P. xanthii*; (2) *P. fugax*, *P. epilobi*, and *P. aphanis*; (3) *P. aphanis, P. filipendulae, P. ferruginea, P. dipsacacearum, P. macularis,* and *P. spiraeae*; and (4) *P. filipendulae* and *P. plantaginis* respectively.

Figure 3.6: Part 1 of BI using 193 sequences from the Cysotheceae tribe of the ITS region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny.

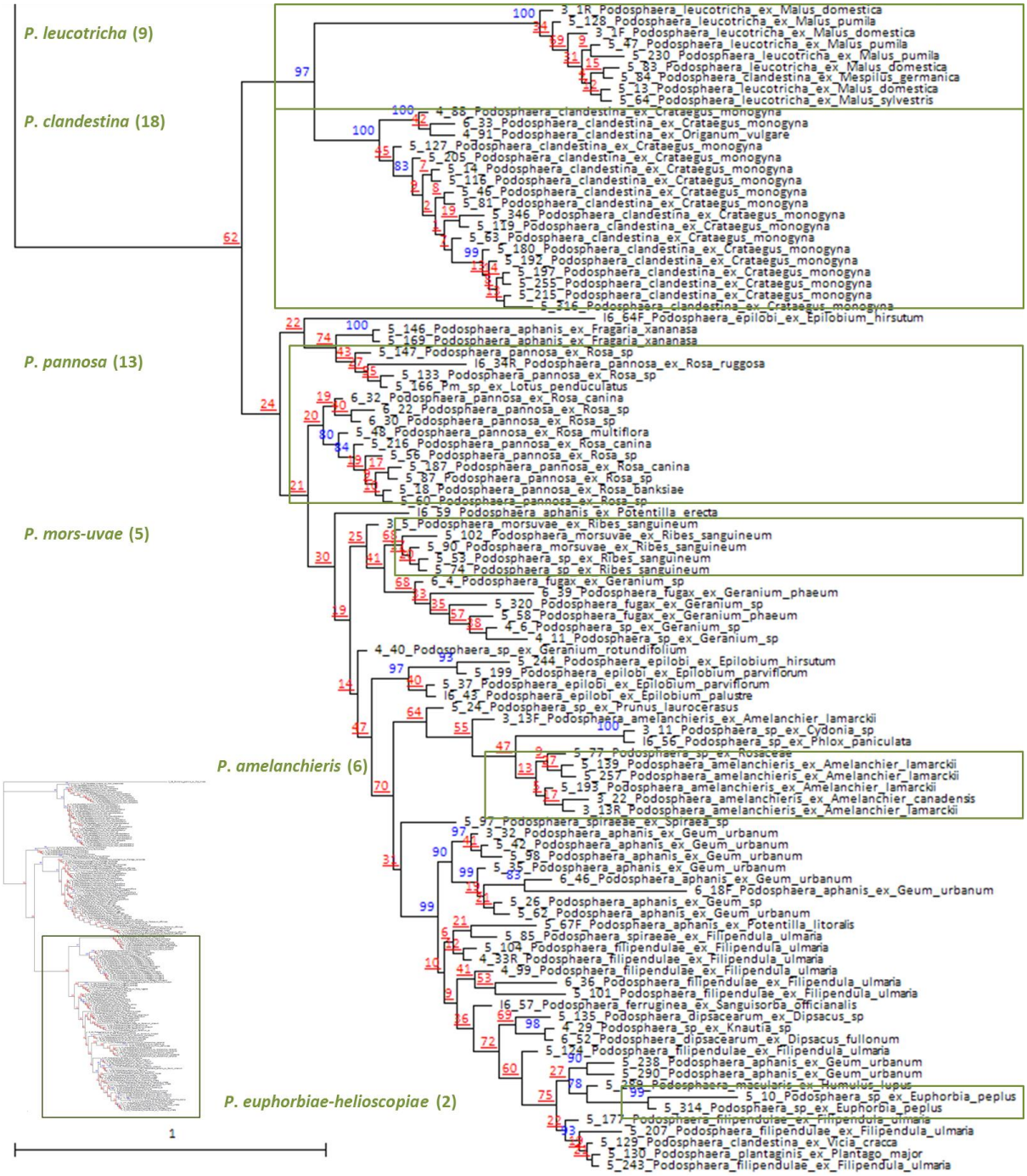

Figure 3.7: Part 2 of BI using 193 sequences from the Cysotheceae tribe of the ITS region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny.

3.3.2.2 Golovinomyceteae

The division of the dataset into Golovinomyceteae resulted in 102 taxonomic units of a 753 bp region within the ITS region (Figure 3.8). BI of this dataset resulted in splitting of the three genera, from the rooted *Blumeria graminis* accession, into monophyletic groups. The three *Arthrocladiella* accessions were *A. mougeotti* found on *Lycium barbarum* and had PP of 100%, the 41 *Neoerysiphe* accessions had PP of 99%, and the 57 *Golovinomyces* accessions had PP of 36%.

Within the *Neoerysiphe*, all four species were shown to be monophyletic (Table 3.1).

Within the *Golovinomyces*, seven of the 11 putative taxa were monophyletic (Table 3.1). Potential groups of *G. cichoracearum* and *G. sordidus* were shown to arise polyphyletically. The *G. cichoracearum sensu lato* initially grouped into five separate clades: seven accessions on Asteraceous hosts (*Cosmos, Cirsium, Osteopermum, Aster,* and *Solidago*) with 61% PP which could be identified as *G. asterum*, two accessions on the Asteraceous *Pilosella aurantiaca* with 100% PP, six accessions found on *Sonchus* sp. with 100% PP which could be identified as *G. sonchicola*, two accessions on *Verbascum* (of the Scrophulareaceae) with 100% PP which could be identified as *G. verbasci*, and three accessions parasitising *Senecio vulgaris* with 85% PP which could be identified as *G. fischeri* or *G. senecionis*. The *G. cynoglossi* grouped into three separate clades: five accessions on *Myosotis* sp. with 96% PP, three more accessions found on *Myosotis* sp. with 100% PP, and five accessions parasitising the Boraginaceae (*Symphytum, Pulmonaria,* and *Onosma*) with 100% PP. Accessions 5_279 *G. magnicellulatus* ex *Epilobium hirsutum*, 4_81 *G. cichoracearum* ex *Rosa gallica* positioned within the *G. sonchicola* clade, and 5_43 *G. cynoglossi* ex *Silene dioica* are placed within groups of high PP, however, PM species of these species or genus have not been recorded on these hosts previously.

Table 3.1: Monophyletic PM groups from ITS data within the Golovinomyceteae.

Species within group	Number of Accessions	PP of group*
A. mougeotii	3	100
N. galii	2	99
N. nevoi	3	100
N. geranii	5	100
N. galeopsidis	31	99
G. depressus	6	100
G. biocellaris	1	
G. cichoracearum	2	61
G. sp.	2	
G. asterum	3	
G. artemisiae	1	
G. cynoglossi	5	96
G. cichoracearum	2	100
G. magnicellulatus	5	100
G. cynoglossi	3	100
G. sonchicola	7	100
G. orontii	6	81
G. sp.	1	
G. cichoracearum	2	
G. verbasci	2	100
G. fischeri	3	85
G. cynoglossi	5	100

*Groups with a single accession have no PP and are shaded in grey.

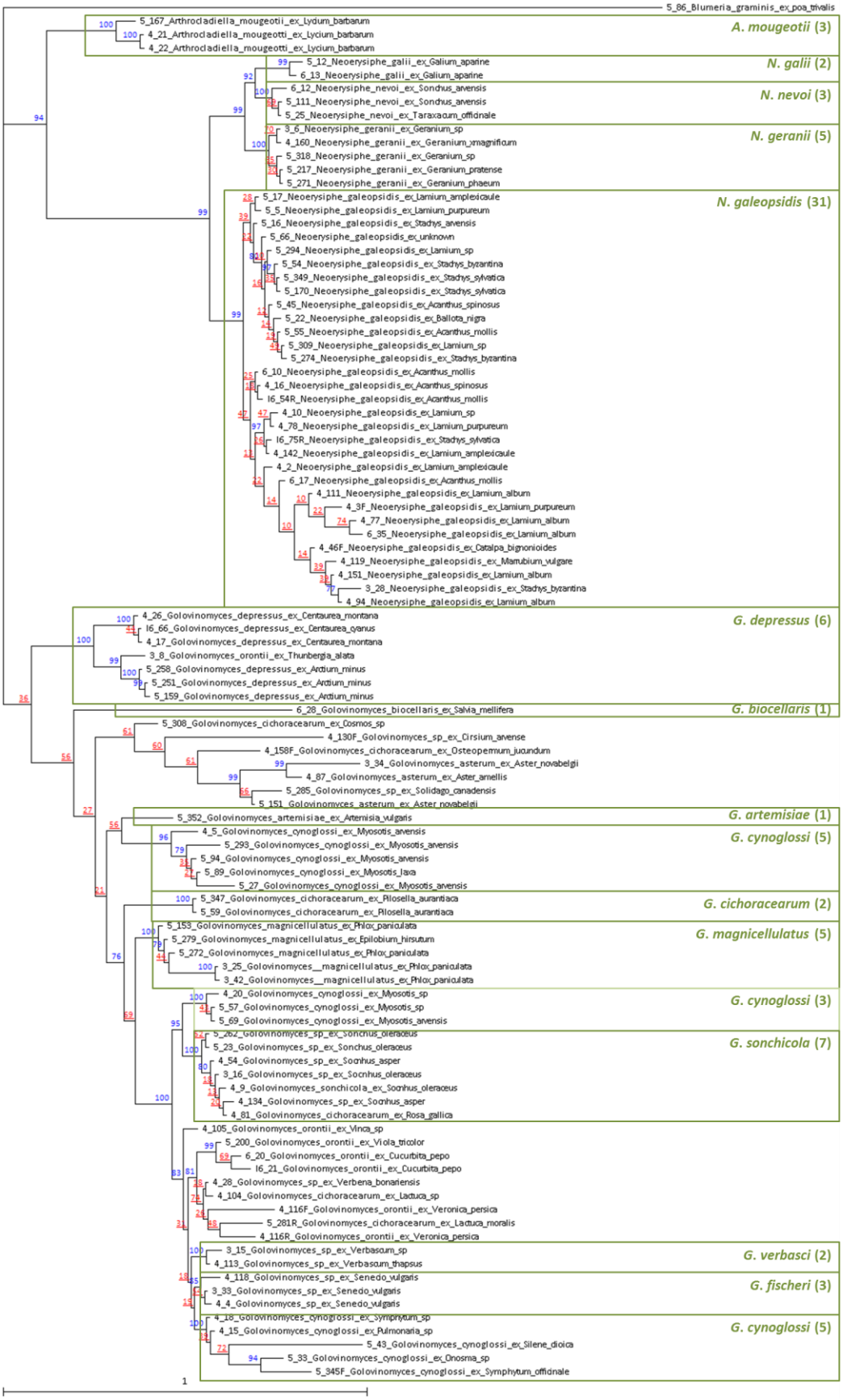

Figure 3.8: BI using 102 sequences of the Golovinomyceteae tribe of the ITS region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny.

3.3.2.3 Erysipheae

The division of the dataset into Erysipheae resulted in 201 taxonomic units of 802 bp region within the ITS region. BI resulted in numerous splits of the single genus *Erysiphe* into various different species clades and species complexes (Figure 3.9 & Figure 3.10). Of the approximately 28 different *Erysiphe* species included in the analysis, 15 were shown to be monophyletic (Table 3.2). The remaining accessions grouped within monophyletic groups of different species (Table 3.2).

Table 3.2: Monophyletic PM groups from ITS data within the Erysipheae.

Species within group	**Number of Accessions**	**PP of group**
E. prunastri	2	100
E. adunca	6	100
E. arcuata	3	100
E. necator	3	100
E. hedwigi	4	100
E. symphoricarpi	2	100
Erysiphe sp. ex *Lonicera* sp.	6	57
E. elevata	5	98
E. platani	3	94
E. cruciferarum	7	97
E. berberidis	11	96
E. convolvuli	3	100
E. buhrii	3	100
E. polygoni	6	98
E. heraclei	21	69
E. aquilegiae	28	87
E. trifoliorum	1	
E. buhrii	1	
E. circaeae	3	
E. simulans	1	
E. catalpae	2	
E. alphitoides	22	89
E. platani	3	
E. euonymicola	9	
E. tortilis	1	
E. akebiae	3	
E. simulans	1	
E. trifoliorum	1	
E. trifoliorum	24	57
E. pisi	8	

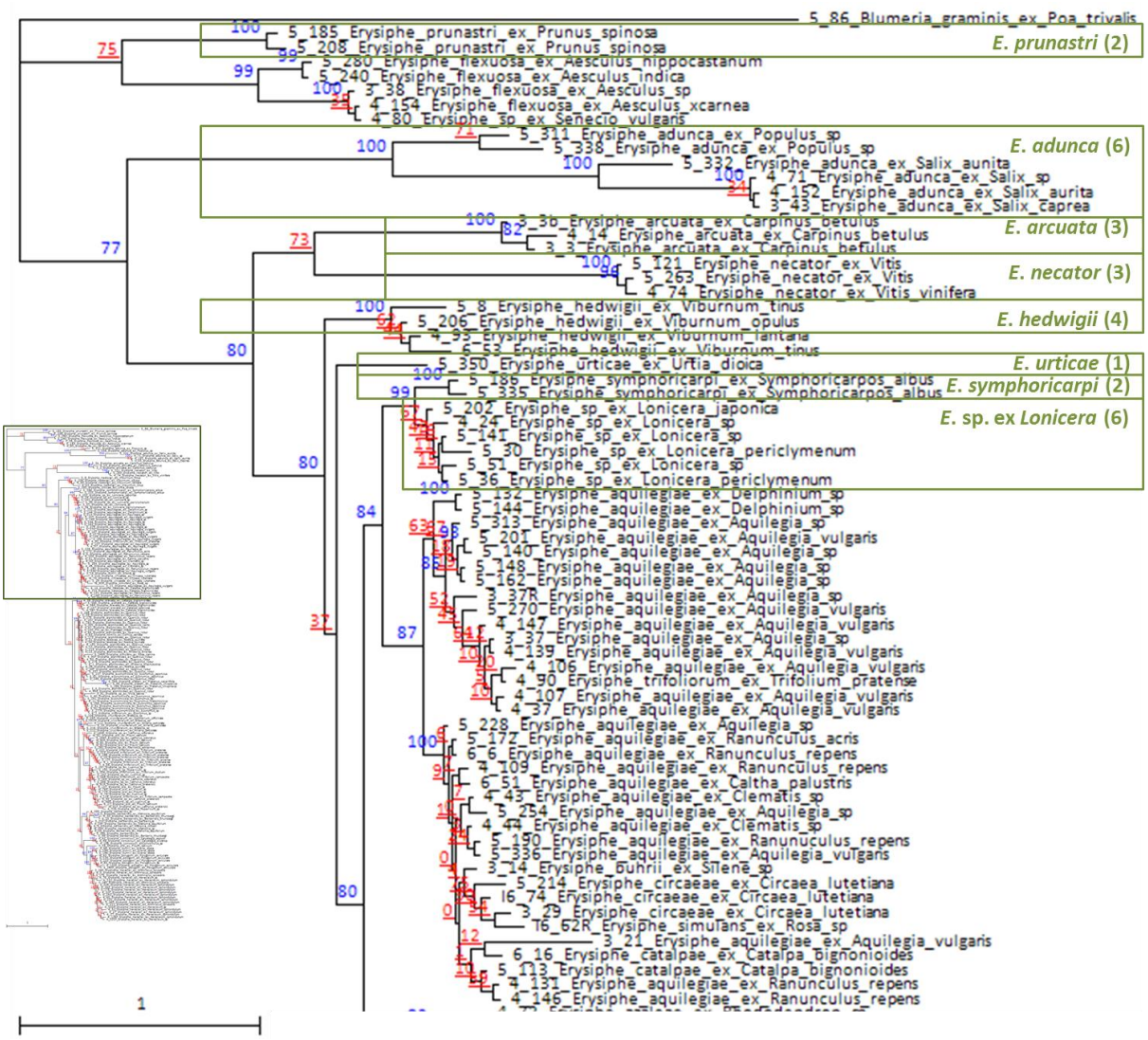

Figure 3.9: Part 1 of BI using 201 sequences of the Erysipheae tribe of the ITS region. Accession names include sample code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green line separations show species.

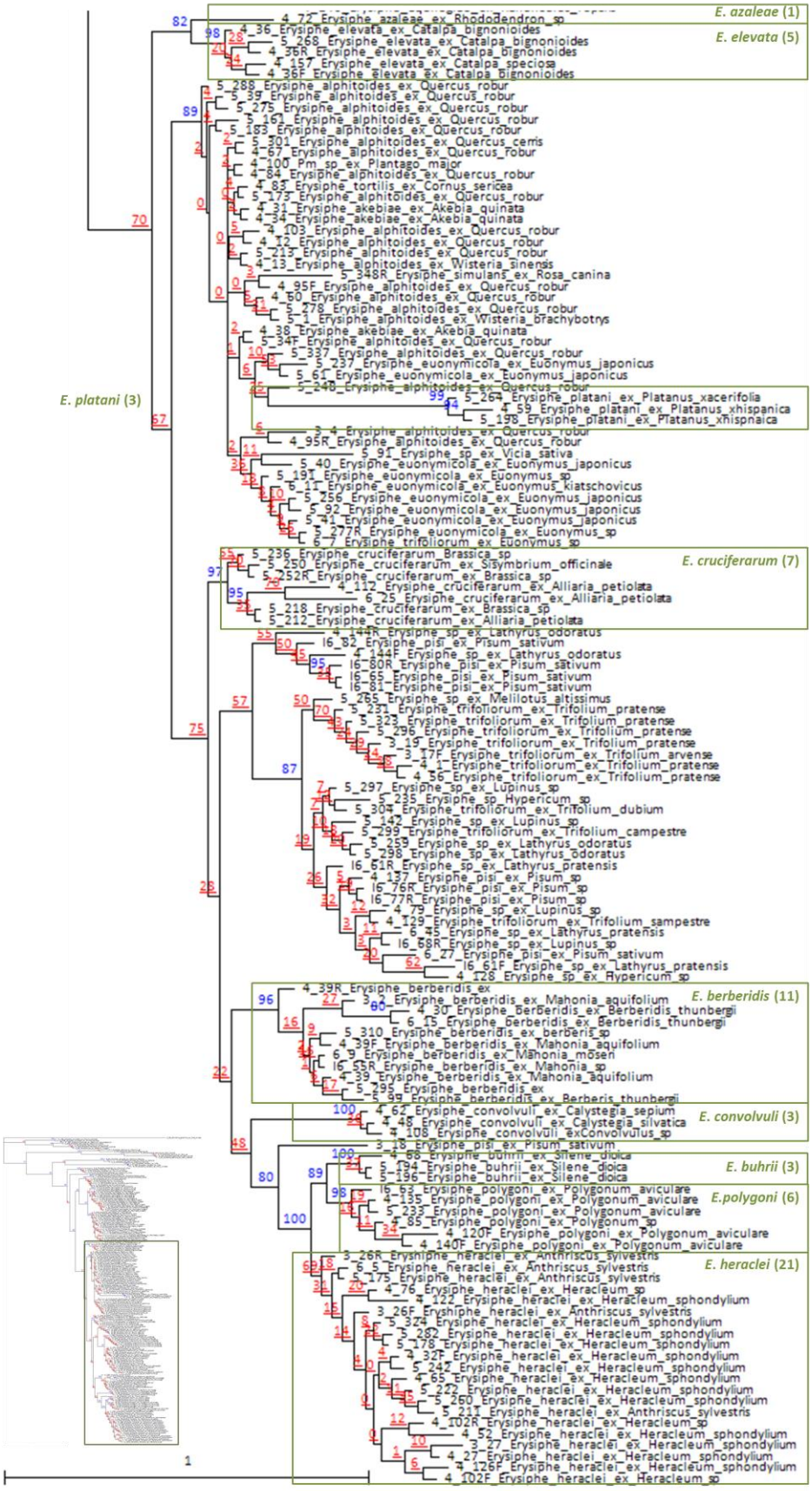

Figure 3.10: Part 2 of BI using 201 sequences of the Erysipheae tribe of the ITS region. Taxon names include sample code, PM name, and host identity. Posterior probabilities (PPs) above 75% are shown in blue and below in red. Green line separations show species.

3.3.3: DNA barcoding analyses

Intra and interspecific differences were quantified for the 507 samples and 35 species of the ITS region. This resulted in a total overlap of 17.08% of inter and intraspecific distances (from 0.0% to 17.08%, covering 96.46% of all intra and interspecific but intrageneric sequences) (Figure 3.11). The samples with a mean of more than 5% intraspecific difference were *P. clandestina, P. xanthii, P. plantaginis, P. tridactyla, P. leucotricha, P. epilobi, N. galeopsidis, G. depressus, G. sordidus, G. cichoracearum, G. cynoglossi, E. buhrii, E. adunca, E. aquilegiae, E. simulans, E. trifoliorum, E. pisi,* and *E. flexuosa*. Of the interspecific, congeneric distances 48.7% (30,471 of 62,617) fell below 5%, 69.8% of these were between samples of the *Erysiphe* and 26.9% were between samples of the *Podosphaera*. The congeneric, interspecific pair distances which were below 0.5% belonged to the pairs of species shown in Table 3.3.

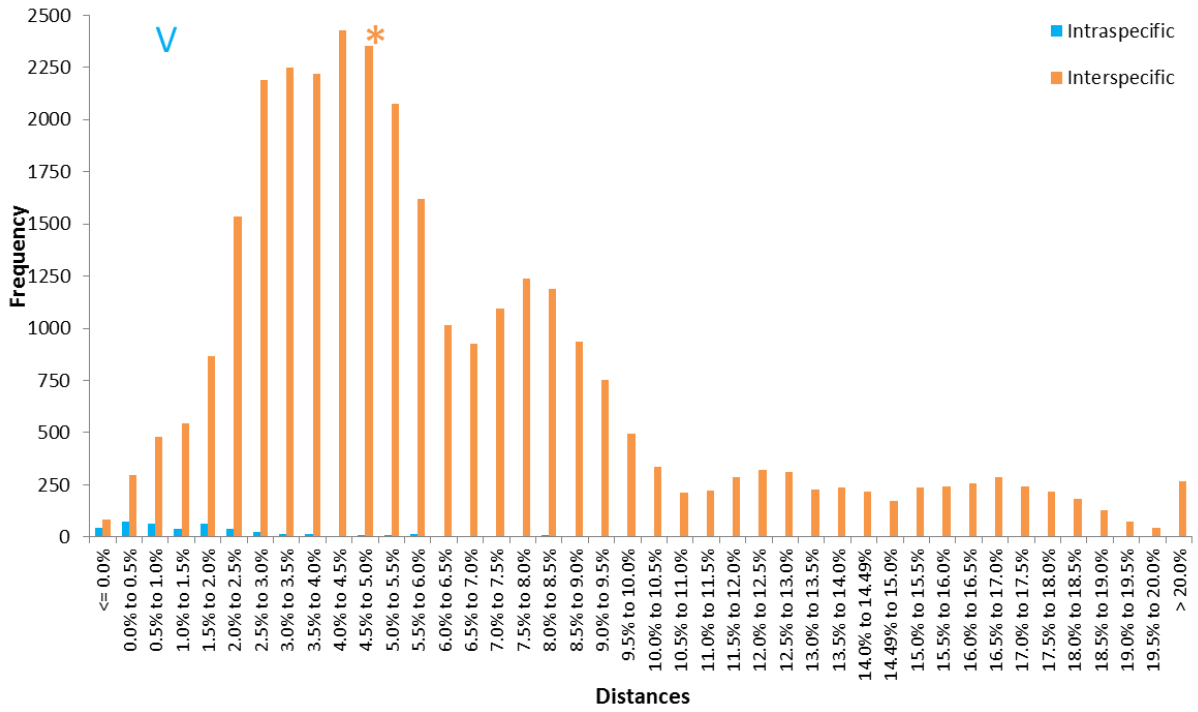

Figure 3.11: The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of 507 ITS samples. A blue chevron (V) marks the mean intraspecific variation per locus and an orange asterisk (*) marks the mean interspecific variation per locus.

Table 3.3: Congeneric, interspecific pair distances below 0.5%

Interspecific pairs below 0.5%	
P. clandestina	*P. dipsacacearum*
	P. xanthii
	P. plantaginis
	P. spiraeae
	P. filipendulae
	P. pannosa
	P. leucotricha
P. leucotricha	*P. amelanchieris*
	P. tridactyla
P. erigerontis-canadensis	*P. xanthii*
	P. plantaginis
	P. macrospora
P. plantaginis	*P. spiraeae*
	P. filipendulae
P. macularis	*P. spiraeae*
	P. filipendulae
P. aphanis	*P. spiraeae*
	P. filipendulae
P. fusca	*P. macrospora*
P. amelanchieris	*P. tridactyla*
P. mors-uvae	*P. pannosa*
G. cichoracearum	*G. sonchicola*
	G. orontii
E. aquilegiae	*E. buhrii*
	E. catalpae
	E. trifoliorum
	E. circaeae
E. buhrii	*E. circaeae*
	E. catalpae
E. alphitoides	*E. euonymicola*
	E. simulans
	E. akebiae
	E. tortilis
E. akebiae	*E. tortilis*
	E. euonymicola
E. euonymicola	*E. tortilis*
E. trifoliorum	*E. pisi*

3.4: Discussion

3.4.1: PM identification

Numerous host plants have only a single PM species recorded to parasitise them. In such cases it was tempting to accept any PM present must be of that identity. However, due to the fast-evolving nature of PM species it was important to ensure that this was indeed the case.

With many different features possible to examine, morphological analyses can be extremely effective (Braun & Cook, 2012). However, morphological analyses of these features will often require samples to be in optimum condition; fresh and with both anamorph and teleomorph available for examination. Such characteristics were often not available because of lag time between sample collection and examination (while samples were in transit), collection by non-experts, and the tendency of PMs to reproduce asexually the majority of the time and sexually only when adverse conditions approach, meant that teleomorphs were rarely available for examination.

Regardless of potential shortcomings, the identification of PM accessions via morphological analyses has proven its utility; having enabled identifications to genus and species levels. Anamorphic features such as conidiogenesis type and conidial characteristics were readily available and characterisation enabled immediate delimitation of potential species. Similarly, teleomorphic feature characterisation aided in delimitation. However, teleomorphs were observed in just 20% of samples and thus cannot be relied upon.

With the necessary time, apparatus, knowledge and quality of sample fungal morphology has been shown to enable fungal identification. Indeed, these studies underpin mycological taxonomy. This was proven by the identification via morphology of *P. macrospora* on *Heuchera* cvs. (Ellingham *et al.*, 2016). The resultant sequence data will enable its future identification to be made exclusively via molecular comparisons. This has served to further highlight the necessity for morphological characterisation of taxa as an important baseline. This will remain critical as molecular characterisation continues. However, the eventual characterisation of multiple regions of

all PM species is an achievable aim. This has the potential to greatly reduce the ambiguities that have remained in 35% of cases in the present study after morphological characterisation

Sequencing of the ITS enabled augmentation of morphological analyses. In 33 cases (6.5% of sequenced samples) ITS analyses yielded contradictory results to those provided by morphological analyses. For ten of these samples ITS analyses identified species previously recorded on the host plant. This adds weight to the possibility of presence of more than one PM on the host plant (Cook *et al.*, 2006); a single species viewed using the microscope, and a separate species amplified and sequenced. The possibility of accidental mixing of samples was regularly ruled out as samples with questionable results were reanalysed. In such cases, multiple species were noted on stored Fungarium samples. Even so, this combination of techniques resulted in samples being identified to species 80% of the time and to genus 95% of the time and is consistent with literature showing the need for an additional method for discrimination of closely related PM species (Meeboon & Takamatsu, 2015a, Meeboon & Takamatsu, 2015c, Meeboon & Takamatsu, 2015b, Pirondi *et al.*, 2015, Takamatsu *et al.*, 2015a). Confusion regarding PM species names was particularly evident when BLAST of the ITS was used for identification (Kovács *et al.*, 2011). The result provided numerous different species of a single genus as well as additional samples of unrelated Fungi. Examples of unrelated fungal results yielded from BLAST were: *Albugo laibachii* (99% identity, 97% query cover) for a sample of *G. cichoracearum*, *Helotiales* (86%, 98%) for a sample of *G. sordidus*, *Neofabraea* (97%, 99%) for a sample of *G. cynoglossi*, and *Tetracladium* (98%, 91%) for a sample of *Podosphaera*. These are likely to have been amplified and sequenced from environmental samples and incorrectly identified. They are therefore difficult to trust.

Schoch *et al.* (2014) investigated this shortfall of sequence databases, advising caution in reliance upon BLAST due to 'dark taxa' (Page, 2013, Page, 2016) and inaccuracy of naming (Nilsson *et al.*, 2006, Bidartondo, 2008). Schoch *et al.* (2014) summarised that DNA sequence data should be tied to correct taxonomic names and clearly annotated specimen data (Wieczorek *et al.*, 2012). Such

standards have been followed where possible in the current study as PM identification utilised currently accepted techniques alongside additionally informative regions, and specimens were preserved and stored in RNG (University of Reading Herbarium).

3.4.2: Phylogenetic analyses

The ITS repeat region has been used extensively in fungal identification and phylogenetic reconstructions (White *et al.*, 1990, Bruns *et al.*, 1991, Lieckfeldt & Seifert, 2000, James *et al.*, 2001). The use of ITS in the PMs (Hirata & Takamatsu, 1996, Wang *et al.*, 2013) is therefore unsurprising.

The weaknesses of the ITS region have been shown in the literature (Takamatsu *et al.*, 2015a) and confirmed in this chapter. Groupings of different species into inseparable clades is due to the lack of DNA sequence variation inherent in the ITS. Other species, grouped polyphyletically, such as *G. cichoracearum* and *G. cynoglossi* must be explained differently. *G. cichoracearum s. lat.* has been recorded previously as heterothallic (Schnathorst, 1959b), occurring on several hosts (Lebeda & Mieslerová, 2011), and grouped polphyletically (Matsuda & Takamatsu, 2003). Data of such previous groupings have remained artefacts in current PM knowledge. The characterisation of individual groups such as *G. sonchicola*, *G. verbasci*, and *G. asterum*, formerly known as *G. cichoracearum* is therefore another important aspect of the present study.

Resolution of species is an important goal of taxonomists (Gürtler & Stanisich, 1996, CBOL Plant Working Group *et al.*, 2009, Schoch *et al.*, 2009, Medina *et al.*, 2011, Ratnasingham & Hebert, 2013). Within fungal taxonomy it has been stated that species resolution equates to the discovery, description, and classification of all species of Fungi; providing tools for their identification along the way (Hibbett *et al.*, 2011). In order to achieve this, a mixture of broad- and narrow-scale studies is required, and analyses of particular narrow clades, such as the Erysiphales, require greater focus. Methods for greater resolution of newly-evolved, phylogenetically-close species are available and have been trialled within the Animalia and Plantae (Savolainen *et al.*, 2000, Meier *et al.*, 2006), as well as the Fungi (Taylor *et al.*, 2000, Reeb *et al.*, 2004, Tretter *et al.*, 2013, Zelski *et al.*, 2014). This

can be as simple as amplifying more variable parts of a genome. However, any recommended region must offer additional information to that already provided by the ITS. The regions flanking the ITS, particularly the 28S region, have been trialled and since adopted by certain researchers (Meeboon & Takamatsu, 2015a, Takamatsu *et al.*, 2015b), however this has resulted in only slight improvements and polytomies remain in PM phylogenies. The search for new regions is well documented in certain clades (Tretter *et al.*, 2013, Tretter *et al.*, 2014b) but negative results are rarely shared (Fanelli, 2011) and thus little progress has been made within the PMs; identical regions may be trialled by different researchers with similar negative outcomes.

However, the clear discrimination of all PM tribes, genera, and numerous species in this chapter and numerous papers to date confirm the strength and utility of ITS for Order-wide reconstruction of the relationships of hundreds of species. The identification of regions to complement or replace the ITS will enable a shift towards concatenated alignments (Medina *et al.*, 2011, Tretter *et al.*, 2014a) resulting in increasingly accurate phylogenies of species trees rather than single-gene trees (Mallo & Posada, 2016).

3.4.3: DNA barcoding analyses

The K2P distribution graphs illustrate the intra and interspecific distances per locus corresponding to the barcoding gap (Hebert *et al.*, 2003a). A useful barcoding locus should have no overlap between intra and interspecific K2P distances (Quaedvlieg *et al.*, 2014). The analysis of ITS did not produce this gap. This contrasts with BI analyses which form numerous monophyletic groupings of distinct taxa and highlights the limitations of the barcoding technique (Rubinoff, 2006, Valentini *et al.*, 2009). Failing a clear barcoding gap, a low K2P overlap is desired. The ITS has been proposed as a universal DNA barcode marker for Fungi (Schoch *et al.*, 2012), the result of this chapter shows that although it is a good candidate for the PMs, the K2P overlap means that additional regions are required in order to further delimit closely related species. Similar DNA sequences of PM species within the ITS may be the result of clade barcodes mixing with cases where species barcodes are present. This result is

congruent with that of Quaedvlieg *et al.* (2014) who compared the ITS region with the ITS large subunit (LSU), β-tubulin, Actin, the second largest subunit of RNA polymerase II (*RPB2*), elongation factor 1-α (EF1-α), and Calmodulin for identification of the *Teratosphaeriaceae*. Both ITS and LSU were shown to have a higher K2P overlap than the other five loci tested and were therefore less suitable to serve as reliable identification loci. Numerous other studies have attempted to improve on ITS results (Reeb *et al.*, 2004, Roe *et al.*, 2010, Groenewald *et al.*, 2013) using other DNA regions, with the LSU, small subunit (SSU), β-tubulin, Actin, *RPB2,* EF1-α, Mcm7, histone H3 gene (HIS), Chitin synthase (*Chs*), and Calmodulin regions regularly being trialled. However, within the PMs, few of these have been recorded in publications and only the ITS, LSU, SSU, and flanking regions of the ITS, D1 and D2, are used for phylogenetic reconstruction. More work is required in this field as there is currently no other region to compare this result with.

3.5: Conclusions

The use of morphological analyses for the identification of PM fungi set a strong foundation which has since been complemented with rDNA ITS sequence data. The abundance, diversity, and quality of PM accessions received from the citizen science scheme have proven sufficient for testing these established identification techniques and it has been possible to consistently discriminate between PM tribes, genera, and a high proportion (ca. 80%) of PM species. The ITS region should be augmented with additional markers in order to improve analyses for fungal identification such as phylogenetic reconstruction and DNA barcoding. Implementation of such methods for species identification, alongside proactive testing of plant material entering the country, will enable species to be monitored with greater ease, efficiency, and accuracy. In turn, limiting the spread of potentially detrimental species.

Chapter 4: Augmenting current ID techniques with novel gene *Mcm7*

4.1: Introduction

The advent of fungal phylogenomics, gave rise to an increase in the use of single-copy protein-coding genes for resolving deep or species-level phylogenies by fungal systematists (Aguileta *et al.*, 2008, Schmitt *et al.*, 2009, Curto *et al.*, 2012). Low-copy number nuclear genes, those which are not repeated or repeated only a few times, are useful because of their sometimes rapid evolutionary rate (Sang, 2002, Small *et al.*, 2004, Choi *et al.*, 2006). However, given the high evolutionary rate inherent in low-copy regions, developed markers may not work consistently through evolutionary time as the sites at which amplification begins are also subject to evolution and therefore may change. In cases of rapid speciation, genomic DNA may not have diverged sufficiently to resolve a phylogeny using a single locus (Beltrán *et al.*, 2002, Seehausen *et al.*, 2003). This has been shown to be the case in numerous clades (Reeb *et al.*, 2004, Raja *et al.*, 2011, Morgenstern *et al.*, 2012), including the PMs (as shown in Chapter 3 and other PM publications including: Heluta *et al.* (2010), Kabaktepe *et al.* (2017)). However, multiple independent loci can often provide the necessary variability for reliable species identification via phylogenetic analyses and DNA barcoding (Beltrán *et al.*, 2002, Sang, 2002) due to the greater level of sampling of the genome. New, easy to use, computer software and analytical phylogenetic methods have been developed to provide the capability for analyses using concatenated datasets (Murphy *et al.*, 2001, Li *et al.*, 2007, Rowe *et al.*, 2008, Edwards, 2009).

Primers can be developed for amplification and sequencing of low-copy, highly variable regions providing relevant sequence data is available. This may come from sequenced genomes or through the use of generic primers, sourced from studies on closely related clades, for a targeted region. In the case of the PMs, potentially useful, understudied regions, can be mined from four published genomes (*Blumeria graminis* (Spanu *et al.*, 2010), *Erysiphe necator* (Jones *et al.*, 2014), and *Erysiphe pisi* and *Golovinomyces orontii* (Max Planck Institute for Plant Breeding Research)). Through

analyses of alignments of the PM genomes alongside closely-related Ascomycota, primers can be developed for exclusive amplification of PMs.

Progress in this field has been largely driven by phylogenetic reconstructions of the PMs spanning the Erysiphaceae (Takamatsu *et al.*, 2008a, Takamatsu *et al.*, 2008b, Takamatsu, 2013b) and increasingly specific taxonomic levels (Inuma *et al.*, 2007). Understanding of the evolution and relatedness of PM species has progressed from purely morphological observations to complementary studies of morphology and genomic DNA, and allowed phylogeographic theories to be developed and repeatedly tested (Brewer & Milgroom, 2010, Troch, 2012, Takamatsu *et al.*, 2016). Remaining discrepancies among phylogenies of the ITS region are highlighted in the literature (section 1.5) and confirmed in this study (section 3.3). The use of additional DNA regions in tandem with ITS can increase phylogenetic resolution and stability at multiple taxonomic levels. A standard, broadly applicable, set of sequence markers would be a valuable resource in constructing robust PM phylogenies using only a few loci (Schmitt *et al.*, 2009) without the expense of whole genome sequencing, while also informing studies of other fungi.

Numerous different regions have been used routinely to identify fungal species and to infer evolutionary relationships within the ascomycete fungi. However, the protein-coding genes most commonly used, such as the β-tubulins, the elongation factor EF1-α, the γ -actin, heat shock proteins, chitinases, chitin synthases, RNA polymerases, dehydrogenases, and histones were not found in the list of the best-performing genes, for accurate phylogenetic reconstruction and discrimination of species, when tested against 246 single-copy orthologous genes extracted from 30 fungal genomes (Aguileta *et al.*, 2008). Two single-copy orthologues, *Mcm7* and *Tsr1* (reviewed in chapter 6), outperformed all others in the study of Aguileta *et al.* (2008). These were trialled, alongside gene regions established for use in other fungal clades, for the PMs in this study.

Mcm7 (minichromosome maintenance protein (Schoch *et al.*, 2012)) is a gene coding for the replication licensing factor required for DNA replication, initiation, and cell proliferation (Moir *et al.*,

1982). The protein encoded by this gene is one of the highly conserved mini-chromosome maintenance proteins (MCM) that are essential for the initiation of eukaryotic genome replication (Kearsey & Labib, 1998). Since 2008 (Aguileta *et al.*) the phylogenetic utility of *Mcm7* has been tested across the Ascomycota (Raja *et al.*, 2011), and more specifically in the Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes and Sordariomycetes (Schmitt *et al.*, 2009), *Xanthoparmelia* (Leavitt *et al.*, 2011), *Montanelia* (Divakar *et al.*, 2012), thermophilic fungi (Morgenstern *et al.*, 2012), *Geomyces* (Minnis & Lindner, 2013), the Kickxellomycotina (Tretter *et al.*, 2013, Tretter *et al.*, 2014b), and the Caliciaceae (Prieto & Wedin, 2016), amongst others.

In this chapter the possibility of developing working markers for the *Mcm7* region for PMs was investigated and the value of resultant data for phylogenetic reconstruction explored. The resolution of this region in DNA barcoding studies was compared with the standard ITS region.

4.2: Materials and methods

4.2.1: Sample collection – The Powdery Mildew Citizen Science Scheme

Samples were collected via the powdery mildew citizen science scheme (Chapter 2). One hundred and ninety-five of these were successfully amplified (Appendix 5) in the study outlined in this chapter.

4.2.2: Putative species identification

The techniques outlined in Chapters 2 and 3 formed the basis of the identities of PM species used in this chapter.

4.2.3: DNA extraction

The DNA extracted for initial PM species identification (Chapter 3) was used for these further analyses.

4.2.4: Data mining for *Mcm7* molecular markers

PM genomes (Max Planck Institute for Plant Breeding Research, Spanu *et al.*, 2010, Jones *et al.*, 2014) were aligned with 22 closely related species (Table 4.1) in order to identify the location of the *Mcm7* region and then develop primers to trial in the amplification of samples. Primers were designed manually with the aid of the web-based software Primer-BLAST (Ye *et al.*, 2012) and Primer3 (Untergasser *et al.*, 2012). The general primer-choice conditions were set for an optimal primer of approximately 20 bases, annealing temperature (Tm) of 60 °C, and GC % of around 50. Primer pairs were developed to produce amplicons with expected lengths of 400-600 bp. Some degenerate base pairs were necessary in order to match the diversity of PMs. To ensure the specificity of markers to PMs, potential marker sequences were compared with the alignment of nucleotide sequences using the search function in BioEdit 7.2.5 (Hall, 1999). If these matched sequences of non-PM samples, they were discounted. Particular attention was paid to the 3′ end of primers to ensure they

consisted of a sequence unique to the PMs (Innis *et al.*, 1990). A total of 13 primers (five forward, eight reverse) were designed (Table 4.2) and ordered from Sigma-Aldrich.

Table 4.1: *Mcm7* sequences imported from GenBank for identification of *Mcm7* region in PM genomes and design of PM specific primers

Species	**GenBank Accession No.**
Bisporella citrina	JN672971.1
Chalara sp.	KM495490.1
Chalara sp.	KM495491.1
Chlorencoelia torta	JN672985.1
Cudoniella clavus	JN672988.1
Geomyces destructans	KF212372.1
Geomyces sp.	KF212363.1
Graddonia coracina	JN672993.1
Hymenoscyphus fructigenus	JN672997.1
Lachnellula sp.	JN673005.1
Lambertella hicoriae	KF545473.1
Lambertella subrenispora	KF545466.1
Lambertella viburni	KF545443.1
Lanzia sp.	KF545444.1
Leotiomycetes sp.	KF545450.1
Leotiomycetes sp.	KF545458.1
Leotiomycetes sp.	KF545474.1
Poculum sydowianum	KF545465.1
Rutstroemia cunicularia	KF545445.1
Rutstroemia firma	KF545461.1
Strossmayeria basitricha	JN673019.1
Vibrissea filisporia f. *filisporia*	JN673023.1

Table 4.2: Primers designed for trialling amplification and sequencing of *Mcm7* region of PMs

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
Mcm7F1	F	ACVTGTGATCGRTGYGGDTGTG	22	63.08	54.55
Mcm7F2	F	TGTGATCGRTGYGGDTGTGA	20	59.03	50
Mcm7F3	F	ACYTWYGSRCCMCTWAMYGAATG	23	59.01	43.48
Mcm7F4	F	CCMCTMAMYGAATGYCCHTC	20	56.83	50
Mcm7F5	F	CAACTRCAYCAYTCWACYCG	20	56.17	50
Mcm7R1	R	ATWGCYTTRAATCCDGTATA	20	51.55	35
Mcm7R2	R	AGRTATTCGTARATRTGTCC	20	48.6	35
Mcm7R3	R	TTGCKAGRTATTCGTARAT	19	50.89	36.84
Mcm7R4	R	TTGCKAGRTATTCGTARATRTGTCC	25	59.77	40
Mcm7R5	R	TGSCCATAWATTTCHGGRGCRATKGA	26	65.19	46.15
Mcm7R6	R	TGSCCATAWATTTCHGGRGC	20	58.51	50
Mcm7R7	R	CCATAWATTTCHGGRGCRATKGA	23	59.29	43.48
Mcm7R8	R	TCATYCCRTCRCCCATYTCYTTWG	24	62.57	50

4.2.5: PCR and sequencing protocol

PCR was carried out using the newly designed PM specific primers of the *Mcm7* region (Table 4.2). All 40 possible combinations of these were trialled in 25 µl mixes of 12.5 µl BioMix™ Red (Bioline), 0.75 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 ng μl^{-1}, 9 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}. Cycling parameters were adapted from Amrani and Corio-Costet (2006) with an initial denaturation step of 95 °C for five minutes, followed by 37 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for one minute, and elongation at 72 °C for one and a half minutes and a final elongation at 72 °C for five minutes. Four samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003) were initially trialled.

The PCR products were separated and visualised as in 3.2.4.

The four primer combinations exhibiting the highest amplification success (number of products x product strength) were Mcm7F1 and Mcm7R5, Mcm7F1 and Mcm7R7, Mcm7F2 and Mcm7R5, and Mcm7F2 and Mcm7R8. These were trialled at a gradient of annealing temperatures

from 52-62 °C. The most successful, single primer combination was Mcm7F2 and Mcm7R8 at an annealing temperature of 54 °C. This amplified a region of approximately 550 bp. Reducing the ambiguity of base pairs was trialled for greater accuracy in amplification and sequencing of the *Mcm7* region and resulted in the design of primers Mcm7F2a and Mcm7R8a (Table 4.3). Reducing primer length in order to increase sequencing success was also trialled, resulting in the design of primers Mcm7F2seq and Mcm7R8seq (Table 4.3). Application of these modified primers produced sequences of lesser quality (this is discussed in section 4.4.1: *Mcm7* amplification and sequencing). Primers Mcm7F2 and Mcm7R8 were therefore used in all future amplifications. Single amplicons were sequenced and assembled as in section 3.2.4.

Table 4.3: Primers adapted from Mcm7F2 and Mcm7R8 for more efficient amplification and sequencing of *Mcm7* products

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
Mcm7F2a	F	TGTGATCGGTGTGGGTGTGA	20	69.4	56
Mcm7R8a	R	TCATTCCGTCGCCCATTTCTTTWG	24	59.8	57.5
Mcm7F2seq	F	GADCAAGTNCCWGTDGG	17	50.3	53.9
Mcm7R8seq	R	GCYTCYAARTAAGTRTC	17	45.7	41.2

4.2.6: Sequence alignment

Sequence alignment of *Mcm7* data, and their complementary ITS sequences, was performed as in section 3.2.6. The dataset of 106 sequences of *Mcm7* for which there were ITS equivalents was concatenated using Mesquite (Maddison & Maddison, 2017). The alignment files of the *Mcm7* and the *Mcm7* combined with the ITS rDNA were deposited in TreeBASE as S20952.

4.2.7: Phylogenetic analyses

Phylogenetic analyses were performed as in section 3.2.7.

For BI of *Mcm7* the HKY+I+G model was used and was run for 10,000,000 generations. For BI of the ITS accessions for which there were *Mcm7* equivalents the GTR+I+G model was used and was run for 5,000,000 generations. For BI of *Mcm7* accessions for which there were ITS equivalents the

HKY+G model was used and was run for 5,000,000 generations. For BI of the combined dataset (ITS and *Mcm7*) the separate models of individual datasets were used for each region and were run for 5,000,000 generations, at a temperature of 0.1 in order to reach the optimal solution most efficiently.

4.2.8: DNA barcoding analysis

Accessions were renamed, to species where possible, according to results *Mcm7* phylogenetic analysis. Datasets of all *Mcm7* accessions, ITS accessions for which there were *Mcm7* equivalents, *Mcm7* accessions for which there were ITS equivalents, and concatenated ITS and *Mcm7* accessions were analysed and treated as in section 3.2.8.

4.3: Results

4.3.1: *Mcm7* amplification and sequencing

Nineteen of the 40 possible combinations of 13 primers (five forward and eight reverse) resulted in at least a single product from the five initial PM accessions trialled. The combination of Mcm7F2 and Mcm7R8 produced the most bands of greatest intensity (Figure 4.1). After optimisation of PCR, 84% of 299 trialled accessions resulted in a product being visualised using gel electrophoresis. Sequencing resulted in 187 of 251 (74.5%) accessions producing readable sequences. These were contributed to GenBank (Accession numbers KY786340 – KY786476 (presented in Appendix 5)). Sequencing worked in both forward and reverse directions, however forward sequences tended to be of poor quality. Those which were unsuccessful were characterised by weak reads, resulting in little or no sequence data, or messy reads, potentially contaminated with more than one PM species or additional conspecific fungi.

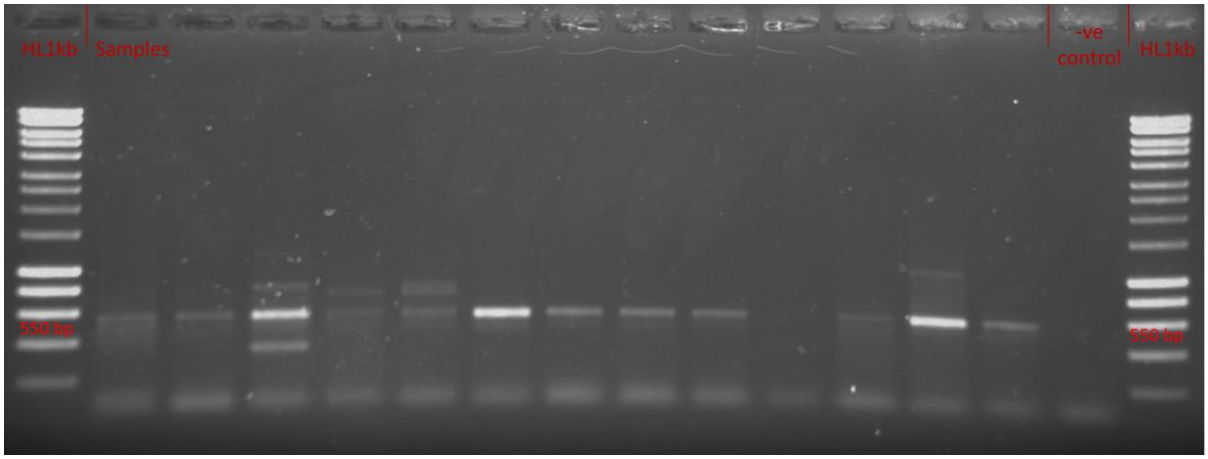

Figure 4.1: Amplification of 550 bp product of *Mcm7* with primers Mcm7F2 and Mcm7R8 with HyperLadder™ 1kb and negative control.

Samples were successfully amplified and sequenced from the *Blumeria, Podosphaera, Sawadaea, Arthrocladiella, Golovinomyces, Neoerysiphe,* and *Erysiphe*. Although DNA from accessions identified as *Phyllactinia* was amplified on two occasions, sequencing was never successful for the four accessions in the collection.

GenBank sequence data from the *Mcm7* region of PMs is solely based upon available PM genomes (*B. graminis, G. orontii, E. pisi,* and *E. necator*). This dearth of GenBank data meant that identifications of PM samples based on NCBI GenBank Nucleotide BLAST were not possible. When BLAST was optimised for finding 'highly similar sequences' searches returned 'no significant similarity' 39% of the time (73 out of 187). All other sequences matched the *Mcm7* region of various Ascomycetous fungi (including *Botrytis*, *Chlorociboria*, *Collema*, *Cosmospora*, *Cudoniella*, *Lambertella*, *Lobothallia*, *Pertusaria*, *Strossmayeria*, *Tetrapisispora*, and *Trapelia*) with identities and query covers ranging from 20-99% of the submitted sequence.

4.3.2: Sequence alignment

All 187 sequences were included in the initial sequence alignment. This was reduced to 151 sequences as a result of poor sequence quality and short sequence reads. Included in this reduction were the only four sequences of *Sawadaea Mcm7* DNA. Alignment resulted in a region of 604 bp. This was trimmed to 495 bp in order to remove gaps and poor quality sequence reads near the primer sites and leave sequences of equal size for later analyses. The region was 38.8% conserved. This compared to 75.6% in the ITS. There were 106 accessions with both *Mcm7* and ITS sequences. *Mcm7* sequences were concatenated with ITS (810 bp) for analysis resulting in 1315 bps.

4.3.3: Phylogenetic analyses

4.3.3.1 Mcm7

BI of PM phylogeny using 151 samples of the 505 bp region within the *Mcm7* region resulted in clear discrimination of each PM genus. Support for the overall topology was high. Tribes Cystotheceae (PP 99%) and Erysipheae (PP 100%) were monophyletic (Figure 4.2 and Figure 4.3). However, genera of the Golovinomyceteae were grouped paraphyletically: *Arthrocladiella* and *Golovinomyces* shared an exclusive common ancestor but the *Neoerysiphe* were grouped as sister to the Erysipheae tribe. The node separating *Neoerysiphe* and Erysipheae from *Arthrocladiella* and *Golovinomyces* had a PP of 92%. Each genus included in the sampling proved to be monophyletic and had high posterior

probability: *Podosphaera* (PP 100%), *Arthrocladiella* (PP 99%), *Golovinomyces* (PP 100%), *Neoerysiphe* (PP 99%), and *Erysiphe* (PP 99%).

Within the Cystotheceae (clade A) species of *Podosphaera* were shown to be distinct from each other. Samples 5_13 and 5_47R found on *Malus* sp. showed themselves to be closely related. This highlighted a previously incorrect identification; the closely related yet distinct species *P. leucotricha* and *P. clandestina* generally show a clear host divide, growing on species of the tribe Maleae and the genus *Crataegus* respectively. *Podosphaera* species occurring on *Taraxacum officinale* were not previously identified to species level due to the similarities of potential species *P. xanthii* and *P. erigerontis-canadensis*. They formed a cluster here which included sample 6_44 *P. plantaginis* on *Plantago lanceolata* and because of the single host and monophyletic clustering were identified conclusively as *P. erigerontis-canadensis*. Monophyletic groups are listed in Table 4.4.

Within the *Golovinomyces*, seven of the nine species included in the analysis were shown to be monophyletic (Table 4.4). Sample 5_267 on *Monarda didyma* was previously unidentified as it could have been either *G. biocellaris* or *G. cichoracearum.* As it is grouped monophyletically with *G. depressus* ex *Salvia officinalis* and separate from *G. cichoracearum* accessions, it is now identified as *G. biocellaris*. *G. cynoglossi* appears to be polyphyletic, although the accessions form host-specific monophyletic groups: two separate groups ex *Myosotis* sp., of two and three accessions respectively, were monophyletic while three of the four remaining accessions (on *Pulmonaria, Symphytum,* and *Silene*) formed a separate monophyletic group. These could be two cryptic species; taxa that are morphologically identical to each other but belong to different species.

Neoerysiphe separated into four monophyletic species (Table 4.4). The varying accessions of *N. galeopsidis* showed no separation according to host species.

Tribe Erysipheae (clade C) separated into individual species. Of the 18 putative species included in the analysis, 13 were shown to be monophyletic (Table 4.4). The *E. aquilegiae* group, 18 accessions (PP 100%), was paraphyletic; containing two other species (*E. catalpae* and *E. circeae*)

within it. There was no clear difference between var. *aquilegiae* found on *Aquilegia* sp. and *Caltha palustris* and var. *rannunculi* found on *Delphinium* sp. and *Rannunculus repens*. Further separations were observed as the *Mcm7* region showed phylogenetic difference between *E. heraclei* on *Heracleum* (PP 93%) and *Anthriscus* (PP 16%). *E. prunastri* and *E. necator* were distinct and quite separate from the rest of the tribe. Species *E. hyperici*, *E. ludens*, and *E. trifoliorum* are known to be species with near identical appearance. It was not possible to show clear separation between these using ITS (section 3.3) however this is improved by the *Mcm7* region; *E. trifoliorum* and *E. hyperici* are monophyletic while *E. ludens* is paraphyletic around the *E. hyperici* sample.

