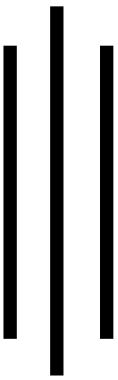
EXPLORATION OF SOIL FUNGAL DIVERSITY OF MANASLU CONSERVATION AREA (MCA), NEPAL AND BIOTECHNOLOGICAL AND MOLECULAR CHARACTERIZATION OF SELECTED SPECIES

M.Sc. Thesis
2013



For the partial fulfillment of M.Sc. degree

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Sunita Khanal
Exam Roll No.: BT 050-067
T.U. Regd. No.: 5-2-553-19-2006

EXPLORATION OF SOIL FUNGAL DIVERSITY OF MANASLU CONSERVATION AREA (MCA), NEPAL AND BIOTECHNOLOGICAL AND MOLECULAR CHARACTERIZATION OF SELECTED SPECIES

M.Sc. Thesis
2013

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Sunita Khanal
Exam Roll No.: BT 050-067
T.U. Regd. No.: 5-2-553-19-2006

Supervisors

Dr. Rajani Malla
Head of Department
Central Department of Biotechnology
Tribhuvan University (T.U.)
Kirtipur, Kathmandu, Nepal.

Dr. Sangita Shrestha
Unit Chief & Chief Scientific Officer
Molecular Biotechnology Unit
Nepal Academy of Science & Technology (NAST)
Khumaltar, Lalitpur, Nepal.

Acknowledgements

First and foremost, I would like to express my sincere thanks and heartfelt gratitude to my supervisors, Dr. Rajani Malla, Head, Central Department of Biotechnology, Tribhuvan University (TU), Kirtipur and Dr. Sangita Shrestha, Unit Chief and Chief Scientific officer, Molecular Biotechnology Unit, Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur for their continued support and guidance, critical comments and valuable suggestions which have nurtured me to develop research skills and learn how to work independently in laboratory. They were always available to help me from the very beginning to the end of my research.

I am many grateful to Prof. Dr. Surendra Raj Kafle, Vice-Chancellor of NAST, Prof. Dr. Prakash Chandra Adhikari, Secretary of NAST and Mr. Iswor Prasad Khanal, Chief of science faculty, NAST for allowing me to carry out my research work at Molecular Biotechnology Laboratory, NAST. My sincere thanks also go to Dr. Jyoti Maharjan, Mrs. Neesha Rana and Mrs. Jaishree Sijapati, Senior Scientific Officers of Molecular Biotechnology Unit, NAST for their help and valuable suggestions. I would like to thank Dr. Anjana Giri, Unit Chief and Senior Scientific Officer, Ms. Prabina Rana, Scientific Officer of Biological Resources Unit, NAST for allowing me to carry out a part of my work in Biological Resources Laboratory, NAST. I would also like to acknowledge NAST lab members: Ranjan Koirala, Poonam Yadav, Smita Shrestha, Jagat Krishna Chippi Shrestha, Om Basukala, Nabin Narayan Munankarmi, Gaurav Chandra Gyanwali, Surendra Neupane, Gorakh Raj Giri and Rajesh Lamichhane for their practical assistance and creating lab environment productive.

I would also like to thank University Grant Commission (UGC), Sanothimi, Bhaktapur for awarding Masters thesis support to carry out this research. It is my pleasure to thank all the faculties of Central Department of Biotechnology, Prof. Dr. Tribikram Bhattarai, Prof. Dr. Krishna Das Manandhar, Prof. Dr. Mohan Kharel, Prof. Dr. Ganga Prasad Kharel, Prof. Dr. Sreerama Lakshmaiah, Dr. Sampoornanand Jha, Ms. Jarina Joshi, Mr. Bal Hari Poudel and Ms. Smita Shrestha for their valuable suggestions throughout my research. I am also indebted to my friends Usha, Jyoti, Sunita, Prabin, Prashanna, Santosh, Rajiv, Kushal Shrestha, Pratap, Janardan, Nawneet, Rejeena, Meena, Surendra, Suman, Prakash and Pradeep and other seniors, juniors and staffs of Central Department of Biotechnology for their constant support and encouragement to carry out this research.

It is always my great pleasure to acknowledge my parents Suresh Pd. Khanal and Sudha Khanal and my beloved sister Sujita Khanal for their unconditional love, tremendous moral support and constant encouragement. Last but not the least, I would like to thank GOD for listening to my prayers and giving me strength to carry on in any situation.

Glossary Acronyms

AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
asl	above sea level
bp	base pair
CMC	Carboxy Methyl Cellulose
cpDNA	chloroplast Deoxy-ribose Nucleic Acid
CTAB	Cetyl trimethyl ammonium bromide
D1/D2	Divergence region of Large Subunit nuclear ribosomal DNA
DAF	DNA Amplification Fingerprinting
DDW	Double Distilled Water
DNA	Deoxy-ribose Nucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium Bromide
ETS	External Transcribed Spacer
HGT	Horizontal Gene Transfer
IGS	Inter Genic Spacer
ITS	Internal Transcribed Spacer
LCB	Lactophenol Cotton Blue
LSU	Large Sub-unit
MCA	Manaslu Conservation Area
mDNA	mitochondrial Deoxy-ribose Nucleic Acid
ME	Minimum Evolution
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MP	Maximum Parsimony
MSAs	Multiple Sequence Alignments
mV	milli-Volts
NA	Nucleic Acid

NCBI	National Center for Biotechnology Information
nDNA	nuclear Deoxy-ribose Nucleic Acid
NJ	Neighbor Joining
nrDNA	nuclear ribosomal Deoxy-ribose Nucleic Acid
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RAPD	Random Amplified Polymorphic DNA
rDNA	ribosomal Deoxy-ribose Nucleic Acid
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribose Nucleic Acid
rRNA	ribosomal Ribose Nucleic Acid
SCARs	Sequence Characterized Amplified Regions
SDS	Sodium Dodecyl Sulphate
SDW	Sterile Distilled Water
SSCP	Single-Strand Conformational Polymorphism
ssNA	single strand Nucleic Acid
SSRs	Simple Sequence Repeats
SSU	Small Sub-unit
STRs	Short Tandem Repeats
TAE	Tris-Acetate-EDTA
TES	Tris-EDTA-SDS
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Contents

List of Tables

List of Figures

List of Plates

ABSTRACT

Fungi are achlorophyllous chemoheterotrophic eukaryotic organisms. Within their varied natural habitats fungi usually are the primary decomposer organisms and can be used for bioremediation. Fungal species are studied as model organisms to gain knowledge of basic processes such as genetics, physiology, biochemistry, and molecular biology. Some of the fungi are most important plant pathogens, parasites of animals and disease causing agents in Humans. Despite this, fungi can be used to produce human antibiotics, act as source of protein in human and animal food and are used to prepare biopesticides, bioherbicides and bioferitizers. Besides, fungi are widely used for production of biotechnologically important extracellular enzymes which can be applied in several industries such as paper/pulp industry, textile industry, food industry, baking and brewery industry, etc.

The present study was designed to explore soil fungal diversity of Manaslu Conservation Area (MCA) starting from 1700 m to 4300 m asl. Fungi were also isolated from rhizospheric soils of some selected medicinal plants. Three main activities were undertaken. Firstly, fungi were isolated and identified up to genus level using standard morphological techniques. Secondly, dominant as well as industrially and medically important fungal isolates were selected to screen for the production of six different biotechnologically important extracellular enzymes *viz.,* amylase, cellulase, pectinase, protease, xylanase and lipase. Thirdly, the selected species were characterized by PCR and DNA sequencing-based molecular techniques by using nuclear ribosomal DNA (nrDNA) Internal Transcribed Spacer (ITS) region and D1/D2 domains of large subunit (LSU) (28S) ribosomal DNA as DNA barcodes and study their phylogenetic relationships.

A total of fifty-nine isolates were obtained from soil samples of MCA region. Out of these, 23 fungal species were from soil samples of five different altitudinal variations and 36 fungi were from seven different rhizospheric soils. On the morphological identification of fungal isolates, *Penicillium* spp. were most dominant in both sets of soil samples. It was followed by *Aspergillus, Mucor* and *Nigrospora* spp. Besides, *Penicillium* spp. and *Aspergillus* spp. were found to be distributed in each of the altitudes examined.

A total of eleven isolates including six *Penicillium* spp. and five *Aspergillus* spp. were selected and were tested for extracellular enzymes. The present study revealed that all the isolates considered for enzyme study, except *Penicillium ruqulosum* (31c), have shown extracellular enzyme activity with maximum isolates showing positive to cellulase (~64%) and Protease (55%). *Penicillium aurantiogriseum* (A277) showed positive reaction to all six extracellular enzymes screened, followed by *Penicillium ochrochloron* (A12) with positive for five enzymes tested.

For the molecular species identification and phylogenetic study, nrDNA ITS regions and LSU D1/D2 domains were amplified using their respective Universal Primers. The successful amplified products were sequenced at Macrogen Company, South Korea. Following retrieval of double stranded sequencing, the sequences were edited, aligned and analyzed using Codon Code Aligner *v.4.2.2*. Since the sequence success rate of the ITS regions was very low which wasn't able to be analyzed further, the following analyses were done on the basis of D1/D2 domains. The D1/D2 sequences obtained for these regions facilitated the identification of fungi at species level by comparing the nucleotide sequences with GenBank database using bioinformatic tools. The Blastn search of the D1/D2 sequences of nine species under study were identical to *Penicillium ochrochloron, Penicillium aurantiogriseum* strain DAOM 214787, *Penicillium aurantiogriseum* strain CBS 324.89, *Penicillium ruqulosum, Penicillium chrysogenum, Penicillium simplicissimum, Aspergillus niger Aspergillus parasticus* and *Aspergillus protuberus.* The sequences were then analyzed using MEGA *v.5.2.2.* The smallest difference of 8 nucleotides in D1/D2 regions was found between *Penicillium simplicissimum* and *Penicillium ochrochloron* and the largest difference, 299 nucleotides, between *Aspergillus protuberus* and *Penicillium chrysogenum*. This data suggested that the D1/D2 domain is sufficiently variable for the identification of filamentous fungi and relevant species. The Maximum Parsimony (MP) tree was obtained from the D1/D2 sequence data using the Subtree-Pruning-Regrafting (SPR) algorithm with most parsimonious tree length of 490 and parsimony-informative sites of 301. The consistency index and the retention index value were 0.906122 and 0.936022 respectively. The constructed tree revealed that *Penicillium* spp. and *Aspergillus* spp. formed separate clusters. But, the two species, *Aspergillus parasticus* (A13) and *Penicillium aurantiogriseum* (A277) formed a single clade. However, *Penicillium aurantiogriseum* (A16) showed the distant relationship with the other species. The phylogenetic analyses were also done using some reference species and the results were congruent. Thus, the analysis of the D1/D2 domain of the LSU rDNA of *Penicillium* and *Aspergillus* species showed distinguished intergenus and interspecies sequence differences.

Hence, the results obtained from present research have the potential to enhance our understanding of fungal communities of MCA and their functional roles in soil ecological processes. Furthermore, this study has highlighted the potentiality of executing future biotechnological project on each of these individual species.

Keywords: Soil fungi, Manaslu Conservation Area (MCA), Internal Transcribed Spacer (ITS), D1/D2 domain, Extracellular enzymes, *Penicillium* and *Aspergillus* species.

CHAPTER 1
INTRODUCTION

1.1 Background

Manaslu Conservation Area (MCA), declared as the second conservation area of Nepal on December 28, 1998 is under NTNC (National Trust for Nature Conservation) management. Manaslu, a mountainous region in northern part of Gorkha District (Figure 1.1), has a fragile but diverse natural resource base and a rich cultural environment. MCA encompasses a 1,663 sq. km. area with seven Village Development Committees (VDCs) and eleven forest types are found in this area (NTNC, 2013). There are about 9,000 inhabitants living in MCA and 2,000 species of plants, 33 mammals, 110 birds, 3 reptiles and 11 butterflies have been reported from the area. So far, total numbers of microorganisms (fungi, bacteria and virus) found within MCA regions have not been reported. The altitude rises from a mere 600m to the summit of Mt. Manaslu (8,163), the eighth highest peak in the world. The climatic zone varies from sub-tropical to alpine. The temperatures in the area also vary widely with the climatic zone: in the subtropical zone, the average summer and winter temperatures vary in the range of 31–34 °C and 8–13 °C respectively; in the temperate climatic zone, the summer temperatures are between 22–25 °C and winter temperatures are between -2 to 6 °C when snow and frost are also experienced. The alpine zone, above 5,000 meters (16,000 ft) elevation, is distinct and falls within the permanent snow line, where the temperatures lie much below freezing zone (NTNC, 2001).

MCA is one of the most suitable areas for the search of microorganisms adapted to low temperatures. The organisms living here (psychrophiles and psychrotolerants) present adaptations in their enzymatic systems, in their membranes and therefore in their genes that represent a great biotechnological potential. Over the last few years this region has been investigated mainly for the exploration and exploitation of medicinal plants and very little or no research has been carried out in the field of microbial biotechnology (Bhuju et al., 2007).

Study of biodiversity has an intrinsic value focusing on the conservation of all species. A gram of fertile agricultural soil may contain 2.5 billion bacteria, 400,000 fungi, 50,000 algae and 30,000 protozoa. All these organisms have particular functions and interact with each other and with their physical environment to create the fertile soil that humans depend on for agricultural production (Trevors, 1998). The diversity helps to understand the role of each organism in the ecosystem. It is well known that soil harbors bacteria, actinomycetes, algae, protozoa, virus and fungi in abundance (Paul, 2007). Even tinier soil microorganisms and fungi are responsible for cycling essential nutrients like nitrogen, phosphorus and sulfur and making them available to higher plants (Quebec Biodiversity Website, 2013). Fungal floras in soils or associated with plant roots have been studied worldwide (Watanabe, 2002). Fungal diversity is an essential part of biological research especially in the context of its ecological and economic implications

(Shenoy et al*.,* 2007). In pathogical aspects, soil fungi have found to be associated to different types of infections or diseases in plants, animals and other microbes as well as have been used in production of antibiotics. Investigation on fungal infections to plants and environmental factors for soil-borne fungal disease occurrence (Watanabe, 2002), furnish information on aspects of the disease process that can be advantageous for developing improved disease control methods in plants (Bioinformatica, 2013). Besides, soil fungi are also the cause of wide varieties of diseases in human. Aspergillosis caused by *Aspergillus* spp and Histoplasmosis by *Histoplasma capsulatum* are few example of systemic fungal infection in Human (Georgopapadakou and Walsh, 1994; Doctor fungus, 2013). In addition, fungi are responsible for food spoilage and the production of toxic secondary metabolites (May, 1997). Aflatoxins and Ochratoxin A (OTA), mainly produced by species belonging to *Aspergillus* are the one of the causes for food poisoning (JECFA 2001). However, an antibiotic Penicillin is the best example of production of useful drugs from fungi. Commercially produced fungal biopesticides from *Trichoderma* spp. (Agropedia, 2010); various species of *Aspergillus* and *Fusarium* as bioremediation agents for various pollutants (Satyanarayana, 2009); microfungi (filamentous fungi e.g., *Fusarium venenatum*) as a source of mycoprotein and macrofungi (mushrooms e.g., *Agaricus, Ganoderma, Pleurotus, Auricularia, Boletus*) as edible fungi, are the major economic applications of fungi from soil and other sources. The study of soil fungal floras also involves role of fungi in soil biomass and ecology. Various fungal species such as *Mucor, Rhizopus, Aspergillus, Rhizoctonia, Cladosporium,* etc are known to secrete gums and provide mechanical support by aggregation of soil particles (Beare et al., 1997) and enhance nutrient acquisition, water relations, pH tolerance and disease and pest resistance in higher plants (Douds et al., 2000).

In addition, fungi are very useful organisms in biotechnology (Quinn, 2005; Seidl, 2006). Fungi are well known for their ability to produce various potential enzymes. Many enzymes produced by fungi have found their application in industrial sector. These enzymes have found advantages over chemically processed compounds that generate various toxic chemicals and some undesirable products that are difficult to eliminate. Over the past 50 years, fungi have been used in the production of industrial enzymes (Dalboge, 1997). Different enzymes like amylase, pectinases, proteases, cellulases, xylanases, lipases, and recently cutinases have been reported to be produced by fungi. These extracellular enzymes are applied in the various industries producing detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products (Prabakaran et al*.,* 2009; Thota et al., 2012). Amylases industrially produced from *Aspergillus* have applications in food, detergents, drinks, animal feed and baking. Pectinases produced and characterized from species of *Aspergillus, Penicillium, Fusarium* and *Rhizopus* are used in the preparation of wine and fruit juices (Rangarajan

et al*.,* 2010). *Aspergillus* spp. and *Trichoderma* spp. produced xylanases that have potential application in food, feed, paper, pulp and textile industries (Guimarães et al*.,* 2006). Lipases are enzymes that catalyze a variety of reactions such as hydrolysis, transesterification, and ester synthesis. Industrial applications of lipases from *Rhizopus* spp. is in the detergent, food, flavour industry, biocatalytic resolution of pharmaceuticals (Prabakaran et al*.,* 2009), esters and amino acid derivatives, making of fine chemicals, agrochemicals, use as biosensor, bioremediation and cosmetics and perfumery (Hama et al. 2006).

Fungi grow in diverse habitats in nature and are cosmopolitan in distribution. In laboratory, they can be isolated using various culture media for cultivation, preservation, microscopic examination for identification and biochemical, physiological and molecular characterization. Different concepts have been used by the mycologists to characterize the fungal species, of which morphological observation of reproductive stages is the classic approaches and generate baseline information of fungal species identification and nomenclature (Sharma and Pandey, 2010). Nowadays, fungal taxonomy is in a state of rapid flux, because of the recent researches based on molecular approaches (Sharma and Pandey, 2010).

Species identification is the fundamental task to proceed for other advanced research in molecular biology and biotechnology. However, taxonomic identification of micro fungi to the species level based on microscopic characters and standard cultural methods can be extremely difficult and may result in a species list that misrepresents the fungal community and prone to error. Identification by traditional methods may not even be possible for isolates that fail to sporulate (Watrud et al., 2006). The phenotypic characteristics are strain specific and inadequate for recognition of either species or genera (Kurtzman and Robnett, 1995). Furthermore, the combined availability of Polymerase Chain Reaction (PCR) and DNA sequencing methods with sequence data from a large number of organisms have facilitated the application of these technologies for microbial identification (Weber et al*.,* 2006).

For fungi, several target regions of the rRNA genes are currently being used for identification, such as the Internal Transcribed Spacer (ITS) between genes encoding the 18S and the 28S rRNA, or the D1/D2 region of the Large Subunit (LSU) of the 28S rRNA gene. Several studies have shown that sequencing of the D1/D2 region has great potential for the identification of several of the filamentous fungi (Hall et al*.,* 2004). The ITS region is a multi-copy, transcribed but non-coding region of the ribosomal DNA which can be easily amplified by PCR. Both ITS and D1/D2 are approximately 600 to 800 base pair (bp) long. These regions are chosen as target sequences because both exist in high copy number and amplification primers for both discriminate between the

mycological species. These features provided a sensitivity and specificity sufficient for the detection and identification of fungi (Kwiatkowski et al., 2012).

The biotechnology related researches like exploration of the microbial diversity in soil ecology, molecular genetics characterization, and the biotechnological study on microbial enzymes have very high scopes in Nepal. Therefore, an attempt has been made to undertake some kind of research in this area. Thus, the present study was carried out to explore soil fungal diversity harbored at different altitudinal gradients and rhizospheric environment of Manaslu Conservation Area, to screen the biotechnologically important fungal enzymes produced by different selected species and molecular characterization of selected isolated species.

1.2 Objectives

1.2.1 General Objective

The overall objective of this study is to explore the fungal diversity of Manaslu Conservation Area (MCA) and to select prospective species for biotechnological and molecular characterization.

1.2.2 Specific Objectives

The specific objectives of the study are:

1. To isolate soil fungi harbored at different altitudes and from some rhizospheric environments of some medicinal plants of MCA.
2. To identify the isolates based on morphology and select commercially and industrially important species.
3. To conduct enzyme screening of selected species.
4. To characterize many of them at molecular level using PCR and DNA sequencing based techniques.

1.3 Rationale and Scope of the study

Over the last few years this region has been investigated mainly for the presence and exploitation of medicinal plants and very little or no research has been carried out in the field of biotechnology (Bhuju et al., 2007).

Fungi fulfill a range of important ecological functions, yet current understanding of fungal biodiversity in Nepalese soil is limited. Direct DNA extraction from soil, coupled with polymerase chain reaction amplification and community profiling techniques, has proven successful in investigations of bacterial ecology; however, it still needs to be applied for elucidating the taxonomic and functional characteristics of soil fungal communities (Anderson et al., 2004).

Microorganisms perform their myriads of biochemical reactions under ambient conditions with little or no toxic by-products. Considering the present status and future prospects on the use of microbial by-products as effective bio-reagents in various areas, isolation, identification and molecular characterization of the fungi are parts of fundamental research (Guimarães et al*.,* 2006).

Thus, the present research has been designed to explore the diversity of fungi in soil samples of MCA starting from the altitudes of 1700 m and reaching up to 4300 m asl. The fungal isolates are first identified by morphological characteristics and the biotechnologically potent fungi are further used to screen for the production of extracellular enzymes. These isolates are then characterized by using molecular techniques such as PCR and DNA sequencing.

The scope of this study includes the exploration of the fungal diversity in soil samples of various alttitudinal gradients of MCA representing the diversity in Central Nepal. This study will reveal industrially important fungal species found in the soil of MCA. Furthermore, this study will generate DNA sequences specific to some fungal diversity of MCA that will be baseline database for future molecular study in fungi. Additionally, this research will also open research avenues for the production of the fungal by-products and their application in different industries.

CHAPTER 2
LITERATURE REVIEW

2.1 Fungi

The fungi (singular: fungus) are members of a large group of eukaryotic organisms and classified under the kingdom, Fungi (Pelczar et al., 2003; Madigan and Martinko, 2006). The Kingdom Fungi encompasses an enormous numbers of morphological diversity ranging from single-celled aquatic chytrids to large macroscopic mushrooms (Pelczar et al., 2003; Lian, 2005). One major difference of fungal cells to that of plants is former have cell walls that contain chitin, in contrast to those of plants which are mostly made up of cellulose. The storage carbohydrate of fungi is glycogen while starch is the storage molecule in case of plants (Alexopoulos and Mims, 1985; Lian, 2005).

Fungi are achlorophyllous thallophytes (Hawksworth, 2006). Fungi are heterotrophs, meaning that they can't manufacture their food by themselves and obtain their nutrients by absorption. Saprophytic fungi secrete enzymes to break down dead organic matter in recycling. Many other fungi are parasitic and obtain their nutrients from living hosts (Bilgrami and Verma, 1978; Alexopoulos and Mims, 1985; Pelczar et al., 2003).

Classically, there are two broad groups of fungi: yeasts and moulds. Mould spores germinate to produce the branching filaments known as hyphae. They reproduce by means of spores. Both sexual (meiotic) and asexual (mitotic) spores may be produced, depending on the species and conditions (Lian, 2005). Yeasts, on the other hand, are solitary rounded forms that reproduce by making more rounded forms through such mechanisms as budding or fission (Lian, 2005; Pelczar et al., 2003).

First attempt to obtain laboratory cultures of fungi was made by the great Italian botanist, Micheli (1679-1737). He could succeed in growing three dominant mould, *viz., Mucor, Aspergillus* and *Botrytis*, on freshly cut surfaces of melon, quince and pear. Many of others followed Micheli's lead and obtained different kinds media which are cheap, easier to make and support a wide variety of fungi (Bilgrami and Verma, 1978). Now-a-days, varieties of commercial media for the growth of fungi are found such as Potato Dextrose Agar (PDA), Sabouraud's Dextrose Agar (SDA), Czapek-Dox Agar, Emmon's culture Medium, Raulin's Medium.

Fungi are abundant in soil. They generally make up 10–20% of the total microorganisms in the soil rhizosphere (Hoorman, 2011). Fungi tend to dominate over bacteria and actinomycetes in acid soils as they can tolerate a wide pH range. Fungi can survive in the soil for long periods even through periods of water deficit by living in dead plant roots and/or as spores or fragments of hyphae (Jenkins, 2005). Soil fungi carry out three main functions in the ecosystem: (i) as saprotrophs that digest and dissolve litter and detritus; (ii) as predators and parasites of soil organisms; and (iii) as symbionts of plants (as mycorrhizae or lichens) and insects (Adl, 2003).

2.2 Morphology of Fungi

All fungi have typical eukaryotic morphology. The outermost cell wall of most fungi contains chitin, a polymer of glucose derivative, N-acetylglucosamine (Madigan and Martinko, 2006). Inner to the chitinous cell wall, is the bi-layered plasma membrane (Alexopoulos and Mims, 1985; Barbara, 2013). Fungal membranes possess ergosterol in contrast to cholesterol found in mammalian cells. The cytoplasm consists of various organelles such as mitochondria, golgi apparatus, ribosomes, endoplasmic reticulum, lysosomes, microtubules and a membrane enclosed nucleus. However, no vacuoles are found. Nuclear membrane persists throughout the metaphase of mitosis, unique property in fungi, unlike in plant and animal cells where it dissolves and re-forms. The nucleus possesses paired chromosomes. (Adl, 2003; Rao, 2006).

The thallus of fungi is made up of hyphae, which are cylindrical tube like structures, elongating by growth at tips. These hyphae are responsible for the filamentous nature of mould. A mass of hyphae is known as mycelium. The hyphae may be branched or unbrached, septate or aseptate. Hyphae (septate) usually have cross walls, called septa that divide them into numerous cells. All moulds are septate, except zygomycetes (Alexopoulos and Mims, 1985; Rao, 2006). The details of septa can be a class characteristic. For example, in Ascomycetes, there are frequent septa that are perforated by pores which allow passage of cytoplasm and organelles including nuclei. In some classes of Basidiomycetes, the pore is associated with modified endoplasmic reticulum, called dolipore septa (L. *dolium*, a large jar or cask i.e., barrel) (Alexopoulos and Mims, 1985). The dolipore regulates passage of material between the cells; notably, nuclei do not pass through (Adl, 2003). The role of septa is crucial in preventing whole hyphal damage after a hypha is broken or invaded by a predator or parasite. Septal pores are blocked by cell wall deposition, and a new branch initiates a growing tip. Non-septate hyphae are considered to be more primitive because if a hyphal strand is damaged the entire strand dies. (Adl, 2003; Rao, 2006).

Mycelium generally are of three kinds viz., Vegetative mycelium (penetrate the surface of the medium and absorbs nutrients), Aerial mycelium (grow above the agar surface) and Fertile mycelium (aerial hyphae that bear reproductive structures such as conidia or sporangia) (Figure 2.1).

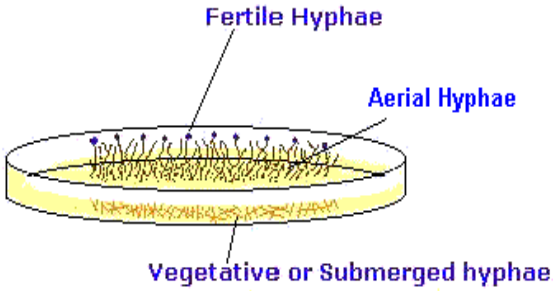


Figure 2.1: Types of mycelium (Source: Rao, 2003)

2.3 Reproduction in Fungi

Fungi reproduce by asexual and sexual means. Asexual reproduction is the commonest mode in most fungi with fungi participating in sexual mode only under certain circumstances. The form of fungus undergoing asexual reproduction is known as anamorph (or imperfect stage or mitosporic fungus) and when the same fungus is undergoing sexual reproduction, the form is said to be teleomorph (or perfect stage). The whole fungus, including both the forms is referred as holomorph (Sigler, 2012).

2.3.1 Asexual reproduction

Asexual propagules are termed as spores or propagules (TutorVista, 2013) depending on their mode of production. Asexual spores are produced following mitosis. Asexual spores arise either by budding off or by differentiation of hyphae. These develop following mitosis of a parent nucleus and are formed in any manner except involving cytoplasmic cleavage. This exogenous process is known as conidiogenesis, a process that occurs both in yeasts and moulds (Rao, 2003).

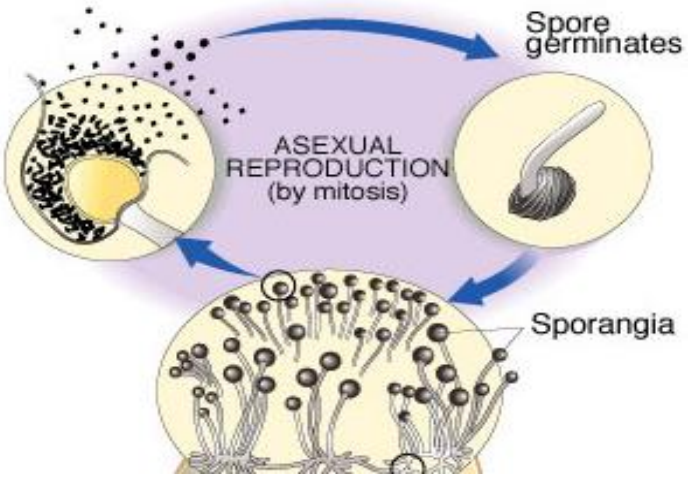


Figure 2.2: Representation of asexual reproduction in fungi (Source: Biology Active Learner, 2013)

The different kinds of asexual spores shown in Figure 2.3, are Zoospores, Sporangiospores, Conidiospores, oidia and chlamydospores (Pelczar et al., 2003;

TutorVista, 2013). Zoospores are flagellated, motile spores produced inside structures called zoosporangia. These spores do not have a cell wall. Such spores are produced in lower fungi such as *Achyla* (water mould) and *Saprolegnia* (fresh water mould). Sporangiospores are single celled spores formed within sacs called sporangium at the end of special hyphae sporangiophores in fungi such as *Rhizopus* and *Mucor* (TutorVista, 2013; WordPress, 2013). Small single-celled conidia (microconidia) and large multicellular conidia (macrconidia) are borne on specialised structures called conidiophore (Rao, 2003). Such spores are produced in fungi like *Aspergillus* and *Penicillium* (TutorVista, 2013). Oidia or arthrospores are single-celled spores formed by disjoining of hyphal cells. They do not store reserve food and hence cannot survive under unfavourable conditions. Such spores are produced in *Rhizopus* (Pelczar et al*.,* 2003; TutorVista, 2013). Chlamydospores are thick walled resting spores which arise directly from vegetative hyphal cells. They store reserve food and highly resistant to adverse conditions (TutorVista, 2013).

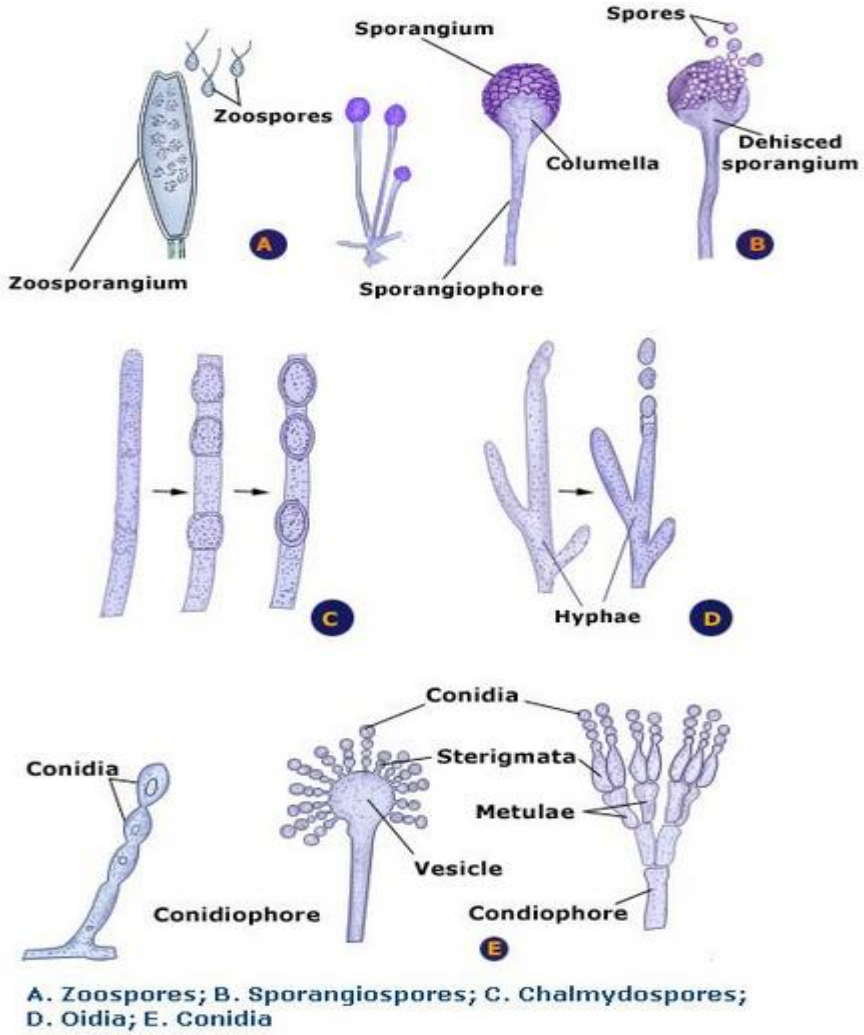


Figure 2.3: Modes of asexual reproduction (Source: TutorVista, 2013)

2.3.2 Sexual reproduction

The sexual lifecycle of fungi is different from all other eukaryotic organisms in that the zygote is the only diploid cell. When two mating types fuse, the two nuclei do not fuse but coexist until the right conditions are present. Fusion results in the diploid zygote, which immediately undergoes meiosis to return to the haploid state. The coexistence of two different mating types of nuclei is the dikaryotic stage, which is unique to fungi (Lian, 2005). The sex organelles of fungi if present are called gametangia. They may form differentiated sex cells. If the male and female gametangia are morphologically different the male gametangium is called antheridium and female gametangium is called ogonium (Pelczar et al., 2003).

Based on the compatibility in sexual reproduction the fungal hyphae can be distinguished into two types homothallic and heterothallic. In homothallic forms, fusion occurs between the genetically similar strains or mating types. In such forms, meiosis results in the formation of genetically identical spores. In the heterothallic forms, fusion occurs between the genetically different mating types or strains. The strains are genetically compatible and are designated as + strain and - strain. In such forms meiosis results in the formation of both the strains, in equal numbers. Heterothallism is a device to prevent inbreeding and promote out breeding (TutorVista, 2013).

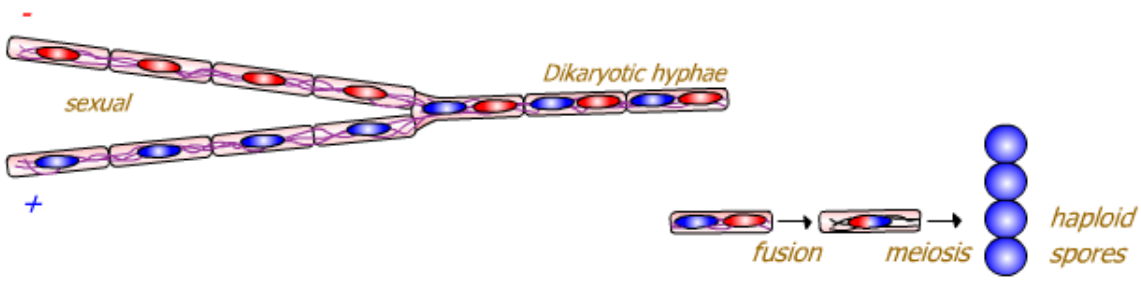


Figure 2.4: Schematic diagram of Sexual cycle in Fungi (Source: Barbara, 2013)

Sexual propagules are produced by the fusion of two nuclei that then generally undergo meiosis. The first step in sexual methods of reproduction involves plasmogamy (cytoplasmic fusion of two cells). The second step is karyogamy (fusion of two compatible nuclei), resulting in production of diploid or zygote nucleus. This is followed by genetic recombination and meiosis. The resulting four haploid spores are said to be sexual spores, e.g. zygospores, ascospores, basidiospores and oospores (Pelczar et al., 2003; Madigan and Martinko, 2006).

Zygospores, which are the sexual spores of zygomycetes are round, thick walled reproductive structures that result from the union of two gamentagia. Ascomycetes produce sexual spores called ascospores in a special sac like cell known as ascus. In basidiomycetes the basidiospores are released from club shaped structure called basidium, which are formed exogenously at the tip of special outgrowth called

sterigmata. Oospores are formed by fertilization of female eggs or oosphores by male gametes and found within the special female structure called oogonium (Pelczar et al., 2003; Rao, 2003).

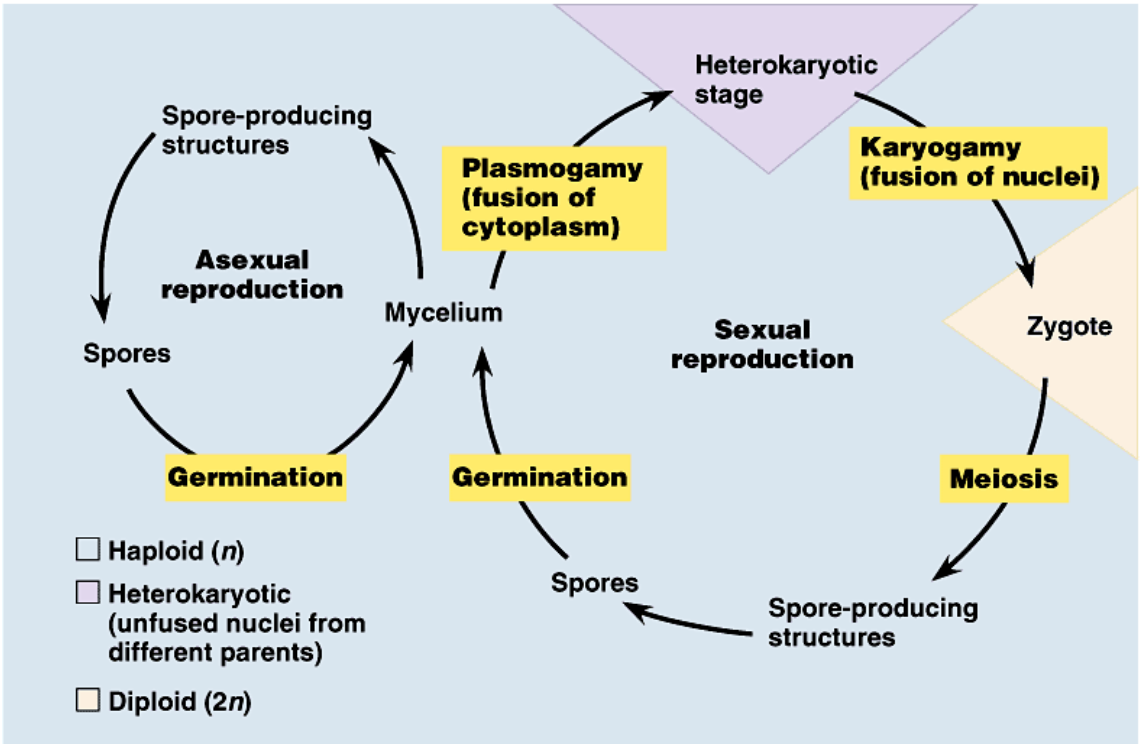


Figure 2.5: Schematic diagram representing life cycle of fungal species (Source: WordPress, 2013)

2.4 Classification of fungi

Taxonomy of the fungi follows the recommendation of the Committee on International Rules of Botanical Nomenclature. Accordingly, the various taxa have endings as follows (Volk, 2000; Pelczar et al., 2003):

Domains:	Eukarya
Divisions/ Phyla:	*-mycota*
Subdivisions:	*-mycotina*
Classes:	*-mycetes*
Subclasses:	*-mycetidae*
Orders:	*-ales*
Families:	*-aceae*

Genera and species have no standard endings (Enclycopaedia Britannica, 2013).

