Taxonomy, molecular phylogeny and taxol production in selected genera of endophytic fungi
by Jeerapun Worapong

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
This study examined the taxonomy, molecular phytogeny, and taxol production in selected genera of endophytic fungi associated with tropical and temperate plants. These common anamorphic endophytes are Pestalotiopsis, Pestalotia, Monochaetia, Seiridium, and Truncatella, forming appendaged conidia in acervuli. Sexual states of these fungi, including Amphisphaeria, Pestalosphaeria, Discostroma and Lepteutypa, are in a little known family Amphisphaeriaceae, an uncertain order of Xylariales or Amphisphaeriales (Pyrenomycetes, Ascomycota).

The classification of the anamorph is based primarily on conidial morphology i.e. the number of cells, and appendage type. However, UV irradiation can convert typical conidia of Pestalotiopsis microspora (5 celled, 2-3 apical and 1 basal appendage) into fungal biotypes that bear a conidial resemblance to the genera Monochaetia and Truncatella. The single cell cultures of putants retain 100% homologies to 5.8S and ITS regions of DNA in the wild type, suggesting that no UV induced mutation occurred in these regions. These results call to question the stability of conidial morphology and taxonomic reliance on this characteristic for this group of fungi. Therefore, a molecular phylogenetic approach was used to clarify their taxonomic relationships.

Teleomorphs of these endophytes were previously placed in either Xylariales or Amphisphaeriales. Based on parsimony analysis of partial 18S rDNA sequences for selected anamorphic and teleomorphic taxa in Amphisphaeriaceae, this research supports the placement of these fungal genera in the order Xylariales sharing a common ancestor with some taxa in Xylariaceae. The 18S rDNA region is helpful in resolving phylogenetic relationships at or above the ordinal level for these sampled taxa.

Parsimony analyses of the ITS1-5.8S-ITS2 sequences suggest that selected genera in Amphisphaeriaceae are a monophyletic group sharing the synapomorphic characteristic of an anamorphic state which has an annellidic holoblastic type of conidiogenesis in acervuli of textura angularis cells. These taxa were delineated into 5 groups somewhat consistent with previous classical classifications. Many of the taxa in this group of fungi are either closely related or identical according to the 97-100 % homologies of the complete ITS1-5.8S-ITS2 regions. Results reflect a correlation between host and the geographical origin for some clades of the inferred tree. A majority of temperate taxa are with gymnosperm hosts, and one group is on tropical palm. Taxol production was detected only in anamorphs, particularly in Seiridium, and for genera on palm or yew, but this may reflect sampling bias.

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APPROVAL

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Jeerapun Worapong

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date 18 May 2001

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ABSTRACT

This study examined the taxonomy, molecular phylogeny, and taxol production in selected genera of endophytic fungi associated with tropical and temperate plants. These common anamorphic endophytes are *Pestalotiopsis, Pestalotia*, *Monochaetia*, *Seiridium*, and *Truncatella*, forming appendaged conidia in acervuli. Sexual states of these fungi, including *Amphisphaeria*, *Pestalosphaeria*, *Discostroma* and *Lepteutypa*, are in a little known family Amphisphaeriaceae, an uncertain order of Xylariales or Amphisphaeriales (Pyrenomycetes, Ascomycota).

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Teleomorphs of these endophytes were previously placed in either Xylariales or Amphisphaeriales. Based on parsimony analysis of partial 18S rDNA sequences for selected anamorphic and teleomorphic taxa in Amphisphaeriaceae, this research supports the placement of these fungal genera in the order Xylariales sharing a common ancestor with some taxa in Xylariaceae. The 18S rDNA region is helpful in resolving phylogenetic relationships at or above the ordinal level for these sampled taxa.

Parsimony analyses of the ITS1-5.8S-ITS2 sequences suggest that selected genera in Amphisphaeriaceae are a monophyletic group sharing the synapomorphic characteristic of an anamorphic state which has an annellidic holoblastic type of conidiogenesis in acervuli of textura angularis cells. These taxa were delineated into 5 groups somewhat consistent with previous classical classifications. Many of the taxa in this group of fungi are either closely related or identical according to the 97-100 % homologies of the complete ITS1-5.8S-ITS2 regions. Results reflect a correlation between host and the geographical origin for some clades of the inferred tree. A majority of temperate taxa are with gymnosperm hosts, and one group is on tropical palm. Taxol production was detected only in anamorphs, particularly in *Seiridium*, and for genera on palm or yew, but this may reflect sampling bias.

CHAPTER 1

LITERATURE REVIEW

Endophytic Fungi

The term "endophyte" is derived from two Greek words, which are "endon" meaning within, and "phyton" meaning plant (Chanway, 1996). Translated literally, an endophyte is an organism that lives inside a plant. Chanway (1996) also proposed that endophytes could not only be fungi but also bacteria that reside in plant tissues without causing disease. However, many mycologists refer to endophytes as fungi that asymptotically infect the healthy tissues of a wide variety of plants ranging from conifers to grasses (Carroll, 1988; Clay, 1988a; Petrini, 1986).

Endophytes are distinguished from "mycorrhizal fungi" by virtue of the location of the fungus in the plant tissues where the fungus resides. Endophytes live entirely inside plant tissues but cause no disease symptoms, whereas the hyphae of mycorrhizal fungi emanate into the soil surrounding infected roots (Chanway, 1996). In addition, endophytic fungi enhance host plant immunity by providing defensive chemicals and protection from animals or pathogens of plants (Carroll, 1988; Clay, 1988; Petrini, 1986). We still do not know much about their exact roles in their respective hosts. Some endophytic species may also help host plants compete for nutrients with other plants. This kind of relationship between fungi and plants is recognized as a mutualistic symbiosis (Carroll, 1990), which is discussed below.

Mutualistic symbiosis implies the exchange of benefits and costs between associated organisms (Carroll, 1990). For example, food donation is the direct and prime

benefit that endophytic fungi derive from host plants since fungi are heterotrophs relying on other organisms for food such as sugar. Moreover, endophytic fungi do not destroy cells or tissues of the host, but enhance cycling of nutrients and compounds, increasing the longevity and the photosynthetic ability of the plant (Sinclair and Cerkauskas, 1996). Most endophytic growth is limited within host plant tissues to avoid host defense mechanisms that typically prevent colonization by virulent pathogens. A number of endophytes produce compounds that are active as antagonists to plant pathogens and insects. For instance, alkaloids, which can be toxic to livestock as well as to insect pests, are produced by grass endophytes (*Balansiae* species of Clavicipitaceae), both in the infected plant and in culture (Clay, 1988b). Pederson *et al.* (1988) reported that chemical compounds provided by endophytes could even attack pathogenic nematodes and fungi in the soil. Another example is taxol, a compound isolated from endophytes of a yew tree (*Taxus* sp.) and other plant species residing in damp environments (Stierle, 1993; Strobel, 1996 a&b). It is believed that taxol may protect those hosts from root pathogenic fungi, such as *Pythium* and *Phytophthora*.

Evolution of Endophytes from Latent Pathogens to Mutualistic Symbiosis

There are several lines of evidence that support a close relationship between the endophytic lifestyle and that of a pathogen. Endophytes have evolved directly from plant pathogenic fungi with a long latent phase that infect hosts (Clay, 1988 a&b; Carroll, 1988). Many leaf pathogens, like *Rhabdocline spp.*, a disease agent of Douglas-fir, infect

young leaves of the host, but lesions only appear a few months later. The longest latency periods are found with coniferous endophytes. Endophytes of coniferous needles infect young needles, but grow slowly and sporulate a few years later.

Second, avirulent strains of a pathogen may be considered endophytic since they are sister species to the virulent pathogen on the same or closely related hosts (Carroll, 1988). For example, the avirulent strain *Acremonium coenophialum*, an important grass endophyte, is closely related to *Epichloe typhina*, a pathogen of grass. Both *Clavicepitous* pathogenic fungi and *Balansiae* endophytes share the same the same method of vertical transmission and are found in the ovarian tissue of the host. Moreover, both *Clavicepitous* pathogenic fungi and *Balansiae* endophytes are capable of producing ergot alkaloids (Clay, 1988 a&b). In douglas-fir, *Rhabdocline pakeri* an endophytes has morphological characters similar to *R. wierii* and *R. pseudotsugae*, virulent needle pathogens.

Third, innocuous endophytes can cause pathogenic symptoms when the host is under stress (Carroll, 1988). Moreover, the tendency towards host specificity is similar to biotrophic fungi, but greater than that seen in necrotrophic fungal pathogens. The *Balansiae* endophytes, *Atkinsonella* and *Epichloe*, infect only cool season grasses whereas *Balansia*, *Balansiopsis*, and *Myriogenospora* infect only warm season grasses (Clay, 1988b).

Strategies of Mutualism in Endophytes

Two patterns of endophytic mutualism are constitutive mutualism and inducible mutualism (Carroll, 1988). Constitutive mutualism involves a transmission of endophytes from one generation to the next via the germ line. The constitutive endophyte infects host ovules and is carried through the seed (seed-borne). This type of transmission may be called vertical transmission in some literature (Bayman *et al.* 1998). These endophytes develop a systemic infection throughout the aerial parts of the plant with considerable mycelium biomass. Fungal toxins, giving immediate and direct benefits to the endophyte's host, are produced. Grass endophyte is an example of constitutive mutualism. Inducible mutualism or horizontal transmission involves an infection from one host to another by spores through air or in water (Bayman *et al.*, 1998). Inducible mutualism involves a much looser association between endophyte and host. Inducible endophytes, such as *Rhabdocline parkeri* in Douglas-fir, develop little fungal biomass in only the vegetative parts of the host, and remain metabolically inactive for long periods.

Taxonomy of Endophytes

Fungi isolated from surface-sterilized plant tissues are primarily *Ascomycetes* or asexual stages (deuteromycetes), with a few Basidiomycetes (Carroll, 1986). Some oomycetes have been isolated as endophytes as well (Sinclair and Cerkauskas, 1996). Endophytic Ascomycetes include member of the classes Pyrenomycetes, Discomycetes

and Loculoascomycetes. Regardless of the host plants, most of the ascomycete endophytes are species of *Cryptocline*, *Cryptosporiopsis*, *Phomopsis*, *Phoma*, *Fusarium*, *Alternaria*, *Hypoxylon*, *Xylaria*, *Taxomyces*, *Monochaetia*, *Discostroma* and *Pestalotiopsis* (Bayman *et al.*, 1998; Carroll, 1988; Li, *et al.*, 2000; Okane *et al.*, 1996; Strobel *et al.*, 1996 a&b).

Rainforest Endophytes

It is estimated that only 5 % of the world's fungi are known to science (Hawksworth, 1991; Hawksworth and Rossman, 1997). The unknown fungi definitely include endophytes, especially endophytes existing in the rainforest around the world. Consistently, most of the rainforest endophytes are Xylariales (Petrini, 1986). Humid tropical forests seem to be a place for these endophytic fungi where they are especially common and diverse in living plants (Rodrigues, 1992). Thus, it is essential to understand and clarify the taxonomic diversity of xylariacious endophytes, particularly some members of Amphisphaeriaceae and their presumed anamorphic coelomycetous forms because this group of fungi may produce potentially useful and important compounds.

Although studies on endophytic fungi in higher plants from temperate and rainforest regions are becoming more common, there is still much to know about the astonishing and useful microbial metabolites produced by these endophytes. Some of these potentially useful bioactive compounds are pestaloside, pestalopyrone, pestalotiopsin-A and pestalopsin-B, taxol, ambuic acid, jesterone, and cryptocin (Pulici *et*

al., 1996 a&b; Li *et al.*, 2001b; Harper *et al.*, 2000). All of these compounds have antifungal bioactivity.

Taxol effectively stops growth of root rot pathogens, such as *Pythium*, *Phytophthora* and *Aphanomyces* (Strobel *et al.*, 1996 a&b). Therefore taxol may be an important defense mechanism that endophytic fungi provide to the host in damp conditions. Taxol is also an extremely active antifungal agent as well as an effective compound against breast cancer. *Taxomyces andreanae*, (Stierle & Strobel) was the first deuteromycetous endophyte shown to produce taxol (Stierle *et al.*, 1993). However, *T. andreanae*, which was isolated from the stem of a yew tree (*Taxus brevifolia*) from Glacier National Park, produce only small amounts of taxol, i.e. about 50 ng/l as compared to *Pestalotiopsis microspora*, an endophyte from Himalayan yew which can produce 50 μg/l (Strobel *et al.*, 1996b). Other endophytic species that can produce taxol, but in lower quantities include *Pestalotia*, *Monochaetia* and *Seimatoantlerium* (Table 1). Taxol producing endophytic fungi can be found not only in yew, but also from other plant species such as pine and stinking yew that grow in moist and shaded environments. These host plants are listed in Table 1.

Pestalotiopsis microspora isolated from *Torreya taxifolia* (stinking yew) also produced torreyanic acid, an unusual dimeric quinone with selective cytotoxicity against human cancer cell lines (Lee *et al.*, 1996). It is very interesting that the fungus *Pestalotiopsis microspora* and closely related genera such as *Monochaetia* can produce not only taxol but also ambuic acid (Li *et al.*, 2001a). Ambuic acid, a cyclohexenone, has an antimycotic function against the Oomycetes (*Pythium*) which cause plant disease.

These *P. microspora* strains were isolated from a variety of hosts and different regions, such as *Taxus baccata* (German yew), *Torreya taxifolia* (Florida stinking yew), *Taxodium disticum* (South Carolina bald cypress), *Wollemia nobelis* (Australian Wollemi pine), and *Dendrobium speciosum* (Papua New Guinea orchid) (Strobel *et al.*, 1996a&b, 1997 & 1999)

Table 1. Endophytic fungi from different hosts that producing taxol (Strobel *et al.*, 1996 a&b, 1997& 1999; Lee *et al.*, 1995 & 1996)

Host plant	Geographical origin	Fungus	Taxol[a] (ng/Litre)
Taxus brevifolia (pacific yew)	Glacier National Park, Montana, USA	*Taxomyces andreanae*	50
Taxus baccata	Glacier National Park, Montana, USA	*Pestalotia bicilia*	1081
Taxus baccata	Ross creek, Montana, USA	*Monochaetia* sp.	102
Taxus baccata	Ross creek, Montana, USA	*Fusarium lateritium*	130
Taxus wallachiana	Himalayan mountains, Nepal	*Pestalotiopsis microspora* *Seimatoantlerium nepalense*	500 62-80
Torreya taxifolia (stinking yew)	Florida, USA	*Pestalotiopsis microspora*	42
Wollemia nobilis (pine)	Eastern Australia	*Pestalotiopsis guepinii* *Pestalotiopsis* sp.	485 127-172
Maguireothamnus speciosus	Venezuela	*Seimatoantlerium tepuiense* *Xylaria* sp.	250-350 242

[a]Taxol measured quantitatively using monoclonal antibodies.

Recently, *Pestalotiopsis jesteri*, a species of endophytic fungus isolated from the inner bark of *Fragraea bodenii*, has been described to produce a new cycloxenone named jesterone (Strobel *et al.*, 2000, Li and Strobel, 2001b). Similarly, Jesterone and hydroxy-jesterone can inhibit the growth of plant pathogenic *Oomycetes* (Li and Strobel, 2001b). Another potent compound from the endophytic fungus *Cryptosporiopsis* cf. *quercina*, which was isolated from the inner bark of the stem of *Triptergyium wifordii*, is cryptocin,

a tetramic acid with antifungal activity against plant pathogens (Li *et al.*, 2001b). Cryptocin can inhibit the growth of representative pathogens in the main taxonomic classes such as Ascomycetes (*Sclerotinia* sp. and *Pyricularia oryzae*, a casual agent of rice blast disease), Basidiomycetes (*Rhizoctonia* sp.), fungi imperfecti (*Geotrichum candidum* and *Fusarium oxysporum*) and Oomycetes (*Pythium* sp. and *Phytophthora* sp.).

Because of the value of these compounds, it is important to determine the correct taxonomic relationships of selected *Pestalotiopsis* spp. and taxonomically related genera in the Amphisphaeriaceae. However, classification in Xylariales where this group has been placed is problematic due to the absence of teleomorph or anamorph stages in culture necessary for identification (Petrini, 1986). Many species of xylariacious endophytes will not sporulate in agar culture even after inoculation of cultures back onto host tissue or other plant material. Some of the species that do not produce ascospores in culture are also in the family Amphisphaeriaceae. It became apparent that only limited information on this group could be gained from laboratory culture. Its taxonomy was not well established because hardly any teleomorph cultural data is available for many of the predominant taxa. Generally, endophytic fungi in Amphisphaeriaceae form only anamorphic states in culture, and, as a result, less is known about their total ecology and their life cycle.

Therefore, in this dissertation research I have undertaken several studies to advance the knowledge of the coelomycetous endophytes, emphasizing some anamorphic genera of Amphisphaeriaceae (*Pestalotiopsis*, *Pestalotia*, *Monochaetia*, *Truncatella*, and

Seiridium) and some teleomorphic genera in Amphisphaeriaceae (*Amphisphaeria* sp., *Discostroma* sp., and *Pestalosphaeria* sp.). The objectives of this research include:

-To present evidence that ecological factors such as UV irradiation can change morphological characteristics important in species classification.

-To test whether selected genera in the Amphisphaeriaceae form a monophyletic group in the Pyrenomycetes (Ascomycota) using 18S rDNA.

-To examine the phylogenetic relationships among selected genera in Amphisphaeriaceae, and compare results from the complete sequences of the ITS 1&2 regions and 5.8S rRNA gene with morphology-based classification schemes.

-To investigate whether phylogenetic taxonomy can provide clues for determining which fungal taxa might have potential for producing taxol or other unique compounds.

In order to describe the research, it is first necessary to provide background material on fungal classification, species concepts, and taxonomy of Amphisphaeriaceae. I conclude this chapter with remarks and objectives of the research, following this background material.

The Fungal System

The Hierarchical Classification

In general, fungi as well as plants are hierarchically classified into Kingdom, Phylum, Class, Order, Family, Genus and Species (mentioned in Alexopoulos, 1959).

The kingdom may be comprised of many phyla, and each phylum may include many classes, orders, family, genera and species. Each category may be broken down into sub-phyla, sub-classes, or down into varieties and biological strains in species. The standard ending of the names of phyla, classes, orders and families of fungi are **-mycota**, **-mycete**, **-ales** and **-aceae**, respectively (Alexopoulos, 1959). Names of genera and species have no standard ending. An example of this hierarchical order is as follows:

Kingdom: Fungi
Phylum: Asco**mycota**
Class: Pyreno**mycetes**
Order: Xylari**ales**
Family: Amphisphaeri**aceae**

Taxonomy is an attempt to classify and name organisms with an internationally accepted system, based on the relationships among organisms, with the least amount of confusion (Haeckel, 1894; Copeland 1956). However, the system for each organism is always changing, and will continue to change until the gaps in knowledge have been filled. For example, the classification of fungi is difficult due to the great differences in opinions and interpretation of fungal classification among mycologists. It was mentioned by Alexopoulos in 1959 that fungi were once classified in the plant kingdom, but at present are relegated to their own kingdom by Whittaker (1969). Whittaker (1969) established the tradition of a tree-kingdom system of classification where organisms were placed into one of five kingdoms: animals, plants, fungi, protists, and monera. Classification into these categories was based totally on phenotypic characteristics. The monera were identified as prokaryotes because of the absence of a nuclear membrane whereas the other four multicellular forms were defined as eukaryotes because of the

presence of a nuclear membrane. A new classification suggests a three domain system of Eubacteria, Archeae, and Eukaryota which includes fungi as well as plants, animals and simpler eukaryotes (Guarro, 1999).

Definition of Fungi

Fungi are comprised of eukaryotic cells with a defined nucleus surrounded by a nuclear membrane and containing other organelles (i.e. mitochondria and golgi apparatus). They are spore producing, achlorophyllous organisms with absorbtive nutrition. Their branched multicellular filamentous forms, known as hyphae, are typically surrounded by cell walls, which are mostly comprised of chitin and glucans (Alexopoulos *et al.*, 1996).

Classification of Fungi

The kingdom fungi, based on ribosomal DNA (rDNA) sequence data and ultrastructural characters, is comprised of four phyla: Chrytidiomycota, Zygomycota, Ascomycota, and Basidiomycota (Forster *et al.*; 1990, Bruns *et al.*, 1991&1993; Berbee and Taylor, 1993&1995 Bowman *et al.*, 1992; Patterson and Sogin, 1992). For instance, the structure of the thallus, composition of cell wall polysaccharides, type of lysine synthesis, and also the molecular phylogeny by analysis of 18S rDNA sequence are evidence that Chrytridiomycota is linked firmly with Ascomycota and Basidiomycota (see review by Cripps, 2001). Basidiomycota is classified into three large classes: Hymenomycetes, Urediniomycetes, Ustilaginomycetes (Table 2). This modern

classification is based on 18S DNA and septal pore apparatus. Thus, it is important to note that not only the phenotypic approach, but also the molecular phylogeny, provides evidence of relationships at various taxonomic levels. *Ascomycetes* are divided into several large classes based on small subunit rDNA and potentially important morphological and biochemical characters, such as shape of the ascocarp, forcibly discharged asci, presence of a simple septal pore and Woronin bodies (Alexopoulos *et al.*, 1996). Six major groups of ascomycetes are Archiascomycetes, Hemiascomycetes, Plectomycetes, Pyrenomycetes, Discomycetes and Loculoascomycetes.

Table 2. Phyla and classes of the kingdom fungi based on rDNA analysis and ultrastrucural features as adapted from Alexopoulos *et al.*, (1996) and Cripps (2001).

Kingdom	Phylum	Class
Fungi	Chrytidiomycota	Chytridiomycetes
	Ascomycota	Hemiascomycetes Archiascomycetes Plectomycetes Pyrenomycetes Discomycetes Loculoascomycetes
	Basidiomycota	Hymenomycetes Urediniomycetes Ustilaginomycetes
	Zygomycota	Zygomycetes

Nomenclature

An organism's name must be given in a Latin binomial as assigned by taxonomists (as mentioned in Alexopoulos, 1959 and Alexopoulos *et al.*, 1996). It is comprised of two words. The first word dictates the classified genus while the second one denotes species. The naming of all groups of fungi including yeast, lichen-forming fungi, slime molds, and Oomycota is regulated under the rules of the International Code of Botanical Nomenclature (ICBN) (Alexopoulos *et al.*, 1996). The objective of the code is to provide internationally accepted rules for naming taxonomic groups. The ICBN is governed by committees at each International Botanical Congress is held every four years. The code of nomenclature belongs to the Botanical Code since fungi were traditionally classified as "plants" in the past. A committee for fungi will consider which proposed code should be conserved or changed under the rules of ICBN relating to fungal nomenclature. Any updated changes to the code are published in Taxon, the official journal of the International Association for Plant Taxonomy.

A Dual Nomenclature

Fungi have had a dual nomenclature since the last century because fungi can propagate in either an asexual or a sexual way, and one can be reported without evidence of the existence of the other phase (reviewed by Alexopoulos *et al.*, 1996 and Guarro, 1999). Because of those reasons, ICBN's rules denote that it is legal to name the same fungus with separate binomials according to states: one for the sexual state and the other

one for the asexual state. In general, each state of a fungus occurs at different times and on different substrates, except in Zygomycetes. In Zygomycetes, asexual and sexual states often develop together.

The asexual state of the fungus is called the anamorph whereas the sexual state of the fungus is known as the teleomorph. Holomorph refers to a fungus that has both sexual and asexual states. Fungi that show the asexual state alone have been classified as Deuteromycetes. However, recently some mycologists have tried to unite the dual system into a single nomenclature on the basis of the phylogenetic species concept or on the basis of the rDNA sequences. After the Holomorph Conference in 1993, it was agreed to conserve the term deuteromycetes with a lowercase "d" for identification purposes, and formally not recognize deuteromycetes as a high-level taxon rank.

Classical Classification

For centuries, the classification and identification of fungi have been mainly based on observable characteristics and the fungal life cycle (de Bary 1887), a method known as the phenotypic approach. Other features that have been used lately for classification include susceptibility to chemical and antifungal drugs, physiological and biochemical tests, secondary metabolites, ubiquinone systems, fatty acid composition, and cell wall composition (reviewed in Guarro *et al.*, 1999).

The types of conidia (asexual spores) and the process involved in conidium formation are considered the most important characteristics for the identification and classification of fungi. Unfortunately, some fungi have a limited number of

morphological characteristics that can be used for taxonomic purposes. For instance, sterile fungi show only the vegetative phase; that is, they do not sporulate and only hyphal elements or other nonspecific structures such as chlamydospores, sclerotia or particular hyphal are observed. While they may be useful in identification, they are generic features of limited value. Chlamydospores are thick-walled thallic conidia that function as resting spores, whereas sclerotia are hard resting bodies. Both chlamydospores and sclerotia, which are resistant to harsh environmental conditions, can remain dormant for a long period, and will germinate on the return of suitable growth conditions (Alexopoulos *et al.*, 1996).

However, morphological and physiological characteristics often are dependent on cultural conditions. Ascomycetes offer the most distinctive examples of large changes in characteristics on different nutrition (Minter, 1987). Such Ascomycetes have a high degree of plasticity when grown on artificial media. For example, a species of *Neurospora* produces asci containing four brown ornamented ascospores with germ pores on malt agar, while the same species grown on biotin-deficient agar produces many asci with no spores.

Thus classifying fungi by their morphological characters can cause problems when a single very plastic characteristic is used. Even if many characters are used for identification and classification, problems may occur if characters conflict. Conflicting characteristics can lead to alternative classifications depending upon which character is emphasized. For instance, the Erysiphales have closed ascomata (cleistothecia), yet forcibly discharge their ascospores after the uptake of water by asci. When the closed

cleistothecium is emphasized, Erysiphales are classified in Plectomycetes (Berbee and Taylor, 1992) whereas they are classified in Pyrenomycetes according to the forcible discharge of ascospores (Ainsworth *et al.*, 1971). Saeng (1998) used nucleic acid data to solve the classification problem that stemmed from relying on the conflicting characteristics. The results of rDNA sequences show that the Erysiphales are closely related to the inoperculate Discomycetes, quite a stunning revelation.

Advantages of Morphological Characteristic Data

Morphological characteristics are the easiest traits to use for identification of fungi. Morphological identification is still the most common procedure practiced worldwide. It is an inexpensive and quick method that requires only the naked eye and the light microscope. Moreover, morphological characteristics depict features that are easily recognized. In other words, morphological data is a good way for the mind to organize taxa. In fact phylogeneticists apply advanced biochemical techniques and statistical computer programs to test the previous classifications done by morphological mycologists. However, it may be a good idea to consider all the available information, both morphological and molecular data, to achieve a more accurate picture of evolutionary relationships of these fascinating fungi.

Molecular Phylogeny

Molecular phylogenetics, also called molecular systematics, is the study of evolutionary relationships among organisms by using molecular data such as DNA and

protein sequences, or other markers such as single nucleotide polymorphisms (SNP) and restriction fragment length polymorphisms (RFLP) (Graur and Li, 1999). Phylogenetic classification is based on the evolution of a genetically related group of organisms, as distinguished from development of an individual organism (Guarro and Li 1999). Molecular phylogeny involves the use of molecular and morphological data with the support of statistical methods to infer evolutionary relationships among organisms (Li, 1997). The aim of phylogenetic studies is to rebuild correct genealogical ties among biological entities, to determine when organisms last shared a common ancestor, and to follow the development of the sequence change events along evolutionary lineages.

Before 1800, scientists tried to detect, describe and explain the diversity of organisms using systematics (mentioned by Moritz and Hillis, 1996). The goal of systematics is to build a classification of organisms that best reflects their evolutionary history (phylogeny). The study of phylogeny had begun before Mendel's Laws were rediscovered in 1900 (Mentioned by Graur and Li, 1999). Taxonomists first tried to use as many morphological, physiological and biochemical characters as possible for determining groups, until they proposed a system to produce a classification based on phylogenic relationships in the twentieth century. For example, Nuttall (1902, 1904) inferred the phylogenetic relationships among primates by using serological cross-reactions. If the serological cross-reactions were strong, it showed that those lineages were closely related. He found that the closest relatives of humans are the apes, the Old World monkeys, the new World monkeys, and the prosimians (in order of decreasing relatedness).

Mitchell *et al.* (1995) mentioned that molecular phylogeny helps taxonomists explain horizontal gene transfer and homoplasy in the evolution of fungal species. These two events occur frequently, and make delineation of fungal taxa difficult. Horizontal gene transfer is the transfer of genetic material between unrelated species. Homoplasy is a situation where a trait has evolved convergently in many times in unrelated lineages.

Fungal Molecular Data

Since the 1960's molecular biology has been adopted by evolutionary biologists. The data available for phylogeny continues to increase because of the power of molecular biological methods for examining the molecular structure of proteins and nucleic acids. New molecular techniques, like the Polymerase Chain Reaction (PCR), have been developed and are now being used by mycologists to aid in constructing fungal taxonomy (for example see Moritz and Hillis, 1996). The increasing availability of nucleotide data is helping mycologists to review the evolutionary relationships among fungi. Informative sequences come from a single copy region of genes that perform the same function in all taxa, and evolve at a fast enough rate to separate taxa into monophyletic groups (Bruns *et al.*, 1991). Those genes that fit these criteria are ribosomal RNA (rRNA) genes from the nuclear and mitochondrial genomes, cytochrome oxidase genes and some protein synthesis elongation factors. Introns of several protein-encoding genes, like the β-tubulin, actin, chitin synthase, acetyl coenzyme A synthase, glyceraldehydes-3-phosphate dehydrogenase, lignin peroxidase or orotidine 5′-monophosphate decarboxylase genes, also provide useful phylogenetic information for building molecular-based trees (Guarro

et al. 1999). However, one particular gene cannot determine all taxonomic evolutionary relationships of an organism.

The rDNA cluster is comprised of three structural regions, the 5.8S, 18S rRNA and 28S rRNA genes, as shown in Figure 1 (Mitchell, 1995). These three regions are transcribed into RNA molecules that form part of a 40S ribosome. Internal spacers (ITS) sitting between these three genes are transcribed and spliced out during post-transcriptional processing. Another spacer sitting upstream of the 18S gene is also transcribed, and is called the external transcribed spacer (ETS). The region that separates ribosomal gene clusters is a non-transcribed spacer (NTS). Both ETS and NTS regions comprise the intergenic spacer region or IGS. Each genomic region has an informative sequence for delineating taxa at a certain level, order, genus and species.

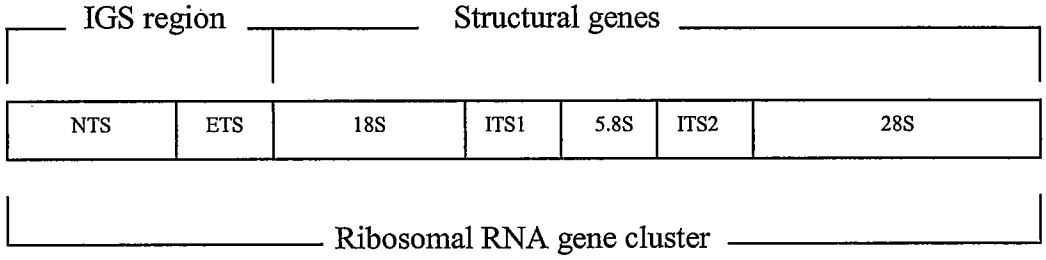


Figure 1. The nuclear ribosomal RNA gene cluster is comprised of a Non-Transcribed Spacer (NTS), External Transcribed Spacer (ETS), 18S rRNA, Internal transcribed Spacer 1 (ITS1), 5.8S, Internal transcribed Spacer 2 (ITS2) and 28S rRNA genes. IGS stands for the intergenic spacer region (adapted from Mitchell, 1995).

The rDNA genes are frequently targeted for fungal phylogenetic studies (Bruns *et al.*, 1991 and Mitchell 1995). The important reasons are that, first the rDNA genes are highly conserved. Second, the rDNA genes are found in all organisms, with a common

function of evolutionary origin. Third, the rDNA genes do not encode protein, and a single-copy nuclear gene is easy to amplify by PCR techniques.

Moreover, several variable regions, including the spacers between genes, evolve at rates different enough to recognize taxa at different ranks (Bruns *et al.*, 1991&1993; Berbee and Taylor, 1993&1995, Seifert *et al.*, 1995 and Bruns *et al.*, 1991). For example, in fungal molecular phylogenetics the nuclear small subunit 18S region has sufficient statistically significant divergence for studying relationships at or above the generic level whereas the large nuclear subunit 28S gene, especially the D2 domain, has enough variable nucleotide base pairs to examine the relationships of organisms near the generic level. The internal transcribed spacers, ITS1 and ITS2, are useful to discriminate relationships near the species level in fungi. In contrast, the 5.8S rDNA region has few base pairs with little variability for phylogenetic resolution.

Advantages of Molecular Data in Phylogenetic Studies

Graur and Li (1999) mentioned that molecular data, specifically DNA and amino acid sequence data, are much more reliable for evolutionary studies than morphological and physiological data for many reasons. For example, DNA and protein sequences are strictly heritable. This statement cannot be said for many morphological traits that can be affected by the environment, such as colors of the fruiting bodies of mushrooms which can depend on substrates in the soil. Another distinctive advantage of molecular data is that the description of molecular characters and character states is clearly identified; e.g. the nucleotide base in the 5.8S rDNA of the *Roussoella* species at the position 154 is U

whereas it is C in *Xylaria hypoxylon* (Eriksson, 1999a). In contrast, morphological descriptions are often ambiguous, such as the color and shape described of an ascospore as "hyaline to brown," and "slightly elongate," respectively. Usually molecular data can be quantitatively processed using mathematical and statistical methods, whereas morphological and physiological data cannot be quantitatively assessed. Moreover, molecular traits evolve more regularly than do morphological and physiological characters, thus molecular data can present a clearer picture of the relationships at all levels of classification of an organism (e.g., kingdom, phylum, family, genus and species). For instance, the ribosomal DNA sequence can be used to explain evolutionary relationships among distantly related phyla of bacteria, fungi, algae, plants and animals. Finally, molecular data are becoming more available than morphological data in twentieth century. This abundant molecular data is very useful and powerful for phylogenetic studies with organisms, which possess only a limited number of morphological or physiological characteristics, like bacteria, algae and protozoa.

The Concept of Species in Fungi

According to Taylor (Taylor *et al.*, 1999), there are three ways to examine the species concept in fungi: the Morphological Species Concept (MSC), the Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC). Classification by MSC is based on the morphological characters or other phenotypic characters. The BSC criterion uses the ability to interbreed whereas the PSC relies on the concordance of genetic data.

Morphological Species Concept (MSC)

Fungi are classified as the same species on the basis of observed similarities, and distinguished from one another on the basis of different characteristics (Alexopoulos, 1996 and Taylor *et al.*, 1999). MSC is a historical fungal classification system using observable characteristics that have been used worldwide. Types of conidia and conidiogenesis are the most important observable characteristics used for identification and classification of anamorphic fungi (Guarro *et al.*, 1999). Conidiogenesis is the process of conidia formation, and the cells that produce conidia are called conidiogenesis cells. Blastic and thallic are two major types of conidiogenesis. In blastic conidiogenesis, there is a little scar on the conidiogenous cells after the formation of conidia. In contrast to blastic conidiogenesis, in thallic conidiogenesis the entire conidiogeneous cells are completely changed to one or more conidia.

In teleomorphic fungi, the type of fruiting body is used for identification at the level of phylum: ascoma in ascomycota, and basidioma in basidiomycota. The shape, color and septation on ascospores and basidiospores help mycologists identify these fungi at the level of genus and species. Other structures such as sclerotia or chlamydospores are somewhat useful for identification when both asexual and sexual states are absent. Furthermore, ultra-structures taken by scanning electronic microscopy and transmission electronic microscopy reveal details of differences of special taxonomic significance. For example, examining the ultra-structure of hyphae reveals that there is a septum pore in Basidiomycetes, versus a Woronin body in the septum of Ascomycetes.

Biological Species Concept (BSC)

The biological species concept classifies a species as individuals that interbreed (Guarro, 1999, Taylor *et al.*, 1999). This concept emphasizes sexual and parasexual reproduction within species, and also the presence of barriers that prevent cross breeding of species. Only sexually-reproducing fungi like Basidiomycetes, Ascomycetes and Zygomycetes can be classified based on this concept. A single spore is cultured on the media, and combined with another single spore isolate to determine compatibility. After a suitable time, the culture is examined for signs of sexual reproduction, which is evidence the spores are from one heterothallic species. Distinctively, the BSC is limited to teleomorphic fungi, and many imperfect fungi (anamorph) cannot be classified by the BSC at all for taxonomic study because they lack sexual reproduction. For example, Basidiomycetes classified by BSC include *Armillaria*, *Pleurotus* and *Xeromphalina*, and *Omphalotus* (Peterson and Hughes, 1999). These fungi are all teleomorphs. Since the molecular methods that have been developed can be applied to any fungus, the BSC is used mostly by fungal population biologists.

Phylogenetic Species Concept (PSC)

The Phylogenetic species concept defines species as monophyletic groups of individuals that share a derived character state (Alexopoulos *et al.*, 1996, Taylor, 1999). PSC is determined by combining phylogenetic approaches and molecular biological techniques. PSC has provided new information to mycologists to delineate fungal taxa in

which sexual reproduction has not been observed. PSC depends on cladistic analysis of variable characters. Character states are the different expressions of a character, which serve as the basis for comparison among groups of organisms. Those characters are morphology, ultra-structural features and nucleic acid sequences. Each character state can be informative at only a certain taxonomic level. For example, nucleic acid sequences are the most frequently used in phylogenetic methods. DNA nucleotide sequences were first applied to study phylogenetic relationships in yeasts, which have few morphological characters (Kurtzman, 1984). Kurtzman showed that a variable region of the 25S rDNA gene in *Saccaromyces* is sufficiently different to classify species of yeast. In contrast to 25S rDNA, 18S rDNA can be used to discriminate between taxa at or above the generic level (Guarro *et al.*, 1999).

Advantages and Disadvantages of MSC, BSC and PSC

The three concepts, MSC, BSC and PSC, which mycologists use to define each species have their own limitations (Berbee and Talor 1995; Bruns *et al.*, 1991; Guarro *et al.*, 1999; Seifert *et al.*; 1995, Mitchell *et al.*, 1995; Taylor *et al.*, 1999). For example, a species of fungus classified by MSC often comprises more than one biological or phylogenic species. The morphological species *Armillaria mellea* can be comprised of more than a dozen biological species and two to three phylogenetic species (Taylor *et al.*, 1999). MSC provides insufficient taxonomic resolution with sterile fungi that are not able to replicate either via sexual or asexual reproduction. MSC also gives ambiguous classifications caused by the extreme morphological plasticity displayed by fungi.

However, the MSC has a strong point in that it has been applied widely to compare and identify fungi among existing taxa and between new and existing taxa.

BSC can be applied to fungi that can be mated, but approximately 20 % of fungi engage only in asexual reproduction (Reynolds, 1993). For mating studies, most fungi need special media, host plants and conditions, and many fungi cannot be cultured at all. Thus it is impossible to use the BSC criteria for all fungi. Moreover, homothallic fungi producing meiospores without a mating partner and cannot be classified by BSC.

The afore-mentioned problems from MSC and BSC can be solved by the PSC. Nevertheless, PSC also has some weak points. One of the problems for PSC is that one has to decide how many base pair differences are sufficient to decide or indicate different species (Guarro *et al.*, 1999, Seifert *et al.*, 1995). The number of base differences in a particular gene needed to specify individual species varies from gene to gene, and organism to organism. Taylor *et al.* (1990) suggested that nucleotide divergence in one fungus cannot be used to define taxonomic rank throughout for taxa.

Another problem is when a species has a polymorphic gene with two alleles, and the gene is the character state used to make the phylogenetic analysis. The question arises whether individuals sharing allele A would be called a species to the exclusion of those sharing allele B (Taylor *et al.*, 1999). An uncertainty about the PSC is that the number of determined species will rise as the genetic markers become more polymorphic. However, this concern will be invalid if more than one polymorphic locus has been used to formulate the determination of species using the PSC (Taylor *et al.*, 1999). Taylor *et al.* (1999) proposed a way to solve this problem by using more than one polymorphic

locus. Even though the definition of the phylogenetic species concept is complicated, this approach seems to fit well for fungal classification. Since there are different ways to recognize a species, mycologists should clearly understand which concepts they are using.

Taxonomy of Amphisphaeriaceae

The family Amphisphaeriaceae was first established by Winter (1887) to describe genera that produce ascocarps with partially immersed ascostroma in the substratum (mentioned by Kang *et al.*, 1998). These genera are *Amphisphaeria, Caryospora, Ohleria, Strickeria, Trematosphaeria, and Winteria.* Höhnel (1919) added *Anisostomula*, *Physosporella*, *Pemphidium*, *Merrilliopeltis*, *Oxydothis*, *Ceriospora*, *Griphosphaeria*, and *Leiosphaerella* under a Physosporellen group (mentioned by Kang *et al.*, 1998). Petrak (1919) combined the Physosporellen and *Hyponectria* into a new family, Hyponectriaceae based on the close relationships between *Hyponectria* with *Anisostomula*. *Amphisphaeria* was placed in the Phaeodidymae of the Sphaeriaceae because of the carbonaceous ascomata with an ostiole and two celled ovoid brown ascospores. Arx and Müller reclassified these genera into the Polystigmataceae. The taxonomic position of *Amphisphaeria* was unclear until Müller and Arx (1962) reintroduced Amphisphaeriaceae by combining some genera from Physosporellen, Hyponectriaceae and Polystingmataceae. This classification was based on the distinguishing characteristics of Amphisphaeriaceae: which were the presence of an amyloid ring in the ascus apex and ascomata immersed under a clypeus. At that time the

Amphisphaeriaceae included *Amphisphaeria, Apiorhynchostoma, Apiospora, Apiothyrium, Cainia, Cainiella, Ceriophora, Ceriospora, Chaetapiospora, Leiosphaerella, Oxydothis, Pseudomassaria, Roussöella* and *Seynesia.*

In 1973 Müller and Arx placed the Amphisphaeriaceae in the order Sphaeriales or Xylariales (mention by Hawksworth *et al.*, 1995), class Pyrenomycetes, phylum Ascomycota. They described the Amphisphaeriaceae as ascomycetous fungi that have an opening cylindrical asci within ascomata comprised of a carbonaceous wall. The ascostroma may be solitary or aggregated bearing spherical to cylindrical asci which have a single wall and a typical ring like structure at the apex. The ascospores are one celled or septate and hyaline or brown color. Genera classified in Amphisphaeriaceae by Müller and Arx (1973) were *Vialaea, Physalospora, Myelosperma, Xylochora, Urosporela, Anisostomula, Hyponectria, Amphisphaerella, Pemphidium, Chaetapiospora, Entosordaria, Apiothyrium, Apiospora, Pseudomassaria, Lejosphaerella, Ceriospora, Oxydothis, Amphaeria, Seynesia, Roussoella, Ceriophora, Cainia, Cainiella, Monographus, Exarmidium, Griphosphaerioma, Discostroma, Griphosphaeria, Broomella, Chitonospora, Phragmodiscus, Mycothyridium* and *Lepteutypa.*

Subsequently, Hawksworth and Eriksson (1986) proposed the order Amphisphaeriales containing four families; Amphisphaeriaceae, Cainiaceae, Clypeosphaeriaceae and Hyponectriaceae. They proposed Amphisphaeriales as an order to accommodate a pyrenomycetous group with a distinctive amyloid ring in the ascus. *Cainiaceae* was a family established for *Cainia* species, which have longitudinal germ slits in the ascospores and a series of rings on the apex of the ascus. Clypeosphaeriaceae

are xylariaceous fungi that have poorly developed stromatal tissue, solitary or aggregated perithecia with a thick-walled region at the ostiole, cylindrical asci with a negative iodine apical ring, and transversely septate hyaline or brown ascospores sometimes with a germ pore and mucous sheath (Hawksworth *et al.*, 1995). Genera that belong to Clypeosphaeriaceae are *Anthostomella*, *Clypeosphaeria*, *Hypospila*, *Linospora* and *Trabutia* (mentioned by Kang *et al.*, 1998). Barr (1989) classified six genera of *Apiiorhynchostoma, Clypeosphaeria, Melomastia, Pseudovalsaria, Saccardoella* and *Urosporella* into Clypeosphaeriaceae, but Hawksworth *et al.* (1995) reclassified eight genera of *Apiorhynchostoma, Ceratostomella, Clypeosphaeria, Crassoascus, Duradens, Frondicola, Melomastia and Pseudovalsaria* in Clypeosphaeriaceae based on ascostroma and ascospore morphology. As described by Hawksworth *et al.* (1996), Hyponectriaceae is an ascomycete that produces immersed or erumpent thin-walled perithecia with papillate ostioles, thin-walled asci with either a small amyloid reaction (giving blue color with iodine Melzer's solution) or negative amyloid reaction at the apical ring and possessing hyaline to pale brown ascospores. Ascospores of Hyponectriaceae are simple to transversely septate, sometimes thick-walled and sometimes with a mucous sheath.

