DOCTOR OF PHILOSOPHY

Investigating the Ecology and Infection Biology of Gymnopus fusipes, a Fungal Root Rot Pathogen of Woodland Trees

Pettifor, Bethany

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Investigating the Ecology and Infection Biology of *Gymnopus fusipes,* a Fungal Root Rot Pathogen of Woodland Trees

Bethany J Pettifor

2019 - 2023

A thesis submitted to Bangor University in candidature for the degree of

Doctor in Philosophy

School of Natural Sciences

Bangor University, Deiniol Road, Bangor, LL57 2UW

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Summary

Gymnopus fusipes (syn. *Collybia fusipes*), is an understudied basidiomycete fungus commonly found in woodlands across Europe. *G. fusipes* is a slow-growing primary pathogen, with the ability to destroy whole root systems, and has been linked to episodes of oak decline in Europe and the UK. Orange lesions on large central roots are characteristic of *G. fusipes* infection and are often accompanied by white mycelial fans. Above the ground level, typical decline symptoms (such as poor crown condition and presence of fruiting bodies) are not always correlated with infection status, leading to ineffective detection and diagnosis, leaving young and mature trees at higher risk of being wind thrown due to a lack of anchoring roots. To address the paucity of information on the infection biology and ecology of *G. fusipes,* the current study focused on four main objectives; (i) to conduct a systematic literature review to highlight existing knowledge and identify key knowledge gaps in order to collate and analyse existing information on *G. fusipes* into an up-to-date resource on the species, (ii) to optimise methods for isolation, culture, nucleic acid extraction and phylogenetic analysis of *G. fusipes*, (iii) to develop a rapid molecular-based diagnostic assay, suitable for use on field samples, to allow for accurate diagnosis of *G. fusipes*, without the need for pure culture, and (iv) to investigate disease progression at a molecular level using transcriptomic analysis of *G. fusipes* at different infection stages, including an early infective state (through a seedling inoculation trial), an established infective state (through sampling at a site heavily impacted with *G. fusipes*) and a non-infective state (through vegetative mycelial cultures).

A systematic review of 96 publications revealed that *G. fusipes* is associated with numerous species of tree host, mainly *Quercus* spp. and is suggested to have a presence across the northern hemisphere, although a lack of molecular validation of its identity and occasional spurious citation in the small amounts of focused literature makes this unclear. Optimisation of culture dependant methods and molecular analyses identified the best practice for isolation of *G. fusipes* from environmental samples and cultivation in the lab. Optimal methods for nucleic acid extraction were also documented. A growth rate study of five geographically diverse *G. fusipes* strains across five ecologically relevant temperatures suggested that temperature and the *G. fusipes* isolate selected both had a significant effect on the growth rate of *G. fusipes*, however there was no significant interaction between the temperature and isolate, indicating that there is no localised temperature adaptation in this species. A *G. fusipes* specific qPCR assay, based on the fungal 18S rRNA gene was developed to be applicable to a range of environmental sample types without the need for pure culture, resulting in accurate detection of this species in mycelial plate cultures, fruiting bodies and infected woody tissues. Genome sequencing combined with comparative transcriptomic analysis of *G. fusipes* in different infective stages highlighted differences in gene expression profiles between a non-infective agar culture, and an active infection in living tissue, illustrating that genes involved with processes such as enzyme production and transcription promotion are highly expressed in active infection, and those encoding carbohydrate binding and chitin production being highly expressed when in a non-infective state.

This body of work represents important progression in understanding the biology, ecology and infection biology of *G. fusipes.* The data presented in this study are crucial to informing management and potential methods to combat this important root rot pathogen.

CHAPTER 1

An Introduction to the Thesis

1.1 | Introduction

Gymnopus fusipes (Bull.:Fr.) Gray (syn. *Collybia fusipes*) is an agaricomycete fungus in the family Omphalotaceae, responsible for causing root rot on oak (*Quercus* L.) species across the UK and Europe. *Gymnopus fusipes* is suggested to have a distribution spanning most of the northern hemisphere and is thought to parasitise a number of host species, including both broadleaf and coniferous varieties. Although having been described almost 250 years ago and being mentioned frequently in grey literature, such as popular science books and forest guides as the cause of *Collybia* root rot, there is a noticeable lack of primary peer-reviewed research on *G. fusipes*. Further to this, much of the existing research is outdated, and with changes to taxonomy and the advent of molecular biology, the existing information is in need of being updated.

Therefore, the overarching aim of this study was to explore, assess and advance the available information regarding *Gymnopus fusipes*, specifically relating to the ecology and infection biology of the species. The current work addresses this aim through the completion of the following objectives: (i) to conduct a systematic literature review to highlight existing knowledge and identify key knowledge gaps in order to collate and analyse existing information on *G. fusipes* into an up-to-date resource on the species, (ii) to optimise methods for isolation, culture, nucleic acid extraction and phylogenetic analysis of *G. fusipes*, (iii) to develop a rapid molecular-based diagnostic assay, suitable for use on field samples, to allow for accurate diagnosis of *G. fusipes*, without the need for pure culture, and (iv) to investigate disease progression at a molecular level using transcriptomics (gene expression analysis).

By addressing these objectives, this study will not only provide an evaluation of existing information relevant to *G. fusipes*, but will also offer important novel information and applied methods, crucial to informing management and increasing understanding of this widespread root rot pathogen.

1.2 | Using a Systematic Approach to Synthesise Existing Knowledge on *Gymnopus fusipes* (syn. *Collybia fusipes*), the Cause of *Collybia R*oot Rot

Pettifor, B. J., Denman, S., & McDonald, J. E. (2022). Using a systematic approach to synthesize existing knowledge on *Gymnopus fusipes* (syn. *Collybia fusipes*), the cause of *Collybia* root rot. *Forest Pathology*, 00, e12766. https://doi.org/10.1111/efp.12766

The first step in this program of research was to collate and analyse existing knowledge on *Gymnopus fusipes* and form and up-to-date informational resource on the species. This was accomplished through the employment of a systematic search method, which allowed for thorough exploration of the existing literature regarding *G. fusipes*. After strict search and inclusion criteria were utilised, the resulting ninety-six publications, were analysed in depth, and any information pertaining to *G. fusipes*, including data on host range, distribution, ecology, molecular analysis, phylogeny and taxonomy, morphology and life cycle, infection biology, and biochemistry, was recorded. The collation of this information provides a comprehensive up to date resource on the current status of *Gymnopus fusipes* knowledge. This work also provided the opportunity to identify key knowledge gaps and aided in the planning of subsequent experiments and chapters.

1.3 | Optimising Methods for Isolation, Culture, Nucleic Acid Extraction and Phylogenetic Analysis of *Gymnopus fusipes*

The main aim of this chapter was to optimise culture-dependant and molecular methods for working with *Gymnopus fusipes*, including isolation, culture, nucleic acid extraction and phylogenetic analysis. *G. fusipes* is a very slow growing species and therefore is difficult to isolate and work with. This chapter describes the challenges and solutions to be considered when working with *G. fusipes* in the lab. This chapter includes empirical data from small scale pilot experiments, as well as qualitative notes from exploratory observations regarding obtaining environmental samples and isolates, isolating from environmental samples, germination of *G. fusipes* from basidiospores and tissue samples, cultivating *G. fusipes* on different media types, as well as molecular methods such as optimising nucleic acid extraction protocols, along with a basic phylogenetic analysis of the *ITS* gene region.

1.4 | The Impact of Temperature on Growth Rate of the Root Rot Fungus, *Gymnopus fusipes*

The main aim of this section was to assess the growth rate of geographically diverse *Gymnopus fusipes* isolates at five ecologically relevant temperatures, in order to identify the optimal growth temperature of the species. This experiment involved using five *G. fusipes* isolates from different locations and growing them at five different temperatures for a four-week period, recording growth of each replicate at specific time points. This experiment was run in parallel by two independent laboratories (Bangor University, Gwynedd, UK and Forest Research, Alice Holt Forest, Surrey, UK). Initial analysis showed similar trends, therefore the data was pooled and analysed further. Statistical analysis of the data obtained was used to determine the optimal growth temperature of *G. fusipes* from those tested. Understanding the growth rate of *G. fusipes* and the effects of temperature on this growth rate will provide crucial information for models to determine distribution of this pathogen, as well as any potential changes to geographical range that may occur as a result of climate change.

1.5 | Development and Application of a qPCR-Based Diagnostic Tool for Accurate Detection of *Gymnopus fusipes,* the Cause of *Collybia* Root Rot in Oak

This chapter describes the development of a novel diagnostic assay using quantitative real-time PCR (qPCR), that could be used to accurately identify *Gymnopus fusipes* from environmental samples including fruiting bodies and infected tree tissues. The process involved obtaining nucleotide sequences for *G. fusipes* and 112 related species from NCBI GenBank, aligning the sequences and identifying potential primer regions, designing suitable primers and testing *in silico* using the NCBI PrimerBLAST tool. After this, the primers were tested with a range of *G. fusipes* DNA extracts as well as a range of synthetically produced non-target sequences (7 nucleotide sequences that had been returned in the PrimerBLAST search, with 3-10 bp mismatches to the target sequence), before being tested on extracts from unprocessed environmental samples. Reliable positive results were obtained from plate cultures, fruiting bodies and infected root tissue, meaning there is no need for a pure culture (which could take weeks), allowing the diagnostic to be completed in less than 48 hours. Not only will this assay help identify problematic cases of *G. fusipes* infection, but could also be used in targeted distribution investigations, in order to confirm the suggested distribution in the literature.

1.6 | Whole Genome Sequencing and Transcriptomic Analysis of *Gymnopus fusipes,* at Different Stages of Infection Development

This chapter describes work to elucidate the process of *G. fusipes* infection development at different stages of infection. This chapter presents results of a successful seedling inoculation trial, which was completed to obtain samples for an early-stage infection, as well as a field sampling campaign, to collect samples from infected mature trees to represent an established infection. RNA was extracted from these samples, as well as axenic *G. fusipes* mycelial cultures (representing a non-infective state), and sequenced. These transcripts were mapped to the first draft *G. fusipes* genome (completed in this study) in order to understand gene expression at different stages of infection. The transcriptomic profiles at different infection stages were compared for upregulation or downregulation of pathogenicity genes to aid in understanding the mechanisms of infection utilised by *G. fusipes* in the infection process.

1.7 | Final Synthesis and Future Research Priorities

This chapter summarises the work completed in this study illustrating how this research exhibits the most comprehensive review of *Gymnopus fusipes* literature to date, valuable empirical data and qualitative research notes on the isolation, cultivation, and molecular analysis of *G. fusipes* in the lab. The work presents an investigation into the impact of temperature on growth rate, development of an effective qPCR-based diagnostic assay, and the first instance of whole genome sequencing and gene expression analysis of *G. fusipes* through transcriptome analysis. This chapter also addresses future research priorities in the field that should be addressed to close key knowledge gaps.

CHAPTER 2

Using a Systematic Approach to Synthesise Existing Knowledge on *Gymnopus fusipes* (syn. *Collybia fusipes*), the Cause of *Collybia* Root Rot

Pettifor, B.J., Denman, S., Mcdonald, J.E., 2022. Using a systematic approach to synthesize existing knowledge on *Gymnopus fusipes* (syn . *Collybia fusipes*), the cause of *Collybia* root rot. For. Pathol. 52 (5), e12766. https://doi.org/10.1111/efp.12766

Abstract

Gymnopus fusipes (syn. *Collybia fusipes*; syn. *Agaricus fusipes*) is an agaricomycete fungus known to cause root rot on a number of economically important tree species, including oak, where it has been linked to the development of chronic oak decline. Due to lack of correlation between above ground decline symptoms and *G. fusipes* infection, its presence can often go undiagnosed until mortality. Although *G. fusipes* was first described over 200 years ago, there is still a paucity of information on the biology and ecology of this species, which represents a barrier to understanding its impacts on tree health. The aim of this review was to synthesise existing knowledge on the biology, ecology, host range and host interactions of *G. fusipes*. Using a systematic search, five online databases were used to obtain published literature resulting from the search terms "*Gymnopus fusipes", "Collybia fusipes"* and *"Agaricus fusipes"*. After a strict filtering process, the papers were examined for data pertaining to the biochemistry, distribution, ecology, genomic information, host range, infection biology, morphology, and phylogeny of the species. The results reveal that there is a large amount of ambiguous and sometimes spurious citation of *G. fusipes* in the literature. However, it can be confirmed that *G. fusipes* is a facultative saproparasite, found in several countries, mainly in Europe, and is associated with several socioeconomically important host species, including oak, chestnut, and fir. *Gymnopus fusipes* has repeatedly been investigated with regards to oak decline in Europe, where it is believed to play a crucial role in the early stage of decline development. Key knowledge gaps highlighted in this review include a lack of information on the basic biology of the species, including its life cycle, which is crucial to fully understanding *G. fusipes* infection and epidemiology. Further work is needed to assess *G. fusipes* distribution, phylogeny, and host range through molecular identification. There is also a need to characterise the pathogen-host interaction at a molecular level, with identification of active genes and therefore the mechanisms of infection. A combination of culture-based and molecular techniques should be utilised in order to close these key knowledge gaps.

2.1 | Introduction

Gymnopus fusipes (Bull.:Fr.) Gray (syn. *Collybia fusipes*), is a mushroom forming agaricomycete fungus from the family Omphalotaceae (Ványolós et al., 2016). The species *G. fusipes*, first appeared in the literature under the name *Agaricus fusipes,* in Volume III of Pierre Bulliard's "Herbier de la France" (1783), a descriptive collection of plants and fungi native to France, complete with hand drawn images, anatomical description and details on properties and uses. *Gymnopus fusipes,* or *Agaricus fusipes*, was described as a mushroom, common in French woodlands, with a morphological description mentioning the stipe which decreases in thickness from the top to a point at the base (Bulliard, 1783; Campbell, 1939). In 1821, the species was reclassified as *Gymnopus fusipes* (Gray, 1821), however in 1872, the species was again reclassified, placing it in the genus *Collybia* (Quelet, 1872). This nomenclature remained for 125 years, when a more rigorous classification system was developed (Antonin et al., 1997). The new classification included a more in-depth morphological analysis including spore-print typing, the study of stipe shape and measurements of fruiting bodies at full maturity. This work along with the advent of molecular phylogeny, providing greater phylogenetic resolution, supported previous claims that the genus *Collybia* should be divided into three genera, *Collybia, Rhodocollybia* and *Gymnopus*, with *Rhodocollybia* and *Gymnopus* being more closely related (Antonin et al., 1997; Mata et al., 2004; Mata and Petersen, 2003).

Current analysis describes species within the genus *Gymnopus* as fungi with fruiting bodies that grow on substrates from basal mycelia, forming a non-insititious or pseudoinsitituous stipe, a cap with a pileipellis made up of smooth or irregular hyphae and a white to cream coloured spore print (Antonin et al., 1997). The genus *Gymnopus* contains approximately three hundred plant-associated, saprotrophic, mushroom-forming species with an almost global distribution (Jang et al., 2016).

Gymnopus fusipes fruiting bodies*,* have typical agaricomycete morphology (Figure 2.1), are often brown to reddish-brown in colour, and can appear individually, but more often form in small groups at the base of trees and stumps (Marçais et al., 2000b) . The distinctive tapering stipe of the species has led to the common name of the Spindle-shank mushroom (Ványolós et al., 2019).

Figure 2.1. Photograph of *Gymnopus fusipes* fruiting bodies (basidiocarps), after being pulled from beneath the tree host. The stipe clearly decreases in width from the cap to the base, the tapered section is darker in colour due to being buried beneath the groundline. These features can make it difficult to identify without specialist knowledge. Photo Credit: David Humphries.

Gymnopus fusipes was first noted to cause root rot in oak (*Quercus* L.) trees in France in the early 1980s (Delatour and Guillaumin, 1984; Guillaumin et al., 1985), prior to which, there was no established link between the presence of *G. fusipes* and an impact on tree health (Marçais et al., 2000b). Since this time, *G. fusipes* has been established as a primary pathogen capable of infecting both young and mature oak trees (Marçais et al., 2000b; Marçais and Delatour, 1996), as well as suggested associations with tree species in other genera including beech (*Fagus* L.), chestnut (*Castanea* Mill.) and hornbeam (*Carpinus* L.) (Marçais and Caël, 2000; Piou et al., 2002). Infection by *G. fusipes* typically causes deterioration of host health, vigour, and root condition, which can occur over several decades (Camy et al., 2003a). Symptoms of infection include distinctive orange lesions on main roots below ground level, with small white mycelial fans dispersed within the necrotic tissues, as well as hypertrophy of host cells, in which bark can increase in thickness up to 4 cm (Guillaumin et al., 1985; Marçais et al., 2000b). There is little information regarding the exact mechanisms used by *G. fusipes* when causing infection, however it is thought that this pathogen may kill large lateral and collar roots, and central sections of the root system where the deep anchoring

roots are found, which in turn leads to impaired water uptake and in some cases higher rates of wind failure (Marçais et al., 1999).

Gymnopus fusipes, as well as other fungal or oomycete pathogens, such as those in the genera *Armillaria* and *Phytophthora,* have been associated with oak decline in Europe (Ragazzi et al., 1995; Thomas, 2008; Thomas et al., 2002). In the UK specifically, *G. fusipes* has been linked to two oak decline syndromes, acute oak decline (AOD), and chronic oak decline, otherwise known as COD (Denman and Webber, 2009; Gagen et al., 2019). Whilst AOD is a rapidly progressing decline caused by a combination of bacterial and insect elements and is characterised by vertical cracks and stem bleeding (Brown et al., 2015; Denman et al., 2014, 2012), COD is a slowly progressing decline disease, linked primarily to fungal infection, poor root health and prolonged weakening of host condition (Denman and Webber, 2009; Gagen et al., 2019; Lonsdale, 2015). Both forms of decline are caused by interactions between biotic and abiotic factors and ultimately lead to deterioration of the host and eventually tree mortality (Finch et al., 2021; Marçais et al., 2011).

COD, as with other decline syndromes, can be conceptualised using Manion's decline spiral (Manion, 1981). In this process, numerous biotic and abiotic factors are grouped into different levels representing their contribution to the decline. In the first phase of decline, predisposing factors are biotic factors such as host age, genetic potential and host susceptibility to infection, along with abiotic factors such as soil compaction and air pollution. The next stage of the decline spiral, the inciting factors, include major tipping events such as severe insect defoliation (biotic) and environmental factors such as drought and frost. In the final stage of the spiral, the contributing factors take hold, and ultimately lead to death of the host. These are mainly biotic factors, such as canker fungi, viruses, and root rot fungi, including species from the genus *Armillaria. Gymnopus fusipes* may therefore also represent a major contributing factor to tree declines, but its specific role and impacts on the decline process currently represents a major knowledge gap.

The overarching aim of this review is to synthesise existing knowledge on the distribution, ecology and infection biology of *Gymnopus fusipes*. This resource will provide key insights into the activities of *G. fusipes*, which will help inform future research priorities and guide management of the species.

2.2 | Materials and Methods

The specific objectives of this review were to firstly conduct a systematic literature search to identify published literature concerning *Gymnopus fusipes*; secondly, to filter the published works through a strict selection process, to collate information on key topics, and finally to synthesise a resource on the current state of *Gymnopus fusipes* research and identify key knowledge gaps.

A systematic literature search was conducted to identify peer-reviewed published works on *Gymnopus fusipes* (syn. *Collybia fusipes* or *Agaricus fusipes*). For this analysis, five publication databases were selected (based on relevance to biological/environmental sciences and microbial ecology) to obtain literature, which was then analysed (Figure 2.2). These databases were BioOne, CAB Direct, ProQuest, Bangor University Library Catalogue and Google Scholar.

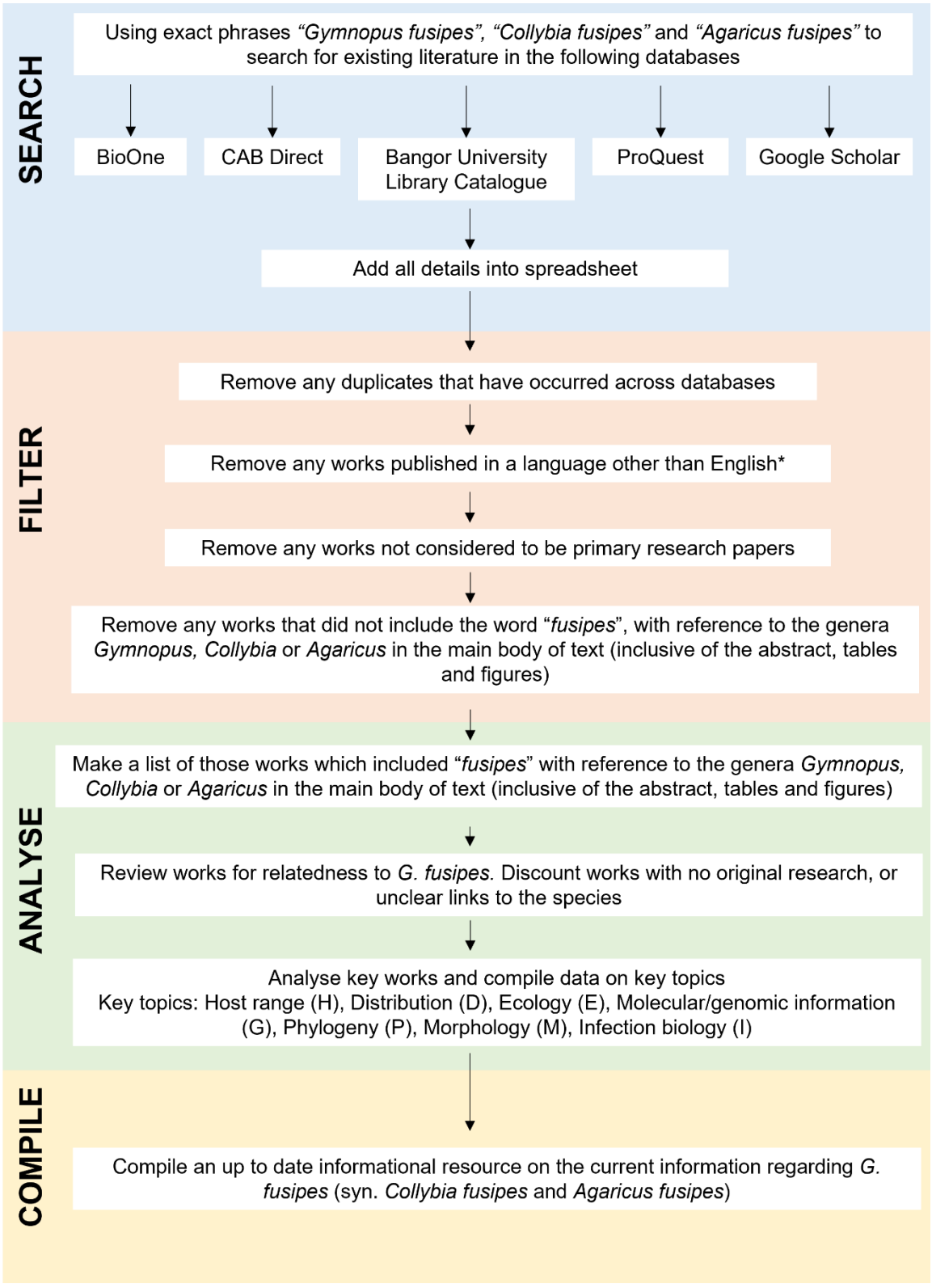

Figure 2.2. Schematic of the methods used in this review, from the literature search to obtain literature through the filtering of non-target works, analysis of content, and finally to compilation of an up-to-date informational resource on *G. fusipes*. *There were a high number of non-English publications in the collection at this stage, and it was considered unfeasible to obtain accurate translations for this number of publications, therefore this filtering step was added to the methods.

The exact phrases "*Gymnopus fusipes", "Collybia fusipes"* and *"Agaricus fusipes"* were searched in all five databases, and all search results were compiled into a spreadsheet using Microsoft Excel. The filtering process for the review was as follows: firstly, any publication with occurrence in two or more databases was retained only once as a single copy. Secondly, any works that were published in a language other than English were removed. Many of these publications were not relevant and were collated by the search due to the word *"fusipes"* being mentioned in the reference list and not the main text. Consequently, translation of these sources (across numerous languages) was deemed unfeasible. Thirdly, due to a large amount of grey literature and anecdotal mentions of *G. fusipes*, any publications that weren't considered primary peer-reviewed research were also removed from the collection. Numerous publications were found to mention *G. fusipes* only in the reference list, therefore the next stage of the filtering process was to only preserve publications which made mention of *"Gymnopus fusipes" "Collybia fusipes"* and *"Agaricus fusipes"* in the main body of the text. This process was inclusive of the abstract, any figures and tables, but excluded the reference list. These publications were compiled into a separate spreadsheet for analysis (Supplementary Information 2.1).

The publications obtained from this systematic search were then reviewed for their accuracy and relatedness to the topic, and the purpose of this review. Publications which were deemed appropriate to the subject included novel information on *G. fusipes,* or clear results associated with the species. These publications were given a coded letter (Table 2.1) to highlight the key topics covered by the existing literature.

Table 2.1. Coded letters assigned to publications when each of the particular topics was mentioned with reference to *Gymnopus fusipes*.

Key Topic	**Coded Letter**
Host range of *G. fusipes*	H
Distribution of *G. fusipes*	D
Ecology of *G. fusipes* and ecological strategy	E
Genomic analysis of *G. fusipes*	G
Phylogeny and taxonomy of *G. fusipes*	P
Morphology of *G. fusipes* and any information on the life cycle of the species	L
Infection biology of *G. fusipes* and its links with Chronic Oak Decline (COD)	I
Biochemistry of *Gymnopus fusipes*	B

Graphics were produced to depict the breadth of topics covered in the existing literature on *G. fusipes* and information collated to form an up-to-date resource on current literature and knowledge on *G. fusipes.*

2.3 | Results and Discussion

2.3.1 | Systematic Literature Search and Data Analysis

The results from each stage of the systematic literature search are detailed in Table 2.2.

Table 2.2. The number of search results from each stage of the systematic literature review.

Search / Selection Criteria	**Total no. of publications**
Number of initial search results across all five databases	1000 *"Gymnopus fusipes" – 252* *"Collybia fusipes" – 706* *"Agaricus fusipes" – 42*
Number of publications after removing duplicates	765
Number of publications after removing non-English language works	396
Number of publications when filtered for primary, peer-reviewed research papers only	257
Number of publications where "*fusipes*" (with reference to the genera *Gymnopus*, *Collybia* or *Agaricus*) appears in the main body of text (inclusive of figures and tables)	184
Number of publications containing novel information or clear results directly associated to *Gymnopus fusipes* (or its synonyms)	96

The number of publications found across the five databases regarding *G. fusipes* was 1000, however after applying the strict selection criteria, few remained, including only 15 publications with *"fusipes"* in the title. Almost a quarter of the original 1000 publications were lost after removing duplicates, and of the 765 publications that remained, approximately 48% were lost after removing works published in a language other than English. Although some information may have been lost in this step, it was deemed as appropriate, as after the final stages of analysis, less than 25% of the English-language publications were considered relevant. Further to this, the majority of the key

publications cited in the research were already included in the analysis, indicating that few important publications had been missed.

After removing any works not considered to be primary research, only 25.7% of the original 1000 works remained and after selecting for works where the species name "*fusipes*" appeared in the main body of text (inclusive of figures and tables, but excluding the reference list) the number of publications suitable for analysis was 184, 18.4% of the original 1000 publications. These publications were then reviewed and assessed for their accuracy and relatedness to *G. fusipes*. This process included removing any publications not containing novel information, or results without a clear link to *G. fusipes* (Figure 2.3).

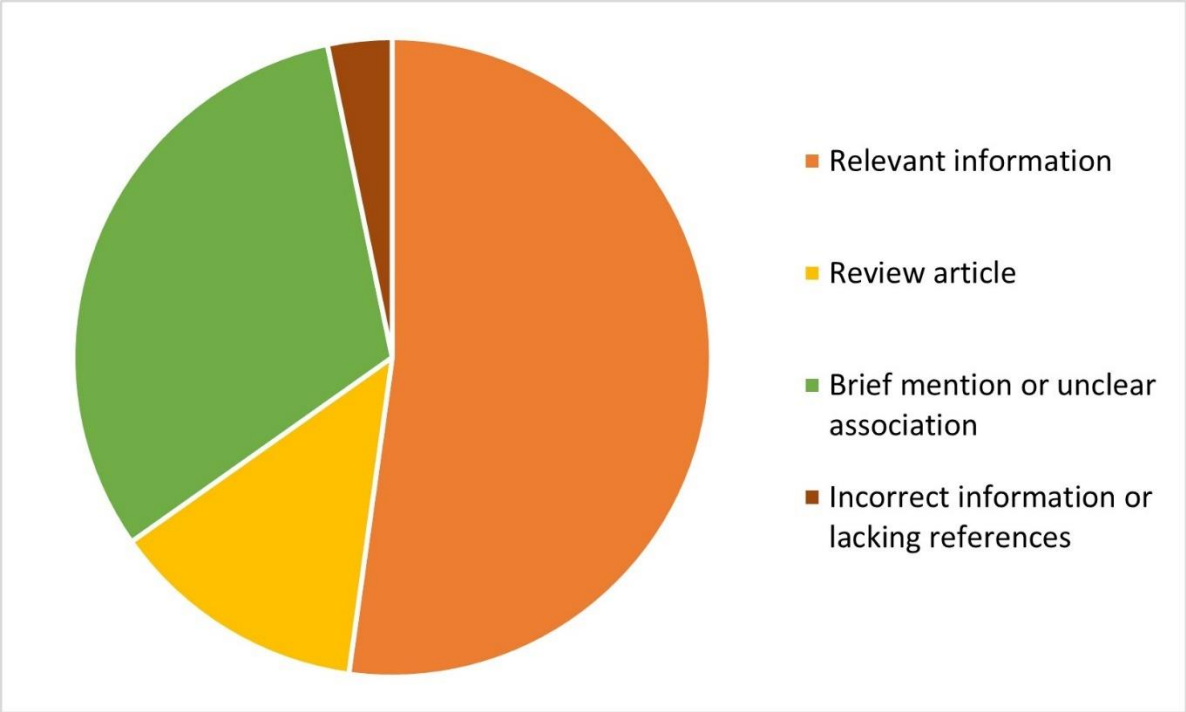

Figure 2.3. Chart depicting the status of available information on *G. fusipes.* Only 96 out of the original 184 publications could be considered relevant and appropriate to provide information for the up to date resource.

Just over half of the 184 publications from the systematic search were deemed relevant for the purpose of this review (Supplementary Information 2.1), less than 25% of the English-language publications and just 9.6% of the original 1000 search results.

At the time of writing, search results from Google Scholar returned 227 results for *G. fusipes.* The same database returned approximately 1690 results for *Dothistroma septosporum*, a causal agent of *Dothistroma* needle blight in pine species (Mccarthy et al., 2022) and 3820 results for *Fusarium*

circinatum, the causal agent of pitch canker in pine species (Wingfield et al., 2008). These results indicate a clear lack of research on *G. fusipes,* in comparison to other fungal forest pathogens.

Figure 2.4 indicates how many times the species name "*fusipes*" was mentioned in the 96 publications. The majority of the publications, even with the inclusion of research with direct and clear links to *G. fusipes*, mentioned the species name "*fusipes*" only once, indicating how *G. fusipes*, although seemingly well-known, is understudied as a focus organism.

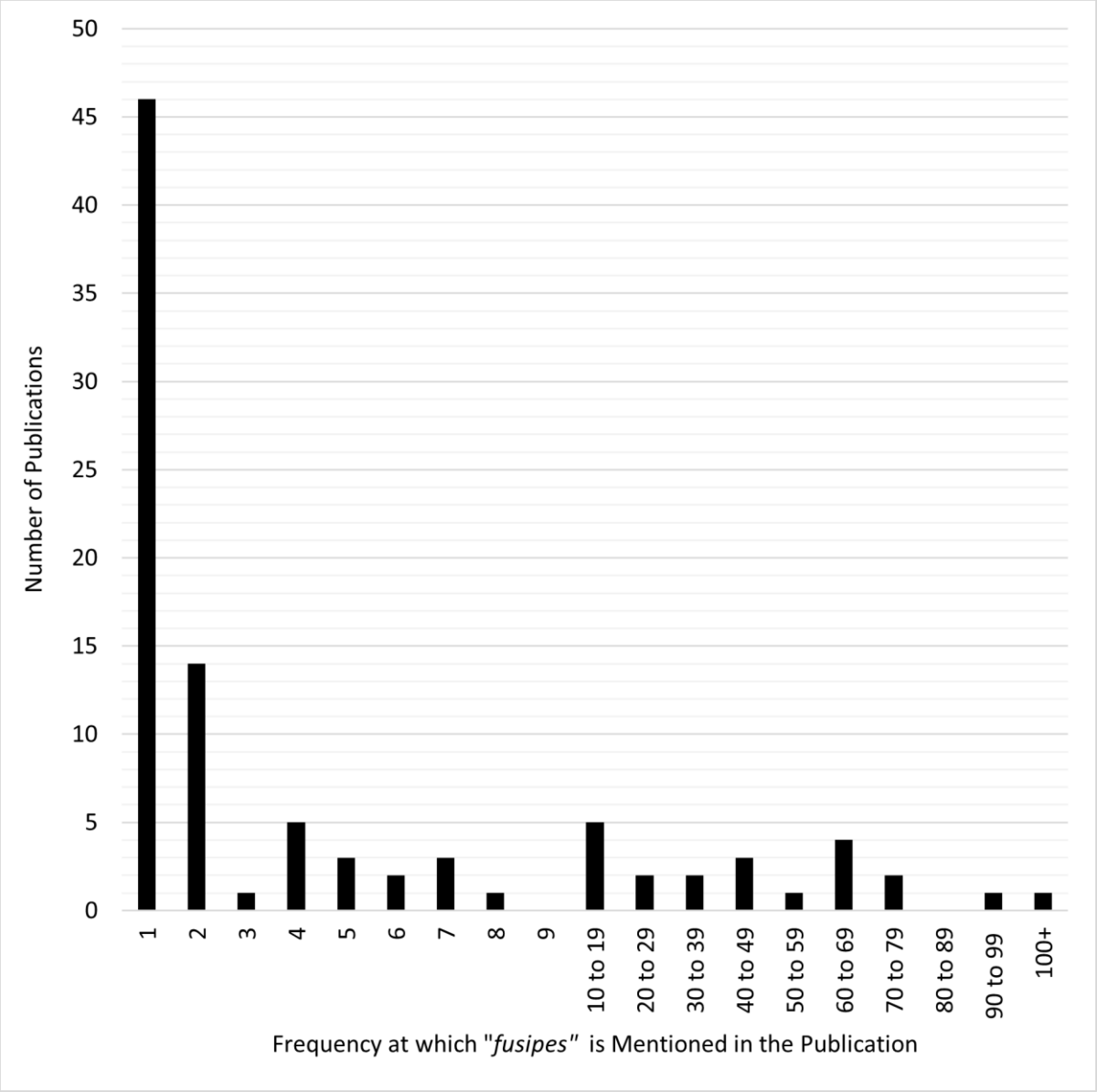

Figure 2.4. Frequency with which the species name "fusipes" is mentioned in the 96 analysed publications.

The ninety-six published works were analysed, and the literature examined to identify any information on *G. fusipes* pertaining to its biochemistry (B), distribution (D), ecology (E), molecular information and genomic analysis (G), host range (H), infection biology and its links to chronic oak decline (I), morphology including links to the lifecycle of the species (M), and the phylogeny and taxonomy of *G. fusipes* (P); the information was coded based on the topics referenced (Figure 2.5).

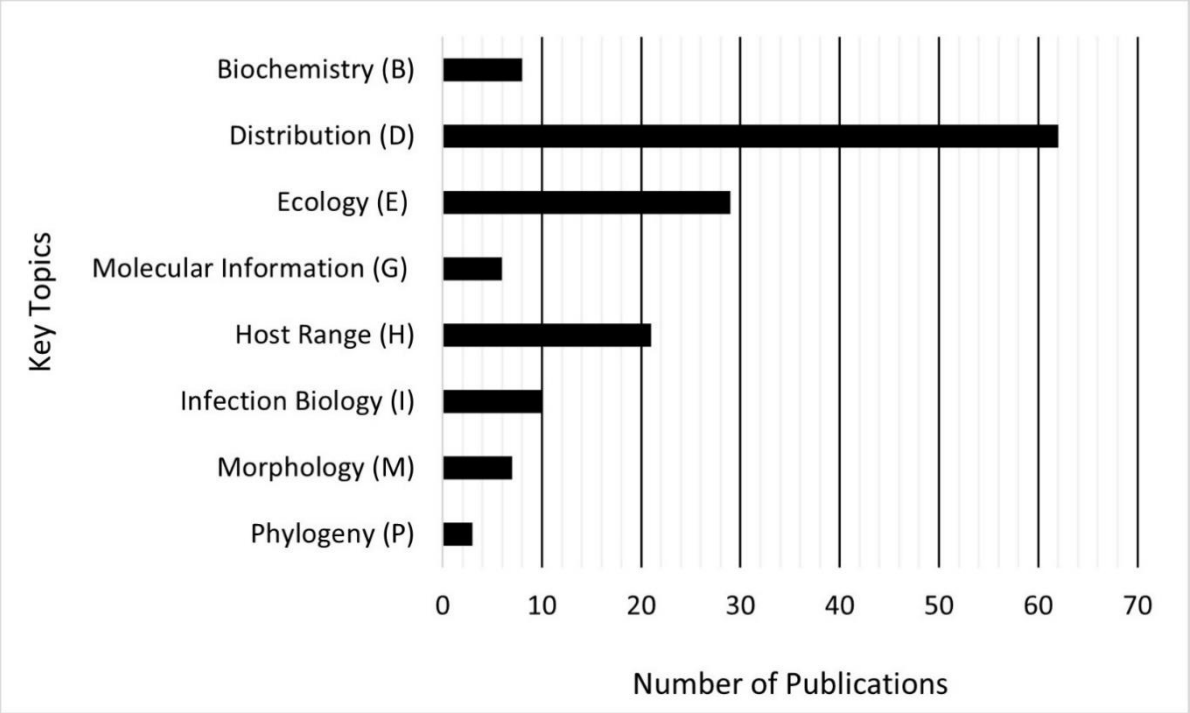

Figure 2.5. The number of publications mentioning each of the identified key topics in the analysed publications. Some publications covered more than one topic and were counted for each topic covered.

2.3.2 | Distribution of *Gymnopus fusipes*

Analysis of the existing literature on *G. fusipes* revealed that distribution is the most extensively reported aspect of the species, with 62 publications indicating a location of presence (Figure 2.6). Of these 62 publications, 49 used only macroscopic or microscopic analysis to confirm species presence, often in the field. This process requires high levels of skills in fungal identification and is often subject to seasonal sampling and environmental conditions. Ten publications reported isolation of *G. fusipes* from fruiting bodies or infected wood tissues as an identification method, which is reasonably reliable, but only 3 publications used molecular analysis to confirm species identification. Molecular identification is the gold standard for species identification, as once a new species

evolves, gene sequences change and can be identified before changes to morphology or mating behaviours occur (Taylor et al., 2000).

Much of the research concerning *G. fusipes* has been focused in Europe, with molecular based identification confirming the presence in France (Aguayo et al., 2021), Germany (Schmidt et al., 2012) and Belgium (Chandelier et al., 2021). Studies that isolated *G. fusipes* have been concentrated in France (Marçais et al., 1998, 1999, 2000b; Marçais and Caël, 2001), the UK (Boddy and Thompson, 1983; Campbell, 1939; Denman et al., 2017; Gibbs and Greig, 1990) and Poland (Przybyl, 1994). The presence of *G. fusipes* in these areas can therefore be considered with confidence. If macroscopic analysis is taken into account (with the aforementioned caveats), *G. fusipes* is suggested to have a distribution covering most of Europe (Piou et al., 2002), areas in the USA (Gabel et al., 2004), Mexico (Reverchon et al., 2010), northern Africa (Ben et al., 2013) and parts of Asia (Semwal and Bhatt, 2019).

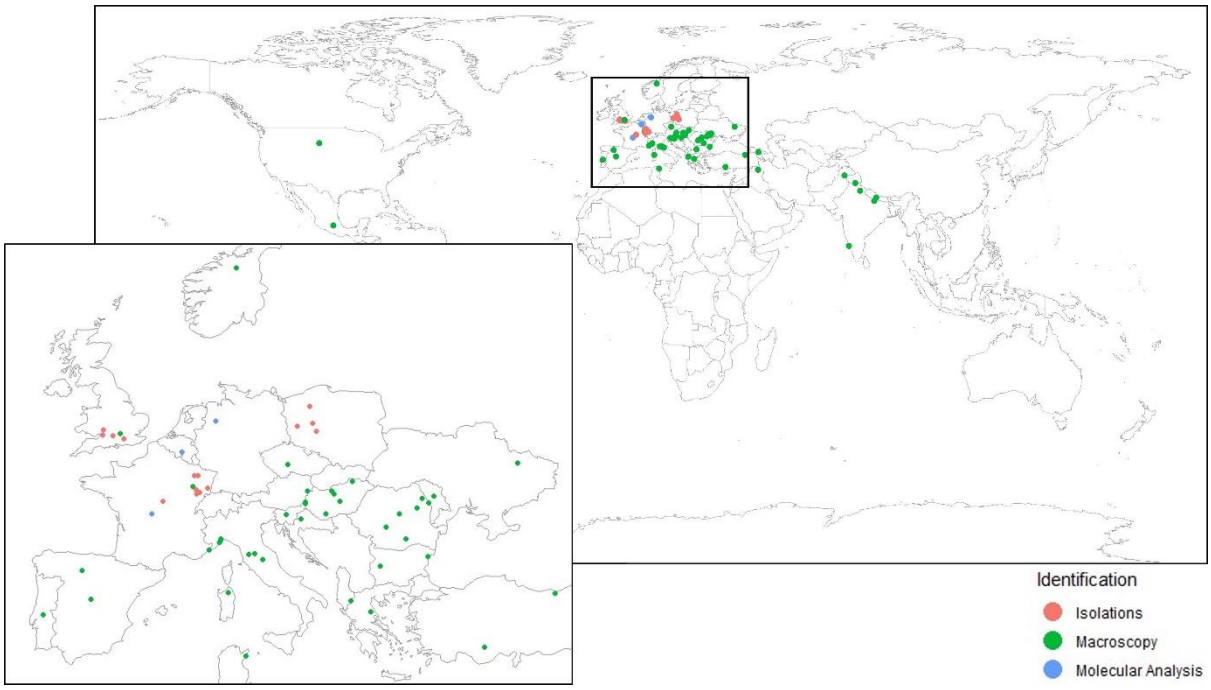

Figure 2.6. Currently known distribution of *Gymnopus fusipes*, created using data from the published literature (Supplementary Information 2.1). Molecular based identifications depicted in red, isolation-based identifications in green and macroscopic based identifications in blue.

2.3.3 | Ecology of *Gymnopus fusipes* in Forest Environments

Gymnopus fusipes is widespread where the environment is suitable, and although sometimes documented as a typical member of the oak associated fungal community (Watling, 2014), *G. fusipes* is actually categorised as a forest pathogen (Aguayo et al., 2021; Chandelier et al., 2021). *Gymnopus*

fusipes is most often found on tree stumps or at the base of living trees (Sardariu, 2013; Tortic and Lisiewska, 1978), however macroscopic identifications have suggested that *G. fusipes* may also occur in soil (Ambrosio et al., 2018) and leaf litter (Diamandis and Perlerou, 2001), although this finding may result from the stipe of the fruiting body being attached to a suitable organic matter substrate below the ground level, such as decaying wood or root tissue (Campbell, 1939).

Species in the genus *Gymnopus* are generally saprotrophic organisms, with some parasitic species, which utilise leaf litter and other forms of vegetation as nutrient sources (Arenal et al., 2006). It has been suggested that *G. fusipes* has two different ecological strategies (Przybyl, 1994). Firstly, as a parasitic biotroph, obtaining nutrients from the tissues of living hosts (Tortic and Lisiewska, 1978), and secondly, as a saprotroph, obtaining nutrients from dead tissues (Reis et al., 2011).

As a saprotroph, *G. fusipes* obtains nutrients from decaying wood (Chandrawati and Narendra Kumar, 2014; Murvanishvili et al., 2010; Vishwakarma et al., 2017). As a biotrophic parasite, *G. fusipes* obtains nutrients from the roots of the living host (Angelini et al., 2012; Thomas et al., 2002). The mechanisms utilized by *G. fusipes* in these scenarios are currently unknown.

Gymnopus fusipes is thought to be lignicolous (Laganà et al., 2002), suggesting it produces extracellular enzymes capable of degrading lignin, cellulose and xylans (Petre and Tanase, 2013). Lignicolous fungi are crucial for wood decay and carbon cycling, and can be saprotrophic, when metabolising dead organic matter, or saproparasitic, when obtaining nutrients from the roots of the living host, therefore referred to as a "saproparasite" (Sardariu, 2013; Sardariu and Mititiuc, 2009).

Aspects of *G. fusipes* ecology are linked very closely to its distribution, as geographical factors and climate have a considerable influence on the success of many species. *Gymnopus fusipes* thrives in soils with a low pH, low nutrient availability and a high sand content, a coarse soil texture is also preferred, presumably for the increased drainage provided (Camy et al., 2003c). These conditions are crucial to the survival of *G. fusipes* as well as many other species of plants and fungi (Camy et al., 2003c), as waterlogging can lead to hypoxia, a condition whereby gas exchange between the organism and the atmosphere is drastically reduced, leading to build-up of toxic compounds such as metal ions and organic and volatile acids (Bourgeade et al., 2018). It has been demonstrated that there is increased presence of *G. fusipes* in non-waterlogged soils, and the fitness and survival of *G. fusipes* is negatively affected when waterlogging occurs (Camy et al., 2003a, 2003c), or groundwater levels are high (Piou et al., 2002).

It was confirmed, through somatic incompatibility tests (Marçais et al., 2000a), that *G. fusipes* spreads via basidiospores, which germinate on the root collar of potential host trees, resulting in a scattered distribution of the pathogen throughout a forest ecosystem (Marçais et al., 1998).

2.3.4 | Host Range of *Gymnopus fusipes*

The host range of *G. fusipes* has a strong influence on distribution and ecology, and the species has been reported in association with 13 genera of host species, including both angiosperms and gymnosperms (information from the literature in Supplementary Information 2.1). Unfortunately, the majority of these reports do not demonstrate a clear and direct link between *G. fusipes* and a specific host, and it is merely noted to be associated with a forest dominated by a particular host species. From the literature in this analysis (Supplementary Information 2.1), direct associations between *G. fusipes* and three tree genera were made, *Quercus, Castanea* and *Abies,* with *Quercus* spp. making up the majority of associations. Molecular identifications have been made only from *Quercus robur* and isolations have been made only from other *Quercus* species (Figure 2.7). Different *Quercus* species appear to vary in susceptibility to *G. fusipes* infection (Marçais and Caël, 2000), with *Q. rubra* (red oak) being more susceptible than *Q. robur* (pedunculate oak) which is more susceptible than *Q. petraea* (sessile oak). This observation further highlights the importance of both fungal isolation studies and molecular identification to improve our understanding of the distribution and host range of *G. fusipes*.

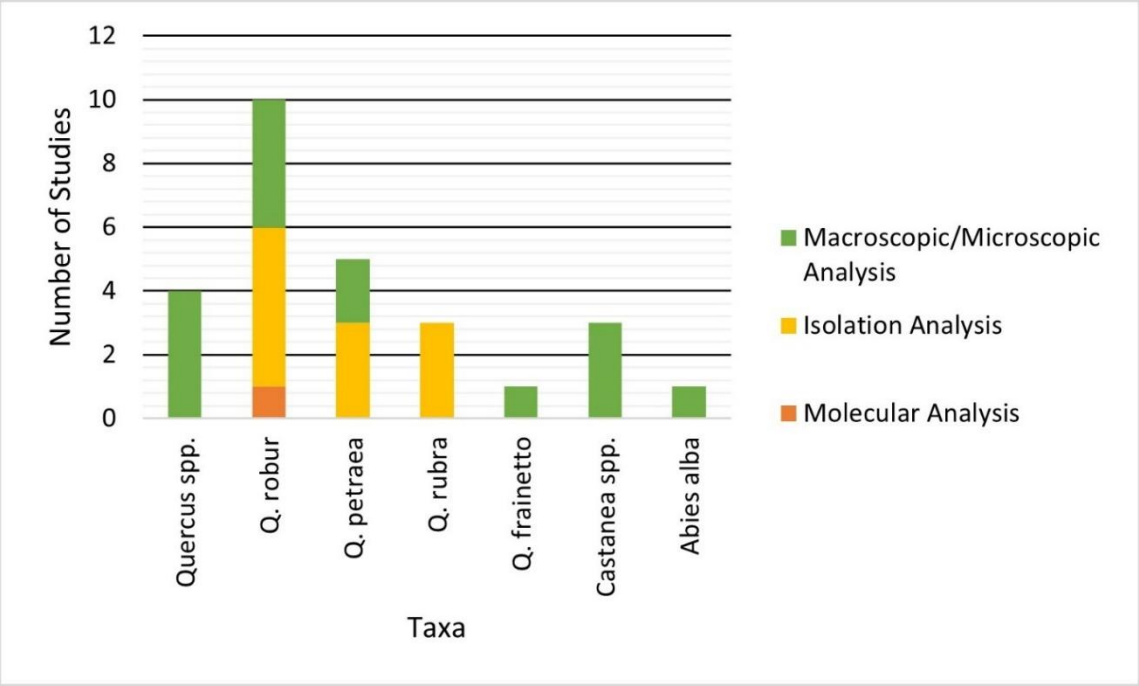

Figure 2.7. Number of studies that have focussed on *Gymnopus fusipes* association with different tree host genera. Colour is indicative of methods used for identification (macroscopy, isolations, or molecular analysis).

2.3.5 | Infection Biology of *Gymnopus fusipes* and links to Chronic Oak Decline

Although described in the 1780s, *G. fusipes* was only documented as a pathogen in the 1980s, when it was reported to cause root rot on oak trees (Marçais et al., 2000b). Prior to this, *G. fusipes* was suggested to be a miscellaneous oak decline agent, responsible only for the initial weakening of the tree host and increasing susceptibility to attack by secondary pathogens (such as those in the genus *Armillaria*), or a typical member of the oak associated fungal community (Watling, 2014). However, since being described as a pathogen, *G. fusipes* has been documented as a primary root pathogen, capable of colonizing young and healthy root systems as well as mature hosts (Camy et al., 2003a; Marçais and Delatour, 1996). The species is now known be involved in chronic oak decline, with a crucial role in disease development through deterioration of the root system and eventual disruption of water conduction (Marçais et al., 2000b).

Oak decline, including chronic oak decline in the UK, involves the combination of abiotic and biotic factors which lead to reduced vigour and eventual mortality of a tree host (Camy et al., 2003b; Manion, 1981; Marçais et al., 1999). Decline factors that are particularly important in *G. fusipes* infection include soil type and condition, weather events, and moisture levels (Camy et al., 2003d), as these factors not only weaken the tree host, but are important in infection success. *Gymnopus fusipes* is negatively affected by waterlogging and high levels of ground water, leading to impaired growth, survival and infectivity success (Camy et al., 2003a; Piou et al., 2002).

Gymnopus fusipes infection develops at the root/soil interface under favourable soil conditions (Camy et al., 2003a) and progresses deeper into tissues of the tree, including the vascular cambium, where less susceptible hosts are able to defend against infection (Marçais and Caël, 2000). *Gymnopus fusipes* infection then progresses to the sapwood, causing large amounts of damage to the functional tissues of the host before spreading through the entire root system (Przybyl, 1994).

