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Understanding the drivers of emergence of *Curreya pithyophila* and associated impacts on Caledonian pine

Project Final Report

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Forest Research

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Content

1 Executive summary

1.1 Background and objectives

This project followed a previous project 'PHC2022/07: Understanding a new health threat to Caledonian Scots pine (*Pinus sylvestris*)' caused by a new and unusually widespread infestation of a previously rare stroma-forming fungus *Curreya pithyophila* (syn. *Cucurbidothis pithyophila*). There are two morphologically and genetically distinct forms of this fungus which are most easily differentiated by spore type (phragmospore and dictyospore). Both forms are infesting shoots and branches of Scots pine across Scotland in a symbiotic association with the native Scots pine adelgid, *Pineus pini*. Affected Scots pine also exhibits abundant blackened branch cankers resulting from secondary infections by the native pine pathogen *Crumenulopsis sororia*. The primary cause of these cankers appears to be *P. pini* in association with the two forms of *C. pithyophila*, with feeding wounds subsequently colonised by *C. sororia*. This project investigated the potential drivers of emergence of *C. pithyophila* and its impact on Caledonian Scots pine with the following specific objectives:

- Investigate the genetics and breeding system of phragmospore and dictyospore specimens of *C. pithyophila* from Britain.
- Compare the morphology of British specimens of *C. pithyophila* to global herbarium specimens collected from Pinaceae hosts.
- Determine the incidence and impact of *C. pithyophila* and cankers in two native pine populations in eastern and western Scotland.
- Investigate whether host genetic factors influence the responses of Caledonian Scots pine to *C. pithyophila* and cankers.
- Examine whether *C. pithyophila* develops on Scots pine in the absence of *P. pini*.

1.2 Research undertaken

Reference genomes based on long read (PacBio) sequencing were assembled for a phragmospore isolate and a dictyospore isolate of *C. pithyophila* from Scotland and gene content analysed. Illumina assemblies were produced for 20 phragmospore and 20 dictyospore isolates from Britain. Analysis of single nucleotide polymorphisms (SNPs) was undertaken to investigate genetic diversity and population structure in *C. pithyophila*. Vegetative compatibility tests were also carried out to determine whether *C. pithyophila* exhibits a vegetative self × nonself recognition system and to shed further light on the ecology and gene flow of phragmospore and dictyospore populations. A total of 48 herbarium specimens comprising both forms of *C. pithyophila* were examined from *Abies, Larix, Picea* and *Pinus* hosts across continental Europe, Asia and North America. Morphological assessments of these specimens were conducted in comparison with British specimens.

Field surveys were carried out at two native Caledonian Scots pine populations, Glen Affric in the west and Rothiemurchus in the east, to determine the incidence and impact of *C. pithyophila* and cankers. Within each population, 50 young trees and 50 mature trees were surveyed for crown dieback, presence of *C. pithyophila,* presence of cankers and percentage visible disease on the six lowest live branches. Cankers were dated using dendrochronological methods. To investigate the influence of host genetic factors, the same variables were surveyed at three Caledonian Scots pine common garden trials located in the east, west and south of

Scotland. Inoculation experiments were carried out to determine whether both forms of *C. pithyophila* can infect Scots pine in the absence of *P. pini*.

1.3 Main findings

Significant functional gene divergence between dictyospore and phragmospore forms of *C. pithyophila* was revealed. Both forms of *C. pithyophila* contain ligninolytic genes not yet found in an ascomycete species. Both forms are heterothallic (outcrossing) requiring two different mating type strains for sexual reproduction. Almost all isolates of *C. pithyophila* were incompatible with each other regardless of whether they were the same mating type or not. This suggests that sexual reproduction occurs at a high frequency within each population. The fungus was unable to grow in the tissues of Scots pine without the presence of *P. pini*.

There was significant divergence between dictyospore and phragmospore isolates in SNP analyses providing further evidence for their separation into distinct species. There was greater divergence among dictyospore isolates from Britain than phragmospore isolates, with dictyospore isolates from south-west Scotland separating from other Scottish isolates. This suggests that the dictyospore form has been present in Britain for longer than the phragmospore form. The presence of genetically similar strains in widely differing geographical locations suggests that introductions have occurred through non-natural pathways, such as planting. Ascospore sizes in British specimens of *C. pithyophila* most closely matched specimens from Scots pine in continental Europe, although there was no consistent variation in spore size based on either host or region.

Incidence of *C. pithyophila* was higher in Caledonian pine at Glen Affric than at Rothiemurchus which may reflect the wetter site conditions at Glen Affric. The low overall incidence of this fungus and the high incidence of blackened cankers typical of secondary *C. sororia* infections in both pine populations suggests higher levels of past infections. This is because native Scots pine growing on good sites are not typically susceptible to *C. sororia*. Most cankers sampled within these two populations dated back to the mid-1990s onwards indicating that disease may have started earlier here than at planted sites in Scotland. Surveys of Caledonian pine common garden trials revealed some heritability in susceptibility to *C. pithyophila* potentially allowing future adaptation to increased, climate-driven prevalence of *C. pithyophila/P. pini* infestations. Incidence of cankers may be more affected by site conditions than genetic factors.

1.4 Recommendations

- Plant only healthy stock which is visibly free of adelgids or fungal infection and pay particular scrutiny to any Pinaceae hosts imported from overseas.
- Plant Scots pine only on sites deemed suitable for this species.
- Monitor Scots pine plantings for signs of *C. pithyophila* infestations and report any findings to Forest Research via Tree Alert Tree Alert (forestresearch.gov.uk).
- Report any *C. pithyophila* stromata observed on other Pinaceae hosts in Scotland.
- Surveys for *C. pithyophila* on Scots pine should be conducted in England and Wales.

1.5 Next steps

Further exploration of the *C. pithyophila* genome data will shed more light on the differences between phragmospore and dictyospore forms of *C. pithyophila* and their respective

evolutionary processes, allowing a better understanding of whether one, or both forms, are likely to have been recent introductions. Similarly, the application of molecular clock analyses to SNP data may reveal the length of time each population has been evolving for in Britain. Data from the common garden trials should be analysed further to test for local adaption of source populations at the environmentally contrasting trial sites.

2 Introduction

A new canker disease of Scots pine (*Pinus sylvestris*) formed the focus of a previous project 'PHC2022/07: Understanding a new health threat to Caledonian Scots pine (*Pinus sylvestris*)'. This disease is due to a new and unusually widespread infestation of two distinct forms of a previously rare stroma-forming fungus currently assigned to *Curreya pithyophila* (syn. *Cucurbidothis pithyophila*) (Green et al. 2024b). The two forms of this fungus are most easily differentiated by spore type (phragmosporous and dictyosporous). Both forms are infesting shoots and branches of Scots pine across Scotland in an apparently symbiotic association with the native Scots pine adelgid, *Pineus pini*. Affected Scots pine also exhibits abundant blackened shoot and branch cankers from which the native pine pathogen *Crumenulopsis sororia* has been isolated (Green et al. 2024b). The primary cause of these cankers appears to be *P. pini* in association with the two forms of *C. pithyophila*, with feeding wounds subsequently colonised by the canker-causing pathogen, *C. sororia*.

This project followed the outcomes of Green et al. (2024b) to gain a better understanding of what is driving this newly widespread disease on one of Scotland's most iconic tree species. The specific objectives are outlined below:

- Further investigate the genetics and breeding system of phragmospore and dictyospore specimens of *C. pithyophila* from Britain to assess whether one or both forms represent recent introductions into the country and are thereby driving the outbreak.
- Compare the morphology of British specimens of *C. pithyophila* to herbarium specimens collected from Pinaceae hosts in Europe, North America and Asia.
- Carry out field surveys of Caledonian Scots pine within two native pine populations sites of differing rainfall levels; Glen Affric in the west and Rothiemurchus in the east, to determine the extent and impact of disease on young and old trees.
- Investigate whether genetic factors such as provenance of origin and /or family influence Caledonian Scots pine susceptibility to disease through surveys of three common garden trials located in Scotland.
- Undertake inoculation studies to determine whether *C. pithyophila* can grow within tissues of Scots pine in the absence of *P. pini*.

3 Methods

3.1 Investigate the genetic diversity, mating system and evolutionary history of British specimens of C. pithyophila

3.1.1 Whole genome sequencing

With funding from the Defra-funded GAP DC2 project, reference genomes were assembled by Edinburgh Genomics and NS Bio Limited for one phragmospore isolate (18.1) and one

dictyospore isolate (19.1) of *C. pithyophila* following long read (PacBio Hifi) sequencing (Edinburgh Genomics). A further 20 isolates of each phragmospore and dictyospore form were sequenced using Illumina (Edinburgh Genomics). Isolates were collected from a range of geographical locations in Scotland (see Green et al. 2024b), with the exception of a single phragmospore isolate from Devon, England.

Genomic analyses were carried out by NS Bio Limited. The 18.1 and 19.1 reference genomes were annotated using Funannotate v1.8.15 (Palmer and Stajich, 2020) and Augustus (Stanke et al. 2008). Functions were added to gene predictions using InterProScan5 (Jones et al. 2014) and EggNOG v6.0 (Hernández-Plaza et al. 2023) alongside further functional annotation with CAZy, MEROPS and PFAM domains. Illumina reads were assembled for each of the 40 isolates, mating type genes identified, and, following initial filtering, SNP (single nucleotide polymorphisms) analyses were performed both with each isolate's respective reference genome and a separate all-to-one analysis with the 19.1 reference genome (being the larger of the two). Principal Component Analyses were performed on independent bi-allelic SNP sites present in all isolates.

3.1.2 Vegetative compatibility tests

Vegetative compatibility tests were carried out to determine whether *C. pithyophila* exhibits a vegetative self × nonself recognition system analogous to other ascomycete species and to shed further light on the ecology and gene flow of phragmospore and dictyospore populations.

Pairing tests were carried out by Douglass (2024) between i) three phragmospore isolates and three dictyospore isolates, ii) six sibling isolates (i.e. from the same ascoma) obtained from each of two phragmospore specimens and two dictyospore specimens, and iii) seven dictyospore isolates and five phragmospore isolates from different specimens representing different geographical locations and ITS or TEF1-α sequence variants (Green et al. 2024b). In short, 2 mm diameter plugs of mycelium were taken from the growing margin of colonies derived from single ascospore isolates and placed 10 mm apart in the centre of malt agar plates, two replicate plates per pairing. Plates were placed at 15 °C in the dark for 12-14 weeks for visual analysis of vegetative compatibility. Each test was repeated once (Douglass, 2024).

3.2 Compare British specimens of C. pithyophila with specimens collected from Abies, Larix, Picea and Pinus hosts in Europe, North America and Asia

3.2.1 Background and investigations into culture collection isolates

For taxonomic clarity, the fungus that is currently known as *Curreya pithyophila* had been assigned to the genus *Cucurbidothis* in 1921 but was (incorrectly) reassigned to the genus *Curreya* in 1975 (Green et al. 2024b). Therefore, most historic records refer to this fungus as '*Cucurbidothis pithyophila*' and a taxonomic paper has been published which moves this fungus back into the correct genus *Cucurbidothis* (Taylor et al., 2026).