Thereafter Cainiaceae was combined with Amphisphaeriaceae and considered to be in Xylariales by Eriksson and Hawksworth (1987). They described Amphisphaeriales as a synonym of Xylariales because there were two genera in these orders that were closely related. These two genera were *Astrocystis* in Xylariales and *Collodiscula* in Amphisphaeriales. The Amphisphaeriaceae were then classified in Xylariales (Eriksson and Hawksworth, 1993). Amphisphaeriaceae are described as ascomycetous fungi that

have crustose stroma, immersed or erumpent perithecium with a papillate ostiole, interascal periphysate, numerous thin-walled paraphyses, cylindrical asci with an amyloid positive ring on the apex, hyaline to brown septate ascospores sometimes with a germ pore (Hawksworth *et al.*, 1995). The anamorphs of Amphisphaeriaceae are mostly Coelomycetes that produce acervuli bearing conidia. There are 37 genera (+22 synonyms) in Amphisphaeriaceae (Eriksson, 1999b). The thirty seven genera of Amphisphaeriaceae are *Amphispaerella, Amphisphaeria, Arecophila, Ascotaiwania, Atrotorquata, Blogiascospora, Broomella, Cainia, Capsulospora, Ceriophora, Ceriospora, Chitonospora, Clypeophysalospora, Discostroma, Dyrithium, Ellurema, Flagellosphaeria, Frondispora, Griphosphaerioma, Idosphaeria, Lanceispora, Lleiosphaerella, Lepteutypa, Lindquistomyces, Manokwaria, Mukhakesa, Neobroomella, Neohypodiscus, Ommatomyces, Oxydothis, Paracainiella, Pemphidium, Pestalosphaeria, Reticulosphaeria, Urosporella, Urosporellopsis,* and *Xylochora.*

According to Barr (1975, 1989, 1990, 1993 and 1994), Amphisphaeriaceae was considered to belong under the order Xylariales because this family shared a number of characteristics that are typically xylariaceous. These general characteristics were: single or gregarious ascostroma which are immersed or erumpent in the substrate beneath a small or well developed, blackened clypeus; cylindrical, short stipulate asci containing an amyloid or nonamyloid apical ring and a short pulvillus; and radially symmetric ascospores. The genera accepted in Amphisphaeriaceae are *Amphisphaerella, Amphisphaeria, Broomella, Crassoascus, Discostroma, Dyrithium, Griphosphaerioma, Lepteutypa* and *Pestalosphaeria.* Barr (1993&1994) removed some other genera that

Eriksson and Hawksworth (1987) accepted for the Amphisphaeriaceae. For example, *Ceriospora, Leiosphaerella, Oxydothis, Vialaea* and *Urosporella* were moved from Amphisphaeriaceae to Hyponectriaceae on the basis of asci the forming a basal layer, whereas *Iodosphaeria* was place in the Lasiosphaeriaceae (Sordariales) on the basis of peridium and centrum structures (Barr, 1993&1994). *Diapleella* was considered in the family Phaeosphaeriaceae, order Pleosporales, class Loculoascomycetes (Barr, 1993& 1994). *Cainiella* and *Ceratostomella* were classified under the family Diaporthaceae, order Xylariales, class Pyrenomycetes (Barr, 1993&1994).

Table 3. Ascospore characteristics of the genera of the Amphisphaeriaceae (adapted from Barr, 1975&1994).

Genus	Amyloid reaction on apex of ascus.	Ascospores			
		Germ pores	No. of septation	Pigmentation	Wall surface
Amphisphaerella,	not recorded	several germ pores in equatorial ring	none	brown	not recorded
Amphisphaeria	amyloid	none	1	brown	smooth, entire
Broomella	nonamyloid	none	3	brown	smooth, appendage
Crassoascus	not recorded	none	3-4-7	terminal cells often lighter brown	ornamented, gel coating
Discostroma	amyloid	none	2	brown	smooth, entire
Dyrithium	not recorded	none	3-5	terminal cells brown	not recorded
Griphosphaerioma	nonamyloid	none	1-(3+)	hyaline	smooth, entire
Lepteutypa	amyloid	none	3	brown	longitudinally, striate, entire
Pestalosphaeria	amyloid	none	2	brown	longitudinally, striate, entire

Characteristics of ascospores are a major basis not only for separating Amphisphaeriaceae from other families (Xylariaceae, Boliniaceae, and Diatrypaceae), but also for recognizing individual genera in this family (Barr, 1994). The features of ascospores considered to be important include pigmentation, shape, septation, amyloid reaction, and wall ornamentation (Table 3). The uniseriate ascospores are elliptic or ovoid, and differ from one genus to another by the intensity of pigmentation and/or appendages, and by septation (Table 3). Certain Coelomycetes are observed as anamorphs of the Amphisphaeriaceae (Table 4) (Barr, 1975; De Hoog, 1979). These coelomycetous anamorphs have holoblastic, monoblastic or percurrently proliferating conidiogenous cells bearing hyaline or versicolor, several-septate conidia with appendages.

Based on both molecular and morphological criteria (Eriksson and Winka, 1997 and 1998; Eriksson 1999 a&b and 2000 a&b), Amphisphaeriaceae is classified in the order Xylariales, subclass Xylariomycetidae, class Sordariomycetes, subphylum Pezizomycotina, phylum Ascomycotina. Based on DNA sequence data of the 5.8S rRNA region and internal transcribed spacer ITS2, Kang *et al.* (1998) reorganized Amphisphaeriales to accommodate three families: Amphisphaeriaceae, Cainiaceae and Clypeosphaeriaceae. From the results of molecular studies, they concluded that the family Amphisphaeriaceae (sensu stricto) was comprised of *Amphisphaeria*, *Discostroma*, *Ellurema*, *Lepteutypa*, *Pestalosphaeria* and other genus possessing *Pestalotia*-like anamorphs. The family Clypeosphaeriaceae included *Apioclypea*, *Capsulospora, Clypeosphaeria, Oxydothis* and other related genera. The Cainiaceae was

reproposed for the genera *Atrotorquata* because *Atrotorquata*'s branch separated from the branch of Amphisphaeriaceae in a teleomorph-anamorph connection dendrogram. Moreover, *Atrotorquata* has ascospores and asci, which resemble ascospores of *Cainia* in having ascus apparatus comprised of a series of rings and brown bi-celled ascospores with longitudinal germ slits.

Table 4. The genera of Amphisphaeriaceae with their anamorphs compiled from Barr, 1975; De Hoog, 1979; Nag Raj, 1977.

Teleomorph (Sexual state)	Anamorph (Asexual state)
Amphisphaeria	*Bleptosporium, Dendryphiopsis*
Amphisphaeria argentinensis	*=Bleptosporium pleurochaetum*
Amphisphaeria angustata	*=Dendryphiopsis atra*
Amphisphaerella	Not known
Apiospora	*Arthrinium, Nigrospora, Pteroconium*
Blogiascospora	*Seiridium*
Broomella	*Pestalotia, Pestalotiopsis, Truncatella*
Crassoascus	Not known
Ceriospora	*Chaetoconis*
Discostroma	*Seimatosporium*
Discostroma tricellular	*= Seimatosporium azaleae*
Dyrithium	Not known
Griphosphaeria	*Fusarium, Seimatosporium*
Griphosphaerioma	*Labridella*
Griphosphaerioma kansensis	*= Labridella cornu-cervae*
Hymenopleella	*Seiridium-like (Monochaetia)*
Lepteutypa	*Hyalotiella, Seiridium*
Lepteutypa cupressi	*= Seiridium unicorne*
Pestalosphaeria	*Pestalotiopsis, Pestalotia*
Pestalosphaeria hansenii	*= Pestalotiopsis foedans & Pestalotiopsis microspora* (NE32)
Physalospora	*Arthrinium*
Pseudomassaria	*Beltraniella*

Generic synonyms for *Discostroma* are *Clethridium, Clathridium, Curreyella, Phragmothella, Griphosphaeria, Paradidymella* and *Discostromopsis* (Eriksson and Hawksworth, 1993).

Concluding Remarks and General Objectives

Fungi that exist within various parts of healthy plants are known as endophytes. Frequently, the greatest numbers of diverse fungi come from living plants in rainforests of every continent. The most common endophytic genera that are isolated as anamorphic endophytes of temperate and tropical plants are *Pestalotiopsis*, and its allies such as *Pestalotia*, *Monochaetia*, *Seiridium*, *Seimatoantlerium*. One importance of these groups of fungi is their unique and potentially useful secondary metabolites such as taxol and ambuic acid. Differentiation of these anamorphic genera is based primarily on the location and number of appendages developed on conidia as well as on the number of cells comprising the conidia (Guba, 1961; Sutton, 1980; Nag-Raj, 1993). Certain representatives in this group of appendage-bearing coelomycetous fungi are linked to sexual states of Amphisphaeriaceae (Pyrenomycetes, Ascomycota). For example, *Pestalotiopsis* sp. is the asexual state of *Pestalosphaeria* sp. whereas *Seimatosporium* sp. is the mitotic state of *Discostroma* sp. (Barr, 1994; Sutton, 1987; Metz, 2000).

Even though the relationship of these fungi is based on their asexual spore morphology, and acervular characteristics, they have been placed in different taxonomic categories dependant on different criteria by various researchers. The taxonomic problem associated with teleomorphs of these coelomycetous fungi is that there are many versions of classification for Amphisphaeriaceae depending on which morphological characteristics are emphasized. To classify genera in Amphisphaeriaceae, order Xylariales, Barr (1994) considered the major characteristics of the uniseriate ascospores

to be septation, intensity of pigmentation, and the presence or absence of wall ornamentation and/or appendages, whereas Eriksson and Hawksworth (1987) considered stromata, shape of perithecium, peridia and centrum structures as major characteristics. In addition, another problem that makes classification in Amphisphaeriaceae confusing is that most genera in Amphisphaeriaceae have been linked with different genera of coelomycetous anamorphs (Table 4), and there is not a one-to-one correspondence. Moreover the presence of anamorphs and teleomorphs of Amphisphaeriaceae rarely occur at the same time where cultured on agar (Barr, 1975 & 1994).

There is some evidence that historically important characteristics are not stable. Christensen (1932) reported that the genus *Monochaetia* could not be separated from *Pestalotia* since he found both *Monochaetia* and *Pestalotia* type conidia in the same acervulus of *Pestalotia funereal* isolated from *Pinus palustris* Mill, but Guba (1961) and the International Code of Botanical Nomenclature did not accept this evidence. Interestingly, Christensen's discovery is consistent with our observation of variation in conidial morphology of *Pestalotiopsis microspora*WT98 to include *Monochaetia*- and *Truncatella*- like conidia after irradiation with a short wave germicidal UV lamp. These two observations imply that conidial morphology of *Pestalotiopsis* is prone to morphological change in the environment.

To some degree the use of molecular phylogeny is now solving these taxonomic problems. Molecular phylogenetic data may be particularly helpful in supporting or disproving historical fungal taxonomic arrangements. Based on the 5.8S rDNA and ITS2 sequences, Kang *et al.* (1998&1999) concluded that the Amphisphaeriales should be

revised as an order, and the Amphisphaeriaceae were restricted to *Amphisphaeria*, *Discostroma*, *Ellurema*, *Lepteutypa*, *Pestalosphaeria* and other genera possessing *Pestalotia*-like anamorphs. However, Eriksson (1999b) examined the classification done by Kang *et al.* (1998), suggesting that the 5.8S r DNA and ITS2 sequences were too divergent to infer relationships at the order and family levels, and that the nucleotide number in those two regions was not sufficient for statistical significance. Therefore, I undertook a partial phylogenetic study of *Pestalotiopsis* sp., and selected genera in Amphisphaeriaceae, using the sequences of the nuclear encoded small subunit ribosomal DNAs (18S rDNA, 5.8S rDNA) and internal transcribed spaces (ITS1&2), and compared the results from molecular phylogeny with available anamorphic and teleomorphic characteristics of sampled genera in Amphisphaeriaceae to infer evolutionary relationships among these fungi. The hypotheses and objectives of this dissertation are listed as follows:

1. Hypothesis: Environmental factors such as UV irradiation can change *Pestalotiopsis microspora*WT98's conidial morphologies, which are basic characteristics in classical classification. Preliminary data showed that UV irradiation of *P. microspora* spores with appendages at both apices can result in a variety of spore types including those representative of two closely related genera, *Monochaetia* and *Truncatella*. Objectives of this experiment were to irradiate *Pestalotiopsis microspora*WT98 spores, and to evaluate the various changes in conidial morphology in resulting UV irradiated biotypes.

2. Hypothesis: Selected genera in Amphisphaeriaceae form a monophyletic group in the Pyrenomycetes. To test the hypothesis, the 18S rDNA sequences from some representative ascomycota genera in Pyrenomycetes and Amphisphaeriaceae were determined, aligned and processed for phylogenetic reconstruction.
3. Hypothesis: Molecular analysis of the complete sequences of the ITS1-5.8S rDNA-ITS2 and comparison of the results from the complete sequences of the ITS1-5.8S rDNA-ITS2 with available morphological phenotypes are useful information to explore evolutionary relationships among chosen genera in Amphisphaeriaceae. To obtain insight into the evolutionary relationships among representative genera in Amphisphaeriaceae, the complete sequences of the internal transcribed spacers, including the 5.8S RNA gene from twenty-six taxa, were investigated, aligned and assessed for inferred phylogenetic trees, and critically compared with anamorphic and teleomorphic characteristics including geographical origins, hosts and nutritional modes.
4. Hypothesis: An understanding of possible genetic relatedness of representative genera in Amphisphaeriaceae may facilitate the discovery of new taxol producers or producer of novel unique compounds. To investigate the correlation between genetic relatedness and taxol production, the ability to produce taxol was screened and mapped onto the single most parsimonious cladogram inferred from the internal transcribed spacers including 5.8S RNA gene.

CHAPTER 2

UV LIGHT-INDUCED CHANGES IN CONIDIAL MORPHOLOGY OF *PESTALOTIOPSIS MICROSPORA*, AND IMPLICATIONS FOR CLASSIFICATION OF RELATED TAXA

Introduction

Pestalotiopsis microspora is a common endophyte found in tropical and subtropical plants and has been determined to be a producer of taxol, an anticancer drug (Strobel *et al.*, 1996 a & b). It has been suggested that taxol produced by this endophyte may protect the host from root diseases caused by *Phytophthora* and *Pythium* (Strobel *et al.*, 1996a). However, the role of this fungus in the host plant remains unclear. In spite of semisynthetic production methods, taxol is still an expensive drug to obtain. *P. microspora* has been considered a possible candidate as a source of taxol. Potentially, it would be extremely useful, more cost effective and more widely available, if a fermentable microbial source of taxol could be developed. Dr. Eugene J. Ford mutated *P. microspora* trying to find putants that produce more taxol than the *ca* 100 ng L^{-1} produced by wildtype isolates. One isolate of *P. microspora* (WT98) was selected for use in these studies seeking clones that produce and accumulate increased taxol. He found that some conidia of *P. microspora* WT98 were changed to *Monochaetia*-, *Seiridium*- and *Truncatella*-like conidia after mutation by irradiation. This suggests that the number of appendages and septa in conidia of this fungus may not be a stable characteristic in morphological classification.

Historically *Seiridium*, *Pestalotia*, *Monochaetia*, *Pestalotiopsis* and *Truncatella* have a taxonomic connection. All are Coelomycetes, and are the imperfect state of Ascomycota (deuteromycetes). The genus *Seiridium* was first established in 1831 by Nees ex Krombh to describe fungi that have six celled conidia produced in chains held together by slender filaments (mentioned by Guba, 1961). However, Guba (1961) placed *Seiridium* under the Sexloculate section of the genus *Monochaetia* because *Seiridium* produces six celled conidia with a single apical appendage and four intermediate colored cells.

Later the genus *Pestalotia* was first found as a single species on grape canes (*Vitis vinifera*) in Italy, and was named to honor Fortunato Pestlozza by de Notaris in 1839 (mentioned by Guba, 1961). The genus *Pestalotia* was established on the basis of the fungus *Pestalotia pezizoides* de Not. which has six-celled conidia with four dark colored middle cells and two terminal hyaline cells. The apical hyaline cell holds two or more simple or branched bristles at the apex, or with one branched appendage. The conidia are formed in an acervulus with a gelatinous well-developed stroma. A synonym was *Petalozzia*. In 1884 Saccardo created *Monochaetia* as a subgenus of *Pestalotia* since this taxon differs from *Pestalotia* by having only one appendage rising from the superior hyaline cell. *Monochaetia* was given generic rank by Allescher about twenty years later.

In 1914 Klebahn divided *Pestalotia* into three sections according to the number of cells constituting the conidia. These three sections were designed Quadriloculatae, Quinqueloculatae and Sexloculatae. Quadriloculatae was a section that included four-celled conidia having with two intermediate colored cells. Quinqueloculatae included a

group of 5-celled conidia with three intermediate colored cells. Section Sexloculatae included six-celled conidia having with four intermediate colored cells. The number of the appendages on the apical hyaline cell was 2 or more, and the pattern of could be simple or branched.

Christensen (1932) proposed combining the genera *Pestalotia* and *Monochaetia* into one genus because he found one-appendaged conidia in different sectors of *Pestalotia funereal* cultures as well as normal two and three appendaged forms. However, Guba (1961) debated Christensen's assertion, and suggested that Christensen had misdetermined those monoconidial isolations as *Pestalotiopsis funerea.*

Steyaert (1949) divided the genus *Pestalotia* into two genera: *Pestalotiopsis* and *Truncatella* according to the number of the cells in the conidia. Steyaert defined *Pestalotiopsis* as fungi that produce five-celled conidia containing three colored middle cells and two terminal hyaline cells with two or more appendages rising from a superior hyaline cell and one basal appendage, and *Truncatella* as fungi that produce four-celled conidia containing two colored middle cells and two terminal hyaline cells with branched-appendage rising from the superior cell with no basal appendage. However, Guba (1961) did not accept Steyaert's classification.

Guba (1961) accepted the system proposed by Klebahn, and applied it to both *Pestalotia* and *Monochaetia.* Guba (1961) explained that for his classification of primary importance was the number of apical appendages. If the conidia have one apical appendage, that fungal specimen belongs to the genus *Monochaetia.* Consequently, *Seiridium* and *Hyaloceras* were classified in the genus *Monochaetia* (Guba, 1961). If the

conidia have two or more apical appendages, that fungus was classified as *Pestalotia*. As a result *Labridella*, *Truncatella* and *Pestalotiopsis* were included under the genus *Pestalotia* by Guba (1961). Secondly, Guba (1961) considered the number of cells in the conidia of these fungi. The total number of cells in conidia, including the two end hyaline cells, and the colored median cells would be 4, 5, or 6 cells, thus placing the specimens in either the Ouadri-, Quinque-, or Sexloculate sections of *Monochaetia* or *Pestalotia* (Guba, 1961).

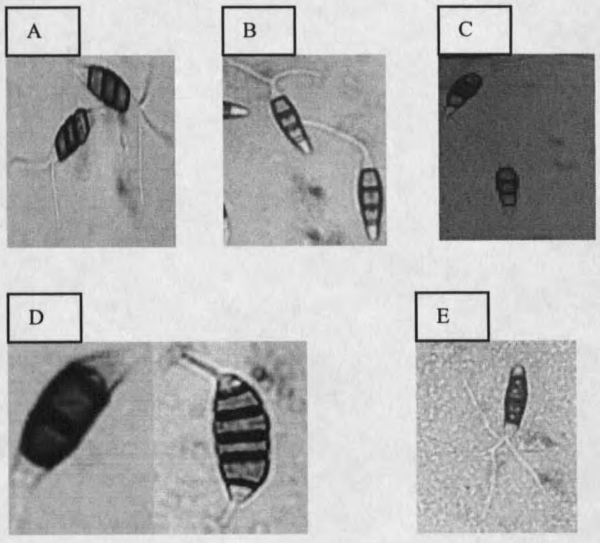


Figure 2. Conidia of *Pestalotiopsis*, *Monochaetia*, *Seiridium* and *Truncatella*. A. Conidia of *Pestalotiopsis microspora* WT98. B. Five-celled conidia with one or two appendages of *Monochaetia camelliae* (ATCC 60625). C. Five-celled conidia with one or two appendages of *Monochaetia* isolate 162. D. Four-celled and six-celled conidia of *Seiridium unicorne* (ATCC 48159). E. Four-celled conidia of *Truncatella angustata* (ATCC 96024). Magnification of all figures was 630X using ZEISS light microscope.

Nag Raj (1993) explained the morphological differences in conidia of *Pestalotia sp.*, *Pestalotiopsis* sp., *Monochaetia* sp., *Seiridium* sp. and *Truncatella* sp. as follows:

Pestalotia spp. produce 6-celled conidia with intermediate colored median cells bearing 2-4 dichotomously branched-apical appendages and a single centric basal appendage. Nag Raj (1993) gave only one example, *Pestalotia pezizoides* whose conidia contain six cells bearing 2-4 dichotomously branched-apical appendages and a single centric basal appendage. The rest of the *Pestalotia* species, which have conidia of five cells bearing 2-4 apical appendages and a single centric basal appendage were excluded and put into the *Pestalotiopsis*. *Pestalotiopsis* spp. produce 5-celled conidia with concolor or versicolor median cells. Generally, conidia of *Pestalotiopsis* spp. have two or more simple or branched apical appendages and a single centric basal appendage (Figure 2A). *Monochaetia* spp. have euseptate conidia, in which each cell is separated by multi-layered walls similar in structure to lateral walls. The median cells are thick with pigmentation in various shades of brown. Septation of conidia may be variable. The apical cell of *Monochaetia* spp. bears a single branched appendage while the basal cell does not bear any basal appendage (Figure 2B&C). *Seiridium*'s conidia are 5- or 6-distoseptate (sometime two can be found) with each cell possessing a thick secondary wall separated from the outer wall (Figure 2D). The median cells have thick-walls, and are brown to dark brown in color. Teleomorphs of *Seiridium* spp., when known, are in *Blogiascospora* and *Lepteutypa* (Barr, 1994, Nag Raj 1993). Conidia of *Truncatella* possess four cells comprised of two colored middle cells and hyaline cells at both ends (Figure 2E). The modified apical cell holds thin-walled branched appendages while the basal cell has no appendage.

A serendipitous observation in seeking increased taxol production induced through mutation with a short-wave germicidal UV lamp led to the discovery of conversion of *Pestalotiopsis* conidia to *Monochaetia*- and *Truncatella*-like conidia. These results are consistent with my hypothesis that appendage and septum number are not stable characteristics for morphological classification. These UV irradiated cultures of *Pestalotiopsis microspora* WT98 are called putative mutants or putants (or variants or biotypes) because mutants by definition are any heritable biological entity that differs from wildtype passing through meiosis process. *Pestalotiopsis microspora* WT98 and these UV irradiated cultures cannot produce sexual stage.

To test the hypothesis I evaluated and compared the change in conidial morphology among *Pestalotiopsis microspora* WT98 and putants after exposure to UV irradiation. In addition I used molecular data, ITS1-5.8S-ITS2, to confirm that the putants were not contaminants because the DNA is strictly heritable and independent of the environment. The ITS spaces have been successfully used for the analysis of relationships at the infrageneric and species levels (Burns *et al.*, 1991 and Lee and Taylor, 1992).

This research intends to show that conidial morphology (appendage and cell number in conidia), so important in the historical classification of genera in Amphisphaeriaceae, is an unstable characteristic. To show this, UV irradiated cultures of *Pestalotiopsis microspora* WT98 will first be characterized as to conidial morphology before and after treatment. Second, a comparison of the complete ITS1-5.8S-ITS2 sequences is intended to show that UV irradiation does not affect ITS spacers and 5.8S

RNA gene, and that those putants are not contaminants. To test these hypotheses, the ITS spacers and 5.8S RNA gene from *Pestalotiopsis microspora* WT98, putants and taxonomically related genera of *Pestalotiopsis* were sequenced, aligned and compared in pairwise.

Materials and Methods

Fungal Strains

The fungal strains used in this experiment were an endophytic isolate of *Pestalotiopsis microspora*, designated CP4 and WT98 in our laboratory. Both isolates were isolated from *Cephalotaxus fortunei*, in China by Jia-Yoa Li and Gary Strobel, Montana State University (unpublished). *P. microspora* isolate CP4 was used for kill curve determination whereas WT98 was used for UV irradiation. Other fungal strains were either purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, or the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. Species considered in this study, including original substrate and geographic origin are listed in Table 5.

Preliminary Experiment to Determine 50 % Kill Curve, Mutation and Isolation

Pestalotiopsis microspora CP4 was cultured on water agar plates with sterile (γ-irradiated) carnation leaves, provided by the *Fusarium* Lab at Pennsylvania State University. The plates were incubated at 20 ° C under 12 hours light/12 hours dark cycles of fluorescent light for three weeks to produce acervuli and conidia. The conidia from

one acervulus were diluted to a concentration of 10^3conidia/ml with sterile distilled water, and 1 ml was spread on 1/10X potato dextrose agar (PDA). The conidia were allowed to initiate the germination process for 1-2 hours at room temperature, and then exposed to a short-wave germicidal UV lamp (254 nm, PHILIPS TUV 8W/GB T5) at 8.5 inches distances. To determine a balance of mutation rate and surviving cells a kill curve were measured. Initially exposure times were for one to seven minute intervals and the kill curve was constructed at 24, 48, 72, and 96 hours intervals after exposure. After UV exposure for four to five minutes, conidia were allowed to germinate for 24-48 hour at room temperature. Each putant was isolated by cutting out a single hyphal tip of a germinating conidium. Some 4,000 single spores UV irradiated cultures were cultured and examined for morphological change.

Table 5. Selected taxa of *Pestalotiopsis* and taxonomically related genera in Amphisphaeriaceae subjected to ITS1, 5.8S rDNA and ITS2 regions sequencing.

Organisms	Strain no.	Original substrate	Origin
Monochaetia camelliae	ATCC60625	*Camellia japonica*	New Zealand
Monochaetia sp.	Isolate #162	*Taxus baccata*	China
Pestalosphaeria hansenii	ATCC48245	*Pinus carribaea*	New Guinea
Pestalotia rhododendri	ATCC24306	*Rhododendron sp.*	Italy
Pestalotia thujae	CBS303.75	*Thuja occidentalis*	The Netherlands
Pestalotiopsis funereoides	CBS175.25	*Juniperus sp.*	England
Pestalotiopsis microspora	NE32	*Taxus wallachiana*	Nepal
Pestalotiopsis microspora	CP4, WT98	*Cephalotaxus fortunei*	China
Pestalotiopsis microspora	CBS171.26	Not mentioned	Italy
Pestalotiopsis microspora	CBS364.54	Bath Towel at Florida beach	USA
Pestalotiopsis neglecta	CBS200.65	*Taxus baccata*	U.K.
Seiridium cardinale	ATCC52521	*Juniperus communis*	Italy
Seiridium unicorne	ATCC48159	*Cryptomeria japonica*	New Zealand
Truncatella angustata	ATCC96024	*Prunus avium*	USA (Washington)

Microscopic Observation

The putants (UV3, 6, 9, 10, 15, 12, 20) were provided by Dr. Eugene J. Ford. The three hundred conidia produced by either *Pestalotiopsis microspora* WT98 or the putants were examined for morphological change under a light microscope (Zeiss). The selected putants with altered conidia morphology were photographed and examined by Dr. Wilford Hess with laser and scanning electron microscopy with an argon laser at 488 and 514 wavelengths. The fungal material was critically point dried, gold sputter coated and examined with a JEOL 6100 scanning electron microscope (Strobel *et al.*, 1996a). Results were calculated as the percentage of conidia with typical appendage numbers, branching and number of cells. The percentages of conidia with atypical spore morphology were also determined.

Fungal DNA Isolation

For DNA isolation, all fungi were grown in 1.5 ml of potato dextrose broth for 18 to 24 h at 23 ° C. The mycelium was harvested by centrifugation and washed twice with sterile double distilled H_2O. Total genomic DNA was extracted by the methods of Lee and Taylor (1990).

Amplification of ITS1, ITS2 and 5.8S rDNA

The ITS regions of each fungus were amplified using PCR and the universal ITS primers ITS5 (5'GGA AGT AAA AGT CGT AAC AAG G) and ITS4 (5' TCC TCC CTT

ATT GAT ATG C) (White *et al.* 1990). PCR was performed in a 50 µl reaction containing 0.1 µg genomic DNA, 0.4 µM of each primer, 0.16 mM four dNTPs and 5uM *Taq* polymerase (Promega) in a buffer of 10 mM tris-HCl (pH 9.0 at 25 ° C), 50 mM KCl, 3 mM $MgCl_2$, 0.1 % Triton X-100. PCR cycling conditions consisted of denaturation at 94 ° C for 1.5 min, annealing at 55 ° C for 2.5 min, and extension at 72 ° C for 3 min for 40 cycles, with a final extension at 72 ° C for 10 min (Willits, 1999). The PCR products were gel purified and desalted using a QuickStep PCR purification kit (Edge Biosystems).

Cycle Sequencing ITS Regions and 5.8S rDNA

10-40 ng of PCR product was sequenced using ABI prism BigDye terminator chemistry (Perkin-Elmer) and primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3'), ITS2 (5' GCT GCG TTC TTC ATC GAT GC 3'), ITS3 (5' GCA TCG ATG AAG AAC GCA GC 3'), ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3'). Sequencing conditions were 25 cycles of 96 ° C for 10 sec, 50 ° C for 5 sec and 60 ° C for 4 min. Isopropanol was used to precipitate and purify extension products in microcentrifuge tubes (Protocol ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, 1998). The final isopropanol concentration was 60 ± 5 %. For example, add 80 µl of 75% isopropanol to 20 µl of extension products. Leave the extension product tubes at room temperature for exactly 15 minutes to precipitate the extension products. After precipitation, the extension products were centrifuged at 3000x g with the Eppendorf Centrifuge 5804 for 30 minutes. The

supernatant was immediately discarded by inverting the microcentrifuge tubes onto paper towels. To dry the precipitates, the inverted microcentrifuge tubes were placed with the towel into the Eppendorf Centrifuge 5804 and spun at 700x g for 2 minutes. The reactions were resolved on an ABI prism 310 Genetic Analyzer (Perkin-Elmer) at the Montana State University Sequencing Center. Nucleotide sequences from both strands were merged with the Sequencher program (Gene Codes Corporation, Inc., 1995). The sequences were submitted to GenBank, with accession numbers shown in Table 15, Chapter 4.

Sequence Analysis

Alignments of the ITS regions and 5.8S rDNA between *Pestalotiopsis microspora* WT98 and other species pairs were performed using both the multiple alignment program CLUSTALW (Thomson *et al.*, 1994) followed by manual alignment. The sequence analysis of ITS 1 & 2 regions and 5.8S rDNA was complete for the genera of *Pestalosphaeria*, *Pestalotia*, *Pestalotiopsis*, *Monochaetia*, *Truncatella*, *Seiridium* and the putants. The total number of different base pairs was reported as percentage of base differences. Percentage of nucleotide divergence in each region of ITS1-5.8S-ITS2 between *Pestalotiopsis* microspora WT98 and other species pairs were also calculated.

Results and Discussion

50 % Kill Curve

Pestalotiopsis microspora CP4 was used in preliminary experiments to determine a kill curve under a short-wave germicidal UV lamp (254 nm, PHILIPS TUV 8W/GB T5). The 50 % kill curve showed that at 96 hours there is 50 % germination of conidia after the conidia were exposed for 2.5 minutes. This result suggests that a dose of UV exposure at 2.5 minutes at a distance of 8.5 inches gives surviving cells with several putants.

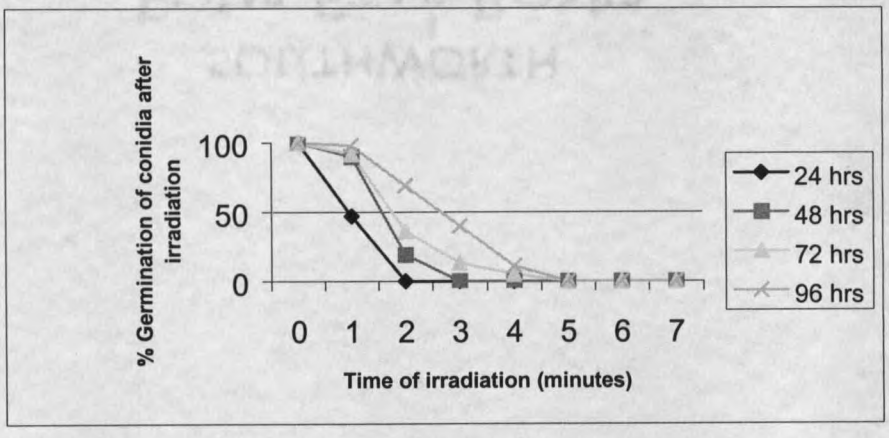


Figure 3. Kill curve of *P. microspora* (CP4) using UV (254 nm) irradiation at 8.5 inches distances.

Microscopic Observation

Microscopic examination of conidia from wildtype *P. microspora* strain #98 and putants (UV3, 6, 9, 10, 15, 12, 20) revealed two major categories of conidial types from three hundred conidia produced by either *Pestalotiopsis microspora* WT98 or a putant

with altered conidial morphology. In the first category, the number of apical appendages was changed while the second category possessed an abnormal number of cells in the conidia (Table 6 & 7). It is important to note that the conidia of these putants were derived from the initial single spore (single nucleus) or single hyphal tip after UV radiation. Eighty-nine percent of the conidia produced by the wildtype were five-celled and possessed two apical appendages and one basal appendage. Ten percent were five-celled having three apical appendages and one basal appendage and one percent had a single appendage and one basal appendage. Thus, the majority (89 %)of conidia produced by the wildtype were five-celled and possessed two apical appendages and one basal appendage and eleven percent exhibits natural variation.

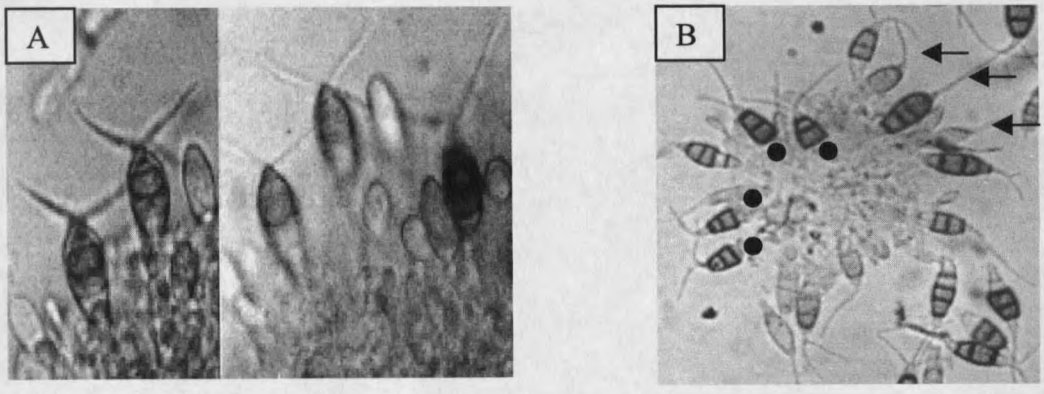


Figure 4. A. Acervulus of *Pestalotiopsis microspora* WT98 illustrating homogeneous pattern of morphology of young five-celled conidia with two apical appendages (magnification 630X). B. Acervulus of UV15 showing different morphologies of young conidia such as five-celled conidia with one and two apical appendages (magnification 200X). Circles mark five-celled conidia with two apical appendages, and arrows depict five-celled conidia with one apical appendage.

The conidia of putants still produce spores in their acervuli and in that respect, are identical to the wildtype. The conidia morphology of the wildtype and each putant seemed to be constant within in a single acervulus (Figure 4A & 4B), except UV15. The acervulus of UV15 contained multiple types of conidia, including: five-celled conidia with one apical appendage, five-celled conidia with two apical appendages and four-celled conidia with one apical appendage (Figure 4C). In addition only progenies of UV15 seems to maintain abnormal number of appendage and number of cell per conidium after transferring on the culture media for at least ten times.

Table 6. Percentage of different appendage morphologies in conidia from *P. microspora* WT#98 and the UV irradiate biotypes.

Morphology of conidia	Percentage of conidia with different morphologies							
Appendage types	WT	UV3	UV6	UV9	UV11	UV12	UV15	UV20
Terminal Appendages								
Obviously none	0	0.5	1	5	0.5	4	0	1
At least one appendage	0	1	3	8	1	2	0	9
One appendage -each end	1	39	35	33	5.0	15.5	46	21
Two apical appendages and a basal appendage	89	53.5	43	41	37	28.5	43	58
Triple apical appendages and a basal appendage	10	10	18	13	56.5	50	11	11
Total each column 100%	100	100	100	100	100	100	100	100

In each putant studied, with one exception (UV15), between 1% and 9 % of the conidia possessed one appendage (Table 6). Many of these conidia, with 4 euseptations and one terminal appendage (Figure 5A, 5B) bear similarities to the fungus *Monochaetia kansensis* (Sutton, 1980, Figure 8A). Similarly, in all other cases for irradiated fungi,

conidia appeared with no appendages, but having 3-4 euseptations (Figure 5C, 5D) making the spores appear as if they belonged to *M. saccardiana* (Sutton, 1980, Table 6, Figure 8B).

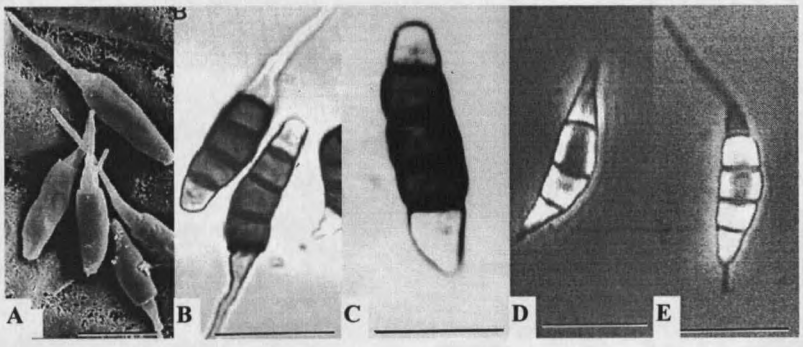


Figure 5. *Monochaetia*-like conidia of putants of *Pestalotiopsis microspora*WT98. 5A and 5B. Conidia with 4 euseptations and one apical appendage. 5C and 5D. Conidia without appendages at both ends. 5E. Conidia bearing a single appendage at each end of the conidium. A, B, C, D and E are pictures from the scanning electron microscope. Bar = 10 μm. Courtesy of W. M. Hess.

Another type of conidia from irradiated fungi was a large percentage of conidia (5.0 and 46 %) bearing a single appendage at each end of the conidium (Table 6, Figure 5E). This is quite unlike the wildtype in which only 1% of the conidia possessed one apical and one basal appendage (Table 6). In addition, *M. ceratoniae*, *M. monochaeta* (Figure 8C) and *M. carissae* each possess a single apical and a basal conidial appendage comparable to the examples in Figure 5C and in UV-3 (Table 6) (Sutton, 1980). In yet another example, conidia appeared with two apical appendages of either normal or abnormal length, comparable to the number of appendages in *P. microspora* WT98, but

having no basal appendage (Figure 6A, 6B, 6C & 6D). This is somewhat characteristic of conidia in both *Monochaetia karstenii* and *M. nattrassii* (Figure 8D).

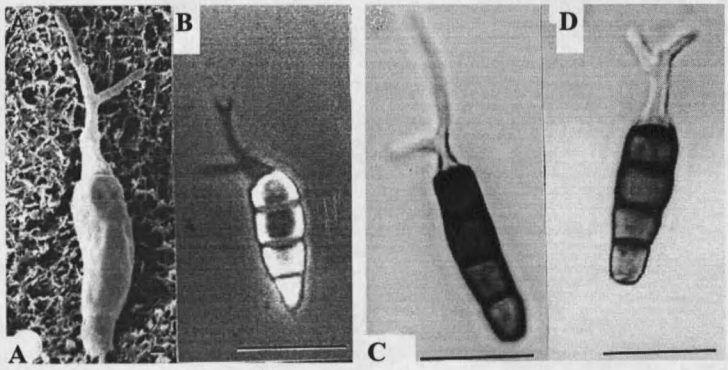


Figure 6. A, B, C and D illustrate conidia bearing two apical appendages either normal or abnormal in length, comparable to the number of appendages in *P. microspora*WT98, but having no basal appendage. A, B, C and D are pictures from the scanning electron microscope. Bar = 10 μm. Courtesy of W. M. Hess.

Figure 7. A. Conidia with multiple apical appendages that are extremely shortened. B. Conidia with shortened appendages at both ends on a spore that is narrow in width compared to its length. A and B are pictures from the scanning electron microscope. Bar = 10 μm. Courtesy of W. M. Hess.

