Molecular Taxonomy and Population Genetics of *Lophodermium* on *Pinus sylvestris* in Scotland

Sabrina N. A. Reignoux

PhD Thesis
University of Edinburgh
2010

Declaration

I, Sabrina N. A. Reignoux, hereby declare that this thesis represents entirely my own work unless otherwise clearly acknowledged. This thesis has not been previously presented for any other degree or professional qualification and was conducted between September 2006 and August 2010.

Sabrina N. A. Reignoux

Acknowledgements

I am ever so grateful to both of my supervisors Richard Ennos and Sarah Green for their excellent support throughout my PhD. I would also like to thank Elizabeth Aitken, Graham Stone, James Nicholls and Martin Jones for their contribution to Chapter 2, Roberta Bergero for her generous help across a wide range of issues, Fiona Oliver for her help with the development of ISSR genotyping, Grace McAskill for valued assistance with storage of fungal isolates and help with the growth rate experiment, Steven Hendry, Heather Steel and Bridget Laue for their illuminating discussions.

I would also like to thank Darrell and my family for their emotional support.

Abstract

Endophytes are organisms which infect plants asymptomatically for at least part of their life cycle. The vast majority of endophytes are ascomycete fungi. They show high species diversity and are often closely related to economically important pathogens. There is some evidence that endophytes protect their host from pathogen and herbivores. Little is known about the evolutionary relationship between potentially mutualistic endophytes and related pathogens and how these two groups differ in population biology and genetics. The *Lophodermium/Pinus sylvestris* endophyte/host system is ideal for investigating these questions since native pine forests harbour closely related *Lophodermium* species which range from endophytic (*L. pinastri, L. conigenum*) to parasitic (*L. seditiosum*). Current taxonomy recognises only one species, *L. pinastri,* fruiting on senescent needles. However there are recent indications that this taxon includes cryptic species. In this thesis molecular approaches were used to clarify the taxonomy of *Lophodermium* on Scots pine. Genealogical Concordance Phylogenetic Species Recognition based on ACTIN and ITS sequences revealed three cryptic species within *L. pinastri* two of which were widely distributed. The existence of these taxa was supported by AFLP genetic marker analysis and differences in culture growth rate. Analysis of Inter-SSR markers revealed that *L. seditiosum* and *L. conigenum* are both out-crossing species and that unlike one of the cryptic *L. pinastri* species, their populations are genetically differentiated. Implication of the discovery of cryptic *L. pinastri* species and opportunities offered by the clarification of the taxonomy of *Lophodermium* on Scots pine are discussed.

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Chapter 1: Introduction

1.1 Introduction: The Diversity of Fungi

The fungal kingdom includes economically important plant pathogens, mycorrhizal fungi, animal pathogens and insect associated fungi. However since all plants grow in company with fungi, and relationships between fungi and plants are much more common than associations of fungi with other groups, it is reasonable to make estimates of fungal diversity on the basis of the plant-fungal ratio. Hawksworth (1991) initially proposed that every higher plant species is associated with approximately six fungal species and on this assumption estimated fungal diversity to amount to 1.5 million taxa. This estimation was deliberately conservative and accounted for host specificity.

Within ten years of his original paper Hawksworth (2001) felt the need to revise his estimate of the number of fungal taxa to 5.1 million for two main reasons. Advances in molecular biology had provided the opportunity for

mycologists to define species more accurately by using methods such as Genealogical Concordance Phylogenetic Species Recognition which uses phylogenetic concordance between different loci in order to define species (Taylor *et al.*, 2000). Using these techniques, species that would otherwise have been cryptic with almost indistinguishable morphology had been recognised in many different genera. Cryptic species groups were seen to be ubiquitous. Moreover, a recent interest in the more obscure plant associations, such as the endophytes living asymptomatically within the plant, had led to the conclusion that the 1:6 ratio adopted by Hawksworth (1991) was likely to be underestimating the real diversity of fungi (Arnold, 2007; Arnold *et al.*, 2000; Arnold *et al.*, 2007).

1.2 Fungal-Plant Associations

The lifestyles of fungi associated with plants can vary dramatically from one another. At one end of the spectrum are parasites which are of huge economic importance due to the crop losses that they cause. At the other end of the spectrum are mycorrhizal fungi which are essential to plant growth and development as well as playing an important role in plant protection against parasites. Mycorrhizal fungi have what would be defined as a mutualistic relationship with their hosts because they mutually exchange nutrients, which improves the fitness of both partners (Siddiqui *et al.*, 2008). Another important lifestyle of fungi is saprotrophy, which involves the decomposition of dead organic matter, such as leaf litter.

Although parasites, mycorrhizal fungi and pure saprotrophs represent a substantial fraction of total fungal diversity, a large contribution to total diversity, especially in the ascomycetes, is made by the lesser known endophytes. Endophytes can be difficult to define because of the wide variety of lifestyles that they adopt. Etymologically the word endophyte means: "organism living within a plant host". The term can be used to refer to fungi, bacteria or even insects (Schulz & Boyle, 2005). A more meaningful definition has been proposed by Wilson (1995), regarding endophytic fungi as "fungi living asymptomatically in a plant for at least part of their life cycle". This definition does not necessarily imply mutualism and includes a continuum of interactions between fungus and host which can range from latent pathogen to beneficiary of the host. The definition excludes mycorrhizae since they grow externally as well as internally within their hosts. A more detailed account of the different lifestyles of endophytes, which may span the spectrum from parasite to mutualist to saprophyte in different parts of the life-cycle of one single organism, will be given in the following sections.

1.2.1 Endophytes and Mutualism

Mutualism can be defined as an exchange of benefit between two organisms of which both equally gain increased fitness. The mis-use of this term and clarification of the definition of this type of interaction was reviewed by Boucher *et al.* in 1982. Although it is a misconception to think that all endophytes have a mutualistic relationship with their host, at least some

degrees of mutual benefits have been observed in many cases (Sieber, 2007; Bayman, 2007; Saikkonen *et al.*, 1998; Carroll, 1988). Yet emphasis has been laid on the continuum characteristics of these relationships which has been identified as the result of a balanced antagonism between host and symbiont (Shulz & Boyle, 2005). The different reviews of endophytes cited above have highlighted benefits to the host which can vary from protection against pathogens, insects and herbivores to growth enhancement in exchange for shelter and sometimes food.

1.2.1.1 Towards a classification of endophytes

Rodriguez *et al.* (2009) undertook the important and challenging task of classifying endophytes according to their biological and ecological characteristics. This is very important for a coherent discussion of this diverse group of organisms. Indeed, one cannot classify endophytes by following their taxonomical class. Rodriguez *et al.* (2009) therefore defined four groups according to their mode of transmission and colonisation, and the tissues that they infect.

1.2.1.2 Class I: The grass endophytes

Before the review by Rodriguez *et al.* (2009), Schulz & Boyle (2005) distinguished the Clavicipitaceae (Lindau) O.E. Erikss endophytes (*Epichloë* (Fr.) Tul. & C. Tul. and *Balansia* Speg. species) from all other endophytes based on their unique relationship with their host. They are systemic

colonisers of grass which reproduce asexually through vertical transmission (Clay & Schardl, 2002; Schardl *et al.*, 2004). This intimate relationship has led to strong coevolution with their grass hosts. As a result this group is taxonomically and phylogenetically restricted to one family, the Clavicipitaceae. The grass endophytes form part of a unique interaction for which mutualism is essential. The endophyte needs to keep its host alive and healthy and fit to reproduce since it is transmitted through the seeds (Clay & Schardl, 2002). Evidence for host benefits is strong. The most well known host benefit is from the production of alkaloids by the endophyte. Alkaloids are toxic to herbivores (Clay, 1988). The mutualistic grass endophytes are closely related to parasitic endophytes which reproduce sexually and are transmitted horizontally. They prevent their grass host from producing flowers in order to produce their fruiting bodies. In fact there is strong evidence that asexual species of parasitic *Neotyphodium* Glenn, C.W. Bacon & Hanlin have originated from inter-specific hybridization of endophytic *Epichloë* species (Clay & Schardl, 2002). While extensive knowledge about evolution of grass endophytes has been accumulated over the past decades, an understanding of the ecology and evolution of non-grass endophytes is still in its infancy. The next section will be discussing recent advancements in this domain.

1.2.1.3 Endophytes of Class II, III, and IV: The Non-clavicipitaceous group

The remaining types of fungal endophytes are not restricted phylogenetically

and can even include Basidiomycetes, although most of them are Ascomycetes and have a broad host range (Rodriguez *et al.*, 2009). A further three groups can be recognised according to the location of the infection and the level of colonisation while in the endophytic stage. Of these three classes only class III and IV are strictly horizontally transmitted.

Class II can colonise any part of the plant, from roots to seeds. Their main attributes are that they confer stress tolerance to their hosts. As well as being horizontally transmitted they are able to reproduce in a vertical transmission manner via seeds and rhizomes, yet they do not appear to be as host specific as grass endophytes. Lifestyles can vary from mutualistic symbionts to pathogenic to saprophytic. For more details on this class Rodriguez *et al.* (2009) should be consulted.

Class IV endophytes are restricted to roots and are characterised by dark pigments and septate hyphae (Rodriguez *et al.*, 2009). These endophytes have never been associated with disease at any stage of their life-cycle (Rodriguez *et al.*, 2009). They form systemic infections and are horizontally transmitted. Although there is no empirical evidence of their mutualistic status, they are always associated with hosts which are in high stress environments (Rodriguez *et al.*, 2009). Their taxonomy is poorly understood. Consequently their research is lagging behind the other classes of endophytes (Rodriguez *et al.*, 2009)

The final group of endophytes, Class III, are thought to be the most diverse

group (Arnold, 2007; Arnold *et al.*, 2000; Rodriguez *et al.*, 2009) and form the subject of this thesis. They infect aerial parts of plants such as stems or leaves. They are strictly horizontally transmitted yet they show some levels of host specificity (Rodriguez, *et al.*, 2009). Three sub-classes can be distinguished based on the continuum of interactions with their hosts. The first sub-class infects the host and shows limited growth until tissues naturally senesce, at which point they colonise saprophytically. The second sub-class also show initial limited growth, but engage in saprophytic colonisation upon the imposition of stress on the host which is external to the symbiosis. The third sub-class is the virulent sub-class which induces disease symptoms after infection, though the length of the latency stage may vary.

The wider ecological role of this group of endophytes is poorly understood although some leaf endophytes have been shown to be efficient in decomposing the litter (Korkama-Rajala *et al.*, 2008; Osono *et al.*, 2010). There is some limited evidence of mutualism in this group which has been characterised as being of benefit to the endophyte because it represents "turf guarding", preventing herbivores and more virulent pathogens from damaging the substratum on which the endophyte will later fruit (Herre *et al.*, 2005, Arnold. *et al.*, 2003; Minter, 1981b, Carroll, 1988; Miller *et al.*, 2002).

1.2.2 Endophytes and Parasitism

What is common to all endophytes is their close phylogenetic relationship to pathogens. In fact most pathogens have an asymptomatic phase which is

more or less extended. The difference between a potentially mutualistic lifestyle as opposed to a parasitic one is essentially in the capacity to colonise and produce fruiting bodies to the detriment of the host rather than producing fruiting bodies via vertical transmission or following colonisation of naturally senescing tissues. In some species the potential to act more virulently has been lost, in other it appears to have been gained (Clay & Schardl, 2002; Herre *et al.*, 1999; Wilkinson & Sherratt, 2001, Ortiz-Garcia *et al.*, 2003). Other endophytes can have both lifestyles, sometimes occurring in different hosts and sometimes in the same host. In the latter case the pathogen will take the opportunity to colonise and complete its life cycle when its host is stressed or vulnerable. It has been shown that mutation of a single gene can render a pathogenic species of a class II endophyte asymptomatic or even beneficial to its host (Freeman & Rodriguez, 1993).

A question which naturally arises, and which is of great evolutionary interest is how endophytes with mutualistic and parasitic lifestyles differ from one another in their biology, ecology and evolution potential? Many studies in this area have focussed on related pathogens and endophytes of grasses because of their economical importance for crop losses and livestock welfare respectively. Although studies have compared population genetics of hybrids to non hybrids in *Epichloë/Neotyphodium* populations, none to date have compared the population genetics and evolution of pathogenic and non virulent species of class III endophytes.

The motivation for this study is to learn more about the population biology

of potentially mutualistic class III endophytes and how they differ from related parasites. One other interest of this study would be to learn more about the evolutionary relationships between these two sub-classes of plant symbionts.

If any random pathogenic and non pathogenic species were compared in terms of their population biology, they could differ in many ways which were not associated with differences in lifestyles. In order to avoid these difficulties and pinpoint differences between endophytes and parasites that are associated with their different lifestyles, an ideal system would require fungal species which are related and sympatric, with species representing each of the three sub-classes of Class III endophytes described above. Only then can fundamental questions about association of lifestyle with differences in genetic diversity, breeding systems and population structure be answered. Such a system would also allow investigation of the phylogenetic relationships between endophytes and related parasites. Fortunately such a system does exist and has been previously characterised. The next section will describe current knowledge of such a system, *Lophodermium* Chevall. on *Pinus sylvestris* L., and explain why it is suitable for investigating these questions. At the same time we will examine whether the current taxonomy of *Lophodermium* on *Pinus sylvestris* is likely to be adequate for these studies.

1.3 Lophodermium on Pinus sylvestris

1.3.1 Benefits from Studying *Lophodermium* on *P. sylvestris*

A good example of a system for investigating the comparative biology and ecology of endophytic and parasitic fungi is provided by the ascomycete genus *Lophodermium* associated with *Pinus sylvestris*. It includes three closely related species originally lumped together into one taxon. One of these is a pathogen and the remaining two are endophytes. The overall aim of this thesis is to study the phylogenetic relationships among these fungi, and to compare aspects of their genetic diversity, breeding systems and population structure in natural populations. We ask whether differences between species in these attributes can be explained by their differences in life style. I begin by reviewing what is already known about this system.

1.3.2 Biology of *Lophodermium* on *Pinus sylvestris*

Lophodermium is an ascomycete genus which comprises at least 145 species (Kirk *et al.*, 2008). Recent work on the genus has shown that it is polyphyletic, with only one of the multiple lineages being associated with pines (Lantz *et al.*, 2010). Other lineages are associated with a range of diverse hosts including spruces, podocarps, grasses and rhododendrons.

In pines, *Lophodermium* species are generally isolated from secondary needles showing no sign of disease (Diwani & Millar, 1980). However *Lophodermium*

also includes an economically important primary pathogen of pine, *Lophodermium seditiosum* Minter, Staley & Millar. It attacks mostly seedlings and young pines causing premature needlecast followed by death in severe outbreaks (Diwani & Millar, 1987). The following sections will review current and previous understanding of the taxonomy of *Lophodermium* with emphasis on those species occurring on *Pinus sylvestris*. It will also present current knowledge of their ecology and biology.

1.3.2.1 Taxonomy and Ecology of *Lophodermium*

Originally, only one species of *Lophodermium* (*L. pinastri* (Schrad.) Chevall.) was thought to infect *P. sylvestris* needles. Millar and Watson (1971) later recognised two biotypes based on culture morphology and habitat, while Stephan (1973) recognised three biotypes solely based on culture morphology. Minter (1977) undertook the important task of linking those three culture biotypes to fruiting body morphology, fruiting habitat, and time of spore release. Four species were then recognised to be associated with *P. sylvestris* of which three were observed in greater frequency (Minter *et al.*, 1978). The main morphological differences were the level of embedment and size of both perfect and imperfect fruiting bodies respectively ascocarp (Figure 1.1 and 1.3) and pycnidia (Figure 1.2 and 1.3) within the host tissue and the production as well as colour of zone lines (Figure 1.3). Variation in the degree of embedment of the ascocarp can be observed with the naked eye as the differences in black to grey shades when the ascocarp is dry. With little embedment of the ascocarp, it appears black when dry, whereas deeply

embedded ascocarps appear grey. Other characters which were found to be informative were the ascocarp placement relative to the stomata and the morphology of the paraphysis apex. Asci are mostly cylindrical and ascospores may be coiled, forming a helix, or straight (Minter, 1981a; Minter *et al.*, 1978). The three most frequently observed species were *L. pinastri*, *L. conigenum* (Brunaud) Hilitzer and *L. seditiosum*_Minter, Staley & Millar. A fourth species was first identified as *L. pini-excelsae* S. Ahmad later corrected to be a novel species named *L. staleyi* Minter (Minter, 1981a).

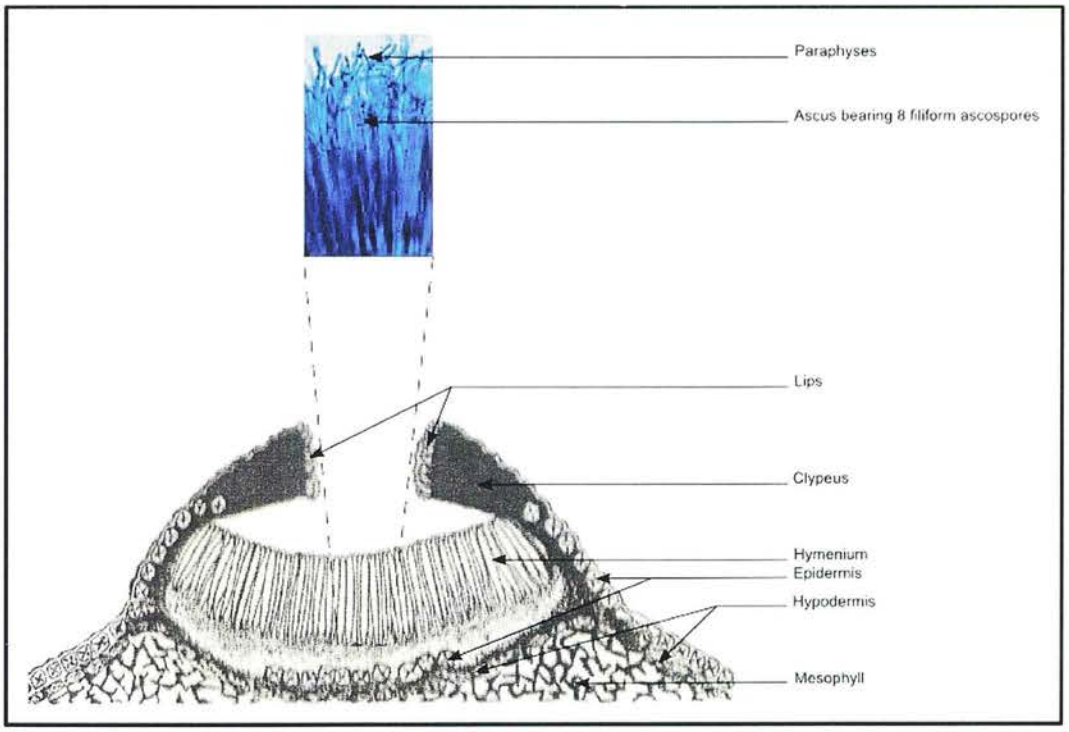


Figure 1.1 Explanatory diagrams of the sexual fruiting body (ascocarp) of the perfect stage. Illustration was taken from Minter (1981a). Photograph of the hymenium taken using an Olympus SE 300 light microscope fitted with a digital camera. The content of a mature ascocarp of *Lophodermium* was crushed under a microscope slide and a cover slide. It was stained with lactophenol cotton blue and observed with a magnification x400.

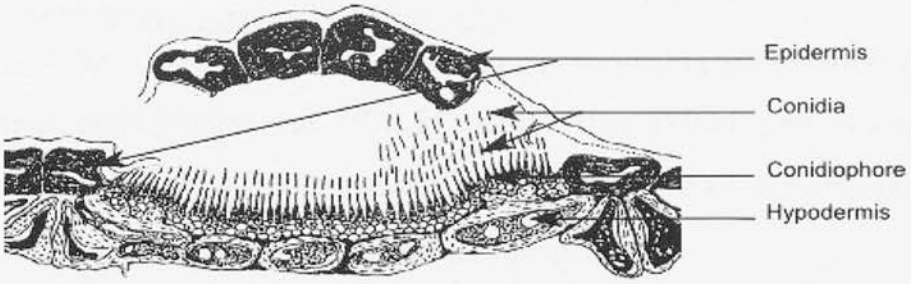


Figure 1.2 Diagrams of the asexual fruiting body (pycnidia) of the imperfect stage *Leptostroma* (Jones 1935).

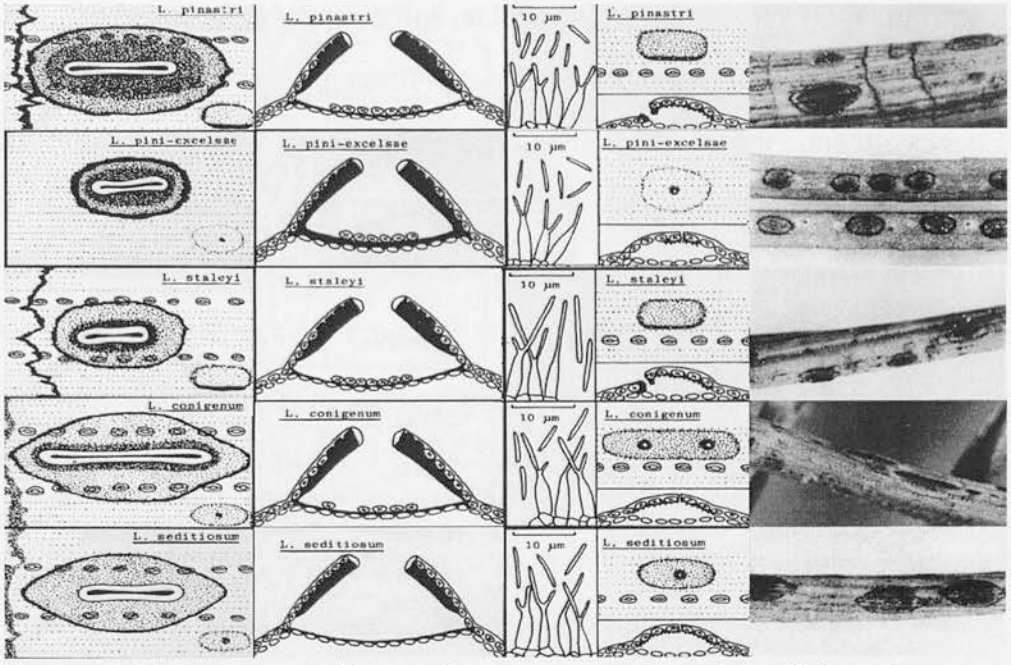


Figure 1.3 Diagrams and picture of ascocarps and pycnidia. Column 1 represents ascocarps of five *Lophodermium* species (*L. pinastri*, *L. pini-excelsae*, *L. staleyi*, *L. conigenum* and *L. seditiosum*) as it seen on the surface of a needle. The second column illustrates vertical section representing the displacement of the host epidermal cells of each species. The third and fourth columns illustrate conidiophores and the pycnidia of the imperfect stage as seen on the needle and in a vertical section. The last column contains the photographs of each corresponding species. All diagrams and pictures were taken from the manuscript "*Lophodermium* on pines" by D W Minter (1981a). diagrams and pictures were taken from the manuscript "*Lophodermium* on pines" by D W Minter (1981a).

Lophodermium pinastri (Schrad.) Chevall.

Lophodermium pinastri isolates are slow-growing in culture (3mm/day on 2% malt agar) (Minter *et al.*, 1978; Minter & Millar, 1980b). Young cultures are white, and at the end of their growth they produce a black stromatic circle at the periphery of, or sometimes all over the colony.

On the host Scots pine, conidiomata, known as pycnidia, start developing on naturally cast needles in autumn and throughout winter. Though *L. pinastri* can also be found on the needles on broken branches, they develop here mainly on needles which would have been old enough to naturally senesce. At the same time as pycnidial development occurs, black zone lines start to form on either side of the pycnidium. These are complete when pycnidia are fully developed in spring (Minter & Millar, 1980a). Conidiomata are sub-epidermal and appear as grey blister-like masses with longitudinal slit openings (Minter, 1980b). The pycnidia are 0.3 to 0.4mm long. Conidia are bacillar shaped measuring 4.5 to 6.25µm long (Minter *et al.*, 1978).

Ascocarps (Figure 1.2) start to develop in January and become mature and open between April and September with a peak between May and July (Minter & Millar, 1980a). *Lophodermium pinastri* forms small oval ascocarps that are amphigenous, *i.e.* in line with the length of the needle with a longitudinal slit in the centre. The slit is surrounded by lips which are visible when wet. Ascocarps measure 0.6 to 1.2 mm long. They are partially sub-epidermal. Therefore, they appear grey at the periphery and black in the centre when dry. When wet the ascocarp becomes black with hyaline,

sometimes red lips. Asci are cylindrical bearing eight ascospores that sometimes form a helix near the extremity of the ascus. Each spore is surrounded by a gelatinous sheath. Paraphyses are filiform hyphae which are sometimes swollen at their apex and are the same length as asci (Minter *et al.*, 1978).

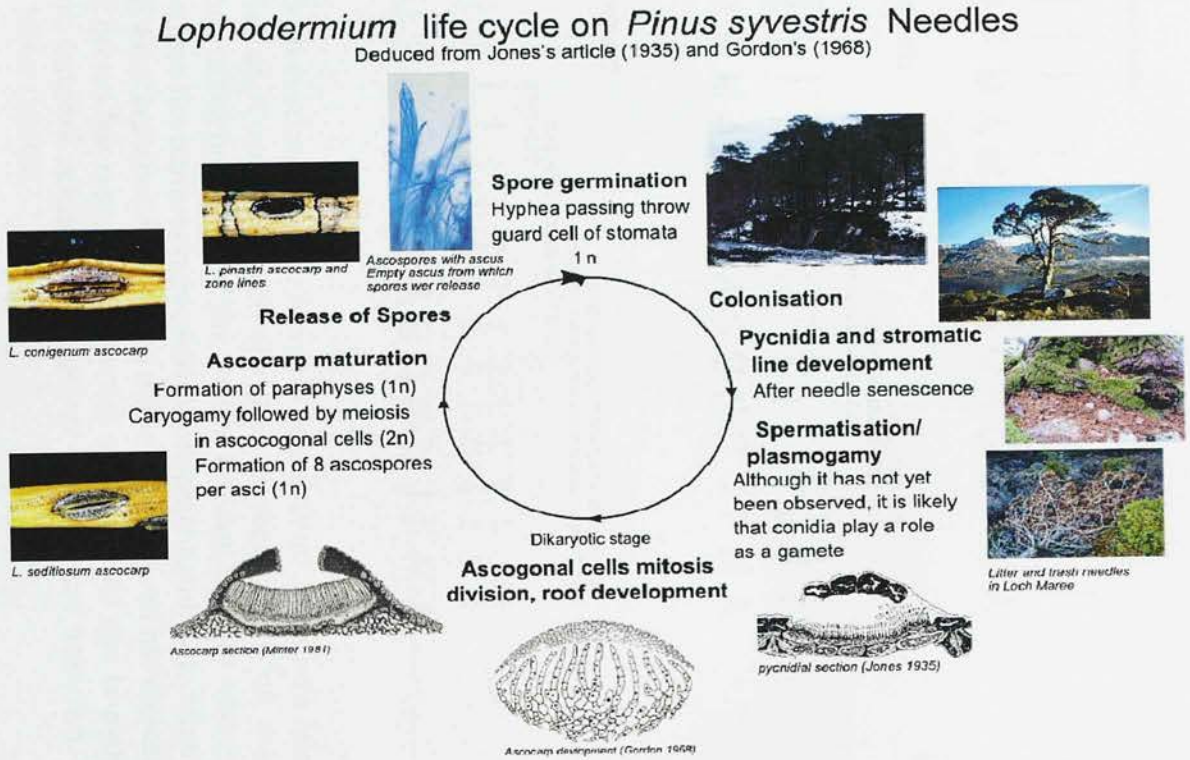


Figure 1.4 Illustration of *Lophodermium* life-cycle on pine needles of *P. sylvestris*.

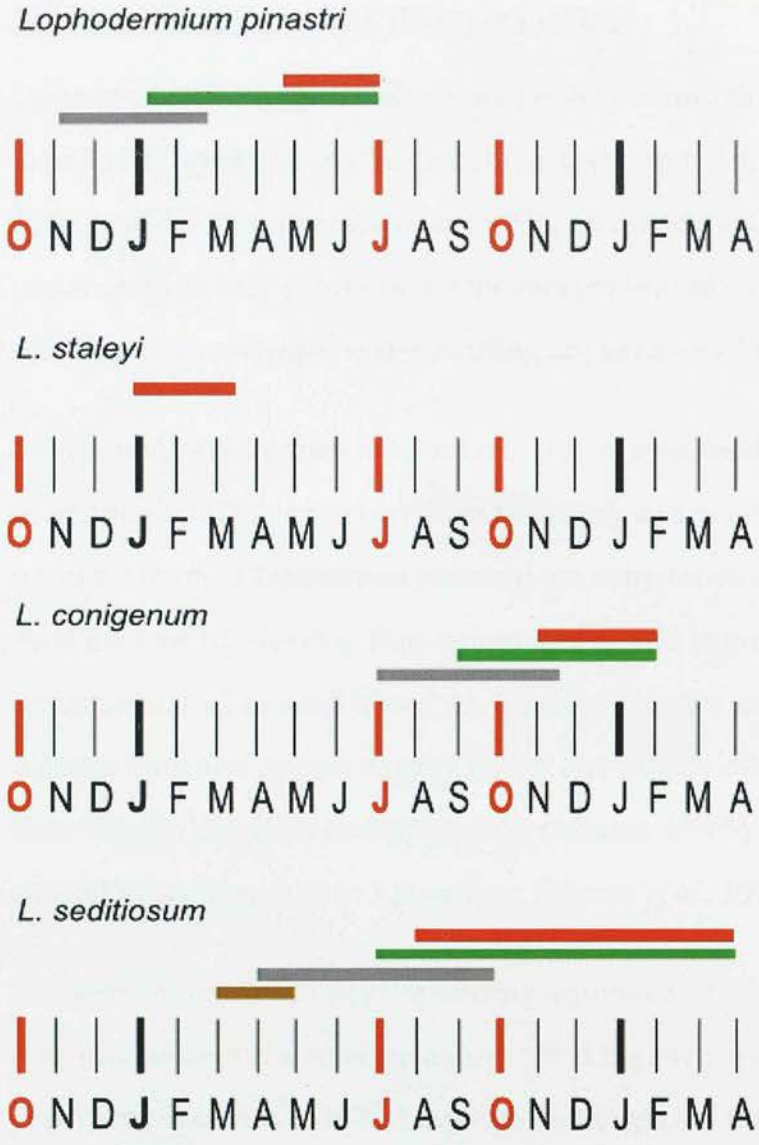


Figure 1.5 Illustration of *Lophodermium* colonisation and spore release stages on the needles of *P. sylvestris* over one year as represented on the scale; letters corresponds to the first letter of the month, July and October months are coloured in red. The grey bar represents pycnidia development and maturation; the green bar represents ascocarp development and maturation; the red bar represents ascospore discharge; brown bar represents browning of the needle due to pathogenicity of *L. seditiosum;* Detailed colonisation stages of *L. staleyi* are unknown. Deduced from Minter & Millar (1980a)

Lophodermium conigenum (Brunaud) Hilitzer

Lophodermium conigenum isolates are faster growing than *L. pinastri* in culture (4 or 5mm/day on 2% malt extract media) (Minter *et al.*, 1978; Minter & Millar, 1980b). Young cultures are white on the top and cream to light brown underneath as they grow older. One morphological characteristic of young colonies of *L. conigenum* is the swirling appearance of hyphae.

Lophodermium conigenum is found fruiting on needles that have been prematurely killed e.g. when branches break as a result of wind or snow. The pycnidia (named *Leptostroma pinorum*) are sometimes accompanied by brown zone lines which develop throughout spring and summer on needles which remain attached to dead branches. Conidiomata are sub-epidermal 0.35 to 0.45mm long and appear as grey blister like masses often coalescing with no clear margin and with ostiole opening (Minter, 1980b). Conidia are bacillar shaped measuring 5.25 to 7.5μm long (Minter *et al.*, 1978).

Ascocarps (Figure 1.2) develop during autumn and mature over the winter, releasing ascospores between autumn and the early months of the following year (Minter & Millar, 1980a). Mature ascocarps are the longest of all those found on needles of Scots pine (0.9 to 2mm). The slit is surrounded by hyaline or green lips which are visible when wet. Ascocarps are partially sub-epidermal and appear black in the centre with a grey surrounding when dry. Zone lines are diffuse and dark brown (Minter, 1981a).

Lophodermium seditiosum Minter, Staley & Millar

In culture *L. seditiosum* isolates grow faster than the other two taxa, approximately 5mm/day on 2% malt extract media (Minter & Millar, 1980b). Young cultures are white becoming dark brown. Abundant dark pigment is released into the media below growing cultures.

Lophodermium seditiosum is found on cones (Minter & Millar, 1980a) and on needles that have been prematurely killed, the same habitat as that occupied by *L. conigenum*. Indeed Minter (1977; 1980a) found that on the Island of Rhum on the west of Scotland, *L. conigenum* was completely replaced by *L. seditiosum* on broken branches. *Lophodermium seditiosum* is also found on needles that it has invaded as a primary pathogen. In the latter case, infection of *P. sylvestris* by *L. seditiosum* causes browning of one-year-old needles in the early spring, followed by needle fall in summer, especially in young trees (Diwani & Millar, 1980). Pycnidial development of the anamorph *Leptostroma rotrupii* occurs from May until October (Minter & Millar, 1980a ; Minter, 1980b). Rarely, brown zone lines develop on either side of pycnidia. Conidiomata are very similar to *Leptostroma pinorum* but slight longer, 0.30 to 0.5mm (Minter *et al.*, 1978; Minter, 1980b). Conidia are bacillar shaped measuring 6 to 8μm long (Minter *et al.*, 1978).

Ascocarps (Figure 1.2) develop over the summer and open in August releasing ascospores in autumn and throughout winter (Minter & Millar, 1980a). *Lophodermium seditiosum* forms elliptical ascocarp 0.8 to 1.5 mm long (Minter *et al.*, 1978). Ascocarps are totally sub-epidermal appearing grey

when dry and black with blue or green lips when wet. When stromatic lines are present they can be recognized as brown diffuse circles around the needle (Minter, 1981a; Minter *et al.*, 1978).

Lophodermium pini-excelsae Ahmad

Lophodermium pini-excelsae is usually associated with five needle pines and is found at low frequency on *P. sylvestris* in Scotland (Minter *et al.*, 1978). In culture its colonies on 2% Malt Extract are very similar to those of *L. pinastri*.

Conidiomata of the *Leptostroma* stage are sub-epidermal 0.15 to 0.3mm long with similar morphology to *L. pinastri*. However it produces longer conidiospores, 8 to 12µm long (Minter, 1980b). The ascocarp (Figure 1.2) is much smaller than for any of the other taxa, with a length of 0.3 to 0.8 mm, and it is partly sub-epidermal and oval. Mature ascocarps are black in the centre with grey lips. More than half of the clypeus is covered by epidermal cells which can be observed as a grey area on the perimeter of the structure when dry. Paraphyses are the same length as the asci and are threadlike with neither hooked nor swollen tips. Asci are cylindrical and bear 8 straight and filiform ascospores (Minter *et al.*, 1978). *Lophodermium pini-excelsae* releases spores in mid-winter.

Lophodermium staleyi Minter

Lophodermium staleyi is very similar to *L. pinastri* and *L. pini-excelsae*. Minter (1981a) revised the previous findings relating to the presence of *L. pini-*

excelsae on *P. sylvestris*. *L. pini-excelsae* is normally associated with *P. excelsa* and other haploxylon pines whereas *L. staleyi* is associated with diploxylon pines such as *P. sylvestris*. Like *L. pini-excelsae*, *L. staleyi* produces smaller ascocarps than *L. pinastri*, measuring 350 to 800 μm (Figure 1.3). The ascocarps bear only grey lips and are rarely accompanied by zone lines. Mature fruiting bodies can be found first on needles attached to branches deprived of light, and can be found on the pine litter in January. Cultures are similar to that of *L. pinastri* although *L. saleyi* generally grows more slowly. It produces the largest condia (8-12 μm) of all three species (Minter, 1981a).

1.3.2.2 The Need for a Taxonomic Re-assessment

Molecular Data and Fungal Barcoding

Minter's treatment was essential for the fields of forest pathology and mycology and has helped in answering pressing inconsistencies which used to exist in our understanding of the taxon *L. pinastri*. However, in the light of recent advances in molecular tools such as nucleotide sequence analysis, further questions remain concerning the taxonomy of *L. pinastri*. These have been prompted by the application of molecular phylogenetic analysis which has been used to confirm or clarify the current taxonomic arrangements of fungal species which are largely based on morphology. The next section will focus on those molecular approaches and how recent work on phylogeny of *Lophodermium* has influenced the current study.

DNA sequencing has led to the emergence of molecular barcoding as a way

of defining taxa (Hebert *et al.*, 2003). Here a standard barcode locus of DNA is sequenced and sequences are compared among individuals to define taxa. It relies upon the fact that individuals of the same species will share more nucleotide identity (low intraspecific variation) than individual belonging to different species (high interspecific variation). The wider the interval between the two types of variation the less is the risk of misidentification. This interval is known as the barcoding gap (Meyer & Paulay, 2005; Chase & Fay, 2009).

Application of molecular barcoding varies across disciplines. It can be used to define molecular taxonomic units in order to detect the true species diversity as opposed to the visible diversity which is often underestimated especially in organisms in which size is less than 1 mm (Blaxter *et. al.*, 2005; Arnold *et. al.*, 2007; Higgins *et. al.*, 2007). It is also useful in routine identification of organisms as well as in the detection of cryptic species and recently diverged species (Meyer & Paulay, 2005). On the other hand molecular barcoding alone has many limitations, particularly where it relies on information from a single locus (Funk & Omland 2003; Meyer & Paulay, 2005). For instance single species may contain multiple lineages particularly if they are recently formed and there has been insufficient time for lineage sorting of ancestral polymorphisms. Alternatively different species may share the same barcode because there has been insufficient time for fixation of new mutations to distinguish the lineages in the two species.

