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Analysis of Indoor Fungal Contaminants Using Internal Transcribed Spacer Sequence Variability

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ABSTRACT

Fungi present in indoor environment always create serious health risks for the individual dwellers. The present study was focused on the enumeration of fungal contaminants in the newly constructed lab. Totally 8 different fungi were isolated from the indoor environment by using sedimentation culture plate method. The isolated species were identified conventionally, as well as using the sequence homology of internal transcribed spacer regions. This ribosomal non-coding unit sequence based analysis is the most popular locus for species identification and to study the phylogenetic variation. The fungal species isolated and described are already found to be reported as pathogens present in indoor air. Based on the sequences obtained, phylogenetic tree was constructed using both maximum likelihood and distance matrix depicts that the distance method using ClustalW2 offers better resolution and relates the related genera. The study reveals the methodology in fungal taxonomy and also for environmental monitoring of fungi.

Key words: Indoor air contamination, internal transcribed spacer, fungal contamination, air-borne fungi

INTRODUCTION

Fungi produces air borne dispersal units called spores which are common in both indoor and outdoor environments. Frequent exposure to these spores brought series of health consequences such as allergy, infection, irritation or toxic reactions (Lee *et al*., 2006; Wu *et al*., 2003; Fischer and Dott, 2003). Nearly 10% of world populations are allergic to fungal spores and their concentration in the environment depends on several factors like weather, vegetation, air quality and various anthropological effects (Burge, 1986, 2001; Oliveira *et al*., 2010). In general population, the incidence of mold allergy ranges from 6-24%, reaches up to 44% among atopic individuals and 80% in asthmatic patients; it was found more severe in immune-compromised individuals (Simon-Nobbe *et al*., 2008; Oliveira *et al*., 2010).

The increased presence of fungal contamination in the air causes vigorous health effects (Chakraborty *et al*., 2000) especially the burden of illness from fungi present in homes and public buildings are controversial because of limited study in this sector (Dales *et al*., 1997). The majority

of the fungi detected in the indoor air are the most commonly existing in outdoor environment. Ventilation and water incursions in buildings, paws of pets, shoes and clothing of human harbor these fungal particles into the indoor environment (Gots *et al*., 2003). *Cladosporium*, *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium* are the most common indoor fungi followed by genera like *Paecilomyces*, *Mucor*, *Rhizopus*, *Trichoderma*, *Ulocladium*, *Stachybotrys*, *Walneria* and *Curvularia* were also reported (Fischer and Dott, 2003; Wu *et al*., 2003).

Uninvited entry of biological hazards in a specific environment can be listed out as contamination (Gorny, 2004). Monitoring the microorganisms and quality assessment of air is essential and the simplest method for estimating is that sedimentation culture plate method was adopted (Chakraborty *et al*., 2000). In this case, identifying the fungus is more important that describing them without knowing the taxon. This gives a clear indication on fungal contamination and disease possibilities. Conventional and molecular techniques were adopted for the identification of clinical and environmental samples (Henry *et al*., 2000). The molecular analysis of DNA helps in better understanding of fungal biology and the related aspects (Crespo *et al*., 1997). The molecular analysis of DNA helps in better understanding of fungal biology and the related aspects (Crespo *et al*., 1997) 18s RNA, mitochondrial DNA and the intergenic spacer and the Internal Transcribed Spacer (ITS) region offers targets for the genus level identification. Varying sequence pattern of ITS region can be used in the phylogenetics analysis of different organisms (Henry *et al*., 2000; Guarro *et al*., 1999) and also offers a higher sensitivity than other molecular targets being existing in hundreds of copies per genome approximately. Nearly, 51,000 fungal ITS sequences where available so far and is the most popular locus for the species identification and sub generic phylogenetic reference in sequence based mycological research. These spacers occupy the small subunit coding sequence (SSU) and the large subunit coding sequence (LSU) of the ribosomal operon (Martin and Rygiewicz, 2005; Burge, 1986). Both 12 spacer regions are useful in species level identification and also in phylogenetic analysis among related species (Subramanian *et al*., 2003; Henry *et al*., 2000; Guarro *et al*., 1999). In the present work the newly constructed lab were found to be contaminated with the fungal propagules. The prepared solid media was frequently encountered fungal contamination and it creates an alarm of fungal occurrences in the lab environment. The goal of the study was to isolate and identify the fungal contaminants by both conventional and molecular methods and to analyze the relatedness among the isolates.

