

Table of Contents

TITLE PAGE	1
1 INTRODUCTION	2
1.1 Objectives of the Study	4
1.2 Significance of the Study	4
1.3 Scope and Limitation of the Study	5
2 REVIEW OF RELATED LITERATURE	6
2.1 Microcavities	6
2.2 Fabry-Perot Microcavity	8
2.3 Microcavity Polaritons	9
2.4 Polariton Spin and Polarization	12
2.5 Silicon-based microcavity	12
2.6 Transfer Matrix Formalism	13
2.6.1 Advantages and drawbacks of the Transfer Matrix Method	14
3 RESEARCH METHODOLOGY	16
3.1 Microcavity Structure	16
3.2 Simulation Process	17
3.3 Run: Algorithm	18
3.4 Reflectivity Measurement of Multilayered Structures	19
3.5 Microcavity polaritons	19
REFERENCES	21

CHAPTER 1

INTRODUCTION

With the advancement of today's technology, the realizations of crystalline microstructures with unusual and interesting optical properties are made possible. Microstructures, such as optical microcavities, can be used as controlled "laboratories" for observing large varieties of intriguing optical phenomena in semiconductor quantum optics and photonics [1].

An optical microcavity is basically an optical resonator which confines light to small volumes close to or below the dimension of the light's wavelength. It has a wide range of applications in both fundamental and applied fields of research including cavity quantum electrodynamics, optical communication, resonant cavity optoelectronics, micromechanical sensors and biosensing [2]. Fabrication of such optical microcavities can be achieved by growing multilayered structures. It can be fabricated in a way that the active medium, cavity and two mirrors consist of thin films. The light is confined to a small volume, even up to the nanometric scale of the cavity. For better confinement of light, a high refractive index contrast is needed at the interface between the layers of the structures. This results to resonant frequencies distributed at the spectrum, which is highly dependent on the cavity dimension.

There are many optical microcavities made with different semiconductor materials, but silicon-based microcavities are very interesting for optoelectronics applications such as fabrication of efficient low cost optoelectronic devices [3]. Examples of these are, vertical-cavity surface emitting lasers (VCSELS), low-threshold diodes, polariton lasers, optical switches and spin memory element [2, 4]. In particular, amorphous Silicon Nitride ($a-Si_{1-x}N_x : H$) are very suitable materials to grow optical microcavity base on multilayered structures such as Fabry-Perot microcavity. The reason is that they do not present the lattice parameter mismatch problem. Hence, you get layers with lesser effects. Also the tunable optical gap and refractive index contrast can be tuned

by variation of nitrogen content in the silicon alloy [5].

Most of the researches done in microcavities are gallium - based [5, 7] made of active III-V semiconductor materials which control laser emission spectra for data transmission [1]. In this study a silicon-based microcavity is modeled. This is possible by using transfer matrix method (TM), can be useful tool in simulation and characterization of microcavities for reflectance, transmittance and absorbance. A multilayered structure can be simulated to predict optical properties before fabrication by applying the transfer matrix algorithm. To test the validity of the TM algorithm used in for ongoing studies with, amorphous silicon nitride ($a-Si_{1-x}N_x : H$) microcavity from a journal by Ballarini et al. [4] is modeled. The computed transmittance spectrum of the microcavity is then compared to their experimental result. This is to test if the transfer matrix program works for such multilayered structure. Aside from modeling an established journal, this study also simulate multilayered structures of the grown and fabricated samples from NIP, UP-Diliman for reflectivity. Reflectivity measurements of the samples will be also considered. After which is the comparison of the simulated result to the experimental result. This is also to test the TM algorithm. The program is useful for designing other silicon-based microcavities like SiGe before it goes for fabrication.

After the test of transfer matrix algorithm for various multilayered structures is the design and simulation of silicon based microcavity, specifically composed of silicon germanium ($Si_{1-x}Ge_x$) alloy, where quantum wells are embedded inside. The design parameters of the microcavity will be varied to ensure optimum overlap between exciton and photon field. Transfer matrix algorithm will be used. Acquiring the optimum resonance between exciton and photon in this microcavity may be used as parameters in future experiment for efficient and low cost optoelectronics applications. Overall, this study is an optical investigation of silicon-based microcavities and multilayered structures both in theory and experiment.

1.1 Objectives of the Study

This study investigates silicon-based microcavities for future optical applications. This also examines the reflectivity of grown and fabricated multilayered structures. Specifically, it aims the following:

1. Design a simulation algorithm using transfer matrix method.
 - a. Simulate the transmittivity of a silicon-based ($a - Si_{1-x}N_x : H$) microcavity by using transfer matrix method and compare the experimental results to the established result obtained by Ballarini et al. [4].
 - b. Characterize the reflectivity of grown and fabricated multilayered structures from NIP, UP-Diliman.
 - c. Simulate the reflectivity of the given multilayered structures and compare results from the experiment.
2. Design silicon-based microcavities with an optimum coupling between photon and exciton using transfer matrix method.

1.2 Significance of the Study

For the past decades, strong light-matter interactions in solid-state devices are objects of increasingly intense research [8] due to coherent and stimulated effects in such system which can lead to new optical devices [9]. The first step of this study is to create a simulation algorithm for multilayered structures or microcavities. After which is the validation of the program to various multilayered structures. If the program works, then is the design and simulation of silicon-based microcavities, particularly silicon germanium (SiGe). This is the very aim of this study. Through simulation, the silicon-based microcavity can be characterized and varied with its thickness in which there is optimum resonance between exciton and photon. The result may be used as parameters in silicon-based microcavity fabrication for future experiments. Eventually, this may lead to various and useful optical applications that are efficient and low cost. Production

of such semiconductor (SiGe) material for the silicon-based microcavities will not be a problem since silicon is believed to be the optimum material for optical components fabrication due to mainly economical and practical reasons [10]. Silicons are cheaper than other group III-V and II-VI materials. It is still abundant in sources since it's the most common element in the earth's crust. Mostly, silicons are less toxic where health risks will be lesser than other semiconductor materials.

1.3 Scope and Limitation of the Study

The focus of this study is to design and simulate Silicon-based microcavities. Primary, this will consider a simulation of a microcavity made from amorphous silicon nitride ($a - Si_{1-x}N_x : H$) [4] for transmittance using transfer matrix method (TM). Another is the simulation of multilayered structures of grown and fabricated samples from NIP, UP-Diliman for reflectance. Reflectivity measurement of the given samples will be also considered. The results from the simulation and experiment will be then compared. This is to test the TM algorithm. Then is the design and simulation of silicon-based microcavities, particularly the SiGe. This will simulate, design and characterize the microcavity using transfer matrix method. Thickness of microcavity will be varied to get the optimum resonance between exciton-polaritons. Matlab computing software will be used as the simulation tool.

On the other hand, only reflectivity experiment will be done on the grown and fabricated multilayered samples from NIP, UP-Diliman for reflectivity. This study will simply use transfer matrix method for the simulation part.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 Microcavities

Microcavities have wide range of applications in both fundamental and applied fields of research including cavity quantum electrodynamics, optical communication, resonant cavity optoelectronics, micromechanical sensors and biosensing [2]. Basically, a microcavity is an optical resonator which confines light to small volumes close to or below the dimension of light's wavelength. It could be a micron- or sub-micron sized optical resonator which has two different schemes to confine light (Figure 2.1). The first scheme is by using reflection of a single-interface like a metallic surface. The second scheme is by using microstructures periodically patterned on the scale of the resonant optical wavelength like Distributed Bragg Reflectors (DBRs). Microcavity has also diverse modes such as resonant optical modes, longitudinal resonant mode and transverse mode. But this traditional feature or mode can lose its precision since all modes exist at the same footing.

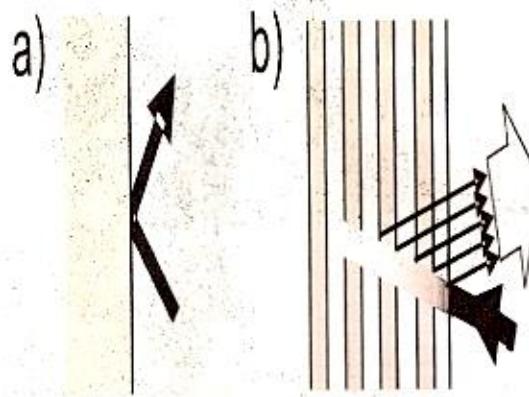


Figure 2.1: (a) single interface reflection and (b) interference from multiple interfaces [11].

Among the properties of microcavities are the quality factor (Q-factor) and finesse, intracavity field enhancement and field distribution, angular mode pattern, low

threshold lasing, Purcell factor and lifetimes, strong coupling and weak coupling. This strong coupling in a microcavity will be dealt with in detail in Section 2.3.

There are different types of microcavities according to its application. The most common of all is the planar microcavity which uses two flat mirrors to confine light. These mirrors are close to proximity so only a few wavelengths of light can fit in between. Under with planar microcavity are the metal microcavities and Dielectric Bragg mirrors (DBRs). The finesse of DBRs cavity is based on the reflectivity of each mirror dependent on the number of pair repeats and the refractive index contrasts between the two materials used. So each layer is just a quarter of the desired centre wavelength of reflection. In this study we will use Silicon- based (e.g. $a - Si : N$) as DBRs.

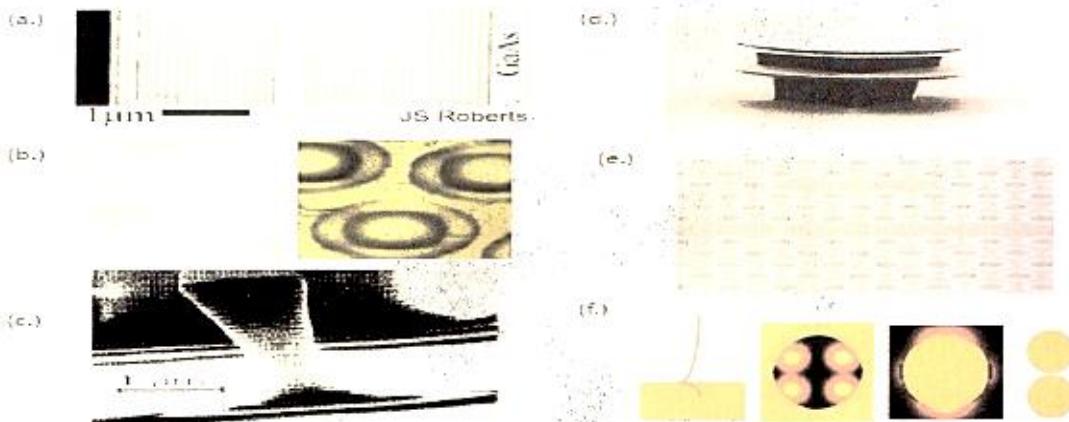


Figure 2.2: (a) Scanning electron micrograph of GaAs/AlGaAs DBR microcavity on a GaAs substrate, from Savvidis et al. (2000) (b) SEM and optical micrograph, of $5 \mu m$ diameter, $5 \mu m$ radius of curvature mirrors, from Prakash et al. (2004) (c) Pillar microcavity from an etched planar DBR semiconductor microcavity from Gerard et al. (1996) (d) microdisk (lower ring) with upper electrical contact, from Frateschi et al. (1995) (e) Plasmon localisation: on flat noble metals, metallic voids, metal spheres, and between metal spheres [11].

Other than planar are spherical mirror microcavities which control the photonic modes in the microcavity where the light has to be confined in the other two spatial directions. Another is the Pillar microcavities which are used to confine the lateral extent of the photonic modes inside planar microcavities by etching them into discrete mesas. Whispering gallery modes is an alternative approach which uses total internal reflection within a high refractive index convex body. This can exist within spheres (3D modes)

or disks (2D modes), or more complicated topological structures. For photonic crystals, it employs periodic patterning in 2 or 3 dimensions to confine light to a small volume surrounding a defect of the structure. They are advantageous for many applications since it can show Q-factors exceeding 10^5 while producing extremely small mode but such cavity are hard to fabricate. Lastly is the new plasmonic cavity which is based on plasmons localized to small volumes close to metals.

Along with these are the key issues when considering microcavities. They are the optical losses or finesse, coupling to incident light, optical mode volume, fabrication complexity and tolerance, incorporation of active emitters and practicality of electrical contacting.

2.2 Fabry-Perot Microcavity

Fabry-Perot (FP) interferometer is mainly composed of two high reflecting surfaces spaced out by a spacer. It is called interferometer in the sense that the large number of rays, interfering at the two reflecting surfaces, produces an interferometer with extremely high resolution.

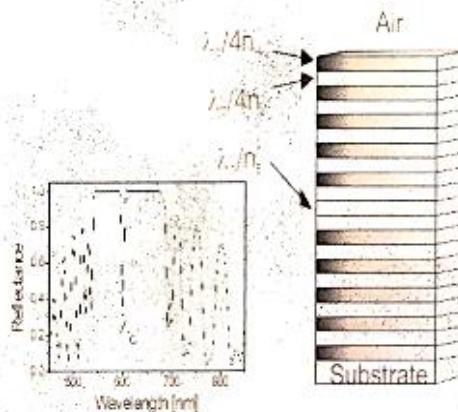


Figure 2.3: Sketch of a Fabry-Perot Microcavity [5].

The whole structure of FP filter will be a multilayered structure if two DBRs are used as reflecting surfaces and thin film as the spacer. This is commonly referred as Fabry Perot microcavity which works on low interference order. Sometimes, FP microcavity

structure can also be described as a DBR structure in which the central layer has been replaced by a different refractive index layer. Figure 2.3 shows an example sketch of Fabry Perot microcavity in which two DBRs are spaced by a thin film of different refractive index with respect to those of the DBR's layers. These central layer or spacer should be of λ_c or $\lambda_c/2$ thickness for it to work on the second or first interference order. On its side is the FP reflectance spectrum centered at λ_c .

2.3 Microcavity Polaritons

A microcavity is composed of Perot-Fabry cavity sandwiched between two reflectors with quantum wells of narrower band gap embedded on it. The reflectors are quarter-wave layers of semiconductor called distributed Bragg reflectors (DBRs) with high and low indices of refraction. Conversely, quantum wells (QWs) are potential wells that confines particle like excitons to two-dimensional motion thus occupying a planar region at low temperatures [12]. When trapped in an optical cavity, photon energy is quantized in the growth direction while the in-plane photon states remain unaffected. Similarly, exciton energy states show same quantization in the growth direction and continuous states in the free in-plane motion. The strong coupling between the exciton and cavity modes gives rise to a new half light and half matter quasi-particle called polaritons. In this strong coupling regime, the photon and exciton dispersion repel each other at resonance (Figure 2.4). This eventually leads to two distinct dispersion called the polariton branches with upper and lower polariton mode [13, 14].

Considering the DBRs force of the axial wave vector k_z to be quantized (see Fig 2.5), the bare cavity dispersion can be derived as,

$$E_{ph} = \hbar c k = \hbar c \sqrt{k_z^2 + k_{\parallel}^2} = \hbar c \left[\left(\frac{N\pi}{n_{eff} L_c} \right)^2 + k_{\parallel}^2 \right]^{1/2} \quad (2.1)$$

where k_z is along the epitaxial growth direction, k_{\parallel}^2 is the wave vector parallel to the quantum well, L_c is the effective cavity length, n_{eff} is the effective intracavity index of refraction and N is the mode number or the number of half-wavelengths in the cavity.

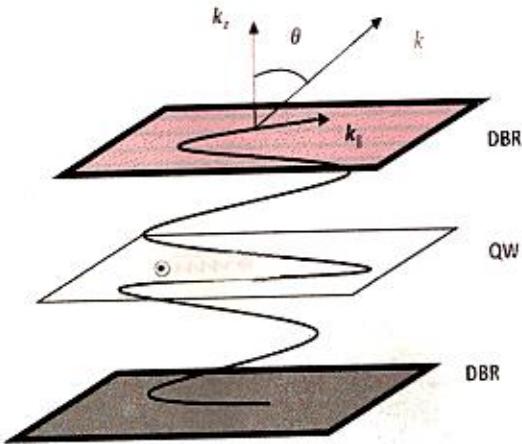


Figure 2.4: Basic structure of microcavities and illustration of the photon-exciton oscillator coupling [7].

Consequently, the exciton energy is,

$$E_{ex} = E_0 + \frac{\hbar^2 k_{\parallel}^2}{2(m_e + m_h)} \quad (2.2)$$

where E_0 and $m_e(m_h)$ is the ground state exciton energy and the electron in-plane mass respectively. For momentum to be conserved as required by the translational invariance of the system, the photon and exciton is given the same in-plane momentum k_{\parallel} . As a result, there is a strong coupling between exciton and photon modes with the same in-plane wave vector.

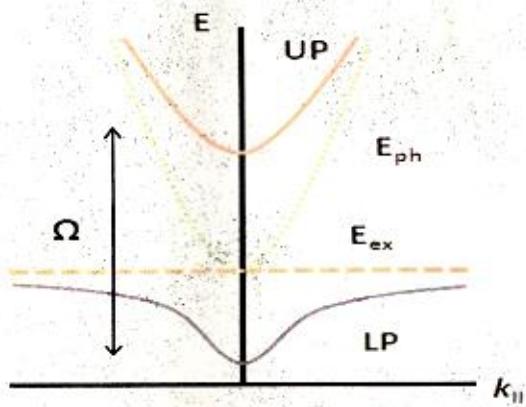


Figure 2.5: Dispersion relation of the upper and lower polariton (solid curves) [7].

The exciton and photon modes can be treated as coupled oscillators with coupling matrix element Ω . The coupling is expressed by matrix Hamiltonian with exciton states $|ex\rangle$ and photon states $|ph\rangle$ as basis. This gives us,

$$H = \begin{bmatrix} E_{ex} & \Omega/2 \\ \Omega/2 & E_{ph} \end{bmatrix} \quad (2.3)$$

where E_{ex} and E_{ph} are the energies of the exciton and cavity photon mode respectively. The eigenvectors of this Hamiltonian is expressed as a superposition of the exciton and photon states,

$$\begin{aligned} |UP\rangle &= C|ex\rangle + X|ph\rangle \\ |LP\rangle &= X|ex\rangle - C|ph\rangle \end{aligned} \quad (2.4)$$

where X and C are just the standard Hopfield coefficients which describe the fraction of the exciton and photon content of the polariton given by,

$$\begin{aligned} X^2 &= \frac{1}{2} + \frac{E_{ph} - E_{ex}}{2\sqrt{(E_{ph} - E_{ex})^2 + \Omega^2}} \\ C^2 &= 1 - X^2 \end{aligned} \quad (2.5)$$

UP and LP are the two coupled mode eigenstates with higher and lower energy states. Eigen energies are derived by diagonalizing the Hamiltonian. We have,

$$E_{LP}^{UP} = \frac{E_{ex} + E_{ph}}{2} \pm \left[\left(\frac{E_{ex} - E_{ph}}{2} \right)^2 + \left(\frac{\Omega}{2} \right)^2 \right]^{1/2} \quad (2.6)$$

The anticrossing or energy splitting between the exciton and photon modes at resonance is the Rabi splitting ($\Omega/2$) pr coupling constant (Ω). Basically, it is just a function of the quantum oscillator strength f containing the electric dipole matrix elements of the atomic transition. It is also dependent on the number of atomic oscillators

as well as the number of quantum wells.

2.4 Polariton Spin and Polarization

Some of the features of Microcavity polariton are its weakly interacting, light mass, lifetime variation in momentum space, bottleneck effect, magic angle, polariton spin and polarization.

Excitons have total spin of ± 1 and ± 2 . These are possible since they are formed by an electron and heavy-hole with $\pm 1/2$ and $\pm 3/2$ spin projections respectively. Exciton with ± 2 spin cannot be optically excited given that photon has spin ± 1 . Spin ± 2 are optically inactive, "dark" states while spin ± 1 are optically "bright" states which can be excited by ω^+ and ω^- circularly-polarized light. Only these "bright" exciton states that are couple to light are shifted in energy by the Rabi splitting while the "dark" states remain unchanged. This eventually increases the exciton binding energy since the excited states does not couple to light.

2.5 Silicon-based microcavity

Over the last decade, optical properties of silicon nanocrystals along with its electronic properties have been a very challenging and promising field of research for technological and scientific reasons [15]. Studies have even extended over other "silicon-like" (e.g. Ge) and silicon based like SiGe nanocrystals. This is due to the poor optical properties of the bulk crystals like Si where it has a small band gap and a resulting phonon-assisted light emission.

Similarly, silicon-based multilayered structures have been intensively studied in the framework of all silicon optoelectronics [16]. A study done by Giorgis [17] shows that microcavity entirely based on hydrogenated amorphous Silicon Nitride ($a-Si_{1-x}N_x : H$) posses appealing optical properties such as resonant enhancement of photoluminescence. Also, Fabry-Perot microcavities were used for the enhancement and inhibition of photoluminescence (PL) in a hydrogenated amorphous silicon nitride ($a-SiN_x : H$) [18].

2.6 Transfer Matrix Formalism

Transfer Matrix (TM) method is generally used in optics and acoustics to analyze the propagation of electromagnetic or acoustic waves through stacked layers of same or different reflectivity indices. This is important for the design of anti-reflective coatings and dielectric mirrors. Thus, these materials give us a wide range of applications in instrumentation, military, medical, scientific, display, vision and space.

As stated, TM method allows the simulation of reflectivity, absorption and transmission of periodic structures [7]. Consider a system of stack dielectric materials of various thicknesses t_j and reflectivity indices n_j where a field is incident. This field will have reflected and transmitted components such as a sum of forward and backward moving waves.

$$E = E_+ e^{ikx} + E_- e^{-ikx} \quad (2.7)$$

The stack of layers can be represented as a system matrix which is the effective contribution of all the layers and interfaces (TM). Transfer matrix equation is then,

$$E' = T_M E \quad (2.8)$$

The Transfer matrix across an interface and a layer are given by

$$T_{int} = \frac{1}{2} \begin{pmatrix} n+1 & -(n-1) \\ -(n-1) & n+1 \end{pmatrix} \quad (2.9)$$

$$T_{layer} = \begin{pmatrix} e^{ik_j t_j} & 0 \\ 0 & e^{-ik_j t_j} \end{pmatrix} \quad (2.10)$$

T_M is then,

$$T_M = \begin{pmatrix} t_{11} & t_{12} \\ t_{12} & t_{22} \end{pmatrix} = T_1 T_2 T_3 \dots T_n \quad (2.11)$$

Now, the transmitted and reflective electric fields are just

$$E_{trans} = \frac{\det(T)}{t_{22}} E_{inc} \quad (2.12)$$

$$E_{ref} = -\frac{t_{21}}{t_{22}} E_{inc} \quad (2.13)$$

With reflectivity as

$$R = \frac{E_{ref}^2}{E_{trans}^2} \quad (2.14)$$

There are also studies using transfer matrix method of multilayer to investigate the transmittance at the central wavelength 720 nm of Si when using various multilayer thin film coatings [19].

2.6.1 Advantages and drawbacks of the Transfer Matrix Method

Transfer Matrix method is a very useful algorithm and has many advantages. But like any other numerical method, it has also its own drawbacks. One of its advantages is, it is fitted for reflectivity and transmission calculations multilayer structures. In this sense, this is very useful in this study where a multilayer specifically a Fabry-Perot microcavity will be simulated for these two optical properties: reflectance and transmittance. With this method, either real or complex values for the refractive index can be taken. Lossless material is represented by real refractive index while one of the two types of materials is represented by the complex refractive index. The negative imaginary part of the refractive index gives us the idea that the material is absorptive while the positive indicates a gain medium.

Another pro of TM method is its ability to handle any number of layers in a multilayer structure. With this, it is better than plane method [20] in which its key method is the matrix diagonalization. The computer time in this method required scales like N³ where N is just the number of plane waves in the expansion. This is inefficient and time consuming if large N is required for more complex structures. With TM there is no requirement that multilayer should be periodic. There is no restriction in TM

regarding the order of any layer's thickness. It can define independently the thickness. So, for modeling structures with different periodic multilayer stacked together, obviously not fully periodic, TM method is the most suitable.

Now for TM's method drawbacks, first it assumes an infinite plane perpendicular to the propagation. This means that each layer in a multilayer structure extends infinitely in both of its dimensions. In order to avoid errors from this assumption, the layers to be modeled have to be wide. Next drawback is it calculates the field throughout the structure by layer by layer propagation with matrix relations. With this, it is computational speed dependent and lacks a mathematical expression that can relate the field between multiple layers. If this mathematical expression is present then it would reduce the mathematical calculation required as well as the computational time.

Lastly, TM method is only limited to continuous propagation, not for pulse propagation. Solution to model such propagation is to combine Fourier Transform with TM. But Finite Difference Time Domain Method is better for pulse propagation.

CHAPTER 3

RESEARCH METHODOLOGY

Presented in this chapter are the methods in modeling and simulating a Si-based microcavity to get the optimum resonance between the exciton and cavity mode. Furthermore, this presents the reflectivity experiment of already grown and fabricated multilayered structures from NIP, UP-Diliman.

3.1 Microcavity Structure

The general structure of the microcavity to be used in this study is a repetition of amorphous silicon nitride ($a-Si_{1-x}N_x : H$) as shown in Figure 3.1. This consists of three sets of distributed Bragg reflectors (DBRs) namely DBR1, DBR2 and DBR3 with alternating high and low index of refraction. There are also two emitter layers spaced out by DBR2. Specifically, H for high refractive index with $n_H = 2.22$ ($x = 0.41$) and L for low refractive index with $n_L = 1.8$ ($x = 0.57$). The two spacers or cavity have refractive index of $n_c = 1.9$ ($x = 0.50$). DBR1 and DBR3 have six pairs of layers while DBR2 has 2.5 pairs. Each layer has an optical thickness of $\lambda/4n_H$ or $\lambda/4n_L$ (where $\lambda = \lambda_c$ - is the central wavelength of AL radiation spectrum). The cavity has thickness of λ_c/n_c . Only consider that the laser is at normal incidence to the top of microcavity.

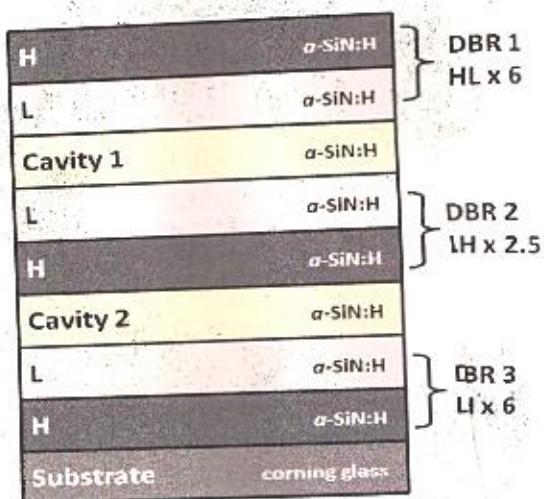


Figure 3.1: Schematic Structure of the SiGe-based microcavity.

The structure is designed so that the resonance modes would be centered at the maximum of the cavity spacers that is why wavelength λ_c is designed to be 685nm. The numbers of pairs LH of DBR2 is also chosen to check the absorption of H layers so C2 would not be excited.

3.2 Simulation Process

The first thing to do in the simulation is the construction of the Si-based microcavity polariton using the structure in Figure 3.1 with corresponding given parameters. After which is the running of the program which will be further discussed in Section 3.3. Then finally, is the gathering of data.

These parameters are:

- Incident angle: This is the angle (θ) between the propagation of incident wave and the normal to the surface of the layer. This ranges from 0 to 90 degrees. Here, we consider that the angle is only 0 degrees which means that the incident wave is at normal incidence.
- Temperature: This considers that the microcavity is at Room temperature (300 K).
- Wavelength range: Range of the wavelength from the initial values to its final values. Also included is the step size.
- Central wavelength: This central wavelength of radiation spectrum. Transmission and reflectivity is centered at this wavelength.
- Refractive index of the ambient medium: This is the first medium before the wave arrives at the surface of the layer. Normally, ambient medium is air.
- Refractive index of the Distributed Bragg reflectors and the spacer or cavity: This is dependent in the x content of a given alloy. Here the refractive indices are dependent on the Nitrogen content of the amorphous silicon Nitride alloy. As the Nitrogen content increases, the refractive index decreases.

