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# MOLECULAR DIVERSITY OF POTATO RHIZOSPHERE FUNGI ASSESSED THROUGH 18S rRNA SEQUENCE

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**ABSTRACT: Fungi play an important role in the soil ecosystem by manipulating the plant growth as well as yield. Only a small portion of soil fungi can be cultured through customary microbiological techniques yielding limited information on the composition and dynamics of soil fungal communities. We tested the potentiality of variable region (V4 and V5) of 18s rDNA of the fungi using 10 primers targeting the region. Cloning and sequencing of the targeted sequence was followed and diversity was analysed based on the sequence variation. Primers nu-SSU-0817 and nu-SSU-1196 gave the best results yielding amplicon size of 420-450 bp. Further, sequence and phylogenetic analyses revealed their distribution among four phyla i.e., Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota along with a small portion representing arthropods and protistans as well. The study revealed, phylogentic relations and novel insights to assess biodiversity of fungal community inhabiting the potato rhizosphere and the selected primers are well suited to study the fungal diversity**

**Key Words:** Potato, Rhizosphere, Metagenomics, 18S rDNA, Variable region

## INTRODUCTION

Soil has a composite, dynamic and heterogeneous environment containing a major reservoir of microbial genetic diversity occupied with many key processes obligatory for ecosystem operation. A gram of soil has been reported to hold up to 10 billion microorganisms and thousands of diverse species (Robe *et al.,* 2003). The rhizosphere microbial population is of immense importance as it helps to promote growth and health of the plant by catabolizing organic matter, mineralizing nutrients, fixing $N_2$ and play a role in the suppression of soil borne pathogens (Nadella *et al.,* 2019). Furthermore, the composition of the microbial community in the rhizosphere is influenced by soil type, growth stage, crop rotation, and other ecological factors. Understanding the structure and diversity of microbial community in the rhizosphere leads to a better understanding of pathogen-antagonist interactions.

The soil fungi are important component of the soil microbiota playing a intricate and varied roles in ecosystems in decomposing plant residues, promoting nutrient cycling and stimulating plant growth via mycorrhizal and parasitic associations (Hatti *et al.,* 2018). Although some fungi are well known to cause a range of plant diseases and in some cases devastate crops, others are known to alienate plant pathogens. Knowledge of fungal structure and diversity in soils is essential for suitable crop soil management and production. Regardless of their important role in soil ecosystem functioning and their huge biodiversity (1.5 to 3.0 million species), studies of soil fungal communities represents only about 30% of total investigations of soil microbial communities reported in the literature (Schmit and Mueller, 2007). The

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genetic sequence database for soil fungi is also smaller when compared to soil bacteria (Patil *et al.,* 2017). Moreover, richness and distribution of only certain fraction of the fungi in soil has been studied due to difficultly to culture axenically. Therefore, fungal community in natural habitats is poorly known (Vandenkoornhuyse *et al.,* 2002). The knowledge of the fungal community diversity in soil ecosystems can be used as a useful indicator of soil quality used for sustainable agriculture management and production.

With the development of new molecular techniques, significant advances have been made in determining the diversity of fungal communities. The ITS (Internal Transcribed Spacer) region of the rRNA genes are well suited for analyzing microbial diversity, as they are present in all known organisms containing well conserved variable regions and available in plenty at public database (Ranjard *et al.,* 2001). Targeting of specific regions of 18S rDNA allows analysis of only fungal diversity even in presence of non fungal rDNA and therefore, its appropriate method for analyzing the soil fungal diversity. In recent years, the small-subunit ribosomal RNA gene (SSU rDNA) has been extensively applied to survey the huge bacterial and fungal diversity found in many common and extreme habitats including soil rhizospheres (Chaudhary *et al.,* 2012; Pfeiffer *et al.,* 2017; Gumiere *et al.,* 2019). The study of 18S rDNA genes provides greater knowledge of the diversity of fungi in the environment and has revolutionized fungal systematics. Therefore, molecular approaches based on the analysis of 18S rRNA genes are getting more widely used for the assessment of fungal communities in soils. The present study aims to explore 18S rDNA-based molecular approaches to investigate fungal community in potato fields located at Shimla, Himachal Pradesh comprising sandy loam soil.

## MATERIALS AND METHODS

### Soil samples

The soil samples selected for study was taken from potato fields of ICAR - Central Potato Research Institute, Shimla, situated in the north-western ranges of Himalaya viz., 31.61°N 77.10°E with an average altitude of 2397.59 meters (7866.10 ft). The field contain sandy loam soil with 0.8 to 1.0% organic carbon and N: $P_2O_5$: $K_2O$ (approx 400:120:300Kg) and the samples were taken from the rhizospheres of 2 month old potato crop (Kufri Jyoti). Each sample was placed in sterile plastic bag, sealed, transported to lab, passed through 2.0 mm sieve and stored at -20° C. Soil pH was determined using digital pH meter in soil-water suspensions (1: 2.5) after shaking at 35 rpm for 1 hr.

### Culture conditions

Potato Dextrose Agar (HiMedia, India) was chosen as growth medium for fungal culture using soil dilution technique by suspending 5 gm of soil in 45 ml of sterile distilled water (Waksman, 1922). A dilution series were made viz. $10^{-2}$, $10^{-3}$ and $10^{-4}$ and aliquots (0.5 ml) were spread on PDA plates amended with 3 combinations of antibiotic viz. chloramphenicol (100 $\mu gml^{-1}$), streptomycin (50 $\mu gml^{-1}$) and chloramphenicol + streptomycin and incubated at 28°C for 3-7 days. The fungal species were counted and identified based on their colony morphological characters like colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows.