MATERIALS AND METHODS

Sampling site: The indoor environment of a newly constructed lab was selected as the sampling site. The lab was encountered with 20-30 people daily. Total area of the lab was 22×15×12 sq. ft. The average temperature during the sampling period was 28-35°C. The humidity was found to be very low in that season. The external environment temperature was around 35-45°C as it was a period of hot summer i.e., April-June 2010. These days are the initial phase of commencing work in the lab and were examined for fungal exposure. Initially, the fungi were found to contaminate the plates that were kept in the open environment which were prepared for other cultivation purposes. The grown fungi were found to be different from the already existed isolates.

Morphological characterization: The fungi grown as contaminants and by using sedimentation culture method, the molds present in the lab were examined using Saboraud's Dextrose Agar (SDA) plate. The plates were incubated at 28-30°C for 3-7 days and the grown fungi were isolated in a fresh SDA plate. Stock of the isolate was prepared and stored in SDA slant at 4EC

for future use. The fungi grew for 4-5 days were examined for both naked and microscopic morphology. The fungal filaments were studied using light microscope (Optix, Italy) after staining with lactophenol cotton blue.

Total DNA extraction: Total cellular DNA was extracted from the fungal mycelium grown on SDA plate. Spores were collected by pouring 50 µL of triton-X100 over the grown propagules. This was done several times over the same spot to collect enough material. The spore/triton-X 100 mixture was transferred to a 1.5 mL microcentrifuge tube containing 500 µL of CTAB buffer [Stock (pH 8): 100 mL 1 M Tris; 280 mL 5 M NaCl; 40 mL 0.5 M EDTA; 20 g Cetyl Trimethyl Ammonium Bromide (CTAB) store at room temperature] (CTAB buffer: 5 mL of stock; 0.2 g polyvinylpyrrolidone; 25 µL of $-mercaptoethanol prepared right before extraction) and a scoop of sterile glass powder. The mixture was agitated using vortex mixer for 2 min following incubation in water bath at 65°C for 30 min. Five hundred microliter of chloroform-isoamyl alcohol (24:1) was added and mix well by inversion. Cellular debris was removed by centrifugation at 12000 rpm for 5 min. The aqueous phase was removed and transferred to a new 1.5 mL microcentrifuge tube and the above steps were repeated one more time. The DNA was precipitated by the addition of 233 µL of isopropanol along with 32 µL of 4 M ammonium acetate to the aqueous phase. After a gentle mix, the content was centrifuged at 12000 rpm for 10 min. The pellet was rinsed with 70% ethanol and dried. The dried pellet was dissolved in 100 µL TE buffer and stored at -20ºC. The isolated DNA was verified using 0.8% agarose gel electrophoresis.

DNA amplification: The internal transcribed spacer region of the rRNA gene cluster was amplified from genomic DNA obtained from fungal mycelia by PCR with the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4-R (5'-TCCTCCGCT TATTGATATGC-3') (White *et al*., 1990). The reaction mixture contained 20 µL 2X PCR premix (GENET BIO, Korea), 1 µL of DNA (25-50 ng), 1 µL forward and reverses primer each. PCR conditions consisted of an initial denaturation at 94°C for 4 min; 35 cycles at 94°C for 60 s, 50°C for 45 s and 72°C for 60 s and a final 7 min extension at 72°C. The amplified products were examined by agarose gel electrophoresis and the amplified DNA was sequenced.