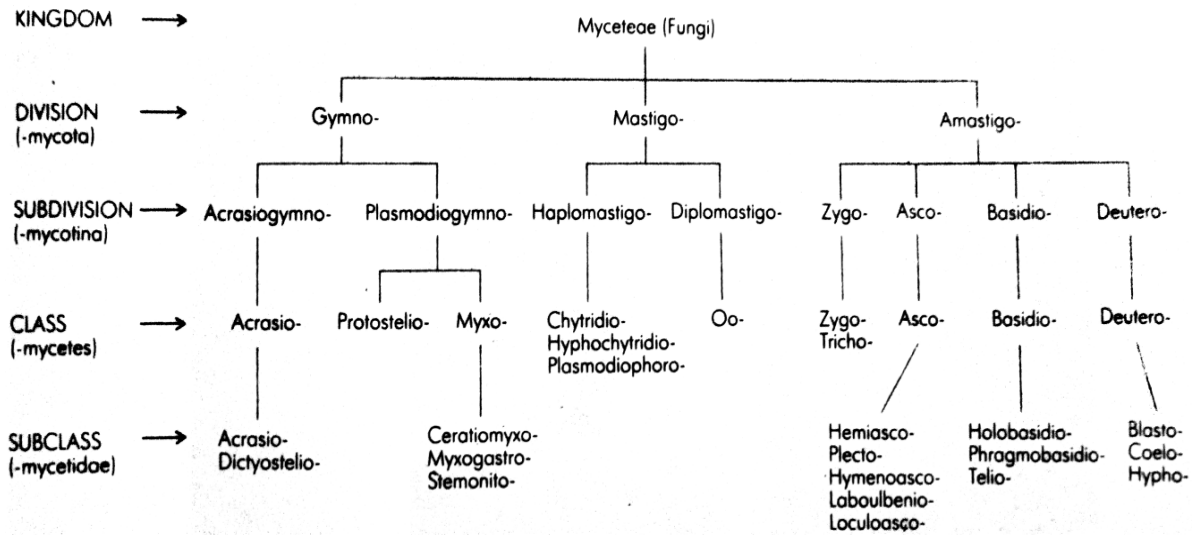


Figure 2.6: Main outline of fungal classification showing principal taxa (Source: Pelczar et al., 2003)

Before in 1973, the Kingdom Fungi (Myceteae) was only divided into Myxomycota (slime moulds) and Eumycota (True moulds). The Mastigomycotina was the Sub-divisions under the Division Eumycota (Aneja, 2008). However, in this review, the classification scheme (Figure 2.6) proposed by Alexopoulos, 1985, the eminent American mycologist is used. The division *Gymnomycota* are slime moulds and *Mastigomycota* are aquatic flagellated lower fungi. The *Amastigomycota* are the terrestrial fungi without flagella (Pelczar et al., 2003). Soil mycoflora as well as the medically important fungi are under the division *Amastigomycota* (Tortora et al., 2010). However, the classification of fungus-like organisms, particularly that of species belonging to the Mycota, is in a state of flux. Gene sequence analyses are resulting in the redefining of some phyla and the reassigning of some genera to different phyla (Fungi Online, 2013).

The classification of terrestrial fungi (*Amastigomycota)* is primarily based on the characteristics of the sexual spores and fruiting bodies present during the sexual stages of their life cycles. However, many of the fungi produce sexual spores and fruiting bodies only under certain environmental conditions. Thus, the complete or perfect life cycles are yet unknown to many fungi. For these imperfectly described fungi, the classification is based on the morphology of their asexual spores and thalli (Pelczar et al., 2003).

Based on sexual reproduction, fungi are classified into four following groups (Gilman, 1975; Alexopoulos and Mims, 1985; Adl, 2003; Pelczar et al., 2003; Rao, 2003):

2.4.1 Zygomycetes

The term zygomycetes refers to the production of a thick-walled sexual resting spore called a zygospore that develops within zygosporangium formed as a result of the complete fusion of two equal or unequal gametangia (Alexopoulos and Mims, 1985). Asexual spores include chlamydoconidia, conidia and sporangiospores. Zygomycetes are

commonly known as bread moulds. These are fast growing, terrestrial and largely saprophytic fungi. Hyphae are coenocytic and mostly aseptate. Genera include *Mucor, Rhizopus, Rhizomucor, Absidia,* etc (Gilman, 1975; Adl, 2003; Rao, 2003). Phycomycetes are an obsolete taxon for certain fungi with nonseptate hyphae. These fungi are currently classified under Zygomycota (Encyclopaedia Britannica, 2013).

2.4.2 Ascomycetes

The one distinguishing character in ascomycetes is the formation of endogenous sexual spores called ascospores, typically eight, in an ascus, a sac-like structure. Other characteristics are septate mycelium with simple septal pores and asexual reproduction via conidia (Alexopoulos and Mims, 1985). They exist as saprophytes and parasite of plants. The genera includes *Trichophyton, Microsporum, Blastomyces, Leptoshaeria, Piedraia,* etc (Rao, 2003).

2.4.3 Basidiomycetes

These species by definition form exospores (meiotic products) called basidiospores by budding of the cell membrane and new cell wall deposition, on a specialized spore-producing structure, the basidium. Basidiospores are generally uninucleate and haploid (Alexopoulos and Mims, 1985). Hyphae of Basidiomycetes are dikaryotic and can often be distinguished by the presence of clamp connections over the septa. The septum is a typical, commonly reffered to as the dolipore septum, characterized by a barrel-shaped swelling in the center of the septal wall surrounding a central pore. Genera includes *Amanita, Lepiota, Coprinus, Agaricus,* etc (Adl, 2003).

2.4.4 Deuteromycetes

Since sexual phase (perfect phase or teleomorph) of these fungi aren't yet identified, they are commonly called imperfect fungi or technically Fungi Imperfecti (University of Exeter, 2013). They have septate hyphae and asexual reproduction is by conidia. This class contains majority of medically important fungi. The genera includes *Candida, Cryptococcus, Rhodotorula, Torulopsis, Aspergillus, Penicillium, Monilia, Neurospora, Botrytis, Verticillium, Trichoderma, Sporotrichum, Geotrichum, Coccidioides, Microsporum or Trichophyton, Alternaria, Drechslera, Curvularia, Phialophora,* etc (Alexopoulos and Mims, 1985).

2.5 Economic Importance of Fungi

2.5.1 Fungi in Laboratory Research

Fungi are very useful organisms in biotechnology. They are important experimental organisms easily cultured, occupy little space, multiply rapidly and have a short life cycle. Many fungi are used as model organisms for genetics, cell biology and molecular biology research (Quinn, 2005). Fungi produce unique complex molecules, metabolites, using

established metabolic pathways. These metabolites may be biologically active with high medical or industrial importance (EMLab, 2006; Alomari, 2009). Manipulation of the genome and optimization of environmental conditions enable the high production of compounds by enhancing their capacity to form or release the target molecules (Alomari, 2009).

2.5.2 Drugs from Fungi

Currently there are about 1,600 antibiotics commercially produced and a number of medical drugs are manufactured using various fungi. Penicillin from *Penicillium chysogenum,* Cephalosoporins from *Cephalosporium,* Griseofulvin from *Penicillium griseofulvin* are some of the examples of antibiotics which are used to treat various ailments of human (Alomari, 2009). The other medicinally importance products are those of anti-cholesterol statins, the immunosuppressant and steroids (Quinn, 2005; EMLab, 2006). Lovastatin as secondary metabolite of soil-borne fungus, *Aspergillus terreus* has been used to reduce or remove low density lipoproteins from blood vessels in humans (Alomari, 2009). Cyclosporin A, a primary metabolite of several fungi, including *Trichoderma polysporum* and *Cylindrocarpon lucidum,* has proven to be a powerful immunosuppressant during and after bone marrow and organ transplants in humans. Gliotoxins also have immunological and antibiotic activity, produced by *Aspergillus fumigates* (Quinn, 2005; EMLab, 2006; Alomari, 2009).

2.5.3 Fungi as Decomposers and for Bioremediation

Fungi are also used as decomposers and help to remove dead organic matter from the ecosystems (Quinn, 2005). Fungi release degradative enzymes that break down structurally complex substance in wood and other organic matters (Mtui, 2008). Certain fungi, in particular white rot fungi (*Phanerochaete chyrsosporium*), can degrade soil pollutants such as insecticides, herbicides, pesticides (e.g., DDT and lindane), azo dyes, pentachlorophenol (PCP), polyaromatic hydrocarbons (PAHs), chlorinated aromatic hydrocarbons (CAHs) (Bioremediation Service Providers, 2013), creosote, coal tars, and heavy fuels and turn them into carbon dioxide, water, and basic elements suggesting that they may have application in the bioremediation (Christian, 2005; Sace, 2010). Various species of *Aspergillus* and *Fusarium* are reported to degrade various pollutants and hence can be employed in bioremediation (Satyanarayana, 2009).

2.5.4 Fungi as Biopesticides / Bioherbicides and Biofertilizers

Different fungal biopesticides have been used to control plant diseases (caused by other fungi, bacteria or nematodes), as well as some insect pests and weeds. Two of the most common commercial fungal biopesticides are *Trichoderma* spp. and *Beauveria bassiana* (Bpia, 2013). First commercially available mycoherbicide named ‘Collego’ contains the spores (conidia) of *Collectotrichum gleosporiodes f. sp. Aeschynemone* and used to

control the weeds in annual crops. Further, the rust fungus *Puccinia canalicuta is* commercialized under the name Dr. Biosedge for control of *Cyprus esculantus L.* (yellow nut sedge) (Agropedia, 2010). Similarly many pathogens possessing mycoherbicidal properties have been identified. The pathogens derived mycoherbicides normally initiate disease in specific weed population and kill the weeds within 3-5 weeks (Agropedia, 2010). A rust fungus, *Puccinia abrupta* have been used as biological control agent against *Parthenium hysterophorus,* an aggressive annual weed threatening to natural and agro ecosystems. Mycoherbicide products from *Fusarium pallidoroseum, F. oxy-sporum, F. moniliforme, Puccinia melampodii, Alternaria alternate, A. dianthi, A. macrospora, Colletotrichum* sp., *Rhizoctonia solani* and *Sclerotinia sclerotiorum* are reported to cause pathogenesis on *Parthenium* weed (Gnanavel, 2013).

Versicular-arbuscular mycorrhizal (VAM) fungi are symbiotic soil fungi colonizing in the roots of many plants. They impart to their hosts a variety of benefits which include increased growth and yield due to enhanced nutrient acquisition, water relations, pH tolerance and disease and pest resistance (Douds et al., 2000). Basically, VAM Facilitates solubiliization of unavailable forms of Phosphorus (P) and other immobile nutrients that can be taken up by the plant roots (Douds et al., 2000; Agriculture Information, 2012) reducing dependence on chemical fertilizers and acting as the biofertilizers. For an example, work of Plenchette and Morel, 1996 showed that *Glycine max* colonized by the VAM fungus *Glomus intraradices* produced 80% of maximum growth at a soil with low P concentration (Plenchette and Morel, 1996).

2.5.5 Fungi as Food

Mycoprotein is healthy, meat-free form of protein. It is an alternative protein source for Vegetarians, contains all the essential amino acids, low in fat, no cholesterol or trans fats and good source of dietary fibre. A filamentous fungus, *Fusarium venenatum,* is the main source of mycoprotein (Mycoprotein, 2013). In addition to this, macrofungi, belonging to Basidiomycetes contain many wild as well as cultivable edible mushrooms which have high level of dietary fiber, substantial amount of protein, vitamins and minerals with low fat level. They also have various properties for health benefits such as antioxidative, antitumour and hypercholesterolic effects (Yang, 2002). Examples of wild as well as cultivated edible mushrooms include the species belonging to genera such as *Agaricus, Ganoderma, Pleurotus, Auricularia, Boletus, Clavulina, Lentinula, Volvariella, Laccaria, Anellaria,* and *Amanita hemibapha* (Pokhrel et al., 2010; Rana and Giri, 2010).

2.5.6 Fungi in Enzyme technology/ Industry

Many enzymes produced by fungi have relevant biotechnological applications in several industrial areas (Guimarães et al*.,* 2006). These fungal enzymes can convert wood, plastic, paints and jet fuel, among other materials, into nutrients. Some of these enzymes have been used in pulp and paper processing (biopulping and biobleaching), in the synthesis of fine chemicals (biocatalysis), and in destruction of persistent pollutants (bioremediation and biodegradation) (Fungal Genomics Project, 2005; Guimarães et al*.,* 2006). Enzymes provide a potent, diverse set of specialized tools for food and beverage formulators. They can function uniquely to control process time, enhance flavor, improve texture, extend shelf life and decrease the use of chemical food additives. Some of the important industrial enzymes which play a major role in food industries include Amylases, Cellulase, Lipase, Pectinase, Protease and Xylanase (Otles, 2013).

Fungi such as *Aspergillus* spp., *Trichoderma reesei, Penicillium chrysogenum* are capable of producing cellulase enzyme for potential use for commercial biomass saccharification of cellulosic materials into simple sugars (Mathew et al., 2008; Jayant et al., 2011). Various forms of amylases are used to convert starch into dextrins and sugars (Otles, 2013). Amylases, commonly produced by various species of *Aspergillus*, are used as sources of industrial amylases (Prabakaran et al., 2009). They play a major role in the food (baking) and brewing industries (Otles, 2013). Amylases also have applications in detergents, drinks, animal feed and baking (Guimarães et al., 2006). Lipase breaks down natural lipids and oils to free fatty acids and glycerol. It is useful in baking and egg processing with other enzymes (Otles, 2013). Furthermore, lipases from fungi have been widely used for biotechnological applications in dairy industry, oil processing, and production of surfactants and preparation of pure pharmaceuticals (Prabakaran et al., 2009).

Pectinases act on pectins and their derivatives and play a major role in the food and beverages industry. In production of fruit drinks, pectinase is a unique enzyme used for the peeling of citrus fruit. It can be used to produce fresh fruit segments from oranges and grapefruit and are less likely to damage fruits than conventional cutting (Otles, 2013). Thus, Pectinases are used in the preparation of wine and fruit juices (Guimarães et al., 2006). Xylanases have potential application in food, feed, paper, pulp and textile industries (Polizeli et al., 2005). These enzymes degrade plant fibers made of xylan hemicelluloses producing xylose monomers. Xylanase is used in the pretreatment of pulps, prior to bleaching, in pulp and paper industries (Mishra and Dadhich, 2010). Xylanases produced by *Aspergillus caespitosus* and *Aspergillus Phoenicis* showed good potential for pulp bleaching (Guimarães et al., 2006).

Among the increasingly valuable roles of fungi in the biotechnology industry, one is their ability to produce enzymes capable of releasing sugars from plants, trees, and other forms of complex cellulosic biomass, which can then be converted to biofuels and biobased chemicals. Advances in fungal biology and in bioengineering fungal systems industrial applications have facilitated to efficiently degrade a wide range of non-crop plant materials, such as switchgrass, corn stover, sorghum and energy cane which makes it an interesting but yet-rarely used candidate for biofuel production (fuelfix, 2013). The inability of many microbes to metabolize the pentose sugars abundant within hemicellulose creates specific challenges for microbial biofuel production from cellulosic material. Although engineered strains of *Saccharomyces cerevisiae* can use the pentose xylose, the fermentative capacity pales in comparison with glucose, limiting the economic feasibility of industrial fermentations. Thus, to better understand xylose utilization for subsequent microbial engineering, the genomes of xylose –fermenting fungi *viz., Spathaspora passalidarum* and *Candida tenuis* are been studied (Wohlbach et al., 2011).

Table 2.1: Fungal enzymes of commercial importance (Source: Wallenstein and Weintraub, 2008)

Enzymes	Main Source
Amylase	*Aspergillus niger, Aspergillus. oryzae*
Cellulase	*A. niger, Trichoderma reesei, T. viride, Penicillium finiculosum*
Hemicellulase	*A. niger, A. oryzae, T. reesei, T. viride, P. emersonii*
Lipase	Several species including *A.niger, A. oryzae*
Pectinase	Several species including *A. niger, Rhizopus oryzae*
Protease	Several species including *A. niger, A. oryzae*
Xylanase	*A. niger, T. reesei*

2.5.7 Fungi as Foes

Some of the fungal genus are pathogens of man or are responsible for food spoilage and the production of toxic secondary metabolites (May, 1997). An aflatoxin, mainly produced by *Aspergillus flavus* is associated with the majority of the agricultural contamination (Perrone et al., 2007) and is highly carcinogenic to human (Yan et al., 2012). Ochratoxin A (OTA), mainly produced by species belonging to *Aspergillus*, is a potent nephrotoxin which may contaminate various food and feed products such as grains, legumes, coffee, dried fruits, beer and wine and meat (Perrone et al., 2007). Various reports evidenced that members of the *A. niger* species complex, together with *A. carbonarius* and *A. japonicus/aculeatus* are frequently responsible for post-harvest decay of fresh fruit (apples, pears, peaches, citrus, grapes, figs, strawberries, tomatoes, melons, etc.) and some vegetables (especially onions, garlic, and yams). Furthermore it

is also among the commonest fungi isolated from dried fruits, beans, oil seeds and nuts (peanuts, pecans, pistachios, hazelnuts, almonds, walnuts etc.) (JECFA 2001).

Fungi cause a wide variety of diseases in human. These cover aspergillosis, blastomycosis, candidiasis, coccidiodomycosis, crytococcosis, histoplasmosis, sporotrichosis, chromoblastomycosis and various other miscellonous syndromes (Doctor fungus, 2013). Fungal infections are important causes of morbidity and mortality of hospitalized patients. Invasive pulmonary aspergillosis is a leading cause of mortality in bone-marrow transplant recipients, while *Pneumocystis carinii* pneumonia is the cause of death in many patients with acquired immunodeficiency (Georgopapadakou and Walsh, 1994). The increase in frequency of patients attacked by fungal infections explains the need for additional, basic as well as applied studies within medical mycology (Lange, 2010).

Fungi also constitute the largest number of plant pathogens and are responsible for a range of serious plant diseases. Most vegetable diseases are caused by fungi. They damage plants by killing cells and/or causing plant stress. Sources of fungal infections are infected seed, soil, crop debris, nearby crops and weeds (Arbico-organics, 2013; AusVeg, 2013). Downy mildews, Powdery mildews, and White blister/ White rust (*Albugo candida*) are some of the highly prevalent foliar diseases. Fungi such as *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., *Rhizoctonia* spp., *Sclerotinia* and *Sclerotium* spp. are responsible for soilborne diseases infecting most of the vegetable crops (Ellis et al., 2008). Some of the fungi are specific to a particular crop group, e.g. Clubroot (*Plasmodiophora brassicae*) in brassicas, Leaf blight (*Alternaria dauci*) in carrots, and Red root complex in beans (AusVeg, 2013).

2.6 Identification of Fungi

Identification of fungal species is fundamental of any research endeavor. A rapid and accurate method of fungal identification is essential to use the selected strain in an industry and also for disease surveillance and implementing a disease management strategy in case of pathogenic fungi (Anderson and Cairney, 2004).

Basically, fungi are identified by two methods, viz., Morphological and Molecular identification. These methods are reviewed in the following sections.

2.6.1 Morphological Identification

The morphological identification in fungi involves description of morphology of spores and reproductive structures. The identification is possible when the dispersal structures are formed in culture (Tiwari et al., 2011). One of the most common media used to culture fungi in laboratory is Potato Dextrose Agar (PDA). It consists of potatoes infusion, dextrose and agar. High concentration of sugar and a low pH (5.5) prevents growth of

most bacteria and makes it selective for fungi (Rao, 2006). However, this is the traditional method as it depends on the visual aspects. The morphology of asexual hyphae and spores does not provide sufficient characters for identification. Most cannot be assigned to a taxonomic group without sequencing of DNA regions for molecular phylogenic analysis (Adl, 2003). Thus, this method has certain drawbacks such as not all fungi produce fruiting bodies simultaneously, some fruiting bodies are inconspicuous and easily overlooked (Anderson and Cairney, 2004), inability of certain fungi to be grown in culture media and the difficulty in identifying fungi to the species level based on microscopic features, many of the hyaline and dematiaceous fungi do not sporulate (Hall et al., 2003) and these methods are time consuming, labor intensive and subject to varying environmental conditions during the experiments (Mirhendi et al., 2007).

The quick and reliable species identification tools are necessary for interpretation of results in any research fields. In this context, recently developed molecular diagnostic tools aid in rapid, accurate and reliable identification of the fungi at the species level (Anderson and Cairney, 2004; Kirk et al., 2004).

2.6.2 Molecular Identification

Molecular techniques, based on Polymerase Chain Reaction (PCR), have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies and diagnosis of several fungal species (Williams et al., 1990; Clulow et al., 1991; Nasir and Hoppe, 1991; EI-Fadly et al., 2008). Molecular approaches have been introduced to provide simple, rapid and objective methods of identification of fungi than phenotypic methods. Mainly, ribosomal targets such as Large Subunit (LSU) RNA genes (D1/D2 regions) and Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2 regions) between the small and large subunit RNA genes have been targeted for molecular identification of fungi (Hinrikson et al., 2005). The use of DNA sequence diversity in the ribosomal regions as an aid to species identification has been exploited using PCR amplification of targets followed by either Restriction Fragment Length Polymorphism (RFLP) or DNA hybridization with specific probes or DNA sequence analysis (Hinrikson et al., 2005). Regions of ribosomal DNA (rDNA) have also been used in phylogenetic studies of fungal genomes (Tiwari et al., 2011). The overview of molecular markers are listed below (Michelmore and Hulbert , 1987; Clapp, 1996; Semagn et al., 2006; Agarwal et al., 2008; Shrestha, 2013):

1. Non-PCR based markers:

A) Restriction analyses or Restriction Fragment Length Polymorphisms (RFLPs)

B) Nucleic acid Hybridization Methods (Southern Hybridization; Northern Blot, Dot Blots, DNA microarrays).

2. PCR-based Markers

A) Arbitrary primed PCR technique: Random Amplified Polymorphic DNA (RAPD); DNA Amplification Fingerprinting (DAF); Arbitrarily-Primed PCR (AP-PCR); Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLPs)

B) Site-targeted PCR techniques: Sequence Characterized Amplified Regions (SCARs), Simple Sequence Repeats (SSRs) or microsatellites or Short Tandem Repeats (STRs), PCR-RFLP or Cleaved Amplified Polymorphic sequences (CAPs)

C) DNA Sequencing based techniques: DNA sequences of molecular barcodes in cpDNA, mtDNA and nDNA

D) Other molecular marker techniques: Real Time Polymerase Chain Reaction testing; Single-Strand Conformational Polymorphism (SSCP); Single Nucleotide Polymorphism (SNPs)

The molecular methods those are applied to the fungal identification and characterization are summarized in the following section.

2.6.2.1 Restriction Fragment Length Polymorphism (RFLP)

The publication of Botstein et al. (1980) about the construction of genetic maps using restriction fragment length polymorphism (RFLP) was the first reported molecular marker technique in the detection of DNA polymorphism. A Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms are differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme (Merriam-Webster, 2013). The similarity of the patterns generated can be used to differentiate species and strains from one another. RFLP is reported to be the first DNA profiling technique which has wide application in genome mapping, localization of genes for genetic disorders, determination of risk for disease and paternity testing (Clapp, 1996; Biology-online, 2013). RFLP is the sensitive, specific and fast method for the detection of medically important fungi like *Cryptococcus neoformans, Aspergillus fumigates, Fusarium solani, Candida* sps (Mirhendi et al., 2001; Mousavi et al., 2007) and identification of decay Fungi in Spruce Wood (Jasalavich et al., 2000).

2.6.2.2 Nucleic-acid Hybridization techniques: Southern Blotting/ Northern Blotting/ Dot Blotting/ DNA Microarray

Southern Blotting, Northern Blotting, Dot Blotting and DNA Microarray are all nucleic acid hybridization-based techniques. The theoretical basis of nucleic acid hybridization is that under suitable conditions of temperature and salt concentration, complementary sequences of single stranded molecules anneal to form a stable double –stranded structures or hybrids (Clapp, 1996). To be used as a diagnostic tool, target nucleic acid is

fixed usually on to a solid support and the complementary nucleotide sequence (called probe) which carry a label, provides the necessary signal whereby successful hybridization can be recognized. In northern and southern blots, nucleic acid fragments are first separated by gel electrophoresis then transferred to a membrane by capillarity in which they are immobilized by expressing to UV light (nylon) or by baking (nitrocellulose) whereas in dot-blot or spot hybridization, the target sample (eg. Crude sap extract) is spotted onto a membrane filter.

Nucleic acid hybridization technique is one of the most exciting areas of modern techniques being used in fungal classification (Aneja, 2008). Nucleic acid probing is useful for differentiating closely related species (Clapp, 1996). In this method, one type of nucleic acid (NA) is extracted from a fungus, separated (if necessary) into single strands (ssNA) and then compared with the ssNA obtained from similar type of fungus. After mixing the single strands together and encouraging the formation of new double strands (hybrids), the genetic relatedness of the two fungal types can be determined (Aneja, 2008).

Probe hybridization is useful for identifying slow growing organisms such as the dimorphic fungi *Histoplasma capsulatum, Coccidioides immitis* and *Blastomyces dermatitidis* which has been facilitated by commercially available probes such as AccuProbes, produced by Gen-Probe, Incorporation, San Diego, California (Pfaller, 2001; Hologic Gen-Probe, 2013). AccuProbes provide rapid results and have high sensitivity and specificity (Pounder et al*.,* 2006) with the following exceptions. The hybridization probe requires the pure culture of fungus to achieve their reported sensitivities (Huffnagle and Gander, 1993; Gromadzki and Chaturvedi, 2000; Pounder et al., 2006). The test relies on the sequence specific hybridization of a chemiluminescent single stranded DNA probe with complementary ribosomal RNA released from the target organism to form a stable DNA:RNA hybrid (Gromadzki and Chaturvedi, 2000). The probe for *B. dermatitidis* gives a false-positive result with *Paracoccidioides brasiliensis* (Pounder et al., 2006). The limitation of the *Coccidioides* AccuProbe is that it cannot be used to differentiate between two recognized species, *Coccidioides immitis* and *C. posdasii,* because it requires the use of live *Coccidioides* culture for positive reaction which is hazardous to laboratory personnel. Formalin killed cultures have shown the false negative results with the *Coccoidioides* probe and are not recommended (Gromadzki and Chaturvedi, 2000).

Currently there are no commercial sources of fungal hybridization probes other than AccuProbe system. Reports have been found for the development of genus and species specific probes for a number of fungal organisms in addition to the dimorphic pathogens detected by AccuProbe system. However these probes are not commercially available

and are not yet routinely used in most diagnostic laboratories (Gromadzki and Chaturvedi, 2000; Lindsley et al., 2001; Pounder et al., 2006; Hologic Gen-Probe, 2013).

2.6.2.3 Arbitrarily primed PCR methods

Introduction of arbitrarily primed PCR methods *viz*. Random Amplified Polymorphic DNA (RAPDs) (William et al., 1990), Arbitrarily Primed-PCR (AP-PCR) (Welsh and McClellan, 1990) and DNA Amplification Fingerprinting (DAF) (Caetano-Anolles and Bassam, 1993) have revolutionized genetic diversity studies in various disciplines for various purposes including cultivar identification, species identification, strain identification etc. in bacteria, fungi, plants and animals. Among these three technologies, which are all arbitrarily primed PCR techniques, RAPDs remained the most popular (Agarwal et al., 2008).

Random Amplified Polymorphic DNA (RAPD), a type of Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) (William et al., 1990, Espinasa and Borowsky, 1998), is a type of PCR reaction in which DNA fragment is amplified using single primer with an arbitrary nucleotide sequence (Zakaria et al., 2005). The need for sequence information from the DNA template, in order to design the primers, limits the applicability of the method to a few organisms or to highly conserved genomic DNA regions. This is an obstruction if highly variable regions need to be analyzed for the identification of organisms at the strain or isolate level. To circumvent this problem, the basic PCR method has been modified by using short (9-13 mers) oligonucleotides of arbitrary sequence (Clapp, 1996). Generally, decamer primers are commercially available to amplify the total genomic DNA under low annealing temperatures by PCR. The single random primer hybridizes to homologous sequences in the genome and DNA region between the two hybridization sites will be amplified using Taq polymerase. Each random primer gives a different pattern of PCR products in agarose gels (Brandt et al., 1998). RAPD markers have been used in identification of *Collectotrichum fragariae* from diseased strawberry and in the characterization of *Fusarium moniliformea* (Zakaria et al*.,* 2005). It also finds its application in the identification and genetic variation of different species of *Fusarium* (Sabir, 2006; EI-Fadly et al., 2008). RAPD markers can also be used to determine the degrees of genetic relatedness between different strains on the basis of similarity coefficients (Soll, 2000).

2.6.2.4 Site-targeted PCR techniques (SCARs/ SSRs / CAPs)

Sequence Characterized Amplified Regions (SCARs) are derived from individual RAPD markers. For this, RAPD markers (for eg. Species-specific markers or markers linked to gene of interests) have to be cloned and sequenced in order to design longer SCAR primers (usually 20-24 mers) for the amplification of specific DNA fragment (Vosman, 1998).

Microsatellites also known as Simple Sequence Repeats (SSRs) (Zietkewicz et al., 1994) or Short Tandem Repeats (STRs) are tandemly arranged repeats of mono-, di-, tri- tetra- and penta- nucleotides with different lengths of repeat motifs (Chawla, 2002). PCR-based assays of microsatellites have become probably the most popular and powerful of the currently available techniques for identifying highly polymorphic markers. They are widely dispersed throughout eukaryotic genomes and are often highly polymorphic due to variation in the number of repeat units. These SSR length polymorphisms at individual loci are detected by PCR using locus-specific flanking region primers where the sequence is known. Due to the high information content in the SSR derived genetic data and the advantage that material for SSR analysis can be derived from non-invasive sampling from free living organisms, this tool has become one of the molecular tools of choice for many population and biodiversity studies (Avise, 2004).

DNA-based molecular techniques combining the polymerase chain reaction (PCR) with the analysis of restriction fragment length polymorphisms (RFLP), also known as Cleaved Amplified Polymorphic sequences (CAPs) (Shrestha et al., 2010) or Amplified fragment length polymorphism (AFLP) (Vos et al. 1995), represent new tools to aid in properly identifying fungi. The PCR-RFLP technique couples two known procedures to detect polymorphism in DNA regions which have been amplified by specific oligonucleotide primers and restricted with different endonucleases (Gomes et al., 2002). The PCR-RFLP approach has already been widely used for fungal species identification by analyzing regions of ribosomal DNA of fungi (Michelmore and Hulbert, 1987; Gomes et al., 2002). For example, the work published by Gomes et al., 2002 detected the polymorphism in fungal isolates by using PCR-RFLP technique applied to the ITS regions of these fungi. Thus, the presence of different restriction sites in fungal DNA have suggested that PCR-RFLP can be used for carrying out genetic heterogeneity studies and identification without the need for sequencing (Gomes et al., 2002; Dagar et al., 2011).

2.6.2.5 Real Time Polymerase Chain Reaction testing

The Real time PCR, also known as Quantitative PCR (q-PCR) has been used for the quantification of fungal DNA and RNA in soil (Anderson and Cairney, 2004). This technology combines nucleic acid target amplification using standard PCR chemistry and detection using fluorescent probes in a simultaneous, single-well reaction. This technology offers several advantages over conventional methods (Palladino et al., 2001). First, real-time PCR testing has demonstrated equivalent sensitivity and specificity to conventional PCR testing and in many instances has been shown to be more sensitive than culture-based detection (Raggam et al., 2002; Uhl et al., 2003). Second, real-time PCR testing decreases the turnaround time in comparison with conventional PCR testing by eliminating the necessity to perform post amplification electrophoretic processing

and detection. Third, by combining target amplification and detection in a single, closed reaction vessel, real-time PCR testing reduces the possibility for environmental contamination with amplified nucleic acids. It has been reported that real-time PCR testing has been used for the detection and identification of *Aspergillus* spp., *Candida* spp., *Coccidioides* spp., *Histoplasma capsulatum* and *Pneumocystis jirovecii* (Wengenack and Binnicker, 2009).

2.6.2.6 Single-Strand Conformational Polymorphism (SSCP)

Among the different methods devised to detect genetic polymorphisms, the analysis of single-strand conformation polymorphism (SSCP) (Orita et al., 1989) is simple and yet exquisitely sensitive (Hinrikson et al., 2005; Makino et al., 1992). It has been reported that even single base substitution can be detected between DNA fragments ranging in size from 100-450 bp (Hayashi, 1991).

Because very high resolution polyacrylamide gel electrophoresis is required to detect the small differences in migration rate between homologous fragments, minimal amounts of sample have to be analyzed and a sensitive method has to be used to visualize the bands, like silver staining, autoradiography of radio labeled fragments or laser- induced fluorescence of fluorescently labeled fragments (Clapp, 1996).

For identifying fungi, the taxon-specific primers as well as more generic primers targeting a highly variable region of the same 18S gene can be used. Coupled with PCR amplification, SSCP analysis can be used to rapidly identify variants of a defined DNA segment, without relying on prior knowledge of their DNA sequences (Clapp, 1996). ITS amplicons have been characterized using single-strand conformation polymorphism (SSCP) analysis exploiting both size and sequence differences (Walsh et al., 1995); however, clinical diagnostic applications may be compromised by intra-species SSCP pattern variability such as that described recently for strains of *A. fumigatus* and *A. flavus* (Rath and Ansorg, 2000). Multiple SSCP patterns are also predicted to occur within species of *A. flavipes*, *A. nidulans*, *A. niger*, *A. ustus*, and other *Aspergillus* species that exhibit sequence differences among strains of the same species (Aguirre et al., 2004; Hinrikson et al., 2005).

2.6.2.7 Sequence based DNA tools

DNA sequencing-based analysis methods are reproducible and rapid means of identification. When attempts are made to identify the microorganisms using DNA sequencing, the gene targeted must contain highly conserved regions that can serve as primer binding sites and these conserved regions should flank regions with enough sequence variability to allow for discrimination to the genus or species level. In addition, the target gene should be present at high copy numbers whenever possible to increase the sensitivity of the PCR amplification prior to sequence analysis. DNA sequencing has

enabled the identification of fungi that would be difficult, if not impossible, to identify by conventional methods alone (Davis, 2010). Fungal species identification by molecular methods relies on the amplification and sequencing of various regions (Walker et al., 2012) such as Internal Transcribed Spacer (ITS), D1/D2 (Li et al., 2008), mitochondrial (mt) DNA cytochrome *b* (Wang et al., 1998), cytochrome *c* oxidase I (COI), β-tubulin (Mostert et al., 2005) and other Protein coding genes (Walker et al., 2012).

DNA barcoding systems employ short standardized segments to identify species in all the Kingdoms of life (Seifert et al., 2007; CBOL Plant Working group, 2009; Fungal Barcoding, 2013). The concept of applying DNA barcoding for the identification of global species was first proposed by Hebert et al. (2003) and has gained significant momentum since then (Li et al., 2011). Fungal barcoding are currently being used extensively for phylogenetic reconstruction as well as species recognition (Fungal Barcoding, 2013). DNA Barcoding differs from molecular phylogeny in that the main goal is not to determine classification but to identify an unknown sample in terms of a known classification (Seifert et al., 2007). According to Li et al. (2011), DNA barcode should meet the following criteria: (i) high universality so that it can be sequenced routinely across species , (ii) high sequence quality and coverage that is amenable to the production of bidirectional sequences with minimum ambiguous base pairs, (iii) high discriminatory power to enable most species to be distinguished.

The choice of loci as barcoding for the animals and other eukaryotes are mitochondrial DNA such as CO1 (Cytochrome *c* oxidase 1) gene. Due to slower rate of CO1 gene evolution in plants with insufficient variations and low species discrimination power, there was a need of some other alternative barcodes. Therefore, for the land plants concatenation of trnH-psbA intergenic spacer, large subunit of ribulose-bisphosphate carboxylase (*rbc*L) and maturase K (*mat*K) of cpDNA (Chloroplast DNA) are used (Kress et al., 2009; Groot et al., 2011).

DNA-based systems for species identification of fungi have variously used a barcode-like 400 to 600 bp region of the nuclear ribosomal 28S Large SubUnit (LSU) region (Abliz et al., 2004), the internal transcribed spacer (ITS) cistron (Weber et al., 2006), partial β-tubulin A (*BenA)* or *Calmodulin* gene sequences (Begerow et al., 2004; Kwiatkowski et al., 2012), or partial elongation factor 1-α (EF-1α) sequences and sometimes other protein-coding genes (Schoch et al., 2012; Seifert et al., 2007). Moreover, fungal mitochondria are not maternally inherited, as is the case in animals. Consequently, initial efforts to barcode fungi involve testing a number of mitochondrial and nuclear candidate sequences (Fungal Barcoding, 2013). While there is ample evidence that mtDNA marker is indeed suitable across a broad taxonomic range to delineate species, it has also become clear that a complementation by a nuclear marker system could be

advantageous. Ribosomal RNA genes (rDNA) could be suitable for this purpose, because of their global occurrence and the possibility to design universal primers. However, it has so far been assumed that these genes are too highly conserved to allow resolution at, or even beyond the species level. On the other hand, it is known that rDNA regions harbor also highly divergent parts (Sonnenberg et al., 2007).

