ENDOPHYTIC FUNGI OF *CASSIA FISTULA* L.

NUTTHAPORN RUCHIKACHORN

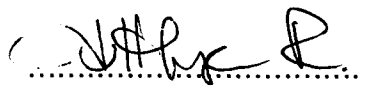
A thesis submitted in partial fulfillment of the requirement of Liverpool John Moores University for the degree of Doctor of Philosophy

March 2005

Any maps, pages, tables, figures graphs,or photographs, missing from this digital copy, have been excluded at the request of the university.

Declaration

I declare that while registered as a candidate for the degree of Doctor of Philosophy I have not, in the duration of this research programme, been registered as a candidate for another award from any other academic or professional institute.



(Nutthaporn Ruchikachorn)

Abstract

Endophytic fungi from *Cassia fistula* or golden shower, a well known medicinal plant in Thailand and Asia, were isolated from trees growing in three geographical separate sites. These locations were Kanchanaburi, Nakhon Ratchasima and Bangkok and were selected to allow comparisons between their endophytic assemblages and to evaluate these data in relation to differences in plant diversity and density and local environment. Kanchanaburi which was the site closest to a natural forest situation provided the highest number of isolates with Bangkok, where the trees were isolated individuals, having the least.

Members of the Xylariaceae proved to be common and frequent isolates especially species of *Xylaria* and *Daldinia* but *Nemania* and *Hypoxylon* were also obtained. *Phomopsis* was also well represented and clearly was dominant at the Kanchanaburi site. Species of *Fusarium*, *Colletotrichum*, *Penicillium*, *Nigrospora*, *Coprinus* and *Psathyrella* were also identified but were occasional isolates.

Differences in endophytic assemblages between samples obtained early in the rainy season (July, 2001) with those sampled towards the end of the rainy season (December, 2001) were found to occur in the Nakhon Ratchasima samples with over twice as many isolates obtained from the December samples. This is likely to be a reflection on the longer exposure period to the potential inoculum of these leaves. A total of 956 endophytic isolates were obtained from the three sites with samples from Kanchanaburi (December 2000) and Bangkok and Nakhon Ratchasima in July 2001 with a further samples from Nakhon Ratchasima in December, 2001. Isolations were also made from different anatomical regions of the leaf, leaf lamina, midrib and veins. There were no appreciable differences in either the number of isolates obtained or an association between leaf area and specific fungal species.

Identification of many xylariaceous endophytic isolates is well known to be problematic since *Xylaria* species rarely produce their anamorphic form in culture and virtually no members of the Xylariaceae develop their teleomorph in culture. Therefore molecular techniques were used to compare DNA sequences of the ITS region from a selection of endophyes with sequences obtained from teleomorphic material, or cultures derived from teleomorphs of identified and authenticated Xylariaceae. Comparisons were also made with data held in GenBank. This enabled

the identity of a number of taxa to be made although more sequences from *Xylaria* species are required for future investigations. A number of non-xylariaceous taxa were also named as a result of DNA sequence comparisons.

Secondary metabolites from the xylariaceae were also investigated and their metabolite profiles used to support identifications. The metabolite profiles proved to be a useful tool to confirm doubtful endophytic isolates when their DNA sequences could not place them with certainty in a right group. Together with extracts from other endophytic species, their inhibitory effects on bacteria and fungi were tested. *Cassia* endophytes were found to show low antimicrobial activity. However, they may later be shown to have other activities when when tested e.g. anti-malarial, anti-cancer and anti-HIV.

Acknowledgements

I would like to thank Professor Anthony Whalley, my superb supervisor, for his kindness, constant support and unevaluated suggestion throughout this project. He also introduced me into the fascinating world of fungi and mycologists. I am deeply appreciated for everything that you did. Thank you very much for having me as part of the best 'Thaiteam'.

I also would like to express my sincere gratitude to my co-supervisors, Dr. George P Sharples, Associated Professor Dr. Prakitsin Sihanonth and Dr. Surang Thienhirun, for their incessant supports. Their advices have always been useful. Thank you very much indeed.

My appreciation is also extended to the wonderful staffs of School of Biomolecular Sciences, Dr. Stan Lambert, Mr. Peter Hamlett, Mrs. June Lewis and Mr. John Bridson for their helpfulness in laboratory facilities and techniques and also their supports throughout my study. Although I do not use many Scanning Electron Microscopic pictures in the thesis, but I obtained a very good lesson about SEM from Mr Paul Gibbons. Thank you for sharing me a very useful knowledge.

Despite the fact that it is the first time for me to live far away from home, but I never feel lonely. Thank you to Tony, Dr. Margaret Whalley, Gareth Whalley and Rhys Whalley for giving me the warmest feeling that I could ever have dreamt here. The warmest thank you is also extended to Professor Colin Reynolds and his family for their encouragements. I always feel that Liverpool is my second home.

My molecular approach will never reach the goal without the help of these two fantastic ladies, Dr. Jittra Piapukiew and Nuttika Suwannasai. Thank you for sharing your precious knowledge and helping me with interpreting the molecular results.

Living here will be very boring without these super friends and colleagues of mine. Thank you to Dr. Glyn Hobbs, Dr. Piyarat Parinyapong, Dr. Nawasit Rakbamrung, Dr. Podjana Chumkhunthod, Dr. Lianne Nugent, Dr. Cherdchai Phosri, Dr. Anirach Mingkhwan, Panvipa Lekluanngarm, Ek Sangvichien, Sirirath Sodngam, Sayampong Pongdam, Kullakunya Chansri, Apinun Choothavornchaikul, Pattama Ritruechai, Elsie Gaskell, Louise McCulloch, Michelle Lea, Ismini Nakouti, Irene Nantume and Clare Watson for being nice and friendly. Furthermore, I also wish to

give my warmest thanks to Jean-Louis Bourdon for sharing with me the fabulous time and giving me his support and friendship.

My very special thanks is also for to my super duper best friends, Benjamas Aiamkitsumrit, Pornsawan Aramsri, Chanachok Srangaiam, Santi Panin and Chaiyapong Sirikittisup, for their comfort and encouragement whenever I need it. You make me appreciate the meaning of 'Friendship'!

I am indebted to the Royal Thai Government for the financial support and also to the British Mycological Society for supporting part in the last four months of my study.

Lastly, I would like to express my deepest gratefulness to my wonderful family for supporting me in every possible way, understanding and giving me all the love and moral support. You are the real 'Wind beneath my wings'!

Abbreviations

°C	Degree Celcius
A_{260nm}	Absorbance at 260 nm
A_{280nm}	Absorbance at 280 nm
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	gram
µg	microgram
µm	micron
h	hour
ITS	Internal transcribed spacer
kb	kilobase
l	Litre
M	Molar
MEA	Malt extract agar
MEB	Malt extract broth
MHA	Mueller-Hinton agar
mg	Milligram
min (s)	Minute(s)
ml	Millilitres
mM	Millimolar
nm	Nanometre
ng	Nanogram
PCR	Polymerase chain reaction
pmol	Picomol
rpm	Revolutions per minute
RFLP	Restriction fragment length polymorphism
RNA	Ribonuleic acid
RNAse	Ribonuclease
SEM	Scanning Electron Microscope
SDS	Sodium dodecyl sulphate
sec(s)	Second(s)
SSU	Small subunit
Taq	*Thermus aquaticus*

TAE	Tris-acetate-EDTA buffer
Tris-HCl	Tris (hydroxymethyl) aminoethane hydrochloride
U	Unit
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume

Related studies undertaken in conjunction with the research programme

Meetings, visits and workshops attended

During this research programme I have attended symposia and presented my work as follows:

- Bioactive fungal metabolites - Impact&Exploitation, BMS/FEMS symposium, University of Wales, Swansea, UK. 22-27 April, 2001.
- Ruchikachorn, N. (2001) **Endophytic fungi of selected species of *Cassia* in Thailand**. Oral presentation at the Biomolecular Sciences Postgraduate symposium at Liverpool John Moores University, Liverpool, UK. 4 June, 2001.
- Ruchikachorn, N. (2001) **A preliminary study of endophytic fungi in selected Thai medicinal plant**. Oral presentation at the Howard Eggins Meeting organized by British Mycological Society at the Linnean Society, Piccadilly, London, UK. 15 November, 2001.
- Ruchikachorn, N. (2002) **A preliminary study of endophytic fungi in selected Thai medicinal plant**. Oral presentation at the Howard Eggins Meeting and Postgraduate symposium organized by British Mycological Society at Liverpool John Moores University, Liverpool, UK. 10-11 July, 2002.
- The 7th International Mycological Congress, University of Oslo, Norway, 11-17 August, 2002.
- Ruchikachorn, N., Sihanonth, P. & Whalley, A.J.S. (2002) **Endophytic fungi from *Cassia fistula* L.** Poster presentation at the 7th International Mycological Congress, University of Oslo, Norway. 11-17 August, 2002.
- Workshop session, Chemotaxonomy in the Xylariaceae. At the Chemical and Physical Methods for the study of Biologically important materials Workshop organized by Research Centre for Bioorganic Chemistry (RCBC) Chulalongkorn University, Liverpool John Moores University and British Council, Chulalongkorn University, Thailand. 15-16 January, 2003.
- Ruchikachorn, N. (2003) **Molecular Identification of Xylariaceae**. Oral presentation at the Chemical and Physical Methods for the study of Biologically important materials Workshop, Chulalongkorn University, Thailand. 15-16 January, 2003.

- Ruchikachorn, N., Whalley, A.J.S & Sihanonth, P. (2004). **Molecular study of endophytic fungi from *Cassia fistula* L.** Poster presentation at Howard Eggins and Postgraduate symposium organized by British Mycological Society, University of Nottingham, Nottingham, UK. 11-14 September,2003.

Paper published

- Edwards, R.L., Jonglaekha, N., Kshiragar, A., Maitland, D.J., Mekkamol, S., Nugent, L.K., Phosri, C., Rodtong, S., Ruchikachorn, N., Sangvichien, E., Sharples, G.P., Sihanonth, P., Suwannasai, N., Thienhirun, S., Whalley, A.J.S. & Whalley, M.A. (2003) The xylariaceae as phytophatogens. *Recent Research Development in Plant Science* 1-19.

Table of Contents

Table of Figures

Page

Table of Tables

CHAPTER 1

Introduction

1.1 Endophytes

On leaf surfaces there are usually a range of organisms that colonize the plant surface known as 'epiphytes'. It has also been known for many years that all normal plant tissues are colonized by, or harbour internally, fungi, which are called 'endophytes' ('endo' is derived from the Greek word 'endon' which means 'within' and 'phyte' is also derived from the Greek word 'phyton' which means plant). Endophytes are contained entirely within the plant substrate whereas epiphytes are strictly confined to the outer surface of plant tissues.

The term endophyte was first coined by De Bary in 1866. He described them as any fungus whose hyphae invade tissues or cells of living autotropic organisms. Many authors have since tried to produce a more accurate and precise definition. Carroll (1986) recognized endophytes as organisms that cause asymptomatic infections within plant issues, excluding pathogenic fungi and mutualistic fungi such as mycorrhizal fungi. Later Carroll summarized the term 'endophytes' as fungi that form inapparent infections within leaves and stems of healthy plants and are closely related to pathogens but are not harmful to host plants (Carroll, 1988). However, the most widely used definition is the one given by Petrini (1991) who defined endophytes as "all organisms that live inside plant tissues for at least part of their life cycle without causing any disease symptoms in the host". Wilson (1995) more recently proposed the definition of endophytes as fungi or bacteria which invade the tissue of living plants and cause asymptomatic infection entirely within plant tissues.

The transmission of endophyte has been recently reviewed by Saikkonen et *al.* (2004). They reported that there are two important ways of transmission from endophyte-plant host-endophyte; horizontal and vertical transmission (Figure 1.1). Horizontal transmission is via asexual or sexual spores of the fungus. This type of transmission forms local latent infections in plant tissues and represents the most common type of infection and occurs in all types of plants. By contrast, systemic and vertical

transmission, which is from plant to offspring by host seeds, are only rarely described from woody plants (Bloomberg, 1966; Petrini, 1991). The important factors which affect the vertical transmission are size, morphological complexity and the longer time taken to maturity of woody plants. Fungal endophytes which infect one part of the crown of a tree are unlikely to grow systematically through highly differentiated and woody plant tissues to reach another branch or crown of a tree. In other small trees and herbaceous plants their morphology and branch pattern allows fungi to grow throughout the host. The earlier time taken to their reproduction in perennial grasses, compared with woody perennials, increases the opportunities for vertical transmission of the fungus (Saikkonen *et al.*, 2004).

The relationship of endophytes and plants is another unanswered question. According to all definitions, endophytic infection in plants can not be considered as a disease causing factor since the plant disease is an interaction between host, parasite, vector and environment over time (Figure 1.2) which will result in production of disease signs/symptoms (Sinclair & Carkauskas, 1996). Latent infection is the stage where the host is infected with a pathogen but does not show disease symptom and remains healthy until the signs or symptoms are induced by environmental factors or the state of maturity of pathogens or their hosts (Agrios, 1988). Some researchers believe that they are pathogens which live their life in the form of a latent infection which will be

presented when the host is stressed (Miller, 1980; Andrews *et al.*, 1985). Minter and Ingham (1984) found leaf spotting on *Chrysenthemum* was associated with ejected ascospores of *Podospora* which is non-pathogenic being a saprobic fungus instead.

The distinction between an endophyte and a pathogen is not clear. Freeman and Rodriguez (1993) found that only a single mutation in a single genetic locus can entirely change the status of a fungus from pathogenic to non-pathogenic without having any effect on host specificity. Many pathogens live in plant tissues as an asymptomatic stage through latent infection before the symptom appears. Both endophytic and latent infecting fungi can infect host plants and remain in the tissue after their penetration. However the infection does not necessarily result in the development of visible symptoms. There is some taxonomic evidence which supports a very close relationship between endophytes and pathogens (Funk, 1985; Sherwood-Pike *et al.*, 1986). From either a close, or the same host, the endophyte is frequently a sister species to the virulent pathogen. For instance, an endophyte of Douglas-fir, *Rhabdocline parkeri* Sherw., is morphologically similar to two needle pathogens, *R. weirii* A.K. Parker & J. Reid and *R. pseudotsugae* Syd.(Funk 1985; Sherwood-Pike *et al.* 1986). In some cases, one or more physiological alterations during the fungal lifetime will cause a switch from one type of lifestyle to another. Also, changes in host susceptibility caused

by the presence or absence of stress factors, such as, drought or poor nutrient supply will induce the transition from one stage to another, such as, mutualism to neutral or antagonism to mutualism, etc. This factor will also determine the duration of the period during which an individual will remain in the same state (Petrini, 1991).

On the other hand, Carroll (1988, 1995) stated that endophytes have a relationship with the host as 'protective mutualists' and that endophytes act against herbivorous insects and pathogenic microorganisms. There is some evidence or reports that support his suggestion. Webber (1981), for example, showed that the elm bark beetle avoids trees in which its bark has been colonized by *Phomopsis oblonga* (Desmaz.) Traverso. When the beetles were induced to breed in an infected log in the laboratory only a few progeny emerged from the logs. Yang *et al.* (1994) showed that the Nepalese yew, *Taxus wallachiana* Zucc., and an endophyte, *Phoma* sp., have a mutualistic relationship. They found that the endophyte lived in the intracellular host tissues acquiring support and food from the phloem of *T. wallachiana* but at the same time they produced one or more antibiotics which protected the host from bacterial infection. Carroll (1988) also categorized the mutualism relationship into 2 groups; Constitutive Mutualism and Inducible Mutualism:

1. **Constitutive Mutualism** is usually associated with grass endophytes. The endophyte is transmitted via the seed and develops a systemic infection throughout the host parts. The fungus produces toxins which affects herbivores and provides benefits to the host plant.
2. **Inducible mutualism** involves in much looser association with the host such as that of *P. oblonga* in elm or *R. pakeri* in Douglas-fir. The fungi normally colonized inactive tissue such as bark but will develop and become apparent when the host is stressed by herbivores or pathogens. When infected wound host tissues are consumed by herbivores the fungi affect the insects and the number of herbivores decreases. In this term, the endophyte is not directly deterring herbivores but protects the host once the herbivore attacks the host tissue.

The mutualism characteristics among endophytes were also considered by Carroll (1988). He suggested that, firstly, the endophyte is prevalent in its host over a wide geographical range and causes minimal disease symptoms in the host. Secondly, the vertical transmission of fungus occurs via host seeds or vegetative propagules. If seed

transmission does not occur, the horizontal transmission will be efficient. Thirdly, the fungus grows entirely through host tissues. If the infection is dispersed, they should be numerous, if limited to a particular organ, a high proportion of the organs should be infected. Fourthly, the endophyte should produce secondary metabolites which are likely to be antibiotic or toxic in nature. Finally the fungal endophyte is taxonomically related to known herbivore or pathogen antagonists such as *Acremonium* or *Phomopsis* (Carroll, 1988).

The mode of fungal infection in the plant during early stages has been studied by many groups. The infection process in any host symbiont interaction involves the germination of spores on the leaf surface, with attachment occurring before or after the germination process. Finally penetration of host cuticle by infection hyphae takes place (Petrini, 1991). There is, however, a question about host specificity and recognition mechanisms of endophytes to specific host plants. Sieber (1985) first demonstrated organ specificity in relation to infection by endophytes of wheat. Later Sieber (1988, 1989) studied the same specificity but this time on Norway Spruce, *Picea abies* (L.) Karst.. Petrini (unpublished data) also confirmed organ specificity of endophytes when he studied beech leaves, twigs and roots and found that there were differences in the fungal community structure in these different plant organs.

Carroll *et al.* (1977) was the first to report tissue specificity by endophytes. He found that the fungi isolated from petiole segments of European coniferous were restricted to that part and were rarely detected again in the other parts of needles. Stone (1986) investigated *R. parkeri* and a *Phyllosticta* species which coexist in needles of *Pseudotsuga menziesii* (Mirbel.) Franco.. He found that *R. parkeri* was restricted to epidermal and hypodermal cells while *Phyllosticta* was found intercellularly in the mesophyll. These findings confirm that endophytic fungi show a degree of tissue specificity. Tissue and also organ specificity along with development of specialized food preferences (Carroll & Petrini, 1983; Sieber, 1989) are probably a mechanism to prevent competition among endophytes which occupy the same host. However, this specificity maybe a fungal response to surrounding environment (Petrini,1991).

Surface sterilization is used to eliminate epiphyte spores or mycelium which are attached to the surface of plant host prior to isolation of the endophytes (Luginbühl & Müller, 1980; Petrini & Dreyfuss, 1981; Clark *et al.*, 1983; Petrini, 1986; Kreisel &

Schauer 1987). Schulz *et al.* (1993) evaluated many surface sterilization methods to find the most effective way to eliminate epiphytes and to isolate endophytes from different types of herbaceous plants and shrubs growing near Brunswick, Germany. Only one method proved to be ineffective, using 50% ethanol for 5 minutes (Schulz *et al.*, 1993). However, there is no certainty that any surface sterilization method will kill all fungi on plant surfaces (Petrini, 1986). Furthermore, it is not possible to find a suitable method for sterilization of all types of plant surface. The sturdy leaves of *Vaccinum myrtillus* L., and the delicate leaves of *Anemone nemorosa* L., and stems of plants will react differently to the solutions used in surface sterilization (Schulz *et al*, 1993).

There are also a number of taxa which are known to be epiphytes that can live internally within plant tissue as endophytes as well. O'Donnell and Dickinson (1980) found that under appropriate conditions some epiphytes e.g. *Alternaria alternata* (Fr.) Keissl., *Cladosporium cladosporioides* (Fresen.) de Vries and *C. herbarum* Link ex Fr. may penetrate into the tissue of their host plant (*Phaseolus*). The switch from epiphyte to endophyte enable epiphytes to protect themselves from negative ecological conditions such as irradiation or desiccation and also to avoid antagonistic activities by other competitive surface organisms (Petrini, 1991). Verhoeff (1974) noted that some epiphytic fungi are capable of forming subcuticular appressoria or hyphae that can survive surface sterilization. This maybe the explanation of why we occasionally find some common epiphytes in the inner plant tissues. Canavesi (1987), Legault *et al.* (1989) and Cabral (1985) also supported the hypothesis that certain phylloplane fungi will colonize the interior of plant tissues only when the number of true endophytes decreased. Facultative endophytes are probably fungal species which are mainly involved in litter degradation and are likely to be found in older plant tissues. The epiphytic colonization of leaves occurs rapidly via fungal spores. Kinkel (1987, 1989) considered air spora to be the major determinant of the composition and quantity of fungal community on apple leaves.

Colonization of plant tissues by endophytes may occur by :

1. Infection of aerial plant tissues by air spora
2. Bose (1947) and Bloomberg (1966) showed that some endophytes of *Casuarina equisetifolia* L. and *Pseudotsuga menziesii* (Mirbel)Franco. are seed-borne. Some grass endophytes colonize their host in the same way (Clay 1986).

3. The endophyte inoculum is carried by insects (Carroll, 1986, Petrini *et al.*, 1989) which demonstrates the close relationship existing between the fungus and the insect.

Members of the Ascomycotina, Basidiomycotina, Deuteromycotina and some Oomycetes have been isolated as endophytes (Petrini, 1986; Clay, 1991). Endophytic fungi have been isolated from a wide range of plant groups such as mosses, ferns, orchids, lichens, grasses, and woody plants with a broad geographical range from the temperate zone to the tropical zone (Rodrigues & Petrini, 1997). The symptomless endophytes have been categorized into two distinct groups based on host type.

1. Clavicipitaceous endophytes or grass endophytes
2. Non-clavicipitaceous endophytes or non-grass endophytes or woody plant endophytes.

1.1.1 Grass endophytes

Members of the grass endophyte group comprise the family *Clavicipitaceae*, which belongs to the Ascomycotina, tribe Balansiae (Diehl, 1950). There are approximately 30 species including five genera *Atkinsonella*, *Balansia*, *Balansiopsis*, *Epichloë* and *Myriogenospora*. The largest genus is *Balansia* with 15-20 species. When compared to *Claviceps*, *Balansia* fungi are systemic and perennial whereas *Claviceps*, well-known grass parasites, are transient. Most species are endophytes and their hyphae occur intracellularly in stem and leaf tissues. Only a few species are epiphytes. Their hyphae surround the meristems of young leaves in a dense mat form. Neither endophyte nor epiphyte produce haustoria which are a very common characteristic among parasitic fungi. Fruiting bodies of the genus *Balansia* are formed on leaves or aborted inflorescences of host species (Clay, 1988)

For their transmission and reproduction, grass endophytes tend to transmit vertically and maternally by growing hyphae into seeds when they are in their anamorphic form or asexual form. In *Epichloë*, which can produce both sexual and asexual stages, they can be transmitted either vertically (by growing into seeds) or horizontally (by spores) (Carroll, 1988; Carroll *et al.*, 1997)

The growth of grass endophytes, *Epichloë* and *Neotyphodium*, are regulated by hormonal activity and production of chitinase by host plant. Also seasonal change may regulate their growth rate (Saikkonen *et al.*, 2004). Pan and Clay (2002) showed that *Epichloë* infection can alter reproduction functions such as vegetative growth that promote the spread of fungus.

Clavicipitaceous endophytes have proved to be mutualistic. Their symbiosis is interpreted as a defence mutualism to the herbivores and other enemies of hosts. It protects the host by production of fungal alkaloids. This association has been shown to improve nitrogen utilization and drought resistance by enhancing osmotic adjustment and lower stomatal conductance (Selosse & Le Tacon, 1998). It is well-established that clavicipitaceous endophytes in grasses can have broad and dramatic biological effects on growth and reproduction of host grasses, pathogens and herbivory and natural enemies of herbivores (Saikkonen *et al.*, 1998). Lethal effects of grass endophytes on herbivores have been reported by many scientists. Toxicity of *Claviceps*-infected grasses has been known for a long time and the role of *Balansia* and *Myriogenospora* infected grasses in cattle poisoning has been studied in depth (Luttrell & Bacon, 1977). Not only insects but also livestock such as cattle, horses and sheep have been affected from eating infected grasses and exhibit alkaloid poisoning. In the 1970s livestock in USA and New Zealand were affected by alkaloids which were produced by endophytes belonging to *Balansiae* family. The alkaloid which is produced by these endophytes is insect resistance and toxic to mammals. *Claviceps* and *Balansia* are responsible for the production of this substance because there is no record of ergot alkaloid in non-infected grasses, the alkaloids are only found in infected grasses and in pure culture of the fungi. Clay and Schardl (2002) noted that systemic endophytes which are host-specific are now thought to affect virtually every type of plant-plant, plant-pathogen and plant-herbivore interaction in grassland communities.

In mammals the action of ergot alkaloids is vasoconstrictive. It causes contraction of small blood vessels to the brain and other organs and can cause dementia and convulsion and gangrene of the extremities (Clay, 1988). However, some ergot alkaloids have medicinal usefulness. Ergotamine can be used in treatment of migraine and some other similar compounds can cause hallucinatory effects (Clay, 1988). These compounds could be lethal if they are used in high concentration (Bacon *et al.*,1986). Normally ergot alkaloid is known only from the seed of morning glory (Convolvulaceae)

but it is interesting that this substance can be isolated from endophytes as well. Other types of alkaloids are produced by the *Acremonium* endophytes of fescue and rye grass. In infected tall fescue, there are pyrrolizidine alkaloids which if the alkaloid dose in cattle diet is high, the cattle will be affected by the fescue toxicity, whereas low doses have no effect on them (Jones *et al.*, 1983). Peramines, one type of alkaloids, have been detected in endophyte-infected perennial ryegrass and it is shown to be the major insect feeding deterrent (Rowan *et al.*, 1986). This suggests that mammalian toxicity and insect resistance have different chemical bases.

However, some grasses are able to produce alkaloids independently of fungal endophytes. Canary grass, *Phalaris*, produces high level of tryptamine indole alkaloids (Wood & Clark, 1971; Ball & Hoveland, 1978). These alkaloids are toxic to livestock. Some grasses, such as barley, produce alkaloids during the seedling stage when herbivory might have the greatest effect on plant fitness (Culvenor, 1973; Waller & Dermer, 1981).

Endophytes also greatly affect plant fitness. There is some evidence that suggests that endophytic infection increases plant fitness in some species. The Balansiae fungi which sterilize their host are certainly parasites but eventhough the infected plant is sterile, it can be vegetatively healthy and expand clonally even in mixed populations (Bradshaw, 1959; Harberd, 1961; Clay, 1984; Kelly & Clay, 1987). On the other hand, some endophytes do not sterilize their host cells and are seedborne. An experiment conducted by Clay (unpublished) found that the infected clones of tall fescue which were planted in natural grassland exhibited significantly higher survival, growth and reproductive rates over a 3-5 year period. Mortimer and di Menna (1983) and Read and Camp (1986) also showed that plants infected with seed-borne endophytes had increased productivity rates.

In the absence of herbivores endophytes also enhance the growth of host plants. Latch *et al.* (1985) found that under controlled environment conditions pyrennial ryegrass grew significantly faster when they were infected with endophytes. Clay (1987) also demonstrated similar results for ryegrass and tall fescue which were infected with endophytes in the greenhouse. Belesky and Fedders (1996) studied tall fescue infected with endophytes and found that endophyte infection influenced leaf mass and also relative growth rates. The endophytes provided a growth and size advantage for the

host but not for uninfected plants. Ahmed *et al.* (2001) experimentally grew east Indian lemongrass (*Cymbopogon flexuosus* (Stued.) Wats.) which was infected by *Balansia sclerotica* (Pat.) Hohn. under glasshouse conditions. They found that endophyte-infected lemongrass produced 195% more shoot biomass and 185% more essential oil when compared to the endophyte-free lemongrass. Furthermore the quality of essential oil produced from endophyte-infected lemongrass was equal to the one from endophyte-free lemongrass. Generally when considering the situation between endophyte and plant it has been assumed that the plant will be the one to lose energy and therefore its growth would be reduced. Contrastingly the host plant substantially benefits from the endophytes and its growth rate increases.

The rate of endophytic infection increases with age in dominant pasture and forage grasses which frequently have endophytes. Latch *et al.* (1987) reported that, in central Europe, the frequency of endophyte infection is much higher in wild populations and old pastures than in commercial cultivars and younger pastures for forage grasses. Lewis and Clement (1986) studied the perennial ryegrass in Britain and obtained a similar result noting that the frequency of endophyte-infected ryegrass is higher in old pastures and lower in young pastures.

1.1.2 Non-grass endophytes

Non-grass or non-clavicipitaceous endophytes are endophytes which are harboured within the tissue of woody plants or shrubs. The woody endophytes include fungi belonging to Ascomycotina, Basidiomycotina, Deuteromycotina and the Oomycetes (Petrini, 1991). Non-grass endophytes exhibit more diversity than grass endophytes and they infect a much broader taxonomic range of host plants (Saikkonen, 1998). However, Petrini (1991) noted that many wood endophytes are likely to be highly specific regarding host species and among host populations and in this respect are very much like systemic endophytes of grasses. Furthermore the infection of woody endophytes is highly localized within leaves, petioles, barks and stems (Petrini, 1991).

One factor which greatly affects endophyte population is the mode of arrival. Carroll (1995) stated that "the endophytic infection in trees must be initiated through fungal propagules and also the conditions that favor dispersal and germination of spores should also favor high infection frequencies". The arrival is not only from dispersed

propagules, which can be either identical or dissimilar genetically as stated before, but also from vegetative mycelium (Rayner & Boddy, 1986).

1. Arrival by mycelium is important for root-infection fungi such as *Heterobasidion annosum* (Fr.) Bref. and *Armillaria* species. This form of colonization bestows several advantages on the fungus including the possibility for synergism between individual hyphae within the mycelium, the possibility of multiple infection foci for the same genotype and subsequent coalescence between these, and also the opportunity for importation of nutrients and water from exogenous sources.

Mycelium colonization provides an opportunity for the same genotype to spread from tree to tree. Therefore it is no coincidence that the population of adjacent trees often have the same genotype.

2. Arrival by propagules in the form of spores has a limitation for importing resources for colonization. This colonization will therefore be localized and the variation of propagule depends on whether they are genetically identical or different.

- genetically identical propagules are often asexual conidiospores. Conidia are generally produced by Ascomycotina, including wood-decaying fungi of the family Xylariaceae and also some Basidiomycotina.
- genetically different propagules are ascospores and basidiospores which developed meiotically from heterokaryotic hyphae.

Fröhlich *et al.* (2000) studied endophytes associated with palms and found that most endophytes entered the petiole via leaf and that the transmission of palm endophytes is likely to be horizontal (via air spora) rather than vertical (via seeds).

Endophytes may avoid substrate competition or antagonism by occupying tight compartmentalization within the same plant tissue with the host not reacting to the endophytes in the same way as to pathogens. Host-fungal recognition mechanism is very complex. It allows only symbiont partners to penetrate into host tissues without triggering defense barriers of the host plant (Petrini, 1991). Collmer and Keen (1986) examined the production of endophytic extracellular enzymes involved in infection and penetration processes of endophytes. The potential of endophytes to produce extracellular enzymes needed for cell wall degradation supports the hypothesis by Carroll (1988) that fungal endophytes of aerial plant tissues represent a highly specialized group of microorganisms.

Carroll and Petrini (1983) found that the tolerance of endophytes to gallic acid presumably results from their ability to cope with phenolic compounds which are normally present in host plant tissues. The production of extracellular enzymes which are responsible for plant invasion and colonization as well as of an auxin-like growth factor by endophytes indicates that these fungi are likely to have closely evolved with the host plant and have adapted to their environment (Petrini, 1991).

Surprisingly there are fewer reports on endophytes from woody plants than reports on grass endophytes. Most of the grass endophytes reported are concerned with ecological aspects of herbivory or toxicity. Studies on woody endophytes tend to be on the number and diversity of endophytes from different kinds of hosts and environments. The relationship of endophytes and herbaceous hosts is different from those of grass endophytes. The endophytes in trees appear to be more tissue specific within the host, transmission is mostly horizontally via asexual spores from plant to plant, and highly diverse among hosts and the diversity is especially high in tropical plants. Non-grass endophytes have also been found in seeds and acorns, thus they may also be able to transmit vertically and maternally via seeds.

Endophytes are thought to have evolved from parasitic or pathogenic fungi. Most woody endophytes are closely related to pathogenic fungi and presumably evolved from them by an extension of latency periods and reduction of their virulence. The relationship of woody endophytes and plants are considered to be mutualistic. Carroll (1988) found that endophytes from non-grass hosts provide protection for the host through the production of a wide range of mycotoxins and enzymes that can inhibit growth of microbes and invertebrate herbivores. However in some situations there is not clear difference between latent pathogens and endophytes. Significantly Petrini (1991) noted that the host-endophyte symbiosis cannot be presented as a static relationship. Dreyfuss and Petrini (1984) isolated *Colletotrichum gloeosporioides* (Penz.)Sacc., *Colletotrichum* sp., *Fusarium solani* (Mar.) Sacc., another *Fusarium* sp., and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., all well known as pathogens, as endophytic fungi from tropical plants.

Carroll and Carroll (1978) in their studies on endophytes of coniferous needles in over 200 sites throughout western Oregon and southern Washington found endophytes such as *Chloroscypha* sp., *Cryptocline* sp., *Leptostroma* sp., *Naemacyclus* sp., *Phomopsis*

sp., *Phyllosticta* sp., and some unidentified *Coelomycetes* as the dominant fungi. They also found that there were many endophytes which were restricted to the host and were host-specific. Petrini (1986), however, noted that some endophytes maybe specific only at family level of host plants. Leuchtmann and Clay (1989) investigated variation of *Atkinsonella hypoxylon* (Perk) Diehl. isolated from different hosts using isozyme electrophoresis. They found that the variation may correlate with the ability of each strain to infect a particular host. Sieber *et al.* (1991) isolated wood-inhabiting *Melanconium* sp. from different species of Alders (*Alnus* spp.) in Europe and Canada. Sieber-Canavasi *et al.* (1991) also studied the *Leptostroma* sp. from conifer needles.

Some endopytes exhibit a degree of tissue-specificity. Dreyfuss and Petrini (1984) found that species of *Acremonium* and *Fusarium* were restricted to roots whilst others, such as *Pestalotia* sp., or *Colletotrichum* sp., can be found only from aerial plant organs. Bettucci and Alonso (1997) studied the fungal population in healthy and symptomatic twigs of *Eucalyptus grandis* in Uruguay. They isolated *Fusicoccum eucalypti* Sousa da Camara and *Cytospora chrysosperma* (Pers.:Fr.) Fr. commonly from bark and xylem of symptomatic twigs. Therefore, they concluded that certain fungi are more common in certain tissues. Sieber *et al.* (1999) investigated the needles of *Pinus mugo* sp. *uncinata* from 2 sites in the montane zone and isolated endophytes which seemed to be tissue specific. Thus *Lophodermium pinastri* (Schrad. Ex. Hook.) Chev. was isolated mainly from the needle tip but *Cyclaneusma minus* (Butin) Di Cosmo, Peredo & Minter was frequently isolated from the middle segment of needles, whereas endophytes were rarely isolated from the base of the needles. Cannon and Simmons (2002) also demonstrated a very interesting result for tissue-specific endophytes isolated from leaf samples from the Iwokrama Forest Reserve, Guyana. They found that colonization of endophytes was greater in samples from midribs than from those from laminar tissue. The number isolated was also greater at the tip of the lamina than the base. They explained this phenomenon by suggesting that the leaf tip is more prone to get infected as rainwater draining of the apex washes un-established fungal spores or mycelium on the leaf surface towards their tips.

Carroll and Petrini (1983) investigated substrate utilization of endophytes isolated from coniferous foliage in Europe and Oregon, USA. They tested the capability of the endophytes in substrate utilization and found that most of the isolates utilized xylan and pectin and also showed lipolytic activity. Endophytes from the same hosts exhibited

similar variety in their substrate utilization capacity. They suggested that endophytes from a single host may co-exist within a single leaf by biochemical partitioning resources (Carroll & Petrini, 1983).

The degree of fungal colonization of alpine plants by endophytes is very much dependent on ecological conditions (Petrini,1987). High endophytic colonization occurred when there was long-lasting snow and the host plant was poorly supplied with nutrients. Whereas low colonization occurred when there was increasing wind exposure. The number of endophyte species as expected was low in extreme conditions (Petrini, 1987).

Bettucci and Alonso (1997) and Bettucci *et al.* (1999) found that conditions such as drought influence the fungal population. Drought may cause wound lesions on the host surface and this encourages the fungal invasion of twigs and bark. In their studies on endophytic communities Espinosa-Garcia and Langenheim (1990) found that the endophytic communities of same tree species growing at the same location are generally similar but differences in species richness and distribution of selected fungal species cannot be detected between young and old individuals of same host.

Petrini and Carroll (1981) studied foliage endophytes of some Cupressaceae in Oregon and found that in a given tree infection rates tended to increase with increasing foliage age. Fisher *et al.* (1986) noticed a similar result for fungal endophytes in *Ulex europeaus* L. and *U. gallii* Planchon. They reported that the incidence of colonization with different endophytes significantly increased with advancing age of host tissue. Arnold *et al.* (2000) in their investigation of tropical fungal endophytes also reported that endophyte infection increases with leaf age. Similar results for the endophyte assemblages in leaves of *Rhizophora apiculata* Blume were found by Kumaresan and Suryanarayanan (2002) which they suggested was due to repeated re-infection of leaves over time by the air spora.

The plant canopy also has an influence on endophytic populations. Thus samples obtained from homogenous stands with a closed canopy had higher colonization rates (Petrini & Carroll 1981; Petrini *et al.* 1982; Legult *et al.* 1989). Arnold and Herre (2003) also studied the endophytes of *Theobroma cacao* Linn. and found that endophytes colonized leaves of *T. cacao* more rapidly beneath the forest canopy then in cleared

sites which they stated reflected an increased abundance of aerial and epiphytic propagules.

Endophytic infections maybe also correlate with elevation above sea level and/or humidity and moisture. Sieber (1988) investigated Norway Spruce (*P. abies*) and its endophytes. It was found that for *Tiarosporella parka* (Bert.et.Broome) Whitney the infection was greater at low elevation and at a humid site but at the higher elevations and drier site, *Lophodermium piceae* (Fuckel) Höhn. was more frequent. Carroll and Carroll (1978) compared endophytic incidence with host distribution of *P. menziesii* and found that infection rate decreased at high elevation and drier sites. Sieber *et al.* (1999) studied the endophytes in needles of *Pinus mugo* sp *uncinata* which was sampled from two different sites in the montane zone; one on a mountain ridge and the other in a peat bog. At these two sites there were slightly different endophytic populations. At the mountain ridge *Cenangium ferruginosum* Fr. and *Cyclaneusma minus* (Butin) Di Cosmo, Peredo & Minter were dominant, whereas at the peat bog the dominant species were *C. ferruginosum* and *Lophodermium pinastri* (Schrad.)Chevall..

Seasonal changes also have an influence on endophytic populations and community. Widler and Müler (1984) demonstrated that season influenced the occurrence of some endophytes of *Arctostaphylos uva-ursi* (L) Sprangel but species with seasonal variation were never host-specific or the frequently observed taxa. Rodrigues (1994) studied the foliar endophytes of Amazonian palm (*Euterpe oleracea* Mart.). She found that environmental factors such as rainfall and atmospheric humidity might influence the occurrence of endophytes. It could be speculated that a particular nutrient within the host tissue could be more available during a certain season of the year thus affecting fungal activity.

Elamo *et al.* (1999) in their studies on the endophytic fungi in birch leaves over 3 years (1994-1996) found that environmental factors such as weather greatly affected the endophytic population. The number of endophytes was high in 1995 which was rainy and warm, when compared to the years 1994 and 1996 which were drier and cooler. However it was assumed that foliar endophytes of birch remained in fallen leaves during the winter and that their spores were spread through wind or rain splash onto new leaves the following season. They also concluded that rain is important because it

helps in promoting spore dispersal and in germination and subsequent development (Elamo *et al.*, 1999).

Mswaka and Megan (1999) investigated *Trametes* and other wood-decay fungi from a Zimbabwe forest and found that there was no significant difference between growth of the same species isolates in relation to temperature and that the geographic distribution correlated very well with the temperature relationship of endophytes. Therefore a broad optimum temperature range allows these fungi to rapidly decay the wood in tropical climates.

However, in a study of beech leaves, no distinctive seasonal patterns in the endophytic community was observed but there was marked seasonal variation in colonization rate on the trees (Sieber & Hugentobler, 1987).

Rodrigues and Samuels (1990) demonstrated the importance of the morphology of palm leaves in relation to their endophytic population. They discovered that endophytes in unopened leaves only occurred in the veins because unopened leaves are very tightly rolled and it is therefore unlikely that infection is via airborne spores. They believed that endophytes found in the unopened leaves maybe 'true' endophytes possibly transmitted through seeds. Whereas the opened leaf contained fungi living endophytically and that these endophytes are air-borne and infect the leaf once it has opened (Rodrigues & Samuels, 1990). In experiments on fast-expanding leaves such as *Ouratea lucens* (H.B.K.)Engl. it was found that there was a higher mean rate of damage caused by herbivores than in slow-expanding leaves such as *Heisteria concinna* Standl. (Arnold *et al.*,2001). Isolation from fast-expanding leaves produced greater diversity of fungi. However the rates of species accumulation from both leaf types proved to be similar. Thus other factors such as leaf chemistry would appear to be involved (Arnold *et al.*,2001).

Clear-cutting and plantations tend to reduce the transmission of endophytic fungi (Sieber-Canevesi & Sieber 1988). Johnston (1998) found differences between natural stands and planted stands when he worked on endophytes of manuka (*Leptospermum scoparium* Forst.). In natural stands a *Phyllosticta* sp. was the single dominant endophyte whilst in the planted stands this species was absent or presented at low frequency. Even though the planted stands were close to natural stands and had been

established for several years there was still a clear difference between them. The common endophytes in planted stands were *Botryosphaeria* and an *Alternaria* sp. which exhibited a low level of presence in natural stands. Differences in endophytic populations between natural and planted stands was said to be related to some unrecognizable environment factors.

Air pollution can also affect species composition of, and frequency of colonization by a given species, in endophytic communities (Petrini, 1991). Forest and fungal community are equally affected by pollutants. Riesen and Close (1987) studied endophytic colonization in barley leaves. They found that it was reduced significantly after propiconazole treatment of barley fields. Lappalainen *et al.* (1999) over a two year period examined density of endophytes on birch growing close to a large copper-nickel smelter in Finland. They found that total endophyte numbers were low near to the factory in both years. The leaves near to the factory contained heavy metals and SO_2. This may be important in the decline of endophytic fungi near to the smelter since both heavy metals SO_2 are known to be toxic to many fungi.

Rodrigues (1994) found that leaves of a tropical palm were more highly infected when collected during the wet season than during the dry season. This finding is not surprising because most endophytic infection in tree hosts must be initiated through fungal propagules. Leaf samples that were collected during the dry season were more likely to be uninfected than those of mature trees of the same species sampled during the rainy season. This is probably a result of higher relative humidity and the higher incidence of propagules from fungi within, or beneath, the canopy i.e the available inoculum.

Petrini (1991) stated that the endophytes from the same host species, and the same location were similar but that there are differences in species richness and distribution of fungal species between young and old leaf samples from the same host.

There have been many studies on the distribution of endophytic fungi with subjects very varied in terms of host or location. The temperate plant *Ulex* sp was found to contain up to 369 endophyte isolates (Fisher *et al.*, 1986). In coniferous needles Carroll and Pertini (1978) isolated as the dominant fungi *Chloroscypha* sp., *Cryptocline* sp, *Leptostroma* sp., *Naemacyclus* sp., *Phomopsis* sp., *Phyllosticta* sp., several unidentified

Coelomycetes and also an undescribed taxon in the Hemiphacediaceae from 19 hosts sampled in over 200 sites throughout western Oregon and southern Washington. Foliage endophytes were studied by Petrini and Carroll (1981) from four species of Cupressaceae sampled from 19 sites in Oregon. *Chamaecyparis lawsoniana* (A. Murr.) Parl. and *Thuja plicata* Donn. ex D. Don showed high overall infection rates (~30-50%) while *Calocedrus decurrens* (Torr.) Florin. and *Juniperus occidentalis* Hook. had lower infection rates (~10-35%). Elamo *et al.* (1999) studied the commonest endophyte, a *Fusicladium* species, of birch leaves and found out that this endophyte was affected by the environment. However Sieber *et.al.* (1999) in an examination of *Pinus mugo* sp. *uncinata* needles from 2 sites in the montane zone in Switzerland found that about 35-40% of needles were colonized by species common to both sites. Pirttila *et al.* (2003) investigated the endophytic fungi in different tissues of *Pinus sylvestris* L. (Scot Pine Buds) and found *Hormonema dematoides* and *Rhodotorula minuta* (Saito) Harrison occurred in different tissues at different frequencies. Ganley *et al.* (2004) however found that the majority of endophytes (90% of 2019 cultures) in western white pine (*Pinus monticola* Douglas ex D. Don) belonged to the family Rhytismataceae.

Moreover endophytes can even be isolated from plants growing in extreme conditions like gypsum sand or clay with high salinity and a basic pH (Pelaez *et al.*, 1998).

There have, however, been fewer studies on tropical endophytes. In their investigations of a tropical palm, *Manilkara bidentata* (A.DC.) Chev., or members of the Orchidaceae group, *Lepanthes*, from Puerto Rico (Lodge *et al.*,1996), Bayman *et al.* (1997) found that *Xylaria* and *Rhizoctonia*-like fungi were the dominant groups. Leaves from *Tectona grandis* L. (Teak) from the Northern part of Thailand were examined by Mekkamol (1997) and she isolated a high number of endophytes including many members of the Xylariaceae. She also found differences between isolations made during the early part of the rainy season compared with those made towards the end. Dreyfuss and Petrini (1984) investigated 36 tropical plants belonging to the families Pteridophyta, Araceae, Bromeliaceae, Orchidaceae, Piperaceae and Crassulaceae in Columbia. Some noteworthy endophytes included *Botryodiplodia piperina* Syd., *Dicyma* cf. *olivacea*, *Hadromena* sp. and *Zygosporium* cf. *echinosporum*. Rodrigues and Samuels (1990) studied the tropical palm tree, *Licuala ramsayi* (Mueler.) Domin. in Brazil. There were 11 fungi isolated from the samples and most of them were Xylariaceous anamorphs. Rodrigues (1994) investigated Amazonian palm, *E. oleracea*, and recovered 57 species

and 6 taxa which were mainly in the Ascomycotina and Deuteromycotina. *Xylaria cubensis* (Mont.) Fr. and *Letendraeopsis palmarum* K.F.Rodrigues&Samuels were the most common species. Manuka leaves (*Leptospermum scoparium* Forst.) were found to be different in their endophyte populations from natural and planted stands (Johnston, 1998). Fröhlich *et al.* (2000) also isolated endophytes from palms, which included 3 unidentified palms *Licuala* spp from Brunei Darussalam and 3 *L. ramsayi* from Australia. Colonization rates in both species were up to 81-89% and the communities were composed of dominant xylariaceous species. Cannon and Simmons (2002) studied the leaves sampled from Iwokrama Forest Reserve, Guyana. There were 64 fungal mycotaxa from an overall 2492 cultures. The common species belonged to the genera *Colletotrichum, Nodulisporium, Pestaliopsis* and *Phomopsis.*

Fröhlich and Hyde (1999) studied the biodiversity of tropical palm fungi and reported a total of 189 species isolated from 6 palm species. In addition 53 sterile mycelia were isolated to give a total of 242 taxa from 2672 isolates. They challenged the estimation of 1.5 million fungal species and the 5.7 species to 1 host ratio proposed by Hawksworth (1991) suggesting that from their data for tropical palms the ratio should be 33 species to 1.

Arnold *et al.* (2000) in their examination of two plant species, *Heisteria concinna* Standl. and *Ouratea lucens* (Kunth) Engl. isolated 418 endophyte morphospecies (estimated 347 genetically distinct taxa) from 83 leaves. They suggested that tropical endophytes comprise an important and quantifiable component of fungal biodiversity and the high richness they found led them to postulate that tropical endophytes are hyperdiverse. They also noted that with continuing exploration of tropical endophytes the 1.5 million species of fungi proposed by Hawksworth (1991) will underestimate fungal biodiversity. In a later study of dicotyledonous neotropical trees in Panama the abundance and diversity of tropical endophytes was again recognized (Arnold, Maynard, & Gilbert, 2001). They concluded that tropical endophytes are a vital component of diversity estimates and contribute substantially to fungal biodiversity.

Arnold and Herre (2003) studied the foliar endophytes of the tropical tree (*Theobroma cacao* L., Malvaceae). They found that canopy cover is related to density of endophyte infection over the short term and that the leaves remained receptive to endophytic infection after initial infection had occurred. Moreover, age-specific leaf toughness and

chemistry were not associated with colonization and growth. Young and mature leaves proved to be equally susceptible to infection by foliar endophytes. When they tested the effects of extracts from forest and clearing grown leaves, and young and mature leaves to endophytes isolated from *T. cacao*, they were found to have no effect. These results suggest that neither phenolics nor other chemical factors which vary with leaf age have a direct influence on endophytic hyphal growth *in vitro*. They concluded that high inoculum, beneficial conditions for survival of epiphytic propagules and multiple applications throughout the lifetime of the leaf and plant should increase colonization of healthy tissues by foliar endophytes (Arnold & Herre, 2003).

Arnold *et al.* (2003) found that the endophytic population of *T. cacao* can limit damage from pathogens. They demonstrated that when cacao leaves were infected with endophytes prior to infection with a pathogenic *Phytophthora* species the leaves exhibited less necrosis and mortality when compared with endophyte-free leaves. They found that the protection was primarily localized to endophytic-infected tissues. Furthermore, endophytic-mediated protection was greater in mature leaves which have fewer defenses against fungal pathogen than young leaves. *In vitro* studies suggested that the protection maybe mediated by direct interactions of endophytes with the pathogen (Arnold *et al.*, 2003).

1.2 Xylariaceae

The family Xylariaceae is very well represented throughout the world occurring mainly as wood inhabitants and exhibits its greatest diversity in the tropics (Ju & Rogers, 1996; Whalley, 1996; Rogers, 2000). Petrini and Petrini (1985) demonstrated the regular occurrence of Xylariaceae as endophytes of temperate hosts but later emphasized that they exhibited their greatest diversity and abundance in tropical plants where they appear to be universally present (Petrini *et al.* 1995). Most Xylariaceae are saprotrophic and are important in the decay of litter and woody substrata but there are also a number of species, mainly belonging to the genera *Biscogniauxia* and *Rosellinia* which cause diseases (Whalley, 1996; Edwards *et al.*, 2003). To what extent species of *Biscogniauxia* which cause various canker diseases in their tree hosts can be considered to be endophytes or whether they should be considered latent pathogens has been discussed in detail by Rogers (2000). Regardless of definition these and other Xylariaceae are frequently isolated from healthy tissues and their presence in their host

is often only apparent in the field once the host is stressed or a branch has become detached. The fungus then readily produces its stromata on the dead or dying wood.

The Xylariaceae is a family of fungi belonging to the Ascomycota. Most of its members develop asci in perithecia beneath the surface of a stroma. Most asci have an iodine-positive ascus apical ring. The ascospores of the Xylariaceae are usually depicted as a more or less phaseoliform single cell with a smooth, brown wall which possesses a conspicuous full-length germ slit. In most Xylariaceae their stomal surface is hard, black, rough and carbonaceous, especially when it is maturing and most species produce a conidial state (Ju & Rogers, 1996). Conidia are produced blastically from conidiogeneous area of conidiophores. Each conidium is cut off from the conidiogenous cell by a septum. Conidia have a flattened area at the former site of their attachment to the conidiogeneous cell (Rogers, 1978).

The Xylariaceae can colonize a wide range of substrates but in some species they tend to be host-specific. Thus *Hypoxylon fragiforme* (Bull. ex Fr.) Kick and *Biscogniauxia nummularia* (Bull: Fr.) Kuntze are associated with *Fagus, H. multiforme* (Fr.) Fr. with *Betula* and *Daldinia concentrica* (Bolton) Ces.& De Not. with *Fraxinus* (Whalley, 1996).

The Xylariaceae occupy a wide range of habitats but in general they can be grouped into wood-habitats, dung-habitats, litter-habitats and a few are associated with insects (Rogers, 1979; Whalley, 1996). Wood-inhabiting Xylariaceae are the most common as they are associated with bark (eg: *H. fragiforme*), and decorticated wood (eg: *Nemania confluens* (Tode) Læssøe & Spooner.) which is also well-decomposed and those which show no preference (Rogers, 1979; Whalley, 1996). The dung inhabiting Xylariaceae are *Poronia*, *Podosordaria* and *Hypocopra*. *Poronia punctata* (L.:Fr.) Fr. always produces a number of antibiotic compounds, the punctatins, but *P. oedipus* (Mont.) Mont. which is closely related to *P. punctata* does not (Whalley & Edwards, 1995). Normally xylariaceous fungi are not considered to be litter fungi but some of them are found growing in the leaf litter layer or fruit litter such as *Xylaria carpophila* (Pers.) Fr., which grows on beech cupules or *X. magnoliae* J.D.Rogers on magnolia fruit (Whalley, 1985).

Xylariaceae in the ecosystem can be categorized by the position of their host or the substrate invaded and by the timing of the invasion (Rogers, 2000)

1. Species that invade living leaves and stems. They often fruit on living host materials. An example of this kind of lifestyle is *Anthostomella*.
2. Species that invade living stems, remaining dormant until host gets stressed and then rapidly colonizing host and fruiting on it. *Daldinia*, *Biscogniauxia*, *Camillea* and *Hypoxylon* are all in this group.
3. Species that decay living roots and wood, moving from the dead material to the living one. *Kretzschmaria calvus*, *Rosellinia necatrix* Prill. and various *Xylaria* species are saprophytes which can become plant pathogens when they are allowed to build up in the litter.
4. Species that often fruit on decayed material, but are isolated as endophytes from living hosts. Most species of *Xylaria* and *Nemania* are in this category.
5. Species that fruit on seeds and fruits. Mostly in this category are *Xylaria* which have a specific relationship with host.
6. Species that invade dung. *Hypocopra*, *Podosordaria* and *Poronia* also have special relationships with animals. Many taxa have dormant ascospores which will pass through the mammalian digestive tracts.
7. Species that are associated with ant and termite nests such as *X. melanaxis* Ces. and *X. nigripes* (Kl.) Sacc.. These fungi are associated with dead or dying insect nests. It is assumed that these fungi are accumulated by insect and start to fruit after the nests are abandoned.
8. Species that inhibit litter and organic soils. Generally, xylariaceous fungi are isolated as anamorphs such as *Nodulisporium* and *Geniculosporium* from soil litter. Most of these fungi do not produce a teleomorph in culture.
9. Species that are damaging pathogens such as *Camillea tinctor* (Berk.) Læssøe, Rogers & Whalley., *Biscogniauxia capnodes* (Berk.) Ju & Rogers. and *B. mediterranea* (De Not.) Kuntze.. These species appear to be latent pathogens which invade plant tissues and remain quiescent until host stress allows the pathogens to rapidly colonize.

1.2.1 Morphological characteristics of Xylariaceae

Morphological characteristics of Xylariaceae are described as followed (Ju & Rogers, 1996; Whalley, 1996).

- *Stromata and ascomata*

Stromata may be bipartite. In this case the upper layer that hides the stromatal surface disappears at maturity. This character can be deduced by the presence of remnants at the margins, even at maturity. It is encountered in *Biscogniauxia* and *Obolarina*.

Stromata may be more or less immersed in their substrate (some *Anthostomella*, *Euepixylon*), erumpent (*Entoleuca*), superficial broadly attached (most cases), superficial loosely attached (*Kretzschmaria*), constricted at base (*Daldinia*) or stipitate (*Xylaria*). In most genera, the stromatal tissue is homogenous. It is hollow and filled with liquid in *Entonaema* and presents concentric zones in *Daldinia*.

Stromata are most often multiperitheciate and perithecia arranged in one layer. In *Lopadostoma* subgenus *Lopadostoma*, perithecia are in valsoid configuration. Uniperitheciate stromata occur in *Anthostomella*,*Creosphaeria*, *Hypocopra*, *Lopadostoma* subgenus *Anthostomopsis*, *Nemania* and *Rosellinia*, frequently with a tendency to become coalescent.

- *Asci and ascospores*

At the generic level, the most distinctive feature of asci lies in the apical apparatus. It may be broader than high (ring-like or discoid) or higher than broad (inverted hat-shaped to urn-shaped), easily seen when bluing in Melzer's reagent (amyloid). Amyloidity is not constant within a genus and is helpful at the specific level.

Ascospores are typically one-celled, ellipsoid, provided with a germination site and pigmented. *Biscogniauxia anceps* (Sacc.) Rogers, Ju & Cand. is noteworthy in having two-celled ascospores that frequently remains hyaline, even when mature. In most *Anthostomella* taxa, young ascospores are conspicuously two-celled and keep a dwarf hyaline cell when mature. This also occurs in *Nemania*, *Rosellinia* and *Xylaria*, but much less conspicuously.

The germination site is slit-like (sigmoid or bilateral at times), except in *Euepixylon* where it is pore-like. The shape, the relative length of the germ slit and its position on the ascospore's wall is often diagnostic at the specific level.

In *Daldinia* and *Hypoxylon* the perispore may be dehiscent in 10% KOH. Unfortunately this character is not constant in all taxa and does not allow a reliable distinction of these two genera.

Under the microscope most members of the family can be recognized by having: **1)** dark unicellular spores with more or less evident germination sites, mostly in the form of a slit (pale line). In some cases the immature spores are bicellular and may leave a cellular appendage on the mature spore. Some have gel-coats or appendages; and **2)** a mostly blue reaction to the apical apparatus of the asci in iodine reagents (The Funga, Læssøe, Web resource)

1.2.2 Conidial states of the Xylariaceae

Most xylariaceous fungi have conidial states. In nature *Xylaria* conidia may have been produced on the surface of immature perithecial stromata, on anamorphic appendages borne on developing perithecial stromata, or on separate structures distinct from teleomorphic stromata. Anamorphic and teleomorphic structures are different in appearance and are also separated physically and temporally (Callan & Rogers 1993).

