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NATIONAL AGRICULTURAL HIGHER EDUCATION PROJECT

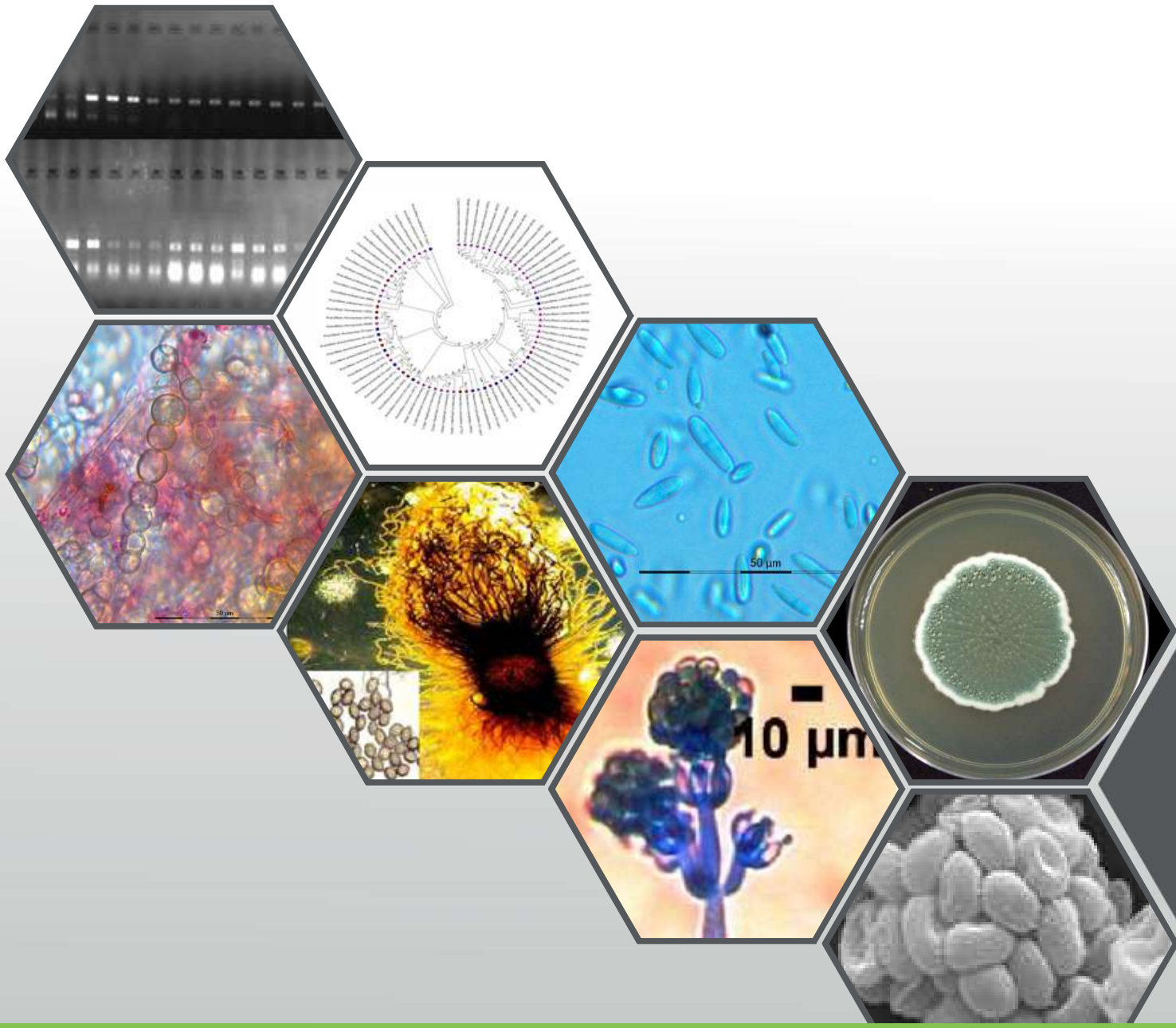
Centre for Advanced Agricultural Science and Technology (CAAST) on

Genomics-Assisted Crop Improvement and Management

Practical Manual

"GENOMICS-ASSISTED MOLECULAR SYSTEMATICS OF FUNGI"

"Shaping human resources to harness the global gene pool for sustainable agriculture"



Division of Plant Pathology

ICAR-Indian Agricultural Research Institute, New Delhi

http://nahep-caast.iari.res.in/

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on
Genomics-Assisted Molecular Systematics of Fungi
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About NAHEP-CAAST at IARI, New Delhi

Centre for Advanced Agricultural Science and Technology (CAAST) is a new initiative and student centric subcomponent of World Bank sponsored **National Agricultural Higher Education Project (NAHEP)** granted to The Indian Council of Agricultural Research, New Delhi to provide a platform for strengthening educational and research activities of post graduate and doctoral students. The ICAR-Indian Agricultural Research Institute, New Delhi was selected by the NAHEP-CAAST programme. NAHEP sanctioned Rs 19.99 crores for the project on "**Genomic assisted crop improvement and management**" under CAAST programme. The project at IARIspecifically aims at inculcating genomics education and skills among the students and enhancing the expertise of the faculty of IARI in the area of genomics.

Objectives:

1. **To develop online teaching facility and online courses for enhancing the teaching and learning efficiency, and scientific communications skills**
2. **To develop and/or strengthen state-of-the art next-generation genomics and phenomics facilities forproducing quality PG and Ph.D. students**
3. **To develop collaborative research programmes with institutes of international repute and industries inthe area of genomics and phenomics**
4. **To enhance the skills of faculty and PG students of IARI and NARES**
5. **To generate and analyze big data in genomics and phenomics of crops, microbes and pests for genomicsaugmentation of crop improvement and management**

IARI's CAAST project is unique as it aimed at providing funding and training support to the M.Sc. and Ph.D.students from different disciplines who are working in the area of genomics. It will organize lectures andtraining programmes, and send IARI students and covering students from several disciplines. It will provideopportunities to the students and faculty to gain international exposure. Further, the project envisagesdeveloping a modern lab named as **Discovery Centre** that will serve as a common facility for students' researchat IARI.

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Foreword

The ICAR-Indian Agricultural Research Institute, New Delhi has made significant contributions in developing crop protection and production technologies for all major crops in India. The institute has core strength in the area of genomics and modern research facilities for conducting advanced genomics programmes. ICAR- IARI has major focus on research and academic programme on fungal taxonomy, fungal genomics and molecular biology.

The Herbarium Cryptogamae Indiae Orientalis (HCIO) is a national herbarium situated at ICAR-Indian Agricultural Research Institute, New Delhi. At present nearly 50000 fungal diseased specimens comprising of different groups of fungi are being maintained and preserved in their natural and digitized form in HCIO. The Indian Type Culture Collection (ITCC) is an affiliate member of the World Federation for Culture Collections (WFCC; 430) established in 1936 in the Division of Plant Pathology, ICAR-Indian agricultural Research Institute, New Delhi, with a view to furnish a knowledge on living fungi. About 4000 fungal cultures are being maintained at this collection. ITCC and HCIO are the show windows of Division of Plant Pathology, ICAR-IARI, New Delhi. Mycology unit of Division of Plant Pathology are actively involved in the research programmes relating to microbial diversity, ecology and fungal taxonomy using both classical and molecular approaches. DNA Barcoding of fungi has been done in a big way to augment the digital database of Indian Type Culture Collection, one of the oldest fungal collections in this country.

Presently research efforts are underway on metagenomics, delineation of fungal species complexes utilizing genomic tools, DNA barcoding, development of molecular markers and whole genome sequencing of fungi. With this background the Centre for Advanced Agricultural Science and Technology (CAAST) under NAHEP is organizing a Short Training course on "Genomics-assisted molecular systematics of fungi" in Division of Plant Pathology, ICAR-IARI for the benefit of students of SAUs and other traditional government and private universities. I am sure that the training will impart the basics of molecular taxonomy to the Post Graduate and Ph.D. students of relevant area.

Date: 30th July 2019

Dean and Joint Director (Education)
ICAR-IARI, New Delhi

Preface

Fungi are one of the most important groups of organisms on the planet. They are used in food (Mushrooms and Morels); baking industry; production of enzymes, antibiotics, hormones and different organic acids; organic matter decomposition; symbiotic relation with plants and are potential biotic threats to crop production. Authentic identification of fungi is essential as wrong identification would result in wrong interpretations in research programmes. Moreover most of these fungal groups are vast, diverse and complex, making the identification task equally complicated and difficult. Diversity studies on various groups of fungi will facilitate the Indian scientists to update the latest developments in fungal taxonomy through detailed descriptions of genera /species; establishment of evolutionary relationships; taxon-based through development of databases and whole genome sequencing. This area can also be utilized further for need based molecular characterization / DNA barcoding and chemotaxonomy through chemo-profiling for bioactive compounds produced by fungi and utilization of next generation sequencing technology.

The discovery of first microbial genome by Craig Venter in the year 1996 has culminated in the birth of science of genomics. In the last two decade, 'omics science' and genomic data has enables us to understand diverse plant associated microbial communities, pathogens of crop plants and their behavior on plants associated niches.Recently, fungal taxonomy group at division of Plant Pathology, ICAR-IARI has been instrumental in successfully demonstrating the genomics-assisted molecular systematic and whole genome sequencing.

The major objective of the training programme is to train young students on the application of genomic tools for fungal taxonomy with special relevance to DNA barcoding, molecular phylogeny and whole genome sequencing. The recent advances on fungal taxonomy will facilitate students to update their skill on fungal systematics. The proposed training program would, therefore, be an opportunity for researchers on a national level to have active interactions and experiences to hone their skills in the area of genomics assisted fungal taxonomy. Hands-on training in this topic will be imparted in addition to lectures by eminent experts so that the participants could apply the same in their research programmes.

Date: 30.07.2019

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Contents

Diversity and Taxonomy of Fungi

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Biodiversity of fungi forms an important component as it forms resource material for biotechnology. 1.5 -5.0 million is the estimated total fungal species count and of which 93843 species are reported from world. Around 29,000 Fungal species are reported from India indicating that 1/3 of fungal diversity exists in India. It is also known that 5-7% Fungi are culturable and out of which 40% are soil fungi. Biodiversity contributes to the existence of human race and is the resource material for biotechnology. Loss of bioresources leads to economic crisis and vanishing of mankind.

Ex-situ and *In-situ* conservation of germplasm has become essential in order to protect the bioresources. Nature is bountiful of living communities/biotic communities which offer food security, nutritional security and health security besides adding economic strengths and sustainable development. Bioresource management in India has become a critical issue. If the *in-situ* and *ex-situ* conservation including legal restrictions are not followed, world's biota may become extinct. 21st century is the era of proteomics, genomics and metabolomics. Application of all such modern technologies may bring out novel organisms and their functional chemical molecules.

Species Concept in Fungi

The basic rank in taxonomy is the species. However, exactly what different mycologists consider to be a species can vary widely, and there are different approaches for delineating them. Attempts to reach a consensus for a universal definition of species have been unsuccessful, and consequently several concepts have been used. However, the genetic basis for some of these concepts is largely unknown. The widespread occurrence of asexual reproduction by asexual propagules (conidia) and of hyphal anastomosing can cause confusion because a mycelium in its natural environment seems to be a single physiological and ecological unit but in reality is a genetic mosaic. Therefore, in mycology, the distinction between a population and an individual is not always easy, and this can create confusion in genetic studies.

Different concepts have been used by mycologists to define the fungal species. The morphological (phenetic or phenotypic) concept is the classic approach used by mycologists; in this approach, units are defined on the basis of morphological characteristics and ideally by the differences among them. The polythetic concept is based on a combination of characters, although each strain does not have to have the same combination. The ecological concept, which is based on adaptation to particular habitats rather than on reproductive isolation, is often used for plant pathogenic fungi. The biological concept, which was developed before the advent of modern phylogenetic analysis, emphasizes gene exchange (i.e., sexual and parasexual reproduction) within species and the presence of barriers that prevent the crossbreeding of species. A species is considered to be an actual or potential interbreeding population isolated by intrinsic reproductive barriers. However, application of the biological species concept to fungi is complicated by the difficulties in mating and in assessing its outcome. Also, whether a cross is considered fertile or sterile depends on the frame of reference. In this sense, published accounts of crosses between different species are often difficult to interpret because authors have failed to specify the type of infertility and its severity. The biological species concept cannot be applied to organisms that do not undergo meiosis. It is applied only to sexual fungi, whereas asexual ones need only possess similar characteristics to each other. However in asexual fungi, genetic

exchange through somatic hybridization is a theoretical possibility, although it is limited by vegetative incompatibility. For asexual dermatophytes, the cohesion species concept, based on a demographic exchangeability of phenotypes, has been used to explain the proliferation of disjunct phenotypes. The demographic exchangeability would be the ecological analogue of the genetic exchangeability of sexual species.

Two recent and important developments have greatly influenced and caused significant changes in the traditional concepts of systematics. These are the phylogenetic approaches and incorporation of molecular biological techniques, particularly the analysis of DNA nucleotide sequences, into modern systematics. Molecular techniques, which were previously used only in research laboratories, are now commonplace. These developments have provided new information that has caused the biologicalspecies concept to come under criticism in favor of the phylogeneticspecies concept. This new concept has been found particularly appropriate for fungal groups in which no sexual reproduction has been observed (deuteromycetes). Hence, new concepts, specifically formulated within the field of phylogenetics, are becoming familiar to mycologists. Population studies and molecular data are increasingly showing that many widely used morphospecies actually comprise several biological or phylogenetic species. One of the problems for morphologists involves deciding how many base differences are required for strains to be considered different species. This has been partly solved by the phylogenetic-species concept, especially when based on cladistic analysis of molecular characteristics, which offers consistency in the delineation of species. Cladogram topology indicates the existence of monophyletic groups, which may represent species or supra- or sub-specific taxa. Peterson and Kurtzman correlated the biological species concept with the phylogenetic-species concept by comparing the fertility of genetic crosses among heterothallic yeasts. They demonstrated that the D_2 region, a variable region of the 25S rDNA gene, is sufficiently variable to recognize biological species of yeasts and that conspecific species generally show less than 1% nucleotide substitution.

However, the definition of the phylogenetic-species concept is also complex, and several different definitions have been proposed. There appear to be two main approaches. The character-based concept, or diagnostic approach, defines a species as a group of organisms that have common observable attributes or combinations of attributes. In contrast, the history-based concept insists that organisms must be historically related before they can be considered members of any given species. Some of the different species concepts currently in use are difficult to distinguish, and so mycologists should be familiar with them and clearly define which they are using to recognize species.

Evolution and Phylogeny of Fungi

Little is known about evolutionary relationships among fungi. Only recently have some data become available, although they are still sparse. The simple morphology, the lack of a useful fossil record, and fungal diversity have been major impediments to progress in this field. Classically, studies on fungal evolution have been based on comparative morphology, cell wall composition, cytologic testing, ultra-structure, cellular metabolism, and the fossil record. More recently, the advent of cladistic and molecular approaches has changed the existing situation and provided new insights into fungal evolution.

The phylogenetic relationship among higher fungal taxa remains uncertain, mainly because of a lack of sound fossil evidence, and remains a source of much controversy. The proposed phylogenetic relationships among the Animalia, Plantae, and Fungi kingdoms depend on the molecular regions and

methods used by different investigators. Phylogenetic analysis has shown that the fungal kingdom is part of the terminal radiation of great eukaryotic groups which occurred one billion years ago. Surprisingly, although mycology has been classically considered a branch of botany, there is also evidence that the kingdom Fungi is more closely related to Animalia than to Plantae. The analysis of amino acid sequences from numerous enzymes indicated that plants, animals, and fungi last had a common ancestor about a billion years ago and that plants diverged first. Another former hypothesis, i.e., that fungi are derived from algae, has been definitively abandoned.

The number of nucleotide substitutions in DNA sequences is directly proportional to the time passed, and so the number of base changes can be used to estimate the date of evolutionary radiation. On this basis, and using reference points such as the appearance of fossilized fungal clamp connections from the fossil record, the absolute timing of the origin of fungal groups has been estimated. The three main fungal phyla, Zygomycota, Ascomycota, and Basidiomycota, are thought to have diverged from the Chytridiomycota approximately 550 million years ago. The Ascomycota, Basidiomycota split occurred about 400 million years ago, after plants invaded the land. Many ascomycetes have evolved since the origin of the angiosperms in the last 200 million years. These results, with a few exceptions, are broadly supported by fossil evidence.

Nomenclature

To be formally recognized by taxonomists, an organism must be described in accordance with internationally accepted rules and given a Latin binomial. The rules that control the bio-nomenclature are very diverse and depend on the type of organism. Biological nomenclature is regulated by five different codes devoted to plants, cultivated plants, bacteria, viruses, and animals. However, in an attempt to harmonize the different codes, some efforts are being made to find a unified system. Meanwhile, the nomenclature of fungi (including yeasts) is governed by the International Code of Botanical Nomenclature (ICBN) as adopted by each International Botanical Congress. Any proposed changes to the Code are published in Taxon, the official journal of the International Association for Plant Taxonomy, and then debated in the Congress for approval. The Code aims to provide a stable method for naming taxonomic groups, avoiding and rejecting names which may cause error, ambiguity, or confusion. However, strict application of the Code frequently leads to name changes for nomenclatural rather than scientific reasons. This causes annoyance among users, who do not usually understand the reasons for the changes. The taxonomy of some pathogenic fungi is particularly unstable and controversial at present. Changes to the names of taxa and their consequent diseases are potentially confusing. For example, the name of the fungus *Allescheria boydii* (so called in the early 1970s) was changed to *Petriellidium boydii* and then to *Pseudallescheria boydii* within a very short period. Recently Melbourne code with some major changes has come into existence in 2013.

The dual modality of fungal propagation, i.e., sexual and asexual, has meant that since the last century there has been a dual nomenclature. The fungus, as a whole, comprises a teleomorph (sexual state) and one or more anamorphs (asexual states). The term “holomorph” has been reserved for fungi with teleomorphic sporulation together with all their sporulating or vegetative anamorphs. The anamorph and teleomorph generally develop at different times and on different substrates, although in zygomycetes they often occur together. Since each phase has been described in total ignorance of the existence of the other in many cases, the ICBN maintains that it is legal to give them separate binomials. For a long time, the anamorphs that occurred alone have been grouped into anamorph genera because they share some morphological features. These anamorphs have been placed in a separate major high-level taxon called Deuteromycotina or Deuteromycetes. With the advent of

molecular approaches in fungal taxonomy, some mycologists have advocated abandoning the dual system of naming because unified classification of all fungi may be possible on the basis of the rDNA sequences of the anamorphs. Other authors do not agree with this proposal and have considered it absolutely necessary to conserve deuteromycete taxon names, at least for identification purposes. During the Holomorph Conference, it was agreed to maintain the term deuteromycetes with a lowercase "d" and not to formally recognize this group of organisms at a particular rank.

Another controversy resulted from the replacement of the terms "anamorph" and "teleomorph" with "mitosporic fungus" and "meiosporic fungus", respectively, in the last edition of Ainsworth and Bisby's Dictionary of the Fungi; this source is considered a fundamental framework for fungal terminology and taxonomy. These changes have not been accepted by numerous authors, who consider that the anamorph and teleomorph phases of a fungus are determined not simply by the type of cellular processes (meiosis or mitosis) that precede sporulation but also by morphological features. Additionally, it has been argued that the cytological events preceding sporulation have not been investigated in sufficient depth to correlate teleomorph morphology with sexual recombination.

Mycological Methods Adopted for Taxonomy

The correct identification of fungi is of great practical importance not only in the clinical setting but also in plant pathology, biodeterioration, biotechnology, and environmental studies. An enormous number of species of fungi are already known, and so taxonomists are being kept very busy with recognizing and describing new species and grouping taxa. Hence, most species have received only limited study, so that classification has been mainly traditional rather than numerical and has been based on readily observable morphological features. However, some groups of fungi, because of their economical or pathological importance, have been studied more extensively. Other features beside morphology, such as susceptibilities to yeast killer toxins, susceptibilities to chemicals and antifungal drugs, the use of morphograms, molecular techniques, physiological and biochemical tests, secondary metabolites, ubiquinone systems, fatty acid composition, cell wall composition, and protein composition, have been used in classification and also in identification. The increased use and availability of modern techniques have opened up many new areas within systematics and have enabled more traditional ones to be developed further. Some of these approaches are detailed below.

Morphology

Classification systems of organisms are historically based on observable characteristics. This is the phenotypic approach. The classification and identification of fungi, unlike other important pathogens such as bacteria or viruses, relies mainly on morphological criteria. The fungi of medical importance are microscopic, and the study of their morphology requires the use of the light microscope. The classical light microscopic methods have been enhanced by Nomarski differential interference contrast, fluorescence, cytochemistry, and the development of new staining techniques such as those for ascus apical structures. Unfortunately, during infections most pathogenic fungi show only the vegetative phase (absence of sporulation); in host tissue, only hyphal elements or other nonspecific structures are observed. Although the pigmentation and shape of these hyphae and the presence or absence of septa can give us an idea of their identity, fungal culture is required for accurate identification. Species-specific antibodies and the use of probes can be very useful in such cases. Although some commercial probes exist, these techniques are not yet available or convenient for routine use in medical mycology. Therefore, the growth of isolates in appropriate culture media, enabling their most characteristic features to be recognized, is still the most common procedure used in practice.

For the identification and classification of imperfecti fungi, the type of conidia and conidiogenesis (the process involved in conidium formation) are considered the most important sets of characteristics to be observed. Cells that produce conidia are conidiogenous cells. Often a different structure which bears one or more conidiogenous cells is present; this structure is the conidiophore. There are two basic types of conidiogenesis, blastic and thallic. In blastic conidiogenesis, there is a small spot on the conidiogenous cell from which the conidia are produced. In thallic conidiogenesis, the entire conidiogenous cell is converted into one or more conidia.

Electronic microscopy techniques allow the recognition of several details of special taxonomic significance. Cross sections of cell walls observed by transmission electronic microscopy (TEM) reveal significant differences between ascomycetous and basidiomycetous yeasts. The value of differences in the ultrastructure of the septum at the base of the ascus and in ascogenous hyphae has become apparent as a result of the studies of Kimbrough. In general, the correlation between septal type and family is high in discomycete groups but still requires investigation and analysis in other ascomycetes.

The increased availability of scanning electronic microscopy (SEM) has resulted in a number of significant findings and, in some ascomycetes, has greatly facilitated identification at the species level by enabling differences in the surface detail of the ascospores to be clearly visualized. Freeze fracturing has revealed fine details of outer wall layers of conidia or ascospores.

In recent years, morphological techniques have been influenced by modern procedures, which allow more reliable phenotypic studies to be performed. Numerical taxonomy, effective statistical packages, and the application of computer facilities to the development of identification keys offer some solutions and the possibility of a renaissance of morphological studies. Automated image analyzers, electronic particle sizing, and fractal geometry may have a lot to offer in the analysis of fungal morphology.

The phenotypic approach has been largely criticized for its lack of standardized and stable terminology and for its high subjectivity. Moreover, some phenotypic characteristics have been considered to be unstable and dependent on environmental conditions, as with growth in artificial culture. A clear limitation of phenotypic approaches is that they cannot be applied to fungi that do not grow in culture. Consequently, there are many fungi that will remain unclassified as long as taxonomists rely solely on phenotypic characteristics. Another notable problem of classification based on morphological criteria is the above-mentioned dual system of classification, with no consistent correlation between the taxonomies of the ascomycetes and deuteromycetes. This is an important difficulty in establishing the taxonomic concept of the fungus as a whole.

Molecular Techniques

Since the distinguishing morphological characteristics of a fungus are frequently too limited to allow its identification, physiological and biochemical techniques are applied, as has been routinely done for the yeasts. However, for poorly differentiated filamentous fungi, these methods are laborious, time-consuming, and somewhat variable and provide insufficient taxonomic resolution. In contrast, molecular methods are universally applicable.

Comprehensive and detailed reviews of the use of molecular techniques in fungal systematics have been provided.

Two important technical advances have stimulated the use of molecular techniques. Firstly, the advent of PCR has allowed the analysis of small numbers of fungal cells or even single spores, dried herbarium material, or extinct organisms. Second, the selection of universal oligonucleotide primers specific to fungi has provided easy access to nucleotide sequences.

The aim of molecular studies in biodiversity is fourfold: (i) phylogenetic studies, i.e., tracing back the most probable course of evolution and the historic coherence between groups at higher taxonomic ranks; (ii) taxonomic studies, mostly at the level of genera and species; (iii) diagnostic applications, i.e., recognition of defined taxonomic entities; and (iv) epidemiology and population genetics, i.e., monitoring outbreaks of subspecific entities with respect to the analysis of populations and their mode of reproduction. Each of these broad aims and levels of diversity has its own set of optimal techniques. In this review, only phylogenetic and taxonomic studies are discussed.

One of the groups of genes which is most frequently targeted for phylogenetic studies is the one that codes for rRNA. Introns of several protein-encoding genes, such as the β-tubulin, actin, chitin synthase, acetyl coenzyme A synthase, glyceraldehyde-3-phosphate dehydrogenase, lignin peroxidase or orotidine 5′-monophosphate decarboxylase genes, can also be applied and can provide important information. The main reasons for the popularity of rDNA are that it is a multiple-copy, non-protein-coding gene, whose repeated copies in tandem are homogenized by concerted evolution, and it is therefore almost always treated as a single-locus gene. Furthermore, ribosomes are present in all organisms, with a common evolutionary origin. Parts of the molecule are highly conserved and serve as reference points for evolutionary divergence studies. The conserved regions alternate with variable regions or divergent domains. The 5.8S, 18S, and 25S rDNAs are transcribed as a 35S to 40S precursor, along with internal and external transcribed spacers (ITS and ETS). All spacers are spliced out of the transcript. Between each cluster is a nontranscribed or intergenic spacer (NTS or IGS) that serves to separate the repeats from one another on the chromosome. A 5S gene takes a variable position and is transcribed in the opposite direction. The total length of one DNA repeat is between 7.7 and 24 kb.

Comparisons of the 18S (also called the small-subunit [SSU]) rRNA sequences have been performed to assess the relationships of the major groups of living organisms. For phylogeny of filamentous fungi, the 18S sequence is mostly used completely or in subunits of over 600 bp. In the yeasts, the D1 and D2 variable regions of 25S rDNA regions are almost exclusively used. This technique is currently being extended to Heterobasidiomycetes and sometimes also to filamentous ascomycetes. In only a limited number of fungi have both regions been sequenced. Due to this different choice of target regions, comparison of fungi to all possible relatives is hampered. The 25S variable domains are very informative and allow comparisons from high taxonomic levels down to the species level, although only a limited number of variable positions remain. In the 18S gene, the variable domains mostly provide insufficient information for diagnostic purposes, and thus large parts of the molecule must be sequenced to obtain the resolution required. The ITS regions are much more variable, but sequences can be aligned with confidence only between closely related taxa. These regions are generally used for species differentiation but may also demonstrate patterns of microevolution. In contrast, 5.8S rDNA is too small and has the least variability. 5S has been used mainly to infer relationships at the ordinal level, where differences could be traced back to the secondary structure of the molecule.

Among the electrophoretic methods, restriction fragment length polymorphism (RFLP) is particularly significant for taxonomy. This technique involves digesting DNA samples with a panel of restriction enzymes. The patterns can be tabulated and compared or phenetic trees can be constructed. The first RFLP technique widely used in taxonomy compared patterns of mitochondrial DNA (mtDNA). However, some authors have sequenced the mtSSU rDNA instead. mtDNA is generally indicative of differences somewhat below the species level, but in groups where microspecies are currently distinguished, such as in the dermatophytes, the differences seem to correspond to teleomorph species. RFLP-based typing methods have been used to reveal anamorph-teleomorph connections. Most commonly, the RFLP of PCR-amplified rDNA is used. This technique is also known as amplified rDNA restriction analysis and provides a quick insight into relationships between moderately distant fungi. Therefore, homogeneity of ITS profiles corresponds well to final ITS sequencing diversity. The method is primarily confirmatory; i.e., new strains are quickly assigned to sequenced strains with the use of restriction maps to study *Phialophora*. Amplified rDNA restriction analysis is particularly useful as an inexpensive and simple alternative to SSU rDNA sequencing when broad relationships have already been determined. However, the frequent occurrence of introns in SSU rDNA may hamper quick comparison of strains.

A popular technique is random amplified polymorphic DNA (RAPD) with 10-mer primers. However, this method is gradually being abandoned because of poor reproducibility. Microsatellites are a special class of tandem repeats, which have a base motif of up to 10 bp that is frequently repeated (up to about 100 times); they are found in many genomic loci with an almost ubiquitous distribution. A general profile comparison of microsatellites enables species recognition. In addition, due to the high level of polymorphism, individual bands can be informative for the characterization of strains.

DNA bar-coding is the recent addition to understand the taxonomy of fungi. The fungal taxonomy has changed with modern concepts besides the morphology and reproductive criteria.DNA barcoding is a systematic way to link DNA data with reference specimens to facilitate identification. There is a lack of DNA sequence data associated with the record number of specimens in Kew's fungal collection; Currently only about 400 out of its ~1.25 million specimens are represented in GenBank. This neglect masks the true value of Kew's fungarium, which, being the largest and one of the most extensive collections of fungi from around the world, offers a tremendous opportunity for generating voucher based DNA barcodes.

Other Techniques (Physiological & Chemical Profiling)

Because numerous fungi grow relatively rapidly in pure culture, it is possible to use physiological and biochemical techniques to identify and classify them. These techniques have been successfully used in the study of black yeasts. The different ranges of growth temperature have been used as a complementary tool in the identification both of asexual and sexual fungi. Growth rates on defined media under controlled conditions are also valuable in studies of complex genera such as *Penicillium*. Commercially available kits such as the API system have also been used to identify filamentous fungi. Paterson and Bridge have published a compilation of the physiological techniques used in the identification of filamentous fungi. They list a variety of biochemical methods which range from simple agar-based tests to more sophisticated chromatographic and electrophoretic methods.

Secondary metabolites are compounds neither essential for growth nor key intermediates of the organism’s basic metabolism but presumably playing some other role in the life of fungi. They are

usually found as a mixture of closely related molecules with a peculiar and rare chemical structure. The most common are steroids, terpenes, alkaloids, cyclopeptides, and coumarins, and some of these are mycotoxins. The advent of thin-layer chromatography, especially the simple technique of directly spotting thin-layer chromatography plates with small samples of culture cut from petri dishes, has made it possible to qualitatively assess secondary metabolites much faster than by conventional extraction, purification, and concentration techniques. This improvement has resulted in huge amounts of new secondary-metabolite data, which is now being incorporated in databases. The pattern of secondary-metabolite production has for a long time been of great use in the identification and classification of lichens. This pattern has been used less in the taxonomy of ascomycetes and basidiomycetes, although it is well known that these organisms produce a vast array of such compounds. The use of this method in fungal taxonomy has been questioned because the production of these compounds can be affected by environmental conditions and the detection procedure presents some difficulties. However, its potential in ascomycete systematics is well illustrated by the chemotaxonomic studies performed in Eurotiales and in Xylariales. In these orders, individual species can often be recognized on the basis of particular metabolite profiles. Integrated approaches involving morphology, physiology, and secondary metabolites have been used in several attempts to clarify the systematics of some fungi.

Ubiquinone systems:Besides the use of secondary metabolites in taxonomy, some use has also been made of other compounds which play an essential role in metabolism and which are primary rather than secondary metabolites, e.g., ubiquinones (coenzyme Q). These compounds are important carriers in the electron transport chain of respiratory systems. The number of isoprene units attached to the quinone nucleus varies, and such differences in ubiquinone structure are excellent indicators in the classification of genera and subgeneric taxa in bacteria and yeasts. Although less common, these techniques are also being used in the taxonomy of black yeasts and filamentous fungi. The results provided by these techniques sometimes correlate with those provided by molecular techniques, although conclusions based purely on ubiquinone systems are debatable and, depending on the method used, can provide different sets of data.

Fatty acid composition: Recently, gas chromatography, combined with methods of multivariate statistical analysis, has successfully been used to study the fatty acids of numerous and varied filamentous fungi, including oomycetes, zygomycetes, basidiomycetes, and even sterile mycelia. These techniques have also proved to be useful at intraspecific level.

However, several technical aspects of the procedure used must be highly controlled to minimize sources of variation, which can influence the results enormously. The culture conditions and temperature are among the most important factors to be standardized.

Cell wall composition*:* Numerous studies have shown differences in the structure and composition of the cell wall of fungi which have been used in the definition of high fungal taxa. For example, cellulose is a particular component of the alkali-insoluble fraction of oomycetes, while ascomycetes and basidiomycetes contain both chitin and glucan in these fractions. In contrast, the zygomycetes have chitosan and polyglucuronic acid. Significant variations in the sugar composition of cell walls have been observed in the different genera of dermatophytes. The walls of yeast cells that have been subjected to hydrolysis yield substantial amounts of glucose and mannose, but species differ in the presence or absence of smaller amounts of other polysaccharides (fucose, galactose, rhamnose, and xylose). This feature has also been used in classification of fungi. Bartnicki-Garcia has reviewed the

biochemical and physiological characteristics of the cell wall components which have been used to delimit high taxa.

Protein composition:Isoenzyme patterns produced by electrophoretic techniques (zymograms) have determined generic relationships and differentiated species. Apart from electrophoresis, immunological techniques and protein sequencing also have suitable resolution for interspecific characterization. Isozyme analysis is considered to be an economical and practical technique for screening large populations. What is more, the characteristics determined by this technique are generally accepted to be of independent genetic origin. Allozyme (allelic isozyme) data are commonly used in phylogenetic studies.

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Trends in the taxonomy of Oomycetes with reference to *Phytophthora*

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Introduction

The Class Oomycetes includes some of the devastating plant pathogens like *Phytophthora, Pythium* and downy mildewsand a few pathogens of insects, crustaceans and animals. The genera *Phytophthora* is an important pathogen in India on both agricultural and horticultural crops and attracted the attention of scientists and policy makers resulting in launching network projects and being investigated all across the country (www.phytofura.net.in). Formerly considered as lower fungi and water molds now the oomycetes find a separate phylogenetic group separate from fungi and placed closer to brown algae and diatoms and elevated to phylum Oomycota.

The name *Phytophthora,* a Greek word meaning the 'plant destroyer', was coined by Anton de Bary in 1876. As the name indicates, *Phytophthora* is one of the most destructive plant pathogen ever known. Being an alga-like organism it requires free water for critical stages in its life cycle. The asexual reproducing structures of *Phytophthora* calledsporangia, release large number of motile biflagellate zoospores which swim in a film of water and enter the host tissues through infection process are capable of causing explosive epidemics. Downy mildew of grapes and potato blight are typical examples. *Phytophthora* also possess a range of long-lived propagules which can remain in a latent phase for many years and difficult to manage. *Phytophthora* species have remarkably flexible mating systems allowing clonal and sexual reproduction and the generation of novel species via hybridisation. Its genetics thus enable an evolutionary plasticity that supports its adaption to novel plant hosts and environments. *Phytophthora* can infect any plant part; majority of *Phytophthora* species infect below-ground plant parts, causing crown and root rots. Infections on roots and basal stem cankers. The soil phase is very difficult to detect till considerable damage is caused and symptom appear. Some pathogens infect all parts of the plant as in potato, black pepper and cocoa. Regarding host range, *P. cinnamomi* has a wide host range infecting over 300 species of flowering plants and *P. infestans* has a narrow host range like potato and tomato.

Species of *Phytophthora*

Since the report and naming of *P. infestans,* the notorious Irish famine pathogen, several plant pathogenic species have been described. Erwin & Ribeiro (1996) reviewed the species of the genus *Phytophthora* in their book '*Phytophthora* worldwide' and described 59 species. With the advent of pathogenomics and whole genome sequencing and annotation of species of *Phytophthora,* several new species have been described from different parts of the world and by 2012 over one hundred species were described (Sikora *et al.,* 2012). At a recent conference of the International Union of Forest Research Organizations (IUFRO) many other species awaiting formal description were reported making total to about 150 species (Cooke, 2015). *Phytophthora* has been found to occur not only as pathogens but in several trophic levels.

Unique characters of *Phytophthora*

The vegetative phase of the pathogen is multinucleate coenocytic mycelium with occasional septa delimiting reproductive structures both asexual and sexual. The cell wall comprises mainly of insoluble polymers of glucans with ß, 1-3 and ß, 1-6 linkages. The protoplast is enveloped in a double layered plasma membrane with other cell organelles typical of eukaryotic cell such as nuclei, mitochondria, endoplasmic reticulum and dictyosomes. There are four unique organelles in

Phytophthora, they are; peripheral vesicles that later relocate in to the zoospores, fingerprint vesicles containing water soluble reserve polysaccharide laminarin which is made up of ß, 1-3 linkages of glucans, bullet shaped micro bodies with crystalline fibrils and microtubules in the cisternae of endoplasmic reticulum that later form flagellar hairs. The nutritive requirement varies and for all species and external source of thiamine is required. Glucose, sucrose and other glucose containing saccharides serve as carbon source, whereas, trehalose, cellobiose or cellose are poorly utilized. Organic acids and amino acids are unsuitable and useful only for buffering. Nitrate, ammonia or single amino acid such as asparagine is utilized as nitrogen sources. Sterols are not essential for vegetative growth but required for reproduction. Some species such as *P. infestans* are highly demanding in their nutritional requirements also have a narrow host range (Erwin *et.al*, 1983). The availability of nutrients determines the vegetative and reproductive phase of this pathogen. Under in vitro conditions the mycelial growth of pathogen shows various morphologic features depending up on the medium and other cultural conditions such as temperature. The common features such as white cottony, stellate or chrysanthemum like floral patterns are produced by several species. Asexual reproduction is by the production of sporangia.

Classification of *Phytophthora*

For long this genus has been traditionally included under the kingdom fungi under the class oomycetes and studied with other plant pathogenic fungi. The genus though filamentous and absorptive in nutrition as other fungi, they were considered unique because of several characters. Primarily, based on their cell wall composition that is mainly of ß 1- 3 and 1-6 glucans unlike chitin in fungi as mentioned earler. The vegetative phase is diploid and meiosis occurs only in the gametangia. They also differ in their asexual reproductive structures, nutritional requirements and their sensitivity to fungicides. The oomycetes pathogens have long been considered as unique group because of their special characteristics under the kingdom fungi. The use molecular tools opined that the photosynthetic straminipiles namely brown algae, diatoms and golden brown algae are closer to the oomycetes than to the true fungi. Hence they are no longer considered as ‘fungi’ but many scientists refer to as oomycete pathogens.

Phytophthora can be readily differentiated from its closely associated genus *Pythium* by the morphology of sporangia. The formation of zoospores is within the sporangium of *Phytophthora* whereas in *Pythium*, zoospores form after protoplasm has flown out from the sporangium into a vesicle.

The life cycle of *Phytophthora* includes both sexual and asexual phases. This genus *is* reported to possess some of the most sophisticated weaponry known to plant pathogens (Gregory 1983). The asexual spores called sporangia are the most characteristic infectious propagules of *Phytophthora.* These are usually produced under suitable environmental conditions which includes optimum temperature and moisture. The sporangia are borne on sporangiophores, which are usually dispersed by air currents to neighbouring plants and can travel easily to nearby fields when the air is moist. They dry out and die at high temperatures or after travelling long distances. The sporangium germinates in aqueous solutions or on agar media by the production of a germ tube that usually emerges from the tip of the sporangium (direct germination) or, in an aqueous medium after the temperature is reduced, by the production of uninucleate, biflagellate zoospores within the sporangium (indirect germination) (Erwin and Ribeiro, 1996). The zoospores emerge from the sporangium in a membranous vesicle that soon breaks, allowing zoospores to swim away (Gisi *et al.,* 1979). Zoospores are reniform in shape with heterokont flagella emerging from the concave side.

These are considered to be the major infectious propagules of *Phytophthora.* Zoospores swim for hours (Bimpong and Clerk, 1970) and eventually cease to swim, round up and within minutes develop a cell wall (Bartnicki-Garcia 1973) to form a cyst. Cysts germinate by producing germ tubes which infect the plant. From the infection sites, an extensive network of thread like hyphae penetrates between the cells, absorbing nutrients to feed the growing mycelium. If the environmental conditions are conducive, the disease develops very rapidly.

Survival structures of *Phytophthora*

The significance of plant pathogens in agriculture reflects their ability to survive an adequate amount of time to be redistributed as potential inoculum. *Phytophthora* species are well adapted to the diverse environments that they encounter in different seasons. They produce several types of microscopic structures that are very efficient in initiating diseases on their own. *Phytophthora* produces two kinds of spores designed for long-term survival: chlamydospores and oospores. Chlamydospores are asexual spores, which may form terminally at the tips of hyphae or may be intercalary and are thin or thick walled with spherical to oval in shape. An oospore is a thick walled sexual spore that is resistant to desiccation, cold temperatures and other extreme environmental conditions, and can survive in the soil, in the absence of a host plant, for many years. These structures can remain latent in the soil for years and become activated when conditions are ideal. The wall of the chlamydospores (0.5 – 1.5 µm) are usually not as thick as the oospore wall (up to 3 µm). Chlamydospores have been observed to survive up to six years and oospores up to 13 years (Erwin and Ribeiro, 1996).Besides these, *Phytophthora* can also survive in the form of zoospores or hyphal swellings. They can survive for up to a few weeks. Tsao (1983) reported that the encysted zoospores can survive up to a period of several months.

Mating types

Phytophthora species are divided into two categories, which are delimited by their sexual properties, homothallic (self fertile) and heterothallic (self sterile). Homothallic species are capable of sexual reproduction in single mating type cultures, while heterothallic species generally require two mating types (A1 and A2) for sexual reproduction. In the homothallic species, oospores function as persistent propagules in diseased plant material. The role of oospores in heterothallic species is not well understood, but evidence strongly indicates that the crossing of A1 and A2 mating type isolates can be a source of new races or biotypes (Erwin and Ribeiro, 1996). Oospores in heterothallic species are incited by an external factor or by a hormone like substance produced by the opposite mating type.

Identification of *Phytophthora*

The genus *Phytophthora* has been widely acknowledged as taxonomically ‘difficult’ (Brasier 1983), as many of the characters used for species identification are plastic, highly influenced by environment, show overlap between species, and have an unknown genetic basis. Waterhouse (1963), a renowned mycologist at the commonwealth Mycological Institute at Kew, United Kingdom, performed a major review of the genus and used morphological characters as the basis for species identification and taxonomy. He classified species into six morphological groups based primarily on papillation and caducity (easy detachment) of sporangia, type of anthredial attachment, and mating system. This taxonomic key provided the framework for a number of traditional identification keys like Stamps *et al., (*1990). A revision of Waterhouse (1963) key included six new species and was based on the same parameters as the 1963 key, but was presented in a tabular key containing 67 species, 19 of which have been described since 1978 (Stamps *et al.,* 1990). It incorporates the original

groups 1-6 of the previous key with the addition of group 7 for the marine species. Further revision of a total of 54 species was described by Erwin and Ribeiro (1996).

Previous identification of *Phytophthora* was purely based on the morphological aspects and other growth-temperature relationships. Along with this, other criteria such as breeding systems and gametangial structure, were used to provide insights into behaviour and adaptation.

Most common keys encountered for identification of *Phytophthora* are dichotomous key. These keys usually form a series of numbered questions arranged in "couplets". Each time a question is answered, the user is directed to the number of a new question-couplet. This continues until, instead of a number, the name of the species (or other taxon) is given. Gallegly and Hong (2008) developed a dichotomous like key for the identification of *Phytophthora* as exemplified below

Heterothallic species

PAPILLATE SPORANGIA

Colony growth at 35 °C or higher

a. Non caducous sporangia, usually ovoid to spherical, usually with one papilla; chlamydospores usually present; arachnoid mycelium in most isolates……***P nicotianae***

b. Caducous sporangia, many with two or more papillae, distorted shapes, long pedicels (up to 100 pm long); usually no chlamydospores…….***P capsici***

No colony growth at 35 °C or very slight growth

a. Caducous sporangia with short pedicels; sporangia of variable shape, ovoid to ellipsoid, avo1/w ratio <1.6:1; chlamydospores abundant……..***P palmivora***

b. Caducous sporangia with medium pedicels 5 to 10 pm long; sporangia mostly ovoid or obpyriform but some ellipsoid, avo1/w ratio <1.6:1; chlamydospores in most isolates; tapered oogonial stalk…….***P megakarya***

Lucid keys

Lucid tools are powerful and highly flexible knowledge management software applications designed to help users with identification or diagnostic tasks. Lucid key is an interactive matrix comprising of (1) a list of entities and a list of features and states describing or otherwise associated with them (2) score data relating the feature states to the entities, andvarious images and/or HTML pages attached to the features, states and/or entities. This software helps in developing and publishing electronic keys; either dichotomous or multi-access keys, for identification of various organisms. Despite the dichotomous key, multi-access keys allows the user to start anywhere in the key with any character. It has the freedom from missing or obscure structures. Lucidcentral.org (www.lucidcentral.org) provides the platform for designing and publishing of these keys. The website also allows the user to search for different keys.

Ristiano (2012) developed a lucid key for the identification of common *Phytophthora* sp. This key and includes important morphological and molecular characters that are useful for identification of 55 common species of *Phytophthora*. A set of 20 features are used to make a correct species identification. Once a culture is obtained, the user enters responses to known character state options into Lucid Player, and the correct species is identified. Illustrations of each character state for a feature are included in the key. The main morphological features included in the key are: asexual structures, sexual structures, and chlamydospore, hyphae, and cultural characteristics. The user can

read an illustrated "Fact Sheet" on each species that includes pictures of morphological characters, disease symptoms, host range, and relevant references. A cross-linked glossary of terminology is included in each fact sheet. In addition, a DNA search function that contains a simple search of internal transcribed spacer (ITS) and Barcode of Life (BOL, 5′ end of the *cox 1* gene) sequences for each species can be queried. The key was created to provide teachers, diagnosticians, and regulatory personnel with easily accessible tools to distinguish common species in the genus *Phytophthora* based on a number of important morphological and molecular characteristics. The key is available for purchase from APS PRESS and provides another useful tool for the identification of this oomycete plant pathogen.

Multi locus sequence typing (MLST) advantages

Housekeeping genes are rich in variability than 16s rDNA and the long length of the analysed sequence allows better differentiation of the organism. ITS sequences in some circumstances fail in discriminate among closely related taxa. Similarly, phylogenetic analyses and were based on coding sequence (Martin and Tooley, 2003 and Kroon *et al.*, 2004), with a relatively low mutation rate and therefore real limited target sites for diagnostic development. Introns and intergenic portions of the nuclear and mitochondrial genome used in MLST are more variable and therefore more promising targets for diagnostic development.

MLST in *Phytophthora*

By analyzing four mitochondrial genes and intergenic spacer regions of ITS markers to diffenentiate about 15 species have been developed. Further by analyzing 225 genetic markers using genomic data from seven loci of 82 species of *Phytophthora* were grouped into ten clades. Recently, 166 isolates belonging to 92 species and 17 provisional species have been analyzed for four mitochondrial genes and a total of 11 loci and further discriminate the phylogenetic relationships among the ten clades and new species described.

Genomics

The complete genome of two *Phytophthora* isolates (05-06 and 98-93) infesting black pepper was sequenced using Illumina/Roche 454 platforms. The cross-platform sequence data was *de novo* assembled and annotated structurally and functionally to curate all possible gene by gene information. Isolate 05-06 and 98-93 consist of a total genome size of 63.8Mb and 46.1Mb, respectively in hybrid assembly, obtained from 20.96 million high quality reads with average length of 101 bp generated using next generation sequencing method - Illumina- Solexa 1G Genetic Analyzer and Roche 454 GSFLX. Quality checks revealed that 99.54% HQ bases are present in 05-06 isolate while it is 99.71% in 98-93 isolate. *De novo* assembly resulted in 32044 and 9831 contigs for 05-06 and 98-93 with peak depth of 5 and 6, respectively. Structural annotation provided a total of 16356 genes, 38947 exons and 16356 CDS for 05-06 isolate compared to 13068 genes, 33813 exons and 13068 CDS for 98-93 isolate. The number of predicted proteins was 7154 in 05-06 and 9344 in 98-93 isolate. SSR statistics was 1344 in 05-06 and 2496 in 98-93. Whole genome alignment with the reference genome revealed that 05-06 is 95.35% similar while 98-93 is 87.90% similar to the reference genome. Variant annotation revealed that there are 113068 SNPs in 05-06 compared to 37839 SNPs in 98-93. About 2039 genes are conserved between 05-06 and 98-93 isolates. Genes unique to 98-93 are 6095 in numbers while 4034 genes are unique to 05-06. Functional annotation using BLAST search revealed the presence of various proteins important for the survival of *Phytophthora* sp. in host plants and virulence associated proteins crucial for its infection.

Conclusion

The whole genome sequence data provides vast information about the pathogen. The single nucleotide polymorphism (SNPs) present in the genome with reference to the reference *P. capsici* can be utilized for deciphering the pathogenic variability in *P. capsici.* This can also aid in evolutionary studies. Unique conserved domains can be identified which will help in identifying newly acquired characteristics specific to Host Pathogen interaction. Classification of domains based on homeostasis, metabolic pathway and host pathogen interaction can be done. Effectors can be identified and ranked according to pathogenicity.

Accurate identification is the first step in managing the pathogens. The modern science of "omics" especially transcriptomics helps in discerning the intraspecific variation in pathogenicity. The pathogenic variability in two black pepper isolates of *P.capsici* that are morphologically similar but varied in their pathogenicity was studied by the transcriptome and the data indicated the differential interaction with the host defence mechanisms.

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Taxonomic Characterization of Zygomycetous Fungi of Agriculture and Industrial use

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Zygomycota, or zygote fungi, is a phylum of fungi, approximately 1050 species are known (Krogh 2010), which is approximately 1% of the described species of true Fungi (more than900 described species; Kirk *et al.* 2001). They are mostly terrestrial in habitat, living in soil or on decaying plant or animal material and in aquatic ecosystems. Some are parasites of plants, insects, and small animals, some of them are used as bio-control agents while others form symbiotic relationships with plants (Raven *et al.*, 2005).

Characteristic features:
Hyphae are non septate or coenocytic (i.e., non-septate - no cross-walls), cell walls contain chitin, chitosan, and polyglucuronic acid, flagellated spores are absent, reproduce asexually by producing sporangiospores within a special sac called the sporangium. Sporangiums are four types: 1. True sporangium, 2. Sporangioles-much smaller than true sporangia, no columella and few spores, 3.monosporous sporangium (one-spored sporangium) and 4. Merosporangium - sac containing 10-15 sporangiospores that occur in a linear sequence - superficially it looks a lot like an ascus. Sexual spores are called zygospore(s) contained within a zygosporangium. Blakeslee (1904) discovered heterothallism, for which sex hormones facilitate sexual reproduction. For example *Mucor* species have (+) and (-) mating strains, produce trisporic acids which are volatile (+ strain yields 4-hydroxymethyltrisporates and - strain yields trisporins) and diffuse through the air. Volatiles stimulate progametagia production and the synthesis of carotene (a precursor for trisporic acids) and trisporic acids. A positive feedback mechanism is formed between the two compatible strains leading to physical contact of progametangia and sexual reproduction.Heterothallism (Gr. *heteros* = different from, *thallos* = shoot; the condition of being self-sterile, requiring a partner for sexual reproduction) and homothallism (Gr. *homos* = alike, *thallos* = shoot; the condition of being self-fertile; able to reproduce sexually without a partner) exhibited among species in this phylum.