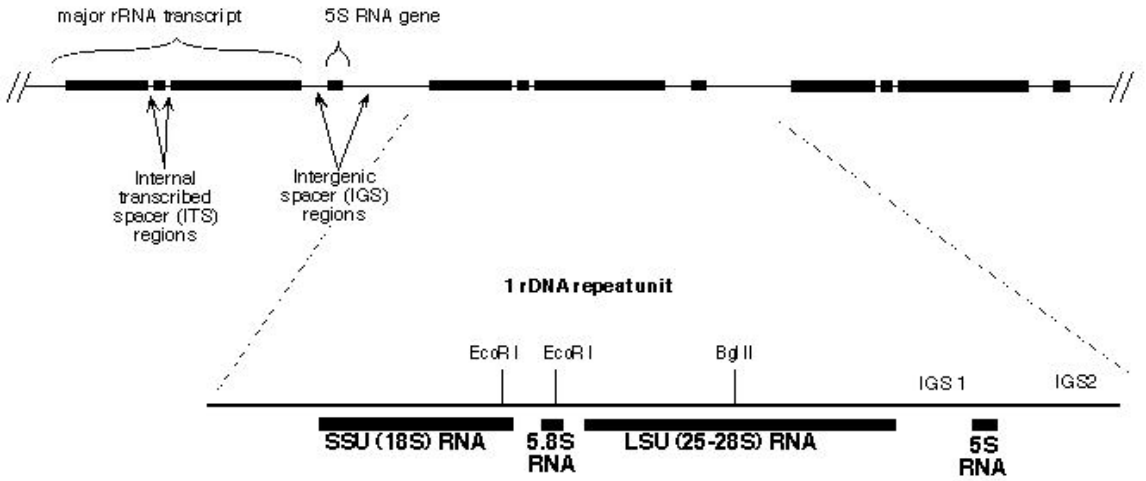


Figure 2.7: General organization of eukaryotic nuclear ribosomal DNA (nrDNA) (Source: Vilgalys lab, 2013)

All eukaryotes show a stereotypic arrangement, with often hundreds of tandemly repeated ribosomal transcription units, separated by intergenic spacers (IGS). Each produces a large transcript which is processed into the small subunit, the 5.8S subunit and the large subunit. The external transcribed spacer (ETS) and the internal transcribed spacers (ITS1 and ITS2) do not become part of the mature rRNA, but their sequences and structures are required for the correct processing. It is evident that the SSU is more conserved; interrupted by a few less conserved regions, while the LSU show larger regions of divergence (Sonnenberg et al., 2007).

The two most widely used fungal DNA barcoding regions are explained in following section.

2.6.2.7.1 Internal Transcribed spacer (ITS)

Internal Transcribed spacer (ITS) region is composed of ITS1 intergenic spacer, 5.8S rDNA and ITS2 intergenic spacer (ITS1-5.8S rDNA-ITS2), with the size ranging from 600 to 800 bp (Hinrikson et al., 2005). The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis (Martin and Rygiewicz, 2005). Out of the various regions of rDNA, the internal transcribed spacer (ITS) and Intergenic spacer (IGS) of the nrDNA repeat units have been reported to evolve fast and may vary among species within a genus or among populations of the species and hence can be used for phylogenetic study (Tiwari et al., 2011). Therefore, the ITS region has been considered a

convenient target for the molecular identification of fungi at species level (Gardes and Bruns 1993; Bin, 2008; Peay et al., 2008). The genetic diversity of many fungi is studied by sequencing Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA). ITS regions of ribosomal DNA also have been used in phylogenetic studies of fungal genomes (Tiwari et al., 2011).

The earliest PCR primers to gain wide acceptance for work with fungal Internal Transcribed Sequences (ITS) were ITS1 and ITS4 which amplify the highly variable ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and situated between the 18s Small SubUnit-coding sequence (SSU) and the 28s Large SubUnit-coding sequence (LSU) of the ribosomal operon. These primers amplify a wide range of fungal species (Martin and Rygiewicz, 2005). Some samples required PCR amplification with primer pair ITS5 and ITS4 or ITS5 and ITS2 or ITS3 and ITS4 to generate sufficient quantities of PCR amplicon for DNA sequencing (Hinrikson et al., 2005).

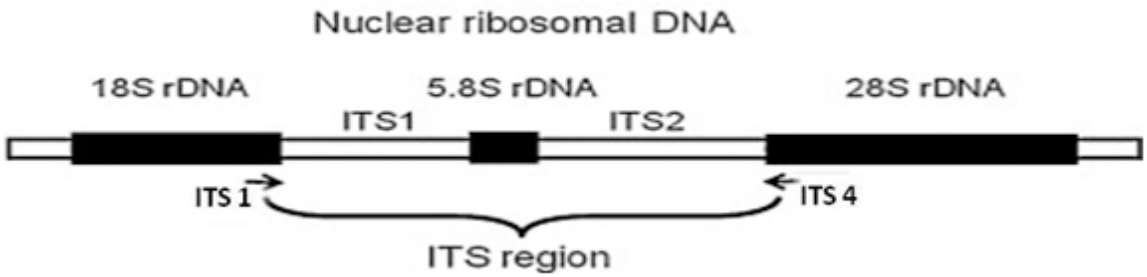


Figure 2.8: The three coding and two internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA repeat unit of eukaryotes. Arrows indicate approximate binding locations of the primers ITS1/4 used for PCR amplification. (Source: Oqata et al., 2013).

2.6.2.7.2 D1/D2

The Large Subunit (LSU) containing ~3000 to 3500 nucleotides is a part of the ribosomal DNA (rDNA) gene complex which occurs in tandem repeats, arranged in ribosomal clusters in the nuclear genome (Sonnenberg et al., 2007). The divergence region, symbolic representation as D, is numbered in 5'- 3' direction in the mature LSU region of rDNA gene complex. The highly variable part of the nuclear rDNA, the LSU D1-D2 fragment permits its use in DNA barcoding (Hall et al., 2004; Sonnenberg et al., 2007). The D1/D2 domains of LSU rDNA have been reported to be useful for identification of most ascomycetes, zyomycetes and yeasts (Abliz et al*.,* 2003). It is evident that the D1-D2 region belongs to the most divergent parts, although it contains also some highly conserved stretches that allow the selection of universal primers (Sonnenberg et al., 2007; Dagar et al., 2011). The 5'D1 - 3'D2 region is about 600-nucleotides (Kurtzman and Robnett, 1997; Hinrikson et al., 2005). PCR using broad-range primer pairs ITS1 and D2R or D1 and D2R generate the D1/D2 region amplicons for sequencing (Hinrikson et al., 2005). However, it has been highly reported that D1/D2 domains of fungal LSU-rDNA can

be well amplified with primers NL1 and NL4 (Abliz et al., 2003; Dagar et al., 2011; Kurtzman and Robnett, 1997; Kwiatkowski et al., 2012; Scorzetti et al., 2002).

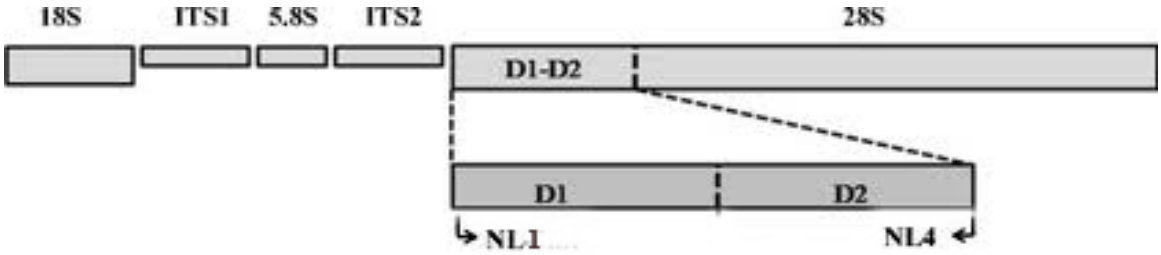


Figure 2.9: D1/D2 regions of the part of 28S Large Subunit (LSU) region of nuclear ribosomal DNA (Source: Pinheiro et al., 2013).

The combined uses of PCR/DNA sequencing methods in the D1/D2 LSU regions have facilitated the identification of the fungi (Weber et al., 2006). Several studies have shown that sequencing the D1/D2 region has great potential for the identification of yeasts and several species of the filamentous fungi (Kurtzman and Robnett, 1995; Hall et al., 2004; Weber et al., 2006).

2.7 Phylogenetic analysis

PAUP, PHYLIP and MEGA are major programs that are used for phylogenetic construction. Due to easy accessibility of MEGA, it is used for analysis purpose in this research. Therefore, MEGA software is described in upcoming section.

MEGA software provides tools for exploring, discovering, and analyzing DNA and protein sequences from an evolutionary perspective. The first version of MEGA was developed for the limited computational resources that were available on the average personal computer in early 1990s. As with previous versions, MEGA 5 is specifically designed to reduce the time needed for mundane tasks in data analysis and to provide statistical methods of molecular evolutionary genetic analysis in an easy-to-use computing workbench. It emphasizes the integration of sequence acquisition with evolutionary analysis. It contains an array of input data and multiple results explorers for visual representation. The results explorers allow users to browse, edit, summarize, export, and generate publication-quality captions for their results. MEGA 5 also includes distance matrix and phylogeny explorers as well as advanced graphical modules for the visual representation of input data and output results (Tamura et al., 2011). To assess the reliability of a phylogenetic tree, MEGA provides the Bootstrap test. This test uses the bootstrap re-sampling strategy, according to the number of replicates entered (Hillis and Bull, 1993; Tamura et al., 2011).

The process of carrying out sequence-based phylogenetic analysis can be divided into four key steps listed as follows:

1. Select a sequence of interest. This could correspond to a whole gene, a region of a gene (coding or noncoding regions can be used), a regulatory region for a gene, a transposable element, or even a whole genome.
2. Identify homologs. Acquire sequence data for objects that are homologous to the sequence of interest
3. Align sequences. Align the sequence of interest and the homologous regions to generate a sequence data matrix.
4. Calculate phylogeny. Carry out phylogenetic inference on the alignment.

2.7.1 Sequences Alignment

In bioinformatics, a sequence alignment is a way of arranging the sequences of DNA, RNA or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences (Mount, 2004). For the phylogenetic data analysis, multiple sequences have to be aligned in a designated file format. Multiple sequence alignments (MSAs) are essential in most bioinformatics analyses that involve comparing homologous sequences (Sievers et al., 2011). Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns (Mount, 2004). The multiple alignment systems that are mostly targeting proteins or short DNA sequences and are based on progressive alignment, include CLUSTALW (Higgins et al., 1994), MULTALIGN (Barton and Sternberg, 1987), T-COFFEE (Notredame et al., 2000), MAFFT/ PartTree (Katoh et al., 2002), MUSCLE (Edgar RC, 2004), PROBCONS (Do et al., 2005) and Clustal Omega (Sievers et al., 2011). The best-known, easily available and reliable system based on progressive multiple alignment is perhaps CLUSTALW (Batzoglou, 2005). Therefore, for the sequence alignment in this study, ClustalW has been used which is described in following section.

For aligning any number of homologous nucleotide or protein sequences, ClustalW is a widely used system. ClustalW provides a number of options of data presentation, homology matrices and presentation of phylogentic trees (Higgins et al., 1994). For the Multiple Sequence Alignments (MSAs), the exact way of computing an optimal alignment between N sequences has a computational complexity of $O(L^N)$ for N sequences of length L making it prohibitive for even small numbers of sequences. Most automatic methods are based on the progressive alignment heuristic which aligns sequences in larger and larger sub-alignments, following the branching order in a guide tree (Sievers et al., 2011). And, ClustalW uses progressive alignment methods for MSAs. In these, the most similar sequences, that is, those with the best alignment score are aligned first. Then progressively more distant groups of sequences are aligned until a

global alignment is obtained. This heuristic approach is necessary because finding the global optimal solution is prohibitive in both memory and time requirements (Higgins et al., 1994; Jeanmougin et al., 1998). This approach can routinely make alignments of a few thousand sequences of moderate length, but it is tough to make alignments much bigger than this with an increase in complexity (Sievers et al., 2011).

ClustalW uses command line or command console interface for the alignment while ClustalX uses Graphical User Interface (GUI) for the sequence alignment (Jeanmougin et al., 1998; ClustalW / ClustalX, 2013). Clustal Omega is a new multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments (Sievers et al., 2011).

2.7.2 Constructing Phylogenetic Trees from Sequence Data

There are numerous methods for constructing phylogenetic trees from molecular data (Nei and Kumar, 2000). They can be classified into Distance methods, Parsimony methods, and Likelihood methods (Tamura et al., 2011).

2.7.2.1 Distance-based Method

The overall distance between all sequence pairs is calculated to construct a tree (Wiley online, 2013). The organisms sharing a recent common ancestor should, on average, be more similar to each other than organisms whose last common ancestor was more ancient. Therefore, it should be possible to infer evolutionary relationships from the patterns of similarity among organisms. This is the principle that underlies the various distance methods of phylogenetic reconstruction (Evolution, 2013). A distance matrix (i.e., a table of evolutionary distances between each pair of taxa) is generated. In the simplest case, the distances represent the dissimilarity between each pair of taxa. The distance-based method can be broadly categorized as Minimum Evolution (ME), Neighbor Joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Tamura et al., 2011). Each method is explained briefly in following section.

In Minimum Evolution (ME) method, a topology showing the smallest value of the sum of all branches (S) is chosen as an estimate of the correct tree. However, the construction of a minimum evolution tree is time-consuming because, in principle, the S-values for all topologies must be evaluated. The number of possible topologies (unrooted trees) rapidly increases with the number of taxa so it becomes very difficult to examine all topologies (Tamura et al., 2011).

Neighbor Joining (NJ) is one of the most widely used distance-based methods for building phylogenetic trees (Evolution, 2013). NJ method (Saitou and Nei 1987) is a simplified version of the minimum evolution (ME). In the case of the NJ method, the S-value is not computed for all or many topologies, but the examination of different

topologies is embedded in the algorithm, so that only one final tree is produced. The NJ method produces an unrooted tree because it does not require the assumption of a constant rate of evolution. Finding the root requires an outgroup taxon (Nei and Kumar, 2000).

UPGMA method constructs a tree by identifying the shortest distance (D) in the matrix, clustering those two taxa into a single OTU (Operational Taxonomic Unit) for use in all subsequent calculations, calculating a new distance matrix, and then repeating these steps (Evolution, 2013). This tree-making method assumes that the rate of evolution has remained constant throughout the evolutionary history of the included taxa. Therefore, it produces a rooted tree (Nei and Kumar, 2000).

2.7.2.2 Parsimony Method

Maximum Parsimony (MP) is a character-based method that is one of the most widely used and accepted in systematics. MP seeks the phylogenetic tree that minimizes the total number of changes (or branches) to illustrate evolutionary relationships (Wiley online, 2013). MP tree construction is based on principle of Occam's Razor. The principle known as Occam's (or Ockham's) Razor states that lacking any other factors, the most parsimonious one should be chosen. The parsimony methods for the phylogentic inference work in the following manner: in a given set of data (e.g., a multiple sequence alignment), phylogenetic trees that represent alternative possible relationships among the OTUs in the dataset are given a score. The score is a measure of the number of evolutionary changes (e.g., A changing to T) that would be required to generate the data given for that particular tree. Of the possible trees, the one with the lowest score (i.e., the one requiring the fewest evolutionary changes) is known as the most parsimonious tree or Maximum Parsimony (MP) tree (Evolution, 2013). For a given topology, the sum of the minimum possible substitutions over all sites is known as the tree Length. In this term, the topology with the minimum tree length is known as the Maximum Parsimony tree. The phylogenetic tree(s) inferred using this criterion are unrooted trees (Tamura et al., 2011). The MP use subtree pruning and regrafting (SPR) technique for the construction of tree. In such methods, a branch along with all of its sub-branches is moved from one location on a tree to another spot on the same tree, thus enlarging the searched space. The scores are then compared between the two trees and the tree with the better score is selected as the next starting point (Evolution, 2013).

2.7.2.3 Likelihood Method

One limitation of both the distance and parsimony methods is that although they may select one tree over another on the basis of some criterion, it is not possible to say how much more probable one tree is than another. Likelihood methods have been designed to provide such a statistical framework for phylogenetic reconstruction (Evolution,

2013). Maximum likelihood (ML), as implemented in phylogenetics, uses a stochastic model that gives the probability of a particular character changing at any given point on a tree. The method searches for the tree with the highest probability or likelihood. Maximum Likelihood (ML) trees are scored based on a character dataset, and the tree with the best score is selected (Tamura et al., 2011).

2.7.3 Testing Best-fit model

This option tests a data file (nucleotide or amino acid) for goodness of fit to some popular models of evolution under several criteria which can be used to pick the most appropriate evolutionary model for the given analysis (Tamura et al., 2011).

jModelTest is a tool to carry out statistical selection of best-fit models of nucleotide substitution. It implements five different model selection strategies: hierarchical and dynamical likelihood ratio tests (hLRT and dLRT), Akaike and Bayesian information criteria (AIC and BIC), and a decision theory (DT) method. It also provides estimates of model selection uncertainty, parameter importances and model-averaged parameter estimates, including model-averaged tree topologies (Sullivan and Joyce, 2005; Posada, 2008). jModelTest2 includes High Performance Computing (HPC) capabilities and additional features like new strategies for tree optimization, model-averaged phylogenetic trees (both topology and branch length), heuristic filtering and automatic logging of user activity (jModelTest2, 2013).

The results of the best-fit model analysis show the estimated values of all parameters for each model (frequencies, transition probabilities, rate variation parameters, etc.), plus the count of total parameters. The model which has a low number of parameters (to keep variance low) yet is accurate enough (as measured by the goodness-of-fit criteria) is picked for the construction of phylogenetic tree (Tamura et al., 2011).

Consensus trees are a convenient way to summarize the agreement between two or more trees. jModelTest provides two types of consensus (clustering) methods. They are Strict and majority rule consensus methods (jModelTest2, 2013). Both of these methods are based on simple counts of the frequency of occurrence of clusters in the set of trees. The strict consensus tree contains only those clusters found in all the trees evaluated and the majority rule consensus tree or median tree contains all clusters occurring in at least half (> 50%) of the trees. Strict consensus tree is useful primarily to see which clusters are always supported by all the trees in the profile. Majority rule consensus has often been used to summarize bootstrap trees (COMPONENT User's Guide, 2013).

2.8 Soil fungi Research in Nepal

The fungi associated with the soil are known as soil borne fungi. Many biotic and abiotic factors influence growth and biomass of soil fungi (Vaidya et al., 2007). Soil is the source of organic and inorganic nutrients. The decomposition process of the organic matter contributes to the maintenance of the nutrient status of soil providing an environment suitable for different types of fungi (Vaidya et al., 2007; Khatri, 2012). The high elevation barren soils actually harbor significant microbial diversity but have remained mostly unstudied in all of the major mountain ranges of the Earth (King et al., 2010). It was also illustrated by Bhuju et al., 2007 stating that the high altitude soil mycoflora of Nepal has not been adequately investigated so far. However, very few reports are available about soil fungi of Nepal. The most dominant fungi in soil of Nepal include *Penicillium, Aspergillus, Fusarium* and *Mucor* (Luitel and Koirala, 2009). It has been reported that microorganisms are neither uniformly distributed throughout the soil profile nor throughout a single soil horizon. The various factors such as soil, soil texture, soil depth, pH, organic and inorganic nutrients play important role in the distribution and growth of soil fungi (Vaidya et al., 2007). According to Waksman, 1916; *Mucor* and *Penicillium* commonly occur in soil of temperate or cool-climate areas, *Aspergillus* in tropical soil, and *Trichoderma* occurs frequently in wet or acidic soils. Bhuju et al. (2007) have reported that the organisms living in the high altitudes of Manaslu Conservation Area (MCA) are psychrophiles and psychrotolerants adapting to low temperatures. Similarly, soils at high altitudes >3,000m in the Annapurna Mountain, has low water activity due to dry climate and consequently these soils are found to contain psychrophilic fungi with xerophilic characteristics (King et al., 2010). The most extreme xeroophiles belong to the ascomycetes genera *Aspergillus* and *Eurotium* (Margesin and Miteva, 2011). King et al. (2010) also indicated that soil water availability is the primary limiting factor for life in high-elevation (>5100 m) soils.

CHAPTER 3
MATERIALS AND METHODS

3.1 Collection of Soil Sample

Manaslu Conservation Area (MCA) was the selected site for the collection of sample. The altitude of MCA ranges from 600 m to the summit of Mt. Manaslu (8,163). Soil samples from five different altitudinal ranges (1700 m, 2200 m, 2700 m, 3200 m and 3600 m) and the soil from Rhizosphere of the different medicinal plants of MCA from the altitude 3500 m to 4300 m (asl) were collected. For each soil sample, about 500 gram of soil from 10-15 cm depth was taken with sterilized gloves in hand. The samples were collected in zip-lock bags and were brought to molecular biotechnology laboratory at NAST at normal temperature and stored in refrigerator until use.

3.2 Isolation of fungi

Isolation of fungi was carried out by Soil dilution plate method (Henderson, 1961). One gram of soil sample was mixed with 10 mL of sterile distilled water and a series of dilutions (10^{-1} to 10^{-5}) were made. From different dilutions, 1 mL volumes was pipetted onto sterile petri plate and molten (about 45^{o}C) Potato Dextrose Agar (PDA) was added to it and swirled slowly to mix them (Pour Plate method) and incubated (Biological Incubator, Beeta, India) at 28°C for a week, observing the growth of mycelium in plate.

After a week, fungal isolates obtained in each dilution were recorded and pure culture of each of the isolates was obtained on PDA media (Hi media, Laboratories Ltd. Bombay, India). The Stock cultures for each isolates were maintained on PDA slant in culture tubes at 4^{0}C. Sub culturing of isolates were carried out periodically till entire research was done.

3.3 Identification of fungi

Two methods were used for the identification of the isolated fungi. They were:

A. Morphological Method B. Molecular Method

3.3.1 Morphological Method

The identification of the fungi by morphological method was based on colony characteristics on the media and the shape and arrangement of spores (Conidial ontogeny) under the microscopic observation. For the microscopic observation, the isolates were stained with Cotton Blue (Human Diagnostics and Surgichem Laboratory reagent, Kolkata, India). A small portion of fungal mass at its proper stage was mounted in a drop of cotton blue stain on the slide. It was left for 15 mins and observed under microscope (Olympus Biological Microscopes, India).

The identification of the isolates was carried out using criteria of the following standard books and online internet resources:

1. A manual of soil fungi (Gillman, 1975)

2. Dematacеous Hyphomycetes (Ellis, 1971)
3. Genera of Hyphomycetes (Barron, 1968)
4. Pictorial Atlas of Soil and Seed fungi: Morphologies of Cultured Fungi and Key to Species (Watanabe, 2002)
5. Experiments in Microbiology Plant pathology and Biotechnology (Aneja KR, 2008)
6. mold.ph (2013)
7. Mycology online (2013)

The diversity of identified isolates was expressed in percentage distribution by formulation ($\frac{No.of\ each\ isolates}{Total\ number\ of\ isolates} * 100$) in MS-Excel 2007.

3.3.2 Molecular Method

The selected fungi were also identified by the amplification and sequence analysis of the D1/D2 region of the large subunit of the 28S ribosomal RNA gene and the Internal Transcribed Spacer (ITS) region using standard molecular and bioinformatics tools.

3.3.2.1 DNA extraction by Modified CTAB Extraction Method (Moller et al., 1992)

The fungi were cultured on PDA plate for a week and mycelia was taken for the extraction of DNA by Modified CTAB extraction method (Moller *et. al*., 1992)

Approximately 50 mg of the fungal mycelia was scraped from a week old PDA culture and manually grounded in sterile mortar and pestle with pre-warmed (60^0C) TES lysis buffer (500 µL). It was then transferred into Eppendorf tube (2mL). 10mg/mL ProteinaseK (10 µL) was added to the grounded material and incubated in waterbath at 60^0C for 60 min. 5 M NaCl (140 µL) and 10% (w/v) of CTAB (65 µL) were added to the suspension and incubated at 65^0C for 15 mins. The supernatant was taken in new microfuge tube after centrifugation (Eppendrof, Germany) at 26000g for 10 minutes at 4^0C. DNA was extracted by adding equal volume (~600 µL) of Chloroform:Isoamylalcohol (24:1, v/v) centrifuged at 26000g for 15mins. The supernatant (~400 µL) from the centriguged tube was taken in the new microfuge tube. DNA was precipitated by adding 0.6 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and kept at -20^0C for at least two hours. Thereafter, the mixture was centrifuged at 26000g for 15 mins and the pellet obtained was washed twice with 70% ethanol. Thus obtained pellet was re-suspended in 100 µL of TE with RNase A and stored at -20^0C until use.

3.3.2.2 DNA Quantification

After extraction of DNA, quantification and purity assessment of fungal DNA was measured with the dilution factor of 10 µL /90 µL (sample /SDDW) by using UV Biophotometer (Eppendorf-AG22331, Germany).

3.3.2.3 Polymerase Chain Reaction (PCR)

For the fungal species identification and phylogenetic study, two main genomic loci: D1/D2 region of the large subunit or the 28S ribosomal RNA gene and the Internal Transcribed Spacer (ITS) region, were amplified by PCR (Eppendorf, Germany). Various constituents of the PCR mixture, along with the annealing temperature were varied for the amplification and the standard constituents with standard cycling conditions were maintained.

3.3.2.4.1 ITS amplification

The used primer sequence for the amplification of ITS1-5.8s-ITS2 region (Kwiatkowski et al*.,* 2012) was:

Primer	Sequence
ITS 1 (Forward. 19 mers)	5'-TCC GTC GGT GAA CCT GCG G-3'
ITS 4 (Reverse, 20 mers)	5'-TCC TCC GCT TAT TGA TAT GC-3'

The PCR was done with 25 µL final volume. The constituents of PCR mixture was according to Khatri, (2012). The optimized PCR reaction conditions comprised of 25 ng of template DNA, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 1 U/µL of Taq Polymerase and 0.4 µM of each primers. Similarly, the PCR program used for the amplification of ITS was as given by Kwiatkowski et al*.,* (2012). PCR cycling condition is: initial denaturation of 95^{o}C for 3mins followed by 32 cycles of 95^{o}C for 1 min, 60^{o}C for 1 min and 72^{o}C for 3 mins, with a final extension at 72^{o}C for 5 mins.

3.3.2.4.2 D1/D2 amplification

For the amplification of the D1/D2 region of the fungal DNA, the extracted DNA was amplified by PCR method. For the PCR, the ready to use 2X PCR Master Mix (Promega) was used. The 2X master mix includes 50 U/mL Taq DNA Polymerase (pH 8.5), 400µM each dNTPs (dATP, dGTP, dCTP, dTTP) and 3 mM $MgCl_2$. 12.5 µL of 2X PCR Master Mix (Promega) is used in 25 µL reaction volume to make 1X concentration. Various aspects of the PCR were varied for the amplification of the D1/D2 region. The varied parameters were:

1. $MgCl_2$ (1.5 to 4.0 mM) 2. Template DNA (12.5 to 62.5 ng/µL)

3. Annealing temperature (48 to 68^{0}C in gradient PCR)

The used primer sequence for the amplification of D1/D2 region (Kwiatkowski et al*.,* 2012) was:

Primer	Sequence
NL1 (Forward, 24 mers)	5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'
NL4 (Reverse, 19 mers)	5'-GGT CCG TGT TTC AAG ACG G-3'

The PCR was done with 25 µL final volume. The optimized PCR reaction conditions comprised of 25 ng/µL of template DNA, 12.5 µL of 2X PCR Master Mix (Promega), final concentration of 2.5 mM $MgCl_2$ and 1 µM each primer concentration. Similarly, the

optimized PCR cycling condition is: initial denaturation of 95^{o}C for 4 mins followed by 30 cycles of 94^{o}C for 1 min, 65^{o}C for 2.5 mins and 72^{o}C for 2.5 min, with a final extension at 72^{o}C for 10 mins.

3.3.2.5 Agarose Gel Electrophoresis

10µL of amplified PCR products from both sets were separately mixed with 2 µL of 6X gel loading dye (Fermentas) (final 1X in 12 µL mixture) and mixture was loaded on to the well using micropipette. Gene ladder (5 µL) was also loaded on to a well. It was run at about 12.5 V/cm for 1 hr and the DNA bands were visualized on Gel documentation system (INGENIUS, Syngene Bio Imaging, SYIGLHR/1546, UK).

3.3.2.6 Sequencing and Phylogenetic Analysis

The amplified DNA samples of the selected species were sent to Macrogen, South Korea for bidirectional sequencing. The retrieved bidirectional sequences of ITS and D1/D2 regions were edited, aligned and assembled using CodonCode Aligner *v.4.2.2* software. Each sequence from the ITS and the D1/D2 regions was separately used to perform individual nucleotide-nucleotide searches with the BLASTn algorithm at the NCBI website: http://www.ncbi.nlm.hih.gov/BLAST/ (Zhang et al*.,* 2000; Romanelli et al*.,* 2009; NCBI, 2013).

Assembled sequence data of D1/D2 genes of isolated fungi were used to perform a phylogenetic analysis. The phylogentic analysis of the assembled and aligned sequences was performed by Maximum Parsimony (MP) analysis with MEGA *v.5.2.2* software in which gaps were treated as missing data (Jeanmougin et al*.,* 1998; MEGA, 2013). Similarly, the best-fit model was tested and the consensus tree was built accordingly using jModelTest *ver.2.1.4* software (Sullivan and Joyce, 2005; jModelTest, 2013).

3.4 Screening for extracellular enzyme production

The selected samples (expected to be as *Penicillium spp* and *Aspergillus spp* from morphological identification) were used for the screening of six different enzymes: cellulase, amylase, pectinase, xylanase, lipase and protease. PDA media supplemented with 0.5% of respective substrates such as CMC (Carboxy Methyl Cellulose), Soluble starch, Pectin, Birch wood xylan, Tributyrin and Skim milk powder were prepared for cellulose, amylase, pectinase, xylanase, lipase and protease screening. Each plate was inoculated with respective sample in the center with the help of inoculating wire. The inoculated plates were incubated at 28^{o}C for 48 hours. After incubation, 1% iodine solution was flooded to the starch, pectin and tributyrin plates and 0.5% congo red to the cellulose and xylan plates (Guimarães et al., 2006; Prabakaran et al., 2009; Jayant et al., 2011). The zone of hydrolysis was observed around the culture sample. The plates showing halozone were taken as positive for enzyme production.

CHAPTER 4
RESULTS

The present investigation was undertaken in order to study the fungal diversity of Manaslu Conservation Area (MCA), Nepal using morphological and molecular analysis tools. Also, the Screening of selected isolates was performed for the production of extracellular enzymes of biotechnological interest. The results of experiments performed to study the diversity of the soil fungi by morphological and molecular identification and to study their potential of producing extracellular enzymes are systematically represented below.

4.1 Isolation of fungi

A total of 59 fungal strains were isolated from soil sample collected from Manaslu Conservation Area (MCA), Nepal. Out of these, 23 were isolated from the altitude ranging from 1700 to 3600 meters m (asl) with the difference of 500 m; and 36 were isolated from seven different rhizospheric regions of different medicinal plants found above 3600 m altitude.

4.2 Morphological study of the fungal isolates

In the present investigation, the morphological identification of the fungi was based on colony characteristics on the media and the shape and arrangement of spores (Conidial ontogeny) under the microscopic observation.

4.2.1 Growth character of isolates

The Potato Dextrose Agar (PDA) was used to isolate the strain incubating at 28^0C and the colony study was done in 3 to 7 days incubated PDA plates. The morphological characters such as growth rate, fruiting structures, spores formation, pigment exudation, and color of the colony on surface and reverse of the media plates were studied. The colony characteristics that were observed on PDA for respective fungal isolates are represented in Table 4.1 and Colony observation of two fungal isolates studied on PDA medium is shown in Plate 4.1.

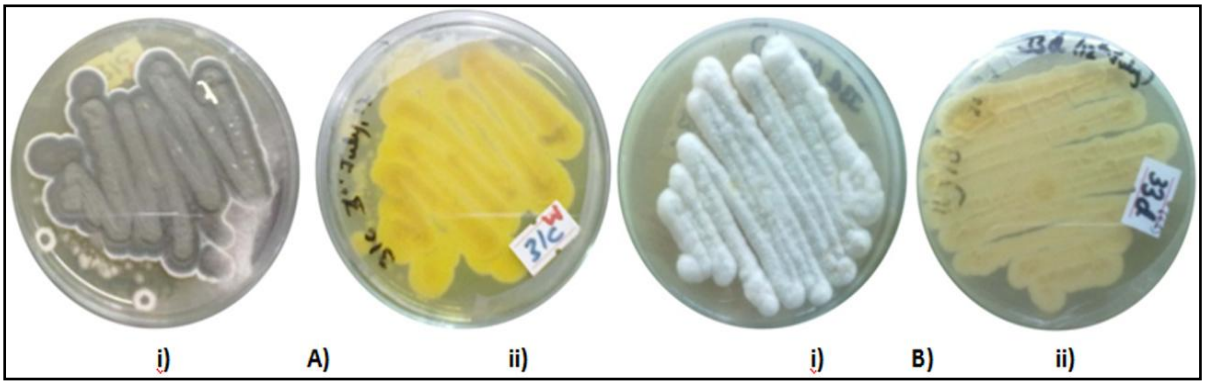


Plate 4.1 i) Front and ii) Backward view of PDA plates showing the growth of fungi A) 31c and B) 33d after 7 days of incubation at 28^0C.

Table 4.1 Colony characteristics of the fungal isolates

Isolated species	Colony Characteristics on PDA plate
Mucor spp.	White to dark gray mycelium widespread fast in and on the media (Separated by microscopic observation)
Rhizopus spp.	
Aspergillus spp.	Greenish-blue, black or green colonies with dusty spores
Penicillium spp.	Greenish or blue green colonies with dark yellow from backward view
Fusarium spp.	Woolly white to pinkinsh-purple colonies
Chysosporium spp.	Cottony white colonies
Alternaria spp.	Black colonies
Nigrospora spp.	Gray colonies
Trichoderma spp.	Fast growing white to green colonies
Cladosporium spp.	Greenish black powdery colonies
Verticillium spp.	Pinkish brown
Gliocladium spp.	Dark green mass with mucilaginous character

4.2.2 Microscopic Observation

The cotton blue stained isolates were observed under compound microscope. The spore and hyphal arrangements help to compare the species with the pre-determined fungi for the identification of the isolates. The microscopic observations of some isolates are represented in Plate 4.2 and the microscopic characteristics that helped to identify the represented fungi are presented in Table 4.2. They were further confirmed by molecular method.

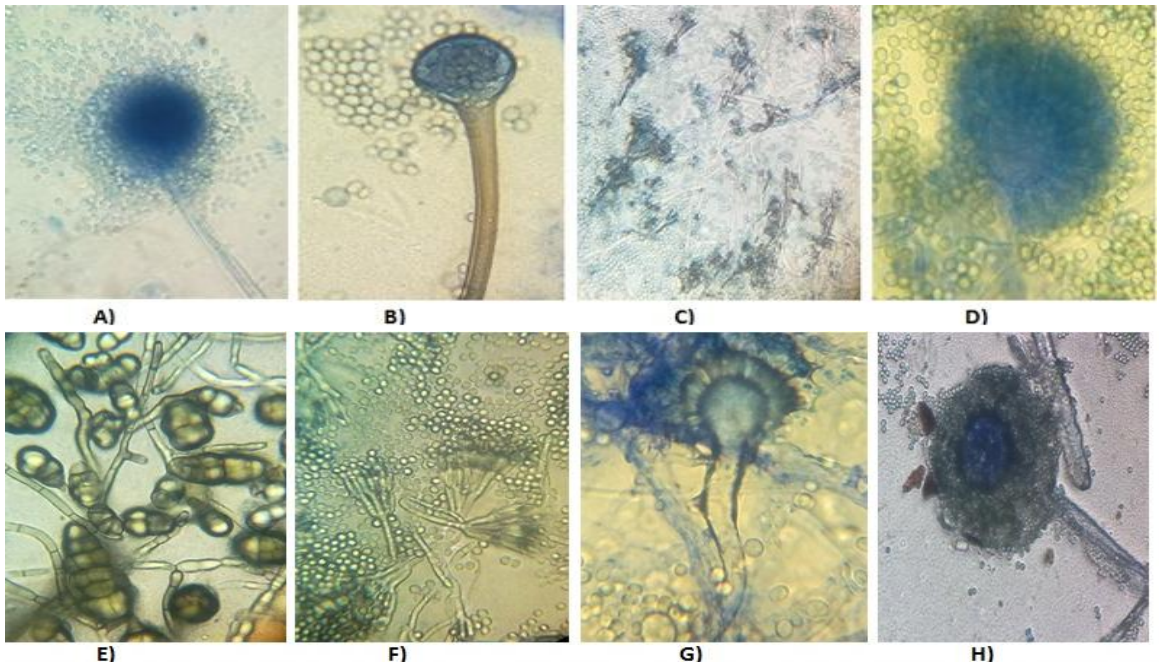


Plate 4.2 Light Microscopic observation of isolated strains **A)** 33d: *Aspergillus* **B)** 26: *Mucor* **C)** 31c: *Peniillium* **D)** 63a: *Aspergillus* **E)** 103a: *Alternaria* **F)** 41a: *Penicillium* **G)** 112a: *Penicillium* **H)** A12: *Aspergillus.*

Table 4.2 Microscopic characteristics of the major fungal isolates

Fungal isolates	Microscopic observation
Penicillium spp.	Conidia in long chains on repeatedly branched conidiophores resembling a brush-like head
Aspergillus spp.	Series of conidia on phialides arise from vesicle at the tip of conidiophore
Mucor spp.	Non-septate mycelium without rhizoids with single columellate sporangiophore
Rhizopus spp.	Septate mycelium with rhizoids with sporangiophores in clusters
Alternaria spp.	Horizontally septate hyphae with branched acropetal chains of multicelled conidia
Fusarium spp.	Sickle-shaped septate macrocondia produced from conidiophores
Chysosporium spp.	Pear-shaped condia laterally or terminally occurring from conidiophore
Nigrospora spp.	Unicellular spherical conidia arising from septate mycelium
Trichoderma spp.	Group of conidia as a ball at the tip of aseptate mycelium
Cladosporium spp.	Variable sized conidia arising from branched conidiophore
Verticillium spp.	Phialides arranged in verticals around the conidiophore. Conidia are solitary or form clusters at the tips of the phialides.
Gliocladium spp.	Conidia appear in a group forming a globule at the end of branched conidiophores.

The Taxonomic classification of each species are (Source: Doctor fungus, 2013):

Aspergillus spp.
Kingdom: Fungi
Phylum: Ascomycota
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*

Penicillium spp.
Kingdom: Fungi
Phylum: Ascomycota
Class: Euascomycetes
Order: Eurotiales
Family: Trichomaceae
Genus: *Penicillium*

Mucor spp.
Kingdom: Fungi
Phylum: Zygomycota
Order: Mucorales
Family: Mucoraceae
Genus: *Mucor*

Rhizopus spp.
Kingdom: Fungi
Phylum: Zygomycota
Order: Mucorales
Family: Mucoraceae
Genus: *Rhizopus*

Alternaria spp.
Kingdom: Fungi
Phylum: Ascomycota
Class: Euascomycetes
Order: Pleosporales
Family: Pleosporaceae
Genus: *Alternaria*

Fusarium spp.
Kingdom: Fungi
Phylum: Ascomycota
Order: Hypocreales
Family: Hypocreaceae
Genus: *Fusarium*

Chysosporium spp.
Kingdom: Fungi
Phylum: Ascomycota
Class: Euascomycetes
Order:Onygenales
Family: Onygenaceae
Genus: *Chrysosporium*

Nigrospora spp.
Kingdom: Fungi
Phylum: Ascomycota
Order: Trichosphaeriales
Family: Trichosphaeriaceae
Genus: *Nigrospora*

Trichoderma spp.	Kingdom: Fungi Phylum: Ascomycota Class: Euascomycetes Order: Hypocreales Family: Hypocreaceae Genus: *Trichoderma*	*Cladosporium spp*	Kingdom: Fungi Phylum: Ascomycota Subphylum: Ascomycotina Genus: *Cladosporium*
Verticillium spp.	Kingdom: Fungi Phylum: Ascomycota Family: P*lectosphaerellaceae* Genus: *Verticillium*	*Gliocladium spp.*	Kingdom: Fungi Phylum: Ascomycota Order: Hypocreales Family: Hypocreaceae Genus: *Gliocladium*

4.2.3 Morphologically identified strains

The fungal species isolated and identified on the basis of morphology are shown in Table 4.3 and 4.4. The diversity of fungi observed from different altitudes is represented in Table 4.3. The pure cultures of the 23 isolates from altitudinal variation were previously obtained in the laboratory and were maintained on PDA slant at 4^0C. The further processing as identification and characterization were carried out by sub-culturing the isolates. Therefore, the study of number of colonies weren't done for these isolates. However, their individual distribution in different altitudes is represented in tabular form below.