To date there is no agreement on which universal markers should be used for barcoding in fungi. The main candidates proposed as molecular barcoding

markers for fungi are the ITS region of ribosomal DNA and *cox*I mitochondrial cytochrome oxidase (Seifer 2009). ITS has particularly been favoured both for barcoding and in phylogenetic analysis of closely related species because of its practicality and because of the informative quality of its variation. It is a multigene family and therefore includes several copies which are made identical through the process of concerted evolution. For this reason it has lent itself to DNA sequencing (Álvarez and Wendel, 2003). Only a small amount of DNA is required to obtain good quality sequences. This is particularly useful for environmental samples and herbarium specimens. The universality of primers also made it an ideal candidate for its use across a wide range of taxa (Gardes and Bruns 1993). Moreover it is thought to bear the right amount of variation and to have a wide enough barcoding gap in order to define taxa (Seifer 2009). For plants and fungi a wealth of ITS sequence data is already available in databases such as Genebank (Seifer, 2009).

However there remains a problem with using ITS as a barcoding locus because there does not seem to be a universal threshold of intraspecific variation across all fungal lineages. In the case of ascomycetes, the 3% threshold which was thought to apply to all fungi appears to be unsatisfactory according to Nilsson *et. al.* (2008) who corrected it to 1.96% with a wide standard deviation of 3.73. They concluded that automatic delineation of taxa by simple barcoding would be difficult while using such a marker. Nevertheless ITS has been applied for defining MOTUs (Molecular Taxonomic Units) by Higgins *et. al.* (2007). They used a conservative 5% or

more nucleotide divergence threshold to define individuals belonging to different species.

Molecular Sequence Variation in *Lophodermium*

Phylogenetic analysis of molecular sequences has been applied to help clarify the taxonomy of *Lophodermium* on pines by Ortiz-Garcia *et al.* (2003). They used the ribosomal DNA Internal Transcribed Spacer (ITS) region and morphological data relevant to classification of 11 *Lophodermium* species to compare the results of morphological and molecular approaches. Embedment of the ascocarp within host tissues has been a major character in delimiting species with pine-associated species of *Lophodermium* in Minter's treatment and was one of the characters compared in the ITS phylogenies. Species delimitation between *L. conigenum, L. seditiosum* and *L. pinastri* proved to be concordant using morphological and molecular classification. The sequence gap between these taxa was greater than 5%. However it was noted in their results that nucleotide variation among the two isolates of *L. pinastri* was very high. The 5.6% nucleotide variation found between two individuals classed as *L. pinastri* is more comparable to that expected of levels of interspecific variation. This suggests that there may be more than one species within the taxon currently known as *L. pinastri*. Since it is crucial to establish the taxonomy of a group before biological and ecological comparisons can be made among its members, the first part of this thesis is aimed at clarifying the taxonomy of *Lophodermium* on *P. sylvestris* using new molecular tools unavailable to Minter.

1.3.2.3 Explanations for High ITS sequence variation within *L. pinastri*

Two explanations can be offered for the high level of ITS sequence variation observed between *L. pinastri* isolates. The first is that the 3% to 5% nucleotide divergence threshold used for defining species is inappropriate for the ITS locus used (Nilsson *et. al.* 2008). The presumed barcoding gap does not exist using this marker; intraspecific variation explains the variation observed among individuals of *L. pinastri*. In this case the present taxonomy holds. The second explanation is that the high sequence divergence at ITS is due to the lumping of cryptic species. In this case the taxonomy is inappropriate and in need of revision.

In order to distinguish between these two explanations a number of different and complementary approaches can be taken. A first step in the process is to obtain more sequences from within *L. pinastri*. These data can be subject to phylogenetic analysis to determine whether different clades can be distinguished within *L. pinastri*, between which there is a significant barcoding gap. If this is the case, then these clades could represent cryptic species. However an alternative explanation for such a result is that these clades arise within a single taxon as a consequence of incomplete sorting of ancestral polymorphism (Taylor *et al.* 2003; Rosenberg, 2003).

To eliminate ancestral polymorphism as an explanation for multiple clades within *L. pinastri*, independent sequence data from a number of unlinked loci must be obtained. If phylogenetic analysis data from different loci reveal the same clades, there is phylogenetic concordance. This provides very strong evidence that the clades represent different cryptic species. This is the basis

for the Genealogical Concordance Phylogenetic Species Recognition (GSPR) approach (Taylor *et al.*, 2000; Dettman *et al.*, 2003; Alamouti *et al.*, 2011).

It should be recognised that the situation may not always be this simple, particularly where species have recently evolved. If ancestral polymorphism has been retained at a locus, or there has been insufficient time for mutations to be fixed in different lineages, there will not be phylogenetic concordance (Taylor *et al.*, 2000). Thus GSPR is not a foolproof method for defining species. This reasoning argues for the use of many loci when applying GSPR. If phylogenetic concordance is found among a high proportion of them, this is very powerful proof for the presence of different species (Alamouti *et al.*, 2011).

Further confirmation that the clades recognised by GSPR represent different cryptic species can be obtained using a population genetic approach. This relies on the fact that individuals within a biological species share in the same gene pool which is different from that shared by another biological species (Mayr 1970). By scoring selectively neutral marker allele frequencies at many loci across the genome, and grouping together individuals which share the same alleles, biological species should be recognisable. These should correspond to the same groupings as those revealed by GSPR.

The first experimental part of this thesis is concerned with applying the approaches outlined above to clarify the taxonomy of *Lophodermium* species inhabiting the needles of *Pinus sylvestris* in Scotland. Once the task of clarifying the taxonomy using molecular methods has been conducted, it is

then possible to look for previously unrecognised morphological characters by which the cryptic species can be distinguished. It also becomes possible to study whether the taxa that have been identified differ in other aspects of their biology and distribution. These topics are dealt with in later chapters of this thesis. In the remaining part of this chapter I outline and justify in more detail the experimental approaches that have been followed in this thesis.

1.3.2.3 Molecular Approaches to Clarify *Lophodermium* Taxonomy

Choice of Molecular Sequences and Sample Sites

Indication that the entity currently known as *L. pinastri* may comprise more than one taxon came originally from analysis of sequence variation at the ITS locus (Ortiz-Garcia *et al.* 2003). This, together with the widespread use of the ITS locus for both molecular barcoding and phylogenetic analysis means that ITS is an obvious first choice for a more detailed phylogenetic analysis of molecular sequences within *L. pinastri* (Siefert, 2009). The choice of a second complementary locus to analyse, ACTIN, is determined by availability of published universal primers for ascomycetes, and previous successful use of the marker for species delimitation (Carbone and Kohn 1999).

The size of the barcoding gap needed for delineating species relies greatly on the distribution of both intraspecific and interspecific sequence variation. If both distributions overlap then the barcoding gap will be limited or even non-existent. Overlapping distributions are likely to occur more often where sampling covers wide geographical locations, and is less likely where

individuals are sampled within more restricted sites (Meyer & Paulay, 2005; Nilsson *et. al.*, 2008). Over a greater geographic area more intraspecific variation will be encountered giving rise to a wider distribution which is more likely to overlap with interspecific nucleotide variation. In this study analysis restricted to local populations in Scotland. It will provide the best opportunity for detecting a barcoding gap if it exists.

Phylogenetic Analysis

Having obtained a collection of ITS and Actin sequences from within populations of *L. pinastri* in Scotland they can be subject to phylogenetic analysis. As a first step in determining whether the variation in these sequences can be accounted for by a single variable taxon, or multiple taxa, the structure of the trees can be analysed. Paraphyly or polyphyly between variants of the putative species would suggest either incomplete lineage sorting or rejection of the hypothesis that high intraspecific variation is due to overlumping of cryptic species (Meyer & Paulay, 2005; Rosenberg 2003). However if the sympatric putative species form concordant, fully monophyletic clades across different loci than it is likely that these belong to reproductively isolated units and therefore represent distinct species (Funk & Omland, 2003).

Phylogenetic analysis helps not only in defining taxa, but also provides a chance to investigate the evolutionary relationship between taxa. In the case of *Lophodermium*, a large number of ITS GenBank sequences are available for the genus from closely related species (Ortiz-Garcia *et al.* 2003). Once the taxa within *L. pinastri* have been established, further phylogenetic analysis

including new and existing ITS sequences can be used to evaluate the phylogenetic relationships within the genus and determine for this clade whether endophytes have been derived from parasites or vice-versa. This could form part of a data set, which would include additional information from many other phylogenetically independent clades, to determine whether any general conclusions about the evolutionary relationships between endophytes and parasites can be established.

Population Genetic Analysis

An alternative approach to defining cryptic species is to use population genetic analysis (Lee *et al.* 2007). This relies on the fact that individuals of different species should share considerably fewer alleles than individuals within the same species. Variation at selectively neutral markers distributed across the whole genome, for example AFLP (Amplified Fragment Length Polymorphism) markers, can be scored to determine the multilocus genotypes of individual isolates. A matrix of genetic distances among isolates can then be established, and this can be used in multivariate analysis to determine whether isolates cluster into discrete groups with high genetic similarity. If the groups so defined concur with the concordant clades detected using phylogenetic analysis (above) then this is powerful confirmatory evidence that these represent good biological species.

1.3.2.4 Detection and Morphological Characterisation of Taxa

After the *Lophodermium* species encountered on *P. sylvestris* have been redefined by molecular analyses it is important to establish whether there are

any morphological characters by which these newly defined species differ, and learn about their distribution in natural population. The second part of this thesis will use the molecular information on any newly defined taxa to design appropriate species-specific primers to identify isolates from several natural populations according to the species defined previously. Using these results, relative species frequency from these populations will be inferred.

A large collection of isolates classified by species will result from this work. This will provide the opportunity to compare culture characters, such as growth rates, for each of the newly defined taxa using a statistically sound design. In this way it may be possible to find morphological characters in culture which could be used to identify the newly defined taxa without the need to undertake molecular analysis. This would also help to confirm that the taxa defined by molecular methods differ for characters that are likely to be ecologically important.

1.3.3 Mating System and Population Genetics

Ascomycetes are haploid for most of their life cycle. Jones (1935) speculated that anastosmosis occurred between conidia and trichogynes from observing their presence within pycnidia. However he did not ascertain the direct origins of ascogenous cells. Minter (1977) investigated the function of conidia as spermatia with no success. Conidia failed to germinate *in vitro*. Although the mechanism by which these fungi have sex is unknown, this stage in their lifecycle is important. Fusion of either the same or different mating type

thalli induces the development of ascogenous cells and karyogamy (short diploid stage, refer to Figure 1.4) occurs. This is followed by meiosis which re-establishes a haploid nucleus which is incorporated into the ascospores (Jones, 1935).

The ability for these *Lophodermium* species to self is unknown although some speculations have been proposed. The frequency of the parasitic species *L. seditiosum* is often low in natural populations, compared with the population size of the endophytic species. Where there is a lack of mating partners one might anticipate that selfing through homothallism might evolve. Thus we might anticipate homothallism in *L. seditiosum* but not in the endophytes. On the other hand culture variation has been observed among single spore isolates of *L. conigenum* and *L. seditiosum* derived from single ascocarps. Therefore one could speculate that these two species are heterothallic (Minter & Millar 1980b). However all *Lophodermium* species may not have the same mating system. Given the uncertainty indicated above, one of the objects of this thesis will be to determine the mating systems in the *Lophodermium* species on Scots pine using molecular methods.

A simple way of determining the mating system in ascomycete fungi is to look for segregation of genetic markers in families derived from single ascocarps (Ennos & Swales, 1987; Czembor & Arseniuk, 2000). In homothallic species no segregation occurs, whereas segregation occurs in heterothallic species for markers that are polymorphic in the population. Thus, segregation of RAPD markers in *L. nitens* among single spore isolates from

individual ascocarps was used to indicate heterothallism in that more distantly related species (Deckert *et al.*, 2002).

Previous authors have encountered difficulties in obtaining single spore isolates of *L. pinastri* to study mating system in this species (Minter & Millar, 1980b; Osorio & Stephan, 1989). Further attempts to obtain single ascospore isolates will be undertaken in this taxon, along with spore isolation of *L. seditiosum* and *L. conigenum* to study their mating systems using a similar method to that used by Deckert *et al.* (2002).

Genetic Diversity, Population Structure and Gene Flow

Two major processes are important in determining the amount of genetic diversity for selectively neutral alleles in populations. The first is mutation, which increases diversity. The second important factor is random genetic drift, which is especially important in small populations (Wright, 1931). Drift leads to loss of genetic variation from populations. It is particularly important where populations undergo bottlenecks (Nei *et. al.*, 1975) due to population fragmentation or when only a few migrants form a new colony in a new habitat or area via a founder event (Mayr, 1954).

The effective population sizes of *Lophodermium* taxa are expected to differ because of their different ecologies. In particular the pathogen *L. seditiosum* is anticipated to have a smaller population size than the remaining *Lophodermium* taxa on *Pinus sylvestris* because the number of needles that it is able to colonise is much smaller than those available to the endophytic

species. If the *Lophodermium* species differ in their population size, this may be evident in the amount of genetic diversity that they show, lower genetic diversity being expected in *L. seditiosum* than in *L. pinastri* or *L. conigenum*. This prediction can be tested using the collections of isolates that have been identified to species in earlier work.

While ascospore release in *Lophodermium* has been well characterised (Minter & Cannon, 1984), it is not so easy to determine how far these spores can travel. Lanier *et. al.* (1969) estimated that spores from litter needles would travel no further than 20 m if a wind velocity of 0.5 m/s is applied. However suspended needles which would correspond to trash and senescent needles remaining attached to the tree would be likely to disperse spores greater distances because they are subject to greater air turbulence. As a consequence spores released in those two latter habitats would travel further than in the litter when subject to the same wind velocity. We may therefore expect differences in the ability of different *Lophodermium* taxa to disperse their spores depending on where their fruiting bodies are located at the time of spore release.

A spatial study of allele frequency could inform us about the capacity of each species to exchange genetic material between populations and therefore allow us to evaluate their ability to disperse spores. If spore dispersal and therefore gene flow is limited between populations, we expect that populations will share fewer alleles and become more genetically differentiated. Population differentiation has previously been used to

estimate gene flow and the ability for ascomycete species to disperse (Ennos and Swales, 1991; Rousset, 1997). Work of this kind on populations of *L. piceae*, an endophyte and litter saprophyte, do not show any genetic differentiation, implying that their effective population size is very large and/or gene flow is unrestricted (Müller *et al.*, 2007). It will be important to determine whether the endophytes on *P. sylvestris* show similar patterns, and whether this contrasts with the parasite *L. seditiosum*. The third part of this thesis will therefore utilise multi-locus bi-allelic molecular markers to investigate the mating systems of the *Lophodermium* species and their genetic structure among native populations of *P. sylvestris*.

1.4 Aim and Plan of the Thesis

The ultimate aim of this work is to understand better the population biology and ecology of related endophytes and pathogens of forest systems. In order to achieve this, the taxonomy, evolutionary relationships, morphological attributes, mating systems and population genetic structure of *Lophodermium* species associated with *P. sylvestris* will be investigated. The areas of research to be addressed are outlined below. This project concentrates largely on local populations of native Scots pine in Scotland, which represent an isolated geographic outlier at the Northwestern edge of the range of *P. sylvestris* in a climate that is unusually oceanic for the host species (Salmela *et al.* 2010). Thus the results obtained may not be directly applicable to the *Lophodermium* taxa occupying the wider range of Scots pine across continental Europe.

Nevertheless the project will give valuable information which can be built upon both with regard to the findings which concern *Lophodermium* in particular and to the wider question of this thesis. These considerations will be discussed in the final chapter.

1. Taxonomy

The taxonomy of these species, in particular that of *L. pinastri*, needs to be re-investigated using a combination of molecular methods including: molecular barcoding, multigene genealogy and population genetics. If more than the currently recognised number of species are present, their phylogenetic relationship will be investigated.

2. Characterisation and Distribution of Cryptic Species

In the event that cryptic species are recognised based on molecular evidence, rapid and specific tools will be designed to identify them. An attempt will then be made to establish meaningful morphological differences among the taxa, and determine their distribution in natural populations.

3. Population Genetics

Having established a refined taxonomy, differences between these species in terms of breeding systems, genetic diversity, and the extent of genetic differentiation among populations will be investigated.

Chapter 2: Molecular Detection of Cryptic Species Within Endophytic *Lophodermium*

2.1 Introduction

The fungal kingdom is characterised as highly diverse comprising an estimated 1.5 million species. This estimate has been derived from knowledge of plant diversity based on the assumptions that most fungi are associated with plants, and that on average each plant species is associated with six fungal species (Hawksworth, 1991). Associations with plants include various kinds of symbioses (mycorrhizae, lichen forming and endophytic) together with saprophytic and parasitic associations. The majority of research effort has been focussed on mycorrhizal and parasitic fungi because of their economic importance for plant growth and health. More recently, endophytes have attracted the attention of scientists. Many definitions of an endophyte have been proposed because interactions with host species vary

from plant mutualist to latent pathogen (Carroll, 1988; Shulz and Boyle, 2005; Wilson, 1995). The definition most appropriate for this current study is from Wilson (1995); "organisms which live within the tissue of their host without causing them harm for at least part of their life cycle". This implies some mutualistic relationship with the host but does not exclude latent pathogens.

Due to the wide diversity of interactions that occur between hosts and endophytes, it is helpful to develop a classification of endophytes based on fungal taxonomy, host taxonomy, type of tissue infected and ability to colonise the host systemically. Rodriguez *et al.* (2009) distinguished four classes of endophytes:

Class I endophytes are restricted to grass hosts and are known to protect plants from herbivores. They are taxonomically distinct from other endophytes and are vertically transmitted whereas all other classes of endophytes are primarily horizontally transmitted.

Class II endophytes systemically infect a variety of host tissues and have been shown to confer protection against stress. They have a wide taxonomic range and include related pathogens such as *Colletotrichum* and *Fusarium*.

Class III endophytes infect aerial tissues non-systemically until the time when the host tissue naturally senesce (yearly shedding of leaves and bark formation on the stem).

Class IV endophytes correspond to the root infecting Dark Septate

Endophytes (DSE). They systemically infect healthy roots and are distinct from mycorrhizal fungi since they do not produce mycorrhiza. However they are thought to help their hosts in challenging environments.

This study focuses on class 3 foliar endophytes. The fungi involved are usually ascomycetes from a wide taxonomic spectrum (Higgins *et al.*, 2007). Class 3 foliar endophytes are often genetically closely related to fungal pathogens and are potentially competing with them (Arnold, 2007; Arnold *et al.*, 2009; Minter, 1981). The distinction between endophytes and parasites is often not clear cut. Indeed, in some instances an endophytic species may become pathogenic when its host becomes stressed (Desprez-Loustau *et al.*, 2006; Slippers & Wingfield, 2007; Stanosz *et al.*, 2001).

The ecological role of class 3 endophytes is still unclear (Arnold *et al.*,2003). It has been suggested by Minter (1981) that in situations where endophytes and closely related pathogens infect the same aerial tissue, the endophytes protect their hosts from pathogen invasion. For example, there is some evidence that the highly diverse endophytes within the leaves of *Theobroma cacao* protect against infection by the unrelated pathogen, *Phytophthora* sp. (Arnold *et al.*,2003).

Studying fungal endophytes can be challenging since many taxa remain undescribed, making identification of this flora demanding. Even with the recent increase in knowledge of their diversity, it remains difficult to identify the range of endophytic fungal species represented within host leaves, and

their relative abundance. Ecological surveys have generally relied upon analyses of cultural morphotypes to define species (Arnold *et al.*, 2001). This restricts study to those species that grow in axenic culture. Moreover, difficulties can arise when differences in culture morphology do not correspond to the degree of genetic divergence that occurs among individuals (Arnold *et al.*, 2007).

Some fungal species can show high morphological variability in culture. For instance, Minter and Miller (1981) derived colonies of two distinct morphological types from spores taken from a single fruiting body of *Lophodermium*. Thus identification of taxa based on colony morphology can be misleading. On the other hand, since lineage divergence can occur with little change in morphology, especially fruiting body morphology, it is not unusual to come across what is known as cryptic species (Taylor *et al.*, 2000). These species represent different genetic lineages but have so few morphological characters with which to differentiate them that they are hidden within a species complex.

Molecular tools have successfully been used to detect cryptic fungal species which show differences in culture morphotype (Guo, 2010; Taylor *et al.*, 2000). A good starting point for recognising cryptic species is to adopt a DNA barcoding approach. DNA barcoding involves sequencing a universal target locus and comparing sequences to known reference sequences previously lodged in databases such as Genbank. Individuals of the same species should have low nucleotide variation in the order of 1.96% sd 3.73 in the case of the

widely used ITS of Ascomycete fungi (Nilsson *et al.*, 2008). Ideally when comparing individuals of different species, nucleotide variation should be much higher, allowing for a gap between intraspecific and interspecific variation known as a "barcoding gap". The commonly used barcoding marker for fungi is the ITS region which is surrounding the 18s ribosomal DNA, although it is still being debated whether it really should be the standard choice (Nilsson et al., 2008; Seifert, 2009). ITS was first used by mycologists because of its ease of amplification since it has multiple copies. Databases such as Genbank contain an extensive record of ITS sequences from fungal taxa which makes ITS a primary candidate for barcoding (Seifert, 2009). In recent environmental studies, such as the estimation of endophyte species richness, the conservative estimate of 5% nucleotide variation was used as a threshold to define MOTUs. This value was overall coherent with morphology and congruent with phylogenies (Arnold *et al.*, 2007; Higgins *et al.*, 2007). More than 5 % nucleotide variation between the unknown isolate and the closest database reference might suggest that this species has not yet been deposited in the database. The reliability of this analysis depends on the richness of the database for a given taxa at a particular locus as well as the correct identification or isolation of the individual with which it is being compared (Seifert, 2009). It is also compromised because high intraspecific variation can exist within a species due to poor lineage sorting in recently diverge species, and this may be interpreted as a signal of the presence of cryptic species. Poor sampling of the reference taxon could also underestimate the anticipated variation within

individuals of the same species (Meyer & Paulay, 2005). Using a multilocus barcoding approach would help to alleviate some of these problems.

Species delimitation based solely on sequence similarity at a target locus is useful for guidance and for community ecological studies. However, genetic studies which aim to compare the evolutionary and ecological genetics of particular endophytes and pathogens require more precise species delimitation. If cryptic species are not recognised, there is a risk of overestimating genetic diversity within taxa, and of misinterpreting genetic divergence as due to of lack of gene flow within a species when this is in fact due to incompatibility of mating between biological but cryptic species. If a species complex is suspected, further investigation using phylogenetic and population genetic approaches is necessary (Dohan & Rizzo, 2005; Pavlic *et al.*, Samuel *et al.*, 2006 ; 2009; Kauserud *et al.*, 2006; Saleh & Leslie, 2004).

While concordance between multilocus barcoding may help with determining the cause of the variation a better understanding would be aided by the use of phylogenetic inference which involves sequencing of multiple genes from a range of variants from the species investigated. Individuals falling within the same monophyletic clade at each unlinked locus are then taken to belong to the same species (Taylor, 2000). This approach has been termed Genealogical Concordance Phylogenetic Species Recognition (GCPSR). If paraphyly or polyphyly occur, it would either suggest that the species have only recently diverged or that the intraspecific variation that is assumed is underestimated and should be revised (Meyer &

Paulay, 2005; Rosenberg 2003).

The population genetics approach to recognition of cryptic species makes use of the fact that different species should share fewer selectively neutral alleles than individuals from the same species. If a genome-wide sample of allele frequencies can be surveyed by techniques such as AFLP (Amplified Fragment Length Polymorphism) analysis, then groups of individuals sharing genetic markers can be recognised. Results of this type of analysis can be visualised on principal coordinate plots where individuals of the same species should cluster independently from individuals of different species. Unlike phylogenetic analysis, this phenetic approach does not provide information on the evolutionary history of a species but nonetheless can successfully be used to describe distinct genetic entities.

The problem of delimiting cryptic species is one which is of great practical importance in the genus *Lophodermium*, where a range of closely related species inhabit the needles of Scots pine, *Pinus sylvestris*. One of these species, *L. seditiosum*, is a pathogen causing a serious needle cast disease of seedlings (Diwani and Millar, 1987; Nicholls and Skilling, 1974). Two endophytes have also been recognised on Scots pine needles; *L. conigenum*, which fruits on needles that have been prematurely killed, and *L. pinastri*, which fruits on naturally senesced needles. If the interactions among these species within the pine needle can be determined, there is the potential to make use of the endophytes for biological control of the pathogen. However a major limitation to realising this potential is uncertainty over species delimitation

in the endophytes.

Recent studies of *Lophodermium* Chevall. indicate that *L. pinastri* may be a species complex (Johnston *et al.*, 2003; Ortiz-Garcia *et al.*, 2003). A PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) assay of ITS designed to distinguish between species of *Lophodermium* resulted in two banding patterns for isolates of *L. pinastri* as opposed to one each for *L. seditiosum* and *L. conigenum* (Johnston *et al.*, 2003). In addition, ITS sequence analysis of *Lophodermium* from pines found only 94.4% similarity among *L. pinastri* isolates (Ortiz-Garcia *et al.*, 2003). This is comparable to interspecific levels of sequence similarity found elsewhere in the genus. It is therefore important to determine how many endophytic *Lophodermium* species are associated with *P. sylvestris* in the native pine forests of Scotland before embarking on studies of interactions between these species and the possible utility of endophytes in biological control.

The objectives of this study are, firstly, to determine the number of *Lophodermium* taxa associated with needles of *P. sylvestris* sampled from a native pine forest. The second objective is to investigate the phylogenetic relationships among the species and to place this within the context of a wide range of *Lophodermium* isolates from other hosts and geographical locations. The final objective is to investigate the evolutionary relationships between pathogen and endophyte species. In particular, we are interested in determining whether there is evidence that the pathogen has been derived from the endophytic species. A combination of multi-gene genealogy and

population genetic approaches will be used to answer these points.

Needle samples have been collected from litter and from broken branches in two native *P. sylvestris* populations in Scotland. The fungi isolated from these needles have been classified on the basis of fruiting body and colony morphology using the descriptions by Minter *et al.* (1978). Bayesian phylogenetic analyses of ITS and Actin gene sequences as well as population genetic analysis of AFLP polymorphism have been used to delimit the cryptic species within *Lophodermium* on *P. sylvestris* within Scotland. Database sequences from worldwide collections have then been added and further phylogenetic analysis has been undertaken to establish the evolutionary relationships of taxa from Scotland with related taxa worldwide.

2.2 Material and Methods

2.2.1 Fungal Isolates

Lophodermium individuals were isolated in the lab from needles collected in the spring of 2005 from two native pine forests in Scotland; Glen Affric (NH 278 278) and Amat (NH 467 894) (Figure 2.1). At Glen Affric, a total of 18 isolates were obtained. These included 12 isolates of *L. pinastri* obtained from litter needles, as well as three isolates of *L. seditiosum* and three of *L. conigenum* which were obtained from needles on broken branches (Table 2.1). At Amat, two isolates of *L. pinastri* were obtained from litter needles (Table 2.1). Collections were made using labelled single use paper bags under 20 Scots pine at least 50m apart. Needles were laid to dry at room temperature and placed back in their paper bag where they were kept at room temperature in the lab until being isolated. Although isolations were made from needles bearing or not fully mature ascocarps, visual recognition of the different species was made primarily by the use of literature and by observing specimens at the Royal Botanical Garden Edinburgh. Needles were surface sterilized in 1.5% sodium hypochloride for 5 minutes then cut into 2-10mm sections removing the first 2 mm from the edge of the needle under aseptic conditions. Two needle sections per plate were placed onto Petri dishes containing 2% Malt Extract Agar (MEA)(Sigma-Aldrich Company Ltd., Gilligham, UK). The plates were incubated for four weeks at room temperature. Individuals of *Lophodermium* were selected on the basis of morphology and transferred onto fresh 2% MEA.

Figure 2.1: Location of *Pinus sylvestris* native populations used for the collection material of *Lophodermium* isolates.

Table 2.1: List of isolates used in both sequence and AFLP. analysis - refers to absence of genetic data; + refers to presence of genetic data; GN refers to green needles, BB refers to broken branches

Taxon and putative species	Host	Needles/ fascicle	Isolate	Origin	Fruiting habitat	Genebank nb		AFLP	Authors
						ITS	Actin		
Cenococcum geophilum		NA			-	AM084698	-	-	Anderson & Parkin unpub.
Lophodermium eucalypti	*Leptospermum scoparium*	NA	ICMP16796	New-Zealand	-	EF191235	-	-	Johnston and Park 2006
Lophodermium actinothyrium	*Poaceae*	NA	ICMP14599	Argentina	-	AY100663	-	-	Ortiz-Garcia *et al.* 2003
Lophodermium agathidis	*Metroideros fulgens*	NA	ICMP14958	New-Zealand	-	AY100662	-	-	Ortiz-Garcia *et al.* 2003
Lophodermium minor	*Nothofagus menziesii*	NA	ICMP13973	New-Zealand	-	AY100665	-	-	Ortiz-Garcia *et al.* 2003
Lophodermium piceae	*Picea abies*	NA	III/32	Finland	Litter/GN	EF592050	-	-	Korkama-Rajala *et al* 2008
Lophodermium piceae	*Picea mariana*	NA	Lope32	Quebec	Litter/GN	AY971734	-	-	Sokolski *et. al.* unpub.
Lophodermium baculiferum	*Pinus montezumae*	5	mon2zem	Mexico	-	AY100656	-	-	Ortiz-Garcia *et. al.* 2003
Lophodermium baculiferum	*Pinus montezumae*	5	mon3cp	Mexico	-	DQ406801	-	-	Salas-Lizana & Pinero unpub.
Lophodermium nitens	*Pinus ayacahuite*	5	122_Oa	Mexico	-	DQ406798	-	-	Salas-Lizana & Pinero unpub.
Lophodermium nitens	*Pinus monticola*	5	BPI843548	USA Washington	-	AY465526	-	-	Ganley and Newcombe 2003
Lophodermium indianum	*Pinus greggii*	3	loingr	Mexico	Litter/BB	AY100642	-	-	Ortiz-Garcia *et al.* 2003
Lophodermium australe	*Pinus palustri*	2 – 3		USA Florida	Litter/BB	U92308	-	-	Gernandt et al. 1997
Lophodermium conigenum	*Pinus radiata*	2 – 3	ICMP13970	New-Zealand	BB	AY100646	-	-	Ortiz-Garcia *et. al.* 2003
Lophodermium conigenum	*Pinus sylvestris*	2	L242*	Germany	BB	AF473559	-	-	Ortiz-Garcia *et. al.* 2003
Lophodermium seditiosum	*Pinus sylvestris*	2	93-C2	Sweeden	BB/Litter/GN	AF473553	-	-	Stenstrom & Ihrmark 2005
Lophodermium seditiosum	*Pinus sylvestris*	2	L30	Finland	BB/Litter/GN	AY775703	-	-	Muller *et al* 2007
Lophodermium kumaunicum	*P. kesiya, P. roxburghii*	3 – 5	Isolate 22		-	EU696777	-	-	Liu *et al* unpublished
Lophodermium macci	*Pinus strobus*	5	s-171	Quebec Valcartier	Litter	AF540560	-	-	Sokolski *et. al.* 2004

Taxon and putative species	Host	Needles/ fascicle	Isolate	Origin	Fruiting habitat	Genebank nb		AFLP	Authors
						ITS	Actin		
Lophodermium pinastri	*Pinus ponderosa*	2 – 5	Iopon	USA Oregon	Litter	AY100649	-	-	Ortiz-Garcia *et. al.* 2003
Lophodermium pinastri	*Pinus sylvestris*	2	L26	Finland	Litter	AY775701	-	-	Muller *et al* 2007
Lophodermium pinastri	*Pinus sylvestris*	2	BBA L230	Germany Luneburg	Litter	AY100650	-	-	Ortiz-Garcia *et al.* 2003
Lophodermium pinastri	*Pinus pinaster*	2	NZFS785	New-Zealand	Litter	AY247753	-	-	Johnston *et al* 2003
Lophodermium pinastri	*Pinus nigra*	2	LUBC	British Colombia	Litter	AF462434	-	-	Catal & Adams unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa13	Scotland Glen Affric	Litter	HM060659	HM060677	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa4	Scotland Glen Affric	Litter	HM060662	HM060679	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa6	Scotland Glen Affric	Litter	HM060661	HM060680	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa23	Scotland Glen Affric	Litter	HM060660	HM060678	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa1	Scotland Glen Affric	Litter	HM060657	HM060673	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa28	Scotland Glen Affric	Litter	HM060656	HM060674	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa29	Scotland Glen Affric	Litter	HM060658	HM060675	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa8	Scotland Glen Affric	Litter	HM060655	HM060676	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa7	Scotland Glen Affric	Litter	HM060663	HM060683	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa2	Scotland Glen Affric	Litter	HM060665	HM060682	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa9	Scotland Glen Affric	Litter	HM060664	HM060684	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Aa12	Scotland Glen Affric	Litter	HM060666	HM060681	+	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Ba11	Scotland Amat	Litter	HM122037	-	-	Reignoux *et. al.* unpub.
Lophodermium pinastri	*Pinus sylvestris*	2	Ba8	Scotland Amat	Litter	HM122036	-	-	Reignoux *et. al.* unpub.
Lophodermium seditiosum	*Pinus sylvestris*	2	Ac2	Scotland Glen Affric	BB/GN/Litter	HM060653	HM060668	+	Reignoux *et. al.* unpub.
Lophodermium seditiosum	*Pinus sylvestris*	2	Ac1	Scotland Glen Affric	BB/GN/Litter	HM060652	HM060667	+	Reignoux *et. al.* unpub.
Lophodermium seditiosum	*Pinus sylvestris*	2	Ac4	Scotland Glen Affric	BB/GN/Litter	HM060654	-	-	Reignoux *et. al.* unpub.
Lophodermium conigenum	*Pinus sylvestris*	2	Ab2	Scotland Glen Affric	BB	HM060648	HM060669	+	Reignoux *et. al.* unpub.
Lophodermium conigenum	*Pinus sylvestris*	2	Ab3	Scotland Glen Affric	BB	HM060649	HM060670	+	Reignoux *et. al.* unpub.
Lophodermium conigenum	*Pinus sylvestris*	2	Ab4	Scotland Glen Affric	BB	HM060650	HM060671	+	Reignoux *et. al.* unpub.
Phaeoisariopsis griseola	Phaseolus vulgaris	NA	CPC 12242	Colombia	NA	DQ289828	DQ289895		Crous *et al.* 2006

2.2.2 DNA preparation, PCR and sequencing

5mm x 5mm squares of agar with active mycelium were cut from agar plates and transferred to 50 ml flasks containing 20ml 2% malt extract broth and cultures were grown at room temperature for four weeks. Approximately 200mg of mycelia were dried on paper towels and ground in liquid nitrogen using a clean mortar and pestle. DNA was extracted using the Plant DNAeasy kit (QIAGEN GmbH, Hilden, Germany) following the protocol provided by the manufacturer. The same isolates were used for all subsequent genetic analyses (Table 2.1).

Unless otherwise stated, all PCR reactions were carried out in a GeneAmp® PCR system 9700 (Applied Biosystems, Foster city, USA). Left over primers were digested by adding of 2µl of ExoSAP-IT® (Cleveland, USA) to 5µl of the amplified product. Sequencing reactions were set up for both strands with 2µl of clean product using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, USA) following the protocol provided. Sequencing was done on an ABI3730 capillary sequencer at The Gene Pool, School of Biological Sciences, University of Edinburgh.

2.2.2.1 Amplification of a partial fragment of the ACTIN gene.

The partial ACTIN locus was chosen for sequencing because it contains introns which are useful for detecting intraspecific and interspecific variation when establishing genetic relationships between closely related taxa. It also

contains very conserved exons which are likely to be variable only at synonymous codon sites. DNA of the partial ACTIN gene was amplified using ACT-512F and ACT-783R primers (Carbone and Kohn 1999) (Table2.2). The PCR reaction was prepared in a total volume of 25µl containing 0.65U of BioTaq™ DNA Polymerase (Bioline Ltd., London, UK), 1x NH_4 reaction buffer, 2mM $MgCl_2$, 200uM of each dNTPs (Roche diagnostic Ltd., Burgess Hill, UK) and 0.4 µM of each primers. An initial denaturation at 95°C for 8min was followed by 35 cycles at 95°C for 15s, 55°C for 20s, 72°C for 60s and a final extension at 72°C for 5 min.

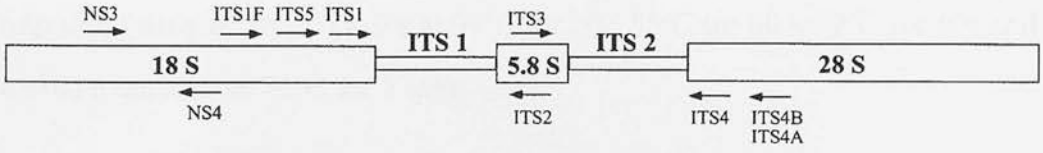
Table 2.2: List of primers used for sequencing and AFLP. 6FAM, VIC, NED and PET refers to colours of labelled primers

Primer name	Primer sequence	author
ACT-512F	5' ATGTGCAAGGCCGGTTTCGC 3'	Carbone and Kohn 1999
ACT-783R	5' TACGAGTCCTTCTGGCCCAT 3'	Carbone and Kohn 1999
ITS1F	5' CTTGGTCATTTAGAGGAAGTAA 3'	Gardes and Burns, 1993
ITS4A	5' CGCCGTTACTGGGGCAATCCCTG 3	Larena et al. 1999
EcoRI-A	5' GACTGCGTACCAATTCA 3'	Vos et al. 1995
MseI-C	5' GATGAGTCCTGAGTAAC 3'	Vos et al. 1995
B-EcoRI-AAC	5' 6FAM-GACTGCGTACCAATTCAAC 3'	Vos et al. 1995
G-EcoRI-AAC	5' VIC-GACTGCGTACCAATTCAAC 3'	Vos et al. 1995
Y-EcoRI-AAC	5' NED-GACTGCGTACCAATTCAAC 3	Vos et al. 1995
R-EcoRI-AAC	5' PET-GACTGCGTACCAATTCAAC 3	Vos et al. 1995
MseI-CC	5' GATGAGTCCTGAGTAACC 3'	Vos et al. 1995
MseI-CG	5' GATGAGTCCTGAGTAACG 3'	Vos et al. 1995
MseI-CT	5' GATGAGTCCTGAGTAACT 3'	Vos et al. 1995
MseI-CAA	5' GATGAGTCCTGAGTAACAA 3'	Vos et al. 1995
MseI-CAG	5' GATGAGTCCTGAGTAACAG 3'	Vos et al. 1995
MseI-CCG	5' GATGAGTCCTGAGTAACCG 3'	Vos et al. 1995
MseI-CTA	5' GATGAGTCCTGAGTAACTA 3'	Vos et al. 1995
MseI-CAGA	5' GATGAGTCCTGAGTAACAGA 3'	Vos et al. 1995

2.2.2.2 Amplification of Internal Transcribed Spacer (ITS) of the Ribosomal DNA

Like the partial ACTIN locus, the Ribosomal DNA locus contains highly variable ITS1 and ITS2 regions and more conserved regions coding for the 5.8S subunit which can be used to resolve genetic differences between closely related species (Figure 2.2). The ITS region was amplified using the universal primers ITS1F and ITS4A (Gardes and Burns, 1993 & Larena *et al.* 1999) (Table 2.2). The PCR reaction was prepared in a total volume of 25µl as described for the partial ACTIN gene except that 2.5mM $MgCl_2$, 160µM of each dNTPs and 0.2µM of each primer were used. An initial denaturation at 94°C for 4min was followed by 32 cycles at 94°C for 40s, 55°C for 40s, 72°C for 60s and a final extension at 72°C for 10 min.