DNA sequencing and analysis: Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The sequences of spacer region obtained were used to search the GenBank database with BLASTN algorithm to determine the phylogenetic positions of the isolates. The aligned sequences were incorporated to construct phylogenetic tree using maximum-likelihood method (Saitou and Nei, 1987). MEGA 4 software (Tamura *et al*., 2007). To determine the variation among the isolated strains; the tree was also constructed using online multiple alignment tool ClustalW2.Manipulation and tree editing was done by using TreeView. The sequences obtained were deposited in GenBank for accession.

RESULTS AND DISCUSSION

Isolation and morphological characterization: To study the molds present in the newly constructed lab, fungi grown as contaminants and the fungal growth obtained from the open plate method were purified and identified. Both morphological and sequence based methods were used in identification. Morphological characterization was carried out based on the description given in the standard manual for soil fungi (Gilman, 1959). Since there is no standard manual for air borne fungi, the manual for soil fungi helps in partial identification of the fungus. Colour, nature of mycelium in both obverse and reverse position, mycelial and spore morphology observed under

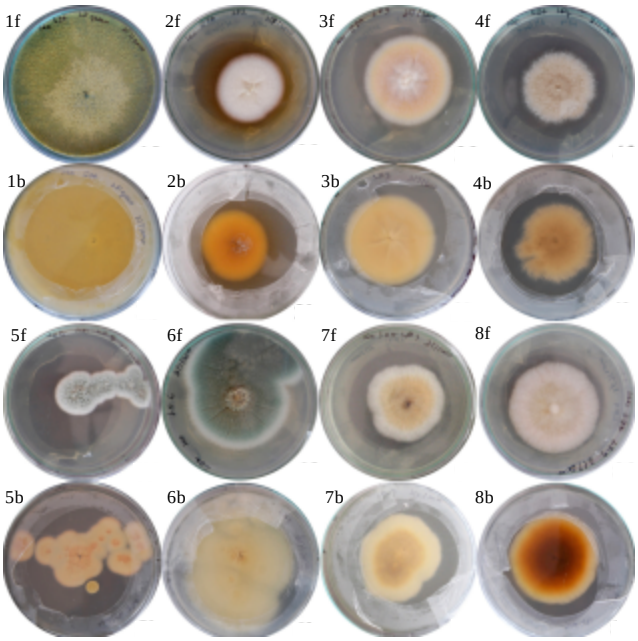

Fig. 1: Overview of the fungi isolated from the study, f: Front view, b: Back view, 1-NTLF01 *T. longibrachiatum*, 2: NTLF02 *A. terreus*, 3: NTLF03 *E. nidulans*, 4: NTLF04 *Nodulisporium* sp. 5: NTLF05 *P. citrinum*, 6: NTLF06 *A. fumigatus*, 7: NTLF07 *Fusarium* species, 8: NTLF08 *Fusarium* species

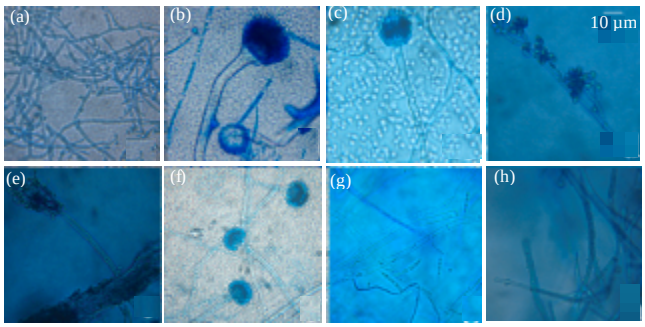

Fig. 2(a-h): Microscopic view o f the fungi stained with lactophenol cotton blue, Magnification: x400 a: NTLF01 *T. longibrachiatum*, b: NTLF02 *A. terreus*, c: NTLF03 *E. nidulans*, d: NTLF04, *Nodulisporium* sp. e: NTLF05 *P. citrinum*, f: NTLF06 *A. fumigatus*, g: NTLF07 *Fusarium* sp., h: NTLF08 *Fusarium* species

microscope were used for morphological characterization. Morphological characteristics of the fungi were detected and documented by plating and microscopic analysis (Fig. 1, 2), respectively. Six genera were noted from the eight isolated species from the indoor environment.