Sample 5_193R on *Amelanchier lamarckii* was identified as *Podosphaera amelanchieris* after morphological and ITS analyses. Its position in the Mcm7 phylogeny showed it to be amongst the *Golovinomyces* and specifically within the *Golovinomyces sordidus* species complex.

Long branches arose for accessions 5_14 Podosphaera_clandestina, 5_90R Podosphaerea_morsuvae, and 4_13R Erysiphe_alphitoides. Each of these accessions was grouped within the appropriate genus, and 4_13R Erysiphe_alphitoides within the appropriate species complex. These were excluded from barcoding analyses due to their clear difference from their closely related species.

Particular identifications made after morphological and ITS analysis and after *Mcm7* analysis were disparate. These were: 5_160 on *Taraxacum officinale* (identified as *G. cichoracearum* after morphological and ITS analysis, but as a *Podosphaera* species after amplification of *Mcm7* DNA), 5_179 on *Plantago lanceolata* (identified as *P. plantaginis* after morphological and ITS analysis, but as *G. sordidus* after amplification of *Mcm7* DNA), and 5_193 on *Amelanchier lamarckii* (identified as *P. amelanchieris* after morphological and ITS analysis, but as a *Golovinomyces* species after amplification of *Mcm7* DNA), and 5_249 on *Plantago major* (identified as *E. cruciferarum* after morphological and ITS analysis, but as *G. sordidus* after amplification of *Mcm7* DNA).

Table 4.4: Monophyletic PM groups from *Mcm7* data within the Erysiphales.

Species within group	Number of Accessions	PP of group*
P. macrospora	1	
P. erigerontis-canadensis	3	55
P. plantaginis	1	
P. leucotricha	2	100
P. euphorbiae-helioscopiae	1	
P. dipsacearum	1	
P. epilobi	1	
A. mougeotii	1	
G. depressus	3	100
G. cynoglossi	2	100
G. biocellaris	2	100
G. cichoracearum	1	
G. magnicellulatus	3	100
G. cynoglossi	4	64
G. sonchicola	4	100
G. verbasci	1	
G. cynoglossi	3	78
G. orontii	1	
G. sordidus	9	52
P. amelanchieris	1	
N. galii	3	100
N. geranii	5	100
N. nevoi	5	100
N. galeopsidis	17	100
E. prunastri	1	
E. necator	1	
E. hedwigii	1	
E. lonicerae	4	92
E. aquilegiae	15	100
E. catalpae	1	
E. circeae	1	
E. platani	1	
E. elevata	1	
E. alphitoides	13	99
E. euonymicola	6	
E. cruciferarum	3	100
E. berberidis	4	100
E. ludens	2	100
E. hyperici	1	
E. trifoliorum	5	
E. buhrii	1	
E. heraclei	11	99

*Groups with a single accession have no PP and are shaded in grey.

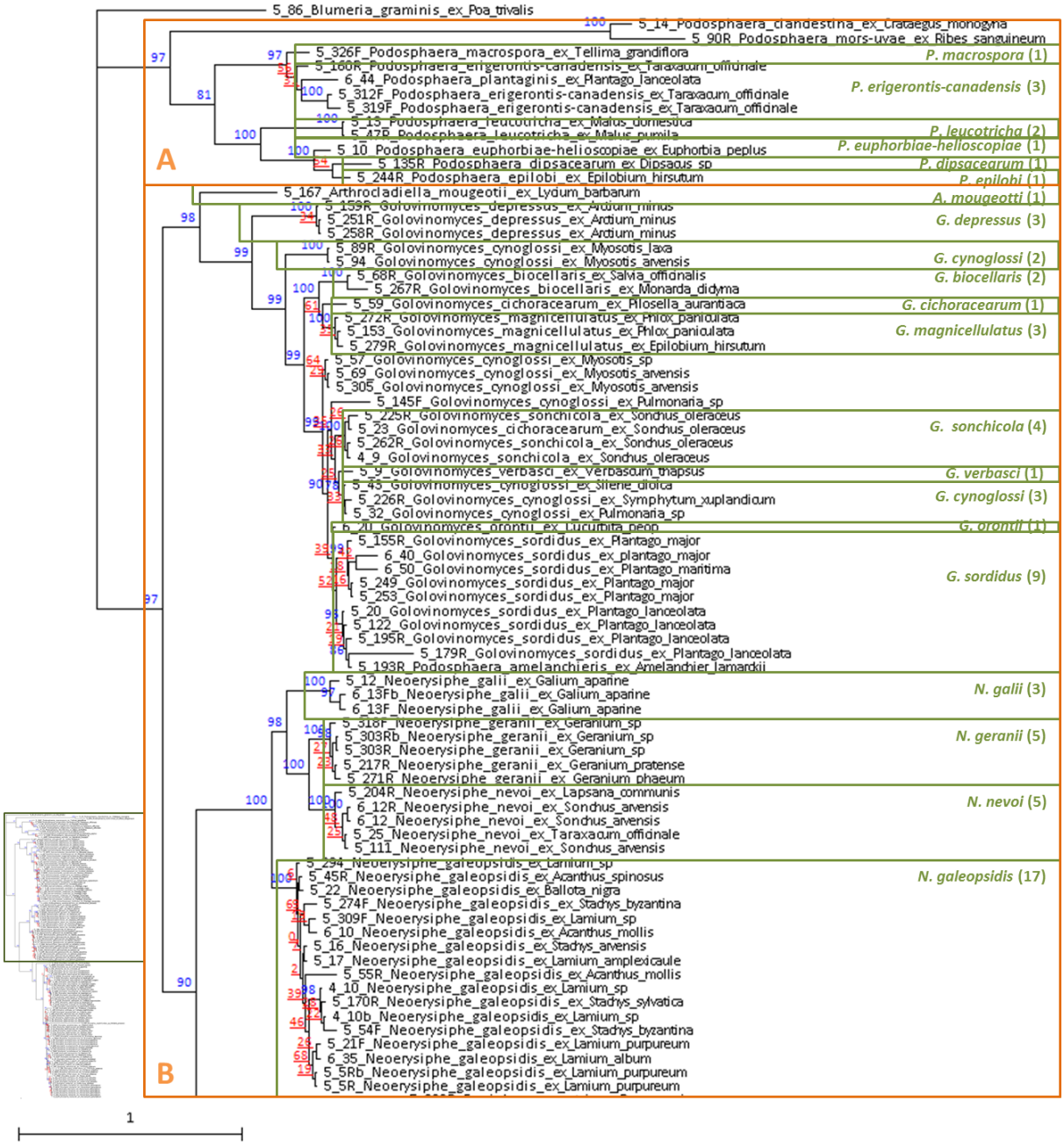

Figure 4.2: Part 1 of BI using 151 sequences of the *Mcm7* region. Accession names include sample code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

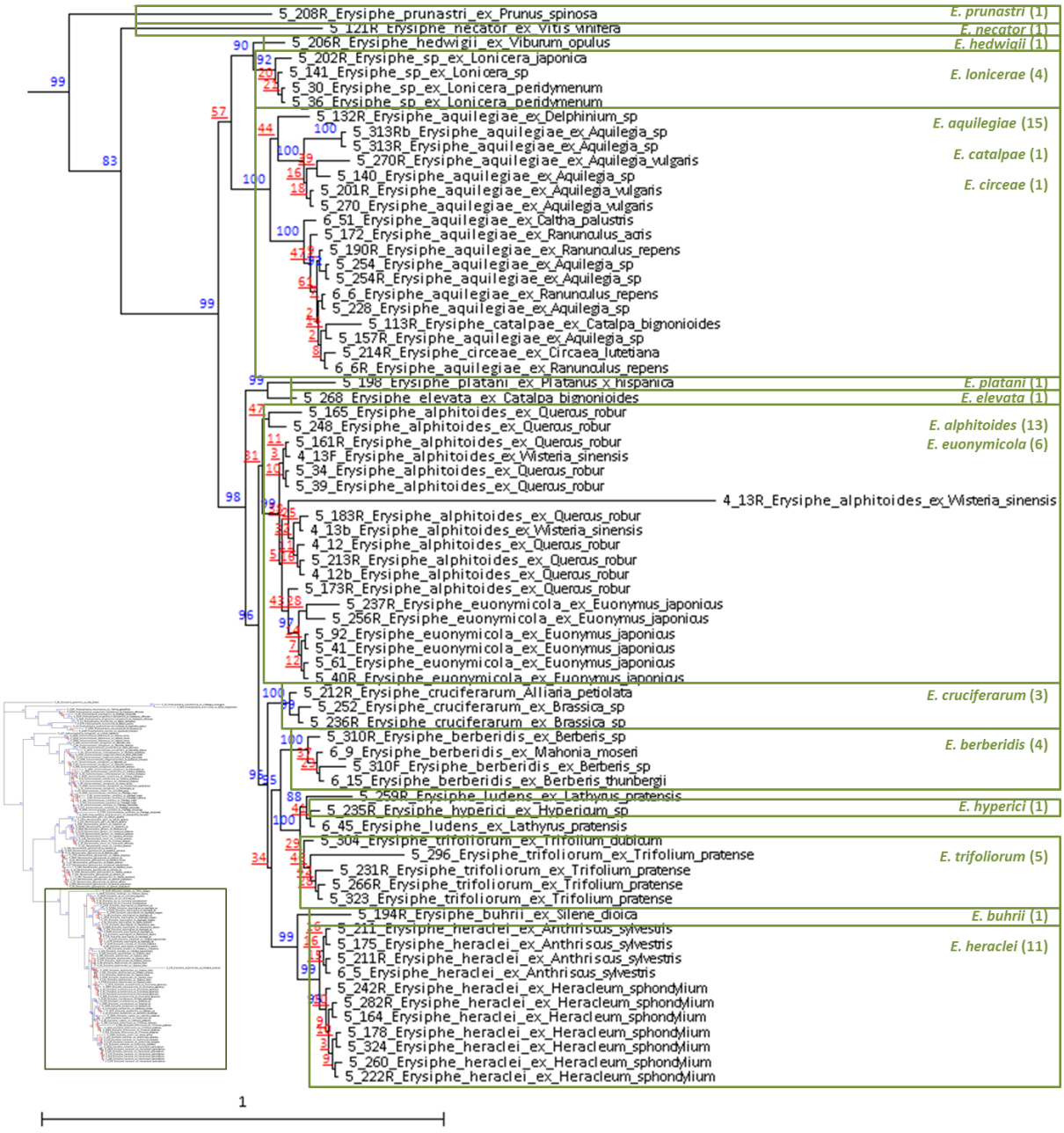

Figure 4.3: Part 2 of BI using 151 sequences of the *Mcm7* region. Accession names include sample code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny.

4.3.3.2 Direct comparison of ITS and *Mcm7*

BI of the PM phylogeny of 106 accessions of the *Mcm7* region (Figure 4.5) resulted in an overall topology similar to that of the BI of the PM phylogeny of 106 accessions of the ITS region (Figure 4.4). The main differences came in the positioning of genera within the Golovinomyceteae (clade B). The *Mcm7* phylogeny was similar to that of section 4.3.3.1 and as such *Arthrocladiella*, *Golovinomyces*, and *Neoerysiphe* were grouped paraphyletically with the *Neoerysiphe* being sister to the Erysipheae tribe. The ITS phylogeny showed Golovinomyceteae to be monophyletic (PP 92%). Both regions showed the *Neoerysiphe* within this tribe to be monophyletic and individual species were separated. The ITS phylogeny showed *Arthrocladiella* to be grouped between *G. depressus* and the remaining *Golovinomyces* species.

Comparison of the ITS and *Mcm7* trees confirmed their relative variability as branch lengths varied. The mean branch length was greater in the *Mcm7* tree than that of the ITS tree. This greater variation in sample sequences resulted in a shorter scale bar, longer individual branches and fewer polytomies in the *Mcm7* tree.

The differential identifications of sample 5_160 as *G. cichoracearum* with ITS and a *Podosphaera* species with *Mcm7*, 5_179 as *P. plantaginis* via ITS and *G. sordidus* via *Mcm7*, 5_193 as *P. amelanchieris* when using ITS and a *Golovinomyces* species when using *Mcm7,* and 5_249 as *E. cruciferarum* via ITS and *G. sordidus* via *Mcm7* resulted in their exclusion from the combined analysis. These accessions were renamed accordingly to their *Mcm7* identity for *Mcm7* TaxonDNA analyses.

Figure 4.4: BI using 106 sequences of the ITS region. Accession names include sample code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

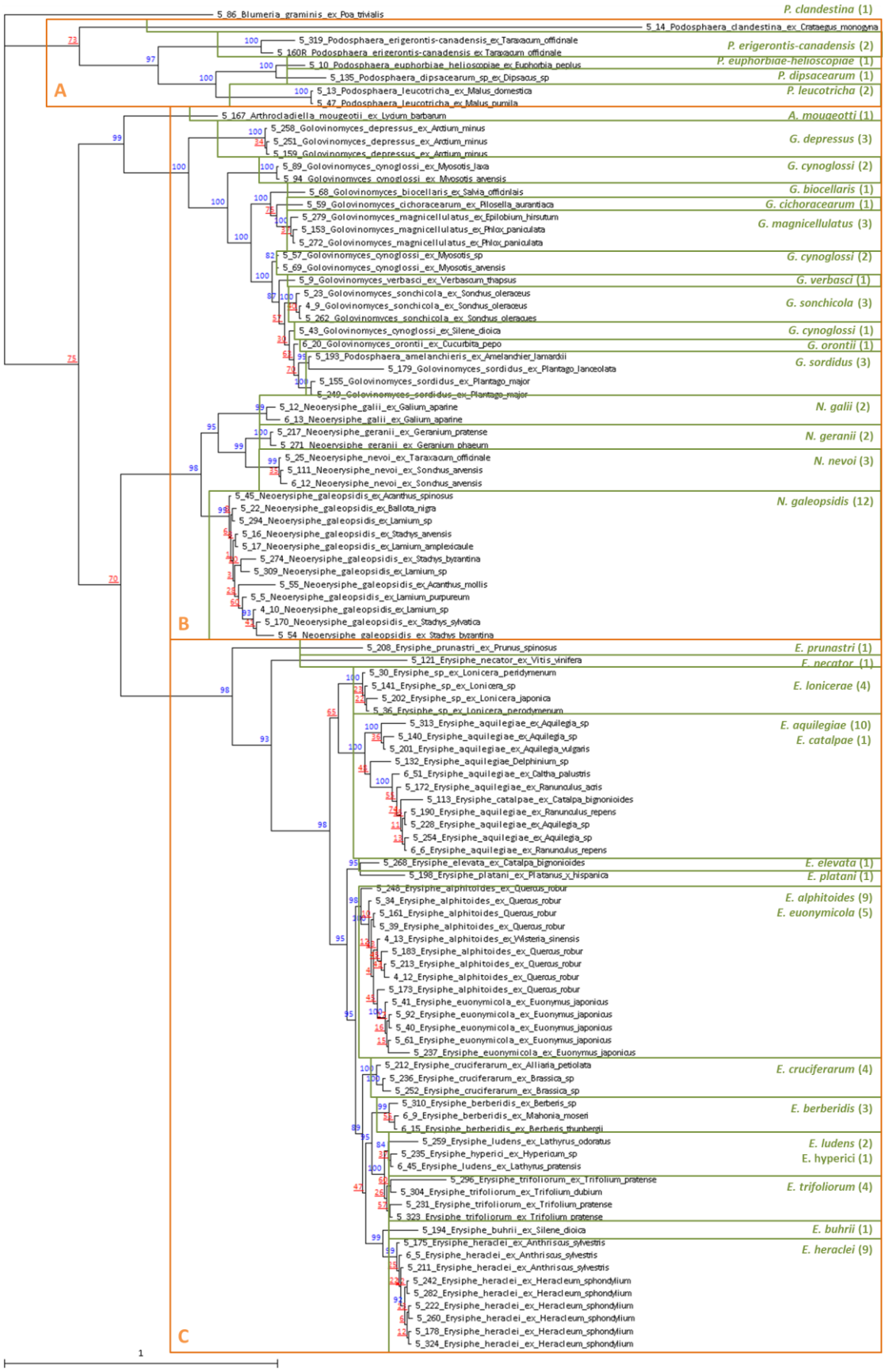

Figure 4.5: BI using 106 sequences of the *Mcm7* region. Accession names include sample code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

4.3.3.3 Combined ITS and *Mcm7* phylogeny

BI of PM phylogeny using 102 accessions of the combined 1315 bp region of the ITS and *Mcm7* regions resulted in clear discrimination of each PM genus. PP for the overall topology was high. Tribes Cystotheceae (Clade A) and Erysipheae (Clade C) were monophyletic, both with PP of 99% (Figure 4.6). However genera within the Golovinomyceteae (Clade B) tribe (*Arthrocladiella*, *Golovinomyces*, and *Neoerysiphe*) were paraphyletic: each genus was monophyletic but *Arthrocladiella* and *Golovinomyces* were paraphyletic to *Neoerysiphe* which was sister to the Erysipheae tribe. The node separating *Neoerysiphe* and Erysipheae from *Arthrocladiella* and *Golovinomyces* had PP of 94%. Each genus included in the sampling was shown to be monophyletic as *Podosphaera* (PP 99%), *Arthrocladiella* (PP 99%), *Golovinomyces* (PP 96%), *Neoerysiphe* (PP 100%), and *Erysiphe* (PP 99%).

Within the *Golovinomyces*, eight of the ten different species included in the analysis were shown to be monophyletic (Table 4.5). *G. cynoglossi* arose two separate times. The polyphyletic nature of *G. cynoglossi* could be related to its hosts, although two individual groups of two accessions on *Myosotis* sp. arose monophyletically (PP 99% and 100%), with an additional accession on *Silene diocia* as sister to *G. sonchicola*. Once again this shows support for a cryptic species.

Neoerysiphe continued to separate into four monophyletic species (Table 4.5).

Of the the 15 putative *Erysiphe* species, 12 were shown to be monophyletic (Table 4.5). The *E. aquilegiae* group, 10 accessions (PP 99%), contained *E. catalpae*. There remained no clear difference between var. *aquilegiae* and var. *rannunculi*. Eight of the nine *E. alphitoides accessions* formed a monophyletic group. The ninth was polyphyletic to this group and sister to *E. euonymicola.* Similarly, one of the four *E. trifoliorum* accessions was grouped polyphyletically to the main group; sister to *E. hypericic* and *E. ludens*. Further separations were observed as the combined regions showed phylogenetic difference between *E. heraclei* on *Heracleum* (PP 100%) and *Anthriscus* (PP 100%).

Table 4.5: Monophyletic PM groups from *Mcm7* data within the Erysiphales.

Species within group	Number of Accessions	PP of group*
P. erigerontis-canadensis	1	
P. clandestina	1	
P. leucotricha	2	100
P. euphorbiae-helioscopiae	1	
P. dipsacearum	1	
A. mougeotii	1	
G. depressus	3	100
G. cynoglossi	2	100
G. biocellaris	1	
G. cichoracearum	1	
G. magnicellulatus	3	100
G. orontii	1	
G. sordidus	1	
G. verbasci	1	
G. cynoglossi	3	99
G. sonchicola	3	100
N. galii	2	99
N. geranii	2	100
N. nevoi	3	300
N. galeopsidis	12	98
E. prunastri	1	
E. necator	1	
E. lonicerae	4	100
E. aquilegiae *E. catalpae*	10 1	99
E. elevata	1	
E. platani	1	
E. alphitoides *E. euonymicola*	9 5	96
E. berberidis	3	100
E. trifoliorum *E. hyperici* *E. ludens*	4 1 2	100
E. buhrii *E. heraclei*	1 9	100

*Groups with a single accession have no PP and are shaded in grey.

Figure 4.6: BI using 106 sequences of the ITS and *Mcm7* regions combined. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

4.3.4: DNA barcoding analysis

4.3.4.1 Mcm7

Intra and interspecific differences were quantified for the 146 accessions and 40 species of the *Mcm7* region. This resulted in a total overlap of 17.88% (from 0.43% to 18.31%, covering 93.79% of all intra and interspecific but intrageneric sequences) (Figure 4.7a). Accessions with a mean of more than 5% intraspecific difference were *G. sordidus*, *E. aquilegiae*, and *E. trifoliorum*. There were 1,412 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (82.9%) and *Golovinomyces* (16.8%). The only interspecific pair below 0.5% difference was *E. aquilegiae* and *E. circeae*.

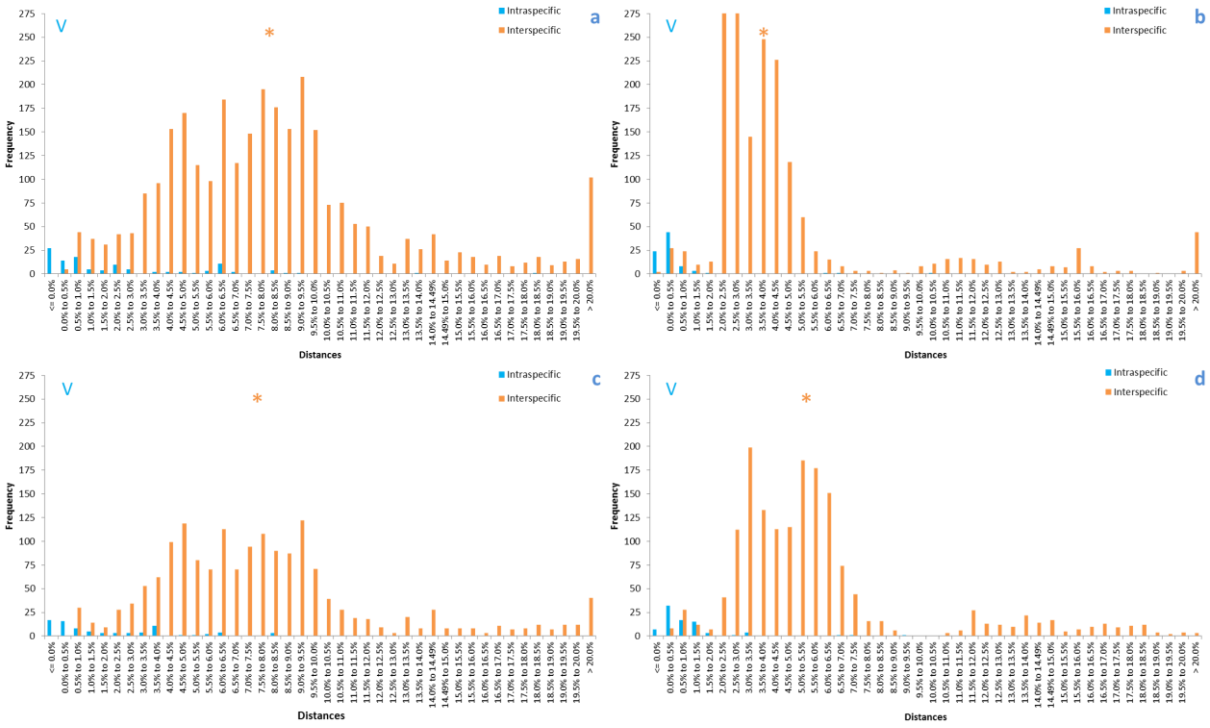

Figure 4.7: The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of (a) 146 *Mcm7* accessions, (b) 102 ITS accessions common to the ITS and *Mcm7* regions, (c) 102 *Mcm7* accessions common to the ITS and *Mcm7* regions, and (d) 102 accessions of the ITS and *Mcm7* regions combined. Blue chevrons (V) mark the mean intraspecific variation per locus and orange asterisks (*) mark the mean interspecific variation per locus.

4.3.4.2 Direct comparison of ITS, *Mcm7*, and combined datasets

4.3.4.2.1 ITS

Intra and interspecific differences were quantified for the 104 accessions and 35 species of the ITS region. This resulted in a total overlap of 10.42% (from 0.0% to 10.42%, covering 89.48% of all intra and interspecific but intrageneric sequences) (Figure 4.7b). The only accessions with a mean of more than 5% intraspecific difference were *G. cynoglossi*. There were 2,756 interspecific, congeneric distances which fell below 5%; 98% of these were between accessions of the *Erysiphe*. The interspecific pairs which were below 0.5% difference were: *E. aquilegiae* and *E. catalpae*, and *E. alphitoides* and *E. euonymicola*.

4.3.4.2.2 Mcm7

Intra and interspecific differences were quantified for the 103 accessions and 34 species of the *Mcm7* region. This resulted in a total overlap of 7.57% (from 0.61% to 8.18%, covering 61.82% of all intra and interspecific but intrageneric sequences) (Figure 4.7c). Accessions with a mean of more than 5% intraspecific difference were *E. aquilegiae*, *E. trifoliorum*, and *G. cynoglossi*. There were 896 interspecific, congeneric distances which fell below 5%; 91% of these were between accessions of the *Erysiphe*. *E. alphitoides* and *E. euonymicola*, *G. cynoglossi* and *G. sonchicola*, and *G. cynoglossi* and *G. orontii* were the species pairs with closest interspecific congeneric distances falling between 0.5%-1% difference.

4.3.4.2.3 Combined ITS and *Mcm7*

Intra and interspecific differences were quantified for the 101 accessions and 33 species of the combined ITS and *Mcm7* regions. This resulted in a total overlap of 8.98% (from 0.33% to 9.31%, covering 86.47% of all intra and interspecific but intrageneric sequences) (Figure 4.7d). The only accessions with a mean of more than 5% intraspecific difference were *G. cynoglossi*. There were 1,536 interspecific, congeneric distances fell below 5%; 97.5% of these were between accessions of the *Erysiphe*. The only interspecific pair below 0.5% difference was *E. alphitoides* and *E. euonymicola*.

4.4: Discussion

4.4.1: *Mcm7* amplification and sequencing

Markers for this promising region and a protocol for their use were successfully designed. Given the dearth of previous sequence data for the *Mcm7* region, it was vital to locate and identify the region using previously amplified samples of closely related, ascomycetous fungi. The use of 22 of these ensured that the region was accurately located and their alignment proves them to be reliably identified. After locating the region, primer design was reliant upon the accuracy of sequence data within the four available PM genomes. The challenge was then to ensure a designed primer would be specific only to the PMs, such that other fungi in the environmental sample of DNA were not amplified, and general enough to amplify and sequence the full diversity of PMs. This could not be guaranteed as sample genomes were of just three out of 12 genera: *Blumeria, Erysiphe,* and *Golovinomyces* genera. The use of ambiguous base pairs within the primers was necessary in order to maximise the likelihood of amplification and sequencing and it was hoped that these would also accommodate for the remaining PM genera whose DNA sequences were unknown at this point. Samples from the most common PM genera have been amplified and sequenced. The lack of success with *Phyllactinia* species may be due to sequencing error and must be trialled further. The primers must now be tested on herbarium specimens and rarer and more exotic PM genera and species such as *Cystotheca, Pleochaeta,* and *Leveillula.*

The amplification of multiple products in certain accessions may indicate that the primer combination is not as specific to PMs as hoped; amplifying additional accessions of congeneric or mycoparasitic fungi as well as the targeted PM. However, there was no correlation between the samples with faint additional bands sent for sequencing and poor sequence data. Instead poor sequence data proved to be associated with a weak initial product (signified by low intensity band on the agarose gel). Sequence data with multiple peaks present in trace files may have resulted from amplification of more than one PM species on a single host. This is exemplified by the four disparate

identifications of ITS and *Mcm7* (section 4.3.3.1). There was evidence for preferential amplification of *Golovinomyces* spp. by the promising primers Mcm7F2 and Mcm7R8: accessions identified as *P. plantaginis, P. amelanchieris,* and *E. cruciferarum* based on morphology and ITS were identified as *G. sordidus , Golovinomyces* sp., and *G. sordidus* respectively when analysed with *Mcm7*.

The reduction in ambiguous bases and length of Mcm7F2 and Mcm7R8 after additional sequence data was acquired resulted in a reduction in amplification and sequencing success. These adapted primers may require further optimisation. Design of alternate primers from the diverse alignment of data was problematic due to the low level of conservation in *Mcm7*; with no stretch proving to be better than that originally used for the design of Mcm7F2 and Mcm7R8. The proposed markers have been shown to consistently provide positive outcomes in amplification and sequencing. Their continued success should be monitored to ensure their efficacy.

4.4.2: Phylogenetic analyses

The *Mcm7* region was useful for phylogenetic analyses, confirming the findings of Aguileta *et al.* (2008) and numerous other studies which had confirmed its utility in other fungal groups. Computation time is slow; a dataset of approximately 150 sequences of 500bp for 5,000,000 generations taking approximately a day on a 3.20 GHz processor. The result was similar to accepted phylogenies of Braun and Takamatsu (2000). The main topological difference came in the placement of the *Neoerysiphe*. Based on morphology and ITS sequence data this has been known to be a clade within the tribe Golovinomyceteae. Combining *Mcm7* with ITS sequence data did not resolve this placement, therefore confirming the close relationship of the *Neoerysiphe* and *Erysiphe* clades in the *Mcm7* region.

The combined regions performed well under BI. Species within a genus grouped together and as sister to their congenerics. The use of *Mcm7* enabled greater discrimination of individual species: confirming the identity of certain accessions and enabling revisions elsewhere: particularly within *Erysiphe* (species *E. trifoliorum, E. ludens,* and *E. hyperici*) and *Golovinomyces* (species

G.cichoracearum, *G.sonchicola*, and *G.verbasci*). The greater sequence variation of *Mcm7* than the ITS, resulted in longer branch lengths and greater inter- and intra-specific separation, which could be vital for resolving PM species complexes such as those in *Phyllactinia* species, *G. cichoracearum*, *P. fuliginea*, and *P. tridactyla*, in future. Greater sample numbers are needed of these clades in order to do so.

No clear improvement of the resolution of relationships has been seen within species with numerous accessions yet: the *E. heraclei* remain clearly grouped as potential *formae speciales* onto different host plants in both *Mcm7* and ITS analyses; *E. aquilegiae* accessions remain scattered between var. *aquilegiae* and var. *ranunculi* in both *Mcm7* or ITS analyses; and *N. galeopsidis* accessions remain scattered between host genera in both *Mcm7* and ITS analyses. Accession 5_113R consistently grouped amongst *E. aquilegiae*. This may be due to the close relationship of *E. elevata* and *E. aquilegiae* or *Catalpa* being host to more than one *Erysiphe* species (Cook *et al.*, 2006). If so, this would be a new record of PM on this host.

4.4.3: DNA barcoding analysis

The K2P distribution graphs visualise the intra and interspecific distances per locus corresponding to the barcoding gap (Hebert *et al.*, 2003a). An ideal barcoding locus should have no overlap between intra and interspecific K2P distances (Quaedvlieg *et al.*, 2014). None of the analyses produced this gap. Failing this, a low K2P overlap is desired: the individual regions showed varying degrees of overlap of intra and interspecific distances. The ITS had a higher K2P overlap than the *Mcm7* suggesting the ITS was more conserved. This made it less suitable as a reliable identification locus for PM fungi across the whole scale of tested sequences. The *Mcm7* showed far greater natural variation within a species and between different species than the ITS and is therefore a strong candidate for efficient and reliable PM identification and population studies. As in numerous other fungal clades (Crous *et al.*, 2000, Câmara *et al.*, 2002, Kang *et al.*, 2002, Keča *et al.*, 2006, Maphosa *et al.*, 2006, Schena *et al.*, 2008, Amatulli *et al.*, 2010, Bensch *et al.*, 2012, Groenewald *et al.*, 2013,

Birkebak *et al.*, 2016, Haight *et al.*, 2016), this region could then be used regularly as an additional 'identifier' to the 'anchor' of the ITS (Kõljalg *et al.*, 2013). This method is extremely efficient in computation time; taking just a few seconds to calculate intra and interspecific distances.

Extensive K2P overlaps across all analyses were the result of closely related species of *Erysiphe* and *Golovinomyces* having very similar sequences and wide variation within certain species. The difference within *E. aquilegiae* has been recognised previously as distinct varieties *aquilegiae* and *ranunculi* (Braun & Cook, 2012). However the difference within *G. cynoglossi* is thus far undocumented.

4.5: Conclusions

The *Mcm7* region has proved to be a valuable addition to the currently established techniques for the identification of PM fungi. Amplification and sequencing of the *Mcm7* region was made possible by previous sequence data of PM genomes and *Mcm7* regions of other fungi. BI and K2P analyses have helped to prove the utility of *Mcm7* as an addition to ITS. Due to the historical weight of sequence data based around the ITS it has become a necessary tool for PM identification as samples are compared with this extensive library. However, *Mcm7* has been shown to achieve a greater level of discrimination of PM species and genotypes than the ITS. This is shown in its greater branch length under BI and lower K2P overlap in DNA barcoding. If amplification success can be improved close to that of the ITS, then this region should be adopted for future identification of PM species, particularly those of closely-related, phylogenetically young, recently evolved species such as those in the genera *Erysiphe, Golovinomyces*, and *Podosphaera*. Adoption of such an efficient region, alongside other technologies, could help to support rapid identification techniques that, if implemented alongside greater screening, might limit the spread of potentially harmful plant diseases; major threats to the UK horticultural and agricultural industries.

Chapter 5: Augmenting current ID techniques with β-tubulin

5.1: Introduction

The identification of additional regions to complement the ITS is key to the improvement of the discrimination process of PM species (Inuma *et al.*, 2007). Approaches have been developed to evaluate concatenated datasets for their phylogenetic utility (Murphy *et al.*, 2001, Li *et al.*, 2007, Rowe *et al.*, 2008, Edwards, 2009). New data from potentially informative regions such as the *Mcm7* and β-tubulin regions alongside the ITS may then result in phylogenies that better reflect the species tree rather than simply gene trees (Mallo & Posada, 2016). Concatenated alignments of PM species have been shown to provide results of greater approximation to the probable evolutionary tree than individual regions (Medina *et al.*, 2011, Tretter *et al.*, 2014a).

β-tubulin is a region which has received moderate attention within the field of fungal diagnostics (McKay *et al.*, 1999, Fraaije *et al.*, 2001) and more recently the PMs (Troch *et al.*, 2014, Vela-Corcía *et al.*, 2014). It is one of seven tubulins, which constitute a small family of globular proteins (McKean *et al.*, 2001). In a eukaryotic cell, the most abundant members are α-tubulins and β-tubulins, the proteins that are the primary constituents of microtubules (Einax & Voigt, 2003). The β-tubulin gene is said to be conserved, with "at least 60 % amino acid similarity between the most distantly related lineages" (Juuti *et al.*, 2005). It has been used as a molecular target in real-time PCR technologies for the accurate and reliable quantification of fungal DNA in environmental samples (Schena *et al.*, 2004) and as a reference gene in quantitative gene expression analysis in fungi (Yan & Liou, 2006). More relevant to the current study is that this gene has been reported to amplify using universal primers for fungi (Glass & Donaldson, 1995). Its use as a molecular marker for addressing intraspecific genetic diversity at varying taxonomic levels for fungi (Ayliffe *et al.*, 2001) and intraspecifically in population genetics studies of PM fungi (Cunnington *et al.*, 2003, Inuma *et al.*, 2007, Brewer & Milgroom, 2010, Troch *et al.*, 2014) are also promising for its diagnostic use in PMs.

Chapter 5: Augmenting current ID techniques with β-tubulin

Key to the success of β-tubulin when ITS can no longer successfully discriminate PM species is the greater divergence of its DNA sequences, due to a faster mutation rate (reported within the *Blumeria graminis* complex) (Wyand & Brown, 2003). This has also been reported in the phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's eye rot of apple (de Jong *et al.*, 2001), and the *Gibberella fujikuroi* (*Fusarium*) species complex (O' Donnell *et al.*, 1998b). The intraspecific variation of β-tubulin is contested in the study of Pirondi *et al.* (2015) as it shows eight 'housekeeping genes' of *Podosphaera xanthii* to have near identical sequences. In this case genetic diversity within and among populations was very low and isolates did not group according to geographical origin, host plants, climate areas, cultivation systems or mating types. They therefore claim that such a result suggests a clonal population structure of this PM caused by reproduction predominantly by asexual reproduction.

The potential for improving PM identification using an additional region, the *Mcm7*, has been shown in Chapter 4. Chapter 5 will continue to investigate the potential for complementing the morphological and ITS analyses which have become ubiquitous in PM identification. The study used accessions sourced from the Powdery Mildew Survey (Chapter 2).

In this chapter the possibility of using primers sourced from the literature for the β-tubulin region for PMs was investigated. The possibility of developing new working markers for the β-tubulin region for PMs was also investigated and the value of resultant data for phylogenetic reconstruction explored. The resolution of this region in DNA barcoding studies was compared with the standard ITS region.

5.2: Materials and methods

5.2.1: Sample collection – The Powdery Mildew Citizen Science Scheme

Samples were collected via the powdery mildew citizen science scheme (Chapter 2). One hundred and nine of these were successfully amplified (Appendix 5) in the study outlined in this chapter.

5.2.2: Putative species identification

The techniques outlined in Chapters 2, 3, and 4 formed the basis of the identities of PM species used in this chapter.

5.2.3: DNA extraction

The DNA extracted for initial PM species identification (section 3.2.3) was used for these further analyses.

5.2.4: Generic primer trials

5.2.4.1 Sourcing primers

Primers for the amplification of the β-tubulin region were sourced from previous publications (Glass & Donaldson, 1995, Ayliffe *et al.*, 2001, Amrani & Corio-Costet, 2006, Brewer & Milgroom, 2010, Vela-Corcía *et al.*, 2014) and ordered from Sigma-Aldrich (Table 5.1).

Table 5.1: Generic primers sourced from literature for trialling amplification and sequencing of β-tubulin region of PMs

Primer name	Direction	Sequence (5' - 3')	Reported Tm (°C)	Product size (bp)	Source
Tub3	F	GGCXAARGGXCAYTAYACXGA	58	600	Amrani and Corio-Costet (2006)
Rtub4	R	TGYTGXGTXARYTCXGGXAC			
tubA	F	GCRTCYTGRTAYTGYTGRTAYTC	58	1000	Ayliffe *et al.* (2001)
tubB	R	TGGGCNAARGGNCAYTAYACNGA			
Bt2c	F	CAGACTGGCCAATGCGTA	56	500	Brewer and Milgroom (2010)
Bt2d	R	AGTTCAGCACCCTCGGTGTA			
Bt2a	F	GGTAACCAAATCGGTGCTGCTTTC	58-68	402	Glass and Donaldson (1995)
Bt2b	R	ACCCTCAGTGTAGTGACCCTTGGC			
BtubF	F	ATGCGTGAAATTGTTCATCT	N/A	1800	Vela-Corcía *et al.* (2014)
BtubR	R	TTATTCTTCCGGTTGCATGGGTG			

5.2.4.2 PCR and sequencing

Amplification of the five primer combinations was trialled according to published protocols (Glass & Donaldson, 1995, Ayliffe *et al.*, 2001, Amrani & Corio-Costet, 2006, Brewer & Milgroom, 2010, Vela-Corcía *et al.*, 2014). The PCR products were separated and visualised as in section 3.2.4.

Multiple products per sample were consistently amplified (Figure 5.1). Individual bands were excised, purified using the QIAquick Gel Extraction Kit, and sent to Source BioScience via courier for sequencing.

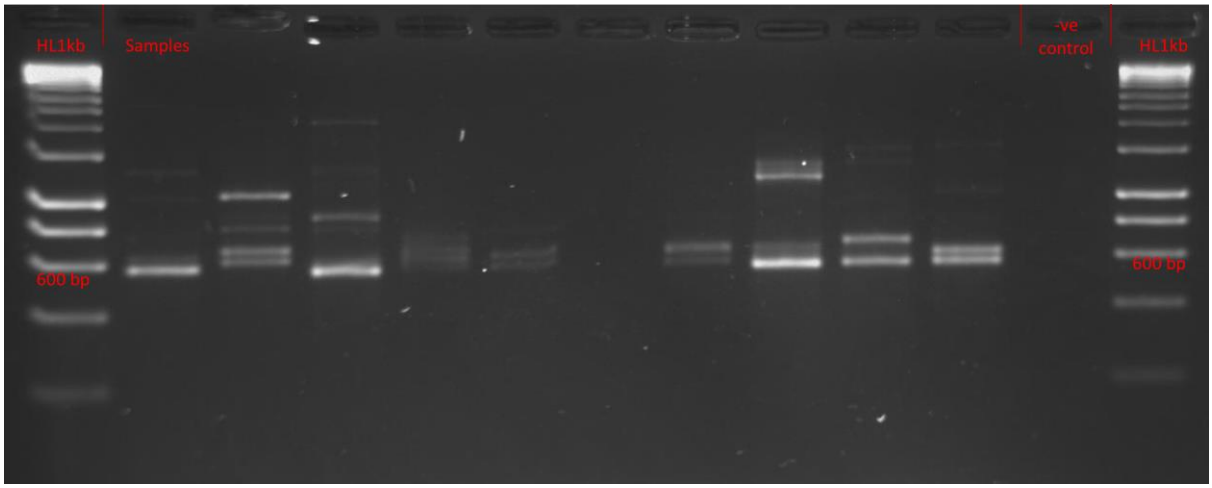

Figure 5.1: Amplification of 600 bp product of β-tubulin with primers Tub3 and Rtub4 before optimisation of PCR protocol.

Annealing, and extension temperatures of PCR protocols were explored using gradient PCR and $MgCl_2$ concentrations were increased in increments of 0.25mM up to 1mM in order to optimise amplification for single products. Single products were amplified for the primer combinations Tub3 and Rtub4 and tubA and tubB (Figure 5.2) with the PCR protocol from Amrani and Corio-Costet (2006) at an annealing temperature of 58°C and 0.5mM $MgCl_2$. Products of more than 10ng per band of preliminary sample amplifications, lacking strong additional amplified products, were purified using the QIAquick PCR Purification Kit and sent to Source BioScience via courier for sequencing.

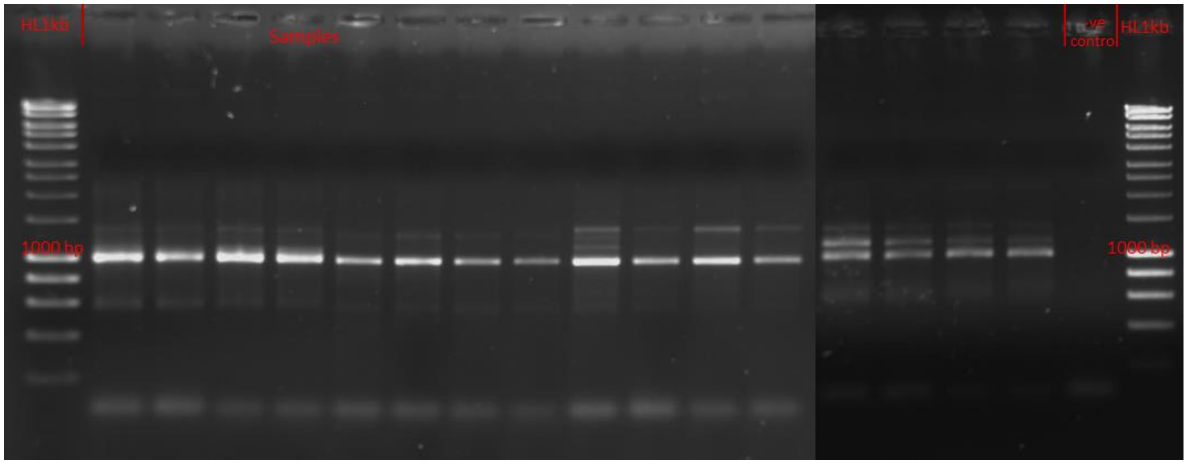

Figure 5.2: Amplification of 1000 bp product of β-tubulin with primers tubA and tubB after optimisation of PCR protocol.

Complementary forward and reverse sequences generated in this study as in section 3.2.4. This resulted in products of 470-590 bp for Tub3 and Rtub4 and 850-920 bp for tubA and tubB. NCBI GenBank Nucleotide BLAST was performed on samples. The results showed no significant similarity to β-tubulin PM sequences. Instead results showed similarity to contaminants from the environmental accessions such as: *Passalora fulva, Sclerotinia sclerotiorum, Sclerotinia homeocarpa, Didymium squamulosum,* and *Phaeosphaeria avenaria*.

5.2.5: Data mining for β-tubulin molecular markers

PM genomes (Max Planck Institute for Plant Breeding Research, Spanu *et al.*, 2010, Jones *et al.*, 2014) were aligned with two fungal species (*Cistella spicicola* (GenBank Accession No. GU727565.1) and *Melampsora lini* (GenBank Accession No. AF317682.1) and 12 PM samples in order to identify the location of the β-tubulin region and then develop primers to trial in the amplification of samples. A total of 14 primers (seven forward, seven reverse) were designed (Table 5.2) and ordered as in section 4.2.4.

Table 5.2: Primers designed for trialling amplification and sequencing of β-tubulin region of PMs.

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
BtubF1	F	GTTCACCTCCARACTGGCCAATG	23	62.42	52.17
BtubF2	F	AAYCARATYGGDGCYGCNTTCT	22	60.88	45.45
BtubF3	F	GAAYGTWTAYTTYAAYGAGGT	21	49.04	28.57
BtubF4	F	TGTGAYTGTCTTCARGG	17	48.33	41.18
BtubF5	F	ATGATGGCDACMTTYTCRGTTGT	23	61.67	43.48
BtubF6	F	TGTATGAGRACDTTRAAGCT	20	54.11	40
BtubF7	F	GGTGTRACYACHTGTCT	17	48.97	47.06
BtubR1	R	AGCTTYAAHGTYCTCATACA	20	55.85	45
BtubR2	R	AGACADGTDGTYACACC	17	50.39	47.06
BtubR3	R	ACCATGTTAACHGCYAAYTT	20	55.45	40
BtubR4	R	AAWCCAACCATRAARAARTG	20	47.86	25
BtubR5	R	GAVGCWGCCATCATRTTYTT	20	49.36	41.18
BtubR6	R	GTRAATTGATCHCCRACRCG	20	57.44	50
BtubR7	R	TCCATYTCRTCCATTCCTTC	20	54.19	45

5.2.6: PCR and sequencing protocol

PCR was carried out using the newly designed PM specific primers of the β-tubulin region (Table 5.2). The 31 possible combinations expected to amplify a product of more than 200 bp were trialled in 25 µl mixes of 12.5 µl BioMix™ Red (Bioline), 0.5 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 ng μl^{-1}, 9.25 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}. Cycling parameters were adapted from Amrani and Corio-Costet (2006) with an initial denaturation step of 95 °C for five minutes, followed by 37 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for one minute, and elongation at 72 °C for one and a half minutes and a final elongation at 72 °C for five minutes. Three samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003), were trialled initially.

The PCR products were separated and visualised as in section 3.2.4.

The seven primer combinations exhibiting the highest amplification success (number of products x product strength) were BTF1 and BTR2, BTF1 and BTR3, BTF1 and BTR6, BTF5 and BTR6, BTF5 and BTR7, BTF6 and BTR6, and BTF6 and BTR7. These were trialled for amplification of five different samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003). The three primer combinations exhibiting the highest amplification success were BTF1 and BTR3, BTF1 and BTR6, and BTF5 and BTR7.These were trialled at a gradient of annealing temperatures from 53-62 °C. The most successful temperature was 55 °C. The three primer combinations were then trialled with seven new samples at this optimised annealing temperature. The most successful primer combination was BTF5 and BTR7. This amplified a region of approximately 800 bp. Reducing the degeneracy of base pairs was trialled for greater accuracy in amplification and sequencing of the β-tubulin region and resulted in the design of primers BTF5a, BTF5b, and BTR7a (Table 5.3). Application of these modified primers produced greater amplification success and sequences of greater quality. Primer combination BTF5b and BTR7a was best and was used in all future amplifications with an annealing temperature of 55 °C. Single amplicons were sequenced and assembled as in section 3.2.4.

Table 5.3: Primers adapted from BTF5 and BTR7 for more efficient amplification and sequencing of β-tubulin products

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
BtubF5a	F	ATGATGGCSACATTTTCGGTTGT	23	61.63	43.48
BtubF5b	F	ATGATGGCSSACATTTTCGGTTGT	24	63.68	45.83
BtubR7a	R	TCCATTTCGTCCATTCCTTC	20	55.44	45

5.2.7: Sequence alignment

Sequence alignment of β-tubulin data, and their complementary ITS sequences, was performed as in section 3.2.6. The dataset of 85 sequences of β-tubulin accessions for which there were ITS equivalents was concatenated using Mesquite (Maddison & Maddison, 2017). The alignment files of the β-tubulin and the β-tubulin combined with the ITS rDNA were deposited in TreeBASE as S20944.

5.2.8: Phylogenetic analyses

Phylogenetic analyses were performed as in section 3.2.7.

For BI of each separate data set (all β-tubulin accessions, ITS accessions for which there were β-tubulin equivalents, and β-tubulin accessions for which there were ITS equivalents) the GTR+I+G model was used and was run for 5,000,000 generations. For BI of the combined dataset (ITS and β-tubulin) the separate models of individual datasets were used for each region and were run for 5,000,000 generations.

5.2.9: DNA barcoding analysis

Datasets of all β-tubulin accessions, ITS accessions for which there were β-tubulin equivalents, β-tubulin accessions for which there were ITS equivalents, and concatenated ITS and β-tubulin accessions were analysed and treated as in section 3.2.8.

5.3: Results

5.3.1: β-tubulin amplification and sequencing

Seventeen of the 31 possible combinations of 14 primers (seven forward and seven reverse) resulted in amplification of at least a single product from the three initial PM samples trialled. The combination of BTF5 and BTR7 produced the most bands of greatest intensity, this was refined to make BTF5b and BTR7a (Figure 5.3). After optimisation of PCR, 74% of 146 trialled samples in 2014 resulted in a product being visualised using gel electrophoresis. Sequencing resulted in 115 of 116 samples producing readable sequences. In 2016 18% of 82 trialled samples produced a product which could be visualised using gel electrophoresis. Sequencing resulted in seven of 15 samples producing readable sequences. These were contributed to GenBank (Accession numbers KY786690 – KY786781 (presented in Appendix 5)). Sequencing worked in both forward and reverse directions in 2014, but only in reverse, via the BTR7a primer, in 2016. Those which were unsuccessful were characterised by weak reads, resulting in little or no sequence data, or messy reads, potentially contaminated with more than one PM species or additional conspecific fungi.

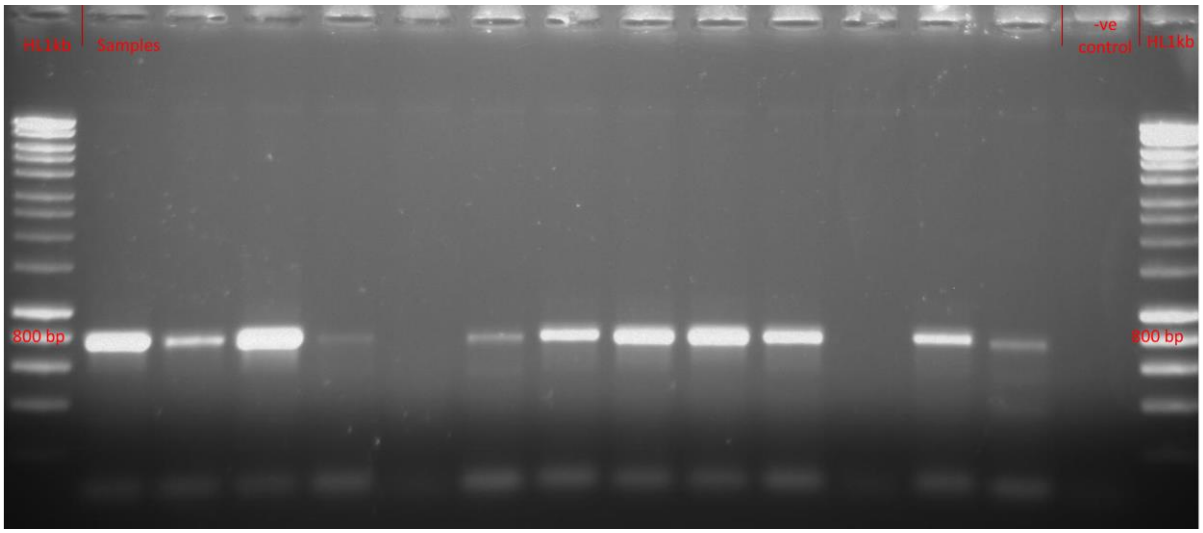

Figure 5.3: Amplification of 800 bp product of β-tubulin with primers BTF5b and BTR7a with HyperLadder™ 1kb and negative control.

Samples were successfully amplified and sequenced from the *Blumeria, Podosphaera, Sawadaea, Phyllactinia, Arthrocladiella, Golovinomyces, Neoerysiphe,* and *Erysiphe* genera.

β-tubulin sequence data from the PMs is sparse in GenBank; the 419 sequences available (April 2017) are from the following PM species: *Blumeria graminis*, *Podosphaera fusca*, *Golovinomyces orontii*, *Oidium heveae*, *Erysiphe pisi*, *Erysiphe necator* (called *Uncinula necator* in sequences on GenBank), *Erysiphe syringae-japonicae, Erysiphe ligustri,* and *Erysiphe syringae*. This dearth of GenBank data meant that identifications of PM samples based on BLAST were not possible. When BLAST was optimised for finding 'highly similar sequences' searches matched the nine PM species present with identities of 95-100% and sequence cover of 80-99% of the submitted sequence. BLAST also returned best matches to non-PM samples such as *Cercophora*, *Chaetomidium*, *Monilinia*, *Neofabraea*, *Peziza*, and *Botryotinia*; identities matching 65-99% and covering 77-98% of the submitted sequence.

5.3.2: Sequence alignment

All 115 sequences were included in the initial sequence alignment. This was reduced to 103 sequences as a result of poor sequence quality and short sequence reads. The full breadth of PM genera in the present study were included in this. Alignment resulted in a region of 824 bp. This was trimmed to 768 bp in order to remove gaps and poor quality sequence reads near the primer sites and leave sequences of equal size for later analyses. The region was 50.6% conserved. This compared to 75.6% in the ITS and 38.8 % in the *Mcm7*. There were 85 accessions with both β-tubulin and ITS sequences. β-tubulin sequences were concatenated with ITS (881 bp) for analysis resulting in 1649 bps.

5.3.3: Phylogenetic analyses

5.3.3.1 β-tubulin

BI of PM phylogeny using 103 accessions of the 768 bp region within the β-tubulin region resulted in separation of the genera as expected after ITS and *Mcm7* analyses. However multiple outliers were

also evident. Support for the overall topology was moderate. Tribes Cystotheceae (clade A, PP 98%), Phyllactinieae (PP 63%), and Erysipheae (clade C, PP 67%) were monophyletic (Figure 5.4). Genera of the Golovinomyceteae tribe (*Arthrocladiella*, *Golovinomyces*, and *Neoerysiphe*) were grouped paraphyletically with *Arthrocladiella* and *Golovinomyces* as sister to the Erysipheae tribe. The node separating *Arthrocladiella, Golovinomyces* and Erysipheae from *Neoerysiphe* had a PP of 92%. Each genus included in the sampling showed strong support: *Sawadaea* (PP 100%), *Podosphaera* (PP 99%), *Arthrocladiella* (PP 96%), *Golovinomyces* (PP 100%), *Neoerysiphe* (PP 99%), and *Erysiphe* (PP 67%), however *S. tulasnei* was grouped amongst *Podosphaera* spp., *Oidium longipes* grouped between *Golovinomyces* spp., accessions of *E. pisi* were more closely related to the *B. graminis* outgroup than any other accession, and accessions of *E. lonicerae, E. polygoni*, and *E. elevata* were also grouped amongst *Podosphaera* spp.