Figure 2.2: Location of universal primers amplifying the ribosomal DNA 5.8S subunit and internal transcribe spacers ITS1 and ITS2 of fungi (Larena *et al.* 1999).



2.2.2.3 AFLP

AFLP (Amplified Fragment Length Polymorphism) markers were obtained as described by Vos *et al.* (1995). Approximately 0.5µg of genomic DNA was digested with 5 U of *Eco*RI and *Mse*I enzymes (Promega, Fitchburg, USA) in NEB buffer 2 (New England BioLabs©inc., Ipswich, UK) and 0.5µg of BSA for 4H at 37°C. Digested genomic DNA was ligated to the *Eco*RI and *Mse*I adapters using NEB T4 ligase (New England BioLabs©inc., Ipswich, UK) and

1x of NEB ligase buffer at 4°C for two days.

*Eco*RI-adapter:

5-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5

*Mse*I-adapter:

5-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5

For the first PCR step, fragments from the digest were amplified with the selective primers *Eco*RI-A and *Mse*I-C which comprise the adapter sequences plus one base (Table 2.2).

The PCR reaction was prepared in a total volume of 13µl, containing 0.5U of BioTaq™ DNA Polymerase, 1x NH_4 reaction buffer, 1.92mM $MgCl_2$, 200µM of each dNTPs and 0.3µM of each primer. An initial denaturation at 94°C for 2 min was followed by 20 cycles at 94°C for 30s, 56°C for 60 s, 72°C for 60s and a final extension at 72°C for 2 min.

A second selective PCR amplification step was carried out using primers comprising *Eco*RI-A and *Mse*I-C to which had been added one, two or three more selective bases (Table 2.2) and 2µl of a 10 x dilution of the first step amplification product. The eight *Mse*I-C primers were chosen from 100 tested primer combinations on the basis of peak quality and ease of scoring. One *Eco*RI primer was used (Table 2.2) which was labelled with a unique fluorescent dye for detection using the capillary sequencing ABI3730 system.

The PCR reaction was prepared in a total volume of 10µl, containing 0.5U of BioTaq™ DNA Polymerase, 1x NH_4 reaction buffer, 1.5mM $MgCl_2$, 200uM of each dNTPs and 0.5µM of each base selective primer. Amplification was carried out using the MJ Research Dyad (Bio-Rad Life Science, Hemel-Hempstead, UK) with initial denaturation at 94°C for 2 min followed by 10 cycles at 94°C for 20s, 66°C for 30 s decreased by 1°C for every cycle, 72°C for 2 min, plus 20 cycles at 94°C for 20 s, 56°C for 30 s and 72°C for 2min. Bands were detected using the ABI3730 capillary sequencer.

2.2.3 Sequence analyses of the ACTIN and ITS loci

Sequences were trimmed and checked for errors using Sequencher® 4.5 (GeneCodes Corporation, Ann Arbor, USA). Nucleotide pair wise comparison and search for close nucleotide sequence matches was carried out using NCBI Blastn http://www.ncbi.nlm.nih.gov/blast/ (Altschul *et al.*, 1997). The latter was based on the ITS region which were the most similar to the queried sequence. The parameters used for the search were 10% threshold of statistical significance; reward and penalty for match and mismatch was based on a ratio of 0.5 (1,-2); the gap cost was set to 5,2. The choice of sequences to be used for later analysis was based on at least 95% nucleotide identity with the query and coverage of the order of 98% or more. Sequences were aligned using ClustalX2 (Thompson *et al.* 1997, Larkin *et al.* 2007) and manually adjusted in BioEdit 7.0.5.3 (Hall, 1999). In Clustal, sequences were first aligned in a pair-wise manner using slow/accurate parameters (gap opening: 15; gap extend: 6.66). Sequences were then

grouped by similarity in order to produce the final multiple alignment. Unless otherwise stated the Dothideomycetes *Phaeoisariopsis griseola* DQ289895 was chosen as an outgroup. The reason for this choice was based on the ACTIN sequence which included the same introns and exons as isolates in this study. In order to allow the application of different evolutionary models on parts of the sequences which have different level of variation, sequences were partitioned. The choice of the model of evolution for each partition was based on the application of the most complex model in a preliminary run. The substitution rate model (GTR +Γ+ I) was a general time reversible (GTR) model with six different substitution rates with gamma distributed substitution rates (Γ) and proportion of invariable sites (I). After running the complex model the output was examined in Tracer v1.4 (MCMC Trace Analysis Tool Version v1.5.0, 2003-2009, Rambaut and Drummond, http://beast.bio.ed.ac.uk/). If the parameters were not appropriately distributed appropriately, the model was simplified. Burn-in time was determined by observing the number of generations required for stability of the log likelihood values in Tracer. Convergence was determined when the average standard deviation of splits frequencies was less than 0.01. Where necessary, sampling of the trees was carried out every 10 generations in order to resolve the parameter estimation.

2.2.3.1 ACTIN

Introns were located after translation of the nucleotide sequences of the partial ACTIN gene into amino-acid sequences using the internet application

Virtual Ribosome http://www.cbs.dtu.dk/services/VirtualRibosome/ (Wernersson 2006). The protein sequences were aligned and the nucleotide sequences realigned according to the protein alignment. The alignment was partitioned into 1 intron and 3 exons to allow analysis of the prior parameters input for the Bayesian phylogenetic analysis.

The model used for the phylogenetic analysis was a nucleotide substitution (4 by 4) with the prior parameters of substitution GTR (General Time Reversible) + Γ + I. The analysis was performed in Mr Bayes 3.1.2 (Huelsenbeck and Ronquist 2001). The GTR model expects that all 6 substitutions have different rates. The parameter Γ means that the variation rate follows a gamma distribution. If this is true the mean Γ over all the sampled trees should be below 1. The parameter I stands for the proportion of invariable sites. We should expect it to be close to 0 in an intron and closer to 1 when it is in a conserved region such as an exon or ribosomal coding sequence. Four Markov Chain Monte Carlo (MCMC) chains were set to run for 2000000 generations. Trees were sampled every 10 generations. The temperature of the chain was set at 0.01. The first 250 trees were removed from the analysis by setting the burn-in. Parameters were analysed using the P output files from 2 simultaneous runs of the Bayesian analysis in Tracer.

2.2.3.2 Internal Transcribed Spacer (ITS) of Ribosomal DNA

Since the ribosomal DNA sequences comprised hypervariable regions at the ITS and conserved regions at the 18S, 5.8S and 28S subunits, data were

partitioned in two (hypervariable and conserved regions). This allowed the application of different evolutionary models for each partition. Identification of hypervariable regions was aided by the internet application G-Blocks 0.9b http://molevol.cmima.csic.es/castresana/Gblocks_server.html (Castresana 2000, Talavera and Castresana 2007). Regions comprising 10 bases or more were considered to be conserved when more than half of the sequences were identical. Regions were considered hypervariable when no more than 18% of the sequences were identical. Some manual adjustments were made to group some conserved partitions together.

The phylogenetic analysis was carried out using the same model as described for the ACTIN gene, except that the four MCMC chains were first set to run for 1000000 generations with sampling every 1000 generations then increased to 2000000 sampling every 10 generations in order to resolve the parameter estimation.

2.2.3.3 Combined ACTIN ITS phylogenetic analysis

The combined analysis of the ACTIN and ITS loci was carried out using the same model as described for each independent analysis except that the four MCMC chains were first set to run for 1000000 generations then increased 5 times, sampling trees every 1000 generations increased 10x by sampling every 100 generations in order to resolve the parameter estimation.

2.2.3.4 Inclusion of Lophodermium sequences from Genbank

The Dothideomycetes *Cenococcum geophilum* (AM084698) was chosen as an outgroup for this analysis. *Lophodermium* ITS sequences obtained from Genbank were added to the ITS alignment in order to obtain more information about the wider phylogenetic relationship of *Lophodermium* species. The sample comprised one individual per taxon, per host and per location available in the database Genbank. The phylogenetic analysis was carried out as described for the ITS alignment except that the four MCMC chains were set to run for 5000000 generations and trees were sampled every 1000 generations in a first analysis and increased 10x by sampling every 100 generations in a second analysis in order to resolve the parameter estimation.

2.2.4 AFLP analysis

ABI output files were analysed in GeneMapper v3.7. Peaks were scored for presence or absence at each locus of size "x". A distance matrix between individuals was calculated from these data using the Jaccard coefficient. The matrix of genetic distances was used in principal coordinate analysis (PCO) employing the programme PAST v1.70 (Hammer *et al.*, 2001) to visualise the genetic relationships among the isolates.

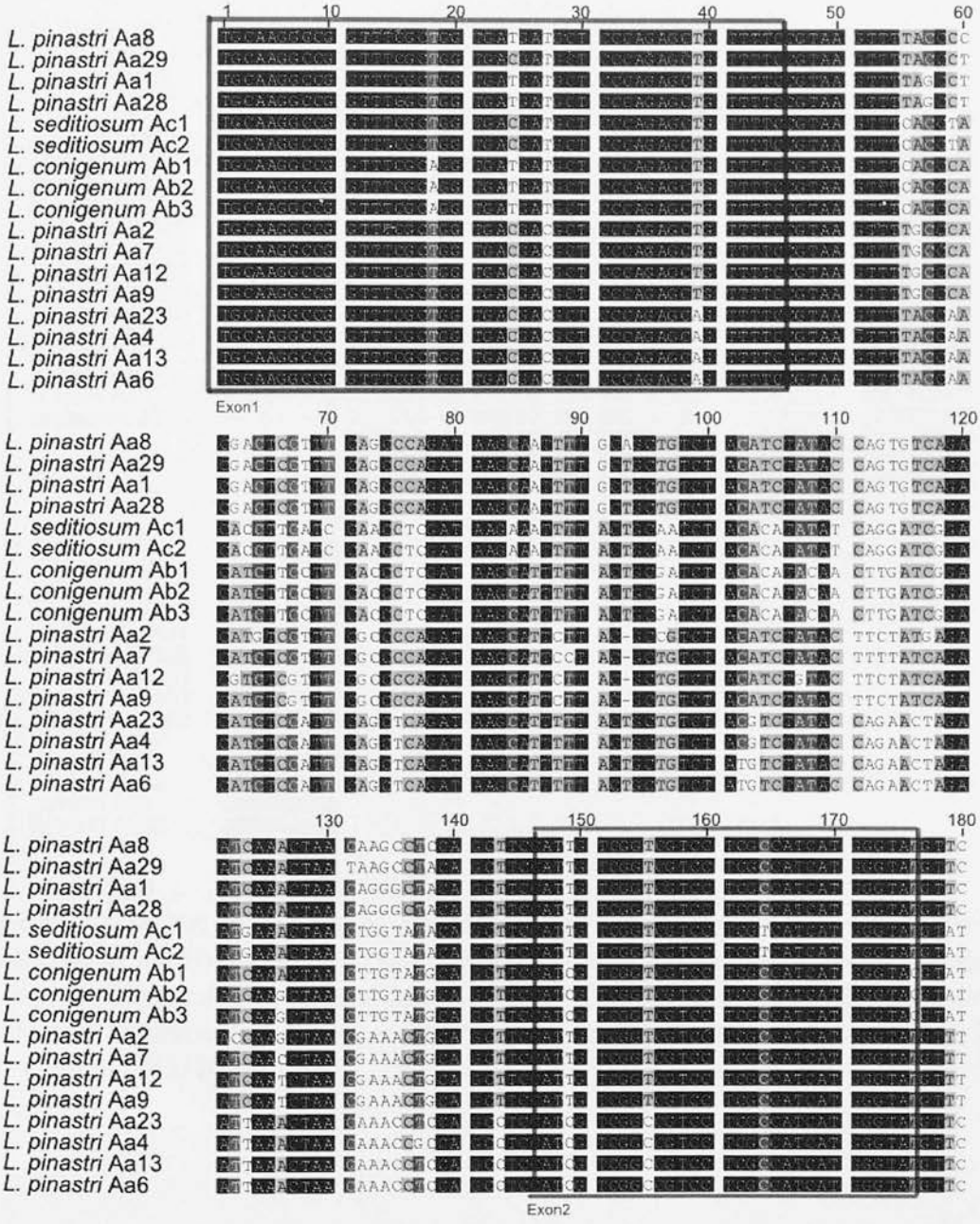
2.3 Results

2.3.1 Analysis of the ACTIN sequences

The ACTIN nucleotide sequences ranged in size from 267 to 280 bp. No size variation was observed between the sequences of *L. conigenum* and *L. seditiosum,* which were all 276bp in length. The sequences of *L. pinastri* varied from 267-280 bp. All ACTIN sequences included 3 exons separated by 2 introns. The total number of variable sites was 101 of which 97 were parsimony informative. *L. seditiosum* and *L. conigenum* differed by about 10% (90% nucleotide similarity) whereas they both differed from *L. pinastri* by approximately 20% (80% similarity). Isolates within species *L. seditiosum* and *L. conigenum* were very similar at this locus with similarity values no less than 99%. However isolates within *L. pinastri* species varied from 83% to 100% nucleotide similarity (Table 2.3). Nucleotide variation mostly occurred within introns and at synonymous sites which code for the same amino acid (Figure 2.3).

Table 2.3: Nucleotide variation based on BLASTn of 2 nucleotide sequences of ACTIN sequences.

		L. pinastri												*L. seditiosum*		*L. conigenum*		
		Aa1	**Aa8**	**Aa28**	**Aa29**	**Aa2**	**Aa7**	**Aa9**	**Aa12**	**Aa4**	**Aa6**	**Aa13**	**Aa23**	**Ac2**	**Ac1**	**Ab2**	**Ab3**	**Ab4**
L. pinastri	**Aa1**	1.00																
L. pinastri	**Aa8**	0.97	1.00															
L. pinastri	**Aa28**	1.00	0.97	1.00														
L. pinastri	**Aa29**	0.97	0.97	0.97	1.00													
L. pinastri	**Aa2**	0.86	0.87	0.86	0.87	1.00												
L. pinastri	**Aa7**	0.86	0.87	0.86	0.87	0.96	1.00											
L. pinastri	**Aa9**	0.86	0.87	0.86	0.87	0.97	0.98	1.00										
L. pinastri	**Aa12**	0.86	0.87	0.86	0.87	0.96	0.97	0.99	1.00									
L. pinastri	**Aa4**	0.84	0.85	0.84	0.85	0.84	0.84	0.84	0.83	1.00								
L. pinastri	**Aa6**	0.84	0.85	0.84	0.85	0.84	0.84	0.84	0.83	0.98	1.00							
L. pinastri	**Aa13**	0.84	0.85	0.84	0.85	0.84	0.84	0.84	0.83	0.98	1.00	1.00						
L. pinastri	**Aa23**	0.84	0.85	0.84	0.85	0.84	0.84	0.84	0.84	0.99	0.99	0.99	1.00					
L. seditiosum	**Ac2**	0.82	0.81	0.82	0.82	0.79	0.79	0.79	0.78	0.78	0.78	0.78	0.79	1.00				
L. seditiosum	**Ac1**	0.82	0.81	0.82	0.82	0.79	0.79	0.79	0.78	0.78	0.78	0.78	0.79	1.00	1.00			
L. conigenum	**Ab2**	0.80	0.81	0.80	0.80	0.81	0.81	0.81	0.80	0.78	0.78	0.78	0.78	0.89	0.89	1.00		
L. conigenum	**Ab3**	0.80	0.81	0.80	0.80	0.81	0.81	0.81	0.80	0.78	0.78	0.78	0.78	0.89	0.89	1.00	1.00	
L. conigenum	**Ab4**	0.81	0.82	0.81	0.81	0.81	0.81	0.81	0.81	0.78	0.78	0.78	0.79	0.89	0.89	0.99	0.99	1.00


1
10
20
30
40
50
60
L. pinastri Aa8
L. pinastri Aa29
L. pinastri Aa1
L. pinastri Aa28
L. seditiosum Ac1
L. seditiosum Ac2
L. conigenum Ab1
L. conigenum Ab2
L. conigenum Ab3
L. pinastri Aa2
L. pinastri Aa7
L. pinastri Aa12
L. pinastri Aa9
L. pinastri Aa23
L. pinastri Aa4
L. pinastri Aa13
L. pinastri Aa6
Exon1
70
80
90
100
110
120
130
140
150
160
170
180
Exon2

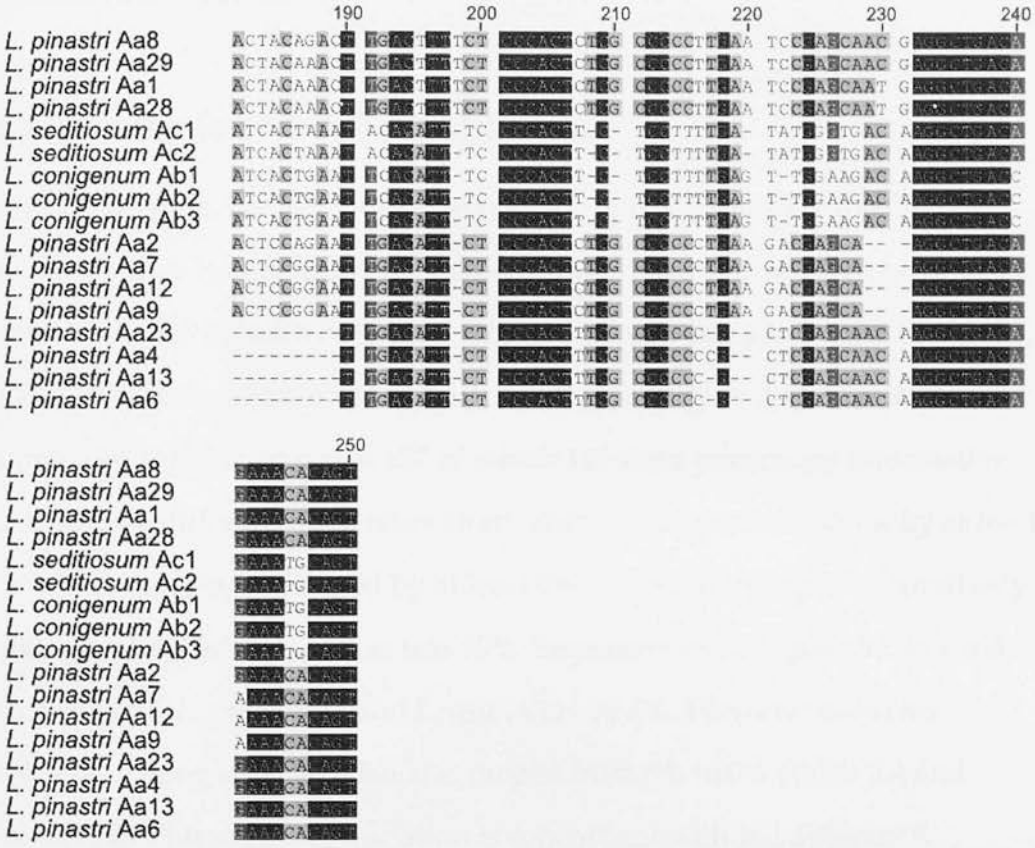


Figure 2.3: ACTIN alignment of *Lophodermium* isolates from litter and broken branches of the native forest of Glen Affric (Scotland). Dark shade represent bases which are identical between isolates and lighter shades represent bases which are different between isolates. Box shade was compiled using Geneious 5.0 2 (Drummond et al 2007)

The translated peptide sequence was:

CKAGFAGDDAPRAVFRSIVGRPRHHGIMIGMGQKDS

This peptide sequence did not vary among isolates of the different *Lophodermium* species.

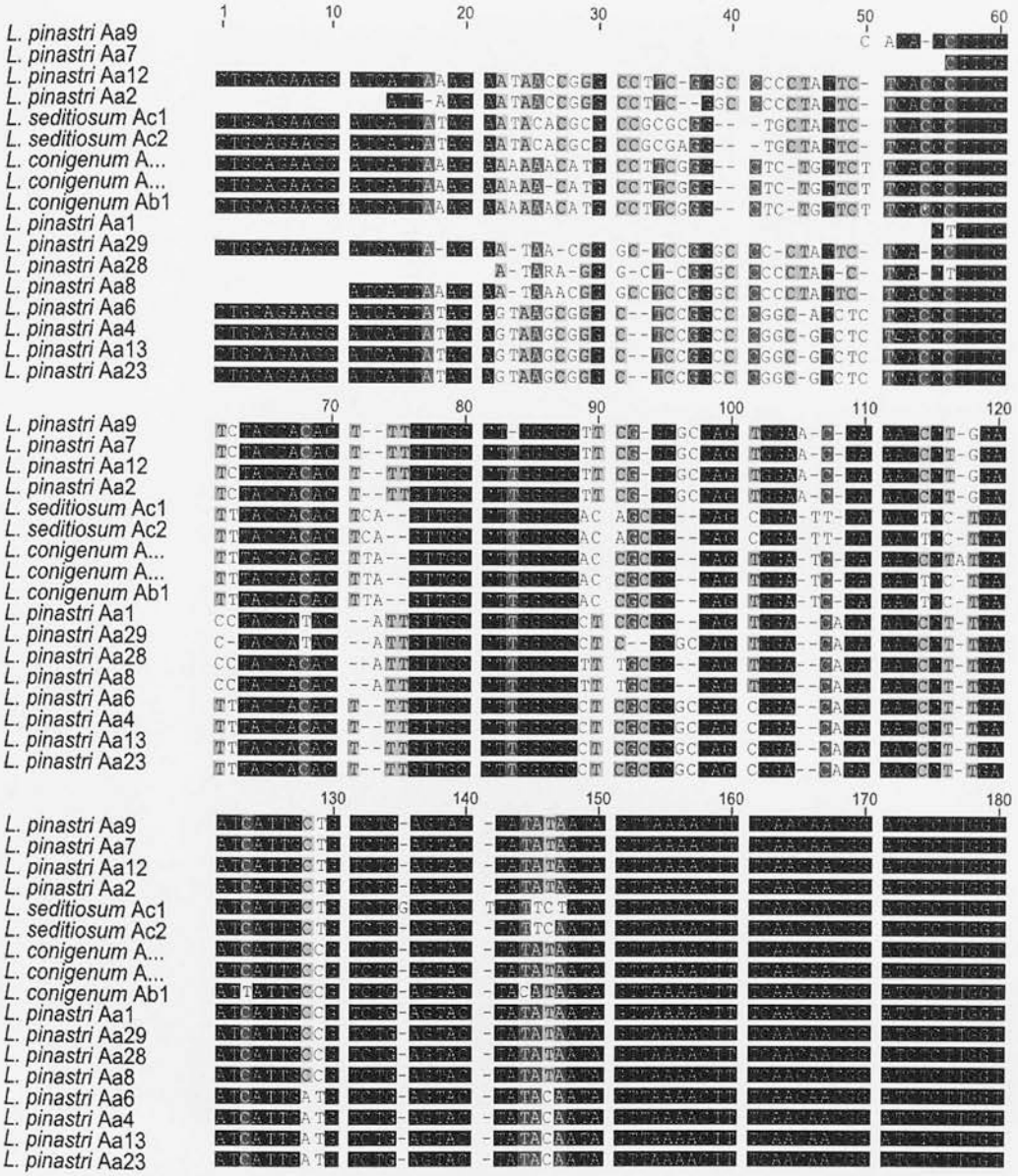
2.3.2 Analysis of the ITS sequences

For *L. conigenum,* the ITS sequences ranged from 475bp to 477bp and for *L. seditiosum* they ranged from 452 to 494 bp. The *L. pinastri* sequences ranged from 431 to 505 bp with no consistent difference in size among the lineages I, II and III as recognised by phylogenetic analysis (Figure 2.5). The total number of variable sites was 127 of which 115 were parsimony informative. *L. seditiosum* differed in sequence from other *Lophodermium* species by at least 7% and *L. conigenum* differed by at least 9%. For these two species, similarity among isolates within species was 99%. Sequences from *L. pinastri* differed from those of *L. conigneum* and *L. seditiosum* by 8%. However sequence variation among *L. pinastri* isolates ranged from 8% to 0% (Table 2.4 and Figure 2.4). This sequence variation is concordant with the different *L. pinastri* lineages inferred from the ACTIN phylogeny (Figure 2.5). Variation was no more than 3% within lineages and usually only 1%. Nucleotide similarity among the *L. pinastri* lineages was no greater than 95% (Table 2.4). Nucleotide variation was concentrated in the hypervariable region but was also present in the conserved regions (Figure. 2.4).

Table 2.4: Nucleotide variation based on BLASTn of 2 nucleotide sequences of ITS sequences.

		Aa1	Ba11	AY775701	Aa2	Ba8	AY100649	EU696777	Aa4	Aa6	AF540560
L. pinastri	Aa1	1.00									
L. pinastri	Ba11	0.99	1.00								
L. pinastri	AY775701	0.99	0.99	1.00							
L. pinastri	Aa2	0.93	0.92	0.92	1.00						
L. pinastri	Ba8	0.93	0.93	0.93	0.98	1.00					
L. pinastri	AY100649	0.92	0.92	0.92	0.99	0.99	1.00				
L. kumaunicum	EU696777	0.92	0.92	0.92	0.97	0.98	0.98	1.00			
L. pinastri	Aa4	0.95	0.92	0.92	0.92	0.92	0.93	0.92	1.00		
L. pinastri	Aa6	0.95	0.92	0.92	0.93	0.92	0.91	0.92	0.99	1.00	
L. macci	AF540560	0.92	0.92	0.93	0.94	0.95	0.94	0.94	0.91	0.91	1.00
L. conigenum	Ab2	0.89	0.88	0.89	0.90	0.91	0.90	0.90	0.88	0.88	0.90
L. conigenum	AF473559	0.89	0.87	0.89	0.90	0.91	0.90	0.90	0.88	0.88	0.90
L. australe	U92308	0.90	0.89	0.89	0.90	0.89	0.89	0.87	0.88	0.88	0.89
L. indianum	AY100642	0.91	0.89	0.89	0.90	0.91	0.90	0.89	0.91	0.91	0.90
L. seditiosum	Ac2	0.89	0.87	0.90	0.92	0.90	0.91	0.89	0.89	0.89	0.90
L. seditiosum	AF473553	0.89	0.87	0.90	0.92	0.91	0.91	0.90	0.89	0.89	0.90
L. baculiferum	DQ406801	0.88	0.88	0.88	0.91	0.91	0.91	0.88	0.86	0.86	0.91
L. nitens	DQ406798	0.94	0.94	0.94	0.94	0.95	0.95	0.94	0.94	0.94	0.94
L. piceae	AY971734	0.94	0.94	0.94	0.94	0.95	0.94	0.94	0.93	0.93	0.95
L. minor	AY100665	0.90	0.90	0.90	0.90	0.90	0.89	0.92	0.89	0.89	0.91

		Ab2	AF473559	U92308	AY100642	Ac2	AF473553	DQ406801	DQ406798	AY971734	AY100665
L. pinastri	Aa1										
L. pinastri	Ba11										
L. pinastri	AY775701										
L. pinastri	Aa2										
L. pinastri	Ba8										
L. pinastri	AY100649										
L. kumaunicum	EU696777										
L. pinastri	Aa4										
L. pinastri	Aa6										
L. macci	AF540560										
L. conigenum	Ab2	1.00									
L. conigenum	AF473559	0.99	1.00								
L. australe	U92308	0.94	0.94	1.00							
L. indianum	AY100642	0.96	0.96	0.95	1.00						
L. seditiosum	Ac2	0.90	0.90	0.92	0.93	1.00					
L. seditiosum	AF473553	0.90	0.90	0.91	0.93	0.99	1.00				
L. baculiferum	DQ406801	0.89	0.89	0.88	0.87	0.87	0.87	1.00			
L. nitens	DQ406798	0.91	0.91	0.90	0.92	0.93	0.93	0.89	1.00		
L. piceae	AY971734	0.90	0.90	0.90	0.90	0.92	0.92	0.90	0.92	1.00	
L. minor	AY100665	0.86	0.86	0.91	0.86	0.89	0.89	0.85	0.93	0.85	1.00


L. pinastri Aa9
L. pinastri Aa7
L. pinastri Aa12
L. pinastri Aa2
L. seditiosum Ac1
L. seditiosum Ac2
L. conigenum A...
L. conigenum A...
L. conigenum Ab1
L. pinastri Aa1
L. pinastri Aa29
L. pinastri Aa28
L. pinastri Aa8
L. pinastri Aa6
L. pinastri Aa4
L. pinastri Aa13
L. pinastri Aa23
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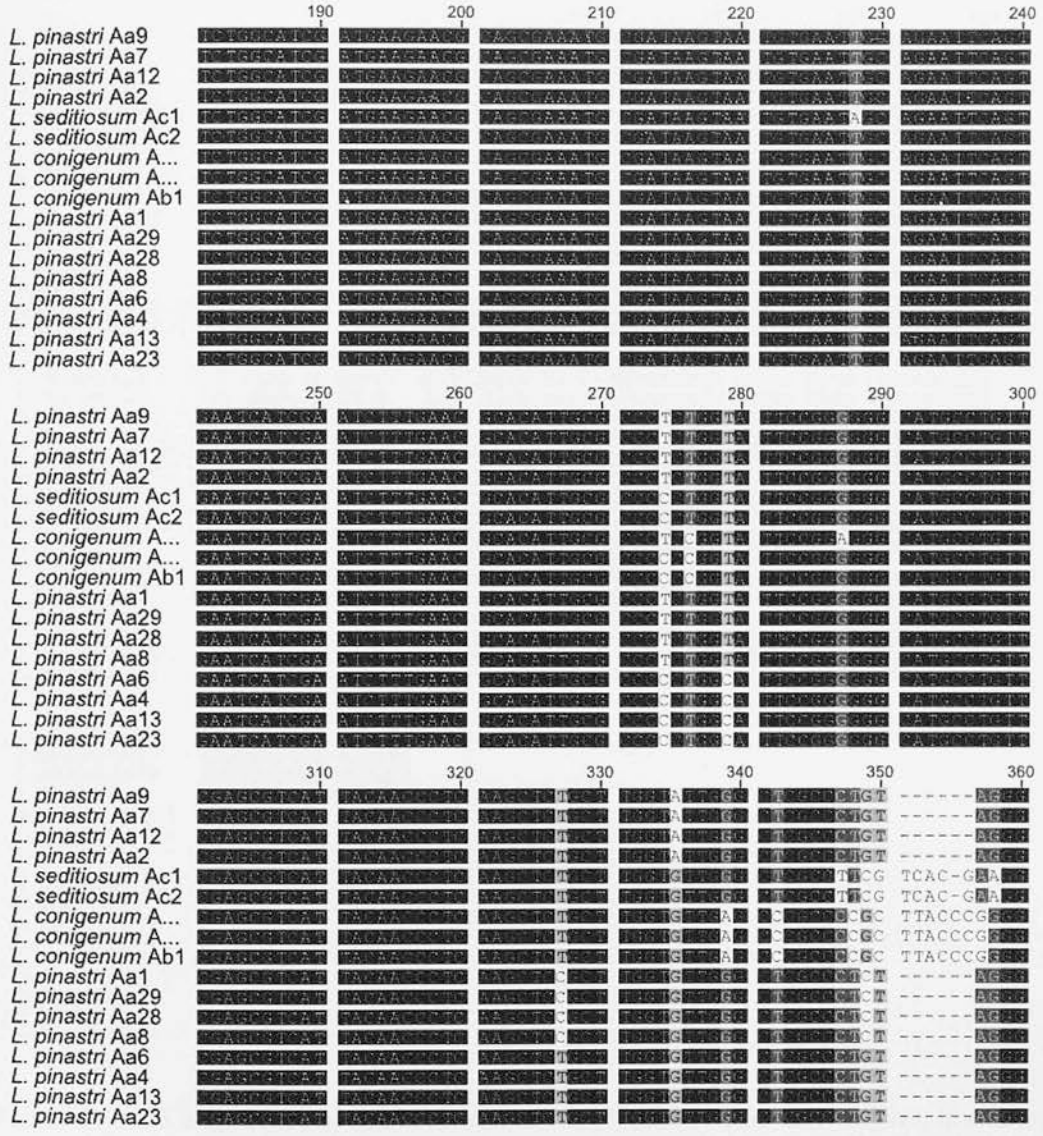
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L. pinastri Aa9
L. pinastri Aa7
L. pinastri Aa12
L. pinastri Aa2
L. seditiosum Ac1
L. seditiosum Ac2
L. conigenum A...
L. conigenum A...
L. conigenum Ab1
L. pinastri Aa1
L. pinastri Aa29
L. pinastri Aa28
L. pinastri Aa8
L. pinastri Aa6
L. pinastri Aa4
L. pinastri Aa13
L. pinastri Aa23

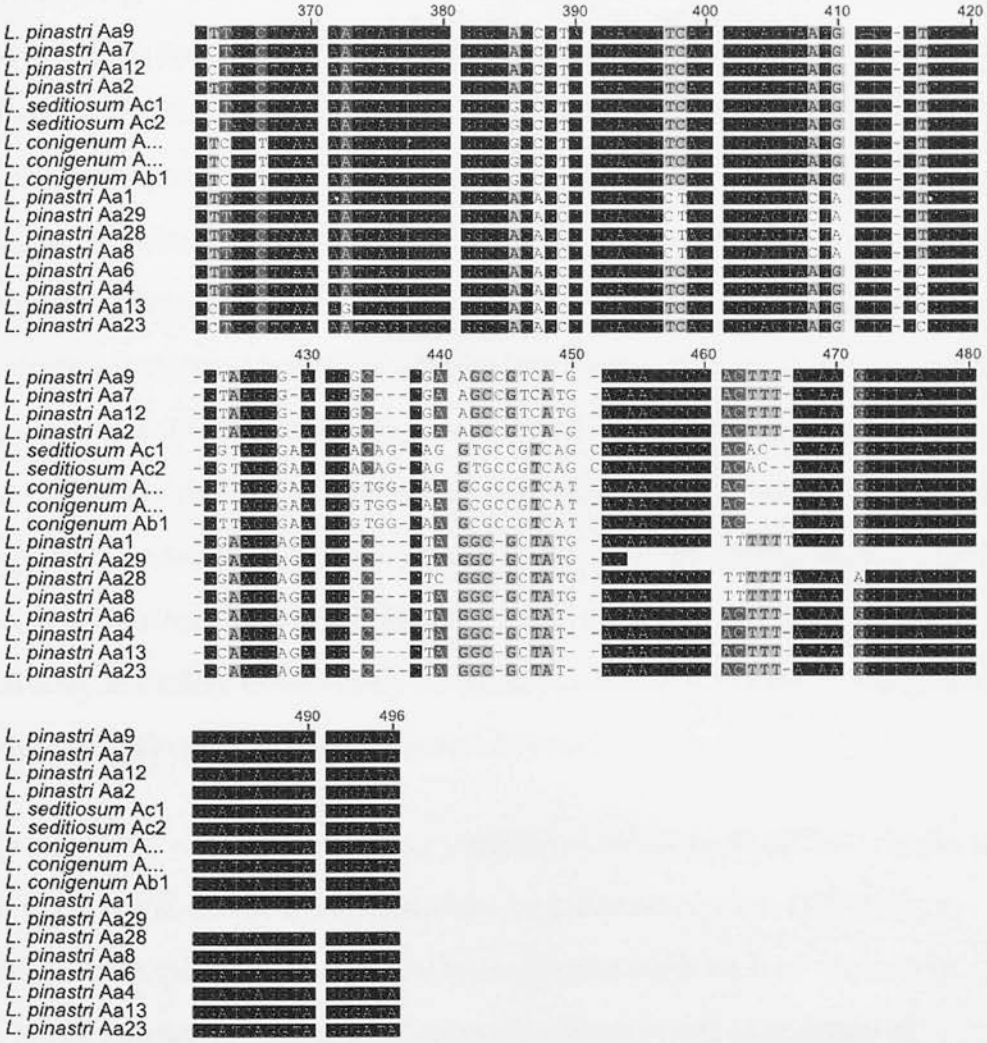

Figure 2.4: ITS alignment of *Lophodermium* isolates from litter and broken branches of the native forest of Glen Affric (Scotland). Dark shade represent bases which are identical between isolates and lighter shades represent bases which are different between isolates. Box shade was compiled using Geneious 5.0 2 (Drummond et al 2007).

2.3.3 Phylogenetic Analysis of the ACTIN Sequences

The phylogenetic tree revealed five monophyletic clades (Figure 2.5) which were well supported by posterior probabilities of over 95 % for four of the clades and 77% for one clade (Figure 2.5). Three of the clades comprised isolates collected from litter needles which had similar characters to *L. pinastri* in culture. Thus, the isolates comprising these clades were named *L. pinastri* I, *L. pinastri* II and *L. pinastri* III (Figure 2.5). Isolates from broken branches formed a separate monophyletic clade which included two species, *L. conigenum* and the pathogen *L. seditiosum*, which formed sister lineages nested within this clade. According to this phylogenetic tree (Figure 2.5) the different *L. pinastri* lineages diverged at the same time as the lineage from broken branches. This latter lineage diverged further into the endophyte fruiting on broken branches (*L. conigenum*) and the pathogen *L. seditiosum* which occasionally fruits in the same habitat.

