2.1 Ecological Importance of the Hymenochaetaceous Fungi

The hymenochaetaceous fungi include non-lamellate hymenomycetes which inhabit wood and decompose lignin or cellulose ingredients of wood. Species of Hymenochaetales are mainly saprobiotic on dead wood (Donk, 1964; Talbot, 1973; Kendrick, 1992; Alexopoulos et al., 1996) and are considered as the major wood decomposers. They decay forest trees and wood products and cause great economic loss to wood industries. They can be divided into two groups by rotting habits, white-rotting fungi which can degrade lignin as well as cellulose and brown-rotting fungi can degrade cellulose but only slightly modify lignin and leave the decayed wood brown-colored. Three kinds of enzymes are associated with lignolysis. Phenol oxidases such as laccase, lignin peroxidase and manganese-peroxidase have been found in white rot fungi (Eriksson et al., 1990).

As per the studies by Hansen and Goheen (2000), Phellinus weirii is a cause of root rot in conifer forests of western North America, are examined. This pathogen is found to kill trees in slowly expanding mortality centers, creating gaps in the forest canopy. It is widespread, locally abundant, and very long-lived. Phellinus noxius causes brown root rot to fruit and ornamental trees in Taiwan (Ann et al., 2002).

Wood rotting nature of the Hymenochaetaceae members was confirmed by Sharma J.R. (2006). He studied hymenochaetaceous fungi of temperate Himalaya and commented that there is lot of diversity in species composition and richness, host preferences and type of wood rots caused by them.

The diversity of Hymenochaetaceae and its relationship with native plants of Caatinga was investigated by Santos et al. (2010). As per their investigations it
was concluded that Hymenochaetaceae diversity was not significantly related to humidity and a total of 14 species belonging hymenocheataceae were confirmed.

Dai (2012), studied the polypore diversity in China with annotated checklist of Chinese polypores and concluded that majority of the Chinese polypores are white rot fungi whereas 14% cause brown rot.

2.2 Medicinal Importance of the Hymenochaetaceous Fungi

Hymenochaetaceous mushrooms are widely known and consumed by the people due to their nutritive and medicinal properties. Many Basidiomycetes mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium, culture broth. They possess antioxidant, antitumor and antibacterial properties (Ganeshpurkar et al., 2010).

In Korea, 35 species of the Hymenochaetaceae reported having medicinal properties (Lee, 1990; Jung, 1994; Jung, 1995). Among them Phellinus linteus and Phellinus igniarius have been known to have anti-cancer properties, especially against uterine cancer. Inonotus cuticularis, Inonotus tabacinus and Onnia orientalis have been known to be effective as antibiotics and anti-cancer agents (Ahn, 1992).

Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against various allogeneic and syngeneic tumors, and prevent tumor metastasis. Antitumor and hypoglycemic activities of polysaccharides from the sclerotia and mycelia of Inonotus obliquus was confirmed by Mizuno et al. (1999).

Polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host (Wasser, 2002). As per this review in Japan, China, Russia and Korea, several different polysaccharide antitumor drugs have been developed from the fruiting
bodies, mycelia and culture media of mulberry yellow polypore (*Phellinus linteus* (Berk. At Curt.) Teng.) and Chaga or cinder conk (*Inonotus obliquus* (Pers.:Fr.) Pilat.) (Wasser, 2002).

Examination of proteoglycan derived from *Phellinus linteus* by giving oral administration to prevent or treat collagen-induced arthritis (CIA) in mice as experimental model of autoimmune disease was carried out by Kim *et al.* (2003). Their findings suggested that immune-modulating proteoglycan isolated from *P. linteus* may be crucially involved in the prevention and treatment of autoimmune joint inflammation such as rheumatoid arthritis.

Lu *et al.* (2009) confirmed anti-proliferative effects activity in breast and bladder cancer cells from *Phellinus linteus*.

The medicinal mushroom Chaga, has been used as folk medicine in Russia, Poland and Baltric countries, as a cleansing and disinfecting measure; as decoctions for stomach diseases, intestinal worms; liver and heart ailments; and in cancer treatment. It has been reported that chaga provides health promoting functions like antibacterial, hepatoprotective, anti-inflammatory, immunomodulatory, antitumor and anti-oxidant activities (Kim, 2005; Lemieszek *et al.*, 2011).

Medicinal importance of *Phellinus* was confirmed by Ajith and Janardhanan (2007). They studied different medicinal mushrooms occurring in Southern parts of India and indicated that *Phellinus rimosus* have antioxidant and antitumor activities. Their investigations also revealed that fungus had significant antimutagenic and anticarcinogenic activities and thus they stated that Indian medicinal mushrooms are potential sources of antioxidant and anticancer compounds.
2.3 Systematics

Main activities of the systematic can be specified as classification and identification, which fundamentally aim:
1) to discover all branches of the evolutionary tree of life,
2) to document all the changes that occurred during the evolution, and
3) to describe all species (Judd et al., 2002).