Classification: Prior to use of molecular phylogenetics in defining taxa, the phylum was divided in to two classes are*Zygomycetes* and *Trichomycetes*, but now there are no class and class *Trichomycetes* is non-existent. Among four of its orders two (*Amoebidiales* and *Eccrinales*) are now placed in kingdom *Protozoa* and the other two (*Asellariales* and*Herpellales*) are kept in sub phylum *Kickxellomycotina* of *Zygomycotina*. The order *Glomales* is removed from *Zygomycetes* and elevated to phylum *Glomeromycota*, due to lack of zygospores formation, mycorrhizal habit and lack of DNA sequence homology (Schuessler *et al.* 2001). Molecular phylogenyalso reveals that the phylum*Myxomycota* is poly phyletic and could see a split in to several new phyla in coming years. Recently (Kirk *et al.* 2008) the phylum*Zygomycota* classified in to four subphyla, which are monophyletic, but the relationship among them are not resolved. The subphyla have 9 orders and 27 families.

1. Phylum*Zygomycota*
1. Sub phylum-*Mucoromycotina*- 3 orders, *Mucorales* (9 families), *Endogonales* (1 family) and *Mortierellales* (1 family).
2. Sub phylum-*Entomophthoromycotina* - 1order, *Entomophthorales* (6 families).
3. Sub phylum-*Kickxellomycotina* - 4 orders, *Asellariales*, *Kickxellales, Dimargaritales* (1 family in each)and *Herpellales* (2 families)

4. Sub phylum-Zoopagomycotina- 1 order, Zoopagales (5 families).
2. Phylum Glomeromycota- (Coenocytic, Arbuscular Mycorrhizal (AM) Fungi.
In the present text description is given on members of Mucorales.

Order Mucorales:

Mucorales belong to the subdivision Zygomycotina, class Zygomycetes, is an assemblage of fungi which reproduce asexually by non-motile aplanospores usually produced in sporangia and sexually by gametangial copulation which is typically isogamous resulting in the formation of zygospore. The mycelium is haploid, coenocytic, septa is formed to delimit reproductive organs. The order comprised 12 families, 47 genera and 130 spp.The Mucorales are widely distributed in soil, mostly saprophytic, some cause spoilage of food or weak parasites of fruits and vegetables or mycoparasites or cause diseases of animals including man.The classification of Mucorales was attempted by several authors with modifications. Lendner (1908) recognized 6 families on the basis of morphological characters as well as growth limiting temperatures. Zycha (1935), Naumov (1939), Martin (1940) and Hesseltine (1955) classified it into six, eight, seven and nine families respectively. Hesseltine & Ellis (1973) recognized 14 families, included *Radiomycetaceae* and *Saksenaeaceae*. Benjamin (1959) recognized three main patterns of sporangial development among the mucoraceous fungi. (i) relatively large multispored, columellate, sporangia (ii) few spored sporangiola, columellae absent or rudimentary and (iii) sporangiola containing one or two spores. On the basis of asexual and sexual characteristics, *Syncephalastraceae* (merosporangiferous) included in *Mucorales*, while *Piptocephalidaceae* in the order *Zoopagales*. The families *Dimargaritaceae* and *Kickxellaceae* were raised to orders *Dimargaritales* and *Kickxellales*. Recent classification is according to Ainsworth & Bisby's Dictionary of the Fungi (9^{th}ed. 2001) which recognizes 12 families, 47 genera and 130 species.Synthetic Mucor agar (SMA) is widely used as a standard medium for the taxonomic description of most of the *Mucorales* (Hesseltine 1954). Potato Dextrose Agar (PDA), Yeast Extracy Agar (YXT) and Rabbit Dung Agar (for coprophilous fungi) are also used. Present classification of Mucoralesis based on Ainsworth & Bisby's (A dictionary of fungi 1995), where they include a total of 13 families, 56 genera and 299 species.

1. Chaetocladiaceae : 2 genera, 7 species
2. Choanephoraceae : 3 genera, 5 species
3. Cunninghamellaceae : 1 genus, 7 species
4. Gilbertellaceae : 1 genus, 1 species
5. Mortierellaceae : 7 genera, 106 species
6. Mucoraceae : 20 genera, 122 species
7. Mycotyphaceae : 2 genera, 6 species
8. Phycomycetaceae : 1 genous, 3 species
9. Pilobolaceae : 3 genera, 13 species
10. Radiomycetaceae : 2 genera, 4 species
11. Saksenaeaceae : 1 genus, 2 species
12. Syncephalastraceae : 1 genus, 2 species
13. Thamnidiaceae : 12 genera, 22 species

1. Zygospores present in sporocarp ……...….*Mortierellaceae*
1. Sporocarp absent ……..……. ……2
2. Sporangia, sporangiola or monosporous sporangiola present, borne on thripsofsporangiophores…… …….……3
2. Sporangia or sporangiola absent, only monosporous sporangiola (conidia) present, borne on head vesicles of sporangiophores……...……*Cunninghamellaceae*
3. Multispored merosporangia produced on a fertile vesicle……...….*Syncephalastraceae*
3. Merosporangia not produced, sporangia and / or sporangiola present …….. 4
4. Sporangia lageniform……*Saksenaeaceae*
4. Sporangia usually globose to obpyriform never lageniform……...……5
5. Sporangial wall densely cutinized above, sporangia forcibly discharged, trophocyst present or absent……..….*Pilobolaceae*
5. Trophocyst always absent; deliquescent sporangium; sporangiola may be present …..…6
6 Sporangiola uni or multispored; both sporangia and sporangiola present on same sporangiophore or on different sporangiophore …….…….....……7
6. Multispored sporangia present, sporangiola always absent ….……..…… 8
8. Sporangiophore unbranched, zygospore suspensors tong-like ……....*Phycomycetaceae*
8. Sporangiophore branched or unbranched, zygospore suspensor opposed…....*Mucoraceae*
7. Sporangiospores with hyaline hair like polar appendages; sporangial wall persistent splitting into two equal halves ……..…….. 9
7. Sporangiospores not with appendages; sporangial wall deliquescent if persistent, fracturing irregularly ……..……...10
9. Sporangiospores fusiform, pale brown to reddish brown; both multispored sporangia and sporangiola present but on separate sporangiophores; zygospore suspensor apposed ……*Choanephoraceae*
9. Sporangiospores hyaline, oval to irregular in shape; only sporangia present; zygospore suspensors opposed …….……*Gilbertellaceae*
10. Only sporangiola present; sporangiaabsent ……..…….11
10. Both sporangia (diffluent and columellate) and sporangiola (few or one spored persistent walled) borne on the same or separate morphologically identicalsporangiophores ……..*Thamnidiaceae*
11. Sporangiolaborneonpedicels……. ……..……12
11. Sporangiola borne on complex ampullae ……...…..*Radiomycetaceae*
12. Pedicellate unispored sporangiola formed on fertile vesicles, borne on branches which end in spines; sporangiophores terminating in sterile spines……...……*Chaetocladiaceae*
12. Sporangiola uni-or multispored borne on dehiscent pedicels; no sterile spines ……..*Mycotyphaceae*

Key to the Important Genera of Mucoraceae based on Hesseltine, C.W. (1955). *Mycologia* 47: 344-363

la. Sporangia pyriform …….. …….*Absidia*
lb. Sporangia sphaerical ……2
2a. Sporangiophores united into groups on a rhizoidiferous stolon ……..3
2b. Sporangiophores arising singly from the mycelium, not from rhizoidiferous …...…….4
3a. Sporangiophores unbranched, at the nodes opposite rhizoids; spores usually longitudinally striate ……..…….*Rhizopus*

3b. Sporangiophores branched, with terminal sporangium large; side branches in whorls; lateral sporangia smaller than the principal sporangium; spores smooth….....…*Actinomucor*
4a. Sporangiophores branched, side branches strongly curved ……..……*Circinella*
4b. Sporangiophores unbranched or branched, side branches not markedly curved…..…….. 5
5a. Zygospores on separate hyphae, mostly heterothallic, isogamous ……*Mucor*
5b. Zygospores on short side branches of the sporangiophores, homothallic, heterogamous …….....*Zygorhynchus*

Description of some important species of Mucorales:

1. *Absidia* van Tieghem

Aerial mycelium hyaline or coloured, much branched, producing arched stolons with rhizoids and sporangiophores determinate or indeterminate in length; sporangiophores usually erect, arising singly or in fascicles or whorls, often branched and septate, arising along the stolons but not opposite the rhizoids; sporangia small, pyriform, terminal, usually deliquescing, often light in colour; columellae commonly with projections, variously shaped with a well-developedapophasis; sporangiospores minute, one-celled hyaline, non-striate; zygospores borne aerially, are surrounded by curved unbranched appendages which may arise from one or both suspensors.

2. *Choanephora cucurbitarum* (Berkeley & Ravenel) Thaxter

Colonies white to greyish white, later becoming sterile; sporangiophores unbranched, often bent or circinate below the sporangium, hyaline, becoming darkened above; sporangia spherical to slightly flattened, at first white, later black, up to 156 (120)µm in diam, multispored; sporangial wall persistent, breaking from above to base into two equal parts; columellae pyriform to globose, with a small collar, up-to 120 x 108µm; sporangiospores faintly striate, light coloured to brown with hyaline hair-like bristles, 1 to 1.5 times as long as spores, ovoid to elliptical to almost triangular, 18-27 x 9-12.6µm; conidiophores consisting of primary vesicle from which secondary vesicle arise on short stalks, secondary vesicle bear single spored sporangiola, such monosporous sporangiola termed as conidia, which are brown, ovoid, distinctly striate,15-21 x 9-11.51µm.

3. *Circinella* van Tieghem & Le Monnier

Mycelium hyaline or coloured; sporangiophores branched sympodially, indefinite in length; branches with one or many sporangia, sometimes with a sterile spine; sporangia always borne circinately at the end of branches; sporangia spherical or globose with persistent and incrusted sporangial wall many spored, sporangiola never present, columellae variously shaped, always with a well-defined collar; sporangiospores usually spherical or globose, smooth; zygospores *Mucor* like.

4. *Cunninghamella echinulata* (Thaxter) Thaxter

Colonies at 25°C growing rapidly, powdery in appearance, at first white, becoming pale smoke grey; mycelium hyaline, hyphae up-to 20µm in diam; conidiophores erect, hyaline, up-to 18µm in diam, irregular to pseudo-verticillately branched; vesicles globose to sub-globose, usually smooth, the terminal vesicles sometimes verrucose, up-to 50µm in diam, the lateral vesicles 15-30µm; conidia globose to sub-globose, 10-14µm in diam, oval ones 7-15 x 15-20µm, echinulate or sometimes smooth; giant conidia present, 14-28µm in diam, pale brown, shortly echinulate or verrucose; chlamydospores not observed; heterothallic.

5. *Gilbertella persicaria Hesselt.*

Sporangiophores often slightly roughened a short distance below the sporangium, frequently branched, generally with a septum below the branch, up-to 16.5-39.6µm in diam., up-to 1.2 cm in height; sporangia black, globose to dorsiventrally flattened, generally circinately borne, 84-126µm in diam; sporangia I wall granular, minutely roughened, breaking into two equal halves; columellae hyaline, oval to cylindrical and sometimes pyriform, 14.8-135x13.2-99µm; sporangiospores oval to irregular in shape with, a papilla at either end, with 4 or 5 appendages, 4.5-21 x 4.0-16.5µm .

6. *Mucor circinelloides* V. Tieg hem. f. *circinelloides* Schipper

Colonies up to 6mm in height at 25°C on PDA covering the petriplates in four days, light greyish, composed of tall and short sporangiophores, tall sporangiophores sympodially branched with, long and short branches, latter circinate; the short sporangiophores profusely branched with circinate branches with slightly incrusted walls; the younger parts of the sporangiophores filled with droplets; sporangia up-to 80µm in diam with slightly incrusted walls, the larger sporangia having deliquescent walls, leave small collarettes. The smaller sporangia having persistent walls, leaving large basal membranes; columellae obovoid to ellipsoidal in the larger sporangia and globose in the smaller sporangia, up-to 53 x 49µm; sporangiospores ellipsoidal, 4.3-6.8 x 3.7-4.7µm; chlamydospores in the substrate hyphae, 15.7-23.0µmin diam. The growth and sporulation were good at 20-25°C and poor at 15°C and 37°C.

7. *Rhizopus oryzae* Went. and Prinsen Gerl.

Colony greyish brown, filling the petriplates in 4 days on PDA at 25°C, rhizoids brownish, sporangiophores on stolons up-to 1000-1500µm in length, 10-20µm in width, with swellings and dichotomous branching; sporangia greyish black, powdery in appearance, up-to 175µm in diam, columellae ellipsoidal with truncate base, up-to 130µm in height, mouse grey, Sporangiospores angular, sub-globose to ellipsoidal, with ridges on the surface, up to 8(-10)µm in length. Good growth and sporulation at 15-40°C; no growth at 45°C.

8.*Syncephalastrum racemosum* (Cohn) Schroeter

Turf at first white later grey, about 6mm in height; sporangiophores vigorous, at first unbranched, later richly branched with strongly curved laterals; fruiting head globose or oval, 22-70µm in diam, brown or grey, with numerous small warts to which the merosporangia are attached; merosporangia 5-10 spored.

9. *Thamnidium* Link.

Sporangiophores erect, terminated by a sporangium resembling that of the genus *Mucor*. They are formed at definite points on single or verticillate branches, which in turn are dichotomously branched and terminated by small sporangia or sporangioles. The sporangium is terminal, multisporous, with a diffluent membrane, incrusted with calcium oxalate, and possessing a large columella. Sporangioles small, spherical, containing four to ten spores, with an incrusted membrane, persistent, not diffluent, without columellae. The spores are of the same size in both sorts of sporangia; colourless, smooth. Zygospores naked, formed on the mycelium. Suspensors without appendages.

10. *Zygorhynchus* Vuillemin

Hyphae continuous, branched, unequal, often nodose, immersed, prostrate or forming a cottony aerial turf. Chlamydospores smooth, intercalary or terminal. Sporangiophores solitary or in an irregular sympodial system, bearing typical sporangia or abortive sporangia and zygospores; not apophysate. Sporangia uniform; wall diffluent, with the base concrescent with the columella. Upon its

disappearance a collarette remains. Spores numerous, minute, smooth. Zygospores homothallic, warted. Gametangia very unequal, produced on unequally bifurcated hyphae, one straight and small, the other curved and thicker, at the end a reflexed pear-shape.

11. Gongronella butleri (Lendner) peyronel & Dal Vesco

Colony growing slowly on SMA, about 3-5mm high with white turf. Sporangiophores 2.1-3.1 μm wide, hyaline, smooth to very faintly roughened, always with a septum under the apophysis beneath the sporangium, branching simply or irregularly. Rhizoids present. Sporangia globose, 16.6-22.7 in diam, with thin, smooth and easily dissolved wall,borne on straight to curved sporangiophores. Apophyses oval, 7.0-10.3 μm in length and 8.0-8.7 μm in diam with smooth surfaces. Columella reduced, hemispherical to dome-shaped, smooth, 2.1-4.2 μm in height, with collar around the base. Sporangiospores oval to flattened on one side to reniform, smooth, 2.5-7.2 × 1.7-4.7 μm in size. Chlamydospores observed in substrate mycelium, globose to ovoid with smooth surfaces, 4.9-6.8 × 4.6-10.7 μm in size. Homothallic, zygosporangium globose, 16-36 μm in diam, roughened with warts about 2μm in height, with two nearly unequal suspensors.

Order Entomophthorales:

Members of this order are predominately parasites of insects and other arthropods, *Entomophthora* (more than 40 species recognized) and *Massospora* are insect parasites, *Entomophthora muscae* kills house flies.Fungus invades insect after contact with sporangium. A germ tube forms and penetrates the host. Insect becomes restless and exhibits behavioral changes. After 5 to 8 days, host crawls to elevated position (e.g., blade of grass, twigs, window panes, etc.) Death often occurs between 3:00 to 7:00pm.

Host is attached to surface by rhizoids, sporangiophores form and push out between the abdominal segments sporangia are forcibly discharged as turgor pressure builds up in the sporangiophore. The sporangium may be shot as far as 1.0 to 1.5cm away from host.

Trichomycetes (Obligate commensals of arthropods):

Trichomycetes are obligately associated with an arthropod host, these fungi do not grow independently of their hosts in nature.Arthropod hosts include, insect larvae, crayfish, millipedes, beetles and isopods; not found in predacious arthropods, only detritivores, hosts may live on land or in fresh or marine waters. These fungi are usually found in the gut but one genus *Amoebidium* (probably not really a Trichomycetes) is found on the outer surface of the host, thalli are attached to host by a holdfast that penetrates the lining of the gut or exoskeleton only and does not penetrate living tissue. Trichomycetes are generally considered to be commensals (trichomycete benefits from interaction but neither benefits nor harms the host), although exceptions may exist.

Studies suggest that under sub optimal nutritional conditions the fungus (*Smittium culisetae*) may aide in survival and development of the host. Mosquito larvae, *Aedes aegypti*, raised axenically on semidefiied media not give adequate amounts of riboflavin do not survive well and none pupate, however with fungus were present about 50% of infested larvae were still alive at the 4th instar and about half pupated. One species, *Smittium morbosum*, kills mosquito larvae (midgut epithelium is penetrated and larvae die due to an inability to shed their molts). Four Orders are found in this group; only members from one order (Harpellales) has been cultured. The asexual propagative structure is the "trichospore" - consists of an elongated sporangium that contains a single sporangiospore. Trichospores have a collar and an appendage that serves to entangle it in debris. Trichospores explosively extrude their spore as it passes through the gut of a suitable host. This is followed by

holdfast formation. Spores are released form gut when host defecates, molts, or dies. For example, in species of *Smittium*, germination and attachment occur within a half hour of ingestion of the spores. In *Smittium* species extrusion of the sporangiospore and holdfast formation occur in two stages. Trichospores are initially preconditioned in the midgut by exposure to potassium largely supplied by the insect's excretory organs and high pH (pH 10). The pH drops to 7 as spores pass through the hindgut and the "spores" extrude and become attached to the cuticle of the gut. Zygospores may be produced, but are seen only in dissected gut material, and only once in culture.

Role of Zygomycetous fungi in Agriculture:

1. Fungi in humus formation: Different groups of fungi including Zygomycetes produces *humus* and this is one among natural sources of plant nutrients, actually locked in plant and animal bodies in a complex form. Fungi degenerates these and makes them available in simpler form which is called *humus***.** During the process, CO_2 is also formed, which is utilized by green plants during photosynthesis. *Humus* is basically a degenerative product of cellulose, hemicellulose, lignin, proteins, nucleic acid, etc. The major part of the *humus* consists of *humic acid*, *humins*, *fulvic acid*, etc. Because of its nutrient-rich nature, *humus* maintains physical and chemical properties of soils supporting various biological activities. During *humus* formation, all those complex organic molecules are degraded in steps, Cellulose, Hemicellulose, Pectin and Lignin etc.

2. Biological pest controlling: The concept was developed in the late 1800's to early 1900's. Since chemical sprays were relatively cheap and efficient, however, in the last several decades, problems such as carcinogenic compounds in many sprays, gradual resistance, in the case of insects and their slow biodegradability in the environment have brought about a renaissance in bio-control agents. Most species of fungi that have been studied belong to the Entomophthorales of *Zygomycota* (Hajek*et al.*,1995). *Entomophaga maimaiga*, has been somewhat successful against the Gypsy Moth in France and *Medicago sativa*, alfalfa a forage have a number of common pathogens, among them the Alfalfa Weevil controlled by introduction of various species of *Erynia* (Entomophthorales).

3.Mycorrhizal associations: Arbuscular mycorrhizas are found in 85% of all plant families, and occur in many crop species. The fungi involved are members of the*Zygomycota*(related to *Mucor*), presently *Glomeromycota*, classified currently in six genera (*Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora*) and they seem to be obligate symbionts, none of them can be grown in axenic culture, i.e. in the absence of their hosts. Their important role is in mineral nutrient uptake and sometimes in protecting against drought or pathogenic attack.The hyphae of arbuscular mycorrhizal fungi produce the glycoprotein glomalin, which may be one of the major stores of carbon in the soil. Arbuscular mycorrhizal fungi have (possibly) been asexual for many millions of years and, unusually, individuals can contain many genetically different nuclei (a phenomenon called heterokaryosis). The hyphae of AM fungi extend into soil, where their large surface area and efficient absorption enable them to obtain mineral nutrients, even if these are in short supply or are relatively immobile. AM fungi seem to be particularly important for absorption of phosphorus, a poorly mobile element, and a proportion of the phosphate that they absorb has been shown to be passed to the plant. Structures resembling those of the present-day AM fungi have been found in fossils of primitive/present Pteridophytes of the Devonian period and Bryophytes. It is thought that these fungi colonized the earliest land plants and that mycorrhizal associations could have been essential for development of the land flora.

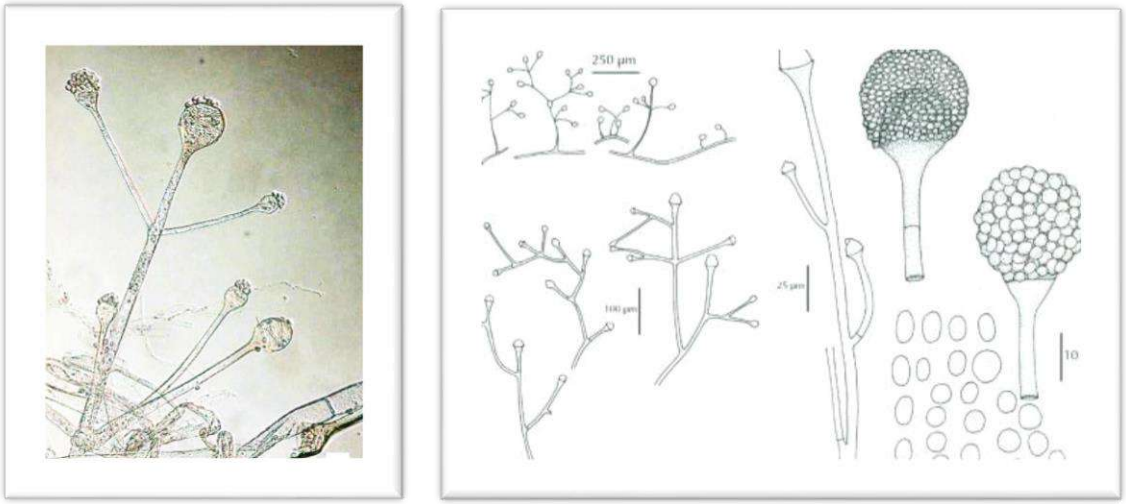
Role of Zygomycetous fungi in industrial sector:

Zygomycetes fulfil all requirements for being utilized as core catalysts in bio refineries, and would be useful in creating new sustainable products. Apart from the extended use of Zygomycetes in preparing fermented foods, industrial metabolites such as lactic acid, fumaric acid, and ethanol are produced from a vast array of feed stocks with the aid of Zygomycetes.

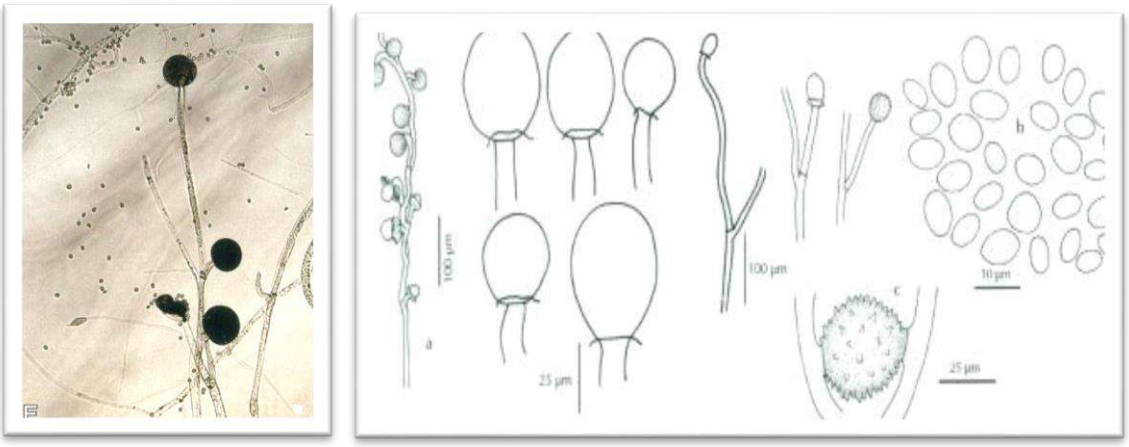
1. Production of organic acids: Various species of*Rhizopus*and*Mucor* i.e. *M. indicus, M. corticolous, M. hiemalis, and M. indicus,Rhizopus oryzae*, *Rhizomucor pusillus or Rhizomucor miehei* etc.used to make sake, the rice wine of Asia. Another species of Rhizopus is used in the commercial production of ethanol and glucocorticoids.

2. Production of enzymes: Zygomycetes fungi are the source of a great diversity of enzymes, hydrolytic enzymes, such as those needed for the degradation of plant carbohydrates, including amylases, cellulases, and xylanases. Proteases and lipases, Pectinases needed for isolation, purification, and application of different relevant industrial processes produced from *Rhizopus microspores* var. *rhizopodiformis. Mucor circinelloides* and various Zygomycetes genera, e.g. *Gilbertella, Mucor, Rhizomucor*, and *Rhizopus* have presence of a complete cellulase system, comprising endoglucanase, exoglucanase and β-glucosidase.Several species of Rhizopus, *Mortierella romanniana*, *M. vinacea*, *M. alpina,Blakeslea trispora* and *Mucor indicus produces* Linolenic acid, Arachidonic acid and β-carotene.

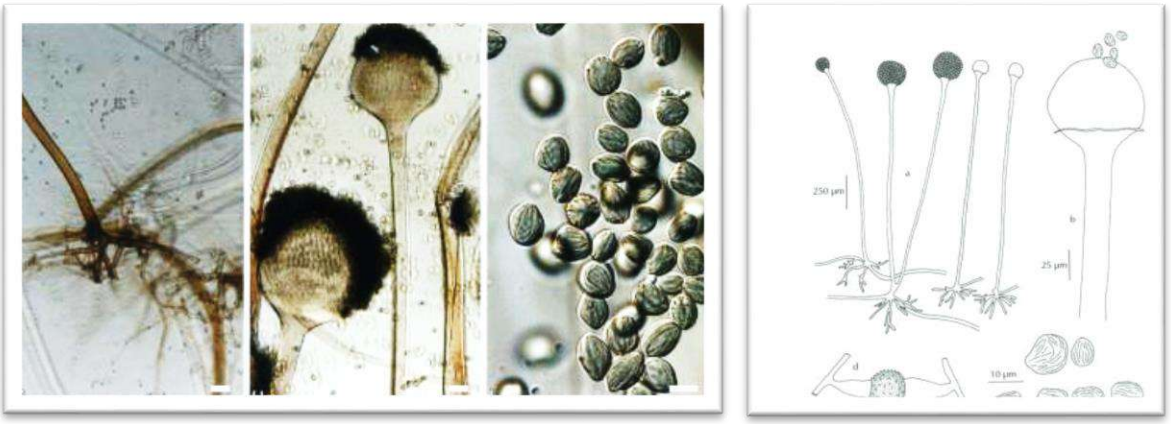
Identification of Zygomycetous fungi



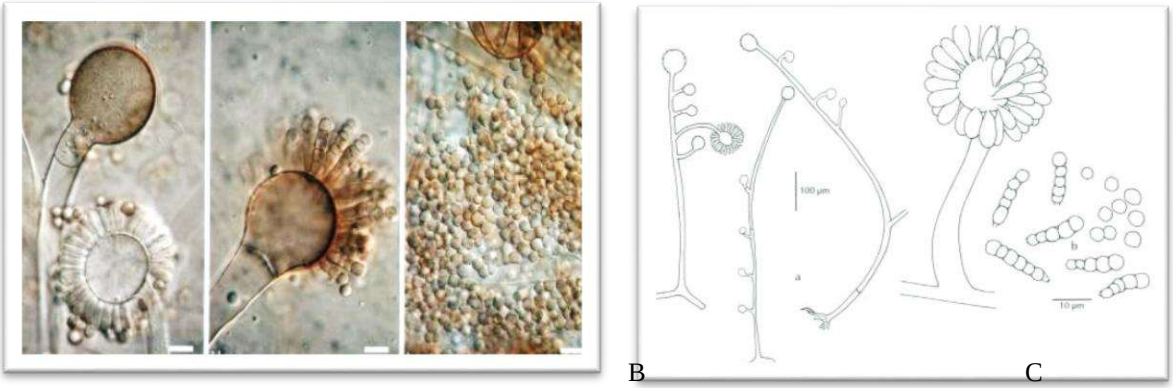
Absidia: Sporangiophores, sporangia, columellae and sporangiospore



Mucor: Sporangiophores, sporangia, columellae, sporangiospores & zygospore

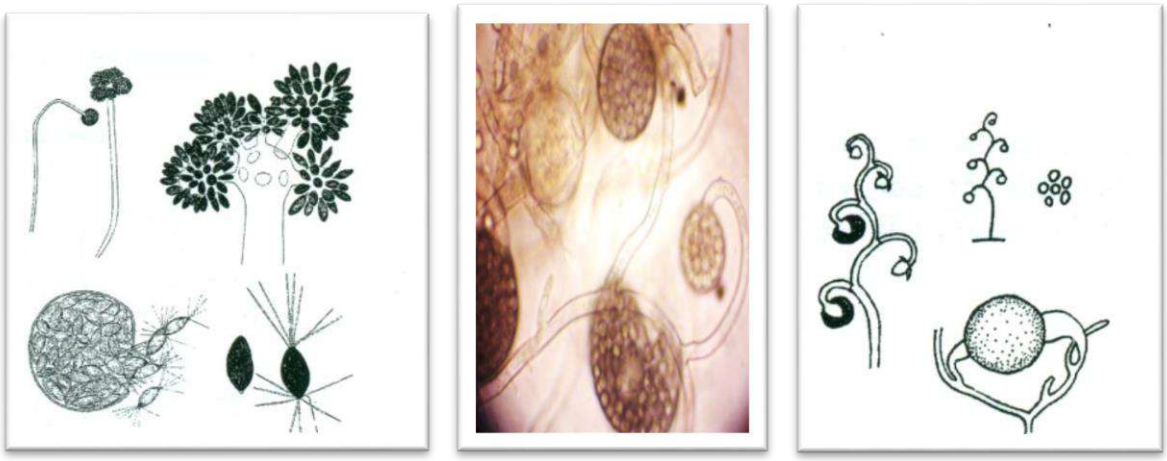


Rhizopus: Rhizoides, stolons, sporangiophores, sporangia, columellae, sporangiospore & zygospore



A B C

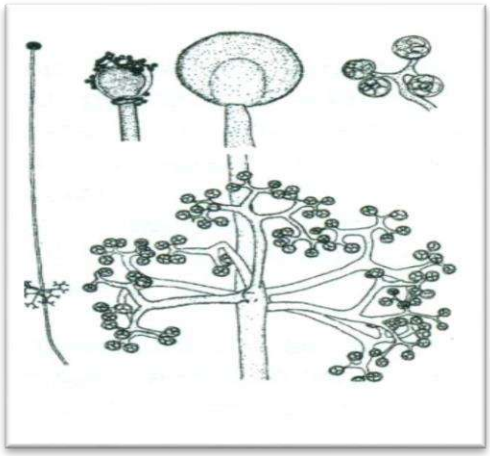
Syncephalastrum: A. Sporangiophores, sporangia, columellae & sporangiospores, B. sporangiophore bearing vesicle with merosporangia all over their surface C. Merosporangia & Merospores



Choanephora: Sporangiophores,**Circinella**: Sporangiophoree,sporangia,columellae,sporangia,sporangiospore and sporangiospore and zygosporessporangiola

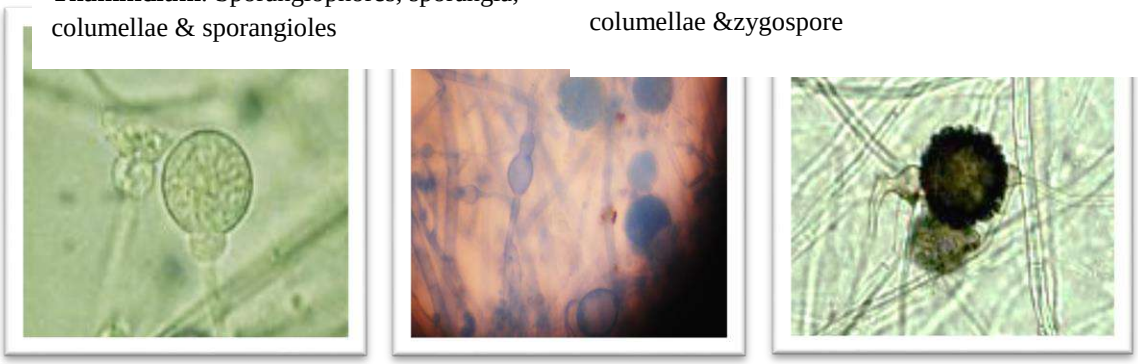
Cunninghamella: conidiophore,vesicle & conidia

Gilbertella: Sporangiophoree, sporangia, columellae & sporangiospore

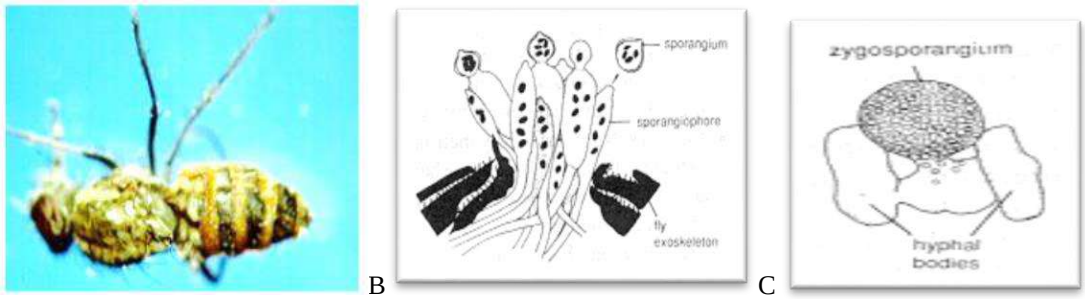


Thamnidium: Sporangiophores, sporangia, columellae & sporangioles

Zygorhynchus: Sporangiophores, sporangia, columellae &zygospore

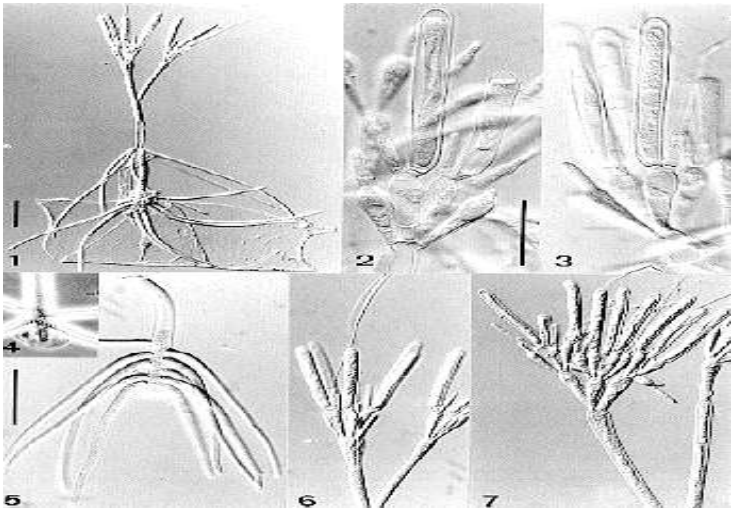


Gongronella butleri: Sporangiophore with bulging base(apophysis)and zygospore



A B C

Entomophthora muscae: A.dead house flies due to fungal infection B.exoskeleton burstsand produces tightly-packed masses of sporangiophores C. zygospore.



Orphella catalaunica:A Trichomycete at hindgut of a stonefly nymph (*Plecoptera, Insecta*).1. Mature thallos, 2 & 3. Speculating heads with trichospores, 4&5. Holdfast cells with branches, 6&7. Sporulating heads

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Taxonomical Studies on Macro–Fungi

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Nature has provided us the beautiful ecosystem of which macro fungi are its integral parts. Macrofungi are important economically due to their importance in food, medicine, bio-control, chemical, biological and other industries. Medicinal values of the mushrooms are known to human beings since ages (CharakSamhita 3000BC).

Macro fungi are also called as fleshy fungi popularly known as mushrooms. About 15000 species of mushroom are found world over of which 7000 species are edible, amongst 2000 species are neutracitically important, 200 species are mycorrhizal of which 90% are endomycorrhizal and 10% are ectomycorrhizal, nearly 200 species are experimentally cultivated and 10 are grown on industrial scale. So not all the mushrooms found in nature are edible some are poisonous also. Cases of accidental mushroom poisoning were also known to ancient Greeks and Romans as early as 500 B.C. The name "evil ferments of the earth" was given to some of the mushrooms. The edible members were called "mushrooms" while the poisonous varieties were termed "toadstools". The Roman emperor Cladius Caesar (A.D. 54) was murdered by his wife who mixed his food with *Amanita phalloides.* Sporophores of mushrooms were popular among the Roman potters and sculptures in a series of ancient stone sculptures (1000 B.C.-200 A.D.). Mushrooms have fascinated human beings since very beginning. Mushrooms belong to kingdom fungi. These are defined as fruiting bodies (Carpophores) of the larger fungi or macromycetes (Singer, 1961) and are commonly termed as fleshy fungi. Persoon (1801) and Fries (1821-1832) were among the first who gave authentic description of larger fungi. Since then tremendous progress has taken place in the description and identification of macromycetes. The word truffle is also used to refer the fruiting bodies of the fleshy fungi but these are characterized as growing below ground. Theophrastus 300 B.C. was the first to mention about Truffles, this is an edible, subterranean fungus in the genus *Tuber*, order Tuberales, of the class Ascomycetes (Division Mycota).The most valued truffle is the Perigord (*T. melanosporum*). The English truffle, *T. aestivum,* is found principally in beech woods. False truffles (*Rhizopogon*) form small, underground, potato-like structures under coniferous trees, in parts of North America. Discorides in first century AD divided fungi into edible and poisonous varieties. It was assumed that only few of the fleshy fungi are safe to eat this lead to call edible fungi as mushrooms and poisonous fungi as toadstool. However Singer (1961) in his book "*Mushrooms and Truffles*" concluded that toadstool is synonymous to Mushrooms only. But not all the species of same genus are poisonous as Amanita phalloides is deadly poisnou while *Amanita muscaria*and*A.caesaria* are hallucinogenic.

Some mushrooms belong to Ascomycota while most of these are from Basidiomycota (Lakhanpal et al. 2016). Mushrooms have fascinated human beings since very beginning. Mushrooms belong to kingdom fungi. Singer (1986) in "The Agaricales in modern taxonomy" gave the most comprehensive treatise based on morphoanatomical features, which was modified by Kirk *et al.* (2008) based on molecular characterization.

Medicinal values of the mushrooms are known to human beings since ages (Charak Samhita 3000BC). Francia *et al.* (1999) listed six groups, (1) six species which reduce the total cholesterol level, *Auricularia auricula-judae, Cordyceps sinensis, Ganoderma lucidum, Grifola frondosa, Pleurotus ostreatus, and Tremella fuciformis;* (2) two species which reduce the "bad cholesterol – (LDL) the low-density lipoprotein level", *Auricularia auricula-judae and Tremella fuciformis;* (3) three species which reduce the triglyceride level, *Cordyceps sinensis, Grifola frondosa,* and *Lentinua edodes;* (4) six species which are reported to reduce platelet binding under *in vitro* condition,

Auricularia auricula-judae, Calyptella sp., *Ganoderma lucidum, Kuehneromyces* sp., *Neolentinus adhaerens* and *Panus* sp.*;* (5) three species which may reduce the arterial pressure, *Ganoderma lucidum, Grifola frondosa* and *Tricholoma mongolicum* and (6) six species which may decrease glycemia, *Agaricus bisporus, Agrocybe aegerita, Cordyceps sinensis, Tremella aurantia, Grifola frondosa and Coprinu scomatus*.

Macro fungi are diverse in their uses as food and medicine and several species serve as decomposers and also form mycorrhizal associations. Ectomycorrhizal association was found in *Agaricus anguistus, Amanita caesria*, while *Boletus, Lepiota* and *Russula* are ectomycorrhizal. Taxonomists describe about 1 lakh species of fungi, but till now the fungal global biodiversity is not fully understood. About 1.5 million fungi have been projected on earth surface. Of these, approx 5-7% (nearly 1 lakh) of the fungi are described till now. Morphology is studied at four levels: macroscopic, microscopic, ultra-structure, and molecular biology. The order Agaricales comprises of 3 sub-orders, 17 families, 230 genera and approximately 5000 species. From India only 283 edible species have been reported (Purukayastha and Chandra, 1985 and Lakhanpal 1990). Sathe (1979) indicated 275 species of Agarics existed in India. Manjula (1983) recorded 538 valid species belonging to 115 genera of 20 families of the oreder Agaricalels deposited in Kew from India and Nepal. Sarbhoy (1997) stated that till 1979 about 450 species from the oreder Agaricales have been reported from India. However Walting and Abraham (1992), was of the view that 650 species have been recorded from India.

Walting (1973) gave simple key for identification of fungi. Those having spore borne externally on clavate to cylindrical cell were classified in Basidimycota while macrofungi in which spores are produced in a clavate, cylindrical and subgloobose cells were grouped in Ascomycota. In Ascomycota common type is cup or modified cup on log stalk or sessile. *Morchella* is *on* stalk and epigeous. In Hellotiales fungi are like earths tongue while in sphaeriales like *Xylaria* shape is like man finger. Truffles are hypogeous (below ground).

Basidomycota:

1. Hymenomycetes a. Agaricales b. Aphyllophorales

Spores are forcibly discharged; basidia present when spore mature and spore print can be taken on maturity.

Hymenomycetes have two sub classes:

A. Phragmobasidiomycetdae: jelly like fungi and maturebasidia are septate.

B. Holobasidiomycetdae: Basidia have only one basal septum.

2. Gasteromycetes eg. Puffballs, earth stars, stink horns etc.

Spores are not forcibly discharged, spore print not obtainable. Hymenium closed at maturity. In some cases basidia disintegrate prior to spore dispersal it includes gasteroidesagaricales also. Auricularia auricular is different as it is gelatinous.

Parts of mushroom

Mushroom can be divided mainly into two parts ; 1. Cap (pileus) and 2. stalk or stem (stipe). On the underside of the cap in basidiomycetous mushrooms are found spore-making reproductive surfaces called as hymenia : most species have gills (lamellae), but some have pores or tubes, while others have ridges, teeth or other structures. In ascomycetous mushrooms (morels, false morels and their allies and close relatives), most or the entire upper surface of the cap is reproductive, and is folded, broadly pitted or wrinkled in most species.

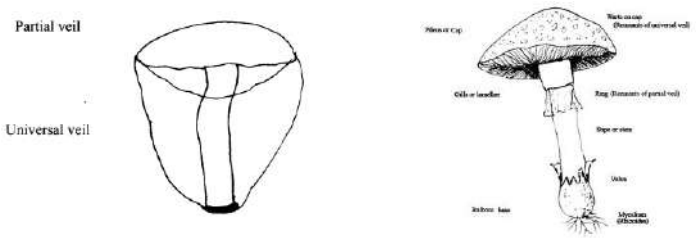


Fig. 1. Parts of Mushrooms

Fig. 2. Collection and processing of mushrooms. Source: Internet website. 2. Diversity of mushrooms.

Some mushrooms are initially enveloped in a membrane that goes around the entire mushroom. The mushroom then breaks out of this membrane, the "universal veil," as it expands.The ruptured universal veil may leave remnants such as warts or granules on the cap surface, a cup-like structure (volva) at the base of the stalk, or thick slime or thin fibrils on the cap and the lower part of the stalk. Another membrane found in some mushrooms which initially connects the margin of the cap to the middle of the stalk or stem, covering or sealing off the gills or other structures bearing the reproductive surface (hymenium). This membrane is known as the "partial veil," (Fig. 1) then ruptures, usually leaving a ring (annulus) on the stalk, and sometimes some fringe-like ("appendiculate") fragments of tissue on the edge of the cap (Fig. 1&2).

Most of the commercially cultivated mushroom belongs to order Agaricales:

The fungal order Agaricales, also known as gilled mushrooms (for their distinctive gills) or euagarics, contains some of the most familiar types of mushrooms. The order has 33 extant families, 413 genera, and over 13000 described species, along with six extinct genera known only from the fossil record. In three volumes of *Systema Mycologicum* published between 1821 and 1832, Elias Fries put almost all of the fleshy, gill-forming mushrooms in the genus *Agaricus.* Fries based his classification on macroscopic characters of the fruit bodies and color of the spore print. His system had been widely used as it had the advantage that many genera could be readily identified based on characters observable in the field. Fries's classification was later challenged when microscopic studies

of basidiocarp structure, initiated by Fayod and Patouillard, demonstrated several of Fries's groupings were unnatural.

Rolf Singer's work The Agaricalesin Modern Taxonomy, published in four editions g from 1951 to 1986, used both Fries's macroscopic characters and Fayod's microscopic characters to reorganize families and genera; his most recent classification included 230 genera within 18 families. Singer treated three major groups within the Agaricalessensulato: the Agaricalessensustricto, Boletineae, and Russulales. These groups are still accepted by modern treatments based on DNA analysis, as the euagarics clade, bolete clade, and russuloid clade.

Ainsworth in 1973 divided Agaricales order in 15 families: Boletaceae, Hygrophoraceae, Tricholomataceae, Entolomataceae, Pluteaceae, Lepiotaceae, Agaricaleceae, Bolbitiaceae, Strophariaceae, Copriniaceae, Cortinariaceae, Paxillaceae, Russulaceae, Amanitaceae and Gomphidiceae.

Order Agaricales: Characteristic features

Fruit body development is hemingiocarpous, hymenium covering lamellae on the lower surface of the pileus or lining tubes which are easily seperable from the pileus.

It has been classified by Smith (1973) and Singer (1975) into 16-18 families. Singer considered Schizophyllaceae and Polyporaceae family in the Agaricales While Webster (1980) have placed these two families in the Aphyllophorales order.

The families and genera are based on the following characters

1. Presence or absenceof annulus, volva etc.
2. Spore colour and ornamentation
3. Mode of attachment of gill to the stipe
4. Composition of Trama (homo or heteromerous)
5. Chemical reaction of the spores and flesh (many spores give amyloid reaction with iodine).

Vilgalys *et al.* (1993) have reviewed the various system of classification of Agaricales. Earlier ones were based on microscopic characters as defined by E.M. Fries (1794-1878) while later ones involved the use of light & electron microscopy and biochemistry. Further refinement of taxonomy of Agaricales involve use of molecular techniques (Burn *et al.* 1989.1990)

Singer (1986) recognised 17 families in Agaricales including Polyporaceae and Bondarziaceae which are now included in the order Aphyllophorales (Alexopolous, Mims and Blackwell, 1996). Agaricales have nearly 300 genera and about 5000 species.

Simple Key to the Families of the Order Agaricales

A. Basidiocarp soft and putrescent; hymenophore usually separating readily from context.
B. Hyrnenophore consisting of tubes, sometimes of shallow pits .....Boletaceae
BB.Hymenophore consisting of lamellae which are often united by conspicuous veins
.....Paxillaceae
AA.Basidiocarp fleshy to tough, or membranous, hyrnenophore not separating readily
From context.
C. Context of the sporophore containing nests of sphaerocysts .....Russulaceae
CC. Sphaerocysrs absent.
D. Gills waxy, broadly triangular in section .....Hygrophoraceae
DD. Gills not waxy, narrow in section .....Agaricaceae

1. BOLETACEAE. Characteristic feature is presence of vertically arranged tubes on the underside of the Pileus instead of gills. Pores visible on under surface of the pileus are actually opening of these tubes. Size & depth of these tubes vary from species to species. Other feature is the soft decaying

nature of the pileus which is mostly infested with worms separating it from the Polyporaceae family of Aphyllophorales. These are eaten after removal of the tubes. Mostly mycorrhizal with conifers and woody plants.13 genera .Type genus *Boletus.* Smith & Thiers (1971), in "Boletes of Michigan" have reported 11 genera.

2. RUSSULACEAE. These differ from other Agarics by production of heteromeroushymenophraltrama containing nests of sphaerocysts bounded by hyphal elements. These produce white to yellowish spores. Hyphae lack clamp connections. Produce simple basidiocarp consisting of pileus and stipe. The gills are attached to the stalk. Flesh of the fruit body is granular or fibrous, fruit body is brittle and breaks like a chalk (due to marble like cells called sphaerocysts in the flesh and stipe). Basidiospores are large and ornamented with spines and ridges which show amyloid reaction. *Russula, Lactarius.* Pegler & Young (1979) have placed some gasteromycetes genera like *Elasmomyces, Zelleromyces, Martellia* and *Gymnomyces*in order Russulales. Red head & Norvell.1993 concluded that genus *Bonderzewea* of Aphyllophorales is related to non mycorrhizal species of *Lactarius.*

3. HYGROPHORACEAE. These produce white thin walled basidiospores from long cylindrical basidia best known genus is Hygrophorus, characterized by thick gills which are andante to decurrent. Gills are characteristically covered with waxy material. Some members of the genus produce colourfull basidiocarp having yellow, orange or red caps.

4. TRICHOLOMATACEAE. Stipe is confluent with the pileus, colour of the spore is variable, (White, yellow, pink or brown)Lamellae attached with the stipe rarely free. Hyphae lack clamp connections. i.e. genus *Pleurotus, Marasmius,Lentinus , Termitomyces, Flammulina, Clitocybe, Tricholoma, Laccaria* & *Armillaria* etc.
Termitomyces is associated with the termites, while *M. oreades*is fairy ring mushrooms. It is edible with small cap& thin stalk. *Clitocybe* is edible but *C. delbata*is deadly poisonous. Pleurotus species is most common are either sessile (lack stalk) or have very short lateral stalk. But *P.ulmarius* is edible large white mushroom having thick central stalk.