Within the Cystotheceae (clade A), *Podosphaera* species did not form polytomies and intraspecific variation was evident. Five of the 11 putative species included in the analysis were monophyletic (Table 5.4). Five *P. fugax* accessions grouped with a *P. mors-uvae* accession with 98% PP and five *P. erigerontis-canadensis* accessions grouped with three *P. xanthii* accessions and four outliers (*S. tulasnei, E. lonicerae, E. polygoni,* and *E. elevata*). A fourth *P. xanthii* accession was separate from these; the host *Senecio jacobaea* is also known to harbour *P. senecionis* and *P. pericallidis* and the accession is therefore likely to be one of these two species. Two accessions of *P. tridactyla* on *Prunus* spp. were paraphyletic.

Phyllactinia fraxini was the only accession representative of the Phyllactinieae. This was monophyletic and placed between the Cystotheceae and Golovinomyceteae.

Within the Golovinomyceteae (clade B), *Neoerysiphe* (four accessions of *N. galeopsidis* (PP 100%) were monophyletic. There was a single accession from *Arthrocladiella* (*A. mougeotti*) which was grouped alone. The only *Oidium* accession was grouped amongst four *Golovinomyces* accessions. Within this group all four taxa were monophyletic (Table 5.4).

Tribe Erysipheae (clade C) separated into individual species. Of the 19 putative species, 17 were monophyletic (Table 5.4). *E. aquilegiae* separates into the two known varieties var. *aquilegiae* and var. *ranunculi*. Accessions on Fabaceae hosts and *Hypericum* are not completely resolved: two *E. ludens* ex *Lathyrus* spp., an *E. hyperici* ex *Hypericum* sp., three *E. trifoliorum* ex *Trifolium* spp., and an *E. intermedia* ex *Lupinus* sp. are grouped together (PP 98%). Within this group the *E. trifoliorum* and *E. intermedia* are monophyletic with PP of 58%.

Table 5.4: Monophyletic PM groups from β-tubulin data within the Erysiphales.

Species within group	Number of Accessions	PP of group*
S. bicornis	5	100
P. clandestina	1	
P. filipendulae	3	99
P. fugax	5	98
P. mors-uvae	1	
P. macrospora	1	
P. xanthii	1	
P. plantaginis	1	
P. erigerontis-canadensis	5	27
P. xanthii	3	
S. tulasnei	1	
E. lonicerae	1	
E. polygoni	1	
E. elevata	1	
Ph. fraxini	1	
N. galeopsidis	4	100
A. mougeotii	1	
G. montagnei	1	
O. longipes	1	
G. cichoracearum	2	100
G. sordidus	1	
E. adunca	1	
E. arcuata	1	
E. necator	1	
E. hedwigii	1	
E. aquilegiae	14	99
E. platani	1	
E. elevata	1	
E. akebiae	3	94
E. alphitoides	6	89
E. cruciferarum	1	
E. berberidis	4	100
E. ludens	2	98
E. hyperici	1	
E. trifoliorum	3	
E. intermedia	1	
E. convolvuli	2	100
E. buhrii	1	
E. polygoni	2	100
E. heraclei	9	100

*Groups with a single accession have no PP and are shaded in grey.

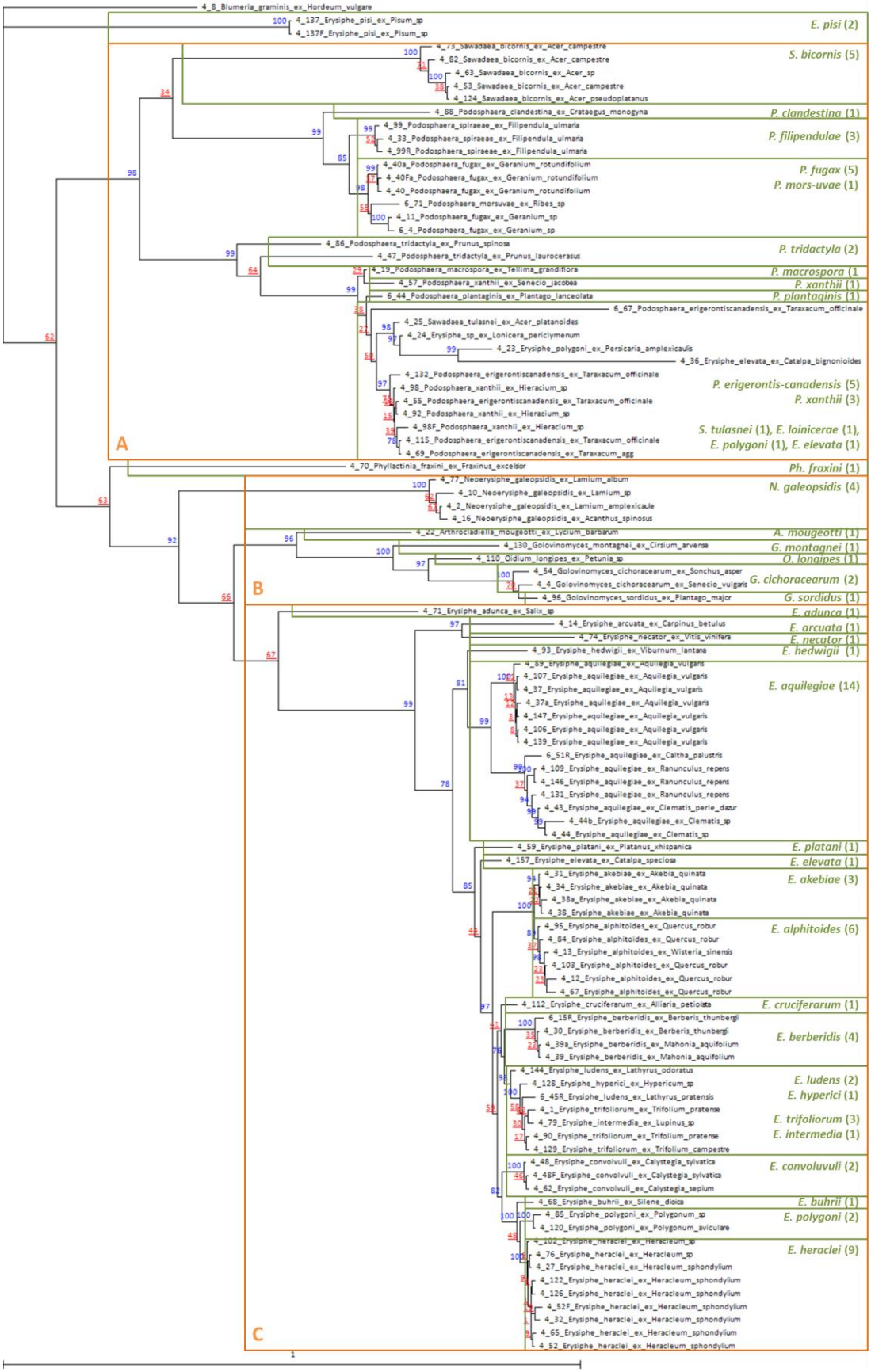

Figure 5.4: BI using 103 sequences of the β-tubulin region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

5.3.3.2 Direct comparison of ITS and β-tubulin

BI of the PM phylogeny of 85 accessions of the β-tubulin region (Figure 5.6) resulted in an overall topology very similar to that of the BI of the PM phylogeny of 85 accessions of the ITS region (Figure 5.5). Differences came in the positioning of genera within the Cystotheceae (clade A) and Golovinomyceteae (clade B). Both phylogenies were similar to that of section 4.3.3.1. The ITS phylogeny showed Golovinomyceteae to be monophyletic (PP 92%). Both regions showed the *Neoerysiphe* within this tribe to be monophyletic and individual species were separated. The ITS phylogeny showed *Arthrocladiella* to be grouped between *G. depressus* and the remaining *Golovinomyces* species.

β-tubulin performed better when discriminating between certain closely related species: *E. akebiae* and *E. alphitoides* separate monophyletically while they are clustered together in the ITS phylogeny; and the same is true of *E. ludens, E. hyperici, E. trifoliorum,* and *E. intermedia*. Neither region manages to discriminate between *P. erigerontis-canadensis* and *P. xanthii*; these species are grouped together in both analyses.

Comparison of the ITS and β-tubulin trees showed that, despite their relative variability (24.4% and 49.4% variable respectively), mean branch length was similar.

Accession 4_89 was identified as *E. aquilegiae* with ITS and a potential *Leveillula taurica* with β-tubulin and 4_86 was identified as *P. tridactyla* via ITS and *E. prunastri* via β-tubulin. These accessions were renamed accordingly to their β-tubulin identity for β-tubulin TaxonDNA analyses.

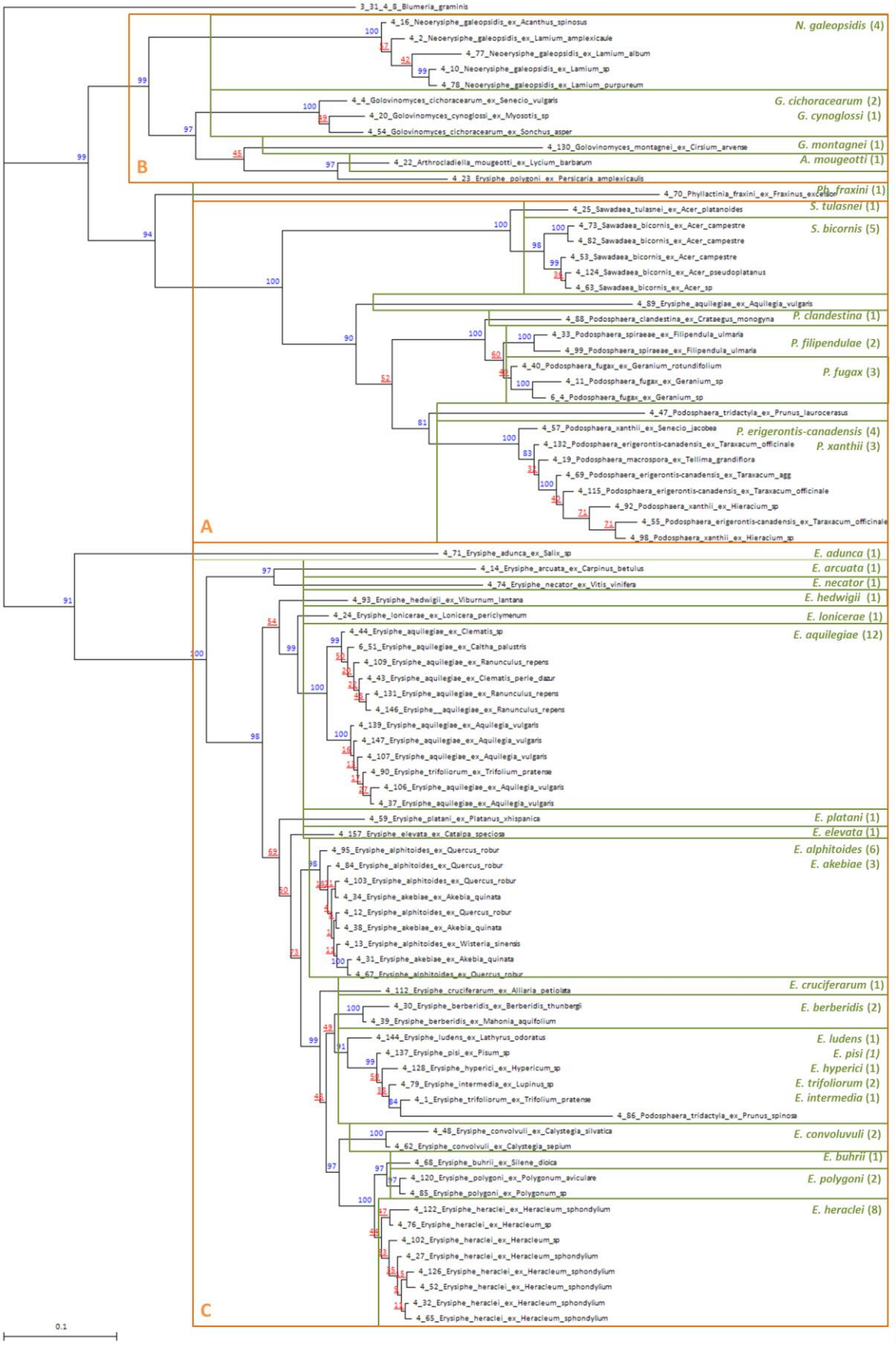

Figure 5.5: BI using 85 sequences of the ITS region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

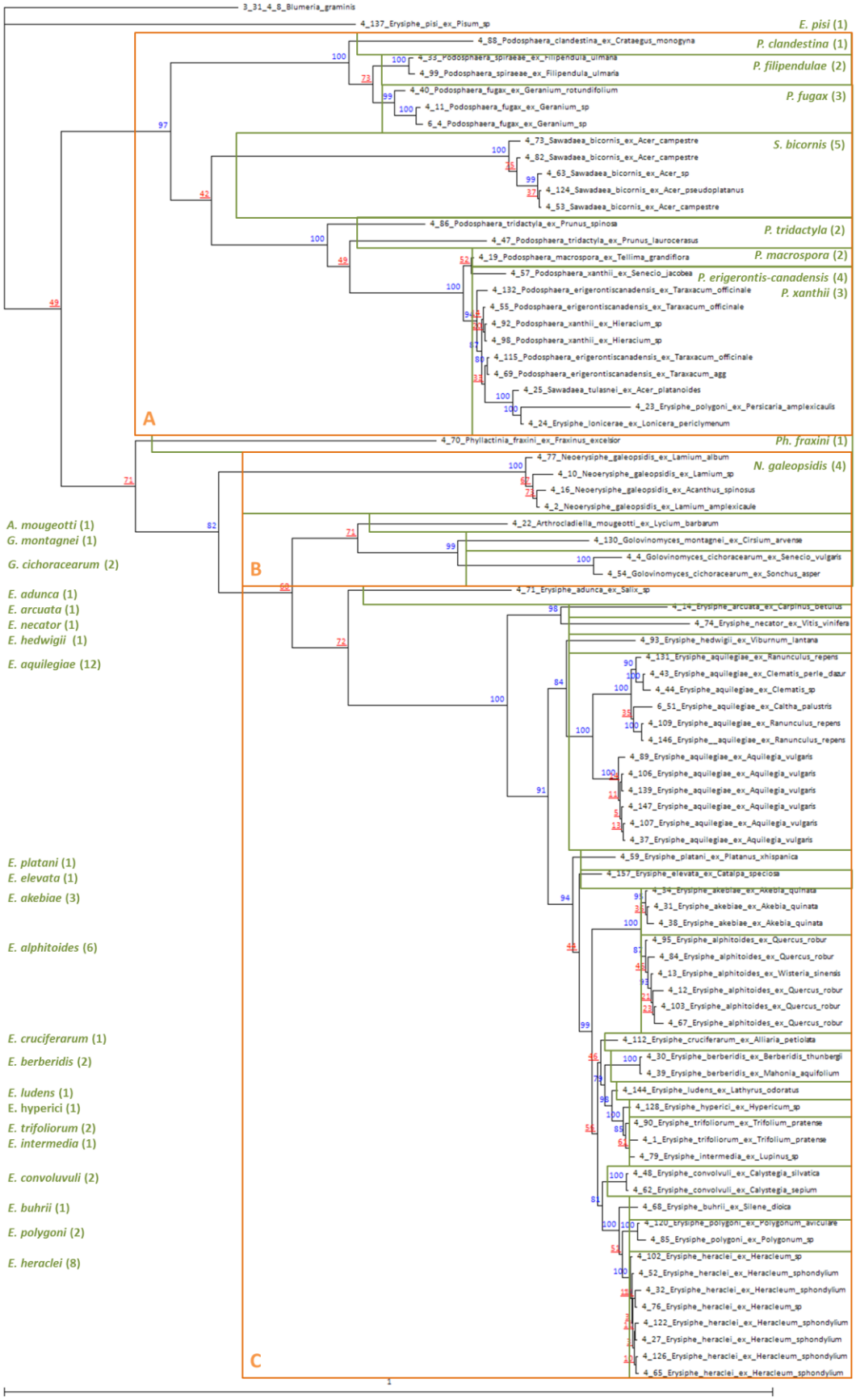

Figure 5.6: BI using 85 sequences of the β-tubulin region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

5.3.3.3 Combined ITS and β-tubulin phylogeny

BI of PM phylogeny using 85 accessions of the combined 1649 bp region of the ITS and β-tubulin regions resulted in clear discrimination of PM genera. Support for the overall topology was high. All tribes were monophyletic: Phyllactinieae (one accession), Golovinomyceteae (clade B), nine accessions (PP 96%), Cystotheceae (clade A), 22 accessions (PP 94%), and Erysipheae (clade C), 51 accessions (PP 94%) (Figure 5.7). When outliers (in this case accessions with disparate identifications based on ITS and β-tubulin) were excluded, each genus included in the sampling was shown to be monophyletic: *Phyllactinia* (one accession), *Neoerysiphe* (PP 100%), *Arthrocladiella* (one accession), *Golovinomyces* (PP 100%), *Sawadaea* (PP 99%), *Podosphaera* (PP 94%), and *Erysiphe* (PP 94%).

Species remained monophyletic with the exception of *P. xanthii* and *P. erigerontis-canadensis* and *E. trifoliorum* and *E. intermedia*. The latter pair had been resolved by β-tubulin but was clustered in this combined analysis. The newly resolved monophyly with β-tubulin of *E. akebiae* and *E. alphitoides* and *E. ludens* and *E. hyperici* are maintained.

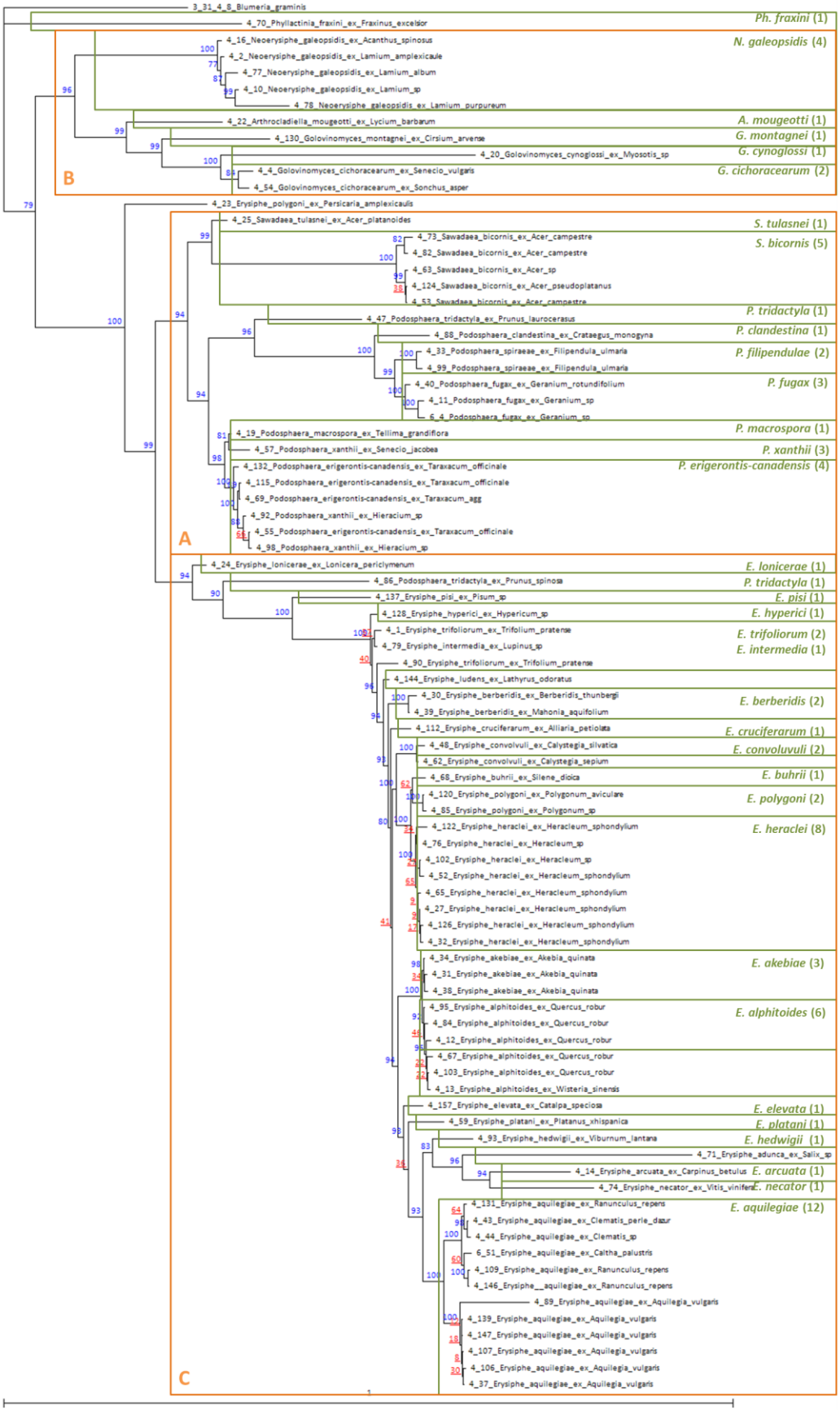

Figure 5.7: BI using 85 sequences of the ITS and β-tubulin regions combined. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

5.3.4: DNA barcoding analysis

5.3.4.1 β-tubulin

Intra and interspecific differences were quantified for the 102 accessions and 39 species of the β-tubulin region. This resulted in a total overlap of 15.12% (from 0.0% to 15.12%, covering 73.67% of all intra and interspecific but intrageneric sequences) (Figure 5.8a). Accessions with a mean of more than 5% intraspecific difference were *E. aquilegiae*, and *P. xanthii*. There were 510 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (86.3%) and *Podosphaera* (12.9%). The only interspecific pair below 0.5% difference was *P. erigerontis-canadensis* and *P. xanthii*.

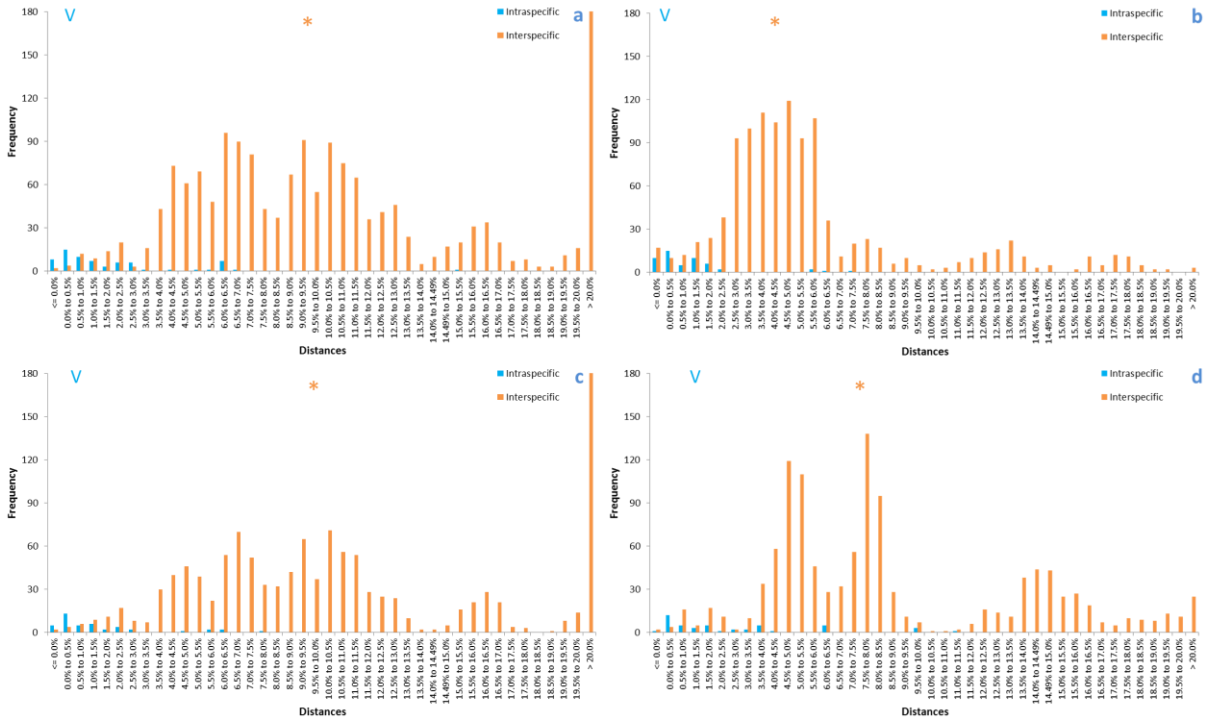

Figure 5.8: The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of (a) 102 β-tubulin accessions, (b) 82 ITS accessions common to the ITS and β-tubulin regions, (c) 82 β-tubulin accessions common to the ITS and β-tubulin regions, and (d) 82 accessions of the ITS and β-tubulin regions combined. Blue chevrons (V) mark the mean intraspecific variation per locus and orange asterisks (*) mark the mean interspecific variation per locus.

5.3.4.2 Direct comparison of ITS, β-tubulin, and combined datasets

5.3.4.2.1 ITS

Intra and interspecific differences were quantified for the 82 accessions and 35 species of the ITS region. This resulted in a total overlap of 7.13% (from 0.0% to 7.13%, covering 81.44% of all intra and interspecific but intrageneric sequences) (Figure 5.8b). The only accessions with a mean of more than 5% intraspecific difference were *E. trifoliorum*. There were 1,264 interspecific, congeneric distances which fell below 5%; 94.6% of these were between accessions of the *Erysiphe*. The interspecific pairs which were below 0.5% difference were: *E. aquilegiae* and *E. trifoliorum*, *E. alphitoides* and *E. akebiae*, and *P. erigerontis-canadensis* and *P. xanthii*.

5.3.4.2.2 β-tubulin

Intra and interspecific differences were quantified for the 82 accessions and 35 species of the β-tubulin region. This resulted in a total overlap of 7.62% (from 0.0% to 7.62%, covering 37.76% of all intra and interspecific but intrageneric sequences) (Figure 5.8c). The only accessions with a mean of more than 5% intraspecific difference were *E. aquilegiae*. There were 356 interspecific, congeneric distances which fell below 5%; 90.4% of these were between accessions of the *Erysiphe* and 9.6% were between accessions of the *Podosphaera*. The only interspecific pair below 0.5% difference was *P. erigerontis-canadensis* and *P. xanthii*.

5.3.4.2.3 Combined ITS and β-tubulin

Intra and interspecific differences were quantified for the 82 accessions and 35 species of the combined ITS and β-tubulin regions. This resulted in a total overlap of 11.04% (from 0.0% to 11.04%, covering 72.47% of all intra and interspecific but intrageneric sequences) (Figure 5.8d). No accessions had a mean of more than 5% intraspecific difference. There were 552 interspecific, congeneric distances fell below 5%; 92.4% of these were between accessions of the *Erysiphe* and 7.6% were between accessions of the *Podosphaera.* The interspecific pairs which were below 0.5% difference were: *P. erigerontis-canadensis* and *P. xanthii* and *E. alphitoides* and *E. akebiae*.

5.4: Discussion

5.4.1: β-tubulin amplification and sequencing

Markers for this promising region and a protocol for their use were successfully designed. As sequence data for the β-tubulin region are available for just nine PM species, it was vital to locate and identify the region using previously amplified samples. The use of two fungal species alongside the available PMs ensured that the region was accurately located. The ease of alignment proves them to be reliably identified as samples from this region. After locating the region, primer design was reliant upon the accuracy of sequence data within the nine available PM species. The challenge was then to ensure a designed primer would be specific only to the PMs, such that other Fungi in the environmental sample of DNA were not amplified, and general enough to amplify and sequence the full diversity of PMs. This could not be guaranteed as sample species were of the *Blumeria, Erysiphe, Oidium,* and *Golovinomyces* genera; just three holomorphic genera out of 16.

The amplification of multiple products in certain accessions may indicate that the primer combination is not as specific to PMs as hoped; amplifying additional samples of conspecific or mycoparasitic fungi as well as the targeted PM. However, there was no correlation between the samples with faint additional bands sent for sequencing and poor sequence data. Instead poor sequence data proved to be associated with a weak initial product (signified by low intensity band on the TAE gel). Messy sequence data may have resulted from amplification of more than one PM species on a single host. This is exemplified by the disparate identifications of ITS and β-tubulin: samples identified as *E. aquilegiae* and *P. tridactyla* based on host, fungal morphology, and ITS were identified as *Leveillula taurica* and *E. prunastri* respectively when analysed with β-tubulin; these identifications are in line with their respective known host ranges.

Samples from the most common PM genera have been amplified and sequenced. This includes underrepresented genera such as the *Arthrocladiella* and *Phyllactinia*, which failed to

amplify in the *Mcm7* region (Chapter 4). The primers must now be tested on herbarium specimens and rarer and more exotic PM genera and species such as the *Cystotheca, Pleochaeta,* and *Leveillula.*

The use of degenerate base pairs within the primers was necessary in order to maximise the likelihood of amplification and sequencing as these would also accommodate for the remaining PM genera whose DNA sequences were unknown at this point. The most successful initial primer combination contained three ambiguous bases in the 23 base pair forward primer (BTF5) and two in the 20 base pair reverse primer (BTR7). The reduction of ambiguity when the primers were refined (to just one and zero ambiguous bases respectively) resulted in increased amplification and sequencing success when first trialled. However, the huge reduction in sequencing success from 2014 (74% success) to 2016 (18% success) could have been a result of over-refinement of these primers. This could be tested by focusing on amplification and sequencing success rates within particular genera and examining existing sequence data for variability at the priming site. The existing data show a spread of unsuccessful amplifications from *Podosphaera*, *Sawadaea, Golovinomyces, Neoerysiphe,* and *Erysiphe*; across the full spread of available genera. The β-tubulin alignment generated from the current study shows variability at the beginning of sequence reads; the priming site. This is to be expected close to primer binding sites making conclusions ambiguous. However, the bases refined within refined primers BTF5b and BTR7a are complementary to those in the final alignment. As such a switch back to unrefined primers BTF5 and BTR7 did not improve this success rate.

The β-tubulin region codes for the globular proteins (McKean *et al.*, 2001) making up microtubules (Einax & Voigt, 2003). This functionality means that the region is subject to ongoing evolution (Li *et al.*, 2004), but at a rate slower than that of non-coding regions (Holst-Jensen *et al.*, 1997, Ponting *et al.*, 2009). The sequence of base pairs targeted by the designed primers may therefore vary from species to species and over time. This may result in the loss of PCR and sequencing success evident in the current study. However, fungal evolution is not rapid enough to

satisfy this (Berbee & Taylor, 2001, Hirsh & Fraser, 2001). It is therefore hypothesised that the change of sequencing companies and their PCR product requirements (from Source BioScience requiring less than 10 µl to GATC requiring at least 20 µl) was the most significant factor in the reduced success of sequencing. Anecdotal evidence has since arisen of further poor quality sequences from the company GATC. This was particularly the case when samples were purified by GATC rather than by the researcher. Further studies are needed to resolve this issue. However, future studies may make use of either primer combination BTF5 and BTR7 or BTF5b and BTR7a. Their continued success or failure should be monitored to ensure their efficacy, as the sequences within this region of PMs will evolve further over time.

5.4.2: Phylogenetic analyses

The β-tubulin region was useful for phylogenetic analyses and produced a result similar to accepted phylogenies of Braun and Takamatsu (2000). Like the *Mcm7*, β-tubulin was able to discriminate between samples indistinguishable by the ITS. Continued research into the region is likely to continue to elucidate other species which can be resolved using a region such as this. The study therefore concurs with numerous others within fungal systematics (Ayliffe *et al.*, 2001, Cunnington *et al.*, 2003, Inuma *et al.*, 2007, Brewer & Milgroom, 2010), which promote its usage. Computation time for BI is similarly slow to that of Chapter 4 (and all other phylogenetic analyses of large datasets). The main drawback of the β-tubulin region proved to be that of potentially erroneously sequenced data. For certain accessions such as 4_36 on *Catalpa bignonioides* it is possible that the β-tubulin primers may have favourably amplified a coexisiting sample of *Podosphaera catalpae* rather than the *Erysiphe elevata* identified by morphological and ITS analyses. Each of the other outlying samples cannot be explained in this way as no species matching the sample's placement within the phylogeny have been recorded on the respective hosts. These could therefore be multiple copies of the β-tubulin gene (Cleveland *et al.*, 1981).

The combined regions performed well under BI. Species grouped together and as sister to their congenerics. The use of β-tubulin enabled greater discrimination of individual species: confirming the identity of certain accessions and enabling revisions elsewhere: particularly within the *Erysiphe* (species *E. trifoliorum, E. ludens,* and *E. hyperici*). Accessions within this chapter have also shown clear divide between *E. aquilegiae* var. *aquilegiae* and var. *ranunculi*. However, this is true for both β-tubulin and ITS.

5.4.3: DNA barcoding analysis

None of the analyses within this chapter produced the barcoding gap between intra and interspecific K2P distances (Quaedvlieg *et al.*, 2014). However, the low K2P overlap desired was evident in the β-tubulin region as it covered just 37.76% of all intra and interspecific distances; lower than analyses of the ITS (81.44%) and the concatenated β-tubulin and ITS (72.47%) analyses. This is further highlighted by the 1,264 interspecific, congeneric distances which fell below 5% in ITS barcoding analysis in comparison to 356 in β-tubulin analysis and 552 when the regions are combined. This was also superior to the result from the *Mcm7* region, which had covered just 61.82% of all intra and interspecific and 896 interspecific, congeneric distances below 5% for a dataset of the same size. The β-tubulin region also showed greater variation within a species and between different species than the ITS and is therefore another strong candidate for efficient and reliable PM identification. Like the *Mcm7* reviewed in Chapter 4, it could therefore be used regularly as an additional 'identifier' to the 'anchor' of the ITS (Kõljalg *et al.*, 2013).

The extensive K2P overlaps were once again evident across all analyses; this time the result of closely related species of the *Erysiphe* and *Podosphaera* having very similar sequences and wide variation within certain species. The difference within *E. aquilegiae*, recorded previously as distinct varieties (Braun & Cook, 2012), is again evident through β-tubulin analysis.

The similarity of *E. alphitoides* and *E. akebiae* is clear from ITS analysis, but has been resolved with β-tubulin. These species parasitise hosts of different plant families (Fagaceae and

Ranunculaceae respectively) and the similarity of these PM species is therefore interesting. It is likely that one of these species evolved as the result of a host jump (Matsuda & Takamatsu, 2003) onto its new host family. There is an apparently strong affinity between oaks and Erysiphales. More than 50 PM species are listed on oaks in various regions of the world in the Systematic Mycology and Microbiology Laboratory Fungus-Host Database (Farr *et al.*, 2010), one of these is *E. alphitoides*. As such, there is a great deal of research on the *Quercus* – *E. alphitoides* relationship (Limkaisang *et al.*, 2006, Takamatsu *et al.*, 2007, Topalidou, 2008, Desprez-Loustau *et al.*, 2010) but relatively little about the *Akebia* – *E. akebiae* relationship (Garibaldi *et al.*, 2004, Li *et al.*, 2010, Siahaan & Takamatsu, 2016). *E. alphitoides* and *E. akebiae* are consistently grouped closely to each other. The species have been separated via β-tubulin analysis, however the present study cannot elucidate which species may be derived. A molecular clock (Takamatsu & Matsuda, 2004, Takamatsu *et al.*, 2008a, Takamatsu *et al.*, 2010) approach is necessary for such inferences.

The similarity of *P. erigerontis-canadensis* and *P. xanthii* is previously documented (Braun, 1987, Braun, 1995) and was evident from analysis of ITS. This is backed up by β-tubulin as this superior barcoding region also fails to separate the species. A lack of accessions of *P. xanthii* from the *Mcm7* region meant that its resolution of these species went untested. The separation of putative *P. xanthii* ex *Senecio jacobea* shows that this may well be a separate species: *P. senecionis*. PMs ex *Hieracium* spp. of the current study were previously identified as *P. xanthii* due to morphological and ITS identification of a *Podosphaera* and the generalist nature of this species. Otherwise the only previous record of PM ex *Hieracium* spp. is *G. cichoracearum* (Braun & Cook, 2012). However, due to the lack of separation from *P. erigerontis-canadensis* under both barcoding and phylogenetic analyses and the lack of previous records of *Podosphaera* on *Hieracium* the two accessions of PM on *Hieracium* can now be putatively identified as *P. erigerontis-canadensis* as well; a new record of PM-host relationship [*yet to be published*]. This species has been documented to be separate from closely related *P. fusca* (Ito & Takamatsu, 2010) and contain several *formae speciales*; this requires further gene sequence analyses as well as cross-infection assays such as those

described in Whipps *et al.* (1998) and Vági *et al.* (2007) in order to describe the highly specific forms of the species.

The new reference sequences generated from this study for the β-tubulin region and efficient computation of results could be paired with new technologies outlined in Chapter 10 to increase speed and efficiency of plant-disease diagnostics. This would enable superior control against harmful new PM diseases entering the country and causing economic losses to the horticultural and agricultural industries.

5.5: Conclusions

The β-tubulin region has proved to be another valuable addition to the currently established techniques for the identification of PM fungi. Although generic primers were unable to produce PM sequences, newly designed, PM specific primers aided in generating multiple PM β-tubulin sequences. BI and K2P analyses of these sequences have helped to prove the utility of β-tubulin as an addition to ITS, and *Mcm7*. The historical weight of sequence data continues to favour the ITS and thus it remains a necessary tool for PM identification and will continue to be an intermediary to other fungal species. However, like *Mcm7*, β-tubulin has achieved a greater level of discrimination of PM species. The region also maintains the previously accepted overall phylogenetic topology of PMs under BI. Amplification success has, at times been close to the level of success seen in PCR of ITS, however this must be trialled and optimised further in order to consistently amplify and sequence PM DNA. Providing this occurs, this region could be adopted for future identification of PM species, particularly those of closely-related, phylogenetically young, recently evolved species such as those in the *Podosphaera*, *Golovinomyces*, and *Erysiphe* genera.

Chapter 6: Augmenting current ID techniques with novel gene *Tsr1*

6.1: Introduction

The wide use of protein-coding genes in inferring evolutionary relationships among the ascomycete fungi and PMs has shown variable performance from gene to gene (Aguileta *et al.*, 2008). Meta-analysis of studies which aimed to compare the utility of different genes has shown definitions of phylogenetic informativeness to vary from study to study. This is largely due to the varying methods employed for testing gene phylogenetic utility. However, Aguileta *et al.* (2008) managed to compare gene based trees to an ideal tree, Townsend (2007) used character rates projected backwards in time applied to ascomycetous taxa, Collins *et al.* (2005) used base compositional stationarity, and Graybeal (1994) used empirical saturation plots. From these studies it became clear that different genes behave differently and offer varying utility for discovering older or younger divergences. These largely showed protein-coding genes to be more informative than ribosomal genes, but an identical method of assessment of regions would enable more meaningful comparisons. However, Aguileta *et al.* (2008) showed several protein-coding genes used routinely in fungal phylogenetic studies to perform poorly when tested against single-copy homologous genes from fungal genomes. Two single-copy homologues of protein coding gene loci outperformed all other protein-coding genes in their study. These were MS456 (*Mcm7* (recommended in Chapter 4)) and MS277 (*Tsr1* (studied in this Chapter)).

Continued analysis of such new genes to compare their diagnostic utility with regions currently in use can help to improve robustness and resolution of phylogenetic and barcoding analyses among ascomycete fungi and improve cost management of molecular studies (Raja *et al.*, 2011). The number of loci required to resolve a phylogeny can vary greatly (Lemmon & Lemmon, 2013). Hundreds of loci may be necessary for certain clades depending on factors, such as population size, time between speciation events, and properties of the loci being considered (Leaché & Rannala, 2010, Knowles & Kubatko, 2011, Liu & Yu, 2011). As DNA sequencing becomes more

common in phylogenetics, excess data of questionable utility may quickly arise. It is therefore important to be selective of regions to sequence and analyse; those offering the most efficient steps towards species resolution should be targeted. Due to varying evolution rates inherent within different loci, only a subset of loci are suitable targets for phylogenetic questions. Knowledge and evidence of precisely which genes or regions of genomes sequenced in future are useful for certain questions is therefore becoming increasingly important as data begins to exceed the rate at which it can be analysed appropriately (Lemmon & Lemmon, 2013). This knowledge will ease the process of separating phylogenetically informative data from non-informative data. Hence, while Chapter 4 tested the efficacy of *Mcm7* for PM diagnostics, this chapter will continue the investigation by analysing the region *Tsr1* in an identical fashion.

Tsr1 is a gene required for rRNA accumulation during biogenesis of the ribosome (Gelperin *et al.*, 2001). Since its phylogenetic utility was highlighted in 2008, it has been tested and proven across a wide range of Pezizomycotina (Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes and Sordariomycetes (Schmitt *et al.*, 2009)), the Kickxellomycotina (Tretter *et al.*, 2013), and *Lasallia* (Sadowska-Deś *et al.*, 2013) amongst few others. Schmitt *et al.* (2009) in particular showed *Tsr1* was able to resolve both large and fine scale phylogenetic relationships and that sequences were alignable across a wide range of unrelated taxa while having sufficient variability to resolve within-genus relationships. However, hypervariable introns were evident and greatly reduced phylogenetic utility of the region (Schmitt *et al.*, 2009).

In this chapter the possibility of developing working markers for the *Tsr1* region for PMs was investigated and the value of resultant data for phylogenetic reconstruction explored. The resolution of this region in DNA barcoding studies was compared with the standard ITS region.

6.2: Materials and methods

6.2.1: Sample collection – The Powdery Mildew Citizen Science Scheme

Samples were collected via the powdery mildew citizen science scheme (Chapter 2). Eighty-eight of these were successfully amplified (Appendix 5) in the study outlined in this chapter.

6.2.2: Putative species identification

The techniques outlined in Chapters 2, 3, and 4 formed the basis of the identities of PM species used in this chapter.

6.2.3: DNA extraction

The DNA extracted for initial PM species identification (section 3.2.3) was used for these further analyses.

6.2.4: Data mining for *Tsr1* molecular markers

PM genomes (Max Planck Institute for Plant Breeding Research, Spanu *et al.*, 2010, Jones *et al.*, 2014) were aligned with eight closely related species (Table 6.1) in order to identify the location of the *Tsr1* region and then develop primers to trial in the amplification of samples. A total of 14 primers (eight forward, six reverse) were designed (Table 6.2) and ordered as in section 4.2.4.

Table 6.1: *Tsr1* sequences imported from GenBank for identification of *Tsr1* region in PM genomes and design of PM specific primers

Species	**GenBank Accession No.**
Ceratocystis adiposa	KC590615.1
Ceratocystis coerulescens	KC590618.1
Ceratocystis platani	KC590627.1
Ceratocystis smalleyi	KC590632.1
Neurospora crassa	XM_951859.2
Verticillium dahliae	XM_009657978.1
Thielaviopsis australis	KC405314.1
Thielaviopsis basicola	KC405318.1

Table 6.2: Primers designed for trialling amplification and sequencing of *Tsr1* region of PMs

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
Tsr1F1	F	GGWGTCTTACTRGAYGAYCAYCATTA	26	59.51	42.31
Tsr1F2	F	GGWGTCTTACTRGAYGAYCA	20	53.08	45
Tsr1F3	F	TCTTACTRGAYGAYCAYCATTA	22	53.59	36.36
Tsr1F4	F	CTRCAYCCAMAAGTDCTRGC	20	56.05	50
Tsr1F5	F	CGGTAYCGAGGAYTRAAGAG	20	55.1	50
Tsr1F6	F	TGCTVCGYCATGARCAWAA	19	54.58	42.11
Tsr1F7	F	AGYTCYGAYTAYCCRGARCC	20	58.3	55
Tsr1F8	F	ATMAARTCTAAARCYGA	17	41.17	23.53
Tsr1R1	R	TTWTGYTCATGRCGBAGCA	19	60.45	52.63
Tsr1R2	R	GGYTCYGGRTARTCRGARCT	20	58.3	55
Tsr1R3	R	TCRGYTTTAGAYTTKAT	17	44.15	29.41
Tsr1R4	R	ACGRGRTCCGCAYTGWAG	18	58.72	61.11
Tsr1R5	R	AYGCGYTTAGCAATYACYCT	20	57.92	45
Tsr1R6	R	AGYTGYAGDGCCTTRAACCAWTC	23	60.62	47.83

6.2.5: PCR and sequencing protocol

PCR was carried out using the newly designed PM specific primers of the *Tsr1* region (Table 6.2). All 36 possible combinations expected to amplify a product of more than 200 bp were trialled in 25 µl mixes of 12.5 µl BioMix™ Red (Bioline), 0.5 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 ng μl^{-1}, 9.25 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}. Cycling parameters were adapted from Amrani and Corio-Costet (2006) with an initial denaturation step of 95 °C for five minutes, followed by 37 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for one minute, and elongation at 72 °C for one and a half minutes and a final elongation at 72 °C for five minutes. Four samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003), were initially trialled.

The PCR products were separated and visualised as in section 3.2.4.

The five primer combinations exhibiting the highest amplification success (number of products x product strength) were Tsr1F1 and Tsr1R5, Tsr1F1 and Tsr1R6, Tsr1F2 and Tsr1R5, Tsr1F2 and Tsr1R6, and Tsr1F3 and Tsr1R6. These were trialled with five new PM samples previously shown to have been successfully amplified and sequenced using PM specific ITS primers with the same PCR protocol as before. The most successful, single primer combination was Tsr1F1 and Tsr1R6. This amplified a region of approximately 1150 bp. The combination was trialled at a gradient of annealing temperatures from 50-60 °C with 52 °C amplifying a strong, single product most frequently. Reducing the degeneracy of base pairs and length of primer was attempted for greater accuracy in amplification and sequencing of the *Tsr1* region and resulted in the design of primers Tsr1R6a and Tsr1R6b (Table 6.3). Reducing primer length further for sequencing was also trialled, resulting in the design of primers Tsr1F1seq and Tsr1R6seq (Table 6.3). Application of these modified primers produced sequences of lesser quality (this is discussed in section 6.4.1). Primers Tsr1F1 and Tsr1R6 were therefore used in all future amplifications. Single amplicons were sequenced and assembled as in section 3.2.4.

Table 6.3: Primers adapted from Mcm7F2 and Mcm7R8 for more efficient amplification and sequencing of *Mcm7* products

Primer name	**Direction**	**Sequence (5' - 3')**	**Length**	**Mean Tm (°C)**	**Mean GC %**
Tsr1R6a	F	AGYTGYAGGGSCCCTGAACCATTC	24	66.2	50
Tsr1R6b	R	TGYAGGGSCCCTGAACCATTC	21	60.4	52.38
Tsr1F1seq	F	CCAGATGATGARYYYGA	17	48.2	47.1
Tsr1R6seq	R	CARGTRAGYGGTGCCAC	17	55.1	61.8

6.2.6: Sequence alignment

Sequence alignment of *Tsr1* data, and their complementary ITS sequences, was performed using MUSCLE (Edgar, 2004), manually edited such that all bases were in the correct amino acid reading frame. The dataset of 60 sequences of *Tsr1* for which there were ITS equivalents was concatenated using Mesquite (Maddison & Maddison, 2017). The alignment files of the *Tsr1* and the *Tsr1* combined with the ITS rDNA were deposited in TreeBASE as S20955.

6.2.7: Phylogenetic analyses

Phylogenetic analyses were performed as in section 3.2.7.

For BI of *Tsr1* the HKY+I+G model was used and was run for 5,000,000 generations. For BI of the ITS accessions for which there were *Tsr1* equivalents the SYM+G model was used and was run for 5,000,000 generations. For BI of *Tsr1* accessions for which there were ITS equivalents the HKY+I+G model was used and was run for 5,000,000 generations. For BI of the combined dataset (ITS and *Tsr1*) the separate models of individual datasets were used for each region and were run for 3,000,000 generations.

6.2.8: DNA barcoding analysis

Datasets of all *Tsr1* accessions, ITS accessions for which there were *Tsr1* equivalents, *Tsr1* accessions for which there were ITS equivalents, and concatenated ITS and *Tsr1* accessions were analysed and treated as in section 3.2.8.

6.3: Results

6.3.1: *Tsr1* amplification and sequencing

Fifteen of the 36 possible combinations of 14 primers (eight forward and six reverse) resulted in at least a single product from the five initial PM samples trialled. The combination of Tsr1F1 and Tsr1R6 (Figure 6.1) produced the most bands of greatest intensity. After optimisation of PCR, 291 (76%) of 385 trialled samples resulted in a product being visualised using gel electrophoresis. Sequencing resulted in 83 (64%) of the 130 samples sent for sequencing producing readable sequences. However, this could be broken down to 79 (75%) of 105 samples successfully sequenced in 2015 and just four (16%) of 25 samples successfully sequenced in 2016. These were contributed to GenBank (Accession numbers KY786477 – KY786550 (presented in Appendix 5)). Sequencing worked in both forward and reverse directions. Just six (24%) of 25 samples sent for sequencing in 2016 produced sequences and only two of these were longer than 400 bp. Across the whole study period, those which were unsuccessful were characterised by weak reads, resulting in little or no sequence data, or messy reads, potentially contaminated with more than one PM species or additional conspecific fungi.

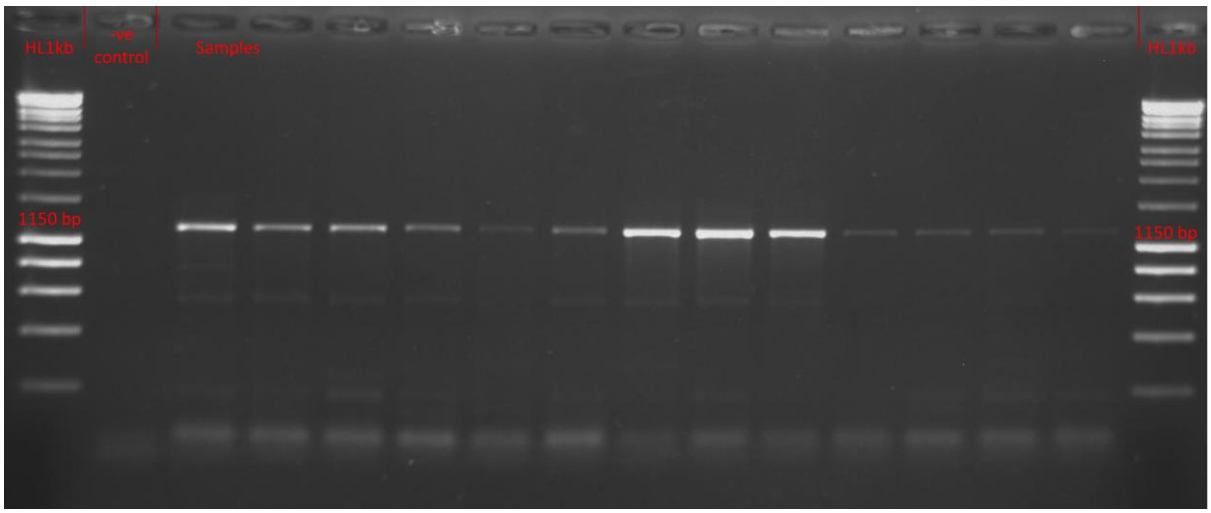

Figure 6.1: Amplification of 1150 bp product of *Tsr1* with primers Tsr1F1 and Tsr1R6 with HyperLadder™ 1kb and negative control.

Accessions were successfully amplified and sequenced from the *Podosphaera, Sawadaea, Golovinomyces, Neoerysiphe,* and *Erysiphe* genera. Accessions were amplified but failed to produce

readable sequences from genera *Blumeria, Phyllactinia* and *Arthrocladiella,* potentially due to the specificity of the used primers.

GenBank sequence data from the *Tsr1* region of PMs is based solely upon available PM genomes (*B. graminis, G. orontii, E. pisi,* and *E. necator*). This dearth of GenBank data meant that identifications of PM accessions based on BLAST were not possible. When BLAST was optimised for finding 'highly similar sequences' searches returned 'no significant similarity' 90% of the time (75 out of 83) and matched the *Tsr1* region of various ascomycete fungi (including *Aspergillus*, *Fusarium*, *Glarea*, *Metarhizium*, and *Sphaerulina*) with identities and query covers ranging from 20-99% of the submitted sequence.

6.3.2: Sequence alignment

All 83 sequences were included in the initial sequence alignment. This was reduced to 77 sequences as a result of poor sequence quality and short sequence reads. Alignment resulted in a region of 1258 bp. This was trimmed to 1058 bp in order to remove gaps and poor quality sequence reads near the primer sites and leave sequences of equal size for later analyses. The region was 23.8% conserved for all species, 34.2% conserved within tribe Cystotheceae, 36.7% conserved within tribe Golovinomyceteae, and 53.0% conserved within tribe Erysipheae. This compared to 75.6% in the ITS, 38.8 % in the *Mcm7*, and 50.6% in the β-tubulin. There were 60 accessions with both *Tsr1* and ITS sequences. *Tsr1* sequences were concatenated with ITS (791 bp) for analysis resulting in 1849 bps.

6.3.3: Phylogenetic analyses

6.3.3.1 Tsr1

BI of PM phylogeny using 77 samples of the 1258 bp region within the *Tsr1* region resulted in clear discrimination of each PM genus. The Cystotheceae (Clade A) (PP 100%) and Erysipheae (Clade C) (PP 100%) had strong support (Figure 6.2). Genera of the Golovinomyceteae (Clade B) (*Arthrocladiella*, *Golovinomyces*, and *Neoerysiphe*) were underrepresented; with just four accessions. However the tribe also had strong support (PP 100%). Accessions of *Golovinomyces* and *Neoerysiphe* were

grouped paraphyletically, alongside two suspected outlying accessions of *P. clandestina*. Within clade B, the three accessions were shown to be monophyletic (Table 6.4). However a previously unidentified accession on *Arctium minus* also grouped within the clade and is therefore most likely to be *G. depressus*. Such identification would mean that the two accessions of *G. depressus* were polyphyletic. A second *N. galeopsidis* accession grouped amongst the *Erysiphe* spp.

Within the Cystotheceae (clade A), *Podosphaera* species formed few polytomies. Five of the ten taxa were shown to be monophyletic (Table 6.4). While three of the four accessions of *P. fugax* were monophyletic, the fourth was sister to this group and the three accessions of *P. pannosa*. Accession 5_77 ex Rosaceae was newly identified as *P. tridactyla* and the three accessions of this species were paraphyletically grouped within this tribe. Accession 5_109 ex *Filipendula ulmaria* can now be identified as *P. filipendulae* rather than *P. spiraeae*. This accession forms a cluster with *P. aphanis* (PP 54%). Three accessions previously identified as *Sawadaea* spp. were positioned in clade C, amongst the *Erysiphe* spp. and three accessions previously identified as *P. clandestina* were positioned in clade B and C.