Fungal systematic starts with the identification, then continues with determination of relationship of the identified fungus with that of known fungi (Shenoy et al., 2007). This process is further followed by naming and classification of the fungus (Talbot, 1971).

Figure 2.1: Relationships between identification, determination, classification, nomenclature taxonomy, and systematics (Talbot, 1971).

Fungal systematics is an essential part of biological research especially in the context of its ecological and economic implications. Classification systems of organisms are historically based on observable characteristics. This is the phenotypic approach. Classification has been mainly traditional rather than
numerical and has been based on readily observable morphological features. Fungal systematics is still based mainly on morphological criteria, and pathogenic fungi are usually recognized and identified basically by their phenotypes. Numerous alternative approaches have been developed, including nutritional and physiological studies, serologic tests, secondary metabolites, ubiquinone systems, and fatty acids. Although some of these are very useful for identifying poorly differentiated fungi such as yeasts and black yeasts, they are only complementary tools of morphological data in most cases.

2.4 History and classification of Hymenochaetales and Hymenochaetaceae

Friesian system of classification of fungi was modified and changed by many researchers amongst them authors like Quelet (1886) and Karsten (1889) made some changes which finally resulted in the erection of major Hymenochaetaceous genera like Phellinus Ouel. and Inonotus Karst. The genera of Hymenochaetaceae has non-lamellate fruit bodies and has been regarded as a natural family. The dark coloured context which turns permanently black (rarely dark reddish at first) on application of KOH solution and completely clampless generative hyphae have formed a basis for the recognition of the Hymenochaetaceae.

Patouillard (1900) was the first to recognize the recent Hymenochaetaceae as "Série des Igniaires" in his essay and included the group within the Aphyllophoracés. He practically applied microscopic characters such as basidia and basidiospores in the classification of fungi.

Corner (1948) admitted genera only with haplosetae in the “xanthochroic series”, which later corresponded to the subfamily Hymenochaetoideae Donk. Corner (1950) described "xanthochroic series" and included Aurificaria, Coltricia,
Coltriciella, Cyclomyces, Inonotus, Onnia, Phellinus and Phylloporia in the series based on the characteristic of the basidiocarps blackened on KOH solution.

Donk (1948) thoroughly revised and supplemented the classification system with microscopic details. The groups “Serie des Igniaires” and “Serie des Astrostromes” of Patouillard were accepted under subfamily Hymenochartoidae and a family Hymenochaetaceae.

The naturalness of the family Hymenochaetaceae Donk was further emphasized by Corner (1948, 1950); Nobles (1958) and Oberwinkler (1977). The family Hymenochaetaceae was created by Donk, which was further raised to ordinal level (Hymenochaetales) by Oberwinkler (1977).

The unique clampless septum and xanthochroic reaction has rendered many taxonomists to consider the Hymenochaetaceae as a natural monophyletic group. The demarcations between the genera of the family, however, are not clear owing to frequent homoplasies of characters. Characters have been treated differently in importance according to taxonomists. Characters considered to be important in one genus may be ignored in another genus. Inevitable subjectivity in the descriptions of character made the present classifications much more complicated. Descriptions on characters such as the shape and colour of basidiospores, the shape of basidiocarps and the hyphal systems of context are often poorly defined. Phellinus ribis was described to have a monomitic hyphal system and was transferred to Phylloporia by Ryvarden (1972). However, it was not accepted by Larsen and Cobb-Poule (1990) and Larsen et al. (1994). Thus, a lot of synonyms are frequently found in Phellinus and related genera such as Inonotus and Hymenochaete.

According to Ryvarden (1991) this family is one of the most homogeneous among the basidiomycetes and a prime example of strong macromorphological variation over a common set of microscopical characters. The monophyletic origin of the hymenochaetaceous fungi is suggested by the
widespread presence of clampless hyphae, dolipore apparatuses with imperforate parenthesome, the xanthochroic reaction and the production of white rot. The genera of Hymenochaetaceae are complex group of species club together with different characters like morphology of basidiocarp, spore colour, pileal surface and many more.

Overlapping and variable morphological characteristics have made the classification of hymenochaetaceae unreliable and unstable, which has been always a nuisance to mycologists (Hibbett and Donoghue 1995).