In the altitudinal variation, the fungi in certain altitude were moreover specific. For example, in altitude of 1700 m, there was 100% *Mucor.* Similarly, in 3200 m, 5 out of 8 isolates were *Penicillium.* However, the maximum of the isolates were found to be *Penicillum* (8/23 i.e. ~35%). The unidentified isolates were due to absence of sporulation on the medium and these weren't able to identify microscopically.

Table 4.3 Morphologically identified soil mycoflora from different altitudes (total isolates = 23)

Altitude (metres, asl)	Colony code	Fungi isolated
1700	10 ie S1P22	*Mucor* sp.
	26 ie S1P14	*Mucor* sp.
2200	A16	*Penicillium* sp.
	A19	*Crysosporium* sp.
	A35	*Nigrospora* sp.
	A36	Unidentified
	A163	*Crysosporium* sp.
2700	A12	*Penicillium* sp.
	A13	*Aspergillus* sp.
	A39	*Penicillium* sp.
	A123	*Mucor* sp.
	A124	Unidentified

3200	A7	Unidentified
	A10	*Penicillium* sp.
	A24	*Penicillium* sp.
	A25	*Penicillium* sp.
	A30	*Fusarium* sp.
	A31	Unidentified
	A241	*Penicillium* sp.
	A277	*Penicillium* sp.
3600	14 ie S2P11	*Gliocladium* sp.
	21 ie S2P22	*Nigrospora* sp.
	23 ie S2P23	*Aspergillus* sp.

Diverse mycoflora was isolated from rhizospheric soils of different medicinal plants and is represented in Table 4.4. As illustrated in Table 4.4, there were variations in the fungal strains in different Rhizospheric regions which are in contrast to the altitudinal variation. The regions also differ with the number of total isolates in different rhizospheres. Overall, *Penicillium* spp. (6 isolates) outstands maintaining the trend from altitudinal variation. It was found that soil microbial communities didn't show a consistent altitudinal change along the studied elevation gradient.

Table 4.4 Morphologically identified soil mycoflora from different rhizospheric regions (total isolates = 36)

Reference Medicinal plants	Altitude (meters, asl)	Colony code	Fungi isolated	No. of colony/gm
Aconitum sp.	3721	31c	*Penicillium* sp.	$5.6x10^2$
		33a	*Cladosporium* sp.	$1.01x10^5$
		33b	*Fusarium* sp.	$1x10^3$
		33c	*Nigrospora* sp.	$2x10^3$
		33d	*Aspergillus* sp.	$1.01x10^5$
Nardostachys grandiflora (Jatamasi)	3769	41a	*Penicillium* sp.	$7x10^1$
		42c	*Penicillium* sp.	$1x10^2$
		45a	*Fusarium* sp.	$1x10^5$
Rhododendron anthopogon	3627	61a	*Trichoderma* sp.	$6.5x10^2$
		62a	*Crysosporium* sp.	$4x10^2$
		62b	Unidentified	$1x10^2$
		63a	*Aspergillus* sp.	$1x10^3$
		63b	*Verticillium* sp.	$6x10^3$
		63c	Unidentified	$1.3x10^4$
Dactylorhiza hatagirea (Panchaule)	3600	81a	*Mucor* sp.	$1x10^1$
		81b	Unidentified	$1x10^2$
		81d	*Penicillium* sp.	$6.1x10^2$
Swertia sp.	3668	91a	Unidentified	$1.26x10^3$
		91b	*Nigrospora* sp.	$8x10^1$
		92a	Unidentified	$2x10^2$
		93a	*Monilia* sp.	$1x10^3$
		94a	Unidentified	$1x10^4$
		94b	*Penicillium* sp.	$1x10^4$
		95a	*Rhizopus* sp.	$1x10^5$

Podophyllum hexandrum	3826	101a	*Verticillium* sp.	$1.12x10^3$
		101b	*Rhizopus* sp.	$5x10^1$
		102b	Unidentified	$7x10^2$
		103a	*Alternaria* sp.	$1x10^3$
		103b	*Monilia* sp.	$2.52x10^5$
		103d	*Fusarium* sp.	$1x10^3$
		104a	*Verticillium* sp.	$1x10^4$
		105a	*Nigrospora* sp.	$3x10^5$
Neopicrorhiza scrophylariiflora	4300	111a	*Monilia* sp.	*TNTC (Too Numerous to be Counted)*
		112a	*Penicillium* sp.	$1.2x10^3$
		113a	*Mucor* sp.	$1.03x10^5$
		113b	*Aspergillus* sp.	$1x10^3$

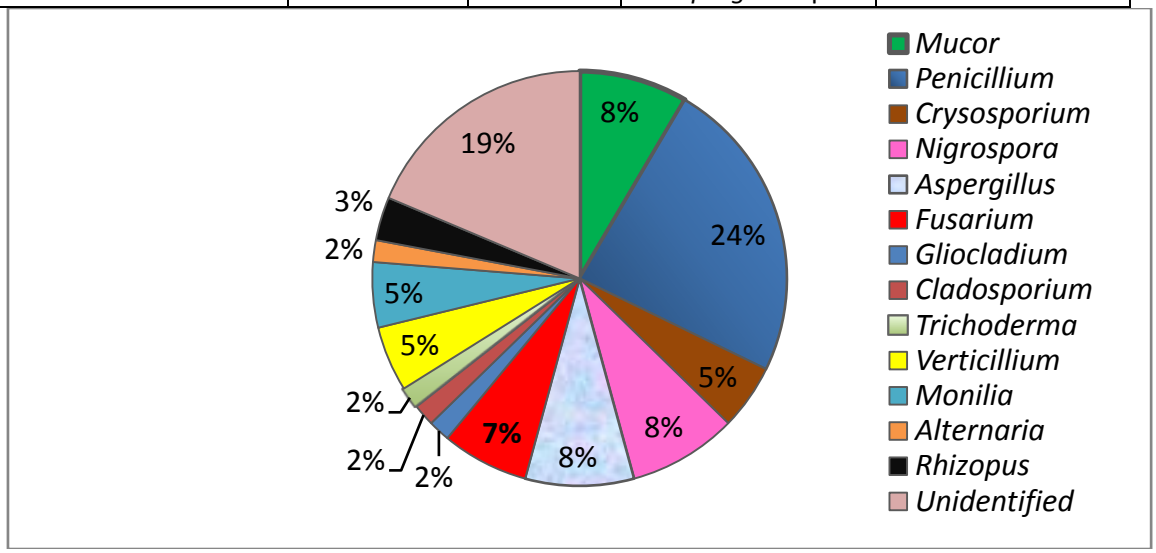


Figure 4.1 Percentage distributions of the different fungal isolates from MCA

The diversity of mycoflora in the MCA region is represented in Figure 4.1. In a total of 59 isolates, 14 (~ 24%) were *Penicillium* species, the dominant soil fungi. It is followed by 5 each of *Mucor, Nigrospora* and *Aspergillus* species. 11 isolates were yet unidentified by visualization techniques. The unidentified result may be due to lack of fruiting body or spore formation.

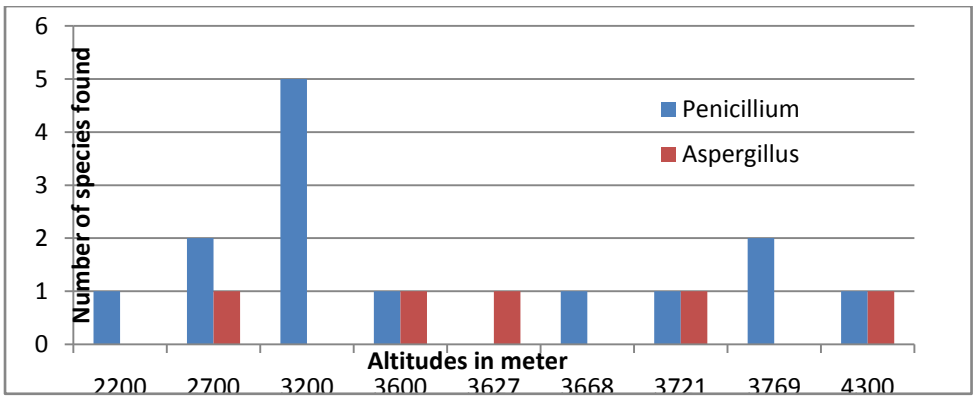


Figure 4.2 Graphical representation of distribution of *Penicillium* spp. and *Aspergillus* spp. in soil of different altitudes of MCA

The altitudinal variation in distribution of *Penicillium* and *Aspergillus* is shown in Figure 4.2. It represents the presence of *Penicillium* and *Aspergillus* species in each of the altitudes studied with higher rate of occurrence of *Penicillium* spp. at certain altitudes.

4.3 Screening of selected fungi for the production of extracellular enzymes

A total of 11 fungi that were morphologically identified as *Penicillum* spp. and *Aspergillus* spp. were tested for the production of biotechnologically important six different enzymes (Appendix 6) and the results are presented in Table 4.5.

Table 4.5 Enzyme screening of selected fungal strains

Sample code	**Source**	**Species**	Appearance of Halozone in different media for Enzyme Screening					
			Amylase	**Cellulase**	**Pectinase**	**Protease**	**Xylanase**	**Lipase**
A10	Altitudinal gradients	*Penicillium* sp.	-	+	+	-	-	-
A12		*Penicillium* sp.	+	+	+	+	+	-
A16		*Penicillium* sp.	-	-	-	+	-	-
A277		*Penicillium* sp.	+	+	+	+	+	+
A13		*Aspergillus* sp.	-	-	+	+	-	-
23		*Aspergillus* sp.	+	+	-	+	+	-
112a	Rhizospheric regions	*Penicillium* sp.	-	+	-	-	-	-
31c		*Penicillium* sp.	-	-	-	-	-	-
33d		*Aspergillus* sp.	-	+	-	+	-	-
63a		*Aspergillus* sp.	-	-	-	-	+	+
113b		*Aspergillus* sp.	+	+	-	-	+	-

The present study (Table 4.5) revealed that all the isolates, except 31c (*Penicillium* sp.), have shown extracellular enzyme activity with maximum isolates showing positive to cellulase (~64%) and Protease (55%). From the Table 4.5, it can be seen that the maximum positive enzyme assay was observed in A277 (*Penicillium* sp.), showing positive reaction to the entire enzymes tested; closely followed by A12 (*Penicillium* sp.) with positive towards five enzymes. However, strain 31c didn't respond to any of the enzymes.

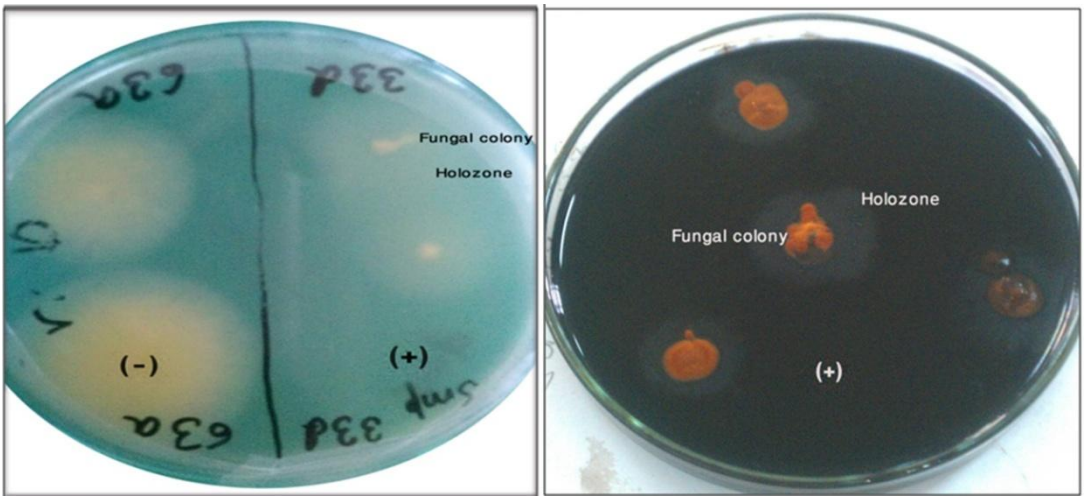


Plate 4.3 An example of enzyme screening A) Protease screening for sample 63a (*Aspergillus* sp.) and 33d (*Aspergillus* sp.) showing holozone (+) in 33d B) Amylase screening for sample A12 (*Penicillium* sp.) showing positive (+) with holozone.

4.4 DNA Extraction and Quantification

The DNA was successfully extracted from the mycelia of 59 samples. The quantification of extracted DNA was done by UV Biophotometer. The DNA concentration was found from 31 ng/µl to 2294.7 ng/µl. The OD_{260}/OD_{280} ratio was found to be more or less 1.8 showing good purity of samples (Appendix 9).

4.5 Molecular identification

Molecularly the isolates were studied by using two nuclear ribosomal regions. One was Internal Transcribed Spacer (ITS) region gene sequence (ITS1-5.8s-ITS2) flanked by 18S Small Subunit (SSU) and 28S Large Subunit (LSU). The second region was D1/D2 (5' D1-D2 3') region in 28S LSU rDNA. The results are presented in following section.

4.5.1 Molecular study based on nuclear ribosomal ITS region

4.5.1.1 PCR amplification

The PCR was done with universal primers ITS1 (forward) and ITS4 (reverse) amplifying ITS1-5.8s-ITS2 region. For the amplification of the ITS region, PCR condition was previously optimized in the laboratory by varying different parameters and the best condition was used as explained in methodology chapter.

The size of amplified ITS region in 1% agarose gel was approximately 600-700 bp which is shown in Plates 4.4 and 4.5. Out of 59 samples, 48 were successfully amplified.

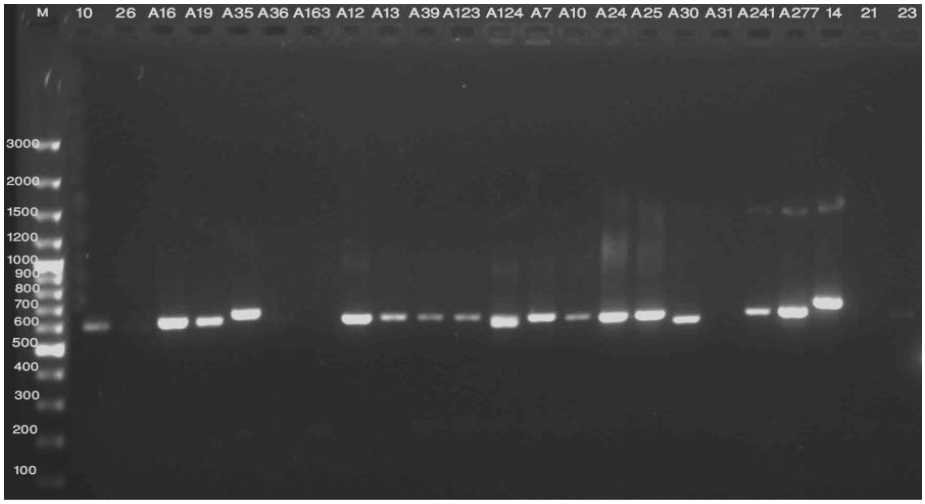


Plate 4.4 1% agarose gel electrophoresis of ITS region amplification of the 23 strains isolated from altitudinal variations with ITS primers ITS1/4. Lane marked with M is 100 bp plus molecular weight marker. Lanes 1 to 23 represent the respective labeled samples.

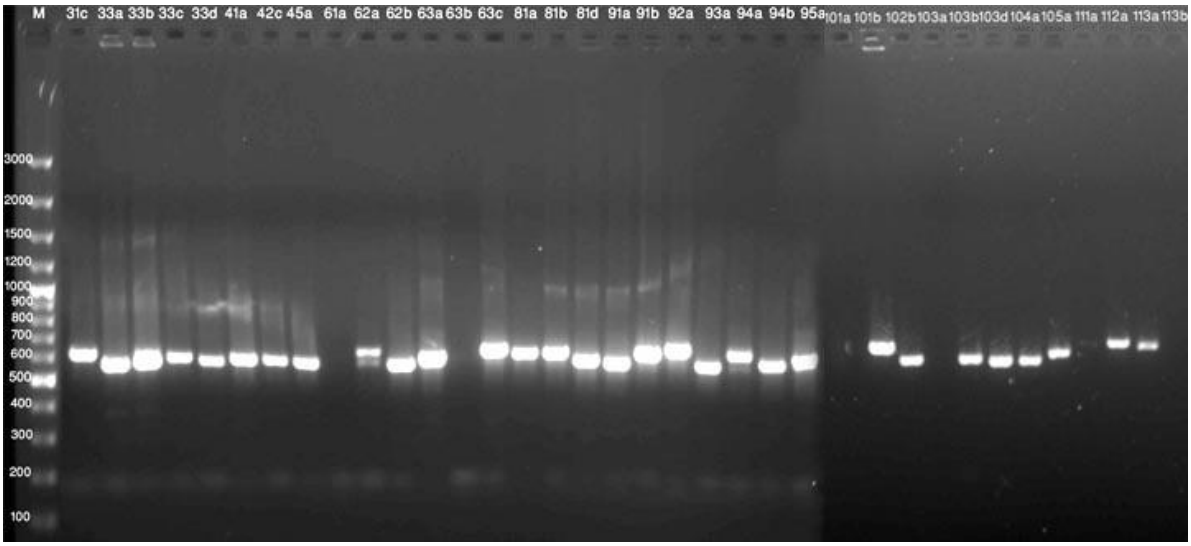


Plate 4.5 1% agarose gel electrophoresis showing ITS region amplification of the 36 strains isolated from different rhizospheric regions with ITS primers ITS1/4. Lane marked with M is 100 bp plus molecular weight marker. Lanes 1 to 36 represent the respective labeled samples.

A total of 11 strains, which were morphologically identified as *Penicillum* spp. and *Aspergillus* spp. and screened for the production of extracellular enzymes, were selected for the PCR amplification. From the selected 11 samples, 9 clearly amplified bands with ITS primers (Plate 4.6) were selected for sequencing. These include sample named A12, A16, A277, 23, 31c, 33d, 63a, 112a and A10 presented in Table 4.6.

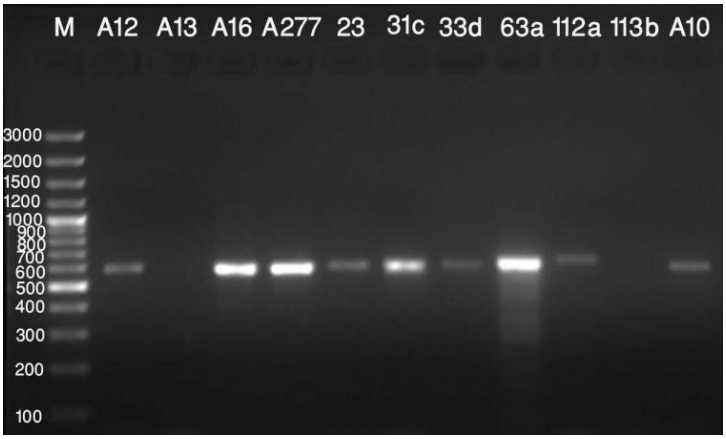


Plate 4.6 1% agarose gel electrophoresis of ITS region amplification of the selected species with ITS primers ITS1/4. Lane marked with M is 100 bp plus molecular weight marker. Lanes 1 to 11 represents the respective labeled samples.

Table 4.6 Generalization of the PCR success for the selected fungal species

Sample code	Source	Species	PCR Success	Sent for Sequencing
A12	Altitudinal variation	*Penicillium* sp.	Yes	Yes
A10		*Penicillium* sp.	Yes	Yes
A16		*Penicillium* sp.	Yes	Yes
A277		*Penicillium* sp.	Yes	Yes
A13		*Aspergillus* sp.	No	No
23		*Aspergillus* sp.	Yes	Yes
112a	Rhizospheric regions	*Penicillium* sp.	Yes	Yes
31c		*Penicillium* sp.	Yes	Yes
33d		*Aspergillus* sp.	Yes	Yes
63a		*Aspergillus* sp.	Yes	Yes
113b		*Aspergillus* sp.	No	No

The overall PCR success rate of 11 selected samples was 81.8% and thus 9 clearly amplified samples were sent for sequencing.

4.5.1.2 Nucleotide Sequencing

The success rate of sequences obtained from Macrogen Company, South Korea for the ITS region was only 22.22% (2 out of 9 samples were sequenced). The retrived sequences were edited, assembled and consensus sequences were prepared using CodonCode Aligner *v.4.2.2* software. The consensus sequences obtained are shown below.

Fungal Isolate 23 (*Aspergillus* sp)

1-
TCCGTTACGGGTGTTTTTTTTTTTGTTGGCGGCACCAAAAAGATCCCTCGTGAAATTGTTAATGGAATTGG
ATTACAATTACAACAGACCTCACCCCTTCTGAATATCTCAAAGGAATTCTAGAGGGCGGGGGCCGCGGTT
AAAACCCCCCGCGGCCCTGAAAGGGGGACCGCCGCCAGCAATTTAGGTACCGCAAACACAGGTGTGTGT
TGGGCTCGCTAGGAACCCTACACTCGGTAATGATCTTCCTCTTCTTATTGATATGAAAAAGTTCAGCGGGT
TTCCCTACCTGAAGCGAGCCCAACCTCCAACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCA
TTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCT
AGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCG
ATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAAC
GCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGC
TTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCG
ATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAAT
SKTTCCAAGGTGACCTCSGAAAAAGTAGGGACACCCTTTGATCTTTTTTTTTTATCAAAAAAAGGAGGAAG
ATCATTACCGTCGTGTAGGGATCCTAGCGAGACCAAATAACAACGTGTTGTGCTGTACCAAAAATGCGTC
GCGTGCCGCCTCTCGAGACCGTCTGAATATCTAAACCAGCCCCTGCGCCTT -964

Fungal Isolate 33d (*Aspergillus* sp)

1-
TGGTTGGACGTCGACTGGCGCCCGGCCGGCCCTAAATCGAGCGGGTGACAAAGCCCCATACGCTCGAGG
ACCGGACACGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGGGGGGAACAACCACCCAAACAAAACC
GGGATTGGAGGGCTTCAAAATTCTTTGGCATGATATGCCCTGAGAATTGCAGGGGTCACTGTTTCCTCTC
CTCTAATAAAGAATTTTGAATTCCTGAATTCCCATTACTTATCCCAGTTCGAAAGGGCTGCTGCCCATCAA
GACCCGTTAGATTGCTTTTCCAAGATAAAGACCGAGGTTATATTCCTACTTCAAAAGGGGCCCTCGTAAGC
TTTCCCTTTATTTACCGCGGGGGAGATCTCTTTTGATAAAGAGCCGAGCTTTTCCTTGCTGACTAAAAAAC
AGTTTTGGCAGTTTGAAAACCAAAAGGGTTGCAAACCCTTAACTTAAATATATTGATAAACGTACGGACG
GATCATTACTGAGTGCGGGCTGCCTTCGGGCCCAACCTCCACCCGCTGACTACCTAACCATWGATTGCTT
CGGCGGGGAGCCCTCTCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGT
CTGAGTCTGAATATAAAATCAGTCAAAACTTTCAACCATGGATCTCTTGGTTCCGGTATCATAAAAAACAC
ATTGAAAAGCATTGTGGAGGTAAATGATTTTTTTCGCATTTCCCTCCGTACTATTGCAAATTTTTTTCCCCC
CCTTACTACATAACCTATCACCTCTAGGGAGTGATGGGAATGATGGCCGAATTGCTTTATTTATTCCCTTTC
CGAGTGAAAGCACAGGGGTGCATAACTTTCTCGAAAGGTGACAAGGGAATATTTCCAGTTATTCACCAAA
GGAGATGTATCTTTTGAAAAAAAAGAGATTTTCCTTGATCCTAACAAAATGAATGACAGGCTCTTTGAACA
ACCAGAGGTTAGTTCGAAGGACCTTACCAACAGTTTTGGCAGGACGGAAGGATCTTT -1042

The assembled sequences were then compared to the available sequences in the NCBI database using NCBI-BLAST search tool. The sequences were confirmed with *Aspergillus* strains in comparison of the top hits from GenBank database. The BLASTn results for each isolate yielded identification to the species level with 98% and 94% identities respectively (Table 4.7). Thus, the result from ITS region was unclear.

Table 4.7 NCBI-BLAST analysis of sequenced strains

Sample ID	Length (bp)	Strain Name	GenBank Accession Number	Max Score	Total Score	Query Cover	E value	Ident
23	964	*Aspergillus flavus* isolate UOA/HCPF 5774	FJ878681.1	902	1010	66%	0.0	98%
33d	1042	*Aspergillus* sp. 7MM-2011 isolate MS57	JN021555.1	316	541	34%	2e-82	94%

4.5.1.3 Phylogenetic inferences

The pairwise and multiple alignments of the two species were done in MEGA5 and analysis is expressed in Table 4.8 and Appendix 10.1.

Table 4.8 Evaluation of ITS sequences and sequences alignment analysis

Parameters	Analysis
Universal ability of ITS1/ITS4 primers for amplification of ITS regions	Yes
Percentage sequencing success	22.22
Aligned sequence length (bp)	997
Number of conserved sites	383
Number of variable sites	552
Distribution of variable sites	Dispersive
Number of sample (individuals)	2

The present data from ITS sequencing contains insufficient phylogenetic information to provide strong relationship as the phylogenetic analysis for these samples was not possible because at least 3 samples are required to test the phylogeny.

Due to sequencing failure, the phylogenetic tree construction wasn't possible with ITS sequences. Therefore, sequences of ITS regions of the related species of present study were retrieved from NCBI GenBank database. The evolutionary relationship of the obtained sequences was analyzed by using MEGA5. The Maximum Parsimony (MP) tree was constructed using the Subtree-Pruning-Regrafting (SPR) algorithm with 1000 bootstrap replication for the ITS region reference sequences. The single tree was formed with tree length of 330 (Figure 4.3). The parsimony-informative (Pi) sites are 221 out of 704. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

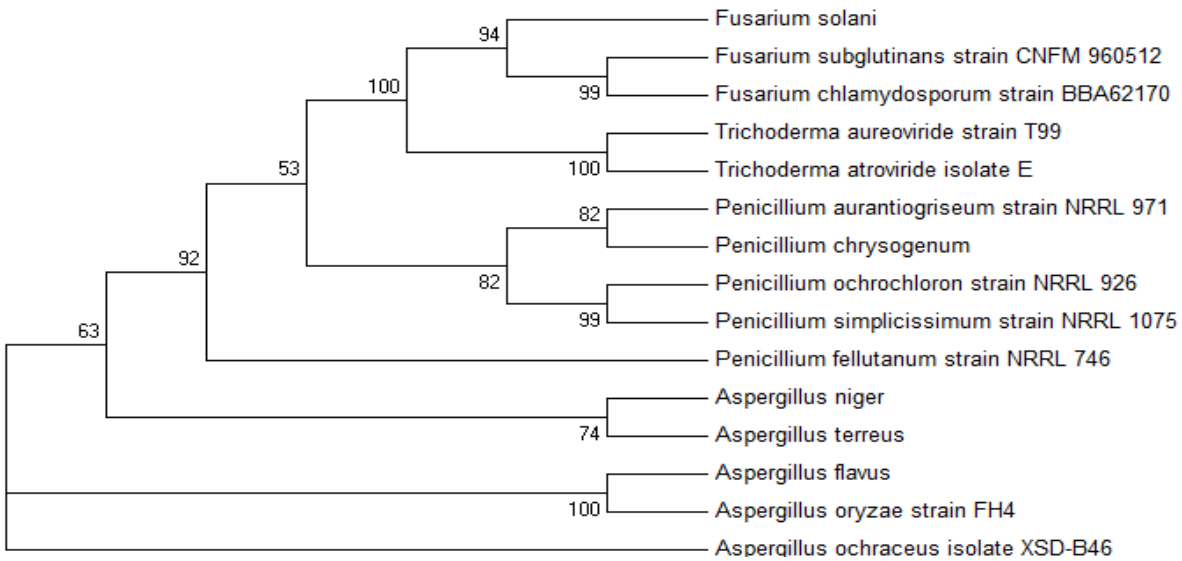


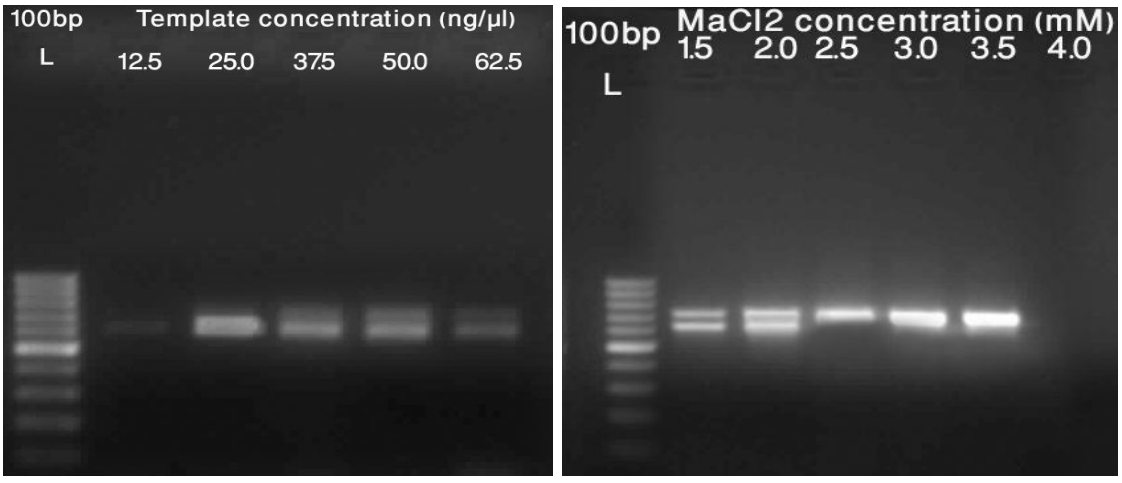
Figure 4.3 The Maximum Parsimony (MP) tree for ITS regions was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm in MEGA5.

Figure 4.3 showed that *Penicillium, Aspergillus, Trichoderma* and *Fusarium* form their own separate clade with over 75% bootstrap value. This stated that ITS regions are suitable to infer the evolutionary relationship between and within different fungal species. However, *Aspergillus ochraceus* showed distant relationship to the other species forming outgroup. *Fusarium* and *Trichoderma* formed sister clades with 100% bootsrap value while *Penicillim* spp. is sister to both *Fusarium* and *Trichoderma* with 53% clade support.

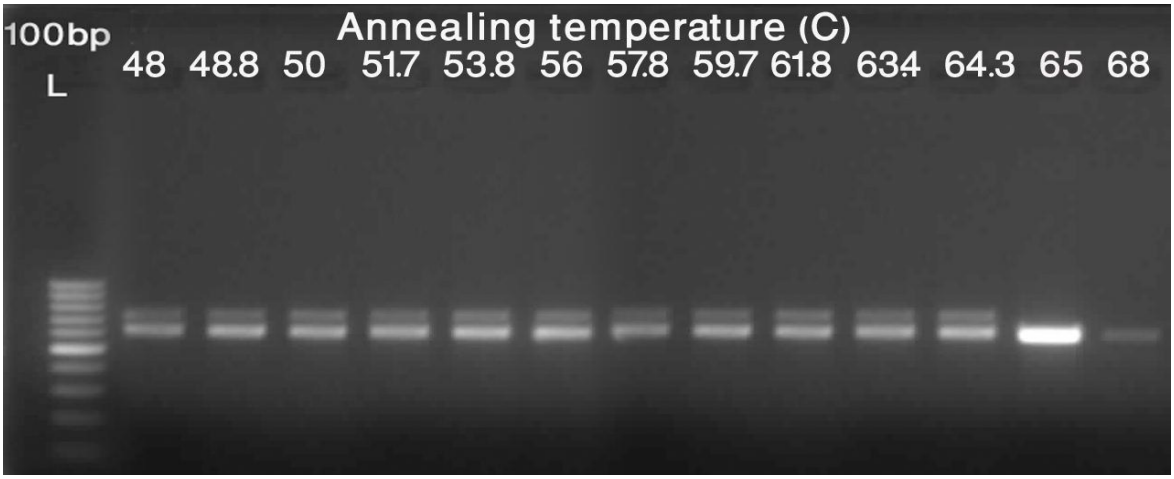
4.5.2 Molecular study based on D1/D2 domains of LSU nrDNA

4.5.2.1 PCR amplification

For the amplification of the D1/D2 region, PCR condition was optimized by varying various parameters and the best condition was selected for the amplification of D1/D2 region (Plate 4.7 and Table 4.9).



A) **B)**



C)

Plate 4.7: PCR conditions optimization of the D1/D2 domains of LSU nrDNA **A)** Template concentration (ng/µl) **B)** $MgCl_2$ concentration (mM) **C)** Annealing temperature (^{0}C)

Table 4.9 Summarization of tested and optimized PCR condition for D1/D2 domains of LSU nrDNA

PCR parameters	Tested Ranges	Optimized conditions	Remarks
Template concentration (ng/µl)	12.5, 25.0, 37.5, 50.0, 62.5	25 ng/µl	Absence of band in concentration of 12.5 ng and faint and double bands in higher concentrations (37.5 to 62.5 ng)
$MgCl_2$ concentration (mM)	1.5, 2.0, 2.5, 3.0, 3.5, 4.0	2.5 mM	Faint band was seen in lower concentration (1.5 and 2.0 mM) and no bands in 4.0 mM. The brighter single band with lower concentration (2.5 mM) of $MgCl_2$ was selected.
Annealing temperature (^{0}C)	48, 48.8, 50.0, 51.7, 53.8, 56.0, 57.8, 59.7, 61.8, 63.4, 64.3, 65, 68	65^{0}C	Single distinct band was observed at 65^{0}C. Double bands in lower temperatures (45 to 64.3 ^{0}C) and faint band in higher temperature (68^{0}C) was seen.

Thus, the optimized PCR reaction conditions comprised of 25 ng of template DNA, 12.5µl of 2X PCR Master Mix (Promega), 2.5 mM $MgCl_2$ and 1 µM each of forward and reverse primers. Similarly, the optimized PCR cycling condition comprises of initial denaturation of 95^{o}C for 4 mins followed by 30 cycles of 94^{o}C for 1 min, 65^{o}C for 2.5 mins and 72^{o}C for 2.5 min, with a final extension at 72^{o}C for 10 mins.

The size of the PCR amplified fragment of D1/D2 regions of various isolate varied from 600 to 700 bp. Out of 59 samples, 45 were successfully amplified (Plate 4.8).

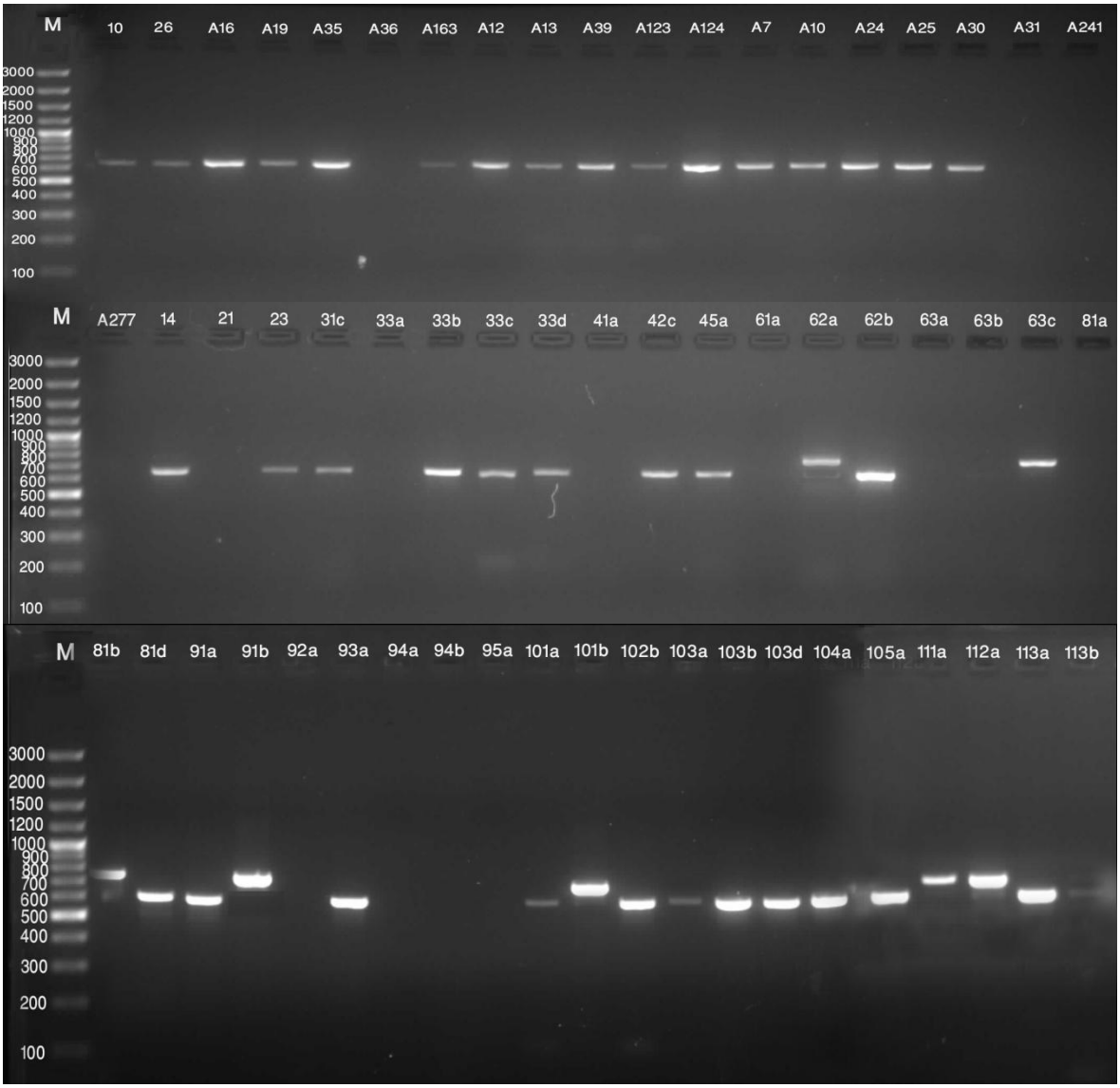


Plate 4.8 Agarose gel electrophoresis of D1/D2 region amplification of the 23 strains isolated from altitudinal variations and the 36 samples isolated from rhizospheric regions with D1/D2 primers NL1/NL4. Lane marked M is 100 bp plus molecular weight marker. Other lanes represent the respective labeled samples.