### Soil DNA Extraction and PCR amplification

Genomic DNA was extracted from 0.1 gm of soil using MO BIO Ultraclean® Soil

DNA kit (Mo Bio Laboratories Inc., USA) as recommended by manufacturer. The 18S rRNA gene was chosen as the target gene because, conversely to the ITS region as it contains regions unaffected by length polymorphism. To identify and evaluate suitable primer set, a two step procedure was chosen. Firstly, 10 primer sets targeting the 18S rRNA gene were compared *in silico* for the length of the amplicon. Secondly, selected 4 primer sets commercially synthesized by Sigma Aldrich were used for PCR amplification to identify better amplifiable primer set, to ensure good accuracy, reproducibility and to facilitate better cloning for sequencing. Highly proficient set of primers nu-SSU-0817 (5′ TTAGCATGGAATAATRRAATAGGA 5′) and nu-SSU-1196 (5′ TCTGGACCTGGTGAGTTTCC 5′) (Borneman and Hartin, 2012) which amplifies a 420 to 450 bp region and contain the V4 (partial) and V5 variable regions of small sub unit of 18S rRNA gene (**Fig. 1a**). The PCR reaction was carried out with 20µl consisting of 2X Taq buffer, 40ng of template DNA, 2µM of dNTPs, 2.5 U of polymerase, 0.8µl of primer. Thermal cycling was performed using Gen Amp® PCR system 9700 (Applied Biosystems, USA) with an initial denaturation step at 94°C for 4 min, then 35 cycles were run at 94°C for 45 sec, 56°C for 45 sec, 72°C for 1 min and final extension at 72°C for 30 min. Amplified products were electrophoresed in 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light.

## Cloning and sequencing of PCR products

The PCR product was purified from 1% agarose gel electrophoresis using QIAquick

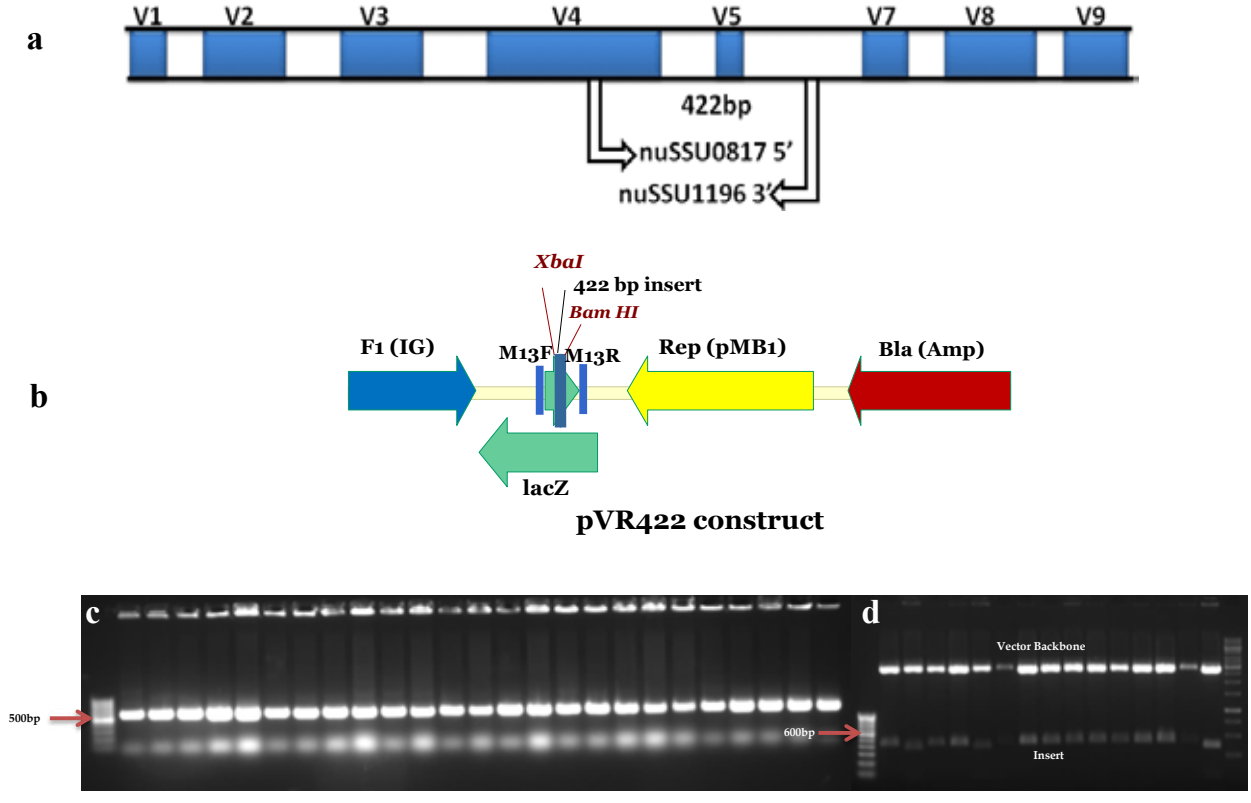


*Figure 1a: Positions of the primers in eukaryotic 18S rDNA gene. The variable regions (V) are highlighted in blue, 1b: pVR422 vector construct with ≅422bp insert, restriction and M13 primer site, 1c: Clone confirmation by a colony PCR with M13 primers and 1d: restriction digestion with Xba I and Bam HI*

gel extraction kit (Qiagen, India). The eluted fragments were cloned by TA cloning method using InsTAclone™ PCR cloning kit (Fermantas) following the instructions of the manufacturers protocol. The recombinant plasmid vector (pTZR/T) was transformed into competent cells (*E. coli* $DH_{5\alpha}$) by heat shock method and the cells were plated on LA (Luria Bertini Agar) plates with Ampicillin/ IPTG/X-Gal and incubated overnight at 37°C. The recombinant cells were initially checked for insert by colony PCR with universal M13(F) (5′ GTTGTAAAACGACGGCCAGT 3′) and M13(R) (5′ CAGGAAACAGCTATGACC 3′) primer and then plasmid DNA was extracted using GenElute™ Plasmid miniprep kits (Sigma Aldrich) according to the manufacturer's instructions. The recombinant vector pVR422 **(Fig. 1b)** was checked for insert by restriction digestion with *XbaI* and *BamHI* enzymes and run on 1% agarose gel for insert clone confirmation. The sequencing reactions of the selected clones were performed using 3500 Genetic Analyzer (Applied Biosystems, USA) using M13(F) primer. A 14µl reaction volume containing 6µl Big dye reaction mix, 4µl M13(F) (1pM) primer and 4µl of the template DNA was used for 25 cycles PCR run with the following program: 96°C for 1min, 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. The cycle reaction product was purified using Dye Ex 2.0 Spin kit (Quiagen, India), vacuum dried, re-suspended in 20 µL HiDi, denatured and sequenced.