- Refractive index of the substrate: The substrate can be in the form of a material or medium.
- Thickness of Distributed Bragg reflectors: This is just a quarter of the central wavelength. Also dependent on its refractive index.
- Thickness of spacer or cavity: The thickness can be either same as the central wavelength or half of it. Also dependent on refractive index. The thickness of the spacer we use is half of the wavelength.
- Data plotted: Any combination of reflectivity or transmission.

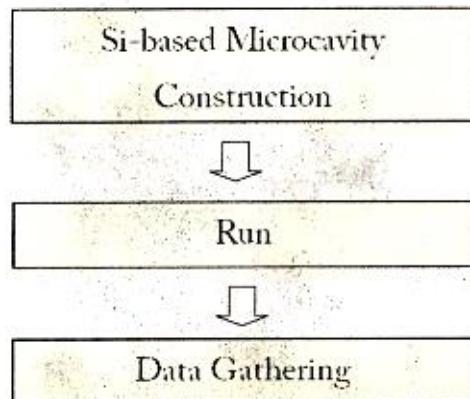


Figure 3.2: Simulation process of the SiGe-based microcavity.

3.3 Run: Algorithm

The algorithm of the program will be discussed in this section. First is the initialization of environment, the Si-based microcavity. Then is the Transfer matrix construction which will return the propagation matrix from one medium to another. Next is the list construction of the length. After which is the calling Transfer matrix functions. Reflectivity and transmittance will be then computed. Finally, the data output or the graphical data output.

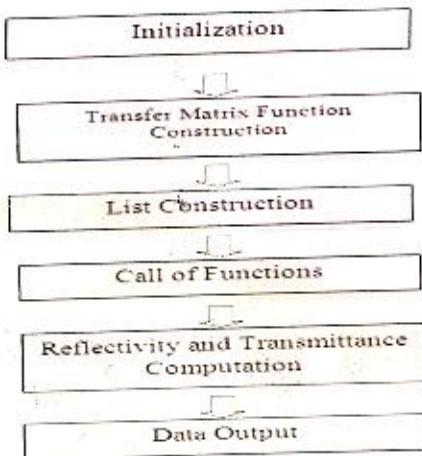


Figure 3.3: Algorithm to be used in the simulation of Silicon-based microcavity.

3.4 Reflectivity Measurement of Multilayered Structures

Figure 3.4 shows the experimental set-up for measuring reflectance of the Multilayered Structures sample at Room Temperature. The sample is attached to a plastic sample holder. Two UV fused silica Plano-convex lenses are used with different focal length, 25mm and 50mm respectively, to focus the light. An input beam is aligned parallel to the optical axis and is focused into a spot on the sample surface with 0 incident angle. The reflected beam is picked up by a 50% beam splitter and sent into HR4000 ocean optics spectrometer which records its $I(\lambda)$. A 50 W halogen lamp is used as the input light, which has relatively flat spectral distribution and supplies sufficient intensity.

3.5 Microcavity polaritons

Same process with the previous method in simulating Silicon-based microcavity will be done on this microcavity the difference is that we consider embedding quantum wells. An example of structure of the microcavity to be used is consist of three sets of four Si quantum wells embedded inside. Only consider that the laser is at normal incidence to the top of microcavity as shown in Figure 3.5. The design could be varied depending on the x component of the SiGe alloy until maximum resonance is achieve.

For the simulation part, the parameters temperature and incident angle are not

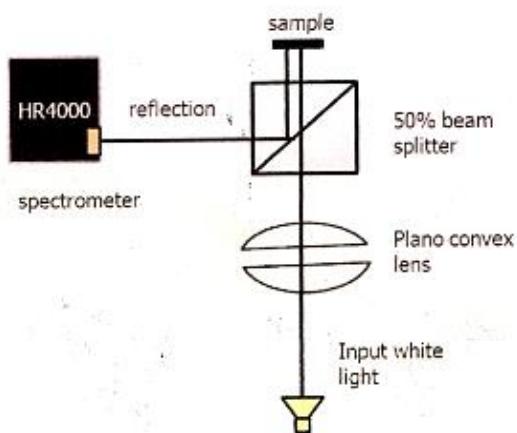


Figure 3.4: Set up for reflection measurement.

change. Note that the refractive index of the $Si_{1-x}Ge_x$ alloy is highly and directly dependent on its Germanium content. So with higher Germanium content, refractive index is high. As Germanium content decreases, the refractive index also decreases.



Figure 3.5: Schematic Structure of the SiGe-based microcavity..

REFERENCES

- [1] Kavokin, A. *Exciton-polaritons in microcavities:recent discoveries and perspectives.*
- [2] Vahala, K. *Optical microcavities*, Nature, **424** (2003) 839-844.
- [3] Dubey, R.S. and Gautam, D.K. "Photoluminescence and Surface Morphology of Nanostructured Porous Silico", *Chalcogenide Letters*, **6**, 10 (2009) 523-528.
- [4] Ballarini, V., Ricciardi, C., Gioorgis, F., Lotti, R.C. and Rossi, F. *Silicon-based microcavities: theory and experiment*, Semicons. Sci. Technol. **19** (2004) S489-S491.
- [5] Ballarini V. *Growth and Characterization of Semiconductor/Insulating Films and Nanostructures for Electronic and Photonic Applications*. PhD thesis, Politecnico di Torino, Corso Duca degli Abruzzi, Torino (2004).
- [6] Deng, H. *Dynamic Condensation of Semiconductor Microcavity Polaritons*. PhD thesis, University of Standford, (2006).
- [7] Balili, R. *Bose-Einstein Condensation of Microcavity Polaritons*. PhD thesis, University of Pittsburgh, (2009).
- [8] Deveaud, B. (Eds.)(2007). *Physics of Semiconductor Microcavities: From Fundamentals to Nanoscale Devices*. Weinheim: WILEY-VCH Verlag GmbH and Co. KGaA.
- [9] Lanty, G., Brhier, A., Parashkov, R., Lauret, J. S., and Deleporte, E. *New Journal of Physics* **10**, 065007 (2008).
- [10] Dohnalova, K. *Study of optical Amplification in Silicon-based Nanostructures*, PhD thesis, Charles University in Prague and Universite Louis Pasteur - Strasbourg I in France (2007).

- [11] Kavokin, A., Baumberg, J., Malpuech, G. and Laussy, F., (2006) *Microcavities* ; Clarendon Press, Oxford.
- [12] Deng, H., Weihs, G., Snoke, D., Bloch, J., and Yamamoto, Y. *Proc. Nat. Acad. Sci.* **100**, 15318 (2003)
- [13] Weisbuch, C., Nishioka, M., Ishikawa, A. and Arakawa, Y. *Phys. Rev. Lett.* **69**, 3314 (1992).
- [14] Houdre, R., Weisbuch, C., Stanley, R. P., Oesterle, U., Pellandini, P. and Illegems, M. *Phys. Rev. Lett.* **73**, 2043
- [15] Zdetsis, A. *Optical and Electronic Properties of Small Size Semiconductor Nanocrystals and Nanoclusters*. Rev. Adv. Mater. Sci. **11** (2006) 56-78.
- [16] Descrovi, E. et. al., *Field localization and Enhanced Second-Harmonic Generation in Silicon-Based Microcavities*. Optics Express. **15**, 7 (2007).
- [17] Giorgis,F. *Optical microcavities based on amorphous Silicon Nitride Fabry-Perot Structures* Appl. Phys. Lett. **77**, 522 (2000).
- [18] Serpengzel, A. Aydinli, A. Bek, A. Gre, M. *Visible Photoluminescence from Planar Amorphous Silicon Nitride Microcavities*. JOSA B, Vol. 15 Issue 11, pp.2706-2711 (1998)
- [19] Salih, K. and Ahmed, N. M. *Int. J. Nanoelectronics and Materials.* **2** No. **1** (2009) 109-118.
- [20] Perez E., *Design, Fabrication and Characterization of Porous Silicon Multilayer Optical Devices*.

Molecular Identification of Locally Isolated *Mycelia Sterilia* Using the Internal Transcribed Spacer (ITS) Region DNA Sequences

INTRODUCTION

Mycelia Sterilia is a large group of fungi with various morphological types. The group reproduces via hyphal fragmentation and shares the property of being defective in forming true spores (Fisher *et al.*, 1994). The classical fungal systematics uses conventional methods based on comparative morphological features which rely heavily on reproductive structures. This method cannot be applied for identifying fungal isolate categorized as *Mycelia Sterilia* due to its failure to sporulate in culture (Lacap *et al.*, 2003). Bills (1996) introduced a practical means of estimating endophytic fungal diversity by grouping *Mycelia Sterilia* into 'morphological species', later termed morphotypes, based on colony surface textures, exudates, growth rate, hyphal pigments and margin shapes. Since these morphotypes are not real taxonomic entities, it do not reflect species phylogeny and may comprise from distantly related taxa (Guo *et al.*, 2000). In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al.*, 2006). Molecular methods are therefore needed to identify *Mycelia Sterilia*.

Nucleotide sequences of the ribosomal RNA (rRNA) provide a means of analyzing phylogenetic relationship over a wide range of taxonomic levels (Woese and Olsen, 1986). Several recent studies have shown that most of the endophytic fungi were detected and identified by comparative analyses of the ribosomal DNA (rDNA) sequences, especially the internal

transcribed spacer (ITS) region of the isolate (Gao *et al.*, 2005; Wang *et al.*, 2005; Arnold *et al.*, 2007; Ligrone *et al.*, 2007 Sánchez Márquez *et al.*, 2007; Morakotkarn *et al.*, 2007). DNA sequences have been used by fungal taxonomists as a basis for reclassification of all fungal taxa and have more recently utilized ITS sequencing as the "gold standard".

OBJECTIVES

Published studies exclusively on the taxonomic position of *Mycelia Sterilia* in the Philippines are rare. This study aims to identify locally isolated *Mycelia Sterilia* by molecular analysis of the internal transcribed spacer (ITS) region DNA sequences including the ITS1, 5.8S, and ITS2, and comparing the sequences with those established fungal species in the GenBank. Specifically, this study aims to: (1) isolate high quality genomic DNA of thirty locally isolated *Mycelia Sterilia* by comparing the efficiency of different DNA isolation kits, (2) amplify and sequence the ITS region and (3) analyze the sequences of the ITS region of the isolates. The experiment will be conducted at the Molecular Biology and Biotechnology Laboratories, Institute of Biological Science, University of the Philippines, Los Baños on June 2010 to under the supervision of Dr. Rina B. Opulencia, Dr. Teresita U. Dalisay and Dr. Asuncion K. Raymundo.

REVIEW OF LITERATURES

Mycelia Sterilia

Mycelia Sterilia of fungi is an artificial taxonomic group characterized by their inability to produce spores of any kind (Hawksworth *et al.*, 1983). The lack of sporulation is the only unique trait shared by all sterile mycelia. The nomenclature of the members of *Mycelia Sterilia* is unidentifiable since fungal systematics is mainly based on morphology of spores and spore bearing structures. Limited taxonomic and genetic information has been available on *Mycelia Sterilia* due to the problems with their classification. Historically, the group was considered a part of Fungi Imperfecti, a questionable higher taxon of Kingdom Fungi. Members of this group also require a more complicated propagation, maintenance and preservation compared to sporulating fungi (Currah and Tsuneda, 1993).

Distribution of *Mycelia Sterilia* in Nature

Fungi of the *Mycelia Sterilia* group are ubiquitous and have been isolated from diverse ecological niches. They commonly prefer decaying wood as habitat but can also be found in soil, plants and plant debris. *Mycelia Sterilia* which resides asymptotically inside living plant tissues is endophytic. Surveys of various host plants have demonstrated that these fungal endophytes are ubiquitous in plant species (Kumar *et al.*, 2004; Zhang *et al.*, 2006; Sánchez Márquez *et al.*, 2007; Huang *et al.*, 2008; Oses *et al.*, 2008) and even lichens (Li *et al.*, 2007).

An average 20-54% of the population for a given host of a fungal endophyte belongs to the *Mycelia Sterilia* group (Fisher *et al.*, 1994).

Mycelia Sterilia is also routinely isolated from household environments. Heterogeneous group of *Mycelia Sterilia* was the only airborne fungi found present at higher concentrations in the homes of wheezing children. Wheezing is a high-pitched whistling sound produced when there is a blockage to the regular flow of air from the lungs. Air pollution can cause wheezing in young children. These *Mycelia Sterilia* isolates may include a potent source of allergens which cause wheezing. Several fungi of this group are known to be associated with human allergies and skin infections in humans. *Mycelia Sterilia* was also found to be present in the ear canal of cattle with external parasitic otitis. Future studies may confirm and elucidate the importance of these agents in the etiology of bovine otitis (Vinnere, 2004).

Importance of *Mycelia Sterilia*

Members of *Mycelia Sterilia* group show unexpected characters such as being both plant deleterious and plant growth promoter. Among sterile fungi, *Rhizoctonia solani* and *Sclerotium rolfsii* are well-known plant pathogens of high economical importance. *R. solani* is a difficult-to-control fungus, responsible for severe yield losses in potatoes, cereals and other agricultural crops worldwide while *S. rolfsii* is the causal agent of southern blight on a great range of agricultural crops. There are also reports of less common plant pathogenic sterile fungi causing stem rot of bean seedlings, crown canker of pigeon pea (*Cajanus cajan*) and several others (Howard *et al.*, 1977).

On the other hand, Sterile Red Fungus isolated from Australia has the potential for biological control of *Gaeumannomyces graminis* var. *tritici* – a pathogen of worldwide importance, the causal agent of take-all disease (Rowland *et al.*, 1994). Another *Mycelia Sterilia* member, the Sterile Dark Mycelia fungus, when incorporated in the soil infested with *Gaeumannomyces graminis* var. *tritici* also showed reduced infection of the take-all fungus of wheat crops in Hokkaido.

Several other sterile isolates have also shown good biocontrol efficacies against root pathogens of agricultural crops. Table beets planted on *Laetisaria arvalis* amended soils showed reduced infection by *Phoma betae*, a seed borne pathogen. An unidentified *Mycelia Sterilia* was also found to be a hyper parasite on the mycelia of *Macrophomina phaseolina*, the cause of damping-off and charcoal root rot disease on slash pine seedlings (*Pinus elliottii* Engelm var. *elliottii*). A sterile septate fungus has the potential to suppress damping-off caused by *Ralstonia solani*. A sterile fungus isolated from zoysiagrass rhizosphere was found to promote growth of spring wheat.

Information about the ecological role of *Mycelia Sterilia* is scarce (Hall, 1987). Sterile fungi may have symbiotic association in plant families that usually lack it. Such isolates are able to cause asymptotically colonize root cortex and in some cases also the stele. It was speculated that, among other cortical fungi, *Mycelia Sterilia* could contribute to plant fitness including its defense against pathogens, improving nutrition conditions, fecundity, etc.

Taxonomy of *Mycelia Sterilia*

Fungal Morphotypes. *Mycelia Sterilia* members are not usually given taxonomic status since conventional classification of fungi relies heavily on reproductive structures, which they lack (Lacap *et al.*, 2003). Mycologists have adopted the concept of morphotypes to provide a practical means to estimate fungal diversity. Since then several other studies followed the same concept in determining diversity of fungi in the absence of reproductive structures (Taylor *et al.*, 1999, Fröhlich and Hyde, 1999, Guo *et al.*, 2000 and Fröhlich *et al.*, 2000).

The organization of taxa into morphotypes does not reflect species phylogeny because these morphotypes are not real taxonomic entities (species/genus etc). A morphotype which has similar cultural character may comprise distantly related taxa. *Mycelia Sterilia* has been and remains an enormously difficult group of fungi for taxonomists. Various identification methods including morphology, physiological properties, nucleic acid amplification, restriction fragment length polymorphism analysis, and sequencing have been proposed in the past to better discriminate and understand the possible classification of *Mycelia Sterilia* (Chen *et al.*, 2002).

Morphology. *Mycelia Sterilia* can be assigned to one of the classes of the Fungal Kingdom - Ascomycetes or Basidiomycetes using very few mycelial features that are available. The principal differences between mycelia of fungi belonging to these classes are: 1) the structure of hyphal septa (simple septum in Ascomycetes and dolipore septum in Basidiomycetes) and 2) presence or absence of clamp connections, which are a characteristic

feature of Basidiomycetes (Alexopoulos *et al.*, 1996). Both presence of dolipore septum and presence or absence of clamp connections can be used as important features for separation of the sterile basidiomycetous isolates.

Other hyphal characters that are informative for the characterization of sterile strains include presence of moniloid cells (enlarged hyphae of irregular shape), formation of sclerotia, differences in hyphal diameter, length, character of hyphal branching, overall colony appearance, margins, texture, color, growth rate, temperature response, etc. Most of these features, however, are dependent on growth conditions such as media composition, light intensity, etc.

Several diagnostic keys were also developed based on morphological characters to aid proper identification of the Basidiomycetes that are unable to produce spores under laboratory conditions. Furthermore, several comprehensive descriptions of the most abundant sterile fungi are available.

Biochemical studies. Color reactions in response to adding certain chemicals such as KOH, peroxidase and dyes are some simple chemical test for many non-sporulating isolates especially the ones belonging to the Basidiomycetes are widely used (Stalpers, 1978; Desjardin, 1990). The genus *Rhizoctonia* is frequently characterized using isozyme analysis (Sweetingham *et al.*, 1986; Yang *et al.*, 1994; Worth, 2002).

Molecular analyses. The advent of molecular techniques such as sequencing of the ribosomal genes and their spacers could be a potential approach for the identification of *Mycelia Sterilia* (Guo *et al.*, 2000). Molecular approaches have been developed to provide more rapid and accurate identification of fungi compared to traditional morphological methods. Various

analyses of the DNA are used as a powerful tool for separation of sterile strains and their analyses of the DNA are used as a powerful tool for separation of sterile strains and their tentative identification. DNA sequencing is widely used for this purpose. Sequence analysis is a relatively simple procedure. In addition, interpretation of nucleic acid sequences is straightforward and does not depend on too much expertise compared to morphological analyses (Ciardo *et al.*, 2006).

Despite all difficulties in identification of sterile strains, there has been several works attempting to resolve taxonomic position of biologically active (deleterious or beneficial) *Mycelia Sterilia*, and as it was expected, all of them employ rDNA analysis for this particular purpose. Several sterile fungi have been tentatively identified based on the ITS region, small subunit rRNA gene and large subunit rRNA gene.

Polymerase Chain Reaction (PCR)

PCR is technique developed by Kary Mullis in 1985 as an in vitro method for replicating a defined DNA sequence so that its amount is increased exponentially. It consists of repetitive cycles of denaturation to convert double-stranded DNA to single stranded, annealing of oligonucleotide primers to the target DNA, and extension of the DNA by nucleotide addition from the primers by the action of DNA polymerase. This technique is capable of amplifying 10^5 to 10^6 -fold from nanogram amounts of template DNA within a large background of irrelevant sequences.

PCR has a diverse application in molecular biology, clinical diagnosis, forensic analysis and population genetics due to its sensitivity, specificity and rapidity. It offers the opportunity to

characterize fungi by amplification of specific sequences. Polymerase chain reactions (PCR)-based systems in a variety of formats have been extensively used for identification of fungi. These methods include PCR (Li *et al.*, 2003), ITS fragment length polymorphism (Chang *et al.*, 2001), restriction fragment length polymorphism (Frutos *et al.*, 2004), DNA probe hybridization (Coignard *et al.*, 2004), and DNA sequencing. Among these molecular methods, ITS sequence analysis has been proven to be an accurate method for species delineation. However, until now only a limited number of species or just a specific genus has been evaluated for species identification by ITS sequence analysis (Chen *et al.*, 2000; Hinrikson *et al.*, 2005; Sugita *et al.*, 1999; 2002).

In recent years, several DNA-based molecular identification methods have been established which make use of the variable domains of the 18S or 28S rRNA gene (Kurtzman and Robnett, 1997; Makimura *et al.*, 1994). Since the variability of 18S and 28S rRNA genes is limited, it can be difficult to differentiate between species (Fell *et al.*, 2000). The ITS region, located between the 18S and 28S rRNA genes, is more promising for species discrimination because of its higher variability (Iwen *et al.*, 2002). Although attempts to identify fungi by focusing on either the ITS1 or the ITS2 region may be successful for some species and genera (Chen *et al.*, 2000; 2001), analysis of the complete ITS region offers greater promise for molecular identification (Chen *et al.*, 2001). The reliability of identification by sequencing not only depends on the length of the sequence determined, but also on the quality and availability of reference sequences. High sequence similarity of the ITS regions may be evidence, but it does not provide definite proof for the identity of two taxa. However, for a large majority of species, the similarity tree of the ITS region is identical to the phylogenetic tree, allowing good identification (Scorzetti *et al.*, 2002).

Internal transcribed spacer region (ITS). Since the variability of 18S and 28S rRNA genes is limited, it can be difficult to differentiate between species due to its conserved nature. (Fell *et al.*, 2000).

The internal transcribed spacer (ITS) region is located between the highly conserved genes coding for 18S and 28S rRNA. The ITS encompasses the two non-coding regions, ITS1 and ITS2, in the DNA genes encoding the fungal ribosomal RNA (rRNA) "operon". The ITS regions are separated by the highly conserved 5.8S rRNA gene (White *et al.*, 1990). ITS1 is found between the 18S and the 5.8S rRNA genes while ITS2 is found between the 5.8S and the 28S rRNA genes (Fig. 1). The entire rRNA "operon" is transcribed, but, after transcription, the two ITS sequences are excised and, therefore, not used for any functional purpose (Waddington, 2009).

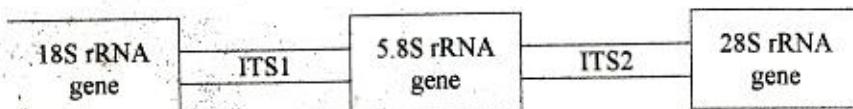


Figure 1. Schematic representation of fungal ribosomal genes bridged by ITS regions.

The internal transcribed spacer (ITS) region evolves fast and is often highly variable among distinct fungal species (Gardes *et al.*, 1991). It is allowed to accumulate mutations at a faster rate than the 5.8S, 16S, and 28S rRNA genes since it is not used for any functional purpose other than as spacer. It is this slightly increased rate of accumulated mutations that allows the ITS sequences to provide an improved level of resolution compared with the rRNA gene sequences (Waddington, 2009). ITS region also has less selective pressure than do ribosomal gene regions and thus changes more rapidly. However, some selective pressure exists, probably

from the constraint to maintain the RNA secondary structure required for post-transcriptional processing (Shinohara *et al.*, 1999). The ITS1 and ITS2 must continue to fold in such a way that nucleases can cut the transcript at the appropriate points.

The rDNA ITS (ITS1, ITS2 and the 5.8S gene) regions are often used for taxonomy at the genus and species levels. It is a convenient target for molecular identification of fungi since the entire ITS region is often between 500 and 800 bp and can be readily amplified with 'universal primers' that are complementary to sequences within the rRNA genes. Primers make use of the conserved regions of the 18S, 5.8S and 28S rRNA genes to amplify the non-coding regions between them (White *et al.*, 1990). It can easily be amplified due to its multicopy nature even from small, dilute, or highly degraded DNA samples. The ITS region is also often highly variable among morphologically distinct fungal species (Gardes *et al.*, 1991; Gardes & Bruns 1991; Baura *et al.*, 1992; Chen *et al.*, 1992; Lee & Taylor 1992) but, with one known exception (O'Donnell 1992), intraspecies variation is low (Gardes *et al.*, 1991; Gardes & Bruns 1991; Anderson & Stasovski 1992; Baura *et al.*, 1992; Chen *et al.*, 1992; Lee & Taylor 1992).

Species concept in *Mycelia Sterilia*

There are currently at least 28 genera and around 200 species of *Mycelia Sterilia* known to science according to the current edition of the Dictionary of Fungi (Kirk *et al.*, 2001). However, it is very difficult to justify these numbers. Some of these sterile mycelia were found to be just a mycelial stage of already known fungi with mainly basidiomycetous teleomorphs (including such well-known examples as *Rhizoctonia* and *Sclerotium*), others fail to sporulate on artificial substrata, etc. The structures observed in vegetative fungal thallus are usually too few to create a reliable key system for separation of different mycelia into species. The level of variation exhibited by the isolates belonging to the same species of *Mycelia Sterilia* is unknown as well as if variation level is the same among different species of sterile fungi. Uniqueness of certain traits is also unknown (Parmeter *et al.*, 1964). Historically, the tendency very often was to name sterile isolates by the closest species of sterile fungi (Howard *et al.*, 1977). Accumulation of DNA sequencing data in the future can shed some light upon problems of systematics of *Mycelia Sterilia* and will definitely lead to revision of several existing taxa.

MATERIALS AND METHODS

Collection of *Mycelia Sterilia*. The Mycology Laboratory of the Crop Protection Cluster, College of Agriculture, U.P. Los Baños, headed by Dr. Teresita U. Dalisay, houses isolates declared as *Sterile Mycelia* after 2-month incubation on an in-house strength of Potato Dextrose Agar (PDA) at room temperature. Thirty cultures isolated from various sources will be collected for this study. Provenances of these cultures are detailed in Table 1. The isolates will be sub-cultured on Sabouraud dextrose agar (SDA). Stock and working cultures will be stored at 4°C and 30°C, respectively.

Cultural Characterization. Each *Mycelia Sterilia* isolates will be inoculated to two (replicate) SDA plates and will be incubated at 30°C for 2 weeks. Cultural characteristics of the isolates will be observed such as colony shape, color (above and reverse), elevation, texture, type of mycelium, margin shape, density, zonation and effect on the medium will be examined after two weeks of incubation. Colony diameter (cm) will also be measured on the 7th and 14th day of incubation. Cultures which are presumptively carrying spores after an extended two-month incubation period under laboratory conditions will not be included in the study.

Table 1. Geographic location, ecosystem and associated organism from where Sterile *Mycelia* are collected.

ISOLATE CODE	GEOGRAPHIC LOCATION	ECOSYSTEM	ASSOCIATED ORGANISM
BD1	Marinduque	Soil near Mine tailing	Corn
BD2	Marinduque	Soil near Mine tailing	Corn
BD3	Marinduque	Soil near Mine tailing	Corn
BD4	Marinduque	Soil near Mine tailing	Corn
BD5	Marinduque	Soil near Mine tailing	Corn
BD6	Marinduque	Soil near Mine tailing	Corn
BD7	Batangas	Lowland soil treated with organic fertilizer	Rice
BD8	Batangas	Lowland soil treated with organic fertilizer	Rice
BD9	Batangas	Lowland soil treated with organic fertilizer	Rice
BD10	Batangas	Lowland soil treated with organic fertilizer	Rice
BD11	Batangas	Lowland soil treated with organic fertilizer	Rice
BD12	Batangas	Lowland soil treated with organic fertilizer	Rice
BD13	Batangas	Lowland soil treated with organic fertilizer	Rice
BD14	Laguna	Tiller/Root/Leaf	Rice
BD15	Laguna	Tiller/Root/Leaf	Rice
BD16	Laguna	Tiller/Root/Leaf	Rice
BD17	Laguna	Tiller/Root/Leaf	Rice
BD18	Laguna	Tiller/Root/Leaf	Rice
BD19	Laguna	Tiller/Root/Leaf	Rice
BD20	Laguna	Tiller/Root/Leaf	Rice
BD21	Laguna	Tiller/Root/Leaf	Rice
BD22	Laguna	Tiller/Root/Leaf	Rice
BD23	Laguna	Tiller/Root/Leaf	Rice
BD24	Laguna	Tiller/Root/Leaf	Rice
BD25	Laguna	Meat	Kuhol
BD26	Laguna	Meat	Kuhol
BD27	Laguna	Meat	Kuhol
BD28	Laguna	Meat	Kuhol
BD29	Laguna	Meat	Kuhol
BD30	Laguna	Meat	Kuhol

Table 2. Cultural characteristics on Sabouraud Dextrose Agar (SDA) of the 30 isolates of *Mycelia Sterilia* from various sources.