A total of 11 strains, which were morphologically identified as *Penicillum* spp. and *Aspergillus* spp. and that were screened for the production of extracellular enzymes, were chosen for the PCR amplification of D1/D2 region and 10 successfully amplified products were selected for sequencing (Plate 4.9). The selected samples include A12, A13, A16, A277, 23, 31c, 112a, 113b and A10 as presented in Table 4.9.

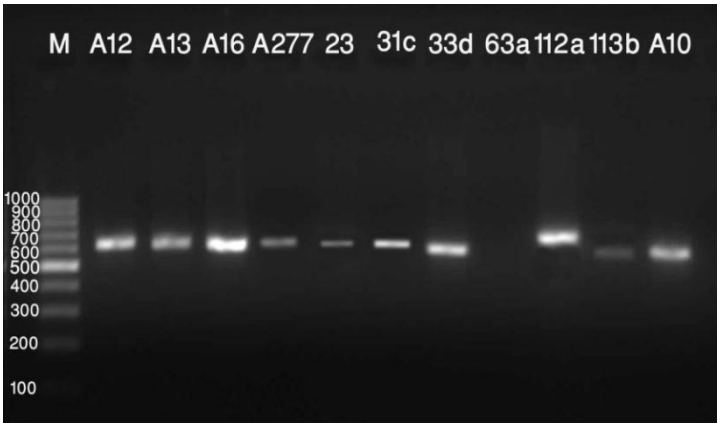


Plate 4.9 1% agarose gel electrophoresis of D1/D2 region amplification of the selected species with D1/D2 primers NL1/4. Lane marked with M is 100 bp plus molecular weight marker. Lanes 1 to 11 represents the respective labeled samples.

Table 4.10 Generalization of the PCR success for D1/D2 domains for the selected fungal species

Sample code	Source	Species	PCR Success	Sent for Sequencing
A12	Altitudinal variation	*Penicillium* sp.	Yes	Yes
A10		*Penicillium* sp.	Yes	Yes
A16		*Penicillium* sp.	Yes	Yes
A277		*Penicillium* sp.	Yes	Yes
A13		*Aspergillus* sp.	Yes	Yes
23		*Aspergillus* sp.	Yes	Yes
112a	Rhizospheric regions	*Penicillium* sp.	Yes	Yes
31c		*Penicillium* sp.	Yes	Yes
33d		*Aspergillus* sp.	Yes	Yes
63a		*Aspergillus* sp.	No	No
113b		*Aspergillus* sp.	Yes	Yes

The overall PCR success rate for the 11 selected samples was 90.9% and 10 clearly amplified samples were sent for sequencing.

4.5.2.2 Nucleotide Sequencing

The retrieved sequences from the Macrogen Company, South Korea were edited, assembled and consensus sequences were prepared using CodonCode Aligner *v.4.2.2* software. The assembled consensus sequences are given as follows:

Fungal Isolate A12 (*Penicillium* sp)

1-
TTGGTCCGTGGTTCAAGACGGGTCGCTTACGACCATTATGCCAGCGTCCGAGCCGAAGCGCGTTCCTCGG

TCCGGGCTGGCCGCATGGCACCCTTGGCTATAAGACGCCCCGGGGGGCGTTACATTCCAAGGGCCTTTG
ACCGGCCGCCCAAACCGACGCTGGCCCGCCCGCAGGGAAGTACACCGGCACGAATGCCGGCTGAACCCT
GCGAGCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACAATTTCACGTGCTGTTTAACTCTCTTTTCAAAGTG
CTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCCGGCCAGTATTTAGCTTTAGATGAAATTTAC
CACCCATTTAGAGCTGCATTCCCAAACAACTCGACTCGTCGAAGGAGCTTCACACGGGCGTGGGCACCCC
ATCCCAGACGGGATTCTCACCCTCTATGACGGCCCGTTCCAGGGCACTTAGATGGGGGCCACTCCCGAAG
CATCCTCTGCAAATTACAATGCGGACCCCGAGGGGGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTC
GCCGTTACTGAGGCAATCCCTGTTGGTTTCTTTTCCTCCGCTTTTTGATATGCAAAA - 620

Fungal Isolate A13 (*Aspergillus* sp)

1-
TTTTCCATATCCAATAGCGGAGGAAAAGAAACCAACCGGGATTGCCTCAGTAACGGCGAGTGAAGCGGC
AAGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGGTGCG
GCCCCTGTCTAAGTGCCCTGGAACGGGCCGTCAGAGAGGGTGAGAATCCCGTCTGGGATGGGGTGTCCG
CGCCCGTGTGAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCA
TCTAAAGCTAAATACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTT
GAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCGACCAGACTCGCCTCCAGG
GTTCAGCCGGCATTCGTGCCGGTGTACTTCCCTGGGGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAA
AGGCTCCCGGAATGTAGTGCCCTCCGGGGCACCTTATAGCCGGGAGTGCAATGCGGCCAGCCTGGACCG
AGGAACGCGCTTCGGCACGGACGCTGGCATAATGGTCGTAAACGACCCGTCTGAA -608

Fungal Isolate A16 (*Penicillium* sp)

1-
GGTCCGTTGTTCAAGACGGGTCGCTTACGACCATTATGCCAGCGTCCGAGCCGAAGCGCGTTCCTCGGTC
TAGGCAGGTCGCATTGCACCCTCGGCTATAAGACGCCCCTAGGGGCGTTACCTTCCGAGGGCCTTTGACC
GACCGCCCAAACCGACGCTGGCCCGCCCGCGGGGAAGTACACCGGCACGAATGCCGGCTGAACCCCGCG
AGCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACAATTTCACGTGCTTTTTAACTCTCTTTTCAAAGTGCTTT
TCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCCGGCCAATATTTAGCTTTAGATGAAATTTACCAC
CCATTTAGAGCTGCATTCCCAAACAACTCGACTCGTCGAAGGAGCTTCACACGGGCGCGGACACCCCATC
CCATACGGGATTCTCACCCTCTATGACGTCCCGTTCCAGGGCACTTAGATGGGGACCGCTCCCGAAGCAT
CCTCTGCAAATTACAATGCGGACCCCGAAGGAGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCC
GTTACTGGGGCAATCCCTGTTGGTTTCTTTTCCT -598

Fungal Isolate A277 (*Penicillium* sp)

1-
TTGCATATCCATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCA
AGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGGAGCG
GTCCCCATCTAAGTGCCCTGGAACGGGACGTCATAGAGGGTGAGAATCCCGTATGGGATGGGGTGTCCG
CGCCCGTGTGAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCA
TCTAAAGCTAAATATTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTT
GAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCGACCAGACTCGCTCGCGGG
GTTCAGCCGGCATTCGTGCCGGTGTACTTCCCCGCGGGCGGGCCAGCGTCGGTTTGGGCGGTCGGTCAA

AGGCCCTCGGAAGGTAACGCCCCTAGGGGCGTCTTATAGCCGAGGGTGCAATGCGACCTGCCTAGACCG
AGGAACGCGCTTCGGCTCGGACGCTGGCATAATGGTCGTAAGCGACCCGTCTTGAA -607

Fungal Isolate 31c (*Penicillium* sp)

1-
TTTGCATATCCAAAAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAACGGCGAGTGAAGCGG
CAAGAGCTCAAATTTGAAATCTGGCTCCTTCGGGGCCCGAGTTGTAATTTGGAGAGGATGCTTCGGGCGT
GGCCCCCATCTAAGTGCCCTGGAACGGGCCGTCATAGAGGGTGAGAATCCCGTCTGGGATGGGTGGTCC
CGCCCGTGTGAAGCTCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGAGGGTGGTAAATTTCA
TCTAAAGCTAAATATTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTT
GAAAAGAGAGTTAAACAGCACGTGAAATTGTTGAAAGGGAAGCGTTGGCAACCAGACTCGCCCGGGAG
GGCTCAGCCGGCACGTGTGCCGGTGCACTCCCCCCCGGGCGGGCCAGCGTCGGTTTGGGCGGTCGGTCA
AAGGCCCCGGGAATGTAGCACCCTCCGGGGTGCCTTATAGCCCGAGGCGCCATGCGACCTGCCCGGACC
GAGGAACGCGCTTCGGCTCGGACGCTGGCGTAATGGTTGTCAACGGCCCGTCTT -606

Fungal Isolate 33d (*Aspergillus* sp)

1-
TTTGGTCCGGGTGTTAAGACGGGTCGTTTGCAACCATTACGCCAGCGTCCGTGCCGAAGCGCGTTCCTCG
GTCCAGGCTGGCCGCATTGCACCCCAGGCTATAAGACGTCCCGGAGGACGATACATTCCTGGGGCCTTTG
ACCGGCCGCCCGAACCGACGCTGGCCCGGCCCCGGGGAAGTACACCAGCACGAATGCTGGCTGAACCCC
GAGGCCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACAATTTCACGTGCTGTTTAACTCTCTTTTCAAAGTG
CTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCCGGCCGGTATTTAGCTTTAGATGAAATTTA
CCACCCATTTAGAGCTGCATTCCCAAACAACTCGACTCGTCGAAGGAGCTTCACACGGGCACGGGCACCC
TGCCCAAGACGGGATTCTCACCCTCTCTGACGGCCCGTTCCAGGGCACTTAGACAGGGGCCGCACCCGAA
GCATCCTCTGCAAATTACAACTCGGACCCCGGAGGGGCCAGATTTCAAATTTGAGCTCTTGCCGCTTCACT
CGCCGTTACTGGGGCAATCCCGGTTGGTTTCTTTTCCTCCGCTAT -608

Fungal Isolate 112a (*Penicillium* sp)

1-
TTTGCATTTGCAATTCCAATAGCGGAGGAAAAGAAAATAACAATGATTTCCCTAGTAACGGCGAGTGAAG
AGGAAAGAGCTCAAAGTTGGAAACTGTTTGGCTTAGCTAAACCGTATTGTAAACTGTAGAAACATTTTCC
TGGCACGCCGGATTAATAAGTCCTTTGGAACAAGGCATCATGGAGGGTGAGAATCCCGTCTTTGATCCGA
GTAGTTGTCTTTTGTGATATGTTTTCAAAGAGTCAGGTTGTTTGGGAATGCAGCCTAAATTGGGTGGTAA
ATCTCACCTAAAGCTAAATATTTGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAG
AACTTTGAAAAGAGAGTTAAACAGTATGTGAAATTGTTAAAAGGGAACCGTTTGGAGCCAGACTGGTTT
GACTGTAATCAACCTAGAATTCGTTCTGGGTGCACTTGCAGTCTATACCTGCCAACAACAGTTTGATTTGG
AGGAAAAAATTAGTAGGAATGTAGCCTCTCGAGGTGTTATAGCCTACTATCATACTCTGGATTGGACTGA
GGAACGCAGCGAATGCCATTAGGCGAGATTGCTGGGTGCTTTCGCTAATAAATGTTAGAATTTCTGCTTC
GGGTGGTGCTAATGTTTAAAGGAGGAACACATCTAGTATATTTTTTATTCGCTTAGGTTGTTGGCTTAATG
ACTCTAAATGACCCGTCTTGAAACACGGAACCAAACAAAA -740

Fungal Isolate 113b (*Aspergillus* sp)

1-
ATAGCACCAACCCGAAGCTTGGTACCGTGTCTCAAGACGGGTCGTTTACGACCATTATGCCAGCGTCCGT
GCCGAAGCGCGTTCCTCGGTCCAGGCTGGCCGCATTGCACCCCTGGCTATAAGGTGCCCCGGAGGGCAC
TACATTCCAGGGGCCTTTGACCGGCCGCCCAAACCGACGCTGGCCCGCCCACGGGGAAGTACACCGGCA
CGAATGCCGGCTGAACCCCGCGGGCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACAATTTCACGTGCTGT
TTAACTCTCTTTTCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCCGGCCAGTATT
TAGCTTTAGATGAAATTTACCACCCATTTAGAGCTGCATTCCCAAACAACTCGACTCGTCGAAGGAGCTTT
ACACGGGCACGGACACCCCGCCCAAGACGGGATTCTCACCCTCTCTGACGGCCCGTTCCAGGGCACTTAG
ACGGGGGCCGCACCCAAAGCATCCTCTGCAAATTACAATGCGGACTCCGAAGGAGCCAGCTTTCAAATTT
GAGCTCTTGCCGCTTCACTCGCCGTTACTGAGGCAATCCCGGTTGGTTTCTTTTCCTCCGCTTAATGATATG
CAACGG -640

Fungal Isolate A277 (*Penicillium* sp)

1-
CCGGGTTTTTAAGACGGGTCGCTTACGACCATTATGCCAGCGTCCGAGCCGAAGCGCGTTCCTCGGTCCG
GGCTGGCCGCATGGCACCCTCGGCTATAAGACGCCCCGGGGGGCGTTACATTCCGAGGGCCTTTGACCG
GCCGCCCAAACCGACGCTGGCCCGCCCGCGGGGAAGTACACCGGCACGAATGCCGGCTGAACCCCGTGG
GCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACAATTTCACGTGCTGTTTAACTCTCTTTTCAAAGTGCTTTT
CATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCCGGCCAGTATTTAGCTTTAGATGAAATTTACCACC
CATTTAGAGCTGCATTCCCAAACAACTCGACTCGTCGAAGGAGCTTCACACGGGCGCGGGCACCCCATCC
CAGACGGGATTCTCACCCTCTATGACGGCCCGTTCCAGGGCACTTAGATGGGGGCCGCTCCCGAAGCATC
CTCTGCAAATTACAATGCGGACCCCGAAGGGGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCG
TTACTGAGGCAATCCCTGTTGGTTTCTTTTCCTC -597

Then the assembled sequences were compared to already available sequences in the NCBI database using NCBI-BLAST search tool. The sequences showed high similarities to *Penicillium* and *Aspergillus* strains when compared with the top hits from GenBank database. The BLASTn results for each isolate yielded identification to the species level with >99% identities (Table 4.11).

The length of the nucleotide sequence for each strain is summarized in Table 4.11. The length of D1/D2 region of 28S nrRNA gene in *Penicillium* species varied from 597 to 740 bp and in *Aspergillus* species from 606 to 640 bp.

Table 4.11 The NCBI-BLAST search result for the species identification of the selected isolates of present study

Sample code	Length (bp)	Strain Name	Accession Number	Max Score	Total Score	Query Cover	E value	Ident
A12	620	*Penicillium ochrochloron* strain UWFP 720	AY213619.1	1090	1090	99%	0.0	99%
A16	598	*Penicillium aurantiogriseum* strain DAOM 214787	JN938945.1	1094	1094	100%	0.0	100%
A277	607	*Penicillium aurantiogriseum* strain CBS 324.89	AB479286.1	1112	1112	99%	0.0	99%
31c	606	*Penicillium ruqulosum* strain DAOM 216317	JN938937.1	1083	1083	99%	0.0	99%
A10	597	*Penicillium simplicissimum* strain KUC5153	HM469430.1	1064	1064	98%	0.0	99%
112a	740	*Penicillium chrysogenum* strain CBS 306.48	AY213615.1	1312	1312	96%	0.0	99%
A13	608	*Aspergillus parasticus* strain BBEF4	KC589438.1	1098	1098	99%	0.0	99%
33d	608	*Aspergillus protuberus* isolate AFTOL-ID 5007	FJ176897.1	1101	1101	99%	0.0	99%
113b	640	*Aspergillus niger* strain KAML02	KC119204.1	1123	1123	96%	0.0	99%

The isolate named 23 wasn't successfully sequenced with D1/D2 but with ITS region sequencing and analysis, has enabled it to be identified as the strain *Aspergillus flavus* (Table 4.7). In contrast, the ITS region for species 33d was not clearly able to identify the species for *Aspergillus* isolate (Table 4.7) but D1/D2 sequencing helped to identify it as *Aspergillus protuberus* (Table 4.11). In addition, for 112a, the alignment of D1/D2 sequences with pre-existing sequences of NCBI database showed 99% similarlity to *Penicillium* species as well as to *Mucor* species (not shown), which showed unclear decision for the identification even in genus level. This was clearly identified as *Penicillium* species by morphological analysis (Plate 4.2G, Table 4.7). Thus, it was supposed to be *Penicillium chrysogenum* (Table 4.10). Therefore, our results suggest that both molecular and morphological analyses need to be carried out for reliable fungal species identification.

4.5.2.3 Phylogenetic inferences

4.5.2.3.1 Evaluation of Nucleotide differences

A matrix of the nucleotide differences for nine strains was generated using MEGA5 to evaluate the usefulness of these sequences for identification. Table 4.12 showed the pairwise differences in the number of nucleotides in the studied species. Evolutionary

analyses were conducted in MEGA5. The analysis involved 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 555 positions in the final dataset.

Table 4.12 Estimates of Evolutionary Divergence between Nucleotide Sequences

	Penicillium ochrochloron (A12)	*Aspergillus parasticus* (A13)	*Penicillium aurantiogriseum* (A16)	*Penicillium aurantiogriseum* (A277)	*Penicillium ruqulosum* (31c)	*Aspergillus protuberus* (33d)	*Penicillium chrysogenum* (112a)	*Aspergillus niger* (113b)
Penicillium ochrochloron (A12)								
Aspergillus parasticus (A13)	265							
Penicillium aurantiogriseum (A16)	21	269						
Penicillium aurantiogriseum (A277)	270	26	273					
Penicillium ruqulosum (31c)	281	44	284	39				
Aspergillus protuberus (33d)	36	272	42	278	286			
Penicillium chrysogenum (112a)	294	155	290	155	156	299		
Aspergillus niger (113b)	28	271	33	275	286	24	296	
Penicillium simplicissimum (A10)	8	262	17	267	278	30	296	24

We can observe from Table 4.14 that generally the number of differences was distributed in the range of 20-40 nucleotides. The smallest difference of 8 nucleotides was found between *Penicillium simplicissimum* (A10) and *Penicillium ochrochloron* (A12). The largest difference, 299 nucleotides, was between *Aspergillus protuberus* (33d) and *Penicillium chrysogenum* (112a).

4.5.2.3.2 Study of Nucleotide substitution pattern

The nucleotide substitution pattern was estimated in MEGA5 and represented in Table 4.13. Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100.

Table 4.13 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	**T**	**C**	**G**
A	-	*5.78*	*7.22*	**21.91**
T	*5.96*	-	**4.29**	*7.45*
C	*5.96*	**3.43**	-	*7.45*
G	**17.53**	*5.78*	*7.22*	-

Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. The transition rate is highest for G to A substitution while the transversional substitutions are minimal.

Disparity Index Test of Substitution Pattern Homogeneity was performed using MEGA5 (Table 4.14). The sequences that have evolved with the same pattern of substitution was judged from the extent of differences in base composition biases between sequences (Disparity Index test) and a Monte Carlo test (1000 replicates) was used to estimate the P-values.

Table 4.14 Test of the Homogeneity of Substitution Patterns between Sequences

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	*Penicillium ochrochloron* (A12)		2.41	0.07	2.64	2.33	0.00	8.63	0.00	0.00
2	*Aspergillus parasticus* (A13)	**0.00**		3.23	0.00	0.00	2.74	5.22	2.80	2.35
3	*Penicillium aurantiogriseum* (A16)	**0.04**	**0.00**		3.44	3.26	0.01	8.28	0.01	0.19
4	*Penicillium aurantiogriseum* (A277)	**0.00**	1.00	**0.00**		0.07	2.99	4.77	3.06	2.60
5	*Penicillium ruqulosum* (31c)	**0.00**	1.00	**0.00**	0.14		2.63	6.50	2.70	2.22
6	*Aspergillus protuberus* (33d)	1.00	**0.00**	0.35	**0.00**	**0.00**		9.12	0.00	0.00
7	*Penicillium chrysogenum* (112a)	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**		9.03	9.16
8	*Aspergillus niger* (113b)	1.00	**0.00**	0.28	**0.00**	**0.00**	1.00	**0.00**		0.02
9	*Penicillium simplicissimum* (A10)	0.32	**0.00**	**0.00**	**0.00**	**0.00**	1.00	**0.00**	0.29	

The Table 4.13 showed the probability of rejecting or accepting the null hypothesis by estimating the P-values which are shown below the diagonal. P-values smaller than or equals to 0.05 (marked with **Bold**) are considered significant. The estimates of the disparity index per site are shown for each sequence pair above the diagonal. Higher the disparity index per site, lower is the P-value and test is more significant. The result

showed that the null hypothesis can be rejected in 31% of the genes at the 5% significance level.

4.5.2.3.3 Multiple and Pair-wise Alignment of sequences

A total of 670 bp of nrDNA D1/D2 nucleotide sequence from identified *Penicillium* spp. and *Aspergillus* spp. were aligned in MEGA5. The pairwise and multiple alignments of the nine species were performed using CLUSTAL W. The result from Clustal W is summarized in Table 4.15 and Appendix 10.2.

Table 4.15 Evaluation of D1/D2 domains sequences and sequences alignment analysis

Parameters	Analysis
Universal ability of NL1/NL4 primers for amplification of D1/D2 domains	Yes
Percentage sequencing success	90
Aligned sequence length (bp)	670
Number of conserved sites	117
Number of variable sites	423
Distribution of variable sites	Dispersive
Number of sample (individuals)	9

The number of singleton sites was determined in the aligned sequences by MEGA5. The 81 singleton site highlighted with the respective base pair location is shown in Figure 4.4. A singleton site contains at least two types of nucleotides with at most one occurring multiple times. MEGA identifies a site as a singleton site if at least three sequences contain unambiguous nucleotides.

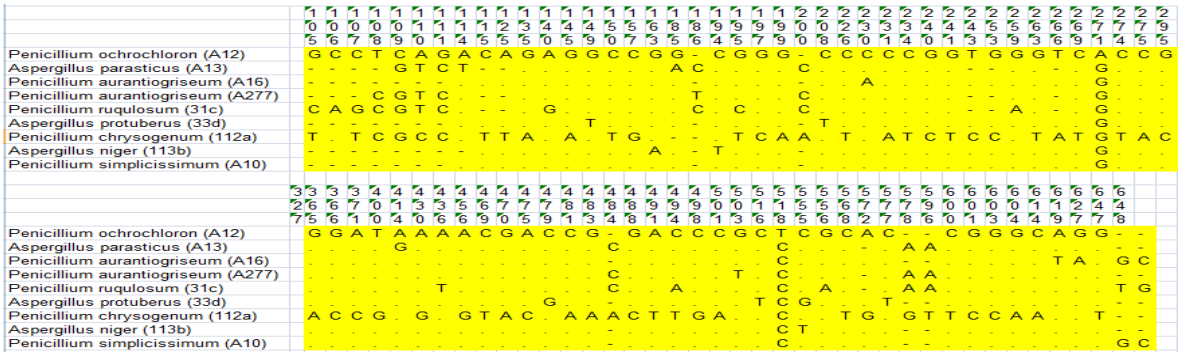


Figure 4.4 Singleton sites (81/670) for the D1/D2 domain.

4.5.2.3.4 Construction of Phylogenetic tree

The likelihood score was computed in jModelTest *ver.2.1.4* software with number of substitution schemes 7 (i.e. NumModels 56) including models with equal/unequal base frequencies (+F), including models with/without a proportion of invariable sites (+I) and including models with/without rate variation among sites (+G). The base tree for likelihood calculations was ML (Maximum likelihood). After completing the computation of likelihood scores, Bayesian information criteria (BIC) calculations was done in

jModelTest with confidence interval of 95% to find the best-fit DNA model. There were 8 models in the 95% confidence interval *viz.,* K80, K80+G, TPM1, TPM1+G, TrNef, K80+I, TrNef+G and TIM1ef. The K80 (Kimura 1980) model was determined as best-fit model with the least value of BIC (Figure 4.5).

```
  Sample size: 538.0

Model selected:
 Model = K80
partition = 010010
-lnL = 2543.2854
K = 17


Tree for the best BIC model = ((112a:0.40116816,31c:0.04082871):0.02784463,A277:0.03005491,
((A10:0.00000001,(A12:0.01493054,(A16:0.02878129,(33d:0.03854125,113b:0.01786650):0.02727795):
0.00474934):0.00252553):1.06446871,A13:0.00381858):0.03017595);


* BIC MODEL SELECTION : Selection uncertainty

Model             -lnL    K         BIC      delta      weight cumWeight
-----------------------------------------------------------------------
K80          2543.2854   17   5193.4645     0.0000      0.3788    0.3788
K80+G        2540.5357   18   5194.2529     0.7885      0.2554    0.6341
TPM1         2541.3941   18   5195.9696     2.5052      0.1082    0.7424
TPM1+G       2538.6456   19   5196.7605     3.2961      0.0729    0.8152
TrNef        2542.0913   18   5197.3641     3.8997      0.0539    0.8691
K80+I        2542.2570   18   5197.6954     4.2310      0.0457    0.9148
TrNef+G      2539.6614   19   5198.7921     5.3276      0.0264    0.9412
TIM1ef       2540.1972   19   5199.8638     6.3993      0.0154    0.9566
```

Figure 4.5 BIC calculations carried out in jModelTest. K80 with lowest BIC value (5193.4645) was the best-fit model.

After determining the best model, model-averaged phylogeny was computed with K80 model. In phylogenetic averaging, criterion for tree weights was BIC and strict consensus type was chosen with confidence interval 95%. The unrooted strict consensus tree was constructed accordingly using K80 model (Figure 4.6).

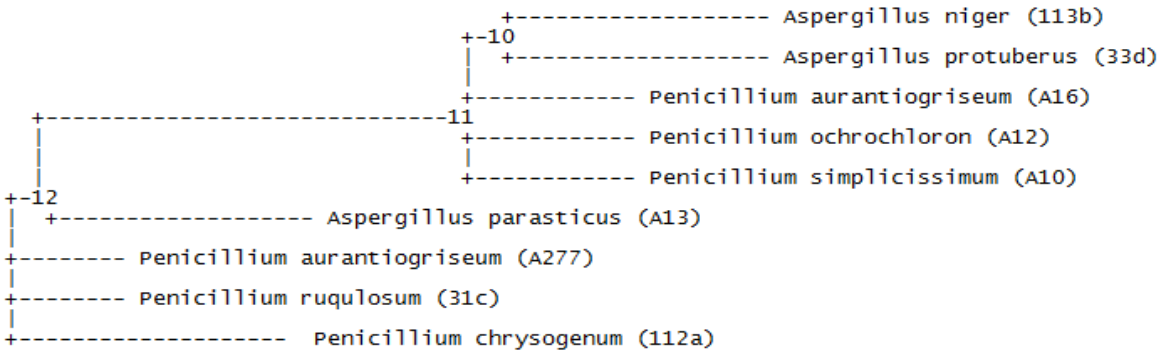


Figure 4.6 Strict consensus tree generated with confidence interval of 0.95 and selection criterion by BIC using K80 model. The number labeled indicates the branch length. Branch lengths are the expected number of substitutions per site. Analysis was done in jModelTest.

Three major clades were visible in strict consensus tree from Figure 4.6. The *Aspergillus* spp. and *Penicillium* spp. formed separate clade with branch length 10 and 11 respectively. However, *Aspergilllus parasticus* has shown to be sister species to other *Penicillium* spp and *Aspergillus* spp.

The Maximum Parsimony (MP) tree was constructed using the Subtree-Pruning-Regrafting (SPR) algorithm with 1000 bootstrap replication. The most parsimonious tree length of 490 is shown in Figure 4.7.

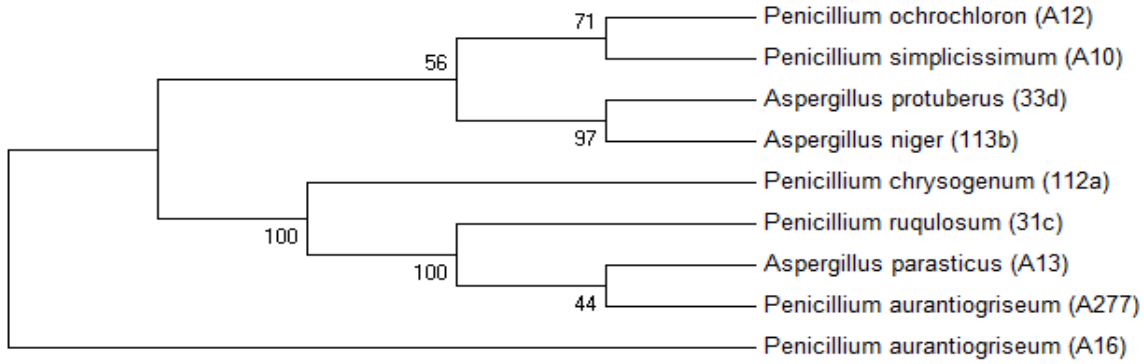


Figure 4.7 The Maximum Parsimony tree for D1/D2 domains obtained using the Subtree-Pruning-Regrafting (SPR) algorithm in MEGA5.

The consistency index and the retention index value is 0.906122 and 0.936022 respectively. The parsimony-informative (Pi) sites are 301 out of 670. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. This data suggested that the D1/D2 domain can be used for the identification of filamentous fungi and related species. The analysis involved 9 fungal isolate sequences. All positions containing gaps and missing data were eliminated. There were a total of 556 characters in the final dataset.

The phylogenetic analyses, conducted in MEGA5 (Figure 4.7) showed that *Penicillium* spp. and *Aspergillus* spp. under study are mostly monophyletic. In the cladogram, two nodes majorly represent the group of sub-clades with 56% and 100% bootstrap values respectively. However, the sub-clades didn't represent the clear inter- and intra- specific nucleotides difference patterns. Nevertheless, the tree of Figure 4.7 showed that *Penicillium ochrochloron* is the sister terminal to *Penicillium simplicissimum* and *Aspergillus protuberus* to *Aspergilllus niger* spp. While, the clades of *Aspergillus protuberus* and *Aspergilllus niger* with *Penicillium ochrochloron* and *Penicillium simplicissimum* form a sister groups. But, the two species *Aspergillus parasticus* and *Penicillium aurantiogriseum* (A277) formed a single sub-clade showing paraphyletic relationship. The farthest distance/ relationship was shown by *Penicillium aurantiogriseum* (A16). *Penicillium ochrochloron* and *Penicillium simplicissimum* formed a single clade in both the phylogeny of ITS region (Figure 4.3) and D1/D2 domains (Figure 4.7), though the strains of the species were different. Thus, the phylogenetic relationships recovered by the two markers show general congruence, but are not identical for all nodes.

The strict consensus tree from jModel Test (Figure 4.6) and the Maximum Parsimony tree from MEGA5 (Figure 4.7) using different substitution models, gave similar tree topologies but former differ slightly in regard to the position of some of the isolates within the bottom clade.

Furthermore, the D1/D2 domain sequences for some of the reference fungi (*Fusarium* spp. and *Trichoderma* spp.) were pulled from NCBI GenBank database and were aligned by Clustal W and included in the phylogenetic analysis using MEGA5 (Figure 4.8).

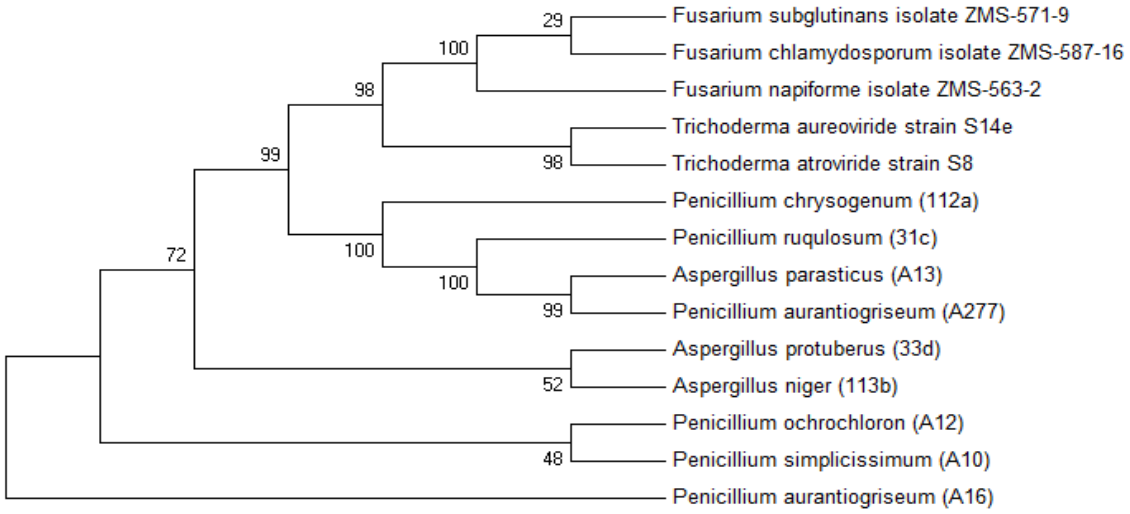


Figure 4.8 The MP tree with reference species of *Fusarium* and *Trichoderma,* obtained using the Subtree-Pruning-Regrafting (SPR) algorithm in MEGA5.

The evolutionary history was inferred using the Maximum Parsimony method. Five MP trees were generated and the MP tree with most parsimonious trees length of 333 is shown in Figure 4.8. The consistency index is 0.869841 and the retention index is 0.934921. Out of 670 characters used in the analysis, 387 sites were parsimony-informative (Pi) sites. Four major clades were visible from Figure 4.8. The results for the nine species under studies were principally not affected by adding other examined species of *Trichoderma* and *Fusarium*. The nine species under study formed three different clades while all the examined species were separated in its distant clade. In addition, *Trichoderma* and *Fusarium* form sister groups with each other with 98% bootstrap value and related to other species of *Penicillium* and *Aspergillus*. This result was also observed in case of ITS region phylogeny (Figure 4.3). The *Penicillium aurantiogriseum* (A16) still showed the distant relationship to the other species (Figure 4.7 and 4.8).

CHAPTER 5
DISCUSSION

5.1 Fungal diversity in soil sample of Manaslu Conservation Area (MCA) based on morphology

5.1.1 Site for sample collection

Manaslu Conservation Area (MCA) is the ongoing thematic project of NAST working on working on various aspects of flora, medicinal plants and microbes of high altitudinal regions of MCA. The present research was designed to study the soil fungi from high elevation regions of MCA. The soil samples were collected from the altitudes of 1700 to 3600 m at the difference of 500 m and also from rhizospheric regions of seven different reference medicinal plants. Altitudinally-defined climate conditions provide specific vegetation types and soil environments that could influence soil microbial communities, which in turn may affect microbial residues (Zhang et al*.,* 2013). The fungal diversity obtained from these regions represents the diversity of soil fungi in MCA region, Gorkha, Nepal.

5.1.2 Isolation and morphological identification of soil fungi

The different types of fungi were isolated from 12 different soil samples following soil plate dilution method from 1 gm of soil. The isolates were identified by morphological analysis. In this study, total of 59 fungi were isolated. Out of these 23 were from soil of different altitudes with variation of 500 m and 36 from rhizospheric regions of seven different reference plants. Hyphae, filamentous body, interact with soil particles near plant roots that promote foraging for food (Hoorman, 2011) and this might be the reason for the increase in number and types of fungi found in plant roots.

Prior to the development of molecular tools, morphological identification was the sole technique used for fungal species identification (Shrestha, 1989; Watanabe, 2002). And, the morphology-based species identification of fungi is the fundamental task to proceed for other advanced research in molecular biology and biotechnology (Watrud et al., 2006). Fruiting structures, spores, mycelia, growing habits, and morphologies of various organs in culture plates (Watanabe, 2002; Aneja, 2008) are basically observed for the morphological identification of fungi. By compound microscope, identification of fungi is possible and the names of fungi may be determined by comparing with already known species. Based on spores, hyphal septum and arrangement of nucleus, fungi are easily classified (Watanabe, 2002). In the present study, the total fungal isolates were identified up to genus level. While the identification up to species level is also possible by morphological analysis (Shrestha, 1989) but it is troublesome. Fungal isolates from both the cases i.e. altitudinal and rhizospheric regions, the *Penicillium* spp. were most dominant. In total strains, there were 14 *Penicillium* spp., have most dominant which was then followed by species of *Aspergillus, Mucor, Nigrospora, Alternaria, Monilia, Fusarium, Verticillium, Trichoderma, Cladosporium.* The isolates were determined as

saprophytic fungi having high industrial, medical and biotechnological importance. This result is also supported by many reports such as Watanabe (2002) and Paul (2007) who expressed that *Penicillium* species are the dominant soil fungus found in most of the soil type. Waksman (1916) stated that among soil mycoflora, four genera of fungi viz. *Penicillium, Aspergillus, Mucor* and *Trichoderma*, live commonly in soil of any location. Besides, other dominant genera include *Alternaria, Cephalosporium, Cladosporium, Fusarium, Rhizopus and Verticillium*. Various types of vegetation are found in different climatic zones in higher altitudes (Rana and Giri, 2010). The vegetation is either harmed (acting as pathogens) or enhanced (acting as biofertilizers) by the soil fungal communities. Besides, soils of high altitudes may harbor tremendous diversity of economically important fungi that can be exploited for commercial purpose.

5.2 Enzyme assay of selected fungi of MCA

Fungi found in higher altitude produce extracellular enzymes as the basis of their adaptation (Bhuju et al., 2007) in harsh climatic conditions. Fungi have the ability to produce various potential enzymes having their application in industrial sector (Dalboge, 1997). Different enzymes like amylase, pectinases, proteases, cellulases, xylanases, lipases, and recently cutinases have been reported to be produced by fungi with the application in the various industries producing detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products (Prabakaran et al*.,* 2009). Therefore, fungi are important industrial microorganisms for the large-scale production of enzymes.

In this investigation, *Penicillium* spp. were dominant in the studied areas of MCA region and it is of major importance in the environment, food and drug production (Mathew et al*.,* 2008; Jayant et al*.,* 2011; Tiwari et al*.,* 2011). Similarly, it has been reported that *Aspergillus* species have high potential to produce industrially important extracellular enzymes such as amylase, cellulase, lipase, protease and pectinase (Guimarães et al*.,* 2006; Prabakaran et al*.,* 2009). Significantly higher levels of production of extracellular enzyme were observed for the *Aspergillus* and *Penicillium* spp. among the different soil isolates in the research by Mishra and Dadhich (2010). Thus, a total of 11 fungi that were morphologically identified as strains of *Penicillium* and *Aspergillus* were chosen to screen for the production of extracellular enzymes of biotechnological important.