### Sequence analysis

The clone sequences were trimmed for their vector back bone with VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and were blast searched for similarity hits. All the nucleotide sequences were submitted to NCBI/Genbank with accession numbers from KC808030 to KC808134 (**Table 1**). The phylogenetic analysis was performed on the Phylogeny.fr platform (http://www.phylogeny.fr/) (Dereeper *et al.,* 2008) where the sequences were aligned with MUSCLE alignment tool (v3.7) configured for highest accuracy and ambiguous regions were removed with Gblocks (v0.91b). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites and 4 γ-distributed rate categories to account for rate heterogeneity across sites. The γ-shape parameter was estimated directly from the data (γ = 0.775) and reliability for internal branch was assessed using the aLRT test ($Chi^2$-based parametric). Graphical representation of phylogenetic tree was performed with TreeDyn (v198.3).

## RESULTS AND DISCUSSION

Rhizosphere is the volume of soil adjacent to plant roots that plays significant role in plant health and soil fertility. Actively growing roots secret a diverse array of organic root exudates that stimulate the microbial populations present in rhizosphere resulting in a community structure at soil-root interface, which is distinct and richer from that in the bulk soil (Hinsinger *et al.,* 2005). During the soil fungal diversity analysis using traditional culturing methods, the dilution factor plays a major role in the growth of the fungal species. The higher dilutions may miss the organisms which represent scarcely in the soil. In the present study the $10^{-2}$ dilution had the maximum fungal colonies of different species than the higher dilutions of $10^{-3}$ and $10^{-4}$. The combination of chloramphenicol and streptomycin were proved to be better for preventing the bacterial contamination than using individually (results not shown). The primer pairs used to amplify rDNA from all four major phyla of fungi: *Ascomycota*, *Basidiomycota*, *Chytridomycota*,

**Table 1: Diversity analysis of fungal community and clones from potato rhizosphere**

| Clone No. | Fragment size (bp) | Fungal species with most similar sequences (NCBI Acc. No.) | Order | % Identity | Phylum | Acc. No. of Clones |
|---|---|---|---|---|---|---|
| NTNR-1 | 443 | *Bionectria ochroleuca* (NCBI Acc. No. GU112755.1) | Hypocreales | 97% | Ascomycota | KC808010 |
| NTNR-2 | 442 | *Basidiobolus meristosporus* (NCBI Acc. No. JX242609.1) | Basidiobolales | 92% | Zygomycota | KC808011 |
| NTNR-3 | 443 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808012 |
| NTNR-6 | 445 | *Descolea tenuipes* (NCBI Acc. No. HQ832432.1) | Agaricales | 98% | Basidiomycota | KC808013 |
| NTNR-8 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808014 |
| NTNR-9 | 441 | *Cercozoa sp.* (NCBI Acc. No. AB585966.1) | Unclassifed | 97% | Protista | KC808015 |
| NTNR-10 | 444 | *Pseudallescheria fimeti* (NCBI Acc. No. AM409202.1) | Microascales | 99% | Ascomycota | KC808016 |
| NTNR-11 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808017 |
| NTNR-12* | 445 | *Acremonium recifei* (NCBI Acc. No. HQ232206.1) | Hypocreales | 99% | Ascomycota | KC808018 |
| NTNR-13 | 64 | *Porostereum crassum* (NCBI Acc. No.) | Pezizales | 100% | Basidiomycota | - |
| NTNR-15 | 443 | *Cercozoa sp.* (NCBI Acc. No. AB585966.1) | Unclassified | 99% | Protista | KC808019 |
| NTNR-16 | 443 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808020 |
| NTNR-17* | 118 | *Sparassis brevipes* (NCBI Acc. No.) | Unclassified | 99% | Basidiomycota | - |
| NTNR-18 | 434 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 99% | Ascomycota | KC808021 |
| NTNR-19 | 445 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 96% | Ascomycota | KC808022 |
| NTNR-20 | 471 | *Gibberella pulicaris*(NCBI Acc. No. AF081467.1) | Hypocreales | 97% | Ascomycota | KC808023 |
| NTNR-21 | 440 | *Eimeriidae sp* (NCBI Acc. No. FJ716244.1) | Eucoccidiorida | 99% | Protista | KC808024 |
| NTNR-22 | 444 | *Chaetomium sp.* (NCBI Acc. No. KC790427.1) | Sordariales | 99% | Ascomycota | KC808025 |
| NTNR-23 | 445 | *Pyrenochaeta lycopersici* (NCBI Acc. No. DQ898289.1) | Pleosporales | 98% | Ascomycota | KC808026 |
| NTNR-24 | 444 | *Stilbella fimetaria* (NCBI Acc. No. FJ939395.1) | Hypocreales | 99% | Ascomycota | KC808027 |
| NTNR-25 | 444 | *Bionectria ochroleuca* (NCBI Acc. No. GU112755.1) | Hypocreales | 100% | Ascomycota | KC808028 |
| NTNR-26 | 445 | *Glomerella acutata* (NCBI Acc. No. JN939859.1) | Glomerellales | 99% | Ascomycota | KC808029 |
| NTNR-27 | 446 | *Chrysamoeba tenera* (NCBI Acc. No. EF165102.1) | Chromulinales | 94% | Protista | KC808030 |
| NTNR-29 | 468 | *Trichosporon laibachii* (NCBI Acc. No. JQ008926.1) | Tremellales | 96% | Basidiomycota | KC808031 |
| NTNR-30 | 939 | *Mortierella sp.* (NCBI Acc. No. JF895929.1) | Mortierellales | 91% | Zygomycota | KC808032 |
| NTNR-31 | 548 | *Fusarium oxysporum* (NCBI Acc. No. JN604549.1) | Hypocreales | 99% | Ascomycota | KC808033 |
| NTNR-32 | 1031 | *Penicillium sp.* (NCBI Acc. No. KM222262.1) | Eurotiales | 99% | Ascomycota | KC808034 |
| NTNR-33 | 514 | *Ceriporiopsis aneirina* (NCBI Acc. No. AB084589.1) | Polyporales | 99% | Basidiomycota | KC808035 |
| NTNR-34 | 459 | *Basidiomycete sp* (NCBI Acc. No. AF202278.1) | Unclassified | 94% | Basidiomycota | KC808036 |
| NTNR-35 | 512 | *Fusarium oxysporum* (NCBI Acc. No. HM210090.1) | Hypocreales | 99% | Ascomycota | KC808037 |
| NTNR-36 | 501 | *Sterkiella sp.* (NCBI Acc. No. KC182573.1) | Sporadotrichida | 98% | Protista | KC808038 |
| NTNR-37 | 459 | *Psathyrella gracilis* (NCBI Acc. No. DQ851582.1) | Agaricales | 100% | Basidiomycota | KC808039 |
| NTNR-38 | 510 | *Bionectria ochroleuca* (NCBI Acc. No. KJ145325.1) | Hypocreales | 99% | Ascomycota | KC808040 |
| NTNR-39 | 510 | *Cercomonadida sp.* (NCBI Acc. No. HQ007035.1) | Cercomonadida | 96% | Protista | KC808041 |
| NTNR-40 | 512 | *Mortierella sp.* (NCBI Acc. No. KC833483.1) | Mortierellales | 99% | Zygomycota | KC808042 |
| NTNR-41 | 511 | *Seiridium sp.* (NCBI Acc. No. AF346559.1) | Xylariales | 99% | Ascomycota | KC808043 |
| NTNR-42 | 512 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1.) | Hypocreales | 99% | Ascomycota | KC808044 |
| NTNR-43 | 511 | *Fusarium fujikuroi* (NCBI Acc. No. KM276792.1.) | Hypocreales | 99% | Ascomycota | KC808045 |