The Erysipheae (clade C) separated largely into individual species. Nine of the 14 taxa were monophyletic (Table 6.4). A large clade based largely on *E. alphitoides* (12 accessions) also included *E. euonymicola* (four accessions), *E. lonicerae* (two accessions), and *E. aquilegiae* (one accession), as well as outlying accessions of *P. clandestina* (one accession), *S. bicornis* (two accessions), and *N. galeopsidis* (one accession). An accession of *E. buhrii* was grouped amongst ten accessions of *E. heraclei*.

Table 6.4: Monophyletic PM groups from *Tsr1* data within the Erysiphales.

Species within group	Number of Accessions	PP of group*
P. tridactyla	2	96
P. leucotricha	3	
P. aphanis	5	
P. filipendulae	1	
P. epilobi	1	
P. mors-uvae	2	100
P. fugax	4	41
P. pannosa	3	
G. depressus	1	
G. verbasci	1	
N. galeopsidis	1	
E. arcuata	1	
E. necator	1	
E. lonicerae	1	
E. alphitoides	12	100
E. euonymicola	4	
P. clandestina	1	
E. aquilegiae	1	
S. bicornis	2	
N. galeopsidis	1	
E. platani	1	
E. cruciferarum	4	100
E. berberidis	1	
E. pisi	1	
E. ludens	1	
E. trifoliorum	3	89
E. heraclei	10	100
E. buhrii	1	

*Groups with a single accession have no PP and are shaded in grey.

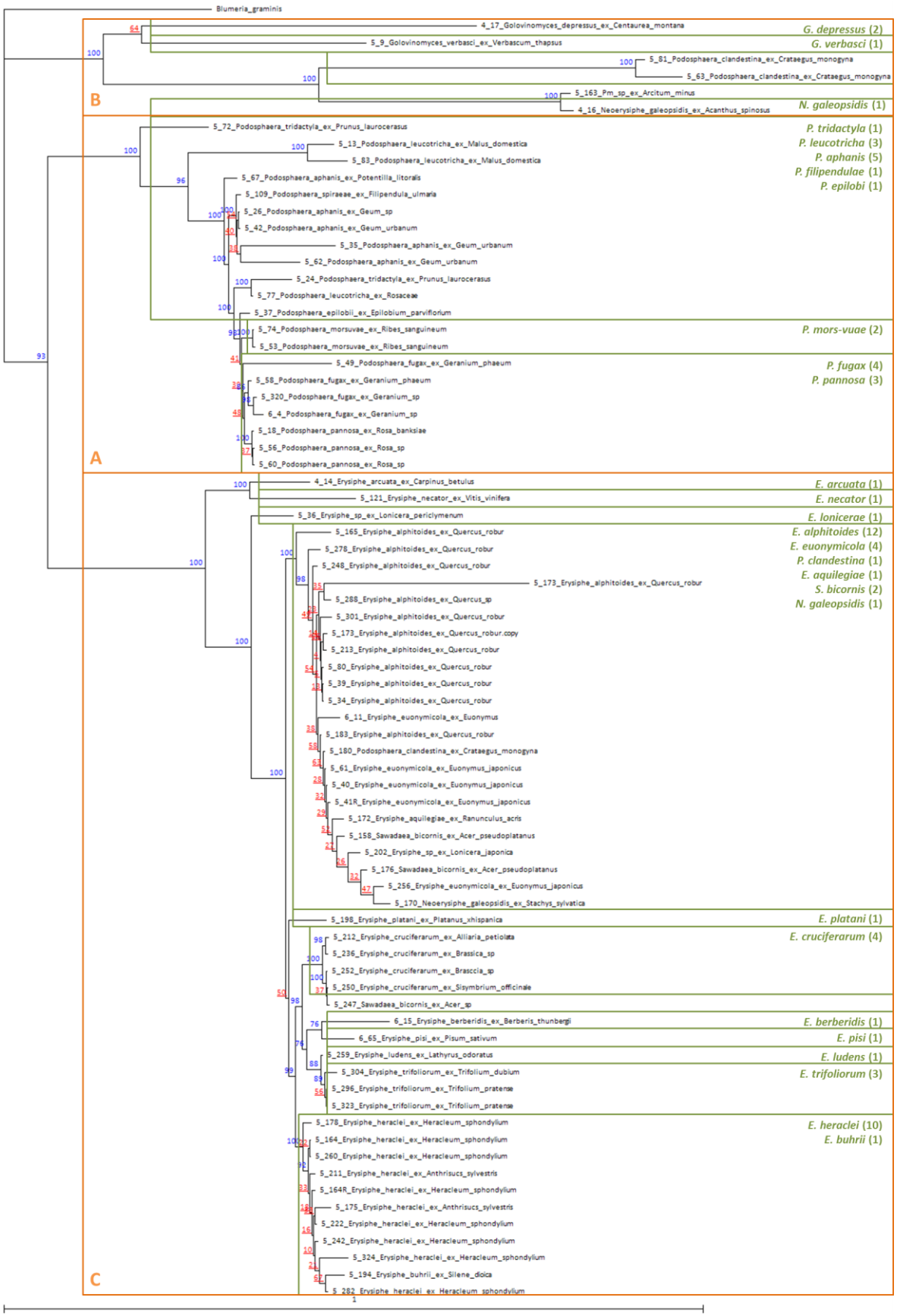

Figure 6.2: BI using 77 sequences of the *Tsr1* region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

6.3.3.2 Direct comparison of ITS and *Tsr1*

BI of the PM phylogeny of 60 accessions of the *Tsr1* region (Figure 6.4) resulted in an overall topology similar to that of the phylogeny of 60 accessions of the ITS region (Figure 6.3). The main difference was the number of outliers. As in Figure 6.2, Figure 6.4 also placed two accessions of *P. clandestina* within the Golovinomyceteae (clade B), an accession of *P. clandestina*, *E. aquilegiae, N. galeopsidis,* and three accessions of *S. bicornis* within tribe Erysipheae (clade C). Each of these accessions was positioned as expected, amongst its congenerics in Figure 6.3.

The remaining differences regard the grouping of accessions of different species: ITS outperforms *Tsr1* as it shows clear separation between *E. heraclei* and *E. buhrii*, as well as *E. lonicerae* and *E. aquilegiae* from the *E. alphitoides – E. euonymicola* group. However, unlike ITS, *Tsr1* separates *E. platani* from this *E. alphitoides – E. euonymicola* group. Neither region consistently separates *E. alphitoides* from *E. euonymicola*.

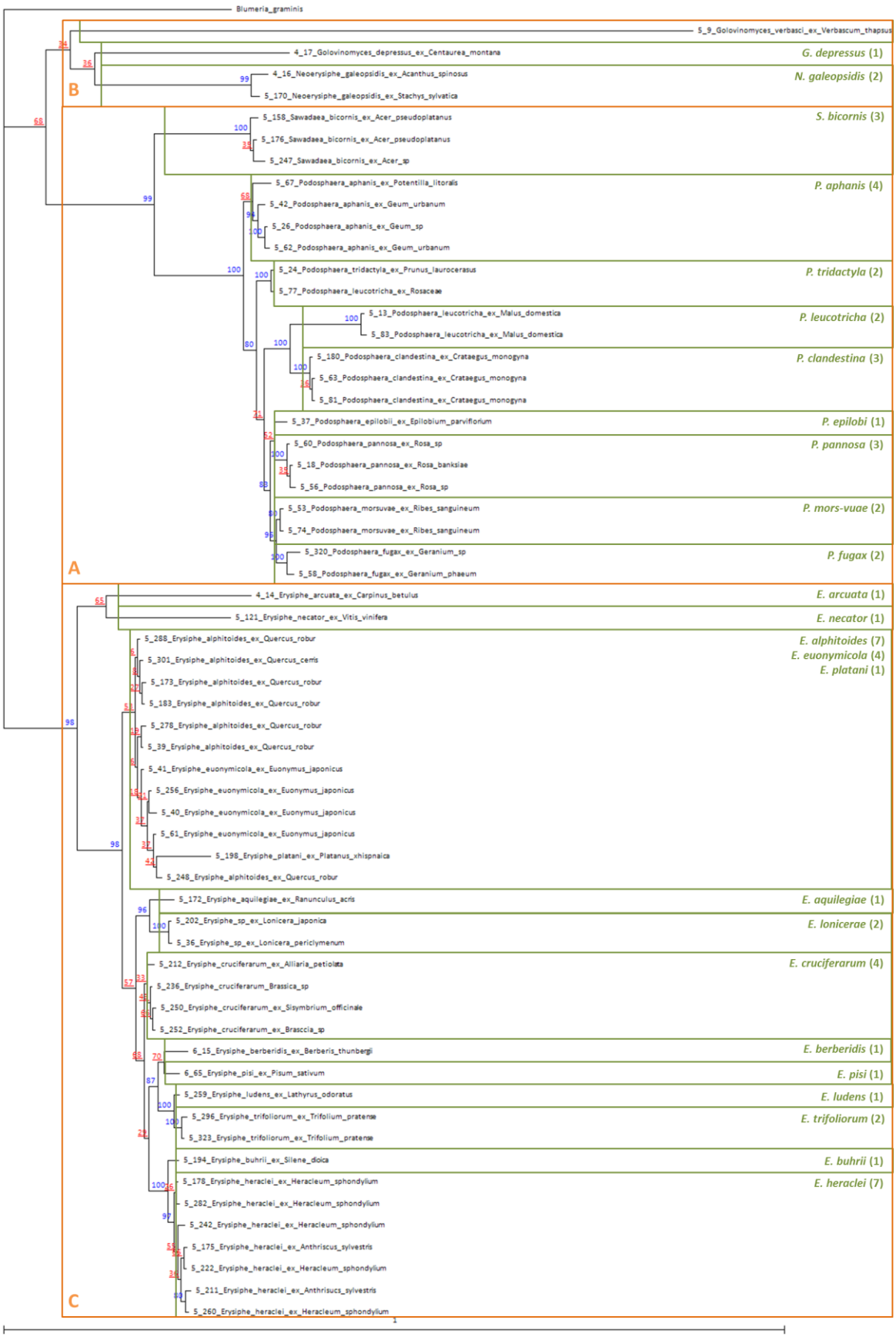

Figure 6.3: BI using 60 sequences of the ITS region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

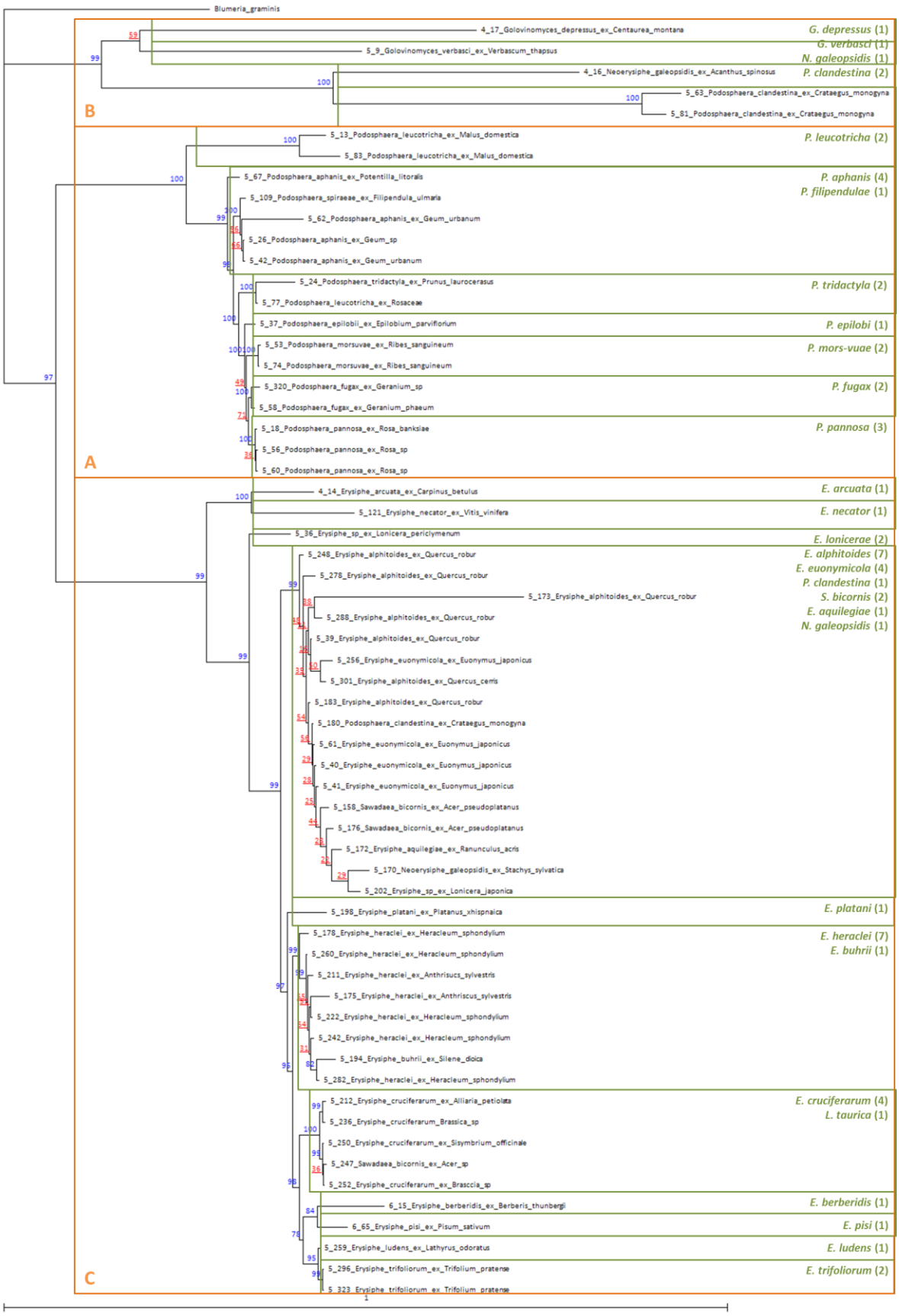

Figure 6.4: BI using 60 sequences of the *Tsr1* region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

6.3.3.3 Combined ITS and *Tsr1* phylogeny

BI of PM phylogeny using 60 accessions of the combined 1849 bp region of the ITS and *Tsr1* regions resulted in clear discrimination of each PM genus (Figure 6.5). The Cystotheceae (A) (PP 98%) and Erysipheae (C) (PP 84%) were monophyletic. Outlying accessions for *Tsr1* remained problematic: the similarity of *Tsr1* sequences of an accession of *P. clandestina*, *E. aquilegiae, N. galeopsidis,* and three accessions of *S. bicornis* resulted in their placement at the base of the Erysipheae (clade C). Two accessions of *P. clandestina* remained within the Golovinomyceteae (clade B).

Within the Cystotheceae (clade A), *Podosphaera* species formed few polytomies. Seven of the 11 taxa were monophyletic (Table 6.5). Newly identified *P. filipendulae* remains grouped with the *P. aphanis* and three of the four *P. aphanis* accessions are grouped together while the fourth forms a polyphyletic sister group. The three accessions previously identified as *Sawadaea* spp. and one of *P. clandestina* were positioned at the base of the Erysipheae (clade C), and two accessions of *P. clandestina* remain grouped amongst the Cystotheceae (clade A).

Within Erysipheae (clade C) nine of the 13 taxa remain separated into monopyletic groups of individual species (Table 6.5). Groupings of *E. alphitoides* (seven accessions), and *E. euonymicola* (four accessions) sit at the base of numerous monophyletic taxa with two accessions of *E. lonicerae* placed monophyletically either side of it. Seven accessions of *E. heraclei* form a monophyletic group with one of *E. buhrii*.

Table 6.5: Monophyletic PM groups from *Tsr1* data within the Erysiphales.

Species within group	Number of Accessions	PP of group*
P. clandestina	2	97
P. leucotricha	2	100
P. aphanis	1	
P. aphanis *P. filipendulae*	3 1	100
P. tridactyla	2	99
P. epilobi	1	
P. mors-uvae	2	100
P. fugax	2	99
P. pannosa	3	100
S. bicornis	3	97
G. depressus	1	
G. verbasci	1	
N. galeopsidis	1	
E. aquilegiae	1	
E. lonicerae	1	
E. lonicerae	1	
E. arcuata	1	
E. necator	1	
E. platani	1	
E. heraclei *E. buhrii*	7 1	100
E. cruciferarum	4	97
E. berberidis	1	
E. pisi	1	
E. ludens	1	
E. trifoliorum	2	100

*Groups with a single accession have no PP and are shaded in grey.

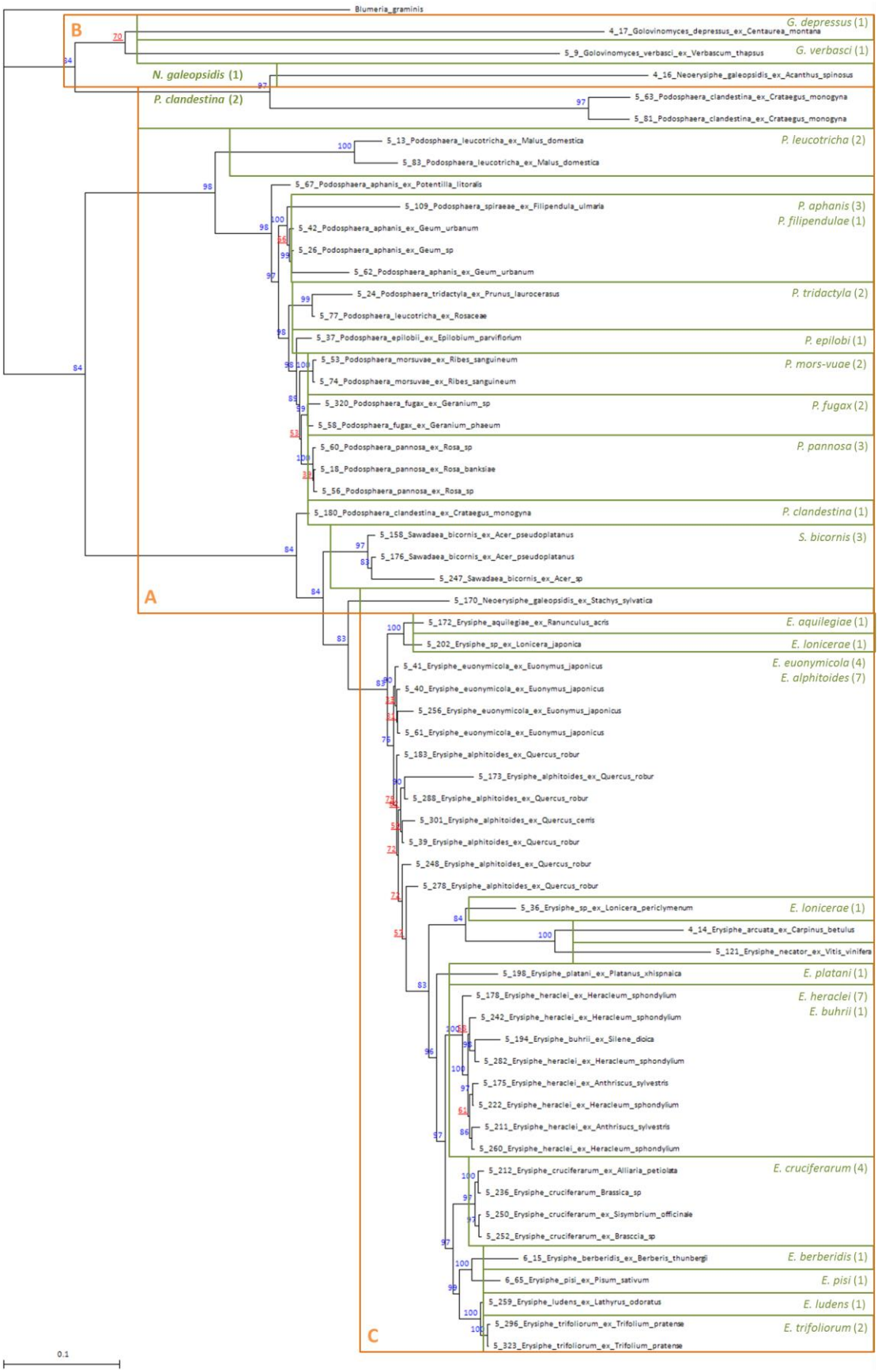

Figure 6.5: BI using 60 sequences of the ITS and *Tsr1* regions combined. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

6.3.4: DNA barcoding analysis

6.3.4.1 Tsr1

Intra and interspecific differences were quantified for the 73 accessions and 28 species of the *Tsr1* region. This resulted in a total overlap of 22.73% (from 0.3% to 23.04%, covering 86.52% of all intra and interspecific but intrageneric sequences) (Figure 6.6a). Accessions with a mean of more than 5% intraspecific difference were *S. bicornis*, *P. leucotricha, P. pannosa*, *P. aphanis*, *P. clandestina*, *E. alphitoides*, and *E. lonicerae*. There were 432 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (62.5%) and *Podosphaera* (37.5%). The only interspecific pair below 0.5% difference was *E. alphitoides* and *E. euonymicola*.

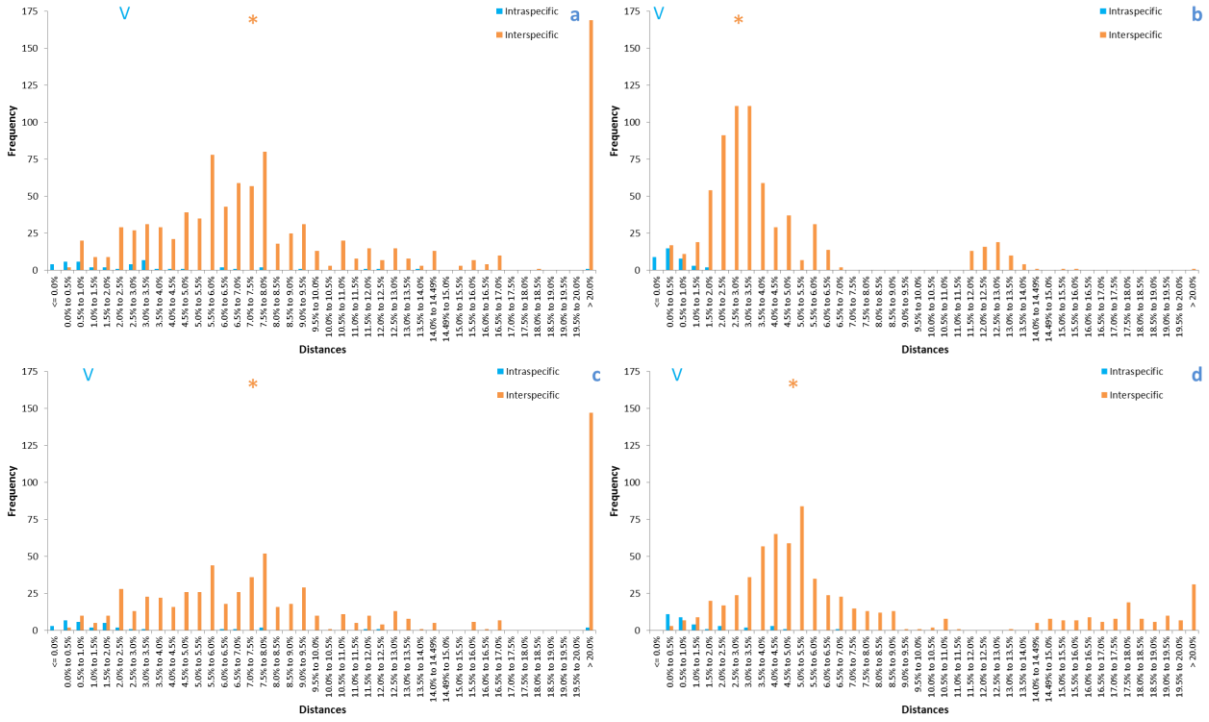

Figure 6.6: The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of (a) 73 *Tsr1* accessions, (b) 60 ITS accessions common to the ITS and *Tsr1* regions, (c) 60 *Tsr1* accessions common to the ITS and *Tsr1* regions, and (d) 60 accessions of the ITS and *Tsr1* regions combined. Blue chevrons (V) mark the mean intraspecific variation per locus and orange asterisks (*) mark the mean interspecific variation per locus.

6.3.4.2 Direct comparison of ITS, *Tsr1*, and combined datasets

6.3.4.2.1 ITS

Intra and interspecific differences were quantified for the 60 accessions and 26 species of the ITS region. This resulted in a total overlap of 1.42% (from 0.14% to 1.56%, covering 11.06% of all intra and interspecific but intrageneric sequences) (Figure 6.6b). There were no accessions with a mean of more than 5% intraspecific difference. The largest mean intraspecific difference was 1.57% in *P. aphanis*. There were 1,078 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (77.7%) and *Podosphaera* (22.3%). The only interspecific pair below 0.5% difference was *E. alphitoides* and *E. euonymicola*.

6.3.4.2.2 Tsr1

Intra and interspecific differences were quantified for the 60 accessions and 26 species of the *Tsr1* region. This resulted in a total overlap of 25.58% (from 0.3% to 25.89%, covering 88.61% of all intra and interspecific but intrageneric sequences) (Figure 6.6c). Accessions with a mean of more than 5% intraspecific difference were *S. bicornis*, *P. tridactyla, P. leucotricha, P. pannosa*, *P. aphanis*, *P. clandestina*, *E. alphitoides*, and *E. lonicerae*. There were 310 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (55.5%) and *Podosphaera* (44.5%). The only interspecific pair below 0.5% difference was *E. alphitoides* and *E. euonymicola*.

6.3.4.2.3 Combined ITS and *Tsr1*

Intra and interspecific differences were quantified for the 60 accessions and 26 species of the combined ITS and *Tsr1* regions. This resulted in a total overlap of 6.38% (from 0.41% to 6.8%, covering 68.96% of all intra and interspecific but intrageneric sequences) (Figure 6.6d). There were no accessions with a mean of more than 5% intraspecific difference. The largest mean intraspecific differences were 4.5% in *E. alphitoides* and *P. leucotricha*. There were 594 interspecific, congeneric distances that fell below 5%; the most common of these were between accessions of the *Erysiphe*

(80.5%) and *Podosphaera* (19.5%). The only interspecific pair below 0.5% difference was *E. alphitoides* and *E. euonymicola*.

6.4: Discussion

6.4.1: *Tsr1* amplification and sequencing

Markers for this region and a protocol for their use were successfully designed. Given the dearth of previous sequence data for the *Tsr1* region, it was vital to locate the region within available PM genomes using samples of closely related, ascomycetous fungi. *Tsr1* sequences from other fungi ensured that the region was accurately located and their alignment proves them to be reliably identified. The available PM genomes have proven to be of great use for design of PM primers for each region tested thus far; *Tsr1* was no exception as NCBI GenBank Nucleotide megaBLAST showed amplified and sequenced samples to match those of the same region of other Ascomycetes. The specificity of designed primers could not be guaranteed as sample genomes covered just three out of 12 genera: *Blumeria, Erysiphe,* and *Golovinomyces*. The use of degenerate base pairs within the primers was necessary in order to maximise the likelihood of amplification and sequencing and it was hoped that these would also accommodate for the remaining PM genera whose DNA sequences were unknown at this point. Samples from the most common PM genera have been amplified and sequenced. The lack of success with *Phyllactinia* and *Arthrocladiella* spp. may be due to sequencing error and must be trialled further. Amplification and sequencing of a wider array of accessions is necessary in order to fully evaluate the performance of the protocol and primers designed in the current study. Tests on herbarium specimens and rarer and more exotic PM genera and species such as the *Cystotheca, Pleochaeta,* and *Leveillula*, will complete the evaluation.

Amplification of multiple products in most accessions before optimisation may indicate that the primer combination is not as specific to PMs as hoped; amplifying additional accessions of conspecific or mycoparasitic fungi as well as the targeted PM. This was overcome through optimisation of the PCR. Reduction of ambiguity and length within the primer pair Tsr1F1 and Tsr1R6 after additional sequence data was acquired, resulted in a reduction in amplification and sequencing success; potentially due to the variability within the region. Primer refinement should be trialled

further with primers designed for individual PM tribes if necessary. Throughout 2015 the proposed markers were shown to consistently provide positive outcomes in amplification and sequencing. However, a change of sequencing company in 2016 and their PCR product requirements (from Source BioScience requiring less than 10 µl to GATC requiring at least 20 µl) affected sequencing success significantly, as discussed in Chapter 5.

6.4.2: Phylogenetic analyses

Analyses of the *Tsr1* region resulted in a phylogeny close to that expected and therefore confirmed the findings of studies evaluating this region (Aguileta *et al.*, 2008, Schmitt *et al.*, 2009, Sadowska-Deś *et al.*, 2013, Tretter *et al.*, 2013), however, whether it improves on established regions such as the ITS is debatable. Phylogenetic computation time is similarly slow to other phylogenetic analyses of this size (see Chapter 4) and although the result was similar to accepted phylogenies of Braun and Takamatsu (2000) certain differences are evident. The overall topology is similar to that of the accepted ITS region. However, sequences previously identified as *P. clandestina, N. galeopsidis,* and *S. bicornis* were shown grouped in unexpected positions within the phylogeny. Their new positions were unlikely to be indicative of sequencing of alternative PM species, as their hosts have not been known to harbour the species inferred. Mixing or contamination of accessions could explain the erroneous sequence of *N. galeopsidis*. However, this was tested for and unexpected results remained on all three *P. clandestina* and all three *S. bicornis* accessions inferred another meaningful explanation; the accessions may have also hosted additional PM species. Two of the *P. clandestina* accessions were grouped within tribe Golovinomyceteae, as sister to *N. galeopsidis*, while the third was placed within the *E. alphitoides – E. euonymicola* group. Two *S. bicornis* accessions were placed within the *E. alphitoides – E. euonymicola* group, while the third was most closely related to *E. cruciferarum*. Product sizes after amplification and sequencing of these accessions are similar to those of all other amplified and sequenced PM samples, therefore discounting erroneous sequences of alternative Fungi. Presence of alternative copies of the *Tsr1* gene are also unlikely to explain these discrepancies, as the region is known to be single-copy (Gelperin *et al.*, 2001, Aguileta *et al.*, 2008).

This was confirmed via observation of the whole genome alignments. The hypervariable nature of the region is therefore the most plausible explanation. This has been cited in the past and is therefore known to be the most problematic feature of the region when considering it for phylogenetic reconstruction (Schmitt *et al.*, 2009).

Tsr1 showed evidence that it was inferior to ITS for phylogenetic reconstruction. Although the overall *Tsr1* phylogeny was accurate, it did not perform better than the ITS at any phylogenetic level and grouped closely related *Erysiphe* and *Podosphaera* spp. together when ITS showed them to be distinct. Combined BI of ITS and *Tsr1* was superior to that of the *Tsr1* alone, but inferior to the ITS as indistinct groupings remnant from *Tsr1* and sequences of hypervariable samples resulted in a less accurate, less resolved phylogeny. Due to its variability, *Tsr1* may be able to improve the resolution of closely related species of unsampled PM genera, however its current performance within the *Podosphaera* and *Erysiphe* genera shows little to no evidence to support this. This is contrary to the findings of Schmitt *et al.* (2009) who predicted *Tsr1* to perform as well as *Mcm7* within the Ascomycetes and both to be superior to other regions in current usage. Along with *Mcm7*, the variable nature of *Tsr1* should set it apart from commonly used ribosomal markers, such as ITS or the mitochondrial small subunit (mtSSU); all these regions have the power to resolve phylogenetic relationships at generic levels, but ribosomal markers are likely to yield ambiguous and saturated alignments, when used to compare distantly related taxa. The use of *Tsr1* alongside the routinely used dataset of ITS failed to improve the power of phylogenetic resolution for PM fungi. The use of sequences from the distantly related taxon *Homo sapiens* as outgroups by Aguileta *et al.* (2008) has indicated that *Tsr1* might be useful for phylogenetic studies outside of the fungal kingdom. This should be tested further, however the current result implies it to perform worse than other tested experimental and established regions.

6.4.3: DNA barcoding analysis

None of the analyses within this chapter produced the barcoding gap between intra and interspecific K2P distances (Quaedvlieg *et al.*, 2014). The low K2P overlap desired for a useful barcoding region was more evident in ITS than the experimental region *Tsr1*. This was characterised by the large intraspecific variation of *Tsr1* resulting in 88.6% of sample sequences overlapping with up to 25% variation both intra- and interspecifically. This compares with just 11.0% of ITS samples overlapping with just 1% variation and 69% of combined ITS and *Tsr1* samples overlapping with 6.4% variation. This result shows ITS to be the most suitable region for DNA barcoding, therefore agreeing with its proposal as a universal barcode for Fungi (Schoch *et al.*, 2012). However, Chapters 3, 4, and 5 do not substantiate this result and thus the sampling within this chapter has resulted in a favourable barcoding result for the ITS. This result is due to the hypervariable nature of *Tsr1* (Schmitt *et al.*, 2009) compared to the highly conserved nature of ITS (Takamatsu *et al.*, 2015a); neither is a suitable barcoding region. Great variability is seen intra- and interspecifically in *Tsr1*. Species known commonly to lack clear boundaries due to their intraspecific variation and interspecific similarities, such as *E. alphitoides*, are included in those contributing to the high level of K2P overlap. Other species, which usually show clear separation from conspecifics, thanks to conserved intraspecific sequences, such as *S. bicornis*, *P. tridactyla, P. leucotricha, P. pannosa*, *P. aphanis*, *P. clandestina*, and *E. lonicerae* are also included in this.

This result contrasts with the few studies to have explored the utility of *Tsr1* (Schmitt *et al.*, 2009, Sadowska-Deś *et al.*, 2013, Tretter *et al.*, 2013). This is largely down to the 'hypervariability' of *Tsr1* sequences, particularly within the Cystotheceae which showed just one third of all bases to be conserved; far less than half the bases conserved within the Erysipheae.

6.5: Conclusions

The *Tsr1* region has proved to be a far less valuable addition than regions *Mcm7* and β-tubulin for improvements to PM diagnostics. Amplification and sequencing of the *Tsr1* region was made possible by previous sequence data of PM genomes and *Tsr1* regions of other fungi. However, the success of this process has varied across the years of study, potentially due to varying sequencing protocols of different sequencing companies. Obtained *Tsr1* sequences result in a topology similar to that accepted for PMs. However, numerous outliers were also sequenced and BI was therefore less accurate than that of the established ITS region. ITS also outperforms *Tsr1* when analysed for K2P overlap. With greater time and resources the *Tsr1* region may prove to be useful for differentiation of closely related PM species and differentiation of species complexes. However, regions *Mcm7* and β-tubulin should receive greater initial attention due to their greater performance on all levels.

Chapter 7: Augmenting current ID techniques with Actin

7.1: Introduction

Many fungal ascomycetous clades have been shown to require numerous regions to fully resolve phylogenies (Trierveiler-Pereira *et al.*, 2014, Trouillas *et al.*, 2015, Birkebak *et al.*, 2016). This chapter will continue to explore potential regions for improving this process.

The Actin gene is typically a highly expressed gene (McElroy *et al.*, 1990) with a coding region of at least 1000 bp (Reece *et al.*, 1992). It is conserved throughout the eukaryotes and its function in host plants has been shown to be a major contributor to non-host resistance; exemplified by studies in *Arabidopsis* (Yun *et al.*, 2003) and barley (Opalski *et al.*, 2005). Due to its variation over time, the region has received attention as a molecular clock gene for studies examining organism phylogeny (Reece *et al.*, 1992) and has proven to be superior to ITS to identify closely related taxa of ascomycetous yeasts (Daniel & Meyer, 2003), genera of the Mucorales and Mortierellales (Voigt & Wöstemeyer, 2001), and *Candida* species (Daniel *et al.*, 2001). It has also been used for deep level phylogenies (Baldauf *et al.*, 2000, Voigt & Wöstemeyer, 2000). Other studies however, have shown Actin gene sequences to be insufficient for the differentiation of sugar beet pathogen species (Weiland & Sundsbak, 2000) and not to provide species-level resolution in *Mycosphaerella* (Hunter *et al.*, 2006).

The phylogenetic use of Actin within the PMs is yet to be quantified. The research reported in this chapter therefore aimed at investigating the potential of Actin DNA sequences for complementing the morphological and ITS analyses which have become ubiquitous in PM identification. The study used accessions sourced from the Powdery Mildew Survey (Chapter 2).

The possibility of using primers sourced from the literature for the Actin region for PMs was investigated in this chapter. The possibility of developing new working markers for the Actin region for PMs was also investigated and the value of resultant data for phylogenetic reconstruction

explored. Finally, the resolution of this region in DNA barcoding studies was compared with the standard ITS region.

7.2: Materials and methods

7.2.1: Sample collection – The Powdery Mildew Citizen Science Scheme

Samples were collected via the powdery mildew citizen science scheme (Chapter 2). One hundred and eighty eight of these were successfully amplified (Appendix 5) in the study outlined in this chapter.

7.2.2: Putative species identification

The techniques outlined in Chapters 2, 3, and 4 formed the basis of the identities of PM species used in this chapter.

7.2.3: DNA extraction

The DNA extracted for initial PM species identification (section 3.2.3) was used for these further analyses.

7.2.4: Generic primer trials

7.2.4.1 Sourcing primers

Primers for the amplification of the Actin region were sourced from previous publications (Carbone & Kohn, 1999, Weiland & Sundsbak, 2000) and ordered from Sigma-Aldrich (Table 7.1).

Table 7.1: Generic primers sourced from literature for trialling amplification and sequencing of Actin region of PMs

Primer name	Direction	Sequence (5' - 3')	Reported Tm (°C)	Product size (bp)	Source
ACT-512F	F	ATGTGCAAGGCCGGTTTCGC	61	300	Carbone and Kohn (1999)
ACT-783R	R	TACGAGTCCTTCTGGCCCAT			
5FWDACT	F	GTATGTGCAAGGCCGGTTT	50-55	1200	Weiland and Sundsbak (2000)
MIDREVACT	R	ATGAGGCAGACCTAGCCACCAAG			

7.2.4.2 PCR and sequencing

Amplification of the two sourced primer combinations was trialled according to published protocols (Carbone & Kohn, 1999, Weiland & Sundsbak, 2000). The PCR products were separated and visualised as in section 3.2.4.

Multiple products per sample were consistently amplified (Figure 7.1). Individual bands were excised, purified using the QIAquick Gel Extraction Kit, and sent to Source BioScience via courier for sequencing.

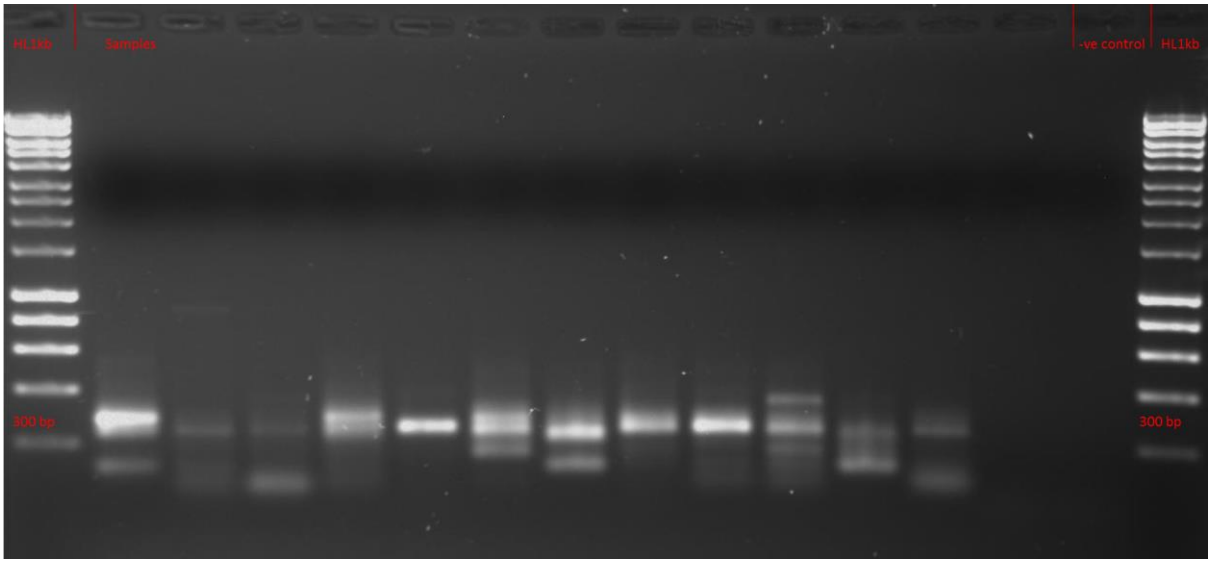

Figure 7.1: Amplification of 300 bp product of Actin with primers ACT-512F and ACT-783R before optimisation of PCR protocol.

Annealing and extension temperatures of PCR protocols were explored using gradient PCR in order to optimise amplification for single products. Single products were amplified for the primer combinations ACT-512F and ACT-783R with PCR protocol from Amrani and Corio-Costet (2006) at an annealing temperature of 60°C (Figure 7.2). Products of more than 10ng per band of preliminary sample amplifications, lacking strong additional amplified products, were purified using the QIAquick PCR Purification Kit and sent to Source BioScience via courier for sequencing.

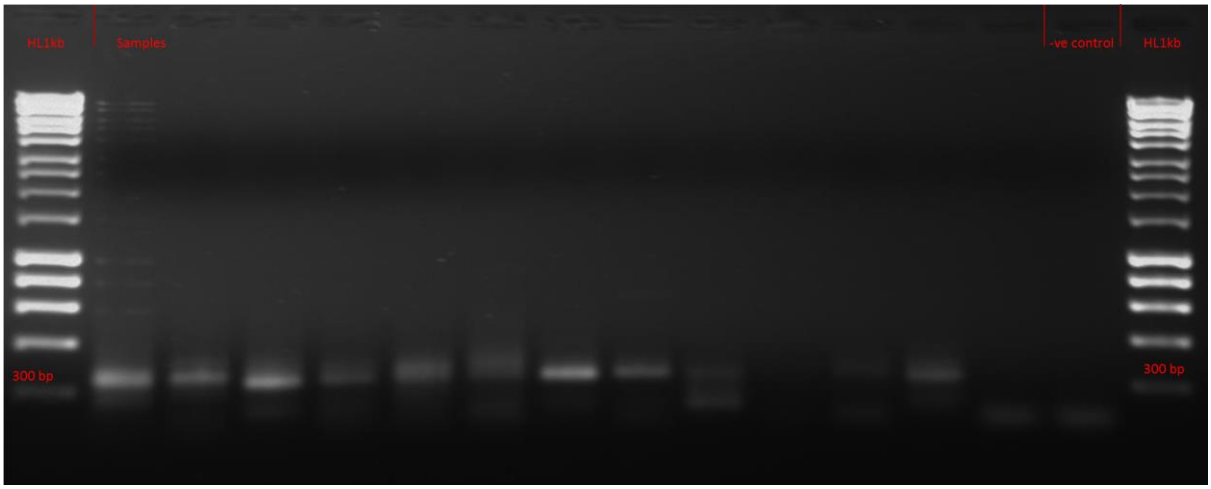

Figure 7.2: Amplification of 300 bp product of Actin with primers ACT-512F and ACT-783R after optimisation of PCR protocol.

Complementary forward and reverse sequences generated in this study were assembled and manually edited using MegAlign software (DNASTAR, Madison, WI, USA). This resulted in products of 240-300 bp for ACT-512F and ACT-783R. NCBI GenBank Nucleotide BLAST for highly similar sequences (megablast) was performed on samples. The results showed no significant similarity to Actin PM sequences. Instead results showed similarity to contaminants from the environmental samples such as: *Ramularia vizellae, Botrytis fuckeliana,* Leotiomycetes spp., and *Lophodermium australe*.

7.2.5: Data mining for Actin molecular markers

PM genomes (Max Planck Institute for Plant Breeding Research, Spanu *et al.*, 2010, Jones *et al.*, 2014) were aligned with six closely related fungal species (Table 7.2) and seven PM samples in order to identify the location of the Actin region. Primers were then developed to trial in the amplification of accessions. A total of 13 primers (eight forward, five reverse) were designed (Table 7.3) and ordered as in section 4.2.4.

Table 7.2: Actin sequences imported from GenBank for identification of Actin region in PM genomes and design of PM specific primers

Species	GenBank Accession No.
Lambertella himalayensis	KF545190.1
Sclerotinia sclerotiorum	KF545187.1
Rutstroemia echinophila	KF545176.1
Lanzia sp.	KF545147.1
Ciboria amentacea	KF545177.1
Leotiomycetes sp.	KF545191.1

Table 7.3: Primers designed for trialling amplification and sequencing of Actin region of PMs

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
ActF1	F	CGTGTTGACATGGCTGGYCGTGATTT	26	68.29	53.85
ActF2	F	TAGCWGARCGYGGCTATAC	19	56.43	52.63
ActF3	F	AAAGARAARYTKTGTTACGTDGC	23	61.3	37
ActF4	F	CTATTGGWAAYGARMGATTYCG	22	62.22	41
ActF5	F	CTYGGYCTCGAAAGYGGTGGYATTC	25	61.18	56
ActF6	F	GAAAGYGGTGGYATTCATGT	20	60.07	47.62
ActF7	F	CAGACCGTATGCAGAAAG	18	57.4	50
ActF8	F	GCWCCATCRTCCATGAAGGTC	21	60.07	52.38
ActR1	R	CTTTCTGCATACGGTCTG	18	57.4	50
ActR2	R	GACCTTCATGGAYGATGGWTC	21	58.72	52.38
ActR3	R	CWGAGTACTTTCKCTCRGGCGG	22	60.11	55
ActR4	R	GAGAGATGCAAGAATAGATCCACC	24	65.7	46
ActR5	R	CTTGYTTRGAAATCCACATYTGCTG	25	64.12	42

7.2.6: PCR and sequencing protocol

PCR was carried out using the newly designed PM specific primers of the Actin region (Table 7.3). All 16 possible combinations of primers more than 250 bp apart were trialled in 25 µl mixes of 12.5 µl BioMix™ Red (Bioline), 0.5 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 ng μl^{-1}, 9.25 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}. Cycling parameters were adapted from Amrani and Corio-Costet (2006) with an initial denaturation step of 95 °C for five minutes, followed by 37 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for one minute, and elongation at 72 °C for one and a half minutes and a final elongation at 72 °C for five minutes. Three

samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003), were initially trialled.

The PCR products were separated and visualised as in section 3.2.4.

The two primer combinations exhibiting the highest amplification success (number of products x product strength) were ActF1 and ActR3 and ActF1 and ActR4. These were trialled for amplification of 18 different samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003). The most successful primer combination was ActF1 and ActR3. This amplified a region of approximately 500 bp. Reducing the degeneracy of base pairs was trialled for greater accuracy in amplification and sequencing of the Actin region and resulted in the design of primers ActF1a, ActR3a, and ActR3b (Table 7.4). Application of these modified primers produced greater amplification success and sequences of greater quality. Primer combination ActF1a and ActR3b was best and was used in all future amplifications with an annealing temperature of 60 °C. Single amplicons were sequenced and assembled as in section 3.2.4.

Table 7.4: Primers adapted from ActF1 and ActR3 for more efficient amplification and sequencing of Actin products

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
ActF1a	F	CGTGTTGACATGGCTGGTCGTGATTT	26	66.16	50
ActR3a	R	CAGAGTACTTTCGCTCGGGCGG	22	65.38	63.64
ActR3b	R	TCAGAGTACTTTCGCTCGGGCGG	23	66.47	60.87

7.2.7: Sequence alignment

Sequence alignment of Actin data, and their complementary ITS sequences, was performed as in section 3.2.6. The dataset of 125 accessions of Actin for which there were ITS equivalents was concatenated using Mesquite (Maddison & Maddison, 2017). The alignment files of the Actin and the Actin combined with the ITS rDNA were deposited in TreeBASE as S20956.

7.2.8: Phylogenetic analyses

Phylogenetic analyses were performed as in section 3.2.7.

For BI of Actin the GTR+I+G model was used and was run for 10,000,000 generations. For BI of the ITS accessions for which there were Actin equivalents the SYM+I+G model was used and was run for 5,000,000 generations. For BI of Actin accessions for which there were ITS equivalents the GTR+I+G model was used and was run for 5,000,000 generations. For BI of the combined dataset (ITS and Actin) the separate models of individual datasets were used for each region and were run for 10,000,000 generations, at a temperature of 0.8 in order to reach the reach the optimal solution most efficiently.

7.2.9: DNA barcoding analysis

Datasets of all Actin accessions, ITS accessions for which there were Actin equivalents, Actin accessions for which there were ITS equivalents, and concatenated ITS and Actin accessions were analysed and treated as in section 3.2.8.

7.3: Results

7.3.1: Actin amplification and sequencing

Thirteen of the 16 possible combinations of 13 primers (eight forward and five reverse) resulted in at least a single product from the three initial PM accessions trialled. The combination of ActF1a and ActR3b produced the most bands of greatest intensity. After optimisation of PCR, 211 (93%) of 226 trialled samples resulted in a product being visualised using gel electrophoresis (Figure 7.3). Sequencing resulted in 146 (86%) of 170 samples producing readable sequences of at least 200 bp. Forty-two of these produced poor sequences when initially sequenced. They therefore required altered sequencing protocols which resulted in longer sequences of greater quality. These were contributed to GenBank (Accession numbers KY786551 – KY786689 (presented in Appendix 5)). Sequencing worked in both forward and reverse directions. Those which were unsuccessful were characterised by short, weak reads. Samples were successfully amplified and sequenced from the *Blumeria, Podosphaera, Sawadaea, Phyllactinia, Arthrocladiella, Golovinomyces, Neoerysiphe,* and *Erysiphe* genera.

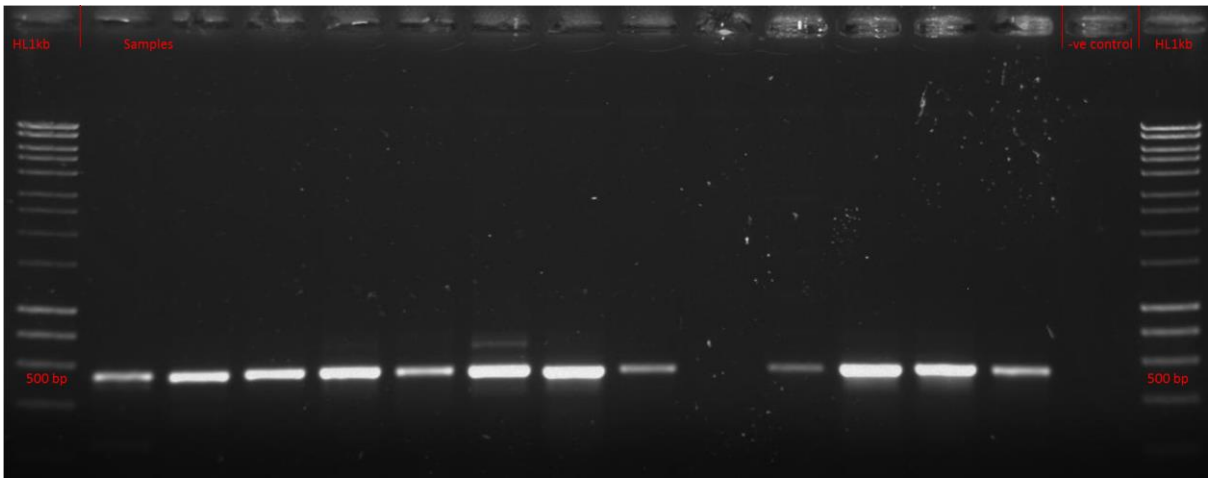

Figure 7.3: Amplification of 500 bp product of Actin with primers ActF1a and ActR3b with HyperLadder™ 1kb and negative control.

GenBank sequence data from the Actin region of PMs is solely based upon PM genomes *B. graminis* and *E. necator*. This dearth of GenBank data meant that identifications of PM samples based on NCBI GenBank Nucleotide BLAST were not possible. When BLAST was optimised for finding 'highly similar sequences' searches returned 'no significant similarity' 6% of the time (9 out of 146). All other sequences matched the Actin region of various Ascomycetous fungi (including *Alternaria, Botrytis*, *Exophiala*, *Fusarium*, *Kluyveromyces*, *Nectria*, *Sarocladium*, and *Trichoderma*) with identities and query covers ranging from 64-99% of the submitted sequence.

7.3.2: Sequence alignment

All 188 sequences were included in the initial sequence alignment. This was reduced to 187 sequences as a result of poor sequence quality. Alignment resulted in a region of 598 bp. This was trimmed to 508 bp in order to remove gaps and poor quality sequence reads near the primer sites and leave sequences of equal size for later analyses. The region was 27.2% conserved. This compared to 75.6% in the ITS. There were 124 accessions with both Actin and ITS sequences. Actin sequences were concatenated with ITS (874 bp) for analysis resulting in 1382 bps.

7.3.3: Phylogenetic analyses

7.3.3.1 Actin

BI of the PM phylogeny using 187 samples of the 508 bp region within the Actin region resulted in phylogenies of low support. The majority of species were grouped polyphyletically across the entire phylogeny. Separation of accessions into three groupings similar to the large PM tribes Cystotheceae (clade A), Golovinomyceteae (clade B) (Figure 7.4), and Erysipheae (clade C) (Figure 7.5), clear in phylogenies of the ITS phylogeny, was apparent, however numerous outliers and low support meant that very little significance could be drawn from these. The single accession of tribe Phyllactinieae (4_70 *Phyllactinia fraxini*) showed evidence of monophyly as it was grouped alone.

A putative clade of species predominantly of the Golovinomyceteae (clade B) had a PP of 58% (Figure 7.4). Within this clade *A. mougeotii,* four accessions (PP 98%), *O. longipes* (one accession), *G.*

cichoracearum (one accession), and *G. fischeri,* two accessions (PP 100%) were monophyletic. The remaining accessions true to the tribe (*G. cynoglossi* (two accessions)*, G. sordidus* (thee accessions)*, G. sonchicola* (three accessions)*,* and *G. orontii* (five accessions)) were polyphyletic. Six accessions on hosts of *Plantago* spp. previously identified as *G. sordidus* (three accessions) and *P. plantaginis* (two accessions), as well as one unidentified accession, were grouped monophyletically. The clade also included two accessions of *E. heraclei* and lacked all *Neoerysiphe* accessions seen within the group when ITS, *Mcm7,* β-tubulin*,* and *Tsr1* regions are used*.*

A putative clade of species predominantly of the Cysotheceae (clade A) had a PP of 33% (Figure 7.4). Species of *Podosphaera, Neoerysiphe, Golovinomyces, Sawadaea,* and *Erysiphe* were scattered throughout this clade. The species did not cluster together and the *Neoerysiphe, Golovinomyces,* and *Erysiphe* are not normally considered part of it.

A putative clade of species predominantly of the Erysipheae (clade C) had a PP of 95% (Figure 7.5). The base of this clade included various species of *Posphaera, Sawadaea, Neoerysiphe, Golovinomyces,* and *Erysiphe*. *Erysiphe* clades and support are presented in Table 7.5.

Table 7.5: Monophyletic PM groups from Actin data within the *Erysiphe*.

Species within group	Number of Accessions	PP of group*
E. arcuata	2	100
E. lonicerae	2	100
E. hedwigi	1	
E. lycopsidis	1	
E. berberidis	2	100
E. cruciferarum	1	
E. convolvuli	3	100
E. buhrii	1	
E. polygoni	2	100
E. heraclei	10	85
E. catalpae *E. aquilegiae*	1 9	99
E. intermedia *E. trifoliorum*	1 3	100
E. euonymicola *E. akebiae*	2 3	28
E. tortilis *E. alphitoides*	1 10	40

*Groups with a single accession have no PP and are shaded in grey.

