Chapter 2

Reappraisal of Literature

The genus *Suillus* Gray was described by Samuel Frederick Gray with *Suillus luteus* (L.) Roussel as type species (Gray 1821). Later, the genus has been emended by Smith and Thiers (1964). They are commonly known as "slippery jacks" because of the pileus being slimy especially under wet conditions. The genus epithet is derived from the Latin word “sus”, which means "pig". The genus is generally characterized by viscid, slimy and readily decaying pileus, usually with sudecurrent to decurrent tubular hymenophore and central to excentric stipe often with conspicuous punctuate or glandular dots on its surface. Spore prints vary from light brown through yellow brown to dark chocolate brown and finally to olive shades. Microscopically, clustered cystidial elements usually occur in fascicles and contain colored amorphous pigments around the base of bundles or over the cystidia themselves. Generally, clamp connections are absent from the hyphae of the *Suillus* sporocarps. Mostly they form mycorrhizas with members of family the *Pinaceae* (particularly *Pinus*, *Larix* and *Pseudotsuga*), but a few species are also found associated with hardwoods. Main distinguishing inter-specific characters for species delimitations are color and ornamentation of the pileal surface, color and color change of flesh, tubes, pores and stipe, the presence/absence of a partial veil or annulus, pore shape and distribution, host, as well as the habitat. All *Suillus* species are edible, but many consider them undesirable due to their slimy cap cuticle. Removal of slimy cap cuticle and tube layers is highly advised before their consumption (Weber and Smith 1980). Their odor is mild and taste is mild or slightly acidic. There are about 30 edible *Suillus* species known worldwide (Boa 2012). Typical example of edible *Suillus*

is *S. brevipes* (Orr and Orr 1979), which contains good amount of lipid content mainly linoleic acid, oleic acid and palmitic acid (Sumner 1973).

2.1 Systematic position of *Suillus*

Suillus is a genus of basidiomycete fungi belonging to the family *Suillaceae* and order *Boletales*. Before 1997, the genus *Suillus* was placed under the family *Boletaceae* of order *Boletales*. There is relatively extensive literature documenting the systematic and taxonomic organization of the order *Boletales* using morpho-anatomical characteristics (Singer 1986; Agerer 1999), pigment chemistry (Gill and Steglich 1987; Besl and Bresinsky 1997), and the sequence analysis (Bresinsky et al. 1999; Kretzer and Bruns 1999) of the basidiocarps. These findings showed remarkable variance in morpho-anatomy, hymenophores and pigment composition of basidiocarps within the *Boletales*. As a result, numerous new families and genera have been generated.

Scientific Classification (Kirk et al. 2008)**:**

Kingdom: Fungi

Subkingdom: Dikarya

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Boletales

Family: *Suillaceae*

Genus: *Suillus*

On the basis of chemotaxonomic findings, *Suillus* species were found to be closely related to the *Gomphidiaceae* and *Rhizopogonaceae* as compared to the remaining *Boletales* (Besl and Bresinsky 1997). These results led to the formation of a new

suborder *Suillineae* within the *Boletales*, which included *Gomphidiaceae*, *Suillaceae*, and *Rhizopogonaceae*. Thus, the genus *Suillus* was carved out from the family *Boletaceae* and now placed in the newly formed family *Suillaceae* along with the genera *Psiloboletinus* and *Truncocolumella*.

2.2 Diversity

About 50 species of *Suillus* have been reported from different parts of the world (Kirk et al. 2008). Among them majority of the species are described by Smith and Thiers (1964, 1971), Corner (1972) and Thiers (1976, 1979). Thereafter, several other investigations on diversity and evolutionary phylogenetics of *Suillus* species from different parts of the world have been reported (Kretzer et al. 1996; Kretzer and Bruns 1997; Wu et al. 2000; Manian et al. 2001; Beatriz et al. 2006; Feng et al. 2008; Bruns et al. 2010; Min et al. 2014; Sarwar and Khalid 2014). Kretzer et al. (1996) derived the ITS sequences from 47 isolates belonging to 38 recognized species of the genus *Suillus* from America, Canada, Europe and Asia. They also revealed that the generic and species concepts of *Suillus*, *GastroSuillus*, *Boletinus* and *Fuscoboletinus* should be reevaluated and suggested the collapsing of genera *Boletinus* and *Fuscoboletinus* into the genus *Suillus*. Later on, the genus *Gastrosuillus* was also collapsed into *Suillus* on the basis of ITS data (Kretzer and Bruns 1997). The phylogenetic relationship between fourteen eastern Asian and twenty two eastern North American S*uillus* species has been determined by Wu et al. (2000) and interrelationship among thirty four common European *Suillus* isolates representing eight species was studied by Manian et al. (2001). Moreover, nineteen different isolates representing seven species were recorded from Mediterranean area of central Spain (Beatriz et al. 2006). Also, the molecular identification and genetic diversity of twenty seven *Suillus* strains isolated from Inner Mongolia have been studied (Feng et al. 2008).

A new *Suillus* species, *S. quiescens* T.D. Bruns & Vellinga, was described from California and Oregon by Bruns et al. (2010). Recently, a world-wide key to the genus *Suillus* was given by Klofac (2013). There are very few reports of this particular genus from India. *Suillus brevipes*, *Suillus pallidiceps*, *Suillus punctatipes* and *Suillus subluteus* were reported from *Pinus patula* forests of Tamilnadu, India (Natarajan and Raman 1983). *Suillus sibiricus* (Sagar and Lakhanpal 2005) and *Suillus granulatus* (Dar et al. 2010) have been shown to be ectomycorrhizal with Himalayan blue pine (*Pinus wallichiana*) from the northwestern Himalayan region of India.

2.3 Distribution and habitat

Most of the *Suillus* species known so far are documented from northern hemisphere, but some have been also reported from southern hemisphere associated with the introduced pine species (McNabb 1968; Watling and Gregory 1989; Dunstan et al. 1998). In Western Australia, *S. granulatus* and *S. luteus* are among the most abundant and frequently encountered *Suillus* species. In general *Suillus* species are confined to the temperate, boreal and Mediterranean regions, although there are few reports of their occurrence from tropical areas (Natarajan and Raman 1983; Halling and Mueller 2002). *Suillus* species are common root symbionts of members of the family *Pinaceae* and also some deciduous species (Singer 1986; Kretzer et al. 1996; Wu et al. 2000). Researchers have reported strong host specificity in basidiomycetes (Begerow et al. 2004; Shefferson et al. 2007) displaying different levels of specialization. Majority of the ectomycorrhizal (ECM) fungi are associated with a broad range of hosts (Trappe 1962; Molina et al. 1992; Kårén et al. 1997; Smith and Read 1997; Bruns et al. 1998; Horton and Bruns 1998; Cairney and Chambers 1999; Cullings et al. 2000; Kennedy et al. 2003), whereas others are associated with narrow range of hosts (Massicotte et al. 1994;

Molina and Trappe 1994; Kretzer et al. 1996). *Suillus* species exhibit narrow host range, which are almost exclusively associated with *Pinaceae* (Kretzer et al. 1996; Wu et al. 2000). Based on the ITS sequence analysis, Kretzer et al. (1996) have proposed that *Larix* association in the genus *Suillus* seems to be primitive and associations with pines, Douglas-fir, and hardwoods seem to be derived. In fact, host shifts of basidiomycetes are considered to be major driving forces in their evolution (Refrégier et al. 2008; Li et al. 2009; Vercken et al. 2010; Li et al. 2011; Rochet et al. 2011).

Both the habitats and the symbiotic hosts are crucial while considering the conservation of ECM fungi. Many *Suillus* species has entered regional red-lists due to habitat loss, loss of symbiotic hosts, climatic changes, pollution, catastrophes etc. A few *Suillus* species have been enlisted as endangered or vulnerable species under regional red lists (Arnolds and Kuyper 1996). *Suillus sibiricus* has been listed as threatened species by seven European countries (Dahlberg and Croneborg 2006). In addition, *S. flavidus, S. tridentinus, S. collinitus, S. plorans* and *S. lakei* are included in individual country's red-lists, as threatened species.