Xylariaceous fungi produce conidia blastically from conidiogenous cells of conidiophores. The conidial wall is originally holoblastic. Each conidium is separated from the conidiogenous cells by a septum and there is also additional wall material deposited on each side of the septum. The conidium is eventually separated from the conidiogenous cells by the rupture in the plane of the septum. Conidia still have a flattened area which corresponds to where they were formerly attached to the conidiogenous cells.

After the conidium has separated, the conidiogenous area reveals scars or denticles as an evidence of conidial production (Greenhalgh 1967; Greenhalgh & Chesters 1968; Jong & Rogers 1972). The morphology of the secession scar is characteristic for some species.

The morphology and arrangement of conidiogenous cells and conidiophores is an important characteristic for species, groups of species and, probably, entire genera. For *Hypoxylon* and Daldinia, their conidial states are usually referred to the form-genus

Nodulisporium Preuss but in *Nemania* and *Rosellinia*, their conidial states are generally referred to the form-genus *Geniculosporium* Chesters & Greenhalgh (Ju & Rogers, 1996).

Nodulisporium is subdivided into four types based on the branching patterns of conidiogenous structures. Listing in ascending order of complexity, the four types are *Sporothrix*-like, *Virgariella*-like, *Nodulisporium*-like and *Periconiella*-like (Ju & Rogers, 1996) (Figure 1.3).

1. *Sporothrix*-like: Conidiophores are simple and often aseptate. They also terminate in a condiogenous region, or are sparingly branched from the conidiogenous region and form an overlying conidiogenous region on the additional branches. There is one conidiogenous cell on each terminus.
2. *Virgariella*-like: Conidiophores do not have a dominant main axis. They are unbranched or dichotomously branched one or two, or sometimes more, when they form a level of conidiogenous regions; additional branches arising from the first level of conidiogenous cells and terminating in a second level of conidiogenous regions. This process of producing additional branches usually continuing several times to result in several levels of conidiogenous regions. There are one or two conidiogenous cells on each terminus.
3. *Nodulisporium*-like: It is very much like a *Virgariella*-like type. However, the differences are the original conidiophores and additional branches. They are more complex and have a bushy structure. Several levels of conidiogenous regions are often produced. On each terminus, there are usually two or three, or sometimes more, conidiogenous cells.
4. *Periconiella*-like: Conidiophores are composed of a main axis and sometimes there are one or more major branches. These terminate with three or four conidiogenous cells arising in whorls from the main axis and/or from major branches or from additional subordinate branches. Additional branches are normally lacking or rarely produced.

Ju and Rogers (1996) also noted that the hallmark of the Periconiella-like type is the property of 'apical dominance' of the main axis, which differentiates it from the other three types. The *Periconiella*-like type is similar to the *Nodulisporium*-type in having the tendency to produce more than two conidiogenous cells at each branch terminal. Both *Nodulisporium*-like and *Virgariella*-like are also alike in frequently producing additional branches but they differ in the simplicity of branching. The *Virgariella*-like type is simpler since they are unbranched or dichotomously branched. While the *Sporothrix*-like type is so reduced that the conidiogenous cells are the entire conidiogenous structures. The additional branches are found in all four types but they are very much less found in *Sporothrix*-like and *Periconiella*-type.

To summarize the conidial state of xylariaceae, their conidiophore morphology is distinctive for each form genus. All xylariaceous anamorphs usually have hyaline to light brown conidiophores but there are different in branching, the position of conidiogenous cells, and the arrangement of conidiogenous loci within the conidiogenous cells. These can be good differential characters and for many xylariaceous endophytes are the only features available for identification purposes (Petrini & Petrini, 1985).

1.2.3 Cultural characteristics

Most xylariaceous fungi can be cultured on many media such as 2% malt agar, potato dextrose agar and oatmeal agar. Their characteristics on these agar media such as growth rate, texture of the colony surface, mycelial colour and also conidiomata are stable within a species. Conidia are produced on conidiophores which develop freely on mycelium or on special structures such as small pustular areas or well-developed stromata.

Even though Martin (1967) stated that there are six types of xylariaceous growth form, Van der Gucht's observations indicated only 4 growth forms as follows (Van der Gucht, 1994) (Figure 1.4) :

1. Colony surface is powdery, coloured (brown-olive green-grey), and there usually is a small amount of white aerial mycelium. Carbonization is absent. Conidia are formed on freely developed conidiophores on mycelium. There are numerous conidia covering the whole colony surface. Growth rate is

rapid (covering 9 cm diameter Petri dish within 2 weeks). *Daldinia* and *Hypoxylon* are characteristic for this group.

2. Colony surface is coarse, powdery. It is characterized by ropy, straggling hyphae and also the aggregation of mycelium. The colony is coloured ochre-yellow, olive-green to brown. There is no carbonization. Conidia are formed on conidiophores which develop freely on mycelium. The conidia are abundant. Growth rate is rapid. *Biscogniauxia* belongs in this group.
3. Colony is felty to fleecy. It is uniform to zonate, white and also smooth. The carbonization is often presented. Conidia are formed on conidiophores which develop freely on mycelium. The number of conidia is not abundant. Growth rate is moderate (covering 9 cm Petri dish within 3-4 weeks). *Nemania* is representative of this group.
4. Colony is felty to fleecy or floccose. It is uniform or zonate, smooth or rough and also white. Carbonization is very marked. Conidia are produce on conidiophores which is packed tightly in a palisade, on conidiomata. Growth rate is moderate to slow. *Kretzchmaria* and *Xylaria* represent this group.

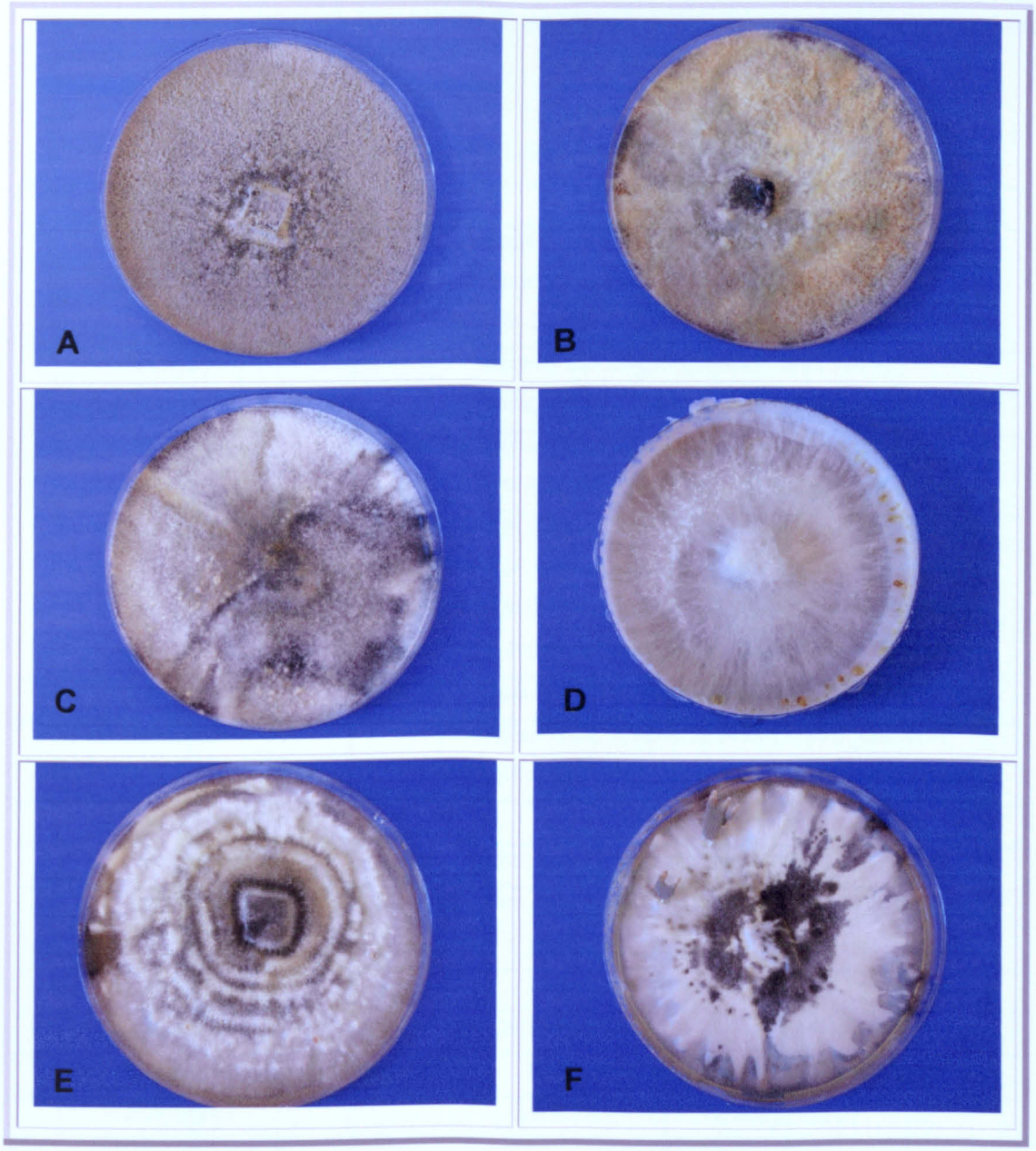


Figure 1.4 Anamorphic forms of selected Xylariaceae growing on Malt Extract Agar (MEA); A = *Daldinia concentrica*, B = *Hypoxylon fragiforme*, C = *Hypoxylon multiforme*, D = *Rosellinia necatrix*, E = *Xylaria polymorpha*, F = *Xylaria* sample 1

There is a possibility that anamorphic form in culture can be linked with the definite morphological types of teleomorph. For example species of *Nemania* and *Rosellinia* constantly exhibit anamorphs belonging to *Geniculosporium* and the species *Periconiella* has been reported to link with *Biscogniauxia* (Petrini & Petrini, 1985).

Important known anamorph-teleomorph connections of the Xylariaceae are summarized in Table 1.1.

Table 1.1 Anamorph-Teleomorph connections

Teleomorph	***Anamorph***
Anthostomella	Geniculosporium *Chester & Greenh. (Martin 1969a, =* Nodulisporium *type 2a),* Nodulisporium *Preuss and* Virgariella *S.Hughes (Francis, Minter & Caine,1980)*
?Ascotricha	Dicyma *Boulanger (Hawksworth, 1971)*
?Ascotrichella	?Humicola-*like (Valdoserra & Guarro, 1988)*
Astrocystis	Acanthodochium *Samuels, JD Rogers&Nagas (Samuels, Rogers & Nagasawa, 1987; Ju & Rogers,1990)*
Biscogniauxia	Nodulisporium *(Greenhalgh&Chester, 1968; Callan&Rogers, 1986; San Martin&Rogers, 1993),* Geniculosporium *(Eckblad&Granmo, 1978; Whalley&Edwards, 1985),* Periconiella *(Petrini&Muller, 1986)*
Calceomyces	Nodulisporium *(Udagawa&Ueda, 1988)*
Camillea	Xylocladium *Syd. (Crane&Dumont, 1975; Læssøe, Rogers&Whalley, 1989; Ju, Gonzalez & Rogers, 1993)*
?Chaeocarpus	*Unknown*
Collodiscula	Acanthodochium *(Samuels, Rogers&Nagasawa, 1987)*
Daldinia	Nodulisporium *(Chester&Greenhalgh, 1964; Petrini&Muller, 1986)*
Engleromyces	*Unknown*
Entoleuca	Geniculosporium, Dematophora *(Rogers&Ju, 1996)*
Entonaema	Nodulisporium *(Rogers, 1982)*
Euepixylon	Geniculosporium *(Whalley, 1976)*
Helicogermslita	*Unknown*
Holttumia	*Unknown*
Hypocopra	*Unknown*
Hypoxylon	Nodulisporium, Virgariella, Hadrotrichum *Fuckel,* Rhinocladiella *Nannf. (Martin, 1967a; Greenhalgh&Chesters, 1968; Jong&Rogers, 1972; Petrini&Muller, 1986)*

Table 1.1 (Continue II) Anamorph-Teleomorph connections

Teleomorph	***Anamorph***
Induratia	Nodulisporium *(Samuels, Muller & Petrini, 1987)*
Jumillera	Libertella-*like*, Geniculosporium *(Rogers, Ju & San Martin, 1997)*
Kretzschmaria	Hadrotrichum *(Petrini & Muller, 1986)*
Kretzschmariella	Mirandinia *Matsushima (Ju & Rogers, 1994)*
Leprieuria	Geniculosporium *(Samuels & Muller, 1980)*
Lopadostroma	*Scolecosporous anamorph*, Libertella-*Like (Ju, Gonzalez & Rogers, 1993)*
Nemania	Geniculosporium *(Chester & Greenhalgh, 1964; Petrini & Muller, 1986)*
Obolarina	Rhinocladiella-*like (Candoussau & Rogers, 1990)*
?Penzigia	*Unknown*
Phaeosporis	Sporothrix *Hektoen & CF Perkins (Jong & Davis, 1974)*
Phylacia	Geniculosporium *(Rodrigues & Samuels, 1989)*
Podosordaria	Lindquistia *Subram & Chandrash. (Subramanian & Chandrashekara, 1977; Rogers & Læssøe, 1992)*
Poronia	Lindquistia *(Subramanian & Chandrashekara, 1977; Stiers, Rogers & Russell, 1973)*
Pulveria	*Unknown*
Rhopalostroma	Nodulisporium *(Hawksworth & Whalley, 1985)*
Rosellinia	Geniculosporium, Dematophora *R.Hartig,* Nodulisporium *(Petrini, 1992)*
Sarcoxylon	*Unknown*
Seynesia	Acanthodochium *(Hyde, 1995)*
Stilbohypoxylon	*Unknown*
Stromatoneurospora	*Unknown*
Thamnomyces	Nodulisporium *(Samuels & Muller, 1980)*
Theissenia	*Unknown*
Theumenella	Nodulisporium *(Samuels, 1989; Samuels & Rossman, 1992)*
Versiomyces	*Unknown*

Table 1.1 (Continue III) Anamorph-Teleomorph connections

Teleomorph	*Anamorph*
Vivantia	Nodulisporium *(Rogers, Ju & Candoussau, 1996)*
Wawelia	*Anamorph described by Minter & Webster (1983) as being geniculate but not assigned to a form genus.*
Whalleya	Geniculosporium-*like but with dry scolecosporous conidia (Rogers, Ju & San Martin, 1997)*
Xylaria	*Typically produced on developing stromata but no form genus yet assigned.* Xylocoremium flabelliforme *is associated with* X. cubensis *(Rogers, 1984, 1985b)*

1.2.4 Distribution of Xylariaceae

The current data shows that most of the Xylariaceae are distributed in the tropics, but some species are cosmopolitan and are widespread throughout the world. They are, as stated earlier, common as endophytes especially in tropical plants (Whalley,1997). In Puerto Rico a *Xylaria* sp. was isolated from more than half of the shoots of *C. equisetifolia* and from nearly all of the mature *Manikara bidentata* (A. DC.) Chev. leaves sampled (Bayman *et al.*, 1998).

Tropical palms have been studied by several groups. Rodrigues and Samuels (1990) studied the Australian fan palm (*L. ramsayi*) in lowland rainforest of Australia. They isolated mostly xylariaceous anamorphs. Again in the Amazonian palm (*E. oleracea*), Rodrigues (1994) isolated *Xylaria cubensis* (Mont.) Fr. as one of the most common species but other Xylariaceae taxa were also found. Fröhlich *et al.* (2000) isolated endophytes from palms in Brunei Darussalam (*Licuala* sp.) and palms in Australia (*L. ramsayi*). They found an overall colonization rate of 81-89% with the most common fungi being taxa of the Xylariaceae. Thus Tropical palms harbour a significant number of xylariaceous endophytes.

The percentage recovery of *Xylaria* species from leaves of *Manilkara bidentata* in Puerto Rico was very high occurring in 73-74% of leaves (Lodge *et al.*, 1996). *Xylaria* cf *multiplex*, *X.* cf. *adscendens*, *X. mellisii* (Berk.) Cooke and members of the *X. arbuscula* Sacc. complex were identified. These *Xylaria* species were rarely found in the petiole

and the authors suggested that these endophytic fungi may appear to have originated from airborne or waterborne propagules (Lodge *et al.*, 1996).

Lepanthes, a genus of the Orchidaceae was studied by Bayman *et al.* (1997). Roots and leaves were investigated and the most frequently isolated endophytes were *Xylaria* species and *Rhizoctonia*-like fungi. They also noted that the *Xylaria* from *Lepanthes* fitted at least three of the criteria of Carroll for assuming that endophytes are mutualist Carroll (1988). Firstly, they are widespread and cause no obvious damage; secondly, they grow throughout host tissues; thirdly, there are many reports that these species can produce toxic secondary metabolites. Xylariaceous fungi have also been isolated from other tropical plants such as manuka leaves (*Leptospermum scoparium* Forst.&G.Forst.) (Johnston 1998), from healthy twigs of *Eucalyptus globules* Labil. and *E. grandis* Hill ex. Maiden in Uruguay (Bettucci *et al.*, 1999) and from symptomless leaves of tropical plants in the Iwokrama Forest Reserve in Guyana (Cannon & Simmons, 2002).

In Thailand there have been a few studies on the Xylariaceae. However an earlier study by Mekkamol found that Xylariaceous fungi are one of the most common endophytes. Mekkamol (1997) studied endophytes from teak (*T. grandis*) leaves from a natural stand and from a teak plantation. Members of the Xylariaceae were recovered as 20-60% of all isolates from each site depending on the year sampled. Thienhirun (1997) also demonstrated the great number and diversity of Thai Xylariaceae and noted that there are a considerable number of new species waiting to be described. Continuing studies on Thai Xylariaceae strongly support this earlier view and the dominant taxa are *Xylaria* and *Hypoxylon* species (Thienhirun & Whalley, 2004) which is in agreement with other studies of tropical Xylariaceae. Phottita *et al.* (2001) studied endophytes from wild banana (*Musa acuminate* Colla.) at Doi Suthep Pui National Park, northern Thailand. They found Xylariaceous fungi to be the most frequently isolated endophytes from the leaves. Chareprasert (2001) investigated the endophytic fungi from leaves of teak (*T. grandis*) and rain tree (*Samanea saman* Merr.) growing in the campus of Chulalongkorn University, Bangkok. Surprisingly members of xylariaceae were found only in the mature leaves of teak and were not at all present in rain tree leaves. This may be because the rain tree produces some toxins which inhibit conidia or ascospores of xylariaceous fungi. It might also be relevant that the leaves are thin and delicate unlike the much more robust teak leaves and this might account for these differences.

1.3 Study of secondary metabolites of endophytic fungi

Fungi are a large diverse group and they have been known for a long time to be a rich source of important bioactive metabolites and they can be used as antimicrobial agents (Moss, 1984). For instance, *Penicillium chrysogenum* Thom produces the antibiotic penicillin; *Monascus rubber* van Tieghem and *Aspergillus terreus* Thom produce lovastatin which lowers the level of blood cholesterol or *Trichoderma polysporum* (Link:Fries) Rifai. which produces the immunosuppressive agent, cyclosporin A (Kingston,1996).

Plants are a promising source of antimicrobial, immunosuppressive, anti-cancer, and anti-arthritic substances. Ironically, microorganisms which are associated with plants, rather than the plants themselves, sometimes offer raw materials with promising therapeutic potential (Strobel & Long, 1998). These materials or secondary metabolites are not essential for the growth of the producing culture but serve diverse survival functions in nature. Secondary metabolism occurs best at submaximal growth rates. These metabolites are produced after growth and are not involved in the growth of the producing organisms. Many secondary metabolites have antibiotic activity and could kill the producing culture if made too soon. The capability of the culture to resist their own metabolites is still unknown. There are, however, some theories that could explain why they can survive their antibiotic substances; 1) enzymatic detoxification, 2) alteration of the antibiotic's normal target in the cell or 3) modification in permeability to allow the antibiotic to be pumped out of the cell and restrict its re-entry (Demain, 1996).

In today's world there are increasing health problems for the world population, such as AIDS, drug resistance, cancer and emerging viruses so that there is an increasing need for new drugs. Thus novel drug discovery is important and endophytic fungi residing in plant tissues as mutualists are seen as a potential source. There are numerous reports on these fungi producing novel and interesting compounds which are sometimes extremely valuable (Strobel, 2002; Tan & Zou, 2001; Schulz *et al.*, 2002; Huang & Kaneko, 1996).

Strobel and Long (1998) suggested guidelines for selecting plants and associated endophytes to study for potential novel drug producer.

- Select plant species from which one or more notable drugs have already been discovered. It could be assumed that endophytes that live inside plants may produce the same compounds.
- Observe the ecological setting and health of the plant for clues about microorganisms which play a role in protecting plant hosts.
- Investigate plants that occupy unusual ecological niches such as the plants that grow in extreme conditions. These plants may support endophytes that produce novel bioactive compounds.

Studies of secondary metabolites from endophytes were already in focus when Stierle *et al.* (1993) firstly discovered the valuable anticancer agent, Taxol, from an endophyte of Pacific Yew, *Taxomyces andreanae* Strobel, Stierle & Hess. Taxol is a highly derivatized diterpenoid and has shown to be an anti-tumor agent for breast and ovarian cancer (Segal, web resource). Pacific Yew, *Taxus brevifola* Nutt., is however, a slow-growing tree and Taxol occurs as only 0.01-0.03% of the dry phloem while the full regimen of ovarian treatment requires 2 g of purified Taxol. Unfortunately the amount of taxol produced by *Taxomyces* is also low. Therefore scientists are investigating new hosts and isolating novel endophytes with an expectation of obtaining higher yields of Taxol (Strobel *et al.*, 1994; Stierle *et al.*, 1995; Strobel *et al.*, 1996; Li *et al.*, 1996; Li *et al.*, 1998; Strobel *et al.*, 1999; Metz *et al.*, 2000).

Pestalotiopsis microspora (Spegazzini) Batista & Peres. is one of the most widely distributed endophytic fungi in nature. This fungus produces many potentially useful compounds. It produces taxol and also isospectin (a bioactive isobenzofuranone) which has potential antifungal and antioxidant activities (Strobel *et al.*, 2002) and more recently pestacin has been discovered and this exhibits antioxidant and antimycotic activities (Harper *et al*, 2003).

Marine fungi are also a good source of secondary metabolites. Isocoumarin glucosides, Haloresellins A and B, have been isolated from the marine fungus *Halorosellinia oceanica* Whalley, EBG Jones, Hyde & Laessoe (Chinworrungsee *et al*, 2002). In addition, halorosellinic acid was also isolated from the same fungus (Chinworrungsee *et al*, 2001). This compound has antimalarial activity but also weak antimycobacterial activity. Extracts from a marine endophytic fungus from the South China Sea coast exhibited cytotoxicity toward NCI14460 and Bel-7402 and high activities against *Heliothis armigera* (Huhner) and *Sinergasilus* sp. (Chen *et al*, 2003).

Fisher *et al.* (1984) obtained three novel antibiotics from an endophytic *Cryptosporiopsis* species isolated from *Vaccinium myrillus* (Bilberry) which were detected on Thin-Layer Chromatography plates. These substances inhibited *Candida albicans* (Robin)Berkhout and it was suggested that further studies would be worthwhile. Now that *Candida* infections are becoming a worldwide problem especially for immunodeficiency patients further studies on this and similar fungi could be rewarding.

Preussomerin exhibits very significant antifungal and antibacterial activity and was firstly isolated from the coprophilous fungus *Preussia isomera* Cain. (Weber *et al.*, 1990). Polishook *et al.* (1993) isolated the derivative of this substance, preussomerin D, from the yeast *Hormonema dematioides* Lagerb. & Melin which has been shown to have broad spectrum antimicrobial activity against filamentous fungi, clinical yeasts and Gram-positive bacteria.

Cytochalasins are a class of mould metabolites of which there are now more than 20 types. Cytochalasins have been isolated from a variety of fungal species including *Helminthosporium* spp., *Phoma* spp., *Xylaria* spp., *Hypoxylon* spp., and *Chalara* spp. The cytochalasins exhibit a broad spectrum of activity. They have been used as biological probes but their therapeutic application has been limited because of their toxicity. Wagenaar *et al.* (2000) isolated three new cytochalasins, as well as cytochalasin E from the endophytic fungus *Rhinocladiella* sp.. They found that only cytochalasin E had significant activity when it was tested against human cancer cell lines but there was no significant activity with the three new cytochalasins.

Colletotrichum species are common endophytes and an isolate from the plant *Artemisia annua* L. belonging to the family Asteraceae was found to produce new bioactive metabolites (Lu *et al.*, 2000). The new compounds were found to be ergosterol derivatives and had an inhibitory effect on Gram-negative and Gram-positive bacteria. Some of the sterol was shown to be antifungal and antiviral as well. These findings suggested the possibility that this endophytic *Colletotrichum* could protect its host by producing these metabolites which maybe toxic or lethal to phytopathogens.

The Xylariaceae are also a rich source of secondary metabolites. Their ability to produce novel metabolites with unusual structures has attracted chemical investigations

which have proved to be most rewarding with 30 new compounds isolated from 30 species. The major metabolites from xylariaceous fungi can be grouped as dihydroisocoumarins and derivatives, succinic acid and derivatives, butyrolactones, cytochalasins, sesquiterpene alcohol (punctaporonins), griseofulvin and griseofulvin derivatives, naphthalene derivatives, and long chain fatty acids (Whalley & Edwards, 1998).

Rosellinia necatrix Prill. which causes a white root rot in tea plants and many types of orchard trees and has also been isolated as an endophyte from plant produced three kind of diketopiperazines which have retarditive activity for growth of plant seeding and plant roots (Chen, 1960).

Xylaria obovata (Berk.) Fr. is another source of novel metabolites. Cytochalasins and some phytotoxins were isolated from this fungus (Abate *et al.*, 1997). Extracts from some *Xylaria* spp are active against *Plasmodium falciparum* (K1, multidrug resistant strain). Those compounds were isolated as (-)-depudecin, (+)-phaseolinone, (+)-phomenone, 19-20-epoxycytochalasin Q, and (E)-methyl3-(4methoxyphenoxy) propenoate (Isaka *et al.*, 2000).

A mangrove *Xylaria* from the South China Sea produced five unique metabolites, Xyloketals A,B,C,D and E. It was found that Xyloketal A exhibited the activity of inhibiting acetylcholine esterase (Lin *et al.*, 2001).

Xylaria multiplex (Kunze) Fr. BCC1111 produced two new 10-membered lactones, multiploides A and B, which exhibited antifungal activity against *Candida albicans* but had no activity toward the malarial parasite *P. falciparum* (Boonphong *et al.*, 2001).

Stadler *et al.* (2001a and 2001b) have extensively studied the secondary metabolites in *Daldinia* and other Xylariaceae. They isolated a number of metabolites which proved to be consistent with the data from Whalley and Edwards (1995). However, the *Daldinia* strains that Stadler *et al.* have studied to date failed to produce cytochalasins in culture even though these compounds were present in substantial amounts in the stromata of *D. eschscholzii* (Ehrenb.:Fr.) Rehm. Stadler *et al.* (2001) agreed with Whalley and Edwards (1995) that the production of secondary metabolite in Xylariaceae is a constant and reliable feature which can be used in chemotaxonomy of the family.

1.4 Identification of Xylariaceae

Xylariaceous fungi have teleomorphic or sexual stages and anamorphic or asexual stages. Teleomorphic stages produce ascospores in the ascus and at the tip of ascus there is an apical ring which will be turned blue when stained with iodine. Spores of xylariaceae are phaseoliform single cells with smooth brown walls and full-length germ slits. Some strains of Xylariaceae such as *Camillia* spp. show distinctive ornamentation on their cell walls which can be used in their identification (Rogers, 1979). All Xylariaceous anamorphs usually have hyaline to light brown conidiophores, but the branching, the position of the conidiogenous cells and the arrangement of the conidiogenous loci within the conidiogenous cells form good differential characters (Petrini & Petrini, 1985).

The identification of xylariaceous endophytes on the basis of morphological characteristics alone is difficult because they seldom produce a teleomorph in culture. The confident identification of Xylariaceous endophytes is still problematic even after comparing them directly with cultures derived from known teleomorphs (Petrini & Petrini, 1985). A combination of morphological characters with biochemical analyses is one way to achieve successful identification of species (Rodrigues, Leuchtmann & Petrini, 1993). This however may work well for temperate species but for tropical ones there are still many which are as yet undescribed (Thienhirun & Whalley, 2004).

Secondary metabolites are structurally diverse compounds but in the Xylariaceae generally exhibiting taxonomic specificity. However secondary metabolite formation provides another possible feature of use in identification even though there is a major criticism that the same secondary metabolite can be produced by unrelated species. In some cases secondary metabolites are produced by very closely related species only and can indicate a close phylogenetic relationship. Studies on *Fusarium* and *Penicillium* species have proved the value of chemotaxonomy in characterizing fungal species and for their subsequent identification (Frisvad *et al*, 1998).

The Xylariaceae produce secondary metabolites in a constant and reliable manner even when they are grown on different culture media or under slightly varying condition (Figure 1.5). Only *Biscogniauxia atropunctata* (Schwein.:Fr.) Pouzar. produced different metabolites when growing on different culture media. Moreover, little variation was

detected between isolates of the same species or from different geographical regions or host substrates (Whalley & Edwards, 1995). Specific groups of secondary metabolites, especially dihydroisocoumarins, succinic acid derivatives, butyrolactones, cytochalasins, punctaporonins, naphthalenes, mitorubrins and griseofulvins have phylogenetic significance among *Hypoxylon*, *Biscogniauxia, Camillea, Ustulina, Xylaria* and *Rhopalostroma*. Other compounds are produced in small quantities in the stromata as found in the nature. These compounds are difficult to detect but they maybe very useful addition to secondary metabolite profiles of the colorful species.

Hypoxylon species produce dihydroisocoumarins with 5-methylmellein as a common metabolite. Mellein, iso-ochracein and ramulosin are characteristic for a limited number of species of *Hypoxylon*. *Biscogniauxia* was erected for applanate species of *Hypoxylon* sensu Miller (Miller, 1961) and for cup-shaped stromal *Nummularia* species (Pouzar, 1979) Metabolite production, especially the presence of 5-methyl mellein amongst members of species formerly in *Nummularia* confirmed the close relationship between members of *Biscogniauxia* regardless of their stromal characteristics (Whalley & Edwards, 1986; Whalley, 1996).

Beside the dihydroisocoumarins which are associated with *Hypoxylon* and *Biscogniauxia*, succinic acid and its derivatives are associated with *Xylaria* and its relatives. Therefore the presence of 2-hexylidene 3-methyl succinic acid in *Poronia pileiformis* (Berkeley) Fries. suggested that this fungus really belongs in *Xylaria* (Paden, 1978). Taxonomic significance of other chemical compounds such as cytochalasins is still not known but it may be a useful marker for identification at species level (Whalley & Edwards, 1986)

Stadler *et al.* (2001) used secondary metabolite profiles to differentiate *Daldinia* and its allies. They extracted from the stromata of *Daldinia* spp. and studied their chemical profiles by HPLC. They found that some metabolites such as BNT (1,1'-Binaphthalene-4,4'-5,5'-tetrol) or mitorubins might be useful in identifying xylariaceous fungi; *Daldinia* and *Hypoxylon* at species level but not at the generic level. Interestingly these compounds were not found in the other genera; *Biscogniauxia, Entoleuca, Kretzschmaria, Rosellinia*, and *Xylaria*.

However the characterization of xylariaceous fungi by secondary metabolite pattern depends on the amount of metabolites produced. There should be sufficient amounts to achieve taxonomic identification. It is very important to recognize that no one class of chemical compound alone can provide reliable, sufficient data for taxonomic evaluation. It is the overall picture of metabolite presence which appears to be important (Whalley & Edwards, 1995).

At the molecular level DNA techniques have been applied to characterize and identify xylariaceous fungi. This approach has been accelerated with the application of the polymerase chain reaction (PCR) which has made it much easier to sequence DNA

from small amounts of ascomycete material. The most commonly used gene in phylogenetic studies has been the encoding small subunit ribosomal RNA (SSU rRNA, 18S rRNA, 16S-like rRNA). Different kinds of RNA data can be used for reconstructing phylogenetic tree of the ascomycetes (Eriksson, 1995). Moreover the internal transcribed spacer region and intergenic spacer of the nuclear rRNA repeat unit evolve fastest and may vary among species within a genus or among population. The PCR method and direct sequencing offer several advantages over cloning and direct rRNA sequencing. Because PCR method utilizes relatively crude preparation of total DNA such as the ones from minipreps, only small amounts of DNA are required (about only 0.1 to 10 ng per amplification). It reduces error by sequencing both stands of gene, and this method is compatible with automated DNA sequencing instruments that utilize fluorescently labeled sequencing primers or dioxynucleotide triphosphates (White *et al.*, 1990).

A molecular approach has been used to identify serious pathogenic fungi of crayfish, *Aphanomyces astaci* Schikora (Oidtmann *et al.*, 2002) and also the pathogenic fungi in ocular infections (Ferrer *et al.*, 2001). This method proved to be rapid and reproducible that can be useful to identify and classify pathogenic fungi in the laboratory and in the field. Loppnau and Breuil (2003) combined PCR and restriction fragment length polymorphism technique to identify coniferous associated *Ceratocystic* fungi. The β-tubulin gene from over 200 species of this fungus was amplified and sequenced. The selected restriction enzymes were used to create the pattern which will be used to identify fungus. This is proved to be a time-saving method.

Studies on grass endophytes using the PCR techniques to detect and define the diversity of *Epichloë* by using specific primers (microsatellite-containing locus; MS primers) to amplify the tiny amount of fungal genomic DNA from infected plant materials have been undertaken. This method proved to be useful in planta detection for a variety of related species (Groppe *et al.*, 1995; Groppe & Boller, 1997).

Now the internal transcribed spacer (ITS) region is probably the most widely sequenced DNA region in fungi. It has been very useful for molecular systematic at species level. The two internal spacers, ITS-1 and ITS-2, are located between genes encoding the 5.8s, 18s and 28s nuclear ribosomal RNA subunits. The ITS-1 and ITS-2 spacers in addition to the 5.8s RNA are referred as ITS region (Slotta, 2000) (Figure 1.6). Several

factors make the ITS region a good target for molecular identification of fungi (Sanchez-Ballesteros *et al.*, 2000). Firstly, the ITS region is usually between 600-800 bp in length and can be amplified using universal primers (White *et al.*, 1990) which are specific for fungal rRNA genes. Secondly, because of the multicopy nature of rDNA repeat, ITS regions are easy to amplify from small, diluted or degraded DNA samples, and finally, the ITS regions are highly variable between fungal species.

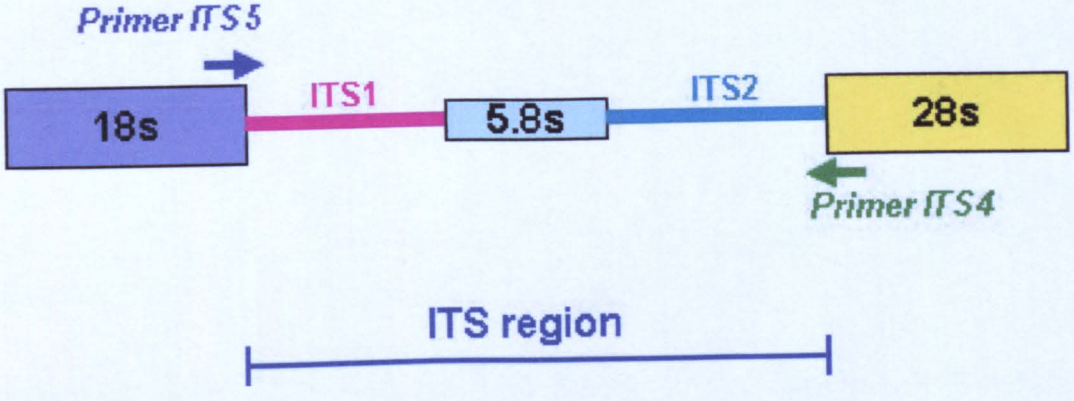


Figure1.6 ITS regions and specific primers

Larena *et al.* (1999) tested the specificity of special design primers, ITS4A, which will specifically amplify ascomycete ITS regions. They found that this primer is a useful tool in determining the affinities of many mitosporic fungi with their perfect states.

Guo *et al.* (2000) used ITS primers and the PCR method to identify 19 unknown endophytic species from Chinese fan palm *Livistona chinensis* (Jacq.) R.Br. ex Mart. and with this molecular based rRNA sequence method could place 9 morphospecies into genus level, another 5 morphospecies into family level and the remaining 5 morphospecies were inferred to their ordinal placement. The problem that occurred in this study was the limitation of DNA sequence data in GenBank and EMBL.

Daldinia species have been examined by Johannesson *et al* (2000) and Stadler *et al.* (2001). Johannesson *et al.* (2000) investigated 35 taxa of *Daldinia* from northern Europe and studied their diversity. They found that the diversity of *Daldinia* is wider than reflected in the literature. Stadler *et al.* (2001a, 2001b) studied *Daldinia* and other

xylariaceae from around the world. They used chemotaxonomy combined with molecular techniques using specific primers to discriminate all taxa at the species level. They also showed that restriction analysis of the amplified nuclear small subunit rDNA (ARDRA) was suited to distinguish species that showed an insertion into their SSU.rRNA, and microsatellite-PCR (MIS PCR) provided specific genetic fingerprints which allowed for recognition of varieties or sub-populations within a given morphological species.

Lee *et al.* (2000) examined the phylogenetic relationship of 18 species of *Xylaria* (22 strains). They found that they formed a monophyletic group and are phylogenetically homogenous except for *X. cubensis* which developed an independent lineage from other xylariales. The characteristics such as ascospores, perithecia and stromata, support the *Xylaria* grouping from molecular method, but there seems to be no specific character that can justify the phylogenetic results. They suggested that the evolution of these characters may happen many times within *Xylaria*.

Sanchez-Ballesteros *et al* (2000) presented preliminary DNA databases of Xylariaceae which can be used to identify unknown Xylariales. Their data were composed of DNA sequences of ITS1-5.8s-ITS2 region. Their results were consistent with the current concept of the genus *Hypoxylon* which separated its allied genera (*Biscogniauxia, Camillea, Whalleya, Creosphaeria, Nemania* and *Kretzchmaria*) from *Hypoxylon*. They also demonstrated the importance of anamorphs in fungal classification as evidenced by the correlation between the type of anamorph and the relative placement of the teleomorphs in the phylogenetic tree derived from sequence analysis.

Mazzaglia *et al.* (2001) used the ITS region on 5.8srDNA of *Hypoxylon* species to construct a phylogenetic tree. They found that the results from ITS sequencing were consistent with classical morphological methods. They suggested that ITS sequence analysis is an efficient and accurate method for discriminating *Hypoxylon* and related genera (*Biscogniauxia* and *Entoleuca*).

For xylariaceous endophytes the absence of a teleomorph makes the identification process difficult because of the need to rely on colony morphology and anamorphs. In many cases these are not sufficiently distinctive to separate closely related species or in the case of many tropical isolates there are no cultures obtained from known

teleomorphs for comparison. Ribosomal DNA subunit sequence analysis of the isolates and comparison with an appropriate database would help to identify unknown or problematic isolates. Most important for future application will be to enlarge the DNA analysis databases by adding more genera and increasing the number of studied isolates for each species (Mazzaglia *et al.*, 2001).

Platas *et al.* (2001) studied the tandem repeat in the ITS1 region of the Xylariales, and suggested that the repetitions could have occurred through slipped strand mispairing. The tandem repeat increases the divergence rate in the ITS1 of the Xylariaceae. Later they investigated the heterogeneity in ITS1 of *Xylaria hypoxylon* (L.) Grev. (Platas *et al.*, 2004). They found the repeated domain which suggested that it might be derived from the fusion of ITS1 with a fragment of the γ-glutamyltranspeptidase gene. From this evidence, they suggested that the rate of evolution of ITS1 can be independent from the rate of evolution of other genes. They also indicated that recombination with other nuclear genes could participate in the evolution of the ITS.

1.5 The subject : *Cassia fistula* L. (Golden Shower)

Cassia fistula L., known as Khoon (in Thai) or golden shower, is a deciduous tree, which is found all over Thailand, and it has been a Thai National plant as well. Normally it is often planted as an ornamental tree. Now it is also known as a useful medicinal plant. Native folks use all parts of this plant in native remedies. It is thought to be good for the kidney as those who use it remain free of kidney stones. For large doses, the leaves and barks can cause vomiting, nausea, abdominal pain and cramps. Leaves of *C. fistula.* are used for curing boils, coughs, skin disease, eczema, ringworm; relieving burning sensation while urinating, and sometimes pastes made from leaves can be used for rheumatism. Other parts of this plant are also used; roots for blood dysentary, pain in the ribs, snakebite and tumor; fruit for spleen disease and tonsils; seeds for constipation, typhoid and pediatric worm infections.

There are numerous reports about the other uses of this plant. Sircar *et al.* (1970) obtained gibberellic acid from the floral parts of *C. fistula*; 5 mg in the petals and 2 mg each in the filaments and anthers per kilogram fresh weight. Besides being a plant growth regulator producer, this plant was found to significantly reduce the total

cholesterol in hypercholesterolaemic male albino rats (El-Saadany *et al.*, 1991). Furthermore, it also reduced blood and liver total lipids.

C. fistula was also evaluated for the effects reported in Panamanian folk medicine for diabetic treatment (Esposito Avella *et al.*, 1991). They found that the aqueous fraction of *Cassia* leaves produced a significant decrease in glycaemia.

In term of antimicrobial effects, Perumal Samy *et al.* (1998) screened Indian medicinal plants including *Cassia* for antibacterial properties against *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris* and *Pseudominas aeruginosa* and this plant exhibited significant antibacterial activity.

Bhakta *et al.* (1999) evaluated hepatoprotective activity of *Cassia* leaf extracts. They found that the extract had shown to process a great protective effect by lowering the serum levels of transminases (SGOT, SGPT).

Wijaya *et al* (2000) investigated seed of *C. fistula* and found a novel trypsin inhibitor. This *C. fistula* L. trypsin inhibitor is the first known example of a plant defensin with protease inhibitory activity. They suggested that a possible role of this substance against herbivores and fungal pathogens.

Antioxidant activity was also detected in *C. fistula* L (Siddhuraju *et al.*, 2002). But among the various plant fraction extractions, the aqueous alcoholic extracts from stem, bark and leaves, show significant antioxidant activity. This isolation would help to ascertain the potency of these compounds which could be used further in the food and pharmaceutical industries.

Gupta *et al.* (2000) reported the effect of a methanolic extract (ME) of *C. fistula* seeds on the growth of Ehrlich ascite carcinoma (EAC). They found that ME treatment increased life span and decreased tumour volume and viable tumour cell count. Moreover, there was an improvement in the haematologist parameter following ME treatment. They therefore suggested that ME of *C. fistula* seed has an antitumour activity.

Although this *Cassia* plant has very interesting properties there are no reports on fungal endophytes of this plant. The endophytes of this plant may be significant in some of these properties outlined above.

1.6 Aims and Objectives.

1. To isolate endophytic fungi from *C. fistula* trees.
2. To compare endophytic assemblages from Cassia trees from three different geographical sites
3. To develop a PCR based protocol for comparison of ITS regions of endophytic fungi to establish taxonomic relationships and to enable identifications to be made.
4. To attempt to initiate teleomorphic development in selected Xylariaceae
5. To investigate secondary metabolites from selected endophytic Xylariaceae for comparison of metabolite profiles for taxonomic and identification purposes
6. To screen metabolites from selected endophytic isolates for their antimicrobial activity.

CHAPTER 2

Materials and Methods

2.1. Sampling

Healthy *Cassia fistula* L. leaves were collected from three different sites in Thailand, the Plant Genetic Conservation Project at Kanchanaburi Province, from Nakhon Ratchasima Province and finally from Bangkok Province (Figure 2.1-2.3). The leaves sampled from Kanchanaburi Province were tested first and the samples were made during the winter season. The samples from Nakhon Ratchasima Province and Bangkok Province were made during the rainy season. In the following year fresh samples were obtained from Nakhon Ratchasima Province again during the winter season. Five *Cassia* trees were randomly selected at each site and five leaves were collected from each tree. The leaves were placed in sterile plastic bags and stored at 4°C until processed. All specimens were processed within one week of sampling.

Each leaf sample was cut into 5 pieces measuring 1x1 cm^2 before the surface sterilisation process and each piece was then divided into 5 equal pieces measuring 1 x 0.2 cm^2 before placing them onto the surface of the culture media.

2.2. Media

Malt Extract agar (MA) (Oxoid) was selected as the primary isolation medium since this has been a preferred choice for many previous investigations of endophytes. For identification purposes Potato dextrose agar (PDA) (Oxoid) and Oatmeal agar (OA) were also used. In the teleomorph induction method, MA was the medium of choice. The antibiotics, streptomycin and tetracycline were used in the isolation procedure to counter bacterial growth.

2.3. Fungal isolation and culture method

Leaf samples were surface sterilised according to the method described by Petrini (1986) and to make sure that there was no epiphytic contamination following the

process leaf surface impressions on agar followed by incubation were made as recommended by Schultz *et al.* (1993). Leaf pieces were submerged in 96% ethyl alcohol for 1 minute and were transferred to sodium hypochlorite solution containing 5% available chlorine and (1 drop per 15 ml of) Tween 80 for 5 minutes. After that, they were then transferred to 96% ethyl alcohol for 30 seconds. They were finally washed in sterile distilled water and the sterile leaf pieces were then surface dried with sterile filter paper and immediately placed onto the surface of 2% Malt Extract agar plates containing antibiotics; 25µl/ml tetracycline and 50µg/ml streptomycin, to prevent bacterial contamination. The plates were then incubated at room temperature 21ºC – 25ºC for up to 1 month and examined daily for growth of fungi from the leaf samples. As necessary endophytic fungi were transferred to fresh Malt Extract agar, Potato Dextrose agar, and Oatmeal agar plates, incubated as before to ensure pure cultures and to encourage sporulation.

2.4. Identification of organisms

- ***Microscopic features***

The microscopical features were carried out with bright field microscopy and Differential Interference Contrast (DIC) light microscopy with an Olympus BH2 research microscope (Olympus BH2-RFCA, Japan). The specimens were mounted with lactophenol cotton blue and were observed using x10, x40 and x60 dry objectives for examination of spores, sporing structures and any other characteristics that could be used for their identification.

All photographic illustrations were made using a Nikon Coolpix 4500 digital camera.

The fungal cultures which appeared to belong to the Xylariaceae on the basis of their colony characteristics were separated from the others for further study including attempted teleomorph induction (Mekkamol, 1997)

- ***Slide culture method***

This method was used to aid identification of fungi from the *Cassia* leaves. The way in which spores are attached to their conidiophores is of great importance for their

identification and this method reduces damage to the material and maintains the attachment of the conidia and the spatial arrangement of mycelial material.

First of all, cover slides were attached to petri dishes with a drop of molten agar. After that 2% Malt Extract agar was poured into the plate and left to set. The set Malt Extract Agar was then cut in the shape of cover slips in the area where they were located to form a well. Each species of endophyte was inoculated onto the agar well and incubated at 25°C for at least one month or until its spores were formed. The slides were then removed, mounted on a slide with a drop of lactophenol cotton blue stain and observed by light microscopy.

- ***Macroscopic features***

Characters such as colour, colony and shape were recorded using an Olympus stereomicroscope (Olympus SZ60, Japan) fitted with a Nikon Coolpix 4500 digital camera. Mycelial structure and conidiospores were critical point dried (Polaron Critical Point Dryer) and examined using a scanning electron microscope (JEOL-SEM 840) following coating with gold applied by a Polaron diode sputter coater.

- ***Teleomorph induction method***

One possible way to identify endophytic fungi likely to be xylariaceous, based on colony form, is through the comparison of the unknown with colonies of known species. This certainly is accurate to generic level but has too many limitations at species level for reliable identification. The technique developed by Mekkamol (1997) to investigate *Xylaria* and related species was used in this study. Pure cultures of the unknown species were inoculated onto selected twigs and then incubated in sterile sand bags or jars in attempts to induce them to form their teleomorphs. This can usually allow identification to species level in many cases where the teleomorph is produced (Mekkamol, 1997). Some of the cultures in this study were initially identified as *Xylaria* species on the basis of their colony form and immature stromata which were sometimes covered with their anamorphs. Although the *Xylaria*-type characteristics developed in culture they never developed maturity but a few produced anamorphic structures. However, in the absence of the mature teleomorph, it is rarely possible to identify these isolates to species and for this reason teleomorph induction was attempted (Figure 2.4)

1. The selected isolates were inoculated onto 2% Malt Extract Agar in glass jars and incubated for 10 days at room temperature (about 25 °C).
2. Twigs* of about 3 cm in diameter were freshly cut into about 8 cm long pieces and then put in autoclavable bags (Fisher). They were then autoclaved at 121°C (15 lb/inch^2) for 1 hour. One piece of sterilised twig was put vertically on the surface of grown culture in the jar and the cultures were then incubated for a further month.
3. The well-colonised twigs were then transferred from the jar to sterilised moist sandbags where the depth of sand was about 4 cm. The bags were tied with tape or rubber bands and then kept at room temperature for one or two months. Stromata of each fungus were expected to develop at this stage.
4. The twigs with young stromata were transferred to moist sand pots and watered daily. The infected twigs without any noticeable stromata were incubated further in the moist sand bag for one more month before the bag were cut horizontally at which stage they were incubated further with water application once or twice a week as necessary.

* Twigs used in this study were from Ash trees.
Fraxinus excelsior. (white ash)

2.5. Molecular study of Endophytic fungi

All isolates that were categorised as members of the Xylariaceae and *Phomopsis* and were studied further using molecular techniques; Polymerase Chain Reaction and DNA sequencing.

2.5.1. *DNA preparation*

1. All fungal isolates were inoculated into 100 ml Malt Extract Broth (MEB) (Oxoid) and incubated at 30°C for 7 days.

2. Mycelium was harvested and put into 1.5 ml microcentrifuge tube to fill about one third of each tube.

3. 500 microlitres (µl) of 1M Tris (pH 7.2) (Sigma) was added into each microcentrifuge tube.

4. The tube was then centrifuged for 5 minutes and the supernatant was removed. The eppendorf was kept at -20°C until used.

2.5.2. *DNA extraction protocol* (modified from Lee & Taylor, 1990)

1. The frozen mycelium was ground by micropestle and 500 µl of fresh lysis buffer (50mM Tris-HCl (pH 7.2), 50mM EDTA, 3% SDS and 1% 2-mercaptoethanol) was added into the tube. The microcentrifuge tube was then incubated in the 65ºC water bath for 1 hour.

2. 500 µl of phenol-chloroform-amyl alcohol was added and the tube was then centrifuged (Sanyo Micro Centaur) at 13000g for 25 minutes or until the aqueous phase was clear.

3. 400 µl of supernatant was removed and transferred to a new clean microcentrifuge tube, being careful not to transfer the debris at the interface.

4. 40 µl of 3M sodium acetate (pH 8.0) (NaOAC) and 440 µl of cold Isopropanol (Sigma) were added to the tube containing the supernatant. The tube was then gently inverted several times to mix the contents and placed in a deep freeze at -20ºC for at least 3 hours to aid DNA precipitation.

5. The tube was then centrifuged at 13000g for 30 minutes at 4ºC. Supernatant was rinsed off and then 480 µl of cold 70% Ethanol was added. The tube was centrifuged again at 13000g for 5 minutes at 4ºC and the ethanol rinsed off.

6. The pellet was left to dry on a heat block (about 50-55 ºC) (Techne DRI-BLOCK® DB-2P) for 5-10 minutes and then was re-suspended in 100 µl of sterile distilled water.

7. The DNA suspension was stored at -20ºC until used.

8. To check the presence of DNA, gel electrophoresis of chromosomal DNA product (cDNA) was performed by using 1% agarose (Molecular Grade, Bioline) which contained 5µg/ml ethidium bromide (EtBr) (Sigma). The gel was run in 1X Tris-Acetate buffer (0.04M Tris-Acetate, 0.001M EDTA) at 90V for 1 hour. A fifty base-pair (bp) DNA ladder (Perfet DNA™ 50 bp ladder, Novagen) was used as a marker in the gel. The illuminated bands of extracted DNA product were detected using an UV illuminator (320 nm, UVP,inc.).

9. RNAse treatment: DNA samples were treated with Ribonuclease A (Novagen) prior to PCR protocol to digest RNA that was present in the solution. 1 µl of Ribonuclease A was put into each tube and the tubes were incubated in a 37ºC water

bath for 30 minutes. The tube was then immediately put into an ice bucket and kept at -20 ºC until used.

2.5.3. *Assessment of DNA concentration and qualification* (Maniatis *et al.*, 1982).

The concentration and purity of DNA samples were obtained using a spectrophotometer (Lambda 40, Perkin Elmer), measuring the optical density at 260 nm/280 nm. A dilution of 1:150 was initially made up, by diluting stock extracted DNA with distilled water (10 µl stock cDNA + 1490 µl distilled water). The purity of DNA was calculated by the OD260 nm/OD280 nm ratio; the proportion value should be between 1.8-2.0. If the value is below 1.8, the phenol:chloroform extraction would be re-performed.

OD260 nm was used to calculate the DNA concentration using the following formula:

DNA concentration (µg/ml) = OD260nm x 50 x dilution factor

E.g. If OD260nm = 0.025 at the dilution 1:150

So DNA concentration = 0.025 x 50 x 150 = 187.5 µg/ml = 187.5 ng/µl

Note: Spectrophotometer conversions: Double-stranded DNA (dsDNA); $A^{260} = OD^{260} = 1$ for a 50 µg/ml solution (Maniatis *et al.*, 1982)

Following this the DNA suspension was diluted to 100 ng/µl for the Polymerase Chain Reaction procedure.

2.5.4. *Polymerase Chain Reaction (PCR) of Endophytic fungi DNA*

The Polymerase Chain Reaction (PCR) is the method used to produce large number of copies of a gene. It is important to have enough templates for sequencing. There are 3 major steps in PCR protocol which were repeated, in this study, 35 times in an automated cycler. This cycler would heat and cool the reaction mixed tube continuously. The cycling reactions are described as follows (Figure 2.5) (Principle of

the PCR, Web resource, http://allserv.rug.ac.be/~avierstr/principles/pcr.html; modification of Lee & Taylor, 1990).

1. **Denaturation**: The denaturation process was started at 95ºC. At this temperature, the double stranded DNA melted to be single stranded (ss) DNA and all the enzymatic reaction stopped.

2. **Annealing**: This process was done at 52ºC. At this stage, the primers were moving around by Brownian movement. The hydrogen bonds were continuously formed between ssDNA and ss primers. When primer fits perfectly to ssDNA, polymerase enzyme consequently attached and started copying the templates and this resulted in stronger hydrogen bond.

3. **Extension**: This process was performed at 72ºC. This temperature is ideal for polymerase enzyme to work after the primer and DNA have attached to each other and the extension with dNTPs occurred at this stage. The bases which are complementary to the template were attached to the primer on the 3'side. The polymerase added dNTP from 5' tp 3', while reading the template from 3' to 5' side, bases were added complementary to the template.

PCR of selected species of endophytes was carried using 2 universal primers according to White *et al.* (1990); ITS5 (forward primer) and ITS4 (reverse primer) (Life Technologies).

Primers	GenePrimer	Product size (bp)	Tm (ºC)
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	315	63
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	N/A	58

Each PCR tube contained 50 µl of PCR reaction mix consisting of 25 µl ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma), 5 µl DNA, 1 µl ITS4, 1 µl ITS5 and 18 µl H_2O. The tube was mixed gently and placed in the PCR machine (GeneAmp PCR system 9600, Perkin Elmer Cetus). The PCR cycle was adapted from the method used by Mazzaglia *et al.* (2001) as follows; initial denaturation at 95ºC for 2 minutes 30 seconds, followed by 35 cycles consisting of denaturation at 95 ºC for 30 seconds,

annealing at 52 ºC for 30 seconds and extension at 72 ºC for 1 minute, and finished by a final extension at 72 ºC for 5 minutes. The PCR products were then detected by gel electrophoresis using the same conditions as for the DNA extraction protocol but the concentration of agarose was increased to 2%.

2.5.5. *Purification of PCR products*

PCR products were purified using QIAquick® PCR purification kit (QIAGEN) according to manufactures instructions as follows (QIAquick spin handbook, 2002).

1. 250 ul of PB Buffer was added to 45 microlotres of the PCR samples and mixed carefully.
2. QIAquick spin column was placed in a provided 2 ml collection tube.
3. The sample was applied to QIAquick column for DNA binding and centrifuged at 13,000 rpm for 60 seconds.
4. The flow-through was discarded. The QIAquick column was placed back into the same tube.
5. To wash DNA, 0.75 ml of PE buffer was added to the QIAquick column and the tube was centrifuged at 13,000 rpm for 60 seconds.
6. The flow-through was discarded and the QIAquick column was then placed back in the same tube. The column was centrifuged for an additional 1 minute.
7. The QIAquick column was transferred to a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, fifty microlitres of EB buffer (10mM Tris-HCl (pH 8.5)) or H_2O was added to the centre of the QIAquick membrane. The column was centrifuged for 1 minute.
9. The column was discarded. The microcentrifuge tube was kept at -20 ºC until being sequenced.
10. The presence of Purified PCR product was investigated by gel electrophoresis using 2% agarose which contained 5µg/ml ethidium bromide. The gel was run using 1X tris-acetate buffer (0.04M tris-acetate, 0.001M EDTA) at 90V for 1 hour. Fifty base-pair (bp) DNA ladder (Perfet DNA™ 50 bp ladder, Novagen) was used as a marker in the gel. The illuminated bands of DNA product were detected using an UV illuminator (320 nm, UVP,inc.).

The isolates were inoculated into Malt Extract Broth (MEB) and incubated at room temperature (20-25 °C) for 6 weeks as static cultures. The metabolites from each isolate were then separated from the mycelium by filtration through 4 layers of muslin gauze. The mycelium and culture medium were both retained and stored frozen as separate entities until the test was being taken.