Prior to the current outbreak, *C. pithyophila* had rarely been collected in Britain with only nine previous records in the UK's Fungal Conservation Database (CATE2) and the Fungal Records Database of Britain and Ireland (FRDBI), with 12 records in the Global Biodiversity Information Facility (GBIF). Across the Northern Hemisphere, there are 277 observations and voucher specimens listed in GBIF (accessed 04 September 2025). These records are from assumed Pinaceae hosts (the data are not given but random checked records were all

Pinaceae) in Europe (138 records plus one record for Morocco), Asia (8 records) and North America (57 records) (note that 74 records lack country information), and include the following genera: *Abies, Larix, Picea* and *Pinus*. Of the 277 records, only 182 have the year of collection provided. In recent years there have been many records from Switzerland (47 of the 54 from 2010 to present day). All North American records are from 1911 to 1968 and represent 54 of the 67 records of *C. pithyophila* in that period. It is difficult to make any inferences due to missing data, but these provide potential evidence for the occurrence of previous outbreaks similar to the current outbreak in Scotland. However, note also that these records only include digitised data, with potentially more records of observations and voucher specimens not yet digitally recorded.

There has been an assumption that all the records represent one species and in most cases no distinction has been made between the two known spore forms, dictyospore and phragmospore. A recent report based on specimens collected in Britain has confirmed that the two spore forms are in fact two species (Green *et al.*, 2024b). The same authors hypothesised that one, or both, of these fungi may represent relatively recent introductions into Britain and proposed that a study of global herbarium specimens of *C. pithyophila* on different conifer hosts could provide significant insights into where the British specimens are likely to have evolved.

Given the widespread records of *C. pithyophila* in the Northern Hemisphere and the wide host range (requiring the fungus to associate with different host-associated adelgids), it is possible that the two species represented by the different spore forms might in fact both be species complexes, comprising multiple cryptic species. To investigate the extent of the two spore forms and their morphological similarity with British specimens, herbarium specimens were obtained from different global geographic locations for the four Pinaceae genera.

Strains listed in various international culture collections as *C. pithyophila* were also ordered as part of this project, plated onto agar media, their DNA extracted, and ITS sequences obtained for comparison with British isolates. The outcome was that all the strains had been incorrectly identified, and none were *C. pithyophila*. The strain ordered from CBS (CBS955.68, listed as *Curreya pityophila* from *Pinus cembrae* in Switzerland) was in fact *Sarea difformis* (*Sareales*), a pine inhabiting fungus. A second strain was obtained (MAFF no. 410060 named *Cucurbidothis pityophila*) but its DNA matched *Devriesia* and *Extremus* in the *Mycosphaereales*. Other strains with sequences in GenBank named as *Curreya pithyophila* (IARI-RPF-1 [KF530860, KF530856], clone G-jav1-LSU1_OTU-0-043_307 [MF337705]) did not match isolates from Britain and are also considered incorrectly identified. In addition, strain CBS149.32 has been sequenced previously and is shown to be a member of the *Didymosphaeriaceae* (Valenzuela-Lopez *et al.* 2018), so it is incorrectly identified as is CBS986.69, UTHSC:DI16-357 also phylogenetically in the *Didymosphaeriaceae*.

3.2.2 Examination and processing of herbarium specimens

Records of preserved voucher specimens of *C. pithyophila* on GBIF were examined to identify a selection from different tree hosts and different continents, and additionally some non-databased specimens were available too. Eleven specimens were requested from various herbaria by staff at Royal Botanic Gardens Edinburgh (RBGE) and after a week in the freezer were held in the herbarium. The specimens included type material of both the dictyospore and

phragmospore forms. A large, non-databased collection of around 37 specimens was also sent from ETH Zurich (ZT). In total 48 specimens were received (Supplementary Table 1).

Morphological examinations were made of all the specimens, and all were photographed. All specimens were sampled for DNA extraction for phylogenetic studies funded elsewhere, unless they were too scant or had been misidentified. Extreme care was taken to sample from only one stroma per specimen (as both spore forms can occur in stroma on the same host branch for instance) and, in the case where a specimen was composed of multiple pieces of material, the exact stroma from which DNA samples were taken was labelled with a jeweller's tag. Finally, more detailed examinations were made of a representative selection of 15 herbaria specimens as well as the British phragmospore and dictyospore specimens (Appendix 1). Measurements of 30 ascospores and/or conidia and 20 ascomata were made. Varying numbers of measurements were made of other structures depending on availability. Measurements in italics are the mean values (= $\bar{x}$); otherwise ranges are given (Appendix 1).

3.3 Surveys of Caledonian Scots pine at Glen Affric and Rothiemurchus to determine disease impacts and likely time frame of infections

3.3.1 Site surveys and statistical analyses

This study investigated *C. pithyophila/P. pini* infestation and cankers in young and mature trees of native Caledonian Scots pine within two populations with differing rainfall histories. These were Glen Affric located to the west of Scotland's 'Great Glen' (latitude 57.246952, longitude -5.0562599) and Rothiemurchus, located further to the east in the Cairngorm region (latitude, 57.148195, longitude -3.7965605). Although both sites have oceanic climates, Glen Affric has a higher long term average rainfall for the period 1991-2020 (1359 mm/yr) compared with Rothiemurchus (985 mm/yr) (Location-specific long-term averages - Met Office).

Each site was pre-surveyed using aerial imagery and five areas selected for assessment based on accessibility and having a mix of young and mature trees (e.g. Fig. 1a). Rothiemurchus was surveyed in June 2024 and Glen Affric in July 2024. Within each of the five areas per site, ten young trees (up to 4 m height) and ten mature trees (e.g. 'granny' pine of large girth) were assessed for; i) stem diameter (DBH) at 1·3 m above ground, ii) approximate height (m), iii) percentage crown dieback, iv) presence/absence of *C. pithyophila,* v) presence/absence of blackened cankers typical of *C. sororia* infection (Green et al. 2024b), and vi) the percentage area covered by *C. pithyophila* and/or cankers (referred to hereafter as 'visible disease') on the six lowest living branches. If some, or all, of the lowest six living branches could not be visibly assessed due to being out of physical reach, then these were treated as missing values.

R (v. 4.1.2) was used for data manipulation and statistical modelling. Graphics were produced using 'ggplot2' (Wickham, 2016). Differences in percentage crown dieback, presence/absence of *C. pithyophila* and presence/absence of cankers in association with biotic and abiotic factors were analysed using generalised linear mixed models from the 'lme4' package (Bates et al, 2015). Crown dieback was analysed using a binomial distribution. Mean percentage cover of visible disease was calculated from the scores across the six branches. Site and tree size were analysed as two-level factors, and presence/absence of *C. pithyophila* and cankers as binary variables. Separate analyses were run for each response variable and factor, and their interactions. All models included plot nested within site as a random effect.

To test the association between presence/absence of *C. pithyophila* and presence/absence of cankers, a Pearson's Chi-squared test with Yates' continuity correction was used (R Core Team, 2024). To explore the relationship between mean percentage cover of visible disease and tree size and site, Wilcoxon rank sum tests were used (Kassambara, 2023). To explore the relationship between mean percentage cover of visible disease and tree size, a new variable, lower branch average, was calculated due to missing data by averaging the percentage cover of visible disease from the two branches per tree.

3.3.2 Dendrochronological analyses

Branches containing the largest, and theoretically oldest, blackened cankers typical of *C. sororia* infection (e.g. Fig. 1b) were sampled, each sample from a different tree, for dendrochronological dating. Branches bearing cankers were cut into cross-sections of approximately 2–3 cm using a handsaw and a knife. The samples were then prepared using a microtome to obtain thin micro-sections between 15 and 25 µm, ensuring sufficient clarity for the precise identification of tree-ring boundaries. High-resolution images of the micro-sections were captured using a microscope (4×). These images were subsequently analysed using CooRecorder software, where tree-ring widths were systematically measured and dated.

The timing of cambial death was reconstructed by dating the outermost ring of each branch-ring series. However, due to the occurrence of partial cambial mortality, the date of the outermost tree ring varied between trees and across different portions of branches. Tree-ring dating was conducted from the bark inwards, with the youngest ring designated as the outermost and the oldest located near the pith. To align with the sample collection date, tree-ring zero was assigned to the year 2024.

Figure 1 – (a) Caledonian pine at Rothiemurchus surveyed in June 2024, illustrating young and mature trees assessed in the study, and (b) blackened canker typical of Crumenulopsis sororia *infection collected from Caledonian pine for dendrochronological analysis.*

3.4 Survey of C. pithyophila *and cankers in three Caledonian Scots pine provenance/progeny trials*

3.4.1 Survey details

This survey was conducted to test whether genetically inherited factors such as source population, and/or family influence the response of Caledonian Scots pine to *C. pithyophila/ P. pini* infestation and subsequent canker development caused by secondary *C. sororia*

infection. The survey involved three Caledonian Scots pine common garden trials planted in Scotland in 2012 at Glensaugh (east; latitude 56.893567, longitude −2.535736), Inverewe (west; latitude 57.775714, longitude −5.597181) and Yair (south; latitude 55.603625, longitude −2.893025). Mean annual rainfall (1991-2020) for Inverbervie (the closest weather station to Glensaugh) is 703 mm/yr, for Aultbea (the closest weather station to Inverewe) is 1448 mm/yr, and for Galashiels (the closest weather station to Yair) is 833 mm/yr (Location-specific long-term averages - Met Office). The Inverewe trial is also within 1 km of the coast and planted on a forestry restock site. Each trial comprised 3-4 replicate half-sib offspring from eight mother trees selected from each of 21 source populations of Caledonian Scots pine representing seven seed zones in Scotland. Details of provenance locations, selection of mother trees, seed collection and propagation, field site and planting information including experimental design (randomised block) are presented in Beaton et al. (2022).

The Yair trial was surveyed in September 2024 and the Glensaugh and Inverewe (Fig. 2a) trials were surveyed in October 2024. Every tree in each trial was assessed for; i) presence/absence of *C. pithyophila* (e.g. Fig. 2b,c), ii) presence/absence of blackened cankers typical of *C. sororia* infection (e.g. Fig. 1b), and iii) the percentage area covered by *C. pithyophila* and/or cankers ('visible disease') on the six lowest living branches.

3.4.2 Statistical analyses

All analyses were performed in R v. 4.4.2 (R Core Team, 2024). Data visualisations were created using *ggplot2* (Wickham, 2016). Generalised linear mixed models (GLMM) were used to partition variance in disease traits, using Markov Chain Monte Carlo methods via the 'MCMCglmm' package (Hadfield, 2010). Traits were analysed separately in univariate models. Binary disease traits (incidence of *C. pithyophila* and cankers) were analysed using threshold models, and visible disease (expressed as a mean percentage of visible disease across the lower six branches of each tree) was analysed using a binomial model with a logit link. All models comprised an intercept, with block and family (nested within source population) included as random effects. MCMCglmm models ran for 220,000 iterations, with a burn-in of 2000 iterations and a thinning interval of 100, giving an effective sample size of around 2000 iterations to estimate posterior distributions of the random effects. Parameter expanded non-informative priors were used for all models with residual variance fixed to 1 for binary data (de Villemereuil et al., 2013; de Villemereuil, 2018).

Narrow-sense heritability (the total phenotypic variability explained by additive genetic effects) was estimated on the latent scale as follows:

$$h^2 = \frac{V_A}{V_P} = \frac{RV_{family}}{V_{family} + V_{forest} + V_{block} + V_{residual}}$$

where V_A represents additive genetic variance and V_P represents total phenotypic variance. For threshold models, residual variance was fixed to 1 (de Villemereuil, 2013) and for binomial logit models, residual variance was set to $\boldsymbol{\pi}^2/3$ (Nakagawa & Schielzeth, 2010). Heritability estimates were converted from the latent scale to the observed scale using the *QGglmm* package (de Villemereuil et al. 2016). For each trait, three different relatedness scenarios were considered where families were assumed to be comprised of half-siblings (R = 4), full siblings (R = 2) or 50% full- and 50% half-siblings (R = 3) (Perry et al. 2016). Narrow-sense heritability

estimates are presented as summary statistics of the posterior distribution (mean, median, mode and 95% credible interval).