2.4 Abundance and persistence

Suillus species form abundant basidiocarps in nature and are easy to culture as compared to the other ECM fungi. Advantage of their abundance and easy culturing has been employed in several studies focusing on the genetic structure and genet sizes of *Suillus* populations (Fries 1987; Dahlberg and Stenlid 1990, 1994; Dahlberg 1997; Bonello et al. 1998; Zhou et al. 1999). Dahlberg and Stenlid (1990, 1994) studied the spatial distribution of *Suillus bovinus* clones isolated from basidiocarps collected in different aged *Pinus sylvestris* stands and found that the size of genets increased with

increasing forest age, suggesting that this species spread its population by mycelial growth. Increase in size of genets and their persistence over many years have been also reported in *S. granulatus* (Jacobson et al. 1993), *S. variegatus* (Dahlberg 1997) and *S. pungens* (Bonello et al. 1998) populations. On the contrary, a few *Suillus* species with small genet sizes in young forests or after disturbance act as early colonizers and are suggested reproducing mainly by spore dispersal (Dahlberg and Stenlid 1995; Zhou et al. 1999; Bruns et al. 2002). Thus, *Suillus* species are good competitors that can persist for long time and extend their distribution either by mycelial growth or by spore propagation. These two ecologically favorable attributes, abundance and persistence, encourages *Suillus* species as potential candidates for mycorrhizal applications in forestry purposes (El Karkouri et al. 2006).

2.5 Taxonomy

The classical approach of basidiomycetes systematics mainly relies upon phenotypic examination of sporocarps supplemented by microscopy and *in vitro* culturing. In addition to the morpho-anatomical descriptions of sporocarps, morphology of spindle pole bodies and septa as well as the physiological and biochemical characteristics of basidiomycetes fungi have remarkably contributed towards the basidiomycetes systematics (Yang 2011). Morphological, biochemical and physiological studies some time led to many ambiguities during identification and classification of basidiomycetes due to the insufficiency of characters (Hibbett 2007). Therefore, an authentic, reliable and supporting taxonomic tool is crucial for the documentation and classification of the fungal biodiversity. The limitation of identification of basidiomycetes based on a few morphological and physiological characters can be overcome by the molecular methods, which are becoming increasingly important for studying taxonomic and phylogenetic

relationships among the fungi. During last two decades, the emergence of various molecular and phylogenetic methods has greatly accelerated the study of molecular systematic of fungi. Especially, the invention of polymerase chain reaction (PCR) and the availability of large number of universal oligonucleotide primers specific to the fungi (Vilgalys and Hester 1990; White et al. 1990; Gardes et al. 1991; Gardes and Bruns 1993) have greatly increased our potential to study the fungal systematics (White et al. 1990; Bruns et al. 1992; Nei and Kumar 2000). Further, the advancements in statistical methods and computational technology have made the assessment of phylogenetic relationship easier and more convenient. Molecular techniques such as, DNA sequence analysis, RAPD, RFLP, AFLP etc., have provided valuable information for understanding relationship among different groups of basidiomycetes/fungi and their classification (Hibbett and Vilgalys 1993; Bunyard et al. 1994; Bresinsky et al. 1999; Drehmel et al. 1999; Liu et al. 1999; Zhang et al. 2004; Binder and Hibbett 2006; Buyck et al. 2008). Sequences derived from ribosomal DNA (i.e. nSSU and nLSU rDNA), mitochondrial DNA (mtDNA) and protein coding genes (e.g. tef1, rpb1, rpb2, gpd) have been used to investigate the diversity and molecular evolution of the fungi in number of studies (Swann and Taylor 1995; Fell et al. 2000; Lacourt et al. 20001; Lutzoni et al. 2004; Nuytinck et al. 2006, 2007; Matheny et al. 2007a, 2007b; Nuytinck and Verbeken 2007; Geml et al. 2009; Stubbe et al. 2010; Van de Putte et al. 2010; Park et al. 2013, 2014). Thus, phylogenetic analysis of DNA or protein sequences has evolved as a powerful tool in fungal systematics very quickly in the last 20 years.

Despite the fact that molecular methods have become prevalent and necessary in fungal taxonomy and systematics (Seifert 2009; Begerow et al. 2010; Nilsson et al. 2011), classical methods still are of equal importance in understanding the evolution of

the basidiomycetes. In the 20th century, many hypotheses were postulated regarding the evolution of basidiomycetes based on morphology, ultrastructure, and structure of pigments or metabolites that have been shown to be true by molecular analysis in the last two decades (Yang 2011). Therefore, multiple methods, including classical as well as molecular, should be used to document and classify the diversity of basidimomycetes. Although there are number of molecular methods that can be used along with morphological method, but ribosomal DNA (rDNA) sequence analysis always have been a most widely used methodology for authentic and reliable identification of fungal species and have greatly enhanced our present knowledge on phylogeny and evolution of fungi. In the present work, a combination of classical and rDNA based molecular taxonomy is used to study the intra-genus variation within *Suillus* species that are representatives of conifer forests in north western Himalayan region of India and a phylogenetic analysis is performed to determine their evolutionary relationships.

2.6 Internal transcribed spacers (ITS) and its limitations

In eukaryotes, ribosomal DNA (rDNA) comprises of various coding genes (18S, 5.8S, 28S and 5S genes) that are clustered in tandem repeat units separated by spacers, *viz*. internal transcribed spacer (ITS) and intergenic spacer (IGS) regions (Fig. 2.1). The ribosomal coding genes and the spacers have evolved at different pace during the evolution and have been used as the key targets for fungal identification purposes. These regions own the benefits of high copy number, high magnitude of conserved coding regions and variable spacer regions. The coding regions of rDNA exhibit a higher magnitude of conserved region that confers greater homogeneity within the species than between the species (Arnheim 1983) and provide us with the sites for

primer designing. For example, PCR primers for identification and detection of *Rhizopycnis vagum* were developed by Ghignone et al. (2003) based on the alignment of ITS sequences. On the contrary, the spacer regions show remarkable sequence variations even within the species. The variations in the spacer regions have provided framework for identification, differentiation and classification of different ranks of the global biodiversity of kingdom fungi. At the same time, differences at inter-specific or intra-specific level have proven fruitful in distinguishing difficult-to-identify taxa. Thus, the rDNA is a convenient region to design nucleic acid markers for taxonomic purposes. The most widely sequenced rDNA locus for molecular systematics of fungi at the subgeneric level for species identification is the ITS region (Horton and Bruns 2001; Bridge et al. 2005). The ITS region comprises of the 3' end of the 18S gene, the ITS1 spacer, the 5.8S gene, the ITS2 spacer and the 5' end of the 28S gene (Fig. 2.1). In recent years, it has been consistently used for molecular systematics, phylogeny and ecology of fungi at the species level as well as within the species.

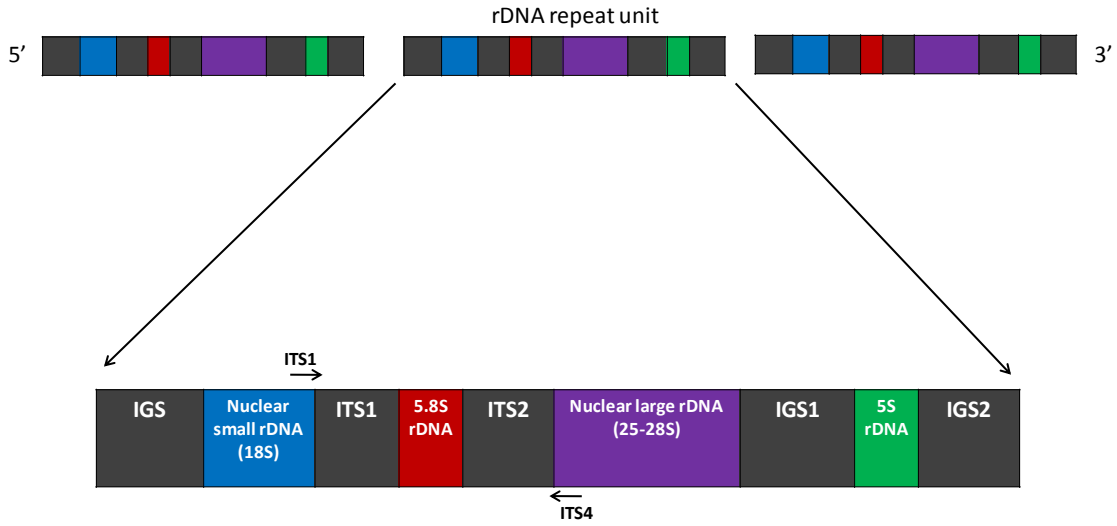

Fig. 2.1 Diagrammatic representation of fungal ribosomal DNA (rDNA) repeat unit. The universal primers used to amplify the internal transcribed spacer (ITS) region in this study are represented by arrows in 5' to 3' direction