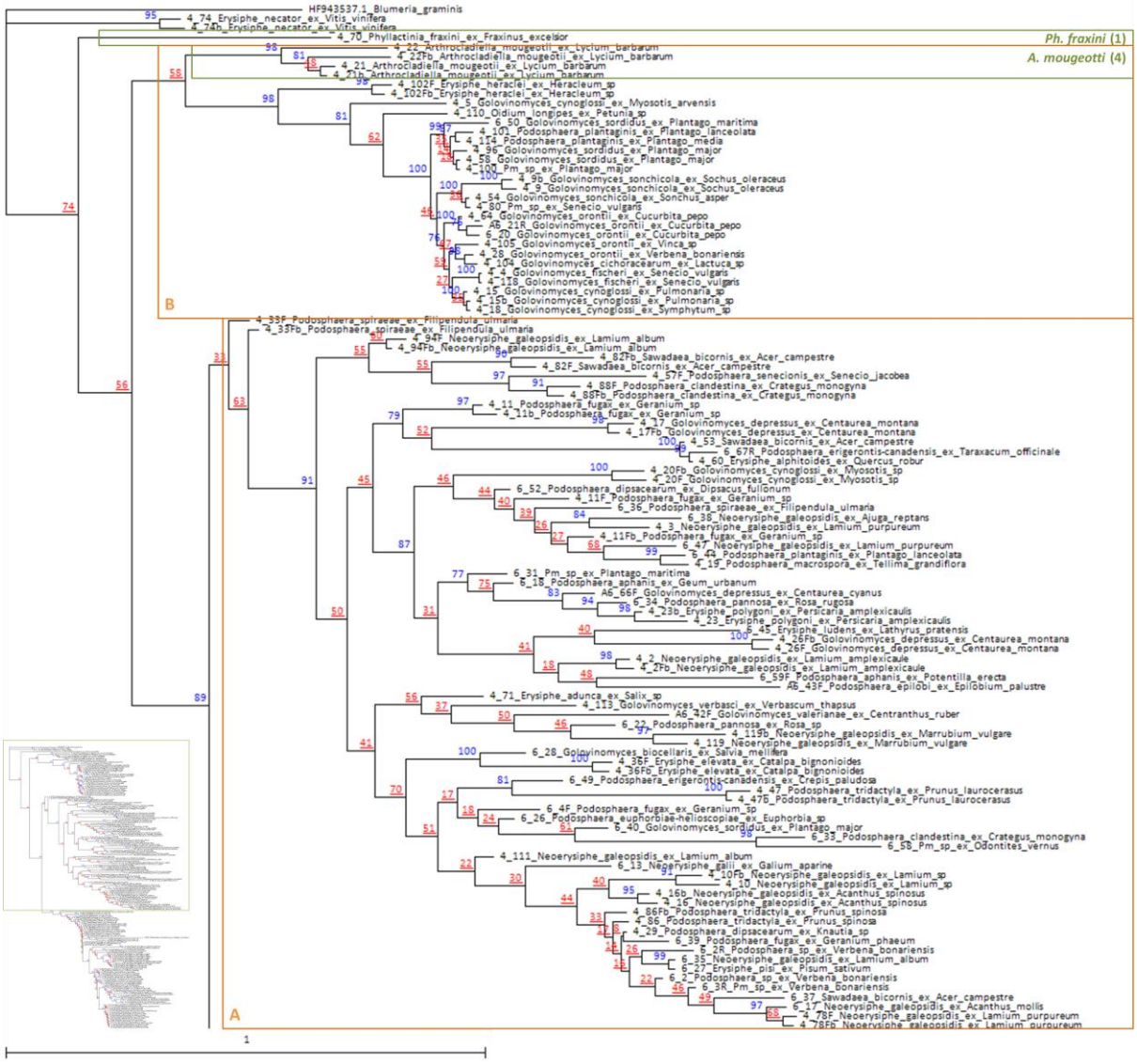

Figure 7.4: Part 1 of BI using 187 sequences of the Actin region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

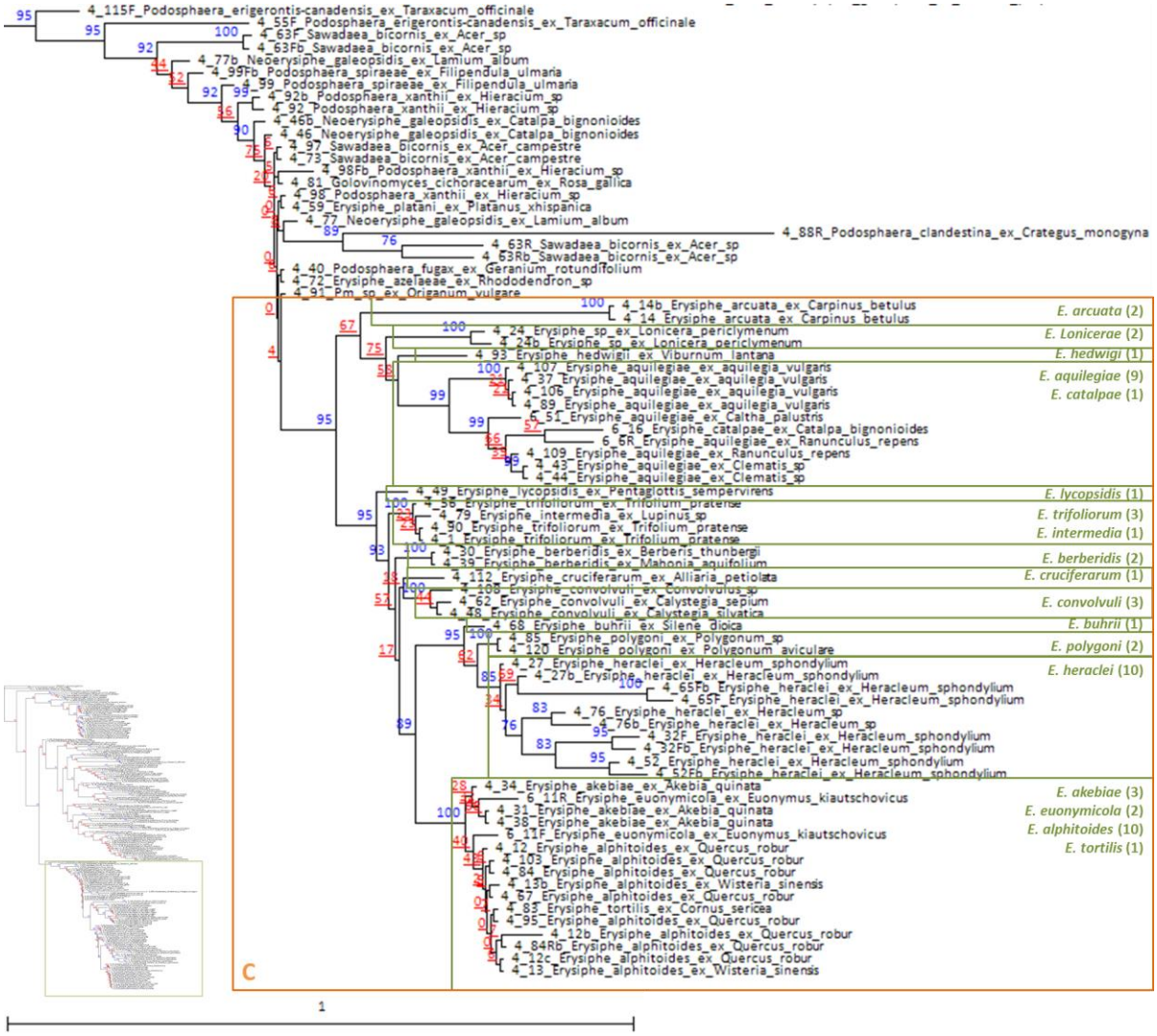

Figure 7.5: Part 2 of BI using 187 sequences of the Actin region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

7.3.3.2 Direct comparison of ITS and Actin

BI of the PM phylogeny of 124 samples of the Actin gene (Figure 7.7) resulted in an overall topology very different to that of the BI of the PM phylogeny of 124 samples of the ITS region (Figure 7.6). While the ITS phylogeny performed as expected, with clear separation of PM tribes and genera and strong support for monophyletic groups, the Actin phylogeny performed similarly to that of section 7.3.3.1.

Three rough clades were observed in the Actin phylogeny and tended towards the groupings of the PM tribes Cystotheceae (clade A), Golovinomyceteae (clade B) (Figure 7.4), and Erysipheae (clade C) (Figure 7.5). These once again included numerous unexpected, confusing, and erroneous samples. Clade C, the Erysipheae is made up largely of species of *Erysiphe*, but also includes four *Podosphaera* of various species, two *N. galeopsidis* accessions, three *S. bicornis* accessions, and an accession of *G. cichoracearum*. Many *Erysiphe* accessions are positioned in clades A and B. Clade B, the Golovinomyceteae is made up largely of species of *Arthrocladiella* and *Golovinomyces*, but also includes an accession of *E. heraclei,* and two accessions of *P. plantaginis* ex *Plantago* spp. which can be identified as *G. sordidus.* The clade has no *Neoerysiphe* spp. Clade A, the Cysotheceae is made up largely of species of *Podosphaera* and *Sawadaea* but also includes 11 accessions of *Neoerysiphe* spp., six accessions of *Golovinomyces* spp., and six accessions of *Erysiphe* spp. Many *Podosphaera* and *Sawadaea* accessions are positioned in clades B and C. Accessions within this clade are scattered with no clear relationships and low support.

Three clear clades were seen in the ITS phylogeny: Cystotheceae (Clade A) (PP 72%), Golovinomyceteae (Clade B) (PP 99%), and Erysipheae (Clade C) (PP 77%). Each genus was monophyletic and all species, apart from the 15 presented in Table 7.6 were monophyletic.

Table 7.6: Non-monophyletic PM species from ITS data within the Erysiphales.

PM species
P. erigerontis-canadensis
P. xanthii
P. plantaginis
G. cynoglossi
G. chicoracearum
G. sonchicola
G. orontii
G. fischeri
G. verbasci
E. aquilegiae
E. trifoliorum
E. catalpae
E. alphitoides
E. euonymicola
E. akebiae

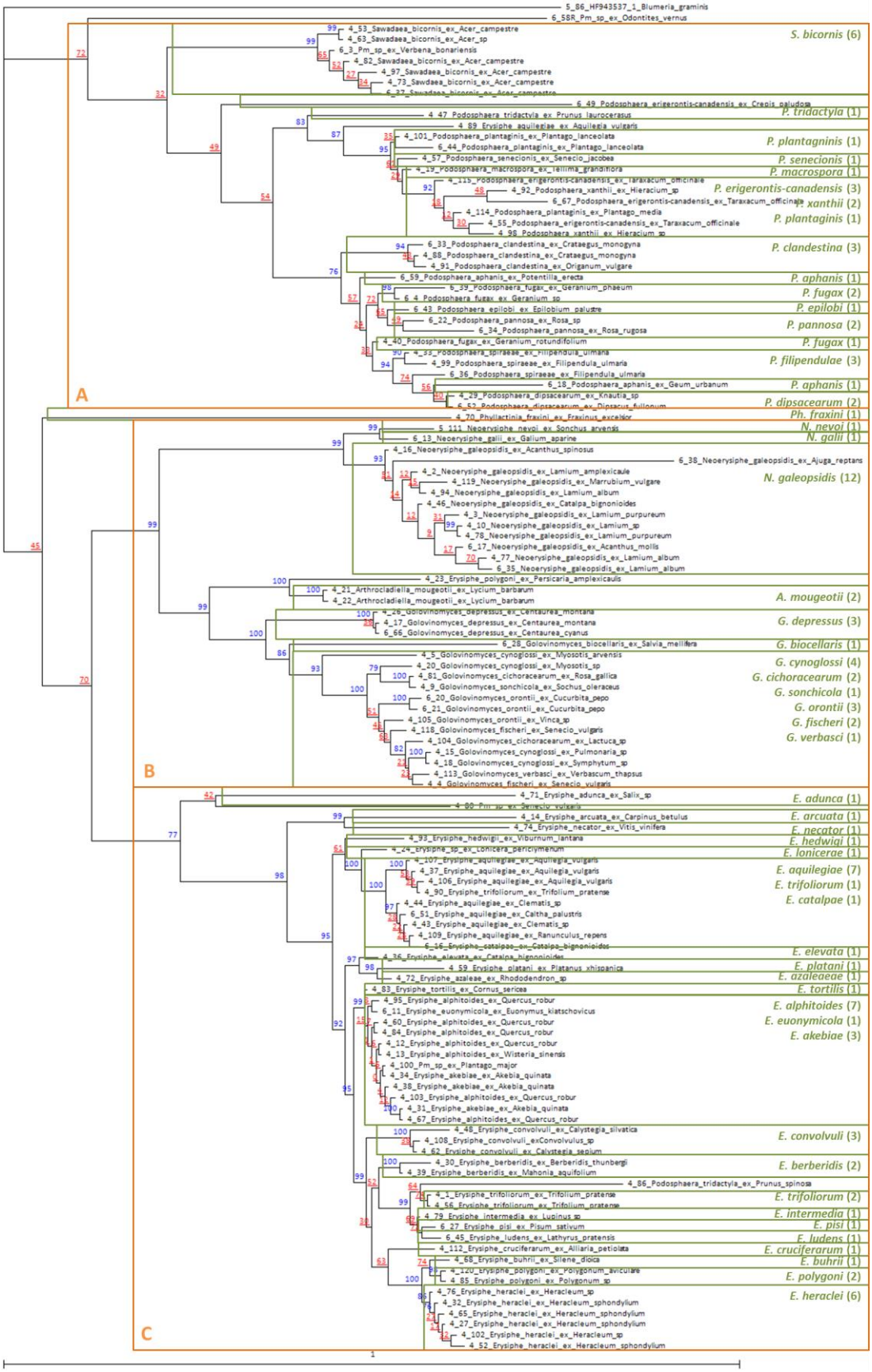

Figure 7.6: BI using 124 sequences of the ITS region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

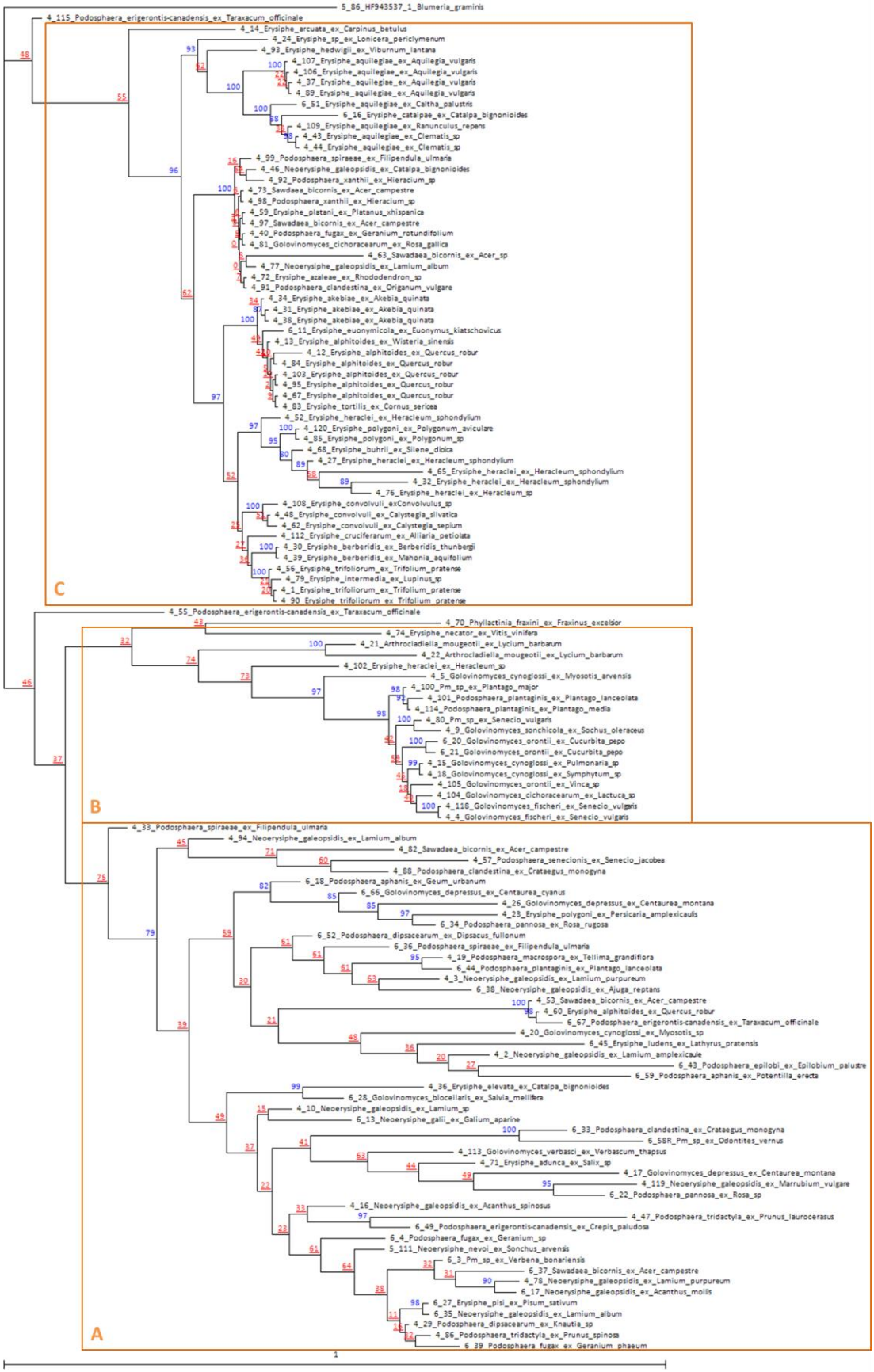

Figure 7.7: BI using 124 sequences of the Actin region. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

7.3.3.3 Combined ITS and Actin phylogeny

BI of PM phylogeny using 102 samples of the combined 1315 bp region of the ITS and Actin regions resulted in a phylogeny with the accuracy of the ITS region combined with numerous artefacts of the inaccuracies of the variable Actin region (Figure 7.8).

Unresolved ITS samples *P. erigerontis-canadensis, P. plantaginis, P. xanthii, G. cynoglossi, G. cichoracearum, G. sonchicola, G. orontii, E. aquilegiae, E. catalpae, E. alphitoides, E. euonymicola,* and *E. akebiae* remained unresolved. In contrast, after satisfactory resolution via the ITS, samples of *P. tridactyla, P. macrospora, P. senecionis, P. clandestina, P. fugax, P. filipendulae, S. bicornis,* and *E. tortilis* became unresolved within larger groups of their genera or polyphyletic across different genera. The *Podosphaera, Golovinomyces,* and *Erysiphe* genera remained clustered within their expected tribes, close to other closely related congeneric species. Improvement was seen in the resolution of accessions of *G. fischeri, G. verbasci,* and *E. trifoliorum*, which were monophyletic via analysis of the concatenated ITS, Actin dataset.

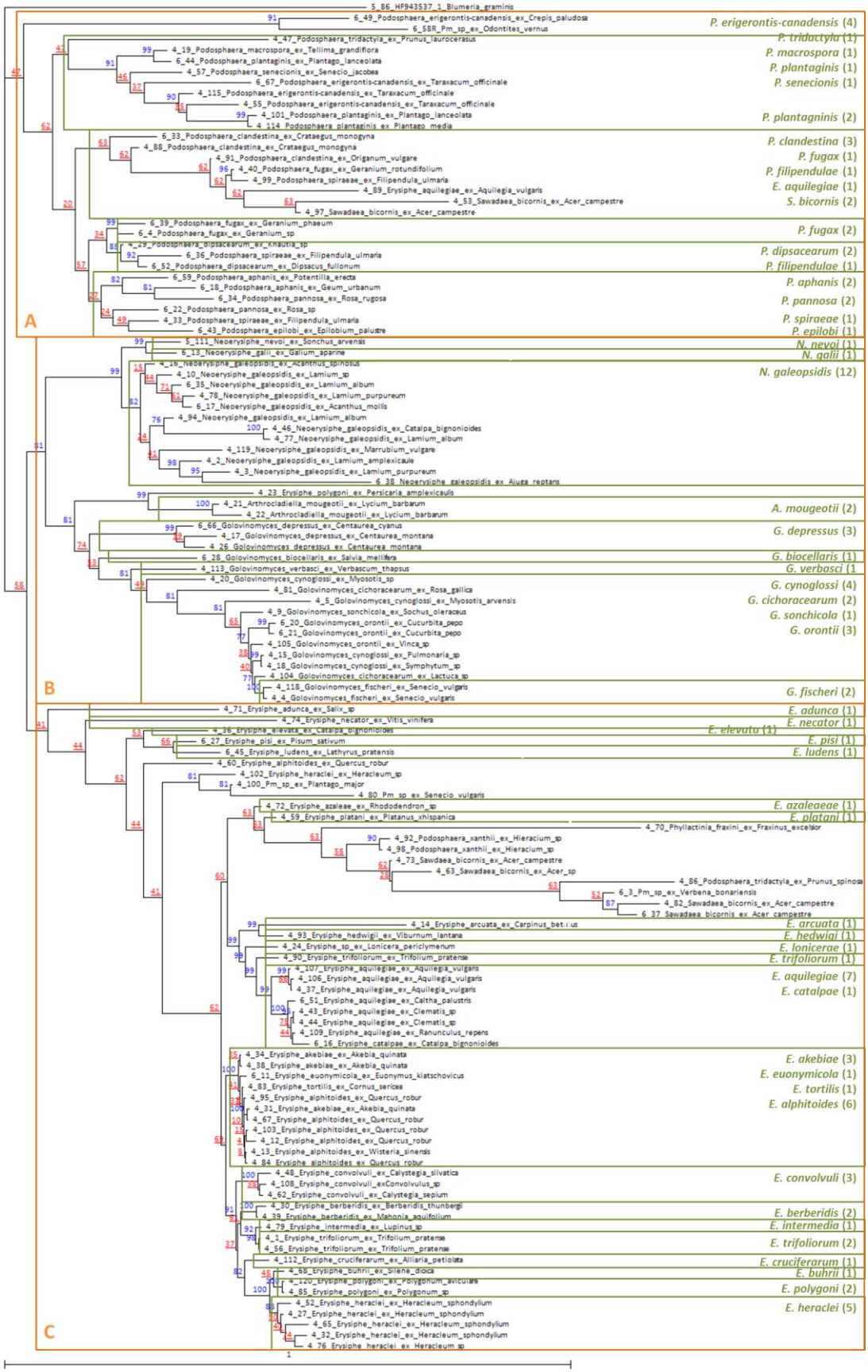

Figure 7.8: BI using 124 sequences of the ITS and Actin regions combined. Accession names include accession code, PM name, and host identity. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

7.3.4: DNA barcoding analysis

7.3.4.1 Actin

Intra and interspecific differences were quantified for the 187 accessions and 53 species of the Actin region. This resulted in a total overlap of 47.94% (from 0.0% to 47.94%, covering 99.94% of all intra and interspecific but intrageneric sequences) (Figure 7.9a). Numerous accessions had a mean of more than 5% intraspecific difference. Accessions below this threshold were *E. alphitoides, E. akebiae, E. arcuata, E. polygoni, E. berberidis, E. convolvuli, E. elevata, E. lonicerae, E. heraclei, E. necator, P. tridactyla, P. fugax, S. bicornis, A. mougeotti, G. sordidus,* and *G. orontii*. There were 502 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (52.2%), *Golovinomyces* (36.7%), and *Podosphaera* (10.8%). Interspecific pairs below 0.5% difference were *P. xanthii* and *P. dipsacacearum, P. xanthii* and *P. fugax, P. xanthii* and *P. tridactyla, E. platani* and *E. azelaeae, E. alphitoides* and *E. euonymicola, E. alphitoides* and *E. akebiae, E. alphitoides* and *E. tortilis,* and *G. biocellaris* and *G. cichoracearum*.

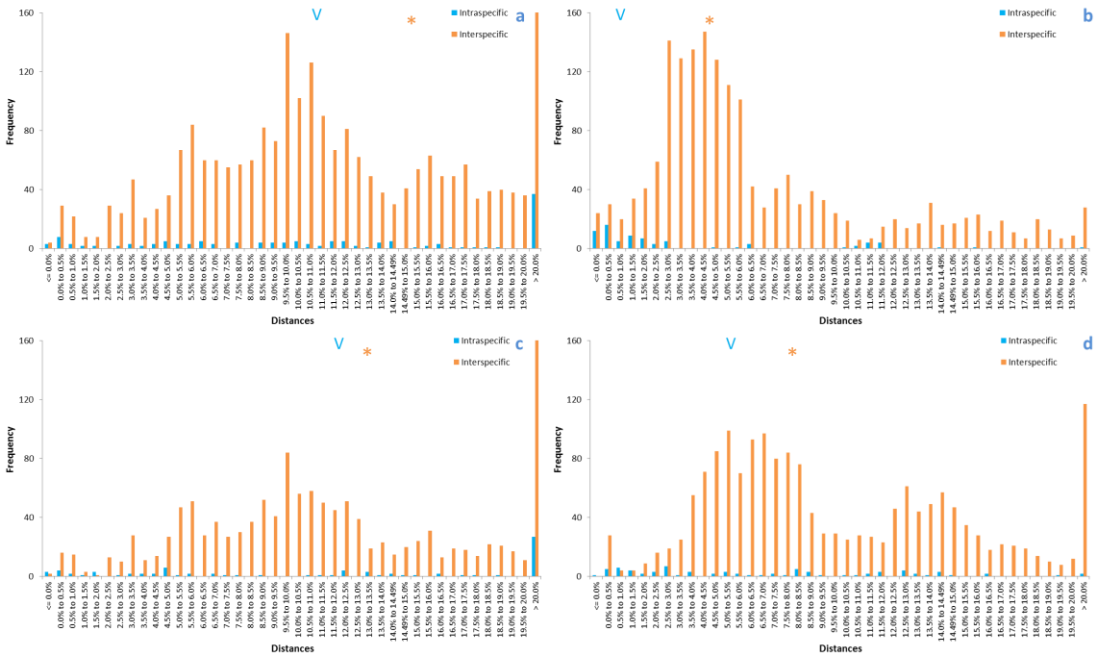

Figure 7.9: The frequency distribution of the intra and interspecific K2P distance values (barcoding gaps) of (a) 187 Actin accessions, (b) 124 ITS accessions common to the ITS and Actin regions, (c) 124 Actin accessions common to the ITS and Actin regions, and (d) 124 accessions of the ITS and Actin regions combined. Blue chevrons (V) mark the mean intraspecific variation per locus and orange asterisks (*) mark the mean interspecific variation per locus. The greater than 20% value reaches 1,240 on (a) and 587 on (c).

7.3.4.2 Direct comparison of ITS, Actin, and combined datasets

7.3.4.2.1 ITS

Intra and interspecific differences were quantified for the 124 accessions and 50 species of the ITS region. This resulted in a total overlap of 21.3% (from 0.0% to 21.3%, covering 98.99% of all intra and interspecific but intrageneric sequences) (Figure 7.9b). Accessions with a mean of more than 5% intraspecific difference were *E. polygoni, E. trifoliorum, P. tridactyla, N. galeopsidis,* and *G. cynoglossi*. There were 1,728 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (79.2%), *Golovinomyces* (9.0%), and *Podosphaera* (11.0%). The interspecific pairs which were below 0.5% difference were: *E. alphitoides* and *E. tortilis*, *E. alphitoides* and *E. euonymicola, E. alphitoides* and *E. akebiae, E. euonymicola* and *E. akebiae, E. euonymicola* and *E. tortilis, E. aquilegiae* and *E. catalpae, E. euonymicola* and *E. tortilis, P. xanthii* and *P. plantaginis, P. xanthii* and *P. erigerontis-canadensis,* and *G. cichoracearum* and *G. sonchicola*.

7.3.4.2.2 Actin

Intra and interspecific differences were quantified for the 124 accessions and 50 species of the Actin region. This resulted in a total overlap of 33.8% (from 0.0% to 33.8%, covering 98.34% of all intra and interspecific but intrageneric sequences) (Figure 7.9c). Numerous accessions had a mean of more than 5% intraspecific difference. Accessions below this threshold were *E. aquilegiae, E. alphitoides, E. akebiae, E. polygoni, E. berberidis, E. convolvuli, E. heraclei, P. fugax, S. bicornis, G. cichoracearum,* and *G. orontii*. There were 276 interspecific, congeneric distances which fell below 5%; the most common of these were between accessions of the *Erysiphe* (62.3%), *Golovinomyces* (22.5%), and *Podosphaera* (10.9%). Interspecific pairs below 0.5% difference were *P. clandestina* and *P. fugax, E. platani* and *E. azelaeae, E. alphitoides* and *E. euonymicola, E. alphitoides* and *E. akebiae,* and *E. alphitoides* and *E. tortilis.*

7.3.4.2.3 Combined ITS and Actin

Intra and interspecific differences were quantified for the 124 accessions and 50 species of the combined ITS and Actin regions. This resulted in a total overlap of 20.73% (from 0.09% to 20.83%, covering 95.06% of all intra and interspecific but intrageneric sequences) (Figure 7.9d). Accessions with a mean of more than 5% intraspecific difference were *E. aquilegiae*, *E. polygoni, E. heraclei, E. trifoliorum, P. clandestina, P. aphanis, P. pannosa, P. plantaginis, P. xanthii, P. filipendulae, P. fugax, P. tridactyla, S. bicornis, N. galeopsidis, G. cichoracearum,* and *G. cynoglossi*. There were 632 interspecific, congeneric distances that fell below 5%; the most common of these were between accessions of the *Erysiphe* (82.6%), *Golovinomyces* (8.2%), and *Podosphaera* (7.9%). The interspecific pairs which were below 0.5% difference were: *E. alphitoides* and *E. tortilis*, *E. alphitoides* and *E. euonymicola, E. alphitoides* and *E. akebiae, E. euonymicola* and *E. akebiae, E. akebiae* and *E. tortilis, E. euonymicola* and *E. tortilis*.

7.4: Discussion

7.4.1: Actin amplification and sequencing

Markers for this region and a protocol for their use were successfully designed. Given the dearth of previous sequence data for the Actin region, it was vital to locate and identify the region using previously amplified samples of closely related, ascomycetous fungi. The use of six of these ensured that the region was accurately located and the four available PM genomes ensured primers could be designed accurately.

Accessions were consistently amplified and sequenced; however, it was not certain whether the primers were specific to PMs. Lack of PM Actin sequences available in GenBank meant that accurate, informative BLAST was not possible. However, the consistent amplification and sequencing of numerous products of a similar size and their alignment with available PM genomes indicated that they were indeed Actin of PMs. The variation of accessions within the region was shown to be far greater than for ITS (and other analysed regions) via BI and K2P analyses. Favourable sequencing of *Golovinomyces* sp. was evident for accessions found on *Plantago* sp. as accessions previously identified as a mixture of *G. sordidus* and *P. plantaginis* were shown to be similar through both BI and K2P analyses; accessions of *G. sordidus* had therefore been amplified and sequenced each time.

7.4.2: Phylogenetic analyses

The grouping of samples from *Plantago* spp. enabled identification of these accessions to species level (*G. sordidus*). Otherwise, the Actin region proved to be largely uninformative for phylogenetic analyses. These findings are therefore in line with studies suggesting it to be insufficient for differentiation of fungal species (Weiland & Sundsbak, 2000, Evans, 2014). Combining Actin with ITS resulted in a phylogeny far more similar to that expected and managed to resolve a few extra PM species to that of the phylogeny built purely on ITS sequence data. However, the fact that the combined phylogeny was less resolved than one of ITS with many additional discrepancies meant that it could not even be proposed as an additional identifier to the anchoring ITS (Kõljalg *et al.*,

2013). The variation within the region, within genera and within species has resulted in this inability to produce informative phylogenetic data. It will be interesting to analyse the utility of Actin when combined with other, more informative, regions (such as the *Mcm7* and β-tubulin); its large variation of seemingly uninformative DNA may help to generate a fully resolved phylogeny when analysed alongside regions of greater individual phylogenetic utility.

7.4.3: DNA barcoding analysis

None of the analyses produced the barcoding gap (Hebert *et al.*, 2003a) related to a good barcoding region (Quaedvlieg *et al.*, 2014). The Actin region showed a greater level of intraspecific variation than the ITS, and that of all regions tested thus far. For a coding region, conserved throughout all eukaryotes and known to be involved in non-host resistance (Yun *et al.*, 2003, Opalski *et al.*, 2005), Actin varies more than other studies have reported; both within and between PM species. This variation is rarely informative of species limits. It is not a good barcoding region.

Extensive K2P overlaps across all analyses, particularly those including Actin data, were the result of closely related species of the *Podosphaera*, *Golovinomyces,* and *Erysiphe* and the great variation of DNA bases within this region.

7.5: Conclusions

Previously designed generic primers were unable to produce PM sequences, however newly designed, PM specific primers aided in generation of multiple PM Actin sequences. These sequences have thus far proved not to be the solution for PM diagnostics. While a small amount of informative data has been revealed in the region, the amplification and sequencing of numerous questionable results from previously identified accessions has been shown through both BI and K2P analyses. This is coupled with the high level of variation within PM tribes, genera, and species for this region. Its greatest utility may be as a complementary region to other, more individually informative regions. This will be explored in Chapter 9.

Chapter 8: Failed augmentation of current ID techniques with novel DNA gene regions

8.1: Introduction

In addition to the regions trialled in previous chapters three further regions were trialled and abandoned due to lack of suitable results: Calmodulin, Chitin synthase (*Chs*), and Translation elongation factor 1 alpha (EF1-α).

Calmodulin is a multifunctional intermediate calcium-binding messenger protein expressed in all eukaryotic cells (Stevens, 1983). The region is intronless and encodes a protein of 148 amino acid residues (444 bp) (LeJohn, 1989). Calmodulin has been shown to be important in defence of host plants against pathogens such as PMs (Panstruga, 2005) as loss of calmodulin binding in the plant can halve the ability of the mildew resistance locus (MLO) to negatively regulate defence against PM *in vivo* (Kim *et al.*, 2002). PCR-based techniques for Calmodulin have been used as a powerful diagnostic method for distinguishing mycotoxigenic fungi involved in food spoilage at a species level with species-specific primers (Edwards *et al.*, 2002). Phylogenies based on the Calmodulin gene have also aided species diagnostics in *Gibberella* (O' Donnell *et al.*, 2000), *Fusarium* (Mulè *et al.*, 2004), *Penicillia* (Wang & Zhuang, 2007), *Sporothix* (Madrid *et al.*, 2009, Romeo *et al.*, 2011), and *Aspergillus* (Samson *et al.*, 2014); all ascomycete fungi.

Chitin is a major component of the fungal cell wall. Its production from glucose involves approximately six enzyme-catalysed reactions; *Chs* catalyses the last of these reactions (Zhang *et al.*, 2000). Thus potential inhibitors of *Chs* activity can affect antifungal activity (Debono & Gordee, 1994). Plant pathogenic fungi normally have multiple *Chs* genes (Kong *et al.*, 2012), each of approximately 600 bp (Roberts *et al.*, 1986). The variation of these genes among certain Fungi have shown the region to have potential taxonomic use (Mehmann *et al.*, 1994). The conservation of specific motifs in the core region of *Chs* across all eukaryotes except plants suggest that it originated

before the so-called `crown kingdoms' (Fungi, Plantae, Animalia, Alveolates (comprising ciliates, dinoflagellates and apicomplexans), and Stramenopiles (where diatoms, oomycetes, labyrinthulids, brown algae and chrysophytes are included) (Sogin *et al.*, 1996, Van de Peer & De Wachter, 1997)) (Ruiz-Herrera *et al.*, 2002). Separation of these eukaryotic groups occurred approximately 1 billion years ago, it may be concluded that *Chs* have their origin as a branch of antique L-glycosyl-transferases, once the plant kingdom had diverged about this time. Later diversification within the Fungi has resulted in varying copies within different fungal groups and has enabled phylogenetic studies to yield promising results in dermatophyte species (Kano *et al.*, 1997) and *Magnaporthe oryzae* (Kong *et al.*, 2012). Within the PMs each *Chs* gene has been shown to be present as single copies within the barley powdery mildew genome (Zhang *et al.*, 2000).

EF1-α is a highly conserved gene region coding for enzymatic delivery of aminoacyl tRNAs to the ribosome. It exhibits low rates of amino acid substitutions and is a single or low copy number gene (Roger *et al.*, 1999). As such it has been shown to produce promising results in phylogenetic studies of *Fusarium* (O' Donnell *et al.*, 1998a, Seifert & Lévesque, 2004, Kristensen *et al.*, 2005, Amatulli *et al.*, 2010), *Mycosphaerella* (Hunter *et al.*, 2006), *Armillaria* (Maphosa *et al.*, 2006), the entire Basidiomycota phylum (Matheny *et al.*, 2007), and across the four Kingdoms of Eukaryota (Baldauf *et al.*, 2000).

In this chapter samples from the Powdery Mildew Survey (Chapter 2) were used to investigate the possibility of using primers sourced from the literature for the Calmodulin, *Chs*, and EF1-α regions for amplification of PMs. The possibility of developing new working markers for these regions for PMs was also investigated and the value of resultant data for phylogenetic reconstruction explored. The resolution of this region in DNA barcoding studies was compared with the standard ITS region.

8.2: Materials and methods

8.2.1: Accessions

Accessions were collected via the powdery mildew citizen science scheme (Chapter 2). The DNA extracted for initial PM species identification (section 3.2.3) was used for the amplification of the different regions trialled in this chapter.

8.2.2: Generic primer trials

8.2.2.1 Sourcing primers

Primers for the amplification of the Calmodulin, *Chs*, and EF1-α genes were sourced from previous publications (Carbone & Kohn, 1999, O' Donnell *et al.*, 2000, Brewer & Milgroom, 2010) and ordered from Sigma-Aldrich (Table 8.1).

Table 8.1: Generic primers sourced from literature for trialling amplification and sequencing of Calmodulin, *Chs* and EF1-α regions of PMs.

Region	Primer name	Direction	Sequence (5' - 3')	Product size (bp)	Source
Calmodulin	CAL-228F	F	GAGTTCAAGGAGGCCTTCTCCC	500	Carbone and Kohn (1999)
	CAL-737R	R	CATCTTTCTGGCCATCATGG		
	CL11	F	ACCATGATGGCGGCAAG	400	O' Donnell *et al.* (2000)
	CL22	R	TCCTTCATCTTGCGCGCC		
Chs	CHS-79F	F	TGGGGCAAGGATGCTTGGAAGAAG	300	Carbone and Kohn (1999)
	CHS-354R	R	TGGAAGAACCATCTGTGAGAGTTG		
EF1-α	EF 1-5	F	ATAGCGACGATGAGCTGCTT	500	(Brewer & Milgroom, 2010)
	EF 1-6	R	TCGAAAAGGTTTGTTGCAGA		
	EF1-728F	F	CATCGAGAAGTTCGAGAAGG	350	Carbone and Kohn (1999)
	EF1-986R	R	TACTTGAAGGAACCCTTACC		

8.2.2.2 PCR and sequencing

Amplification of the sourced primer combinations was trialled for each region according to published protocols (Carbone & Kohn, 1999, O' Donnell *et al.*, 2000, Brewer & Milgroom, 2010). The PCR products were separated and visualised as in section 3.2.4.

Multiple products per sample were amplified consistently for each region. Individual bands were excised, purified using the QIAquick Gel Extraction Kit, and sent to Source BioScience via courier for sequencing. Annealing and extension temperatures and primer, DNA, and magnesium concentrations of PCR protocols were explored in order to optimise amplification for single products. Single products were rarely amplified.

8.2.3: Data mining for molecular markers

PM genomes (Max Planck Institute for Plant Breeding Research, Spanu *et al.*, 2010, Jones *et al.*, 2014) were aligned with closely related fungal species in order to identify the location of the regions and then develop primers to trial in the amplification of samples. The Calmodulin and *Chs* regions did not show sufficient variability from closely related ascomycete fungi for development of PM specific primers. For the EF1-α region, a total of nine primers (five forward, four reverse) were designed (Table 8.2) and ordered as in section 4.2.4.

Table 8.2: Primers designed for trialling amplification and sequencing of EF1-α region of PMs

Primer name	Direction	Sequence (5' - 3')	Length	Mean Tm (°C)	Mean GC %
EF1aF1	F	TCACATYAAYGTGGTCGTSATCGG	24	63.19	50
EF1aF2	F	GAAYTMGGAAAAGGATCYTTC	21	51.6	38.1
EF1aF3	F	GACAAGCTWAAGGCMGARCG	20	59.96	50
EF1aF4	F	CATYGCDGCYGGTACYGGTG	20	61.09	60
EF1aF5	F	GGTGTYAARCARTTRATYGTYGC	23	61.02	47.83
EF1aR1	R	CACCRGTACCRGCHGCRATG	20	61.09	60
EF1aR2	R	GCRACRATYAAYTGYTTRACACC	23	61.02	47.83
EF1aR3	R	ACATCYTGDARWGGKAGRCG	20	58.52	55
EF1aR4	R	CGTGATGCATTTCBACVGAYTT	22	61.8	50

8.2.4: PCR and sequencing protocol

PCR was conducted using the newly designed PM specific primers of the EF1-α region (Table 8.2). All 10 possible combinations of primers more than 400 bp apart were trialled in 25 µl mixes of 12.5 µl BioMix™ Red (Bioline), 0.5 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 ng μl^{-1}, 9.25 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}. Cycling parameters were adapted from Amrani and Corio-Costet (2006) with an initial denaturation step of 95 °C for five minutes, followed by 37 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for one minute, and elongation at 72 °C for one and a half minutes and a final elongation at 72 °C for five minutes. Three samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003), were initially trialled.

The PCR products were separated and visualised as in section 3.2.4.

The three primer combinations exhibiting the highest amplification success (number of products x product strength) were EF1aF1 and EF1aR1, EF1aF1 and EF1aR2, and EF1aF1 and EF1aR4. These were trialled for amplification of four different samples of DNA, spanning the PM clade and previously shown to have been successfully amplified and sequenced using PM specific ITS primers PMITS1 and PMITS2 (Cunnington *et al.*, 2003). After amplification of multiple products extension and annealing temperatures and concentrations of primer, DNA and magnesium were explored. No combination was shown to consistently produce a single product and the region was therefore explored no further.

8.3: Results

After attempted optimisation of PCR protocols amplification success using generic primers of all three regions remained low (Table 8.3). Single bands of expected PM length of the most successful region, *Chs*, were sequenced and resulted in products of 100-300 bp. Identification using BLAST returned non-PM ascomycete fungi such as, *Alternaria*, *Arthrogpahis*, *Aureobasidium*, *Drechslera*, *Mycosphaerella*, and *Pyrenophora* with sequence identities of 82-100% and sequence query covers of 83-92%.

Table 8.3: PCR results of Calmodulin, *Chs*, and EF1-α regions.

Region	Generic primer combination	Number of accessions trialled	Number of samples producing:		
			Multiple bands	Single bands	Single bands of expected PM length
Calmodulin	CAL-228F & CAL-737R	27	16	8	3
	CL11 & CL22	14	10	2	1
Chs	CHS-79F & CHS-354R	27	11	13	13
EF1-α	EF 1-5 & EF 1-6	27	25	0	0
	EF1-728F & EF1-986R	14	9	1	0

8.4: Discussion

Regions Calmodulin, *Chs*, and EF1-α have each been shown to be useful for fungal diagnostics in numerous publications (Kano *et al.*, 1997, O' Donnell *et al.*, 1998a, Baldauf *et al.*, 2000, O' Donnell *et al.*, 2000, Mulè *et al.*, 2004, Seifert & Lévesque, 2004, Kristensen *et al.*, 2005, Hunter *et al.*, 2006, Maphosa *et al.*, 2006, Matheny *et al.*, 2007, Wang & Zhuang, 2007, Madrid *et al.*, 2009, Amatulli *et al.*, 2010, Romeo *et al.*, 2011, Kong *et al.*, 2012, Samson *et al.*, 2014). Data for these regions have not been obtained in the present study and therefore previous assertions of the utility of their DNA cannot be challenged. However, part of the utility of a region is down to its ease of amplification and sequencing (Quaedvlieg *et al.*, 2014) and the current study can therefore strengthen claims that Calmodulin, *Chs*, and EF1-α are not optimal regions for PM diagnostics. The current chapter has highlighted: the three regions in question do not amplify PMs when generic ascomycete primers are used; it was not possible to design primers specific to Calmodulin or *Chs* of the PMs; and primers designed for amplification of EF1-α did not produce single products. These failures can be put down to the environmental nature of sourced samples (Martin & Rygiewicz, 2005, Bellemain *et al.*, 2010) meaning that PM conspecific ascomycete fungi were preferentially amplified. The problems may be overcome through the use of single culture PM specimens gained from cloning or culturing. However, culturing trials (Morrison, 1960, Kenyon *et al.*, 1995, Álvarez & Torés, 1997, Nicot *et al.*, 2002) have rarely resulted in reproducible results (section 2.3.4) and cloning can be a protracted process, particularly when the rapid PM diagnostics sought from the current study are considered.

8.5: Conclusion

The low amplification success of regions Calmodulin, *Chs*, and EF1-α via the use of generic ascomycete primers means that they have been of little use to the current study. Sequence variation of the PM Calmodulin and *Chs* regions was insufficient to allow design of primers intended to specifically amplify them. Although EF1-α seemed to offer sufficient variation from PM conspecifics and primers were therefore deemed sufficiently specific in design, amplification of environmental samples sourced from the PM citizen science scheme also resulted in multiple products and preferential amplification of non-PM ascomycetes. This was not solved by PCR optimisation.

Given sufficient sequence data, the regions may be useful as PM identifiers, however the failure to amplify single products with any primer combination has meant that research ceased for each region. Resources were instead spent on generating DNA sequence data of successfully amplified regions *Mcm7,* β-tubulin, *Tsr1*, and Actin alongside ITS.

Chapter 9: Combined analyses

9.1: Introduction

Phylogenies of protein coding genes and rRNAs often conflict as currently available protein data for phylogenetic reconstructions have regularly included uneven taxonomic sampling, wide disparities in evolutionary rates among lineages, and/or inadequate characterisation at the risk of combining paralogues in a single analysis (Manso-Silván *et al.*, 2007, Medina *et al.*, 2011, Tretter *et al.*, 2014b, Trierveiler-Pereira *et al.*, 2014). Combining regions aims to overcome these flaws and result in greater precision of species resolution, in order to reflect the ideal species tree, more accurately than when accounting for regions alone as single gene phylogenetic trees (Medina *et al.*, 2011, Tretter *et al.*, 2014b, Mallo & Posada, 2016). Data from regions generated in Chapters 3-7 were therefore combined to produce multi-gene phylogenies.

It has been shown that many loci may be necessary for resolution of certain clades (Leaché & Rannala, 2010, Knowles & Kubatko, 2011, Liu & Yu, 2011), particularly when DNA may not have diverged sufficiently to resolve a phylogeny using a single locus (Beltrán *et al.*, 2002, Seehausen *et al.*, 2003). Multiple independent loci have often been shown to provide the necessary variability for reliable species identification (Beltrán *et al.*, 2002, Sang, 2002) using software and analytical phylogenetic methods for analysing concatenated datasets (Murphy *et al.*, 2001, Li *et al.*, 2007, Rowe *et al.*, 2008, Edwards, 2009).

The diagnostic utility of each combination of the regions trialled in the current study must therefore be quantified by phylogenetic and barcoding analyses. The current study used data generated in individual chapters, initially sourced from material from the Powdery Mildew Survey (Chapter 2). This chapter investigated the possibility of developing phylogenies most similar to an ideal species tree in which tribes and genera have monophyletic roots and the species within them are clearly separated, using each combination of the five regions. Combinations of regions were also

tested in order to assess which were most informative for barcoding analyses at generic and species levels.

9.2: Materials and methods

9.2.1: Sampling

DNA sequence data generated in Chapters 3-7 were used in this chapter.

9.2.2: Sequence alignment

Sequence alignments of ITS, *Mcm7*, β-tubulin, *Tsr1*, and Actin, were concatenated in the 22 different combinations remaining (see concatenated regions in Table 9.1 & Table 9.2), after combinations of individual regions with ITS in each chapter, using Mesquite (Maddison & Maddison, 2017). This tool combined identically named accessions from different datasets. Initial concatenation of combinations of two regions resulted in numbers considered too low to allow sufficient analyses of the hundreds of potential PM species including those known to be closely related and therefore difficult to discriminate (Table 9.1). Therefore sample names were simplified within sequence alignments of ITS, *Mcm7*, β-tubulin, *Tsr1*, and Actin data: accession number and host data were removed, while PM species name identified by the techniques outlined in previous chapters remained and were augmented with additional identifying numbers 1 - *n* (n = the number of samples of any one PM species in a given alignment) such that accessions confirmed as conspecific based on data from morphology, ITS and at least one other DNA region were combined to provide complete DNA datasets for most species studied regardless of whether they came from the same original accession or not (Table 9.1). Concatenated alignments were then manually edited such that gaps without data did not remain and those displayed in section 9.3: Results were deposited in TreeBASE (Accession S20957). The method was repeated for all combinations of three, four, and five regions (Table 9.2).

Table 9.1: Numbers of samples concatenated for combinations of two regions combined when concatenated by sample (Original names) and PM species (Simplified names).

Concatenated regions	Original names	Simplified names
Mcm7, β-tubulin	4	55
Mcm7, *Tsr1*	28	43
Mcm7, Actin	11	78
β-tubulin, *Tsr1*	3	35
β-tubulin, Actin	66	73
Tsr1, Actin	6	46

Table 9.2: Number of taxa and characters concatenated for combinations of three, four, and five regions

Number of regions	Concatenated regions	Number of taxa	Characters per region	Total characters
3	ITS, *Mcm7*, β-tubulin	55	771, 495, 754	2020
	ITS, *Mcm7*, *Tsr1*	43	770, 495, 1043	2308
	ITS, *Mcm7*, Actin	74	775, 496, 506	1777
	ITS, β-tubulin, *Tsr1*	34	769, 759, 1044	2572
	ITS, β-tubulin, Actin	71	817, 757, 501	2075
	ITS, *Tsr1*, Actin	40	777, 1050, 502	2329
	Mcm7, β-tubulin, *Tsr1*	26	494, 753, 1024	2271
	Mcm7, β-tubulin, Actin	47	497, 752, 498	1747
	Mcm7, *Tsr1*, Actin	27	493, 1025, 494	2012
	β-tubulin, *Tsr1*, Actin	29	756, 1050, 498	2304
4	ITS, *Mcm7*, β-tubulin, *Tsr1*	26	767, 492, 750, 1025	3034
	ITS, *Mcm7*, β-tubulin, Actin	48	771, 493, 754, 500	2518
	ITS, *Mcm7*, *Tsr1*, Actin	27	766, 493, 1025, 494	2778
	ITS, β-tubulin, *Tsr1*, Actin	32	769, 758, 1043, 498	3068
	Mcm7, β-tubulin, *Tsr1*, Actin	25	493, 751, 1043, 492	2760
5	ITS, *Mcm7*, β-tubulin, *Tsr1*, Actin	25	767, 492, 750, 1024, 492	3525

9.2.3: Phylogenetic analyses

Phylogenetic analyses were performed as in section 3.2.7.

Separate models were specified for each dataset within concatenated alignments and were: GTR+I+G for ITS, HKY+G for *Mcm7*, GTR+G for β-tubulin, GTR+I+G for *Tsr1*, and GTR+I+G for Actin. Analyses of two combined regions were run for 10,000,000 generations. Analyses of three, four, and five combined regions were run for 5,000,000 generations. The most suitable tree had, in each case, been reached after said number of generations.

Two phylogenetic approaches are known in measuring corroboration: taxonomic congruence and total evidence (Yassin *et al.*, 2010). Resultant data of the 50% majority rule consensus tree were analysed for accuracy by measuring corroboration with an ideal species or total evidence tree (TET). Corroboration among data sets for a particular node is indicated by replication of that node in topologies derived from the separate data sets to infer taxonomic congruence (Miyamoto & Fitch, 1995). Phylogenies inferred for each combination of regions were compared to the TET and relative consensus fork indexes (RCFI) from 0 to 1 were estimated to give the proportion of nodes shared (Colless, 1980).

9.2.4: DNA barcoding analyses

Concatenated datasets of all possible combinations of ITS, *Mcm7*, β-tubulin, *Tsr1*, and Actin were analysed and treated as in section 3.2.8. Total overlap of inter- and intra-specific distances and their overlap with 5% error margins on both ends were recorded in order to allow comparison between region combinations. Barcoding analyses are usually accession and not taxon based due to the necessity of measuring within-taxon variability. However, this was included to enable an additional analysis of datasets and within-taxon variability will therefore be within-species variability in the current analyses.

9.3: Results

9.3.1: Sequence alignment

Concatenation of identical accessions of all combinations of two regions resulted in datasets of 4, 28, 11, 3, 66, and 6 taxa (Table 9.1). Given the great diversity of PM species and repeats of particular species within the dataset (such as *P. clandestina*, *S. bicornis*, *N. galeopsidis*, *E. alphitoides*, *E. aquilegiae*, *E. trifoliorum*, and *E. heraclei*) the current accessions were considered inadequate as closely related PM species rarely remained within the datasets. Combining samples of different origin but the same species resulted in larger concatenated datasets (Table 9.1 & Table 9.2) which provided more informative phylogenetic results and barcoding results providing within-species rather than within-taxon variability.

9.3.2: Phylogenetic analyses

Phylogenetic reconstruction of concatenated datasets produced phylogenies of varying accuracy (Table 9.3). Samples were identified in Chapter 3 using the combination of established techniques for host identification, fungal morphology, and BLAST, BI, and K2P analyses of rDNA ITS sequence data with 80% success rate of applying a name to an accession. Of the remaining 20%, 6% were misidentified as species closely related to their actual identity, 5% could not be identified beyond being a PM, and 9% were identified no further than genus. An update of species identities based upon data gathered in subsequent chapters has shown that 88% of samples within the complete ITS phylogeny of Chapter 3 were positioned accurately as samples thought to be conspecific were grouped and nodes were regularly replicated from the TET (RCFI = 0.88). This benchmark has been improved upon in the individual use of the *Mcm7* region (RCFI = 0.974), as well as numerous combinations of regions (Table 9.3). Further improvement upon the *Mcm7* phylogeny was observed when combining the three regions ITS, *Mcm7*, and *Tsr1* (RCFI = 0.977) (Figure 9.1 & Table 9.3), in which the *E. alphitoides* – *E. euonymciola* complex was the only part of the topology to remain unresolved. RCFI reached 1 when combining the four regions of ITS, *Mcm7*, β-tubulin, and *Tsr1*

(Figure 9.2 & Table 9.3) and ITS, *Mcm7*, β-tubulin, and Actin (Figure 9.3 & Table 9.3) as well as when all five regions were combined (Figure 9.4 & Table 9.3).

Table 9.3: Comparative success of all region combinations for phylogenetic reconstruction of PM samples.