3.5 *Inoculation trial to determine whether* C. pithyophila *develops on Scots pine in the absence of* P. pini

In this study, 6 mm diameter mycelial plugs were removed from the margins of colonies of one phragmospore isolate and one dictyospore isolate of *C. pithyophila* actively growing on malt agar and inoculated onto young Scots pine without the presence of *P. pini*. This simple experiment was carried out to determine whether the fungus can invade host tissues in the absence of the adelgid.

Each plant was inoculated in three places; i) at the mid-point on the main stem, ii) at the mid-point of a side shoot and iii) at the tip of a different side shoot. Three wounding treatments were each applied to four replicate plants at the three inoculation points per plant. The wounding treatments were; i) no wounding, ii) shallow wounding whereby the area was marked with a cork borer and wounded with needle several times and iii) deep wounding whereby the outer bark was pierced with a 6 mm diameter cork borer and removed using a scalpel. Thus, a total of twelve plants were inoculated with each isolate and twelve plants inoculated with sterile malt agar plugs as negative controls. The inoculation points were wrapped in damp cotton wool following placement of a droplet of distilled water onto the inoculum plug, then sealed with parafilm and tin foil. The plants were incubated in a greenhouse (Fig. 2d) at 15 °C for six weeks, before being examined for the presence of lesions or stromata.

Figure 2 – (a) Caledonian pine common garden trial at Inverewe surveyed in October 2024, (b and c) Curreya pithyophila *on Caledonian pine at the Inverewe trial in October 2024, (d) Scots pine inoculated with* C. pithyophila *and incubating in a greenhouse.*

4 Results

4.1 Investigate the genetic diversity, mating system and evolutionary history of British specimens of C. pithyophila

4.1.1 Whole genome sequencing

The 18.1 (phragmospore) and 19.1 (dictyospore) reference genomes were assembled to high levels of completeness (94.4 % and 94.7 % complete, respectively). The 18.1 genome consists of ~ 69 million base pairs (bp) assembled into 21 contigs containing 9,345 genes. The 19.1 genome is larger; 70.5 million bp assembled into 17 contigs containing 10,806 genes. A Non-metric Multidimensional Scaling plot generated from the InterPro gene function data positioned each isolate on opposite quadrants of the plot (data not shown), indicating significant functional gene divergence between dictyospore and phragmospore forms.

The InterProScan results revealed genes putatively associated with lignin degradation. Proteins annotated as fungal ligninases were found in both forms but are slightly more abundant in the 19.1 dictyospore genome (3 occurrences) compared to the 18.1 phragmospore genome (2 occurrences). Other genes with multiple occurrences but which differ between the two forms also have a putative role in lignin degradation and include those encoding the following enzymes; glucose-methanol-choline (GMC) oxidoreductases, cellobiose dehydrogenase, galactose oxidases, glyoxal oxidase, multicopper oxidases, quinone reductase, manganese superoxide dismutase, monooxygenases and phenol hydroxylase. The phragmospore and dictyospore genomes also differed in relation to self/non-self-recognition, controlled by a suite of heterokaryon incompatibility genes (*het* genes). InterProScan annotation revealed 96 genes with the *het* domain in the 19.1 dictyospore genome and 57 genes with the *het* domain in the 18.1 phragmospore genome.

Each of the 40 isolates with Illumina assemblies were found to contain either the *MAT1-1-1* gene (mating type 1 or Mat1) or the *MAT1-1-2* gene (mating type 2 or Mat2), but not both. This proves that *C. pithyophila* is heterothallic (outcrossing). Mating type segregated 1:1, with eleven phragmospore isolates of mating type 1 and nine of mating type 2, and ten dictyospore isolates per mating type.

A total of 147,363 core SNPs were identified in the phragmospore population (aligned to reference genome 18.1) and 178,461 core SNPs were identified in the dictyospore population (aligned to reference genome 19.1). The largest number of SNPs in any pairwise comparison was 37,424 for phragmospore isolates and 87,679 for dictyospore isolates. PCA of core biallelic SNP data filtered for independence (based on being more than 50,000 bp between SNPs) showed significant divergence between dictyospore and phragmospore isolates with PCA1 explaining 95% of all the variation observed among isolates and PCA2 explaining just 1% of the variance (Fig. 3a). All phragmospore isolates cluster very closely while dictyospore isolates are more diverged (Fig. 3a). Sibling isolates (from the same ascoma) cluster closely as expected. PCA of dictyospore isolates show four isolates from SW Scotland which cluster separately from other dictyospore isolates with PCA1 and PCA2 explaining only 16% and 13% of the variance, respectively (Fig. 3b). For phragmospore isolates geographical origin of collection did not explain isolate position in the PCA plots (Fig. 3c). Here, PCA1 and PCA2 explained only 11% and 8% of the variance, respectively. The only phragmospore isolate from England (Devon) clustered most closely to an isolate from south Scotland (Fig. 3c).

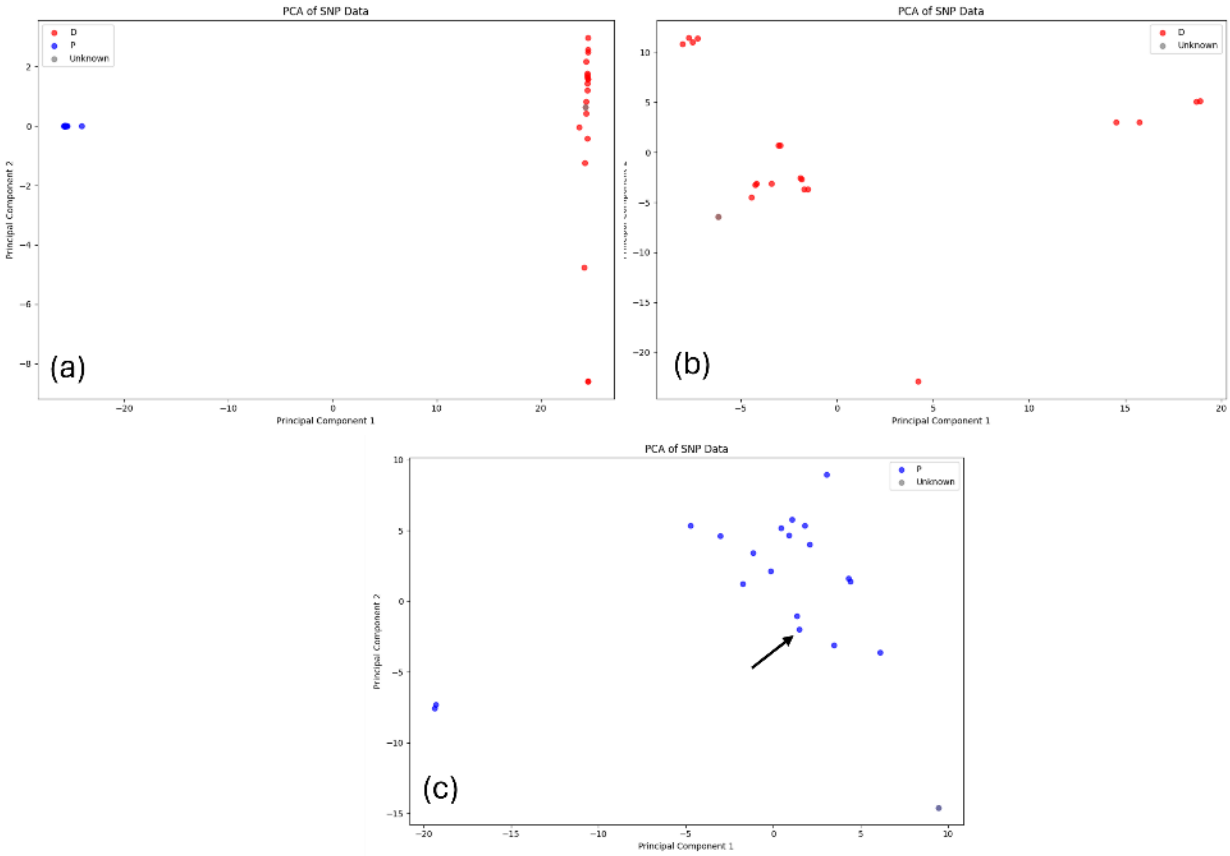


Figure 3 – Principal component analyses of SNP data from twenty dictyospore (D) isolates (red dots) and twenty phragmospore (P) isolates (blue dots) of Curreya pithyophila *from Scots pine in Britain. (a) Dictyospore isolates are more divergent than phragmospore isolates but the two forms are most divergent from each other. (b) Divergence among dictyospore isolates showing a cluster of four isolates from SW Scotland to the right of the plot. (c) Divergence among phragmospore isolates with isolate from Devon (arrow) clustering with isolates from Scotland.*

4.1.2 Vegetative compatibility tests

Distinct compatible and incompatible reactions were observed between paired isolates. The reaction was regarded as incompatible when growth of colonies stopped upon meeting, often with a dark reaction line visible at the junction when viewed from below (e.g. Fig. 4a), or an aerial hyphal barrage when viewed from above (e.g. Fig. 4b,f,h,j), and the two colonies remained distinct. A reaction was regarded as compatible when colonies merged fully or partially along the junction line and could not be distinguished as separate when viewed from below (e.g. Fig. 4c) or above (Fig. 4d,e,g,i) (Douglass, 2024).

All self x self pairings produced reactions that were generally regarded as compatible with colonies merging across most or all of the junction line (e.g. Fig. 4c,d). There was clear and consistent incompatibility between all dictyospore x phragmospore pairings (e.g. Fig. 4b); the two forms are confirmed as genetically incompatible. Of the thirty combinations of dictyospore sibling pairings from the two specimens, only one pairing was regarded as compatible (Fig. 4e). All pairings of dictyospore isolates from different specimens were incompatible (e.g. Fig. 4f) Phragmospore sibling compatibility differed between the two specimens. For one of the specimens, all sibling pairings were compatible (e.g. Fig. 4g). For the other specimen, two of the sibling isolates were incompatible with each other and with all other four siblings (e.g. Fig 4h) whereas the remaining four siblings were compatible with each

other (e.g. Fig. 4i). All pairings of phragmospore isolates from different specimens were incompatible.

Seven of the phragmospore isolates and eleven of the dictyospore isolates used in these tests subsequently had their mating type confirmed through genome sequencing. All pairings of isolates of known mating type were incompatible, regardless of whether they were Mat1 vs Mat2 pairings (e.g. Fig. 4j), Mat1 vs Mat1 or Mat2 vs Mat2.

