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STUDIES IN THE INCIDENCE OF *ASPERGILLUS FLAVUS*, *A. NIGER* AND A RARE NEW STRAIN OF *A. SILVATICUS* IN TWO LOCALITIES OF BENGALURU

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ABSTRACT
Sampling for incidence of airborne *Aspergillus* species for investigation in to their seasonal occurrence was carried out in the intramural environments (bedrooms) of two localities in Bengaluru. The sampling was carried out by using nutrient media petriplates for a one year period from April, 2017 to March 2018. The occurrence of airborne *Aspergillus* species showed considerable variation in both the localities. The samplings showed maximum number of *Aspergillus* species in early winter (November). *Aspergillus flavus* showed the highest prevalence followed by *A. niger* and *A. silvaticus*. The present study also includes the first time molecular characterization of a rare, new strain of *A. silvaticus* isolated Colony Forming Unit from the bedrooms. "Lp1af" is the strain which was identified by 18S rDNA sequencing. The sequence has been submitted to the Gene Bank and accession number obtained.

KEYWORDS: *Aspergillus*, Aeromycoflora, CFUs, Gene Bank accession number.

INTRODUCTION

All over India *Aspergillus* genera are isolated most commonly from the air (Wadhwani *et al*, 1986, Agashe and Anuradha 1996). Many species of *Aspergillus* in the aeromycoflora are known to be allergenic (Peat *et al*, 1993, Ebrahimi *et al*, 2011, Infante *et al*,1992 *and* Twaroch *et al* 2015). These allergenic species have increased these days due to the various anthropogenic activities. Fungal aeroallergens can cause a number of respiratory diseases. *Aspergillus* genus is isolated most commonly from the air (Sinha *et al*, 1998, Majumdar and Bhattacharya, 2004, Manikpuri *et al*, 2018, Marigoudar and Abraham, 2018). Many species of *Aspergillus* in the aeromycoflora are known to be allergenic (Shivpuri and Agarwal, 1969, Wadhwani *et al*, 1986, Simeray Joel *et al*, 2001, Goldman and Huffnagle, 2009, and Ghosh *et al*, 2011). The dominance of this fungus in the air may be due to its thermophilic nature and its stability to thrive well during un-favorable environmental conditions.

Bengaluru is one of the fastest growing cities in India and perhaps in Asia. It is situated in the south eastern part of Karnataka. It lies at an elevation of 900 meters above sea level and comes between 12^0 $151^{-}13^0$ 15^1 North latitude with $77^0$4-77^0 59^1 east longitude. Although Bengaluru is blessed with a good climate, past studies on the air-spora have proved the presence of several allergenic spores in the atmosphere (Agashe and Mathew, 1998). The present study is an investigation into the prevalence and seasonality of common airborne *Aspergillus* species in the bedrooms of houses in the Northern part of Bengaluru, India.

MATERIALS AND METHODS

The study was carried out in the bedrooms of two houses in north Bengaluru (Subramanyanagar and Malleshwaram) for a period of one year from April 2017 to March 2018.

Study sites

Set 1- This site was the ground floor of a house in Malleshwaram, in the northern part of Bengaluru. Small buildings (with 3-4 floors) are generally present in this area. All of them have natural light and ventilation. Some trees like *Artocarpus heterophyllus, Nyctanthes sp., Cocos nucifera* and *Carica papaya are* found near the study site. The average bedroom temperature was 22-28^0C during the study.

Set 2-This study site was the eighth floor apartment in Subramanyanagar, also in the northern part of Bengaluru. The area is cleanly maintained with good ventilation. It is 100 meters away from a train track.

Samplings: Every Monday between 6pm to 8pm, two petriplates were exposed for a period of 10 minutes simultaneously at a height of 2 feet from the floor to avoid dust.