Isolate Code	Size (cm) of colony after		Shape	Color		Elevation	Texture	Mycelium	Edge	Density	Zonality	Effect on Medium
	7 days	14 days		Above	Reverse							
BD 1												
BD 2												
BD 3												
BD 4												
BD 5												
BD 6												
BD 7												
BD 8												
BD 9												
BD 10												
BD 11												
BD 12												
BD 13												
BD 14												
BD 15												
BD 16												
BD 17												
BD 18												
BD 19												
BD 20												
BD 21												
BD 22												
BD 23												
BD 24												
BD 25												
BD 26												
BD 27												
BD 28												
BD 29												
BD 30												

Culture Preparation. A hyphal tip will be obtained from each fresh culture using a dissecting microscope and will be incubated on Potato Dextrose Broth (PDB) for 7 days at 30°C with shaking. After incubation, mycelia will be separated from the broth by centrifugation at 30,000 rpm for 5 minutes. Harvested mycelia will be stored at -80°C until needed.

PDB will be prepared by mixing 20g each of dextrose and agar, and 200g potato infusion in 1 litter of distilled water. The resulting mixture will be heated until all the ingredients are completely dissolved. Potato infusion will be prepared by boiling 200g potato in 1000ml of distilled water.

DNA Extraction. Three commercially available DNA extraction kits will be compared based on their efficiency to extract a good quality DNA indicated by a clean band on 1% w/v agarose gel in 0.5 x TAE buffer (40mM Tris, 1mM EDTAm pH 8.0) which will be visualized using UV light after staining with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$).

Two tough to lyse fungi will be used for the evaluation of the following kits: ZR Fungal/Bacterial DNA Kit™ (Zymo Research Corporation, USA), GF-1 Plant DNA Extraction Kit (Vivantis Technologies, USA) and GenElute DNA Extraction Kit (Sigma-Aldrich, USA). Genomic DNA will be extracted from each harvested mycelia using mini-columns according to the manufacturer's protocol. Appendix 1 shows the protocols from each manufacturer.

PCR Amplification. Universal primers, pITS1 and pITS4, will be used to amplify the ITS regions including the 5.8S rRNA gene of the thirty *Mycelia Sterilia* isolates. Primer sequences are as follows: pITS1 = TCCGTAGGTGAAACCTGCGG and pITS4 = TCCTCCGCTTATTGATATGC. An approximately 500-800bp product is expected after

amplification (White *et al.*, 1990). The primers will be synthesized by Aitbiotech Pre. Ltd. (Singapore). The ITS region will be amplified using G-Storm GS-1 automated thermal cycler (G-Storm, UK). Amplification will be performed in a 50 μ L reaction volume which will contain 25 μ L 2X Taq Master Mix (Vivantis Technologies, USA), 0.3 μ M of each primera, ~5ng template DNA and 1.5-3.0mM MgCl₂. The thermal cycling program will be as follows: 5 minute initial denaturation at 95°C, followed by 40 cycles of 30 seconds denaturation at 94°, 50s primer annealing at 52°C - 60°C, 1 min extension at 72° and a final 10 min extension at 72°. A negative control using water instead of template DNA will be included in the amplification process. Four microlitres of PCR products from each PCR reaction will be analyzed by electrophoresis at 100 V for 2 h in a 1% (w/v) agarose gel in 0.5X TAE buffer and will be visualized using UV light after staining with ethidium bromide (0.5 μ g ml⁻¹). The concentration of MgCl₂ and annealing temperature will be varied until optimized conditions are attained as indicated by a single band on 1% agarose gel.

Sequencing. The unpurified PCR amplicons will be sent to Macrogen, Inc., South Korea which uses the machine ABI3730XL (Applied Biosystems, USA) for sequencing. DNA strands will be sequenced bi-directionally using the primers pITS1 and pITS4.

DNA Sequence Analysis. Contiguous sequences will be assembled and manually edited using Sequencher® 4.10.1 (USA). Discrepancies will be resolved by verifying the chromatogram for both forward and reverse sequences. These *Mycelia Sterilia* sequences will then be used as query sequence to search for similar sequences from GenBank and EMBL using BLAST program. The most similar reference sequences with query sequences will be obtained

and will be used for subsequent phylogenetic analyses with several standard reference sequences. The complete ITS region sequences will be aligned using Clustal W2 program (Larkin *et al.*, 2007) and the results will be adjusted manually where necessary to maximize alignment. The alignment data will be subsequently used for maximum-parsimony analysis in which searches for most parsimonious trees will be conducted with the heuristic search algorithms with tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b1a (Swofford 1998). For each search, 1000 replicates of random stepwise sequence addition will be performed and 100 trees will be saved per replicate. Gaps will be treated as missing data. Character states will be treated as unordered. Statistical support for the internal branches will be estimated by bootstrap analysis with 1000 replications.

To establish the general origin of the *Mycelia Sterilia* in the kingdom Fungi, the 5.8S genes of 17 representative reference taxa as recommended by Guo *et al.*, 2003 will be obtained from GenBank and EMBL for phylogenetic analysis, of which nine reference species, will be of *Ascomycota* (*Dermea acerina* AF141164, *Dothidea hippophaeos* AF027763, *Gelasinospora nigeriensis* AJ002400, *Colletotrichum graminicola* AF059676, *Hypocrea rufa* X93980, *Morchella crassipes* AF008232, *Sclerotinia borealis* AF067644, *Tryblidiopsis pinastri*, and *Xylaria hypoxylon*), four reference species of *Basidiomycota* (*Lentinula edodes* U33093, *Pisolithus tinctorius* AF004737, *Pleurotus pulmonarius* U60648, and *Tremella foliacea* AF042452), and four reference species of *Zygomycota* (*Endogone pisiformis* AF006511, *Gigaspora margarita* AY035661, *Glomus mosseae* U31996 and *Mucor recurvus* AF412294).

To further identify these sequences to as low a taxonomic level as possible; ITS region sequences will be included in the subsequent maximum parsimony, which will include more closely related taxa.

LITERATURE CITED

- ALEXOPULOS, C.J. MIMS, C.W. AND BLACKWELL, M. 1996. Introductory mycology. 4th edition. John Wiley & Sons, Inc., New York -...- Singapore.
- ANDERSON J.B. AND E. STASOVSKI. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria*. *Mycologia*, 84: 505-516.
- ARNOLD, A.E., D.A. HENK, R.L. EELLS, F. LUTZONI AND R. VILGALYS. 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* 99: 185-206.
- BAURA G, T.M. SZARO AND T.D. BRUNS. 1992. *Gastrosuillus laricinus* is a recent derivative of *Suillus greuillei*: molecular evidence. *Mycologia*, 84: 592-597.
- BILLS, G.F. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: Endophytic Fungi in Grasses and Woody Plants (eds. S.C. Redlin, L.M. Carris and M.N. St Paul). APS Press, USA: 31-65.
- CHANG, H. C., S. N. LEAW, A. H. HUANG, T. L. WU, AND T. C. CHANG. 2001. Rapid identification of yeasts in positive blood cultures by a multiplex PCR method. *J. Clin. Microbiol.* 39:3466-3471.
- CHEN W., J.W. HOY AND R.W. SCHNEIDER. 1992. Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythlium* species. *Experimental Mycology*, 16: 22-34.
- CHEN, Y. C., J. D. EISNER, M. M. KATTAR, S. L. RASSOULIAN-BARRETT, K. LAFE, S. L. YARFITZ, A. P. LIMAYE, AND B. T. COOKSON. 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J. Clin. Microbiol.* 38:2302-2310.
- CHEN, Y. C., J. D. EISNER, M. M. KATTAR, S. L. RASSOULIAN-BARRETT, K. LAFE, U. BUI, A. P. LIMAYE, AND B. T. COOKSON. 2001. Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. *J. Clin. Microbiol.* 39:4042-4051.
- CIARDO, D. E., G. SCHÄR, E.C. BÖTTGER, M. ALTWEGG AND P. P. BOSSHARD. 2006. Internal Transcribed Spacer Sequencing versus Biochemical Profiling for Identification of Medically Important Yeasts. *Journal of Clinical Microbiology*. 44(1): 77-84.
- COIGNARD, C., S. F. HURST, L. E. BENJAMIN, M. E. BRANDT, D. W. WARNOCK, AND C. J. MORRISON. 2004. Resolution of discrepant results for *Candida* species identification by using DNA probes. *J. Clin. Microbiol.* 42:858-861.

CURRAH, R.S. AND A. TSUNEDA. 1993. Vegetative and reproductive morphology of *Phialocephala fortinii* (Hyphomycetes, *Mycelium radicis atrovirens*) in culture. Transaction of Mycological Society of Japan 34: 345-356.

FELL, J. W., T. BOEKHOUT, A. FONSECA, G. SCORZETTI, AND A. STATZELL-TALLMAN. 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evol. Microbiol. 50:1351-1371.

FISHER, P.J., O. PETRINI, L.E. PETRINI AND SUTTON, B.C. 1994. Fungal endophytes from the leaves and twigs of *Quercus ilex* L. from England, Majorca and Switzerland. New Phytologist, 127: 133-137.

FRÖHLICH, J. AND K.D. HYDE. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? Biodiversity and Conservation 8: 977-1004.

FRÖHLICH, J., HYDE, K. D. & PETRINI, O. 2000. Endophytic fungi associated with palms. Mycological Research 104: 1202-1212.

FRUTOS, R. L., M. T. FERNANDEZ-ESPINAR, AND A. QUEROL. 2004. Identification of species of the genus *Candida* by analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Antonie Leeuwenhoek 85:175-185.

GAO, X.X., H. ZHOU, D.Y. XU, C.H. YU, Y.Q. CHEN AND L.H. QU. 2005. High diversity of endophytic fungi from the pharmaceutical plant, *Heterosmilax japonica* Kunth revealed by cultivation-independent approach. FEMS Microbiology Letters 249: 255-266.

GARDES M. AND T.D. BRUNS. 1991. Rapid characterization of ectomycorrhizae using RFLP pattern of their PCR amplified-ITS. Mycological Society Newsletter, 41: 14.

GARDES M., T.J., WHITE, J.A. FORTIN, T.D. BRUNS AND J.W. TAYLOR. 1991. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Canadian Journal of Botany, 69: 180-190.

GUO, L. D., HYDE, K. D. & LIEW, E. C. Y. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytologist 147: 617-630.

GUO, L. D., HUANG G.R., WANG, Y., HE, W.H., AND ZHENG, W.H. 2003. Molecular Identification of white morphotype strains of endophytic fungi *Pinus tabulaeformis*. Mycol. Res. 107 (6): 680-688.

HALL, G. 1987. Sterile fungi from roots of winter wheat. Transactions of British Mycological Society 89: 447-456.

HAWKSWORTH, D.L., B.C. SUTTON AND G.C. AINSWORTH. 1983. Ainsworth and Bisby's Dictionary of Fungi. CMI, Kew, UK.

HINRIKSON, H. P., S. F. HURST, T. J. LOTT, D. W. WARNOCK, AND C. J. MORRISON. 2005. Assessment of ribosomal large-subunit D1–D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. *J. Clin. Microbiol.* 43:2092–2103.

HOWARD, C.M., CONWAY, K.E. & ALBREGTS, E.E. 1977. A stem rot beam seedlings caused by a sterile fungus in Florida. *Phytopathology*. 67:430-433.

IWEN, P. C., S. H. HINRICHES, AND M. E. RUPP. 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Med. Mycol.* 40:87–109.

KIRK, P.M., CANNON, P.E., DAVID, J.C. & STALPERS, J.A. (2001) Ainsworths & Bisby's Dictionary of Fungi, 9th edition. CABI Bioscience, Egham, UK.

KURTZMAN, C. P., AND C. J. ROBNETT. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5'end of the large-subunit (26S) ribosomal DNA gene. *J. Clin. Microbiol.* 35:1216–1223.

LACAP, D.C., K.D. HYDE AND E.C.Y. LIEW. 2003. An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. *Fungal Diversity* 12: 53-66.

LARKIN MA, BLACKSHIELDS G, BROWN NP, CHENNA R, MCGETTIGAN PA, MCWILLIAM H, VALENTIN F, WALLACE IM, WILM A, LOPEZ R, THOMPSON JD, GIBSON TJ, HIGGINS DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947-2948.

LEE S.B. AND J.W. TAYLOR. 1992. Phylogeny of five fungus-like prototistian *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biology and Evolution*, 9: 636-653.

LI, Y. L., S. N. LEAW, J. H. CHEN, H. C. CHANG, AND T. C. CHANG. 2003. Rapid identification of yeasts commonly found in positive blood cultures by amplification of the internal transcribed spacer regions 1 and 2. *Eur. J. Clin. Microbiol. Infect. Dis.* 22:693–696.

LIGRONE, R., A. CARAFA, E. LUMINI, V. BIANCIOTTO, P. BONFANTE AND J.G. DUCKETT. 2007. Glomeromycetean associations in liverworts: A molecular cellular and taxonomic analysis. *American Journal of Botany* 94: 1756-1777.

MAKIMURA, K., S. Y. MURAYAMA, AND H. YAMAGUCHI. 1994. Detection of a wide range of medically important fungi by the polymerase chain reaction. *J. Med. Microbiol.* 40:358–364.

MORAKOTKARN, D., H. KAWASAKI AND T. SEKI. 2007. Molecular diversity of bamboo-associated fungi isolated from Japan. *FEMS Microbiology Letters* 266: 10-19.

- O'DONNELL, K.L. 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). Current Genetics 22: 213-220.
- PARMETER, J.R., JR. 1964. The taxonomy of Sterile Fungi. Phytopatology. 55:826-828.
- SÁNCHEZ MÁRQUEZ, S., G.F. BILLS AND I. ZABALGOGEAZCOA. 2007. The endophytic mycobiota of the grass *Dactylis glomerata*. Fungal Diversity 27: 171-195.
- SCORZETTI, G., J. W. FELL, A. FONSECA, AND A. STATZELL-TALLMAN. 2002. Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. FEMS Yeast Res. 2:495-517.
- SETTE, L.D., M.R.Z. PASSARINI, C. DELARMELINA, F. SALATI AND M.C.T. DUARTE. 2006. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. World Journal of Microbiology and Biotechnology 22: 1185-1195.
- SHINOHARA, M.L., K.F. LOBUGLIO AND S.O. ROGERS. 1999. Comparison of ribosomal DNA ITS regions among geographic isolates of *Cenococcum geophilum*. Curr Genet 35: 527-535.
- SUGITA, T., A. NISHIKAWA, R. IKEDA, AND T. SHINODA. 1999. Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. J. Clin. Microbiol. 37:1985-1993.
- SUGITA, T., M. NAKAJIMA, R. IKEDA, T. MATSUSHIMA, AND T. SHINODA. 2002. Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. J. Clin. Microbiol. 40:1826-1830.
- TAYLOR, J.E., K.D. HYDE, AND E.B.G. JONES. 1999. Endophytic fungi associated with the temperate palm *Trachycarpus fortunei* both within and outside of its natural geographic range. New Phytologist 142: 335-346.
- VINNERE, O. 2004. Approaches to Species Delineation in Anamorphic (mitosporic) Fungi: A Study on Two Extreme Cases. Acta Universitatis Upsaliensis. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 917. 72 pp. Uppsala.
- WADDINGTON, M. 2009. Identification of Fungi Using Ribosomal Internal Transcribed Space DNA Sequences. Retrieved June 15, 2010, from Pharmaceutical Technology: <http://pharmtech.findpharma.com/pharmtech/article/articleDetail.jsp?id=595863&sk=&date=&pageID=3>.

- WANG, Y., L.D. GUO AND K.D. HYDE. 2005. Taxonomic placement of sterile morphotypes of endophytic fungi from *Pinus tabulaeformis* (Pinaceae) in northeast China based on rDNA sequences. *Fungal Diversity* 20: 235-260.
- WHITE TJ, BRUNS T, LEE S, TAYLOR J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: a Guide to Methods and Applications*, 315-322. Academic Press, New York.
- WOESE, C.R. AND G.J. OLSEN. 1986. Archaebacterial phylogeny: perspectives on the Urkingdoms. *Syst. Appl. Microbiol.* 7: 161-177.

**MOLECULAR IDENTIFICATION OF LOCALLY ISOLATED
MYCELIA STERILIA BY ANALYSIS OF THE INTERNAL
TRANSCRIBED SPACER (ITS) REGION DNA SEQUENCES AND
SCREENING OF PROTEASE AND XYLANASE ENZYMES**

BRYAN EDWARD A. DEVANADERA

**SUBMITTED TO THE FACULTY OF GRADUATE SCHOOL
UNIVERSITY OF THE PHILIPPINES LOS BAÑOS
IN PARTIAL FULLFILMENT OF THE
REQUIREMENTS FOR THE
DEGREE OF**

**MASTER OF SCIENCE
(Microbiology)**

MAY 2011

The thesis attached hereto, entitled "MOLECULAR IDENTIFICATION OF LOCALLY ISOLATED MYCELIA STERILIA BY ANALYSIS OF THE INTERNAL TRANSCRIBED SPACER (ITS) REGION DNA SEQUENCES AND SCREENING OF PROTEASE AND XYLANASE ENZYMES", prepared and submitted by BRYAN EDWARD A. DEVANADERA, in partial fulfillment of the requirements for the of MASTER OF SCIENCE (MICROBIOLOGY) is hereby accepted.

ASUNCION K. RAYMUNDO
Member, Guidance Committee

Date Signed

TERESITA U. DALISAY
Member, Guidance Committee

Date Signed

RINA B. OPULENCIA
Chair, Guidance Committee

Date Signed

Accepted as partial fulfillment of the requirements for the degree of MASTER OF SCIENCE (MICROBIOLOGY).

DAMASA M. MACANDOG
Director
Institute of Biological Sciences

Date Signed

OSCAR B. ZAMORA
Dean, Graduate School
University of the Philippines
Los Baños

Date Signed

DEVANADERA, BRYAN EDWARD A. University of the Philippine Los Baños, May 2010. Molecular Identification of Sterile Mycelia by Analysis of the Internal Transcribed Spacer (ITS) Region DNA Sequences and Screening of Protease and Xylanase Enzymes

Major Professor: Dr. Rina B. Opulencia

ABSTRACT

Mycelia sterilia of fungi is an artificial group of filamentous fungi where members are morphologically unidentifiable due to their failure to produce any kind of spores. In this study, 32 fungal isolates that exhibit sterile mycelia were obtained from the Mycology Laboratory of the Crop Protection Cluster, College of Agriculture, U.P. Los Baños. The fungal isolates were identified by amplification using polymerase chain reaction and subsequent analysis of the internal transcribed spacer 1 (ITS1)-5.8S gene-ITS2 DNA sequence. Phylogenetic analysis of the 5.8S gene sequences revealed that the 26 isolates belong to phylum Ascomycota while the rest belong to Basidiomycota. Comparison of the nucleotide sequences of the ITS region and 5.8S gene with sequences available in GenBank allowed the identification of 12 and 20 isolates to the species and genus levels, respectively. The isolates were screened for its capability to produce protease and xylanase enzymes using specific plated medium for each enzyme tested. Fifteen isolates were positive for xylanase enzyme. BD30 showed better performance based on the R ratio (diameter of the colony / diameter halo of hydrolysis) on the screening medium. Seven isolates were positive for protease enzyme with similar R ratio. BD30 is recommended to be assayed quantitatively to further explore its capability to produce xylanase enzyme.

INTRODUCTION

Mycelia Sterilia is a large group of fungi that shares property of being defective in forming reproductive structures essential for its morphological identification (Fisher *et al.*, 1994). The species concept in fungi has been mainly based comparative morphological features which rely heavily on reproductive structures. This method is inappropriate for fungal isolates categorized as *Mycelia Sterilia* (Lacap *et al.*, 2003). The ubiquity of *Mycelia Sterilia* pushed mycologist to offer ways of estimating its diversity. The morphological species concept (Bills, 1996) based on colony surface textures, exudates, growth rate, hyphal pigments and margin shapes provides a practical means of estimating the diversity of *Mycelia Sterilia*. This method does not reflect species phylogeny because it is not a real taxonomic entity and may comprise from distantly related taxa (Guo *et al.*, 2000).

Advances in molecular biology provided mycologists with powerful tools that can be used for delineation of *Mycelia Sterilia*. These techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al.*, 2006). The cultivation-independent polymerase Chain Reaction (PCR) provided means to identify *Mycelia*

Sterilia by amplification of specific regions from a genome. Sequencing of the resulting nucleotide fragment made the analysis of phylogenetic relationship over a wide range of taxonomic levels possible (Woese and Olsen, 1986).

DNA sequences have been used by fungal taxonomists as a basis for reclassification of all fungal taxa and have more recently utilized internal transcribed spacer (ITS) sequencing as the "gold standard". ITS is a region in the ribosomal RNA operon can accumulate mutation at a slightly faster rate than 5.8S, 18S and 28S rRNA, which makes it useful for molecular systematic at the species or even within species level (Baldwin, 1992; Schmidt and Moreth, 2002). Molecular sequencing offers an effective method for the identification of Mycelia Sterilia.

Several filamentous fungi are frequently being utilized in various fields because of the economically valuable metabolites and important substances like enzymes. Protease and xylanase are enzyme which are preferred to be extracted from fungi because of its superior characteristics compared to its bacterial counter part (Rao *et al.*, 1998).

Published studies exclusively on the taxonomic position and enzyme screening of isolate declared as Mycelia Sterilia in the Philippines are rare. This study generally aims to molecularly identify locally isolated Mycelia Sterilia by analysis of the internal transcribed spacer (ITS) region DNA sequences and to screen these isolates for protease and xylanase enzymes. Specifically, this study sought to:

- (1) do partial cultural characterization of thirty two Mycelia Sterilia isolates,
- (2) compare the efficiency of three commercially available DNA extraction methods by visual reading on the basis of the quality of band produced on 1% agarose gel
- (3) optimize the Polymerase Chain Reaction (PCR) conditions for the amplification of the ITS1-5.8S-ITS2 region using universal primers, pITS1 and pITS4 and
- (4) determine the ITS1-5.8S-ITS2 region DNA sequences and compare the sequences with those established fungal species on GenBank and
- (5) perform Neighbor-Joining phylogenetic analyses on the Mycelia Sterilia isolates.

The experiment was conducted at the Molecular Biology and Biotechnology Laboratories, Institute of Biological Science, University of the Philippines, Los Baños on June 2010 to May 2011 under the supervision of Dr. Rina B. Opulencia, Dr. Teresita U. Dalisay and Dr. Asuncion K. Raymundo.

REVIEW OF LITERATURES

Mycelia Sterilia

Mycelia Sterilia of fungi is an artificial taxonomic group characterized by their inability to produce spores of any kind (Hawksworth *et al.*, 1983). The lack of sporulation is the only unique trait shared by all Mycelia Sterilia. The group reproduces via hyphal fragmentation and contains various morphological types (Fisher *et al.*, 1994). Mycelia Sterilia are not usually given taxonomic placement since classical fungal systematic uses traditional cultivation-dependent methodologies which are based on comparative morphological features which rely heavily on reproductive structures such as spores and spore bearing structures (Lacap *et al.*, 2003). Therefore, the traditional cultivation-dependent process of fungal identification cannot be applied on isolates categorized as Mycelia Sterilia.

Distribution of Mycelia Sterilia

Mycelia Sterilia is ubiquitous and has been isolated from diverse ecological niches even in heavy metal contaminated sites of Marinduque Island. Twenty four percent of fungi isolated from the sites are Mycelia Sterilia. This indicates their tolerance to heavy metals (Flores, 2010).

Fungi of the *Mycelia Sterilia* group are ubiquitous and have been isolated from diverse ecological niches. They commonly prefer decaying wood as habitat but can also be found in soil, plants and plant debris. *Mycelia Sterilia* which resides asymptotically inside living plant tissues is endophytic. Surveys of various host plants have demonstrated that these fungal endophytes are ubiquitous in plant species (Kumar *et al.*, 2004; Zhang *et al.*, 2006; Sánchez Márquez *et al.*, 2007; Huang *et al.*, 2008; Osces *et al.*, 2008) and even lichens (Li *et al.*, 2007). An average 20-54% of the population for a given host of a fungal endophyte belongs to the *Mycelia Sterilia* group (Fisher *et al.*, 1994).

Mycelia Sterilia can also be found in host plants. They reside asymptotically inside living plant tissues as endophyte. Various percentages of Mycelia Sterilia have also been reported in several endophyte studies from different plant hosts. Mycelia Sterilia can take up an average of 20%, which can increase up to 54% in *Quercus ilex* (Holm oak), of the fungal population for a given plant host (Fisher *et al.*, 1994). Table 1

shows the proportions of Mycelia Sterilia found in various plant hosts (Lacap *et al.*, 2003).

**Table _____. Proportion of Mycelia Sterilia from various plant hosts
(Lacap *et al.*, 2003).**

Host	Location	Proportion of Mycelia Sterilia (%)
<i>Cuscuta reflexa</i>	India	23
<i>Hippohau rhamnoides</i>	Spain	18-24
<i>Licuala</i> sp. and	Australia and	11-16
<i>Trachycarpus fortunei</i>	Brunei	
<i>Livistonia chinensis</i>	Hong Kong	16
<i>Picea mariana</i>	Canada	33
<i>Quercus ilex</i>	Switzerland	54
Red Mangroves	USA	12-14
<i>Sequoia sempervirens</i>	USA	26

Mycelia Sterilia is also routinely isolated from household environments which are known to be associated with human allergies and eye and skin infections and even animal mycoses. Heterogeneous group of Mycelia Sterilia was the only airborne fungi found present at higher concentrations in the homes of wheezing children characterized by high-pitched whistling sound produced when there is a blockage to the regular flow of air from the lungs (Strachan *et al.*, 1990). Accurate fungal species identification is a prerequisite for the effective management of a pathogenic disease causing fungi, biological studies especially for epidemiology, surveillance and in anti-fungal choice due to species specificity. However, identification of Mycelia Sterilia can be difficult or nearly

impossible morphologically since it doesn't produce the needed structures for identification.

Identification of Mycelia Sterilia

Identification of Mycelia Sterilia to the species level is essential in descriptive, taxonomical, ecological and biodiversity studies. Since spores and reproductive structures are necessary for fungal identification, methods of spore formation induction were developed for Mycelia Sterilia. The method can be performed in the laboratory using very specific conditions such as growing these fungi on modified artificial media and inclusion of host tissue in plate cultures or incubation under various conditions to promote sporulation. Mycelia Sterilia isolated from the palm *Livistona chinensis* was induced of sporulation by inoculating the isolates into flasks containing malt extract agar and a sterilized petiole fragment of palm which was incubated in alternating near-UV and darkness for 12h. This method increased the initial identifiable/sporulating isolates from 48% to 83.5% after 3month incubation at room temperature (Guo *et al.*, 1998). Spore induction on Mycelia Sterilia also led to the discovery of distinct but rare sexual forms and revealed that the group is not homogenous. Some isolates belong to different, distantly related classes of fungi (Vinnere *et al.*, 2010). Despite the spore induction method, several fungi are still unable to produce spores. The sexual stage of these fungi is extremely hard to induce due to incorrect growth conditions or the lack of mating partner such as those heterothallic species which require mating with a compatible strain

for spore production (Borman *et al.*, 2008). In order to classify and estimate the diversity of these fungi, morphotype grouping was developed.

Morphotype. “Morphotype”, preferably used over “morphological species” or “morphospecies”, is a concept of grouping Mycelia Sterilia on the basis of similarity in colony surface textures, exudates, growth rate, hyphal pigments, margin shapes and sporulating structures (if present) (Bills, 1996). This concept has been useful in classifying and estimating fungal diversity in the absence of reproductive structures (Lacap *et al.*, 2003). However, the arrangement of Mycelia Sterilia according to morphotype does not reflect species phylogeny because morphotypes are not real taxonomic entities (Guo *et al.*, 2000). A single morphotype may comprise of distantly related fungi with similar cultural characteristics.

Mycelia Sterilia has been and remains an enormously difficult manage group of fungi for taxonomists due to the inherent problems with the identification and classification, in addition to a more complicated propagation, maintenance and preservation compared to sporulating fungi (Currah and Tsuneda, 1993). This led to limited taxonomic and genetic information about Mycelia Sterilia. Various identification methods including morphology, physiological properties, nucleic acid amplification and sequencing have been proposed in the past to better discriminate and understand the possible classification of Mycelia Sterilia (Chen *et al.*, 2002).