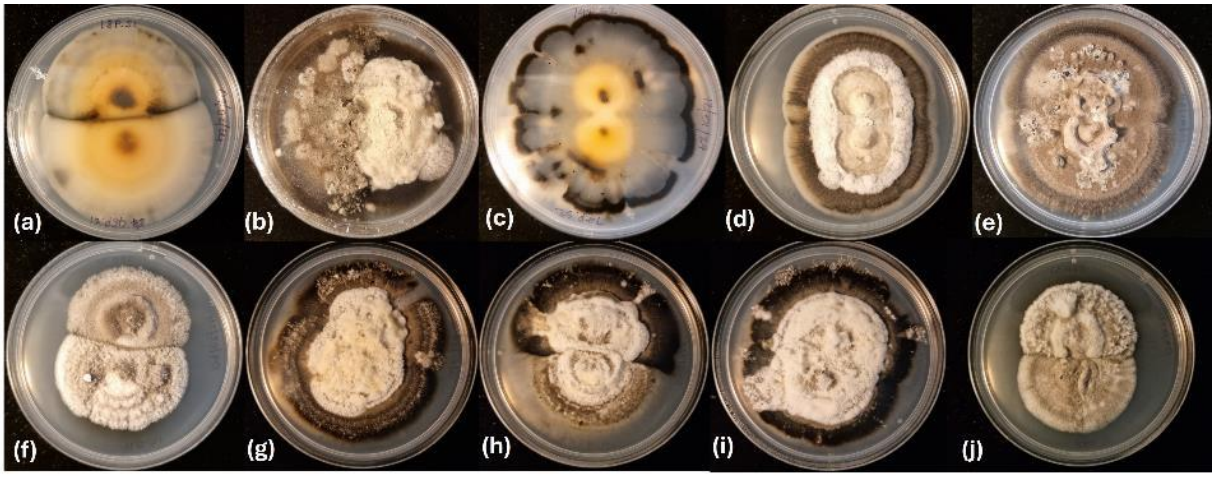


Figure 4 - Vegetative compatibility tests between paired isolates of Curreya pithyophila *on malt agar after 12-14 weeks incubation. (a) Phragmospore isolates 18 and 45 from different geographical locations showing an incompatible reaction with a dark reaction line when viewed from below. (b) Incompatible reaction between phragmospore isolate 18 and dictyospore isolate 19, both from the same geographical location, with aerial barrage along the junction line. (c) Compatible self x self reaction with phragmospore isolate 74.S2 viewed from below. (d) Compatible self x self reaction with phragmospore isolate 48. (e) Compatible reaction observed between sibling dictyospore isolates 52.S1 and 52.S5. (f) Incompatible reaction between dictyospore isolates 71 and 73 from different locations. (g) Compatible reaction between sibling phragmospore isolates 52.S2 and 52.S4. (h) Incompatible reaction between sibling phragmospore isolates 74.S1 and 74.S4. (i) Compatible reaction between sibling phragmospore isolates 74.S3 and 74.S5. (j) Incompatible reaction between dictyospore isolate 25 of mating type 2 and dictyospore isolate 53.S1 of mating type 1.*

4.2 Compare UK specimens of C. pithyophila with specimens collected from Pinus, Picea and Abies hosts in Europe, North America and Asia

4.2.1 Morphological analyses

Morphological examination of all 48 received herbarium specimens revealed a mixture of spore forms in the different geographic locations and on the different hosts (Table 1; Supplementary Table 1). However, it should be noted that other than *Pinus* and *Picea* in Europe, all records are represented by a single or only two specimens. The selected specimens for which detailed morphological examinations were carried out are given in Appendix 1. Ascospore mean length and width data for both phragmospores and dictyospores across hosts and regions, in comparison with British specimens, are shown in Fig. 5a and b.

Table 1 - Summary of the occurrence of each C. pithyophila *spore form (D = dictyospore, P = phragmospore, A = anamorph) by tree species and continental distribution for 48 received herbaria specimens. For further information on morphology for a subsample of specimens see Appendix 1.*

Host	**Asia**	**Britain**	**Continental Europe**	**North America**
Abies alba			P	
Abies concolor				D
Abies pindrow	PA			
Abies veitchii	D			
Larix decidua			P	
Picea abies		P*	D, A?	
Picea excelsa			D, A?	
Picea sitchensis				D
Pinus cembrae			D, DA, P, PA	
Pinus pumila	D			
Pinus strobus				D
Pinus sylvestris		D, DA, P, PA	D, P, PA	

* = host identified as 'spruce fir', assumed here to be *Picea abies*

? = anamorph identity (e.g. P or D-form) could not be confirmed

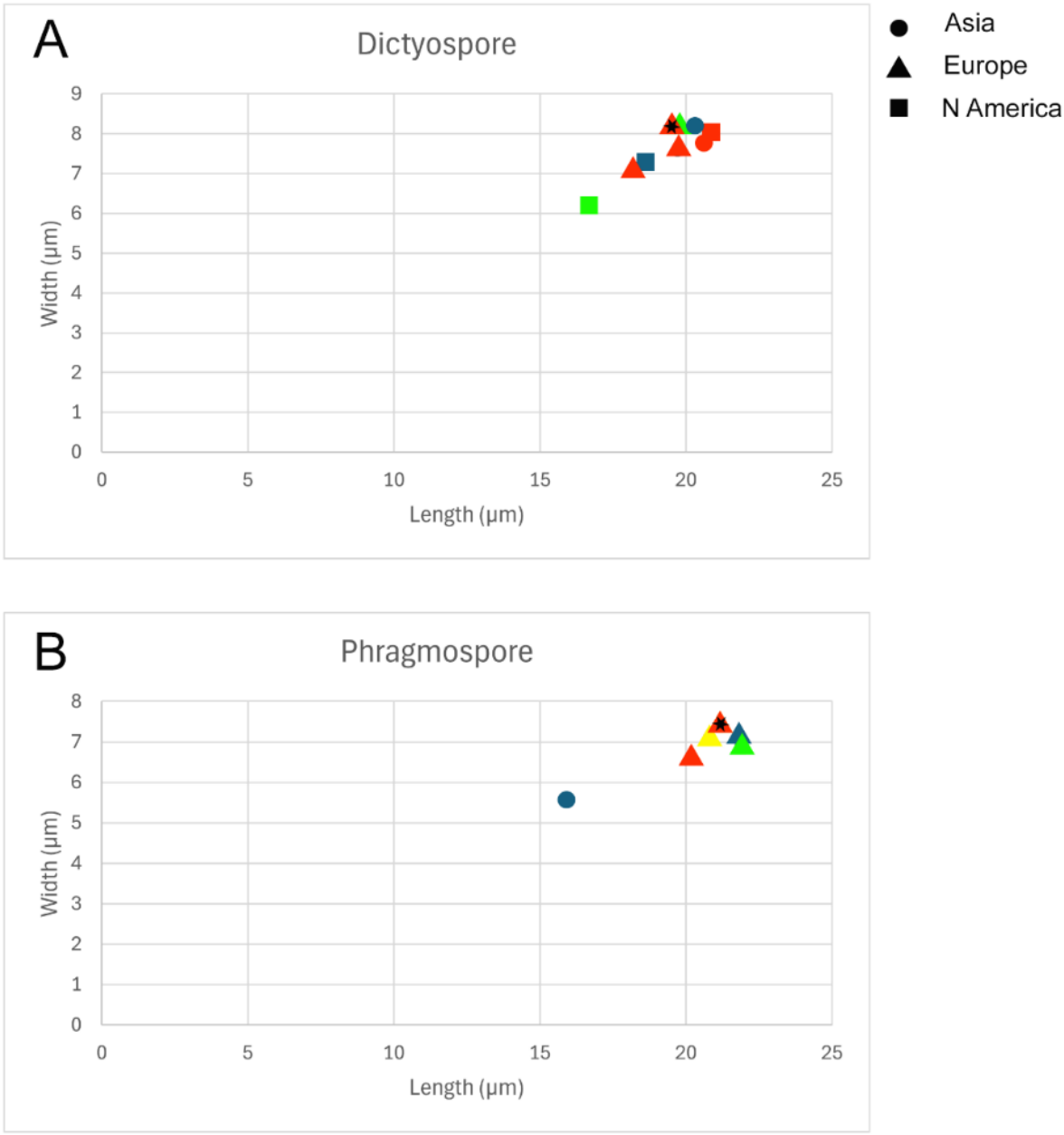


Figure 5 – Mean ascospore lengths and widths of selected specimens of C. pithyophila *across hosts and regions for dictyospores (A) and phragmospores (B). British specimens are indicated with a black star. For further information see Appendix 1. Symbol colours are as follows: red* = Pinus*; blue* = Abies*; green* = Picea*; yellow* = Larix.

Illustrated in Fig. 6 are stromata and ascospores of specimens showing the taxonomic 'type' of each species (Fig. 6B,G), representatives of the samples from Britain (Fig. 6A,F), several representatives where the stroma had an unusual morphology (Fig. 6C,D), and two samples where the ascospores were particularly small (Fig. 6E,H); plus the only sample from *Larix* is shown (Fig. 6I). There was considerable variation in the morphology of the stroma, varying from encircling small twigs, often near branch junctions, to occurring on much larger branches often with quite flaky, thin bark. For several specimens there was a distinctive pattern, either radiating (Fig. 6D) or reticulate (Fig. 6C).

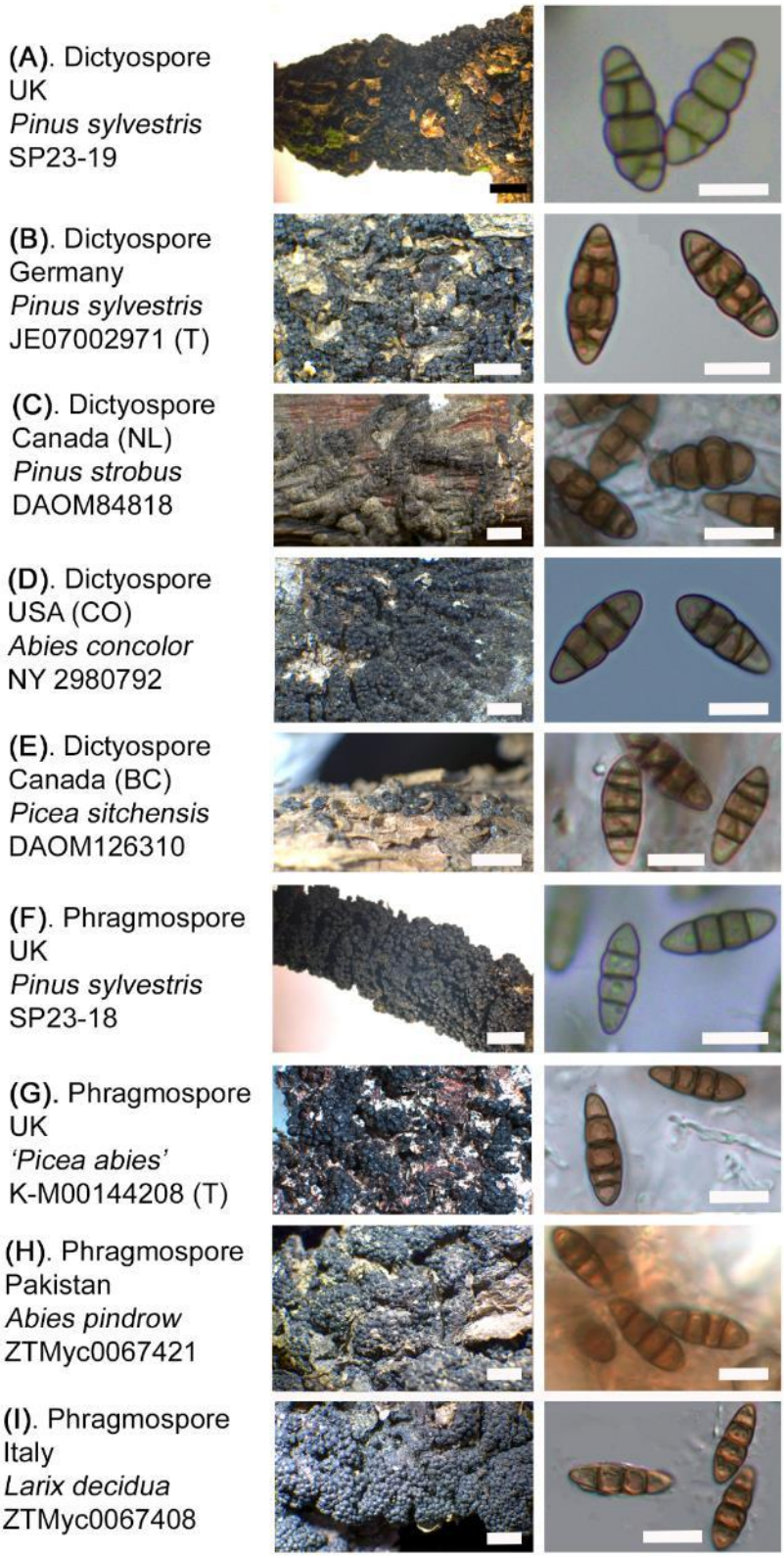


Figure 6 - A comparison of the morphology of the stroma and ascospores of herbarium specimens of C. pithyophila *from different Pinaceae hosts and regions. For further information see Appendix 2.*

4.3 Surveys of Caledonian Scots pine at Glen Affric and Rothiemurchus to determine disease impacts and likely time frame of infections

4.3.1 Site surveys and statistical analyses

At Rothiemurchus, 10 of the 50 young trees had *C. pithyophila* but the fungus was not observed on any of the 50 mature trees. At Glen Affric, 7 of the 50 young trees and 24 of the 50 mature trees had *C. pithyophila*. At Rothiemurchus, 48/50 young trees and 40/50 mature trees had cankers, and at Glen Affric 47/50 young trees and 42/50 mature trees had cankers. Mean percentage crown dieback was 24.7 % in young trees and 19.6% in mature trees at Rothiemurchus, and 23.5% in young trees and 31.1% in mature trees at Glen Affric. Figure 7 presents the combined data for trees of both sizes at each site.