Five replicated petriplates (9cm diameter) containing Martin's Rose Bengal Streptomycin Agar (MRBA) medium were carried to the study sites in sterilized container and exposed to the indoor air for ten minutes to receive the sedimentation of the indoor air borne fungal spores on the media plates. The exposure time was standardized to get countable number of fungal colonies/colony forming units (CFUs) per plate. After exposure, each set (two petriplates from each site) were brought separately to the Mycology Laboratory, Department of Botany, Joseph's college (Autonomous), Bengaluru with utmost care and incubated at room temperature for a total of 15days with constant observation. After 3-4 days of incubation at room temperature, fungal colonies (CFUs) that developed in the exposed plates were counted and individual species were identified. The fifth petriplate was not exposed (kept unopened) but incubated with the exposed petriplates as a

control to check, if there was any contamination during the preparation of the medium.

Microscopic slides stained with Lactophenol blue were prepared from each of the CFUs and observed microscopically (10x- 40x) to identify them up to the species level. The CFUs that could not be identified directly from the plates were sub cultured in MRBA again and identified. The laboratory experience and taxonomic literature by Barnett (1960), Domsch and Anderson (1980), Sutton *et al.* (1998), Kirk *et al.* (2008), and Dube (2010) were employed to identify the fungal taxa. Annual, monthly and seasonal percentage occurrences of individual *Aspergillus* species were determined.

Description of the identified *Aspergillus* species

Aspergillus flavus Link ex Fries

Colonies are citron-green to yellow-green in color. Conidiophores are wide with pitted walls, rough, spiny in appearance broad upwards and gradually enlarging in to vesicles. Conidia are pyriform to almost globose, nearly colorless to yellow green in color. Conidia size: 40-50µ in diameter. They are found in soil, food and dairy products.

Aspergillus niger Van Tieghem.

Colonies are colorless, some strains with yellow colored hyphae; rapidly growing with abundant submerged mycelium, reverse colourless. Conidiophores are yellow to brown near the vesicle with thick walls, smooth and septate. Conidial heads are fuscous, blackish brown smooth walled, short and columnar. Phialides are borne directly on the vesicle or on Metulae. Conidia are globose, smooth at first, later rough or spinulose and 25-40µ in diameter.

Aspergillus silvaticus Fennell and Raper.

Colonies attaining a diameter of 4-5 cm in 10days to 12 weeks, generally raised in central areas, radially furrowed, faintly zonate, with narrow growing margins white, shading quickly to Deep Colonial Buff .Central colony area producing conidial structures in a dense strand in deep forest-green shades. Reverse in buff to flesh-pink shades. Conidial heads very abundant. Conidiophores mostly 250-270 µ in length, 5.5-8.5 µ in diameter, with walls smooth and definitely brownish. vesicles nearly globose, colored as the conidiophores. Bearing sterigmata nearly their entire surface in two series, crowded. Conidia globose to sub-globose, deep yellow-green conspicuously roughened.

RESULTS AND DISCUSSION

The results of the experiments obtained during the period of investigation revealed a wide range of airborne *Aspergillus* species. Among them *Aspergiilus flavus, A. niger* and *A. silvaticus* were dominant. A total number of 1080 *Aspergillus* CFUs were formed during present study.

TABLE 1:- Colonies of *Aspergillus* isolated (April 2017- March 2018) Set-1.

SL No	FUNGI	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	TOTAL
1	*Aspergillus flavus*	19	3	7	3	32	20	24	104	66	22	14	51	365
2	*A. Niger*	68	18	3	76	30	7	10	1	1	2	13	1	230
3	*A. silvaticus*	7	10	5	0	17	22	8	4	30	19	14	4	140
	Total	94	31	15	79	79	49	42	109	97	43	41	56	735

TABLE 2:-Seasonal difference in fungal CFUs on MRBA Medium

Season	Months	CFUs
Summer	Mar, Apr, May and Jun	196
Rainy	Jul, Aug, Sep and Oct	249
Winter	Nov, Dec, Jan and Feb	290
TOTAL		735