Number of regions	Regions	Samples in analysis	RCFI
1*	ITS	175 or 507	0.879 or 0.886
	Mcm7	151	0.974
	β-tubulin	103	0.874
	Tsr1	77	0.857
	Actin	187	0.684
2	ITS, *Mcm7**	106	0.972
	ITS, β-tubulin*	85	0.953
	*ITS, Tsr1**	60	0.850
	ITS, Actin*	124	0.798
	Mcm7, β-tubulin	55	0.927
	Mcm7, *Tsr1*	43	0.930
	Mcm7, Actin	78	0.949
	β-tubulin, *Tsr1*	35	0.857
	β-tubulin, Actin	73	0.808
	Tsr1, Actin	46	0.870
3	ITS, *Mcm7*, β-tubulin	55	0.909
	ITS, Mcm7, *Tsr1*	43	0.977
	ITS, Mcm7, Actin	74	0.973
	ITS, β-tubulin, *Tsr1*	34	0.853
	ITS, β-tubulin, Actin	71	0.887
	ITS, *Tsr1*, Actin	40	0.875
	Mcm7, β-tubulin, *Tsr1*	26	0.923
	Mcm7, β-tubulin, Actin	47	0.936
	Mcm7, *Tsr1*, Actin	27	0.926
	β-tubulin, *Tsr1*, Actin	27	0.889
4	ITS, *Mcm7*, β-tubulin, *Tsr1*	26	1.000
	ITS, *Mcm7*, β-tubulin, Actin	48	1.000
	ITS, *Mcm7*, *Tsr1*, Actin	27	0.963
	ITS, β-tubulin, *Tsr1*, Actin	32	0.906
	Mcm7, β-tubulin, *Tsr1*, Actin	23	0.870
5	ITS, *Mcm7*, β-tubulin, *Tsr1*, Actin	25	1.000

*Phylogenies taken from their respective Chapters.

Orange text displays values of greater accuracy than the ITS.

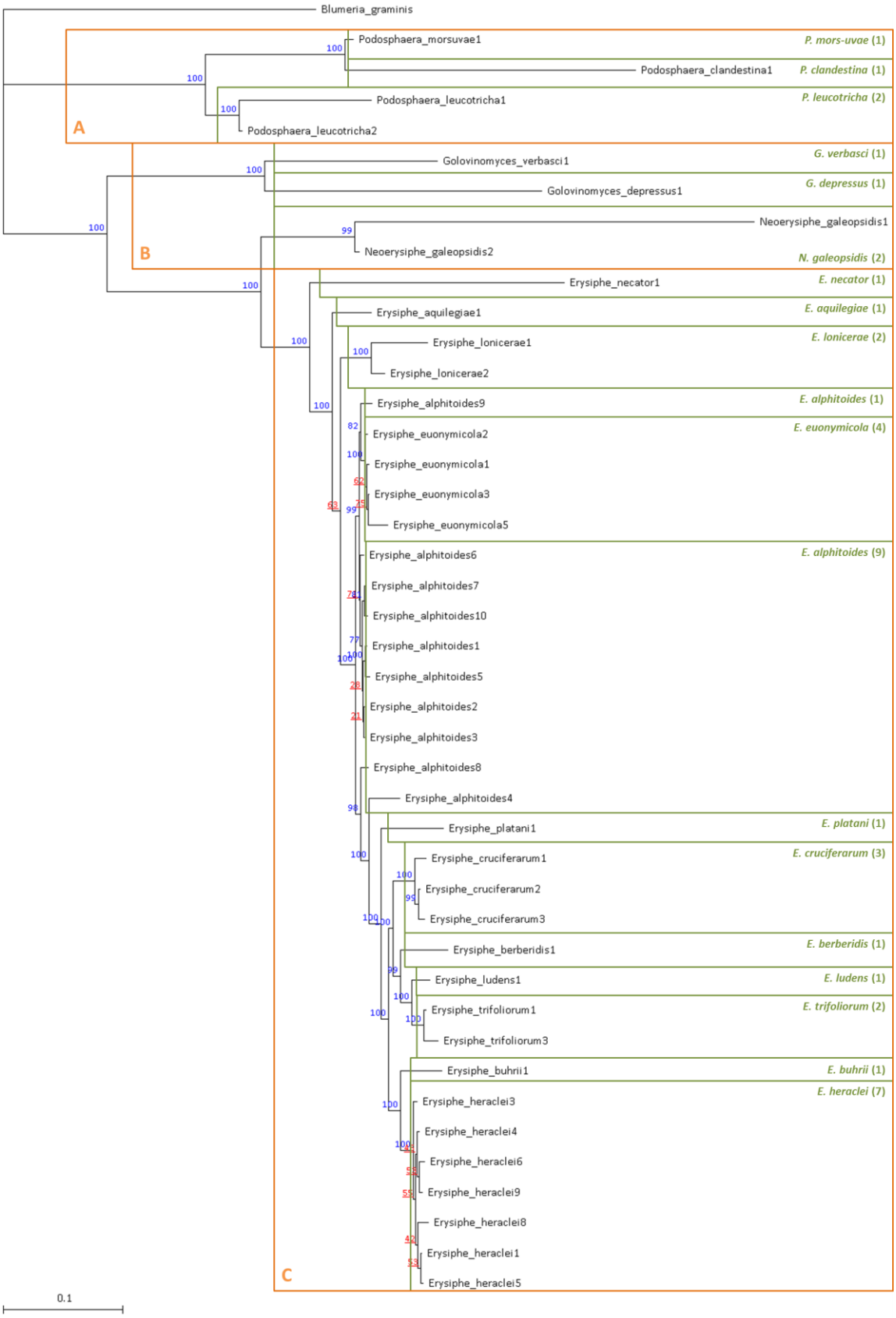

Figure 9.1: BI using 43 sequences of three regions combined (ITS, *Mcm7*, and *Tsr1*). Accession names include PM name with additional identifying numbers 1 — *n*. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

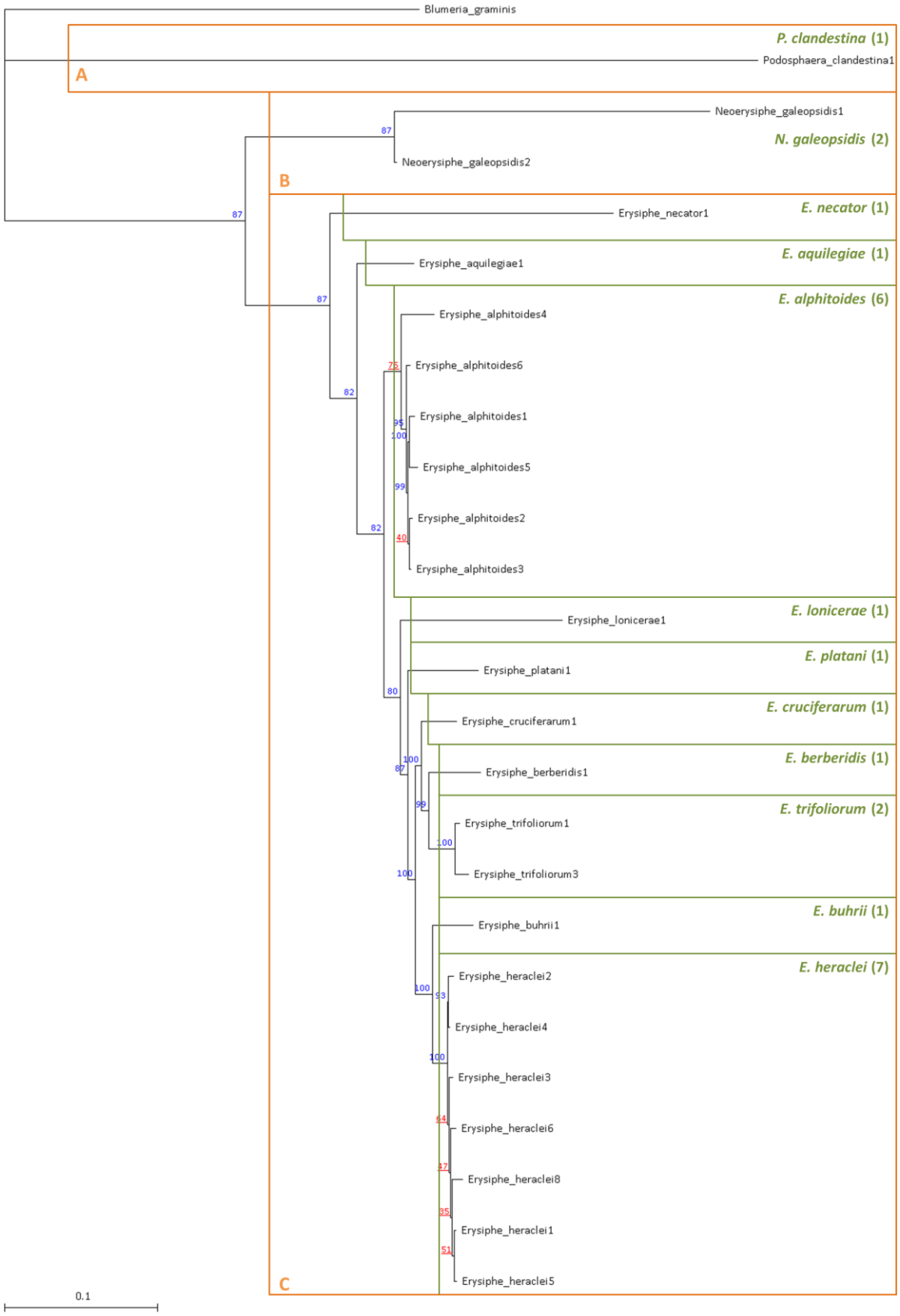

Figure 9.2: BI using 26 sequences of four regions combined (ITS, *Mcm7*, β-tubulin, and *Tsr1*). Accession names include PM name with additional identifying numbers 1 — *n*. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

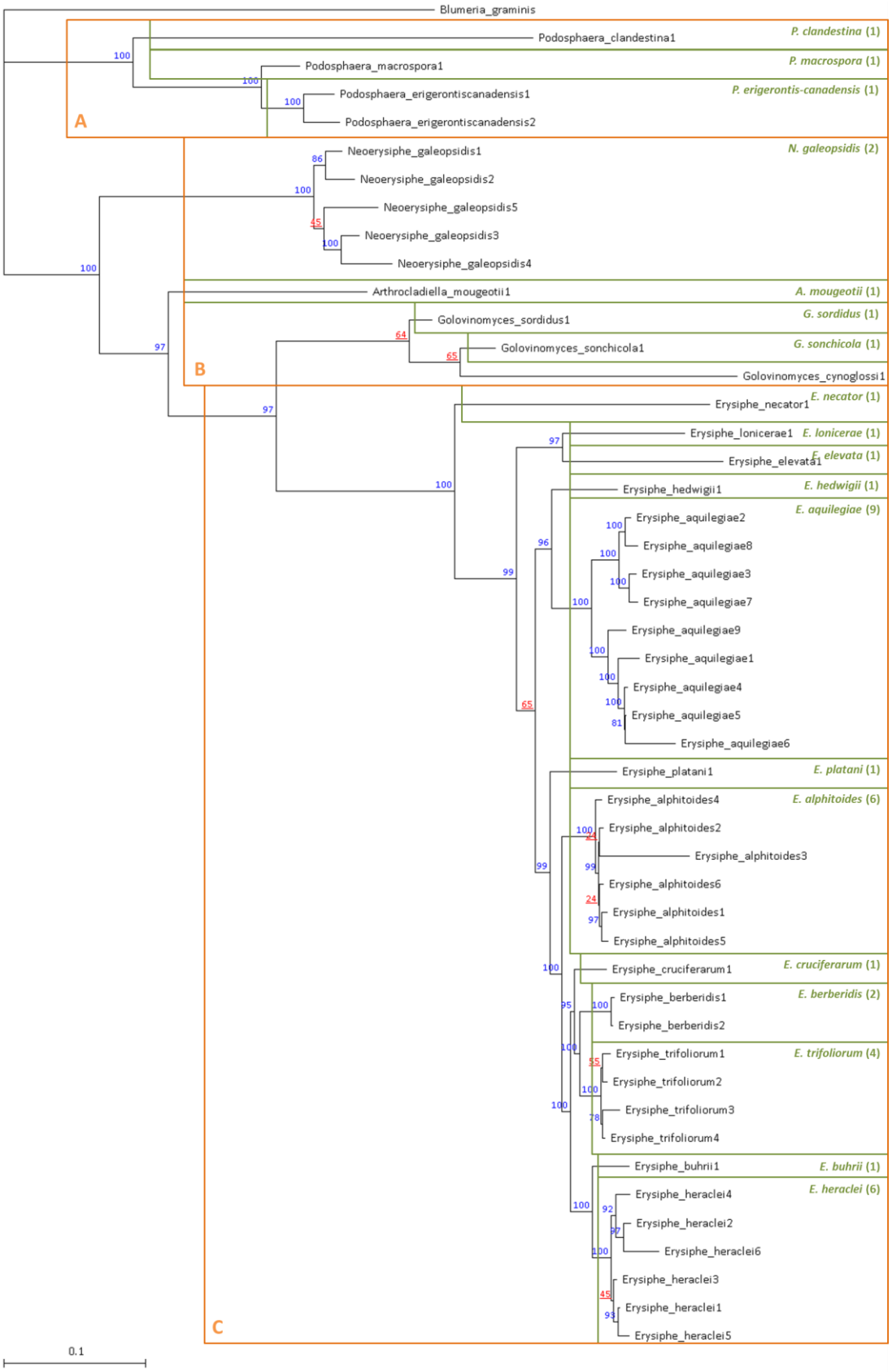

Figure 9.3: BI using 48 sequences of four regions combined (ITS, *Mcm7*, β-tubulin, and Actin). Accession names include PM name with additional identifying numbers 1 — *n*. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

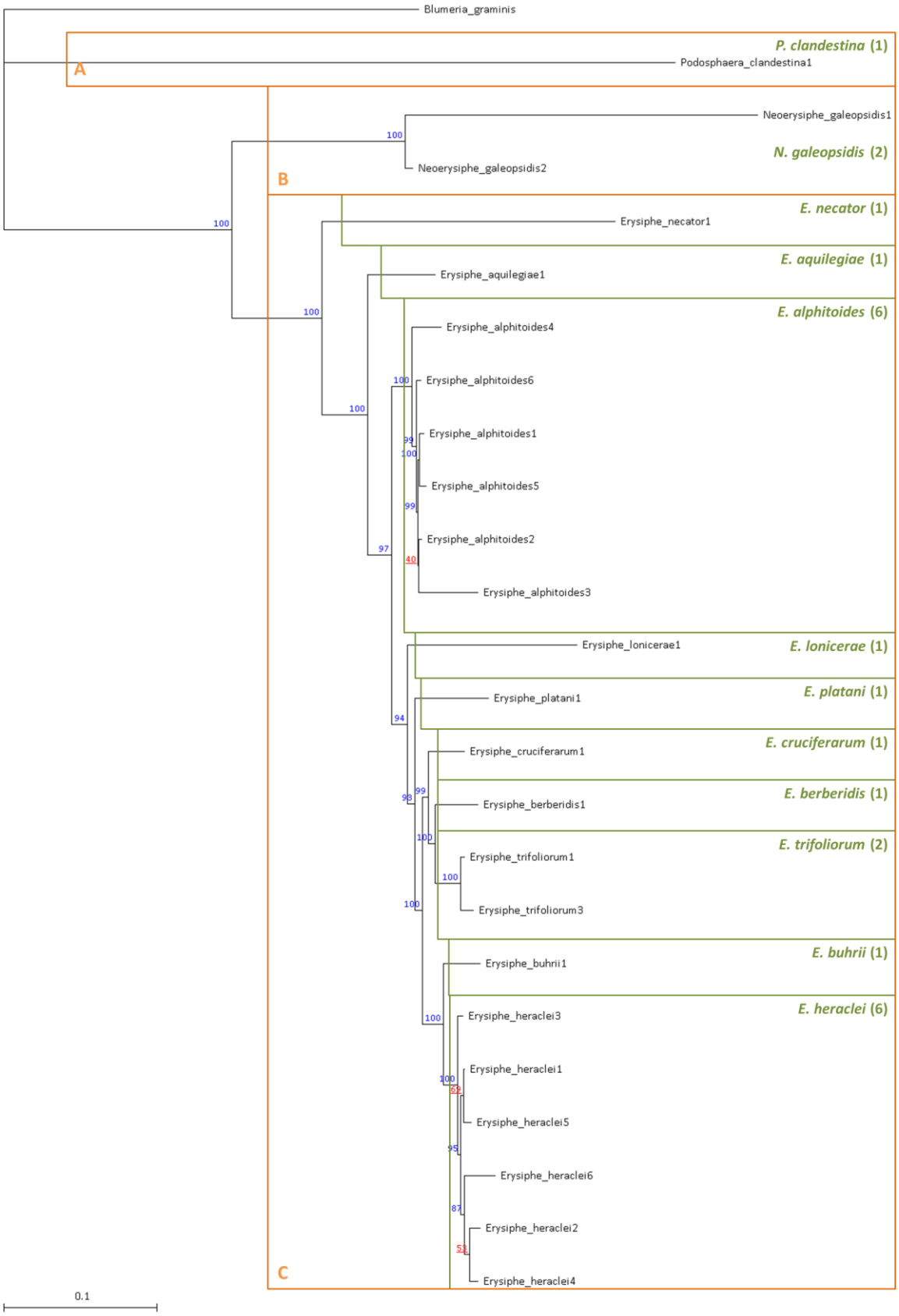

Figure 9.4: BI using 25 sequences of five regions combined (ITS, *Mcm7*, β-tubulin, Tsr1, and Actin). Accession names include PM name with additional identifying numbers 1 — *n*. PPs above 75% are shown in blue and below in red. Green lines and names show species separation by phylogeny. Orange boxes denote PM tribes.

9.3.3: DNA barcoding analyses

Inter- and intra-specific differences were quantified for combined species accessions for each possible combination of the five regions. A barcoding gap (clear separation of inter- and intra-specific distances) was not observed within any combination of regions (Table 9.4). However, addition of 5% error margin to both ends of sequences resulted in concatenated regions *Mcm7*, β-tubulin, *Tsr1* and ITS, *Mcm7*, β-tubulin, *Tsr1* displaying the desired gap (Figure 9.5 & Table 9.5).

The ITS region has been used extensively for PM and fungal diagnostics. Chapter 3 of the current study produced sequence overlap of 17.1% between inter- and intra-specific taxa which included 96.5% of all taxa or 6.5% including 70.1% of all taxa when 5% error margins were added. This benchmark was improved upon by the *Mcm7* and β-tubulin regions (Figure 9.5, Table 9.4, & Table 9.5). Furthermore, concatenation of various region combinations has been shown to enhance species discrimination when using DNA barcoding analyses. Evidence shows the most promising of these are likely to be combinations of *Mcm7*, β-tubulin and ITS, *Mcm7*, β-tubulin, Actin as the overlap of similar inter- and intra-specific sequences includes the least accessions (41.9% for both combinations (Table 9.4)). Results for these two concatenations remain promising when 5% error margins are added (Table 9.5).

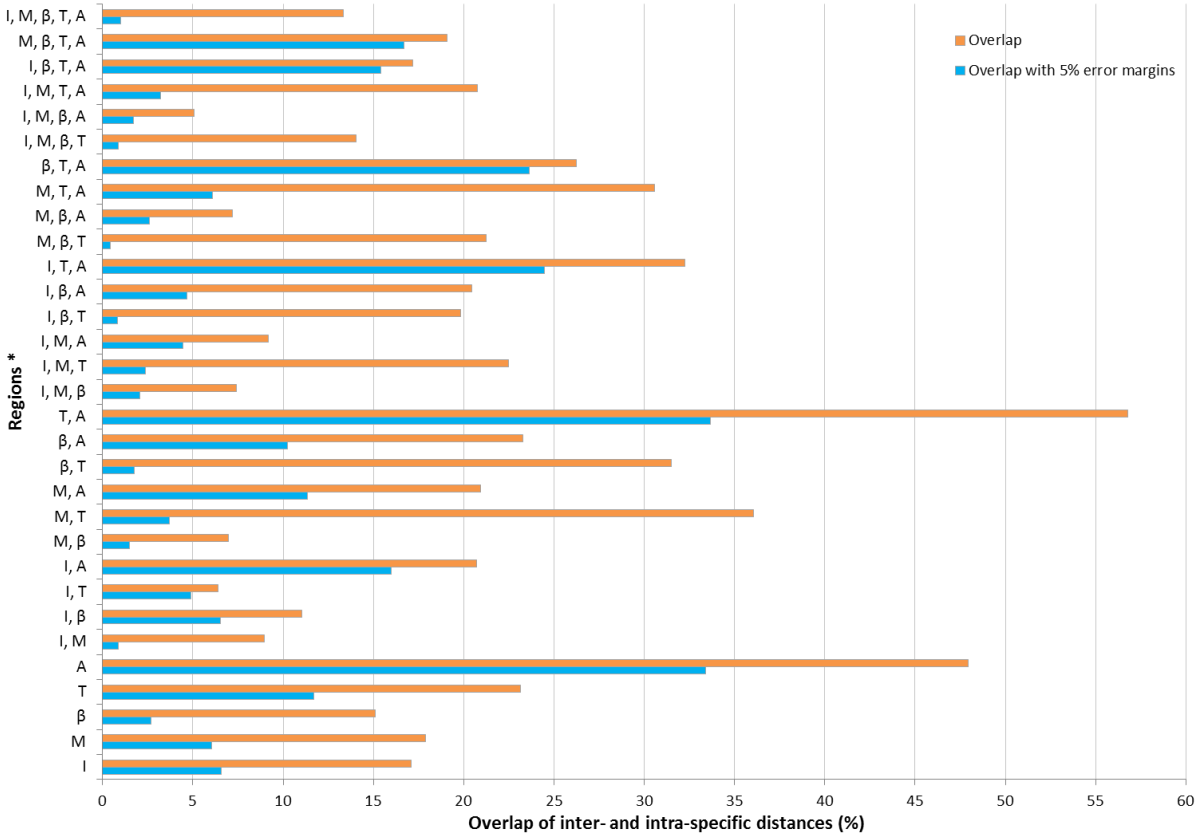

Figure 9.5: Total overlap and overlaps with 5% error margins of concatenated regions of PM accessions by DNA barcoding. *Abbreviated regions combinations: I = ITS, M = *Mcm7*, β = β-tubulin, T = *Tsr1*, and A = Actin.

Table 9.4: Total overlap of inter- and intra-specific distances of all region combinations.

Concatenated regions	Total overlap	
	Total overlap (%)	% covering all inter- and intra-specific accessions
ITS	17.08 (from 0.0 to 17.08)	96.46
Mcm7	17.89 (from 0.41 to 18.31)	93.89
β-tubulin	15.12 (from 0.0 to 15.12)	73.67
Tsr1	23.16 (from 0.3 to 23.47)	87.22
Actin	47.94 (from 0.0 to 47.94)	99.94
ITS, *Mcm7*	8.97 (from 0.33 to 9.31)	86.47
ITS, β-tubulin	11.04 (from 0.0 to 11.04)	72.47
ITS, Tsr1	6.38 (from 0.41 to 6.8)	68.96
ITS, Actin	20.73 (from 0.09 to 20.83)	95.06
Mcm7, β-tubulin	6.97 (from 1.05 to 8.02)	41.87
Mcm7, *Tsr1*	36.05 (from 0.61 to 36.66)	88.27
Mcm7, Actin	20.95 (from 1.05 to 22.0)	94.94
β-tubulin, *Tsr1*	31.49 (from 1.07 to 32.56)	86.19
β-tubulin, Actin	23.27 (from 0.66 to 23.93)	84.54
Tsr1, Actin	56.8 (from 1.27 to 58.07)	94.26
ITS, *Mcm7*, β-tubulin	7.39 (from 1.05 to 8.44)	63.08
ITS, Mcm7, *Tsr1*	22.5 (from 0.6 to 23.1)	87.5
ITS, Mcm7, Actin	9.18 (from 1.03 to 10.21)	79.4
ITS, β-tubulin, *Tsr1*	19.82 (from 1.68 to 21.5)	82.55
ITS, β-tubulin, Actin	20.43 (from 0.42 to 20.86)	89.71
ITS, *Tsr1*, Actin	32.25 (from 0.98 to 33.24)	92.52
Mcm7, β-tubulin, *Tsr1*	21.25 (from 2.12 to 23.37)	80.3
Mcm7, β-tubulin, Actin	7.21 (from 3.08 to 10.29)	51.2
Mcm7, *Tsr1*, Actin	30.56 (from 1.15 to 31.72)	91.69
β-tubulin, *Tsr1*, Actin	26.24 (from 2.66 to 28.91)	88.63
ITS, *Mcm7*, β-tubulin, *Tsr1*	14.06 (from 3.1 to 17.17)	67.77
ITS, *Mcm7*, β-tubulin, Actin	5.05 (from 3.34 to 8.39)	41.86
ITS, *Mcm7*, *Tsr1*, Actin	20.77 (from 0.96 to 21.73)	91.69
ITS, β-tubulin, *Tsr1*, Actin	17.18 (from 3.24 to 20.43)	81.91
Mcm7, β-tubulin, *Tsr1*, Actin	19.1 (from 2.84 to 21.95)	82.41
ITS, *Mcm7*, β-tubulin, *Tsr1*, Actin	13.32 (from 3.44 to 16.77)	70.78

Orange text displays values of total overlap less than 10; the most promising concatenated regions for DNA barcoding.

Green text displays values of low overlap cover of inter-and intra-specific accessions; the most promising concatenated regions for DNA barcoding.

Table 9.5: Total overlap with 5% error margins on both ends of inter- and intra-specific distances of all region combinations.

Concatenated regions	Total overlap with 5% error margins on both ends	
	% Overlap	% covering all inter- and intra-specific accessions
ITS	6.57 (from 1.58 to 8.16)	70.14
Mcm7	6.03 (from 2.16 to 8.2)	51.79
β-tubulin	2.66 (from 3.68 to 6.34)	18.45
Tsr1	11.68 (from 2.16 to 13.85)	72
Actin	33.41 (from 3.46 to 36.88	93.33
ITS, *Mcm7*	0.85 (from 2.38 to 3.23)	13.29
ITS, β-tubulin	6.52 (from 3.3 to 9.82)	64.87
ITS, Tsr1	4.87 (from 1.93 to 6.8)	62.06
ITS, Actin	15.98 (from 3.11 to 19.09)	85.96
Mcm7, β-tubulin	1.47 (from 4.24 to 5.71)	15.02
Mcm7, *Tsr1*	3.68 (from 1.8 to 5.49)	25.15
Mcm7, Actin	11.33 (from 4.36 to 15.7)	69.83
β-tubulin, *Tsr1*	1.77 (from 3.57 to 5.35)	14.11
β-tubulin, Actin	10.23 (from 4.24 to 14.48)	55.68
Tsr1, Actin	33.67 (from 4.94 to 38.61)	80.89
ITS, *Mcm7*, β-tubulin	2.06 (from 3.94 to 6.01)	12.56
ITS, Mcm7, *Tsr1*	2.38 (from 1.45 to 3.84)	8.64
ITS, Mcm7, Actin	4.46 (from 3.95 to 8.41)	48.01
ITS, β-tubulin, *Tsr1*	0.81 (from 3.67 to 4.48)	5.6
ITS, β-tubulin, Actin	4.69 (from 4.39 to 9.09)	43.12
ITS, *Tsr1*, Actin	24.46 (from 4.09 to 28.55)	89.39
Mcm7, β-tubulin, *Tsr1*	0.44 (from 4.36 to 3.92)	0
Mcm7, β-tubulin, Actin	2.6 (from 5.41 to 8.02)	24.55
Mcm7, *Tsr1*, Actin	6.09 (from 4.37 to 10.47)	59.86
β-tubulin, *Tsr1*, Actin	23.63 (from 5.27 to 28.91)	78.78
ITS, *Mcm7*, β-tubulin, *Tsr1*	0.86 (from 4.1 to 3.23)	0
ITS, *Mcm7*, β-tubulin, Actin	1.72 (from 4.75 to 6.47)	20.48
ITS, *Mcm7*, *Tsr1*, Actin	3.2 (from 4.38 to 7.58)	49.05
ITS, β-tubulin, *Tsr1*, Actin	15.43 (from 5.0 to 20.43)	75.27
Mcm7, β-tubulin, *Tsr1*, Actin	16.68 (from 5.26 to 21.95)	70.85
ITS, *Mcm7*, β-tubulin, *Tsr1*, Actin	1.02 (from 4.89 to 5.92)	18.1

Orange text displays values of total overlap with 5% error margins less than five; the most promising concatenated regions for DNA barcoding.

Green text displays values of low overlap cover of inter-and intra-specific accessions; the most promising concatenated regions for DNA barcoding.

9.4: Discussion

9.4.1: Sequence alignment

Initial concatenation of proposed markers from identical accessions yielded final sequence alignments of low numbers of accessions. Given that the total number of PM species identified globally is almost 900 and those recorded in the UK number 196, these small datasets were inadequate as they regularly failed to include the closely-related species that the study aimed to improve discrimination of. The novel nature of PM markers used within the current study meant that generated data could not be supplemented by using sequences from online sequence databases. Sourcing outgroups or additional related taxa from databases such as GenBank is standard practice for numerous phylogenetic, barcoding, biogeographic and other such studies reliant upon sequence data. The grouping of identical species from separate accessions in order to gain additional concatenated sequences to analyse was therefore considered the best solution to this issue of a dearth of data. The result was datasets of far greater size than otherwise possible which could offer more species comparisons during data analysis.

9.4.2: Phylogenetic analyses

Present day PM (Wang *et al.*, 2013, Meeboon & Takamatsu, 2015b, Meeboon & Takamatsu, 2017b) and fungal (Alvarado *et al.*, 2016, Barge *et al.*, 2016, Birkebak *et al.*, 2016) phylogenetic analyses rely upon the ITS as an informative anchoring region. It has been necessary to supplement ITS data with additional identifying regions, which have come from across the fungal genome (Crespo *et al.*, 2007, Faircloth *et al.*, 2012, Quaedvlieg *et al.*, 2013, de Campos-Santana *et al.*, 2016). However, PM studies have most recently begun to rely upon a flanking region of the ITS — 28S (Meeboon *et al.*, 2015, Meeboon & Takamatsu, 2015a, Meeboon & Takamatsu, 2015b, Meeboon & Takamatsu, 2015c, Meeboon & Takamatsu, 2017a, Meeboon & Takamatsu, 2017b). This addition has improved species resolution across the Order, however discrepancies show further improvement is still possible, and even necessary (Meeboon *et al.*, 2015, Meeboon & Takamatsu, 2015a, Meeboon & Takamatsu,

2015b, Meeboon & Takamatsu, 2015c, Meeboon & Takamatsu, 2017a). Concatenation of regions in the current study has been shown to improve PM phylogenetic resolution by more than 10%; certain region concatenations resolving 100% of accessions.

The reduction in number of accessions within datasets of concatenated regions is likely to have been a positive influence on phylogenetic accuracy; species difficult to resolve using molecular or phylogenetic methods, such as *E. euonymicola*, lacked the required spread of data from additional regions and were therefore not included in certain analyses. In the current study a trade-off between number of regions concatenated and number of accessions within the resultant dataset is therefore evident. However, the step to 100% accuracy, in certain cases, shows great promise. Future studies hoping to employ additional regions in order to reproduce results of the current study in order to gain the greatest possible accuracy must also be aware of a trade-off between additional costs from additional sequencing and the diminishing increases in accuracy gleaned from each additional region. This is highlighted in examples of phylogenetic resolution from the ITS (RCFI = 0.88), to ITS and *Mcm7* (RCFI = 0.972), to ITS, *Mcm7*, and *Tsr1* (RCFI = 0.977), to ITS, *Mcm7*, *Tsr1*, and β-tubulin (RCFI = 1). However, such a trade-off is likely to be overcome with the use of next-generation sequencing (Rizzo & Buck, 2012).

Various region combinations yielded similarly positive results and the next logical step was to discount certain regions, in favour of those with the greatest utility. Regions such as Actin, which when analysed alone, seem to be poor candidates, are particularly challenging as they can become ideal 'identifiers' when combined with certain other region combinations. The sub-optimal consensus tree of the Actin region lacked phylogenetic resolution; a result of its great inter- and intra-specific diversity. This was easily resolved when combined with other phylogenetic regions shown to provide greater phylogenetic resolution.

When analysed alone, β-tubulin was shown to produce a phylogeny with similar accuracy to that of the ITS (Chapter 5 & Table 9.3), whereas it tended to reduce the accuracy of phylogenies when combined with all combinations of the other four regions (with the exception of Actin).

Analyses of the *Mcm7* region have proven consistently to produce phylogenies similar to those expected, regardless of the additional regions they are combined with. Future phylogenies of PMs, and fungi in general, should consider this region in the search for greater reliability and accuracy from phylogenetics. As ever, further studies are required to test the performance of region combinations on a greater diversity of accessions.

9.4.3: DNA barcoding analyses

While the ITS region has been proposed as the universal fungal barcode (Schoch *et al.*, 2012), it cannot accurately identify species in many genera of plant-pathogenic fungi (e.g., *Alternaria*, *Botryosphaeria*, *Calonectria*, *Cercospora*, *Diaporthe*, *Fusarium*, *Ilyonectria*, *Teratosphaeria*, etc.). However, it has been shown to consistently reach at least the generic level (Schoch *et al.*, 2014). The necessity for additional regions is therefore widely accepted within DNA barcoding practice (Steciow *et al.*, 2014, Crous *et al.*, 2015, Mallo & Posada, 2016). Due to the lack of crossover of sequence data from individual PM accessions (see Appendix 5), conspecific accessions were combined such that there were few intra-accessional distances typical of DNA barcoding analyses; these were replaced by intra-specific distances. Although this is a breach of the regular barcoding technique, combining identical species has ensured that the results remain relevant.

Regions trialled in the present study have shown promise for diagnostic practices by increasing the number of resolved PM species accessions. Regions β-tubulin and *Mcm7* improved barcoding results seen in the 'universal fungal barcoding region' ITS (Schoch *et al.*, 2012) by showing evidence of consistent, reproducible, unambiguous species discrimination for rapid data processing by computer programmes (in this case the TaxonDNA/SpeciesIdentifier 1.8). These should be considered for future barcoding analyses of the PMs and the wider fungal clade.

9.5: Conclusions

PM DNA regions have been concatenated and trialled, using BI and DNA barcoding analyses, for their combined species diagnostic utility. Results show clear improvements from the existing benchmark set by the fungal and PM standard region — the ITS. Additional costs associated with sequencing and analysing additional regions can be justified by the greater accuracy of PM species discrimination which comes as a result of augmentation of the ITS region. While β-tubulin outperforms other regions when analysed for barcode potential, the *Mcm7* region outperforms all other regions, including the ITS, for phylogenetic reconstruction via BI, and all regions apart from β-tubulin as a species barcode. As such, the *Mcm7* region is deemed to be the most suitable region to complement the ITS. Given, the accuracy of the *Mcm7* region when analysed alone, there is also a case for using it exclusively; instead of the ITS. However, the quantity of ITS sequence data already deposited within online sequence databases, ensures that it will remain a necessary marker for fungal diagnostics and particularly to allow comparison of potential novel species to past accessions.

Chapter 10: General Discussion

10.1: Introduction

PMs continue to have substantial detrimental effects on important global crops (Curtis *et al.*, 2002, Sabaratnam, 2012) such as cereals (Rabbinge *et al.*, 1985, Liu & Shao, 1995, Curtis *et al.*, 2002), vegetables (Sabaratnam, 2012, Zheng *et al.*, 2013), ornamentals (Denny, 2014), fruit trees (Boesewinkel, 1980, Boesewinkel, 1981, Polk *et al.*, 1997, Sijaona *et al.*, 2001, Jones & Aldwinckle, 2002) and amenity trees (Cook *et al.*, 2004). The ability to improve the identification process of the different species known to cause such damage will continue to aid control and monitoring. Key aspects of this include targeted defence against specific PMs (Kim *et al.*, 2002, Pessina *et al.*, 2014, Jiang *et al.*, 2016) and limiting pathogen spread (Bebber *et al.*, 2014); both practices are aided by accurate and efficient diagnostics. Diagnostic practices and taxonomic detail have increased as PM biology, host ranges, distributions, and phylogenetic relationships are better understood and as a result, new PM species continue to be described (Ale-Agha *et al.*, 2008, Tang *et al.*, 2017). However, a global inventory is incomplete, with numerous more PM fungi likely to be found in understudied tropical regions (Limkaisang *et al.*, 2006) and detailed descriptions of holomorphic PMs still lacking for the majority of described species. The identification of informative molecular markers can augment current information in order to increase reliability of species determination.

The current study enlisted volunteer help to contribute PM accessions affecting their own gardens and immediate surroundings to enable broad-scale sample collection. The efficacy of established identification techniques for PM species discrimination was then examined and attempts to improve the process were explored. Protocols for obtainment of additional PM accession data from seven, previously neglected, regions were developed with varying success. Resultant data were then analysed for their PM species diagnostic utility using established methods of analyses, also with varying success.

10.2: Sampling

PM accession collection from the University of Reading campus and RHS Garden Wisley was supplemented by the three year Powdery Mildew Survey. This provided a more complete understanding of PM presence across the UK through a range of accessions of plants hosting PMs potentially novel to the host, the study, the country, or the world. Greater effort in publicising the scheme successfully increased public awareness of the effect of PMs, and the need for samples to improve diagnostics; peak sampling coming in the autumn of 2014 and the summer of 2015 when the scheme was promoted at numerous flower shows and science fairs, as well as within relevant specialist society publications.

The success of the current citizen science scheme shows the great potential for further, similar scientific sampling projects and highlights the willingness of the public to engage with science. Small-scale, independent citizen science schemes, such as this, have become more achievable as social media platforms ensure the possibility of immediate connection to potential audiences. This aspect of scheme promotion was well utilised with regular blogs and online outreach, however, the physical outreach could have been improved by organising collection events and being present at applicable society outreach events. A strong case study of scientific outreach is that of Cape Citizen Science from South Africa, which aims to develop local knowledge of *Phytophthora* in order to keep natural areas healthy. Public engagement and communication of the project uses a suite of online and printed media as well as through organising collecting days and highlights the fact that, through these means of sampling, anyone can contribute to science (Hulbert, 2016).

Distribution data generated from the current project was submitted to the National Biodiversity Network Atlas (National Biodiversity Network, 2017) and the British Mycological Society checklist of fungi of the British Isles (British Mycological Society, 2017). Such contributions have provided the project with a legacy; serving as references for future studies and highlighting the presence of PMs across the UK.

Future success could be achieved simply by continuing to promote and maintain it. This would present an ideal opportunity to collect data over a large spatial and temporal extent, by continuing to log the distributions of currently known PMs of the UK and identifying new PMs as threats to native flora. The scheme could include a mobile-friendly app in future such as that used by the OPAL Tree Health Survey (OPAL Forest Research FERA, 2017) in order to pin the sample and its later identification to its specific location and enable updates to species presence and distribution data. However, such developments may have a negative impact on contributions, as participants begin to believe it to be obligatory to possess the app in order to contribute.

Creating projects similar to Cape Citizen Science and the Powdery Mildew Survey is achievable. Project convenors may alter the studied pathogen and location of study in order to increase knowledge of specific plant diseases in the UK, PMs in other countries, or specific plant diseases in other countries. Given appropriate and prompt feedback on receipt of samples, contributors will continue to provide further samples and feel that the project is worthwhile. Any future citizen science based study should ensure feedback of this nature in order to maximise the possibility of receiving repeat samples from specific collectors.

10.3: Species identification using established techniques

Analysis of host plants (Chapter 2), fungal morphology, and ITS sequence data (Chapter 3) in the current study enabled PM species identification in 80% of samples. Despite the sequence data currently available in GenBank for the ITS region, many accessions such as those on *Heuchera* cvs. (Ellingham *et al.*, 2016) yielded ambiguous results due to a lack of previous ITS data and a lack of variability in deposited ITS data. This was the case for the majority of PM accessions; requiring prior knowledge of expected PM species in order to select potential appropriate ITS matches from the database. Morphology and host data were therefore required in order to identify PM species; morphological measurements (specifically asexual spore dimensions) enabled the *Heuchera* PM species to be identified. Linking such precise, repeat morphological measurements to sequence data

may enable future identifications to be made from this ITS data, providing it is unique to the PM species. ITS data of *P. macrospora* ex *Heuchera* was therefore submitted to the NCBI GenBank along with all other PM ITS sequences generated and will serve as references for future PM identifications.

Twenty percent of accessions could not be identified from the combination of host, morphological and ITS data due to shared fungal morphological features as well as ITS rDNA and the nomenclatural and taxonomic limitations of certain closely-related, newly separated taxa. Of these, the most common issue was a lack of resolution beyond genera as species were too similar to tell apart. This was evident in phylogenetic reconstructions and was consistent with the literature, which has frequently highlighted examples of different PM species with indistinguishable ITS rDNA (Takamatsu *et al.*, 1999, Khodaparast *et al.*, 2012). This serves to further highlight the urgent need for robust, multi-gene phylogenies to clarify species limits and their lineages.

Cases in which this is evident arise within *Podosphaera* and particularly *Erysiphe*. The multitude of species within these groups (approximately 90 and 380 respectively) and their relatively recent adaptive radiations (Takamatsu & Matsuda, 2004, Takamatsu *et al.*, 2010) mean that species have not had sufficient time for consistent molecular divergence within the ITS. There are therefore numerous cases within the PMs of 'compound' species such as *G. cichoracearum* (Matsuda & Takamatsu, 2003, Lebeda & Mieslerová, 2011), or *P. erigerontis-canadensis (Braun & Cook, 2012)* particularly within the *Erysiphe* such as *E. trifoliorum* (Braun & Cook, 2012). These may prove to merit finer delimitation, into *formae speciales* or varieties for instance, however these are not recognised by the Botanical Code (Voss *et al.*, 1983). 'Cryptic species', different forms indistinguishable by most means (Hebert *et al.*, 2004, Saito *et al.*, 2016), are also found in the PMs. Examples include *G. cynoglossi* shown in the current study to be separated by molecular analyses. It would be of great interest to investigate the signature enabling the Erysipheae to readily jump to new hosts (Matsuda & Takamatsu, 2003). If a gene or suite of genes responsible for this were identified, they could be silenced in order to limit the capacity of PMs to spread to new hosts.

In contrast the Phyllactinieae clade and accessions within it were consistently discriminated, potentially due to the low number of collected accessions of the few species in the study. Many more Golovinomyceteae accessions were collected, but these were also consistently discriminated. The rate of diversification since the clade arose from other PMs approximately 40 million years ago (Takamatsu *et al.*, 2008a) has been relatively slow; with just 57 extant species (one *Arthrocladiella*, 45 *Golovinomyces*, and 11 *Neoerysiphe*).

As in the present study, previous efforts to test the barcoding ability of the ITS have proved its variable diagnostic utility across different fungal genera (Crous *et al.*, 2015). The ability of even short reads to identify certain fungal species, as in the study of Min and Hickey (2007), has not been shown in this study. Longer reads, including fragments spanning the ITS2, the LSU-D1 and LSU-D2 domains as in Stockinger *et al.* (2010), or additional genetic markers as in Irinyi *et al.* (2015) may therefore be required for reliable species identification. Future studies are likely to use next-generation approaches in order to enable diagnostics. These will generate the sequence data needed for comparison with past studies concurrently with additional data. Studies analysing the diagnostic utility of DNA regions as additional identifiers will help explore and elucidate the taxonomic and phylogenetic uncertainties currently evident.

10.4: Improving PM diagnostics

It is demonstrated in this thesis that sequencing of regions additional to the ITS considerably aids unambiguous species identifications. The 18S (Saenz *et al.*, 1994, To-anun *et al.*, 2005), β-tubulin (Inuma *et al.*, 2007, Seko *et al.*, 2010), *Chs* (Seko *et al.*, 2010), and 28S (Takamatsu & Matsuda, 2004, Meeboon & Takamatsu, 2017b) regions have been used in the past with varying success. Similarly, the regions *Chs*, Calmodulin, EF1-α, Actin, *Tsr1*, β-tubulin, and *Mcm7* trialled in the current study have offered varying levels of useful, additional diagnostic data to the ITS.

The wealth of fungal rDNA data available from previous studies initially ensured that appropriate regions were identified and could be evaluated for potential diagnostic utility. All

regions trialled in the current study, apart from the *Mcm7* and *Tsr1*, have been widely used for increasingly refined identification of species within other fungal clades (Table 10.1). Generic primers were therefore sourced with ease, but their use favoured amplification of fungi other than the PMs within the environmentally sourced DNA (Martin & Rygiewicz, 2005, Bellemain *et al.*, 2010). Alignment of the Calmodulin and *Chs* gene regions provided insignificant variability between PM species and their fungal conspecifics for attempts at specific primer design (Chapter 8). The other candidate genes, including *Mcm7* and *Tsr1*, provided numerous priming sites with potential specificity to PMs. Developed primers of EF1-α consistently amplified multiple products and the gene was therefore excluded from later amplifications (Chapter 8). However, optimal primers and protocols were developed for reproducible amplification and sequencing of the four remaining gene regions.

The Actin gene (Chapter 7) was amplified and sequenced with the greatest success (approximately 90% of samples), but provided negligible identification utility to complement the ITS. The region was just 30% conserved and similar to the studies of Weiland and Sundsbak (2000) and Hunter *et al.* (2006) did not enable species discrimination. Phylogenetic reconstructions produced sub optimal results as genera and species were scattered throughout the topology. Although the study of Quaedvlieg *et al.* (2014) showed Actin to provide important barcoding data, the current analyses showed inter- and intra-specific distances to be mixed such that similar sequences could have been from accessions of the same species, but equally different genera of different tribes. This uncertainty may be a result of the amplification and latter sequencing of a different PM species present in the leaf microbiome to those amplified by most other regions. However, it is more likely to be due to sequencing of substantially different copies, pseudogenes or paralogous copies, of the Actin gene may have been sequenced from different accessions of particular PM species.

Table 10.1: Well resolved fungal clades of previous studies.

Fungal clade	**Reference**
Armillaria	Maphosa *et al.* (2006)
Aspergillus	Samson *et al.* (2014)
Basidiomycota	Matheny *et al.* (2007)
Caliciaceae	Prieto and Wedin (2016)
Candida	Daniel *et al.* (2001)
Dermatophyte species	Kano *et al.* (1997)
Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes and Sordariomycetes	Schmitt *et al.* (2009)
Geomyces	Minnis and Lindner (2013)
Gibberella/*Fusarium*	O' Donnell *et al.* (1998a), O' Donnell *et al.* (1998b), O' Donnell *et al.* (2000), Mulè *et al.* (2004), Seifert and Lévesque (2004), Kristensen *et al.* (2005), Amatulli *et al.* (2010)
Kickxellomycotina	Tretter *et al.* (2013), Tretter *et al.* (2014b)
Lasallia	Sadowska-Deś *et al.* (2013)
Magnaporthe oryzae	Kong *et al.* (2012)
Montanelia	Divakar *et al.* (2012)
Mucorales and Mortierellales	Voigt and Wöstemeyer (2001)
Mycosphaerella	Hunter *et al.* (2006)
Neofabraea	de Jong *et al.* (2001)
Penicillia	Wang and Zhuang (2007)
Sporothix	Madrid *et al.* (2009), Romeo *et al.* (2011)
Thermophilic fungi	Morgenstern *et al.* (2012)
Xanthoparmelia	Leavitt *et al.* (2011)

Amplification and sequencing of the *Tsr1* gene (Chapter 6) was successful approximately 70% of the time and was significantly affected by alteration of sequencing companies from 2014 and 2015 to 2016. This is likely due to the optimisation of initial protocols to the low volume requirements of Source BioScience. An alteration of PCR product volume required for sequencing with GATC meant that the optimised PCR protocol had to be adjusted for a greater final volume. Resultant DNA data enabled identification of 86% of accessions to species level, when accompanied by host, morphological, and ITS data (a 6% improvement from established techniques). Schmitt *et al.* (2009) showed the region to be alignable across different fungal orders ("a wide range of unrelated taxa") and at the same time have sufficient variability to resolve within-genus relationships, however PM sequences proved to be difficult to align, particularly as a result of hypervariable regions also

identified by Schmitt *et al.* (2009). Studies investigating the utility of *Tsr1* have regularly failed to compare these data with established regions, such as the ITS (Schmitt *et al.*, 2009, Tretter *et al.*, 2013). However the study of Sadowska-Deś *et al.* (2013) confirmed that ITS has insufficient variability for intraspecific studies within populations and the use of protein-coding genes, particularly *Tsr1* (and *Mcm7*), may enable assessments of variability within and among populations. This region offers useful additional information to aid in species discrimination and thus could be used as an identifier, alongside the ITS. However phylogenetic and barcoding analyses from the current study showed it was not as informative as other trialled regions and resolved few additional species when analysed alone and combined with the ITS.

Sequencing of the β-tubulin gene (Chapter 5) was also affected by the change of sequencing companies. However, resultant data showed evidence of the high efficacy of the region as a reliable identification locus for PM fungi across the whole scale of tested sequences via DNA barcoding and concurs with the study of Quaedvlieg *et al.* (2014). Direct comparison of β-tubulin with other tested regions showed it to be the best candidate as a reliable marker for barcoding analyses of PM. However, the result did not produce a barcoding gap and thus PM species of *Podosphaera* and particularly *Erysiphe* remained mixed. Phylogenetic analyses proved β-tubulin was as informative as ITS and augmented barcoding ability and phylogenetic resolution of the ITS when the two were concatenated. The region is therefore a strong candidate as a PM identifier to be used alongside currently established techniques.

For the *Mcm7* gene (Chapter 4) 80% of accessions trialled were successfully amplified and sequenced. While the ability of *Mcm7* to discriminate inter- and intra-specific distances was little better than the ITS, the combination of *Mcm7* and ITS reduced the overlap of inter- and intra-specific distances to less than 10% of accessions. Phylogenetic analyses proved the great potential of *Mcm7* for improving PM diagnostics; resolving 97% of all accessions as expected from an ideal tree. The region could be adopted to aid future identification of PM species.

While *Mcm7* has shown the greatest accuracy at solving current phylogenetic discrepancies, β-tubulin proved superior for DNA barcoding. Region concatenation (Chapter 9) can improve diagnostics and factors, such as population size, time between speciation events, and properties of the loci must be considered (Leaché & Rannala, 2010, Knowles & Kubatko, 2011, Liu & Yu, 2011) as the number of loci required to resolve a phylogeny can vary greatly (Lemmon & Lemmon, 2013). When concatenated and analysed the *Mcm7* and β-tubulin regions were amongst the best performing combinations for DNA barcoding analyses. Phylogenetic analyses showed *Mcm7* to be the most accurate single region. Accuracy was improved when combining the region with ITS and *Tsr1* and reached 100% when combining these three regions with either β-tubulin or Actin.

The difference in results between phylogenetic and barcoding techniques is particularly interesting. Bayesian analyses of the current study have generally provided high species resolution with good PPs and yet the barcoding results have been poor; rarely providing unambiguous discrimination between inter- and intra-specifics. It is uncommon for a barcoding region to offer sufficient phylogenetic signal to resolve evolutionary relationships, especially at deeper levels (Hajibabaei *et al.*, 2006). The opposite is also true of phylogenetically informative regions and thus the hope that the same region could offer increased certainty for PM species identifications through both methods may have been poorly founded.

Greater discrimination of accessions when using Bayesian analyses rather than barcoding is not uncommon (Rubinoff *et al.*, 2006, Heimeier *et al.*, 2010, Dai *et al.*, 2012). The difference, evident in computational time, stems from the use one of the most basic phylogenetic methods available (simple pairwise distances interpreted through clustering to produce tree-like representations of species clusters (Neighbour Joining phenograms)) in typical barcoding. This has led to sustained criticism that barcoding uses bad phylogenetic practice and therefore its conclusions are suspect (e.g., Will and Rubinoff (2004)). The Bayesian method enlisted in the current study for phylogenetic reconstruction also has limitations as posterior probability may support false phylogenetic

hypotheses (Douady *et al.*, 2003) and ambiguous data has been shown to have deleterious effects on topological conservation (Lemmon *et al.*, 2009), however, BI has been shown to require less computation time than other molecular systematic methods such as maximum parsimony and maximum likelihood (Leaché & Reeder, 2002, Douady *et al.*, 2003) and incorporates appropriate models into any analysis (Posada & Buckley, 2004).

However, on balance, BI has produced results with far greater utility than that of DNA barcoding. The nature of the barcoding analysis means that outliers such as incorrectly identified PM species, a potential result of more than one PM infecting a single host, can easily skew resulting data. It is therefore rarely useful as the sole analysis tool. Instead DNA barcoding of PMs and similar phylloplane fungi can serve as an initial tool for taxa placement into tribes and genera and taxa selection before later analyses. In this way, appropriate accessions could be selected for BI of individual clades of closely related taxa (Hajibabaei *et al.*, 2007).

Mcm7, β-tubulin, *Tsr1*, and Actin have each augmented the accuracy of diagnostics as additional informative features by enabling greater discrimination of closely related PMs. However, the *Mcm7* region has proven to be the single most informative region; bettering ITS-based results such that it could be considered as an ITS alternative. Nevertheless, more research is required in this field in order to evaluate whether the *Mcm7* region is superior to other regions and whether analysis of this region alone can precede others when whole genome sequences become easily producible.

Identifications have also served to further understanding of the specificity of PMs. It is understood that more than 90% of PMs are specific to an individual genus or species of plant host (Braun & Cook, 2012). These can be considered specialist pathogens. Why pathogens would stray from a generalist nature to limit themselves to just a few potential hosts is debatable, but there are likely to be trade-offs impeding optimal adaptation to several host species at the same time (Van Tienderen, 1991, Barrett & Heil, 2012, Bruns *et al.*, 2014). These trade-offs may be constraints or a lower coevolutionary rate when tracking a particular host species (Whitlock, 1996). Therefore, if a

principal host is sufficiently abundant, specialist species may be advantaged as they can exploit their hosts more efficiently (Soler *et al.*, 2009) and will therefore be the most common type. Generalist PMs have been considered to be uncharacteristically abundant amongst biotrophic pathogens with species such as the *E. alphitoides* (Desprez-Loustau *et al.*, 2010), *E. trifoliorum*, *G. orontii*, *P. erigerontis-canadensis* known to occur on numerous host plants spanning different plant families. However, with ever greater detailed gleaned from studies, the similarities tying such forms to a single denomination become more distinguished and studies therefore begin to separate PM species into separate species, varieties, or *formae speciales*. This is perfectly exemplified within the current study as initial morphological investigations point the researchers towards a specific PM clade. Data, such as the rDNA ITS, then augment initial data and can confirm initial delimitations, however, ambiguities remain and similar accessions can easily be attributed to the same clade. It is only with further investigation, in this case the sequencing of additional regions, that such delimitations are proved insufficient and PMs occurring on a specific host family, shown to appear morphologically the same, and possess similar ITS rDNA are in fact more than one species.

10.5: Future work

Knowledge of the regions shown to be diagnostically informative in the current study, *Tsr1*, *Mcm7*, and β-tubulin, and efficient computation of results via DNA barcoding and BI will aid in future unambiguous identification of PM species from around the world. These stable, reproducible methods for PM identification will aid in field trials for resistant agricultural and horticultural plant varieties. Currently, specific PM species known to infect a host are targeted in the development of host resistance (for example *E. pisi* infecting *Pisum sativum*). The possibility of additional PM species infecting a host (for example *E. trifoliorum* and *E. baeumleri* infecting *Pisum sativum* (Fondevilla *et al.*, 2011, Fondevilla & Rubiales, 2012)) can therefore confuse comparisons of infection methods of PM on susceptible and resistant varieties. Efficient and accurate PM identification at various stages of field trials will therefore enable comprehensive conclusions of host resistance to be made.