2.7.1 Preparation of media

The medium used in this study was Mueller Hinton Agar (MHA) (Oxoid) for testing bacteria and 2% Malt Extract Agar (MA) was used for testing fungi.

2.7.2 Preparation of inoculum

One loopful of bacteria and the yeast were inoculated into 5 ml sterile normal saline solution (NSS) (Oxoid) and incubated at 37°C in a water bath for 2-3 hours. The turbidity of bacterial and yeast suspensions was adjusted with NSS to match the turbidity of 0.5 McFarland (OD 0.1 at 625 nm).

Fungi for testing were grown on MA slopes for 2 weeks or until spores were produced. Five mls of NSS containing 0.05% Tween 80 were poured into each tube and the spore suspension obtained by gently removing the spores from the surface growth. The final inoculum was adjusted with NSS containing 0.05% Tween 80 to obtain a turbidity of McFarland no 0.5 (OD 0.1 at 625 nm).

2.7.3. Preparation of sample discs and antibiotic discs

Forty to fifty mls of each broth sample were placed in a freeze dryer (Super Modulyo, Edwards) and dried. It was then re-suspended in 95% Ethyl Alcohol. After that the fungal solution was filter sterilized (Single use syringe filter pore size 0.20 µm, Sartorius) and kept at -20ºC until used.

For mycelial samples, the frozen material was ground and soaked in sterile Phosphate Buffer Saline (PBS) (Sigma) overnight and the sample

was then filtered to obtain only the liquid extract. The broth was then filter sterilized (Single use syringe filter pore size 0.20 µm, Sartorius) and maintained at -20ºC until used.

Six mm diameter filter papers (Whatman No17) were sterilized at 121°C for 15 min and 50 µl of each fungal extract was put on to each disc and left to dry in a 30°C incubator. The same method was used to prepare the control antibiotic discs (0.167 g/l ampicillin (Sigma) for testing the bacteria and 1000 U nystatin (Merck) for testing the fungi.

2.7.4. Disc diffusion method (Figure 2.6.1)

Mueller Hinton Agar (MHA) and 2% Malt Extract Agar (MA) were prepared as described in Appendix I and left to set. They were then seeded with the test organisms according to Kirby-Bauer disk diffusion method. After that, 2 sample discs, 1 positive control (selected antibiotics; ampicillin disc for bacteria and nystatin disc for fungi) and 1 negative control (sterilised distilled water disc) were placed onto the surface of the medium and incubated at 30°C for 24 hrs for bacteria and yeasts and 48 hrs for fungi. Inoculated plates were observed for the presence of an inhibition zone surrounding the discs (Figure 2.6.2). If the inhibition zone appeared it was reported as a positive result. The tests were carried out in duplicate.

The following microorganisms were used: Gram-positive bacteria: *Bacillus cereus* and *Staphylococcus aureus*; Gram-negative bacteria: *Escherichia coli* and *Pseudomonas fluorescens*; yeast: *Saccharomyces cerevisiae* Hansen and *Candida albicans* (Robin) Berkhout; filamentous fungi: *Penicillium digitatum* Saccardo and *Aspergillus niger* van Tieghem.

2.8. Secondary metabolite as a herbicidal agent

Cress seeds were grown in moist chamber for 3 days until their shoots emerged. Five cress seedlings were placed onto each filter paper and then 500 µl of metabolite, distilled water, bleach and ethanol were put onto the filter papers with the seedlings. Cotton balls, moistened with water, were put into each plate containing the seedlings to maintain a high humidity in the atmosphere. The plates were then incubated at room temperature for 24 hrs. The seedlings were observed for any inhibition of their growth. If the seedlings were brown, distorted, discoloured or stopped growing when compared to seedlings in distilled water, this was interpreted as inhibition from metabolites.

Figure 2.3 Sampling sites

Sai Yok District, Kanchanaburi Province

Non Soong District, Nakhon Rachasima Province

Figure 2.3 Sampling sites (continued)

Bangkapi District Ratchathewi District

Bangkok Province

Figure 2.4 Procedure of induction of teleomorph of Xylariaceous fungi

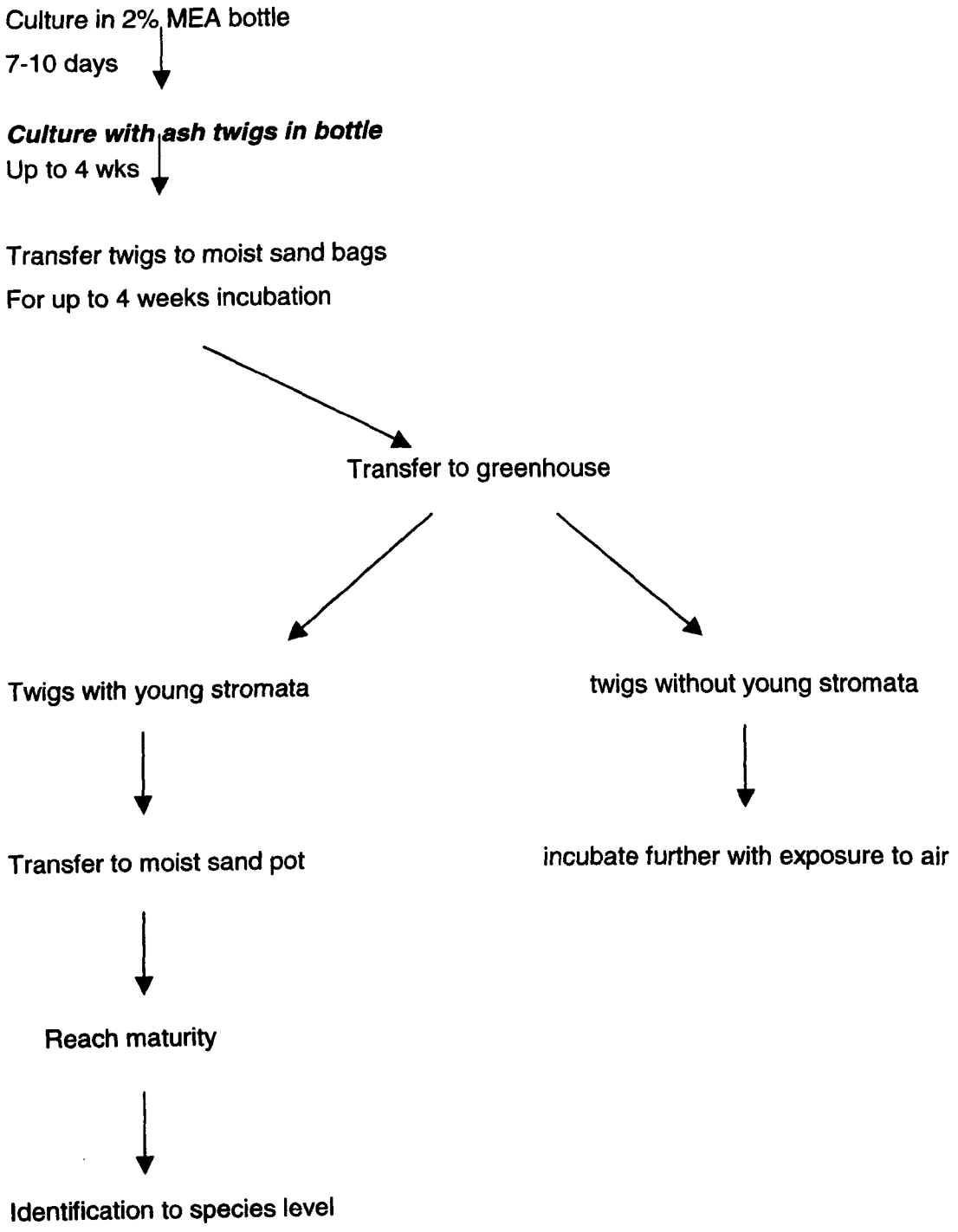
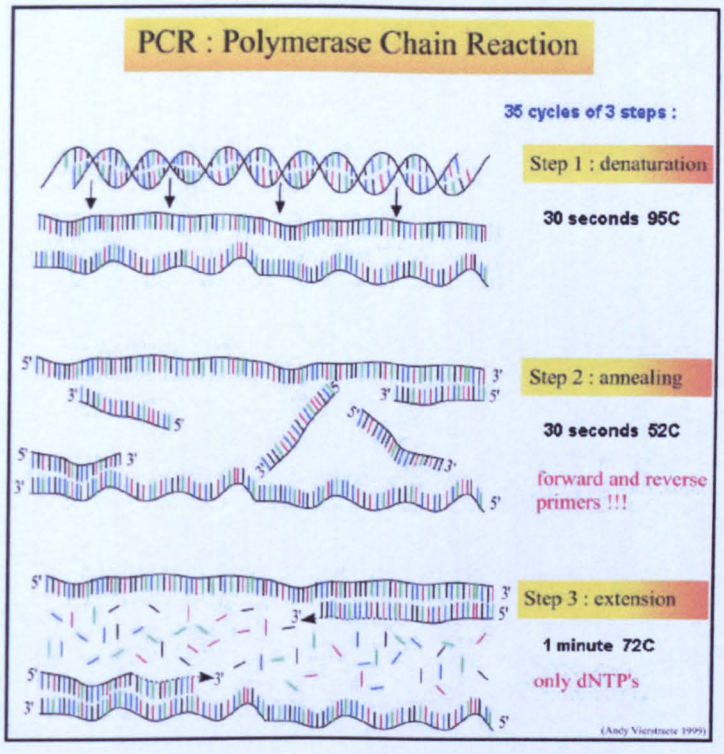


Figure 2.5 Principles of the PCR reaction



(modified from http://allserv.rug.ac.be/~avierstr/principles/pcr.html)

CHAPTER 3

Diversity of endophytic fungi from *Cassia fistula*

3.1 General geography and vegetation of Thailand

Thailand, situated in South East Asia, covers an area of 517,000 km^2, and is roughly about the size of France. Thailand shares borders with Myanmar in the north and the west, with Laos in the north and the east, with Cambodia in the east and with Malaysia in the south. It has long coastlines in the south along the Indian Ocean and in the east bordering the Gulf of Thailand.

Thailand consists of four distinct geographic regions. The northern part of Thailand is mountainous with the highest point Doi Inthanon reaching 2576 m above sea level and has forests and fertile valleys. The northeastern region consists of a high, semi-arid plateau used for cattle grazing and for growing crops such as maize. It has been subject to bad droughts in the past, and is economically the poorest region of Thailand. The central part of the country is mainly flat and fertile and surrounds the Chao Phraya river. This region is also the main rice-growing area of Thailand. The southern region is hilly and mountainous and was once covered with rainforests. This area has the highest annual rainfall due to the influence of monsoons.

3.1.1 Vegetation

The first national survey of Thailand's forest resources was undertaken in 1951 and this indicated that 53% of the entire country was covered with forest. However, the figure was reduced to 30% by 1972 and this was further reduced down to 26% by 1993. In summary, Thailand had lost almost 50% of natural forest in 32 years. (Charuppat, 1994)

The severe loss of Thai natural forest prompted the Thai government to launch the National Conservation Plan in 1962. In 1965 the first forest which was chosen for conservation was Khao Yai National Park, a mainly virgin evergreen forest covering an area of 2,100 km^2. This park is now recognized as a world heritage site of international importance. In 1991 the Royal Forest Department had estimated that the national conserved area covered 113,080 km^2 which represented approximately

12% of the total area of Thailand. Moreover, through the re-forestation programme, it was proposed to raise the forested area to about 36% of the total land mass.

The Royal Forest Department had carried out the first survey of the forest vegetation of Thailand in 1936 and, as a result, forest types were grouped and divided into seven floristic regions. There were the northern, northeastern, eastern, central, southeastern, southwestern, and southern, or peninsular regions. This publication provided the very first detailed account of vegetation characteristics of each region of Thailand (Smitinand, 1997). The Thai forest vegetation was classified as :

1. **Evergreen forest**: Approximately 30% of Thailand is covered by evergreen forest which is characterized by the distinguishing feature of no leaf shedding period; thus the trees stay green all year round. The climax forest is uniform in general appearance and major characteristics. Rainfall is heavy with annual precipitation rates ranging from approximately 800 to 1,500 mm in the northeast, 1,500-2,000 mm in the north and 2,000 to 4,500 mm in the south. Six evergreen forest types are recognized on the basis of species composition, altitude and geographical environment. This includes hill evergreen forest, pine forest, mangrove forest, and peat-swamp forest.

2. **Deciduous forest**: 55% of total forest in Thailand is covered with different types of deciduous forest. This type of forest is characterized by its species shedding their leaves in the dry season of January to May with new leaf growth starting at the onset of the following rainy season. Their distribution can be either entire or scattered within the ecosystem. This kind of forest has an open canopy with their understorey thinly developed and with sparsely developed ground cover. The forest is strongly influenced by geographical factors and rainfall. The subdivisions of deciduous trees are dry dipterocarp forest, mixed deciduous forest, bamboo forest and savanna forest.

All three study sites were situated in deciduous forest or where deciduous forest had once occurred.

3.1.2 Climate

The climate in Thailand is influenced by the monsoons from the Pacific and Indian oceans. It is also modified by regional topography, especially the Himalayan ridges

in the centre of the country. The general climatic pattern of the country depends on seasonal winds which blow from China during the winter and from the Pacific and Indian oceans during the remaining part of the year. There are three seasons in Thailand as follows (Thienhirun, 1997).

1. **Winter season**: This lasts from mid-November to the end of January. The weather is cool and mainly dry; the coolest period starts from December to mid January varying from 8-16ºC. The regular occurrence of a cold front, which reaches Thailand from Mongolia via central China plays an important role especially in the northern part of Thailand at the top of Doi Inthanon, Chiang Mai province, where the minimum temperature ranges from 1-2 ºC, and in the northeastern part of Thailand, at Phuluang Wildlife Sanctuary, where the minimum temperature ranges from -1 to 4 ºC.

2. **Dry season**: This normally lasts from March to June when the weather is mainly dry and hot. The hottest period is in May with the average temperature varying from 26-36 ºC. Monsoon and variable winds are usually not involved in this season. The hottest area is the Khorat plateau in Nakhon Ratchasima province which is situated in the north eastern part of Thailand where the maximum temperature ranges from 35-45 ºC.

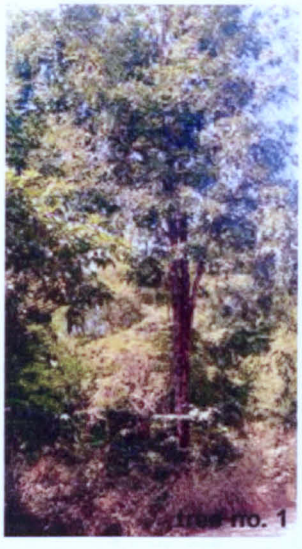
3. **Rainy season**: This lasts from June to November when the weather is humid and the temperature is rather stable around 17-25 ºC. The average rainfall is approximately 1,500-2,000 mm per year. Peninsular Thailand, in the south, is situated between the Andaman Sea of the Indian Ocean and the South China Sea of the Pacific Ocean, experiences the highest annual rainfall. From May to October, the climate is affected by the southwest monsoon from the Andaman Sea. Ranong province, which is situated between these two oceans, receives the direct effect from the monsoon and also experiences the maximum rainfall recorded in Thailand, averaging 4,500 mm per year. High mountainous ranges such as Doi Inthanon in the north, Phuluang and Phu Kradueng in the northeast and Khao Tanawsri in the west can obstruct and change the wind direction and precipitation levels. This also has a strong effect on species and vegetation types of the whole country.

3.2 Site details

Site 1

Kanchanaburi province is the third largest of 76 provinces and is situated in the central part of Thailand. It is about 129 kms west of Bangkok. It covers an area of 19480 km^2. This province is characterized by mountains and fertile river valleys (The Kwae Yai and Kwae Noi). The west of Kanchanaburi is the border of Thailand and Myanmar. Sai Yok, one of the 13 districts of Kanchanaburi, is located about 50 kms from the provincial town. Its landscape is generally rich in forests, mountains, and waterfalls. The sampling site is in the deciduous forest.

Figure 3.2 The Kanchanaburi sampling site showing individual localities



Site 2

Nakhon Ratchasima, commonly known as Khorat, is the large province on the northeastern plateau. It is the gateway to the northeastern part of Thailand (E-San). It is about 259 kms from Bangkok and occupies about 20,494 km^2. There are 32 Amphoes (districts) in Nakhon Ratchasima. The sampling site at Nakhon Ratchasima was in a small village Ban Rai which is in Amphoe Non Sung and the distance between Amphoe Muang to Amphoe Non Sung (the sampling site) is 37 kms. The *Cassia* trees at this amphoe were of the original natural vegetation.

Figure 3.4 The Nakhon Ratchasima sampling site

Site 3

Bangkok, the last sampling site, is the capital city of Thailand. It is situated in the central part of Thailand. It is located on the flat and fertile region resulting from the Chao Phraya river. The sampling sites for Bangkok were at Wang Thonglang district and Ratchathewi district. There were only 3 trees included for this site because of difficulties in locating fully matured *Cassia* trees in Bangkok since in this province, people simply plant *Cassia* as ornamental trees.

3.3 Results

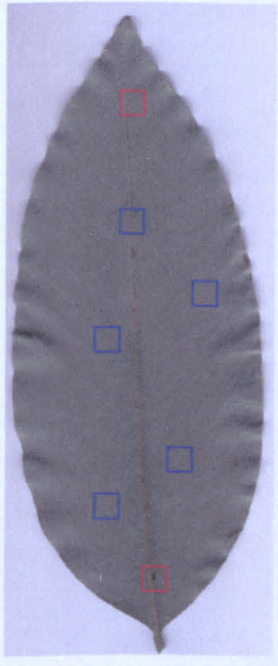
3.3.1 Endophytic fungi from *Cassia* trees

The three sites selected were sampled and endophytic fungal endophytes recovered from the *Cassia* trees (Figure 3.6).

In December 2000, five *Cassia* trees from the Plant Genetic Conservation Project area, Sai Yok District, Kanchanaburi Province (Figure 3.3) were randomly chosen. Five leaves from each tree were picked, wrapped in tissue paper and stored at 4°C in a refrigerator before further processing at the Mycology Laboratory at Liverpool John Moores University, UK.

Leaves were washed with 95% ethanol and sodium hypochlorite according to the surface sterilization method of Petrini (1986) They were then cut in the selected areas into 5 pieces measuring 1x1 cm^2 (Figure 3.7) using a sharp blade and each piece was then cut into 5 smaller pieces measuring 1x 0.2 cm^2.

Figure 3.7 Diagram of sampling pattern on each leaf



Note : ☐ Blue box : usual pattern for tissue selection

☐ Red box : In some samples isolates were obtained from tip and basal regions of the leaf

These were then placed on the surface of 2% MEA containing streptomycin and tetracycline. These were examined daily for up to 1 month following incubation at room temperature (around 20°C - 25°C), and endophytes growing from the tissue were recorded and subcultured for further study. Isolates which could be recognised as species of *Xylaria*, *Daldinia,* and *Phomopsis* were separated for teleomorph induction (Mekkamol, 1997) in ash twigs and for continuing study. The rest of the isolates were maintained on malt agar slopes at 4°C and also in skim milk at -20°C. This formed the protocol used for all future sampling and isolation. Overall numbers of isolate from the three sites are shown in Table 3.1.

3.3.2 Kanchanaburi (Site 1)

A total of 465 fungal isolates were obtained from the Kanchanaburi site and for each tree the number of isolates ranged from 86-97 isolates (Table 3.1). The most common endophytes from this area were *Phomopsis* and *Penicillium* species which were found in high numbers (Table 3.2). The other endophytes were *Xylaria* spp,

Daldinia spp, *Hypoxylon* spp, and *Colletotrichum* spp. The other isolates remained sterile; in spite of prolonged incubation and exposure to black light, no sporulation was induced.

All *Cassia* trees from this site are located within or near the main forest which has a relatively high tree density. The number of isolates obtained from the 5 trees sampled did not differ significantly from one tree to the next and ranged in numbers from 86-97. The isolates were then grouped as 'morphotypes' and, from these, representatives were selected. Thirty-seven isolates were subsequently chosen for further study. The selection was based on macroscopical characteristics of cultures grown on malt agar with *Xylaria*, *Daldinia*, and *Phomopsis* being the most frequently recognized taxa (Table 3.3, Figure 3.8).

Table 3.1 Isolates obtained from the 3 sampling sites

Sampling sites	**Tree No.**					*Total*
	1	*2*	*3*	*4*	*5*	
Kanchanaburi (4^{th} December 2000)	**97**	**86**	**95**	**90**	**97**	**465**
Nakhon Ratchasima (29^{th} July 2001)	**11**	**41**	**22**	**23**	**30**	**127**
Bangkok * (30^{th} July 2001)	**25**	**17**	**15**			**57**
Nakhon Ratchasima (1^{st} December 2001)	**55**	**67**	**48**	**82**	**55**	**307**

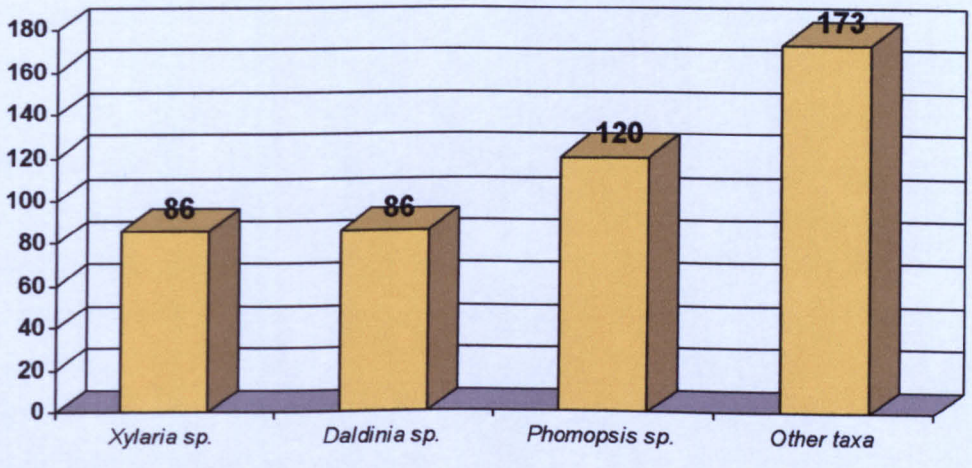
Note: * Three trees only sampled at this site

Table 3.2 Isolates from Kanchanaburi (December 2000)

Tree No.	*Fungal Taxa*				*Total*
	Xylaria sp.	*Daldinia sp.*	*Phomopsis sp.*	*Other taxa	
1	8	5	26	58	97
2	17	26	19	24	86
3	21	14	28	32	95
4	23	16	27	24	90
5	17	25	20	35	97

*Other taxa : Other species beside *Xylaria* sp., *Daldinia* sp. and *Phomopsis* sp. They were such as *Penicillium* sp., *Fusarium* sp., *Nigrospora* sp. *etc.*

Figure 3.8 Total recorded types and numbers of the isolated species from *C. fistula* L. from the Kanchanaburi site.



3.3.3 Nakhon Ratchasima (Site 2) and Bangkok (Site 3).

In late July 2001, a study was carried out after 2-3 weeks of the start of the rainy season in another 2 different sites, Nakhon Ratchasima Province (Figure 3.3) and Bangkok Province (Figure 3.5), to determine their endophyte populations. The samples were processed using the same method as stated earlier.

The endophytic population at the Nakhon Ratchasima site consisted of 127 isolates and for the Bangkok site 57 isolates were obtained (Table 3.1). The common endophytes were generally similar for both sites, but it was noted that *Daldinia* spp. were more prominent at the Nakhon Ratchasima site (Table 3.3). This fungus occurred in many of the leaf segments although *Phomopsis* and *Penicillium* species were also frequent (Table 3.3). In this sample one isolate was later identified as a *Fusarium* sp. on the basis of its form, colour and in the production of soluble red pigment in the medium. This was separated for additional study of its metabolite. There were a total of 52 isolates chosen for further investigations. Selection was based on the same characteristics as stated earlier (3.2.1).

During the winter season (December 2001) more samples were taken from Ban Rai, Non Sung district, Nakhon Ratchasima in order to compare the number of endophytes obtained during the different seasons. This sampling resulted in a total of 307 isolates with *Xylaria*, *Daldinia* and *Phomopsis* species being dominant. Sixty-four isolates were selected for further study. *Fusarium* and *Nodulisporium* species were also obtained at this sampling time with the *Nodulisporium* likely to represent the anamorphs of xylariaceous fungi.

Figure 3.9 compares the endophytic populations from the different sampling sites and demonstrates that Kanchanaburi is the site from which most endophytes were isolated with Bangkok being the site from which the least isolates were obtained. This would almost certainly be the result of differences in the diversity of the plant species present at each of the sampling site. Thus trees which are at a site surrounded by natural forest tend to harbour more microorganisms than trees at the stand-alone sites (Arnold & Herre, 2003). *Phomopsis* spp. proved to be one of the most common endophytes. This may be because *Phomopsis* spp. produce large number of asexual conidia within a short period of time following the start of the rainy season. In this situation they would represent the dominant airspora. *Daldinia* spp. also produce large amounts of spores in nature but these are ascospores and will occur after a much longer period of time following the onset of the rainy season but because they are ascospores they will be much more resistant and persistent.

Table 3.3 Isolates from Nakhon Ratchasima and Bangkok (July 2001)

Tree No.	*Endophytes*				*Total*
	Xylaria sp.	*Daldinia sp.*	*Phomopsis sp.*	Other taxa*	
[1]NK					
1	0	4	1	6	11
2	8	7	10	16	41
3	2	8	3	9	22
4	3	5	3	12	23
5	3	7	7	13	30
[2]BKK					
1	5	3	10	7	28
2	5	1	3	8	17
3	5	3	5	2	12

Note : [1] NK : Nakhon Ratchasima

[2] BKK : Bangkok

*Other taxa : Other species beside *Xylaria* sp., *Daldinia* sp. and *Phomopsis* sp. They were such as *Penicillium* sp., *Fusarium* sp., *Nigrospora* sp. *etc.*

Figure 3.9 The endophytic population from 3 sampling sites

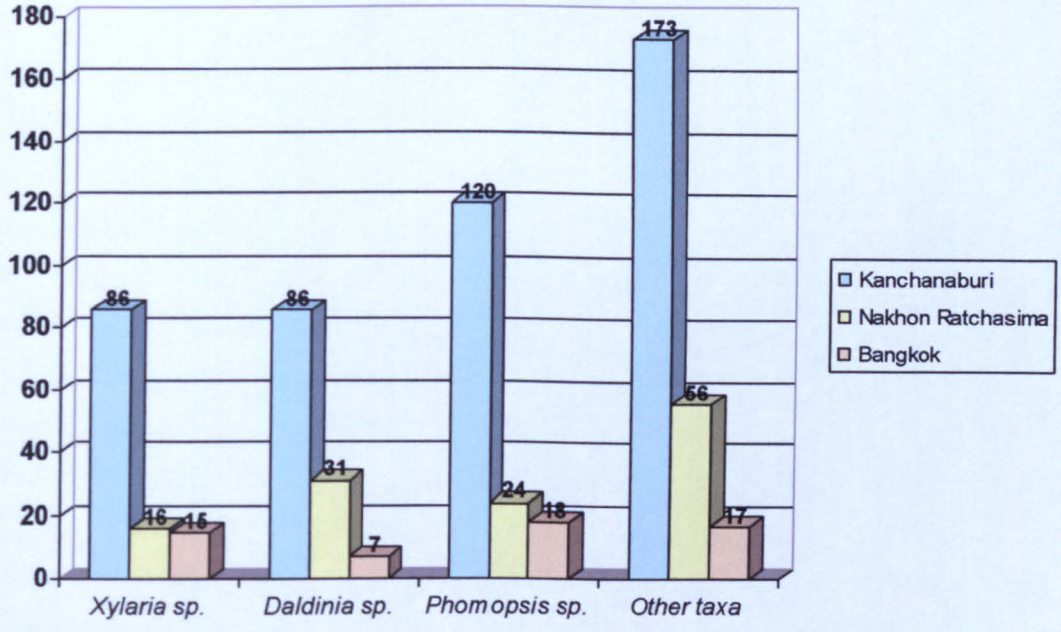


Table 3.4 Summary of isolation of endophytic fungi from different plants and different sample sites.

Type of Plants	Study Areas	Mean of number of Isolates
Cassia fistula L.	***Kanchanaburi***	93
(Golden Shower)	Nakhon Ratchasima (July 2001)	25.4
	Nakhon Ratchasima (December 2001)	60.8
	Bangkok	19
Tectona grandis L.	Mae Rim Forest	277.7 [a]
(Teak)	Chiang Dao plantation	286 [a]
	Bangkok	90 [b]

Note : a : Mekkamol, 1998

b: Chareprasert, 2001

3.4 Discussion

In this chapter the diversity of fungal species making up the endophytic communities of *Cassia* trees at three different and distinctive sites are compared (Table 3.1). A number of clear features emerge with the Kanchanaburi site being the most productive in terms of total numbers of isolates and the Bangkok site exhibiting the least. On the basis of total numbers of isolates over 8 times as many isolates were obtained from the Kanchanaburi site compared to the Bangkok site (Table 3.1) but on the basis of mean number of isolates for each tree this ratio drops to 4-5 (Table 3.4). Arnold and Herre (2003) studied the endophytic communities in leaves of *T. cacao* (Malvaceae), an understorey tree, in a neotropical rainforest in Panama, Central America. They examined canopy cover and age of leaf in relation to endophytic fungal colonization. They established that transmission is horizontal and that if the leaves were protected from surface wetting then the level of colonization was low or in some cases endophyte free leaves could be obtained. They also showed that endophytes colonized the leaves of *T. cacao* more readily beneath the forest canopy than from areas where the trees were in cleared or partially cleared sites (Arnold & Herre, 2003). This is more or less in broad agreement with the results presented here where the greatest colonization of *Cassia* leaves was at the Kanchanaburi site where the tree density was greatest and least at the Bangkok site which was much more open and could be equated to a cleared forest area. Arnold and Herre (2003) also concluded that although canopy density is related to density of endophyte infection over the short term which they related to patterns of inoculum abundance. They also reported that eventually endophytic infection densities in forest and in clearing plants became more similar and suggested that leaves remained receptive to endophytic infection after the initial infection period. This differs from the usual pattern for plant-fungus interactions where following the primary infection further infection by different species or even strains is reduced (Norman, Atkinson & Hooker, 1996). However it should be noted that endophytic infection of non-grass hosts is not usually seen as a pathogen-host situation (Petrini, 1991) although Arnold and Herre (2003) found that endophytes isolated from mature *T. cacao* leaves grew more rapidly on media supplemented with leaf extracts from the plant than on media containing extracts from other tree species growing in the same forest area. They suggested that interspecific differences in leaf chemistry influenced the endophytic assemblages (Arnold & Herre, 2003). However, it has been shown that inoculation of endophyte-free leaves with endophytes isolated frequently from naturally infected, asymptomatic hosts significantly decreased both

leaf necrosis and leaf mortality when T. cacao seedlings were challenged with a major pathogen ,*Phytophthora* spp. (Arnold *et al.*, 2003). Regardless of opinion it does seem that from studies in Panama, endophytes inhabit every mature leaf which was sampled amongst 24 species of taxonomically diverse woody angiosperms (Arnold, 2001), and it is not surprising that for all leaves sampled from the three diverse sites in Thailand that a substantial endophytic fungal population could be identified.

Although most published data is on endophytes from temperate regions there are an increasing number of reports from tropical plants. Arnold and Herre (2003) reported that in contrast to temperate forests "endophytic fungi associated with leaves of woody angiosperms, especially in tropical forests, are poorly known" (Arnold & Herre, 2003). However there are significant publications such as those of Petrini and Dreyfuss (1981), Dreyfuss and Petrini (1984), Rodrigues and Samuels (1990), Pereira, Azevedo and Petrini (1993), Rodrigues, (1994), Lodge, Fisher and Sutton (1996), Rodrigues and Petrini (1997); Arnold *et al.*, (2000), Arnold, Maynard, and Gilbert (2001), Gamboa and Bayman (2001), Cannon and Simmons (2002) and Suryanarayanan, Murali, and Venkatestan (2002), Suryanarayanan, Venkatestan and Murali (2003) and Arnold *et al.* (2003) which have contributed to a better understanding of endophytes from tropical plants.

Studies on endophytic fungi in Thailand are even more limited. There are few reports on fungal endophytes with the only major studies to date being those of an investigation of the endophytic fungi of teak (*T. grandis*) leaves (Mekkamol *et al*, 1997; Mekkamol, 1997; Chareprasert, 2001) and a study of endophytes of wild banana leaves (*Musa acuminate* L .A. Colla) by Photita *et al.* (2001). Mekkamol (1998) compared the endophytic assemblages of natural forest trees with plantation stands and found high numbers and diversity in samples from the natural stands in Mae Rim Forest and from the Chiang Dao plantation, both in Chiang Mai province in northern Thailand, for samples obtained in two out of the three years of the investigation. In a study of teak endophytes sampled on the campus of Chulalongkorn University, Bangkok, Chareprasert, (2001) demonstrated the presence of a substantial endophytic community but with much lower numbers than found by Mekkamol. The results from Chiang Dao and Mae Rim were found by application of the Sorenson index to exhibit a high species similarity. Most other studies of endophyte assemblages from the same plant species growing in different geographical locations usually show them to be completely different (Petrini, 1986,

1991; Fisher & Petrini, 1990; Fisher *et al.*, 1993, 1994). This has been explained to be the result of differences in the surrounding vegetation, temperature, rainfall, humidity and possibly pollution (Petrini, 1991). These reports are however on temperate plants and probably are not directly comparable. Mekkamol (1998) pointed out that her two sites were only 80 km apart and that the surrounding vegetation was similar although less diverse and extensive in the Chiang Dao plantation area (Mekkamol, 1998). The results from the teak leaves sampled in Bangkok (Chareprasert, 2001) is therefore in line with the results obtained during the current study on *Cassia* where the lowest number of endophytes recovered was from the Bangkok samples (Table 3.4). The sites chosen for sampling the *Cassia* were all different in terms of their immediate vegetation, local climatic conditions and especially in the case of Bangkok, atmospheric pollutants. This is considered to be important in relation to the differences in the endophyte communities at these sites.

Thus there are major differences between the *Cassia* tree samples from the different sites. In Kanchanaburi the trees are growing in a natural forest but in Bangkok, and also in Nakhon Ratchasima, the trees there had been planted for ornamental purposes. It is therefore not so surprising to find considerable differences in the endophytic communities from these different tree sites. In some ways the Nakhon Ratchasima site is between that of Kanchanaburi and Bangkok with greater plant diversity and cover in the immediate area but much less so than at Kanchanaburi and greater than in Bangkok. In consideration of endophytic populations the source of the fungi isolated is clearly of great importance and it can be argued that for each site sampled there is likely to be a decreasing inoculum present from Kanchanaburi, Nakhon Ratchasima to Bangkok. Arnold and Herre (2003) stressed the importance of abundance of the inoculum in the immediate vicinity of the trees sampled and related colonization density to level of sporefall noting that after 7 days of exposure, leaves in the forest area exceeded those from the cleared area by a factor of 1.5. However after 15 days exposure the relative densities of colonization were similar. In the present study it is only possible to compare populations at the time of sampling.

There is also the influence of local climatic conditions which might be considerable when comparing these three sites in Thailand with the Kanchanaburi site generally having higher humidities than the other sites. Mekkamol (1998) sampled over 3 years and also at the onset of the rainy season as well as late in the rainy season. She found that the sampling time was very important in relation to the fungal species

isolated. For example, *Phomopsis* and *Colletotrichum* species proved to be the dominant taxa early in the rainy season whilst *D. eschscholzii* and *Xylaria* species become much more significant later in the rainy season. This can be explained on the basis of the potential endophytic inoculum. *Phomopsis* and *Colletotrichum* grow rapidly on vegetation following the first rain and within a short period are sporulating with the production of large numbers of conidia. *Daldinia* and *Xylaria* are believed to enter healthy leaves and plant tissues as ascospores or conidia (Gowan & Vilgalys, 1991) and since the conidia of a number of *Xylaria* species do not germinate readily (Greenhalgh & Roe, 1984) the production of ascospores in nature will be highly significant since these are likely to be the originators of the endophytic assemblages of xylariaceous species. It is known that these take a considerable period of time to develop and discharge their ascospores. Mekkamol (1998) found that for *Daldinia eschscholzii* inoculated onto sterile teak branches, at least 12 weeks was required for development through to maturity. The results for *Xylaria* indicated a period of 18 to more than 34 weeks depending on the species. These times are however based on development from fresh inoculum in the branches and in nature this would not be the exact situation. Members of the Xylariaceae impregnate their woody substrata and then develop rapidly once the conditions are suitable (Whalley, 2001). Nevertheless it can be seen that the time for development of ascospores on natural substrata in the surrounding forest from where the trees were sampled will be at least 2-3 months again depending on the species involved. Certainly if ascospores are the main source of inoculum for members of the Xylariaceae this would explain their increasing importance as the rainy season progresses. Another important aspect is the time of exposure of the leaf to potential fungal propagules. Arnold & Herre (2003) and others have noted that there is a considerable difference in endophyte density between young and mature leaves. Mekkamol (1997) found that in teak leaves there were greater numbers of endophytes isolated from mature leaves and also in younger leaves *Phomopsis* and *Colletotrichum* spp. were more prevalent but in mature leaves *Daldinia* and *Xylaria* were more frequent. Rodrigues (1994) in her study of the Amazonian palm, *E. oleracea*, recorded larger numbers from mature leaves pointing out that "expanded leaves represent a better physical trap for spores than folded leaves". Mekkamol (1998) reported young teak leaves to be about one third the size of mature leaves which could attain a size of 40 cm in length and 30 cm at their widest point. Clearly these would present a much greater physical surface for capturing spores and would be present on the trees for a much greater time period. In this study of *Cassia* endophytes samples of leaves were obtained from Nakhon Ratchasima on 29 July 2001 i.e. early in the rainy season

and on 1 December 2001 i.e. towards the end of the rainy season. The total number of isolates obtained was 127 for July and 307 for December. Thus the longer exposure time and effect of the rainy season on general fungal sporulation and therefore potential inoculum could account for this clear difference which is in agreement with the findings of Mekkamol (1997) and Rodrigues (1994). In their study of endophytes of wild banana endophytes from Doi Suthep in Chiang Mai Province Photita *et al.*, (2001) also found fewer isolates recovered from younger leaves.

At all three sites *Daldinia* and *Xylaria* species were regular isolates but identification on the basis of morphological features alone is insufficient to enable confidant assignment to species level. Although it is possible to recognize some species on their characteristic growth form in culture (Chacko & Rogers, 1981) this is rare and alternative approaches are required (Petrini & Petrini, 1985). To overcome this identity problem two experimental methods have been applied, PCR based methodology (Chapter 4) and secondary metabolite profiles (Chapter 5). These methods together with morphological data have allowed a considerable number of the xylariaceous isolates to be successfully identified thus increasing our knowledge of these fungi as endophytes in tropical plants.

Although at all three sites, *Daldinia, Phomopsis* and *Xylaria* proved to be the most frequent isolates a number of other fungi were also identified. These included *Penicillium* spp., *Colletotrichum* spp., *Nigrospora sphaerica* (Sacc.)Mason., *Fusarium* sp., several basidiomycetes and sterile mycelia.

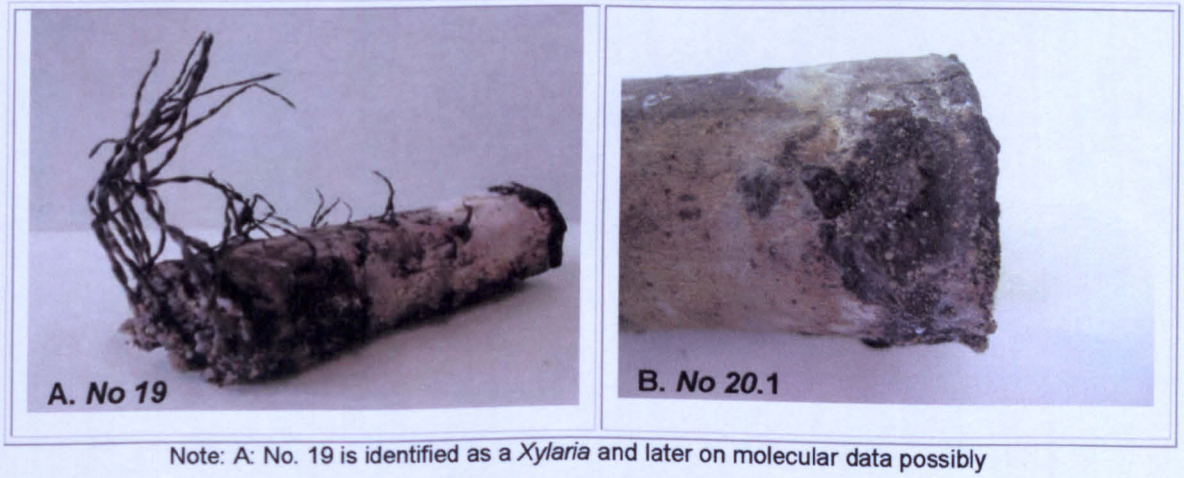
CHAPTER 4

Molecular study of Endophytic fungi

4.1 Introduction

A total of 956 endophytic isolates were obtained from the three sampling sites and from these 153 isolates (approximately 16%) were chosen and studied further. These were selected, as detailed in Chapter 2, on their overall cultural features and grouped as 'morphotypes'. The teleomorph induction method developed by Mekkamol (1997) was followed in an attempt to induce those cultures assigned to the Xylariaceae, on the basis of their cultural features, to produce their sexual stages (teleomorphs) which are necessary for accurate identification to species level. Unfortunately there were only 2 isolates which started to produce their teleomorph and in both cases these failed to develop to full maturity with the production of asci and ascospores. (Figure 4.1). The remaining species failed to produce their sexual stage even though they were incubated for prolonged periods of time. However a number produced stromatal initials which could be recognized as belonging to species of Xylaria whilst isolates of *Rosellinia, Nemania* and *Daldinia* were recognized as being xylariaceous by their cultural characteristics. A number of isolates produced a *Nodulisporium* anamorph and were therefore considered to be xylariaceous.

Figure 4.1 Immature teleomorph of isolate no 19 and no 20.1



Note: A: No. 19 is identified as a *Xylaria* and later on molecular data possibly AF163027 *Xylaria apiculata*

B: No. 20.1 is identified as a *Daldinia* and is probably *Daldinia eschscholzii*

Therefore a molecular approach was undertaken in an attempt to identify these xylariaceous isolates and also selected morphotypes of other fungi e.g. *Phomopsis* species were examined. Attempts were also made to induce teleomorph formation in *Phomopsis* by inoculation onto oatmeal agar followed by incubation at 25ºC under a black light (345-400 nm) illumination (Punithalingam, E; Pers comm.). This also proved to be unsuccessful although numerous conidia were formed and from these data and cultural characteristics at least seven morphotypes were recognized.

4.2 Results and discussion

The target in identification using PCR was rRNA especially ITS regions. ITS5 (forward primer) and ITS4 (reverse primer) were used to amplify the whole ITS region. The ITS primers make use of conserved regions of the 18s, 5.8s and 28s rRNA genes to amplify the non coding regions between them (White *et al.*, 1990). Endophytes were cultured in 2% MEB for 4-7 days or until enough mycelium was obtained, and fungal DNA extraction was then performed according to the method described by Lee and Taylor (1990) but with some modifications (Chapter2).

Chromosomal DNAs from all chosen isolates (63 isolates, Table 4.1) were extracted and precipitated using 3% SDS and phenol-chloroform and isopropanol, respectively. DNA from all selected endophytes was obtained successfully (Figure 4.2). They were then amplified using universal primers (ITS5 and ITS4, White *et.al.*, 1990). Size of PCR products ranged from 550-700 bps depending on the species of endophytes (Figure 4.3). After that, PCR products were purified using QIAquick PCR purification kit (Figure 4.4). At this stage, any primers, smaller DNA fragments and any other contaminants were removed from the DNA solution. Gel electrophoresis was performed at every step to confirm the presence of chromosomal DNA and PCR products.

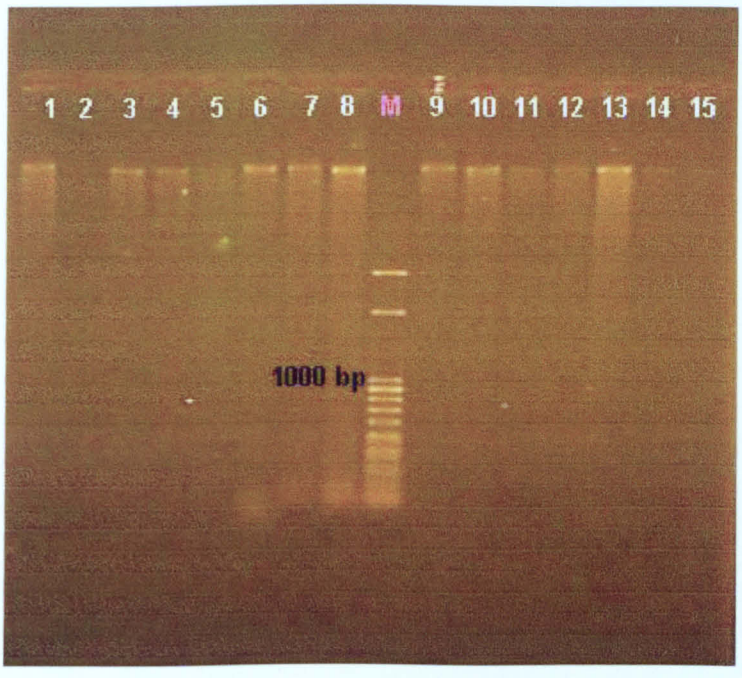
Table 4.1 Selected *Cassia* endophytes to be studied using molecular approach.

Isolate number	Source	Grouping
3 (1.5.1/4)	Kanchanaburi	*Phomopsis*
8 (2.6.1/4.1)	Kanchanaburi	Other taxa
11 (2.6.3/5)	Kanchanaburi	*Phomopsis*
16 (3.4.2/5)	Kanchanaburi	*Xylaria*
19 (3.6.4/3)	Kanchanaburi	*Xylaria*
20.1 (3.8.4/3.1)	Kanchanaburi	*Daldinia*
21 (4.1.1/5)	Kanchanaburi	*Xylaria*
22 (4.1.5/3)	Kanchanaburi	*Phomopsis*
25 (5.1.1/3)	Kanchanaburi	*Nodulisporium*
29 (5.3.3/4)	Kanchanaburi	*Xylaria*
33 (5.5.1/2)	Kanchanaburi	*Daldinia*
34 (5.5.4/4)	Kanchanaburi	*Xylaria*
38 (2.6.2/5)	Kanchanaburi	*Xylaria*
SC3 (NK1.2.5/5)	Nakhon Ratchasima	Xylaria
SC4 (NK1.3.2/3)	Nakhon Ratchasima	*Daldinia*
SC6 (NK2.4.1/4)	Nakhon Ratchasima	*Phomopsis*
SC8 (E3/2.1)	Nakhon Ratchasima	*Phomopsis*
SC10 (BKK3.3.2/2)	Bangkok	Other taxa
SC18 (BKK1.1.3/2)	Bangkok	*Daldinia*
SC19 (BKK1.2.1/3)	Bangkok	*Daldinia*
SC25 (BKK1.5.3/1)	Bangkok	*Daldinia*
SC27 (BKK1.5.5/5)	Bangkok	*Phomopsis*
SC31 (BKK2.3.3/5)	Bangkok	*Xylaria*
SC32 (BKK2.3.4/3)	Bangkok	Other taxa
SC33 (BKK2.4.5/5)	Bangkok	*Daldinia*
SC40 (BKK3.2.5/3)	Bangkok	*Daldinia*
SC42 (BKK3.6/6)	Bangkok	*Daldinia*
SC43 (NK1.4.1/1)	Nakhon Ratchasima	Other taxa
SC44 (NK2.3.2/2.2)	Nakhon Ratchasima	*Daldinia*
N4 (NK2.1.2/1)	Nakhon Ratchasima	*Xylaria*
N24 (NK3.1.4/1)	Nakhon Ratchasima	*Xylaria*
N26 (NK3.2.3/1)	Nakhon Ratchasima	*Daldinia*
N37 (NK4.2.4/1)	Nakhon Ratchasima	*Daldinia*
N46 (NK5.1.2/5)	Nakhon Ratchasima	*Phomopsis*

Table 4.1 (Continued) Selected *Cassia* endophytes to be studied using molecular approach.

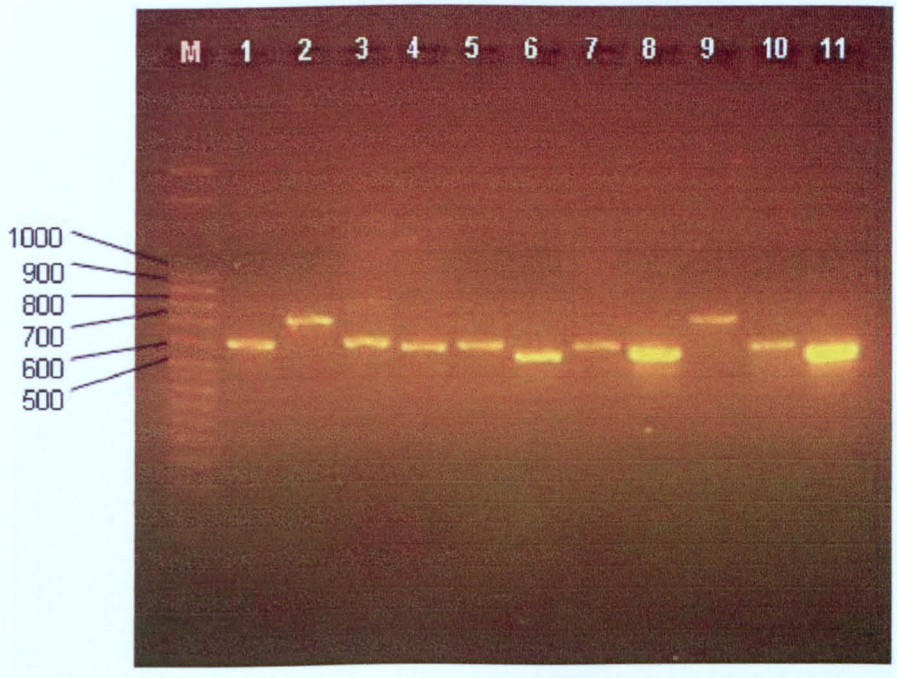
Isolate number	Source	Grouping
U1 (WNK4.4.2/1)	Nakhon Ratchasima	Other taxa
U41 (WNK2.1.4/5)	Nakhon Ratchasima	Other taxa
U59 (WNK5.3.2/5)	Nakhon Ratchasima	*Nodulisporium*
UX1 (WNK5.5.5/1)	Nakhon Ratchasima	*Xylaria*
UX2 (WNK5.2.4/4)	Nakhon Ratchasima	*Xylaria*
UX3 (WNK2.5.5/5)	Nakhon Ratchasima	*Xylaria*
UX5 (WNK5.5.3/2)	Nakhon Ratchasima	*Xylaria*
U65X (WNK5.4.5/1.1)	Nakhon Ratchasima	*Xylaria*
UD1 (WNK2.5.1/2)	Nakhon Ratchasima	*Daldinia*
UD2 (WNK1.1.5/3)	Nakhon Ratchasima	*Daldinia*
UD3 (WNK5.4.1/3)	Nakhon Ratchasima	*Nodulisporium*
UD4 (WNK2.5.1/5)	Nakhon Ratchasima	*Nodulisporium*
UD5 (WNK5.4.4/2)	Nakhon Ratchasima	*Nodulisporium*
U65D (WNK5.4.5/1.2)	Nakhon Ratchasima	*Nodulisporium*
UP3 (WNK4.1.1/4)	Nakhon Ratchasima	*Phomopsis*
UP4 (WNK2.2.3/4.2)	Nakhon Ratchasima	*Phomopsis*
P2 (WNK4.5.1/5)	Nakhon Ratchasima	*Phomopsis*
P8 (WNK3.2.4/4)	Nakhon Ratchasima	Other taxa
P12 (WNK1.2.2/1)	Nakhon Ratchasima	*Phomopsis*
P22 (WNK2.4.3/3)	Nakhon Ratchasima	Other taxa
P24 (WNK4.4.3/3.2)	Nakhon Ratchasima	*Nodulisporium*
PX1 (WNK3.4.1/2)	Nakhon Ratchasima	*Xylaria*
PD1 (WNK4.3.2/3)	Nakhon Ratchasima	*Nodulisporium*
PD2 (WNK4.4.1/1.1)	Nakhon Ratchasima	*Daldinia*
PD3 (WNK4.5.3/4)	Nakhon Ratchasima	*Nodulisporium*
PD4 (WNK4.2.3/4)	Nakhon Ratchasima	*Nodulisporium*
PP1 (WNK3.4.4/1)	Nakhon Ratchasima	*Phomopsis*
PP2 (WNK1.3.1/2)	Nakhon Ratchasima	*Phomopsis*
PP3 (WNK1.2.4/5)	Nakhon Ratchasima	*Phomopsis*

Figure 4.2 Chromosomal DNA from selected endophytic fungi



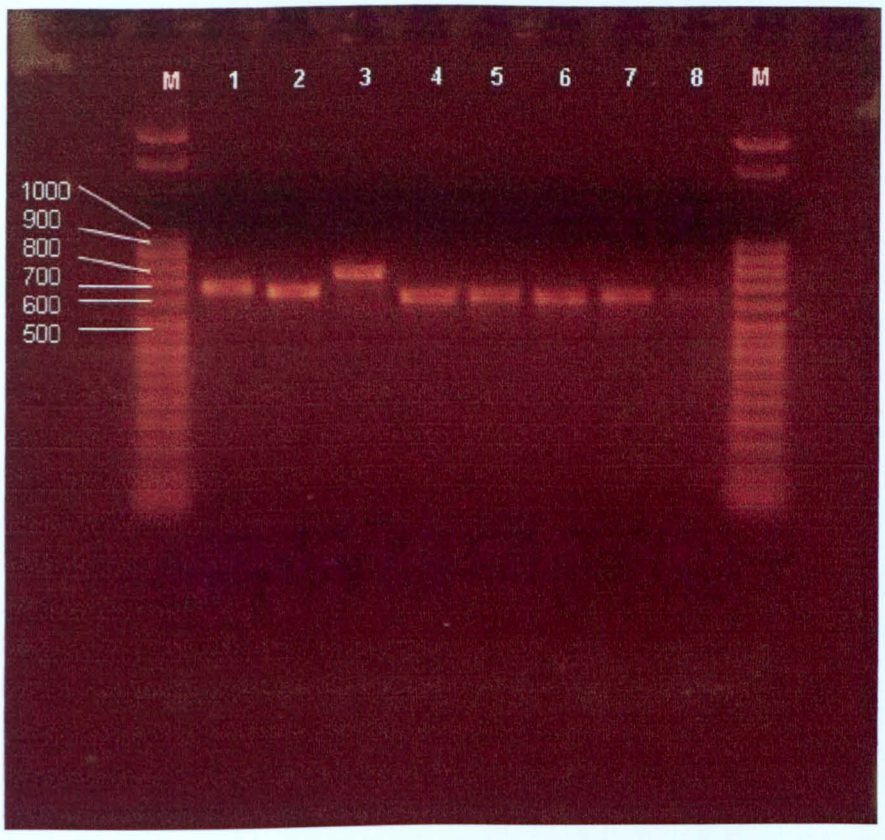
Lane number	content	Source
M	Perfect 50bp Ladder	
1	8	Kanchanaburi
2	SC11	Nakhon Ratchasima
3	SC13	Nakhon Ratchasima
4	U1	Nakhon Ratchasima
5	U41	Nakhon Ratchasima
6	U59	Nakhon Ratchasima
7	P15	Nakhon Ratchasima
8	P22	Nakhon Ratchasima
9	19	Kanchanaburi
10	21	Kanchanaburi
11	29	Kanchanaburi
12	34	Kanchanaburi
13	UX5	Nakhon Ratchasima
14	P2	Nakhon Ratchasima
15	P12	Nakhon Ratchasima

Figure 4.3 PCR products of endophytic fungi amplified using ITS5 and ITS4



Lane number	content	PCR product size (approximately; bp)	Source
M	Perfect 50bp Ladder		
1	29	600	Kanchanaburi
2	N4	700	Nakhon Ratchasima
3	N24	600	Nakhon Ratchasima
4	UX5	600	Nakhon Ratchasima
5	20.1	600	Kanchanaburi
6	SC43	550	Nakhon Ratchasima
7	U1	600	Nakhon Ratchasima
8	3	570	Kanchanaburi
9	SC10	700	Nakhon Ratchasima
10	P8	610	Nakhon Ratchasima
11	SC44	600	Nakhon Ratchasima

Figure 4.4 Purified PCR products



Lane number	content	PCR product size (approximately; bp)	Source
M	Perfect 50bp Ladder		
1	SC19	600	Bangkok
2	SC25	580	Bangkok
3	SC10	700	Nakhon Ratchasima
4	P8	560	Nakhon Ratchasima
5	P22	560	Nakhon Ratchasima
6	U41	560	Nakhon Ratchasima
7	SC6	570	Nakhon Ratchasima
8	UP3	570	Nakhon Ratchasima

The purified PCR products were then sent to the Liverpool School of Tropical Medicine to perform DNA sequencing with a Beckman CEQ8000 capillary sequencer. Sequencing results were analyzed using computer programmes; Chromas (Technelysium Pty Ltd) and BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA). The blast website (http://www.ncbi.nlm.nih.gov/BLAST/) is where databases for almost all fungal DNA sequences are held. The sequences which had been analyzed with computer programmes were entered into Blast programme (BLASTN 2.2.10 [Oct-19-2004], Altschul *et al*, 1997) to find the most probable match and the results are shown in Tables 4.2-4.5.