5. AMANITACEAE. Represented by genus Amanita, characterized by white spores, free gills and presence of annulus and volva. Most genus are ectomycorrhizal encountered near woody areas both of coniferous and hardwood .Lamellae are free from stipe and have bilateral trama , spores and gills are white , stipe is neatly separable from the pileus. Both volva and annulus are present in the mature fruit body. *Amanita* and *Limacella* are important genera.
A. virosa (destroying angel); pure white
A. caesaria (Caesars mushroom) yellow to orage cap.
A. muscaria (fly agaric) yellow, orane or brilliant red cap.
A.phalloides (death cap) is deadly poisnous.
Mycetismus is better known as mushroom poisoning. Litten 1975 reported that 90-95 % of all deaths due to mushroom poisoning are due to single species *A.phalloides.* This species have olive green pileus up to 12 c.m. wide and stipe also up to 12-15 c.m. long. The stipe, gills, annulus and volva all are white.
Amanita produce some deadly toxinslikeamatoxin (9 type) and phallotxins (7 type) *A.phalloides* produce ά-amanatin which is specific inhibitor of the RNA polymerase found in eukaryotic cells so important in elucidating the enzyme action causing slow lingering death. Only amatoxins are poisonous when ingested but as intravenous injections both the compounds are lethal.

Weiland (1978) developed the Meixner test to check whether a particular mushroom contain the amatoxin or not. In this test the stalk or cap of the mushroom is pressed to piece of the news paper or any paper containing lignin. This area is then allowed to dry and then a drop of conc. HCL is added. If blue colour appears in 5-10 minutes amatoxin are assumed to be present.

6. AGARICACEAE. Large family has about 25 genera. Best known is *Agaricus*. It has white to grey brown cap, free gills an annulus and volva. Stalk is readily separable from the pileus. In young fruit body gills are light in colour often pink or white. Later on become darker assuming the colourthe spores.(Characterized by black or brown spores, presence of annulus, easily separable nature of the stipe with the pileus .Free lamellae).type genus *Agaricus.*
A. campestris, A. rodmani and *A. brunnescens (A. bisporus)* are edible while *A. xanthodermis* is poisonous causing gastrointestinal disorders.
Genus *Lepiota* and *Chlorophyllum* are placed by some in lepiotaceae family but Singer 1986, has placed them in Agaricaceae family. Lepiota genus is edible but superficially resembles Amanita in possessing white spores, free gills and annulus. But Volva is lacking in Lepiota and annulus is conspicuous and persistent.

7. STROPHARIACEAE. Singer (1986) placed 9 genera in it including *Pholiota,* Stropharia and Psilocybe. Members have attached gills produce dark spore (Purple – brown colour). No one is mycorrhizal .Stipe is confluent with the pileus. There flesh not separable. Basidiospores are brown, the thin layer covering the pileus or stipe is composed of tubular gelatinous hyphae. *Psilocybe*was studied by Guzman1983, - It has small basidiocarp with conic to companulate cap and long thin stalk. On maturirymasy or may not posses annulus. 144 species are recognized by Guzman 1983, out of which 81 are hallucinogenic. These are characterized by bluing reaction when bruised or broken. Most widely cultivated species of *Psilocybe* is *P.cubensis.*

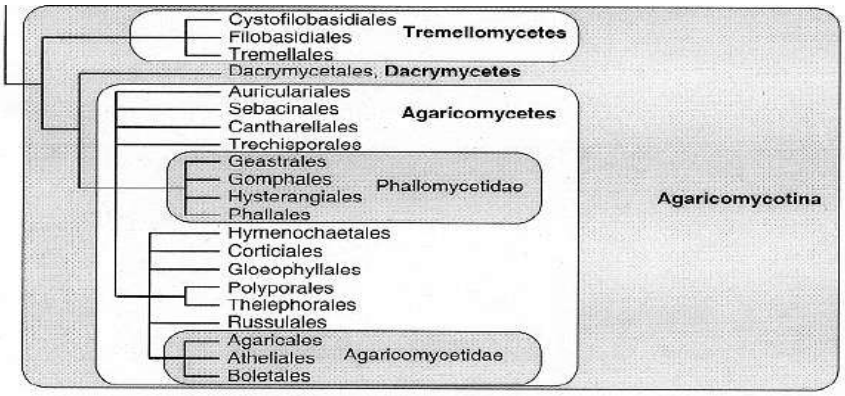
8. COPRINACEAE. Basidiocarp are fragile, grow on dung of herbivorous animals. *Coprinus* is known genera. All dark spored genera producing black to purple brown or purple black spores. Members are found on dead twigs, wood, dung, soil and litter and few athers are parasites on other agarics. Genus Coprinus is characterized by black spores with hymeniferousgills that tend to dissolve into black inky liquid that tends to drop from the disintegrating cap.
C. comatus (Shaggy mane) is edible mushroom Its cylindrical cap has many scales and annulus. *Podaxis* genus is also placed in this family.

9. PLUTACEAE. Members produce pink spores and found on wood and litters of all kinds. Pluteus and Volvariella are best known members. *P. cervinus* is edible species common on sawdust piles. *Volvariellavolvaceae* is better known as paddy straw mushroom and is edible.

10. CORTINARIACEAE. Large family produces brown spores which are rusty brown to cinnamon brown. Species with bright yellow brown to clay brown spores are placed in BOLBITACEAE family.. Genus Cortinarious is delicate, spider web like veil or cortinaalso called as partial veil this structure extends from margin of the pileus to the stipe. Many species are ectomycorrhizal and some are poisonous i.e, *C. orellanus* (Cause of mass poisoning in Poland in 1952). Genus *Pholiota* also belong to this family. These have attached gills and mayor may not possess annulus and are found on woods. These have scaly cap.

11. ENTOLOMATCEAE; 12. LEPIOTACEAE; 13. BOLBITACEAE; 14. PAXILLACEAE; 15. GOMPHIDIACEAE

A New Phylogenetic Classification of True Fungi (Hibbett *et al. 2007*)



Molecular characterization

Molecular phylogenetics research has demonstrated that the euagarics clade is roughly equivalent to Singer's Agaricalessensustricto. A recent (2006) large-scale study by Brandon Matheny and colleagues used nucleic acid sequences representing six gene regions from 238 species in 146 genera to explore the phylogenetic grouping within the Agaricales.The analysis showed that most of the species tested could be grouped into six clades that namely the Agaricoid, Tricholomatoid, Marasmioid, Pluteoid, Hygrophoroid and Plicaturopsidoid clades.

i. DNA Extraction

DNA for ITS sequencing is extracted from petri dish using CTAB method. A typical ECM mushroom DNA extraction protocol is given in Fig. 9.

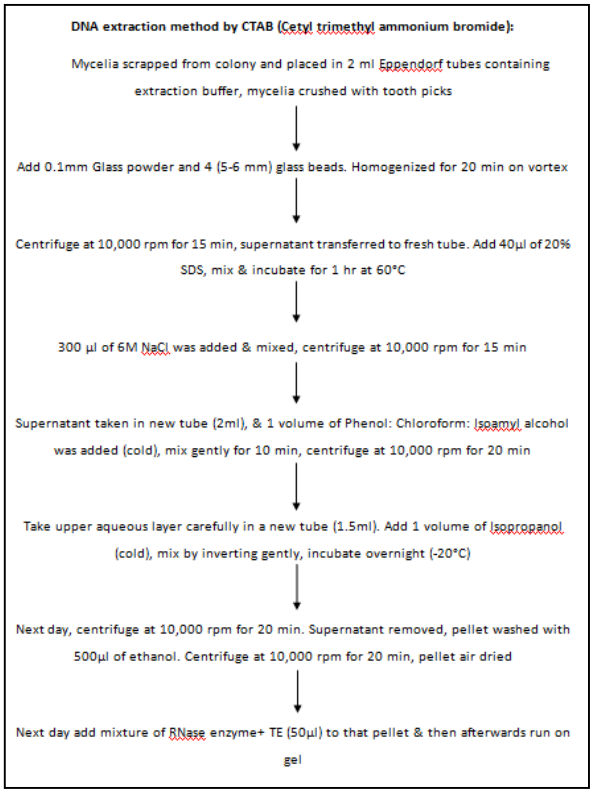


Fig. 3. DNA extraction from mushroom specimen or culture.

ii. PCR

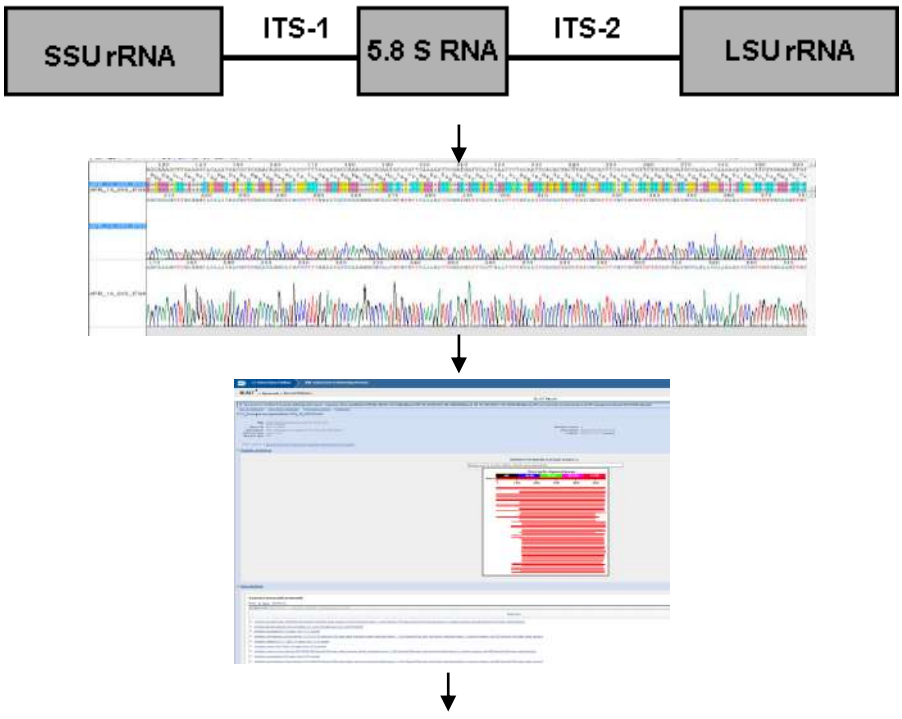
A typical PCR of ITS region is is achieved by the following reaction mixture and conditions.

Requirements (for 1 reaction)	Volume (µl)
DD PCR water	16.3
Taq Buffer+ 15mM MgCl2 (1x)	2.5
dNTPs (10mM)	2.5
Primers: (1PM)	2.5
Forward Primer (ITS1)	1.5
Reverse Primer (ITS4)	1.5
Taq DNA Polymerase	0.2
Template (Isolated DNA)	1

35 CYCLES	Denaturation at 94°C for 1 min. ↓ Annealing at 56°C for 30sec ↓ Extension at 72°C for 1min.

iii. Sequencing

The sequencing reactions of PCR product were carried out using Taq DNA polymerase dye terminator cycle applying automated DNA sequencing method based on dideoxynucleotide chain termination method. The sequencing reactions were carried out using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's protocol. This Kit contains the four ddNTPs with different fluorescence labels termed as BigDye Terminators. 2 µl PCR product and 3 pmol of the sequencing primer were used in a 20 µl sequencing reaction. The sequencing primers for LSU were LR5R (TCCTGAGGGAAACTTCG), LROR (ACCCGCTGAACTTAAGC), LR3R (GTCTTGAAACACGGACC) and LR16R (TTCCACCCAAACACTCG) for sequencing (Vilgalys and Hester 1990). The sequencing reaction mixes were subjected to 25 cycles in a Perkin Elmer thermal cycler 9700. Each cycle consisted of 95°C for 10 min, 50°C for 5 min and 60°C for 4 min. DNA sequencing was carried out on ABI 3730xl Automated Sequencer.



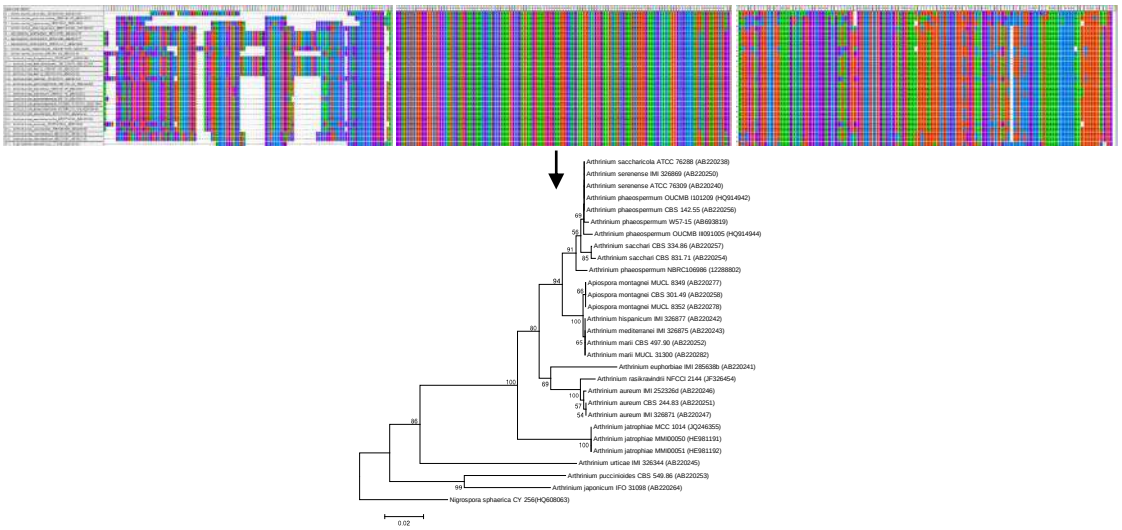


Fig. 4. Sequencing of Internal Transcribed Spacer (ITS) and phylogenetic tree construction necessary for phylogenetic species identification.

Suggested reading:

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Morpho-molecular characterization of wheat fungal pathogens

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Wheat (*Triticum aestivum* L.), is second most important staple food crop of the world accounts nearly 30 per cent global cereal production covering an area of 220 million hectares. In India, during 2018-19 Rabi season, wheat was cultivated in 29.55 mha. Wheat production in India has increased many folds from 6.4 mt in 1950 to 101.20 mt in 2018-19 with average national productivity i.e., 3424 kg/ha (ICAR-IIWBR, 2019). Since wheat is grown in different agro climatic conditions in our country, the constraints to its production vary from one zone to other. Biotic stresses are the main constraints in wheat production worldwide. The most serious biotic stresses affecting wheat are rusts, bunts and smuts.

Rusts

Rusts are the most serious diseases of wheat crop in India. The causal agents of rusts are highly specialized plant pathogens with narrow host ranges. These are fungal organisms which belong to genus *Puccinia*, family Pucciniaceae, order uredinales and class basidiomycetes. Of the rust diseases of wheat, the most common these days is called leaf or brown rust and is caused by *P. triticina* Eriks. develops rapidly at temperatures between 10° and 30°C. Stem rust, caused by *P. graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., is also known as black rust is favoured by humid conditions and warmer temperatures of 15° to 35°C. Stripe or yellow rust, caused by *P. striiformis* West. f. sp. *tritici* Eriks. & E. Henn., is principally a disease of wheat grown in cooler climates (2° to 15°C), which are generally associated with higher elevations or cooler years. Wheat rust pathogens are generally obligate in nature. In principle, they produce five distinct fruiting forms (pycnium, aecium, uredium, telium and basidiospores) that appear in a definite sequence. Some of the spore stages parasitize one host, whereas the others may infect and parasitize a different alternate host. Rust fungi producing only teliospores and basidiospores are called microcyclic, and those additionally producing pycniospores, aeciospores and urediospores are called macrocyclic. Basidiospores, aeciospores and urediospores can infect host plants, and teliospores serve only as sexual, overwintering stage, which upon germination produces basidium. Aeciospores are formed after dikaryotization of pycniospores which appear after infection of basidiospores. Aeciospores upon infection produce dikaryotic mycelia, which then bear urediospores. Some macrocyclic rusts complete their life-cycle on a single host and are called autoceious. Others, such as stem rust of wheat, require two different hosts (wheat and *Berberis*) are called heteroecious**.**

Symptoms

Rusts may kill young wheat plants, but more often, they reduce foliage, root growth and yield by reducing the rate of photosynthesis. Stem rust appears as long, narrow, elliptical pustules or blisters parallel along axis of stem, leaf or leaf sheath in wheat. Blisters also may appear on neck and glumes. Later on, epidermis covering pustules rupture irregularly and can be seen around mass of brick-red colored spores, the urediospores. Telial pustules arise later in the season, which are black in color. Uredopustules of brown rust of wheat are of bright orange color, gathered in irregular small clusters on entire or portion of leaf, and burst on the upper surface. Stripes of yellow rust form on the leaves and in severe cases, symptoms appear on the sheath, stalk, glumes and awns. The green color of leaves fades in long streaks on which rows of urediosori appear. Urediosori of yellow rust rupture the epidermis as enabling the wind dispersal of the spores.

Stem (black rust)

Leaf (brown rust)

Stripe (yellow rust)

Stem rust

Uredia of *P. graminis* f. sp. *tritici* occur on wheat stems, leaves and leaf sheaths and rarely on glumes, awns and seed. Uredial pustules may erupt through both surfaces and tend to be larger on the underside. The pustules are oval, elongate, or spindle shaped and upto 3 x 10 mm in size. Numerous infections may weaken stems and cause plants to lodge. Urediospores are 15-24 x 21-40 µm, orange red and oval, oblong or ellipsoid. Four median germ pores indent their thick and spiny walls. Host maturity and the aeging of uredia initiate the formation of black-brown teliospores in uredial sori or in separate erumpent telial sori. Teliospores are ellipsoid to clavate, 15-20 x 40-60 um and two celled. They are tapered at their apex and have smooth, thick walls and a slight constriction at their septum. Teliospores germinate after several weeks of dormancy and a basidium is emerged and four basidiospores develop on sterigmata. Pycnia on barberry are small, flask shaped and sunken except for the ostiole. Pycnia exude slender, hyaline spores (pycniospores) and receptive hyphae in small droplets attracts insects. Aecia on the underside of barberry leaves are yellow and horn like. Aeciospores in long chain are subglobose, 15-19 X 16-23 µm, smooth and light orange-yellow (Roelfs *et al*., 1992).

Leaf rust

Uredia of *P. triticina* are upto 1.5 mm in diameter and are scattered or clustered primarily on the upper surface of leaf blades. They are round to ovoid, orange-red and erumpent. Uredospores are 15-30 µm in diameter, subgloboid and red brown with 3-8 germ pores scattered in their thick, echinulate walls. Telia sori develop beneath the epidermis, principally on leaf sheaths and blades. They are the size of uredia, glossy black and not erumpent. Teliospores are round or flattened at the apex like those of *P. striiformis* (Roelfs *et al*., 1992).

Stripe rust

Uredia are yellow, appear mainly on leaves and heads and often arranged into stripes. Individual pustules measure 0.3-0.5 X 0.5-1 µm but their linear orientation between vascular bundles and the development of runner hyphae can results in stripes as long as leaf blade. Urediospores are 20-30 µm in diameter, yellow orange and spherical. They have thick and echinulate walls and 6-12 germ pores. Urediospores are one celled, spiny walled and dikaryotic. They are nutrient independent and germinate in contact with water films. Germ tube penetrate stomata directly (stripe rust) or via an appresorial peg. In wheat heads, uredia normally occur on the ventral surface of the glumes. Urediospore production on host plants may be followed by teliospore development within uredia or separate telial sori. Teliospores are brown black, binucleate and two celled and have thick, smooth walls. Telial pustules, prevalent on leaf sheaths are persistently subepidermal. They are dark brown and often form long, dark streaks (Roelfs *et al*., 1992).

Karnal bunt

Karnal bunt of wheat was first reported in 1931 from Karnal (Haryana) in 1931 by Mitra and is hence named after the place. The disease is caused by a fungus, *Tilletia indica* (Mitra) and is also called as partial bunt. The disease has been reported from several countries worldwide and the teliospres of the fungus have been intercepted in wheat stocks from nine countries. The disease occurs frequently in the areas with a temperature range from 5-30^{0}C and relative humidity between 45-100 % during the crop cycle. Under natural conditions the disease caused by fungus (*T. indica*) infects bread wheat, durum wheat and triticale.

Symptoms

Karnal bunt pathogen infects wheat at the flowering stage prior to seed formation, hence the symptoms are visible only when the grains have fully developed in ear heads. In a stool all the ear heads are not affected and also all the grains in a spike. A careful examination of individual ear reveals bunt infection in the field. In the standing wheat crop, the infected spike can be detected by the shiny silvery black spikelets, with glumes spread apart and swollen ovaries. The spikes of infected plants generally are reduced in length and in number of spikelets. In seeds, the infection starts at the embryonic end and spreads in all directions but to different degrees. Occasionally, random localized infection has been observed over the grain other than embryonic end. The groove portion of the seed gets infected while the dorsal side remains unaffected. The pericarp of the infected grains ruptures with release of black powdery mass which gives a pungent fishy odour. This odour is specific to Karnal bunt pathogen. Most of the seeds show partial infection but in severely bunted seeds, the whole endosperm material may be converted into the mass of bunt spores, which comes out during harvesting and threshing operations and seed looks hollow.

Teliospores of *T. indica* are dark brown to black, globose to sub-globose in shape having hyaline sheath of 2-4 µm thickness and measure 22-49 µm in size, average being 35 µm in diameter. Mixed with the spores are globose to elongate, yellowish sterile cells which have smooth wall. They are smaller in size (15-28 µm) than normal teliospores. Scanning electron microscopy of teliospore shows three distinct layers: the perisporium (sheath), the episporium and the endosporium. The perisporium is a fragile, fractured structure and the episporium is reticulated having numerous curved projections with blunt margins, due to which the surface of the spore looks rough. With the advancing maturity the perisporium ruptures at a few places and projections become visible. The surface projections are 3.1-4.0 µm thick in mature teliospore. An individual projection is composed of two double strands which are near the apex. The endosporium is thick and lamellate structure (Aggarwal *et al*., 2016).

KB infected earhead

KB infected grains

Teliospore of *Tilletia indica*

Flag smut

Flag smut, a minor disease is India is caused by *Urocystis agropyri* which is soilborne and externally seed borne pathogen. The disease was first reported in Australia in 1868. Prior to this report, flag smut was observed on *Agropyron* sp. in Europe in 1848 and the causal organism described as *Uredo agropyri* Preuss. Thus the presence of flag smut on grass in Europe was observed 20 years before it was first observed on wheat in Australia. Since then it has been reported from almost all the wheat growing regions of the world including Japan, India, Pakistan, Europe, South Africa and U.S.A. It is believed to be introduced from Australia and was first reported by Butler from Lyallpur in Punjab now in Pakistan. Flag smut occurs in Punjab, Haryana, Himachal Pradesh. Madhya Pradesh, Uttar Pradesh, Delhi, Bihar and Rajasthan.

The symptom is characterized by long, black raised streaks on the leaves, leaf sheaths, awns and sometimes on the stems. These black streaks break through the plant tissue, revealing a mass of powdery grey-black spores which easily rub off onto a finger. Infected plants are often stunted so they are not always easily identified in the crop during the season. Infected leaves do not expand fully and remain rolled and twisted. Infected plants can produce many tillers, but not all tillers on a plant will exhibit symptoms. During harvest the black spores are released from the plant contaminating seed and soil. Typically spores survive in soil for 3 years, but can survive for up to 7 years. Soil or seed borne spores infect the new wheat plant before emergence. Infection is favored by early sowing into relatively dry and warm soils. Optimal temperature for infection is 20°C, but infection may occur at as low as 5°C and as high as 28°C. The fungus grows inter and intra cellularly between vascular bundles of the leaf tissue and other effected plant parts.

Loose smut

Loose smut of wheat, caused by *Ustilago segetum tritici*, occurs in India wherever the crop is grown. Smut was known to the Romans, who named it *Ustilago*, which comes from the Latin word for burn. Loose smut is more common in regions with a cool, moist climate during flowering of the host. In India, the disease incidence is more in northern parts than in southern wheat growing areas. The first record of occurrence of *loose smut disease* in India was from Punjab. A specimen in HCIO (Herbarium Cryptogramae Indiae Orientalis) No. 7675 collected on 3rd April, 1897 is apparently the first record in the country. The incidence of the disease was quite high in traditional Indian wheat before the semi-dwarf era started. In 1967, with introduction of Kalyansona, a variety derived in mid sixties, loose smut started to decline. In the eighties and early nineties, this disease once again got aggravated due to cultivation of susceptible genotypes, but is on the decline now. Symptoms of loose smut are not apparent until head emergence. Before heading, dark green, erect leaves may be visible on plants infected by loose smut. Heads of infected plants emerge earlier, are darker in color, and are slightly taller than heads of healthy plants. Spikelets of infected heads may be completely replaced by masses of olive-black spores, which may be removed and blown away by wind, leaving an empty rachis. When spikelet tissues are not completely destroyed, remnants of glumes or awns may remain attached to the rachis. Loose-smutted heads may occur together in groups.

Loose smut survives as dormant mycelium in the embryo of infected grain. When grain infected by the loose smut fungus is used as seed for the next crop, the mycelium grows following germination of the seed and systemically infects the plant and eventually colonizes and replaces the ovaries. Spores from the smutted heads are blown by wind and/or rain splash, and when they land on healthy flowering heads, they germinate, forming mycelium, which penetrates the ovary or stigma directly, and grows and colonizes the embryo of the developing seed or grain. The optimum temperature for teliospore germination and further growth is 20° to 25°C and 95 percent relative humidity.

Flag Smut

Loose Smut

Conclusion

Visual identification of plant disease is the most rapid and cost effective method of disease diagnosis, but it is difficult for inexperienced personal and is also limited particularly to disease affecting aerial parts of the plants. Other method of pathogen identification is based on observed morphological characteristics by microscopic examinations of the diseased tissue, this method also require highly specialized taxonomists. Various problems associated with microscopical detection of wheat pathogens can be overcome by protein/ Nucleic acid based detections. These methods are very robust and repeatable. The nucleic acid based detections can be used at any developmental stage of plant since all living cells contain entire set of genome and not affected by environment. The Polymerase chain reaction based assays may be used as tool for fungal detection.

Precise identification and diagnosis of plant pathogens during early stages of infection can help a lot in effective management of the diseases. Currently more sensitive methods like Real Time PCR and Microarrays are being used. Real Time PCR has emerged as one of the promising and very sensitive method of plant pathogen detection. Generally pathogen variability is studied by using differential host reactions, culture characteristics, morphological markers and biochemical tests. These markers distinguish pathogens on the basis of their physiological characters. i.e pathogenicity and growth behaviors are and are highly influenced by the host age, inoculums quality and environmental conditions. Use of different hosts for pathotyping of plant pathogens is a time consuming and laborious process. Moreover, differential hosts are available only for a few host pathogen systems thus limiting the analysis of pathogen variability. In such cases use of molecular markers has been advocated for characterization for genetic variability.

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Morphological Characterisation of *Trichoderma* species

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Taxonomy of *Trichoderma* has gone through a remarkable transformation in the past 40 years. Today, approximately 150 species were recognized, and most of them were described after 2000, many as anamorphs of *Hypocrea* species (Samuels *et al.* 2012). The important species of *Trichoderma* found in Indian soils are described here under.

***Trichoderma aggressivum* (**Section – Pachybasium)

The anamorphs of the different *H. lixii* lineages are morphologically difficult to distinguish from each other, from *T. harzianum* s. stricto, but also from other species of the Harzianum clade such as *T. aggressivum*. Due to these difficulties in recognising phenotypic differences no formal taxonomic implications have been published yet.

Colony

7.0-8.5 cm growth in 4 days on PDA medium. White and raised compact pustules becoming yellowish green after 48hr coalescing and forming large cushions. Bright yellow colour pigmentation of agar at the reverse of the petriplate.

Conidiophores

Conidiophores arising mainly singly from main axis with the branches in 2-3 whorls at less than 90° to the axis with long 'internodes'.

Phialides

Phialides flask-shaped, enlarged in the middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base. Cylindrical, ampulliform and laginiform phialides are also seen. Solitary phialides are common.

Conidia

Often ovoidal and smooth 4.0-6.0 x 2.5-3.0 µm, green.

A B C D

E F G H

Trichoderma aggressivum (A) Growth on PDA (B) Yellow green cushioned pustules (C) Reverse of the plate (D) Conidiophore branching (E,F,G) Phialide disposition (H) Spores

***T. asperellum* (Section Trichoderma)**

The filamentous fungus *T. asperellum* is the genetically distinct agamospecies with cosmopolitan distribution. *T. asperellum* is frequently isolated from root-free soil, soil litter, rhizosphere of various plants, healthy plant tissues, fungal biomass and dead wood.

Colony

Forming up to 5 concentric rings of dense conidial production, with conidia toward the center dark green and conidia toward the margin just beginning to form, aerial mycelium lacking. Pustules formed in abundance throughout the colony. No yellow pigment diffusing through the agar.

Conidiophores

Conidiophores have a symmetric aspect terminating in two or more phialdies, and primary branches arising below the tip frequently paired and projecting at nearly 90 degrees to the main axis. Primary branches progressively longer as the distance from the tip increases, members of a pair tending to have the same length, producing secondary branches that do not rebranch.

Phialides

Typically produced at the tips of primary, secondary and tertiary branches, typically in whorls of 2-4 phialides, straight, ampulliform, only slightly enlarged in the middle,

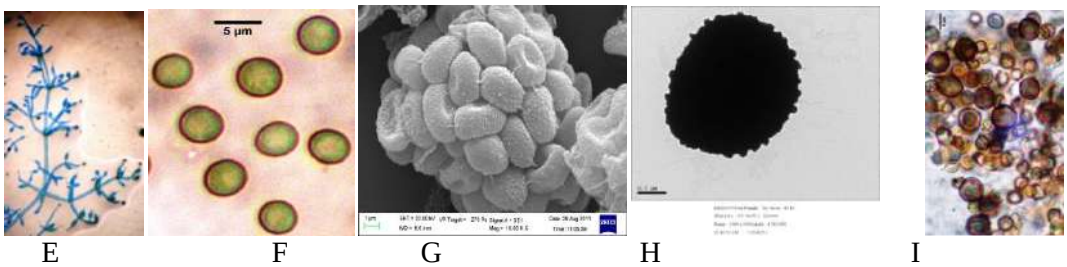
Conidia

Globose to subglobose or ovoidal, finely spinulose (ornamentation could be seen in SEM and TEM only), dark green, 3.5-6.0 x3.0-5.0 µm

Chlamydospores

Abundant, terminal or infrequently intercalary, on immersed hyphae, subglobose to ovoidal, smooth, pale green, 5.0-15.0 µm diam.

A B C D



E F G H I

T. asperellum(A) Growth on PDA, (B) pustules, (C) Reverse of the plate, (D) Conidiophore branching, (E) Phialide disposition, (F) Spores, (G) Spores under SEM, (H) Spore under TEM (Chlamydospores)

***Trichoderma atroviride* (Section – *Trichoderma*)**

Trichoderma atroviride has been confused in the literature with the superficially similar species, *T. harzianum* Rifai, which also has smooth, globose to subglobose conidia. A common soil fungus.

Colony

7.0-8.5 cm growth was observed in 4 days on PDA medium. Small spreading yellow pustules turning to green on age. Pustules concentrating on the edge of petriplate. Dense sporulation at the centre of the plate. Reverse of the petriplate is drab coloured.

Conidiophores

Fertile and paired branches are common, branches are typically arising from 90^0 or less with respect to the branch above the point of branching.

Phialides

Phialides flask-shaped, enlarged in the middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base. Cylindrical, ampulliform and laginiform phialides are also seen. Solitary phialides are common.

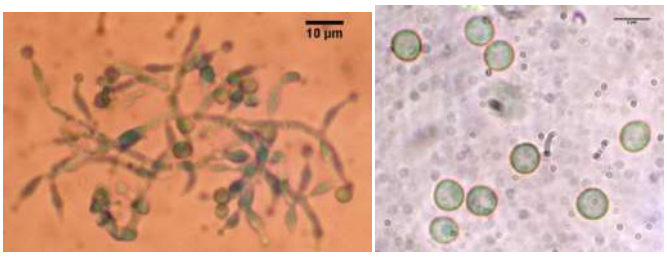
Conidia

Globose to ovoidal and smooth 3.00-3.50X3.80-4.00 µm, green.

Chlamydospores

Globose and smooth.

A B C D



E F

Trichoderma atroviride (A) Growth on PDA (B) Pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores

***Trichoderma brevicompactum (*Section – Pachybasium)**

Trichoderma brevicompactum is characterized by a pachybasium-type morphology, morphologically resembling other small-spored species referable to *Trichoderma* section *Pachybasium* but with essentially subglobose conidia.

Colony

7-7.5 cm growth was observed in four days. White mycelium growing mostly at the edge of the petriplate with white small pustules. Reverse of the plate was colourless. Pustules scattered around the periphery of the Petri dish, yellow-green or gray-green, uniformly cottony.

Conidiophores

Conidiophores are thick, branching along the entire length, often at base of conidiophores or at interior of pustules. Branches loosely paired at a node when arising toward the tip of the conidiophores.

Phialides

Phialides arising in verticals of 3-6 from the tip of long conidiophores and are ampulliform, globose to sub globose. The broad and short phialides and branches giving a compact, compressed appearance to the conidiogenous structures.

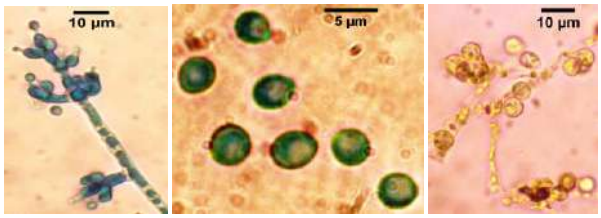
Conidia

Conidiasubglobose or short ellipsoidal, mostly 3.00-5.00X 2.00-3.50 µm, smooth-walled, appearing pale grey-green.

Chlamydospores

Subhyaline, intercalary or terminal, solitary, globose and pyriform,5.00-7.00 µm.

A B C D



E F G

Trichoderma brevicompactum(A) Growth on PDA (B) Pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores (G) Chlamydospores

Trichoderma citrinoviride (Section – Longibrahiatum)

Trichoderma citrinoviride (teleomorph *Hypocrea schweinitzii*, Ascomycota, Dikarya) is a very frequent soil fungus from the Longibrachiatum clade of the genus *Trichoderma*.

Colony

6-9cm cm growth was observed in 4 days on PDA medium. White small pustules forming in radial manner and turning to dark green with age. Sporulation mostly concentrated 2cm away from the edge and forming like a ring. Bright yellow colour pigmentation of agar was observed at the reverse of the petriplate.

Conidiophores

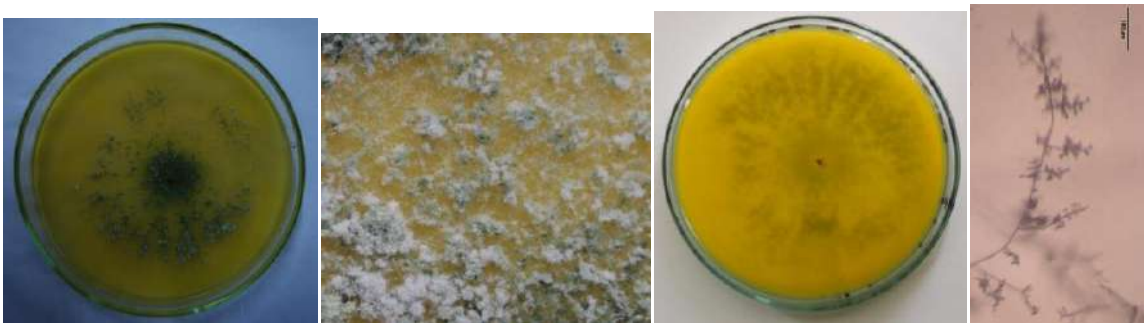
Conidiophores fertile with less secondary branches. Mostly the phialides arising singly on the main axis.

Phialides

Langeniform to ampuliform, long and slender, horn shaped, their base is little narrower than the middle part which projects into conical or sub-cylindrical neck.

Conidia

Cylindrical to ellipsoidal, Smooth, 4.00-5.25X2.45-2.75 µm, green.



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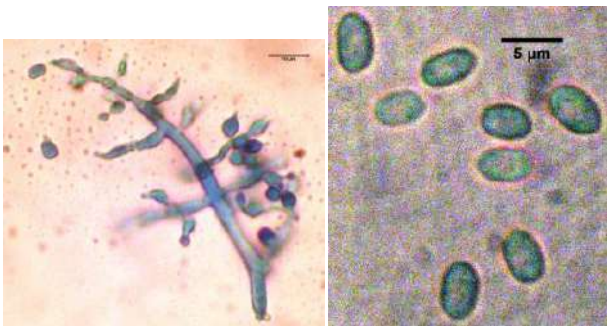


E F

Trichoderma citrinoviride (A) Growth on PDA (B) Pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores

***Trichoderma harzianum* (Section - Pachybasium)**

The filamentous fungus *Trichoderma harzianum sensu stricto* is the genetically distinct temperate agamospecies belonging to the group of closely related (cryptic), albeit diverse, species of the *Harzianum* clade of *Trichoderma* (teleomorph*Hypocrea*, Ascomycota, Dikarya). In the broad taxonomic sense these fungi (*T. harzianum sensu lato*) are the most frequent *Trichoderma* species cultivated from soil worldwide.

Colony

7.0-8.5 cm growth was observed in 4 days on PDA medium Two types of strains were observed in the study. 1.Brown colour pigment producers and pigment non-producers. Pustules are loosely aggregated into flat, spreading pustules.Conidiation effuse,appearing powdery due to dense conidiation, rapidly turning yellowish-green to dark green. Colourless to dark brown at the reverse of the petriplate.

Conidiophores

The conidiophores branching and phialides in the non-pigment producers was resembled *T. asperellum* in some points. They are irregularly branched and narrower and bear laginiform or subulate phialides. The pigment producers are less extensively and verticillate branching.

Phialides

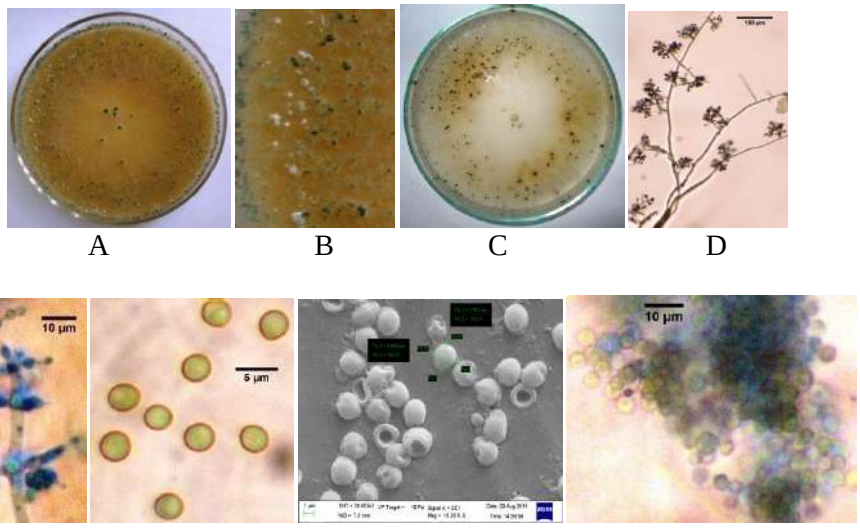
Phialides are ampulliform in pigment producers and laginiform to subulate in non-producers. Usually 3-4, verticillate

Conidia

Sub globose to obovoid, smooth, 2.5-3.0 X 2.0-2.5 µm, pale green.

Chlamydospores

Chlamydospores terminal and intercalary, globose, 4.0-8.0 µm diam.



A B C D

E F G H

Trichoderma harzianum (A)Growth on PDA (B) pustules (C) Reverse of the plate;(D) Conidiophore branching (E) Phialide disposition (F) Spores (G) Spores under SEM, (H) Chlamydospores

Trichoderma hamatum *(*Section: Pachybasium)

Trichoderma hamatum has been identified as effective biocontrol agent and consistently induce suppression of diseases caused by a broad spectrum of soil borne plant pathogens. Bissett (1991b) distinguished *T. hamatum* from the morphologically similar *T. strigosum* and *T. pubescens* by nonfertile conidiophore elongations, which are stout, coiled and undulate or hamate.

Colony

7.0-8.5 cm growth was observed in 4 days on PDA medium. Mycelium is mostly submerged, less aerial mycelium which is floccose and white. Compact cushioned pustules looking velvety due to the

presence of numerous, flexuous sterile conidiophore apices. These pustules are white at first, becoming yellow, then pea green followed by olive green. Colourless at the reverse of the petriplate.

Conidiophores

Conidiophores in pustules are broad, comprising a regular, undulate and humate sterile elongation with the phialides arising near the base. The lateral branches typically comprise one or a few, broad cells with phialides arising at the tip and along the length. The internodes between branches are short and the phialides densely clustered.

Phialides

Phialides are short, broadly ellipsoidal to ovoidal, pyriform and ampuliform, formed on small branches, smooth walled, hyaline or pale green colour.

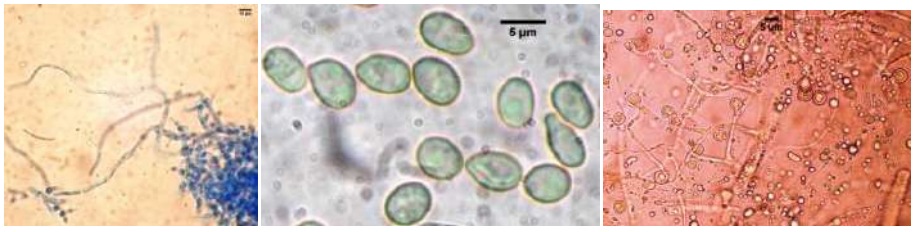
Conidia

Oblong to ellipsoidal and smooth, 5.0-6.0X3.0-4.0 µm, green.

Chlamydospores

Chlamydospores terminal and intercalary, globose, 4.0-8.0 µm diam.

A B C D



E F G

Trichoderma hamatum(A) Growth on PDA (B) pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores (G) Chlamydospores

***Trichoderma koningiopsis (*Section – Trichoderma)**

Trichoderma koningiopsis is a common and cosmopolitan species, but it is more common at tropical than at temperate latitudes. *Trichoderma koningiopsis* is the most commonly encountered species having a *T. koningii*-like morphology (Samuels *et al.* 2006).

Colony

6.0-7.5 cm growth was observed in 4 days on PDA medium. Compact to cottony 1-2 mm diam bluish green pustules are formed with conidial production restricted to the margin of the colony. The pustules are formed along the edge of the plate in circle. Reverse of the plate is colourless.

Conidiophores

Fertile branches arising along the length of the main axis, with longer or shorter internodes; terminal part of conidiophore sparingly branched and with long internodes between branches; branches sometimes formed pustules with short, pachybasium-like crowded phialides. Branches arising at an angle of slightly less than 90° with respect to the main axis, longer branches near the base and short branches or solitary phialides arising near the tip.

Phialides

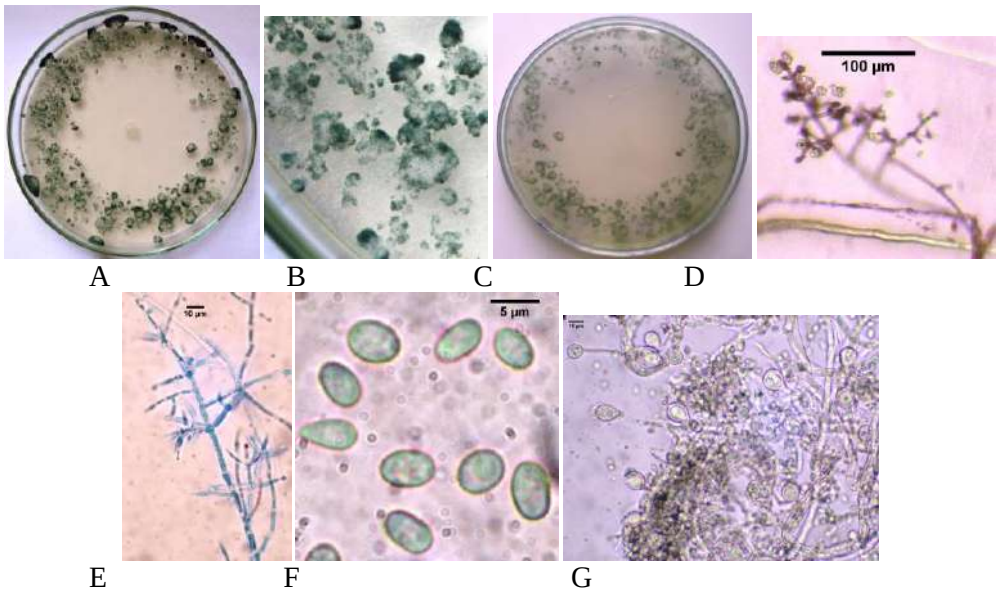
Phialides straight, sometimes hooked or sinuous, lageniform or sometimes conspicuously swollen in the middle, in whorls of 2-5, sometimes several phialides arising from the same point.

Conidia

Conidia dark green, ellipsoidal and smooth, 4.50-6.50X3.00-4.00 µm.

Chlamydospores

Chlamydospores abundant, terminal and intercalary, globose to subglobose and pear shaped, 10.00-15.00 µm in diameter.



A B C D

E F G

Trichoderma koningiopsis (A) Growth on PDA (B) pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores (G) Chlamydospores

***Trichoderma longibrachiatum* (Section – Longibrachiatum)**

The cosmopolitan filamentous fungus *Trichoderma longibrachiatum* is the genetically distinct agamospecies belonging to the Longibrachiatum clade of *Trichoderma* (teleomorph Hypocrea, Ascomycota, Dikarya). *T. longibrachiatum* is usually a common component of *Trichoderma* communities isolated from soil and other environments such as mushrooms and food rotting fungi, marine and soil animals and dead wood.

Colony

7.0-8.5 cm growth was observed in 4 days on PDA medium. Limited aerial mycelium, floccose. Tufts are loose and dark green, sometimes mottled with white flecks. Reverse yellowish green and sometimes colourless

Conidiophores

Conidiophores typically consisting of a strongly developed central axis, sparingly branched, primary branches long, secondary branches usually short and rarely re-branched from which arise, toward the tip, solitary phialides.

Phialides

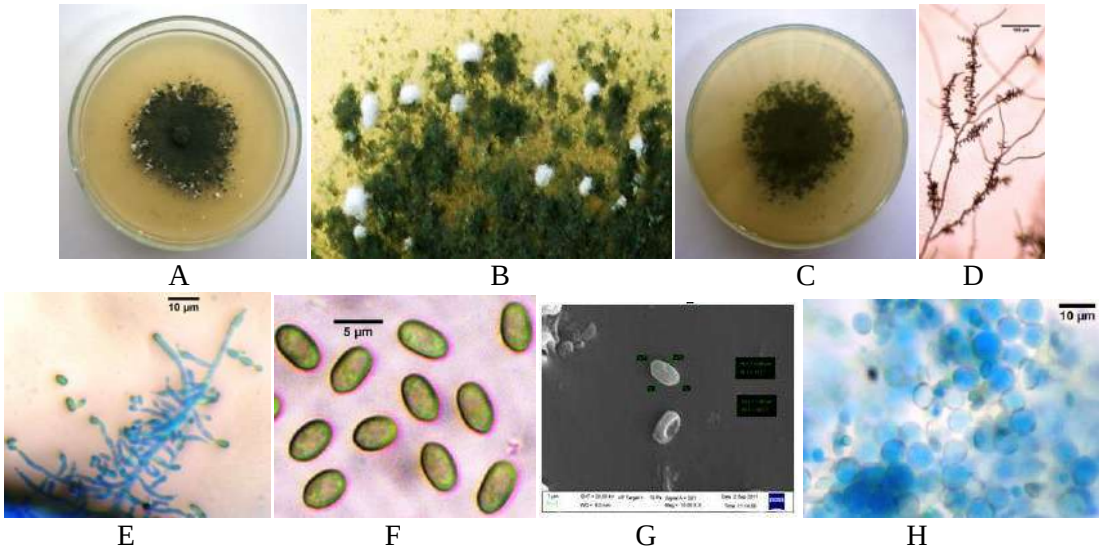
Phialides arising directly, mostly solitary, occasionally in verticils of 2-3. Phialides are ampuliform to laginiform or cylindrical but, when in whorls, enlarged in the middle or squat, straight or hooked to sinuous,. Intercalary phialides common and conspicuous.

Conidia

Oblong to ellipsoidal and smooth, 3.5-8.0 X 3.0-5.0 µm, green.

Chlamydospores

Sometimes present.Terminal or intercalary, subglobose to globose 8-10µm diam.



A B C D

E F G H

Trichoderma longibrachiatum (A) Growth on PDA, (B) pustules, (C) Reverse of the plate, (D) Conidiophore branching, (E) Phialide disposition, (F) Spores, (G) Spores under SEM, (H) Chlamydospores

***Trichoderma reesei* (Section –** Longibrahiatum)

Trichoderma reesei is an industrially important cellulolytic filamentous fungus. In light of *T. reesei's* capacity to secrete large amounts of cellulases and hemi cellulases, *T. reesei* as a host to produce low cost enzymes for the conversion of plant biomass materials into industrially useful bioproducts such as sugars and bioethanol (Jaklitsch, 2009).

Colony

7-8.5 cm in four days forming white pustules at first turning to green then greyish green on age. The small pustules at first concentrating at the centre then equally distributed throughout the plate. Reverse of the petriplate is bright yellowcoloured.

Conidiophores

Comprising a well-defined main axis rarely re-branched. Phialidese arise singly toward the tip of the conidiophore; each branch terminating in one or two phialides and phialides arising singly from

intercalary cells of the branch. Branches typically arising at 90° or less with respect to the branch above the point of branching. Paired branching systems rare.

Phialides

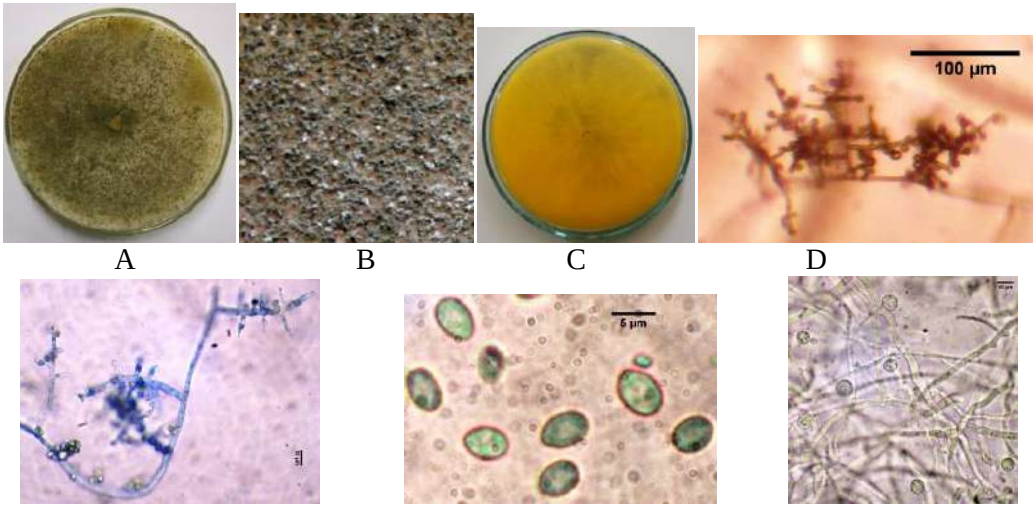
Phialides typically solitary, straight or sinuous or hooked; some phialides are cylindrical and ampulliform and sometimes flask-shaped, constricted to the tip and slightly at the base. Intercalary phialides are common.