Sequence analysis of ITS region in fungi, generally divulges a high level of inter-specific polymorphism and low or infrequent intra-specific divergence (Liu et al. 1997; Martin et al. 1998; Manian et al. 2001; Horton 2002; Singla et al. 2004; Leonardi et al. 2005; Mello et al. 2005; Park et al. 2014). For example, Manian et al. (2001) examined genetic diversity of common European *Suillus* species based on ITS data and found that *S. plorans* and *S. bovinus* exhibited maximum inter-specific divergence (13.6 and 13.3, respectively) when compared to other *Suillus* species. Analogously, the inter-specific divergence between a few pairs of *Suillus* species was found low, e.g. 2.4 between *S. collinitus* and *S. granulatus* and 3.8–4.2 between *S. plorans* and *S. variegatus*. Intra-specific divergence in ITS region differs considerably among the fungi, which ranges from 0-2% in some fungi (Gardes et al. 1991; Cooke et al. 1999; Manian et al. 2001) to 15% in others (Muthumeenakshi et al. 1998; Roy et al. 1998; Balardin et al. 1999; Manian et al. 2001). Phylogenetic analysis of *Suillus* species by Manian et al. (2001) revealed that *S. laricinus* (0.5–4.4) and *S. granulatus* (0–11) from Europe, Asia and North America showed high degrees of intra-specific divergence. On the other hand, *S. bovinus, S. variegatus* and *S. luteus* showed very low intra-specific divergence (0–0.7). Similarly, intra-specific homology and species-specific conservation of ITS region has been illustrated in different isolates of some *Suillus* species, such as *S. collinitus*, *S. mediterraneensis*, *S. luteus*, *S. albidipes*, *S. brevipes*, *S. tomentosus*, and *S. umbonatus* (Horton 2002; Beatriz et al. 2006).

Discrepancies between morphological and molecular identification of many ECM fungi including *Suillus* species have been revealed by many authors (Kretzer et al. 1996; Kårén et al. 1997; Farmer and Sylvia 1998; Wipf et al. 1999; Manian et al. 2001; Bruns et al. 2010). A review on exploration of physiology and biodiversity of ECM fungi was

made by Buscot et al. (2000) who hypothesized four possible mechanisms for contradictions between phenotypic and genetic characters, which include paucity of phenotypic characters for species delimitation, intra-specific phenotypic flexibility, convergent evolution and cryptic speciation. The phylogenetic analysis based upon ITS data separated *S. granulatus* isolates from Europe, Asia, and North America into different groups (Kretzer et al. 1996; Manian et al. 2001; Bruns et al. 2010), probably suggesting that a cryptic speciation process has taken place between these isolates. It was suggested that the isolates reported as *S. granulatus* from Europe, Asia, and North America might be representing at least two different taxa. On the contrary, the ITS locus may be unsuccessful in distinguishing some well identified phenotypic species. Bruns et al. (2010) have mentioned that a few pairs of *Suillus* species (*S. pseudobrevipes* and *S. volcanalis*, *S. glandulosipes* and *S. neoalbidipes*, *S. brevipes* and *S. weaverae*) are not distinguished by the ITS locus. This might be due to over-description (=synonymy) or to the lack of ITS divergence among the sibling species. Thus, there are limitations of using ITS locus for species-level determinations in the genus *Suillus*.

Irrespective of the ITS limitations mentioned above, we have selected the ITS region for molecular identification and phylogenetic examination of the *Suillus* species, mainly for two reasons. First, the ITS region has been sequenced from large number of *Suillus* species from different regions of the world (Kretzer et al. 1996; Kretzer and Bruns 1997; Wu et al. 2000; Manian et al. 2001; Beatriz et al. 2006; Feng et al. 2008; Bruns et al. 2010; Sarwar et al. 2011, 2012; Sarwar and Khalid 2014). Second, several investigations on phylogenetics and bio-geographical analysis of the genus *Suillus* have demonstrated the advantage of using nuclear ribosomal RNA (nrRNA) region to diagnose and figure out the phylogenetic relationships among the species in this genus

(Kretzer et al. 1996; Wu et al. 2000; Manian et al. 2001; Feng et al. 2008). Until now, there was no study documenting the ITS sequences of *Suillus* species from India and this study will provided a phylogenetic framework for biogeographic analysis of Indian *Suillus* species as well as to infer phylogenetic relationships at the species level in this genus. Further, the ITS data may be a successful tool for distinguishing the cryptic species within this genus.

2.7 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is a method for differentiation of organisms in which restriction patterns are obtained by the cleavage of DNA with restriction enzymes, followed by the segregation of the resulting fragments by gel electrophoresis to produce RFLP patterns. The technique is often applied to the fields of molecular ecology and systematics of fungi for species delimitations. Molecular ecological studies on ECM fungi have mainly employed restriction analysis of the ITS region (Horton and Bruns 2001) for identification and differentiation at the species level. Comparisons of ITS-RFLP patterns is an extremely simple, subtle and widely used method for determining inter-specific similarities or dissimilarities. Usually, two or three restriction enzyme patterns are adequate to discriminate most of the species (Gardes and Bruns 1996; Dahlberg et al. 1997; Pritsch et al. 1997, 2000; Mahmood et al. 1999; Methvyn et al. 2000).

Diversity of ECM symbionts associated with *Pinus halepensis* trees after out-planting at a fire-disturbed site (El karkouri et al. 2004) and containerized *Pinus nigra* trees established naturally (El karkouri et al. 2005) have been investigated in Mediterranean region of France. The nuclear ribosomal ITS region was PCR amplified with ITS1 and ITS4 primers and amplified PCR products were cut at least with three

restriction enzymes, such as *Hinf*I, *Msp*I, *Mbo*I, and *Taq*I. Molecular typing using PCR-RFLP analysis and sequencing of ITS region of the nuclear rDNA detected twelve ITS-RFLP taxa on non-inoculated, *S. collinitus*-inoculated, and naturally regenerated trees in a fire-disturbed *P. halepensis* plantation (El karkouri et al. 2004). The ECM fungus *S. collinitus* was found to be the most dominant (45.8–59.7%) symbiont in all three treatments indicating its strong ectomycorrhizal competitivity in *P. halepensis* plantation of Mediterranean region. *Suillus mediterraneensis* occurred at moderate frequency (11.7–31.9%) and was confined to non-inoculated and naturally regenerated trees, suggesting its low ectomycorrhizal competitivity in contrast to *S. collinitus*. Dominance of both these *Suillus* species in *P. halepensis* plantation supports them as prospective ECM fungi for Mediterranean *P. halepensis* forest management. Ten other ITS-RFLP taxa observed on *P. halepensis* trees were rare (0.0–9.6%), demonstrating their poor competitivity against both the *Suillus* species. In naturally established *P. nigra* nursery, RFLP patterns of ITS region resulted in typing of four ITS-RFLP taxa (El karkouri et al. 2005). All of them were identified as *Boletales*, namely *Rhizopogon rubescens*, *R. luteolus*, *S. bovinus* and *S. variegatus*. *Rhizopogon rubescens* was most abundant (37.5%) among all the ectomycorrhizal *Boletales* detected, while *S. bovinus* (25%) and *S. variegatus* (26.4%) appeared at moderate frequency. The ECM symbiont *R. luteolus* was rare and occurred with low frequency (2.8%) on *P. nigra* seedlings. In addition, the ITS-RFLP along with inter simple sequence repeat (ISSR) amplification and specific sequence-characterized amplified region (SCAR) sequencing allowed the detection of *S. collinitus* strain Sc-32 on *P. halepensis* seedlings after inoculation and outplanting in a Mediterranean plantation (El karkouri et al. 2006). The survival of *S. collinitus* Sc-32 was monitored for 56 months after inoculation and it was demonstrated

that inoculant can survive on seedlings even after 4 years of outplanting. This suggested that *S. collinitus* Sc-32 is a suitable and persistent inoculant for *P. halepensis* forestry in adverse Mediterranean ecosystems.

