Diversity of AM (Arbuscular Mycorrhizal) fungi in two geographically and ecologically different states of India

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Abstract

Newly created phylum Glomeromycota includes arbuscular mycorrhizal fungi (AMF), which symbiotically associate with plant roots and benefit the plant nutrition, growth and survival, due to their greater exploitation of soil for nutrients. These associations represent a key factor in the below ground dynamics which influence species diversity and plant community structure. Knowledge of AM fungal diversity in any geographical region is of prime importance in understanding ecosystems functioning and forms the basis in many conservation priority approaches. The main objective of the present study was to estimate AMF diversity in terms of abundance, species richness and evenness in 54 samples from 6 different sites falling in forests of Delhi ridge area and Himachal region. The AM fungal spore count, species richness, most frequent species, and intra-radical colonization were studied in various samples drawn from these regions. The spores were quantified and characterized after the first trap culture cycle. Single spore cultures could only be raised for five of the isolated species and the identification based on morphological characters was confirmed with signature molecular sequences for these species. Various parameters used for diversity estimation and statistical calculations included Margalef's species richness index, Shannon Weiner Index, AMF Abundance, Analysis of variance (ANOVA), done with software SPSS 17, means were separated using DMRT (Duncan's Multiple range test) at 5% and significance (LSD, p= 0.05). In the Himachal and Delhi region at least 21 species of AMF are reported to be occurring belonging to seven genera and several of them remains unidentified because they did not propagate in single spore culture or isolating genome DNA from them was difficult from their single spore. The genus Glomus occurred most frequently and Glomus macrocarpum and Funneliformis mosseae were the most commonly occurring species.

KEYWORDS: *biodiversity, AM fungi, arbuscular, Glomus, Glomeromycota*

Introduction

AM (Arbuscular mycorrhizal) fungi are ubiquitously present throughout the world soils and have significant contribution in plant growth. These fungi symbiotically associated with the plant rhizosphere and are the main components of the soil micro biota in most of the forest-ecosystems and form 25% of the biomass of the soil micro flora and fauna combined (Smith and Read 2008). They contribute to the plants by increasing the absorption and translocation of mineral nutrients from the soil to the host plant (Blaszkowski 2012), by improving the biotic and abiotic stress tolerance of the host plant and and to build up the macro-porous structure of the soil that allows penetration of water as well as air and prevents erosion (Singh and Adholeya 2013). These associations represent a key factor in the below ground dynamics which influence species diversity and plant community structure (Chaudhary *et al.* 2008, Turrini and Giovannetti 2012, Opik *et al*., 2010;2013). Understanding distribution and occurrence of AM fungi in forest ecosystems is hampered due to their

microscopic nature, hidden habitat in soil, tedious lengthy procedures for pot culturing and accurate/correct identification as they are mostly obligate symbionts and most of their taxonomic characters are lost in the mature spores isolated from forest soils (Brundrett *et al.* 1996; Walker 1999; Oehl *et al.* 2003; 2014).

India as a country boasts of its climatic and soil variations, and hence scope for much diversity in agriculture. These practices affect mycorrhizae both quantitatively and qualitatively. An understanding of their taxonomic status, geographical distribution, occurrence and abundance is of prime importance in understanding ecosystems functioning (Fitter 2005, Turrini and Giovannetti 2012) and exploiting them as biofertilizers and to increase the bioavailability of immobilized soil minerals. Several checklists such as catalogue of life (Roskov *et al.* 2013), INVAM (http://invam.wvu.edu/) and species lists (Schüßler & Walker 2010, http://schuessler.userweb.mwn.de/amphylo/), AMF biodiversity in India, a checklist (Gupta *et al.* 2014) or identification manuals (Schenck and Pérez 1990; Oehl *et al.* 2011 a, b; Błaszkowski 2012) have proven as effective tools for mycorrhizal research and application.

Even with such a huge ecological importance, AMF species diversity in India is poorly known despite the fact that India is one of the major biodiversity centres. Earlier studies on AMF distribution indicate the uneven distribution of Glomeromycota among states, climatic zones and ecosystem (Gupta *et al.* 2014). Presence of AMF has been recorded from 88 % of the sites examined in India with *Glomus fasciculatum* (currently named as *Rhizophagus fasciculatus*) and *Glomus macrocarpum* being the most commonly recorded species (Rani and Mukerji 1990). More than 148 Glomeromycota species were known to exist up to 2014, but a systematic record of occurrence and diversity of AMF in certain regions including northern and north-eastern India is poorly attended to and more studies need to be conducted or made available from these regions. The names whatever exists need to updated based on the availability of type specimens and molecular sequence data and should follow the consensus classification (Oehl *et al.* 2008, 2011 a, b; Schüßler and Walker 2010; Goto *et al.* 2012; Redecker *et al.* 2013; Gupta *et al.* 2014).