Conidia

Conidia green, oblong to ellipsoidal, smooth, 4.50-6.00X3.50-4.00 µm.

Chlamydospores

Abundant within 7 days, globose to subglobose, terminal or intercalary 8.00-10.00 µm diam.



A B C D

E F G

Trichoderma reesei (A) Growth on PDA (B) pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores (G) Chlamydospores

Trichoderma saturnisporum (*Section – *Longibrachiatum)

Section *Saturnisporum* has been amalgamated with section *Longibrachiatum*. The species in section *Saturnisporum* morphologically resemble some isolates in section *Trichoderma*, which have tuberculate conidia and flexuous conidiophores. But the isolates in section *Saturnisporum* produced a conspicuous yellow-green reverse pigment in culture that does not occur in section *Trichoderma*.

Colony

7.0-8.5 cm growth was observed in 4 days on PDA medium. White fluffy pustules turing to grey-green colour with the age. The pustules uniformly spreaded throughout the plate. Reverse of the plate is yellow-green.

Conidiophores

Conidiophores arising from the aerial mycelium of developed pustules, asymmetrically branched, the branches producing phialides directly or rebranching, the secondary branches producing phialides along the length and ending in a single phialide; sometimes the main axis of a conidiophore terminating in a sterile, septate, hypha-like elongation.

Phialides

Phialides mainly arising singly, less frequently in appressed to divergent whorls of 2-3, typically curved, ampulliform to broadly lageniform, sometimes hooked or sinuous.

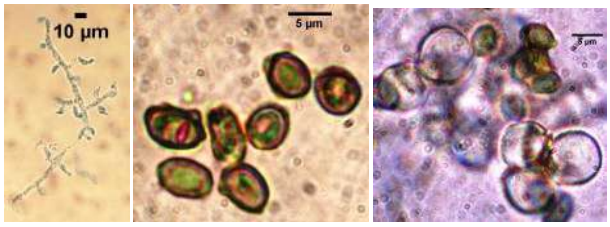
Conidia

Conidia green, smooth walled but with conspicuous sinuate, bullate or wing like inflations of the outer wall, ellipsoidal, 4.5-7.5X3.5-4.5 µm.

Chlamydospores

Chlamydospores present, globose, 6.0-12.0 µm dia.

A B C D



E F G

Trichoderma saturnisporum (A) Growth on PDA (B) Pustules (C) Reverse of the plate (D) Conidiophore branching (E) Phialide disposition (F) Spores(G) Chlamydospores along with the spores

Trichoderma virens **(Section** Pachybasium**)**

Colony

6.0-7.0 cm growth was observed in 4 days on PDA medium.conidiation effuse covering the entire surface of the plate, or forming spreading, light yellowish green flat pustules concentrated near the margin, later turning dark green. Reverse colourless and some times drab coloured.

Conidiophores

Conidiophores arising in clusters in lateral branches from undifferentiated aerial mycelium, at the base sterile and unbranched, but the upper part fertile toward the apex, each branch terminating in a penicillus of (2-)3-6 closely appressed phialides. The entire branching system irregular and uncrowded.

Phialides

Phialides mainly arising in closely appressed whorls of 2-5 on terminal branches, less frequently in pairs or singly, straight, lageniform to ampulliform and sometimes laginiform to subulate, base constricted, swollen in the middle, attenuate at the tip.

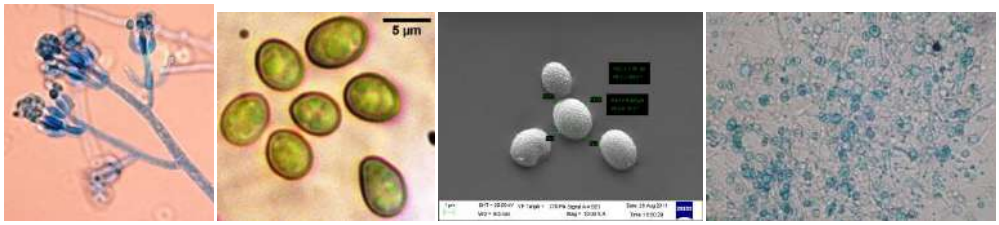
Conidia

Broadly ellipsoidal to obvoidal and minutely warted at high magnifications(SEM), 3.5-8.0 X 3.0-5.0 µm, dark green. Conidia from adjacent phialides often coalescing into large gloeoid masses.

Chlamydospores

Chlamydospores abundant, terminal and intercalary, globose to subglobose, 6.0-12 µm.

A B C D



E F G H

Trichoderma virens (A) Growth on PDA, (B) pustules, (C) Reverse of the plate, (D) Conidiophore branching, (E) Phialide disposition, (F) Spores, (G) Spores under SEM, (H) Chlamydospores

Diversity in *Aspergillus*

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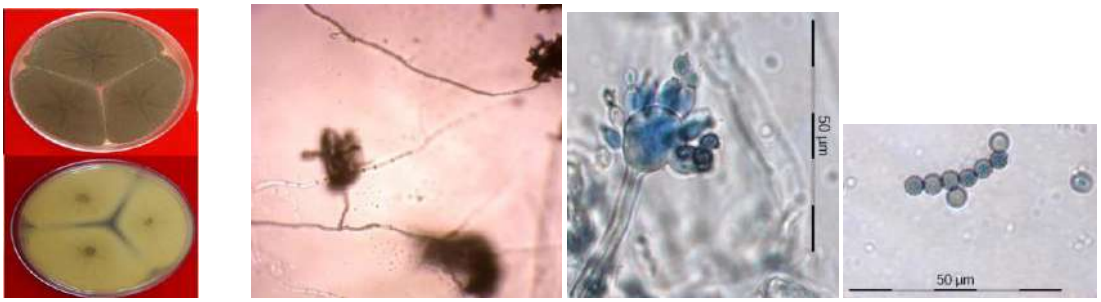
1.Aspergillus aculeatus

Colony: Fast growing, purple brown to purple black and Uncoloured at the back of the plate.
Conidiophores: Globose, 500-1000um
Conidial Head: 1000-2000um long
Vesicle: Elongate to globose, 35-100um entirely fertile
Sterigmata: Uniseriate, 6.5-10x 3-4.5um
Conidia: Elliptical to globose, 3.5-4.0x4.5-7.0um, echinulate
Sclerotia: May be present



1 2 3 4

Aspergillus aculeatus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

2.A. amstelodami

Colony: Slow growing, plain or wrinkled, yellow to dull yellow gray, bright yellow and Yellow at the back of the plate
Conidiophores: 275-350um long
Conidial Head: Radiate to columnar, 120-150um
Vesicle: Sub globose 18-25um
Sterigmata: Uniseriate, 5-6.5umx 2.5-3.5um
Conidia: Finely spinulose, subglobose to elliptical with end flattened, 3.5-5.2x3.5-4um
Sclerotia: Clistothecia abundant and cluster in mass, bright yellow, globose to subglobose 115-140um, ascospore lenticular 4-7.5x3.6-3.8, prominent V-shape equatorial furrow with to irregular ridge, rough

1 2 3 4 5 6

A. amstelodami1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3,4) Vesicle & Sterigmata, 5) Conidia 6)Ascospore

3. *A. clavatus*

Colony: Fast growing, floccose, white and blue green and colourlessat the back of the plate.
Conidiophores: Abundant, erect 1.5-3um long
Conidial Head: Clavate, large 300-400x150-200um
Vesicle: Clavate, fertile over an area 200-250x40-60um
Sterigmata:: Uniseriate 2.5-3.5um x2-3um(at base and 7-10x2.5-3 at apex), phailides with short neck
Conidia: Elliptical, smooth, 3-4.5x2.5-4.5um

1 2 3 4 5

A. clavatus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3,4) Vesicle & Sterigmata,5) Conidia

4.*A. fischeri*

Colony: Medium growing, pale blue green to olive gray,Colourless at the back of the plate.
Conidiophores:300-500um long
Conidial Head: Columnar, 120-135um
Vesicle: Flask shape, 12-18um, sterigmata one-half to three-fourths
Sterigmata: Uniseriate, 5.5-7x2-2.5um
Conidia: Subglobose to elliptical, 2-2.5um
Sclerotia: Clistothecia abundant, globose, 400um, ascospore biconvex, 7x4um, with too ruffled *equatorial bands*

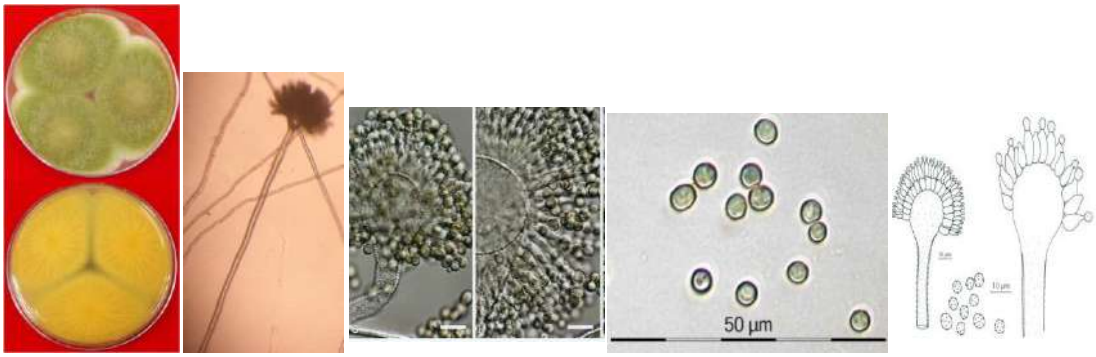
1 2 3 4 5

A. fischeri1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia, 5) Sclerotia

5. *A.flavus*

Colony character: Colonies are Fast growing,Floccose, jade green intense yellow or yellow green in colour.Creamish dull yellow to pinkish drab at the reverse of the petriplate.
Conidiophores: Conidiophores are Long, coarsely roughened
Conidial Head: Pale to intense yellow or yellow green, Radiate are very loosely columnar,300-500um (-1000 um-2000)

Vesicle: Vesicles are Globose, 10-65um, Elongate when young, later stage
Sterigmata: Sterigmata are single or mostly double, Phailides are cylindrical tapering to distinct neck, 6.5-10 um x 3-5um in size
Conidia: Conidia are Echinulate, Globose to sub globoseFinely rough walled, 3.0-6.0um
Sclerotia: Sclerotia ranges 400-700um, black covered by exudate droplets



1 2 3 4

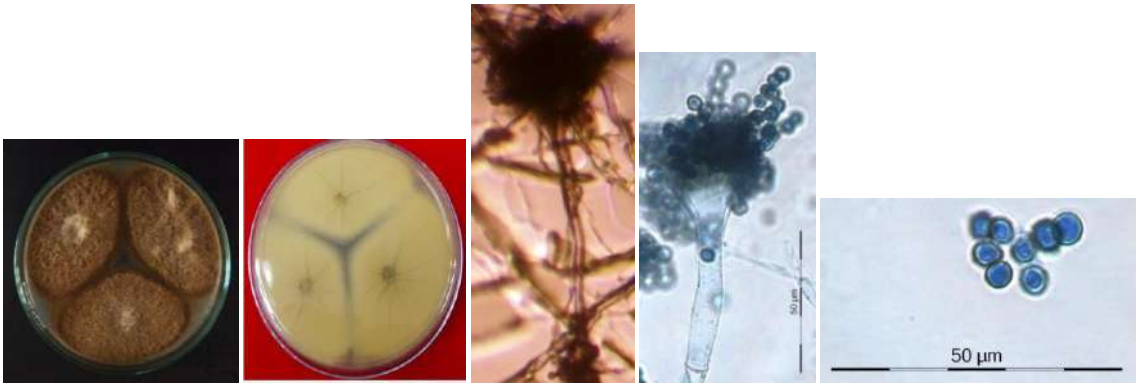
A.flavus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head,3) Vesicle & Sterigmata,4) Conidia

6. *A. fumigatus*
Colony: Velvety to floccose, lily green to castor gray andyellow to red brown at the back of the plate
Conidiophores: Sharpe, 300um long, arising from very short branches
Conidial Head: Columnar, 400-450um, Fertile upper part
Vesicle: Flask shaped, 20-30um
Sterigmata: Uniseriate, 5-10x2-3um
Conidia: Echinulate, globose to subglobose, 2-3.5um

1 2 3 4 5

Fig1:1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3,4) Vesicle & Sterigmata, 5) Conidia

7.*A.funiculosus*
Colony: Slow growing, thick mate, funiculose, olive buff and brownish purple at the back of theplate
Conidiophores: 400-600um long
ConidialHead: Heads projecting above the mycelial felt, large, radiate 100- 300um
Vesicle: Globose, 8-35um, sterigma entire
Sterigmata: Uniseriate 5-7x2-2.5um
Conidia: Elliptical, smooth, 3-3.5x2-2.5um, globose or subglobose



1 2 3 4

A.funiculosus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata,4) Conidia

8. *A. japonicus*

Colony: Fast growing, purple brown to purple black and Colourless to yellow green tinge at the back of the plate

Conidiophores: 500-1000 long,

Conidial Head: Small, radiate to columnar 600-700um long

Vesicle: Elongate to globose, 15-50um

Sterigmata: Uniseriate, 5.5-8x3-4.5um

Conidia: Globose to subgloboseechinulate

Sclerotia: Sclerotia abundant, white to cream, globose upto 500um in diameter

1 2 3 4

A. japonicus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

9. *A. neveus*

Colony: Slow growing, plane or radially furrowed dense mycelium, dull ivory
Brown to greenish black at the back of the plate

Conidiophores: 600-1000um occasionally branched

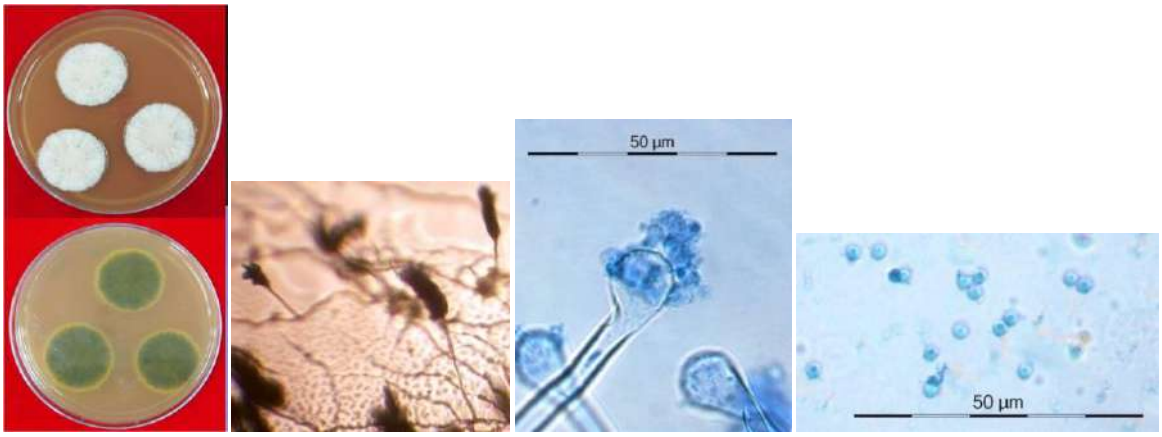
Conidial Head: Loose coloums 200-300x 20-60um

Vesicle: Hemispherical, 300-1000um 8-15um

Sterigmata: Biseriate, Primary 5-8x2.5-3.0um, secondary 5-7x2-2.5

Conidia: Globose, 2-2.5um, smooth

Sclerotia: Exudate abundant



1 2 3 4

A. neveus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

10. *A. nidulans*

Colony: Fast growing, dark cress green, Purplish red at the back of the plate

Conidiophores: 75-100um long,

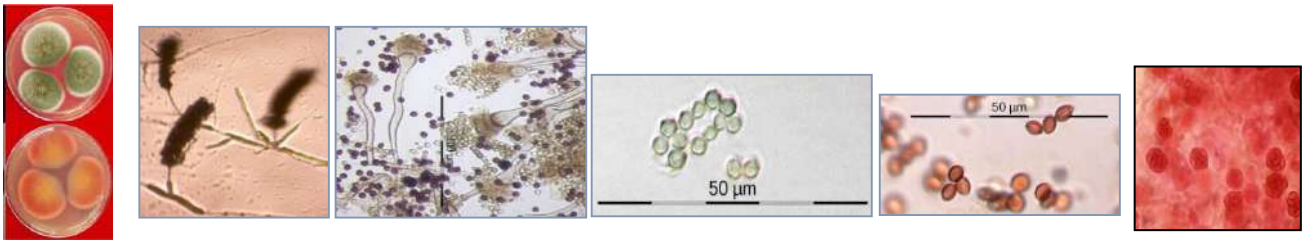
Conidial Head: Short, columnar, 60-70x30-35um

Vesicle: Hemispherical 8-10um

Sterigmata: Biseriate, (primary)5-6x2-3um, (Secondary) 5-6x2-2.5um,

Conidia: Globoserugulose 3-3.5um

Sclerotia: Clistothecia abundant, globose, 125-150um, hulli cell 125um, radish brown, ascospore purple red, lenticular smooth with to equatorial crests



1 2 3 4 5 6

A. nidulans1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia 5)Ascospores 6) Cleistothecia

11. *A. niger*

Colony: Colony is fast growing, purple black brown black or black in colour, Cream dull yellow to yellow at the reverse of the petri plate

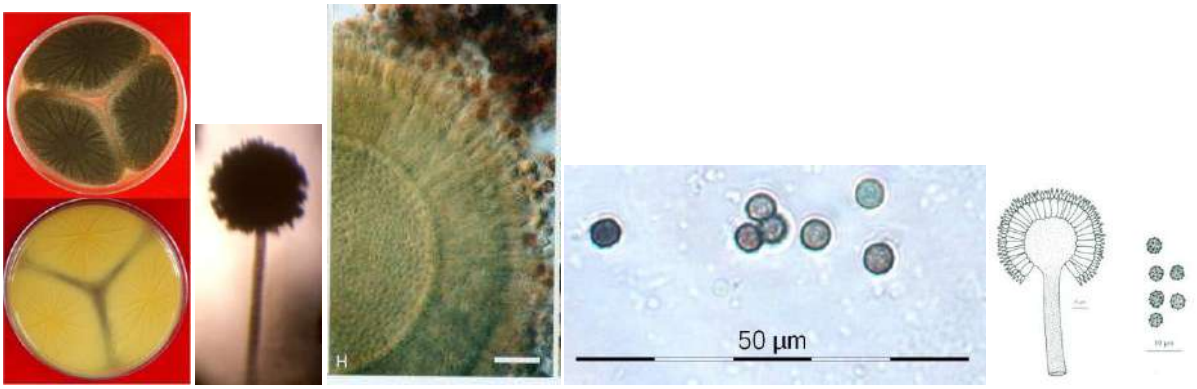
Conidiophores: Conidiophors are Smooth, long and coarse

ConidialHead: Conidial head are large and black Globose to Radiate, 300-1000um in size.

Vesicle: Vesicles are Globose in shape,25-75um in size

Sterigmata: Sterigmata are biseriate, metulae present, phailied cylindrical tapering to distinct neck, 7-10 x3-3.5um in size.

Conidia: Conidia are globose rough wall with black bars,3.5-4.5um



1 2 3 4

A. niger1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

12. *A. ochraceus*

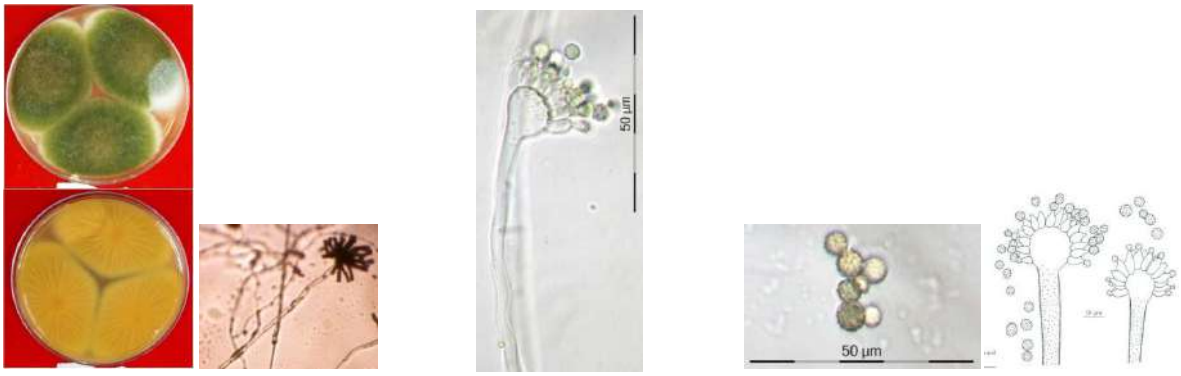
Colony: Medium growing, plain or slightly furrow or zonate, tuff basal mycelial, colorless to dull yellow orange, light ochraceous buff and yellowish to greenish brown at the back of the plate.
Conidiophores: 1-1.5mm long, coarsely roughened
Conidial Head: Globose, conidial chains adhering, 750-800um,
Vesicle: Globose, 35-50um, entire
Sterigmata: Biseriate, (primary) 15-25x5-6um, (secondary) 7-11x2-3.3um
Conidia: Globose to subglobose 2.5-3.5um, smooth to slightly rough
Sclerotia: Present, gloose, ovate, cylindrical 1mm dia.

1 2 3 4

Fig:1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

13. *A. parasiticus*

Colony: Colonies areFloccose, bright yellow to ceadar green to dark dull yellow green.Creamish dull yellow to pinkish drab at the reverse of the plate.
Conidiophores: Conidiophores are long, Smooth or roughened
Conidial Head: Conidial head are Globose to Radiate, 300-500um
Vesicle: Vesicles are globose,20-35um
Sterigmata: Sterigmata are biseriate or biseriate cylindrical tapering to distinct neck, 7-9 x 3-4um
Conidia: Conidia are globose to subglobose, rough walled, 3.5-5.5um in size
Sclerotia: Sclerotia are rare, 400-700um, black covered by exudate droplets.



1 2 3 4

A. parasiticus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata,4) Conidia

14. *A. puniceus*

Colony: Colony are medium growing, vinaceous pink, Yellow to reddish brown at the back of the plate.

Conidiophores: 150-300um in size

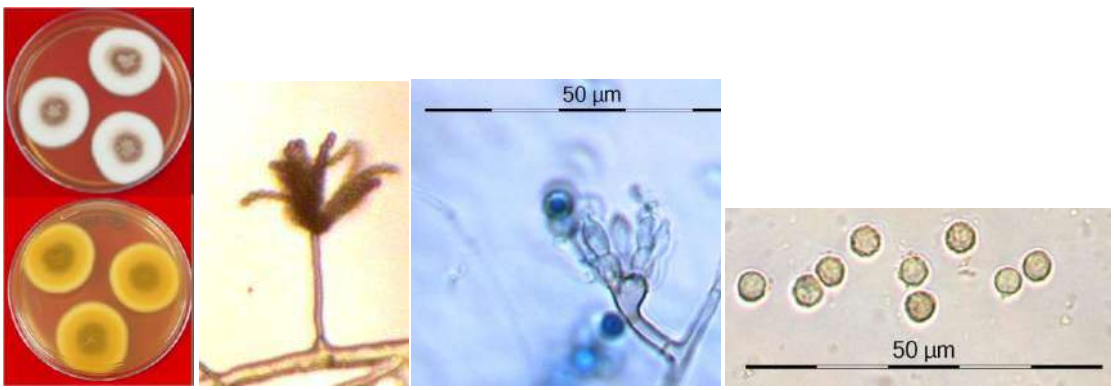
Conidial Head: Radiate to hemispherical, rarely loosely columnar,90-135um

Vesicle: Sub globose(8-16um) to elliptical (15-18umx13-15um

Sterigmata: Biseriate, 4-7um

Conidia: Globose, 2.5-3.3, slightly roughened

Sclerotia: Exudate wine red, abundant hulli cell elongate, crescent shaped or twisted and aggregated



1 2 3 4

A. puniceus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

15. *A. quadrilineatus*

Colony: Coplony are fast growing, Slightly wrinkled, dark purple brown, olive green conidial areas. Purplish red to purplish brown at the back of the petri plate.

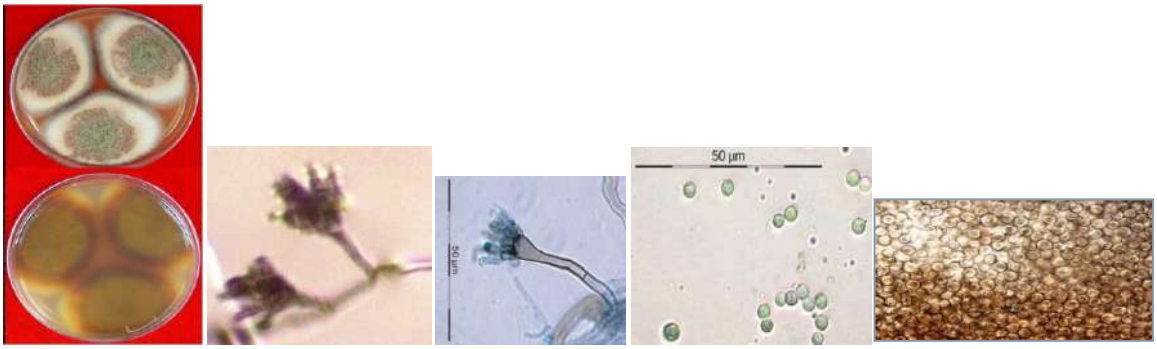
Conidiophores: Conidiophores are Sinuate, smooth walled, dull brown,50-75um long

Conidial Head: Conidial head is Short columnar, 60- 70x30-35um

Sterigmata: Sterigmata isbiseriate, primary 5-6x2-3um, secondary 2-2.5um

Conidia: Conidia are globose,rugulose, yellow green, 3-4um

Sclerotia: Sclerotia are clistothecia with hulle cell abundant, 125-150um,ascospore purple red,lenticular 4-4.8umx 3.4-3.8um



1 2 3 4 5

A. quadrilineatus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia, 5) Hulle cells

16. *A. sulphureus*

Colony: Colonies are Slow growing, cream to yellow, Pale yellow at the reverse of petri plate.

Conidiophores: Conidiophores are Long Smooth to finely rough up 1mm long

Conidial Head: Conidial head is pale pure yellow Loosely Radiate, spore chains adherence, head 500um

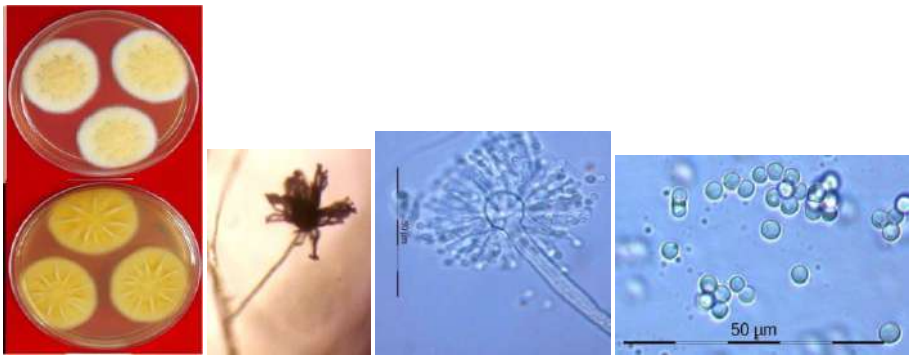
Vesicle:globose to alongate, 12-25um

Sterigmata: Bseriate, (primary) 4.5-8x3.3-4.4um (Secondary) 6.5-8.0x2-2.5um

Conidia: Fusiform toglobose, slightly roughened, 2-2.5um

Sclerotia: Abundant cream to pale yellow, Globoseto subglobose

300-450um, exudate present clear.



1 2 3 4

A. sulphureus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata,4) Conidia

17. *A. sydowi*

Colony: Slow growing colony, velvety intensively blue green in colour, Reddish brown to dark red at the reverse of the plate.

Conidiophores: Hyaline, smooth walled conidiophores

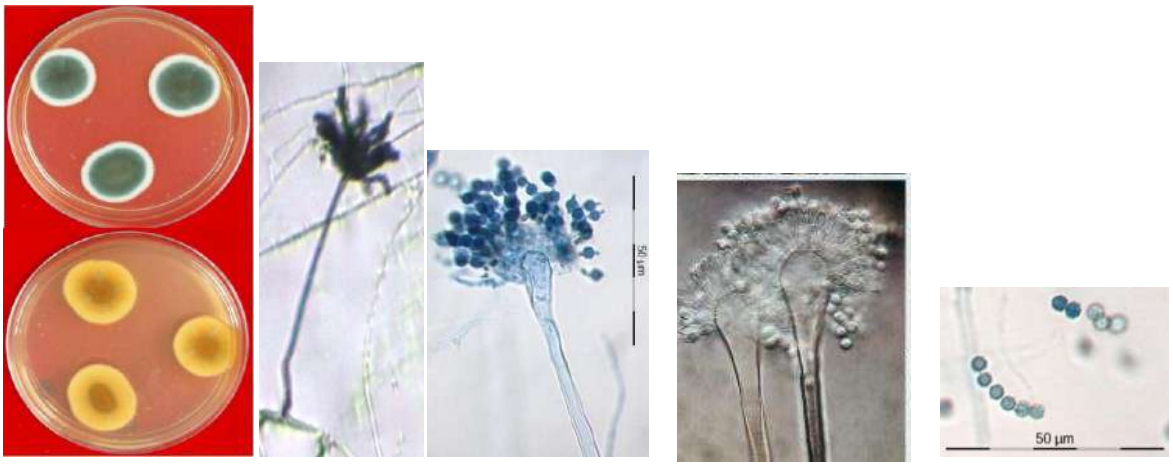
Conidial Head: Conidial head is typical radiate to nearly globose, 100-150um

Vesicle: Spathulate to sub clavate, 10-20um, Globose fertile over almost entire surface.

Sterigmata: Biseriate, phailied cylindrical with short neck, 05-10um x2-3.5um

Conidia: Globose to sub globose, 2.5-4um,Conspicuouslyspinulose

Sclerotia: No sclerotia exudate abundant, reddish brown



1 2 3 4 5

A. sydowi1) Growth on PDA & reverse of the plate, 2) Conidiophores&Conidial head, 3,4) Vesicle & Sterigmata, 5) Conidia

18. *A. terreus*

Colony: Medium growing, velvety, cinnomom buff to woody brown and Dull yellow at the back of the plate

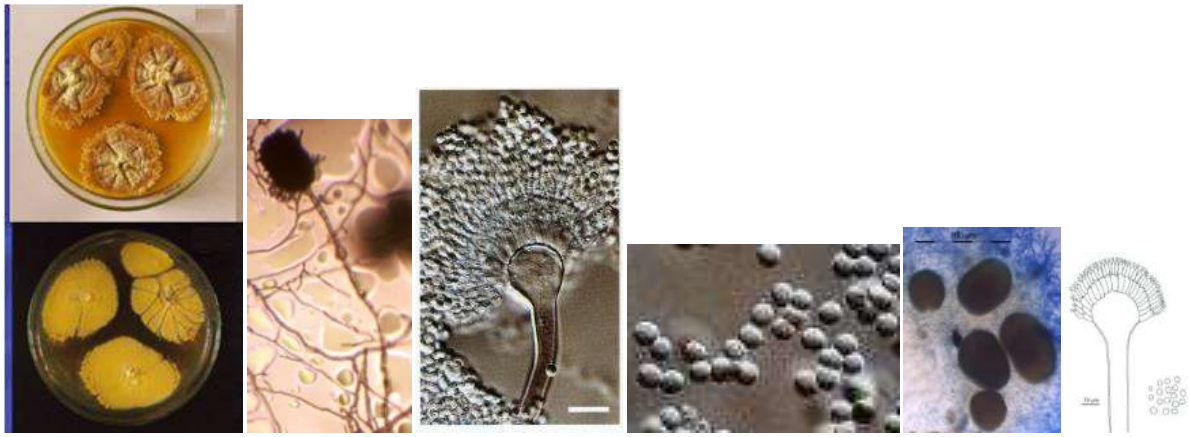
Conidiophores: Short, 100-500x5-8um

ConidialHead: Long, compactly columnar, globose 100-150x30-50um

Vesicle: Hemispherical, globose to subglobose, 10-20um

Sterigmata: Biseriate becoming columnar, (primary) 5-7x2.2.5um (secondary) 5.5-7.5x1.5-2.0um

Conidia: Globose to ellipsoidal, smooth, 1.5-2.5um



1 2 3 4 5

A. terreus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia 5) Sclerotia

19. *A. terricola*

Colony: Fast growing, deeply floccose, to brown old gold

Pale yellow at the back of the petri plate

Conidiophores:0.5-1mm

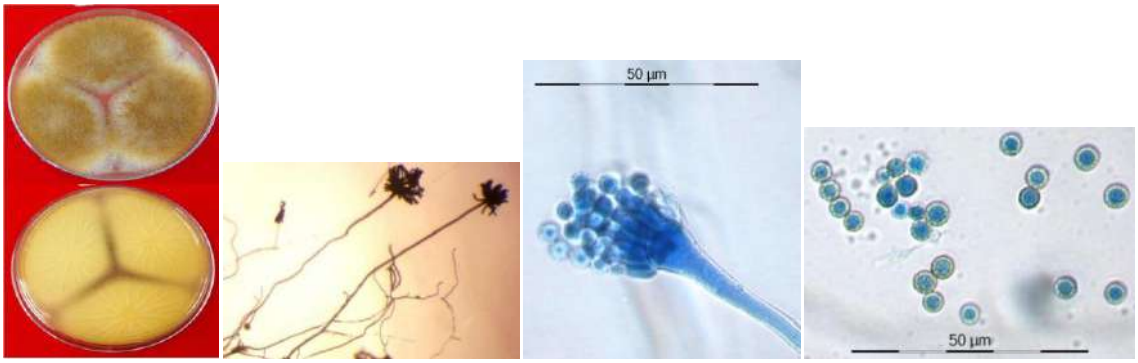
Conidial Head: Loosely radiate, 100-400um

Vesicle: Sub globose hemispherical, 20-45um

Sterigmata: Uni orBiseriate (primary) 5-15x4.5-8.0um (secondary)7-13x4-7um

Conidia: Elliptical to pyriform or cylindrical or sub globose to globose, 4.5-9.0um, echinulate

Sclerotia: No sclerotia

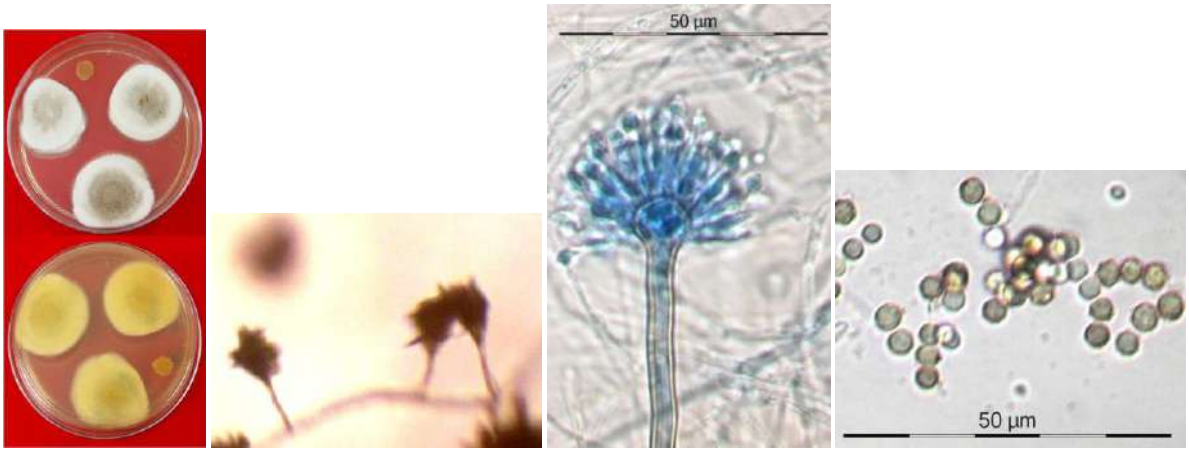


1 2 3 4

A. terricola1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

20. *A.ustus*

Colony: Medium growing, Olive gray to red brown
Conidiophores: 400um, rarely septate
Conidial Head: Radiate to loosely columnar, 100-125um
Vesicle: Upright, hemispherical to sub globose, 7-15um
Sterigmata: Biseriate primary 4-7x3-4um, secondary(5-7x2.5-3.0um
Conidia: Globose, 3.2-4.5um echinulate
Sclerotia: Hulli cell present, ovate to elongate, helicoids are twisted



12 3 4

A. ustus1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

21. *A. versicolor*

Colony: Slow growing, velvety to floccose, orange yellow to green, pea green and Cream yellow to reddish brown at the back of the plate.
Conidiophores: 700 um long
Conidial Head: Hemispherical, radiate100-125um, roughened
Vesicle: Hemi-spherical, 12-16um
Sterigmata: Biseriate Primary 5.5-8x3um secondary 5-7.7x2-2.5um
Conidia: Globose, echinulate 2-3um
Sclerotia: Hulli cell rare

1 2 3 4

A. versicolor1) Growth on PDA & reverse of the plate, 2) Conidiophores & Conidial head, 3) Vesicle & Sterigmata, 4) Conidia

Identification of commonly occurring *Fusarium* species

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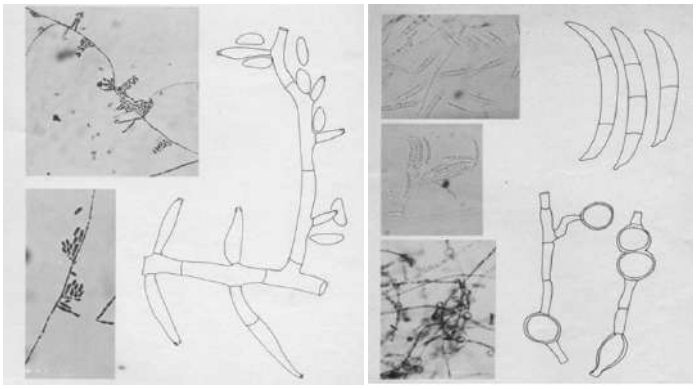
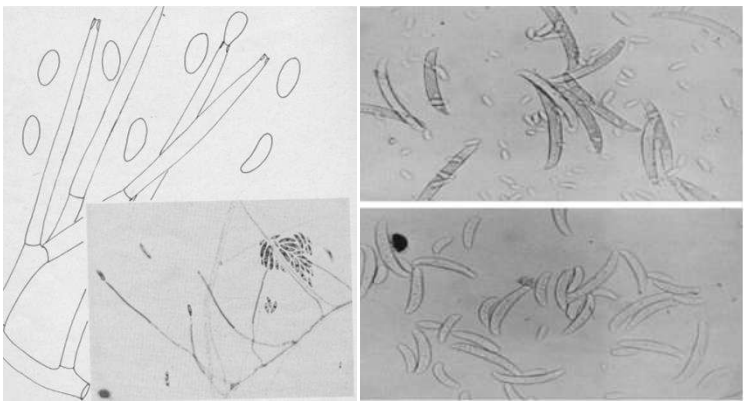
Division of Plant Pathology, ICAR-Indian Agricultural Research Institute,New Delhi- 110012

***Fusarium oxysporum* Schlecht.,**
Growth rate : 4.5 cm.
Culture pigmentation: White,peach, salmon, vinaceous grey to purple, violet
Microconidia: oval-ellipsoidal, cylindrical, straight or curved, 5-12 x 2.2-3.5µ, produced from simple, short, lateral phialides often grouped forming tubercularia like sporodochia.
Macroconidia : Generally 3-5 septate, 27-60 x 3-5µ,thin walled, fusoid.
Chlamydospores: Globose, formed singly or in pairs, intercalary or on short lateral branches
Dignostic characters: The short simple phialides producing the microconidia together with the presence of chlamydospores.



***2.Fusarium solani* (Mart.)Sacc.,**
Growth rate : 3.2cm.
Culture pigmentation: Greyish-white to white,light brown
Microconidia: 8-16 x 2-4 µ, cylindrical to oval and may become 1-septate, produced from long slender, lateral phialides 45-80 x 2.5-3.0 µ,laterally borne or on branched conidiophores.

Macroconidia : Generally 3-5 septate,27-60 x 3-5µ
Chlamydospores: Globose, formed singly or in pairs, intercalary or on short lateral branches
Dignostic characters: The long phialides, the branched and elaborate microconidiophores and the shape of the macroconidia.



***3.Fusarium verticilloides*(Sacc.) Nirenberg**

Growth rate : 4.6cm.

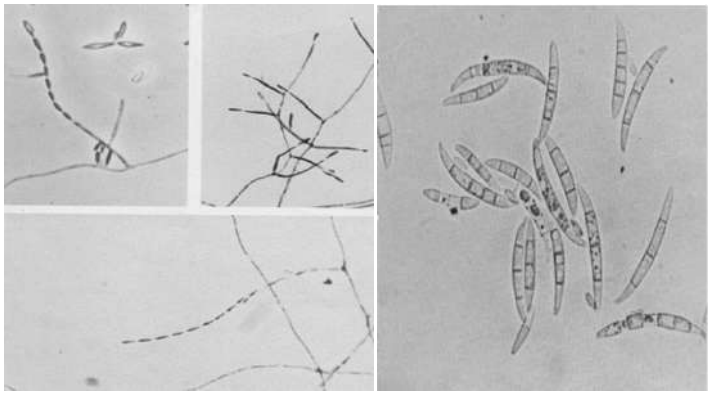
Culture pigmentation: Peach salmon,vinaceous purple to violet

Microconidia: Fusoid to clavate**,** 5-12 x 1.5-2.5 µ,occationally becoming 1-septate and produced in chains from subulate lateral phialides, 20-30 x 2.0-3.0 µ, at the base

Macroconidia : Some strains do not readily form macroconidia but when present they are in equilaterally fusoid, thin walled,3-7 septate,25-60 x 2.5-4.0µ

Chlamydospores: Absent but globose stromatic initial cells may be present in some cultures

Dignostic characters: The presence of the chains of microconidia which can be best observed in situ and the absence of chlamydospores



***4.Fusarium decemcellulare* Brick.**

Growth rate : 3.2 cm.

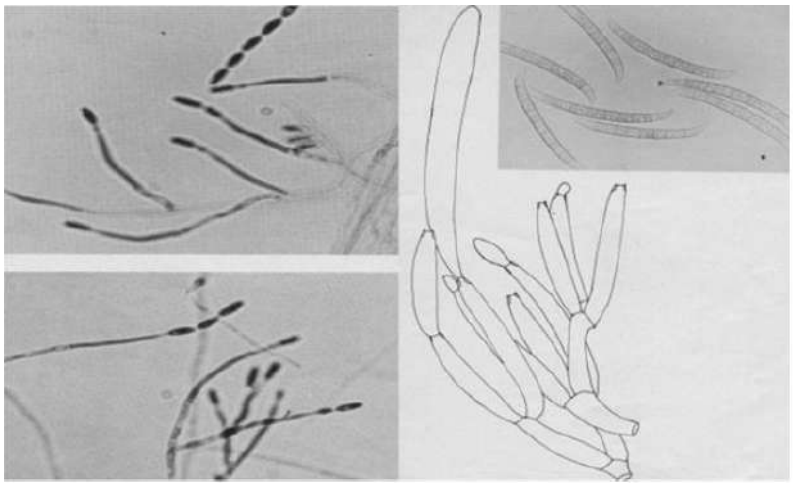
Culture pigmentation: Rose darkening to red, aerial mycelium white but pustules of macroconidia cream to yellow

Microconidia: Formed in chains from well developed phialides. They are oval, aseptate, to 1-septate, 10-15 x 3.0.-5.0 µ

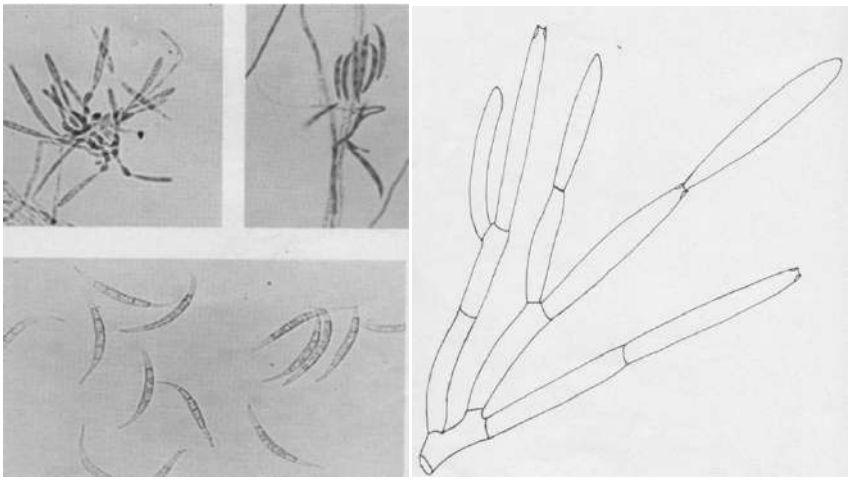
Macroconidia : Formed on sporodochia fromwell developed phialides. They are 7-10 septate, 55-130 x 6-10µ

Chlamydospores: Absent

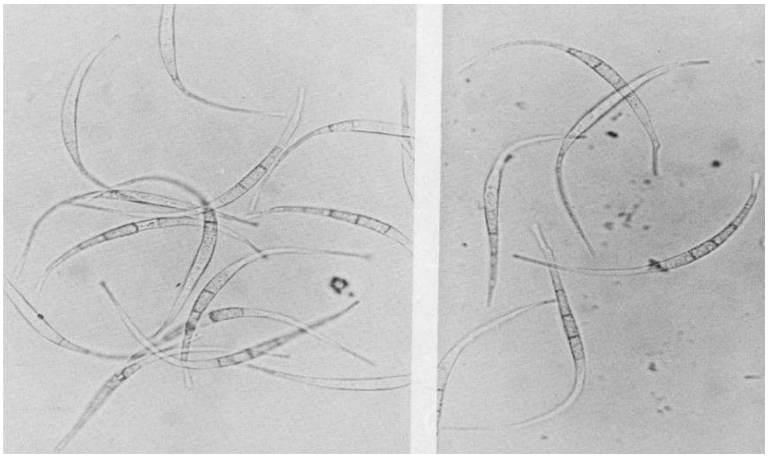
Dignostic characters: The presence of the chains of microconidia, distinct spore shape and size, pigmentation



5.Fusarium equiseti(Corda)Sacc.
Growth rate : 5.9 cm.
Culture pigmentation: Peach usually changing to avellaneous and finally becoming buff brown
Microconidia: Absent
Macroconidia : only are produced and these may be variable in size and are produced from single solitary or grouped phialides; conidia4- 7septate, 22-60 x 3.5-9.0µ
Chlamydospores: Globose, 7-9 µ diam., intercalary, solitary, in chains or clumps.
Dignostic characters: The absence of microconidia and pigmentation



***6.Fusarium acuminatum* Ellis&Everhart.**
Growth rate : 4.5 cm.
Culture pigmentation: Saffron to bay to caramine red
Microconidia: Absent
Macroconidia : only are produced and these may be variable in different isolates,3-7 septate,30-70 x 3.5-5.0µ often with an incurved elongation of the apical cell,and are produced from phialides.
Chlamydospores: Intercalary in knots or in chains.
Dignostic characters: Spore shape and caramine red pigmentation



***7.Fusarium udum* Butler.**

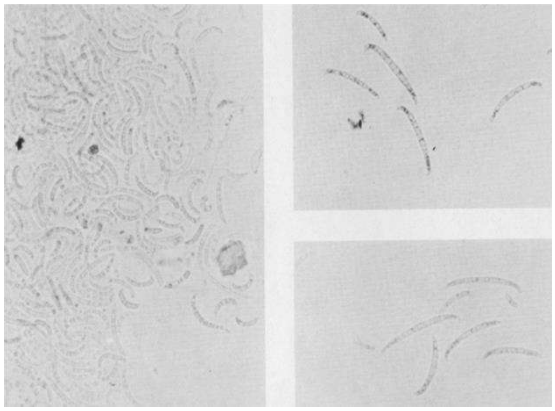
Growth rate : 4.2 cm.

Culture pigmentation: Pale sulphureus to rose buff becoming salmon orange with production of conidia, occational strains have purple pigmentation

Conidia: No clear distinction between microconidia and macroconidia**.** Conidia variable with a strongly curved or hooked apex,6-8 x 3-3.5µ and 30-40 x 3-3.5µ

Chlamydospores: Sparse,oval to globose,8-11 x 8-12µ

Dignostic characters: Extremely variable conidia with strongly curved apex and limited host range on *Cajanus* and *Crotalaria*



***8.Fusarium palledoroseum* Berk & Rav.**

Growth rate : 6.1 cm.

Culture pigmentation: Peach changing to avellaneous and finally becoming buff brown.

Microconidia: Absent

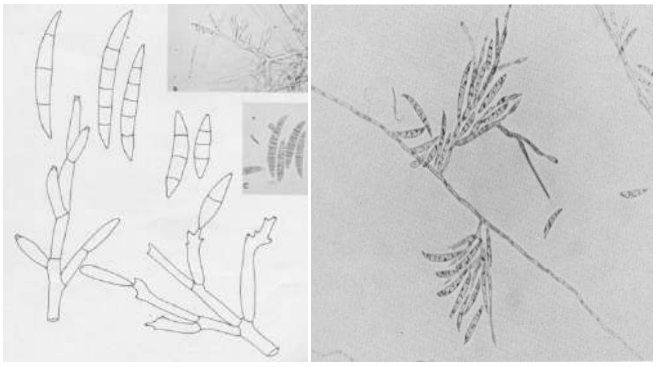
Macroconidia : of two types, primary and secondary

Primary macroconidia: with wedge – shape foot cell, 0-5 septate,7.5-35 x 2.5-4.0µ,formed as blastospores from polyblastic sympodial cells, upto 5 separate spores formed by each cell.

Secondary macroconidia : with typical heeled foot cell, 3-7 septate, 20-46 x 3.0-5.5 µ formed from phialides usually grouped in sporodochia

Chlamydospores: Often sparse, globose, 10-12 µ diam.,becoming brown, intercalary, single or in chains.

Dignostic characters: The presence of primary and secondary macroconidia, pigmentation,spore form, and presence of chlamydospores.



***9.Fusarium graminearum* Schwabe**

Growth rate : 8.9 cm.