In addition, the monophyly of the Hymenochaetaceae has been doubted by some mycologist which was supported by the phylogenetic study of mitochondrial ribosomal DNA sequences (Hibbett and Donoghue 1995). The monophyly of this group was supported by characters such as xanthochroic reaction, yellow to deep brown trama, absence of clamp connections and cause of white rot (Fiasson and Niemelä 1984, Oberwinkler 1977, Ryvarden 1991). Hymenochaetaceae members are morphologically distinct and therefore considered as separate monophyletic unit by most of the authors (Larsson et al. 2006; Hibbett et al., 2007).

The distinction between Phellinus and its closest relative, Inonotus, is the occurrence of perennial fruitbodies and dimitic hyphal systems in Phellinus. However, these characters are not always consistent. In several taxa, the dimitic hyphal system is vague or lacking, for instance, in Phellinus robustus and Phellinus ribis complexes.

The family Hymenochaetaceae is currently classified in the Agaricomycetes and is one of the largest families in Basidiomycota (Kirk et al. 2008) and order hymenochaetales contains 2 families, 48 genera and 610 species (Hibbett et al., 2007; Kirk et al., 2008). As per the estimations by Kirk et al. (2001), Hymenochaetaceae contained 14 Genera and 298 species whereas latest knowledge estimated 27 genera and 487 species (Kirk et al. 2008).
2.5 Recent developments in classification of Hymenochaetaceae

Morphological characters significantly assist in identification of fungi as evident from a plethora of identification keys and morphological descriptions. Morphological characters, however, may not reflect phylogenetic relationships as many of them are subject to plasticity, parallelism, and reversal (homoplasy) (Judd et al., 2002). Phylogenetic Species Concepts and molecular characters, combined together, can overcome most of the limitations associated with Biological Species Concept and the other species concepts. The identification and classification of hymenochaetaceous fungi was dependent mainly on morphological criteria.

In recent years, morphological techniques have been influenced by modern procedures, such as numerical taxonomy, effective statistical packages, and the application of computer facilities, which allow more reliable phenotypic studies to be performed. The phenotypic approach has been largely criticized for its lack of standardized and stable terminology as well as for its high subjectivity. Moreover, some phenotypic characteristics have been considered to be unstable and dependent on environmental conditions. Consequently, there are many fungi that will remain unclassified as long as taxonomists rely solely on phenotypic characteristics.

Molecular biology techniques, especially the analysis of rRNA sequences, are currently used for reliable phylogenetic studies, which enable a more natural classification system to be established. However, despite the effective application of these techniques in PCR mediated identification systems, they are not yet currently available in the routine clinical mycology environment.

The studies based on the North American and European collections it was suggested that *Phellinus s.l.* and *Inonotus s.l.* are heterogeneous. For these studies numerous characters from morphology, anatomy, sexuality, nuclear behavior, pigmentation, and ecology were taken into consideration (Fiasson, 1982; Fiasson
and Niemelä, 1984; Parmasto, 1986; Donk, 1974; Dai, 1996, 1999; Fischer 1996a, 1996b; Parmasto, 2001). Based upon such examinations several attempts made to split *Phellinus* and *Inonotus* into a number of smaller, more natural genera.


Fiasson and Niemelä (1984) made attempt and revised the taxonomy of the European poroid Hymenochaetales and proposed to split the Hymenochaetales into two families, *i.e.*, 1) the Phellinaceae, comprising *Phellinus* sansu stricto, *Fomitiporia*, *Fulvifomes*, *Fuscoporia*, *Inonotopsis*, *Ochroporus*, *Onnia*, *Phellinidium*, and *Porodaedalea*, and 2) the Inonotaceae, comprising *Inonotus* sansu stricto., *Inocutis*, and *Phylloporia*. This system was accepted by Jahn and Jahn (1986), Nuss (1986), as well as Hansen and Knudsen (1997).

The circumscription of Hymenochaetaceous genera is imprecise even with the traditional systematics (Corner 1991). Fiasson and Niemelä (1984) proposed two new suborders for Hymenochaetales, Phaeolinae (comprising only one family, Phaeolaceae) and Hymenochaetinae, comprising families Hymenochaetaceae, Inonotaceae and Phellinaceae (Fiasson and Niemelä 1984).

Since the split suggested by Fiasson and Niemelä, (1984) was only based on European collections (temperate species), and no members from tropical areas, the suggested family concept had been mostly rejected (Gilbertson and Ryvarden, 1986–1987; Parmasto, 1988; Larsen and Cobb-Poulle, 1990; Ryvarden and Gilbertson 1993–1994).
The generic status of the *Fomitiporia* was verified by Fischer (1996b) using analyses of mitochondrial DNA and ribosomal DNA combined with results from sexuality, DNA content of nuclei, and karyology. Dai (1999) accepted the genera *Fomitiporia* and *Phellinidium*, but only granted subgeneric level to *Phellinus* s.s., *Fulvifomes*, *Fuscoporia*, *Phellinidiopsis*, and *Porodaedalea*.