***Trichoderma*:** *Trichoderma longibrachiatum* isolated from the study, are the common species abundant in nature, saprophytes and also reported as indoor air pollutant (Druzhinina and Kubicek, 2005; Thrane *et al*., 2001). The mycelium of the isolate was initially dark green and upon maturation became yellow to white color, forming a flat, firm turf. Conidiophores were erect, rising from short, branched sides, branching usually opposite, apex were not swollen with bore terminal

conidial heads. Conidia are small, mostly globose, oblong to ellipsoidal with bright colored hyphae (Tang *et al*., 2003). Kredics *et al*. (2003) reported that *Trichoderma longibrachiatum* can act as an opportunistic pathogen for mammals as well as humans especially severe under immunocompromised state. They are known to be major causative agent of *Trichoderma* associated mycoses and Allergic Fungal Sinusitis (AFS). Tang *et al*. (2003), later Druzhinina *et al*. (2008) from his studies emphasized that the relationship between the clinical and wild *T. longibrachiatum* strains was unclear.

Aspergillus: Four different *Aspergillus* species were detected and were found to be dominated in the sampled site. *Aspergillus* has already been reported several times as a part of indoor air fungal communities. It has been proved that the major causative agent of many diseases like fungal allergy, aspergillosis, allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis and invasive infection in many individuals (Hyvarinen *et al*., 2010).

The species identified from the study *Aspergillus terreus*, *E. nidulans* (anamorph *A. nidulans*) and *A. fumigatus* are the one reported several times as opportunistic pathogens (Simon-Nobbe *et al*., 2008).

Aspergillus terreus forms white, aerial mycelium. Sometimes it forms brown mycelium whereas new mycelium always white in color. Reverse and agar occurs bright yellow to deep brown. Conidia are elliptical to globose (Gilman, 1959). It is one of the causative agents of invasive aspergillosis and capable of producing toxin called ochratoxin (Henry *et al*., 2000; Simon-Nobbe *et al*., 2008).

Emericella nidulans (anamorph: *Aspergillus nidulans*) were typical in morphology and a representative of perfect *Aspergillus*. They are homothallic fungi, able to produce 'nest-like' fruiting body called cleistothecium (Han, 2009). In the initial culturing phase it looks green which on sub culturing forms orange color hyphae. Whitish new mycelium upon maturation forms pink colored mycelial mass. On the reverse it forms a light yellow to white color, upon aging it forms a liquid over the mycelium in the form of dew drops (Matsuzawa *et al*., 2010). In case of *A. fumigatus*, mature hyphae produce aerial mycelium and the new forms have flattened mycelium. It forms green to dark green colonies. Reverse and substratum is colorless to yellow. Conidia are crowded. This fungus can grow in mucous secretions in the lung and cause type I and III hypersensitivities and severe bronchopulmonary infections and reported to be grown in indoor environment (Srikanth *et al*., 2008). *A. fumigatus* is one of the major species associated with invasive aspergillosis and *Aspergillus* associated diseases (Zhao *et al*., 2001; Benneth and Klich, 2003; Matsuzawa *et al*., 2010).

Penicillium: Both *Aspergillus* and *Penicillium* are the most studied ascomycetes and are already described to be major contaminants of indoor air (Burge, 1986). Spores of *Aspergillus* and *Penicillium* are readily airborne when compared to many other fungal spores and the main indoor fungal contaminants (Burge, 2001). These two are xerophilic molds, dominates in indoor air when compared to outdoor air (Thrasher and Crawley, 2009). *Penicillium* rarely causes diseases in human but excessive inhalation of the spores cause atopic asthma in sensitive persons (Simon-Nobbe *et al*., 2008). The only *Penicillium* species predominant in the lab was *P. citrinum* which produces characteristic red pigment but the property was lost on subsequent cultivation. It forms aerial mycelium, pale green hyphae, on the reverse it forms wrinkled white which turns to red color upon maturation. They produce a characteristic two series of elements called phialides and forms thick sclerotium and can consistently grow at high temperature (Houbraken *et al*., 2010). *Penicillium citrinum* is ubiquitous in the environment rarely cause human infection (Mok *et al*.,

1997). According to the report of Wei *et al*. (1993), *P. citrinum* is the most predominant *Penicillium* species detected in the indoor environment. It also reported that they can produce toxin called citrinin which acts as a nephrotoxin in test animals but its significance with human disease was unconfirmed (Benneth and Klich, 2003).