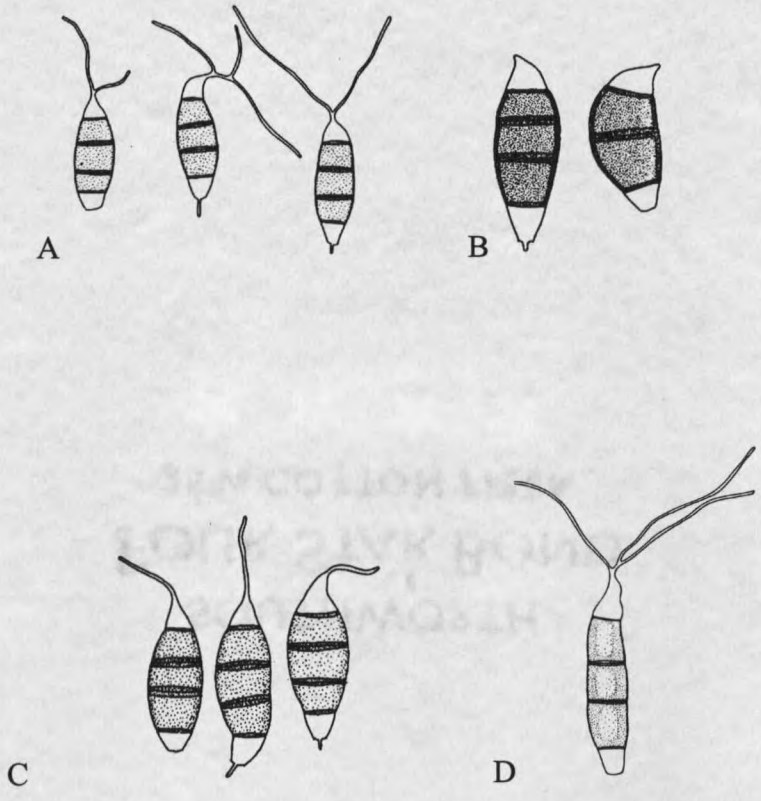


Figure 8. A. conidia of *Monochaetia kansensis*. B. conidia of *Monochaetia saccardiana*. C. conidia of *Monochaetia monochaeta* and D. conidia of *Monochaetia nattrassii*. (modified from Sutton, 1980).

In addition, other variants were noted that seem to have no resemblance to any previously described fungal species. These included a form with multiple apical appendages that are extremely shortened (Figure 7A) and one that had shortened appendages at both ends on a spore that is narrow in width compared to its length (Figure 7B). Also, one putant line (UV 11) consistently produced more apical appendages than the wildtype. In this case, UV 11 notably had 56.5 % as triple apical appendages vs. the wildtype that had only 10% (Table 6).

Table 7. Percentage of normal and abnormal number of cells per conidium in the *P. microspora* WT #98 and the putants.

Number of cells in each conidium:	WT	UV3	UV6	UV9	UV11	UV12	UV15	UV20
Five cells (normal)	100	91	99	78	100	66	98.5	98
Single cell	-	-	-	5	-	3	-	-
Two cells	-	1	1	15	-	8	-	1
Three cells	-	1	-	-	-	-	1.5	-
Four cells	-	1.5	-	-	-	20	-	-
Five celled distorted	-	3	-	-	-	3	-	-
Six & more than six cells	-	2.5	-	-	-	-	-	-
Spores with no cytoplasm	-	-	-	2	-	-	-	1
Total each column 100%	100	100	100	100	100	100	100	100

(-) denotes no observed spores with the number of cells in this group.

In the second category, especially with putants UV9, UV12 and UV15, some variation in cell number was noted in each conidium. It ranged from five cells to as little as one e.g. 5% and 3 % one-celled conidia in UV 9 and UV12, respectively (Table 7, Figure 9). Certain of these conidial types bore resemblance to those in such fungal genera as *Truncatella*, namely, 20.0 % of UV12 conidia which were four celled with triple distorted or branched - apical appendages (Table 7, Figure 8, at the arrow of a right corner) (Sutton, 1980). In UV 9 and UV12, 15 % and 8 % of the conidia had two celled whereas 3 % of the UV 12 conidia had five distorted cells. Finally, UV3 produced 2.5 % six celled conidia (Table 7). In some conidial types, the conidia possessed no cytoplasm or empty cells as in UV 9 and UV 20 (Table 7).

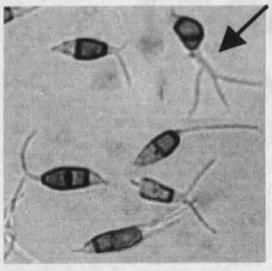


Figure 9. Variation in cell number of the conidia was noted and it ranged from two, three and four cells. The arrow depicts four celled conidia with triple distorted or branched - apical appendages, which could be found in UV12 and UV15. (light microscope, magnification = 630X)

To confirm that each uncommon type of conidia from putants produced spores that were identical to the spore types from which they were derived, I cultured 5-10 single spore replicates. Each spore from putants was placed on carnation leaves on the surface of water agar and incubated for 3-4 weeks prior to observation under the light microscope at 400X and 630X magnification. The resulting microscopic examination of conidia showed that the unusual number of appendages and atypical number of cells in the conidia from the sampled putants were not heritable. From sampled putants UV3, UV9 and UV15, replicates had different numbers of appendages and cells within each acervulus. For example, six replicates of a single five-celled conidium with a single appendage from putant UV15 generated five-celled conidia with one and two appendages in one acervulus. The same putant of a single five-celled conidium with two appendages produced five-celled conidia with one, two or three appendages in one acervulus in six cultures. Five cultures from a single five-celled conidium with two appendages produced

five-celled conidia with one appendage, two appendages or three appendages in one acervulus.

Results show putants of *P. microspora* WT98 to be various taxonomically accepted genera. This research suggests that we cannot distinguish the genera *Pestalotia* and *Pestalotiopsis* from the genus *Monochaetia* on the basis of number of apical appendages, nor on the number of cells in the conidia of the fungi examined. UV irradiation of *Pestalotiopsis microspora* spores, which have appendages at both apices, resulted in a variety of spore types including those representative of the closely related genus *Monochaetia*.

The results of this experiment are consistent with the discovery of Christensen in 1932. Christensen (1932) described how he obtained mono-appendaged conidial cultures from a single acervulus of a species (conidia typically with four appendages) isolated from *Pinus pavulstris* Mill. *Pestalotia funerea* is a Pestalotia species usually producing conidia with 3-4 apical appendages and a single basal appendage. The two races conformed with the characters of both *Pestalotia* and *Monochaetia*, and as a result he suggested that the number of appendages was not a good characteristic to distinguish the two genera, and that the genus *Monochaetia* could not be maintained as a genus distinct from *Pestalotia*. To quote Christensen (1932, p 532 and 540),

> "...The cultures described above, which in turn were obtained from the same acervulus as were other spores which gave rise to race that produced spores with three setae. It is not likely that two genera of fungi would be fruiting in the same acervulus. Nor is it likely that *Monochaetia* spores would, by chance, so frequently lodge on an acervulus of *Pestalozzia*., ... There are two possible explanations of this: either the new race is a previously undescribed variant of *Pestalozzia funerea*, and

> the limits of the species must be widened accordingly, and the genus *Monochaetia* considered no longer valid, or else the author has observed the birth of a species of *Monochaetia* from a species of *Pestalozzia*."

It is possible that *Pestalotiopsis* is likely to be predecessor of *Monochaetia*, not the other way around from the results of UV irradiation in this report and circumstantial evidence if *Monochaetia* is truly a separate genus. Furthermore the endophytic isolations, which produce a single appendage conidia were found with less frequency than *Pestalotiopsis* isolations. Two isolations of the *Monochaetia* form were obtained from five hundred *Pestalotiopsis* isolates. Guba (1961) mentioned that the deuteromycetes are extremely variable under different conditions in nature. It is possible in nature, that an ancestor like one of these fungal taxa gave rise to all of the other fungal genera mentioned above. UV irradiation may cause deletions, transition and transversion mutations onto the DNA sequences giving rise to errors in either DNA replication or repair. These mutations may have occurred and then one of the spore types eventually gave rise to one or more fungal biotypes that stabilized into a biotypical form that could be recognized at the genus level. DNA sequencing has been shown to confirm the links between generic or higher level (Berbee and Taylor, 1992; Rehner and Samuels, 1998). A high similarity of the ITS1-5.8S-ITS2 nucleotide sequences of *Monochaetia*, *Pestalotia* and *Pestalotiopsis* could be an indication that these three taxa might represent the same genus.

Variability within the ITS1-5.8S-ITS2 Regions

To confirm identicalness of biotypes to wildtype and each other, sequence analysis of the ITS1-5.8S-ITS2 regions belonging to the genera *Pestalotia*, *Pestalosphaeria*, *Pestalotiopsis*, *Monochaetia*, *Truncatella*, *Seiridium* and the UV treated biotypes were compared. The entire aligned ITS1-5.8S-ITS2 region for all studied taxa was between 517-530 base pairs (Table 8). Boundaries of the ITS1 and ITS2 regions were determined by comparison with the published sequences of the ITS region (Arenal *et al.*, 2000, Willits and Sherwood, 1999). The last five base pairs of 18S rRNA gene are "5′ CATTA 3′", and the first six base pairs of 28S r RNA gene are "5′ TTGACC 3′". The amplification products of ITS1 for sampled taxa in this experiment ranged in length from 188 to 195 base pairs, except *Pestalotiopsis microspora* CBS 364.54 possessing 142 base pairs (Table 8), and the ITS2 nucleotide length ranged from approximately 165 to 168 base pairs. Expectedly, the 5.8S rDNA sequences from all of the sampled taxa in this study were totally conserved. There is no variation in the whole region of 5.8S r DNA (Table 9). The first seven base pairs and the last six base pairs of 5.8S rDNA sequence of sampled taxa are"5′ AAACTTT 3′" and "5′ TCATTT 3′", respectively.

The results of sequence comparison showed that there was 100% identical nucleotide sequence in those regions among *P. microspora* WT98 and the putants: UV3, 6, 9, 10, 12 15 & 20 (Table 8). The 100% identity of ITS 1 & 2 regions and 5.8S rDNA shows that the putants were not contaminants, i.e. stray conidia of other genera. A comparison of *Pestalotiopsis microspora* WT98 with other isolations of *Pestalotiopsis*

microspora revealed 1.74-3.27 % differences in the ITS regions and 5.8S rDNA while *P. microspora* (CBS 364.54) showed a 17.95 % difference in the ITS regions and 5.8S rDNA (Table 8). The majority of the 17.95 % difference comes from an insertion or deletion (indel) in the ITS1 region (Table 9). There were 57 insertion or deletion positions out of 97 base pair difference; the remainder was 23 sites of transition (A/G =7, C/T=16) and 13 sites of transversion (A/T=9, G/C= 1, G/T=3). Comparing the two spacers independently, there are 32.30 % and 18.18 % nucleotide divergence in ITS 1 and ITS 2, respectively (Table 9).

Table 8. Base pair differences in the nuclear ITS regions and 5.8S rDNA, reported between *Pestalotiopsis microspora* WT98 and other selected species pairs.

Taxon pairs	Total	No. of different base pairs	% difference
P. microspora WT98 vs. Putants	517	0	0
P. microspora WT98 vs. *Monochaetia camelliae*	518	11	2.12
P. microspora WT98 vs. *Monochaetia* isolation162	520	15	2.88
P. microspora WT98 vs. *Pestalotiopsis neglecta*	517	8	1.55
P. microspora WT98 vs. *Pestalosphaeria hansenii*	518	9	1.74
P. microspora WT98 vs. *Pestalotia rhododendri*	518	9	1.74
P. microspora WT98 vs. *Pestalotiopsis microspora* (NE32)	518	9	1.74
P. microspora WT98 vs. *Pestalotiopsis funereoides*	519	16	3.08
P. microspora WT98 vs. *Pestalotia thujae*	519	16	3.08
P. microspora WT98 vs. *P. microspora* (CBS 171.26)	520	17	3.27
P. microspora WT98 vs. *P. microspora* (CBS 364.54)	518	93	17.95
P. microspora WT98 vs. *Seiridium cardinale*	530	77	14.53
P. microspora WT98 vs. *Seiridium unicorne*	527	77	14.61
P. microspora WT98 vs. *Truncatella angustata*	517	70	13.54
Monochaetia camelliae vs. *Monochaetia* isolation162	519	12	2.31

The highly variable regions of the ITS among the same species *P. microspora* might correlate with geographical origin or original substrate of isolates (Table 5). *P. microspora* WT98 was an endophyte isolated from *Cephalotaxus fortunei*, in China, but *P. microspora* (CBS 364.54) was isolated from a towel on the beach of Florida. It is possible that UV light on the beach of Florida causes the wide range of deletions or insertions in ITS1 region of *P. microspora* (CBS 364.54). However, the other strains of *P. microspora* studied were not significantly different enough to conclude that geography plays a role in the divergences of the ITS regions. The sequencing of more collections of *P. microspora* are needed. It is possible to find a considerable range of variation in ITS regions when these two spacers have evolved faster than any other regions such as 18S rDNA and 5:8S rDNA (Bruns *et al.*, 1991).

There is no exact method for indicating how much sequence divergence is needed for the recognition of species in fungi because the amount of sequence divergence within a specific taxa is variable. For example, in *Fusarium sambucinum* divergences have been reported between 0-15% in the two spacers of ITS (O'Donnell, 1992); *Rhizoctonia solani* AG 2 isolates, with 0-22 % divergence in ITS1-5.8S-ITS2 (Salazar *et al.*, 1999); and *Beauveria brongniartii*, with 0.7-18.7% divergence in ITS1 and 1.8-16.7% divergence in the ITS2 (Neuveglise *et al.*, 1994). In addition, there are several reports that show different taxa of fungi are synonymous because of the identical sequence of the ITS1 space alone or of the ITS1-5.8S-ITS2 regions. Viljoen *et al.* (1993) has reported the equivalence of *S. cardinale*, *S. unicorne* and S. *cupressi* based on 89-93 % homology of ITS1 sequences. Arenal *et al.* (2000) reported that *Epicoccum nigrum* and *Phoma*

epicoccina, which were morphologically indistinguishable from *Epicoccum nigrum*, are the same biological species based on the high similarity (98-100 %) of ITS1-5.8S-ITS2 regions. *Epicoccum nigrum* and *Phoma epicoccina* are synanamorphs even if there is no evidence that they are connected with the same teleomorph state (Arenal *et al.*, 2000). Khuls *et al.* (1996) showed that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina* based on the identical ITS1-5.8S-ITS2 regions and the high similarity of PCR-fingerprinting patterns. Khuls *et al.* (1996) also mentioned that their results implied that any sexual population might generate one or more asexual clones or species.

Variation in the entire region of ITS1-5.8S-ITS2 between *Pestalotiopsis microspora* WT98 versus *Pestalosphaeria hansenii, Pestalotiopsis neglecta, Pestalotiopsis funereoides* and *Pestalotia thujae* ranged from 1.55-3.08 % while nucleotide divergence between *P. microspora* WT98 and *Monochaetia camelliae* and *Monochaetia* isolation 162 were 2.12 % and 2.88 %, respectively (Table 8). The divergence percentage was also very low, 2.31 % in comparison of the entire region of ITS1-5.8S-ITS2 between *Monochaetia camelliae* and *Monochaetia* isolate 162, (Table 8). Wildtypes *Truncatella angustata* and *Seiridium cardinale* and *Seiridium unicorne* revealed base sequence differences from *P. microspora* of WT98 of 13.54 %, 14.53 % and 14.61 %, respectively in the complete sequences of the ITS1&2 and 5.8S rDNA (Table 8).

Considering each separately, percentage divergence between species of *Pestalotiopsis* (including *Pestalotiopsis microspora* WT98) and the genus *Monochaetia*

were still very low, ranging from 4.12 % - 6.19 % in ITS1, and 1.81–2.98 % in ITS2 (Table 9).

Table 9. Base pair differences in each region of ITS1-5.8S rDNA-ITS2 reported between *Pestalotiopsis microspora* WT98 and other species pairs.

Taxon pairs	Nucleotide divergence (%)		
	ITS1 (142, 194-205 bp)	5.8 S (158 bp)	ITS2 (165-168 bp)
P. microspora WT98 vs. Putants	0	0	0
P. microspora WT98 vs. *Monochaetia camelliae*	4.12	0	1.81
P. microspora WT98 vs. *Monochaetia* isolation162	6.19	0	1.79
P. microspora WT98 vs. *Pestalotiopsis neglecta*	3.09	0	1.21
P. microspora WT98 vs. *Pestalosphaeria hansenii*	3.09	0	1.81
P. microspora WT98 vs. *Pestalotia rhododendri*	3.09	0	1.81
P. microspora WT98 vs. *Pestalotiopsis microspora* (NE32)	3.09	0	1.81
P. microspora WT98 vs. *Pestalotiopsis funereoides*	6.19	0	2.39
P. microspora WT98 vs. *Pestalotia thujae*	6.19	0	2.39
P. microspora WT98 vs. *P. microspora* (CBS 171.26)	6.19	0	2.98
P. microspora WT98 vs. *P. microspora* (CBS 364.54)	32.30	0	18.18
P. microspora WT98 vs. *Seiridium cardinale*	23.41	0	17.37
P. microspora WT98 vs. *Seiridium unicorne*	23.15	0	18.07
P. microspora WT98 vs. *Truncatella angustata*	15.08	0	24.24
Monochaetia camelliae vs. *Monochaetia* isolation162	2.59	0	4.17

The overall range of variation which is below 3% for the entire ITS 1&2 region and below 7 % in individual spaces is within the limits of the infra and inter-species delimitation identified for many other fungal species from different orders mentioned previously. Thus, the high homology of ITS1-5.8S-ITS2 regions (97-96%) between species of *Pestalotia*, *Pestalotiopsis* and *Monochaetia* strongly suggests that the demarcation between species is not clear, and perhaps boundaries should be removed.

The exception is the large divergence between *Pestalotiopsis microspora* WT98 and *Pestalotiopsis microspora* (CBS 364.54), which was explained previously.

Table 9 shows the percentage of nucleotide divergence in each region of ITS1-5.8S rDNA-ITS2 between *Pestalotiopsis microspora* WT98 and other species pairs. There is quite a high degree of conservation in the ITS2 compared with the ITS1 region among the genera examined. In general, a majority of the nucleotide divergence (ranging from 4.12 % - 32.30 %) happened in the ITS1 space in most species pairs, except the pairs of *P. microspora* WT98 vs. *Truncatella angustata* and *Monochaetia camelliae* vs. *Monochaetia* isolate 162. Only for *P. microspora* WT98 and *Truncatella angustata* were based differences larger in ITS2 (24.24%) than in ITS1 (15.08%). *Monochaetia camelliae* and *Monochaetia* isolate 162 differ by 4.17 % in the ITS1 region and 2.59 % in the ITS2 region.

Figure 10 shows that there are 12 variable positions in the entire region of ITS1-5.8S-ITS2 between *Pestalotiopsis microspora* WT98 and *Monochaetia camelliae*, and 15 variable positions between *Monochaetia camelliae* and *Monochaetia* isolate 162. Transitions occurred more frequently than transversions and indels (twelve transitions of ten C/T and two A/G; eight transversions of three A/T, four G/T, one G/C, plus seven insertions/deletions), indicating that these sequences have diverged only recently. Of these different sites, eight (4.12 %) and three (1.81 %) were detected in ITS1 and ITS2 of *Pestalotiopsis microspora* WT98 and *Monochaetia camelliae*, respectively (Table 9). Likewise twelve (6.19 %) and three (1.79 %) were detected in ITS1 and ITS2 of *Pestalotiopsis microspora* WT98 and *Monochaetia* isolate 162, respectively (Table 9).

```
Pestalotiopsis micosporaWT98 CATTATAGAGTTTTCTAAACTCCCAACCCATGTGAACTTACCATTGTTGCCTCGG
Monochaetia camelliae        ...................................................
Monochaetia isolation 162    ...................................................

Pestalotiopsis micosporaWT98      CAGAAGCTGCTCGGCGCGCCTTACCTTGGAACGGCCTACCCTGTAGCGCC
Monochaetia camelliae             ........A.C...TA--..T.........................
Monochaetia isolation 162         ........A.CT..TTA-..C.........................

Pestalotiopsis micosporaWT98      TTACCCTGGAACGGCTTACCCTGCAACGGCTGCCGGTGGACTACCAAACT
Monochaetia camelliae             ..............T.......T.......................
Monochaetia isolation 162         ..............C.......T.......................

Pestalotiopsis micosporaWT98      CTTGTTATTTTATGGTTATCTGAGCGTCTTATTTTAATAAGTCAAAACTT
Monochaetia camelliae             ............T..T..............................
Monochaetia isolation 162         ............T..A..............................

Pestalotiopsis micosporaWT98      TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
Monochaetia camelliae             ...................................................
Monochaetia isolation 162         ...................................................

Pestalotiopsis micosporaWT98      CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
Monochaetia camelliae             ...................................................
Monochaetia isolation 162         ...................................................

Pestalotiopsis micosporaWT98      GCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCAT
Monochaetia camelliae             ...................................................
Monochaetia isolation 162         ...................................................

Pestalotiopsis micosporaWT98      TTCAACCCTTAAGCCTAGCTTAGTGTTGGGAGCCTACTGCTTTTGCTAGC
Monochaetia camelliae             ..................................T..............
Monochaetia isolation 162         ..................................C..............

Pestalotiopsis micosporaWT98      TGTAGCTCCTGAAATACAACGGCGGATCTGCGATATCCTCTGAGCGTAGT
Monochaetia camelliae             ...................................................
Monochaetia isolation 162         G..................................................

Pestalotiopsis micosporaWT98      AATTTTTAT-CTCGCTTTTGACTGGAGTTGCAGCGTCTTTAGCCGCTAAA
Monochaetia camelliae             .......T.T.....................................
Monochaetia isolation 162         .......A.-.....................................

Pestalotiopsis micosporaWT98      CCCCCCAA--TTTTTAATGG TTGACC
Monochaetia camelliae             T.......--..................
Monochaetia isolation 162         C.......AT..................
```

Figure 10. Sequence alignment of ITS1, 5.8S and ITS2 region of *Pestalotiopsis microspora* WT98, *Monochaetia camelliae* and *Monochaetia* isolate 162. The conserved positions are indicated with dots, and insertions/deletions positions with dashes. The 5.8S region (158 base pairs) is indicated with bold and underlined letters. The **CATTA** represents the last five nucleotides of 18S rDNA while the **TTGACC** represents the first five nucleotides of 28S rDNA.

We cannot generalize that the ITS2 spacer of genera *Truncatella* and *Monochaetia* is more variable than ITS1 spacer because only one or two species were tested, and sequencing of more species is required. Studying the nucleotide divergence of intraspecies and interspecies of one particular gene could tell the threshold or the limit of variation of nucleotide sequence necessary to identify species. In addition one particular gene sequence cannot determine all taxonomic evolutionary relationships of this group of fungi, and different genomic regions may help to resolve an unclear species identity. The 18S RNA and 28S RNA genes of these selected genera both intraspecies and interspecies may give additional informative data about the evolutionary relationships among the taxa. Other molecular techniques, for instance, the pattern of Restriction Fragment Length Polymorphism (RFLP) and polymerase chain reaction-fingerprints, could also provide more valid evidence for the investigation of the pattern of speciation in selected genera in Amphisphaeriaceae.

Secondary metabolites are another evidence supporting that these clones were affected and possibly mutated by the UV radiation, rather than being contaminants. Secondary metabolites of wildtype # 98 and selected putants mentioned previously were examined by thin layer chromatography. It was noted that some putant strains produce, not only new, but apparently greater quantities of secondary metabolites than the wildtype strain # 98 (Dr. Eugene Ford, personal communication).

In conclusion, UV radiation of *Pestalotiopsis microspora* WT98 conidia could resulted in putants that can produce conidia similar to those of *Monochaetia* with five-cells with a single apical appendage. Therefore UV irradiation altered basic

morphological characteristics important in species classification, the number of apical appendages and to a lesser extent the number of septa in five-celled conidia of *Pestalotiopsis microspora* WT98. Our ability to change the morphology of various taxonomically accepted genera by UV irradiation represents another example of the difficulty of predicting relationships based upon morphology alone. All those putants of *Pestalotiopsis microspora* WT98 producing conidia similar to those of *Monochaetia* are not contaminants according to the identical sequences of ITS1-5.8S-ITS2 regions of *Pestalotiopsis microspora* WT98 and putants. Both the results of the UV irradiation experiment and the high similarity of ITS1-5.8S-ITS2 sequences of *Monochaetia*, *Pestalotiopsis* and *Pestalotia* suggest that the number and type of appendages in fungal conidia are not stable characteristics for morphological classification. A species of *Pestalotiopsis or Pestalotia* can give rise to conidia conforming to the description given for *Monochaetia*, and the significant similarity (96-97%) of ITS1-5.8S-ITS2 regions are valid evidence that the genera of *Monochaetia*, *Pestalotiopsis* and *Pestalotia* are very closely related.

CHAPTER 3

A PHYLOGENETIC STUDY OF SELECTED GENERA IN AMPHISPHAERIACEAE USING 18S rDNA

Introduction

There are two controversial aspects concerning classification of the family Amphisphaeriaceae, (Pyrenomycetes, Ascomycota). The first relates to the order in which the Amphisphaeriaceae should be placed: Xylariales or Amphisphaeriales. Based on the characteristics of ascostroma and ascospores, the family Amphisphaeriaceae has been classified in the order Xylariales (Eriksson and Hawksworth, 1993; Barr, 1994; Hawksworth *et al.*, 1995). In contrast, based on the 5.8S rDNA and ITS2 sequence Amphisphaeriales was reestablished to accommodate Amphisphaeriaceae, Cainiaceae and Clypeosphaeriaceae (Kang *et al.*, 1998). Kang *et al.* (1998) justified reestablishing Amphisphaeriales because Cainiaceae and Clypeosphaeriaceae were shown to be more closely related to the Amphisphaeriaceae than to the Xylariaceae (Figure 11). Also, Kang *et al.* explained that the consensus tree derived from the alignment of the 5.8S rDNA and ITS2 spacer showed two separate lineages, Xylariales and Amphisphaeriales (Figure 13). *Xylaria hypoxylon* and *Hypoxylon fragiforme*, representing the Xylariaceae, order Xylariales, have hyphomycetous anamorphs with the conidia simply generated directly from the hyphae. The hyphomycetous anamorphs in this group are *Nodulisporium* and *Geniculosporium* (mentioned by Kang *et al.*, 1998). Unlike the Xylariaceae, the Amphisphaeriaceae have coelomycetous anamorphs in which the conidia are generated within pycnidia or acervuli comprised of both fungal hyphae and

host tissue (mentioned by Kang *et al.*, 1998). These coelomycetous anamorphs are *Pestalotia* and *Hyalotiopsis* (mentioned by Kang *et al.*, 1998).

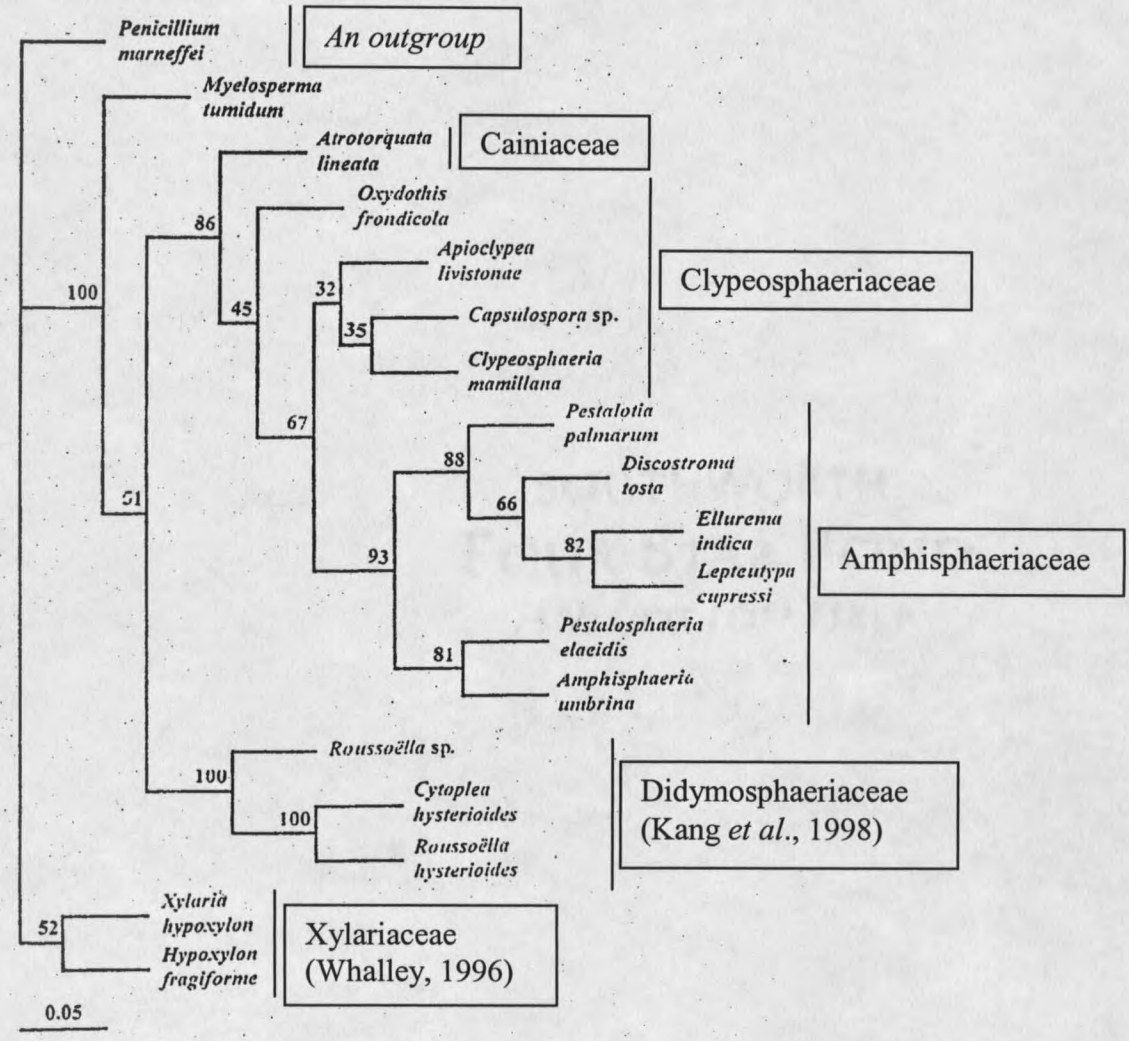


Figure 11. The neighbor-joining tree with bootstrap values above the branches using 5.8S rDNA and ITS2 spacer of 18 taxa (modified from Kang *et al.*, 1998). The bar indicates 5 substitutions per 1000 nucleotides. Didymosphaeriaceae is a family proposed by Kang *et al.* (1998) to accommodate genera *Roussoella* and *Cytoplea*.

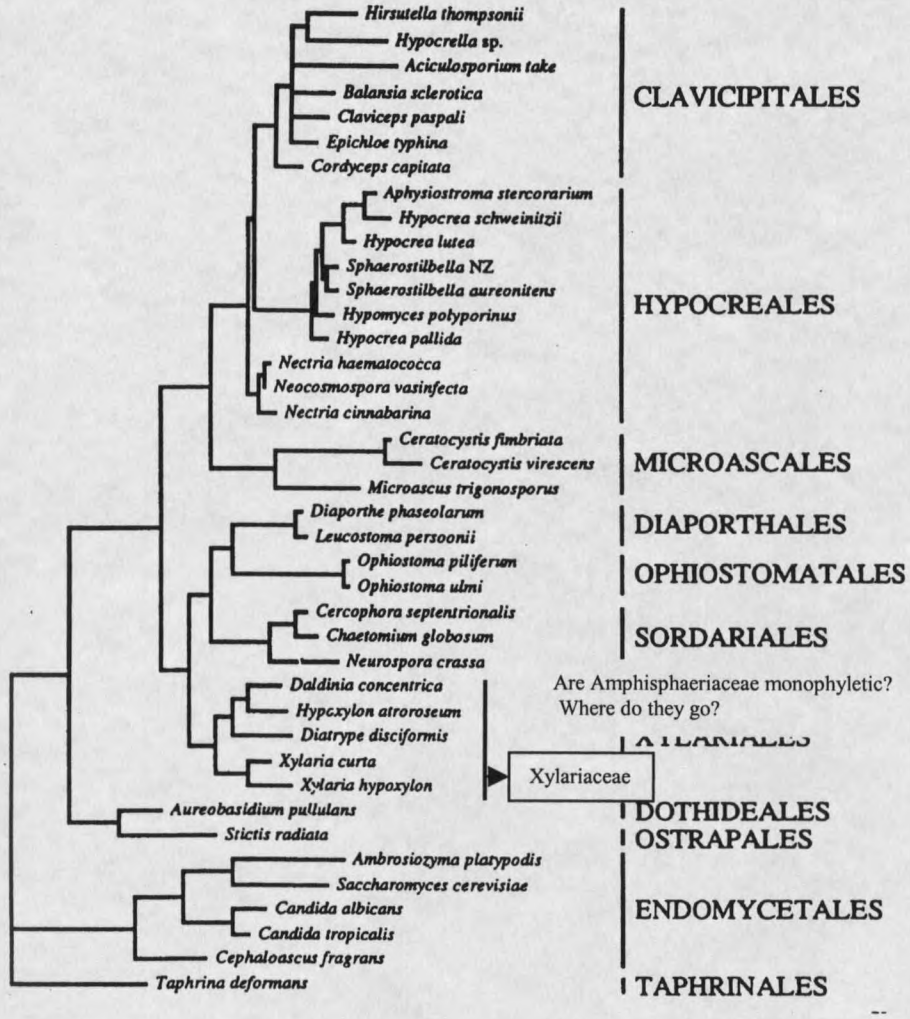


Figure 12. Strict consensus of 10 equally most parsimonious cladograms containing 40 taxa of Pyrenomycetes. This phylogenic tree depicts evolutionary relationships of the orders of Pyrenomycetes (Ascomycota), based on 18S rDNA analysis (modified from Spatafora *et al.*, 1993).

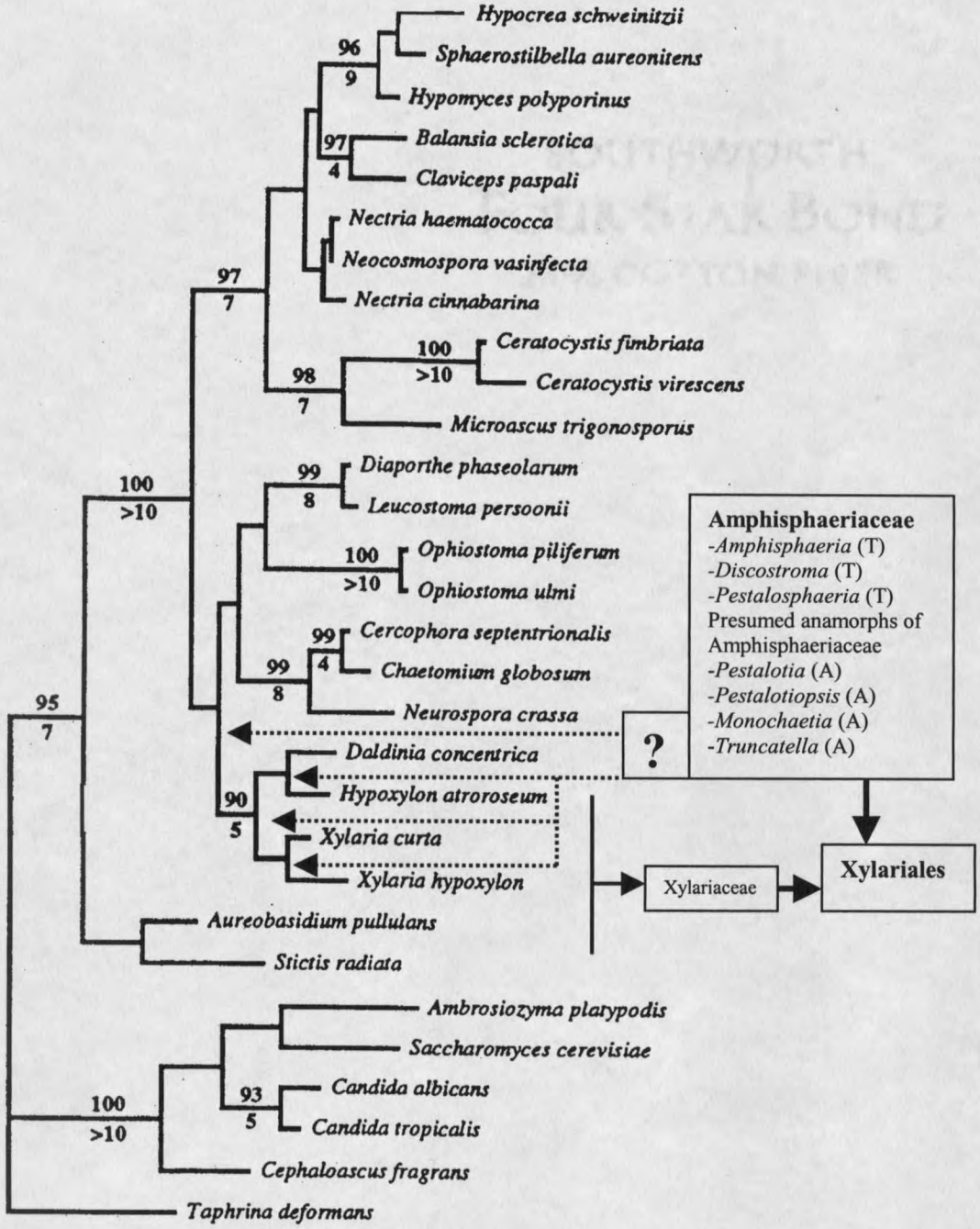


Figure 13. Single most parsimonious cladogram containing 30 taxa of Pyrenomycetes base on 18S rDNA analysis (modified from Spatafora, 1993). This phylogenetic tree shows where the family Xylariaceae is placed and where the family Amphisphaeriaceae might be. Both Xylariaceae and Amphisphaeriaceae are placed in the order Xylariales by Barr (1994). Bootstrap values are given above the nodes and decay indices are presented below their respective nodes. T=Teleomorph. A=Anamorph.

However, Eriksson (1999) disagreed with the classification by Kang *et al.* (1998) noting that the 5.8S r DNA and ITS2 sequences were too divergent to infer relationships at the order and family levels, and the numbers of nucleotides showing identity in those two regions were not sufficient for statistical significance. More conserved regions are needed for the placement analysis of Amphisphaeriaceae within an order.

In order to help resolve this problem, I studied a more conserved region and more informative sequence, which is the nuclear encoded small subunit ribosomal DNA (18S rDNA). This information would help provide nucleotide characters to resolve ambiguous classifications. The research undertaken for this study is a molecular phylogenic analysis of the 18S rDNA used as a basis to determine ordinal level ranking and placement of selected Amphisphaeriaceae (Figure 12 & 13). I tested the hypothesis that selected genera in Amphisphaeriaceae form a monophyletic group within the Xylariales, Pyrenomycetes. To analyze the phylogenetic relationships among genera in the family Amphisphaeriaceae, I sequenced thirteen species considered to be in Amphisphaeriaceae, and aligned these sequences with those of other Ascomycetes, which were generously given by J. W. Spatafora (Fourteen) and GenBank (Six), fourteen and six, respectively. Those from Spatafora consisted of genera of Pyrenomycetes from orders: Clavicipitales, Hypocreales, Microascales, Diaporthales, Sordariales and Xylariales (Figure 12 & 13). Maximum parsimony methods were used to infer the phylogenetic relationships of selected genera in the Amphisphaeriaceae. On this basis it was possible to resolve the issue as to placement of the Amphisphaeriaceae in the order Xylariales.

Materials and Methods

Fungal Strains

Fungal strains used in this experiment were either purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, or the Centraalbureau voor Schimmelcultures (CBS), Barrn, The Netherlands, or provided by Gary Strobel (Montana State University). Margaret E. Barr kindly provided *Discostroma fuscella* isolated from *Rosa* sp., Sydney, British-Columbia. Two species of *Discostroma tricellular* (T. Kobayashi 6-(4) or MAFF235878) and *Amphisphaeria* sp. (T. Kobayashi – 30 (5) or MAFF237482) were given by the Genebank Administration Division of national Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki, Japan. The species, original substrates, geographical origins and size in basepairs of 18S rDNA sequenced are listed in Table 12.

Fungal DNA Isolation

For DNA isolation, all fungi were grown in potato dextrose broth in 1.5 ml for 18 to 24 h at 23 ° C. The mycelium was harvested by centrifugation and washed twice with sterile ddH_2O. Total genomic DNA was extracted by the methods of Lee and Taylor (1990).

Amplification of 18S rDNA

The nucleotide base pair fragment of the 18S r DNA gene from each fungus was amplified via the polymerase chain reaction (PCR) as a single fragment with the primers UK4F (5' CYG GTT GAT CCT GCC RG) and UREV (5' GYT ACC TTG TTA CGA CTT). PCR was performed in a 50 µl reaction containing 0.1 µg genomic DNA, 0.4 µM each primer, 0.16 mM four dNTPs and 5u *Taq* polymerase (Promega) in a buffer of 10 mM tris-HCl (pH 9.0 at 25 °C), 50 mM KCl, 3 mM $MgCl_2$, 0.1 % Triton X-100. Amplification was for 30 cycles (45 sec at 94.5 °C, 45 sec at 53.5 °C, 90 sec at 72.5 °C).

Table 10. Primers used for determining 18S rDNA sequences of selected genera in Amphisphaeriaceae.

Primers	Sequences from 5' to 3'
UK4F	5' CYG GTT GAT CCT GCC RG 3'
NE32F1	5' CTG CCC TAT CAA CTT TCG 3'
NE32F2	5' CTC GTA GTT GAA CC T TGG 3'
NE32F3	5' AGA CTA ACT ACT GCG AAA GC 3'
NE32F4	5' GGA TTG ACA GAT TGA GAG C 3'
NE32F5	5' GTA AGC GCA AGT CAT CAG 3'
UREV	5' GYT ACC TTG TTA CGA CTT 3'
NE32R1	5' AGA CCC TGC CAT CGA AAG 3'
NE32R2	5' TTG GAG CTG GAA TTA CCG 3'
NE32R3	5' GCT TTC GCA GTA GTT AGT C 3'
NE32R4	5'GGT CTC GTT CGT TAT CGC 3'
NE32R5	5' AGC ACG ACG GAG TTT AAC 3'

Cycle Sequencing of 18S rDNA

The PCR product (10-40 ng) was sequenced using ABI prism BigDye terminator chemistry (Perkin-Elmer) and primers shown in Table 10. Sequencing conditions were 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. Isopropanol precipitation was used to purify extension products (Protocol ABI Prism BigDye Terminator Cycle

Sequencing Ready Reaction Kit, 1998). The reactions were resolved on an ABI prism 310 Genetic Analyzer (Perkin-Elmer). Nucleotide sequences were determined on both strands with Sequencher program (Gene Codes Corporation, Inc., 1995).