Both *Phellinus* s.l. and *Inonotus* s.l. exhibit the typical characteristics of the order, such as the xanthochroic reaction (darkening of the trama with KOH), the yellow to deep brown trama, the clampless hyphae, the occurrence of setae (not in all species), and the cause of a white rot. The available data on the ultrastructure of the septal pores showed non-perforated parenthesomes to be a common character for the Hymenochaetales (Moore, 1980; Keller, 1997; Müller et al., 2000; Hibbett and Binder, 2002).

It is difficult to distinguish the species in the Hymenochaetaceae even after having good number of collections mainly because of the enormous intra-specific variability, the small and vague differences between the species, and different “concept” of species used by different mycologists. A taxonomic study will be quite fruitful only when there is complete clarity about which species concept and species criteria which is to be used (Parmasto, 1985). Morphological, biochemical, geographical and ecological are the four main groups of criteria which can be used for species discrimination in the Hymenochaetaceae.

It is difficult to study the distinct and constant character between the species, as all the quantitative and most qualitative characters are variable in all species, for example spore size and spore colour, therefore use of statistical methods definitely help to study the variability of characters and to evaluate the significance of the difference between the taxa under study.
2.6 Diversity of morphological characters in Hymenochaetaceous fungi

The natural selection tends to favor morphological elaboration, this was evidenced from the studies on repeated evolution of complex forms (Bonner 1988). Within the fungi, some of the most conspicuous and elaborate forms that have evolved are the fruiting bodies of homobasidiomycetes. The Homobasidiomycetes is a group of Fungi with approximately 16,000 described species (Kirk et al., 2008), including such familiar forms as gilled mushrooms, polypores, coral fungi and gasteromycetes. The fruiting bodies of homobasidiomycetes include some of the most complex forms that have evolved in the fungi, such as gilled mushrooms, bracket fungi and puffballs (‘pileate-erect’) forms.

Species recognition and species concepts are intriguing subjects in fungal biology (Petersen and Hughes 1999). Speciation is a dynamic evolutionary process affected by factors, including geographical distribution, host range and host specificity, genetic compatibility, hybridization events, horizontal gene transfer or gene duplication, that result in phenotypic expression. Taylor et al., (2000) revised the term “species concept” and suggested that diagnosis of species depends on the application of operational concepts such as morphological species recognition, biological species recognition and phylogenetic species recognition.

Hymenochaetaceous fungi are known to have many variations of the basidiocarps some of the variations can be enlisted are effused, effused-reflexed basidioma, few stipitate mushroom-like (agaricoid), coral-like (clavarioid) and spathulate basidiomata. The hymenial surfaces also are variable, ranging from smooth, poroid, to lamellate. Such fruit body forms and hymenial types at one time formed the basis for the classification of fungi.
Micro-morphological characteristics also are exceedingly variable. Generative and Skeletal are the two basic kinds of hyphae which are known to be involved in construction of basidiocarp. Spores are mainly smooth but vary in shape from the large globose to the extremely ovoid. Most species have some kind of vegetative (sterile) cells in the fruit body tissue, often sharing the space with the basidia in the hymenium. They collectively could be called cystidia but because some of them have a distinctive form, unique terms have been introduced for them. The majority of species in Hymenochaetaceae have a characteristic kind of cystidia called setae. Their function is possibly to protect the hymenium from insects (Larsson et al., 2006).

2.7 Taxonomical characters of Hymenochaetaceous fungi

2.7.1 Macro morphology:

Macro morphological characters of such as attachment of basidiocarp, morphology of basidiocarp are important taxonomic characters. It shows xanthocronic reaction to context and dissepiments.

a. **Basidiocarp:** It is the part of fungus that is visible and also called as sporophore, fruiting body, carpophores, fructification, basidiocarp, basidiome. Basidiocarps vary from resipunate to effused-reflexed to pileate in the hymenochaetaceae.

b. **Shape:** Shape of the fruiting body ranges from applanate, convex, effused reflexed, triquetrous, ungulate, and imbricate. The type of attachments can also be described as broadly attached, semicircular, broadly attached elongated, dimidiate, flabelliform, spathulate, central stipe, eccentric type lateral stipe.
c. **Consistency:** this can be understood as per the life span of the fruit body like annual, biennial and perennial. Consistencies of the perennial fruiting bodies shows dominance of skeletal hyphe and are woodier while annual once are softer, fleshy because of monomitic hyphae and usually shrink considerably on drying.