The present study is aimed to study and compare the biodiversity patterns of arbuscular mycorrhizal fungi in two ecologically and geographically different states of India i.e. Himachal Pradesh and New Delhi.

Material and Methods

Strategy for selecting field sites

The study was conducted in 54 samples from 6 different sites 3 each from Delhi forest sites (DL1, DL2, DL3) and 3 sites from Mandi, Himachal Pradesh (sites HP4, HP5,HP6). From each region, 3 replicates were selected and for each of the 3 collection fields were chosen for each site. Total of 9 undisturbed core samples (500 g each) were collected (soil and roots) from each site from a depth of 0-30 cm using a core sampler. The samples were air-dried in the shade to the point where there was no free moisture and were stored in zip bags. The samples were used for three different purposes: (1) Propagation of AM fungal isolate of each collection field for their characterization using pot culture and monoculture and single spore cultures, (2) Analysis of AM fungal parameters (3) Soil physico chemical analysis. The steps followed are summarised in flow diagram in figure 1.

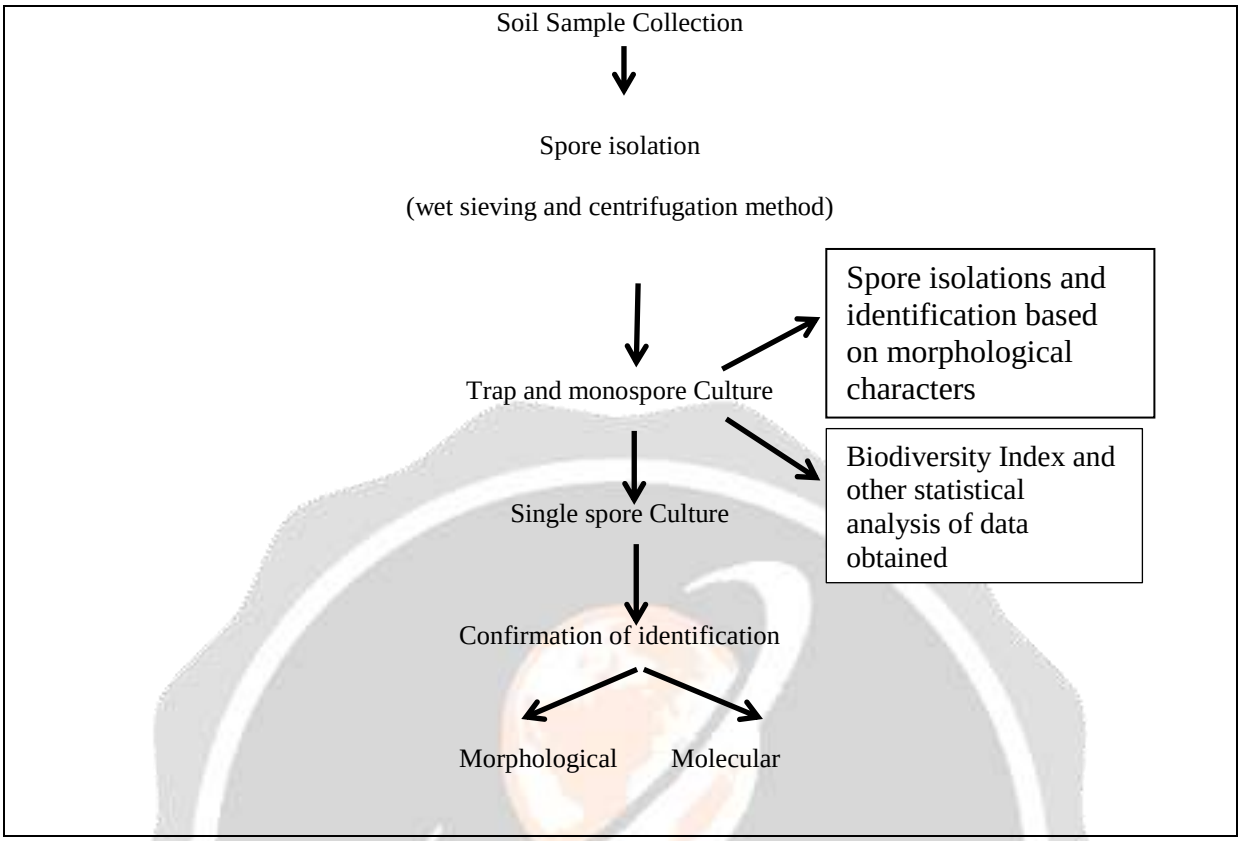

Figure 1 - Flow Chart showing the steps in isolation and identification of AMF propagules and Biodiversity Index Analysis

Propagation of AM fungi in trap cultures, monocultures and single spore cultures

Method used for pot culturing was same as developed by Dr. L. K. Abbot at University of Western Australia (Brundrett *et al.* 1996) using coarse textured soils with moderate nutrient level in non-draining pots. The soil was pasteurised by steaming for 1 hour at 90 ^{0} C on two consecutive days and watered to field capacity with minimal nutrient solution. Trap cultures were initiated using all spores separated from 50 g of soil samples by wet sieving and decanting using onion and *Trigonella* as host plants. In another set of culture, healthy spores uniform appearance were picked using dissecting microscope (Olympus Magnus MSZ-BI) and collected on a small filter paper and were used for initiating the pot cultures. Monospore cultures were raised using 50 spores of same type in sterile soil with *Trigonella* as host plant. Single spore cultures were raised in culture rooms in absolute sterile environment by inoculating individual spores using maize as host plants.

AM fungal spore isolation, quantification, and characterization