These results were obtained after 2000000 generations of MCMC chains. Split frequency was 0.005940 and the mean Log likelihood was -1153 with an effective sample size of 6258. Analysis of parameters such as nucleotide frequency, substitution rates, Gamma distribution and proportion of invariable sites revealed that the choice of GTR + Γ + I was the most suitable model for all partitions.

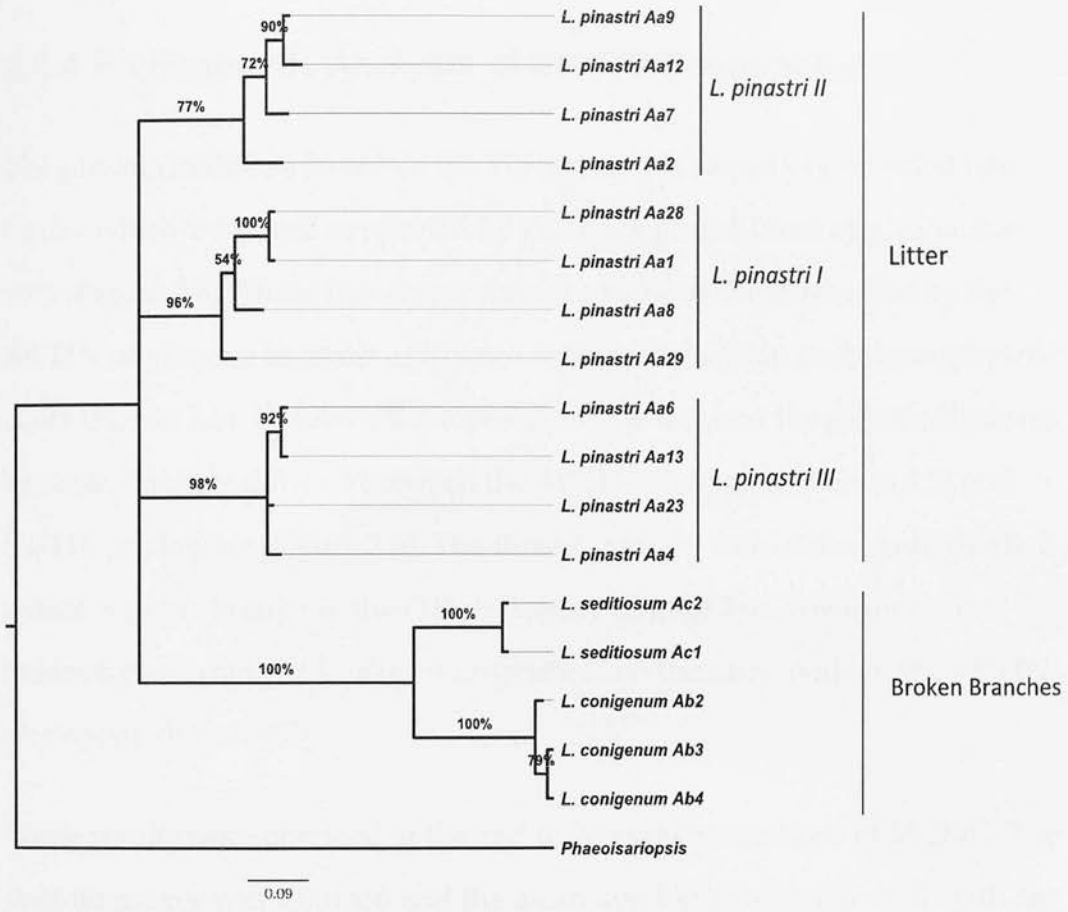

Figure 2.5: Bayesian inference of phylogeny at the ACTIN locus from 9295 trees. Posterior probabilities are represented above the branches. The outgroup is *Phaeiosariopsis*. 'Litter' and 'Broken Branches' represent the habitat where these species are commonly found.

2.3.4 Phylogenetic Analysis of the ITS Sequences

The phylogenetic tree based on the ITS nucleotide sequences revealed five clades which were well supported by posterior probabilities of greater than 90% (Figure 2.6). These five clades were the same as those revealed by the ACTIN phylogeny in terms of isolates represented within each monophyletic clade (Figure 2.5). However the topology of the tree and the genetic distances between lineages differed between the ACTIN phylogeny (Figure 2.5) and the ITS phylogeny (Figure 2.6). The three *L. pinastri* were sister clades nested within a single lineage in the ITS phylogeny (Figure 2.6), however each monophyletic clade of *L. pinastri* originate from the same node in the ACTIN phylogeny (Figure 2.5).

These results were obtained at the end of 2000000 generations of MCMC. The split frequency was 0.001455 and the mean Log likelihood was -1687 with an effective sample size of 5444. As with the ACTIN, Bayesian analysis revealed that GTR + Γ + I was the most suitable model for all partitions (hypervariable and conserved).

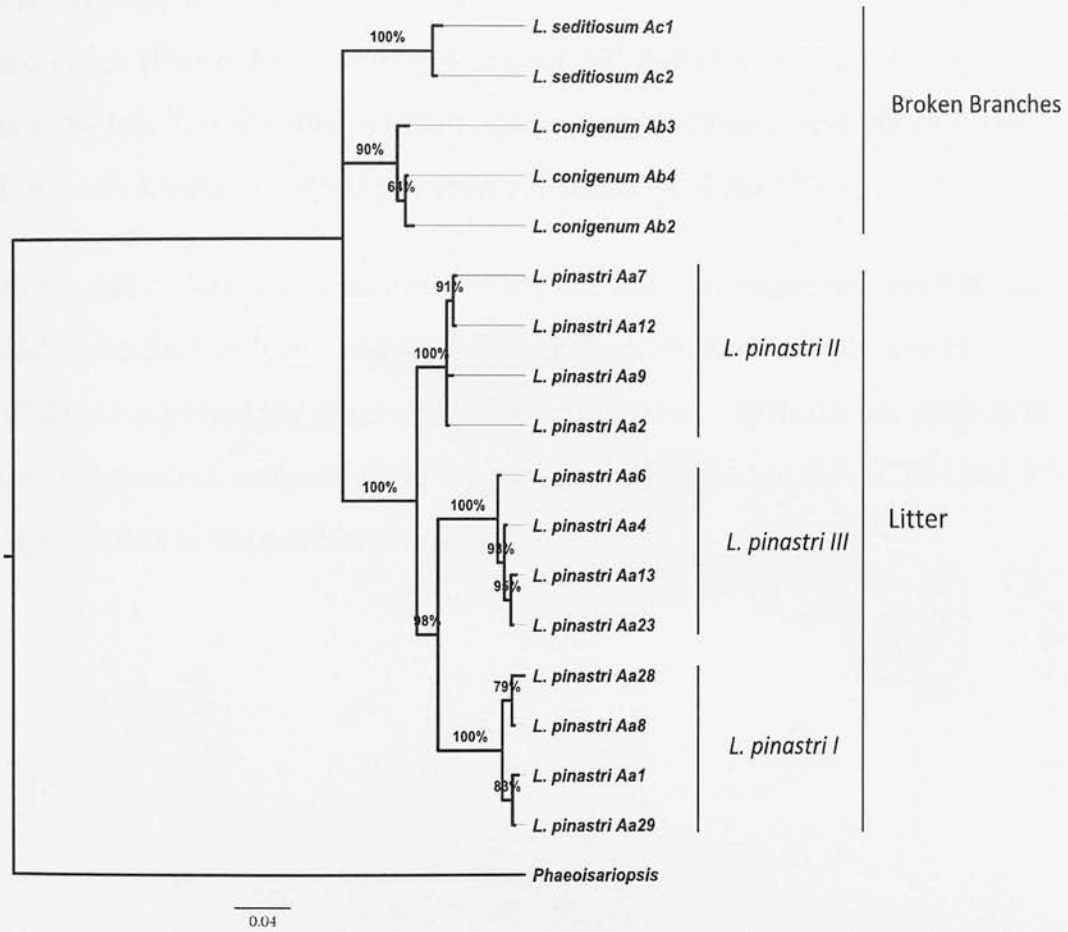

Figure 2.6: Bayesian inference of phylogeny of the partial ribosomal DNA from 6075 trees. Posterior probabilities are represented above the branches. The outgroup is *Phaeiosariopsis*. 'Litter' and 'Broken Branches' represent the habitat where these species are commonly found.

2.3.5 Combined analysis of ACTIN and ITS sequences

The topology of the phylogenetic tree based on the combined ACTIN and ITS sequences (Figure 2.7) most closely resembled that of the ITS phylogeny (Figure 2.6). The five monophyletic clades were congruent and the all three *L. pinastri* lineages retained posterior probabilities of over 95%.

At the end of 5000000 generations of MCMC the split frequency was 0.001424 and the mean Log likelihood was -2876 with an effective sample size of 13884. As expected the parameters estimation were very much the same as in the independent analyses. Only the pinvar of the exon 1 of the ACTIN had a higher value in the combined analysis.

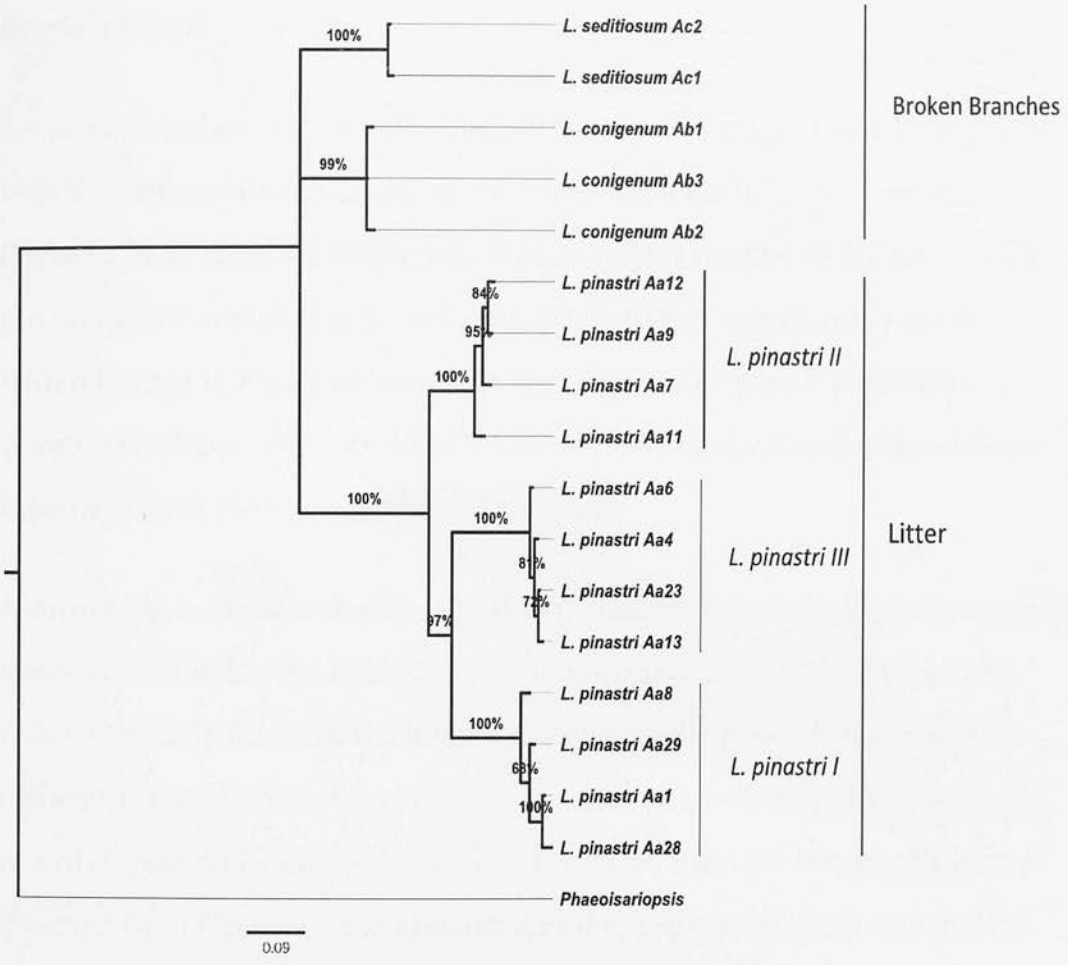

Figure 2.7: Bayesian inference of phylogeny of the combine analysis of both partial ACTIN and partial ribosomal DNA from 2128 trees. Posterior probabilities are represented above the branches. The outgroup is *Phaeiosariopsis*. 'Litter' and 'Broken Branches' represent the habitat where these species are commonly found.

2.3.6 Inclusion of *Lophodermium* sequences from Genbank

The phylogenetic tree based on ITS sequences from the 18 Scottish isolates as well as *Lophodermium* ITS sequences from Genbank revealed that the two sister lineages of *L. pinastri* (I and III) formed a separate clade from the lineage II, and included another clade represented by *L. macci* Sokolski &

Bérubé (2004)(Figure 2.8).

All of these isolates in clades containing *L. pinastri* lineages I and III, together with the clade containing *L. pinastri* II, were collected from litter needles (Figure 2.8). *L. macci* AF540560 was found on litter needles of the five-needle pine species *P. strobus.* Likewise *L. pinastri* AY100649, which was placed within lineage II, is a symbiont of another five needle pine *P. ponderosa.* Another Genbank sequence AY247753 from New Zealand was isolated from litter needles of the two needle pine *P. pinaster.*

A surprising result was that lineage II of *L. pinastri* included a *Lophodermium* species described in the Himalayas (*L. kumaunicum* EU696777) (Figure 2.8) which is usually found in the litter of the five needle pines *P. kesiya* and *P. roxburghii.* The exact source of this isolate was not specified in the Genbank record. *L. pinastri* I included records of *L. pinastri* from the two needle pine *P. sylvestris* from Germany and Finland. Another intriguing result was that no records of *L. pinastri* lineage III were present in the Genbank sample.

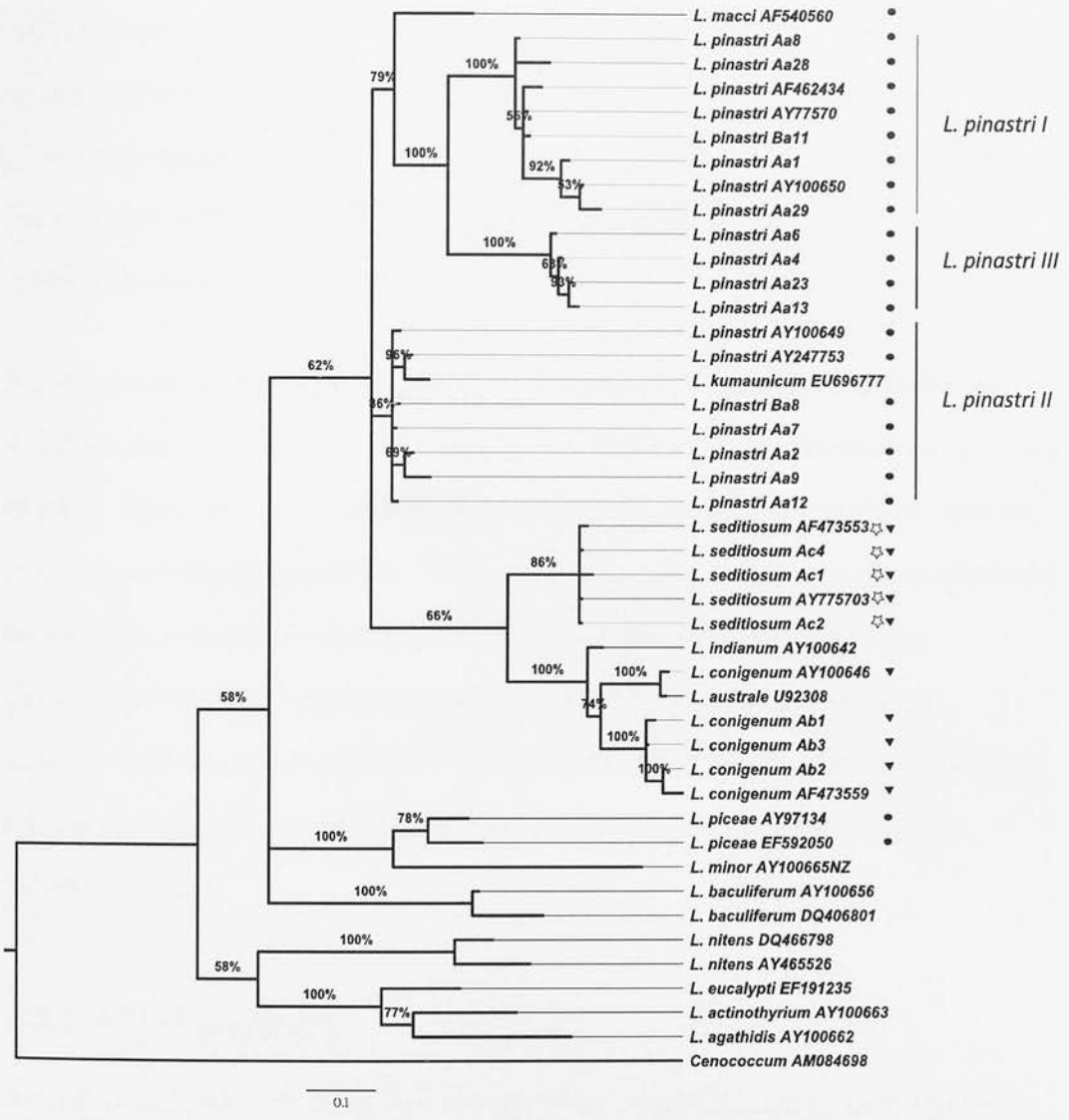

Figure 2.8: Bayesian inference of phylogeny of the partial ribosomal DNA from 99247 trees. Posterior probabilities are represented above the branches. The outgroup is *Cenococcum*. Full circles represents taxon which are mostly found in the litter; full triangle represent those which occur mostly on broken branches; stars represents those which are known to cause diseases.

The Scottish *Lophodermium* isolates collected from needles on broken branches formed a separate clade to isolates from litter needles. The broken

branch clade included Genbank sequences of the pathogen *L. seditiosum* and the endophyte *L. conigenum* (Figure 2.8). This clade appears to have diversified earlier than the clades representing *Lophodermium* isolates derived from litter needles. This would need to be confirmed upon application of a molecular clock to the analysis.

For this analysis the split frequency at the end of 5000000 generations was 0.020785 and the mean Log likelihood was -3803 with an effective sample size of 2479. There were five substitution rates in the conserved partition and six in the hypervariable partition. These rates were different from those obtained by analysis of the ITS sequence based on Scottish isolates alone. The nucleotide frequencies remained very similar to those in the previous analysis and the gamma shape of the substitution rates was more convincing than that of the phylogenetic analysis of ITS based on a more restricted number of taxa.

2.3.7 AFLP analysis

A total of 822 peaks of Amplified Fragment Length Polymorphism (AFLP) markers were scored ranging between 50 and 500bp. Each *Lophodermium* taxon produced between 155 and 257 peaks. The primer combination with the highest scores was AAC/CT with 139 peaks (Table 2.5) and the average per primer combination was 103±30. On average, markers were very polymorphic for all primer combinations (Table 2.6). In terms of percentage polymorphic loci, *L. conigenum* was the least polymorphic (77.17%) and *L.*

seditiosum the most polymorphic (98.36%). Among the three *L. pinastri* lineages, *L. pinastri* III was the least polymorphic (84.92%) compared with *L. pinastri* I (96.24%) and *L. pinastri* II (94.81%) (Table 2.6).

Table 2.5: AFLP marker number per species per primer combinations

primer combination	Total	*L. pinastri* I	*L. pinastri* II	*L. pinastri* III	*L. conigenum*	*L. seditiosum*
AAC/CAA	107	27	24	23	24	25
AAC/CG	135	31	33	33	43	26
AAC/CT	139	24	48	39	45	23
AAC/CC	130	26	46	33	40	25
AAC/CAGA	93	26	28	7	31	22
AAC/CTA	80	18	34	10	23	9
AAC/CCG	79	20	32	13	17	11
AAC/CAG	59	12	12	5	21	14

Two Principal Coordinate (PCO) plots were derived from the matrix of Jaccard coefficient similarity indices based on AFLP data. The 17 of the 18 isolates from Glen Affric included in the phylogenetic analyses were used in these plots. One plot included isolates of *L. conigenum* and *L. seditiosum* as well as *L. pinastri* (Figure 2.9). The second (Figure 2.10) only included *L. pinastri* isolates. Both PCO plots clearly distinguished the different lineages of *L. pinastri* recognised in phylogenetic analyses.

Table 2.6: Percentage of polymorphic loci for each *Lophodermium* taxon

primer combinations	*L. pinastri* I			*L. pinastri* II			*L. pinastri* III			*L. conigenum*			*L. seditiosum*		
	total number of peaks	number of polymorphic peaks	% poly morphic	total number of peaks	number of polymorphic peaks	% poly morphic	total number of peaks	number of polymorphic peaks	% poly morphic	total number of peaks	number of polymorphic peaks	% poly morphic	total number of peaks	number of poly morphic peaks	% poly morphic
AAC/CC	39	37	95	70	67	96	36	27	75	35	22	63	51	47	92
AAC/CAA	30	30	100	33	33	100	26	26	100	17	13	76	23	23	100
AAC/CT	23	17	74	32	24	75	32	29	91	35	29	83	39	39	100
AAC/CG	23	20	87	39	38	97	29	19	66	27	20	74	38	36	95
AAC/CAG	22	19	86	18	15	83	12	9	75	23	20	87	26	26	100
AAC/CTA	22	21	95	36	35	97	26	24	92	17	14	82	14	14	100
AAC/CAGA	18	18	100	27	27	100	10	9	90	24	21	88	25	25	100
AAC/CCG	22	22	100	19	19	100	11	10	91	14	9	64	22	22	100
Average	25±7	23±7	92±9	34±16	32±16	94±9	23±10	19±9	85±12	24±8	19±6	77±10	30±12	29±11	98±3

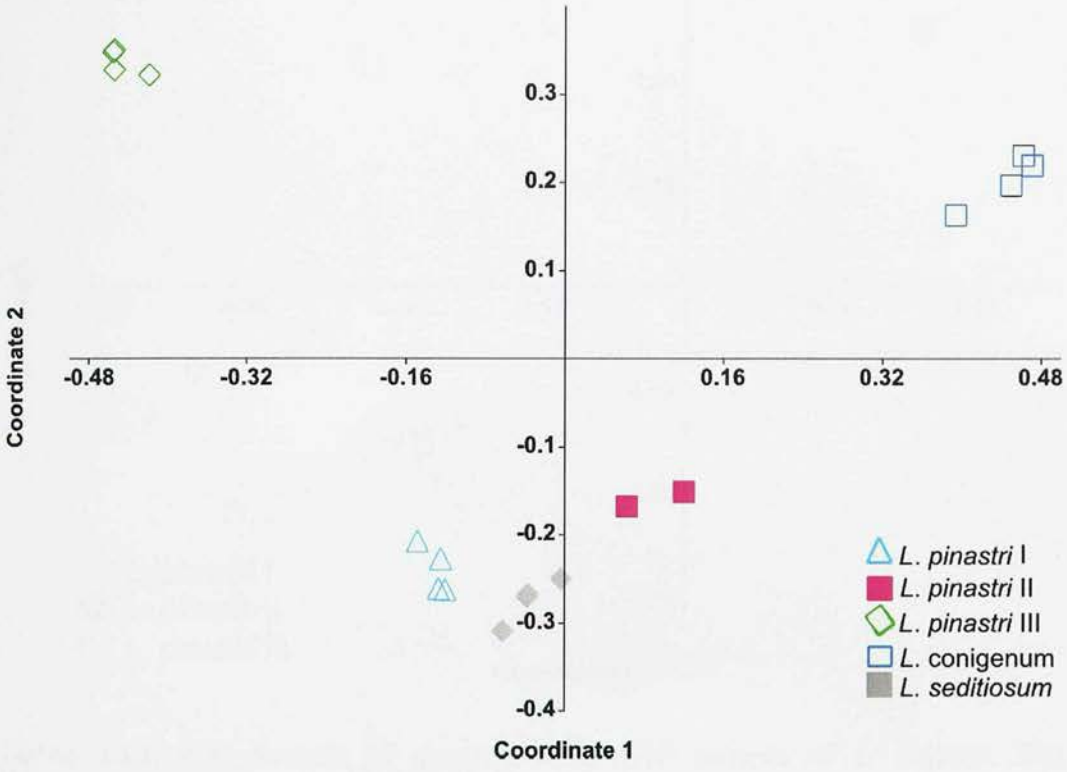

Figure 2.9: PCO analysis of genome wide AFLP analysis of *Lophodermium* species from *P. sylvestris.* The percentages of explained variation are 25% and 16.5%. Phylogenetic lineages are colour coded: Cyan: *L. pinastri* I, Magenta: *L. pinastri* II, Green: *L. pinastri* III, Grey: *L. seditiosum,* Blue: *L. conigenum*.

Figure 2.10: PCO analysis of genome wide AFLP analysis of *L. pinastri*. The percentages of explained variation are 26% and 20%. Lineages are colour coded: Cyan: *L. pinastri* I, Magenta: *L. pinastri* II, Green: *L. pinastri* III.

2.4 Discussion

The most important result to emerge from this study is that five different *Lophodermium* taxa can be identified from needles of a single Scots pine population in Scotland using molecular methods. Two of these taxa correspond to *L. seditiosum* and *L. conigenum* described by Minter and Millar (1980a). The remaining three taxa fall within the previously described taxon *L. pinastri*. These three cryptic taxa are hereafter referred to as *L. pinastri* I, II and III.

The three cryptic taxa within *L. pinastri* have been identified using two independent molecular techniques. In the first, sequence information from two independent loci, ITS and ACTIN has been utilised in Bayesian phylogenetic analysis for Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor *et al.* 2000). The same three strongly supported clades have been found within *L. pinastri* using each of the two loci independently and in analysis of the combined sequences, though the topology of the trees is slightly different in each case. The advantage of Bayesian probability with Markov Chain Monte-Carlo chain is that parameters of a chosen evolutionary model can be estimated and the model can be changed if necessary. This is especially important when different loci with different rates of evolution are being analysed to produce a joint phylogeny.

GCPSR has been used in a number of other studies to detect cryptic

endophytic species. One of these, *Trichoderma koningii,* is known to protect *Theobroma cacao* from pathogens (Samuel *et al.*, 2006). Bayesian phylogenetic analysis of three genes (partial ACTIN, Translation elongation factor EF-1-α and partial Calmodulin) detected as many as eleven cryptic species within *Trichoderma koningii* (Samuel *et al.*, 2006). GCPSR has also been used to clarify classification of *Botryosphaeria*. Here a complex of two species was found within *Botryosphaeria lutea,* an endophyte of acacia and eucalyptus species (Slippers *et al.*, 2004).

The second method employed in this study to recognise cryptic species uses a population genetic approach. Genome wide genetic markers generated by AFLP analysis have been used to estimate genetic distance between isolates, and this information has been used in a phenetic clustering procedure. The three clusters found within *L. pinastri* using this analysis correspond exactly to the three lineages within *L. pinastri* found in the phylogenetic analysis. The concordance between the two approaches provides very powerful evidence that the three genetically distinct groups found by molecular methods within *L. pinastri* represent real taxonomic entities and should be treated as true, though cryptic, species. These results, if extrapolated to other groups, add further weight to the argument that fungal diversity, especially in endophytic fungi, has been substantially underestimated as suggested by Hawksworth (2001).

Although the number of isolates used in this analysis was small, it is nevertheless clear that all three *L. pinastri* lineages co-occur within a single

native Scots pine population in Scotland. In addition lineages I and II have been detected both at Glen Affric and at Amat suggesting widespread distribution of the cryptic species. The fact that only two isolates from Amat were used might explain why *L. pinastri* III is missing from the Amat sample. The incorporation of Genbank sequences of *L. pinastri* in the phylogenetic analysis allows these isolates to be classified into the three lineages detected here. Before this study *L. pinastri* II had not been found in continental Europe, and no sequences of *L. pinastri* III had been lodged in Genbank. This emphasises that using Genbank records to assess the geographical distribution of endophyte taxa can be misleading. The fact that *L. pinastri* III sequences have not previously been deposited in Genbank may suggest that it exists at a low frequency and has been overlooked in previous sampling. It would therefore be interesting to determine the relative frequency of the *L. pinastri* cryptic species in Scotland, continental Europe or even world wide.

One unexpected finding in the wider ITS phylogenetic analysis is that an isolate determined as *L. kumaunicum* Minter & M.P. Sharma (1982) was included within the *L. pinastri* II clade. Could this mean that *L. pinastri* II is in fact *L. kumaunicum*? The description of this species is quite different from that of *L. pinastri* (Minter *et al.*, 1978; Minter and Sharma, 1982). When wet or dry, *L. kumaunicum* has a totally black, elongated rhombic ascocarp with a clear margin. In contrast *L. pinastri* has an ascocarp which is black in the middle with a grey surround and an outer black line when it is dry. From Minter and Sharma's description of the species it appears that the fruiting bodies of *L. kumaunicum* co-occur with another *Lophodermium* species which resembles *L.*

pinastri morphologically (Minter and Sharma, 1982). Since the present analysis was undertaken, some more sequences of *L. kumaunicum* (GU138702 and FJ861981) have become available in Genebank. Alignment of these using Blastn gives a nucleotide similarity of 88% to *L. pinastri* II (AY100649) and *L. kumaunicum* (EU696777) as opposed to 98% similarity between *L. pinastri* II (AY100649) and the *L. kumaunicum* isolate (EU696777) used is this study. Therefore it is very likely that in the earlier work *L. pinastri* II was isolated and sequenced instead of *L. kumaunicum* and the isolate (EU696777) is in fact *L. pinastri* II. This finding emphasises the need for caution in relying on the taxonomic information associated with fungal sequences deposited in Genbank.

Having used detailed phylogenetic and population genetic analysis to delimit cryptic species in *L. pinastri,* it is informative to determine whether a barcoding approach based on ITS sequence similarity could be used to recognise these cryptic species. The use of less than 5% nucleotide variation between ITS sequences appears to be a reasonable threshold for identifying and delimiting species of *Lophodermium*. This would allow for variation not accounted for because of the sampling strategy used in this study. However high intraspecific variation can exist within a species due to poor lineage sorting in recently diverge species, this may be mis-interpreted as a signal of the presence of cryptic species. Poor sampling of the reference taxon could also underestimate the anticipated variation among individuals of the same species (Meyer & Paulay, 2005). Further practical problems with single locus barcoding are that the lack of species representation and the presence of

misidentified sequences in Genbank means that care and common sense should be used when making a decision on the identification of an isolate based on DNA barcoding methods. The potential of the partial ACTIN gene to act as another barcoding locus should be noted. 5% nucleotide variation threshold appears to be applicable for the ACTIN gene to. However very few ACTIN sequences are present in Genbank or other databases making its use restricted at present. Using more loci with a greater sampling scheme covering wider geographical area and host would enable a more accurate taxon delimitation and may highlight the true reliability of the markers used in this study.

With the aim of identifying closely related sympatric *Lophodermium* species an alternative option to sequencing may be to use species-specific primers based on barcoding markers such as ITS to identify the species. This approach has already been followed in an attempt to distinguish *L. seditiosum* and *L. pinastri* based on the ITS locus (Stentrom and Ihrmark, 2005). However the *L. pinastri* primers designed for this assay are not ideal because they fail to amplify *L. pinastri* lineages II and III. This emphasises how different these lineages are from *L. pinastri* I, and indicates the importance of clarifying the taxonomy of the fungal group being studied before designing molecular assays for their detection. A more traditional method for identification of the cryptic species on a large scale would be to find reliable characters in culture that can be used to discriminate between cryptic species. This will be attempted in the next chapter.

Although all the *Lophodermium* species which are associated with the two needle pine *P. sylvestris* are grouped within one clade, one of these taxa, *L. pinastri* II, is also found on five needle pines (Table 1 and Figure 8). Although host sampling is limited this suggests that *Lophodermium* species are mostly host specific but with some low levels of host switching occurring among pine hosts. Host switching is most likely to occur in artificial situations such as in arboreta where a variety of exotic species are grown together, or after introduction of the normal host into areas containing other indigenous pine species at high density. An example of this might be the introduction of *P. sylvestris* into North America for the production of Christmas trees.

From the phylogenetic analyses that include only the isolates from Scottish pine populations it is difficult to infer the evolution history of the cryptic *L. pinastri* taxa because of the inconsistency of node placements between all species across the two loci and the occurrence of polytomy (nodes with more than two branches). It is unlikely that the phenomenon of polytomy is due to a recent rapid speciation event giving rise to multiple branching at one particular node. This is because the clades within the polytomy are both well supported and congruent between the ITS and Actin loci, indicating that lineages are well sorted and new mutations have been fixed between taxa.

One other problem is the movement of the *L. pinastri* II clade from within the nested *L. pinastri* clade in the ITS and combined ITS/ACTIN tree (Figures 6 and 7) to a position in an independent clade in the large ITS tree and the ACTIN tree (Figures 5 and 8). The ITS phylogenetic tree which includes

database sequences is better resolved. However the posterior probability for the clade which includes all taxa infecting *P. sylvestris* is only 62%. This is likely to be due to the difficulty of placing *L. pinastri* II. It is possible that if more *Lophodermium* taxa were sampled and incorporated in this phylogeny, better resolution would be obtain and *L. pinastri* II would be positioned with more certainty.

One consistent result that emerges from the phylogenetic analyses is that in this particular system *L. conigenum* and *L. seditiosum* which tend to fruit on broken branches have diversified separately from the litter species. This would suggest that different modes of selection are imposed on these different groups of species, and the ecological niche of fruiting may play an important role in this selection. For instance the nutrient availability in naturally senesced needles will be much lower than that in prematurely killed needles. There will be greater selection for ability to 'retain territory' to exploit in naturally senesced needles. There may therefore be greater selection for barriers to keep out competitors, and this may account for the greater production of zone lines shown by *L. pinastri* than in *L. conigenum* and *L. seditiosu*m. Although these results do not categorically confirm that endophytes are precursor to pathogens in the evolution of these organisms as suggested by Ortiz-Garcia *et al.* (2003), this trend is noticeable in the large ITS tree which includes Genbank isolates (Figure 2.8).

Lophodermium is a genus that is ubiquitous in pines needles. It includes an ever growing number of species which have been morphologically described

(Minter *et al.*, 1978; Minter and Sharma, 1982) and in which, as in this study, a molecular approach has been combined with a morphological approach to recognise cryptic taxa (Sokolski *et al.*, 2004; Hou *et al.*, 2009). This is adding to the understanding of the diversity of fungal endophytes and fungi in general. In the genus *Lophodermium* saprophytes and endophytes which often represent different stages of their life cycles dominate this diversity compared to pathogens such as *L. seditiosum*. In order to determine why these endophytes are so speciose and successful, and to understand their ecological role, more information is required on their distribution among Scots pine populations, their genetic diversity and their population genetic structure. These topics will be addressed in a later chapter of this thesis.

Finally, the lack of complete congruence between the two loci could be explained by ancestral polymorphism for the ACTIN and/or history of gene duplication and extinction for ITS (Álvarez and Wendal, 2003; Maddison, 1997; Pamilo and Nei, 1988). Improvement of this study could be made by using many more loci and a more thorough sampling both at the species level as well as geographical in order to look for the most correct phylogeny.

Chapter 3: Cryptic Taxa Within *Lophodermium pinastri*: detection, frequency, and growth rate

3.1 Introduction

Endophytes are organisms which live asymptomatically within plants for at least part of their life cycle (Wilson, 1995). Endophytes are ubiquitous to all plants and are present in every plant organ (Rodriguez *et al.*, 2009; Arnold, 2007). They comprise a wide range of taxa from bacteria to fungi. Furthermore they display a wide spectrum of interactions with their hosts which can range from latent pathogenicity to strictly obligate symbiosis such as that seen in the well studied group of grass endophytes (Carroll, 1988).

While grass endophytes show many mutualistic interactions with their host, such as protection of the host plant against herbivores and enhancement of growth, the ecological roles of other distinct groups of endophytes are not always so clear (Rodriguez *et al.*, 2009). It has been suggested that some pine

needle endophytes may compete with related pathogen species and prevent invasion of host tissue by the pathogen. If this were true, then there is the potential for use of these endophytes in the control of *Lophodermium* needlecast disease (Minter, 1981). Other studies have provided evidence that the diverse community of endophytes of *Theobroma cacao* leaves helps to protect them against *Phytophthora* species (Arnold *et al.*, 2003). Furthermore there are a number of examples where endophytes have been shown to confer protection against herbivores (Carroll, 1988).

A major challenge in understanding the functioning and ecological role of these communities lies in accurately identifying the taxa involved and estimating the diversity of these communities. Only when this has been achieved is it possible to explore how endophyte species relate to and interact with pathogens and hosts, and to understand how these interactions are affected by environmental factors such as climate.

The ascomycete genus *Lophodermium* (anamorph *Leptostroma*) includes a large number of taxa that are ubiquitous endophytes of pine (Hata & Futai, 1996). *Lophodermium* also includes one pathogenic species, *L. seditiosum*, causing a potent disease which attacks vulnerable young seedlings (Skilling & Nicholls, 1975; Diwani & Millar, 1987). The disease is characterised by premature needle loss which causes death and growth reduction of seedlings leading to great losses in nurseries. One major interest in this system comes from the suggestion that the endophytic *Lophodermium* species could potentially be used to control needle-cast disease of one particular pine

species, *Pinus sylvestris* L.(Minter, 1981).