In the enzyme screening study, the examined species of *Penicillium* and *Aspergillus* showed high potential of producing celluase and protease. This result was also supported by findings of Khatri, 2012 who showed that the *Aspergillus* spp. from high altitudes of MCA have high potential of producing cellulase enzyme. In case of sample A277 (morphological identified as *Penicillium*) showed highly positive towards all the six enzymes tested. This result was supported by findings of Petruccioli et al. (1988). He

found that the *Penicillium* spp. have high potential for the production of extracellular cellulase enzyme. However, the capacity of microorganisms to produce extracellular enzymes is influenced by environmental conditions such as temperature, pH, aeration, inoculum age and the presence of inducer or repressor substrates (Piccoli-Valle et al., 2001; Khatri, 2012). Thus, the higher production of these enzymes in different environmental conditions can be studied and can be utilized further for the commercial purpose. In addition, the temperature of soil from where fungi were isolated was about 10-15^{0}C and the isolates are also capable of growing at temperature as high as 30-32^{0}C during summer. Thus, these cold tolerant high altitude microorganisms may have acquired some adaptive modification in response to change in temperature such as changes in enzymes, membrane structure or resistant genetic makeup. Very few researches have been carried out in the production and characterization of the high altitude fungal enzymes from Nepal, so this research is one of the initiatives which stimulate the researchers to carry out further research on such field.

5.3 Molecular study of soil fungi of MCA

The eleven selected fungal species were further characterized by molecular techniques using ITS and D1/D2 domains of nuclear ribosomal DNA.

5.3.1 DNA extraction and quantification

The DNA was extracted by using Modified CTAB Method from the mycelium of the 3-5 days fungal culture. The present investigation found that gentle crushing of mycelia on mortar and pestle with buffer offer quicker and more prominent lysis of fungal cell wall. The quantification of extracted DNA was done by using UV-Biophotometer. Nucleic acids absorb ultraviolet light in a specific pattern. For a 1-cm pathlength of the uvette in biophotometer, the optical density at 260 nm (OD_{260}) equals 1.0 corresponds to a concentration of 50 µg/ml for double-stranded DNA. To determine the concentration of DNA in the original sample, calculation involves: dsDNA concentration = 50 µg/mL × OD_{260} × dilution factor (Carlos et al., 2007) which is calculated and displayed in UV-biophotometer. The ratio of absorptions at 260 nm vs 280 nm is commonly used to assess DNA contamination of protein solutions, since proteins (in particular, the aromatic amino acids) absorb light at 280 nm (Carlos, et al., 2007; Sambrook and Russell, 2001). Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8; pure RNA has an OD_{260}/OD_{280} ratio of ~2.0. Low ratios could be caused by protein or phenol contamination (Carlos et al., 2007). Contamination of nucleic acid solutions makes spectrophotometric quantitation inaccurate (Carlos et al., 2007) and the quality and purity of the extracted nucleic acids is vital for successful PCR amplification of target genomic DNA/RNA (Anderson and Cairney, 2004). For the extracted DNA of fungal isolates in this investigation, the concentration ranged from 31 ng/µl to 2294.7 ng/µl. For some of the fungi, the

arbitrarily taken mycelium amount may not be sufficient enough to extract higher DNA or the process following the standard kit can enhance the concentration and quality of DNA extracted. Similarly, the OD_{260}/OD_{280} ratio was found to be more or less 1.8 with few towards 2.0, which might be due to RNase concentration incorporated into Tris-EDTA (TE) buffer that was used to resuspend the precipitated DNA. The RNase in TE might not have been enough.

5.3.2 PCR conditions

PCR-based molecular approaches are now being increasingly used to provide a more rapid and objective identification of fungi compared to traditional phenotypic methods. Ribosomal targets, especially the large-subunit RNA gene (D1-D2 region) and internal transcribed spacers 1 and 2 (ITS1 and ITS2 regions), have shown particular promise for the molecular identification of some fungi (Hinrikson et al., 2005). For the correct species identification, sequences other than ITS and D1/D2 *viz.,* nuclear small ribosomal subunit (SSU) gene, partial *β-tubulin A* (*BenA)* or *Calmodulin* gene sequences, or partial elongation factor 1-α (EF-1α) sequences or other protein-coding genes (Khatri, 2012; Kwiatkowski et al*.,* 2012; Seifert et al*.,* 2007) can also be employed to generate species specific DNA barcodes. The genetic polymorphisms at the molecular level can also be studied by other PCR-based molecular techniques such as Random Amplified Polymorphic DNA (RAPD) marker or PCR-RFLP (PCR-Restriction Length Polymorphic DNA) marker techniques (Michelmore and Hulbert, 1987).

5.3.2.1 Optimization of PCR conditions

A successful PCR assay requires efficient and specific amplification of the product (Bip-rad, 2013). The use of polymerase chain reaction (PCR) to generate large amounts of a desired product can be a double-edged sword. Failure to amplify under certain conditions can lead to the generation of multiple undefined and unwanted products, even to the exclusion of the desired product, the presence of nonspecific background bands due to mis-priming or mis-extension of the primers, the formation of primer-dimers that compete for amplification with the desired product, and mutations or heterogeneity due to mis-incorporation (Strachan and Read, 1999; Roux, 2009; Babec, 2012). A typical response at this point is to vary one or more of the many parameters (PCR reactions and cycling conditions) that are known to contribute to primer-template fidelity and primer extension (Roux, 2009). High on the list of optimization variables are Mg++ concentrations, template DNA concentrations, or buffer pH and cycling conditions. In regard to the last, the annealing temperature is most important (Roux, 2009).

In this investigation, the PCR reactions and annealing temperature were optimized for D1/D2 domains of fungal nrDNA LSU region.

5.3.2.1.1 Optimization of PCR reactions

In order to maintain reproducible banding patterns optimization of PCR reaction parameters is necessary. High on the list of optimization variables are Mg++ concentrations, template DNA (tDNA) concentrations, or buffer pH, Primer concentration, dNTPs concentration (Palumbi, 1996; Roux, 2009; Bio-rad, 2013; Shrestha, 2013). In the present study, the tDNA concentration and $MgCl_2$ concentrations were critically optimized.

The template carries the DNA segment or target that is wished to amplify. The quality and quantity of the extracted DNA is vital for successful PCR amplification of target sequences. High or low amount of DNA usually inhibit PCR-amplification creating hindrance for primer annealing (Anderson and Cairney, 2004). For most of the species, good results have been achieved for concentrations from 50-100 ng in 25-50 µl PCR reaction volume (Shrestha, 2001). Therefore, in present research the template DNA was varied from 12.5 to 62.5 ng/ µl. The minimum concentration that gives the best crispy banding patterns was observed for 25 ng DNA and hence it was used in further PCR process.

$MgCl_2$ is a cofactor of polymerase enzyme therefore determines the DNA amplification process and governs the reproducibility of banding patterns. Its concentration must be optimized for every primer:template system (Palumbi, 1996; Strachan and Read, 1999). Magnesium is usually supplied to PCR amplification in the form of magnesium chloride. As this compound is a salt, in the water environment of a DNA synthesis reaction, it dissociates into the two ions Mg++ and Cl-. Because of its positive charge, the magnesium ion interacts with negatively charged molecules (primers, template, PCR products and dNTPs) in the reaction. Of critical importance, however, are the dNTPs. Each carries a total of 4 negatively charged oxygen molecules attached to the triphosphate group. Because it is necessary for free magnesium ion to serve as an enzyme cofactor in PCR, the total magnesium ion concentration must exceed the total dNTP concentration (Babec, 2012). The $MgCl_2$ can be optimized either by determining the optimal primer/template combination (primer screening) in the fixed $MgCl_2$ concentration or varying $MgCl_2$ concentration for fixed primer/template combination (Shrestha, 2001). In the present investigation the later strategy was followed. The range of $MgCl_2$ concentration tested were 1.5 mM to 4.0 mM and the distinct bright and single band in agarose gel electrophoresis was observed in concentration of 2.5 mM which was then taken for further amplification.

5.3.2.1.2 Optimization of PCR cycle

Optimizing the annealing temperature of PCR assay is one of the most critical parameters for reaction specificity. Setting the annealing temperature too low may lead

to amplification of nonspecific PCR products. On the other hand, setting the annealing temperature too high may reduce the yield of a desired PCR product (Bio-rad, 2013). Optimization of annealing temperature begins with calculation of the T_m values of the primer-template pairs. The *T*m is defined as the temperature at which half of the potential binding sites of template DNA are thought to have primer bound to them. A long primer, or one with greater GC content, has a higher *T*m because of the greater number of hydrogen bonds (Palumbi, 1996). The simplest rule of thumb is that *T*m (in degree centigrade) of perfect primer (i.e. one that has a perfect sequence match to the template) is sum of four times the (G + C)'s and two times (A + T)'s (Palumbi, 1996; Roux, 2009). Even after calculating the T_m of a primer, it may need to determine the annealing temperature empirically. This involves repeating a reaction at many different temperatures above and below the calculated T_m of the primers. The optimal annealing temperature for an assay can be easily determined using PCR instruments that have a thermal gradient feature. The optimal annealing temperature is the one that results in the highest yield with no nonspecific amplification (Bio-rad, 2013). In the present investigation, the T_m value for the D1/D2 primers, NL1/NL4 was calculated as 60^0C and thus the annealing temperature was varied from 48^0C to 68^0C in the thermal gradient conditions of PCR. The optimized annealing temperature with distinct single band was found at 65^0C.

5.3.3 PCR amplification of nrDNA ITS regions and LSU D1/D2 domains

The PCR was performed in the optimized conditions of $MgCl_2$, tDNA and annealing temperature. In the total 59 samples, the PCR amplification rates of the two complementary ITS regions and D1/D2 domains were respectively 81.4% and 76.2%. While in case of eleven selected fungal isolates, the PCR amplification success was 81.8% (9/11) and 90.9% (10/11) respectively for ITS and D1/D2 regions.

The PCR conditions may not be same for all the species (Kwiastkowski et al., 2012), thus the percentage of PCR success wasn't cent. Sometimes, even though using an established PCR protocol that had been optimized and successful for the amplification of a particular isolate, use of that same protocol on a different isolates can result in a less than desirable outcome (Babec, 2013). Poor amplification, however, can result from one of several causes: the temperatures used for thermal cycling may not be the best, the template may be of poor quality, or reagents may be at suboptimal concentrations (Babec, 2013; Palumbi, 1996). PCR works readily with a DNA template of up to two to three thousand base pairs in length and above this size product yields often decrease, as with increasing length premature termination by the polymerase begin to affect the efficiency of the PCR (Strachan and Read, 1999). In addition, helices with a high G-C content possess a higher melting temperature, often impairing PCR (Strachan and Read,

1999; Babec, 2013). Also, numerous inhibitors such as ionic detergents (e.g., SDS), phenol, chloroform, etc may be present in the template preparation which interfere the PCR amplification of the tDNA (Roux, 2009). The Proteinase K carryover from DNA extraction can digest *Taq* polymerase. However, these problems can be resolved by re-extraction, ethanol precipitation, and/or centrifugal ultrafiltration (Roux, 2009). Further studies on the assessment of efficacy of nrDNA ITS and D1/D2 LSU nrDNA as potential DNA barcodes for Nepalese soil fungi need to be undertaken. Also, for the samples which didn't amplify using ITS1 and ITS2 primers, other pairs of universal ITS primers specific to fungi can be used and assessed. This can be applied to D1/D2 regions as well.

5.3.4 Identification of species by nucleotide sequencing

The nine and ten successfully amplified fungal isolates for ITS regions and D1/D2 domains respectively were sent for sequencing to Macrogen Company, South Korea. However, only 22.22% amplified ITS fragments and 90% of amplified D1/D2 domains could generate unambiguous bidirectional sequences. The amplified products <50 ng concentration is difficult in sequencing resulting into insufficient sequences (DNA Sequencing Core, 2013). The insufficient sequences are those that are either completely blank or two simultaneous and superimposed (unreadable) sequences (DNA Sequencing Core, 2013). This happens when only one of the primers acted on both ends with illegitimate amplification as sequencing uses one primer, while PCR utilizes two (Wang et al*.,* 2012; DNA Sequencing Core, 2013). Clark and Whittam (1992) used heuristic approaches to quantify the influence of sequencing errors and found that sequencing error rates of < 1 nucleotide/ kb probably have little effect on conclusions about the evolutionary history of highly polymorphic organisms but organisms with very low nucleotide diversity require greater sequencing accuracy. Aware of the large impact of sequencing quality on downstream analysis, several groups have attempted to detect, quantify and understand errors that arise from next-generation sequencing pipelines (Wang et al*.,* 2012).

The sequences retrieved for ITS and D1/D2 regions were separately tested for best hits in NCBI-BLAST and the best aligned of the query sequence to pre-deposited sequences in GenBank database was used to identify the species. By the sequence analysis of both ITS and D1/D2 regions, the identified species includes *Aspergillus flavus* (23)*, Aspergillus parasticus* (A13), *Aspergillus protuberus* (33d)*, Aspergillus niger* (113b)*, Penicillium ochrochloron* (A12)*, Penicillium aurantiogriseum* (A16), *Penicillium aurantiogriseum* (A277), *Penicillium ruqulosum* (31c)*, Penicillium chrysogenum* (112a) and *Penicillium simplicissimum* (A10)*.* This genotypic identification was also confirmed by phenotypic traits based on colonies aspects on solid media and microscopic characterization of microconidia and phialides in the aerial mycelium. In addition, the species which showed

uncertain identification with sequencing were re-identified on the morphological basis. In the comparative study of ITS and D1/D2 regions sequences, *Aspergillus flavus* (fungal isolate named 23) was only be enabled to be identified by ITS but not by D1/D2 sequences. In contrast, *Aspergillus protuberus* (isolate 33d) was only identified by D1/D2. Hence, combined sequence analysis of the D1/D2 and ITS regions is recommended for correct species identification. In addition, species definition also requires the classical biological information such as life cycles and phenotypic characterization (Scorzetti et al*.,* 2002; Pisani et al*.,* 2007; Romanelli et al*.,* 2007; Schoch et al*.,* 2012).

5.3.5 Phylogenetic inferences

5.3.5.1 Nucleotide variation

The length of D1/D2 domains varied from 597 to 740 base pairs in the studied sample. It has been reported that D1/D2 are approximately 600 to 800 base pair (bp) long (Abliz et al*.,* 2004; Hall et al*.,* 2004; Kwiatkowski et al*.,* 2012) and this study showed approximation to the reported data. The nucleotide difference between and within the species was analyzed by MEGA5 (Kimura, 1980; MEGA, 2013) in D1/D2 region. The sequence variability can be illustrated by the number of base changes between closely related species (Scorzetti et al., 2002). Estimation of the number of nucleotide substitutions is one of the most important subjects in the study of molecular evolution. This measure of evolutionary distance is routinely used to infer phylogenetic trees and estimate divergence times among genes, individuals, populations, and species (Tamura and Kumar, 2002). In this study, the number of nucleotides differences was distributed in the range of 20-40. These results suggested that the sequences of the D1/D2 domains might be useful for identification of these filamentous fungi. According to Abliz et al., (2004) and Sonnenberg et al*.,* (2007), for the species in which the number of nucleotide difference is less than three, the sequences of D1/D2 domains are considered to be insufficient criteria to identify confidently each species and there were no below three nucleotide differences in the pairwise comparisons between the studied species. Thus, these results provide the evidences that the D1/D2 region can be readily amplified for the *Penicillium* spp. and *Aspergillus* spp. and other related filamentous fungi.

Similarly, the nucleotide substitution pattern was evaluated and the disparity index per substitution was tested using MEGA5. The rate of transition was found higher than the rate of transversion. The extent of differences in base composition biases between sequences called as Disparity Index test, does not require a priori knowledge of the pattern of substitutions, extent of rate heterogeneity among sites, or the evolutionary relationship among sequences (Kumar and Gadagkar, 2001; Tamura and Kumar, 2002). P-values are estimated by Monte-Carlo test and p-values less than 0.05 is considered to

be significant (Kumar and Gadagkar, 2001). In this study, 69% of inter- and intra- species nucleotide variation in D1/D2 regions showed significance of phylogeny analysis with p-value less than 0.05. This indicates that the neutral evolutionary sites are potentially evolving with significantly different substitution patterns between fungal species.

5.3.5.2 Construction of Phylogenetic tree

There is ample evidence, however, that real-world gene sequences evolve heterogeneously and are not identically distributed. The maximum likelihood and Bayesian Markov chain Monte Carlo (BMCMC) can become strongly biased and statistically inconsistent when the rates at which sequence sites evolve change non-identically over time. Maximum parsimony performs substantially better than current parametric methods over a wide range of conditions tested, including moderate heterogeneity and phylogenetic problems not normally considered difficult (Kolaczkowski and Thornton, 2004). Maximum parsimony describes a particular non-parametric statistical method for constructing phylogenies. In this study, the preferred phylogenetic tree is the tree that supposes the least evolutionary change (maximally parsimonious) to explain observed data (Tamura et al., 2011).

The information from ITS sequencing wasn't enough for the phylogenetic analysis between the identified strains. However, D1/D2 phylogenetic analysis revealed the evolutionary relationship between the species of *Penicillium* and *Aspergillus*. The polyphyletic characteristics in *Penicillium* spp. and *Aspergillus* spp. were inferred from their clade formations, most of the *Penicillium* spp. and *Aspergillus* form a single-membered clade independently (paraphyletic) but *Aspergillus parasticus* (A13) and *Penicillium aurantiogriseum* (A277) showed close relationship forming a single clade (monophyletic) (Figure 4.7). Therefore, the cladogram didn't represent the inter- and intra- specific relationship clearly as strains were not specific to a particular clade. It can be explained by Kwiatkowski et al. (2012) stating that some of the fungi exhibit limited interspecies diversity at the ITS and D1/D2 regions and are identified to the genus level only or with inconclusive species level identification. For example, the species identification of *Aspergillus,* using ITS primers, was inconclusive in the study of Khatri, 2012 as the strain showed 99% similarity to *Aspergillus niger* as well as *Aspergillus tubingensis.*

The close relationship observed between *Aspergillus parasticus* (A13) and *Penicillium aurantiogriseum* (A277) was non monophyletic and shown to be sister species. They showed gene analog, a feature that appears similar in two taxa which have originated from two different ancestors. This may be explained by gene transfer or Gene flow. The news report on May 22, 2010 Science Daily suggests that fungi have the capacity to rapidly change the make-up of their genomes and become infectious to plants and

possibly animals, including humans (Science Daily, 2010). There was a belief that fungi were generally confined to vertical gene transfer or conventional inheritance, a slower type of genetic change based on the interplay of DNA mutation, recombination and the effects of selection (Science Daily, 2010). Besides, fungi also have significant potential for Horizontal Gene Transfer. Horizontal gene transfer (HGT) refers to the transfer of genetic material between species using mechanisms other than inheritance from ancestors (Bioinformatica, 2013) i.e. fungi are able to transfer an adaptable capability to a different strain in a single generation. In fungi, HGT has been used to explain the similarity gene clusters among species (Science Daily, 2010). Horizontally transferred genes may allow the pathogen to mimic either the plant's own proteins, or those of other organisms (Bioinformatica, 2013). Detail study of individual genera, such as among the Aspergilli, reveals that many recent changes have occurred in genome expansions (Kelkar and Ochman, 2011). Based on a genome-wide analysis of Fusarium species, it was shown experimentally that complete chromosomes were being transferred between different fungal strains, along with the ability to cause infection (Science Daily, 2010).

Thus, highly pleomorphic morphological features and unsuitability of ITS regions for the genus- and species- level differentiations have necessitated the need for better alternative genetic markers (Dagar et al., 2011). Although the D1/D2 region displays enough sequence variability to allow the identification of many fungi to the species level, for some fungi the sequence of the D1/D2 region alone is not sufficient for accurate identification to the species level (Romanelli et al., 2007). In this study, the success rate of ITS sequencing was very low so we couldn't compare the sequences of D1/D2 with ITS (Schoch et al., 2012; Scorzetti et al*.,* 2002) for the confirmation of the species identification. In such a case, a second locus (e.g. D1/D2) can be sequenced (Romanelli et al., 2007) and in the meantime, the success rate of the sequence of ITS can be increased by repeated amplification with optimized conditions for each individuals (Kwiatkowski et al., 2012).

Phylogeny-centric fungal systematics is an ideal approach for discovering unknown branches of the fungal tree of life and better understanding evolutionary changes in the known fungal lineages (Shenoy et al*.,* 2007). It is rightly stated that proper identification reveals the correct biology of a fungus. Consequently, incorrect identification can potentially cause some problems, especially in disease diagnostics, evolutionary relatedness and genetic mapping. Thus, in order to identify the species correctly, various fungal barcode regions of the nuclear small ribosomal subunit (SSU) gene (Weber et al*.,* 2006), partial *β-tubulin A* (*BenA)* or *Calmodulin* gene sequences, or partial elongation factor 1-α (EF-1α) sequences or other protein-coding genes can be used (Abliz et al*.,* 2004; Begerow et al*.,* 2004; Weber et al*.,* 2006; Seifert et al*.,* 2007; Kwiatkowski et al*.,*

2012; Schoch et al., 2012). The PCR-RFLP approach has also been used for fungal species identification (Michelmore and Hulbert, 1987; Gomes et al., 2002). The polymorphisms at the molecular level have been studied by Random Amplified Polymorphic DNA (RAPD) marker technique (Tiwari, 2011), however it isn't suitable for robust-molecular diagnostics.

Kirk et al. (2004) stated that there are problems associated with studying fungal diversity in soil. These arise not only from methodological limitations, but also from a lack of taxonomic knowledge. It is difficult to study the diversity of a group of microorganisms when it is not understood how to categorize or identify the species present (Kirk et al*.,* 2004). When studying microbial diversity, replicates of 1 to 5 g of soil are often used to measure diversity and then conclusions about the community are made. There are numerous problems with this approach. One is the innate heterogeneity of soil and thus of spatial distribution of the microorganisms (Trevors, 1998). Insufficient characters for identification and the laboratory culture problem are the basic hindrances to the morphological characterization (Adl, 2003; Anderson and Cairney, 2004) and molecular techniques based on PCR have been used to overcome the limitations of culture-based methods. However, molecular analyses also have their own limitations (Kirk et al., 2004). Lysis efficiency of cells or fungal structures varies between and within microbial groups (Prosser, 2002). Lysis efficiency also varies for different fungal cells. Spores will lyse differently than mycelia and mycelia of different ages will also have different lysing efficiency (Prosser, 2002). The variation in the ability to break open cells or fungal structures can lead to biases in molecular-based diversity studies (Kirk et al*.,* 2004). In addition, molecular analyses can also be affected by degradation of the gene products over time due to inadequate or inappropriate storage (Hillis, 1987). However, in this investigation, the diversity of fungal isolates from soils of MCA were studied by morphological analyses and PCR-based molecular techniques.

Due to the time, resources and budget limitations research on evaluation of growth of fungal isolates in different media, optimization for the enzyme production, re-sequencing of ITS region for the unsuccessful sequences and sequencing of ITS and/ or D1/D2 regions for all isolated fungi could not be carried out.

CHAPTER 6
SUMMARY

Fungi are ubiquitous in the environment and fulfill a range of important ecological functions, particularly those associated with nutrient and carbon cycling processes in soil (Christensen, 1989). Despite this, our understanding of soil fungal community diversity and functioning remains poor relative to that of soil bacterial communities (Anderson and Cairney, 2004). The purpose of present investigation was to determine the diversity of soil fungi in Manaslu Conservation Area (MCA), Nepal and to study the importance of isolated fungi. During this investigation altogether 59 species of fungi were isolated from soil samples collected from different regions of MCA. Isolation and estimation of mycofloral population was accomplished by the serial dilution plate technique. By morphological and cultural differences the following species of fungi were identified: *Penicillium, Aspergillus, Mucor, Rhizopus, Crysosporium, Nigrospora, Fusarium, Gliocladium, Cladosporium, Trichoderma, Verticillium, Monilia* and *Altenaria.* Among the isolated fungi, *Penicillium* spp. was predominant, occupying 24% of total isolates, which was followed by *Aspergillus, Nigrospora* and *Mucor* spp., representing 8% each.

Eleven of these isolated fungi representing *Penicillium* and *Aspergillus* species were selected for the study of ability of production of biotechnological importance enzymes. The enzymes tested were amylase, protease, pectinase, cellulase, xylanase and lipase. Out of eleven fungi, *Penicillium aurantiogriseum* (A277) showed the ability of production of all six different extracellular enzymes tested. It was followed by *Penicillium ochrochloron* (A12) showing positive response to five enzymes. The enzymes that were highly produced by most of the fungi were cellulase and protease. Thus, these fungal isolates can be further utilized for the commercial production of these extracellular enzymes specially cellulase and protease.

The selected fungi were further characterized by PCR amplification and sequencing of nuclear ribosomal ITS regions and D1/D2 domains of LSU rDNA to identify the isolates up to species level and to study their phylogeny. The consensus sequence was assembled using CodonCode Aligner *v.4.2.2* and was compared with the GenBank Database. The sequence showed >99% identical to *Penicillium ochrochloron, Penicillium aurantiogriseum* strain DAOM 214787, *Penicillium aurantiogriseum* strain CBS 324.89, *Penicillium ruqulosum, Penicillium chrysogenum, Penicillium simplicissimum, Aspergillus niger Aspergillus parasticus, Aspergillus protuberus* and *Aspergillus flavus.* Thus, the sequence information helped to identify the isolates up to species level.

Since, the sequence success rate for ITS was very low (22.22% i.e. 2/9), further analysis in the isolates were done using D1/D2 domains. However, ITS analysis was performed by retrieving sequences from GenBank databases for the comparative fungal species. The sequences of D1/D2 domains were then aligned and analyzed by using MEGA v.5.2.2. The inter- and intra- species nucleotide differences were found to range from 8 to 299.

This result suggested the sufficient variability of D1/D2 domains for the identification and characterization of fungal species. Besides, the homogeneity of substitution patterns was tested between sequences to check the probability of rejecting or accepting the null hypothesis by estimating the P-values. The result showed that the null hypothesis can be accepted in 69% of the genes at the 5% significance level with p-value <0.05. This also determined the significance of the use of D1/D2 domains to test the relatedness between and within the species.

The Clustal W alignment using D1/D2 sequences was done with nine individuals with the total sequence length of 670bp. The number of conserved and variable sites was found to be 170 and 391 respectively. The number of singleton sites was 86 and parsimonious informative sites were 301. The Maximum Parsimony (MP) tree was constructed with 1000 bootstrap test. By the phylogenetic analysis of D1/D2 domains of LSU rDNA, the *Penicillium* and *Aspergillus* showed close relationship forming sister groups with common lineage. The non-monophyletic clade formed by *Aspergillus parasticus* (A13) and *Penicillium aurantiogriseum* (A277) can be explained by gene transfer or gene flow.

In summary, our results show that DNA barcodes can be very effective in the context of fungal species identification and that additional data, such as geography and morphology may be required to obtain higher rates of success in species identification in other contexts. Furthermore, various well established gene loci play vital role towards fungal systematic and genetics.

CHAPTER 7
CONCLUSION

As a result of the survey, 59 species were isolated from soil of MCA out of which 23 were from different altitudinal gradients and 36 were from rhizospheric regions of seven different medicinal plants. Twelve genera of fungi were identified by morphological analysis and few were unidentified. Out of these, *Penicillium* spp. was most dominant followed by *Aspergillus, Nigrospora, Mucor, Rhizopus, Fusarium, Chysosporium, Trichoderma, Cladosporium, Verticillium* and *Gliocladium* spp. The unidentified isolates can be sporulated in other specific selective media to identify species based on morphological techniques. Thus, the result showed the high diversity of fungal communities in MCA.

The fungal ability to produce industrially important enzymes was also studied. For this purpose, *Penicillium* spp. and *Aspergillus* spp. isolated from the study were used. In this investigation, the tested *Penicillium* spp. and *Aspergillus* spp. have shown high potentiality to the production of examined industrially important extracellular enzymes. Thus, various *Penicillium, Aspergillus* or other related species isolated in this study have high potential of producing biotechnological important enzymes. Further studies are to be carried out with these fungal isolates for optimization of culture conditions and characterization of the enzyme activity at various physico-chemical conditions so that potentiality of these new isolates for industrial application will be established.

Furthermore, the selected biotechnologically important eleven fungi were identified up to species level by using sequences of ITS (ITS1-5.8S-ITS2) and D1/D2 (Variable domain of Large Subunit) regions of fungal ribosomal genome. The D1/D2 sequences were also used to infer the phylogeny of the selected species. The inter- and intra- species nucleotide differences in the D1/D2 regions showed that D1/D2 domains can be used for the species-based identification of filamentous fungi and for studying the phylogenetic relationships among different fungal species. However, it must be emphasized that no single set of primers of gene locus or community profiling techniques (such as morphological studies) will be optimal for the assessment of fungal diversity in all instances, but rather they should be chosen with the aims of research and the target group of fungi in mind.

Thus, this research has the potential to enhance further our understanding of fungal communities and their functional roles in soil ecological processes. The results of this project have opened up research avenues in the sectors of soil fungal ecology and biotechnology as well as fungal taxonomy and genetics.

REFERENCES

Abliz P, Fukushima K, Takizawa K and Nishimura K (2004) Identification of pathogenic dermatiaceous fungi and related taxa based on large subunit ribosomal DNA D1/D2 domain sequence analysis. *FEMS Microbiol. Immunol.* **40**: 41-49.

Adl SM (2003) *The Ecology Of Soil Decomposition,* Pub CAB International Wallingford, UK: 34-39 pp.

Agarwal M, Shrivastava N and Padh H (2008) Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Rep.* **27**: 617-631.

Agriculture Information (2012) Mycorrhiza: Best biofertilizer [Online] <http://www.agricultureinformation.com/forums/sale/86571-mycorrhiza-best-biofertilizer-sale-wholesale-price.html>

Agropedia (2010) Mycoherbicides [Online] <http://www.agropedia.iitk.ac.in/content/mycoherbicides-0>

Aguirre L, Hurst SF, Choi JS, Shin JH, Hinrikson HP and Morrison CJ (2004) Rapid Differentiation of *Aspergillus* Species from other Medically important opportunistic molds and yeasts by PCR-Enzyme Immunoassay. *J. of Clin. Microbiol.* **42**(8): 3495-3504.

Alexopoulos CJ and Mims CW (1985) *Introductory Mycology.* Second Edition. Pub M.S. Sejwal, Wiley Eastern Limited, India: 3, 5-10, 191, 235, 414, 534 pp.

Alomari H (2009) Uses of fungi in Biotechnology [online] <http://forums.ksu.edu.sa/showthread.php?4793-Uses-of-fungi-in-biotechnology >

Anderson IC and Cairney JWG (2004) Diversity and ecology of fungal communities: increased understanding through the application of molecular techniques. *Appl. Environ. Microbiol.* **6**(8): 769-779.

Aneja KR (2008) *Experiments in Microbiology Plant pathology and Biotechnology,* Pub New Age International Pvt. Ltd., New Delhi, India. 87-88, 286-291 pp.

Arbico-organics (2013) Fungal Diseases [Online] < http://www.arbico-organics.com/category/plant-fungal-disease/28>

AusVeg (2013) Fungal diseases [Online] <http://ausveg.com.au/intranet/technical-insights/cropprotection/fungal-diseases.htm>

Avise JC (2004) *Molecular markers natural history and evolution: Second Edition.* Sinauer Associates, Inc. Publishers Sunderland, Massachusetts.

Babec (2012) PCR optimization [Online] < http://babec.org/node/13>

Barbara L (2013) Wisc-Online: The Fungi Kingdom: Common Characteristics of Fungi [Online] < http://www.wisc-online.com/Objects/ViewObject.aspx?ID=BIO304>.

Baron GL (1968) *The Genera of Hyphomycetes from Soil* Pub Robert E. Krieger, Huntington, NewYork.

Barton GJ and Sternberg MJE (1987) A strategy for the rapid multiple alignment of protein sequences. *J. Mol. Biol.* **198**: 327–337.

Batzoglou S (2005) The many faces of sequence alignment. *Brief bioinform.* **6**(1): 1467-5463.

Beare MH, Hu S, Coleman DC and Hendrik PF (1997) Influences of mycelial fungi on soil aggregation and organic matter storage in conventional and no-tillage soils. *Appl. Soil Ecol.* **5**(3): 211-219.

Begerow D, Beate J and Franz O (2004) Evolutionary relationships among β-tubulin gene sequences of basidiomycetous fungi, *Mycol. Res.* **108**(11): 1257-1263.

Bhuju UR, Shakya PR, Basnet TB and Shrestha S (2007) Nepal Biodiversity Resource Book. Protected Areas, Ramsar Sites, and World Heritage Sites.

Bilgrami KS and Verma RN (1978) *Physiology of Fungi*. Vikas Publishing House Pvt. Ltd. Ansari Road, Delhi, India. 1-12, 294-295 pp.

Bin L (2008) PCR-based sensitive detection of the edible fungus *Boletus edulis* from rDNA ITS sequences. *Electro. J. of Biotechnol.* **11**(3)

Bioinformatica (2013) Fungal Bioinformatics and Genetics Research [Online] <http://bioinformatica.vil.usal.es/?page_id=13>

Biology-online (2013) Restriction fragment length polymorphism [Online] <http://www.biology-online.org/dictionary/Restriction_fragment_lengthpolymorphism>

Biology Active Learner (2013) Why do fungi reproduce both sexually and asexually? [Online] < http://163.16.28.248/bio/activelearner/25/ch25c3.html>.

Bioremediation Service Providers (2013) White-rot Fungus [Online] <http://www.hawaii.edu/abrp/Technologies/fungus.html>

Bio-rad (2013) PCR assay design and optimization [Online] <http://www.bio-rad.com/en-np/applications-technologies/pcr-assay-design-optimization>

Botstein D, White RL, Skolnick M and Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet*. **32**:314–333.

Bpia (2013) Microbial Biopesticides: Fungi [Online] <http://www.biopesticideindustryalliance.org/microbialfungi.php>

Brandt ME, Arvind AP, Mayer LW and Holloway BP (1998) Utility of random amplified polymorphic DNA PCR and TaqMan automated detection in molecular identification of *Aspergillus fumigates. J. of Clin. Microbiol.* **36**: 2057-2062.

Caetano-Anolles G and Bassam BJDNA (1993) Amplification fingerprinting using arbitrary oligonucleotide primers. *App Biochem Biotechnol*. **42**:189–200.

Cai L, Guo XY and Hyde KD (2008) Morphological and molecular characterisation of a new anamorphic genus *Cheirosporium*, from freshwater in China. *National Herbarium Nederland Persoonia.* **20**: 53–58.

Carlos F, Barbas III, Burton DR, Scott JK and Silverman GJ (2007) Quantification of DNA and RNA. *Cold Spring Harbor Protocol.* doi: 10.1101/pdb.ip47.

CBOL Plant Working group (2009) A DNA barcode for land plants. *PNAS.* **106**(31): 12794–12797.

Chawla HS (2002) *Introduction to Plant Biotechnology.* Third Edition. Science Pub. Inc., USA.

Christensen M (1989) A view of fungal ecology. *Mycologia.* **81**: 1-19.

Christian V, Shrivastava R, Shukla D, Modi HA and Vyas BR (2005) Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: enzymology and mechanisms involved. *Indian J Exp Biol.* **43** (4): 301–312.

Clapp JP (1996) *Methods in molecular biology, Vol. 50: Species diagnostics protocols: PCR and other nucleic acid methods*, Humana Press, Totowa, New Jersey: 177-207 pp.

Clark AG and Whittam TS (1992) Sequencing Errors and Molecular Evolutionary Analysis. *Mol. Biol. Evol.* **9**(4): 744-752.

Clulow SA, Lewis BC and Matthews P (1991) A pathotype classification for *Mycosphaerella pinodes*. *J Phytopathol.* **131**: 322-332.

ClustalW / ClustalX (2013) ClustalW / ClustalX: Multiple alignment of nucleic acid and protein sequences [Online] <http://www.clustal.org/clustal2/>

COMPONENT User's Guide (2013) Chapter 4: Consensus Trees [Online] <http://www.taxonomy.zoology.gla.ac.uk/rod/cplite/ch4.pdf>

Dagar SS, Kumar S, Mudgil P, Singh R and Puniya AK (2011) D1/D2 Domain of Large-Subunit Ribosomal DNA for differentiation of *Orpinomyces* spp. *Appl. Environ. Microbiol.* **77**(18): 6722-6725.

Dalboge H (1997) Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance *FEMS Microiol Rev.***21**:29-42.

Davis S (2010) DNA sequencing of fungi in a microbiology laboratory. *Microbiol. Australia.* **3**:142-144.

Destination Nepal Tours and and Travels Pvt. Ltd. (2013) Nepal National Parks Map [Online] < http://dntt.com.np/packages.php?id=190>.

DNA Sequencing Core (2013) Direct Sequencing of PCR Products [Online] <http://seqcore.brcf.med.umich.edu/doc/dnaseq/pcr.html>

Do CB, Mahabhashyam MSP, Brudno M and Batzoglou S (2005) ProbCons: probabilistic consistency-based multiple sequence alignment. *Genome Res.* **15**: 330–340.

Doctor fungus (2013) Human Mycoses [Online] <http://www.doctorfungus.org/mycoses/human/human_index.php>

Douds DDJr, Gadkar V and Adholeya A (2000) *Mass production of VAM fungus biofertilizer* in Mycorrhzal Biology. Kluwer Academic/Plenum Publishers.

Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**(5): 1792–1797.

EI-Fadly GB, EI-Kazzar MK, Hassan MAA and EI-Kot GAN (2008) Identification of some *Fusarium* spp. Using RAPD-PCR Technique. *Eqypt J Phytopathol.* **36**(1-2): 71-80.

Ellis MB (1971) *Dematiaceous Hyphomycetes* Kew, Surrey: Common Wealth Mycological Institute.

Ellis SD, Boehm MJ and Mitchell TK (2008) Fungal and Fungal-like Diseases of Plants. *Fact Sheet- Agriculture and Natural Resources, The Ohio State University:* 401-407 pp.

EMLab (2006) Industrial Uses of Fungi, Report of Dr. Michelle Seidl. *The Environmental Reporter, Vol. 4, Issue 9. EMLab.*

Encyclopaedia Britannica (2013) Outline of classification of fungi [Online] <http://www.britannica.com/EBchecked/topic/222357/fungus/57975/Outline-of-classification-of-fungi#toc275020>

Espinasa L and Borowsky R (1998) Evolutionary divergence of AP-PCR (RAPD) patterns. *Mol. Bios. Evol.* **15**(4): 408-414.

Evolution (2013) Chapter 27: Phylogenetic Reconstruction [Online] <http://evolution-textbook.org/content/free/contents/ch27.html>

Fuelfix (2013) Researchers studying biofuels production with unique fungus [Online] <http://fuelfix.com/blog/2013/06/08/researchers-studying-biofuels-production-with-unique-fungus/>

Fungal Barcoding (2013) Fungal Barcoding Database, International Fungal Working Group [Online] < http://www.fungalbarcoding.org/**>**

Fungal Genomics Project (2005). Fungal Genomics Project, Concordia University (2005) [online] <http://fungalgenomics.concordia.ca/home/indappl.php>

Fungi Online (2013) Classification of Fungi [Online] <http://www.fungionline.org.uk/1intro/4classification.html>

Gardes M and Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhiza and rusts. *Mol. Ecol.* **2** (2): 113-118.