A characteristic symptom of oak decline is a deteriorating crown condition, although with *G. fusipes* infection, this symptom does not always correlate with a severe infection (Camy et al., 2003d; Marçais and Caël, 2001). In an extensive infection, a large percentage of the host root system can be damaged or killed (Marçais et al., 1999), however, trees can survive long after infection due to the surviving roots continuing to absorb water from deep beneath the ground, on occasion, adventitious roots develop to mitigate the effects of root loss (Marçais et al., 2000b, 1999; Marçais and Caël, 2001). This response could explain why a mature host it can take approximately thirty years from initial infection for *G. fusipes* to cause such a drastic infection (Camy et al., 2003a).

Although fruiting bodies could be considered the most distinctive sign of *G. fusipes* infection, these are not always present (Piou et al., 2002; Thomas et al., 2002). In some cases, high numbers of trees

in a forest ecosystem may present no fruiting bodies over a number of years, but still have lesions on the root system (Marçais et al., 2000b). The most definitive symptom of *G. fusipes* infection is the presence of yellow, or orange coloured necrotic lesions on the main roots of the host beneath the soil line (Camy et al., 2003a). In more advanced stages of infection, this presents as dark orange wet rot, which extends deep into the sapwood of the tree (Marçais et al., 1999; Przybyl, 1994). Consequently, *G. fusipes* lesions are likely to be undetected due to the lack of obvious above ground symptoms, potentially resulting in infected hosts remaining undiagnosed for long periods of time, leading to underestimations of the impacts of *G. fusipes*.

It is proposed that *G. fusipes* can work in tandem with numerous other species in the complex aetiology of oak decline, including *Armillaria* species, with which *G. fusipes* shares an ecological niche, and *Phytophthora* species (Marçais et al., 2011). For example, mature oak trees co-infected with *G. fusipes* and *Armillaria* species, present severe decline symptoms, including twig shedding and poor crown density (Denman et al., 2017).

2.3.6 | Morphology and Lifecycle of *Gymnopus fusipes*

The genus *Gymnopus*, includes approximately 350 species, each with a small brown fruiting body, tough stipe and white spore print (Coimbra et al., 2015; Dutta et al., 2015). Species in this genus are also often compared to members of closely related genera, especially *Collybia* (Vinet and Zhedanov, 2011), due to the similar morphological characteristics of many genera in the family Marasmiaceae/Omphalotaceae (Putra, 2020).

A distinguishing feature of *G. fusipes* fruiting bodies is the tough tapering stipe, which decreases in diameter from the top (the cap) to the base and led to the common name of "spindle-shank" (Pegler, 2001; Ványolós et al., 2016). The stipe, which appears rooted into the ground, is sometimes referred to as a pseudorhiza (Petersen and Hughes, 2017), or can be considered a pseudosclerotium, a dense group of hyphae that connect to colonized roots beneath the ground level (Campbell, 1939). The stipe of *G. fusipes* is tough in texture and able to withstand adverse weather conditions such as wind (Halbwachs et al., 2016). This tough texture and rigidity is potentially due to the presence of sarcodimitic tissues in the stipe (tissues which contain two types of interacting hyphae in a single tissue), especially at the cap end of the pseudorhiza, which results in the increased width at the top (Redhead, 1987). The pseudorhiza of *G. fusipes* is thought to be perennial which can result in fruiting bodies appearing in the same locality repeatedly (Norvell, 1998).

Gymnopus fusipes is thought to utilise two ecological strategies, parasitic and saprophytic, which may influence the morphology of the fruiting bodies. As a saprophyte on decaying tree stumps and roots, the basidiocarp is usually darker in colour, and smaller than when in the parasitic mode,

fruiting as individual basidiocarps. When the species is acting as a parasite on the base of living hosts, the basidiocarps are lighter in colour, larger in size, and produced in groups. The parasitic phase fruits around the beginning of September, approximately two weeks later in the season than when in the saprotrophic life strategy which tends to fruit towards the end of August (Przybyl, 1994).

Gymnopus fusipes does not produce traditional cords (Boddy and Thompson, 1983), however it can sometimes be found to produce cord-like structures on the bark surface, which have been suggested to aid in the spread of *G. fusipes* at the bark level (Marçais et al., 1999). *Gymnopus fusipes* spreads through the forest ecosystem via basidiospores, which has been proven by somatic incompatibility testing (Marçais et al., 2000a).

The lifecycle of *G. fusipes* has not been extensively investigated, therefore it can only be assumed that the species follows a similar life cycle to other agaricomycetes (Figure 2.8). In this type of lifecycle, basidiospores are dispersed and germinate to form monokaryotic hyphae. Monokaryotic hyphae, with a single haploid nucleus in each cell compartment, separated by a septum (in the case of *G. fusipes*, as with most basidiomycetes, a septum with a dolipore system and perforated septal pore cap to prevent the movement of nuclei between the compartments) meet, and if compatible, fuse through anastomosis, the process by which single hyphae fuse to form a branching network, crucial for many fungal processes such as the exchange and migration of nuclei, nutrient flow and mycelial homeostasis, which is imperative for survival (Novais et al., 2017). This process leads to the development of dikaryotic hyphae, with two haploid nuclei in each cell compartment and the formation of clamp connections between cells, which is extremely stable during vegetative growth (Gao et al., 2019). Environmental conditions stimulate the production of a dikaryotic fruiting body (mushroom), on which form specialised structures called basidia: the process of karyogamy (fusion of two haploid cells to form one diploid cell) and subsequent meiosis (cell division to produce four haploid basidiospores) occurs. The resulting basidiospores are dispersed and the cycle continues (Casselton and Olesnicky, 1998).

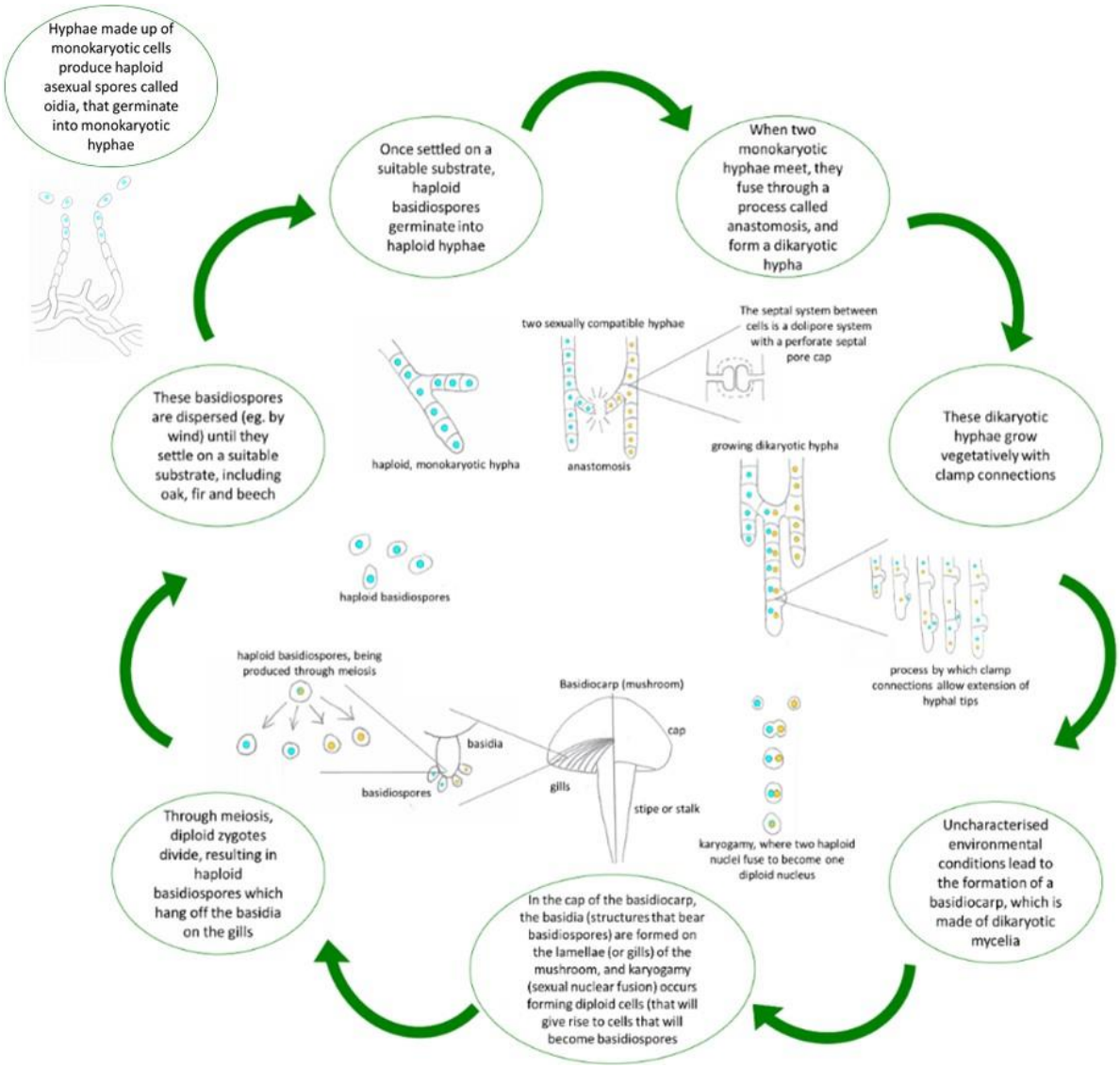

Figure 2.8. Diagrammatic representation of the typical agaricomycete lifecycle. This hypothetical framework was compiled to demonstrate the probable lifecycle of *Gymnopus fusipes*.

2.3.7 | Somatic Incompatibility and Genomic Analysis of *Gymnopus fusipes*

There is a noticeable lack of genomic analyses of *G. fusipes*, with only 6 publications presenting molecular data. This problem is reflected in databases such as NCBI GenBank and Unite which, at the time of writing, contained only 15 and 1 nucleotide sequences for *G. fusipes* respectively.

The majority of genetics-based work on the species has focused on somatic incompatibility, the prevention of fusion between two genetically incompatible hyphae (Worrall, 1997). Somatic incompatibility in *G. fusipes* is suggested to be controlled by at least three multiallelic gene loci responsible for the response exhibited in vegetative incompatibility reactions (Marçais et al., 1998), and that a strong incompatibility response is controlled by just one locus out of a possible three or more loci involved in compatibility (Marçais et al., 2000a). Somatic incompatibility experiments

illustrated that isolates from neighbouring tree root systems are mostly incompatible, indicating that they are genetically distinct, providing evidence to show that *G. fusipes* does not spread via root-to-root contact, and supporting the hypothesis that *G. fusipes* spreads via basidiospores (Marçais et al., 2000a).

The lack of genomic analysis specifically relating to *G. fusipes* has resulted in an incomplete and changing phylogeny depending on the gene being analysed. Research using the nuclear ribosomal internal transcribed spacer (nrITS) and large subunit regions proved that the genus *Collybia* falls into a completely different clade to *Gymnopus* and *Rhodocollybia*, despite these genera previously all being classified as *Collybia* (Antonin et al., 1997; Mata et al., 2004). Single gene phylogenetic analysis can provide different results depending on the gene targeted; for example, *G. fusipes* grouped with different species in analyses using the large ribosomal subunit (LSU) compared with the internal transcribed spacer (ITS) region (Saar et al., 2009).

Traditionally the classification of fungal species (phylogeny, taxonomy, systematics) was determined by morphological analysis of macroscopic and microscopic characteristics as well as chemical characteristics (Pfyffer et al., 1986). However, it is suggested that there are too many variables in morphological analysis for this process to be totally accurate. For example, *G. fusipes* was documented to have morphological differences (size, colour, fruiting schedule, number of fruiting bodies) depending on whether it is acting as a saprotroph or a parasite (Przybyl, 1994).

A more complete and state-of-the-art approach to classification is therefore needed, which includes both morphological data and molecular information to provide more accurate identification of fungal species. A comprehensive database storing all of the relevant details on fungal species, such as “Faces of Fungi” would be an ideal scenario (Jayasiri et al., 2015), but would need to be widely used and regulated to be effective and accurate.

2.3.8 | Biochemical Properties of *Gymnopus fusipes* and Potential for Commercial Application

Although there is a lack of information on the biochemistry of *G. fusipes,* there have been some investigations of the biochemical properties of this species. For example, it is known that *G. fusipes* possesses genes encoding laccase production, which is a class of enzymes important in lignin degradation in white-rot fungi (Luis et al., 2005, 2004).

Wood decay fungi can be grouped by the different types of decay caused. Brown-rot and soft-rot fungi, degrade mainly cellulose, whereas white-rot fungi degrade both cellulose and lignin, leading to a more complete degradation of host tissues (Pandey and Pitman, 2003). White-rot fungi produce

a number of classes of extracellular enzymes such as lignin peroxidases, manganese peroxidases and laccases (Hatakka, 1994).

Fungi produce secondary metabolites, compounds with bioactive properties which help the fungus adapt to the environment (Hautbergue et al., 2018). *Gymnopus fusipes* produces exudates when grown in culture on different types of media (Petre and Tanase, 2013). These exudates contain compounds with antimicrobial properties and are often produced in the environment when in competition with other microbes. *Gymnopus fusipes* produced antimicrobial compounds (Wilkins, 1952) active against the bacterial species *Pseudomonas aeruginosa,* a widely antimicrobial-resistant pathogen known to cause infection in plants and animals (including humans) with a high mortality rate (Poole, 2011), *Serratia marcescens,* a pathogen affecting immunocompromised humans in various capacities (Cristina et al., 2019), and *Bacillus subtilis,* a ubiquitous bacteria used as a model organism for studying numerous prokaryotic processes (Su et al., 2020; Suay et al., 2000).

In recent years, the biochemical properties of *G. fusipes* have been studied closely in relation to human health. Compounds extracted from *G. fusipes* have the potential to block G-protein-coupled inwardly rectifying potassium (GIRK) channels (Ványolós et al., 2019), the abnormal functioning of which has been linked to cardiac arrhythmia as well as other disorders such as neuropathic pain, and drug addiction (Walsh, 2011). *Gymnopus fusipes* has also been studied in connection with the recently isolated and biosynthesised Gymnopeptides A and B, natural products originally extracted from the species, and found to have potential anti-cancer activities (Ványolós et al., 2016). With further analysis, these gymnopeptides were found to be members of the borosin RiPP family of peptides, with the potential to be up to one thousand times more potent in antiproliferative cancer activities than cisplatin, which is currently used as the positive control in anti-cancer studies (Quijano et al., 2019) . These gymnopeptides were effective in antiproliferative activities against a number of human cancer cell lines including cervical, skin and breast (Ványolós et al., 2016).

2.4 | Conclusions and Research Priorities

In this literature review, existing knowledge on the distribution, ecology and infection biology of the root rot pathogen *Gymnopus fusipes* has been synthesised. A systematic literature search across five databases revealed that there are relatively few primary publications pertaining to *G. fusipes* compared to other tree associated pathogens, such as *Dothistroma septosporum* and *Fusarium circinatum,* highlighting the need for more focussed work on the species.

Gymnopus fusipes is confirmed to be common throughout Europe, however there are records linking it to much of the northern hemisphere including countries in Europe, North America, northern Africa

and parts of Asia. The pathogen has been confirmed to associate with 3 genera of host tree, although it has been linked to tree species across 13 genera including both broadleaf and coniferous species. The most common association documented in the literature is with the oak species, *Quercus robur.* Many factors relating to the distribution and host range of *G. fusipes* affects its survival and infection success, such as temperature, soil type and weather. *Gymnopus fusipes* has the typical morphology of an agaricomycete with a stipe tapering in width towards the base, however this tapering is often below the ground level. The basidiocarps can appear individually (saprophytic lifestyle) or as small clusters (parasitic) and vary in size and colour, although the caps are always brown.

Gymnopus fusipes is a saproparasite, with the ability to function as either a saprophyte or parasite, although the factors determining the ecological strategy are currently unknown. As a slow growing primary pathogen, *G. fusipes* has a key role in the early stages of oak decline, following the decline spiral. Infection by *G. fusipes* often goes unnoticed due to the lack of correlation between the above ground symptoms and the infection status of the host. Symptoms of infection by *G. fusipes* can include a deterioration of the crown and the presence of fruiting bodies (although neither are always present), but always includes orange necrotic lesions on the root tissues and small white mycelial fans beneath the bark. In a severe infection, whole root systems can be destroyed, leaving the tree host vulnerable to wind failure.

Gymnopus fusipes spreads from host to host via basidiospores, which has been confirmed through somatic incompatibility tests that illustrated that neighbouring trees are infected by genetically distinct isolates. Other genomic information regarding *G. fusipes* is lacking, and there is a severe deficit of molecular data present in databases such as NCBI GenBank and Unite Fungal database. Although there have been some reports on gene expression in the existing literature, it is basic and requires further attention. The biochemical properties of *G. fusipes* are of interest, as certain fungal products have antiproliferative activities against a number of human cancer cell lines.

Future work should aim to use molecular techniques to confirm the distribution of *G. fusipes,* and also to clarify host range. It is crucial that more focussed research is performed to increase understanding of the factors influencing the *G. fusipes* life strategy of saprophyte or parasite, and whether the former can change into the latter. This investigation could help to explain why there is protracted delay between infection and mortality of the tree host. The mechanisms by which *G. fusipes* degrades tree root tissues, and the genes expressed at each stage of infection also need to be examined in order to provide a timeline of infection and the processes occurring at each stage which would be highly beneficial to informing management. There would also be a benefit to

increasing the number of nucleotide sequences available in databases such as NCBI GenBank and in preparing an up to date phylogeny based on multiple genes. This work would help to create a rapid diagnostic tool that could be deployed both in the field and the laboratory to quickly and accurately identify *G. fusipes* infection.

In conclusion, *G. fusipes* is an understudied root rot pathogen, capable of destroying almost the whole root systems of host trees, leading to a high risk of wind failure. *Gymnopus fusipes* not only impacts tree health, but with a reasonably large number of potentially susceptible host trees being present on lands open to the public (country parks and gardens), *G. fusipes* also has the potential to threaten human safety. More research is required to fill the knowledge gaps in the literature, in order to help inform management.

2.5 | Acknowledgements

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CHAPTER 3

Optimising Methods for Isolation, Culture, Nucleic Acid Extraction and Phylogenetic Analysis of *Gymnopus fusipes*

Abstract

Gymnopus fusipes (syn. *Collybia fusipes*) is an understudied basidiomycete fungus, known to be the cause of severe root rot on a number of socioeconomically important tree species, in forests across temperate regions. It is therefore important to understand *Gymnopus fusipes*, so we can begin to develop strategies to limit its spread and combat the negative impacts of this pathogen. As alluded to in Chapter 2, there is a lack of research dedicated to this forest pathogen and therefore a paucity of information on how best to isolate and culture this species. This work aims to address this, by documenting the optimisation of methods for culture-dependant and independent techniques for *G. fusipes*, including best practice for isolation from environmental samples, culturing conditions, and extraction of nucleic acids (DNA and RNA) from isolates, fruiting bodies and infected woody tissues, this is accompanied by the construction of a phylogenetic tree of *G. fusipes* based on the internal transcribed spacer (*ITS*) gene region. This work is crucial to develop a methodological framework for studying the ecology and biology of *G. fusipes* in this thesis and provide optimised protocols that enable future research studies on *G. fusipes*.

3.1 | Introduction

Through exploration of the existing literature conducted in Chapter 2 (Pettifor et al., 2022), it was found that *Gymnopus fusipes* (syn. *Collybia fusipes*) is a relatively understudied wood decay fungus, with a broad distribution potentially spanning much of the northern hemisphere (Gabel et al., 2004; Kholfy et al., 2014; Piou et al., 2002; Senthilarasu, 2014) and a host range potentially encompassing 13 genera of broadleaf and coniferous trees (Akata et al., 2014; Ambrosio et al., 2018, 2015; Boddy and Thompson, 1983; Ghate et al., 2014; Piou et al., 2002; Ramshaj et al., 2021; Thomas and Polwart, 2003; Zervakis et al., 1998). *G. fusipes* is documented to cause drastic root rot and has been linked to chronic oak decline (COD) in the UK (Quine et al., 2019), and similar decline episodes across Europe (Thomas et al., 2002).

Infection by *G. fusipes* develops from the collar of the tree host, after germination of a basidiospore, and progresses downwards through the root system (Marçais et al., 1998). Orange lesions and white mycelial fans in the root tissues are characteristic of infection (Guillaumin et al., 1985), however these are only visible with excavation of the root structure, and above ground signs such as a deteriorated crown and fruiting body presence are not always observed, even with a high level of infection (Camy et al., 2003c). Severe infection and a deteriorated root system coupled with a full crown, can result in an increased chance of wind failure. The potential for *G. fusipes* infection to remain undetected for long periods of time results from the lack of above ground symptoms of infection, and the often incorrect assumption that the decline is caused by species from the genus *Armillaria,* as the two species have been found to co-exist in the same host (Marçais et al., 1999).

Only fifteen published works provide information on isolation of *G. fusipes* from the environment, as well as best practices for cultivation of this species in the lab. Only one publication provides a method for long term storage of *G. fusipes* (Delatour, 1991), however there is little information on short-term maintenance of cultures and its effect on culture viability, which represent critical steps when working with this slow growing species. There is also a significant lack of DNA-based analyses of *G. fusipes*, with (as of January 2023) only 15 nucleotide sequences being available on the NCBI GenBank Nucleotide Database (https://www.ncbi.nlm.nih.gov/genbank/), and no specific phylogenetic analysis having been completed on different isolates of the species. This is a major barrier to understanding the ecology, biology and genetic diversity of *G. fusipes*.

The main aim of this chapter is to develop a suite of optimised methods for isolation, cultivation and nucleic acid extraction for *G. fusipes*, and to provide some molecular analysis on the species in the form of a phylogenetic tree to determine relatedness of isolates. The aim of the study will be met through the completion of the following objectives:

i. To isolate *G. fusipes* strains from environmental samples including fruiting bodies and infected woody tissues.

ii. To optimise agar-based techniques for the growth of *G. fusipes*, including growth medium type and lighting conditions.

iii. To test and optimise methods for extracting nucleic acids from different *G. fusipes* sample types, including mycelial cultures, fruiting bodies and infected woody tissues.

iv. To optimise PCR assays for commonly used fungal housekeeping genes and use these to produce a phylogeny of different *G. fusipes* isolates.

This work presented in this chapter describes a number of small exploratory experiments which resulted in a combination of empirical data and qualitative observations. Where possible, statistical analyses have been conducted, and these are outlined with the relevant methods.

3.2 | Materials and Methods

3.2.1 | Building a Culture Collection of *Gymnopus fusipes* Strains

Attempting to Obtain *Gymnopus fusipes* from Culture Collections and Previous Studies

Obtaining isolates of *G. fusipes* from global culture collections was a significant challenge. After an extensive search, it was found that *G. fusipes* was not present in any of the widely used culture collections such as DSMZ (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell cultures GmbH (https://www.dsmz.de/)) or the CABI Genetic Resource Catalogue (The UKBRCN CAB International (CABI) Culture Collection (https://www.ukbrcn.org/)). Although records of *G. fusipes* strains were found on the WFCC (World Federation for Culture Collections (https://wfcc.info/)) site in the Global Catalogue of Microorganisms database (https://gcm.wdcm.org/), technical issues with the webpage and database prevented procurement from this site.

Corresponding authors of 21 publications (Supplementary information 3.1) mentioning work on *G. fusipes* were contacted in an attempt to obtain viable isolates of the species. Responses from authors were limited, and those that did respond stated that the work was based primarily on visual observations of macro- and microscopic characteristics, and that they did not perform any culture-based work or isolations. In Chapter 2, it was highlighted how there is a real black of primary research on this species, and in searching for isolates of *G. fusipes* to work with this was further emphasised.

Dr Benoit Marcais (French National Institute for Agriculture, Food and Environment (INRAE)), the corresponding author for much of the earlier work done on *G. fusipes*, was contacted, and after being revived from long term storage, three strains of *G. fusipes* (C41, C49 and C52) were provided as mycelial plate cultures. Each of the three strains was originally isolated from the tissues of an infected oak host at three different sites in France.

Field Sampling Campaigns for *Gymnopus fusipes*

In the years of the project, between 2019 and 2022, various field sampling campaigns were conducted by members of the wider UK Oak decline research group. If *G. fusipes* infection was observed at the sites, members of the group were instructed to collect any fruiting bodies and take woody tissue samples if permissions allowed. A dedicated field sampling campaign was held specifically to obtain samples of *G. fusipes* in June 2022, which is documented in Chapters 5 and 6.

Collecting *Gymnopus fusipes* Samples, Including Fruiting Bodies and Infected Woody Tissues

As described in Chapter 2, *G. fusipes* infection does not always present above ground symptoms, such as the presence of fruiting bodies and host crown deterioration, typical of declining trees. This means it can be challenging to identify and diagnose *G. fusipes* infection. Further to this, the fruiting triggers of *G. fusipes* are currently unknown, and the fruiting season in the UK appears to be variable, meaning that some fruiting bodies observed in the typical sampling season (summer) can already be degraded and unsuitable for collection.

It was noted in this study, that when taking samples of fruiting bodies, it is important to consider the condition of the sample. In the following exploratory work, it was found that obtaining fresh fruiting bodies with little to no signs of decay was crucial, as fruiting bodies that had already begun to deteriorate in situ degraded rapidly once removed from the site. Fruiting bodies were collected as soon as possible after identification and were stored in paper bags or envelopes, as this prevented the build-up of water when in transit, which was found to compromise the condition of the fruiting body and led to difficulty when attempting to isolate. Fruiting bodies were processed immediately after collection only, due to the rapid degradation in sample quality that was often observed.

Woody tissue samples were collected from previously identified hosts impacted with *G. fusipes* infection, using as close to sterile techniques as possible. This involved brushing away any debris or topsoil from the root system, and then using sterile chisels and forceps to cut and take small pieces of infected tissue from the live/dead junction on the lesion margin, where there were often white mycelial fans. Woody tissue samples were stored and sealed in suitable containers, such as 50 ml Falcon tubes, or small plastic bags and kept in a cool box in transit. Infected woody tissues were processed immediately after sample collection, and again after a number of weeks having been stored at 4˚C (between 8 and 12 weeks).

Testing Different Surface Sterilisation Methods

Due to the high number of contaminants often present in environmental samples, such as fruiting bodies and infected woody tissue, isolation from these tissues whilst avoiding contamination from other non-target microorganisms can be challenging. Slow growing species, such as *G. fusipes*, are

more difficult to isolate, due to the high risk of bacterial or fungal contaminants overgrowing them and quickly colonising the medium (Indunil Chinthani et al., 2020), making later subculturing difficult. To address these issues, various steps are followed to ensure the best attempt at isolation. After appropriate storage (outlined above), samples were cleaned thoroughly, by removing any debris from the field, and then surface sterilising before being plated to help reduce any potential contamination, and isolation plates must be checked regularly for growth of non-target microorganisms.

Various methods of surface sterilisation were tested for their effectiveness in sterilising environmental samples including fruiting bodies and infected woody tissue. These methods involved using a 10% bleach solution to surface sterilise the whole sample before cutting into smaller pieces and subjecting to one of five further wash steps before being plated onto ½ strength malt extract agar. These treatments included; a non-wash control, a water wash involving dipping the sample in sterile water for 10 seconds before drying with clean soft tissue (fresh sterile water was prepared for each individual piece of sample), an ethanol wash involving dipping the sample in 70% ethanol for 10 seconds before allowing to air dry, a bleach wash involving dipping the sample in the 10% bleach solution for 10 seconds before drying with clean soft tissue, and a flame treatment which involved passing the sample through the flame of a Bunsen burner 3 times before plating. These methods were trialled on a selection of the environmental samples, including fruiting bodies and infected woody tissue, in the autumn of 2021.

Collection and Germination of *Gymnopus fusipes* Basidiospores

Fungal isolates can also be obtained via collection of basidiospores (Lakkireddy and Kües, 2017), however this can also be difficult if fruiting bodies are not present, or if the fruiting bodies have degraded. In this work, caps from fresh fruiting bodies were placed gills down on an empty petri dish and covered with a glass jar. A piece of slightly dampened tissue was placed in the glass jar before it was placed over the top of the cap, and the caps were left over one or two days. Once a spore print had been produced, spores were collected by scraping a sterile inoculation loop over the spores and transferred into a 1.5 ml Eppendorf tube containing 50 µl of sterile nuclease free water. A fresh inoculation loop was used to collect approximately 10 µl of this suspension and spread over ½ strength malt extract agar plates or stabbed approximately 1 cm deep into an agar slant (25 ml of agar in a 50 ml falcon tube set at an angle), where the lid was not sealed fully during incubation.

Culture Collection Curation

Strains that were successfully isolated in this study were maintained on ½ strength malt extract agar, on agar plates, that were sub-cultured approximately once per month, and on agar slants that could be stored at 4˚C for up to 3 months.

3.2.2 | Cultivation of *Gymnopus fusipes* Strains

Culture medium containing malt extract, was found to be the most frequently used medium for the cultivation of *G. fusipes* in the literature (Camy et al., 2003a; Marçais and Caël, 2000; Marçais and Delatour, 1996). Isolates were initially cultured and maintained on full strength malt extract agar (Malt Extract Agar (Merck) composition: Malt extract, 30 g/L; Mycological Peptone, 5 g/L; Agar 15 g/L), however in exploratory work, the strains were found to grow slightly better on half-strength Malt extract medium (composition: Malt Extract Agar (Merck) 25 g/L; Agar Technical No. 2 (Oxoid) 25 g/L). Using half strength malt extract agar resulted in a slightly thicker appearing biomass. This was therefore used for isolation and culture maintenance throughout the study. Small scale experiments were designed and conducted to determine whether changing the culture medium or lighting conditions would increase the growth rate of the isolates.

Testing Different Culture Media for the Growth of *Gymnopus fusipes*

Different media types were tested in order to find the optimal conditions for the growth of *G. fusipes*. The experiment involved triplicate cultures of *G. fusipes* being grown on three different media types. This was repeated for each of the following 5 isolates, C41, C49, C52, AH1 and GMW83, along with triplicate non-inoculated plates (Figure 3.1). The experiment was run for 28 days at ambient room temperature, under naturally occurring day/night lighting conditions, and the diameter of the colonies was measured approximately once per week.

Media conditions were as follows; half-strength malt extract agar (as above), half-strength malt extract agar with the addition of 1 ml/L ethanol and carrot agar (composition: Agar Technical No. 2 (Oxoid) 15 g/L, juice from 350 g carrots (carrots washed, chopped, and blended and then passed through a muslin cloth), made up to 1 L with distilled water). The addition of ethanol to the culture medium was derived from Weinhold (1963), who found that adding ethanol stimulated growth in *Armillaria* species. As *G. fusipes* and *Armillaria* spp. occupy the same ecological niche in the tree host system, it made sense to include this in the experiment. Carrot agar is another medium used to cultivate root rot organisms such as *Armillaria* and *Phytophthora* (Ford et al., 2017; Fraser et al., 2020; Rees et al., 2022), therefore this was also selected for the experiment.

Testing Different Lighting Conditions for the Growth of *Gymnopus fusipes*

Light availability is a requirement for a number of processes crucial to survival (Pawlik et al., 2019). Therefore, the following experiment was designed to test whether lighting conditions had an effect on the growth of *G. fusipes* on agar plates. The previously described experimental set up (used for the culture media type testing) was used as the "light" condition, as cultures had been subject to natural day/night schedules. An identical experimental set up (Figure 3.1) was created and conducted simultaneously at ambient temperature under complete darkness.

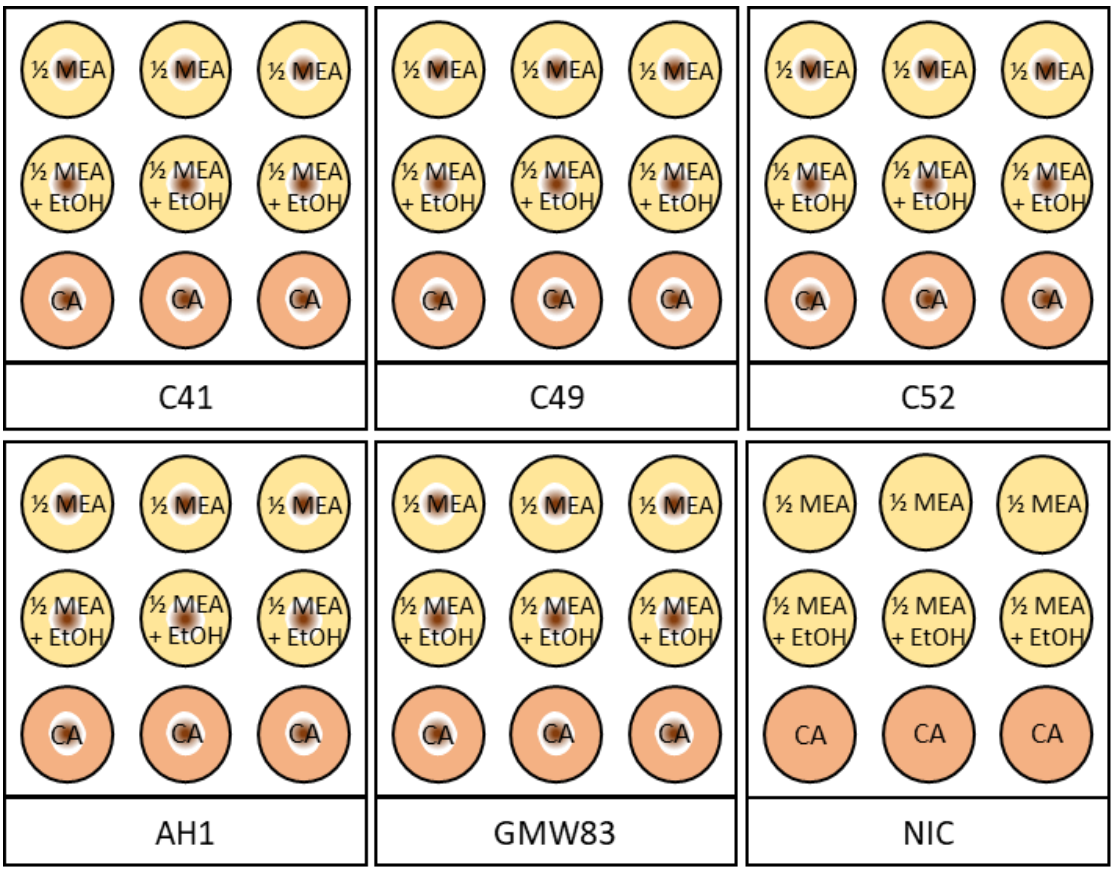

Figure 3.1. Experimental set up for media testing and lighting experiments for optimal growth of *G. fusipes*. Triplicate replicates of each of the five isolates (as well as a non-inoculated control) were cultured on each of the media types, ½ malt extract agar, ½ strength malt extract agar with the additional of ethanol and carrot agar medium. An identical experiment was conducted under complete darkness.

Data analysis

A general linearised model was generated in R (version 4.1.1) to determine whether the medium type or light conditions had a significant effect on the measurement of colony diameter at day 28 of the experiment. As the data appeared to be normally distributed, the Guassian (symmetrical

response) variable was designated, and this was tested with a log link and also a square root link (as the data was area related), however both links resulted in an insignificant result when the Chi Square test was completed.

3.2.3 | Reliable Methods for Nucleic Acid Extraction from Environmental Samples

Extracting high quality nucleic acids (DNA and RNA) from environmental samples such as soils and woody tree tissues can be difficult, due to the presence of chemical compounds which can inhibit reactions in downstream processing. Methods for extracting nucleic acids from samples such as soils, fruiting bodies and infected woody tissues must generally be optimised depending on the compounds present. Soils are known to contain high levels of polysaccharides, phenolic compounds and humic acids, which are often co-extracted with DNA and cause issues in down-steam reactions such as inhibition of qPCR (Lim et al., 2017). Tree tissues contain high levels of polysaccharides, phenolic compounds and tannins, which also inhibit downstream processing by binding to the DNA and causing further issues in DNA based processing (Healey et al., 2014; Rossi et al., 2021).

In this project, a wide range of methods were attempted to obtain high quality DNA and RNA. Initially, for DNA, very basic temperature-based extraction methods were tested, including a boil preparation, a microwave preparation, and a thermolysis protocol. Although these methods are considered relatively basic and crude, they can be efficient and cost effective. The MP Biomedicals FastDNA® Spin Kit (MP Biomedicals, SKU: 116540600-CF) was also trialled and although, after optimisation worked well on mycelial cultures, the extracts from the kit required a separate clean up protocol, resulting in a reduced yield. The FastDNA® Spin kit was also less effective on fruiting body samples and was unsuitable for the environmental samples such as woody tissue and soil. Finally, for both DNA and RNA isolations, precipitation-based extractions were trialled, and it was found that after homogenisation, the best route was a modified phenol-chloroform extraction method, which was found to effectively isolate high-quality DNA, and with only slight modifications, RNA, from all sample types. Each extraction method was analysed through electrophoresis, using a 1% agarose gel, which was run for around 50 minutes at 100 volts (for DNA) or for 60 minutes at around 80 volts (for RNA).

Testing Temperature Based DNA Extraction Methods

In the temperature-based extraction methods, 12 mycelial cultures were utilised for each method, a boil preparation (adapted from Pettifor et al., 2020) was tested. This involved taking a small piece of mycelial tissue and placing in 50 µl of molecular grade H_2O. This was then incubated at 95˚C for 2.5 minutes, vortex mixed at a high speed for 10 seconds, and then incubated at 95˚C for a further 2.5 minutes.

Secondly, an extraction method utilising a microwave, taken from (Ramya et al., 2018), was trialled. This involved taking a small amount of mycelial tissue, transferring to 1 ml of TE buffer, before vortex mixing briefly at a high speed. This suspension was then centrifuged at 10000 x *g* for 1 minute, and the supernatant discarded. The cell pellet was then washed with 1 ml of TE buffer by pipetting up and down approximately 10 times and centrifuged at 10000 x*g* for 1 minute. The wash step was repeated using 1 ml of molecular grade H_2O and again centrifuged at 10000 x*g* for 1 minute. The washed cell pellet was then resuspended in 200 µl of TE buffer and was microwaved at 900 W for 1 minute. The suspension was briefly vortexed at a medium speed and microwaved again at 900 W for a further 1 minute.

Next, also taken from (Ramya et al., 2018) was a thermolysis extraction method. In this, a small amount of mycelial tissue was transferred to 100 µl of molecular grade H_2O before being vortex mixed thoroughly at a high speed (approximately 10 seconds). The suspension was centrifuged at 10000 x*g* for 1 minute, and the supernatant discarded. The pellet was resuspended in 100 µl of cell lysis buffer and incubated at 85˚C for 25 minutes.

Testing and Optimising the FastDNA® Spin Kit

Due to the lack of success with basic temperature-based methods, a column-based kit extraction was tested and optimised. The MP Biomedicals FastDNA® Spin Kit was tested and after 2 rounds of optimisation, was found to work well for mycelial and fruiting body samples.

The kit was firstly used following the manufacturers provided protocol. The first round of protocol optimisation of this method, half the amount of tissue (approximately 0.25 g) was used. The second round of optimisation for this method involved increasing homogenisation of the sample, by increasing homogenisation from one cycle to two cycles in a Qiagen PowerLyzer24 instrument (at 2500 m/s for 40 sec) before running through the spin column.

Testing and Optimising a Precipitation Based Extraction Method

The unreliable results obtained, and the unsuitability of the FastDNA® Spin kit for soils and infected woody tissues led to the testing of a precipitation-based extraction method.

In this, a 4% CTAB buffer was prepared (NaCl, 8.18 g/100 ml (Sigma); CTAB, 4.00 g/100 ml (Sigma); PVP, 1.00 g/100 ml (Fluka); Tris-HCl (1 M), 20 ml/100 ml (Invitrogen); EDTA (0.5 M), 4 ml/100 ml (Invitrogen); up to 100 ml with ultrapure H_2O), along with a 6 M solution of NaCl and a 3 M solution of sodium acetate.

Samples, including mycelial cultures, fruiting bodies, woody tissues and soils, were homogenised by adding 1 ml of the 4% CTAB buffer to 0.5 g of sample in a 2 ml tube, and after adding a 3 mm

ceramic bead to the tube, samples were homogenised in a Qiagen PowerLyzer24 instrument at a speed of 2500 m/s, for 30 seconds (this was run twice).

After homogenisation, samples were incubated at 60°C for 1 hour, mixing by inversion every 15 minutes. After allowing to cool to just above room temperature, homogenised samples were centrifuged at 15000 *x g* for 15 minutes, after which the supernatant was removed and transferred to a new tube, and the remaining homogenate discarded.

At this point, an approximately equal volume of chloroform:isoamyl alcohol (IsoAA) (24:1) was added to the tube, and this was mixed using a rotating mixer for 5 minutes. The tubes were then centrifuged at 15000 *x g* for 15 minutes at room temperature. The upper aqueous phase was then transferred to a fresh tube, and 250 µl (6 M) NaCl, 50 µl (3 M) sodium acetate and 500 µl ice-cold isopropanol were added. This was mixed by inverting ten times, and the tubes were incubated at –20°C for between 30 minutes and overnight. After the incubation, tubes were centrifuged at 15000 *x g* for 15 minutes at room temperature, to pellet the nucleic acids, and the supernatant was removed and discarded. The nucleic acid pellet was washed with 70% ethanol and allowed to air dry for between 15 and 30 minutes. The pellet was then resuspended in 50 µl nuclease free water.

The extract was treated with RNase A solution and incubated at 37°C for 25 – 30 minutes, and this was subsequently followed by a secondary precipitation involving the addition of 25 µl (6 M) NaCl, 5 µl (3 M) sodium acetate and 50 µl ice-cold isopropanol and the inversion of tubes ten times. The tubes were incubated at -20°C for between 30 minutes and 2 hours, followed by centrifugation at 15000 *x g* for 15 minutes at room temperature, to re-pellet the DNA, the supernatant was removed and discarded, and the DNA pellet was washed with 70% ethanol and allowed to air dry for between 15 and 30 minutes. The pellet was resuspended, once again, in 50 µl nuclease free water.

The homogenisation step initially used for this method was found to cause severe shearing of DNA when applied to mycelial cultures. The homogenisation methods were therefore tailored to specific sample types as follows. For mycelial plate cultures, no homogenisation was required, the samples were added to the buffer and went straight into the 60°C heating step. For soil samples, the ceramic bead was replaced with 0.5 g of glass beads, the remainder of the protocol (including Qiagen PowerLyzer24 instrument parameters) was unchanged. For fruiting bodies and woody tissue, samples were frozen and ground in liquid nitrogen before being added to the tube for homogenisation, along with 0.5 g glass beads instead of a ceramic bead, homogenisation was conducted with the above parameters, and the remainder of the protocol was unchanged.

It was also found that on the more contaminated samples, a second chloroform:isoAA was step was required, immediately after the first. This involved taking the upper aqueous phase, after centrifugation, into another fresh tube and again adding equal volumes of chloroform:isoAA before mixing with the rotating mixer and then centrifuging the mixture at 15000 *x g* for 15 minutes. The sodium acetate precipitation was then followed as above (Figure 3.2).

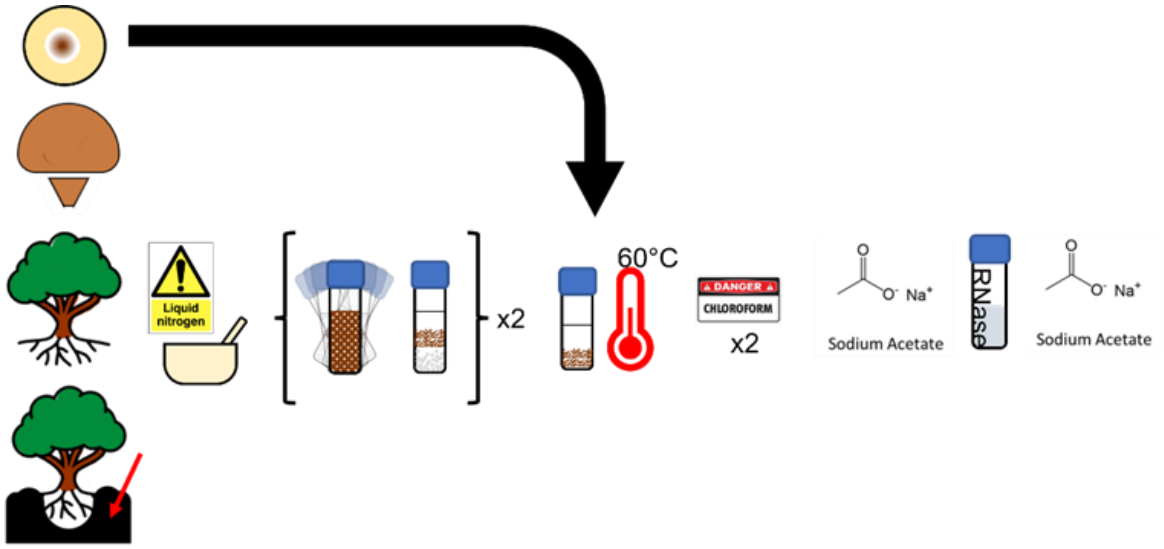

Figure 3.2. An overview of the DNA extraction method optimised in this study for extraction of high-quality DNA from mycelial cultures, fruiting bodies, infected woody tissues and soils. Extraction method includes a homogenisation step (not for mycelial cultures), an incubation at 60 degrees, before two chloroform:isoamyl alcohol wash steps followed by precipitation using sodium acetate. The resulting co-extraction of DNA and RNA was then treated with RNase A solution and followed with a secondary precipitation, resulting in high yields of pure DNA.

Reliable methods for RNA Extraction from Environmental Samples

For the following RNA extraction methods, an extremely high level of cleanliness was adhered to. This involved thorough bleaching of all surfaces and equipment prior to the extractions, and at regular intervals throughout the extraction period. RNase-*Zap*™ RNase Decontamination Solution (Invitrogen™) was also frequently to spray down surfaces and equipment. Pipettes and pipette tips, along with all pestles, mortars and spatulas (used to take the ground sample from the mortar) were UV treated for a minimum of 1 hour before the extraction took place and were wiped with RNase-*Zap*™ Decontamination wipes (Invitrogen™) before touching any of the samples. All samples were kept in liquid nitrogen if they were not being processed, and the temperature was maintained throughout the extraction protocol with the addition of liquid nitrogen, and the use of chipped ice.

Following the previous success of the sodium acetate precipitation for DNA extractions from environmental samples, this protocol was modified for the extraction of RNA.

Briefly, samples, approximately 3 – 5 mm^2 were ground in a pestle and mortar, the temperature was maintained with the consistent addition of liquid nitrogen, and the ground sample was transferred to a 2 ml screw cap tube containing 0.5 g of glass beads and 1 ml of 4% CTAB extraction buffer (with the addition of 2% V/V β-mercaptoethanol). Samples were frozen in liquid nitrogen whilst other samples were being processed and were removed from the liquid nitrogen and allowed to thaw slightly. Once thawing had begun, samples were homogenised further using the Qiagen PowerLyzer24 instrument at a speed of 2500 m/s, for 30 seconds. This was repeated in order to ensure all the sample and buffer had thawed and been homogenised.

After homogenisation, samples were centrifuged at 14000 RPM for 10 minutes at 4˚C. The supernatant was removed and transferred to a new 1.5 ml microcentrifuge tube, where an equal volume of chloroform:isoAA (24:1) was added. The tubes were shaken for approximately 10 seconds in order to form an emulsion. The tubes were again centrifuged at 14000 RPM for 10 minutes at 4˚C. the supernatant was removed and transferred to a new 1.5 ml microcentrifuge tube. This, the first of two, precipitation step involved the addition of 250 µL of NaCl (6M), 50 µL of sodium acetate (3M) and 500 µL of ice-cold isopropanol. The tubes were inverted twice to allow thorough mixing of the reagents, and then were incubated at -20˚C overnight.

After the overnight incubation, where the nucleic acids were precipitated, samples were centrifuged at 14000 RPM for 10 minutes at 4˚C, to pellet the nucleic acids at the bottom of the tube. At this point, the supernatant was removed, and the pellet was washed with 500 µL of ice-cold 70% ethanol by pipetting up and down. Without removing the ethanol from the tube, the tubes were once again centrifuged at 14000 RPM for 10 minutes at 4˚C, to rebind the pellet to the tube. After this, all alcohol was removed from the tube using a pipette. Any residual alcohol was allowed to evaporate from the tube by allowing the pellets to air dry for 15 to 20 minutes, after which, the nucleic acids were resuspended in 50 µL of ultra-pure, nuclease free H_2O, which had been UV treated prior to the extraction.

The extract (containing both DNA and RNA at this point) was treated with turbo DNase to remove the DNA in the sample. This involved adding 5 µL of the DNase Buffer and 8 µL of the DNase treatment to the extract and incubating at 37˚C for 25 to 30 minutes. After this, a second precipitation was performed to remove the digested DNA from the sample, to leave just RNA in the tube. This involved adding 25 µL of NaCl (6M), 5 µL of sodium acetate (3M) and 50 µL of ice-cold isopropanol, due to the smaller volume of the extract. The tubes were once again inverted twice to

allow thorough mixing of the reagents, and then were incubated at – 20˚C for a minimum of 2 hours. After this incubation, tubes were centrifuged at 14000 RPM for 10 minutes at 4˚C, to pellet the RNA. The supernatant was removed, and the pellet was washed with 500 µL of ice-cold 70% ethanol by pipetting up and down. Again, without removing the ethanol from the tube, the tubes were centrifuged at 14000 RPM for 10 minutes at 4˚C, to rebind the RNA pellet to the tube. After this, all ethanol was removed, and any residual alcohol was allowed to evaporate by allowing the RNA pellet to air dry for 15 to 20 minutes, after which, the RNA was resuspended in 30 µL of ultra-pure, nuclease free H_2O, which had been UV treated prior to the extraction.

The RNA extracts were analysed for potential salt, polysaccharide and protein contamination using a Nanodrop spectrophotometer. The values of this analysis indicated contamination, therefore the protocol went through two processes of optimisation (as follows) in an attempt to remove the contamination.

In the first optimisation, a second chloroform:isoamyl alcohol wash step was introduced immediately after the first. Changes were as follows; immediately after the first chloroform wash step, and subsequent centrifugation, the supernatant was transferred to a new 1.5 ml microcentrifuge tube and again, equal volumes of chloroform:isoAA (24:1) were added. The tubes were shaken to form an emulsion, and centrifuged at 14000 RPM for 10 minutes at 4˚C. The supernatant was removed again and transferred to a new 1.5 ml microcentrifuge tube. From here, the first of the precipitation steps was followed, and the remainder of the protocol unchanged.

The second optimisation trial for RNA extractions, involved using a lithium chloride (LiCl) precipitation method for the second precipitation step. This was due to LiCl precipitation being biased towards RNA molecules (Korolenya et al., 2022; Walker and Lorsch, 2013), meaning it should precipitate more RNA than other methods. The modification to the protocol included the second chloroform wash step as described previously and was as follows; 0.5 volumes (approximately 30 µl) of chilled LiCl was added to each reaction immediately after the Turbo DNase treatment, and tubes were incubated at -20˚C for 2 hours. The remainder of the protocol was as previously described.

The inclusion of a second ethanol wash step (washing the extracted pellet with 500 µl of 70% ethanol and centrifuging to rebind the pellet to the wall of the tube, before removing all alcohol from the tube and allowing to air dry for approximately 20 minutes) to clean the pellet of co-extracted DNA and RNA was utilised for samples that provided a particularly unclean pellet, however this was only performed before the DNase treatment.