Winter season witnessed the maximum number of *Aspergillus* CFUs (39.45% Table-2 and 43.48% Table-4) in the bedroom environment. This may be attributed to low temperature which is suitable for fungal proliferation. Moreover the bedrooms were not airy and spacious. The average occupancy in each house was six. The houses occupied an area of about 850 square feet and the bedrooms measured approximately 120 square feet. Each bedroom had an average occupancy of 2-3. These cramped living conditions would have lead to more Carbondioxide concentration, micro temperature and humidity. The North-east monsoon, accompanying humidity, availability of dead, decaying matter from nearby vegetation, dampness of indoor walls, ventilation, leaky roofs, indoor-maintenance, indoor-plumbing problems would have led to increased fungal growth in the indoor environment. Summer season nurtured the minimum number of air borne *Aspergillus* (In Set: 1 26.67% CFUs and in Set: 2 19.42% CFUs). The rainy season retained moderate CFUs (In Set: 1, 33.88% CFUs and in Set: 2 37.10% CFUs). This might be due to restricted entry of fungal spores in to the houses as rain and water flow would have caused settling and draining of the fungal spores from atmosphere. This could be the reason why there were less CFUs during rainy season. The results of the present study are also supported by observations by Agarwal and Shivpuri (1974) who have reported that extreme heat and cold are unfavourable for the sporulation of fungal species.

A perusal of Table-5 indicates *Aspergillus flavus* to be the most prolific air-borne species (504 CFUs). The species had the highest concentration in November (25.99%) and December (14.48%). May (1.58%) and July (0.99%) showed the least concentration months. The next dominant *Aspergillus* species was *A. niger* (Figure-3) was the most predominant in both the sets. Similar observations were made by Chauhan and Kulshrestha (2006), and Nayak *et al.* (2013). Studies on monthly occurrence of *A. niger* (Graph:-2) showed almost similar fluctuation in the number of CFUs. The data given in the Table-6 suggests, the relative incidence of *A. niger* was high in April (22.71%) and July (27.44%). It was least in March (0.31%) and followed by November (0.94%).

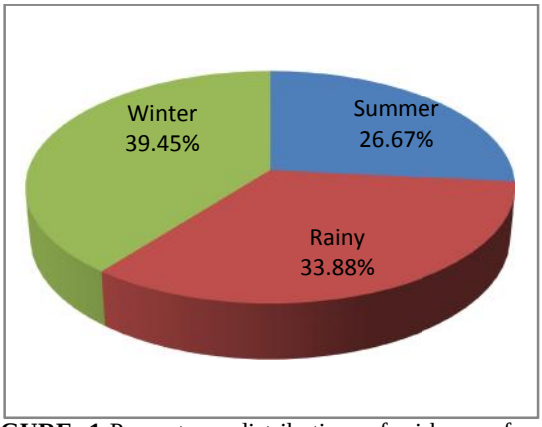


FIGURE 1-Percentage distribution of airborne fungal spores based on season (April 2017-March 2018) Set-1