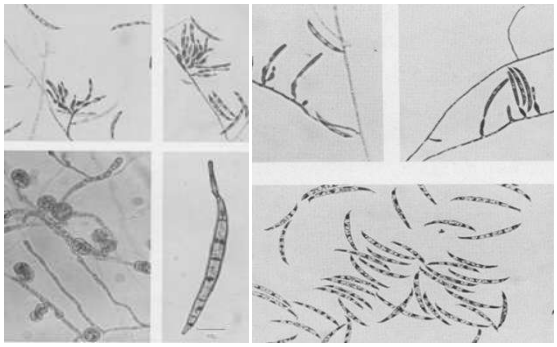
Culture pigmentation: Rose,coral becoming vinaceous with a brown tinge

Microconidia: Absent

Macroconidia : only are produced from simple lateral phialides which may or may not become grouped on branched conidiophores. Macroconidia falcate generally with an elongated epical cell narrowing gradually to a point; 3 septate, 30-50 x 3.5-4.0µ,5-7 septate, 36 x 3.5-5.0 µ

Chlamydospores: Absent or rare; If present intercalary,10-12 µ diam. in knots or in chains.

Dignostic characters: The long falcate macroconidia often formed sparsely in many strains are characteristic. Many isolates of thisspecies with floccose aerial mycelium and rose to coral pigmentation produce neither macroconidia nor chlamydospores until surface of colony is washed clean of mycelium and culture reincubated.



***10.Fusarium dimerum* Penz.**

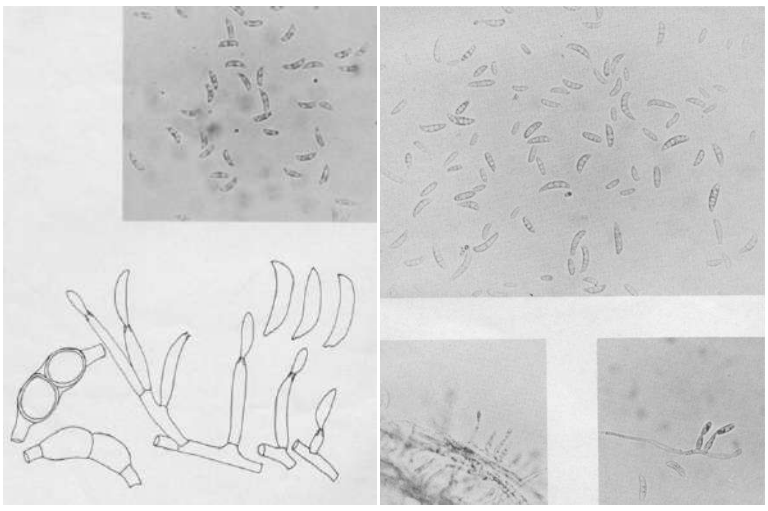
Growth rate : 2.7 cm.

Culture pigmentation: Orange beige to apricot

Conidia: Somewhat heterogenous probably representing primary and secondary conidia as occational phialides develop,0-septate,6.5-10.5 x2.3-2.5µ1-2 septate,10-12 x 3.0-3.5µ

Chlamydospores: Globose,oval to smooth, 8-12µ diam., intercalary, formed singly or in chains

Dignostic characters: Conidial form and presence of chlamydospores separate it from the related species.



Taxonomy of Genus *Penicillium*

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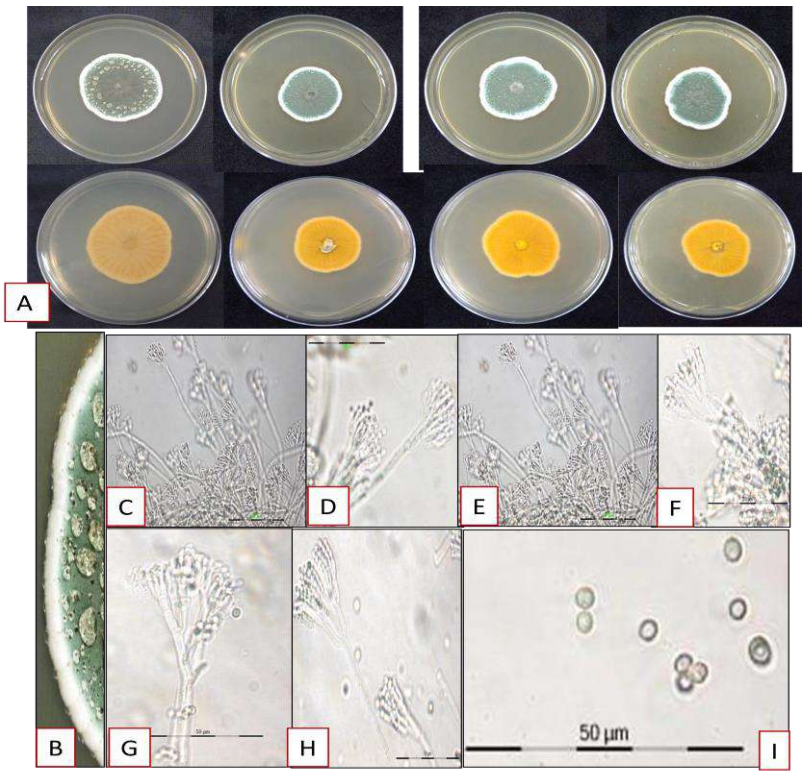
1. *Penicillium aethiopicum* Frisvad

In *Penicillium* subgenus *Penicillium* section *Chrysogena* series *Aethiopica*

Diagnostic features: Griseofulvin, viridicatumtoxin, tryptoquialanins, geosmin, ellipsoidal smooth-walled conidia, markedly sulcate colonies with a golden yellow reverse, growth at 37°C.

Description:

Conidia: Smooth-walled, ellipsoidal, 2.8-3.2 x 3.3-3.8 µm, in long columns
Phialides: 7-9 µm, short collula
Metulae: 12-17 µm
Rami: 15-25 µm
Stipes: 200-350 µm, smooth to rough-walled
Synnemata or fasciculation: Weakly fasciculate
Sclerotia: None
Colony texture: Sulcate on CYA
Conidium colour CYA: Dull green.
Exudate droplets on CYA: Copious, clear
Reverse colour on CYA: Golden yellow
Diffusible colour: Occasional; pale orange
Ehrlich reaction: No reaction
Odour and volatile metabolites: Griseofulvin, Dechlorogriseofulvin,9,12-Octadecadienoic acid, 1,2-Benzenedicarboxylic acid and Hexadecane.



***Penicilliumaethiopicum*; A. Colonies on CYA, B. Colony Texture, C,D,E. Conidiophore on 40X, F,G,H. phialides on 100X, I. spore on 100X**

2. ***Penicillium chrysogenum*** **Thom:**

In *Penicillium* subgenus *Penicillium* section Chrysogena series *Chrysogena*

Diagnostic features: Roquefortine C & D, chrysogine, penicillin F & G, globose to subglobose to broadly ellipsoidal smooth-walled conidia, relatively short phialides with short broad collula, high growth rate on YES with a yellow reverse and strong sporulation.

Description:

Conidiophores: Bi-, ter- and quarterverticillate, both appressed and divergent rami born from aerial and subsurface hyphae

Conidia: Smooth-walled, globose to subglobose to broadly ellipsoidal, 2.5-4 µm x 2.3-3.5µm

Phialides: Cylindrical, with short broad collula, 7-9 µm x 2.3-2.5 µm

Metulae: Cylindrical, 8-12 µm x 2.5-4 µm

Rami: Cylindrical, 15-20 µm x 3-4 µm

Stipes: 200-300 µm x 3-4 µm

Synnemata or fasciculation: None

Sclerotia: None

Colony texture: Floccose to velutinous

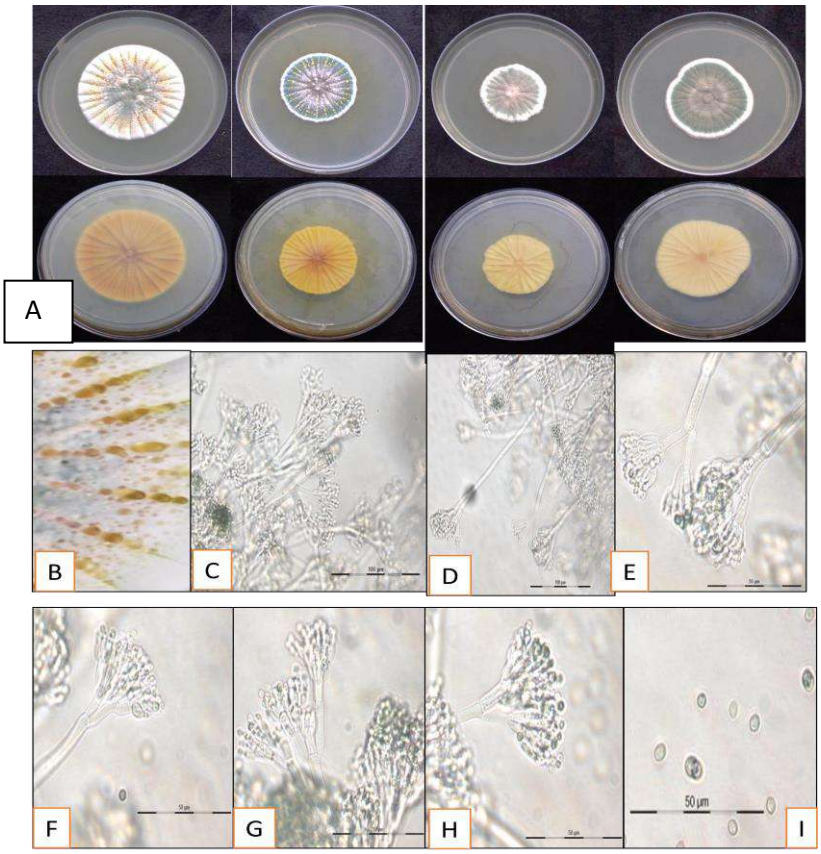
Conidiumcolour on CYA: Blue green to green

Exudate droplets on CYA: Often present, copious, yellow

Reverse colour on CYA: Cream, yellow, rarely brown

Diffusible colour: Yellow pigment often produced

Odour and volatile metabolites: Hexadecene, Octadecene, Eicosene, Hexadecanoic acid, Docosanol, Pentadecyl-heptafluorobutyrate, Cyclohexadecane, Octadecanoic acid and Griseofulvin.



Penicilliumchrysogenum; **A. Colonies on CYA, B. Colony texture, C,D. conidiophore on 40X, E,F,G,H. phialide on 100X, I. spore on 100X**

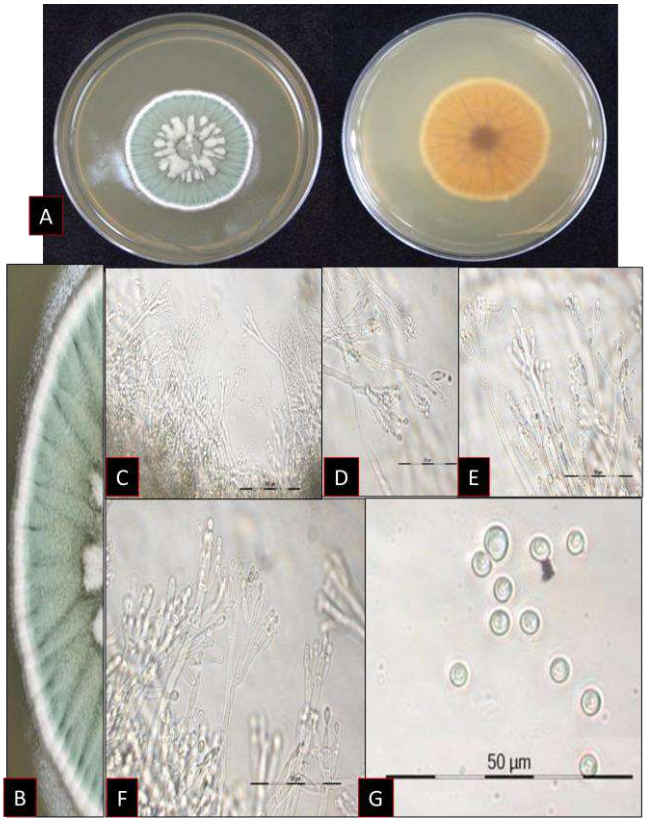
3. *P. crustosum* Thom:

In *Penicillium* subgenus *Penicillium* section *Viridicata* series *Camemberti*

Diagnostic features: Penitrem A, roquefortine C, terrestric acid, viridicatin, smooth-walled conidia, crustose on CYA and MEA after 7-10 days, high growth rate, good growth on CREA. Strong sporulation and high growth rate on YES agar.

Description:

Conidiophores: Terverticillate, appressed elements, born from subsurface hyphae
Conidia: Smooth-walled, globose to subglobose, 3-4 µm.
Phialides: Cylindrical tapering to a distinct collulum, 9-12 µm x 2.5-3 µm
Metulae: Cylindrical, 10-15 µm x 3-3.5 µm
Rami: Cylindrical, 15-25 µm x 3.5-4 µm
Stipes: Rough-walled, 200-400 µm x 3.5-4.5 µm
Synnemata or fasciculation: Weakly fasciculate
Sclerotia: None
Colony texture: Velutinous to weakly fasciculate, becoming crustose
Conidiumcolour on CYA: Dull green to grey green or blue green at the colony margin
Exudate droplets on CYA: Copious, clear or brown
Reverse colour on CYA: Cream-coloured to yellow brown
Diffusible colour: Pale brown or none
Odour and volatile metabolites: 9,12-Octadecadienoic acid, Hexadecane, Octadecene, E-15-Heptadecenal,Pyrrolo-pyrazine-1,4-dione, Chloro-2-phenoxyethylene, 3-Methyl-4-nitro-benzenamine, Octadecyltrifluoroacetate, 2,5-Piperazinedione, Docosene, 1,2-Benzenedicarboxylic acid and Hexacosene.



***Penicilliumcrustosum;* A. Colonies on CYA, B. Colony texture, C. conidiophore on 40X, D,E,F. phialide on 100X, G. spore on 100X**

4. *Penicillium janthinellum* Biourge:

Diagnostic feature: Monoverticilate, ampuliform, long slender collula, pyriform to ellipsoidal conidia.

Similar species: *P. ochrochloron*is closely related *P. janthinellum*, although *P. simplicissimum*in its typical form produces penicilli which are quite different from those of *P. janthinellum*, intermediate between the two species.

Description:

Conidiophore: Monoverticillate from surface or aerial hyphae

Conidia: Spheroidal,2.2-3.0µm in diameter, but sometimes short pyriform to ellipsoidal,2.2-3.0×2.0-2.5µm with smooth to finely roughened walls.

Phialide: ampuliform, 7-11×2.o-2.5µm with long slender collula

Metulae: terminally measuring 12-20×2-2.5µm, sometimes longer if intercalary.

Stripe: smooth and thin walled, easily bent, typically long and slender

Synnemata and fasciation: Absent

Sclerotia: Absent

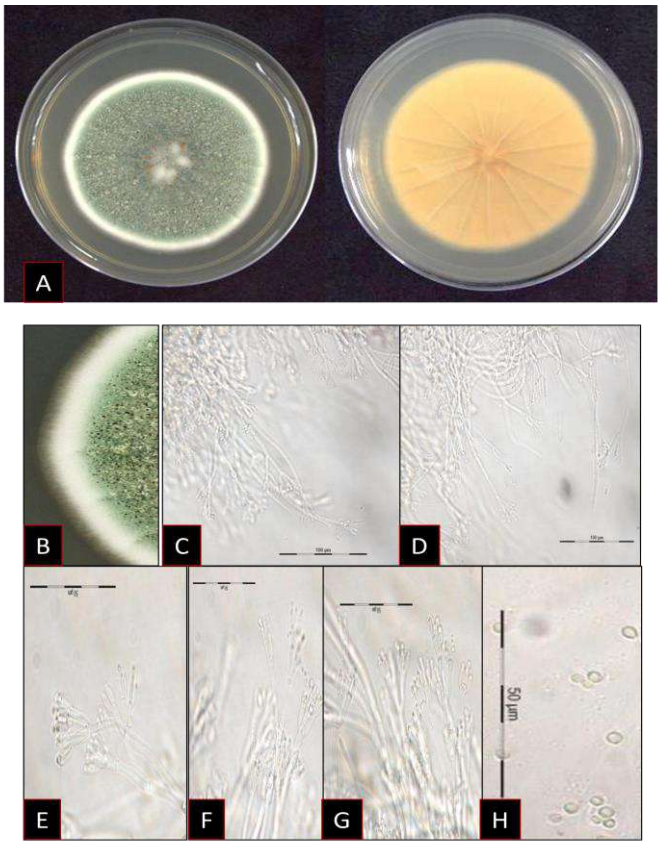
Colony texture: Floccose usually conspicuous layer of mycillium.

Conidiumcolour on CYA: Greyish green to dull green

Exudate droplet on CYA: Clear to brown or reddish brown.

Reverse colour on CYA: Pale, yellow to yellow brown or reddish brown.

Odour and volatile metabolite: Hexadecene, Octadecane,Pyrrolo-pyrazine-1,4-dione, 2H-Pyrrol-2-one, 1,2-Benzenedicarboxylic acid,Nonadecyl-trifluoroacetate, 2,5-Piperazinedione, 2-benzyl-3,6-dioxo-5-isopropyl-piperazine, Eicosene, 1,2- Benzenedicarboxylic acid, Cyclotetracosane and 17-Pentatriacontene.



***Penicilliumjanthinellum;* A. Colonies on CYA, B. Colony texture, C. conidiophore on 40X, D,E,F,G. phialide on 100X, H. spore on 100X**

5. ***Penicillium mononematosum*** **(Frisvad, Filt. & Wicklow) Frisvad:**

In *Penicilliums*ubgenus *Penicillium* section *Chrysogena* series *Mononematosa*

Diagnostic features: Fumitremorgins, verrucologen, cyclopaldic acid, divergent structures and often 2 rami, smooth-walled conidia, very good growth at 30°C

Description:

Conidiophores: Terverticillate to quaterverticillate, appressed and divergent elements, born from subsurface hyphae

Conidia: Smooth-walled, subglobose to broadly ellipsoidal, 3.2-3.7 µm x 2.5-3.2 µm

Phialides: Flask shaped with a distinct broad collulum, 7.5- 10 µm x 2.5-3.2 µm

Metulae: Cylindrical, 10-15 µm x 3-4 µm

Rami: Cylindrical, 15-25 µm x 3-4 µm

Stipes: Broad smooth-walled 200-500 µm x 3-4.5 µm

Synnemata or fasciculation: None

Sclerotia: None

Colony texture: Velutinous

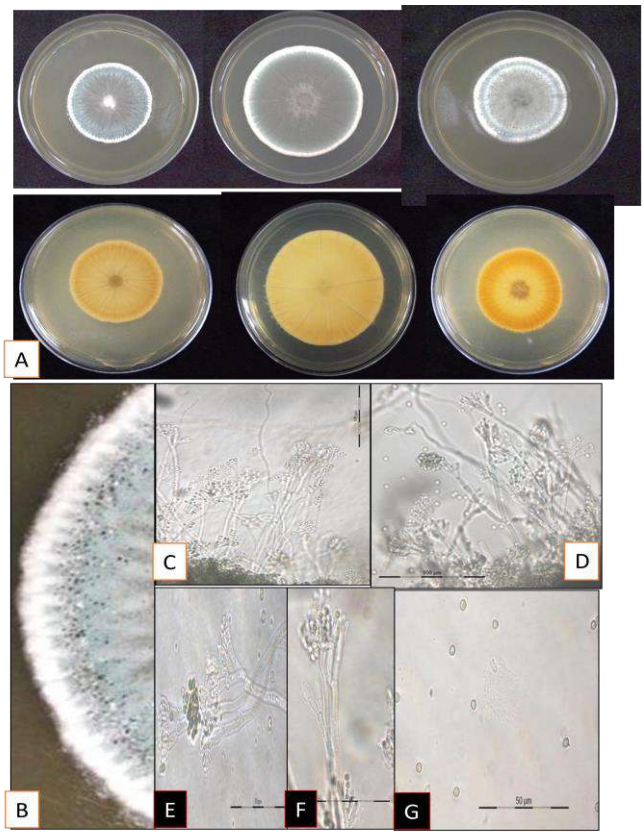
Conidiumcolour on CYA: Blue green to green

Exudate droplets on CYA: Copious, clear

Reverse colour: Beige to greyish cream

Diffusible colour: None

Odour and volatile metabolites: 9,17-octadecadienal, Tridecene, Hexadecene, β-Eudesmol, Octadecene, Pyrrolo-pyrazine-1,4-dione,9,12-Octadecadienoic acid-ethyl ester, Tetradecen-1-ol trifluoroacete, Docosene, 2,5-Piperazinedione, Octacosyl-acetate, Octacosyl-heptafluorobutyrate, Nonadecene, 17-Pentatriacontene and Oleic acid- propyl ester.



Penicilliummononematosum; **A. Colonies on CYA, B. Colony texture, C,D. conidiophore on 40X, E,F. phialide on 100X, G. spore on 100X**

6. *Penicilluium polonicum* K.M. Zalessky:

In Penicillium subgenus Penicillium section Viridicata series Viridicata

Diagnostic features: Penicillic acid, puberuline / verrucofortine, verrucosidin, cyclopenin, cyclopenol, smooth walled conidia,

Description: Conidiophores terverticillate, few biverticillate and quarterverticillate from subsurface hyphae

Conidia: Smooth-walled, globose to subglobose, 3-4 x 2.5- 3.5 µm

Phialides: Flask-shaped tapering to a distinct collulum, 7.5 - 10 µm x 2.5-2.8 µm

Metulae: Cylindrical, 10-13 µm x 2.8-3.5 µm

Rami: Cylindrical, 15-25 µm x 3-3.5 µm

Stipes: 180-400 µm x 3-4 µm, walls smooth to finely roughened

Synnemata or fasciculation: None

Sclerotia: None

Colony texture: Velutinous

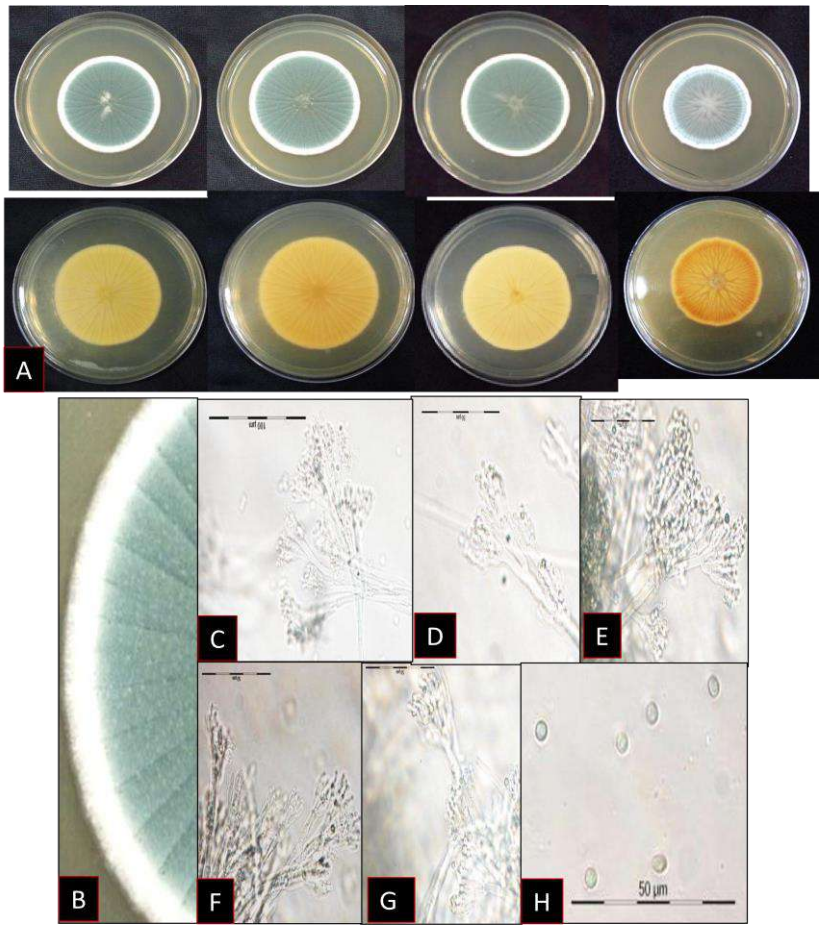
Conidiumcolour on CYA: Blue green

Exudate droplets on CYA: Present, clear

Reverse colour on CYA: Pale to cream or yellow brown to red brown

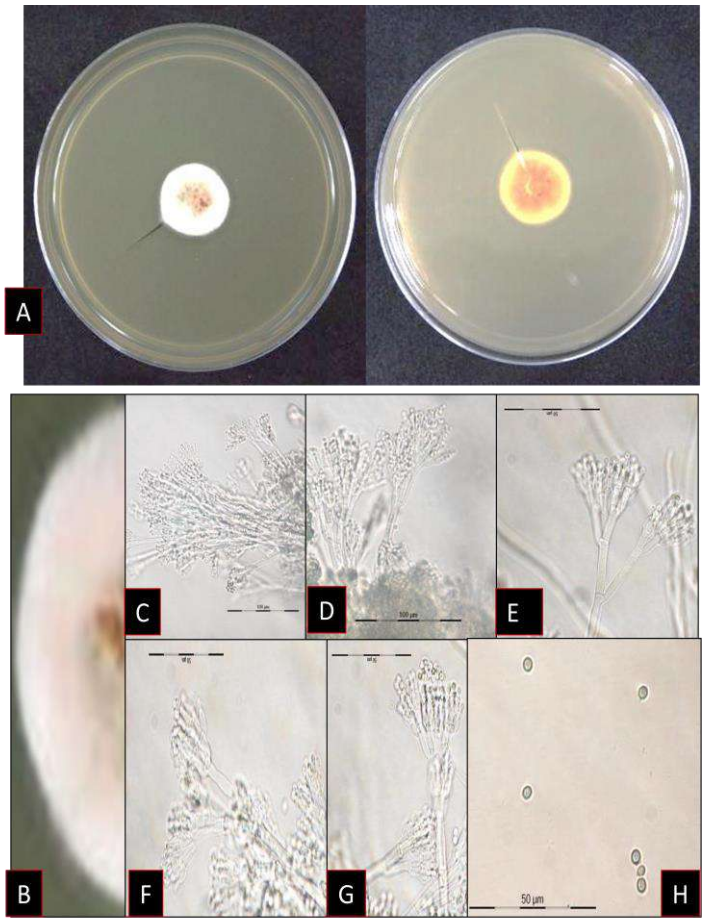
Diffusible colour: None or beige brown to red brown

Odour and volatile metabolites: Hexadecene, Eicosene, Docosene, Pyrrolo-pyrazine-1,4-dione and 9,12-Octadecadienoic acid-ethyl ester.



***Penicilliumpolonicum;* A. Colonies on CYA, B. Colony texture, C,D,E,F,G. Phialides on 100X, H. spore on 100X**

7. *Talaromyces pinopilus* (Hedgc.) Samson, Yilmaz, Frisvad & Seifert:
Syn. *Penicillium pinophilum*
Dignostic feature: Mitorubrinic acid, biverticillate, stipes smooth walled, conidia smooth, globose to subglobose.
Description:
Conidiophores: Biverticillate;
Conidia: Smooth, globose to subglobose,2–3 × 2–3 µm
Phialide: Acerose
Metulae: three to eight,
Rami: divergent, 10–11 × 2.5–3 µm;
Stripe: Smooth walled
Synnemata or fasciculation: None
Sclerotia: Absent
Colony texture: loosely funiculose and floccose especially in the centre
Conidiumcolour on CYA: Greyish green to dull green
Exudate droplet on CYA: Small clear and red droplets
Reverse colour on CYA: Greyish orange to orange
Odour and volatile metabolite: 9,12-Octadecadienoic acid, 2,5-di-Butyl-Phenol, Hexadecene, Benzofuran-2-one, Octadecene, 1,2-Benzenedicarboxylic acid, Eicosene, Hexadecanoic acid, Docosene, Hexacosene, Methyl 10,12-octadecadinoate and Griseofulvin.



***Talaromycespinophilus;* A. Colonies on CYA, B. Colony texture, C,D. conidiophore on 40X, E,F,G. phialide on 100X, H. spore on 100X**

Diversity in Hyphomycetous fungi

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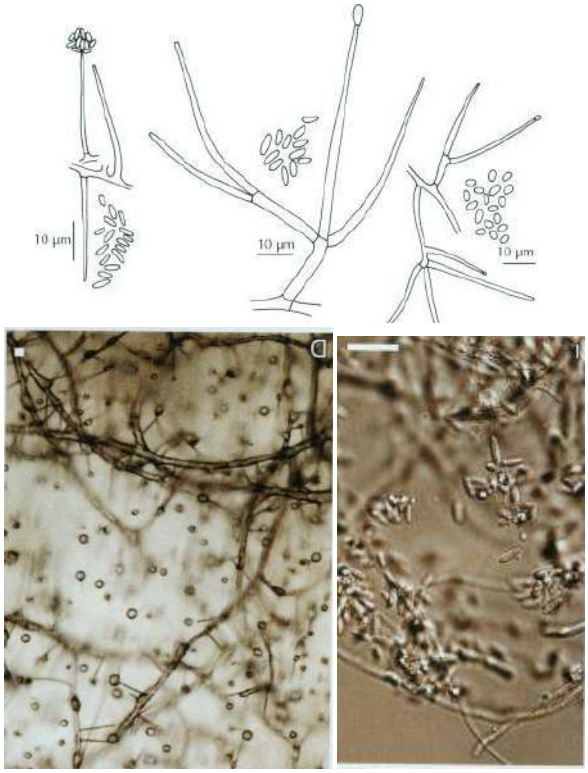
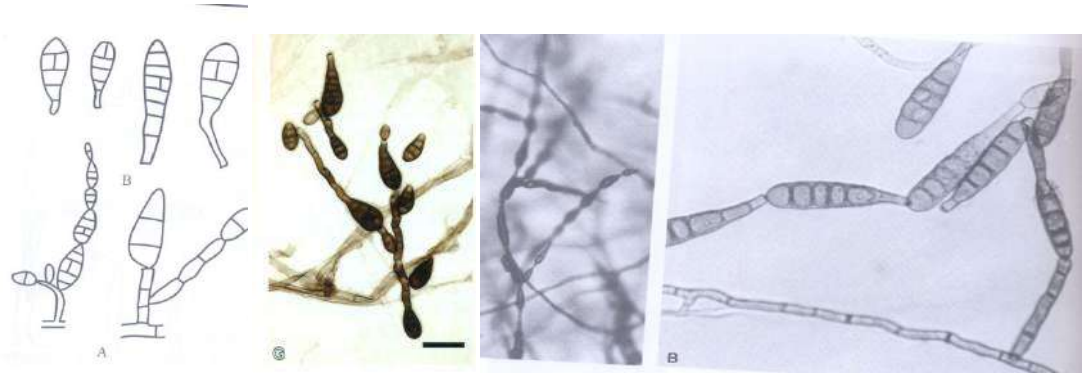
Hyphomycetes are a form classification of Fungi, part of what has often been referred to as Fungi imperfecti, Deuteromycota, or anamorphic fungi. Hyphomycetes lack closed fruit bodies, and are often referred to as moulds (or molds). Most hyphomycetes are now assigned to the Ascomycota, on the basis of genetic connections made by life-cycle studies or by phylogenetic analysis of DNA sequences; many remain unassigned phylogenetically. Identification of hyphomycetes is primarily based on microscopic morphology including: conidial morphology, especially septation, shape, size, colour and cell wall texture, the arrangement of conidia as they are borne on the conidiogenous cells (e.g. if they are solitary, arthrocatenate, blastocatenate, basocatenate, or gloiosporae), the type conidiogenous cell (e.g. non-specialized or hypha-like, phialide, annellide, or sympodial), and other additional features such as the presence of sporodochia or synnemata. The Hyphomycetes draw nourishment from living or dead organic matter and are adapted to grow, reproduce, and survive in a wide range of ecological situations. Many also cause economically important diseases in agriculture and forestry crops. These are primary pathogens of plants causing root, stem and leaf necrosis, die-backs, cankers, wilts and blights. About 1400 genera comprising more than 11,500 species are recognized.

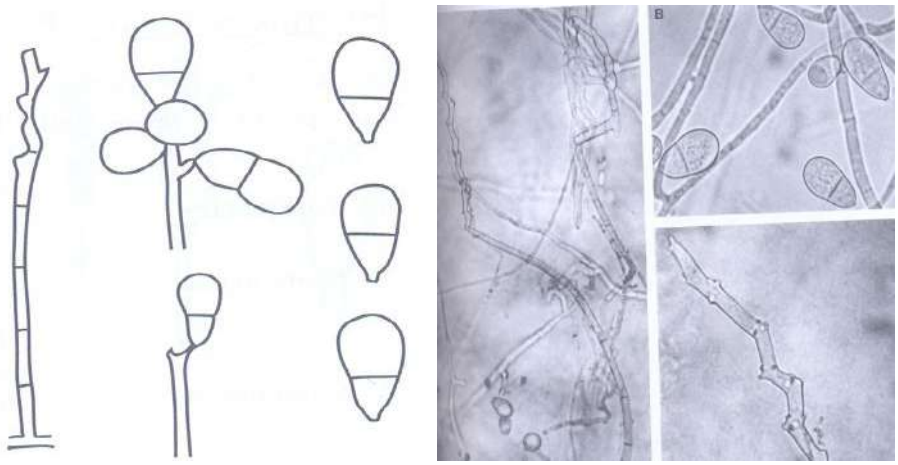
1. Acremonium Link
Type species: A. *alternatum* Link
Morphology:
Conidiophores (phialides): erect, simple, septate, hyaline, gradually tapering toward apexes with terminal slimy conidial masses
Conidia : phialosporous, clavate, cylindrical or ellipsoidal, hyaline, I-celled, usually biguttulate. Hyphe often crustaceous. Conidial masses 12-40 µm in diameter.



2. Alternaria Nees ex Fr.
Type species: *A. alternata* (Fr.) Keissler
Morphology:
Conidiophores : pale brown, simple orirregularly and loosely branched bearing catenulate conidia at the apex and apical fertile parts.
Conidiogenous cells : Integrated, terminal, becoming intercalary, polytretic, sympodial, or some times monotretic, cicatrized.
Conidia : catenulate, or solitary, dry, typically ovoid or obclavate, often rostrate, pale or midolivaceous brown, or brown, sooth or verrucose, with transverse and oblique or longitudinal septa.

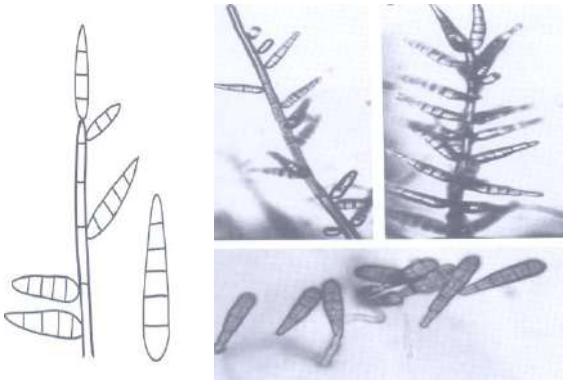


3. ArthrobotroysCorda
Type species: *A. superba* Corda
Morphology:
Conidiophores : hyaline, erect, simple or rarely branched, bearing 2-6 conidia sympodially on sterigmata in the apical parts.
Conidia : sympodulosporous, hyaline, ovate, 2-celled, composed of large apical cells and small basal cells, apiculate and truncate at base.



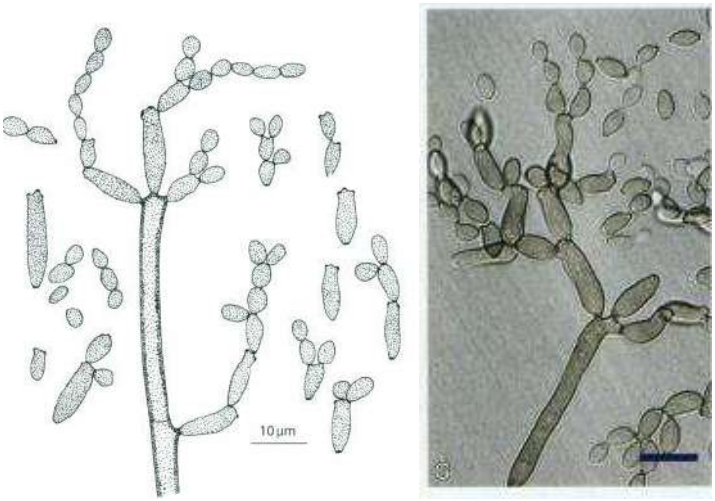
4. Bipolaris *Link* ex Fr.
Type species *H. velutinum* Link: Fr.

Conidiophores : Macronemqtous, mononematous, straight or flexuous, cylindrical orsbulate, mid to very dark brown, smooth or occationally verruculose, bearing conidia apically and laterally on the fertile apical parts in alternate, opposite, or verticillate fashion.
Conidiogenous cells: Polytretic, integrated, terminal and intercalary,, determinate, cylindrical.
Conidia: Solitary, acro - pleurogenous, developing laterally often in verticals through very small pores, beneath the septa. Growth of the conidiophore caesing with the formation of terminal conidia,simple, obclavate, rostrate, subhyaline to brown, smooth, pseudoseptate, Frequently with dark brown or black scar at the base.



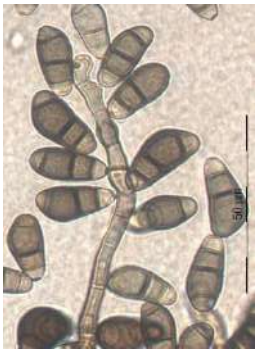
5. Cladosporium Link
Type species: C. *herbarum* Link ex Fr.

Conidiophores : Macronematous, and some times micronematous. Straight or flexuous, mostly unbranched or with branches restricted to the epical region forming a stipe and head, olivaceous brown or brown, smooth or verrucose. Ramo-conidia often present.
Conidiogenous cells: Polyblastic, usually integrated, terminaland intercalary, sometimes discrete, sympodial, more or less cylindrical, scars prominent.
Conidia : Blastosporous, often not well differentiated from branches, hyaline or pale brown, ovate, spherical or sub spherical, ellipsoidal, cylindrical, subglobose, irregular in shape, apiculate at one end, often truncate at another end,verruculose or echinulate with 0-3 or more septa.



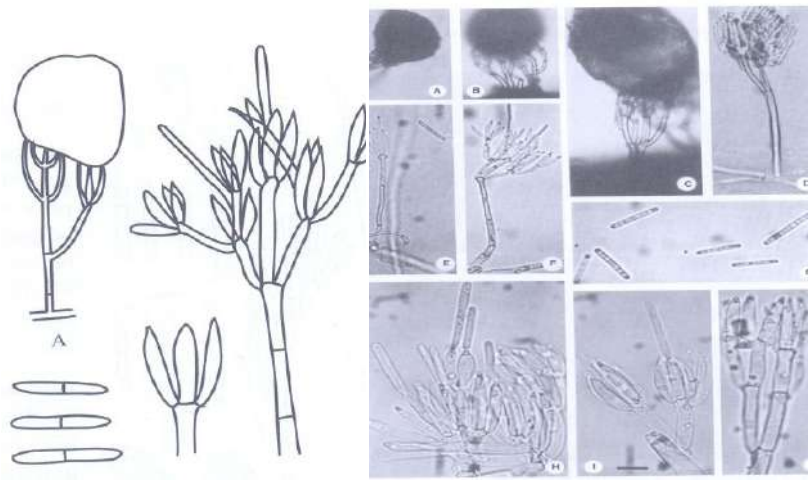
6. Curvularia Boedijn
Type species: *C. lunata*

Conidiophores : Stright or flexuous, often geniculate, smooth, dark brown, simple, erect, thick walled, bearing conidia apically and laterally.
Conidiogenous cells: Polytretic, integrated, terminal, sympodial
Conidia: Solitary, acropleurogenous, simple often curved, clavate, ellipsoidal, broadly fusiform, obovoid or pyriform, with 3 ormore transverse septa, pale or bark brown often with some cells, usually the end ones are paler than others, some times with dark bands at the septa, smooth orverrucose, hilum in some species protruberant.



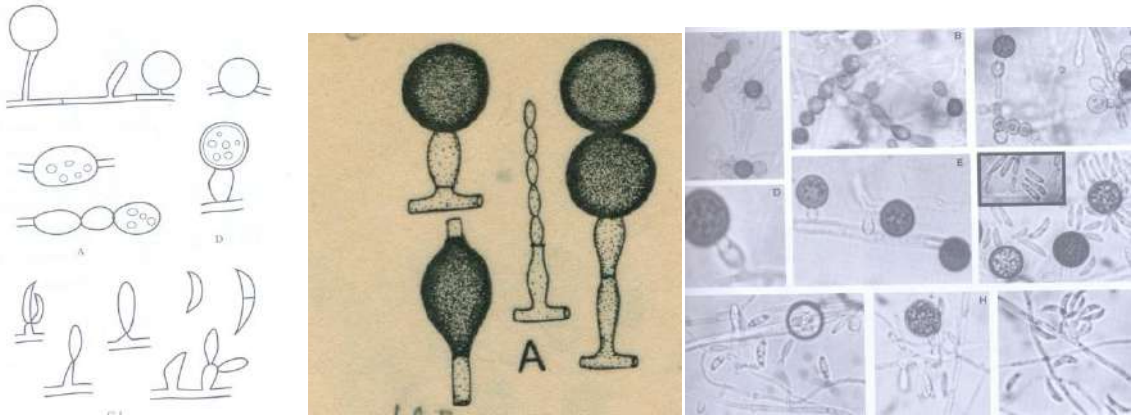

7. CylindrocladiumMorgan
Type species: *C. scoparium*

Conidiophores : erect, dichotomously branched, near the apex, the ultimate branches bearing the phialides, the main axis mostly forming a long, unbranched thread terminating to a globose to club-shaped apex. hyaline, erect, branched 1-3 times, mainly verticillate or penicillate at the apical parts, bearing spore masses at phialides on the respective branches, without stipes and terminal vesicles characteristic of most of *Cylindrocladium* species.
Conidia : Solitary on the phialides, phialosporous, hyaline, cylindrical, 1-many septate, mostly glued together by a colourless substance



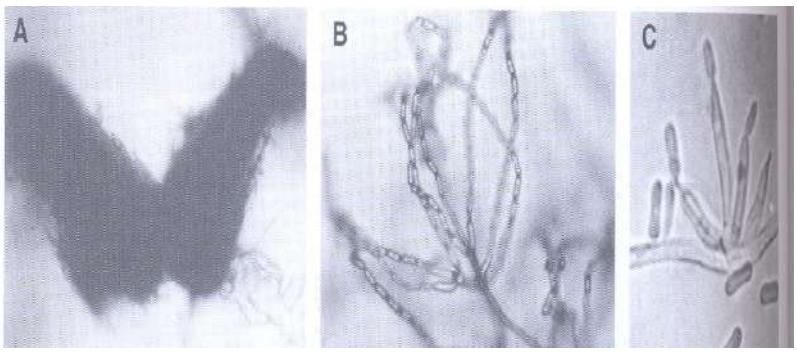
8. *Humicola* T. Watanabe
Type species: *H.fuscoatra*

Conidiophores: Unbranched or irregularly branched, straight or flexuous, colourless to pale golden brown, smooth.
Conidiogenous cells: Monoblastic, integrated,terminal, determinate, cylindrical, doliiform, pyriform
Conidia: Solitary, dry, acrogenous, simple, typically spherical, obovoid or pyriform, pale to midgolden brown, smooth, aseptate,
Humicola also has a phialidic state, the phialides being discrete, subulate, colourless, smooth. Phialoconidia catenate, or in slimy heads, very small, colourless, smooth, aseptate.



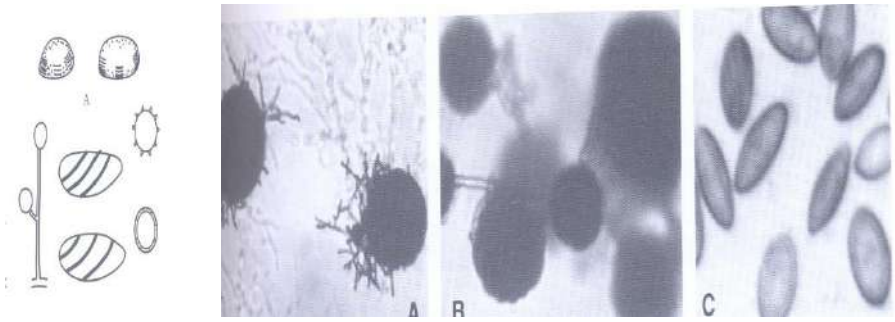
9.*Metarhizium*Sorokin
Type species: *M. anisopliae* (Metschn.) Sorokin

Morphology:
Conidiophores : Hyaline, short, erect, branched, terminating in a small group, or cluster of phialides, or single phialides.often united forming sporodochia, simpIe or branched, bearing catenulate conidia at the phialides in the branches: phialides apically pointed.
Conidia : Abstricted from the tips of the phialides, and forming long chains, (some times columns), long, ovoid to cylindrical, rounded at the ends, 1-celled, hyaline,, olive green in mass, forming a layer over the phialides, phialosporous. Entomogenous.



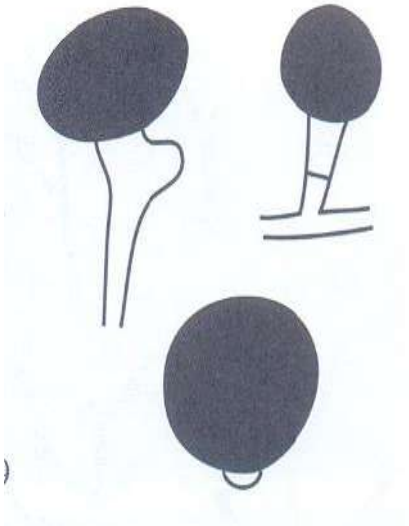
10. *Myrothecium*(Corda) Sacc.
Type species: *M. inundatum*

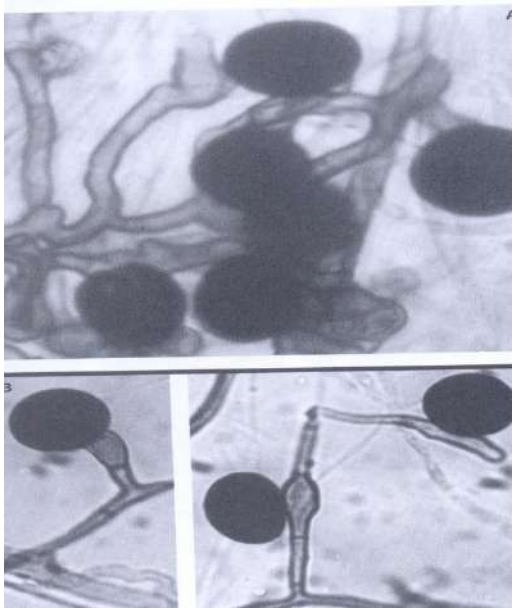
Sporodochia: Sessile or stalked, green to black mass of conidia is usually surrounded by a zone of white, flocculent, contorted hyphae.
Conidiophores : Macronematous, mononematous, closely packed together to form sporodochia branched, straight or flexuous, colourless or olivaceous, smooth or verruculose.
Conidiogenous cells: Monophialidic, discrete, cylindrical, clavate or subulate.
Conidia: Aggregated, in dark green or black slimy masses, semi-endogenous or acrogenous, simple, cylindrical rounded at the ends, navicular, limoniform, or broadly ellipsoidal, with projecting truncate base, hyaline to pale olive, smooth or stritely marked, 0-septate



11.Nigrospora Zimm.
Type species: *N. panici*

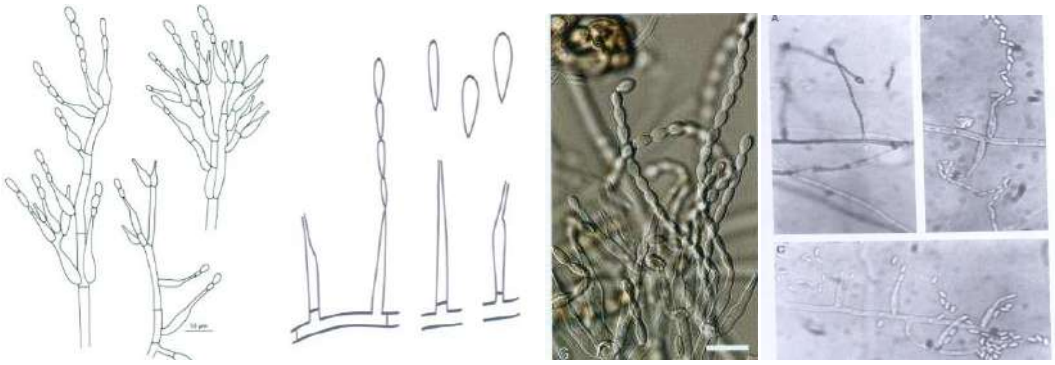
Conidiophores : Micronematous, flexuous, colourless to brown, smooth
Conidiogenous cells: Descrete, solitary, determinate, ampulliform, or sub-spherical, colourless with a violent discharge mechanism,
Conidia :acrogenous, simple, spherical, aleuriosporous, black, sub globose or disc-shaped, occasionally apiculate in the upper part.





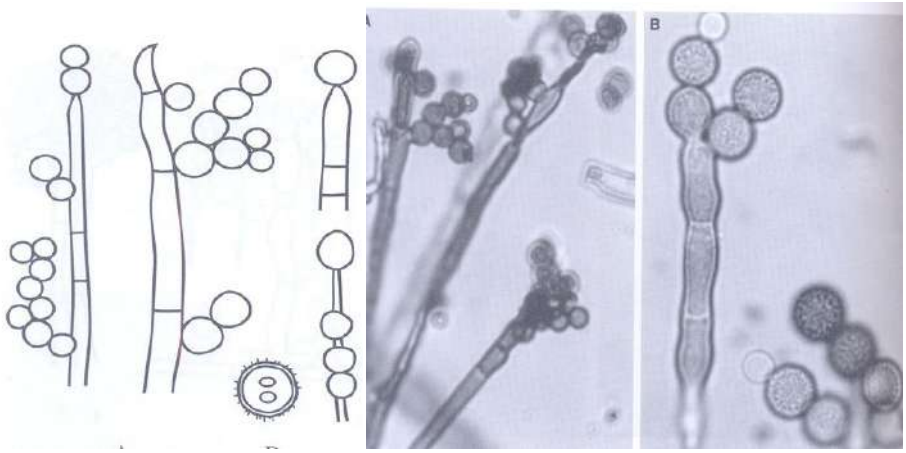
12. Paecilomvces(Holm: Gray) A. H. S. Brown &G. Smith
Type species: *P. fusispora*

Conidiophores (phialides) : Araising singly terminally and laterally on the hyphae, erect or ascending, straight or slightly flexuous, almost cylindrical, but tapering towards apex, septate, pale brown, to brown, echinulate or verrucose, bearing a terminal phialide and other phialides singly, in pairs, or vertices, just beneath the apex and on the branches.
Phialides: Flsk-shaped, with a narrow some times curved, cylindrical neck, colour less, smooth or echinulate
Conidia : Produced basipetally very long chains at the tips of the phialides, limoniform, 0-septate,, colourless, finely cchinulate,



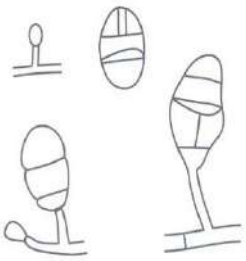
13. Periconia Tode ex Fries
Type species*: P.lichenoides*

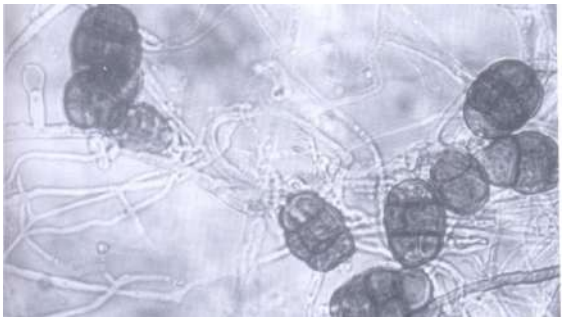
Conidiophores: Macronematous, micronematous, and mononematous. Macronematous conidiophores mostly with a stipe and spherical head, looking like round headed pins, branches present or absent, stipe straight or flexuous, pale to dark brown, often black, smooth or rarely verrucose, some times the apex is sterile.
Conidiogenous cells: Monoblastic or polyblastic, discrete on stipe and branches, determinat, ellipsoidal, spherical or sub spherical.
Conidia : Catenate, chains often branched, araising at one or more points, on the curved surface of the conidiogenous cell, simple, spherical orsub spherical, ellipsoidal, ablong, broadly cylindrical, pale to dark brown, verruculose or echinulate, 0-septate.