3.2.4 | Phylogenetic Analysis of *Gymnopus fusipes*

Sequencing the *ITS* Region for Initial Identification of *Gymnopus fusipes* Strains

For initial identification of the isolates obtained throughout this study, the *ITS* gene region was used, and strains were confirmed to be *G. fusipes* if they retuned a sequence similarity of 97% or more (this is a recognised threshold for species delineation, Pettifor et al., 2020) when analysed using the "Nucleotide BLAST" program on the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Although identification using the internal transcribed spacer (*ITS*) gene and associated subregions (*ITS1* and *ITS2*) is considered the "gold standard" for fungal species (Badotti et al., 2017; Iquebal et al., 2021), there are a number of other genes are available for identification. The most common include, the large ribosomal subunit, *28SrRNA* (Vilgalys and Hester, 1990; Ye et al., 2020), the small ribosomal subunit, *18SrRNA* (White et al., 1990; Ye et al., 2020), the RNA polymerase II gene, *RPB2,* the translation elongation factor 1-α, *Tef1-α*, β-tubulin, *tub2* (Větrovský et al., 2016), and the cytochrome oxidase I, *COI* (Dentinger et al., 2011).

PCR and Sequencing of Five Housekeeping Genes for *Gymnopus fusipes* MLSA

While some genes are more appropriate than others when identifying fungi, due to divergence between species, absence in some fungal genomes and also the potential occurrence of paralogous genes (Dentinger et al., 2011; Větrovský et al., 2016), the lack of molecular information on *G. fusipes* meant that any of the genes may have the potential for identifying intraspecific variations. The genes selected for this experiment were, *ITS*, *18SrRNA*, *28SrRNA*, *Tef1-α*, and the *RPB2* gene. Primer sequences were derived from literature where the genes were amplified in fungal species (Table 3.3).

All reactions, regardless of the gene region of interest were prepared as follows; after being mixed briefly with a vortex mixer, 1 µl of extracted (and cleaned if necessary) DNA was used with the GoTaq® Green Master Mix. Reactions were 50 µl in volume, and along with the 1 µl of DNA template, contained 25 µl of GoTaq® Green Master Mix, 22 µl of nuclease-free, PCR-grade water, 1 µl of 10 pmol of forward primer and 1 µl of 10 pmol reverse primer (Table 3.3). Conditions of the PCR reactions varied depending on the gene region of interest and the primers being used (Table 3.3). Ten microliters of the PCR products (length dependant on the gene region of interest, see Table 3.3) were then visualised using a 1% agarose gel electrophoresis at 100 V for 45 to 50 minutes. One microliter of each of the PCR products was used to quantification using the Qubit™ dsDNA HS Assay kit. Unpurified PCR products were sent to GENEWIZ (UK) for Sanger sequencing, where both forward and reverse sequencing was conducted using the primers from the PCR reactions. Returned nucleotide sequences were analysed using the "Nucleotide BLAST" program on the NCBI BLAST database, to confirm species identification.

Table 3.1. Primer pairs and initial PCR cycling conditions for the five chosen gene regions in this study are outlined, along with primer sequences, references and approximate PCR product length.

Gene Region		*ITS*	*18SrRNA*	*28SrRNA*	*Tef1-α*	*RPB2*
Forward Sequence		ITS5: GGA AGT AAA AGT CGT AAC AAG G	NS1: GTA GTC ATA TGC TTG TCT C	LROR: AGA TCT TGG TGG TAG TA	EF1-983F: TAC AAR TGY GGT GGT ATY GAC A	6F: GAY GAY MGW GAT CAY TTY GG
Reverse Sequence		ITS4: TCC TCC GCT TAT TGA TAT GC	NS8: TCC GCA GGT TCA CCT ACG GA	LR7: TAC TAC CAC CAA GAT CT	EF1-156R: CAN GAC TTG ACY TCA GTR GT	7CR: CCC ATR GCT TGY TTR CCC AT
Reference		(White et al., 1990; Ye et al., 2020)	(Marshall et al., 2003; White et al., 1990)	(Vilgalys and Hester, 1990; Ye et al., 2020)	(Morehouse et al., 2003; Ye et al., 2020)	(Reeb et al., 2004; Ye et al., 2020)
PCR Cycling	**Initial Denaturation**	95˚C (3 mins)	95˚C (2 mins)	94˚C (1 min)	95˚C (3 mins)	95˚C (3 mins)
	Denaturation (35 cycles)	94˚C (40s)	95˚C (30s)	94˚C (1 min)	94˚C (40 s)	94˚C (40 s)
	Annealing (35 cycles)	54˚C (45s)	55˚C (30s)	50˚C (1 min)	54˚C (45s)	58˚C (45s)
	Extension (35 cycles)	72˚C (1 min)	72˚C (1 min)	72˚C (1 min)	72˚C (1 min)	72˚C (1 min)
	Final Extension	72˚C (10 mins)	72˚C (10 mins)	72˚C (10 mins)	72˚C (10 mins700)	72˚C (10 mins)

3.3 | Results

3.3.1 | Building a Culture Collection of *Gymnopus fusipes* Strains

Attempting to Obtain *Gymnopus fusipes* from Culture Collections and Previous Studies

The attempt to obtain *G. fusipes* isolates from global culture collections was a significant challenge. After an extensive search, it was found that *G. fusipes* was not present in any of the widely used culture collections such as DSMZ or the CABI Genetic Resource Catalogue. Although records of *G. fusipes* strains were found on the WFCC site in the Global Catalogue of Microorganisms database, technical issues with the webpage and database prevented procurement from this site.

Responses from corresponding authors were limited, and those that did respond stated that the work was based primarily on visual observations of macro- and microscopic characteristics, and that they did not perform any culture-based work or isolations. In Chapter 2, it was highlighted how there is a real black of primary research on this species, and in searching for isolates of *G. fusipes* to work with this was further emphasised.

Contact with Dr Benoit Marcais was positive, and after being revived from long term storage, three strains of *G. fusipes* (C41, C49 and C52) were provided as mycelial plate cultures. Each of the three strains was originally isolated from the tissues of an infected oak host at three different sites in France.

Field sampling campaigns for *Gymnopus fusipes*

Through the sampling campaigns conducted between 2019 and 2022, Dr Nathan Brown (Rothamsted Research) kindly assisted with provision of several *G. fusipes* fruiting body samples through the summer of 2020, including samples from Winding Wood, Great Monks Wood and Hatchlands. Dr Sandra Denman and her team at Forest Research were responsible for obtaining a number of fruiting bodies, infected tissue samples and soil samples in the summers of 2020 and 2021, including samples from Alice Holt Forest, Crabtree Plantation and Lingfield College. David Humphries (City of London Council, not a part of the wider research group) was responsible for providing samples of fruiting bodies, infected wood tissue and soils from Highgate Woods. Ed Pyne (Bangor University) was responsible for obtaining the fruiting body sample EP1. Samples of soil, fruiting bodies and infected woody tissue were also collected from a site in the UK heavily impacted with *G. fusipes* infection in the summer of 2022 (this work is detailed in Chapters 5 and 6).

Collecting *Gymnopus fusipes* Samples, Including Fruiting Bodies and Infected Woody Tissues

It was noted in this work that fruiting body samples should be as fresh as possible when collected and should be stored in paper bags or envelopes to prevent the build-up of moisture when in transit.

Improper sample storage prevented isolation attempts from samples from one of the sites in this work, as the fruiting bodies had severely deteriorated before making it to the lab. Fruiting bodies were processed as soon as possible, as even with storage at 4°C with paper wrappings, the samples could begin to deteriorate within a week. This was variable across fruiting body samples, with those being viable the longest having been collected when they were fresh.

Woody tissue samples, it was found, had more successful isolation if the sample had been stored at 4˚C for a number of weeks prior to isolation, as this reduced the number of contaminants on the tissue sample which led to less contamination when the sample was plated onto agar.

Testing Different Surface Sterilisation Methods

The surface sterilisation methods tested in this work had varying levels of success, but the most effective technique involved removing any soil or debris from the sample, surface sterilising by dipping the whole sample in 10% bleach solution for 10 seconds, before cutting a small inner section of tissue (approximately 3 mm^2) from the sample and sterilising by dipping in 70% ethanol for 10 seconds. The sample was then allowed to air dry on a piece of clean tissue, before being placed onto ½ strength malt extract agar and incubated at 25˚C until growth was observed. If any contamination was detected, the plates were removed from the incubator and disposed of. If no contamination was detected, the plates were replaced in the incubator and regularly checked for contamination until the *G. fusipes* colonies were clear enough to be sub-cultured onto fresh ½ strength malt extract agar plates (approximately 2 – 3 weeks), which was completed by taking a 5 mm mycelial plug from the hyphal edge. Three *G. fusipes* strains were successfully isolated using this method (AHPC from fruiting body tissue, and MP2 and MP5 from infected woody tissues).

Collection and Germination of *Gymnopus fusipes* Basidiospores

The production of spore prints was variable in its success, with a number of fruiting bodies producing no spore print (this could potentially be explained by the samples not being as fresh when they were collected, meaning the spores may have already been released).

Plating of the spore suspension proved to be unsuccessful in this experiment, however the agar slant approach seemed to be effective, allowing isolation of two strains of *G. fusipes* using this method (AH1 and GMW83). This could be explained by the increased aeration in the headspace of the tube compared to the shallowness of the agar plate.

Culture Collection Curation

Although numerous samples were collected over the four-year project, obtaining existing strains and isolation attempts from environmental samples were often unsuccessful. A total of five *G. fusipes*

strains were isolates in this project, bringing the total number of *G. fusipes* strains in this work to 8 (Table 3.3).

Table 3.2. Isolates obtained for use in this project and those isolated from work conducted in this project. The isolate name, origin and date of collection are detailed.

Strain Name	Origin of Sample	Date of Isolation	Notes
C41	Siarrouy, France	1994	Strain originally isolated from infected *Q. robur* tissue (isolate revived from long term storage by B. Marcais, INRAE in 2019)
C49	Loriet, France	1992	Strain originally isolated from infected *Q. rubra* tissue (isolate revived from long term storage by B. Marcais, INRAE in 2019)
C52	Haute-Saône, France	1993	Strain originally isolated from infected *Q. robur* tissue (isolate revived from long term storage by B. Marcais, INRAE in 2019)
AH1	Alice Holt Forest, Surrey, UK	2020	Fruiting bodies collected from *Q. robur*, isolate germinated from spore prints
GMW83	Great Monks Wood, Essex, UK	2020	Fruiting bodies collected from *Q. robur*, isolate germinated from spore prints
AHPC	Alice Holt Forest, Surrey, UK	2021	Fruiting bodies collected from *Quercus* sp., strain isolated from fruiting body tissue
MP2	UK	2022	Strain isolated from infected woody tissues from a *Quercus* host
MP5	UK	2022	Strain isolated from infected woody tissues from a *Quercus* host

3.3.2 | Cultivation of *Gymnopus fusipes* Strains

Testing Different Culture Media for the Growth of *Gymnopus fusipes*

The data obtained from this small experiment yielded important results (Figure 3.3). Results were calculated as an overall growth, combining results for all isolates, to represent *G. fusipes* as a species in general. The carrot agar resulted in the largest growth over the 28-day experiment, with a mean diameter increase of 52.2 mm at T_{21}, and all isolates (apart from GMW83) unmeasurable at T_{28}, as the colonies had reached the limits of the agar plates. The half strength malt extract agar condition resulted in a mean diameter growth of 41.7 mm. The half strength malt extract agar with the additional ethanol resulted in a mean diameter growth of 42.4 mm.

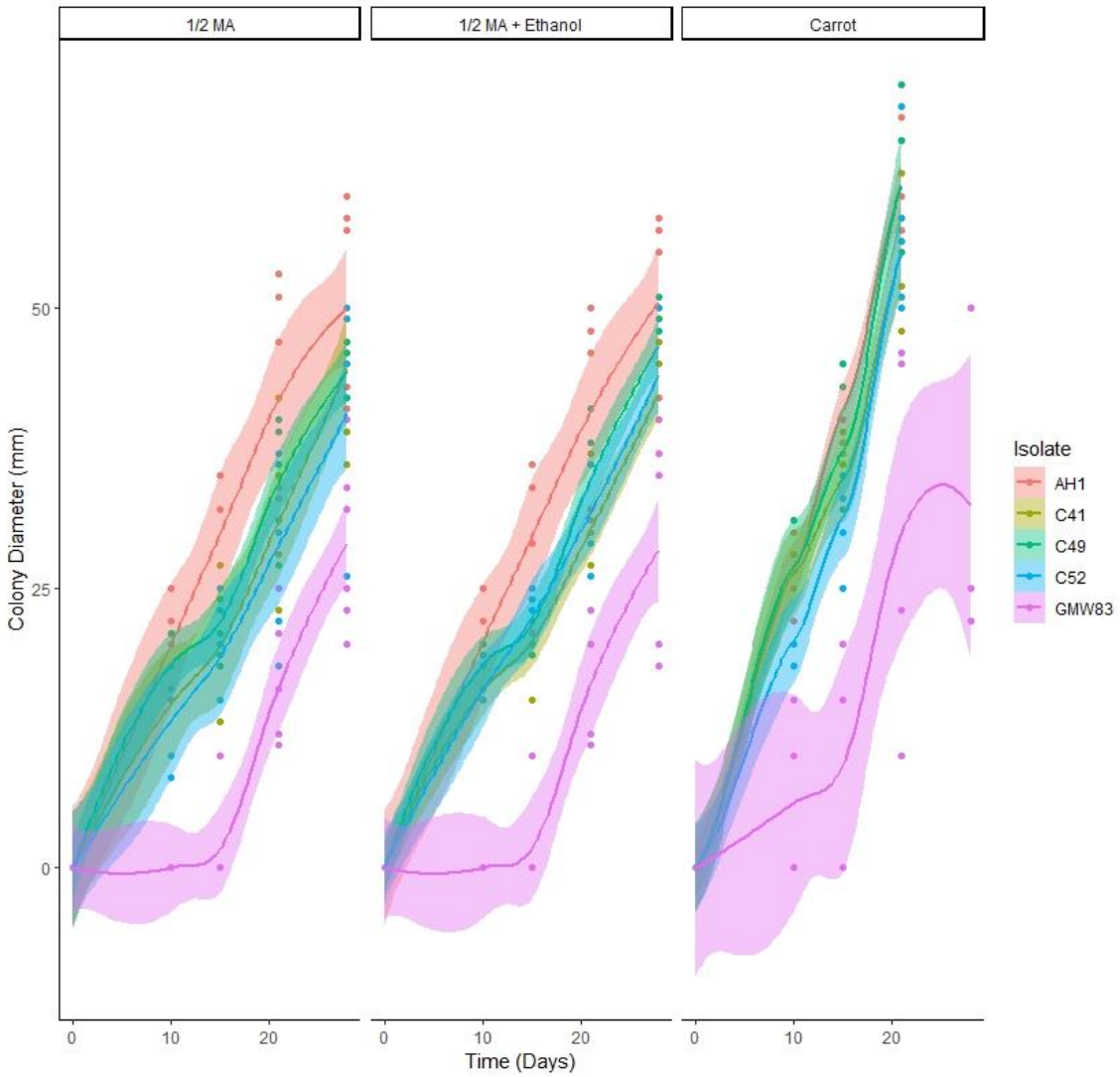

Figure 3.3. Weekly growth measurements of five G. fusipes isolates on three different medium types, half strength malt extract agar, half strength malt extract agar with ethanol added, and carrot agar, over the course of the 28-day experiment. On carrot agar, data from T_{28} was unreliable for all but strain GMW83, as the colonies had reached the limits of the agar plate.

It is important to note that the diameter measurements of the colonies are not the only factor that needs to be taken into account. The results of this test also revealed that biomass of *G. fusipes* can appear very differently depending on which medium is being used (Figure 3.4). By observing the isolates growing on the three different media types, it was clear that the larger growth obtained by using the carrot agar had a much smaller biomass than expected. Although the colonies were larger, the mycelial mat was much thinner. The ½ strength malt extract agar with added ethanol produced biomass with a dark brown pigment which pooled on the top of the mycelial mat and soaked into the medium surrounding the colony. This was only observed after 2 - 3 weeks of growth when there was no ethanol added to the medium, and only in very small amounts. The medium that produced

standard looking biomass (thick, white colonies with little to no pigmentation) were found on the ½ strength malt extract agar.

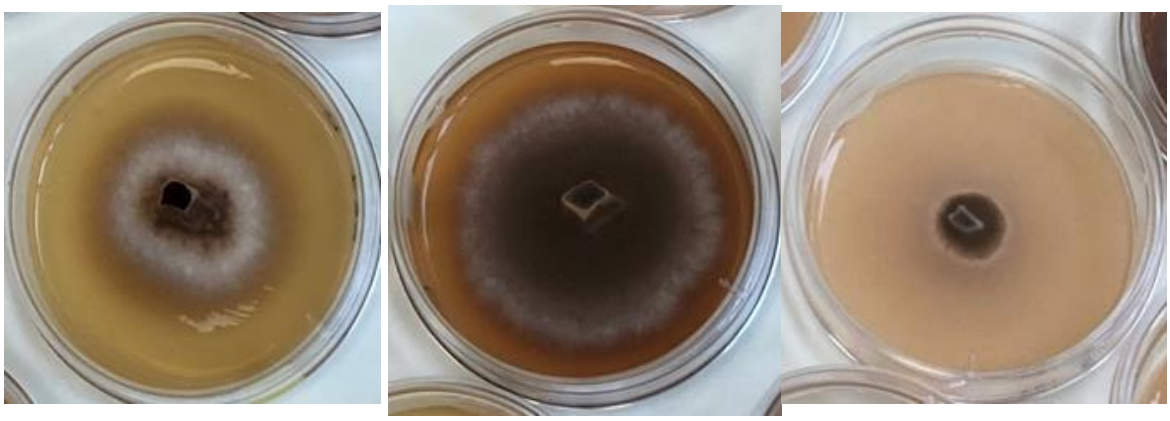

Figure 3.4. *G. fusipes* growing on three different media types. Far left: half strength malt extract agar, resulting in a thick white mycelial mat, with little to no pigmentation. Middle: half strength malt extract agar with the addition of ethanol, resulting in a thick dark brown colony, with the production of brown pigments which pooled on the top of the mycelial mat and also seeped into the agar plate itself. Far right: carrot agar, which resulted in a large, but extremely thin mycelial mat and biomass with less dense growth.

Testing Different Lighting Conditions for the Growth of *Gymnopus fusipes*

When considering the growth of the isolates under the different lighting conditions, regardless of the media type (Figure 3.5), there is a stark different between the growth of isolates cultured in complete darkness, compared with those cultured under standard day/night lighting conditions. The *G. fusipes* replicates that were incubated in complete darkness, at room temperature, grew a mean of 50.7 mm throughout the experiment, however those grown with standard day/night conditions grew only 36.3 mm on average, over the 28-day experiment.

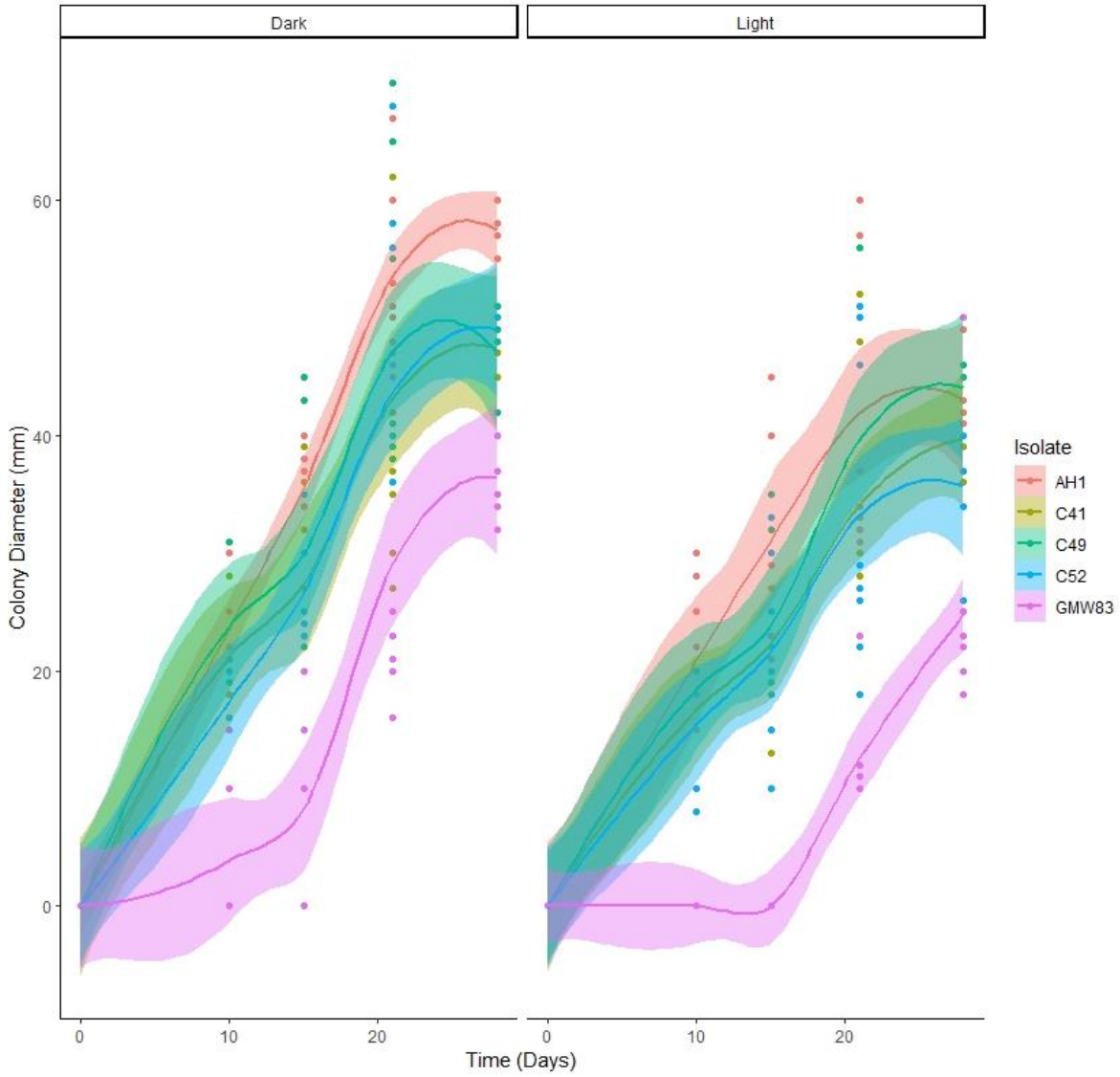

Figure 3.5. Weekly growth measurements of five *G. fusipes* isolates under dark or light conditions. Colonies that were grown in complete darkness (Left) grew to a larger size than those grown in daylight conditions (Right).

Data analysis

After performing a Chi Square test for significance, it was determined that overall (pooling data for all of the five isolates together to look at *G. fusipes* as a whole) there was no significant difference in the growth of *G. fusipes* across the three different media types. However, a significant difference was observed between those cultures grown under light conditions and those grown in complete darkness ($P < 0.001$). It was observed that *G. fusipes* when grown in complete darkness demonstrated increased growth and produced larger colonies within the 4-week timeframe of the experiment. Therefore, it was decided that cultures of *G. fusipes* would be grown on ½ strength malt

extract agar, in complete darkness for isolation and culture maintenance for the remainder of the project.

3.3.3 | Reliable Methods for Nucleic Acid Extraction from Environmental Samples

Testing Temperature Based DNA Extraction Methods

Gel electrophoresis of the extracted DNA revealed that none of the temperature-based methods were effective in extracting high quality DNA from mycelial cultures, and in some cases (Thermolysis) also resulted in genomic DNA degradation (Figure 3.6). This could possibly be explained by the complex cell walls existing in fungi, indicating that a better method of cell lysis is required for this sample type.

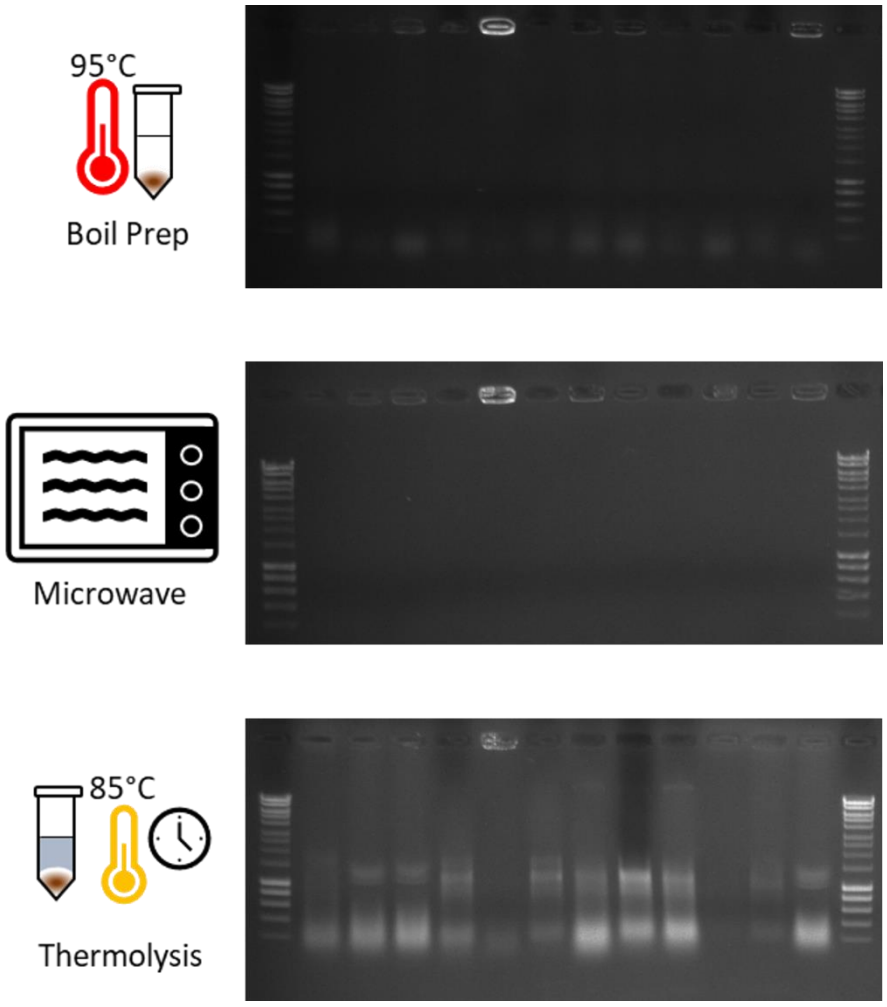

Figure 3.6. Gel electrophoresis images of the temperature-based extraction methods. The boil prep method and microwave method resulted in no extraction of DNA, and the thermolysis method resulted in DNA that was heavily degraded.

Testing and Optimising the FastDNA® Spin Kit

Following the provided protocol for the FastDNA® Spin Kit it was found that mycelial tissue often clogged the filter in the spin column, and although some bands were visible when visualised through gel electrophoresis, these were faint (Figure 3.7), suggesting that the sample was not being processed fully, and that the DNA extraction was inefficient.

When the FastDNA Less method was followed, the mycelial tissues did not clog the filter of the spin column, however this method also resulted in a lack of observable DNA when analysed through gel electrophoresis, and a mean of only 1.05 ng/µl was measured when analysed with a Qubit dsDNA high sensitivity kit (ThermoFisher. Catalogue number: Q32854).

The final optimisation of this kit (FastDNA Lyze method) was found to be successful for mycelial tissues, resulting in bright genomic DNA bands when analysed through gel electrophoresis and a mean of around 30 ng/µl when measured with the Qubit dsDNA high sensitivity kit.

Although resulting in a better yield of DNA, the FastDNA® Spin Kit did not provide DNA which, according to Nanodrop spectrophotometry, was free from contamination with proteins, salts or polysaccharides, often leaving extracts unreadable through spectrophotometry. This required a separate clean-up process, and was less than ideal.

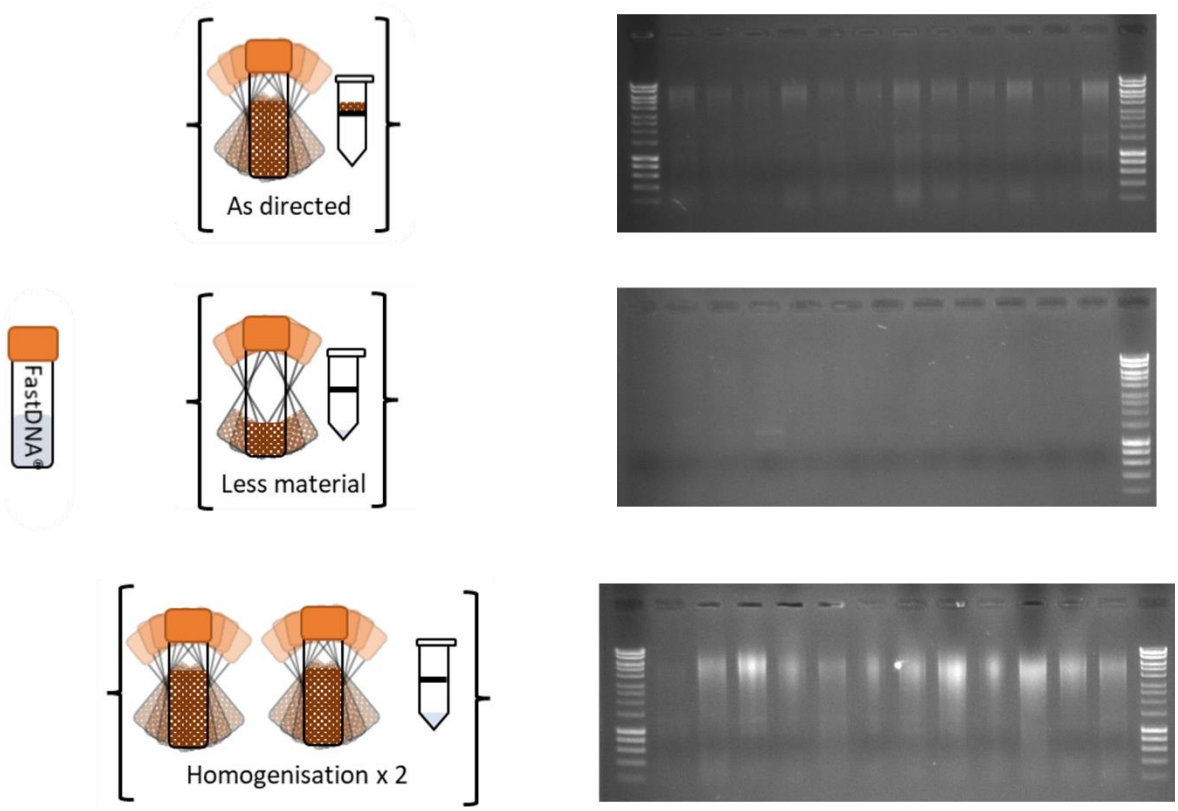

Figure 3.7. Gel electrophoresis images of the DNA extractions conducted using the FastDNA® Spin Kit. The initial method (as directed) resulted in low yield DNA that had high levels of contamination. The first optimisation (less material) resulted in an almost indeterminable yield of DNA. The final method (homogenisation x 2) resulted in a higher yield of DNA, however this was still highly contaminated.

Testing and Optimising a Precipitation Based Extraction Method

It was found that although homogenisation using the Qiagen PowerLyzer24 instrument was effective when extracting from fruiting bodies, infected woody tissues, and soils, this homogenisation caused severe shearing of the DNA when extracting from mycelial cultures. It was identified that no homogenisation was required for the mycelial cultures, and that these samples should go straight into the 60°C incubation (Figure 3.8).

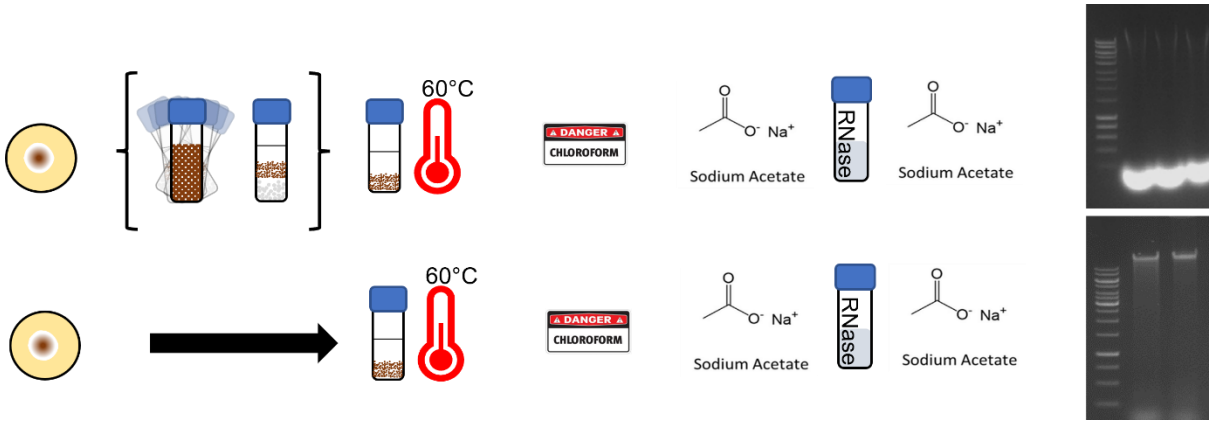

Figure 3.8. Summary of method using homogenisation on mycelial cultures, with gel image showing extreme shearing of the DNA. Optimised method shows mycelial cultures bypassing the initial homogenisation step, and being transferred directly into the 60˚C incubation. This resulted in clear genomic DNA bands when visualised with gel electrophoresis.

The CTAB/chloroform DNA extraction method trialled in this work, with few alterations was found to be effective across various sample types, including mycelial cultures, fruiting bodies, tree tissues, and soils (Figure 3.9).

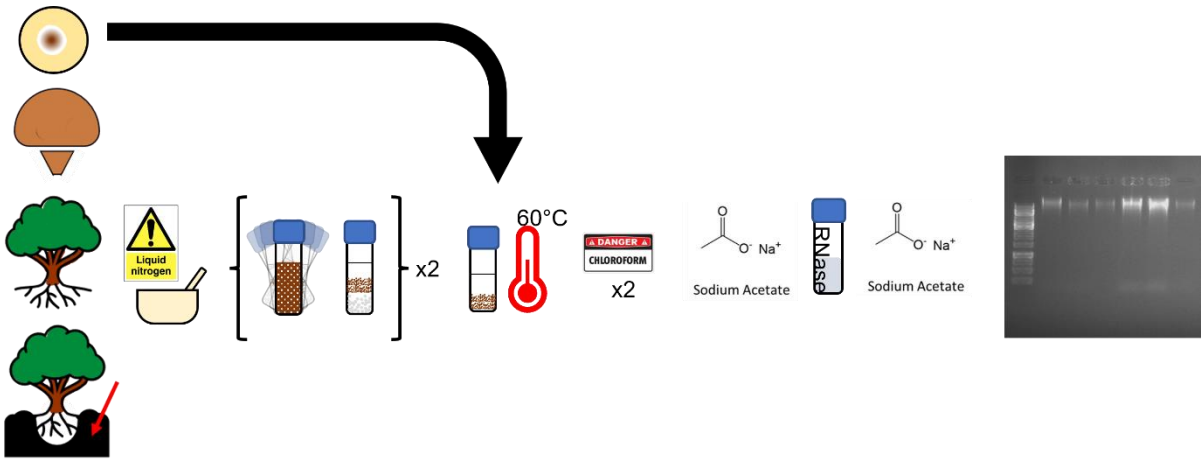

Figure 3.9. Summary of the DNA extraction method developed in this project, including homogenisation, heated incubation, chloroform washing and sodium acetate precipitations. Gel electrophoresis image shows high-quality, high molecular weight DNA with bright bands when visualised.

Reliable methods for RNA Extraction from Environmental Samples

RNA extractions from samples including mycelial cultures, fruiting bodies and infected woody tissues were overall very successful. Two optimisations to the initial method were conducted, one which involved a second chloroform wash step, and one which involved using a lithium chloride

precipitation for the second precipitation. After optimising the initial method with two chloroform wash steps, high yields of RNA were extracted which were of a good purity.

The initial method of RNA extraction, following a similar protocol to the DNA extraction above, was found to extract RNA from around 50% of the samples. However, when analysed with Nanodrop spectrophotometry, the 260/280 and 260/230 ratios indicated high levels of contamination with salts, proteins and polysaccharides (Figure 3.10A).

The first optimisation of the method involved the addition of a second chloroform wash step. This improved the effectiveness of the method, increasing the success rate from 50% to 75% . According to Nanodrop spectrophotometry analysis, protein contamination was also reduced, resulting in an increased 260/280 ratio (Figure 3.10B).

The second optimisation involved using the second chloroform wash step, as above, and also changed the method in the final precipitation from sodium acetate to lithium chloride. This method was successful in isolating RNA from 4 of the 7 samples, however the yield of RNA was reduced, indicated by faint bands on the gel and low quantity values (Figure 3.10C) . Nanodrop spectrophotometry values for the 260/280 ratios were deteriorated compared to those previously obtained, although the values for the 260/230 ratio appeared to be greatly improved.

It was determined that the method developed with the first optimisation (Figure 3.10B), with two chloroform wash steps, and the use of sodium acetate for both precipitation steps was to be used for the samples in this study. The inclusion of a second ethanol wash step (washing the extracted pellet with 500 µl of 70% ethanol and centrifuging to rebind the pellet to the wall of the tube, before removing all alcohol from the tube and allowing to air dry for approximately 20 minutes) to clean the pellet of co-extracted DNA and RNA was also utilised for samples that provided a particularly unclean pellet, however this was only performed before the DNase treatment.

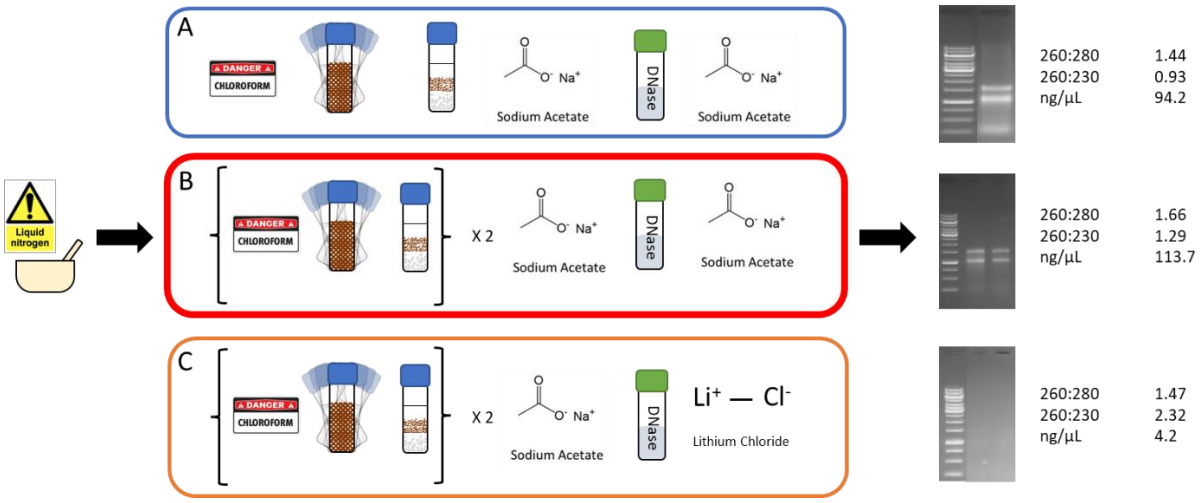

Figure 3.10. Summary of RNA extraction methods. A. Initial method, with one chloroform wash step and using sodium acetate for both precipitations. B. Second trial method, with two chloroform wash steps and using sodium acetate for both precipitations. C. Third trial method, with two chloroform wash steps and using sodium acetate for the first precipitation and Lithium Chloride for the second precipitation. 1% agarose gel electrophoresis shows that methods A and B produce the clearest bands and Nanodrop spectrophotometry indicates that although method C produces slightly purer RNA, the loss in yield is large.

3.3.4 | Phylogenetic Analysis of *Gymnopus fusipes*

Sequencing the *ITS* Region for Initial Identification of *Gymnopus fusipes* Strains

When analysed using the NCBI BLAST program, all the ITS gene regions amplified using DNA extracted from samples taken in this study returned a similarity of 97% or more to *G. fusipes*.

PCR and Sequencing of Five Housekeeping Genes for *Gymnopus fusipes* MLSA

Optimisation of the PCR reactions was difficult for a number of the genes, with some strains having amplification, and other isolates having no amplification under the recommended reaction conditions. This was addressed through the attempted optimisation of cycling conditions, including changing annealing temperature and extension length, however for a number of strains, the PCR under a number of different optimised processes was still unsuccessful. Issues also occurred when some of the PCR products were sent for sequencing, resulting in incomplete sequences, and therefore incomplete datasets for the samples (Table 3.3).

Table 3.3. Genes that had successful (✓) or unsuccessful (✕) sequencing, for each isolate DNA sample.

Sample	18S rRNA	ITS	Tef1-α	28S rRNA	RPB2
AH1	✓	✓	✕	✕	✕
AH2	✕	✓	✕	✕	✕
AH3	✓	✕	✕	✕	✕
AH4	✕	✓	✕	✕	✕
AHPC	✓	✓	✕	✕	✕
AHPC-S	✕	✓	✕	✕	✕
AHPC-W	✕	✓	✓	✕	✕
C41	✓	✓	✕	✕	✕
C49	✓	✓	✕	✕	✕
C52	✓	✓	✕	✕	✕
CP1	✕	✓	✕	✕	✕
CP2	✕	✕	✕	✕	✕
CP3	✕	✓	✕	✕	✕
CP4	✕	✕	✕	✕	✕
EP1	✕	✕	✕	✕	✕
GMW83	✕	✓	✕	✕	✕
GMW83-FB	✕	✓	✕	✕	✕
HL3	✕	✓	✕	✕	✕
HL1298	✓	✓	✓	✕	✕
HL1314	✕	✓	✓	✕	✕
HW1	✓	✓	✓	✕	✕
HW2	✕	✓	✕	✕	✕
HW3	✕	✓	✕	✕	✕
HW4	✕	✓	✓	✕	✕
HW5	✓	✓	✓	✕	✕
WW134A	✕	✓	✕	✕	✕
WW134B	✕	✕	✕	✕	✕
WW200	✕	✓	✕	✕	✕
WW295	✓	✓	✕	✕	✕
G. erythropus	✓	✓	✓	✕	✕
A. bisporus	✓	✓	✓	✕	✕

Resulting nucleotide sequences were analysed in the program Geneious Prime® (version: 2023.0.3), where sequences were aligned (using the Geneious Alignment tool, with automatically determined parameters), along with a number of nucleotide sequences from NCBI GenBank (Table 3.4) for a selection of species related to *G. fusipes*, covering all four subsections (Jang et al., 2016), and a phylogenetic tree was constructed to determine whether this gene could highlight differences in the relatedness of the *G. fusipes* isolates and related species. The only gene region, which was obtained for the full set of isolates, was the *ITS* gene region (Figure 3.11). This prevented the construction of a multi-locus sequence analysis for the strains, which would have provided a deeper understanding of the relatedness of strains from different geographical regions.

Table 3.4. Species names and sequences accession numbers for the fifteen *ITS* sequences retrieved from the NCBI GenBank database to be used in alignment with the *G. fusipes* strains obtained in this study.

Species	Accession Number	Reference
Armillaria mellea	AM269761.1	Guglielmo et al., 2007
Gymnopus androsaceus	DQ444311.1	Mata et al., 2006
Gymnopus cremeostipitatus	NR_152898.1	Antonín et al., 2014
Gymnopus dryophilus	AF505787.1	Mata et al., 2002
Gymnopus dysodes	AF505778.1	Mata et al., 2002
Gymnopus erythropus	KY950460.1	Zhu, 2017
Gymnopus foetidus	KY026682.1	Petersen and Hughes, 2016
Gymnopus fusipes	KF897021.1	Ma and Lei, 2013
Gymnopus ocior	AF505782.1	Mata et al., 2002
Gymnopus omphalinoides	MW134040.1	Li et al., 2022
Gymnopus schizophyllus	NR_182367.1	Li et al., 2022
Gymnopus spongiosus	AF505784.1	Mata et al., 2002
Gymnopus subsupinus	KM975399.1	Cooper and Park, 2014
Gymnopus variicolor	NR_152921	Ryoo et al., 2020
Marasmius otagensis	MN007021.1	Hood and Lewis, 2019

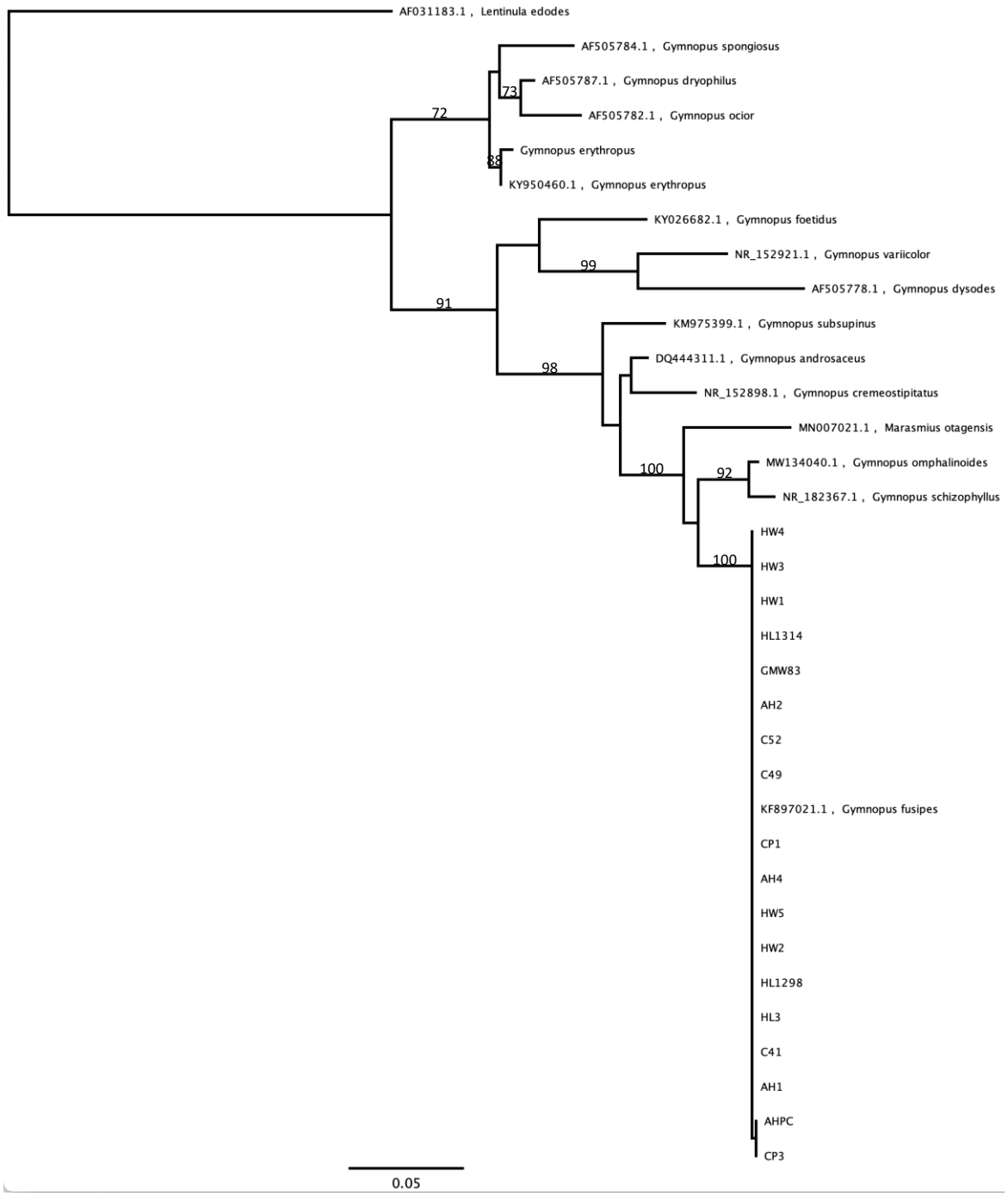

Figure 3.11. Phylogenetic tree of *ITS* sequences created in Geneious Prime® using a PhyML tree build method. Bootstrap values above 70% are indicated on the tree.

3.4 | Discussion

The overarching aim of this chapter was to optimise methods for isolation, culture and nucleic acid analyses for *G. fusipes.*

As explored in Chapter 2, there is a severe lack of research regarding *G. fusipes* in the literature, and this was reflected in the presence, or lack thereof, of viable isolates available for experimentation.

After searching multiple databases for cultures of *G. fusipes*, there were found to be little to none available. This resulted in the development of the first objective for this chapter, to isolate *G. fusipes* from environmental samples, including fruiting bodies and infected woody tissues.

Initially, identifying how to obtain samples of *G. fusipes* was troublesome, the lack of concise information regarding fruiting seasons for the species made it difficult to obtain fruiting body samples from the field. It is important to note that the main factor that has been highlighted to affect the fruiting of *G. fusipes* specifically, is that of groundwater levels (Piou et al., 2002). *G. fusipes* has been determined to be sensitive to waterlogging, which causes a reduction in infection success and growth (Camy et al., 2003b). After multiple unsuccessful fruiting seasons (potentially affected by the high levels of precipitation in the summers of 2018 and 2019), fruiting body samples and infected woody tissue samples were collected from sites across the UK in the summer and autumn of 2020, the summer and autumn of 2021 and the summer of 2022. It has been noted throughout this study that the current fruiting season for *G. fusipes* is much longer than originally described for the species (Przybyl, 1994), with fruiting bodies making an appearance from late May through to September, and sometimes even later in the year in the UK. The differences between the documented fruiting season and the current fruiting season could be explained by the lack of reliable information on the fruiting season specific to the UK, however numerous grey literature sources (fungal forums and fungal information websites) do describe similar timings to that of Przybyl (1994). The apparent changes that have developed in the phenology of *G. fusipes* fruiting over the years, could in part be due to a changing climate, as the UK has in the last 50 years, experienced a mean annual temperature increase of 1.01˚C (Met Office, 2021). This means that the ground can become warmer earlier in the season, allowing more growth and earlier fruiting, and remains warm throughout the summer and late into the autumn, altering the end of the fruiting season, which is usually caused by a drop in temperature (Kauserud et al., 2012). These discrepancies between the literature and the occurrence in the field means that it is crucial to have a number of knowledgeable individuals consistently looking for signs of fruiting body presence in the field throughout the summer and autumn seasons, and the ability to collect samples as soon as possible to prevent degradation, which can severely impact the success of isolating from the samples.

The work carried out in Section 3.3 aimed to document the most efficient way of isolating *G. fusipes* from environmental samples. After collecting good quality samples of infected woody tissue, and fruiting bodies, it was determined that a double sterilisation method was the most successful. It was found, due to the slow growing nature of *G. fusipes*, that isolating from infected woody tissues was an arduous task. Contamination of agar plates with bacterial and fungal species can be common when isolating slow growing fungi, some common fungal contaminants can reach a colony diameter

of up to 10 cm in less than 4 days (Shi et al., 2019), whereas the research carried out in Section 3.5 found that at ambient temperature, *G. fusipes* takes approximately 4 weeks to grow to this size, meaning overgrowth of contaminants was rapid. Even with the double sterilisation method described in Section 3.3, contamination, and overgrowth by secondary organisms (bacteria and potentially fungal endophytes) was common. This resulted in decision to store the woody tissue samples at 4˚C for a number of weeks before isolation attempts, which greatly diminished the number of both bacterial and fungal contaminants.