The spores were quantified and characterized after the first trap culture cycle. The spores were isolated following the modified technique of wet sieving and decanting (Daniels and Skipper 1982) and were quantified as given by Brundrett *et al.* (1996). The diagnostic slides of different species of AM fungal spores were prepared in PVLG and Melzer reagent (Morton 1992). The initial observation of spores (colour, shape, etc. in water) was recorded under stereomicroscope (Olympus). The measurements of AM fungal spores (spore diameter, wall thickness, hyphal thickness and thickness at the attachment point) were done using a compound microscope (Nikon E200). Once the data on the spores was generated they were characterized up to the level of species using the manual for the identification of VA mycorrhizal fungi (Schenck and Perez 1990). Some species were also characterized by the monograph (Hall 1984), the INVAM http://invam.caf.wvu.edu/fungi/taxonomy/speciesID), www.phylogeny.com and BEG

(http://www.kent.ac.uk/bio/beg/) websites, and the existing published taxonomic literature. Collections, which could not be thus determined, were characterized up to the genus level and labelled sp.1, sp. 2, and so on.

Molecular identification

Genome DNA was extracted from 20 spores from single spore cultures collected in 0.5 ml tube and crushed thoroughly using a micropestle. To each sample 100 µl of lysis buffer (SP Fungal DNA Mini Kit from High Media, MB543 HiPurATM) was added and mixed thoroughly. DNA purification was done as per the instructions of the kit and the genome DNA was quantified.

Figure 2 - The parameters used for DNA isolation and amplification

Primer Details:

Fungal 18s FP-5'- GTAGTCATATGCTTGTCTC-3'
Fungal 18s RP-5'- GAAACCTTGTTACGACTT-3'

PCR conditions:

94°C	94°C	55°C	72°C	72°C
5min	30 sec	30 sec	1.30min	10min(Final extention)

X35 cycles

PCR amplification :

DNA		1 µl (100ng)
Forward primer	200ng	
Reverse primer	200ng	
dNTPs (2.5mM each)		2 µl
10x Taq pol assay buffer	10 µl	
Taq Polymerase (3u/µl)	0.5 µl	
Water		34.5µl
Total reaction volume		50 µl

DNA amplification, cloning and sequencing was done by Biotech Pvt. Ltd. Steps and conditions followed for PCR amplification and sequencing of the amplified products are given in figure 2. The sequence data as *.ab1 file was processed with bioedit and the extracted sequences was identified with BLASTn and Clustal omega.

Intraradical colonization by AM fungi

The roots were cleared by heating in KOH (Phillips and Hayman 1970; Brundrett *et al.* 1996) and were stained with Trypan blue and Chlorozal black E (without chloral anhydrous). Gridline intersection method was used for calculation of root length colonised (Brundrett *et al.*1996).