The number of endophytic *Lophodermium* species associated with *P. sylvestris* has been subject to revision over the years. *L. pinastri* was first thought to be the only species associated with the production of ascocarps on *P. sylvestris* needles. It was believed that this was a very variable species which could act as an endophyte, a saprophyte and a pathogen. It was then demonstrated that four species of *Lophodermium* were able to colonise needles of *P. sylvestris* and that these were ecologically distinct. The first of these was *L. seditiosum* which was shown to be the causal agent of *Lophodermium* needle-cast disease (Minter *et al.*, 1978; Diwani & Millar, 1987). The other two common *Lophodermium* species associated with *P. sylvestris* needles were *L. pinastri* and *L. conigenum* which can be isolated from asymptomatic needles. The ascocarps of these three taxa can be distinguished on the basis of their morphology. *L. pinastri* fruits on litter needles, while both *L. conigenum* and *L. seditiosum* fruit on needles from damaged branches (Minter & Millar, 1980). A fourth species, *L. staleyi* previously thought to be *L. pini-excelsae*, was found at low frequency on litter needles (Minter & Millar, 1980; Minter, 1981a).

In chapter two of this thesis it was demonstrated that *P. sylvestris* litter needles are the substratum for three species of endophytic *Lophodermium* which were previously described as the single taxon *L. pinastri*. These cryptic taxa, tentatively named *L. pinastri* I, *L. pinastri* II and *L. pinastri* III were recognised based on sequence variation and phylogenetic analysis of two unlinked loci, as well as genome wide analysis of AFLP markers. It is

assumed that each of these cryptic species is able to infect healthy needles. Such an assumption is based on the widespread ability of taxa within *Lophodermium* for adopting an endophytic lifestyle (Hata & Futai, 1995; Diwani & Millar, 1987; Kowalski, 1982).

Having clarified the taxonomy of the endophytic species of *Lophodermium* on *P. sylvestris,* there is now the opportunity to investigate the population biology and genetics of *Lophodermium* endophytes and compare them to their related pathogen species in order to learn about the factors influencing their ecology and evolution. To make use of this opportunity it is crucial to have an identification procedure in place that is capable of distinguishing among species easily and accurately. Species-specific primers have previously been designed to specifically amplify the Internal Transcribed Spacer (ITS) region of *L. seditiosum* and *L. pinastri* (Stenstrom & Ihrmark, 2003). However these primers were designed before the discovery of three cryptic species within *L. pinastri*. The first aim of this study was therefore to design and test species-specific primers for amplifying each of the cryptic *L. pinastri*.

Another use for such species-specific PCR assays is to survey the distribution of these cryptic species in natural populations of *P. sylvestris*. There is already some information on the distribution of cryptic species from an analysis of ITS sequences lodged in Genbank. Sequences now recognisable as belonging to *L. pinastri* I have been derived from isolates taken from *P. sylvestris* in Germany. Sequences from *L. pinastri* II have come from *P. ponderosa* in Oregon (USA). However there is no previous Genbank record of a sequence

from *L. pinastri* III. The results from chapter 2 demonstrate that all three species co-occur within the same *P. sylvestris* native stand at Glen Affric. However beyond this we have no understanding of the natural distribution of these three cryptic taxa. A second aim of the work reported here is therefore to determine the distribution of the three cryptic taxa among three native *P. sylvestris* populations in Scotland, as well as a population of this host in France.

Previous work on leaf endophytes of evergreen broad leaves and conifers using traditional culturing and morphological recognition techniques have compared the isolation frequencies of taxa on native and managed host populations (Fisher *et al.*, 1994) and on different hosts (Hata & Futai, 1996). They have also documented changes in the endophyte community composition (Collado *et al.*, 1999) over the seasons and on needles of different age (Kowalski, 1982). Some pioneering endophyte community ecology studies gave evidence for the effect of environmental factors on infection rates (Petrini *et al.*, 1982). However they did not compare endophyte assemblage between the sites investigated. Yet there is evidence that environmental differences such as climate and micro-climate can affect endophyte communities (Sieber *et al.*, 1999; Higgins *et al.*, 2007). In the work described here molecular recognition techniques were used to survey variation in the relative frequency of the three cryptic *Lophodermium* endophytes which we have detected. We ask whether their frequency differs among the populations sampled, and whether the relative frequency of taxa varies among years. This is a first step in investigating how environmental

factors such as the density of host trees, climate etc. might affect the composition of endophyte communities.

Because it is not always possible to have access to molecular methods to identify species and because of the tradition of morphology based description in mycology, it is important to determine whether it is possible to characterise some phenotype differences between the three cryptic *L. pinastri* species that have been detected. In other studies in which cryptic species have been revealed using a molecular approach, significant differences in culture growth rate have been found (Samuel *et al.*, 2006; Grunig & Sieber, 2005). A further objective of this study is therefore to compare the growth rates of the three cryptic species in culture to assess whether this can be used as a means for identifying cryptic species without the need for molecular methods. Finally, in the original description of *Lophodermium* species inhabiting pine needles, Minter *et al.* (1978) made extensive use of ascocarp morphology and the presence of zone lines on needles. A final aim of the work was therefore to see whether ascocarp morphology and presence of zone lines differs among the three cryptic species of *L. pinastri*.

To summarise, the aim of this chapter was to develop a fast and accurate method to distinguish each cryptic species of *L. pinastri*. The method developed consisted of the design of species-specific primers to be used directly on mycelium of young isolated individuals from needles. The design made use of alignments produced for the phylogenetic analysis of ITS sequences described in chapter 2. By scoring large samples of isolates from

four different native pine forests from two countries, Scotland and France, information about the spatial distribution of the taxa was determined. The data were used to find whether co-occurrence of the three *L. pinastri* species is the norm. Isolate collection over three years for two of the Scottish populations also allowed an assessment of dynamic changes in relative frequency of the species over time. Estimations of growth rate in culture for each species were achieved by randomly selecting isolates of the three cryptic species previously identified by species-specific PCR from the three Scottish populations. These were grown under controlled conditions. Finally, attempts were made to detect differences among species in appearance of ascocarps and banding on host needles.

3.2 Material and Methods

3.2.1 Field Collections

L. pinastri isolates were collected from litter needles underlying twenty different Scots pine trees in each of three native forests in Scotland, Loch Maree, Glen Affric, and Abernethy, and one in France, Arlanc (Table 3.1 and Figure 3.1).

Table 3.1: Summary of location and environmental conditions for each of the sites from which isolates of *L. pinastri* were obtained

Sites	Latitude	Longitude	Annual Rain Fall	Temperature	Altitude
Loch Maree	57° 38' 55.84" N	5° 23' 13.48" W	1778-2032 mm	5 – 12°C	10-80 m
Glen Affric	57° 18' 46.23" N	4° 51' 6.23" W	1778 mm	5 – 12°C	180-450 m
Abernethy	57° 13' 33.68" N	3° 40' 32.21" W	762-889 mm	3 – 11°C	210-450 m
Arlanc	45° 24' 9.77" N	3° 41' 43.58" E	275 mm	3 – 14°C	700-800 m

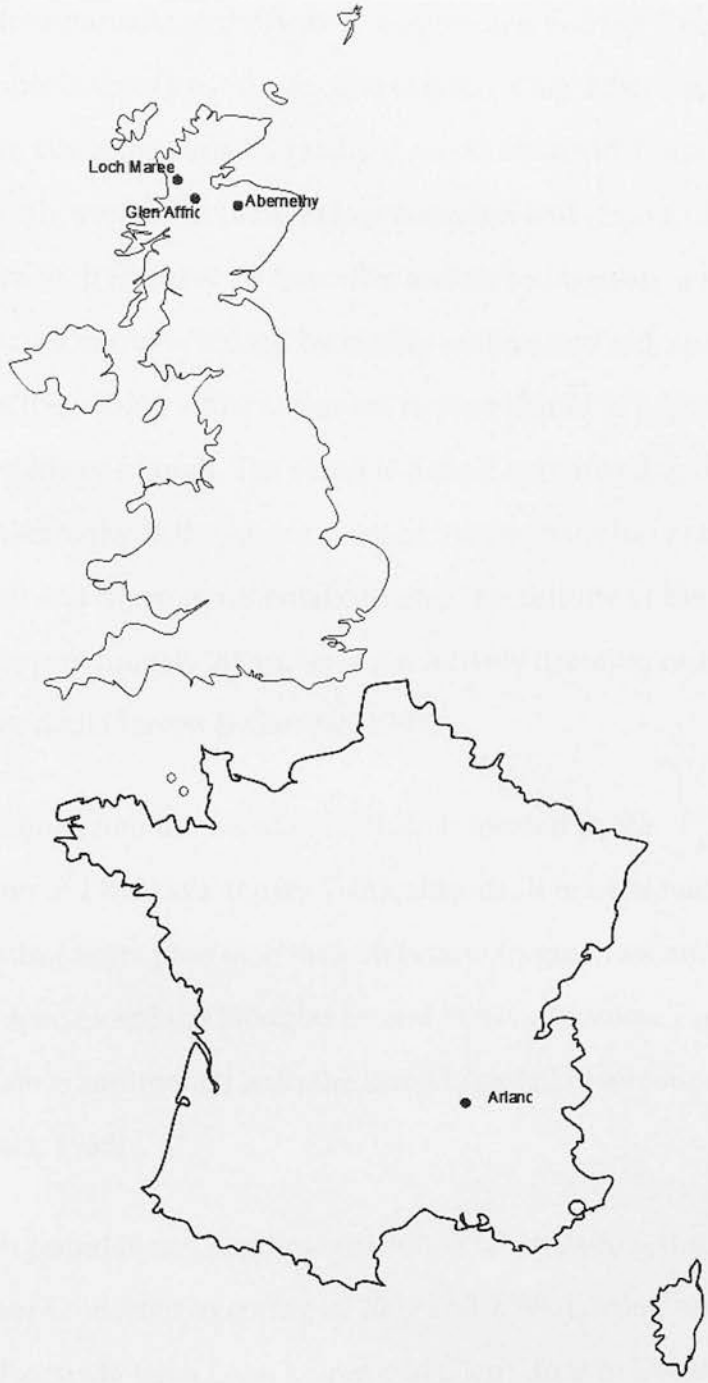

Figure 3.1: Native Scots pine populations used for the collections of *Lophodermium* from litter needles

All Scottish sites are remnants of the heavily fragmented ancient Caledonian forest which contains trees of mixed age class (Mason *et al.*, 2004). The populations chosen cover the climatic gradient across Scotland. Loch Maree is located to the north west of Scotland at low elevation and close to the sea. It has the highest rainfall levels of all four sites and its soil is peaty and poor in nutrients. The stand is characterised by mostly mature and old, sparsely spaced Scots pines trees. Glen Affric is located further inland at higher elevation and has a lower rainfall. The stand is denser and mixed with birch, *Betula pubescens*. Abernethy is the easternmost of the Scottish sites and has a much lower rainfall and more continental climate. The altitude of the Abernethy site is approximately 200m. Its soil is a freely draining nutrient poor humus iron podzol (Steven & Carlisle, 1959).

The French population from the woods of Arlanc is located in the mountainous region of Livradois at over 700m altitude. It is a remnant of the ancient Massif Central Scots pine forest which is now fragmented and under competition from species such as Douglas fir and Norway spruce. The soil is poor and the climate is continental with the lowest rainfall of all four sites (Table 3.1) (Thebaud, 1988).

In all three Scottish populations, needles which had fallen during the previous season were collected in spring of 2005 and 2008. Further needle collections were also made from Loch Maree and Glen Affric in December 2008. Needles from Arlanc were collected in October 2008.

3.2.2 Isolation of *L. pinastri* from needles

Fifty to 200 randomly chosen needles per year and collection site were surface sterilised in 0.75% sodium hypochlorite for 10 min and cut into three or four 1 cm sections. All sections from each needle were placed in the same Petri dish containing 2% MEA (Malt Extract Agar). These were incubated at room temperature for approximately 2-3 weeks until mycelial growth typical of *L. pinastri* (Minter *et al.*, 1978) was visible. One *L. pinastri* isolate per needle section was selected based on morphological characteristics, sub-cultured onto 2% MEA and incubated for four weeks at room temperature.

3.2.3 Identification of *L. pinastri* species using species-specific PCR

ITS sequences were obtained from each of the five *Lophodermium* species that had been identified from needles of Scots pine in Scotland in an earlier study (see chapter 2). These species were *L. seditiosum, L. conigenum, L. pinastri* I, *L. pinastri* II and *L. pinastri* III. Manual alignment of these sequences permitted selection of nucleotide sites for the design of species-specific primers *i.e.* primers that would only amplify ITS from the chosen species. This choice was aided by the application of Primer 3 http://frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky, 1999). The choice of the primer site was informed by the nucleotide variability across the five *Lophodermium* species and the invariability of sequences within each species. Optimisation of combinations of primers using pure DNA of isolates representing each cryptic species resulted in the choice of one set of primers for uniquely identifying each

species. The sequences of these primers are given in Table 3.2. Tests using DNA derived from pure cultures demonstrated that amplification of ITS sequences only occurred when DNA derived from pure cultures of the appropriate species was used.

Table 3.2: Species-specific ITS primer pairs used to distinguish *L. pinastri* cryptic species. TM is melting temperature.

Primer name	species	5' to 3' Oligo	TM	%GC	Fragment length
ITSLpIF	*L. pinastri* I	CCCTTGAATCATTGCCGTCTG	56	52	334bp
ITSLpIR	*L. pinastri* I	CCTTGTAAAAAAGGGGGTTG	53.4	51	
ITSLpIIF	*L. pinastri* II	CCTATTCTCACCCTTTGTC	51.7	50	394bp
ITSLpIIR	*L. pinastri* II	CCTTGTAAAGTGGGGGTTG	50.2	53	
ITSLpIIIF	*L. pinastri* III	GATGTCGAGTACTATACAF	50	47	252bp
ITSLpIIIR	*L. pinastri* III	GCATTACTGCGCTGAAGGTCGGGCTGTG	71	62	

3.2.4 Assessment of frequency of *L. pinastri* taxa using colony PCR

The species-specific ITS primers developed above for the three *L. pinastri* taxa were used to determine the taxonomic identity of the 471 *L. pinastri* isolates isolated as described from the Scottish and French populations of Scots pine. In order to deal with taxonomic identification of this large number of isolates, a rapid colony PCR method was developed. This made use of the 90°C PCR initiation step to break cell walls and extract DNA from a small amount of mycelia from colonies grown on agar.

To conduct the colony PCR the surface of the culture was scraped with a 200µl micro-pipette tip (EasyLoad® 200, Greiner Bio-One GmbH) and the mycelia transferred to 50µl of sterile distilled water. The mixture was

vortexed vigorously for approximately 20 seconds until the suspension was homogeneous and residues of agar had become detached from the mycelium. At this stage the suspension was frozen at -20 °C for up to one month. Just before PCR, the mycelial suspensions were thawed and vortexed for a second time.

Identification of each cryptic *L. pinastri* species was based on the positive amplification of the ITS region using the three species specific primer pairs (Table 3.2). For each isolate, 1 µl of each mycelial suspension was added to the three PCR reactions comprising 0.025 µl of BioTaq™ DNA polymerase, 1x NH_4 reaction buffer, 160 mM of di-nucleotide mix and 0.2 µM of each primer. For the reactions amplifying *L. pinastri* I and III, 2 mM of MgCl2 was added, whereas 3 mM $MgCl_2$ was added to the reaction amplifying *L. pinastri* II. The total volume was 20 µl. PCR was carried out in a GeneAmp PCR system 9700. The thermal program was the same for all reactions and was as follows: an initiation at 94 °C for 4 min, 32 cycles of denaturation at 94 °C for 40 sec., annealing at 60 °C for 40 sec., an extension at 72 °C and a final extension at 72 °C for 10 min.

For a number of colonies tested there was a lack of amplification with all three species specific primers. For these isolates the ITS region was amplified using ITS primers ITS1F and ITS4A (Table 2.2). Both strands of the resulting product were sequenced using reagents from the BigDye kit and run through an ABI3730 capillary sequencer at GenePool (School of Biological Sciences, University of Edinburgh). The taxonomic identity of the isolate was then

determined from its sequence similarity to known ITS sequences HM060657 from *L. pinastri* I, sequence HM060665 from *L. pinastri* II and sequence HM060662 from *L. pinastri* III.

3.2.5 Comparison of Species Frequencies among Populations and among Years

Species frequencies were calculated by dividing the number of isolates identified as one species by the total number of isolates for each year and location. Chi-square tests were applied to test two null hypotheses. The first is that in any given year the frequency of the three *L. pinastri* species does not differ significantly amongst the pine populations. The second is that within a site there is no variation in the relative frequencies of the three species among different years.

3.2.6 Comparison of Growth Rate in culture among *L. pinastri* taxa and among Populations

After classification of the Scottish *L. pinastri* isolates into cryptic species by species-specific colony PCR, randomly chosen isolates taken from each of the three cryptic species from each of the three sites were used to analyse variation in growth rate under standard conditions on artificial media. The experiment was conducted as a mixed model randomised block design with replication. Species and site of origin were fixed effects in the model and isolates within species was a random factor. In total, 120 isolates were used. These comprised a total of 40 isolates of *L. pinastri* I, 58 isolates of *L. pinastri* II

and 18 isolates of *L. pinastri* III derived from the three Scottish populations. These isolates were randomly allocated into two groups of 60 isolates (Table 3.3). Each group of 60 isolates was used to set up a replicate growth rate trial on each of two different days. Each group of 60 isolates inoculated on a particular day constituted a separate block. Each of the four blocks was arranged on a different shelf of the incubator.

Table 3.3: Number of isolates of each *L. pinastri* cryptic species from each of three Scottish populations used in experiment to measure growth rate of cultures. N= total number of isolates; n_I, n_{II}, n_{III} = number of isolates of *L. pinastri* I, *L. pinastri* II and *L. pinastri* III respectively.

Block 1 & 3

Population	N	n_I	n_{II}	n_{III}
Loch Maree	14	4	10	0
Glen Affric	22	6	9	7
Abernethy	22	10	10	2
N	58	20	29	9

Block 2 & 4

Population	N	n_I	n_{II}	n_{III}
Loch Maree	14	4	10	0
Glen Affric	24	6	9	9
Abernethy	20	10	10	0
N	58	20	29	9

Total

Population	N	n_I	n_{II}	n_{III}
Loch Maree	28	8	20	0
Glen Affric	46	12	18	16
Abernethy	42	20	20	2
N	116	40	58	18

To conduct the growth rate measurement, mycelial plugs were cut from the growing margins of cultures of each isolate using a sterile 5mm diameter cork borer and transferred onto 2% malt agar in a 90mm Petri dish. Isolates were grown in a controlled environment within an incubator set for day night cycles at 20°C for 12H of light and at 15°C for 12H of dark. The colony radius was measured in mm from the centre of the colony once a week. The radial growth of the colony between weeks 2 and 4, which corresponded to the active growth period of the colonies, was used as the measure of growth rate in the analysis.

The effects of the factors Species, Site, Species x Site Interaction, Blocks and Isolates within species were analysed by appropriate Analysis of Variance using R 2.8.0 (R Development Core Team 2008). *A posteriori* Tukey tests were used to determine the significance of differences in mean growth rate among isolates from different species. The nature of the interaction between Species and Site was explored using an interaction plot.

3.2.7 Linking Ascocarp and colony morphotypes

Attempts were made to recover viable spores from ascocarps of *L. pinastri* and to germinate these spores to produce colonies in culture. However, although it was possible to recover ascospores of *L. pinastri* from ascocarps, there were problems with successful germination of ascospores. These difficulties had previously been noted by Minter (1977), Diwani & Millar, (1987) and Osorio & Stephan (1989). Minter (1977) encountered low and slow germination rates of *L. pinastri* ascospores which caused contamination problems by the time the germination had occurred. Diwani & Millar (1987) observed spore disintegration in water. In a study of ascospores germination in the Rhytismataceae, Osorio and Stephan (1989) observed the exudation of *L. pinastri* spore content.

To overcome these problems and provide a link between ascocarp morphology, culture morphology and ITS sequence, an alternative approach based on Minter (1977) was used. Needles bearing *L. pinastri* ascocarps were photographed and surface sterilised. One of the ascocarps that had been

photographed was cut out and placed on a Petri dish containing 2% MEA. The Petri dishes containing the ascocarps were incubated at room temperature for four weeks. The mycelia growing from the section of the needle containing the ascocarp was isolated. Species-specific colony PCR was carried out on this culture as described in section 3.2.2. Isolations from a total of 28 ascocarps with *L. pinastri* morphology were attempted from needles found on broken branches (trash), from senescent needles remaining attach to lower branches which are deprived of light (shoot tip) and from litter needles from the three Scottish Scots pine populations (Table 3.4).

Table 3.4: Numbers of individual ascocarps of *L. pinastri* from trash, shoot tips and litter in three Scottish populations for which culturing and genetic identification was attempted. A total of 28 fruiting bodies were tried but only 4 were successful.

Population	Trash	Shoot Tips	Litter
Loch Maree	4	2	2
Glen Affric	2	6	5
Abernethy	1	2	4

3.3 Results

3.3.1 Reliability of species-specific colony PCR

Colony PCR using the species-specific ITS primers was conducted on the 471 isolates of *L. pinastri sensu lato* from the four populations. Sixty two of these tested positive for *L. pinastri* I only, 247 tested positive for *L. pinastri* II only, none tested positive for *L. pinastri* III, and 124 did not show any amplification for any of the three species-specific primers. For those giving no positive results with any of the species-specific primers, the ITS region was amplified using universal primers ITS1 and ITS4A.

Forty two of the isolates that showed no amplification with species-specific primers gave successful ITS amplification directly from colonies using the universal ITS primers. The amplified DNA was then sequenced for both strands using the ABI3730 capillary system. Isolates were then identified to *L. pinastri* taxon on the criterion of no less than 95% identity to a reference sequence of the relevant taxon using Geneious Basic 5.0.2. The reference sequences were derived from an earlier phylogenetic analysis described in chapter 2. The reference sequences were from isolates representing each of the cryptic species of *L. pinastri* from the native pine forest of Glen Affric.

Fifteen of the non-amplifying isolates were identified as *L. pinastri* III using the reference sequence HM060662, 12 were identified as *L. pinastri* I using the reference sequence HM060657, 11 were identified as *L. pinastri* II using the

reference sequence HM060665, and 4 of the sequences showed more than 95% similarity to the *L. conigenum* ITS sequence HM060648.

3.3.2 Species frequency in four native pine populations

The most commonly isolated species in all the pine populations were *L. pinastri* I and *L. pinastri* II (Table 3.5). *L. pinastri* II was the most abundant in Scotland whereas *L. pinastri* I appeared to be the dominant species in the pine population located in France. *L. pinastri* III was not isolated from any of the 200 needles collected from the French pine population of Arlanc. It was successfully isolated and identified in two out of three pine populations in Scotland. Although *L. pinastri* III tended to be present at low frequencies overall (< 2%), in Glen Affric its frequency (up to 30%) was not negligible and was similar to that of the more abundant *L. pinastri* I (Table 3.5).

Table 3.5: Frequencies of three cryptic species of *L. pinastri* isolated from needles collected in different years from three Scottish Scots pine populations. Identification of species was based on species- specific ITS sequence; N = Total number of *L. pinastri* isolates in sample.

Year	Population	*L. pinastri* I	*L. pinastri* II	*L. pinastri* III	N
2004	Loch Maree	0.00	1.00	0.00	35
2007	Loch Maree	0.40	0.60	0.00	10
2008	Loch Maree	0.06	0.94	0.00	82
2004	Glen Affric	0.20	0.52	0.28	25
2007	Glen Affric	0.09	0.82	0.09	34
2008	Glen Affric	0.31	0.39	0.30	23
2004	Abernethy	0.46	0.54	0.00	24
2007	Abernethy	0.35	0.64	0.01	124
2008	Arlanc	0.74	0.26	0.00	161

Isolates which were obtained in spring were from needles that had fallen in autumn of the previous year, whereas isolates obtained in autumn were from needles that had fallen in that year. In the following analyses, all isolates will be referred to by their needle fall year rather than by their collection year. *L. pinastri* I and III were combined in all these analysis because of the low numbers of *L. pinastri* III observed in certain locations.

For the Scottish populations, differences in species frequencies were first compared among years (2004, 2007 and 2008). Data from each pine population were tested independently with a chi-square test (Table 3.6a) The hypothesis that species frequencies differed between sample location within Scotland was then tested with a chi-square test treating each year independently (Table 3.6b).

Table 3.6: Results of chi-square tests used to determine significance of difference in frequency of *L. pinastri* cryptic species a.) between different years and b.) between populations within years. Data from *L. pinastri* I and *L. pinastri* III were combined in the analysis; the year represents the needle fall year as opposed to the collection year.

Population	Year	X^2	Df	P	Significance
Abernethy	2004/2007	0.4257	1	0.5141	
Glen Affric	2004/2007/2008	16.9002	2	$2.4e^{-4}$	***
Loch Maree	2004/2007/2008	19.2437	2	$6.63e^{-5}$	***

Year	Populations	X^2	Df	P	Significance
2004	Abernethy, Glen Affric, Loch Maree	22.6519	2	$1.2e^{-5}$	***
2007	Abernethy, Glen Affric, Loch Maree	8.4932	2	0.01431	*
2008	Arlanc (Fr), Glen Affric, Loch Maree	100.8992	2	$<2.2e^{-16}$	***
Signif. codes:	0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ' '

Relative frequencies of the *L. pinastri* species differed significantly among years at Glen Affric and Loch Maree ($P<0.001$ in both cases) but not at Abernethy ($P=0.51$). For any given year, relative species frequencies differed significantly among the three Scottish populations ($P<0.05$). For the year 2007 this difference was less marked. This is accounted for by the similar species frequencies in Abernethy and Loch Maree. At Loch Maree and Abernethy *L. pinastri* II tended to be the predominant species, with a significant representation of *L. pinastri* I. At Glen Affric there was a good representation of all three *L. pinastri* taxa.

3.3.3 Culture growth rate

Prior to analysis the growth rate measurements were Log transformed to

ensure homogeneity of variances, a necessary condition for applying the analysis of variance. The ANOVA table describes the effect of cryptic species identity, the sample location, and the interaction of these two factors on the growth rate of the colonies (Table 3.7). Both taxonomic identity and location of sample had a significant effect on the growth rate of isolates ($P<0.001$). There was also a significant interaction between these two factors ($P<0.001$). There was no significant block effect, suggesting that growth conditions in the incubator were very uniform (Table 3.7).

Table 3.7: ANOVA for growth rate of *L. pinastri* isolates from three cryptic species sampled from three populations of Scots pine in Scotland. The significance of the factors species, population and the interaction between these two factors is shown. *** $p<0.001$, n.s. non significant.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance
Species	2	48.436	24.2181	159.7166	$<2.2e^{-16}$	***
Population	2	2.515	1.2573	8.2918	0.0004344	***
Block	3	0.789	0.2629	1.7336	0.1640732	
Species:Population	3	4.082	1.3607	8.9740	$2.191e^{-05}$	***
Species:Isolates	107	72.456	0.6772	4.4658	$1.812e^{-14}$	***
Residuals	114	17.286	0.1516			

The growth rate of each *L. pinastri* cryptic species is illustrated in the box plot of the Log Growth rate measured during the active growth between week 2 and 4 (Figure 3.2). *L. pinastri* I showed the fastest growth with an average rate of 0.8 mm/day (Table 3.8). It is followed by *L. pinastri* II with a mean growth rate of 0.5mm/day. The slowest growth was shown by *L. pinastri* III with a mean growth rate of 0.18mm/day (Table 3.8). Tukey multiple comparison tests indicated that for each species, growth rate was significantly different from all others ($P<0.001$ for all combinations) (Table 3.9). The growth rate of an isolate affected the size of the mature colony as illustrated in Figure 3.3. *L.*

pinastri I tended to produce the largest mature colonies, and *L. pinastri* III the smallest mature colonies after eight weeks (Figure 3.3).

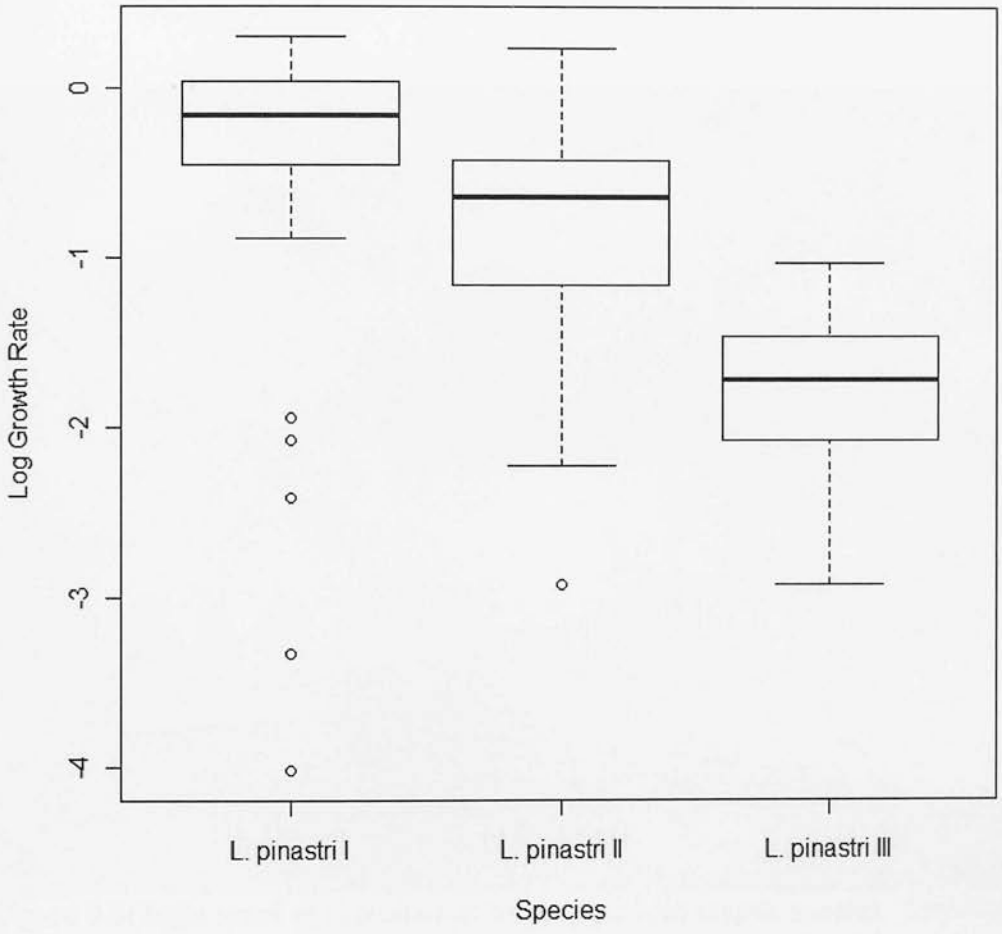

Figure 3.2: Whisker and Boxplots showing variation in colony growth rate for isolates of three cryptic species of *L. pinastri* isolated from three populations of Scots pine in Scotland. The empty circle represents outliers; the standard deviations are represented by the whiskers and the mean by the thick horizontal lines.

Table 3.8: Mean and standard error for growth rate (mm/day) of each cryptic species of *L. pinastri* found in the controlled environment experiment.

Species	Median	Mean	Sdt dev
L pinastri I	0.638	0.800	0.327
L pinastri II	0.317	0.500	0.236
L pinastri III	0.125	0.181	0.072

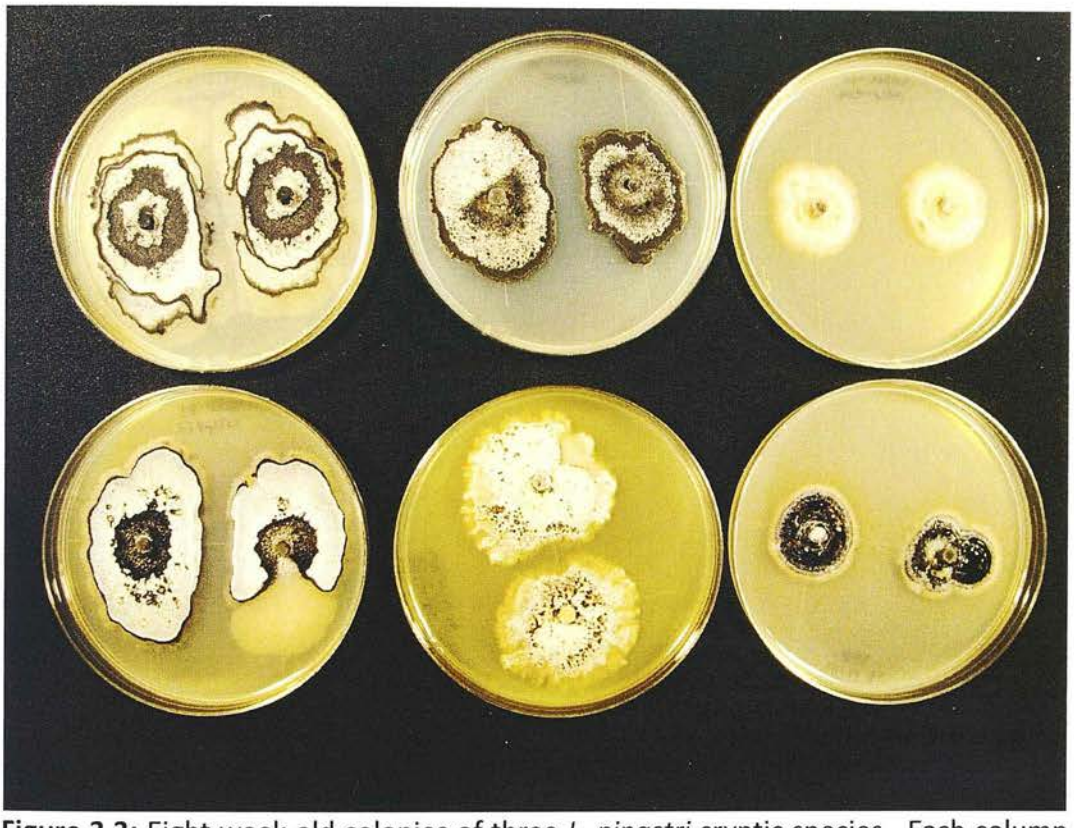

Figure 3.3: Eight week old colonies of three *L. pinastri* cryptic species. Each column contains two isolates from a single *L. pinastri* cryptic species. Replicate colonies of the same isolate are present on each plate. Columns from left to right are; *L. pinastri* I, *L. pinastri* II, and *L. pinastri* III.

The growth rate of isolates also varied significantly among pine populations ($P<0.001$) (Table 3.7). Despite this variation among populations within species, the overall difference between the species was still highly significant.

The variation among sites for each species is illustrated in the interaction plot (Figure 3.4). *L. pinastri* I from Glen Affric, which is located between the other two pine populations, grew slower than those from Loch Maree in the west and Abernethy in the east. The *L. pinastri* II growth rates for the three pine populations decreased from east to west. In contrast, the *L. pinastri* III growth rate was greatest for isolates from Glen Affric located in the west (Figure 3.4).

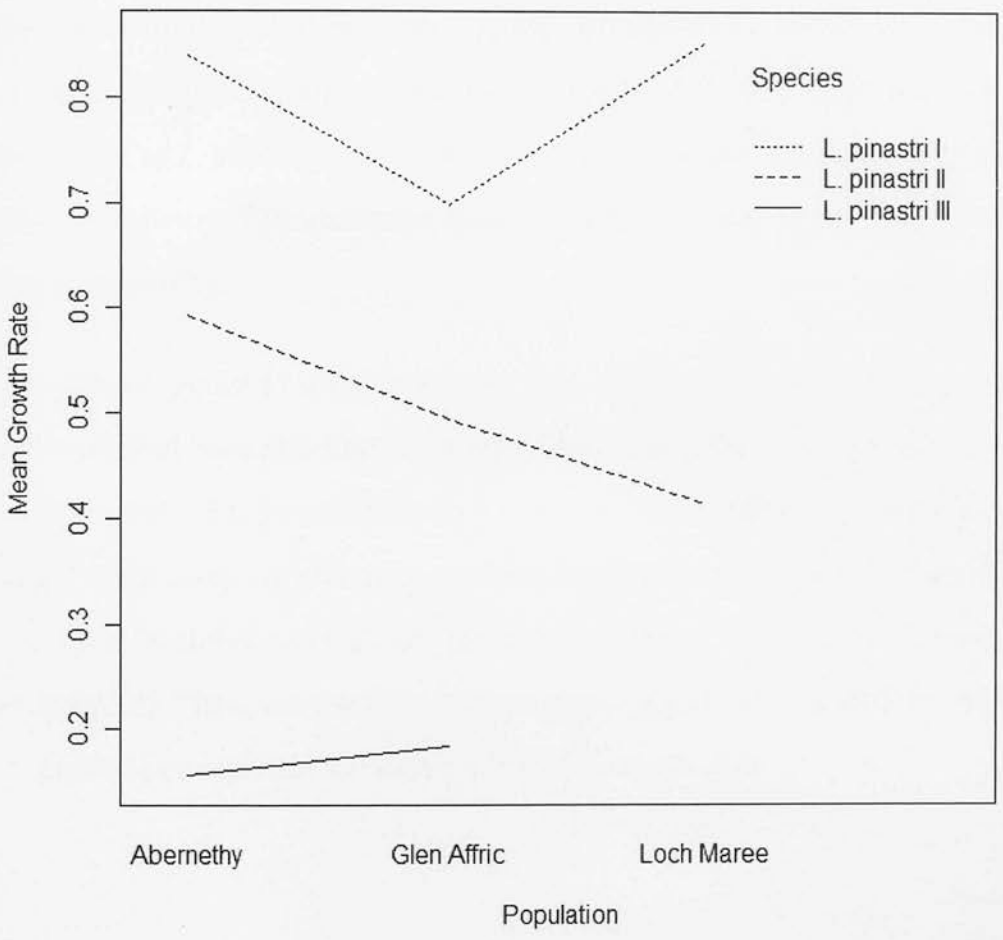

Figure 3.4: Interaction plot showing the variation in growth rate for three cryptic species of *L. pinastri* (*L. pinastri* I, *L. pinastri* II and *L. pinastri* III) taken from three different Scots pine populations within Scotland (Abernethy, Glen Affric and Loch Maree.

3.3.4 Ascocarp morphology characters on needle

Twenty eight *L. pinastri*-like ascocarps from litter needles, shoot tips and trash needles of Loch Maree, Glen Affric and Abernethy were set up on 2% Malt extract agar after surface sterilisation of the needle. Only ascocarps derived from the litter produced hyphae. A total of four ascocarps formed colonies identified using species-specific colony PCR. Three of these were identified as *L. pinastri* I. One was from Glen Affric and the other two were from Abernethy. The remaining isolate was identified as *L. pinastri* II and was from Abernethy.