Phylogenetic trees were constructed to determine the relationship of each fungal endophyte within and between groups. The Neighbour-joining method was the model to build the tree with 1000 bootstraps. *Rhizopus oryzae* Went & Prinsen Geerligs was included in the phylogenetic tree as an outgroup. The constructed trees are shown as Figures 4.5 to 4.8.

Figure 4.5 Neighbour-joining analysis of ITS1-5.8s-ITS2 sequences alignment of *Cassia* endophytes (isolates with number in front of their species names) and reference strains from GenBank (isolates with accession number followed species names) and herbarium. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. The topology was rooted with Rhizopus oryzae as an outgroup. Numbers on branches are bootstrap value from 100 replications.

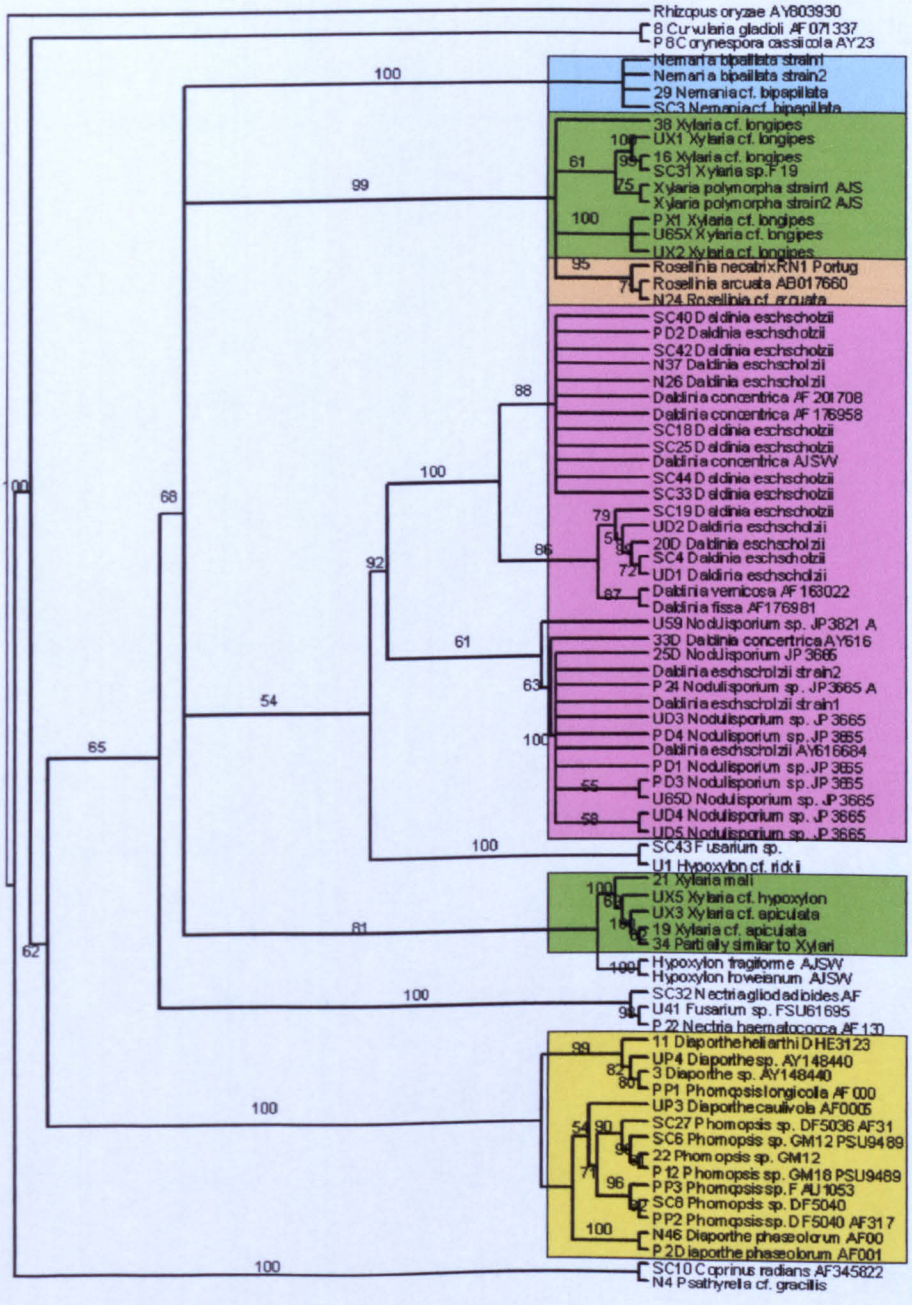


Figure 4.6 Neighbour-joining analysis of ITS1-5.8s-ITS2 sequences alignment of *Xylaria*-like group (isolates with number in front of their species names) and reference strains from GenBank (isolates with accession number followed species names) and herbarium. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. The topology was rooted with Rhizopus oryzae as an outgroup. Numbers on branches are bootstrap value from 1000 replications.

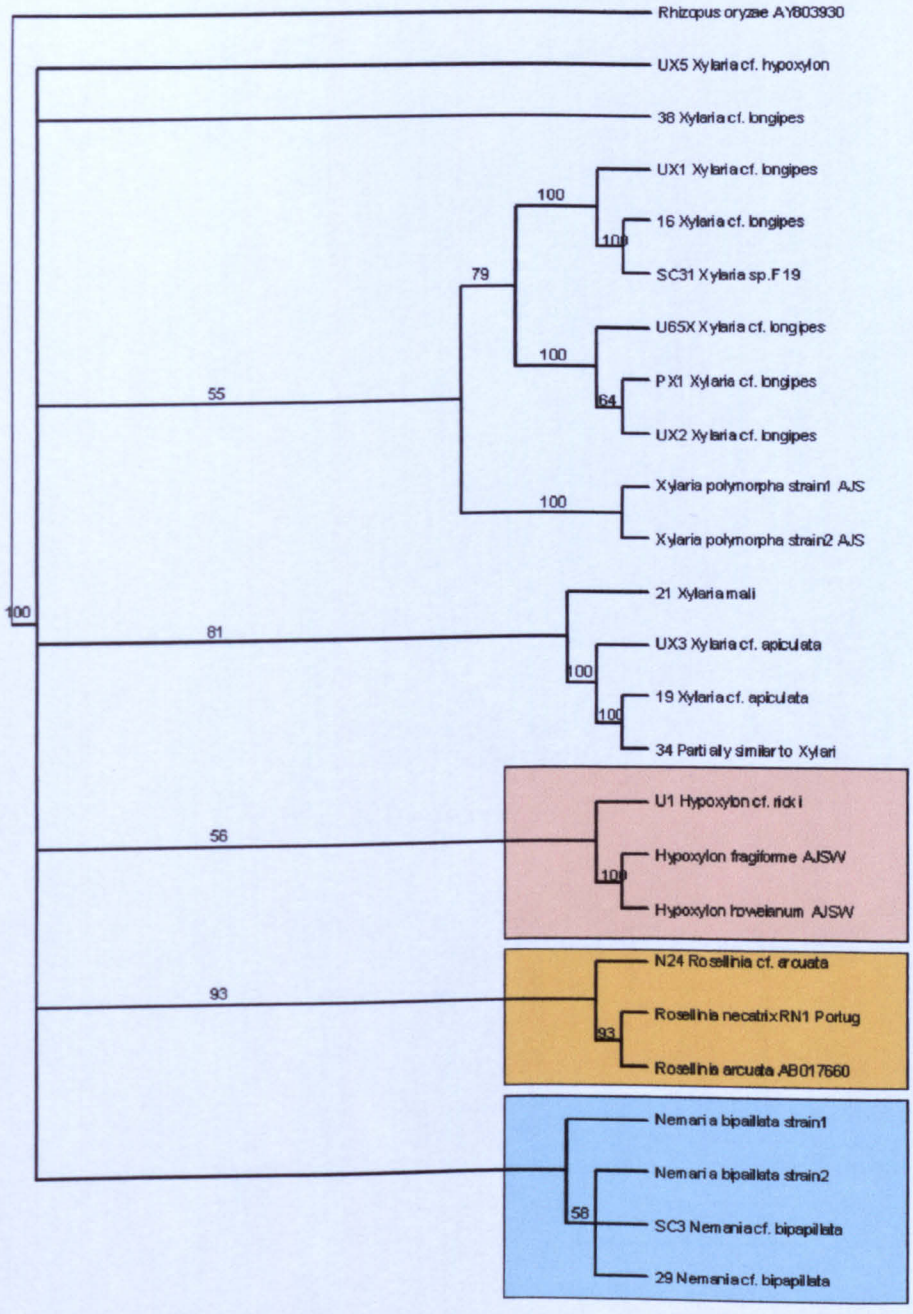


Figure 4.7 Neighbour-joining analysis of ITS1-5.8s-ITS2 sequences alignment of *Daldinia*-like and *Nodulisporium*-like group (isolates with number in front of their species names) and reference strains from GenBank (isolates with accession number followed species names) and herbarium. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. The topology was rooted with Rhizopus oryzae as an outgroup. Numbers on branches are bootstrap value from 1000 replications.

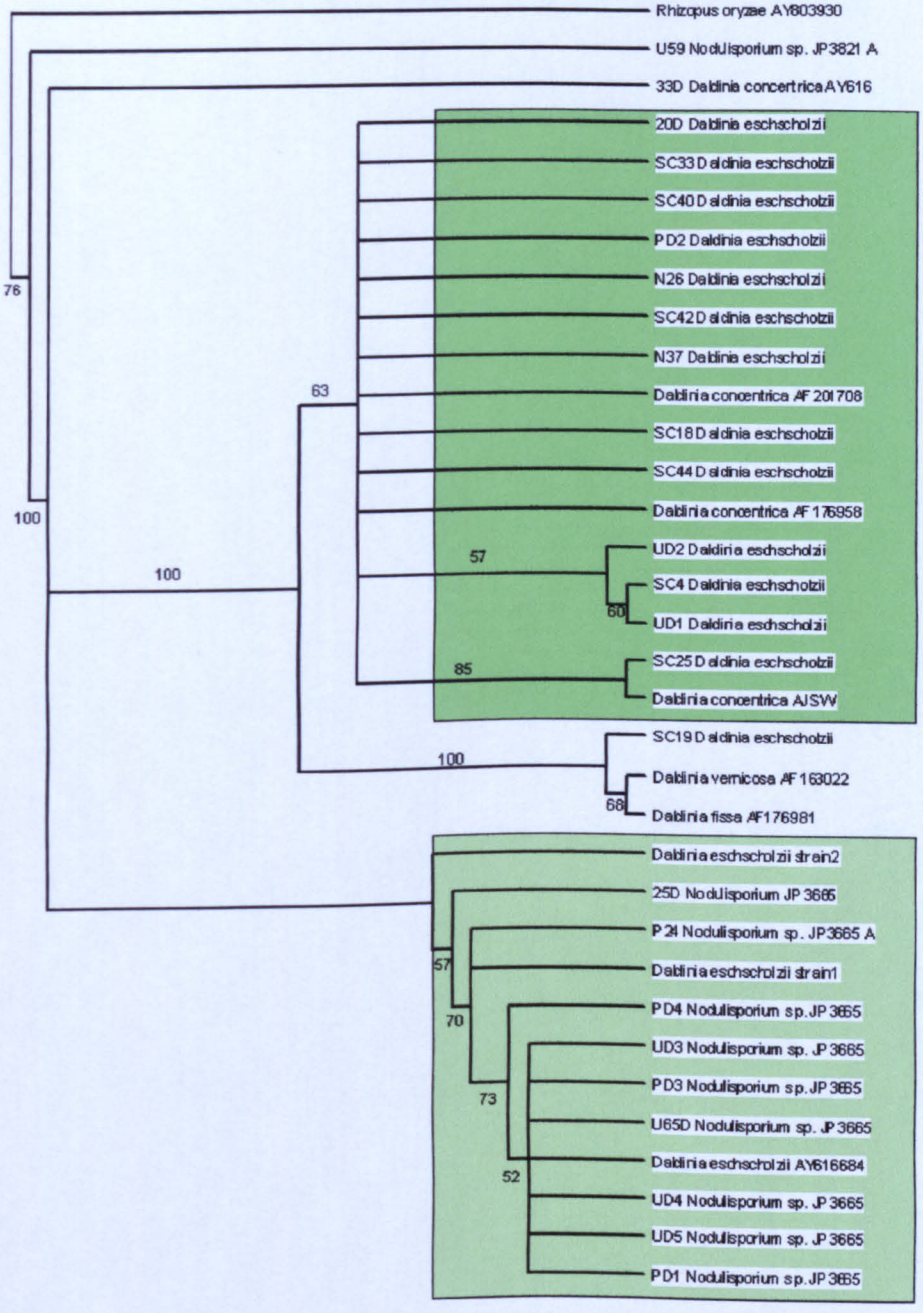


Figure 4.8 Neighbour-joining analysis of ITS1-5.8s-ITS2 sequences alignment of *Phomopsis*-like group (isolates with number in front of their species names). Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. The topology was rooted with Rhizopus oryzae as an outgroup. Numbers on branches are bootstrap value from 1000 replications.

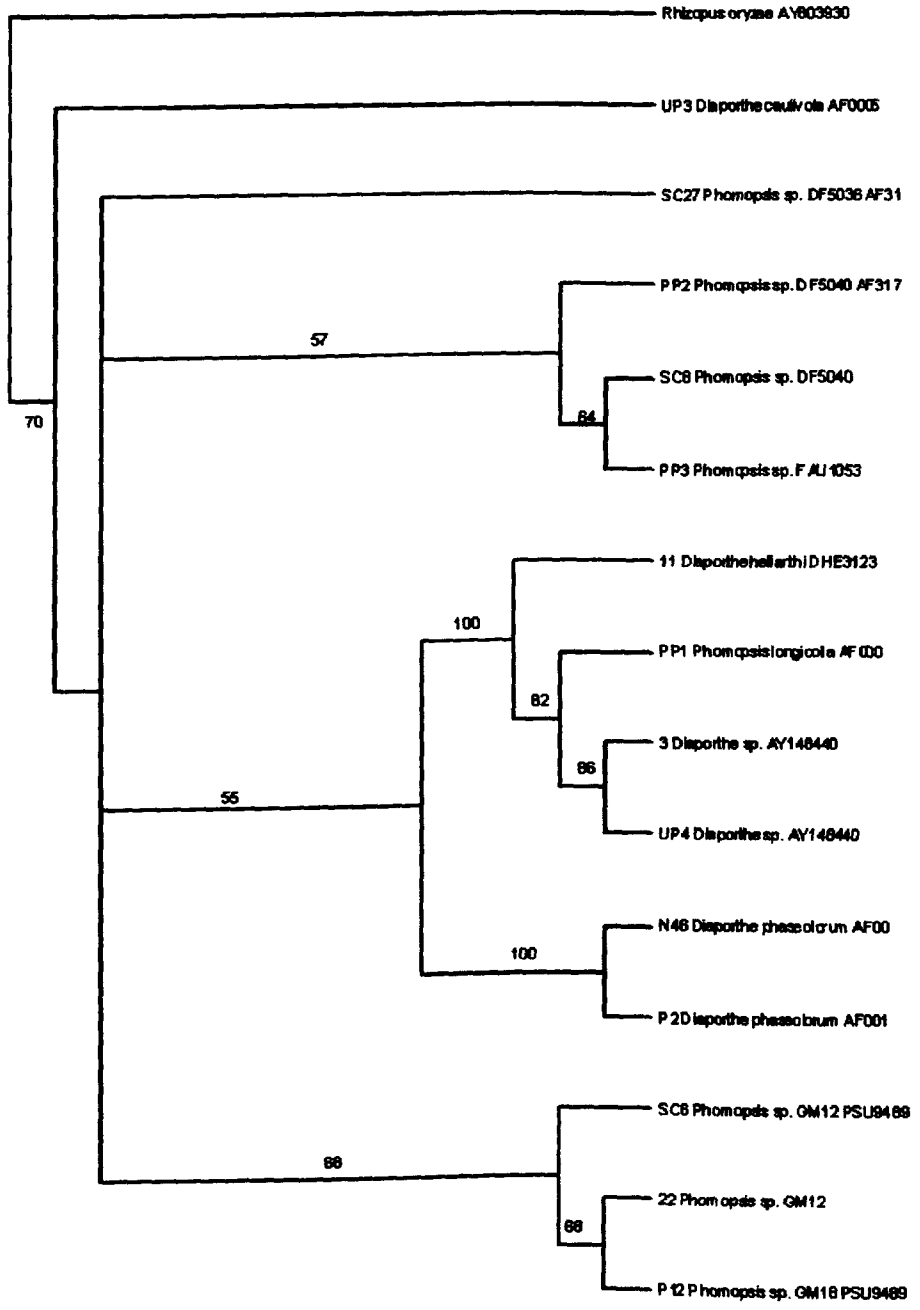


Table 4.2 Possible matches for unknown fungal endophytes from *Cassia*.

Group I : *Xylaria*-like (15 isolates)

Isolate number	Source	Possible matches/ GenBank Accession Number	Percentage of Similarity
16 (3.4.2/5)	Kanchanaburi	*Xylaria longipes* / AF163038	88%
19 (3.6.4/3)	Kanchanaburi	*X. apiculata* / AF163027	88%
21 (4.1.1/5)	Kanchanaburi	*X. mali* / AF163040	86%
29 (5.3.3/4)	Kanchanaburi	*Nemania bipapillata* / AJ390429	93%
34 (5.5.4/4)	Kanchanaburi	Unknown Partially similar to *X. apiculata* / AF163027	74%
38 (2.6.2/5)	Kanchanaburi	*X. longipes* / AF163038	86%
N24 (NK3.1.4/1)	Nakhon Ratchasima	*Rosellinia arcuata* / AB017660	85%
SC3 (NK1.2.5/5)	Nakhon Ratchasima	*N. bipapillata* / AY541610	95%
SC31 (BKK2.3.3/5)	Bangkok	*X. longipes* / AF163038	85%
PX1 (WNK3.4.1/2)	Nakhon Ratchasima	*X. longipes* / AF163038	84%
UX1 (WNK5.5.5/1)	Nakhon Ratchasima	*X. longipes* / AF163038	86%
UX2 (WNK5.2.4/4)	Nakhon Ratchasima	*X. longipes* / AF163038	86%
UX3 (WNK2.5.5/5)	Nakhon Ratchasima	*X. apiculata* / AF163027	87%
UX5 (WNK5.5.3/2)	Nakhon Ratchasima	*X. hypoxylon*/ AJ309350	86%
U65X (WNK5.4.5/1.1)	Nakhon Ratchasima	*X. longipes* / AF163038	87%

Table 4.2 provides the most likely identifications for each of the endophytic isolates grouped on their cultural and general morphological features as species of *Xylaria* or other closely related genera. It can be seen that the majority were confirmed as *Xylaria* species with *Nemania bipapillata* and *Rosellinia arcuata* also included. These three genera are known to be interlinked (Rogers, 1994; Whalley & Edwards, 1995; Rogers, 2000) and in many ways they possess similar cultural features although most *Xylaria* cultures are readily recognized to generic level by their

attempt to produce their upright, although immature stromata (Chacko & Rogers, 1981) The majority of the *Xylaria* isolates are identified here as a result of comparison of their sequences with those in GenBank with *X. longipes* Nits. being the most frequently matched species. However *X. longipes* is mainly a temperate species (Rogers, 1993) and although Dennis (1970) recorded it from Venezuela it has not as yet been reported from Thailand or Malaysia (Thienhirun, 1997; Whalley *et al.*, 2000). However Thienhirun (1997) recorded *X. longipes* var. *tropica* San Martin & Rogers from Khao Soi Dao Wildlife Reserve in Chanthaburi Province, Thailand. It is suggested here that in the absence of a suitable sequence for this undoubtedly tropical and very closely related taxon that the GenBank match of *X. longipes* is rightly the closest fit but in fact these endophytic isolates from Thailand may well prove to be the tropical form, *X. longipes* var. *tropica*. To confirm this authentic material of this taxon will in future be sequenced for a direct comparison. Two isolates were matched via GenBank as *X. apiculata* Cooke. This species was described from New Zealand where it was reported to be the most common *Xylaria* species there (Rogers & Samuels, 1986) and it was noted that it is related to *X. arbuscula* Sacc. Dennis (1970) also reported *X. apiculata* from Trinidad although it has not yet been reported from Thailand or Malaysia (Thienhirun, 1997; Whalley *et al.*, 2000). It should also be noted that Mekkamol (1998) did not report *X. apiculata* or *X. arbuscula* as either teleomorphs from the forest or as endophytes. However, she described a number of immature endophytic *Xylaria* species which may represent one or both of these taxa but in the absence of their teleomorphs or molecular data their specific identity remains unknown. It has also been shown that both *X. apiculata* and *X. arbuscula* are closely related on the basis of their ITS regions sequences (Lee, Ko & Jung, 2000).

One isolate from Kanchanaburi (21) was matched as first choice with *Xylaria* MS259 (GenBank) with the closest named taxon being *X. mali* Fromme. The identity of this isolate remains uncertain since MS259 has not been clearly linked with a known species and *X. mali* is apparently a mainly temperate species causing serious disease of apple trees (Rogers, 1984; Edwards *et al.*, 2003). Isolate UX5 although linking with *X. mali* and *X. apiculata* in the same clade of the phylogenetic tree (Fig. 4.5) was matched most closely with *X. hypoxylon* (L.:Fr.)Grev. followed by *X. arbuscula* (Appendix II). *Xylaria hypoxylon* is however normally considered to be a temperate species although a collection from the tropical house, University of Leeds, UK was identified as a *Xylaria* species closely related to *X. hypoxylon* and was

thought to have been imported with tropical plants from Africa (Cannon & Whalley, 1985).

Cultures with similarities to *Xylaria* were identified through their sequences as either *Nemania bipapillata* (Berk. & M.A.Curt.) Pouzar or as *Rosellinia arcuata* Petch. *N. bipapillata* is widely distributed throughout the world and occurs on both dicots and monocots (Ju & Rogers, 2002). In the UK it commonly occurs on decorticated wood which is well rotted (Whalley, 1996). Collections from both Thailand and Malaysia are usually characterized by their distinctive raised rims surrounding the ostioles (Thienhirun, 1997; Whalley, 2001) and Miller placed such material in his section Annulata of *Hypoxylon* as *Hypoxylon subannulatum* Henn.(Miller,1961). Abe and Liu (1995) transferred *H. subannulatum* to *Nemania* as *N. subannulata* (Henn. & Nyman) Abe & Liu. Henning described *H. subannulatum* from the Philippines but Ju and Rogers (2002) in their monograph of *Nemania* stated that "the type material of *H.subannulatum* was not located; from the original diagnosis, it is likely that this name is a synonym of *N. bipapillata*" Thienhirun (1997) considered collections of *Nemania* from Thailand to belong to *N. subannualata* since the raised rim and disc were very distinctive and appeared quite different to material collected in Europe and identified as *N. bipapillata*. Mekkamol (1998) and Whalley (2001) also followed this concept and here it is suggested that without further molecular studies the amalgamation or separation of these two taxa must remain uncertain. However for the present the results for the Thai endophytes indicate that they are *N. bipapillata* and comparison of the Thai sequences for these endophytes, sequences from Thai collections exhibiting the prominent raised rim and UK collections will be necessary to resolve this problem. Interestingly Van der Gucht (1995) stated that "within the genus *Nemania* it seems to be identical to type material of *N. bipapillata*. Surprisingly, however, is that the well-developed small annular disc surrounding the ostiolum has never been mentioned before in any description of *N. bipapillata* (see Pouzar, 1985; Petrini & Muller, 1986; Petrini & Rogers, 1986)".

The *Rosellinia* isolate N24 from Nakhon Ratchasima was closely matched with the sequence for *R. arcuata* Petch in GenBank. This is an interesting result since *R. arcuata* and *R. necatrix* Prill. are very closely related and there has been speculation that they may be the same (Francis, 1985). *Rosellinia arcuata* is an important pathogen of tea and is seen as being mainly tropical and subtropical whereas *R.necatrix* is more temperate (Edwards *et al.*, 2003). The molecular data here indicates *R.* arcuata as the first match but includes *R. necatrix* as being closely

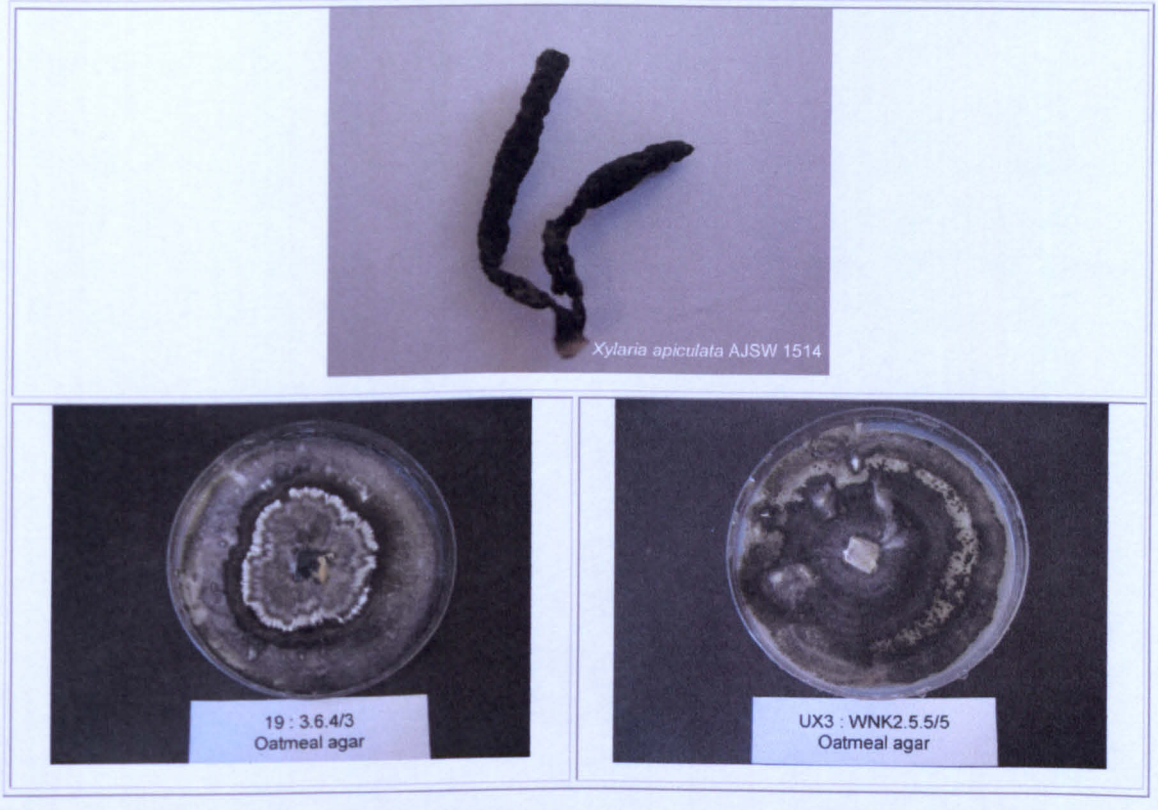
related (Appendix II). Interestingly Petrini (2003) considered *R. arcuata* and *R. necatrix* to be separate taxa but also drew attention to the need to undertake molecular studies on these taxa. She also reported that macroscopically *R. arcuata* resembles *R. necatrix* but its ascospores are statistically significantly larger (Petrini, 2003). It had been reported that the geographical distribution of *R. arcuata* is confined to tropical and humid regions of Africa and Asia (Saccas, 1956) but Petrini noted that the Petch specimens were from a mountainous region of Sri Lanka and that in New Zealand it was mainly collected in the northern part of North Island in national parks and state forests with subtropical vegetation and had not been reported from cultivated trees (Petrini, 2003). *Rosellinia necatrix* has been reported from Doi Inthanon, Chiang Mai Province in Northern Thailand (Thienhirun, 1997) and was also collected in rain forest in Los Baños, Laguna, Luzon, Philippines (Whalley, unpublished). The current molecular data for the Thai isolate indicates *R. arcuata* on the basis of the GenBank sequence and comparison with *R. necatrix* from Portugal (Figure 4.5) places them in the same clade but it is not clear whether they are the same or distinct species. Whether or not the Thai and Philippine collections are *R. arcuata* or *R. necatrix* remains to be seen. The need for further molecular studies with additional collections is clearly required to resolve this. Petrini and Petrini (in press) attempted to use morphological combinations to allocate *Rosellinia* species into 6 morphological groups with *R. arcuata* and *R. necatrix* grouping together in character combination 5 but it is still unclear whether these are really the same or separate taxa. Secondary metabolites from *R. necatrix* from Europe have been isolated and their structures determined with cytochalasin E and rosnecatrone being found to be characteristic compounds for this taxon (Edwards *et al.*, 2001; Edwards *et al.*, 2003). The isolate from Thailand designated here as *R.arcuata* has been found to contain cytochalasin E but not rosnecatrone and contains other compounds instead (Chapter 5).

A single isolate (34) from Kanchanaburi was not assigned but on the basis of the BLAST search was found to be partially similar to *X. apiculata* (Table 4.2, Figure 4.5). The majority of the *Xylaria*-like isolates were equally divided between the Kanchanaburi and Nakhon Ratchasima sites with only a single *Xylaria* isolate being obtained from Bangkok.

The identified *Xylaria*-like endophytes are described below with descriptions taken from Rogers (1984), Rogers, Calan and Samuels (1987), Rogers *et al.* (1988), Gonzalez & Rogers (1989), Thienhirun (1997), Van der Gucht (1995) or from

authenticated herbarium material. Images are from material held in the Herb. AJS Whalley or as given as per individual acknowledgements.

***Xylaria apiculata* Cooke**



Stromata: upright, stipitate; 1-6 cm long; 2-3 cm diam., gregarious and sometimes fasciculate to solitary and scattered, unbranched, dichotomously branched, or palmately branched, perithecia inconspicuous; ostioles lower than or the same level as the stromatal surface; stromatal surface wrinkled; dark brown (brown vinaceous), or blackish; KOH-extractable pigments lacking; tissue below perithecia conspicuous, white and homogeneous. Perithecia : more or less globose; 0.4-1 mm diam.

Asci cylindrical 229-280 µm total length x (5-)7-9(-10) µm, sporing part 120-180 µm; 8-spored, apical ring J+, cylindrical, (3.5-)4-5 µm high x 3-4 µm wide. Ascospores: (16-)20-24.5(-30) x (5-)6-8(-9) µm; inequilateral, flattened on one side, not curved, or slightly curved; ends narrowly rounded, germ slit straight, full length of spore or occasionally less than full length; on flattened side of the spore, perispore indehiscent in 10% KOH. Conidiogenous cells forming a palisade, consisting of branched, light brown, smooth conidiophores; each branch terminating in a conidiogenous cell; conidiogenous cells 11-19 x 2.5-3 µm, cylindrical; conidiogenous loci terminal, with a 0.5-1.0 µm diam., refractive circular scar remaining after conidial

dehiscence. Conidia (4.0-)5.2-7.7(-10.0) x (1.5-)1.8-2.3(-3.0) µm, ellipsoidal to oblong, the apex slightly hooked or beaked; pale brown, smooth; each conidium with a protuberant, 0.5-1.0 µm wide, refractive, frill-like, basal abscission scar.

In culture colonies grow 3-5 cm on oatmeal agar over a 2 week incubation at 20ºC in diffuse daylight, mycelium dark green to black with a white margin. Sterile stromata forming within one month, conidia rarely forming in culture.

Occurs widely on decaying, decorticated wood of dicotyledonous trees. Although this species has not been reported from Thailand Rogers *et al.* (1987) recorded it from Sulawesi, Indonesia and Van der Gucht (1995) from Papua New Guinea and many other countries.

***Xylaria arbuscula* Sacc.**

Stromata: Solitary to gregarious, Unbranched or rarely branched near base and then fasciculate, (1-)2-3(-4) cm long; fertile portion lanceolate, circular to elliptic in section, 2.3 mm; Stipe to 10 mm long x 1.0-1.5 mm diameter, not sharply delimited from fertile portion, slightly wrinkled, glabrous or with fine brown hairs; at first with a cinereous coating on surface of stroma, the cinereous colouration eventually disappearing and then stroma surface becoming black, smooth or slightly wrinkled; Perithecia completely immersed, 300-400 µm diameter, each opening through an inconspicuous papilla; internal tissue of stroma solid, white. Asci, 150-210 µm total length x 5-9 µm, the soporiferous part 65-100 µm, cylindrical; Apical ring J+, cylindrical, 2-3 µm wide x 3-4 µm high; Asci 8-spored, ascospores uniseriate with overlapping ends. Ascospores (11-)13-16(-19) x (4.0-)5.0-6.0(-7.5) µm inequilateral with one side flat and one side curved; elliptic in top view; one-celled or with inconspicuous cellular appendage (primary appendage) on one end, transparent brown; slit full length or less than full length and then 7-12 µm long, diagonally inserted, or parallel to long axis of ascospore, straight or broadly sigmoidal.

In culture colonies grow 5 cm on oatmeal agar over a 2 week incubation at 20ºC in diffuse daylight, with pronounced concentric rings of slightly raised, mycelium yellow-tan to orange-tan alternating with broader bands of appressed white mycelium. Stromata not forming in culture.

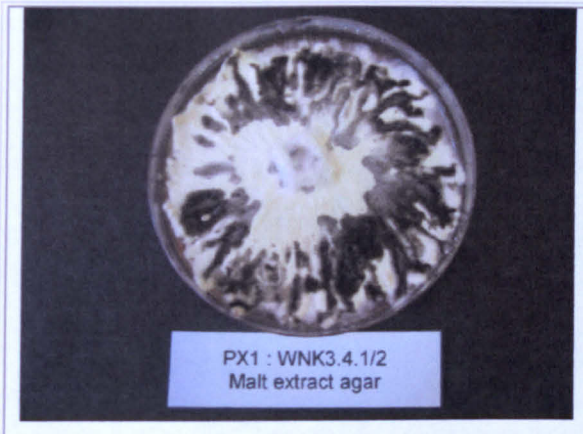
Occurs on bark or decorticated wood of angiosperms and gymnosperms. This species has not been reported from Thailand but occurs widely being recorded from Mexico (Gonzalez & Rogers, 1989), Venezuela (Rogers *et al.*, 1988), New Zealand (Rogers & Samuels, 1986) and North Sulawesi, Indonesia (Rogers *et al.*, 1987).

Xylaria hypoxylon **(L.:Fr.) Grev.**

Stromata: stiptate, erect, tough, pliant, clavarioid in shape, usually branched near the top, occasionally simple, up to 8 cm tall by 3-5 mm broad, often flattened in cross section above, rounded below; the base dark brown to black, often tomentose, branch tips white from asexual spores (conidia) or concolorous with the base and minutely pimpled with perithecial pores; Ascospores: 10-14 x 4-6 µm, black, smooth, strongly inequilateral; Conidia: hyaline, smooth, elliptical to elongate.

Occurs on rotting wood. Widely distributed in the temperate region and does not appear to be reported from the tropics.

Xylaria longipes **Nits.**



Stromata: cylindrical to clavate with rounded fertile apices, with short to long stipes, 4-6 cm total height x 3-5 mm broad; externally blackish with sloughing brown scales associated with cracking; internally white, becoming hollow; texture woody; surface roughened with wrinkles, scales, and ostioles papillae. Perithecia 0.4-0,5 mm diameter. Ostioles conical to hemispherical, often coated with white granular material. Asci 8-spored, cylindrical, stipitate, 137-150 µm total length x 6-7 µm broad, the spore-bearing part, 88-95 µm long, the stipe, 45-50 µm long, with apical apparatus, quadrate to rectangular, 2.5-3 µm high x 2-2.5 µm broad, J+. Ascospores brown, unicellular, ellipsoid-inequilateral to navicular with narrow ends, smooth, 13.8-16.3 x 5-6 µm, with spiraling germ slit spore length.

Occurs on wood. Reported from Thailand as *X. longipes* var. *tropica* by Thienhirun (1997).

***Xylaria mali* Fromme**

Xylaria mali
http://maple.dnr.cornell.edu/images/diseases/dis27.jpg

Stromata: cylindric to clavate, to highly irregular, usually somewhat to strongly flattened, unbranched or branched from bases or near apices, forming grotesque configurations, solitary or many arising from reddish pannose bases, with ill-defined stipes, total length 2-8 cm x 0.5-2 cm diameter, at first chalky white, yellowish or grayish brown, often yellow orange at apices, becoming brown and finally dull black, interior at first white, becoming brownish, and finally hollow, very smooth to somewhat wrinkled surface, ostiole papillae very inconspicuous and widely separated; Perithecia: o.5-1 mm diameter; Asci: long-stipitate, up to at least 200 µm total length (spore-bearing part 110-120 µm) x 6-8 µm, with apical ring urn-shaped or inverted hat-shaped in optical section, 1.5-2.5 µm broad x 2-3 µm high, J+; Ascospores: smooth, brown to chestnut brown, ellipsoid-inequilateral to crescentic, (12-)13-17.5(-19) x 4.5-6 µm, with long straight germ slit, immature ascospores with cellular appendages which degenerate and disappear as spores mature; Conidia: hyaline, ellipsoid with tapering ends, 8-10 x 3-4 µm.

Occurs throughout the southeastern and Midwestern USA and probably elsewhere, most often from apple (root rot) and less frequently on angiospermous hosts. There are no reports of this *Xylaria* species from tropical counties.

***Nemania bipapillata* (Berk. & M.A.Curt.) Pouzar**

Stromata: erumpent to superficial, small to widely effused, occasionally separating into individual perithecial stromata, 1-2 x 0.5-1 cmx 1-1.5 mm thick; externally dark brown to black; internally dark brown; surface smooth, uneven due to protruding perithecia. Perithecia completely immersed, widely dispersed in an undulating stroma, subglobose, 0.5-1 mm diam.; ostioles papillate, each in the centre of a convex annular disc (0.2-0.3 mm diameter), surrounded by a wide flattened border, slightly raised above the disc. Paraphyses filiform, tapering towards the apex,

remotely septate, 2.5 µm diameter near the top. Asci cylindrical, 8-spored 135-260 x 6.5-7 µm (spore bearing part 55-80 µm long); apical apparatus rectangular, 3-4 µm high x 2.5 µm broad,J+. Ascospores obliquely uniseriate, inaequilaterally ellipsoid with rounded ends, medium to dark brown, smooth, 11-13.5(14.5) x 4.5-5.5(6) µm (average ± SD : 12.67 ± 0.75 x 5.38 ± 0.25 µm) ; germ slit straight, on the straight of the spore, almost full spore length.

Occurs on dead wood lying on the forest floor in lowland rain forest. This species, often as *N. subannulata* has been reported from Thailand (Thienhirun, 1997), Papua New Guinea (Van der Gucht, 1995), Malaysia (Whalley, 2001) as well as from many other countries (Ju & Rogers, 2002).

Rosellinia arcuata **Petch**

Stromata: Subiculum persistent, brown to dark brown, wiry, appressed, with synnemata, stromata size (1250)1766 ± 330(2500) µm high, (1125)1740 ± 312(2250) µm wide (n = 30), globose to subglobose with almost flattened top, cupulate, often with a short cylindrical base immersed in the subiculum, copper brown, dark brown, black around the ostioles, smooth, solitary to crowded and laterally compressed. Ostioles finely to coarsely papillate. Ectostromata (75)100 – 125 µm thick, black. Entostroma white, confined to base. Perithecia detached and collapsed in mature material. Ascus apical ring (6.7)8.3 ± 0.8(9.6) µm high, upper width 5.7-6.2 µm, lower width 3.8-5.7 µm (n=16), J+, dark blue. Ascospores (37.4)48.6 ± 4.2(58.5) µm long, (4.8)7.6 ± 0.8(9.6) µm wide (n = 167), inequilateral, narrowly ellipsoidal to needle shaped, with narrowly wounded ends, brown to dark brown, with straight, 8-12 µm long germ slit centred on the flat side, both extremities and flat side surrounded by a slimy sheath, 2-3 µm thick at ends, 1 µm thick at the side. Synnemata up to 2000 µm, conidia 4-5 x 3-4 µm.

In culture colonies grow 7 cm on oatmeal agar over 20 day incubation under 12 h dark and 12 h UV and fluorescent light. In the centre, (3 cm) black from confluent tufts of black hyphae shaped as witches' brooms, otherwise white, hyphae very short, sterile. Colonies grow covering the whole plate (9 cm) on CMD after 43 day incubation under the same condition, black with cottony white margin and on the original inoculum, 0.5-1 mm long, acute, unbranched, black, without stromatic base, composed of 3-4 µm wide smooth, brown, branching, septate hyphae, often sterile. Free conidiophores forming on one area of the original inoculum, long heavily and irregularly branched. Conidiogenous cells 18-48 x 3-4 µm (n = 9), terminal, geniculare, smooth, subhyaline to pale tan towards the base. Conidia 3.5-5(7) x 2-3.5 µm (n = 22), forming at the tip of each synnema as loose, white-grey areas, oblong with flat, non-protuberant base bearing a minute frill being more refractive than the rest of the conidial wall, smooth, subhyaline.

Occurs on corticated twigs and roots and although not reported from Thailand Thienhirun recorded the closely related *R. necatrix* (Thienhirun, 1997).

Table 4.3 Possible matches for unknown fungal endophytes from *Cassia*. Group II : *Daldinia*-like and *Nodulisporium* spp. (25 isolates)

Isolate number	Source	Possible matches / GenBank Accession Number	Percentage of similarity
20.1 (3.8.4/3.1)	Kanchanaburi	*Daldinia concentrica* /AF176958	88%
25 (5.1.1/3)	Kanchanaburi	*Nodulisporium* sp. JP3665 / AF280628	86%
33 (5.5.1/2)	Kanchanaburi	*D. concentrica* /AY616682	69%
SC4 (NK1.3.2/3)	Nakhon Ratchasima	*D. concentrica* /AF201708	94%
SC18 (BKK1.1.3/2)	Bangkok	*D. concentrica* /AF176958	94%
SC19 (BKK1.2.1/3)	Bangkok	*D. concentrica* /AF201708	84%
SC25 (BKK1.5.3/1)	Bangkok	*D. concentrica* / AF176958	95%
SC33 (BKK2.4.5/5)	Bangkok	*D. concentrica* /AF176958	94%
SC40 (BKK3.2.5/3)	Bangkok	*D. concentrica* /AF176958	90%
SC42 (BKK3.6/6)	Bangkok	*D. concentrica* /AF176958	96%
SC44 (NK2.3.2/2.2)	Nakhon Ratchasima	*D. concentrica* /AF176958	97%
N26 (NK3.2.3/1)	Nakhon Ratchasima	*D. concentrica* /AF176958	93%
N37 (NK4.2.4/1)	Nakhon Ratchasima	*D. concentrica* /AF176958	95%
U59 (WNK5.3.2/5)	Nakhon Ratchasima	*Nodulisporium* sp. JP3821 /AF280627	86%

P24 (WNK4.4.3/3.2)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	96%
PD1 (WNK4.3.2/3)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 / AF280628	95%
PD2 (WNK4.4.1/1.1)	Nakhon Ratchasima	*D. concentrica* /AF176958	91%
PD3 (WNK4.5.3/4)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	94%
PD4 (WNK4.2.3/4)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	96%
UD1 (WNK2.5.1/2)	Nakhon Ratchasima	*D. concentrica* /AF201708	94%
UD2 (WNK1.1.5/3)	Nakhon Ratchasima	*D. concentrica* /AF176958	94%
UD3 (WNK5.4.1/3)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	92%
UD4 (WNK2.5.1/5)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	94%
UD5 (WNK5.4.4/2)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	95%
U65D (WNK5.4.5/1.2)	Nakhon Ratchasima	*Nodulisporium* sp. JP3665 /AF280628	94%

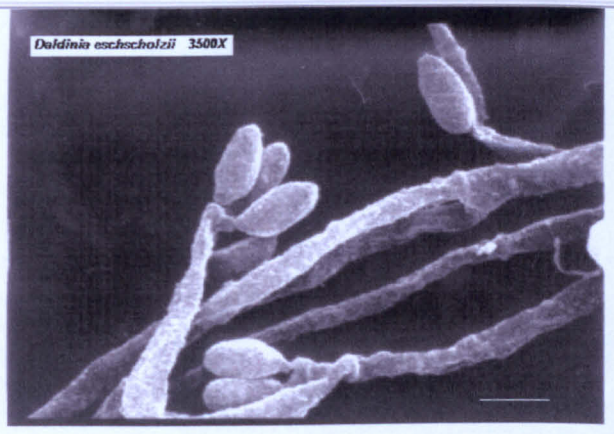
Reference to Table 4.3 for examination of *Daldinia* and *Nodulisporium*-like isolates indicates that the majority are a *Daldinia* species. Thienhirun (1997) found *D. eschscholzii* to be widespread and common in Thailand and Mekkamol (1998) frequently isolated it as an endophyte from teak leaves. Sequences of authenticated *D. eschschozii*, *D. concentrica* and *D. caldariorum* Henn. (as *D. vernicosa* (Schwein.)Ces. & De Not) were also included in the phylogenetic tree (Figure 4.5). These data together with the BLAST searches (Table 4.3) confirm the identity of *Daldinia* including isolates designated as *Nodulisporium*-like isolates. *Nodulisporium* is one of the most common form genera occurring as anamorphs in *Daldinia*, *Hypoxylon* and other closely related genera (Ju & Rogers, 1996).

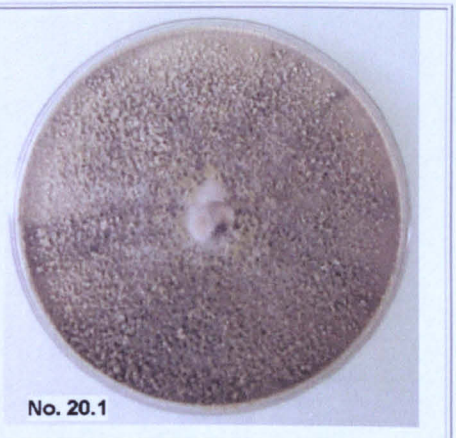
In the GenBank database sequences from *D. concentrica*, *D. petrini* Y.-M. Ju, J.D.Rogers, & San Martin and *D. fissa* C.G.Lloyd were available for comparison. In most cases *D. eschscholzii* was found to be the majority species although from the GenBank data many were matched with *D. concentrica*. However this is mainly an European and temperate zone species and has not been reported from Thailand (Thienhirun, 1997). It is well known to be closely related to *D. eschscholzii* which is mainly tropical and subtropical Dennis, 1970; Stadler *et al.*, 2001a). Typical *D. eschscholzii* cultures are fairly distinctive and produce aromatic volatile compounds

which are also characteristic. Therefore sequences from authentic *D. eschscholzii* from Malaysia (M.A. Whalley) were obtained and compared with the Thai sequences. Figure 4.5 indicates two main groups of *Daldinia*; DI which includes in the clade the sequences from *D. eschscholzii* obtained from teleomorphic material from Malaysia and also a match with AF280628 *Nodulisporium* species (GenBank) which has subsequently been matched with *D. eschscholzii* from Phuket, Thailand (Triebel *et al.*, In Press). The other *Daldinia* isolates D2 all closely group and according to Genebank sequence data represent *D. concentrica* AF176957 to AF176958 and AF201708. The later was matched by Triebel *et al.*, (In press) with their sequences for *D. concentrica* from Germany and Wales. However all the cultures of Thai endophytes grouped in D2 were distinctive and unlike all cultures of *D. concentrica* isolated in the UK. It is suggested that in light of the lack of confirmed collections of *D. concentrica* in Thailand and indeed Malaysia (Thienhirun, 1997; Mekkamol, 1998; Whalley, 2001; Thienhirun & Whalley, 2004) that these Thai endophytic isolates are in fact *D. eschscholzii* and further molecular data on this and closely related taxa are required. The majority of these isolates are from the Nakhon Ratchasima site followed by Bangkok and finally Kanchanaburi.

The identified *Daldinia*-like endophytes are described below:-

***Daldinia eschscholzii* (Ehrenb.:Fr) Rehm**





Stromata: turbinate to placentiform, seesile or with short, stout stipe, solitary to infrequently aggregated, smooth, 1.5-6 cm. diameter x 1-4 cm. high; surface brown vinaceous, dark brick, sepia, grayish sepia, or vinaceous grey, blackened and varnishes in age; dull reddish brown granules immediately beneath surface, with KOH-extractable pigments livid purple, dark livid, or vinaceous purple; the tissue between perithecia brown, pithy to woody; the tissue below the perithecial layer composed of alternating zones, the darker zones dark brown, pithy to woody, 0.2 - 1mm thick, the lighter zones white, grey or grayish brown, gelatinous and very hard when dry, becoming pithy to woody, persistent, 0.3-1 mm thick. Perithecia tubular, 0.8-1.5 mm high x 0.3-0.4 mm diam. Ostioles obsolete or slightly papillate. Asci 160-195 µm total length x 7-9 µm broad, the spore-bearing part 70-80 µm long, the stipe 90-120 µm long, with apical apparatus, discoid, 0.5 µm high x 2-2.5 µm broad, bluing in Melzer's reagent. Ascospores brown to dark brown, unicellular, ellipsoid-inequilateral, with narrowly rounded ends, 11.3-13.8 x 5-6.3 µm, with straight germ slit spore-length on convex side; perispore dehiscent in 10%KOH, conspicuous coil ornamentation; epispore smooth.

Colonies growing rapidly on mail extract agar, becoming brown grey and granular as conidia are produced, dark brown with green on the underside, profilic conidial production, conidiophores and conidia very similar to those described by Ju *et..al.* (1997)

Occurs on wood, often large logs or branches.

Table 4.4 Possible matches for unknown fungal endophytes from *Cassia*.

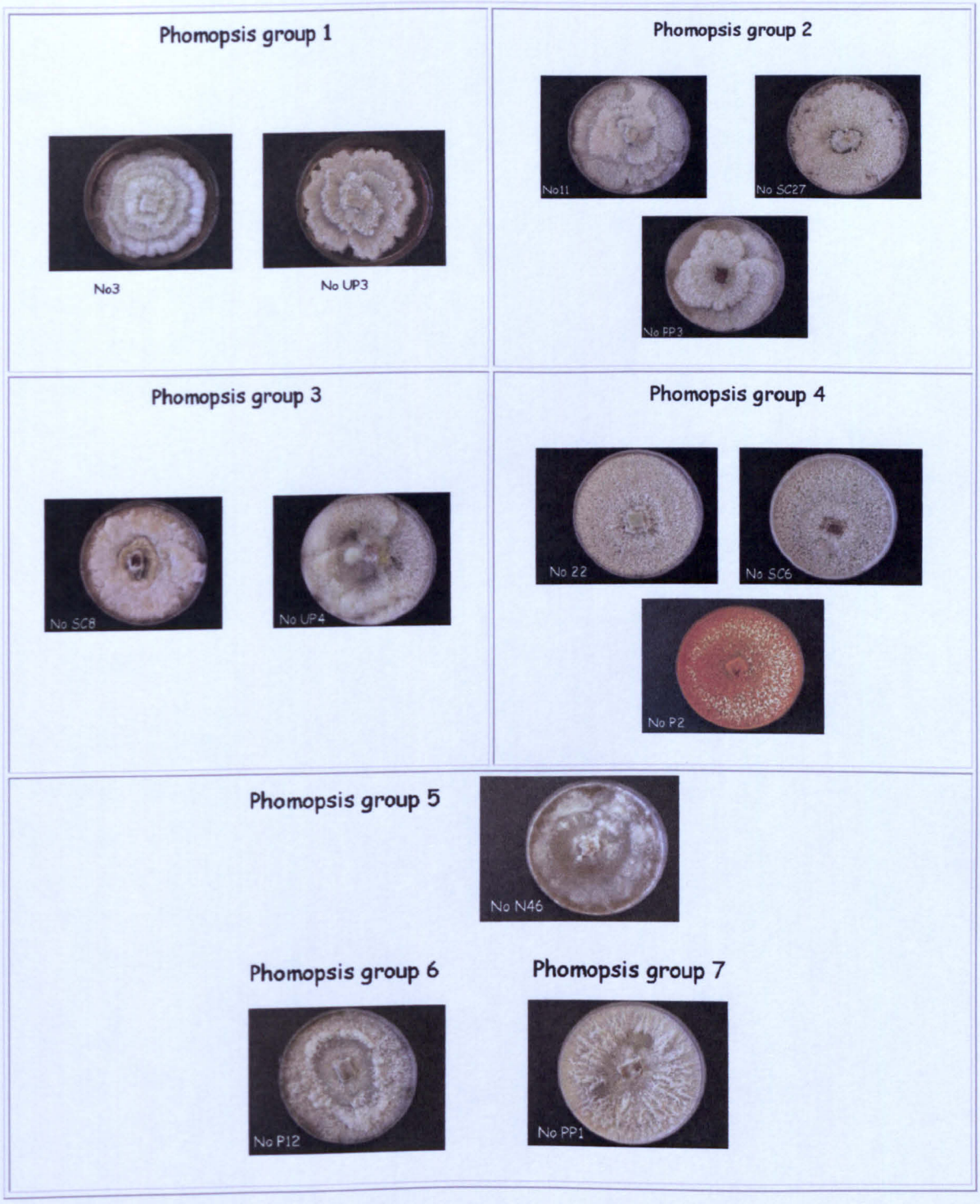
Group III : *Phomopsis* spp and *Diaporthe* spp (14 isolates)

Isolate number	Source	Possible matches / GenBank Accession Number	Percentage of similarity
3 (1.5.1/4)	Kanchanaburi	*Diaporthe* sp./ AY148440	91%
11 (2.6.3/5)	Kanchanaburi	*Diaporthe helianthi* /AJ312366	90%
22 (4.1.5/3)	Kanchanaburi	*Phomopsis* sp. GM12 / PSU94898	88%
SC6 (NK2.4.1/4)	Nakhon Ratchasima	*Phomopsis* sp GM12 / PSU94898	86%
SC8 (E3/2.1)	Nakhon Ratchasima	*Phomopsis* sp. DF5040 /AF317584	86%
SC27 (BKK1.5.5/5)	Bangkok	*Phomopsis* sp. DF5036 /AF317583	88%
N46 (NK5.1.2/5)	Nakhon Ratchasima	*Diaporthe phaseolorum* / AF001017	95%
P2 (WNK4.5.1/5)	Nakhon Ratchasima	*Diaporthe phaseolorum* / AF001017	98%
P12 (WNK1.2.2/1)	Nakhon Ratchasima	*Phomopsis* sp. GM18 / U94898	89%
PP1 (WNK3.4.4/1)	Nakhon Ratchasima	*Phomopsis longicolla* / AF000207	96%
PP2 (WNK1.3.1/2)	Nakhon Ratchasima	*Phomopsis* sp. DF5040 / AF317584	86%
PP3 (WNK1.2.4/5)	Nakhon Ratchasima	*Phomopsis* sp. FAU1053/AF103000	88%
UP3 (WNK4.1.1/4)	Nakhon Ratchasima	*Diaporthe caulivola* / AF000567	96%
UP4 (WNK2.2.3/4.2)	Nakhon Ratchasima	*Diaporthe* sp. 36-1 / AY148440	93%

Species of *Phomopsis* were common isolates and in agreement with Mekkamol (1998) it was found that identification to species level was impossible with only cultural and anamorphic characteristics available. Initially at least 7 morphotypes (Figure 4.9) were designated and following sequence comparisons with data on GeneBank 7 or more taxa could be recognized. There is also the likelihood that there are undescribed species occurring in Thailand. Species of *Phomopsis* and *Diaporthe* are based on their host, such as, *Diaporthe helianthi* Munt.Cvet.*et al.* and its host, sunflower, *Helianthus annuus* L. A number of the Thai isolates were

matched with their teleomorph and these are known to have phytopathogenic ability and are usually viewed as pathogens However, these fungal pathogens can be isolated from diseased as well as from healthy plant tissue at any given time (Chase, 2002) For instance, isolate no 3 in which its ITS sequence was matched with *Diaporthe helianthi* DHE312366 commonly causes severe stem canker and leaf necrosis in sunflower (*H. annuus*). It is responsible for the yield losses of up to 40% in many European and non-European countries (Debaeke, Estragnat & Reau, 2003). Isolate numbers N46 and P2 were identified as *Diaporthe phaseolorum* (Cooke&Ellis)Sacc. AF001017. These two isolates differ in their cultural characteristics but their DNA sequences was found to be quite similar and using the BioEdit programme, when they were aligned together, there was up to 91% similarity in their ITS sequences. If they are not conspecific they are certainly very closely related. *Diaporthe phaseolorum* is reported to attack soybean (*Glycine max* L. Merr.) in Alabama causing pod and stem blight and the variety *D. phaseolorum* var. *meridionalis* causes severe stem canker in soybean and is widespread throughout the state. The degree of damage causing by this fungus depends on the state of the plant when the infection starts, the soybean cultivar, crop stress, and also climatic conditions (Integrated Pest Management, 2004). Isolate number UP3 which was identified as *D. caulivola* AF000567, usually referred to as the variety *D. phaseolorum* var. *caulivola*, also causes stem canker in soybean and, in recent years, the damage has been widespread in the southeastern part of the United States. The disease can be very critical because it kills plants before they reach maturity, and the incidence of disease is greater in the wet season (Soybean Disease Atlas, 2nd Edition, web resource). Isolate no PP1 was matched with the rRNA sequence of *Phomopsis longicolla* Hobbs AF000207. *Phomopsis longicolla* is the imperfect form (anamorph) of a *Diaporthe* sp. which primarily causes seed decay in soybean. This disease is also widespread and causes endemic disease problems (Chase, 2002). The remaining isolates could be identified to genus level (*Phomopsis* sp. or *Diaporthe* sp.) but could not be matched on their sequences with determined species. It may be because of the lack of species information in GenBank or because they represent previously unknown species. Identification of *Diaporthe*/*Phomopsis* is based on their substrata or hosts as well as on morphological characteristics and at present there is insufficient detailed information to resolve many of the taxonomic and identification problems in these genera.

Figure 4.9 Morphotype-grouping of *Phomopsis* species.