Data analysis

AM fungi community structure was analysed using the following ecological parameters: mean population abundance for a species, total population abundance, species richness, species diversity and species evenness at each site. Population abundance at each site was defined as the sum of individuals of a particular species, counted at each site during all the observations.

Total population abundance was calculated as the population abundance of all component species at each site. Species richness was expressed by the number of species found in each study site during the observation period. Detailed formula for calculation of these characters is given in table 1.

Table 1 - Different parameters used for biodiversity estimation

S.No.	Parameter	Formula
1.	Margalef's species richness index	DMG= (S-1)/ln N WHERE, S=Total no. of species N= Number of individuals summed all over species
2.	Diversity Index or Shannon Weiner Index	$\mu=-\sum_{i=1}$ Ni/N.ln (Ni/N) WHERE, Ni=Number of species of i species N=Number of individuals summed all over species
3.	AMF Abundance	Total no. of spores /50gm of soil

Results and Discussion

In the present study where biodiversity and distribution of AM fungi in Delhi ridge area was compared with that of Himachal Pradesh Mandi region revealed 16 species and more than 21 isolates respectively of AM fungi in the 54 study sites scattered in 6 different reserved forest regions of India. Isolation from the spores as trap culture was necessary since many of the identification characters used in AM fungal spore identification are lost in the field samples (Walker 1999; Singh and Adholeya 2013). Also, it had advantage that not all AM fungi produced sufficient quantities of spores in field soils to allow isolation or identification. It also served as a step in purification of AM fungal spores from other saprophytic and parasitic fungal and bacterial species which remain infested on the spore surface (Brundrett *et al.* 1996; Smith and Read 2008) and produces better freshly produced spores for monospace and single spore cultures. Many spores being dormant in the forest soil and multiply in trap cultures, so trap cultures reveal additional species, otherwise not observed in field soils. Thus the number of species isolated into trap cultures exceeded those identified from field-collected spores suggesting the inaccuracy of fungal surveys based solely on field spore observations.

Table 2 - Comparison of AMF diversity parameters in different regions of Delhi and Himachal. The detailed formula for calculation of the parameters is given in table 1 and the site detail is explained in material and methods.

Collection sites	Diversity index	Mean abundance	Species richness index
DL1	1.3133 ± 0.385^{bc}	76.00 ± 10.53^{d}	1.0800 ± 0.3034^{bc}
DL2	1.5933 ± 0.192^{ab}	162.3333 ± 50.00^{ab}	1.2733 ± 0.025^{abc}
DL3	1.6867 ± 0.133^{ab}	78.00 ± 22.5^{d}	1.3933 ± 0.302^{abc}
HP4	1.7500 ± 0.204^{a}	103.00 ± 7.81^{cd}	1.7190 ± 0.361^{ab}
HP5	1.8700 ± 0.95^{a}	146 ± 60.00^{abc}	1.8553 ± 0.389^{a}
HP6	1.940 ± 0.277^{a}	187.00 ± 29.51^{a}	1.5910 ± 0.246^{abc}

Of the several biodiversity parameters studied, the difference in the diversity index was statistically most significant in Himachal soil sample HP4, HP5 and HP6 compared to Delhi soils (DL1, DL2 and DL3) (Table 2). Mean abundance and species richness of AM fungi was much higher (Fig 3 a and b) and statistically significant in Himachal soils as compared to Delhi ridge area (Singh and Adholeya, 2013). Of all the regions studied, maximum AM fungal diversity i.e., 20 species were recorded in the Mandi, Himachal region as compared to delhi where up to 14 species were recorded (Table 3). This number however is much lower in comparison to India overall where more than 148 are known to occur (Gupta *et al.* 2014) and which is much less in comparison to global AM flora which consists of up to ~270 species are reported to exist (Oehl *et al*. 2014). However, the reasons for increased spore population in Himachal soils can be known after a more extensive study and whether the increased diversity in Himachal soil confers more benefits to plants is a matter of further investigation.