Beatriz et al. (2006) studied the molecular characterization of nineteen *Suillus* isolates from different pine forests of central Spain. Variation within ITS region of *Suillus* was examined by PCR (using primers ITS5 and ITS4B) coupled with RFLP analysis. PCR products amplified from all the *Suillus* isolates were approximately 800 bp in length. Restriction digests were produced using three different restriction enzymes *i.e*., *Alu*I, *Hinf*I and *Tqq*I. Cluster analysis based on restriction digests grouped the isolates into six different groups. Different isolates of the same species were grouped together, confirming the low intra-specific variability in the ITS region. Moreover, the sequence analysis of ITS region resulted in accurate identification of some *Suillus* isolates, which were misidentified earlier by morphological methods. These studies discussed herein confirm the potential of ITS-RFLP for the identification, molecular characterization, species delimitation and ecological studies of *Suillus* species. Using this technique for documentation of *Suillus* taxa from an un-explored region, like northwestern region of India, will further extend our knowledge on taxonomic diversity of the genus *Suillus*.

2.8 Ectomycorrhizae of *Suillus* species

According to an information system for Characterization and **DE**termination of **E**cto**MY**corrhizae (DEEMY) (2004–2014), ectomycorrhizae of eight *Suillus* species have been characterized from different parts of the world. Among them, ectomycorrhizae for five *Suillus* species (*S. bovinus, S. collinitus, S. plorans, S. sibiricus*

and *S. variegatus*) have been described in association with *Pinus* species (Uhl 1988; Agerer 1990; Treu 1990; Mleczko and Ronikier 2007) and rest three have been characterized from *Larix* roots. Recently, ectomycorrhizae of *S. flavidus* with *P. wallichiana* were studied from Pakistan (Sarwar et al. 2012). In context of India, ectomycorrhizae formed by different ECM fungi, including *S. brevipes,* on *Pinus patula* roots have been described from southern region of India (Mohan et al. 1993a, 1993b, 1993c). All these studies reveal that ectomycorrhizal systems of different *Suillus* species vary remarkably in morphology and anatomy. Mleczko and Ronikier (2007) have demonstrated that the ectomycorrhizal features of *Suillus* species are more congruent with their phylogenetic relationships as compared to the classical basidiocarp-based systematics.

2.9 Role of ectomycorrhizal fungi and *Suillus* species in pine establishment

Ectomycorrhizal (ECM) fungi generally improve growth and survival of host plants, increase their nutrient and water uptake, and provide them resistance against biotic (e.g. plant pathogens) and abiotic (e.g. heavy metals) stresses (Garbaye 2000; Chalot et al. 2002; Hall 2002). There are many studies demonstrating the positive effects of ECM fungi on growth and nutrient contents of plants, especially the pine trees. Following mycorrhizal inoculation of *P. sylvestris* with *Boletus* species, a significant increase in dry weight and phosphorus content of mycorrhizal seedlings was observed by Mejstřík (1975). Inoculation of *Pinus rigida* seedlings with *Pisolithus tinctorius* showed superior growth and normal foliar ion composition even under phosphorus limitation compared to the un-inoculated seedlings (Cumming 1993). *Pinus densiflora* seedlings inoculated with *Tricholoma matsutake* showed a significant increase in total seedling dry weight compared to the control treatments (Guerin-Laguette et al. 2004). In a nursery

experiment, Rincón et al. (2005a) found *Rhizopogon roseolus* as the best ECM fungi among the five ECM fungi tested for production of containerized mycorrhizal *Pinus pinea* seedlings. Compared with non-mycorrhizal seedlings, *P. densiflora* seedlings inoculated with different ECM fungi in single or multiple treatments exhibited increased seedlings growth (Sim and Eom 2006; Dalong et al. 2011) and nutrient contents (Dalong et al. 2011). Similarly, ectomycorrhizal inoculation of *P. wallichiana* seedlings with different ECM fungi showed an increase in growth, biomass and nutrient contents of the mycorrhizal seedlings (Dar et al. 2007, 2010; Ahangar et al. 2012; Itoo and Reshi 2014a). Additionally, highest plant growth response of pine seedlings has been observed when treated with a combination of multiple ECM fungi in contrast to single ECM fungus (Sim and Eom 2006; Dalong et al. 2011; Ahangar et al. 2012; Sousa et al. 2012).

While considering the ectomycorrhizal *Suillus* species, the fungi have been shown to promote plant height, root biomass, shoot biomass as well as nitrogen and phosphorus uptake in pine trees (Read and Boyd 1986; Bending and Read 1995; Colpaert et al. 1999; Beatriz et al. 2006; El Karkouri et al. 2006). Timonen et al. (1997) investigated root colonization and growth responses of *P. sylvestris* seedlings after inoculations with indigenous genets of *S. bovinus* (SBK4b and SBK5b) and *S. variegatus* (SVK3b), both in single inoculated treatments (SITs) and multiple inoculated treatments (MITs). All the mycorrhizal seedlings in SITs and MITs exhibited significant positive plant growth responses as compared to un-inoculated seedlings. Short-term phosphorus uptake rates in ectomycorrhizal and non-mycorrhizal roots of *P. sylvestris* seedlings have been studied following interactions with four ECM fungi, namely *Paxillus involutus*, *S. luteus*, *S. bovinus* and *Thelephora terrestris* (Colpaert et al. 1999). The results revealed a marked increase in phosphorus-uptake capacity of all the

mycorrhizal plant root systems with heterogeneity in affinity for phosphorus-uptake among the different ECM fungi. Increased phosphorus uptake has also been shown in mycorrhizal *Pinus radiata* seedlings infected with *R. rubescens* and *S. luteus* (Liu et al. 2008). *Suillus collinitus,* forming ectomycorrhizas mainly with *P. halepensis* trees (Beatriz et al. 2006; El Karkouri et al. 2004) in harsh Mediterranean environments with water and nutrient poor soils, is used as a persistent mycorrhizal inoculant in nurseries and experimental plantations to improve pine growth, mineral nutrition and survival (El Karkouri et al. 2006). Moreover, stimulation of bacterial survival in *S. granulatus* colonized root elongation zone and their synergistic positive effect on *P. halepensis* seedlings has been observed by Rincón et al. (2005b). Furthermore, *Suillus* isolates exhibit metal tolerance to many toxic metals, such as Zn, Cu and Cd (Colpaert et al. 2000, 2004; Adriaensen et al. 2005) and these metals tolerant *Suillus* isolates have been shown to protect mycorrhizal pine seedlings from metal stress (Colpaert and Van Assche 1993; Adriaensen et al. 2004, 2005; Krznaric et al. 2009). Thus, metal adapted *Suillus* isolates can also be used as an excellent mean for large-scale afforestation and regeneration of pine seedlings at metal polluted and industrial sites.

2.10 Selection of efficient ectomycorrhizal isolates

The ECM fungi differs in their physiological attributes, such as morphology, growth rates and mycorrhizal ability and this is certainly true for *Suillus* species, which exhibit remarkable inter-specific as well as intra-specific variations for a wide range of physiological traits (Dahlberg and Finlay 1999; Cazzoli 2002, Beatriz et al. 2006). On the basis of these differentiating features, suitable and efficient ECM isolates can be selected for mass inoculum production for forestry purposes. *In vitro* mycorrhizal capacity of the different local *Suillus* isolates has been evaluated for the growth of a

typical Mediterranean pine species (*P. halepensis*) with an aim to select suitable isolates for afforestation programmes (Beatriz et al. 2006). Although there are a few studies reporting positive effects of different ECM fungi on growth, biomass, and nutrient contents of *P. wallichiana* seedlings (Dar et al. 2007, 2010; Ahangar et al. 2012; Itoo and Reshi 2014a), studies focusing on isolation and evaluation of mycorrhizal capacity of indigenous *Suillus* species to promote growth of *P. wallichiana* seedlings are still lacking. Owing to different advantages provided by *Suillus* species to the host plant, particularly pine species, it is of utmost importance to isolate local *Suillus* species and study their effects on plant growth so as to select suitable isolates for mass inoculum production.