A description of ***Diaporthe phaseolorum* (Cke.&Ell.) Sacc.** is given as a generic example

Surface more or less blackened over wide area; ostiole short conic to elongate-filiform, sinous, 120-400 x 50-80 µm, erumpent separately. Dorsal zone along bark surface; ventral zone usually absent, occasionally present laterally or along the pitch; perithecia small, 160-350 x 110-200 µm, scattered or crowded; asci clavate, 28-46 x 5.5-8 µm; spore biseriate, broad-fusoid, two-celled, hyaline, constricted at the septum, 8-12 x 2-3.5 µm

Hosts: *Phaseolorus lunatus*, *Phaseolus* sp.

Distributed in New Jersey, USA (reported from many points along Atlantic seaboard, according to Harter)

Table 4.5 Possible matches for unknown fungal endophytes from *Cassia*.

Group IV : Other taxa (9 isolates)

Isolate number	Source	Possible matches / GenBank Accession Number	Percentage of similarity
8 (2.6.1/4.1)	Kanchanaburi	*Curvularia gladioli* /AF071337	90%
SC10 (BKK3.3.2/2)	Bangkok	*Coprinus radians* / AF345822	92%
SC32 (BKK2.3.4/3)	Bangkok	*Nectria gliocladioides* /AF106532	99%
SC43 (NK1.4.1/1)	Nakhon Ratchasima	Fungal endophyte / AF413049	89%
N4 (NK2.1.2/1)	Nakhon Ratchasima	*Psathyrella* cf. *gracilis* / AY228352	82%
U1 (WNK4.4.2/1)	Nakhon Ratchasima	*Hypoxylon rickii* / HAN390408	72%
U41 (WNK2.1.4/5)	Nakhon Ratchasima	*Fusarium* sp./ FSU61695	92%
P8 (WNK3.2.4/4)	Nakhon Ratchasima	*Corynespora cassiicola* / AY238606	95%
P22 (WNK2.4.3/3)	Nakhon Ratchasima	*Nectria haematococca* / AF130142	98%

Some of endophytes which have been isolated from *C. fistula* were put into "other taxa group" and identified using molecular techniques. Isolate no.8 was matched to *Curvularia gladioli* AF071337 (*Curvularia trifolli* f. sp. *gladioli* Parmelee&Luttrell) and this fungus causes leaf spot in gladiolus. It also produces corm rot but infrequently foliage and slower infections occur. *Curvularia* is a soil fungus which can live in soil for more than three years. The source of the disease inoculum comes from spores

produced in the spring time. Spores of the fungus can be spread by rain splash or irrigation water and also by leaf movement and air currents. (Miller & Thompson, web resource).

Isolate P8 was identified as *Corynespora cassiicola* (Berk.&Curt.)Wei. AY238606 which is found in tropical and subtropical areas (*Corynespora* Leaf Spot, web resource). This fungus is a phytopathogen which causes brown ring spot illness (Wei, 1950) and leaf spots on *Coleus barbatus* Benth (Fernandes & Barreto, 2003). A survey by the International Rubber Research and Development Board (IRRDB) showed that *C. cassiicola* is a causative agent of *Corynespora* leaf fall disease (CLFD) in rubber plants and this problem is serious and endemic in Asia especially in Indonesia, Sri Lanka and Thailand. This fungus infects rubber leaves at various ages and the symptoms which develop depend on the age of the rubber leaves (International Rubber Research & Development Board, web resource). It produces spores which are spread through wind and rain splash. The conidia are produced throughout the year. Research in Malaysia found that the maximum number of spores are trapped during August to September with maximum liberation at about noon. The climate has an important influence on this disease as well; hot and humid climate will encourage the disease (International Rubber Research & Development Board, web resource). In addition, this fungus also causes target leaf spot in cucumber (*Cucumis sativus* L.). However, this fungus has a particularly wide host range which includes cucumbers, cowpea, soybean, sesame, okra, cotton, tomato and other cultivated and weed species (Abul-Hayja & Williams, web resource). To summarize, *C. cassiicola* is considered to be an aggressive, facultative parasite which sporulates readily on plant debris (Kingsland - International Society for Horticultural Science, web resource).

Isolate numbers SC43 and U41 were both identified as *Fusarium* species which are often initially recognized by their characteristic pink/red soluble pigment which diffuses into the culture medium. SC43 constantly produced red soluble pigment and U41 produce pink soluble pigment. *Fusarium* is a filamentous fungus that is commonly distributed on plants and in soils. It is found as part of the normal mycoflora of commodities, for instance, rice, bean, soybean and other crops (Pitt *et. al.*, 1994). Even though most species of *Fusarium* are more common in tropical and subtropical areas it can also be found in soil in temperate regions. *Fusarium* species are well known to be phytopathogenic but they can also cause various infections in human. The genus *Fusarium* is currently considered to contain more

than 20 species, and the most common ones are *F. solani* (Mart.)Sacc. , *F. oxysporum* Schlcct.:Fr., and *F. chlamydosporum* Wollenw & Reinking(de Hoog *et.al.*, 2000).

Some *Fusarium* species also have a known sexual stage, such as, *Nectria haematococca* Berk. & Br. which is the teleomorphic stage of *F. solani.* Isolate no. P22 was identified as *Nectria haematococca* AF130142. In British Columbia and Ontario, Canada, it has been found that *F. solani /N. haematococca* is the main cause of stem and fruit rot of greenhouse pepper. The yield losses were approximately 5% and this fungus can attack wide variety of plants which including most greenhouse vegetables. The ascospores produced from *N. haematococca* is the key to natural spread in British Columbia greenhouses. The ascospores are released at night and these are more favorable for disease development since periods of high relative humidity and dew occur during this time. Whereas the conidia which are produced by *F. solani* are less important in dissemination of disease (*Fusarium* Stem and Fruit Rot of Greenhouse Pepper, web resource). Another report on root rot caused by *N. haematococca* is from Argentina. Barreto *et al.* (2003) reported root rot of greenhouse olive (*Olea europaea* L.). They found that the anamorphic *F. solani* was frequently isolated while the teleomorph, *N. haematococca*, was rarely found. Other *Nectria* species were identified in this study. Isolate no SC32 was identified as *Nectria gliocladioides* Smalley & H.N.Hansen AF106532. It is a decay fungus which has also been isolated from soybean (*G. max*).

Mostly of the endophytes placed in the "other taxa" group have therefore been identified as phytopathogens such as *Fusarium / Nectria* species, *Corynespora cassiicola* and *Curvularia gladioli.* The reason that they habitat *C. fistulosa.* is still unknown but there is a possibility that these endophytes may also be pathogenic to *Cassia* sp. especially *C. cassiicola* which is reported to have a wide host range (Abul-Hayja & Williams, web resource) and can also cause damage in rubber plantation in Thailand (International Rubber Research & Development Board, web resource). Another possibility is that these fungi are inhabiting *Cassia* and produce the propagules which can then be transferred to other plants nearby and that these propagules will then spread the diseases throughout the area. These pathogens can be seen to follow the description of an endophyte as given by Petrini (1991) in that an endophyte is an organism that can live inside plant tissue for at least part of their life cycle without causing any symptom disease. In this study of *Cassia* trees all

samples were healthy and did not show any disease symptoms but perhaps if they had been left longer then disease symptoms might have become apparent.

Isolate no U1 was identified as *Hypoxylon rickii* Ju & Rogers which is a good member of the Xylariaceae (Ju & Rogers, 1996). It was originally placed in the "other taxa" group because its cultural characteristics were atypical Xylariaceae and it did not fit with other *Xylaria* species isolated at first. It produces a soluble blackish green pigment into the culture medium when it is growing. It is also a slow-growing fungus which covers the whole 9cm Petri dish within one month. It failed to produce any spores and remained sterile. There is no report of this fungus as a phytopathogen.

Interestingly, 2 basidiomycetes were identified in this study. The first one is isolate number SC10 which was identified as *Coprinus radians* (Desm.:Fr.)Fr. AF345822 and the second one is N4 which was identified as *Psathyrella gracilis* (Fries)Quëlet. AY228352. *Coprinus radians* normally grows indoors and often forms a fuzzy yellowish orange mat of mycelium (*Coprinus radians*, web resource). *Psathyrella gracillis* is ubiquitous in parks. Even though it is fragile and short-lived, it can be found throughout the mushroom season, with fresh flushes appearing after the rain. This mushroom is recognised by a brown hygrophanous cap which soon fades to a pale buff-brown. The disc is usually dingy yellowish-brown, and the stipe is slender with a pruinose apex.

The identified *"other taxa"* endophytes are described below:

***Coprinus radians* (Desm.:Fr.) Fr.**

Coprinus radians
Copyright © by Hans Bender
http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/radians_.htm

Pileus: up to 30 x 25 mm when still closed, subglobose, ovoid or ellipsoid, expanding to conical or convex and then up to 50 mm wide, centre golden yellow or ochre, covered with a layer of felty, whitish veil, breaking up into small, white to cream woolly flocks, those becoming yellow- to red-brown at centre of pileus; Lamellae, L = c.60-70, l = 3-5, free, first white then grey-brown to black, 3-8 (-10) mm broad, stipe 30-80 x 2-7 mm, white, base clavate, sometimes with a volva-like margin and often attached to a rust coloured ozonium; Spores 8.5-11.5 x 5.5-7 µm, Q = 1.5-1.9, cylindrical-ellipsoid or ellipsoid, in side view a few phaseoliform, with rounded base and apex, medium to dark red-brown, germ spore eccentric, c. 1.3 µm wide; Basidia 18-34 x 8-9 µm, 4-spored, surrounded by 3-6 pseudoparaphyses; Pleurocystidia 50-120 x 30-65 µm, subglobose, ellipsoid, broadly utriform or subcylindric; Cheilocystidia lageniform and 30-6 µm0 x 12-20 x 5-10 µm or (sub)globose, ellipsoid, ovoid, broadly utriform and 40-100 x 25-50 µm; Caulocystidia 35-100 x 10-28 x 7-12 µm, lageniform; Veil 20-80 x 5-45 µm, made up of chains of cylindrical to ellipsoid, fusoid or (sub)globose cells, if (sub)globose than towards end of the chain and 25-45 µm in diameter, thin-walled to somewhat think-walled and brownish towards end of chain and from centre of pileus; Pileipellis a epithelioid hymeniderm; Clamp-connections absent, only pseudoclamo found.

Occurs in fasciculate groups, sometimes around trunks or on logs, and branches of deciduous trees. Wide-spread in Europe but not common.

***Corynespora cassiicola* (Berk. & Curt.) Wei**. (Ellis, 1971)

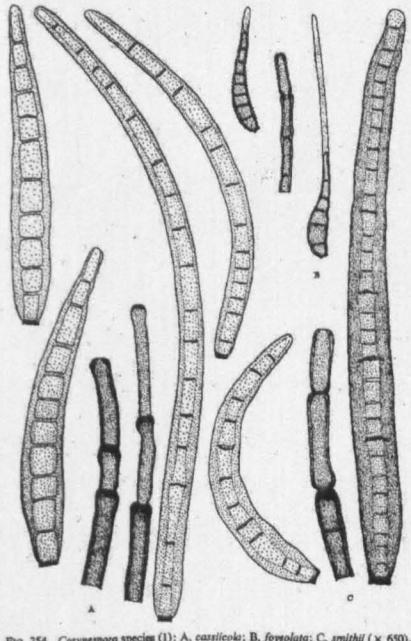


FIG. 254. *Corynespora* species (1): A, *cassiicola*; B, *fawcettii*; C, *smithii* (× 650).

(Ellis, 1971)

Colonies: effuse, grey or brown, thinly hairy; viewed under binocular dissecting microscope the conidiophores appear iridescent; Mycelium: mostly immersed, no stroma; Conidiophores: pale to mid brown, with up to 9 successive cylindrical proliferations, 110-850 µm long, 4-11 µm thick; Conidia: solitary or in chains of 2-6, very variable in shape, obclavate to cylindrical, straight or curved, subhyaline to rather pale olivaceous brown or brown, smooth, with 4-20 pseudosepta, 40-220 µm long (up to 520 µm in culture), 9-22 µm thick, 4-8 µm wide at the truncate base.

Common and cosmopolitan species especially in the tropics occurring on a wide range of host plants.

***Curvularia* Boedijn**. (Ellis, 1971)

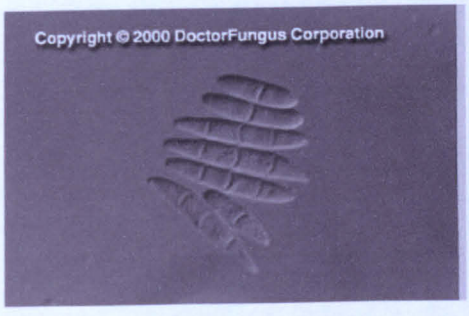
***Curvularia* sp.**
http://www.doctorfungus.org/thefungi/curvularia.htm

Colonies: effuse, brown, grey or black, hairy, cottony ot velvety; Mycelium: immersed in natural substrata; Stromata: often large, erect, black, cylindrical, sometimes branched, formed by many species in culture, especially on firm substrata such as rice grains; Conidiophores: macronematous, monomatous, straight or flexuous, often geniculate, sometimes nodose, brown, usually smooth; Conidiogenous cells: polytretic, integrated, terminal, sometimes later becoming intercalary, sympodial, cylindrical or occasionally swollen, cicatrized; Conidia: solitary, acropleurogenous, simple, often curved, clavate, ellipsoidal, broadly fusiform, obovoid or pyriform with 3 or more transverse septa, pale or dark brown, often with some cells, usually the end ones, paler than the others, sometimes with dark bands at the septa, smooth or verrucose, hilum in some species protuberant. In many species occasional triradiate stauroconidia are formed at the same time as normal conidia.

***C. trifolii* f. sp. *gladioli* Parmelee & Luttrell** (Ellis, 1971)

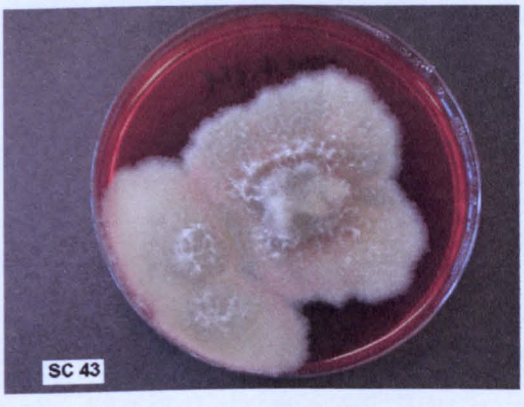
Occurs on *Gladiolus* in Canada, Malaya, Rhodesia and Sierra Leone.

Fusarium sp.



Fusarium sp. ***Fusarium solani***

http://www.doctorfungus.com/thefungi/fusarium.htm

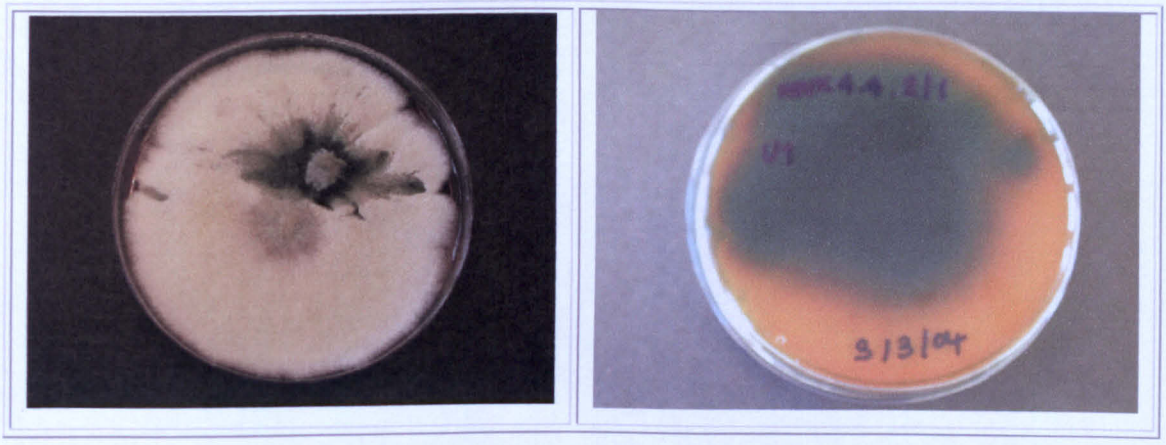


Colonies growing rapidly on Sabouraud dextrose agar incubating at 25°C, woolly to cottony, flat, spreading colonies. *Fusarium dimerum* is the only slow-growing species. From the front, the colour of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink, or purple. From the reverse, it may be colourless, tan, red, dark purple, or brown.

Phialides: cylindrical, with a small collarette, solitary or produced as a component of a complex branching system, monophialides or polyphialides (in heads or in chains); Macroconidia,3-8 x 11-70 µm, from phialides on unbranched or branched conidiophores, 2- or more celled, thick-walled, smooth, cylindrical or sickle- (canoe-)shaped, distinct basal foot cell, pointed distal ends, accumulate in balls or rafts; Microconidia,2-4 x4-8 µm, on long or short simple conidiophores, 1-celled (occasionally 2- or 3-celled), smooth, hyaline, ovoid to cylindrical, arranged in balls (occasionally occurring in chains); Chlamydospores, when present, sparse, in pairs, clumps or chains, thick-walled, hyaline, intercalary or terminal.

Occurs in plants and in soil

***Hypoxylon rickii* Ju & Rogers** (Ju & Rogers, 1996)



Stromata: effused-pulvinate, plane or with inconspicuous to conspicuous perithecial mounds, 0.8-1.5 mm thick, surface rust (39) or sienna (8); orange red granules immediately beneath surface and between perithecia, with KOH-extractable pigments orange (7); the tissue below the perithecial layer black, 0.3-0.9 mm thick; Perithecia: tubular, 0.3-0.5 mm diameter x 0.5-0.8 mm high; Ostioles: lower than the stromatal surface; Asci: 100-150 µm total length x 4-6 µm broad, the spore-bearing parts 45-55 µm long, the stipes 55-100 µm long, J+, discoid, 0.2-0.5 µm high x 1.2-1.5 µm broad; Ascospores: brown, unicellular, ellipsoid-inequilateral, narrowly round ends, 6.5-8 x 3-4 µm, with slightly sigmoid germ slit spore-length; perispore dehiscent in 10% KOH, with inconspicuous coil-like ornamentation; smooth epispore.

Colonies: grow covering Oatmeal agar over 4 week-incubation, whitish at first, then becoming fulvous (43), velvety to felty, azonate, with diffuse margins, reverse dull green (70); Sporulating regions: scattered over entire surface of colony, honey (64); Conidiogenous structure: Nodulisporium-like, hyaline to yellowish, smooth to finely roughened; Conidiogenous cells: hyaline, smooth, 10-20 x 3-3.5 µm; Conidia: hyaline, smooth to finely roughened, subglobose to ellipsoid, 4-6 x 3-4 µm.

Occurs on decorticated wood.

***Psathyrella gracilis* (Fries) Quëlet.**

Psathyrella gracilis
Photo: © Michael Wood
http://www.mykoweb.com/CAF/species/Psathyrella_gracilis.html

Pileus: cap 1.0-4.0 cm broad, obtuse-conic, campanulate to convex-umbonate at maturity, margin striate at first, obscurely so in age, surface moist, hygrophanous, medium-brown fading to pale buff-brown, the disc usually dingy yellowish-brown, flesh very thin, colored like the cap, unchanging, odor indistinct, taste mild; Lamellae, gills adnexed, moderately broad, close to crowded, pale dingy-buff, becoming brownish-grey in age; Stipe, 4-8 cm tall, 1.5-3 mm thick, slender, thin, fragile, stuffed at maturity, more or less equal, sometimes twisted, occasionally continuing as pseudorhiza with sparse white mycelium and adhering substrate, surface pruinose at the apex, often colored purple-brown from adhering spores, amooth elsewhere and concolorous with the cap, veil absent; Spores 11-13 x 5.5-7.0 µm, elliptical, smooth, with an apical pore, spore print purple brown.

Occurs gregariously in disturbed habitats such as wood chips, leaves, open grassy areas; fruiting year-round when moisture is available but especially abundant from late fall to mid winter.

CHAPTER 5

Secondary Metabolites and Antimicrobial Assays

5.1. Secondary metabolites for profiling and identification

"The terrestrial mycelial fungi amongst the eukaryotes, and the actinomycetes amongst the prokaryotes, share a number of physiological and ecological features. The filamentous morphology of both groups is associated with the efficient utilization of exogenous, macromolecular, and often insoluble substrates, as well as the ability to produce a remarkably diverse range of secondary metabolites" (Moss, 1984). Furthermore fungal secondary metabolites have a great diversity of molecular structure and frequently show taxonomic specificity in their production which usually occurs during the stationary phase of growth or idiophase (Bu'lock, 1980). "The best known secondary metabolites have medicinal, industrial, or agricultural impact as antibiotics, toxins, anticancer drugs, growth promoters, hallucinogens, immunosuppressants and so on" (Bentley & Bennett, 1988). Without doubt the fungi are prolific producers of secondary metabolites. Turner (1971) reported almost 4000 fungal compounds in 1971 but by 1983 this had more than doubled (Turner & Aldridge, 1983) and at the current time is believed to be in excess of 10,000 products (Frisvad, Pers Comm).

Dreyfuss and Chapela (1994) stated that many non-taxonomically closely related fungi can produce the same secondary metabolites. In this case, the horizontal transmission of genetic information of fungi may be significant (Metzenberg, 1991; Rodriguez-Valera, 1992). On the other hand, for certain compounds, phylogenetic relationships among metabolite producers may play a role (Bills *et.al.*, 1994). Dreyfuss and Chapela (1994) proposed that metabolites and metabolic production patterns which are specially produced by single strain or by one taxon are the exception. Bills *et al.* (1994) and Wildman (1997) noted that not all isolates of a fungal species known to be a secondary metabolite producer is actually producing the compound.

The value of secondary metabolite production in making systematic decisions depends therefore very much on the fungal genera investigated and on the conditions under which they are cultured. Frisvad, Thrane and Filtenborg (1998) in an extensive review of the role and use of secondary metabolites in fungal

taxonomy concluded that secondary metabolites have been shown to be very reliable and highly diagnostic taxonomic characters in some of the few genera of filamentous fungi, where they have been applied on a large scale. They state that "Combined morphological and secondary metabolite data have yielded clearly circumscribed species in cases where a large number of isolates from different geographic regions and habitats have been examined". They further noted "that in *Penicillium*, *Aspergillus*, and *Fusarium*, which are regarded as especially difficult to classify and identify, secondary metabolites have been particularly effective" (Frisvad *et al.*, 1998). They do however recognize that there are some problems in using secondary metabolites in the taxonomy of filamentous fungi resulting from the apparent inability of a particular taxon or isolate to produce some of their metabolites under the culture conditions employed.

The Xylariaceae have received extensive examination regarding their secondary metabolites as reviewed by Whalley & Edwards (1999). Their major metabolites were grouped as dihydroisocoumarins and derivatives (Anderson, Edwards & Whalley, 1983), succinic acid derivatives (Anderson, Edwards & Whalley, 1985; Adeboya *et al.*, 1996), butyrolactones (Edwards & Whalley, 1979; Anderson *et al.*, 1982), cytochalasins (Edwards, Maitland & Whalley, 1989), sesquiterpine alcohols (punctaporonins) (Edwards, Poyser & Whalley, 1988; Edwards *et al.*, 1989), griseofulvin and griseofulvin derivatives (Whalley & Edwards, 1995), naphthalene derivatives (Whalley & Edwards, 1995), and long chain fatty acids (Edwards *et al.*, 1995; Adeboya *et al.*, 1995). They concluded that in general the occurrence of specific compounds is closely related to the systematic position of the species from which they originated and that chemical data has proved to be invaluable in recognizing associations between species, species groups, and genera (Whalley & Edwards, 1995). Subsequently rosnecatrone, a bicyclo [4.1.0]hept-3-en-2-one, has been reported as unique to *R.necatrix* (Edwards *et al.*, 2001), and Hashimoto & Asakawa (1998) identified more than 20 novel secondary metabolites from stromatal collections of *Daldinia* species Stadler *et al.* (2001) then reported on the secondary metabolites of *Daldinia* species from different geographical locations noting that stromata of *Daldinia* generally contained binaphthyls and that most cultures produced naphthalene and chromane derivatives. Whereas *Hypoxylon* and associated genera produced mellein and derivatives (Stadler *et al.*, 2001). The xylariaceous taxon, *Creosphaeria sassafras* (Schwein.:Fr.) Y.-M. Ju, San Martin, & J.D. Rogers has recently been found to contain the sassafrins (azaphilones) Quang *et al.* (2005) and five unique compounds, the xyloketals, have been isolated from a

seed inhabiting *Xylaria* from a mangrove angiosperm in the South China Sea (Lin *et al.*, 2001a). Later a novel *N*-cinnamoylcyclopeptide containing an allenic ether was isolated from culture filtrates of the same fungus (Lin *et al.*, 2001b) and from *X. multiplex* (Kunze.)Berk. two new 10-membered lactones with antifungal activity have been obtained (Boonphong *et al.*, 2001). Further investigation of metabolites of the Xylariaceae is expected to result in the discovery of further compounds previously unknown. Although the recent emphasis has been on detecting bioactivity in members of the Xylariaceae (Isaka *et al.*, 2000; Lin *et al.*, 2001a; 2001b; Boonphong *et al.*, 2001) their application in systematic studies and use in identification should not be overlooked.

5.2 Results

Therefore in this study the first 9 selected isolates from the *Xylaria*-like groups were grown in malt extract broth, supplemented with 6% glucose, at room temperature (20-25ºC) for 8 weeks (Table 5.1).

Table 5.1 Selected isolates of the Xylaria-like group for secondary metabolite profiling

Isolate number	Possible identification
16	*Xylaria longipes*/AF163038
19	*X. apiculata*/AF163027
21	*X. mali*/AF163040
SC31	*X. longipes*/AF163038
UX1	*X. longipes*/AF163038
UX3	*X. apiculata*/AF163027
UX5	*X. hypoxylon*/XHY309350
PX1	*X. longipes*/AF163038
U65X	*X. longipes*/AF163038

The culture broths were extracted with ethyl acetate and evaporated to concentrate the extracts. The samples were then examined with the collaboration of the natural product chemists, Dr R L Edwards and Dr D J Maitland, at the University of Bradford where chemical profiles were obtained and individual compounds identified from a number of these extracts.

The first nine isolates belonging to the *Xylaria*-like group were analysed and chemical profiles obtained and compounds identified when possible. Isolate number 16 produced phloroglucinol, 5-carboxymellein and cytochalasin D. Isolate number 19 was shown not to belong to the *X. cubensis* (Mont.)Fr. group as it failed to

produce the key compound, cubensic acid (Edwards *et al.*, 1991) and it was also found to produce few, or a low yield of, metabolites. Isolate number 21 was also found not to be a member of the X. *cubensis* group as it also lacked cubensic acid and on the basis of its metabolite pattern is probably related to *X. feejeensis* (Berk.)Fr. However the molecular study on this isolate indicated that the first match on GenBank was *X. mali* AF163040 (see Chapter 4). Isolate number SC31 produced a variety of metabolites which were identified as, cytochalasin D, phloroglucinol, 5-carboxymellein and coriloxin. Isolate number UX1 did not produce coriloxin but instead 40 mg of phloroglucinol was obtained. Isolate UX3 produced cytochalasin D and 4.8 g of phloroglucinol in the culture broth. Isolate UX5 and PX1 were identified by their metabolites not to be in the *X. cubensis* group and although isolate U65X is also not a member of the *X. cubensis* group on the basis of its metabolites it also has a different TLC metabolite profile to the others and as it also produced 1.1 g of an unknown compound it would appear to be a different *Xylaria* species to all the others examined.

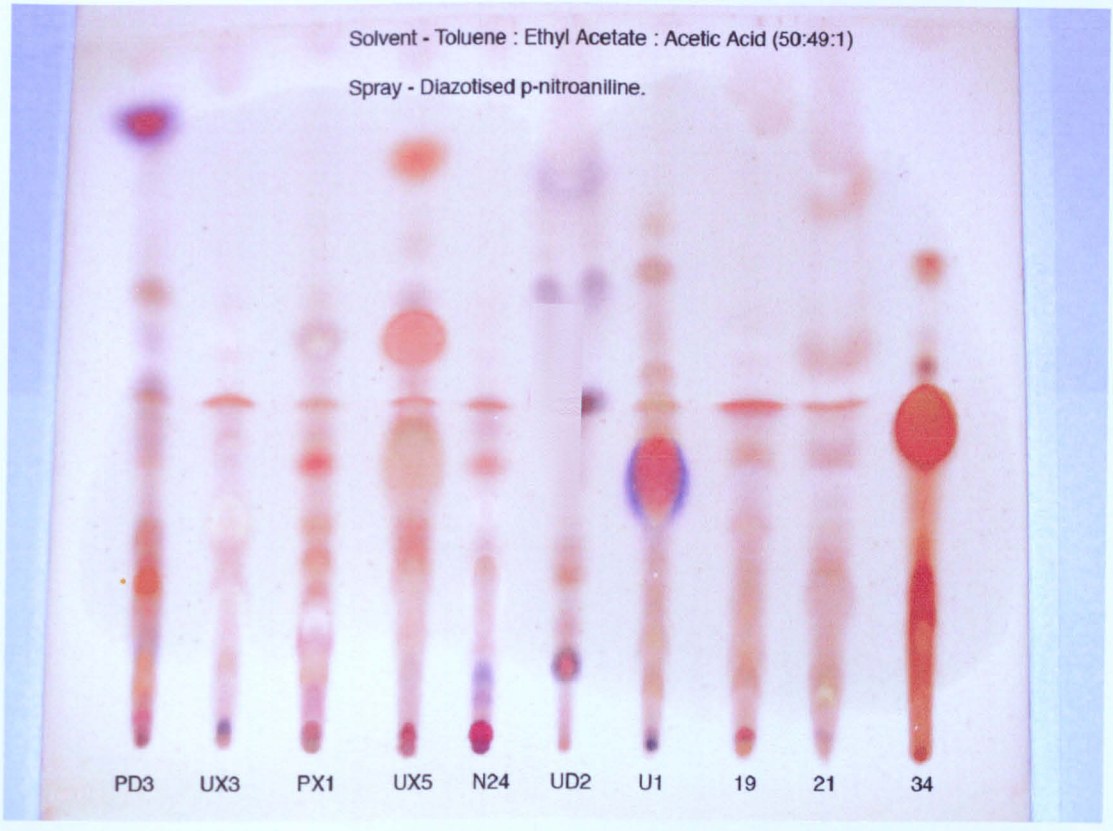
The second batch of endophytes (Table 5.2),10 selected isolates from the *Xylaria*-, *Daldinia*-, and *Nodulisporium*-like groups, were grown in the same conditions (malt extract broth, supplemented with 6% glucose, at room temperature (20-25ºC) for 8 weeks), in an attempt to study metabolites from different species of Xylariaceae taking into account the identifications based on molecular methods (Chapter 4).

Table 5. 2 Selected isolates for the study of their secondary metabolites

Isolate number	Possible identification
19	*X. apiculata* / AF163027
21	*X. mali* / AF163040
34	Partially similar to *X. apiculata* / AF163027
UX3	*X. apiculata* / AF163027
UX5	*X. hypoxylon* / XHY309350
PX1	*X. longipes* / AF163038
N24	*Rosellinia arcuata* / AB017660
UD2	*D. concentrica* /AF176958
PD3	*Nodulisporium* sp. JP3665 /AF280628
U1	*Hypoxylon rickii* / HAN390408

Thin Layer Chromatography was performed with isolates from different groups in order to differentiate these isolates using patterns of secondary metabolites. The results are shown in Figure 5.1 and also in Appendix IV.

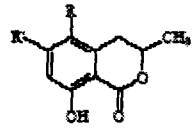
Figure5.1 TLC patterns of *Xylaria*-like endophytes



As in Figure 5.1 it was clearly shown that most of the endophytic isolates exhibited different secondary metabolite patterns. The most outstanding ones are numbers 34 and N24. Isolate number 34 did not match any of results in GenBank (Chapter 4). It was classified as unknown but some part of its ITS region resembles *X. apiculata*. On the TLC plate it was seen to display a distinctive big dark orange spot. For isolate number N24 which has been identified as *R. arcuata* or *R. necatrix* from GenBank a number of interesting compounds were found. These include the 19-20 epoxide of cytochalasin C described originally from *X. obovata* (Abete *et al.*, 1997) and ά-glucoside 1-8 dihydroxynaphthalene. The presence of these compounds in the absence of cytochalasin E and rosnecatrone strongly supports the identification of N24 as *R. arcuata* and nor *R. necatrix*. Isolate UD2 and PD3 are from *Daldinia* and *Nodulisporium* group respectively. It can be seen that they produced different metabolites and it is possible that using metabolite profiles along with DNA blast results some species problems can be resolved. For isolate number U1 which was identified previously on the basis of its DNA sequences as *H. rickii*. Its TLC pattern clearly showed the profile which was distinctive from the others. Within the *Xylaria*-like group (isolate number UX3, PX1, UX5, 19, 21 and 34), the results also showed variation in metabolite profile patterns.

In this study some interesting metabolites from endophytic fungi were isolated and studied. Describtions of the metabolite are described as follows:

5-Carboxymellein (Pittayakhajonwut, 2000)



(1) R=H, R'=H
(2) R=CH_3, R'=H
(3) R=CHO, R'=H
(4) R=CO_2H, R'=H
(5) R=CO_2CH_3, R'=H
(6) R=CH_2OH, R'=H
(7) R=CH_3, R'=OCH_3

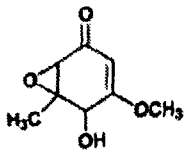
(5) = 5 carboxymellein

(Whalley & Edwards, 1998)

This compound gave a yellow coloration with diazotized p-nitroaniline and bromocresol green on the TLC plate. The first reagent indicated the substance as a phenol or an enol and the second reagent indicated the substance as an acid.

5-Carboxymellein has been isolated earlier in low yield from xylariaceous fungi; *Hypoxylon illitum* (Schwein.)M.A. Curtis, *H. mammatum* (Wahlenberg) P. Karst. and *Nummularia discreata* (Schwein.)Tul. & C. Tul. (Anderson, Edwards & Whalley, 1983). Later, this compound was found to be produced in culture of *Phomopsis oblonga*, the fungus which causes Dutch Elm Disease (Claydon, Grove & Pople, 1985). There also is a suggestion that 5-Carboxymellein has anti-boring and anti-feeding deterrent activity. Chinworrungsee *et.al.* (2001) also isolated this substance from the marine fungus *Halorosellinia oceanica* and also found that this compound possesses antimalarial activity with IC_{50} value of 4 µg/ml and moderate cytotoxicity against KB and BC-1 cell line with IC_{50} value of 1-13 µg/ml. This compound has also been isolated from wood infected by unknown fungi (Alvarenga & Braz-Fo, 1978) and from the shake cultures of *Valsa ceratosperma* (Tode:Fr.) Maire, the pathogenic fungus of apple (Okuno *et.al.*, 1986).

Coriloxin (Pittayakhajonwut, 2000)

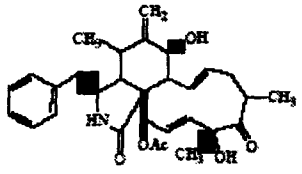


(Edwards *et al.*, 2001)

This compound gave a yellow coloration on TLC plate with the anisuldehyde spray reagent after heating and a violet coloration on TLC plate when sprayed with diazotized p-nitroaniline.

Coriloxin was first isolated from the fungus *Coriolus vernicipes* (Berk.) Murrill (Nissan Chemical Industries Ltd. Patent 1980). It was also later isolated from the xylariaceous fungus, *Xylaria obovata* Berk. (or *X. fibula* Massee) (Adeboya,1995).

Cytochalasin D (Pittayakhajonwut, 2000)



(Whalley & Edwards, 1998)

Cytochalasin D gave a yellow coloration with the anisaldehyde spray reagent.

Cytochalasin D from *Zygosporium mansonii* S. Hughes is available commercially (Vederas *et.al.*, 1975). This is one of the most commonly found cytochalasins from various xylariaceous fungui such as *Hypoxylon terricola* Mill (Edwards, Maitland & Whalley, 1989) and *Xylaria cubensis* (Edwards, Maitland & Whalley, 1991).

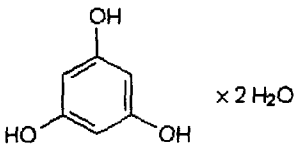
Cytochalasins are a group of fungal metabolites which inhibit the addition of G actin to a nucleation site and thus perturb labile microfilament arrays. Cytochalasin B inhibits at approximately 1 µg/ml but at about 5 g/ml it begins to inhibit glucose transport. Cytochalasin D, on the other hand, affects only the microfilament system and therefore is preferable to use (Betina, 1989).

Cytochalasin D also known as Zygosporin A, is an alkaloid drug and is a potent inhibitor of actin-dependent cellular processes such as cell motility and cell division. It is also said to be an antimitotic drug (Betina, 1989).

Cytochalasin D is produce from many fungi such as *Helminthosporium* sp, or *Zygosporium mansonii.* It blocks actin dependant process in cells. However, cells treated with cytochalasin round up and the lamellipodia and microvilli disappear. The total F-actin content of treated cells diminished as stress fibers and cortical thin filaments are no longer visible under the microscope. Therefore, cytochalasin D is believed to bind to G actin and prevent polymerization of actin monomers. Existing

F-actin fibers then depolymerize as the effective concentration of free G-actin become limiting. In some cells, binding of cytochalasin D to G-actin also results in the proteolytic degradation of monomeric actin (Betina, 1989).

Phloroglucinol



The chemical formula of this substance is 1,3,5-trihydroxybenzene. It is used as phloroglucin stain in conjunction with hydrochloric acid and alcohol to detect the presence of mechanical wood pulp in paper which is turned red (Phloroglucinol, web resource). In terms of medicinal purposes it is used in France in the formulation 1,3,5-trimethoxybenzene as an antispasmodic (Pharmaceutical Technology, web resource). This substance is used to treat the pains in digestive functional disorders, in urinary tract and in gynecology (Doctissimo, web resource; BIAM-Phloroglucinol, web resource).

Phloroglucinol is usually isolated from plants such as musuki, *Hagenia abyssinica* (Bruce)Gmelin, (Lounassma, Widen & Huhtikangas, 1973), root of *Leucanthemopsis pulverulenta* (Lag.)Heywood (de Pascual *et.al.*,1982), South African *Helichrysum* species (Jakupovic *et.al.*,1986), *Eucalyptus grandis* W. Hill ex Maiden (Umehara *et.al*, 1998), leaves of *Mallotus pallidus* (Airy Shaw) Airy Shaw (Supudompol, Likhitwitayawuid & Houghton, 2004) in the form of phloroglucinol and its derivatives. It has also been isolated from the brown alga *Sargassum spinuligerum* Sond. (Keusgen *et al.*,1997). Although this compound has been widely found in nature there is no report on phloroglucinol production in fungi.

5.3. Antimicrobial Assays

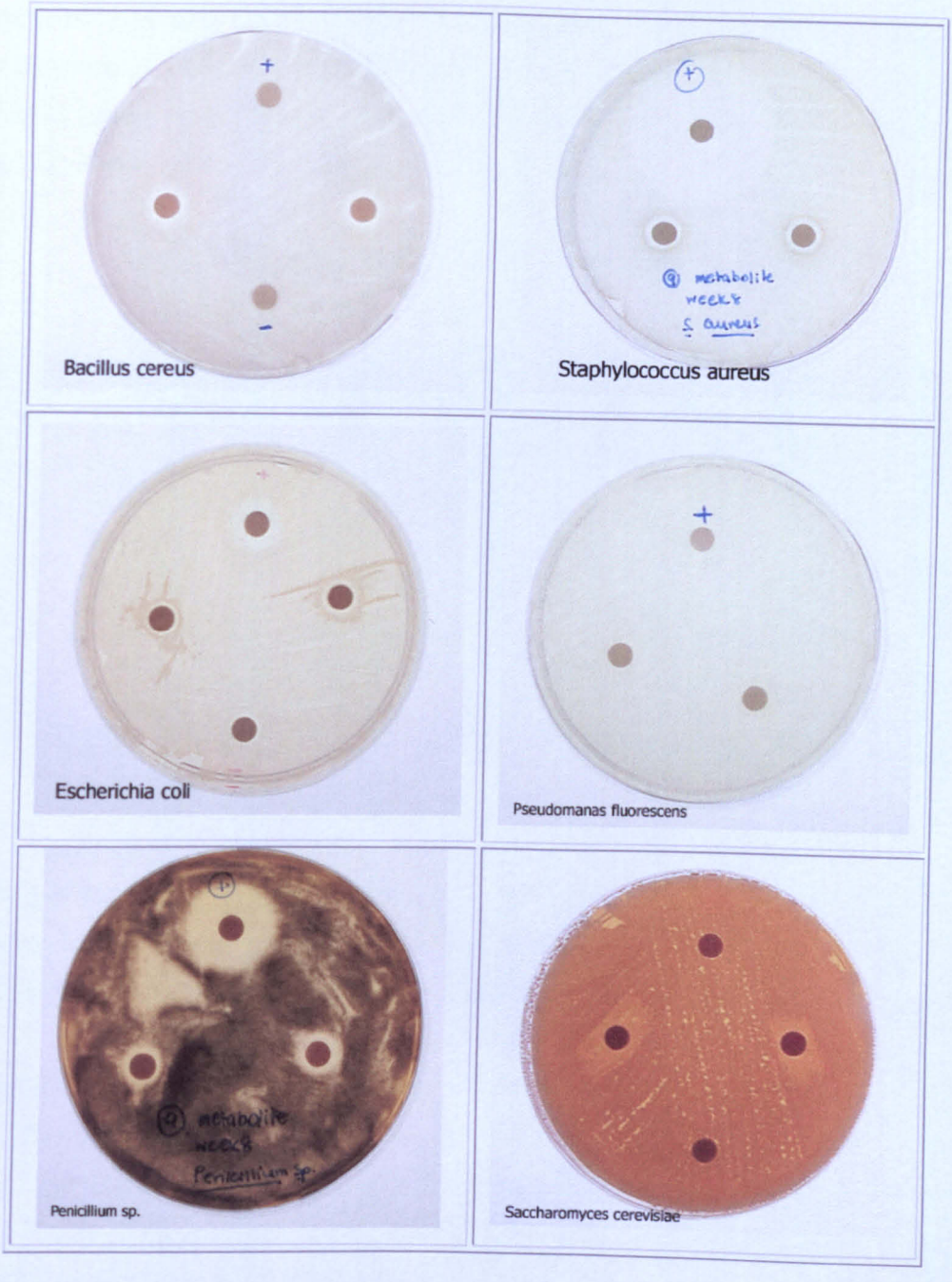
Twenty-two endophytic isolates from *C. fistula* were selected on the basis of their morphological characters to do primary testing for antimicrobial activities. The positive result was determined by the presence of inhibition zone around fugal extract disc. There were 5 isolates from the *Xylaria*-like group, 7 isolates from the *Daldinia*-like group, 4 isolates in the *Phomopsis*-like group, and 6 isolates from other taxa group (Table 5.3 - 5.5). There were 10 isolates showing antimicrobial activities

to tested organisms (Table 5.6, Figure 5.2). Isolates from the *Xylaria*-like group, number 16 inhibited growth of *Escherichia coli* and number 21 and SC3 inhibited growth of *Pseudomonas fluorescens*. These isolates showed their activities in 2 week-incubation culture broth extract (for 16 and 21) and mycelium extract (for SC3) (Table 5.6).

Two isolates from the *Daldinia*-like group showed antibacterial activity. At 6-week incubation, culture broth extract of number SC25 inhibited growth of *Staphylococcus aureus* and after 8-weeks incubation it also showed strong inhibition to both *Bacillus cereus* (from mycelium extract) and *S. aureus* (from culture broth extract). At 2-week incubation, mycelium extract of isolate number SC44, showed inhibition to *E. coli* (Table 5.6).

Another 4 isolates in "other taxa" group showed inhibition at various stages. Isolate number 9 inhibited the growth of *S. aureus* and *Penicillium* sp. with 8-week incubation culture broth extract. Isolate number 20.2 showed the inhibition of 2-week incubation culture broth extract to *E. coli*, *Saccharomyces cerevisiae* and *Penicillium* sp. Culture broth extract of isolate number SC32 showed inhibition to *B. cereus* in both 2-week and 6-week incubation samples. It also inhibited *S. aureus* in all 2-week, 6-week, and 8-week culture broth samples. *Escherichia coli* and *Ps. fluorescens* were also inhibited by 2-week culture broth extracts and 2-week mycelium extract respectively. Finally, isolate number SC43 showed inhibition against *B. cereus* with both 2-week and 4-week culture broth and mycelium extracts. *Escherichia coli* was also inhibited by 2-week incubation culture broth extract (Table 5.6).

Figure 5.2 Primary antimicrobial assay



Subsequently the antimicrobial assay was conducted again with 13 *Xylaria*-like group isolates, *Daldinia*-like group (13 isolates), *Phomopsis*-group (9 isolates) and one isolate of a *Fusarium* endophyte. All 36 isolates were cultured in malt broth for 8 weeks and the mycelium and culture broth were then separated and extracted using ethyl acetate. *Aspergillus niger* and *Candida albicans* were added as additional test microorganisms.

Table 5.7 - 5.9 illustrate the results of antimicrobial assays of all three groups. It is clearly shown in Table 5.7 that members in the *Xylaria*-like group did not exhibit antimicrobial activity. Isolate number SC43 which was later identified as a *Fusarium* sp. (Chapter 4) was also tested against the microorganisms. It was found that a mycelial extract from SC43 showed inhibition to *B. cereus* and *Sac. cerevisiae* and also minor inhibition to *Ps. fluorescens* (Figure 5.3).

Figure5.3 Antimicrobial activity of isolate number SC43

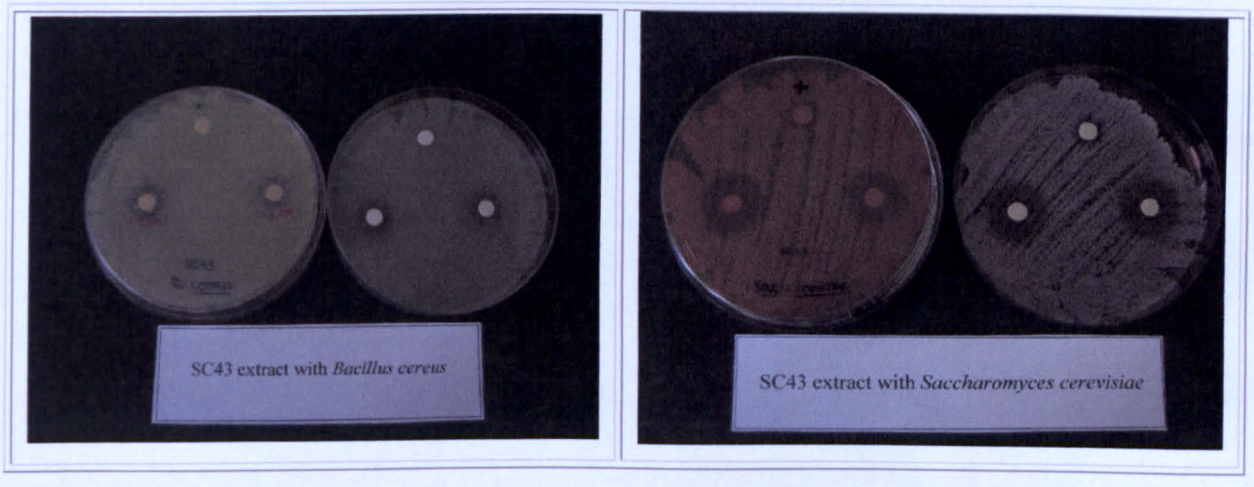


Table 5.8 shows the results from *Daldinia*-like group. It illustrates in the table that all isolates in this group but number UD2 did not show any antimicrobial activity. Mycelium extract from number UD2 exhibited antibacterial activity against *B. cereus*.

Table 5.9 demonstrated the results from *Phomopsis*-like group. Isolate number SC8, PP1, P2, P12, UP3 and UP4 did not show antimicrobial activity. However, there were 3 isolates showing sign of antifungal activity in the form of a non-sporulation zone (Figure 5.4). Mycelial extract from number 22 showed antifungal activity to *Aspergillus niger* whilst its culture broth extract inhibited *Penicillium* sp. For isolate number SC27, both mycelial and culture broth extracts could inhibit *A. niger*. At the same time, isolate number PP3 also displayed the same results, both mycelium and culture broth extract showed inhibition of *A. niger*.

Figure5.4 *Phomopsis*-like isolates showing antifungal activity as non-sporulation zone

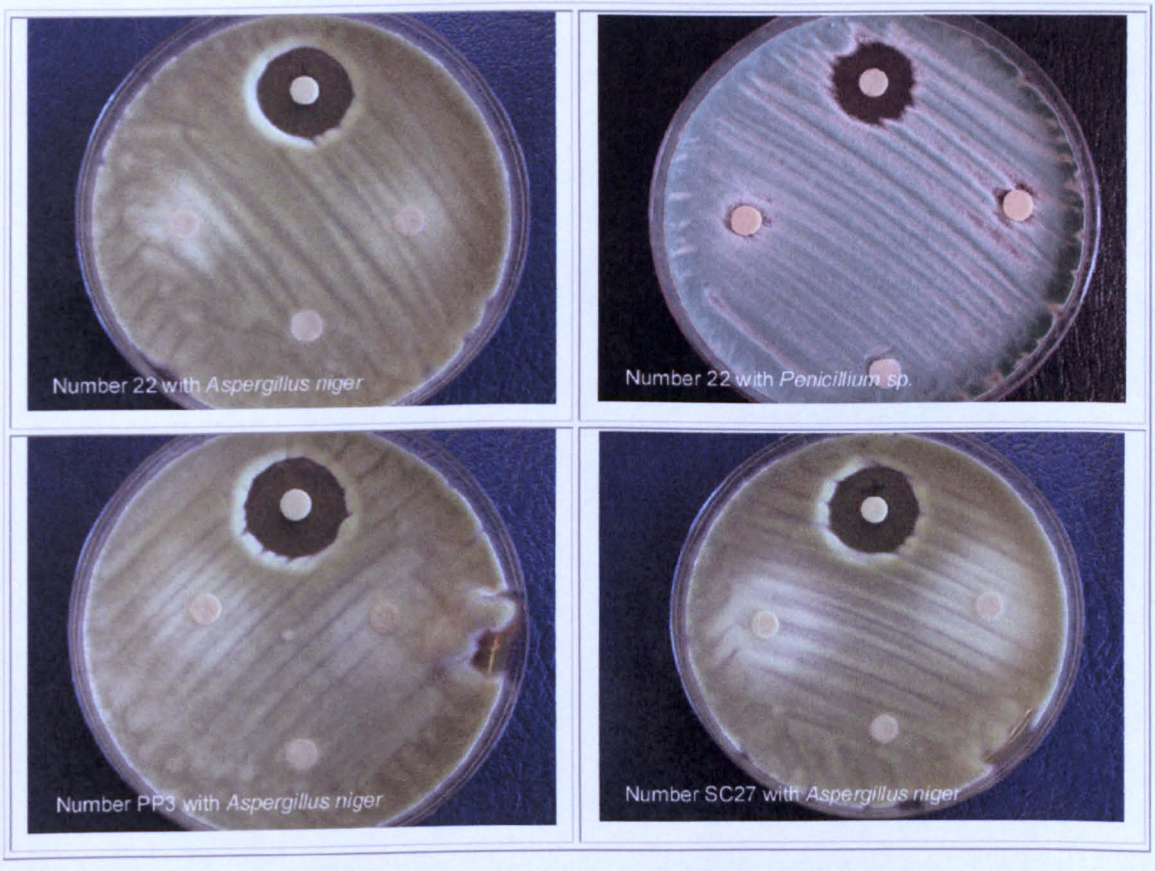


Table 5.3 The result of disc assay of mycelium and metabolite of tested isolates on week 2 (October 25 , 2001)

Tested isolates		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Pennicillium sp.*
3 (1.5.1/4)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	+	-	-	-	-
16 (3.4.2/5)	Mycelium	-	-	-	+/-	-	-
	Metabolite	-	+/-	+	-	-	+/-
19 (3.6.4/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
20.2 (3.8.4/3.2)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	+/-	+	-	+	+
21 (4.1.1/5)	Mycelium	-	-	-	+/-	-	-
	Metabolite	-	-	+/-	+	-	-
SC25 (BKK1.5.3/1)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	+/-	-	-	-
SC27 (BKK1.5.5/5)	Mycelium	-	-	-	-	-	-
	Metabolite	+	-	-	+/-	-	+/-
SC31 (BKK2.3.3/5)	Mycelium	-	-	-	+/-	-	-
	Metabolite	-	-	-	-	-	-
SC43 (NK1.4.1/1)	Mycelium	+	-	-	-	-	-
	Metabolite	+	-	+	-	-	-
SC44 (NK2.3.2/2.2)	Mycelium	-	-	+	-	-	-
	Metabolite	-	+/-	+/-	-	-	+/-

Note: + = positive, - = negative, +/- = weak positive

Table 5.3 The result of disc assay of mycelium and metabolite of tested isolates (batch 2) on week 2 (January 30 , 2002)

Tested isolates		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Pennicillium sp.*
9 (2.6.1/4.2)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
11 (2.6.3/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
20.1 (3.8.4/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
22 (4.1.5/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
25 (5.1.1/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
28 (5.1.4/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC3 (NK1.2.5/5)	Mycelium	-	-	-	+	-	-
	Metabolite	-	-	-	-	-	-
SC20 (BKK1.3.1/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC32 (BKK2.3.4/3)	Mycelium	-	-	-	+	-	-
	Metabolite	+	+	+	-	-	-
SC33 (BKK2.4.6/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC40 (BKK3.2.5/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
N26 (NK3.2.3/1)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-

Note: + = positive, - = negative, +/- = weak positive, N/A = not being tested

Table 5.4 The result of disc assay of mycelium and metabolite of tested isolates on week 6 (November 22, 2001)

Tested isolates		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Pennicillium sp.*
3 (1.5.1/4)	Mycelium	-	+/-	+/-	-	-	-
	Metabolite	-	+/-	-	+/-	-	-
16 (3.4.2/5)	Mycelium	-	+/-	-	+/-	-	-
	Metabolite	-	+/-	-	+/-	-	-
19 (3.6.4/3)	Mycelium	-	+/-	+/-	+/-	-	-
	Metabolite	-	-	-	-	-	-
20.2 (3.8.4/3.2)	Mycelium	+/-	-	-	-	-	-
	Metabolite	-	+/-	-	+/-	-	-
21 (4.1.1/5)	Mycelium	-	+/-	-	+/-	-	-
	Metabolite	-	-	-	-	-	-
SC25 (BKK1.5.3/1)	Mycelium	-	+/-	-	-	+/-	-
	Metabolite	+/-	+	-	+/-	-	+/-
SC27 (BKK1.5.5/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	+/-	-	-	-	-
SC31 (BKK2.3.3/5)	Mycelium	+/-	+/-	+/-	-	-	-
	Metabolite	+/-	-	-	+/-	-	-
SC43 (NK1.4.1/1)	Mycelium	+	+/-	-	-	-	-
	Metabolite	+	+/-	-	+/-	-	-
SC44 (NK2.3.2/2.2)	Mycelium	-	+/-	-	-	-	-
	Metabolite	-	-	-	-	-	-

Note: + = positive, - = negative, +/- = weak positive

Table 5.4 The result of disc assay of mycelium and metabolite of tested isolates (batch 2) on week 6 (February 27, 2002)

Tested isolates		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Pennicillium sp.*
9 (2.6.1/4.2)	Mycelium	-	-	-	-	-	-
	Metabolite	N/A	N/A	N/A	N/A	N/A	N/A
11 (2.6.3/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
20.1 (3.8.4/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
22 (4.1.5/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
25 (5.1.1/3)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	-	-	-	-	-
28 (5.1.4/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC3 (NK1.2.5/5)	Mycelium	-	-	-	-	-	-
	Metabolite	N/A	N/A	N/A	N/A	N/A	N/A
SC20 (BKK1.3.1/5)	Mycelium	-	-	-	-	-	-
	Metabolite	N/A	N/A	N/A	N/A	N/A	N/A
SC32 (BKK2.3.4/3)	Mycelium	-	-	-	-	-	-
	Metabolite	+	+	-	+/-	-	+
SC33 (BKK2.4.6/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC40 (BKK3.2.5/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
N26 (NK3.2.3/1)	Mycelium	-	-	-	-	-	-
	Metabolite	-	+/-	+/-	-	-	-

Note: + = positive, - = negative, +/- = weak positive, N/A = not being tested

Table 5.5 The result of disc assay of mycelium and metabolite of tested isolates on week 8 (December 7, 2001)

Tested isolates		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Pennicillium sp.*
3 (1.5.1/4)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	+/-	+/-	-	-
16 (3.4.2/5)	Mycelium	-	-	-	+/-	-	-
	Metabolite	-	-	+/-	+/-	-	-
19 (3.6.4/3)	Mycelium	-	-	+/-	-	-	-
	Metabolite	+/-	-	-	-	+/-	-
20.2 (3.8.4/3.2)	Mycelium	-	-	+/-	-	-	-
	Metabolite	+/-	-	+/-	-	-	-
21 (4.1.1/5)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	-	+/-	-	-	-
SC25 (BKK1.5.3/1)	Mycelium	+	+/-	-	-	+/-	+/-
	Metabolite	-	+	+/-	+/-	-	-
SC27 (BKK1.5.5/5)	Mycelium	-	-	-	-	+/-	-
	Metabolite	-	-	+/-	+/-	-	-
SC31 (BKK2.3.3/5)	Mycelium	-	-	-	+/-	+/-	-
	Metabolite	-	-	-	-	-	-
SC43 (NK1.4.1/1)	Mycelium	-	-	-	+/-	+/-	-
	Metabolite	-	-	+/-	+/-	-	-
SC44 (NK2.3.2/2.2)	Mycelium	-	-	-	+/-	-	-
	Metabolite	-	-	-	-	-	-

Note: + = positive, - = negative, +/- = weak positive

Table 5.5 The result of disc assay of mycelium and metabolite of tested isolates (batch 2) on week 8 (March 13, 2002)

Tested isolates		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Pennicillium sp.*
9 (2.6.1/4.2)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	+	-	-	-	+
11 (2.6.3/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
20.1 (3.8.4/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
22 (4.1.5/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
25 (5.1.1/3)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	-	-	-	-	-
28 (5.1.4/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC3 (NK1.2.5/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC20 (BKK1.3.1/5)	Mycelium	-	-	-	-	-	-
	Metabolite	+/-	-	-	-	-	-
SC32 (BKK2.3.4/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	+	-	-	-	+
SC33 (BKK2.4.6/5)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
SC40 (BKK3.2.5/3)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-
N26 (NK3.2.3/1)	Mycelium	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-

Note: + = positive, - = negative, +/- = weak positive, N/A = not being tested

Table 5.6 Summary of positive result in Antimicrobial activity assays

	Tested organisms																	
Isolate code	*B. cereus*			*S. aureus*			*E. coli*			*Ps. fluorescens*			*S. cerevisiae*			*Penicillium* sp.		
week	2	6	8	2	6	8	2	6	8	2	6	8	2	6	8	2	6	8
3	☺																	
9						☺												☺
16							☺											
20.2							☺						☺			☺		
21										☺								
SC3										☺								
SC25			☺		☺	☺												
SC32	☺	☺		☺	☺	☺	☺			☺							☺	
SC43	☺☺	☺☺					☺											
SC44							☺											

Note: ☺ culture broth extract
☺ mycelium extract

Table 5.7 The result of disc assay of mycelium and metabolite of tested isolates incubating for 8 weeks
Xylaria-like group

Isolate No.		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Candida albicans*	*Aspergillus niger*	*Penicillium sp.*
16	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
19	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
21	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
29	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
34	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
N4	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
N24	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UX1	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UX2	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UX3	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UX5	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
PX1	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
U65X	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC43	Mycelium	-	-	-	+/-	-	-	-	-
(*Fusarium* sp.)	Metabolite	+	-	-	-	+	-	-	-

Note: + = positive, - = negative, +/- = weak positive, N/A = not being tested

Table 5.8 The result of disc assay of mycelium and metabolite of tested isolates incubating for 8 weeks *Daldinia*-like group

Isolate No.		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Candida albicans*	*Aspergillus niger*	*Penicillium sp.*
20.1	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC4	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC18	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC19	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC25	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC33	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC40	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC42	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC44	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
N26	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
N37	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UD2	Mycelium	+/-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
PD2	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-

Note: + = positive, - = negative, +/- = weak positive, N/A = not being tested

Table 5.9 The result of disc assay of mycelium and metabolite of tested isolates incubating for 8 weeks *Phomopsis*-like group

Isolate No.		*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas fluorescens*	*Saccharomyces cerevisiae*	*Candida albicans*	*Aspergillus niger*	*Penicillium sp.*
22	Mycelium	-	-	-	-	-	-	+	-
	Metabolite	-	-	-	-	-	-	-	+
SC6	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
SC27	Mycelium	-	-	-	-	-	-	+	-
	Metabolite	-	-	-	-	-	-	+	-
PP1	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
PP3	Mycelium	-	-	-	-	-	-	+	-
	Metabolite	-	-	-	-	-	-	+	-
P2	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
P12	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UP3	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-
UP4	Mycelium	-	-	-	-	-	-	-	-
	Metabolite	-	-	-	-	-	-	-	-

Note: + = positive, - = negative, +/- = weak positive, N/A = not being tested

5.4 Discussion and Conclusions

Secondary metabolites are the products of cellular metabolism that are more restricted in their distribution than primary metabolites and are found in most species but being generally constant in a single fungal family (Campbell, 1984). Fungal species have conventionally been characterized by similar morphological differentiation in representative isolates and therefore chemical differentiation products can also be expected to be species specific (Frisvad, Thrane & Filtenborg, 1998). Each fungus therefore produces signature metabolites which can be used as a key characteristic. Secondary metabolites have a potential for the characterization of species and for phylogenetic relationships and have proved to be very valuable as shown by chemotaxonomic studies in *Penicillium, Fusarium* and the Xylariaceae (Frisvad, 1994, Whalley & Edwards, 1995). Furthermore, chemical analysis of secondary metabolites also provides more comparative results than traditional descriptions of colour and odor as seen in the lichens (Huneck & Yoshimura, 1996). In many filamentous fungi genera e.g. *Penicillium* morphological data are limited or very variable, and in these cases, secondary metabolites have helped in resolving taxonomic problems (Frisvad, Thrane & Filtenborg, 1998).