Isolating from fruiting body tissues resulted in less secondary contamination, however obtaining a spore print from a good quality basidiocarp was found to be the optimal method for clean isolation of *G. fusipes.* Both of these methods require a good quality fruiting body to be present on the infected root system at the time of collection, which is not always the case, as it is documented that fruiting bodies are not always present on infected tree systems (Marçais et al., 2000). When fruiting bodies are present, the physical condition can be varied depending on the environmental factors in play at the time of collection, as unfavourable weather conditions and high levels of moisture can cause the fruiting body to degrade. In the field (Chapter 6, Section 6.2.4), it was found that if the individual had fruited early in the season, the fruiting bodies were often already in a state of decay. Fruiting body condition was found to be critical to the success of the isolation attempt or spore print attempt, and only those in optimal condition (as fresh as possible) on collection, resulted in a successful spore print or isolation attempt.

The second objective in this chapter was to optimise cultivation techniques for the growth of *G. fusipes*, including media type and lighting conditions (Section 3.4). After consulting the limited literature and carrying out some preliminary experiments, it was determined that *G. fusipes* strains should be isolated and maintained on half strength malt extract agar plates and incubated in complete darkness. Agar plates should be poured approximately 1 cm thick (so that there is no risk of the plates drying out in the long incubation time required for *G. fusipes* to grow). Although agar plates should always be prepared with the highest standard of aseptic technique, isolates, whether from environmental samples, or from previous cultures, should be checked regularly for any potential contamination. As previously stated, this is due to the long growth time required for *G. fusipes*, approximately three to four weeks, meaning that there is a risk of contaminants quickly overgrowing the target species.

The third objective in this chapter was to test and optimise methods for effectively extracting high quality nucleic acids from different sample types, including mycelial cultures, fruiting bodies and infected woody tissues. After a large range of methods were tested, it was found that the highest

quality of both DNA and RNA are best extracted from *G. fusipes* by co-extraction using homogenisation with liquid nitrogen, a series of chloroform washes, a sodium acetate precipitation, and subsequent DNase/RNase treatments followed by a further sodium acetate precipitation. This method results in the best and cleanest yield of nucleic acids, regardless of whether the end goal is DNA or RNA.

The final objective for this chapter was to optimise PCR assays for commonly used fungal housekeeping genes and then to use these to produce an MLSA (multi-locus sequence analysis) of different *G. fusipes* isolates and related species. This objective was decidedly difficult to meet. Although a number of housekeeping genes were selected for the MLSA analysis, PCR was unsuccessful with a number of primer pairs, resulting in incomplete data sets. This in turn meant that the MLSA could not be completed in the time frame of the project. A phylogenetic tree based on the *ITS* region was completed, as a full data set (sequences for all of the isolates obtained in this study) was obtained for this gene region.

This data in this chapter provides a useful starting point for those working with *G. fusipes* in a laboratory setting, with information on best practice for isolation, culture and nucleic acid extraction. However, in light of the information presented in this chapter, it is clear that more work needs to be completed and must prioritise obtaining empirical data on the responses of *G. fusipes* to various environmental factors, as well as continuing to increase the breadth of molecular information on *G. fusipes*, including the optimisation of PCR assays for phylogenetic analysis. The combination of culture and molecular based work will be crucial to understanding this pathogen on a much deeper level, which will result in information that could aid future researchers and inform management practices.

3.5 |References

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CHAPTER 4

The Impact of Temperature on Growth Rate of the Root Rot Fungus, *Gymnopus fusipes*

Abstract

Gymnopus fusipes is an understudied root rot pathogen associated with multiple tree species and is linked to episodes of oak decline across the UK and Europe. The reported distribution of *G. fusipes* potentially spans most of the northern hemisphere, however much of these observations are based on visual identification of fruiting bodies and there is a lack of confirmatory molecular and/or isolation data to validate this ecological range. Given the paucity of information regarding the true ecological distribution of *G. fusipes*, it is difficult to predict and model the potential distribution of the species under both current and future climate scenarios. In this study, to determine the growth capabilities of *G. fusipes* across a range of ecologically relevant temperatures, five geographically diverse isolates of *G. fusipes* were grown at five different temperatures (ranging from 4˚C to 37˚C), in order to determine the optimal incubation temperature for *G. fusipes* growth, and to establish whether geographically diverse isolates showed any signs of local adaptation to temperature tolerance, by having different growth rates at different temperatures. *G. fusipes* isolates grew with varying success at 4˚C, 10˚C, 20˚C and 25˚C, with minimal growth observed with incubation at 37˚C. Statistical analysis, and a generalised linear mixed-effects model fitted to the growth data, illustrated that incubation temperature had a significant effect on *G. fusipes* growth rate, with 25˚C found to be the optimum ($P < 0.001$). Isolates were found to have different growth rates at each of the temperatures, with a UK isolate (originating from the south of England) having the highest overall growth rate across all five temperatures tested ($P < 0.001$), increasing by a mean value of over 4915 mm^2 over the 28-day experimental period (at the optimal growth temperature of 25˚C). Local adaptation to temperature was not found in the isolates tested, as there was no significant interaction between temperature and isolate. These data demonstrate the optimal incubation temperature for future laboratory studies on *G. fusipes* and provide the first data on the growth rate of this pathogen across ecologically relevant climate ranges that may inform land managers, modellers, and policy makers in predicting the current and potentially future geographical limits of this widespread root rot pathogen.

4.1 | Introduction

Gymnopus fusipes is a primary fungal pathogen, responsible for causing root rot on a number of economically important tree hosts, mainly oak, across the UK and Europe (Aguayo et al., 2021; Boddy and Thompson, 1983; Chandelier et al., 2021; Marçais et al., 1998; Piou et al., 2002; Przybyl, 1994; Schmidt et al., 2012). *G. fusipes* has been linked with various episodes of oak decline in the UK and Europe since it was first determined to be a primary pathogen in the mid 1980's (Delatour and Guillaumin, 1984; Guillaumin et al., 1985). Although the confirmed distribution of *G. fusipes* (using molecular analysis or fungal isolation) is limited to the UK and Europe, other literature that utilises less conclusive macroscopic and microscopic identification methods suggest a much larger distribution (Pettifor et al., 2022), with *G. fusipes* predicted to exist in much of the northern hemisphere, including Europe, America, Asia, and northern Africa (Ben et al., 2013; Gabel et al., 2004; Reverchon et al., 2010; Semwal and Bhatt, 2019). *G. fusipes* is reported to be the cause of root rot in a number of oak species (*Quercus* L.), as well as being present on beech (*Fagus* L.), hornbeam (*Carpinus* L.), chestnut (*Castanea* L.) (Marçais and Caël, 2000; Piou et al., 2002) and silver fir (*Abies alba* Mill.) (Ambrosio et al., 2015).

Gymnopus fusipes infection presents below ground-level as orange lesions on the main roots of the host tree, with white mycelia scattered throughout and black cord-like structures close to the bark surface (Marçais et al., 1999). Trees infected with *G. fusipes* often go undiagnosed due a frequent lack of visible symptoms, such as fruiting bodies and a deteriorating crown condition, even with a severe infection (Marçais et al., 2000, 1999). This lack of diagnosis, coupled with the destruction of large anchoring roots, results in both young and mature trees being at a higher risk of being wind thrown (Marçais and Delatour, 1996). While infection with *G. fusipes* is a slow process, taking up to 30 years from infection to mortality of the host (Camy et al., 2003a), it can be devastating, destroying large central roots, and often whole root systems (Marçais et al., 1999).

Climatic factors, such as temperature, rainfall, and extreme weather significantly influence various ecological processes, ecosystem services and biodiversity (Grimm et al., 2013; Stenseth et al., 2002). A changing climate, and specifically a change in temperature, can have both direct and indirect effects on the distribution and activity of forest pathogens, due to the multitude of complex temperature-sensitive biological processes involved in infection success and host/pathogen survival (Voyles et al., 2017). Features of pathogen biology such as growth, reproduction and dispersal can be directly affected by temperature, along with indirect effects such as changes in host distribution, as well as hosts being exposed to non-optimal conditions that cause stress and increase susceptibility to infection (Dukes et al., 2009). An example of this can be seen in *Dothistroma pini*, a

causal agent of *Dothistroma* needle blight (DNB) in France, where a gradual increase in mean temperature was identified as the key factor explaining the recent increased prevalence of the pathogen. *Dothistroma pini*, which although present in the country for a number of decades, had previously been unable to exist further north due to its need for a warmer climate (Desprez-Loustau et al., 2016; Fabre et al., 2012).

There is much uncertainty surrounding how pathogens and tree hosts will respond to a changing climate and this is due in part to the lack of empirical data determining tolerances and limits of pathogens and their hosts to different climatic elements (Dukes et al., 2009). This is made more difficult due to the fact that fungal species can survive at a wide range of temperatures, but that optimal growth rate and metabolic processing can require a much narrower margin, even when other factors, such as nutrient availability, remain constant (Dukes et al., 2009; Li et al., 2009). It is suggested that local adaptation to particular climates can cause geographic variation in pathogenicity, which can be observed in forest pathogens, whereby isolates originating from different locations may have variations in their response to temperature as a variable. For example, in the case of *Phytophthora infestans*, isolates from warmer climates were found to be less virulent at colder temperatures, and similarly, isolates from colder climates were less aggressive under warmer temperatures, with temperature differences for peak aggressiveness between isolates being up to 4°C (Wu et al., 2022). This can be explained by genetic differentiation, developing from local adaptation to environmental variables, which leads to a trade-off between enzyme stability and function, with those optimised for high temperatures being less effective at low temperatures and vice versa (Zhan and McDonald, 2011). *Puccinia striiformis* f. sp. *tritici,* the causal agent of stripe rust (yellow rust) on wheat, previously known to prefer cooler climates, has in recent years become more prevalent in warmer areas including the eastern USA and Australia (Nnadi and Carter, 2021). These isolates appear to not only have evolved to survive at these higher temperatures, thus increasing the pathogen's distribution, but have also been found to be much more aggressive at the higher temperature across numerous variables when compared to the isolates from cooler climates, including growth rate (88% increase), lesion size (50% wider) and spore production (370% more spores) (Milus et al., 2009).

In the case of *G. fusipes*, key environmental factors impacting its infection biology include waterlogging and temperature. Waterlogging, which has been identified as a limiting factor of *G. fusipes* for a number of years, is known to affect the survival of *G. fusipes* inoculum and infection success, due to the intolerance of the species to hypoxia (Camy et al., 2003a, 2003c, 2003b; Marçais and Caël, 2001, 2000; Piou et al., 2002). The effects of temperature as a limiting factor have not been researched as intensively, although predictive species distribution modelling, herbarium data,

and 75 environmental predictor variables, have determined temperature to be a limiting factor for the spread of *G. fusipes* in Norway, where *G. fusipes* did not reach the northern limits of the *Quercus* host range (Wollan et al., 2008).

There is a lack of research into the impact of temperature on the growth of *G. fusipes*, therefore the aim of this study was to firstly identify the optimal growth temperature of *G. fusipes* across a range of ecologically relevant temperatures, using a culture-based approach, and secondly to determine whether isolates from different geographical origins were locally adapted to temperature, using culture-based data and statistical modelling. This work employed traditional culture-based methods in order to address the following hypotheses: (i) temperature will have a significant effect on the growth rate of *G. fusipes* isolates, and (ii) there will be no interaction between isolate and temperature that may suggest that geographically diverse isolates have localised temperature adaptation.

Although this research focuses on *G. fusipes* growth in a laboratory setting, the resulting empirical data will still aid in understanding the growth rate of *G. fusipes* in different climates. The results of this work will help inform management techniques by offering suggestions of temperature tolerances and limits for *G. fusipes* and will also provide key information for modelling for the potential spread and overall distribution of the root rot pathogen *G. fusipes* in a changing climate.

4.2 | Materials and Methods

4.2.1 | Obtaining Isolates of *Gymnopus fusipes* and Confirmation of their Identity using *ITS* Sequencing

Strains of *Gymnopus fusipes* were obtained from two areas of the UK and three regions of France (Figure 4.1). Isolates were maintained on half strength malt extract agar (½ MEA: 25 g/L malt extract agar (Merck), 25g/L Technical Agar (Oxoid), pH 5 ± 2) at ambient room temperature (20 – 23˚C). DNA was extracted from *G. fusipes* strains using the MP Biomedicals FastDNA® Spin Kit with a modified protocol (as detailed in Chapter 3, Section 3.5.1). Briefly, 200 - 300 mg of mycelial tissue (double the quantity of tissue suggested in the manufacturers protocol), was added to a lyzing matrix A tube (containing a 5 mm ceramic bead and garnet shards, MP Biomedicals), before adding 1 ml of CLS-Y and being homogenised a total of three times at 3700 m/s for 40 seconds in a PowerLyzer24 instrument (Qiagen), the remainder of the protocol stayed as directed by the manufacturer. Extracted DNA was purified using the Zymo Clean and concentrate kit (Zymo Research) according to the manufacturer's instructions. Using the purified DNA extracts, the fungal Internal Transcribed Spacer region (*ITS)* was amplified via PCR and sequenced to confirm species identity. Briefly, after vortex mixing, 1 µl of extracted and cleaned DNA was added to GoTaq® Green Master Mix, in a PCR

reaction as follows. Each reaction was 50 µl in volume, and along with the 1 µl of DNA template, contained 25 µl of 2 x GoTaq® Green Master Mix, 22 µl of PCR-grade water, 1 µl each of 10 pmol of *ITS* gene specific oligonucleotide primers (White et al., 1990); ITS1 (5' – TCCGTAGGTGAACCTGCGG – 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3'). PCR reaction conditions were as follows: initial denaturation at 95˚C for 2 mins, followed by 35 cycles of; denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s, and extension at 72˚C for 10 s, with a final extension at 72˚C for 5 min. This produced a PCR amplification product approximately 350 bp in length. Ten microliters of the PCR product was then visualised using a 1% agarose gel electrophoresis at 100V for 45 mins. One microliter of PCR product was used for quantification using the Qubit™ dsDNA HS Assay Kit. The unpurified PCR product was sent to GENEWIZ (GENEWIZ, Takeley, United Kingdom) for sequencing, where the GENEWIZ *ITS1* primer was used in the sequencing reaction. The resulting sequences were analysed in Geneious Prime® (version 2023.0.4), where sequences were aligned, quality clipped by eye and realigned. The quality-controlled sequences were fed into the "Nucleotide BLAST" program on the NCBI BLAST database (National Center for Biotechnology Information (NCBI)). *G. fusipes* isolates were confirmed by analysing the resulting *ITS* gene sequence, whereby a sequence similarity of 97% was used as a cut off for species delineation (Pettifor et al., 2020). Each of the five isolates returned a similarity to *G. fusipes* of 99% or above, therefore were considered with confidence to be *G. fusipes*.

Each of the five strains were sub-cultured, and copies of each of the five *G. fusipes* stains were transported to an independent laboratory where from this point onwards, two parallel experiments were conducted. One experiment was completed at Bangor University, UK (BU) and the parallel experiment was run at Forest Research, Alice Holt Lodge, UK (FR).

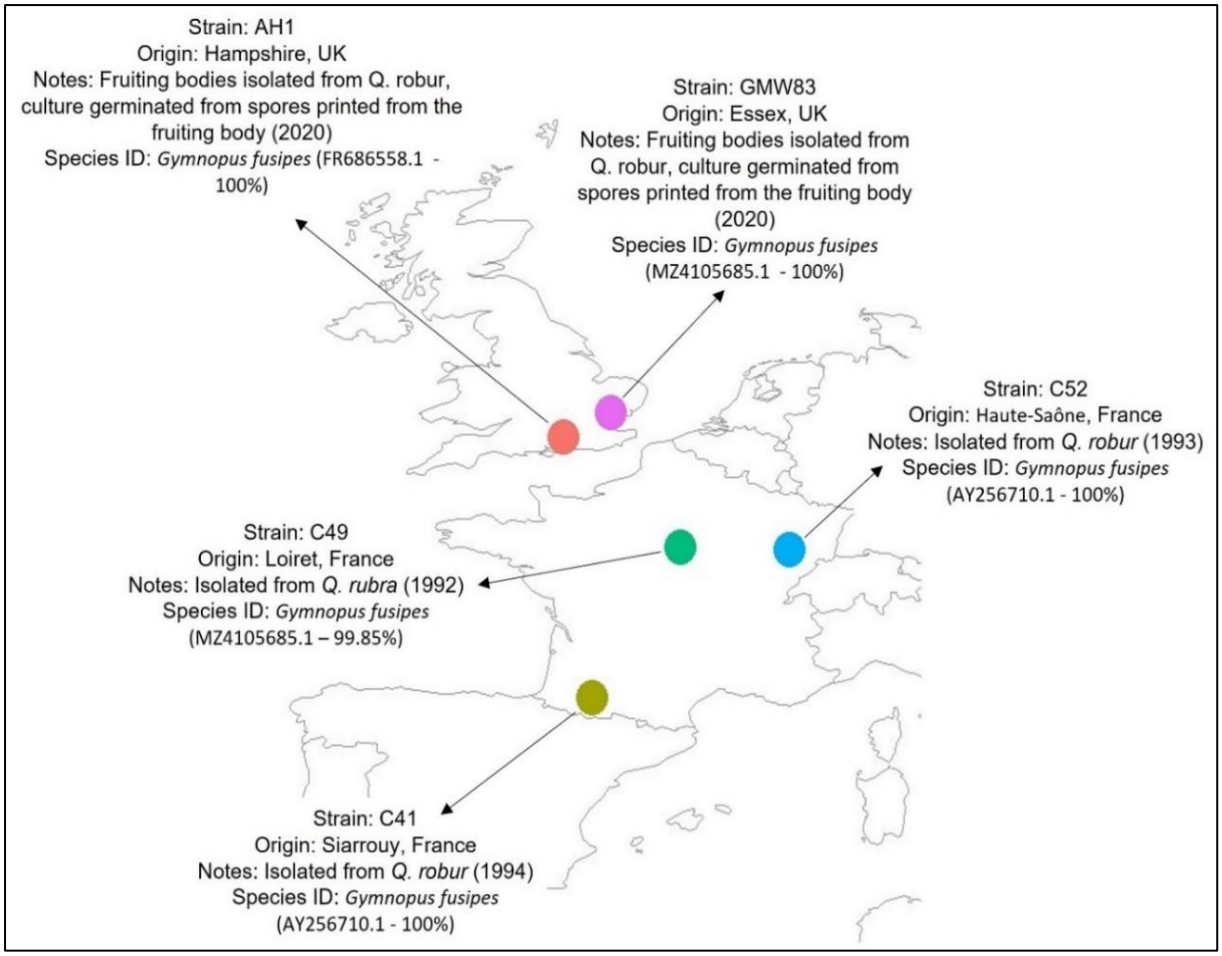


Figure 4.1. Location and information on the five isolates of *Gymnopus fusipes* that were used in this study. Strain name, place of origin and notes on isolation are detailed (along with BLAST search result identity accession number and similarity percentage for species identification).

4.2.2 | Producing *Gymnopus fusipes* Inoculum and Media Preparation for the Growth Rate Experiment

Starter cultures were prepared by filling sterile 90 mm petri dishes with 40 ml of half-strength MEA (25 g/L malt extract agar (BU used Merck/ FR used Oxoid), 25g/L Technical Agar (Oxoid), pH 5 ± 2). Three agar plugs of each isolate (C41, C49, C52, AH1 and GMW83), were inoculated onto 10 agar plates, resulting in thirty inoculation points per isolate (Figure 4.2). These starter cultures were incubated at 25˚C for 14 days. After the 14-day incubation period, one hundred and fifty sterile 90 mm petri dishes were each filled with 40 ml of ½ strength MEA, using the Fisherbrand bottle top dispenser, and perpendicular axes were drawn onto the bottom of the plates.

4.2.3 | Identifying Growth Temperature Optima for *Gymnopus fusipes*

Twenty-five experiment replicates for each of the five strains were inoculated, in the centre of the perpendicular axis (Figure 4.2). Twenty-five non-inoculated plates were kept for the duration of the experiment as non-inoculated negative controls to confirm the absence of contamination in the experimental process. After drawing around the circumference of the 5 mm agar plug (BU) or the 10 mm agar plug (FR) with a thin black permanent marker, all plates were incubated at 25˚C for 7 days, as an acclimatisation period.

After the 7-day acclimatisation period, the plates were removed from the incubator, and the circumference of the growth was outlined. At this point, 5 replicates of each strain were placed in different incubators maintained at five different temperatures, 4˚C, 10˚C, 20˚C, 25˚C and 37˚C. The layout of the replicates in each of the incubators was completely randomised, using a random number generator, in order to avoid any effects of incubator positioning. The plates were arranged in two layers of 15 (Figure 4.2), and this layout was consistent across the five different incubation temperatures, and at both sites.

Isolates were incubated at these temperatures for 7 days before the colony circumference was once again outlined. Colony outlines were recorded every 7 days; T_0, T_7 and then after being transferred to different temperatures on days T_{14}, T_{21} and T_{28} of the experiment. After the experiment, measurements were taken along each of the axis on the base of the plate, giving a total of four radial measurements per plate. Data was compiled into an excel spreadsheet for further analysis.

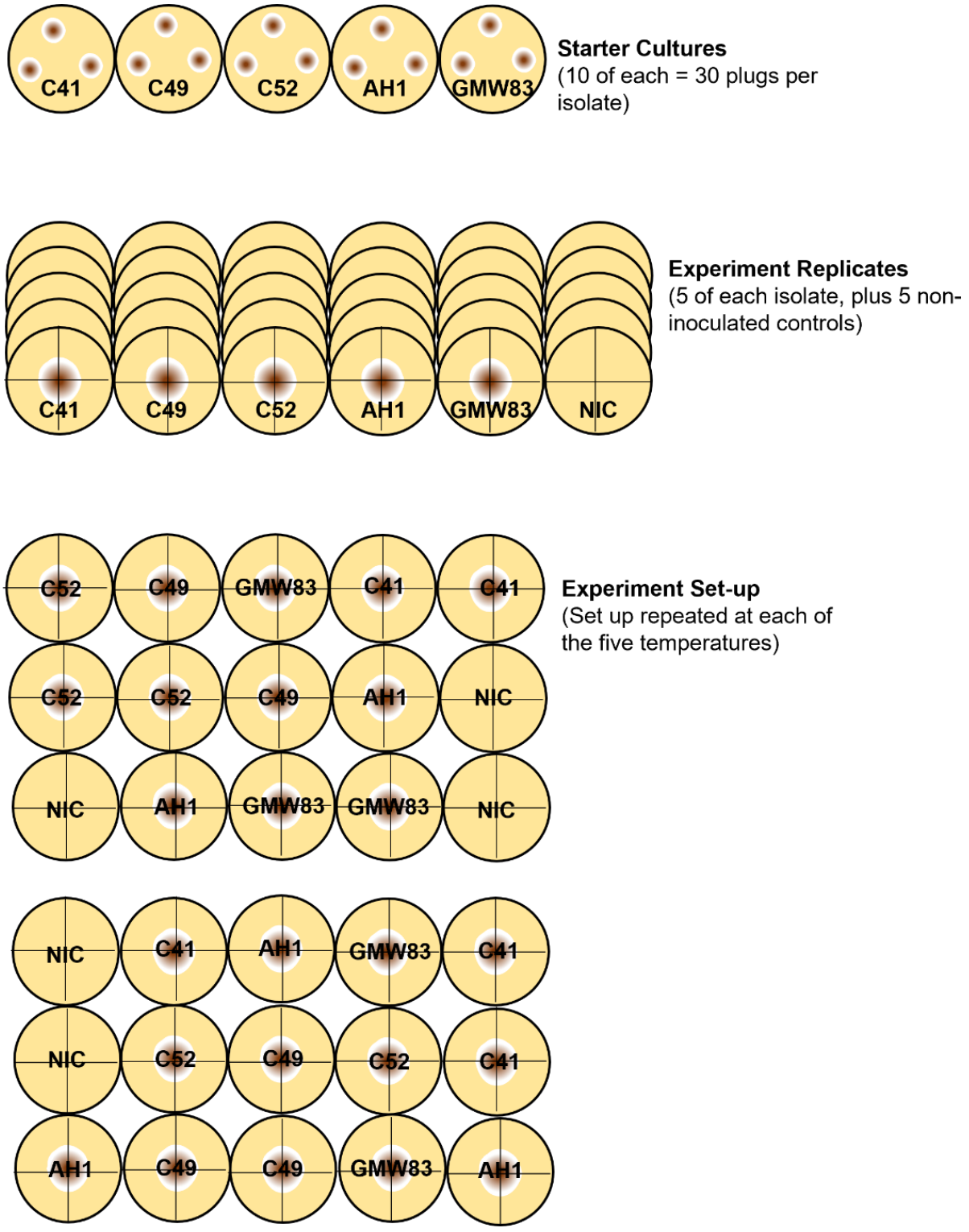


Figure 4.2. Diagrammatic representation of the experiment set up, including number of replicates for starter cultures, experiment replicates and the layout of the experimental set up which was determined using a random number generator to avoid experimental effects of incubator placement.

4.2.4 | Data Analysis

Firstly, the difference in radius between T_7 and T_{28} of the experiment was calculated, to give the overall growth of each replicate in the experimental period, excluding the initial 7-day acclimatisation period at 25˚C. The four radial measurements for each experiment isolate replicate, at each timepoint, were converted into an estimate of colony area (using πr^2) , and the mean colony area was fitted to a linear model in order to visualise trends more easily. Separate plots were made for the two parallel independent experiments at Bangor University and Forest Research.

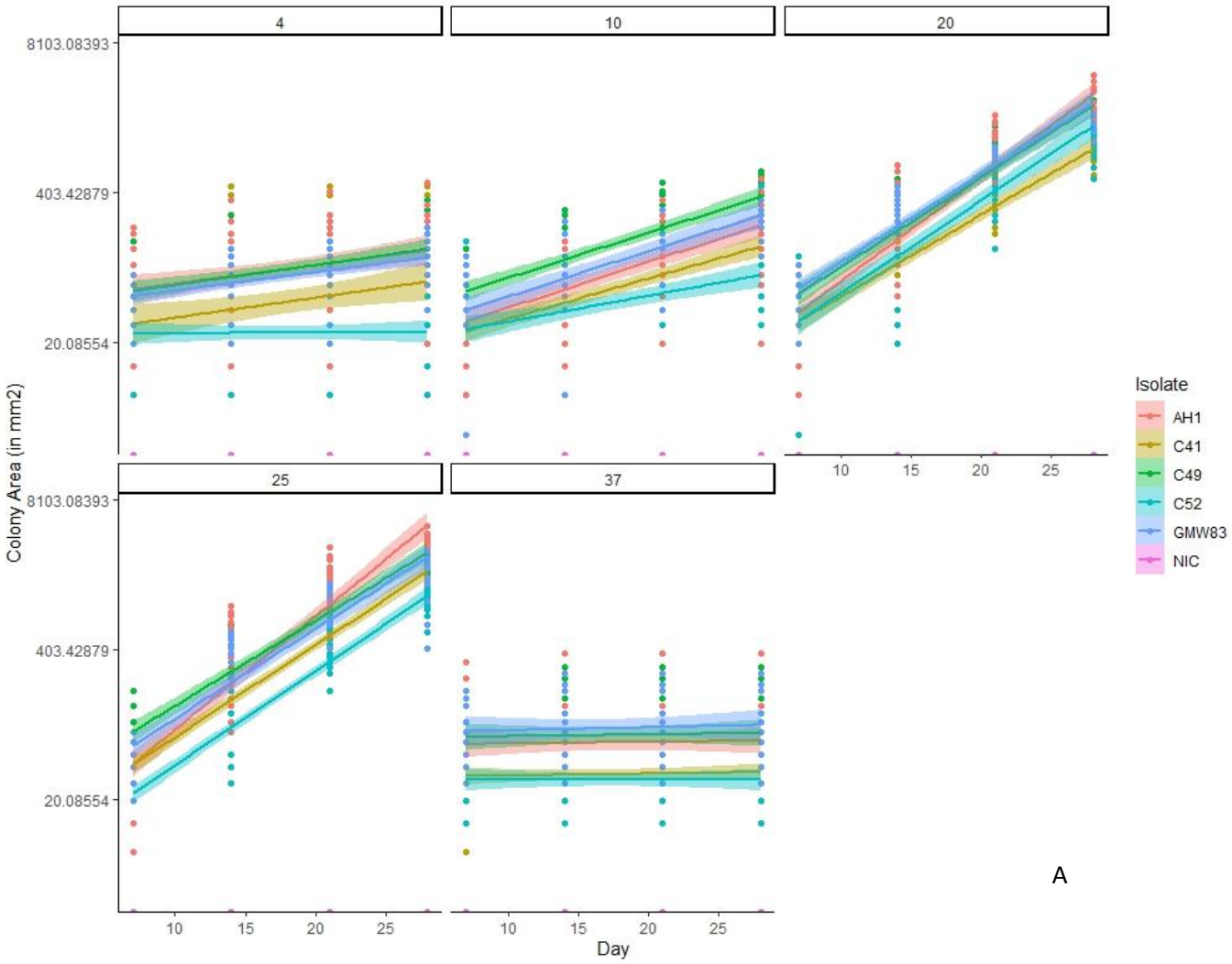
As the trends in the growth data were very similar across the two sites, the two datasets of colony area growth over the experimental period (T_7 to T_{28}) were then combined. Statistical analysis of the data was conducted, firstly to determine whether there was any significant effect of temperature on the growth rate of *G. fusipes*, whether the individual isolates had different growth rates, and to determine whether there was any significant interaction between isolate and temperature that may suggest that geographically diverse isolates have localised temperature adaptation.

This analysis was completed through the use of a generalized linear mixed-effects model. Initially considering temperature as a fixed effect, with isolate and site (BU or FR) as random effects, in order to determine the effect of temperature on growth of *G. fusipes*. As the data presented a Gamma distribution, and was a measurement of area, this was addressed in the family link section of the model parameters, whereby Gamma family with a "sqrt" link was determined. This analysis was then repeated, altering the parameters to consider the *G. fusipes* isolate as a fixed effect, to determine whether the isolate had an effect on growth rate, regardless of the temperature considered. Finally, the model was fitted to include the interaction between isolate and temperature. Statistical values for each of the models were calculated using Chi Squared tests. All statistical analyses were performed in R Studio (version 2022.02.3).

4.3 | Results

4.3.1 | Impact of Temperature on the Growth Rate of *Gymnopus fusipes*

Growth of the *G. fusipes* isolates was measured by taking four radial measurements for each experimental replicate, which were then converted to an estimate of area. Measurements were taken at T_0 of the experiment to provide a baseline figure for comparison, and then at T_7 after the acclimatisation period at 25 °C, and then on T_{14}, T_{21} and T_{28} of the experiment when the experimental replicates were being incubated at the different temperatures. These values were used to create growth over time plots for data collected at each site (Figure 4.3).



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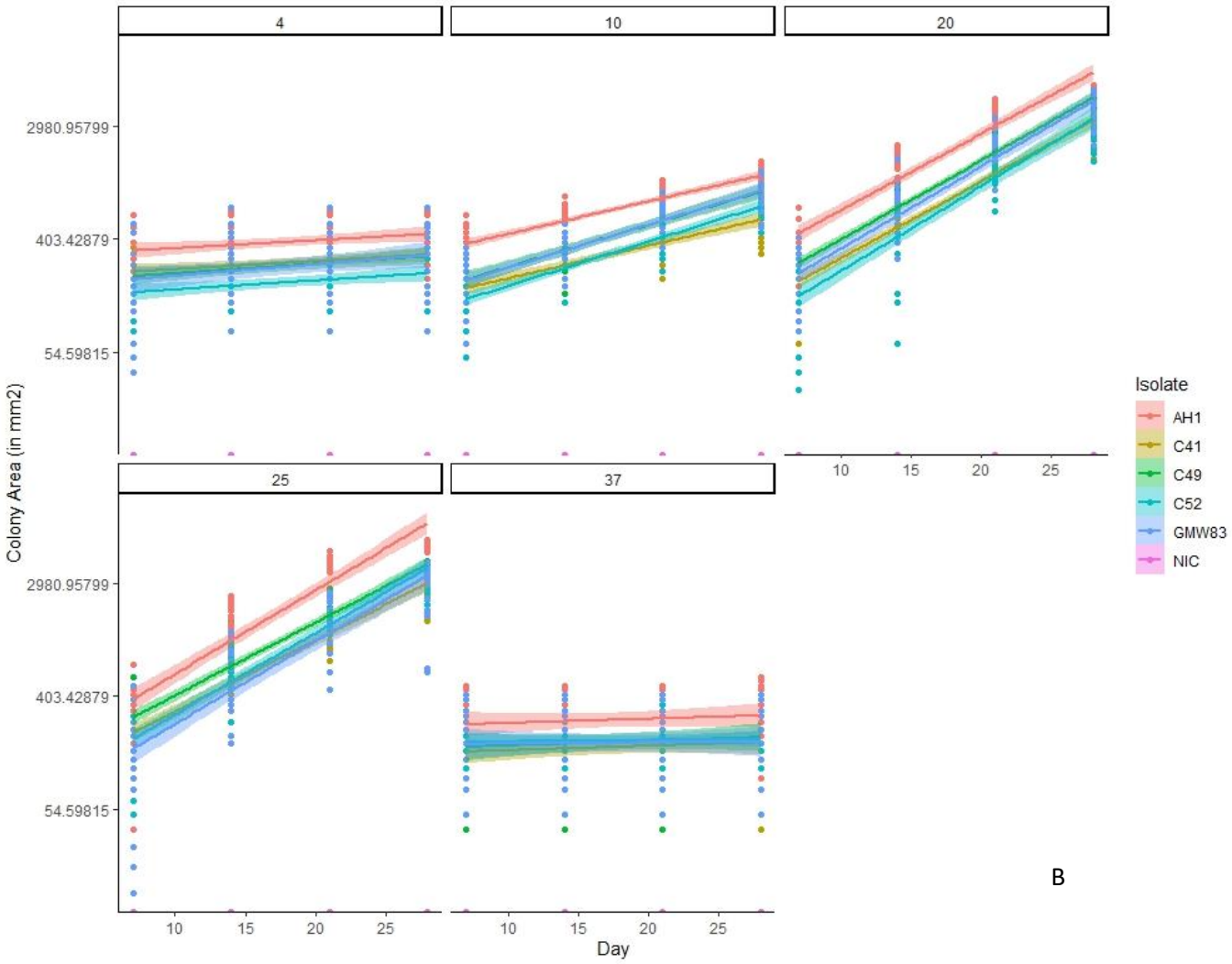

Figure 4.3. (A) Colony area (mm^2) of the *G. fusipes* isolates recorded between day 7 and day 28 of the growth rate experiment. Measurements taken at Bangor University. (B) Colony area (mm^2) of the *G. fusipes* isolates recorded between day 7 and day 28 of the growth rate experiment. Measurements taken in the parallel experiment at Forest Research.

The trends in both data sets were very similar, with all isolates undergoing a clear increase in colony area at 10 °C, 20°C and 25°C with the largest increase being at 20°C and 25 °C. Colony area increase at 4°C and 37°C was much less clear. The mean difference in colony area between T_7 and T_{28} for each of the isolates was calculated (including data from both sites) at each to provide a mean area increase for each of the experiment replicates over the experimental period. Over the 28-day experimental period, growth of *G. fusipes* isolates varied greatly between the different incubation temperatures, with growth of up to 402.6 mm^2 at 4 °C, between 8.0 mm^2 and 1003.2 mm^2 at 10 °C, between 703.3 mm^2 and 5400.2 mm^2 at 20 °C, between 671.9 mm^2 and 5568.5 mm^2, and growth of up to 86.2 mm^2 at 37 °C. Across the 5 temperatures, *G. fusipes* isolate AH1 had the largest growth over the 28-day experiment, growing between 21.2 mm^2 and 185.3 mm^2 at 4 °C, between 106.6 mm^2 and 1003.2 mm^2 at 10 °C. At the higher temperatures of 20°C and 25 °C, isolate AH1 had a growth of 1728.3 - 5400.2 mm^2 and 2430.0 – 5568.5 mm^2 respectively. At 37 °C, isolate AH1 still maintained the largest growth rate of the five isolates, growing between 0 and 86.2 mm^2 over the course of the experiment.

4.3.2 | Statistical Analysis

Statistical analyses were completed using generalised linear mixed-effects models with different parameters testing the two hypotheses, and significance was tested using Chi squared tests. Firstly, analysis was conducted to determine whether the incubation temperature had a significant effect on the growth rate of *G. fusipes.* The results of this analysis were significant ($p < 0.001$) indicating that incubation temperature does have an effect on the growth rate of *G. fusipes*, with 25°C having the largest effect, and therefore being considered the optimal temperature for *G. fusipes* growth of those tested in this experiment.

Analysis was also conducted to determine whether the isolate of *G. fusipes* influenced the growth rate observed. The results of this analysis were also significant, with isolate AH1 (isolated from a site in the south of England, UK) having the most significant effect on growth rate ($p < 0.001$), indicating that different isolates do have different growth rates, regardless of the temperature being observed. The data highlighted that at 25˚C, the optimal temperature for growth, isolate AH1 had the highest increase in mean area size over the experiment, with an overall mean increase (across the total 10 replicates, 5 from BU and 5 from FR) of approximately 4915 mm^2 (Figure 4.4).

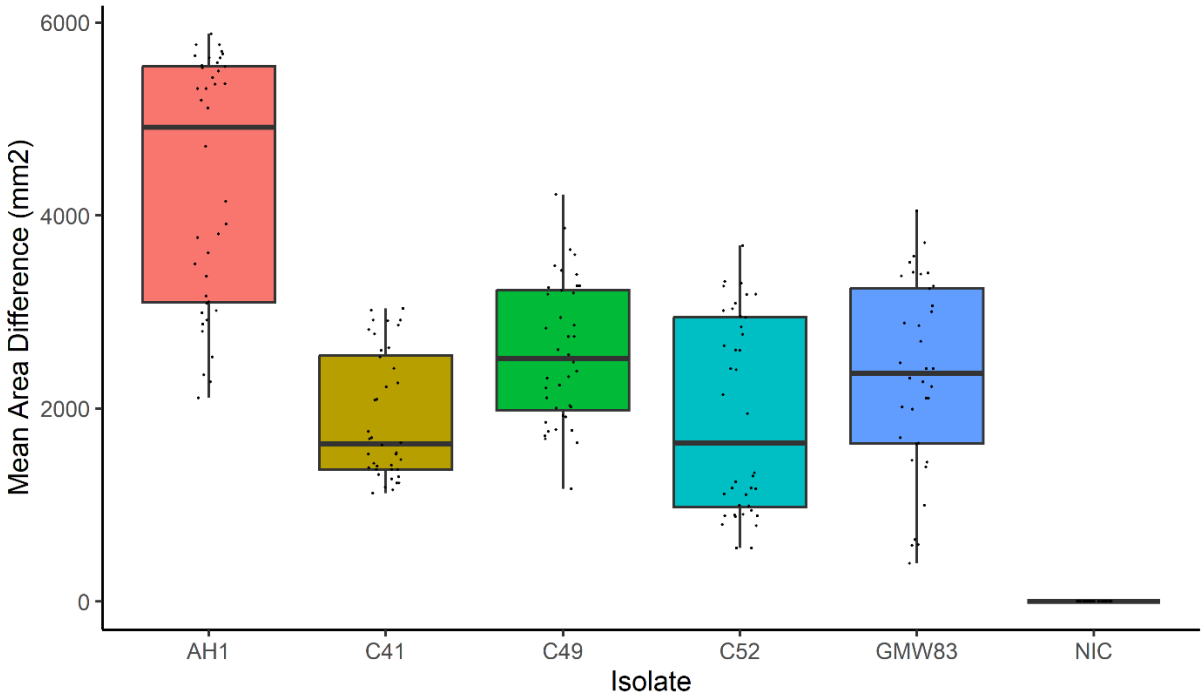

Figure 4.4. Mean area increase of the five *G. fusipes* isolates at 25˚C. Isolate AH1 had a significantly larger increase in mean area size then the other isolates tested in the experiment at this optimal temperature.

The final statistical analysis was conducted to determine if there was a relationship between incubation temperature and isolate on the growth of *G. fusipes*, which may indicate localised adaptation to temperature between the different isolates. The five isolates experienced different growth rates across the incubation temperatures, with AH1 having the largest growth rate overall, and isolates C49, C52 and GMW83 maintaining an intermediate level of growth, and isolate C52 which had overall the smallest change in area size over the experimental period. In this analysis, the relationship between isolate and temperature was used in the generalised linear mixed-effects model (Figure 4.5), with temperature as a polynomial number. This analysis provided an insignificant result, indicating that there is no significant interaction between the *G. fusipes* isolate and incubation temperature on the growth rate of *G. fusipes.* This illustrates that there is no indication of localised adaptation to temperature in the *G. fusipes* isolates tested in this experiment. There is some discrepancy between the actual data and the model predictions, particularly at 20˚C and 25˚C, where the growth of the isolates was more variable.

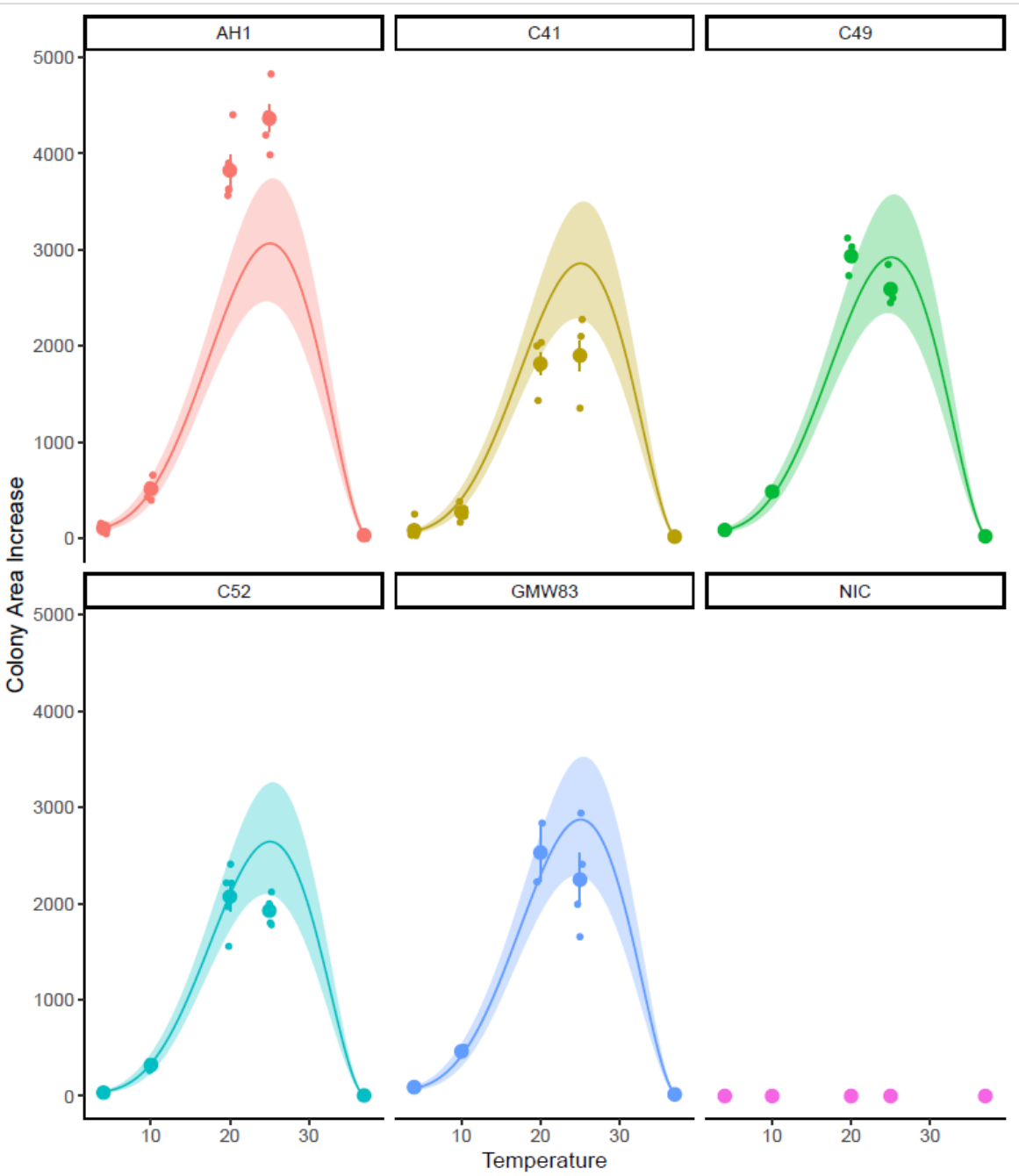

Figure 4.5. Effect of *G. fusipes* isolate, and temperature on the colony area increase of *G. fusipes* in the experimental period (with mean and standard error indicated), a generalised linear mixed-effects model (with temperature as a polynomial number) has been fitted to the data and is indicated by the continuous line along with the 95% confidence intervals for the model.

4.4 | Discussion

The main aim of this study was initially to determine the optimal growth temperature for *G. fusipes* in vitro, using traditional culture-based methods, and secondly to determine whether geographically diverse *G. fusipes* isolates showed any signs of local adaptation to temperature tolerance, using

culture-based data and statistical modelling. The data obtained from this experiment have addressed the following two hypotheses (i) incubation temperature will have a significant effect on the growth rate of *G. fusipes* isolates, and (ii) geographically diverse isolates will not display signs of any local adaptation to varying temperatures.

A significant effect was found when incubating the *G. fusipes* isolates at different temperatures, with a p value of < 0.001 indicating that incubation temperature alone is significant in its effects on the growth of *G. fusipes*, regardless of the isolate in question. The optimal incubation temperature for *G. fusipes* growth, of the five temperatures tested, was found to be 25 °C, having the largest influence on the growth rate across all five of the isolates. Isolate AH1 was found to have to fastest growth rate of all five *G. fusipes* isolates tested, regardless of the incubation temperature ($p < 0.001$), with isolates C49, GMW83 and C41 having a similar, intermediate rate of growth, and isolate C52 having the slowest rate of growth overall. The analysis to determine whether geographically diverse isolates would display signs of any local adaptation in temperature tolerance was insignificant. The results of this work showed that there was no significant combined effect of *G. fusipes* isolate and incubation temperature on the growth rate of *G. fusipes*.

Ideally, the growth rate of *G. fusipes* would have been measured in an infected root system by assessing lesion size, and not on agar plates, in order to provide a realistic expectation for growth rate, however this is not necessarily feasible for this pathogen. *G. fusipes* infection occurs below the ground level, meaning that it would be difficult to make continual measurements without disrupting the infection system. *G. fusipes* infection is difficult to identify (Chapter 2, Chapter 5), and more difficult to determine infection severity without unrooting the tree host, therefore it would be unwise to attempt to directly measure growth rate in naturally occurring infections, due to the inherent variability of the system. In artificial inoculation experiments, *G. fusipes* can take between 6 months (Chapter 6) and two years (Camy et al., 2003a; Marçais and Delatour, 1996) to develop lesions that can be measured. Measuring growth *in Planta* has been conducted in some tree pathogens, including some species from the genus *Phytophthora*, that are known to infect species of *Rhododendron* (Taylor and Grünwald, 2021), however these are fast growing leaf pathogens, and as such are more accessible for continued measurements of growth.

The data obtained from this study does not necessarily correlate with the climate data for the UK and France (Weather Atlas, 2023), where the *G. fusipes* isolates used in this study were obtained, as in the south of the UK (where isolates AH1 and GMW83 were obtained), the temperature reaches around 22°C in the peak of summer, 3°C lower than the 25°C optimum. In France, the temperature in the summer reaches around 24 °C, which is closer to the 25°C optimum. This indicates that *G.*

fusipes isolates, particularly in the UK, may be generally growing slower than isolates in warmer climates, such as Italy, Turkey and Greece (identified in Chapter 2, Section 2.3.2).

Infection success at different temperatures has been investigated for a number of other plant pathogens, often on plants with a much shorter life span, such as agricultural crops. Optimal temperatures can be identified in a laboratory setting for all plant-pathogen interactions, however it is often found that in the field, infections will often occur at a wider range of temperatures, due to adaptation to the natural fluctuations of the day. For example, *Puccinia striiformis*, a causal agent of leaf rust in wheat plants, is known to have an optimal infection temperature of 21 °C, however in the field was found to successfully infect anywhere between 18°C and 30°C (Castroverde et al., 2015; Park, 1990). In some cases, small daily fluctuations in temperature, as little as 5 °C, can negatively affect the resistance of the host plant, making them more susceptible to infection, as in the case of potato late blight caused by *Phytophthora infestans* (Castroverde et al., 2015; Shakya et al., 2015).

Phenotypic variation in temperature sensitivity is documented in a number of other fungal plant pathogens, including *Mycosphaerella graminicola*, known to cause leaf blotch of wheat (Zhan and McDonald, 2011). The lack of evidence for *G. fusipes* could be explained by the lack of globally representative isolates from more varied climatic zones, more representative of the transcontinental distribution of the species. This was, however, unfeasible due to a severe lack of *G. fusipes* isolates in global culture collections, and a limited amount of culture-based work having been completed by previous researchers, who were therefore unable to provide isolates representative of other locations (highlighted in Chapter 3). The study would benefit from the addition of globally representative isolates.

This experiment has provided empirical data that could potentially be used in processes such as predictive modelling which could to help determine the current and potential future distribution of *G. fusipes.* Environmental niche modelling, or species distribution modelling are key tools providing analysis of biotic and abiotic factors such as environmental conditions and host availability, to identify potential distribution of a species, which can be used to determine effects of a varying factors including climate change (Wang et al., 2018). Although this can be considered a crucial task in the understanding of the spread of phytopathogens, the majority of environmental niche modelling has been done on plants, animals and insects (Chaloner et al., 2020). It has been suggested that the low number of phytopathogen environmental niche models could be due to the difficult nature of phytopathogen study, including identification difficulty, lack of visible symptoms, and life-cycle effects, as well as the lack of available data on factors such as global distribution (Ireland and Kriticos, 2019). In order to utilize existing modelling software, such as CLIMEX, to determine the

potential distribution of a phytopathogen such as *G. fusipes*, numerous environmental factors must be considered. These parameters include moisture, heat and cold stress, dry and wet stress, and cold-wet stress, as well as temperature (Yonow et al., 2013).

Future research on *G. fusipes* should aim to prioritise obtaining empirical data regarding the effect of various environmental factors on *G. fusipes*, in order to inform distribution modelling. This could include repeating this experiment with more temperature increments between 25°C and 37 °C, to determine the temperature limits. An effort should also be made to increase accurate identification of *G. fusipes* in the field, on a global scale, as this will provide evidence to support the somewhat questionable distribution claims made about this species. It is important that a deeper understanding of the effects of a changing climate on this species is acquired, as particularly an increase in temperature and a decrease in waterlogging occurrences may have a significant effect on the spread and also aggressiveness of this pathogen.