| Clone No. | Fragment size (bp) | Fungal species with most similar sequences (NCBI Acc. No.) | Order | % Identity | Phylum | Acc. No. of Clones |
|---|---|---|---|---|---|---|
| NTNR-44 | 513 | *Fusarium fujikuroi* (NCBI Acc. No. KM276792.1.) | Hypocreales | 99% | Ascomycota | KC808046 |
| NTNR-45 | 508 | *Fusarium fujikuroi* (NCBI Acc. No. KM276792.1.) | Hypocreales | 99% | Ascomycota | KC808047 |
| NTNR-46 | 512 | *Geotrichum sp.* (NCBI Acc. No. JQ320368.1) | Saccharomycetales | 99% | Ascomycota | KC808048 |
| NTNR-47 | 445 | *Sclerotium hydrophilum* (NCBI Acc. No. KC354147.1) | Thelephorales | 99% | Basidiomycota | KC808049 |
| NTNR-49* | 177 | Eladia saccula | Eurotiales | 98% | Ascomycota | - |
| NTNR-50 | 443 | *Pestalotiopsis sp.* (NCBI Acc. No. EU089663.1) | Xylariales | 99% | Ascomycota | KC808050 |
| NTNR-51 | 445 | *Psathyrella gracilis* (NCBI Acc. No. DQ851582.1) | Agaricales | 99% | Basidiomycota | KC808051 |
| NTNR-52 | 442 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 99% | Ascomycota | KC808052 |
| NTNR-53 | 444 | *Chaetomium sp.* (NCBI Acc. No. KC790427.1) | Sordariales | 99% | Ascomycota | KC808053 |
| NTNR-54 | 443 | *Eimeriidae sp.* (NCBI Acc. No. FJ716244.1) | Eucoccidiorida | 99% | Protista | KC808054 |
| NTNR-55 | 442 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 97% | Ascomycota | KC808055 |
| NTNR-56 | 441 | *Mortierella sp.* (NCBI Acc. No. KC833483.1) | Mortierellales | 99% | Zygomycota | KC808056 |
| NTNR-57 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808057 |
| NTNR-58 | 443 | *Mortierella sp.* (NCBI Acc. No. KJ867233.1) | Mortierellales | 96% | Zygomycota | KC808058 |
| NTNR-59 | 444 | *Acremonium recifei* (NCBI Acc. No. HQ232206.1) | Hypocreales | 100% | Ascomycota | KC808059 |
| NTNR-60 | 441 | *Aplanochytrium sp.* (NCBI Acc. No. EU851170.1) | Labyrinthulomycetes | 97% | Protista | KC808060 |
| NTNR-61 | 445 | *Seiridium sp.* (NCBI Acc. No. AF346559.1) | Xylariales | 98% | Ascomycota | KC808061 |
| NTNR-62 | 445 | *Pyrenochaeta lycopersici* (NCBI Acc. No. DQ898289.1) | Pleosporales | 98% | Ascomycota | KC808062 |
| NTNR-79 | 444 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 99% | Ascomycota | KC808063 |
| NTNR-80 | 443 | *Bionectria ochroleuca* (NCBI Acc. No. KJ145325.1) | Hypocreales | 100% | Ascomycota | KC808064 |
| NTNR-81 | 444 | *Pseudodifflugia cf. gracilis* (NCBI Acc. No. AJ418794.1) | - | 94% | Protista | KC808065 |
| NTNR-82 | 444 | *Acremonium recifei* (NCBI Acc. No. HQ232206.1) | Hypocreales | 100% | Ascomycota | KC808066 |
| NTNR-83 | 441 | *Mortierella sp.* (NCBI Acc. No. KC833483.1) | Mortierellales | 99% | Zygomycota | KC808067 |
| NTNR-84 | 441 | *Mortierella sp.* (NCBI Acc. No. KC833483.1 ) | Mortierellales | 99% | Zygomycota | KC808068 |
| NTNR-85 | 961 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808069 |
| NTNR-86 | 444 | *Chaetomium sp.* (NCBI Acc. No. KC790427.1) | Sordariales | 98% | Ascomycota | KC808070 |
| NTNR-87 | 444 | *Fusarium sp.* (NCBI Acc. No. KM222310.1 ) | Hypocreales | 99% | Ascomycota | KC808071 |
| NTNR-88 | 444 | *Sporendonema purpurascens* (NCBI Acc. No. GQ280417.1) | Unclassified | 98% | Ascomycota | KC808072 |
| NTNR-89* | 973 | *Bodomorpha sp.* (NCBI Acc. No. HM536172.1) | Cercomonadida | 99% | Protista | - |
| NTNR-90 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808073 |
| NTNR-91 | 443 | *Pestalotiopsis sp.* (NCBI Acc. No. EU089663.1) | Xylariales | 99% | Ascomycota | KC808074 |
| NTNR-92 | 444 | *Rhizophydium sp.* (NCBI Acc. No. AY601710.1 ) | Rhizophydiales | 96% | Chytridiomycota | KC808075 |
| NTNR-93 | 446 | *Psathyrella gracilis* (NCBI Acc. No. DQ851582.1) | Agaricales | 100% | Basidiomycota | KC808076 |
| NTNR-94 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808077 |
| NTNR-99 | 445 | *Pyrenochaeta lycopersici* (NCBI Acc. No. DQ898289.1) | Pleosporales | 99% | Ascomycota | KC808078 |
| NTNR-100 | 444 | *Cheilymenia vitellina* (NCBI Acc. No. EU940044.1) | Pezizales | 99% | Ascomycota | KC808079 |
| NTNR-101 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808080 |
| NTNR-102 | 443 | *Cercozoa sp.* (NCBI Acc. No. AB585966.1) | Unclassfied | 99% | Protista | KC808081 |
| NTNR-103 | 444 | *Cheilymenia vitellina* (NCBI Acc. No. EU940044.1) | Pezizales | 99% | Ascomycota | KC808082 |