The three *L. pinastri* I isolates possessed ascocarps which showed clear and full marginal lines also known as zone lines. These lines are considered characteristic of *L. pinastri* (Figure 3.5 to 3.7). The isolate of *L. pinastri* II was from an ascocarp which had some zone lines around it. However these lines were much fainter and did not appear to produce a ring around the needle (Figure 3.8). These are the only clear morphological differences that could be discerned between the ascocarps of the two cryptic taxa.

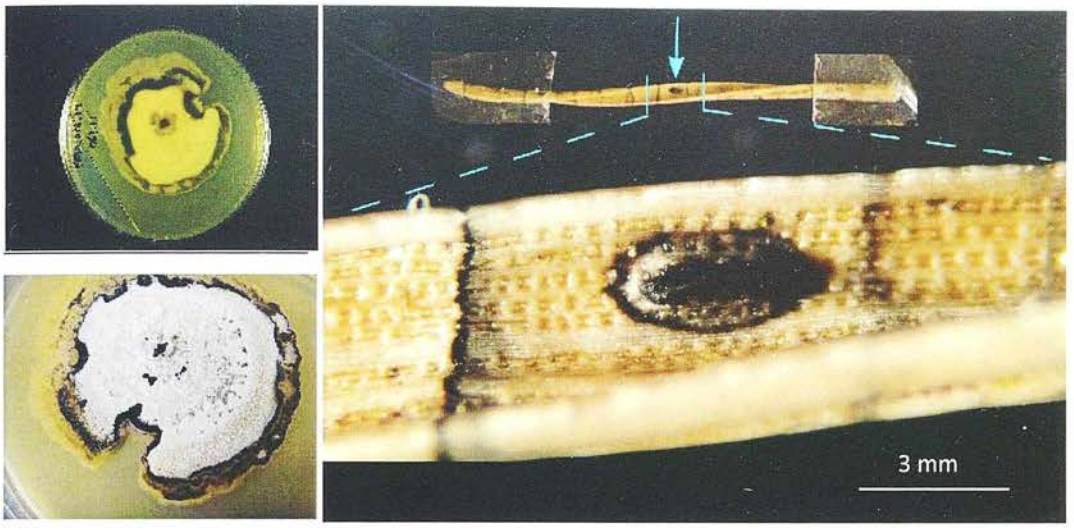

Figure 3.5: *L. pinastri* I colony (isolate FR90.067.11) and corresponding ascocarp from which it was derived.

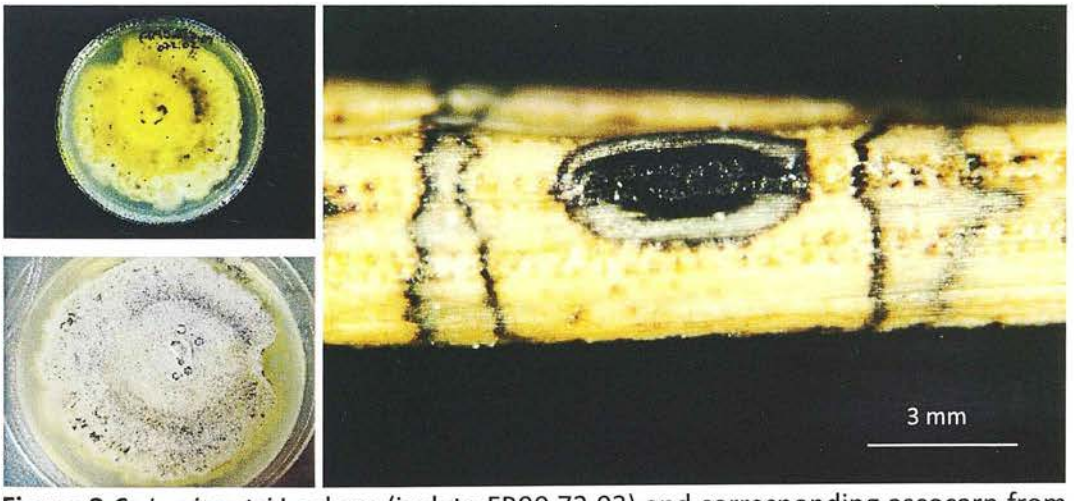

Figure 3.6: *L. pinastri* I colony (isolate FR90.72.02) and corresponding ascocarp from which it was derived.

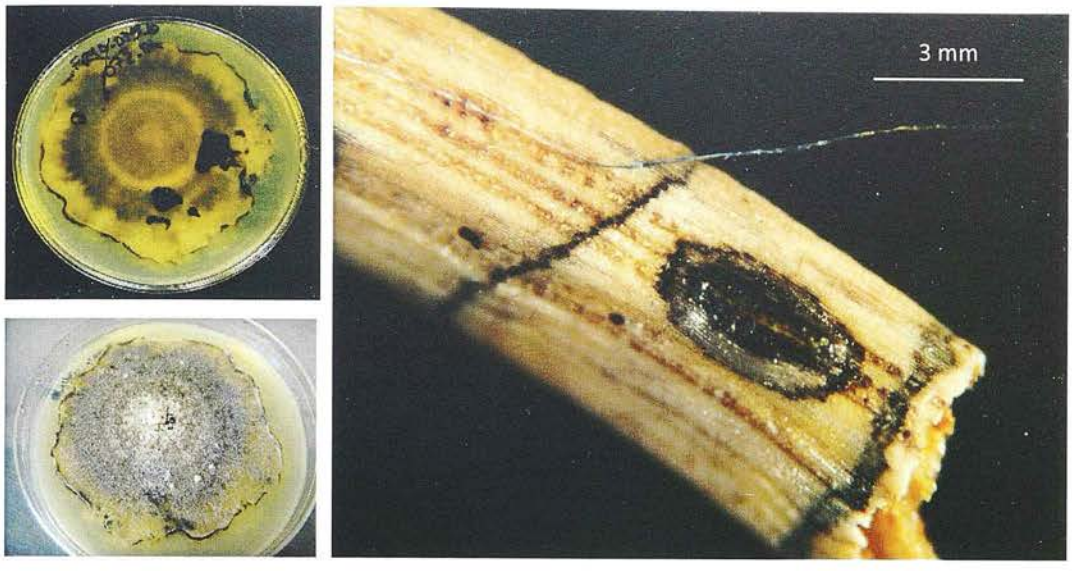

Figure 3.7: *L. pinastri* I colony (isolate FR90.72.04) from Abernethy and corresponding ascocarp from which it was derived.

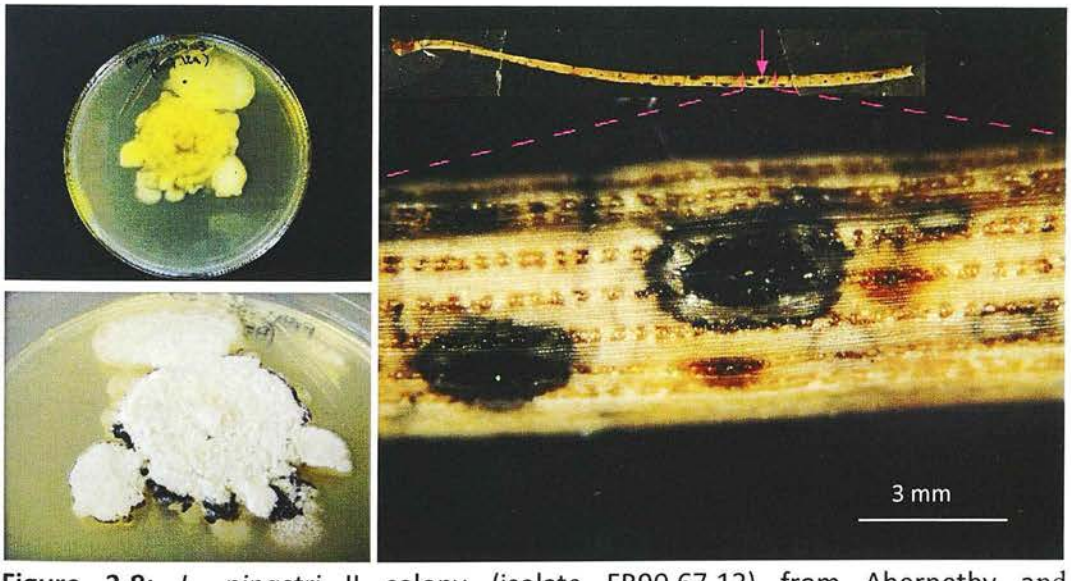

Figure 3.8: *L. pinastri* II colony (isolate FR90.67.12) from Abernethy and corresponding ascocarp from which it was derived.

3.4 Discussion

Chapter 2 of this thesis gave evidence that *P. sylvestris* litter needles are host to three *Lophodermium* species, based on genetic data, in at least one native pine population in Scotland. With the taxonomy of *Lophodermium* species associated with *P. sylvestris* defined, it is now possible to begin investigating biological questions concerning the endophyte species with the ultimate aim of understanding the relationship between the pathogen and its related endophytes, and the interactions that occur between them.

To facilitate such studies, it was first essential to develop a method which would enable accurate and reliable identification of each of the three species. Other authors have used species-specific PCR to identify the pathogen *Lophodermium seditiosum* and the endophyte *L. pinastri* directly from green needles. Their objective was early detection of the pathogen for the purpose of disease management (Stenstrom & Ihrmark, 2003). Our aim was to identify each *Lophodermium* species associated with *P. sylvestris* accurately after isolation and culture on 2% Malt Extract Agar. The existing primers used for the detection of *L. pinastri* from green needles had been designed prior to the discovery of cryptic species within the *L. pinastri* complex. This is why a new set of primers which identified each cryptic species specifically were designed based on the alignments of ribosomal DNA from the ITS region used in the phylogenetic analysis of chapter 2.

In order to screen isolates quickly and bypass lengthy DNA extractions,

direct PCR on mycelium suspension (colony PCR) was carried out. The outcome of species-specific colony PCR was that litter needle isolates could be sorted reliably into three groups: *L. pinastri* I, *L. pinastri* II and no amplification. The primer set which aimed to amplify specifically *L. pinastri* III did not perform well when used on DNA in crude mycelial suspensions. Further work is required to redesign a suitable set of primers for amplifying *L. pinastri* III ITS sequences using crude DNA extracts.

Although some isolates remained unknown, the subsequent sequencing using universal ITS1 and ITS4A permitted us to identify further isolates as *L. pinastri* III, *L. pinastri* I, *L. pinastri* II and even *L. conigenum*. This was achieved by comparison to reference sequences, and identification was based on a criterion of 95% sequence similarity. Direct detection in the needle was not the purpose of this study. Such assays are required to detect low quantities of DNA from very small amounts of mycelium. This is more achievable using Real-Time PCR, also known as quantitative PCR.

Isolate identification using species-specific PCR and sequencing has enabled the investigation of the distribution and relative frequency of each cryptic species. It has revealed that it is misleading to use records of sequences deposited in Genbank either to estimate the geographic distribution of a species or to make a judgement on the association of species with particular hosts. If one followed the guidance of presence of sequences in Genbank to determine the actual distribution of isolates identifiable as *L. pinastri* II, then it should not have been found in Europe.

In terms of *Lophodermium* species distribution among Scots pine populations, Glen Affric is the only native pine forest which has a significant presence of *L. pinastri* III. The detection of the species was not a chance event since its presence at Glen Affric was evident in all three years sampled. In contrast, *L. pinastri* III was not isolated as frequently or as consistently in Abernethy or Loch Maree and was not isolated at all from the 200 needles of the French population. On the other hand, the significant presence of *L. pinastri* I and *L. pinastri* II in both Scotland and France demonstrates that these two species often co-occur within the same host and pine populations. More populations would need to be surveyed to draw general conclusions about the distribution of these species in Europe or elsewhere in the range of Scots pine. These results reveal that restricting identification to the level of the genus when surveying the communities of endophytes will lead to a severe underestimation of the level of diversity of these endophyte communities.

In terms of range of host species, isolates deposited in Genbank which can now be recognised as belonging to *L. pinastri* II, have been isolated from a diversity of *Pinus* hosts which can range from two to five-needle pines. It would be interesting to know whether this is the case for the other two cryptic species. Since *L. pinastri* III appears to be less abundant on *P. sylvestris*, it is conceivable that this tree is of secondary importance as a host for *L. pinastri* III. This endophyte may be far more abundant on another *Pinus* host species.

Dynamic changes in the populations of these different endophyte species

were investigated by estimation of their relative frequencies over a number of different years of needle fall. The significant difference in frequency of each endophytic *Lophodermium* species between years suggests the influence of environmental factors, both abiotic and biotic, on the endophytic, the saprophytic, or both stages of their life cycle. This is the first time that different species from the same genus have been surveyed from one single host species at the same site over different years. Other studies have provided evidence for the influence of canopy density and humidity on infection rates of *Lophodermium* (Petrini *et al.*, 1982). *Lophodermium* depends greatly on humidity to allow opening of ascocarps for release of spores. It is possible that some species perform slightly better at lower humidity levels (Minter & Cannon, 1984). This may be the case for *L. pinastri* I, which is seen to have a higher relative frequency in the French population than in the Scottish sites.

It is possible that climate plays a role in the ability of individuals to establish and fruit within senescent needles. It was shown that only a few genotypes remained within one needle at the saprotrophic stage of *L. piceae* compared to a high genotypic diversity observed among isolates from green needles (Müller *et al.*, 2001). This phenomenon could extend to species diversity within the *L. pinastri* complex, and lead to lower species diversity of fruiting ascocarps than of isolates within green needles.

Another environmental influence could be competition with other species. Negative correlation in frequency between *L. pinastri* and *Cyclaneusma minus*,

both as endophytes and as saprophytes in *P. sylvestris* and *P. mugo*, provides evidence that inter-specific interaction affects species frequency (Gourbiere *et al.*, 2001; Seiber *et al.*, 1999). It should be noted that identification of *L. pinastri* in both studies was not based on genetic data. Thus it is unclear which *L. pinastri* species was scored in each study.

Studies on *Lophodermium* endophyte communities, such as those referred to above, have been based on morphological identification which did not recognise the three cryptic species of *L. pinastri.* Therefore, these studies will need to be re-evaluated in this light. In the mean time it may be possible to use growth characters in culture to identify the cryptic species without the need for molecular assessments. For instance Samuel *et al.* (2006) found that growth rate in culture was the best morphological character for differentiating species within the *Trichoderma koningii* aggregate, and that this was superior to differentiation based on conidiophore morphology.

Using randomly chosen isolates which were identified using species-specific colony PCR, it was possible to compare the growth rate among each of the cryptic species of *L. pinastri*. Growth rate of isolates from different locations with different environmental characteristics was also compared in order to demonstrate that differences observed between taxa were not due to local population variation. Growth rates between the *L. pinastri* cryptic species were highly significantly different, backing up the genetic evidence of chapter 2 that these entities represent different species. Large differences in culture growth rate, though not directly relevant to the performance of the

species in nature, do suggest that the three *L. pinastri* cryptic species will differ significantly in their ecology. Within each species culture growth rates also varied according to sample location. This is a possible sign of local adaptation of each species. However this adaptation does not override the differences observed between species. This contrasts with the situation for *Trichoderma,* in which cryptic speciation occurred as a consequence of spatial isolation (Samuel *et al.,* 2006). Since there is some evidence of local adaptation which is translated to differences in growth rate in culture, it is plausible to suggest that growth rate must be different within the needle. It would be difficult at this stage to infer whether this is an adaptation of growth rate in the living needle as opposed to the saprotrophic stage of their life-cycle or both. However the coexistence of the three species confirms their potential to compete at some stage of their life-cycle.

Co-existence of these genetically distinct species within the same populations and findings of significantly different growth rates independent of geographical location suggests that evolution of these species may not have been through geographical separation. Other factors of speciation may have been involved in bringing about reproductive isolation in sympatry. Such factors could be different timing of fructification already observed between some of these species (Minter and Millar, 1980). Host preference may also have played a role. In this context it will be important to learn more about the host specificity of all the *L. pinastri* cryptic species.

Using the method described by Minter (1977), to link culture morphology of

L. pinastri to ascocarp morphology, it was possible to demonstrate the potential use of the character zone line to differentiate *L. pinastri* I and *L. pinastri* II. However a much larger sample would be required for more certainty and statistical assurance of this result. A bigger sample size may also enable us to identify other meaningful ascocarp characters.

While comparing specimens used in this study to the collection deposited by David Minter in RBGE as well as to illustrations from his published work (Minter, 1981a), it has become apparent that *L. pinastri* I may correspond to *L. pinastri* and that *L. pinastri* II may correspond to *L. staleyi*. The latter occurs on needles attached to branches of two needle pines which are deprived of light as well as on the litter. *L. staleyi* forms smaller ascocarp than *L. pinastri* and lack defined black zone lines. It is different from *L. pini-excelsae* in terms of its fine ascocarp perimeter line.

The most likely reason for the lack of *L. pinastri* III isolates recovered in this manner from ascocarps could be due to the low frequency of this species and the small sample size. However it is also possible that this species fruits at a different time of the year. Further characterisation of this species is required.

An attempt to use molecular methods to find the identity of *L. pinastri* III by matching its ITS sequence with that of GenBank records was unsuccessful. In the course of this work it became clear that some sequences deposited in Genbank had been incorrectly identified. Thus, BLAST analysis of the ITS sequence of entry EU520183, which is labelled as *L. pini-excelsae*, showed that

it had 98% identity to a *Stagonospora* species and to strains of various environmental endophytes as well as 91% similarity to *Phaeosphaeria nodorum*. Both *Stagonospora* and *Phaeosphaeria* are genera in the family Pleosporaceae whereas *Lophodermium* is a genus in the family Rhytismatacea. The other two records for *L. pini-excelsae,* FJ861987 and FJ861988, are 98 to 100% identical to *L. macci* sequences deposited as reference sequences based on both morphological and DNA analysis (Sokolski *et al.,* 2004). These sequences are only 91% identical to *L. pinastri* III ITS sequences. This indicates that at present there are no reliable ITS or other sequences in Genbank from the entity named *L. pini-excelsae*. It is therefore not possible to determine whether this is synonymous with any of the cryptic taxa that we have identified within *L. pinastri*. These results highlight the importance of ensuring that fungal records in the Genbank database are from properly verified specimens so that in the future it will be possible to link genetic data to morphological characters already used to identify and name fungal species.

Chapter 4: Breeding System & Population Structure of *Lophodermium* on *Pinus sylvestris*

4.1 Introduction

A very high proportion of fungal species have life cycles which include intimate interactions with plants. Arguably the most numerous of these fungal groups which interact with plants are the endophytes. Endophytes are defined as organisms which live asymptomatically within plants for at least part of their life cycle (Wilson, 1995). Endophytes are ubiquitous to all plants and are present in every plant organ (Rodriguez *et al.*, 2009; Arnold, 2007). It has been estimated that every plant species is associated with it at least six fungal species, many of which are endophytes that are yet to be described. A high proportion of these fungal endophytic species reside in the phylum Ascomycota.

For simplification fungal endophytes can be divided into two broad classes

based on their degree of colonisation of host tissue. The first group colonises host tissues systemically and is often vertically transmitted. Typical examples are the grass-infecting Claviceps. Evolution in such endophytes is typically towards mutualism, and there is good evidence for fungal production of compounds such as alkaloids which provide plants with protection against herbivores (Clay & Schardl, 2002; Schardl *et al.*, 2004).

In contrast, the second group of endophytes show limited colonisation of aerial host tissues and are typically horizontally transmitted (Rodriguez *et al.*, 2009). They are ubiquitous and are represented in most ascomycete phyla (Arnold, 2007; Higgins *et al.*, 2007). Species diversity within their host is often underestimated, especially in leaves or needles of trees as exemplified in chapter 2 of this thesis. Moreover, the host specificity of these endophyte taxa can be variable (Arnold, 2007).

The lifestyles of this second group of horizontally transmitted endophytes vary across a wide spectum. At one extreme are species that remain asymptomatic within host tissues until these senesce naturally, at which point colonisation of tissues and fruiting occurs. These taxa may be regarded as mutualists because there is some evidence that they benefit their host during their endophytic stages. For instance, Arnold *et al.* (2003) demonstrated the importance of the diverse community of endophytes in *Theobroma cacao* leaves for the protection against *Phytophthora* species. Miller *et al.* (2002) demonstrated that rugulosin production by endophytes in spruce needles protected the host against a bud worm pest.

At the other extreme are species that are capable of acting as primary parasites, but may also behave as endophytes and remain asymptomatic in host tissues in the absence of host stress. When host tissues become stressed these endophytes colonise stressed tissues, acting as primary pathogens and fruiting on tissue that they have been actively involved in killing. In the middle of this spectrum lie endophytes which remain asymptomatic in host tissue unless it is killed prematurely. Premature death of tissue is the trigger for tissue colonisation and fruiting by this third group of horizontally transmitted endophytes.

Where endophytes adopt different life styles, this is likely to have consequences for many other aspects of their population biology. For instance, if endophytes evolve to occupy a more restricted niche, this will have consequences both for their ability to find other individuals with which to mate, and for the size and degree of fragmentation of their populations. Endophytes occupying stressed or prematurely killed host tissue are likely to occupy a more restricted niche than those fruiting on naturally senescing host tissue. They may have difficulty in finding mating partners, and the evolution of self-fertilisation may therefore be favoured in this group.

This group of endophytes is also expected to have populations that are both lower in size and more fragmented than those occupying naturally senescing host tissue. If this is the case it will be evident in the genetic structure of their populations. Smaller, more fragmented populations are more affected by genetic drift than larger, more continuous populations. Thus endophytes

occupying restricted niches should show greater genetic marker differentiation among sites than endophytes occupying large host niches. We therefore predict increased selfing and greater population genetic structure in endophytes that are facultative parasites, or fruit on stressed tissue, than in endophytes that fruit on naturally senesced host tissue.

In order to test these predictions we ideally need a system in which we have a closely related group of endophyte species which show the full spectrum of lifestyles from parasite to mutualist. If the predicted differences in mating systems and genetic structure are found among these species then it is reasonable to ascribe these differences to the different lifestyles that they possess, because they otherwise are very similar in their biological and ecological characteristics.

A system which is well qualified for such investigation is the *Lophodermium* complex of species which colonises Scots pine needles. As we know it, the ascomycete genus *Lophodermium* (anamorph *Leptostroma*) includes a large number of taxa that are ubiquitous endophytes of pine (Hata & Futai, 1996). *Lophodermium* also includes an economically important pathogen, *L. seditiosum*. It induces significant loss in Scots pine nurseries with premature needle-cast in young seedlings, and growth reduction followed by death (Skilling & Nicholls, 1975; Diwani & Millar, 1987).

It is now recognised that five *Lophodermium* species are able to colonise *P. sylvestris* from native forests (Chapter 2 and 3). Three endophyte species, *L.*

pinastri sensu lato fruit on litter needles that have senesced naturally. The endophyte *L. conigenum* fruits on needles found on damaged branches. The final species *L. seditiosum* is a primary pathogen but can also overlap in its fruiting niche with *L. conigenum* and fruit on prematurely killed needles. For simplicity *L. seditiosum* will be referred to as a pathogen and the other taxa as endophytes in the strict sense of the term, *ie* an organism which does not induce diseases.

This comparative investigation of the mating system and population genetic structure of pathogen and endophytes of *P. sylvestris* will focus on one endophyte from litter needles *L. pinastri* II, and both species fruiting on broken branches, *L. seditiosum* and *L. conigenum*. The choice of using *L. pinastri* II is based on an assessment of the relative frequencies of the three cryptic *L. pinastri* taxa in Scotland and France that has been described in chapter 3.

This chapter will therefore attempt to compare the mating system, genetic diversity and population structure of these three genetically related, sympatric but distinct species of *Lophodermium* which comprise two endophytes and a pathogen. Previous population genetics studies of pathogens have helped to inform us about evolution in species and the risks of a species becoming more virulent. This has been an important tool in epidemiology (McDonald *et al.*, 1989; Abang *et al.*, 2006; Hayden & Howlett, 2005; Hayden *et al.*, 2007; Zhan *et al.*, 2005; Stukenbrok *et al.*, 2006; Zhang *et al.*, 2009; Zhou *et al.*, 2006; Lee *et al.*, 2007; Paavlainen *et al.*, 2001; Perez *et al.*,

2010; Burgess *et al.*, 2004; Hamelin *et al.*, 1998). Population genetics of individual endophytes has also been investigated (Ahlholm *et al.*, 2002; Kraj, 2008; Müller, 2007; McCutcheon & Carroll, 1994; Sulivan & Faeth, 2004; Wali *et al.*, 2007; Zhang *et al.*, 2010). However, none of these studies provide a comparative analysis of mating systems and genetic structure in a system which includes both pathogen and endophytes coming from the same sites and present on the same host.

In order to carry out these studies it is necessary to develop an appropriate set of polymorphic genetic markers. Many population genetic studies use co-dominant markers such as microsatellites also known as SSR (Simple Sequence Repeats) markers (Zane *et al.*, 2002). Microsatellite markers are very useful since they are abundant in most genomes and very polymorphic due to variation in the number of repeats. Each microsatellite locus can be amplified with the assistance of primers which are designed on its flanking regions. However microsatellite markers can be difficult to obtain in fungi due to their smaller genomes and and the lower quantity of SSRs present (Santana *et al.*, 2009; Dutech *et al.*, 2007).

With the difficulties in obtaining such markers in *Lophodermium* species it was decided to use a fingerprinting method, inter-SSR markers, to assess the mating system and genetic structure of all three *Lophodermium* species using data from Scottish and French populations of Scots pine. ISSRs, also known as microsatellite primed PCR and RAMS (Randomally Amplified Microsatellites), have been used in Ascomycetes since the mid 1990's

(Hantulla *et al.*, 1996) and were first described by Zietkiewicz *et al.* (1994). ISSR analysis utilises primers which include a microsatellite and a degenerate anchor region. This allows amplification of regions between microsatellites. Unlike RAPDs this method does not rely on low annealing temperature to produce polymorphism. Instead, polymorphism of these markers is the result of presence or absence of microsatellites and length variation (Wolfe et al 1998).

ISSR (Inter-SSR) markers are dominant and bi-allelic and combine the high polymorphism of microsatellite and the simplicity of RAPDs (Randomly Amplified Polymorphism DNA). Since *Lophodermium* species are haploid like most ascomycete fungi, using a dominant marker does not remove valuable information on heterozygosity which would be important in diploid organisms. ISSRs have been successfully used to estimate the reduction of genotype diversity between *L. piceae* within green needles and as a saprophyte from litter needles (Muller *et al.*, 2001). ISSRs have also been used to estimate population structure in *Botrytis cinerea* which is a soft fruit pathogen and *Gremmeniella abietna*, a pine pathogen (Ma & Michailides, 2005; Hamelin *et al.*, 1999).

In order to assess the mating system, naturally produced families of isolates taken from single ascocarps have been scored for their ISSR banding pattern. If the taxon is homothallic and self-fertilising, all individuals derived from a single ascocarp will be identical in ISSR pattern. If the species is heterothallic and outcrossing, the families from a single ascocarp are expected to segregate

for at least one of the polymorphic ISSR alleles. Mating system has been assessed in two native pine populations for each of the species for which progeny arrays could be obtained

For assessment of genetic structure, the ISSR banding patterns of individuals sampled from three native Scots pine populations in Scotland have been scored, together with a single population of *L. pinastri* II sampled from France. Frequencies of the bands, here interpreted as the products of single loci, have been estimated. From these data estimates of genetic diversity for ISSR loci, and genetic differentiation among populations have been calculated.

4.2 Material and Methods

4.2.1 Field Collections

Isolates were obtained from both litter and trash (broken branches) needles. Litter needles were collected from below twenty different Scots pine trees in each of three native forests in Scotland, Loch Maree, Glen Affric, and Abernethy, and one in France, Arlanc (Table 3.1 and Figure 3.1). Trash needles were collected from five broken branches in each of three native forests in Scotland.

All Scottish sites are remnants of the heavily fragmented ancient Caledonian forest which contains trees of mixed age class (Mason *et al.* 2004). The populations chosen cover the climatic gradient across Scotland. Loch Maree is located to the north west of Scotland at low elevation and close to the sea. It has the highest rainfall levels of all four sites and its soil is peaty and poor in nutrients. The stand is characterised by mostly mature and old, sparsely spaced Scots pines trees. Glen Affric is located further inland at higher elevation and has a lower rainfall. The stand is denser and mixed with birch, *Betula pubescens*. Abernethy is the easternmost of the Scottish sites and has a much lower rainfall and more continental climate. The altitude of the Abernethy site is approximately 200m. Its soil is a freely draining nutrient poor humus iron podzol (Steven & Carlisle 1959).

The French population from the woods of Arlanc is located in the mountainous region of Livradois at over 700m altitude. It is a remnant of the

ancient Massif Central Scots pine forest which is now fragmented and under competition from species such as Douglas fir and Norway spruce. The soil is poor and the climate is continental with the lowest rainfall of all four sites (Thebaud, 1988).

4.2.2 Fungal isolation

4.2.2.1 Mating system estimation

Ascopore isolation was performed for the study of the mating system. Attempts were made to recover viable spores from ascocarps of *L. pinastri* and to germinate these spores to produce colonies in culture. However, although it was possible to recover ascospores of *L. pinastri* from ascocarps, there were problems with successful germination of ascospores. These difficulties had previously been noted by Minter (1977), Diwani & Millar, (1987) and Osorio & Stephan (1989). Minter (1977) encountered low and slow germination rate of *L. pinastri* which caused contamination problems by the time the germination had occurred. Diwani & Millar (1987) observed spore disintegration in water. In the study of ascospore germination in the Rhytismataceae , Osorio and Stephan (1989) observed the exudation of *L. pinastri* spore content. In contrast to the situation in *L. pinastri*, recovery of viable ascospores from single ascocarps was possible for both *L. conigenum* and *L. seditiosum*.

Mature ascocarps from trash needles, recognised as *L. conigenum* and *L.*

seditiosum based on descriptions in Minter *et al.* (1978), were soaked in water until ascocarps opened. Each ascocarp was cut out and placed on petroleum jelly in the middle of a Petri dish lid held over 2% malt extract agar and left over night at room temperature in order for the ascospores to be ejected onto the media and to germinate. The surface of the media was then examined using a dissecting microscope. Sharp tungsten needles were used to isolate three3 to 9 single ascospores siblings from 7 to 13 parental ascocarps for each of the two *Lophodermium* species from two *P. sylvestris* populations, Loch Maree and Abernethy (Table 4.1).

Table 4.1: Isolate numbers for each population, each isolation method (needle surface sterilisation and spore isolation) and each species used in the population genetic assay.

species	Loch Maree			Glen Affric			Abernethy			Arlanc		
	Spore	Surface sterilised	Total	Spore	Surface sterilised	Total	Spore	Surface sterilised	Total	Spore	Surface sterilised	Total
L seditiosum	21	14	35	0	22	22	15	16	31	0	0	0
L conigenum	8	20	28	0	31	31	15	14	29	0	0	0
L pinastri II	0	27	27	0	34	34	0	34	34	0	34	34

4.2.2.2 Genetic Diversity and Population Structure

Fifty to 200 randomly chosen needles from the litter and trash per collection site were surface sterilised in 0.75% sodium hypochlorite for 10 min and cut into three or four 1 cm sections. All sections from each needle were placed in the same Petri dish containing 2% MEA (Malt Extract Agar). These were incubated at room temperature for approximately 2-3 weeks until mycelial growth typical of *L. pinastri, L. conigenum* and *L. seditiosum* (Minter *et al.*, 1978) was visible. One isolate per needle section was selected based on

morphological characteristics, sub-cultured onto 2% MEA and incubated for four weeks at room temperature.

4.2.3 Isolate identification

Primers used to identify specifically isolates of each species are given in table 4.2. Design of species-specific primers amplifying *L. conigenum* ITS followed the same procedure as in chapter 3. Identification of *L. pinastri* II used the same primer set as in chapter 3. *L. seditiosum* was identified using species specific primers designed by Stenstrom and Ihrmark (2005). In order to deal with taxonomic identification of a large number of isolates, the same colony PCR method as in chapter 3 was applied with the exception of the magnesium chloride concentration and annealing temperature in the PCR specific for *L. seditiosum* and *L. conigenum*. A summary of these conditions is detailed in table 4.3.

Table 4.2: Species-specific ITS primers used for the identification of each species of *Lophodermium* used for the study of their population genetics; primer description includes the species they specifically identify, their melting temperature (Tm) and their GC content (GC%).

Primer name	species	5' to 3' Oligo	TM	GC%	Authors
ITSLpIIF	*L. pinastri* II	CCTATTCTCACCCTTTGTC	51.7	50	
ITSLpIIR	*L. pinastri* II	CCTTGTAAAGTGGGGGTTG	50.2	53	
ITSLc4F	*L. conigenum*	ATGCCTTCGGGCTCTGTTCTTC	63.7	55	
ITSLc1R	*L. conigenum*	GTTGTATGACGGCGCTTGC	62.7	58	
Ls11	*L. seditiosum*	CACCCTTTGTTTACCACACTCA	60.1	45	Stenstrom and Ihrmark, 2005
Ls12	*L. seditiosum*	CGGCACCTGCTGTCCTTC	60.8	67	Stenstrom and Ihrmark, 2005

Table 4.3: PCR conditions differing for each species-specific PCR assay using BioTaq reagents (Bioline, London UK).

PCR	$MgCl^2$ (mM)	Annealing (°C)
L. pinastri II	3	60
L. conigenum	1.5	57
L. seditiosum	2	64

4.2.4 DNA extraction

Randomly chosen isolates of each species were grown in 2% Malt Extract Agar for three weeks at room temperature. One cm^2 of mycelium was scraped from the surface of the culture with a clean, sterilised razor blade. The mycelium was transferred into a 2.0mL screw cap tube for use with bead mill FastPrep® Instrument (Qbiogene, Montreal, Canada) to which was added a ceramic bead and sand. The mix was then processed for 20 second at 4m/s. The mix was then added to 400µl 65°C warm Cetyltrimethyl Ammonium Bromide CTAB (Sigma-Aldrish, St Louis, USA). The DNA was extracted using an adapted method of the CTAB DNA Miniprep for Plant DNA Isolation (Clarke, 2009). Centrifugation after each extraction steps was conducted for at least 3min at maximum speed until each phases were properly separated. If the phenol or chloroform extraction produced a large amount of precipitate it was sometimes necessary to centrifuge a second time in order to recover more aqueous phase. The remaining part of the protocol followed that of Clarke (2009).

DNA was quantified by measuring the spectrophotometry reading of 2µl of DNA extract using NanoDrop 1000 spectrophotometer (Wilmington, USA). Dilutions to obtain a DNA concentration of 50 to 100ng/µl were prepared in

standard strength TE buffer.

4.2.5 Inter-SSR development and application

4.2.5.1 Amplification of Bands

Five ISSR primers were chosen out of 24 initially included in trial runs (Table 4.4). The choice was first based on the positive amplification of ISSR using isolates of *L. conigenum* and *L. seditiosum*. Reproducibility and polymorphism were further tested with sibling spore isolates of *L. conigenum*. Difficulties with optimisation of the PCR were overcome with the use of a touchdown thermal cycle program (table 4.5) using a DNA engine system Dyad and PTC-200 stations (Bio-Rad, Hertfordshire UK). In touchdown PCR there is a decrease in the annealing temperature every cycle. Once the primers anneal specifically at the optimum temperature there is enough template of the PCR product to compete with less specific priming locations and allow specific amplification at lower temperature. Therefore this method increases yield and reproducibility of products from difficult primers. In the case where primers did not perform well with the standard Touchdown 62 program, the Grad 58 program was implemented and tested. The reaction mix included between 0.05 to 0.1µg of DNA in 25µl total volume. The GoTaq Green polymerase Kit (Promega, Madison USA) was used for this assay. The final concentration of MgCl2 was 1.5mM, 160mM of each dNTPs, 0.025U/µl of GoTaq polymerase and 0.2µM of the ISSR primer.

Table 4.4: InterSSR primers used for the genotyping of *Lophodermium* species; Description includes their melting temperature (Tm), the thermal cycle programme used, the fluorescent labels for automation using capillary sequencing system ABI3730; Primers included a degenerated anchor region at the 5' end.

Primer	Sequence	TM	Program	Label	Authors
issrCCA	5'DDBCCACCACCACCACCA	59°C	Touchdown 62	6FAM	Hantula *et al.*, 1997; Muller *et al.*, 2001
issrCGA	5'DHBCGACGACGACGACGA	59°C	Touchdown 62	not used in automated assays	
issrGT	5'YHYGTGTGTGTGTGTGTG	55°C	GRAD 58	NED	
issrAG	5'HBHAGAGAGAGAGAGAG	51°C	GRAD 58	VIC	Chadha and Gopalakrishna, 2007
issrAC	5'DBD ACA CAC ACA CAC AC	51°C	GRAD 58	PET	

Table 4.5: InterSSR touchdown thermal cycle programmes used to produce ISSR genotypes; The first thermal cycle "Touchdown 62" starts annealing at 62°C in the first cycle, the following annealing temperature is decreased by 0.5°C in every cycle until it reaches the 26th cycle. It was used with the primer issrCCA and issrCGA; The thermal cycle programme Grad 58 is a variation of touchdown which is often preferred for primers of lower melting temperature, this programme was used with the primers issrGT, issrAG and issrAC.