4.5 | References

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CHAPTER 5

Development and Application of a qPCR-Based Diagnostic Tool for Accurate Detection of *Gymnopus fusipes*, the cause of *Collybia* Root Rot in Oak

Abstract

Gymnopus fusipes (syn. *Collybia fusipes*) is a facultative, saproparasitic fungus causing severe root rot on oak trees across Europe. *G. fusipes* infection develops slowly, but can decimate entire root systems, resulting in hosts being windthrown, with significant impacts on woodland habitats, ecosystem services and risks to people and property. Often above ground symptoms of infection, such as deterioration of host crown condition and presence of fruiting bodies are absent. Consequently, there is a paucity of information on the distribution and severity of *G. fusipes* infection in oaks, highlighting the need for a rapid molecular diagnostic for *G. fusipes*. The aim of this research was therefore to develop a *G. fusipes*-specific quantitative Polymerase Chain Reaction (qPCR) assay that can confirm the identity of *G. fusipes* isolates, in addition to culture independent identification of *G. fusipes* fruiting bodies and detection in infected woody tissues. All available 18S rRNA gene sequences for *G. fusipes* and related fungal species were used to design *G. fusipes*-specific 18S rRNA gene qPCR primers. Primers were initially validated using *in silico* PCR and subsequently, the specificity of the assay was confirmed in qPCR assays using synthetic DNA control fragments with 3-10 bp mismatches with the target sequence. To validate the molecular diagnostic, the qPCR assay was tested on *G. fusipes* fruiting bodies, soil and woody tissues from infected and asymptomatic trees at a parkland impacted by *G. fusipes* infection. The qPCR assay successfully identified all fruiting body samples as *G. fusipes* (n=6) and detected *G. fusipes* infection in 67% of woody tissue samples from infected trees (n=4/6). *G. fusipes* was not detected in soil samples or the woody tissues of asymptomatic trees. The qPCR assay designed in this study provides a useful diagnostic and monitoring tool to identify the distribution and prevalence of *G. fusipes* infection in host trees.

5.1 | Introduction

Gymnopus fusipes (Bull.:Fr.) Gray (syn. *Collybia fusipes*), is a slow-growing primary pathogen capable of colonising whole root systems of both young and mature trees (Camy et al., 2003a; Marçais and Delatour, 1996), resulting in disruption of life-critical functions such as water and nutrient uptake and deterioration of crown condition that can lead to mortality (Camy et al., 2003b). *G. fusipes* is primarily connected to infection in oak (*Quercus* L.) species; however, it is also thought to affect other broadleaf and coniferous host species (Marçais and Caël, 2000; Piou et al., 2002). *G. fusipes* is known to occupy woodlands throughout Europe (Aguayo et al., 2021; Boddy and Thompson, 1983; Chandelier et al., 2021; Marçais et al., 1998; Przybyl, 1994; Schmidt et al., 2012), however it potentially has a distribution spanning much of the northern hemisphere (Ben et al., 2013; Gabel et al., 2004; Piou et al., 2002; Reverchon et al., 2010; Semwal and Bhatt, 2019). In recent years, *G. fusipes* has been linked with chronic oak decline (COD) in the UK (Quine et al., 2019), where this pathogen along with numerous other biotic and abiotic factors interact to cause deterioration and eventually mortality of the host (Denman and Webber, 2009; Gagen et al., 2019).

G. fusipes fruiting bodies are distinctive, with a stipe that tapers from the cap to the base (Bulliard, 1783), however the tapering section of the stipe is often close to the ground, sometimes appearing to be rooted into the soil, therefore making it difficult for a non-specialist to confirm identity without excavation (Petersen and Hughes, 2017). Further to this, there is often a lack of fruiting body presence, even with severe *G. fusipes* infection, and consequently the absence of fruiting bodies is not necessarily a good indication of host health status (Camy et al., 2003d). Typical decline symptoms, including a deterioration of crown condition, are also not always correlated with *G. fusipes* infection status, with some heavily infected individuals maintaining a full crown until mortality (Marçais and Caël, 2001; Thomas et al., 2002). Infection is often only clearly apparent below the ground level, as the fungus spreads downwards through the root system after settling and infecting at the base of the tree host as a basidiospore (Camy et al., 2003c; Casselton and Olesnicky, 1998). Below the ground, on the main roots of the host, *G. fusipes* infection appears as yellow or orange-coloured lesions often with white mycelial fans throughout the necrotic tissues (Camy et al., 2003d, 2003c).

Diagnosis of fungal pathogens in the forest ecosystem is usually done through macroscopic and microscopic analysis of fruiting bodies by knowledgeable individuals (Kimic et al., 2022), often accompanied by isolation of a pathogen from infected tissue for subsequent DNA extraction and sequencing. However, isolation of pure isolates from environmental samples can be challenging, and culture time can range from a few days to several weeks, making cultivation-based diagnostics a laborious process that could yield false negative results (Luchi et al., 2020). With a large proportion

of *G. fusipes* host species (oak species, for example) being found not only in forests, but also in gardens, public parks and grounds, there is a heightened sense of urgency to accurately diagnose potential *G. fusipes* infection. There is a real risk of injury to people and damage to properties that comes with the increased risk of trees being windthrown due to a deteriorated root system (Camy et al., 2003a; Jactel et al., 2009; Marçais et al., 1999). Consequently, there is a need for a rapid and accurate diagnostic tool for identifying *G. fusipes* in environmental samples without the requirement for pure culture.

The main aim of this study was to develop a *G. fusipes* specific quantitative Polymerase Chain Reaction (qPCR) assay will the ability to confirm the identity of cultured *G. fusipes* isolates, as well as the culture independent identification of *G. fusipes* fruiting bodies and detection in infected woody tissues. This will be done through the completion of the following objectives: (i) to design species-specific qPCR primers for *Gymnopus fusipes*, and (ii) to optimise a qPCR assay to accurately detect *G. fusipes* in environmental samples such as fruiting bodies and infected woody tissue.

5.2 | Materials and Methods

5.2.1 | Primer Design

After checking relevant databases for *G. fusipes* nucleotide sequences, all available 18S rRNA nucleotide sequences belonging to members of the genus *Gymnopus*, and the closely related genera *Collybia, Rhodocollybia* and *Dendrocollybia*, were obtained from the NCBI GenBank. The 18S rRNA gene nucleotide sequence for *Armillaria mellea* (Accession number: MH855148.1) was also included as an outgroup. Sequences were aligned using the MUSCLE alignment tool in Geneious Prime (version 2022.2.2), using standard program-defined parameters, in order to find polymorphisms between the species.

Using regions conserved within the *G. fusipes* sequences, but variable in all other species, five primer pairs for the 18S rRNA gene were designed. Potential primer pairs were checked against accepted primer design rules (Dieffenbach et al., 1993), and their specificity was then tested *in silico* using the NCBI PrimerBLAST tool (www.ncbi.nlm.nih.gov/tools/primer-blast/), using a *G. fusipes* sequence (accession number: AY256711.1) as the PCR template (Figure 5.1). The three most specific primer pairs (those that returned the least amount of cross-reacting non-target sequences in the PrimerBLAST search), along with a synthesised DNA fragment of the *G. fusipes* 18S rRNA gene sequence used for primer design, and a selection of 8 non-target but potentially cross-reacting 18S rRNA sequences identified in the alignment (with 3-10 bp mismatches with the primer pairs), were ordered from Eurofins Genomics (Ebersberg, Germany).

5.2.2 | Primer Testing and Assay Optimisation

Each qPCR reaction contained 12.5 µl of 2 x QuantiFast SYBR Green PCR Master Mix, 2.5 µl of forward primer Gf_qPCR_F1 (10 µM), 2.5 µl of reverse primer Gf_qPCR_R1 (10 µM), 6.5 µl of nuclease-free H_2O and 1 µl of DNA template. Reactions were performed using the Applied Biosystems QuantStudio™ 6, with set-up and analysis completed with the accompanying QuantStudio™ Real-Time PCR System software (v1.7.1).

Initial reaction conditions consisted of an initial denaturation step at 95˚C for 5 minutes, followed by 40 cycles of denaturation at 95˚C for 10 seconds and annealing at 60˚C for 30 seconds. This was followed by a melt curve analysis as follows; 95˚C for 15 seconds, 60˚C for 1 minute and a slow increase (0.05˚C/second) to 95˚C for 15 seconds.

Using the non-target 18S rRNA gene sequences from the NCBI PrimerBLAST search (Table 5.1) that had a low number of base pair mismatches to the target primer sequence binding site, the conditions of the reaction were optimised to validate the specificity of the assay. This was done through systematically increasing the primer annealing temperature from 60˚C to 66˚C, until accurate *G. fusipes* amplification was achieved and no amplification of the non-target sequences occurred in 40 cycles of the reaction (Figure 5.1).

Table 5.1. G. fusipes sequence and non-target sequences from the NCBI PrimerBLAST search ordered to be synthetically produced for testing in the lab. Base pair sequence match to primer sequence indicated by red underlined bases. Total number of base-pair mismatches to the sequence are also highlighted. Sequence

Sequence	NCBI Accession Number	Forward Primer Sequence (with mismatches highlighted)	Reverse Primer Sequence (with mismatches highlighted)	Total BP mismatches
Gymnopus fusipes	AY256711.1	GCACGTCTTATTTCTAATCC	GACTTCGAAAAAAAAGGGC	0
Agaricus bisporus	CP039879.1	***A***CAC***T***T***G***TT***T***TTTCTAATC***G***	GACTTCGAA***T***A***C***AAAG***ACA***	10
Melanopsichium pennsylvanicum	HG529617.1	GCACGTCTT***TG***TTC***G***AAT***T***C	***A***ACTTCGAAA***G***AAAAG***C***G***G***	8
Coprinopsis cinereal okayama	XM_002910848.1	GCACGTCTTATTTCTAATCC	***C***AC***A***TC***A***AAAA***C***AAAGGGC	4
Melampsora larici-populina	XM_007409322.1	GCACGTCTTATTTCTAATCC	***T***A***A***T***GG***GAAAAAAAAGGG***A***	5
Cryptococcus neoformans var. neoformans	AE017345.1	GCACGTCTTATTTCTAATCC	***TC***C***A***TCGAAAAAAAA***G***GGC	4
Apoitrichum mycotoxinovorans	CP053620.1	GCACGTCTTATTTCTAATCC	***AT***C***AG***CGAAAAAAAAGG***T***C	5
Termitomyces sp.	MH725798.1	GCACGTCTTATTTCTAATCC	***CT***CTTC***C***AAAAAAAAGGGC	3
Termitomyces sp.	MH725798.1	GCACGTCTTATTTCTAATCC	G***T***CT***A***CGAAAAAAAAGGG***G***	3

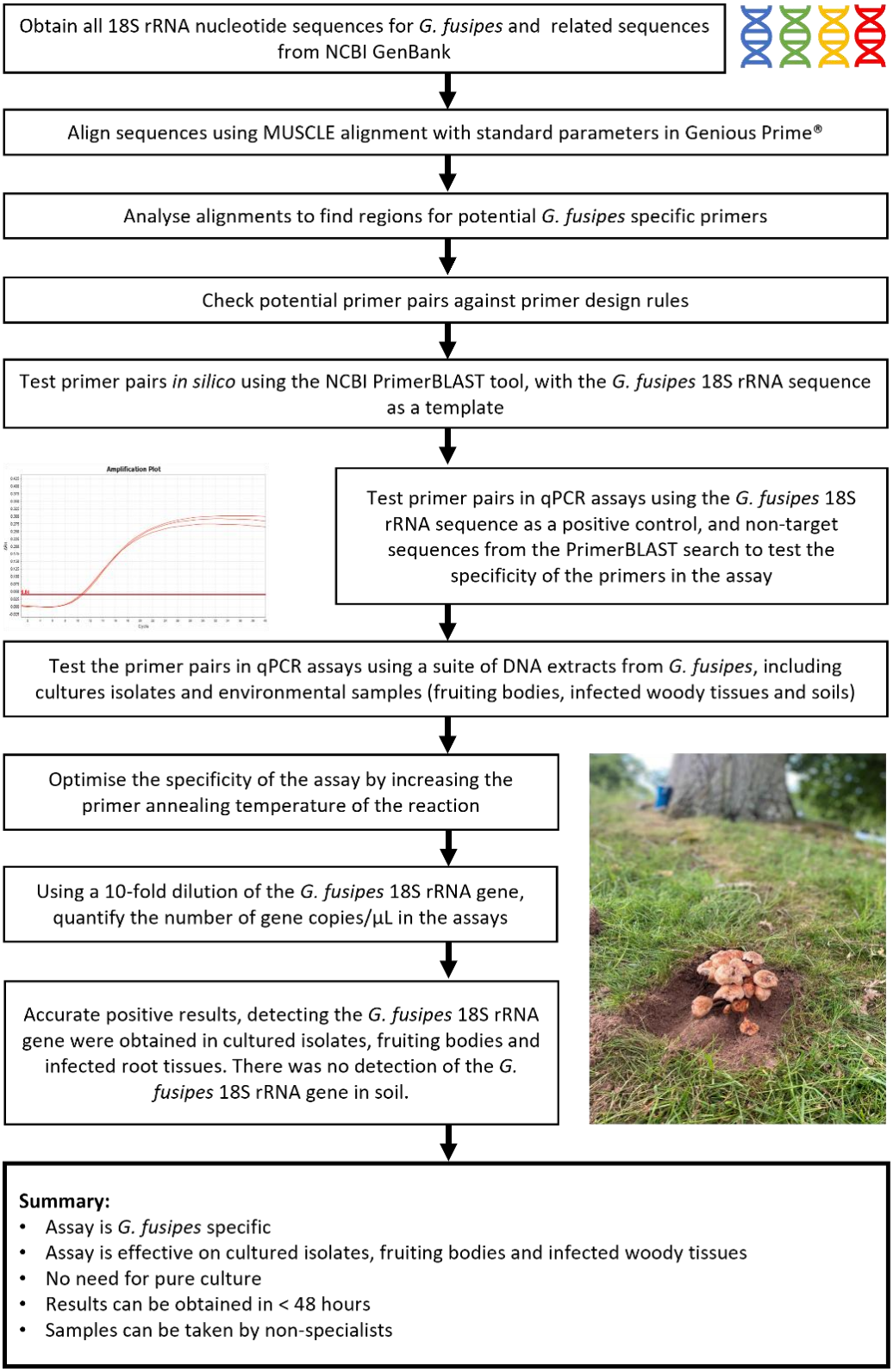

Figure 5.1. Primer design workflow summary. After obtaining all nucleotide sequences available of the 18S rRNA gene for *G. fusipes* and related species, sequences were aligned, and the alignment was used to identify potential regions for primer sites. Primer pairs were tested firstly *in silico*, and then using synthetic 18S rRNA gene fragments of *G. fusipes* and non-target sequences with 3-10 bp mismatches with the target sequence. The specificity of the assay was optimised through a temperature increase, and the final assay was used to successfully identify *G. fusipes* from in various samples from a parkland impacted by *G. fusipes* infection.

5.2.3 | DNA Extraction from *Gymnopus fusipes* Isolates, Fruiting Bodies and Environmental Samples (soil and woody tissues) to Validate the *G. fusipes*-specific qPCR Assay

DNA was isolated from *G. fusipes* mycelial cultures using the FastDNA™ Spin kit (MP Biomedicals) with modifications described in Chapter 3, and subsequent clean up using the Zymo DNA Clean & Concentrator kit (following the manufacturer's protocol), which was found to be a crucial step when using this extraction method.

DNA was extracted from fruiting bodies, infected woody tissues and soils using a modified phenol-chloroform extraction method (also detailed in Chapter 3). Briefly, wood tissue and soil samples were homogenized either by grinding with liquid nitrogen in a pestle and mortar (wood tissue), or by bead-beating with a 5 mm ceramic bead (fruiting bodies and soil). Homogenized samples were then added to a tube containing 1 ml of 4% CTAB buffer (8.18 g NaCl; 4.00 g CTAB (Sigma); 1.00 g PVP (Fluka); 20 ml 1M Tris-HCl; 4 ml 0.5M EDTA (Invitrogen); made up to 100 ml with ultrapure H_2O) and heated at 60˚C for 1 hour (inverting every 15 mins). The homogenate was then centrifuged at 14000 x g for 15 minutes at room temperature. Equal volumes of chloroform:isoamyl alcohol (24:1) were added to the tube and this was mixed on a rotating mixer for 5 minutes, before being centrifuged again at 14000 x g for 15 minutes at room temperature. The supernatant was once again placed in a new 1.5 ml tube, and the chloroform wash step was repeated. After the second wash, the supernatant was transferred again to a new 1.5 ml tube, and nucleic acids were precipitated with the addition of 250 µl NaCl 6M, 50 µl sodium acetate 3M and 500 µl ice-cold isopropanol and incubated at -20˚C overnight. After the overnight incubation, reactions were centrifuged at 14000 x g for 15 minutes at room temperature. The supernatant was removed, and the pellet was carefully washed with 500 µl of 70% ethanol. Without removing the alcohol from the tube, the tubes were centrifuged again at 14000 x g for 15 minutes at room temperature to rebind the pellet to the tube wall. After this, all alcohol was removed from the tubes and the DNA pellets were allowed to air dry at room temperature for 15 to 20 minutes. DNA pellets were resuspended in 50 µl of ultrapure nuclease-free H_2O. After the nucleic acids were resuspended, the tube was treated with RNase A Solution and incubated at 37˚C for 30 minutes, after which a secondary precipitation step was followed. To the 50 µl of nucleic acids, 25 µl of NaCl 6M, 5 µl of sodium acetate 3M and 50 µl ice-cold isopropanol were added, and this was incubated at -20˚C for a minimum of 2 hours. After the incubation, extractions were centrifuged at 14000 x g for 15 minutes at room temperature. Once again, the supernatant was removed, and the pellet was carefully washed with 500 µl of 70% ethanol. Without removing the alcohol from the tube, the tubes were centrifuged again at 14000 x g for 15 minutes at room temperature to rebind the pellet to the tube wall. Again, alcohol was removed from the tubes and the DNA pellets were allowed to air dry at room temperature for 15 to 20 minutes. DNA pellets were resuspended in 30 µl of ultrapure nuclease-free H_2O.

5.2.4 | Quantitative PCR (qPCR) Detection of *Gymnopus fusipes* in Environmental Sample Types

Gymnopus fusipes was detected in environmental samples (fruiting bodies and infected wood tissue) using the qPCR assay as stated in section 2.2, using 1 µl of environmental DNA extracts as template in each of three replicate qPCR assays. Cycling conditions were as follows; an initial denaturation step at 95˚C for 5 minutes, followed by 40 cycles of denaturation at 95˚C for 10 seconds and annealing at 66˚C for 30 seconds. This was followed with a melt curve analysis as above. The C_t value (the cycle number at which the fluorescence of the SYBR green passes the threshold level of 0.04) was measured in each reaction, and complimentary melt curve analysis was conducted.

5.2.5 | Preparation of DNA Standards for Abundance

DNA standards for absolute quantification using a standard curve were produced using the *G. fusipes* 18S rRNA sequence (accession number: AY256711.1), which was used to design the qPCR primers. Briefly, the concentration of DNA in the standard was measured using the Qubit dsDNA HS Assay Kit (Invitrogen) and the Qubit 3.0 fluorimeter (Invitrogen). Using calculations from (Qiagen, 2010) and a 10-fold serial dilution method, *G. fusipes* standards from 3 x 10^6 to 3 gene copies were produced. One microliter of each dilution was run in triplicate on each reaction plate and used to create a standard curve.

5.2.6 | Field Sample Analysis

Fruiting bodies (from symptomatic hosts only, identified through morphological analysis), woody tissues and soil samples were collected from paired symptomatic and asymptomatic oak trees infected with *G. fusipes* at a parkland site in the UK (Figure 5.2). Fruiting bodies and infected woody tissues were collected from infected hosts and immediately frozen in liquid nitrogen before being stored at – 80˚C once back in the lab. To assess if mycelia of *G. fusipes* could be detected in the soil adjacent to infected trees, soil samples were collected from the area touching infected tree roots, 15 cm away from the infected root and 30 cm away from the infected root.

Figure 5.2. Map of samples taken from field site in the UK. In some cases, multiple samples were taken from the same tree system, therefore the numbers correspond to the sample identification number. The blue icon represents a dead tree, the yellow icon represents a tree with no above ground symptoms but with root rot present, the green icons represent healthy trees, and the red icons represent trees with G. fusipes infection.

DNA extractions were carried out using the modified phenol chloroform method as above, and DNA extracts were run in triplicate through the optimised qPCR assay protocol. For fruiting body and infected woody tissue extracts, DNA was diluted 1:10 before being used in the qPCR assay.

5.3 | Results

5.3.1 | Primer Design and Specificity

Seven hundred and eighty-eight nucleotide sequences for the 18S rRNA gene (Figure 5.3), covering *G. fusipes* and 112 related species (Supplementary Information 5.1) were aligned. Polymorphisms between *G. fusipes* and related species were identified and used to design species specific primers with identical nucleotide sequences to *G. fusipes* (Figure 5.4).

Results of the PrimerBLAST search (Supplementary Information 2) highlighted that primer pair 1 (Gf_qPCR_F1/Gf_qPCR_R1) had the smallest number of non-target sequences returned (n=4), with between 8 – 10 bp mismatches with the target sequence. A selection of non-target sequences from

the search across all five potential primer pairs, containing between 3 and 10 bp mismatches with the target *G. fusipes* 18S rRNA sequence were ordered for testing in the lab.

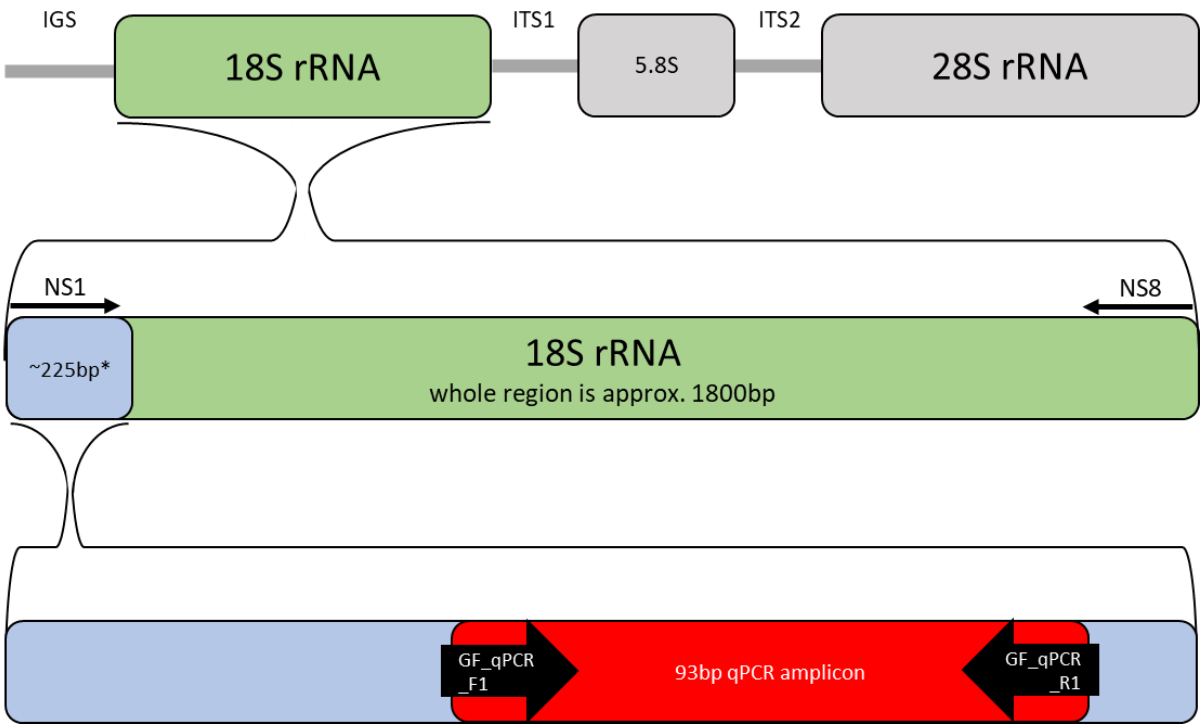

Figure 5.3. Schematic of the 18S rRNA gene region, approximately 1800 bp in length when amplified using the NS1 and NS8 primer set. The region of interest for this study (*) is in the first section of the gene (~225bp), where the forward and reverse qPCR primers are located, flanking the 93 bp qPCR amplicon.

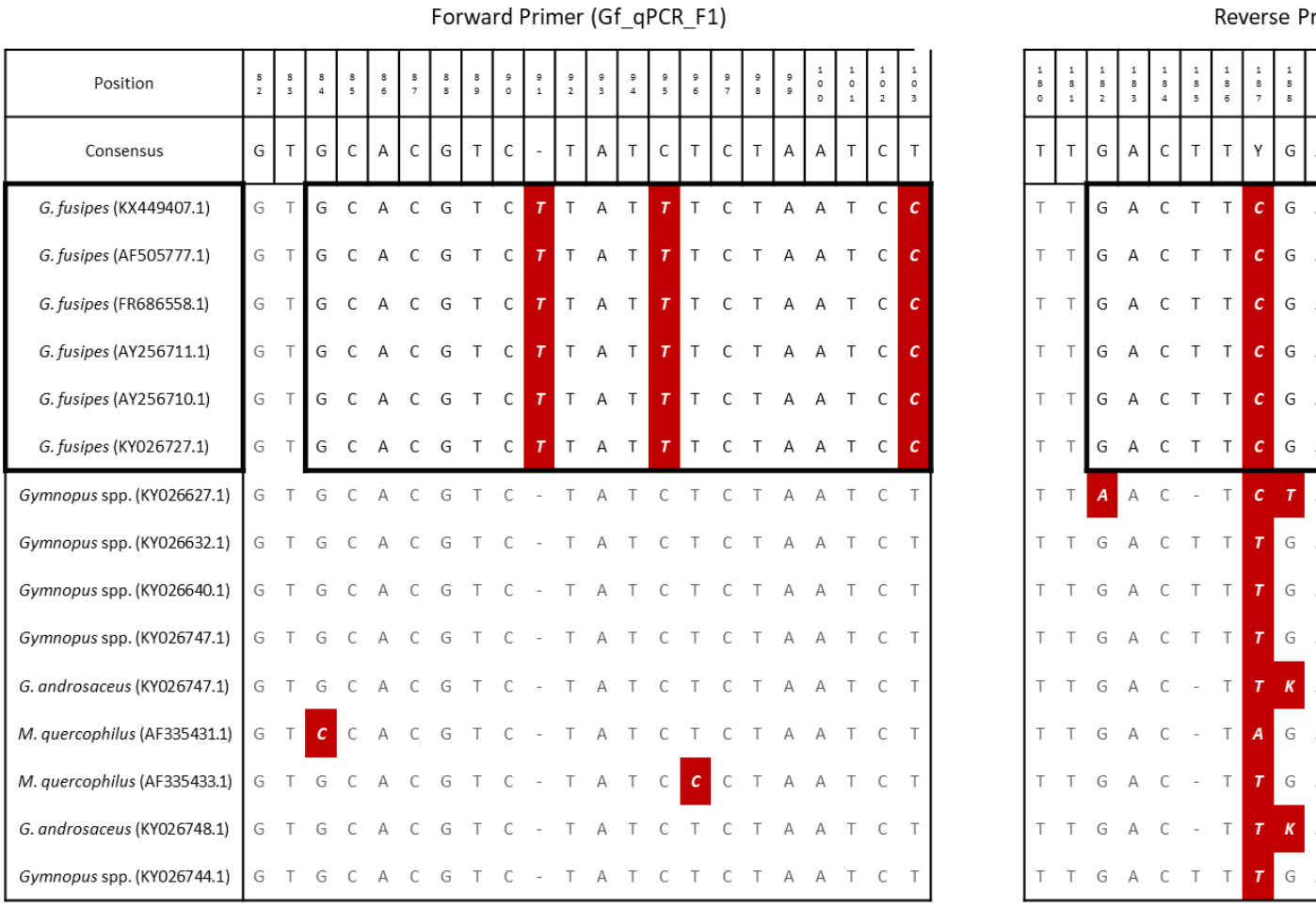

Figure 5.4. Section of the multiple alignment of the 18S rRNA gene sequence with *G. fusipes* and related species, highlighting binding sites for forward primer (Gf_qPCR_F1) and reverse primer (Gf_qPCR_R1) used to detect *G. fusipes* 18S rRNA gene. Sequences were obtained from the NCBI GenBank database, and corresponding accession numbers are detailed in brackets.

5.3.2 | Primer Testing and Assay Optimisation

Initially, amplification of non-target control sequences was observed when the manufacturer's recommended annealing temperature of 60˚C was used, and so a series of increases to the primer annealing temperature were tested to optimise the assay and make it more specific to *G. fusipes*. When the annealing temperature was increased from 60˚C to 66˚C, amplification of the non-target sequences was eliminated during the 40-cycle reaction. The increase in temperature caused an increase in C_t value for all samples in the reaction, including environmental samples and standards, however the specific *G. fusipes* samples were still successfully detected (Figure 5.5).

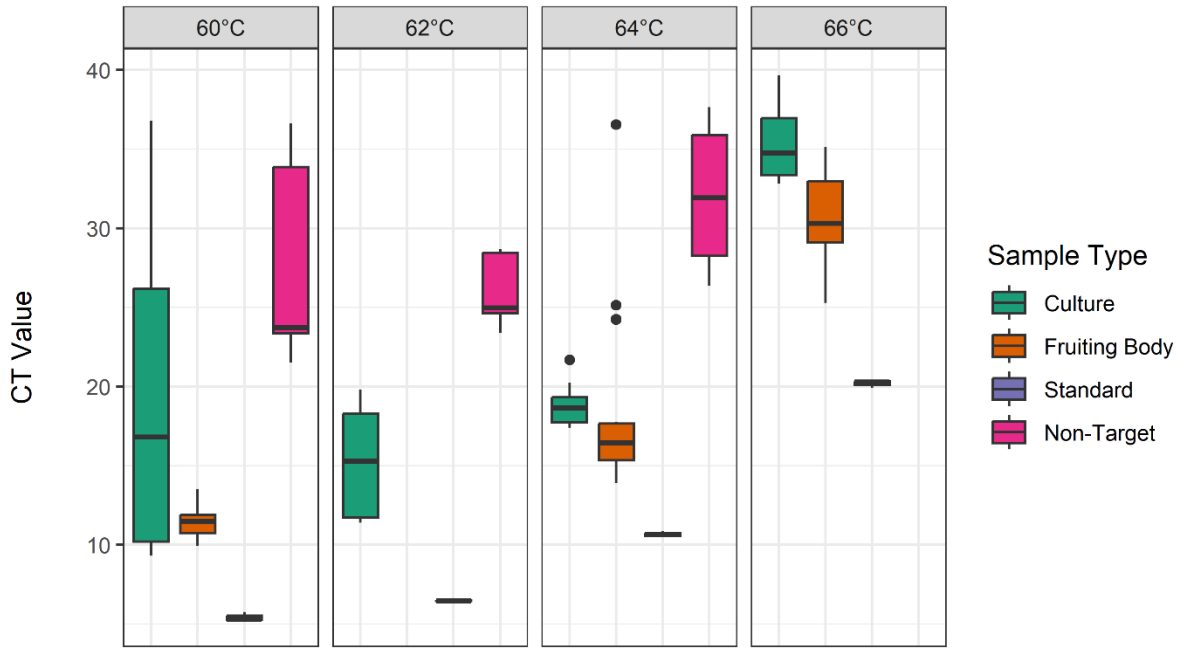

Figure 5.5. C_t values for the different sample types with increasing annealing temperature from 60°C to 66 °C. C_t value increases across all sample types with annealing temperature, however the increase to 66°C eliminates the non-target cross-reacting sequences from being able to cross the threshold within the 40-cycle reaction.

The sensitivity of the assay, at the new annealing temperature, was determined by running triplicate replicates of known concentrations of the *G. fusipes* 18S rRNA gene, from 3 x 10^6 gene copies/µL to 3 gene copies/µL . The results of this test showed that the assay under optimised conditions was able to detect the *G. fusipes* 18S rRNA as low as 3,000 gene copies per microliter (Figure 5.6).

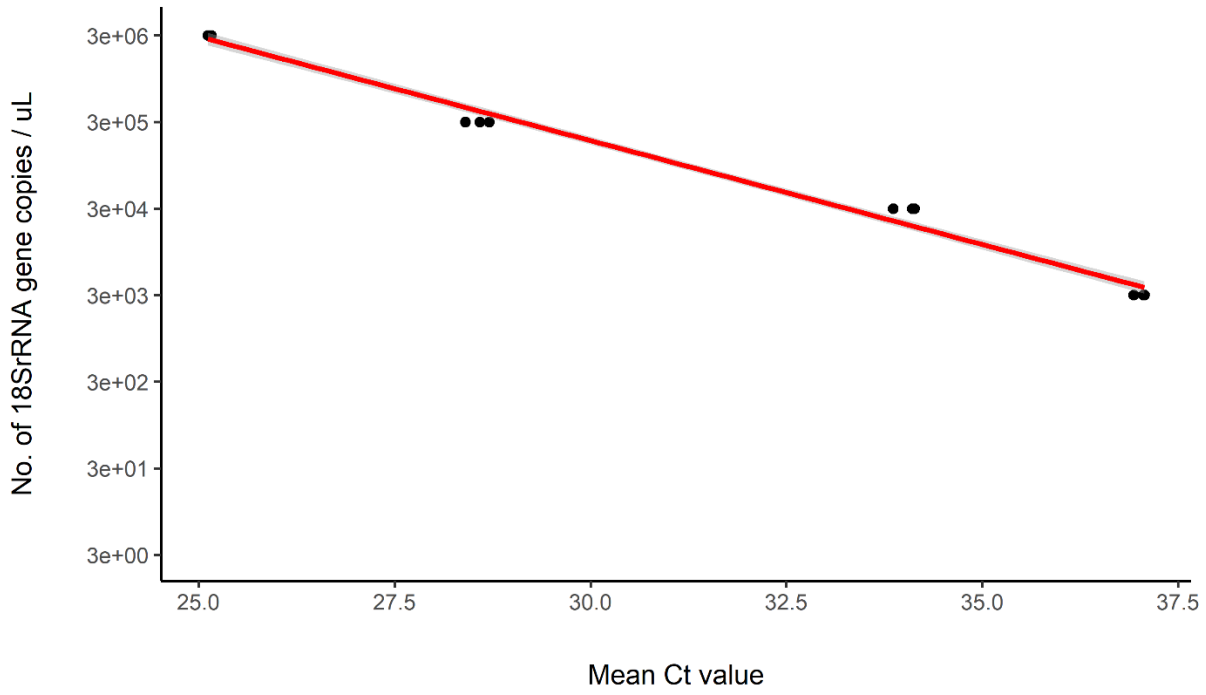

Figure 5.6. Standard curve demonstrating the efficiency of detection of the *G. fusipes* 18S rRNA gene in qPCR assays. A serial dilution from 3 x 10^6 to 3 *G. fusipes* 18S rRNA gene copies was produced, and the C_t value for each of these dilutions is highlighted. 3 x 10^3 gene copies/µL were consistently detected, at a C_t value between 37 and 37.5. Detection below this quantity was unreliable. Linear regression revealed that log(No. of 18S rRNA gene copies/µL) = 28.696 + -0.553 x Mean C_t value.

5.3.3 | Detecting *Gymnopus fusipes* in Field Samples

The qPCR diagnostic assay, with the optimised annealing temperature of 66 °C, was successful in detecting the *G. fusipes* 18S rRNA gene in DNA extracts from all 6 fruiting bodies and in 4 out of 6 infected root tissue samples (Figure 5.7). *G. fusipes* was not detected in any of the soil samples or DNA extracted from healthy tree tissue at the site, confirming its specificity for *G. fusipes* and its capacity to diagnose mycelia of *G. fusipes* in infected woody tissues.

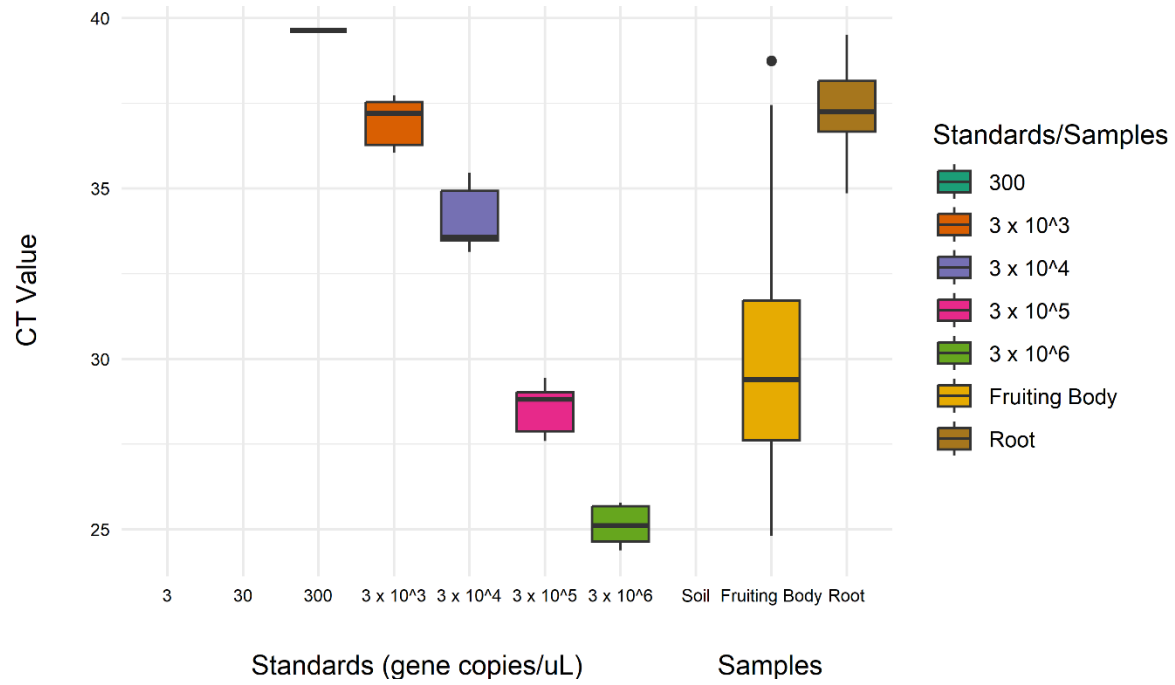

Figure 5.7. C_t values for the standards (serial dilutions from 3 x 10^6 to 3 gene copies/µL) and the three different environmental sample types, soil, fruiting bodies and infected woody tissue (Root). There were no positive results for the standards below 300 gene copies/µL, and there were no positive results for any of the soil samples. Fruiting bodies gave positive results over a range of C_t values, indicating a range of 18S rRNA gene copies/µl. Infected woody tissues appeared to have a low level of 18S rRNA gene copies/µl.

A summary of the environmental samples tested with the diagnostic can be found in Table 5.2. A total of 36 soil samples were tested (24 from the symptomatic trees and 12 from the asymptomatic trees), none of which gave a positive result. Six fruiting bodies from symptomatic trees were successfully identified as *G. fusipes* through detection of the *G. fusipes* 18S rRNA gene. Ten woody tissue samples were taken from trees across the parkland site (4 from symptomatic host; 1 from a dead host; 1 from a host that had no above ground symptoms but had root rot below the ground level; 4 asymptomatic hosts) the qPCR diagnostic was successful in detecting *G. fusipes* in 4 of the 6 samples from infected hosts.

Table 5.2. Environmental samples taken from parkland site impacted by *G. fusipes* infection. Sample type, status of tree host and absolute quantification from qPCR assay. Soil samples are labelled as A (in contact with the infected host root), B (15 cm from the tree root), and C (30 cm from the tree root). R2/R3, and R10/R11 were samples taken from the same host trees, therefore one positive result from these hosts confirms *G. fusipes* presence in the host root system. Limit of quantification for the assay was between 3×10^2 and 3×10^3.

Sample ID	Sample Type	Host Infection Status	Diagnostic Result	Approximate *G. fusipes* 18S rRNA gene copies / µL
S2 A, B and C	Soil	Symptomatic	Negative	-
S3 A, B and C	Soil	Symptomatic	Negative	-
S4 A, B and C	Soil	Asymptomatic	Negative	-
S5 A, B and C	Soil	Symptomatic	Negative	-
S6 A, B and C	Soil	Asymptomatic	Negative	-
S7 A, B and C	Soil	Early symptomatic (no above ground symptoms but colonised root system)	Negative	-
S8 A, B and C	Soil	Asymptomatic	Negative	-
S9 A, B and C	Soil	Symptomatic	Negative	-
S10 A, B and C	Soil	Symptomatic	Negative	-
S11 A, B and C	Soil	Symptomatic	Negative	-
S12 A, B and C	Soil	Asymptomatic	Negative	-
FB1	Fruiting Body	Symptomatic	Positive	7.73×10^5
FB2	Fruiting Body	Symptomatic	Positive	6.33×10^5
FB3	Fruiting Body	Symptomatic	Positive	2.21×10^5
FB5	Fruiting Body	Symptomatic	Positive	4.76×10^5
FB9	Fruiting Body	Symptomatic	Positive	5.34×10^5
FB11	Fruiting Body	Symptomatic	Positive	3.03×10^6
R1	Root Tissue	Dead	Positive	3.229×10^3
R2	Root Tissue	Symptomatic	Negative	-
R3	Root Tissue	Symptomatic	Positive	9.327×10^3
R4	Root Tissue	Asymptomatic	Negative	-
R5	Root Tissue	Symptomatic	Positive	1.456×10^3
R6	Root Tissue	Asymptomatic	Negative	-
R7	Root Tissue	Early symptomatic (no above ground symptoms but colonised root system)	Negative	-
R8	Root Tissue	Asymptomatic	Negative	-
R9	Root Tissue	Symptomatic	Negative	-
R10	Root Tissue	Symptomatic	Negative	-
R11	Root Tissue	Symptomatic	Positive	1.898×10^3
R12	Root Tissue	Asymptomatic	Negative	-

5.4 | Discussion

The overarching aim of this work was to develop a *G. fusipes*-specific qPCR assay that could confirm, through detection of the *G. fusipes* 18S rRNA gene, the identity of cultured *G. fusipes* isolates, in addition to culture independent identification of *G. fusipes* fruiting bodies, and detection in infected woody tissues. The current work presents the development and optimisation of such an assay, successful at detecting the *G. fusipes* 18S rRNA gene in cultured isolates, fruiting bodies and infected woody tissues, which can be completed in less than 48 hours.

After multiple optimisation and validation processes, including *in silico* testing, primer annealing temperature increase and testing the assay against a full suite of sample types, before being applied to a parkland site impacted by *G. fusipes* infection, the final qPCR diagnostic was able to accurately detect the *G. fusipes* 18S rRNA gene in all cultured *G. fusipes* isolates (n = 5), all fruiting body samples (n = 6) and 67% of the infected woody tissue samples (n = 4/6). As expected, there was no detection of the *G. fusipes* 18S rRNA gene in woody tissue samples from healthy tree hosts, or in any of the soil samples.

Two woody tissue samples from trees known to be infected with *G. fusipes*, did not amplify in the reactions, with an undeterminable C_t value and no quantitative information. However, one of the tree hosts did present fruiting bodies, which tested positive for detection of the *G. fusipes* 18S rRNA gene, confirming the presence of *G. fusipes* in this host root system. The samples which did not give a positive result, and had no fruiting bodies present, should be closely monitored and further woody tissue samples be taken for analysis, which could include a complimentary culture-based approach as well as the use of the qPCR diagnostic. It should be understood that the lack of a positive result (amplification of the *G. fusipes 18SrRNA* gene above the threshold level) does not necessarily mean *G. fusipes* is not present in the system, it could be that there is an issue with the DNA extract, or that there may not be enough copies of the *G. fusipes* 18S rRNA gene in the sample.

The assay was able to confidently quantify as low as 3×10^3 gene copies / µL. Some samples passed the threshold for detection at lower levels ($300 – 1.76 \times 10^3$ gene copies / µL), however this was not always repeatable, therefore could not be considered with confidence. Similar assays for other pathogens have been found to be sensitive to much lower concentrations (Chandelier et al., 2021; Demontis et al., 2008), however as the first instance of a diagnostic for this illusive fungal species, it can still be considered useful. Without a deeper understanding of *G. fusipes* cell structure in different tissue types (vegetative fruiting bodies, compared to actively infecting hyphae), it is difficult to determine what level of infection a certain gene copy number might relate to (Black et al., 2013).

A deeper understanding of *G. fusipes* fruiting triggers and sporulation would increase the potential applications of this diagnostic assay, as it could then be used, along with spore trapping, to measure the spread of basidiospores in a forest ecosystem. The primers designed in this project have the potential to be developed further and could potentially provide the starting point for the development of a LAMP assay, a diagnostic tool that is similar to a qPCR assay but can be carried out with specialist equipment onsite/in the field.

In conclusion, this work presents the first instance of a *G. fusipes*-specific qPCR assay that can be used to accurately identify *G. fusipes* in cultured isolates, fruiting bodies and infected woody tissues, without the need for pure culture. This assay has been found to be effective on up to 100% of cultured isolates, up to 100% of the fruiting bodies and 67% of infected woody tissues tested. Therefore, this diagnostic will be useful in improving the chances of accurately diagnosing a *G. fusipes* infection, even when typical symptoms (e.g. fruiting bodies) are absent. Although the diagnostic must be carried out in a laboratory, with DNA extraction and specialised equipment, samples can be taken by non-specialists, and the whole process can be completed to give a result in less than 48 hours. This rapid diagnostic tool will help identify *G. fusipes* infection in previously unknown hosts and could also be used for targeted distribution analysis to confirm the presence of *G. fusipes* in uncertain geographical ranges. This data is critical to understanding and developing strategies to manage and prevent the spread of this root rot pathogen.

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CHAPTER 6

Whole Genome Sequencing and Transcriptomic Analysis of *Gymnopus fusipes*, at Different Stages of Infection Development

Abstract

Gymnopus fusipes is an understudied basidiomycete fungus and primary causal agent of root rot linked to episodes of oak decline throughout Europe. Due to the enigmatic nature of *G. fusipes* root rot in oak, there is currently no information on its pathology at the molecular biological level. Here, we sequenced and annotated the genome of *G. fusipes* and performed comparative transcriptome analysis of both natural infections of mature oak trees and oak seedlings inoculated with *G. fusipes,* compared with axenic growth of *G. fusipes* on agar. Whole genome sequencing of *G. fusipes* resulted in 26 contigs, and a genome length of 57.9 Mbp and 1644 complete BUSCO genes. Transcriptomic analysis of infection development revealed that there is a large variation in differentially expressed genes between *G. fusipes* in a non-infective state, and *G. fusipes* in an active infection. This provides important information on molecular mechanisms of infection for this important root rot pathogen, which could help form the foundation of plans to combat the impacts of *G. fusipes* infection

6.1 | Introduction

Gymnopus fusipes (syn. *Collybia fusipes*) is a poorly studied basidiomycete fungus from the family Omphalotaceae (Ványolós et al., 2016). It is established that *G. fusipes* is a primary pathogen causing root rot on a number of socioeconomically important tree species, including oak, chestnut and fir (Pettifor et al., 2022) and is able to affect both young and mature trees (Marçais and Caël, 2001; Wainhouse et al., 2016). The time between initial infection and mortality of the host can span decades (Camy et al., 2003a), and above ground symptoms (fruiting body presence and host crown deterioration) are often only apparent in the later stages of infection, if at all (Camy et al., 2003b; Marçais et al., 2000b; Thomas et al., 2002). This lack of detection of *G. fusipes* infection has potentially resulted in an underestimation of its prevalence and role in oak declines across Europe and a lack of focus on its infection biology (Marçais et al., 1999; Pettifor et al., 2022).

Trees infected with *G. fusipes* do not necessarily have visible symptoms, such as fruiting bodies present at the base of the trunk, and the fruiting triggers of this species are currently unknown (Pettifor et al., 2022). Fruiting bodies can appear in small clusters, or individually (Figure 6.1 C and D), depending on whether the fungus is existing as a parasite (usually occurring in clusters) or a saprotroph (usually occurring as individuals) (Przybyl, 1994). Signs of decline in the tree host, such as crown dieback and leaf wilting, as well as damaged or missing buttress roots (Denman and Webber, 2009; Finch et al., 2021), are also unpredictable with *G. fusipes* infection, as some infected hosts maintain a full and healthy crown until mortality (Camy et al., 2003b). Below the ground level, infection is more apparent, with distinctive orange lesions and patches of necrotic tissue, and white mycelial fans running throughout (Figure 6.1 E and F). Infection occurs when a *G. fusipes* basidiospore lands on the collar of the host tree and germinates, penetrating the outer bark, and colonising the root system (Marçais et al., 2000a). Infection spreads from the tree collar down to the main buttress roots and eventually further out to the lateral roots, which leads to a high risk of trees being windthrown due to a deterioration of anchoring roots (Camy et al., 2003a; Marçais et al., 1999; Przybyl, 1994).

Oak trees in particular are known to be hotspots of biodiversity, with a suggested 2300 species associated with oak, 326 of which being obligate associates (Mitchell et al., 2019). Many of the species affected by *G. fusipes*, such as oak, chestnut and fir (Clark et al., 2022; Gazol et al., 2020; Mitchell et al., 2019), are keystone species across temperate climates, with crucial roles in ecosystem services, including biodiversity maintenance, soil formation, and also in economic services such as timber production (Boyd et al., 2013; Fisher et al., 2009).

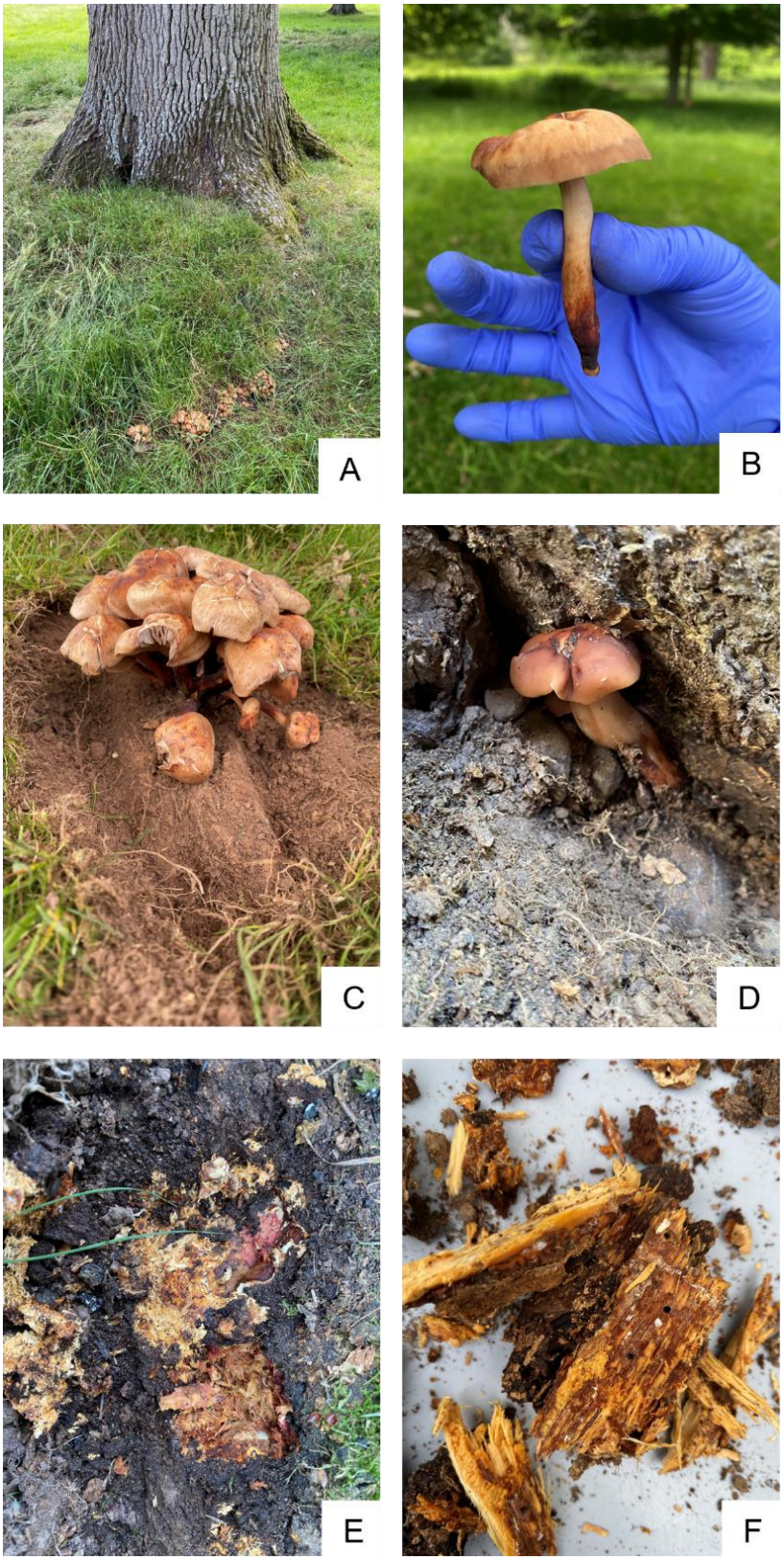

Figure 6.1. A. *G. fusipes* fruiting bodies at the base of an oak tree (*Quercus* L.). B. *G. fusipes* fruiting body, with tapering stipe from cap to base. C. Cluster of *G. fusipes* fruiting bodies attached to a living root, typical of a parasitic infection. D. Singular *G. fusipes* fruiting body on the base of a fallen oak tree, typical of saprotrophic infection. E. Oak root infected with *G. fusipes*, after removing soil and outer bark. Orange lesions and white mycelia can be seen. F. Panel of infected tissue taken from an oak root infected with *G. fusipes*. Small white mycelia can be seen throughout.