Furthermore, promotion of PM resistance loci of hosts such as the MLO (Jørgensen, 1992) may be specific to certain PMs. Identification of PM species within trials is therefore critical.

Numerous pathogens other than PM are also threatening global food security and biodiversity. *Phytophthora* is a pertinent pathogen known to cause disease in numerous plant species worldwide. This is a genus of hundreds of plant destroying Oomycetes (Brasier, 2009) which also require the ITS for identification of genera and species (Martin *et al.*, 2012, Scibetta *et al.*, 2012). Like PM identification, *Phytophthora* identification faces similar pitfalls due to reliance on the ITS region (Kang *et al.*, 2010, Scibetta *et al.*, 2012). Studies furthering potential markers for species diagnostics within this problematic clade, as well as numerous others, could therefore aid in disease prevention in multiple host-disease relationship examples. This would in turn greatly reduce pressures on plants important for agriculture, horticulture, and biodiversity.

Improved efficacy and accuracy of diagnostically useful molecular markers of plant pathogens could be paired with new technologies such as lateral flow microarrays (Carter & Cary, 2007) or direct PCR (Werle *et al.*, 1994) and on-site sequencing of targeted regions using nanopore technologies such as the MinION (Eisenstein, 2012, Mikheyev & Tin, 2014, Mitsuhashi *et al.*, 2017). These could offer a fast and efficient method for disease identification for plant health practitioners. If put into practice, they would boost the identification of old and new PM species on old and new hosts in the field and provide border checkpoints at main points of entry such as those working with the government's Animal and Plant Health Agency (APHA) with an augmented toolkit for ensuring new, unwanted threats to the UK horticultural and agricultural industries do not enter the country.

The sequences generated from this study were deposited in the NCBI GenBank and will serve as references for future PM identifications (providing the regions come into common usage). Ultimately, a comprehensive database of accurately identified sequences is central to the molecular identification of PMs. The generation of sequence data for multiple gene regions, using a range of PM samples from varying geographic distributions, has enabled progress towards this long-term aim.

Knowledge of different elements of the PM genome and their utility for varying purposes will aid in future genome characterisation when next generation methods are likely to come into common usage. In order to aid this, PMs spanning the globe and their entire Order should be sequenced, with particular attention on species complexes of the *Podosphaera* and Erysiphe, such as that of *E. euonymicola* - *alphitoides* highlighted in the current study or the oak PM complex from Feau *et al.* (2012), in order to complete a holistic study of PMs of the world; their taxonomy and phylogeny, and their evolutionary history.

The ITS region has historically underpinned fungal diagnostics, and due to the present quantity of ITS sequence data deposited within online sequence databases, is likely to continue to do so. The β-tubulin region can offer additional diagnostic utility to the PM identification process; serving as an 'identifier'. The *Mcm7* offers greater promise though and could replace the ITS. While much work remains, the results obtained in the current study have confirmed that molecular techniques show promise in the major effort of documenting and understanding the diversity of PMs by using diagnostics. Implementation of the methods outlined in the current study has the potential to limit economic damage caused to horticultural and agricultural industries as well as biodiversity.

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Appendices

Appendix 1: Powdery Mildew Survey launch blog posts from 2014, 2015, and 2016.

Powdery Mildew Survey

Posted on May 21, 2014 by Oliver Ellingham

Please refer to the 2015 survey for updated info.

As part of the Powdery Mildew citizen science scheme, I am asking YOU to keep an eye open for powdery mildews.

Powdery mildews commonly occur on garden plants, are unsightly, and can cause serious damage. To help understand how widespread powdery mildews are, both in terms of geography and hosts, the Royal Horticultural Society and University of Reading are working together to identify and map as many powdery mildews as possible over the next two growing seasons. You can help by supplying us with infected plant samples and in exchange we will do our best to tell you what mildew is infecting your plant.

Powdery Mildew Survey 2015

Posted on March 3, 2015 by Oliver Ellingham

Please refer to the 2016 survey for updated info.

The inaugural 2014 Powdery Mildew Survey produced a total of 160 powdery mildew samples resulting in 51 different species. Samples were received from all around the UK.

With two years of collection records, a baseline of species recorded in the UK and spring arriving, what better time to launch 2015's Powdery Milwho? So please send in your powdery mildew infected plant material for identification: will yours be a common UK species, a species shifting to a new host, an invasive species, or even a new species?

Follow the guidelines on how to send in your samples here.

Powdery mildews commonly occur on garden plants, are unsightly, and can cause serious damage. To help understand how widespread powdery mildews are, both in terms of geography and hosts, the Royal Horticultural Society and University of Reading are working together to identify and map as many powdery mildews as possible over the next two growing seasons. You can help by supplying us with infected plant samples and in exchange we will do our best to tell you what mildew is infecting your plant.

Powdery Mildew Survey 2016

Posted on March 17, 2016 by Oliver Ellingham

Now entering its third year the powdery mildew citizen science scheme is gaining more momentum, more followers, and more samples.

Can we hope for more again this year?

The inaugural 2014 Powdery Mildew Survey produced a total of 160 powdery mildew samples resulting in 51 different species. This was followed in 2015 by a further 353 samples: 54 species.

In 2015 we identified powdery mildew on new hosts, and concluded with the adoption of the scheme by GCSE course conveners; students will now have the opportunity to contribute to science and learn via this novel method.

So, with two years of citizen science collection records, a baseline of species recorded in the UK, and spring arriving, what better time to launch 2016's Powdery Milwho? So please send in your powdery mildew infected plant material for identification: will yours be a common UK species, a species shifting to a new host, an invasive species, or even a new species?

Follow the guidelines on how to send in your samples here.

Appendix 2: Science and Plants for Schools - The powdery mildew survey - teacher and student notes

The powdery mildew survey
Technical & Teaching Notes

Introduction and context

In this practical and data analysis activity students collect samples of leaves showing samples of infection with powdery mildew; the samples can be sent for analysis as part of the *Powdery Mildew Survey* citizen science project. Students analyse data from the survey at the end of this activity.

Ideas about communicable diseases in plants are included in the updated programmes of study for Key Stage 4 science published in December 2014. These ideas will be included in GCSE Science courses from 2016 (for first assessment in summer 2018).

This activity will help students to demonstrate the following learning outcomes at KS4:

- describe ways to identify a common plant disease in the lab and in the field
- explain how the spread of communicable diseases may be reduced or prevented in plants.

It also enables students to practice aspects of the 'Analysis and evaluation' strand of *Working Scientifically*, and to practice the following KS4 mathematical skills:

- M1c: Use percentages
- M2c: Construct and interpret frequency tables and bar charts
- M4a: Translate information between numeric and graphical form

Background information

In this activity, powdery mildew is used as an example of a plant disease common in Britain. Powdery mildew is caused by pathogenic fungi of almost 900 different species belonging to various genera in the family *Erysiphaceae*. The symptoms of infection include white, powdery patches of fungus that spread over the upper and lower surfaces of leaves, stems, and sometimes also flowers and fruit. The white, powdery patches can be seen easily with the naked eye.

The disease affects many different plant species in the UK. Each fungus has a limited range of host plants, but the large number of fungal species means the disease is commonly seen in apples, blackcurrants, gooseberries, grapes, courgettes, marrows, cucumbers, peas, honeysuckle, rhododendrons, azaleas, roses, oak trees, barley and other grasses.

Mildews are just one of the many fungal diseases that threaten our crops, particularly as climate change affects temperatures and rainfall. Powdery mildew can reduce wheat yields, for example, by up to 20%.

The *Powdery Mildew Survey* is run by the Culham Research Group at the University of Reading and the Royal Horticultural Society. They are asking people to send samples of infected plant material as part of a citizen science project. The data contribute to a national database of powdery mildew fungal species, the host plants infected, and the locations in which they are found. This helps scientists to track the prevalence of UK species, the introduction of new species, and examples of fungal species shifting to new host plants.

Safety Notes

Fungal spores are usually present in normal air, so the risk of adverse reactions to the spores is minimal. However, keeping infected plant material inside plastic food bags as much as possible will eliminate most of the safety concerns. Transporting the infected material in sealed bags will prevent infection of other plants. It is not necessary to wear gloves when handling the infected plant material, but hands must be cleaned immediately after doing so. Students with a known allergy to fungal spores should avoid handling the infected plant material.

For any infected plant material not sent to the Powdery Mildew Survey:
Sealed bags should be disposed of after use. Infected plant material could be composted, as the composting process will sterilise the spores produced by the fungus.

Apparatus and materials

Before the practical activity, the teacher/technician will have to find a location in which some plants are showing symptoms of powdery mildew infection.

Per student/pair:

- student activity sheet
- clear, sealable plastic food bag
- marker pen and sticky label
- access to:
  - plant species identification key (appropriate for the chosen location)
  - scissors (to remove infected leaves from plant)
  - antibacterial hand gel
  - smartphone, or a record of the GPS coordinates or postcode of the chosen location.

Suppliers

Susceptible species grow in all parts of the British Isles, and powdery mildew infection is widespread all year round. Powdery mildew is most likely to be found in damp locations that are sheltered from the wind. It is commonly found on roses, aquilegia, geraniums, hawthorn, oak, birch, willow, acers and fruit trees such as apple and cherry.

The following photograph of geranium leaves infected with powdery mildew is provided to aid identification when sourcing material for this activity. (Photograph courtesy of Oliver Ellingham, University of Reading)

Teaching Notes

The OCR Gateway specification for GCSE Biology requires students to describe barley powdery mildew (*Erysiphe graminis*).

Students could work in pairs to collect the infected leaves. However, each student should answer the questions on their own.

There are many free smartphone apps available that will give GPS coordinates of the user's location (including the compass app that comes pre-installed on an iPhone). Alternatively, the GPS coordinates of the chosen location can be determined in advance using Google Maps (right click on the chosen location and select "What's here?" to view the coordinates in decimal degrees).

More information about the *Powdery Mildew Survey* and photographs of plants displaying typical symptoms of infection are available at https://blogs.reading.ac.uk/crg/powdery-mildew-survey-2016/.

Sending samples to the Powdery Mildew Survey

Infected plant material should be placed in a sealed bag marked with the GPS coordinates or postcode of where the sample was found, the species or common name of the host plant, and your email address (so that a link to the record in the national database can be emailed back to you).

Post samples to: Powdery Mildew Survey, Oliver Ellingham, Harborne Building, School of Biological Sciences, University of Reading, RG6 6AS.

When species identifications have been received back from the survey, students can compare them to the list of species identified in the 2015 survey; the list can be found at http://blogs.reading.ac.uk/whiteknightsbiodiversity/campus-species-lists/fungi/campus-species-listsfungipowdery-mildew-2015/. Discuss whether the species in the students' samples are common to the UK, or are (potentially invasive/new) species that have not been recorded in the UK before.

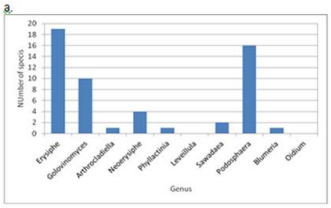
Additional support

Some students may benefit from the following additional support:

- During step 1 of the collection procedure, remind the students to look for white, powdery patches of fungus spread over the upper and lower surfaces of leaves and stems.
- In question 2a, point out that the table contains frequency data, and the data are discontinuous; if necessary, remind the students that this suggests they should draw a bar chart.

Answers to questions

1. Step 4 (seal the bag) and step 8 (keep the bag sealed during transport) help to reduce the risk of spores from the sample being carried away by the wind, which reduces the risk of spreading them to other plants/areas. Step 5 (use hand gel to clean hands after touching infected leaves) will kill or remove fungus/spores, to reduce the risk of transferring them to other plants by direct contact.
2. a.



b. *Erysiphe*

c. 54/150 x100% = 36%

d. Lower ability students may agree with the conclusion and should support this with relevant data from the table. Higher ability students should discuss the idea that the conclusioin cannot be made from the 2015 survey data alone because e.g. the survey only looked at 353 samples, it's only one year, did not (it's impossible to) sample every infected plant/site in the UK, species from the *Oidium* genus may be present in the UK but weren't present in the 2015 samples, etc.

3. Global trade / infected plant material imported from East Asia.
4. a. Sequence the DNA/genome and compare to sequences from known species of fungi, or use gene probes/DNA finger-printing to test for particular sequences/alleles/genetic

Appendices

variants in the DNA. Also the idea that this is more accurate that identifying species from symptoms/appearance.

b. It helps scientists to: track the spread of different fungus species around the UK; identify invasive species that are new to the UK; give advice to farmers/gardeners/public/the government to help stop the spread.

Acknowledgements

This activity was developed by Alistair Moore at the *University of York Science Education Group* (www.uyseg.org) with the generous assistance of Oliver Ellingham at the University of Reading.

The powdery mildew survey
Students' Sheet

Introduction

Powdery mildew is a plant disease caused by many different species of fungi. These fungi cause disease in many different species of plants, including food crops such as apples, blackcurrants, cucumbers, peas and barley, and popular ornamental plants such as honeysuckle, roses and oak trees.

When food plants suffer from diseases like mildew, they will often produce a smaller crop, making less food available for farmers to sell and for consumers to buy and eat. This can make food more expensive.

The symptoms of mildew infection include white, powdery patches of fungus that spread over the upper and lower surfaces of leaves, stems, and sometimes also flowers and fruit.

The fungus produces spores (structures that help it to survive in unfavourable conditions). It can be spread from one plant to another when the spores are carried by the wind.

Plants infected with powdery mildew are found all over the UK. The **Powdery Mildew Survey** is collecting data on which species of powdery mildew fungi are found where. This will help scientists to track the spread of the pathogens, and to give advice to help reduce the spread.

You are going to collect some samples for the survey.

Instructions

Your teacher will take you to a place where there have been reports of powdery mildew.

1. Look for a plant that has symptoms of powdery mildew infection.
2. Use an identification key to identify the species of the plant.
3. Use scissors to snip one or two leaves off the plant. Place the leaves in a small plastic bag.
4. Seal the bag.
5. Use hand gel to clean your hands.
6. Ask your teacher to help you find the GPS coordinates or postcode of your location.
7. Write the GPS coordinates or postcode on the bag containing the infected leaves.
8. Keep the bag sealed while you transport it back to school.

Your teacher will send the samples of infected leaves to the Powdery Mildew Survey.

Scientists working on the survey will record the appearance of the symptoms and pathogens present in the samples. They will also analyse the DNA of the pathogens to identify the species.

The results will be added to a national database, and your teacher will be sent a link to view the data (this may take a few weeks).

Safety notes

Keep the infected leaves inside the small plastic bag at all times. Clean your hands immediately after handling the infected leaves.

When doing fieldwork, work carefully to minimise the risk of harm to you and the organisms in the ecosystem being studied.

Questions

1. It is important to try to prevent the spread of powdery mildew.

   Look again at the instructions for collecting your sample of infected leaves.

   Which steps in the instructions reduce the risk of you spreading the disease? Explain why they reduce the risk.

2. A common cause of powdery mildew around the world is the fungus *Erysiphe graminis*. It infects barley, which is a cereal crop.

   Erysiphe is the name of the genus, and *graminis* identifies the species.

   However, many different species of fungi cause powdery mildew.

   This table shows how many species in each genus have been found in the UK historically, and in the *Powdery Mildew Survey* in 2015.

Genus of fungus	Number of species found in UK	
	Historical data	2015 survey
Erysiphe	85	19
Golovinomyces	13	10
Arthrocladiella	1	1
Neoerysiphe	4	4
Phyllactinia	7	1
Leveillula	1	0
Sawadaea	2	2
Podosphaera	22	16
Blumeria	1	1
Oidium	14	0
Total	150	54

The data for the 2015 survey were collected from 353 samples from all over the UK.

a. Draw an appropriate graph or chart to display the data from the 2015 survey.

b. Which genus is found most frequently in the UK?

c. The 2015 survey found 54 of the 150 species of powdery mildew fungi that have been recorded historically in the UK.

   Write this proportion as a percentage.

d. Dan looks at the table of data. He concludes that the *Oidium* genus of fungi has completely disappeared from the UK.

   Do you agree with his conclusion? Explain your answer.

3. Matilda sends a sample of infected leaves to the Powdery Mildew Survey.

   The survey identifies the species of fungus in her sample as *Erysiphe syringae-japonica*. This is an invasive species from East Asia that is new to the UK.

   Explain how this species may have been introduced to the UK.

4. There are over 900 different species of fungi that cause powdery mildew. Most of them produce very similar symptoms in the plants they infect.

   a. Suggest how DNA analysis could help to identify the species of fungus present in a sample.

   b. The survey collects data on the species of fungi causing powdery mildew, the plant species they are infecting, and the locations of infected plants.

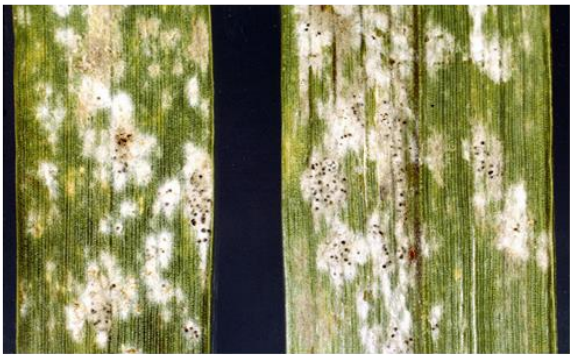
      Suggest why it is helpful to collect this kind of data.

Appendix 3: BSPP printed flyer disseminated at trade fairs.

Find a Fungus!

Powdery Mildew Citizen Science Project

Go outside and see if you can spot one of the 150 already known 'powdery mildews' found on plants around the UK, and then send your fungal infected plant samples to be identified using modern DNA technologies. Has your sample been found on a previously unrecorded host plant? Is it a powdery mildew new to the UK? Or a species new to science?



Now entering its third year the powdery mildew citizen science project is gaining more momentum, more followers, and more samples. The first survey in 2014 produced a total of 160 powdery mildew samples resulting in 51 different species being identified. This was followed in 2015 by a further 353 samples which allowed the detection of 54 species and the identification of powdery mildew on new host plants.

To help understand how widespread powdery mildews are, both in terms of geography and hosts, the Royal Horticultural Society and University of Reading are working together to identify and map as many powdery mildews as possible and they need your help.

With over 900 named species, believed to occur on more than 10,000 different plant hosts even experts struggle to ID them effectively..

The development of DNA sequence technology allows the researchers to identify and map their occurrences more confidently to discover where and when they are most prevalent.

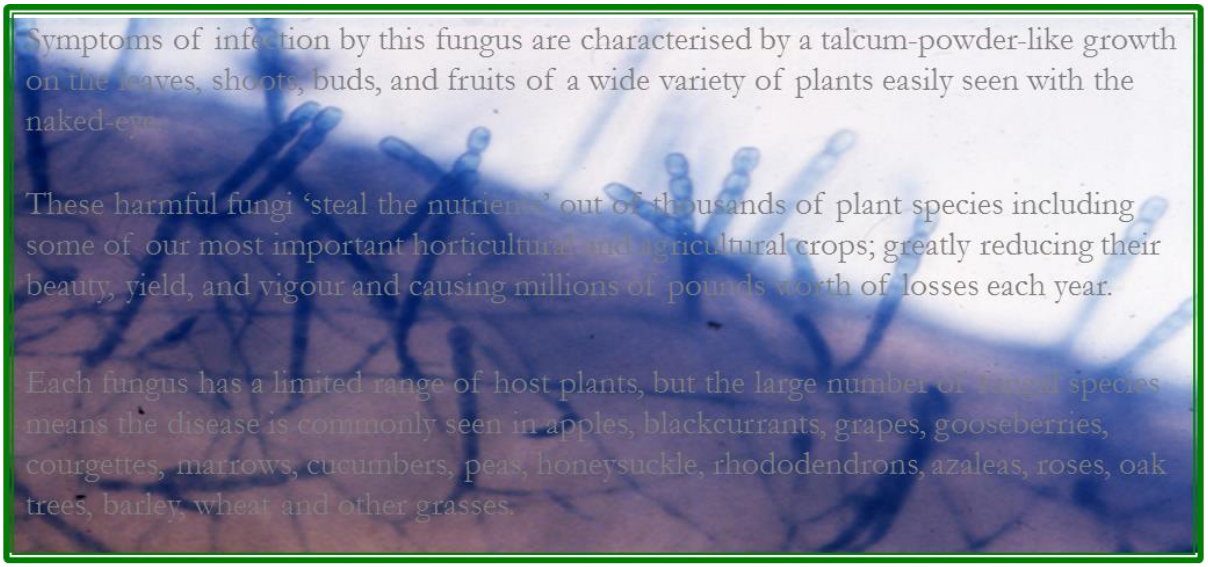
Powdery mildew (*Erysiphales, Ascomycota*) is a fungus reliant upon its plant hosts to survive and thrive, a so-called obligate parasite. Further research into this host-pathogen relationship by inspecting infected plants and analysing DNA sequences could help to prevent the pathogen in the future. This project aims to develop a set of molecular markers to aid in quick and easy identification of the Powdery Mildews, which will help to increase awareness of the species most prevalent within Britain and potentially further afield.

More overleaf about finding & sampling powdery mildews

University of Reading

BSPP

Royal Horticultural Society



Collecting your samples

Have a look in gardens, fields, hedgerows, waste ground or allotments to find something white/grey and powdery on the plant.

Prune off several whole leaves, not just the bit that appears infected. Put the fresh leaves in a slightly inflated sealed bag .

What information do I need to send with my infected plant?

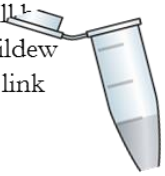
The postcode/grid reference/GPS of where the sample was found, your email address and the name of the host plant (if known). Photos of the plant are also happily received.

Send to:

Powdery Mildew Survey
Oliver Ellingham
Harborne Building
School of Biological Sciences
University of Reading, RG6 6AS

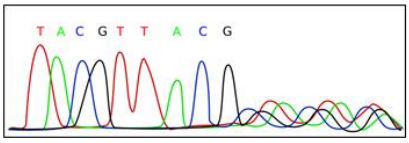
What happens to your samples?

Leaves are ground up and the DNA extracted (plant and fungus). DNA sequence information is used to identify it..



Once identified your sample will be added to a national powdery mildew database and you will be sent a link to the relevant record.

We will email you when results are available. This may take several weeks. This information will help to form a more complete picture of powdery mildew presence in the UK and to develop cutting-edge, molecular identification techniques.



Appendix 4: Accessions of the study

Study accession	Host ID	Date	Site	Final PM ID
OE2013PM1	*Malus domestica*	06/07/2013	Harris Garden, University of Reading	*Podosphaera leucotricha*
OE2013PM2	*Mahonia aquifolium*	09/08/2013	RHS Wisley	*Erysiphe berberidis*
OE2013PM3	*Carpinus betulus*	09/08/2013	RHS Wisley	*Erysiphe arcuata*
OE2013PM4	*Quercus robur*	09/08/2013	RHS Wisley	*Erysiphe alphitoides*
OE2013PM5	*Ribes sanguineum*	09/08/2013	RHS Wisley	*Podosphaera mors-uvae*
OE2013PM6	*Geranium* sp.	09/08/2013	RHS Wisley	*Neoerysiphe geranii*
OE2013PM7	*Acer pseudoplatanus*	12/08/2013	Harris Garden, University of Reading	*Sawadaea bicornis*
OE2013PM8	*Thunbergia alata*	15/08/2013	Whiteknights House, University of Reading	*Golovinomyces orontii*
OE2013PM9	*Mahonia* x *wagneri*	16/08/2013	Harris Garden, University of Reading	*Erysiphe berberidis*
OE2013PM11	*Cydonia* sp.	16/08/2013	Harris Garden, University of Reading	*Podosphaera leucotricha*
OE2013PM12	*Cucurbita maxima*	16/08/2013	RHS Wisley	*Golovinomyces orontii*
OE2013PM13	*Amelanchier lamarckii*	16/08/2013	Harris Garden, University of Reading	*Podosphaera amelanchieris*
OE2013PM14	*Silene*sp.	17/08/2013	Harris Garden, University of Reading	*Erysiphe buhrii*
OE2013PM15	*Verbascum*sp.	20/09/2013	RHS Wisley	*Golovinomyces verbasci*
OE2013PM16	*Sonchus oleraceus*	23/09/2013	Harris Garden, University of Reading	*Golovinomyces sonchicola*
OE2013PM17	*Trifolium arvense*	23/09/2013	Trial Plots, University of Reading	*Erysiphe trifoliorum*
OE2013PM18	*Pisum* sp.	23/09/2013	Trial Plots, University of Reading	*Erysiphe pisi*
OE2013PM19	*Trifolium pratense*	23/09/2013	RHS Wisley	*Erysiphe trifoliorum*
OE2013PM21	*Aquilegia vulgaris*	23/09/2013	Harris Garden, University of Reading	*Erysiphe aquilegiae*
OE2013PM22	*Amelanchier canadensis*	23/09/2013	RHS Wisley	*Podosphaera amelanchieris*
OE2013PM23	*Populus* sp.	23/09/2013	Wilderness Area, University of Reading	*Erysiphe adunca*
OE2013PM24	*Monarda* sp.	02/10/2013	Harris Garden, University of Reading	*Golovinomyces biocellaris*
OE2013PM25	*Phlox paniculata*	02/10/2013	Harris Garden, University of Reading	*Golovinomyces magnicellulatus*
OE2013PM26	*Anthriscus sylvestris*	04/10/2013	RHS Wisley	*Erysiphe heraclei*
OE2013PM27	*Heracleum sphondylium*	04/10/2013	Harris Garden, University of Reading	*Erysiphe heraclei*
OE2013PM28	*Stachys byzantina*	04/10/2013	Harris Garden, University of Reading	*Neoerysiphe galeopsidis*
OE2013PM29	*Circaea lutetiana*	04/10/2013	Harris Garden, University of Reading	*Erysiphe circaeae*
OE2013PM30	*Plantago major*	04/10/2013	Wilderness Area, University of Reading	*Golovinomyces sordidus*
OE2013PM31	*Poaceae* sp.	04/10/2013	Harris Garden, University of Reading	*Erysiphe* sp
OE2013PM32	*Geum urbanum*	04/10/2013	Harris Garden, University of Reading	*Podosphaera aphanis*
OE2013PM33	*Senecio vulgaris*	07/10/2013	Wilderness Area, University of Reading	*Golovinomyces fischeri*
OE2013PM34	*Aster novi-belgii*	07/10/2013	Harris Garden, University of Reading	*Golovinomyces asterum*
OE2013PM35	*Rosa* sp.	07/10/2013	Harris Garden, University of Reading	*Podosphaera pannosa*
OE2013PM36	*Geranium* sp.	13/10/2013	RHS Wisley	*Neoerysiphe geranii*
OE2013PM37	*Aquilegia* sp.	13/10/2013	RHS Wisley	*Erysiphe aquilegiae*
OE2013PM38	*Aesculus* sp.	13/10/2013	Wilderness Area, University of Reading	*Erysiphe flexuosa*
OE2013PM39	*Mitella*sp.	13/10/2013	RHS Wisley	*Podosphaera macularis*
OE2013PM40	*Verbascum*sp.	13/10/2013	RHS Wisley	*Golovinomyces verbasci*
OE2013PM41	*Acer pseudoplatanus*	16/10/2013	Wilderness Area, University of Reading	*Sawadaea bicornis*
OE2013PM42	*Phlox paniculata 'Peacock White'*	05/11/2013	RHS Wisley	*Golovinomyces magnicellulatus*

OE2013PM43	*Salix caprea*	11/11/2013	Trial Greenhouses, University of Reading	*Erysiphe adunca*
OE2014PM1	*Trifolium pratense*	04/03/2014	Trial Greenhouses, University of Reading	*Erysiphe trifoliorum*
OE2014PM2	*Lamium amplexicaule*	14/04/2014	Experimental plots, University of Reading	*Neoerysiphe galeopsidis*
OE2014PM3	*Lamium purpureum*	14/04/2014	Experimental plots, University of Reading	*Neoerysiphe galeopsidis*
OE2014PM4	*Senecio vulgaris*	14/04/2014	Experimental plots, University of Reading	*Golovinomyces fischeri*
OE2014PM5	*Myosotis arvensis*	30/04/2014	Harris Garden, University of Reading	*Golovinomyces cynoglossi*
OE2014PM6	*Geranium* sp.	09/05/2014	Harris Garden, University of Reading	*Podosphaera fugax*
OE2014PM7	*Taraxacum officinale*	12/05/2014	Christchurch Road, Reading	*Podosphaera erigerontis-canadensis*
OE2014PM8	*Hordeum vulgare*	14/05/2014	Experimental plots, University of Reading	*Blumeria graminis*
OE2014PM9	*Sonchus oleraceus*	14/05/2014	Harborne building, University of Reading	*Golovinomyces sonchicola*
OE2014PM10	*Lamium* sp.	09/06/2014	RHS Wisley	*Neoerysiphe galeopsidis*
OE2014PM11	*Geranium* sp.	09/06/2014	RHS Wisley	*Podosphaera fugax*
OE2014PM12	*Quercus robur*	09/06/2014	RHS Wisley	*Erysiphe alphitoides*
OE2014PM13	*Wisteria sinensis*	09/06/2014	RHS Wisley	*Erysiphe alphitoides*
OE2014PM14	*Carpinus betulus*	09/06/2014	RHS Wisley	*Erysiphe arcuata*
OE2014PM15	*Pulmonaria* sp.	09/06/2014	RHS Wisley	*Golovinomyces cynoglossi*
OE2014PM16CS	*Acanthus spinosus*	09/06/2014	Alexandra Road, Reading	*Neoerysiphe galeopsidis*
OE2014PM17CS	*Centaurea montana*	12/06/2014	Shades of Green, Stirling	*Golovinomyces depressus*
OE2014PM18CS	*Symphytum* sp.	12/06/2014	Shades of Green, Stirling	*Golovinomyces cynoglossi*
OE2014PM19CS	*Tellima grandiflora*	12/06/2014	Shades of Green, Stirling	*Podosphaera macrospora*
OE2014PM20CS	*Myosoits* sp.	12/06/2014	Shades of Green, Stirling	*Golovinomyces cynoglossi*
OE2014PM21CS	*Lycium barbarum*	13/06/2014	Lincoln	*Arthrocladiella mougeotii*
OE2014PM22CS	*Lycium barbarum*	16/06/2014	31 Sea Crest Road, Lee-on-the-Solent	*Arthrocladiella mougeotii*
OE2014PM23CS	*Persicaria amplexicaulis*	16/06/2014	31 Sea Crest Road, Lee-on-the-Solent	*Erysiphe polygoni*
OE2014PM24CS	*Lonicera periclymenum 'Graham Thoms'*	16/06/2014	31 Sea Crest Road, Lee-on-the-Solent	*Erysiphe lonicerae*
OE2014PM25	*Acer platanoides*	16/06/2014	Wilderness area, University of Reading	*Sawadaea tulasnei*
OE2014PM26CS	*Centaurea montana*	17/06/2014	Moray	*Golovinomyces depressus*
OE2014PM27CS	*Heracleum sphondylium*	18/06/2014	Maidstone	*Erysiphe heraclei*
OE2014PM28CS	*Verbena bonariensis*	22/06/2014	Blackpool	*Golovinomyces orontii*
OE2014PM29CS	*Knautia 'melton hybrid'*	23/06/2014	Blackpool	*Podosphaera dipsacearum*
OE2014PM30CS	*Berberis thunbergii atropurpurea*	23/06/2014	Merseyside	*Erysiphe berberidis*
OE2014PM31	*Akebia quinata*	23/06/2014	Luckmore Drive, Reading	*Erysiphe akebiae*
OE2014PM32CS	*Heracleum sphondylium*	24/06/2014	Suttons Park Avenue, Reading	*Erysiphe heraclei*
OE2014PM33CS	*Filipendula ulmaria*	25/06/2014	Middlesborough	*Podosphaera filipendulae*
OE2014PM34	*Akebia quinata*	02/07/2014	Whiteknights House, University of Reading	*Erysiphe akebiae*
OE2014PM35	*Trifolium sp.*	07/07/2014	RHS Wisley	*Erysiphe trifoliorum*
OE2014PM36	*Catalpa bignonioides*	07/07/2014	RHS Wisley	*Erysiphe elevata*
OE2014PM37	*Aquilegia vulgaris*	09/07/2014	Harris Garden, University of Reading	*Erysiphe aquilegiae*

OE2014PM38	*Akebia quinata*	14/07/2014	Secret Garden, University of Reading	*Erysiphe akebiae*
OE2014PM39	*Mahonia aquifolium*	17/07/2014	Harris Garden, University of Reading	*Erysiphe berberidis*
OE2014PM40CS	*Geranium rotundifolium*	31/07/2014	Earley Gate, University of Reading	*Podosphaera fugax*
OE2014PM41CS	*Verbena bonariensis*	06/08/2014	Chippenham, Wiltshire	
OE2014PM42CS	*Rosa 'Madame Alfred Carrière'*	17/08/2014	London	*Podosphaera pannosa*
OE2014PM43CS	*Clematis Perle d'Azur'*	17/08/2014	London	*Erysiphe aquilegiae*
OE2014PM44CS	*Clematis* sp.	17/08/2014	London	*Erysiphe aquilegiae*
OE2014PM45	*Monarda* sp.	20/08/2014	Harris Garden, University of Reading	*Golovinomyces biocellaris*
OE2014PM46	*Catalpa bignonioides*	07/07/2014	RHS Wisley	*Neoerysiphe galeopsidis*
OE2014PM47CS	*Prunus laurocerasus*	26/08/2014	Allcroft Road, Reading	*Podosphaera tridactyla*
OE2014PM48CS	*Calystegia silvatica*	26/08/2014	Allcroft Road, Reading	*Erysiphe convolvuli*
OE2014PM49CS	*Pentaglottis sempervirens*	26/08/2014	Allcroft Road, Reading	*Erysiphe lycopsidis*
OE2014PM50CS	*Cucurbita pepo*	04/08/2014	Farnborough	*Golovinomyces orontii*
OE2014PM51CS	*Quercus robur*	04/08/2014	Farnborough	*Erysiphe alphitoides*
OE2014PM52	*Heracleum sphondylium*	28/08/2014	West side of Whiteknights Lake, University of Reading	*Erysiphe heraclei*
OE2014PM53CS	*Acer campestre*	02/09/2014	St. Andrews	*Sawadaea bicornis*
OE2014PM54CS	*Sonchus asper*	02/09/2014	St. Andrews	*Golovinomyces sonchicola*
OE2014PM55CS	*Taraxacum officinale*	02/09/2014	St. Andrews	*Podosphaera erigerontis-canadensis*
OE2014PM56CS	*Trifolium pratense*	07/09/2014	APD, University of Reading, University of Reading	*Erysiphe trifoliorum*
OE2014PM57CS	*Senecio jacobaea*	07/09/2014	APD, University of Reading, University of Reading	*Podosphaera senecionis*
OE2014PM58CS	*Plantago major*	07/09/2014	APD, University of Reading, University of Reading	*Golovinomyces sordidus*
OE2014PM59CS	*Platanus* x *hispanica*	07/09/2014	Wilderness area, University of Reading, Whiteknights Campus	*Erysiphe platani*
OE2014PM60CS	*Quercus robur*	11/09/2014	Kerry	*Erysiphe alphitoides*
OE2014PM61CS	*Quercus robur*	11/09/2014	Kerry	*Erysiphe alphitoides*
OE2014PM62CS	*Calystegia sepium*	15/09/2014	Pepper Lane entrance, University of Reading	*Erysiphe convolvuli*
OE2014PM63	*Acer* sp.	16/09/2014	Kew Gardens	*Sawadaea bicornis*
OE2014PM64CS	*Cucurbita pepo*	17/09/2014	Pennant, Wales	*Golovinomyces orontii*
OE2014PM65CS	*Heracleum sphondylium*	17/09/2014	Pennant, Wales	*Erysiphe heraclei*
OE2014PM66CS	*Mentha* sp.	17/09/2014	Pennant, Wales	*Golovinomyces biocellaris*
OE2014PM67CS	*Quercus robur*	17/09/2014	Pennant, Wales	*Erysiphe alphitoides*
OE2014PM68CS	*Silene dioica*	17/09/2014	Pennant, Wales	*Erysiphe buhrii*
OE2014PM69CS	*Taraxacum agg.*	17/09/2014	Pennant, Wales	*Podosphaera erigerontis-canadensis*
OE2014PM70CS	*Fraxinus excelsior*	13/09/2014	Foxlease, Hampshire	*Phyllactinia fraxini*
OE2014PM71CS	*Salix* sp.	13/09/2014	Foxlease, Hampshire	*Erysiphe adunca*
OE2014PM72CS	*Rhododendron* sp.	13/09/2014	Foxlease, Hampshire	*Erysiphe azaleae*
OE2014PM73CS	*Acer campestre*	14/09/2014	Whiteknights Lake	*Sawadaea bicornis*
OE2014PM74CS	*Vitis vinifera*	21/09/2014	Dereham, Norfolk	*Erysiphe necator*
OE2014PM75CS	*Cucurbita pepo*	21/09/2014	Dereham, Norfolk	*Golovinomyces orontii*
OE2014PM76CS	*Heracleum* sp.	21/09/2014	Dereham, Norfolk	*Erysiphe heraclei*
OE2014PM77CS	*Lamium album*	21/09/2014	Dereham, Norfolk	*Neoerysiphe galeopsidis*
OE2014PM78CS	*Lamium purpureum*	21/09/2014	Dereham, Norfolk	*Neoerysiphe galeopsidis*

OE2014PM79CS	*Lupinus* sp.	21/09/2014	Dereham, Norfolk	*Erysiphe intermedia*
OE2014PM80CS	*Senecio vulgaris*	21/09/2014	Dereham, Norfolk	Pm sp
OE2014PM81CS	*Rosa gallica*	21/09/2014	Dereham, Norfolk	Pm sp
OE2014PM82CS	*Acer campestre*	21/09/2014	Dereham, Norfolk	*Sawadaea bicornis*
OE2014PM83CS	*Cornus sericea*	21/09/2014	Dereham, Norfolk	*Erysiphe tortilis*
OE2014PM84CS	*Quercus robur*	21/09/2014	Dereham, Norfolk	*Erysiphe alphitoides*
OE2014PM85CS	*Polygonum* sp.	21/09/2014	Dereham, Norfolk	*Erysiphe polygoni*
OE2014PM86CS	*Prunus spinosa*	21/09/2014	Dereham, Norfolk	*Podosphaera tridactyla*
OE2014PM87CS	*Aster amellus*	21/09/2014	Dereham, Norfolk	*Golovinomyces asterum*
OE2014PM88CS	*Crataegus monogyna*	21/09/2014	Dereham, Norfolk	*Podosphaera clandestina*
OE2014PM89CS	*Aquilegia vulgaris*	21/09/2014	Dereham, Norfolk	*Erysiphe aquilegiae*
OE2014PM90CS	*Trifolium pratense*	21/09/2014	Dereham, Norfolk	*Erysiphe trifoliorum*
OE2014PM91CS	*Origanum vulgare*	21/09/2014	Dereham, Norfolk	Pm sp
OE2014PM92CS	*Hieracium* sp.	21/09/2014	Dereham, Norfolk	*Podosphaera xanthii*
OE2014PM93CS	*Viburnum lantana*	21/09/2014	Dereham, Norfolk	*Erysiphe hedwigii*
OE2014PM94CS	*Lamium album*	21/09/2014	Martiott's Way, Norfolk	*Neoerysiphe galeopsidis*
OE2014PM95CS	*Quercus robur*	21/09/2014	Martiott's Way, Norfolk	*Erysiphe alphitoides*
OE2014PM96CS	*Plantago major*	21/09/2014	Martiott's Way, Norfolk	*Golovinomyces sordidus*
OE2014PM97CS	*Acer campestre*	21/09/2014	Martiott's Way, Norfolk	*Sawadaea bicornis*
OE2014PM98CS	*Hieracium* sp.	21/09/2014	Martiott's Way, Norfolk	*Podosphaera xanthii*
OE2014PM99CS	*Filipendula ulmaria*	23/09/2014	JIC, UEA	*Podosphaera filipendulae*
OE2014PM100CS	*Plantago major*	23/09/2014	JIC, UEA	Pm sp
OE2014PM101CS	*Plantago lanceolata*	23/09/2014	JIC, UEA	*Podosphaera plantaginis*
OE2014PM102CS	*Heracleum* sp.	23/09/2014	JIC, UEA	*Erysiphe heraclei*
OE2014PM103CS	*Quercus robur*	23/09/2014	JIC, UEA	*Erysiphe alphitoides*
OE2014PM104CS	*Lactuca* sp.	23/09/2014	JIC, UEA	*Golovinomyces cichoracearum*
OE2014PM105CS	*Vinca* sp.	23/09/2014	Royal Holloway University, London	*Golovinomyces orontii*
OE2014PM106CS	*Aquilegia vulgaris*	24/09/2014	Eastern Avenue, Reading	*Erysiphe aquilegiae*
OE2014PM107CS	*Aquilegia vulgaris*	24/09/2014	Eastern Avenue, Reading	*Erysiphe aquilegiae*
OE2014PM108CS	*Convolvulus* sp.	25/09/2014	Royal Holloway University, London	*Erysiphe convolvuli*
OE2014PM109	*Ranunculus repens*	26/08/2014	Harborne Bulding, University of Reading	*Erysiphe aquilegiae*
OE2014PM110CS	*Petunia* sp.	26/08/2014	Luckmore Drive, Reading	*Euoidium longipes*
OE2014PM111CS	*Lamium album*	30/09/2014	Newbury	*Neoerysiphe galeopsidis*
OE2014PM112CS	*Alliaria petiolata*	30/09/2014	Newbury	*Erysiphe cruciferarum*
OE2014PM113CS	*Verbascum thapsus*	30/09/2014	APD, University of Reading	*Golovinomyces verbasci*
OE2014PM114CS	*Plantago media*	01/10/2014	APD, University of Reading	*Podosphaera plantaginis*
OE2014PM115CS	*Taraxacum officinale*	01/10/2014	APD, University of Reading	*Podosphaera erigerontis-canadensis*
OE2014PM116CS	*Veronica persica*	01/10/2014	APD, University of Reading	*Golovinomyces orontii*
OE2014PM117CS	*Senecio vulgaris*	02/10/2014	Experimental plots, University of Reading	*Golovinomyces fischeri*
OE2014PM118CS	*Senecio vulgaris*	02/10/2014	Experimental plots, University of Reading	*Golovinomyces fischeri*
OE2014PM119CS	*Marrubium vulgare*	02/10/2014	Martiott's Way, Norfolk	*Neoerysiphe galeopsidis*
OE2014PM120CS	*Polygonum aviculare*	02/10/2014	Dereham, Norfolk	*Erysiphe polygoni*
OE2014PM121CS	*Acer campestre*	06/10/2014	Unviersity of Reading campus	*Sawadaea bicornis*

OE2014PM122CS	*Heracleum sphondylium*	06/10/2014	Unviersity of Reading campus	*Erysiphe heraclei*
OE2014PM123CS	*Plantago major*	06/10/2014	Unviersity of Reading campus	Pm sp
OE2014PM124CS	*Acer pseudoplatanus*	06/10/2014	East Tuddenham	*Sawadaea bicornis*
OE2014PM125CS	*Centaurea montana*	06/10/2014	East Tuddenham	*Golovinomyces montagnei*
OE2014PM126CS	*Heracleum sphondylium*	06/10/2014	Hockering	*Erysiphe heraclei*
OE2014PM127CS	*Artemisia* sp.	06/10/2014	Hockering	*Golovinomyces artemisiae*
OE2014PM128CS	*Hypericum* sp.	06/10/2014	Hockering	*Erysiphe hyperici*
OE2014PM129CS	*Trifolium campestre*	06/10/2014	Hockering	*Erysiphe trifoliorum*
OE2014PM130CS	*Cirsium arvense*	04/10/2014	Warwick services M40	*Golovinomyces montagnei*
OE2014PM131CS	*Ranunculus repens*	04/10/2014	Warwick services M40	*Erysiphe aquilegiae*
OE2014PM132CS	*Taraxacum officinale*	04/10/2014	Leighton Buzzard, Bedfordshire	*Podosphaera erigerontis-canadensis*
OE2014PM133CS	*Senecio vulgaris*	04/10/2014	Leighton Buzzard, Bedfordshire	*Golovinomyces fischeri*
OE2014PM134CS	*Sonchus asper*	04/10/2014	Leighton Buzzard, Bedfordshire	*Golovinomyces sonchicola*
OE2014PM135CS	*Polygonum aviculare*	04/10/2014	Leighton Buzzard, Bedfordshire	*Erysiphe polygoni*
OE2014PM136CS	*Lathyrus odoratus*	12/10/2014	3 Princes Street, Norwich	*Erysiphe trifoliorum*
OE2014PM137CS	*Pisum* sp.	18/10/2014	Burscough	*Erysiphe pisi*
OE2014PM138CS	*Pisum* sp.	18/10/2014	Chorley, Lancashire	*Erysiphe pisi*
OE2014PM139CS	*Aquilegia vulgaris*	18/10/2014	Lancashire	*Erysiphe aquilegiae*
OE2014PM140	*Polygonum aviculare*	21/10/2014	Dereham, Norfolk	*Erysiphe polygoni*
OE2014PM141CS	*Aquilegia vulgaris*	18/10/2014	Yorkshire	*Erysiphe aquilegiae*
OE2014PM142CS	*Lamium amplexicaule*	18/10/2014	Yorkshire	*Neoerysiphe galeopsidis*
OE2014PM143CS	*Solenostemon scutellarioides*	24/10/2014	RHS Wisley	*Golovinomyces biocellaris*
OE2014PM144CS	*Lathyrus odoratus*	28/10/2014	Grove Road, Legihton Buzzard	*Erysiphe trifoliorum*
OE2014PM145CS	*Corylus avellana*	29/10/2014	Northcourt Avenue, Reading	*Phyllactinia guttata*
OE2014PM146CS	*Ranunculus repens*	29/10/2014	South Kesteven, Peterborough	*Erysiphe aquilegiae*
OE2014PM147CS	*Aquilegia vulgaris*	29/10/2014	South Kesteven, Peterborough	*Erysiphe aquilegiae*
OE2014PM148CS	*Myosotis arvensis*	29/10/2014	South Kesteven, Peterborough	*Golovinomyces cynoglossi*
OE2014PM149CS	*Plantago coronopus*	29/10/2014	Dawlish, Devon	*Golovinomyces sordidus*
OE2014PM150CS	*Syringa* sp.	29/10/2014	West Deeping Churchyard, Peterborough	*Erysiphe syringae*
OE2014PM151CS	*Lamium album*	02/11/2014	Hessle, East Yorkshire	*Neoerysiphe galeopsidis*
OE2014PM152CS	*Salix aurita*	12/11/2014	Redlands Road, Reading	*Erysiphe adunca*
OE2014PM153CS	*Corylus avellana*	12/11/2014	Tower Hill, London	*Phyllactinia guttata*
OE2014PM154CS	*Aesculus* x *carnea*	12/11/2014	Pymmes Park, London	*Erysiphe flexuosa*
OE2014PM155CS	*Betula papyrifera*	12/11/2014		*Phyllactinia betulae*
OE2014PM156CS	*Betula pendula*	16/11/2014	Swinely Forest	*Phyllactinia betulae*
OE2014PM157CS	*Catalpa speciosa*	16/11/2014		*Erysiphe elevata*
OE2014PM158CS	*Osteospermum jucundum*	16/11/2014		*Golovinomyces cichoracearum*
OE2014PM159CS	*Veronica chamaedrys*	16/11/2014		*Golovinomyces orontii*
OE2014PM160CS	*Geranium* x *magnificum*	16/11/2014		*Neoerysiphe geranii*
OE2015PM1CS	*Wisteria brachybotrys* 'Murasaki-kapitan'	01/08/2014	RHS Garden Hyde Hall	*Erysiphe alphitoides*
OE2015PM2CS	*Wisteria frutescens* 'Amethyst Falls'	01/08/2014	RHS Wisley	*Erysiphe alphitoides*

OE2015PM3CS	*Heuchera* 'Caramel'	17/01/2015	RHS Wisley	*Podosphaera macrospora*
OE2015PM4CS	*Heuchera* 'Galaxy'	17/01/2015	RHS Wisley	*Podosphaera macrospora*
OE2015PM5CS	*Lamium purpureum*	14/01/2015	Sonning, Reading University Farm	*Neoerysiphe galeopsidis*
OE2015PM6CS	*Prunus laurocerasus*	24/01/2015	South Tottenham, London	*Podosphaera tridactyla*
OE2015PM7CS	*Hedera algeriensis* 'Gloire de Marengo'	24/01/2015	South Tottenham, London	
OE2015PM8CS	*Viburnum tinus*	24/01/2015	South Tottenham, London	*Erysiphe hedwigii*
OE2015PM9CS	*Verbascum thapsus*	14/03/2015	Whiteknights campus	*Golovinomyces verbasci*
OE2015PM10CS	*Euphorbia peplus*	22/03/2015	Maiden Erleigh School, Reading	*Podosphaera euphorbiae-helioscopiae*
OE2015PM11CS	*Mahonia aquifolium*	16/03/2015	Northcourt Avenuce, Reading	*Erysiphe berberidis*
OE2015PM12CS	*Galium aparine*	16/03/2015	Northcourt Avenuce, Reading	*Neoerysiphe galii*
OE2015PM13CS	*Malus domestica*	30/04/2015	St. Albans	*Podosphaera leucotricha*
OE2015PM14CS	*Crataegus monogyna*	30/04/2015	Widnes	*Podosphaera clandestina*
OE2015PM15CS	*Taraxacum officinale*	04/05/2015	Hayle, Cornwall	*Podosphaera erigerontis-canadensis*
OE2015PM16CS	*Stachys arvensis*	05/05/2015	Sonning	*Neoerysiphe galeopsidis*
OE2015PM17CS	*Lamium amplexicaule*	05/05/2015	Sonning	*Neoerysiphe galeopsidis*
OE2015PM18CS	*Rosa banksiae*	05/05/2015	Lucton, Leominster, Herefordshire	*Podosphaera pannosa*
OE2015PM19CS	*Acanthus spinosus*	09/05/2015	Colchester	*Neoerysiphe galeopsidis*
OE2015PM20CS	*Plantagolanceolata*	10/05/2015	Crown Place, Reading	*Golovinomyces sordidus*
OE2015PM21CS	*Lamium purpureum*	10/05/2015	Sonning, Reading University Farm	*Neoerysiphe galeopsidis*
OE2015PM22CS	*Ballota nigra*	10/05/2015	Sonning, Reading University Farm	*Neoerysiphe galeopsidis*
OE2015PM23CS	*Sonchus oleraceus*	10/05/2015	Upper Readlands Road, Reading	*Golovinomyces sonchicola*
OE2015PM24CS	*Prunus laurocerasus*	11/05/2015	Northcourt Avenuce, Reading	*Podosphaera pannosa*
OE2015PM25CS	*Taraxacum officinale*	11/05/2015	Tickenor Drive, Finchampstead	*Neoerysiphe nevoi*
OE2015PM26CS	*Geum* sp.	11/05/2015	Tickenor Drive, Finchampstead	*Podosphaera aphanis*
OE2015PM27CS	*Myosotis arvensis*	11/05/2015	Tickenor Drive, Finchampstead	*Golovinomyces cynoglossi*
OE2015PM28CS	*Euonymus* sp.	10/05/2015	Kingston Upon Thames	*Erysiphe euonymicola*
OE2015PM29CS	*Aquilegia vulgaris*	16/05/2015	Chester Zoological Gardens	*Erysiphe aquilegiae*
OE2015PM30CS	*Lonicera periclymenum*	26/05/2015	Newport, Saffron Walden	*Erysiphe lonicerae*
OE2015PM31CS	*Centaurea montana*	26/05/2015	Newport, Saffron Walden	*Golovinomyces depressus*
OE2015PM32CS	*Pulmonaria* sp.	26/05/2015	Newport, Saffron Walden	*Golovinomyces cynoglossi*
OE2015PM33CS	*Onosma* sp.	26/05/2015	Newport, Saffron Walden	*Golovinomyces cynoglossi*
OE2015PM34CS	*Quercus robur*	27/05/2015	Burghfield Common, Berkshire	*Erysiphe alphitoides*
OE2015PM35CS	*Geum urbanum*	27/05/2015	Burghfield Common, Berkshire	*Podosphaera aphanis*
OE2015PM36CS	*Lonicera periclymenum*	27/05/2015	Burghfield Common, Berkshire	*Erysiphe lonicerae*
OE2015PM37CS	*Epilobium parviflorium*	27/05/2015	SBS, Whiteknights	*Podosphaera epilobii*
OE2015PM38CS	*Prunus laurocerasus*	27/05/2015	South Croydon, Surrey	*Podosphaera tridactyla*
OE2015PM39CS	*Quercus robur*	28/05/2015	APD, Whiteknights Campus	*Erysiphe alphitoides*
OE2015PM40CS	*Euonymus japonicus*	28/05/2015	APD, Whiteknights Campus	*Erysiphe euonymicola*
OE2015PM41CS	*Euonymus japonicus*	28/05/2015	APD, Whiteknights Campus	*Erysiphe euonymicola*
OE2015PM42CS	*Geum urbanum*	22/05/2015	Runcorn, Cheshire	*Podosphaera aphanis*
OE2015PM43CS	*Silene dioica*	25/05/2015	Woolton, Liverpool	*Golovinomyces cynoglossi*