Step	Temperature	Time	cycle
1	95°C	15min	
2	95°C	30sec	
3	**62°C**	**45sec**	-0.5deg/cycle
4	72°C	2min	Step 2 x27
5	95°C	30sec	
6	**52°C**	**45sec**	
7	72°C	2min	Step 5 x12
8	72°C	10min	

Touchdown 62

Step	Temperature	Time	cycle
1	95°C	5min	
2	95°C	1min	
3	**58°C**	**1min**	
4	72°C	1min	Step 2 x5
5	94°C	30sec	
6	**55°C**	**1 min**	
7	70°C	1min	Step 5 x5
8	93°C	30sec	
9	**50°C**	**1 min**	
10	70°C	1min	Step 8 x15
11	93°C	30sec	
12	**47°C**	**1 min**	
13	70°C	1min	Step 11 x15
14	72°C	10min	

4.2.5.2 Scoring ISSR Bands

Mating system estimation

In the first assay which compared genotypes of different sets of siblings from Loch Maree and Abernethy (Figure 4.1), PCR products were separated on a gel system. The gel used was a mix of polymer 0.4% Agarose and 0.4% Synergel in 1x TBE (10.8g/l TRIS, 5.5g/l Boric Acid, 0.2mM EDTA). DNA bands were allowed to migrate for 45 min at 120V on an Apollo™ 23/14 cm system (CLP Genesee Scientific, San Diego USA). Bands were visualised by adding 12µl of Safeview fluoresecent dye (NBS Biologicals Ltd., Huntingdon UK) into 120ml 1x TBE. A picture of the electrophoretic banding patterns was taken under the UV light of an AlphaImager gel imaging system (Alpha Innotech Cell Bioscience, Santa Clara Canada) at 320 µm wavelength (Figure 4.1). Reproducibility of the assay was evaluated by repeating at least two PCRs for each species from both location sampled. Bands were scored as presence or absence of bands for each specific size. A montage of digital images were used onto which guiding lines were drawn in Open Office Calc spreadsheet (http://www.openoffice.org/http://www.openoffice.org/). Variation in migration across the gel was taken into consideration when tracing the guiding lines. Scored bands were recorded in the same spreadsheet.

Genetic diversity and genetic structure

In the second set of assays which involved genotyping isolates of unknown

relatedness, each of the four ISSR primers was labelled with a different fluorescent label (Table 4.5). This enabled automated scoring of band size using the capillary sequencer ABI3730 from GenePool (School of Biological Sciences, University of Edinburgh). Since each PCR product from a particular ISSR primer was identifiable with a unique fluorescent dye, it was possible to multiplex all the PCR products before reading by the sequencer. PCR products were diluted fifty times and combined in sterile distilled water. One microlitre of the diluted multiplexed PCR product was added to 9μl of the size standard GeneScan™ 1200 Liz™ (Applied Biosystem, Carlsbad USA) and buffer which was prepared as 5μl of the size standard in 1ml of Hi-Di™ Formamide (Applied Biosystem, Carlsbad USA) per reading plate.

Capillary sequencing automation of ISSR genotypes produced a reading of peaks of different sizes which was analysed using GeneMapper 3.7 (Applied Biosystem, Carlsbad USA). Size standards were manually checked and corrected where incorrect size was assigned. The method for scoring the presence or absence of peaks made use of panel management where each of the four labeled PCR products corresponded to one marker. This allowed positioning of bins within which peaks were scored. Peaks of sizes below 50bp and above 1200bp were not scored. Allocation of bins was conducted after combining all samples from one species and one primer together. Only those regions which were easy to score were used. Regions which showed too much overlap between peaks were omitted from scoring. If several peaks were systematically close together only the tallest peak would be scored within one sample.

4.2.5.3 Genetic Analysis of ISSR data

The first estimation of genetic variation within each population was based on the average percentage polymorphism. This was calculated in OpenOffice Calc as the number of loci which had both alleles (present and absent) in one population over the total number of loci.

The genetic diversity H and measures of population structure G_{ST} and Theta were performed using the software FSTAT 2.9.3.2 (Goudet, 1995; Goudet, 2002). FSTAT was originally designed to be used with co-dominant markers such as microsatellites in diploid organisms. Since this study uses dominant markers in haploid organisms, the input table needed some modification. There were only two alleles for each loci (presence or absence). Each loci was given a unique name based on the primer which generated the peak and its size. The presence of the peak was given the number 1 and the absence was given the number 2. Presence 1 or absence 2 was duplicated as 11 or 22 so that marker dominance and haploidy is transcribed as homozygosity in the input format. In a first instance allele frequencies, number of alleles and genetic diversities were computed as well as estimation of population structure. The mean genetic diversity per population was calculated in OpenOffice Calc using the ouput data from FSTAT. Estimation of genetic structure between populations were estimated using Nei's F statistics as well as Weir and Cockerman'F statistics (Nei, 1977; Weir and Cockerman, 1984). Finally, Fst per pair of samples was computed in order to investigate the pattern of genetic differentiation among the populations.

4.3 Results

4.3.1 Application of Inter-SSR analysis to the study of genetic variation in *Lophodermium* species

4.3.1.1 Reproducibility

Reproducibility of the ISSR assay scored on a gel system was determined from a total of five *L. conigenum* and three *L. seditiosum* single spore isolates for which repeat PCRs were conducted. In the case of *L. seditiosum,* repeats were loaded away from each other on the gel. The PCR reproducibility itself was very good as shown in Figure 4.1., with all repeat isolates showing identical sets of bands on the gel. The only inconsistency likely to arise in the scoring is for bands of smaller size located at the far end of the gel which does not receive as much UV light.

4.3.1.2 Band scoring on agarose

Agarose based genotyping for the study of the mating system of *L. conigenum* used two primers (issrCCA and issrCGA). These produced a total of 24 scorable polymorphic bands when run out on agarose. Five primers were used for the analysis of the *L. seditiosum* mating system (issrCCA, issrCGA, issrAC, issrAG and issrGT). With this increase in the number of primers used, the number of polymorphic bands scored on agarose was increased to 54.

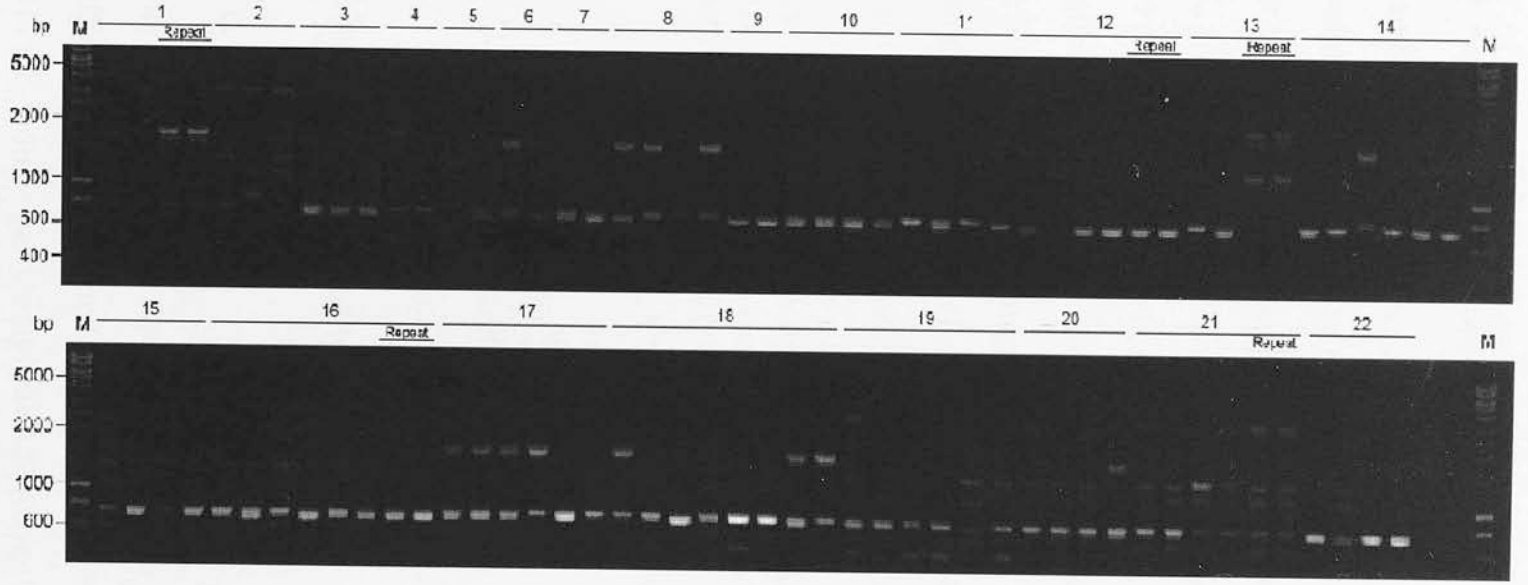

Figure 4.1: Electrophoresis image of the CCA ISSR PCR of *Lophodermium conigenum* sets of sibling spores visualised under UV (320 wavelength) using 12µl safeview in 100ml 1x TBE; 14 families from Abernethy annotated from 1 to 14 which includes three PCR repeats; 8 families from Loch Maree annotated from 15 to 22 which includes two repeat PCR; M = Hyperladder I (Bioline, London UK)

4.3.1.3 Automated band scoring

Using automated band scoring on the ABI3730 sequencer with four fluorescent labelled primers on isolates of unknown relatedness the total number of scorable bands was 200 for *L. pinastri* II, 174 for *L. conigenum* and 80 for *L. seditiosum*. Over all three species the total number of bands scored was 409 (Table 4.6). This number is a conservative estimate based on the assumption that peaks of the same size correspond to the same locus across species. However, if this assumption was relaxed only 45 loci would be added to this total. The primer which produced the most scorable bands (155) was issrAG which is an AG microsatellite primer (Table 4.6).

Table 4.6: Number of Loci per PCR and species; the total number of loci across species (Nloci) is a conservative total since it assumes that the same peak size across all species corresponds to the same locus.

Primer	Nloci	*L. pinastri II*	*L. conigenum*	*L. seditiosum*
issrCCA	100	43	41	30
issrAG	155	72	58	42
issrAC	105	57	48	8
issrGT	49	28	27	0
Total	409	200	174	80

4.3.2 Mating system analysis

4.3.2.1 L. conigenum

Ascospore isolations from *L. conigenum* ascocarps yielded 14 families from Abernethy and 8 families from Loch Maree (Tables 4.7 and 4.8). The average number of individuals per family was 3.28 (range 2-6) at Abernethy and 5.5

(range 4-8) at Loch Maree. Individuals were scored for presence/absence of bands at 24 ISSR loci. At Abernethy, 20 of these loci were polymorphic, whereas at Loch Maree only 14 showed polymorphism.

In haploid ascomycete fungi, self-fertilisation leads to the production of genetically identical offspring from a single ascocarp. The presence of genetic variation within a family indicates that outcrossing must have taken place. At Abernethy 12 of the 14 families showed segregation for at least one of the ISSR loci, whereas at Loch Maree all 8 families showed variation for at least one ISSR locus.

4.3.2.2 L. seditiosum

Seven families of *L. seditiosum* were isolated from Abernethy and 8 from Loch Maree (Tables 4.9 and 4.10). Mean family size was 6.14 (range 4-9) at Abernethy and 5.38 (range 3-7) at Loch Maree. Individuals were scored for variation at 54 polymorphic loci. At Abernethy 46 of these loci were polymorphic, whereas at Loch Maree polymorphism was present at 42 ISSR loci. At both Abernethy and Loch Maree every family showed segregation for at least one of the ISSR loci scored.

Table 4.7: Mating system of *L. conigenum*; ISSR genotypes of spore sibling isolates of *L. conigenum* from Loch Maree; each table corresponds to each family of sibling spores; 0 represents absence of a band and 1 represents presence of a band; the two rows for each family represent frequency of both alleles.

FLMLc01

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc01/2	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0
Flmlc01/3	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
Flmlc01/4	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc01/9	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
Allele 1 freq	0	0	0	0	0	0.25	0	1	0.25	0	0	1	0	0.5	0	0	0	0	0	0.25	0.75	0	0	0
Allele 0 freq	1	1	1	1	1	0.75	1	0	0.75	1	1	0	1	0.5	1	1	1	1	1	0.75	0.25	1	1	1

FLMLc04

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc04/1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc04/3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc04/5	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc04/6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc04/7	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc04/8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc04/9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Allele 1 freq	0	0	0	0	0	0	0	0.29	0.14	0	0	1	0	0.14	0	0	0	0	0	1	0	0	0	0
Allele 0 freq	1	1	1	1	1	1	1	0.71	0.86	1	1	0	1	0.86	1	1	1	1	1	0	1	1	1	1

FLMLc06

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc06/1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0
Flmlc06/2	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc06/5	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc06/6	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc06/7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc06/9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Allele 1 freq	0	0	0	0	0	0.67	0	0	0	0	0	1	0	0	0	0	0.17	0.17	0	1	0	0	0	0
Allele 0 freq	1	1	1	1	1	0.33	1	1	1	1	1	0	1	1	1	1	0.83	0.83	1	0	1	1	1	1

FLMLc08

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc08/1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0
Flmlc08/2	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0
Flmlc08/3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0
Flmlc08/4	0	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc08/5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0
Flmlc08/7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0
Flmlc08/8	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc08/9	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
Allele 1 freq	0	0	0	0	0	0.5	0	0.13	0.13	0.13	0	1	0	0.13	0.25	0	0.25	0.25	0.63	0.5	0	0	0	0
Allele 0 freq	1	1	1	1	1	0.5	1	0.88	0.88	0.88	1	0	1	0.88	0.75	1	0.75	0.75	0.38	0.5	1	1	1	1

FLMLc09

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc09/1	1	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0
Flmlc09/2	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc09/5	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0
Flmlc09/6	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0
Flmlc09/8	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc09/9	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0
Allele 1 freq	0.17	0	0	0	0	0	0	0.5	0.17	0.33	0	1	0	0.83	0.67	0	0	0	0	1	0	0	0	0
Allele 0 freq	0.83	1	1	1	1	1	1	0.5	0.83	0.67	1	0	1	0.17	0.33	1	1	1	1	0	1	1	1	1

FLMLc10

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc10/1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc10/3	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc10/4	1	0	1	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1	1	0	0	0
Flmlc10/7	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Allele 1 freq	0.5	0	0.25	0	0	0.25	0	0.75	0.5	0	0	1	0	1	0	0.25	0.25	0	0	1	0.25	0	0	0
Allele 0 freq	0.5	1	0.75	1	1	0.75	1	0.25	0.5	1	1	0	1	0	1	0.75	0.75	1	1	0	0.75	1	1	1

FLMLc11

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc11/4	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc11/5	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc11/6	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Flmlc11/7	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc11/8	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
Allele 1 freq	0.2	0	0	0	0	0	0	0.8	0.8	0	0	1	0	0.8	0	0	0	0	0	1	0	0	0	0
Allele 0 freq	0.8	1	1	1	1	1	1	0.2	0.2	1	1	0	1	0.2	1	1	1	1	1	0	1	1	1	1

FLMLc12

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Flmlc12/2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc12/4	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1	1	0	0	0
Flmlc12/6	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Flmlc12/8	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Allele 1 freq	0.25	0	0	0	0	0	0	0.75	0.25	0	0	1	0	0.25	0	0.25	0.25	0	0	0.75	0.25	0	0	0
Allele 0 freq	0.75	1	1	1	1	1	1	0.25	0.75	1	1	0	1	0.75	1	0.75	0.75	1	1	0.25	0.75	1	1	1

Table 4.8: Mating system of *L. conigenum*; ISSR genotypes of spore sibling isolates of *L. conigenum* from Abernethy; each table corresponds to each family of sibling spores; 0 represents absence of a band and 1 represents presence of a band; the two rows for each family represent frequency of both alleles.

FAbLc01

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabIc01/3	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
FabIc01/4	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
FabIc01/5	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
Allele 1 freq	0.33	0	0	0	0.33	0.33	0.33	0.33	0.33	0	0.33	1	0	0	0	0	0	0	0.33	0	0	0	0	0.33
Allele 0 freq	0.67	1	1	1	0.67	0.67	0.67	0.67	0.67	1	0.67	0	1	1	1	1	1	1	0.67	1	1	1	1	0.67

FAbLc02

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabIc02/2	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
FabIc02/4	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0
FabIc02/7	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Allele 1 freq	1	0	0	0	0	0	0	0.67	1	0.33	0.33	0.33	0	0	0	0	0	0.67	0.33	0.67	0	0	0	0
Allele 0 freq	0	1	1	1	1	1	1	0.33	0	0.67	0.67	0.67	1	1	1	1	1	0.33	0.67	0.33	1	1	1	1

FAbLc03

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabIc03/2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0
FabIc03/4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
FabIc03/5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
Allele 1 freq	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0.33	0	0.33	0	0	0	1	0
Allele 0 freq	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0.67	1	0.67	1	1	1	0	1

FAbLc04

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabIc04/3	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0
FabIc04/4	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Allele 1 freq	0	0	0	0	0.5	0	0	0.5	0	0	0	1	0	0	0.5	0	0	0	1	0	0	0	0	0
Allele 0 freq	1	1	1	1	0.5	1	1	0.5	1	1	1	0	1	1	0.5	1	1	1	0	1	1	1	1	1

FAbLc05

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabLc05/1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
FabLc05/2	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Allele 1 freq	0	0	0	0	0	0	0	0.5	0.5	0	0	1	0.5	0	0	0	0	0	1	0	0	0	0	0
Allele 0 freq	1	1	1	1	1	1	1	0.5	0.5	1	1	0	0.5	1	1	1	1	1	0	1	1	1	1	1

FAbLc06

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabLc06/4	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
FabLc06/8	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0
Allele 1 freq	0.5	0	0	0	0	0.5	0	0.5	0	0	0	0.5	0.5	0	0	0.5	0.5	0.5	0.5	0	0	0	0	0
Allele 0 freq	0.5	1	1	1	1	0.5	1	0.5	1	1	1	0.5	0.5	1	1	0.5	0.5	0.5	0.5	1	1	1	1	1

FabLc07

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabLc07/2	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1
FabLc07/7	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1
Allele 1 freq	0.5	0	0	0	0	0	0	0.5	0	0	0	0.5	1	0	0	0	0	0	1	0	0	0	0	1
Allele 0 freq	0.5	1	1	1	1	1	1	0.5	1	1	1	0.5	0	1	1	1	1	1	0	1	1	1	1	0

FabLc08

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabLc08/1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0
FabLc08/4	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
FabLc08/7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
FabLc08/9	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Allele 1 freq	0	0	0	0	0	0.75	0	0	0	0	0	0.75	0.25	0	0	0.25	0.25	0	0.5	0.5	0	0.25	0	0
Allele 0 freq	1	1	1	1	1	0.25	1	1	1	1	1	0.25	0.75	1	1	0.75	0.75	1	0.5	0.5	1	0.75	1	1

FabLc09

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
FabLc09/7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
FabLc09/8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Allele 1 freq	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Allele 0 freq	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1

FabLc10

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Fablc10/3a	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Fablc10/4a	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Fablc10/5	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Fablc10/7	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Allele 1 freq	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Allele 0 freq	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1

FabLc11

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Fablc11/3	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Fablc11/4	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Fablc11/5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Fablc11/6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Allele 1 freq	0.25	0.25	0	0	0	0	0	0	0	0	0	0.75	0.5	0	0	0	0	0	1	0	0	0	0	0
Allele 0 freq	0.75	0.75	1	1	1	1	1	1	1	1	1	0.25	0.5	1	1	1	1	1	0	1	1	1	1	1

FabLc12

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Fablc12/1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Fablc12/3	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Fablc12/4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Fablc12/5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Fablc12/8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Fablc12/8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0
Allele 1 freq	0	0	0	0	0	0.17	0	0.33	0.17	0	0	0.17	0.83	0	0	0.17	0.17	0	0.83	0	0	0	0	0
Allele 0 freq	1	1	1	1	1	0.83	1	0.67	0.83	1	1	0.83	0.17	1	1	0.83	0.83	1	0.17	1	1	1	1	1

FabLc15

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Fablc15/2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Fablc15/5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0
Fablc15/8	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Allele 1 freq	0	0	0.33	0.33	0	0	0	0.33	0	0	0	0.33	0.33	0	0	0.33	0.33	0	0.67	0	0	0	0	0
Allele 0 freq	1	1	0.67	0.67	1	1	1	0.67	1	1	1	0.67	0.67	1	1	0.67	0.67	1	0.33	1	1	1	1	1

FabLc17

	CCALc1	CCALc2	CCALc3	CCALc4	CCALc5	CCALc6	CCALc7	CCALc8	CCALc9	CCALc10	CCALc11	CCALc12	CCALc13	CCALc14	CCALc15	CGALc1	CGALc2	CGALc3	CGALc4	CGALc5	CGALc6	CGALc7	CGALc8	CGALc9
Fablc17/4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Fablc17/5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0
Fablc17/6	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Fablc17/7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Fablc17/8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Fablc17/8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Allele 1 freq	0	0	0	0	0.17	0	0	0	0	0	0	0.17	0.83	0	0	0.17	0.17	0	0.5	0.5	0	0	0	0
Allele 0 freq	1	1	1	1	0.83	1	1	1	1	1	1	0.83	0.17	1	1	0.83	0.83	1	0.5	0.5	1	1	1	1

Table 4.9: Mating system of *L. seditiosum*; ISSR genotypes of spore sibling isolates of *L. seditiosum* from Loch Maree; each table corresponds to each family of sibling spores; 0 represents absence of a band and 1 represents presence of a band; the two rows for each family represent frequency of both alleles.

Flmls01

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Flmls01/2	0	0	1	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	1
Flmls01/3	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Flmls01/4	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1
Flmls01/5	1	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Flmls01/6	0	0	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	1
Allele 1 freq	0.4	0	1	0	0	0	0	1	0.6	0.4	0	0	1	0.4	0	0	0	0.2	0.6	1	0	0.4	0	0.2	0	0	1
Allele 0 freq	0.6	1	0	1	1	1	1	0	0.4	0.6	1	1	0	0.6	1	1	1	0.8	0.4	0	1	0.6	1	0.8	1	1	0

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Flmls01/2	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	1
Flmls01/3	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1	1
Flmls01/4	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1
Flmls01/5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
Flmls01/6	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0.6	0	0	0	0	0.4	0	0	0.6	0	0.6	0	0	0	0.8	0.4	0.8	0.6	0.2	0.2	0	0.8	0	0	0.2	0.8	1
Allele 0 freq	0.4	1	1	1	1	0.6	1	1	0.4	1	0.4	1	1	1	0.2	0.6	0.2	0.4	0.8	0.8	1	0.2	1	1	0.8	0.2	0

Flmls02

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Flmls02/1	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1
Flmls02/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Flmls02/3	1	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Flmls02/4	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Flmls02/6	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Flmls02/7	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Flmls02/8	1	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Allele 1 freq	0.29	0	0.86	0	0.43	0	0	0.86	0	0.86	0	0	0.71	0	0	0	0	0	1	0.14	0	0	0	0	0	0.14	0.29
Allele 0 freq	0.71	1	0.14	1	0.57	1	1	0.14	1	0.14	1	1	0.29	1	1	1	1	1	0	0.86	1	1	1	1	1	0.86	0.71

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Flmls02/1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	1	1
Flmls02/2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Flmls02/3	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	1	1	0	1	0	0	0	0	1	1	1
Flmls02/4	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	1	0	0	0	1	1
Flmls02/6	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	1
Flmls02/7	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	1
Flmls02/8	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1
Allele 1 freq	0	0.14	0	0	0	0	0.57	0.57	0.14	0	0.43	0.14	0	0	1	0	0.29	1	0.29	0	0.57	0.29	0	0	0.43	1	1
Allele 0 freq	1	0.86	1	1	1	1	0.43	0.43	0.86	1	0.57	0.86	1	1	0	1	0.71	0	0.71	1	0.43	0.71	1	1	0.57	0	0

FImls03

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
FImls03/1	0	1	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0
FImls03/2	1	1	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0
FImls03/3	0	1	0	0	0	0	0	1	0	1	0	0	1	1	0	0	1	0	1	1	0	0	0	0	0	1	0
FImls03/4	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
FImls03/5	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
FImls03/6	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Allele 1 freq	0.67	0.67	0	0	0.33	0	0	1	0	1	0	0.33	0.5	0.67	0	0	0.17	0	1	0.33	0	0	0	0	0	0.5	0
Allele 0 freq	0.33	0.33	1	1	0.67	1	1	0	1	0	1	0.67	0.5	0.33	1	1	0.83	1	0	0.67	1	1	1	1	1	0.5	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
FImls03/1	0	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1
FImls03/2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FImls03/3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1
FImls03/4	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FImls03/5	0	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1
FImls03/6	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0	0.5	0	0	0	0.17	0	0	0.67	0	0.5	0.67	0	0	0.67	0	0	1	0	0	0	1	0.5	0	0	0.5	1
Allele 0 freq	1	0.5	1	1	1	0.83	1	1	0.33	1	0.5	0.33	1	1	0.33	1	1	0	1	1	1	0	0.5	1	1	0.5	0

FImls04

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
FImls04/1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	0	0
FImls04/2	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0
FImls04/3	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0
FImls04/5	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0
FImls04/6	1	1	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0
FImls04/7	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
FImls04/8	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Allele 1 freq	0.14	0.29	0	0	0	0	0	1	0	1	0	0	0.71	1	0	0	0	0	0	0.71	0	0	0.57	0	1	0.29	0
Allele 0 freq	0.86	0.71	1	1	1	1	1	0	1	0	1	1	0.29	0	1	1	1	1	1	0.29	1	1	0.43	1	0	0.71	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
FImls04/1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FImls04/2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
FImls04/3	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1	1
FImls04/5	0	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	1	0	0	0	1	0	0	0	1	1
FImls04/6	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0	1	1	0	0	0	1	0	0	0	1	1
FImls04/7	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1	1
FImls04/8	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0	0	0	0	0	0.71	0	0	0.57	0	1	0.29	0	0.14	0.86	0.14	0.57	0.86	0	0	0	0.86	0	0	0.14	0.86	0.86
Allele 0 freq	1	1	1	1	1	0.29	1	1	0.43	1	0	0.71	1	0.86	0.14	0.86	0.43	0.14	1	1	1	0.14	1	1	0.86	0.14	0.14

FImls06

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
FImls06/1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0
FImls06/3	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0
FImls06/4	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0
FImls06/5	0	1	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0
FImls06/6	0	1	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0
Allele 1 freq	0	1	0	0	0	0	0	0.6	0.6	0.8	0	0.8	0.4	0.2	0	0	0	0	1	1	0	0	0	0	0	1	0
Allele 0 freq	1	0	1	1	1	1	1	0.4	0.4	0.2	1	0.2	0.6	0.8	1	1	1	1	0	0	1	1	1	1	1	0	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
FImls06/1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FImls06/3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
FImls06/4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
FImls06/5	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
FImls06/6	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0	0	0.2	0	0	0.2	0	0.2	0	0	0.6	0.4	0	0	0.2	0	0	1	0	0	0	1	0	0	0	1	1
Allele 0 freq	1	1	0.8	1	1	0.8	1	0.8	1	1	0.4	0.6	1	1	0.8	1	1	0	1	1	1	0	1	1	1	0	0

FImls07

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
FImls07/1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
FImls07/3	0	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
FImls07/4	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
FImls07/6	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Allele 1 freq	0	0.75	0.25	0	0	0	0	1	0	1	0.25	0.25	0.75	0	0	0	0	0	0.75	0.25	0	0	0	0	0	0.5	0
Allele 0 freq	1	0.25	0.75	1	1	1	1	0	1	0	0.75	0.75	0.25	1	1	1	1	1	0.25	0.75	1	1	1	1	1	0.5	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
FImls07/1	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FImls07/3	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1
FImls07/4	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FImls07/6	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0.5	0.5	0	0	0	0	0.25	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0.75	1
Allele 0 freq	0.5	0.5	1	1	1	1	0.75	1	1	1	0	0	1	1	0	1	1	0	1	1	1	0	1	1	1	0.25	0

Flmls11

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Flmls11/1	0	1	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Flmls11/4	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Flmls11/6	1	0	1	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Allele 1 freq	0.67	0.33	0.67	0	0	0	0	1	0	1	0	0.67	0.33	0.67	0	0	0	0	1	0	0	0	0	0	0	0	0
Allele 0 freq	0.33	0.67	0.33	1	1	1	1	0	1	0	1	0.33	0.67	0.33	1	1	1	1	0	1	1	1	1	1	1	1	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Flmls11/1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	1
Flmls11/4	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	1	0	1	0	1	0	0	0	1	1
Flmls11/6	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1
Allele 1 freq	0	0.33	0	0	0	0	0.33	0	0.33	0	1	0.33	0	0	1	0.67	0	1	0	0.67	0	1	0	0	0	1	1
Allele 0 freq	1	0.67	1	1	1	1	0.67	1	0.67	1	0	0.67	1	1	0	0.33	1	0	1	0.33	1	0	1	1	1	0	0

Flmls27

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Flmls27/1	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Flmls27/2	1	0	1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0
Flmls27/4	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Flmls27/5	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Flmls27/6	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Flmls27/8	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Allele 1 freq	1	0	1	0	0	0	0	1	0	1	0.17	0.5	0	0.17	0	0	0	0	1	0	0	0	0	0	0	0.83	0
Allele 0 freq	0	1	0	1	1	1	1	0	1	0	0.83	0.5	1	0.83	1	1	1	1	0	1	1	1	1	1	1	0.17	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Flmls27/1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1
Flmls27/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Flmls27/4	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Flmls27/5	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Flmls27/6	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Flmls27/8	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1
Allele 1 freq	0	0.5	0	0	0	0	0	0.17	0	0	0.83	0	0	0	0.17	0.17	0	0.83	0	0	0	1	0	0	0.33	1	1
Allele 0 freq	1	0.5	1	1	1	1	1	0.83	1	1	0.17	1	1	1	0.83	0.83	1	0.17	1	1	1	0	1	1	0.67	0	0

Table 4.10: Mating system of *L. seditiosum*; ISSR genotypes of spore sibling isolates of *L. seditiosum* from Abernethy; each table correspond to each family of sibling spores; 0 represents absence of a band and 1 represents presence of a band; the two rows for each family represent frequency of both alleles.

FabIs01

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
FabIs01/1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
FabIs01/2	1	0	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
FabIs01/3	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
FabIs01/4	0	0	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Allele 1 freq	0.75	0	1	1	0	0	0.5	0	1	0	0	1	0	0.75	0	0	0	1	0	0	0	0	0	0	0	0	0.25
Allele 0 freq	0.25	1	0	0	1	1	0.5	1	0	1	1	0	1	0.25	1	1	1	0	1	1	1	1	1	1	1	1	0.75

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
FabIs01/1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	1
FabIs01/2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	0	0	1	1
FabIs01/3	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
FabIs01/4	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0.5	0	0.5	0	0	0	0	0	0.75	0.25	0.5	0	0	0	0.75	0	0	1	0	0	0	1	0.5	0	0	1	1
Allele 0 freq	0.5	1	0.5	1	1	1	1	1	0.25	0.75	0.5	1	1	1	0.25	1	1	0	1	1	1	0	0.5	1	1	0	0

FabIs03

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
FabIs03/1	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1
FabIs03/2	1	0	1	0	0	0	1	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1
FabIs03/3	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
FabIs03/4	1	0	1	0	1	0	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
FabIs03/6	1	0	1	0	0	0	1	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
FabIs03/7	1	0	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1
Allele 1 freq	0.83	0	0.83	0	0.5	0	1	0	1	0	0.5	0.67	0	0.5	0.5	0	0	1	0	0.5	0	0	0	0	0	0.17	0.5
Allele 0 freq	0.17	1	0.17	1	0.5	1	0	1	0	1	0.5	0.33	1	0.5	0.5	1	1	0	1	0.5	1	1	1	1	1	0.83	0.5

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
FabIs03/1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
FabIs03/2	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
FabIs03/3	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
FabIs03/4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
FabIs03/6	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
FabIs03/7	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0.33	0	0	0	0	0.67	0.83	0	0	0.33	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
Allele 0 freq	0.67	1	1	1	1	0.33	0.17	1	1	0.67	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0

Fabls08

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Fabls08/1	0	0	1	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls08/2	1	1	0	0	0	0	1	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Fabls08/3	0	1	0	0	0	0	1	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Fabls08/4	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1
Fabls08/6	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Allele 1 freq	0.4	0.8	0.2	0.2	0	0	1	0	1	0	0	0.6	0.8	1	0	0.6	0	0.4	0	0	0	0	0	0	0	0	0.2
Allele 0 freq	0.6	0.2	0.8	0.8	1	1	0	1	0	1	1	0.4	0.2	0	1	0.4	1	0.6	1	1	1	1	1	1	1	1	0.8

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Fabls08/1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1
Fabls08/2	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1
Fabls08/3	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1
Fabls08/4	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	1
Fabls08/6	1	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0.2	0	0	0	0	0	0	0.4	0.2	0.8	0.2	0.6	0	0.6	0.2	0	0	1	0	0	0	0.8	0	0.6	0	1	1
Allele 0 freq	0.8	1	1	1	1	1	1	0.6	0.8	0.2	0.8	0.4	1	0.4	0.8	1	1	0	1	1	1	0.2	1	0.4	1	0	0

Fabls11

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Fabls11/1	1	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0
Fabls11/3	0	1	1	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0
Fabls11/4	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls11/5	1	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0
Fabls11/6	1	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Fabls11/8	0	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Allele 1 freq	0.5	0.67	0.83	0	0	0	1	0	1	0	0	0	1	0.33	0	0	0	0.83	0.17	0	0	0	0.33	0.33	0	0	0
Allele 0 freq	0.5	0.33	0.17	1	1	1	0	1	0	1	1	1	0	0.67	1	1	1	0.17	0.83	1	1	1	0.67	0.67	1	1	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Fabls11/1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1
Fabls11/3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1
Fabls11/4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls11/5	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	1
Fabls11/6	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1
Fabls11/8	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1
Allele 1 freq	0	0	0	0	0	0	1	0	0	0	0.5	0	0	0	0.33	0.33	0	1	0	0.67	0	0.33	0	0	0	1	1
Allele 0 freq	1	1	1	1	1	1	0	1	1	1	0.5	1	1	1	0.67	0.67	1	0	1	0.33	1	0.67	1	1	1	0	0

Fabls13

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Fabls13/1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Fabls13/3	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Fabls13/4	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Fabls13/5	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls13/6	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls13/8	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1
Allele 1 freq	0.83	0	0	0	0	0	1	0	1	0	0	0.33	0.67	0	0	0	0	0.33	0.67	0	0	0	0	0	0	0.33	0.17
Allele 0 freq	0.17	1	1	1	1	1	0	1	0	1	1	0.67	0.33	1	1	1	1	0.67	0.33	1	1	1	1	1	1	0.67	0.83

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Fabls13/1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	1	0	0	1	0	0	0	1	0
Fabls13/3	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	0	0	0	1	1
Fabls13/4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	1	0	0	0	1	0
Fabls13/5	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1	1	0	0	1	0	0	0	1	1
Fabls13/6	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	0	0	0	1	0
Fabls13/8	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	1	0	0	1	0	0	0	1	0
Allele 1 freq	0.5	0	0	0	0	0.17	0	0	0.33	0	1	0	0	0	1	0	0.83	1	1	0	0	1	0	0	0	1	0.33
Allele 0 freq	0.5	1	1	1	1	0.83	1	1	0.67	1	0	1	1	1	0	1	0.17	0	0	1	1	0	1	1	1	0	0.67

Fabls15

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Fabls15/1	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1
Fabls15/2	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls15/3	1	1	1	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls15/4	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1
Fabls15/5	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls15/6	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1
Fabls15/7	1	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Allele 1 freq	0.71	0.29	0.71	0	0	0	1	0	1	0	0	0	1	0.29	0	0	0	1	0	0.29	0	0	0	0	0	0	0.43
Allele 0 freq	0.29	0.71	0.29	1	1	1	0	1	0	1	1	1	0	0.71	1	1	1	0	1	0.71	1	1	1	1	1	1	0.57

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Fabls15/1	0	1	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Fabls15/2	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls15/3	1	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls15/4	0	1	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Fabls15/5	0	0	1	0	1	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Fabls15/6	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls15/7	1	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Allele 1 freq	0.29	0.57	1	0	0.43	0.57	0.86	0	0	0	0.14	1	0.29	0	0.57	0	0	1	0	0	0	1	0	0	0	1	1
Allele 0 freq	0.71	0.43	0	1	0.57	0.43	0.14	1	1	1	0.86	0	0.71	1	0.43	1	1	0	1	1	1	0	1	1	1	0	0

Fabls16

Band	CCALs1	CCALs2	CCALs3	CCALs4	CCALs5	CCALs6	CCALs7	CCALs8	CCALs9	CCALs10	CCALs11	CCALs12	CCALs13	CCALs14	CGALs1	CGALs2	CGALs3	CGALs4	CGALs5	CGALs6	CGALs7	CGALs8	CGALs9	CGALs10	CGALs11	CGALs12	CGALs13
Fabls16/1	0	0	0	1	0	1	1	0	1	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0
Fabls16/2	1	0	0	1	0	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls16/3	0	0	0	1	0	0	1	1	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0
Fabls16/4	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls16/5	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0
Fabls16/6	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0
Fabls16/7	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fabls16/8	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
Fabls16/9	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Allele 1 freq	0.22	0	0	0.44	0	0.33	1	0.44	1	0	0	0.44	0.44	0.11	0	0	0	1	0.44	0.11	0.11	0	0	0	0.33	0.22	0
Allele 0 freq	0.78	1	1	0.56	1	0.67	0	0.56	0	1	1	0.56	0.56	0.89	1	1	1	0	0.56	0.89	0.89	1	1	1	0.67	0.78	1

Band	AG1	AG2	AG3	AG4	AG5	AG6	AG7	AG8	AG9	AG10	AG11	AG12	AG13	AC1	AC2	AC3	AC4	AC5	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	GT9
Fabls16/1	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls16/2	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Fabls16/3	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
Fabls16/4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls16/5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1
Fabls16/6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1
Fabls16/7	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1
Fabls16/8	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	1
Fabls16/9	1	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1
Allele 1 freq	0.56	0	0	0.22	0.11	0.33	0	0	0.22	0.11	0.11	0.22	0	0.11	0.33	0	0.22	1	0	0	0	0.89	0	0	0.11	1	1
Allele 0 freq	0.44	1	1	0.78	0.89	0.67	1	1	0.78	0.89	0.89	0.78	1	0.89	0.67	1	0.78	0	1	1	1	0.11	1	1	0.89	0	0

4.3.3 Genetic diversity of three *Lophodermium* species

The complete data on ISSR allele frequencies for *L. pinastri* II, *L. conigenum* and *L. seditiosum* at Abernethy, Glen Affric and Loch Maree , and for *L. pinastri* II at Arlanc are shown in Table 4.11. Allele frequencies were often less than 0.2 or more than 0.8, especially for *L. pinastri* II and *L. conigenum.* Polymorphism and the mean gene diversity over loci per population are given in Table 4.12. Both measures of genetic diversity are in concordance with one another. The most genetically diverse species is the pathogen *L. seditiosum*. *L. seditiosum* shows an average of 54% polymorphism compared with 49.5% and 39.5% for *L. conigenum* and *L. pinastri* II, respectively. *L. seditiosum* is also the most genetically diverse according to its gene diversity which is 0.11 as opposed to 0.07 and 0.04 for *L. conigenum* and *L. pinastri* II, respectively. Both measures also indicated that the amount of genetic diversity does not differ greatly between populations.