How reliable and useful the application of secondary metabolites is for taxonomy purposes is open to debate as there are different opinions regarding whether they are strain-specific (Engel *et al.*, 1982; Vining, 1992) or depending on environment factors (Bu'Lock, 1980; Moss, 1984; Brunner & Petrini, 1992; Monaghan *et al.*, 1995). However, the problem concerning environmental factors can be solved by optimizing and standardizing the best culture conditions (Frisvad, Thrane & Filtenborg, 1998). There is also the criticism raised against the use of secondary metabolites to differentiate between fungi since in many cases the same metabolite can be produced from unrelated species. However, in some cases, secondary metabolites are produced by closely related fungal groups which can indicate a close phylogenetic relationship (Frisvad, Thrane & Filtenborg, 1998). While individual secondary metabolites are important in chemotaxonomy the profile of secondary metabolites is considered more reliable in fungal characterization. The secondary metabolite profile (SMP) concept was introduced by Frisvad and Filtenborg (1983) to *Penicillium* taxonomy but it has later been modified to include other expressions of differentiation. The Xylariaceae is one of the only families of the Ascomycotinia to have received extensive studies of its secondary metabolites (Frisvad *et al.*, 1998). The members in this group, *Hypoxylon*, *Daldinia*

Biscogniauxia, *Entonaema* *Camillea*, *Kretschmaria*, *Nemania*, *Xylaria*, and *Rhopalostroma* have been found to produce compounds which have systematic significance such as dihydroisocoumarins, succinic acids derivatives, butyrolactones, cytochalasins, punctaporonins, naphthalenes, mitorubrins and griseofulvins. Some minor compounds are also produced in small quantities in the stroma. Even though they are difficult to detect it is still very useful to include them to get the full picture of secondary metabolite profile of the fungi in this group (Whalley & Edwards, 1987, 1995). Whalley and Edwards (1995) noted that the secondary metabolites should be taken along with other methods and they may be valuable in separating closely related species and predicting intra- and intergeneric associations. In a series of recent studies on the chemicals in both stromata and produced in culture Stadler *et al.* (2001a) have described new species of *Daldinia* based on chemical profiles, ascospore morphology and molecular data. They have also investigated metabolites in many of the Xylariaceae using HPLC-UV visible (diode array) detection and mass spectroscopy (Stadler *et al.*, 2001a, b; Quang et al., 2005).

Thin layer chromatography was used in the current study since it has an excellent track record for similar chemotaxonomic studies concerning non-volatile secondary metabolites (Culberson & Kristinsson, 1970; Filtenborg & Frisvad, 1980; Filtenborg, Frisvad & Svendsen, 1983; Frisvad & Filtenborg, 1983; Culberson & Elix, 1989; Paterson & Bridge, 1994). Furthermore nearly all known secondary metabolites can also be detected by TLC (Frisvad, Thrane & Filtenborg, 1998).

Since the identification of xylariaceous fungi in culture is a major problem, for reasons discussed earlier, secondary metabolite profiles of the unknown isolates were compared with those from known species and matched when possible (Whalley & Edwards, 1998).

One of the most interesting metabolites which was obtained from *Cassia* endophytes is phloroglucinol (1,3,5-trihydroxybenzene). It is widely used in wood staining and, in France, it has been used as an antispasmodic agent. It is mostly isolated from a wide ranges of plant species (Lounassma, Widen & Huhtikangas, 1973; de Pascual *et al.*, 1982; Jakupovic *et al.*, 1986; Umehara *et al*, 1998; Supudompol, Likhitwitayawuid & Houghton, 2004) and a single species of an alga (Keusgen *et al.*, 1997). This is as far as it is known the first report of phloroglucinol from a fungus. It was found to occur only in species of *Xylaria,* including *X. longipes*

(cf *X. longipes* var. *microspora* and *X. apiculata*). It could therefore be a useful marker compound for these and closely related species.

5-Carboxymellein, coriloxin and cytochalasin D were the main compounds isolated from the *Xylaria* endophytic species. These are chemicals which have been regularly isolated from xylariaceous species. *Xylaria obovata* produced and secreted coriloxin into culture media and could be detected by TLC and HPLC (Adeboya, 1995). Coriloxin was isolated from only one isolate in this study, isolate number SC31 which according to the DNA analysis matched it with *X. longipes* AF163038 (Chapter 4).

5-Carboxymellein has been shown to be a metabolite frequently isolated from a wide range of fungi including many xylariaceous genera. It was detected in low yield from *Hypoxylon illitum*, *H. mammatum* and *Nummularia discreta* (Anderson, Edwards & Whalley, 1983). Whalley and Edwards (1986) noted that the dihydroisocoumarins with 5-methymellain as a common compound are usually associated with *Hypoxylon* species. Even though mellein has been isolated elsewhere, mellein, iso-ochracein and ramulosin are key characteristic components in *Hypoxylon* (Whalley & Edwards, 1995). It has also been isolated from phytopathogens e.g. *Phomopsis oblonga* (Claydon, Grove & Pople, 1985) and *Valsa ceratosperma* (Okuno *et.al.*, 1986). 5-Carboxy mellein found in a mangrove species of the Xylariaceae, *Halorosellinia oceanica* showed antimalarial activity (Chinworrungsee *et.al*, 2001). This compound has now been isolated from 2 isolates included in this study, isolate numbers 16 and SC31, both of which have been matched with *X. longipes* AF163038 (Chapter 4).

The cytochalasins are usually associated with the genera *Xylaria*, *Rosellinia* and *Kretzschmaria* (Whalley & Edwards, 1998, Edwards et al., 2003) and also *H. terricola* Mill (Edwards, Maitland & Whalley, 1989). Isolate numbers 16, SC31 and UX3 all produced cytochalasin D in broth culture

Isolate number 16 and SC31 which were previously identified by DNA analysis as *X. longipes* AF163038 gave results resembling those from a seed inhabiting *Xylaria* sp. (392) from Thailand which was investigated by Pittayakhajonwut (2000). *Xylaria* sp. 392 was found to produce a variety of metabolites including 5-carboxymellein and cytochalasin D. whilst coriloxin was found only in SC31. Thus coriloxin is probably a useful marker for the identitification of *X. longipes* when examined along with other

metabolites and most *X. cf longipes* from this study (16, SC31 and UX1) produced coriloxin, cytochalasin D, 5-carboxymellein and phloroglucinol. Interestingly phloroglucinol has never been found before in xylariaceous fungi, including teak *Xylaria* endophytes which have been studied previously (Pittayakhajonwut, 2000). Possibly as a result of genetic variation resulting from continued subculturing solate number UX3, *X. apiculata* AF163027. produced only cytochalasin D and phloroglucinol. Interestingly isolate number 21 produced key metabolites suggesting that it should be *X. feejeensis* which is in disagreement with DNA sequence match which indicated *X. apiculata*. However there is still a lack of unique key metabolites of each known isolate to identify unknown *Cassia* endophytes although they do add useful data. Thus detection of some metabolites provides sufficient information to group these endophytes together even though there is insufficient data to identify them to species level. It is likely that these phloroglucinol producing *Xylaria* species are either the same or are at least very closely related species.

However, in the case of isolate number N24 for which the DNA sequence results matched with *Rosellinia arcuata* and *R. necatrix* metabolite data can be very helpful in making a decision. These two species are very closely related (Petrini, 2003) and the GenBank sequences of these two are identical. In making a decision whether N24 should be placed with *R. arcuata* or *R. necatrix* the presence of absence of specific compounds now becomes useful. The key metabolite is rosnecatrone which has been detected from every isolate of *R. necatrix* so far examined and this includes a tropical isolate from the Philippines (Edwards *et al.*, 2001; Edwards *et al.*, 2003). However, N24 did not produce rosnecatrone and therefore *R. arcuata* is considered to be the more likely candidate.

In term of antimicrobial activity it is interesting that the *Xylaria* species from this study rarely possessed any antimicrobial ability. Some of the *Xylaria* isolates (16, 21 and SC3) exhibited slight antibacterial activity after two weeks incubation only. It seems likely that *Xylaria* species exhibit biological activity of a different kind. Thus Isaka *et al.* (2000) isolated antiplaspodial compounds from an unidentified *Xylaria* and two novel cytochalasins from *X. obovata* were found to be cytotoxic (Abate *et al.*, 1997). The Xyloketals, isolated from a mangrove *Xylaria* species (Lin *et al.*, 2001) include one which inhibited acetylcholine esterase and a novel cyclic peptone isolated from a seed inhabiting *Xylaria* is being investigated for its bioactivity and Sing *et al.* (1999) presented the structure of a novel eremophilane sesquiterpenoid with HIV-1 Intergrase inhibiting activity. However there are also reports of antifungal

activity from *Xylaria* or closely related fungi. The multiplolides, the new antifungals, were reported from *X. multiplex* (Boonphong *et al.*, 2001) and Abbanat *et al.* (1999) earlier reported on antifungal compounds from *Halorosellinic oceanica*. This latter fungus has also been reported to produce the antimalarial halorosellinic acid (Chinworrungsee *et al.*, 2001) and antimicrobial azaphilones from *Creosphaeria sassafras* (Schwein.:Fr.) Y.-M. Ju, San Martin, & J.D. Rogers have recently been isolated (Quang et al., 2005). Although in this study the bioactivity of the Xylariaceae endophytes was disappointing there is, however, good evidence to indicate that the Xylariaceae, especially *Xylaria* and its allies are a potential source of novel, bioactive metabolites.

In terms of antimicrobial activity species of *Fusarium* proved to be the most effective with activity against bacteria and fungi. Interestingly an endophytic *Fusarium* species isolated from *Croton oblongifolius* Roxb. in Thailand has recently been found to have strong antibacterial activity and activity against a number of fungi (Puriso *et al.*, 2004).

Chapter 6

General Discussion and Conclusions

This study on fungal endophytes of *C. fistula* from Thailand has three main themes. Firstly the examination and comparison of the endophytic assemblages from the three different sampling sites to assess their composition and diversity. Secondly to develop a molecular protocol for identification of isolates considered to be members of the Xylariaceae and, when possible, the identification of *Phomopsis* species and selected non-sporulating isolates. Finally secondary metabolites produced by selected Xylariaceae were extracted and their metabolite profiles examined as an alternative approach to identification and these, together with extracts from other isolates, were tested for their bioactive capabilities. It is recognized that ideally more samples at different times of the year would have been valuable and that it would also have been useful to have examined all isolates regarding their identity and metabolite production but this was clearly not possible because of both time and resource constraints. Nevertheless important data on the diversity of the fungal endophytes of *Cassia* is provided and a successful identification protocol for xylariaceous isolates based on molecular techniques is presented. These techniques were also used to identify a number of other endophytic species which lacked suitable diagnostic features in culture. Additional data for identification of members of the Xylariaceae was based on their secondary metabolite profiles with comparison of metabolites with those obtained from known species previously isolated from their teleomorphic stage. Furthermore primary testing for bioactive properties for some of these fungi was undertaken.

The three sites selected for the investigation proved to be quite different regarding their endophytic fungal populations as was originally anticipated since they had been chosen on the basis of different plant diversity, different tree density and entirely different local environments. The site at Kanchanaburi proved to have the greatest number of endophytes and also the greatest range of species. This site was part of a natural forest, designated a plant genetic preservation area under the auspices of HRH Crown Princess Maha Chakri Sirindhorn. This site contained considerable plant diversity with 84 species identified at this time. *Cassia fistula* is found here as a frequently occurring tree and is very conspicuous with distinctive

growth form and flowers (Gardner, Sidisunthorn & Anusarnsunthorn, 2000). This site although the most natural from which samples were obtained is nevertheless fairly dry and has low rainfall in relation to many of the forests in Thailand (Thienhirun, 1997).

This deciduous forest as illustrated (Chapter 3 Figure 3.4 & 3.4) does however lack the high tree density seen in many other forests but was selected partially because of its importance as part of a conservation project and also because *Cassia* occurred in combination with a recorded high plant diversity consisting of the original natural vegetation. The results on endophytic species and their diversity show this to be the most populous site both in terms of their total numbers and range of species recorded. In accordance with most recent endophytic studies the leaf surface sterilization protocol was tested and the method selected (Petrini, 1986) as later endorsed by Schultz *et al.* (1993) proved to be successful and was chosen as the method used throughout this study. Furthermore leaf samples were obtained from different parts of the leaves in an attempt to recognize differences between fungal endophyte assemblages of the leaf lamina, midrib, and basal region as a number of studies have indicated differences in the sampled area and the species recovered (Bertoni & Cabral, 1988; Petrini & Fisher, 1988; Rodrigues & Samuels, 1990; Rodrigues, 1994; Mekkamol, 1998; Frohlich, Hyde & Petrini 2000; Photita *et al.*, 2001; Arnold & Herre, 2003; Nugent, 2004). As far as the *Cassia* leaves investigated are concerned no appreciable differences in numbers of fungi isolated occurred between the specific parts of the leaf. One sampling was made at this site in December 2000 and a total of 465 fungal endophytic isolates were obtained. It is interesting to note that there was little variation between the 5 tree samples investigated in terms of their total endophytic populations with a range only 86-97 just over 11% between trees with a mean percentage of 93% (Table 3.4). Published data on variation in endophytic mycofloras in samples is not always in clear agreement with some studies showing differences in endophyte infection frequency whilst others do not. Carroll (1995) in reviewing the available data listed factors such as geographically disjunct sites of collection, liquid precipitation, ecological diversity and environmental and climatic conditions. Bernstein and Carroll (1977) found no correlation between height in the crown and endophytic infection frequencies but Johnson and Whitney (1989) found the opposite. Bayman *et al.* (1997) in their investigation of orchid endophytes found that there was considerable variation within individual plants and within individual roots and leaves of seven species of *Lepanthes* sampled. There were significant differences among orchid species in

number and types of endophytes but the heterogeneity of endophytes in single plants and plant organs overshadowed differences occurring between species (Bayman *et al.*, 1997). In the current study there was remarkable similarity between the leaves samples at each site with the exception of one plant sampled at Nakhon Ratchasima (July 2001) where a considerably lower number of isolated were obtained.

The most common species belonged to the genera *Phomopsis* and *Penicillium* but *Daldinia*, *Xylaria* and *Colletotrichum* were also frequently isolated. Most of the other isolates obtained failed to sporulate or to produce distinctive features which would enable an identification to be made. Later as a result of molecular techniques some of these isolates were identified as species of *Curvularia* and a non-sporulating *Daldinia* species.

The results from Nakhon Ratchasima obtained in July 2001 and December 2001 are different in a number of ways. The samples from July were from early in the rainy season and here the total number of isolates was only 127 or 25 per tree (Table 3.4). These were mainly species of *Phomopsis*, *Penicillium* and *Daldinia* species. Mekkamol (1998) found that in teak leaves in northern Thailand *Phomopsis* and *Colletotricum* species dominated early in the rainy season whereas *Daldinia* and *Xylaria* species became more important as the rainy season progressed and by December had become the dominant fungi isolated. Mekkamol used a series of inoculation experiments to induce teleomorph formation in xylariaceous species and was able to assign a large number of her isolates to *D. eschscholzii* (Mekkamol, 1998). She also found that a number of the *Xylaria* isolates would develop to maturity and could be identified as *X. aristata* Mont., *X. cubensis*, *X. feejensis*, *X. grammica* (Mont.)Fr., *X. multiplex*, and *X. juruensis* var *microspora* P. Henn. on the basis of their ascospore dimensions and other features (Mekkamol, 1998).

Samples taken in December, i.e well into late rainy season, resulted in a considerable increase in numbers with a total of 307 isolates with a mean of 61.4 isolates per tree (Table 3.4). Unfortunately it was not possible to compare populations at the start of the rainy season with the end of the rainy season for the other two sites. However the results from Nakhon Ratchasima demonstrate a considerable increase in endophytic isolates from July to December or an increase of 142%. A number of studies on tropical plant endophytes has shown that the age of the leaf is important with young leaves having a considerably lower infection

frequency than older more mature leaves (Rodrigues, 1994; Carroll, 1995; Mekkamol, 1998; Kumaresan & Suryanarayanan, 2002; Arnold & Herre, 2003). Nugent (2004) found that infection rates in *Fraxinus* leaves increased steadily from young to mature leaves indicating that this was mainly a result of a much longer exposure to the potential inoculum in the surrounding environment. Arnold and Herre (2003) had reached a similar conclusion in their studies in a tropical lowland rainforest in Panama.

Daldinia, Xylaria and *Phomopsis* proved to be the dominant fungi from the December samples with *Fusarium* and *Nodulisporium* species as well. It is likely that the *Nodulisporium* isolates represent xylariaceous taxa, especially species of *Daldinia* and *Hypoxylon*, as these are the common anamorph occurring throughout the family (Ju & Rogers, 1996; Whalley, 1996). It has been suggested that the high presence of *Daldinia* and other Xylariaceae as endophytes relates to their ability to infect as ascospores (Gowan & Vigalys, 1991; Whalley, 1996). These are likely to be more resistant to uv light and desiccation than the thin walled and pale conidia of many fungi and therefore have greater survival potential. Ascospores of *Daldinia concentrica* have been shown to be discharged over a several month period (Ingold, 1971) and ascospores in dried herbarium specimens can be germinated up to 25 years from their date of discharge (Nugent, 2004). It was suggested in Chapter 3 that the high presence of *Phomopsis* early in the rainy season might relate to their presence as asexual conidia in the air spora at that time and this is at a time when ascospores of *Daldinia* and its relatives would be absent or in very low numbers since there would be a period of at least 2-3 months before stromata producing ascospores had developed in the immediate forest area (Mekkamol, 1998; Whalley, 2001). Also from this site a number of isolates which remained sterile were later shown following DNA sequence comparisons, to be basidiomycete fungi including of *Coprinus*.and *Psathyrella* species.

The Bangkok site had been selected as being from a very different environmental situation to provide an interesting comparison with natural habitat (Kanchanaburi) and the more rural but not completely urbanized (Nakhon Ratchasimma); and Bangkok representing a fully urban site with different vegetation, tree density, climate and pollution aspects. The total number of isolates was by far the lowest with only a total of 57 isolates from three tree samples or only 19 per tree sampled. The results in terms of species identified is similar to that from Nakhon Ratchasima with *Daldinia, Phomopsis* and *Penicillium* as the main taxa recognized. It is

suggested that the low number of isolates recovered from the Bangkok *Cassia* samples is a result of the individuality of the tree since they were growing as planted ornamentals and were separated from a range of other trees which might provide inoculum of a more diverse nature or make the local environment more favourable for the production of fungal propagules and their subsequent spread and survival. Arnold and Herre, (2003) and other workers have shown that the potential fungal inoculum is related to tree diversity, canopy cover and climatic conditions. In Bangkok the *Cassia* trees samples were from a site lacking in both plant diversity and canopy cover and were also likely to be subjected to atmospheric pollutants not present in either Kanchanaburi or Nakhon Ratchasima. There is little detailed information on urbanization or pollutant effects on endophytic populations although there are reports of air pollution and simulated acid rain affecting the endophytic fungi in pine needles (Helander, 1995; Helander *et al.*, 1994) and birch leaves (Helander *et al.*, 1993). There do not appear to have been parallel studies on any tropical plants. Furthermore the limited studies on Thai endophytic fungi show the Xylariaceae to be regular, and sometimes dominant, members of the endophytic community (Mekkamol, 1998; Chareprasert, 2001; Photita *et al.*, 2001). This has also been shown for studies of tropical plants from other countries as summarized by Rodrigues and Petrini (1997).

It is well known that fungal endophytes isolated from many plants are primarily common genera found to be either necrotrophic or saprotrophic e.g. *Phomopsis*, *Colletotrichum* Corda, *Fusarium*, *Coniothyrium* Corda, *Alternaria* Nees, *Septoria* Sacc., *Hypoxylon* (Bassett & Fenn, 1984; Petrini, 1994; Petrini & Fisher, 1988; Rodrigues & Samuel, 1990; Sieber *et al.*, 1991; Fisher & Petrini, 1992; Fisher *et al.*, 1992; 1994; Whalley, 1996; Edwards *et al.*, 2003). Mekkamol (1998) found *Phomopsis* and *Colletrotricum* species to be major endophytic isolates in teak leaves sampled early in the rainy season but there is no indication that these later became necrotrophic. In the present study on *C. fistula* leaves *Phomopsis* species were regular isolates and later a number of these were assigned to their teleomorphic species of *Diaporthe* by comparison of DNA sequences with those deposited at GenBank (Chapter 4). A number of these are known to be phytopathogens although the diseases they cause appear to be mainly of temperate plants. Other pathogenic fungi identified through their DNA sequences were *Fusarium solani*, or as its teleomorph *Nectria haematococca*, *Curvularia gladioli* which causes leaf spot in gladiolus and *Corynespora cassiicola* which can cause leaf spot in a number of plants but most interestingly in rubber plants. *Corynespora*

cassiicola is the causative agent of so called *Corynespora* leaf fall disease in rubber plants and is endemic in Asia being serious in Indonesia, Sri Lanka and Thailand (International Rubber Research Board). Its occurrence as an endophyte in *C. fistula* is of special interest as it indicates that it might be present as an asympomless infection in a range of plants in Thailand. It was however only isolated from Nakhon Ratchasima during this study and this might relate to the more dry conditions occurring at all of the sites where the *Cassia* trees were sampled. In the south of Thailand where there are extensive rubber plantations the humidity is generally high for much of the year and the rainfall is considerably greater. Studies in Malaysia show that *C. cassiicola* produces conidia throughout the year but the maximum numbers are found during August to September with maximum liberation at about noon. This would relate to the high humidity during that period. *Curvularia* was again found as a single isolate but from Kanchanaburi and although this site has a higher plant density it is still in relation to many forests in Thailand a dry site. A *Curvularia* species was isolated by Mekkamol (1998) from a mature teak leaf from Chiang Dao Plantation but only two isolates were obtained and these only in one out of the three years of sampling. *Nigrospora sphaerica* was also isolated on a single occasion from Kanchanaburi and this has also been recorded by Mekkamol again from Chiang Dao Plantation (Mekkamol, 1998) and was also isolated from teak leaves in the campus of Chulalongkorn University in Bangkok by Chareprasert (2001). Surprisingly *Colletotrichum* was only isolated in low numbers, but occurred in each site, and never became a major endophytic isolate as found by Mekkamol (1998). Chareprasert (2001) also recorded *Colletotrichum* from teak leaves at Chulalongkorn University.

Generally basidiomycete species are uncommon as endophytes but that may partially be an underestimate as identification of these usually 'sterile isolates' is extremely difficult. However in this study a species of *Coprinus* (*C. radians* on the basis of its GenBank match) was obtained from the Bangkok site and a species of *Psathyrella* (as *P. gracilis* on its GenBank match) was isolated from Nakhon Ratchasima. It could be that the basidiospores produced, which are usually thin walled are less resistant to drying and UV radiation than the pigmented and more robust ascospores, have less chance of surviving long periods and therefore have reduced potential to infect. As there are a high number of Xylariaceae isolated as endophytes from tropical plants it has been suggested that their presence is a result of high numbers of ascospores providing the inoculum together with their known ability to survive adverse conditions for a considerable time (Rodrigues & Petrini,

1997). Mekkamol (1998) found that they dominated after several months of the rainy season and concluded that this was a result of their increased presence in the airspora following several months after the onset of the rainy season when *Daldinia* and other Xylariaceae would have developed to maturity and be discharging ascospores. Studies in Malaysia indicated a 3 month or more period required for development of *D. eschscholzii* and *Hypoxylon* species following artificial inoculation and subsequent incubation in the forest (Whalley, 2001). In spite of an apparent lack of basidiomycete species occurring as endophytes for example Santos *et al.*, (2003) isolated one basidiomycete (not yet identified) but they also noted there were a number of Mycelia sterilia which may subsequently prove to be basidiomycete in nature. However they do occur even if occasionally as Chareprasert (2001) isolated *Schizophylum commune* Fr. as an endophyte of teak at Chulalongkorn and fortunately this could be identified as it developed to maturity in culture.

In common with many investigations of tropical endophytic fungi members of the Xylariaceae (Rodrigues & Petrini, 1997) this study demonstrated their constant presence as endophytes of *C. fistula* and in some cases exhibiting a high presence. This is in agreement with previous studies in Thailand by Mekkamol (1998), Chareprasert (2001) and Photita *et al.* (2001) as well as studies from other tropical countries (Strobel, 2003). Isolates of the genus *Daldinia* proved to be the most frequent species of the Xylariaceae, being obtained in samples from all three sites.
It was assumed that these were most probably isolates of *D. eschscholzii* as this proved to be the common species isolated by Mekkamol and identified following induction of their teleomorph resulting from their inoculation into sterilized teak branches and subsequent incubation in a forest or under simulated natural conditions (Mekkamol, 1978). Unfortunately it proved impossible to simulate the necessary environmental conditions in Liverpool to allow the full development of stromata of these tropical isolates and therefore molecular data and comparison of cultures with DNA and cultures of *D. concentrica* from the UK were undertaken. It was shown that all the isolates from Thailand considered to be a *Daldinia* species separated into two clades (Figure 4.5 & 4.7). In clade 1 all cultures were characteristic and compared well with cultures obtained from authentic teleomorph material of *D. eschscholzii* from Thailand and Malaysia but on the basis of their DNA sequences they matched with two sequences from GenBank and one from North Wales. Clade 2 contained isolates whose cultures were originally designated as *Nodulisporium* species but not initially considered to be clearly those of *D. eschscholzii.* Their DNA sequences however matched closely with sequences from

D. eschscholzii stromata from Malaysia and also with a sequence from Thailand provided by Dr M. Stadler (Bayer AG). It is suggested that the DNA sequence data in GenBank and also obtained in the current study is insufficiently discriminating for these two very closely related species. Thus the match with *D.concentrica* is considered to be misleading and further sequences obtained from different parts of the fungal genome would be necessary to confidently separate these two very closely related taxa. There are differences in their cultural characteristics and their metabolites (Stadler *et al.*, 2001a; b) but at present identifications based on current GenBank data is insufficient to distinguish between them. As a single species they represented in Kanchanaburi 18.5% of all isolates, in Nakhon Ratchasima 25% and in Bangkok 12.5%.

The *Nemania* clade (Figures 4.5 & 4.6) demonstrates a close relationship between the two endophytic isolates from Thailand and two collections from North Wales. Interestingly the two temperate samples linked together as did the two tropical samples. It is however premature to suggest that the tropical material represents *N. subannulata* and the temperate *N. bipapillata* until further collections and isolates have been investigated and perhaps other sequences from a different part of the genome will be required to resolve this question. Ju and Rogers (2002) considered *N. bipapillata* and *N. subannulata* (as *H. subannulatum*) to be the same although Thienhirun (1997) and Whalley (2001) suggested that they might be different. The two *Nemania* samples from Thailand were from Nakhon Ratchasima and Kanchanaburi.

A single isolate from Nakhon Ratchasima originally grouped with the Xylaria-like group of isolates was, on the basis of its DNA sequence, matched with *Rosellinia arcuata/necatrix* on GenBank. This is a very interesting result since there is no clear agreement on the separation of these two species (Francis, 1985) although in a recent revision of *Rosellinia* species from New Zealand Petrini (2003) considered them to be different on the basis of their morphological characteristics. Petch (1916) described *R. arcuarta* as an important pathogen of tea with its distinctive *Dematophthora* conodial state and pointed out that perethecial material was rare in the field. Certainly a major problem in recognizing *R. necatrix* in the past has been the lack of mature perithecial material (Teixeira de Sousa & Whalley, 1991; Teixeira de Sousa *et al.*, 1995; Edwards *et al.*, 2003)) and this also appears to be a similar problem for *R .arcuata* (Petrini, 2003). However *R. necatrix* was reported from Thailand on the basis of a teleomorphic collection by Thienhirun (1997) which

closely fitted published descriptions and in addition it has been collected in the Philippines (Whalley unpublished). They are both undoubtedly closely related and Petrini (2003) stated that "macroscopically *R.arcuata* resembles *R. necatrix* but its ascospores are statistically larger" and pointed out that according to Saccas (1956) the geographical distribution of *R. arcuata* is confined to tropical and humid regions of Africa and Asia. However the Petch specimens are from a mountainous region of Sri Lanka (Petrini, 2003) and interestingly the collection from Thailand is from the highest mountain there, Doi Inthanon (Thienhirun, 1997). Petrini (2003) also pointed out that in New Zealand, *R. arcuata* was mainly collected in the northern part of the North Island in national parks and state forests with subtropical vegetation and had not been reported from cultivated plants. Doi Inthanon is an area where a number of horticultural stations are operating under the auspices of the Royal Highland Project. Here several cultivated plants known from elsewhere to be susceptible to *R. necatrix* root rot e.g. apple, pear, plum, are grown but the only collection of the *Rosellinia* was obtained from natural forest although close to cultivated plants. Studies on *R. necatrix* metabolites from isolates from several European countries have confirmed the presence of cytochalasin E (Whalley & Edwards, 1997) and established rosnecatrone as a characteristic species specific metabolite (Edwards *et al.*, 2001). The culture obtained from the teleomorphic collection from the Philippines was also found to produce these characteristic metabolites. Examination of the *Rosellinia* endophytic isolate (Chapter 5) showed that although it contained cytochasin E it lacked the rosnecatrone but also contained 19-20 epoxide of cytochalasin C and the novel ά-glucoside 1-8 dihydroxynaphthalene. This suggests that it differs from *R.necatrix* and may well represent the true *R. arcuata*. This can only be clarified by examination of DNA samples from other collections and probably, as in the case of *D. concentrica* and *D. eschscholzii*, comparison of sequences from a different part of the fungal genome. It could be that the sequence from the ITS region is insufficient to separate with confidence what are closely related species when other characteristics are considered. In Figure 4.5 and 4.6 the clade containing the *Rosellinia* sequences splits into two with one Japanese strain, as *R. arcuata,* linking with *R. necatrix* from Portugal. It is possible that the *Rosellinia* from Japan is in fact *R. necatrix* since it is a common plant pathogen of many types of fruit tree in that country (Sivanesan & Holliday, 1972). The separate branch of the clade was formed from the *R. arcuata* from Thailand and although similar does exhibit differences in its DNA sequence.

The only species identified as a *Hypoxylon* was placed close to *H. rickii* as its closest match and linked with two other *Hypoxylon* species, *H. fragiforme* (Pers.:Fr.)J. Kickx Fil. and *H. howeianum* Peck used as markers. *H. rickii* has been reported from South and Central America and Louisiana (USA) but not as yet from Asia (Ju & Rogers, 1996).

Xylaria species were amongst the most frequent isolates and although they possess distinctive cultures these are insufficiently unique to allow identification to species although *X. cubensis* can be recognized by its *Xylocoremium flabelliforme* (Schwein.:Fr.) J.D. Rogers anamorph (Rogers, 1984; 1985). Surprisingly this was one species of *Xylaria* expected since it is a common endophyte of many tropical plants (Rodrigues, 1994, Rodrigues & Petrini, 1997) and occurs widely in Thailand as a teleomorph in the forests (Thienhirun, 1997) and was a common endophytic species in teak from northern Thailand (Mekkamol, 1998). As stated in Chapter 4 a number of isolates (seven) were matched with GenBank as close to *X. longipes*. This is a temperate species but a tropical variety *X. longipes* var. *tropica* was described from Mexico by San Martin & Rogers (1989) and reported from Thailand by Thienhirun (1997). It is believed that these isolates from Cassia represent this tropical variety and in the absence of sequence data for it in GenBank the nearest match was the closely related *X. longipes*. Two isolates were matched as *X. apiculata* which is widely reported from tropical, subtropical and temperate regions (Van der Gucht). Thienhirun (1997) and Mekkamol (1998), however, did not record the species from Thailand but it can be expected to occur there. Two isolates were matched to *X. hypoxylon* and *X. mali* and In both cases these matches are unlikely since they are species usually associated with a temperate climate (Dennis, 1981; Rogers, 1984). Cannon and Whalley (1988) reported on a *Xylaria* growing in a hothouse in England and believed that it had been imported along with tropical plants. They considered it to be close to *X. hypoxylon* but suggested that this is a species complex. The identity of these two species must remain uncertain until sequences from many more known tropical *Xylaria* species have been obtained.

The application of DNA sequences for identification of endophytes has so far been limited. Guo, Hyde and Liew (2000) identified endophytic fungi from *Livistoma chinensis* (Jacq.)R. Brown using morphological data and rDNA sequences. Mazzaglia et al (2001) applied sequence analysis of the 5.85S rDNA and ITS regions to evaluate genetic relationships among some species of *Hypoxylon* and related genera with a view to detecting the latent pathogen *Biscogniauxa*

mediterranea (De Not.) Kunze in its host tree. Sanchez-Ballesteros *et al.* (2000) undertook a phylogenetic study of *Hypoxylon* and related genera based on ribosomal ITS sequences and demonstrated a high correlation between the type of anamorph and the relative placement of the teleomorphs in the phylogenetic tree. Since nearly all endophytic Xylariaceae need to be identified on their anamorphic features this is an important finding regarding future application of sequence analysis for their identification. The only molecular study on *Xylaria* is that of Lee, Ko and Jung (2000) who used nuclear ribosomal ITS1-5.8s-ITS2 sequences to demonstrate that *Xylaria* formed a phylogenetically homogeneous group except for *X. cubensis*. Unfortunately only a small number of species were examined and none of these are truly tropical taxa. The lack of suitable sequences especially for *Xylaria* is a major limitation. at the current time when applying sequence data for their identification. Miss N. Suwannasai (Suranaree University of Technology, Nakhon Ratchasima, Thailand) is now establishing a tropical *Xylaria* and related xylariaceous genera DNA database which is based on DNA samples extracted from teleomorphs and their cultures belonging to, or isolated from, identified and authenticated species, most of which are tropical. This will then enable identification of many endophytic xylariaceous isolates to be made with the availability of a suitable DNA sequence database.

Attempts have also been made to apply chemical profiling for the identification of a number of selected xylariaceous isolates (Chapter 5). This approach has been made possible since the Xylariaceae have been subjected to extensive chemical analyses with useful systematic data being obtained (Whalley & Edwards, 1995; 1999). Chemical profiles and presence or absence of certain metabolites has indicated an identity for a number of isolates and has eliminated others which were expected.

Continued interest in metabolites from the Xylariaceae has centered on their potential as useful bioactive agents and in the last few years there have been a number of publications relating to compounds from *Xylaria* and related genera, and their bioactivity (Chapter 5). Unfortunately none of the endophytic isolates exhibited bioactivity of note but as discussed in Chapter 5 it may be that antimalarial activity, antiviral activity and other activities might be expected. The already proven diversity of metabolites and indeed novel metabolites from the Xylariaceae makes them an attractive group for potential exploitation. It has, for example, been found that

endophytic Xylariaceae are strong producers of lignolytic enzymes (Urairuj, Khanongnoch & Lumyong, 2003).

This study is the first thorough study of *C. fistula*, a plant of medicinal value in Thailand. The presence of a range of endophytic fungi has been demonstrated. In agreement with many other studies on such fungi it is shown that the endophyte assemblages are greater as the leaves mature. Also in agreement with Mekkamol (1998), the importance of the rainy season in relation to the potential inoculum, especially *Daldinia* and *Xylaria* species, is confirmed. There were also clear differences between samples obtained from the three sites with the Kanchanaburi site producing the most endophytic isolates both in number and diversity, followed by Nakhon Ratchasima and finally Bangkok. Thus endophytic populations are greater where there is highest plant diversity and density and the lowest where there are the least. A major problem investigation of tropical endophytes concerns the inability to identify many of them especially to species level. This is partially a result of a high proportion of unidentified species represent probably previously undescribed species (Hawksworth, 2001) but also the lack of distinguishing of characters in cultures of existing tropical endophytic fungi.

References

Abate, D., Abraham, W-F. & Meyer, H. (1997) Cytochalasins and phytotoxins from fungus *Xylaria obovata*. *Phytochemistry* **44**(8):1443-1448.

Abul-Hayja, Z. & Williams, P.H. Cuke Disease-Target Spot. *Corynespora cassiicola*. http://cuke.hort.ncsu.edu/cucurbit/cuke/dshndbk/tls.html

Adeboya, M.O. (1995) Metabolites from members of the fungus genus *Xylaria*. Ph.D.Thesis. University of Bradford. Bradford.

Agrios, G.N. (1988) *Plant Pathology*. 3rd edition. Academic Press: New York USA. p803.

Ahmad, A., Alam, M. & Janardhanan, K.K. (2001) Fungal endophyte enhances biomass production and essesntial oil yield of East Indian Lemongrass. *Symbiosis* **30**:275-285.

Alcamo, I.E. (1994) *Fundamental of Microbiology*. 4th edition. The Benjamin/Cummings Publishing Company Inc. California.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research* **25**:3389-3402.

Alvarenga, M.A., Braz Fo, R., Gottlieb, O.R., de P. Dias, J.P., Magalhaes, A.F. & Magalhaes, E.G. (1978) Dihydroisocoumarins and phthalide from wood samples infested by fungi. *Phytochemistry* **17**(3):511-516.

Anderson, J.R., Edwards, R.L. & Whalley, A.J.S. 1983. Metabolites of the higher fungi. 21.3-methyl-3,4-dihydroisocoumarins and related-compounds from the ascomycete family Xylariaceae. *Journal of the Chemical Society-Perkin Transactions 1* **9**:2185-2192.

Andrews, J.H., Hecht, E.P. & Bashirian, S. (1985) Association between the fungus *Acremonium curvulum* and Eurosian water milfoil, *Myriophyllum spicatum*. *Canadian Journal of Botany* **60**: 1216-1221.

Arie, T., Namba, S., Yamashita, S., Doi, Y. & Kijima, T. (1987) Stem blight, a new disease of exacum by *Nectria gliocladioides* Smalley et. Hanson. *Annals of the Phytopathological Society of Japan* **53**(5):570

Arnold, A.E.& Herre, E.A. (2003) Canopy cover and leaf age affect colonization by tropical fungal endophytes : Ecological pattern and precess in *Theobroma cacao* (Malvaceae). *Mycologia* **95**(3):388-398.

Arnold, A.E., Maynard, Z., Gilbert, G.S., Coley, P.D. & Kursar, T.A. (2000) Are tropical fungal endophytes hyperdiverse? *Ecology letters* **3**:207-274.

Arnold, A.E., Maynard, Z. & Gilbert, G.S. (2001) Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycological Research* **105**(12):1502-1507.

Arnold AE, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA. 2003. Fungal endophytes limit pathogen damage in tropical tree. *PNAS* **100**(26):15649-15654.

AVRDC International Cooperators. *Corynespora* Leaf Spot. http://www.avrdc.org/LC/cucurbits/coryn.html

Bacon, C.W., Lyons, P.C., Porter, J.K. & Robbins, J.D. (1986) Ergot toxicity from endophyte-infected grasses: a review. *Agronomy Journal* **78**: 106-16.

Ball, D.M. & Hoveland, C.S. (1978) Alkaloid levels in *Phalaris aquatica* L. as affected by environment. *Agronomy Journal* **70**:977-981.

Barreto, D., Babbitt, S., Gally, M. & Perez, B.A. (2003) *Nectria haematococca* causing root rot in olive greenhouse plants. *RIA* **32**(1):49-55.

Bassett, E.N. & Fenn, P. (1984) Latent colonization and pathogenicity of *Hypoxylon atropunctatum* on Oaks. *Plant Disease* **68**:317-319.

Bayman, P., Angulo-Sandoval, Baez-Ortiz, Z. & Jean Lodge, D. (1998) Distribution and dispersal of Xylaria endophytes in two tree species in Puerto Rico. *Mycological Research* **102**(8): 944-948.

Bayman, P., Lebron, L.L., Tremblay, R.L. & Jean Lodge, D. (1997) Variation in endophytic fungi from rods and leaves of Lepanthes (Orchidaceae). *New Phytologist* **135**: 143-149.

Belesky, D.P., Fedders, J.M. (1996) Does endophyte influence regrowth of Tall fescue? *Annals of Botany* **78**:499-505.

Bernstein, M.E. & Carroll, G.C. (1977) Internal fungi in old-growth Douglas fir foliage. *Canadian Journal of Botany* **55**:644-653.

Bertoni, M., Cabral, D. (1988) Phyllosphera of *Eucalyptus viminalis* II : Distribution of endophytes. *Nova Hedwigia* **46**:491-502.

Betina, V. (1989) *Mycotaxins:Chemical, Biological and Environmental Aspects.* Elsevier:Amsterdam.

Bettucci, L., Alonso, R. & Tiscornia, S. (1999) Endophytic mycobiota of healthy twigs and the assemblage of species associated with twig lesions of *Eucalyptus globules* and *E. grandis* in Uruguay. *Mycological Research* **103**(4):468-472.

Bettuci, L. & Alonso, R. (1997) A comparative study of fungal populations in healthy and symptomatic twigs of *Eucalyptus grandis* in Uruguay. *Mycological Research* **101**(9):1060-1064.

Bhakta, T., Mukherjee, P.K., Mukherjee, K., Banerjee, S., Mandal, S.C., Maity, T.K., Pal, M. & Saha, B.P. (1999) Evaluation of hepatoprotective activity of *Cassia fistula* leaf extract. *Journal of Ethnopharmacology* **66**(3): 277-282.

Bills, G.F., Pelaez, F., Polishook, J.D., Diez-Matas, M.T., Harris, G.H., Clapp, W.H., Dufresne, C., Byrne, K.M., Nallin-Omstead, M., Jenkins, R.G., Mojena, M., Huang, L. & Bergstrom, J.D. (1994) Distribution of zaragozic acids (squalestatins) among filamentous ascomycetes. *Mycological Research* **98**:733-739.

Bloomberg, W.J. (1966) The occurrence of endophytic fungi in Douglas Fir seedlings and seeds. *Canadian Journal of Botany* **44**:413-420.

Boonphong, S., Kittakoop, P., Isaka, M., Pittayakhajonwut, D., Tanticharoen, M. & Thebtaranonth, Y. (2001) Multiplolides A and B, new antifungal 10-membered lactones from *Xylaria multiplex*. *Journal of Natural Products* **64**:965-967.

Bose, S.R. (1947) Hereditary (seed-borne) symbiosis in *Casuarina equisetifolia*. *Nature (London)* **159**:512-514.

Bradshaw, A.D. (1959) Population differentiation in *Agrostis tenuis* Sibth II. The incidence and significance of infection by *Epichloë typhina*. *New Phytologist* **58**:310-315.

Brunner, F. & Pertini, O. (1992) Taxonomy of some *Xylaria* species and xylariaceous endophytes by isozyme electrophoresis. *Mycological Research* **96**:723-733.

Bu'Lock, J.D. (1980) Mycotoxins as secondary metabolites. In *The biosynthesis of mycotaxins. A study in secondary metabolism*. (PS Steyn ,ed.): 1-16. Academic Press,New York.

Cabral D. (1985) Phyllosphere of *Eucalyptus viminalis*. Dynamics of fungal populations. *Transactions of the British Mycological Society* **85**:501-511.

Callan, B.E. & Rogers, J.D. (1986) Cultural characters and anamorphs of *Biscogniauxia* (= *Nummularia*) *marginata*, *B. dennisii* and *B. repanda*. *Canadian Journal of Botany* **64**: 842-847.

Callan, B.E. & Rogers, J.D. (1993) A synoptic key to *Xylaria* species from continental United States and Canada based on cultural and anamorphic features. *Mycotaxon* **46**:141-154.

Canavasi, F. (1987) Beziehungen zwischen Endophytischen Pilzen von *Abies alba* Mill. und den Pilzen der Nadelstreue. Dissertation ETH no. 8325. Swiss Federal Institute of Technology, Zurich, Switzerland.

Candoussau, F. & Rogers, J.D. (1990) Notes on *Obolarina dryophila* from France. *Mycotaxon* **39**: 345-349.

Cannon, P.F. & Simmons, C.M. (2002) Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia* **94**(2):210-220.

Cannon, P.F. & Whalley, A.J.S. (1988) Notes on an unusual *Xylaria* growing in a British hothouse. *Transactions of the British Mycological Society* **91**:176-179.

Carroll, F.E., Müller, E. & Sutton, B.C. (1977) Preliminary studies on the incidence of needle endophytes in some European conifers. *Sydowia* **29**:87-103.

Carroll, G. & Petrini, O. (1983) Patterns of substrate utilization by some fungal endophytes from coniferous foliage. *Mycologia* **75**(1):53-63.

Carroll, G. (1988) Fungal endophytes in stems and leaves: From latent infection to mutualistic symbiont. *Ecology* **69**(1): 2-3.

Carroll, G. (1995) Forest endophytes: Pattern and process. *Canadian Journal of Botany* **73**(Suppl1): S1316-S1324.

Carroll, G.C. & Carroll, F.E. (1978) Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. *Canadian Journal of Botany* **56**: 3034-3043.

Carroll, G.C. & Petrini, O. (1983) Patterns of substrate utilization by some endophytes from coniferous foliage. *Mycologia* **75**:53-63.

Carroll, G.C. & Tudzynski, P. (eds) (1997) *The Mycota V. Plant relationships.* Part B. Berlin: Springer-Verlag.

Carroll, G.C. (1986) The Biology of endophytism in plants with particular reference to woody perennials. In *Microbiology of the phyllosphere* (N.J. Fokkema & J. von den Heuvel, eds.): 205-222. Cambridge University Press, Cambridge, United Kingdom.

Cerkauskas, R. (Agriculture and Agri-food Canada), *Fusarium* stem and fruit rot of greenhouse pepper. http://www.gov.on.ca/OMAFRA/english/crops/facts/01-083.htm

Chacko, R.J. & Rogers, J.D. (1981) Cultural characteristics of some species of *Xylaria*. *Mycologia* **73**:415-428.

Chareprasert, S. (2001) Endophytic fungi of Teak leaves *Tectona grandis* L. and Rain Tree leaves *Samanea saman* MERR. in Chulalongkorn University Campus. M.Sc. Thesis. Chulalongkorn University.

Charuppat, T. (1994) Using Landsat-5™ imagery for monitoring the changes of forest area in Thailand. Report of the Forest Research Office, Royal Forest Department, Bangkok, Thailand.

Chase, T. (2002) Occurrence of soybean stem canker in South Dakota: Using molecular techniques for pathogen identification. Plant Science Mini Seminar (21 November 2002). South Dakota State University. http://plantsci.sdstate.edu/miniseminar/chase/chase_handout.html

Chen, G., Lin, Y., Wen, L., Vrijmoed, L.L.P & Jones, E.B.G. (2003) Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the South China Sea coast. *Tetrahedron* **59**:4907-4909.

Chen, Y-S. (1960) Studies on the metabolic products of Rosellinia necatrix Berlese. Part I. Isolation and characterization of several physiologically active neutral substances. *Bulletin of the Agricultural Chemical Society of Japan* **24**(4) Suppl:372-381.

Chesters, C.G.G & Greenhalgh, G.N. (1964) *Geniculosporium serpens* gen. et sp. nov., the imperfect state of *Hypoxylon serpens*. *Transactions of the British Mycological Society* **47**:393-401.

Chinworrungsee, M., Kittakoop, P., Isaka, M., Chanphen, R., Tanticharoen, M. & Thebtaranonth, Y. (2002) Halorosellins A and B, unique isocoumarins glucosides from the marine fungus *Halorosellinia oceanica*. *Journal of the Chemical Society, Perkins Transactions 1* 2473-2476.

Chinworrungsee, M., Kittakoop, P., Isaka, M., Rungrod, A., Tanticharoen, M. & Thebtaranonth, Y. (2001) Antimicrobial halorosellinic acid from the marine fungus *Halorosellinia oceanica*. *Bioorganic & Medicinal Chemistry Letters* **11**:1965-1969.

Chow, L.M. http://personal.cityu.edu.hk/~bhqef/xy/index.html

Clark, E.M., White, J.W. & Patterson, R.M. (1983) Improved histochemical techniques for the detection of *Acremonium coenophialum* in Tall Fescue and methods of *in vitro* culture of the fungus. *Journal of Microbiological Methods* **1**:149-155.

Clay, K. & Schardl, C. (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist* **160**:s99-s127.

Clay, K. (1984) The effect of the fungus *Atkinsonella hypoxylon* (Clavicipitaceae) on the reproductive system and demography of the grass *Danthonia apicata*. *New Phytologist* **98**:165-175.

Clay, K. (1986) Grass Endophytes. In *Microbiology of the phyllosphere*. (N.J. Fokkema & J. van den Heuvel ,eds): 188-204. Cambridge University Press: Cambridge, UK

Clay, K. (1987) Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*. *Oecologia* **73**:358-362.

Clay, K. (1988) Fungal endophytes of Grasses: A defensive mutualism between plants and fungi. *Ecology* **69** (1): 10-16.

Clay, K. (1991) Fungal endophytes, grasses, and herbivores. In *Microbial mediation of plant-herbivore interactions*. (P. Barbosa, V.A. Krischik & C.G. Jones ,eds) : 199-252. John Wiley & Sons, Inc., New York.

Claydon, N., Grove, J.F. & Pople, M. (1985) Elm bark beetle boring and feeding deterrents from *Phomopsis oblonga*. *Phytochemistry* **24**(5):937-943.

Collmer, A. & Keen, N.T. (1986) The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**:383-409.

Coprinus radians. http://www.mushroomthejournal.com/ima/Taxa/copriradia720.html

Crane, J.L. & Dumont, K.P. (1975) Hyphomycetes from the West Indies and Venezuela. *Canadian Journal of Botany* **53**: 843-851.

Culberson, C.F. & Elix, J. (1989) Lichen substances. *Methods in Plant Biochemistry* **1**:509-535.

Culberson, C.F. & Kristinsson, H. (1970) A standardized method for the identification of lichen substances. *Journal of Cheomatography* **46**:85-93.

Culvenor, C.C. (1973) Alkaloids. In *Chemistry and biochemistry of herbage. Volume 1.* (G.W. Butler & R.W. Bailey ,eds) :375-446. Academic Press, New York.

Cytochalasin reference. http://cytochalasin.4mg.com/

D.S.R Travel Co., Ltd. http://www.welcomethai.com

De Bary, A. (1866) Morphologie und physiologie der Pilze, Flechten und Myxomyceten. Hofmeisster's Handbook of Physiological Botany Vol2. Leipzig.

de Hoog, G.S., Guarro, J., Gene, J. & Figueras, M.J. (2000) Atlas of clinical fungi. 2nd edition, Vol1. Centraalbureau Voor Schimmelcultures:Utrecht, The Netherlands.

de Pascual, J-T., Moreno Valle, M.A., Gonzalez, M.S. & Bellido, I.S. (1982) Phloroglucinol derivatives from *Leucanthemopsis pulverulenta. Phytochemistry* **21**(3):791-792.

Debaeke, P., Estragnat, A. & Reau, R. (2003) Influence of crop management on sunflower stem canker (*Diaporthe helianthi*). *Agronomie* **23**:581-592.

Demain, A.L. (1996) Fungal secondary metabolism: Regulation and Functions. In *A century of Mycology.* (B. Sutton, ed): 233-254. Cambridge: Cambridge University Press.

Dennis, R.G.W. (1970) *Fungus flora of Venezuela and adjacent countires.* Kew Bulletin, Additional Series 3. HMSO.

Dennis, R.W.G. (1981) *British Ascomycetes.* J.Cramer:Vaduz.

Diehl, W.W. (1950) *Balansia* and the *Balansiae* in America. Agriculture Monograph 4. United States Department of Agriculture. Washington D.C., USA.

Doctor Fungus: *Fusarium* sp. http://www.doctorfungus.org/TheFungi/fusarium.htm

Dreyfuss, M. & Petrini, O. (1984) Further investigations on the occurrence and distribution of endophytic fungi in tropical plants. *Botanica Helvetica* **94**(1):33-40.

Dreyfuss, M.M. & Chapela, I.H. (1994) Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In *The discovery of natural products with therapeutic potential.* (V.C. Gullo, ed.):49-80. Butterworth - Heinemann, London.

Dreyfuss, M.M. & Petrini, O. (1984) Further investigations on the occurrence and distribution of endophytic fungi in tropical plants. *Botanica Helvetica* **94**:33-40.

Eckblad, F-E., Granmo, A. (1978) The genus *Nummularia* (Ascomycetes) in Norway. *Norwegian Journal of Botany* **25**: 69-75.

Edwards, R.L., Jongkaekha, N., Kshirsagar, A., Maitland, D.J., Mekkamol, S., Nugent, L.K., Phosri, C., Rodthong, S., Ruchikachorn, N., Sangvivhien, E., Sharples, G.P., Sihanonth, P., Suwannasai, N., Thienhirun, S., Whalley, A.J.S. & Whalley, M.A. (2003) The xylariaceae as phytopathogens. *Recent Research Developments in Plant Science* **1**:1-19.

Edwards, R.L., Maitland, D.J., Scowen, I.J., De Sousa, A.J.T. & Whalley, A.J.S. (2001) Metabolites of the higher fungi. Part 32. Rosnecatrone, a phytotaxic bicycle [4.1.0] hept-3-en-2-one from the fungus *Rosellinia necatrix* Prill. *Journal of Chemical Society, Perkin Transactions 1* 537-542.

Edwards, R.L., Maitland, D.J. & Whalley, A.J.S. (1989) Metabolites of the higher fungi. 24. Cytochalasin-N, cytochalasin-O, cytochalasin-P, cytochalasin-Q, and cytochalasin-R. New cytochalasins from the fungus *Hypoxylon terricola* Mill. *Journal of Chemical Society, Perkin Transactions 1* **1**:57-65.

Edwards, R.L., Maitland, D.J. & Whalley, A.J.S. (1991) Metabolites of the higher fungi. 26. Cubensic acid, 3,7,11,15-tetrahydroxy-18-(hydroxymethyl)-2,4,6,10,14,16,20-heptamethyldocosa-4E,8E,12E,16E-Tetraenoic acid, a novel polysubstituted C22 fatty acid from the fungus *Xylaria cubensis* (Mont.) Fr. with

substituents and substitution pattern similar to the macrolide antibiotics. *Journal of Chemical Society, Perkin Transactions 1* **6**:1411-1417.