In order to identify the mechanisms of infection used by pathogens such as *G. fusipes*, and to characterise the interactions between pathogen and host, gene expression analysis or transcriptomic analysis is often employed (Nibedita and Jolly, 2017). Transcriptomic analysis has been used to identify genes associated with disease on a range of tree host-pathogen systems, where a difference in pathogen gene expression can be found across hosts with differences in susceptibility, and also in early and late stage infection (Chittem et al., 2020; Gaspar et al., 2020). Fungal pathogens are known to express various genes when actively infecting, that may not be present in a laboratory culture, as pathogens are often highly influenced by the environment that they are in (Miguel-Rojas et al., 2023). For example, when in an infective state, genes associated with plant defence evasion, production of enzymes affecting cellulose, hemicellulose, as well as other woody tissue components, and carbohydrate binding molecules, will be highly expressed (Yan et al., 2018). Genes are often differentially expressed at different stages of infection, with avoidance and defence against the host defences being upregulated in the early stages of infection, and genes associated with host cell death often upregulated in later stages of infection (Chittem et al., 2020).

Given the propensity for *G. fusipes* to cause primary disease associated with severe root-rot, chronic decline and mortality in oak trees, the lack of information on the molecular basis for *G. fusipes* pathogenicity is a major knowledge gap that prevents understanding of the mechanisms by which it infects host tissues and hinders future efforts to manage the disease. Therefore, the main aim of this work was to elucidate the process of *G. fusipes* infection at a molecular level through analysis of gene expression of infection at different stages; non-infective (mycelial plate culture), early infection (a seedling inoculation trial < 6 months) and established infection (a field site with a known occurrence of *G. fusipes* infection for a number of years). This was achieved by generating the first draft genome of *G. fusipes,* to allow comparative transcriptome mapping of both natural infections of mature oak trees and oak seedlings inoculated with *G. fusipes,* compared with axenic growth of *G. fusipes* on agar. We hypothesised that; (i) there will be a difference in transcriptome profile between the non-infective control, early-stage infection condition and the established infection condition, and that (ii) *G. fusipes* will express pathogenicity genes when infecting oak tissue (*in Planta* analysis of natural infections in mature trees and oak seedling inoculations) when compared with axenic grown on artificial medium (*in vitro*).

Transcriptome analysis of *G. fusipes* at different stages of infection (non-infective mycelial cultures, early infection in a seedling infection trial, and established infection in mature oak trees at a field site) will provide information as to which genes are unique and common to each stage. This data could provide crucial information to help understand how this pathogen causes disease through

elucidating the mechanisms of infection and could provide key information to aid in the development of new management techniques.

6.2 | Methods

6.2.1 | *Gymnopus fusipes* isolation and confirmation of identity

Gymnopus fusipes strain "AHPC", was isolated in summer 2021, from a fruiting body beneath an oak tree (*Quercus* sp.) in Alice Holt Forest, Farnham, UK. After surface sterilisation for 10 seconds in 10% bleach, the fruiting body was cut into small sections, approximately 3 to 5 mm^2, and the pieces were washed for 10 seconds in 70% ethanol before drying on tissue paper. These small fruiting body pieces were then placed on half strength malt extract agar (½ MEA: 25 g/L malt extract agar (Merck), 25g/L Technical Agar (Oxoid), pH 5 ± 2) and incubated at 25˚C for approximately 4 weeks. Isolate AHPC was maintained on half-strength agar at 25˚C and sub-cultured approximately once per month.

For taxonomic identification and confirmation that strain AHPC was an isolate of *G. fusipes*, DNA was isolated from mycelial biomass using the MP Biomedicals FastDNA™ Spin Kit (optimised by repeating the initial homogenisation step three times, see Chapter 3: Section 3.6.1 for full details), and the internal transcribed spacer (*ITS)* region was amplified with PCR using standard primers *ITS1* and *ITS4*. Briefly, after vortex mixing, 1 µl of extracted and cleaned DNA was added to GoTaq® Green Master Mix, in a PCR reaction as follows. Each reaction was 50 µl in volume, and along with the 1 µl of DNA template, contained 25 µl of 2 x GoTaq® Green Master Mix, 22 µl of PCR-grade water, 1 µl each of 10 pmol of *ITS* region specific oligonucleotide primers (White et al., 1990); ITS1 (5' – TCCGTAGGTGAACCTGCGG – 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3'). PCR reaction conditions were as follows: initial denaturation at 95˚C for 2 mins, followed by 35 cycles of; denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s, and extension at 72˚C for 10 s, with a final extension at 72˚C for 5 min. This produced a PCR amplification product approximately 350 bp in length. Ten microliters of the PCR product was then visualised using a 1% agarose gel electrophoresis at 100V for 45 mins. One microliter of PCR product was used for quantification using the Qubit™ dsDNA HS Assay Kit.

The unpurified PCR product was then sent to Azenta/GENEWIZ (Leipzig, Germany) for purification and subsequent Sanger sequencing. The resulting *ITS* sequence was compared to documented nucleotide sequences using the NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

6.2.2 | Whole genome sequencing of *Gymnopus fusipes* strain AHPC

After 4-weeks of growth, DNA was extracted from mycelial plate cultures of *G. fusipes* strain AHPC (Figure 6.2), using a modified 4% CTAB chloroform extraction method with a sodium acetate precipitation (details of this optimised extraction method are outlined in Chapter 3, Section 3.6). Five microliters of the extracted DNA were visualised using 1% agarose gel electrophoresis at 85V for 60 mins and checked for purity using a NanoDrop Spectrophotometer. HiFi sequencing libraries and DNA sequencing was conducted by Novogene, UK, using Pacific Biosystems HiFi sequencing technology. In this, libraries were prepared, and sequencing was run using 1 SMRT cell and a CCS sequencing mode. After initial sequncing, HiFi polishing of the data occurred. The resulting Hifi sequencing data was assembled using Hifiasm (version: 23102020), using program default parameters, and the resulting primary assembly was then used in the following analysis. The assembly was then assessed for contiguity using QUAST (version: 5.2.0, with the “rna-finding” and “fungus” options selected), and completeness using BUSCO (version 5.4.5), with the following parameters; lineage dataset as “basidiomycota_odb10” (Creation date: 2020-09-10, number of genomes: 133, number of BUSCOs: 1764, mode as “euk_genome_met” and the gene predictor used was “metaeuk”. Kraken2 (version: 2.1.2) was used to identify bacterial contaminants, and the data was then fed through the purge_dupes pipeline (version: 1.2.5) to remove duplicated genes. The new quality-controlled assembly was checked again using quast and BUSCO.

The genome was processed before annotation using Funannotate (version 1.8.4) clean and sort (with default parameters). Soft masking of the assembled genome was completed with RepeatModeler (version: 2.0.4) to identify repeats and create a custom repeat library and RepeatMasker (version: 4.1.4) using the custom repeat library to generate the masked version of the genome. The final soft-masked genome was annotated with Funannotate train (parameters as follows; --stranded RF, --jaccard_clip, --species "Gymnopus fusipes", --strain AHPC, --cpus 12) to train the ab-initio gene predictors. Finally, Funannotate predict (with the BUSCO database “basidiomycota_odb10” selected).

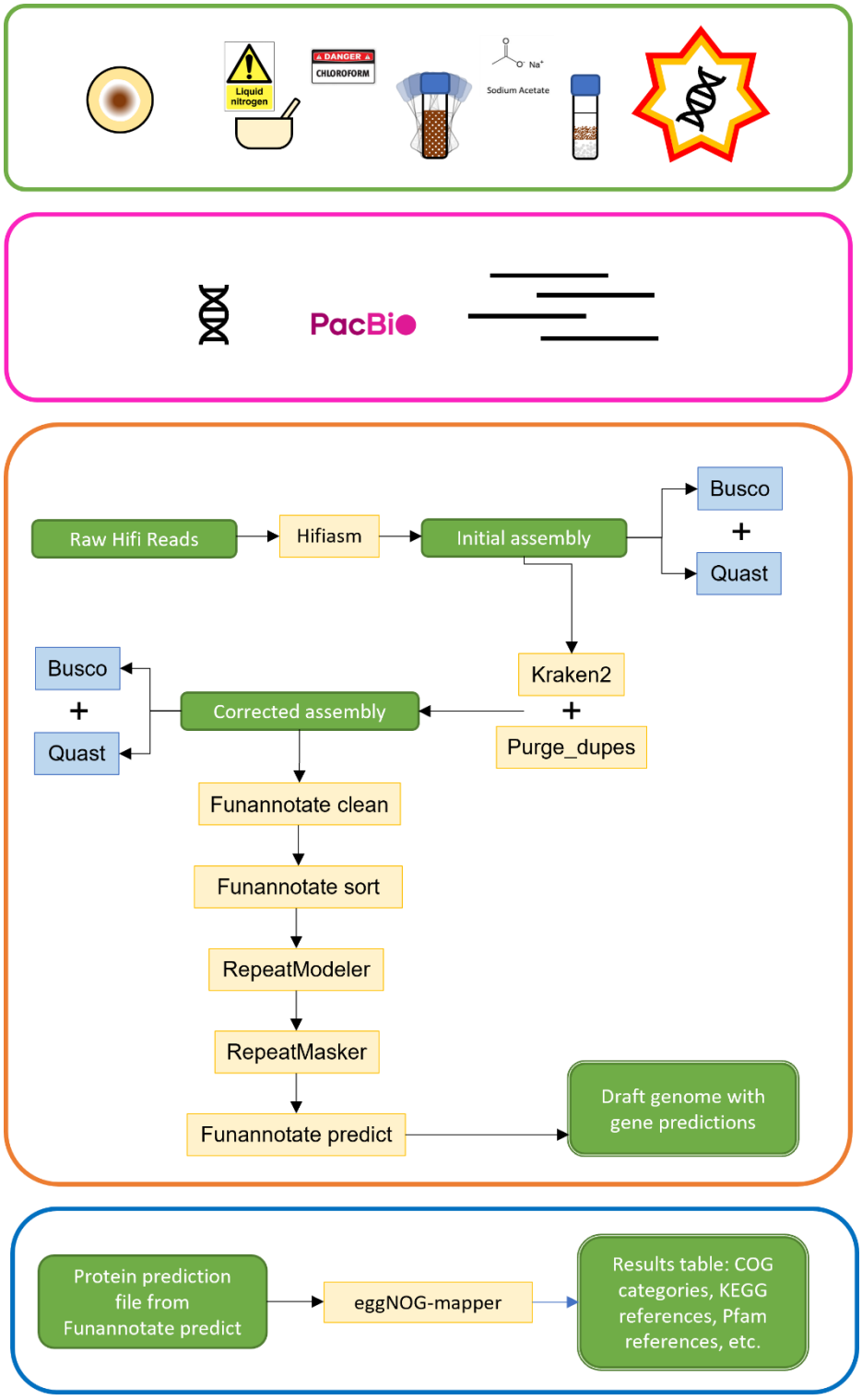

Figure 6.2. Summary of workflow for whole genome sequencing. *G. fusipes* mycelial cultures were grown for 4 weeks before DNA was extracted using a sodium acetate precipitation method. DNA was sent to Novogene (UK) for PacBio Hifi sequencing, where 1 SMRT cell and a CCS sequencing mode was used. The sequencing was followed by Hifi polishing. The resulting data was then analysed using various bioinformatics tools, including hifiasm for assembly, Quast and Busco for quality checking, Kraken2 and purge_dupes for quality control, and then funannotate clean and sort, followed by RepeatModeler and RepeatMasker, before being run through the funannotate predict pipeline to produce a draft genome with gene predictions. Bioinformatic analysis was conducted using Super Computing Wales (SCW).

The resulting protein predictions from the funannotate predict step, were analysed using the eggNOG-mapper public online web resource (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019), where suggested functional annotations were obtained.

6.2.3 | Oak seedling greenhouse inoculation tests

The following experiment was conducted to obtain *G. fusipes* samples representative of an early infection. Inoculum for this experiment was produced by inoculating 5 mm agar plugs of *G. fusipes* strain AHPC onto ½ strength malt extract agar and incubating at 25˚C for 3-4 weeks. To improve the chances of infection success on the young seedlings, three different wounding methods were trialled. All methods included surface sterilisation of the outer bark with 10% bleach before the seedlings were processed. These methods included a no wound method (which involved applying the 5 mm agar plug directly to the outer bark on the collar of the sapling, just above the soil level), a shallow wound method ((adapted from Rizzo et al. (2002), which involved slicing the outer bark upwards vertically to create a flap, underneath which the agar plug was situated), and a deep wound method (modified from Terhonen et al. (2019), which involved drilling a 5 mm hole into the collar of the seedling, approximately 5 mm deep, in which the agar plug was placed). Each method was used to inoculate 8 seedlings with *G. fusipes* and a further 4 seedlings were treated with a non-inoculated agar plug to act as a non-inoculated wound control. Seedlings were arranged in the greenhouse using a random number generator for placement, this was to negate any unavoidable effects of the greenhouse positioning (Figure 6.3).

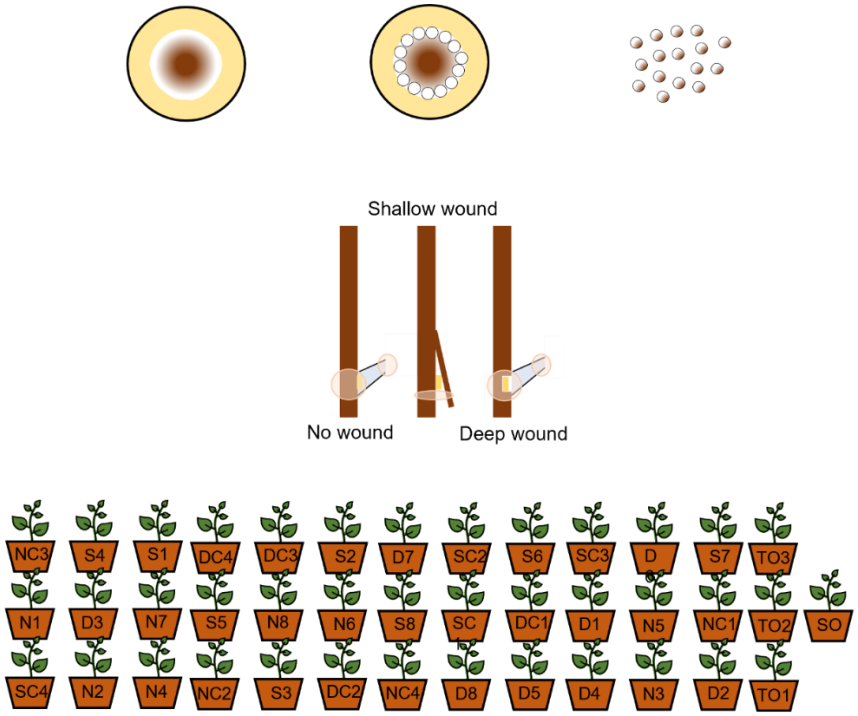

Figure 6.3. Schematic diagram of the greenhouse-based seedling inoculation trial. *G. fusipes* mycelial cultures were grown for approximately 3-4 weeks, before 5 mm plugs were taken from the white growing edge. These plugs were inoculated onto seedlings using one of three methods (no wound, shallow wound, or deep wound), and were arranged in a greenhouse using a random number generator for positioning to avoid any greenhouse effects.

Initially all agar plugs were secured in place with a modified 0.5 ml microcentrifuge tube. This involved removing the cap of the tube and cutting the base of the tube at a 45° angle. The agar plug was then placed onto the seedling and the tube placed over it, and held in place with moulded wax (Figure 6.4). This was to ensure sufficient air flow to the agar plug. However when the infection sites of the seedlings were assessed approximately half-way through the experimental period, these securings were removed, and wound sites were brushed clean with a soft paintbrush and protected by wrapping gently with parafilm. This remained until the end of the experiment.

Figure 6.4. Left: Modified 0.5 ml microcentrifuge tube used to secure the agar inoculum plugs in place in the no wound method (where agar plugs were placed directly onto the outer bark) and the deep wound method (where a 0.5 mm hole was drilled into the bark and the inoculum plug placed into it). The tube was secured to the seedling using moulded wax. Right: Wax seal used to secure the flap of outer bark under which the agar plugs were placed.

Seedlings were inoculated in February 2022, and were housed at ambient temperature in a greenhouse (with temperatures between 4.3˚C and 36.5˚C), however, due to high temperatures experienced around April 2022 (with temperatures reaching a hgih of 50˚C), the seedlings were moved outside, where temperatures were slightly cooler. Soil moisture levels were measured using a Theta probe to take triplicate measurements of each seedling pot, on a biweekly bases, and this soil moisture content was maintained at approximately 15% througout the experimental period. The infection trial was run for approximately 6-and-a-half months, ending on the 1st September 2022.

After the 6 and a half month experimental period, the parafilm was removed, and the wound sites were observed. Photographs of the lesions were taken for digital analysis, and pieces of tissue (3 - 4 pieces of tissue each approximately 5 mm^2) along the lesion margin were collected in 0.5 ml microcentrifuge tubes, and immediately frozen in liquid nitrogen for RNA extraction.

Lesion images for all seedlings were analysed using Digimizer (Version 6), where the outline of each lesion was measured and used to calculate the area. Using a generalised linear model (with a gamma family due to the data distribution and square root link as the data was considering area), the area for all 8 inoculated seedlings and 4 non-inoculated seedlings were used to determine whether the presence of *G. fusipes* in the inoculations influenced the lesion size across each of the wounding conditions. Analysis of these models was completed using a Chi squared test for significance. All statistical analyses were performed in R Studio (version 2022.02.3).

6.2.4 | Sampling mature oak trees naturally infected with *G. fusipes* at a heavily infected site in the UK

In May 2022, a field site in the UK known to be heavily infected with *G. fusipes* was visited, and over the course of two days, samples of fruiting bodies, root tissue and soils were collected from 10 trees (Figure 6.5). Healthy and diseased trees were paired according to proximity to try and make them as similar as possible.

Firstly, soil samples were taken by brushing away any topsoil and debris from the infected root and fruiting bodies (if present), then a sterile trowel (washed with 10% bleach, rinsed with sterile distilled water and dried) was used to dig for soil at three distances from the infected root, (i) immediately adjacent to the root, 15 cm away from the infected root, and 30 cm away from the root. Soil was collected in 50 ml falcon tubes and was placed in a cool box immediately after collection. Secondly, fruiting bodies (if present) were collected and placed in paper envelopes before being stored in a cool box. Finally, root tissue (from where the fruiting body emerged on the infected roots, or on the roots close to the collar of the tree for healthy trees) was taken using a sterile chisel (washed with 10% bleach, rinsed with sterile distilled water and dried) and a mallet. Root tissue pieces were approximately 1.5 – 2 cm^2 in diameter, although some of the heavily infected roots were difficult to measure as the root tissue was already degraded, root tissue samples were split into two aliquots for further processing, one set (for isolation attempts) were stored in a cool box, one set (for RNA analysis) was immediately frozen in liquid nitrogen within moments of being collected.

Figure 6.5. Trees sampled at a parkland site heavily impacted with *G. fusipes* infection. Samples were paired as follows: 2/3 and 4, 5 and 6, 8 and 9, 10/11 and 12. Samples 1 and 7 were not paired with any of the others.

Once the various samples had been returned to the lab, soil samples were stored at -20°C, some infected root samples, and fruiting body samples were stored at 4°C. Any tissues taken for RNA analysis were immediately transferred from liquid nitrogen to -80°C, until processing occurred.

6.2.5 | Isolation and qPCR diagnostic analysis to confirm the presence of *G. fusipes* in field site samples

A selection of samples of *G. fusipes* from the parkland impacted by heavy infection were used to firstly confirm the presence of *G.* fusipes, using the qPCR diagnostic developed previously (Chapter 5), and also for isolation attempts (Figure 6.6). *G. fusipes* was confirmed to be present in the fruiting body samples and a number of infected woody tissue samples, through the use of the qPCR-based diagnostic (developed in Chapter 5). DNA was extracted from all samples (for detailed methods, see Chapter 3: Section 3.6.1 and Chapter 5: Section 2.3) and run through the qPCR assay.

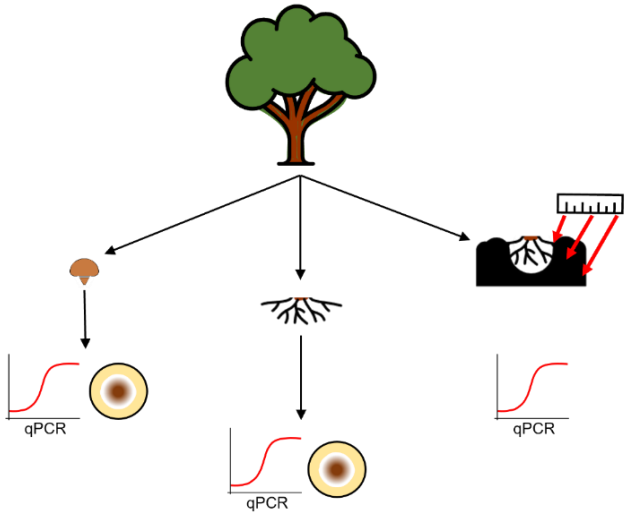

Figure 6.6. Summary of sample processing for confirmation of G. fusipes in the field site samples. Fruiting bodies were used with the qPCR diagnostic and also used for isolation attempts. Infected root tissue samples were used with the qPCR diagnostic and also used for isolation attempts. Healthy root tissue samples were used with the qPCR diagnostic to provide evidence for the absence of G. fusipes in the samples. Soil samples taken from three increasing distances from the roots of healthy and infected trees were used with the qPCR diagnostic to indicate the presence of G. fusipes in the surrounding soils.

6.2.6 | Preparation of *G. fusipes* mycelial cultures for the non-infective condition

G. fusipes strain AHPC was sub-cultured onto fresh ½ strength malt extract agar. After 3 to 4-weeks growth at 25˚C, mycelial tissue was removed from the plate, using a sterile scalpel, and RNA was extracted from agar cultures using the method outlined below (Section 6.2.7). RNA was isolated from four replicates of the growing strain.

6.2.7 | Extraction of high-quality RNA for transcriptomic analysis

RNA was extracted from samples above (paired healthy and infected seedlings, paired healthy and infected mature trees, and non-infective agar controls, see Figure 6.7), following the optimised protocols described in Chapter 3 (Section 3.6.2). Briefly, samples were homogenised with a pestle and mortar (maintaining temperature with the addition of liquid nitrogen) before being transferred to a 2 ml screw cap tube containing 1 ml of 4% CTAB buffer (with 2% v/v β-mercaptoethanol added). Samples were processed further in a PowerLyzer (Qiagen) at 2500 m/s for 30 seconds, which was repeated twice to ensure thawing of the buffer and full homogenisation of the contents of the tube. The homogenised samples were then centrifuged at 14,000 RPM for 10 minutes at 4˚C. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube, where two chloroform wash steps were performed. Precipitation of nucleic acids was done by sodium acetate precipitation and overnight incubation at – 20˚C. After an ethanol wash, nucleic acids were resuspended with ultra-

pure, nuclease free water. This extract was DNase treated using the Turbo™ DNase (Invitrogen™), and a second sodium acetate precipitation, with a 2 to 3-hour incubation at – 20˚C was carried out to remove the digested DNA from the extracts. After further ethanol washes and final resuspension, gel electrophoresis (80 V for 60 minutes) and purity analysis using the Nanodrop spectrophotometer, of the final RNA extracts was completed prior to submitting for RNA sequencing. RNA extracts were sent to Novogene, UK, for mRNA sequencing, where after library preparation consisting of an mRNA enrichment using Poly(A) capture, sequencing was conducted using Illumina PE150 technology.

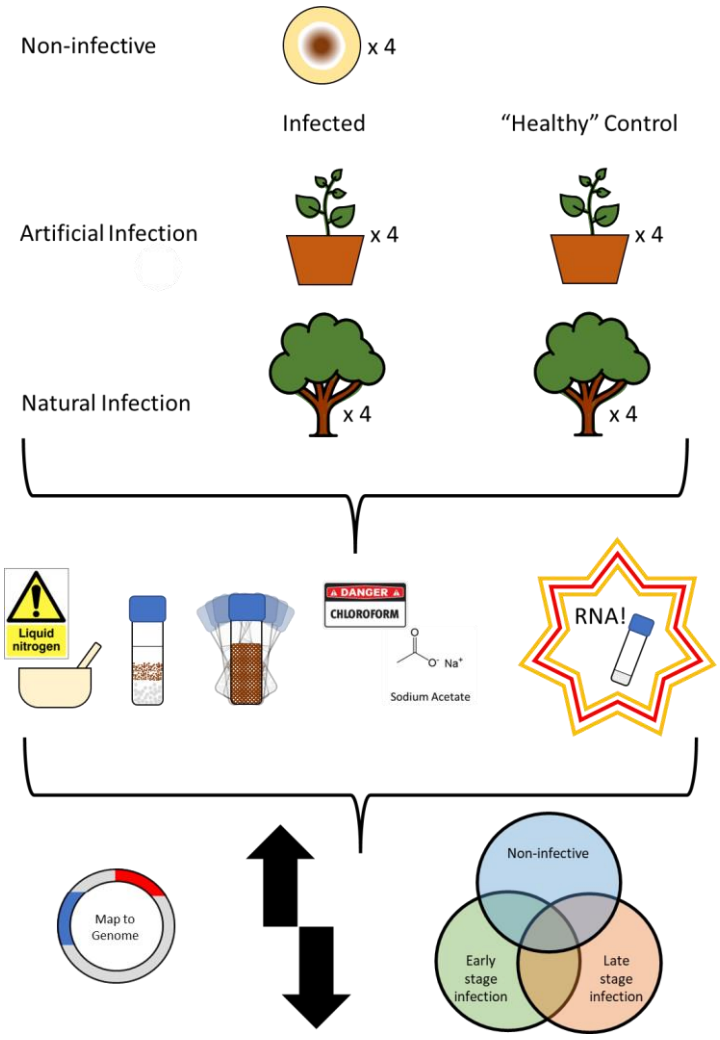

Figure 6.7. Overview of the experimental plan and methodology used in this work. The first stage outlines the experimental plan, including four replicates of *G. fusipes* mycelia, along with infected and non-infected seedlings (via a seedling infection trial), and then infected and "healthy" samples from mature trees at an infected field site. The second stage represents the RNA extraction protocol that was used to extract RNA from the early infection seedling samples, the established infection root tissue samples, and the axenic mycelial cultures. The final stage represents the bioinformatic analysis that took place, including the assembly of a genome for *G. fusipes*, then mapping RNA-seq data to this genome, before assessing upregulation and downregulation of pathogenicity genes and then finally to compare the gene expression at different infection stages.

6.2.8 | Mapping transcriptomic data to *G. fusipes* genome for gene expression analysis

The RNA reads obtained from the Illumina paired end sequencing were 150 bp in length. These reads were fed through the FastP pipeline (version: 0.23.2) under program defined parameters, in order to assess the read quality and to trim and filter any potentially unreliable reads (Figure 6.8). FastP was used to assess quality, filter low quality reads, remove low quality bases, trim reads, and cut adaptors from the sequences (Chen et al., 2018).

The draft genome was processed using Hisat2 (version: 2.1.0). Hisat2 build was used to index the genome ready for RNA transcript mapping, using program default parameters. The indices from the genome were then used to allow mapping of the corrected RNA reads along the genome, using hisat2 count (the previously determined indices were used and the corrected forward and reverse RNA reads were used, the BAM file output mode was selected). Samtools (version: 1.14) sort was used to sort the mapped reads into the order of the genome. HTSeq (version: 0.13.5) count was used to count the number of reads from the transcriptomic data that mapped the each of the genes in the genome. In this, the sorted BAM file output from samtools sort and the gene prediction file from Funannotate predict were used to provide the count numbers for each gene.

Using the count data produced by using HTSeq, along with a basic metadata table (defining the ID of the sample and the infective condition), the following differential gene expression analysis was conducted in R Studio (version: 4.1.1), using the DESeq2 package from Bioconductor. After compiling data for all of the samples into a table, defining the column names for the “Gene” from the genes Funannotate predict program, and “Counts” for the number of counts each of the *G. fusipes* samples had for each gene, the DESeq program was run. This included automatic normalisation of the gene counts, and statistical analysis to determine differentially expressed genes. A principle component analysis was conducted on the data, and a heatmap of sample similarity, as well as a heatmap of differentially expressed genes across the different infective conditions were produced, before a selection of the differentially expressed genes were identified by cross-referencing with the results table from the eggnog-mapper analysis.

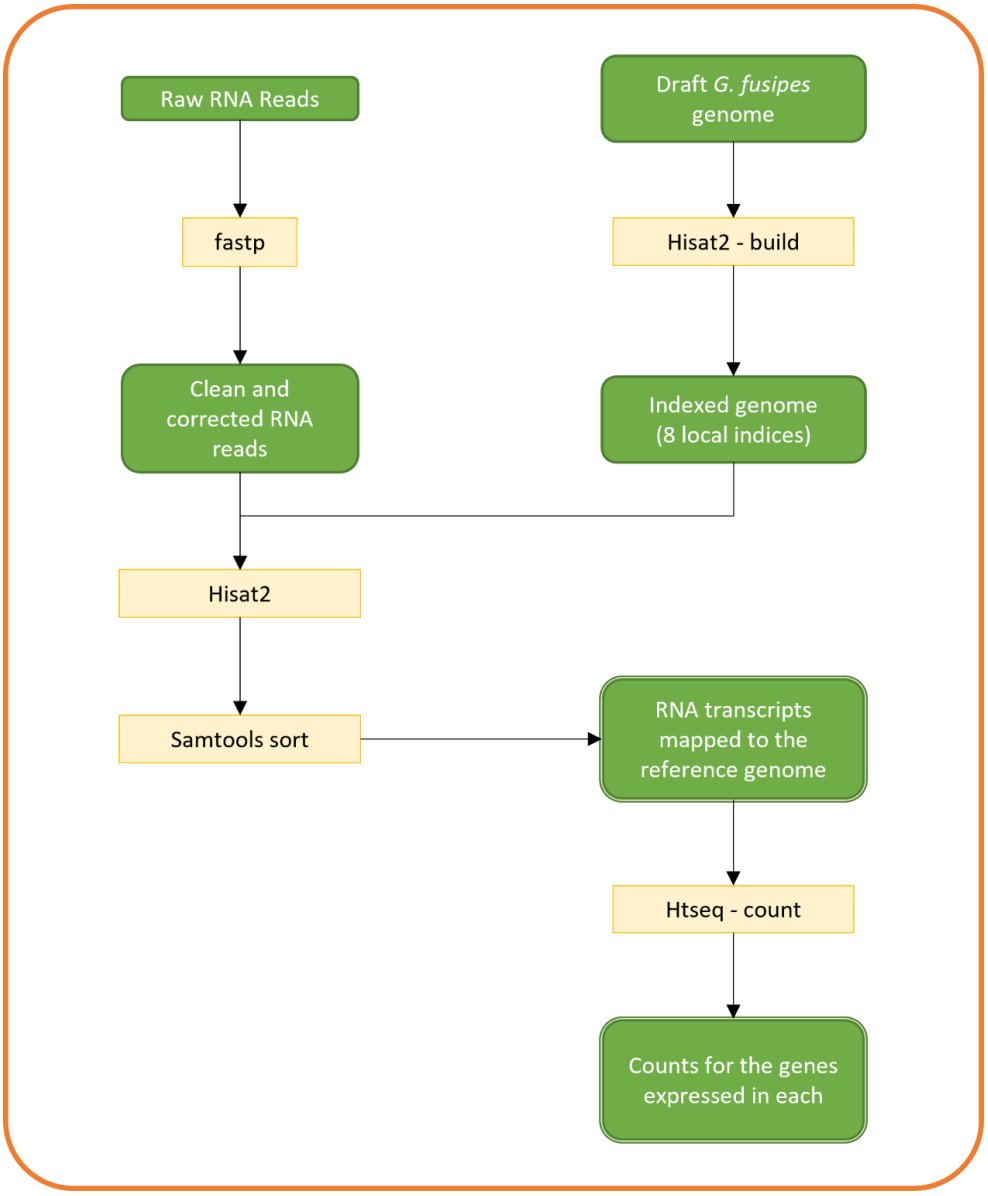

Figure 6.8. Overview of the bioinformatics analysis conducted in the RNA-seq analysis pipeline. The resulting gene counts were then processed further in R studio using the package DESeq2 to produce figures representing differential gene expression.

6.3 | Results

6.3.1 | *Gymnopus fusipes* isolation and confirmation of identity

The *G. fusipes* strain used in this study, "AHPC", was successfully isolated in summer 2021, from a fruiting body beneath an oak tree (*Quercus* sp.) in the UK. Surface sterilisation followed by a secondary wash step resulted in successful isolation of the strain.

Following successful DNA extraction, and subsequent PCR amplification of the *ITS* gene region, the resulting *ITS* sequence was analysed using the NCBI BLAST program, and was identified as *Gymnopus fusipes,* with a sequence similarity of 100% (Accession number: KX449407.1).

6.3.2 | Whole genome sequencing of *Gymnopus fusipes* strain AHPC

The data resulting from the hifiasm analysis consisted of numerous files, including primary and alternate contigs. The primary assembly was therefore used for the following analyses. The assembly was assessed for contiguity (using QUAST) and completeness (using BUSCO), after noting a high frequency of duplicated BUSCO genes, the assembly was then fed through the Kraken2 and purge_dupes pipelines to remove the duplications, and the resulting corrected assembly was assessed again with QUAST and BUSCO (Table 6.1).

Table 6.2. Quality assessment scores for the primary hifiasm assembled *G. fusipes* assembly, before and after correcting by removing duplications. Quality was assessed using QUAST for contiguity and BUSCO for completeness.

	Initial Primary Assembly	**Corrected Assembly**
Total Length	100.8 Mbp	57.9Mbp
Total Contigs	200	26
N50	5.2 Mbp	6.13 Mbp
Complete BUSCOs	1702 (96.4%)	1644 (93.2%)
Complete and Single Copy BUSCOs	680 (38.5%)	1612 (91.4%)
Complete and Duplicated BUSCOs	1022 (57.9%)	32 (1.8%)
Fragmented BUSCOs	23 (1.3%)	16 (0.9%)
Missing BUSCOs	39 (2.3%)	104 (5.9%)
Total BUSCO Groups Searched	1764	1764

The eggnog-mapper analysis of the protein predictions provided results for 8578 queries and produced a number of potential functional results. The frequency at which each of the categories of

COGs (Clusters of Orthologous Groups) occurred is illustrated in Figure 6.9. Some queries matched to multiple COG categories, and this is presented in the data.

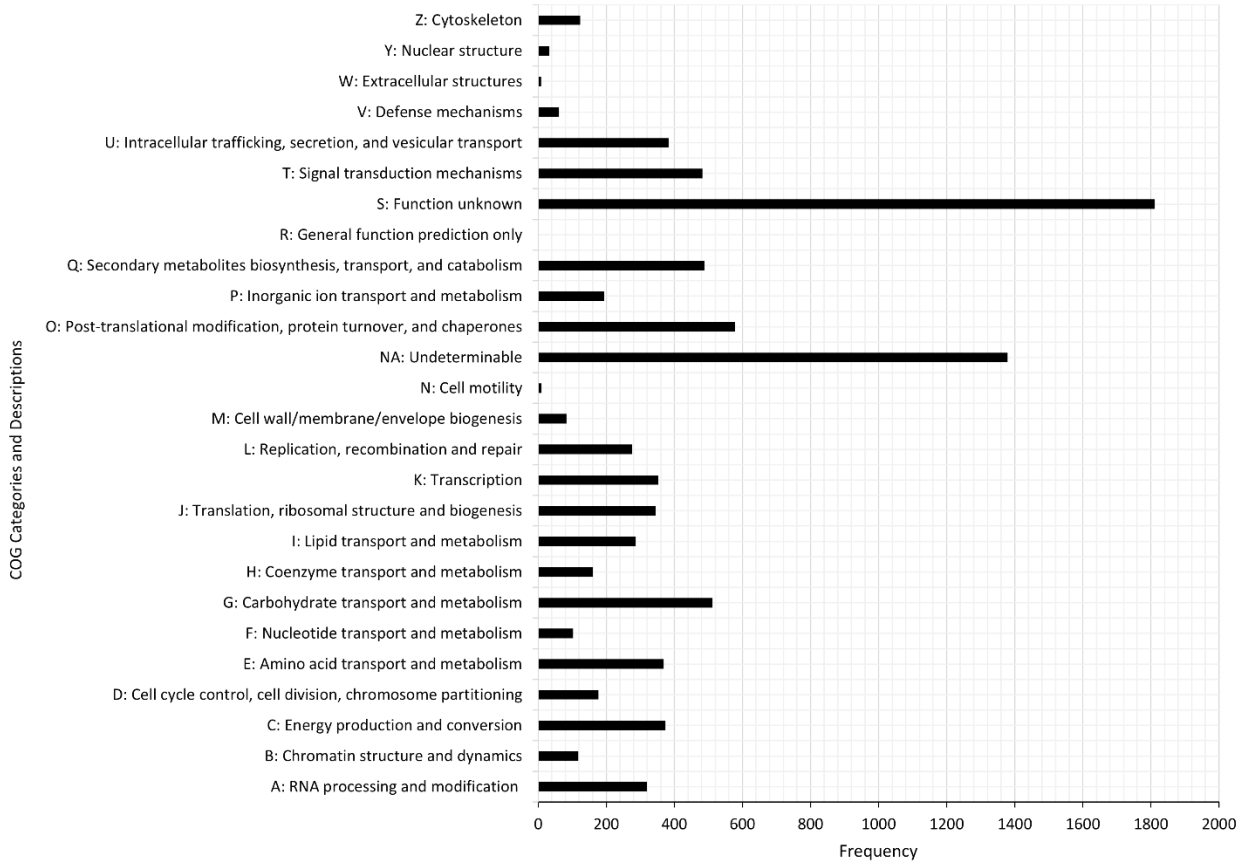

Figure 6.9. Frequency of genes in the *G. fusipes* draft genome associated with COG categories, using the eggnog-mapper public online web resource.

The analysis resulted in specific COG predictions for 5388 of the 8578 queries, with the remaining 3190 queries being undeterminable (n = 1379) or falling into COG category “S: Function unknown” (n = 1811). There were no queries falling into category “R: General function prediction only”. If the results of these categories are removed from the analysis (Figure 6.10), it can be seen that all other categories have representation in the dataset, with COG categories involving energy production (C), carbohydrate transport (G), post-translational modification and protein turnover (O), biosynthesis of secondary metabolites (Q) and signal transduction mechanisms (T), being most heavily represented.

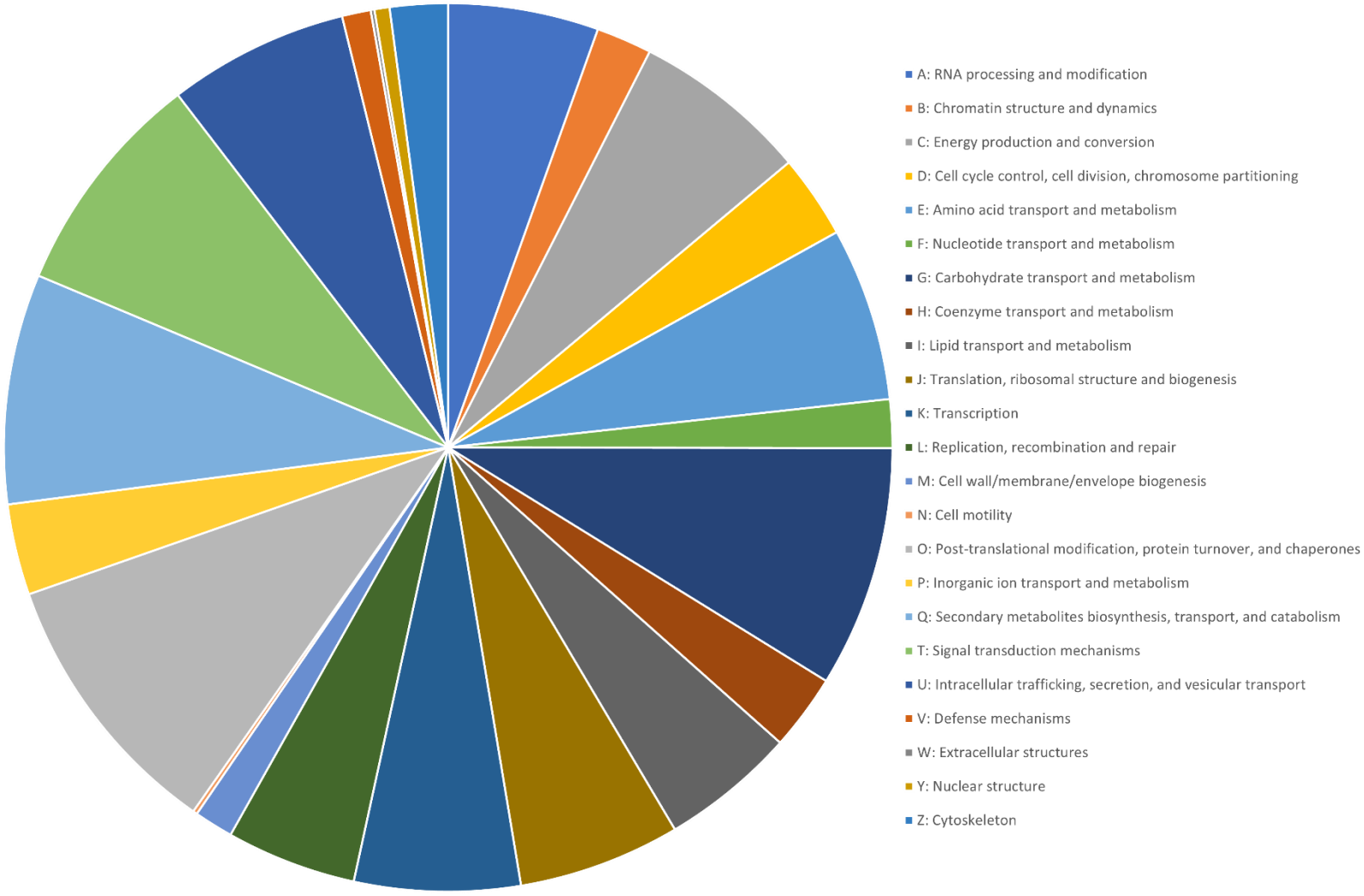

Figure 6.10. COG categories represented by the predicted genes in the *G. fusipes* draft genome, according to the eggNOG-mapper analysis.

6.3.3 | Oak seedling greenhouse inoculation tests

After the 6-and-a-half-month trial, lesions were observed in the deep wound treatments and the shallow wound treatments (Figure 6.11). Lesions were visually similar to those observed in infected root systems in the field.

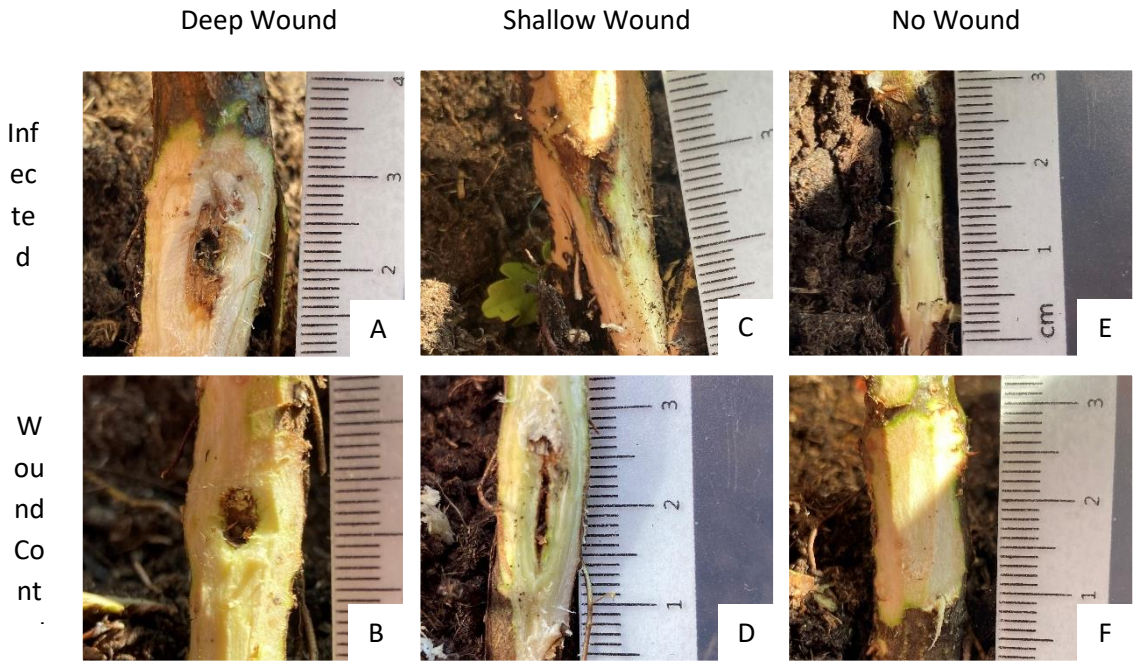

Figure 6.11. Lesions observed on seedlings after the 6.5-month experimental period. The deep and shallow wound methods produced lesions similar to those observed in the field. **A.** Seedling wounded with the deep wound method, infected with *G. fusipes* **B.** Seedling wounded with the deep wound method, treated with a non-inoculated agar plug control **C.** Seedling wounded with the shallow wound method, infected with *G. fusipes* **D.** Seedling wounded with the shallow wound method, treated with a non-inoculated agar plug control **E.** Seedling with the no wound method, infected with *G. fusipes* **F.** Seedling with the no wound method, treated with a non-inoculated agar plug control.

The no wound method resulted in no lesions on either the inoculated or non-inoculated seedlings. The shallow wound method resulted in lesions between 0.12 cm^2 and 1.17 cm^2, and the deep wound method resulting in lesions between 0.33 cm^2 and 0.89 cm^2 (Figure 6.12). The generalised linear model of the lesion area measurements indicated that there was not a significant difference in size between the deep wound seedlings inoculated with *G. fusipes* and those that were treated with a non-inoculated agar plug ($P = 0.06$). The analysis of the shallow wound method, however, found that there was a significant difference in the lesion size of seedlings inoculated with *G. fusipes* and those treated with a non-inoculated agar plug ($P = 0.01$).

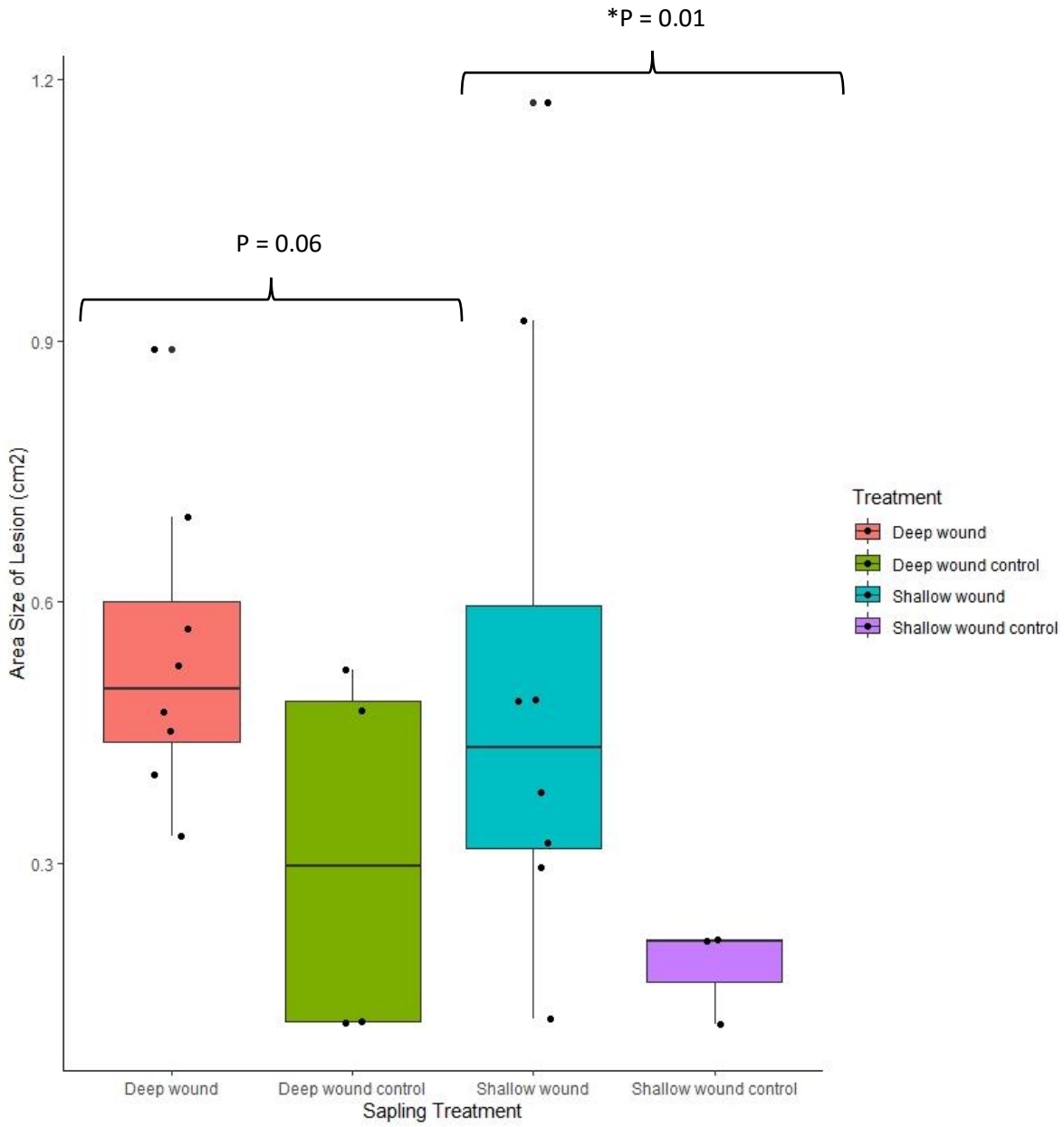

Figure 6.12. Results of the lesion analysis for each of the three wounding methods trialled in the seedling infection trial. There were no lesions present on the "No wound" seedlings, therefore they were not included in the analysis. There was a difference in lesion size between deep wound inoculated seedlings and deep wound control seedlings, however this was found to be insignificant (P = 0.06). There was a significant difference in lesion size between the shallow wound inoculated seedling and the shallow wound control seedlings (P = 0.01).