2.11 *Pinus wallichiana* and need for indigenous ectomycorrhizal inoculants

Pinus wallichiana A.B. Jacks., commonly known as blue pine, is a five needle pine with an average height and width ranging from 30–36 m and 2.5–3.0 m, respectively (Troup 1921). Blue pine is native to the Himalayan region and occurs naturally in moist and dry temperate forest ecosystems of many adjacent countries of the region, such as Afghanistan, Pakistan, India, Nepal, Bhutan, Myanmar and China (Troup 1921; Critchfield and Little 1966). In these diverse forest ecosystems, it is distributed from middle to high altitudinal zones ranging from 1500–3600 m and extends longitudinally from 69^{o}–75^{o}E and latitudinally from 26^{o}–36^{o}N (Thapliyal et al. 2008; Singh and Thapliyal 2012). *Pinus wallichiana* is one among the most important and over-exploited pine species in the Himalayan region, which is mainly used as a source of commercially valuable timber in variety of purposes, such as construction, agriculture, horticulture, furniture manufacturing, fuel wood, religious practices etc. In terms of timber usage, blue pine is considered next to deodar (*Cedrus deodara*) among the coniferous plants of

Himalayas. Additionally, the species is important as it is also known for its turpentine and resin yield (Coppen et al. 1988; Shuaib et al. 2013). Thus, the blue pine holds a great potential for socio-economic uplift of people inhabiting the Himalayan regions. Recently, *P. wallichiana* has attained international attention for its resistance to blister rust disease (Khan 2004; Lu et al. 2007; King et al. 2010). Pertaining to its economic, aesthetic and ecological importance, *P. wallichiana* is a promising pine species for forestry programmes.

Pine trees are mycotrophic and mainly rely on mycorrhizal fungi for their early establishment, survival and growth (Smith and Read 1997). There is a relatively extensive literature (Cumming 1993; Timonen et al. 1997; Rincón et al. 2005b; Sim and Eom 2006; Dar et al. 2007; Ahangar et al. 2012; Sousa et al. 2012) reporting positive effect of ECM fungi on pine seedlings growth and survival under nursery conditions. These studies reveal that root colonizing and plant benefitting abilities of different ECM fungal strains are variable and it is advantageous to select specific strains as inoculants for production of mycorrhizal seedlings in nurseries. Selection of suitable ECM fungal isolates for afforestation practices in a defined area is a highly resource-consuming task. Moreover, the competition between different ECM fungi plays a significant role in the structuring of ectomycorrhizal assemblages (Koide et al. 2005). The survival of introduced inoculants in the nurseries or forest may get challenged by other indigenous ECM fungi, which further present a crucial problem in selection of mycorrhizal inoculants (Browning and Whitney 1992; Le Tacon et al. 1992, 1997; El Karkouri et al. 2002; Quoreshi et al. 2008). A decrease in inoculated ECM fungi after two years of inoculation has been observed in black spruce and jack pine seedlings (Browning and Whitney 1992). Similarly after five years of outplanting, most of the ECM fungi

inoculated to conifer and hardwood species were replaced by several indigenous ECM fungi (Quoreshi et al. 2008). On the contrary, the efficiency of eco-specific and species-specific native ECM isolates is always superior than the introduced ones due to their environmental adaptation to the concerned site. The local isolate *S. collinitus* has been shown to survive on inoculated *P. halepensis* seedlings even after 56 months of inoculation (El Karkouri et al. 2006) and therefore used as a mycorrhizal inoculant in Mediterranean plantation. Improvement of *P. halepensis* seedlings establishment with selected *S. collinitus* isolates as inoculants has been also observed by Rincón et al. (2007) after 2 years of outplanting in a degraded gypsum soil. Kennedy et al. (2007) have suggested that the outcomes of ectomycorrhizal competition are strongly influenced by the local environment under which it occurs. Thus, ecological adaptability of introduced ECM fungi to the transplantation site is an important criterion to select well adapted ECM isolates for nursery inoculation programmes. For successful afforestation of a defined region, it is therefore advisable to identify and select native and efficient ECM strains for inoculum production, which are best adapted to the local environmental conditions. This practice, often known as "mycorrhization control", generally improves survival, field performance and plant productivity of mycorrhizal seedlings (Castellano and Molina 1989; Marx and Cordell 1989; Selosse et al. 2000; Rossi et al. 2007). In view of the above, the present investigation aimed at evaluating the effectiveness and influence of various indigenous *Suillus* isolates on growth, biomass and nutrient contents of *P. wallichiana* seedlings was undertaken.

2.12 Extracellular enzymes of ECM fungi involved in nitrogen and phosphorus uptake

Nitrogen and phosphorus are the major growth limiting nutrients in most of the natural forest soils. In these forest soils, nitrogen and phosphorus are present mainly as organic macromolecules that are not directly available to plants or fungi. Due to such nutrient constraints in many forest ecosystems, formation of mycorrhizas is considered a vital symbiotic interaction as they have been frequently reported to increase nutrients content in mycorrhizal plants as compared to non-mycorrhizal plants (Chalot et al. 2002; Smith and Read 2008). Mycorrhizal fungi allow access to unavailable forms of nutrients, especially phosphorus and nitrogen, by breaking down the organic macromolecules into simple accessible forms. ECM fungi utilize nutrients from organic compounds of soils by producing extracellular enzymes (Aučina et al. 2007; Courty et al. 2009, 2010b; Pritsch and Garbaye 2011). In light of the ecological importance of organic nutrient resources in many forest ecosystems and the potential of extracellular enzymes secreted by ECM fungi to break down these organic macromolecules into plant accessible forms, a few extracellular enzyme activities of *Suillus* isolates were evaluated in the present study so as to check their efficiency for pine establishment and afforestation.

Nitrogen uptake: Nitrogen (N) is a primary plant nutrient essential for plant growth and development. About 95% of soil total N is associated with the soil organic matter (proteinaceous material, amino acids, amino sugars, heterocyclic N compounds etc.) that is unavailable to plants. Rest of the soil N is present as nitrate (NO_3^-), nitrite (NO_2^-) and the ammonium (NH_4^+) ions, which constitute the major inorganic pool of soil total N (Schulten and Schnitzer 1998). Most of the plants utilize N in nitrate form and a few in ammonium (NH_4^+) form. The soil organic matter is continuously being converted to

inorganic forms, by soil micro-flora, in a process termed as ‘mineralization’. The task is accomplished by a number of hydrolytic enzymes produced by the soil microbes. Most of the major hydrolytic enzymes involved in the mobilization of N from organic matter have been detected in ericoid and some in ECM fungi (Leake 1996; Chalot and Brun 1998). Among different hydrolytic enzymes, extracellular proteases are considered as key enzyme for mineralization of organic matter (Ramstedt and Söderhäll, 1983; Nygren et al. 2007) as 40% of the total soil N is present as protein N (Schulten and Schnitzer 1998). Numerous studies have highlighted the production and/or characterization of extracellular protease in ericoid and ECM fungi (Ramstedt and Söderhäll, 1983; El-Badaoui and Botton 1989; Leake and Read 1990a; Zhu et al. 1990, 1994; Leake and Read 1991). The efficiency of ericoid and ECM fungi to promote organic N uptake is correlated mainly with the extracellular protease activities (Bajwa et al. 1985; Leake and Read 1989; Leake and Read 1991; Leake 1996).