Sequence and Phylogenic Analysis

The sequences of 18S rDNA regions from different strains were first aligned by the multiple alignment program CLUSTAL W (Thomson *et al.*, 1994). The alignment was visually and manually adjusted afterward. Gap positions were introduced to the alignment to account for nucleotide insertion-deletion (indel) event. The data were analyzed using the parsimony heuristic search, neighbor joining, and bootstrap parsimony of the Phylogeny Using Parsimony Analysis (PAUP*) program version 4.0b4a (Swofford, 1999). Reference taxa were *Protomyces inouyei* (GenBank serial number D11377), *Taphrina wiesneri* (D12531), *Taphrina deformans* (U00971) and *Taphrina pruni-subcordatae* (AB000957). The 18S rDNA sequence set consists of 32 taxa including an outgroup, *Taphrinales* as used by Spatafora (Table 11). Those of other ascomycetes were generously given by J. W. Spatafora and GenBank, fourteen and six, respectively (Table 11). Those from Spatafora consisted of genera of Pyrenomycetes from orders: *Clavicipitales*, *Hypocreales*, *Microascales*, *Diaporthales*, *Sordariales* and Xylariales (Table 11). The stepwise addition option was used to find the most parsimonious bootstrap trees. Bootstrap by using the full heuristic search option of PAUP* was performed to calculate the robustness of each branch. The analysis was set with the following parameters: 100 bootstrap replicates (Felsenstein, 1985), with gaps

treated as missing data, tree bisection-reconnection branch swapping, and random sequence addition. All characters were weighted equally. The percentage of bootstrap replicates that confirmed each clade is indicated in the figures.

Table 11. List of additional taxa kindly given by J. W. Spatafora (1993) and * from GenBank.

Genera	Source	GenBank accession numbers
Clavicipitales		
Balansia sclerotica	ATCC 16582, J. W. Spatafora	U32399
Claviceps paspali	ATCC 13892, J. W. Spatafora	U32401
Hypocreales		
Hypomyces polyporinus	ATCC 46844, J. W. Spatafora	U32410
Hypocrea schweinitzii	C. T. Rogerson, J. W. Spatafora	L36986.
Microascales		
Ceratocystis fimbriata	T. C. Harrington C89, J. W. Spatafora	U32418
C. virescens	T. C. Harrington C89, J. W. Spatafora	U32410
Sordariales		
Chaetomium globosum	ATCC 44699, J. W. Spatafora	U20379
Neurospora crasa	J. W. Spatafora	X04917
Diaporthales		
Diaporthe phaseolarum	F. A. Uecker 458, J. W. Spatafora	L36985
Leucostroma persoonii	Berbee and Taylor, 1992	M83259
Xylariales		
Diatrype disciformis	CBS 197.49, J. W. Spatafora	U32403
Hypoxylon atroroseum	J. D. Rogers, J. W. Spatafora	U32411
*Hypoxylon fragiforme**	Kawasaki, H. and Miyado, S.	AB014045, AB014046
Xylaria curta	J. D. Rogers, J. W. Spatafora	U32417
Xylaria hypoxylon	ATCC 42768, J. W. Spatafora	U20378
Taphrinales		
*Taphrina deformans**	Berbee,M.L. and Taylor, J.W., 1993	U00971
*Taphrina wiesneri**	Nishida, H. and Sugiyama, J., 1993	D12531
*Taphrina pruni-subcordatae**	Sjamsuridzal, W., *et al.*, 1997	AB000957
*Protomyces inouyei**	Nishida, H., Blanz, P.A. and Sugiyama, J., 1993	D11377

Table 12. Selected Amphisphaeriaceae taxa for the 18S rDNA region sequenced and size in basepairs of 18S rDNA sequences. T and A stand for Teleomorph and Anamorph, respectively.

Taxa	Strain number	Original substrate and original geographic	Genbank Accession number	No. of basepairs
Amphisphaeria incrustans (T)	ATCC18007	Decayed *Acer* sp. wood, Quebec Canada	AF346547	1673
Amphisphaeria sp.° (T)	MAFF235878	*Diospyros kaki Thunberg* var. domestica *Makino,* Japan	AF346545	1760
Discostroma fuscella♠ (T)	10071	*Rosa* sp., Sydney, BC, Canada	AF346548	1767
Discostroma tricellular° (T)	MAFF237482	*Rhododendron indicum,* Japan	AF346546	1766
Monochaetia camelliae (A)	ATCC60625	*Camellia japonica*, New Zealand	AF346549	1765
Pestalosphaeria hansenii (T)	ATCC48245	*Pinus carribaea*, New Guinea	AF242846	1765
Pestalotia rhododendri (A)	ATCC24306	*Rhododendron* sp.	AF346551	1756
Pestalotia thujae (A)	CBS303.75	*Thuja occidentalis*, The Netherlands	AF346552	1758
*Pestalotiopsis jesteri** (A)	NG6b	*Frragraea bodenii*, New Guinea	AF346553	1754
*Pestalotiopsis microspora** (A)	NE32	*Taxus wallachiana*, Nepal	AF115396	1732
Seiridium cardinale (A)	ATCC52521	*Juniperus communis,* Italy	AF346556	1763
Seiridium. Unicorne (A)	ATCC48159	*Cryptomeria japonica,* New Zealand	AF346557	1743
Truncatella angustata (A)	ATCC96024	*Prunus avium* (cherry fruit), Washington, USA	AF346560	1754

* fungi isolated by Li & Strobel, Montana State University; ♠ fungus given by Barr, Canada; °fungi provided by Genebank Administration Division of national Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki, Japan. Those without *, ♠, ° were purchased from ATCC and CBS.

Results and Discussion

Sequence Analysis

Sequences ranging from 1673-1767 base pairs for 13 species typically placed in Amphisphaeriaceae, were used for this study (Table 12). These thirteen sequences plus the nineteen sequences, provided by J. W. Spatafora (1998) and GenBank (Table 11) were initially aligned and compared by CLUSTALW alignment program. Minor adjustments of the 32 sequences were done manually and visually. The data set consisted of 1800 alignable sites from thirty-two taxa; 1439 constant characters and 107 variable characters are parsimony-uninformative, while 255 characters are parsimony informative. There is 98-100 % similarity of 18S rDNA sequences of selected genera in Amphisphaeriaceae, which included *Amphisphaeria sp.*, *Amphisphaeria incrustans, Discostroma fuscella, Discostroma tricellular, Monochaetia camelliae, Pestalosphaeria hansenii, Pestalotia rhododendri, Pestalotia thujae, Pestalotiopsis jesteri, Pestalotiopsis microspora* (isolate NE32), *Seiridium cardinale, Seiridium unicorne,* and *Truncatella angustata.*

Initially, I started with a sequence of *Pestalotiopsis microspora* (isolateNE32) and, then searched for similar sequences by using the BlastSearch program of GenBank. The search hit a sequence of *Hypoxylon fragiforme.* A comparison of 18S rDNA sequences between *Pestalotiopsis microspora* (isolateNE32) and *Hypoxylon fragiforme* showed 98 % homology. There was 96-97 % homology of 18S rDNA region between *Pestalotiopsis microspora* (isolate NE32), *Hypoxylon atroseum, Diatype disciformis,*

Xylaria hypoxylon and *Xylaria curta* (data not shown) while there were 92-95 % homology of 18S rRNA gene between *Pestalotiopsis microspora* (isolate NE32) and *Clavicipitales* (*Balansia sclerotica* & *Claviceps paspali*), *Hypocreales* (*Hypomyces polyporinus* & *Hypocrea schweinitzii*), *Microascales* (*Ceratocystis fimbriata* & *Ceratocystis virescens*), *Diaporthales* (*Diaporthe phaseolarum* & *Leucostroma persoonii*), and *Sordariales* (*Chaetomium globosum* & *Neurospora crassa*). The high similarity of 18S rDNA sequences between Amphisphaeriaceae and Xylariaceae seems to indicate that the representatives of Amphisphaeriaceae in this study are very closely related to the studied genera in Xylariaceae.

Phylogenetic Analyses

Phylogenetic analysis based on 18S sequences indicated that selected genera in Amphisphaeriaceae, which included *Amphisphaeria sp., Amphisphaeria incrustans, Discostroma fuscella, Discostroma tricellular, Monochaetia camelliae, Pestalosphaeria hansenii, Pestalotia rhododendri, Pestalotia thujae, Pestalotiopsis jesteri, Pestalotiopsis microspora, Seiridium cardinale, Seiridium unicorne* and *Truncatella angustata* form a monophyletic group in the Xylariales, Pyrenomycetes. This result was strongly supported by 100 % bootstrap values and identical topologies of all dendrograms derived from the bootstrap analysis, neighbor joining, and strict consensus heuristic search (Figure 14, 15 & 16). There are two lines of evidence that suggest that the selected taxa should remain united in the family of Amphisphaeriaceae, which should be placed in Xylariales. First, cladistic analysis of the 18s rDNA sequences from selected genera in

Amphisphaeriaceae formed a sister clade to *Hypoxylon fragiforme*, a species in Xylariaceae suggesting two separate families (Figure 16). The other evidence is the high homology (96-98 %) of 18s rDNA sequences between selected Amphisphaeriaceae and Xylariaceae that supports placement of both in Xylariales. In addition, our results, inferred from 18S rDNA sequences, corroborate the morphological classifications which were done by Eriksson and Hawksworth, 1993; Barr, 1994; Hawksworth *et al.*, 1995, in which the Amphisphaeriaceae was classified in the order Xylariales. Under no circumstances does the analyses show support for the order Amphisphaeriales as proposed by Kang *et al.* (1998). Also, the transfer of Amphisphaeriaceae to Amphisphaeriales is not supported by the phylogenic analysis of 18S rDNA from this study. The selected taxa of Amphisphaeriaceae would have been transferred to Amphisphaeriales if all of them formed a monophyletic clade outside of Xylariaceae, and this did not occur. It would have taken several extra steps to force a placement of these selected Amphisphaeriaceae in a monophyletic clade outside of Xylariales (Figure 16).

Phylogenetic analysis of the 255 phylogenetically informative positions produced 82 equally most parsimonious cladograms. A strict consensus yielded the most parsimonious cladograms shown in Figure 14. Every topology from the strict consensus inferred tree matched up to all topologies from the neighbor-joining tree that were derived from an analysis employing either the Jukes-Cantor or the Kimura two-parameter model for nucleotide substitutions (Figure 15). These orders were Clavicipitales (*Balansia sclerotica* & *Claviceps paspali*), Hypocreales (*Hypomyces polyporinus* & *Hypocrea schweinitzii*), Microascales (*Ceratocystis fimbriata* & *Ceratocystis virescens*),

Diaporthales (*Diaporthe phaseolarum* & *Leucostroma persoonii*), Sordariales (*Chaetomium globosum* & *Neurospora crassa*) and Xylariales (*Diatype disciformis*, *Hypoxylon atroseum*, two strains of *Hypoxylon fragiforme*, *Xylaria hypoxylon*, *Xylaria curta*, *Amphisphaeria sp.*, *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne*, *Truncatella angustata*) (Figure 15). Together all fungal orders examined in this research formed clades identical to those reported by Spatafora *et al.*, (1993; Figure 12). Support for the strength of each clade in the parsimony and neighbor-joining tree was assessed by bootstrap analysis of 100 resampled data sets (Felsenstein, 1985). To facilitate discussion, I have defined these clades as groups 1-4 (Figure 16). Group 4 was divided into subgroups 4.1-4.8 (Figure 16).

Group 1 represents reference taxa, including *Protomyces inouyei*, *Taphrina wiesneri*, *Taphrina deformans* and *Taphrina pruni-subcordatae*. Monophyly of the Taphrinales clade is strongly supported by 100% bootstrapping. However, a clade of *Taphrina deformans* and *Taphrina pruni-subcordatae* received only 68% of the bootstrap value. The weak support of bootstrap may come from incorrect in-sequence alignment since I did not make manual and visual adjustment to these four sequences of reference taxa. It is reasonable that *Protomyces inouyei* did not group with *Taphrina* spp. because *Protomyces inouyei* is classified in Ascomycota, Taphrinomycetes, Protomycetales, Protomycetaceae, *Protomyces* while *Taphrina* spp. are classified in Ascomycota,

Taphrinomycetes, Taphrinales, Taphrinaceae, *Taphrina*. Both *Protomyces* and *Taphrina* genera are placed in the same class Taphrinomycetes.

Group 2 and 3 are genera in Pyrenomycetes, which were chosen as a basal clade for selected Amphisphaeriaceae in case selected Amphisphaeriaceae would have formed a monophyletic group or several separate groups outside Xylariales. These taxa are included in the cladistic analysis of selected Amphisphaeriaceae using 18S rDNA as an internal indication of reliable alignment. The results showed that the alignment in this analysis is quite reliable since all topologies with strong support in statistical bootstrapping are consistent to that of previous reports such as Spatafora *et al.*, (1993; Figure 12 & 13). The control clades include taxa sampled from the orders Clavicipitales, Hypocreales, Microascales (*Ceratocystis fimbriata* & *Ceratocystis virescens*), Diaporthales, and Sordariales. Group 2 is a super clade of Clavicipitales, Hypocreales and Microascales while group 3&4 are a super clade of Diaporthales, Sordariales and Xylariales. These two super clades received 92, and 59 % bootstrapping, respectively (Figure 16). Each pair of representative orders received bootstrap confidence levels at 99% for Clavicipitales, and 100% for Hypocreales, Microascales, Diaporthales and Sordariales, respectively. In the group 2, the cladogram shows that Clavicipitales is a monophyletic sister group to the Hypocreales. Moreover, genera in both orders Clavicipitales (*Balansia sclerotica* & *Claviceps paspali*) and Hypocreales (*Hypomyces polyporinus* & *Hypocrea schweinitzii*) are more closely related to each other than genera in Microascales.

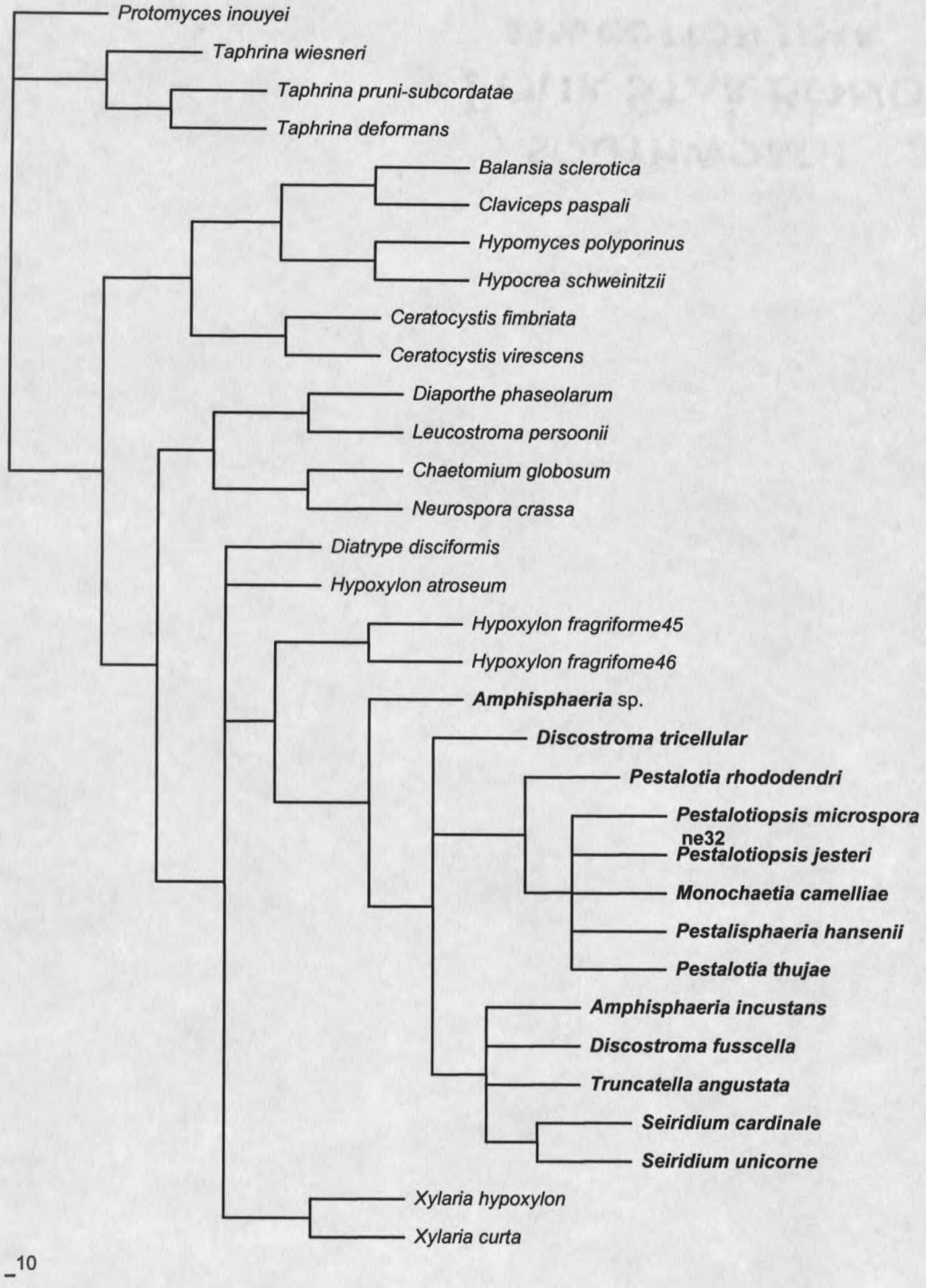


Figure 14. Neighbor-joining based on 18S rDNA analysis containing 32 taxa. The designated outgroups are *Taphrina deformans*, *Taphrina wiesneri*, *Taphrina pruni-subcordatae* and *Protomyces inouyei*. Bold = taxa of interest. Bar = 10 substitutions.

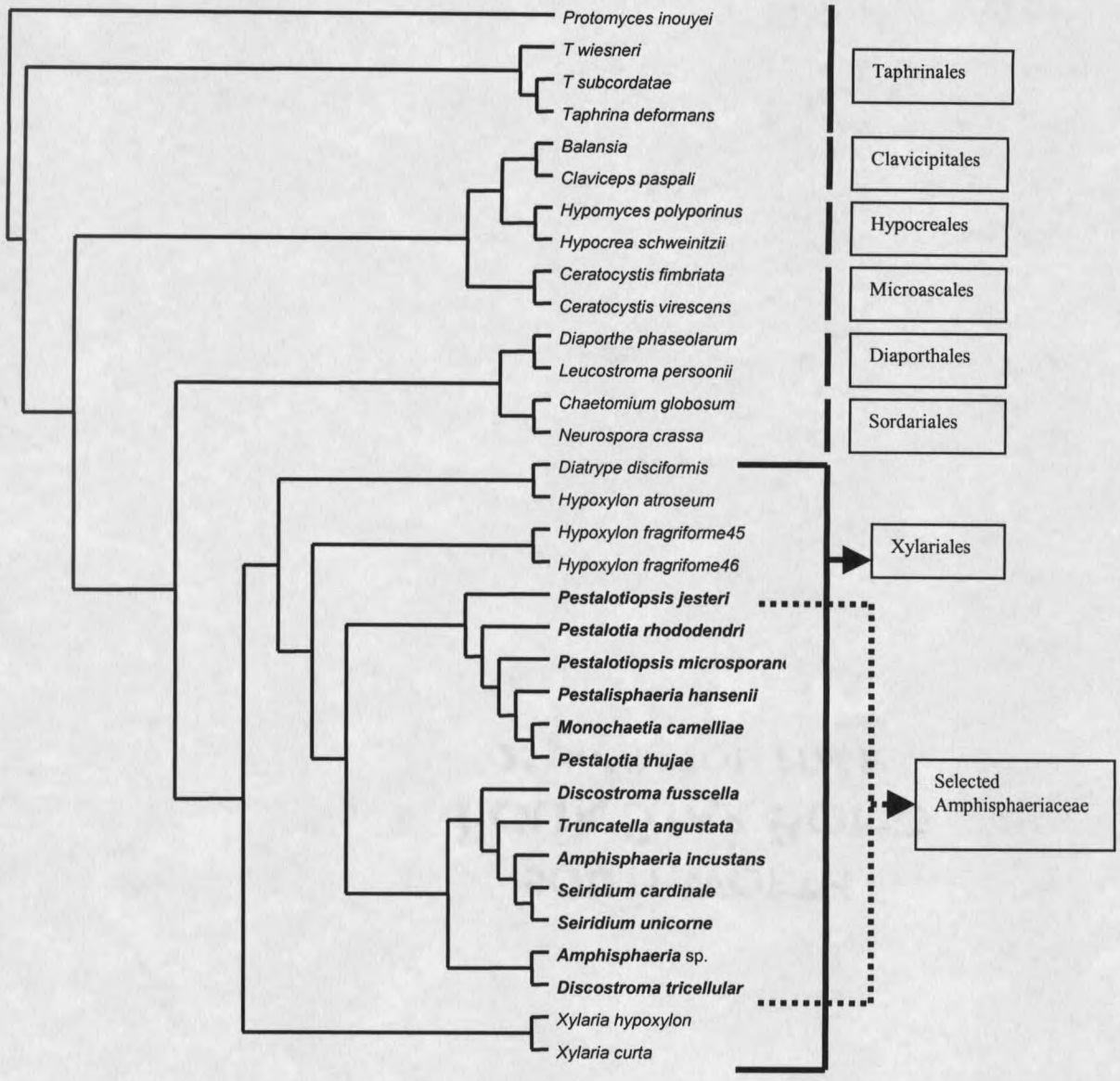


Figure 15. Strict consensus of 82 equally most parsimonious 18s rDNA cladograms shows selected Amphisphaeriaceae genera should be placed in Xylariales and still remain as family rank. Bold letters stand for teleomorph. Taphrinales is an outgroup.

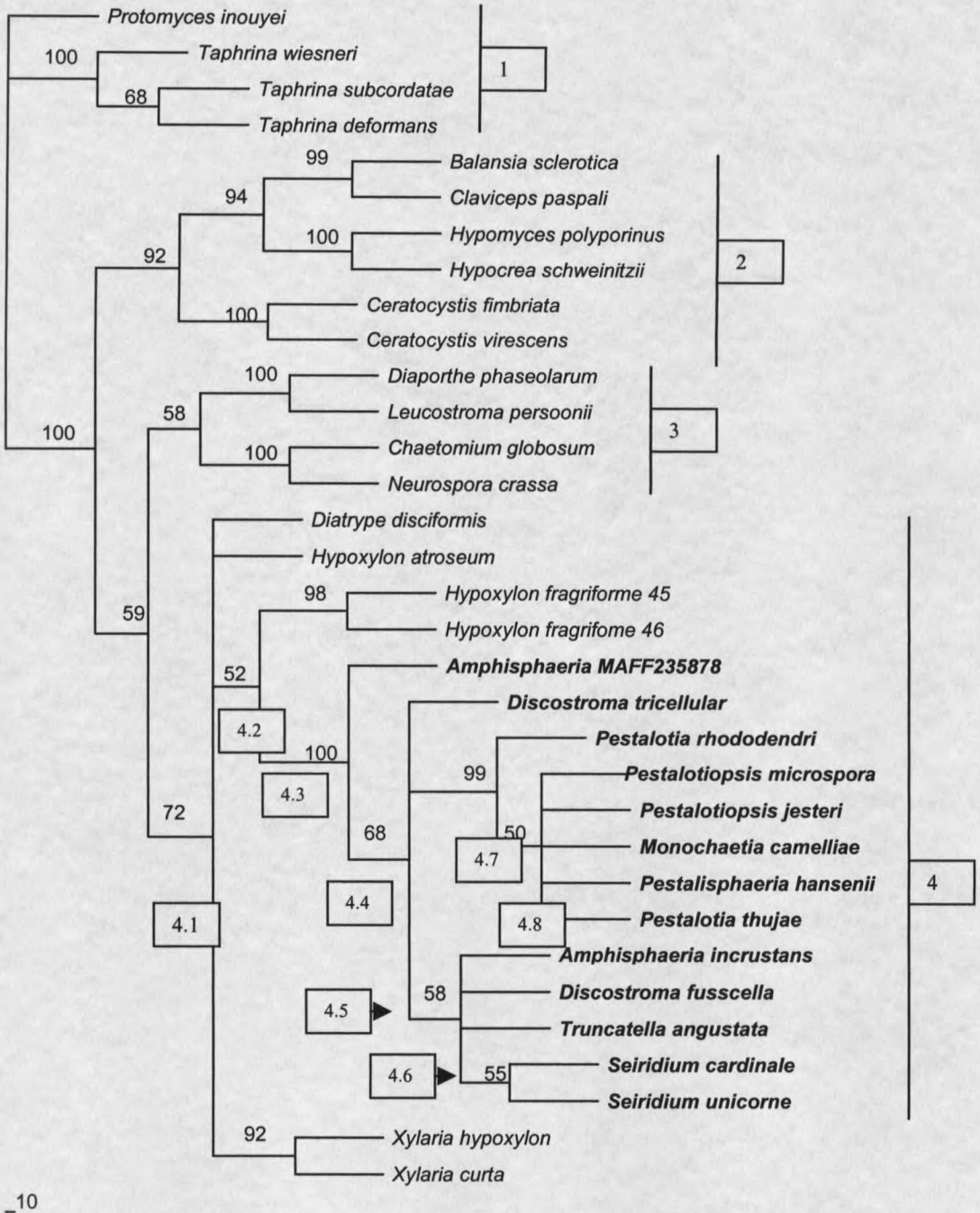


Figure 16. Maximum parsimony phylogram of 18S rDNA sequences for selected genera in Amphisphaeriaceae and other genera in Pyrenomycetes. Reference taxa are *Taphrina deformans*, *Taphrina wiesneri*, *Taphrina pruni-subcordatae* and *Protomyces inouyei*. Bootstrap confidence measures greater than 50 % from 100 replications are indicated at internodes. Nodes and clades are presented as numbers in the squares in the order that they are described in the text. Bar = 10 substitutions.

Group 3 is a weakly supported clade (bootstrapping 58%) composed of four species in two orders in Diaporthales and Sordariales. Diaporthales encompasses *Diaporthe phaseolarum* and *Leucostroma persoonii* while Sordariales encompasses *Chaetomium globosum* and *Neurospora crassa*. Both branches of these orders received high support, shown by 100 % bootstrapping (Figure 16). The cladogram showed that Diaporthales and Sordariales are closely related to the Xylariales, and all of them share the same common ancestor with 59 % bootstrap value (Figure 16). Although this internode was supported with a low confidence value, Spatafora *et al.* and Chen *et al.* (1993, 1999) mentioned that these three orders always form a super clade in Pyrenomycetes. Genera in Ophiostromatales may be required to polarize and raise the bootstrap value of this internode.

Group 4 represents Xylariales, which is composed of two large families, Amphisphaeriaceae and Xylariaceae. It is moderately supported as monophyletic (bootstrapping = 72 %; Figure 16 at internode 4.1). Genera in Xylariaceae include *Diatype disciformis*, *Hypoxylon atroroseum*, two strains of *Hypoxylon fragiforme*, *Xylaria hypoxylon* and *Xylaria curta*. The cladogram suggests that these two families are very closely related and derived from the same common ancestor. A morphological characteristic that is possibly congruent with the 18S rDNA gene tree of this topology is a type of conidiogenous cell in the anamorph state. All of the Xylariales representatives in this study are associated with anamorphs that have holoblastic conidiogenous cells (Bar, 1990 and Nag Raj, 1993). Furthermore, the conidiogenous cell of coelomycetous anamorphs of Amphisphaeriaceae and xylarialian anamorphs is most frequently

holoblastic (Barr, 1990; Alexopoulos, 1996). This is in contrast to other orders, which have a type of enteroblastic conidiogenesis (Alexopoulos, 1996).

At internode 4.2, two strains of *Hypoxylon fragiforme* (98 % bootstrapping) formed a monophyletic group with genera in Amphisphaeriaceae, including *Amphisphaeria* sp., *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne* and *Truncatella angustata*. The internode 4.2 received low support, confirmed by 52 % bootstrapping. Although the bootstrap value was quite low, the three inferred phylograms from three molecular phylogeny methods showed that the representatives of Amphisphaeriaceae in this study are very closely related to *Hypoxylon fragiforme* (Figure 14, 15 & 16). In addition the high homology (about 97-98 %) of 18S DNA sequences among these genera, provides other evidence to support the close relationship of taxa of representative Amphisphaeriaceae in this study to the species *Hypoxylon fragiforme* in Xylariaceae. It is interesting to note that *Hypoxylon fragiforme* is a common endophytic fungus that is usually isolated from beech, oak, willow and birch tress (Jong and Roger, 1972). *Hypoxylon fragiforme* was used as a model for a study of a host–specific recognition mechanism by (Chapela *et al.*, 1993). A novel HIV-1 protease inhibitor cytochalasin was isolated from fermentations of the fungus *Hypoxylon fragiforme* (ATCC20995) (Ondeyka *et al.*, 1992). Additional sequences from more species of *Xylaria*, *Daldinia* and especially *Hypoxylon* are needed to delineate families. The long-branch length of selected genera in Amphisphaeriaceae could be an indication

that the 18S rDNA sequences of these genera might be more primitive than the 18S rDNA sequence of *Hypoxylon fragiforme*. Morphological characteristics, required by Rogers (1979), show agreement with respect to this hypothesis. To quote Rogers (1979)

> "The presence of cellular appendages on immature ascospores, in some cases persisting on the mature spore, indicating that the ancestors of the Xylariaceae had two-celled ascospores."

Ascospores of Amphisphaeriaceae, *Discostroma* & *Pestalosphaeria*, and *Lepteutypa* contain two, three and four cells, respectively whereas ascospores of *Hypoxylon* and *Daldinia* are aseptate and associated with dehiscent perispores (an outer wall of ascospores, opening at maturity). Thus it may be possible that selected Amphisphaeriaceae taxa might be an ancestor to *Hypoxylon* and *Daldinia*. While *Daldinia* and *Hypoxylon* are typically placed in Xylariaceae with *Xylaria* having black carbonaceous stroma, many *Xylaria* spp. have a stroma that is corky or leathery, freshy or woody in consistency (Alexopoulos *et al.*, 1996).

Group 4.3 includes the representative genera in Amphisphaeriaceae, which are *Amphisphaeria* sp., *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne* and *Truncatella angustata*. Both anamorphs and teleomorphs are included. Monophyly of selected Amphisphaeriaceae received a robustness of 100 % bootstrapping (Figure 16). The phylogram from 18S rDNA analysis in this study showed that selected genera in Amphisphaeriaceae are a monophyletic group in Xylariales, and are closely related to *Hypoxylon fragiforme*, a genus in Xylariaceae. One morphological

characteristic that is possibly congruent with the 18S rDNA gene tree of the selected Amphisphaeriaceae clade is the conidiogenous cell of coelomycetous anamorphs of Amphisphaeriaceae. *Pestalotia* (anamorphs of *Pestalosphaeria*), *Pestalotiopsis* (an anamorphs of *Pestalosphaeria*), *Monochaetia*, *Truncatella*, *Seiridium* (anamorphs of *Lepteutypa*) and *Seimatosporium* (anamorphs of *Discostroma*) are all correlated with the annellidic type of holoblastic conidiogenous cell (Sutton, 1980). For xylariacious groups, particularly *Hypoxylon*, the type of conidiogenous cell is acropleurogenous (Alexopoulos *et al.*, 1996) suggesting two separate families in Xylariales.

In group 4.3, a branch separates *Amphisphaeria* sp. from *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne*, *Truncatella angustata*. The ascospores of the genus *Amphisphaeria* are typically hyaline; two celled with a smooth wall surface and the ascus apex is amyloid. The one-septate ascospores of *Amphisphaeria* sp. may explain why *Amphisphaeria* sp. was separated at the node 4.3 from genera exhibiting more than two septate ascospores. For example, the genus of *Pestalosphaeria* (anamorphs= *Pestalotia*, *Pestalotiopsis*, *Monochaetia*) produces two septate ascospores, *Discostroma* produces three to five septate ascospores (an anamorph = *Seimatosporium*), *Broomella* (anamorph = *Truncatella*) produces three septate ascospores, and *Lepteutypa* (anamorph = *Seiridium*) produces three septum ascospores (Bar, 1975). I could not find conidia of *Amphisphaeria* sp. on any of the cultures, which are available in Plant Pathology and Microbiology departments. It should also be noted

that the asexual state of *Amphisphaeria* sp. was not known by Barr, 1994. However, Nag Raj (1977) reported that the anamorphs of *Amphisphaeria argentinensis* is *Bleptosporium pleurochaetum*. The unknown asexual spore of *Amphisphaeria* sp. is another example that anamorphs and teleomorphs of Amphisphaeriaceae rarely occur at the same time when cultured on agar.

Group 4.4 is a weakly supported sub-internode (68 % bootstrapping). This group includes *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne* and *Truncatella angustata*. This analysis was unable to resolve some relationships within groups 4.4 to 4.9. The unresolved branches may be an explanation of why sequence-derived relationships are not congruent or consistent with morphological ones. For example, most conidia in these genera are fusiform, four or more cells with intermediate colored cells and hyaline cells at both end, with a varying number of appendages, (except for *Amphisphaeria incrustans*). *Amphisphaeria incrustans* conidia are dark brown 2-4 multi septate without any appendages at both ends. The morphological characteristics of ascospores are an unclear characteristic for determining generic level relationships in the Amphisphaeriaceae according to the 18S rRNA gene tree.

Group 4.5 includes *Discostroma tricellular*, *Amphisphaeria incrustans*, *Discostroma fusscella* and *Truncatella angustata*. *Discostroma fusscella Truncatella angustata*, *Seiridium unicorne* and *Seiridium cardinale*. *Discostroma tricellular*, which

produces three septate ascospores, should have clustered with an unresolved 4.5 node (bootstrap = 58 %; Figure 16), composed of *Amphisphaeria incrustans*, *Discostroma fusscella* and *Truncatella angustata*. *Discostroma fusscella Truncatella angustata*, *Seiridium unicorne* and *Seiridium cardinale*, have a sexual state with three septate ascospores whereas the sexual state of *Amphisphaeria incrustans*, which appeared within this sub-internode, is unknown. However, morphological characteristics of ascospores among *Truncatella angustata*, *Discostroma fusscella Truncatella angustata*, *Seiridium unicorne* and *Seiridium cardinale* are different. *Truncatella* sp. (an anamorph of *Broomella*) produces three septate brown ascospores with appendages at both ends and a nonamyloid apical ascus, whereas *Discostroma* sp. and *Seiridium* sp. (an anamorph of *Lepteutypa*) produce two and three septate brown ascospores with a smooth surface and has an amyloid apical ascus (Barr, 1975).

In the selected Amphisphaeriaceae clade, group 4.6 represents *Seiridium cardinale* and *Seiridium unicorne*. It is supported with the low bootstrap value 55 % (Figure 16). According to Graur and Li (2000), topologies in which all branches with low bootstrap values are collapsed into polytomies and a result of the collapsed tree is similar to the original inferred tree. Therefore, reducing the sub-internode 4.6 into the group 4.5 does not change any difference from the original inferred tree.

Group 4.7 (bootstrap = 99 %) is composed of *Pestalotia rhododendri* and an unresolved cluster of *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia thujae*, *Pestalotiopsis jesteri* and *Pestalotiopsis microspora* (NE32). All of the conidia of group 4.7 have three versicolored intermediate cells. The unresolved group 4.8

(bootstrap = 50 %; Figure 16) puts genus *Monochaetia camelliae* with *Pestalosphaeria hansenii*, *Pestalotia thujae*, *Pestalotiopsis jesteri* and *Pestalotiopsis microspora* (NE32). Since bootstrap values supports group 4.8 only 50 %, unresolved group 4.8 can be collapsed into the group 4.7. In addition, there is no clear delineation of *Monochaetia* from the other three genera, which are considered closely related to each other, accordingly to 18S rDNA data. There is also no morphological feature that tends to distinguish *Monochaetia camelliae* from other related genera. Although *Monochaetia camelliae* produces five-celled conidia with a single appendage at the apical hyaline cell while *Pestalosphaeria hansenii*, *Pestalotia thujae*, *Pestalotiopsis jesteri* and *Pestalotiopsis microspora* (NE32) produce five-celled conidia with two to three appendages at the apical hyaline cell and one appendage at the basal hyaline cell. It has been shown in chapter 2 that number of the appendages is not useful for distinguishing the difference between *Pestalotia* or *Pestalotiopsis* and *Monochaetia* (Bar, 1975).

The low percentage confidence intervals within the branch of *Pestalosphaeria*, *Pestalotia*, *Pestalotiopsis* and *Monochaetia* and the branch of *Amphisphaeria incrustans*, *Discostroma fusscella* and *Truncatella angustata* using the bootstrap analysis reaffirms the ambiguity of relationships between these species, and the inability of 18S rDNA to distinguish the differences at the generic level of certain genera in the selected Amphisphaeriaceae.

In summary, parsimony analysis of 18S rDNA sequences of selected genera in Amphisphaeriaceae including *Amphisphaeria* sp., *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria*

hansenii, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne*, *Truncatella angustata*, suggests that the selected genera in Amphisphaeriaceae are monophyletic forming a sister group to *Hypoxylon fragiforme*, a genus in Xylariaceae within the Pyrenomycetes. In addition parsimony analysis of 18S rDNA sequences revealed that the selected species in Amphisphaeriaceae should be classified in the order Xylariales which agrees with the morphological classification done by Eriksson and Hawksworth, 1993, Barr, 1994, Hawksworth *et al.*, 1995.

Unresolved inter-branches and uneven distribution of morphological features imply that 18S rRNA gene is not divergent enough to distinguish certain genera. Other regions or genes such as ITS 1&2 regions and mitochondrial DNA gene, which evolve faster than 18S rRNA gene, may diverge enough to analyze the differences among these species. It seems that for the analysis in this study the region of 18S rDNA sequences is more helpful in resolving phylogenetic relationships at or above the ordinal level. This result is consistent with other literature that the 18S rDNA sequence analysis generally produces distinct clades at the ordinal level (Bruns *et al.*, 1991; Spatafora *et al.*, 1993; Mitchell *et al.*, 1995). Thus other regions or genes such as ITS 1&2 spaces and mitochondria DNA gene, which evolve faster than the 18S rRNA gene, may be useful to resolve phylogenetic relationships among selected species of Amphisphaeriaceae.

Since Amphisphaeriaceae is a big and complex family, one may contest that the selected representatives of Amphisphaeriaceae in this phylogenetic study are not complete enough to be indicative of the whole taxonomy. Certainly any taxonomic

conclusion would be perfectly appropriate if all good representative species of Amphisphaeriaceae could be analyzed. Thus, this result should be interpreted within the limits of the given data set and not for the group as a whole. Further analyses of more taxa as well as analysis of different genes is required along with a more complete morphological approach for fully resolving relationships within the Amphisphaeriaceae. Thus, other regions or genes such as ITS 1&2 spaces and mitochondria DNA gene, which evolve faster than 18S rRNA gene, may be useful to resolve phylogenetic relationships among selected species of Amphisphaeriaceae.

It is important to understand the genetic relatedness of these endophytic fungi because most of them produce potentially antimicrobial compounds, which may be applied to pharmaceutical and agricultural uses. For example, the selected taxa *Pestalotiopsis jesteri* and *Pestalotiopsis microspora* are related because they produce jesterone and taxol compounds that can inhibit growth of oomycete pathogens. *Amphisphaeria sp.* and *Discostroma tricellular* are endophytes found in tropical regions, and come from the Genebank Administration Division, National Institute of Agrobiological Resources, Japan. Some of them are pathogens of important trees. *Monochaetia camelliae, Seiridium cardinale, Seiridium unicorne* and *Truncatella angustata,* which were purchased from American Type Culture Collection, USA, were recorded as pathogens of *Camelliae* (tea), *Cypress* and *Prunus* (cherry), respectively.

CHAPTER 4

A PHYLOGENETIC STUDY OF SELECTED *PESTALOTIOPSIS* SPECIES, AND RELATED GENERA IN AMPHISPHAERIACEAE USING THE ITS1 AND ITS2 REGIONS AND 5.8S rDNA

Introduction

A group of taxonomically related and presumed anamorphic genera of Amphisphaeriaceae are ubiquitous endophytes from temperate and tropical plants (Strobel, 1996 a & b). Despite an ongoing interest in these fungi because of their potential to yield pharmaceutical compounds, the classification of Amphisphaeriaceae remains confusing, particularly at the generic level because of their great diversity. The main confusion stems from disagreement among various authors regarding the criteria used to define and delimit the genera in this family. For example, Muller and Arx (1962 and 1973) delineated the family Amphisphaeriaceae as pyrenomycetes with a distinctive amyloid ring in the ascus apex and an ascostroma immersed under a clypeus. Muller and Arx classified genera in Amphisphaeriaceae, which are also included by other researchers, are listed in Table 13. Additional genera included in this family by Muller and Arx (1973) that are not included in other classifications are *Anisostomula*, *Apiospora*, *Apiothyrium*, *Cainiella*, *Chaetapiospora*, *Entosordaria*, *Exarmidium*, *Griphosphaeria*, *Hyponectria*, *Lejosphaerella*, *Monographus*, *Myelosperma*, *Mycothyridium*, *Pemphidium*, *Phragmidiscus*, *Physalospora*, *Pseudomassaria*, *Rousso*ëlla, *Seynesia* and *Vialaea*.

Barr separated genera within Amphisphaeriaceae by relying on morphological characteristics of the ascospores (e.g. intensity of pigmentation, presence or absence of wall ornamentation, appendage type, and septation). The basic characteristics of

ascospores of Amphisphaeriaceae accepted by Barr are radial symmetry, hyaline coloration, brown coloration (if distoseptate), the presence of one or more germ pores (if brown coloration is present) and cylindrical asci with or without amyloid ascus ring. Genera she included are *Amphisphaerella, Amphisphaeria*, *Broomella*, *Crassoascus*, *Discostroma*, *Dyrithium, Griphosphaerioma, Lepteutypa* and *Pestalosphaeria.*

In 1998, Kang *et al.* used molecular studies to verify the relationships between the genera in Amphisphaeriaceae by comparing the 5.8S rDNA and ITS2 sequences. The species used to verify the relationships between the genera in the Amphisphaeriaceae were *Amphisphaeria umbrina*, *Apioclypea livistonae*, *Atrotorquata lineata*, *Capsulospora* sp., *Clypeosphaeria mamillana*, *Cytoplea hysterioides*, *Discostroma tosta*, *Ellurema indica*, *Hypoxylon fragiforme*, *Lepteutypa cupressi*, *Myelosperma tumidum*, *Oxydothis frondicola*, *Pestalosphaeria elaeidis*, *Pestalotia palmarum*, *Rousso*ë*lla hysterioides, Rousso*ë*lla* sp. and *Xylaria hypoxylon*. Based on the 5.8S rDNA and ITS 2 sequences of those taxa, Kang *et al.* (1998; Figure 17) concluded that the family Amphisphaeriaceae is restricted to *Amphisphaeria*, *Discostroma*, *Ellurema*, *Lepteutypa*, *Pestalosphaeria*, and other genera possessing *Pestalotia*-like anamorphs.