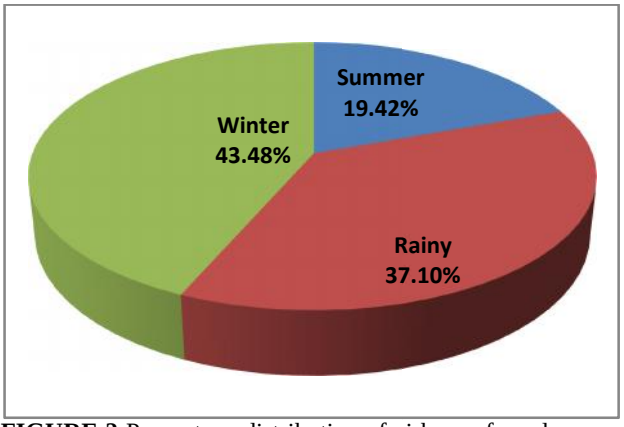


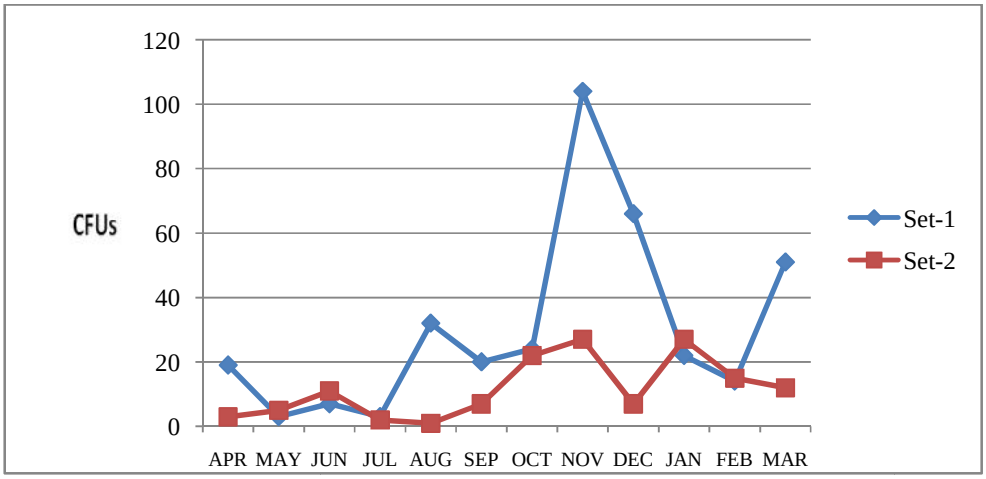
FIGURE 2-Percentage distribution of airborne fungal spores based on season (April 2017-March 2018) Set-2**.**

TABLE 3:- Colonies of *Aspergillus* isolated (April 2017- March 2018) Set-2.

SL No	FUNGI	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	TOTAL
1	*Aspergillus flavus*	3	5	11	2	1	7	22	27	7	27	15	12	139
2	*A. Niger*	4	7	6	11	15	7	8	2	7	0	20	0	87
3	*A. silvaticus*	3	5	9	5	23	14	13	31	9	2	3	2	119
	Total	10	17	26	18	39	28	43	60	23	29	38	14	345

TABLE 4:-Seasonal difference in Set-1 fungal CFUs on MRBA Medium

SEASON	MONTHS	CFUs
Summer	Mar, Apr, May and Jun	67
Rainy	Jul, Aug, Sep and Oct	128
Winter	Nov, Dec, Jan and Feb	150
TOTAL		345



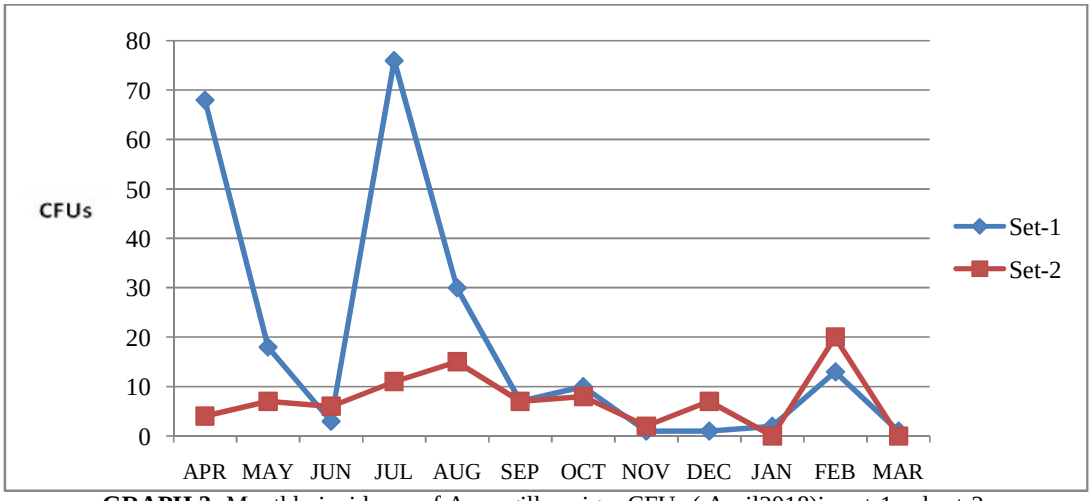
GRAPH 1- Monthly incidence of *Aspergillus flavus* CFUs (April 2017- March 2018) in set-1 and set-2.