d. **Pileus:** The basidiocarps are distinctly coloured, which is because of the presence of polyphenols and the colour remains reasonably unchanged throughout the life span in Hymenochaetaceae. Such polyphenols are very resistant to degradation by natural causes. Old specimens are usually found to have more intense colour than young once because of increased pigmentation. The pileus cover serves as a protective layer from external influences. The surface can be glabrous or hairy. The degree of hairiness varies from glabrous, velvety, hirsute, to tomentose. It may be dull, glossy or cuticular (Ryvarden and Johansen, 1980). If the pileus is glabrous it can be either completely smooth, with or without any zonation, radial lines, or raised ridges.

e. **Margin:** Most species in the hymenochaetaceae family have more or less continuous margin of several millimeters wide, usually paler than rest of upper surface. Hyphae grow at equal rate and margin become tomentose. Sometimes margin can end abruptly with an almost vertical slope such basidiocarp often have tendency to curl up and loosen along margin under drying.

f. **Pore Surface:** Pore surface of the fruiting body, also known as hymenium, is important character in the morphological study. The colour of pore surface is characteristics and diagnostic feature for many species. Number of pores per mm is important character. With age, the dissepiments in some species may
become thick as they develop at different rates. The outline of pore may be angular and irregular (Ryvarden and Johansen, 1980).

g. **Context:** The context is the tissue of the pileus that lies between the upper surface and the tube layer. It is sterile part of basidiocarp and is homogeneous with regards to both colour and consistency. However in some species context is too distinctly duplex, the lower part being dense and without apparent structural direction, while the upper part is more soft and fibrous which often integrate with a pileus tomentum. The context in Hymenochaetaceae is pale to reddish brown and blackens rapidly and permanently on the application of potassium hydroxide.

### 2.7.2 Micro morphology

Micro morphological characters consist of hyphal system, setae, basidia, and Basidiospore.

a. **Hyphal System:** Types of hyphal systems are fairly consistent to the species and are applied on a wide scale to characterize genera of Hymenochaetaceae. The basidiocarp consists of hyphae. Corner (1932 a, b) clarified and well defined the skeletal hyphae, generative hyphae and this system was major step in the classification of a wood rotting Hymenochaetaceae. He introduced and well described the concept and principles of mono-di-trimitic hyphal system. In this family there are only two types of hyphal systems *i.e.* Monomitic and Dimitic. This system consists of two types of hyphae. Simple septate generative hyphae without clamp connection and thick skeletal hyphae.

b. **Generative hyphae:** These are always septate, thin-walled to thick walled, branched or unbranched to variable degree, hyphae without clamp connections. They are mostly hyaline to pale yellow or golden brown. These hyphae are of
unlimited growth. In dimitic specimens thin walled generative hyphae collapse easily during drying. These are difficult to observe because of dominance of skeletal hyphae in dimitic or perennial species. These are essential component of basidiocarp producing clamp connections and basidia. These are usually freely branched and multisepate.

c. **Skeletal hyphae**: These are originating from generative hyphae. In dimitic species generative hyphae are rapidly replaced by skeletal hyphae. They are normally unbranched or rarely terminally branched, thick walled to solid, septate, aseptate, without clamp, may be hyaline or colored often react with Melezer's solution showing strong reddish brown colour (dextrinoid reaction). Skeletal hyphae often dominate in the context. They are aligned longitudinally in growing region and their tips usually protrude into the hymenium or subhymenium in basidiocarp. Principle element of basidiocarp originating from generative hyphae. Usually not freely branched although certain kinds may show some branching occasionally

d. **Basidiospore**: These are very important in taxonomic point of view. Characters like shape, size, colour, wall thickness and the chemistry are usually checked. Basidiospores are smooth, thin-walled or may be thick-walled and warted. A range of spore size is large. The shape of basidiospore ranges from globose, subglobose, oblong, ellipsoid, ovoid, respectively. They are hyaline or pigmented, pale yellow to golden yellow, light brown to reddish brown or rusty brown.

e. **Setae**: Setae are sterile element occurring in many species of hymenochaetaceae. They occur mostly in hymenial or tramal region, accordingly they are designated as hymenial and tramal setae. They are dark
coloured, thick walled, rigid with spines like organs, usually having more or less pointed apex and the darken (xanthocronic) in alkali. They are yellow to reddish brown in colour; simple or branched varies in size and shape. The setae are subulate or ventricose. Setae may be straight or distinctly bend or hook. Hymenial setae arise terminally from skeletal hyphae or may also be laterally. In such cases, setae will be extended laterally on both sides and may often also be somewhat swollen at base. The tramal setae normally embedded on the trama and run parallel to tube wall or may project obliquely into hymenium. They are usually of considerable, length and are often called giant setae.