Elamo, P., Helander, M.L., Saloniemi, I. & Neuvonen, S. (1999) Birch family and environmental conditions affect endophytic fungi in leaves. *Oecologia* **118**:151-156.

Ellis, M.B. (1971) *Dermatiaceous Hyphomycetes.* 1st ed. The Eastern Press: London.

El-Saadany, S.S., El-Massry, R.A., Labib, S.M., Sitohy, M.Z. (1991) The biological role and hypocholeterolaemic potential of the legume *Cassia fistula* in hypercholeresterolaemic rats. *Nahrung* **35**(8):807-815.

Engel, G., von Milczewski, K.E., Prokopek, D., Teuber, M. (1982) Strain-specific synthesis of myophenolic acid by *Penicillium roqueforti* in blue-veined cheese. *Applied Environmental Microbiology* **43**:1034-1040.

Eriksson, O.E. (1995) DNA and Ascomycetes systematics. *Canadaian Journal of Botany* **73**(Suppl1): S784-S789.

Espada, A.R.S, De la Fuente, J.M, Hueso-Rodriguez, J.A. & Elson. S.W. (1997) New cytochalasins from the fungus *Xylaria hypoxylon. Tetrahedron* **53**(18): 6485-6492.

Espinosa-Garcia, F.J. & Langenheim, J.H. (1990) The leaf fungal endophytic community of a coastal redwood population-diversity and spatial patterns. *New Phytologist* **116**:89-98.

Esposito Avella, M., Diaz, A., De Gracia, I., De Tello, R., Gupta, M.P. (1991) Evalutation of traditonal medicine: effect of *Cajanus cajan* L. and of *Cassia fistula* L. on carbohydrate metabolism in mice. *Revista Medica de Panama* **16**(1): 39-45.

Etherington&Roberts.Dictionary – phloroglucinol. http://palimpsest.stanford.edu/don/dt/dt2566.html

Fernandes, R.C. & Barreto, R.W. (2003) *Corynespora cassiicola* causing leaf spots on *Coleus barbatus. Plant Pathology* **52**(6):786.

Ferrer, C., Colom, F., Frases, S., Mulet, E., Abad, J.L. & Alio, J.L. (2001) Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8s ribosomal DNA typing in ocular infections. *Journal of Clinical Microbiology* **39**(8):2873-2879.

Filtenborg, O., Frisvad, J.C. & Svendsen, J.A. (1980) A simple screening method for toxigenic fungi in pure cultures. *Lebensmittel Wissenschaft und Technologie* **13**:128-130.

Filtenborg, O., Frisvad, J.C. & Svendsen, J.A. (1983) Simple screening method for moulds producing intracellular mycotoxins in pure cultures. *Applied Environmental Microbiology* **45**:581-585.

Fisher, P.J., Anson, A.E. & Petrini, O. (1984) Novel antibiotic activity of an endophytic *Cryptosporiopsis* sp. isolated from *Vaccinium myrtillus*. *Transaction of the British Mycological Society* **83**(1): 145-148.

Fisher, P.J. & Anson, A.E. & Petrini, O. (1986) Fungal endophytes in *Ulex europaeus* and *Ulex gallii*. *Transaction of the British Mycological Society* **86** (1): 153-156.

Francis, S.M., Minter, D.W. & Caine, T.S. (1980) Three new species of *Anthostomella*. *Transactions of the British Mycological Society* **75**: 201-206.

Francis, S.M. (1985) Rosellinia necatrix-fact or fiction? *Sydowia* **38**:75-86.

Freeman, S. & Rodrigues, R.J. (1993) Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. *Science* **260**:75.

Frisvad, J.C. & Filtenborg, O. (1983) Classification of terverticillate penicillia based on profiles of secondary metabolites. *Applied Environmental Microbiology* **46**:1301-1310.

Frisvad, J.C., Thrane, U. & Filtenborg, O. (1998) Role and use of secondary metabolites in fungal taxonomy. In *Chemical Fungal Taxonomy*. (J.C. Frisvad, P.D. Bridge & D.K. Arora, eds): 289-319. Marcel Dekker Inc, New York.

Frisvad, J.C. (1994) Classification of organisms by secondary metabolites. In. *The identification and characterization of pest organisms.* (D.L. Hawksworth ,ed.) :303-320. CAB International, Wallingford.

FrÖhlich, J., Hyde, K.D. & Petrini, O. (2000) Endophytic fungi associated with palms. *Mycological Research* **104**(10):1202-1212.

FrÖhlich, J. & Hyde, K.D. (1999) Biodiversity of palm fungi in the tropics are global fungal diversity estimates realistic? *Biodiversity and Conservation* **8**:977-1004.

Funk A. (1985) *Foliar fungi of western trees.* Canadian Forestry Service. Victoria, British Columbia, Canada.

Ganley, R.J., Brunsfeld, S.J. & Newcombe, G. (2004) A community of unknown endophytic fungi in western white pine. *PNAS* **101**(7):10107-10112.

Gardner, S., Sidisunthorn, P. & Anusarnsunthorn, V. (2000) *Field guide to the forest trees of Northern Thailand.* Kobfai Publishing Project: Bangkok.

Geris dos Santos, R.M., Rodrigues-Fo, E., Rocha, W.C. & Teixeira, M.F.S. (2003) Endophytic fungi from *Melia azedarach. World Journal of Microbiology and Biotechnology* **19**:767-770.

Gombao, M.A. & Bayman, P. (2001) Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*:Meliaceae). *Biotropica* **33**:352-360.

Gonzalez, F.S.M. & Rogers, J.D. (1989) A preliminary account of *Xylaria* of Mexico. *Mycotaxon* **34**:283-373.

Gowan, S.P. & Vilgalys, R. (1991) Ribosomal DNA length polymorphisms within populations of *Xylaria magnoliae* (Ascomycotina). *American Journal of Botany* **78**:1603-1607.

Greenhalgh, G.N. & Chesters, C.C.G. (1968) Conidiophore morphology in some British members of the Xylariaceae. *Transactions of the British Mycological Society* **51**: 57-82.

Greenhalgh, G.N. (1967) A note on the conidial state scar in the xylariaceae. *New Phytologist* **66**:65-66.

Groppe, K. & Boller, T. (1997) PCR assay based on a microsatellite-containing locus for detection and quantification of *Epichloë* endophytes in grass tissue. *Applied Environmental Microbiology* **63**(4): 1543-1550.

Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995) A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied Environmental Microbiology* **61**(11): 3943-3949.

Guo, L.D., Hyde, K.D. & Liew, E.C.Y. (2000) Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytologist* **147**: 617-630.

Gupta, M., Mazumder, U.K., Rath, N., Mukhopadhyay, D.K. (2000) Antitumor activity of methanolic extract of *Cassia fistula* L. seeds against Ehrlich Ascites Carcinoma. *Journal of Ethnopharmacology* **72**(1): 151-156.

Hall, B.G. (2001) *Phylogenetic trees made easy: A How-to manual for molecular biologists.* Sinauer Associates:Massachusettes, USA.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis programme for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**:95-98.

Harberd, D.J. (1961) Note on choke disease of *Festuca rubra. Scottish Plant Breeding Station Report* **1961**:47-51.

Harper, J.K., Arif, A.M., Ford, E.J., Strobel, G.A., Porco, Jr. J.A., Tomer, D.P., Oneil, K.L., Heider, E.M. & Grant, D.M. (2003) Pestacin: a 1,3 dihydroisobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. *Tetrahedron* **59**:2471-2476.

Hawksworth, D.L. & Whalley, A.J.S. (1985) A new species of *Rhopalostroma* with a *Nodulisporium* anamorph from Thailand. *Transactions of the British Mycological Society* **84**: 560-562.

Hawksworth, D.L. (1971) A revision of the genus *Ascotricha* Berk. *Mycological Papers* **126**:1-28.

Hawksworth, D.L. (1991) The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycological Research* **95**:641-655.

Helander, M.L., Neuvonen, S., Sieber, T. & Petrini, O. (1993a) Simulated acid rain affects birch leaf endophyte populations. *Microbial Ecology* **26**:227-234.

Helander, M.L., Ranta, H. & Neuvonen, S. (1993b) Responses of phyllosphere microfungi to simulated sulphuric and nitric acid deposition. *Mycological Research* **97**:533-537.

Helander, M.L., Sieber, T.N., Petrini, O. & Neuvonen, S. (1994) Endophytic fungi in Scots pine needles: spatial variation and consequences of simulated acid rain. *Canadian Journal of Botany* **72**:1108-1113.

Helander, M.L. (1995) Responses of pine needles endophyte to air pollution. *New Phytologist* **131**:223-229.

Holliday, P. (1980) *Fungus Diseases of Tropical Crops.* Cambridge University Press, Cambridge, UK.

Horn, W.S.S.M., Schwartz, R.E.S. & Blaney, W.M. (1995) Phomopsichalasin, a novel antimicrobial agent from an endophytic *Phomopsis* sp. *Tetrahedron* **51**(14): 3969-3978.

Huang, L.H. & Kaneko, T. (1996) Pyrenomycetes and Loculoascomycetes as sources of secondary metabolites. *Journal of Industrial Microbiology* **17**:402-416.

Huneck, S. & Yoshimura, I. (1996) *Identification of Lichen-Substances.* Springer, Berlin.

Hyde, K.D. (1995) Fungi from palms.XXI. The genus *Seynesia. Sydowia* **47**: 180-198.

Ingold, C.T. (1971) *Fungal spores their liberation and dispersal.* Clarendon Press, Oxford.

Integrated Pest Management. 2004. Soybean: Insect, Disease, Nematode, and Weed Control Recommendations for 2004. Alabama Cooperative Extension System/ Alalbama A&M and Auburn Univeristies. IPM-0413 Soybean:10-11. http://www.aces.edu/pubs/docs/A/ANR-0500/VOL-0001/SOYBEAN.pdf

Isaka, M., Jaturapat, A., Kladwang, W., Punya, J., Lertwerawat, Y., Tanticharoen, M. & Thebtaranonth, Y. (2000) Antiplasmodial compounds from the wood-decayed fungus *Xylaria* sp. BBC 1067. *Planta Medica* **65**: 473-475.

Jakupovic, J., Kuhnke, J., Schuster, A., Metwally, M.A. & Bohlmann, F. (1986) Phloroglucinol derivatives and other constituents from South African *Helichrysum* species. *Phytochemistry* **25**(5):1133-1142.

Johannesson, H., Læssøe, T. & Stenlid, J. (2000). Molecular and morphological investigation of *Daldinia* in Northern Europe. *Mycological Research* **104**(3): 275-280.

Johnson, J.A. & Whitney, N.J. (1989a) An investigation of needle endophyte colonization patterns with respect to height and compass direction in a single crown of balsam fir (*Albies balsamea*). *Canadian Journal of Botany* **67**:723-725.

Johnson, J.A. & Whitney, N.J. (1989b) A study of fungal endophytes of needles of balsam fir (*Albies balsamea*) and Red Spuce (*Picea rubens*) in New Brunswick, Canada, using culture and electron microscope techniques. *Canadian Journal of Botany* **67**:3513-3516.

Johnston, P.R. (1998) Leaf endophytes of manuka (*Leptospermum scoparium*). *Mycological Research* **102**(8):1009-1016.

Jones, T.A., Buckner, R.C., Burrus, P.B. I.I., Bush, L.P. (1983) Accumulation of pyrrolizidine alkaloids in benomyl-treated tall fescue parents and their untreated progenies. *Crop Science* **23**: 1135-1140.

Jong, S.C. & Davis, E.E. (1974) *Areolospora*, a new humicolous genus in the Xylariaceae. *Norwegian Journal of Botany* **21**:23-30.

Jong, S.C. & Rogers, J.D. (1972) Illustrations and descriptions of conidial states of some *Hypoxylon* species. *Washington State University Agricultural Experiment Station Technical Bulletin* **71**: 1-49.

Ju, Y-M., Rogers, J.D. (1994) *Kretzschmariella culmorum* (Cooke) Y.-M. Ju & J.D. Rogers and notes on some other monocot-inhabiting xylariaceaous fungi. *Mycotaxon* **51**: 241-255.

Ju, Y-M., Gonzalez, F.S.M., Rogers, J.D. (1993) Three xylariaceous fungi with scolecosporous conidia. *Mycotaxon* **41**: 219-228.

Ju, Y-M., Rogers, J.D. (1990) *Astrocystis* reconsidered. *Mycologia* **82**: 342-349.

Ju, Y-M., Rogers, J.D. (1996) *A revision of the genus Hypoxylon.* Mycologia Memoir No 20. APS Press, Minnesota.

Ju, Y-M., Rogers, J.D. (2002) The genus *Nemania* (Xylariaceae). *Nova Hedwigia* **74**:75-120.

Kelley, S.E. & Clay, K. (1987) Interspecific competitive interactions and the maintenance of genotypic variation within the populations of two perennial grasses. *Evolution* **41**:92-103.

Keusgen, M., Falk, M., Walter, J.A. & Glombitza, K-W. (1997) A phloroglucinol derivative from the brown alga *Sargassum spinuligerum. Phytochemistry* **46**(2):341-345.

Kingston, D.G.I. (1996) Natural products as pharmaceuticals and sources for lead structures. In *The Practice of Medicinal Chemistry.* (C.G. Wermuth, ed):101-116. London, Academic Press.

Kinkel, L.L., Andrews, J.H., Berbee, F.M. & Nordheim, E.V. (1987) Leaves as island for microbes. *Oecologia* **71**:405-408.

Kinkel, L.L., Andrews, J.H. & Nordheim, E.V. (1989) Fungal immigration dynamics and community development on apple leaves. *Microbial Ecology* **18**:45-58.

Kreisel, H. & Schauer, F. (1987) *Methoden des mykologischen laboratoriums.* Gustav Fisher Verlag, Jena.

Kumaresan, V. & Suryanarayanan, T.S. (2002) Endophytic assemblages in young, mature and senescent leaves of *Rhizophora apiculata* : evidence for the role of endophytes in mangrove litter degradation. *Fungal Diversity* **9**:81-91.

Læssøe, T. & Peterson, J.H. The Funga : Xylariales the dominant group. http://www.mycokey.com/AAU/WestAfrica/Xylariales.htm

Laessoe, T., Rogers, J.D., Whalley, A.J.S. (1989) *Camillea, Jongiella* and light-spored species of *Hypoxylon*. *Mycological Research* **93**: 121-155.

Lappalainen, J.H., Koricheva, J., Helander, M.L. & Haukioja, E. (1999) Densities of endophytic fungi and performance of leafminers (Lepidoptera : Eriocraniidae) on birch along a pollution gradient . *Environmental Pollution* **104**:99-105.

Larena, I., Salazar, O., Gonzalez, V., Julian, M.C. & Rubio, V. (1999) Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *Journal of Biotechnology* **75**:187-194.

Latch, G.C.M., Hunt, W.F. & Musgrave, D.R. (1985) Endophytic fungi affect growth of perennial ryegrass. *New Zealand Journal of Agricultural Research* **28**:165-168.

Latch, G.C.M., Potter, L.R. & Tyler, B.F. (1987) Incidence of endophytes in seeds from collections of *Lolium* and *Festuca* species. *Annals of Applied Biology* **111**:59-64.

Lee, J.S., Ko, K.S. & Jung, H.S. (2000) Phylogenetic analysis of *Xylaria* based on nuclear ribosomal ITS1-5.8s-ITS2 sequences. *FEMS Microbiology Letters* **187**:89-93.

Lee, S.B. & Taylor, J.W. (1990) Isolation of DNA from fungal mycelia and single spores. In *PCR protocols : A guide to methods and applications.* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White ,eds): 282-287. Academic Press, London.

Legault, D., Dessureault, M. & Laflamme, G. (1989a) Mycoflore des aiguilles de *Pinus banksiana* et *Pinus resinosa* I Champignons endophytes. *Canadian Journal of Botany* **67**:2052-2060.

Leuchtmann, A. & Clay, K. (1989) Isozyme variation in the fungus *Atkinsoniella hypoxylon* within and among populations of its host grasses. *Canadian Journal of Botany* **67**:2600-2607.

Lewis, G.C. & Clements, R.O. (1986) A survey of ryegrass endophyte (*Acremonium loliae*) in the U.K. and its apparent ineffectuality on a seedling pest. *Journal of Agricultural Science* **107**:633-638.

Li, J.Y., Sidhu, R.S., Bollon, A. & Strobel, G. (1998) Stimulation of Taxol production in liquid cultures of *Pestalotiopsis microspora*. *Mycological Research* **102**(4): 461-464.

Li, J.Y., Strobel, G., Sidhu, R., Hess, W.M. & Ford, E.J. (1996) Endophytic Taxol-producing fungi from bald cypress, *Taxodium distichum*. *Microbiology* **142**: 2223-2226.

Lin, Y., Wu, X., Feng, S., Jiang, G., Luo, J., Zhou, S., Vrijmoed, L.L.P., Jones, E.B.G., Krohn, K., Steingrover, K. & Zsila, F. (2001a) Five unique compounds: Xytoketals from mangrove fungus *Xylaria* sp. from the South China Sea Coast. *Journal of Organic Chemistry* **66**:6252-6256.

Lin, Y., Wu, X., Feng, S., Jiang, G., Zhou, S., Vrijmoed, L.L.P., Jones, E.B.G. (2001b) A novel N-cinnamoylcyclopeptide containing an allenic ether from the fungus *Xylaria* sp. (Strain # 2508) from the South China Sea. *Tetrahedron letters*.**2**:449-451.

Lodge, J.D., Fisher, P. J. & Sutton, B.C. (1996) Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia* **88**(5): 733-738.

Loppnau, P.A. & Breuil, C. (2003) Species level identification of conifer associated ceratocystis sapstain fungi by PCR-RFLP on a β-tubulin gene fragment. *FEMS Microbiology Letters* **222**(1):143-147.

Lounasmaa, M., Widen, C.J. & Huhtikangas, A. (1973) Phloroglucinol derivatives of Hagenia abyssinica. *Phytochemistry* **12**(8):2017-2025.

Lu, H., Zou, W-X., Meng, J-C., Hu, J. & Tan, R-X. (2000) New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. *Plant Science* **151**:67-73.

Lüginbuehl, M. & Müeller, E. (1980) Endophytische pilze in den oberirdischen organen von vier gemeinsam an gleíchen standorten wachsenden pflanzen (*Buxus, Hedera, Ilex, Ruscus*). *Sydowia* **33**:185-209.

Luttrell, E.S. & Bacon, C.W. (1977) Classification of Myriogenospora in the Clavicipitaceae. *Canadian Journal of Botany* **55**: 2090-2097.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (eds). (1982) *Molecular Cloning : A laboratory manual.* Cold Spring Harbor,New York.

Martin, P. (1967) Studies in the xylariaceae: I New and old concepts. *Journal of South African Botany* **33**:205-240.

Martin, P. (1969) Studies in the Xylariaceae V. Euhypoxylon. *Journal of South African Botany* **35**: 149-206

Mazzaglia, A., Anselmi, N., Vicario, S. & Vannini, A. (2001) Sequence analysis of the 5.8s rDNA and ITS regions in evaluating genetic relationships among some species of *Hypoxylon* and related genera. *Mycological Research* **105**(6): 670-675.

Mekkamol, S., Jonglaekha, N., Hywel-Jones, N., Whalley, A. (1997) Endophytic fungi of Teak in Thailand: A preliminary account. *BIOTEC News* **3**(3): 4.-5.

Mekkamol, S. (1998) Endophytic fungi in *Tectona grandis* L. (Teak). Ph.D. Thesis. Liverpool John Moores University.

Metz, A.M., Haddad, A., Worapong, J., Long, D.M., Ford, E.J., Hess, W.M. & Strobel G.A. (2000) Induction of the sexual stage of *Pestalotiopsis microspora*, a Taxol-producing fungus. *Microbiology* **146**: 2079-89.

Miller, C.S. (1980) Infection processes on conifer needles. In *Microbial ecology of Phylloplane.* (J.P. Blakeman ,ed):185-209. Academic Press, London.

Miller, J.H. (1961) *A monograph of the world species of Hypoxylon.* University of Georgia Press:Athen, USA.

Miller, R.W. & Thompson, J.A. Curvularia leaf spot of Gladiolus. http://www.growit.com/bin/Problems.exe?Mytype=264

Minter, D.W. & Ingham, J.C. (1984) Leaf spotting on chrysanthemum associated with ejected spores of a species of *Podospora*. *Bulletin of the British Mycological Society* **18**:133-134.

Minter, D.W. & Webster, J. (1983) *Wawelia octospora* sp. nov., a xerophilous and coprophilous member of the Xylariaceae. *Transactions of the British Mycological Society* **80**: 370-373.

Monaghan, R.L., Polishook, J.D., Pecore, V.J., Bills, G.F., Nallin-Ilmstead, M. & Streicher, S.L. (1995) Discovery of novel secondary metabolites from fungi-is it really a random walk through a random forest? *Canadian Journal of Botany* **73**:S925-S931.

Mortimer, P.H. & di Menna, M.E. (1983) Ryegrass staggers: further substantiation of a *Lolium* endophyte aetiology and the discovery of weevil resistance of ryegrass pastures infected with *Lolium* endophyte. *Proceedings of the New Zealand Grassland Association* **44**:240-243.

Moss, M.O. (1984) The mycelial habit and secondary metabolite production. In *The ecology and physiology of fungal mycelium.* (D.H. Jennings & A.D.M. Rayner ,eds): 127-142. Cambridge University Press, Cambridge.

Mswaka, A.Y. & Magan, N. (1999) Temperature and water potential relations of Tropical *Trametes* and other wood-decay fungi from the indigenous forest of Zimbabwe. *Mycological Research* **103**(10):1309-1317.

Nissan Chemical Industries Ltd., 1980 Abstract. 94(3): patent number 15539x, Japan.

Norman, J.R., Atkinson, D. & Hooker, J.E. (1996) Arbuscular mycorrhizal fungal-induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytopthora fragariae*. *Plant Soil* **185**:191-198.

Nugent, L.K. (2004) Latent invasion by selected xylariaceous fungi. Ph.D. Thesis. Liverpool John Moores University.

O'Donnell, J. & Dickenson, C.H. (1980) Pathogenicity of *Alternaria* and *Cladosporium* isolates on *Phaseolus*. *Transactions of the British Mycological Society* **74**:335-342.

Oidtmann, B., Bausewein, S., HÖlzle, L., Hoffmann, R. & Wittenbrink, M. (2002) Identification of the crayfish plaque fungus aphanomyces astaci by polymerase chain reaction and restriction enzyme analysis. *Vetrinary Microbiology* **85**:183-194.

Okuno, T., Oikawa, S., Goto, T., Sawai, K., Shirahama, H. & Matsumoto, T. (1986) Structures and phytotoxicity of metabolites from valsaceratosperma. *Agricaltural and Biological Chemistry* **50**:997-1001.

Paden, J.W. (1978) Morphology, growth in culture and conidium formation in *Poronia pileiformis*. *Canadian Journal of Botany* **56**: 1774-1776.

Page, R.D.M. (1996) Treeview: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**:357-358.

Pan, J.J. & Clay, K. (2002) Infection by the systemic fungus *Epichloë glyceriae* and clonal growth of its host grass *Glyceria striata*. *Oikos* **98**(1): 37-46.

Parungao, M.M., Fryar, S.C. & Hyde, K.D. (2002) Diversity of fungi on rainforest litter in North Queensland, Australia. *Biodiversity and Conservation* **11**:1185-1194.

Paterson, R.R.M. & Bridge, P,D. (1994) *Biochemical techniques for filamentous fungi*. CAB International,Wallingford.

Pelaez, F., Collado, J., Arenal, F., Basilio, A., Cabello, A., Diez Mataz, M.T., Garcia, J.B., Gonzalez del Val, A., Gonzalez, V., Gorrochategui, J., Hernandez, P., Marti, I., Platas, G. & Vincente, F. (1998) Endophytic fungi from plants living on Gypsum soils as a source of secondary metabolite with antimicrobial activity. *Mycological Research* **102** (6): 755-761.

Pereira, J.O., Azavedo, J.L., Petrini, O. (1993) Endophytic fungi of Stylosanthes: a first report. *Mycologia* **85**:362-364.

Perumal Samy, R., Ignacimuthu, S. & Sen, A. (1998) Screening of 34 indian medicinal plants for antibacterial properties. *Journal of Ethnopharmacology* **62**(2): 173-182.

Petrini, L. & Petrini, O. (1985) Xylariaceous fungi as endophytes. *Sydowia* **38**:216-234.

Petrini, L. & Rogers, J.D. (1986) A summary of the *Hypoxylon serpens* complex. *Mycotaxon* **26**:401-436.

Petrini, L. & Petrini, O. (1985) Xylariaceous Fungi as Endophytes. *Sydowia* **38**: 216-235.

Petrini, L.E. & Muller, E. (1986) Haupt- und Nebenfruchtformen europaischer *Hypoxylon*-Arten (Xylariaceae, Sphaeriales) und verwandter Pilze. *Mycologia Helvetica* **1**:501-627.

Petrini, L.E., Petrini, O. & Laflamme, G. (1989) Recovery of endophytes of *Abies balsamea* from needles and galls of *Paradiopsis tumifex*. *Phytoprotection* **70**:97-103.

Petrini, L.E. & Petrini, O. (2004) Morphological studies in *Rosellinia* (Xylariaceae): the first step towards molecular investigations. *In Press.*

Petrini, L.E. (1992) *Rosellinia* species of the temperate zones. *Sydowia* **44**:169-281.

Petrini, L.E. (2003) *Rosellinia* and related genera in New Zealand. *New Zealand Journal of Botany* **41**:71-138.

Petrini, O. & Carroll, G.C. (1981) Endophytic fungi in foliage of some Cupressaceae in Oregon. *Canadian Journal of Botany* **59**: 629-636.

Petrini, O. & Dreyness, M. (1981) Endophytische pilze in epiphytischen Araceae, Bromeliaceae und Orchidaceae. *Sydowia* **34**:135-145.

Petrini, O. & Fisher, P.J. (1988) A comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. *Transactions of the British Mycological Society* **91**:233-238.

Petrini, O., Petrini, L.E. & Rodrigues, K.F. (1995) Xylariaceous endophytes: an exercise in Biodiversity. *Fitopatologia Brasiliera* **20**:531-539.

Petrini, O., Stone, J. & Carroll, F.E. (1982) Endophytic fungi in evergreen shrubs in Western Oregon: a preliminary study. *Canadian Journal of Botany* **60**:789-796.

Petrini, O. (1986) Taxonomy of endophytic fungi of aerial plant tissues. In *Microbiology Ecology of Phyllosphere.* (N. Fokkema & J. Van Der Heural ,eds): 175-187. Cambridge University Press, Cambridge, UK.

Petrini, O. (1987) Endophytic fungi of alpine Ericaceae. The endophytes of *Loiseleuria procumbens.* In *Arctic and Alpine Mycology II. Environmental Science Research. Vol. 34.* (G.A. Laursen, J.F. Amirati & S.A. Redhead ,eds): 71-77. Plenum Press,New York.

Petrini, O. (1991) Fungal endophyte of tree leaves. In *Microbial Ecology of Leaves.* (J.H. Andrews & S.S. Hirano ,eds.):179-97. Springer Verlag, New York.

Pharmaceutical Technology, Phloroglucinol, Jupiter Bioscience Ltd. http://www.jupiterbioscience.com

Phloroglucinol – Doctissimo http://www.doctissimo.fr/medicament-PHLOROGLUCINOL.htm

Phloroglucinol : http://www.biam2.org/www/Sub2438.html

Photita, W., Lumyong, S., Lumyong, P. & Hyde, K.D. (2001) Endophytic fungi of wild banana (*Musa acuminate*) at Doi Suthep Pui National Park in Thailand. *Mycological Research* **105**:1508-1513.

Pirttila, A.M., Pospiech, H., Laukkanen, H., Myllyla, R. & Hontola, A. (2003) Two endophytic fungi in different tissues of scots pine buds (*Pinus sylvestris* L.). *Microbial Ecology* **45**:53-62.

Pitt, J.I., Hocking, A.D., Bhudhasamai, K., Miscamble, B.F., Wheeler, K.A. & Tanboon-Ek, P. (1994) The normal mycoflora of commodities from Thailand. 2 Beans, rice. Small grains and other commodities. *International Journal of Food Microbiology* **23**:35-43.

Pittayakhajonwut, P. (2000) Secondary metabolites from *Xylaria* endophytes. Ph.D. Thesis. University of Bradford, Bradford, UK.

Plant Industry Division – Seed Section. Phomopsis Seed Decay of Soybeans Increased by Weathering. Tests to Assess Weathering Effects on Soybean Seed Quality. North Carolina Department of Agricultural & Consumer Services. http://www.ncagr.com/plantind/Seed/Soytest.htm

Platas, G., Acero, J., Borkowski, J.A., Gonzalez, V., Portal, M.A., Rubio, V., Sanchez-Ballesteros, J., Salazar, O. & Palaez, F. (2001) Presence of a simple tandem repeat in the ITS1 region of the Xylariales. *Current Microbiology* **43**:43-50.

Platas, G., Ruibal, C. & Collado, J. (2004) Size and sequence heterogeneity in the ITS1 of *Xylaria hypoxylon* isolate. *Mycological Research* **108**(1):71-75.

Polishook, J.D., Dombrowski, A.W., Tsou, N.N., Salituro, G.M. & Curotto, J.E. (1993) Preussomerin D from the endophyte *Hormonema dematioides*. *Mycologia* **85**(1): 62-64.

Pouzar, Z. (1979) Notes on the taxonomy and nomenclature of *Nummularia* (Pyrenomycetes). *Česká Mycologie* **33**:207-219.

Pouzar, Z. (1985) Reassessment of *Hypoxylon serpens*-complex I. *Česká Mycologie* **39**:15-25.

Puriso, B., Piapukiew, J., Pornpakakul, S., Petsom, A., Roengsumran, S., Whalley, A.J.S. & Sihanonth, P. (2004) *Antimicrobial compounds produced by Fusarium sp., endophytic fungus from Croton oblongifolius in Thailand.* Abstract. The 9th Biological Science Graduate Congress. 16th-18th December 2004. Chulalongkorn University, Thailand.

Quang, D.N., Hashimoto, T., Fournier, J., Stadler, M. & Radulovic Asakawa, Y. (2005) Sassafrins A-D, new antimicrobial azaphilones from the fungus *Creosphaeria sassafras*. *Tetrahedron In Press.*

Rayner, A.D.M. & Boddy, L. (1986) Population structure and the infection biology of living trees. *Advances in Plant Pathology* **5**:119-160.

Read, J.C. & Camp, B.J. (1986) The effect of fungal endophyte *Acremonium coenophialum* in tall fescue on animal performance, toxicity, and stand maintenance. *Agronomy Journal* **78**:848-850.

Riesen, T.K. & Close, R.C. (1987) Endophytic fungi in propiconazole-treated and untreated barley leaves. *Mycologia* **79**:546-552.

Rodrigues, K.F., Leuchtman, A. & Petrini, O. (1993) Endophytic species of *Xylaria*: Cultural and Isozymic studies. *Sydowia* **45**: 116-38.

Rodrigues, K.F. & Petrini, O. (1997) Biodiversity of Endophytic fungi in Tropical regions. In *Biodiversity of Tropical Microfungi.* (K.D. Hyde, ed): 57-69. Hong Kong University Press, Hong Kong.

Rodrigues, K.F. & Samuels, G.J. (1989) Studies in the genus *Phylacia* (Xylariaceae). *Memoirs of the New York Botanical Garden* **49**: 290-297.

Rodrigues, K.F. & Samuels, G.J. (1990) Preliminary study of endophytic fungi in tropical palm. *Mycological Research* **94**(6):827-830.

Rodrigues, K.F. (1994) The foliar fungi endophytes of the Amazonian Palm *Euterpe oleracea*. *Mycologia* **86**(3): 376-385.

Rogers, J.D., Callan, B.E., Rossman, A.Y. & Samuel, G.J. (1988) *Xylaria* (Sphaeriales, Xylariaceae) from Cerro De La Neldina, Venezuela. *Mycotaxon* **31**:103-153.

Rogers, J.D., Callan, B.E. & Samuels, G.J. (1987) The xylariaceae of the rain forests of North Sulawesi (Indonesia). *Mycotaxon* **29**:113-172.

Rogers, J.D., Ju, Y-M. & Candoussau, F. (1996) *Biscogniauxia anceps comb. nov.* and *Vivantia guadalupensis gen. et sp. nov. Mycological Research* **100**: 669-674.

Rogers, J.D., Ju, Y-M. & San Martin, F. (1997) *Jumillea* and *Whalleya*, new genera segregated from *Biscogniauxia*. *Mycotaxon* **64**: 39-50.

Rogers, J.D. & Ju, Y-M. (1996) *Entoleuca mammata* comb. nov. for *Hypoxylon mammatum* and the genus *Entoleuca*. *Mycotaxon* **59**: 441-448.

Rogers, J.D. & Læssøe, T. (1992) *Podosordaria ingii* sp. nov. and its *Lindquistia* anamorph. *Mycotaxon* **44**: 435-443.

Rogers, J.D. & Samuels, G.J. (1986) Ascomycetes of New Zealand 8. *Xylaria*. *New Zealand Journal of Botany* **24**(4):615-650.

Rogers, J.D. (1979) The Xylariaceae:systematic, biological and evolutionary aspects. *Mycologia* **71**(1):1-42.

Rogers, J.D. (1982) *Entonaema liquescens*: description of the anamorph and thoughts on its systematic position. *Mycotaxon* **23**: 429-437.

Rogers, J.D. (1984a) *Xylaria acuata*, *Xylaria cornu-damage*, and *Xylaria mali* in Continental United States. *Mycologia* **76**(1):22-33.

Rogers, J.D. (1984b) *Xylaria cubensis* and its anamorph *Xylocoremium flabelliforme*, *Xylaria allantoidea* and *Xylaria poitei* in Continental United States. *Mycologia* **76**: 912-923.

Rogers, J.D. (1985b) Anamorphs of *Xylaria*: taxonomic considerations. *Sydowia* **38**:255-262.

Rogers, J.D. (1994) Problem genera and family interfaces in the eupyrenomycetes. In *Ascomycete Systematics: Problems and Perspectives in the Nineties*. (D.L. Hawksworth ,ed.) :321-331. Plenum Press, New York.

Rogers, J.D. (2000) Thoughts and musings on tropical Xylariaceae. *Mycological Research* **104**(12):1412-1420.

Rowan, D.D., Hunt, M.B. & Gaynor, D.L. (1986) Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. *Journal of the Chemical Society D: Chemical Communications* **142**: 935-936.

Saikkonen, K., Faeth, S.H., Helander, M. & Sullivan, T.J. (1998) Fungal endophytes: A continuum interactions with host plants. *Annual Reviews of Ecology and Systematics* **29**: 319-343.

Saikkonen, K., Wäli, P., Helander, M. & Faeth, S.H. (2004) Evolution of endophyte-plant symbioses. *TRENDS in Plant Science* **9**(6):275-280.

Samuels, G.J. & Muller, E. (1980) Life history studies on Brazilian ascomycetes. 8. *Thamnomyces chordalis* (anam.: *Nodulisporium*) and *Camillea bacillum* (anam.: *Geniculosporium*) with notes on taxonomy of the Xylariaceae. *Sydowia* **33**: 274-281.

Samuels, G.J., Rogers, J.D & Nagasawa, E. (1987) Studies in the Amphisphaeriaceae (sensu lato). 1. *Collodiscula japonica* and its anamorph, *Acanthodochium collodisculae*. *Mycotaxon* **28**: 453-459.

Samuels, G.J. & Rossman, A.Y. (1992) *Thuemenella* and *Sarawakus*. *Mycologia* **84**:26-40.

Samuels, G.J. (1989) *Thuemenella cubispora*, a xylariaceous ascomycete and its biogeography. *Mycological Society of America Newsletter* **40**:46.

San Martin, F. & Rogers, J.D. (1993) *Biscogniauxia* and *Camillea* in Mexico. *Mycotaxon* **47**: 229-258.

Sánchez-Ballesteros, J., González, V., Salazar, O., Acero, J., Portal, M-A., Julián, M., Rubio, V., Bills, G.F., Polishook, J.D., Platas, G., Mochales, S. & Paláez, F. (2000) Phylogenetic study of *Hypoxylon* and related genera based on ribosomal ITS sequences. *Mycologia* **92**(5):964-977.

Schulz, B., Boyle, C., Draeger, S., RÖ mmert, A.K. & Krohn, K. (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* **106**(9): 996-1004.

Schulz, B., Wanke, U., Draeger, S. & Aust, H.J. (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research* **97**(12):1447-1450.

Segal, M. FDA Consumer Reprint--Ovarian Cancer. http://www.fda.gov/fdac/reprints/ovarian.html

Selosse, M-A & Le Tacon, F. (1998) The land flora : a phototroph-fungus partnership? *Tree* **13**(1):15-20.

Sherwood-Pike, M., Stone, J.K. & Carroll, G.C. (1986) *Rhabdoeline parkeri*, a ubiquitous foliar endophyte of Douglas fir. *Canadian Journal of Botany* **64**: 1849-1855.

Siddhuraju, P., Mohan, P.S. & Becker, K. (2002) Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chemistry* **79**:61-67.

Sieber, T.N. & Hugentobler, C. (1987) Endophytische Pilze in Blättern und Ästen gesunder und geschädigter Buchen (*Fagus sylvatica* L.). *European Journal of Forest Pathology* **17**:411-425.

Sieber, T.N., Rys, J. & Holdenrieder, O. (1999) Mycobiota in symptomless needles of *Pinus mugo* sp. *uncinata*. *Mycological Research* **103**(3): 306-310.

Sieber, T.N., Sieber-Canavesi, F., Petrini, O., Ekramoddoullah, A.K.M. & Dorworth, C.E. (1991) Characterization of Canadian and European Melanconium from some *Alnus* species by morphological, cultural, and biochemical studies. *Canadian Journal of Botany* **69**(10): 2170-2176.

Sieber, T.N. (1985) Endophytische pilze von winterweizen (*Triticum aestivum* L.). Dissertation ETH no 7725. Swiss Federal Institute of Technology, Zürich, Switzerland.

Sieber, T.N. (1988) Endophytische pilze in nadeln von gesunden und geschadigten Fichten (*Picea abies* (L.) Karsten). *European Journal of Forest Pathology* **18**:321-342.

Sieber, T.N. (1989a) Endophytic fungi in twigs of healthy and diseased Norway spruce and white fir. *Mycological Research* **92**:322-326.

Sieber-Canavesi, F., Petrini, O. & Sieber, T.N. (1991) Endophytic Leptostroma species on *Picea abies*, *Abies alba* and *Abies balsamea*: a cultural, biochemical, and numerical study. *Mycologia* **83**:89-96.

Sieber-Canavesi, F. & Sieber, T.N. (1988) Endophytische pilze in Tame (*Abies alba* Mill.) – Vergleich zweiser Standorte im Schweizer Mittelland (Naturwald Aufforstung). *Sydowia* **40**:250-273.

Sinclair, J.B. & Cerkauskas, R.F. (1996) Latent infection vs. Endophytic colonization by Fungi. In *Endophytic fungi in grasses and woody plants: Systematics, Ecology, and Evolution.* (S.C. Redin & L.M. Carris ,eds): 3-29. APS Press, Minnessota, USA.

Sircar, P.K., Dey, B., Sanyal, T., Ganguly, S.N. & Sircar, S.M. (1970) Gibberellic acid in the floral parts of *Cassia fistula*. *Phytochemistry* **9**:735-736.

Sivanesan, A. & Holliday, P. (1972b) *Rosellinia necatrix*. C.M.I. *Descriptions of pathogen fungi and bacteria No.352.*

Slotta, T.A.B. (2000) Phylogenetic analysis of *Iliamma* (Malvaceae) using the internal transcribed spacer region. M.Sc. Thesis. The Virginia Polytechnic Institute and State University.

Southern Soybean Disease Workers. Soybean Disease Atlas. 2nd Edition. Stem and Pod Rot Disease. http://cipm.ncsu.edu/ent/SSDW/stematlas.html

Stadler, M., Wollweber, H., Mühlbauer, A., Asakawa, Y., Hashimoto, T., Rogers, J.D., Ju, Y.M., Wetzstein, H-G. & Tichy, H-V. (2001a) Molecular chemotaxonomy of *Daldinia* and other Xylariaceae. *Mycological Research* **105**(10): 1191-1205.

Stadler, M., Wollweber, H., Mühlbauer, A., Henkel, T., Asakawa, Y., Hashimoto, T., Ju, Y-M., Rogers, J.D., Wetzstein, H-G. & Tichy, H-V. (2001b) Secondary metabolite profiles, genetic fingerprints and taxonomy of *Daldinia* and allies. *Mycotaxon* **77**: 379-429.

Stierle, A., Strobel, G., Stierle, D., Grothaus, P. & Bignami, G. (1995) The search for a Taxol producing microorganism among the endophytic fungi of the Pacific Yew, *Taxus brevifolia*. *Journal of Natural Products* **58**(9): 1315-1324.

Stierle, A., Strobel, G. & Stierle, D. (1993) Taxol and Taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific Yew. *Science* **260**: 214-216.

Stiers, D.L., Rogers, J.D. & Russell, D.W. (1973) Conidial state of *Poronia punctata*. *Canadian Journal of Botany* **51**: 481-484.

Stone, J.K., Bacon, C.W. & White, J.F. Jr. (2000) An overview of endophytic microbes: endophytism defined. In *Microbial Endophytes*. (C.W. Bacon & J.F. White Jr. ,eds):3-29. Marcel Dekker, New York.

Stone, J.K. (1986) Foliar endophytes of *Pseudotsuga menziesii* (Mirb.) Franco Cytology and ohysiology of the host-endophyte relationship. Dissertation. University of Oregon, Eugene. USA.

Strobel, G., Ford, E., Worapong, J., Harper, J.K., Arif, A.M., Grant, D.M., Fung, P.C.W. & Chau, R.M.W. (2002) Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. *Phytochemistry* **60**:179-183.

Strobel, G., Stierle, A. & Hess, W.M. (1994) The stimulation of Taxol production in *Taxus brevifolia* by various growth retardant. *Plant Science* **101**: 115-124.

Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R.S. & Hess, W.M. (1996) Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiology* **142**: 435-440.

Strobel, G.A., Ford, E., Li, J.Y., Sears, J., Sidhu, R.S. & Hess, W.M. (1999) *Seimatoantlerium tepuiense* gen. nov., a unique epiphytic fungus producing Taxol from the Venezuelan Guyana. *Systematic and Applied Microbiology* **22**: 426-433.

Strobel, G.A. & Long, D.M. (1998) Endophytic microbes embody pharmaceutical potential. *ASM News* **64**(5):263-268.

Strobel, G.A. (2002) Rainforest endophytes and bioactive products. *Critical Reviews in Biotechnology* **22**(4):315-333.

Strobel, G.A. (2003) Endophytes as sources of bioactive products. *Microbes and Infection* **5**:535-544.

Subramanian, C.V. & Chandrashekara, K.V. (1977) *Lindquistia*, a new hyphomycete genus. *Buletin de la Sociedad Argentina de Botanica* **18**: 145-151.

Supudompol, B., Likhitwitayawuid, K. & Houghton, P.J. (2004) Phloroglucinol derivatives from *Mallotus pallidus*. *Phytochemistry* **65**(18):2589-2594.

Suryanarayanan, T.S., Murali, T.S. & Venkatesan, G. (2002) Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. *Canadian Journal of Botany* **80**:818-826.

Suryanarayanan, T.S., Venkatesan, G. & Murali, T.S. (2003) Endophytiv fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. *Current Science* **85**(4):489-493.

Swofford, D.L. (2004) *PAUP*: Phylogenetic analysis using parsimony (*and other methods) version 4.0b10.* Sinauer Associates, Sunderland, Massachusetts, USA.

Tan, R.X. & Zou, W.X. (2001) Endophytes: a rich source of functional metabolites. *Natural Product Reports* **18**:448-459.

Teixeira De Sousa, A.J. & Whalley, A.J.S. (1991) Induction of mature stromata in *Rosellinia necatrix* and its taxonomic implications. *Sydowia* **43**:281-290.

The International Rubber Research and Development Board. *Corynespora* Leaf Fall Disease (CLFD). http://www.trrdb.com/IRRDB/NaturalRubber/Diseases/clfd.htm

Thienhirun, S. & Whalley, A.J.S. (2004) The Xylariaceae. In *Thai Fungal Diversity* (E.B.G.Jones, M. Tanticharoen & K.D. Hyde, eds) BIOTEC:Thailand. *In Press*

Thienhirun, S. (1997) A preliminary account of the Xylariaceae of Thailand. Ph.D. Thesis. Liverpool John Moores Unviersity. United Kingdom.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**:4876-4882.

Tian, X.L., Cao, L.X., Tan, H.M., Zeng, Q.G., Jia, Y.Y., Han, W.Q. & Zhou, S.N. (2004) Study on the communities of endophytic fungi and endophytic

actinomycetes from rice and their antipathogenic activities *in vitro*. *World Journal of Microbiology and Biotechnology* **20**:303-309.

Triebel, D., Persoh, D., Wollweber, H. & Stadler, M. (2005) Phylogenetic relationship within the Xylaraiceae with *Nodulisporium*-like anamorphs as inferred from ITS nrDNA sequences. *Nova Hedwigia In Press.*

Turner, W.B. (1971) *Fungal Metabolites.* Academic Press, London, United Kingdom.

Turner W.B., Aldridge D.C. (1983). *Fungal metabolites II.* Academic Press, London, United Kingdom.

Udagawa,S-I. & Ueda, S. (1988) *Calceomyces*, a new genus of the Xylariaceae with shoe-shaped ascospores. *Mycotaxon* **32**: 447-455.

Ulje, K. *Coprinus radians.* http://www.grzy.pl/coprinus-site-kees-uljee/species/radians_.htm

Umehara, K., Singh, I.P., Etoh, H., Takasaki, M. & Konoshima, T. (1998) Five phloroglucinol-monoterpene adducts from *Eucalyptus grandis*. *Phytochemistry* **49**(6):1699-1704.

Valldosera, G. & Guarro, G. (1988) Some coprophilous ascomycetes from Chile. *Transactions of the British Mycological Society* **90**: 601-605.

Van Der Gucht, K. (1994) The xylariaceae of Papua New Guinea. Ph.D. Thesis. Universiteit Gent.

Van der Gucht, K. (1995) Illustrations and descriptions of xylariaceous fungi collected in Papua New Guinea. *Bulletin Jardin Botanique National de Belguique* **64**:219-403.

Vederas, J.C., Graf, W., David, L. & Tamm, C.H. (1975) Biosynthesis of cytochalasins. Part IV. *Helvetica Chimica Acta* **58**:1866-1898.

Verhoeff, K. (1974) Latent infections by fungi. *Annual Review of Phytopathology* **12**:99-110.

Vining, L.C. (1992) Roles of secondary metabolites from microbes. In *Secondary metabolites: Their function and evolution.* (D.J. Chadwick & J. Whelan, eds): 184-198. Ciba Foundation Symposium 171. John Wiley and Sons, Chichester.

Wagenaar, M.M., Corwin, J., Strobel, G. & Clardy, J. (2000) Three new cytochalasins produced by an endophytic fungus in the genus *Rhinocladiella. Journal of Natural Product* **63**:1692-1695.

Waller, G.R. & Dermer, O.C. (1981) Enzymology of alkaloid metabolism in plants and microorganisms. *In The biochemistry of plants.* Volume 7 (E.E. Conn, ed.): 317-402.. Academic Press, New York.

Wat Bowonnivet Vihara, Bangkok, Thailand. *Cassia* pictures http://www.watbowon.org

Webber, J. (1981) A natural control of Dutch elm disease. *Nature (London)* **292**: 449-451.

Weber, H.A., Baenziger, N.C. & Gloer, J.B. (1990) Structure of preussomerin A, an unusual new antifungal metabolite from the coprophilous fungus *Preussia isomera. Journal of the American Chemical Society* **112**: 6718-6719.

WebWright Asia Co. Ltd. Thailand Map http://www.amazing-thailand.com

Wei, C.T. (1950) Notes on *Corynespora. Mycological Papers* **34**:1-10.

Whalley, A.J.S. & Edwards, R.L. (1985) *Nummulariella marginata*: its conidial state, secondary metabolites and taxonomic relationships. *Transactions of the British Mycological Society* **85**: 385-390.

Whalley, A.J.S. & Edwards, R.L. (1986) Xylariaceous fungi: Use of secondary metabolites. In *Evolutionary biology of the fungi.* (A.D.M. Rayner, C.M. Brasier & D. Moore, eds): 423-434.Cambridge University Press, Cambridge, UK.

Whalley, A.J.S. & Edwards, R.L. (1995) Secondary metabolites and systematic arrangement within the Xylariaceae. *Canadian Journal of Botany* **73**(Suppl1): S802-S810.

Whalley, A.J.S. & Edwards, R.L. (1999) The Xylariaceae: A case study in biological and chemical diversity. *Pure and Applied Chemistry* **70**(11):2123-2134.

Whalley, A.J.S. (1976) Notes on the conidial state of *Hypoxylon udum*. *Transactions of the British Mycological Society* **67**: 515-517.

Whalley, A.J.S. (1985) The Xylariaceae: Some ecological considerations. *Sydowia* **38**: 369-382.

Whalley, A.J.S. (1997) Xylariaceae. In *Biodiversity of Tropical Microfungi.* (K.D. Hyde, ed.): 279-296. Hong Kong University Press, Hong Kong.

Whalley, M.A., Ju, Y-M., Rogers, J.D. & Whalley, A.J.S. (2000) New xylariaceous fungi from Malaysia. *Mycotaxon* **74**:135-140.

Whalley, M.A. (2001) Studies of tropical xylariaceae. Ph.D.Thesis. The University of Portsmouth, Porthsmouth, UK.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A guide to Methods and Applications.* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White ,eds.): 315-322. Academic Press, London.

Widler, B. & Müller, E. (1984) Untersuchungen über endophytische pilze von *Arctostaphylos uva-ursi* (L.) Spreng (Ericaceae). *Botanica Helvetica* **94**:307-337.

Wijaya, R., Neumann, G.M., Condron, R., Hughes, A.B. & Polya, G.M. (2000) Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. *Plant Science* **159**:243-255.

Wildman, H.G. (1997) Potential of tropical microfungi within the pharmaceutical industry. In *Biodiversity of tropical microfungi.* (K.D. Hyde ,ed.): 29-46. Hong Kong University Press, Hong Kong.

Wilson, A.D. (1995) Endophyte – the evolution of a term and clarification of its use and definition. *Oikos* **73** (2): 274-276.

Wiyakrutta, S., Sriubolmas, N., Panphut, W., Thongon, N., Danwisetkanjana, K., Ruangrungsi, N. & Meevootisom, V. (2004) Endophytic fungi with anti-microbial, anti-cancer, and anti-malarial activities isolated from Thai medicinal plants. *World Journal of Microbiology* **20**:265-272.

Wood, M. & Stevens, F. California Fungi: *Psathyrella gracilis.* http://www.mykoweb.com/CAF/species/Psathyrella_gracilis.html

Woods, D.L. & Clark, K.W. (1971) Variation in the context of tryptamines in clones of *Phalaris arundinacea* L. *Crop Science* **11**:121-122.

Xylariaceous fungi: Evolution of morphological characters 1. http://pyrenomycetes.free.fr/xylariaceous/keydir/morphchar.htm

Yang, X., Strobel, G., Stierle, A., Hess, W.M., Lee, J. & Clardy, J. (1994) A fungal endophyte-tree relationship: *Phoma* sp. in *Taxus wallachiana. Plant Science* **102**: 1-9.

Appendix I

2% Malt Extract Agar

malt extract (Oxiod)	20 g
peptone bacteriological (Oxoid)	1 g
Glucose	20 g
Oxoid agar No.1	15 g
H_2O	1 litre

Malt Extract Broth

Malt extract (Oxoid)	17 g
Peptone	3 g
H_2O	1 litre

Potato Dextrose Agar

Potato (infusion form)	200 g
Dextrose	20 g
Oxoid agar No.1	15 g
dH_2O	1000 ml

200 g of diced potato were boiled in about 300 ml of distilled water and the potato infusion was filtrated. Meanwhile, 15 g of agar was put into approximately 700 ml of water and left to dissolve in the steamer. When agar was done, it was mixed with 20 g of dextrose and the potato infusion. The medium was sterile at 121ºC for 15 minutes.

EDTA pH 8.0

diSodium EDTA $2H_2O$	186.1 g
d H_2O	800 ml

adjust the volume to 1000 ml

3M Sodium Acetate pH 8.0

Sodium Acetate 3 H_2O	102 g
d H_2O	200 ml

adjust the volume to 250 ml

1M Tris

Tris base	121.1 g
d H_2O	800 ml

Adjusting the pH by adding concentrated HCl approximately 70-76.1 ml.

10% SDS

SDS (Electrophoresis grade)	25 g
d H_2O	225 ml

Dissolving the SDS solution by heating the solution to 68ºC.