| Clone No. | Fragment size (bp) | Fungal species with most similar sequences (NCBI Acc. No.) | Order | % Identity | Phylum | Acc. No. of Clones |
|---|---|---|---|---|---|---|
| NTNR-104 | 441 | *Geotrichum sp.* (NCBI Acc. No. JQ320368.1 ) | Saccharomycetales | 99% | Ascomycota | KC808083 |
| NTNR-106 | 441 | *Mortierella sp.* (NCBI Acc. No. KJ890360.1) | Mortierellales | 98% | Zygomycota | KC808084 |
| NTNR-107 | 444 | *Bionectria ochroleuca* (NCBI Acc. No. GU112755.1) | Hypocreales | 98% | Ascomycota | KC808085 |
| NTNR-108 | 443 | *Acremonium recifei* (NCBI Acc. No. HQ232206.1) | Hypocreales | 100% | Ascomycota | KC808086 |
| NTNR-109 | 444 | *Ascomycete sp.* (NCBI Acc. No. AF202293.1) | Unclassified | 97% | Ascomycota | KC808087 |
| NTNR-110 | 446 | *Pyrenochaeta lycopersici* (NCBI Acc. No. DQ898289.1) | Pleosporales | 99% | Ascomycota | KC808088 |
| NTNR-111 | 444 | *Minimedusa pubescens* (NCBI Acc. No. DQ915463.1) | Cantharellales | 97% | Basidiomycota | KC808089 |
| NTNR-112 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808090 |
| NTNR-113 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808091 |
| NTNR-114 | 444 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 99% | Ascomycota | KC808092 |
| NTR-1 | 444 | *Chaetomium sp* (NCBI Acc. No. KC790427.1 ) | Sordariales | 99% | Ascomycota | KC808093 |
| NTR-2 | 471 | *Rhizophlyctis rosea* (NCBI Acc. No. NG017175.1) | Rhizophlyctidales | 98% | Chytridiomycota | KC808094 |
| NTR-3 | 528 | *Chaetomium globosum* (NCBI Acc. No. JQ964323.1) | Sordariales | 99% | Ascomycota | KC808095 |
| NTR-4 | 522 | *Phaeosphaeria anchiala* (NCBI Acc. No. AY741066.1) | Pleosporales | 98% | Ascomycota | KC808096 |
| NTR-5 | 445 | *Lecanora achariana* (NCBI Acc. No. DQ973004.1) | Lecanorales | 98% | Ascomycota | KC808097 |
| NTR-6 | 477 | *Endogone pisiformis* (NCBI Acc. No. KC708389.1) | Endogonales | 98% | Zygomycota | KC808098 |
| NTR-7 | 444 | *Histiogaster sp.* (NCBI Acc. No. JQ000118.1) | Astigmata | 99% | Arthropoda | KC808099 |
| NTR-8 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808100 |
| NTR-9 | 443 | *Rhizophydium sp.* (NCBI Acc. No. DQ536492.1) | Rhizophydiales | 99% | Chytridiomycota | KC808101 |
| NTR-10 | 477 | *Rhizophydium sp.* (NCBI Acc. No. DQ536492.1) | Rhizophydiales | 99% | Chytridiomycota | KC808102 |
| NTR-11 | 469 | *Diatrype stigma* (NCBI Acc. No. FJ430579.1) | Xylariales | 97% | Ascomycota | KC808103 |
| NTR-12 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 97% | Ascomycota | KC808104 |
| NTR-13 | 416 | *Fusarium oxysporum* (NCBI Acc. No.) | Hypocreales | 97% | Ascomycota | KC808105 |
| NTR-18 | 438 | *Thamnocephalis sphaerospora* (NCBI Acc. No. AB016013.1) | Zoopagales | 90% | Zygomycota | KC808106 |
| NTR-19 | 443 | *Cercomonadida sp.* (NCBI Acc. No. HQ007035.1) | Cercomonadida | 95% | Protista | KC808107 |
| NTR-20 | 443 | *Rhizophydium sp.* (NCBI Acc. No. DQ536492.1) | Rhizophydiales | 99% | Chytridiomycota | KC808108 |
| NTR-22 | 446 | *Chaetothyriales sp.* (NCBI Acc. No. FJ358320.1) | Chaetothyriales | 99% | Ascomycota | KC808109 |
| NTR-23 | 444 | *Acremonium recifei* (NCBI Acc. No. HQ232206.1) | Hypocreales | 99% | Ascomycota | KC808110 |
| NTR-24 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808111 |
| NTR-25 | 444 | *Cercozoa sp.* (NCBI Acc. No. AB585966.1) | Unclassified | 99% | Protista | KC808112 |
| NTR-27 | 445 | *Dimorpha-like sp.* (NCBI Acc. No. AF411283.1) | Unclassified | 98% | Protista | KC808113 |
| NTR-28* | 75 | *Funneliformis mosseae* (NCBI Acc. No. FR750227.1 ) | Glomerales | 94% | Zygomycota | - |
| NTR-30 | 444 | *Glomerella acutata* (NCBI Acc. No. JN939859.1) | Glomerellales | 99% | Ascomycota | KC808114 |
| NTR-31 | 443 | *Chaetomium sp.* (NCBI Acc. No. KC790427.1) | Sordariales | 97% | Ascomycota | KC808115 |
| NTR-32 | 443 | *Rhizophydium sp.* (NCBI Acc. No. DQ536492.1) | Rhizophydiales | 99% | Chytridiomycota | KC808116 |
| NTR-35 | 445 | *Sistotrema raduloides* (NCBI Acc. No.) | Cortciales | 99% | Basidiomycota | KC808117 |
| NTR-36 | 440 | *Rhizophydium sp.* (NCBI Acc. No. DQ536492.1) | Rhizophydiales | 99% | Chytridiomycota | KC808118 |
| NTR-37 | 445 | *Trechispora farinacea* (NCBI Acc. No. EU909231.1) | Trechisporales | 98% | Basidiomycota | KC808119 |
| NTR-40 | 441 | *Mortierella sp.* (NCBI Acc. No. KC833483.1) | Mortierellales | 98% | Zygomycota | KC808120 |