Morphology. Mycelia Sterilia can be assigned to one of the classes of the Fungal Kingdom - Ascomycetes or Basidiomycetes using few mycelial features that are available. The principal differences between mycelia of fungi belonging to these classes are: 1) the structure of hyphal septa (simple septum in Ascomycetes and dolipore septum in Basidiomycetes) and 2) presence or absence of clamp connections, which are a characteristic feature of Basidiomycetes (Alexopoulos *et al.*, 1996). Both presence of dolipore septum and presence or absence of clamp connections can be used as important features for separation of the sterile Basidiomycetes from sterile Ascomycetes. Other hyphal characters that are informative for the characterization of sterile strains include presence of moniloid cells (enlarged hyphae of irregular shape), formation of sclerotia, differences in hyphal diameter, length, character of hyphal branching, overall colony appearance, margins, texture, color, growth rate, temperature response, etc. Most of these features, however, are dependent on growth conditions such as media composition, light intensity, etc (Vinnere, 2004).

Microscopic observation and characterization of several other structures are needed to further discriminate these fungi up to the species level. Unfortunately, characters observable by light microscopy vary with culture conditions and are sometimes highly pleomorphic (Brookman *et al.* 2000). Differentiation of species using ultrastructural are problematic because ultrastructure depends not only on the age of microorganisms but also on the method and quality of their preparation (Ho and Barr, 1995). Moreover, Mycelia Sterilia fails to produce important structures making morphological identification impossible.

by cultivation. This approach however can be hindered by the presence of potent PCR inhibitors in fungal cultures and difficulties inherent in breaking fungal cell walls and hence difficulties in DNA extraction.

DNA extraction methods

Fungi have cell walls that are not readily susceptible to lysis hence impede cell lysis and the recovery of DNA. These problems can be overcome by using fungal DNA extraction procedures which are usually laborious and costly." Fungal DNA extraction is a critical step of sample processing due to the cell wall's resistance to the usual DNA extraction procedure. Efficient fungal cell lysis is one factor that needs to be considered when employing DNA extraction methods. The quality of extracted DNA will dictate its suitability for several down stream processes (Fredericks *et al.*, 2005).

Grinding of cells frozen with liquid nitrogen using mortar and pestle, mechanical disruption using glass beads, the enzymatic digestion of cell wall polysaccharides to form spheroplasts followed by conventional membrane lysis procedures and sequential freeze-thaw cycles or incubation with hot detergent and proteases are fungal DNA extraction methods that have been developed and promise to produce good quality DNA (Fredericks *et al.*, 2005). However, these conventional (long) methods are not practical where many samples must be processed and where risks of cross contamination must be meticulously avoided. Commercial DNA extraction methods provide ease in sample processing and standardized reagent composition for repeatability of results essential for nucleic acid-based fungal identification.

Biochemical analyses. Color reactions in response to adding certain chemicals such as KOH, peroxidase and dyes are some simple chemical test for many non-sporulating isolates especially the ones belonging to the Basidiomycetes are widely used (Stalpers, 1978; Desjardin, 1990). The genus *Rhizoctonia*, a Mycelia Sterilia, is frequently characterized using isozyme analysis (Sweetingham *et al.*, 1986; Yang *et al.*, 1994; Worth, 2002). Commercially available conventional identification kits based on biochemical reactions can provide ease in fungal identification but became unreliable due to the continuously increasing number of newly discovered fungal species which are not included in the repertoires of these kits.

Molecular analyses. DNA based techniques have the advantage of allowing direct identification of fungi. The advent of molecular techniques such as sequencing of the ribosomal genes and their spacers could be a potential approach for the identification of *Mycelia Sterilia* (Guo *et al.*, 2000). Molecular approaches have been developed to provide more rapid and accurate identification of fungi compared to traditional morphological methods. Various analyses of the DNA are used as a powerful tool for separation of sterile strains and their tentative identification. DNA sequencing is widely used for this purpose. Sequence analysis is a relatively simple procedure. In addition, interpretation of nucleic acid sequences is straight-forward and does not depend on too much expertise compared to morphological analyses (Ciardo *et al.*, 2006). Advances in Molecular Biology, especially the development of the Polymerase Chain Reaction (PCR), led to a more rigorous alternative for the identification of *Mycelia Sterilia*.

Polymerase Chain Reaction (PCR)

PCR is a molecular technique developed by Kary Mullis in 1983 as a fast *in vitro* enzymatic replication of a defined DNA sequence generating 10^5 to 10^6 -fold exponentially amplified copies of template DNA within a large background of irrelevant sequences (Eeles and Stamps, 1994). It relies on thermal cycling which consist of repetitive cycles of heating for denaturation of double-stranded DNA to single stranded and cooling for enzymatic replication of the DNA. Replication involves annealing of oligonucleotide primers to the target DNA and extension of the DNA by nucleotide addition from the primers by the action of DNA polymerase. The primers which are complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. The first cycle is characterized by a product of indeterminate length. The second cycle initiates the exponential production of short products which is cumulative in a series of amplification (Coen, 1994). PCR has a diverse application in molecular biology, clinical diagnosis, forensic analysis and population genetics due to its sensitivity, specificity and rapidity.

PCR is a cultivation-independent method which offers the opportunity to identify fungi by amplification of specific target regions from a genome and sequencing of the resulting PCR products. It exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al.*, 2006). PCR can overcome poor diagnostic sensitivities, potential technical bias and long turnaround times associated with fungal identification

PCR target sites

PCR amplification of coding genes, e.g., cytochrome c oxidase 1 (CO1) gene, beta-tubulin 2 gene (tub2) and 18S rDNA (Makimura, *et al.*, 1994), 28S rDNA (Sandhu *et al.*, 1995) and 5.8S genes of rDNA and non-coding internal transcribed spacer (ITS) regions of rDNA followed by sequencing of resulting amplicons have shown the most promise for the identification of fungi. Most coding genes are highly conserved and have been successfully used to assess phylogenetic relationships at higher taxonomic levels. The variability of 18S and 28S rRNA genes is limited, which makes it difficult to use for differentiation between species (Fell *et al.*, 2000). The non-coding ITS regions are excellent targets for sequence-based approaches because it benefit from a fast rate of evolution, resulting in greater sequence variation which allow discrimination between closely related species. These region sequences therefore generally provide greater lower taxonomic resolution at genus and species level (Guo, 2010).

Internal Transcribed Spacer (ITS). The internal transcribed spacer (ITS) region is located between the highly conserved genes coding for 18S and 28S rRNA. The ITS encompasses the two non-coding regions, ITS1 and ITS2, in the DNA genes encoding the fungal ribosomal RNA (rRNA) "operon". The ITS regions are separated by the highly conserved 5.8S rRNA gene (White *et al.*, 1990). ITS1 is found between the 18S and the 5.8S rRNA genes while ITS2 is found between the 5.8S and the 28S rRNA genes (Fig. 1). The entire rRNA "operon" is transcribed, but, after transcription, the two

ITS sequences are excised and, therefore, not used for any functional purpose (Waddington, 2009).

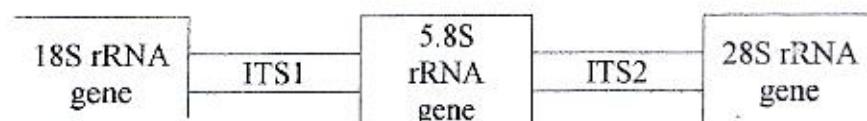


Figure _____. Schematic representation of fungal ribosomal genes bridged by ITS1 and ITS2.

The internal transcribed spacer (ITS) region evolves fast and is often highly variable among distinct fungal species (Gardes *et al.*, 1991). It is allowed to accumulate mutations at a faster rate than the 5.8S, 16S, and 28S rRNA genes since it is not used for any functional purpose other than as spacer. It is this slightly increased rate of accumulated mutations that allows the ITS sequences to provide an improved level of resolution compared with the rRNA gene sequences (Waddington, 2009). ITS region also has less selective pressure than do ribosomal gene regions and thus changes more rapidly. However, some selective pressure exists, probably from the constraint to maintain the RNA secondary structure required for post-transcriptional processing (Shinohara *et al.*, 1999). The ITS1 and ITS2 must continue to fold in such a way that nucleases can cut the transcript at the appropriate points.

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA has generally been considered a convenient marker for molecular identification of fungi at

similarity tree or the ITS region is identical to the phylogenetic tree, allowing good identification (Scorzetti *et al.*, 2002).

species level because of its conserved feature within species and multi-copy number per genome (Sanchez-Ballesteros *et al.* 2000). It is a convenient target for molecular identification of fungi since the entire ITS region is often between 500 and 800 bp and can be readily amplified with 'universal primers' that are complementary to sequences within the rRNA genes. Primers make use of the conserved regions of the 18S, 5.8S and 28S rRNA genes to amplify the non-coding regions between them (White *et al.*, 1990).

Although attempts to identify fungi by focusing on either the ITS1 or the ITS2 region may be successful for some species and genera (Chen *et al.*, 2000; 2001), analysis of the complete ITS region offers greater promise for molecular identification (Chen *et al.*, 2001). ITS1 and ITS2 can identify *Aspergillus* at the species level and differentiate it from other true pathogenic and opportunistic molds which allowed for allowing for early diagnosis and screening of effective antifungal agents (Henry *et al.*, 2000). The ITS rDNA sequences were also used for the development of a method for the detection of *Rhizoctonia solani* isolates and identify various anastomosis groups, pathogenic and nonpathogenic to tulips (Schneider *et al.*, 1997).

The reliability of identification by sequencing not only depends on the length of the sequence determined, but also on the quality and availability of reference sequences. High sequence similarity of the ITS regions may be evidence, but it does not provide definite proof for the identity of two taxa. However, for a large majority of species, the similarity tree of the ITS region is identical to the phylogenetic tree, allowing good identification (Scorzetti *et al.*, 2002).

Molecular analysis of the ITS region can eliminate many of the problems associated with the morphological characters and culturing. Analysis of ITS sequence is usually applied to determine species identity (or sometimes higher taxonomic categories) and to identify and discriminate populations within a species.

Taxonomy of Mycelia Sterilia

Molecular phylogenetic analyses often overturn classifications based on morphological features. DNA-based studies made the taxonomy of Fungi in a state of constant flux.

Historically, fungi which are Mycelia Sterilia belong to order Agonomycetales of Deuteromycota, characterized by the lack of spores. This taxon is no longer formally accepted because they do not adhere to the principle of monophyly which means that members of the group don't have a common ancestor (Kendrick, 1981). However, the taxon names (e.g. Mycelia Sterilia) are used informally for descriptive purposes.

Mycelia Sterilia have at least 28 genera and around 200 species (Kirk *et al.*, 2001). Some of these sterile mycelia were found to be just a mycelial stage of already known fungi with mainly basidiomycetous teleomorphs (including *Rhizoctonia*, certain species of which cause damping-off disease, and *Sclerotium*), others just fail to sporulate on artificial substrata (Vinnerec, 2004). Naming of sterile isolates is historically made by the

closest species of sterile fungi and from the researcher's subjective point of view (Howard *et al.*, 1997).

Molecular phylogenetic analyses are the reference of the 2007 comprehensive phylogenetic classification of the Kingdom Fungi. It includes 5 phyla of basal Fungi and 2 phyla of the subkingdom Dikarya (Hibbett *et al.*, 2007). Figure 1 shows the different phyla of the Kingdom Fungi. Notice the absence of Deuteromycota (i.e Mycelia Sterilia). This implies that its members have already been distributed to the different taxa of the Kingdom.

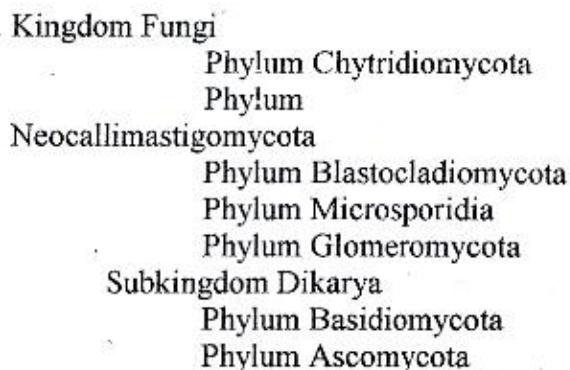


Figure _____. Phyla of the Kingdom Fungi.

Importance of Mycelia Sterilia

Members of Mycelia Sterilia group show unexpected characters such as being both plant deleterious and plant growth promoter. *Rhizoctonia solani* and *Sclerotium*

rolfsii are well-known Mycelia Sterilia plant pathogens of high economical importance. *R. solani* is responsible for the dumping-off disease in potatoes, cereals and other agricultural crops worldwide while *S. rolfsii* is the causal agent of southern blight which affects great range of agricultural crops. A less common plant pathogenic sterile fungi causing stem rot of bean seedlings, crown canker of pigeon pea (*Cajanus cajan*) and several other was also been reported (Howard *et al.*, 1977).

On the other hand, three Mycelia Sterilia isolates form heavy-metals contaminated sites in Marinduque, Philippines were found to have antagonistic effect against *R. solani*. The mechanism can either be antibiosis or space competition (Flores, 2010). Several other Mycelia Sterilia has been reported to be a potential bio-control agent of various plant pathogens. The sterile red fungus from Australia was found effective against *Gaeumannomyces graminis var. tritici*, the causal agent of take-all disease (Rowland *et al.*, 1994). Some Mycelia Sterilia have also been reported to have plant growth capability such as some sterile fungi isolated from Zoysia grass rhizosphere which was found to promote growth of some of soybean varieties in nutrient depleted soil (Shivanna *et al.*, 1995).

Mycelia Sterila may also have symbiotic association to their plant host. Such isolates can asymptotically colonize plant tissues and could contribute to plant fitness including its defense against pathogens, improving nutrition conditions, fecundity, etc.

Mycelia Sterilia isolates used in the Study

The Mycology Laboratory of the Crop Protection Cluster, College of Agriculture, U.P. Los Baños, headed by Dr. Teresita U. Dalisay, houses isolates declared as Mycelia Sterilia. These isolates have been incubated for 2 months on an in-house strength Potato Dextrose Agar (PDA) at room temperature (~30°C) and were unable to produce observable reproductive structures for proper identification. Thirty Mycelia Sterilia cultures isolated from various sources were collected for this study. Provenances of these cultures are detailed in Table 1 ((Dalisay, T.U., personal communication, July 8, 2010). Some isolates were reported to have an antagonistic effect against *Rhizoctonia solani* Kuhn while several others are currently under being screened. Identification of these isolates is important for proper surveillance of Mycelia Sterilia in the ecosystem of isolation. Prevalence and in-depth assessment of fungal diversity will be much appreciated after proper identification of these isolates.

Enzymes

Microorganisms have long been utilized for its products due to its importance in various fields. Filamentous fungi are used in many industrial processes for its economically valuable metabolites and important substances like enzymes (Adrio et al., 2003).

Enzymes (i.e., protease and xylanase) are proteins that catalyze different kinds of biochemical reactions. These highly specific organic catalysts are capable of increasing the velocity of certain reactions which are usually oxidation-reduction, hydrolysis, group transfer, bond-breaking or isomerization (Grisham and Garrett, 1999). Microbial sources of enzymes are preferred over plants and animals due to their rapid growth and limited space required for cell cultivation. Microorganisms can also be genetically manipulated to produce improved enzymes. Microbial based enzymes also possess almost all the desired characteristics for their biotechnological applications (Rao *et al.*, 1998).

Screening for enzyme producing microorganisms is one of the forefront phases in enzyme production process. This crucial step is very important since choosing the excellent microorganism is essential to produce the desired product. Industrial scale enzyme production is usually performed in large bioreactors where culture medium and production condition have already been optimized for maximum enzyme yield.

Protease. A wide variety of proteolytic enzymes are produced by microorganisms which catalyze the peptide bond cleavage in proteins (Polgar, 1989). Proteases are commercially important since it accounts for the 60% of the worldwide sales value of the total industrial enzymes (Godfrey, 1996). Proteases are commonly used as a milk-clotting agent in dairy industry, an agent for meat tenderization, an additive in detergents (Aftab *et al.*, 2006). Depending on the optimal pH where they are active, proteases can be classified as acid, neutral and alkaline protease.

Fungal proteases are preferred over bacterial origin because it can easily be extracted and separated from mycelium (Phadatare *et al.*, 1993). *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* are some protease producing fungi (Aftab *et al.*, 2006). These fungi are known to secrete acid proteases which are stable and have maximum activity acidic conditions. Moreover, thermostable acid proteases are secreted by some thermophilic relatives of *Penicillium* (Hashimoto *et al.*, 1973).

Xylanase. Xylan is the major components of plant hemicelluloses and is next to cellulose being the most abundant renewable polysaccharide in nature. It is the substrate of xylanases (endo-1, 4- β -xylanase, EC 3.2.1.8) which catalyze the endohydrolysis of 1, 4- β -d-xylosidic linkages in xylan (Collins *et al.*, 2005).

One of the most important applications of xylanase is in the paper and pulp industry. Biobleaching using xylanase instead of chlorine based bleaches reduces or eliminates the organo-chlorine wastes production. Another advantage of using xylanase in paper pulp is its specific action on lignin without damaging cellulose thereby preserving paper strength. Microbial xylanases are also useful in the improvement of animal feeds, textile and food processing industries, production of several valuable products like xylitol and ethanol (Salles *et al.*, 2005) and even in the improvement of plant waste material degradability.

Fungal xylanase production is preferred over yeast or bacterial production due to higher enzyme yield and extracellular release of xylanase together with other secondary

MATERIALS AND METHODS

Collection of *Mycelia Sterilia*. A total of thirty *Mycelia Sterilia* cultures isolated from various sources were collected and listed on Table _____. The isolates were sub-cultured on Potato Dextrose Agar (PDA). Stock and working cultures were stored at 4°C and 30°C, respectively.

Cultural Characterization. Each *Mycelia Sterilia* isolates was inoculated to two (replicate) PDA plates and incubated at 30°C for 14 days. Cultural characteristics of the isolates such as colony shape, color (above and reverse), elevation, texture, type of mycelium, margin shape, density, zonation and effect on the medium will be examined after two weeks of incubation. Colony diameter (cm) was measured on the 7th and 14th day of incubation. Cultures which are presumptively carrying spores after an extended two-month incubation period under laboratory conditions will not be included in the study.

Culture Preparation. A hyphal tip was obtained using a dissecting microscope from fresh culture of each isolate, inoculated on Potato Dextrose Broth (PDB) and incubated for 7 days at 30°C with shaking. After incubation, mycelia were separated from the broth by

enzymes which can help for the de-branching of the substituted xylans (Haltrich *et al.*, 1996). *Aspergillus*, *Chaetomium* and *Trichoderma* are some of the known genus of fungi capable of producing xylanase (Collins *et al.*, 2005). Thermostable xylanases produced by *Thermotoga* sp. was reported to be active up to 105°C (Simpson *et al.*, 1991).

Table 1. Geographic location, ecosystem and associated organism from where Sterile *Mycelia* are collected
(Dalisay, T.U., personal communication, July 8, 2010).

Isolate Code	Geographic Location	Ecosystem	Associated Organism
BD01	Marinduque	Soil near Mine tailing	Corn
BD02	Marinduque	Soil near Mine tailing	Corn
BD03	Marinduque	Soil near Mine tailing	Corn
BD04	Marinduque	Soil near Mine tailing	Corn
BD05	Marinduque	Soil near Mine tailing	Corn
BD06	Marinduque	Soil near Mine tailing	Corn
BD07	Batangas	Lowland soil treated with organic fertilizer	Rice
BD08	Batangas	Lowland soil treated with organic fertilizer	Rice
BD09	Batangas	Lowland soil treated with organic fertilizer	Rice
BD10	Batangas	Lowland soil treated with organic fertilizer	Rice
BD11	Batangas	Lowland soil treated with organic fertilizer	Rice
BD12	Batangas	Lowland soil treated with organic fertilizer	Rice
BD13	Batangas	Lowland soil treated with organic fertilizer	Rice
BD14	Laguna	Lowland soil treated with organic fertilizer	Rice
BD15	Laguna	Tiller/Root/Leaf	Rice
BD16	Laguna	Tiller/Root/Leaf	Rice
BD17	Laguna	Tiller/Root/Leaf	Rice
BD18	Laguna	Tiller/Root/Leaf	Rice
BD19	Laguna	Tiller/Root/Leaf	Rice
BD20	Laguna	Tiller/Root/Leaf	Rice
BD21	Laguna	Tiller/Root/Leaf	Rice
BD22	Laguna	Tiller/Root/Leaf	Rice
BD23	Laguna	Tiller/Root/Leaf	Rice
BD24	Laguna	Tiller/Root/Leaf	Rice
BD25	Laguna	Tiller/Root/Leaf	Kuhol
BD26	Laguna	Meat	Kuhol
BD27	Laguna	Meat	Kuhol
BD28	Laguna	Meat	Kuhol
BD29	Laguna	Meat	Kuhol
BD30	Laguna	Meat	Kuhol
BD31	Laguna	Meat	Kuhol
BD32	Laguna	Meat	Kuhol

centrifugation at 30,000 rpm for 5 minutes. Harvested mycelia were stored at -80°C until needed.

PDB was prepared by mixing 20g dextrose and 200g potato infusion in 1 litter of distilled water. The resulting mixture will be heated until all the ingredients are completely dissolved. Potato infusion will be prepared by boiling 200g potato in 1000ml of distilled water.

DNA Extraction Methods. Three commercially available DNA extraction kits will be compared based on their efficiency to recover fungal DNA as assessed by the quality of band produced on 1% w/v agarose gel. Three randomly selected isolated were used to evaluate the three DNA extraction kits. Manufacturers' instructions were followed for all methods.

Method SGM (GenElute™ Plant Genomic DNA Kit [Sigma-Aldrich Co., USA]) uses a detergent and a chaotropic to break open the cells and release DNA from liquid nitrogen grounded samples. Precipitation of proteins, polysaccharides and cell debris followed by centrifugation through a filtration column enables isolation of the DNA. The genomic DNA is purified further by a silica bind-wash-elute procedure in microcentrifuge spin columns.

Method VVN (GF-1 Plant DNA Extraction Kit [Vivantis Technologies, CA, USA]) uses lysis buffer proteinase K and heat at 65°C to break open the cells from a liquid nitrogen grounded sample. The buffer contains denaturing agents to provide lysis of tissue cells, denaturation of proteins and subsequently release of genomic DNA. Insoluble / undigested materials are pelleted by centrifugation. DNA in the supernatant is homogenized in special

buffers to enhance the binding of DNA onto a specially-treated glass filter membrane for efficient recovery of highly pure genomic DNA which is precipitated using ethanol. The DNA is then bound to a spin column, washed and eluted in low salt buffer.

Method ZYM (ZR Fungal/Bacterial DNA Kit™ [Zymo Research Corporation, CA, USA]) uses a bead matrix and lysis buffer (ZR Bashing Bead™ Lysis Tube) to pulverize cells using a Mini-Beadbeater (BioSpec Products, Inc., USA) for high speed cell disruption without using organic denaturants or proteinases. The lysate is filtered using Zymo-Spin™ IV Spin Filter. The DNA is then bound to Zymo-Spin™ IIC Column, washed and eluted.

Quantification of DNA isolate. Four microliters of eluted DNA from each kits were analysed by electrophoresis at 100 V for 2 h in a 1% (w/v) agarose gel in 0.5X TAE buffer (40mM Tris, 1mM EDTAm pH 8.0) which was visualized using UV light after staining with ethidium bromide ($0.5 \mu\text{g ml}^{-1}$). DNA concentration was approximated using ZR 100 bp DNA Marker™ (Zymo Research Corporation, CA, USA). Each fragment has its pre-determined nanogram concentration of digested plasmid.

PCR Amplification. Universal primers, pITS1 and pITS4, were used to amplify the ITS regions including the 5.8S rRNA gene of the thirty Mycelia Sterilia isolates (White *et al.*, 1990). The primers were synthesized by Aitbiotech Pre. Ltd. (Singapore). Table ___ shows the sequences of the primers used.

Table _____. Primer sequences used in the study.

Primers	Sequence (5'→3')	Reference
ITS 1	TCCGTAGGTGAAACCTGCGG	White <i>et al.</i> , 1990
ITS 4	TCCTCCGCTTATTGATATGC	White <i>et al.</i> , 1990

Amplification of the DNA fragment was performed using G-Storm GS-1 automated thermal cycler (G-Storm, UK) in a 50 µL reaction volume which contained 25µL 2X Taq Master Mix (Vivantis Technologies, USA), 0.3µM of each primers, ~5ng template DNA and 1.5-3.0mM MgCl₂. The thermal cycling program used was as follows: 5 minute initial denaturation at 95°C, followed by 40 cycles of 30 seconds denaturation at 94°C, 50s primer annealing at 52°C - 60°C, 1 min extension at 72°C and a final 10 min extension at 72°C. A negative control using water instead of template DNA was included in each amplification batch. Four microlitres of PCR products from each PCR reactions were examined by electrophoresis at 100 V for 2 h in a 1% (w/v) agarose gel in 0.5X TAE buffer and was visualized using UV light after staining with ethidium bromide (0.5 µg ml⁻¹). The concentration of MgCl₂ and annealing temperature were varied until optimized conditions were attained as indicated by a single band on 1% agarose gel.

DNA Sequencing. The PCR amplicons were sent to Macrogen, Inc., South Korea which uses the machine ABI3730XL (Applied Biosystems, USA) for sequencing. DNA strands were sequenced bi-directionally using the primers pITS1 and pITS4 (White *et al.*, 1990).

DNA Sequence Analysis. Contiguous sequences were assembled and manually edited using Geneious Pro 5.3.4 (Drummond *et al.*, 2011). Discrepancies were resolved by verifying

the chromatogram for both forward and reverse sequences. These Mycelia Sterilia sequences were used as query sequence to search for similar sequences from GenBank and EMBL using Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.*, 1997). The most similar reference sequences with the query sequences were obtained and were used for subsequent phylogenetic analyses along with taxonomic reference sequences. The complete ITS region sequences were aligned using Clustal W2 program (Larkin *et al.*, 2007) with 10.00 and 0.10 as gap opening and extension penalties, respectively. The aligned sequences were edited to restrict the phylogenetic analysis to regions of nucleotides that were unambiguously alignable in all sequences. Alignment gaps were treated as missing data. The phylogenetic tree was inferred using the neighbor-joining method (Saitou, 1987) by genetic distance analyses among different sequences. The distances were calculated according to two-parameter method (Kimura, 1980). Statistical support and robustness of the internal branches were estimated by bootstrap analysis (Felsenstein, 1985) with 1000 replications. MEGAS.0 package (Tamura *et al.*, 2007) was used for all the analyses. The ITS sequences of the fungi type strains were obtained from DDBJ/EMBL/GenBank.

Screening for protease production on Czapek's agar medium. The ability of the Mycelia Sterilia isolates to produce extracellular protease during their growth was screened Czapek-Dox agar (Difco) medium containing 20 g·L⁻¹ Casein-Hammersten (Sigma Chemicals, USA). The inoculated plates were incubated at 30°C for 7 days or until the diameter is 30.0 mm. Their ability to hydrolyze casein was evaluated by measuring diameters of both the colony and the halo of hydrolysis. This allowed the calculation of R (diameter of the colony / diameter.halo of hydrolysis).

Screening for xylanase production on Czapek's agar medium. The ability of the 30 Mycelia Sterilia isolates to produce extracellular xylanase during their growth was screened on Czapek's agar medium containing xylan as sole carbon source. The medium composition of Nakamura *et al.*, 1993 protocol was ($\text{g} \cdot \text{L}^{-1}$): beechwood xylan (Sigma-Aldrich, USA), 10.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 and agar, 20.0. The inoculated plates were incubated at 30°C for 7 days or until the colony diameter is 30.0 mm. Their ability to hydrolyze xylan was evaluated by measuring the diameters of both the colony and the halo of hydrolysis. This allowed the calculation of the ratio R (diameter halo of hydrolysis/ diameter of the colony). Optionally, fungal growth can be flooded with 0.1% (w/v) Congo Red and incubated for 30 minutes before washing with 1M NaCL for better visualization of the halo of hydrolysis.