The ability of ECM fungi to secret hydrolytic enzymes, assimilate the products of hydrolytic degradation and mobilize the N from soils is considered possibly to have a great impact on the dynamics of organic N utilization in diverse forest ecological systems. The potential role of ECM fungi in assimilation and mobilization of N from organic matter has been illustrated by many authors (Abuzinadah et al. 1986; Abuzinadah and Read 1986; Bending and Read 1995; Martin and Lorillou 1997; Martin et al. 2001). The capability of several ECM fungi to utilize protein and mobilize its N to *Pinus contorta* plants has been also demonstrated (Abuzinadah et al. 1986; Abuzinadah and Read 1986). Moreover, the protease activities has been shown in many ECM species, such as *Amanita rubescens*, *Amanita muscaria*, *Cenococcum geophilum*, *Hebeloma* spp., *Lactarius subdulcis*, *P. involutus*, *S. bovinus*, *S. variegatus* etc. (El-

Badaoui and Botton 1989; Maijala et al. 1991; Leake 1996; Tibbett et al. 1999; Nehls et al. 2001). Recently, extracellular protease activity of 32 different species of ECM fungi was studied and 29 of the total species were found to exhibit extracellular protease activity (Nygren et al. 2007). Thus, the protease excretion is a prevalent physiological trait of ECM fungi and this ability is of substantial significance for N uptake in forest ecosystems.

Apart from proteins, chitin is another polymeric source of organic N particularly in the forest soils. The potential sources of chitin in soils are fungal cell walls and arthropod exoskeletons. Degradation of chitin and transfer of chitin derived N to the host plant by the ericoid mycorrhizal fungus *Hymenoscyphus ericae* has been demonstrated (Mitchell et al. 1992; Kerley and Read 1995). Also, the utilization of chitin as N source by ECM fungi is a well-known phenomenon (Leake and Read 1990b; Lindahl and Taylor 2004). The ability of ericoid and ECM fungi to promote uptake of chitin derived N may be correlated mainly with the chitinolytic activities of chitinase enzymes secreted by the mycorrhizal fungi (Mitchell et al. 1992; Lindahl and Taylor 2004).

Phosphorus uptake: Phosphorus (P) is one of the nutrients essential for plant growth and plays a censorious role in photosynthesis, energy metabolism, synthesis of nucleic acids and membranes, respiration, enzyme regulation and nitrogen fixation (Raghothama 1999; Vance et al. 2003). The only form of P in soils directly available to plants is inorganic orthophosphate (Pi). Beside this inorganic Pi, soils also contain phosphorus bound to carbon-containing compounds (organic P) that accounts for 20 to 80% of the total phosphorus in the soils (Richardson 1994). Acid phosphatases (ACPases) or phosphomonoesterases are a group of extracellular enzymes that mineralize this organic

P reservoirs of soils into a plant accessible inorganic Pi (Smith and Read 1997). Thus, ACPase improve the phosphate nutrition of plants and have been reported from a number of ECM fungi (Ho 1989; Antibus et al. 1992; McElhinney and Mitchell 1993; Periasamy and Raman 1995; Raman et al. 1998, 2002; Tibbett et al. 1998, Conn and Dighton 2000; Wannet et al. 2000; Jayakumar and Tan 2005; Quiquampoix and Mousain 2005; Alvarez et al. 2006; Courty et al. 2006; Baghel et al. 2009; Hrynkiewicz et al. 2009; Nygren and Rosling 2009; Louche et al. 2010; Bechem 2013). Quiquampoix and Mousain (2005) quantified the ACPase activities in ten isolates of ECM fungi (*Laccaria laccata*, *S. collinitus* (three isolates), *S. granulatus*, *S. luteus*, *H. cylindrosporum* (two isolates), *P. involutu*s and *R. rubescens*) grown in a low-phosphate medium. Among them, only six isolates showed extracellular ACPase activity (*S. collinitus* (one isolate), *S. granulatus*, *S. luteus*; *H. cylindrosporum* (two isolates) and *R. rubescens*). The high level of extracellular ACPase activities was detected in four different fractions separated from culture medium of *H. cylindrosporum* after growing in a pure culture under P-starved conditions and thus, suggested to produce several isoforms of ACPases (Louche et al. 2010). Increased level of extracellular ACPase activity in *Hebeloma* species under P-starved conditions has been also reported by other authors (Tibbett et al. 1998; Quiquampoix and Mousain 2005). ACPase activity has been also shown in some *Suillus* species, such as *S. bovinus*, *S. granulatus*, *S. collinitus* and *S. luteus* (Timonen and Sen 1998; Quiquampoix and Mousain 2005; Cullings et al. 2008; Dar et al. 2010). Moreover, uptake and distribution of P by mycelia of *S. variegatus* strains (Timonen et al. 1996; Wallander 2000) have been demonstrated.

Phytases are the next important extracellular enzymes involved in release of inorganic Pi from organic P. Phytic acid (inositol hexaphosphate) is a principal

constituent of organic P found in the soils (Richardson 1994). These phytates are more stable to decomposition and accumulate in soils (Lim et al. 2007). Ability of ECM fungi to degrade phytates of organic P is attributed to their phytase activities, which allow access to this more recalcitrant organic phosphorus resource (Antibus et al. 1992). Phytase activity of ten ECM isolates has been determined (Quiquampoix and Mousain 2005), out of which only four isolates exhibited extracellular phytase activity that includes one isolate of *S. collinitus* and *S. granulatus* each and two isolates of *H. cylindrosporum*.

2.13 Biochar

2.13.1 Biochar: Definition and properties

Biochar is defined as biomass-derived black carbon produced by the pyrolysis of biomass feedstock in partial or total absence of oxygen. Biochar is a solid, carbonaceous material, which displays a high surface area (Bird et al. 2008) and less concentrations of oxygen and hydrogen level (Abdullah and Wu 2009) as compared to the feedstock. It is a byproduct of the pyrolysis process, which is chiefly composed of recalcitrant organic C and other plant mineral nutrients retained from the original biomass feedstock. Actual elemental composition of biochar depends both upon the source feedstock as well as the pyrolysis conditions (Novak et al. 2009). Carbon contents of biochar vary from 172 g/kg to 905 g/kg (McElligott et al. 2011). Additional elements present in the biochar includes nitrogen ranging from 1.8 g/kg to 56.4 g/kg, phosphorus ranging from 2.7 g/kg to 480 g/kg, potassium ranging from 1.0 g/kg to 58 g/kg and varying amounts of other elements, e.g. oxygen, hydrogen, sulphur, and heavy

metals (Goldberg 1985; Lehmann et al. 2003a, 2003b; Lima and Marshall 2005; Preston and Schmidt 2006; Novak et al. 2009).

A variety of organic feedstocks and different process conditions may be used to produce biochars, which results in products with a diverse range of physico-chemical properties (Baldock and Smernik 2002; Nguyen et al. 2004; Guerrero et al. 2005). In fact, most of the physical and chemical characteristics of the biochar are sensitive functions of pyrolysis feedstock type and process conditions, which subsequently effects the soil properties when used as additive for soil management (Gaskin et al. 2008; Amonette and Joseph 2009; Downie et al. 2009; Novak et al. 2009). Feedstock is a key factor in determining the physicochemical properties of biochar. Gaskin et al. (2010) studied the effect of peanut hull and pine chip biochars on soil nutrients and found that peanut-shell biochar had higher nutrient concentrations than pine chip biochar and increased the pH and nutrients of soil. On the contrary, pine chip biochar decreased soil pH and had little or no effect on soil nutrients. The pore size of biochars, which is possibly important in governing water holding and adsorption capacity of soil, also varies for biochars derived from different feedstocks and exhibits the architecture of the source feedstock (Day et al. 2005; Ogawa et al. 2006; Yu et al. 2006). Novak et al. (2009) derived biochars from different feedstocks such as, peanut hulls, pecan shells, poultry litter and switch grass and observed that they differed entirely in their physical and elemental characteristics. Just as source feedstocks alters the properties of biochar, so too does the pyrolysis temperature. It has been observed that the process temperature is the most significant process parameter. Atkinson et al. (2010) and Joseph et al. (2010) have reviewed the effects of process temperature on biochar properties. The carbon content of biochar derived from different feedstocks (Novak et al. 2009) increased,

while the yield of biochar decreased with an increase in the pyrolysis temperature. Thus, the carbon content of biochar is inversely related to the biochar yield, when pyrolysis temperature is increased. However, above a definite threshold temperature, biochar yield may continue to decrease with no more increase in the carbon content (Sohi et al. 2010). The surface area (Day et al. 2005) and ash content (Novak et al. 2009) of biochar also increases as the temperature is raised and this increase is different in different feedstock types. Moreover, the pyrolysis temperature also affects the pH of biochar, which is generally acidic for low temperatures derived biochar and alkaline for high temperatures derived biochar (Lehmann 2007, Novak et al. 2009).