| Clone No. | Fragment size (bp) | Fungal species with most similar sequences (NCBI Acc. No.) | Order | % Identity | Phylum | Acc. No. of Clones |
|---|---|---|---|---|---|---|
| NTR-41 | 446 | *Pyrenochaeta lycopersici* (NCBI Acc. No. DQ898289.1) | Pleosporales | 99% | Ascomycota | KC808121 |
| NTR-42 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1 ) | Hypocreales | 99% | Ascomycota | KC808122 |
| NTR-43 | 445 | *Rhizophlyctis rosea* (NCBI Acc. No. NG017175.1) | Rhizophlyctidales | 99% | Chytridiomycota | KC808123 |
| NTR-44 | 443 | *Bionectria ochroleuca* (NCBI Acc. No. KJ145325.1) | Hypocreales | 97% | Ascomycota | KC808124 |
| NTR-45 | 442 | *Rhizophydium sp* (NCBI Acc. No. DQ536492.1) | Rhizophydiales | 99% | Chytridiomycota | KC808125 |
| NTR-46 | 444 | *Fusarium oxysporum* (NCBI Acc. No. HM210090.1) | Hypocreales | 99% | Ascomycota | KC808126 |
| NTR-47 | 444 | *Fusarium oxysporum* (NCBI Acc. No. KM276792.1) | Hypocreales | 99% | Ascomycota | KC808127 |
| NTR-48 | 442 | *Mortierella sp.* (NCBI Acc. No. KC833483.1) | Mortierellales | 97% | Zygomycota | KC808128 |
| NTR-49 | 447 | *Lecanora achariana* (NCBI Acc. No. DQ973004.1) | Lecanorales | 98% | Ascomycota | KC808129 |
| NTR-50 | 444 | *Pseudallescheria fimeti* (NCBI Acc. No. AM409202.1) | Microascales | 99% | Ascomycota | KC808130 |
| NTR-51 | 446 | *Pyrenochaeta lycopersici* (NCBI Acc. No. DQ898289.1) | Pleosporales | 99% | Ascomycota | KC808131 |
| NTR-52 | 444 | *Chaetomium sp.* (NCBI Acc. No. KC790427.1) | Sordariales | 98% | Ascomycota | KC808132 |
| NTR-53 | 443 | *Rhizophydium sp.* (NCBI Acc. No. DQ536492.1 ) | Rhizophydiales | 100% | Chytridiomycota | KC808133 |
| NTR-54 | 443 | *Fusarium oxysporum* (NCBI Acc. No. KM250373.1) | Hypocreales | 99% | Ascomycota | KC808134 |

*The sequences not awarded with NCBI accession numbers due to their very short read

and *Zygomycota* (Jumpponen and Johnson, 2005). Approximate sizes of the amplified PCR products were 400–500 bp which show relatively strong specificity for fungal rDNA sequences according to the previous reports (Borneman and Hartin, 2012; Ian *et al.,* 2003) (**Fig 1c and 1d**). PCRs were performed in both the presence and the absence of bovine serum albumin (BSA) for the primer pair and the PCR product yield was considerably increased in the presence of BSA. Partial and full length 168 random clone sequences from library were compared to sequences available in public databases with the NCBI BLAST web application (Nov, 2018). Using BLAST (NCBI), the percentages of identical matches of clone sequences with fungal rDNA sequences in the GenBank database were determined (**Table 1**). However, several sequences from the NTNR fungal clones were initially classified as unidentified fungi in Genbank when compared with their best-matched sequences. Of the 168 clones there were 40 (23.8%) missing or uncultured fungi clones identified (NTNR-4, 5, 7, 14, 33, 48, 63-78, 95-98, 105, NTR-13-17, 21, 26, 29, 33-38, 39). These sequences were considered putative novel sequences which did not have the reference sequences in the Genebank. However clone NTNR-28, blast search showed that there was no significant similar sequence in GenBank and clone NTNR-13, 49 and NTR-28 showed less fragment size (64-177b) so these were removed from further analysis and were also not assigned the accession numbers from NCBI. Chimera check results indicated that no sequence was likely to be a chimera.