6.3.4 | Isolation and qPCR diagnostic analysis to confirm the presence of *G. fusipes* in field site samples

All fruiting bodies collected provided a positive result for present of the *G. fusipes 18SrRNA* gene, and four of the six infected tissue samples also provided a positive result. Two of the infected root

tissue samples did not produce a positive result when analysed with the qPCR diagnostic assay, however fruiting bodies taken from the root systems of these hosts did provide a positive result, so it can be assumed that the root systems were infected with *G. fusipes.* No positive results were obtained from the healthy tree tissue samples, or any of the soil samples. This work is detailed in Chapter 5.

6.3.5 | Extraction of high-quality RNA for transcriptomic analysis

A total of 22 extracts of RNA were obtained and sent to Novogene, UK, for RNA sequencing. These samples consisted of 4 non-infective agar controls, 4 infected seedling samples and 4 healthy seedling samples (to act as wound controls) from the seedling infection trial, 4 infected mature trees from the field site, and 4 healthy mature trees from the field site. The 22 samples also included a sample from a dead host infected with *G. fusipes* and a host that was asymptomatic, with no fruiting bodies or typical signs of decline, but did have apparent *G. fusipes* infection in the root system.

Table 6.2. Details of the 22 RNA extracts sent to be sequenced, and how they relate to the wider gene expression analysis.

No. of samples	Infection Condition	Notes
4	Non-infective	Non-infective control to provide a baseline for transcriptomic analysis
4	Early Infected Seedling	Infected seedlings from the seedling infection trial
4	Healthy Seedling	Healthy seedlings from the seedling infection trial, to act as a wound control
4	Established Infection	Mature infected trees from the impacted field site in the UK
4	Healthy Mature Host	Healthy mature host, with no signs of infection above or below the ground level
1	Asymptomatic Mature Host	Mature tree with infected root system and no above ground signs of infection
1	Dead Host	Dead host colonised by *G. fusipes*

6.3.6 | Mapping transcriptomic data to *G. fusipes* genome for gene expression analysis

mRNA sequencing data obtained from Novogene, UK included approximately 60 million paired end reads at 150 bp in length for each of the samples. After feeding the reads for the 22 samples through the FastP pipeline, a number of reads were removed due to potential unreliability, Hisat2 build was used to index the genome, resulting in 8 local indices to be used for mapping. Hisat2 was used to map the RNA transcripts to the genome (Table 6.3), and samtools sort organised the reads into the order of the genome.

Table 6.3. Overall alignment of the RNA transcripts to the *G. fusipes* genome using hisat2.

Sample Name	Condition	Total reads	Total reads mapped	Overall Alignment
A1	Non-infective	134857963	120909498	92.71%
A2	Non-infective	120385224	108348031	93.06%
A3	Non-infective	136558824	122585063	92.50%
A4	Non-infective	70762848	64376857	93.44%
MP1	Dead Host	149918253	134951602	92.39%
MP3	Established Infection	144116790	112941727	80.14%
MP4	Healthy Mature Host	148183205	1219337	0.82%
MP5	Established Infection	148957344	133626150	91.63%
MP6	Healthy Mature Host	139523515	264814	0.19%
MP7	Asymptomatic Mature Host	167487191	190843	0.11%
MP8	Healthy Mature Host	122340190	6684207	5.47%
MP9	Established Infection	127399358	115834742	92.7%
MP11	Established	119883321	44139033	37.07%

	Infection			
MP12	Healthy Mature Host	122007110	41369	0.03%
S1	Early Infected Seedling	145593652	1808	0%
S2a	Early Infected Seedling	102708336	571815	0.56%
S3	Early Infected Seedling	117288495	1098848	0.09%
S4	Early Infected Seedling	131652044	31333	0.02%
SC1	Healthy Seedling	144918632	12399	0.01%
SC2	Healthy Seedling	170518509	59110	0.03%
SC3a	Healthy Seedling	104045700	5346	0.01%
SC4	Healthy Seedling	118603305	50710	0.04%

The *G. fusipes* conditions resulting in the highest overall alignment to the genome, were the non-infective agar controls (A1 - A3, A4 was discounted from any further analysis due to an error in the running of the hisat2 software), and when present in the infected mature tree hosts. These conditions resulted in alignments between 37.07 and 93.06%. The analysis from the healthy mature hosts did result in some mapping, however these levels were much lower, between 0.03 and 5.47%. The seedling infection trial resulted in the lowest levels of mapping, however there was an observable difference between the infected and control seedlings, with the infected seedlings aligning between 0 and 0.56% and the wound control seedlings aligning between only 0.01 and 0.04% .

The resulting count tables, from HTSeq count, were further analysed with DESeq2 in R Studio. After normalising the data, a principal component analysis was conducted (Figure 6.13), with clear grouping of the non-infective agar transcripts indicating that each of the four replicates had similar gene expression.

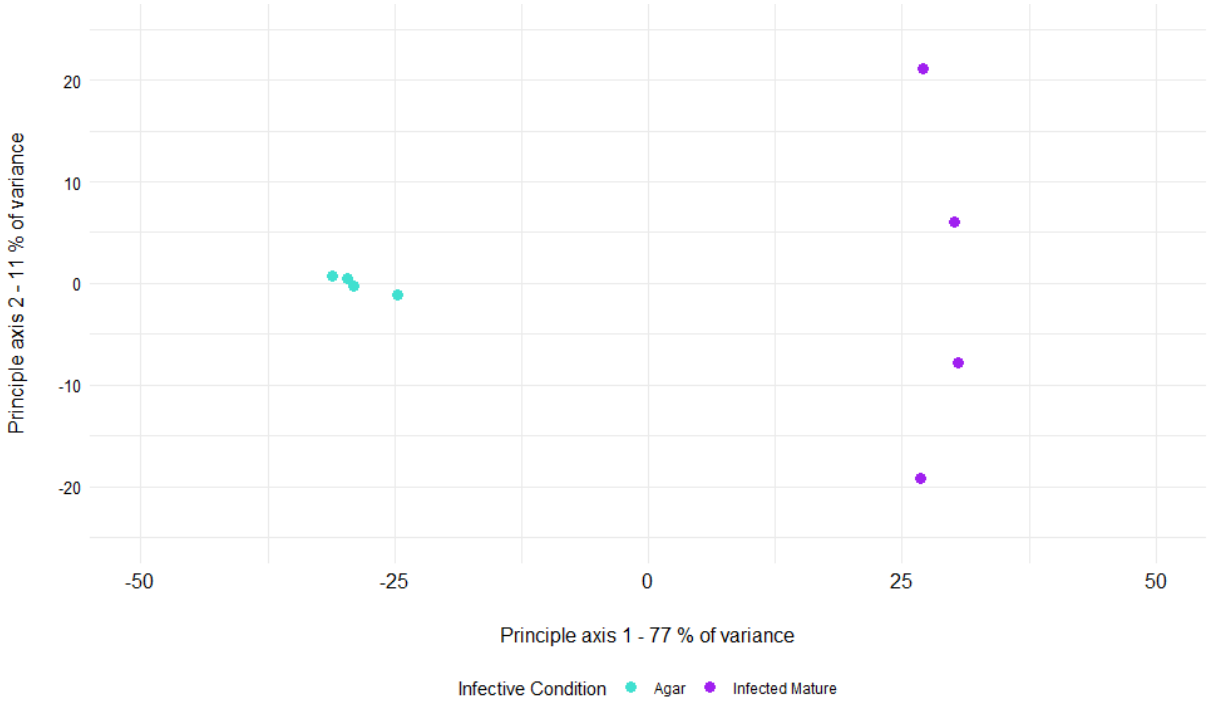

Figure 6.13. Principle component analysis of the differential gene expression analysis conducted using DESeq2. Analysis includes the non-infective agar condition and the established infection in the infected mature trees. Analysis was conducted using DESEq2.

The infected mature tree replicates appear to have a relatively similar transcriptomic profile, but they did not respond as analogously as the non-infective agar controls.

The next stage of analysis focussed on the similarity in differentially expressed genes between the samples (Figure 6.14).

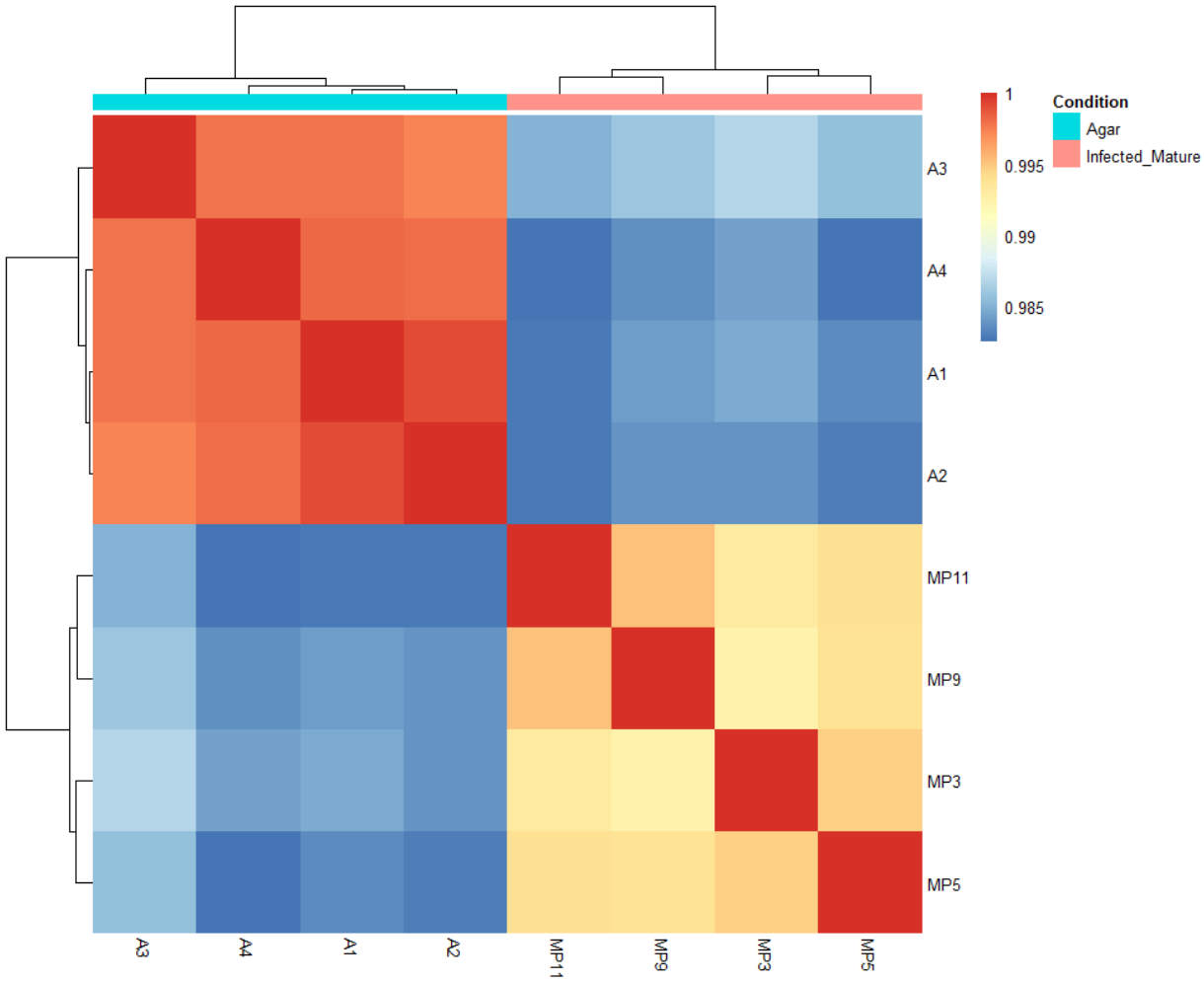

Figure 6.14. A map of similarity between samples in the non-infective agar condition and those from established infection in mature trees. Analysis was conducted using DESeq2.

This analysis showed that, overall, there was a high level of similarity in differentially expressed genes between replicates of the same sample, and a high level of diversity in differentially expressed genes between the two different infective conditions.

After this, analysis was conducted to investigate the most differentially expressed genes between the two infective conditions (Figure 6.15).

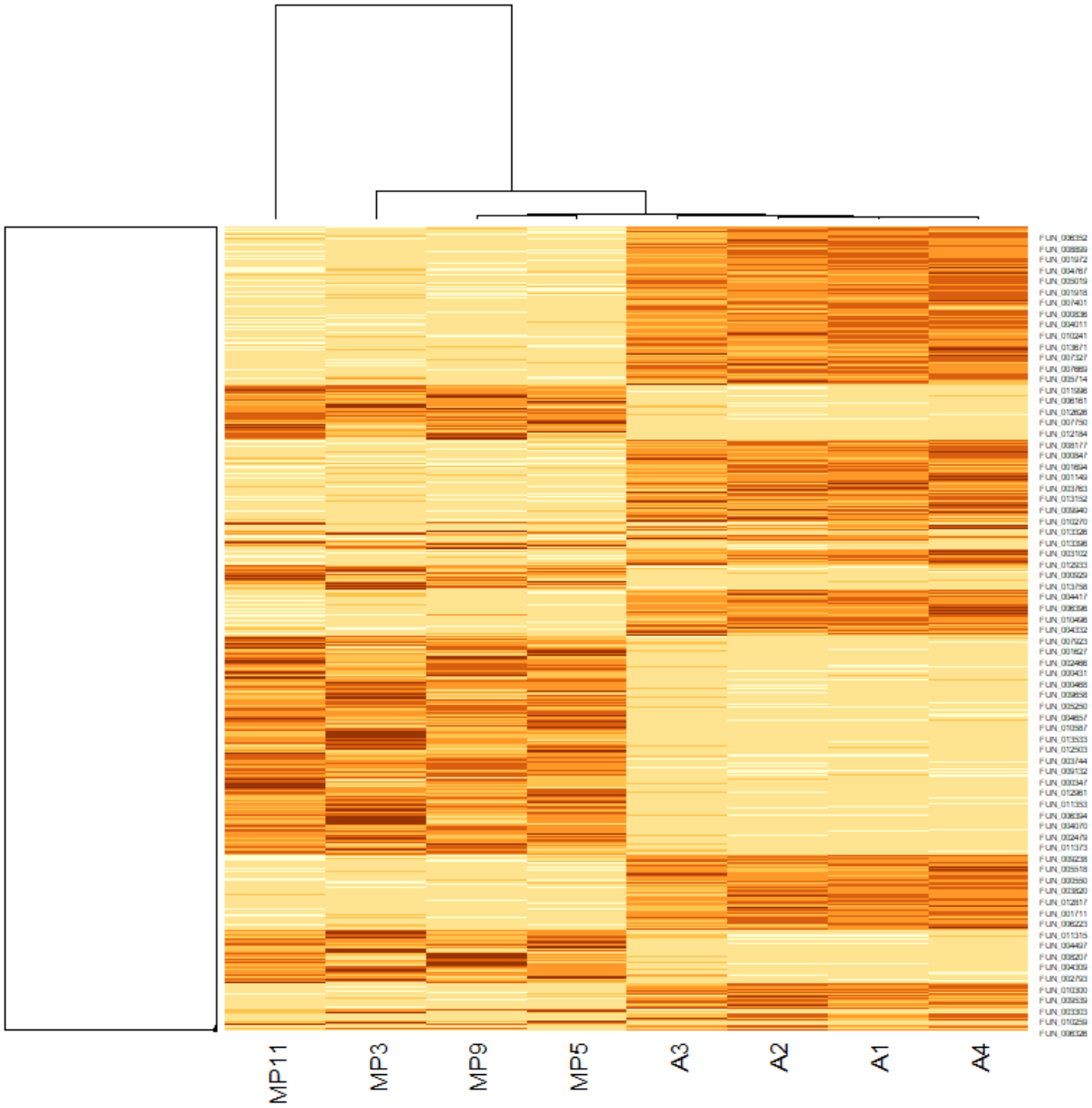

Figure 6.15. Heatmap to show the top differentially expressed genes between the non-infective agar controls and the established infection in mature trees. Analysis was conducted using DESeq2.

The results of this analysis illustrated how the transcriptomic profiles of *G. fusipes* when in a vegetative, non-infective state, and when in an actively infecting state are very different.

The final stage of analysis involved investigating a selection of the differentially expressed genes and identifying their potential role in the biology *G. fusipes.*

Table 6.4. Details from a selection of the genes differentially expressed by *G. fusipes* when in a non-infective state versus an active infection. Analysis was conducted using DESeq2 and gene details were obtained through cross-referencing with results from eggNOG-mapper results.

Gene Name	**Condition with high expression**	**Description**	**PFAMs**
FUN_013908	Non-infective agar	Dual specificity phosphatase, catalytic domain	DSPc
FUN_002762	Non-infective agar		zf-CCCH
FUN_011589	Non-infective agar	Belongs to the adaptor complexes small subunit family	Clat_adaptor_s
FUN_004129	Non-infective agar	carbohydrate-binding module family 19 protein	-
FUN_005019	Non-infective agar	Phenazine biosynthesis-like protein	PhzC-PhzF
FUN_007763	Non-infective agar	Monocarboxylate	MFS_1
FUN_002063	Non-infective agar	O-methyltransferase	Methyltransf_2
FUN_005622	Non-infective agar	Cytochrome p450	p450
FUN_004011	Non-infective agar	- TPR-like protein	CHAT,TPR_10,TPR_8
FUN_012917	Non-infective agar	GAL4-like Zn(II)2Cys6 (or C6 zinc) binuclear cluster DNA-binding domain	Fungal_trans,Zn_clus
FUN_000431	Established infection in mature oak host	L-lysine 6-monooxygenase (NADPH-requiring)	FMO-like,K_oxygenase,Pyr_redox_2
FUN_002959	Established infection in mature oak host	Phosphatidylinositol-4-phosphate 5-Kinase	PIP5K
FUN_003023	Established infection in mature oak host	Arylsulfotransferase (ASST)	Arylsulfotran_2
FUN_011727	Established infection in mature oak host	Alpha beta-hydrolase	Peptidase_S10
FUN_005235	Established infection in mature oak host	Ankyrin repeat	Ank_2,Ank_4,Ank_5,zf-CCCH_2
FUN_010314	Established infection in mature oak host	JAB/MPN domain	JAB
FUN_001157	Established infection in	Alpha-ketoglutarate-dependent taurine	TauD

	mature oak host	dioxygenase	
FUN_004400	Established infection in mature oak host	Interferon-inducible GTPase (IIGP)	IIGP
FUN_008207	Established infection in mature oak host	Peptidase A4 family	Peptidase_A4
FUN_012628	Established infection in mature oak host	Subunit 17 of Mediator complex	Med17

6.4 | Discussion

The main aim of this work was to elucidate and examine the process of *G. fusipes* infection at a molecular level through transcriptomic analysis of infection at different stages. The main objectives of this experiment were to produce the first draft genome for *G. fusipes*, to map transcriptomic reads to the genome and to compare transcriptomes across three different stages of *G. fusipes* infection, non-infective mycelial cultures, early-stage infection in a seedling infection trial, and an established infection in mature tree hosts in the field, in order to identify key genes associated with infection development. The hypotheses in question in this study were that (i) there will be a difference in transcriptome profile between the early-stage infection condition and the established infection condition, and that (ii) *G. fusipes* will express pathogenicity genes when infecting oak tissue (*in Planta* analysis of natural infections in mature trees and oak seedling inoculations) when compared with axenic grown on artificial medium (*in vitro*).

The first objective in this body of work was to produce the first draft genome for *G. fusipes*, this was completed through Pacific Biosystems Hifi sequencing, chosen for the high accuracy and long read length of this technology (Hon et al., 2020). After sequencing, a number of computational analyses and bioinformatics software was used to quality assess, filter out contamination, remove duplication, predict gene placement, and provide functional annotation. A key step in this process was the removal of duplicated genes, as the data was suggestive of two nuclei, which could be due to the DNA being extracted from vegetative mycelial cultures, which often have more than one nucleus in each cell (Gehrmann et al., 2018; Pettifor et al., 2022). By removing the duplicated genes from the analysis, the quality assessment was greatly improved, and it was ensured that analysis of only one nucleus was being conducted. The alternative assembly created in the initial assembly process and the duplicated genes (second nucleus) could be analysed further to provide more molecular information on *G. fusipes* (Duan et al., 2022).

The genome was annotated using the predicted proteins and the eggNOG-mapper online tool, and a basic functional annotation was provided, resulting in representation of all COG categories (clusters of orthologous groups of proteins) in the *G. fusipes* genome. Further analysis of the eggNOG-mapper data, including multiple reference points from databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes) and Pfam (the protein families database), would benefit this study by providing a deeper understanding of the *G. fusipes* genome. Using the results from each of these databases in combination would provide the most accurate gene annotation for the genome (He et al., 2023).

In order to have representative samples of an early *G. fusipes* infection, a seedling inoculation trial was conducted. This trial was considered a success, with measurable lesions, visually similar to those observed in the field, being present on the seedlings approximately 6 months after inoculation. Although this study does not present the first *G. fusipes* inoculation trial, with similar inoculations on more mature seedlings (approximately 2 years old) previously being successful, this trial was able to be conducted much quicker than previous work. This is in part due to the inoculum for this work being a colonised agar plug (taking just 4 weeks to culture), as opposed to a piece of colonised hazel, which could take up to 12 months to obtain (Marçais and Delatour, 1996). The relatively rapid results obtained in this experiment could also be, in part, due to the wounding methods used to allow *G. fusipes* to enter the host system. Although not necessarily representative of a natural infection, the use of wound controls, and statistical analysis showed that the presence of *G. fusipes* did result in significantly larger lesions. A caveat of this work is that *G. fusipes* was not successfully reisolated from the lesions, and so Koch's postulates could not be met, however this could be due to the limiting size of the lesions, and the requirement of the work to obtain lesion samples for RNA extraction and transcriptomic analysis.

In order to have representative samples of an established infection, a field site known to be heavily impacted by *G. fusipes* was visited. Fruiting bodies were present throughout the site, and in varying stages of decomposition. This is unusual as *G. fusipes* is suggested to fruit between August and September (Przybyl, 1994). A number of tree hosts had multiple clusters of fruiting bodies associated with them, up to 4 meters from the tree host, and sometimes more than one cluster on the same buttress root, indicating that high levels of the root system was heavily infected (Marçais et al., 2000b). A majority of the visibly *G. fusipes* infected hosts (those with *G. fusipes* fruiting bodies), also showed typical signs of decline such as a deterioration in crown condition, however without further examination it would not be possible to attribute this solely to *G. fusipes*, as the site is a known area to suffer from both acute oak decline, and chronic oak decline (Sandra Denman, Personal communication, 2021). Further to this, sample MP7, an asymptomatic mature host, did not show

any above ground signs of infection, however this host did have an early-stage *G. fusipes* root infection, suggesting the possibility that a number of apparently healthy tree hosts on the site may harbour an underground infection (Marçais et al., 2000b). Further investigation at this site, potentially over a temporal scale may aid in understanding the ecology of *G. fusipes* infection in this area.

The alignment of RNA transcripts to the *G. fusipes* genome proved difficult, and the percentage of reads that mapped to the *G. fusipes* genome, across the infective conditions were variable. In the non-infective agar condition, up to 93% of reads were effectively mapped to the genome, this is expected as the cultures were pure. The infected mature tree condition also provided results that indicated high levels of effective mapping, between 37% and 92%. Unexpectedly, one of the trees considered to be healthy (MP8), displayed an overall alignment of 5.47%, much higher than any of the other healthy trees, indicating that this host potentially is harbouring an early-stage infection. The extremely low alignments that resulted from the seedling inoculation trial, between 0% and 0.56% for the infected seedlings, led to these samples, and the early-stage infection condition being removed from further analysis.

The first hypothesis in this body of work was to determine that there will be a difference in transcriptome profile between the early-stage infection condition and the established infection condition. Although, due to low read counts in the seedling inoculation trial, the early-stage condition had to be removed from this analysis, the remaining analysis (including a principal component analysis, and heatmaps of similarity between samples and of differentially expressed genes) did suggest that the transcriptomic profiles between non-infective agar cultures and actively infecting *G. fusipes* isolates are very different. Further investigation is required to determine the role of these differentially expressed genes in the infection biology and ecology of *G.* fusipes.

The second hypothesis in this body of work was that *G. fusipes* will express pathogenicity genes when infecting oak tissue (*in Planta* analysis of natural infections in mature trees and oak seedling inoculations) when compared with axenic grown on artificial medium (*in vitro*). Although the seedling inoculation trial was removed from this analysis, there are still a number of differences in *G. fusipes* gene expression profile between the non-infective agar controls and the actively infecting mature tree samples. For example, genes highly expressed in the non-infective agar condition are liked with carbohydrate binding, important for maintaining the non-infective vegetative state, whereas genes expressed in the established infection include a number of enzymes and transcription promotors, both crucial to infection development and host colonisation.

Further to the points already mentioned above, future work should also work to provide more comparisons between different *G. fusipes* infections. For example, if the transcriptomic analysis reveals that different mechanisms of infection are utilised when *G. fusipes* is acting as a necrotroph or as a saprotroph, this may provide information as to how and why *G. fusipes* seems to present more than one trophic behaviour (Suzuki and Sasaki, 2019), therefore increasing available knowledge on the ecology of *G. fusipes*. A better understanding of *G. fusipes* host preference may also inform preventative land management strategies. It is suggested in the literature that the primary host range of *G. fusipes* is oak (Marçais and Caël, 2000; Pettifor et al., 2022), with some publications wrongly suggesting that the host range is limited to oak species and that other host species (as suggested in Piou et al., 2002) may only be very rare occurrences (Wainhouse et al., 2016). Finally, it would be beneficial to the field if the interactions between the host and pathogen were analysed, using transcriptomics, as well as metabolomics and proteomics (Saiz-Fernández et al., 2022), in order to truly understand the complex interactions between pathogen and host. Understanding the interactions between *G. fusipes* and the host tree would benefit this analysis by providing information as to how the host defends against *G. fusipes* attack, which could aid understanding of host colonisation and infection development (Zhang et al., 2019), both important aspects to consider when developing strategies to combat pathogens.

This body of work presents a substantial advancement in the available information regarding *G. fusipes* at a molecular level. Prior to this, the lack of molecular data on *G. fusipes* represented a significant barrier to furthering the understanding of this widespread species. This study has resulted in the first draft genome for *G. fusipes*, along with the first instance of transcriptomic analysis of *G. fusipes* across a range of infective conditions. This work highlighted the differences in gene expression between *G. fusipes* in a non-infective state, and *G. fusipes* in an active infection in a mature tree host. Although further investigation into this data is required for a higher understanding of *G. fusipes*, the data produced in this study could potentially be used to obtain information on the mechanisms of infection and also host responses, and could be utilised to inform management techniques, and help develop strategies to combat this dangerous widespread pathogen.

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CHAPTER 7

Final Synthesis and Future Research Priorities

7.1 | Introduction

The overarching aim of this work was to explore, assess and advance the information currently available on *Gymnopus fusipes,* predominantly, information on the ecology and infection biology of this understudied species. The following objectives were curated to address the central aim of the project, and research was completed to address the objectives.

The first objective of the project was to conduct a systematic literature review to highlight existing knowledge and identify key knowledge gaps in order to collate existing information regarding *G. fusipes* into an up-to-date informational resource on the species. This work was carried out in Chapter 2 and is summarised below (Section 7.2). The second objective involved optimising methods for isolation, culture, nucleic acid extraction and phylogenetic analysis for *G. fusipes.* This work was carried out in Chapters 3 and 4, is summarised below (Sections 7.3 and 7.4). The third objective of the project was to develop a rapid molecular-based diagnostic assay, suitable for use on field samples, to allow for accurate diagnosis of *G. fusipes*, without the need for pure culture. This was designed and optimised in Chapter 5 and is summarised below (Section 7.5). The final objective for this project was to investigate infection progression at a molecular level using transcriptomic analysis. This work was completed in Chapter 6 and is summarised below (Section 7.6).

7.2 | Using a systematic approach to synthesise existing knowledge on *Gymnopus fusipes* (syn. *Collybia fusipes*), the cause of *Collybia* root rot.

Chapter 2 addressed the first objective of the project, to conduct a systematic literature review to highlight existing knowledge and identify key knowledge gaps regarding *G. fusipes*. This work involved obtaining and evaluating all existing literature on *G. fusipes* and after filtering, through the use of strict inclusion criteria, the publications were assessed for their accuracy and reliability. The resulting information was then compiled into an up-to-date resource documenting all current knowledge on *G. fusipes*, covering research in areas of biochemistry, distribution, ecology, genomic information, host range, infection biology, morphology and phylogeny.

The data obtained through the completion of this work indicated that *G. fusipes* appears to have a distribution spanning numerous countries in temperate regions and has been linked to tree species across 13 genera, with 3 being confirmed through molecular or isolation-based methods. *G. fusipes* host range covers both broadleaf and coniferous tree varieties. *G. fusipes* is a typical agaricomycete fungus, with this work providing the first documentation of the probable lifecycle of the species. *G. fusipes* is a primary pathogen, and can be saprophytic or parasitic in its lifestyle, however the triggers which determine this are still unknown. *G. fusipes* has been linked to episodes of oak decline

across the UK and Europe, however infection by this pathogen often presents a lack of symptoms. This is dangerous, as a high level of infection can completely destroy whole root systems, meaning there is a high risk of wind failure for hosts maintaining a full crown. There is a deficit of molecular information available for *G. fusipes* on all databases, with very few nucleotide sequences published (n = 15).

The main conclusion from this work was that there is a substantial lack of focussed research regarding *G. fusipes*, and compared to other forest pathogens, it is extremely underrepresented in the literature. There is also unreliable and sometimes spurious data reporting in the literature, further confusing the little information that is available. The remainder of this project was designed to close some of the key knowledge gaps identified in this work, in particular relating to the ecology and infection biology of the species.

7.3 | Optimising methods for isolation, culture, nucleic acid extraction and phylogenetic analysis of *Gymnopus fusipes*.

Chapter 3 addressed the second objective of this project, which was to optimise methods for isolation, culture, nucleic acid extraction and phylogenetic analysis of *Gymnopus fusipes*. This work involved many small experiments, producing empirical data, as well as qualitative notes to address the following objectives: (i) to isolate *G. fusipes* strains from environmental samples including fruiting bodies and infected woody tissues, (ii) to optimise agar-based techniques for the growth of *G. fusipes,* including growth medium type and lighting conditions, (iii) to test and optimise methods for extracting nucleic acids from different *G. fusipes* sample types, including mycelial cultures, fruiting bodies and infected woody tissues, and finally (iv) to optimise PCR assays for commonly used fungal housekeeping genes and use these to produce a phylogeny of different *G. fusipes* isolates.

It was discovered early in the project that representation of *G. fusipes* in commonly used culture collections was lacking. After contacting 21 corresponding authors of publications identified in Chapter 2 mentioning work on *G. fusipes*, only one contact was able to supply viable cultures and three French strains were obtained. Isolating *G. fusipes* from environmental samples proved to be a very difficult task. As previously documented, above ground signs of infection such as fruiting bodies are not always present on infected trees, therefore identifying fruiting bodies in the field proved difficult, with two of the four summer seasons experienced in this project resulting in no fruiting bodies observed in the field. Furthermore, when fruiting bodies were found to be present, the condition of the fruiting body on collection, and subsequent storage of the fruiting body prior to isolation was a major factor in the success of the isolation attempt, with numerous samples

becoming degraded before being collected, and sometimes degrading in transit if not properly stored. Woody tissue samples were also found to be difficult to isolate from, as *G. fusipes* is a very slow growing pathogen, meaning that even with surface sterilisation, secondary contaminants often grew and rapidly overran the agar plates. The most successful method of isolation was to produce stab slants from basidiospores collected from fruiting bodies, however this does require fruiting bodies to be present and in good condition. Various samples were taken from 32 different infected tree hosts throughout this study, however only 5 strains were successfully isolated from them.

The second objective of this chapter was to optimise agar-based techniques for the growth of *G. fusipes,* including growth medium type and lighting conditions. Testing different media types resulted in clear results, whereby ½ strength malt extract agar was found to give the best biomass growth of *G. fusipes* over four weeks of growth. Culturing *G. fusipes* in complete darkness proved to have a significant positive effect on growth within the 4-week experiment.

The third objective for this chapter was to test and optimise methods for extracting nucleic acids from different *G. fusipes* sample types, including mycelial cultures, fruiting bodies and infected woody tissues. Obtaining clean, high quality nucleic acids from various sample types was not only important for the phylogenetic analysis conducted in this chapter, but was also imperative for the qPCR diagnostic assay development in Chapter 5 and the genomic and transcriptomics work in Chapter 6. Various methods, including simple temperature-based reactions, column-based extraction kits, and chemical precipitations, were tested. These tests resulted in an optimised CTAB/Chloroform based method with sodium acetate precipitation, which with slight alterations to the protocol, can be slightly altered depending on sample type and whether the end product required is DNA or RNA. This method isolates DNA and RNA with a high molecular weight and few impurities, making it an ideal method to be used for downstream applications such as qPCR and sequencing.

The final objective for this chapter was to optimise PCR reactions in order to use five fungal housekeeping genes to produce a multi-locus sequence analysis to determine relatedness of the isolates in the study. This proved to be challenging, with only the *ITS* gene region being obtained for the full data set. Using this gene, it was determined that all the isolates in the study were *G. fusipes*, however the isolates all grouped together, with little differentiation between the sites.

This work, although exploratory, provided some empirical data, as well as qualitative research notes, that were crucial to the remainder of the project.

7.4 | Measuring the growth rate of geographically diverse *Gymnopus fusipes* isolates at a range of temperatures.

Chapter 4 provided further work to address the objective of optimising methods for the culturing of *G. fusipes*, as well as providing evidence to inform the ecology of the species. In this, a growth rate experiment was conducted in order to measure the growth rate of five geographically diverse isolates of *G. fusipes* at five ecologically relevant temperatures. The main aims of this work were to determine the impact of incubation temperature on *G. fusipes* growth, and also to determine whether there was an interaction between isolate and temperature that may suggest localised temperature adaptation in the geographically diverse isolates.

The results of this experiment illustrated that there was a significant impact of temperature on the growth of *G. fusipes* with a 25°C incubation having the largest influence. There was also a significant result when considering the different isolates of *G. fusipes*, with isolate AH1 having the largest growth rate, regardless of the incubation temperature. However, there was no significant interaction between isolate and temperature that would potentially suggest localised adaptation to temperature in the isolates.

It is acknowledged that this experiment was conducted under controlled laboratory conditions, however it would be unfeasible to measure *G. fusipes* growth *in situ* on a host root system. This is due to *G. fusipes* infection occurring only below the ground level and underneath the outer bark of the roots, meaning that the continual measurements required for this experiment would not be obtained without severe disruption to the infection site, which would potentially influence the results.

Despite the controlled nature of this study, the data reported is still highly relevant, as the temperature limits presented in this experiment (the minimal growth at 4°C and 37 °C), highlights the potentially questionable distribution presented in Chapter 2, which included areas likely to reach these temperature limits. This study would have benefitted from more global isolates for full representation, and the inclusion of more temperature increments, in order to fully understand the temperature limits and optima of this understudied species.

7.5 | Development and optimisation of a qPCR-based diagnostic for accurate detection of *Gymnopus fusipes*.

Chapter 5 addressed the third objective of the project, to develop a qPCR-based diagnostic assay, to allow for accurate diagnosis of *G. fusipes* from field samples, without the need for pure culture. This

involved obtaining all available 18S rRNA nucleotide sequences for *G. fusipes*, and 111 related species from the NCBI GenBank database, and aligning the sequences using Geneious Prime®. After identifying a number of potential *G. fusipes* specific primer sites in the 18SrRNA gene region, five potential primer pairs were tested *in Silico*, using the NCBI PrimerBLAST tool. The most efficient three primer pairs were ordered to the lab along with the exact *G. fusipes* sequence (to be used as a positive control) and a selection of the non-target sequences that resulted from the PrimerBLAST search. After testing the primer pairs in the lab using the *G. fusipes* control, the non-target sequences, and a number of isolates in the lab, the most effective primer pair was selected, and the annealing temperature of the reaction was optimised to make the assay highly specific to *G. fusipes* only. This was verified using a number of environmental samples, with and without the presence of *G. fusipes*. The assay, under optimised conditions, was found to be effective in detecting *G. fusipes* in environmental samples, such as fruiting bodies and infected woody tissues, without the need for pure culture, meaning that the process could be completed in less than 48 hours.

A caveat of the works reviewed in Chapter 2 is that a number of publications note the distribution of *G. fusipes*, without confirmation through isolation or molecular analyses. Morphological analysis alone is not always reliable, as there is an expert level of knowledge required for this method of identification, and the possibility of cryptic species. The molecular diagnostic developed in this chapter is highly specific, targeting only the *G. fusipes 18SrRNA* gene. Therefore, this diagnostic tool could be utilised to investigate claims of *G. fusipes* distribution and confirm or disprove its presence, providing further information on the spread of this pathogen on both a local and worldwide scale.

7.6 | Using transcriptomic analysis to compare gene expression of *Gymnopus fusipes* at different stages of infection.

Chapter 6 addressed the final aim of this research, which was to investigate *G. fusipes* infection progression, at a molecular level, using transcriptomic analysis. This work aimed to determine which pathogenicity genes were involved at different stages of *G. fusipes* infection, in order to further the understanding of *G. fusipes* infection biology.

For this, three infective states were developed to compare gene expression at the different stages of infection, these included a non-infective state (mycelial plate cultures), an early infective state (seedling inoculation trial) and an established infective state (mature trees impacted with *G. fusipes* infection). For the non-infective *G. fusipes*, a strain of *G. fusipes* was grown on agar plates for 4 weeks. For the early infective state, a successful seedling inoculation trial was completed, where young seedlings were wounded and subjected to *G. fusipes* infection and left for 6.5 months before

being assessed for presence of lesions, and woody tissue along the lesion margin was taken for RNA analysis. Lesions similar to those observed in the field developed on the seedlings, and there was a significant difference between lesion size in the inoculated seedlings and the non-inoculated wound controls. A field sampling campaign was conducted, in which woody tissue samples from mature trees infected with *G. fusipes* were observed and collected for RNA extraction. RNA was extracted from these samples, and replicates were sent for RNA sequencing.

In order to map the expressed genes, genomic DNA from a *G. fusipes* mycelial culture was extracted and sequenced using Pacific Biosystems HiFi sequencing technology. The genome was assembled and annotated before being used as a reference for the transcriptomic data. After the conduction of a successful sapling infection trial, RNA was extracted from the root tissues. RNA was also extracted from samples of mature trees and axenic *G. fusipes* agar cultures. RNA was sequenced and the reads were aligned to the genome, and after obtaining count numbers for genes in each of the three conditions, differential gene expression analysis was conducted for the non-infective agar controls and the actively infecting *G. fusipes* from infected mature trees.

The results of this study indicated that there is a stark difference in transcriptome profile between an active *G. fusipes* infection, and a non-infecting control. Although further work is required to determine the role of these differentially expressed genes in the infection biology and ecology of the species, this body of work provides a starting point for this further analysis.

7.7 | Future Research Priorities

Although this body of work has begun to close some of the key knowledge gaps identified at the start of the project, more research is required to fully understand this devastating root rot pathogen. Future work in the field should aim to understand environmental factors (other than waterlogging, as this has been explored quite extensively) that affect the growth, fruiting and sporulation of *G. fusipes.* Empirical evidence regarding this would inform a number of areas, including the ecology and spread of the species, as well as confirming the lifecycle of *G. fusipes.* Understanding the impact of these factors on *G. fusipes* would also aid in planning sampling efforts if the fruiting season could be more accurately identified, and also knowledge of the fruiting season would allow for better quality fruiting bodies to be obtained, meaning isolation (from basidiospores) would be more likely to be successful.

Another key topic that should be explored further is the host range of *G. fusipes. G. fusipes* has only been documented to cause root rot on oak species, however has been found to exist on a number of other species. Understanding if *G. fusipes* has a host preference would inform the ecology of the

species. It would be beneficial to know whether *G. fusipes* has a preference for hosts as this would help in targeted isolations, and it may affect the spread and even pathogenicity of this species.

7.8 | Conclusions

The aim of this body of work was to increase the knowledge on *G. fusipes* with regards to its role as a root rot pathogen. This project has successfully fulfilled the overarching aim set out at the beginning of the work, to explore, assess and advance the information currently available on *G. fusipes*. This work has explored and compiled the existing literature, assessed, and optimised current isolation and culturing methods, along with molecular techniques, developed a novel diagnostic assay and has advanced the molecular information available on *G. fusipes*, through genomic and transcriptomic analysis.

The curation of a comprehensive, up-to-date informational resource on *G. fusipes*, coupled with notes on evaluation and optimisation of laboratory methods for working with *G. fusipes*, proved to be an essential starting point for this project, and may prove to be an invaluable resource for those considering research on this species in the future. The development of a novel qPCR assay for detecting *G. fusipes* in field samples such as fruiting bodies and infected woody tissues, has removed the need for pure culture, meaning that the whole process from sample collection to result can be performed in less than 48 hours. This work documents the first instance of whole genome sequencing for *G. fusipes*, and the first instance of transcriptomic analysis being used to assess gene expression in *G. fusipes* infection development.

Although there are still many aspects of *G. fusipes* which require additional research, in order to further develop our understanding of this pathogen, this body of work represents a vast leap forward in the understanding of the ecology and infection biology of *G. fusipes*.

7.9 | References

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Supplementary Information

Supplementary Information 2.1
List of publications mentioning *G. fusipes* in the main text (including abstract, tables and figures), highlighted cells indicate publications directly related to *G. fusipes* used in the final analysis.

No.	**Title**	**Full Reference**
1	A checklist of macrofungi of Gorakhpur District, U.P. India	Vishwakarma, P., Tripathi, N.N., Singh, P., 2017. A checklist of macrofungi of Gorakhpur District, U.P. India. Curr. Res. Environ. Appl. Mycol. 7, 109-120. doi:10.5943/cream/7/2/8
2	A check-list of the Greek macrofungi including hosts and biogeographic distribution: I. Basidiomycotina	Zervakis, G., Dimou, D., Balis, C., 1998. A check-list of the Greek macrofungi including hosts and biogeographic distribution: I. Basidiomycotina. Mycotaxon 66, 273-336.
3	A compendium of generic names of agarics and Agaricales	Kalichman, J., Kirk, P.M., Matheny, P.B., 2020. A compendium of generic names of agarics and Agaricales. Taxon 69, 425-447. doi:10.1002/tax.12240
4	A contribution to the knowledge of larger basidiomycetes of Albania	Assyov, B., 2018. A contribution to the knowledge of larger basidiomycetes of Albania, Phytologia Balcania 24, 187-193.
5	A gold mine for drug discovery: Strategies to develop cyclic peptides into therapies	Jing, X., Jin, K., 2020. A gold mine for drug discovery: Strategies to develop cyclic peptides into therapies. Med. Res. Rev. 40, 753-810 doi:10.1002/med.21639
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Supplementary Information | 3.1

Publications in which corresponding authors were contacted, in an attempt to obtain isolates of *Gymnopus fusipes*.

Reference	Corresponding Author	Notes
Akata, I., Uzun, Y., Kaya, A. (2014) **Macromycetes determined in Yomra (Trabzon) district**. Turk. J. Botany 38(5), 999–1012, Doi: 10.3906/bot-1309-22.	Ilgaz Akata	-
Ambrosio, E., Cecchi, G., Zotti, M., Mariotti, M.G., Piazza, S. Di., Boccardo, F. (2018) **An annotated checklist of macrofungi in broadleaf Mediterranean forests (NW Italy)**. Acta Mycol. 53(2), 1109, Doi: 10.5586/am.1109.	Elia Ambrosio	Informed that this author no longer works in the same lab group and was instructed to contact Mirca Zotti (who had already been contacted regarding a different publication).
Assyov, B. (2018) **A contribution to the knowledge of larger basidiomycetes of Albania**. Phytol. Balc. 24(2), 187–93.	Boris Assyov	-
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Bucşa, L. (2007) **Macromycetes of the Breite Nature Reserve of Ancient Oaks (Transylvania, Romnia)**. Transylv. Rev. Syst. Ecol. Res 4, 33.	Livia Bucşa	-
Doğan, H.H., Öztürk, C. (2006) **Macrofungi and their distribution in Karaman province, Turkey**. Turk. J. Botany 30(3), 193–207.	Hasan Hüseyin Doğan	-
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Karadelev, M., Rusevska, K., Kost, G., Kopanja, D.M. (2018) **Checklist of macrofungal species from the phylum Basidiomycota of the Republic of Macedonia**. Acta Musei Maced. Sci. Nat. 21(1), 23–112.	Mitko Karadelev	Informed that the collection only works with dry samples. Also stated that they could share dry samples, but due to Covid-19 the postal service would be unlikely to transport samples.
Kholfy, S. El., El-Assfouri, A., Touhami, A.O., Belahbib, N., Benkirane, R., Douira, A. (2014) **Bibliographic Catalog of Endemic or Rare Mushrooms of Morocco**. Int. J. Plant, Anim. Environ. Sci. 4(2), 103–16.	Saifeddine El Kholfy (Lead author. No corresponding author identified)	-
Manic, Ş. (2016) **The macromycetes of the downy oak forests from Moldova**. J. Plant Development 23, 139-147.	Ştefan Manic	-
Mihál, I., Bučinová, K., Pavlíková, J. (2009) **Mycoflora of beech forests in the Kremnické vrchy Mts (Central Slovakia)**. Folia Oecologica 36(1).	Ivan Mihál (Lead author. No corresponding author identified)	-
Natcheva, R., Gyosheva, M., Alatas, M., Özdemir, T. (2016) **Contribution to the bryophyte flora and mycota of Bulgaria: I. Bryophytes and larger fungi from Uchilishtna Gora Managed Reserve**. Phytol. Balc. 22(3), 323–30	Rayna Natcheva (Lead author. No corresponding author identified)	-
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Piou, D., Delatour, C., Marçais, B. (2002) **Hosts and distribution of Collybia fusipes in France and factors related to the disease's severity**. For. Pathol. 32(1), 29–41, Doi: 10.1046/j.1439-0329.2002.00268.x.	Claude Delatour	-
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Senthilarasu, G. (2014) **Diversity of agarics (gilled mushrooms) of Maharashtra, India**. Curr. Res. Environ. Appl. Mycol. 4(1), 58–78, Doi: 10.5943/cream/4/1/5.	Senthilarasu G	-
Siller, I., Kutszegi, G., Takács, K., Varga, T., Merényi, Z., Turcsányi, G., Ódor, P., Dima, B. (2013) **Sixty-one macrofungi species new to Hungary in Őrség National Park**. Mycosphere 4(5), 871–924, Doi: 10.5943/mycosphere/4/5/3.	Kutszegi G	-
Stasińska, M., Sotek, Z. (2017) **New data to the knowledge of macrofungi of Wolin National Park**. Acta Mycol. 51(2), Doi: 10.5586/am.1089.	Małgorzata Stasińska	-
Watling, R. (2005) **The fungi of Scottish Western oakwoods**. Bot. J. Scotl. 57, 155–65, Doi: 10.1080/03746600508685094.	Roy Watling	-
Zotti, M., Pautasso, M. (2013) **Macrofungi in Mediterranean Quercus ilex woodlands: relations to vegetation structure, ecological gradients and higher-taxon approach**. Czech Mycol. 65(2), 193–218.	Mirca Zotti	Informed that the group did not have any strains of *G. fusipes* at this time. Offered to search for *G. fusipes* in the next season, however no further contact was received from this group.

Supplementary Information 5.1

Taxa and number of 18S rRNA sequences of each used for alignment to identify potential primer regions.

Species	Number of 18S rRNA sequences	Accession Numbers
Collybia cirrhata	15	>AF274380.1 >AF274381.1 >AF274382.1 >AF361312.1 >AF361313.1 >AF361314.1 >AF361315.1 >AF361316.1 >AF361317.1 >AF361318.1 >DQ830804.1 >DQ830805.1 >KP293582.1 >MK564546.1 >MK671597.1
Collybia cookie	10	>AF065120.1 >AF065123.1 >AF274383.1 >AF361304.1 >AF361305.1 >AF361306.1 >DQ830802.1 >DQ830803.1 >DQ830806.1 >MK573873.1
Collybia hariolorum	2	>JF907991.1

		>MH856329.1
Collybia tuberosa	13	>AY771606.1 >AF065121.1 >AF065124.1 >AF274376.1 >AF274377.1 >AF274378.1 >AF274379.1 >AY854072.1 >AF361308.1 >AF361309.1 >AF361311.1 >DQ830807.1 >KP255470.1
Dendrocollybia racemosa	9	>AF065122.1 >AF274374.1 >AF274375.1 >EU846262.1 >KP454022.1 >DQ644556.1 >DQ825432.1 >KP255472.1 >MF343442.1
Gymnopus acervatus	27	>GU318373.1 >GU318374.1 >GU318375.1 >GU318376.1 >GU318377.1 >GU318378.1 >GU318379.1 >GU318380.1

		>GU318381.1 >GU318382.1 >GU318383.1 >GU318384.1 >GU318385.1 >GU318387.1 >GU318388.1 >GU318389.1 >GU318390.1 >GU318391.1 >GU318392.1 >GU318393.1 >GU318394.1 >GU318395.1 >GU318396.1 >GU318397.1 >GU318398.1 >GU318399.1 >GU318400.1
Gymnopus aff. aquosus	3	>DQ449981.1 >DQ449999.1 >DQ450003.1
Gymnopus aff. biformis	1	>KJ416251.1
Gymnopus aff. brunneigracilis	1	>MF100983.1
Gymnopus aff. dichrous	3	>KY026654.1 >KY026697.1 >KY026696.1
Gymnopus aff. dryophilus	2	>KY026680.1 >KY026679.1
Gymnopus aff. dysodes	1	>DQ480110.1
Gymnopus aff. luxurians	4	>KJ416239.1

		>KJ416238.1 >KJ416237.1 >KJ416236.1
Gymnopus aff. melanopus	1	>KY026699.1
Gymnopus aff. moseri	1	>AY263431.1
Gymnopus aff. nivalis	1	>DQ449982.1
Gymnopus aff. nonnullus	1	>MF061326.1
Gymnopus aff. polygrammus	2	>MF100980.1 >MF100979.1
Gymnopus alkalivirens	2	>DQ480112.1 >DQ450000.1
Gymnopus alnicola	1	>AF505770.1
Gymnopus alpinus	4	>JX536168.1 >DQ480114.1 >DQ480102.1 >DQ480101.1
Gymnopus androsaceus	15	>KY696772.1 >KY026750.1 >KY026749.1 >KY026748.1 >KY026747.1 >KY026663.1 >JN941125.1 >FR717227.1 >GU234007.1 >MH857176.1 >MH857175.1 >MH857174.1 >MH857173.1 >MH856650.1

		>MH856517.1
Gymnopus aquosus	7	>AY256691.1 >DQ449971.1 >DQ449976.1 >DQ449977.1 >DQ450002.1 >DQ480096.1 >KR673434.1
Gymnopus austrosemihirtipes	1	>AY263422.1
Gymnopus barbipes	6	>KJ416265.1 >KJ416268.1 >KJ416266.1 >KJ416267.1 >KJ416269.1 >MK532859.1
Gymnopus biformis	15	>KJ416250.1 >KJ416249.1 >KJ416248.1 >KJ416247.1 >KJ416246.1 >KJ416245.1 >DQ450064.1 >DQ450063.1 >DQ450059.1 >DQ450056.1 >DQ450055.1 >DQ450054.1 >AY256699.1 >AF505771.1 >AF505767.1_

Gymnopus biformis var. lobatus	2	>KJ416254.1 >AF505775.1
Gymnopus billbowesii	2	>MF100989.1 >MF100990.1
Gymnopus ceraceicola	3	>KJ416262.1 >KJ416261.1 >KJ416260.1
Gymnopus cervinus	2	>MF100984.1 >MF100985.1
Gymnopus confluens	53	>MF908467.1 >KY418936.1 >LT716054.1 >KX513743.1 >KP710303.1 >KP710302.1 >KP710301.1 >KP710300.1 >KP710299.1 >KP710298.1 >KP710297.1 >KP710296.1 >KP710295.1 >KP710294.1 >KP710293.1 >KP710292.1 >KP710291.1 >KP710290.1 >KP710289.1 >KP710288.1 >KP710287.1 >KP710286.1

>KP710285.1

>KP710284.1

>KP710283.1

>KP710282.1

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>KP710276.1

>KP710275.1

>KP710274.1

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>KP710272.1

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>KJ026453.1

>HQ604802.1

>HM240527.1

>FJ596784.1

>DQ450053.1

>DQ450052.1

>DQ450051.1

>DQ450050.1

>DQ450049.1

>DQ450048.1

>DQ450047.1

>DQ450046.1

>DQ450045.1

>DQ450044.