RESULTS AND DISCUSSION

Cultural Characteristics of Mycelia Sterilia

Visual evaluation of the cultural and growth characteristics of the thirty two Mycelia Sterilia isolates was performed *in vitro* and summarized in Table ____.

Comparison of DNA Extraction Methods

Three randomly selected Mycelia Sterilia were used to compare the efficiency of three commercially available DNA extraction methods. The fungal genomic DNA yields from the three DNA extraction methods are shown in Figure _____. Based on visual reading, DNA extraction performed using ZYM gave relatively higher yield of good quality, intact DNA compared to VVN and SGM. ZYM employ bead beating for the mechanical disruption of the cells. This method performed well on extracting fungal

Table 2. Cultural characteristics of 30 Mycelia Sterilia isolates on Potato Dextrose Agar (PDA).

Isolate Code	Size (cm) of colony after		Shape	Color		Elevation	Texture	Mycelium	Edge	Density	Zonality	Effect on Medium
	7 days	14 days		Above	Reverse							
BD 1	5	8.5	circular	white	white	raised	woolly	aerial	filamentous	medium	-	-
BD 2	5.3	>	irregular	gray	gray	raised	cottony	aerial	carved	medium	+	-
BD 3	1.5	1.7	irregular	white	white	flat	felly	aerial	undulate	medium	-	-
BD 4	2.4	3.5	irregular	white	white	raised	woolly	aerial	undulate	medium	-	-
BD 5	2.3	3.5	irregular	white	white	raised	woolly	aerial	undulate	medium	-	-
BD 6	8.1	>	circular	white	white	raised	cottony	aerial	undulate	dense	-	-
BD 7	5.0	8.3	irregular	pink	pink	flat	woolly	aerial	filamentous	medium	-	-
BD 8	1.4	1.9	irregular	gray	gray	convex	woolly	aerial	filamentous	medium	-	-
BD 9	4.1	8.4	circular	pinkish	pink	flat	woolly	aerial	undulate	sparse	-	-
BD 10	8.1	>	circular	white	white	flat	woolly	aerial	filamentous	medium	-	-
BD 11	1.4	2.0	irregular	pink	pink	raised	woolly	aerial	undulate	medium	-	-
BD 12	1.6	5.6	irregular	pink	pink	flat	woolly	aerial	undulate	spars	-	-
BD 13	3.9	7.8	irregular	white	white	flat	woolly	aerial	filamentous	medium	+	-
BD 14	1.4	2.1	circular	dirty white	dirty white	flat	woolly	aerial	undulate	spars	-	-
BD 15	2.8	5.3	circular	Pinkish ² , gray ³	pinkish	flat	woolly	aerial	filamentous	medium	-	-
BD 16	7.1	8.3	irregular	white, brown	pale yellow	flat	powdery	aerial	undulate	dense	-	-
BD 17	>	>	circular	gray	gray	raised	cottony	aerial	filamentous	medium	-	-
BD 18	4.3	5.3	circular	white	pinkish	raised	woolly	aerial	entire	dense	+	-
BD 19	3.5	7.5	circular	white	white	flat	woolly	aerial	filamentous	medium	-	-
BD 20	7.5	8.5	irregular	white	white	raised	cottony	aerial	filamentous	dense	-	-
BD 21	>	>	circular	yellowish, white	yellow	flat	felly	aerial	entire	dense	-	-
BD 22	3.3	6.0	irregular	white	white	raised	woolly	aerial	undulate	medium	-	-
BD 23	>	>	circular	gray	gray	raised	cottony	aerial	filamentous	dense	-	-
BD 24	1.5	3.5	irregular	white	white	flat	cottony	aerial	undulate	medium	-	-
BD 25	7.8	8.5	irregular	gray	gray	flat	felly	aerial	undulate	dense	-	-
BD 26	8.8	8.0	irregular	white	pinkish	raised	cottony	aerial	undulate	medium	-	-
BD 27	>	>	circular	gray	gray	flat	felly	aerial	undulate	dense	-	-
BD 28	4.4	7.5	circular	white	white	flat	felly	aerial	entire	medium	+	-
BD 29	5.5	6.5	irregular	gray	gray	raised	cottony	aerial	undulate	medium	-	-
BD 30	5.5	8.3	circular	gold, yellow	gray, brown	flat	felly	aerial	entire	medium	-	-
BD 31	6.1	8.0	circular	gray	gray	flat	woolly	aerial	entire	medium	+	-
BD 32	1.5	2.0	circular	brown	white	raised	woolly	aerial	entire	spars	+	-

¹Completely covering the plate; ² Edge of colony; ³ Center of colony; ⁴ /- present.

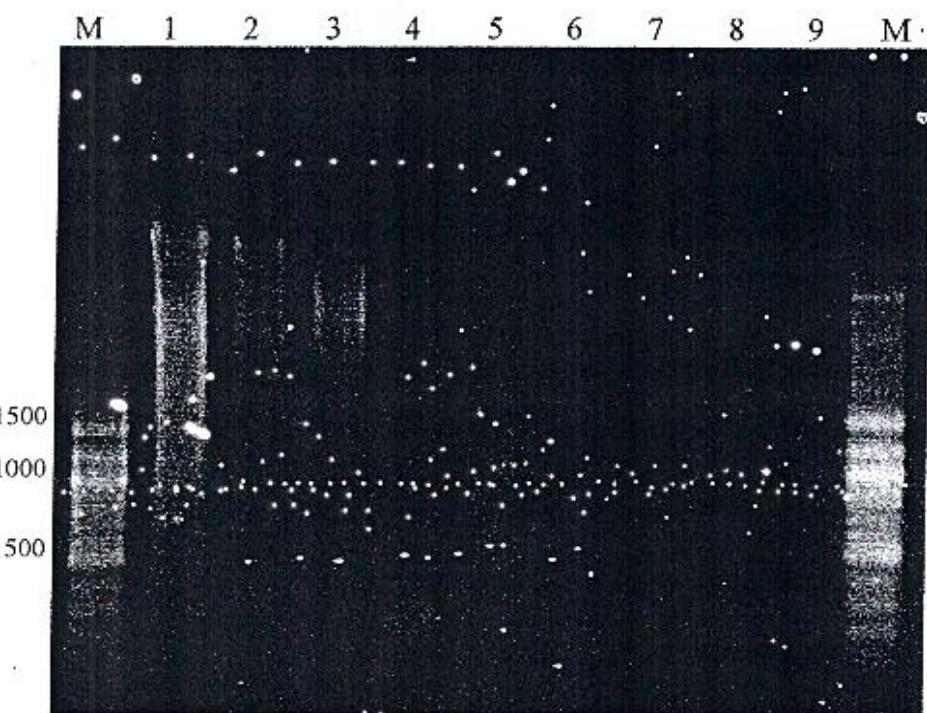


Figure 1. Electrophoretic profile of randomly selected *Mycelia Sterilia* DNA extracts using different extraction methods. 1-3) ZYM, 4-6) VVN, 7-9) SGM. M = 100bp DNA marker.

DNA of the three isolates. ZYM was also able to give a relatively clean DNA as shown by an intense DNA band and a very faint degraded DNA smear due to shearing.

VVN's and SGM's extracted DNA are extremely low in concentration or might have been degraded. DNA bands were not clear and DNA shearing was obvious as indicated by smearing. Although both of these kits were designed to extract DNA from wide variety of plant tissues with complex cell walls, several reports showed the kits compatibility to fungal samples since plants also have similar cell wall characteristics (Pitkäranta, *et al.*, 2008; Charoenporn *et al.*, 2010). A good quality DNA is advisable for downstream processes such as PCR amplification to be able to yield high quality product. This emphasizes the importance of the extraction step in nucleic acid-based fungal identification. Using VVN and SGM extracted DNA would most likely suffer from poor amplification.

Other factors such as kit cost, processing time and additional equipment for each DNA extraction method must also be considered when selecting a DNA extraction method. The VVN method was the least expensive method compared to SGM and ZYM which are both on the same range. VVN requires 1 hour incubation time plus 20 min processing time for each sample while SGM method has a processing time of 40 min and. ZYM method has a 10min processing time plus the use of a bead beater (Mini bead beater***) for disruption of fungal cells. ZYM does not require liquid nitrogen grounded samples unlike the other two, making it less prone to cross contamination. This kit has

short processing time which makes it more amenable to high-throughput sample processing. ZYM method is therefore recommended for succeeding DNA extractions.

ZYM was the method used for the DNA extraction of thirty two Mycelia Sterilia for molecular identification. The ZYM method performed well on some of the isolate while some gave faint DNA bands demonstrating that mechanical disruption of the fungal cell wall is not always the optimal extraction approach. Figure ___ shows the extracted DNA from the 32 Mycelia Sterilia isolates using ZYM.

PCR amplification and Sequencing

The ITS regions (ITS1 and ITS2) including the 5.8S rDNA and a small portion of both 18S and 28S rDNA of Mycelia Sterilia isolates were successfully amplified from genomic DNA with universal primers pITS1 and pITS4. Optimized PCR condition resulted in a single band of PCR product with the expected size range of 500 to 800 bp. The band of each PCR products was of sufficient quality for sequencing reactions. Figure ___ shows the bands produced by amplified target sites.

The PCR products were directly sent to Macrogen, Inc. for sequencing reaction that leads to a chromatogram displaying the complete nucleotide sequence for the DNA fragment. Direct sequencing of ITS combined with BLAST search has proved to be effective and reliable for the identification of fungi (Nicolotti *et al.*, 2010).

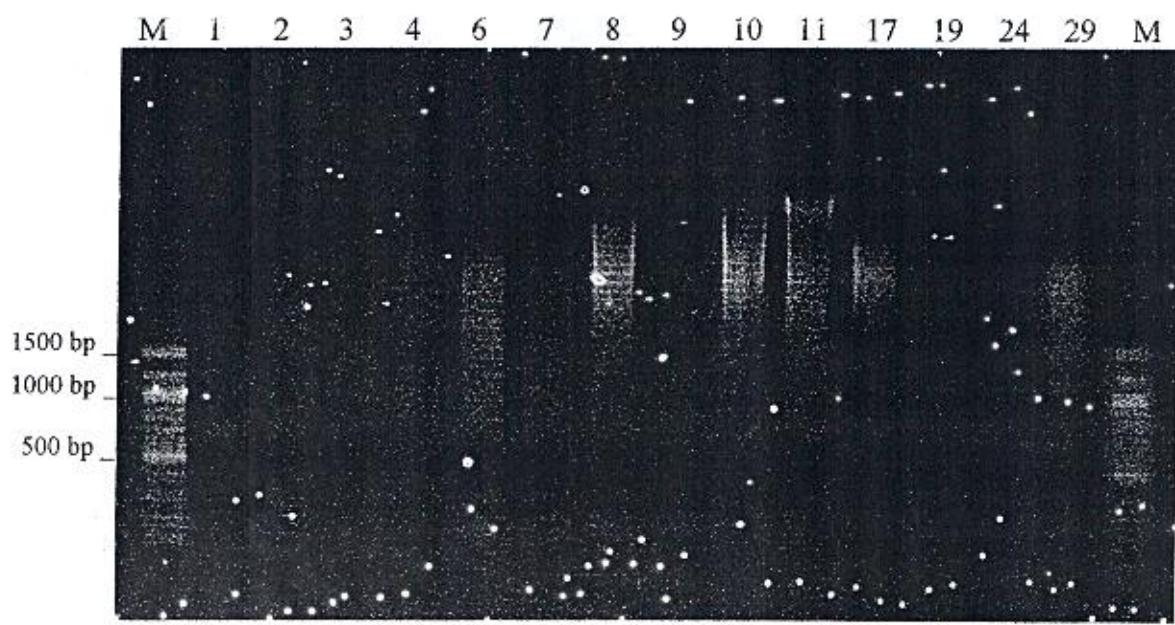
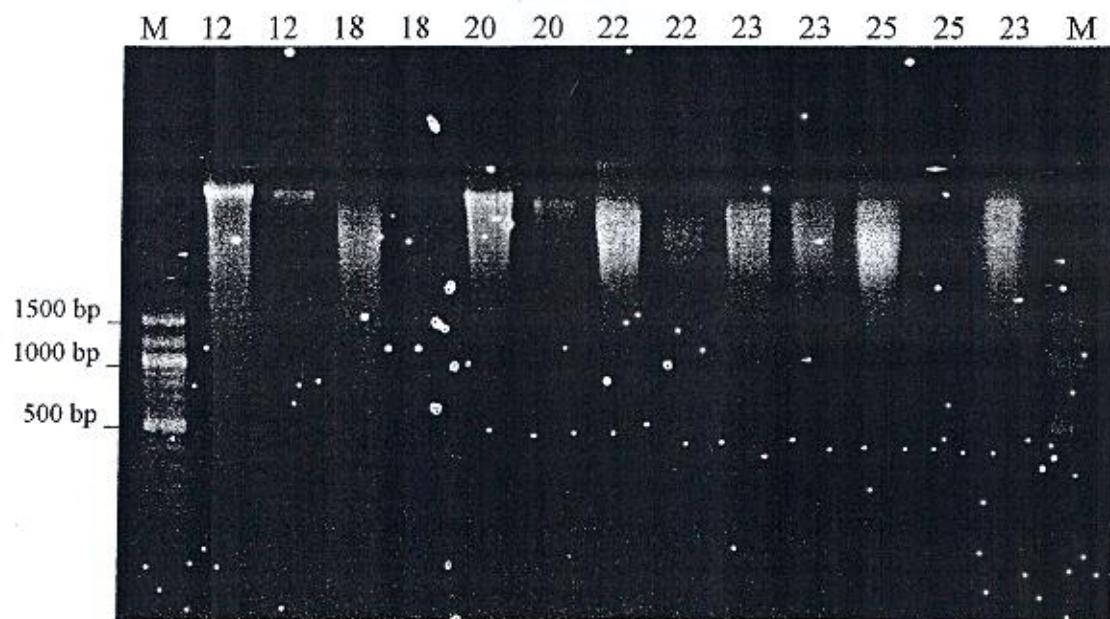


Figure 2. Electrophoretic analysis of the DNA extraction from Mycelia Sterilia isolates using ZYM method. M = 100bp DNA marker.

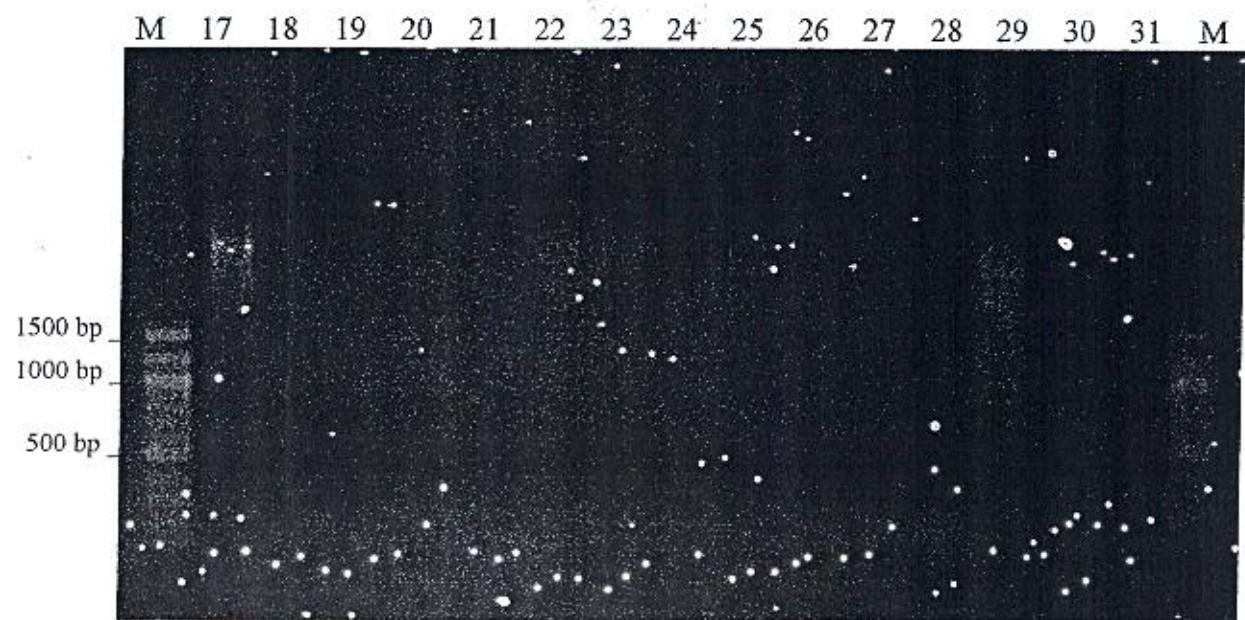
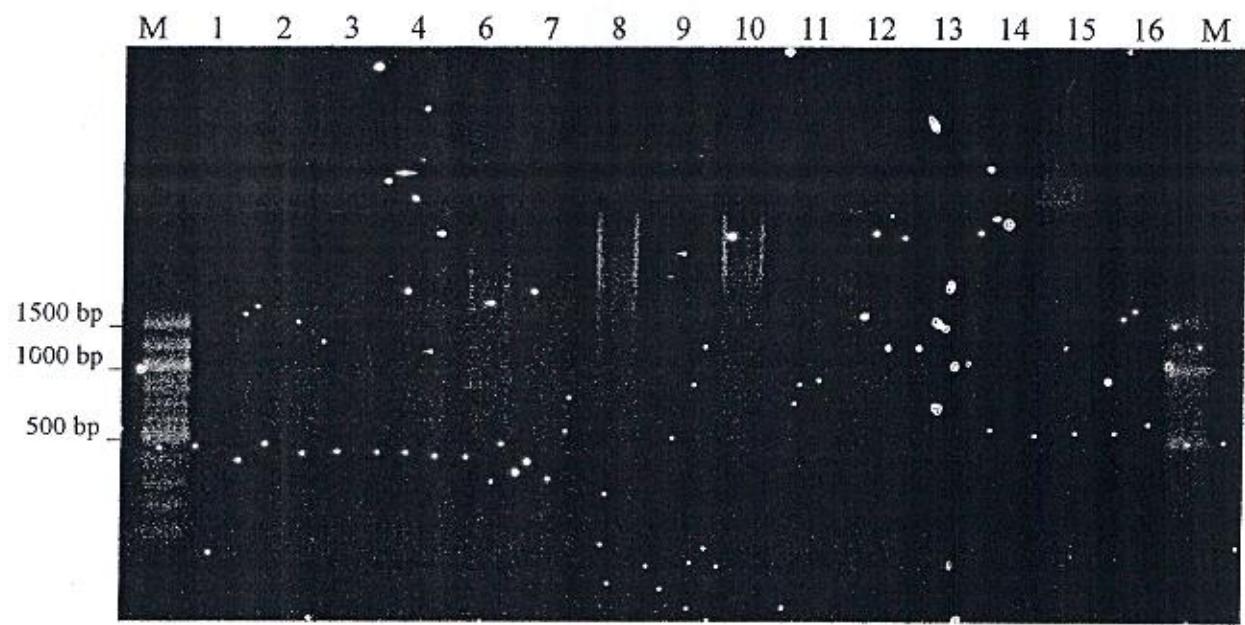


Figure 2. Electrophoretic analysis of the DNA extraction from *Mycelia Sterilia* isolates using ZYM method. M = 100bp DNA marker.

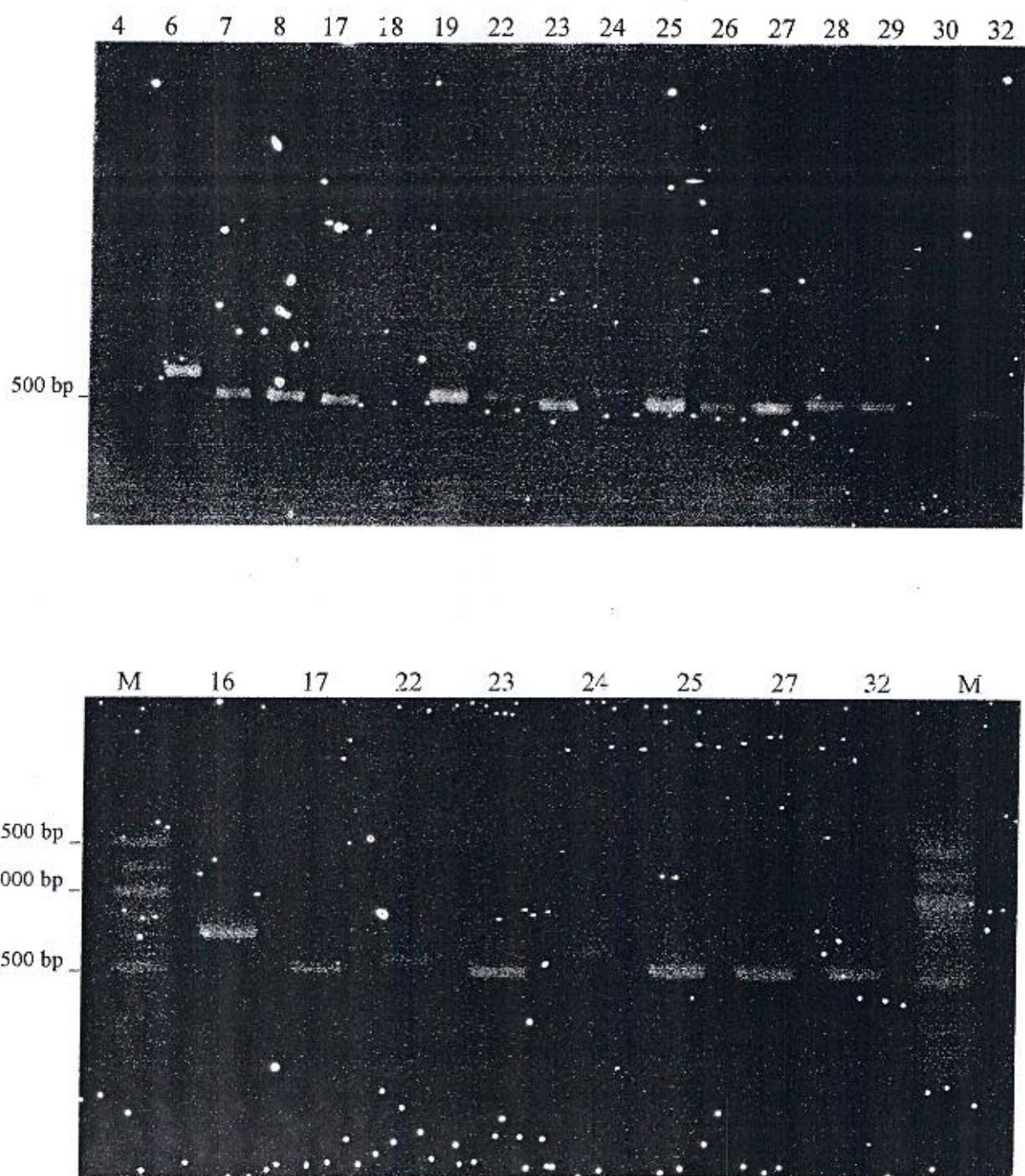


Figure 1. Electrophoretic profiles of ITS sequences amplified from genomic DNA extracted from *Mycelia Sterilia* isolates. M = Marker

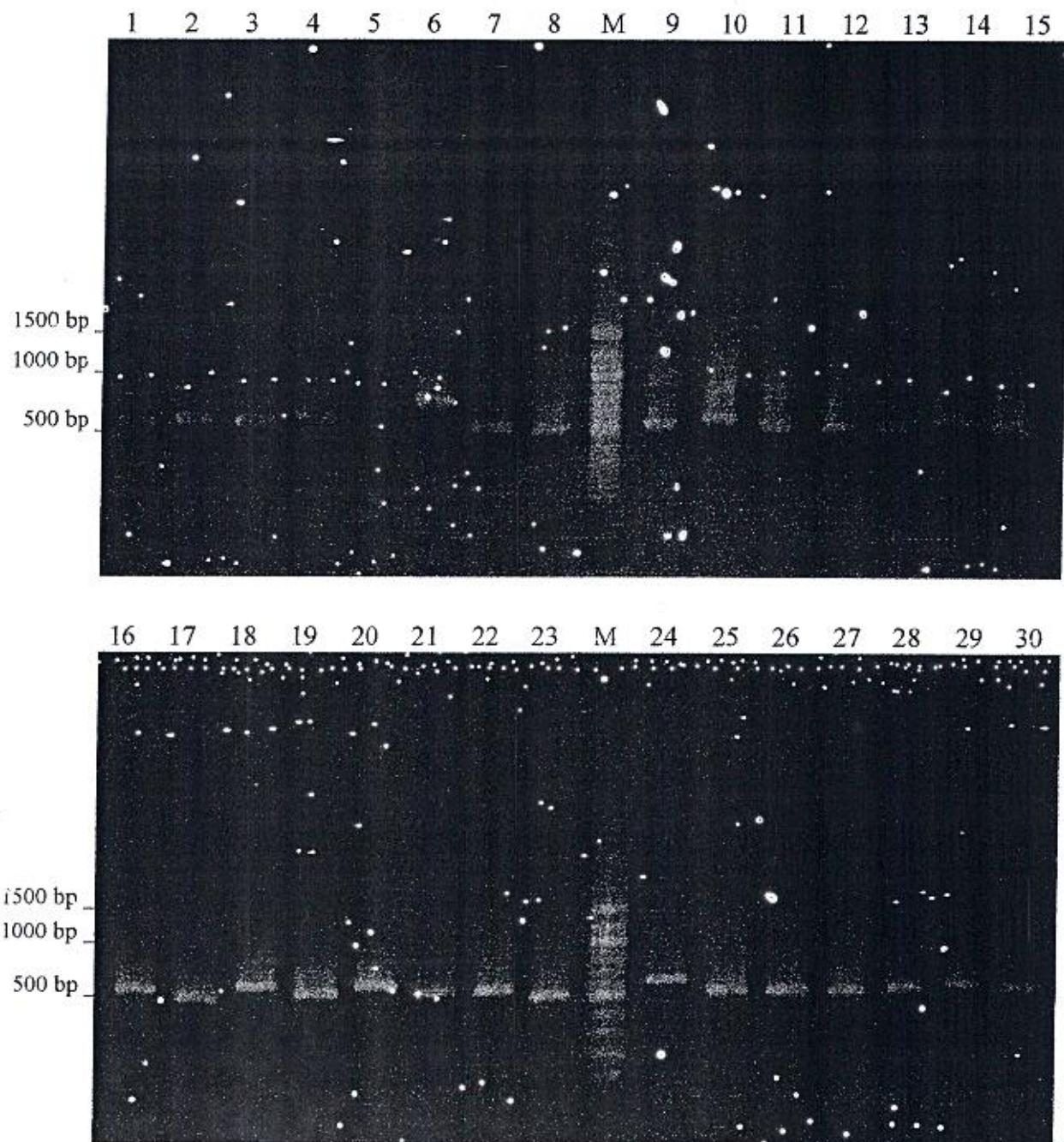


Figure 1. Electrophoretic profiles of ITS sequences amplified from genomic DNA extracted from *Mycelia Sterilia* isolates. M = Marker

The flanking small portion of both 18S and 28S rDNA were removed to get the length of the ITS1-5.8S-ITS2 region. The lengths of ITS1 ranged from 138 nucleotides (nt) (BD08) to 257 nt (BD06), while the lengths of ITS2 ranged from 144 nt (BD17 and BD23) to 237 nt (BD18 and BD24). The lengths of the 5.8S rRNA of all the isolates were consistent with 159 nt. The ITS2 region of BD26 and BD31 was found to be truncated, thus the exact length cannot be determined. BD06 has the longest total length of ITS region with 603 nt while BD32 has the shortest with only 412 nt. Table ___ shows the length of the ITS regions and 5.8S rRNA gene of the isolates).