The preliminary cloning and sequencing results revealed that all the four phyla, i.e., *ascomycota, basidiomycota, zygomycota* and *chytridiomycota* were present. Interestingly 12% sequences were closely related to species of soil arthropods and protista. This also disproves the earlier reports of Ian *et al.* (2003) that nu-SSU-0817 and nu-SSU-1196 are fungal specific primers. Jumpponen and Johnson (2005) have previously obtained the similar results while assessing the rhizospere and root fungal diversity of tall-grass prairie. The result indicated from the 114 clones of fungi kingdom, 78 clones aligned to

phyla ascomycota (with 60% abundance), the largest phylum of fungi associated with potato rhizosphere. Ascomycota is division/phyla of kingdom fungi and are commonly known as *sac* fungi which form the largest phyla of fungi with more than 64,000 species. They are known to form two types of relationship with plants, as mycorrhiza which can be vital importance for growth and persistence of the plants and as endophyte forming mutualistic or commensal association. Even though exact nature of endophytic fungi and host depends on the species involved but in general its colonization of plants can bestow a high resistance against insects, nematodes and bacteria (Hibbett *et al.,* 2007). The major species of fungi belonging to ascomycota that were found in potato rhizosphere were *Fusarium fujikuroi, F. oxysporum, Chaetomium* sp., *Bionectria ochroleuca, Pyrenochaeta lycopersici* and *Acremonium recifei.* The second copious fungal phyla was basidiomycota (10%) which is another phyla of fungi characterized by the presence of basidium, a microscopic reproductive structure and forms second largest phyla (Swann *et al.,* 2007). Ecologically they are important for decaying dead organic matter including plant litter, some even have the mycorrhizal relationship with plants (Hibbett *et al.,* 2007). Zygomycota formed 10% of total fungi from the potato rhizosphere. Approximately 1050 species are reported to be representing the phyla and are mostly present in the decaying plants and animal materials and some even form symbiotic relationship with plants. Least found was chytridiomycota (8%), the phyla of fungi consisting of around 750 species and most of them exist as parasitic on other organisms. Many of these also infect plants like *Synchytriium endobioticum* causing wart disease of potato (Swann and Hibbit, 2007) (**Fig 2a**). The dominant fungal species of basidiomycota was *Psathyrella gracilis* and *Rhizophydium sp.* was the dominant from chytridiomycota in the rhizosphere soils of potato. *Mortierella sp.* was the major representative of Zygomycota (**Fig 2b**). PCRs with these primers showed that these amplify DNA from representatives of all major taxonomic groups of fungi but not with oomycetes including *Phytophthora infestans* even though, the crop later on was heavily infested by late blight and *Phytophthora* very well survives in soil for years (Janmes *et al.,* 2000). This was also confirmed by the direct DNA extraction of the *P. infestans* from infested soils and testing with the PCR (species specific primers *Taqf/Taqr*) (Hussain *et al.,* 2013).

Of 168 clones sequenced, 14 clones belong to the kingdom protista. The clone NTNR-21 and NTNR-54 were found to have 99% sequence similarity to *Eimeriidae sp*. belonging to Apicomplexa. Nine clones had 94-99% sequence similarity to *Pseudodifflugia cf. gracilis*, *Cercomonadida sp*., *Bodomorpha sp.,* Dimorpha-like sp., *Cercozoa sp*. belonging to the Cercozoa taxonomic phyla. The only exception was clone NTR-7 with close relation to *Histiogaster sp* (99% sequence similarity), is a soil arthropod. All these sequences have 90-100% similarity to each other over the entire sequence length, and they clustered together in the phylogenetic tree (**Fig. 3**). The phylogenetic tree has 125 number of taxa with average sequences length of 440 bp. Within the amplified region of V4 and V5, a total of 47 highly conserved (found in 100% sequences) spots having nucleotides from single to five were found. The conserved spots with single nucleotide were maximum with 35 in number, whereas, there was only one conserved spot with five nucleotides. It was observed that the variable regions were also conserved in their positions and were distributed in seven pustules.