Table 4.11: Allele frequencies for each species of the bi-allelic markers ISSR; Only the frequency of the allele represented as presence of an ISSR peak is given; Frequency of absence of an ISSR peak would be the allele frequency subtracted from 1; Not all loci are represented for *L. pinastri* II and *L. conigenum*; occurrence of missing loci is represented "...".

L. pinastri II	Populations			
Loci	**Abernethy N= 33**	**Glen Affric N=31**	**Loch Maree N=23**	**Arlanc N= 32**
AG1020	0.000	0.000	0.043	0.000
AG1026	0.000	0.000	0.000	0.031
AG1034	0.030	0.000	0.000	0.000
AG1035	0.030	0.000	0.000	0.000
AG1038	0.000	0.032	0.000	0.063
AG1041	0.000	0.000	0.043	0.031
AG1044	0.000	0.000	0.043	0.000
AG1048	0.030	0.000	0.000	0.000
AG1050	0.000	0.000	0.000	0.031
AG1052	0.030	0.000	0.000	0.000
GT297	0.030	0.000	0.000	0.000
GT326	0.000	0.000	0.043	0.000
GT342	0.030	0.000	0.000	0.000
GT358	0.000	0.000	0.000	0.031
GT370	0.030	0.000	0.000	0.000
...				
GT809	0.000	0.032	0.000	0.031
GT814	0.000	0.000	0.000	0.031
GT866	0.030	0.000	0.043	0.000
AC262	0.091	0.097	0.043	0.063
AC301	0.030	0.000	0.000	0.000
AC307	0.000	0.032	0.000	0.031
AC310	0.030	0.032	0.000	0.031
AC316	0.000	0.000	0.000	0.031
AC325	0.000	0.000	0.043	0.000
AC368	0.030	0.000	0.000	0.000
AC401	0.000	0.000	0.000	0.031
AC405	0.030	0.000	0.000	0.000
AC451	0.000	0.065	0.000	0.000
AC457	0.000	0.065	0.000	0.000
AC463	0.000	0.065	0.000	0.000
AC514	0.000	0.065	0.000	0.031
AC529	0.000	0.000	0.000	0.031
AC543	0.000	0.000	0.043	0.000
AC545	0.030	0.065	0.000	0.000
AC556	0.061	0.000	0.043	0.031
AC583	0.030	0.000	0.000	0.063
...				
AC1121	0.030	0.032	0.000	0.031
AC1134	0.121	0.032	0.130	0.031
AC1145	0.061	0.032	0.217	0.063

L. pinastri II	Populations			
Loci	Abernethy N= 33	Glen Affric N=31	Loch Maree N=23	Arlanc N= 32
CCA315	0.091	0.065	0.043	0.125
CCA342	0.000	0.000	0.000	0.063
CCA346	0.152	0.065	0.130	0.125
CCA443	0.000	0.065	0.000	0.000
CCA458	0.030	0.032	0.130	0.063
CCA540	0.061	0.032	0.000	0.000
CCA573	0.000	0.000	0.043	0.031
CCA617	0.000	0.000	0.087	0.031
CCA623	0.000	0.000	0.043	0.031
CCA624	0.061	0.000	0.087	0.000
CCA625	0.030	0.000	0.000	0.031
CCA627	0.000	0.000	0.000	0.031
...				
CCA917	0.000	0.000	0.000	0.031
CCA953	0.030	0.065	0.000	0.000
CCA955	0.000	0.000	0.043	0.000
CCA958	0.061	0.000	0.043	0.000
CCA1109	0.030	0.065	0.043	0.000
CCA1110	0.061	0.097	0.043	0.125
CCA1110	0.030	0.032	0.000	0.063
CCA1111	0.030	0.032	0.043	0.031
CCA1112	0.030	0.032	0.000	0.094
CCA1114	0.030	0.032	0.000	0.031
CCA1115	0.000	0.032	0.000	0.000
AG230	0.030	0.000	0.087	0.000
AG233	0.000	0.000	0.000	0.063
AG238	0.030	0.032	0.000	0.000
AG244	0.030	0.000	0.000	0.000
AG251	0.000	0.032	0.000	0.000
AG252	0.000	0.000	0.043	0.000
AG262	0.091	0.032	0.043	0.031
AG270	0.030	0.000	0.000	0.000
AG277	0.030	0.000	0.043	0.031
AG297	0.030	0.000	0.000	0.031
AG299	0.030	0.000	0.000	0.000
AG301	0.030	0.000	0.043	0.000
AG304	0.000	0.000	0.000	0.063
AG309	0.000	0.000	0.000	0.063
...				

L. conigenum	Populations			*L. seditiosum*	Populations		
Loci	Abernethy N=27	Glen Affric N=29	Loch Maree N=23	Loci	Abernethy N=23	Glen Affric N=19	Loch Maree N=30
CCA288	0.000	0.034	0.043	CCA302	0.739	0.684	0.767
CCA397	0.037	0.000	0.000	CCA308	0.043	0.000	0.000
CCA415	0.000	0.000	0.043	CCA385	0.913	0.842	0.733
CCA427	0.000	0.034	0.043	CCA388	0.261	0.421	0.100
CCA443	0.037	0.000	0.000	CCA402	0.000	0.000	0.067
CCA459	0.000	0.034	0.043	CCA404	0.000	0.000	0.067
CCA479	0.037	0.034	0.043	CCA415	0.000	0.053	0.100
CCA482	0.000	0.103	0.043	CCA423	0.043	0.211	0.100
CCA487	0.000	0.000	0.043	CCA427	0.087	0.000	0.033
CCA490	0.037	0.000	0.000	CCA441	0.043	0.000	0.000
CCA549	0.000	0.000	0.043	CCA443	0.043	0.000	0.000
CCA708	0.000	0.034	0.000	CCA451	0.000	0.000	0.033
...				CCA459	0.000	0.053	0.000
CCA809	0.000	0.103	0.000	CCA461	0.000	0.053	0.000
CCA818	0.111	0.000	0.000	CCA482	0.000	0.000	0.033
CCA823	0.148	0.000	0.000	CCA528	0.783	0.579	0.600
CCA854	0.037	0.103	0.000	CCA529	0.174	0.158	0.200
CCA865	0.000	0.000	0.043	CCA531	0.000	0.158	0.167
CCA960	0.000	0.034	0.000	CCA532	0.000	0.053	0.067
CCA962	0.000	0.138	0.130	CCA534	0.000	0.053	0.000
CCA964	0.000	0.069	0.000	CCA574	0.043	0.263	0.300
CCA972	0.000	0.069	0.000	CCA575	0.000	0.000	0.033
CCA100	0.074	0.000	0.043	CCA606	0.000	0.000	0.033
CCA111	0.333	0.276	0.304	CCA730	0.000	0.000	0.033
AG235	0.000	0.000	0.043	CCA752	0.000	0.053	0.000
AG237	0.000	0.034	0.043	CCA781	0.043	0.053	0.100
AG252	0.000	0.103	0.000	CCA784	0.087	0.000	0.000
AG254	0.037	0.103	0.000	CCA854	0.087	0.053	0.167
AG283	0.037	0.000	0.043	CCA860	0.043	0.053	0.100
AG301	0.037	0.000	0.043	CCA865	0.435	0.211	0.267
AG308	0.074	0.000	0.043	AG288	0.043	0.000	0.000
AG337	0.000	0.000	0.043	AG290	0.043	0.000	0.000
AG358	0.037	0.000	0.043	AG317	0.000	0.000	0.033
AG367	0.000	0.000	0.043	AG321	0.043	0.000	0.000
AG371	0.000	0.000	0.043	AG411	0.000	0.000	0.033
AG382	0.000	0.034	0.000	AG433	0.043	0.000	0.000
AG460	0.000	0.000	0.043	AG438	0.043	0.000	0.000
AG469	0.000	0.034	0.000	AG489	0.000	0.000	0.033
...				AG527	0.043	0.000	0.000

L. conigenum	Populations			*L. seditiosum*	Populations		
Loci	Abernethy N=27	Glen Affric N=29	Loch Maree N=23	Loci	Abernethy N=23	Glen Affric N=19	Loch Maree N=30
AG1344	0.000	0.000	0.087	AG528	0.043	0.000	0.000
AG1345	0.037	0.138	0.043	AG530	0.087	0.000	0.033
AG1347	0.185	0.069	0.043	AG531	0.043	0.053	0.100
AG1349	0.074	0.172	0.087	AG532	0.000	0.053	0.033
AG1350	0.000	0.034	0.000	AG533	0.043	0.105	0.067
AG1351	0.000	0.034	0.087	AG534	0.043	0.105	0.000
AG1353	0.000	0.034	0.000	AG543	0.000	0.000	0.033
GT313	0.000	0.034	0.043	AG545	0.000	0.000	0.033
GT318	0.000	0.069	0.043	AG661	0.000	0.053	0.000
GT321	0.074	0.103	0.087	AG665	0.000	0.053	0.000
GT322	0.037	0.069	0.000	AG672	0.087	0.000	0.000
GT370	0.000	0.000	0.043	AG675	0.087	0.000	0.000
GT386	0.000	0.000	0.043	AG678	0.000	0.000	0.033
GT402	0.000	0.103	0.000	AG681	0.000	0.000	0.033
GT403	0.000	0.103	0.000	AG683	0.000	0.000	0.033
...				AG773	0.043	0.000	0.000
GT585	0.000	0.034	0.000	AG776	0.043	0.000	0.033
GT589	0.037	0.000	0.000	AG779	0.000	0.000	0.033
GT593	0.037	0.000	0.000	AG836	0.043	0.000	0.033
GT686	0.000	0.034	0.043	AG840	0.087	0.000	0.033
GT692	0.000	0.034	0.043	AG845	0.043	0.000	0.000
GT758	0.000	0.000	0.043	AG852	0.000	0.000	0.033
GT762	0.074	0.034	0.043	AG854	0.043	0.000	0.000
GT769	0.074	0.034	0.043	AG857	0.000	0.000	0.033
GT1073	0.037	0.000	0.043	AG859	0.043	0.000	0.000
GT1080	0.037	0.000	0.000	AG952	0.000	0.000	0.033
AC303	0.000	0.034	0.000	AG960	0.087	0.000	0.000
AC307	0.000	0.034	0.043	AG962	0.000	0.053	0.000
AC313	0.000	0.034	0.043	AG973	0.043	0.000	0.000
AC319	0.037	0.069	0.043	AG993	0.000	0.000	0.033
AC321	0.111	0.103	0.087	AG1008	0.043	0.000	0.000
AC323	0.000	0.000	0.043	AG1013	0.000	0.053	0.000
AC327	0.000	0.034	0.000	AG1062	0.087	0.105	0.000
AC329	0.037	0.000	0.000	AC303	0.000	0.000	0.067
AC346	0.148	0.069	0.130	AC304	0.522	0.316	0.267
AC361	0.037	0.000	0.000	AC305	0.870	0.895	0.733
AC363	0.148	0.034	0.217	AC306	0.348	0.684	0.533
...				AC309	0.000	0.000	0.067
AC1020	0.074	0.000	0.000	AC310	0.522	0.316	0.267
AC1031	0.074	0.000	0.000	AC312	0.870	0.895	0.733
AC1084	0.037	0.000	0.174	AC313	0.348	0.684	0.533

4.3.4 Population structure of three *Lophodermium* species

Population differentiation, which defines how much one population shares alleles with another, was measured by estimating Fst, the amount of inbreeding due to population subdivision, using two estimators: Gst, which is Nei's estimator of Fst and measures the proportion of gene diversity found between populations and theta, which is Weir & Cockerham's (1984) estimator of Fst and measures the standardised variance in allele frequency among populations. Both statistics gave evidence of significant ($P<0.05$) but low genetic structure for *L. conigenum* ($\theta= 0.028$) and *L. seditiosum* ($\theta= 0.020$) among the three populations in Scotland (Table 4.12). However there was no evidence of significant genetic structure among Scottish populations of *L. pinastri* II, and this lack of structure was also found when the French population from Arlanc was added to the analysis (Table 4.12).

Table 4.12: Mean Gene diversity and population differentiation for each species; Hs = within population mean gene diversity; Ht' = unbiased total gene diversity; Gst' = Nei's estimator of Fst; Theta = Weir & Cockerham's estimator of Fst.

Species	Hs		Ht'		Gst'		θ		95% confidence interval	
	inc Arlanc	exc Arlanc	inc Arlanc	exc Arlanc	inc Arlanc	exc Arlanc	inc Arlanc	exc Arlanc	inc Arlanc	exc Arlanc
L. pinastri II	0.039	0.038	0.039	0.038	0.001	0.001	0.000	0.001	-0.004 – 0.006	-0.005 – 0.008
L. conigenum	NA	0.066	NA	0.068	NA	0.027	NA	0.028	NA	0.014 – 0.044
L. seditiosum	NA	0.105	NA	0.108	NA	0.021	NA	0.020	NA	0.006 – 0.032

Table 4.13: Pairwise comparison of Fst; a.)*L. pinastri II*; b.) *L. conigenum*; c.) *L. seditiosum*

a.)

L. pinastri II	Abernethy	Glen affric	Loch Maree	Arlanc (FR)
Abernethy	0.0000			
Glen Affric	0.0021	0.0000		
Loch Maree	-0.0053	0.0079	0.0000	
Arlanc (FR)	-0.0003	-0.0009	0.0010	0.0000

b.)

L. conigenum	Abernethy	Glen affric	Loch Maree
Abernethy	0.0000		
Glen Affric	***0.0440***	0.0000	
Loch Maree	**0.0064**	***0.0267***	0.0000

c.)

L. seditiosum	Abernethy	Glen affric	Loch Maree
Abernethy	0.0000		
Glen Affric	***0.0333***	0.0000	
Loch Maree	***0.0294***	-0.0020	0.0000

The reason for the *L. seditiosum* population structure is clearly highlighted in the pair wise comparison of Fst in Table 4.13. This indicates that it is the population located in Abernethy which is the most genetically different from the other two Scottish populations located to the west. Loch Maree and Glen Affric, which do not show significant differentiation, are located closer to each other (Figure 3.1). This pattern does not occur for the endophytic species *L. conigenum* where the most genetically divergent population is that from Glen Affric (Table 4.13).

4.4 Discussion

The first result to come from this study is that both the endophyte *L. conigenum* and the pathogen *L. seditiosum* favour outcrossing over selfing. These results would suggest that both species are heterothallic in natural populations. The second conclusion that we can draw is that there is low but significant genetic structuring of populations in *L. conigenum* and *L. seditiosum*, but no significant genetic structuring in the endophyte *L. pinastri* II. Thus population genetic structuring does not differ according to the lifestyle of the species (parasite or endophyte). However the result is consistent with, but does not prove, a coherent model where fruiting habitat is the determining factor which affects structuring of populations of these ascomycete fungi. To demonstrate a robust relationship between fruiting habit and population structure, comparable data would have to be gathered from a number of independent lineages. Nevertheless these results provide an advance in our knowledge to which further results can be added. In this case greater genetic structure is found for the taxa whose fruiting habitat is more restricted (stressed or prematurely killed needles) than for the taxon which fruits on the much more extensive habitat provided by naturally senescing needles.

It has previously been observed that culture morphology within progenies from single ascocarps of *L. seditiosum* and *L. conigenum* differed on 2% Malt Extract agar (Minter & Millar, 1980b). This is compatible with both species being primarily outcrossing. Results of ISSR genotyping reported here reflect

these findings. The original prediction that self-fertilisation may evolve in these taxa was not upheld. This suggests that despite the limited ability of conidiospores (male gametes) to disperse, this does not prevent outcrossing occurring. Conidiospore dispersal is most likely restricted to water films on single needles. The implication is that when needles are stressed, multiple individuals of *L. seditiosum* or *L. conigenum* are able to form pycnidia on the needle, and cross fertilisation is possible.

Because of the constraint of dealing with small family sizes, it was not possible to rigorously test the 1:1 segregation ratio of ISSR bands expected in families of outcrossing species. However agarose scoring of presence/absence of ISSR bands, though relatively insensitive, was a very adequate method for demonstrating segregation of markers and determining the mating system of *L. conigenum* and *L. seditiosum* with a high degree of assurance.

The determination of their mating system as outcrossing was based on the segregation of at least one ISSR band within the progeny of each ascocarp. The challenge faced with the small family size of no more than eight and as few as two sibling spores isolates per family was rectified with the use of a large array of ISSR markers. Their application gave rise to multiple polymorphic markers which increased the chances of observing segregation. For instance, the implementation of PCR with five ISSR primers generating 54 polymorphic bands permitted the observation that all *L. seditiosum* families showed segregation. However for *L. conigenum* the 24 polymorphic bands scored were not sufficient to show segregation in two out of eight

families from Abernethy. It is unlikely that the lack of segregation of markers was due to expression of homothallism. As well as having lower numbers of polymorphic bands, it was more difficult to obtain large family sizes in Abernethy which had the lowest numbers of progeny per families (two to six).

Different approaches to infer the mating system of ascomycete fungi have been used over the years. These include *in vitro* production of sexual fuiting bodies (apothecia, perithecia, ascocarps) and marker segregation among the offspring of fruiting bodies. Obtaining ascocarps of the *Lophodermium* species studied here *in vitro* has proved difficult in the past (Minter, 1977) and therefore this method was not attempted. Studying genetic segregation in progeny from naturally produced ascocarps overcomes this problem. Difficulties with isolating large families of offspring from single ascocarps remain. However this study shows the potential of ISSR markers to assess mating system even when the family size from single ascocarps is small.

There are still more challenges to be overcome to measure the breeding system in *L. pinastri sensu lato*. A method which does not rely on spore isolation should be preferred since single spore isolation has proved unsuccessful with many authors (Minter, 1977; Diwani & Millar, 1987; Osorio & Stephan 1989). Even better knowledge about the biology and ecology of these cryptic species would not necessarily increase chances of obtaining viable and germinating ascospores. Experience shows that the maturity time is crucial when recovering viable spores from ascocarps. However, even after

successfully screening for mature fruiting bodies that were able to release spores, it was discovered that these would still not necessarily germinate. To overcome these problems one approach by which mating system could be inferred would be to design primers which would determine what mating types are present in a population. Presence of ascocarps in the absence of one mating type would suggest that the species is self-compatible and therefore homothallic. On the other hand presence of the sexual stage in populations where both alleles at the mating type locus, designated MAT1 and MAT2, were present, but absence of ascocarps where only one mating type was present, would clearly suggest that the species is heterothallic. This method has been used to demonstrate that the needle blight pathogens *Dothistroma septosporum* and *D. pini* are heterothallic (Groenewald *et al.*, 2007). Although other factors could be causing the absence of sexual fruiting bodies, this method would be particularly useful in finding out the mating system of *L. pinastri sensu lato* since *Lophodermium* relies on the sexual stage for completing its life cycle.

Results of comparative population differentiation revealed that taxa of endophytes, whose population size is inferred to be smaller regardless of their virulence, have greater population differentiation and are therefore more sensitive to forest fragmentation which would restrict their gene flow. These results were inferred from estimations of genetic diversity based on allele frequencies of bi-allelic multilocus ISSR markers. Presence or absence of peaks was scored from PCR reactions which used fluorescent labelled microsatellite primers. This enabled accurate estimation of allele frequencies

at a large number of loci providing the data needed for genetic structure calculations.

Differences between species in terms of their population genetic structure appears to be linked to their fruiting habitat, *ie* litter needles versus broken branches, with structuring occurring for species fruiting on the latter. As well as being a severe pathogen which fruits on needles that it prematurely kills upon infection, *L. seditiosum* (like *L. conigenum*) fruits on needles which have been killed by other means such as bad weather conditions or breakage of branches (Minter & Millar, 1980a; Diwani & Millar, 1987). On the other hand *L. pinastri sensu lato*, which includes *L. pinastri* II, fruits on litter needle (Minter & Millar, 1980a, Chapter 2 and 3 of this Thesis). Both populations of *L. seditiosum* and those of *L. conigenum* had low yet significant genetic differentiation ($P<0.05$) between populations in Scotland. However no significant level of genetic structure was observed between populations of the litter needle species *L. pinastri* II even between populations located in Scotland and in France. Similar results were found for another *Lophodermium* litter species. Muller *et al.* (2007) found no genetic differentiation between European populations of *L. picea* with all the variation existing within populations. These results are equivalent to an Fst value of zero. Although these results are supportive, further evidence would have to be gathered from phylogenetically independent lineages to robustly demonstrate a link between fruiting habitat and population genetic structure.

Several factors could account for population differentiation. Since we have

shown that both *L. seditiosum* and *L. conigenum* are both heterothallic, the effect of homothallism combined with lack of gene flow should be disregarded. Therefore small population size with restricted gene flow is the most likely cause of genetic differentiation. Comparatively, the abundance of substratum required for *L. pinastri* II to fruit, *ie* litter needles, is a lot larger than the broken branches needed for *L. seditiosum* and *L. conigenum* to complete their life cycles. In effect, the lack of substratum reduces their population sizes. Both species had similar genetic structure levels although the endophyte *L. conigenum* had a slightly higher Theta value of 0.028 as opposed to 0.020 for *L. seditiosum*. Similar levels of differentiation were observed for another *P. sylvestris* pathogen, *Crumenulopsis sororia*, from five native pine forests in Scotland (Ennos & Swalles, 1991). Population sizes of this pathogen were even smaller than for *Lophodermium* species.

In order for population genetic differentiation to occur for selectively neutral markers such as those used here, restricted gene flow between populations is needed. The extent of fungal gene flow in natural environments depends on the efficiency of spore dispersal. Two spore types are produced by *Lophodermium*, conidiospores and ascospores. Conidiospores of *Leptostroma* (*Lophodermium* anamorph) are not thought to be involved in asexual reproduction but as spermatia involved in sexual reproduction (Jones, 1935). Conidiospores usually travel in droplets of water and are not usually dispersed very far, probably only to other genotypes on the same needle. Therefore the main dispersal agent would be the ascospores which are violently released into the air. However, the distances travelled by these

ascospores is not known though it was suggested that spores travelled short distances depending on wind velocity (Minter & Cannon, 1984; Lanier, 1969).

The Caledonian forest from which the Scottish populations were sampled was once a more continuous forest but is now heavily fragmented. Forest fragmentation has been shown to affect the endophytic mycobiota (Saikkonen, 2007) such that forest fragments do not necessarily contain all the species found in a large continuous forest. Indeed Minter (1980a) reported an isolated population of Scots pine on Rhum which one of the *Lophodermium* species, *L. conigenum*, was absent, implying restricted interpopulation ascospore dispersal for this species. One could expect similar effects within the other *Lophodermium* species with similar spores and means of spore ejection from ascocarps. Thus this restricted gene flow via ascospores coupled with relatively small population size could account for the population structuring found in *L. seditiosum* and *L. conigenum* occupying native Scots pine populations in Scotland.

Chapter 5: General Discussion

5.1 Taxonomy

In this thesis the use of molecular taxonomy, supported by an assessment of morphological characters of isolates in culture, has enabled us to reveal the presence of more *Lophodermium* species colonising *P. sylvestris* needles than have previously been suspected. The ability to delimit species accurately has meant that it has been possible to investigate aspects of the population genetics of taxa from natural populations in Scotland, and to determine whether there are differences in their population biology associated with different endophyte lifestyles. Although attempts have been made to relate these species to those already described, using both phylogenetic and morphological approaches, much work remains to clarify these matters.

Hibbett (2010) has contended that according to the current code of nomenclature, cryptic species, for which characterisation is based only on molecular taxonomy, cannot be formally named. He has highlighted that the

increasing numbers of cryptic species which cannot be named is not conducive to efficient and essential communication. He proposes a system which would allow a cryptic species to be named based on molecular data only. If it is subsequently proven that the species in question has already been described using traditional methods, then the original name would prevail and replace the cryptic species name. One potential draw back to this approach is the accumulation of synonyms. However Hibbett (2010) estimated that this problem would not be serious.

In this case, it is quite plausible that one of the cryptic species of *L. pinastri* has been described before. On the evidence that gathered, a new hypothesis may be that *L. pinastri* I is synonymous with *L. pinastri,* that *L. pinastri* II corresponds to *L. staleyi* which was described by Minter (1981a), and that *L. pinastri* III may be a new species.

This hypothesis is based on the limited information we have from isolations from fruiting bodies (chapter 3), the fact that the fruiting bodies of *L. pinastri* I, which were mature in the months of May and June, were systematically associated with defined zone lines, and that this cryptic taxon is one of the most frequent species isolated in both Scotland and France.

Regarding the cryptic species *L. pinastri* II, confirmation that this corresponds to *L. staylei* would require DNA sequence analysis of specimens collected and verified as *L. staylei* by David Minter. The most difficult task now required is to identify *L. pinastri* III in the field. Overall, *L. pinastri* III has been less

frequently isolated from needles than the other two species in this studies. This could be due to its slower growth rate in culture which means it is liable to being overgrown by faster growing taxa. Alternatively, the low isolation frequency of this species may truly reflect its abundance within natural populations.

These findings and their limitations stress an important point which has previously been made regarding the taxonomy of *Lophodermium* and other *Rhytismatales* species. This is the necessity of integrating the ecological and biological characters, along with host specificity, into the descriptions of taxa in order to define species accurately (Minter & Millar, 1980a; Ortiz-Garcia et. at., 2003, Lantz *et al.*, 2010).

In the light of these results on the taxonomy of *Lophodermium* species inhabiting *P. sylvestris* litter needles, previous work focussing on *L. pinastri* may need to be reassessed (Goubiere & Debouze, 2003; Hirose & Osono 2006). Further morphological characterisation of fruiting bodies on needles would help in re-evaluating these studies. In previous work, the presence of black zone lines has been used as proof of colonisation by *L. pinastri*. The zone line character may indeed discriminate between the cryptic species within *L. pinastri sensu lato*. However, more work on this matter is crucially needed.

A detailed description of the zone line, rather than presence or absence of a zone line, may be required. If the isolate of *L. pinastri* II from which the

photographed fruiting body (Figure 3.10) was taken is representative of this species regarding zone lines, then a description of this type of zone line would be "faint grey zone lines which form open rings". This would help to distinguish the taxon from *L. pinastri* I where the description of zone lines would be "black zone lines which form a full ring".

Other aspects of their biology and ecology which may be useful to characterise species within the *L. pinastri* complex would be the timing of fruiting. Indeed, this character has previously been used to demonstrate that three to four species rather than one is able to colonise *P. sylvestris* (Minter *et al.*, 1978). It is important to note that if *Lophodermium* species fruit at different times in the year, then this limits or prevents gene flow between them. Indeed, a change in fruiting time may have been one of the first steps triggering the evolution of the cryptic species within *L. pinastri*. Securing knowledge of the timing of fruiting of the cryptic species would greatly improve efficiency in working with this system. This is especially true when attempting to obtain isolates derived from ascospores, since this relies on having ascocarps of the right maturity.

5.2 Phylogeny and Evolution

A larger taxonomic sampling would be required in order to better locate the position of *L. pinastri* II in relation to other *Lophodermium* species in the phylogeny, and to avoid the occurrence of unresolved nodes forming a

polytomy. Taxon sampling should take into consideration the phylogeny of Rhytismataceae. This is especially important because recent phylogenetic work in this group has highlighted the need to revise the genus *Lophodermium*. *Lophodermium* forms polyphyletic clades (Lantz *et. al.*, 2010), only one of which includes pine symbionts together with *Lophodermium* species associated with Juniper.

The phylogeny confirmed that when defining species within this particular group, the ecology of fruiting habitat is a very useful criterion to use as suggested by Minter & Millar (1980a). However more information would be needed to establish the sequence of events leading to diverged species on different fruiting habitats. A working hypothesis is that in *Lophodermium* on pine divergence first occurred from an ancestral species, which would have fruited on naturally senescing needles, to form a clade of species fruiting on broken branches, followed by further divergence to form a species which is able to induce premature needle cast in vulnerable trees. To test this, a dated tree based on data from more loci would be needed.

Although Ortiz-Garcia *et al.* (2003) made a start in establishing the host specificity of *Lophodermium* from pines, further clarification is required using a larger sampling of taxa with respect to both host and symbionts. Such a study also needs to be conducted over a greater geographical distribution. This would enable us to investigate whether all species have the same level of host specificity, and whether some species are evolving more closely with their hosts than others. This would be especially interesting since the preliminary data suggest that *L. pinastri* II has a wider host range than the

other cryptic *L. pinastri* taxa, being associated with both sub-genera of *Pinus*, *Pinus* and *Strobus* (Figure 2.8).

5.3 Population Biology and Ecology

One implicit assumption of this thesis was that all species of *Lophodermium* were able to infect green needles of Scots pine as endophytes. This is not necessarily the case. Although most *Lophodermium* species are endophytes, the recently described litter saprophyte *L. macci* has not been recovered from green needles (Sokolski *et al.*, 2004). In order to determine whether a particular species is in fact endophytic, a species-specific Real-Time PCR assay needs to be developed to detect the presence of each species in the needles. This would avoid the possible neglect of taxa which may be present in low quantities within needles. Such an assay would also be beneficial for evaluating the relative abundance of each species in a single green needle, and avoiding the bias that occurs as a result of preferential isolation of taxa that grow faster in culture.

Such an assay would enable us to know whether species co-occur within a single needle and which species tend to dominate at different stages of the needle's life. Previous studies which investigated these questions relied on isolation into culture. This could bias the results because of the ability of certain species to outgrow others in culture (Kowalski, 1982). The Real-Time PCR assay would furthermore allow for testing the hypothesis that relative growth rate in culture corresponds to relative growth and infection rate in

the needle. Further comparative analyses involving endophytes fruiting on the litter and those fruiting on needles from broken branches could be made.

Another aspect of population biology research which requires expanding is on the distribution of *L. pinastri sensu lato* and the individual cryptic species that it contains. In this study we have concentrated on a geographically outlying population of Scots pine in Scotland which has a postglacial history and origin distinct from that of Scots pine elsewhere. It will be important to extend this study to cover the much more extensive natural host range and determine whether the same taxa of *Lophodermium* are present, and show the same degree of variability and molecular distinction.

5.4 Population Genetics

In this study we found that there was inter-regional genetic differentiation of *L. conigenum* and *L. seditiosum* populations. However no genetic differentiation was found among populations of the litter fruiting *L. pinastri* II, even over national scales. This leads to further questions regarding both the fine and large scale population genetics of these and other *Lophodermium* species. Thus it would be interesting to know whether the populations of other *L. pinastri* species are genetically undifferentiated as we found for *L. pinastri* II. In terms of the taxa that do show genetic differentiation, knowledge of fine scale genetic structure may shed light on their population biology. Looking at genetic differentiation between trees, or between individual broken branches, would allow us to make inferences about the

extent of gene flow within a single pinewood.

One of the original motivations for this study was to investigate Minter's suggestion that the endophytic *Lophodermium* taxa may protect Scots pine trees against attack by *L. seditiosum* (Minter 1981b). If this hypothesis was true, vulnerability of Scots pine to *L. seditiosum* would be greater where the endophytic species were absent. This is most likely where the host species has been recently introduced. For instance, plantations of Scots pine on Rhum lacked *L. conigenum*, and needles on broken branches were colonised exclusively by *L. seditiosum* (Minter 1980a).

In order to better understand the epidemiology of *L. seditiosum* and the potential for endophytes to protect against needle cast disease, it would be helpful to investigate the distribution and genetic diversity of the endophytic *Lophodermium* species in the native and introduced ranges of Scots pine. This could include a study of the genetic diversity and differentiation of all *Lophodermium* species of Scots pine within its native ranges in Europe as well as those introduced in North America in order to understand possible restrictions to endophyte migration and colonisation. This would provide a better understanding of the risks involved with transferring Scots pine outside its native ranges in terms of loss of endophytes and potential increase in vulnerability to outbreaks of *L. seditiosum* and other needle pathogens.

5.5 Conclusion

In recent decades support for taxonomic research has been hard to obtain,

and its relevance in modern biology has been questioned. The first lesson learnt from this thesis is that we are currently ignorant of the scale of fungal biodiversity, and the only way to rectify this is through detailed taxonomic studies. The second lesson to be learned is that taxonomy is an essential foundation for conducting evolutionary and ecological genetics studies. Only when the taxonomy is in place can we identify the species that we wish to study, and only under these circumstances is it possible to make progress in these fields.

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Appendix 1: Glossary

A

Anamorph	Name of the fungus in its imperfect (asexual stage).
Ascocarp	Enclosure of asci into organised sexual fruiting body. There are four types of ascocarps depending on their shape (cleistothecia, perithecia, ascostromata, apothecia)
Ascogenous cell	Cells which are precursor to Asci.
Ascomycetes/ Basidiomycetes	Two of the major phyla within the fungal kingdom. Each are differentiated by the type of reproducing structure ascus and basidia from which sexual spores are formed. Asomycetes includes yeasts used in many food processes such as bread making, moulds, some macrofungi such as morel and truffle and it includes many plant pathogens. Basidiomycetes are well known for producing macrofungal structures (toadstool mushrooms), they also include plant pathogen such as rusts and smuts.
Ascospores	Spores produced after the sexual recombination and meiosis of Ascomycete fungi. They are formed within asci which are often grouped in the hymenium of an ascocarp.
Ascus	(Plural: Asci) Cell which bear on average eight ascospores.
Axenic	Cultures which are entirely free of other contaminating organisms.

D

Diploxylon	Pinus subgenus which included pines of two needles per fascicle.

H

Haploxylon	Pinus subgenus which included pines of 3 to 5 needles per fascicle.
Heterothallic	Fungi with two mating types which reproduce by outcrossing.
Homothallic	Fungi with one mating type which reproduce by self-fertilisation.

I

Imperfect stage	Asexual stage during which spores known as conidia are formed sometimes in a fruiting body called pycnidia. This stage is associated with the anamorph name of the fungus.

P

Perfect stage	Sexual stage during which recombination leads to the production of sexual spores (ascospores) and fruiting body (ascocarp). This stage is associated with the teleomorph name

S

Spermatia	Spores which are able to fertilise specialised hyphae.

T

Trichogynes	Specialised hyphea capable of fusing which spermatia.

Appendix 2: List of Specimens Deposited at RBGE

RBGE nb	Number	Collection Date	Collector	Species	Host	Habitat	Location	Latitude	Longitude
454660	SRC1	23/05/05	R. Ennos	*Lophodermium pinastri* species complex	*Pinus sylvestris*	Litter	Abernethy	57° 13' 33.68" N	3° 40' 32.21" W
454661	SRC2	23/05/05	R. Ennos	*Lophodermium conigenum*	*Pinus sylvestris*	Trash	Abernethy	57° 13' 33.68" N	3° 40' 32.21" W
454662	SRC3	13/12/07	S. Reignoux	*Lophodermium pinastri* species complex (*Leptostroma* stage)	*Pinus sylvestris*	Litter	Abernethy	57° 13' 33.68" N	3° 40' 32.21" W
454664	SRC4	13/12/07	S. Reignoux	*Lophodermium pinastri* species complex (inc. *L. conigenum*)	*Pinus sylvestris*	Shoot tips	Abernethy	57° 13' 33.68" N	3° 40' 32.21" W
463112	SRC5	13/12/07	S. Reignoux	*Lophodermium conigenum*	*Pinus sylvestris*	Trash	Abernethy	57° 13' 33.68" N	3° 40' 32.21" W
463113	SRC6	11/12/07	S. Reignoux	*Lophodermium pinastri* species complex (*Leptostroma* stage)	*Pinus sylvestris*	Litter	Loch Maree	57° 38' 55.84" N	5° 23' 13.48" W
463114	SRC7	11/12/07	S. Reignoux	*Lophodermium seditiosum*	*Pinus sylvestris*	Trash	Loch Maree	57° 38' 55.84" N	5° 23' 13.48" W
463125	SRC8	11/12/07	S. Reignoux	*Lophodermium conigenum* mixed with *L. seditiosum*	*Pinus sylvestris*	Trash	Loch Maree	57° 38' 55.84" N	5° 23' 13.48" W
463127	SRC9	12/12/07	S. Reignoux	*Lophodermium pinastri* species complex (*Leptostroma* stage)	*Pinus sylvestris*	Litter	Glen Affric	57° 18' 46.23" N	4° 51' 6.23" W
463129	SRC10	12/12/07	S. Reignoux	*Lophodermium pinastri* species complex	*Pinus sylvestris*	Shoot tips	Glen Affric	57° 18' 46.23" N	4° 51' 6.23" W
463130	SRC11	12/12/07	S. Reignoux	*Lophodermium conigenum*	*Pinus sylvestris*	Trash	Glen Affric	57° 18' 46.23" N	4° 51' 6.23" W
463131	SRC12	12/12/07	S. Reignoux	*Lophodermium seditiosum* mixed with *L. conigenum*	*Pinus sylvestris*	Trash	Glen Affric	57° 18' 46.23" N	4° 51' 6.23" W
463132	SRC13	21/03/08	S. Reignoux	*Lophodermium pinastri* species complex	*Pinus sylvestris*	Litter	Loch Maree	57° 38' 55.84" N	5° 23' 13.48" W
463168	SRC14	01/05/08	S. Reignoux	*Lophodermium pinastri* species complex	*Pinus sylvestris*	Litter	Glen Affric	57° 18' 46.23" N	4° 51' 6.23" W
463170	SRC15	02/05/08	S. Reignoux	*Lophodermium pinastri* species complex	*Pinus sylvestris*	Litter	Abernethy	57° 13' 33.68" N	3° 40' 32.21" W
463172	SRC16	03/10/08	S. Reignoux	*Lophodermium pinastri* species complex	*Pinus sylvestris*	Litter	Arlanc	45° 24' 9.77" N	3° 41' 43.58" E