a b

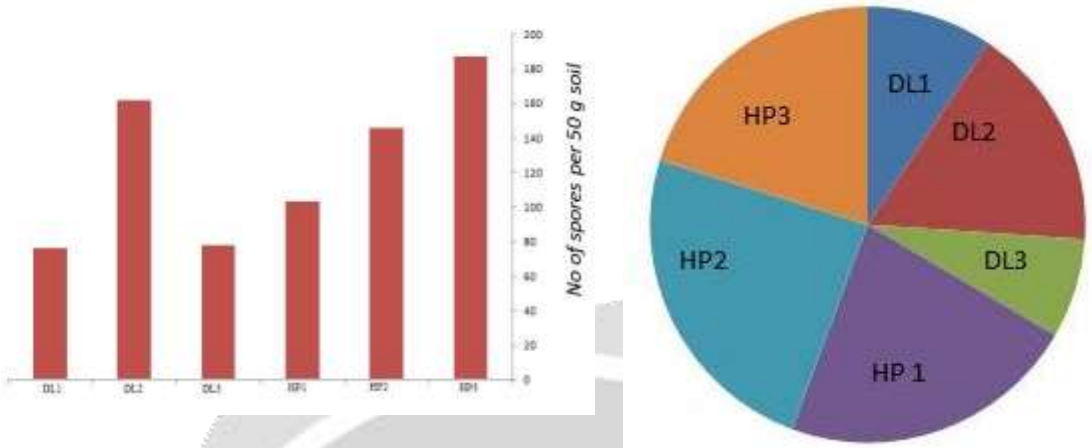

Figure 3 Abundance (a) of AMF and species richness (b) at six different sites

The photographs of AM fungal spores and sporocarps and intraradical colonisation are shown in Figures 4 and 5 respectively. A major highlight of our study is that it is following the latest classification as given by Redecker *et al.* (2013) and is based on molecular data. Genome DNA isolation was very difficult from single spore so at least 20 spores from single spore were used for DNA isolation. Identification of many sequenecs was difficult and we need to use specific primers in our further study. Clustal omega (figure 6 a, b, c, d) turned out to be effective for our sequence where similarity was little less. The genus *Glomus* was recorded as the most frequently occurring followed by *Funneliformis*, which corroborates to our earlier study where, *Glomus macrocarpum* and *Funneliformis mosseae* is found as most the frequently occurring AMF in India (Table 3)(Gupta *et al.* 2014). Although a total of 21 AM fungal species were recorded from 6 different sites falling in two states of India, an average of only 8-10 species per site was found. Another striking observation was the ubiquity and adaptability of a few genera and species in different sites.