>AY256697.1

>AF505773.1

Gymnopus collybioides	1	>AF505772.1
Gymnopus contrarius	3	>KX462133.1 >DQ440643.1 >MH930172.1
Gymnopus cylindricus	3	>AF505776.1 >AY256696.1 >DQ450057.1
Gymnopus densilamellatus	1	>MN258642.1
Gymnopus dichrous	49	>KY026665.1 >KY026656.1 >KY026655.1 >KY242500.1 >KY242499.1 >KY242498.1 >KY242497.1 >KY242496.1 >KY242495.1 >KY242494.1 >KY242493.1 >JF313698.1 >JF313697.1 >JF313696.1 >JF313695.1 >JF313694.1 >JF313693.1 >JF313692.1 >JF313691.1 >JF313690.1 >JF313689.1 >JF313688.1 >JF313687.1

		>JF313686.1 >JF313685.1 >JF313684.1 >JF313683.1 >JF313682.1 >JF313681.1 >JF313680.1 >JF313679.1 >JF313678.1 >JF313677.1 >JF313676.1 >JF313675.1 >JF313674.1 >JF313673.1 >JF313672.1 >JF313671.1 >FJ596783.1 >FJ596782.1 >FJ596781.1 >DQ480115.1 >DQ450009.1 >DQ450008.1 >DQ450007.1 >AY256702.1 >AF505766.1 >MG663260.1
Gymnopus disjunctus	2	>KJ416252.1 >KJ416253.1
Gymnopus dryophilus	44	>AY665779.1 >FJ596766.1 >FJ596767.1

>AF505787.1

>AY256690.1

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>DQ449962.1

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>DQ449974.1

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>DQ480098.1

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>DQ480100.1

>GU256226.1

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>JX536154.1

>KR673450.1

>KR673484.1

>KC152108.1

>KT697965.1

>KT697983.1

>KT697987.1

>KT697990.1

>LC149609.1

>KT874997.1

>KT874998.1

>KT874999.1

>KT875000.1

		>KX513744.1 >LT716055.1 >KY418937.1 >MK571175.1 >MH589962.1 >MH589962.1 >MH589963.1 >MH589965.1 >MH589966.1 >MH589970.1
Gymnopus dysodes	4	>AF505778.1 >DQ449987.1 >KY026666.1 >MF773622.1
Gymnopus earleae	3	>DQ480094.1 >DQ449994.1 >AY256694.1
Gymnopus eneficola	11	>KP710271.1 >KP710270.1 >KJ416257.1 >KJ416256.1 >KJ128268.1 >KJ128267.1 >KJ128266.1 >KJ128265.1 >KJ128264.1 >KJ128263.1 >KJ128262.1
Gymnopus erythropus	6	>AF505783.1 >AF505786.1 >DQ449995.1

		>DQ449996.1 >DQ449997.1 >DQ449998.1
Gymnopus exculptus	1	>DQ449973.1
Gymnopus fagiphilus	1	>JX536127.1
Gymnopus fibrosipes	2	>AY842953.1 >AF505763.1
Gymnopus foetidus	6	>KY026709.1 >KY026682.1 >KY026730.1 >KY026731.1 >KY026739.1 >MG748573.1
Gymnopus foliiphilus	17	>KY026721.1 >KY026705.1 >KY026703.1 >KY026694.1 >KY026690.1 >KY026681.1 >KY026658.1 >KY026652.1 >KY026651.1 >KY026650.1 >KY026647.1 >KY026633.1 >KY026631.1 >KY026630.1 >KY026626.1 >KY026620.1 >MF161164.1

Gymnopus fusipes	6	>KX449407.1 >AY256711.1 >AY256710.1 >AF505777.1 >KY026727.1 >FR686558.1
Gymnopus gibbosus	9	>DQ450020.1 >DQ450019.1 >AY842956.1 >AY263438.1 >AY263437.1 >AY263436.1 >MF100978.1 >KY061203.1 >KY061202.1
Gymnopus graveolens	1	>MH422573.1
Gymnopus hirtelloides	1	>MF100975.1
Gymnopus hirtellus	1	>MF100974.1
Gymnopus hybridus	1	>DQ449980.1
Gymnopus impudicus	6	>AF505779.1 >DQ480109.1 >DQ480109.1 >KJ416264.1 >LT594119.1 >LT594120.1
Gymnopus indoctus	1	>AY263440.1
Gymnopus inusitatus	2	>MH259876.1 >MH259875.1
Gymnopus iocephalus	4	>DQ449984.1 >DQ449986.1 >KX513745.1

		>DQ449985.1
Gymnopus irresoutus	1	>MF100973.1
Gymnopus juniperinus	2	>KY026661.1 >AY256708.1
Gymnopus junquilleus	3	>MG677138.1 >DQ449969.1 >AY256693.1
Gymnopus kauffmanii	1	>DQ450001.1
Gymnopus lodgeae	2	>AF505757.1 >AY256705.1
Gymnopus luxurians	14	>KY026649.1 >KJ416241.1 >KJ416240.1 >DQ480106.1 >DQ480105.1 >DQ450024.1 >DQ450023.1 >DQ450022.1 >DQ450021.1 >AY256709.1 >AF505765.1 >MN523269.1 >MF773597.1 >KM496469.1
Gymnopus luxurians var. copeyi	1	>AF505764.1
Gymnopus macropus	4	>DQ449979.1 >DQ449978.1 >AF505788.1 >MF156258.1
Gymnopus melanopus	4	>KM896875.1

		>AY263442.1 >AY263425.1 >MF100988.1
Gymnopus menehune	4	>KJ778753.1 >JN182864.1 >DQ450043.1 >AY263426.1
Gymnopus mesoamericanus	3	>DQ450036.1 >DQ450035.1 >AF505768.1
Gymnopus micromphaleoides	1	>KJ416243.1
Gymnopus montagnei	2	>MF038943.1 >DQ449988.1
Gymnopus mustachius	1	>MF100987.1
Gymnopus neotripicus	1	>AF505769.1
Gymnopus nonnullus	1	>KY026701.1
Gymnopus nubicola	1	>AF505781.1
Gymnopus obscuroides	2	>KX958399.1 >KX958398.1
Gymnopus ocellus	1	>MF100976.1
Gymnopus ocior	16	>AF505782.1 >DQ449955.1 >DQ449956.1 >DQ449957.1 >DQ449958.1 >DQ449959.1 >DQ449960.1 >DQ449961.1 >DQ449967.1 >DQ449968.1

		>DQ449970.1 >JX536162.1 >JX536163.1 >JX536164.1 >KY026678.1 >MH930144.1
Gymnopus omphalodes	5	>AY256700.1 >DQ450010.1 >DQ450011.1 >AF505761.1 >KJ609160.1
Gymnopus parvulus	4	>DQ450062.1 >DQ450061.1 >DQ450060.1 >AF505774.1
Gymnopus perforans	3	>KY026623.1 >KY026624.1 >KY026625.1
Gymnopus perforans sub. perforans	3	>KY026641.1 >KY026659.1 >KY026662.1
Gymnopus perforans sub. transatlanticus	20	>KY026668.1 >KY026674.1 >KY026669.1 >KY026670.1 >KY026671.1 >KY026672.1 >KY026673.1 >KY026675.1 >KY026710.1 >KY026711.1

		>KY026712.1 >KY026715.1 >KY026717.1 >KY026718.1 >KY026719.1 >KY026720.1 >KY026734.1 >KY026735.1 >KY026742.1 >KY026743.1
Gymnopus peronatus	12	>AF505760.1 >AY256706.1 >DQ450012.1 >DQ450014.1 >DQ450016.1 >DQ450017.1 >KC581300.1 >KP454027.1 >KY026677.1 >KY026755.1 >KY026756.1 >MH855896.1
Gymnopus pinophilus	4	>KY026726.1 >KY026725.1 >KY026724.1 >KY026695.1
Gymnopus pleurocystidiatus	1	>MF100977.1
Gymnopus polygrammus	6	>AY842954.1 >DQ450028.1 >KJ609162.1 >KJ778752.1

		>KY074642.1 >MN258643.1
Gymnopus polyphyllus	6	>FJ596895.1 >FJ596894.1 >DQ480111.1 >DQ480089.1 >DQ449992.1 >AY256695.1
Gymnopus pseudolodgeae	1	>AF505747.1
Gymnopus pseudoluxurians	2	>KJ416242.1 >KY026702.1
Gymnopus pseudoomphalodes	2	>AF505762.1 >AY842957.1
Gymnopus pygmaeus	1	>KX869966.1
Gymnopus readiae	3	>DQ450034.1 >HQ533036.1 >KJ416244.1
Gymnopus rodhallii	2	>MF100981.1 >MF100982.1
Gymnopus semihirtipes	1	>MK607589.1
Gymnopus sequoiae	2	>KY026740.1 >KY026741.1
Gymnopus spongiosus	9	>KY026706.1 >KY026687.1 >KY026686.1 >DQ480113.1 >DQ480093.1 >DQ449993.1 >AF505785.1 >AF505784.1

		>MK532855.1
Gymnopus spp.	79	>KY321575.1 >KY321574.1 >KY321568.1 >KY559328.1 >MK957247.1 >MN483332.1 >MN483329.1 >MN483264.1 >MH589972.1 >MK300725.1 >MK122772.1 >MK122771.1 >MK106030.1 >MH409963.1 >MF061344.1 >MF061342.1 >MG817380.1 >MG817379.1 >KY352520.1 >KY744149.1 >KY026764.1 >DQ450013.1 >DQ450015.1 >KY026660.1 >AY842955.1 >EF062463.1 >KF430218.1 >KX513755.1 >KR673524.1

>KR673453.1

>JX029935.1

>LC198706.1

>DQ480107.1

>DQ480108.1

>DQ480092.1

>DQ480091.1

>DQ480095.1

>DQ480097.1

>DQ480104.1

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>KP133198.1

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>KY026683.1

		>KY026634.1 >KY026619.1 >KY026744.1 >KY026640.1 >KY026632.1 >KY026664.1 >KY026708.1 >KY026707.1 >KY026745.1 >KY026639.1 >KY026648.1 >KY026761.1 >KY026737.1 >KY026736.1 >KY026729.1 >KY026728.1 >KY026760.1 >KY026627.1 >LC327054.1
Gymnopus subcyathiformis	8	>DQ450042.1 >DQ450041.1 >DQ450040.1 >DQ450039.1 >DQ450038.1 >DQ450037.1 >KY404983.1 >KY404982.1
Gymnopus sublaccatus	2	>KY026763.1 >KY026762.1
Gymnopus subnudus	12	>KY026765.1

		>KY026667.1 >KX513748.1 >KX513747.1 >KP004922.1 >KC797153.1 >DQ450018.1 >AY256707.1 >AF505759.1 >MK564556.1 >MK307636.1 >KY777383.1
Gymnopus subpruinosus	5	>AY842952.1 >DQ450025.1 >DQ450027.1 >DQ450026.1 >MK646034.1
Gymnopus subsulphureus	2	>DQ449972.1 >DQ480103.1
Gymnopus talisiae	1	>KT222655.1
Gymnopus termiticola	2	>AY263452.1 >AY263451.1
Gymnopus trabzonensis	1	>KT271754.1
Gymnopus trogioides	1	>AY263428.1
Gymnopus ugandensis	1	>MF100986.1
***Gymnopus* Uncultured**	6	>KU144613.1 >LC013365.1 >LC013344.1 >LC013343.1 >KF639800.1 >FJ197974.1

Gymnopus variicolor	3	>KX926134.1 >KX926133.1 >LT594121.1
Gymnopus villosipes	2	>KJ416255.1 >DQ450058.1
Gymnopus vitellinipes	1	>AY263429.1
Marasmius quercophilus	5	>AF335431.1 >AF335432.1 >AF335433.1 >AF335434.1 >AF519894.1
Rhodocollybia butyracea	1	>GU318386.1
Total number of species	***113***	
Total number of sequences	**788**	

Supplementary Information 5.2

NCBI PrimerBLAST Results FASTA Sequence used as PCR Template: >AY256711.1_G_fusipes_SSU_2. Bold species indicate those that were ordered for testing as target or non-target sequences. Green highlighted species indicate *Gymnopus fusipes* sequences (target), yellow highlighted species indicate non-target species which resulted in a product that was dissimilar to the target (which could be determined using gel electrophoresis of the qPCR product). Red highlighted species indicate a species that is non-target, and a product length close in size to the target *G. fusipes* sequence (which would be difficult to determine using gel electrophoresis).

Pair 1	Name	Sequence (5' - 3')	Template Strand	Length	Start	Stop	Tm	GC%	Self Comp.	Self 3' Comp.
Forward Primer	GF_18SrRNA_qPCR_F1	GCACGTCTTATTTCTAATCC	Plus	20	74	93	51.81	40	4	0
Reverse Primer	GF_18SrRNA_qPCR_R1	GCCCTTTTTTTTCGAAGTC	Minus	19	166	148	53.03	42.11	6	2
Product Length	93									

Results	Database: nr	NCBI Accession	Species	F Sequence Mismatches	R Sequence Mismatches	Product Length
	Organism: N/A	>KX449407.1	*Gymnopus fusipes*	0	0	93
		>KY026727.1	*Gymnopus fusipes*	0	0	93
		>KF897021.1	*Gymnopus fusipes*	0	0	93
		>FR686558.1	*Gymnopus fusipes*	0	0	93
		>AY256711.1	***Gymnopus fusipes***	**0**	**0**	**93**
		>AY256710.1	*Gymnopus fusipes*	0	0	93
		>AF505777.1	*Gymnopus fusipes*	0	0	93
	Database: nr	**NCBI Accession**	**Species**	**F Sequence Mismatches**	**R Sequence Mismatches**	**Product Length**
	Organism: Basidiomycetes	>KX449407.1	*Gymnopus fusipes*	0	0	93
		>KY026727.1	*Gymnopus fusipes*	0	0	93

		>KF897021.1	*Gymnopus fusipes*	0	0	93
		>FR686558.1	*Gymnopus fusipes*	0	0	93
		>AY256711.1	*Gymnopus fusipes*	0	0	93
		>AY256710.1	*Gymnopus fusipes*	0	0	93
		>AF505777.1	*Gymnopus fusipes*	0	0	93
		>HG529617.1	***Melanopsichium pennsylvanicum***	**4**	**4**	**2029**
		>CP039879.1	***Agaricus bisporus***	**5**	**5**	**2408**
		>CP015463.1	*Agaricus bisporus var. bisporus*	5	5	2408
		>CP015476.1	*Agaricus bisporus var. bisporus*	5	5	2403

Pair 2	Name	Sequence (5' - 3')	Template Strand	Length	Start	Stop	Tm	GC%	Self Comp.	Self 3' Comp.
Forward Primer	GF_18SrRNA_qPCR_F2	GACTTCGAAAAAAAAGGGC	Plus	19	148	166	53.03	42.11	6	2
Reverse Primer	GF_18SrRNA_qPCR_R2	CCCAATAGCCATTCCATTCC	Minus	20	220	201	56.2	50	6	0
Product Length	73									

Results	Database: nr	NCBI Accession	Species	F Sequence Mismatches	R Sequence Mismatches	Product Length
	Organism: N/A	>KX449407.1	*Gymnopus fusipes*	0	0	73
		>KY026727.1	*Gymnopus fusipes*	0	0	73
		>KF897021.1	*Gymnopus fusipes*	0	0	73

		>FR686558.1	*Gymnopus fusipes*	0	0	73
		>AY256711.1	*Gymnopus fusipes*	0	0	73
		>AY256710.1	*Gymnopus fusipes*	0	0	73
		>AF505777.1	*Gymnopus fusipes*	0	0	73
		>LR778287.1	*Coregonus sp. 'balchen'*	4	4	2802
	Database: nr	**NCBI Accession**	**Species**	**F Sequence Mismatches**	**R Sequence Mismatches**	
	Organism: Basidiomycetes	>KX449407.1	*Gymnopus fusipes*	0	0	73
		>KY026727.1	*Gymnopus fusipes*	0	0	73
		>KF897021.1	*Gymnopus fusipes*	0	0	73
		>FR686558.1	*Gymnopus fusipes*	0	0	73
		>AY256711.1	*Gymnopus fusipes*	0	0	73
		>AY256710.1	*Gymnopus fusipes*	0	0	73
		>AF505777.1	*Gymnopus fusipes*	0	0	73
		>MH725798.1	*Termitomyces sp.*	3	3	1418
				3	3	1418
		>AE017349.1	*Cryptococcus neoformans var. neoformans*	4	4	3010
		>LT795057.1	*Sporisorium reilianum f. sp. reilianum*	4	4	352
		>XM_002910848.1	***Coprinopsis cinerea okayama***	**4**	**4**	**1116**

		>CP047902.1	*Cryptococcus neoformans var. grubii*	4	5	2915
		>CP048087.1	*Cryptococcus neoformans var. grubii*	4	5	2915
		>CP022321.1	*Cryptococcus neoformans var. grubii*	4	5	2915
		>XM_007409322.1	***Melampsora larici-populina***	**5**	**4**	**1783**
		>CP003820.1	*Cryptococcus neoformans var. grubii*	4	5	2915
		>XM_012191052.1	*Cryptococcus neoformans var. grubii*	4	5	2509
		>FQ311463.1	*Sporisorium reilianum*	5	4	348
		>AE017345.1	***Cryptococcus neoformans var. neoformans***	**4**	**5**	**2667**
		>XM_007005696.1	*Tremella mesenterica*	5	5	740

Pair 3	Name	Sequence (5' - 3')	Template Strand	Length	Start	Stop	Tm	GC%	Self Comp.	Self 3' Comp.
Forward Primer	GF_18SrRNA_qPCR_F2	GACTTCGAAAAAAAAGGGC	Plus	19	148	166	53.03	42.11	6	2
Reverse Primer	GF_18SrRNA_qPCR_R3	GAAGTCCCCAATAGCCATTC	Minus	20	226	207	55.86	50	6	4
Product Length	79									

Results	Database: nr	NCBI Accession	Species	F Sequence Mismatches	R Sequence Mismatches	Product Length
	Organism: N/A	>KX449407.1	*Gymnopus fusipes*	0	0	79
		>KY026727.1	*Gymnopus fusipes*	0	0	79
		>KF897021.1	*Gymnopus fusipes*	0	0	79

		>FR686558.1	*Gymnopus fusipes*	0	0	79
		>AY256711.1	*Gymnopus fusipes*	0	0	79
		>AY256710.1	*Gymnopus fusipes*	0	0	79
		>AF505777.1	*Gymnopus fusipes*	0	0	79
	Database: nr	**NCBI Accession**	**Species**	**F Sequence Mismatches**	**R Sequence Mismatches**	**Product Length**
	Organism: Basidiomycetes	>KX449407.1	*Gymnopus fusipes*	0	0	79
		>KY026727.1	*Gymnopus fusipes*	0	0	79
		>KF897021.1	*Gymnopus fusipes*	0	0	79
		>FR686558.1	*Gymnopus fusipes*	0	0	79
		>AY256711.1	*Gymnopus fusipes*	0	0	79
		>AY256710.1	*Gymnopus fusipes*	0	0	79
		>AF505777.1	*Gymnopus fusipes*	0	0	79
		>MH725798.1	***Termitomyces sp.***	**3**	**0**	**1418**
				3	**0**	**1418**
		>AE017349.1	*Cryptococcus neoformans var. neoformans*	4	4	3010
		>LT795057.1	*Sporisorium reilianum f. sp. reilianum*	4	4	352
		>CP046434.1	*Malassezia globosa*	5	4	845
		>FQ311463.1	*Sporisorium reilianum*	5	4	348
		>CP053620.1	***Apiotrichum mycotoxinovorans***	**5**	**5**	**1813**

Pair 4	Name	Sequence (5' - 3')	Template Strand	Length	Start	Stop	Tm	GC%	Self Comp.	Self 3' Comp.
Forward Primer	GF_18SrRNA_qPCR_F3	GGTTTTCATTAACTTTCTGAAGC	Plus	23	422	444	54.75	34.78	7	3
Reverse Primer	GF_18SrRNA_qPCR_R4	CTTTCCAAAGCGTAGATAAC	Minus	20	559	540	52.04	40	6	2
Product Length	138									

Results	Database: nr	NCBI Accession	Species	F Sequence Mismatches	R Sequence Mismatches	Product Length
	Organism: N/A	>KX449407.1	*Gymnopus fusipes*	0	0	138
		>KY026727.1	*Gymnopus fusipes*	0	0	138
		>KF897021.1	*Gymnopus fusipes*	0	0	138
		>FR686558.1	*Gymnopus fusipes*	0	0	138
		>AY256711.1	*Gymnopus fusipes*	0	0	138
		>AY256710.1	*Gymnopus fusipes*	0	0	138
		>AF505777.1	*Gymnopus fusipes*	0	0	138
		>MT571521.1	*Gymnopus sp.*	3	2	137
		>KY026759.1	*Gymnopus sp.*	3	2	137
		>AY969409.1	*Uncultured Basidiomycota*	3	2	137
	Database: nr	**NCBI Accession**	**Species**	**F Sequence Mismatches**	**R Sequence Mismatches**	**Product Length**
	Organism: Basidiomycetes	>KX449407.1	*Gymnopus fusipes*	0	0	138
		>KY026727.1	*Gymnopus fusipes*	0	0	138

		>KF897021.1	*Gymnopus fusipes*	0	0	138
		>FR686558.1	*Gymnopus fusipes*	0	0	138
		>AY256711.1	*Gymnopus fusipes*	0	0	138
		>AY256710.1	*Gymnopus fusipes*	0	0	138
		>AF505777.1	*Gymnopus fusipes*	0	0	138
		>LC505338.1	*Gymnopus sp.*	2	3	137
		>LC505333.1	*Gymnopus sp*	2	3	137
		>LC505325.1	*Gymnopus sp*	2	3	137
		>LC505290.1	*Gymnopus sp*	2	3	137
		>LC505135.1	*Gymnopus sp*	2	3	137
		>AB859204.1	*Agaricales sp.*	2	3	137
		>MT571521.1	*Gymnopus sp.*	3	2	137
		>KY026759.1	*Gymnopus sp.*	3	2	137
		>AY969409.1	*Uncultured Basidiomycota*	3	2	137
		>MF100972.1	*Setulipes afibulatus*	1	5	149
		>MN992160.1	*Gymnopus androsaceus*	3	3	137
		>MH857175.1	*Gymnopus androsaceus*	3	3	137
		>MH857174.1	*Gymnopus androsaceus*	3	3	137
		>MH857173.1	*Gymnopus androsaceus*	3	3	138

		>MH856650.1	*Gymnopus androsaceus*	3	3	138
		>MH856517.1	*Gymnopus androsaceus*	3	3	137
		>KY352522.1	*Gymnopus androsaceus*	3	3	137
		>KY696772.1	*Gymnopus androsaceus*	3	3	137
		>KY026760.1	*Gymnopus sp.*	3	3	137
		>KY026749.1	*Gymnopus androsaceus*	3	3	137
		>KY026748.1	*Gymnopus androsaceus*	3	3	137
		>KY026747.1	*Gymnopus androsaceus*	3	3	137
		>KY026744.1	*Gymnopus sp.*	3	3	138
		>KY026663.1	*Gymnopus androsaceus*	3	3	138
		>KY026640.1	*Gymnopus sp.*	3	3	138
		>KY026632.1	*Gymnopus sp.*	3	3	138
		>KY026619.1	*Gymnopus sp.*	3	3	138
		>AB859205.1	*Agaricales sp.*	3	3	137
		>JN943605.1	*Marasmius androsaceus*	3	3	137
		>JN021062.1	*Marasmius androsaceus*	3	3	137
		>FR717227.1	*Marasmius androsaceus*	3	3	137
		>GU234007.1	*Marasmius androsaceus*	3	3	137
		>AM902023.1	*Uncultured basidiomycete*	3	3	137

		>DQ444313.1	*Marasmius androsaceus*	3	3	137
		>DQ444314.1	*Marasmius androsaceus*	3	3	137
		>DQ444312.1	*Marasmius androsaceus*	3	3	137
		>DQ444311.1	*Marasmius androsaceus*	3	3	138
		>AF519895.1	*Marasmius quercophilus*	3	3	137
		>AF519893.1	*Marasmius androsaceus*	3	3	137
		>AF335437.1	*Marasmius quercophilus*	3	3	137
		>AF335435.1	*Marasmius quercophilus*	3	3	137
		>AB509591.1	*Micromphale sp.*	4	2	138
		>KY026745.1	*Gymnopus sp.*	3	4	137
		>AF505779.1	*Gymnopus impudicus*	3	4	139
		>AF335438.1	*Marasmius quercophilus*	3	4	137
		>AF335436.1	*Marasmius quercophilus*	3	4	137
		>KT222659.1	*Gymnopus atlanticus*	5	2	137
		>NR_152911.1	*Gymnopus atlanticus*	5	2	136
		>KR673444.1	*Marasmius sp.*	3	4	133
		>MF100988.1	*Gymnopus melanopus*	4	3	133
		>KY591448.1	*Gymnopus melanopus*	4	3	133
		>KY026726.1	*Gymnopus pinophilus*	4	3	134

		>KY026683.1	*Gymnopus sp.*	4	3	134
		>KY026660.1	*Gymnopus sp.*	4	3	134
		>KY026639.1	*Gymnopus sp.*	4	3	134
		>KY026634.1	*Gymnopus sp.*	4	3	134
		>KU529307.1	*Gymnopus melanopus*	4	3	133
		>NR_137539.1	*Gymnopus melanopus*	4	3	133
		>KR348866.1	*Gymnopus melanopus*	4	3	133
		>KM896875.1	*Gymnopus melanopus*	4	3	133
		>AY263442.1	*Gymnopus melanopus*	4	3	133
		>AY263425.1	*Gymnopus melanopus*	4	3	133
		>KT205401.1	*Rhodocollybia pandipes*	3	5	136
		>KM975435.1	*Rhodocollybia incarnata*	3	5	136
		>KM975429.1	*Rhodocollybia incarnata*	3	5	136
		>KM975425.1	*Rhodocollybia incarnata*	3	5	136
		>KM975405.1	*Rhodocollybia incarnata*	3	5	136
		>KM975398.1	*Rhodocollybia incarnata*	3	5	136
		>FJ475743.1	*Uncultured Tricholomataceae*	3	5	135
		>DQ444318.1	*Rhodocollybia sp.*	3	5	135
		>AY313295.1	*Rhodocollybia pandipes*	3	5	136

		>AY313287.1	*Rhodocollybia sp.*	3	5	135
		>AY456079.1	*Uncultured basidiomycete*	3	5	136
		>AF505752.1	*Rhodocollybia pandipes*	3	5	136
		>MT735148.1	*Connopus acervatus*	3	5	139
		>KY366500.1	*Rhodocollybia pandipes*	3	5	137
		>HQ604802.1	*Gymnopus confluens*	3	5	140
		>GU325812.1	*Connopus acervatus*	3	5	138
		>GU325811.1	*Connopus acervatus*	3	5	138
		>GU318400.1	*Gymnopus acervatus*	3	5	140
		>GU318398.1	*Gymnopus acervatus*	3	5	140
		>GU318397.1	*Gymnopus acervatus*	3	5	140
		>GU318396.1	*Gymnopus acervatus*	3	5	140
		>GU318394.1	*Gymnopus acervatus*	3	5	140
		>GU318392.1	*Gymnopus acervatus*	3	5	138
		>GU318391.1	*Gymnopus acervatus*	3	5	138
		>GU318389.1	*Gymnopus acervatus*	3	5	138
		>GU318388.1	*Gymnopus acervatus*	3	5	138
		>GU318387.1	*Gymnopus acervatus*	3	5	138
		>GU318385.1	*Gymnopus acervatus*	3	5	138

		>GU318384.1	*Gymnopus acervatus*	3	5	138
		>GU318383.1	*Gymnopus acervatus*	3	5	138
		>GU318382.1	*Gymnopus acervatus*	3	5	140
		>GU318381.1	*Gymnopus acervatus*	3	5	138
		>GU318380.1	*Gymnopus acervatus*	3	5	140
		>GU318379.1	*Gymnopus acervatus*	3	5	139
		>GU318378.1	*Gymnopus acervatus*	3	5	138
		>GU318377.1	*Gymnopus acervatus*	3	5	138
		>GU318376.1	*Gymnopus acervatus*	3	5	138
		>GU318375.1	*Gymnopus acervatus*	3	5	138
		>GU318374.1	*Gymnopus acervatus*	3	5	138
		>GU318373.1	*Gymnopus acervatus*	3	5	138
		>DQ444310.1	*Gymnopus acervatus*	3	5	140
		>AY313294.1	*Rhodocollybia pandipes*	3	5	136
		>AY313288.1	*Rhodocollybia cf. pandipes*	3	5	137
		>LC505310.1	*Tricholomataceae sp.*	4	4	133
		>MT303151.1	*Marasmius pallidocephalus*	4	4	133
		>MN992164.1	*Gymnopus glabrocystidiatus*	4	4	133
		>MN906136.1	*Gymnopus foliiphilus*	4	4	133

		>MK569406.1	*Dennisiomyces lanzonii*	4	4	134
		>MK214417.1	*Gymnopus glabrocystidiatus*	4	4	133
		>MK268237.1	*Marasmius sp.*	4	4	133
		>MK268235.1	*Gymnopus glabrocystidiatus*	4	4	133
		>MH560579.1	*Gymnopus nidus-avis*	4	4	133
		>MH560578.1	*Gymnopus nidus-avis*	4	4	133
		>MH560577.1	*Gymnopus nidus-avis*	4	4	133
		>MH560576.1	*Gymnopus nidus-avis*	4	4	133
		>MH560575.1	*Gymnopus nidus-avis*	4	4	133
		>MH856221.1	*Micromphale perforans*	4	4	133
		>LS451336.1	*Uncultured Rhodocollybia*	4	4	133
		>MF161164.1	*Gymnopus foliiphilus*	4	4	133
		>NR_152899.1	*Pseudomarasmius glabrocystidiatus*	4	4	133
		>KY321570.1	*Gymnopus sp.*	4	4	135
		>KY321569.1	*Gymnopus sp.*	4	4	135
		>KY321568.1	*Gymnopus sp.*	4	4	135
		>MH016872.1	*Micromphale brevipes*	4	4	133
		>MK020094.1	*Marasmius crinis-equi*	4	4	133
		>KY026763.1	*Gymnopus sublaccatus*	4	4	133

		>KY026762.1	*Gymnopus sublaccatus*	4	4	133
		>KY026758.1	*Gymnopus sp.*	4	4	137
		>KY026753.1	*Micromphale brevipes*	4	4	133
		>KY026746.1	*Gymnopus sp.*	4	4	133
		>KY026743.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026742.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026741.1	*Gymnopus sequoiae*	4	4	133
		>KY026740.1	*Gymnopus sequoiae*	4	4	133
		>KY026735.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026734.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026733.1	*Micromphale brevipes*	4	4	133
		>KY026732.1	*Micromphale brevipes*	4	4	133
		>KY026723.1	*Micromphale brevipes*	4	4	133
		>KY026721.1	*Gymnopus foliiphilus*	4	4	133
		>KY026720.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026719.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026718.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026717.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026715.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133

		>KY026712.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026711.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026710.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026703.1	*Gymnopus foliiphilus*	4	4	133
		>KY026695.1	*Gymnopus pinophilus*	4	4	134
		>KY026694.1	*Gymnopus foliiphilus*	4	4	133
		>KY026693.1	*Gymnopus sp.*	4	4	134
		>KY026692.1	*Gymnopus sp.*	4	4	134
		>KY026691.1	*Gymnopus sp.*	4	4	133
		>KY026690.1	*Gymnopus foliiphilus*	4	4	133
		>KY026685.1	*Gymnopus sp.*	4	4	133
		>KY026684.1	*Gymnopus sp.*	4	4	133
		>KY026681.1	*Gymnopus foliiphilus*	4	4	133
		>KY026675.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026673.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026672.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026671.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KY026670.1	*Gymnopus perforans subsp. transatlanticus*	4	4	134
		>KY026669.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133

		>KY026664.1	*Gymnopus sp.*	4	4	133
		>KY026662.1	*Gymnopus perforans subsp. perforans*	4	4	133
		>KY026659.1	*Gymnopus perforans subsp. perforans*	4	4	133
		>KY026652.1	*Gymnopus foliiphilus*	4	4	133
		>KY026651.1	*Gymnopus foliiphilus*	4	4	133
		>KY026650.1	*Gymnopus foliiphilus*	4	4	133
		>KY026646.1	*Micromphale brevipes*	4	4	133
		>KY026641.1	*Gymnopus perforans subsp. perforans*	4	4	134
		>KY026636.1	*Gymnopus sp.*	4	4	133
		>KY026635.1	*Gymnopus sp.*	4	4	133
		>KY026633.1	*Gymnopus foliiphilus*	4	4	133
		>KY026631.1	*Gymnopus foliiphilus*	4	4	133
		>KY026630.1	*Gymnopus foliiphilus*	4	4	133
		>KY026626.1	*Gymnopus foliiphilus*	4	4	133
		>KY026625.1	*Gymnopus perforans*	4	4	133
		>KY026624.1	*Gymnopus perforans*	4	4	133
		>KY026623.1	*Gymnopus perforans*	4	4	133
		>KY026620.1	*Gymnopus foliiphilus*	4	4	133
		>KY352649.1	*Marasmius sp.*	4	4	134

		>LC014889.1	*Tricholomataceae sp.*	4	4	133
		>KX184795.1	*Marasmiellus sp.*	4	4	134
		>LN909523.1	*Uncultured Gymnopus*	4	4	133
		>KJ831840.1	*Agaricales sp.*	4	4	135
		>KF251072.1	*Gymnopus sp.*	4	4	133
		>JX029948.1	*Micromphale sp.*	4	4	133
		>FJ596763.1	*Tricholomataceae aff. Rhodocollybia*	4	4	133
		>FJ596762.1	*Tricholomataceae aff. Rhodocollybia*	4	4	133
		>AM901982.1	*Uncultured basidiomycete*	4	4	133
		>KY391875.1	*Gymnopus sp.*	5	3	133
		>KY026764.1	*Gymnopus sp.*	5	3	135
		>KY026708.1	*Gymnopus sp.*	5	3	135
		>KY026707.1	*Gymnopus sp.*	5	3	135
		>KY026668.1	*Gymnopus perforans subsp. transatlanticus*	4	4	133
		>KP133198.1	*Gymnopus sp.*	5	3	133
		>AF505766.1	*Gymnopus dichrous*	5	3	135
		>MK607574.1	*Rhodocollybia sp.*	4	5	135
		>MN992636.1	*Rhodocollybia butyracea*	4	5	136
		>MN992504.1	*Rhodocollybia butyracea*	4	5	137

		>MT004785.1	*Rhodocollybia butyracea*	4	5	137
		>MN660937.1	*Rhodocollybia butyracea f. asema*	4	5	137
		>MN660914.1	*Rhodocollybia butyracea f. asema*	4	5	137
		>MN660767.1	*Rhodocollybia butyracea f. asema*	4	5	137
		>MN660716.1	*Rhodocollybia butyracea f. asema*	4	5	137
		>MN258681.1	*Rhodocollybia butyracea*	4	5	137
		>MN258680.1	*Rhodocollybia butyracea*	4	5	137
		>MK627504.1	*Rhodocollybia sp.*	4	5	137
		>MK412391.1	*Rhodocollybia butyracea*	4	5	137
		>MK028510.1	*Rhodocollybia cf. butyracea*	4	5	137
		>MH220536.1	*Rhodocollybia utrorensis*	4	5	138
		>MH930185.1	*Rhodocollybia butyracea*	4	5	136
		>MH255528.1	*Rhodocollybia sp.*	4	5	136
		>MF752705.2	*Rhodocollybia butyracea*	4	5	136
		>KX449468.1	*Rhodocollybia butyracea*	4	5	137
		>KY352518.1	*Collybia butyracea*	4	5	137
		>KY777392.1	*Rhodocollybia butyracea*	4	5	137
		>LT716056.1	*Rhodocollybia butyracea*	4	5	136
		>KY026757.1	*Rhodocollybia butyracea f. asema*	4	5	137

		>KY026754.1	*Rhodocollybia butyracea f. asema*	4	5	137
		>KY026716.1	*Rhodocollybia butyracea*	4	5	137
		>KY026714.1	*Rhodocollybia butyracea*	4	5	137
		>KY026713.1	*Rhodocollybia butyracea*	4	5	137
		>KY026696.1	*Gymnopus aff. dichrous*	5	4	134
		>KT875093.1	*Rhodocollybia butyracea*	4	5	137
		>KT875092.1	*Rhodocollybia butyracea*	4	5	137
		>KT205400.1	*Rhodocollybia olivaceo-grisea*	4	5	136
		>KT205399.1	*Rhodocollybia olivaceo-grisea*	4	5	136
		>LN714597.1	*Rhodocollybia sp.*	4	5	137
		>JF313690.1	*Gymnopus dichrous*	5	4	134
		>JF313685.1	*Gymnopus dichrous*	5	4	134
		>JF313682.1	*Gymnopus dichrous*	5	4	134
		>JF313679.1	*Gymnopus dichrous*	5	4	134
		>JF313678.1	*Gymnopus dichrous*	5	4	134
		>JF313677.1	*Gymnopus dichrous*	5	4	134
		>JF313671.1	*Gymnopus dichrous*	5	4	134
		>GU328571.1	*Uncultured Basidiomycota*	4	5	137
		>GU328560.1	*Uncultured Basidiomycota*	4	5	137

		>EU486454.1	*Rhodocollybia butyracea*	4	5	137
		>DQ450007.1	*Gymnopus dichrous*	5	4	134
		>DQ444317.1	*Rhodocollybia butyracea*	4	5	136
		>AY781251.1	*Collybia butyracea*	4	5	137
		>AY805607.1	*Collybia butyracea*	4	5	137
		>AF505751.1	*Rhodocollybia butyracea*	4	5	137
		>AF505750.1	*Rhodocollybia butyracea*	4	5	137
		>AY313293.1	*Rhodocollybia butyracea*	4	5	136
		>AY313292.1	*Rhodocollybia butyracea*	4	5	136
		>AY313291.1	*Rhodocollybia butyracea*	4	5	137
		>AY313290.1	*Rhodocollybia butyracea*	4	5	137
		>AY313289.1	*Rhodocollybia cf. butyracea*	4	5	137
		>AY256702.1	*Gymnopus dichrous*	5	4	134
		>AY256689.1	*Rhodocollybia cf. butyracea*	4	5	137
		>MK268236.1	*Pseudomarasmius efibulatus*	5	4	134
		>MK268234.1	*Pseudomarasmius efibulatus*	5	4	134
		>JF313681.1	*Gymnopus dichrous*	5	4	134
		>JF313689.1	*Gymnopus dichrous*	5	5	134
		>JF313686.1	*Gymnopus dichrous*	5	5	134

		>JF313683.1	*Gymnopus dichrous*	5	5	134
		>JF313680.1	*Gymnopus dichrous*	5	5	134
		>MT114699.1	*Gymnopus dysodes*	5	5	137
		>MT114698.1	*Gymnopus dysodes*	5	5	137
		>MT939447.1	*Gymnopus dysodes*	5	5	137
		>LC373249.1	*Rhodocollybia butyracea*	5	5	137
		>MF773622.1	*Gymnopus dysodes*	5	5	137
		>KY744145.1	*Gymnopus dysodes*	5	5	137
		>KY026666.1	*Gymnopus dysodes*	5	5	137
		>KP336693.1	*Gymnopus dysodes*	5	5	137
		>KR673491.1	*Gymnopus dysodes*	5	5	137
		>KJ609163.1	*Rhodocollybia butyracea*	5	5	137
		>GU318386.1	*Rhodocollybia butyracea*	5	5	137
		>DQ449987.1	*Gymnopus dysodes*	5	5	138
		>AF505778.1	*Gymnopus dysodes*	5	5	137

Pair 5	Name	Sequence (5' - 3')	Template Strand	Length	Start	Stop	Tm	GC%	Self Comp.	Self 3' Comp.
Forward Primer	GF_18SrRNA_qPCR_F4	GTTATCTACGCTTTGGAAAGTC	Plus	22	540	561	55.23	40.91	6	4
Reverse Primer	GF_18SrRNA_qPCR_R5	GACAGCTAGAAAGCAGAACTTT	Minus	22	651	630	56.65	40.91	4	3
Product Length	112									

Results	Database: nr	NCBI Accession	Species	F Sequence Mismatches	R Sequence Mismatches	Product Length
	Organism: N/A	>KX449407.1	*Gymnopus fusipes*	0	0	112
		>KY026727.1	*Gymnopus fusipes*	0	0	112
		>KF897021.1	*Gymnopus fusipes*	0	0	112
		>FR686558.1	*Gymnopus fusipes*	0	0	112
		>AY256711.1	*Gymnopus fusipes*	0	0	112
		>AY256710.1	*Gymnopus fusipes*	0	0	112
		>AF505777.1	*Gymnopus fusipes*	0	0	112
		>MH409986.1	*Fungal sp.*	2	2	115
		>MH409963.1	*Gymnopus sp.*	2	2	115
		>KP877447.1	*Micromphale foetidum*	2	2	114
		>AB509591.1	*Micromphale sp.*	2	2	113
		>MT023348.1	*Gymnopus sp.*	2	3	133
		>MT023347.1	*Gymnopus sp.*	2	3	133
		>MT023346.1	*Gymnopus sp.*	2	3	133

		>MT023345.1	*Gymnopus sp.*	2	3	133
		>MT023344.1	*Gymnopus sp.*	2	3	133
		>MK028459.1	*Gymnopus foetidus*	2	3	141
		>MH856220.1	*Micromphale foetidum*	2	3	141
		>MG748573.1	*Gymnopus foetidus*	2	3	142
		>KY026731.1	*Gymnopus foetidus*	2	3	141
		>KY026730.1	*Gymnopus foetidus*	2	3	141
		>KY026682.1	*Gymnopus foetidus*	2	3	142
		>KY462299.1	*Gymnopus sp.*	2	3	139
		>KJ416259.1	*Micromphale foetidum*	2	3	141
		>KJ416258.1	*Micromphale foetidum*	2	3	142
		>DQ449988.1	*Caripia montagnei*	2	3	146
		>AY263447.1	*Gymnopus salakensis*	2	3	130
		>MN992160.1	*Gymnopus androsaceus*	3	3	111
		>MH857175.1	*Gymnopus androsaceus*	3	3	112
		>MH857173.1	*Gymnopus androsaceus*	3	3	112
		>MH856650.1	*Gymnopus androsaceus*	3	3	112
		>MH856517.1	*Gymnopus androsaceus*	3	3	112
		>MG916575.1	*Fungal sp.*	3	3	112

		>KY696772.1	*Gymnopus androsaceus*	3	3	112
		>KX401917.1	*Uncultured Agaricales*	3	3	112
		>KY026760.1	*Gymnopus sp.*	3	3	112
		>KY026749.1	*Gymnopus androsaceus*	3	3	111
		>KY026748.1	*Gymnopus androsaceus*	3	3	111
		>KY026747.1	*Gymnopus androsaceus*	3	3	112
		>KY026663.1	*Gymnopus androsaceus*	3	3	112
		>KT222658.1	*Gymnopus talisiae*	3	3	143
		>KT222657.1	*Gymnopus talisiae*	3	3	143
		>KT222656.1	*Gymnopus talisiae*	3	3	143
		>NR_152912.1	*Gymnopus talisiae*	3	3	143
		>KM494194.1	*Uncultured fungus*	3	3	112
		>KJ416262.1	*Gymnopus ceraceicola*	3	3	140
		>KJ416261.1	*Gymnopus ceraceicola*	3	3	140
		>KJ416260.1	*Gymnopus ceraceicola*	3	3	140
		>NR_137112.1	*Gymnopus ceraceicola*	3	3	140
		>KC248392.1	*Gymnopus ceraceicola*	3	3	140
		>KC248400.1	*Gymnopus ceraceicola*	3	3	140
		>KC248389.1	*Gymnopus ceraceicola*	3	3	140

		>KC248395.1	*Gymnopus ceraceicola*	3	3	140
		>KC248394.1	*Gymnopus ceraceicola*	3	3	140
		>KC248391.1	*Gymnopus ceraceicola*	3	3	140
		>JX136413.1	*Uncultured fungus*	3	3	112
		>JX136054.1	*Uncultured fungus*	3	3	112
		>JN032496.1	*Uncultured fungus*	3	3	113
		>JN943605.1	*Marasmius androsaceus*	3	3	112
		>HQ873376.1	*Uncultured fungus*	3	3	112
		>FR717227.1	*Marasmius androsaceus*	3	3	112
		>GU234007.1	*Marasmius androsaceus*	3	3	112
		>AM902023.1	*Uncultured basidiomycete*	3	3	112
		>DQ480110.1	*Gymnopus aff. dysodes*	3	3	143
		>DQ444313.1	*Marasmius androsaceus*	3	3	112
		>DQ444314.1	*Marasmius androsaceus*	3	3	112
		>DQ444312.1	*Marasmius androsaceus*	3	3	112
		>AM260887.1	*Uncultured fungus*	3	3	112
		>AF519895.1	*Marasmius quercophilus*	3	3	112
		>AF519893.1	*Marasmius androsaceus*	3	3	112
		>AF335437.1	*Marasmius quercophilus*	3	3	112

		>AF335435.1	*Marasmius quercophilus*	3	3	112
	Database: nr	**NCBI Accession**	**Species**	**F Sequence Mismatches**	**R Sequence Mismatches**	**Product Length**
	Organism: Basidiomycetes	>KX449407.1	*Gymnopus fusipes*	0	0	112
		>KY026727.1	*Gymnopus fusipes*	0	0	112
		>KF897021.1	*Gymnopus fusipes*	0	0	112
		>FR686558.1	*Gymnopus fusipes*	0	0	112
		>AY256711.1	*Gymnopus fusipes*	0	0	112
		>AY256710.1	*Gymnopus fusipes*	0	0	112
		>AF505777.1	*Gymnopus fusipes*	0	0	112
		>MH409963.1	*Gymnopus sp.*	2	2	115
		>KP877447.1	*Micromphale foetidum*	2	2	114
		>AB509591.1	*Micromphale sp.*	2	2	113
		>MT023348.1	*Gymnopus sp.*	2	3	133
		>MT023347.1	*Gymnopus sp.*	2	3	133
		>MT023346.1	*Gymnopus sp.*	2	3	133
		>MT023345.1	*Gymnopus sp.*	2	3	133
		>MT023344.1	*Gymnopus sp.*	2	3	133
		>MK028459.1	*Gymnopus foetidus*	2	3	141
		>MH856220.1	*Micromphale foetidum*	2	3	141

		>MG748573.1	*Gymnopus foetidus*	2	3	142
		>KY026731.1	*Gymnopus foetidus*	2	3	141
		>KY026730.1	*Gymnopus foetidus*	2	3	141
		>KY026682.1	*Gymnopus foetidus*	2	3	142
		>KY462299.1	*Gymnopus sp.*	2	3	139
		>KJ416259.1	*Micromphale foetidum*	2	3	141
		>KJ416258.1	*Micromphale foetidum*	2	3	142
		>DQ449988.1	*Caripia montagnei*	2	3	146
		>AY263447.1	*Gymnopus salakensis*	2	3	130
		>KY026709.1	*Gymnopus foetidus*	2	4	142
		>MN992160.1	*Gymnopus androsaceus*	3	3	111
		>MH857175.1	*Gymnopus androsaceus*	3	3	112
		>MH857173.1	*Gymnopus androsaceus*	3	3	112
		>MH856650.1	*Gymnopus androsaceus*	3	3	112
		>MH856517.1	*Gymnopus androsaceus*	3	3	112
		>KY696772.1	*Gymnopus androsaceus*	3	3	112
		>KX401917.1	*Uncultured Agaricales*	3	3	112
		>KY026760.1	*Gymnopus sp.*	3	3	112
		>KY026749.1	*Gymnopus androsaceus*	3	3	111

		>KY026748.1	*Gymnopus androsaceus*	3	3	111
		>KY026747.1	*Gymnopus androsaceus*	3	3	112
		>KY026663.1	*Gymnopus androsaceus*	3	3	112
		>KT222658.1	*Gymnopus talisiae*	3	3	143
		>KT222657.1	*Gymnopus talisiae*	3	3	143
		>KT222656.1	*Gymnopus talisiae*	3	3	143
		>NR_152912.1	*Gymnopus talisiae*	3	3	143
		>KJ416262.1	*Gymnopus ceraceicola*	3	3	140
		>KJ416261.1	*Gymnopus ceraceicola*	3	3	140
		>KJ416260.1	*Gymnopus ceraceicola*	3	3	140
		>NR_137112.1	*Gymnopus ceraceicola*	3	3	140
		>KC248392.1	*Gymnopus ceraceicola*	3	3	140
		>KC248400.1	*Gymnopus ceraceicola*	3	3	140
		>KC248389.1	*Gymnopus ceraceicola*	3	3	140
		>KC248395.1	*Gymnopus ceraceicola*	3	3	140
		>KC248394.1	*Gymnopus ceraceicola*	3	3	140
		>KC248391.1	*Gymnopus ceraceicola*	3	3	140
		>JN943605.1	*Marasmius androsaceus*	3	3	112
		>FR717227.1	*Marasmius androsaceus*	3	3	112

		>GU234007.1	*Marasmius androsaceus*	3	3	112
		>AM902023.1	*Uncultured basidiomycete*	3	3	112
		>DQ480110.1	*Gymnopus aff. dysodes*	3	3	143
		>DQ444313.1	*Marasmius androsaceus*	3	3	112
		>DQ444314.1	*Marasmius androsaceus*	3	3	112
		>DQ444312.1	*Marasmius androsaceus*	3	3	112
		>AF519895.1	*Marasmius quercophilus*	3	3	112
		>AF519893.1	*Marasmius androsaceus*	3	3	112
		>AF335437.1	*Marasmius quercophilus*	3	3	112
		>AF335435.1	*Marasmius quercophilus*	3	3	112
		>MH857174.1	*Gymnopus androsaceus*	3	4	112
		>KY026758.1	*Gymnopus sp.*	4	3	111
		>KY026750.1	*Gymnopus androsaceus*	4	3	111
		>KY026745.1	*Gymnopus sp.*	4	3	111
		>KY026648.1	*Gymnopus sp.*	4	3	111
		>KX499211.1	*Uncultured Corticiales*	3	4	143
		>AF335438.1	*Marasmius quercophilus*	4	3	112
		>AF335436.1	*Marasmius quercophilus*	4	3	112
		>JN021062.1	*Marasmius androsaceus*	3	5	111

		>DQ480109.1	*Gymnopus impudicus*	4	4	145
		>AF505779.1	*Gymnopus impudicus*	4	4	145
		>LT980577.1	*Uncultured Cortinarius*	5	5	48

Appendices