Fusarium: Two different types of *Fusarium* were isolated from the indoor environment. Conidiospores were sickle shaped in the isolate *Fusarium* sp. NTLF07 and in NTLF09 no distinct spores was identified. *Fusarium* forms aerial mycelium in which new mycelium forms white to pink color and on maturation it turns to brown. Reverse of the plate is pale brown to dark brown. In NTLF07 the conidia has a maximum of up to 7 septa with irregular conidiophores. It has been reported that *Fusarium* is an important pathogen producing different species specific toxins namely fumonisin, deoxynivalenol, and zearalenone etc. (Benneht and Klich, 2003; Jarvis and Miller, 2005). *Fusarium* species frequently encountered in localized infections in immunocompetent and disseminated in severely immunocompromised patients. Development of skin lesions is the most common infectious aspect of this genus and often used in diagnosis of the infection (Nucci and Anaissie, 2002).

Nodulisporium: *Nodulisporium* sp. forms characteristic brown color with flattened mycelium. Hyphae are irregular and branched. They are the common wood decaying fungi of ascomycetes group of Xylariaceae. They also implicated in clinical reports. They were marked as LF4 *Nodulisporium* sp. in Fig. 2 and identification up to species level is difficult. From the report of Cox *et al*. (1994) and Tang *et al*. (2003) also noted that it can cause allergic fungal sinusitis like *Trichoderma longibrachiatum* which being a common allergic disease caused by *Aspergillus* sp.

Molecular identification: The application of molecular tools helps in identification and to study the relationship among the organisms. ITS variability was examined for the 8 isolates based on the sequence homology of the amplified regions. The sequences of ITS regions were deposited in the GenBank for the accession numbers and was as follows: *Aspergillus terreus* NTLF02-HQ219673, *Emericella nidulans* NTLF03-HQ141711, *Nodulisporium* sp. NTLF04-HQ219674, *Penicillium citrinum* NTLF05-HQ245157, *Aspergillus fumigatus* NTLF06 HQ141712, *Fusarium* sp. NTLF07-HQ141713, *Fusarium* sp. NTLF08-HQ219675 and *Trichoderma longibrachiatum* NTLF01-HQ141714.

The ITS sequences obtained were analyzed to find identical species by using BLAST search. Most of the species matched with the sequences of similar species. The similarity search helps in identification up to species level. From the report of “Planning workshop on all fungi DNA barcoding”, 2007, ITS can be used as a marker for fungal DNA barcoding except *Fusarium* and certain AM fungi, which show variation when compared to all other true fungi (Rossman, 2007). With the help of ITS sequences and the BLAST tool *Nodulisporium* sp. was identified and verified with the report of Cox *et al*. (1994).

Phylogenetic analysis: From the report of Lee and Taylor (1992), 0.3-14.6% of nucleotide difference and comparisons of variable regions permitted the phylogenetic analysis and study of evolutionary relationships. The evolutionary history was inferred using maximum-likelihood method based on Kimura 2 and the phylogenetic tree was observed in Fig. 3. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the