Almost all trees surveyed had cankers and there was no significant association between presence of *C. pithyophila* and presence of cankers (ChiSq 2.3, $P = 0.13$) but presence of *C. pithyophila* did have a significant effect (ChiSq 64, $P < 0.001$) on mean percentage crown dieback across both sites and tree sizes. Estimated overall mean crown dieback was 29.7% in trees with *C. pithyophila* and 22.8% dieback in trees without the fungus. There was no significant effect of site on percentage crown dieback (ChiSq 1.08, $P = 0.3$) and no significant interaction between presence/absence of *C. pithyophila* and site (ChiSq 2.72, $P = 0.1$) for percentage crown dieback.

Presence of cankers was not significantly associated with tree size across both sites (ChiSq 2.65, $P = 0.1$) as 92.1% of young trees and 96.9% of mature trees had cankers. However, presence of *C. pithyophila* was significantly associated with tree size across both sites (ChiSq 6.23, $P = 0.01$) with *C. pithyophila* observed on 8. 6% of young trees and 20.7% of mature trees. Mean percentage cover of visible disease on the lowest six branches per tree had a significant effect on overall percentage crown dieback (ChiSq 41.41, $P < 0.001$) with the trend shown in Fig. 8.

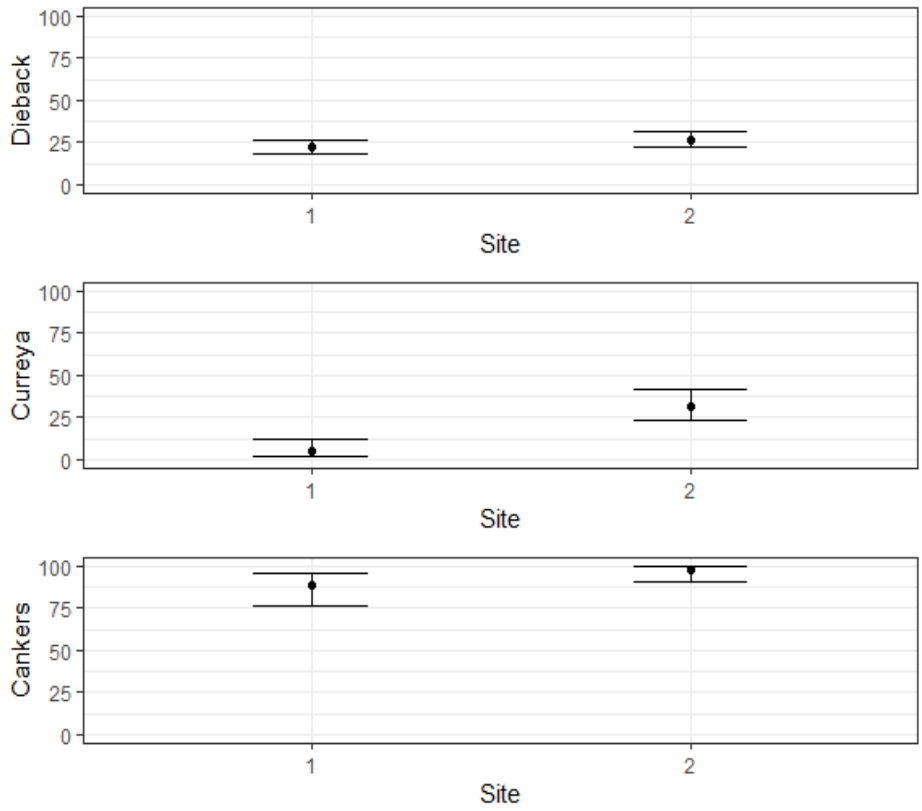


Figure 7 - Overall mean percentage crown dieback, total instances of Curreya pithyophila *and total instances of cankers on Scots pine at Rothiemurchus (site 1) and Glen Affric (site 2). Data for each site combine young and mature trees. Error bars represent 95 % confidence intervals.*

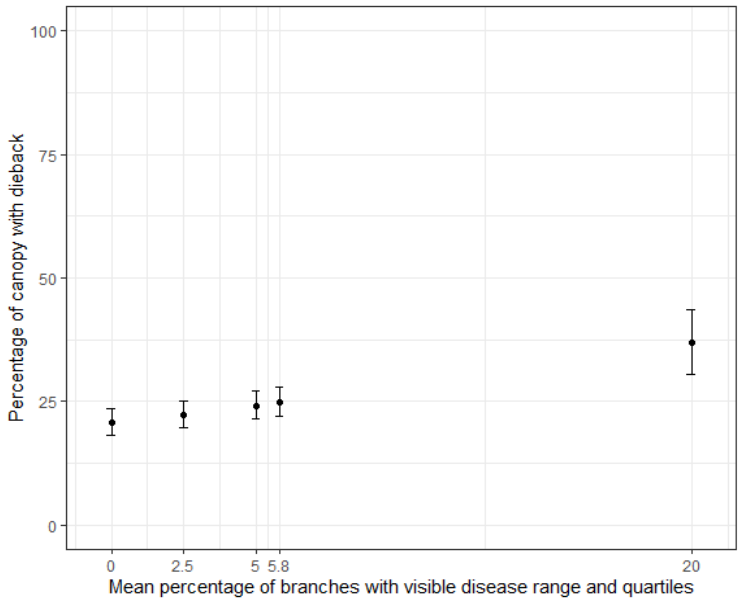


*Figure 8 - Estimated mean percentage crown dieback against mean percentage cover of visible disease (*Curreya pithyophila *and/or cankers) range and quartiles on the six lowest live branches of young and mature Scots pine trees at Rothiemurchus and Glen Affric. Error bars represent 95 % confidence intervals.*

Mean percentage cover of visible disease averaged over six branches per tree was significantly associated with tree size (ChiSq 8.8, $P = 0.005$) and site (ChiSq 12.21, $P = <0.001$) but not with the interaction between tree size and site (ChiSq 0.01, $P = 0.93$). Estimated means for this variable were 2.7% for young trees and 4.2% in mature trees at Rothiemurchus, and 4.5% in young trees and 7.2% in mature trees at Glen Affric.

4.3.2 Dendrochronological analyses

Cankers were found to have black staining in the xylem which is a diagnostic feature of *C. sororia* infection (Vuorinen, 2000). Three cankers were sampled at Rothiemurchus. One from a young tree showed that the cambium was killed in 2013, and the two cankers from mature trees showed cambial death years in 1998 and 2001 (Fig. 9). At Glen Affric, all six cankers were collected from mature trees and cambial death years ranged from 1968 to 2014 (Fig. 9).

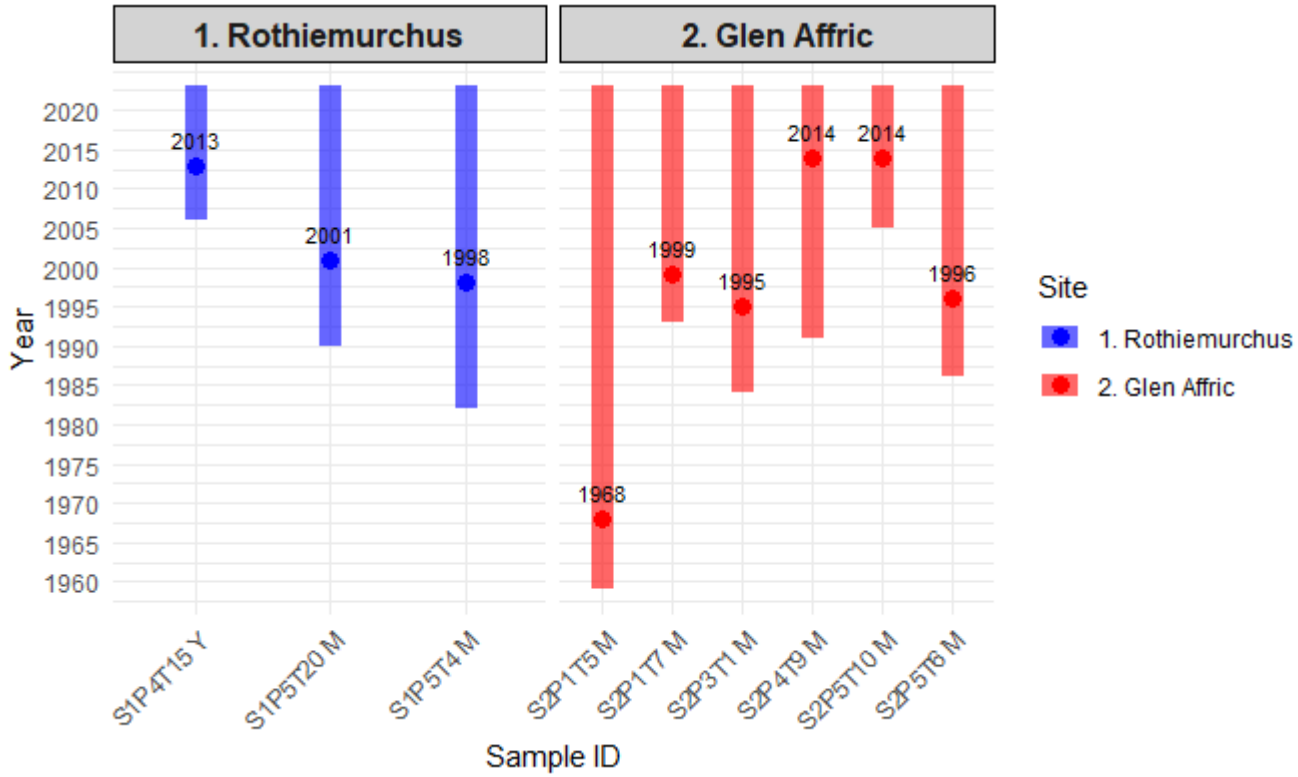
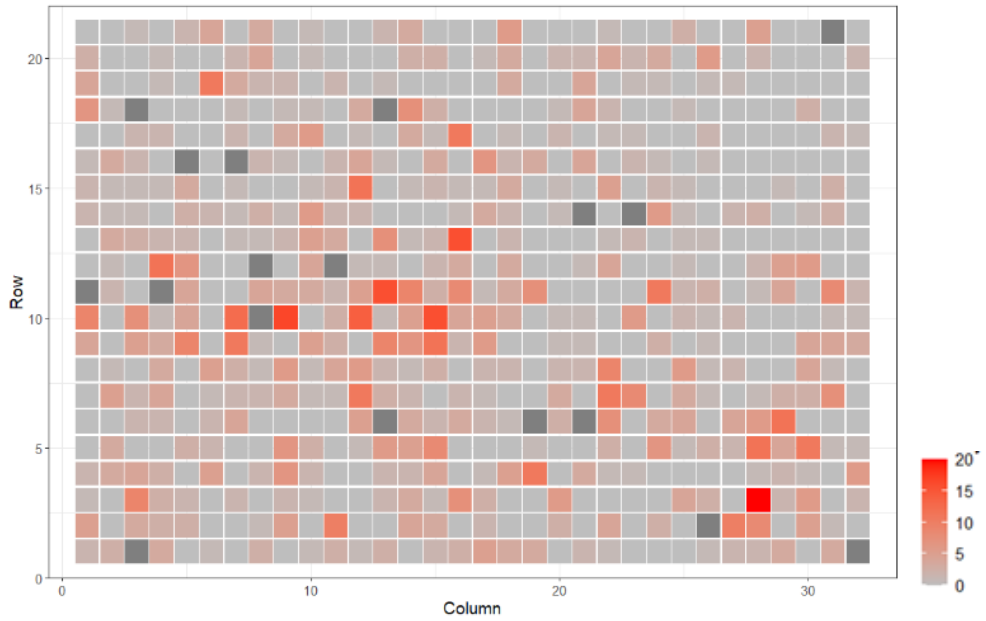