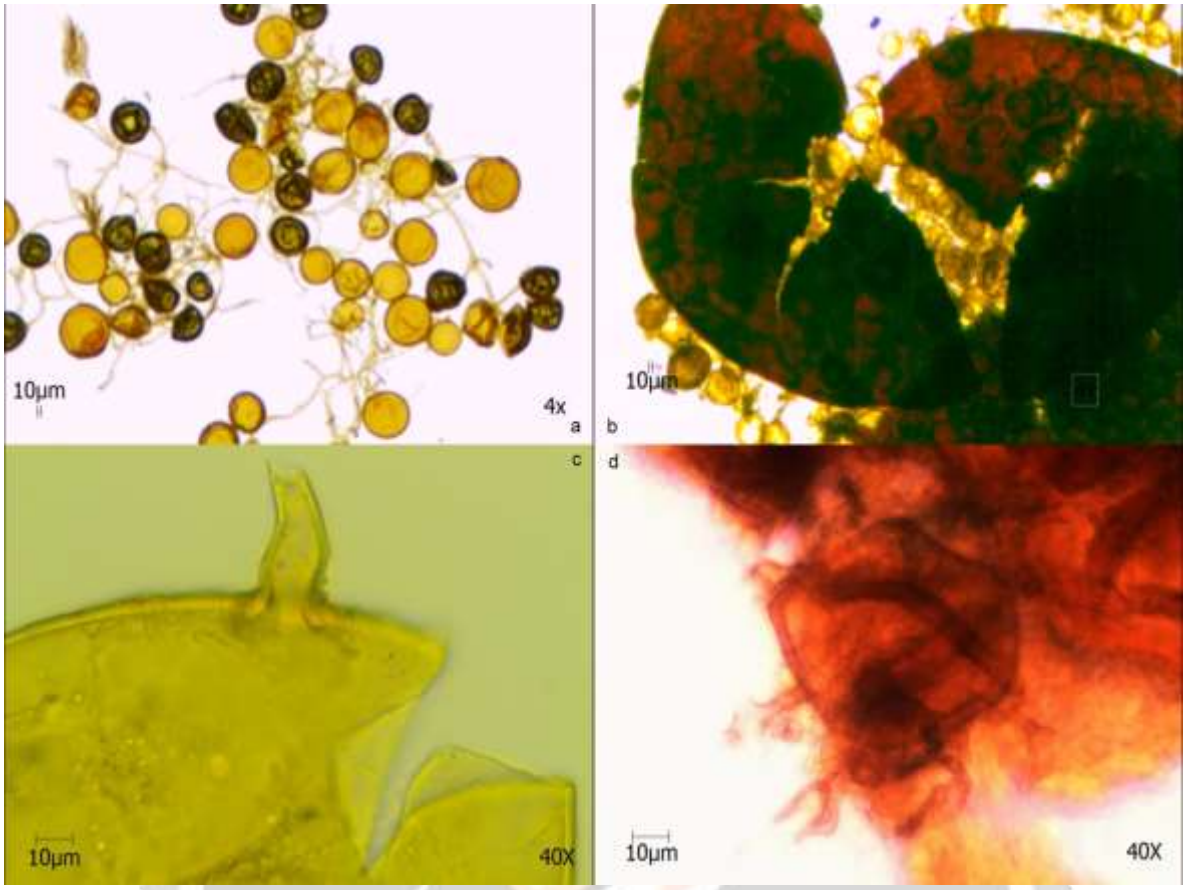

Fig 4 A-D (a). W. M. of *Glomus macrocarpum* spores bunch (b). *Diversispora aurentium* sporocarp crushed sc - sporocarp, p- peridium (c) *Funneiliformas mosseae* spore with subtending hyphae, hyphal wall layer (hwl) having septum (d) subtending hyphae of *Glomus badium* enlarged