50x TAE buffer

Tris base	48.40 g
Glacial acetic acid	11.42 ml
0.5M EDTA (pH8.0)	20.0 ml

Adjust H_2O to 200 ml

PCR cocktail

DNA (10 ng/ml from stock 100 ng/ml)	5 µl
Primer ITS5 (100 pmol)	1 µl
Primer ITS4 (100 pmol)	1 µl
Reddymix	25 µl
dH_2O	18 µl

total volume 50 µl

Nystatin (Sigma)

Nystatin	50 mg = 0.05 g
1M NaOH	200 µl
d H_2O	1800 µl

Concentration of stock solution 25 mg/ml

Ampicillin (Sigma)

Ampicillin	0.01 g
d H_2O	1 ml

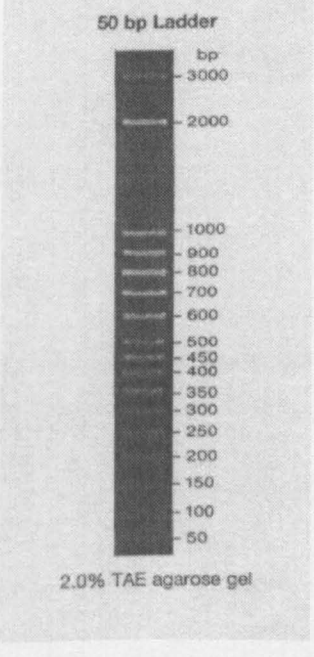
Concentration of stock solution 1 mg/ml

TLC solvent system

Toluene:Ethyl Acetate:Acetic Acid = 50:49:1

Marker ladders used in gel electrophoresis

Perfect DNA 50bp ladder (Novagen)



Appendix II

Possible identification of endophytic fungi from *Cassia fistula* L. using a molecular approach and BLAST programme

Isolate no.	Result
16	1st:AB041994 Unidentified white mycelium 2nd:AF163038 *Xylaria longipes* 3rd:AF163033 *Xylaria enteroleuca*
19	1st:AF163027 *Xylaria apiculata* 2nd:AF153725 *Xylaria* MS358 3rd:AF153733 *Xylaria* MS370 4th:AY183369 *Xylaria arbuscula*
21	1st:AF153726 *Xylaria* MS259 2nd:AF163040 *Xylaria mali* 3rd:AF163035 *Xylaria hypoxylon*
29	HAN390429 *Nemania bipapillata*
38	1st:AB041994.1 unidentified white mycelium 2nd:AF163038 *X. longipes* 3rd:AY315404 *Xylaria* sp F19 4th:AY315405 *Xylaria* sp F4
SC3	1st:AY541610.1 *Nemania bipapillata* strain CL8 2nd:HAN390429 *Nemania bipapillata*
SC31	1st:AY315404 *Xylaria* sp F19 2nd:AY315405 *Xylaria* sp F4 3rd:AB041994.1 unidentified white mycelium 4th:AF163038 *X. longipes*
N24	1st:AB017660 *Rosellinia arcuata* 2nd:AB017658 *Rosellinia necatrix*
PX1	1st:AF163038 *Xylaria longipes* 2nd:AB041994 Unidentified white mycelium 3rd:AF163033 *Xylaria enteroleuca*
UX1	1st:AB041994 Unidentified white mycelium 2nd:AF163038 *Xylaria longipes* 4th:AF163033 *Xylaria enteroleuca*
UX2	1st:AF163038 *Xylaria longipes* 2nd:AF163033 *Xylaria enteroleuca* 3rd:AB041994 Unidentified white mycelium
UX3	1st:AF163027 *Xylaria apiculata* 2nd:AF153725 *Xylaria* MS358 3rd:AF153733 *Xylaria* MS370 4th:AF163029 *Xylaria arbuscula*
UX5	1st:XHY309350 *Xylaria hypoxylon* 2nd:AY183369 *Xylaria arbuscula*
U65X	1st:AF163038 *Xylaria longipes* 2nd:AB041994 Unidentified white mycelium 3rd:AF163033 *Xylaria enteroleuca*

Possible identification of endophytic fungi from *Cassia fistula* L. using a molecular approach and BLAST programme

Isolate	Results
3	1st:AY148440 *Diaporthe* sp. 2nd:AY050627 *Phomopsis sojae* 3rd:AF001020 *Diaporthe phaseolum*
11	1st-3rd:DHE312366 *Diaporthe helianthi* 4th:AF000207 *Phomopsis longicolla*
22	1st: PSU94898 *Phomopsis* sp. GM12 2nd:PSU91617 *Phomopsis* sp. GAP08 7th:AF001205 *Diaporthe phaseolorum*
SC6	1st:PSU94898 *Phomopsis* sp GM12 2nd:PSU91617 *Phomopsis* sp GAP08 3rd:AF079777 *Phomopsis oryzae* 4th:AF001025 *Diaporthe phaseolorum*
SC8	1st:AF317584 *Phomopsis* sp DF5040 2nd:AY620819 *Phomopsis phyllanthicola* 3rd:AY601917 *Phomopsis loropetali*
SC27	1st:AF317583 *Phomopsis* sp. DF5036 2nd:AF001205 *Diaporthe phaseolorum* AK25A
N46	AF001017 *Diaporthe phaseolorum*
P2	1st:AF001017 *Diaporthe phaseolorum* 2nd:AF103000 *Phomopsis* sp.
P12	1st:PSU94898 *Phomopsis* sp. GM18 2nd:PSU91617 *Phomopsis* sp. GAP08 3rd:AF07977 *Phomopsis oryzae*
PP1	AF000207 *Phomopsis longicolla*
PP2	1st:AF317584 *Phomopsis* sp. DF5040 2nd:AF103000 *Phomopsis* sp. FAU 3rd:AF317582 *Phomopsis* sp. DF5028 4th:AF317581 *Phomopsis* sp. DF5041 5th:AB105146 *Phomopsis asparagi*
PP3	1st:AF103000 *Phomopsis* sp. FAU1053 2nd:AF317582 *Phomopsis* sp. DF5028 5th:AF153737 *Diaporthe* sp. MS704 6th:DPH312360 *Diaporthe phaseolus*
UP3	1st:AF000567/AF000563 *Diaporthe caulivola* 2nd:AF000212 *Diaporthe phaseolorum* var *caulivola* 3rd:AY578071 *Phomopsis eucommicola*
UP4	1st:AY148440 *Diaporthe* sp. 36-1 2nd:AF001020 *Diaporthe phaseolorum* strain pssp5 3rd:AY050627 *Diaporthe sojae*

Possible identification of endophytic fungi from *Cassia fistula* L. using a molecular approach and BLAST programme

Isolate	Results
PD1	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 4th:AY354238 *Daldinia petrinae*
PD2	AF176958/AF176957 *Daldinia concentrica*
PD3	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 4th:AY354238 *Daldinia petrinae*
PD4	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 4th:AY354238 *Daldinia petrinae*
UD1	AF201708/AF176958 *Daldinia concentrica*
UD2	AF176958/AF176957 *Daldinia concentrica*
UD3	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 3rd:AY354238 *Daldinia petrinae* 4th:AF176958 *Daldinia concentrica*
UD4	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 3rd:AY354238 *Daldinia petrinae* 4th:AF176958 *Daldinia concentrica*
UD5	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 4th:AY354238 *Daldinia petrinae*
U65D	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AF280629 *Nodulisporium* sp. JP807 4th:AY354238 *Daldinia petrinae*

Possible identification of endophytic fungi from *Cassia fistula* L. using a molecular approach and BLAST programme

Isolate	Results
20.1	AF176958/AF176957 *Daldinia concentrica*
25	AF280628.1 *Nodulisporium* sp. JP3665
33	AF176958/AF176957 *Daldinia concentrica*
SC4	AF201708/AF176958 *Daldinia concentrica*
SC18	AF176958/AF176957 *Daldinia concentrica*
SC19	AF201708/AF176958/AF176957 *D. concentrica*
SC25	AF176958/AF176957/AF176956 *D. concentrica*
SC33	AF176958/AF176957 *Daldinia concentrica*
SC40	AF176958/AF176957 *Daldinia concentrica*
SC42	AF176958/AF176957 *Daldinia concentrica*
SC44	AF176958/AF176957 *Daldinia concentrica*
N26	AF176958/AF176957 *Daldinia concentrica*
N37	AF176958/AF176957 *Daldinia concentrica*
N4	1st:AY228352 *Psathyrella cf. gracilis* 2nd:AJ246160 Oat root associated basidiomycete 3rd:AF345813 *Coprinus lagopus* 4th:AF345810 *Psathyrella candleana*
SC43	1st:AF413049 Fungal endophyte 2nd:AF413047 Fungal endophyte 3rd:AF413048 Fungal endophyte 4th:CSPAJ809 *Fusarium* sp. 5th:FSP289870 *Fusarium* sp. Chiang
U1	1st:HAN390408 *Hypoxylon rickii* 2nd:HAN390398 *Hypoxylon cinnabarinum* 3rd:AF201757.1 *Nodulisporium* sp. 4th:AF201754.1 *Nodulisporium* sp.
SC10	1st:AF345822 *Coprinus radians* 2nd:AF361228 *Coprinus xanthothrix* 3rd:AB176569 mycorrhizal basidiomycetes 4th:AY461815 *Coprinellus radians*
P8	1st:AY238606 *Corynespora cassiicola* 2nd:AY238605 *Corynespora cassiicola* 3rd:AY265333 *Pyricularia variabilis*
P22	1st-4th:AF130142/AF178395/AF178418/AY188918 *Nectria haematococca* 5th:AF178404 *Fusarium* sp.
U41	1st:FSU61695 *Fusarium* sp. 2nd:PPU18954 *Pseudofusarium purpureum*

Possible identification of endophytic fungi from *Cassia fistula* L. using a molecular approach and BLAST programme

Isolate	Results
8	1st:AF071337 *Curvularia gladioli* 2nd:AF455446 *Curvularia trifolii*
SC32	AF106532 *Nectria gliocladioides*
U59	1st:AF280627 *Nodulisporium* sp. JP3821 2nd:AF176969 *Daldinia loculata*
P24	1st:AF280628 *Nodulisporium* sp. JP3665 2nd:AY616684 *Daldinia eschscholzii* 3rd:AF280629 *Nodulisporium* sp. JP807 4th:AY616685 *Entonaema cinnabarinum*

Appendix III

ITS sequences of the *Cassia* endophytes in this study

Group I : *Xylaria*-like group

>16 *Xylaria* cf. *longipes*

CATTAAAGAGTTCTATAACTCCCAAACCCATGTGAACATACCTAACGTTGCCTC
GGCAGGTCGCACCTACCTTGTAGCACCCTACCCTGTAGGGCCTACCCGGGAG
GTGCGGGTGAAGCCTGCCGGCGGCCCACCAAACTCTGTTTGATATTGAATTCT
GAACCTATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCATATTGCGCCCATTAGTATTCTAGTGGGCAGG
ACCGTTCGAGCGTCATTTCAACCCTTAAGCCTTCGTTGCTTAGTGTTGGGAGCC
TACGGCAACGTAGCTCCTCAAAGTTAGTGGCGGAGTTGGTTCACACTCTAGAC
GTAGTAATTTTTTATCTCGCCTATTAGTGGACTAATCCCCTGCCGTAAAACCCC
CAATTTATCAAGGTTGACCTCGAATCGGTTGTGACAAACTCGCTAAATGAAGCA
TATCAATAAGCG

>19 *Xylaria* cf. *apiculata*

CATTAAAGAGTTATACAACTCCTAAACCCATGTGAACCTACCTTTGTTGCCTCG
GGCAGGGCCTGGGCTTACCCTGTGAGCCCCTACCCTGTAGGGACCTTACCCG
GTAGTTGCGGATACAACCTGCCGGTGGTCTACTAAACTCTGTTTACTATGTTAT
TCTGGAATAGTATAACTAAATAAAGAATAAAAACTTTCAACAACGGATCTCTTGG
TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGT
TGGGAGCCTACAGATACTCTCTGTAGTTCCTTAAAGTTAGTGGCGGAGTCGGT
TTCACACTCTAGACGTAGTAAATTTTATCTCGCCTATAGATGAGCCGGTCCCCT
GCCGTAAACCCCCAATATCTAAAGGTGACCTCGGATCAGGTAGGCATACCCG

>21 *Xylaria* sp. MS259

CATTAAAGAGTTTTGATAAAACTCCCAAACCCATGTGAACTTACCTCTTGTTGCC
TCGGCAGGTCGTACCTACCCTGTGGGCTCCTACCCTGTAGGAGCCTACCCGG
TCGTTGCGGTCGTGCCTGCCGGTGGCCCGTCAAAGAAAACTTGTGTATTGTCT
ATGTGATTCTGAGTGGGCAACTAACAATACAGTCAAAACTTTACATACAACGGA
TCTCTTGGTTCTGGCATCGATGAAAAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGACGCACATTGCGCCCATTAGTA
TTCTAGTGGGCATGCCTGTTCGAGCGTCATTTTACCCTTAAGCCCCTGTTGCTT

AGTGTTGGGAGCCTACAGTCCTCTGTAGCTCCTCAAAGTTAGTGGCGGAGTTG
GTTTGCACTTTAGACGCAGTAGATTCTATCTCGTCTACAGTTAAGCCGGTCTCT
CGCCGTTAAAACCCCCTAATTTTTCAAGGTTGACCTCGGATCAGGTAGGAATAC
CC

>29 *Nemania* cf. *bipapillata*

CATTAGAGAGTCTAAACAACTCCCAAACCCACTGTGAACATACCCTACGTTGCC
TCGGCAGGCGCCCGGCTCCGCGACCCCTCGGGGCTGCGGGGTGGCCCTGCC
GGCGGCCCACGAAACTCTTGTCTAGCACTGAATTCTGAGCCCGAGAGGGATAA
AAACAAATTAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GACGCATGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTTGAATCATC
GAATCTTTGAACGCACATTGCGCCCGCCAGTATCTGGCGGGCATGCCTGTACG
ATCGTCATTCTAACCCTCAAGCCTCGTTGCTTGGTGTTGGGAGCCTACGGCTG
TAGCTCCTCAAAGTCAGTGGCGTGGGCTGGCTCGCACCCCAGATGTAGTAGTT
ATTTCTCTCACCTGTGGTCGGGCTAGTCCCCTGCCGTAAAACCCCCCAGACTT
TTTAGTGTTTGACCTCGGATCAGGTAGGGATACCC

>34 Partially similar to *Xylaria* cf. *apiculata*

CATTAAAGAGTTATACATACTCCTAAACCCATGTGATACCTACCTTTGTTGCCTC
GCGCAGGCCTGCAGCTTACCCTGTGAGCCCCTACCCTGTAGGGACCTTACCCT
GGTAGTTGCTGGATACAAGCCTGCCGGTCGGTCCTACTAAACTCTGTTGTACT
ATGATTGATTACTGAATAGTATAACATATAATAAGTTATATAACTATTCATACAAC
CGGATCATCTTGGTTGCTGGCGAGTCAGTATGAAGAACGACAGCGACAATGAC
TGATAATGTAATGATGAATTAGCAGCAATTACATGCTGAATCATCAGTAATCATT
TGTAACGACAGCATCTGCGCCCACTCTAGTATTATTAGTGGGCAGTGACCGTC
GTTGCGTAGCGTCATTTCAACCCTCTCAAGCCCCTCGCTTGCTGTAGCGTTGG
GAGCCGTCACAGCATGATCTCTTCTGTAGGTTCCTTCTAAAGTTAGTGGCGGA
GTCGGTTTGCACACTCTAGACGTAGTAAATTTATATTCTGCTGCTCATCATGAG
TATGACGCGCGGTCCCCTCGCGCGTAAAACCCCAATATCTAAGGTGACTCGGA
TCAGTAGATACCGCTGACTACATTATACGA

>38 *Xylaria* cf. *longipes*

CATTACAGAGTTCTATAACTCCCAAACCCATGTGCACATACCGTACGTTGCCTC
GGCAGGCGGCGCCCACCCCGTAGCGCCCTACACCCGGTAGGGCCTGCCCGG
TGGACGCCGACAAAGCCTGCCGGCGGCCCCTGAAAATTCTGTTTCTTACTGGA
TCTCTGAACATATACCAAAATACGTTAAAACTTTCAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTG
GGAGCCTACCGTCACACGTAGCTCCTGAAAAGTAGTGGCGGAGTCGGTTCTCA

CTCTAGACGTAGTAAATTCTATCTCGCCTATCAGTAGGACCGGTCCCTCGCCGT
AAAACCCCCCCTATATTTTAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCT
GAACTTAAGCATATCAATAAGCGGAAGA

>N24 *Rosellinia* cf. *arcuata*

CATTACAGAGTTATTACAACTCCTAAACCCCATGTGAACATACCTTGCGTTGCC
TCGGCAGGCCGCGTCGTACCTCGCAGTGCCCTTTACGCTGTAAAGGCCTGCTT
GGCGGGCGCGGGTTACCCTGCCGGCGGCTTATAAAACTCTGTTTTAGCATTGT
ACTTCTGAACACATAACTAAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTT
CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTG
GGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCTCGTTGCTTAGTGTTG
GGAGCCTACGGCCTGCCGTAGCTCCCCAAAGTTAGTGGCGGGGTCGGTTCGC
ACTCTAGACGTAGTAGATTTCATCCATCTCGCCTGTAGTGTGTGCCGGTCCTCT
GCCGTAAAACCCCCATATATCAAAGGTTGACCTCGGATCAGGTAGGAATACC

>PX1 *Xylaria* cf. *longipes*

CATTAAAGAGTTTATACTCCCAAACCCCATGTGAACATACCTAACGTTGCCTCG
GCGGGTCGTACCTACCCTGTAGTGCACTTACCTGTAAGTGCCTACCCGGTAGG
CACGGGTAAGCCCGCCGGCGCCCCATTAAACTCTGTTTAATTACTGGATATCT
GAATACAACTAAATAAGTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC
GATGAAGACGCAGCGAAATGCGATAAGTAATGTGAATGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCCGCCCATTAGTATCCTAGTGGGCATGCC
CTGTTCCTGAGCGTCATTTCAACCCTTAAGCCTTCTGTTGCTTAGCGTTGGGGG
CCTACCGTATGGCGGTAGCCCCTTAAAATTAGTGGCGGAGTCGGTTCACACTC
TAGACGTAGTAAATATTATCTCGCCTATTAGTTGGACCGGTCCCCTGCCGTAAA
ACCCCTAATTTATACAAGGTTGACCTCGGATCAGGTAGGAATACC

>UX1 *Xylaria* cf. *longipes*

CATTAAAGAGTTCTATAACTCCCAAACCCATGTGAACATACCAAACGTTGCCTC
GGCAGGTCGCACCTACCTTGTAGCACCCTATCCTGTAGGGCCTACCCGGGAC
GTGCGGGTAAGCCTGCCGGCGGCCCATTAAACTCTGTTTGATATTGAATTCTG
AACCTATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCATATTGCGCCCATTAGTATTCTAGTGGGCAGGA
CCGTTCGAGCGTCATTTCGACCCTTAAGCCTTAGTTGCTTAGTGTTGGGAGCCT
ACGGCGACGTAGCTCCTCAAAGTTAGTGGCGGAGTTGGTTCACACTCTAGACG
TAGTAATTTTTATCTCGCCTATTAGTTGGACCGATCCCCTGCCGTAAAACACCC
AACTTCTCAAGGTTGACCTCGAATCGGTTCAGACAAACTCGCTAATTGAAGCAT
TCATACGG

>UX2 *Xylaria* cf. *longipes*

CATTAAAGAGTTTATAACTCCCAAACCCCATGTGAACATACCTAACGTTGCCTC
GGCGGGTCGTACCTACCCTGTAGTGCACTTACCTGTAAGTGCCTACCCGGTAG
GCACGGGTAAGCCCGCCGGCGCCCCATTAAACTCTGTTTAATTACTGGATATC
TGAATTACAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATG
CCTGTTCGAGCGTCATTTCAACCCTTAAGCCTTCTGTTGCTTAGCGTTGGGGG
CCTACCGTATGGCGGTAGCCCCTTAAAATTAGTGGCGGAGTCGGTTCACACTC
TAGACGTAGTAAATATTATCTCGCCTATTAGTTGGACCGGTCCCCTGCCGTAAA
ACCCCTAATTTATATCAAGGTTGACCTCGGATCA-
GTAGATACCCGCTGAACTAAGCATTATAGCGA

>UX3 *Xylaria* cf. *apiculata*

CATTAAAGAGTTATACAACTCCTAAACCCATGTGAACCTACCTTTGTTGCCTCG
GCAGGTCTGCAGCCTACCCTGTGAGGACCTACCCTGTAGGGACCTTACCCGG
TAGTTGCAGGTTTTAACCTGCCGGTGGTCTACCAAACTCTGTTTACTATGTTATT
CTGAATAATATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGC
ATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGA
GCCTACAGATACCCCCTGTAGCTCCCCAAAGTTAGTGGCGGAGTCGGTTTCAC
ACTCTAGACGTAGTAATTTTTATCTCGCCTATAGATGAGCCGGTCCCCTGCCGT
AAAACCCCCCTATTTCTAAAGGTTGACCTCGGATCAGTGTAGGAACCCGCTGA
ACTTAGCATTATAGCGA

>UX5 *Xylaria* cf. *hypoxylon*

TATTACAACTCCCAAACCCATGTGAACTTACCTTCTGTTGCCTCGGCAGGTCGC
GACCTACCCTGTGAGGCCCTACCCTGTAGGGCCCTACCTGGTAGTCGCGGGT
ACGCCTGCCGGTGGCCCATGAAACTCTGTTTATTCTTGTTATTCTGAATCTATA
ACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCG
AATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAGCCTACAGCCT
TCTGTAGCTCCCCAAAGTTAGTGGCGGAGTCGGTTTACACTCTAGACGTAGTA
AATTTTATCTCGTCTGTAGTTAGGCCGGTCCCTCGCCGTAAACCCCTAATTTAA
GGTGACCTCGGATCAGTAGATACCCGCTGACTTAAGCTTATACGAAAA

>U65X *Xylaria* cf. *longipes*

TATTAAAGAGTTTATAACTCCCAAACCCCATGTGAACATGACCTAACGTGTGCC
TCGGCGGGTCGTACCTACCCTGTAGTGCACTTACCTGTAAGTGCCTACCCGGT
AGGCACGGGTAGGCCCGCCGGCGCCCCATTAAACTCTGTTTAATTACTGGATA
TCTGAATTACAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCA
TGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCTTCTGTTGCTTAGCGTTGGGG
GCCTACCGTATGGCGGTAGCCCCTTAAAATTAGTGGCGGAGTCGGTTCACACT
CTAGACGTAGTAAATATTATCTCGCCTATTAGTTGGACCGGTCCCCTGCCGTAA
ACACCCCTAATTTATACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAA
CTTAAGCTTATACG

>SC3 *Nemania* cf. *bipapillata*

CATTAGAGAGTCTAAACAACTCCCAAACCCACTGTGAACATACCCTACGTTGCC
TCGGCAGGCGCCCGGCTCCGCGACCCTTCGGGGCTGCGGGGTGGCCCTGCC
GGCGGCCCACGAAACTCTTGTCTAGCAGCTGAATTCTGAGCCCGAGAGGGATA
AAAACAAAATTAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTC
CGAGCGTCATTTCAAACCCTCAAGCCTCGTTGCTTGGTGTTGGGAGCCTACGG
CTGTAGCTCCTCAAAGTCAGTGGCGTGGGCTGGCTCGCACCCCAGATGTAGTA
GTTATTTCTCTCACCTGTGGTCGGGCTAGTCCCCTGCCGTACAAACCCCCCAG
ACTTTTTAGTGTTGACCTCGGATCAGTAGGATACCCGCTGAACTTAAGCATATC
AATAAGCGA

>SC31 *Xylaria* sp. F19

CATTAAAGAGTTCTATAACTCCCAAACCCATGTGAACATACCTAACGTTGCCTC
GGCAGGTCGCACCTACCTTGTAGCACCCTACCCTGTAGGGCCTACCCGGGAG
GTGCGGGTAAGCCTGCCGGCGGCCCACCAAACTCTGTTTGATATTGAATTCTG
AACCTATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCATATTGCGCCCATTAGTATTCTAGTGGGCAGGA
CCGTTCGAGCGTCATTTCAACCCTTAAGCCTTCGTTGCTTAGTGTTGGGAGCCT
ACGGCAACGTAGCTCCTCAAAGTTAGTGGCGGAGTTGGTTCACACTCTAGACG
TAGTAATTTTTTTATCTCGCCTATTAGTTGGACTAATCCCCTGCCGTAAAACCCCC
AATTTATTCAAGGTTGACCTCGAATCGGTTCAGACAAACTCGCTAAATTGAAGC
ATATCAATAAGCGAAA

Group II : *Daldinia*- and *Nodulisporium*-like group

>20D *Daldinia eschscholzii*

CATTACCGAGTTATCTACTCAAACACCATAAGAGAAATCAAATCCGAAACGTAG
CCTCGGCGGGCTGCGCTTCCCAGTGCACTCCTGTGTCACCCCGGGCGGCGC
GCCCCCAGACCCGCCGGTGGCACCTTAACTCTGTTTTAATACCGAATCTCTGA
ATGCTTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGC
CTATTCGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCT
GCGCTGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCA
TAACTCTAAGCGTAGTAATATTCTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGC
CGTTAAACCCCTATATTTTCTAGTGGTGACCTCGGATAGTAGAATACCCGCTGA
ACTAAGCATTCAATAGCGAAG

>25D *Nodulisporium* sp. JP3665

TGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGCTCGCTTGCTGAACCAGCG
GAGGGATCATTACTGAGTTATTAAACTCCAACCCTATGTGAACTTACCGCCGTT
GCCTCGGCGGGCCGCGTTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTACA
GGCCCGCCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTC
AACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCG
AATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGTCCC
CAGGGCCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGT
AGTAATACCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCC
TATATCTTTAGTGGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGC
ATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAACGGGGA

>33D *Daldinia concentrica* AY616682

GATTGTGTTGACCAGCAGGAGGGATCATTACTGAGTTATCTAAACTCCCTAACC
CTATGTGAACCTTACCGTCGTTGCCTCGGCGGGTTGGGGAGCTACCCTGTAGT
AGCCCCCCGTACGGCCCGCCGGTGGACTGTAAACTCTTGTTTTTTGTATGGAA
TTCTGAATGCTTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGG
GCATGCCTATTCGAGCGTCATTTCAACCCTTAAGCCCTAGTCGCTTAGCGTGG
GGAGTCTGAGCCCTTCAGGGGGCTCAGTTCCTCAAAGTCATCGGCGGAGTCG
GATCGTGCTCTGAGCGTAGTAATTTTCTCTCGCTTCTGAGGCCGTTCCGGTGA

CTGGCCGTAAAACCCCTATACTTCTAGTGGTTGACCTCGGATTAGGTAGGAATA
CCCGCTGAACTTAAGCATATCAATAAGCGAAAGGAAA

>N26 *Daldinia eschscholzii*

TACCGAGCTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTCGG
CGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCTCC
AAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGCTT
CAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATTCG
AGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGCTGT
ACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACTCTA
AGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGTTAA
ACCCCTATATTTCTAGTGGTTGACCTCGGATTAGGTAGGAATACCCGCTGAACT
TAAGCATTCAATAGCGAA

>N37 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCT
CCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGC
TTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATT
CGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGC
TGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACT
CTAAGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGT
TAAACCCCTATATTTTCTAGTGGTTGACCTCGGATTAGTAGGAATACCCGCTGA
ACTTAAGCATATCATAGCGGA

>SC4 *Daldinia eschscholzii*

TATTACCGAGCTTATCTGAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCC
TCGGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCG
CTCCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAAT
GCTTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTA
TTCGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCG
CTGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATAC
TCTAAGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCG

TTAAACCCCTATATTTTCTAGTGGTGACCTCGGATAGTAGATACCGCTGACTAG
CTTATACG

>SC18 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCT
CCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGC
TTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATT
CGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGC
TGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACT
CTAAGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGT
TAAACCCCTATATTTTCTAAGTGGTTGACCTCGGATTAGGTAGGCATACCCGCA
ACTTGAGCATTCAATAAGCG

>SC19 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCTGTAGCTACCCG
GTAGGCACCCTGTAAGCCACCCCGGTAGGACCAGCTACACACTCTCGTCCGA
GATTGAGCACCAATTCATCGAATGCTTAATCATCCAATATACGTTTAAAATCGTC
TTCAACATACAGAGTATCGTCTATAGGTTCTGGCCTACCAGAAATAACGCATTC
GTAGACTTCGATAGAGATAATCGTCGACTAGCATGAAATCAGTGATTGTGTACA
TAGTCTAAGTAACCGCGCAATCGCCCCACTTTAGTGATCCCACTTGGCATCGC
CTATTGTAAGCGTGTTGGGCATCGCCTATAGCCATGCATTTAGCTTAACCTTGG
GACTTTGCTCGGTAACTCGTTGGGGACTCAGCTCCGCAACATTTATTCGGCGC
AGTTACGGCATACTTATAGGCGTAGTAATGTGCATTCCCCGACTCCTGTAATTA
GTCCGGCTGGCCTGCCGTAAAACCCCTATAT

>SC25 *Daldinia eschscholzii*

CATTACCGAGTTATTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTCG
GCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCTC
CAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGCT
TCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATTC
GAGCGTATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGCTGT
ACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACTCTA
AGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGTTAA

ACCCCTATATTTTCTAGTGGTTGACCTCGGATTAGGTAGGAATACCCGCTGAAC
TTAAGCATATCAATAAGCGGAA

>SC33 *Daldinia eschscholzii*

TATTACCGAGCTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCT
CGGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGC
TCCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATG
CTTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTAT
TCGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCG
CTGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATAC
TCTAAGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCG
TTAAACCCCTATATTTACTAGTGGTTGACCTCGGATAGGTAGGAATACCCGCTG
AACTTAAGCATATCAATAAGCAA

>SC40 *Daldinia eschscholzii*

ACTTACCGTCGTGTGCCTCGGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAG
CTACCCTGTAGCTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCTCC
AAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGCTT
CAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATTCG
AGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTGGGAGTCTGCGCTGTA
CTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACTCTAA
GCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGTTAAA
CCCCTATATTTTCTAGTGGTTGACCTCGGATTAGGTAGGAACCCGCTGAACTAG
CATTCATAGCG

>SC42 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCT
CCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGC
TTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATT
CGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGC
TGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACT
CTAAGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGT

TAAACCCCTATATTTTCTAGTGGTTGACCTCGGATAGGTAGGAATACCCGCTGA
ACTTAAGCATTCATAGCG

>SC44 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCT
CCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGC
TTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATT
CGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGC
TGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACT
CTAAGCGTAGTAATATTTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGT
TAAACCCCTATATTTTCTAGTGGTTGACCTCGGATAGGTAGGAATACCCGCTGA
ACTAAGCATATCAATAAGCG

>PD1 *Nodulisporium* sp. JP3665

CGATTACTGAGTGATCTAAACTCCAACCCTATGTGGAACTTACCGCCGTTGCCT
CGGCGGGCCGCGTTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCC
CGCCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACT
TAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGC
GTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGTCTCCAG
GGCCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTA
ATACCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATAT
CTTTAGTGGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAAA

>PD2 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCTGTAGCTACCCTG
TAGCTACCCGGTAGGCGCGCTCCAAGCCCGCCGGTGGACCACTAAACTCTGT
TTTAATACCGAATCTCTGAATGCTTCAACTTAATAAGTTAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATT
AGTATTCTAGTGGGCATGCCTATTCGAGCGTCATTTCAACCCTTAAGCCTTAGT
TGCTTAGCGTTGGGAGTCTGCGCTGTACTTGTTACGGCGCAGTTCCTCAAAGT
GATTGGCGGAGTTAGGGCATACTCTAAGCGTAGTAATATTTCTTCTCGCTTCTG
TAGTTGTCCTGGCGGCTTGCCGTTAAACCCCTATATTTTCTAAGTGGTTGACCT
CGGATTAGGTAGGCATACCCGCTGAACTAAGCATTCATAGCG

>PD3 *Nodulisporium* sp. JP3665

CATTACTGAGTTATTGCTAAACTCCACCCTATGTGACTTACCGCCGTTGCCTCG
GCGGGCCGCGTTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCCCG
CCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAA
TAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCACATGCGCCCATTAGTATCTAGTGGGCATGCCTGTTCGAGCGTCAT
TTCAACCCTTAAGCCCCTGTTGCTTAGCGTGGGAATCTAGGTCTCCAGGGCCT
AGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAATACC
ATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATATCTTTA
GTGGTTGACCTCGAATCAGGTAGAATACCCGCTGAACTTAAAAGAAAAA

>PD4 *Nodulisporium* sp. JP3665

CATTACTGAGTTATCTAAACTCCAACCCTATGTGAACTTACCGCCGTTGCCTCG
GCGGGCCGCGTTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCCCG
CCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAA
TAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
TGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTC
ATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGTCTCCAGGG
CCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAAT
ACCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATATTC
TTTAGTGGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCTTAATA
C

>UD1 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCACCCTTTGTGAACTTACCGTCGTTGCCTCGG
CGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCTCC
AAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGCTT
CAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATTCG
AGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGCTGT
ACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTTAGGGCATACTCTA
AGCGTAGTAATATTCTTCTCGCTTCTGTAGTGTCCTGGCGGCTTGCCGTTAAAC
CCCTATATTTCTAGTGGTGACCTCGGATAGTAGATACCCGCTGACTAGCATTAT
AC

>UD2 *Daldinia eschscholzii*

CATTACCGAGTTATCTAAACTCCAACCCTTTGTGAAACTTACCGTCGTTGCCTC
GGCGGGCTGCGCTTACCCTGTAGCTACCCTGTAGCTACCCGGTAGGCGCGCT
CCAAGCCCGCCGGTGGACCACTAAACTCTGTTTTAATACCGAATCTCTGAATGC
TTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATT
CGAGCGTCATTTCAACCCTTAAGCCTTAGTTGCTTAGCGTTGGGAGTCTGCGC
TGTACTTGTTACGGCGCAGTTCCTCAAAGTGATTGGCGGAGTGAGGGCATACT
CTAAGCGTAGTAATATTCTTCTCGCTTCTGTAGTTGTCCTGGCGGCTTGCCGTT
AAACCCCTATATTTCTAGTGGTTGACCTCGGATAGGTAGAATACCCGCTGAACT
TAAGCTTATAC

>UD3 *Nodulisporium* sp. JP3665

AGTAAGTCGTAACAAGGTTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAG
TTATCTAACTCCAACCCTATGTGAACTTACCGCCGTTGCCTCGGCGGGCCGCG
TTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCCCGCCGGTGGACT
GCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAGTTAAAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCC
TTAAGCCCCTGTTGCTTAGCGTGGGAATCTAGGTCTCCAGGGCCTAGTTCCCC
AAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAATACCATCTCGCTT
TTGCAGTAGCCCGGCGCTGCCGTAACCCTATATCTTAGTGGTGACTCGATCAG
TAGATACCGCTGAACTAGCTTATAC

>UD4 *Nodulisporium* sp. JP3665

TCGTAACAAGGTTCCGTTGGTGAACCAGCAGAGAGGGATCATTACTGAGTTAT
CTAAACTCCACCCTATGTGAACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCG
CCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCCCGCCGGTGGACTGCTA
AACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAGTTAAAACTTTC
AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAG
CCCCTGTTGCTTAGCGTGGGAATCTAGGTCTCCAGGGCCTAGTTCCCCAAAGT
CATCGGCGGAGTCGGAGCGTACTCTCAGCGTATAATACCATTCTCGCTTTTGC
AGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATATCTTAGTGGTGACCTCGAA
TCAGTAGAATACCCGCTGAACTTAACTTGACAAAAAA

>UD5 *Nodulisporium* sp. JP3665

TGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTATCT
AACTCCACCCTATGTGACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCGCCCT
GTAGTTTACTACCTGGCGGCGCGCTACAGGCCCGCCGGTGGACTGCTAAACT
CTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAGTTAAAACTTTCAACA
ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC
ATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCC
TGTTGCTTAGCGTAGGGAATCTAGGTCTCCAGGGCCTAGTTCCCCAAAGTCAT
CGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAATACCATTCTCGCTTTTGCAG
TAGCCCCGGCGGCTTGCCGTAAAACCCCTATATCTTTAGTGGTGACCTCGAAT
CAGGTAGAATACCCGCTGAACTTAACTTATA

>U59 *Nodulisporium* sp. JP3821 AF280627

GGAGGGATCATTACTGAGTTCTAAACTCCAACCCTATGTGAACTTACCACTGTT
GCCTCGGCGCTGTGCCTGCGAGAGCAGGCCCGCCGGTGGACCACTAAACTCT
GTTATACCTACTGTATCTCTGAATTTATAACTGAAATACGTTAAAACTTTCAACAA
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCA
TTAGTATTCTAGTGGGCATGCCTATTCGAGCGTCATTTCAACCCTTAAGCCCCT
GTTGCTTAGTGTTGGGAATCTGCGTTACGGCGCAGTTCCTTAAAGTGATTTGGC
GGAGCTAGTGCATACTCTAGGCGTAGTAAATACCATTCTCGCTTTTGTAGTAGG
CCTGGCGGCTTGCCGTAAAACCCCTATATTTCTAGTGGTTGACCTCGGATTAG
GTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGAAGG

>U65D *Nodulisporium* sp. JP3665

TCGTAACAAGGTTCCGTGGGTGAACCAGCGGAGGGATCATTACTGAGTTATCT
AAACTCCAACCCTATGTGAACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCGC
CCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCCCGCCGGTGGACTGCTAA
ACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAGTTAAAACTTTCA
ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGC
CCCTGTTGCTTAGCGTGGGAATCTAGGTCTCCAGGGCCTAGTTCCCCAAAGTC
ATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAATACCATTCTCGCTTTTGC
AGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATATCTTAGTGGTTGACCTCGA
ATCAGTAGATACCCGCTGAACTAGCCGAGGAAAGAAAAA

>P24 *Nodulisporium* sp. JP3665 AF280628

ACTCGTATGTGNTGACCAGCAGGAGGGATCATTACTGAGTTATCTAAACTCCAT
CCCTATGTGAACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCGCCCTGTAGTT

TACTACCTGGCGGCGCGCTACAGGCCCGCCGGTGGACTGCTAAACTCTGTTAT
ATATACGTATCTCTGAATGCTTCAACTTAATAAGTTAAAACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGT
ATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGC
TTAGCGTTGGGAATCTAGGTCTCCAGGGCCTAGTTCCCCAAAGTCATCGGCGG
AGTCGGAGCGTACTCTCAGCGTAGTAATACCATTCTCGCTTTTGCAGTAGCCC
CGGCGGCTTGCCGTAAAACCCCTATATCTTTAGTGGTTGACCTCGAATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAATAAGCGAAGGA

Group III : Phomopsis- and Diaporthe- like group

>3 *Diaporthe sp.* AY148440
TGTACAGTTCCGTTGGTTAGCAAGGAGAGGGATCATTGCTGGAACGCGCTTCG
GCGCACCCAGAAACCCTTTGTGAACTTATACCTATCTGTTGCCTCGGCGCAGG
CCGGCCTCTTCACTGAGGCCCCCTGGAAACAGGGAGCAGCCCGCCGGCGGC
CAACTAAACTCTTGTTTCTATAGTGAATCTCTGAGTAAAAAACATAAATGAATCA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCTCTGGTATCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACC
CTCAAGCCTGGCTTGGTGATGGGCACTGCCTTCTAACGAGGGCAGGCCCTGA
AATCTAGTGGCGAGCTCGCTAGGACCCCGAGCGTAGTAGTATATCTCGTCTGG
AAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGAAATTGACCTCG
GATCAGGTAGAATCCCCTGACTACTTAT
>11 *Diaporthe helianthi* DHE312366
GTGTACAGTTCCGTTGTTAGAGGAAGAGGGATCATTGCTGGAACGCGCTTCGG
CGCACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGCAGGC
CGGCCTCTACTGCTGAGGCCCCCCGGAGACGGGGAGCAGCCCGCCGGCGGC
CAACCAAACTCTTGTTTCTACAGTGAATCTCTGAGTAAAAAACATAAATGAATCA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAA
CCCTCAAGCCTGGCTTGGTGTTTGGGGCACTGCCTGTAAAAGGGCAGGCCCT
GAAATCTAGTGGCGAGCTCGCTAGGACCCCGAGCGTAGTAGTATATCTCGTCT
GGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCATGAAATTTTGA
CCTCGGATCTTGTTGGTATAACCCGTCTGACCAG
>22 *Phomopsis* sp. GM12

TAATGTACAAGTTCCGTTGGTGACCAGCGGAGGGATCATTGCTGGACGGCCCC
AGGCACCCAGAACCCTTTGTGACTTATACCTTTTGTTGCCTCGGCGCTGCTGG
TTTAGTAGGCCCTTTGCTTCACAGCAAAGAGACGGCACGCCGGCGGCCAAAAC
AACTCTGTTTTTACACTGAAACTCTGAGAAAAACACAAATGAATCAAAACTTTCA
ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGACGCACATTGCGC
CCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC
ATTGCTTGGTGTTGGGGCACTGCTTTTAACCAAGCAGGCCCTGAAATTTAGTG
GCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAACCCTCGCTCTGGAAGGC
CCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGAAAATTTGACCTCGGAT
CTGTTGGATAC

>SC6 *Phomopsis* sp. GM12 PSU94898

TTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTG
GAACGCGCCCCAGGCGCACCCAGAAACCCTTTGTGAACTTATACCTTTTGTTG
CCTCGGCGCTGCTGGTCTTCACAGGCCCTTTGCTTCACAGCAAAGAGACGGCA
CGCCGGCGGCCAAGTTAACTCTGTTTTTACACTGAAACTCTGAGAAAAAACACA
AATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCG
TCATTTCAACCCTCAAGCATTGCTTGGTGTTGGGGCACTGCTTTTAACGAAGCA
GGCCCTGAAATCTAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAA
CCCTCGCTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCT
GAAAATTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATA
AGCAA

>SC8 *Phomopsis* sp. DF5040

TTTTGTGTAGTAACAGGTTCCGTTGGTGAGCAAGAAGAGGGATCATTGCTGGA
ACGCGCCCTAGGCGCACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGC
CTCGGCGCATGCTGGCCCCCTCGGGGCCCCCTGGAGACAGGGAGCAGGCAC
GCCGGCGGCCAAGTTAACTCTTGTTTTTACACTGAAACTCTGAGAAAAACACAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGT
CATTTCAACCCTCAAGCATTGCTTGGTGTGGGGCACTGCTTTTTACCGAGCAG
GCCCTGAAATTTAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAAC
CCTCGCTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGA
AAATTTGACCTCGGATCATGTTTGGTATAACCCGCTGACTCATACGGGGAGAAA
AA

>SC27 *Phomopsis* sp. DF5036 AF317583

TTTTTGTGTAACAAGTTCCGTTGGAAGCCAGAAGGAGGGATCATTGCTGGAAC
GACGACCCTAGGCGACACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGC
CTCGGCGCATGCTGGCCTCTAGTAGGCCCCTCACCCCGGTGAGGAGACGGCA
CGCCGGCGGCCAAGTTAACTCTTGTTTTTACACTGAAACTCTGAGAAAAACACA
AATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCG
TCATTTCAACCCTCAAGCATTGCTTGGTGTTGGGGCACTGCTTTTTACCGAGCA
GGCCCTGAAATTTAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAA
CCCTCGCTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTTTG
AAAATTTGACCTCGGATCAGTAGATTTTATACCTAGTTAAT

>N46 *Diaporthe phaseolorum* AF001017

TGTACAGATCGTGCTGAACCAGCGAGAGGGATCATTGCTGGAACGCGCTCCA
GGCGCACCCAGAAACCCTTTGTAGAACTTATACCTTACTGTTGCCTCGGCGTA
CGCTGGCCCCTAGGGGTCCCTCTTGTCTCAAGAGGAGCAGGCGCGCCGGCG
GCCAAGTTAACTCTTGTTTTTACAATGAAACTCTGAGAAAAAAAAAACAAAATGAA
TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
CGCACATTGCGCCCTCCGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTT
CAACCCTCAAGCCTGGCTTGGTGTTGGGGCACTGCCTGTAAAAGGGCAGGCC
CTCAAATCTAGTGGCGAGCTCGCCAGGACCCCGAGCGTAGTAGTTAAACCCTC
GCTTTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTCTTTGAAAA
TTTGACCTCGGATCAGGTAGATACCGCTGAACTTACTTCATAA

>P2 *Diaporthe phaseolorum* AF001017

TTTATTAGTAAGTCTTCGTGTGACCAGACGGAGGATCATTTGGCTGGAACGCG
CCCCAGGACGACACCCAGAAACCCTTTGTGAAACTTATACCTTACTGTTGCCTC
GGCGTTACGCTGGCCCCTAGGGGTCCCTCTGTCTACAGAGGAGCAGGCGCGC
CGGCGGCCAAGTTAACTCTTGTTTTTACACTGAAACTCTGAGAAAAAAACAAAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCTCCGGTATTCCGGAGGGCATGCCTGTTCGAGCGT
CATTTCAACCCTCAAGCCTGGCTTGGTGTTGGGGCACTGCCTGTAAAAGGGCA
GGCCCTCAAATCTAGTGGCGAGCTCGCCAGGACCCCGAGCGTAGTAGTTAAA
CCCTCGCTTTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTCTTT
GAAAATTTGACCTCGGATAGTAGGATCCTGAACTAACTTCCGGGAAGAAA

>P12 *Phomopsis* sp. GM18 PSU94898

GGTTCTCCGTTGGTGAACCAGACGGAGGATGCATTGCTGGAACGACCGACCC
TAGGACGACACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCG
CTGCTGGTCTTCACAGGCCCTTTGCTTCACAGCAAAGAGACGGCACGCCGGC
GGCCAAATCAACTCTGTTTTTACACTGAAACTCTGAGAAAAAACACAAATGAAT
CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
CGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTT
CAACCCTCAAGCATTGCTTGGTGTTGGGGCACTGCTTTTAACCAAGCAGGCCC
TGAAATTTAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAACCCTCG
CTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGAAAATT
TGACCTCGGATCAGGTAGGAATACCCGCTGAACCGGGANAA

>PP1 *Phomopsis longicolla* AF000207

TTTTTATAAGTATAGTCGTAATTCCGTTGGTGAACCAGCGGAGGGATCATTGCT
GGAACGCGCCTCGGCGCACCCAGAAACCCTTTGTGAACTTATACCTACTGTTG
CCTCGGCGCAGGCCGGCCTTTGTCAAAGAAGGCCCCCTGGGAACAGGGAGCA
GCCCGCCGGCGGCCAACTAAACTCTTGTTTCTATATTGAATCTCTGAGTAGAAA
AACATAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTT
CGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGATGGGGCACTGCTCTCTC
GCGGGAGCAGGCCCTGAAATCCAGTGGCGAGCTCGCCAGGACCCCGAGCGT
AGTAGTTACATCTCGCTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCC
CAACTTCATGAAAATTGACCTCGGATCAGTAGAATCCCGCTGACTGCATATCAT
ACCCGGAGAAAAA

>PP2 *Phomopsis* sp. DF5040 AF317584

TTTTTTATAGATAGTCGTAACGATCCTTTGCTGAACCAGAGGAGGGATCATTGC
TGGAACGCGCCCTAGGCGCACCCAGAAACCCTTTGTGAACTTATACCTTACTG
TTGCCTCGGCGCATGCTGGCCCCCTCGGGGCCCCCTGGAGACAGGGAGCAG
GCACGCCGGCGGCCAAGTTAACTCTTGTTTTTACACTGAAACTCTGAGAAAAAC
ACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCAAGCATTGCTTGGTGTTGGGGCACTGCTTTTTACCGA
GCAGGCCCTGAAATTTAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTT
AAACCCTCGCTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTT
CTGAAAATTTGACCTCGGATCAGTAGATATGAACTTACGCATATCATTACCGGA
AGGGAA

>PP3 *Phomopsis* sp. FAU1053

TTTTAGTGTAAGTCGTATTCCGTTGGTGACCAGCGGAGGGATCATTGTGGACG
GCCCCAGGGCACCCAGAACCCTTTGTGACTTATACCTTACTGTTGCCTCGGGC
ATGCTGGCCCCCTCGGGGTCCCTTGGAGACAAGGAGCAGGCACGCCGGCGG
CCAAGTTACTCTTGTTTTTACACTGAAACTCTGAGAAAAAACACAAATGAATCTA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAA
CCCTCAAGCATTGCTTGGTGTTGGGGCACTGCCTGTAAAAGGGCAGGCCCTGA
AATTTAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAACCCTCGCTT
TGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTTTGAAAATTTGA
CCTCGGATCAGGTTTGGATAAA

>UP3 *Diaporthe caulivola* AF000567

ATGTAGAAAATTGTAACAAGGTCTCCGTTGCGTGACCCAGCGGAGGGATCATT
GCTGGAACGCGCCCCAGGCGCACCCAGAAACCCTTTGTGAACTTATACCTTAC
TGTTGCCTCGGCGCAGGCCGGCCCCCCAGGGGGCCCCTCGGAGACGAGGAG
CAGGCCCGCCGGCGGCCAACCTAACTCTTGTTTTTACACTGAAACTCTGAGAA
TAAAACATAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTG
TTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGATGGGGCACTGCTTTT
ACCCAAAAGCAGGCCCTGACAATTCAGTGGCGATGCTCGCCAGGACCCCGAG
CGCAGTAGTTAAACCCTCGCTTTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAA
CCCCCAACTTTTGAAAATTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTT
AAGCATATCAATAAGCGGAAA

>UP4 *Diaporthe* sp. 36-1 AY148440

AGTGTAACAGGTTCCGTTGGGTGCCAGAAGAGGGATCATTGCTGGAACGCGCT
TCGGCGCACCCAGAAACCCTTTGTGAACTTATACCTATTTGTTGCCTCGGCGTA
GGCCGGCCTCTTCACTGAGGCCCCCTGGAGACAGGGAGCAGCCCGCCGGCG
GCCAACTAAACTCTTGTTTCTATAGTGAATCTCTGAGTAAAAACATAAATGAATC
AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTCAAGCCTGGCTTGGTGATGGGGCACTGCCTTCTAGCGAGGGCAGGCC
CTGAAATCTAGTGGCGAGCTCGCTAGGACCCCGAGCGTAGTAGTTATATCTCG
TTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGAAAATT
TGACCTCGGATCAGGTAGGATACCCGCTGAACTTACTTCATAC

Group IV : Other taxa group

>8 *Curvularia gladioli* AF071337

TAGTATTGACCTGAGGAGGGATCATTAACAATAACATATGAAGGCTGTACGCG
GCTGCGCTCTTGGGCGCAGTGCTGCTGAGGCTGGATTATTTATTTACCCTTGT
CTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCTCCAGGACCACAT
CATAAACCTTTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAATGTAAATCA
TTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATT
GTACCCTCAAGCTTTGCTTGGTGTGGGCGTTTGTCTTTTGGCTTCTAGCCTACA
GACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACA
TTTTTGCGCTTGCCATCAGCAAAAAGACGCACTCCATCAAGACTCCTTCTCACG
TGACTCGGATCAGTAGGATCCCGCTGACTAGCTTATAGCG

>SC10 *Coprinus radians* AF345822

TGAGTAAGTGTACAGGTTTCCGTAGGTGTACCTGCGGAAGGATCATTAACGAA
TAACTATGGTGTTGGTTGTAGCTGGCTCCTCGGAGCAATGTGCACGCCCGCCA
TTTTTATCTTTCCACCTGTGCACCGACTGTAGGTCTGGATACCTCTCGCCTCCG
GGCGGATGCGAGGGTTGCTCGTAAGGGCTTCCCTTGAACTTCCAGGCTCTAC
GTCTTTTTACACACCCCAATAGTATGATGCAGAATGTAGTCAATGGGCTTCTCA
GCCTATAAAACACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTG
TTTGAGTGTCATTAAATTCTCAACCTCACCAGTTTTCTGAACTGACTGAGGCTTG
GATGTGGGGGTTTGTGCAGGCTGCCTCACCGGCGGTCTGCTCCCCTGAAATG
CATTAGCGAGGTTCATGCTGGACCTCCGTCTATTGGTGTGATAATTATCTACGC
CGTGGACGAGGATACAGACTCGCTTCTAACCGTCCGTCGAGGACCAATACCTT
GAAAATTTGACCTCAAATCAGGTGACTACCGCTGACTAGCTTATAGCG

>SC32 *Nectria gliocladioides* AF106532

TACCGNTTGTTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACT
CCATGTGAACATACCTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTCGTG
TGCCCCGGATCAGGCGCCCGCCTAGGAAACTTAATTCTTGTTTTATTTTGGAAT
CTTCTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTT
CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG
GGCATGCCTGTCTGAGCGTCATTTCAACCCTCATGCCCCTAGGGCGTGGTGTT
GGGGATCGGCCAAAGCCCGCGAGGGACGGCCGGCCCCTAAATCTAGTGGCG

GACCCGTCGTGGCCTCCTCTGCGAAGTAGTGATATTCCGCATCGGAGAGCGA
CGAGCCCCTGCCGTTAAACCCCCAACTTTCCAAGGTTGACCTCAGATCAGGTA
GGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAAG

>SC43 *Fusarium* sp.

CATTAGAGGAAGTAAAAGTCGTAACAGGTCTCGTTGGTGAACCAGCGGAGGGA
TCATTACAGAGTTATCCAACTCCCAAACCCATGTGAACCTATCTCTTTGTTGCCT
CGGCGCAAGCTACCCGGGACCTCGCGCCCCGGGCGGCCCGCCGGCGGACAA
ACCAAACTCTGTTATCTTCGTTGATTATCTGAGCGTCTTATTTAATAAGTCAAAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCC
CTAAGCACAGCTTATTGTTGGGCGTCTACTCCGGTAGTGCCTCAAAGACATTG
GCGGAGCGGCAGTAGTCCTCTGAGCGTAGTAATTCTTTATCTCGCTTTTGTCAG
GTGCTGCCCCCCCGGCCGTAAAACCCCCCAATTTTTTCTGGTTGACCTCGGAT
CAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAA
CCAACAGGGATAGCCCCAGTACG

>N4 *Psathyrella* cf. *gracillis*

CATTATTGAATAAATTGGCGTGGTTGTAGCTGGCTCTTAGGAGCATGTGCACGC
CCGTCATTTTTATCTTTCCACCTGTGCACCCTTTGTAGATCTGGATAACCCTCG
CTTACCCAAGCGGATGCAAGGATTGCTGTGTCGAAAGGCCGGCTCTCTTTGAA
TTTCCAGGTCTATGTCTTTTTACACACCCCAATTGAATGTTGCAGAATGTAGTCA
ATGGGCCTTCACAAGCCTATAAAACTCAATACAACTTTCAGCAACGGATCTCTT
GGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCC
GAGGAGCATGCCTGTTTGAGTATCATTAAATTCTCAACCTTACCAGCTTTTTGT
GTAAGGCTTGGATGTGGGGGTCGTGCAGGCTGCCTCGTATGGAGGCTGGTCT
GCTCCCCTGAAATGCATTAGCGAGTCAAACTTGAGCTCCGTCTATTGGTGTGAT
AATTATCTACACCGTGGATTGGAACTTAGACTTGCTTCTAACCGTCCAACGTGG
ACAATATCTTTGACAATTGATCTCAATCAGTAGACTACCCGCTGACTAGCATTAT
ACCGA

>U1 *Hypoxylon* cf. *rickii*

CATTAGAGGAAGTAAAAGTCGTAACAGGTCCTCGTGCGTGAACCAGCGGAGG
GATCATTAAAGGGTCGCGTGCCTCGCACGCGGCGTCCTGCCAAACCCTATGTG
AACTTACCACTGTTGCCTCGGCGGCGCCGCGGCTACCCGGGAGCTACCCTGT
AGCTACCCTGGAGCTCGCACACCAGGCCCGCCAAAGGACCATAAACTCTATTT
TGACTTGGAAATTCTGAATAAATCAACTTAATACGTTAAAACTTTCAACAACGGA
TCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG

AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAG
TATTCTAGTGGGCATGCCTATTCGAGCGTCATTTCAACCCCTTAAGCCTTGTTG
CTTAGCGTTGGGGGCCTACGTCCACAGCGGCGTAGCCCCTGAAAATTAGTGG
CGGAGTCAGTGAGCACTCTGAGCGTAGTAACTATTCTCGCTTTGGTAGTTTGC
CCCTGGCTGCTGGCCGTTAAACCCCCTATATTTCTAGTGGTTGACCTCGGATTA
GGTAGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAGAAACCAAC
AGGGATGCCCCAGTACGGG

>U41 *Fusarium* sp. FSU61695

AGTATATGTAACAAGGTTCCGTTGGTGACCAGCGGAGGGATCATTACCGAGTT
TACAACTCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGC
CCGCGCCCCGTAAAACGGGACGGCCCGCCGCAGGACCCACAAACCCTGAATT
TTATTGTAACTTCTGAGTTTAAAAAACAAATAAATCAAAACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACACTGCGCCCGCCAGTA
TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTT
TGGTGTTGGGGATCGGGCTGCGGTTCTACCGCGTCCCGGCCCCGAAATCTAG
TGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGGTAACACCTCGCAACTGGA
ACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGAT
CAGGTAGAAATACCCGCTGAACTTAAGCATATCAATAAGCGAG

>P8 *Corynespora cassiicola* AY238606

TTAAAGTCGTAACAAGGTTTCCGTAGGTGACTGCGGAAGGATCATTATCGTAG
GGGCCTCGCCCCCTTCGAGATAGCACCCTTTGTTTATGAGCACCTCTCGTTTC
CTCGGCAGGCTCGCCTGCCAACGGGGACCCACCACAAACCCATTGTAGTACA
AGAAGTACACGTCTGAACAAAACAAAACAAACTATTTACAACTTTCAACAACGG
ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTG
GTATTCCTTAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTAGCTT
GGTGTTGGGCGTCTGTCCCGCCTCCGCGCGCCTGGACTCGCCTCAAAAGCAT
TGGCGGCCGGTTCCCAGCAGGCCACGAGCGCAGCAGAGCAAGCGCTGAAGT
GGCTGCGGGTCGGCGCACCATGAGCCCCCCCACACCAGAATTTGACCTCGGA
TCAGGTAGATACCCGCTGAACTTAAGCATATCAATAAGCGA

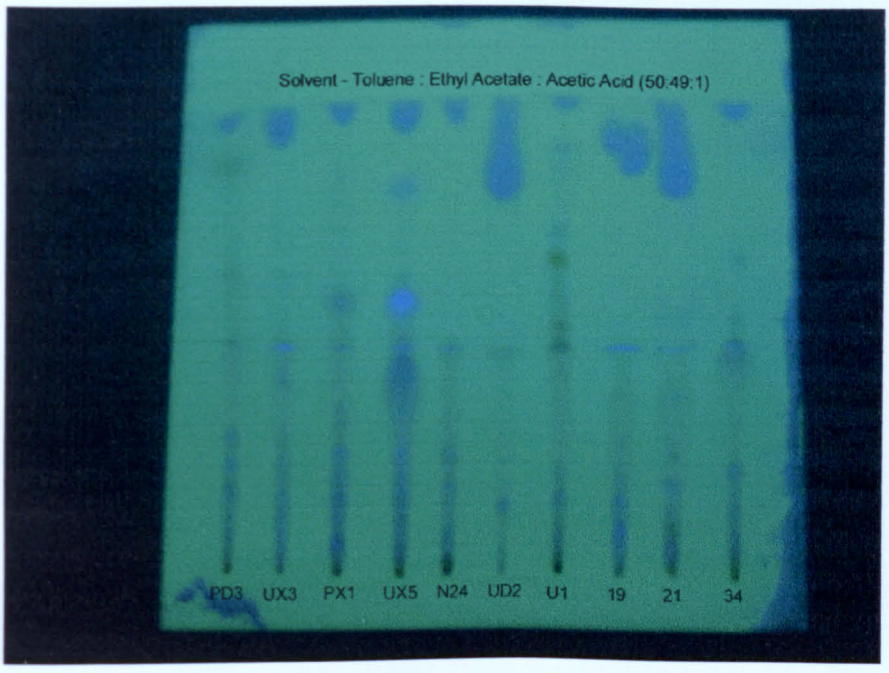
>P22 *Nectria haematococca* AF130142

CTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATACAACTCATCAAC
CCTGTGAACATACCTAAACGTTGCCTCGGCGGGAACAGACGGCCCCGTGAAAA
CGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTCTTCT
GAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA

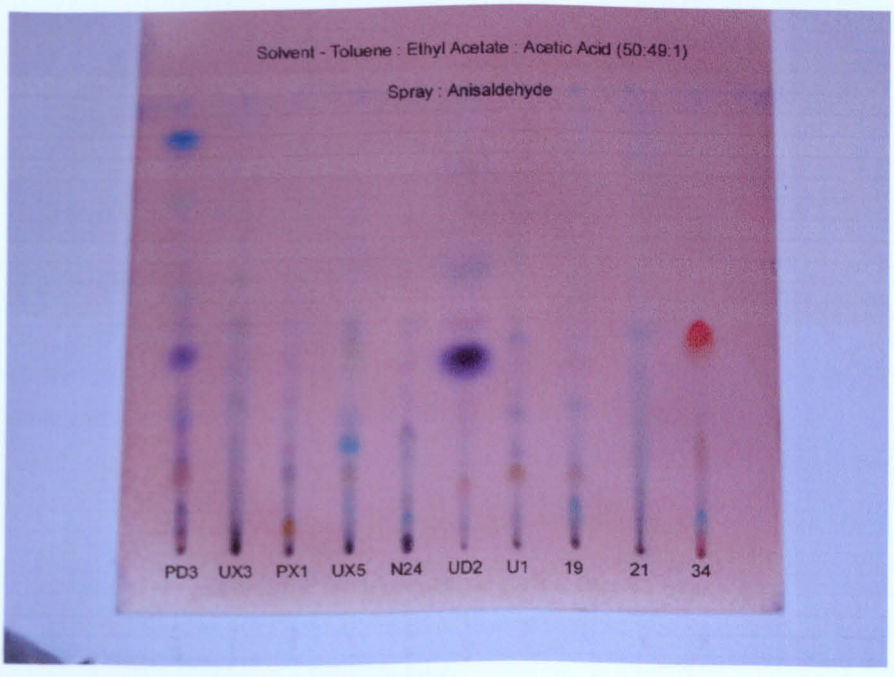
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGC
ATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTGGGG
ATCGGCGGAGGCCCTCCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGT
CCCGCCGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGGAGAGCGG
CGCGGCCACGCCGTAAAACCCCCAACTCTTCTGAAGTTGACCTCGAATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAAAAGCGGAAGG

Appendix IV

TLC plates showing the chromatogram of selected endophytes from *Cassia fislula* L.



Note : The plate was detected under UV light.



Note: The plate was detected by spraying with anisaldehyde solution.

THESIS CONTAINS CD/DVD