Figure 9 - Dendrochronological analyses of cankers on Scots pine at two sites, (1) Rothiemurchus and (2) Glen Affric. Bars show the age of each branch at sampling and the red dots and associated dates indicate the year of cambial death.

4.4 Survey of C. pithyophila *and cankers in three Caledonian pine provenance/progeny trials*

Of the 654 trees surveyed at the Glensaugh trial, 370 had *C. pithyophila* and 63 had cankers with 38 trees having both *C. pithyophila* and cankers. Of the 452 trees surveyed at the Inverewe trial, 247 had *C. pithyophila* and 388 had cankers with 215 trees having both *C. pithyophila* and cankers. Severity of visible disease was generally greater at the Inverewe trial than at Glensaugh (Fig. 10a,b). Only eight trees were found to have *C. pithyophila* at the Yair trial and this trial was excluded from any further analysis.

(a)



(b)

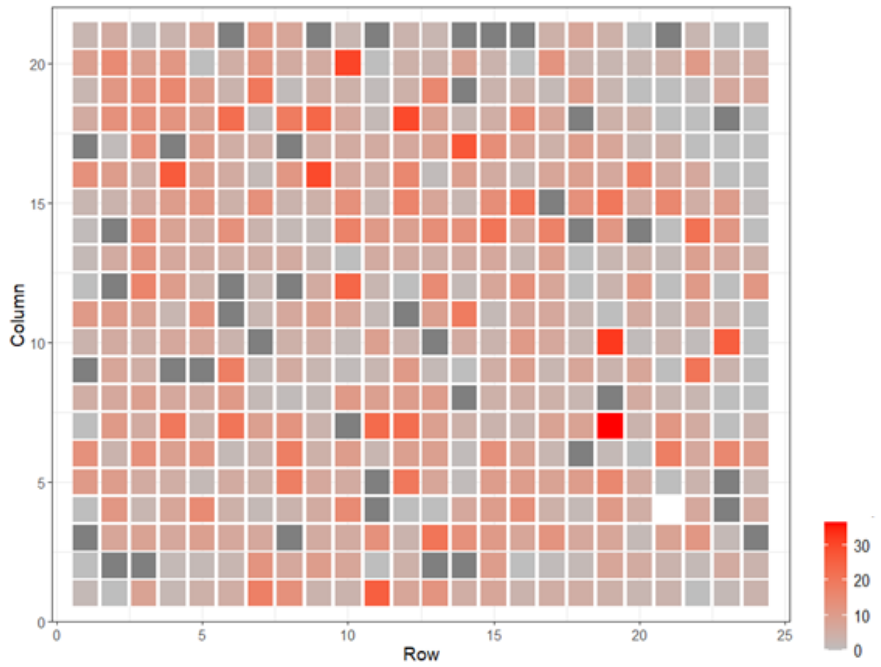


*Figure 10 – Heat maps with legend showing the mean percentage of visible disease (*Curreya pithyophila *and/or cankers) on the six lowest live branches of Caledonian Scots pine planted out in two common garden trials, Glensaugh (a) and Inverewe (b). Each square represents an individual tree in its planted position (figures produced by A. Perry).*

The random effect variances used to estimate narrow-sense heritability are presented in Appendix 2. The posterior means are highest for the effect of block for all three traits (incidence of *C. pithyophila,* incidence of cankers and mean percent visible disease) at both the Glensaugh and Inverewe trials but the credible intervals for the block effect are very wide. Generally, family explained more of the variance than source population (forest) for all three traits in both trials (Appendix 2).

Table 2 shows the mean, median and mode narrow sense heritability estimates (h^2) for both trials. To explain the scale, a heritability estimate of close to zero indicates that almost all of the variability in that trait is due to environmental factors whereas a heritability estimate of 1

means that genetic factors account for all variability in that trait. The median heritability estimate is used to explain the genetic contribution to each trait rather than the mean as it is less likely to be skewed due to outliers. The median h^2 of the proportion of variation due to family was highest for instance of cankers at the Glensaugh trial and instance of *C. pithyophila* at the Inverewe trial (Table 2).

Heritability estimates for instance of *C. pithyophila* were similar for both trials, but heritability estimates for cankers were lower for the Inverewe trial than for Glensaugh (Table 2). At both trials, mean percentage visible disease was the trait with the lowest heritability estimates. The 95% credible intervals for the posterior distribution are large for most of these heritability estimates, particularly when families are assumed to comprise half-siblings compared with the full-sibling relatedness scenario (Table 2).

Table 2 - Posterior distribution of heritability (h^2) *estimates for instance of* Curreya pithyophila*, instance of cankers and mean percentage visible disease on Caledonian Scots pine planted out in two common garden trials, Glensaugh and Inverewe. Estimates are based on assumptions that families comprise half-siblings* (R = *4), full siblings* (R = *2) or an equal mixture of full and half siblings* (R = *3). Columns show the mean, median, mode and 95% credible intervals (CI) for the posterior distribution in each case. All estimates are presented on the original scale following transformation from the latent scale using the QGglmm package. A heritability estimate of close to zero indicates that almost all of the variability in that trait is due to environmental factors whereas a heritability estimate of 1 means that genetic factors account for all variability in that trait.*

	Trait	Relatedness scenario	Mean h^2	Median h^2	Mode h^2	Lower CI	Upper CI
Glensaugh Trial	*C. pithyophila*	*R* = 4	0.191	0.175	0.004	<0.001	0.431
		R = 3	0.143	0.131	0.003	<0.001	0.323
		R = 2	0.095	0.088	0.002	<0.001	0.215
	Cankers	*R* = 4	0.206	0.188	0.002	<0.001	0.459
		R = 3	0.155	0.141	0.002	<0.001	0.344
		R = 2	0.103	0.094	0.001	<0.001	0.230
	% visible disease	*R* = 4	0.056	0.052	0.001	<0.001	0.119
		R = 3	0.042	0.039	0.001	<0.001	0.090
		R = 2	0.028	0.026	0.001	<0.001	0.060
Inverewe Trial	*C. pithyophila*	*R* = 4	0.229	0.202	0.003	<0.001	0.547
		R = 3	0.171	0.152	0.002	<0.001	0.410
		R = 2	0.114	0.101	0.001	<0.001	0.273
	Cankers	*R* = 4	0.090	0.052	0.001	<0.001	0.308
		R = 3	0.067	0.039	0.001	<0.001	0.231
		R = 2	0.045	0.026	0.001	<0.001	0.154
	% visible disease	*R* = 4	0.024	0.016	<0.001	<0.001	0.074
		R = 3	0.018	0.012	<0.001	<0.001	0.056
		R = 2	0.012	0.008	<0.001	<0.001	0.037

4.5 *Inoculation trial to determine whether* C. pithyophila *develops on Scots pine in the absence of* P. pini

Six weeks after inoculation there was no lesion or stromata development in any treatments, indicating that *C. pithyophila* cannot develop within the bark tissues of Scots pine in the absence of *P. pini.*

5 Discussion

Genomic analyses and vegetative compatibility tests support the separation of phragmospore and dictyospore forms of *C. pithyophila* as different species as proposed by Green et al. (2024b), with divergent gene content suggesting separate evolutionary pathways.

Genome sequencing revealed that phragmospore and dictyospore forms of *C. pithyophila* are heterothallic, requiring two separate mating type strains for sexual reproduction to occur, and that both mating types occur at equal ratio. Vegetative compatibility studies also showed that phragmospore and dictyospore forms of *C. pithyophila* have a clear self/non-self recognition system as is common in ascomycete fungi (Leslie, 1993; Saupe, 2000). Vegetative compatibility is the successful fusion of hyphae from two non-self individuals, involving the development of a joint heterokaryotic (containing two or more genetically different nuclei) mycelium (Saupe, 2000). Vegetative incompatibility works to maintain separation of different genotypes within a species by limiting hyphal fusion to prevent horizontal transfer of degraded or infectious cytoplasmic elements such as mycoviruses from one individual to another (Clavé et al. 2024). Heretokaryons are not commonly formed in ascomycetes due to vegetative incompatibility, which is expressed mainly during vegetative growth (Glass and Kaneko, 2003).

Whereas sexual compatibility is controlled by the mating type genes, vegetative compatibility is controlled by a highly polymorphic set of *het* genes (Saupe, 2000; Paoletti and Clavé, 2007; Clavé et al. 2024). Ascomycete fungi are generally known to contain around ten *het* genes (Paoletti, 2016) but the number of annotated *het* domains in fungal genomes can be much higher. For example, 124 genes containing the *het* domain were found in the invasive fungal pathogen *Cryphonectia parasitica*, which causes chestnut blight, yet vegetative compatibility in this fungus is thought to be controlled at only six or seven loci (Crouch et al. 2020). Thus, only a small proportion of the 96 *het* domains in the dictyospore genome and the 57 *het* domains in the phragmospore genome are likely to represent functional *het* genes. A difference at any one of the functional *het* genes is enough to trigger vegetative incompatibility and subsequent death of fused hyphal cells (Saupe, 2000; Paoletti and Clavé, 2007; Clavé et al. 2024). However, while vegetative incompatibility is expressed during vegetative growth it does not necessarily impede sexual reproduction even in heterothallic strains with *het* loci differences (Glass and Kaneko, 2003).

The fact that almost all confrontations between isolates of *C. pithyophila* resulted in incompatibility regardless of mating type suggests that the vegetative portion of each individual stroma may consist of a single genotype. Sexual reproduction and formation of perithecia occur when spores of opposite mating type germinate and undergo fusion with hyphae in the stroma. The high rate of vegetative incompatibility suggests a high frequency of sexual recombination in *C. pithyophila* and reliance on sexually produced ascospores for dissemination rather than asexually produced conidia, as inferred in vegetative compatibility

studies of the ash dieback pathogen *Hymenoscyphus fraxineus* (Brasier and Webber, 2013).