Table 13 provides a comparison of genera classified as Amphisphaeriaceae by Muller and Arx (1973), Barr (1994), Kang *et al.* (1998) and Eriksson (1999 a&b). Genera that are consistently present in the four classification schemes are *Amphisphaeria*, *Discostroma* and *Lepteutypa*. Additional genera included in the three classifications done by Muller and Arx (1973) Barr (1994) and Eriksson (1999) are *Amphisphaerella* and *Broomella*.

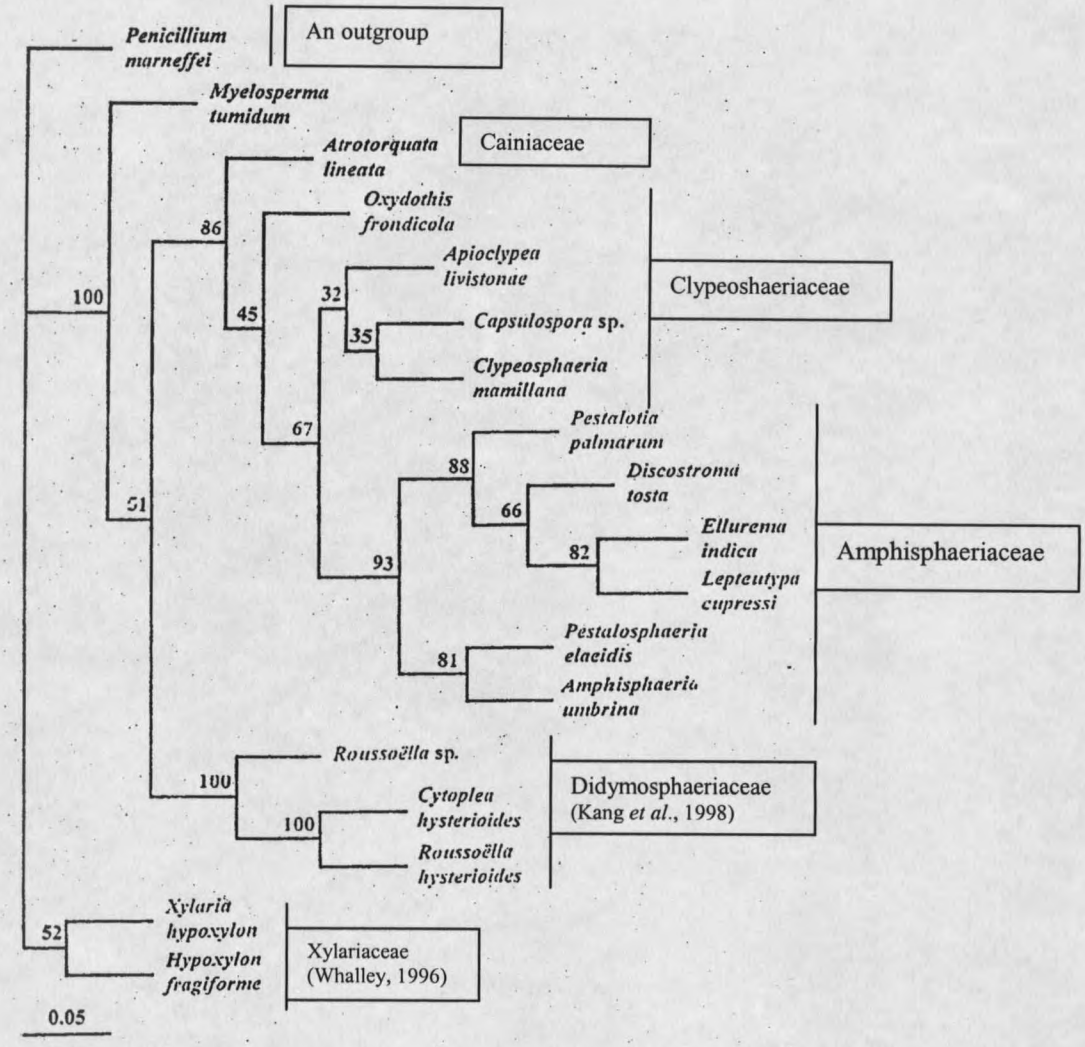


Figure 17. The neighbor-joining tree with bootstrap values above the branches using 5.8S rDNA and ITS2 spacer of 18 taxa (modified from Kang *et al.*, 1998). The bar indicates 5 substitutions per 1000 nucleotides. Didymosphaeriaceae is a family proposed by Kang *et al.* (1998) to accommodate genera *Roussoella* and *Cytoplea*.

Pestalosphaeria is a genus that was classified in the family by Barr (1994), Kang *et al.* (1998) and Eriksson (1983), and *Dyrithium* is a genus that was classified in the family Amphisphaeriaceae by Barr (1994) and Eriksson (1999). Kang *et al.* (1998) and Eriksson (1999) agreed that *Ellurema* should be put in the Amphisphaeriaceae. Genera

also accepted by Muller and Arx (1973) and Eriksson (1999) include *Blogiascospora*, *Ceriophora*, *Ceriospora*, *Chitonospora*, *Oxydothis*, *Urosporella* and *Xylochora*.

Table 13. Comparison of genera classified in Amphisphaeriaceae in several classification schemes.

Muller and Arx (1973)	Barr (1994)	Kang *et al.* (1998)	Eriksson (1999 a&b)
Amphisphaeria	*Amphisphaeria*	*Amphisphaeria*	*Amphisphaeria*
Discostroma	*Discostroma*	*Discostroma*	*Discostroma*
(*Griphosphaerioma*)	(*Griphosphaerioma*)	(*Griphosphaerioma*)	(*Griphosphaerioma*)
Lepteutypa	*Lepteutypa*	*Lepteutypa*	*Lepteutypa*
Amphisphaerella	*Amphisphaerella*	-	*Amphisphaerella*
Broomella	*Broomella*	-	*Broomella*
-	*Pestalosphaeria*	*Pestalosphaeria*	*Pestalosphaeria*
-	*Dyrithium*	-	*Dyrithium*
-	-	*Ellurema*	*Ellurema*
Blogiascospora	-	-	*Blogiascospora*
Ceriophora	-	-	*Ceriophora*
Ceriospora	-	-	*Ceriospora*
Chitonospora	-	-	*Chitonospora*
Oxydothis	-	-	*Oxydothis*
Urosporella	-	-	*Urosporella*
Xylochora	-	-	*Xylochora*

An additional twenty genera that were accepted only by Eriksson (1999 a&b, 2000 a&b) include *Arecophila*, *Ascotaiwania*, *Atrotorquata*, *Capsulospora*, *Clypeophysalospora*, *Flagellosphaeria*, *Frondispora*, *Iodosphaeria*, *Lanceispora*, *Leiosphaerella*, *Lindquistomyces*, *Manokwaria*, *Mukhakesa*, *Neobroomella*, *Neohypodiscus*, *Ommatomyces*, *Paracainiella*, *Pemphidium*, *Reticulosphaeria* and *Urosporellopsis*.

Comparison of the 18S rDNA regions of Amphisphaeriaceae in chapter 3 showed that for the genera tested, Amphisphaeriaceae is a monophyletic group and should be placed in Xylariales. However, 18S rDNA sequences of some teleomorphs and anamorphs of Amphisphaeriaceae were not divergent enough to delineate relationships among the genera within the family. These results suggest that molecular analysis of the ITS1-5.8S rDNA-ITS2 region in conjunction with morphological phenotypes might be a meaningful way to explore evolutionary relationships among selected genera in the Amphisphaeriaceae. To examine the evolutionary relationships among representative genera in Amphisphaeriaceae, the complete sequences of the internal transcribed spacers including 5.8S RNA gene from twenty-six taxa were investigated, aligned and assessed for inferred phylogenetic trees. Sequence data was examined for patterns in anamorphic and teleomorphic characteristics as well as geographic origin, hosts and nutritional modes. Molecular phylogeny, particularly ribosomal DNA sequences, has been proven to be a useful tool for the integration of teleomorph-anamorph or synanamorph systematics, as well as for the study of evolutionary relationships among organisms at different taxonomic levels (Arenal *et al.*, 2000; Burns *et al.*, 1991; Kuhls *et al.*, 1996 and Mitchell *et al.*, 1995). It seems that phylogenetic analysis of the ITS regions and 5.8S genes of selected Amphisphaeriaceae might also be able to distinguish genera or species within these sampled taxa.

The goal of this research is first to infer phylogenetic relationships of the selected species in Amphisphaeriaceae using ITS1-5.8S-ITS2 sequences. Second aim is to evaluate which morphological characteristics best reflect evolutionary relationships for

the selected taxa *Pestalosphaeria* sp. and presumed anamorph taxonomically related genera of Amphisphaeriaceae (e.g. *Pestalotia*, *Pestalotiopsis*, *Monochaetia*, *Seiridium* and *Truncatella*) which share a same common ancestor, as shown by 18S nucleotide data.

Materials and Methods

Fungal Strains

Fungal strains used in this experiment were either purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, or the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, or provided by Gary Strobel (Montana State University). Barr kindly provided *Discostroma fuscella* isolated from *Rosa* sp., Sydney, British-Columbia. Two species of *Discostroma tricellular* (T. Kobayashi 6-(4) or MAFF235878) and *Amphisphaeria* sp. (T. Kobayashi–30 (5) or MAFF237482) were donated by the Genebank Administration Division of the National Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki, Japan. The species, original substrates and geographical origins are listed in Table 14.

Fungal Growth Conditions for Formation of Conidia

Amphisphaeria incrustans, *Pestalotiopsis microspora* (NE-32) *Pestalosphaeria hansenii* (ATCC48245; Shoemaker and Simpson, 1981) and *Pestalotiopsis* isolate NG12-30 were cultured on water agar plates with sterile (γ-irradiated) carnation leaves, provided by the *Fusarium* Lab at Pennsylvania State University. The plates were incubated at 20 °

C under 12 hours light/12 hours dark cycles of fluorescent light for three weeks to produce acervuli and conidia.

Table 14. Species and genera selected for phylogenetic analysis of the ITS1 and ITS2 and 5.8S rDNA sequence data, with original substrate and geographical origin indicated.

Genus	Strain no.	Original substrate	Geographical Origin
Amphisphaeria incrustans (T)	ATCC18007	Decayed Acer sp. wood	Quebec, Canada
Amphisphaeria sp. (T)	T. Kobayashi 6-(4) MAFF235878	*Diospyros kaki thunberg var. domestica Makino* (Ebony family)	Japan
Discostroma fuscella (T)	From fragment 10071	*Rosa sp.*	Canada
Discostroma tricellular (T)	T. Kobayashi –30 (5) MAFF237482	*Rhododendron indicum Sweet* (leaf spot)	Japan
Monochaetia camelliae (A)	ATCC60625	*Camellia japonica* (Tea)	New Zealand
Monochaetia sp. (A)	Isolate #162	*Taxus baccata*	China
Pestalosphaeria hansenii (T)	ATCC48245	*Pinus carribaea*	New Guinea
Pestalotia rhododendri (A)	ATCC24306	*Rhododendron sp.*	Italy
Pestalotia thujae (A)	CBS303.75	*Thuja occidentalis*	Netherlands
Pestalotiopsis funereoides (A)	CBS175.25	*Juniperus* sp. *and Pinus* sp.	England
Pestalotiopsis jesteri (A)	6m, 6t	*Fragraea bodenii*	New Guinea
Pestalotiopsis neglecta (A)	CBS200.65	*Taxus baccata*	U.K.
Pestalotiopsis microspora (A)	CBS171.26	*Royena lucida*	Italy
Pestalotiopsis microspora (A)	CBS364.54	Bath Towel at Florida beach	USA
Pestalotiopsis microspora (A)	NE32	*Taxus wallachiana*	Nepal
Pestalotiopsis microspora (A)	WT98	*Taxus wallachiana*	Nepal
Pestalotiopsis sp. (A)	NG-1230	Moreabina	New Guinea
Seiridium cardinale (A)	ATCC52521	*Juniperus communis* (Cypress Family)	Italy
Seiridium unicorne (A)	ATCC48159	*Cryptomeria japonica* (Cypress Family)	New Zealand
Seiridium sp. (A)	Isolate 51	*Taxus wallachiana*	Nepal
Truncatella angustata (A)	ATCC96024	*Prunus avium*	USA (WT)
*Lepteutypa cupressi** (T)	IMI052255	*Cypress* sp.	Australia
*Pestalosphaeria elaeidis** (T)	IMI061175	*Elaeidis guineensis*, Palm	Nigeria
*Pestalotia palmarum** (A)	ATCC10085	*Cocos* sp. or coconut palm	India

ATCC = American Type Culture Collection. CBS = Centraalbureau voor Schimmelcultures. HKUCC = The University of Hong Kong Culture Collection. IMI = International Mycological Institute. * Genera for which ITS1, ITS2 and 5.8S rDNA sequence was obtained from GenBank. A and T in the parenthesis stand for anamorph and teleomorph, respectively.

Fungal Growth Conditions for Formation of Perithecia and Ascospores

Pestalotiopsis microspora (NE32) and *Pestalosphaeria hansenii* were cultured on water agarose plates containing 1.5 % agarose with sterile yew leaves (Metz *et al.*, 2000) while two species of *Amphisphaeria* sp. and *Discostroma tricellular* were cultured on potato dextrose agar. Incubation was at 20 ° C under 12 hours light/12 hours dark cycles of fluorescent light for three to four weeks to produce perithecia and ascospores (Metz *et al.*, 2000). The light source was a 20 W cool-white fluorescent light (General Electric) and the intensity was 25 $\mu E\ m^{-2}\ S^{-1}$ at the surface of the agarose plates (Metz *et al.*, 2000).

Microscopic Observation and Measurement of Asexual and Sexual spores

For identification purpose, three sets of fifteen conidia, pycnidia, ascospores, ascus, paraphysis and perithecia produced by *Pestalotiopsis microspora* (NE32) and *Pestalosphaeria hansenii* ATCC48245 were examined and measured (length and width) under a light microscope (Zeiss) at a magnification 200X, 400X and 630X. Three sets of fifteen conidia and pycnidia produced by *Pestalotiopsis* isolate NG1230 were examined in the same manner. Raw data (not shown) for each characteristic was used to calculate average sizes. These features were: conidial dimensions, mean of conidium length/width ratio, length of three median cells, apical appendage number, basal appendage number, and apical and appendage length. Conidia of *Amphisphaeria incrustans* and ascospores

of *Amphisphaeria* sp. and *Discostroma tricellular* were observed and photographed under the light microscope camera (Zeiss, model MC 80).

Fungal DNA Isolation

For DNA isolates, all fungi were grown in potato dextrose broth in 1.5 ml for 18 to 24 h at 23 ° C. The mycelium was harvested by centrifugation and washed twice with sterile ddH_2O. Total genomic DNA was extracted by the methods of Lee and Taylor (1990).

Amplification of ITS Regions and 5.8S rDNA

The ITS regions of each fungus was amplified using PCR and the universal ITS primers ITS5 (5' GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5' TCCTCCGCTTA TTGATATGC) (White *et al.*, 1990). PCR was performed in a 50 µl reaction containing 0.1 µg genomic DNA, 0.4 µM each primer, 0.16 mM four dNTPs and 5u *Taq* polymerase (Promega) in a buffer of 10 mM tris-HCl (pH 9.0 at 25 ° C), 50 mM KCl, 3 mM $MgCl_2$, and 0.1 % Triton X-100. PCR cycling conditions consisted of denaturation at 94 ° C for 1.5 min, annealing at 55 ° C for 2.5 min, and extension at 72 ° C for 3 min for 40 cycles, with a final extension at 72 ° C for 10 min (Willits *et al.*, 1999). The PCR products were gel purified and desalted using the QuickStep PCR purification kit (Edge Biosystems).

Cycle Sequencing of ITS Regions and 5.8S rDNA

10-40 ng of PCR product was sequenced using ABI prism BigDye terminator chemistry (Perkin-Elmer) and primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3'), ITS2 (5' GCT GCG TTC TTC ATC GAT GC 3'), ITS3 (5' GCA TCG ATG AAG AAC GCA GC 3'), ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3'). Sequencing conditions were 25 cycles of 96 ° C for 10 sec, 50 ° C for 5 sec and 60 ° C for 4 min. Isopropanol was used to precipitate and purify extension products in microcentrifuge tubes (Protocol ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, 1998). The final isopropanol concentration was 60 ± 5 %. For example, add 80 µl of 75% isopropanol was added into 20 µl of extension products. Extension products tubes were left at room temperature for exactly 15 minutes to precipitate the extension products. After the precipitation, the extension products were centrifuged at 3000x g. with a Eppendorf Centrifuge 5804 for 30 minutes. The supernatant was discarded immediately by inverting the microcentrifuge tubes immediately onto paper towels. The precipitates were dried by placing the inverted microcentrifuge tubes with the towel into the Eppendorf Centrifuge 5804 and spun at 700x g. for 2 minutes. The reactions were resuspended in template suppression reagent (TSR) and resolved on an ABI prism 310 Genetic Analyzer (Perkin-Elmer). Nucleotide sequences from both strands were merged with Sequencher program (Gene Codes Corporation, Inc., 1995). The sequences were submitted to GenBank (accession numbers shown in Table 15).

Sequence and Phylogenetic Analysis

The sequences of ITS1, 5.8S and ITS2 regions from different strains were aligned using both the multiple alignment program CLUSTAL W (Thomson *et al.*, 1994) (IntelliGenetics, Inc., Mountain View, California), and manually aligned afterward.

Phylogenetic analysis of the aligned ITS1, 5.8S rDNA and ITS2 sequences was performed using the maximum parsimony analysis of the Phylogeny Using Parsimony Analysis (PAUP*) program version 4.0b4a (Swofford, 1999). The phylogenetic analysis was performed on twenty-six taxa, including reference taxa. These twenty-six species were *Xylaria mali*, *Xylaria hypoxylon*, *Amphisphaeria incrustans* (Anamorph (A) = *Dendryphiopsis atra*), *Amphisphaeria* sp., *Discostroma fuscella*, *Discostroma tricellular* (A = *Seimatosporium azaleae*), *Pestalosphaeria hansenii* (A = *Pestalotiopsis microspora*), *Lepteutypa cupressi* (A = *Seiridium unicorne*), *Monochaetia camelliae* (A), *Monochaetia* isolate162 (A), *Pestalotia rhododendri* (A), *Pestalotia thujae* (A), *Pestalotiopsis jesteri* (isolate 6m; A), *Pestalotiopsis jesteri* (isolate 6t; A), *Pestalotiopsis funereoides* (A), *Pestalotiopsis microspora* (CBS364.54; A), *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE32; A), *Pestalotiopsis microspora* (WT98; A), *Pestalotiopsis neglecta*, *Pestalotiopsis* isolate NG1230 (A), *Seiridium cardinale* (A), *Seiridium unicorne* (A), *Seiridium* isolate 51 (A), *Truncatella angustata* (A), *Pestalosphaeria eleaidis* (A), and *Pestalotia palmarum* (A). Reference taxa were Xylariaceae: *Xylaria mali* (GenBank accession number AF163040) and *Xylaria hypoxylon* (GenBank accession number AF163035). The full heuristic search was used

to find the most parsimonious trees. Bootstrap by using the heuristic search option of PAUP* was performed to calculate the robustness of each branch. The analysis was set as the following parameters: 100 bootstrap replicates, with gaps treated as missing data, tree bisection-reconnection branch swapping, and random sequence addition (Felsenstein, 1985). All characters were weighted equally. The percentages of bootstrap replicates that confirmed each clade are indicated in the figures.

Results and Discussion

Sequence Analysis

The ITS1-5.8S-ITS2 regions were used for phylogenetic analysis of selected genera in Amphisphaeriaceae because these regions have been successfully used for resolving phylogenetic relationships of fungi at the inter- and intraspecific level (Lee and Taylor, 1992; Egger and Sigler, 1993). Both strands of a fragment containing part of the 3′-end of the 18S rDNA, ITS1, 5.8S, ITS2 and the 5′-end of the 28S rDNA were sequenced. The length of PCR products containing part the of 3′-end of the 18S rDNA, ITS1, 5.8S, ITS2 and the 5′-end of the 28S rDNA are 580-645 base pairs in length. Boundaries of the ITS1 and ITS2 regions were determined by comparison with published sequences of the ITS region (Arenal *et al.*, 2000, Willits and Sherwood, 1999). The last five base pairs of the 18S rRNA gene are "5′ CATTA 3′", and the first six base pairs of the 28S r RNA gene are "5′ TTGACC 3′". The first seven base pairs and the last six base pairs of the 5.8S rDNA sequence of selected Amphisphaeriaceae, *Xylaria mali* and

Xylaria hypoxylon, are "5′ AAACTTT 3′" and "5′ TCATTT 3′", respectively. The sizes of the ITS1-5.8S-ITS2 regions of selected Amphisphaeriaceae are 466-521 base pairs (Table 15). The amplification products of the ITS1 region of sampled taxa in Amphisphaeriaceae ranged from 188 to 195 base pairs in length, except for, *Pestalotiopsis microspora* CBS 364.54 and *Pestalotiopsis* isolate NG1230, which had 142 base pairs each (Table 15).

Table 15. Size in base pairs of the ITS1, 5.8S and ITS2 sequences for selected taxa of Amphisphaeriaceae.

Species	ITS1	5.8S	ITS2	Total	GenBank
Amphisphaeria incrustans	155	158	154	467	AF377283
Amphisphaeria sp.	188	158	154	500	AF375998
Discostroma fuscella	192	158	156	506	AF377284
Discostroma. tricellular	190	158	155	503	AF377285
Monochaetia camelliae	192	158	166	516	AF377286
Monochaetia isolate162	188	158	167	513	AF377287
Pestalosphaeria hansenii	194	158	166	518	AF377290
Pestalotia rhododendri	194	158	167	519	AF377294
Pestalotia thujae	193	158	168	519	AF377295
Pestalotiopsis funereoides	195	158	168	521	AF377289
Pestalotiopsis jesteri (6b)	190	158	165	513	AF377282
Pestalotiopsis jesteri (6m)	190	158	165	513	AF377282
Pestalotiopsis jesteri (6t)	191	158	165	514	AF377282
Pestalotiopsis microspora (CBS171.26)	191	158	165	514	AF377291
Pestalotiopsis microspora (CBS364.54)	142	158	162	462	AF377292
Pestalotiopsis microspora (NE32)	194	158	167	519	AF377288
Pestalotiopsis microspora (WT98)	194	158	165	517	AF377296
Pestalotiopsis microspora (NG12-30)	142	158	166	466	AF377301
Pestalotiopsis neglecta	194	158	166	518	AF377293
Seiridium *cardinale*	195	158	156	509	AF377298
Seiridium unicorne	193	158	156	507	AF377299
Seiridium isolate51	191	158	155	504	AF377297
Truncatella angustata	193	158	153	504	AF377300
*Lepteutypa cupressi**	199	153	156	508	AF009817
*Pestalosphaeria elaeidis**	163	156	193	512	AF009815
*Pestalotia palmarum**	161	156	155	472	AF009818

* represents genera for which ITS1 and ITS2 and 5.8S rDNA sequences were obtained from GenBank.

The ITS2 amplification products of sampled taxa in Amphisphaeriaceae contained 154-168 base pairs (Table 15). Thus the ITS2 region was shorter than the ITS1 by about 10 base pairs for all the species selected. Most selected Amphisphaeriaceae taxa have 158 base pairs in the 5.8S rDNA region, except, *Lepteutypa cupressi*, *Pestalosphaeria elaeidis* and *Pestalotia palmarum*, which had 153, 156 and 156 base pairs, respectively (Table 15).

The sequences of the 5.8S rRNA gene and the flanking ITS1 and ITS2 regions of *Amphisphaeria incrustans* (an anamorph= *Dendryphiopsis atra*) were excluded from the studied alignment because its sequence aligned poorly with the other strains. About 49 % of nucleotide base pairs of *Amphisphaeria incrustans* matched sequences of ITS1&2 and 5.8S region other strains. The low percentage of identity of ITS1&2 and 5.8S rDNA sequences and the differences in conidial characteristics between *Amphisphaeria incrustans* and the other amphisphaeriacious species suggest that *A. incrustans* should not be classified in the family Amphisphaeriaceae. *A. incrustans* produces brown 2-4 septate conidia without appendages (Figure 18A) in contrast to *Pestalotia*, *Pestalotiopsis*, *Seimatosporium* and *Truncatella* that produce conidia with apical and basal appendages. Unlike the conidia of other selected Amphisphaeriaceae, the conidia of *A. incrustans* are generated from acropleuroblastic conidiogenesis cells having no pycnidium or acervulus covering. In addition, Nag Raj (1993) found that an anamorphic state of *Amphisphaeria* was *Bleptosporium* sp. (Figure 18B). Thus, it would make more sense for *A. incrustans* to be identified as Xylariaceae due to the 90 % similarity of 18S rDNA sequence against the selected genera of Amphisphaeriaceae. It is possible that this organism has been

misidentified in the past since the teleomorph form of fungi rarely appears at the same time as the anamorph form. If the sexual stage of *A. incrustans* could be observed on culture media in the lab, such morphological evidence would provide more information about the relationship between this species and selected Amphisphaeriaceae or other Xylariaceae.

Figure 18. A. Acropleuroblastic conidiogenesis cell with young three-septate conidia of *Amphisphaeria incrustans* (anamorph = *Dendryphiopsis atra*). B. Conidiogenous cells of *Amphisphaeria* sp. (*Bleptosporium* sp. = anamorph) with annellidic holoblastic conidiophores (arrow) (modified from Nag Raj, 1992).

The remaining 26 species were: *Xylaria mali*, *Xylaria hypoxylon*, *Amphisphaeria* sp., *Discostroma fuscella*, *Discostroma tricellular*, *Pestalosphaeria hansenii*, *Lepteutypa cupressi*, *Monochaetia camelliae*, *Monochaetia* isolate162, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri* (isolate 6m), *Pestalotiopsis jesteri* (isolate 6t), *Pestalotiopsis funereoides*, *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE-32), *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis* isolate NG1230, *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolate51, *Truncatella angustata*, *Pestalosphaeria eleaidis*

and *Pestalotia palmarum*. All of these sequences were aligned with the CLUSTAL W (Thomson et al., 1994). Minor adjustments of the alignment were done manually. As can be seen from the aligned data set of the whole ITS1-5.8S-ITS2 regions of 26 sampled genera (Appendix B), it can be seen that most of the differences among 26 taxa are due to 41 % (approximately indel 83 base pairs from the whole ITS1 195 base pairs) of insertions and deletions at the position sites 61-144 in ITS1 region from five species: *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis* isolate NG12-30, *Pestalosphaeria eleaidis*, and *Pestalotia palmarum*. Of the variable positions in the ITS2 insertions and deletions about 10% occurred at site positions 41-63, 174-181 and 191-200. Without the inclusion of these four problematic species, the lengths of the sequences are quite homogeneous for the remaining genera of Amphisphaeriaceae used in this study. The two outgroup species *Xylaria mali* and *Xylaria hypoxylon* have 70 % homology of the 5.8 S rRNA gene and the flanking ITS1 and ITS2 regions with the selected Amphisphaeriaceae. The high degree of conservation of the ITS2 compared with the ITS1 region among the studied genera is quite obvious. Similar results have been reported in the study of sensu strictu Amphisphaeriaceae (Kang *et al.*, 1999), and a study of *Hypoxylon* and related genera in Xylariaceae (Sanchez-Ballesteros *et al.*, 2000). Thus the lower degree of conservation in the ITS1 compared with the ITS2 could be a general phenomenon in the Xylariales. Expectedly, the 5.8S rRNA gene was completely conserved among all selected genera of Amphisphaeriaceae because the 5.8S rRNA gene is transcribed into RNA molecules that form part of the ribosome, a critical structure.

Phylogenetic Analysis

Molecular data were derived from the whole ITS1 & 2 and 5.8S rDNA regions of the sequence alignment of 26 taxa. The species were of *Xylaria mali*, *Xylaria hypoxylon*, *Amphisphaeria* sp., *Discostroma fuscella*, *Discostroma tricellular*, *Pestalosphaeria hansenii*, *Lepteutypa cupressi*, *Monochaetia camelliae*, *Monochaetia* isolate162, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri* (isolate 6m), *Pestalotiopsis jesteri* (isolate 6t), *Pestalotiopsis funereoides*, *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE-32), *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis* isolate NG12-30, *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolate 51, *Truncatella angustata*, *Pestalosphaeria eleaidis* and *Pestalotia palmarum*. The number of parsimony-informative characters was 163 of 549. There were many topologies from the bootstrap analysis using the heuristic search option, but only five of them were considered because each topology gave the tree length of 450 or less, and showed reasonable topologies for the inferred tree. Tree length (*TL*), consistency index (*CI*), and retention index (*RI*) were from maximum parsimony analyses using the heuristic search. Options are described in materials and methods. These tree statistic values are described for each possible maximum parsimony (MP) tree in Table 16. Tree length values for the five possible MP tree are 447, 450, 445, 444 and 441, respectively. Therefore, tree number 5 is the maximum parsimony (MP) tree because it has the smallest tree length.

Table 16. Tree length, consistency index, retention index and bootstrap values in each group for five possible maximum parsimony trees.

MP statistic tree values	Tree number				
	1	2	3	4	5
Tree length	447	450	445	444	441
Consistency index	0.7405	0.6649	0.7348	0.7432	0.7392
Retention index	0.8024	0.7905	0.8037	0.8061	0.8024
Group	Bootstrapping at each group				
Group1	75	98	75	75	72
Group 2	80	83	67	74	81
Group 3	100	100	99	100	100
Group 4	98	97	97	96	97
Group5	100	100	100	100	100

All topologies of tree number 1 are identical to tree number 5, but tree number 5 has bootstrapping at each group higher than tree number 1 (Table 16). Moreover the topology of tree number 5 is not different from trees obtained by distance methods (data not shown) when the entire ITS1-5.8S-ITS2 sequences were used. The consistency index is a value which measures the extent of homoplasy (backward and parallel substitutions; Nei and Kumar, 2000). The *CI* values for tree number 1, 2, 3, 4, and 5 were 0.7405 (0.74), 0.6649 (0.66), 0.7348 (0.73), 0.7432 (0.74) and 0.7392 (0.74), respectively. Tree number 2 has the lowest *CI*, and, it is therefore not well supported by the nucleotide configuration. The differences in *CI* between tree number 1, 3, 4, and 5 are very small. This indicates that the nucleotide configuration is supportive for tree numbers 1, 3, 4, and 5 under the maximum parsimony principle. By contrast, the *RI* values for the five trees were 0.8024, 0.7905, 0.8037, 0.8061 and 0.8024, respectively. These values are

negatively correlated with tree length (Nei and Kumar, 2000). Trees with high RI values (tree #1, 3, 4 and 5) have most of the homoplasy occurring down deep in the phylogenetic tree. A tree with a low RI, which is a tree number 2, has most of the homoplasy occurring towards the tips of the tree. Thus, parsimony analysis of the whole ITS1-5.8S-ITS2 region from 165 parsimonious informative characters using bootstrap heuristic search provided the true MP topology of tree having retention index 0.8024 and consistency index 0.7392.

Parsimony analysis of the whole ITS1-5.8S-ITS2 regions with 100 replicates of bootstrap analyses revealed a monophyletic clade for selected genera of Amphisphaeriaceae in this study. It is strongly supported by 100 bootstrapping values (Figure 19). The characteristic that best indicates that these sampled Amphisphaeriaceae come from the same ancestor is the presence of an annellidic type of conidiogenesis cell producing asexual spores. Sutton (1980) classified the genera *Pestalotia* (anamorph of Pestalosphaeria), *Pestalotiopsis* (anamorph of *Pestalosphaeria*), *Truncatella* (presumed anamorph of *Broomella*), *Monochaetia* (presumed anamorph of *Hymenopleella*), *Seiridium* (anamorph of *Lepteutypa*), and *Seimatosporium* (anamorph of *Discostroma*) in the annellidic Blastostromatineae section. The majority of genera in this section are correlated with the annellidic type of conidiogenous cell in an acervulus, textular angularis cell type of acervuli and pigmented conidia (Sutton, 1980). A single most parsimonious tree of 441 steps was divided into five groups to facilitate discussion (Figure 19). The following results and discussion focus on the taxonomic significance of groups one through five.

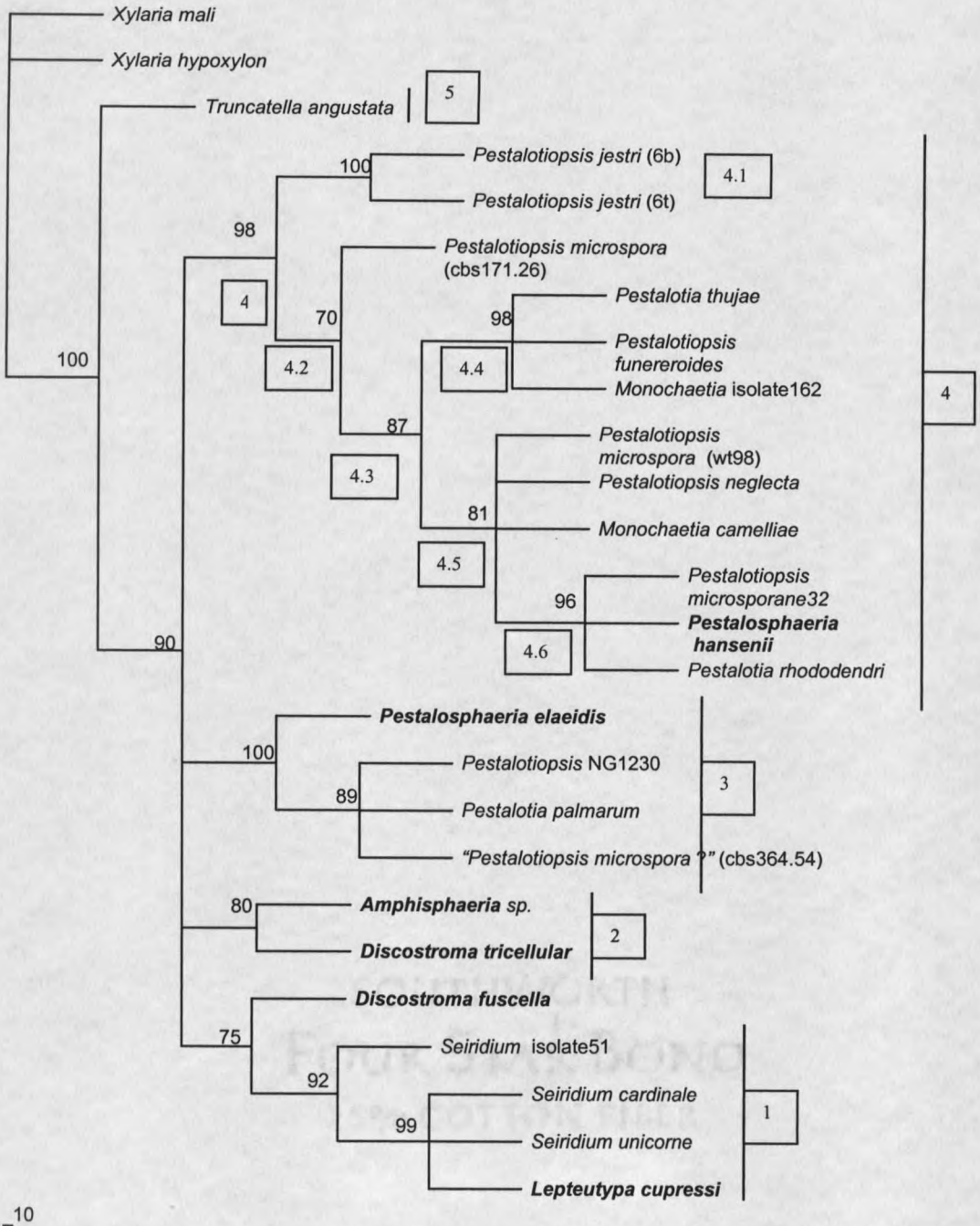


Figure 19. Phylogenetic relationships of selected Amphisphaeriaceae inferred from ITS1-5.8S-ITS2 sequences. Single most parsimonious tree, length = 441 steps, CI = 0.7392, RI = 0.8024. Bootstrap frequencies of more than 50 are indicated on each branch. The reference taxa are *Xylaria mali* and *Xylaria hypoxylon*. Teleomorph species are presented in bold letters. Nodes and clades are numbered in the order that they are described in the text.

Group 1

Group 1 includes the five species *Discostroma fuscella*, *Lepteutypa cupressi*, *Seiridium unicorne*, *Seiridium cardinale* and *Seiridium* isolate51. This monophyletic group is supported by a 75 % bootstrap value obtained through phylogenetic analysis. This result is consistent with Kang *et al.* (1998; Figure 17) in that the genus *Discostroma* forms a sister group with *Lepteutypa cupressi*. Teleomorphic states in this group are *Discostroma fuscella* and *Lepteutypa cupressi*. Conidia of *Discostroma fuscella* are brown and three-celled, whereas conidia of *Lepteutypa cupressi* are brown and four celled. Both *Discostroma fuscella* and *Lepteutypa cupressi* produce asci with amyloid rings. According to Sutton (1980), the genus *Seiridium* produces a confluent acervulus with a thin wall of textular angularis cells. The acervulus bears holoblastic annellidic conidiogenous cells. The conidia of *Seiridium* are 5-euseptate (six-celled) with hyaline apical and basal cells, and four intermediate brown cells. The previous classical classification done by Guba (1961) placed *Seiridium* in Sexloculatae section of *Monochaetia*. Guba (1961) held the genus *Seiridium* to be synonymous with *Monochaetia* because these two genera have a single appendage at the apical and the basal end. Thus about 15 species of *Seiridium* have been placed in *Monochaetia*, in the section Sexloculatae (Guba, 1961). This genus was then reclassified and more than 20 taxa were placed in *Seiridium* sensu strictu (Sutton, 1980). The separation was based on *Seiridium* conidia being five septate with single or several appendages, while the conidia of *Monochaetia* are three to four septate with a single appendage. *Lepteutypa* was found to be the teleomorphic states of *Seiridium* by Swart, 1973.

There are two subgroups at the internode for group 1. *S. cardinale*, *S. unicorne* and *Lepteutypa cupressi* cluster together in the same branch (Bootstrap value = 92%; Figure 19), and are separate from *Seiridium* isolate51 (Bootstrap value = 92%; Figure 19). The topology of this cladogram inferred from ITS1-5.8S-ITS2 in Figure 16 suggests that *Seiridium* isolate 51 is a species distinct from *S. cardinale*, *S. unicorne* and *Lepteutypa cupressi*. The branch with *S. cardinale*, *S. unicorne* and *Lepteutypa cupressi* with 99 % bootstrap value suggests these three taxa are synonymous (Figure 19). The 94-98 % homologies of ITS1-5.8S rDNA-ITS2 nucleotide sequence strongly support the view that *S. cardinale* and *S. unicorne* are synonyms of *L. cupressi* (Table 17 and Figure 20). The important evidence which supports the hypothesis that *S. cardinale*, *S. unicorne* and *Lepteutypa cupressi* are synonymous name for the same species is that *S. unicorne* or *S. cardinale* have been shown to be anamorphic forms of *Lepteutypa cupressi* (Swart, 1973). Swart (1973) found that the *Seiridium* state is regularly accompanied by the perfect state *Lepteutypa*, a canker on cypress tree.

Nucleotide divergence of the ITS1-5.8S-ITS2 sequences support the idea that *Seiridium* isolate51 a species different from *S. cardinale*, *S. unicorne* and *Lepteutypa cupressi*. Nucleotide divergence of whole regions of ITS1-5.8S-ITS2 between *Seiridium* isolate51 and other species (*Lepteutypa cupressi*, *Seiridium unicorne*, *Seiridium cardinale*) is 8.16 %, which is higher than the nucleotide divergence among synonymous taxa of *S. cardinale*, *S. unicorne* and *L. cupressi* (Table 17). Considering both ITS regions independently, there is an 18 % nucleotide divergence for ITS1 and 3.82 % nucleotide divergence for ITS2 (Table 17). Nucleotide differences in each region of ITS1

and ITS2 are still higher than the nucleotide divergence for regions ITS1 and ITS2 among synonyms of *S. cardinale*, *S. unicorne* and *L. cupressi*.

In future work, a study of the nucleotide divergence of the ITS1-5.8S-ITS2 sequences will possibly provide stronger evidence, especially if more sequences from additional species of *Seiridium* and also more isolates of *S. unicorne* and *S. cardinale* from different geographical regions and hosts are compared. Some interesting questions can be hypothesized from the results of the comparison of the ITS1-5.8S-ITS2 sequences among *Seiridium* isolate 51 and *Lepteutypa cupressi*, *Seiridium unicorne*, *Seiridium cardinale*. These questions are: (i) Are the ITS1-5.8S-ITS2 regions useful for resolving the different species of *Seiridium*? (ii) Can the ITS1-5.8S-ITS2 regions distinguish between the different species of *Seiridium*? (iii) How much sequence divergence is sufficient to indicates separate species? (iv) A comparison of morphological characteristics of *Seiridium* isolate 51 and *Lepteutypa cupressi*, *Seiridium unicorne*, *Seiridium cardinale* could possibly confirm species differences in the future.

Table 17. Nucleotide divergence among species of *Seiridium* isolate 51, *Lepteutypa cupressi*, *Seiridium unicorne*, *Seiridium cardinale* for the ITS regions.