14. Pithomyces Berk. & Br.
Type species: *P. flavus*

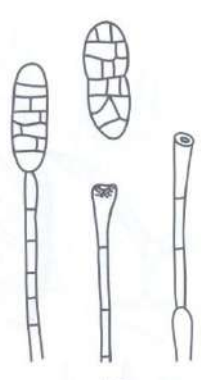
Conidiophores : Micronematous, mononematous, branched, straight or flexuous, hyaline topale olive brown, smooth or verruculose.
Conidiogenous cells: Monoblastic or polyblastic, integrated, intercalary or terminal, determinate, cylindrical, denticulate
Conidia : Solitary, pleurogenous, or acropleurogenous, dry,simple, detached through fracture of the denticle, a part of which always remains with the base of the conidium, ellipsoidal, clavate, limoniform, obovoid, oblong rounded at the ends, pyriform,obpyriform, straw to dark blackish brown, smooth, echinulate, or verruculose, 0-13 tranverse and 1 to manr longitudinal or oblique septa.

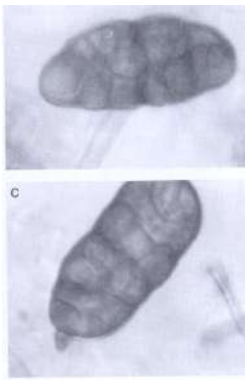




15. Stemphylium Wallr.
Type Species: *S. btryosum*

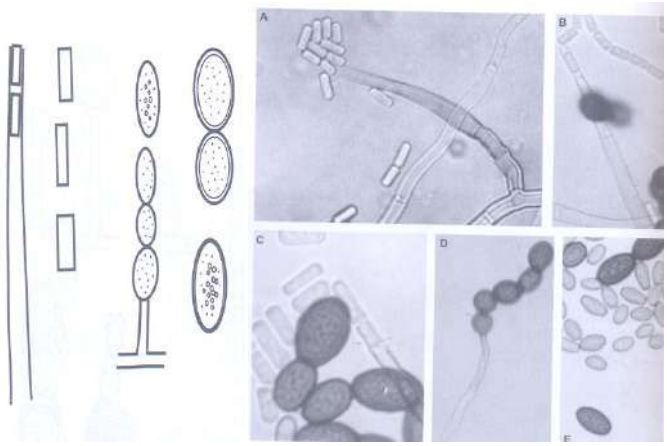
Conidiophores : macronematous, mononematous, scattered, unbranched or loosely branched, straight or flexuous, nodose with a no.of vesicular swellings, pale to midbrown or olivaceous brown, smooth or in part verruculose.
Conidiogenous cells: Monoblastic, integrated, terminal, percurrent, at first clavate or sub spherical, with thewall at the apex thin later often becoming calyciform.
Conidia : Solitary, dry, acrogenous, oblong rounded at the endsellipsoidal, obclavate or sub spherical, some species with a pointed conical apex and one with lateral conical protrusions, pale to mid dark or olivaceous brown, smooth, verrucose, echinulate, muriform, 1-many septa, cicatrized at the base.





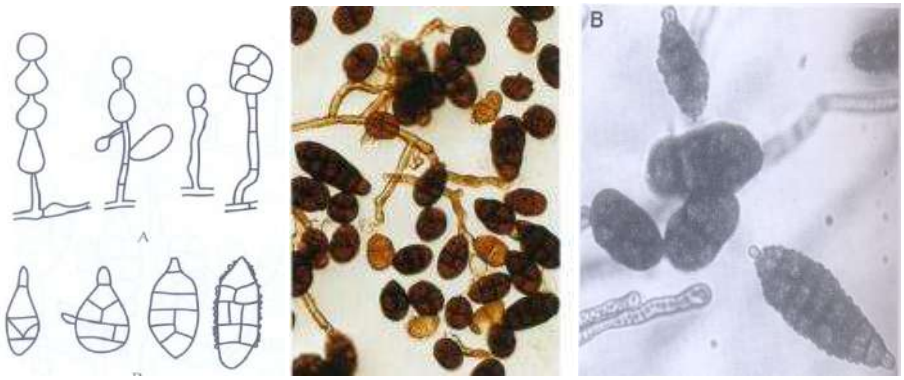
16.ThielaviopsisWent.

Conidiophores (phialides): Macronematous, mononematous, unbranched orirregularly branched, straight or flexuous, hyaline or pale brown, smooth.
Conidiogenous cells: Fragmenting to form arthroconidia, terminaland intercalary, determinate, cylindrical.
Conidia : Catenate, dry, schizogenous, often seceding with difficulty, simple, doliiform, ellipsoidal, obovoid or oblong, mid to very dark brown, smooth,0-septate.
Chlamydospores : darkbrown, ellipsoidal, thick-walled, granulate.



17. Ulocladium Preuss
Type Species: *U.botrytis*

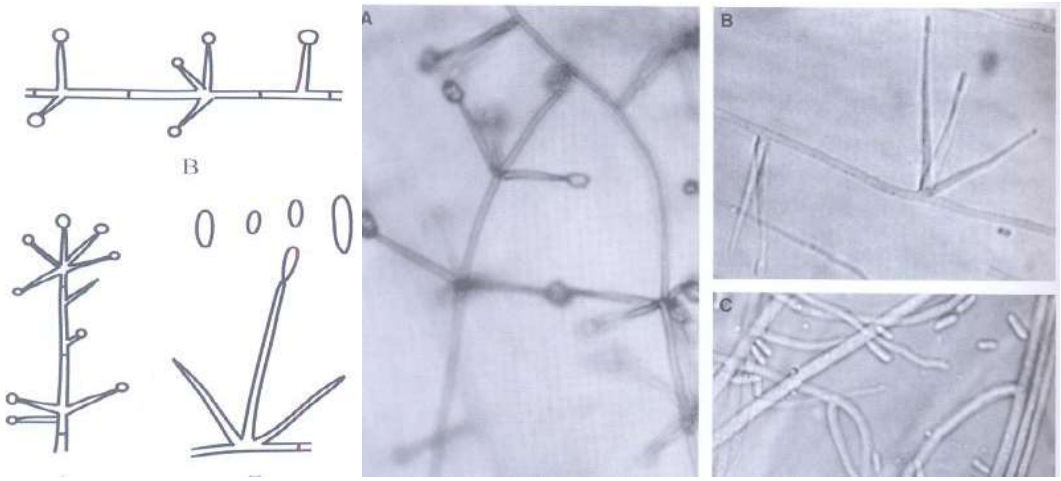
Conidiophores : Macronematous, mononematous, unbranched or branched, straight or flexuous, often geniculate, pale to mid brown, smooth or verruculose.
Conidiogenous cells: Polytretic, integrated, terminal becoming intercalary, sympodial, cylindrical, cicatrized.
Conidia: Solitary, secondary conida on short secondary conidiophores give raise tochains insome, dry, simple, broadly ellipsoidal, o obovoid, clavate, pyriform, sub spherical, frequently with aminute projecting hylum pale to dark blackish brown, smooth or verrucose, with transverse and longitudinal septa. septation some times cruciate.



18. Verticillium Nees ex Link
Type species: *V. tenerum*

Conidiophores (phialides) : Macronematous, mononematous, scattered, each composed of an erect, straight, or flexuous, colourless to dark brown, smooth or verruculose stipe with branches, and phialides commonly in verticels, beneath the septa nearest the apex.

Conidiogenous cells: Monophialidic, discrete, often arranged verticillately, determinate, ampulliform, laginiform, or subulate, ith well defined collarettes.
Conidia ggregated in slimy masses, semi-endogenous or crogenous, simple, allantoid, ellipsoidal or cylindrical, rounded at the ends, colourless to pale brown, smooth 0-septate



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Identification of some important Coelomycetous fungi

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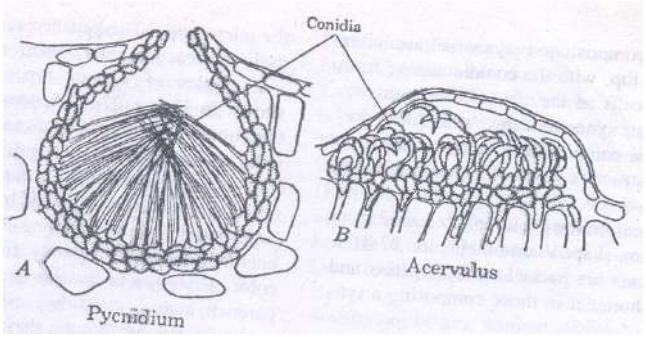
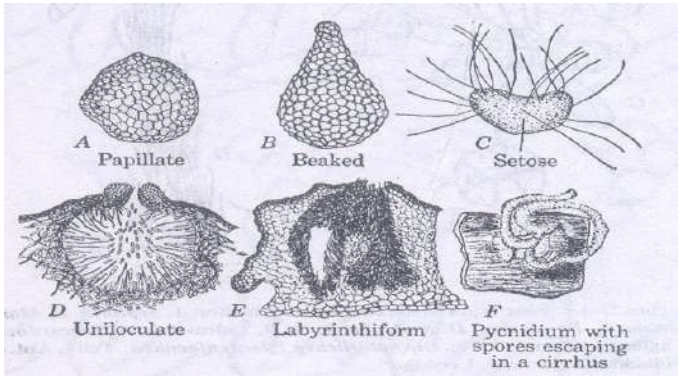
Introduction

Coelomycetes are mitosporic oranamorphic (asexual or imperfect) fungi with sporulation occurring insidefruitbodies (conidiomata) that arise from a thallus consisting of septate hyphae. The fruiting stuructres may be spherical with an apical openig (pynidia) or saucer shaped (acervuli). The production of conidia within a fruiting body distinguishes this group from the hyphomycetes which have "naked" conidia. Pycnidia may be confused with ascocarps so its important to make the distinction between an acsospore and a conidium. Most coelomycetes are now assigned to the Ascomycota, on the basis of life-cycle studies or by phylogenetic analysis of DNA sequences; many remain unassigned phylogenetically. Differences in conidiomatal structure traditionally have been used to separate three orders: the Melanconiales, the Sphaeropsidales, and the Pycnothyriales. However, differences in the ways that conidia are produced are now used in classification and identification. Coelomycetes are known mainly from temperate and tropical regions. They grow, reproduce, and survive in a wide range of ecological situations.They are commonly found in and recovered from soils, leaf litter and other organic debris from both natural and manufactureds ources. Coelomycetes are consistently isolated from or associated with disease conditions in all types of vascular plants. About 1075 genera containing morethan 10000 species are recognized.

The fungi producing pycnidia or acervuli belong to the form-subclass Coelomycetidae. Those forms producing pycnidia are placed in the form-order Sphaeropsidales; those producing acervuli are placed in the form-order Melanconiales.



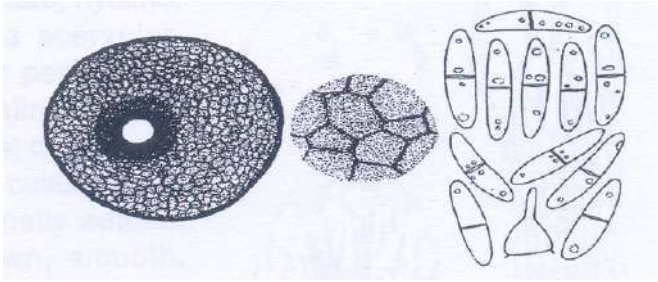
Pycnidia are quite different in appearance from one-genus to the next. They may be superficial or immersed; globose, elongate, or cup-like; unilocular or multi-locular; and light or dark in colour.



The acervuli, may develop in a sub-cuticular or sub-epidermal, becoming erumpent when the conidia are mature, they release them in characteristic droplets that maybe white, cream, pink, orange, black or other colours, depending on the pigmentation of the conidia.

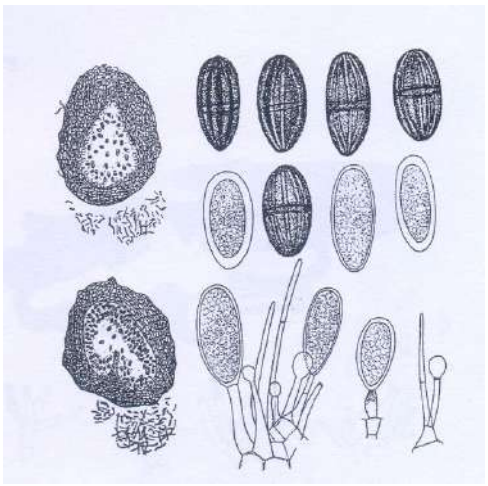
Some important genera of Coelomycetous fungi are given under for the purpose of their identification:

1.*Ascochyta*Lib.



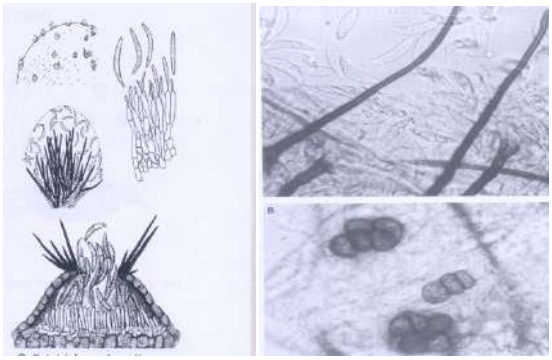
Conidiomata : Pycnidial, amphigenous, globose, brown, seperate immersed, unilocular, thin-walled
Ostiole: Central, circular, slightly papillate
Conidiophore : Absent
Conidiogenous cells: Enteroblastic, phialidic, determinate, descrete, doliiform to lageniform, hyaline, smooth, formed from the inner cells of the pycnidial wall.
Conidia: Hyaline, medianly one septate, thin walled, smooth, continuous or constricted, cylindrical to irregular

2. *Botryodiplodia (Lasiodiplodia)theobromae*Ell.&Ev.



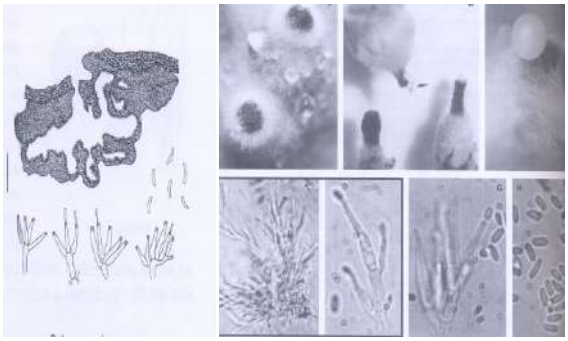
Conidiomata : Eustromatic,Immersed or superficial, globose, carbonous, uni-or multilicular, thick-walled, with dark brown hyphae over the surface, usually with cylindrical necks. Upto 3mm in diam.
Ostiole: Absent, dehiscence by irregular rupture
Conidiophore : Absent
Conidiogenous cells: Holoblastic, determinate, discrete, cylindrical, hyaline, smooth with no percurrent or sympodial proliferation.
Conidia: Acrogenous, hyaline when young, later becoming dark brown with 1-thick transverse septum at the middle, thick-walled, ellipsoid, base truncate, with indistinct longitudinal striations from apex to base often mixed with hyaline, immature conidia.
Paraphyses: Hyaline, cylindrical, septate

3.*Colletotrichum* Corda in Sturm



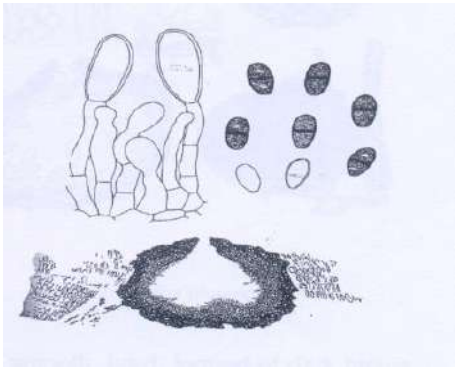
Conidiomata : acervular, subcuticular. epidermal, subepidermal or peridermal, separate or confluent, composed of hyaline to dark brown, thin or thick walled, dehiscence irregular.
Sclerotia : some times present in culture,dark brown to black, often confluent, occationally setose.
Setae : In conidiomata or sclerotia brown, smooth, septate, tapered to the apices.
Conidiophore:Hyaline to brown, septate, branched only at the base, smooth, formed from the upper cells of the conidiomata.
Conidiogenous cells : Enteroblastic, phialidic, hyaline, smooth, determinate, cylindrical, integrated or discrete
Conidia : Hyaline, aseptate, straight or falcate, smooth, thin-walled,sometimes guttulate, multinucleate
Appressoria : Brown, entire or with crenate to irregular margins, simple or repeatedly germinating to produce complex columns of several closely connected appressoria.

4. *Cytospora* Ehrenb. ex Fr.



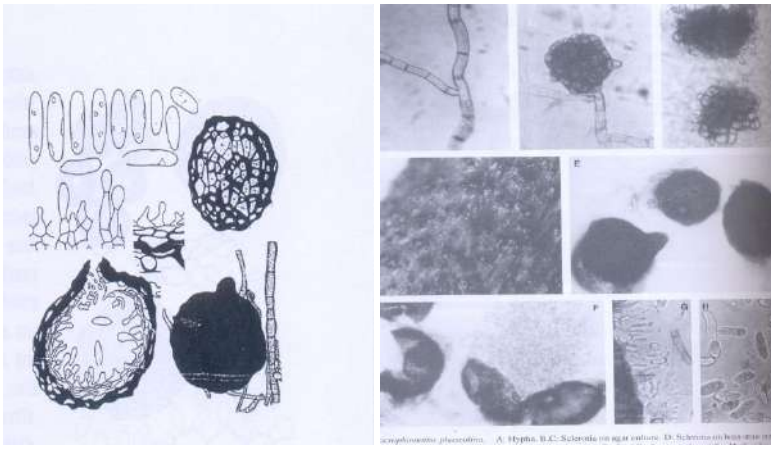
Conidiomata:Eustromatic, separate, sub epidermal,erumpent, dark brown, multi-locular and convoluted, the locules radiating and enlarging from the centre
Ostiole : Single, circular, prominent
Conidiophores : Hyaline,septate, branched irregularly at the base and above, smooth, mostly with apical conidia but some times acro pleurogenous, formed from the inner cells of the locular walls.
Conidiogenous cells : Enteroblastic, phialidic, determinate, integrated, straight, hyaline, smooth, occasionally formed as very small lateral branches immediately below transeverse septa but more often as long distinct branches.
Conidia : Formed in distinct, various coloured masses of globose droplets or tendrils, hyaline, aseptate, thin-walled, eguttulate, smooth, allantoid.

5. *Diplodia* Fr. apud Mont.



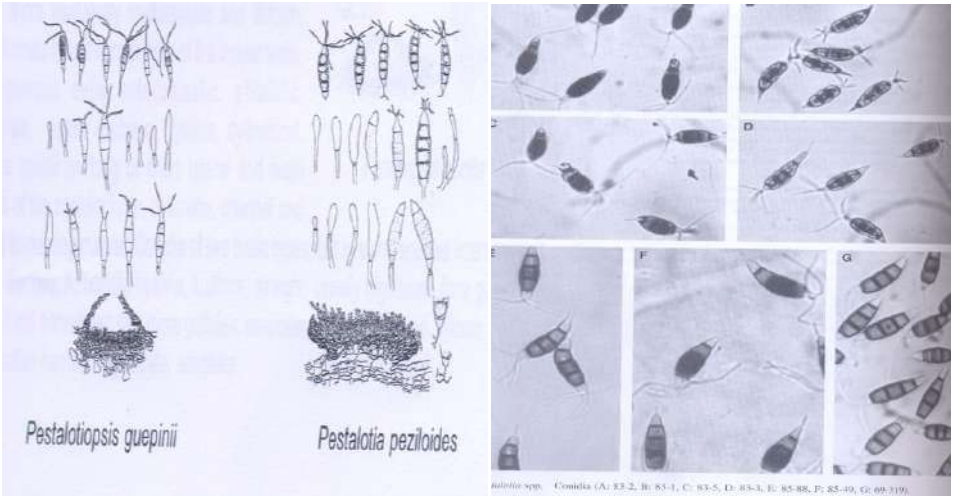
Conidiomata : Pycnidial, separate or aggregated, globose to dark brown to black, immersed, unilocular, thick-walled; wall of an outer layer of dark brown, thick-walled median layer of dark brown thin-walled cells, and an inner layer of thin-walled, hyaline cells.
Ostiole : Single, circular, central, papillate.
Conidiophores : Hyaline, branched and septate above and at the base, smooth, cylindrical, formed from the inner cells of the pycnidial wall.
Conidiogenous cells : Holoblastic, integrated or discrete, determinate, cylindrical, hyaline, smooth, forming a single apical conidium.
Conidia : At first hyaline, with a central guttule, thick-walled, aseptate, smooth, later becoming dark brown and medianly one euseptate, apex obtuse, base truncate.

6. *Macrophomina*Petrak



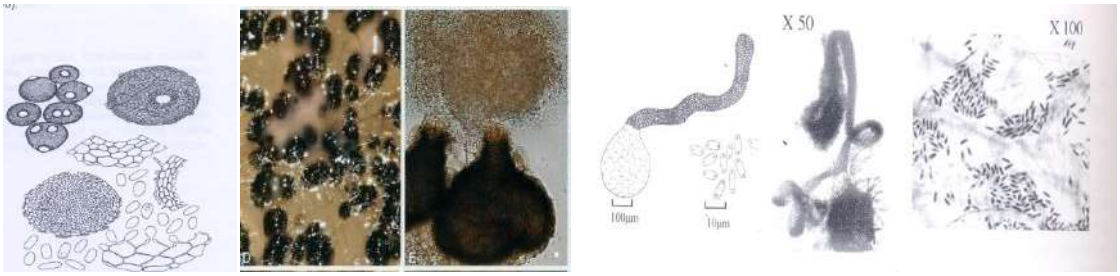
Conidiomata : 130-230 µ in diam. Pycnidia rarely formed on natural media, separate, globose, dark brown, immersed, unilocular, thick-walled
Ostiole : Central,circular, apical, papillate.15-35 µ.in diam.
Conidiophore : Absent.
Conidiogenous cells : Enteroblastic, phialidic, determinate, discrete, lageniform to doliiform, hyaline, smooth
Conidia : Hyaline, aseptate, obtuse at each end, straight, cylindrical to fusiform, thin walled,smooth, + or - guttulate.14-35 x 6-11.5 µ.
Sclerotia : Black, smooth, hard, formed of dark brown, thick-walled cells.60-120 µ.in diam.

7. *Pestalotiopsis*Stey.,



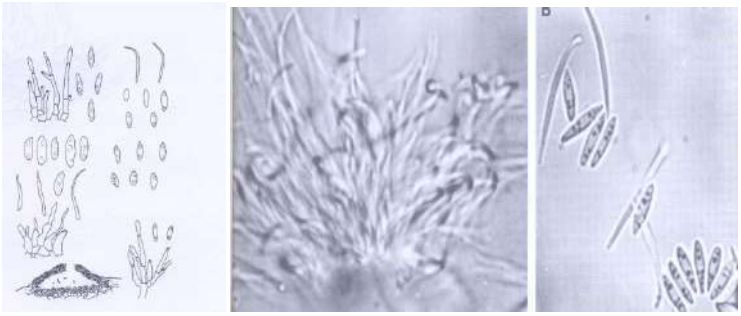
Conidiomata : Acervular, epidermal to subepidermal,separate or confluent, formed of brown. Dehiscence irregular. Conidiophores hyaline, branched and septate atthe base and above, cylindrical or lageniform, formed fromthe upper cells of the pseudoparanchyma.
Conidiogenous cells :Holoblastic, annellidic, indeterminate, integrated, cylindrical, hyaline, smooth, with several percurrent proliferations.
Conidia : Fusiform, straight or slightly curved, 4 – euseptate, basal cell hyaline, truncate, with an endogenous, cellular, simple or rarely branched appendage; apical cell conic, hyaline, with 2 or more apical simple or branched, non-spathulate or euspathulate appendages, median cells brown, sometimes versi coloured, thicker walled, smooth or verruculose.

9. *Phoma* Sacc., nom. cons.,



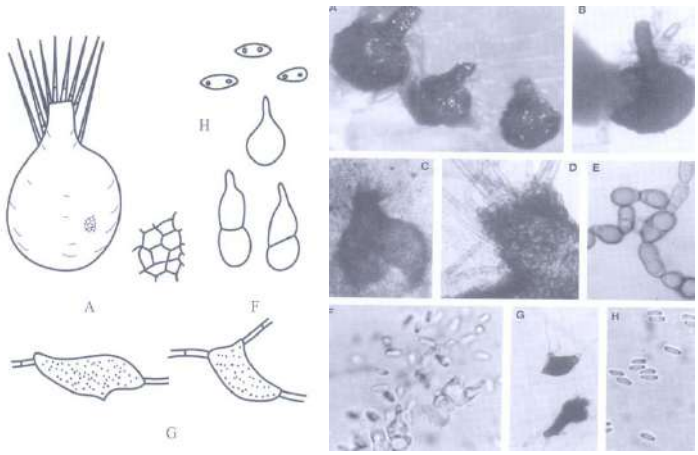
Conidiomata : Pycnidial, immersed, or semi immersed sometimes becoming erumpent, unilocular, brown, globose, separate or aggregated, occasionally confluent, thin walled, pale to medium brown.
Ostiole : Single or several to each pycnidium, central, not papillate.
Conidiophores : Only present in *Phoma cava* and *Phoma tracheiphila* and then either filiform, septate, and branched, or short, irregularly branched, and ramified respectively.
Conidiogenous cells : Enteroblastic, phialidic, integrated or discrete, ampulliform to doliiform, hyaline, smooth.
Conidia : Hyaline, aseptate, thin walled, or occasionally one septate. Thinwalled often guttulate, ellipsoid, cylindrical, fusiform, pyriform or globose.

10. *Phomopsis* (Sacc.) Sacc.,



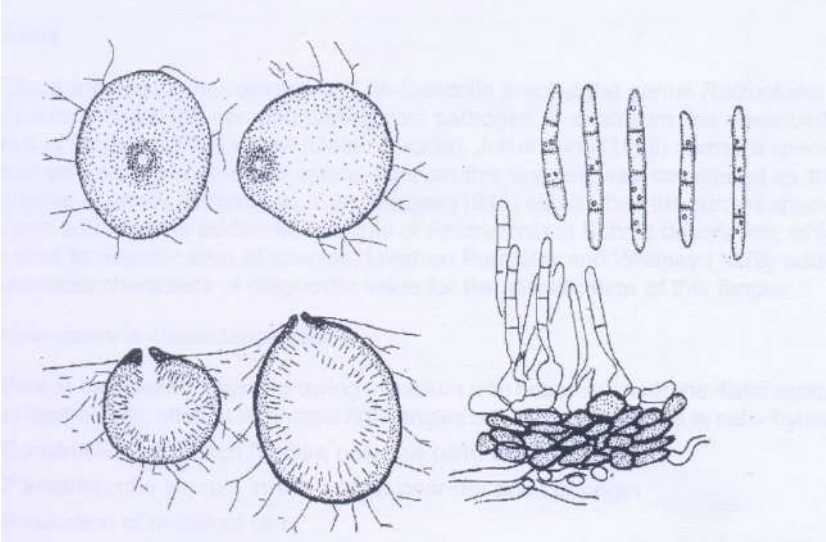
Conidiomata : Eustromatic, immersed, brown to dark brown, separate oraggregated and confluent, globose, ampulliform or applanate, unilocular, multilocular or convoluted, thick walled, often some what darker in the upper region, lined by a layer of smaller-celled tissue.
Ostiole :Single,or several in complex Conidiomata, circular, often papillate.
Conidiophores :Branched and septate at the base and above, occasionally short and only 1-2 septate, more frequently multiseptate and filiform, hyaline, formed from the inner cells of the locular walls.
Conidiogenous cells : Enteroblastic, phialidic, determinate, rarely discrete, hyaline, cylindrical, apertures apical on long or short lateral and main branches of the conidiophore
Conidia : Of two basic types, but in some species intermediate between the two
A. conidia hyaline, fusiform, straight, usually biguttulate (one guttule at
each end) but sometimes with more guttules, aseptate;
B. conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate.

11.*Pyrenochaeta* T.Watanabe



Conidiomata : Pycnidial, 107-297 µ.globose,or sub globose, well necked with setae around
Setae : Brown,thick-walled, tapering from base toward apex, septate,35-200 µ.long,
Conidiophores : Hyaline, simple, ampulliform, with abruptly sharpened or narrowed tips,occationally septate.
Conidiogenous cells :
Conidia : Hyaline, long-ellipsoidal, icelled with two oil globules. 2.8-5.2 x 0.5-1.5 µ.

11. *Septoria* Sacc.



Conidiomata :Pycnidial, immersed, separate or not usually confluent, globose, papillate or not, brown, thin-walled

Ostiole : Single, sometimes papillate.

Conidiophores : Absent.

Conidiogenous cells : Holoblastic, determinate or indeterminate, sympodial proliferations, then each locus with a broad, flat, unthickened scar, discrete, hyaline ampulliform, doliiform or lageniform to short cylindrical.

Conidia : hyaline, multi septate, filiform, smooth, constricted at the septa.

Polyphasic Taxonomy of Endophytic Fungi

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Introduction:

Rising interests in biology and biotechnology of fungi, in general, and endophytes in particular has led to search for diverse group of fungi from various ecological niches and habitats. Fungi colonizing internal plant tissues without causing apparent symptoms of disease are considered to be endophytes, which are generally considered as one of the untapped resources due to lack of comprehensive information of their interaction with the host plants. Endophyte-plant interaction is the balanced symbiotic relationship. Vast genetic diversity of endophytes offers a wealth of possibilities to be used in various sectors like agriculture, health care and industry (Strobel 2003, 2006). Endophytes have been recorded from almost everywhere, from Arctic to Antarctic and temperate to the tropical climates. Their association with every group of plants suggests them to be integral part of plant-microbiome being utilized for various purposes. It has been perceived that metabolically endophytes are more active compared to their general counterparts. It has been hypothesized that endophytic microorganisms are vertically transmitted, have co-evolved with the host, and their associations range from being parasitic to strongly mutualistic.

Endophytic fungal diversity holds promises for academic as well as industrial research. The specific trait of fungal endophytes to synthesize plant metabolites has gained increased attention of researchers world-wide, which has changed the whole approach of screening host plants in different ecological settings and strategies of exploiting endophytic mycobiota are scaling new heights. Among several beneficial properties reported is drought-acclimation, improved resistance to insect pests and herbivores, increased competitiveness, improved tolerance to heavy metal, low pH, high salinity, etc. Recent reports on production of volatile organic compounds (VOCs) by endophytic fungi and their various applications in agriculture and allied sectors has opened a new dimension of research (Strobel et al. 2001, 2011; Singh et al. 2011). Various chemical substances produced by endophytic fungi are broadly grouped into alkaloids, steroids, terpenoids, isocoumarins, quinones, phenylpropanoids, lignans, phenol and phenolic acids, aliphatic metabolites, lactones, hydrocarbons, etc.

Indian Scenario: Singh (2016) reported that only about 248 host plant have been screened so far out of about 17,527 angiosperm and 67 gymnosperm species found in India (Karthikeyan 2009). This record points out that diversity of endophytic fungi in India is meagre, though different aerial and underground parts/tissue types, viz., cotyledons, seed coats, stems, leaf blade, petioles, rhizomes and aerial roots have been screened. However, inclination of researchers in India can be seen towards applied aspects through selected studies conducted on antimicrobial, anticancer, cytotoxicity, anti-inflammatory assays, etc. Synthesis of metal nanoparticles, like silver and gold nanoparticles using fungi is an upcoming area being targeted for developing reliable and ecologically safe processes for applications in various fields.

Isolation of Endophytic Fungi (Protocols)

Isolation of Plant Samples

- Collect the plant samples from target hosts separately either in sterile polythene bags or screw caps tubes

- Wash the samples thoroughly under tap water until the surface adherents are removed.
- Cut into small pieces (5×5 mm approx) from washed samples and subject to surface sterilization as per protocol given below (Table 1).
- Inoculate on fresh suitable agar plate.
- Transfer individual mycelial growth arising out from inoculated plant samples to fresh agar plate by hyphal tipping and cultivate them till full colony growth occur.
- Make multiple sets of agar slants and preserve them for long term using different methods (in 15% glycerol, sterile distilled water, cryo-preservation etc.) for further identification and characterization.
- Evaluate the success of sterilization by plating 100 µl of the last rinsing sterile distilled water (SDW) on potato dextrose agar (PDA), and incubating the plate for, at least 3-days at optimal temperature.
- Terminate and restart the experiment, if any growth is seen on the plate considering them as contaminant.

Internal Tissue Isolation:

- Remove the outer bark of the stems with a sterilized sharp blade and collect into sterile scre-cap tubes and get back to laboratory as soon as possible
- Cut into small pieces of inner tissues of the stems and inoculate on agar plate.
- Incubate inoculated plate at suitable temperature till vegetative growth appears from inoculates tissues
- Transfer individual mycelial growth arising out from inoculated plant tissues to fresh agar plate by hyphal tipping and cultivate them till full colony growth occurs.
- Make multiple sets of agar slants and preserve them for long term using different methods (in 15% glycerol, sterile distilled water, cryo-preservation etc.) for further identification and characterization.

Table 1: Details of surface sterilization used in isolation of endophytic fungi (Crous et al. 1995; Suryanarayanan & Vijaykrishna 2001)

Protocol No.	Chemicals	Concentration	Time
I	Formaldehyde Sterile Distilled Water	40% -	1 min 3 min × 4 times
II	Formaldehyde Sterile Distilled Water	40% -	3 min 3 min × 4 times
III	Ethanol SDW NaOCl SDW Ethanol SDW	96% - 4% - 96% -	1 min 3 min × 4 times 5 min 3 min × 4 times 30 sec 3 min x 4 times
IV	Ethanol SDW NaOCl SDW Ethanol SDW	70% - 4% - 70% -	1 min 3 min × 4 times 1 min 3 min × 4 times 30 sec 3 min x 4 times

Morphological Identification and Characterization of Endophytes

Morphological characters of fungi in general have long served as the basis for mycological taxonomy. Morphological taxonomy starts with the slide preparation and their microscopic observation. A good slide must contain most of the characteristics and developmental stages and taxonomically important structures that will help in recording the data appropriately. In a culture dependent approach, identification starts with the culturing following different methods/protocol. Colony characteristics, colony texture, color determination using standard color charts and describing a colony along with its growth pattern and measurements are very important for understanding the behavior of fungi on different types of media. Different cultural conditions such as temperature, light intensity must be recorded on time scale and must include in all the morphological identification practices. Observation on different fruiting structures and critical comparison with known taxa is crucial step leading to the correct identification of target fungi. Though many saprophytic or pathogenic taxa have their counter parts known as endophytes, their taxonomic characters remains same. Existing monographs, keys and manual are useful in morphological identification of endophytes (Seifert et al. 2011; Bhat 2010; Nagraj 1993; Bhat & Kendrick 1993; Sutton 1980)
Molecular Identification of endophytes

There are numerous fungi with pleomorphic life-cycles. Several characteristic features are not tenable and are highly influenced with various environmental conditions. Therefore, it is difficult to identify up to species level just on the basis of the morphology. Interestingly, in case of some groups of fungi such as *Fusarium* and the allied genera morphological identification frequently fails to distinguish even between the genera. The morphological characters are controlled by the cultural conditions which are provided to the fungi like media, light, humidity etc. So, just relying on the morphological data would not be fair enough. Many conserved gene regions have been identified and established. The ITS gene regions, ribosomal gene regions and other proteins coding gene regions have been established to be helpful in delimiting up to the species level. There is large gap in between the molecular and the morphological work which is to be filled for the correct fungal taxonomy. Moreover, in case of the endophytic fungi, need of molecular taxonomy increases as sometimes the cultures which we isolate are non-sporulating, so the only way to identify is through molecular phylogenetics. So, its high time that we adopt the polyphasic way of identification rather than just practicing the morphological taxonomy (Hyde et al. 2010; 2014)

DNA barcoding has significant impact on taxonomy. It provides more rapid progress then the traditional taxonomic work. DNA barcoding allows taxonomists to rapidly sort specimens by highlighting divergent taxa that may represent new species. DNA barcoding may offer taxonomists, the opportunity to greatly expand, and eventually complete, a global inventory.
The DNA barcoding of the chosen taxa can be carried out by generating the ITS-rDNA sequence data. Ambiguous taxa and/or potential novel taxa can be subjected to multigene phylogeny, to accurately identify the species and place them in the correct taxonomic position in a phylogenetic tree. Following steps may be followed and for detail protocols concern reference may be referred for molecular identification:

- Cultivation of the target fungi on suitable agar plates for a week.
- Scrapping of the mycelial mat of fungi and proceeding for the genomic DNA isolation following a simple and rapid DNA extraction protocol (Aamir et al. 2015).

- Checking the genomic DNA on 0.8% agarose electrophoresis gel and further proceed for the amplification of the desired gene region using suitable primers (White et al. 1990).
- Checking the amplicon on 1.2-1.5% agaorse electrophoresis gel and further proceed for PCR product purification using commercially available kits (Favorgen, Thermofisher, etc.).
- The purified products should be subjected to direct sequencing using BigDye®Terminator v3.1 Cycle Sequencing Kit and ABI 3100 DNA analyzer as per manufacturer's instructions.
- Sequences obtained should be submitted in NCBI GenBank.
- The sequences of the related strains should be retrieved from NCBI after megablast analysis.
- The sequences should be aligned using MAFFT v. 6.864b/MEGA/ClustalW (Katoh & Standley 2013; Kumar et al. 2016).
- The alignments should be checked and adjusted manually using Aliveiw (Larsson 2014).
- Further, in case of the multigene phylogeny, alignments should be concatenated. The same concatenated alignment should be subjected to phylogenetic analysis.
- The best substitution model can be found using ModelFinder (Kalyaanamoorthy et al. 2017).
- Further, windows version MEGA or IQ-tree tool v.1.6.11 (Nguyen et al. 2015) can be used to reconstruct the phylogenetic trees (Fig. 1)

Chemical Characterization: Endophytic fungi are good reservoir of plethora of chemicals. Various chemical substances produced by endophytic fungi are broadly grouped into alkaloids, steroids, terpenoids, isocoumarins, quinones, phenylpropanoids, lignans, phenol and phenolic acids, aliphatic metabolites, lactones, hydrocarbons, etc. However, the specificity of these chemical substances as marker (s) to a particular genus and species are yet to be established majorly barring a few exception (in *Aspergillus* & *Penicillium*). Chemical analyses individual genera and their multiple species/strains have been undertaken e.g. Polizzotto et al. (2010) studied chemotaxonomy of 20 grapevine endophytes which were able to produce number of unknown metabolites. The results show how complementary morphological, molecular and chemical data can clarify relationships among endophyte species-groups of low morphological divergence. As such, chemical profiling of endophytes is important and compliment to the morphological and molecular data.

Outlook & Goal: Endophytic fungi are poorly researched group of organisms, though various studies have established that the endophytic fungi isolated from different tissues, seed, stem, leaf impacts the ecology of the host plants in different ways. It is presumed that vast plant genetic diversity, and seasonal variations in India help in recovering promising endophytes from plant of ecological settings. The rainforests, marine environments, mangrove swamps, can be potential targets for new molecules to be isolated from endophytes which can be useful in different sectors, like agriculture, health and environment. In this regard, interdisciplinary approaches, may lead to realize expected success. Advance tools and techniques also play important role in capturing hidden wealth of endophytes from plant sources. Culture-independent approach of metagenomics needs consideration in order to have valuable and useful gene pool. In India work on endophytic fungi need concerted for systematic mapping of vegetal cover and their endophytes for casing fungal bio-resources into economic wealth. However, rationale for selecting plant in a particular environment for endophytic isolation and their potential screening is the crucial step to be considered wistfully in order to achieve the expected results. Biochemical analysis of endophytes is essential in order to find out the marker metabolites. The morphological, molecular and chemical data will help in providing comprehensive identification and in clarifying relationships among endophyte species-groups of low morphological divergence.

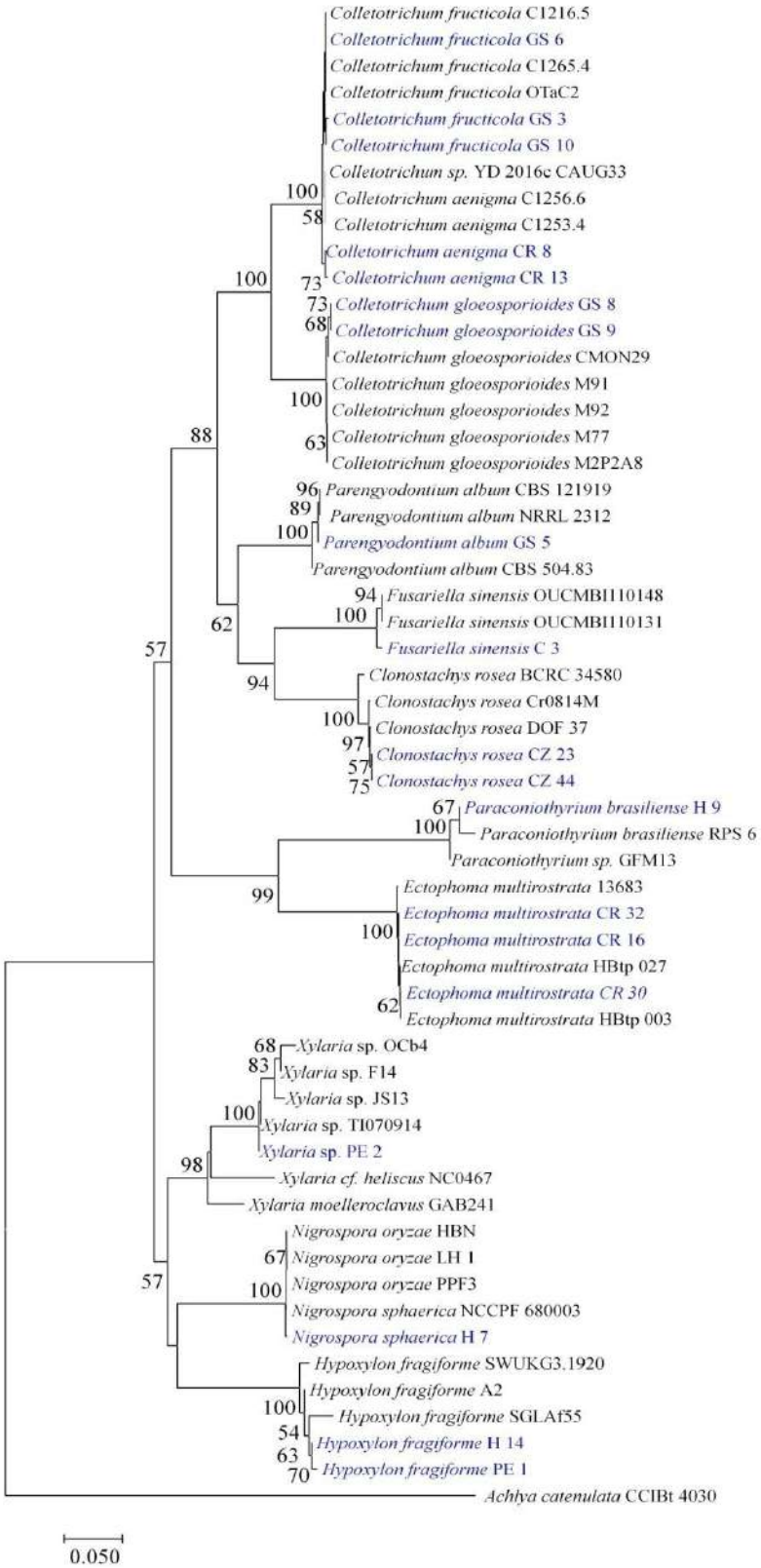

Fig.1. Phylogram generated from maximum-likelihood (ML) method based on Kimura 2-parameter method using ITS sequence data (Kimura 1980). Isolates used in the present study are represented in blue. *Achlya catenulata* was used as outgroup.

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Internal Transcribed Spacer (ITS) based identification

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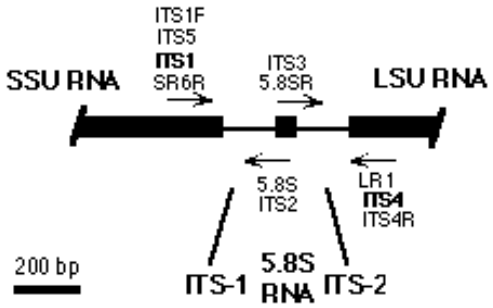
Introduction

The internal transcribed spacer (ITS) is a phylogenetic markerwhich has been of broad use in generic and intrageneric levelclassifications. The length and sequences of ITS regions of ribosomal DNA repeats are believed to be fast evolving and therefore may vary**.** Internal transcribed spacers (ITS) are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 25S rRNA coding regions (ITS2). ITS is of high repetition in nuclear genome, and moreover, there is concerted evolution of intra-site and inter-site among the repetition units by unequal crossing over and gene conversion. That is to say, the sequences among different ITS copies tend to be similar or totally concerted. With high-speed evolution, ITS can provide more variable sites and informative sites, which has been confirmed as an important molecular marker in the study of systematics and evolution concerning fungal populations. Among eukaryotes, including organisms as diverse as protozoa, plants, vertebrates, and fungi, ITS data have been used in constructing phylogenetic trees, estimating genetic population structures, evaluating population level evolutionary processes and determining taxonomic identity. It is useful for phylogenetic analysis among related species



Internal transcribed spacer (ITS) region and their primers for amplification

Table 1. Sequences of ITS primers

Primer name	Sequence (5'->3')	Comments	Reference
ITS1	TCCGTAGGTGAACCTGCGG		White *et al*, 1990
ITS2	GCTGCGTTCTTCATCGATGC	(is similar to 5.8S below)	White *et al*, 1990
ITS3	GCATCGATGAAGAACGCAGC	(is similar to 5.8SR below)	White *et al*, 1990
ITS4	TCCTCCGCTTATTGATATGC		White *et al*, 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	(is similar to SR6R)	White *et al*, 1990
ITS1-F	CTTGGTCATTTAGAGGAAGTAA		Gardes & Bruns, 1993
ITS4-B	CAGGAGACTTGTACACGGTCCAG		Gardes & Bruns, 1993
5.8S	CGCTGCGTTCTTCATCG		Vilgalys lab
5.8SR	TCGATGAAGAACGCAGCG		Vilgalys lab
SR6R	AAGWAAAAGTCGTAACAAGG		Vilgalys lab

Protocol

Materials

Chemicals CTAB buffer, chloroform, isoamyl alcohol, alcohol, chloroform, isopropanol, TE buffer, primers, dNTPs, $MgCl_2$, Taq Polymerase, DD water, PDA (Potato dextrose agar) and PDB (Potato dextrose broth).

Glassware and plastic ware Oakridge tube, 250 ml, borosil flasks, eppendorf tube, PCR tubes

Raising Mycelial Culture

- Multiply the single spore pure fungal cultures in potato dextrose broth (200g peeled potato, 20g dextrose, 1000mL distilled water) on shaking incubator at specific temperature.
- Harvest the mycelium by filtration with the help of Whatman No.1 filter paper and keep at -80°C.

Fungal DNA extraction

DNA is extracted by CTAB method. Steps are given as under:

- Pre-cool a pestle and mortar at 4ºC. Grind 5 g mycelium to a fine powder in liquid nitrogen and transfer to a plastic sterile tube, ensuring that the tissue does not thaw.
- Add to it 10 ml prewarmed isolation buffer and incubate for 60 min with occasional stirring.
- Extract for 10 min with equal volume of chloroform: isoamyl alcohol (24:1).
- Centrifuge at 10,000 rpm for 20 min at room temperature (24ºC).
- Separate the aqueous phase and transfer to a fresh tube.
- To aqueous phase add 0.6 volume of ice-cold isopropanol and 0.1 volume of sodium acetate, and incubate at -20ºC for 30 min.
- Centrifuge at 10,000 rpm for 10 min at 4ºC, discard the aqueous phase.
- Wash the DNA pellet obtained with 70% ethanol (5 ml).
- Centrifuge at 10,000 rpm for 10 min at 4ºC, discard the aqueous phase.
- Dry the DNA pellet and dissolve in 200 µl of TE buffer.
- Genomic DNA dissolves slowly in TE following ethanol precipitation, particularly if the concentration exceeds 1 µg/µl. It may be necessary to resuspend the DNA on a rotary table at 20 rpm overnight at room.

Quantification

a. Spectrophotometery

- Take 1 ml TE buffer in a cuvette and calibrate the spectrophotometer at 260 nm as well as 280 nm wavelength.
- Add 2 to 5 µl of DNA, mix properly and record the optical density (OD) at both 260 and 280 nm.
- Estimate the DNA concentration employing the following formula

$$\text{Amount of DNA (ug/µl)} = \frac{OD_{260} \times 50 \times \text{Dilution factor}}{1000}$$

- Judge the quality of DNA from the ratio of the OD values recorded at 260 and 280 nm the A_{260}/A_{280} ratio around 1.9 (1.85-1.95) indicates best quality of DNA.

b. Nano-drop spectrophotometery

- DNA quantification can also be done directly by loading 1µl of DNA in nanodrop spectrophotometer.