The observation that most pairings between sibling phragmospore isolates were compatible and all except one pairing of dictyospore sibling isolates were incompatible may reflect a greater number of functional *het* genes in the dictyospore genome. Further analyses of the *het* domains in *C. pithyophila* are required to confirm how many are truly functional and to elucidate the variation in *het* loci within each phragmospore and dictyospore population. Advanced understanding of *het* genes in fungal populations may help shed light on their evolutionary processes (Clavé et al. 2024). If *het* genes are highly diverged between dictyospores and phragmospores, it further strengthens the case for their having evolved in geographical or ecological isolation from each other.

SNP analyses revealed very large divergence between dictyospore and phragmospore isolates of *C. pithyophila* which further confirms their separation into distinct species. The greater divergence among dictyospore isolates compared with phragmospore isolates suggests that the phragmospore form may have been evolving over a shorter time scale in Britain. The presence of a SW Scotland cluster of dictyospore isolates and the lack of any geographical clustering of phragmospore isolates would support this hypothesis. However, molecular clock analyses are required to understand better the evolutionary timescales of each sampled population. SNP analyses also suggest that *C. pithyophila* disperses naturally across regions where Scots pine is common and contiguous, probably driven by wind and rain (Green et al. 2024a), with a more distinct dictyospore population in SW Scotland which is geographically isolated from Scots pine populations to the north and east. However, SNP analyses involving a much larger number of isolates are required to be able to confirm this hypothesis as PCA plots were unable to explain much of the variance within each sampled population.

PCA of SNP data showed that the phragmospore isolate from Devon, which had been found on Scots pine planted in the early 2000s, was most similar to a phragmospore isolate from the Scottish Borders, found on Scots pine that had been planted in the 1980s. To date, there have been no other findings of *C. pithyophila* in England and Wales, and although Scots pine has not been intensively surveyed for the fungus in these countries, the lack of a contiguous population of Scots pine between Scotland and Devon strongly suggests either the presence an alternative Pinaceae host, or a non-natural pathway of spread such as the planting of infested material from a similar source.

The survey of herbarium specimens of *C. pithyophila* revealed that both dictyospore and phragmospore forms have been found on *Abies, Picea* and *Pinus* genera with just a single phragmospore specimen from Europe available for *Larix*. Both dictyospore and phragmospore forms have been found on *Pinus sylvestris* in Britain and in continental Europe and on *Pinus cembrae* in continental Europe. All specimens from known hosts of *Picea* are of the dictyospore form. The phragmospore type specimen was collected from a host recorded at the time as 'spruce fir' in Wales in 1876 (Green et al. 2024b); however, there is some ambiguity as to whether the 'spruce fir' is in fact *Picea abies* or not. Both dictyospore and phragmospore forms occur in Asia, Britain and continental Europe. All North American specimens representing *Abies, Picea* and *Pinus* genera were of the dictyospore form (including a specimen observed by Ariyawansa *et al.* (2014), (UBC-F3787), however, no inferences can be made as to whether the lack of phragmospores in North America is significant given the small sample size.

Overall, there was no consistent variation in *C. pithyophila* spore size based on either host or region. Some specimens showed considerably smaller spores for both spore forms, suggestive of different species. These were; ZTMyc0067421 (phragmospore form on *Abies pindrow* from Pakistan, 16 × 5.5 µm compared to 21 × 7.5 µm for the British SP23-18) and DAOM126310 (dictyospore form on *Picea sitchensis* from British Columbia (BC) Canada, 16.5 × 6 µm compared to 19.5 × 8 µm for the British SP23-19). However, it might also reflect other variables such as age of the ascomata sampled and maturity of the spores. The most recent treatment of *C. pithyophila* recorded dictyospore sizes of 15–19 × 3–6 µm (mean = 16 × 5 µm, n = 40), 'on dead wood of *Pinus monticola*' (Ariyawansa *et al.*, 2014) recorded in 1950 in BC, Canada (UBC-F3787). Comparable dictyospore dimensions were also recorded in North America (BC, Canada) but from *Picea sitchensis* as noted above. UBC-F3787 is referred to as a paratype by Ariyawansa *et al.* (2014) but there is no reference to this in the data associated with the specimen (Golinski & Pitblado, 2024) or to the collection being 'dead wood' on the label data.

Mean ascospore sizes for both dictyospore and phragmospore forms from *P. sylvestris* in Britain were closest to those from *P. sylvestris* in Europe, although specimens from all four hosts in Europe have similar sized phragmospores to the British specimens. There are few specimens available from outside Europe that produce the phragmospore forms and it remains to be seen if this reflects the actual distribution. For dictyospores, specimens with similar sized spores to the British specimens occur from a wider geographic range (Europe, Asia and North America) on *Abies, Picea* and *Pinus*. In addition to the Canadian specimen, specimens from Colorado in America (NY 2980792 on *Abies concolor*; 18.5 × 7.5 µm) and from Europe (ZTMyc 0067413 on *Pinus cembrae*; 18 × 7 µm) produced somewhat smaller dictyospores compared to the British specimens. The stroma was conspicuously different in several dictyospore specimens. For example, the specimen from *A. concolor* in Colorado, America, produced large stromata with radiating ascomata, and the sample NY 2980792 from *Pinus strobus* in Newfoundland, Canada produced stroma in narrow reticulate lines. Despite the difference in stroma morphology of the latter, the ascospore dimensions were similar to the British specimens. Genetic analyses of herbarium specimens in comparison with British are currently being undertaken in a separate study to determine the most likely origin of the two British forms.

The annotation of genes coding for lignin peroxidases in *C. pithyophila* may be a novel finding as these genes have not previously been reported in an ascomycete species (e.g. Janusz et al. 2017; Mathé et al. 2019). Ligninases are typically found in basidiomycete fungi and catalyse the first step in lignin degradation (Fungal ligninase (IPR001621) - InterPro entry - InterPro). Both phragmospore and dictyospore forms of *C. pithyophila* also contain most other known fungal gene families with putative roles in lignin degradation as reviewed by Espagne et al. (2008) and Janusz et al. (2017). This almost certainly reflects the unique lifestyle of *C. pithyophila,* which does not penetrate Scots pine below the outer bark layers but is highly effective at separating and pushing up the layers of outer bark to allow *P. pini* direct access to the phloem tissue for feeding (Green et al. 2024b). Scots pine bark contains about 45% lignin (Valentín et al. 2010), and so it appears that bark colonisation by *C. pithyophila* is not a purely physical process but is facilitated by enzymatic activity. It was surmised that the fungus utilises the honeydew from *P. pini* and even possibly dead adelgids for nutrition (Green et al. 2024b). In addition to allowing ingress through the outer bark layers, enzymatic degradation of lignin may serve as a food source for *C. pithyophila* although, as our study showed, it is unable to live in tissues of Scots pine in the absence of the adelgid.

Surveys of two natural Caledonian Scots pine populations, Glen Affric in the west and Rothiemurchus in the east, revealed a low incidence of *C. pithyophila* compared with a 2023 survey of planted Scots pine at six sites in the west of Scotland (Green et al. 2024a). The greater incidence of *C. pithyophila* at Glen Affric compared with Rothiemurchus, particularly on mature trees, may be linked to higher rainfall at this site which will favour spread of the fungus (Green et al. 2024a). Although trees with *C. pithyophila* tended to have higher percentage crown dieback, the overall crown dieback scores were low indicating that this fungus, and its association with *P. pini,* appears to be causing little damage at these native pine sites when considered alone. What is most notable about these surveys, however, is that almost all trees had disfiguring blackened cankers typical of *C. sororia* infection, regardless of site or size, and that the percentage of visible disease, including both *C. pithyophila* stromata and blackened cankers, was greater in mature trees and linked to higher crown dieback scores.

Most of the trees surveyed at Glen Affric and Rothiemurchus were growing on dry, heather-dominated heathland regarded as suitable for the species. The prevalence of typical *C. sororia* cankers on otherwise thriving trees appears to be a recent phenomenon. Although *C. sororia* is a longstanding associate of Scots pine throughout its natural range in Scotland (Ennos and McConnell, 2002), it has historically been regarded as a weak wound parasite (Ennos and Swales, 1987) of no economic importance (Batko and Pawsey, 1964) with infection generally associated with poor host vigour (Batko and Pawsey, 1964). In Scotland, stressed trees growing on bogs are regarded as particularly susceptible and although cankers have occurred historically on low hanging branches of mature Caledonian pine on good sites, young, vigorously growing trees have not typically been affected (R. Ennos, personal communication). Given the prevalence of cankers within these two surveyed populations, it is likely that *C. pithyophila/P. pini* populations have dropped from previously higher levels with stromata dying and flaking off when the branches are no longer suitable feeding habitat for *P. pini.*

The earliest canker on the sampled branches was dated back to 1968 at Glen Affric, however, most cankers examined from both populations were initiated from the mid-1990s onwards. In a similar survey conducted in 2023 of planted Scots pine (P1990s and later) at six sites in the west of Scotland, the oldest cankers were dated back to the late 2000s (Green et al. 2024a). Although other insect species and abiotic factors may cause bark wounds, the feeding wounds left by *P. pini* associated with *C. pithyophila* (Green et al. 2024b) were assumed to have been the conduit for *C. sororia* in most cankers dated given their location at branch and shoot junctions where *C. pithyophila* stromata typically develop (Green et al. 2024a). Based on these assumptions, it was surmised that the current widespread outbreak of *C. pithyophila/P. pini* started during the 2000s (Green et al. 2024a). The current study suggests that *C. pithyophila* may have been present earlier in these native Caledonian pine populations which is very feasible given the reported outbreak of this fungus and adelgid in planted Scots pine in the Moray firth area during the 1960s (Murray and Parry, 1969). The dating of cankers as far back as 1968 on still-living branches confirms the slow, perennating nature of canker development.

In analyses of the Caledonian Scots pine common garden trials, family explained more of the observed variation than source population for all three traits (incidence of *C. pithyophila,* incidence of cankers and mean percentage visible disease). The high block effects for variance and wide credible intervals for most heritability estimates can best be explained by the low sample size, as there were only three to four individuals representing each family. Recent

genotyping of the same Caledonian pine common garden trials has revealed that most trees within families are half-sibs (A. Perry, personal communication). Therefore, the estimates of narrow-sense heritability discussed below are for the half-sib relatedness scenarios. Heritability estimates for incidence of *C. pithyophila* (median 0.18 for the Glensaugh trial and 0.20 for the Inverewe trial) are somewhat lower than the 0.38 estimate for *Dothistroma septosporum* needle blight (DNB) severity in artificially inoculated Caledonian Scots pine families from the same source populations (Perry et al. 2016). Moderate levels of heritability (0.28–0.48) were reported for three needle pathogens (DNB, *Cyclaneusma minus* and *Phytophthora pluvialis*) following assessments of natural infection in four *Pinus radiata* common garden trials in New Zealand (Ismael et al. 2020). The fact that heritability estimates were similar for the two Scottish trials, which are exposed to widely differing rainfall and site conditions, suggests that a proportion of the variation in *C. pithyophila* incidence among genotypes of Caledonian pine may indeed be attributable to host genetic factors.

The Glensaugh trial had a median heritability estimate of 0.19 for incidence of cankers which were observed on less than 10% of the trees in the trial. The heritability estimate for canker incidence at the Inverewe trial, at which 86% of the trees were affected, was lower (0.05). The blackened cankers scored at each trial were typical of those caused by *C. sororia* infection and the heritability estimates for this trait may reflect susceptibility to this (weak) pathogen. The differences in canker incidence between the two trials might be due to higher rainfall and generally less suitable site conditions for Scots pine at Inverewe being more conducive to *C. sororia* infection and canker development, meaning that environmental factors override genetic factors on poorer sites. Certainly, the overall levels of percentage visible disease were also greater at Inverewe. Another factor to consider is that *Hylobius* feeding damage was observed on some trees at the Inverewe trial which is located on a forestry restock site so a proportion of cankers might have developed from weevil feeding wounds.