The sequences were used in BLASTn searches against the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>) to identify the most similar sequences available. The Expect (E) values from the BLAST search, which reports the significance of each match, made possible to detect species displaying the highest sequence similarity with the query sequence. Hits with the lowest e-value were obtained from each query sequence. When more than one sequence had the same e-value, only the first sequence was selected. Initial searches confirms that the sequences are indeed the ITS regions including the 5.8S gene. Table ___ shows the sequences in the GenBank with highest similarity to the Mycelia Sterilia isolates according to BLAST search. Highly similar sequences were obtained and were aligned using the ClustalW program and utilized for phylogenetic analysis using the MEGA5 package (Tamura *et al.*, 2007).

Table 1. Variation in length of the ITS1, ITS2, 5.8S and total length of the Mycelia Sterilia isolates.

Isolate No.	ITS1	ITS2	5.8S	Total length (nt)
BD01	149	168	159	476
BD02	171	154	159	484
BD03	169	150	159	478
BD04	185	156	159	500
BD05	185	156	159	500
BD06	257	220	159	636
BD07	150	161	159	470
BD08	138	156	159	453
BD09	150	161	159	470
BD10	201	153	159	523
BD11	150	161	159	470
BD12	151	161	159	471
BD13	150	161	159	470
BD14	214	152	159	525
BD15	174	169	159	502
BD16	172	164	159	495
BD17	139	144	159	442
BD18	148	237	159	544
BD19	177	153	159	489
BD20	195	189	159	543
BD21	176	153	159	488
BD22	178	153	159	490
BD23	139	144	159	442
BD24	148	237	159	544
BD25	148	148	159	455
BD26	146	>149	159	305
BD27	135	131	159	425
BD28	151	151	159	461
BD29	173	155	159	487
BD30	151	150	159	460
BD31	157	>154	159	316
BD32	154	99	159	412

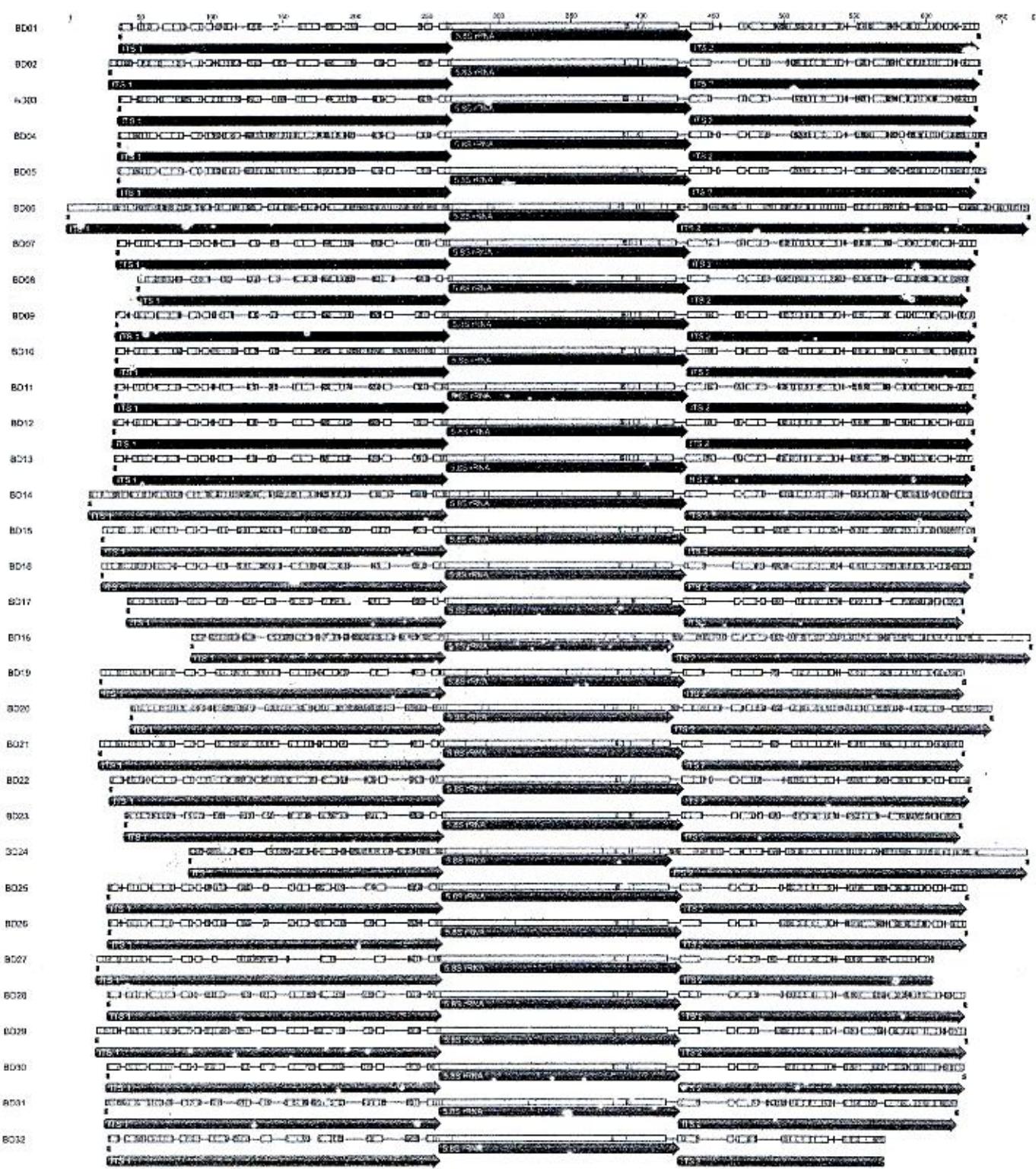


Figure 1. ITS 1-5.8S-ITS 2 sequence length variation.

Table _____. Identity of 32 Mycelia Sterilia isolates based on genetic analysis of the internal transcribed spacer (region). Closest cultured and designate relatives to the isolates according to BLAST search are presented.

Isolate No.	Identity (Accession No.)	Similarity (%)
BD01	<i>Fusarium solani</i> (JF322999)	100
BD02	<i>Phomopsis</i> sp. (GU66663)	98
BD03	<i>Thielavia subthermophila</i> (AJ271575)	100
BD04	<i>Xylaria papulicola</i> (GU300100)	99.6
BD05	<i>Xylaria papulicola</i> (GU300100)	99.6
BD06	<i>Chondrostereum purpureum</i> (JF340265)	85.4
BD07	<i>Stachybotrys bisbyi</i> (AF081480)	91.5
BD08	<i>Stogonospora</i> sp. (AY208791)	95.1
BD09	<i>Stachybotrys bisbyi</i> (AF081480)	91.5
BD10	<i>Trichoderma protrudens</i> (EU330946)	99.6
BD11	<i>Stachybotrys bisbyi</i> (AF081480)	91.5
BD12	<i>Stachybotrys bisbyi</i> (AF081480)	91.5
BD13	<i>Stachybotrys bisbyi</i> (AF081480)	91.5
BD14	<i>Mycoleptodiscus indicus</i> (GU980694)	99.4
BD15	<i>Penicillium steckii</i> (GU994590)	100
BD16	<i>Eupenicillium chrlchii</i> (GU981578)	99.6
BD17	<i>Epicoccum sorghi</i> (HQ450013)	100
BD18	<i>Schizophyllum commune</i> (EU520217)	99.8
BD19	<i>Penicillium citrinum</i> (HQ245157)	99.8
BD20	<i>Rigidoporus</i> sp. (AJ537410)	99.8
BD21	<i>Penicillium citrinum</i> (HQ245157)	100
BD22	<i>Stromatoneurospora pheonix</i> (AY909004)	96.9
BD23	<i>Epicoccum sorghi</i> (HQ450013)	100
BD24	<i>Schizophyllum commune</i> (EU520217)	100
BD25	<i>Fusarium equiseti</i> (HQ718416)	100
BD26	<i>Gibberella moniliformis</i> (GU199422)	100
BD27	<i>Lasiodiplodia pseudotheobromae</i> (FJ545358)	100
BD28	<i>Nigrospora</i> sp. (AM262341)	100
BD29	<i>Diaporthe</i> sp. (EF423549)	100
BD30	<i>Nigrospora</i> sp. (HQ891110)	100
BD31	<i>Cochliobolus lunatus</i> (GQ169765)	99.8
BD32	<i>Cladosporium cladosporioides</i> (HM992497)	100

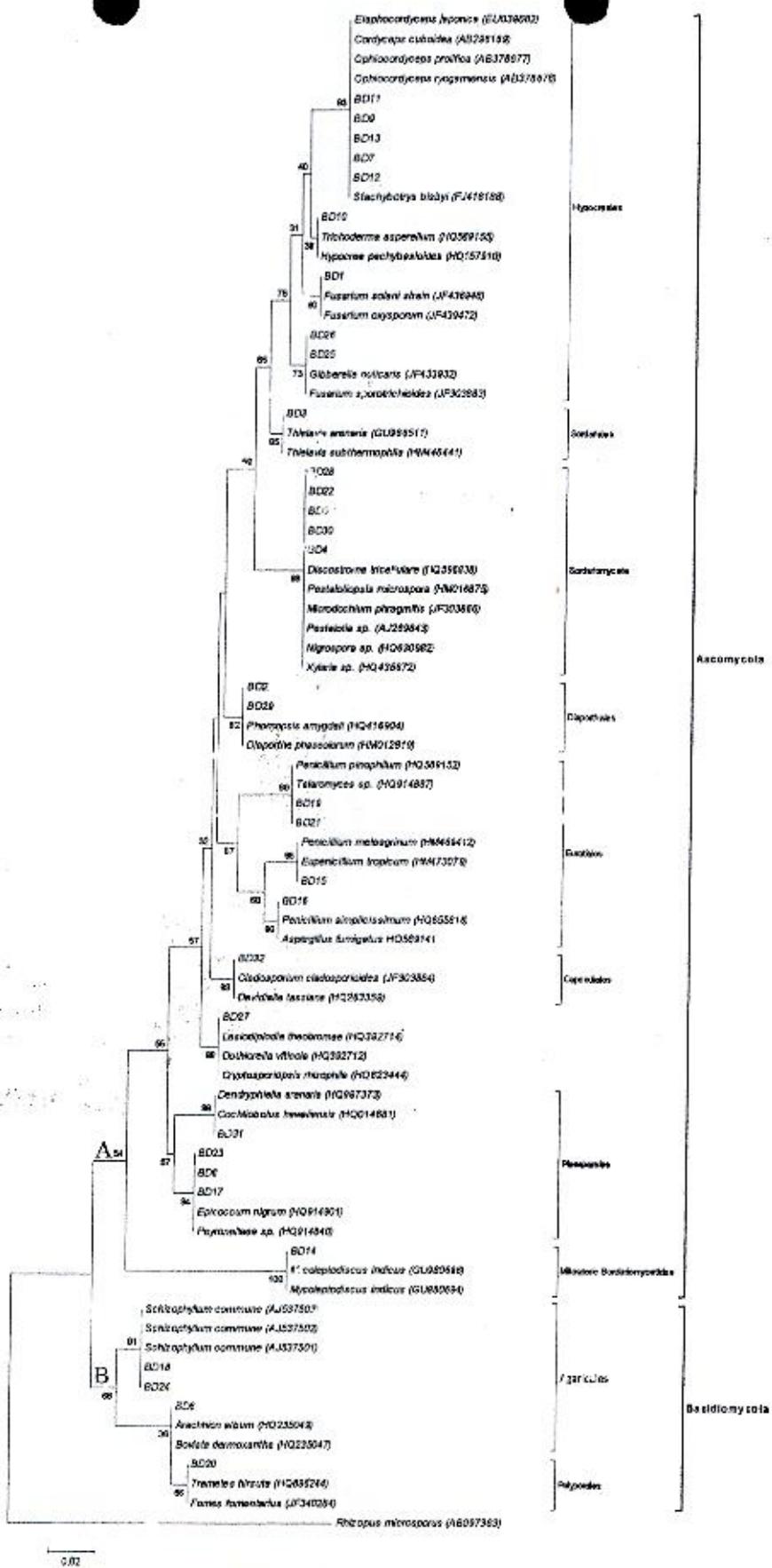
Taxonomic placement of *Mycelia Sterilia* isolates

Sequence information of mycelia sterilia and the determination of its phylogenetic relationship are considered necessary for the identification of these fungi. The ITS1 and ITS2 sequences were found to be highly variable in length which makes alignment between distantly related taxa using the whole ITS region unreliable since ambiguous alignments were observed. Therefore, the 5.8S rRNA gene sequences were initially used in the phylogenetic analysis to determine the general placement of the Mycelia Sterilia in the Kingdom Fungi.

The ITS region was included in the subsequent analyses which includes closely related taxa for the identification of Mycelia Sterilia to as low taxonomic level as possible. In some cases, identification was made to the species level. Results from BLAST search are depicted in Table ____.

Phylogenetics analysis of 5.8S gene sequences. Neighbor-joining phylogenetic tree was made to establish the general taxonomic placement of the 77 aligned 5.8S gene sequences (45 references and 32 isolates) (Figure ____). It was constructed with 1000 bootstrap replications and *Rhizopus microspora* (AB097363) was used as an out-group. The data resulted in two main clades, representing the fungal phylum Ascomycota (clade A) and Basidiomycota (clade B).

Clade A includes 28 isolates belonging to class Dothideomycete, Eurotiomycete and Sordariomycete. The isolates were further grouped into the different orders of phylum Ascomycota. Among these isolates, BD1, BD7, BD9, BD11, BD12, BD13,



BD25 and BD26 clustered with Hypocreales; BD3 clustered with Sordariales; BD4, BD5, BD22, BD28 and BD30 clustered with mitosporic Sordariomycete; BD2 and BD29 with Diaporthales; BD15, BD16, BD19 and BD21 clustered with Eurotiales; BD32 clustered with Capnodiales and BD8, BD17, BD23 and BD31 clustered Pleosporales. BD27 cannot be classified lower than phylum Ascomycota. BD14 clustered with the species of mitosporic Sordariomycetidae. Phylogenetic analysis showed that it is closely related to Dothideomycete than Sordariomycete, where it belongs. This is due to the Sordariomycetidae *incertae sedis* classification of this family. A "group is listed as incertae sedis are taxa of uncertain position which have been placed at the least inclusive level in the hierarchy where they can be assigned with confidence (Hibbett, 2007).

Clade B belongs to class Agaricomycete and includes 4 isolates grouped into two orders of phylum Basidiomycota. Among these isolates, BD6, BD18 and BD24 clustered with the species of the order Agaricales while BD20 clustered with Polyporales.

The 5.8S gene can be used to identify the general taxonomic placement of the 32 isolates to ordinal or higher level due to its highly conserved nature.

Phylogenetic analysis of ITS regions. The ITS regions (ITS1-5.8S-ITS2) were used to further identify the isolates. The ITS sequences can accumulate mutation at a faster rate than 5.8S, 18S and 28S rRNA, which makes it useful for molecular systematic at the species or even within species level (Baldwin, 1992; Schmidt and Moreth, 2002). The ITS1-5.8S-ITS2 sequences of the 32 isolates were compared with sequences listed in GenBank and was analyzed based on the fungal order or classification dictated by the 5.8S rRNA gene.

Hypocreales. Neighbor-joining phylogenetic tree using Hypocreales species which had high similarities to the isolates were constructed based on phylogenetic distance. The eight Hypocreales BD isolates formed three clusters representing Nectriaceae, mitosporic Hypocreales, and Hypocreaceae (Figure ____).

Cluster I contains BD01, BD25 and BD26 which clustered with *Fusarium* (Nectriaceae) species with 99% bootstrap support value (BSV). BD01 and BD25 had 100% ITS sequence similarities to *Fusarium solani* and *F. equiseti*, respectively while BD26 had 100% similarity to *Gibberella* and its anamorph *Fusarium*. Based on phylogenetic analysis, these isolates were placed in genus *Fusarium*.

Cluster II consists of BD7, BD9, BD11, BD12 and BD13. Their ITS regions are of similar sizes with 100% ITS sequence similarity between each other. These isolates clustered with *Stachybotrys* (mitosporic Hypocreales) species with 95% BSV and formed a terminal cluster with *S. bisbyi* with only 91.5% ITS sequence similarity and only 52% BSV. These 5 isolates might presumably represent a new species of *Stachybotrys* lineage but further experiments should be performed to confirm this claim.

Cluster III consists of BD10 which clustered with *Trichoderma* (Hypocreaceae) species and its teleomorph *Hypocrea* with 100% BSV. It had 99.6% ITS sequences similarity to *T. protrudens*. Other similar *Trichoderma* sequences clustered together with BD10. Thus, it was classified under the genus *Trichoderma*.

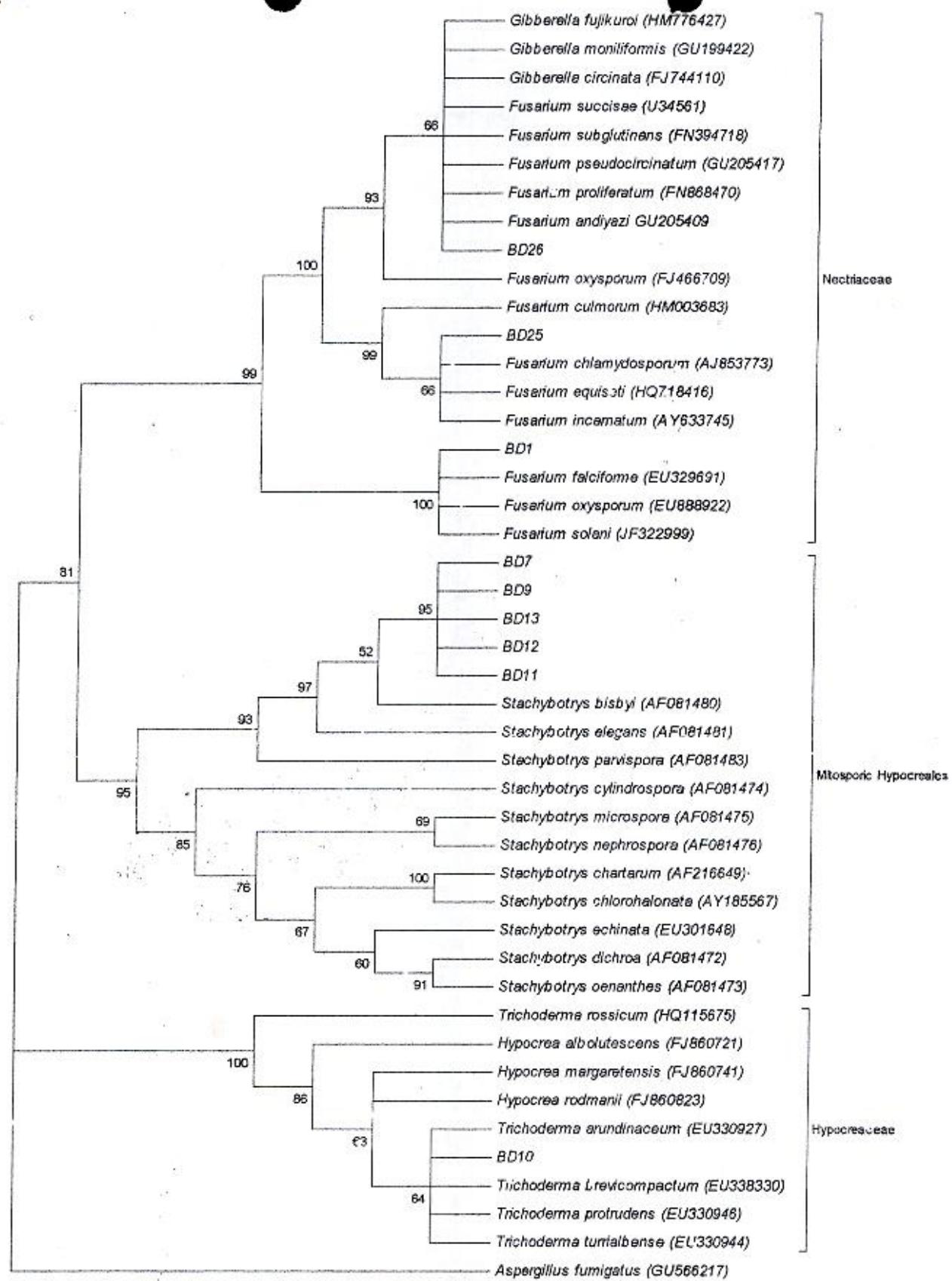


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Aspergillus fumigatus* (GU566217). Bootstrap values >50% (1000 replicates) are shown at the branches.

Sordariales. BD03 clustered with three *Thielavia* (Chaetomiaceae) species with 80% BSV. It formed a terminal clade with *T. subthermophila* with 100% ITS sequence similarity and 97% BSV (Figure ____). BD03 was identified as *T. subthermophila*.

Mitosporic Sordariomycete. Neighbor-joining phylogenetic tree using mitosporic Sordariomycete species which had high similarities to the isolates were constructed based on phylogenetic distance. The four BD isolates formed two clusters representing Xylariaceae and mitosporic Trichosphaerales (Figure ____).

Cluster I consists of BD04 and BD05 which clustered with *Xylaria* (Xylariaceae) species with 96% BSV. Both have 99.6% ITS sequence similarity and formed a terminal clade with *X. papulicola* supported by 100% BSV. BD04 and BD05 have 100% pairwise ITS sequence similarity and both were identified as *X. papulicola*.

BD22 clustered with two Xylariaceae genus (Podosordaria and Stromatoneurospora) with 97% BSV. It had only 96.9% ITS sequence similarity to *Stromatoneurospora phoenix* which is also reflected by its 67% BSV. BLAST search of *Stromatoneurospora* sp. showed that there only one sequence (AY909004) available in the database. BD22 might presumably represent a new species of *Stromatoneurospora* lineage but further experiments should be performed to confirm this claim or if more sequences of *Stromatoneurospora* will be available in the database. BD22 was classified under the genus *Stromatoneurospora*.

Cluster II consists of BD28 and BD30 showed close affiliation with *Nigrospora* (mitosporic Trichosphaerales) species and showed 100% BSV. Both isolates had 100% ITS sequence similarity with *Nigrospora* sp. (AM262341) and *Nigrospora* sp.

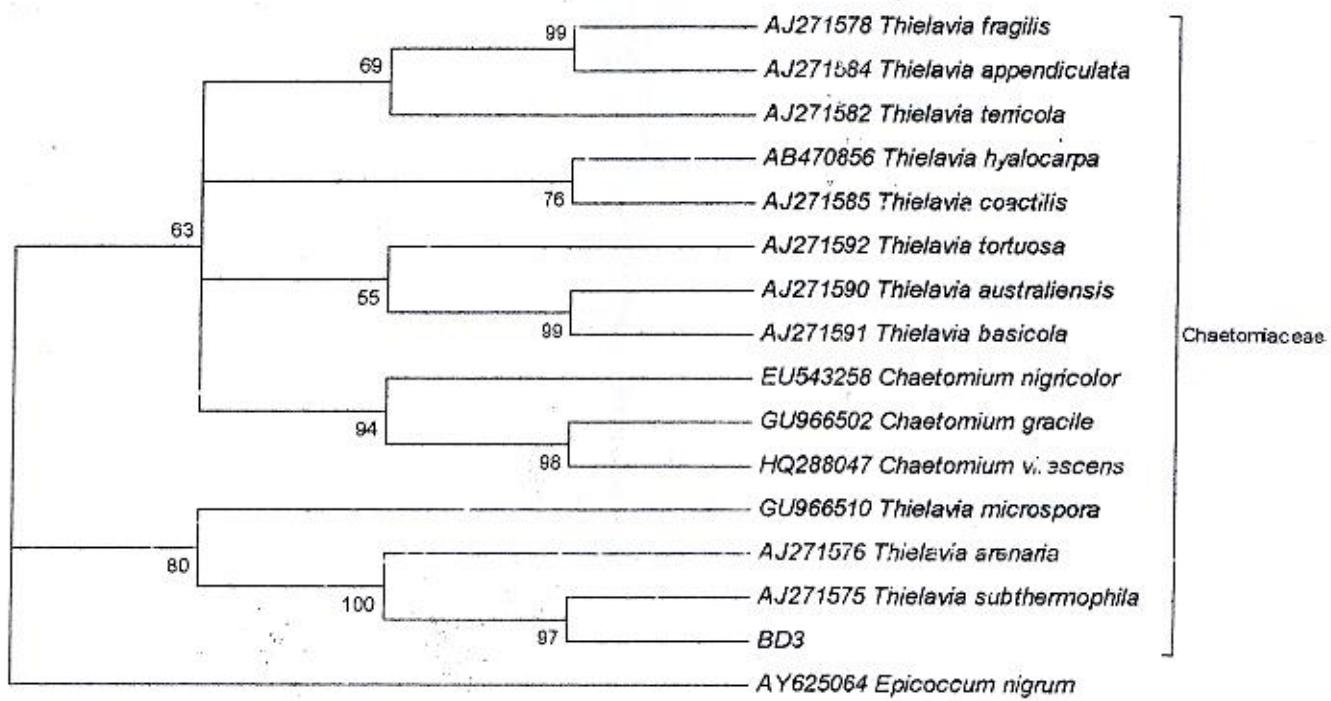


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Epicoccum nigrum* (AY625064). Bootstrap values >50% (1000 replicates) are shown at the branches.

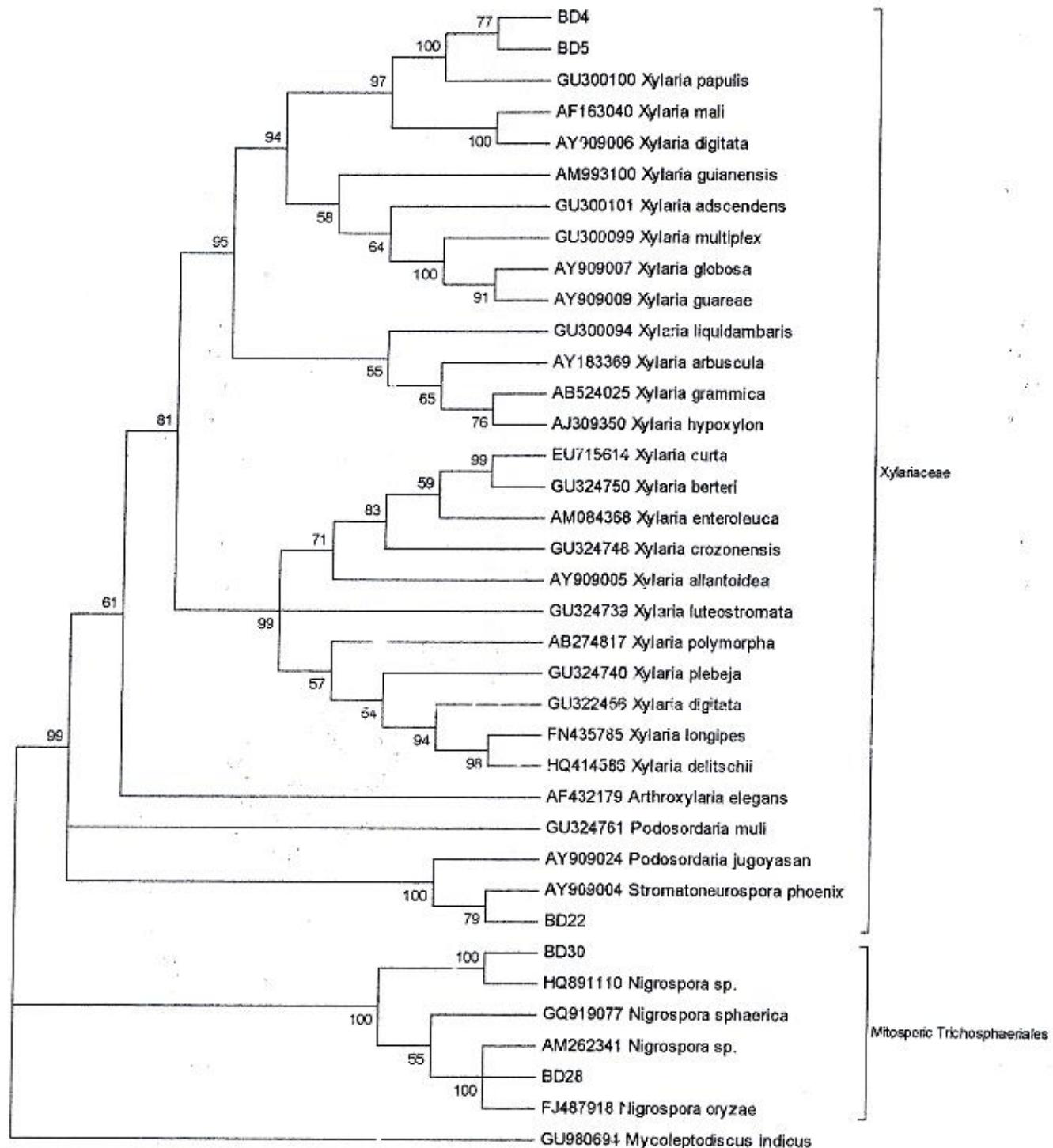


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Mycoleptodiscus indicus* (GU980694). Bootstrap values >50% (1000 replicates) are shown at the branches.