TABLE 5:- Monthly incidence of *Aspergillus flavus* (April 2017- March 2018) from Set-1 and set-2.

SET No	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	TOTAL
Set-1	19	3	7	3	32	20	24	104	66	22	14	51	365
Set-2	3	5	11	2	1	7	22	27	7	27	15	12	139
TOTAL	22	8	18	5	33	27	46	131	73	49	29	63	504

TABLE 6:- Monthly incidence of *Aspergillus niger* (April 2017- March 2018) from Set-1 and set-2.

SET No	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	TOTAL
Set-1	68	18	3	76	30	7	10	1	1	2	13	1	230
Set-2	4	7	6	11	15	7	8	2	7	0	20	0	87
TOTAL	72	25	9	87	45	14	18	3	8	2	33	1	317



GRAPH 2: Monthly incidence of Aspergillus niger CFUs (April2018)in set-1and set-2.

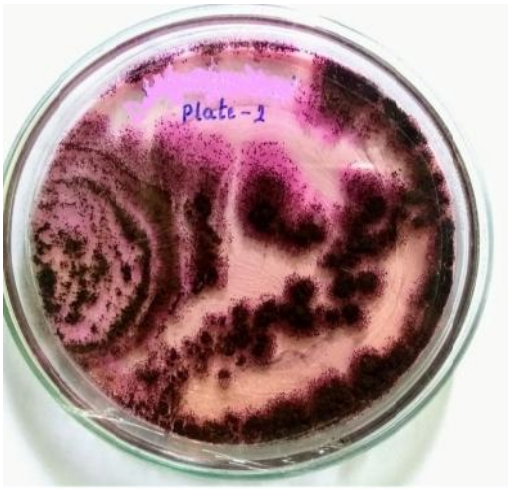
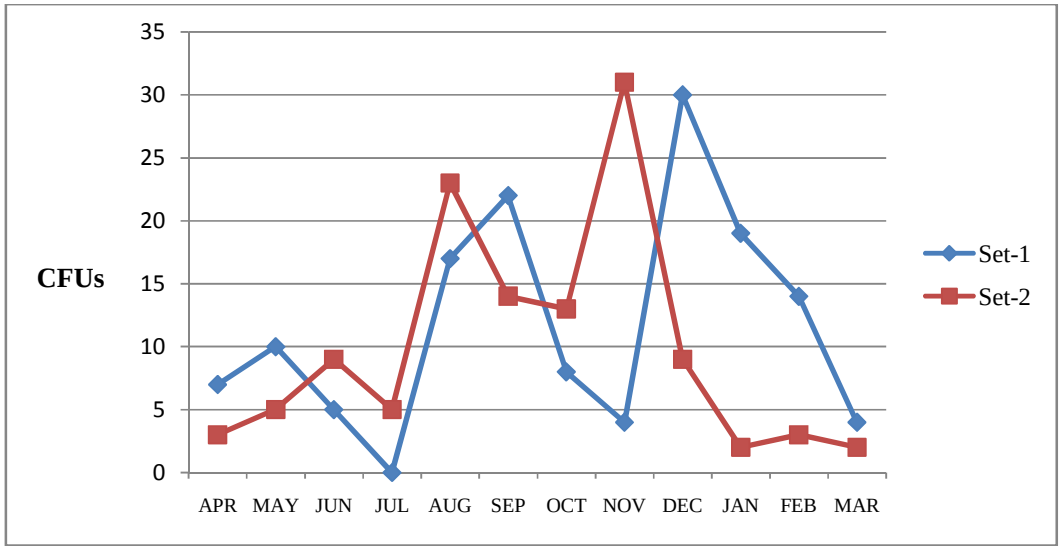