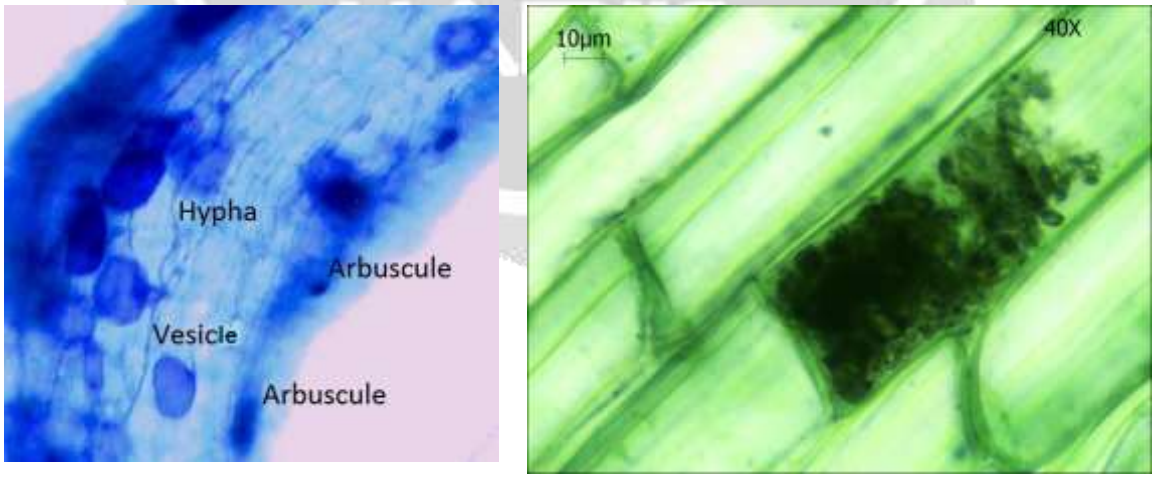

Figure 5 – Root clearing preparation in Trypan blue (left) and Chorozal black

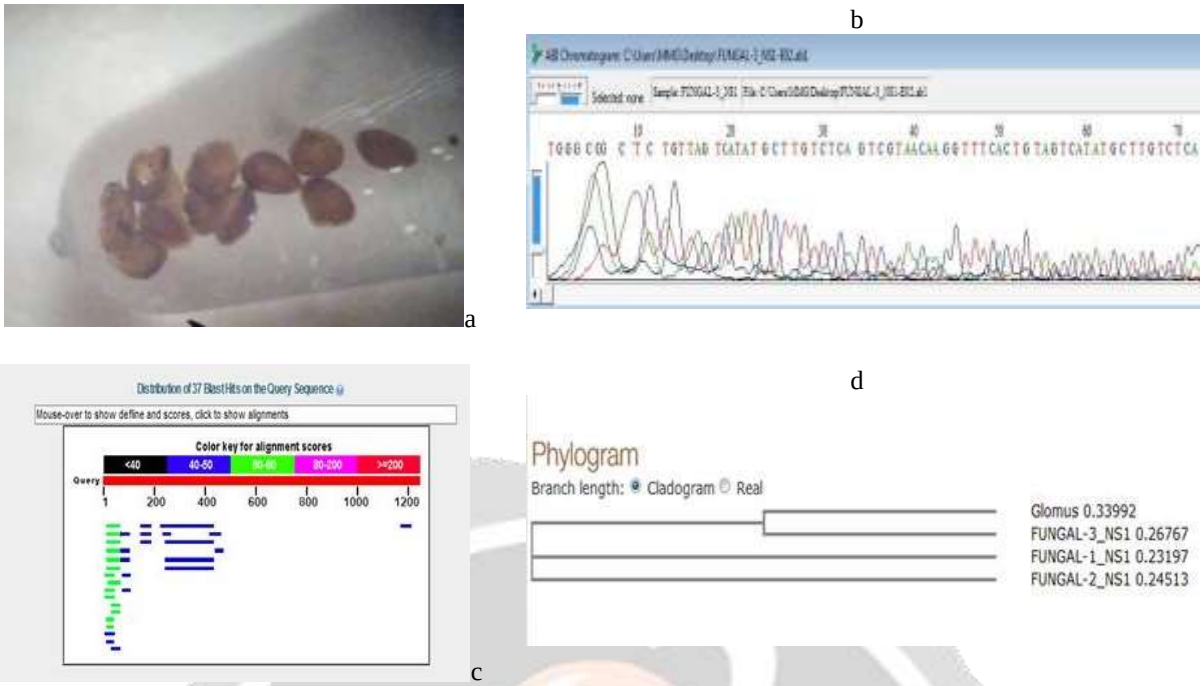

Figure 6 a. spores used for genome DNA isolation, b. sequence as obtained with bioedit, c. Blastn results, d. Phylogram drawn with clustal omega

These findings indicate that the species diversity, abundance and richness present in the Delhi ridge area is lower as compared to Mandi Himachal Pardesh. Two possible explanations could account for this pattern. First, the diversity of AM fungal communities has been related to the diversity of plant communities, although most natural ecosystems contain a great variety of plant species (Bever *et al.* 2001; Rabatin and Stinner 1989; Turrini and Giovannetti 2012). Second, cultural practices and human intervention probably exert strong selective pressures on AM fungal communities (Jansa *et al.* 2002; Singh and Adholeya 2013). All these cause changes in the habitat and substrate availability that may discourage the growth of selected microorganisms, so fungal species and strains most tolerant to these stresses proliferate. Wide spread occurrence of genus *Glomus* indicates its better adaptability to soils in the region of Delhi and Mandi. The comparatively higher frequency of some species, *Funneliformis mosseae* and *G. macrocarpum* among the spore communities indicates their adaptability to varied soil conditions, whereas other species showed a narrow range of their host/environment adaptation.

Table 3 - Mean AM fungal diversity (no of spores /50 g of field spoils) in samples collected from different sites in Himachal and Delhi

S NO	AM fungal species name	DL 1	DL2	DL 3	HP 4	HP5	HP 6
1	*Acaulospora lavies*	13	3		37	49	21
2	*Diversiphora aurantium*		3		5		4
3	*Enterosphospora* sp.			1		2	
4	*Entrophospora infrequens*	2		3			
5	*Funneliformis mosseae*	4	14	5	28	38	58
6	*Gigaspora* sp.					5	
7	*Glomus austral*				2		
8	*Glomus badium*				3	19	39
9	*Glomus caledonium*					5	15
10	*Glomus constrictum*		3		5		18
11	*Glomus intraradices*					5	3
12	*Glomus macrocarpum*	2	9	10	18	28	12

13	*Glomus microaggragatum*				5		
14	*Glomus tenebrosum*					12	5
15	*Rhizophagus aggregatus*				3		
16	*Rhizophagus faciculatus*		16		25	35	50
17	Unidentified species 1	**2**				18	
18	Unidentified species2				11		11
19	Unidentified species 3		4		12		
20	Unidentified species 4		5			14	
21	Unidentified species 5		2			50	

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