	Nucleotide divergence (%)			
	ITS1	5.8S	ITS2	Total
Taxa Size (base pair)	193-200	153-158	156-157	502-515
Seiridium isolate 51 vs. *Lepteutypa cupressi*, *S. cardinale* and *S. unicorne*	18	0	3.82	8.16
Seiridium cardinale vs. *S. unicorne*	2.56	0	1.91	1.57
S. cardinale vs. *Lepteutypa cupressi*	5.64	4.43	1.91	4.12
S. unicorne vs. *Lepteutypa cupressi*	5.64	4.43	2.55	4.31

In order to confirm that *S. cardinale*, *S. unicorne* and *Lepteutypa cupressi* are synonym for the same species, nucleotide divergence among three species of *Seiridium cardinale* vs. *S. unicorne* and *Lepteutypa cupressi* were compared.

```
                    1                                                        60
Seiridium cardinale ---CATTACAGAGTTATCTAACTCCCAAAACCCATGTGAACTTACCTTTGTTGCCTCGGC
S. unicorne         ---CATTA.....................-.............................
Lepteutypa cupressi CACGAGGAT.TTAG...........-AAA--.............................

                   61                                                       120
Seiridium cardinale AGAAGCTACCTGTACCTACCTGGAACAGCCTACCTGGAGCGATCCGGGCTGGCCTACCTG
S. unicorne         .....................................T.........T......
Lepteutypa cupressi ..G......TC.....................................-...........

                  121                                                       180
Seiridium cardinale GAACGGTCTGGTGGTCGACTGCCGGTGGACCATTCAACTCTTGTTATTTTATTGTAATCT
S. unicorne         ............................................................
Lepteutypa cupressi ............................................................

                  181                                                       240
Seiridium cardinale GAGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
S. unicorne         ............................................................
Lepteutypa cupressi ..................................-.........................

                  241                                                       300
Seiridium cardinale TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
S. unicorne         ............................................................
Lepteutypa cupressi ............................................................

                  301                                                       360
Seiridium cardinale TCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATT
S. unicorne         ............................................................
Lepteutypa cupressi ....-.....-....G..-..........C.........-.....................

                  361                                                       420
Seiridium cardinale TCAACCCTTAAGCCTAGCTTAGTATTGGGAGTCTACTGTATTGTAGTTCCTCAAATCCAA
S. unicorne         ............................................................
Lepteutypa cupressi ............................................................

                  421                                                       480
Seiridium cardinale CGGCGGATCTGTGGTATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGTCAGGTGCT
S. unicorne         ...................................................T........
Lepteutypa cupressi ............................................................

                  481                                                524
Seiridium cardinale GCAGCTCCCAGCCGCTAAACCCCCAAATTTTTTAATGGTTGACC
S. unicorne         ........................C....-...........
Lepteutypa cupressi .......T.........................-..........
```

Figure 20. Sequence alignment of ITS1, 5.8S and ITS2 region of *Seiridium cardinale*, S. *unicorne* and *Lepteutypa cupressi*. The conserved positions are indicated with dots, and deletion positions with dashes. The 5.8S region (158 base pairs) is indicated with bold and underlined letters. The CATTA represents the last five nucleotides of 18S rDNA while the TTGACC represents the first five nucleotides of 28S rDNA.

Comparison of the whole region ITS1-5.8S-ITS2 showed diverges between *Seiridium cardinale* vs. *S. unicorne, S. cardinale* vs. *Lepteutypa cupressi* and *S. unicorne* vs. *Lepteutypa cupressi*, to be 1.57 %, 4.12 % and 4.31 %, respectively (Table 16). Only 27 positions were found to be variable, over a total size of 524 base pairs among the three taxa of *Seiridium cardinale* vs. *S. unicorne* and *Lepteutypa cupressi* (Figure 20). Of the 27 total mutations, twelve are insertion-deletion, eleven are transitions (nine C/T and two A/G), four are transversions (three G/T and one A/T). The percentage of nucleotide divergence in the ITS1 space between *Seiridium cardinale* vs. *S. unicorne, S. cardinale* vs. *Lepteutypa cupressi* and *S. unicorne* vs. *Lepteutypa cupressi* are 2.56 %, 5.64 %, 5.64 %, respectively (Table 17). The nucleotide divergence within the ITS2 region is 1.91 % between *Seiridium cardinale* vs. *S. unicorne*, 1.91 % *S. cardinale* vs. *Lepteutypa cupressi*, and 2.55 % *S. unicorne* vs. *Lepteutypa cupressi*. There is no mutation in the 5.8S rRNA gene between *Seiridium cardinale* and *S. unicorne*, but there is a 4.43% difference in this region between *S. cardinale* and *Lepteutypa cupressi* and *S. unicorne* and *Lepteutypa cupressi*. It can be seen that the percent nucleotide difference in all cases is low, always fewer than 6 %. Viljoen *et al.* (1993) reported the equivalence of *S. cardinale*, *S. unicorne* and S. *cupressi* based on the high homology of ITS1 sequences. In addition Viljoen *et al.* (1993) noted that both appendaged and non-appendaged conidia of *S. unicorne* and *S. cardinale* were found in a single acervulus, suggesting that this characteristic, used as a basis for generic concepts, is variable.

Group 2

Group 2 is a miscellaneous group consisting of *Amphisphaeria* sp. and *Discostroma tricellular*, and this internode is supported with an 80 % bootstrap level (Figure 19). There is some correlation of the morphological characteristics of the anamorphic forms of the two genera, including typical eccentric apical appendages and long cylindrical annellidic holoblastic conidiogenous cells. I could not find the anamorph of *Amphisphaeria* sp. in agar culture, but Sutton (1980) mentioned that the anamorph of *Amphisphaeria argentinensis* is *Bleptosporium pleurochaetum* and the anamorph of *Discostroma tricellular* is *Seimatosporium azaleae* (Okane *et al.*, 1995). The acervulus of *Seimatosporium* bears the annellidic type of holoblastic conidiogenous cells the same as *Bleptosporium* does (Sutton, 1980). Conidia of *Seimatosporium* are very variable ranging from 2-5-euseptate cells with or without an appendage at both ends. All species of *Seimatosporium* produce conidia that possess a single simple or branched eccentric appendage, and the truncate basal cell provides a single or branched eccentric appendage (Figure 21A). The ascospore characteristics of *Amphisphaeria* and *Discostroma,* do not reflect the relationships of these two genera. The ascospores of *Discostroma tricellular* and *Discostroma fuscella* are two septate (Figure 21C), and ascospores of *Amphisphaeria* sp. have one septum (two celled ascospore, Figure 21D). The acervulus of *Bleptosporium* bears the annellidic type of holoblastic conidiogenous cells, which produces cylindrical to clavate four-euseptate conidia with three median dark brown cells (Figure 21B). The conidia of *Bleptosporium* also possess an apical hyaline cell with a single eccentric unbranched appendage, and hyaline truncate basal cell with no

appendage (Figure 21B). Examination of more isolates of the anamorphic and teleomorphic state of *Amphisphaeria* and *Discostroma* are necessary for a clearer delineation of this clade.

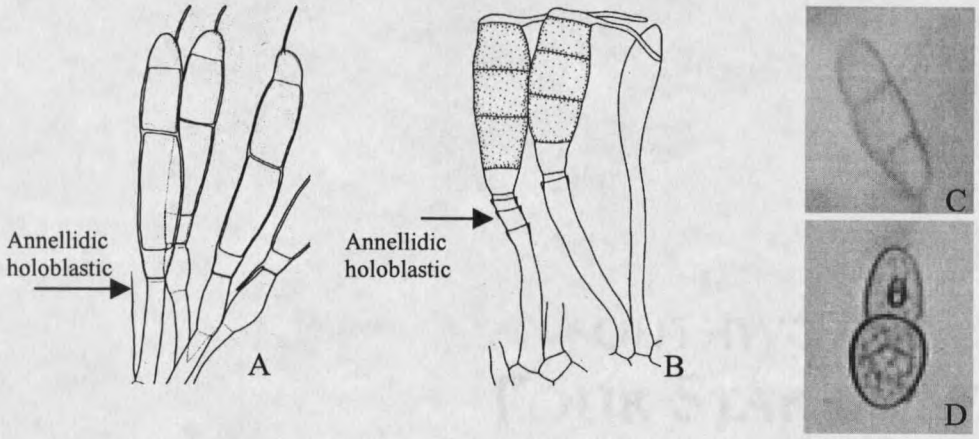


Figure 21. A. conidiogenous cells of *Discostroma tricellular* with conidia (anamorph = *Seimatosporium azaleae*, modified from Okane, 1996). B. conidiogenous cells of *Amphisphaeria* sp. (anamorph = *Bleptosporium* sp.) C. 3-celled ascospore of *Discostroma tricellular*. D. 2-celled ascospore of *Amphisphaeria* sp. (Strain no MAFF235878, Japan).

Group 3

Group 3 is composed of *Pestalosphaeria elaeidis*, *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolateNG-1230 and *Pestalotia palmarum*, which have only 142-150 base pairs in the ITS1 space, fewer than for the other taxa. Guba (1961) described all species in this branch under the section of Quinqueloculatae of genus *Pestalotia* with all possessing five-celled conidia with three intermediate colored cells. This branch was supported with a 100% confidential bootstrap value (Figure 19). The topology of this clade shows that three anamorphic isolates *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolateNG-1230 and *Pestalotia palmarum* are very closely

related to the teleomorphic species *Pestalosphaeria elaeidis*. It is likely that *Pestalotiopsis* isolate 1230 and "*Pestalotiopsis microspora*" (CBS364.54) should be correctly identified as *Pestalotia palmarum*. The evidence is the high homology (94-99%) of the whole regions of ITS1-5.8S-ITS2 (Table 18 and Figure 22), the 98 % bootstrap support (Figure 19) and the similarity of the morphological characteristics of the conidia (Guba, 1961). The nucleotide divergence for the total region sequenced between "*Pestalotiopsis microspora*" (CBS364.54) and *Pestalotiopsis* isolate NG12-30, "*Pestalotiopsis microspora*" (CBS364.54) and *Pestalotia palmarum* and *Pestalotiopsis* isolate NG12-30 and *Pestalotia palmarum* is 0.87 %, 5.93 % and 5.11 %, respectively (Table 18).

Table 18. Nucleotide divergence among species of *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolate NG12-30 and *Pestalotia palmarum* for ITS1-5.8S-ITS2 regions.

	Nucleotide divergence (%)			
	ITS1	5.8S	ITS2	Total
Size (bp)	142-161	153-158	155-166	462-507
Pestalotiopsis microspora (CBS 361.64) VS *Pestalotiopsis* isolate NG12-30	0.70	0	1.81	0.87
Pestalotiopsis microspora (CBS 361.64) VS *Pestalotia palmarum*	7.45	6.33	3.62	5.93
Pestalotiopsis isolate NG12-30 VS *Pestalotia palmarum*	6.83	6.33	1.81	5.11

Examining the sequenced region ITS1-5.8S-ITS2 more closely, there were twelve insertion and deletion positions, nine transversion positions (two A/C, three A/T, three G/C and one G/T), and five transition positions (four A/G, one C/T). Considering each spacer independently for "*Pestalotiopsis microspora*" (CBS364.54) and *Pestalotiopsis*

isolate NG12-30, there are 0.70 % and 1.81 % nucleotide differences in the ITS1 and ITS2, respectively, with 0 % divergence in 5.8S r RNA gene. In contrast 7.45 %, 6.43 % of the nucleotide divergence was found in ITS1, and 3.62 % and 1.81 % in ITS2 between *Pestalotiopsis microspora* (CBS364.54) and *Pestalotia palmarum* and *Pestalotiopsis* isolateNG12-30 VS *Pestalotia palmarum*, respectively. There is 6.33 % nucleotide variation in the 5.8S rRNA gene between both "*Pestalotiopsis microspora*" (CBS364.54) and *Pestalotia palmarum*, and *Pestalotiopsis* isolateNG12-30 and *Pestalotia palmarum*. If the ITS1, ITS2 and 5.8S regions are considered individually, the overall range of variation for all cases varies, from 0-7.54 % (Table 18)

```
P_microsporaCBS36       TAGAGTTTTCTAAACTCCCAACCCATGTGAACTTACCTTTTGTTGCCTCGGCAGAAGTTA
P_microsporaNG1230      ..............................................................G.....
Pestalotia palmarum     ..............................................................G.....

P_microsporaCBS36       -....-.............--..--..--.............-.................TA
P_microsporaNG1230      -....-.............--..--..--.............-.................
Pestalotia palmarum     C....G...........GC...GC...GC............C.................AT

P_microsporaCBS36       TGTAATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCT
P_microsporaNG1230      ............................................................
Pestalotia palmarum     ..................................C...TGCG....................

P_microsporaCBS36       GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
P_microsporaNG1230      ............................................................
Pestalotia palmarum     ..............................C-...-.........................

P_microsporaCBS36       TCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGA
P_microsporaNG1230      ............................................................
Pestalotia palmarum     ........................GC..................................

P_microsporaCBS36       GCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAATCTACTTCTCTTAGGAGTTGT
P_microsporaNG1230      ..........................................................T-......
Pestalotia palmarum     ..........................................................T-......

P_microsporaCBS36       AGTTCCTGAAATACAACGGC-GGATTTGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTC
P_microsporaNG1230      ....................-.......................................
Pestalotia palmarum     ....................C.......................................
```

Figure 22. Sequence alignment of the whole region covering ITS1-5.8S-ITS2 for *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolate NG12-30 and *Pestalotia palmarum*. The conserved positions are indicated with dots, deletion positions with dashes. The 5.8S region (158 base pairs) is represented with bold and underlined letters.

Nonetheless this range of variation is still clearly within the limits of the infraspecific variation found in other fungal species from different orders. For instance, divergence between separate isolates of *Beauveria brongniartii* ranged between 0.7-18.7 % for ITS1 and 1.8-16.7 % for the ITS2 (Neuve'glise *et al.*, 1994). For *Metarhizium anisopliae*, infraspecific divergence ranges from 0-12.1 % in ITS1 and 0-8.5 % in ITS2 (Curran *et al.*, 1994).

Table 19. Conidial characteristic features of "*Pestalotiopsis microspora*" (CBS 364.54), and *Pestalotia palmarum*, which were cited from Guba, 1961, compared with the observed conidial characteristics of *Pestalotiopsis* isolate NG12-30.

Characteristic features	*Pestalotiopsis microspora* CBS364.54 (Guba, 1961)	*Pestalotiopsis* isolate NG12-30	*Pestalotia palmarum* (Guba, 1961)
Conidial dimensions of 5-celled in µm (Width x Length)	19-24 x 5-7	17.6-28.0 x 4.8-7.2	16-22 x 5-7
Mean of conidium length/width ratio	3.6:1	3.4:1	3.2:1
Color of Median cells	Median cells olivaceous, upper two colored cells rarely darker	the two upper cells darker olivaceous brown than the lower cell	the two upper cells umber, the lowest cell olivaceous, sometimes equally colored
Length of three median cells	13-16 µm long	11.2-16.8 (17.6) µm long	11-15 µm long
Apical appendage number, & length in µm	Mostly 3, sometimes2, 3-15 µm long	2- 3, mostly, 11.2-32 µm long	mostly 3, sometimes 2; length up to 16 µm long
Basal appendage number, & length in µm	One basal appendage 4-5 µm in length	One basal appendage 3.2-8.0 (10.4) in length	One basal appendage with up to 6 µm length

The morphological characteristics for conidia of *Pestalotiopsis microspora* (CBS 364.54), *Pestalotiopsis* isolate NG12-30 and *Pestalotia palmarum* were observed, and are shown in Table 19. The morphological characteristics of conidia of *Pestalotiopsis* isolate

NG12-30 and *Pestalotia palmarum* are almost identical, including the size of the 5 celled conidia, the color of the median cells, and the length of both apical and basal appendages (Table 19). The feature that most distinguishes these two species are the two upper median cells, which are a distinctively darker dull olivaceous brown in Pestalotiopsis isolate NG12-30. Tracing back the history of *Pestalotiopsis microspora* (CBS364.54) which was isolated from a bath towel, this species was preserved as *Pestalotiopsis dichaeta* in 1970, and then preserved as *Pestalotia microspora* again in 1989 (CBS database, 2000). The size of the conidia and the length of the apical and basal appendages of *Pestalotiopsis microspora* (CBS364.54) are similar to those of *Pestalotiopsis* isolate NG12-30 and *Pestalotia palmarum*. The main discrepancy is that the upper two olivaceous median cells are occasionally darker in *Pestalotiopsis microspora* (Guba, 1961). This could be accounted for as ecotypic variation. The *Pestalotiopsis* isolate NG12-30 and *Pestalotiopsis microspora* (CBS364.54) appear to be conspecific with *Pestalotia palmarum*. Both of them should be classified as such *Pestalotia palmarum*, and are likely anamorphs for the teleomorphic genus *Pestalosphaeria*.

Group 4

Group 4 from figure 17 encompasses a wide range of morphological diversity (Bootstrapping = 98%; Figure 19), including *Pestalotiopsis jesteri* (isolate 6b and 6t), *Pestalotiopsis microspora* (CBS171.26), *Pestalotia thujae*, *Pestalotiopsis funereoides*, *Monochaetia* 162, *Monochaetia camelliae*, *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis microspora* (NE32), *Pestalosphaeria hansenii*,

and *Pestalotia rhododendri*. Guba (1961) described all species in this branch under the section *Quinqueloculatae* of genus *Pestalotia*, with all possessing five-celled conidia with three intermediate colored cells. These characteristics may be unique for the ingroup taxa studied for groups 3 and 4. The group 4 clade was divided into six subgroups, which are 4.1 through 4.6 (Figure 19).

Group 4.1 contains *Pestalotiopsis jesteri* isolate 6b and *Pestalotiopsis jesteri* 6t. *Pestalotiopsis jesteri* is a new species, isolated from the inner bark of *Fragraea bodenii* in the southern Highlands of Papua New Guinea (Strobel *et al.*, 2000). This species is unique in possessing knobs on the ends of the conidial appendages, reminiscent of a jester hat. *Pestalotiopsis jesteri* produces fusiform shaped 5-celled conidia with three appendages. Each appendage is terminated by a spherical extension. Branches were collected from different parts of the tree, and resulting isolates came from top (6t) and middle (6b) parts. Each level yielded a fungus that produced identical conidia, but the morphologies of the colonies on Potato Dextrose agar varied. The colony of *Pestalotiopsis jesteri* isolated from the top part of the tree (6t) has olivaceous brown mycelium, while the colony of *Pestalotiopsis jesteri* isolated from the middle part (6m) has red brown mycelium. Nonetheless *Pestalotiopsis jesteri*, designated 6m and 6t, showed identical sequences in the ITS1, 5.8S and ITS2 region. The knobs with spherical terminations of *Pestalotiopsis jesteri* 6t & 6m may be a good characteristic for delineation from all other taxa in the clade.

Group 4.2 is comprised of *Pestalotiopsis microspora* (CBS171.26), and a clade of *Pestalotia thujae*, *Pestalotiopsis funereoides*, *Monochaetia* isolate162, *Monochaetia*

camelliae, *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis microspora* (NE32), *Pestalosphaeria hansenii* and *Pestalotia rhododendri* (Figure 19). The internode 4.2 received low support, judging by bootstrap value of 70 % (Figure 19). The olivaceous to brown coloration of intermediate cells of conidia of all members in the internode 4.2 may indicates the relatedness of these taxa.

Group 4.3 from figure 19 contains the subgroup *Pestalotiopsis funereoides*, *Pestalotia thujae* and *Monochaetia* isolate162 and subgroup *Monochaetia camelliae*, *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis microspora* (NE32), *Pestalosphaeria hansenii* and *Pestalotia rhododendri* with 87 % bootstrap value. There are no anatomical features that tend to distinguish *Pestalotiopsis microspora* (CBS171.26) from *Pestalotia thujae*, *Pestalotiopsis funereoides*, *Monochaetia* 162, *Monochaetia camelliae*, *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis microspora* (NE32), *Pestalosphaeria hansenii* or *Pestalotia rhododendri*.

The internode 4.4 includes *Pestalotiopsis funereoides*, *Pestalotia thujae* and *Monochaetia* isolate162, confirmed by a 100 % bootstrap value (Figure 19). The 100% identical ITS1-5.8S-ITS2 sequences of *Pestalotiopsis funereoides*, *Pestalotia thujae* and *Monochaetia* isolate162 reveal that these three isolates are identical (data not shown). This result is consistent with the classification done by Guba (1961). He indicated *Pestalotiopsis funereoides* and *Pestalotia thujae* are in the same subsection of Quinqueloculatae of genus *Pestalotia* which produce five-celled conidia with three intermediate colored cells (Guba, 1961). He also noted that these two species have the same size conidia (22-30 x 7-10 μm), and 4-5 appendages arising from the apical hyaline

cell. The difference between these two species is considered to be the position from which the appendage originates. *Pestalotiopsis funereoides* has simple appendages arising from the apex of the apical cell, while *Pestalotia thujae* has appendages radiating from the side and base of the apical cell. It is reported by Guba (1961) that other Fungi Imperfecti are extremely variable under different conditions in nature. Thus the differences in the genesis of the appendages may correlate to different ecotypes resulting from differences in hosts and location of these two taxa. *Pestalotiopsis funereoides* Steyaert (CBS175.25) was isolated from *Juniperus* in Italy whereas *Pestalotia thujae* Sawada (CBS303.75) was isolated from the cone of *Thuja occidentalis*, in the Netherlands. Nevertheless, to solidify these conclusions, the molecular data needs to be strengthened by examination of more collections of *Monochaetia*, *Pestalotia* and *Pestalotiopsis*.

Another rationale that explains the placement of *Monochaetia* isolate162 with *Pestalotiopsis funereoides* and *Pestalotia thujae* is a report done by Christensen in 1932. Christensen (1932) reported that he obtained races which possessed conidial characters of both *Pestalotia* and *Monochaetia* from monoconidial cultures isolated from the acervuli of *Pestalotia funereoides* on *Pinus pavulstris* Mill. *Monochaetia* conidia are characterized by a single appendage appearing on the hyaline apical and basal cells whereas *Pestalotia* or *Pestalotiopsis* conidia are characterized by more than 2 appendages appearing on the hyaline apical cell and a short appendage at the basal cell (Guba, 1961). Christensen (1932) suggested it was unlikely that two genera of fungi would fruit in the same acervulus, nor was it likely that *Monochaetia* spores would, by chance, so

frequently lodge on an acervulus of *Pestalotia* (Christensen, 1932). In addition Christensen (1932) hypothesized that *Monochaetia* might be derived from *Pestalotia*.

There are no apparent unique morphological conidial features correlated with the group *Monochaetia camelliae*, *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta* at internodes 4.5 with the high bootstrap level 81 % (Figure 19). Polyphyly is suggested for the genera *Monochaetia* and *Pestalotiopsis* are not clearly separated. It is difficult to say whether these problematic results come from misidentification, particularly of *Pestalotiopsis microspora* (WT98). There is a lot of missing data, particularly the teleomorphic forms of *Monochaetia* (*Hymenopleella*), *Pestalotiopsis* (*Pestalosphaeria*) and *Pestalotia* (*Pestalosphaeria*) species. Additional sampling of *Monochaetia* species is absolutely required for a better understanding of the evolutionary relationships among these species.

The 99% homology of the 18S region (=1717 base pair) and 97-98 % of ITS regions and 5.8S of all studied *Pestalotia*, *Monochaetia* and *Pestalotiopsis* species implies that the limits of the species of *Pestalotia* should be widely considered. The high percentage of amenity of 18S rRNA gene and ITS regions and 5.8S among sequences of *Pestalotia*, *Monochaetia* and *Pestalotiopsis* as well as the discovery of *Monochaetia*-type conidia and *Pestalotia*-type conidia in the same acervuli (Christensen, 1932) strongly supports synonymizing these three genera. Until further evidence suggests otherwise, it is convincing that *Pestalotia*, *Monochaetia* and *Pestalotiopsis* species should be placed in the same genus.

The internode 4.6 includes three species: *Pestalotiopsis microspora* (NE32), *Pestalosphaeria hansenii* and *Pestalotia rhododendri* (Figure 16). *Pestalotiopsis microspora* (NE32) is considered to be asexual state of *Pestalosphaeria hansenii*. The monophyly of these species is strongly supported by the molecular characters (Bootstrap = 96 %, Figure 19). The 100 % identical nucleotides in the ITS regions and 5.8S rDNA genes of *Pestalotiopsis hansenii*, *Pestalotiopsis microspora* and *Pestalotia rhododendri* strongly confirm that these are three names are for the same fungus. Moreover, all three species have conidia with three versicolored intermediate cells whose upper two cells are darker in color. However, Guba (1961) described *Pestalotia rhododendri* separately from *Pestalotiopsis microspora* because *Pestalotia rhododendri* produces conidia with chestnut brown to black intermediate cells, and the size of the conidia is longer and broader (25-30 x 7-10 μ) in diameter. In contrast *Pestalotia rhododendri*, *Pestalotiopsis microspora* produce conidia with olivacious to pale brown intermediate cells, and the size of conidia are smaller (18-26 x 5-8 μ) in diameter. The identical sequences of the ITS regions and 5.8S rDNA genes among these three isolates also provides additional support for the integration of anamorph *Pestalotiopsis microspora* (NE32) and teleomorph *Pestalosphaeria hansenii* as has been suggested by Metz, 2000. The identical sequence of the ITS regions and 5.8S rDNA between anamorph and teleomorph in this experiment is another example of research demonstrating that nucleotide data is a useful tool for synanamorph systematics as suggested by Kuhls *et al.* (1996) and Arenal *et al.*, (2000).

Group 5

Group 5 is the species *Truncatella angustata* supported by confidence level of 100 % (Figure 19). Of the genera accepted by Guba (1961), *Truncatella* was placed under the generic name *Pestalotia* in the Quadriloculatae section that described four-celled conidia with two intermediate colored cells. The list of the synonyms for *Truncatella angustata* includes *Pestalotia truncata*, *Seiridium lignicola* and *Truncatella truncata* (Sutton, 1980). *Truncatella angustata* produces conidiophores with annellations holding four-celled conidia. The conidia of *Truncatella angustata* are comprised of two dark brown median cells with no basal appendage. The apical appendages on the hyaline apical cell are variable, being either single or sometimes irregularly or dichotomously branched. The genus *Truncatella* was presumed to be an anamorphic form of *Broomella*, a genus in Amphisphaeriaceae (Barr, 1975). For a large perspective of the evolutionary relationships within Amphisphaeriaceae, an examination of more species of *Truncatella* is necessary.

Examination of Phylogeny in Relation to Host Plant and Geographic Location

Original substrates, original geographic location and nutritional modes were mapped onto a bootstrap single most parsimonious tree inferred from ITS1-5.8S-ITS2 sequences of selected Amphisphaeriaceae (Figure 23). The following is a discussion of the correlations between original geographic locations, original host plants and nutritional modes of groups on the phylogenic tree inferred from ITS1-5.8S-ITS2 sequences.

Group 1 represents the anamorphic genus *Seiridium* and teleomorphs *Lepteutypa cupressi*, *Discostroma fuscella*. *D. fuscella* is a pathogen isolated from *Rosa* species, in Canada (Barr, 1998 personal communication). *Seiridium* isolate 51 is an endophyte isolated from yew trees (*Taxus* sp.) in the Himalayan Mts. of Nepal, and *Seiridium cardinale*, *Seiridium unicorne* and *Lepteutypa cupressi* are pathogens isolated from *Juniperus* (in Italy), *Cryptomeria* (Japanese cedar, Cypress family) in New Zealand, and *Cupressi* in Australia, respectively. It seems that a clade of *Seiridium cardinale*, *Seiridium unicorne* and *Lepteutypa cupressi* depicts a line of pathogens on woody gymnosperm plants with the exception of *D. fuscella* isolated from Rosa, a woody dicot plant (Figure 23). The geographical origins reflect the distribution of these trees, primarily in temperate climates with the possible exception of Australia. It is interesting that isolates from all over the world group so closely.

The taxa sampled for group 2 are pathogenic and endophytic fungi from woody and shrubby dicot plants in Japan. The Amphisphaeriaceae species was isolated from *Diospyros* and *Discostroma tricellular* was isolated from *Rhododendron indicum* (Figure 23).

In group 3 *Pestalotia palmarum* and *Pestalosphaeria elaeidis* are known as pathogens, but nutritional modes are not known for *Pestalotiopsis microspora* (CBS 364.54) (Figure 23). *Pestalotiopsis* isolate NG12-30 is an endophyte isolated from the *Moreabina* plant.

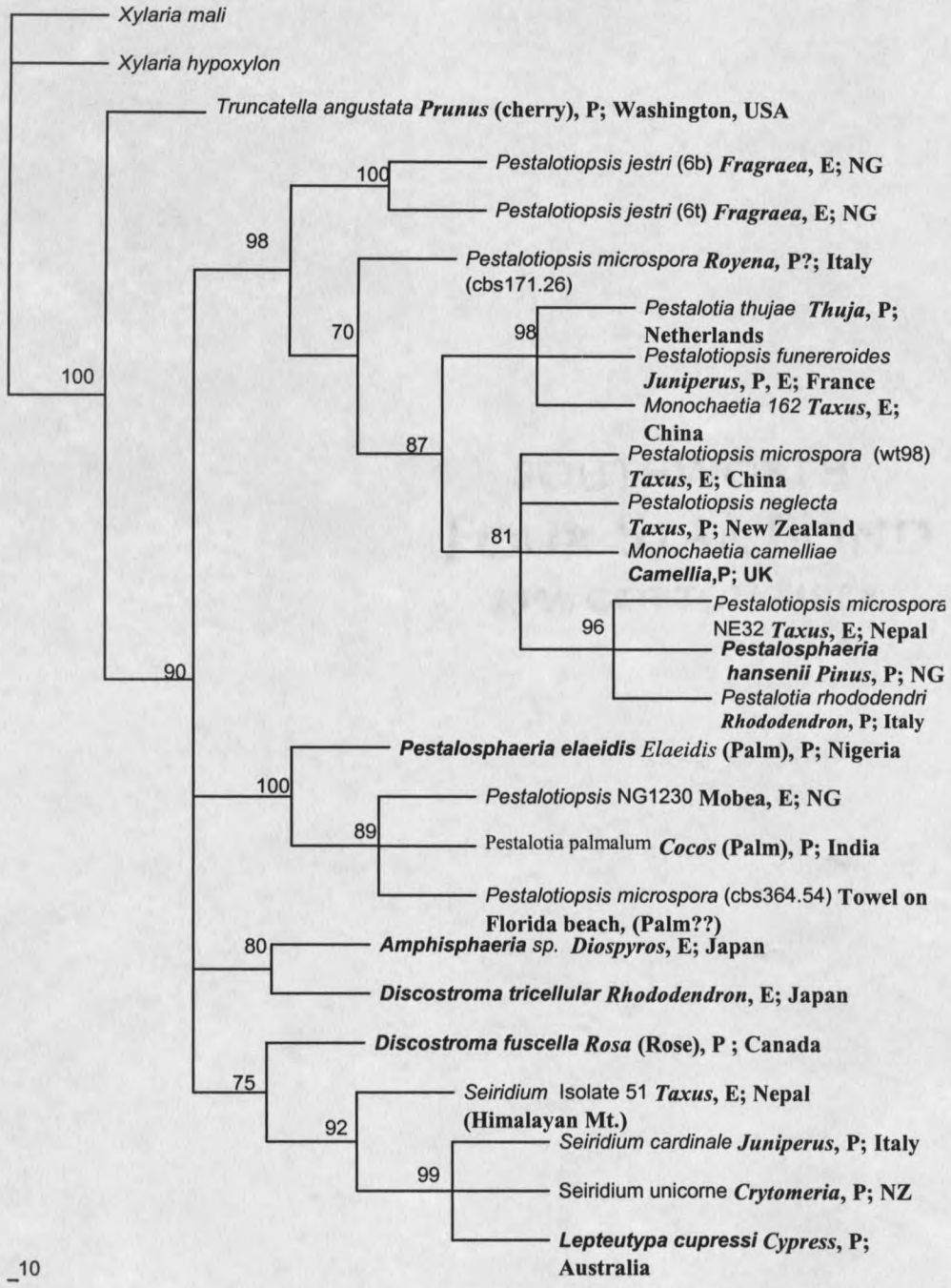


Figure 23. A bootstrap consensus tree inferred from ITS1-5.8S-ITS2 sequences of selected Amphisphaeriaceae. Teleomorph species were presented in bold letters. NG = New Guinea, and NZ = New Zealand. Original substrates, nutritional modes and geographic locations are mapped onto the gene tree.

Group 3 appears to be unique in that all taxa sampled are from tropical climates and are found primarily on palms (a monocot) with the exception of *Pestalotiopsis* isolate NG12-30 which is an endophyte isolated from the inner bark of a tropical tree from the Karvaraur River region of New Guinea. The isolate called *Pestalotiopsis microspora* (CBS 364.54) was found on a towel on the beach in Florida, and could possibly be misidentified. It is interesting that *Pestalotia palmarum* and *Pestalosphaeria elaeidis* were both isolated from palm trees, coconut palm and *Elaeidis* (palm), respectively. Nutritional mode of *Pestalotiopsis microspora* (CBS 364.54) is unknown as it was found on a towel on a beach. *Pestalotiopsis microspora* (CBS 364.54) might be a pathogen of palm trees such as coconut palm (*Cocos*) on the beach in Florida because Lawrence (1951) mentioned that the genus *Cocos* is extensively naturalized in Florida. *Pestalotiopsis* sp. has been isolated from diseased mango trees in Florida, but this isolate did not cause necrosis, tip dieback, gummosis and vascular discoloration on the mango trees, and may be an endophyte (Ploetz *et al.*, 1996). Therefore *Pestalotiopsis microspora* (CBS 364.54) may be an endophyte or a pathogen, but this is impossible to determine unless the original host is known.

Nutritional modes in sampled taxa of group 4 are either endophytic or pathogenic and there is no consistent pattern within this clade (Figure 23). However, information for these taxa is incomplete. Most were isolated from woody plants such as *Taxus*, *Juniperus*, *Pinus* and *Thuja*. The exceptions are *Pestalotiopsis rhododendri*, isolated from *Rhododendron* sp. in Italy, *Pestalotiopsis jesteri* from *Fragraea* in New Guinea, and *Pestalotiopsis microspora* isolate CBS171.26 from *Roneya* in Italy, and *Monochaetia*

camelliae from *Camelliae* (tea) in China. Many isolates in group 4 are from temperate climates, with notable exceptions. It is interesting that the ITS regions and 5.8Sr DNA of *Pestalotiopsis microspora* NE32 are 100 % similar to *Pestalosphaeria hansenii*, considered to be it's teleomorphic analog.

Group 5 represents *Truncatella angustata* isolated from cherry (*Prunus avium*) twigs in USA, and its nutritional mode is not known (Figure 23).

Summary

The ITS1&2 and 5.8S r DNA data generated a single monophyletic clade for selected Amphisphaeriaceae confined to four teleomorphic genera of *Amphisphaeria, Discostroma, Pestalosphaeria* and *Lepteutypa*, and their *Pestalotia*-like anamorphs (*Monochaetia*, *Pestalotia*, *Pestalotiopsis*, *Seiridium* and *Truncatella*). However, the bootstrap value support for several nodes is not strong. Sources of error in our phylogenetic parsimony might be inadequate taxon sampling, misidentifications and incorrect sequence alignment. Furthermore, a working hypothesis of evolutionary relationships of selected genera in Amphisphaeriaceae requires further testing for the effect of gap coding for the larger deletions and how to interpret data. A gap of about forty base pairs is shared by the four taxa of *Pestalosphaeria elaeidis*, *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolate NG-1230 and *Pestalotia palmarum*. To examine the effect of larger gaps against phylogeny estimations, gaps could be evaluated in three ways (Hughes, 1999). First, all gaps are coded as a fifth base. Second, all gap regions are treated as missing data (Jukes-Cantor, 1969 [distance metric]). Third, gap

regions are reduced in size to four informative bases with each informative base representing a set of bases, which are similar across taxa.

In future work, the addition of more sets of representative species in Amphisphaeriaceae and other families might reduce the length of internodes and counteract long branch attraction. Moreover it would be advantageous to add more species of *Amphisphaeria* (Teleomorph=T), *Discostroma* (T), *Broomella* (T), *Amphisphaerella* (T), *Crassoascus* (T), *Dyrithium* (T), *Griphosphaerioma* (T), *Monochaetia* (Anamorph=A), *Seimatosporium* (A) and *Truncatella* (A) for the study of evolutionary relationships of Amphisphaeriaceae. Expanding the phylogenetic study to other genes like the mitochondrial rDNA gene, which evolves faster than the small subunit rRNA genes and internal transcribed spaces, might improve the resolution of unresolved deep branches. The combination of molecular characters from several genes might give meaningful phylogenetic support for evolutionary relationships of taxa in Amphisphaeriaceae. Furthermore, a phylogenetic analysis based on morphology, using key characters that have been used previously might be useful in helping to address the taxonomy of the Amphisphaeriaceae. The pattern of Amphisphaeriaceae groups with an endophytic life style may be more clearly delineated if the integration of both morphological and molecular data is applied to evaluate the phylogenetic relationships of this family.

In conclusion, this research presents progress towards understanding the phylogenetic relationships in selected genera in Amphisphaeriaceae by means of maximum parsimony analysis of the ITS1-5.8S-ITS2 sequences. Parsimony analyses of

these sequences provide strong statistical support for a monophyletic group of the selected species in Amphisphaeriaceae. These selected species include *Amphisphaeria* sp. (isolate MAFF235878), *Discostroma fuscella*, *Discostroma tricellular*, *Pestalosphaeria hansenii*, *Lepteutypa cupressi*, *Monochaetia camelliae*, *Monochaetia* isolate162, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri* (isolate 6m), *Pestalotiopsis jesteri* (isolate 6t), *Pestalotiopsis funereoides*, *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE-32), *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis* isolate NG12-30, *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolate 51, *Truncatella angustata*, *Pestalosphaeria eleaidis*, *Pestalotia palmarum*. Neighbor joining analysis of the same sequence alignments yields an essentially similar tree (data not shown).

Interpretation of the phylogenetic results is restricted to the selected species in Amphisphaeriaceae. Thus, some divisions or groups of chosen genera in Amphisphaeriaceae might be artificial. However, phylogenetic analysis of the ITS spacers and 5.8S gene delineated sampled taxa in Amphisphaeriaceae into five groups. These five groups based on the phylogenic analysis of the ITS1-5.8S-ITS2 sequences are consistent with the nine genera in Amphisphaeriaceae for the classical classifications done by Barr (1994) for teleomorph. In addition, these five groups based on the phylogenic analysis of the ITS1-5.8S-ITS2 sequences are somewhat consistent with classifications done by Kang *et al.* (1998) that the genus *Discostroma* formed a sister clade to the genus *Lepteutypa*. The difference is due to the different taxa used for analysis.

Sequence analysis of the ITS spaces and 5.8S gene of selected taxa in Amphisphaeriaceae revealed a higher degree of conservation in the ITS2 compared with the ITS1, and no divergence in the 5.8S rRNA gene. These two events are also obvious in the sampled taxa in the study of sensu strictu Amphisphaeriaceae by Kang *et al.*, and the study of *Hypoxylon* and related genera in Xylariaceae by Ballesteros *et al.*, 1999.

In addition, the gene tree inferred from ITS1-5.8S-ITS2 sequences was compared with anamorphic and teleomorphic characteristics, geographical origins, hosts and nutritional modes (Figure 22 & 23). A general implication for this comparison is that the five groups separated by ITS spaces and 5.8S rRNA gene tend to be congruent with some anamorphic and teleomorphic characteristics (Figure 24), climate habitats, and type of host plant (Figure 23). The synapomorphic characteristics of all selected taxa in Amphisphaeriaceae seem to be the anamorphic state, which has an annellidic holoblastic type of conidiogenous cell in acervuli of textura angularis cells. There is no clear correlation between nutritional mode in each group of selected taxa in Amphisphaeriaceae because pathogenic and endophytic modes appeared inconsistently in the inferred phylogenetic tree.

Group 1 is represented by *Discostroma fuscella*, *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolate 51 and *Lepteutypa cupressi*. The asexual states of genera *Discostroma* and *Lepteutypa* are *Seimatosporium* and *Seiridium*, respectively. Group 1 is somewhat consistent with the description of Klebahn (1914) and Guba (1961) in the section of Sexloculatae that group conidia with 6-celled and four intermediate brown colored celled; hyaline apical and basal cells together.

Group 2 composed of *Amphisphaeria* sp. and *Discostroma tricellular*. Both anamorphic forms of *Amphisphaeria* (*Bleptosporium*) and *Discostroma* (*Seimatosporium*) have unique an eccentric apical appendages.

Group 3 included *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolate NG12-30, *Pestalosphaeria eleaidis* and *Pestalotia palmarum*. Species in the group 3 distinctively reflects a specific plant host in the palm family in tropical climates.

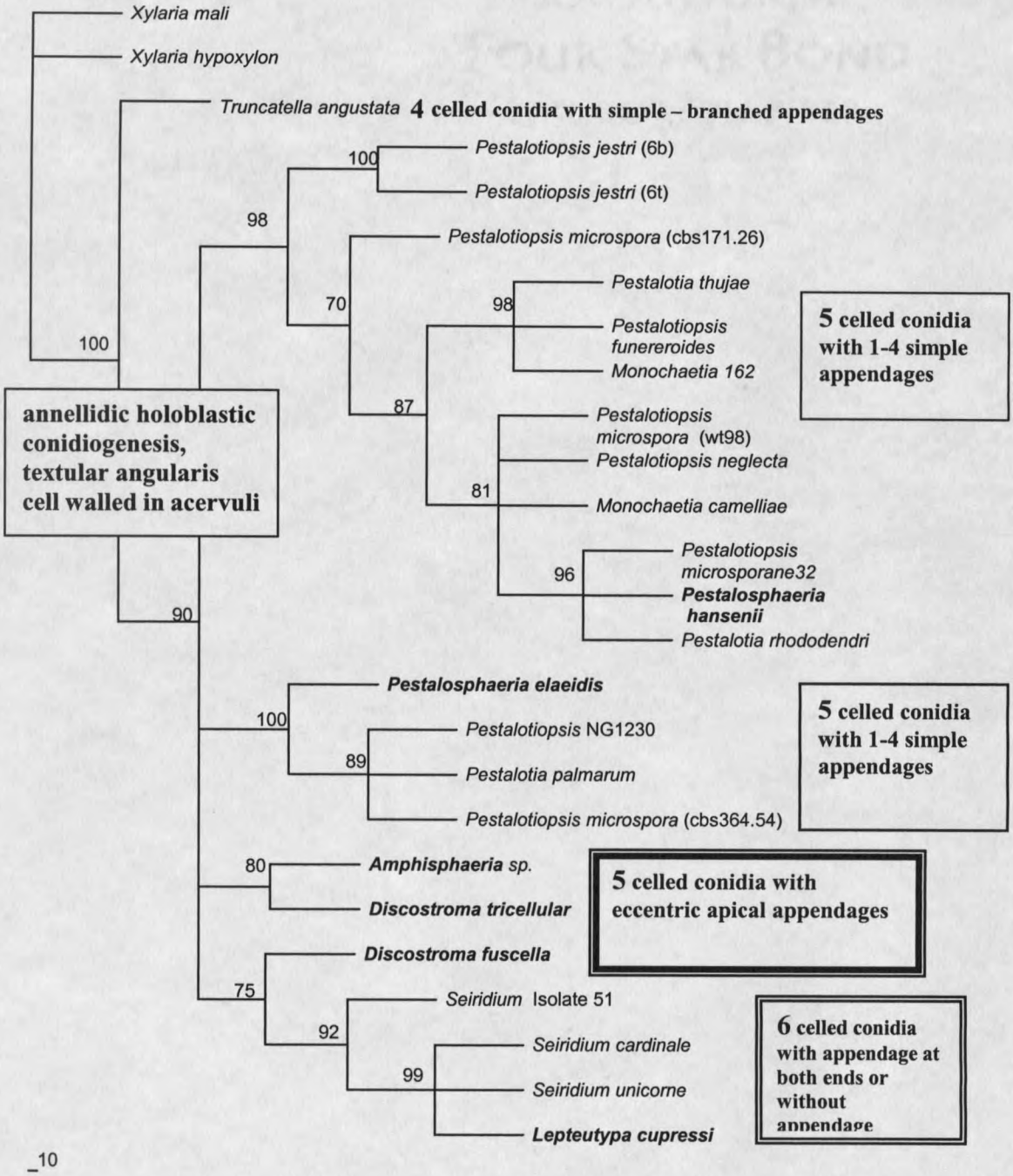
Members in group 4 were *Pestalosphaeria hansenii*, *Monochaetia camelliae*, *Monochaetia* isolate162, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri* (isolate 6m), *Pestalotiopsis jesteri* (isolate 6t), *Pestalotiopsis funereoides*, *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE-32), *Pestalotiopsis microspora* (WT98) and *Pestalotiopsis neglecta*. Most species in the group 4 were isolated from gymnosperm host plants in temperate climates. Both groups 3 and 4 produced 5-celled conidia with three intermediate colored cells and hyaline apical and basal cells (Quinqueloculatae; Klebahn, 1914; Guba, 1961).