2.7.3 Chemical features:

Important chemical test, which have importance in the taxonomic studies are amyloidity or inamyloidity of the spore and hyphal walls. This reaction is most commonly recorded with melzer’s reagent, Spores and hyphae are said to be amyloid if they colour grey or blue and dextrinoid if they turn reddish brown. Except for few species of *Phellinus*, *Fomitiporia* and *Coltricia* where the spore and hyphae show a dextrinoid reaction, the species of Hymenochaetaceae are generally negative in Melzer’s.

Due to presence of styrylpyrone pigments in the basidiocarps of Hymenochaetaceae (Fiasson, 1982) the positive reaction of Potassium Hydroxide permanently darkens the brown hyphae. This reaction is known as xanthochroic reaction and so the name “xanthochroic series” is given to the family.

Hyaline spores of some species of *Inonotus* and *Phellinus* can sometimes be better observed in cotton blue-lactophenol. For the clear observation of setae, tramal setae and setal hyphae, it is better to mount thin sections of basidiocarp in lactophenol.
2.8 Principal Component Analysis

Principal component analysis is useful when one has data on a number of variables and there is some redundancy or some correlation in those variables possibly because they are measuring the same construct. Principal component analysis (PCA) is a standard tool in modern data analysis in diverse fields. PCA provides a roadmap for reducing the complexity in data set and to reveal the hidden relationships between the characters. It can be defined as a linear combination of optimally-weighted observed variables.

Phenetic taxonomy is based on analysis of many unweighted characters. The number of variables that can be analyzed are is so high that it is necessary to use a mathematical tool for grouping them into units corresponding to taxa. PCA enables to reduce the number of possible groupings. Principal Component Analysis replaces many original characters with only a few most significant principal components (PCs) which represent combinations of closely correlated original characters.

Principal Component Analysis was first described by Pearson (1901). In the course of performing a principal component analysis, it is possible to calculate a score for each data set on a given principal component. The Principal Component Analysis (PCA) is widely used in taxonomic research of plants (Sahuquillo et al., 1997; Wolff et al., 1997; Chiapella, 2000; Lilburn and Garrity, 2004; Véla et al., 2007)

The first component extracted in a principal component analysis accounts for a maximal amount of total variance in the observed variables, which means that the first component will be correlated with at least some of the observed variables. The second component will have two important characteristics and this will account for a maximum variance in the data set that was not accounted for by the first component. The second component will be correlated with some of the
observed variables that did not display strong correlations with component 1. A principal component analysis proceeds in this way, with each new component accounting for progressively smaller and smaller amounts of variance (this is why only the first few components are usually retained and interpreted). When the analysis is complete, the resulting components will display varying degrees of correlation with the observed variables, but are completely uncorrelated with one another.

2.9 Molecular systematics studies in Hymenochaetaceous fungi

In the past two decades, molecular investigations of systematic problems have progressed from uncommon curiosities to a standard means of elucidating phylogenetic history. A primary objective of phylogenetic studies is to reconstruct the evolutionary history of a group of organisms. Considering that the organisms under study have a single history, systematic studies of any set of genetically determined characters should be congruent with other such studies based on different sets of morphological characters in the same organisms. Molecular techniques are becoming more important than ever as means to study taxonomic and phylogenetic relationships among fungi.

It was around 90s, molecular systematics began to have a profound influence on the classification of fungi (Bruns et al., 1990; Bruns et al., 1991; Eriksson and Hawksworth, 1991; Hawksworth, 1994; Taylor et al., 1994; Walker and Doolittle, 1982; White et al., 1990; Kim and Jung, 2000). Molecular systematics has contributed to the classification of fungi at all taxonomic levels, being particularly useful in the delimitation of families and orders, primarily using nuclear ribosomal gene sequencing, in particular the large subunit ribosomal DNA (28S rDNA) and small subunit ribosomal DNA (18S rDNA) gene (Berbee and Taylor 1992).
Molecular characters are also known to be subject to convergence and parallelism (Judd et al., 2002). Molecular characters such as DNA sequence-data are essentially advantageous as they offer a greater number of discrete characters, which can be analysed statistically to infer phylogenetic relationships. Molecular methods for studying the taxonomy are now universally applicable. Comprehensive and detailed reviews of the use of molecular techniques in fungal systematics have been provided by Bruns et al. (1991), Hibbett (1992) and Kohn (1992). Two important technical advances have stimulated the use of molecular techniques, firstly, the PCR, which has allowed the analysis of small number of fungal cells or even single spores, dried herbarium material or extinct organisms and secondly, the selection of universal oligo-nucleotide 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 sub-specific entities with respect to the analysis of populations and their mode of reproduction.