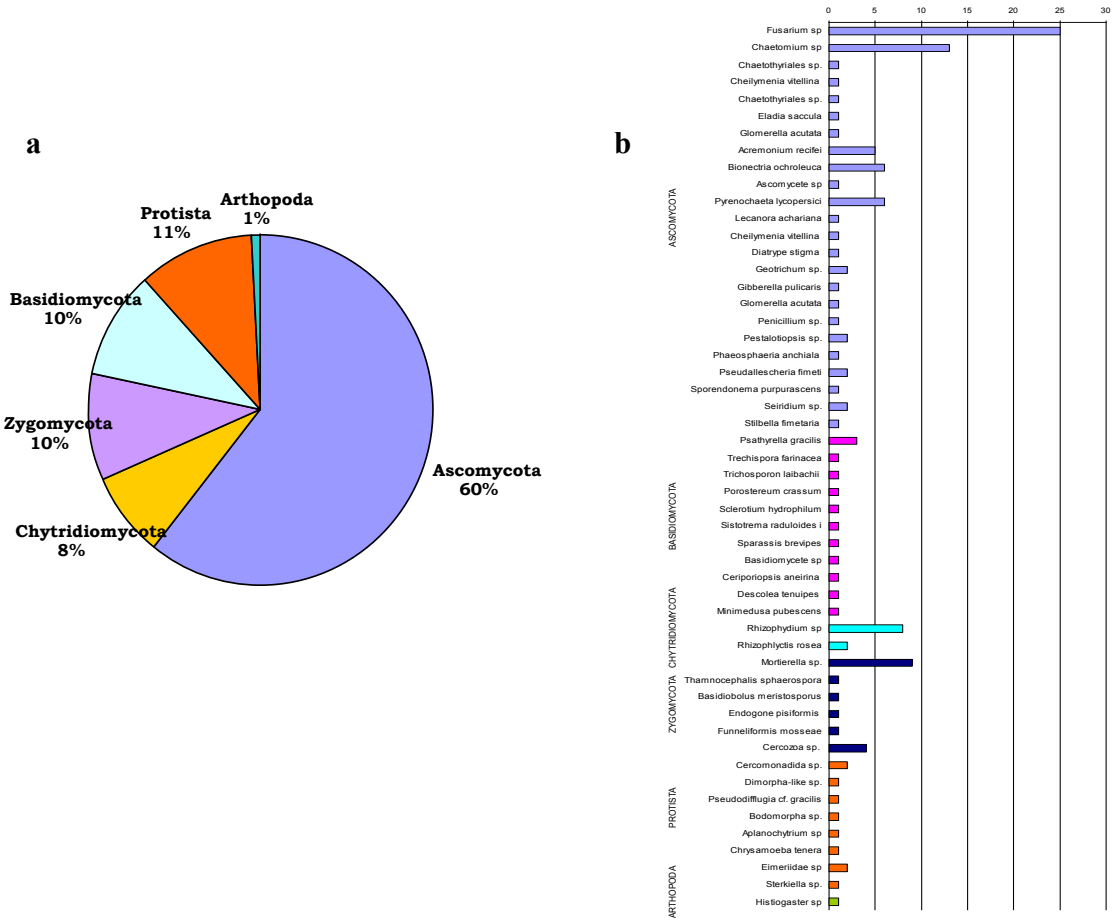


*Figure 2: a) The distribution of fungal population according to their relative abundance in the rhizospheres of potato plant b) Relative abundance of different fungal species in the potato rhizospheres*

To our knowledge, this is the first attempt made to identify the fungal diversity of potato rhizosphere and provides the novel information on the fungal communities inhibiting the potato rhizosphere. The results described in this report provide evidence that PCR primers nu-SSU-0817-5′ and nu-SSU-1196-3′ are not specific to ascomycota, basidiomycota, chytridiomycota and zygomycota as indicated by earlier reports but also amplify orthropods and protistans to some extent. This primer set fails to identify the presence of *Phytophthora* load in the soils infected with late blight. The fungal diversity identification from the studies would be very useful in taking the measures to improve soil fertility and the crop yield. Further, detailed eukaryotic including fungal and prokaryotic diversity from the potato rhizosphere using the deep sequencing technology is being carried out to unearth the influence of different metabiome on plant growth and crop yield.

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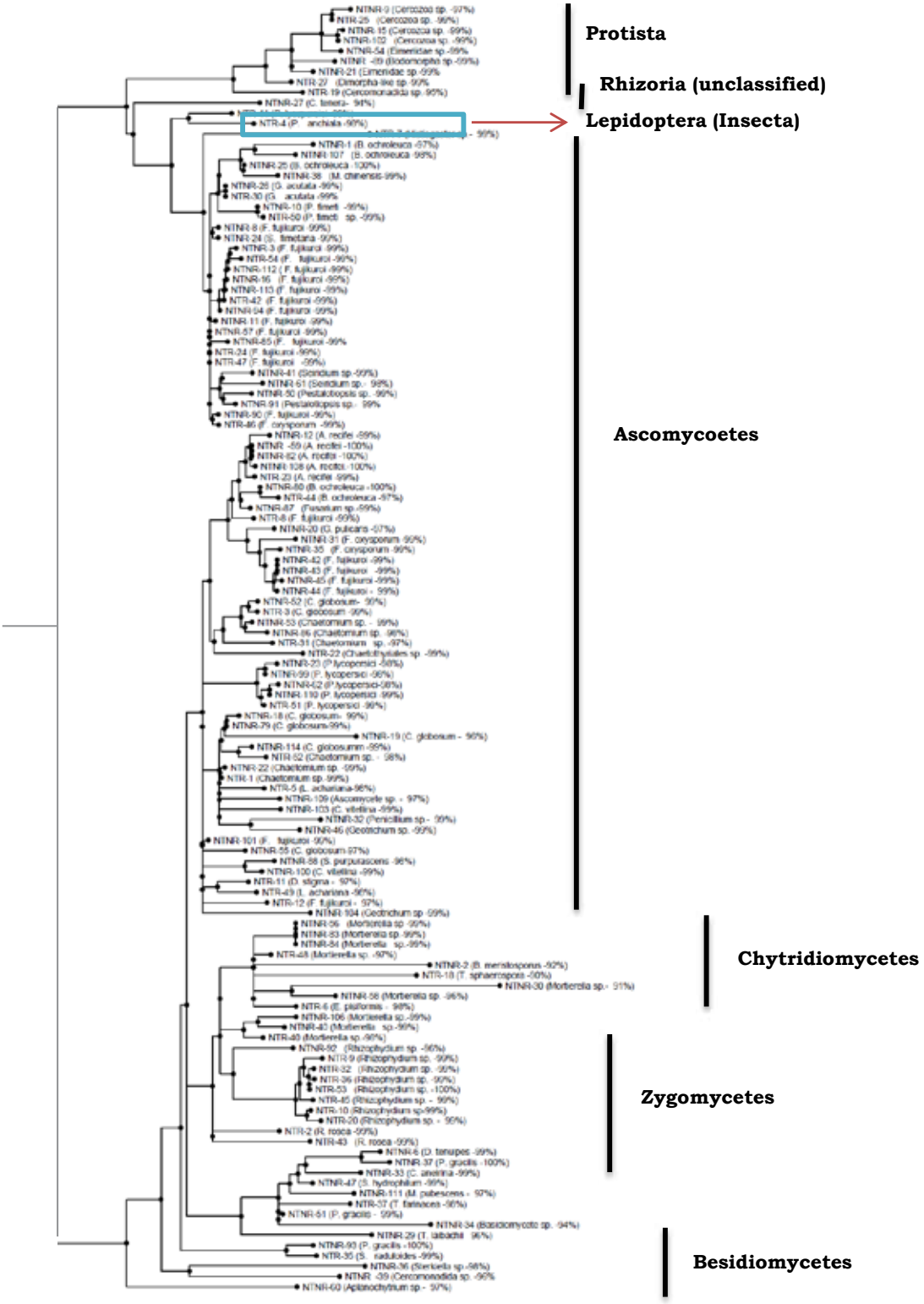


*Fig 3: Dendrogram showing the phylogenetic relationship among the fungal species as assessed by V4 and V5 regions of 18s rDNA (partial)*

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