Georgopapadakou NH and Walsh TJ (1994) Human Mycoses: Drugs and Targets for Emerging Pathoges. *Science.* **264**: 371-373.

Gilman JC (1975) *A Manual of Soil Fungi* Revised Second Edition Oxford and IBH Publishing Company. New Delhi.

Gnanavel I (2013) *Parthenium hysterophorus* L.: A major Threat to Natural and Agro Eco-systems in India. *Sci. Intl.* **1**(5): 124-131.

Gomes EA, Kasuya MCM, de Barros EG and Borges AC (2002) Polymorphism in the internal transcribed spacer (ITS) of the ribosomal DNA of 26 isolates of ectomycorrhizal fungi. *Genet. Mol. Bio.* **25**(4): 477-483.

Gromadzki SG and Chaturvedi V (2000) Limitations of the AccuProbe *Coccidioides immitis* culture identification test: false negative results with formaldehyde killed cultures. *J. Clin. Microbiol.* **38**(6): 2427-2428.

Groot GA, During HJ, Mass JW, Schneider H, Vogel JC and Erken RHJ (2011) Use of *rbcL* and *trnL-F* as a Two-locus DNA Barcode for Identification of NW-European Ferns: An Ecological Perspective. *PLoS ONE.* **6**(1): e16371.

Guimarães LHS, Peixoto-Nogueira SC, Michelin M, Rizzatti ACS, Sandrim VC, Zanoelo FF, Aquino AC, Altino B Junior and Polizeli ML (2006) Screening of filamentous fungi for production of enzymes of Biotechnological interest. *Braz. J. Microbiol*. **37**:474-480

Hall L, Wohlfiel S and Roberts GD (2004) Experience with the MicroSeq D2 Large-Subunit Ribosomal DNA Sequencing Kit for identification of Filamentous Fungi encountered in clinical laboratory. *J. Clin. Microbiol.* **42** (2): 622-626.

Hama S, Tamalampudi S, Fukumizu T, Miura K, Yamaji H, Kondo A and Fukuda H (2006) Lipase Localization in Rhizopus oryzae Cells Immobilized within Biomass Support Particles for Use as Whole-Cell Biocatalysts in Biodiesel-Fuel Production. *J. BioSci. Bioeng.* **101**(4):328-333.

Hawksworth DL (2006) The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycological Res.* **95** (6): 641–655.

Hayashi K (1991) PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Appl.* **1**: 34-38.

Hebert PDN, Cywinska A, Ball SL and deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B*: *Biological Sciences.* **270**: 313-321.

Henderson MER (1961) Isolation, Identification and Growth of some Soil Hyphomycetes and Yeast-like Fungi which utilize Aromatic Compounds related to Lignin. *J. gen. Microbiol.* **26**: 149-154.

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673-4680.

Hillis DM (1987) Molecular Versus Morphological Approaches to Systematics. *Ann. Rev. Ecol. Syst.* **18**: 23-42.

Hillis DM and Bull JJ (1993) An Empirical test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. *Syst. Biol.* **42**(2): 182-192.

Hinrikson HP, Hurst SF, Lott TJ, Warnock DW and Morrison CJ (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**(5): 2092-2103.

Hologic Gen-Probe (2013) Culture Identification/AccuProbe (Fungal) [Online] <http://www.gen-probe.com/>

Hoorman JJ (2011) The Role of Soil Fungus; The Fact Book. *Agri. Nat. Resour.* SAG-14-11.

Huffnagle KE and Gander RM (1993) Evaluation of gen-probe's *Histoplasma capsulatum* and *Cryptococcus neoformans* accuProbes. *J. Clin. Microbiol.* **31**(2): 419-421.

Jasalavich CA, Ostrofsky A and Jellison J (2000) Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. *Appl. and Env. Micro.* **66**(11): 4725-4734.

Jayant M, Rashmi J, Shailendra M and Deepesh Y (2011) Production of cellulase by different co-culture of *Aspergillus niger* and *Penicillium chysogenum* from waste paper, cotton waste and baggase. *JYFR.* **2**(2): 24-27

Jeanmougin F, Thompson JD, Gouy M, Higgins DG and Gibson TJ (1998) Multiple sequence alignment with Clustal X. *Trends in Biochem. Sci.* **23**(10): 403-405

JECFA (2001) Fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives. *WHO Additives Series 47 and FAO Food and Nutrition Paper*. **74**: 281-416.

Jenkins A (2005) Soil fungi. *State of New South Wales Department of Primary Industries, Soil Biology Basics.* <www.dpi.nsw.gov.au>

jModelTest2 (2013) jModelTest 2: HPC selection of models of nucleotide substitution [Online] < https://code.google.com/p/jmodeltest2/>

Katoh K, Misasa K, Kuma K and Miyata T (2002) MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **30**(14): 3059–3066.

Kelkar YD and Ochman H (2011) Causes and Consequences of Genome Expansion in Fungi. *Genome Biol. Evol.* doi:10.1093/gbe/evr124.

Khatri B (2012) *Production, Purification and Characterization of Pectinase produced by Aspergillus niger isolated from Manaslu Conservation Area (MCA).* M.Sc. Thesis submitted in Central Department of Biotechnology, Tribhuvan University, Kirtipur, Nepal.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**(2): 111-120.

King AJ, Karki D, Nagy L, Racoviteanu A and Schmidt SK (2010) Microbial biomass and activity in high elevation (>5100 meters) soils from the Annapurna and Sagarmatha regions of the Nepalese Himalayas. *Himalayan J. Sci.* **6**(8): 11-18.

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H and Trevors JL (2004) Methods of studying soil microbial diversity (Review). *J. Microbiol. Methods.* **58**: 169-188.

Kolaczkowski B and Thornton JW (2004) Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous. *Nature.* **431**(7011): 980-984.

Kress WJ, Erickson DL, Jones A, Swenson NG, Perez R, Sanjur O and Bermingham E (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. *PNAS.* doi/10.1073/pnas.0909820106.

Kumar S and Gadagkar SR (2001) Disparity Index: A simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. *Genetics* **158**: 1321-1327.

Kurtzman CP and Robnett CJ (1995) Molecular relationships among hyphal ascomycetous yeasts and yeast like taxa. *Can. J. Bot.* **73**: S824-S830.

Kwiatkowski NP, Babiker WM, Merz WG, Carroll KC and Zhang SX (2012) Evaluation of Nucleic Acid Sequencing of the D1/D2 Region of the Large Subunit of the 28S rDNA and the Internal Transcribed Spacer Region Using SmartGene IDN Software for Identification of Filamentous Fungi in a Clinical Laboratory. *The Journal of Molecular Diagnostics.* **14** (4).

Lange L (2010) The importance of fungi for a more sustainable future on our planet. *Fungal biology reviews.* **24**: 90-92.

Li HC, Bouchara JP, Hsu MML, Barton R, Su S and Chang TC (2008) Identification of dermatophytes by sequence analysis of the rRNA gene internal transcribed spacer regions. *J. Med. Microbiol*. **57**: 592-600.

Li M, Hui CAO, But PPH and Shaw PC (2011) Identification of herbal medicinal materials using DNA barcodes. *J. Syst. Evol.* **49**(3): 271-283.

Lian B (2005) The Fungi Kingdom: Common Characteristics of Fungi. [Online] <http://www.wisc-online.com/objects/ViewObject.aspx?ID=BIO304>

Lindsley MD, Hurst SF, Iqbal NJ and Morrison CJ (2001) Rapid Identification of Dimorphic and Yeast-like Fungal Pathogens Using Specific DNA Probes. *J. Clin. Microbiol.* **39**(10): 3505-3511.

Luitel KP and Koirala U (2009) Soil-borne Fungi of Cultivated Lands of Biratnagar, Morang District, Nepal. *Our Nature.* **7**: 232-235.

Macdonald PDM (1999) How to interpret a Box Plot in terms of a Normal Distribution. [Online] <http://ms.mcmaster.ca/peter/s2ma3/s2ma3_9798/boxplots.html>

Madigan MT and Martinko JM (2006) *Brock Biology of Microorganisms,* Pub Pearson Peterson Hall, USA: 469-471 pp.

Makino R, Yazyu H, Kishimoto Y, Sekiya T and Hayashi K (1992) F-SSCP: Fluorescence-based polymerase chain reaction-single stand conformation polymorphism (PCR-SSCP) analysis. *PCR Methods Appl.* **2**(1): 10-13.

Margesin R and Miteva V (2011) Diversity and ecology of psychrophilic microorganisms. *Research Microbiol.* **162**(3): 346-361.

Martin KJ and Rygiewicz PT (2005) Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology.* **5**: 25.

Mathew GM, Sukumaran RK, Singhania RR and Pandey A (2008) Progress in research on fungal cellulases fro lignocelluloses degradation. *JSIR.* **67**: 898-907

May GS and Adams TH (1997) The Importance of Fungi to Man. *Genome Research.* **7**: 1041-1044.

MEGA, Molecular Evolutionary Genetics Analysis (2013) Creating Multiple Sequence Alignments [Online] < http://www.megasoftware.net/mega4/m_creating.html>

Meletiadis J, Meis JFGM, Mouton JW and Verweij PE (2001) Analysis of growth characteristics of filamentous fungi in different nutrient media. *J. Clin. Microbiol.* **39**(2): 478-484.

Menna W (2008) Statistical Analysis: How to Read and Interpret a Box Plot [Online] < http://voices.yahoo.com/statistical-analysis-read-interpret-box-2336634.html>.

Merriam-Webster (2013) Restriction Fragment Length Polymorphism [Online] <http://www.merriam-webster.com/dictionary/restriction fragment length polymorphism>.

Michelmore RW and Hulbert SH (1987) Molecular Markers for Genetic Analysis of Phytopathogenic Fungi. *Annual Review of Phytopathology.* **25**(1): 383-404.

Mirhendi H, Diba K, Kordbacheh P, Jalalizand N and Makimura K (2007) Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method. *J. Med. Microbiol.* **56**(11): 1568-1570.

Mishra BK and Dadhich SK (2010) Production of Amylase and Xylanase Enzymes from Soil Fungi of Rajasthan. *J. Adv. Dev. Res.* **1**(1): 21-23

Moller EM, Bahnweg G, Sandermann H and Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues*. Nucleic Acids Res*. **20** (22): 6115-6116.

Mostert L, Groenewald JZ, Summerbell RC, Robert V, Sutton DA, Padhye AA and Crous PW (2005) Species of *Phaeoacremonium* associated with infections in humans and environmental reservoirs in infected woody plants. *J. Clin. Microbiol.* **43**(4): 1752-1767.

Mould.ph (2013) Toxic Black Mold Inspection, Testing, Removal, & Prevention in Midwestern and Eastern USA and Canada by Environmental Hygienists and Industrial Hygienists Phillip & Divine Fry [Online] < http://www.mold.ph/>

Mount DM (2004) *Bioinformatics: Sequence and Genome Analysis* (2nd edition). Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Mousavi SAA, Khalesi E, Bonjar GHS, Aghighi S, Sharifi F and Aram F (2007) Rapid molecular diagnosis for *Candida* species using PCR-RFLP. *Biotechnol.* **6**(4): 583-587.

Mtui G, Masal R (2008) Extracellular enzymes from brown-rot fungus Laetiporus sulphureus isolated from mangrove forests of coastal Tanzania. *SRE.* **3**(4): 154-161.

Mycology online (2013) Mould Identification: A Virtual Self Assessment [Online] <http://www.mycology.adelaide.edu.au/virtual/**>**

Mycoprotein (2013) What is Mycoprotein [Online] < http://www.mycoprotein.org/>

Nasir M and Hoppe HH (1991) Studies on pathotype differentiation within *Mycosphaerella pinodes* (Berk. & Bloxan) Vestergren, a component of the Ascochyta disease complex of Peas (*Pisum sativum*). *Zeitschrift Fur Pflamzen-Krankheiten and Pflanzenschutz.* **98**: 619-626.

NCBI, National Center for Biotechnology Information (2013) BLAST Assembled RefSeq Genomes [Online] <http://www.ncbi.nlm.nih.gov/)

Nei M and Kumar S (2000) *Mol. Phylogenet. Evol*. Oxford University Press, New York. 87, 103 pp.

Notredame C, Higgins DG and Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **302:** 205–217.

NTNC, National Trust for Nature Conservation (2001) [Online] <http://www.ntnc.org.np/national-trust-nature-conservation>

NTNC, National Trust for Nature Conservation (2013) Annual Report of 2012. *National Trust for Nature Conservation (NTNC), Khumaltar, Lalitpur, Nepal.*

Oqata J, Uchiyama N, Kikura-Hanajiri R and Goda Y (2013) DNA sequence analyses of blended herbal products including synthetic cannabinoids as designer drugs. *Forensic Sci. Int.* **227**(1): 33-41.

Orita M, Iwahana H, Kanazawa H, Hayashi K and Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. *Proc Natl. Acad. Sci. USA*. **86**:2766–2770.

Otles S (2013) The Role of The Enzymes [Online] <http://prof.dr.semih.otles.tripod.com/enzymesused/theroleof/theroleof.htm>

Palladino S, Kay I, Fonte R and Flexman J (2001) Use of real-time PCR and the Light Cycler system for the rapid detection of *Pneumocystis carinii* in respiratory speciments. *Diagn. Microbiol. Infect. Dis.* **39**: 2333-2336.

Palumbi SR (1996) *Nucleic Acids II: The Polymerase Chain Reaction: In Molecular Systematics, second edition.* Hillis DM, Moritz C and Mable BM (Editors). Sinauer Associates, Inc. publishers, Massachhusetts, USA. 205-220 pp.

Paul EA (2007) *Soil Microbiology and Biochemistry,* Third Edition, Academic Press, Elsevier, UK: 5-6.

Peay KG, Kennedy PG, and Bruns TD (2008) Fungal community ecology: a hybrid beast with a molecular master. *BioScience.* **58**: 799–810.

Pelczar MJ, Chan ECS, Krieg NR (2003) *Microbiology,* Pub Tata McGraw Hill Company Limited, New Delhi, India: 333-363.

Perrone G, Susca A, Cozzi G, Varga EJ, Frisvad JC, Meijer M, Noonim P, Mahakarnchanakul W and Samson RA (2007) Biodiversity of *Aspergillus* species in some important agricultural products. *SIM.* **59**: 53-66.

Petruccioli M, Federict F and Miller MW (1988) Extracellular enzyme production in species of the genus *Penicillium. Mycologia.* **80**(5): 726-728.

Pfaller MA (2001) Molecular Approaches to Diagnosing and Managing Infectious Diseases: Practicality and Costs. *Emerg. Infect. Dis.* **7**(2): 312-318.

Piccoli-Valle RH, Lopes Passos FML, Vieira Passos FJ and Silva DO (2001) Production of pectin lyase by *Penicillium griseoroseum* in bioreactors in the absence of inducer. *Braz. J. Microbiol.* **32**: 135-140.

Pinheiro AC, Oliveira BP, Veríssimo C, Brandão JC, Tosado L, Jurado V and Macedo MF (2013) Identification of a fungal community on gilded wood carved heritage. *J. Cult. Herit.* **14**(1): 76-81.

Pisani D, Benton MJ and Wilkinson M (2007) Congruence of Morphological and Molecular Phylogenies. *Acta Biotheor.* **55**: 269-281.

Plenchette C and Morel C (1996) External phosphorus requirement of mycorrhizal and non-mycorrhizal barley and soybean plants. *Biology and fertility of soils.* **21**(4): 303-308.

Pokhrel CP, Rana P and Giri A (2010) Domestication of Some Wild Edible and Medicinal Mushrooms of Sagarmatha National Park, Nepal. *Contemporary Research in Sagarmatha (Mt. Everest) Region, Nepal.* 223-227.

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA and Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Review. *Appl. Microbiol. Biotechnol.* **67**: 577-591.

Posada D (2008) jModelTest 0.1.1 [Online] < https://code.google.com/p/jmodeltest2/>.

Pounder JI, Hansen D and Woods GL (2006) Identification of *Histoplasma capsulatum, Blastomyces dermatitidis* and *Coccidioides* Species by repetitive-sequence-based PCR. *J. Clin. Microbiol.* **44**(8): 2977-2982.

Prabakaran M, Thennarasu V, Ayeswariya R M, Bharathidasan R, Chandrakala N and Mohan N (2009) Comparative studies on the enzyme activities of wild and mutant fungal strains isolated from sugarcane field. *Indian J. Sci. Tech.* **2**(11): 46-49.

Prosser JI (2002) Molecular and functional diversity in soil microorganisms. *Plant Soil*. **244**: 9– 17.

Quebec Biodiversity Website (2013) Theory of biodiversity [online] < http://redpath-museum.mcgill.ca/Qbp/2.About%20Biodiversity/importance.html>

Quinn C (2005) Fungi and Industry. *British Mycological Society*. WF04:1-6.

Rao SPN (2006) Introduction to Mycology. JJMMC, Davangere. [Online] <www.microrao.com>

Raggam RB, Leitner E, Mühlbauer G, Berg J, Stöcher M, Grisold AJ, Marth E and Kessler HH (2002) Qualitative detection of *Legionella* species in bronchoalveolar lavages and induced sputa by automated DNA extraction and real-time polymerase chain reaction. *Med. Microbiol. Immunol.* **191**(2): 119-125.

Rana P and Giri A (2010) Diversity, Ethno-mycological Knowledge and Nutritional Value of Some Wild Mushrooms in Sagarmatha National Park, Nepal. *Contemporary Research in Sagarmatha (Mt. Everest) Region, Nepal.* 211-222.

Rangarajan V, Rajasekharan M, Ravichandran R, Sriganesh K and Vaitheeswaran R (2010) Pectinase production from orange peel extract and dried orange peel solid as substrates using *Aspergillus niger. Int J of Biotechnol. And Biochem.* **6**(3): 445-453

Rath PM and Ansorg R **(**2000) Identification of medically important *Aspergillus* species by single strand conformational polymorphism (SSCP) of the PCR-amplified intergenic spacer region. *Mycoses.* **43:** 381–386.

Romanelli AM, Sutton DA, Thompson EH, Rinaldi MG and Wickes BL (2009) Sequence-Based Identification of Filamentous Basidiomycetous Fungi from Clinical Speciments: a Cautionary Note. *J. Clin. Microbiol.* **48**(3): 741-752.

Roux KH (2009) Optimization and Troubleshooting in PCR. *Cold Spring Harbor Protocol.* **4**(4): doi: 10.1101/pdb.ip66.

Sabir JSM (2006) Genotypic identification for some *Fusarium sambucinum* strains isolated from wheat in Upper Egypt. *World J. of Agri. Sci.* **2**(1): 06-10.

Sace J (2010) The Role of Fungi in the Eco System [online] <http://www.brighthub.com/environment/science-environmental/articles/88505.aspx>

Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.

Sambrook J and Russell DW (2001) *Molecular Cloning: A laboratory manual*, Vol 1. Cold Harbor Laboratory Press, New York.

Satyanarayan U (2009) *Biotechnology.* Books and Allied (P) Ltd. Studio.

Schoch C, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W and Fungal Barcoding Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a Universal DNA barcode marker for Fungi. *PNAS.* **109**(16): 6241-6246.

Science Daily (2010) Fungi can change quickly, pass along infectious ability [Online] <http://www.sciencedaily.com/releases/2010/03/100317144634.htm**>**

Scorzetti G, Fell JW, Fonseca A and Statzell-Tallman A (2002) Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Research 2*: 495-517.

Seifert KA, Samson RA, deWaard JR, Houbrakken J, Lévesque CA, Moncalvo JM, Louis-Seize G and Hebert PDN (2007) Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *PNAS.* **104**(10): 3901-3906.

Semagn K, Bjørnstad Å and Ndjiondjop MN (2006) An overview of molecular marker methods for plants. *African Journal of Biotechnology*. **5**(25): 2540-2568.

Sharma G and Pandey RR (2010) Influence of culture media on growth, colony character and sporulation of fungi isolated from decaying vegetable wastes. *JYFR.* **1**(8): 157 – 164.

Shenoy BD, Jeewon R and Hyde KD (2007). Impact of DNA sequence-data on the taxonomy of anamorphic fungi. *Fungal Divers.* **26**: 1-54.

Shrestha S (1981) *Role of Fungal flora in buff meat contamination and spoilage.* MSc Thesis submitted in Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Shrestha S (2001) *Molecular systematics of weedy Sporobulus species of Australia.* PhD Thesis submitted to University of Queensland, Australia.

Shrestha S (2013) Personal Communication (Unit Chief and Chief scientist, Molecular Biotechnology Unit, Nepal Academy of Science and Technology).

Shrestha S, Graham GC, Loch DS and Adkins SW (2010) Molecular identification of weedy *Sporobolus* species by PCR-RFLP. *Int. J. Weed. Biol. Manag.* **50**: 383-387.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD and Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**(539). doi:10.1038/msb.2011.75.

Sigler L (2012) Biology of the Fungi- Conidial Development in Fungi Imperfecti. *University of Alberta Microfungus Collection; Devonian Botanic Garden.*

Soll DR (2000) The ins and outs of DNA fingerprinting the infectious fungi. *Clin. Microbiol. Rev.* **13**: 332-370.

Sonnenberg R, Nolte AW and Tautz D (2007) An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. *Front. Zool.* **4**: 6.

Strachan T and Read AP (1999) *PCR, DNA sequencing and in vitro mutagenesis: In Human Molecular Genetics, 2nd Edition.* Garland Science (Editor), Wiley-Liss, New York, US.

Sullivan J and Joyce P (2005) Model Selection in Phylogenetics. *Annu. Rev. Ecol. Evol. Syst.* **36**: 445-466.

Tamura K and Kumar S (2002) Evolutionary Distance Estimation Under Heterogeneous Substitution Pattern Among Lineages. *Mol. Biol. Evol.* **19**(10): 1727-1736.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony method. *Mol. Biol. Evol.* **28**: 2731-2739.

Thermo Scientific (2013a) 6X DNA Loading Dye [Online] <http://www.thermoscientificbio.com/nucleic-acid-electrophoresis/6x-dna-loading-dye/>

Thermo Scientific (2013b) GeneRuler 100 bp Plus DNA Ladder, Ready-to-Use 100 to 3000bp [online] <www.thermoscientificbio.com/nucleic-acid-electrophoresis/generuler-100-bp-plus-dna-ladder-ready-to-use-100-to-3000-bp/>

Thota P, Bhogavalli PK, Rao VP and Srirangam V (2012) Screening and Identification of Potential Fungal Strains for the Production of extracellular Lipase from Soil. *Plant Sci.* **2**(5): 79-84.

Tiwari KL, Jadhav SK and Kumar A (2011) Morphological and Molecular Study of Different *Penicillium* Species. *MEJSR.* **7** (2): 203-210.

Tortora GJ, Funke BR and Case CL (2010) *Microbiology* Pearson Publication, NewYork, USA: 330-344 pp.

Trevors JT (1998) Bacterial biodiversity in soil with an emphasis on chemically-contaminated soils. *Water Air Soil Pollut*. **101**: 45– 67.

TutorVista (2013) Fungal Reproduction [Online] <http://www.tutorvista.com/content/biology/biology-iii/kingdoms-living-world/fungal-reproduction.php>

Uhl JR, Adamson Sc, Vetter EA, Schleck CD, Harmsen WS, Iverson LK, Santrach PJ, Henry NK and Cockerill FR (2003) Comparison of light cycler PCR, rapid antigen immunoassay, and culture for detection of group A *Streptococci* from throat swabs. *J. Clin. Microbiol.* **41**(1): 242-249.

University of Exeter (2013) A brief introduction to the classification of fungi [Online] <http://www.groups.exeter.ac.uk/devonfungusgroup/overview.htm>

Vaidya GS, Shrestha K, Khadge BR, Johnson NC and Wallander H (2007) Study of Biodiversity of Arbuscular Mycorrhizal fungi in addition with different organic matter in different seasons of Kavre District (Central Nepal). *Scientific World.* **5**(5): 75-80.

Vilgalys lab, (2013) Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA [Online] < http://www.biology.duke.edu/fungi/mycolab/primers.htm>.

Volk TJ (2000) The Kingdom Fungi [Online] <http://www.uwlax.edu/biology/volk/fungi3/sld010.htm>

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*. **23**:4407–4414.

Vosman B (1998) *Variations on a theme. In: Molecular tools for screening biodiversity-plants and animals.* Karp A, Isaac PG and Ingram DS (Editors). Chapman and Hall Publication.

Waksman SA (1916) Soil fungi and their activities. *Soil Sci.* **2**: 103–155.

Walker DM, Castlebury LA, Rossman AY and White JF Jr (2012) New molecular markers for fungal phylogenetics: Two genes for species-level systematic in the Sordariomyctes (Ascomycota). *Mol. Phylogenet. Evol.* **64**(3): 500-512.

Wallenstein MD and Weintraub MN (2008) Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes. *Soil Biol. Biochem.* **40** (9): 2098–2106.

Walsh TJ, Francesconi A, Kasai M and Chanock SJ (1995) PCR and single-strand conformational polymorphism for recognition of medically important opportunistic fungi. *J. Clin. Microbiol*. **33**: 3216–3220.

Wang L, Yokoyama K, Miyaji M and Nishimura K (1998) The identification and phylogenetic relationship of pathogenic species of *Aspergillus* based on the mitochondrial cytochrome *b* gene. *Medical Mycology.* **36**: 153-164.

Wang XV, Blades N, Ding J, Sultana R and Parmigiaani G (2012) Estimation of sequencing error rates in short reads. *BMC Bioinformatics.* **13**: 18.

Watanabe T (2002) *Pictorial Atlas of Soil and Seed fungi: Morphologies of Cultured Fungi and Key to Species:* Second Edition, CRC Press, New York, USA: 1-6, 17-34.

Watrud LS, Martin K, Donegan KK, Stone JK and Coleman CG (2006) Comparison of taxonomic, colony morphotype and PCR-RFLP methods to characterize microfungal diversity. *Mycologia, The Mycological Society of America.* **98**(38): 384-392.

Weber E, Carlotti A and Furtado MR (2006) Microbial identification using a sequencing-based system: bacterial and fungal case studies. *EUFEPS.* **11**(2): 45-52.

Wengenack NL and Binnicker MJ (2009) Fungal molecular diagnostics. *Clin Chest Med.* **30**: 391-408.

Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res*. 18:7213–7218.

Wiley online (2013) Phylogenetic Trees [Online] <http://www.wiley.com/college/student/activities/phylogenetic_trees/>.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV9 (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531-6535.

Wohlbach DJ, Kuo A, Sato TK, Potts KM, Salamov AA, LaButti KM, Sun H, Clum A, Pangilinan JL, Lindquist EA, Lucas S, Lapidus A, Jin M, Gunawan C, Balan V, Dale BE, Jeffries TW, Zinkel R, barry KW, Grigoriey IV and Gasch AP (2011) Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. *PNAS.* **108**(32): 13212-13217.

WordPress (2013) Mycology- all you need to know [Online] <http://mb0804mycology.wordpress.com/page/3/>

Yan S, Liang Y, Zhang J and Liu CM (2012) *Aspergillus flavus* grown in peptone as the carbon source exhibits spore density- and peptone concentration-dependent aflatoxin biosynthesis. *BMC Microbiol.* **12**:106.

Yang ZI (2002) On wild mushroom resources and their utilization in Yunnan province, southwestern China. *J. Nat. Resour.* **17**: 464-469.

Zakaria L, Kulaveraasingham H, Guan TS, Abdullah F and Wan HY (2005) Random Amplified Polymorphic DNA (RAPD) and Random Amplified Microsatellite (RAMS) of Ganoderma from infected oil palm and coconut stumps in Malaysia. *Asia Pacific J. of Mol. Bio. and Biotech.* **13**(1): 23-34.

Zietkiewicz E, Rafalski JA, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*. **20**:176–183

Zhang B, Liang C, He H and Zhang X (2013) Variations in Soil Microbial Communities and Residues along an altitude gradient on the Northern Slope of Changbai Mountain, Chaina. *PLo S ONE.* **8**(6): e66184.

Zhang Z, Schwartz S, Wagner L and Miller W (2000) A greedy algorithm for aligning DNA sequence. *J. Comput. Bio.* **7**(1-2):203-214.

APPENDICES

Appendix 1

The chemicals and reagents used were from following companies:

- HiMedia Laboratories Pvt. Ltd., Mumbai, India
- Human Diagnostics and Surgichem Laboratory reagent, Kolkata, India
- Sigma Aldrich, Germany
- Central Drug House Pvt. Ltd., New Delhi, India
- Rankem Laboratory Reagent, New Delhi, India
- Bioneer, South Korea
- Promega Corporation, USA
- Thermo Scientific, MA, USA
- Fermentas, USA

The instruments used were from following companies:

- Biological Incubator, Beeta, India
- Olympus Biological Microscopes, India
- Eppendorf, Germany
- INGENIUS, Syngene Bio Imaging, SYIGLHR/1546, UK

Appendix 2

Composition of Potato Dextrose Agar (PDA)

[HiMedia REF M096-500G]

Ingredients	Gms/l
Potatoes infusion from	200.00
Dextrose	20.00
Agar	15.00
Final pH (at 25^0C) 5.6 $\pm$ 0.2	
39.0 gm in 1000 ml DW	

Appendix 3

Composition of Lactophenol Cotton Blue (LCB)

[Human Diagnostics and Surgichem Laboratory reagent, Kolkata, India]

- Cotton Blue, Phenol, L(+)-Lactic acid and Glycerol

Appendix 4

Site of sample collection (MCA, Nepal)

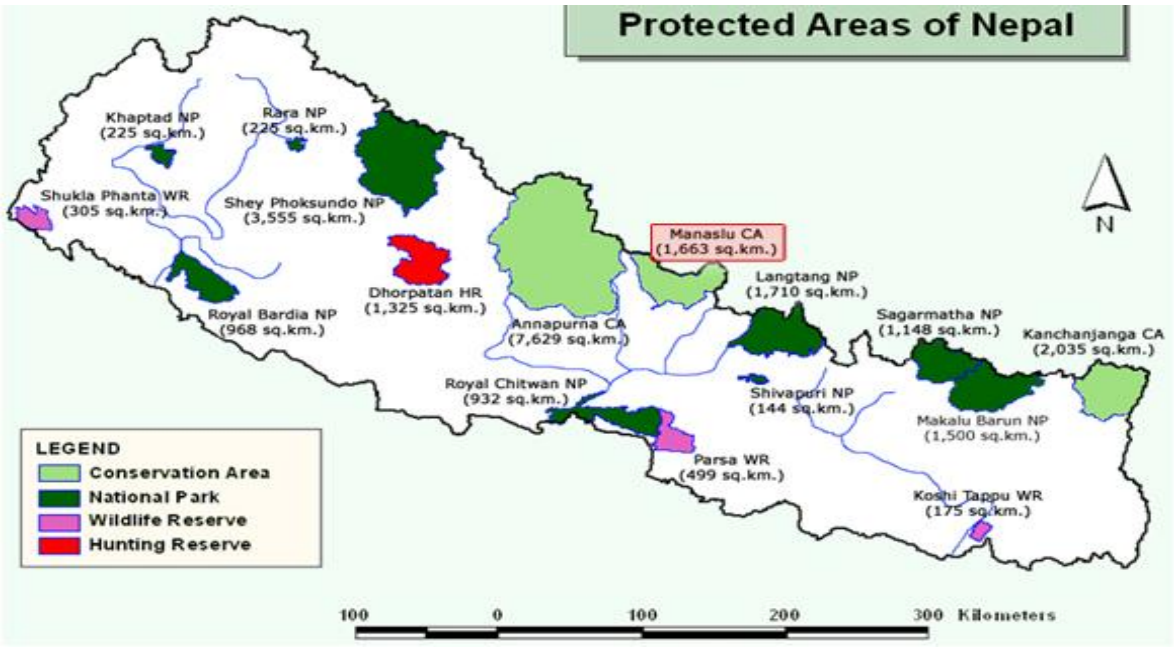


Figure 4.1: Map of Nepal showing the location of Manaslu Conservation Area (MCA), site of sample collection (Source: Destination Nepal, 2013)

Appendix 5

Table 5.1: Details of Soil sample collected from different rhizospheric regions and their Geographical Information

S. N.	Reference Plant	Alt. (m)	Latitude	Longitude	pH	Moisture (%)	Temperature (^{0}C)
1.	*Aconitum* sp.	3721	28^{0}30'2.3"	84^{0}48'26.7"	5.9	35	10.5
2.	*Nardostachys* sp. (Jatamasi)	3769	28^{0}29'58.7"	84^{0}48'26.2"	6.1	32	10.2
3.	*Rhododendron anthopogon*	3627	28^{0}33'36.5"	84^{0}41'50.3"	6.8	10	11.5
4.	*Dactylorhiza hatairea* (Panchaule)	3600	28^{0}30'6.5"	84^{0}48'40.2"	7.0	10	13.1
5.	*Swertia* sp.	3668	28^{0}30'7"	84^{0}48'29.2"	6.6	30	11.7
6.	*Podophyllum hexandrum*	3826	28^{0}43.473"	85^{0}12.214"	Not Available		
7.	*Neopicrorhiza scrophylariiflora*	4300	Information Not Available				

Appendix 6

Table 6.1: Overview of enzymes used for screening

Enzymes Screened	Substrate Used	Substrate Concentration
Amylase	Soluble Starch	0.5%
Cellulase	Carboxy Methyl Cellulose (CMC)	0.5%
Protease	Skim milk powder	0.5%
Xylanase	Birch wood xylan	0.5%
Pectinase	Pectin	0.5%
Lipase	Tributyrin	0.5%

Appendix 7

Reagents and Extraction buffer for DNA extraction

TES lysis buffer (100mM Tris pH 8.0, 10mM EDTA pH 8.0, 2% SDS)

3.0285 gram of Tris Base (Mol. Wt. 121.14 gram, Sigma-Aldrich, USA), 0.9306 gram of EDTA (Mol. Wt.372.24; Promega, Spain) and 5 gram of SDS (Merck Specialities Pvt. Ltd., Mumbai) were weighed and dissolved in 200 mL of DDW and the pH was adjusted to 8.0 by adding concentrated HCl. The final volume was then made upto 250 mL by addition of Sterile Double Distilled Water (SDDW).

Proteinase K (10 mg/ mL)

To prepare 10 mg/mL Proteinase K, 10 mg Proteinase K (Sigma-Aldrich, Germany) was dissolved in 1 mL of Sterile Double Distilled Water (SDDW).

5M Sodium chloride, NaCl (Mol. Wt. 58.44 gram)

29.22 gram of purified NaCl was weighed and dissolved in 50 mL Sterile Double distilled Water (SDDW) by stirring and final volume was made upto 100 mL by addition of SDDW.

10 % (w/v) CTAB (Mol. Wt. 364.46 gram)

10 gram of CTAB (Cetrimide) was weighed and dissolved in 50 mL SDDW in sterile Schott bottle by gentle heating and final volume was made upto 100 mL by adding SDDW.

3M Sodium acetate ,CH_3COONa (Mol. Wt. 136.08 gram)

20.41 gram of sodium acetate was weighed and dissolved in 20 mL SDDW and final volume was made upto 50 mL by addition of SDDW.

TE Buffer (10 mM Tris pH 8.0, 1mM EDTA pH 8.0) **with RNase A**

0.5M EDTA pH 8.0 (2 mL) was added to a bottle containing 1 mL of 1 M Tris buffer pH 8 and the final volume made up to 100 mL. The solution was autoclaved and stored at room temperature. 250 µL RNAse solution (4 mg/mL, Promega, Spain) was added to 100 mL of Tris-EDTA buffer.

Appendix 8

Preparation of gel electrophoresis reagents

TAE stock buffer (50X) Preparation

Tris-Acetate-EDTA (TAE), also called E buffer, is the most common buffer used for agarose gel electrophoresis in the analysis of DNA products resulting from PCR amplification, DNA purification protocols or DNA cloning experiments. There are also other buffers such as Tris-Borate (TBE) and Tris-Phosphate (TPE) that can be used in electrophoresis. TAE is commonly used buffer because of its high resolving power and low cost in comparison to TBE and TPE (Sambrook and Russell, 2001). Tris and Acetate in TAE are to maintain pH of the buffer and EDTA included in the solution binds divalent metal ions and inhibits metal-dependent nucleases (Thermo Scientific, 2013a).

For the preparation of 50X TAE stock buffers, 242 gm Tris base (Mol. Wt. 121.14 gram), was dissolved in approximately 750 mL double distilled water. To this solution, glacial acetic acid (57.1 mL) (Fisher Scientific, Mumbai), was carefully added and then vigorous shaken 0.5 M EDTA (100mL, pH 8.0) was added. The pH was adjusted to 8.0 and final volume was adjusted to 1 L by adding double distilled water. The solution was stored at room temperature.

Gel loading dye (6X)

Samples are mixed with 1X gel loading dye before loading into the well of the agarose gel. These dyes are served for three purposes: to increase the density of the samples so that samples sink evenly into the well, to add color to the sample thereby to track the movement of DNA into the gel, and the dyes in an electric field move toward the anode at predictable rates (Thermo Scientific, 2013a).

The prepared loading dye (6X) of GeNei was used which contain two dyes: [Xylene cyanol FF (4160 bp in agarose gel in TAE) and Bromopheol blue (370 bp in agarose gel in TAE)] for visual tracking of DNA migration during electrophoresis. The presence of glycerol ensures that the DNA in the ladder and sample forms a layer at the bottom of the well (Thermo Scientific, 2013b).

Ladder/ Marker

The 100 bp to 1000 bp ladder (Fermentas Company, Cat. No. #0323) was used to compare the size of the sample DNA.

Agarose Gel (1%)

1.0 gm of Agarose (Promega Cooperation, USA) was dissolved in 100 mL of 1X TAE buffer to make 1% agarose gel. The gel was completely dissolved by heating in microwave. It was then cooled to approximately 55^0C and Ethidium Bromide, EtBr (5 µL from 10 µg/mL stock; Sigma-Aldrich, Germany) was added to final concentration of 0.5 µg/mL. The gel solution was mixed thoroughly by gentle swirling and poured on to the gel casting tray after fixing an appropriate comb (8 or 17 or 25 toothed) for well formation. The gel was allowed to set for 30 mins at room temperature. The comb was carefully removed and the gel was transferred on to the electrophoresis tank (Major Science, California). TAE, an electrophoresis buffer, was added to the mark indicated in the gel tank.