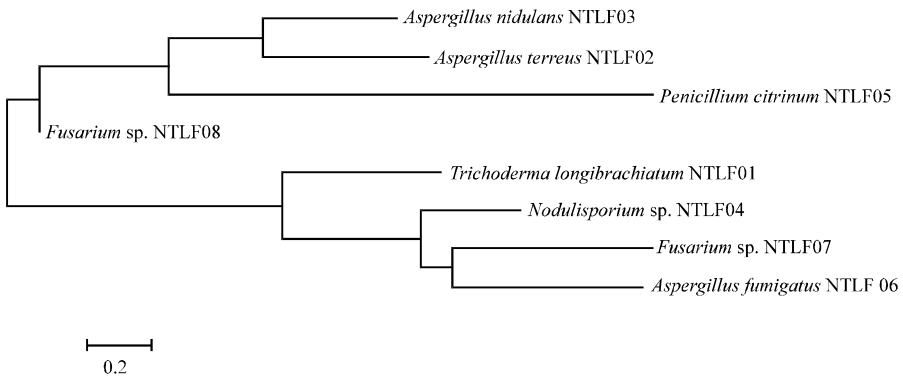

Fig. 3: Phylogenetic tree was constructed using maximum likelihood method from MEGA4 software

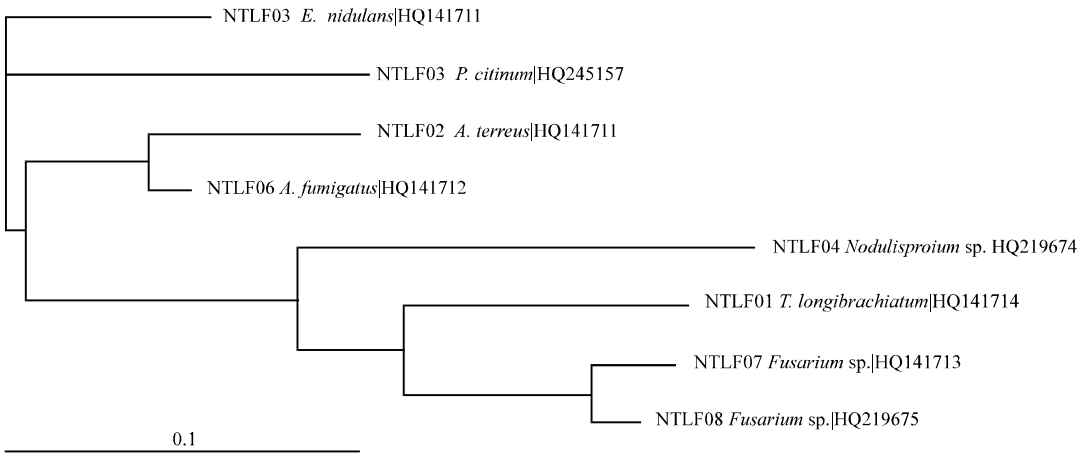

Fig. 4: Phylogenetic tree was constructed using CLUSTALW2 a multiple alignment online tool

taxa analyzed. All positions containing gaps and missing data were eliminated from the dataset. This dataset shows the two *Fusarium* sp. in different clade reveals that ITS region shows much variation to distinguish among species and also not suitable for using as an identity marker for *Fusarium* as per the report of international symposium on DNA barcoding (Rossman, 2007).

The tree was also constructed using ClustalW2 an online alignment tool which depicts the closely related species into a single clade when compared to the evolutionary relationship study using MEGA4. The phylogenetic tree was calculated using distance matrix. Figure 4 clearly distinguishes the *Nodulisporium* NTLF4 from other ascomycetes group. *Trichoderma longibrachiatum* NTLF01 falls in a separate clade in both of the phylograms. It shows a close relationship within the species, whereas *Aspergillus* and *Penicillium* fall separately when compared to other four distinct species. Thus the multiple alignments using ClustalW2 gives a better resolution and joins the associated isolates in a single clade when compared to maximum likelihood method.

CONCLUSION

Of all genera mentioned above, *Aspergillus* was found to be the most dominant ones found in the lab environment. The genera like *Aspergillus, Penicillium, Fusarium* and *Trichoderma* were

common in indoor environment and were analyzed by internal transcribed spacer region and microscopic identification. Partial sequences of ITS region were deposited in GenBank (NCBI) with accession numbers mentioned above. Though the work creates only a rough idea about the fungal contamination over the newly constructed buildings and their environment, it prospect to put more effort to study indoor air contaminants and their relative aspects to avoid risk of infection.

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