(HQ891110), supported by 99% and 100% BSV, respectively. Due to limited *Nigrospora* sp. ITS sequence availability on the database as well as the lack of fully designated sequence where BD28 and BD30 are similar, these isolates can only be classified to the genus *Nigrospora*.

Diaporthales. BD02 and BD29 clustered with *Diaporthe* and *Phomopsis* (Diaporthaceae) species with 99% BSV (Figure __). BD02 had 98% ITS sequence similarity to *Phomopsis* sp. (GU066663) and formed cluster with two *Diaorthe* sp. and four *Phomopsis* sp. supported by 99% BSV. BD29 had 100% ITS sequence similarity to *Diaporthe* sp. (FJ799937) and to *Phomopsis* sp. (GU591548). BD30, *Diaporthe* sp. (FJ799937), *Phomopsis* sp. (GU591548) and *Phomopsis glabrae* clustered together with 100% BSV. BD29 formed a terminal cluster with *Diaporthe* sp. (FJ799937) and *Phomopsis* sp. (GU591548). Telomorph *Diaporthe* Nitschke and anamorph *Phomopsis* (Sacc.) Sacc. connections are well established (Wehmeyer, 1933). The phylogenetic analysis and sequence comparison suggests that BD02 and BD29 can be classified to the genus *Diaporthe* and its anamorph *Phomopsis*.

Eurotiales. BD15, BD16, BD19 and BD21 clustered with *Penicillium* and *Eupenicillium* (both Trichocomaceae) species with 100% BSV (Figure __). BD15 formed a terminal cluster with 99% BSV and has 100% ITS sequence similarity with *P. steckii*. BD16 has 99.6% ITS sequence similarity with *Eupenicillium ehrlichii* and formed a well supported terminal clade. BD19 and BD21 have 99.8 and 100% ITS sequence similarity to *P. citrinum*, respectively and formed a terminal clade with it at

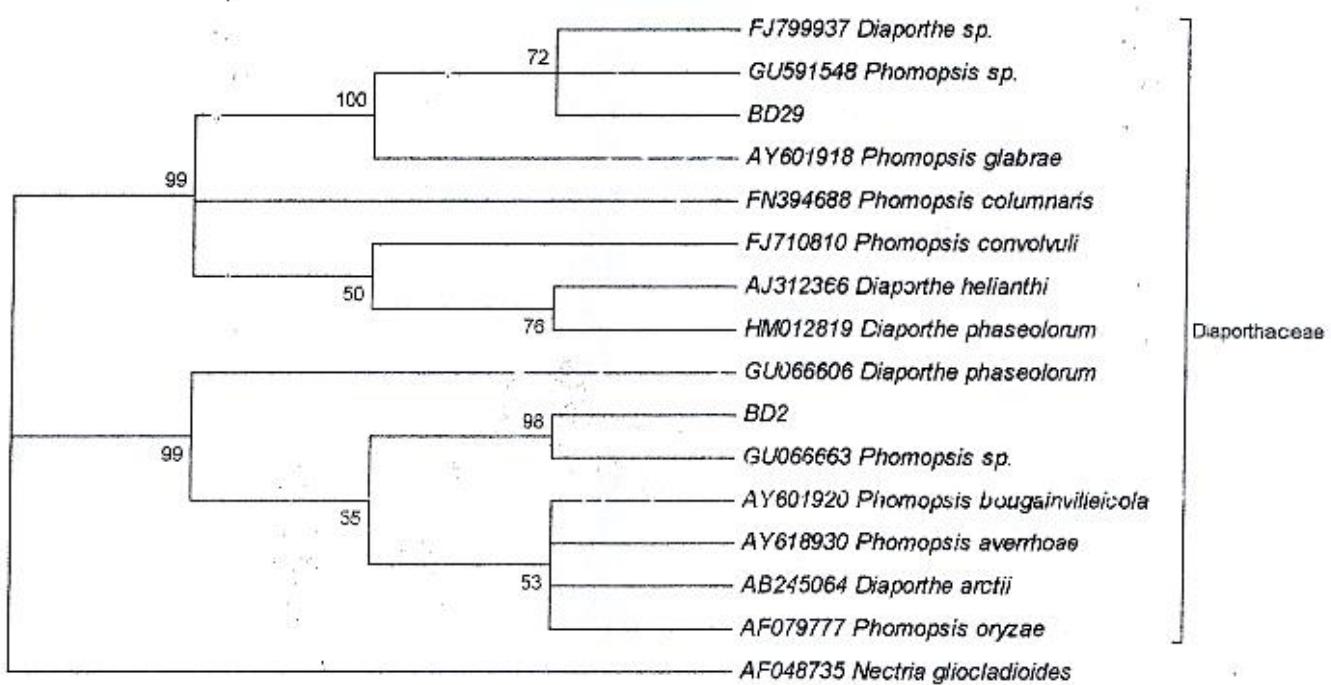


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Nectria gliocladiooides* (AF048735). Bootstrap values >50% (1000 replicates) are shown at the branches.

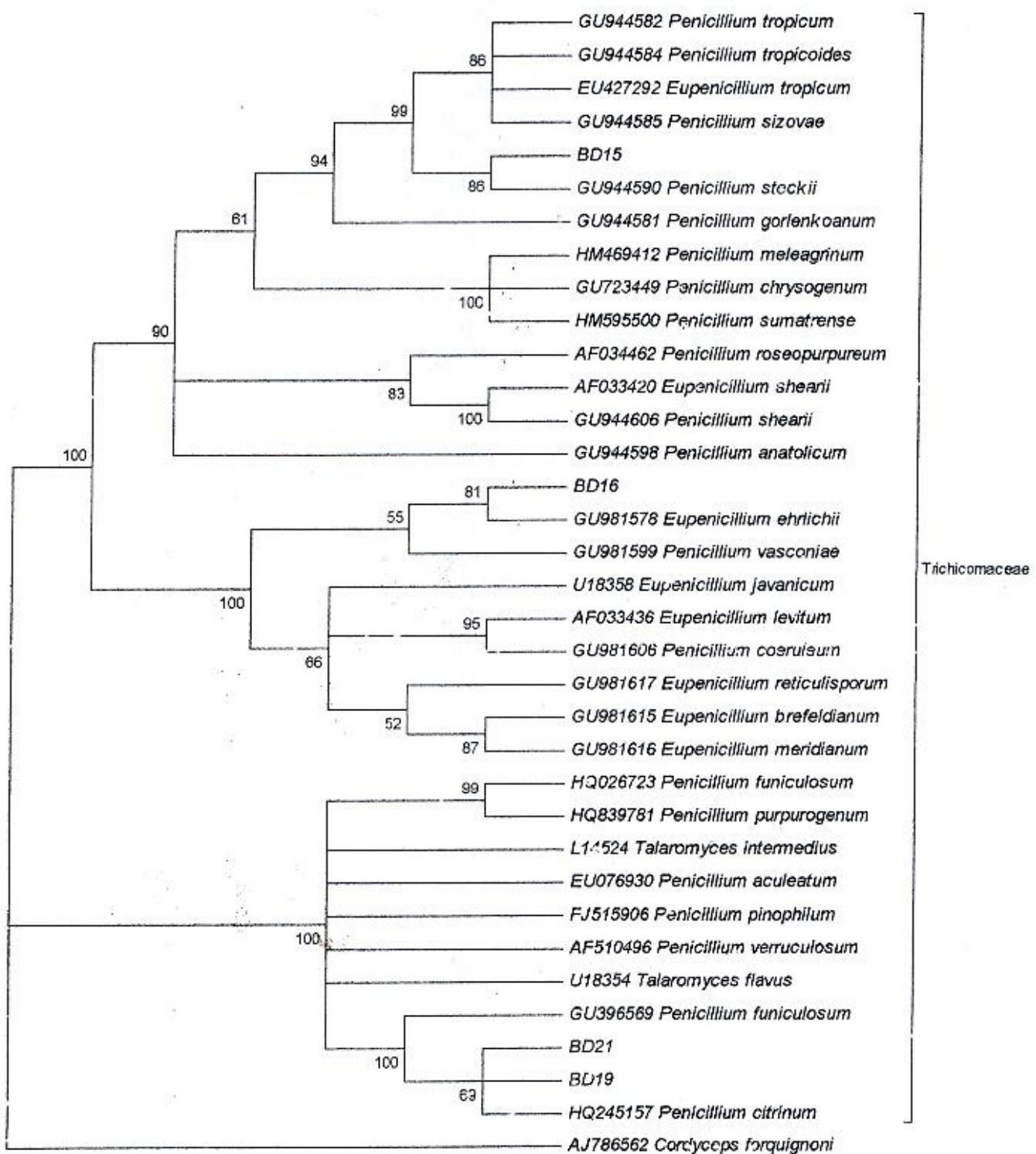


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Cordyceps forquignoni* (AJ786562). Bootstrap values >50% (1000 replicates) are shown at the branches.

100% BSV. BD15, BD16, BD19 and BD21 were classified as *P. steckii*, *Eupenicillium ehrlichii*, *P. citrinum* and *P. citrinum*, respectively.

Capnodiales. BD32 had been identified to Davidiellaceae (Capnodiales) according to 5.8S rDNA sequence analysis and combined with sequences of ITS regions to further reveal that BD32 was a species of genus *Cladosporium* (Davidiellaceae) with 99% BSV (Figure ____). It had very close relationship with *C. cladosporioides* with 100% ITS sequence similarity. BD32 had 100% ITS sequence similarity but did not form a distinct terminal cluster with *C. cladosporioides* and thus classified under genus *Cladosporium*.

Ascomycota. BD27 formed clustered with eight Botryosphaeriaceae species with 86% BSV (Figure ____). It has 100% ITS sequence similarity to both *Lasiodiplodia pseudotheobromae* and *L. theobromae*. BD27 formed a terminal cluster with three *Lasiodiplodia* sp. and its anamorph *Batryosphaeria* sp. supported with 92% BSV. Since BD27 cannot be further differentiated to other *Lasiodiplodia* sp., it was classified as under the genus *Lasiodiplodia*.

Pleosporales. Four BD isolates under order Pleosporales are highly similar to members of Pleosporaceae and Phaeosphaeriaceae (Figure ____). BD08 formed cluster with family Phaeosphaeriaceae with 95% BSV. It had 95.1% ITS sequence similarity with *Stagonospora* sp. (AY208791) and formed terminal cluster with 100% BSV.

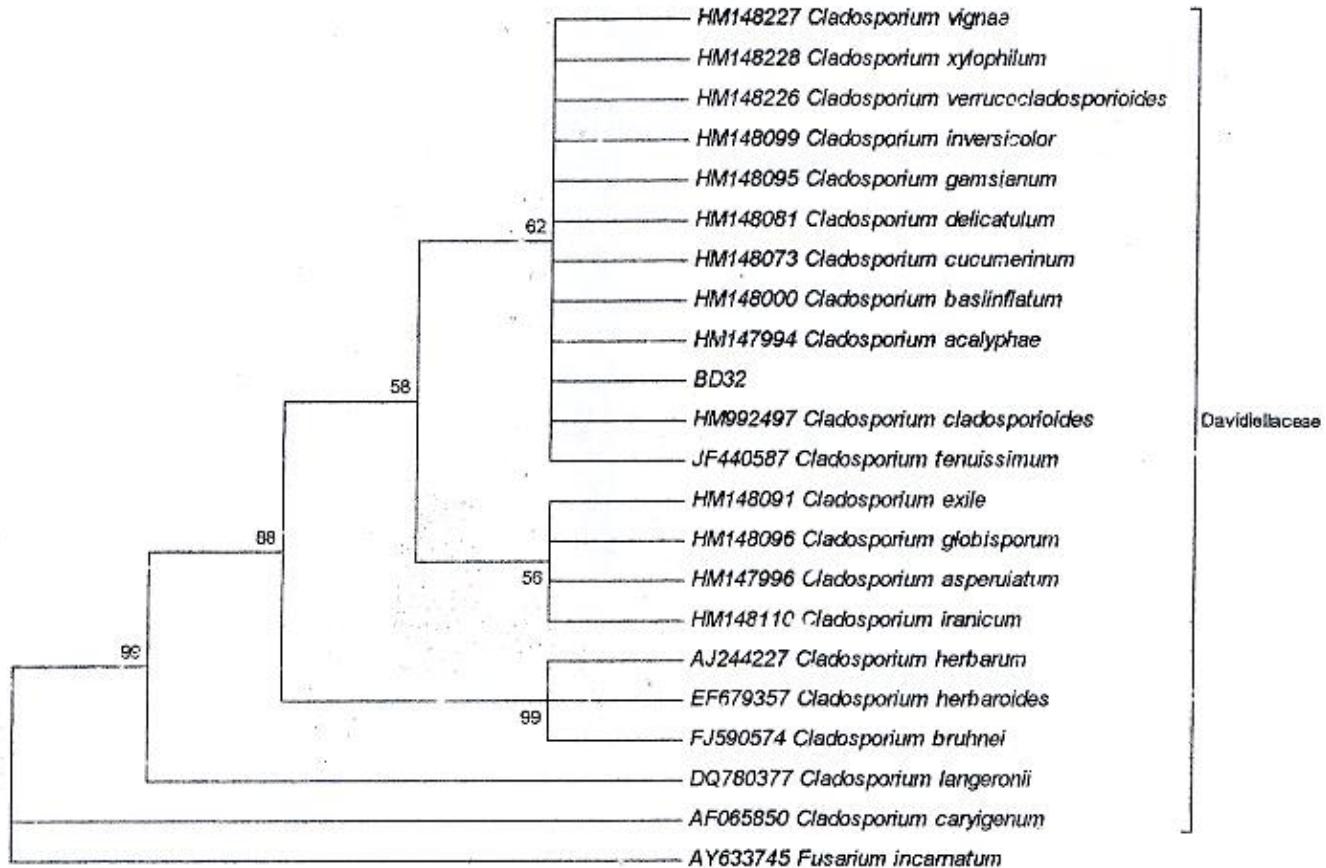


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Fusarium incarnatum* (AY633745). Bootstrap values >50% (1000 replicates) are shown at the branches.

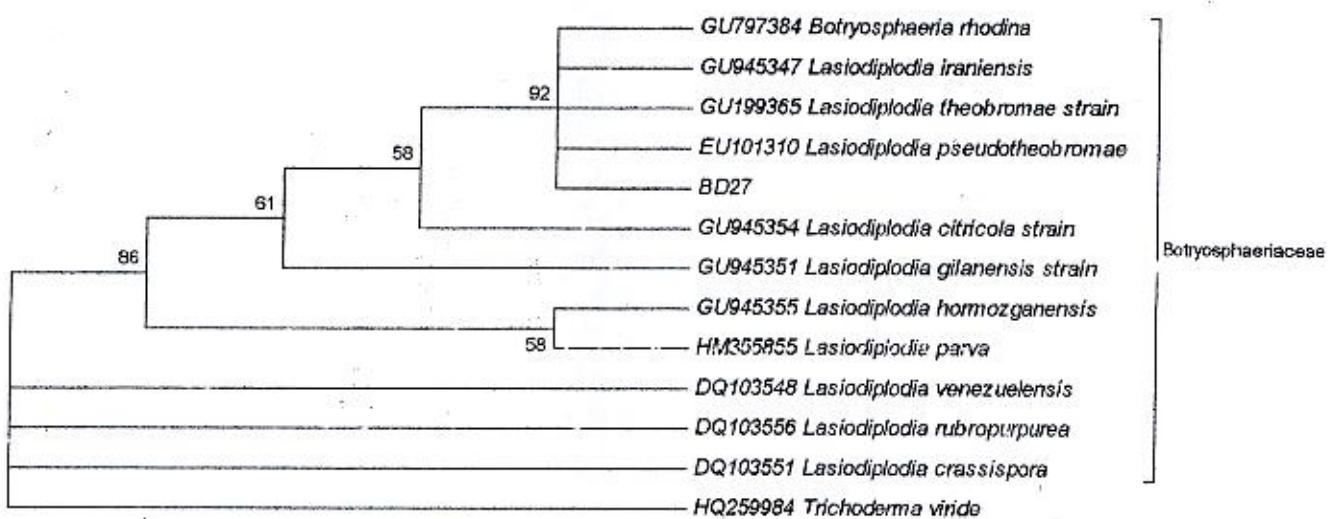


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Trichoderma viride* (HQ259984). Bootstrap values >50% (1000 replicates) are shown at the branches.

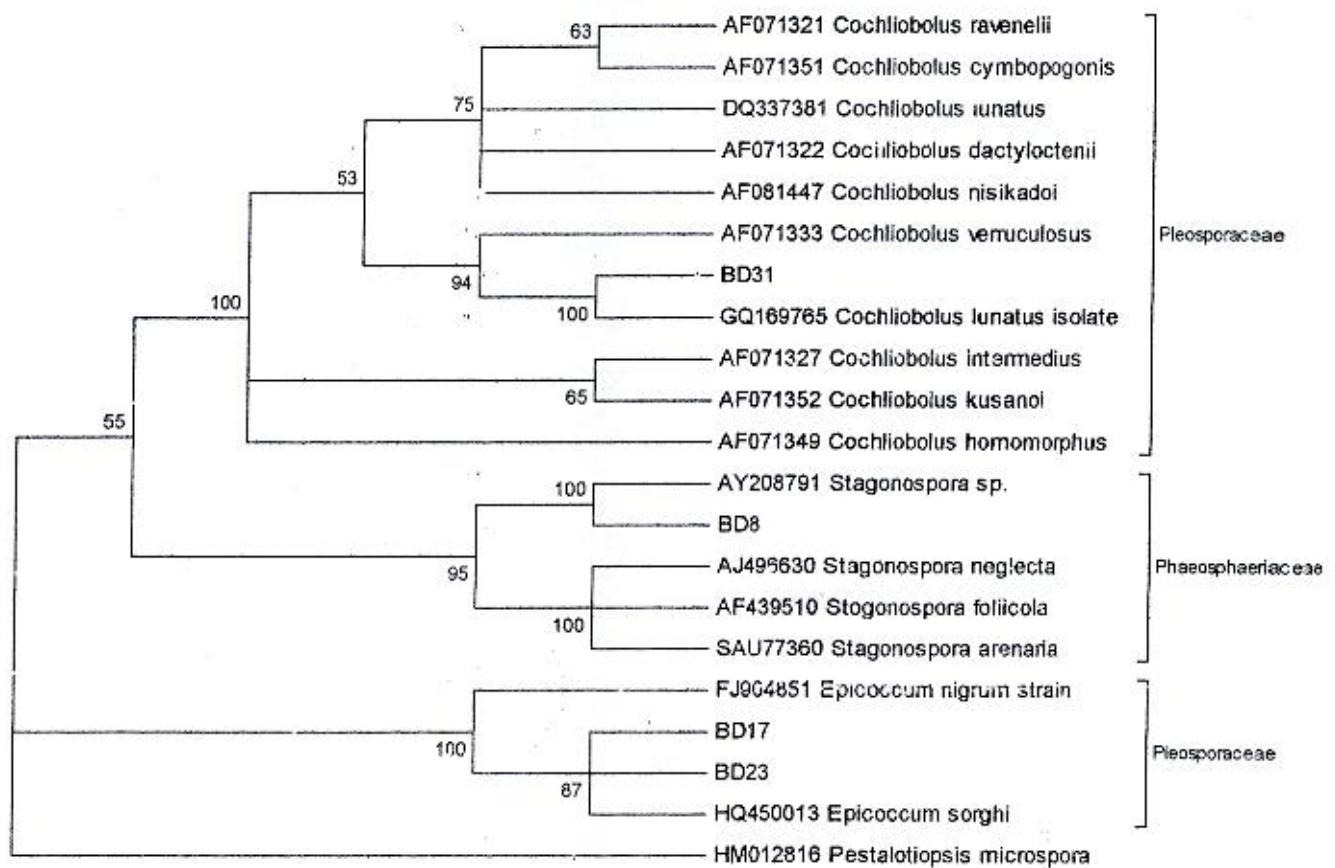


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Pestalotiopsis microspora* (HM012816). Bootstrap values >50% (1000 replicates) are shown at the branches.

BD17 and BD23 formed terminal cluster with *Epicoccum* (Pleosporaceae) species with 100% BSV. Both isolates have 100% ITS sequence similarity with *E. sorghi*, the terminal cluster formed was supported by 87% BSV.

BD31 formed cluster with *Cochliobolus* (Pleosporaceae) species supported by 100% BSV. It had 99.8% ITS sequence similarity with *C. lunatus* and formed terminal cluster supported by 100% BSV.

BD08, BD17, BD23 and BD31 were classified as *Stagonospora* sp., *E. sorghi*, *E. sorghi* and *C. lunatus*, respectively.

Mitosporic Sordariomycetidae. The Magnaportaceae is under mitosporic Sordariomycetidae where BD14 had 99.4% ITS sequence similarity to its member *Mycotodiscus indicus* and formed a terminal clade supported by 98% BSV (Figure ____). BD14 was classified as *M. indicus*.

Agaricales. BD6, BD18 and BD24 belong to the phylum Basidiomycota, order Agaricales (Figure ____). BD6 ITS sequence is only 85.4% similar to *Chondrostereum purpureum* (Cyphellaceae). The low similarity of BD6 to known sequences under order Agaricales is also reflected in the phylogenetic tree. It clusters distinct from other species of family Cyphellaceae. BLAST search of *Chondrostereum* sp. showed that there is only *C. purpureum* sequence available in the database. BD6 might presumably represent a new species of *Chondrostereum* lineage. Further experiments should be performed to confirm this claim or until more sequences become available on the database.

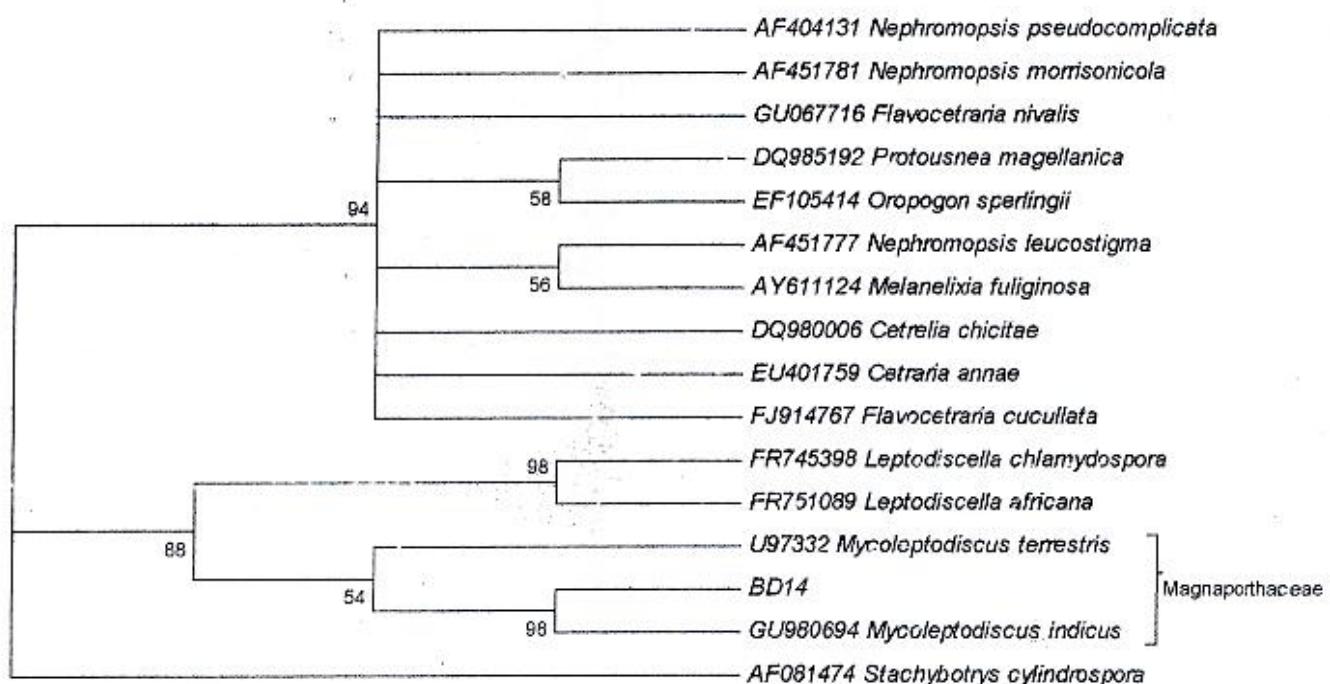


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Stachybotrys cylindrospora* (AF081474). Bootstrap values >50% (1000 replicates) are shown at the branches.

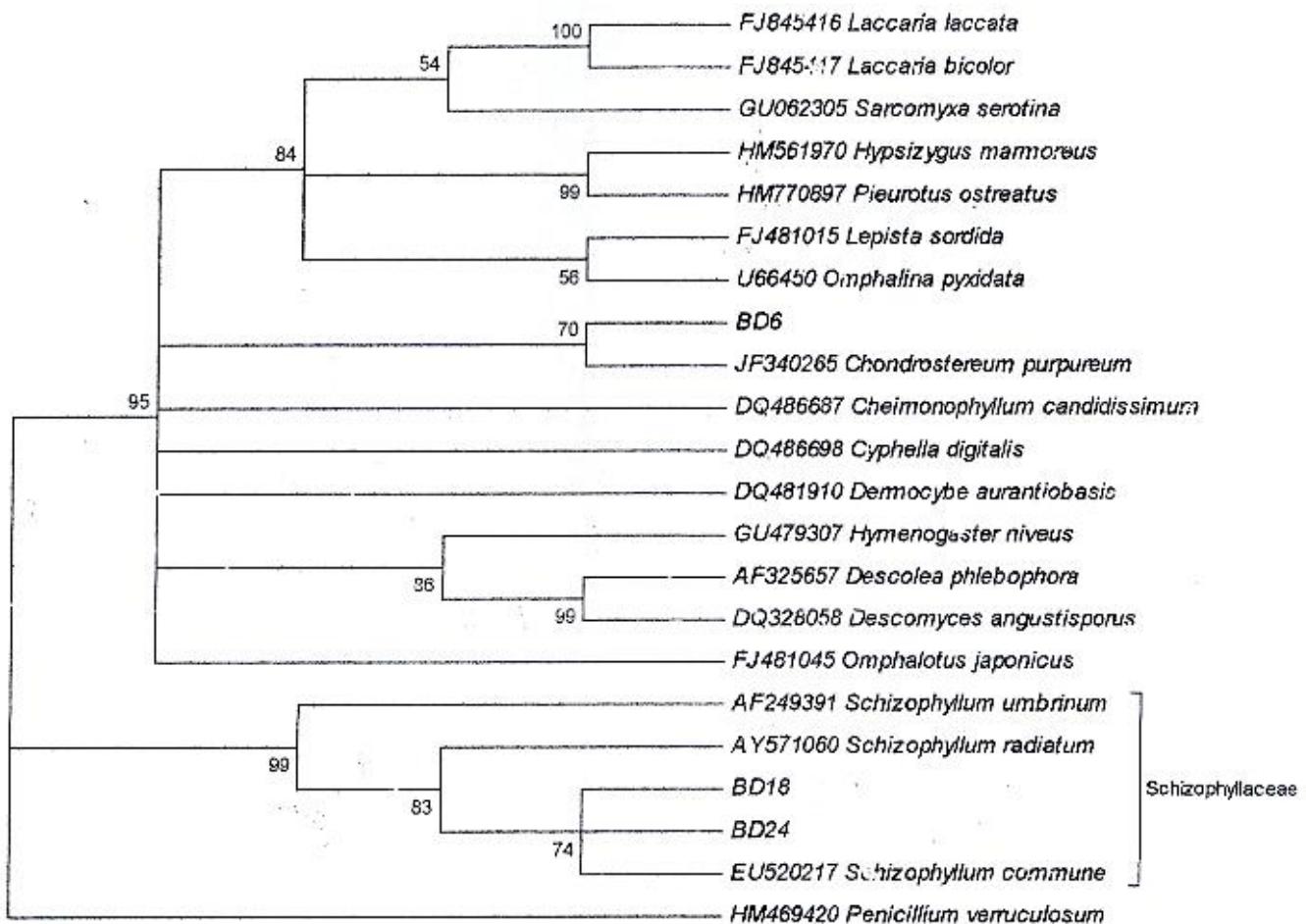


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Penicillium verruculosum* (HM469420). Bootstrap values >50% (1000 replicates) are shown at the branches.