PCR amplification of ITS region

DNA from all isolates is amplified using ITS primers. Steps are given as under:

- Set up a 25 µl reaction in a 0.2 ml micro-centrifuge tube

Template DNA (50 ng)	1.0 µl
10 X Taq polymerase buffer	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
dNTPs (10 mM)	0.5 µl
Primer (10µM)	0.5µl
Taq Polymerase (5 U/µl)	0.5 µl
Autoclaved DD Water	18.5 µl

- Make the final volume up to 25 µl.
- Spin briefly (1 sec.) in a microfuge to mix.
- The mixture is then placed in PCR machine (i Cycler, BIO-RAD). Following are the temperature profile and cycles performed
- Perform the PCR with following conditions:

Initial denaturation	:	94˚C for 5 min	
Denaturation	:	94˚C for 1 min	35 cycles
Primer annealing	:	55˚C for 1 min	
Primer extension	:	72˚ C for 2 min	
Final extension	:	72˚C for 5 min.	

Reactions are stopped at 4ºC.

Gel electrophoresis

Preparation of agarose gel

- For 0.8% agarose gel (size 15 cm x 20 cm) mix the following: 1.6 g of agarose in 200 ml of 1X TAE buffer.
- Dissolve the agarose by heating, mixing several times during heating. Cool to 60ºC and add add 0.05µl of Ethidium bromide solution/ml of gel solution from stock solution (10mg/ml).

Caution: Ethidium bromide is extremely mutagenic wear gloves when handling it and use extra precautions.

- Place tray in the electrophoresis tray with 1 X TAE gel buffer. Pour enough 1X TAE gel buffer into the gel rig to cover the gel by at least 0.5 cm, then remove combs.

Loading the PCR products

- To the PCR product add 1/10 vol. of 10X tracking dye, mix well and carefully load into the wells. Include a λ phage or any other molecular weight marker DNA as control in the adjacent well.
- Run samples at 70 V for 2-4 hr, till the Bromophenol blue dye has migrated 2/3rd of the gel.

  Note: DNA and RNA are negatively charges and thus run from positive to negative or from the black to red side if hooked up properly.

Viewing

- Slide gel into under UV transilluminator or geldoc.
- Approximate of the bands in base pair can be estimated by comparing with molecular weight markers.
- Sequence the PCR product to get the nucleotide sequence of ITS region

Analyze the nucleotide sequences through BLAST using NCBI data base

Construct the phylogeny tree based on the maximum nucleotide sequence similarity by using CLUSTALW 1.8 sequence alignment selecting bootstrap neighboring joint by MEGA 4.1 programme

(Tamura *et al.*, 2007). The strength of the internal branches from the resulting strict consensus trees was tested by bootstrap analysis using 1000 replications.

Steps followed are given as under:

- Select the nucleotide sequence of ITS region and blast in http://blast.ncbi.nlm.nih.gov/blastn
- Select the sequence of accession closely related to ITS region of our query sequence and collect them in notepad
- Open Mega.4 program
- Create a new alignment (√)
- Paste sequences in to alignment explorer and save it
- Open phylogeny in mega 4 and go for neighbor joining boot strep option for preparing phylogenetic tree among our isolates and other isolates downloaded from NCBI in first step.

Expected outcome:

Full length internal transcriber spacer (ITS) region which included ITS 1, 5.8S and ITS 2 regions was amplified and characterized by sequence analysis. The amplification with ITS1 and ITS 4 universal primers showed a single DNA band of 600bp in all the isolates. These amplicons from each isolate were sequenced and phylogenetic analysis of different isolates of *Bipolaris* species from India showed high level of similarity (95%). The analysis grouped the isolates of *Bipolaris* spp. in two major clusters supported by high bootstrap value (99%). Cluster I comprised of 3 sub-clusters, where 24 isolates of *B. sorokiniana* constituted sub-cluster I, *B. oryzae* and *B. maydis* were grouped in sub-cluster II and III respectively with 100% similarity among them.One isolate of *B. sorokiniana* (Bs 53) collected from Himachal Pradesh grouped in Cluster II along with all the 4 isolates of *B. tetramera* and *B. spicifera*

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Points to remember

1. The material (mycelial mat) should not be over dried. A hard, over dried DNA pellet takes very long time to dissolve completely.
2. DNA should not be forced into solution. It might cause shearing.
3. After washing with 70% ethanol, the DNA pellet, which is obtained, should look like white, thick thread like mass.
4. By comparison with the MW marker, a rough estimate of the concentration of DNA can be obtained. Exact spectrophotometric estimation will help in taking known quality of DNA for further use.

Do you know?

Tris interacts with the lipopolysaccharides present on the outer membrane which helps to permeabilize the membrane. This effect is enhanced with the addition of EDTA. **EDTA** chelates divalent cations required for DNAse activity, protecting the DNA from degradation. **Phenoland chloroform/isoamyl** (usually) alcohol extract proteins and lipids away from the DNA. In the presence of sufficiently high NaCl, chilled absolute **ethanol** precipitates the DNA, the last step in a traditional DNA extraction.

Do you know?

chloroform causes proteins to become denatured and become soluble in the organic phase or interphase, while nucleic acids remain in the aqueous phase.

Do you know?

$MgCl_2$is an essential cofactor for the DNA polymerase in PCR. The total magnesium ion concentration must exceed the total dNTP concentration. In general, magnesium ion should be varied in a concentration series from 1.5-4.0 mM, in 0.5 mM steps.

Application of real time PCR technique in mycology and plant pathology

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Introduction

Over last several years, the development of novel chemistries and instrumentation platforms enabling detection of PCR products on real time basis has lead to wide spread adoption of real-time PCR as the method of choice for quantitative changes in gene expression. This is called "real-time PCR" because it allows us to actually view the increase in the amount of DNA as it is amplified. Real-time polymerase chain reaction, also known as quantitative real time polymerase chain reaction (qPCR) is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. Quantitative real-time PCR (qPCR) is a sensitive technique for the detection and quantitation of specific DNA sequence that is useful tool to identify and quantify the microorganisms and pathogens accurately in the environmental samples. In the recent years, qPCR based pathogen quantitation is reported for many plant pathogens including filamentous fungal pathogens such as *Fusarium circinatum* (Schweigkofler, et al., 2004); *Alternaria brassicicola* (Gachon and Saindrenan, 2004); *Rosellinia necatrix, Phytophthora nicotianae, P. citrophthora, and Verticillium dahliae* (Schena et al., 2004; Vandemark and Barker, 2003) and *M. oryzae* (Qi and Yang 2002).

Principle

In Real time PCR, using sequence specific primers, the relative copies of a particular DNA or RNA sequence can be determined. We use the term relative since this technique tends to be used to compare relative copy numbers between tissues, organisms, or different genes relative to a specific housekeeping gene. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR Green) or sequence specific probes (TaqMan Probes). The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle.

Primer designing

RT-PCR primers specific to gene are to be designed using online IDT Primer-Quest software available at http://eu.idtdna.com or Primer 3 plus software available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi with the following parameters: optimal length, 20-25 base pairs; GC content, 50-55%; melting temperature, 52-60°C; amplicon length, 120 to 200 base pairs; maximum self-complementarity at the 3′ end -five nucleotides, and absence of stable hairpins & dimers. Primer specificity and quality parameters were checked with the help of Oligo-Analyzer (https://www.idtdna.com/calc/ analyzer*)*.

Template preparation

A critical aspect of performing real time PCR is to begin with a template that is of high purity. The PCR template DNA is to be prepared critically using the commercial available kits to avoid inhibitors which could potentially interfere with cyclic reactions. The concentration of DNA should be about 5-30 nanograms.

Dyes & Fluorescence detection chemistry in qPCR

Probe based Quantitative PCR

Probe based qPCR relies on the sequence–specific detection of a desired PCR product. Unlike SYBR based qPCR methods that detect all double–stranded DNA, probe based qPCR utilizes a fluorescent–labeled target-specific probe resulting in increased specificity and sensitivity. Additionally, a variety of fluorescent dyes are available so that multiple primers can be used to simultaneously amplify many sequences. This chemistry is ideal for high throughput. Ready mixes contain all necessary components for qPCR you simply add the fluorescent detection chemistry, primers and template.

SYBR® Green based Quantitative PCR

SYBR Green I, a commonly used fluorescent DNA binding dye, binds all double–stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the cycle. SYBR Green I has an excitation and emission maxima of 494 nm and 521 nm, respectively. Specificity of Sigma's SYBR based qPCR detection is greatly enhanced by the incorporation of a hot–start mediated taq polymerase, JumpStart Taq.

Two major applications for qPCR in plant pathology is explained below

i. Gene expression analysis using qPCR

Objectives: To estimate changes in gene expression or transcriptional changes in fungi

Quantitative PCR combines PCR amplification and detection into a single step. With qPCR, fluorescent dyes are used to label PCR products during thermal cycling. Real-time PCR instruments measure the accumulation of fluorescent signal during the exponential phase of the reaction for fast, precise quantification of PCR products and objective data analysis. With the help of qPCR we can analyze the changes in gene expression in a given sample (treated sample) relative to another reference sample such as an untreated control sample.

Gene expression analysis using qPCR involves the following steps

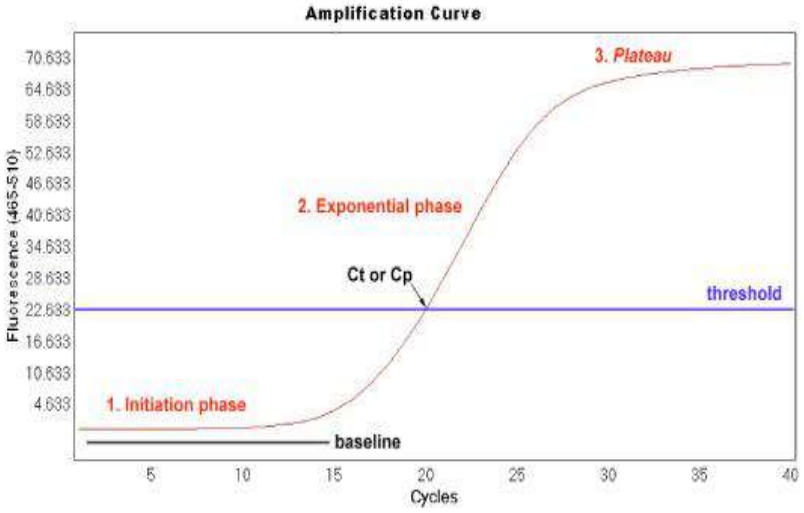
1. Sample Preparation

i. Isolate total RNA
ii. Treat this isolated total RNA with DNAse I to avoid contamination with genomic DNA.
iii. Reverse transcribe this RNA and use the synthesized cDNA as a template for real-time quantitative PCR for gene expression analysis.
iv. Alternatively, nowadays one step qPCR reaction can be performed wherein cDNA synthesis and subsequent amplification can be done in one reaction.

2. qRT-PCR Amplification

i. For PCR amplification, prepare reaction mixture containing cDNA template, gene-specific forward and reverse primers (Design gene-specific RT-PCR primers using primer 3 plus software) and SYBR green mix.
ii. For normalizing expression levels, use a constitutively expressed gene such as housekeeping gene for example 18S rRNA, GAPDH, β Actin etc
iii. Amplify the genes in a Real Time PCR machine. DNA is amplified using an initial denaturation at 95ºC for 3 min, followed by 35 cycles of 95ºC for 15s, annealing for 15s and extension 72ºC for 15s . Reaction is completed with a final extension step of 10 min at 72ºC.
iv. Agarose gel (2.0-2.5%) electrophoresis of the qPCR products can be performed to confirm that the individual qPCR products correspond to a single homogeneous cDNA fragment of expected size.

3. Data Analysis



Amplification plots represent the accumulation of product over the duration of the real-time PCR experiment consists of the following components

1. Baseline: During initial cycles of PCR, there is little change in fluorescence signal. An increase in fluorescence above the baseline indicates detection accumulated PCR product.
2. Threshold line: Point at which a reaction reaches a fluorescent intensity above background. It is set in the exponential phase of the amplification for the most accurate reading.
3. Cycle Threshold, CT: The cycle at which the sample reaches threshold level. CT value of 40 or more means no amplification and cannot be included in the calculations.

i. After visualizing the amplification curve, import the data into Real Time analysis software for further analysis.
ii. The relative expression of genes is calculated using comparative Ct method which involves:
iii. Comparing Ct values of the samples with a control or calibrator such as a non-treated sample.
iv. The Ct values of both the calibrator and the samples are normalized to an endogenous housekeeping gene.
v. This gives ΔCt value of control and the sample.
vi. The comparative Ct method is also known as 2-ΔΔCt method, where ΔΔCt = ΔCt, sample - ΔCt, reference Fold change = Efficiency-ΔΔCt or 2-ΔΔCt (which gives relative gene expression)

ii. Pathogen quantitation using qPCR

Quantitative polymerase chain reaction (qPCR) can detect slow-growing, difficult-to-cultivate, or uncultivatable microorganisms, and can be used when traditional microbiological techniques are inadequate, ambiguous, time-consuming, difficult, and costly. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR Green) or sequence specific probes (TaqMan Probes). The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle. The real-time PCR assay can simultaneously detect and quantitate bacterial, fungal and viral pathogens. Real-time PCR can be a fast diagnostic tool and may be useful as an adjunct to identify potential pathogens.

Objective: To detect and quantify the pathogen in infected tissues using qPCR (Case study: Optimization of Real-time PCR assay for absolute quantitation of *Magnaporthe oryzae)*

Material required:

Real Time PCR reagents
Target specific primers
cDNA of test samples
Micropipettes
Microfuge tubes
PCR tube/plate compatible with thermocycler
RT-PCR thermocycler
Analysis software

Protocol:

Primer designing: qPCR primers specific to gfp- gene are designed using online IDT Primer-Quest software available at http://eu.idtdna.com with the following parameters: optimal length, 25 base pairs; GC content, 50-55%; melting temperature, 60°C; amplicon length, 100 to 160 base pairs; maximum self-complementarity at the 3′ end -five nucleotides, and absence of stable hairpins & dimers. Primer specificity and quality parameters to be checked with the help of Oligo-Analyzer (https://www.idtdna.com/calc/ analyzer).

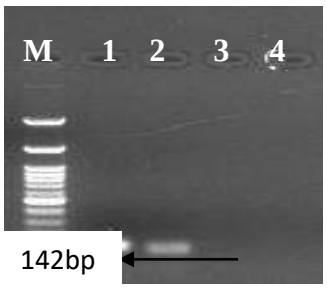
PCR amplification: Real-time PCR will be performed using a 96-well reaction plate (LightCycler® 480 Multiwell Plate 96) and Light Cycler® 96 SW 1.1 (Roche Diagnostics, Switzerland). Each well contains a 20-µl reaction mixture that includes 10 µl of 2× SYBR Green PCR Master Mix (Light Cycler® 480 SYBR Green I Master), final primer concentration of 0.4 µM and three technical replicates with 15 ng of DNA templates. qPCR to be carried out according to the following protocol: denaturation at 95°C for 5 min, 40 repeats of 95°C for 10 s, 61°C for 15 s and 72°C for 15 s. A melting curve analysis will be conducted from 95°C for 10 s, 66°C for 60 s and 97°C for 1 s single time to confirm the amplification of single amplicon. pCAMBgfp vector and wild type *M. oryzae* can be used as positive and negative control in qPCR experiments in order to check the specificity of qPCR based detection assay.

Standardization of absolute quantitation of pathogen biomass: qPCR assay for absolute quantitation of *M. oryzae* is to be optimized using real time PCR primer pairs specific for gfp gene. To construct a standard curve, series of concentration of pCAMBgfp viz., 30000, 3000, 300, 30, 3, 0.3, 0.03, 0.003. 0.0003, 0.00003 pg to be mixed with 15ng of DNA isolated from healthy rice leaves. qPCR to be carried out as described above and a standard graph between amplification threshold values (Cq-values) and template DNA concentration will be prepared. Absolute biomass quantitation to be estimated using the formula (http://cels.uri.edu/gsc/cndna.html) furnished below.

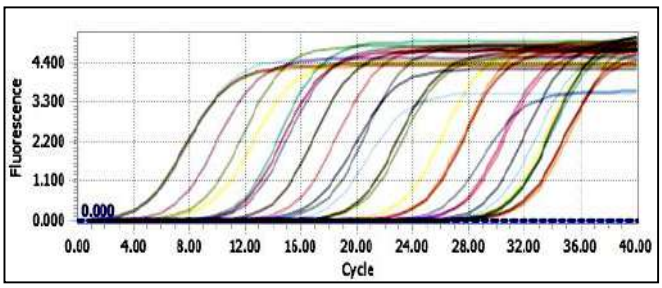
Absolute quantification : $\frac{[\text{DNA concentration X } 6.022\text{x}10^{23}]}{[\text{Product PCR size (bp) X } 10^{9} \text{ X } 650]}$

qPCR based pathogen quantitation by estimation of transgene copy number

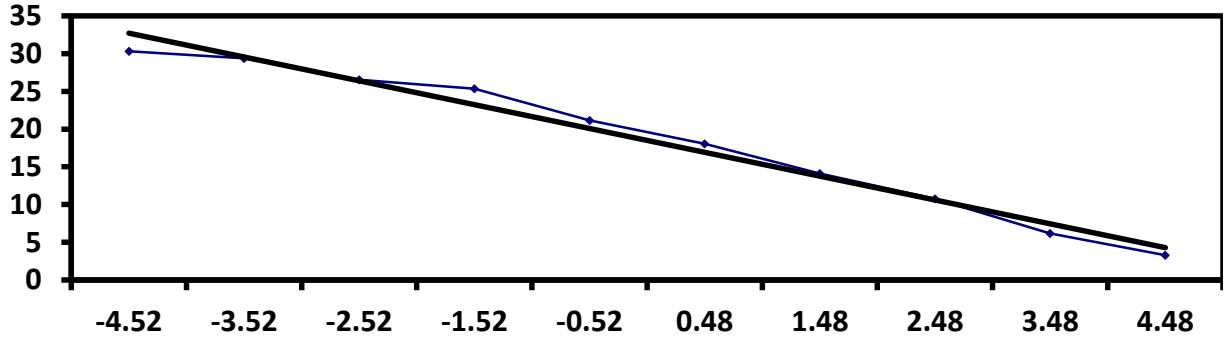
Observation: Primers gfpMgF (5'-GGCCGATGCAAAGTGCCGATAAA-3') gfpMgR (5'-AGGGCGAAGAATCTCGTGCTTTCA-3') will generate a specific 142-bp DNA product for *M. oryzae RMg_Dl::gfp* isolate used in the study. qPCR primers yielded specific signals of *M. oryzae RMg_Dl::gfp* at 19th cycle whereas no amplification was observed with wild type *M. oryzae RMg_Dl* and water control. As far sensitivity, the QPCR assay could detect as low as $30X10^{-8}$ ng (Fig. 1a-c). The standard curve to be constructed based on the DNA of *M. oryzae* ($3.0X10^{1}$ ng, $3.0X10^{0,}$ $3.0X10^{-1}$, $3.0X10^{-2}$, $3.0X10^{-3}$, $3.0X10^{-4}$, $3.0X10^{-5}$, $3.0X10^{-6}$, $3.0X10^{-7}$ and $3.0X10^{-8}$ versus the Ct value obtained in the RT PCR (Fig 1a-c).



a.



b.



c. Gene: gfp; Slope: -2.8802; Efficiency: 2.22; Error: 1.06; R^2: 0.99; Y-intercept: 16.13

Fig. 1a-c. qPCR based method of pathogen quantitation of *M. oryzae*

(a) PCR amplification of 142 bp gfp gene based marker: Amplification by conventional PCR of DNA extracted from pure cultures of *M. oryzae* to assess the specificity of gfpMgF/gfpMgR primers, showing a specific 142-bp product. M. DNA size marker; Lane M 50 bp DNA ladder 1. Vector-pCAMBgfp; Lane 2. *RMg_Dl::gfp*; Lane 3. Wildtype *RMg_Dl*; Lane 4. Water (Negative control)

(b) Real-time PCR amplification and fluorescence readings of DNA extracted from pure cultures of *RMg_Dl::gfp* and wild type *RMg_Dl* which yielded amplification in conventional PCR assays, to assess the specificity of gfpMgF/gfpMgR. The horizontal solid line indicates the cycle threshold

(c) qPCR quantification of *M. oryzae* biomass on rice leaf by plotting cycle threshold (C_t) values from rice leaf DNA against the calibration curve. Each point is the average from three reaction replicates.

Similarly we can quantify the presence of pathogen in infected plants at different time interval during pathogenesis. Sampling can be done at regular time intervals to study the pattern of pathogen behavior within the plant during disease infection.

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Development of diagnostic marker for the identification of fungal pathogens

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Introduction

Accurate and rapid identification of the organism(s) that cause plant diseases is of utmost importance for effective disease management. Conventional methods rely on identification of disease symptoms, isolation and culturing of organisms; and laboratory identification by morphology and biochemical tests. These techniques are used in describing the causal agents of diseases of various crops, however they are too variable, slow, and labour intensive to be of much use in most of the investigations. New, rapid screening methods such as DNA/RNA probe technology and polymerase chain reactions (PCR) technology are being increasingly used in all aspects of fungal diagnostics. The greatest efforts have been on the development of diagnostics based on antibodies and nucleic acid technologies. Nucleic acid-based methods (using probes and/or PCR) have increasingly been used in recent years to develop diagnostic assays for plant pathogens.These methods, particularly those based on PCR, are potentially very sensitive and specific. Two general approaches are used to select target DNA sequences for use in diagnostics: one is to develop a method using known conserved genes, common to all fungi, but which have useful sequence variation within them that can be exploited; the other is to screen random parts of the fungal genome to find regions that show the required specificity. The main DNA region targeted for diagnostic development is ribosomal DNA. Ribosomal DNAs (rDNAs) have a number of useful features that make them well suited as targets for diagnostic methods. They are present in all organisms at high copy number and this allows very sensitive detection. Other genes are also becoming more widely used as targets for diagnostic development and pathogen characterization. The β-tubulin genes are perhaps the second most common targets for diagnostic development in fungi. Targets for development of a diagnostic method can also be sought by screening random regions of the genome to find sequences that are unique to a particular taxon. This was traditionally done by cloning fragments of the pathogen genome and testing individual clones (by DNA probing) to find the ones that are specific to the required target. These specific regions of DNA could then be sequenced to design primers for PCR-based diagnostics. PCR-based techniques such as RAPD (random amplified polymorphic DNA)/ URP (universal rice primers) analysis has been used to generate randomly amplified fragments from the genome. These are then separated by gel electrophoresis and the patterns of bands from related fungi are compared. Any potentially diagnostic bands are then sequenced and used to design specific SCAR (sequence characterised amplified region) primers.

Case Study

Spot blotch of wheat caused by *Bipolaris sorokiniana* is an important disease of wheat, especially in slightly warm (25 ± 1 °C) and humid weather conditions. A quick and reliable PCR-based diagnostic assay has been developed to detect *B. sorokiniana* using a pathogen-specific marker derived from genomic DNA. A PCR-amplified band of 650 bp obtained in *B. sorokiniana* isolates using universal rice primer (URP 1F) was cloned in pGEMT easy vector and sequenced. Based on sequences, six primers were designed, out of which a primer pair RABSF1 (GGTCCGAGACAACCAACAA) and RABSR2 (AAAGAAAGCGGTCGACGTAA) amplified a sequence of 600 bp in B. sorokiniana isolates. The specificity of the marker when tested against 40 isolates of B. sorokiniana, seven isolates of other species of Bipolaris, and 27 isolates of other pathogens infecting wheat and other crops showed a specific band of 600 bp only in B. sorokiniana. The detection limit was 50 pg of genomic DNA. The marker could detect the pathogen in soil and wheat leaves at presymptomatic stage. This sequence characterized amplified region (SCAR) marker designated as SCRABS600 could clearly

distinguish *B. sorokiniana* from other fungal plant pathogens, including *Bipolaris* spp. The utilization of this diagnostic PCR assay in analysis of field soil and wheat leaves will play a key role in effective management of the disease. The materials and protocol for development of SCAR marker is produced below:

Materials

Chemicals CTAB buffer, chloroform, isoamyl alcohol, alcohol, chloroform, isopropanol, TE buffer, primers, dNTPs, $MgCl_2$, Taq Polymerase, DD water, PDA (Potato dextrose agar) and PDB (Potato dextrose broth).

Glassware and plastic ware: Oakridge tube, 250 ml, borosil flasks, eppendorf tube, PCR tubes

Protocols

Isolation of fungal genomic DNA: DNA was extracted from all isolates of *B. sorokiniana* and other species of *Bipolaris* as well as from other pathogens associated with wheat DNA was extracted by CTAB method (described in previous chapters).

PCR amplification using URP

DNA from all isolates is amplified using URP primers. Steps given as under:

- Set up a 25 µl reaction in a 0.2 ml microfuge tube

Template DNA (50 ng)	1.0 µl
10 X Taq polymerase buffer	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
dNTPs (10 mM)	0.5 µl
Primer (10µM)	0.5µl
Taq Polymerase (5 U/µl)	0.5 µl
Autoclaved DD Water	18.5 µl

- Spin briefly in a microfuge to mix.
- The mixture is then placed in PCR machine (i Cycler, BIO-RAD).
- Perform 40 cycles of PCR using the following temperature profile-

Initial denaturation of 94ºC (1 cycle) for 4min

40 cycles of the following:

Denaturation at 94ºC for1min

Annealing at 55ºC for1min

Extension at 72 ºC for1min

Followed by 1 cycle of final extension at 72ºC for10min

Reactions are stopped at 4ºC.

Gel electrophoresis: All PCR products were electrophoresed following the method given in previous chapters

Selection of primers showing monomorphic bands in all BS isolates.

Gel elution: Selected monomorphic band was gel eluted using gel elution kit of Qiagen. Then this product is used for cloning purposes.

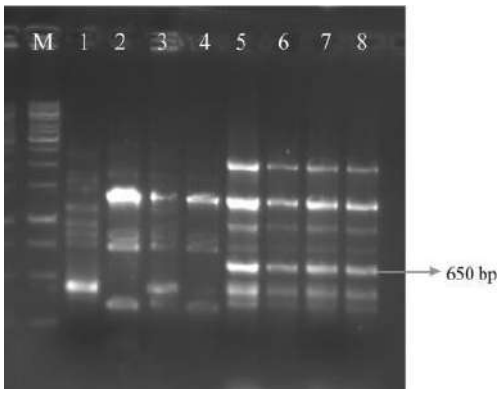

Fig 1 Agarose gel showing the amplified polymorphic DNA band with primer URP 1F in isolates of *Bipolaris* spp. Lanes: M, 1 kb molecular marker (Fermentas); 1, *B. tetramera*; 2, *B. specifera*; 3, *B. maydis*; 4, *B. oryzae*; 5, *B. sorokiniana* BS8; 6, *B. sorokiniana* BS28; 7, *B. sorokiniana* BS34; 8, *B. sorokiniana* BS75.

Cloning of PCR product in pGEM-T Easy Cloning Vector

pGEM®-T Easy vector (3015 bp) from Promega is convenient system for the cloning of PCR products. This vector has single 3'-T overhangs at the insertion site which greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by Taq polymerase which often add a single deoxyadenosine in a template independent fashion to the 3′ end of the amplified fragments. Successful cloning of an insert in the pGEM®-T Easy vectors interrupts the coding sequence of β-galactosidase; recombinant clones are identified by colour screening on indicator plates containing X-gal and IPTG (Fig 2 and 3).

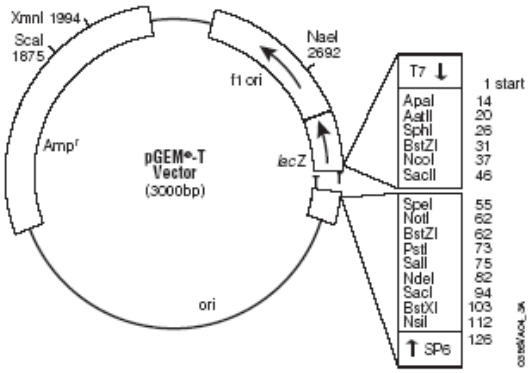

Fig 2 pGEMT vector Map and Sequence Reference point

a. Optimization of insert : Vector molar ratios

1:3 ratio of the vector to DNA insert provides good result. The concentration of PCR product is estimated by comparing with DNA molecular weight markers on a agarose gel.

b. Ligation of PCR product to pGEM-T Easy cloning vector DNA

Ligation reactions are carried out between vector and PCR product. The reaction mix is prepared as follows :

pGEM-T Easy vector (50 ng/µl)	1 µl
PCR amplified product (50 ng/µl)	x µl
2X Ligation buffer	5 µl
T_4 DNA ligase (3U/µl)	1 µl
Sterile distilled water	x µl
Total vol.	10 µl

Despite of standard reaction mix, positive and negative control mix are prepared by adding control insert and no DNA in ligation mix respectively. The ligation mixtures are incubated at 4ºC for 18 hours.

c. Preparation of competent cells

The competent cells are prepared by modified $CaCl_2$ method described by Mandel and Higa (1970).

- 50 ml Luria Broth (LB) is inoculated with overnight grown culture of XLblue strain of *Escherichia coli* and incubated at 37ºC for 1 h. and 15 min. with constant shaking at 200 rpm in a shaker incubator till the bacterial growth as measured by optical density reached 0.3 O.D. at 600 nm
- The culture is then aseptically transferred to 40 ml sterile screw capped tubes and kept on ice for 10 min.
- The cells are centrifuged at 4000 rpm for 10 min. at 4ºC in a Sigma 3K30 centrifuge to pelletize the cells.
- Decant supernatant and re-suspend pellet gently in 10 ml ice cold buffer/100 ml culture aseptically.
- Kept on ice for 1 h.
- The cells are recovered by centrifuging at 3500 rpm for 10 min at 4^0C.
- Decant supernatant re-suspend pellets in 2 ml of chilled buffer and used for transformation after keeping on ice for 1 h.
- Buffer composition: $CaCl_2$ (60mM), PIPES (Piperazine-N, N-bis (2- ethane sulphonic acid) 10 mM (pH 7.0), Glycerol (15 %).

d. Transformation of competent cells

- 200 µl competent cells are added to 20 µl of the ligation mixture in a sterile microfuge tube and is gently mixed and kept on ice for 1 h.
- Then it is given a heat shock at 42ºC for 90 sec. Incubate on ice for 5 min. 1 ml of LB medium is then added and the transformants are allowed to grow at 37ºC for 1 h in shaker incubator at 200 rpm.
- 200 µl of cell suspension are aseptically plated on Luria Agar (LA) plate containing ampicillin, X-gal and IPTG (50 µl of 50 µg/ml ampicillin; 100 µl of 2% X-gal and 10 µl of 0.1 M IPTG in 50 ml LA).
- The plates are incubated overnight at 37ºC.

e. Selection of transformants

The transformants are selected on the basis of blue/white colonies. The white colonies are selected and subsequently streaked on LA (Luria agar) Plates containing IPTG, X-gal and ampicillin.

f. Rapid screening of recombinant by colony PCR Method

Using same primer, white colonies are further screened by polymerase chain reaction. In this case a single colony is taken in each reaction mix in lieu of DNA sample.

Following is the colony PCR reaction master mix.

Set up a 25 µl reaction in a 0.2 ml microfuge tube

Template DNA (50 ng)	1.0 µl
10 X Taq polymerase buffer	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
dNTPs (10 mM)	0.5 µl

Primer (10µM)	0.5µl
Taq Polymerase (5 U/µl)	0.5 µl
Autoclaved DD Water	18.5 µl

The reaction mixture is then placed in PCR machine. The temperature profile and cycle are same as used in amplification of DNA earlier.

g. Isolation of Recombinant Plasmid DNA by Miniprep Method

Isolation of recombinant DNA is done by modified alkaline lysis method (Brinboim and Doly, 1979) or plasmid isolation kit (Xcelris labs, India) as per instruction manual provided in the kit.

- Selected white colonies, positive in colony PCR reaction are individually inoculated in 5 ml of LB medium containing ampicillin (50 µg/ml) in sterile capped culture tubes.
- Tubes are then incubated overnight at 37ºC at 200 rpm in a shaker incubator.
- The overnight grown bacterial cells are then transferred to 1.5 ml sterile eppendorf tube and cells are harvested by centrifuging in a table top centrifuge for 1 min. Care is taken to remove the medium adhering to the cell pellet.
- The pellet is re-suspended in 100 µl of solution I {25mM Tris HCl (pH 8.0), 50mM Glucose, 10mM EDTA} and mixed vigorously by vortexing.
- The 200 µl of freshly prepared lysis solution i.e. solution II (0.2 N NaOH, 1 % SDS) is then added and mixed gently.
- 150 µl of ice cold solution III (3M Sodium acetate pH 4.8) is then added and mixed gently with lysed cell suspension and the mixture is kept on ice for 15 min.
- The chromosomal DNA and the bacterial cell debris are removed by centrifuging at 10,000 rpm for 20 min, at 4ºC in a table top centrifuge (Sigma 112).
- The supernatant is again centrifuged for another 20 min at 10,000 rpm at 4ºC to pelletize any unwanted bacterial debris.
- The supernatant is taken and equal volume of phenol: chloroform : isoamyl alcohol (25:24:1) is added. It was vortexed well, centrifuged in a table top centrifuge for 15 min. at room temperature.
- The clear aqueous phase is transferred to fresh eppendorf tube.
- The DNA in aqueous phase is precipitated by adding 0.8 volume of isopropanol and kept on ice for 10 min.
- The mixture is then centrifuged at 15000 rpm for 20 min at 4ºC.
- To the pellet 200 µl of 70% ethanol is added. The tube is rotated well so that the pellet from the wall gets suspended in 70% alcohol. This ensures removal of adhering salts by 70% alcohol. DNA is then pelletized by centrifuging at 15000 rpm for 5 min.
- The pellet is finally suspended in 30 µl sterile double distilled water.

Gene sequencing of isolated plasmid by DNA sequencer

Primer Designing: New longer and specific primer pairs are designed form the DNA sequence, which is called a **Sequenced Characterized Amplified Region Marker** (SCAR). Primers were designed using Primer-3 software and the primers were synthesized by Xcelris labs (New Delhi, India) to yield a desired PCR product of 600 bp by the set parameters.

Diagnostic PCR

a. Optimization of PCR conditions using designed primer pairs: PCR conditions and cycling pattern are important for specificity and sensitivity of primer amplification.

- Set up a 25 µl reaction in a 0.2 ml microfuge tube 50 ng of fungal DNA with same reaction mix as given earlier.
- The mixture is then placed in PCR machine. Different combinations of temperature profile and cycle durations are standardized for best amplification.
- 40 cycles of PCR using the following temperature profile and duration are performed.

Initial denaturation of 94ºC (1 cycle) for 4min

40 cycles of the following:

Denaturation at 94ºC for 30-50 Sec

Annealing at 55º-68ºC form 30-50Sec

Extension at 72 ºC for 30- 90 Sec

Followed by 1 cycle of final extension at 72ºC for10min

Reactions are stopped at 4ºC.

The optimal annealing temperature was found to be 65°C. One cycle of denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 30s, annealing at 65°C for 40 s and extension at 72°C for 40 s. and a final extension at 72°C for 7 min showed optimal amplification with primer sets i.e., BSF1 and BSR2

Validation of Marker

Testing the specificity, sensitivity and reliability of primers for diagnosis of *B. sorokiniana* at inter and intra specific level as well as at intergenic level.

a. Specificity and sensitivity of BS Primers: The primers were first tested on the all *Bipolaris sorokiniana* isolates. The primer pair which showed best amplification and single band only in *B. sorokiniana* and absent in all others was selected as specific marker. Primer pair BSF1 and BSR2 are selected as specific marker or as SCAR marker. In order to test the sensitivity of this marker for detection of *B. sorokiniana*, 10 pg to 100 ng of serially diluted DNA of the isolate BS-28 was used as DNA templates for PCR amplification (Fig 4).

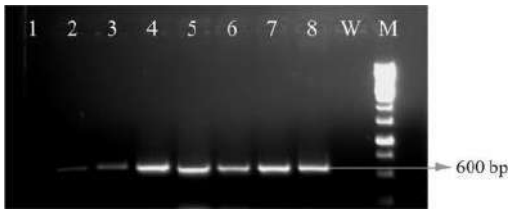

Fig 4. Sensitivity of PCR assays detected by agarose gel electrophoresis using RABSF1 and RABSR2 primers set. Lanes: 1, 25 pg; 2, 50 pg; 3, 100 pg; 4, 400 pg; 5, 1 ng; 6, 10 ng; 7, 50 ng; 8, 100 ng DNA as a template; W, sterile water; M, 1 kb molecular marker (Fermentas)

b. Reliability of BS primers: To test the reliability of *B. sorokiniana* specific primer pair, the PCR assay was conducted with the specific primer pair to detect the pathogen in wheat leaf from 0 to 8 consecutive days after inoculation. The specific primer pair was also used to examine infected samples of wheat leaf and soil from field. The PCR assay was conducted with wheat leaf and soil microbial DNA using specific primer pair to detect the pathogen (Fig 5).

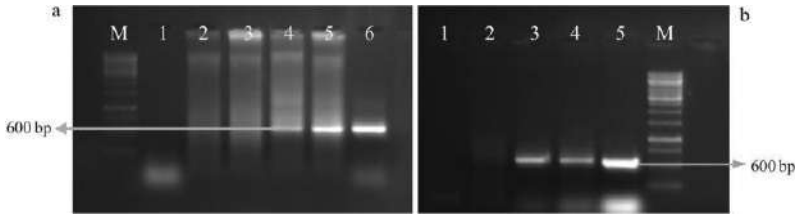

Fig. 5. PCR detection of *Bipolaris sorokiniana* using primer pair RABSF1 and RABSR2 in (a) leaves of wheat seedlings of 'Agralocal' at different times after inoculation (lanes: M, 1 kb molecular marker (Fermentas); 1–5, genomic DNA of wheat after 0, 1, 2, 4, and 8 days, respectively; 6, *B. sorokiniana* DNA template); and (b) wheat leaves and soil samples randomly collected from the Indian Agricultural Research Institute farm (lanes: 1, sterile water; 2, wheat leaves without symptoms (healthy plant); 3, infected wheat leaves; 4, soil sample taken from infected field; 5, *B. sorokiniana* DNA; M, 1 kb molecular marker (Fermentas)).

Exp Expected outcome:

A qA quick and reliable PCR-based diagnostic assay has been developed to detect *B. sorokiniana* using a pathogen-specific marker derived from genomic DNA. Based on sequences, six primers were designed, out of which a primer pair RABSF1 (GGTCCGAGACAACCAACAA) and RABSR2 (AAAGAAAGCGGTCGACGTAA) amplified a sequence of 600 bp in *B. sorokiniana* isolates. This sequence characterized amplified region (SCAR) marker designated as SCRABS600 could clearly distinguish *B. sorokiniana* from other fungal plant pathogens, including *Bipolaris* spp. The utilization of this diagnostic PCR assay in analysis of field soil and wheat leaves will play a key role in effective management of the disease.

Reference

Aggarwal, Rashmi, Gupta, S., Banerjee, S. and Singh, V.B. (2011). Development of a SCAR marker for detection of *Bipolaris sorokiniana* causing spot blotch of wheat. *Can. J. Microbiol*. 57: 934-942.

Points to be remember:

- **If no blue colonies appeared: (a)** Ampicillin is inactive, allowing ampicillin- sensitive cells to grow. Check that ampicillin plates are made properly and used within one month. (b) The bacterial strain (e.g., JM109) has lost its F´ episome. (c) check that the plates have ampicillin/IPTG/X-Gal and are fresh.
- **If not enough clones contain the PCRproduct of interest: (a)** Insufficient A-tailing of the PCR fragment. After the PCRproduct of interest purification of the PCR fragment, set up an A-tailing reaction (8–10). **(b)** Insert:vector ratio is not optimal. **(c)** Multiple PCR products are generated and cloned into the pGEM®-T

Do you know?

10 Units per uL of restriction enzyme solution means *1 uL of enzyme solution should cut 10 ug of DNA*. Normally, for the restriction digests that we perform in lab, 0.5 – 1.0 uL of enzyme is sufficient to cut the amount of DNA in the digest.

How to Optimize Insert: Vector Molar Ratios

To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert: vector (molar ratio)} = \text{ng of insert}$$

DNA Barcoding: A Moderm Tool to Explore Microbial Diversity

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Two and a half centuries after Linnaeus, there are between 1.5 to 1.8 millions of described species, with an estimate that between five and 100 million species await discovery and description (Wilson, 2003). For this reason, the advent of new approaches to stimulate and advance taxonomy, both in terms of investment and popularity, were inevitable (Godfray, 2002; Hebert *et al.,* 2003a; Tautz *et al.,* 2003; Wheeler, 2007; La Salle *et al.,* 2009).

DNA methods aiming to modernize taxonomy were then proposed. Hebert *et al.* (2003a) incited the study of molecular diversity as a means to recognize and identify organisms by bringing up the inherent limitations of morphology, and the steady decrease in the number of specialists available for the task of uncovering our yet unknown diversity. The following limitations of morphology-based taxonomy were mentioned by them:

- Phenotypic plasticity in the characters employed for species recognition lead to incorrect identifications;
- Morphologically cryptic species are often overlooked;
- There is a lack of taxonomic keys to identify immature specimens of many species; and
- Traditional taxonomy requires high levels of expertise in any given group and is therefore restricted to specialists.

Because DNA sequences are unique for each species, they can be viewed as genetic 'barcodes' and have the potential to solve the problems inherent to the kind of taxonomy practiced so far. With a possible nucleotide variation of four nitrogenous bases (A, T, C, G) at each site, there are 4n (where "n" corresponds to the number of nucleotides surveyed) possible codes for any given sequence, making it possible to identify every taxon. The survey of just 15 nucleotide positions can identify 1 billion (4^{15}) species. The identifications can be performed quickly and at low cost, without the need of a taxonomist in the group. Additional advantages of the method would be the possibility of identifying individuals at any stage of development, and the prospect of discriminating between morphologically identical species.

DNA barcoding is a taxonomic system structured on sequence information from a short stretch of a core DNA sequence. A region of approximately 648-bp of the mitochondrial gene cytochrome c oxidase I (COI) was initially proposed as the barcode source to identify and delimit all animal species. The methodology involves the sequencing of that portion of DNA, followed by a comparison with other sequences previously deposited in a database. Species are identified by matching the obtained sequence with sequences of known identity already in the database (Hebert *et al.,* 2003a).

Currently, the molecular identification of species in Fungi is based primarily on nuclear DNA marker (nuclear ribosomal internal transcribed spacer; ITS). But the potential use of mitochondrial markers has also been considered due to their favorable features, among which, above all, their high copy number, the possibility of an easier and cheaper recovering of their sequences. Moreover, the results presented by Seifert *et al.* (2007), would strongly suggest that a mitochondrial gene could really be a good species molecular marker for Fungi, thanks to its appropriate intra and inter-species variability features. Unfortunately, a serious difficulty in the PCR and bioinformatics surveys is due to the presence of mobile introns in almost all the fungal mitochondrial genes and like other mitochondrial genes in these groups, it evolves too slowly for species-level discriminations (Chase and Fay, 2010).

For this reason, alternative stretches of DNA have been proposed as target sequences for the barcoding of these organisms.

DNA Barcoding Initiatives

The Barcode of Life project was proposed to promote DNA barcoding as a global standard for sequence-based identification of eukaryotes. In 2004, this project was formally initiated by the establishment of the Consortium for the Barcode of Life (CBOL), which aims to develop a standard protocol for DNA barcoding and to construct a comprehensive DNA barcode library. Recently, the Barcode of Life project entered a new phase with the launch of the International Barcode of Life Project (iBOL; International Barcode of Life, 2010). The iBOL is a huge international collaboration of 26 countries that aims to establish an automated identification system based on a DNA barcode library of all eukaryotes. In the first five years, the iBOL will focus mainly on developing a barcode library, including five million specimens of 500000 species. The iBOL will also address the development of technologies, including new or improved protocols, informatics, equipment, DNA extraction methods and faster information systems.

The CBOL and iBOL have launched campaigns to build DNA barcode libraries of each animal group. The major targets are fish (Fish-BOL; Ward *et al.*, 2009), birds (ABBI; Hebert *et al.*, 2004a), mammals (Mammalia Barcode of Life), marine life (MarBOL) and insects. The Canadian Barcode of Life Network (BOLNET.ca) was the first national network for DNA barcoding. Subsequently, the following regions or countries have also initiated projects as a part of the iBOL: Europe (ECBOL; http://www.ecbol.org/), Norway (NorBOL; http://dnabarcoding.no/en/), Mexico (MexBOL; http://www.mexbol.org/) and Japan (JBOLI; http://www.jboli.org/). JBOLI provides information and promotes collaborative projects on DNA barcoding in Japan (see http://www.jboli.org/en/projects for relevant projects). There are also thematic programs, such as polar life (PolarBOL), quarantine and plant pathogens (QBOL, as a part of the ECBOL; Bonants *et al.*, 2010) and human health (HealthBOL).

DNA Barcoding and Taxonomy

There is considerable controversy regarding the taxonomic perspective of molecular data, including DNA barcoding (Meier, 2008). There are two principal issues: (i) species identification; and (ii) species discovery. These are sometimes confused. Species identification using barcodes depends on the number of representatives of each species included in the database. The most reliable way to obtain a DNA barcode that accurately represents a species is to base it on the type specimen of that species. The first description of a new species using a DNA barcode from the holotype was by Brown *et al.* (2003), who used this method to describe a new species of *Xenothictis* (Lepidoptera: Tortricidae). Since then, many new species have been described with DNA barcodes from the holotype or paratypes, not only in arthropods, but also in other animals (Burns *et al.*, 2007; Badek *et al.*, 2008; Dabert *et al.*, 2008a,b; Vaglia *et al.*, 2008; Yassin *et al.*, 2008; Yoshitake *et al.*, 2008; Adamski *et al.*, 2009).

Towards Integrative Taxonomy

Everyone knows that traditional taxonomy has serious problems that hinder it progress (May, 2004). Numerous species remain unknown because of the lack of specialists in their groups. Others are only known from their original descriptions, from just the holotype, or have had their type material lost or destroyed. The amount of type material deposited in museums is waiting for a specialist to take interest in it (Padial *et al.*, 2010). Within this context, DNA barcoding cannot be viewed as a threat to taxonomy because it is able to attract interest toward biodiversity studies (Smith, 2005). It is however

necessary to understand that this initiative is not a panacea that will overcome all problems faced by traditional taxonomy. Even though, it can be successively applied when morphology is insufficient.

In fact, one of the main objectives of this initiative, the discovery and description of new taxa, cannot be accomplished with sequence data alone (Ebach and Holdrege, 2005b). As previously mentioned, the superposition of intra- and interspecific variation is a serious problem (Meyer and Paulay, 2005; Cognato, 2006; Meier *et al.,* 2006; Whitworth *et al.,* 2007). This difficulty, however, is not unique to molecular data, and is encountered with other sets of data such as morphology, ecology and other sources (Will *et al.,* 2005). The problems with the sole use of morphology in taxonomy work are also well-known (Packer *et al.,* 2009). Phenotypic plasticity, cryptic species and identification of immature stages are good examples (Hebert *et al.,* 2003b). From this perception that any character system used in taxonomy to the exclusion of others will fall short of the task, the practice of an integrative taxonomy that draws data from different sources is promising. This practice would certainly be superior to the current chaos generated by single data sources and illuminate taxonomic results with complementary sources of data (Will *et al.,* 2005).

A formal proposal for an integrative taxonomy already exists (Dayrat, 2005). This science aims to delimit the units of life's diversity from multiple and complementary perspectives, such as phylogeography, comparative morphology, population genetics, ecology, development, behavior etc. (Dayrat, 2005). One important point of this proposal is, however, the integration of molecular and morphological data. Cryptic species are a good example of the importance of using integrated datasets whenever possible. This procedure can reveal species groups that had not been detected when a given species was initially described based on morphology alone. The use of DNA in addition to morphology helps the recognition of cryptic species that consequently become distinguished based on both sources of characters (Fisher and Smith, 2008, Wiedenbrug *et al.,* 2009, Hamada *et al.,* 2010).

The combination of different sources of data in taxonomy is not new. One good example that dates back to as early as 2003, is Wilson's "encyclopedia of life", a database created with the goal to include information on the natural history, morphology and DNA (EOL, available at http://www.eol.org/) of every species. Lately, the value of an integrative taxonomy has been recognized by the very proponents of the barcoding of life (Smith *et al.,* 2008; Fisher and Smith, 2008; Ward *et al.,* 2009).

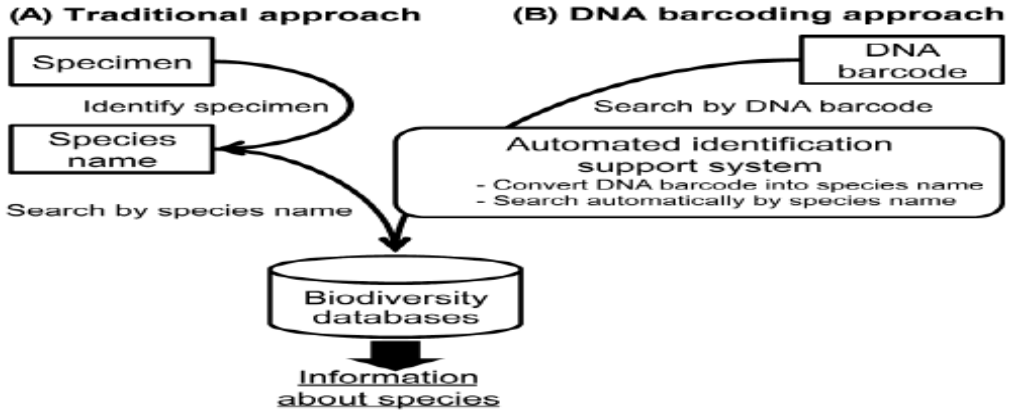

Fig. 1. Workflow for retrieving biodiversity information from databases by (A) traditional approach and (B) DNA barcoding

Conclusion

DNA barcoding has become increasingly common since it was proposed in 2003. Currently, more than one million records are available in the BOLD system, which is the official depository of DNA barcode data. The new large-scale project, iBOL, will accelerate the creation of reference barcode libraries and will facilitate the application of this simple identification method. In the near future, DNA barcoding will become a standard identification protocol for various organisms. As reviewed above, fungal DNA barcoding approach has become less controversial and more supportive in field of taxanomy. However, clear limitations arise from the incomplete coverage of the existing diversity, the inherent characteristics of the mitochondrial DNA (evolutionary rate, inheritance, introns and neutrality) and the single-locus initial strategy. With its enlargement to all eukaryote taxa, the Barcode of life project has also evolved to a more flexible framework. The multi-locus barcoding approach is now commonly accepted, particularly to discriminate between low level taxa and to increase the power of the sequence assignments.

DNA barcoding Protocol:

DNA Extraction

Fungal cultures will be grown at room temperature (25^0 C) in 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth for 3-7 days. Mycelia will be harvested by filtration through filter paper and then this mycelium will be blotted dry with sterile blotting paper and use immediately for DNA extraction. Genomic DNA will be extracted from frozen mycelium of *Fusarium* isolates based on Cetyltrimethyl ammonium bromide (CTAB) extraction method of O' Donnell *et al.* (1998) with partial modification.