Appendix 9

Table 9.1: Results of DNA estimation of the 23 samples from different altitudes

Sample Code	Dilution factor (Sample + diluents) µl	Absorbance (nm)				Ratio 260/280	Ratio 260/230	Concentration (ng/µl)
		230	260	280	320			
10	10+ 90	0.069	0.159	0.102	0.051	1.57	2.32	79.50
26	10+ 90	0.142	0.345	0.223	0.098	1.55	2.42	172.40
A16	10+ 90	1.362	2.688	1.364	0.034	1.97	1.97	1344.0
A19	10+ 90	0.696	0.087	0.057	0.018	1.53	0.12	43.30
A35	10+ 90	0.381	1.159	0.583	0.061	1.99	3.04	579.50
A36	10+ 90	0.100	0.276	0.180	0.093	1.54	2.77	138.10
A163	10+ 90	1.333	1.901	1.082	0.132	1.76	1.43	950.70
A12	10+ 90	0.341	0.474	0.230	0.001	2.06	1.39	273.00
A13	10+ 90	0.233	0.176	0.092	0.012	1.91	0.75	88.10
A39	10+ 90	1.275	1.627	0.827	0.102	1.97	1.28	813.60
A123	10+ 90	1.235	1.154	0.657	0.151	1.76	0.93	577.20
A124	10+ 90	1.705	2.666	1.350	0.073	1.88	1.56	1333.10
A7	10+ 90	0.066	0.520	0.255	0.032	2.04	7.88	260.00
A10	10+ 90	0.562	0.872	0.428	0.010	2.04	1.55	462.20
A24	10+ 90	0.984	1.986	0.948	0.058	2.10	2.02	999.20
A25	10+ 90	0.476	0.860	0.410	0.021	2.10	1.81	430.00
A30	10+ 90	0.843	0.354	0.206	0.075	1.72	0.42	176.90
A31	10+ 90	0.834	0.250	0.215	0.186	1.16	0.30	125.20
A241	10+ 90	0.722	0.212	0.149	0.083	1.42	0.29	105.80
A277	10+ 90	0.633	1.170	0.659	0.031	1.78	1.85	585.00
14	10+ 90	0.100	0.270	0.158	0.046	1.71	2.70	134.80
21	10+ 90	0.874	0.191	0.127	0.075	1.50	0.22	95.30
23	10+ 90	0.242	0.126	0.073	0.005	1.72	0.52	62.90

Table 9.2: Results of DNA estimation of the 36 samples from 7 different rhizospheric regions

Sample Code	Dilution factor (Sample + diluent µl)	Absorbance (nm)				Ratio 260/280	Ratio 260/230	Concentration (ng/µl)
		230	260	280	320			
31c	10+ 90	0.418	0.316	0.224	0.135	1.41	0.76	157.90
33a	10+ 90	0.163	0.062	0.320	0.004	1.96	0.38	31.00
33b	10+ 90	0.180	0.167	0.070	-0.011	2.37	0.92	84.30
33c	10+ 90	0.760	0.539	0.286	0.047	1.88	0.71	269.40
33d	10+ 90	0.207	0.136	0.061	-0.001	2.24	0.66	68.20
41a	10+ 90	0.196	0.133	0.082	0.016	1.61	0.68	66.40
42c	10+ 90	0.150	0.203	0.095	0.000	2.15	1.30	101.70
45a	10+ 90	0.730	1.467	0.661	0.012	2.22	2.01	733.60
61a	10+ 90	0.777	0.743	0.319	-0.081	2.33	0.96	371.70
62a	10+ 90	0.649	0.272	0.148	0.022	1.84	0.42	136.10
62b	10+ 90	0.234	0.239	0.112	0.004	2.13	1.02	119.30
63a	10+ 90	0.213	0.086	0.043	0.001	2.01	0.40	42.80
63b	10+ 90	0.868	0.645	0.324	0.000	2.00	0.74	322.70
63c	10+ 90	0.940	0.387	0.150	-0.030	2.58	0.41	193.30
81a	10+ 90	0.269	0.283	0.123	-0.005	2.30	1.05	141.50
81b	10+ 90	0.689	0.493	0.333	0.158	1.48	0.07	246.70
81d	10+ 90	0.310	0.461	0.212	0.002	2.18	1.49	230.50
91a	10+ 90	0.242	0.320	0.143	-0.080	2.24	1.32	160.00
91b	10+ 90	0.445	0.257	0.217	0.167	1.18	0.58	128.40
92a	10+ 90	0.187	0.133	0.063	0.002	2.12	0.71	66.30
93a	10+ 90	1.003	0.828	0.440	0.085	1.88	0.83	414.20
94a	10+ 90	0.390	0.263	0.174	0.094	1.51	0.67	131.50
94b	10+ 90	0.217	0.091	0.061	0.034	1.48	0.42	45.40
95a	10+ 90	1.823	1.384	0.937	0.427	1.48	0.76	692.00
101a	10+ 90	1.748	0.188	0.153	0.060	1.23	0.25	94.00
101b	10+ 90	0.527	0.866	0.397	0.008	2.18	1.64	433.00
102b	10+ 90	1.416	0.816	0.526	0.166	1.55	0.58	407.80
103a	10+ 90	0.674	0.303	0.105	-0.078	2.88	0.45	151.70
103b	10+ 90	0.743	0.335	0.167	0.016	2.00	0.45	167.70
103d	10+ 90	0.872	0.754	0.311	-0.101	2.43	0.86	377.00
104a	10+ 90	0.869	0.430	0.186	-0.054	2.31	0.49	215.10
105a	10+ 90	0.665	0.479	0.175	-0.101	2.74	0.72	239.30
111a	10+ 90	1.252	0.928	0.520	0.130	1.79	0.74	464.10
112a	10+ 90	0.324	0.134	0.085	0.033	1.58	0.41	67.00
113a	10+ 90	1.014	0.589	0.325	0.059	1.81	0.58	2294.70
113b	10+ 90	0.277	0.098	0.061	0.030	1.61	0.36	49.20

Appendix 10

Table 10.1: Pairwise and Multiple ClustalW alignments of ITS region sequences (Analysis done in MEGA5)

										1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3	3
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3
23	T	T	T	T	T	T	T	T	T	G	T	T	G	G	C	-	G	G	C	A	C	C	A	A	A	A	A	G	A	T	C	C	C
33d	.	G	G	.	.	G	G	A	C	.	.	C	.	A	.	T	.	.	.	G	.	.	C	G	G	C	C	.	G	C	.	.	T

	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6
	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6
23	T	C	G	T	G	A	A	A	T	T	G	T	T	A	A	T	G	G	A	A	T	T	G	G	A	T	-	-	-	T	A	C	A
33D	A	A	A	.	C	G	.	G	C	G	.	G	.	G	.	C	A	A	.	G	C	C	C	C	.	.	A	C	G	C	T	.	G

	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9	9	9
	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9
23	A	T	T	A	C	A	A	C	A	G	A	C	C	T	C	A	C	C	C	C	T	T	C	T	G	A	A	T	A	T	C	T	C
33D	.	G	G	.	.	C	G	G	.	C	.	.	G	G	T	G	.	.	G	.	C	G	.	.	.	C	C	.	T	.	.	G	G

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3
	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2
23	A	A	A	G	G	A	A	T	T	C	T	A	G	A	G	G	G	C	G	G	G	G	G	C	C	G	C	-	-	G	G	T	T
33D	G	C	C	C	.	T	C	C	C	.	C	G	.	G	.	.	.	G	A	A	C	A	A	.	.	A	.	C	C	A	A	A	C

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6
	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
23	A	A	A	A	C	C	C	-	-	-	C	C	C	G	C	G	G	C	C	C	T	G	A	A	A	G	G	G	G	G	A	C	C
33D	.	.	.	.	.	.	G	G	G	A	T	T	G	.	A	.	.	G	.	T	.	C	.	.	.	A	T	T	C	T	T	T	G

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9
	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8
23	G	C	C	G	C	C	A	G	C	A	A	T	T	T	A	G	G	T	A	C	C	G	C	A	A	A	C	A	C	A	G	G	T
33D	.	.	A	T	G	A	T	A	T	G	C	C	C	.	G	A	.	A	.	T	T	.	.	.	G	G	G	G	T	C	A	C	.

	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3
	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1
23	G	T	G	T	G	T	T	G	G	G	C	T	C	-	-	G	C	T	A	G	G	A	A	C	C	C	T	A	C	A	C	T	C
33D	.	.	T	.	C	C	.	C	T	C	.	.	.	T	A	A	T	A	.	A	.	.	.	T	T	T	.	G	A	.	T	.	.

	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	6	6	6	6	6
	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4
23	G	G	T	A	A	T	G	A	T	C	T	T	C	C	T	C	T	T	C	T	T	A	T	T	G	A	T	A	T	G	A	A	A
33D	C	T	G	.	.	.	-	-	.	.	C	C	A	T	.	A	C	.	T	A	.	C	C	C	A	G	.	T	C	.	.	.	.

	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	6	6	6	6	6	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9
	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7
23	A	A	G	T	T	-	-	-	-	-	-	C	A	G	C	G	G	G	T	T	T	C	C	C	T	A	C	-	-	-	-	C	T
33D	G	G	.	C	.	G	C	T	G	C	C	.	.	T	.	A	A	.	A	C	C	.	G	T	.	.	G	A	T	T	G	.	.

	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	9	9	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3
	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0
23	G	A	A	G	C	G	A	G	C	C	C	A	A	C	C	T	C	C	A	A	C	C	C	G	T	G	T	T	T	A	C	T	G
33D	T	T	T	C	.	A	.	.	A	T	A	.	.	G	A	C	.	G	.	G	G	T	T	A	.	A	.	.	C	C	T	A	C

	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	6	6	6
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3
23	T	A	C	C	T	T	A	G	T	T	G	C	T	T	C	G	G	C	G	G	G	C	C	C	G	C	C	A	T	T	C	A	T
33D	.	T	.	A	A	A	.	.	G	G	.	.	C	C	T	C	.	T	A	A	.	.	T	T	T	.	.	C	.	.	T	.	.

	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9
	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6
23	G	-	G	C	C	G	C	C	G	G	G	G	G	-	-	-	-	-	C	T	C	T	C	A	G	C	C	C	C	G	G	G	C
33D	T	T	A	.	.	.	.	G	.	.	.	.	A	G	A	T	C	T	.	.	T	.	T	G	A	T	A	A	A	.	A	.	.

	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	9	9	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9
23	C	C	G	C	G	C	C	C	G	C	C	G	G	A	G	A	C	A	C	C	A	C	G	A	A	C	T	C	T	G	T	C	T
33D	.	G	A	G	C	T	T	T	T	.	.	T	T	G	C	T	G	.	.	T	.	A	A	.	.	A	C	A	G	T	.	T	.

	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	6	6	6	
	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2
23	G	A	T	C	T	A	G	T	G	A	A	G	T	C	T	G	A	G	T	T	G	A	T	T	G	T	A	T	C	G	C	A	A
33D	.	G	C	A	G	T	T	.	.	.	.	A	A	.	C	A	.	A	A	G	.	G	.	.	.	C	.	A	A	C	.	C	T

	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9		
	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
23	T	C	A	G	T	T	A	A	A	A	C	T	T	T	C	A	A	C	A	A	T	G	G	A	T	C	T	C	T	T	G	G	T
33D	.	A	.	C	.	.	.	.	.	T	A	.	A	.	T	G	.	T	.	.	A	C	.	T	A	.	G	G	A	C	.	.	A

	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	9	9	9	9	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8
23	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A
33D	.	.	-	-	-	-	.	.	T	A	C	.	.	.	-	.	T	G	.	.	-	G	.	.	T	G	C	C	.	T	.	.	G

	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	6
	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1
23	T	A	A	C	T	A	G	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	T	C	C	G	T	G	A	A	T	C	A
33D	G	C	C	.	A	.	C	C	T	C	C	.	C	C	C	.	.	T	.	.	C	.	A	.	.	T	-	-	.	.	C	.	.

	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	9	9	9	9	9	
	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4
23	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	G	C	C	C	C	C	T	G	G	T
33D	.	W	.	.	T	.	G	C	.	.	C	G	G	.	.	G	G	G	.	G	C	C	.	T	.	T	.	G	G	G	.	.	C

	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7
23	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	C	T	G	-	-	-	T	C	C	G	A	G	C	G	T	C	A
33D	G	A	G	.	.	.	C	C	.	.	.	G	.	C	T	A	.	.	.	A	A	C	.	T	.	A	T	.	.	C	.	G	.

	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	2	2	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	
	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0
23	T	T	G	C	T	G	C	C	C	A	-	T	C	A	A	G	C	A	C	G	G	C	T	T	G	T	G	T	G	T	T	G	G
33D	G	A	.	T	G	A	T	G	.	.	G	.	.	T	G	A	G	T	.	T	.	A	A	.	A	.	A	A	A	A	.	C	A

	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	9	9	9	9
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3
23	G	T	C	G	T	C	G	T	C	C	C	C	T	C	T	C	C	G	G	G	G	G	G	G	A	C	G	G	G	C	C	C	C
33D	.	.	.	A	A	A	A	C	T	T	T	.	A	A	C	.	A	T	.	.	A	T	C	T	C	T	T	.	.	T	T	.	.

	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2
	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6
23	A	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	C	G	T	C	C	G	-	-	A	T	C	C	T	C
33D	G	G	T	A	T	.	.	T	A	A	A	A	A	A	.	.	.	A	T	T	.	A	A	A	A	G	C	.	.	T	G	.	G

	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	2	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5
	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9
23	G	A	G	C	G	T	A	T	G	G	G	G	C	T	T	T	G	T	C	A	C	-	-	-	C	C	G	C	T	C	T	G	T
33D	.	.	.	G	T	A	.	A	T	.	A	T	T	.	.	.	T	.	.	G	.	A	T	T	T	.	C	.	.	.	C	.	.

	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	9	9	9
	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2
23	A	G	G	C	C	C	G	G	C	C	G	G	C	G	C	T	T	G	C	C	G	-	-	-	-	-	-	A	A	C	G	C	A
33D	.	C	T	A	T	T	.	C	A	A	A	T	T	T	T	.	.	T	.	.	C	C	C	C	C	T	T	.	C	T	A	.	.

	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	9	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2
	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
23	-	A	A	T	C	A	A	T	S	K	T	T	C	C	A	A	G	G	T	G	A	C	C	T	C	S	G	A	A	A	A	A	G
33D	T	.	.	C	.	T	.	.	C	A	C	C	T	.	T	.	.	.	G	A	G	T	G	A	T	G	.	G	.	.	T	G	A

	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	2	2	2	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5
	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8
23	T	A	G	G	G	A	C	A	C	C	C	T	T	T	G	A	T	C	T	T	T	T	T	T	T	T	T	T	A	T	C	A	A
33D	.	G	.	C	C	G	A	.	T	T	G	C	.	.	T	.	.	T	.	A	.	.	C	C	C	.	.	.	C	C	G	.	G

	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	9	9
	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1
23	A	A	A	A	A	G	G	A	G	G	A	A	G	A	T	-	C	A	T	T	A	C	C	G	T	C	G	T	G	T	A	G	G
33D	T	G	.	.	.	.	C	.	C	A	G	G	.	G	.	G	.	.	.	A	.	.	T	T	.	.	T	C	.	A	.	A	.

	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	9	9	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2
	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4
23	G	A	T	C	C	T	A	G	C	G	A	G	A	-	-	-	-	C	C	A	A	A	T	A	A	C	A	A	C	G	T	G	T
33D	.	T	G	A	.	A	.	.	G	.	.	A	T	A	T	T	T	.	.	.	G	T	.	.	T	T	C	.	.	C	A	A	A

	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5
	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7
23	T	G	T	G	C	T	G	T	A	C	C	A	A	A	A	A	T	G	C	G	T	C	G	C	G	T	G	C	C	G	C	C	T
33D	G	.	A	.	A	.	.	.	.	T	.	T	T	T	T	G	A	A	A	A	A	A	A	A	.	A	.	A	T	T	T	T	C

	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9
	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0
23	C	T	C	G	A	G	A	C	C	G	T	C	T	G	A	A	T	A	T	C	T	A	A	A	C	C	A	G	C	C	C	C	T
33D	.	.	T	.	.	T	C	.	T	A	A	.	A	A	.	.	.	G	A	A	.	G	.	C	A	G	G	C	T	.	T	T	.

	9	9	9	9	9	9	9
	9	9	9	9	9	9	9
	1	2	3	4	5	6	7
23	G	C	G	C	C	T	T
33D	.	A	A	.	A	A	C

N.B.: Dot (.) represents same nucleotide as in first accession and Dash (-) represents gap/ missing data.

Table 10.2: Pairwise and Multiple ClustalW alignments of nucleotide sequences of D1/D2 domains of nrDNA LSU region (Analysis done in MEGA5)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Penicillium ochrochloron (A12)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus parasticus (A13)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A16)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A277)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium ruqulosum (31c)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus protuberus (33d)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium chrysogenum (112a)	A	A	C	A	A	C	C	T	A	A	G	C	G	A	A	T	A	A	A	A	A	A	T	A	T	A	C	T	A	G
Aspergillus niger (113b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium simplicissimum (A10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
Penicillium ochrochloron (A12)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus parasticus (A13)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A16)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A277)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium ruqulosum (31c)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus protuberus (33d)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium chrysogenum (112a)	A	T	G	T	G	T	T	C	C	T	C	C	T	T	T	A	A	A	C	A	T	T	A	G	C	A	C	C	A
Aspergillus niger (113b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium simplicissimum (A10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89
Penicillium ochrochloron (A12)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus parasticus (A13)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A16)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A277)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium ruqulosum (31c)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus protuberus (33d)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium chrysogenum (112a)	C	C	G	A	A	G	C	A	G	A	A	A	T	T	C	T	A	A	C	A	T	T	T	A	T	T	A	G	C
Aspergillus niger (113b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium simplicissimum (A10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
Penicillium ochrochloron (A12)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	C	C	T	C	A	G	T	A	A	C	G	G	C	G
Aspergillus parasticus (A13)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	C	C	G	T	-	-	.	.	C
Penicillium aurantiogriseum (A16)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G	T	C	C	G	.	-	-	.	.	C
Penicillium ruqulosum (31c)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	G	C	G	T	C	C	G	.	-	-	.	.	C
Aspergillus protuberus (33d)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	A	A	A	G	C	A	C	C	C	A	G	C	A	A	T	.	T	C	G	C	C	.	.	.	T	.	.	.	A
Aspergillus niger (113b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.
Penicillium simplicissimum (A10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.

	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149
Penicillium ochrochloron (A12)	G	T	G	A	A	G	C	G	G	C	A	A	G	A	G	C	T	C	A	A	A	T	T	T	G	A	A	A	G
Aspergillus parasticus (A13)	A	A	.	C	-	.	.	.	T	T	C	C	T	C	.	G	.	.	C	.	G	G	C	.	.	G	C	C	.
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	A	A	.	C	-	.	.	.	T	T	C	C	T	C	.	G	.	.	T	.	G	G	C	A	.	G	T	C	.
Penicillium ruqulosum (31c)	A	A	.	C	-	.	.	.	T	T	C	C	T	C	.	G	.	.	C	G	G	G	C	A	.	G	T	C	.
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Penicillium chrysogenum (112a)	T	C	.	C	T	.	.	.	T	T	C	C	T	C	A	G	.	.	C	.	.	.	C	C	A	G	.	-	.
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	1 5 1	1 5 2	1 5 3	1 5 4	1 5 5	1 5 6	1 5 7	1 5 8	1 5 9	1 6 0	1 6 1	1 6 2	1 6 3	1 6 4	1 6 5	1 6 6	1 6 7	1 6 8	1 6 9	1 7 0	1 7 1	1 7 2	1 7 3	1 7 4	1 7 5	1 7 6	1 7 7	1 7 8	1 7 9
Penicillium ochrochloron (A12)	T	G	G	C	C	C	C	C	T	C	G	G	G	G	T	C	C	G	C	A	T	T	G	T	A	A	T	T	T
Aspergillus parasticus (A13)	A	T	T	G	.	A	.	T	C	.	C	.	.	C	.	A	T	A	A	G	G	.	.	C	C	C	C	G	G
Penicillium aurantiogriseum (A16)	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	A	T	T	G	.	A	.	.	C	T	C	.	.	C	.	A	T	A	A	G	A	C	.	C	C	C	C	.	A
Penicillium ruqulosum (31c)	A	T	.	G	.	G	.	.	.	.	.	.	.	C	.	A	T	A	A	G	G	C	A	C	C	C	C	G	G
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	A	T	.	A	T	A	G	-	-	T	A	.	.	C	.	A	T	A	A	-	-	C	A	C	C	T	C	G	A
Aspergillus niger (113b)	.	.	.	.	T	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	1 8 1	1 8 2	1 8 3	1 8 4	1 8 5	1 8 6	1 8 7	1 8 8	1 8 9	1 9 0	1 9 1	1 9 2	1 9 3	1 9 4	1 9 5	1 9 6	1 9 7	1 9 8	1 9 9	2 0 0	2 0 1	2 0 2	2 0 3	2 0 4	2 0 5	2 0 6	2 0 7	2 0 8	2 0 9
Penicillium ochrochloron (A12)	C	A	G	A	G	-	G	A	T	G	C	T	T	C	G	G	G	A	G	-	T	G	G	C	C	C	C	C	A
Aspergillus parasticus (A13)	G	G	.	C	A	C	T	.	C	A	T	.	C	.	.	.	.	.	.	C	C	T	T	T	G	A	.	.	G
Penicillium aurantiogriseum (A16)	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	-	C	.	.	T	.	.	.	.	.
Penicillium aurantiogriseum (A277)	G	G	.	C	.	T	T	.	C	C	T	.	C	.	.	A	.	G	.	C	C	T	T	T	G	A	.	.	G
Penicillium ruqulosum (31c)	G	G	.	T	.	C	T	.	C	A	T	.	C	.	C	.	.	G	.	C	C	T	T	T	G	A	.	.	G
Aspergillus protuberus (33d)	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	T	.	-	C	.	.	.	.	.	.	T	G
Penicillium chrysogenum (112a)	A	G	.	C	-	-	T	.	C	A	T	.	C	.	T	A	C	T	A	A	.	T	T	T	T	T	.	.	T
Aspergillus niger (113b)	.	.	.	.	.	-	.	.	.	.	.	.	.	T	.	.	.	T	.	-	C	.	.	.	.	.	.	.	G
Penicillium simplicissimum (A10)	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	-	C	.	.	.	.	.	.	.	.

	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239
Penicillium ochrochloron (A12)	C	T	A	A	G	T	G	C	C	C	T	G	G	A	A	C	G	G	G	C	C	G	T	C	A	T	A	G	A
Aspergillus parasticus (A13)	.	C	G	C	C	C	A	A	A	.	C	.	A	C	G	.	T	.	.	.	.	C	G	.	C	C	C	C	.
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	.	C	G	C	C	C	A	A	A	.	C	.	A	C	G	.	T	.	.	.	.	C	G	.	C	C	G	C	G
Penicillium ruqulosum (31c)	.	C	G	C	C	C	A	A	A	.	C	.	A	C	G	.	T	.	.	.	.	C	G	.	C	C	G	.	G
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
Penicillium chrysogenum (112a)	.	A	.	.	T	C	A	A	A	.	.	.	T	T	G	T	T	.	.	.	A	.	G	T	.	.	.	.	.
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	244	244	244	244	244	244	244	244	244	245	245	245	245	245	245	245	245	245	245	260	266	266	266	266	266	266	266	266	266
	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189
Penicillium ochrochloron (A12)	G	G	T	G	A	G	A	A	T	C	C	C	G	T	C	T	G	G	G	A	T	G	G	G	G	T	G	C	C
Aspergillus parasticus (A13)	.	.	-	A	.	.	T	.	C	A	.	.	-	G	G	C	A	C	.	.	A	T	.	C	C	-	.	G	.
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Penicillium aurantiogriseum (A277)	.	.	-	A	.	.	T	.	C	A	.	.	-	G	G	C	A	C	.	.	A	T	.	C	C	-	.	G	.
Penicillium ruqulosum (31c)	.	.	-	.	.	.	T	G	C	A	.	.	-	G	G	C	A	C	A	C	G	T	.	C	C	-	.	G	.
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	C	A	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	T	.	C	A	.	.	T	G	C	A	.	.	C	A	G	A	A	C	.	.	A	T	T	C	T	A	.	G	T
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	C	.	.	.	.	.	.	T	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299
Penicillium ochrochloron (A12)	A	C	G	C	C	C	G	T	G	T	G	A	A	G	C	T	C	C	T	T	C	G	A	C	G	A	G	T	C
Aspergillus parasticus (A13)	G	A	A	.	.	.	T	G	.	-	A	G	G	C	G	A	G	T	C	.	G	.	T	.	.	C	A	A	G
Penicillium aurantiogriseum (A16)	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	G	A	A	.	.	.	C	G	C	-	.	.	G	C	G	A	G	T	C	.	G	.	T	.	.	C	A	A	G
Penicillium ruqulosum (31c)	G	A	.	.	.	.	T	C	C	C	.	G	G	C	G	A	G	T	C	.	G	.	T	T	.	C	C	A	A
Aspergillus protuberus (33d)	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	G	A	T	T	A	.	A	G	T	C	A	.	.	C	.	A	G	T	C	.	G	.	C	T	C	C	A	A	A
Aspergillus niger (113b)	G	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329
Penicillium ochrochloron (A12)	A	G	T	T	G	T	T	T	G	G	G	A	A	T	G	C	A	G	C	T	C	T	A	A	A	T	G	G	G
Aspergillus parasticus (A13)	G	C	.	.	C	C	C	.	T	T	C	.	.	-	-	.	.	A	T	.	T	C	.	C	G	.	.	C	T
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	G	C	.	.	C	C	C	.	T	T	C	.	.	-	-	.	.	A	T	.	T	C	.	C	G	.	.	C	T
Penicillium ruqulosum (31c)	G	C	.	.	C	C	C	.	T	T	C	.	.	-	-	.	.	A	T	.	T	C	.	C	G	.	.	C	T
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	G	.	.	.	C	C	C	.	T	T	T	.	.	-	-	.	.	A	T	.	T	C	.	C	.	.	A	C	T
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359
Penicillium ochrochloron (A12)	G	G	T	A	A	A	T	T	T	C	A	T	C	T	A	A	A	G	C	T	A	A	A	T	A	C	T	G	G
Aspergillus parasticus (A13)	T	T	.	.	.	C	.	C	.	.	T	.	T	.	C	.	.	A	G	.	G	C	T	.	T	T	C	A	T
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
Penicillium aurantiogriseum (A277)	T	T	.	.	.	C	.	C	.	.	T	.	T	.	C	.	.	A	G	.	G	C	T	.	T	T	C	A	T
Penicillium ruqulosum (31c)	T	T	.	.	.	C	.	C	.	.	T	.	T	.	C	.	.	A	G	.	G	C	T	.	T	T	C	A	T
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
Penicillium chrysogenum (112a)	T	T	.	.	.	C	.	C	.	.	T	.	T	.	C	.	.	A	G	.	T	C	T	.	T	T	C	A	T
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389
Penicillium ochrochloron (A12)	C	G	G	A	G	A	C	C	G	A	T	A	G	C	G	C	A	C	A	A	G	T	A	G	A	G	T	G	A
Aspergillus parasticus (A13)	T	T	T	C	.	.	T	.	A	C	.	C	T	A	C	T	T	G	T	G	C	G	C	T	.	T	C	.	G
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	T	T	T	C	.	.	T	.	A	C	.	C	T	A	C	T	T	G	T	G	C	G	C	T	.	T	C	.	G
Penicillium ruqulosum (31c)	T	T	T	C	.	.	T	.	A	C	.	C	T	A	C	T	T	G	T	G	C	G	C	T	.	T	C	.	G
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	T	T	T	C	C	C	T	.	A	C	G	G	T	A	C	T	T	G	T	T	C	G	C	T	.	T	C	.	G
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419
Penicillium ochrochloron (A12)	C	G	A	A	A	G	A	T	G	A	A	A	A	G	C	A	C	T	T	T	G	A	A	A	A	G	A	G	A
Aspergillus parasticus (A13)	.	T	C	C	G	.	C	C	A	G	T	.	T	T	T	.	G	C	.	.	T	.	G	.	T	.	.	A	.
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	.	T	C	C	G	.	C	C	A	.	T	.	T	T	T	.	G	C	.	.	T	.	G	.	T	.	.	A	.
Penicillium ruqulosum (31c)	.	T	C	C	G	.	C	C	A	.	T	.	T	T	T	.	G	C	.	.	T	.	G	.	T	.	.	A	.
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	.	T	C	T	C	.	C	A	A	.	T	.	T	T	T	.	G	C	.	.	T	.	G	G	T	.	.	.	.
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449
Penicillium ochrochloron (A12)	T	T	A	A	A	C	A	G	C	A	C	G	T	G	A	A	A	T	T	G	-	-	-	T	T	G	A	A	A
Aspergillus parasticus (A13)	.	.	.	C	C	A	C	C	.	.	T	T	.	A	G	.	G	C	.	.	C	A	T	.	C	C	C	.	.
Penicillium aurantiogriseum (A16)	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	.	.	.	C	C	A	C	C	.	.	T	T	.	A	G	.	G	C	.	.	C	A	T	.	C	C	C	.	.
Penicillium ruqulosum (31c)	.	.	.	C	C	A	C	C	.	T	.	T	.	A	G	.	G	C	.	.	C	A	T	.	C	C	C	.	.
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	.	.
Penicillium chrysogenum (112a)	.	.	.	C	C	A	C	C	.	.	A	T	.	T	.	G	G	C	.	.	C	A	T	.	C	C	C	.	.
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	.	.

	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479
Penicillium ochrochloron (A12)	G	G	A	A	G	C	G	-	C	T	T	G	C	G	A	C	C	A	G	A	C	T	C	G	C	T	C	G	C
Aspergillus parasticus (A13)	C	A	.	C	T	.	.	A	.	.	C	.	T	C	G	A	A	G	.	.	G	C	T	T	.	A	.	A	.
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	C	A	.	C	T	.	.	A	.	.	C	.	T	C	G	A	A	G	.	.	G	C	T	T	.	A	.	A	.
Penicillium ruqulosum (31c)	C	A	.	C	T	.	.	A	.	.	C	T	T	C	G	A	A	G	.	.	G	C	T	T	.	A	.	A	.
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	T	.
Penicillium chrysogenum (112a)	C	A	.	C	C	T	.	A	.	.	C	T	T	T	G	A	A	.	A	C	A	.	A	T	.	A	.	A	A
Aspergillus niger (113b)	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.

	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509
Penicillium ochrochloron (A12)	G	G	-	G	T	T	C	A	G	C	C	G	G	C	A	T	T	C	G	T	G	C	C	G	G	T	G	T	A
Aspergillus parasticus (A13)	.	.	C	.	C	G	G	.	C	A	.	C	C	.	.	.	C	.	C	A	.	A	.	.	.	G	A	.	T
Penicillium aurantiogriseum (A16)	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	.	.	C	.	C	G	G	.	C	A	.	C	C	.	.	.	C	.	C	A	T	A	.	.	.	G	A	.	T
Penicillium ruqulosum (31c)	.	.	C	.	-	G	G	.	C	.	A	C	C	.	.	.	C	.	C	A	.	A	.	.	.	G	A	.	T
Aspergillus protuberus (33d)	.	.	-	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Penicillium chrysogenum (112a)	A	.	A	C	A	A	.	T	A	.	T	C	.	G	.	.	C	A	A	A	.	A	.	.	.	G	A	.	T
Aspergillus niger (113b)	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	511	511	511	511	511	511	511	511	511	520	522	522	523	524	525	526	527	527	528	529	530	531	532	533	533	534	535	536	537	538	539
Penicillium ochrochloron (A12)	T	T	C	C	C	T	G	C	G	G	G	C	G	G	G	C	C	A	G	C	G	T	C	G	G	T	T	T	G
Aspergillus parasticus (A13)	.	C	A	.	.	C	T	.	T	C	T	G	A	C	.	G	.	C	C	G	T	.	.	C	A	G	G	G	C
Penicillium aurantiogriseum (A16)	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	.	C	A	.	.	C	T	.	T	A	T	G	A	C	.	T	.	C	C	G	T	.	.	C	A	G	G	G	C
Penicillium ruqulosum (31c)	.	C	A	.	.	C	T	.	T	A	T	G	A	C	.	G	.	C	C	G	T	.	.	C	A	G	G	G	C
Aspergillus protuberus (33d)	.	.	.	.	.	C	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
Penicillium chrysogenum (112a)	.	C	A	.	.	C	T	.	C	A	T	G	A	T	.	.	.	T	T	G	T	.	.	C	A	A	A	G	.
Aspergillus niger (113b)	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	544	544	544	544	544	544	544	544	544	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569
Penicillium ochrochloron (A12)	G	C	G	G	C	C	G	G	T	C	A	A	A	G	G	C	C	C	T	T	G	G	A	A	T	G	T	A	A
Aspergillus parasticus (A13)	C	T	T	A	G	A	C	A	G	G	G	G	C	C	.	.	A	.	C	C	.	A	.	G	C	A	.	-	C
Penicillium aurantiogriseum (A16)	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.
Penicillium aurantiogriseum (A277)	C	T	T	A	G	A	T	.	G	G	G	.	C	C	.	.	T	.	C	C	.	A	.	G	C	A	.	-	C
Penicillium ruqulosum (31c)	C	T	T	A	G	A	T	.	G	G	G	G	C	C	A	.	G	.	C	C	.	A	.	G	C	A	.	-	C
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	T
Penicillium chrysogenum (112a)	C	T	T	A	T	T	A	A	.	.	C	G	G	C	.	T	G	.	C	A	.	.	.	.	A	A	.	G	T
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.

	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599
Penicillium ochrochloron (A12)	G	C	C	C	C	-	-	-	C	C	G	G	G	G	C	G	T	C	T	T	A	T	A	G	C	C	A	A	G
Aspergillus parasticus (A13)	T	.	T	G	.	A	A	A	T	T	A	C	A	A	T	.	C	G	G	A	C	C	-	-	.	.	G	.	A
Penicillium aurantiogriseum (A16)	.	.	.	.	.	-	-	-	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
Penicillium aurantiogriseum (A277)	T	.	T	G	.	A	A	A	T	T	A	C	A	A	T	.	C	G	G	A	C	C	-	-	.	.	G	.	A
Penicillium ruqulosum (31c)	T	.	T	.	.	A	A	A	T	T	A	C	A	A	.	T	C	G	G	G	C	C	-	-	.	.	G	.	A
Aspergillus protuberus (33d)	.	T	.	.	T	-	-	-	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
Penicillium chrysogenum (112a)	T	.	T	A	.	A	G	T	T	T	A	C	A	A	T	A	C	G	G	.	T	.	.	.	.	T	.	.	.
Aspergillus niger (113b)	.	.	.	.	T	-	-	-	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	.	.	G	.
Penicillium simplicissimum (A10)	.	.	.	.	.	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.

	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629
Penicillium ochrochloron (A12)	G	T	G	C	C	A	T	G	C	G	G	C	C	A	G	C	C	C	G	G	A	C	C	G	A	G	G	A	A
Aspergillus parasticus (A13)	.	A	.	.	.	.	G	C	T	T	T	.	A	.	A	T	T	T	.	A	G	.	T	C	T	T	.	C	C
Penicillium aurantiogriseum (A16)	.	.	.	.	A	.	.	.	.	.	A	.	.	T	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.
Penicillium aurantiogriseum (A277)	.	A	.	.	.	.	G	C	T	T	T	.	A	.	A	T	T	T	.	A	G	.	T	C	T	T	.	C	C
Penicillium ruqulosum (31c)	.	A	.	.	.	.	G	A	T	T	T	.	A	.	A	T	T	T	.	A	G	.	T	C	T	T	.	C	C
Aspergillus protuberus (33d)	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Penicillium chrysogenum (112a)	C	A	A	A	.	.	G	T	T	T	C	.	A	.	C	T	T	T	.	A	G	.	T	C	T	T	T	C	C
Aspergillus niger (113b)	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659
Penicillium ochrochloron (A12)	G	C	G	C	T	T	C	G	G	C	T	C	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus parasticus (A13)	C	T	T	.	A	C	T	C	.	.	C	G	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A16)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	G	C	-	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A277)	C	T	T	.	A	C	T	C	.	.	C	G	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium ruqulosum (31c)	C	T	T	.	A	C	T	C	.	.	C	G	T	T	A	C	T	G	A	G	G	-	-	-	-	-	-	-	-
Aspergillus protuberus (33d)	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium chrysogenum (112a)	C	T	T	.	A	C	T	C	.	.	C	G	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus niger (113b)	.	.	.	.	.	.	.	.	.	.	A	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium simplicissimum (A10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	G	C	-	-	-	-	-	-	-	-	-	-	-

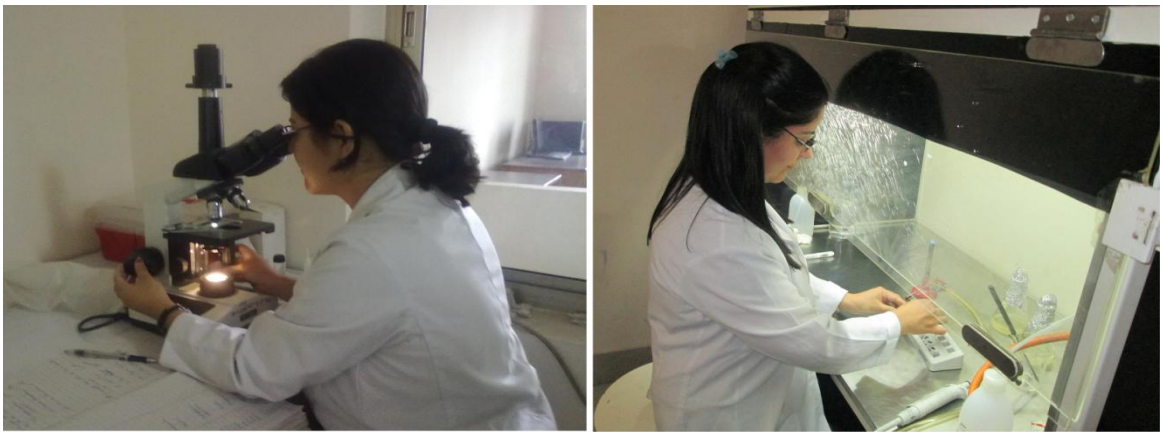
	661	662	663	664	665	666	667	668	669	670
Penicillium ochrochloron (A12)	-	-	-	-	-	-	-	-	-	-
Aspergillus parasticus (A13)	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A16)	-	-	-	-	-	-	-	-	-	-
Penicillium aurantiogriseum (A277)	-	-	-	-	-	-	-	-	-	-
Penicillium ruqulosum (31c)	-	-	-	-	-	-	-	-	-	-
Aspergillus protuberus (33d)	-	-	-	-	-	-	-	-	-	-
Penicillium chrysogenum (112a)	-	-	-	-	-	-	-	-	-	-
Aspergillus niger (113b)	-	-	-	-	-	-	-	-	-	-
Penicillium simplicissi(A10)	-	-	-	-	-	-	-	-	-	-

N.B.: Dot (.) represents same nucleotide as in first accession and Dash (-) represents gap/ missing data.

Appendix 11

Some Photographes

Plate 11.1: Lab members of Molecular Biotechnology Unit, NAST, Khumaltar, Lalitpur, Nepal.



A) **B)**

Plate 11.2: Working on the laboratory **A)** Microscopic observation of stained sample **B)** Extraction of DNA under Laminar Air Flow (LAF)

A) **B)**

Plate 11.3: Working on the laboratory **A)** Preparing Master Mix for PCR under LAF **B)** Loading amplified DNA into agarose gel