FIGURE 3: Colony morphology of *Aspergillus niger*

TABLE 7:- Monthly incidence of *Aspergillus silvaticus* (April 2017- March 2018) from Set-1 and set-2

SET No.	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	TOTAL
Set-1	7	10	5	0	17	22	8	4	30	19	14	4	140
Set-2	3	5	9	5	23	14	13	31	9	2	3	2	119
TOTAL	10	15	14	5	40	36	21	35	39	21	17	6	259



GRAPH 3- Monthly incidence of *Aspergillus silvaticus* CFUs (April 2017- March- 2018) in set-1 and set-2.

FIGURE.4- Colony morphology of *Aspergillus silvaticus.*

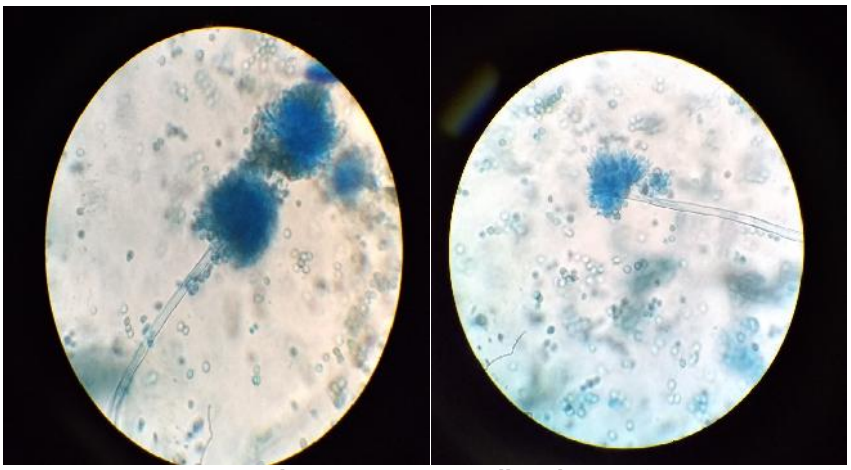


FIGURE 5- Microscopic characteristics *Aspergillus silvaticus* (Microscopic view)