Group 5 consists only of *Truncatella angustata* due to limited taxon sampling. *Truncatella angustata* was classified in section Quadriloculatae because this fungus produces 4-celled conidia with two intermediate colored celled and hyaline cells at both end (Klebahn, 1914; Guba, 1961).

More species of *Amphisphaeria*, *Broomella* (*Truncatella* = anamorph), *Discostroma* (*Seimatosporium* = anamorph), *Hymenopleella* (*Monochaetia* and *Seiridium* -like = anamorph) and *Lepteutypa* (*Seiridium* = anamorph) are required for future work. While taxa of temperate climates were isolated primarily from gymnosperms, this might

reflect a bias in sampling. It is of interest, if it is true that selected genera in Amphisphaeriaceae are primarily associated with gymnosperms and other groups of Xylariaceous fungi outside the Amphisphaeriaceae, particularly *Xylaria* and *Rosellinia* are primarily associated with angiosperms (Rogers, 1979). Then, this association may be an indication that selected genera in Amphisphaeriaceae could be an ancestor of Xylariaceae.

Figure 24. A single bootstrap consensus tree inferred from ITS1-5.8S-ITS2 sequences of selected Amphisphaeriaceae. Teleomorph species are presented in bold letters. Possible shared anamorphic characteristics were mapped onto the gene tree.



CHAPTER 5

TAXOL PRODUCTION BY *PESTALOTIOPSIS* SPECIES AND SELECTED GENERA IN AMPHISPHAERIACEAE

Introduction

Many *Pestalotiopsis* species are common endophytic fungi of rainforest plants. In addition to the endophytic lifestyle some of them are reported as mild to serious pathogens of economically important tropical plants such as palms, pines, mangoes and a large number of ornamental plants (Nag Raj, 1993). Generally, however, *Pestalotiopsis* and taxonomically related genera are isolated from inner tissues of surface sterilized stems, twigs, petioles and leaves of healthy plants such as *Taxus wallichiana* (Strobel, 1996a). Close relatives, such as *Monochaetia*, *Seiridium*, *Seimatosporium* and *Truncatella* also occur as endophytes in the tropics and subtopics, but are not as frequently encountered as species of *Pestalotia*, *Pestalotiopsis*, *Monochaetia*, *Seiridium*, *Seimatosporium* and *Truncatella*. All are presumed the anamorphs of genera in the Amphisphaeriaceae, a large and complex family of fungi. These fungi are taxonomically diverse, and produce a wide variety of secondary metabolites. Both *Pestalotiopsis* sp. and its tree host produce taxol, a diterpenoid secondary metabolite with efficacy in inhibiting growth of oomycetous pathogens. Taxol is also an effective chemotherapeutic agent used in breast cancer in humans (Georg *et al.*, 1994). *Pestalotiopsis microspora* produces taxol in significant but not commercially useful amounts (Strobel *et al.*, 1996 a&b). The taxol biosynthetic pathway in yew plants has been published (Koepp *et al.*,

1995; Wildung and Croteu, 1996), but has not been elucidated in fungi. The evolutionary and genetic basis for this unique host-microbe interaction is poorly understood.

Testing endophytes for taxol production has been an ongoing project in the Strobel lab and numerous isolates have been subjected to the methods which will be described in the next section. In addition, *Pestalotiopsis* spp. and other tested genera in the Amphisphaeriaceae and selected genera in the Xylariaceae have been shown by molecular data to have evolved from a common ancestor. The molecular data shows that the taxa examined are all in one clade in the large order Xylariales. The purpose of this research is to determine if phylogenetic relationships can be used to predict which fungal species might produce a particular metabolite, in this case, taxol. Consequently, this research was undertaken to provide new leads to previously unconsidered fungi as taxol producers.

Materials and Methods

Fungal Strains

Fungal strains used in this experiment were either purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, or the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, or provided by Gary A Strobel (Montana State University) and J. D. Rogers (Washington State University). The species and sources are listed in Table 20. *Daldinia fissa*, *Hypoxylon submonticulosum*, *Hypoxylon haematostroma*, *Hypoxylon mediterraneum*, *Hypoxylon rubiginosum*,

Hypoxylon nummularium were tested for taxol production, but they were not included in the phylogenetic tree.

Fungal Growth

The organisms used in this experiment were grown on Potato Dextrose Agar (PDA) for seven to ten days at room temperature (23 ° C). Each fungal culture was cut into small pieces (0.5 x 0.5 cm) using sterile technique. Several of these mycelium agar plugs were used to inoculate liquid media in flasks. The fungus was grown at 23 ° C in 2 liter Erlenmeyer flasks containing 500 ml M1D medium, containing $Ca(NO_3)_2$ (0.28 g/L), KNO_3 (0.08 g/L), KCl (0.06 g/L), $MgSO_4$ (0.36 g/L), NaH_2PO_4-H_2O (0.02 g/L), $FeCl_3$ (2.0 mg/L), $MnSO_4$ (5.0 mg/L), $ZnSO_4$-$7H_2O$ (2.5 mg/L), H_3BO_3 (1.4 mg/L), KI (0.7 mg/L), Sucrose (30 g/L), Ammonium tartate (5 g/L), Yeast extract (0.5 g/L), Soytone (1.0 g/L) (Pinkerton and Strobel, 1976). The fungi were grown in 2-liter Erlenmeyer flasks (triplicate; test1, test2 and test3) without shaking for twenty-one days.

Crude Extraction Isolation Procedures

After twenty-one days the culture fluid was strained through four layers of cheesecloth to remove the mycelium. The filtrate was extracted with two equal volumes of methylene chloride. The organic phase was concentrated under reduced pressure at 35°C. The residue in the methylene chloride extract of each fungus was used to determine the concentration of taxol and taxanes by a competitive inhibition immunoassay method (CIEIA) using a taxol immunoassay kit (Hawaiian Biotechnology

Group, Aiea, Hawaii) (Grothaus *et al.*, 1993). The CIEIA was performed by Cytoclonal Pharmaceutics Inc., 9000 Harry Hines Blvd, Dallas, Texas.

Table 20. List of taxa included in a study of potential taxol producers.

Species	Sources	Original Substrate	Geological origin
Daldinia fissa	J. D. Rogers	No record	-
Hypoxylon submonticulosum	J. D. Rogers	No record	-
Hypoxylon haematostroma	CBS 255.63	No record	Mexico
Hypoxylon mediterraneum	CBS 259.63	No record	California, USA
Hypoxylon rubiginosum	CBS 263.63	No record	Mexico
Hypoxylon vogesiacum	CBS 266.63	No record	Guatemala
Hypoxylon nummularium	CBS 277.61	No record	South Africa;
Amphisphaeria incrustans	ATCC 18007	Decayed *Acer* sp. wood	Quebec, Canada
Amphisphaeria sp.	MAFF235878	*Diospyros kaki thunberg* var. *domestica Makino*	Japan
Discostroma tricellular	MAFF237482	*Rhododendron indicum*	Japan
Monochaetia camelliae	ATCC 60625	*Camellia japonica*	New Zealand
Discostroma fuscella	From fragment 10071	*Rosa* sp.	Canada
Monochaetia camelliae (A)	ATCC60625	*Camellia japonica* (Tea)	New Zealand
Pestalotia rhododendri	ATCC 24306	*Rhododendron* sp.	Italy
Pestalotiopsis hansenii	ATCC 48245	*Pinus carribaea*	New Guinea
Pestalotia thujae (A)	CBS303.75	*Thuja occidentalis*	Netherland
Pestalotiopsis funereoides (A)	CBS175.25	*Juniperus* sp. *and Pinus* sp.	England
Pestalotiopsis neglecta (A)	CBS200.65	*Taxus baccata*	U.K.
Pestalotiopsis jesteri	6b, 6m, 6t	*Fragraea bodenii*	New Guinea
Pestalotiopsis microspora	CBS171.26	*Royena lucida*	Italy
Pestalotiopsis microspora	CBS364.54	Bath Towel at Florida beach	USA
Pestalotiopsis microspora	NE32	*Taxus wallachiana*	Nepal
Pestalotiopsis microspora (A)	WT98	*Taxus wallachiana*	Nepal
Pestalotiopsis sp.	Isolate NG12-30	Moreabina	New Guinea
Seiridium cardinale	ATCC 52521	*Juniperus communis*	Italy
Seiridium unicorne	ATCC 48159	*Cryptomeria japonica*	New Zealand
Truncatella angustata	ATCC 96024	*Prunus avium*	Washington, USA

The CIEIA was carried out using a monoclonal antibody and an alkaline phosphatase reaction with standardization by a curve constructed with a taxol standard supplied with the immunoassay kit. The accuracy of the assay is + or – 1.0 ng per ml (Grothaus *et al.*, 1993). Fungi producing extracts containing more than 500 ng/L of taxol were recultured and extracted under the same conditions for comparative thin layer chromatography (TLC) analyses and purification. Residue from the last TLC separation was sent for electrospray mass spectrometry (MS) analysis. Dr. Joe Sears in the department of Chemistry, Montana State University, Bozeman, Montana, kindly performed the MS test.

Thin Layer Chromatography Separation

The residue was dissolved in 1 ml methylene chloride and placed on a 3.0 x 50 cm column of Silica Gel particle size 32-63 (Selecto Scientific Silica Gel). The solvent for elution of taxol fractions was a mixture of chloroform and methanol at a ratio of 15:1 v/v. Each fraction eluted from the 3.0 x 50 cm column of Silica Gel particle size 32-63 was spotted on a Merck 1mm (10 x 10 cm) TLC silica gel plate (EM-0.25 mm) and developed in a mixture of chloroform and methanol at a ratio 15:1 v/v as a solvent. Taxol is positively identified via its co-chromatographic mobility with authentic standard taxol (purchased from Sigma) in a ratio of 15:1 v/v of chloroform and methanol solvent system. A reagent consisting of 1 % vanillin in sulfuric acid was sprayed to detect taxol on thin layer chromatographic plates. Taxol appear as a dark blue to dark gray band after gentle heating (Cardellina, 1991). The tentatively positive taxol band was scraped off

the TLC plate, eluted from silica by methanol. The residue was evaporated for concentration, and subjected to separation by rechromatography on Merck 1mm (20 x 20 cm) TLC silica gel plates (EM-0.25 mm) in a multitude of thin layer chromatographic systems. These multi-solvent systems were methylene chloride:methanol at 9:1 v/v (solvent A), chloroform:acetonitrile at 3:2 v/v (solvent B), methylene chloride: tetrahydrofuran at 6:1 v/v (solvent C), ethyl acetate:iso-propanol at 95:1 v/v (solvent D) and ethyl acetate:methanol at 5:1 v/v (solvent E). After each chromatographic separation step, the area of the plate containing putative taxol was carefully removed by scraping off the silica at the appropriate R_F, and then exhaustively eluting it with acetonitrile. Authentic taxol was used as a standard for comparative purposes in each analysis. An ultimate check for the presence of a putative taxol band was screened under the 254 UV light. The taxol band shows a bluish to grayish coloration (Cardellina, 1991). The R_F value of the authentic taxol standard and detected taxol were recorded for each solvent system. Each putative residue from each TLC separation solvent system was weighted and sent to the Cytoclonal Pharmaceutics Inc., 9000 Harry Hines Blvd, Dallas, Texas for CIEIA taxol assays.

Results and Discussion

Eighteen taxonomically related species of Amphisphaeriaceae based on molecular phylogenic study, were sampled for the production of taxol. The sampled species were *Daldinia fissa*, *Hypoxylon submonticulosum*, *Hypoxylon haematostroma*, *Hypoxylon mediteraneum*, *Hypoxylon rubiginosum*, *Hypoxylon vogesiacum*, *Hypoxylon*

nummularium, *Amphisphaeria incrustans*, *Amphisphaeria* sp., *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalotia rhododendri*, *Pestalotiopsis hansenii*, *Pestalotiopsis palmarum*, *Seiridium cardinale*, *Seiridium unicorne* and *Truncatella angustata*. The CIEIA determinations revealed only four taxol producers out of the eighteen sampled species. These taxol-producing fungi are *Hypoxylon submonticulosum*, *Pestalotiopsis* isolate NG12-30, *Seiridium cardinale* and *Seiridium unicorne* (Table 21). Table 22 shows fungal species that can produce taxol as reported by Dr. Jia-Yoa Li (1996) and Dr. Bashyal Bharat (1999).

Notably, however, *Pestalotiopsis* isolate NG12-30 produced 16.4 to 43.4 ng/mg of taxol in crude extract whereas *Hypoxylon submonticulosum*, *Seiridium cardinale* and *Seiridium unicorne* produced less than 10 ng/mg of taxol in crude residue (Table 21). Thus the *Pestalotiopsis* isolate NG12-30 has picked for further tests.

Although the CIEIA test for taxol is extremely sensitive when pure taxol is tested, there may be difficulties when a crude fungal preparation is used in the test. For instance, there is substantial variation for taxol detection by a competitive inhibition immunoassay method using a taxol immunoassay kit. The amount of taxol detected for *Hypoxylon submonticulosum* was inconsistent upon dilution (data not shown, personal communication with a technician who runs the CIEIA test). There is also variation in the amount of taxol produced by *Seiridium cardinale*. The variable results of the CIEIA test might indicate false positive results, no taxol in production by the fungi or it is possible that the false positive result may come from other taxane or other compounds which have a structure similar to taxol. Thus an additional screening test is necessary to confirm that

the results from the CIEIA test are not false positives. The second screening test is a comparison of R_f values between an authentic taxol standard and putative taxol bands from different TLC solvent separation systems.

Table 21. Taxol content of crude extracts of various species of Amphisphaeriaceae detected by a competitive inhibition immunoassay method using a taxol immunoassay kit performed by Cytoclonal Pharmaceutics Inc., 9000 Harry Hines Blvd, Dallas, Texas.

Extraction from taxa of	Dry weight of crude extract (mg)	Taxol ng/mg of extract
Hypoxylon submonticulosum	Test 1=12.75	1=3.3*
	Test 2=13.35	2=3.1*
	Test 3=4.90	3=1.9*
Pestalotiopsis NG12-30	Test 1=33.85	43.4
	Test 2=23.70	16.4
Seiridium cardinale	Test 1=6.55	1=17.1
	Test 2=3.95	2=0.62
	Test 3=7.75	3=0.57
Seiridium unicorne	Test 1=6.55	1=1.35
	Test 2=3.95	2=1.40
	Test 3=7.75	3=1.63

*Results are variable depending upon dilution.

For confirmation, the first extract from *Pestalotiopsis* isolateNG12-30 was chromatographed in at least five thin layer chromatographic systems and yielded a band with the same R_F and color reaction with vanillin/ sulfuric acid as authentic taxol. The R_F of the putative taxol bands was the same as the R_F of authentic taxol under five solvent systems mention in materials and methods (Table 23). The identical R_f value for authentic taxol and putative taxol bands suggest that *Pestalotiopsis* isolateNG12-30 has the potential to produce. The results from CIEIA and R_f values from five solvent systems

of TLC chromatography, confirmed that *Pestalotiopsis* isolate NG12-30 could produce taxol.

Table 22. Taxol content of crude extracts of various species of Amphisphaeriaceae detected by a competitive inhibition immunoassay method using a taxol immunoassay kit performed by the Cytoclonal Pharmaceutics Inc., 9000 Harry Hines Blvd, Dallas, Texas as reported by Li and Bharat.

Extraction from taxa of	Taxol ng/l of extract	Extract residue was isolated by
Pestalotiopsis microspora (NE32)	2751	Jia-Yoa Li
Pestalotiopsis microspora (CBS171.26)	756.24	Jia-Yoa Li
Pestalotiopsis microspora (CBS364.54)	92.14	Jia-Yoa Li
Monochaetia isolate 162	102	Bashyal Bharat
Seiridium isolate51	1.62	Bashyal Bharat

Table 23. R_F of authentic taxol standard and putative taxol band from different TLC solvent separation systems for *Pestalotiopsis* isolate NG12-30.

	R_F of authentic taxol standard	R_F of putative taxol band
TLC using solvent A (chloroform: methanol=15:1)	1.23	1.31±0.1
TLC using solvent B (methylene chloride:methanol=9:1)	1.18	1.20±0.1
TLC using solvent C (chloroform:acetonitile=3:2)	1.84	1.88±0.1
TLC using solvent D (methylene chloride:tetrahydrofuran= 6:1)	2.54	2.57±0.1
TLC using solvent E (ethyl acetate:iso-propanol=95:1)	1.23	1.23±0.1

Pestalotiopsis isolate NG12-30 was then cultured again in two liters of M1D medium for twenty-one days under the same conditions described previously. After extraction with methylene chloride, total dried weight of the crude extract was 244.95 mg (Table 24). In the next step 229.60 mg of crude extraction residue was dissolved in

methanol and run through a silica column with a chloroform: methanol (15:1 v/v) solvent while the15.35 mg of crude extraction was sent for CIEIA test to confirm taxol production. The CIEIA test indicates that the remaining 15.35 mg of crude extract had taxol at 94.6 ng (6.16 ng/mg) (Table 24). Fractions showing putative taxol bands were pooled and concentrated for the next TLC purification steps.

TLC in five solvent systems was performed to purify the putative taxol bands. Extract residue from silica column, 207.65 mg, was dissolved in methanol, then run on the 20x20 cm. TLC plates. The area of the plate containing putative taxol was carefully removed by scraping off the silica at the appropriate R_F and exhaustively eluted with acetonitrile. Authentic taxol was used as a standard for comparative purposes in each analysis. The consecutive separation systems were mentioned in materials and methods. Total amounts of residue obtained from differently sequential separation systems were 207.65, 79.05, 30.50, 1.80, 4.70 mg and 30.0 µ of Methanol (Table 24). By the same token, the residue from each separation was separated and sent for the CIEIA test to confirm putative taxol bands. Total weight of extract residue from each separation (or each solvent system) for CIEIA test is listed in Table 24.

The CIEIA tests revealed that extraction residue from each separation had taxol at 55.6 ng/27.55 mg (2.02 ng/mg), 75.0 ng/4.40 mg (17.05 ng/mg), 70.0 ng/12.55 mg (5.58 ng/mg), 81.8 ng/0.55 mg (148.72 ng/mg), 10.7 ng/0.05 mg (214.0 ng/mg) and 4.04 ng/5 µl of 100% Methanol (0.08 ng/µl.). The positive results of five separation steps indicated that there is putative taxol after consecutive separation systems, and that the specific activity for taxol by the CIEAE test dramatically increased (Table 24).

Table 24. Purification Taxol from NG12-30 based on CIEAE analysis.

	Total weight of extraction residue before purification (mg)	Total weight of extraction residue before next purification (mg)	Total weight of extraction residue for CIEAE analysis (mg)	Taxol/sample (ng)	Taxol in ng /mg of extraction residue
Crude extract from the first separation using methylene chloride	244.95	229.60	15.35	94.6	6.16
Separation 2 Residue from silica gel column	207.65	180.10	27.55	55.6	2.02
Separation 3 Residue from TLC using solvent A (chloroform:methanol=15:1)	79.05	79.05	4.40	75.0	17.05
Separation 4 Residue from TLC using solvent B (methylene chloride:methanol=9:1)	30.50	30.50	12.55	70.0	5.58
Separation 5 Residue from TLC using solvent C (chloroform:acetonitile=3:2)	1.80	1.25	0.55	81.8	148.72
Separation 6 Residue from TLC using solvent D (methylene: chloride:tetrahydrofuran= 6:1	4.70	4.65	0.05	10.7	214.00
Separation 7 Residue from TLC using solvent E (ethyl acetate:iso-propanol=95:1)	30.0 µl of Methanol	25.0 µl of Methanol	5.0 µl of Methanol	4.04	0.808 ng/µl

The 25 µl of residue extraction from the last separation was sent for characteristic electrospray mass spectrometry. The results of the electrospray MS showed no peak of taxol at 854 of taxol plus proton, and 876 of taxol plus sodium ion (Stierle *et al.*, 1993, Strobel *et al.*, 1996) (data not shown). Lacking detection of taxol peaks may have been due to the low amount of taxol present. A reason that might explain why there was no peak of taxol in the mass spectrum is that other groups of taxane compounds may give

false positive results on either the CIEAE analysis or the multiple TLC solvent system, but this unlikely seems given the results in table 22. Possibly, more taxol could be produced by *Pestalotiopsis* isolate NG12-30. For example, experimental conditions might include shaking the culture flask instead of incubating the fungus under the still conditions. Adding supplements or minerals might induce this fungus to produce larger amounts of taxol. Before pursuing electrospray MS, quantities of putative taxol must be estimated by its UV absorption at 273 nm comparatively to authentic taxol standard (Wani et al., 1971). The millimolar absorption coefficient for taxol at 273 nm is 1.7.

Taxol was found inconsistently among taxonomically related genera of *Pestalotiopsis* (Figure 25). This result suggests that molecular phylogeny of *Pestalotiopsis* sp. and selected Amphisphaeriaceae based on the ITS1-5.8S-ITS2 is not a useful tool for tracing taxol producers for all groups in general. However, taxol producers (either taxol^{+} or taxol^{-}) mapped onto a bootstrap single most parsimonious tree inferred from ITS1-5.8S-ITS2 sequences of selected Amphisphaeriaceae reveal some interesting information. For example, taxol production appears restricted to the anamorphic state (Figure 26). These anamorphs are *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolation 51, *Monochaetia* isolation162, *Pestalotiopsis* isolate NG12-30 (or *Pestalotiopsis palmarum*), and *Pestalotiopsis microspora* (CBS364.54) from tropical palms. *Seiridium cardinale* (ATCC52521), *Seiridium unicorne* (ATCC48159) were reported as pathogens on *Juniperus communis* and *Cryptomeria japonica*, respectively (ATCC database at http://www.atcc.org). Most teleomorphs including *Pestalosphaeria hansenii, Discostroma fuscella, Discostroma tricellular* and

Amphisphaeria sp. (MAFF237482) did not produce taxol in detectable quantities. In addition, those cultures purchased from ATCC and CBS are fungal cultures which were isolated from the respective host plants a long time ago. For example, *Pestalotia rhododendri* (ATCC24306) was described in 1929 and *Pestalotiopsis funereoides* (CBS 175.25) in 1925 (ATCC database at http://www.atcc.org and CBS database at http://www.cbs.knaw.nl). Also the taxol-producing pathway may need a biochemical signal from the host plant to initiate taxol production. Separation from the host for a long period of time may result in loss of the ability to produce taxol. It is also quite obvious that almost all *Pestalotiopsis* spp. isolated from yew trees produce taxol, including *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (WT98), *Pestalotiopsis microspora* (NE32), with an exception of, *Pestalotiopsis neglecta.*

It is possible that the genetic relatedness of fungal species could help indicate the production of other biochemical compounds in a general sense (on a broad scale). For example, a novel bioactive organic acid named ambuic acid has been described from *Pestalotiopsis* spp. obtained from representative rainforests on 4 continents (Li *et al.*, 2001). This novel acid was also found in a *Monochaetia* species that was originally isolated as an endophyte from *Taxus walliachiana* in the Himalayan foothills of Nepal. These organisms should be carefully examined for bioactive secondary metabolites as has been done with other *Pestalotiopsis* species. It is interesting that the fungus *Pestalotiopsis microspora* and close related genera such as *Monochaetia* can produce not only taxol but also ambuic acid (Li *et al.*, 2001). Ambuic acid, a cyclohexenone, has an antimycotic function against oomycetous fungi that cause plant disease. Similarly,

jesterone and hydroxy-jesterone, which are produced by *Pestalotiopsis jesteri*, can inhibit the growth of plant pathogenic Oomycetes (Li and Strobel, 2001). Interestingly, most *Pestalotiopsis* spp. and their close relatives produce compounds that can inhibit the growth of the root rot pathogenic *Pythium*. These compounds do have similar structures (Strobel, personal communication). This phenomenon shows that *Pestalotiopsis* spp. and their close relatives exhibit a diversity of secondary metabolites.

In conclusion, the molecular phylogeny of *Pestalotiopsis* species and selected Amphisphaeriaceae based ITS1-5.8S-ITS2 is not a useful tool for tracing taxol producers for all selected taxa in Amphisphaeriaceae, but the genetic relatedness of these fungal species may imply which taxol producers occur in specific groups in sampled taxa in this research. For example, no teleomorphs produce taxol. All *Seiridium* spp. tested produced taxol. *Pestalotiopsis* spp. found in tropical climates and associated with palms showed taxol production. For the large clade of *Pestalotiopsis microspora* (CBS171.26), *Pestalotia thujae*, *Pestalotiopsis funereoides*, *Monochaetia* isolate162, *Monochaetia camelliae*, *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis microspora* (NE32), *Pestalosphaeria hansenii* and *Pestalotia rhododendri*, several taxol producers are found within the clade, and most are formed on yew trees. Thus, these organisms should be as carefully examined for bioactive secondary metabolites as has been done with other *Pestalotiopsis* spp. Several rationales can explain a lack of taxol production in some taxa. First, those fungi may not basically produce taxol. Second, those fungi do not produce taxol under the given conditions. Third, fungal cultures removed from the respective host plants a long time ago might, therefore, lacking a

biochemical signal from the host plant to initiate taxol production. Forth, the amount of taxol produced is too small to be detected. Finally, there is an error in experimental method.

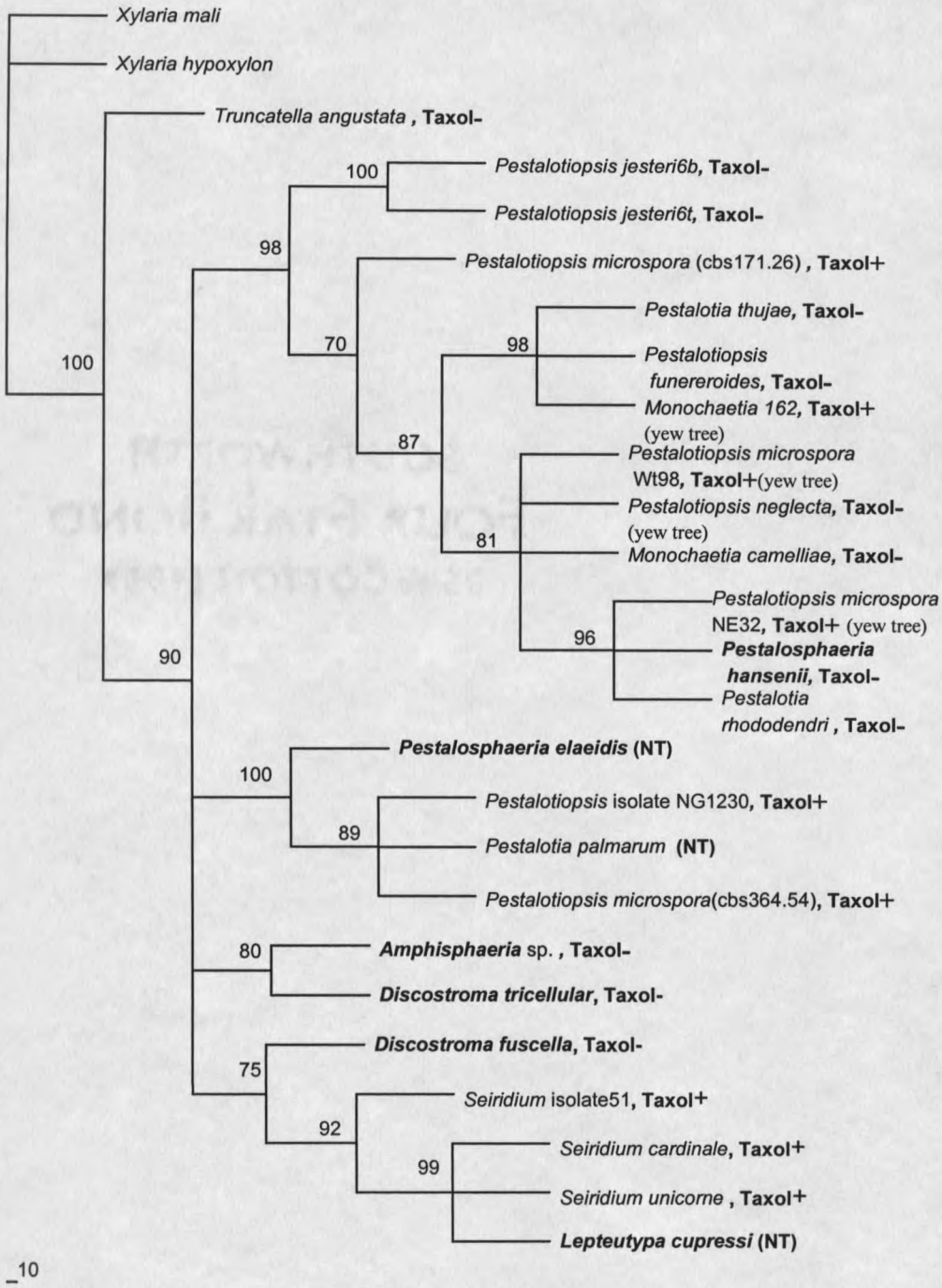


Figure 25. A bootstrap consensus tree inferred from ITS1-5.8S-ITS2 sequences of selected Amphisphaeriaceae. Taxol producer either $Taxol^{+}$ or $Taxol^{-}$ is mapped onto this gene tree. NT depicts those species have not been tested for taxol producing. Teleomorphs represented in bold letters.

CHAPTER 6

SUMMARY

1. UV irradiation of *Pestalotiopsis microspora* WT98 conidia was found to alter basic morphological characteristics important in species classification, which are the number of apical appendages and the number of septa on five–celled conidia with three intermediate cells (2-3 apical and 1 basal appendage per conidium). These biotypes, for example, produce five-celled conidia similar to other fungi such as *Monochaetia* spp. (apical and one basal appendage per five-celled conidium) and *Truncatella* sp. (typically branched apical appendage per four-celled conidium). Thus, the number of appendages and septa is unstable, and both of them are not good characteristics for taxonomic considerations. Nevertheless, cell number seems more stable.
2. Single cell cultures of each of these UV irradiated biotype fungi retain 100% homologies to 5.8s and ITS regions of DNA to the wild type source fungus –*P. microspora* suggesting that no UV induced mutation occurred in this region of the genome.
3. The conversion of *Pestalotiopsis microspora* by UV irradiation into fungal biotypes bearing a conidial resemblance to *Monochaetia* spp. and the significant homology (96-97%) of ITS1-5.8S-ITS2 regions between genera of *Pestalotia*, *Pestalotiopsis*, and *Monochaetia* suggests that these genera are very closely related or even synonymous.

4. Based on phylogenetic analysis of partial 18S rDNA sequences, fungal orders examined in this research formed clades identical to those reported by Spatafora *et al.*, (1993). These orders were Clavicipitales (*Balansia sclerotica* & *Claviceps paspali*), Hypocreales (*Hypomyces polyporinus* & *Hypocrea schweinitzii*), Microascales (*Ceratocystis fimbriata* & *Ceratocystis virescens*), Diaporthales (*Diaporthe phaseolarum* & *Leucostroma persoonii*), Sordariales (*Chaetomium globosum* & *Neurospora crassa*) and Xylariales (*Diatype disciformis*, *Hypoxylon atroroseum*, two strains of *Hypoxylon fragiforme*, *Xylaria hypoxylon*, *Xylaria curta*, *Amphisphaeria sp.*, *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne*, *Truncatella angustata*.

5. Phylogenetic analysis based on partial 18S rDNA sequences suggests that selected genera in Amphisphaeriaceae, which include *Amphisphaeria sp.*, *Amphisphaeria incrustans*, *Discostroma fuscella*, *Discostroma tricellular*, *Monochaetia camelliae*, *Pestalosphaeria hansenii*, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri*, *Pestalotiopsis microspora*, *Seiridium cardinale*, *Seiridium unicorne* and *Truncatella angustata* are a monophyletic group in the Xylariales, Pyrenomycetes. Parsimony analysis of partial 18S rDNA sequences shows that these selected genera in Amphisphaeriaceae are very closely related to *Hypoxylon fragiforme*, a species in Xylariaceae within

the Xylariales, thereby supporting the sharing of a common ancestor. Such a relationship is evident in analyses using neighbor-joining methods based on distance measures.

6. Based on parsimony analysis of partial 18S rDNA sequences a monophyletic group of those selected genera in Amphisphaeriaceae should be placed in the Xylariales, Pyrenomycetes. This phylogenic classification is consistent with classical classifications done by Eriksson and Hawksworth, 1993, Barr, 1994, and Hawksworth *et al.*, 1995, but contrary with the reestablishment of the order Amphisphaeriales by Kang *et al.* (1998) based on 5.8S rDNA and ITS2 region.
7. The anamorphic morphology shared in all showed taxa on the clade Xylariales is the holoblastic conidiogenous cell.
8. It seems that for the analysis in this study the region of 18S rDNA sequence is more helpful in resolving phylogenetic relationships at or above the ordinal level. For example, the relationships among the orders received a robust statistical value. Unresolved inter-branches from 18S rDNA analysis and uneven distribution of morphological features suggest the 18S rRNA gene is not divergent enough to distinguish the selected genera in Amphisphaeriaceae.
9. Parsimony analyses of the complete ITS1-5.8S-ITS2 sequences provided strong statistical support for a monophyletic group of selected species in Amphisphaeriaceae. These selected species include *Amphisphaeria* sp. (isolate MAFF235878), *Discostroma fuscella*, *Discostroma tricellular*, *Pestalosphaeria hansenii*, *Lepteutypa cupressi*, *Monochaetia camelliae*, *Monochaetia* isolate162,

Pestalotia rhododendri, *Pestalotia thujae*, *Pestalotiopsis jesteri* (isolate 6m), *Pestalotiopsis jesteri* (isolate 6t), *Pestalotiopsis funereoides*, *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE-32), *Pestalotiopsis microspora* (WT98), *Pestalotiopsis neglecta*, *Pestalotiopsis* isolate NG12-30, *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolate 51, *Truncatella angustata*, *Pestalosphaeria eleaidis* and *Pestalotia palmarum*. Neighbor joining analysis of the same sequence alignments revealed an essentially similar topology. The synapomorphic characteristic of all selected taxa in Amphisphaeriaceae seems to be an annellidic holoblastic type of conidiogenous cell in acervuli of textura angularis cells of the anamorphic state.

10. Analysis of the complete ITS1-5.8S-ITS2 sequences shows that Amphisphaeriaceae were restricted to *Amphisphaeria*, *Discostroma*, *Lepteutypa*, *Pestalosphaeria* and other genera possessing *Pestalotia*-like anamorphs such as *Monochaetia*, *Pestalotiopsis*, *Seiridium* and *Truncatella*. These genera are consistent within genera of Amphisphaeriaceae for the classical classifications done by Barr (1994), and Kang *et al.* (1998) based on the 5.8S rDNA and ITS 2 sequences.

11. Phylogeny analysis of the complete ITS1-5.8S-ITS2 sequences can delineate selected genera in Amphisphaeriaceae into five groups. Group 1 is represented by *Discostroma fuscella*, *Seiridium cardinale*, *Seiridium unicorne*, *Seiridium* isolate 51 and *Lepteutypa cupressi*. The majority of genera in this section are

correlated with anamorphs producing conidia with 6-cells and four intermediate brown colored cells; hyaline apical and basal cells together. Host plants of group 1 appear to be primarily the cypress family. Group 2 is composed of *Amphisphaeria* sp. and *Discostroma tricellular*. Both anamorphic forms of *Amphisphaeria* (*Bleptosporium*) and *Discostroma* (*Seimatosporium*) have unique eccentric apical appendages. The major hosts of group 2 are dicot angiosperm tropical plants. Group 3 included *Pestalotiopsis microspora* (CBS364.54), *Pestalotiopsis* isolate NG12-30, *Pestalosphaeria eleaidis* and *Pestalotia palmarum*. Species in group 3 distinctively reflect a specific plant host in the palm family in tropical climates. Members in group 4 are *Pestalosphaeria hansenii*, *Monochaetia camelliae*, *Monochaetia* isolate162, *Pestalotia rhododendri*, *Pestalotia thujae*, *Pestalotiopsis jesteri* (isolate 6m), *Pestalotiopsis jesteri* (isolate 6t), *Pestalotiopsis funereoides*, *Pestalotiopsis microspora* (CBS171.26), *Pestalotiopsis microspora* (NE-32), *Pestalotiopsis microspora* (WT98) and *Pestalotiopsis neglecta*. Most species in group 4 were isolated from gymnosperm host plants in temperate climates. Both group 3 and 4 produce 5-celled conidia with three intermediate colored cells and hyaline apical and basal cells. Group 5 consists of only *Truncatella angustata*, a fungus that produces 4-celled conidia with two intermediate colored celled and hyaline cells at both ends.

12. The comparison of the complete ITS1-5.8S rDNA-ITS2 sequences among selected genera in Amphisphaeriaceae is identical for a completely conserved

5.8S rRNA gene, but shows variation in both internally transcribed spacer regions. The high degree of conservation of the ITS2 compared with ITS1 region among the studied genera is quite distinctive.

13. Molecular phylogeny of *Pestalotiopsis* species and selected Amphisphaeriaceae based ITS1-5.8S-ITS2 is not a useful tool for tracing taxol producers for all selected taxa in Amphisphaeriaceae, but could be an indication of tentative taxol producers within specific groups of sampled taxa in this research. The criteria that should be considered for tentative taxol producers are anamorphic genera of *Pestalotiopsis* spp. associated with yew trees from temperate regions and palms from tropical climates, and the anamorphic genera of *Seiridium*.

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APPENDICES

APPENDIX A

PHYLOGENETIC ANALYSIS

Terminology of Phylogenetic trees

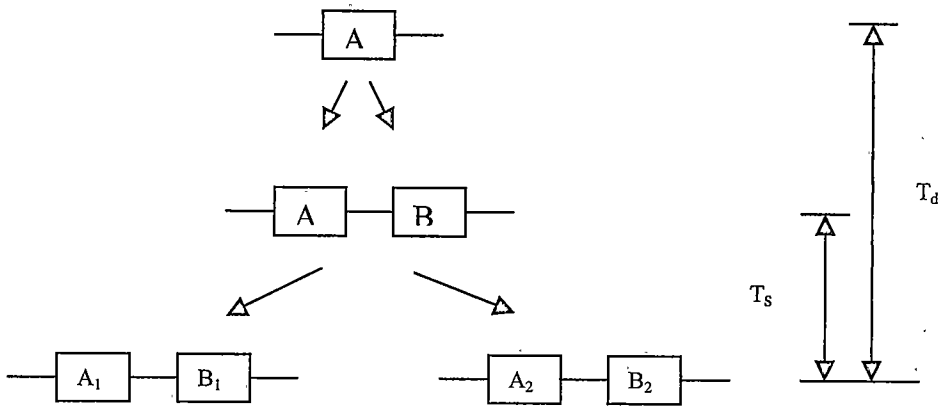
Phylogenetic analysis of DNA or protein sequences becomes useful tools for studying the evolutionary history of organisms, and also for understanding the pattern of genetic evolution. To understand phylogenetic discipline very well, there are many important vocabularies that should be considered before discussing phylogenetic analysis methods. Those terms, which were reviewed from several molecular evolution references (Graur and Li, 2000, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000) will be presented as the following subtopics: a phylogenetic tree, rooted and unrooted trees, gene tree and species tree, taxa and sister taxa, monophyletic groups and paraphyletic groups, type of data, true tree and inferred tree, orthologous genes and paralogous genes, cladistics and phenetics.

A Phylogenetic tree

A way to illustrate the evolutionary relationships among organism is a graph called a **phylogenetic tree** or **dendrogram** (Graur and Li, 1999, and Li, 1997). A dendrogram is comprised of **nodes** and **branches**. The nodes represent the taxonomic units, which can be species, populations, individuals, or genes. One branch will connect any two adjacent nodes. **Terminal nodes** are the nodes at the tips of branches with no descendants whereas **internal nodes** are the nodes between ancestor and the terminal nodes. Terminal nodes represent the **operational taxonomic units (OTUs)** under comparison. **External branches** are branches that end in a tip. The branching pattern of a phylogenetic tree is called **topology**.

There are two patterns of topology: a **bifurcating topology** and a **multifurcating topology** (Graur and Li, 1999, Li, 1997 and Nei and Kumar, 2000). A bifurcating topology is a tree that has only immediate descendant lineage because a DNA sequence splits into two descendant sequences in a gene duplication event or at the time of speciation (Li, 1997 and Nei and Kumar, 2000).

Figure 26. Duplicate genes from two different species. Gene A_1 and A_2, and B_1 and B_2 are orthologous, but the A genes are paralogous to the B genes. Ts represents the time of speciation when the species spitted into two species whereas Td is the time of a gene duplication event (Modified from Nei and Kumar, 2000).



In Figure 26, the time of speciation equals to the time that two species split from one another. A multifurcating topology is a tree that has more than two immediate descendant lineages. The multifurcating tree can be obtained from a short sequence data that have no nucleotide substitution, thus a multifurcating node will show up (Nei and Kumar, 2000). An error of statistic reconstruction of phylogenetic tree may result in the multifurcating tree (Nei and Kumar, 2000).

Rooted and unrooted trees

A rooted tree has a node designated as the root (Figure 2a). The rooted tree has polarity that indicates nodes being ancestor or descendants, and also has an evolutionary path that lead from ancestor to any other descendants (Graur and Li, 2000, and Li, 1997). In a rooted tree, a formula to find number of internal nodes is n-1, of which n is number of terminal nodes. An unrooted tree is a tree that does not define a node as a root; thus it has no evolutionary path. It has been mentioned that an unrooted tree is not a true phylogenetic tree because we cannot specify the direction of evolutionary path. The terminal nodes represent the n OTUs and n-2 internal nodes. The internal branch is called as the **central** branch if an unrooted tree has four external nodes. All methods for building a phylogenetic tree usually give unrooted trees. We can root an unrooted tree by putting sequences of an outgroup into the molecular data. The **outgroup** must have character states that show that the branched off earlier than the taxa under study.

Scaled and unscaled trees

Two ways to draw a phylogenetic tree are as scaled and unscaled trees (Graur and Li, 2000, and Li, 1997). First, a scaled tree has branch lengths that are proportional to the number of changes which happened along that branch (e.g., nucleotide substitutions). Another way to draw a tree is to have an unscaled branch of a tree. It means that the branch lengths are not proportional to the number of changes. For the unscaled tree, we can position OTUs and internal nodes to time of divergence on the unscaled tree.