PCR Amplification

Different genomic regions of proposed species will be amplified using different primers (Table 1) (Gilmore *et al.* 2009, Wang *et al.* 2011).

Table 1**.** Oligonucleotide primers used for the study

S. No.	Genus	Barcode regions	References
1	*Alternaria*	ITS, IGS	Mmbaga*et al.* 2011
2	*Colletotrichum*	ITS, GPDH (glyceraldehyde 3-phosphate dehydrogenase), GS, TUB2	Hyde *et al.* 2009
3	*Drechslera*	ITS, LSU(Large subunit), EF-1α, B-tubulin	Mehta 2011
4	*Fusarium*	*RPB2*,*CAM*, mtSSU and ND6	Schoch *et al.* 2012
5	*Phoma*	ITS,Calmodulin, Actin	Aveskamp *et al.*, 2008

Purification of PCR products, Sequencing and analysis

PCR products of *Fusarium* spp. will be purified from agarose gel using QIA quick gel extraction kit (Qiagen, USA) following manufacturer's instruction. Purified PCR products will be sequenced using respective primers for all targeted barcode region separately in an automated ABI 3100 Genetic Analyser (Applied Biosystems, USA).

The sequence data will be assembled and analyzed using programme BIOEDIT version 7.0.5. Gene products were predicted using GENERUNNER. Multiple nucleotide sequence alignments were done in BIOEDIT. Dendrograms will be constructed from the aligned sequences using the neighbour-joining method and boot strap option of CLUSTAL X.

Barcode Gap:

Barcode gap of targeted loci will be measured by Mega 5 software (Tamura *et al.* 2011).

Bracode gap= Maximum Intraspecific distance – Minimum Interspecific distance

Probability of correct identification (PCI):

Two kinds of sequence alignment will be calculated between every sample pair, namely (i) a global alignment using the Needleman–Wunsch algorithm, which aligns the entire sequence length with penalties for gaps at the alignment ends (Needleman and Wunsch 1970), and (ii) a semiglobal alignment using a variant Needleman–Wunsch algorithm that includes both ends of one sequence and finds the alignment with the highest score without penalizing end gaps in the other sequence.

For the two types of alignment, the p-distance (the proportion of aligned nucleotide pairs consisting of differing nucleotides) will be calculated. The sequence diameter of a species is defined as the greatest p-distance between any two samples from within a species. Based on the sequence diameter, correct identification of a species occurs if, for every sample in the species, no sample from another species lies within the sequence diameter.

The corresponding barcode gap PCI is the fraction of species correctly identified (Hollingsworth *et al.* 2009).

Database Management:

All the data will be submitted to public database (BOLD; NCBI) as well as to reference database developed for Agriculturally Important *Fusarium* species (*Fusarium barcoding*).

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Metagenomic approaches for studying fungal diversity

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Introduction

Microorganisms are omnipresent in nature and can be found virtually everywhere on earth. Their nature of existence and survival may be as a free living organism or as a symbionts, commensal, ammensal or as a pathogen on higher organisms. The higher organisms belonging to both plant and animal kingdoms provides unlimited, large and diverse habitat for a wide range of organisms, both on exterior and interior. The microbial community associated with animal or plant hosts, or with specific tissues or organs in these organisms, is generally known as "microbiota", and the collective genome of the microbiota is referred to as "microbiome" (Hooper and Gordon 2001). Accordingly, the fungal component of these communities constitutes the so-called "mycobiota", and their genomes are referred to as "mycobiome" (Orgiazzi et al 2013; Iliev et al 2012). Among them fungi are very important component of microbial diversity. Fungal diversity studies have traditionally dependent on morphologic and other phenotypic characteristics and these were for decades the main criteria for fungal classification (Bartnicki-Garcia et al 1987). However, due to the instability of morphological traits, the existence of development associated intermediate forms, and the overlapping phenotypic traits between different taxa, these methods alone do not enable a reliable identification and assessment of fungal diversity at lower taxonomic levels, even at the light of modern techniques (Photita et al 2005; Feau et al 2009; Chapela et al 1991). Molecular taxonomy has partially solved this problem, allowing for better species classification of fungi (Photita et al 2005; Feau et al 2009; Guarro et al 1999; Gehlot et al 2012; Fávaro et al 2011). Since it is widely believed and accepted by many that the ribosomal DNA sequence *per se* is not inherently superior to morphological taxonomy (Seifert et al 1995), the use of hybrid approaches has been the methodological choice in many studies.

Approaches used to study fungal diversity by metagenomic analysis:

Choice of marker sequences for fungal diversity analysis: Conventionally the plant associated microbes are analysed by culture based methods. Unfortunately the culture based methods could not reveal the real microbial composition of the environment. Culture based methods are, most often, known to have bias towards few taxonomic groups. In the recent times high-throughput technologies are developed to decipher the interactions between complex fungal communities and their environment including plant associated niches. For prokaryotes, sequencing of 16S rRNA gene is the most common method used for the identification of different species and strains. By analyzing on 16S rRNA gene, it is possible to taxonomically group different bacteria.

Fungal molecular taxonomic studies were accelerated in the early 1990s and have relied on the analysis of the nuclear ribosomal gene cluster, which comprises the 18S or small subunit (SSU), the 5.8S subunit, and the 28S or large subunit (LSU) genes. However, while the SSU and LSU are efficient in the differentiation of high taxonomic levels, they are not as good for intraspecific resolution of fungi. The ITS1 and ITS2 regions are shown to be highly suitable markers for fungal phylogenetic studies due to their high degree of interspecific variability, conserved primer sites, and multicopy nature in the genome. The utilization of the ITS regions as universal DNA barcode markers for fungi was formalized by Schoch and collaborators in 2012. These regions can be easily amplified and sequenced with universal primers and the corresponding ITS sequence data is highly represented in GenBank and other databases (Kõljalg et al 2005; Pruesse et al 2007). The choice of using either ITS1 or ITS2 is optional since these regions share many properties, and enable similar levels of

discrimination (Bazzicalupo et al 2013). However, ITS2 is generally used because it is less variable in length, lacks the problem of co-amplification of a 5`SSU intron, and is better represented in databases than ITS1 sequences (Nilsson et al 2009).

Choice of technology for fungal diversity analysis: Conventional DNA sequencing relies on dideoxy chain termination technique first described more than two decades ago (Sanger *et al.* 1977). Globally many research groups have put efforts for the development of alternative methodology for DNA sequencing. Three alternative methods that emerged as cost and time effective are i. sequencing by hybridization (Bains and Smith 1988), ii. parallel signature sequencing based on ligation and cleavage (Brenner *et al.* 2000), and iii. pyrosequencing (Ronaghi *et al.* 1998). Pyrosequencing is a DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. As pyrosequencing signals are very quantitative in nature and it is possible to use this strategy for the studies of allelic frequency in large population. Pyrosequencing is now being applied for deciphering microbiome (and virome) analysis. Two main methodological strategies have been used in the study of fungal diversity. The first one is referred to as targetedmetagenomicsand is based on the PCR amplification and sequencing of one or more molecular markers (Suenaga 2012). Even though this approach does not involve direct metagenome sequencing, it is very informative with regard to the microbial community composition and it is becoming increasingly useful in the new field of quantitative metagenomics (Frank and Sørensen 2011). The second approach is the random shotgun sequencing of the metagenome, named shotgun metagenomics (Suenaga 2012) which allows for the evaluation of the whole metagenome, and thus the assessment of the community structure and gene content (Gilbert and Dupont 2011).

Metagenomic experimental pipeline and data handling: The use of high quality nucleotide information and associated metadata will improve the systemic understanding of the diversity in an environment. Pipelines such as *Community cyber infrastructure for Advanced Microbial Ecology Research and Analysis* (CAMERA), *Metagenomic-Rapid Annotation using Subsystem Technology* (MG-RAST) and *Metagenome Analyzer* (MEGAN) perform interactive analysis and comparison of the taxonomical and functional content of shotgun and amplicon datasets (Glass *et al.*, 2010; Sun et al 2012). MG-RAST uses *FragGeneScan* (FGS) and a similarity search of ribosomal RNA identification against a nonredundant integration of the SILVA, Greengenes and RDP databases. CAMERA uses *MetaGeneAnnotator* (MGA), while the *Integrated MetaGenome data Management and comparative analysis system* (IMG/M) employs a combination of tools, including FGS and MGA. In IMG/M genes are predicted and putative gene functions are assigned (Markowitz et al 2012). This annotation can be performed on the entire community and relies on unassembled reads or short contigs. The Galaxy pipeline is suitable for a generic taxonomic representation, in which the reads are aligned (megablast) only against the contents of NT and WGS databases (NCBI). MEGAN is used for visualizing annotation results derived from BLAST searches in a functional or taxonomic dendrogram, and also makes analysis of particular functional or taxonomic groups visually easy. Kosakovsky and collaborators directly compared MEGAN with Galaxy, and concluded the results produced were nearly identical. *Quantitative Insights Into Microbial Ecology* (QIIME) and *Cloud Virtual Resource* (CloVR) are applicable for the 16S, 18S, *nihH,* ITS genes and viral metagenomes. Although several function-oriented reference databases are available, none cover all biological functions, and their function classification system does not follow a same standard. In this context, a framework that allows wide visualization and merges interpretations, such as MG-RAST and IMG/M, seems to be more informative.

The length of the reads has to be taken into account. MGRAST requires 75 bp or longer reads for gene prediction and similarity analysis that provides taxonomic binning and functional classification. IMG/M requires the use of assembled contigs for the analysis of more complex genetic elements. However, when studying a complex community with low sequencing depth or coverage, it is unlikely that many reads will cover the same fragment. In this case, the use of the short sequence setting at the filter homology parameters would allow better recovery from the library. Furthermore, data-processing hardware requirements can present another challenge for the analysis of large datasets. In order to address this, QIIME, CloVR, and Boreal Fungi pipelines can ease computational requirements by clustering near-identical reads, resulting in faster execution. Finally, the use of a web interface to perform comparisons using a number of statistical techniques applied to stored computational results is desired. This feature is present in IMG/M and MG-RAST and is useful in beta diversity analyses, for instance, enabling comparison of novel metagenomes and re-analysis of all datasets. Universal metagenomic pipelines, such as Galaxy, MEGAN, CAMERA, MG-RAST and IMG/M, focus on functional analysis using distinct implementations of common genome operations. Other pipelines, such as QIIME 18S, CloVR-ITS and Boreal Fungi, emphasize alternative phylogenetic markers for fungi. Some of the pipelines mentioned here were designed to accept long reads, such as those derived from Sanger and 454/Roche sequencing, as input. Others, such as QIIME and MG-RAST, were designed to directly accept short-read data from the Illumina/Solexa, SOLiD, and Ion Torrent/Applied Biosystems platforms.

Databases for fungal diversity analysis:

Databases for marker used for diversity analysis: *Assembling the Fungal Tree of Life* (AFTOL), *Specific Primers for Phytopathogenic Agents Data Base* (SPPADBASE), *Database of fungal virulence factors* (DFVF), *The Barcode of Life Data Systems* (BOLD SYSTEMS), *User-friendly Nordic ITS Ectomycorrhiza Database*(UNITE), *Comprehensive collection of the fungal ribosomal RNA Internal Transcribed Spacer 1 sequences* (ITSoneDB) and *Internal Transcribed Spacer 2 Database* (ITS2) are some of the databases useful for designing primers for deciphering fungal diversity.

Databases for storing and analyzing metagenome data: Centralizing resources and standardizing annotations are relevant to address questions of microbial ecology, evolution, and diversity. As studies become increasingly more complex day-by-day and comprehensive, the utilization of correct tools for analysis, storage, and visualization is fundamental to ensure the best outcome from metagenomics. Apart from International Nucleotide Sequence Databases such as NCBI, EMBL, and DDBJ, number of speciality databases are available for documenting the data generated from metagenomic analysis. Many databases are available for fungal taxonomic studies. FungiDB is a resource for genomic and functional genomic data across the fungal kingdom. Another important database used for fungal phylogenetic analyses is PHYMYCO-DB.

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Whole Genome Sequencing: Assembly and Annotation

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Genome

In the fields of molecular biology and genetics, a genome is the genetic material of an organism. It consists of DNA (or RNA in RNA viruses). The genome includes both the genes (the coding regions) and the noncoding DNA, as well as mitochondrial DNA and chloroplast DNA. The study of the genome is called genomics.The genome comprises of three components:

a) Nuclear genome: consist of haploid set of genetic material present in the nucleus of an organism in the form of chromosomes.
b) Chloroplast genome: All angiosperms and land plant cell contain specialized organelle called chloroplast. Chloroplast contain circular DNA molecule called cpDNA. Entire set of cpDNA of an organism called chloroplast genome.
c) Mitochondrial genome: Consist of circular DNA molecule located in the energy generating organelles (Mitochondria) of an organism.

Whole Genome Sequencing:

Whole genome sequencing (also known as WGS, full genome sequencing, complete genome sequencing, or entire genome sequencing) is ostensibly the process of determining the complete DNA sequence of an organism's genome at a single time. This entails sequencing all of an organism's chromosomal DNA as well as DNA contained in the mitochondria and, for plants, in the chloroplast. In practice, genome sequences that are nearly complete are also called whole genome sequences.

Whole genome sequencing has largely been used as a research tool, but is currently being introduced to clinics. In the future of personalized medicine, whole genome sequence data may be an important tool to guide therapeutic intervention. The tool of gene sequencing at SNP level is also used to pinpoint functional variants from association studies and improve the knowledge available to researchers interested in evolutionary biology, and hence may lay the foundation for predicting disease susceptibility and drug response.

Whole genome sequencing should not be confused with DNA profiling, which only determines the likelihood that genetic material came from a particular individual or group, and does not contain additional information on genetic relationships, origin or susceptibility to specific diseases. In addition, whole genome sequencing should not be confused with methods that sequence specific subsets of the genome - such methods include whole exome sequencing (1-2% of the genome) or SNP genotyping (<0.1% of the genome). (Fig. 1).

1. Whole genome shotgun sequencing

Whole genome shotgun sequencing developed and preferred by Celera in the leadership of Craig Venter. This approach preferred for sequencing of prokaryotic genomes which are smaller in size and contain less repetitive DNA. In this method whole genomic DNA is sheared randomly into small pieces and all are cloned into plasmids. Both strands of each clone is sequenced. Once the sequences are obtained, they are aligned and assembled using computer assembly algorithm. This method eliminates the BAC library preparation and physical mapping steps.

2. Hierarchical or Clone-by-clone sequencing:

In this method genomic DNA is sheared into 100-150Mb size pieces and cloned into BAC (bacterial artificial chromosome) or YAC (Yeast artificial chromosome) vectors. BAC vectors are then transformed into *E. coli* for replication and storage. The BAC inserts are isolated and their both ends are sequenced. On the basis of end sequence BACs are mapped to determine the order of each cloned 150 Mb fragment, which is called golden tilling path. Each BAC fragment in the golden path is fragmented randomly into smaller pieces of 2 to 5 kb, cloned further into plasmid vectors and sequenced from both sides. These sequences are aligned so that identical sequences are overlap and assembled into finished sequence using computer assembly algorithm. Thus all BAC clones are sequenced and assembled separately and then single contigs of BACs are used for getting complete stretch of minimum tiling path.

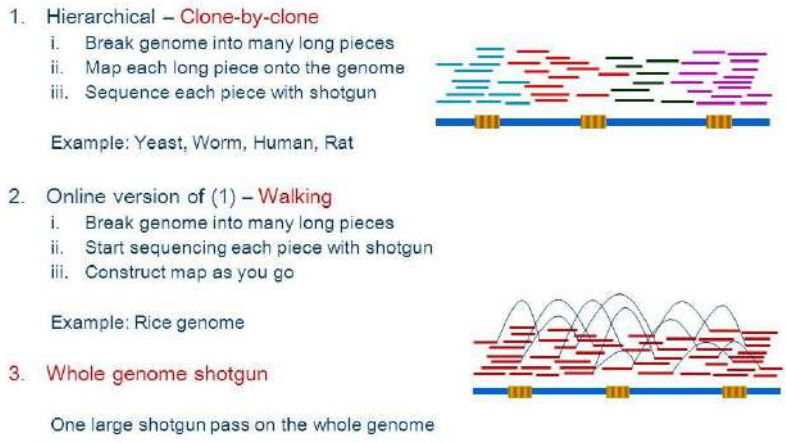

Fig. 1: Strategies for the sequencing of large genomes.

Genome sequencing technologies:

Sanger sequencing

Two DNA sequencing methods were developed in 1977, one by Maxam and Gilbert, which is called chemical degradation method and other was invented by Sanger, which is called chain-termination method. Out of these Sanger method was used for genome sequencing and during Human genome sequencing project, many modifications have been done to convert it into high throughput automated technique. This technique is based on insertion of dideoxynucleotides (ddNTPs) in the chain reaction instead of deoxy nucleotides, which terminate the reaction. The radio labeled or fluorescent dye labeled ddNTPs can help to detect nucleotide at that particular point in DNA fragment. For this first long DNA strands were cut into smaller ones, separated by length in a gel with lanes, and then imaged and read nucleotide sequences. The innovation that made the greatest impact on genomic sequencing was the use of fluorescent dyes and capillaries in an automated sequencing system. Applied Biosystem's ABI 3700, has been the most widely used instrument for large-scale sequencing in many genome sequencing projects before the entry of NGS platforms. This Sanger sequencing machine has 96 capillaries that are fed by robotic loading from microtiter plates. It generates a sequence run every two to three hours for each plate and can read on an average 600-700 bases per run. Celera, the company that produced a rough draft of the human genome in three years, used 200 of these machines running continuously. Similarly, automation was applied to colony picking and plasmid isolation from bacterial colonies to grow up DNA for sequencing.

Next generation sequencing:

Next-generation sequencing methods are group of improved and cost effective techniques compared to Sanger sequencing. These are emerging as the dominant genomics technologies not only for whole genome sequencing but also for expression analysis, epigenetics and marker development. These are new type of sequencing machines with diverse sequencing methods and principles. These new sequencers have capacity to generate genome-scale sequencing even at individual laboratories within short time period. These NGS platforms are described below briefly:

454 Pyrosequencing:

This sequencing technique is based on pyrosequencing, where incorporation of each nucleotide by DNA polymerase results in the release of pyrophosphate (PPi), which in the presence of ATP sulfurylase converts into ATP and ATP in the presence of the firefly enzyme luciferase produce light. The amount of light produced is proportional to the number of nucleotides incorporated. The present platform is Roche/454's GS FLX Titanim, which can generate 0.45 Gb raw data per run with average read length of 330 bp. Pyrosequencing have longer read length as compared to other NGS platforms except single molecule sequencing. Emulsions PCR is used for library preparation in this technique. Long read length and fast run time are advantages of this technique. But main disadvantage is high error rate in homo-polymer repeats.

Illumina (Solexa) sequencing:

This is most extensively used NGS platform for DNA sequencing. PCR clusters are generated on solid phase in this method which are further sequences on same solid phase. Reversible terminator chemistry is used to sequence in this platform. Illumina Solexa's GA instrument the latest instrument which can generate 18 Gb data with read length of 75 bp in 18 days. A low multiplexing capacity of samples is the drawback of this technology.

SOLiDsequencing:

Sequencing of oligonucleotides by ligation and detection (SOLiD) is the sequencing technology where sequencing is done by ligation through cleavable probes. In this technique also emulsions PCR is used for library preparation. Sequence generation capacity of Life/AGP's SOLiD3 is 30 Gb data per run with read length of 50 bp. This sequencing platform takes longest time of 7 days for sequencing one run. This is the most accurate sequencing technology because two-base encoding method provides inherent error correction. The short read length and long run time are the drawbacks of this technology.

Heliscope™ single molecule sequencer

The principle of Heliscope sequencer relies on "*true single molecule sequencing*" (tSMS) technology. The tSMS technology begins with DNA library preparation through DNA shearing and addition of poli-$_{(A)}$ tail to generated DNA fragments, followed by hybridization of DNA fragments to the poli-$_{(T)}$ oligonucleotides which are attached to the flow cell and simultaneously sequenced in parallel reactions. The sequencing cycle consists of DNA extension with one, out of four fluorescently labeled nucleotides, followed by nucleotide detection with the Heliscope sequencer. The subsequent chemical cleavage of fluorophores allows the next cycle of DNA elongation to begin with another fluorescently labeled nucleotide, which enables the determination of the DNA sequence. The Heliscope sequencer is capable of sequencing up to 28 Gb in a single sequencing run and takes about 8 days. It can generate short reads with a maximal length of 55 bases. In a recent development, Helicos announced that it has developed a new generation of "*one-base-at-a-time"* nucleotides which allow more accurate homopolymer and direct RNA sequencing.

Single molecule real time (SMRT™) sequencer

The principle of SMRT sequencer relies on single molecule real time sequencing by synthesis method provided on the sequencing chip containing thousands of zero-mode waveguides (ZMWs). The sequencing reaction of a DNA fragment is performed by a single DNA polymerase molecule, which is attached to the bottom of each ZMW so that each DNA polymerase resides at the detection zone of ZMW.

During the sequencing reaction, the DNA fragment is elongated by DNA polymerase with dNTP's that are fluorescently labeled (each nucleotide is labeled with a fluorophore of different color) at the terminal phosphate moiety. The DNA sequence is determined with CCD array on the basis of fluorescence nucleotide detection, which is performed before nucleotide incorporation, while the labeled dNTP forms a cognate association with the DNA template. The fluorescence pulse is stopped after phosphodiester bond formation, which causes the release of a fluorophore that diffuses out of ZMW. Subsequently, the labeled nucleotide incorporation and detection allow us to determine the DNA sequence. The SMRT sequencer was designed and is still being developed by the Pacific Biosciences (www.pacificbiosciences.com). Although the SMRT instrument has recently been available on the market, the company claims that the SMRT analyzer can be capable of obtaining 100 Gb per hour with reads longer than 1000 in a single run.

Single molecule real time (RNAP) sequencer

A different single-molecule DNA sequencing approach, i.e., *RNA polymerase* (*RNAP*), has been proposed by in which the *RNAP* is attached to one polystyrene bead, whilst the distal end of a DNA fragment is attached to another bead. Each bead is placed in an optical trap and the pair of optical traps levitated the beads. The *RNAP* interacts with the DNA fragment and the transcriptional motion of *RNAP* along the template, changes the length of the DNA between the two beads. This leads to displacement of the two beads that can be registered with precision in the Angstrom range, resulting in single-base resolution on a single DNA molecule. By aligning four displacement records, each with a lower concentration of one of the four nucleotides, in a role analogous to the primers used in Sanger sequencing and for calibration using the known sequences flanking to the unknown sequenced fragment, it is possible to deduce the sequence information. The technique demonstrates that the movement of a nucleic acid enzyme and the very sensitive optical trap method, which may allow extraction of sequence information directly from a single DNA molecule.

Nanopore DNA sequencer

In contrary to all DNA sequencers mentioned above, sequencing a DNA molecule with the Nanopore DNA sequencer is free of nucleotide labeling and detection. This technique was developed from studies on translocation of DNA through various artificial nanopores. The DNA sequencing with Nanopore instrument relies on the converting of electrical signal of nucleotides by passing through a nanopore which is an α-hemolysin pore covalently attached with cyclodextrin molecule – the binding site for nucleotides. The principle of this technique is based on the modulation of the ionic current through the pore as a DNA molecule traverses it, revealing characteristics and parameters (diameter, length and conformation) of the molecule. During the sequencing process the ionic current that passes through the nanopore is blocked by the nucleotide, i.e., the previously cleaved by exonuclease from a DNA strand that interacts with cyclodextrin. The time period of current block is characteristic for each base and enables the DNA sequence to be determined. However, further improvements and modifications in the technique, for example, increasing the number of parameters measured during the translocation of the DNA enabling single-base resolution, could lead to a rapid nanopore-based DNA sequencing technique.

The short read sequences from next-generation sequencers is a major problem for genome assembly particularly in areas associated with repeat regions. Another major problem is accuracy which is slowly improving with new modifications. A main advantage of NGS platforms is its high throughputness. Data generated through these platforms is very high as compared to Sanger sequencing. Other advantage of these technologies over Sanger sequencing is that it can replace the need for *in vivo* cloning by clonal amplification of single molecules individually using either emulsion PCR in 454/Roche and ABI/SOLiD or by bridge amplification on solid surface in Illumina/Solexa platform. These methods use single-molecule templates which can help to detect heterogeneity in a DNA sample.

Genome Assembly:

The genome assembly can be defined as reconstructing the complete sequence of a DNA molecule from short sequence fragments or reads. A schematic presentation of assembly process is shown in Fig. 2. Following are some important terms need to understand assembly in brief.

- Sequence: linear order of nucleotides as they appear onthe DNA molecule
- Read: single observation of the (partial) sequence of aDNA molecule
- Contig: contiguous stretch of sequence, often derivedfrom multiple reads
- Scaffold: linearly ordered and oriented group of contigs
- Coverage**:** the average number of reads that cover a position in the targetsequence

Basic principles of assembly

For the majority of the assembly programmes, the basic scheme is the same, namely the overlap-layout-consensus approach. It consists of the following steps. Sequencing through any platform and quality data in terms of reads. Further overlaps are detected between reads. False overlaps, duplicate reads, chimeric reads and reads with self-matches (including repetitive sequences) are identified and left out for further treatment. The reads are grouped to form a contig layout of the finished sequence. A multiple sequence alignment of the reads is performed, and a consensus sequence is constructed for each contig layout (often along with a computed quality value for each base). Possible sites of misassembly are identified by combining manual inspection with quality value validation. Prior to the assembly, the electropherogram (for Sanger sequencing, images for massively parallel sequencers) for a given sequence is interpreted as a sequence of bases (a read) with associated quality values, these values reflect the log-odds score of the bases being correct. The basecaller PHRED is often used, however alternatives exist, eg. the CATS basecaller. The reads can then be screened for any contaminant DNA such as *Escherichia coli*, cloning or sequencing vector. Low quality regions can be identified and removed. Base quality values can be used in computation of significant overlaps and in construction of the multiple alignments.

Assemblers for genome assembly

In the following a large selection of different assemblers that have been created over time are presented. One of the (relatively) early assemblers is PHRAP, which is still in use, both in itself (for small DNA sequence sets), and as a subcomponent of WGS assemblers, eg. RePS, Phusion, JAZZ, and ATLAS. Other WGS assemblers that also use some variety of the standard overlap-layout-consensus approach are, the Celera assembler, CAP3, RAMEN, PCAP, the TIGR assembler, STROLL, and ARACHNE2. Some new approaches to assembly have been attempted, among them mira and TRAP, which try novel ways to deal with repetitive sequences by checking the trace and quality files. An emerging approach is to use more explicit graph based programs, such as Euler,

Partial ordered alignment (POA), Velvet, Splicing graphs, ASmodeler, and xtract, where the last three are used specifically for ESTs. Other programs that analyze ESTs are TGICL, StackPack, PaCE, Hidden Markov Model (HMM) Sampling, and geneDistiller. Finally, some programs are used in the scaffolding stage, where contigs are processed and put in order, eg. GigAssembler and Bambus (part of the AMOS package).

When different assemblers try to piece the DNA puzzle together they essentially work from the same input, but the assemblers differ in the way they utilize the sequence information, and in the way this is combined with additional information. In general, the differences fall in the following categories. Overlaps: A lot of different methods are used to find potential overlaps between sequences. Some are based on BLAST (eg. geneDistiller), while other assemblers use various other methods to find similarities between reads. Additional information: Depending on how the sequence reads are produced some additional information might be available. This information might consist of read pair information, BAC clone information, base quality information, etc. Some assemblers use this data to impose additional structure on the assembly of the sequences (eg. Gig Assembler). Short read assembly: De novo assembly of the micro reads generated from next generation sequencing platforms is still challenging. While assemblers have been developed and applied to assemble bacterial genomes successfully, on larger genomes the assembly is performed by mapping the micro reads to reference genomes. The major next generation sequencing platforms all have built-in software to handle this task, eg. GS Reference mapper, Gerald for Solexa. In SOLiD systems the mapping tool “mapreads” converts reference sequences into colour space and perform the mapping in colour space. A somewhat related issue is how the sequences are cleaned of contaminant sequences (ie. vector sequences, repeat sequences, etc.). While this can essentially be considered separately and independently from the assembly itself, some assemblers incorporate cleaning in the way they process the reads.

Genome Annotation

The genome annotation is the process oftaking the raw DNA sequences produced by thegenome-sequencing projects and adding the layers ofanalysis and interpretation necessary to extract itsbiological significance and place it into the context ofour understanding of biological processes. The aim of high-quality annotation is to identify the key features of the genome, in particular,the genes and their products.Genomeannotation is a multi-step process, in general falling into three categories: nucleotide-level,protein-level and process-level annotation.

Gene finding is the most visible part ofgenome annotation. In small prokaryotic genomes, gene findingis largely a matter of identifying long open readingframes (ORFs).However, in eukaryotes, defining the precise startand stop position of a gene and the splicing pattern ofits exons among all the non-coding sequence is likefinding a very small and blurry needle in a very largeand distracting haystack. The protein level annotation is sometime appeared to be easier, as annotationgenerally initiate by classifying genes into more manageablegroups or protein families, and by usingsimilarities to better-characterized proteins ofanother species.All the protein signature databases are now integrated into the unified InterProresource. InterPro is across-referencing system for equivalent entries in thePFAM, PRINTS, PROSITE, ProDom, BLOCKS andSMART databases. The most important step toward genome annotation is process level annotation. For this GO is a standard vocabulary fordescribing the function of eukaryotic genes. It consistsof three subparts: molecular function, biological processand cellular component. Molecular function termsdescribe the tasks carried out by individual gene products, such as its enzymatic activity. Biological processterms are used for broader biological goals, such asmeiosis. Cellular component terms describe genes interms of the subcellular structures they are localized to, such as organelles, as well as the macromolecular

complexesthey belong to, such as the ribosome. Blast2GO (www.blast2go.com) is now one stop shop for all type of annotations.

Softwareused for assembly and annotation

As the amount of data generated through genome sequencing projects are growing exponentially, a parallel growth in the tools and methods in data management, visualization, integration, analysis, modeling, and prediction is also increasing. Today bioinformatics tools are the backbone of genome sequencing projects science.

Bioinformatics is used in all the basic areas like sequence analysis, gene prediction, gene expression analysis, protein and metabolite analysis, database management and gene annotation. Many softwares are available for sequence assembly like Phred/Phrap/Consed (http://www. phrap.org), Arachne (http://www.broad.mit.edu/wga/), and GAP4 (http://staden.sourceforge.net/overview.html). TIGR developed a modular, open-source package called AMOS (http://www.tigr.org/software/AMOS/), which is also useful for comparative genome assembly. Many computer programs are available for gene finding and for identifying protein-coding genes by predicting introns and exons in a segment of DNA sequence. Some online programs for gene finding are fGenesh (www.softberry.com), Genscan (http://genes.mit.edu/GENSCAN.html), GeneMarkHMM (http://opal.biology.gatech.edu/GeneMark/), GRAIL (http://compbio.ornl.gov/Grail-1.3/), Genie (http://www.fruitfly.org/seqtools/genie.html), and Glimmer (http://www.tigr.org/softlab/glimmer). For aligning the related genes ClustalX and for proteins ClustalW tool (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) are used. Several sequence-based methods for protein sequences are Pfam (http://pfam.wustl.edu/), ProDom (http://protein.toulouse.inra.fr/prodom/current/html/home.php), and Clusters of Orthologous Group (COG) (http://www.ncbi.nlm.nih.gov/COG/new/). Other methods are based on fingerprints of small conserved motifs in sequences, as with PROSITE (http://au.expasy.org/prosite/), PRINTS (http://umber.sbs.man.ac.uk/dbbrowser/PRINTS/), and BLOCKS (http://www.psc.edu/general/software/packages/blocks/blocks.html).

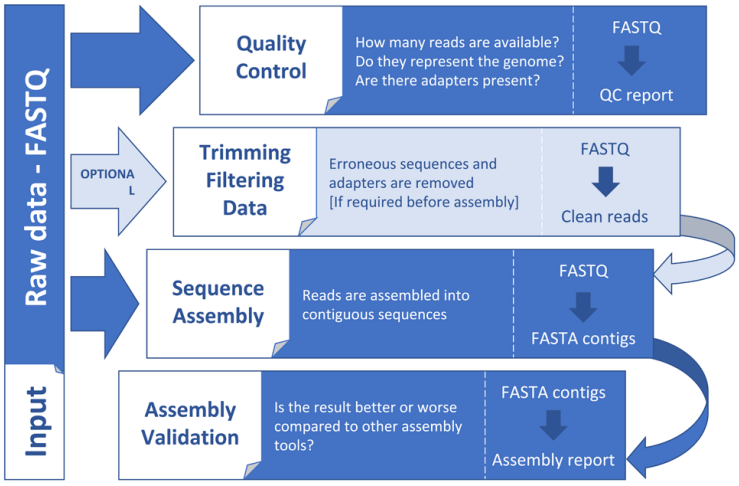

Fig. 2: Overall scheme for whole genome sequencing, assemblyandannotation.

Polyphasic taxonomy and Biosystematics of Fungi

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1. Introduction

Fungi are omnipresent, extraordinarily diverse and perform specialized roles in the environment. They impart many harmful effects such as spoilage and plant and human health damages besides their beneficial role in the production of antimicrobials and bioactive compounds, bioremediation of toxic chemicals and involvement in food, beverages and pharmaceutical industries. Taxonomic information of an unknown microbe is highly essential to establish its biodiversity, relationship among other organisms in the ecosystem and its functional aspects (Gevers *et al.*, 2005). Thus, proper isolation and identification is mandatory before deducing the novel characteristic features of any microbial isolate. With the advent of genomics, the complexity of microbial world is largely understandable. In this regard, recent advancements in microbial systematics have led to a 'polyphasic taxonomic approach' which aims to generate all genotypic, phenotypic and phylogenetic information of a microbial taxon (Vandamme *et al.*, 1996). The resulting biological data of the organisms find relevance and implications in biotechnological research such as production of bioactive molecules, microbial transformations, ecology and bioremediation using microbial isolate or consortium. The prevalent conventional techniques are not sufficient to provide a complete draft for microbial taxonomy as these conventional techniques describe only shape, colour, size, host-range, pathogenicity and assimilation of carbon sources (Prakash *et al.*, 2007). Considerable advancement in the study of microbial taxonomy in the past few decades has taken place such as chemotaxonomy (Minnikin *et al.*, 1975), numerical taxonomy and DNA–DNA hybridization (Johnson, 1991), DNA amplification and sequencing (Konstantinidis and Tiedje, 2007) and whole genome sequencing (Janssen *et al.*, 2003). Despite of this tremendous progress and development of genome based techniques, any individual technique cannot be relied upon solely as a source of taxonomic information. Another problem associated with microbial taxonomic analysis is the existence of some common taxa that cannot be cultured probably due to their protection strategy — viable but not culturable (VBNC) state (Rompre *et al.*, 2002). Hence, no single tool infers definitive assessment of the microbial community, however, it can be studied under two headings i.e. culture dependent and culture independent approaches. Therefore, the use of a polyphasic approach involving a combination of molecular biology techniques and conventional microbiological methods is necessary to obtain a better understanding of microbial diversity.

2. Why use the molecular methods for identification?

Fungi present in the environment can be enumerated, isolated and characterised by various culture dependent classical techniques but they are the most laborious to work with due to their slow growing nature and the conventional practices of identification procedures lead to many ambiguities. Thus, a high level of expertise is necessary for fungal identification (Siqueira and Rocas, 2005). Though the technologies have progressed far beyond the level needed for identification of microorganisms in most laboratories, each procedure has its own lacuna.

3. Catalogue of gene targets for microbial identification

To document different phylogenetic lineages for correlation within genomes of microbes, specific genotypic markers are essential. Varieties of house-keeping genes are targeted in different microbial entities to confer precise genetic fingerprints. There are several genetic markers for rapid cataloguing of gene families having conserved sequences falling within definite microbial clades. Different

genetic targets for microbial identification are 16S rDNA, gyrB, rpoA, rpoB, rpoC, rpoD genes, etc. (Glazunova et al, 2009). 16S rRNA gene is the primary gene target for identification of bacteria as the gene sequences contain conserved, variable and hypervariable regions (Clarridge, 2004). Genetic markers such as DNA metabolic enzyme gyrA and gyrB are also important for identifying microbial diversity in the environment. rpoA, rpoB, rpoC, rpoD are also conserved genes in the ribosomal region effectively used as molecular markers. Multi-locus sequence typing (MLST) is considered to be the "gold standard" for typing of bacterial and fungal species. The MLST scheme identifies internal nucleotide sequences of approximately 400 to 500 bp in multiple house-keeping genes. 26S, 28S and β-tubulin genes are studied for representing molecular relationship within fungal species.

4. Culture dependent identification of fungi

A. Sequence based techniques for fungi

Many techniques have been employed so far for the identification of fungi mainly by using morphological as well as biochemical characteristics such as utilisation of carbon and nitrogen compounds. However, these techniques have less significance due to the occurrence of different morpho/biotypes present within a single species, in addition to being time consuming (Sugita and Nishikawa, 2003). In order to overcome these problems, many advanced techniques have been developed and are described below.

26S rRNA gene sequence:Genetic approaches of studying fungal phylogeny mostly include nuclear rDNA markers. In the case of fungi, rDNA consists of the SSU 18S, ITS, ITS1 + 5.8S + ITS2 and the LSU 25-28S regions (Hibbett *et al.*, 2007). However, ITS has been considered to be the best fungal barcode for identification purposes (Porter and Golding, 2012). It has been reported that the sequence comparison of internal transcribed spacer and D1/D2 26S rDNA spacer sequences using any of the primer sets (Table 1) provide a useful tool for the identification of fungi. Analysis of D1/D2 26S rDNA sequences not only have the advantage of species identification but also permit phylogenetic analysis. However, the major disadvantage is the absence of registered fungal DNA sequences in the DNA data libraries. It is very difficult to phylogenetically analyse ITS sequences as they are more diverse in comparison to the sequences of the 26S rDNA region.

28S rRNA gene sequence:Though many studies of sequence based identification of fungi deal either with the 18S rRNA gene, internal transcribed spacers or the 5′ end of the 28S rRNA gene, the highly conserved 2900 base pair region of the 28S rRNA gene is largely unexplored due to their undefined nature in the genomic database. However, the sequences of the 28S rRNA gene beyond the D1–D2 hypervariable region is a useful tool for the identification procedure of many pathogenic fungi like *Aspergillus candidus,Aspergillus flavus, Aspergillus fumigatus,* and *Candida albicans*. The broad range of fungal rRNA primers can be used in endpoint PCR for amplification and identification of fungi. In this regard, the analytical sensitivity and cross-reactivity testing should be performed for proper validation of the result. Gene sequence based identification of fungi has many advantages over the conventional methods as it does not require viable organisms or sporulation i.e. moulds which are helpful for a rapid identification procedure. Thus the optimal targets for fungal identification are internal transcribed spacer regions i.e. ITS1 and ITS2 and they have been proven to be useful for the identification of yeasts such as *Candida, Cryptococcus, Trichosporon* and mold *Aspergillus* (Petti, 2007). As the ITS regions have many limitations, the alternative gene targets have also been proposed to be useful for fungal identification such as the D1 and D2 domains of the 28S rRNA gene, and elongation factors α-tubulin and β-tubulin (Hall *et al.*, 2003).

β-tubulin gene sequences:Biochemical analysis has suggested the occurrence of two α-tubulin and two β-tubulin genes in fungi. tubA gene is responsible for the production of two alpha tubulin polypeptides i.e. alpha 1 and alpha 2, where alpha 2 is produced by tubB gene. Similarly, beta-tubulin polypeptides beta 1 and beta 2 are produced by benA gene, and beta 3 is produced by tubC gene (Seip *et al.,* 1990). It has been reported that 3.5 times higher phylogenetic relationship can be deduced by analysing β-tubulin genes than by analysing mitochondrial SSU rRNA genes (Craven *et al.,* 2001). Recent analysis of fungal beta tubulin sequences shows that the intron regions of these gene sequences may vary between different lineages (Begerow *et al.,* 2004).

B. PCR based finger printing techniques

Classically, the comparison of DNA fingerprinting banding pattern distribution among taxa is based on the assumption that, bands of the same size are homologous. However, this assumption is correct for the closely related taxa and becomes less reliable for the increasingly separate ones. Thus the assumption of homology can be confirmed by DNA hybridisation (Pillay and Kenny, 1995). In order to enhance the efficiency of PCR fingerprinting techniques, these can be conjugated with hybridisation and southern blotting. The major advantage of this technique involves the considerable easier handling than probing with a single band; however, the disadvantage involves the sharing of sequence similarity with other amplified products of different size (Pillay and Kenny, 1995).

C. Phylogenetic information from whole genome sequences

With the increased availability of completely sequenced genome data, it has become easy to predict the phylogenetic relationship among microorganisms. Though most of the phylogenetic approaches use maximum information available from the whole genome sequence data, there is still the need for selecting the phylogenetic marker genes for individual microorganism (Capella-Gutierrez *et al.,* 2014). Whole genome sequencing approaches can also be applied for analysis of fungal phylogeny as there are now a growing number of complete fungal genomes available (Galagan *et al.,* 2005).

5. Culture independent identification procedures

The vast diversity of living microorganisms in nature are both beneficial and deleterious for plants and human beings and a detailed compact understanding is required for their community structure to deal with their activities, response to environmental stress and climate change (Hirsch *et al.,* 2010). However, only 1% of the total number of microorganisms can be cultured under laboratory conditions and in nutrient rich conditions it can be increased to a mere 10% (Sorensen, 1997). Hence, a new field ‘metagenomics’ has emerged that deals with the investigation of collective microbial genomes obtained directly from the environmental sample without cultivation and without the prior knowledge of the constituent communities (Riesenfeld *et al.,* 2004).

Gradient gel electrophoresis

In order to study the bacterial community structure in a particular environment, culture based approaches don't provide comprehensive information because they fail in cultivation of the predominant microorganisms. There are various other methods available for the culture independent approaches of microbial identification. In culture independent approaches, the total DNA can be isolated from the sample and the desired conserved gene can be amplified by PCR reaction which is of the same size but of different sequences with the aim of generating a distinguishing banding pattern. In DGGE, the organism specific fingerprint is obtained and based upon the banding pattern, the community structure in terms of their quality as well as quantity can be analysed (Fig. 2). Many applications of PCR-DGGE have been described (Bano and Hollibaugh, 2002; Sekiguchi *et al.,* 2002; Crump *et al.,* 2003; Nicol *et al.,* 2003) in the field of soil, sea, river, lake ecosystems. Identification of

species is achieved by purifying and sequencing the bands in the DGGE profile. However, PCR-DGGE analysis is not suitable for the identification of all species, but can be used to screen and group the microbes thus reducing their numbers to be identified by other molecular methods.

6. Polyphasic taxonomic approach: Case study

A polyphasic approach may be adopted for introduction of new fungal species or designing of epitypes which includes molecular, morphological, physiological as well as pathogenic data. In spite of the advancement of the technologies, automation, robotisation and the increasing strength of databases, there should be an international interconnection and accessibility of the technologies available for the development of a better, reliable and sustainable protocol for the identification of any unknown microbial (bacterial, fungal and algal) species (Kampfer and Glaeser, 2012).

Polyphasic taxonomy of the genus Colletotrichum- a case study

Colletotrichum is the causal agent of anthracnose and other diseases on leaves, stems and fruits of numerous plant species, including several important crops. Morphologically-based identification of *Colletotrichum* species has always been problematic, because there are few reliable characters and many of these characters are plastic, dependent upon methods and experimental conditions. Rapid progress in molecular phylogenetic methods is now making it possible to recognise stable and well-resolved clades within *Colletotrichum*. polyphasic approach is recommended for the recognition and identification of species within *Colletotrichum*, matching genetic distinctness with informative morphological and biological characters. An ideal approach for *Colletotrichum* systematics should be based on a multi-gene phylogeny, with comparison made with type specimens, and a well-defined phylogenetic lineage should be in conjunction with recognisable polyphasic characters, such as morphology, physiology, pathogenicity, cultural characteristics and secondary metabolites.

Morphology

Available morphological characters of *Colletotrichum* species include: 1) characters on natural substrata, i.e. size and shape of conidiomata (acervuli), conidia, conidiophores and setae; 2) size and shape of conidia, conidiophores and setae in culture; and 3) size and shape of appressoria. Acervuli, setae and conidial characters (shape and dimensions) on natural substrata can vary due to environmental factors, media and temperature and conidia may be absent from infected host tissues. Some species of *Colletotrichum*, e.g. *C. musae* and *C. gossypii*, consistently fail to produce setae in conidiomata (Sutton and Waterston, 1970) and their presence on natural hosts is often inconsistent for species diagnosis (von Arx, 1957; Sutton, 1966).

Molecular phylogeny

Multi-gene phylogenetics are employed to systematically characterise *Colletotrichum* species relationships and to serve as a base for species diagnosis (Crouch *et al.*, 2006, 2009b; Farr *et al.*, 2006; Damm *et al.*, 2009; Prihastuti *et al.*, 2009). Prihastuti *et al.* (2009) used six genes, the nuclear rDNA internal transcribed spacer (ITS) region, partial Actin (ACT), β-tubulin (TUB2), Calmodulin (CAL), Glutamine synthetase (GS) and Glyceraldehyde 3-phosphate dehydrogenase (GPDH) to study a few closely related *Colletotrichum* species (*C. gloeosporioides* sensu lato) and established that species relationships could be well resolved. Multi-gene phylogenies were also successfully applied to resolve the relationships among *Colletotrichum* species with curved conidia from graminicolous and from herbaceous hosts (Crouch *et al.*, 2009b; Damm *et al.*, 2009). Multi-gene phylogenetics is an accurate and reliable way for the diagnosis of *Colletotrichum* species, but is neither very efficient nor economical. It is currently impractical to apply multiple gene phylogenetics to every *Colletotrichum* species, as different research groups use different gene regions. An international collaborative effort is

essential in order to standardise research being carried out on the genus. Hyde *et al.* (2009) listed all the multi-gene sequences derived from the type or epitype cultures of *Colletotrichum*. This provides an excellent platform for data analysis that aims to study the natural relationships among species.

Physiology, carbon source utilization and growth rate

Colletotrichum acutatum grows significantly slower than *C. gloeosporioides* and can be distinguished by growth rate (Sutton, 1992). Than *et al.* (2008b) and Prihastuti *et al.* (2009) showed a good correlation between growth rate in culture and multi-gene phylogeny in species causing chilli anthracnose and coffee berry disease in Thailand. Colletotrichum asianum could easily be distinguished from *C. fructicola* and *C. siamense* (all from coffee) by its much slower growth rate (Prihastuti *et al.*, 2009).

Pathogenicity testing

Host-specific taxa may have a limited distribution, which may have important biosecurity implications. If the taxon has a wide host range the species is likely to be cosmopolitan and possibly an opportunistic pathogen. Host range studies may provide data useful in classification and future delimitation of species. Pathogenicity tests using *C. acutatum, C. capsici* and *C. gloeosporioides* isolates from chili were carried out on susceptible and resistant varieties of chili (Than *et al.*, 2008b) but names of the *Colletotrichum* species used need to be re-examined in light of a re-assessment of some of these *Colletotrichum* species (Shivas and Tan, 2009).

Secondary metabolite profiles

One of the few studies of secondary metabolites as taxonomic markers for distinguishing *Colletotrichum* spp. involved the use of lectins - proteins or glycoproteins that contain binding sites complementary in shape to particular monosaccharides or oligosaccharides (O'Connell *et al.*, 1998). O'Connell *et al.* (1998) proposed the use of monoclonal antibodies (MAb) in studies of *Colletotrichum* identification and chemotaxonomy because they offer a much wider range of binding sites than lectins. Although antibodies have been used in identification of species such as *C. gloeosporioides* (Peters *et al.*, 1998) their wider applicability in resolving *Colletotrichum* systematics remains to be investigated.

Infraspecific taxonomy

Infraspecific groups within *Colletotrichum* species are poorly understood and have been mostly avoided in the paper on current names (Hyde *et al.*, 2009). The current Code of Botanical Nomenclature provides a few formal infraspecific categories: subspecies, variety and form. Categories such as forma specialis and pathotype have also been used by plant pathologists for infraspecific groups with distinct host specializations or behaviours (Cannon, 2000).

The identification of pathotypes is not only important as a taxonomic tool at the infraspecific level of a *Colletotrichum* species, but has implications for plant breeders trying to develop new improved genotypes with durable resistance to a pathogen. Pathotypic differences actually help to relate the infraspecific taxonomy back to the biological interaction of the pathogen to specific genotypes of a host.

Morphotype, virulence phenotype, phylotype and chemotype were recently used in a polyphasic approach to clarify the taxonomic status of *Colletotrichum* isolates associated with anthracnose disease of yam (Dioscorea spp.) (Abang *et al.*, 2009). Four morphotypes of *C. gloeosporioides* sensu lato were recognised associated with foliar anthracnose of yam: slow growing grey (SGG); fast

growing salmon (FGS); fast-growing grey (FGG); and fast growing olive (FGO). The FGG morphotype showed a greater divergence from the other three morphotypes based on ITS sequence data. Secondary metabolite profiles in high performance TLC (HPTLC) and high performance liquid chromatography (HPLC) showed that the pathogenic SGG and FGS forms had a chemotype (A or B) that was distinct from the non-pathogenic FGG form (chemotype C). A highly phytotoxic HPLC fraction was detected in virulent FGS and SGG strains, but was not detected in the FGG strains. It was not possible to distinguish the pathogenic FGS from SGG forms of *Colletotrichum* based on their ITS based phylotype; however, they could be clearly distinguished based on their combined ITS and metabolite profiles (Abang *et al.*, 2009), which corroborated a previous finding that these strains represented two genetically distinct populations of *C. gloeosporioides* sensu lato on yam (Abang *et al.*, 2005).

7. Concluding remarks

Technological progress has led the advanced techniques to completely dominate the conventional techniques of microbial identification. Despite this, many microorganisms from the environment are yet to be identified and assigned a perfect nomenclature. In the future, more data will be available and more new species of microorganisms will be discovered because of the automation, software development and combination of different databases. Now-a-days there is an increasing access to microbial genomes due to the accumulation of numerous DNA sequences which ultimately leads to a higher level of accuracy and reliability of results. Though polyphasic taxonomy is a useful technique to meet the challenge of identifying any unknown strain, new mathematical and informative strategies should be developed for the possible development of a synthetic taxonomy. Thus, the conventional basis of identification practices cannot be replaced completely and the vast majority of the data should lead to a perfectly reliable and stable identification and classification system. Though many advanced techniques are available now-a-days for the identification of any unknown fungus, a far less number of the total number of microbial species have been discovered and identified till now; many of them are yet to be cultured under laboratory conditions and some of them may possess certain unique characteristic features. Microbial taxonomy and biosystematics is a major modern discipline which needs further financial and intellectual support to determine its role in biotechnology, biodiversity, agriculture, medical science and environmental science.

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