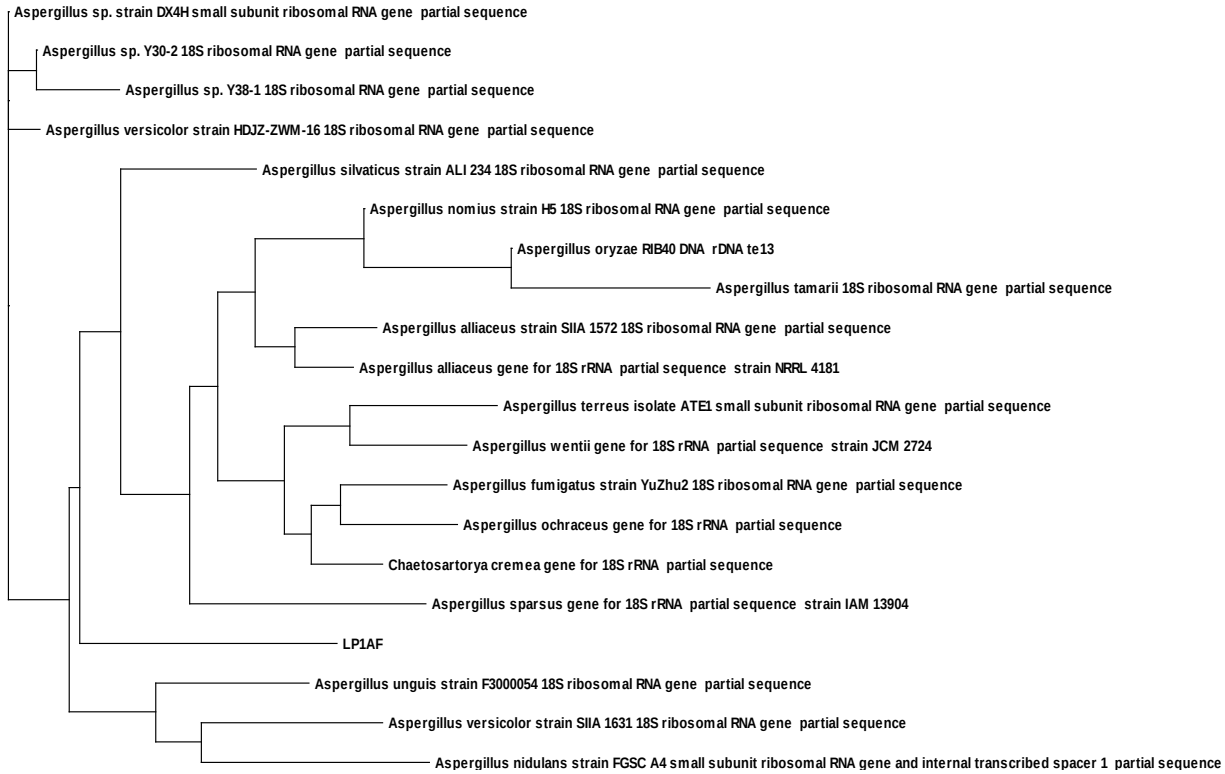


FIGURE 6: Phylogenic tree of *Aspergillus silvaticus* with the Gene bank accession number MH879818.

Like the other two species, *Aspergillus silvaticus* (Figure-4 and 5) also showed relatively high prevalence during winter months. The relative incidence of *A. silvaticus* was high in August (15.44%) and December (15.05%). It was least in July (1.93%) followed by March (2.31%). Quantitative analysis revealed the *A. silvaticus* to be in third (in Set-1) and second (in Set-2) in concentrations compared to *A. niger* and *A. flavus*. Maximum variation can be seen between two sets (Table-7 and Graph-3). *A. silvaticus* is a less familiar air-borne species than *A. flavus* and *A. niger*. As the study has shown *A. silvaticus* to be one of the common airborne *Aspergillus* species and due to the fact that it has been reported less by other workers, it was decided to conduct its molecular study. Molecular study was also carried out to reveal its correct position in the Phylogenetic tree (Figure 6). When the 18S rDNA gene sequencing of *A. silvaticus* was done, it was found that *A. silvaticus* was a new strain. Gene sequence was deposited at NCBI with the accession number MH879818.

CONCLUSION

Aspergillus is the most common fungi recorded from the indoor environment in our study. This result is agreement with the findings of Agashe, S.N., 1994, Dharmage *et al.*, 2002, and Michael *et al.*, 2016 have reported similar results**.** In tropical environments these fungi are dominant and are known for their allergenicity (Cordasco *et al.*, 1995)**.** As the results of our study show *Aspergillus* to be most dominant airborne species. They could be potential aeroallergens (Agarwal and Shivpuri, 1974, Marigoudar and Abraham, 2018). Our findings indicate that small houses with more number of occupance and less cleanliness showed high airborne fungal concentration**.** The vast difference in density of *Aspergillus* species in the two study areas can also be attributed to the height of the residence from the ground level. The ground floor house showed more airborne fungi compare to the eighth floor house. This may be due to the fact that the ground floor house is in close proximity to the surrounding vegetation, dead, decaying litter and garbage.

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