True tree and inferred tree

A **true tree** is a phylogenetic tree from all possible trees, which is obtained from the real number of nucleotide substitution (Nei and Kumar, 2000). A true tree delineates the true evolutionary history of a given number of OTUs (Graur and Li, 1999, and Li, 1997). In contrast, an **inferred tree** is built from observed sequence data or expected number of substitution by certain methods of tree reconstruction (Graur and Li, 2000, Li, 1997, and Nei and Kumar, 2000). It is not necessary that an inferred tree should be the same as true tree.

Orthologous genes and paralogous genes

Othologous genes are genes derived from a speciation event, while **paralogous genes** are genes derived from a duplication event (Li, 1997). For example, in Figure 2, gene A_1 from species 1 and gene A_2 from species 2 are orthologous, and so are gene B_1 from species 1 and gene B_2 from species 2. Both of them were obtained from a process of speciation, the splitting of an ancestral species into two descendant ones. Genes A and gene B are paralogous since both of them were derived from duplication of an ancestral gene.

Gene tree and species tree

Li (1997) described that a **gene tree** is constructed from one gene from each species, but a **species tree** is derived from gene that depicts the evolutionary pathway of a group of species. We can not use gene trees to infer species tree. Graur and Li (1999) rationalized that species trees are good to search for the history of many genes from different species and the routes of inheritance for the species; however, gene trees are useful to trace the evolutionary relationships among different members of a gene family. They also explained that in gene trees, the branching pattern presents pathways of genes from parents to offspring. Different genes may have different routes of inheritance.

Taxa and Sister Taxa

Graur and Li (1999) defined a **taxon** as a given name of a species or a group of species, such as a genus, family, order and class. Every taxon name must be used for only one taxon, for example, *Homo sapiens* (the species name for modern humans). In a monophyletic group, if there are two taxa, both of them are mentioned as a **sister taxa** (Graur and Li, 1999).

Monophyletic groups and paraphyletic groups

Graur and Li (1999) stated that **monophyletic groups** or **natural clades** are a group of all the taxa that share a single common ancestor, including the common ancestor itself. On the other hand, **paraphyletic groups** are terms describe for groups whose common ancestor is shared by any other taxa.

Type of Data

Graur and Li (1999) described that two types of molecular data are **character data** and **distance data**. We can get information about an individual taxon from **character data** such as the nucleotide or amino acid at a particular site. In contrast **distance data** provide quantitative data referring to the dissimilarity between two taxa, e.g., the number of nucleotide or amino acid substitutions (Graur and Li, 1999 and li, 1997). Moreover, we can convert character data in to distance data, but we cannot transform distance data to character data.

In character data, two words, which should be contemplated in meaning, are a character versus character states (Graur and Li, 1999). A character is a well-defined feature and an independent variable unit, such as the ninety-ninth nucleotide position in 5.8S rDNA sequence. In contrast, the character state is the value of the character in a particular OTU, e.g., four nucleotide bases in DNA sequences or twenty amino acids in amino acid sequences. For instance, if nucleotide A is observed at position 155 of 18S rDNA sequence from *Pestalotiopsis microspora*, then the OTU is *Pestalotiopsis microspora*, position 155 is the character, and A is the character state found in the character in *P. microspora*. Graur and Li (1999) referred binary characters to a character that has two state characters such as the form of the presence and absence of a molecular marker, e.g., the presence or absence of retrotransposon at a certain genomic location. In contrast, multi characters are mentioned to a character that has more than two state characters like positions in DNA or amino acid sequences.

Swofford and Olsen (1990) pointed out three advantages of distance data. First, character states themselves are meaningless for study of evolutionary relationships among organisms. Character data will be meaningful if it is converted to distance data by comparing an individual sequence with other sequences. For instance, if the results of comparison show that the similarity between two sequences is 93 %, while the similarity between one of these sequence and a third one is only 50 % homologies, then these results imply a visceral picture of an evolutionary relationships among these three taxa. Second, we can construct a hypothesis about the unseen evolutionary process from distance data such as the number of substitutions between two sequences. Third, there are a lot of fast and efficient statistic methods to reconstruct a phylogenetic tree from distance data.

Graur and Li (1999) also mentioned that distances can be additive. We can calculate the distance between any two OTUs by the sum of the lengths of all the branches connecting them if the branch lengths are estimated from the molecular data (e.g., DNA sequences) according to certain rules. We call this kind of tree an additive tree. However, a tree can not be additive if there are multiple substitutions between sequences at any nucleotide sites, or all the studied OTUs evolve at the same rate (Graur and Li, 1999 and Li, 1997).

Cladistics and Phenetics

Two confusing terms in molecular phylogeny are cladistics and phenetics. Li (1997) explained the meaning of cladistics as the study of the pathways of evolution. A

cladogram is the topology of a rooted phylogenetic tree which presents ancestor and descendant relationships, with emphasis on how many branches there are among a study group of organism, what the branching sequencing is and which branch connects to which other branch (Sneath and Sokal, 1973). In contrast to cladistics, phenetics is the study of relationships among a group of organisms based on the degree of similarity of phenotype, anatomy or molecule (Li, 1997). A phenogram is a treelike network expressing both phenetic relationships and sometimes cladistic relationships. However, phenogram is not necessary identical to cladogram, except, the time of divergence is equal to the degree of genetic or morphological divergence.

Phylogenetic Analysis

At present many statistical methods have been developed for phylogenetic analysis of molecular data. To be able to build correct phylogenetic trees, one should comprehend the methodology of molecular phylogenetics. The following discussion focuses only methods for analysis of DNA sequences that have partial base and codon divergences. Fundamental principles of tree reconstruction from nucleotide sequences will be addressed; more details are reviewed elsewhere (Graur and Li, 2000, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). In fact, Graur and Li (1999) mentioned that amino acid sequences give more reliable molecular data than nucleotide sequences since amino acids evolve slower than nucleotides do. Moreover, the chance of identity at any one location of the amino acid sequence is lower than for nucleotides because there are twenty amino acids, but only four nucleotides (Graur and Li, 1999).

Four basic steps in phylogenetic analysis are alignment, determining the substitution model, tree building, and tree evaluation (Hershkovitz and Leipe, 1998).

Alignment

Many references emphasize that sequence alignments are the first important and critical step in phylogenetic analysis (Graur and Li, 2000, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). Errors in alignment can mislead further analyses. Consequently, we must align sequences carefully and remove poorly aligned sequences before continuing the analysis.

Graur and Li (1999) outlined that sequence alignment is the comparison of two homologous sequences to identify sites of matching, insertions or deletions (gaps) and nucleotide substitutions (mismatches; transitions or transversions) as a result of their divergences from a common ancestor. However, gaps in a sequence alignment can not tell us whether an insertion has occurred in one sequence or an insertion has occurred in the other (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). According to Graur and Li (1999), both terminal gaps (i.e., the missing data from the first sequence) and internal gaps (i.e., introns) are excluded from the alignment of a partial sequence of gene with a complete sequence of homologous gene. In addition, an alignment with a lot of gaps would be unrealistic since insertion and deletion occurred fewer frequencies than nucleotide substitutions (Nei and Kumar, 2000). (Anneke does not clear this point, It means that it just does not happen in realality, Gene is rarely lost, so nucleotide substitutions happen more frequency.) When two sequences

have high similarity, we can do a manual alignment by eye, if the data is examined closely and critically (Graur and Li, 1999 and Nei and Kumar, 2000). However, manual alignment of multiple sequences that have high degree of divergence by visual inspections may increase errors due to bias and inexperience of editors.

In general, phylogenetic sequence data is comprised of a multiple sequence alignment which can de done by heuristic methods of a computer program. CLUSTAL (Higgins and Sharp 1988 and 1999), MACAW (Schuler *et al.*, 1991) and MASH (Chappey *et al.*, 1991) are popular computer programs for distantly related sequences and larger and larger groups of data (Graur and Li, 1999). Most of these programs use a progressive alignment algorithm, which firstly calculates all possible pairwise alignments among the studied sequences, then identifies the pair with the highest score. Results of the progressive algorithm show the aligned sequences in the order of decreasing similarity scores. The progressive algorithm presents fast and quite acceptable results (Hershkovitz and Leipe, 1998). However, many references have shown that phylogenists always carefully "fine tune" the alignment manually afterward to improve the quality of the alignment (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000).

Constructing Phylogenic Trees

Graur and Li (1999) described that an inferred tree is the best estimate of the evolutionary history, which was computed from the incomplete information in the past. They classified methods of constructing phylogenic trees into three categories based on

types of measurement. These three procedures are the distance matrix, maximum parsimony and likelihood methods.

Distance Methods

The distance matrix approach is a method relying on the measurement of the number of nucleotide substitutions or amino acid replacements in a sequence (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). Hershkovitz and Leipe (1998) summarized that distance matrix methods begin the process by counting the number of the differences between two sequences. This number is referred to as the evolutionary distance value. The inferred tree is then computed from the matrix of distance values by running a clustering algorithm that starts with the most similar sequence [i.e. those that have the shortest distance between them] or by trying to minimize the total branch length of the tree. Two common methods of producing a distance tree, which are unweighed pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ), will be discussed here. Minimum evolution (ME) and Fitch-Margoliash will not be discussed in this work because they are rarely used (Hershkovitz and Leipe, 1998).

Unweighed Pair Group Method with Arithmetic Mean (UPGMA)

UPGMA is a phenetic algorithm, which builds tree branches from the greatest similarity among pairs and averages of joined pairs. UPGMA is expected to generate an accurate topology with true branch lengths only when the divergence is according to a molecular clock (all sequences evolve at the same rate).

Strength of this method is that it is a fast method since the OTUs are sequentially clustered to derive a final tree. Weaknesses of UPGMA are, for example, if the sequences used are not long, some branches may by chance happen to have evolved faster than others, thus in effect violating the rate constancy assumption. For this reason, an inferred UPGMA tree often involves errors in branching order, even if the assumption of equal rates holds. In addition, when unequal rates occur, the UPGMA method often becomes inconsistent.

Neighbor Joining (NJ)

Neighbor joining finds the neighbors sequentially that may minimize the total length of the tree. It starts with an unresolved star-like tree, the one that gives the smallest sum of branch lengths. This procedure is continued until N-1 interior branches are found. A distinct advantage is that the computational time is fast since the OTUs are sequentially clustered to derive a final tree. NJ remains consistent if the distances are estimated accurately.

Maximum Parsimony (MP)

Maximum parsimony is a character state method in which the data is calculated from the numbers of deletions or insertions of nucleotides or amino acids at an informative site for constructing a MP tree (Graur and Li, 1999). The principle of maximum parsimony is that firstly MP identifies all the **informative sites**, and then calculates the minimum number of substitutions at each informative site (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). Finally, a tree with the smallest

number of changes will be chosen to explain the differences observed among the taxa under study. Tree branch length in particular MP tree is equal to the total number of substitutions at both informative and uninformative sites.

Nei and Kumar (2000) have explained that only **variable sites** in studied sequences are used for maximum parsimony method while sites that have the same nucleotide for all taxa (**invariable sites**) are excluded. Variable sites can be either informative or uninformative. Many references explained about informative sites for MP. A site is informative only when there are at least two different kinds of nucleotides of the sequences under study (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). For example, sites number 2 and 4 of sequences 1, 2, 3 and 4 shown below as bold letters are informative because these sites possess at least two different kinds of nucleotides and represented at least two times. In contrast, sites number 1, 3 and 5 are uninformative sites because they do not have this condition.

Sites Sequence	1	2	3	4	5
1	G	**G**	A	**C**	A
2	C	**A**	G	**C**	A
3	A	**A**	G	**T**	A
4	A	**G**	G	**T**	A

However, Nei and Kumar (2000) called informative sites in MP analysis **parsimony-informative** sites because invariable sites will be useful or give some informative information in distance and maximum likelihood methods.

Commonly, MP method can be classified in to two categories; unweighed and weighted parsimony (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and

Nei and Kumar, 2000). Unweighed parsimony means that all different nucleotide substitutions are given equal weight. On the other hand, weighted parsimony involves different events at various character state changes. For instance, transversion is weighted greater than transition because in reality transversional substitution happen less frequently than transitional events.

Nei and Kumar (2000) categorized ways of searching MP trees into two strategies. The first one is when the number of OTUs or taxa is less than 10, then the exhaustive search is a suitable way to obtain MP tree. However if we know the correct possible trees, we can calculate the length (L) of potentially correct trees. This type of search is called the specific-tree search. The second strategy is when the number of taxa is greater than 10, in this case Branch-and bound method and heuristic search are efficient ways to get MP trees (Graur and Li, 1999, Nei and Kumar, 2000). The principle of Branch-and bound method is that only trees with short lengths will be evaluated. Nevertheless, the Branch-and-bound method is limited only when there are about 20 taxa under the study. In such a larger (>20) taxa, heuristic search approach is less time consuming for searching MP trees, but the MP tree may not always be found (Nei and Kumar, 2000).

Maximum Likelihood Methods (ML)

Likelihood methods use both distance matrix and character state approaches (Graur and Li, 1997). The goal of ML is to search trees with the highest value (L) of the probability of the nucleotide sequences under a given tree and a substitution pattern. The

likelihood values of ML tree are calculated as the log likelihood (lnL) of the tree, so the highest likelihood value is chosen as the maximum likelihood tree (Graur and Li, 1997). The branch length of ML tree represents either the rate of substitution or the period of evolutionary time. For more detail of how to get ML tree, see Swofford *et al.* (1996), Li (1997) and Nei and Kumar (2000). Each reference noted that the ML method takes the longest time for computing a ML tree. Thus this method is used very often.

Evaluate Tree Reliability

After getting an inferred phylogenetic tree, it is very important to test a reliability of the obtained tree (Graur and Li, 1999, Hershkovitz and Leipe, 1998, Li, 1997, and Nei and Kumar, 2000). The resampling bootstrap method of Felsenstein is the most frequently used method to evaluate a reliability of a tree. The procedure of bootstrapping starts from the resampling data from the original sample dataset with replacement, which means that each site can be resampled again with the same probability as any other sites. The data from the resampling process is called the pseudosample. The pseudosample will be used to build a tree by the same method used for the inferred tree. Theoretically, the bootstrap resampling process is often repeated at least several hundred pseudosamples when many species are involved. However, it is very time consuming to repeat several hundred pseudosamples when the maximum parsimony method is used. A bootstrap value is calculated as percentages, and bootstrap values greater than 50% will be appeared on the relevant internal branch. Bootstrap values greater than 95% show that the true topology has been found or the inferred tree has high confidence of the statistical

reliability. Graur and Li (1999) mentioned that by collapsing branches with bootstrap values lower than 50%, the collapsed trees are more similar to the true tree than the original inferred tree.

APPENDIX B

ALIGNMENT OF ITS1-5.8S-ITS2 SEQUENCES

CLUSTAL W (1.74) multiple sequence alignment

```
Amphisphaeria sp.                CAGAGTTATCTAA-CTCCCAAA-CCCATGTGAACTTACCATTTGTTGCCT
Discostroma tricellular          CAGAGTTATCTAA-CTCCCAAA-CCCATGTGAACTTACCATTTGTTGCCT
Seiridium cardinale              CAGAGTTATCTAA-CTCCCAAAACCCATGTGAACTTACC-TTTGTTGCCT
Lepteutypa cupressi              TATTAGTATCTAA-CTCCAAA---CCATGTGAACTTACC-TTTGTTGCCT
Seiridium unicorne               CAGAGTTATCTAA-CTCCCAA--CCCATGTGAACTTACC-TTTGTTGCCT
Seiridium isolate51              CAGAGTTTTCTAA-CTCCCAAA-CCCATGTGAACTTACC-TTTGTTGCCT
Discostroma fuscella             CAGAGTTATCTAA-CTCCCAAA-CCCATGTGAACTTACC-ATTGTTGCCT
Truncatella angustata            CAGAGTTATCTAA-CTCCCAAA-CCCATGTGAACTTACC-ACTGTTGCCT
Pestalotia rhododendri           TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalosphaeria hansenii         TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalotiopsis microsporaNE32    TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalotiopsis neglecta          TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalotiopsis microsporaWT98    TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Monochaetia camelliae            TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Monochaetia isolate162           TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalotia thujae                TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalotiopsis funereoides       TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCATT-GTTGCCT
Pestalotiopsis microsporacbs17   TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACTATT-GTTGCCT
Pestalotiopsis jesteri6b         TAGAGTTTTTCAAACTCCCAA--CCCATGTGAACTTACCACT-GTTGCCT
Pestalotiopsis jesteri6t         TAGAGTTTTTCAAACTCCCAA--CCCATGTGAACTTACCACT-GTTGCCT
Pestalotiopsis NG1230            TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCTTTTGTTGCCT
Pestalotia palmarum              TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCTTTTGTTGCCT
Pestalotiopsis microsporacbs36   TAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCTTTTGTTGCCT
Pestalosphaeria elaeidis         CAGAGTTTTCTAAACTCCCAA--CCCATGTGAACTTACCTTTTGTTGCCT
Xylaria mali                     AAGAGTTTTGATAACTCCCAAA-CCCATGTGAACTTACCTTCTGTTGCCT
Xylaria hypoxylon                AAGAGTTTTGATAACTCCCAAA-CCCATATGAACTTACCTTCTGTTGCCT
                                  *    * *   * **** **   **** *********     *******

Amphisphaeria sp.                CGGCAGAGCCTACCTG---GTATCTA-CTCTGGAGCGGC-TACCCTGTAG
Discostroma tricellular          CGGCAGAGGCTACCCG---GTACCTA-CCCTGGAGCAGC-TACCCTGTAG
Seiridium cardinale              CGGCAGAAGCTACCT----GTACCTA-CC-TGGAACAGCCTACC-TGGAG
Lepteutypa cupressi              CGGCAGGAGCTACTC----GTACCTA-CC-TGGAACAGCCTACC-TGGAG
Seiridium unicorne               CGGCAGAAGCTACCT----GTACCTA-CC-TGGAACAGCCTACC-TGGAG
Seiridium isolate51              CGGCAGAAGCTACCCT---GTACCTA-CCCTGGAACGAGTTACCCTGTAA
Discostroma fuscella             CGGCAGAACCTACCCG---GTACCTA-CCCTGTAACGACCTACCCTGTAG
Truncatella angustata            CGGCAGATGTTGCTGGGC-GAACCTA-CCCTGTAGCGAGCTACTCTGTAG
Pestalotia rhododendri           CGGCAGAAGCTGCTCGGTGCACCCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalosphaeria hansenii         CGGCAGAAGCTGCTCGGTGCACCCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotiopsis microsporaNE32    CGGCAGAAGCTGCTCGGTGCACCCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotiopsis neglecta          CGGCAGAAGCTGCTCGGTACACCTTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotiopsis microsporaWT98    CGGCAGAAGCTGCTCGGCGCGCCTTA-CCTTGGAACGGCCTACCCTGTAG
Monochaetia camelliae            CGGCAGAAGCTACCCGGT--ACCTTA-CCTTGGAACGGCCTACCCTGTAG
Monochaetia isolate162           CGGCAGAAGCTACCTGGTT-ACCCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotia thujae                CGGCAGAAGCTACCTGGTT-ACCCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotiopsis funereoides       CGGCAGAAGCTACCTGGTT-ACCCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotiopsis microsporacbs17   CGGCAGAGGCTACCCGGTA---CCTA-CCTTGGAACGGCCTACCCTGTAG
Pestalotiopsis jesteri6b         CGGCAGAGGCTACCCGGTA---CCTA-CCTCGGAACGGCCTACCCTGTAG
Pestalotiopsis jesteri6t         CGGCAGAGGCTACCCGGTA---CCTA-CCTCGGAACGGCCTACCCTGTAG
Pestalotiopsis NG1230            CGGC--AGGA------GT------TA---TAGG-TCTTC-----------
Pestalotia palmarum              CGGC--AGGA------GT------TAC--TAGGGTCTTC-----------
Pestalotiopsis microsporacbs36   CGGC--AGAA------GT------TA---TAGG-TCTTC-----------
Pestalosphaeria elaeidis         CGGGCAAGGAAG---GGT------TAACCCTGGGTAACCCTGGGAAGGAA
Xylaria mali                     CGGC--AGGTCGC---GT-----CTA-CCCTGTGG-CACCTACCCTGTAG
Xylaria hypoxylon                CGGC--AGGTCGC---GT-----CTA-CCCTGTGGGCACCTACCCTGTAG
                                 ***                     **     *

Amphisphaeria sp.                C----TA--CCC---TGGAGCGGGTTACCC-------TGTAACG---TCC
Discostroma tricellular          C----TA--CCC---TGGAACGGCCTACCC-------TGTAGCGC-ATCC
Seiridium cardinale              C----GA--TCC----GGGCTGGCCTACCTGGAACGGTCTGGTGGTCGAC
Lepteutypa cupressi              C----GA--TCC----GG-CTGGCCTACCTGGAACGGTCTGGTGGTCGAC
Seiridium unicorne               C----GA--TTC----GGGCTGGCTTACCTGGAACGGTCTGGTGGTCGAC
Seiridium isolate51              C----GAATTCT----TGTGTAGCCTACCC-------TGTAGCAATATTC
Discostroma fuscella             C----GAGTTACC-TGGGAACGGCTTACCC----------TGTAGTGCGC
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Truncatella angustata                C----GACCTACC-CGGGAACGGCTTACCC-------TGTAGCG----CC
Pestalotia rhododendri               C----GCCTTAC-CCTGGAACGGCTTACCC-------TGTAACGG----C
Pestalosphaeria hansenii             C----GCCTTAC-CCTGGAACGGCTTACCC-------TGTAACGG----C
Pestalotiopsis microsporaNE32        C----GCCTTAC-CCTGGAACGGCTTACCC-------TGTAACGG----C
Pestalotiopsis neglecta              C----GCCTTAC-CCTGGAACGGCTTACCC-------TGTAACGG----C
Pestalotiopsis microsporaWT98        C----GCCTTAC-CCTGGAACGGCTTACCC-------TGCAACGG----C
Monochaetia camelliae                C----GCCTTAC-CCTGGAACGGCTTACCC-------TGTAACGG----C
Monochaetia isolate162               C----GCCTTAC-CCTGGAACGGCCTACCC-------TGTAACGG----C
Pestalotia thujae                    C----GCCTTAC-CCTGGAACGGCCTACCC-------TGTAACGG----C
Pestalotiopsis funereoides           C----GCCTTAC-CCTGGAACGGCCTACCC-------TGTAACGG----C
Pestalotiopsis microsporacbs17       C----GCCTTAC-CCGGGAACGGATTACCC-------TGTAGCGG----C
Pestalotiopsis jesteri6b             C----GCCTTAC-CTGGGAGCGG-TTACCC-------TGTAGCGG----C
Pestalotiopsis jesteri6t             C----GCCTTAC-CTGGGAGCGG-TTACCC-------TGTAGCGG----C
Pestalotiopsis NG1230                ------TTATAG--CTG------------------------CTG------
Pestalotia palmarum                  ------TTATAGGCCTGG-----------C-----------CTGG----C
Pestalotiopsis microsporacbs36       ------TTATAG--CTG------------------------CTG------
Pestalosphaeria elaeidis             CAAGGGTTTAACCCCTGGTAAGGCCAAACC----------TTGG----C
Xylaria mali                         GACCCGACCTGG---TGGT--------CGCG-------GTCATGC----C
Xylaria hypoxylon                    GACCCGACCTGG---TGGT--------CGCG-------GTCATGC----C

Amphisphaeria sp.                    TGCCGGTGGACCTT-TAAACTCT-TGT-TATTATATAGTAATCTGAGCCT
Discostroma tricellular              TGCCGGTGGACCTT-TAAACTCT-TGT-TATTTTAAAGTAATCTGAGCGT
Seiridium cardinale                  TGCCGGTGGACCAT-TCAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Lepteutypa cupressi                  TGCCGGTGGACCAT-TCAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Seiridium unicorne                   TGCCGGTGGACCAT-TCAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Seiridium isolate51                  TGCCGGTGGACTAT-TCAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Discostroma fuscella                 TGCCGGCGGACCTC-TTAACTCT-TGT-TATTTTATAGTAATCTGAGCGT
Truncatella angustata                TGCCGGTGGACTAC-TAAACTCT-TGT-TATTTTTAAGTAATCTGAGCGT
Pestalotia rhododendri               TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalosphaeria hansenii             TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis microsporaNE32        TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis neglecta              TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis microsporaWT98        TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATGGTTATCTGAGCGT
Monochaetia camelliae                TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTTATCTGAGCGT
Monochaetia isolate162               TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotia thujae                    TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis funereoides           TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis microsporacbs17       TGCCGGTGGACCAC-TAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis jesteri6b             TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis jesteri6t             TGCCGGTGGACTAC-CAAACTCT-TGT-TATTTTATTGTAATCTGAGCGT
Pestalotiopsis NG1230                --CCGGTGGACCAT-TAAACTCT-TGT-TATTTTAT-GTAATCTGAGCGT
Pestalotia palmarum                  --CCGGTGGACCATCTAAACTCT-TGT-TATTTATT-GTAATCTGAGCGT
Pestalotiopsis microsporacbs36       --CCGGTGGACCAT-TAAACTCT-TGT-TATTTTAT-GTAATCTGAGCGT
Pestalosphaeria elaeidis             --CCGGTGGACTAC-TAAACTCT-TGT-TATTTTAT-GTAATCTGAGCGT
Xylaria mali                         TGCCGGTGGCCCTT-TAAACTTTCTGTGTATTCTATGTTATTCTGAGTTG
Xylaria hypoxylon                    TGCCGGTGGCCCTT-TAAACTTTCTGTGTATTCTATGTTATTCTGAGTTG
                                       **** ** *      **** * *** ****      *  ******

Amphisphaeria sp.                    CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Discostroma tricellular              CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Seiridium cardinale                  CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Lepteutypa cupressi                  CTTATTTTAATAAGTCAAAACTTTCAACA-CGGATCTCTTGGTTCTGGCA
Seiridium unicorne                   CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Seiridium isolate51                  CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Discostroma fuscella                 CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Truncatella angustata                CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotia rhododendri               CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalosphaeria hansenii             CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis microsporaNE32        CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis neglecta              CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis microsporaWT98        CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Monochaetia camelliae                CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Monochaetia isolate162               CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotia thujae                    CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis funereoides           CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
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Pestalotiopsis microsporacbs17      CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis jesteri6b            CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis jesteri6t            CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotiopsis NG1230               CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalotia palmarum                 CTTATTTTAATAAGTCAAAACTCTCATGCGCGGATCTCTTGGTTCTGGCA
Pestalotiopsis microsporacbs36      CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Pestalosphaeria elaeidis            CTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Xylaria mali                        GCAACTAAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
Xylaria hypoxylon                   GCAACTAAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
                                       * *  ******* ****** ***    ********************

Amphisphaeria sp.                   TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Discostroma tricellular             TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Seiridium cardinale                 TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Lepteutypa cupressi                 TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Seiridium unicorne                  TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Seiridium isolate51                 TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Discostroma fuscella                TCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTC
Truncatella angustata               TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotia rhododendri              TCGATGAAGAACGCAGC-AAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalosphaeria hansenii            TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis microsporaNE32       TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis neglecta             TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis microsporaWT98       TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Monochaetia camelliae               TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Monochaetia isolate162              TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotia thujae                   TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis funereoides          TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis microsporacbs17      TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis jesteri6b            TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis jesteri6t            TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotiopsis NG1230               TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalotia palmarum                 TCGATGAAGAACGCAGCGAAATGCGATC-GTA-TGTGAATTGCAGAATTC
Pestalotiopsis microsporacbs36      TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Pestalosphaeria elaeidis            TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Xylaria mali                        TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
Xylaria hypoxylon                   TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAACTGCTGAATTC
                                    ****************** *********  *** ****** *** ******

Amphisphaeria sp.                   AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Discostroma tricellular             AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Seiridium cardinale                 AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Lepteutypa cupressi                 AGTGAATCATCGAATCTT-GAACG-ACATGGC-CCCATTAGTACTCTAGT
Seiridium unicorne                  AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Seiridium isolate51                 AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Discostroma fuscella                AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Truncatella angustata               AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotia rhododendri              AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalosphaeria hansenii            AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis microsporaNE32       AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis neglecta             AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis microsporaWT98       AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Monochaetia camelliae               AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Monochaetia isolate162              AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotia thujae                   AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis funereoides          AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis microsporacbs17      AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis jesteri6b            AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis jesteri6t            AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotiopsis NG1230               AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalotia palmarum                 AGTGAATCATCGAATCTTTGAACGCACATTGGCCCCATTAGTATTCTAGT
Pestalotiopsis microsporacbs36      AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Pestalosphaeria elaeidis            AGTGAATCATCGAATCTTTGAAGCCACATTGCGCCCATTAGTATTCTAGT
Xylaria mali                        AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGT
Xylaria hypoxylon                   AGTGAATCATCGAATCTTTGTTCGCACATTGCGCCCATTAGTATTCTAGT
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Amphisphaeria sp.                    GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Discostroma tricellular              GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Seiridium cardinale                  GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Lepteutypa cupressi                  GG-CATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Seiridium unicorne                   GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Seiridium isolate51                  GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Discostroma fuscella                 GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Truncatella angustata                GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotia rhododendri               GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalosphaeria hansenii             GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis microsporaNE32        GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis neglecta              GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis microsporaWT98        GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Monochaetia camelliae                GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Monochaetia isolate162               GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotia thujae                    GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis funereoides           GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis microsporacbs17       GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis jesteri6b             GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis jesteri6t             GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis NG1230                GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotia palmarum                  GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalotiopsis microsporacbs36       GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Pestalosphaeria elaeidis             GG-CATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCC----TAGCTTAG
Xylaria mali                         GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTCGCTTAG
Xylaria hypoxylon                    GGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTCGCTTAG
                                     ** ***********************************    * ******

Amphisphaeria sp.                    TGTTGGGAATCTACT-----GTAT----TGTAGTTCCTGAAATATAACGG
Discostroma tricellular              TGTTGGGAATCTACT-----GTAT----TGTAGTTCCTGAAATATAACGG
Seiridium cardinale                  TATTGGGAGTCTACT-----GTAT----TGTAGTTCCTCAAATCCAACGG
Lepteutypa cupressi                  TATTGGGAGTCTACT-----GTAT----TGTAGTTCCTCAAATCCAACGG
Seiridium unicorne                   TATTGGGAGTCTACT-----GTAT----TGTAGTTCCTCAAATCCAACGG
Seiridium isolate51                  TATTGGGAATCTACT-----GTAT----TGTAGTTCCTCAAATCCAACGG
Discostroma fuscella                 TGTTGGGAGCCTACT-----GTAT----TGTAGCTCCCCAAATCCAACGG
Truncatella angustata                TATTGGGAACTGGCT-----TTAC----TGCCATTCCTCAAATTCAACGG
Pestalotia rhododendri               TGTTGGGAGCCTACT-GCTTTTGCTAGCTGTAGCTCCTGAAATACAACGG
Pestalosphaeria hansenii             TGTTGGGAGCCTACT-GCTTTTGCTAGCTGTAGCTCCTGAAATACAACGG
Pestalotiopsis microsporaNE32        TGTTGGGAGCCTACT-GCTTTTGCTAGCTGTAGCTCCTGAAATACAACGG
Pestalotiopsis neglecta              TGTTGGGAGCCTACT-GCTTTTACTAGCTGTAGCTCCTGAAATACAACGG
Pestalotiopsis microsporaWT98        TGTTGGGAGCCTACT-GCTTTTGCTAGCTGTAGCTCCTGAAATACAACGG
Monochaetia camelliae                TGTTGGGAGCCTACT-GCTTTTGCTAGCTGTAGCTCCTGAAATACAACGG
Monochaetia isolate162               TGTTGGGAGCCTACC-GCTTTTGCTAGCGGTAGCTCCTGAAATACAACGG
Pestalotia thujae                    TGTTGGGAGCCTACC-GCTTTTGCTAGCGGTAGCTCCTGAAATACAACGG
Pestalotiopsis funereoides           TGTTGGGAGCCTACC-GCTTTTGCTAGCGGTAGCTCCTGAAATACAACGG
Pestalotiopsis microsporacbs17       TGTTGGGAGCCTACT-GCTTTTACTAGCTGTAGCTCCTGAAATACAACGG
Pestalotiopsis jesteri6b             TGTTGGGAGCCTACTAGCCCTCGCGGCCTGTAGCTCCTGAAATACAACGG
Pestalotiopsis jesteri6t             TGTTGGGAGCCTACTAGCCCTCGCGGCCTGTAGCTCCTGAAATACAACGG
Pestalotiopsis NG1230                TGTTGGGAATCTACT-TCT-TTAT-AGTTGTAGTTCCTGAAATACAACGG
Pestalotia palmarum                  TGTTGGGAATCTACT-TCT-TTAT-AGTTGTAGTTCCTGAAATACAACGG
Pestalotiopsis microsporacbs36       TGTTGGGAATCTACT-TCTCTTAGGAGTTGTAGTTCCTGAAATACAACGG
Pestalosphaeria elaeidis             TGTTGGGAATTTACA-G---TTAT--GTAAT--TTCCTGAAATACAACGG
Xylaria mali                         TGTTGGGAGCCTACA-----GTCTT--CTGTAGCTCCCTAAAGTTAGTGG
Xylaria hypoxylon                    TGTTGGGAGCCTACA-----GTCTT--CTGTAGCTCCCTAAAGTTAGAGG
                                     * ******     *                    ***  ***   *  **

Amphisphaeria sp.                    C-GGATCTGCAATATCCTCTGAGCGTAGTAATTTTTTTC-TCGCTTTGGT
Discostroma tricellular              C-GGATCTGTAATGTCCTCTGAGCGTAGTAATTTTTTTCCTCGCTTTGGT
Seiridium cardinale                  C-GGATCTGTGGTATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGT
Lepteutypa cupressi                  C-GGATCTGTGGTATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGT
Seiridium unicorne                   C-GGATCTGTGGTATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGT
Seiridium isolate51                  C-GGATCTGTGGTATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGT
Discostroma fuscella                 C-GGATCTGTGGTATCCTCTGAGCGTAGTAA-TTTTTATCTCGCTTTTGT
Truncatella angustata                C-GGATTTATAGCAATCTCTGAACGTAGTAA-TTTTTTTCTCGTTTTTG-
Pestalotia rhododendri               C-GGATCTGCGATATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGA
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Pestalosphaeria hansenii                C-GGATCTGCGATATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGA
Pestalotiopsis microsporaNE32           C-GGATCTGCGATATCCTCTGAGCGTAGTAAATTTTTATCTCGCTTTTGA
Pestalotiopsis neglecta                 C-GGATCTGCGATATCCTCTGAGCGTAGTAA-TTTTTATCTCGCTTTTGA
Pestalotiopsis microsporaWT98           C-GGATCTGCGATATCCTCTGAGCGTAGTAA-TTTTTATCTCGCTTTTGA
Monochaetia camelliae                   C-GGATCTGCGATATCCTCTGAGCGTAGTAATTTTTTTTCTCGCTTTTGA
Monochaetia isolate162                  C-GGATCTGCGATATCCTCTGAGCGTAGTAA-TTTTTATCTCGCTTTTGA
Pestalotia thujae                       C-GGATCTGCGATATCCTCTGAGCGTAGTAA-TTTTTATCTCGCTTTTGA
Pestalotiopsis funereoides              C-GGATCTGCGATATCCTCTGAGCGTAGTAA-TTTTTATCTCGCTTTTGA
Pestalotiopsis microsporacbs17          C-GGATCTGCGATATCCTCTGAGCGTAGTAA-TTTTTTTCTCGCTTTTGA
Pestalotiopsis jesteri6b                C-GGATCTGCGATATCCTCTGAGCGTAGTAATCTTACCTCTCGCTTTTGT
Pestalotiopsis jesteri6t                C-GGATCTGCGATATCCTCTGAGCGTAGTAATCTTACCTCTCGCTTTTGT
Pestalotiopsis NG1230                   C-GGATTTGTAGTATCCTCTGAGCGTAGTAA-TTTTTTTCTCGCTTTTGT
Pestalotia palmarum                     CCGGATTTGTAGTATCCTCTGAGCGTAGTAA-TTTTTTTCTCGCTTTTGT
Pestalotiopsis microsporacbs36          C-GGATTTGTAGTATCCTCTGAGCGTAGTAA-TTTTTTTCTCGCTTTTGT
Pestalosphaeria elaeidis                C-GGATCTGTGGTATCCTCTGAGCGTAGTAA-ATTATTTCTCGCTTTTGT
Xylaria mali                            C-GGAGTCGGTTC--ACTCTAGACGTAGTAG-AT-CTATCTC--GTCTAT
Xylaria hypoxylon                       C-GGAGTCGGTTTGTACTCTAGACGTAGTAG-ATTCTATCTCTCGTCTAT
                                        * ***           ****   *******   *      **   *

Amphisphaeria sp.                       TAGGTGTTGTAGCTCT--CAGCCGCTAAACCCCCCAA---TTTTAATGG-
Discostroma tricellular                 TAGGTGTTGCAGCTCT--CAGCCGCTAAACCCCCCAA---TTTTAATGG-
Seiridium cardinale                     CAGGTGCTGCAGCTCC--CAGCCGCTAAACCCCCAAAT-TTTTTA-AGG-
Lepteutypa cupressi                     CAGGTGCTGCAGCTCT--CAGCCGCTAAACCCCCAAAT-TTTTTA-TGG-
Seiridium unicorne                      TAGGTGCTGCAGCTCC--CAGCCGCTAAACCCCCAAAC-TTTTAA-TGG-
Seiridium isolate51                     TAGGTGCTGCAGCTCC--CAGCCGCTAAACCCCCCAA--TTTTTA-AGG-
Discostroma fuscella                    TAGGTGCCGCAGCTCT--CAGCCGCTAAACCCCCCAAT-TTTTTAATGG-
Truncatella angustata                   -AAATACTGTAAACCT--CAGCCGCTAAACCCCCCAA--TTTTTAATGG-
Pestalotia rhododendri                  CTGGAGTTGCAGCGTCTTTGGCCGCTAAATCCCCCAA-TTGTTTAATGG-
Pestalosphaeria hansenii                CTGGAGTTGCAGCGTCTTTGGCCGCTAAATCCCCCAA-TT-TTTAATGG-
Pestalotiopsis microsporaNE32           CTGGAGTTGCAGCGTCTTTGGCCGCTAAATCCCCCAA-TT-TTTAATGGG
Pestalotiopsis neglecta                 CTGGAGTTGCAGCGTCTTTAGCCGCTAAATCCCCCAA-TT-TTTAATGGG
Pestalotiopsis microsporaWT98           CTGGAGTTGCAGCGTCTTTAGCCGCTAAACCCCCCAA-TT-TTTAATGG-
Monochaetia camelliae                   CTGGAGTTGCAGCGTCTTTAGCCGCTAAATCCCCCAA-TT-TTTAATGG-
Monochaetia isolate162                  CTGGAGTTGCAGCGTCTTTAGCCGCTAAACCCCCCAAATTTTTTAATGG-
Pestalotia thujae                       CTGGAGTTGCAGCGTCTTTAGCCGCTAAACCCCCCAAATTTTTTAATGGG
Pestalotiopsis funereoides              CTGGAGTTGCAGCGTCTTTAGCCGCTAAACCCCCCAAATTTTTTAATGGG
Pestalotiopsis microsporacbs17          CTGGAGTTGCAGCGTCCTTTGCCGCTAAACCCCCAAA--TTTTTAATGG-
Pestalotiopsis jesteri6b                CAGGATTCCCAGCATC--TAGCCGCTAAACCCCCCAA--TTTTTAATGG-
Pestalotiopsis jesteri6t                CAGGATTCCCAGCATC--TAGCCGCTAAACCCCCCAA--TTTTTAATGG-
Pestalotiopsis NG1230                   TAGGTGCTATAACTCC--CAGCCGCTAAACCCCC-AA--TTTTTTGTGG-
Pestalotia palmarum                     TAGGTGCTATAACTCC--CAGCCGCTAAACCCCC-AA--TTTTTTGTAA-
Pestalotiopsis microsporacbs36          TAGGTGCTATAACTCC--CAGCCGCTAAACCCCC-AA--TTTTTTGTGG-
Pestalosphaeria elaeidis                TAGGTGCTGCAGCTCC--CAGCCGCTAACCC-----A--TTTTCTGTGG-
Xylaria mali                            --AGTTAAGCCGGTCTC-TTGCCGTAAAACCCCCTAA--TTTCTCAAGG-
Xylaria hypoxylon                       --AGTTAAGCCGGTCTC-TTGCCGTAAAACCCCCTAA--TTTCTCAAGG-
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APPENDIX C

AUTHORITY OF FUNGAL STRAINS

Amphisphaeria incrustans (ATCC18007), Hennebert, G. L.

Amphisphaeria sp. (MAFF235878), Unknown.

Discostroma fuscella (Fragment 10071), Barr, E. M.

Discostroma tricellular (MAFF237482), Okane, A., Nakagiri and Ito T.

Fusarium lateritium, Strobel, G. A. and Ford, E. J.

Pestalosphaeria hansenii (ATCC48245), Shoemaker *et* Simpson.

Monochaetia camelliae (ATCC60625), Boesewinkel, H. J.

Monochaetia sp. (Isolate162), Strobel, G. A., Ford, E. J. and Bharat, B

Pestalotia palmarum (ATCC10085), Mundkur, B. B.

Pestalotia bicilia, Strobel, G. A., Ford, E. J. and Li, J. Y.

Pestalotia rhododendri (ATCC24306), Norman, J.

Pestalotia thujae (CBS303.75), van der Aa, H. A.

Pestalotiopsis jesteri (Isolate 6m and 6t), Strobel, G. A., Ford, E. J. and Li, J. Y.

Pestalotiopsis funereoides (CBS175.25), Doyer, C. M.

Pestalotiopsis microspora (CBS171.26), Ciferri, R.

Pestalotiopsis microspora (CBS364.54), Linder, D. H.

Pestalotiopsis microspora (NE32), Strobel, G. A., Ford, E. J. and Li, J. Y.

Pestalotiopsis microspora (WT98), Strobel, G. A., Ford, E. J. and Li, J. Y.

Pestalotiopsis neglecta (CBS200.65), Frankland, J. C. and Sutton, B. C.

Pestalotiopsis sp. (Isolate NG1230), Strobel, G. A. and Worapong J.

Seimatoantlerium nepalense, Strobel, G. A. and Bashyal, B.

Seimatoantlerium tepuiense, Strobel, G. A., Ford, E. J. and Li, J. Y.,

Seiridium cardinale (ATCC52521), Panconesi, A.

Seiridium unicorne (ATCC48159), Boesewinkel, H. J.

Seiridium sp. (Isolate 51), Strobel, G. A., Ford, E. J. and Bharat, B

Taxomyces andreanae, Stierle, A., Strobel, G. A. and Stierle, D.

Truncatella angustata (ATCC96024), Roberts, R. G.