Hymenochaetaceae members are morphologically distinct and therefore considered as separate monopyletic unit by most of the authors (Larsson *et al.* 2006). The monophyly of Hymenochaetaceae cannot be supported by molecular phylogenetic analysis. An attempt was made by Wagner and Fischer (2001; 2002a, 2002b) to classify the hymenochaetales based on molecular phylogenetic
analyses which was further justified by morphological, physiological and ecological characters. Though many authors rejected the monophyly of the group some authors still accepted its monophyly stating the exceptions of genera *Coltricia* and *Coltriciella*. It is also worth noting that Hymenochaetaceae can now only be defined only in terms of molecular data (Larsson *et al.* 2006).

Molecular systematics had been shown to be a valuable tool in modern fungal taxonomy (Bruns *et al.*, 1991). Among various molecular methods including RAPD, RFLP, and sequence analyses, phylogenetic analyses of rDNA sequences are known to have the highest resolving power (Bruns *et al.*, 1991).

RFLP analysis was used as a tool for identification of lignicolous basidiomycetes (European polyporales) by Fischer and Wagner (1999).

Genetic and morphological variability of *Phellinus torulosus* isolates growing on oak wood was studied by Campanile *et al.* (2004a). The random amplified polymorphic DNA (RAPD) technique using polymerase chain reaction (PCR) was used to explore the genetic variability in 138 isolates of *P. Torulosus* from 139 oak woods of Apulia and Basilicata (Italy). Campanile and Luisi (2004b), carried out molecular analysis study with the help of intergenic transcribed spacers-restriction fragment length polymorphism (ITS-RFLP) and sequencing of the ITS region. They confirmed the results obtained with microscopy analysis.

Ribosomal RNA genes form a mosaic pattern of conserved and variable regions which makes taxonomic analysis possible at many levels. Percentage of sequence variability in a given region is different in different fungal taxa and no unique regions can be used to identify all fungal species or to discuss phylogenetic relationships among all fungi. More variable regions are used for systematics at lower taxonomic levels (Vilgalys and Hester, 1990; Zambino and Szabo 1993; Moncalvo *et al.*,1995a, 1995b; Moncalvo *et al.*, 2002).
Due to ubiquitous occurrence and essential functions, regions most commonly used for phylogenetic analyses are nuclear and mitochondrial small subunit rRNAs, nuclear and mitochondrial large subunit rRNAs, and internal transcribed spacers (ITSs) (Zambino and Szabo 1993; Moncalvo et al., 1995a,b). The ITS occurs between coding regions for nuclear small subunit and large subunit rRNAs and intergenic regions (IGR).

The nuclear small subunit rRNA gene region is appropriate for analysis at or above the level of orders (Berbee and Taylor 1992; Hofstetter et al., 2002). The mitochondrial small subunit rRNA gene region may be useful among species or genera of fungi (Bruns et al., 1990). Non-coding portions of the ITS may be more variable than coding regions (White et al., 1990) and are appropriate for the analysis of closely related species in the fungi (Zambino and Szabo 1993).

Sequences of 18S rDNAs are conserved and have been used in phylogenetic analyses of fungi of higher taxonomic ranks such as classes or orders. On the other hand, ITS rDNAs are so variable that they often cannot be
aligned accurately between genera and are now commonly used in the systematics of species within a genus (Moncalvo et al 1995a, b). However, mtSSU rDNAs were reported to evolve 16 times faster than 18S rDNAs, but are less variable than ITS rDNAs (Bruns and Szaro, 1992; Hofstetter, 2002). Thus they are believed to have a potential to fill phylogenetic gaps at a family level between those available from 18S and ITS rDNAs (Hong et al., 2002; Moncalvo et al., 2002).

In addition, the IGR can be used for race identification in some fungal species (Moncalvo et al., 1995a). The ITS region was selected in the phylogenetic study of Phellinus and related genera because it has been recognized to be appropriate in the phylogenetic study of interspecific or intraspecific level (Zambino and Szabo, 1993).

The low rate of polymorphism in the ribosomal DNA (rDNA) transcription unit allows characterization of the rDNA of each species using only a few specimens, and makes this DNA useful for interspecific comparisons. In addition, the different coding regions of the rDNA repeats usually show distinct evolution rates. As a result, this DNA can provide information about almost any systematic level (From Wikipedia, the free encyclopedia).