On the other hand, BD18 and BD24 clustered with *Schizophyllum* (Schizophyllaceae) species with 99% BSV. Both have 99.8% and 100% ITS sequence similarity to *S. commune*, respectively and were supported with 74% BSV.

BD6, BD18 and BD24 were classified under *Chondrostereum* sp., *S. commune* and *S. commune*, respectively.

Polyporales. BD20 formed a terminal clade with 100% BSV and 100% ITS sequence similarity to *Rigidoporus* (Meripilaceae) species (Figure ____). Other *Rigidoporus* sp. formed cluster distinct from BD20. Phylogenetic analysis shows its close affiliation to family Polyporaceae (*Ganoderma* sp, *Dichomitus* sp and *Perenniporia* sp.). BD20 was classified under genus *Rigidoporus*.

Thirty two fungi previous designated as Mycelia Sterilia from various sources were successfully identified on the basis of ITS regions and 5.8S rRNA gene sequence similarity comparison and phylogenetic analyses. A total of twelve isolates were designated into their respective species while twenty isolates were identified to the genus level. This molecular study has shown that the fungi grouped as Mycelia Sterilia contains several phylogenetically distinct entities belonging to the different families of Kingdom Fungi. The 5.8S rRNA sequences can resolve most of the isolates to the family level while the whole ITS region sequences can distinguish the some of the isolates to the species level.

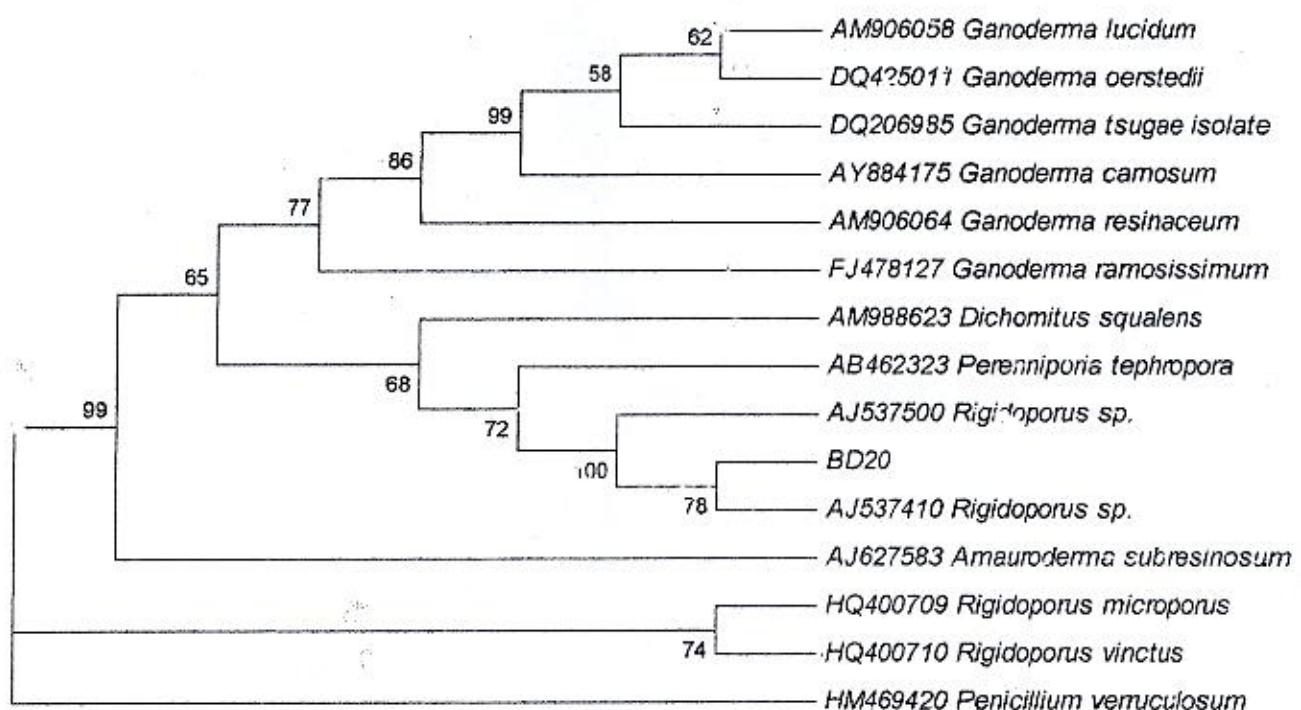


Figure 1. Neighbor-joining phylogenetic tree showing the relationship between *Mycelia sterilia* isolate and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Penicillium verruculosum* (HM469420). Bootstrap values >50% (1000 replicates) are shown at the branches.

Although the Mycelia Sterilia isolates were not able to produce reproductive structure in vitro, based on their molecular identity, it shows that they capable of producing these structures. Necessary culture conditions, nutrient requirement and lighting condition may have not been provided causing them to be sterile.

The major problem which hindered the identification of several isolates to the species level is the limited number of sequences for some species (*Chondrostereum*, *Nigrospora*, *Stromatoneurospora*, etc.) where the query sequence can be compared to. The sequences of these isolates could only be further analyzed when more sequences become available. Although scientists from around the world are frequently submitting sequences of different fungi, still only 1% of the estimated 1.5 million fungal species are available in public databases (Vilgalys, 2003).

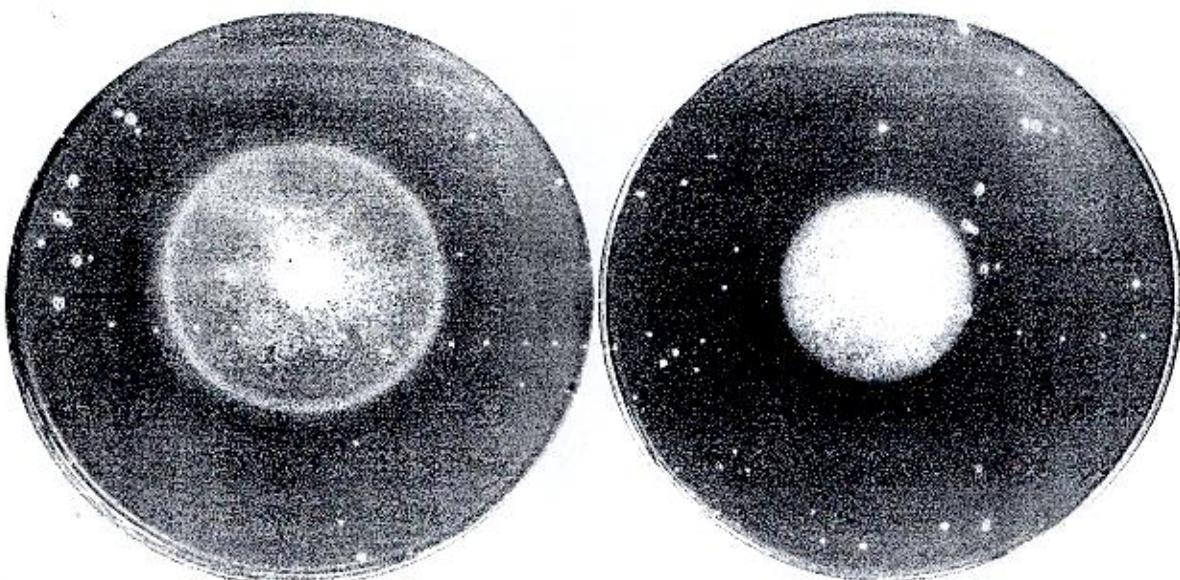
Some query sequences are highly (sometimes 100%) similar to several members of a genus (e.g. *Cladosporium*). In such cases, no further analysis could be performed to assign the isolate to species level. Sequencing of another DNA fragment can be more convenient to properly identify the isolate. Moreover, supplementary data, such as sequence from another DNA fragment or morphological characteristics of a specimen, is necessary to assign a specimen to a particular species with certainty.

However, molecular identification by analysis of the ITS region DNA sequences has proven its capability to determine the taxonomic placement of the thirty two Mycelia Sterilia isolates.

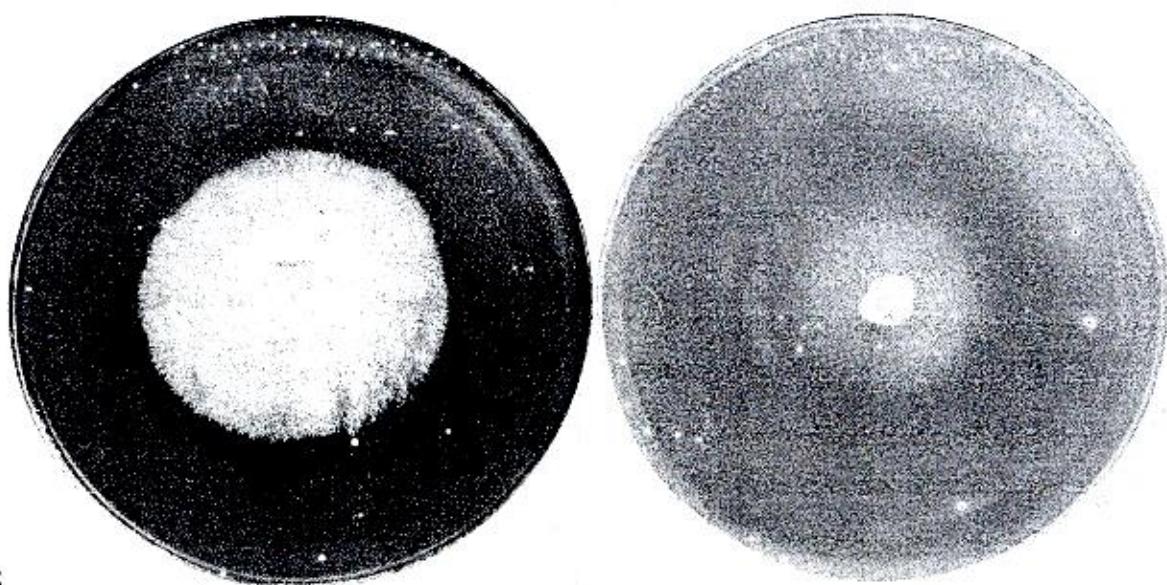
Screening of Mycelia Sterilia for enzyme production using plated medium.

The screening to test the capability of the 32 Mycelia Sterilia isolates was carried out using specific plated medium for each enzyme tested. All isolates were tested for its ability to hydrolyze casein (protease) and xylan (xylanase). These substrates can induce enzyme production by the fungi. Positive qualitative results for both enzymes exhibited distinct halo of hydrolysis around the colony (Figure __). The R ratio value of the positive isolates is a parameter for the evaluation of its ability to produce xylanases and proteases on solid agar media (Djamel *et al.*, 2009).

Based from the results shown in Table __, there were 15 and 7 Mycelia Sterilia that exhibited hydrolytic activity on xylan and casein, respectively. BD03 presented the highest ratio on xylanase screening plate followed by BD30 and BD21. BD03, identified under the genus *Thielavia*, thus, has xylanase activity superior to the others. On the other hand, proteolytic activity of all the Mycelia Sterilia tested did not produce an elevated level of protease enzyme production.



A



B

Figure 1. Protease screening plates. A) positive plates, red arrow shows the zone of clearing and presence of coagulation; B) negative plates.

Table 1. Screening for xylanase and protease production the 30 Mycelia Sterilia isolates.

Isolate Code	Xylanase (R ratio)	Protease
BD01	- ^a	-
BD02	-	-
BD03	+ ^a (1.65)	+ (1.03)
BD04	-	-
BD05	-	-
BD06	-	+ (1.04)
BD07	-	-
BD08	+ (1.19)	-
BD09	-	-
BD10	-	-
BD11	+ (1.05)	-
BD12	+ (1.17)	-
BD13	-	-
BD14	+ (1.03)	-
BD15	+ (1.17)	-
BD16	+ (1.10)	+ (1.03)
BD17	+ (1.06)	-
BD18	-	-
BD19	-	+ (1.03)
BD20	-	-
BD21	+ (1.33)	+ (1.04)
BD22	+ (1.07)	-
BD23	-	-
BD24	-	+ (1.03)
BD25	+ (1.03)	-
BD26	+ (1.05)	-
BD27	-	-
BD28	+ (1.32)	-
BD29	+ (1.03)	-
BD30	+ (1.41)	-
BD31	-	-
BD32	-	+ (1.03)

^a+/- present

SUMMARY AND CONCLUSION

Mycelia Sterilia is an artificial group of fungi unable to produce reproductive structures essential for morphological identification. This group of fungi is almost impossible to identify with classical mycological techniques. Molecular techniques are more appropriate for their proper identification.

PCR amplification followed by sequencing of ITS regions and 5.8S gene of the fungal genome generally provides the ability to discriminate between fungal species. Analysis of the nucleotide sequences of the thirty two Mycelia Sterilia isolates allowed the identification of 12 and 20 isolates to the species and genus levels, respectively. Thus, molecular sequencing of ITS regions and the 5.8S rRNA offer an effective method for the identification of Mycelia Sterilia.

Screening of the Mycelia Sterilia isolates for its capability to produce enzymes resulted to 15 and 7 positive isolates for xylanase and protease enzymes, respectively.

RECOMMENDATION

Quantitative xylanase assay is recommended for BD30 to further explore its ability to produce xylanase enzyme.

LITERATURE CITED

- ADRIÓ, J.E. & DEMAÍN, A.L. 2003. Fungal biotechnology. *Int. Microbiol.* 6:191-199.
- AFTAB S., S. AHMED, S. SAEED AND S.A. RASOOL. 2006. Screening, isolation and characterization of alkaline protease producing bacteria from soil. *Pakistan Journal of Biological Sciences* 9(11): 2122-2126.
- ALEXOPULOS, C.J. MIMS, C.W. AND BLACKWELL, M. 1996. Introductory mycology. 4th edition. John Wiley & Sons, Inc., New York - ... - Singapore.
- ALTSCHUL SF, GISH W, MILLER W, MYERS EW, LIPMAN DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403-410
- BALDWIN BG. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the Compositaogy. *Mol Phylogenet Evol.* 1992;1(1):3-16
- BILLS, G.F. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: Endophytic Fungi in Grasses and Woody Plants (eds. S.C. Redlin, L.M. Carris and M.N. St Paul). APS Press, USA: 31-65.
- BORMAN, A.M., C.J. LINTON, S.J. MILES AND E.M. JOHNSON. 2008. Molecular identification of pathogenic fungi. *Jounal of Antimicrobial Chemotherapy.* 61: Suppl. 1, i7-i12.
- BROOKMAN JL, MENNIM G, TRINCI AP, THEODOROU MK, TUCKWELL DS (2000) Identification and characterization of anaerobic gut fungi using molecular methodologies based on ribosomal ITS1 and 18S rRNA. *Microbiology* 146:393-403.
- CHAROENPORN, C., S. KANOKMEDHAKUL, F.C. LIN, S. POEAIM AND K. SCYTONG. 2001. Evaluation of bio-agent formulations to control Fusarium wilt of tomato. *African Journal of Biotechnology.* 9(36): 5836-5844.

- CHEN W., J.W. HOY AND R.W. SCHNEIDER. 1992. Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythlthium* species. Experimental Mycology, 16: 22-34.
- CHEN, Y. C., J. D. EISNER, M. M. KATTAR, S. L. RASSOULIAN-BAKRETT, K. LAFFE, S. L. YARFITZ, A. P. LIMAYE, AND B. T. COOKSON. 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J. Clin. Microbiol.* 38:2302-2310.
- CHEN, Y. C., J. D. EISNER, M. M. KATTAR, S. L. RASSOULIAN-BARRETT, K. LAFFE, U. EUJI, A. P. LIMAYE, AND B. T. COOKSON. 2001. Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. *J. Clin. Microbiol.* 39:4042-4051.
- CIARDO, D. E., G. SCHÄR, E.C.BÖTTGER, M. ALTWEGG AND P. P. BOSSHARD. 2006. Internal Transcribed Spacer Sequencing versus Biochemical Profiling for Identification of Medically Important Yeasts. *Journal of Clinical Microbiology*, 44(1): 77-84.
- COEN, D.M. 1994. The polymerase chain reaction. In Current Protocols in Molecular Biology. Vol. 1. AUSUBEL F.M., R. BRENT, R.E. KINGSTON, D.D. MOORE, J.D. SEIDMAN, J.A. SMITH AND K. STRUHL (eds.). USA: John Wiley and Sons, Inc.
- COLLINS T., C. GERDAY AND G. FELLER. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews*. 29(1): 3-23.
- CURRAH, R.S. AND A. TSUNEDA. 1993. Vegetative and reproductive morphology of *Phialocephala fortinii* (Hyphomycetes, *Mycelium radicum atrovirens*) in culture. *Transaction of Mycological Society of Japan* 34: 345-356.
- DESJARDIN, D.E. 1990. Culture morphology of Marasmius species. *Sydowia* 42:17-87.
- DJAMEL, C., T. ALI AND C. NELLY. 2009. Acid Protease Production by Isolated Species of *Penicillium*. *European Journal of Scientific Research*. 25(3): 469-477.
- DRUMMOND AJ, ASHTON B, BUXTON S, CHEUNG M, COOPER A, DURAN C, FIELD M, HELED J, KEARSE M, MARKOWITZ S, MOIR R, STONES-HAVAS S, STURROCK S, THIERER T, WILSON A. 2011. Geneious v5.4, Available from <http://www.geneious.com/>
- EELES, R. A. and A. C. STAMPS. 1994. Polymerase Chain Reaction (PCR) - The Techniques and Its Application. RG Landes Company

- FELL, J. W., T. BOEKHOUT, A. FONSECA, G. SCORZETTI, AND A. STATZELL TALLMAN. 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int. J. Syst. Evol. Microbiol.* 50:1351-1371.
- FELSENSTEIN J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- FISHER, P.J., O. PETRINI, L.E. PETRINI AND SUTTON, B.C. 1994. Fungal endophytes from the leaves and twigs of *Quercus ilex* L. from England, Majorca and Switzerland. *New Phytologist*, 127: 133-137.
- FLORES, M.B. 2010. Comparative study on diversity of fungi from mine deposit sites in Marinduque Island and their utilization as potential biological control agent against *Rhizoctonia solani* Kühn. Undergraduate Thesis. University of the Philippines Los Baños: Philippines.
- FREDERICKS D.N., C. SMITH AND A. MEIER. 2005. Comparison of Six DNA Extraction Methods for Recovery of Fungal DNA as Assessed by Quantitative PCR. *Journal of Clinical Microbiology*. 43(1): 5122-5128.
- GARDES M. AND T.D. BRUNS. 1991. Rapid characterization of ectomycorrhizae using RFLP pattern of their PCR amplified-ITS. *Mycological Society Newsletter*, 41: 14.
- GODFREY, T. & WEST, S. 1996. Industrial enzymology. McMillan Publishers Inc. New York, USA.
- GRISHAM, C.M. AND R.H. GARRETT. 1999. *Biochemistry*. Philadelphia: Saunders College Pub. pp. 426-7.
- GUO, L. D., HYDE, K. D. & LIEW, E. C. Y. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytologist* 147: 617-630.
- GUO, L.D. 2010. Molecular Diversity and Identification of Endophytic Fungi. In: Gherbawy, Y and K. Voigt (eds) Molecular Identification of Fungi. Springer-Verlag, Berlin, Heidelberg, pp 277-296.
- GUO, L.D., K.D. HYDE AND E.C.Y. LIEW. 1998. A method to promote sporulation in palm endophytic fungi. *Fungal Divers* 1:109-113.
- HALTRICH D, NIDETZKY B, KULBE KD, STEINER W, ZUPANCIC S (1996). Production of fungal xylanases. *Biores. Technol.* 58: 137-161.

HASHIMOTO, H., KANEKO, Y., IWAASA, T. & YOKOTSUKA, T. 1973. Production and purification of acid protease from the thermophilic fungus, *Penicillium duponti* K1014. *Appl. Microbiol.* 25:584-588.

HAWKSWORTH, D.L., B.C. SUTTON AND G.C. AINSWORTH. 1983. Ainsworth and Bisby's Dictionary of Fungi. CMI, Kew, UK.

HENRY T, IWEN PC, HINRICHSH SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J Clin Microb* 38(4):1510–1515

HIBBETT, D.S. *et al.*, 2007. A higher-level phylogenetic classification of the Fungi. *Mycological Research*. 111 (5): 509-547.

HO, Y. AND D. BARR. 1995) Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from malaysian. *Mycologia* 87:655–677

HOWARD, C.M., CONWAY, K.E. & ALBREGTS, E.E. 1977. A stem rot beam seedlings caused by a sterile fungus in Florida. *Phytopathology*. 67:430-433.

KIRK P.M., P.F. CANNON, J.C. DAVID AND J.A. STALPERS (eds), 2001. Ainsworth & Bisby's Dictionary of the Fungi, 9th edn, CABI Publishing, Wallingford.

LACAP, D.C., K.D. HYDE AND E.C.Y. LIEW. 2003. An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. *Fungal Divers* 12:53–66.

LARKIN MA, BLACKSHIELDS G, BROWN NP, CHENNA R, MCGETTIGAN PA, MCWILLIAM H, VALENTIN F, WALLACE IM, WILM A, LOPEZ R, THOMPSON JD, GIBSON TJ, HIGGINS DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947-2948.

NAKAMURA S, WAKABAYASHI K, NAKAI R, AONO R, HORIKOSHI K. 1993. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. Strain 41 M-1. *App. Environ. Microbiol.* 59(7): 2311-2316

NICOLOTTI, G., P. GONTHIER AND F. GUGLIELMO. 2010. Advances in Detection and Identification of Wood Rotting Fungi in Timber and Standing Trees. In: Gherbawy, Y and K. Voigt (eds) Molecular Identification of Fungi. Springer-Verlag, Berlin, Heidelberg, pp 251 - 276.

PHADATARE, S.U., DESHPANDE, V.V. & SRINIVASAN, M.C. (1993). High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20), Enzyme production and compatibility with commercial detergents. *Enzyme Microb. Technol.* 15:72-76.

PITKÄRANTA, M., T. MEKLIN, A. HYVÄRINEN, L. PAULIN, P. AUVINEN, A. NEVALAINEN AND H. RINTALA. 2008. Analysis of Fungal Flora in Indoor

Dust by Ribosomal DNA Sequence Analysis, Quantitative PCR, and Culture. Applied and Environmental Microbiology. 74(1): 233-244.

POLGAR, L. 1989. Mechanisms of Protease Action. CRC Press. Inc. Boca Raton, Florida. Pp: 43-76.

RAO, M.B., A.M. TANKSALE, M.S. GHATGE AND V.V. DESHPANDE. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.*, 62: 597-635.

ROWLAND, C.Y., D.I. KURTBOKE, M. SHANKAR AND K. SIVASITHAMPARAM. 1994. Nutritional and biological activities of a sterile red fungus which promotes plant growth and suppresses take-all. *Mycological Research* 98(12): 1453-1457.

SALLES BC, MEDEIROS RG, BAO SN, SILVA JR. FG, FILHO EXF (2005). Effect of cellulase-free xylanases from *Acrophialophora nainiana* and *Humicola grisea* var. *thermoidea* on eucalyptus kraft pulp. *Process Biochem.* 40: 343 – 349.

SANCHEZ-BALLESTEROS J, GONZALEZ V, SALAZAR O, ACERO J, PORTAL MA, JULIAN M, RUBIO V, BILLS GF, POLISHOOK JD, PLATAS G, MOCHALES S, PELAEZ F (2000) Phylogenetic study of Hypoxylon and related genera based on ribosomal ITS sequences. *Mycologia* 92(5):964-977.

SANDHU GS, KLINE BC, STOCKMAN L AND G.D. ROBERTS. 1995. Molecular probes for diagnosis of fungal infections. *J Clin Microbiol* 33: 2913-9.

SCHMIDT O, MORETH U. 2002. Data bank of rDNA-ITS sequences from building-rot fungi for their identification. *Wood Sci Technol.* 2002;36:429-433

SCHNEIDER JHM, SALAZAR O, RUBIO V, KEIJER J (1997) Identification of *Rhizoctonia solani* associated with field grown tulips using ITS rDNA polymorphism and pectic zymograms. *Eur J Plant Pathol* 103:607-22.

SETTE, L.D., M.R.Z. PASSARINI, C. DELARMELINA, F. SALATI AND M.C.T. DUARTE. 2006. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. *World Journal of Microbiology and Biotechnology* 22: 1185-1195.

SHINOHARA, M.I., K.F. LOBUGLIO AND S.O. ROGERS. 1999. Comparison of ribosomal DNA ITS regions among geographic isolates of *Cenococcum geophilum*. *Curr Genet* 35: 527-535.

SHIVANNA, M.B., M. S. MEERA, K. KAGEYAMA AND M. HYAKUMACHI. 1995. Influence of zoysiagrass rhizosphere fungal isolates on growth and yield of soybean plants. *Mycoscience*. 36 (1): 25-30.

SIMPSON, H.D., U.R. HAUFLER and R.M. DANIEL. 1991. An extremely thermostable xylanase from the thermophilic eubacterium Thermotoga. Biochem. J. 277: 413-417

STALPERS, J.A. 1978. Identification of wood-ingabiting Aphylophorales in pure culture. Studies of Mycology 16:1-248.

STRACHAN, D.P., B. FLANNIGAN, E. MCCABE AND F. MCGARRY. 1990. Quantification of airborne moulds in the homes of children with and without wheeze. Thorax 45:382-387.

SWEETINGHAM, M.W., R.H. CRUIKSHANK AND D.H. WONG. (1986). Pectic zymograms and taxonomy and pathogenecity of the *Ceratobasidiaceae*. Transactions of British Mycological Society. 86: 305-311.

TAMURA K, PETERSON D, PETERSON N, STECHER G, NEI M, AND KUMAR S. 2011. MEGAS: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution (In Press).

VILGALYS, R. 2003. Taxonomic misidentification in public DNA database. New Phytol 160:4-5

VINNERE, O. 2004. Approaches to Species Delineation in Anamorphic (mitosporic) Fungi: A Study on Two Extreme Cases. Acta Universitatis Upsaliensis. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 917. 72 pp. Uppsala.

VINNERE, O., J. FATEHI AND B. GERHARDSON. The fungal group Mycelia sterilia—its interactions with crop plants and their pathogens. From: <http://www-maaf.slu.se/crop/lasmer6.htm>. Date accessed: December 8, 2010.

WADDINGTON, M. 2009. Identification of Fungi Using Ribosomal Internal Transcribed Space DNA Sequences. Retrieved June 15, 2010, from Pharmaceutical Technology:<http://pharmtech.findpharma.com/pharmtech/article/articleDetail.jsp?id=595863&sk=&date=&pageID=3>.

WHITE TJ, BRUNS T, LEE S, TAYLOR J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a Guide to Methods and Applications, 315-322. Academic Press, New York.

WOESE, C.R. AND G.J. OLSEN. 1986. Archaeabacterial phylogeny: perspectives on the kingdoms. Syst. Appl. Microbiol. 7: 161-177.

YANG, H.A., J. ZHOU, K. SIVASITHAMPARAM, I.C. TOMMERUP, J.E. BARTON AND P.A. O'BRIEN. 1994. Genetic variability in pectic enzymes of *Rhizoctonia solani* isolates causing bare-patch disease of cereals. Journal of Phytopathology 141:259-266.