2.13.2 Historic usage and significance of biochar

Much of our present knowledge regarding biochar has been enlightened from *terra preta de índio* (TP) soils of Amazon. The TP soils are the highly fertile Amazonian dark soils of the central Amazon as compared to the surrounding low fertile soils and are supposed to be created approximately 1000 ybp as illustrated by the Archaeological records and radiocarbon dating (Sohi et al. 2010). High char content of Amazonian dark soils accounts for their greater fertility and dark color (Glaser et al. 2001). The Amazonian dark soils were created by pre-Columbian Indians (Smith 1980; Woods and McCann 1999) and the extent of char content in these soils indicates that the char has been added to the soil purposely so as to increase the fertility of low fertile Amazonian soils. The other cause of increased char content is considered the biochar derived from incomplete combustion of biomass resulting from domestic fires and agricultural burning practices. Studies focusing on the development of TP soils in the Amazon have shown that these soils display enhanced levels of char content relative to the adjacent soils (Sombroek 1966; Glaser et al. 2001; Lehmann et al. 2002), which is resistant to

decomposition and can remain in the soil for thousands of years (Agee 1996; Lehmann and Rondon 2006). TP soils have also been reported from Ecuador and Peru in western South America (Lehmann et al. 2003a, 2003b) and from the savannas of South Africa (Bird et al. 1999). There is relatively extensive literature (Young 1804; Von Liebig 1878; Morley 1927, 1929; Santiago and Santiago 1989; Lehmann and Joseph 2009) documenting the applications of char in traditional horticultural practices and soil improvement from various countries. Use of charcoal for non-fuel purposes like soil amendment in agriculture and forestry has also been a traditional practice in many Asian countries (Okimori et al. 2003). Thus, it may be suggested that the addition of biochar to the soils was probably a worldwide practice to enhance the soil fertility and the gross crop production.

Several recent investigations and reviews on TP soils have emphasized the importance of biochar as a soil additive in long term storage of carbon in soils as well as overall increase in the crop yields (Glaser et al. 2002; Glaser and Woods 2004; Day et al. 2005; Lehmann et al. 2006; Marris 2006; Glaser 2007). In contrast to surrounding soils, these TP soils possess higher amounts of organic matter contents and nutrients (such as nitrogen, phosphorus, potassium, and calcium) as well as the elevated levels of CEC and pH (Sombroek et al. 1993; Glaser et al. 2001; Lehmann et al. 2003a; Liang et al. 2006). The improved nutrient status and enhanced fertility of these TP soils have engendered significant increase in crop yields as compared to the adjacent low fertile soils (Lehmann et al. 2002; Liang et al. 2006; Solomon et al. 2007). These findings have inspired the use of biochar as a propitious soil additive in recent years. Despite global traditional use of biochar, present interest in biochar only began in last few decades. Presently, Japan is the largest commercial producer of charcoal for soil amendments

(Okimori et al. 2003). Biochar exploration significantly escalated in Japan during 1980s (Kishimoto and Sugiura 1980, 1985). As a result, the consumption of carbon in Japan increased from 38,800 t (metric ton) in 1985 to 192,000 t in 1999, out of which the maximum consumption (30.6%) was mainly as soil amendments for agricultural purposes (Okimori et al. 2003).

2.13.3 Biochar and carbon sequestration

The addition of biochar to the soil can contributing towards soil C sequestration (Lehmann 2007; Laird 2008) by restoring the reducing carbon repositories (Lehmann et al. 2006) and increasing the long term storage of carbon in soils (Kuzyakov et al. 2009; McHenry 2009). Biochar produced from organic feedstocks mainly consists of stable aromatic forms of organic carbon and the degree of aromaticity is a function of source feedstock and pyrolysis conditions. Aromaticity is an important governing factor of biochar potential for C sequestration, which stimulates the practice of biochar addition to soils to sequester C (Lehmann 2007). When used as a soil amendment, the aromatic organic carbon of biochar is resistant to decomposition (Amonette and Joseph 2009) and leads to an increase in the recalcitrant C fraction of the soil. Thus, the biochars especially with high aromaticity are resistant to microbial mineralization (Glaser et al. 2002; Lehmann 2007; Novak et al. 2009) and apparently suitable for long-term C sequestration. Increase in soil C fraction further improves the soil quality because soil C plays a crucial role in maintaining chemical, biological and physical processes in the soil (Thompson and Troeh 1978; Stevenson 1994).

2.13.4 Impacts of biochar on plant growth

Soils can be amended with biochar addition to improve plant biomass, crop productivity and soil quality (Blackwell et al. 2009). Reviews on previous studies showed a broad range of biochar application rates and variable plant responses to biochar additions (Glaser et al. 2001, 2002). Glaser et al. (2001) have illustrated that low biochar additions (0.5 t/ha) generally tended to have positive impacts on various crop species, whereas higher biochar rates inhibits crop biomass and/or productivity. In fact, there is relatively extensive literature available documenting the impacts of biochar on plant growth and crop produvtivity (Iswaran et al. 1980; Kishimoto and Sugiura 1985; Chidumayo 1994; Mikan and Abrams 1995; Wardle et al. 1998; Gaur and Adholeya 2000; Hoshi 2001; Lehmann et al. 2002; Chan et al. 2007; Van Zwieten et al. 2007; Kimetu et al. 2008). Majority of these studies (Iswaran et al. 1980; Chidumayo 1994; Wardle et al. 1998; Hoshi 2001; Lehmann et al. 2002; Chan et al. 2007; Van Zwieten et al. 2007; Kimetu et al. 2008) illustrated the beneficial effects of biochar on plant biomass and/or crop yield as a result of biochar additions. Despite this, biochar additions do not always positively effects the soil quality and negative impacts on plant growth have also been revealed due to biochar additions by few authors (Kishimoto and Sugiura 1985; Mikan and Abrams 1995; Gaur and Adholeya 2000).

Furthermore, increased plant growth, nutrient uptake and crop yield have been observed when biochar was added in combination with fertilizer to the soil (Glaser et al. 2002; Lehmann et al. 2002; Yamato et al. 2006; Chan et al. 2007, 2008; Gundale and DeLuca 2007; Steiner et al. 2007; Van Zwieten et al. 2007; Asai et al. 2009; Blackwell et al. 2009; Gaskin et al. 2010). However, a study from Australia on wheat crop didn't show any significant yield response (Blackwell et al. 2007). Thus, fertilizer additions do

not always alleviate the negative impact of fresh biochar additions (Asai et al. 2009). It has been considered that due to high C/N ratios (up to 400) of biochar, it has potential to immobilize plant accessible N causing N deficiency in plants (Lehmann et al. 2006; Chan and Xu 2009; Lehmann and Joseph 2009).

2.13.5 Impacts of biochar on soil properties

Biochar has been used as a soil amendment due to its potential soil conditioning properties and ability to make some desirable changes in the soil physicochemical characteristics. Numerous studies have shown that the addition of biochar to the soil may be advantageous as it can improve soil physical, chemical, and biological properties, increase recalcitrant soil organic carbon fraction and enhance plant growth (Glaser et al. 2002; Lehmann et al. 2002; Lehmann et al. 2005; Rondon et al. 2005; Lehmann and Rondon 2006; Fowles 2007; Rondon et al. 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009). The collaborative abilities of biochar to increase soil pH (Tryon 1948; Mbagwu and Piccolo 1997; Hoshi 2001; Glaser et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rondon et al. 2007; Van Zwieten et al. 2007), improve physical properties (Iswaran et al. 1980; Brady and Weil 2004; Chan et al. 2007; Downie et al. 2009), and retaining soil nutrients and reducing leaching losses (Hoshi 2001; Lehmann et al. 2002, 2003a; Lehmann 2007) are the possible contributing factors responsible for increase in plant productivity.