OE2015PM44CS	*Symphoricarpos albus*	25/05/2015	Woolton, Liverpool	*Erysiphe symphoricarpi*
OE2015PM45CS	*Acanthus spinosus*	25/05/2015	Woolton, Liverpool	*Neoerysiphe galeopsidis*
OE2015PM46CS	*Crataegus monogyna*	25/04/2015	Woolton, Liverpool	*Podosphaera clandestina*
OE2015PM47CS	*Malus pumila*	26/05/2015	Halebank, Widnes	*Podosphaera leucotricha*
OE2015PM48CS	*Rosa multiflora*	26/05/2015	Halebank, Widnes	*Podosphaera pannosa*
OE2015PM49CS	*Geranium phaeum*	01/06/2015	Elmfield Gardens, Newbury	*Podosphaera fugax*
OE2015PM50CS	*Pulmonaria* sp.	01/06/2015	Elmfield Gardens, Newbury	*Golovinomyces cynoglossi*
OE2015PM51CS	*Lonicera* sp.	02/06/2015	Allerton, Liverpool	*Erysiphe lonicerae*
OE2015PM52CS	*Acer campestre*	03/06/2015	Allerton, Liverpool	*Sawadaea bicornis*
OE20153PM53CS	*Ribes sanguineum*	04/06/2015	Allerton, Liverpool	*Podosphaera mors-uvae*
OE2015PM54CS	*Stachys byzantina*	04/06/2015	Woolton, Liverpool	*Neoerysiphe galeopsidis*
OE2015PM55CS	*Acanthus mollis*	07/06/2015	Briggswath, Whitby	*Neoerysiphe galeopsidis*
OE2015PM56CS	*Rosa* 'Dorothy Perkins'	08/06/2015	Tiverton, Devon	*Podosphaera pannosa*
OE2015PM57CS	*Myosotis* sp.	08/06/2015	Tickenor Drive, Finchampstead	*Golovinomyces cynoglossi*
OE2015PM58CS	*Geranium phaeum*	08/06/2015	Elmfield Gardens, Newbury	*Podosphaera fugax*
OE2015PM59CS	*Pilosella aurantiaca*	08/06/2015	Hayle, Cornwall	*Golovinomyces cichoracearum*
OE2015PM60CS	*Rosa* sp.	08/06/2015	Tiverton, Devon	*Podosphaera pannosa*
OE2015PM61CS	*Euonymus japonicus*	07/06/2015	Crosby, Merseyside	*Erysiphe euonymicola*
OE2015PM62CS	*Geum urbanum*	07/06/2015	Crosby, Merseyside	*Podosphaera aphanis*
OE2015PM63CS	*Crataegus monogyna*	07/06/2015	Crosby, Merseyside	*Podosphaera clandestina*
OE2015PM64CS	*Malus sylvestris*	07/06/2015	Crosby, Merseyside	*Podosphaera leucotricha*
OE2015PM65CS	*Sisymbrium officinale*	08/06/2015	Runcorn, Cheshire	*Erysiphe cruciferarum*
OE2015PM66CS	Unknown	08/06/2015	Liverpool, Merseyside	*Neoerysiphe galeopsidis*
OE2015PM67CS	*Potentilla litoralis*	11/06/2015		*Podosphaera aphanis*
OE2015PM68CS	*Salvia officinalis* 'Purpurascens'	11/06/2015	Knaphill, Woking	*Golovinomyces biocellaris*
OE2015PM69CS	*Myosotis arvensis*	11/05/2015	Tickenor Drive, Finchampstead	*Golovinomyces cynoglossi*
OE2015PM70CS	*Taraxacum officinale*	13/06/2015	Liverpool, Merseyside	*Podosphaera erigerontis-canadensis*
OE2015PM71CS	*Taraxacum officinale*	13/06/2015	Liverpool, Merseyside	*Podosphaera erigerontis-canadensis*
OE2015PM72CS	*Prunus laurocerasus*	13/06/2015	Liverpool, Merseyside	*Podosphaera tridactyla*
OE2015PM73CS	*Plantago major*	14/06/2015	St. Helens, Merseyside	Not PM
OE2015PM74CS	*Ribes sanguineum*	14/06/2015	St. Helens, Merseyside	*Podosphaera mors-uvae*
OE2015PM75CS	*Rubus fruticosus*	14/06/2015	St. Helens, Merseyside	*Podosphaera aphanis*
OE2015PM76CS	*Epilobium hirsutum*	14/06/2015	St. Helens, Merseyside	*Podosphaera epilobii*
OE2015PM77CS	Unknown	14/06/2015	St. Helens, Merseyside	*Podosphaera leucotricha*
OE2015PM78CS	*Geranium phaeum*	14/06/2015	St. Helens, Merseyside	*Podosphaera fugax*
OE2015PM79CS	*Quercus robur*	14/06/2015	St. Helens, Merseyside	*Erysiphe alphitoides*
OE2015PM80CS	*Quercus robur*	15/06/2015	Runcorn, Cheshire	*Erysiphe alphitoides*
OE2015PM81CS	*Crataegus monogyna*	15/06/2015	Runcorn, Cheshire	*Podosphaera clandestina*
OE2015PM82CS	*Quercus robur*	15/06/2015	Runcorn, Cheshire	*Erysiphe alphitoides*
OE2015PM83	*Malus domestica*	19/06/2015	Harris Garden, University of Reading	*Podosphaera leucotricha*
OE2015PM84CS	*Mespilus germanica*	19/06/2015	Thorpe Marsh, Norwich	*Podosphaera leucotricha*
OE2015PM85CS	*Filipendula ulmaria*	19/06/2015	Thorpe Marsh, Norwich	*Podosphaera*

				filipendulae
OE2015PM86CS	*Poa trivialis*	19/06/2015	Colwich Crickworks, Staffordshire	*Blumeria graminis*
OE2015PM87CS	*Rosa* sp.	21/06/2015	Elstead, near Guildford	*Podosphaera pannosa*
OE2015PM88CS	*Centaurea montana*	21/06/2015	Allonby, Cumbria	Not PM
OE2015PM89CS	*Myosotis laxa*	21/06/2015	Silloth, Cumbria	*Golovinomyces cynoglossi*
OE2015PM90CS	*Ribes sanguineum*	21/06/2015	Maryport, Cumbria	*Podosphaera mors-uvae*
OE2015PM91CS	*Vicia sativa*	21/06/2015	Allonby, Cumbria	*Erysiphe* sp.
OE2015PM92CS	*Euonymus japonicus*	22/06/2015	Maryport, Cumbria	*Erysiphe euonymicola*
OE2015PM93CS	*Heracleum sphondylium*	22/06/2015	Maryport, Cumbria	Not PM
OE2015PM94CS	*Myosotis arvensis*	22/06/2015	Maryport, Cumbria	*Golovinomyces cynoglossi*
OE2015PM95CS	*Acer pseudoplatanus*	22/06/2015	Maryport, Cumbria	*Sawadaea bicornis*
OE2015PM96CS	*Acer pseudoplatanus*	22/06/2015	Maryport, Cumbria	*Sawadaea bicornis*
OE2015PM97CS	*Spiraea* sp.	19/06/2015	Cabra, Ireland	*Podosphaera spiraeae*
OE2015PM98CS	*Geum urbanum*	24/06/2015	Woodford Gree, Essex	*Podosphaera aphanis*
OE2015PM99CS	*Berberis thunbergii atropurpurea*	24/06/2014	Silloth, Cumbria	*Erysiphe berberidis*
OE2015PM100CS	*Acer pseudoplatanus*	24/06/2014	Crosscanonby, Cumbria	*Sawadaea bicornis*
OE2015PM101CS	*Filipendula ulmaria*	24/06/2014	Crosscanonby, Cumbria	*Podosphaera filipendulae*
OE2015PM102CS	*Ribes sanguineum*	26/06/2015	Workington, Cumbira	*Podosphaera mors-uvae*
OE2015PM103CS	*Acer pseudoplatanus*	26/06/2015	Workington, Cumbira	*Sawadaea bicornis*
OE2015PM104CS	*Filipendula ulmaria*	27/06/2015	Maryport, Cumbria	*Podosphaera filipendulae*
OE2015PM105CS	*Holcus lanatus*	27/06/2015	Maryport, Cumbria	*Blumeria graminis*
OE2015PM106CS	*Raphanus maritiums*	27/06/2015	Maryport, Cumbria	Not PM
OE2015PM107CS	*Achillea millefolium*	27/06/2015	Maryport, Cumbria	Not PM
OE2015PM108CS	*Chenopdoium vulvaria*	27/06/2015	Maryport, Cumbria	Not PM
OE2015PM109CS	*Filipendula ulmaria*	28/06/2015	Cockermouth, Cumbria	*Podosphaera filipendulae*
OE2015PM110CS	*Acer pseudoplatanus*	28/06/2015	Cockermouth, Cumbria	*Sawadaea bicornis*
OE2015PM111CS	*Sonchus arvensis*	28/06/2015	Cockermouth, Cumbria	*Neoerysiphe nevoi*
OE2015PM112CS	*Phleum* sp.	28/06/2015	Cockermouth, Cumbria	*Blumeria graminis*
OE2015PM113CS	*Catalpa bignonioides*	30/06/2015	Harris Garden, University of Reading	*Erysiphe catalpae*
OE2015PM114CS	*Anthriscus sylvestris*	27/06/2015	Maryport, Cumbria	*Erysiphe heraclei*
OE2015PM115CS	*Heracleum sphondylium*	27/06/2015	Maryport, Cumbria	Not PM
OE2015PM116CS	*Crataegus monogyna*	28/06/2015	Cockermouth, Cumbria	*Podosphaera clandestina*
OE2015PM117CS	*Vicia* sp.	29/06/2015	Mawbray, Cumbria	Not PM
OE2015PM118CS	*Senecio jacobaea*	29/06/2015	Mawbray, Cumbria	Not PM
OE2015PM119CS	*Crataegus monogyna*	29/06/2015	Mawbray, Cumbria	*Podosphaera clandestina*
OE2015PM120CS	*Senecio jacobaea*	29/06/2015	Allonby, Cumbria	Not PM
OE2015PM121CS	*Vitis vinifera*	30/06/2015	Adams Road, Cambridge	*Erysiphe necator*
OE2015PM122CS	*Plantago lanceolata*	30/06/2015	Whitehaven, Cumbria	*Golovinomyces sordidus*
OE2015PM123CS	*Centaurea nigra*	01/07/2015	Siddick, Cumbria	Not PM
OE2015PM124CS	*Filipendula ulmaria*	01/07/2015	Siddick, Cumbria	*Podosphaera filipendulae*
OE2015PM125CS	*Vicia cracca*	01/07/2015	Siddick, Cumbria	Not PM
OE2015PM126CS	*Cerastium fontanum*	01/07/2015	Siddick, Cumbria	Not PM

OE2015PM127CS	*Crataegus monogyna*	01/07/2015	Siddick, Cumbria	*Podosphaera clandestina*
OE2015PM128CS	*Malus pumila*	01/07/2015	Siddick, Cumbria	*Podosphaera leucotricha*
OE2015PM129CS	*Vicia cracca*	01/07/2015	Siddick, Cumbria	*Podosphaera clandestina*
OE2015PM130CS	*Plantago major*	01/07/2015	Siddick, Cumbria	Pm sp
OE2015PM131CS	*Silene dioica*	02/07/2015	Siddick, Cumbria	Not PM
OE2015PM132CS	*Delphinium* sp.	02/07/2015	Farnborough	*Erysiphe aquilegiae*
OE2015PM133CS	*Rosa* 'Dorothy Perkins'	03/07/2015	Tiverton, Devon	*Podosphaera pannosa*
OE2015PM134CS	*Symphytum* sp.	07/07/2015	Wigston, Leicester	*Golovinomyces cynoglossi*
OE2015PM135CS	*Dipsacus* sp.	07/07/2015	Wigston, Leicester	*Podosphaera dipsacearum*
OE2015PM136CS	*Prunus laurocerasus*	06/07/2015	South Tottenham, London	*Podosphaera tridactyla*
OE2015PM137CS	*Artemisia vulgaris*	03/07/2015		Not PM
OE2015PM138CS	*Heracleum sphondylium*	03/07/2015	Maidstone	*Erysiphe heraclei*
OE2015PM139CS	*Amelanchier lamarckii*	05/07/2015	Harris Garden	*Podosphaera amelanchieris*
OE2015PM140CS	*Aquilegia* sp.	05/07/2015	Chester Zoological Gardens	*Erysiphe aquilegiae*
OE2015PM141CS	*Lonicera* sp.	05/07/2015	Newport, Saffron Walden	*Erysiphe lonicerae*
OE2015PM142CS	*Lupinus* sp.	05/07/2015	Dereham, Norfolk	*Erysiphe intermedia*
OE2015PM143CS	*Centaurea montana*	05/07/2015	Newport, Saffron Walden	*Golovinomyces depressus*
OE2015PM144CS	*Delphinium* sp.	05/07/2015	Farnborough	*Erysiphe aquilegiae*
OE2015PM145CS	*Pulmonaria* sp.	06/07/2015	Newport, Saffron Walden	*Golovinomyces cynoglossi*
OE2015PM146CS	*Fragaria* x *ananassa*	07/07/2015	South Tottenham, London	*Podosphaera aphanis*
OE2015PM147CS	*Rosa* sp.	07/07/2015	Tiverton, Devon	*Podosphaera pannosa*
OE2015PM148CS	*Aquilegia* sp.	07/07/2015	Chester Zoological Gardens	*Erysiphe aquilegiae*
OE2015PM149CS	*Lychnis coronaria*	07/07/2015		*Erysiphe buhrii*
OE2015PM150CS	*Rosa* sp.	07/07/2015	Tiverton, Devon	*Podosphaera pannosa*
OE2015PM151CS	*Aster nova-belgii*	07/07/2015	Dereham, Norfolk	*Golovinomyces asterum var. asterum*
OE2015PM152CS	*Cucurbita pepo*	07/07/2015	Farnborough	*Golovinomyces orontii*
OE2015PM153CS	*Phlox paniculata*	07/07/2015	Harris Garden	*Golovinomyces magnicellulatus var. magnicellulatus*
OE2015PM154CS	*Acer* sp.	11/07/2015	Worcester Park, Kingston	*Sawadaea bicornis*
OE2015PM155CS	*Plantago major*	11/07/2015	Liverpool, Merseyside	*Golovinomyces sordidus*
OE2015PM156CS	*Taraxacum officinale*	11/07/2015	Liverpool, Merseyside	*Podosphaera erigerontis-canadensis*
OE2015PM157CS	*Aquilegia* sp.	12/07/2015	Liverpool, Merseyside	*Erysiphe aquilegiae*
OE2015PM158CS	*Acer pseudoplatanus*	12/07/2015	Widnes, Halton	*Sawadaea bicornis*
OE2015PM159CS	*Arctium minus*	12/07/2015	Widnes, Halton	*Golovinomyces depressus*
OE2015PM160CS	*Taraxacum officinale*	13/07/2015	Runcorn, Cheshire	*Golovinomyces cichoracearum*
OE2015PM161CS	*Quercus robur*	13/07/2015	Runcorn, Cheshire	*Erysiphe alphitoides*
OE2015PM162CS	*Aquilegia* sp.	13/07/2015	Streatham Hill, London	*Erysiphe aquilegiae*
OE2015PM163CS	*Arctium minus*	14/07/2015	Hundleton, Pembroke	Not PM
OE2015PM164CS	*Heracleum sphondylium*	14/07/2015	Hundleton, Pembroke	*Erysiphe heraclei*
OE2015PM165CS	*Quercus robur*	14/07/2015	Hundleton, Pembroke	*Erysiphe alphitoides*
OE2015PM166CS	*Lotus pedunculatus*	14/07/2015	Hundleton, Pembroke	*Podosphaera clandestina*

OE2015PM167CS	*Lycium barbarum*	14/07/2015	Wigston, Leicester	*Arthrocladiella mougeotii*
OE2015PM168CS	*Rosa* 'Paul's Himalayan Musk'	14/07/2015	Cornwall	*Podosphaera pannosa*
OE2015PM169CS	*Fragaria* x *ananassa*	14/07/2015	Liverpool, Merseyside	*Podosphaera aphanis*
OE2015PM170CS	*Stachys sylvatica*	14/07/2015	Liverpool, Merseyside	*Neoerysiphe galeopsidis*
OE2015PM171CS	*Acer pseudoplatanus*	14/07/2015	St. Helens, Merseyside	*Sawadaea bicornis*
OE2015PM172CS	*Ranunuculus acris*	16/07/2015	Liverpool, Merseyside	*Erysiphe aquilegiae*
OE2015PM173CS	*Quercus robur*	16/07/2015	Liverpool, Merseyside	*Erysiphe alphitoides*
OE2015PM174CS	*Cirsium arvense*	16/07/2015	Liverpool, Merseyside	Not PM
OE2015PM175CS	*Anthriscus sylvestris*	16/07/2015	Liverpool, Merseyside	*Erysiphe heraclei*
OE2015PM176CS	*Acer pseudoplatanus*	16/07/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PM177CS	*Filipendula ulmaria*	16/07/2015	Blyth, Northumberland	*Podosphaera filipendulae*
OE2015PM178CS	*Heracleum sphondylium*	19/07/2015	Hundleton, Pembroke	*Erysiphe heraclei*
OE2015PM179CS	*Plantago lanceolata*	20/07/2015	Crown Place, Reading	*Podosphaera plantaginis*
OE2015PM180CS	*Crataegus monogyna*	20/07/2015	Widnes	*Podosphaera clandestina*
OE2015PM181CS	*Acer campestre*	20/07/2015	Allerton, Liverpool	*Sawadaea bicornis*
OE2015PM182CS	*Artemisia vulgaris*	20/07/2015		*Golovinomyces artemisiae*
OE2015PM183CS	*Quercus robur*	20/07/2015	Hundleton, Pembroke	*Erysiphe alphitoides*
OE2015PM184CS	*Eupatorium cannabinum*	20/07/2015		*Golovinomyces circumfusus*
OE2015PM185CS	*Prunus spinosa*	20/07/2015	Liverpool, Merseyside	*Erysiphe prunastri*
OE2015PM186CS	*Symphoricarpos albus*	20/07/2015	Woolton, Liverpool	*Erysiphe symphoricarpi*
OE2015PM187CS	*Rosa canina*	20/07/2015	Cornwall	*Podosphaera pannosa*
OE2015PM188CS	*Acer pseudoplatanus*	20/07/2015	St. Helens, Merseyside	*Sawadaea bicornis*
OE2015PM189CS	*Acer pseudoplatanus*	20/07/2015	St. Helens, Merseyside	*Sawadaea bicornis*
OE2015PM190CS	*Ranunuculus repens*	20/07/2015	Liverpool, Merseyside	*Erysiphe aquilegiae*
OE2015PM191CS	*Euonymus* sp.	21/07/2015	Preston	*Erysiphe euonymicola*
OE2015PM192CS	*Crataegus monogyna*	21/07/2015	Liverpool, Merseyside	*Podosphaera clandestina*
OE2015PM193CS	*Amelanchier lamarckii*	22/07/2015	Runcorn, Cheshire	*Podosphaera amelanchieris*
OE2015PM194CS	*Silene dioica*	22/07/2015	Runcorn, Cheshire	*Erysiphe buhrii*
OE2015PM195CS	*Plantago lanceolata*	23/07/2015	Angelsey, North Wales	*Golovinomyces sordidus*
OE2015PM196CS	*Silene dioica*	23/07/2015	Angelsey, North Wales	*Erysiphe buhrii*
OE2015PM197CS	*Crataegus monogyna*	23/07/2015	Angelsey, North Wales	*Podosphaera clandestina*
OE2015PM198CS	*Platanus* x *hispanica*	25/07/2015	Victoria Tower Gardens, London	*Erysiphe platani*
OE2015PM199CS	*Epilobium parviflorium*	27/07/2015	Shinfield, Reading	*Podosphaera epilobi*
OE2015PMCS200	*Viola tricolor*	29/07/2015	Sidcup, London	*Golovinomyces orontii*
OE2015PMCS201	*Aquilegia vulgaris*	29/07/2015	Sidcup, London	*Erysiphe aquilegiae*
OE2015PMCS202	*Lonicera japonica* 'Halliana'	29/07/2015	Sidcup, London	*Erysiphe lonicerae*
OE2015PMCS203	*Centaurea montana*	05/08/2015	Boston	Not PM
OE2015PMCS204	*Lapsana communis*	05/08/2015	Allerton Allotments, Liverpool	*Neoerysiphe nevoi*
OE2015PMCS205	*Crataegus monogyna*	06/08/2015	Old Hall, Warrington	*Podosphaera clandestina*
OE2015PMCS206	*Viburnum opulus*	06/08/2015	Leigh, Lancs.	*Erysiphe hedwigii*
OE2015PMCS207	*Filipendula ulmaria*	06/08/2015	Leigh, Lancs.	*Podosphaera filipendulae*

OE2015PMCS208	*Prunus spinosa*	06/08/2015	Leigh, Lancs.	*Erysiphe prunastri*
OE2015PMCS209	*Quercus robur*	06/08/2015	Leigh, Lancs.	*Sawadaea bicornis*
OE2015PMCS210	*Acer pseudoplatanus*	06/08/2015	Leigh, Lancs.	*Sawadaea bicornis*
OE2015PMCS211	*Anthriscus sylvestris*	09/08/2015	Stockport, Cheshire	*Erysiphe heraclei*
OE2015PMCS212	*Alliaria petiolata*	09/08/2015	Stockport, Cheshire	*Erysiphe cruciferarum*
OE2015PMCS213	*Quercus robur*	09/08/2015	Stockport, Cheshire	*Erysiphe alphitoides*
OE2015PMCS214	*Circaea lutetiana*	09/08/2015	Stockport, Cheshire	*Erysiphe circaeae*
OE2015PMCS215	*Crataegus monogyna*	09/08/2015	Stockport, Cheshire	*Podosphaera clandestina*
OE2015PMCS216	*Rosa canina*	09/08/2015	Stockport, Cheshire	*Podosphaera pannosa*
OE2015PMCS217	*Geranium pratense*	09/08/2015	Stockport, Cheshire	*Neoerysiphe geranii*
OE2015PMCS218	*Brassica* sp.	09/08/2015	Stockport, Cheshire	*Erysiphe cruciferarum*
OE2015PMCS219	*Acer pseudoplatanus*	09/08/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS220	*Taraxacum officinale*	09/08/2015	Liverpool, Merseyside	*Podosphaera erigerontis-canadensis*
OE2015PMCS221	*Acer pseudoplatanus*	09/08/2015	Stockport, Cheshire	*Sawadaea bicornis*
OE2015PMCS222	*Heracleum sphondylium*	09/08/2015	Stockport, Cheshire	*Erysiphe heraclei*
OE2015PMCS223	*Epilobium hirsutum*	09/08/2015	Stockport, Cheshire	*Podosphaera epilobi*
OE2015PMCS224	*Plantago major*	09/08/2015	Liverpool, Merseyside	*Golovinomyces sordidus*
OE2015PMCS225	*Sonchus oleraceus*	09/08/2015	Stockport, Cheshire	*Golovinomyces sonchicola*
OE2015PMCS226	*Symphytum xuplandicum*	10/08/2015	Liverpool, Merseyside	*Golovinomyces cynoglossi*
OE2015PMCS227	*Pilosella aurantiaca*	10/08/2015	Liverpool, Merseyside	*Golovinomyces cichoracearum*
OE2015PMCS228	*Aquilegia* sp.	10/08/2015	Liverpool, Merseyside	*Erysiphe aquilegiae*
OE2015PMCS229	*Lycium barbarum*	11/08/2015	Liverpool, Merseyside	*Arthrocladiella mougeotii*
OE2015PMCS230	*Malus pumila*	11/08/2015	Liverpool, Merseyside	*Podosphaera leucotricha*
OE2015PMCS231	*Trifolium pratense*	11/08/2015	Liverpool, Merseyside	*Erysiphe trifoliorum*
OE2015PMCS232	*Anthyllis vulneria*	11/08/2015	Liverpool, Merseyside	*Erysiphe trifoliorum*
OE2015PMCS233	*Polygonum aviculare*	11/08/2015	Liverpool, Merseyside	*Erysiphe polygoni*
OE2015PMCS234	*Acer pseudoplatanus*	11/08/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS235	*Hypericum* sp.	12/08/2015	Liverpool, Merseyside	*Erysiphe hyperici*
OE2015PMCS236	*Brassica* sp.	12/08/2015	Liverpool, Merseyside	*Erysiphe cruciferarum*
OE2015PMCS237	*Euonymus japonicus*	12/08/2015	Liverpool, Merseyside	*Erysiphe euonymicola*
OE2015PMCS238	*Geum urbanum*	12/08/2015	Liverpool, Merseyside	*Podosphaera aphanis*
OE2015PMCS239	*Acer pseudoplatanus*	12/08/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS240	*Aesculus indica*	12/08/2015	Liverpool, Merseyside	*Erysiphe flexuosa*
OE2015PMCS241	*Acer pseudoplatanus*	12/08/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS242	*Heracleum sphondylium*	12/08/2015	Liverpool, Merseyside	*Erysiphe heraclei*
OE2015PMCS243	*Filipendula ulmaria*	12/08/2015	Liverpool, Merseyside	*Podosphaera filipendulae*
OE2015PMCS244	*Epilobium hirsutum*	12/08/2015	Liverpool, Merseyside	*Podosphaera epilobi*
OE2015PMCS245	Unknown	12/08/2015	Liverpool, Merseyside	
OE2015PMCS246	*Acer* sp.	13/08/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS247	*Acer* sp.	13/08/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS248	*Quercus robur*	16/08/2015	Edgbarrow Woods	*Erysiphe alphitoides*
OE2015PMCS249	*Plantago major*	16/08/2015	Edgbarrow Woods	*Erysiphe cruciferarum* & *Golovinomyces sordidus*

OE2015PMCS250	*Sisymbrium officinale*	16/08/2015	Edgbarrow Woods	*Erysiphe cruciferarum*
OE2015PMCS251	*Arctium minus*	17/08/2015	Fairfield Road, Widnes	*Golovinomyces depressus*
OE2015PMCS252	*Brassica* sp.	17/08/2015	Fairfield Road, Widnes	*Erysiphe cruciferarum*
OE2015PMCS253	*Plantago major*	17/08/2015	Fairfield Road, Widnes	*Golovinomyces sordidus*
OE2015PMCS254	*Aquilegia* sp.	17/08/2015	Fairfield Road, Widnes	*Erysiphe aquilegiae*
OE2015PMCS255	*Crataegus monogyna*	17/08/2015	Fairfield Road, Widnes	*Podosphaera clandestina*
OE2015PMCS256	*Euonymus japonicus*	17/08/2015	Fairfield Road, Widnes	*Erysiphe euonymicola*
OE2015PMCS257	*Amelanchier lamarckii*	17/08/2015	Fairfield Road, Widnes	*Podosphaera amelanchieris*
OE2015PMCS258	*Arctium minus*	17/08/2015	Fairfield Road, Widnes	*Golovinomyces depressus*
OE2015PMCS259	*Lathyrus odoratus*	17/08/2015	Liverpool, Merseyside	*Erysiphe trifoliorum*
OE2015PMCS260	*Heracleum sphondylium*	17/08/2015	Fairfield Road, Widnes	*Erysiphe heraclei*
OE2015PMCS261	*Acer pseudoplatanus*	17/08/2015	Fairfield Road, Widnes	*Sawadaea bicornis*
OE2015PMCS262	*Sonchus oleraceus*	17/08/2015	Fairfield Road, Widnes	*Golovinomyces cichoracearum*
OE2015PMCS263	*Vitis vinifera*	18/08/2015	Sherfield Drive, Reading	*Erysiphe necator*
OE2015PMCS264	*Platanus* x *acerifolia*	10/08/2015	Aylesbury	*Erysiphe platani*
OE2015PMCS265	*Melilotus altissimus*	15/08/2015	Marsworth	*Erysiphe trifoliorum*
OE2015PMCS266	*Trifolium pratense*	16/08/2015	Slough	*Erysiphe trifoliorum*
OE2015PMCS267	*Monarda didyma*	20/08/2015	Chilton, Didcot	*Golovinomyces biocellaris*
OE2015PMCS268	*Catalpa bignonioides*	01/09/2015	Campus Central, University of Reading	*Erysiphe elevata*
OE2015PMCS269	*Matricaria discoidea*	06/09/2015	Wilderness Road, Reading	*Podosphaera fusca*
OE2015PMCS270	*Aquilegia vulgaris*	07/09/2015	Harris Garden, University of Reading	*Erysiphe aquilegiae*
OE2015PMCS271	*Geranium phaeum*	07/09/2015	Harris Garden, University of Reading	*Neoerysiphe geranii*
OE2015PMCS272	*Phlox paniculata*	07/09/2015	Harris Garden, University of Reading	*Golovinomyces magnicellulatus* var. *magnicellulatus*
OE2015PMCS273	*Malus domestica*	07/09/2015	Harris Garden, University of Reading	*Podosphaera leucotricha*
OE2015PMCS274	*Stachys byzantina*	07/09/2015	Harris Garden, University of Reading	*Neoerysiphe galeopsidis*
OE2015PMCS275	*Quercus robur*	07/08/2015	Gran Canaria	*Erysiphe alphitoides*
OE2015PMCS276	*Berberis thunbergii atropurpurea*	20/08/2015	Parkfield, Buckinghamshire	*Erysiphe berberidis*
OE2015PMCS277	*Euonymus* sp.	20/08/2015	Parkfield, Buckinghamshire	*Erysiphe euonymicola*
OE2015PMCS278	*Quercus robur*	07/09/2015	Liverpool, Merseyside	*Erysiphe alphitoides*
OE2015PMCS279	*Epilobium hirsutum*	07/09/2015	Liverpool, Merseyside	*Golovinomyces magnicellulatus*
OE2015PMCS280	*Aesculus hippocastanum*	07/09/2015	Liverpool, Merseyside	*Erysiphe flexuosa*
OE2015PMCS281	*Lactuca muralis*	07/09/2015	Liverpool, Merseyside	*Golovinomyces cichoracearum*
OE2015PMCS282	*Heracleum sphondylium*	07/09/2015	Liverpool, Merseyside	*Erysiphe heraclei*
OE2015PMCS283	*Verbascum* sp.	07/09/2015	Liverpool, Merseyside	*Golovinomyces verbasci*
OE2015PMCS284	*Acer pseudoplatanus*	07/09/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS285	*Solidago canadensis*	07/09/2015	Liverpool, Merseyside	*Golovinomyces asterum var. solidaginis*
OE2015PMCS286	Unknown	07/09/2015	Liverpool, Merseyside	*Podosphaera fusca*
OE2015PMCS287	*Epilobium ciliatum*	08/09/2015	Colney Woods Burial Ground, Norfolk	*Podosphaera epilobii*
OE2015PMCS288	*Quercus* sp.	08/09/2015	Thickthorn Roundabout, Norwich, Nofolk	*Erysiphe alphitoides*
OE2015PMCS289	*Humulus lupulus*	09/09/2015	Thurlton, Norfolk	*Podosphaera macularis*

OE2015PMCS290	*Geum urbanum*	09/09/2015	Thurlton, Norfolk	*Podosphaera aphanis*
OE2015PMCS291	*Acer* sp.	10/09/2015	Vauxhall Drive, Woodley	*Sawadaea tulasnei*
OE2015PMCS292	*Taraxacum officinale*	11/09/2015	Experimental plots, University of Reading	*Podosphaera erigerontis-canadensis*
OE2015PMCS293	*Myosotis arvensis*	11/09/2015	Experimental plots, University of Reading	*Golovinomyces cynoglossi*
OE2015PMCS294	*Lamium* sp.	11/09/2015	Experimental plots, University of Reading	*Neoerysiphe galeopsidis*
OE2015PMCS295	*Mahonia aquifolium*	11/09/2015	Experimental plots, University of Reading	*Erysiphe beberidis*
OE2015PMCS296	*Trifolium pratense*	11/09/2015	Experimental plots, University of Reading	*Erysiphe trifoliorum*
OE2015PMCS297	*Lupinus* sp.	12/09/2015	Dereham, Norfolk	*Erysiphe intermedia*
OE2015PMCS298	*Lathyrus odoratus*	13/09/2015	Bramcote, Nottingham	*Erysiphe trifoliorum*
OE2015PMCS299	*Trifolium campestre*	13/09/2015	Everton, Liverpool, Merseyside	*Erysiphe trifoliorum*
OE2015PMCS300	*Plantago lanceolata*	13/09/2015	Everton, Liverpool, Merseyside	*Podosphaera plantaginis*
OE2015PMCS301	*Quercus cerris*	16/09/2015	St. Helens, Merseyside	*Erysiphe alphitoides*
OE2015PMCS302	*Acer* sp.	18/09/2015	Liverpool, Merseyside	*Sawadaea bicornis*
OE2015PMCS303	*Geranium* sp.	20/09/2015	Pennant, Credigion	*Neoerysiphe geranii*
OE2015PMCS304	*Trifolium dubium*	20/09/2015	Pennant, Credigion	*Erysiphe trifoliorum*
OE2015PMCS305	*Myosotis arvensis*	20/09/2015	Pennant, Credigion	*Golovinomyces cynoglossi*
OE2015PMCS306	*Mentha* sp.	20/09/2015	Pennant, Credigion	*Golovinomyces biocellatus*
OE2015PMCS307	*Cucurbita pepo*	24/09/2015	Eden Project, Cornwall	*Golovinomyces orontii*
OE2015PMCS308	*Cosmos* sp.	24/09/2015	Eden Project, Cornwall	*Golovinomyces cichoracearum*
OE2015PMCS309	*Lamium* sp.	25/09/2015	Experimental plots, University of Reading	*Neoerysiphe galeopsidis*
OE2015PMCS310	*Berberis* sp.	25/09/2015	Chestlion Farm, Clanfield	*Erysiphe berberidis*
OE2015PMCS311	*Populus* sp.	25/09/2015	Botley Wood, Hampshire	*Erysiphe adunca*
OE2015PMCS312	*Taraxacum officinale*	04/10/2015	Barkham, Wokingham	*Podosphaera erigerontis-canadensis*
OE2015PMCS313	*Aquilegia* sp.	04/10/2015	Barkham, Wokingham	*Erysiphe aquilegiae*
OE2015PMCS314	*Euphorbia peplus*	04/10/2015	Barkham, Wokingham	*Podosphaera euphorbiae-helioscopiae*
OE2015PMCS315	*Wisteria frutescens* 'Amethyst Falls'	08/10/2015	Addington Road, Reading	*Erysiphe alphitoides*
OE2015PMCS316	*Crataegus monogyna*	08/10/2015	Whiteknights Lake, Reading	*Podosphaera clandestina*
OE2015PMCS317	*Taraxacum officinale*	08/10/2015	Harborne Building, University of Reading	*Podosphaera erigerontis-canadensis*
OE2015PMCS318	*Delphinium* sp.	08/10/2015	Harris Garden, University of Reading	*Neoerysiphe geranii*
OE2015PMCS319	*Taraxacum officinale*	08/10/2015	Whiteknights Lake, Reading	*Podosphaera erigerontis-canadensis*
OE2015PMCS320	*Geranium* sp.	08/10/2015	Harborne Building, University of Reading	*Podosphaera fugax*
OE2015PMCS321	*Taraxacum officinale*	08/10/2015	Whiteknights Lake, Reading	*Podosphaera erigerontis-canadensis*
OE2015PMCS322	*Rosa canina*	08/10/2015	Whiteknights Lake, Reading	*Podosphaera pannosa*
OE2015PMCS323	*Trifolium pratense*	08/10/2015	Whiteknights Lake, Reading	*Erysiphe trifoliorum*
OE2015PMCS324	*Heracleum sphondylium*	08/10/2015	Whiteknights Lake, Reading	*Erysiphe heraclei*
OE2015PMCS325	*Rosa canina*	07/10/2015	Llandover, Carmarthenshire	*Podosphaera pannosa*
OE2015PMCS326	*Tellima grandiflora*	01/06/2011	Kew, Royal Botanic Gardens, Rhododendron Dell, Surrey	*Podosphaera macrospora*
OE2015PMCS327	*Tellima grandiflora*	20/06/2011	Kew, Royal Botanic Gardens, Compt. 323, Surrey	*Podosphaera macrospora*
OE2015PMCS328	*Tellima grandiflora*	02/09/2011	Glasbury, Lower Penylan, Breconshire	*Podosphaera macrospora*

OE2015PMCS329	*Tellima grandiflora*	23/07/2013	Brandon Wood, Warwickshire	*Podosphaera macrospora*
OE2015PMCS330	*Tellima grandiflora* 'purpurea'	12/08/2013	Kew, Royal Botanic Gardens, Cambrigde Cottage, Surrey	*Podosphaera macrospora*
OE2015PMCS331	*Fraxinus excelsior*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Phyllactinia fraxini*
OE2015PMCS332	*Salix aurita*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe adunca*
OE2015PMCS333	*Acer campestre*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Sawadaea bicornis*
OE2015PMCS334	*Plantago lanceolata*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Podosphaera plantaginis*
OE2015PMCS335	*Symphoricarpos albus*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe symphoricarpi*
OE2015PMCS336	*Aquilegia vulgaris*	25/10/2015	Woolton, Liverpool	*Erysiphe aquilegiae*
OE2015PMCS337	*Quercus robur*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe alphitoides*
OE2015PMCS338	*Populus* sp.	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe adunca*
OE2015PMCS339	*Prunus spinosa*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Podosphaera tridactyla*
OE2015PMCS340	*Plantago major*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Golovinomyces sordidus*
OE2015PMCS341	*Taraxacum officinale*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Podosphaera erigerontis-canadensis*
OE2015PMCS342	*Quercus cerris*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe alphitoides*
OE2015PMCS343	*Acer pseudoplatanus*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Sawadaea bicornis*
OE2015PMCS344	*Taraxacum officinale*	25/10/2015	Woolton, Liverpool	*Podosphaera erigerontis-canadensis*
OE2015PMCS345	*Symphytum officinale*	25/10/2015	Woolton, Liverpool	*Golovinomyces cynoglossi*
OE2015PMCS346	*Crataegus monogyna*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Podosphaera clandestina*
OE2015PMCS347	*Pilosella aurantiaca*	25/10/2015	Woolton, Liverpool	*Golovinomyces cichoracearum*
OE2015PMCS348	*Rosa canina*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe simulans*
OE2015PMCS349	*Stachys sylvatica*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Neoerysiphe galeopsidis*
OE2015PMCS350	*Urtica dioica*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe urticae*
OE2015PMCS351	*Plantago major*	25/10/2015	Woolton, Liverpool	*Golovinomyces sordidus*
OE2015PMCS352	*Artemisia vulgaris*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Golovinomyces artemisiae*
OE2015PMCS353	*Ranunuculus repens*	25/10/2015	Spile Island, Lower Church Street, Widnes	*Erysiphe aquilegiae var. ranunculi*
OE2016PMCS1	*Heuchera* 'Caramel'	07/03/2016	RHS Wisley	*Podosphaera macrospora*
OE2016PMCS2	*Verbena bonariensis*	27/03/2016	Stratford, London	*Podosphaera sp.*
OE2016PMCS3	*Verbena bonariensis*	27/03/2016	Stratford, London	Pm sp
OE2016PMCS4	*Geranium* sp.	17/05/2016	Wilderness Road, Reading	*Podosphaera fugax*
OE2016PMCS5	*Anthriscus sylvestris*	17/05/2016	Greensward Lane, Arborfield Cross	*Erysiphe heraclei*
OE2016PMCS6	*Ranunculus repens*	17/05/2016	Greensward Lane, Arborfield Cross	*Erysiphe aquilegiae*
OE2016PMCS7	*Euonymus* sp.	26/05/2016	Wembley, London	*Erysiphe euonymicola*
OE2016PMCS8	*Heuchera* 'Caramel'	01/06/2016	Whiteknights Campus, Reading	*Podosphaera macrospora*
OE2016PMCS9	*Mahonia moseri*	12/06/2016	Derby	*Erysiphe berberidis*
OE2016PMCS10	*Acanthus mollis*	15/06/2016	Hartington Road, London	*Neoerysiphe galeopsidis*
OE2016PMCS11	*Euonymus kiautschovicus*	17/06/2016	Food Sciences, Whiteknights Campus	*Erysiphe euonymicola*
OE2016PMCS12	*Sonchus arvensis*	20/06/2016	Southampton Street, Reading	*Neoerysiphe nevoi*

OE2016PMCS13	*Galium aparine*	20/06/2016	Southampton Street, Reading	*Neoerysiphe galii*
OE2016PMCS14	*Tellima grandiflora*	20/06/2016	Southampton Street, Reading	*Podosphaera macrospora*
OE2016PMCS15	*Berberis thunbergii*	26/06/2016	Christchurch Road, Reading	*Erysiphe berberidis*
OE2016PMCS16	*Catalpa bignonioides*	05/07/2015	Campus Central, Whiteknights	*Erysiphe catalpae*
OE2016PMCS17	*Acanthus mollis*	05/07/2015	Lee, Devon	*Neoerysiphe galeopsidis*
OE2016PMCS18	*Geum urbanum*	05/07/2015	Lee, Devon	*Podosphaera aphanis*
OE2016PMCS19	x *Heucherella*	05/07/2015	Lee, Devon	*Podosphaera macrospora*
OE2016PMCS20	*Cucurbita pepo*	05/07/2015	Guiseley, Leeds	*Golovinomyces orontii*
OE2016PMCS21	*Cucurbita pepo* 'Atena Polka F1'	05/07/2015	Guiseley, Leeds	*Golovinomyces orontii*
OE2016PMCS22	*Rosa* 'Dorothy Perkins'	06/07/2016	Ramsey St. Mary's, Ramsey	*Podosphaera pannosa*
OE2016PMCS23	*Acer pseudoplatanus*	08/07/2016	Horns Drove, Rownhams	*Sawadaea bicornis*
OE2016PMCS24	*Geum urbanum*	08/07/2016	Horns Drove, Rownhams	*Podosphaera aphanis*
OE2016PMCS25	*Alliaria petiolata*	08/07/2016	Horns Drove, Rownhams	*Erysiphe cruciferarum*
OE2016PMCS26	*Euphorbia* sp.	29/07/2016	Tang, N. Yorkshire	*Podosphaera euphorbiae-helioscopiae*
OE2016PMCS27	*Pisum sativum*	20/07/2016	Eden Project, Cornwall	*Erysiphe pisi*
OE2016PMCS28	*Salvia mellifera*	20/07/2016	Eden Project, Cornwall	*Golovinomyces biocellaris*
OE2016PMCS29	*Monarda didyma*	20/07/2016	Eden Project, Cornwall	*Golovinomyces biocellaris*
OE2016PMCS30	*Rosa* 'Black Baccara'	05/08/2016	Thornton-Cleveleys	*Podosphaera pannosa*
OE2016PMCS31	*Plantago maritima*	30/07/2016	Eyemouth, Berwickshire	Pm sp
OE2016PMCS32	*Rosa canina*	30/07/2016	Eyemouth, Berwickshire	*Podosphaera pannosa*
OE2016PMCS33	*Crataegus monogyna*	30/07/2016	Eyemouth, Berwickshire	*Podosphaera clandestina*
OE2016PMCS34	*Rosa rugosa*	30/07/2016	Eyemouth, Berwickshire	*Podosphaera pannosa*
OE2016PMCS35	*Lamium album*	04/08/2016	Lamberton, Berwickshire	*Neoerysiphe galeopsidis*
OE2016PMCS36	*Filipendula ulmaria*	02/08/2016	Greenlaw, Berwickshire	*Podosphaera filipendulae*
OE2016PMCS37	*Acer campestre*	02/08/2016	Chirnside, Berwickshire	*Sawadaea bicornis*
OE2016PMCS38	*Ajuga reptans*	04/08/2016	Studham, Dunstable	*Neoerysiphe galeopsidis*
OE2016PMCS39	*Geranium phaeum*	04/08/2016	Studham, Dunstable	*Podosphaera fugax*
OE2016PMCS40	*Plantago major*	04/08/2016	Stokeinteignhead, Newton Abbot	*Golovinomyces sordidus*
OE2016PMCS41	*Acer campestre*	04/08/2016	Stokeinteignhead, Newton Abbot	*Sawadaea bicornis*
OE2016PMCS42	*Centranthus ruber*	04/08/2016	Mill Lane, Teignmouth	*Golovinomyces valerianae*
OE2016PMCS43	*Epilobium palustre*	07/08/2016	Balerno, Midlothian	*Podosphaera epilobii*
OE2016PMCS44	*Plantago lanceolata*	07/08/2016	Balerno, Midlothian	*Podosphaera plantaginis*
OE2016PMCS45	*Lathyrus pratensis*	07/08/2016	Balerno, Midlothian	*Erysiphe trifoliorum*
OE2016PMCS46	*Geum urbanum*	09/08/2016	Eyemouth, Berwickshire	*Podosphaera aphanis*
OE2016PMCS47	*Lamium purpureum*	10/08/2016	Lamberton, Berwickshire	*Neoerysiphe galeopsidis*
OE2016PMCS48	*Calendula officianalis*	10/08/2016	Lamberton, Berwickshire	*Podosphaera xanthii*
OE2016PMCS49	*Crepis paludosa*	14/08/2016	Threepwood Moss	*Podosphaera erigerontis-canadensis*
OE2016PMCS50	*Plantago maritima*	15/08/2016	Eyemouth, Berwickshire	*Golovinomyces sordidus*
OE2016PMCS51	*Caltha palustris*	15/08/2016	Lamberton, Berwickshire	*Erysiphe aquilegiae*
OE2016PMCS52	*Dipsacus fullonum*	15/08/2016	Lamberton, Berwickshire	*Podosphaera dipsacearum*
OE2016PMCS53	*Viburnum tinus*	16/08/2016	Foxton Dr, Alnwick	*Erysiphe hedwigii*

OE2016PMCS54	*Acanthus mollis*	16/08/2016	Leigh-on-Sea	*Neoerysiphe galeopsidis*
OE2016PMCS55	*Mahonia* sp.	16/08/2016	Leigh-on-Sea	*Erysiphe beberidis*
OE2016PMCS56	*Phlox paniculata*	16/08/2016	Leigh-on-Sea	*Podosphaera collomiae*
OE2016PMCS57	*Sanguisorba officianalis*	19/08/2016	Bowden, Roxburgshire	*Podosphaera ferruginea*
OE2016PMCS58	*Odontites vernus*	19/08/2016	Bowden, Roxburgshire	Not PM
OE2016PMCS59	*Potentilla erecta*	19/08/2016	Eildon Hills, Melrose	*Podosphaera aphanis*
OE2016PMCS60	*Prunus lusitanica*	23/08/2016	Kelso	*Podosphaera tridactyla*
OE2016PMCS61	*Lathyrus pratensis*	26/08/2016	Lindean Moor, Selkirk	*Erysiphe trifoliorum*
OE2016PMCS62	*Rosa 'Dorothy Perkins'*	30/08/2016	Carlisle, Cumbria	*Erysiphe simulans*
OE2016PMCS63	*Polygonum aviculare*	05/09/2016	Thorpe Marsh, Norwich	*Erysiphe polygoni*
OE2016PMCS64	*Epilobium hirsutum*	04/09/2016	Portmoak, Fife	*Podosphaera epilobii*
OE2016PMCS65	*Pisum sativum*	05/09/2016	Lamberton, Berwickshire	*Erysiphe pisi*
OE2016PMCS66	*Centaurea cyanus*	05/09/2016	Lamberton, Berwickshire	*Golovinomyces depressus*
OE2016PMCS67	*Taraxacum officinale*	07/09/2016	Lamberton, Berwickshire	*Podosphaera erigerontis-canadensis*
OE2016PMCS68	*Lupinus sp.*	09/09/2016	Pitlochry, Mid-Perth	*Erysiphe intermedia*
OE2016PMCS69	*Hypericum* sp.	09/09/2016	Pitlochry, Mid-Perth	*Erysiphe hyperici*
OE2016PMCS70	*Lonicera* sp.	10/09/2016	Kincraig, East Inverness	*Erysiphe lonicerae*
OE2016PMCS71	*Ribes* sp.	10/09/2016	Newtonmore, East Inverness	*Podosphaera mors-uvae*
OE2016PMCS72	*Acer campestre*	11/09/2016	Balavil, Kingussie	*Sawadaea bicornis*
OE2016PMCS73	*Aesculus* sp.	11/09/2016	Balavil, Kingussie	*Erysiphe flexuosa*
OE2016PMCS74	*Circaea lutetiana*	16/09/2016	Harestanes, Roxburghshire	*Erysiphe circaeae*
OE2016PMCS75	*Stachys sylvatica*	16/09/2016	Burnmouth. Berwickshire	*Neoerysiphe galeopsidis*
OE2016PMCS76	*Vicia sylvatica*	16/09/2016	Burnmouth. Berwickshire	*Erysiphe pisi*
OE2016PMCS77	*Trifolium campestre*	16/09/2016	Burnmouth. Berwickshire	*Erysiphe pisi*
OE2016PMCS78	*Pisum sativum* 'R'	22/09/2016	Science and Advice for Scottish Agriculture, Edinburgh	*Erysiphe pisi*
OE2016PMCS79	*Pisum sativum* 'R'	22/09/2016	Science and Advice for Scottish Agriculture, Edinburgh	*Erysiphe pisi*
OE2016PMCS80	*Pisum sativum* 'R'	22/09/2016	Science and Advice for Scottish Agriculture, Edinburgh	*Erysiphe pisi*
OE2016PMCS81	*Pisum sativum* 'S'	22/09/2016	Science and Advice for Scottish Agriculture, Edinburgh	*Erysiphe pisi*
OE2016PMCS82	*Pisum sativum* 'S'	22/09/2016	Science and Advice for Scottish Agriculture, Edinburgh	*Erysiphe pisi*
OE2016PMCS83	*Pisum sativum* 'S'	22/09/2016	Science and Advice for Scottish Agriculture, Edinburgh	*Erysiphe pisi*

Appendix 5: Regional GenBank Accessions*

Study accession	GenBank accession				
	ITS	*Mcm7*	β-tubulin	*Tsr1*	Actin
OE2013PM1	KY660725				
OE2013PM2	KY660742				
OE2013PM3	KY660744				
OE2013PM4	KY660747				
OE2013PM5	KY660723				
OE2013PM6	KY660731				
OE2013PM7	KY660727				
OE2013PM8	KY660736				
OE2013PM9					
OE2013PM11	KY660726				
OE2013PM12					
OE2013PM13	KY660729				
OE2013PM14	KY653205				
OE2013PM15	KY660738				
OE2013PM16	KY660735				
OE2013PM17	KY660746				
OE2013PM18	KY660750				
OE2013PM19	KY660739				
OE2013PM21	KY660748				
OE2013PM22	KY660724				
OE2013PM23	KY660741				
OE2013PM24					
OE2013PM25	KY660733				
OE2013PM26	KY660740				
OE2013PM27	KY660749				
OE2013PM28	KY660730				
OE2013PM29	KY653200				
OE2013PM30					
OE2013PM31	KY653161				
OE2013PM32	KY660728				
OE2013PM33	KY660732				
OE2013PM34	KY660737				
OE2013PM35					
OE2013PM36					
OE2013PM37	KY653187				
OE2013PM38	KY660745				
OE2013PM39					
OE2013PM40					
OE2013PM41	KY660722				
OE2013PM42	KY660734				
OE2013PM43	KY660743				

OE2014PM1	KY660798		KY786690		KY786551
OE2014PM2	KY660849		KY786691		KY786552
OE2014PM3	KY660837				KY786553
OE2014PM4	KY660782		KY786692		KY786554
OE2014PM5	KY660769				KY786555
OE2014PM6	KY660816				
OE2014PM7	KY660813				
OE2014PM8			KY786693		
OE2014PM9	KY660770	KY786340			KY786556
OE2014PM10	KY660833	KY786341	KY786694		KY786557
OE2014PM11	KY660817		KY786695		KY786558
OE2014PM12	KY660761	KY786342	KY786696		KY786559
OE2014PM13	KY660754	KY786343	KY786697		KY786560
OE2014PM14	KY660759		KY786698	KY786477	KY786561
OE2014PM15	KY660781				KY786562
OE2014PM16CS	KY660850		KY786699	KY786478	KY786563
OE2014PM17CS	KY660784			KY786479	KY786564
OE2014PM18CS	KY660786				KY786565
OE2014PM19CS	KY660810		KY786700		KY786566
OE2014PM20CS	KY660775				KY786567
OE2014PM21CS	KY660789				KY786568
OE2014PM22CS	KY660790		KY786701		KY786569
OE2014PM23CS	KY660864		KY786702		KY786570
OE2014PM24CS	KY653206		KY786703		KY786571
OE2014PM25	KY660854		KY786704		
OE2014PM26CS	KY660783				KY786572
OE2014PM27CS	KY660826		KY786705		KY786573
OE2014PM28CS	KY660777				KY786574
OE2014PM29CS	KY660844				KY786575
OE2014PM30CS	KY660819		KY786706		KY786576
OE2014PM31	KY660764		KY786707		KY786577
OE2014PM32CS	KY660791		KY786708		KY786578
OE2014PM33CS	KY660846		KY786709		KY786579
OE2014PM34	KY660760		KY786710		KY786580
OE2014PM35					
OE2014PM36	KY660765		KY786711		KY786581
OE2014PM37	KY653192		KY786712		KY786582
OE2014PM38	KY660758		KY786713		KY786583
OE2014PM39	KY660820		KY786714		KY786584
OE2014PM40CS	KY660818		KY786715		KY786585
OE2014PM41CS					
OE2014PM42CS					
OE2014PM43CS	KY653203		KY786716		KY786586
OE2014PM44CS	KY653204		KY786717		KY786587
OE2014PM45					

OE2014PM46	KY660840				KY786588
OE2014PM47CS	KY660815		KY786718		KY786589
OE2014PM48CS	KY660793		KY786719		KY786590
OE2014PM49CS					KY786591
OE2014PM50CS					
OE2014PM51CS					
OE2014PM52	KY660792		KY786720		KY786592
OE2014PM53CS	KY660801		KY786721		KY786593
OE2014PM54CS	KY660772		KY786722		KY786594
OE2014PM55CS	KY660811		KY786723		KY786595
OE2014PM56CS	KY660799				KY786596
OE2014PM57CS	KY660807		KY786724		KY786597
OE2014PM58CS					KY786598
OE2014PM59CS	KY653211		KY786725		KY786599
OE2014PM60CS	KY660762				KY786600
OE2014PM61CS					
OE2014PM62CS	KY660794		KY786726		KY786601
OE2014PM63	KY660800		KY786727		KY786602
OE2014PM64CS					KY786603
OE2014PM65CS	KY660830		KY786728		KY786604
OE2014PM66CS					
OE2014PM67CS	KY660763		KY786729		KY786605
OE2014PM68CS	KY660767		KY786730		KY786606
OE2014PM69CS	KY660842		KY786731		
OE2014PM70CS	KY660851		KY786732		KY786607
OE2014PM71CS	KY660862		KY786733		KY786608
OE2014PM72CS	KY660751				KY786609
OE2014PM73CS	KY660803		KY786734		KY786610
OE2014PM74CS	KY660860		KY786735		KY786611
OE2014PM75CS					
OE2014PM76CS	KY660858		KY786736		KY786612
OE2014PM77CS	KY660841		KY786737		KY786613
OE2014PM78CS	KY660834				KY786614
OE2014PM79CS	KY660797		KY786738		KY786615
OE2014PM80CS					KY786616
OE2014PM81CS	KY660773				KY786617
OE2014PM82CS	KY660857		KY786739		KY786618
OE2014PM83CS	KY660755				KY786619
OE2014PM84CS	KY660757		KY786740		KY786620
OE2014PM85CS	KY660828		KY786741		KY786621
OE2014PM86CS	KY660847		KY786742		KY786622
OE2014PM87CS	KY660788				
OE2014PM88CS	KY660805		KY786743		KY786623
OE2014PM89CS	KY660796		KY786744		KY786624
OE2014PM90CS	KY653193		KY786745		KY786625

OE2014PM91CS	KY660806				KY786626
OE2014PM92CS	KY660848		KY786746		KY786627
OE2014PM93CS	KY653184		KY786747		KY786628
OE2014PM94CS	KY660836				KY786629
OE2014PM95CS	KY660756		KY786748		KY786630
OE2014PM96CS			KY786749		KY786631
OE2014PM97CS	KY660856				KY786632
OE2014PM98CS	KY660814		KY786750		KY786633
OE2014PM99CS	KY660843		KY786751		KY786634
OE2014PM100CS					KY786635
OE2014PM101CS	KY660808				KY786636
OE2014PM102CS	KY660825		KY786752		KY786637
OE2014PM103CS	KY660753		KY786753		KY786638
OE2014PM104CS	KY660779				KY786639
OE2014PM105CS	KY660780				KY786640
OE2014PM106CS	KY653188		KY786754		KY786641
OE2014PM107CS	KY653189		KY786755		KY786642
OE2014PM108CS	KY660795				KY786643
OE2014PM109	KY653197		KY786756		KY786644
OE2014PM110CS	KY660855		KY786757		KY786645
OE2014PM111CS	KY660839				KY786646
OE2014PM112CS	KY660752		KY786758		KY786647
OE2014PM113CS	KY660774				KY786648
OE2014PM114CS	KY660845				KY786649
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OE2016PMCS83					

*Shaded cells have no sequence data.