Another scenario to explain the much lower incidence of cankers at the Glensaugh trial is that *C. pithyophila* arrived at this site more recently and the disease is at an earlier stage allowing genetic influences over canker development to be more apparent. Future monitoring will reveal whether cankers also become more prevalent here. The very low incidence of *C. pithyophila* at the Yair trial in the south of Scotland is interesting as it suggests that the current outbreak of *C. pithyophila* has spread to southern Scotland more recently from Scots pine populations in the north.

At both trials, mean percentage of visible disease had the lowest heritability estimates of all traits (median 0.05 for the Glensaugh trial and 0.02 for the Inverewe trial) indicating that there is greater genetic control over whether an infestation successfully establishes than the extent to which infestations spread on the tree, which may be more influenced by environmental factors. Similar to the conclusions of Perry et al. (2016) in their studies of DNB, these results suggest that Caledonian pine populations may contain sufficient genetic diversity to evolve lower susceptibility to *C. pithyophila/P. pini* if the fungus and its association with *P. pini* continue to become more abundant as predicted with Scotland's changing climate to wetter, warmer winters (Green et al. 2024a). However, environmental factors such as rainfall and site suitability may override genetic factors influencing subsequent canker formation. Further analyses could be done to test for local adaption of source populations at these two environmentally contrasting trial sites.

The current outbreak of *C. pithyophila* is much more widespread compared with the two

previously reported outbreaks in Scotland in the early 1900s and 1960s (Green et al. 2024b). Scotland's shifting climate to wetter, warmer winters will be conducive to this fungus (Green et al. 2025) so it is likely to continue to thrive and expand on Scots pine, as will *C. sororia*. Recommendations arising from this study remain the same as those outlined in Green et al. (2024b) and focus on sourcing and planting trees which are visibly free of *C. pithyophila* and *P. pini* infestations and planting on sites deemed suited to Scots pine. The fungus should also be sought on other Pinaceae hosts in Scotland and on Scots pine in England and Wales.

6 Conclusions

This study confirms the separation of phragmospore and dictyospore forms of *C. pithyophila* as two distinct species which have likely evolved in ecological or geographical isolation from each other. British isolates of the dictyospore form are more diverged than those of the phragmospore form, suggesting that the phragmospore form is a more recent introduction. Both forms are morphologically closest to global herbaria specimens from Scots pine in Europe, although there was no consistent variation in *C. pithyophila* spore size based on either Pinaceae host or region of origin. British isolates of both forms are outcrossing and exhibit a high frequency of sexual recombination and vegetative incompatibility. The fungus appears to be unique as an ascomycete in containing lignin peroxidase genes, reflecting its unusual growth habit within the bark layers of Scots pine. Incidence of *C. pithyophila* was higher in Caledonian Scots pine at Glen Affric than at Rothiemurchus which may reflect the wetter site conditions. Almost all trees surveyed at these sites had cankers typical of *C. sororia* infection. This appears to be a recent phenomenon for native Scots pine on good sites and might indicate previously higher *C. pithyophila/P. pini* infestation levels. Incidence of *C. pithyophila* was moderately heritable in two Caledonian pine common garden trials suggesting that native pine populations that are allowed to freely regenerate have the capacity to adapt if populations of the fungus and adelgid continue to thrive and expand as Scotland's climate changes.

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8 Appendix 1

Comparison of morphological characteristics of voucher specimens of *Cucurbidothis/Curreya pithyophila*. For ascospores/conidia 30 measurements of length and width were made, with 20 made for ascomata and conidiomata. Varying numbers of measurements were made for other structures depending on availability. Measurements in italics = $\bar{x}$; otherwise min–max ranges given. Information is ordered by tree host, Europe/Asia/N America, dictyospore (D)/phragmospore (P).

Voucher specimen number	Tree host	Region; country	Ascospore type; dimensions (µm)	Stroma characteristics	Ascomata diameter (µm)	Asci dimensions (µm)	Pseudo-paraphyses dimensions (µm)	Stroma/ peridium dimensions (µm); comments	Conidia dimensions (µm); width/length	Fig. no.
CUP-069926*	*Abies alba*	Europe; France	phragmospore; *22 × 7*	On small branch 9 mm wide, at branch junction; stroma very enlarged, raised and folded; ascomata 'caviar' like, ostioles often paler	320–510	All deteriorated	2–3	79–138	–	
ZTMyc0067421*	*Abies pindrow*	Asia; Pakistan	phragmospore; *16 × 5.5*	On bark of large branch or trunk, stroma 30–60 mm; stroma convoluted and folded and mixed with bark; some stroma have caviar appearance; in poor condition	240–370	All deteriorated	1.5–3	44–79; stroma inwardly has brown cells (not hyaline)	*6 × 4*; 1.5 Peridium 92 µm	6h
BPI 628608	*Abies veitchii*	Asia; Japan	dictyospore; *20.5 × 8*	On small branches 3–5 mm; at branch junctions,	210–440	Mostly deteriorated 115 × 10.5	1.5–2	64–106	–	

				stroma 12–20 mm long, some flaking bark. No adelgids observed; Ascomata caviar-like to scattered.						
NY 2980792*	*Abies concolor*	N America; Colorado	dictyospore; *18.5 × 7.5*	On bark from thick branch or trunk; stroma radiating out in linear folds, of similar thickness and close together, 30 mm diam.; many ascomata, circular and even.	270–400	*121 × 10.5*	2–3.5	48–87	–	6d
ZTMyc0067408	*Larix decidua*	Europe; Italy?	phragmospore; *21× 7*	On thick branches or bark from tree trunk; up to 45 mm; stromata at branch junctions or not; dense ascomata with caviar-like appearance, with minimal flaking bark and stroma barely visible; stroma highly convoluted and almost radial looking	340–530	All deteriorated	1.5–3	66	–	6i

ZT Myc 0067405*	*Picea abies*	Europe; Switzerland	dictyospore; *20 × 8*	On small branches with stroma mainly occurring between branch junctions21-38 mm long; large ascomata, scattered or grouped, and stroma sometimes very sparse and lacking ascomata	340–650	Only width: 10–12	1–1.5	133	–	
K-M00144208 (phragmospore Type)	*?Picea abies*	Europe; UK, Wales	phragmospore; *22 × 7*	On bark of large branch or trunk; crowded ascomata, caviar-like with flaking of bark and lichen growth	360–520	*140 × 10*	2–3	92–116	–	6g
ZT Myc 0067415*	*Picea abies*	Europe; Switzerland	conidia	On small branches; stroma near but not surrounding branch junctions on up to 20 mm long; conidiomata sparse; lichens present	Conidioma: 340–550; with distinct ostioles	–	–	–	*7 × 5*; 1.4 Peridium 49–61 µm	
DAOM126310	*Picea sitchensis*	N America; Canada, Haida Gwaii	dictyospore; *16.5 × 6*	On small branch 13 mm diam., close to branch junction; very	290–480	All deteriorated	1–2.5	109	–	6e

				sparse, stroma barely visible; host surface raised slightly; no adelgids observed						
ZTMyc 0067413*	*Pinus cembrae*	Europe; Switzerland	dictyospore; *18 × 7*	On small branches 4–8 mm. Associated with branch junctions or not, often multiple in a row; conidiomata on same stroma; mixture of D and P; more ascomata in P stroma on same branch	330–500	Only width: 10–11.5	1–2	70	*5 × 3.5*; 1.4	
ZTMyc 0067413*	*Pinus cembrae*	Europe; Switzerland	phragmospore; *20 × 6.5*	On small branch 4–8 mm. Associated with branch junctions or not, often multiple in a row, mixture of D and P; less ascomata in D stroma on same branch	250–480	*145 × 10*	1.5–3	72–76	–	
SP23-19*	*Pinus sylvestris*	Europe; UK, Scotland	dictyospore; *19.5 × 8*	Encircling shoots and branches, often at branch junctions	250–420	*137 × 10.5*	*2.5*	50-86	*4.5 × 3.5*; 1.3	6a

JE07002971 (dictyospore Type)	*Pinus sylvestris*	Europe; Germany	dictyospore; *19.5 × 7.5*	On bark of thicker branch or trunk; covered in flaky bark and many ascomata; adelgids not observed; 45 ×25 mm	280–430	*115 × 10.5*	1.5–2.5	50–120	–	6b
SP23-18*	*Pinus sylvestris*	Europe; UK, Scotland	phragmospore; *21 × 7.5*	Encircling shoots and branches, often at branch junctions; rarely see on trunk	250–455	*159 × 9.8*	*2.5*	55-85	*6.5 × 4.5*; 1.5	6f
ZTMyc0067417	*Pinus sylvestris*	Europe; Switzerland	Phragmospore; *20.2 × 6.7*	Encircling small branches 3-5 mm. At stem junctions or along twigs	*c.* 400	*142 ×9.4*	*2–3*	70-100	–	
TNS14792	*Pinus pumila*	Asia; Japan	dictyospore; *20.5 × 8*	Encircling a very small branch 3 mm. Not obviously associated with a junction, 26 mm long. Stroma covered in thin host bark, not flaking; large scattered ascomata; no adelgids observed	520–640	All deteriorated	1.5–2	87–103 (–148); stroma inwardly has brown cells (not hyaline)	–	
DAOM84818*	*Pinus strobus*	N America; Canada; Newfoundland	dictyospore; *21 × 8*	On small pine branches 9–12 mm; stroma occurring in reticulate	290–530	*125.7 × 10.5*	1.5–3	84–122	–	6c

				pattern of linear raised areas; host tissue not disrupted or flaking; associated with branch junction or not; ascomata scattered; adelgids look very fresh.						

*adelgids observed (if it was not possible to look for adelgids without damaging the specimen it is noted that 'no adelgids observed' in the 'Stroma characteristics' section).

9 Appendix 2

Random effect variances for instance of *C. pithyophila*, instance of cankers and mean percentage visible disease on Caledonian Scots pine planted out in two commongarden trials, Glensaugh and Inverewe. Columns show the mean and 95% credible interval of the posterior distribution for each random effect, and the effective sample size. All estimates are presented on the latent scale.

	Trait	Random effect	Posterior mean	Lower CI	Upper CI	Effective n
Glensaugh Trial	*C. pithyophila*	Block	0.16	<0.001	0.605	2000
		Forest	0.04	<0.001	0.104	2000
		Family	0.10	<0.001	0.239	2000
	Cankers	Block	0.24	<0.001	0.863	2000
		Forest	0.04	<0.001	0.149	1830
		Family	0.22	<0.001	0.549	2000
	% visible disease	Block	0.67	<0.001	2.047	1504
		Forest	0.03	<0.001	0.092	2000
		Family	0.15	<0.001	0.316	2000
Inverewe Trial	*C. pithyophila*	Block	0.60	0.015	2.051	1820
		Forest	0.06	<0.001	0.165	2000
		Family	0.16	<0.001	0.381	2000
	Cankers	Block	0.63	0.01	2.248	2000
		Forest	0.06	<0.001	0.192	2000
		Family	0.09	<0.001	0.296	2000
	% visible disease	Block	3.12	0.008	13.202	1195
		Forest	0.02	<0.001	0.073	2000
		Family	0.04	<0.001	0.126	2000

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