Fowles (2007) have discussed different attributes of the black carbon that may possibly contribute to increase soil fertility and improve its nutrient status. First, biochar enhances soil cation exchange capacity thus increasing the availability of nutrients for plants. Second, the soil nutrients can bind to the biochar that prevents the subsequent

nutrient run-off from the soil and reduces leaching of nitrogen into the water table. Thus, biochar improves the filtration of percolating soil water (Lehmann and Joseph 2009). Third, the porous nature of biochars provides appropriate conditions for growth and multiplication of soil micro-biota. Soil microorganisms can proliferate within these pores and get protected from soil predators (Saito 1990; Pietikäinen et al. 2000; Ezawa et al. 2002; Samonin and Elikova 2004), such as arthropods, nematodes and protozoan. Thus, biochar acts as a refuge for any soil microorganism colonizing biochar. Further, the soluble organic substrates and nutrients bound to black carbon provide nutrition to the soil microorganisms (Hamer et al. 2004; Atkinson et al. 2010). As consequences, increases in microbial biomass and activities of biochar amended soils have been reported by many authors (Wardle et al. 1998; Pietikäinen et al. 2000; Chan et al. 2008; Steiner et al. 2008; Kolb et al. 2007; Steinbeiss et al. 2009). Soil microbiota is considered as architects of soils (Rajendhran and Gunasekaran 2008) and many ecosystem services, such as the decomposition of organic matter, cycling of nutrients, and suppression of soil-borne diseases and pests, are closely linked to microbial activities and their functional traits (Brussaard 1997). In addition, the potential benefits of incorporating biochar into soil include improvement of soil water retention capacity (Piccolo et al. 1996; Downie et al. 2009) and neutralization of acidic soil (Van Zweiten et al. 2007) by increasing the soil pH.

2.13.6 Impacts of biochar on mycorrhizal fungi

Biochar amendment can transform the total fungal abundance as well as the activity of mycorrhizal fungi in the soils that can in turn affect the mycorrhizal colonization or mycorrhizal efficiency and lead to the altered plant growth. Several investigations have reported both increased root colonization (Harvey et al. 1976; Saito 1990; Ishii and

Kodoya 1994; Mori and Marjenah 1994; Ezawa et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rillig et al. 2010) and stimulated plant growth (Vaccari et al. 2011; Robertson et al. 2012) in response to biochar. This may be attributed to elevated nutrients availability (Ishii and Kadoya 1994; Garcia-Montiel et al. 2000; Lehmann et al. 2003a; Gundale and DeLuca 2006; DeLuca et al. 2006; Nigussie et al. 2012) or improved soil physicochemical properties (DeLuca et al. 2006; Warnock et al. 2007; Chan and Xu 2009) as a result of biochar supplementations. As majority of the literatures available so far illustrate that biochar additions positively affect both mycorrhizas and plant growth, it is noteworthy to discuss possible mechanisms underlying biochar functioning.

Warnock et al. (2007) have explained four possible mechanisms, which describe that how biochar can positively affect mycorrhizal abundance and subsequent plant development. (1) Addition of biochar to the soil induces altered levels of soil physicochemical properties and nutrient availability. Biochar additions increases/decreases soil pH, increases cation exchange capacity (CEC), water holding capacity (WHC), and decreases bulk density. Although the nutrients content in biochar is small, but the alteration of soil physicochemical properties due to biochar addition further led to an increase in soil nutrient availability. This increased level of nutrients can promote the growth of mycorrhizal fungi and the subsequent mycorrhizal colonization of plant roots. (2) Biochar is a potentially diverse niche for large number of soil microorganisms. Biochar facilitate the growth of biochar colonizing bacteria, including mycorrhization helper bacteria (MHBs) and phosphate solubilizing bacteria (PSBs), by providing them reduced carbon compounds, nutrients and protection. Enhanced abundance of MHBs and PSBs in biochar treated soils may further increase

the growth of mycorrhizal hyphae and amount of fungal accessible mineral nutrients, respectively. This ultimately results in increased plant root colonization and plant nutrition uptake. (3) Biochar additions can affect plant–mycorrhizal fungi signaling dynamics by adsorption/desorption of both signaling compounds (carbon dioxide, flavanoids, sesquiterpenes and strigolactones) as well as inhibitory compounds (allelochemicals, other toxic molecules). Thus, biochar can serve as signal reservoirs or sink. When the signaling compounds are adsorbed permanently by biochar, fungal hyphal growth and subsequently the fungal abundance decreases. However if biochar temporarily removes the signal molecules, desorption may take place later on when soil water enters the biochar particles. As a result of desorption, biochar particles can serve as secondary sources of signal molecules and stimulate mycorrhizal colonization of plant roots. Attenuation of allelochemicals or other toxic molecules due to biochar adsorption is also advantageous to mycorrhizal fungi and promotes plant root colonization. In addition to direct effects of biochar on signaling compounds, biochar can indirectly increase stimulating signals and subsequent fungal abundance by altering soil pH. (4) Biochar provides protection to root colonizing fungi and bacteria from soil predators. Thus, biochar serves as refuge from grazing species, such as arthropods, nematodes and protozoan. Phenomenon is purely physical and depends upon pore size of biochar particles. Many of the pores are large enough to get colonized by fungal hyphae and bacteria, but exclude their larger predators. Protection within biochar particles may promote abundance of MHBs, PSBs and mycorrhizal fungi in soil, which further may led to an increase in plant root colonization. According to Warnock (2007), several of these mechanisms are hypothetical and it is difficult to mention which mechanism is likely to be most important under any given environmental conditions. At

present, mechanism 1 is considered to most satisfactory due to vast literature available in its support (Tryon 1948; Ishii and Kadoya1994; Glaser et al. 2002; Lehmann et al. 2002, 2003a, 2005; Matsubara et al. 2002; Rondon et al. 2005; DeLuca et al. 2006; Gundale and DeLuca 2006; Lehmann and Rondon 2006; Yamato et al. 2006; Fowles 2007; Rondon et al. 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009).

Although majority of literature shows that biochar applications can affect both mycorrhizas and plant growth positively, but this is not observed always. Warnock et al. (2010) have found that fungal abundance in roots of *Plantago lanceolata* either decreased or remained unchanged for all the five types of biochar amendments used. Moreover, Gaur and Adholeya (2000) found that the biochar supplementation limited the P uptake in *Zea mays* plants, thus negatively affecting the plant growth. Decrease in soybean yields due to biochar addition has been also reported by Kishimoto and Sugiura (1985). This reduction in root colonization and nutrients uptake because of biochar addition is possibly due to decreased nutrient availability or unfavorable nutrient ratios, such as, very high C/N ratio in the soils (Gaur and Adholeya 2000; Wallstedt et al. 2002). Considering these probabilities of negative effects of biochar amendments on mycorrhizal abundance and plant growth, substantial characterization and evaluation of biochar for plant growth needs to be done before it can be used for afforestation and land reclamation programmes. In the present work, synergistic influence of pine needle derived biochar amendments and *Suillus* mycorrhizal inoculations on *P. wallichiana* seedlings was studied as a practice for afforestation of forest soils.

Thus, the literature survey reveals that there are very few reports of the genus *Suillus* from India. Four species (*S. brevipes*, *S. pallidiceps*, *S. punctatipes* and *S.*

subluteus) have been documented from the southern part of India and only two from (*S. sibiricus* and *S. granulatus*) the northern region of India. Keeping in view the importance of molecular as well as classical methods, a combination of classical and rDNA based molecular taxonomy is used in the present work to study the intra-genus variations among the *Suillus* species that are representatives of conifer forests in the northwestern Himalayan region of India. Further, there is no deposition of the ITS sequences of *Suillus* species from India in any nucleotide database. This study will obtain the ITS sequences for the Indian *Suillus* species and provided a phylogenetic framework for biogeographic analysis of these species. Along with documenting the diversity, it is of great significance to conserve the biodiversity, which can be achieved by isolating and preserving the *Suillus* isolates. In context of India, there is no deposition of any *Suillus* isolates in any internationally recognized culture bank. The present study is focused on isolating the *Suillus* cultures with aim to evaluate their mycorrhizal capacity for improvement of *P. wallichiana* growth. The abilities of indigenous *Suillus* species (except for *S. granulatus*) and biochar additions to promote growth of *P. wallichiana* have not been evaluated, so far. In view of this, there is considerable work that remains to be done especially with reference to *Suillus* diversity, taxonomy, culture isolation and evaluation of their mycorrhizal capacities (with or without biochar addition), because of which the present work has been undertaken.