Furthermore, ribosomes are present in all organisms, with a common evolutionary origin and are highly conserved (van de Peer et al., 1996; van de Peer et al., 1997) as well as they serve as reference points for evolutionary divergence studies. Comparisons of the 18S, the small-subunit (SSU) rDNA sequences have been performed to assess the relationships of the major groups of living organisms (Woese, 1987; Woese et al., 1990).

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. 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 (Walker and Doolittle, 1982).
Hibbett and Donoghue (1995) used sequences of mitochondrial small subunit ribosomal DNA to infer the phylogenetic relationships of the Polyporaceae and the parsimony analyses suggested that macromorphology is evolutionary flexible but certain anatomical and physiological characters contained clues to higher order relationships.

Phylogenetic relationships of mushrooms and their relatives within the order Agaricales was studied by Moncalvo et al., (2000) using nuclear large subunit ribosomal DNA sequences. Their phylogenetic analyses revealed that many groups of agaricoid fungi that are consistent with morphology-based classification schemes. Analyses also support separate placement of the boletes and russules and this study provided a framework for future systematics studies in basidiomycetes for analyzing large molecular data sets.

Molecular analysis of nuclear large subunit of *Phellobilus* was performed by Nimellä et al. (2001) to establish the generic relationship between *Phellobilus*, *Phellinus* s.l. and *Inonotus* s.l. They revised the microscopy of *P. nigrolimitatus* and species turned out to differ enough to be separated into new genus *Phellobilus*.

Studies in the phyllogeny and taxonomy of genus *Phylloporia* with the help of large subunit of the nuclear encoded ribosomal DNA was performed by Wagner and Ryvarden (2002). Molecular sequence data as well as morphological and anatomical features showed the genus to be monophyletic and related to *Fulvifomes*. The phylogenetic relationships of *Phylloporia* were discussed, in addition the genus was monographed and 12 species were accepted and described with a key.

The aim of the study by Tomšovsky and Jankovsky (2007) was to compare extraordinarily grown basidiocarps of *Fuscoporia torulosa* on *Pyrus* with those growing on the base of oak trees according to their DNA sequences to reveal a possible process of speciation. The so-called ITS region of nuclear
ribosomal DNA was chosen for the study. Results did not reveal any significant differences between basidiocarps with the distinct fructification pattern, so all examined specimens belong to one species. The relation of *Fuscoporia torulosa* to *Phellinus senex* is discussed.

**DNA isolation**

DNA from basidiomycetous fungi is usually extracted from living pure cultures. This culture maintenance is a tedious work and furthermore, cultivating even small amounts of fungi requires more time (Blackwell and Chapman, 1993). DNA can be extracted from frozen field collections of fruiting bodies or even dehydrated / dried fruiting bodies therefore cultivating specimen is not the prime requirement of DNA extraction.

However, there are some problems associated with direct DNA extraction from field-collected and herbarium fungal specimens:

(a) high content of polysaccharides and/or secondary metabolites,
(b) action of phenoloxidases and
(c) DNA degradation (Moncalvo *et al.*, 1995a,b).

Several protocols have been proposed for the extraction of DNA from fungal tissues, such as SDS-based (Lee and Taylor, 1990), CTAB-based (Rogers and Bendich, 1985), glass-beadbeating (Smit *et al.*, 1999) and various commercial DNA extraction kits (Loffler *et al.*, 1997). Although the first two methods are time-consuming, the others require the purchase of specialized instrumentation (glass-bead-beating) or have a high cost per sample (commercial DNA extraction kits) and thus may be prohibitive for routine work in Mycology laboratories. The extraction of DNA from fungi has sometimes been notoriously difficult. Fungi often produce polysaccharides and a variety of secondary metabolites, including many phenolic compounds, in copious amounts, that cause problems in DNA
extraction (Rogers, 1994). Furthermore, long-lived basidiomata of many Basidiomycetes species are coriaceous or even woody hard, which poses additional difficulties in DNA extraction.

**Haplotype**

There are many efforts which have identified millions of genetic markers that can be used in genetic studies. Although each marker can be analyzed independently of other markers, it is much more informative to analyze markers in a region of interest simultaneously. The combination of marker alleles on a single chromosome is called a haplotype (*Haploid Genotype*). There is great interest in understanding haplotype structures using identified genetic markers because:

1) haplotype structures may provide critical information on evolutionary history and the identification of genetic variants underlying various traits; and

2) molecular technologies now make it possible to study hundreds of thousands of genetic polymorphisms in population samples of reasonable sizes.

For haplotypes including markers tightly linked with each other, for example, markers within the same gene, alleles at these markers often display statistical dependence, a phenomenon called linkage disequilibrium (LD), or allelic association.